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Ramkissoon, Stacy

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Investigating the variability of *Bordetella pertussis* growth

Stacy Chandra Ramkissoon

A thesis submitted for the degree of Doctor of Philosophy

University of Bath

Department of Biology and Biochemistry

January 2021

Declarations

The material presented here for examination for the award of a higher degree by research and has not been incorporated into a submission for another degree.

....

I am the author of this thesis and the vast majority of the work presented herein was carried out by myself personally. The manuscript included in Appendix A resulted from multi-disciplinary collaborations where some work was performed by colleagues and/or collaborators. Further details can be found in the Statement of Authorship form which accompany the manuscript.

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Abstract

Pertussis is currently ranked fifth in global mortality among vaccine-preventable paediatric infections with cases on the rise over the last 30 years, despite global vaccination programs dating back to the 1940s. To better protect against this disease, the biology and physiology of *Bordetella pertussis* require a better understanding. This includes investigating the variability of *B. pertussis* growth.

This thesis will follow the growth dynamics of *B. pertussis* in several different strains. The growth of *B. pertussis in vivo* during colonisation, *in vitro* during phenotypic modulation, and in response to the antibiotic ampicillin will be explored.

The PMA-qPCR assay was successfully optimised for *B. pertussis* and allowed the growth of *B. pertussis in vivo* to be evaluated during the human colonisation study, while reporter constructs specific to each Bvg phase enabled the sensitivity to modulation during growth *in vitro* to be investigated amongst different strains of *B. pertussis*. Finally, growth of *B. pertussis* in the presence of ampicillin *in vitro,* in broth cultures, was determined and the mechanisms of resistance were investigated. Despite encountering some growth limitations, the results strongly suggest that an increase in the expression of genes encoding for penicillin binding proteins may contribute to ampicillin resistance.

Taken together, the variability of *B. pertussis* growth was observed utilising a variety of tools, *in vivo* and *in vitro,* allowing for a greater appreciation of the biology of this pathogen. This work highlights the importance of studying the growth of *B. pertussis* to better understand how to protect against pertussis infections and supports the determination of new treatment options and methods of prevention to help combat resurgence.

Chapter 1

Introduction
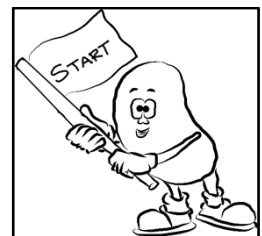
1. Introduction

1.1. *Bordetella* species

Bordetella is a genus of small gram-negative coccobacilli with nine known species, three of which commonly cause respiratory infections in humans. Collectively, these strains are referred to as the *Bordetella bronchiseptica* cluster, classified by sequence analysis, insertion sequence (IS) elements, and multilocus enzyme electrophoresis. These species include: *B. bronchiseptica, Bordetella parapertussis* and *Bordetella pertussis* (Figure 1) [1].
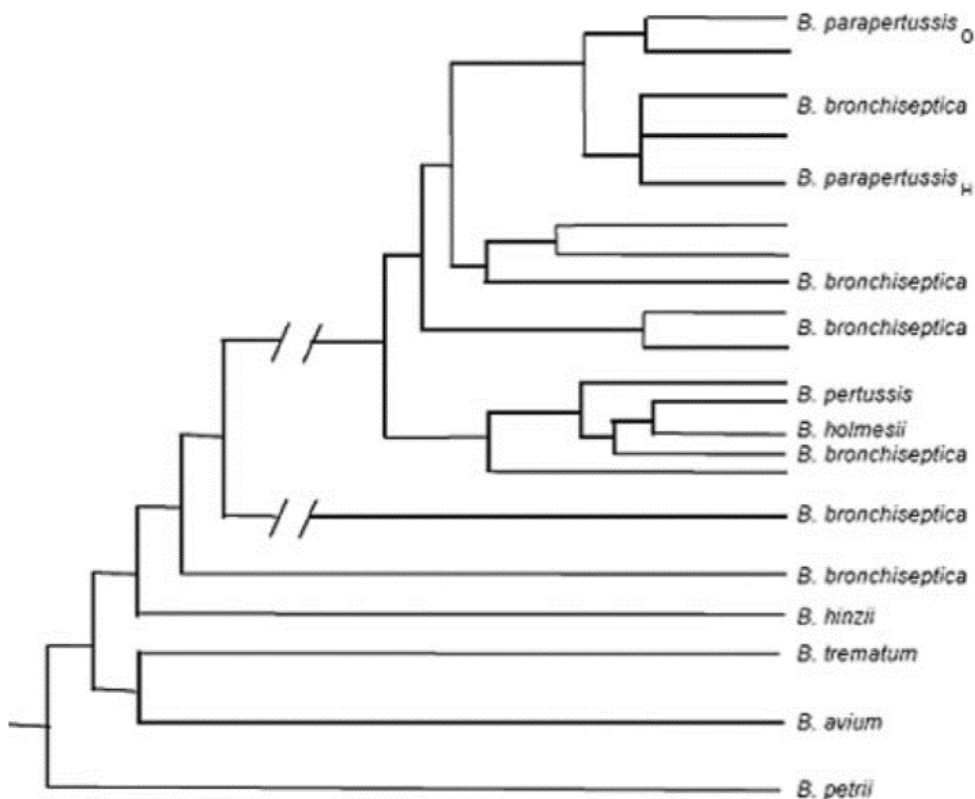
Figure 1. Dendrogram highlighting the relationships among nine Bordetella spp.

Sequence analysis, multilocus enzyme electrophoresis, and insertion sequences were used to determine the relationships and classify the "*B. bronchiseptica*" cluster [1].

B. bronchiseptica has been suggested to be the evolutionary progenitor of *B. pertussis* and *B. parapertussis*$_{hu}$ due to their limited genetic diversity and different host adaptations existing between these strains. *Bordetella holmesii* is also a human pathogen, however it has been excluded from the *B. bronchiseptica* cluster due to it having a different virulent protein expression profile from the rest of the strains in the cluster [1,2].

The clinical presentations of these pathogens have been highlighted in Table 1, with the exception of *B. pertussis*, which will be discussed in greater detail in the following section [1].

Table 1. Human adapted forms of Bordetella species excluding B. pertussis.

Bordetella Species	**Clinical Presentation**
B. bronchiseptica	Can cause severe infections in immunocompromised individuals
B. parapertussis$_{hu}$	Causes milder pertussis-like disease and was not associated with lymphocytosis seen in children infected with *B. pertussis*
*B. holmesii	Can be found in the blood and sputum of young adults and causes respiratory tract infections and septicaemia.

*B. holmesii is not included from the *B. bronchiseptica* cluster

1.2. Clinical course of pertussis

Pertussis or whooping cough, as its more commonly known, is a highly contagious respiratory infection exclusive to humans and is caused by *B. pertussis*.

The incubation period in humans can range from 6-21 days, after which, the clinical course of illness begins and can last for 6-12 weeks or longer in some cases [3]. The clinical course of pertussis can be divided into three stages: 1) Catarrhal Stage, 2) Paroxysmal Stage and 3) Convalescent Stage [3].

Infected individuals enter the 7-14 day 'catarrhal phase' of disease where shedding can occur for up to 6 weeks. Patients are most infectious during this time and are able to transmit the bacteria directly to non-infected individuals. Catarrhal symptoms include a runny nose, sore-throat, nasal congestion and a mild dry cough. In infants, shedding can last for up to 3 months [1,4–6].

During the second week of illness, infected hosts enter the 'paroxysmal phase' phase. Progression into this phase is characterised by repeated coughing fits during a single expiration or paroxysm accompanied by the classic high-pitched "whoop" during inhalation. Paroxysms occur over a period of 2-8 weeks with a frequency of multiple times per hour. Cyanosis, lacrimation, distention of neck veins, and bulging eyes often occur in conjunction with paroxysms and posttussive emesis is common during mucus production that occurs when coughing. Following a paroxysmal episode, children with this infection may lose weight as result of repeated vomiting and develop absolute lymphocytosis [1,4–6].

Severe complications of this disease include pneumonia, seizures, encephalopathy, subarachnoid haemorrhage, rupture of the diaphragm, inguinal hernia, rectal prolapse, and rib fractures. Asymptomatic or mild infections can also occur, however this is more likely in previously vaccinated individuals or those that have had previous *B. pertussis* infections [1]. The clinical manifestations of pertussis depend on age and immune status with milder symptoms developing in older age groups, however neonates are at the greatest risk of

severe complications such as choking and seizures when the disease has progressed to this 'paroxysmal phase'. Pulmonary hypertension leading to cardiac failure and eventual death can occur when additional pathologies such as pulmonary vasoconstriction, hypoxaemia, apnoea and bronchopneumonia are present [1,4–6]. The severity of disease directly correlates with the number of lymphocytes present in these infants [1].

Additionally, the true burden of disease in adults is not known. However, pertussis in adults with a chronic cough has been observed in studies conducted in Canada, Denmark, Korea, Germany, Australia, Estonia and the United States with varied levels of incidence of 2.3%-37% [7–15]. Therefore, testing for pertussis in adults with a persistent cough regardless of age has been recommended to ensure that adults do not become a reservoir of transmission to highly susceptible infants [7,8].

During the 1-2 weeks convalescent phase, coughing is reduced over time and the affected individual is gradually recovering, however coinfections and subsequent respiratory infections can occur [1,4–6].

A summary of the phases of pertussis and clinical manifestations can be found in Table 2.

Table 2. Clinical course of pertussis divided in three stages.

Stage	Length	Clinical Features
Incubation	Usually **7-10 days**; can exceed 28 days	
1. **Catarrhal**	Usually **7-14 days**; range of 4-21 days	• Coryza • Low-grade fever • Mild cough • Lacrimation • rhinorrhoea
2. **Paroxysmal**	Usually lasts **1-6 weeks**, but may persist for up to 10 weeks	• Paroxysmal cough accompanied by a high-pitched "**whoop**" at the end of the paroxysms • Cyanosis • Posttussive emesis • Pulmonary hypertension • Death
3. **Convalescent**	Could last weeks to months	• Gradual recovery • Less persistent paroxysmal coughs • Subsequent respiratory infections with Paroxysms

Adapted from Sealy *et al.*, Elahi *et al.*, and Mattoo and Cherry [1,3,16].

1.3. Disease burden

Despite the introduction of a vaccination in the early 1950s, whooping cough currently ranks fifth in global mortality among vaccine-preventable paediatric infections. [17].

There was an initial reduction of incidence immediately following global vaccination programs, however circulation of pertussis has been restored to a high level with the reported number of global cases increasing significantly over the last 30 years [18]. In the UK for example, Public Health England has reported a ten-fold increase in pertussis cases between the years of 2005–2015 [19]. In the US, there were 10 infant deaths in 7200 reported cases of pertussis in California in 2010, the highest number of reported cases since 1947 [20]. This rise seen in the UK and US has been echoed in other countries including Australia, the Netherlands, and Canada confirming the global resurgence of this preventable illness [21–26].

The reason for this resurgence is not known, however it has been suggested that vaccine escape occurring during the switch from whole-cell vaccines (WCVs) to acellular vaccines (ACVs) may have contributed to the resurgence. It also appears that the immunity afforded by acellular vaccines may wane faster in comparison to the protection provided by whole-cell vaccines. Low vaccine coverage and better laboratory detection methods have also been proposed contributors in this resurgence. [4,27,28].

1.4. Pathogenesis of pertussis

Mattoo *et al*. has described the pathogenesis of *B. pertussis* as consisting of the following four steps: (i) attachment, (ii) evasion of host defences, (iii) local damage, and (iv) systemic manifestations [1].

The pathogenesis of pertussis begins with the inhalation of contaminated respiratory droplets by non-infected individuals. *B. pertussis* organisms have strong tropism for the ciliated cells of the upper respiratory tract and colonise by attaching to epithelial cells of the rear nasopharynx, trachea, and bronchioles where they reproduce and are deposited in the lungs [1,29].

The virulence factors Pertussis toxin (PT), Pertactin (PRN) Filamentous haemagglutinin (FHA), and Fimbriae (FIM), have been implicated in aiding this attachment.

PT also serves to facilitate the evasion of host defences by inhibiting chemotaxis and migration of macrophages, lymphocytes and neutrophils to the site of infection resulting in less efficient phagocytosis and bacterial cell death [1,30].

Colonisation and proliferation are followed by the release of toxins, resulting in paralysis of the cilia in the epithelial cells lining the lungs, and the release of mucus into the lumen of the

respiratory tract. To combat this mucus build-up the distinctive paroxysmal cough and subsequent local damage in the tissues develops [3]. The prime candidate for this damage appears to be tracheal cytotoxin (TCT), rather than PT. Support for this has been seen in *B. parapertussis*$_{hu}$ infections, where identical paroxysmal coughs are elicited by this pathogen, despite not producing PT. Regardless, PT has been deemed responsible for a series of clinical manifestations discussed in greater detail below [1,31,32].

1.4.1. *B. pertussis* virulence factors

There are several key virulence factors that play a major role in pertussis, and a number of them will be highlighted here [33].

1.4.1.1. *Toxins*

Pertussis toxin (PT) is an A-B toxin secreted exclusively by *B. pertussis*. The A subunit of this toxin is composed of the S1 polypeptide encoded by the *ptxA* gene*,* while the pentameric B subunit forms a ring-like structure consisting of polypeptides S2-S5 that are encoded by *ptxB* to *ptxE* genes [34,35]. Secretion of this toxin to the outer membrane occurs utilising nine "Pertussis toxin liberation" (Ptl) proteins that comprise a Type IV secretion system [36].

PT has also been speculated to be involved in the adherence of *B. pertussis* to ciliated respiratory epithelial cells or eukaryotic cell membranes via the B component allowing the S1 subunit of the A component to gain entry into host cells. This mechanism suggests that PT also functions as an adhesin [1,37].

The B component binds ATP, once intercalated into the cytoplasmic membrane, allowing S1 subunits to be released and become activated. The S1 subunit is an ADP-ribosylase that expresses toxic activity through the transfer of ADP-ribose from NAD, to the α-subunit of G-proteins in eukaryotic cells, thus inactivating these G proteins [1,38–40]. Biological consequences of G protein inactivation include a hypersensitivity to histamine, increased insulin secretion and resulting hypoglycaemia in addition to an assortment of immunologic effects. Both B and T lymphocytic cells have been found to be increased in the peripheral blood, and along with neutrophils, have been found to continue to recirculate rather than leave the blood leading to the increased presence of white blood cells or leukocytosis detected in hosts [1,30,41].

Despite the ability to stimulate the host immune response, immunosuppressive activity has been observed in both rat and mouse models. Additionally, PT appears to contribute to the

morbidity in *B. pertussis* infections and the extreme leukocytosis observed in neonates and infants <1 years of age in *B. pertussis* patients.

PT has long been seen as the toxoid responsible for the disease presentation of pertussis, however the *in vivo* role of PT in establishment of infection, disease presentation and transmission In humans is not yet known [1,30,41].

1.4.1.2. Autotransporter

A second virulence determinant is the autotransporter Pertactin. It is a surface-associated protein with the proposed function as an adhesin because it contains both proline and leucine-rich regions, as well as the equally important Arg-Gly-Asp (RGD) tripeptide motif. These molecules form protein-protein interactions and facilitate the *Bordetella* spp. in binding to eukaryotic cells. However, several studies have both contested and supported this proposed role of PRN in attachment [1,42–46]. Therefore, despite PRN appearing to play a role in adhesin and potentially a role as an immunogen, its role in pathogenesis remains unclear.

1.4.1.3. Adhesin factors

Filamentous hemagglutinin (FHA) and Fimbriae (FIM) round out the prominent virulence determinants of the *Bordetella* spp. and are involved in the early stages of bacterial pathogenesis.

FHA is a highly immunogenic surface protein with the ability to adhere to the respiratory mucosa facilitating tracheal colonisation. This dominant attachment factor is synthesized as the precursor FhaB and encoded by the *fhaB* gene. The mature 220 kDa FHA protein is formed by modifying the N-terminus and cleaving the C-terminus of FhaB [1,47].

FHA is proposed to be a hair-pin shaped molecule. This structure facilitates attachment to mammalian cell types via the leukocyte response integrin/integrin-associated protein (LRI/IAP) complex and complement receptor type 3 (CR3). Both act as receptors for FHA via the Arg-Gly-Asp (RGD) triplet. The RGD motif is located in the centre of FHA and localised to the end of the hairpin structure and facilitates binding. Interestingly, in the rat model, FHA alone is not sufficient to colonise healthy, unanaesthetised animals. Therefore researchers suggested that FHA may play a more dominant role in establishing the infection by overcoming clearance mechanisms of the mucociliary escalator [48]. A secondary role of FHA appears to be in facilitating the secretion and dispersal of bacteria promoting bacterial spread [49]. Finally, FHA has also been suggested to perform a series of immunomodulatory functions further facilitating persistence by reducing the Th1 immune responses of the host through the inhibition of T-cell proliferation and suppression of the proinflammatory cytokine,

IL-12. However, in human monocyte-like cells and bronchial epithelial cells FHA has also been shown to elicit proapoptotic and proinflammatory responses [47].

The *Bordetella* spp. also produce FIM, filamentous surface appendages with the proposed function as an adhesin working in concert with FHA to mediate the attachment of *Bordetella* to host airway epithelia and suppress inflammatory airway responses [33]. The two predominant *Bordetella* fimbrial serotypes responsible for this function are Fim2 and Fim3 and are encoded by *fim2 and fim3,* respectively*.*

The Fimbrial subunit FimD, which forms tip adhesin, has also been suggested to mediate the binding of *B. pertussis* to monocytes and cause the activation of CR3 and enhanced binding to FHA [1,50]. The fimbrial biogenesis operon, *fimBCD,* has been suggested to be the only functional fimbrial biogenesis operon on the *B. pertussis* chromosome and is transcriptionally and translationally coupled to the *fha* operon. A study utilising a Fim⁻ *B. bronchiseptica* strain with the expression of FHA and other putative adhesins intact revealed that FIM contributes to the efficient colonisation of the trachea and is necessary for persistence in both the rat and mouse models [48].

Like FHA, Fimbriae also plays an immunomodulatory role by inducing both an IgM and Th2-mediated immune response in the host.

Therefore, the role of Fimbriae during infection is to mediate interaction of *Bordetella* to host epithelial cells, monocytes and macrophages contributing significantly to adherence and in the host immune response to *Bordetella.*

Some or all of these antigens have also been included in the acellular vaccine used to protect against *B. pertussis* infections and will be discussed in future sections.

The expression of these virulence factors is controlled by the BvgAS Two-Component System and will be described in detail in Chapter 4.

1.5. The BvgAS Two-Component System

One of the main proponents allowing *B. pertussis* to cause disease in humans is the conserved *Bordetella* Virulence Regulon encoded by the *bvgAS* locus. It is shared by the members of the *B. bronchiseptica* cluster and is a two-component system that functions as a stimulus-response mechanism enabling the bacteria to adapt to repress or express target genes [51]. These genes enable the secretion of proteins involved in pathogenesis, enzymes required for cellular metabolism, and virulence determinants to promote colonisation of the host. These virulence determinants were discussed in the previous section and include toxins, adhesin factors, and autotransporters.

1.5.1. BvgAS Mechanism

The BvgAS two-component system is composed of a 135-kDa polyhistidine sensor kinase (BvgS) and a 23-kDa cytoplasmic response regulator protein (BvgA). BvgS is fully active at 37°C and initiates a four-step phosphorelay (His-Asp-His-Asp), allowing the phosphorylation of BvgA (BvgA~P), that regulates virulence gene transcription (Figure 2) [51–53].
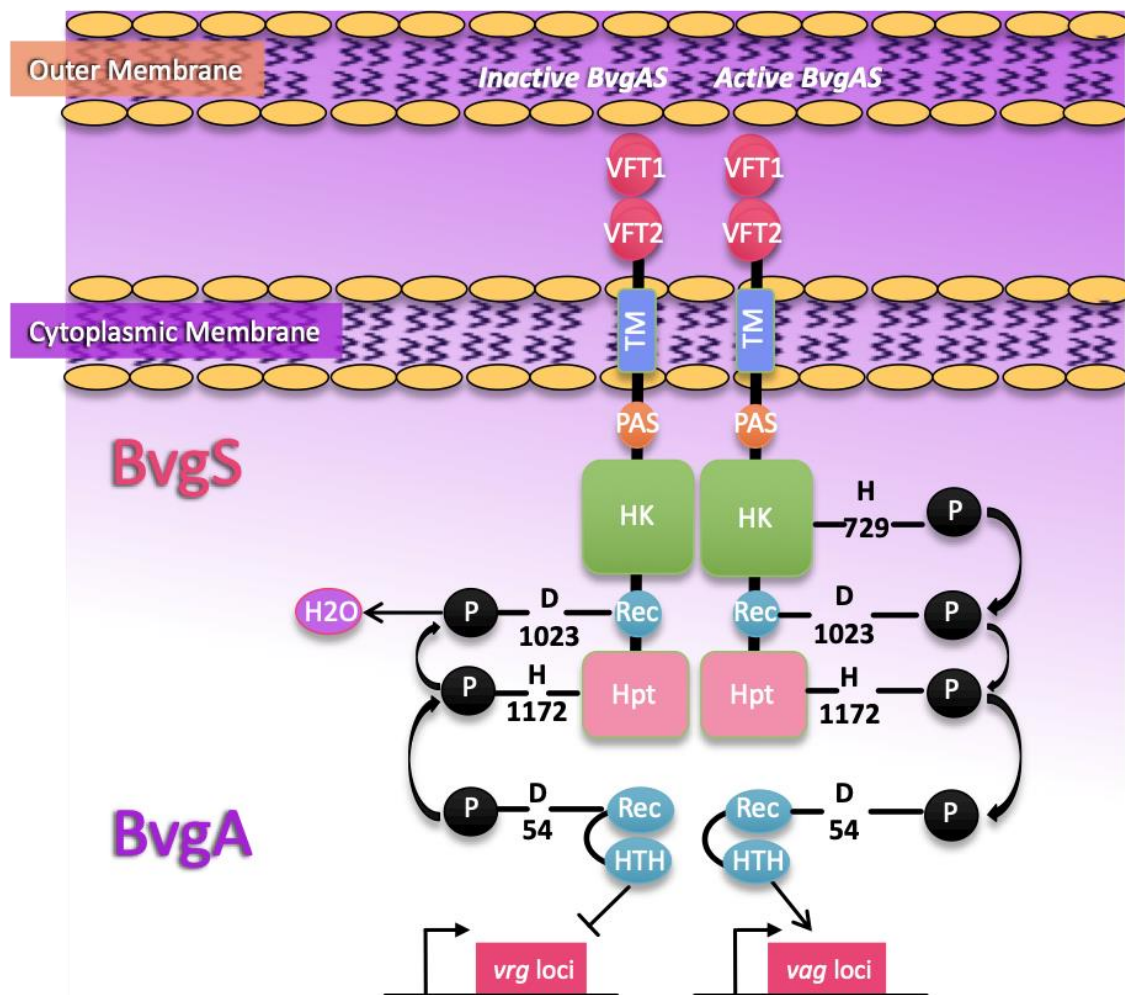
Figure 2. Schematic of the inactive and active BvgAS phosphorelay.

This two-component system comprises of the BvgS histidine sensor kinase and the BvgA response regulator protein. When active at 37°C, the conserved histidine (His729) in the HK domain of the BvgS becomes autophosphorylated. The phosphoryl group gets transferred to Asp1023 in the Rec domain, then to His1172 in the Hpt, eventually phosphorylating Asp54 in the Rec domain of the BvgA. This activates the expression of the *vag* loci, repressing the *vrg* loci. When inactive in the presence of chemical modulators such as magnesium sulphate ($MgSO_4$), nicotinic acid or low temperatures (25°C), the phosphorelay does not occur. Adapted from Sobran and Cotter [54].

The BvgS sensor kinase consists of three domains involved in signal perception: two Venus flytrap (VFT) domains, and a cytoplasmic PAS domain. These domains are followed by the histidine kinase (His-kinase) domain, an Asp-containing receiver domain and a His

phosphotransfer domain (Hpt). Collectively, these domains make up the phosphorylation cascade that ultimately phosphorylates the response regulator, BvgA [2].

BvgS contains two hydrophobic transmembrane sequences, Venus flytrap (VFT) domains 1 and 2. These bi-lobed domains are involved in signal perception and enclose a putative ligand-binding cavity. Environmental signal inputs in the periplasmic domain are detected by VFT2. BvgS is kept active by the VFT2 cavity, which is by default always active and in a closed confirmation until bound to antagonists present in modulators. These modulators include magnesium sulphate ($MgSO_4$) and nicotinic acid. The VFT2 is able to bind antagonists because the charge of the carboxylate group in organic negative modulators is complementary to the charge of VFT2. Once bound, antagonists alter the ligand-binding cavity's electrostatic potential and interrupt signalling, switching BvgS from the 'kinase-on' virulent phase to the 'kinase-off' avirulent phase [55,56]. The cavity of the VFT2 is essential for the perception of negative signals and is therefore conserved [2].

If there are no modulators present, the signal is relayed through a transmembrane segment known as the linker region. The linker region joins the periplasmic domain with the cytoplasmic domains and acts as a molecular relay between the two, allowing the signal to be relayed to the cytoplasmically located PAS domain. This sensing function of the linker region has been confirmed through mutational analysis [57].

Similar to the VFT domains, the PAS domain is also involved in signal perception and regulation. There is a conserved Asn residue (Asn_{608}), which forms hydrogen bonds within the PAS core and allows proper docking to the flanking N-terminal α-helix. Additionally, Asp residue (Asp_{695}) couples the PAS core to its C-terminal α-helix downstream. Dupré *et al*. determined that the structural stability of the tightly folded PAS core needs to be maintained in order to ensure proper coupling to its flanking N- and C-terminal α helices and allow conformational signals generated by periplasmic domain to reach the histidine kinase domain (HK) [51].

The HK domain of BvgS autophosphorylates at histidine residue 729 (His-729) and donates a phosphoryl group to aspartic acid (Asp-1023) of the receiver domain (REC) [51].

The REC acts as a "biochemical checkpoint" that mediates the phosphorylation/ dephosphorylation of a histidine phosphotransferase (Hpt) domain and BvgA. When the transfer of the phosphoryl group is reversed by the REC, it re-phosphorylates itself and postpones BvgA activation. Activation of BvgA can also be completely halted if the REC dephosphorylates the BvgS from the receiver itself. This occurs when Asp_{1023} donates a phosphoryl group to the histidine phosphotransferase (Hpt) domain or to water to form inorganic phosphate. The Hpt domain then transfers phosphate back to BvgS. Alternatively the Hpt domain can phosphorylate and activate BvgA [51,53,57].

The Hpt domain is essential for signal transduction and is highly specific for corresponding regulator proteins [58].

BvgA contains an N-terminal REC and C-terminal DNA-binding helix-turn-helix domain (HTH). The evolutionary conserved HTH module contains two α helices, at the N-terminal end and C-terminal end, to regulate gene expression. It facilitates the binding of specific DNA sequences at cis-acting sequences in promoter regions of *bvg*-activated genes. This HTH-DNA binding is stabilized by 20 amino acid residues. As mentioned previously, the phosphoryl group from the Hpt in the BvgS is transferred to aspartate (Asp_{54}) in the receiver domain of the response regulator BvgA. BvgA then becomes phosphorylated (BvgA~P) and transcriptionally activates the expression of virulence activated genes (*vag* loci) and represses the expression of virulence repressed genes (*vrg* loci) by altering their DNA binding affinity for target promoters [59,60].

The intracellular concentration of phosphorylated BvgA is also responsible for mediating the transcriptional activation of the different phenotypic phases of the BvgAS [2].

1.5.2. Phenotypic phases of BvgAS and associated classes of genes

BvgAS controls four classes of genes that are differentially regulated by environmental signals resulting in three distinct phenotypic phases: 1) Bvg$^+$ (virulent), 2) Bvg^i (intermediate), and 3) Bvg$^-$ (avirulent). This is referred to as 'phenotypic modulation' and can be simulated *in vitro* (Table 3).

Table 3. Phenotypic phases of the BvgAS and associated classes of genes.

Phase	Proposed function	Maximally expressed genes	Encoded genes	Minimally expressed genes
Bvg$^+$ (virulent/ active)	Respiratory tract colonisation	Class 1	**Toxins** (*cyaA-E, ptx-ptl*, *bsc*)	Class 3, 4
		Class 2	**Adhesins** (*fhaB, fim, bvgAS*)	
Bvg^i (intermediate/ partially active)	Respiratory transmission	Class 2	**Adhesins** (*fhaB, fim, bvgAS*)	Class 1, 4
		Class 3	*bipA*	
Bvg$^-$ (avirulent/ not active)	Starvation survival	Class 4	*Vrg, frlAB, flaA, bvgR*	Class 1, 2, 3

Adapted from Mattoo *et al.*, Melvin *et al.*, and Veal-Carr and Stibitz, S.) [1,52,61].

BvgAS is fully active under non-modulating conditions and referred to as the Bvg$^+$ phase. It is in this phase that *B. pertussis* is able to cause respiratory infections in animal models. There is maximal expression of class 1 and class 2 genes in the Bvg$^+$ phase [52,61]. Class 2 genes expressed in this phase include *bvgAS*, which is positively autoregulated, and those encoding adhesins such as *fim*, and *fhaB*. Class 1 genes expressed in this phase encode

toxins and include the *ptx-ptl, cyaA-E* and *bsc* operon, which encodes Pertussis toxin (PT), Adenylate cyclase toxin (ACT), and the Type III secretion system, respectively. Promoters of Class 2 and 3 genes contain high affinity BvgA binding sites that are located close to the RNA polymerase binding site and occupied by BvgA~P even at low concentrations. Class 1 genes promoters contain low affinity BvgA binding sites that are occupied only at higher concentrations of BvgA~P. Class 3 and class 4 genes are minimally expressed in the Bvg+ [62].

The cytoplasmic repressor protein BvgR, which is located downstream and adjacent to the BvgAS locus, is also expressed in the Bvg+ phase. It functions as a phosphodiesterase and mediates repression of virulence repressed genes (*vrgs)* by reducing levels of bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP). c-di-GMP contributes to the virulence of pathogens by stimulating the biosynthesis of adhesins and biofilms, therefore functioning as a second messenger in bacteria [63]. BvgR is transcribed from a BvgA-regulated promoter in the opposite direction of BvgS [53,64].

The BvgAS is partially active with maximal expression of class 2 and 3 genes and minimal expression of class 1 and class 4 genes in the Bvg[i] phase or intermediate phase. The Bvg[i] phases occurs following the switch from Bvg- phase conditions to Bvg+ phase conditions or when bacteria are grown in a low concentration of chemical modulators. This phase has been speculated to play a role in transmission and during the early stages of infection [2]. *BipA*, a class 3 gene that encodes an outer membrane protein, is expressed in the Bvg[i] phase, however it is repressed under Bvg+ conditions [52].

To inactivate BvgS, bacteria are grown at low temperatures ≤25°C, or at 37°C in the presence of chemicals such as ≥40 mM magnesium sulphate ($MgSO_4$) or ≥10mM nicotinic acid [1]. When inactivated, BvgA remains unphosphorylated and transcription of *vag* does not occur [52]. BvgAS is inactive and is in the Bvg- phase or avirulent phase, where there is maximal expression of class 4 genes and minimal expression of class 1,2, and 3 genes. The *vrg* loci are class 4 genes expressed in the Bvg- phase, while the *vag* loci, which are class 1 and class 2 genes, are repressed [2,62,65]. *Vrg* expression is activated when levels of c-di-GMP are increased and the response regulator, RisA, is phosphorylated (RisA~P) by the non-co-operonic sensor kinase RisK [63,64].

The Bvg- phase occurs when the growth conditions of *B. pertussis* are altered allowing the bacteria to enter this phase as a potential mechanism of survival as seen in the model organism of *B. bronchiseptica,* where the Bvg- phase can occur under nutrient-limiting conditions both inside and outside of their host [2,65].

The phenotypic phases of BvgAS are illustrated in Figure 3.
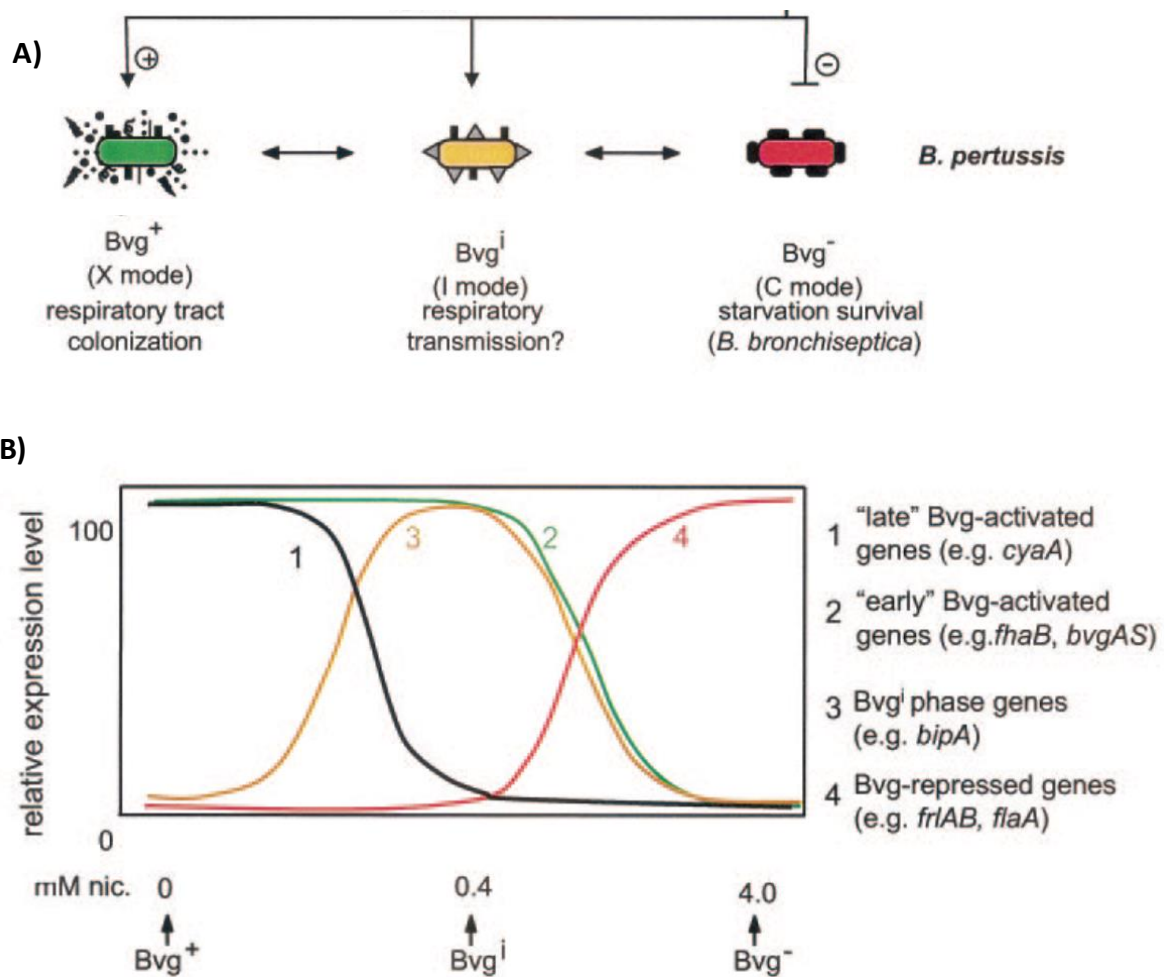
Figure 3. Phenotypic phases of BvgAS.

A) The Bvg+, Bvgi, and Bvg- phase is controlled by the BvgAS in response to environmental conditions. The Bvg+ phase is necessary for respiratory track colonization, while the Bvgi phase is hypothesised to play a role in respiratory transmission. The Bvg- phase is predicted to be important for survival once in the environment.
B) The expression profile for the four classes of genes regulated by the BvgAS are shown. Class one genes are maximally expressed in the Bvg+ phase and are represented by the black curve, labelled as curve 1. These genes are denoted as "late" Bvg-activated genes. Curve 2, or the green curve, represents class 2 genes that are maximally expressed in both Bvg+ and Bvgi phase conditions. These are referred to as "early" Bvg-activated genes. The gold curve of curve 3 represents class 3 genes that are maximally expressed under Bvgi phase conditions. Class 4 genes, represented by the red curve 4, and are maximally expressed under Bvg- phase conditions. The concentration of nicotinic acid (nic) used to modulate these three phenotypic phases are shown below the expression curves [1].

1.5.3. Temporal activation of promoters to regulate gene expression

The BvgAS phosphorelay temporally regulates gene expression through the differential activation of Bvg-activated promoters. Veal-Carr and Stibitz defined the 'Early', 'Late' and middle class of Bvg-activated promoters [61]. Kinnear *et al.* further examined the promoter activation kinetics revealing that after the induction of the Bvg system, transcripts for Bvg-dependent promoters were activated at 10 minutes for early genes (e.g. *fha*), 1 hour for middle genes (e.g. *prn*) and 2-4 hours for late genes (e.g. *ptx*) [66]. Differential Bvg-activated gene expression may provide an advantage for the bacteria to avoid host defenses and

promote the establishment of infections [61]. Smith *et al.* suggested that the difference in transcription at these promoters is due to the specificity of BvgA~P-RNA polymerase interactions such as the different locations of BvgA bindings sites compared to RNA polymerase binding sites [57,62].

1.5.4. Bvg activity in growth cycle

The interaction between the α-subunit of the RNA polymerase and the C-terminus of BvgA has been suggested to be important for the transcriptional activation of virulence-associated genes in *B. pertussis* as previous experiments have proposed that the incorrect positioning of the RNA polymerase relative to BvgA produces a slower growth rate and avirulent mutants. In non-modulating conditions, Bvg$^-$ mutants were found to grow faster and accumulate over generations compared to Bvg$^+$ populations and were more resistant to harsh conditions suggesting a fitness advantage over Bvg$^+$ [67]. The Bvg$^-$ phase occurs when the growth conditions of *B. pertussis* are altered allowing the bacteria to enter this phase as a proposed mechanism of survival [68]. Goffin *et al.* hypothesised that growth rate differences seen between Bvg$^-$ and Bvg$^+$ arose from the differential expression of Bvg regulated virulence factors [68].

The interaction between the α-subunit of the RNA polymerase and BvgA and the apparent fitness advantage between phases strongly suggests an existing relationship between Bvg activity and the growth cycle.

1.6. Protection against pertussis

1.6.1. Immunity

Immunity against *B. pertussis* has been studied using both human and animal models, revealing a complex interplay between humoral and cell-mediated immunity.

Currently the baboon model is preferred because of the baboon's ability to replicate the full spectrum of disease seen in humans including leukocytosis, the paroxysmal cough, and the ability to transmit the disease [69]. Murine models have also been critical in understanding protection against pertussis, despite being unable to replicate all of the clinical symptoms of the human illness.

Using these models have enabled the role of antibodies against factors such as PRN, FIM, FHA and lipopolysaccharide (LPS) to be observed. Response to these antigens have been

shown to contribute to protection collectively, however antibodies on their own do not confer protection to protect against challenge [3].

Cell-mediated immunity, on the other hand, has been shown to provide protection against pertussis enabling bacterial clearance and preventing further dissemination of *B. pertussis.* The LPS of *B. pertussis* interacts with toll-like receptor (TLR)4 activating the innate immunity of mouse and baboon models. In murine models, TLR4 has been found to be play a crucial role in the regulation of interleukin (IL)-10 and Th1 and Th17 cell production in response to *B. pertussis* challenge highlighting the importance of IL-17 against *B. pertussis.* Researchers suspect that IL-17 may be involved in the activation of inflammatory chemokines and cytokines, the recruitment of neutrophils to infection sites, and enhancing the bactericidal activity of macrophages.

Humoral and cell mediated immunity in response to vaccines has been explored in animal models as well [69].

1.6.2. Treatment with antibiotics

Antibiotics are used to treat and manage bacterial infections and have substantially contributed to controlling pertussis globally. Cost, tolerability, safety and effectiveness are some of the topics that need to be considered when selecting an antimicrobial agent.

To treat pertussis, the Centers for Disease Control and Prevention (CDC) guidelines recommends the macrolide agents azithromycin, erythromycin or clarithromycin one to two weeks after the onset of symptoms to lessen their duration and severity, as well as reduce the communicable period [70,71]. Postexposure prophylaxis will also eliminate *B. pertussis* from the nasopharynx of infected persons and can also be administered to prevent secondary infections [70].

Macrolides contain a characteristic macrocyclic lactone ring of 12- 16 atoms bonded to one or more sugars and hydroxyl or alkyl groups [72]. They function to inhibit bacterial cell growth by binding reversibly to the 23S ribosomal RNA (rRNA) in the 50S subunit of the ribosome, blocking protein synthesis [73]. Interestingly, the precise mechanism of these bacteriostatic agents have not been determined, however there are three prominent theories [72]. It has been postulated that they either block elongation, promote the detachment of peptidyl-transfer RNA (tRNA) from ribosomes or prevent the 50S ribosomal subunit from being assembled, all of which result in the inhibition of protein synthesis [72].

The first macrolide of clinical use was erythromycin A, a metabolite of *Streptomyces* spp. comprising of 2 sugar moieties attached to a 14-membered macrocyclic lactone ring.

Erythromycin has been the standard treatment of choice for pertussis for over five decades, where a 14-day course is given to reduce transmission, disease severity and promotes clearance of *B. pertussis* [74]. A key limitation to the use of this antibiotic is the dose-dependent adverse effects affecting the gastrointestinal system [74]. Additional limitations include its short half-life, poor oral bioavailability, and drug-drug interactions.

These limitations have given rise to the semisynthetic derivative clarithromycin and azithromycin, where gastric irritation has been improved [72].

Through the alkylation of the C-6 hydroxyl group of erythromycin, a site prone to degradation by hemiketal metabolites, clarithromycin was produced. Clarithromycin protects against respiratory pathogens with equal or greater activity in comparison to erythromycin. The recommended regimen includes taking two doses per day for 7 days. Like erythromycin, the use of clarithromycin can result in adverse effects such as diarrhea, vomiting, nausea, epigastric distress, hypersensitivity reactions and hepatoxicity [72].

Azithromycin also derives from erythromycin and is an azalide. It forms a 15-membered lactone ring complete with the substitution of a nitrogen in place of a methyl at the C-9a position. A single dose over a five-day course of azithromycin can successfully eradicate *B. pertussis* from the nasopharynx, which is a shorter duration in comparison to erythromycin. This reason for this improved efficacy in clearance could be as a result of a longer serum half-life and increased tissue penetration [74]. It has also been shown to clear carriage in under 48 hours for 88% of participants during the Human Challenge Model [75]. Additionally, azithromycin is more resistant to gastric acid in comparison to clarithromycin and erythromycin resulting in less severe side effects and a greater adherence to the treatment regime, therefore it is the preferred macrolide for persons under 1 month old [74].

Other microbial agents, such as ampicillin, have shown *in vitro* inhibitory action against *B. pertussis,* however the clinical effectiveness has not been illustrated. In a study completed by Bass *et al*., ampicillin was unable to clear *B. pertussis* from the nasopharynx of humans in the paroxysmal state of the disease [76]. This could have been as a result as poor penetration into respiratory secretions [77]. Coupled with high MICs and potentially harmful side effects in children, ampicillin and other β-lactam antibiotics have not been recommended by the CDC for the treatment and prophylaxis of pertussis yet [71].

1.6.2.1. Antibiotic resistance of macrolides in *B. pertussis*

With the advent of antibiotics as therapeutics beginning approximately 70 years ago, antimicrobial resistance mechanisms have co-evolved with these agents resulting in a loss of efficacy against resistant pathogens [78].

Antimicrobial resistance (AMR) is a phenomenon that enables bacteria to resist the toxic action of antibiotics. The bacterium can achieve this using intrinsic, acquired, and adaptive principals. Intrinsic resistance refers to the inherent properties of the microorganism that limit susceptibility to antimicrobials, while acquired resistance refers to microbes that were once susceptible but have become resistant as a result of mutations or the incorporation of new genetic material such as plasmids or integrons. Both principals of resistance can be transmitted vertically to subsequent generations, while adaptive resistance describes a more transient nature of resistance where the bacterium can survive an antibiotic insult by altering genes and protein expression involved in virulence, metabolism, and antibiotic resistance once triggered by an environmental cue [79]. This type of resistance is temporary and once the inducing condition is removed the resistance may also be removed with it [78].

While the macrolides azithromycin and clarithromycin have become the treatment of choice for *B. pertussis* in recent years, erythromycin is still in use globally. *B. pertussis* appears to have acquired resistance to both erythromycin and clarithromycin where erythromycin resistant isolates have been detected in the United States, France, China, Iran and more recently Japan. Clarithromycin and azithromycin resistance have also been detected in Iran and Japan, respectively [80–84]. The resistant *B. pertussis* isolates from the United States, France, China and Japan carried the $A_{2047}G$ mutation in the 23S rRNA gene. This region containing this mutation is critical to erythromycin binding. The resistant isolates from Iran is suspected to have this mutation as well, however it was not determined [80,83–86]. Resistance to quinolones, an alternative to the macrolides inhibiting bacterial DNA replication, has also been found in Japan [87].

1.6.2.2. Ampicillin resistance

Ampicillin is a broad-spectrum β-lactam antibiotic that inhibits bacterial cell wall synthesis. Possible resistance to this antimicrobial agent by *B. pertussis* will be investigated in Chapter 5.

β-lactam antibiotics are among the most used antibiotics worldwide and include the following four different groups of β-lactam antibiotics: penicillin, cephalosporin, monobactam, and carbapenem (Figure 4) [88].

β-**lactams**

Penicillins
Penicillin G (e.g. benzypenicillin)
Penicillin V(e.g. Phenoxymethylpenicillin)
Penicillin M (e.g. Oxacillin, Cloxacilline)
Aminopenicillins (e.g. Amoxicillin)
Caboxypenicillins (e.g. Ticarcillin)
Ureidopenicillins (e.g. Piperacillin)
Aminidopenicillins (e.g. Pivmecillinam)

Cephalosporins
1st generation (e.g. Cefalotin)
2nd generation (e.g. Cefoxitin, Cefuroxime)
3rd generation (e.g. Cefotaxime, Ceftazidime)
4th generation (e.g. Cefepime, Cefpirome)

Monobactam
Aztreonam

Carbapenems
Imipenem
Meropenem
Ertapenem
Doripenem

Figure 4. Chemical structure of four main β-lactams clinically available.

The antibiotics penicillin, cephalosporins, monobactam, carbapenems are represented here [89].

β-lactam antibiotics possess a four-membered β-lactam ring used as a substrate for PBPs [88]. These antibiotics have structural similarity to D-alanyl-D-alanine of the nascent peptidoglycan layer which facilitates their irreversible binding to the Ser403 residue of active PBPs forming an acyl-enzyme intermediate [88]. D-alanyl-D-alanine are the terminal amino acid residues on a pentapeptide subunits attached to N-acetylmuramic acid of the peptidoglycan polymer [89]. When β-lactam antibiotics are present, the pentapeptide lysine residues are sterically blocked from attacking this acyl-enzyme intermediate. This blockage prevents the carbonyl carbon of the acyl-enzyme intermediate from creating covalent bonds between peptides thereby preventing the cross-linking of peptide chains in the peptidoglycan layer [88]. This results in the obstruction of transpeptidation. The inhibition of cross-linkage by β-lactams result in the build-up of peptidoglycan precursors and digestion of existing peptidoglycan by autolytic hydrolases [88,89]. In the absence of β-lactam antibiotics, autolytic cell wall hydrolases are naturally triggered during cell wall synthesis as part of the cocktail of peptidoglycan precursor signals responsible for the reorganisation of the bacterial cell wall. However, in the presence of β-lactam antibiotics, new peptidoglycan is not being produced therefore the structural integrity of the cell wall is diminished until cell lysis and eventual cell death occurs [89–91].

Ampicillin was originally synthesised in 1961 while attempts were being made to extend the antimicrobial activity of penicillin [90]. Ampicillin is an oral penicillin effective against both gram-negative and gram-positive organisms. Structurally, it contains a bulky side chain attached to the 6-aminopenicillanic acid nuclei [92,93]. The chemical structure of ampicillin has been depicted in Figure 5.
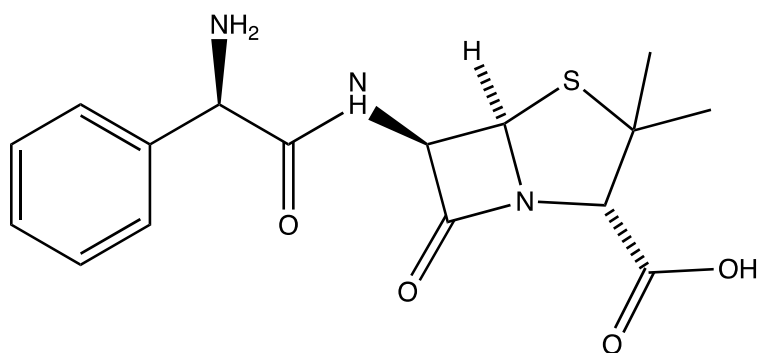
Figure 5. Ampicillin chemical structure containing characteristic β-lactam ring.

Image created with PerkinElmer ChemDraw Professional Version 19.1.1.32 [94].

Although ampicillin is not used to treat *B. pertussis*, ampicillin resistance mechanisms in broth grown cultures will be explored in Chapter 5.

1.6.3. Prevention through the use of vaccines

The treatment of pertussis is generally supportive, and in older populations prophylaxis may be administered, however preventative strategies such as global vaccination programs implemented in developed countries have garnered the most success in controlling the spread of pertussis [95].

Whole-cell vaccinations, introduced in the 1940's, dramatically reduced the incidence of *B. pertussis* by inducing strong protective immunity in neonates. In the 1990s, the switch from whole-cell (WCV) vaccines to acellular (ACV) vaccines was made in order to minimize reactogenic effects of WCV vaccines [16].

WCV vaccines are composed of whole, killed bacteria, while ACV vaccines are comprised of two to five purified *B. pertussis* proteins that induce a strong protection against whooping cough and they are as follows: detoxified Pertussis toxin (PT), Filamentous haemagglutinin (FHA), Pertactin (PRN) and Fimbriae (FIM) (Table 4).

Table 4. Purified B. pertussis proteins contained in ACV vaccines.

Virulence Determinant	Function in Pertussis
Toxin Pertussis toxin (PT)	Exotoxin responsible for lymphocytosis
Autotransporter Pertactin (PRN)	Surface protein responsible for eukaryotic cell binding *in vitro*
Adhesin Filamentous haemagglutinin (FHA) Fimbriae (FIM)	Highly immunogenic surface-associated protein required for tracheal colonisation
	Filamentous cell surface structure required for persistent tracheal colonisation

Adapted from Mattoo *et al.* [1]

Immunity induced by WCVs is considered superior to that induced by ACVs because WCV vaccines induce antibody responses to a wider breadth of antigens as they consist of the whole bacteria compared to limited group of antigens included in ACV.
WCV-induced immune responses are mediated largely by a Th1 cell response in mice, humans and baboons and less so by Th17 in both mice and humans [27]. Th1 cells also confer protection through the clearance of *B. pertussis,* producing a potent proinflammatory response.

ACVs stimulate an antibody response in mice, humans and baboons under the mediation of T helper type (Th) 2 cells induced by IL-4 to prevent adherence of bacteria to respiratory epithelium as well as neutralise toxins. Th17 cells, generated by IL-1 and IL-23, also stimulate an antibody response in the mouse and baboon model, allowing both WCVs and ACVs to induce a robust IgG response to pertussis antigens preventing symptomatic pertussis disease. The Th17 cell response confers protection through the efficient clearance of *B. pertussis* by cells increasing neutrophil and macrophage recruitment to the lungs inducing phagocytosis to kill intracellular bacteria [20].

These distinct mechanisms of immunity in WCV and ACV vaccines in the mouse model are summarized in Figure 6 [20].
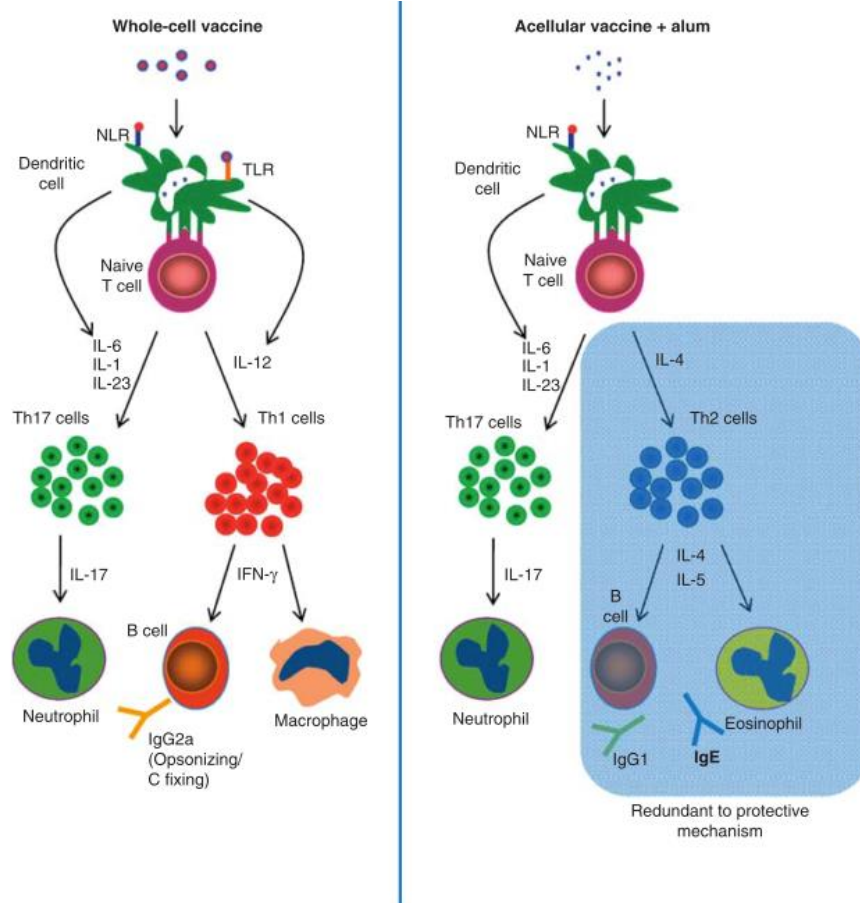
Figure 6. Mechanisms of immunity in response to WCVs and ACVs in the murine model.

WCVs induce Th1 and Th17 cells in response to pro-inflammatory cytokines IL1, IL-6, IL-12, and IL-23 produced by macrophages and dendritic cells. Th1 and Th17 cells secrete interferon (IFN)- ɣ and IL-17, respectively. These cytokines function to kill intracellular bacteria and promote opsonizing antibodies allowing WCVs to confer superior resistance to *B. pertussis* in comparison to ACVs that lack the potent Th1 response. ACVs promote a Th17 and Th2 response, the latter of which prevent adherence of bacteria in the respiratory tract through the promotion of B cell secretion of IgE, IgG1 and IgG3 antibodies. Of note, the Th2 response is not required for the protection of mice from *B. pertussis* infections [20].

The failure of ACV to induce Th1 cells may correlate with the reduced rate of clearance of *Bordetella pertussis* in ACV immunized mice [20]. Therefore, WCV-induced immunity protects against disease, colonisation and transmission, while ACV-induced immunity only protects against disease [27,96]. Some researchers suggest that ACV-induced immunity lasts only for six to ten years [27].

As observed through the baboon model, WCV vaccines confer greater protection against reinfection in comparison to ACV vaccines, natural or convalescent protection [97]. Intriguingly, convalescent baboons were completely protected from reinfection producing an anamnestic response and long-lived antibody responses for up to 7 months. This longevity echoes what is seen in pertussis-infected humans, as the baboons' anti-PT IgG levels could remain elevated for >1-year post infection.

The parallels in T cell response in mice, baboons and humans induced by immunization or infection with *B. pertussis* have allowed us to better understand protective immunity. Despite this, it is clear that, as of yet, there is no single correlate of immunity identified. Specific antibodies are also not sufficient for optimal protective immunity against *B. pertussis*, rather the innate and adaptive immunity system work together to clear *B. pertussis* infections. It can, however, be concluded that Th1 and Th17 cells observed in response to WCV and ACV, respectively, are important to drive the induction of the immunity to *B. pertussis.* Interestingly, Th2 cells, seen only in response to ACV are important for stimulating an antibody response. Collectively, these responses protect against *B. pertussis* infections [20].

In the United States, five doses of the diphtheria, tetanus toxoid and acellular pertussis (DTaP) vaccine are recommended by the CDC at ages 2, 4, 6, 15-18 months, and 4-6 years. While in the UK, maternal vaccination from 16 weeks up to 32 weeks of pregnancy have been recommended by the NHS to maximise the chance of protection at birth, where maternal antibodies are transferred to the fetus prior to birth. After birth, the NHS recommends three doses of the Infanrix-hexa vaccine administered to infants at 8, 12 and 16 weeks. This vaccine is also known as the 6-in-1 vaccine (DTaP/IPV/Hib/HepB) and protects against pertussis, tetanus, diphtheria (DTaP), hepatitis B (HepB), polio (IPV) and haemophilus influenzae type B (Hib) infections.

Vaccination is the most effective way to protect high risk persons and infants from pertussis, therefore vaccination coverage rates need to be maintained among children, adolescents and adults.

1.7. The search for new vaccine candidates: Human Challenge Model

The success of the baboon model of infection has allowed for a greater understanding of *B. pertussis* colonisation, transmission and immunity. In order to explore some of these aspects in humans, a human challenge was conducted by the Periscope Consortium, a group of leading experts in the field of pertussis [28,98]. It is a controlled non-disease colonisation study in humans consisting of two-phases with the goal of identifying biomarkers of protective immunity against colonisation. Healthy vaccinated adult volunteers aged 18-45 years old, who did not have evidence of recent *B. pertussis* exposure, were recruited for phase A. Phase A was a dose-finding stage conducted in June 2017, where volunteers were used to optimise the dosage of live *B. pertussis* cells that would result in the colonisation of approximately 70% of volunteers. They received a nasal inoculation of a low concentration of live *B. pertussis* strain B1917 cells, a strain originally isolated in the Netherlands in 2000

and considered to be representative of currently circulating strains [99]. This strain is sensitive to azithromycin and expresses PRN, PT, and FHA. It is also characterised as *ptxP3-ptxA1-prn2-fim3-2,fim2-1* MLVA27, PFGE BpSR11 [28]*.

Volunteers in this phase were admitted for 17 days and samples such as nasopharyngeal swabs and environmental samples were collected for the identification of *B. pertussis*. The detection methods used will be discussed in greater detail in Chapter 3. At day 14, patients were given azithromycin three days in a row to eradicate *B. pertussis* from the airways. The colonisation period, bacterial shedding, and immunology were assessed during the inpatient stay with follow-up occurring over a 1-year period. This enabled the colonisation period and standard inoculum found in phase A to be used in phase B minimising the duration of infection [28].

In phase B, two groups will be inoculated. The first group will receive the optimised dose for colonisation, while the second group will receive saline or a sham inoculum.

The development of the human challenge model will assist researchers in determining immune signatures that protect against *B. pertussis* colonisation and disease and aid in the development of the next-generation of improved vaccines that might be needed to combat *B. pertussis* resurgence in humans [28,98,100].

1.8. Experimental growth of *B. pertussis*

As a result of this global resurgence, there has been continued interest in creating new correlates of protection to combat pertussis, as well as an interest to better understand the growth dynamics of *B. pertussis in vivo* and *in vitro* amongst strains, which is the scope of this thesis.

B. pertussis a slow-growing bacterium that is fastidious and difficult to grow *in vitro*, however, culture is the gold standard for the diagnosis of pertussis [101]. Despite being highly specific for the detection of *B. pertussis*, it is an insensitive method of detection. Serological and molecular methods are much more sensitive and rapid [102]. This will be discussed in greater detail in Section 1.9.

In a foundational paper published in 1957 by Elizabeth Rowatt, the fastidious nature of *B. pertussis* was highlighted through the many attempts to culture *B. pertussis* on both solid and in liquid media [103].

During *in vitro* growth, issues with reproducibility and low-quality biomass are a constant occurrence. This is due to the fact that *B. pertussis* produces inhibitory substances that prevent their own growth such as the fatty acids oleic or palmitic acid resulting in poor yield

[101,104]. Additional compounds that may affect the growth of *B. pertussis* include sulphur, peroxide, manganese, and peptones; all of which are found in Luria- Bertani (LB) agar [101]. This inhibition has been confirmed to be affected by the lowered activity of AcrABC efflux system in *B. pertussis* in comparison to the highly functional system of *B. bronchiseptica,* which enables growth on LB agar. This was due to two deletions in the gene locus encoding this system, *acrABC*, in *B. pertussis* that affect *in vitro* growth [104].

These inhibitors can be partially overcome through the addition of charcoal, albumin, soluble starch, anion-exchange resins, and blood which act to sequester, neutralise or inactivate inhibitors [101,105].

Two solid media that contain blood and were of early use for the *in vitro* culture of *B. pertussis* include Bordet-Gengou (BG) agar and Regan- Lowe (RL) agar. For the optimal recovery of *B. pertussis,* BG agar containing potato extract with 50% (v/v) blood rabbit or human blood was prepared on the day of culturing. Despite the use of fresh media, BG agar had low selectivity. [101,102]. Regan-Lowe agar is a charcoal agar supplemented with 10% horse blood and 40 µg/ml cephalexin [1]. Both the BG and RL are still in use today, however, charcoal agar has become widely used as a result of its long shelf-life and improved selectivity. It is the solid media of choice for culture of *B. pertussis* in the Preston Lab [106].

Liquid media for the propagation of *B. pertussis* was first described in 1939 by Hornibrook, however many modifications have been made over time to increase the yield of bacteria and to make the medium less complex [103,106,107]. The broth media of choice in the Preston lab is the Stainer-Scholte (SS) broth. Created in 1970, this synthetic medium consists of growth factors, salts, and the amino acids cysteine, proline, and sodium glutamate [107]. Further contributing to the difficulty growing *B. pertussis in vitro*, *B. pertussis* appears to only be able to metabolise amino acids rather than sugars. The amino acids, glutamate and proline in the SS broth are oxidised and utilised as a nitrogen and carbon source by *B. pertussis* during growth [103].

This medium allowed for high biomass of *B. pertussis* to be produced in under 72 hours in shake flasks [107]. To increase yield using SS broth, heptakis (2,6-*O*-Dimethyl)-β-Cyclodextrin (MeβCD) has been utilised in our lab [106,107]. Heptakis has been described as a stimulant for the growth of *B. pertussis* by binding with the hydrophobic acyl residue of the inhibitory fatty acid molecules and forming inclusion complexes around them allowing for normal cell growth [101].

As a result, heptakis can be used to reduce the size of the seed inoculum needed for solid and liquid cultures of *B. pertussis,* where currently very large inoculum are required for seeding cultures [101,106].

Casamino acids, aeration of the medium, and a slightly alkali pH may also facilitate growth [103].

Despite the advances in media preparation, the growth of *B. pertussis in vitro* continues to present challenges. Broth cultures of *B. pertussis* have traditionally been grown in flasks. To increase the throughput of liquid growth assays, 96-microwell plates can be used to culture strains in various growth conditions. However, Kurokawa cautions against the use of microplate assays for slow-growing organisms *in vitro*. The use of the microplate for *B. pertussis* growth assays will be discussed in greater detail in Chapter 6 [108].

1.9. Diagnosis

The laboratory diagnosis of *B. pertussis* has existed for over 100 years. Despite this, diagnostic methods have been plagued with sensitivity and specificity issues, poor specimen collection technique, laboratory medium issues, and confusion interpreting serologic results. Mentioned previously, culture is the gold standard, however serology and polymerase chain reaction (PCR) have been employed to increase confidence in diagnosis [1].

1.9.1. Culture

Culture has traditionally been used as the primary detection method in clinical laboratories, specifically the culture of samples taken from a surface of the upper respiratory tract lined with ciliated epithelium such as nasopharyngeal swabs or other nasopharyngeal samples [5]. Calcium alginate or Dacron swabs are acceptable for nasopharyngeal swabs. Regan-Lowe agar or Ames medium is the appropriate transport medium; however, the latter can only be used with the addition of charcoal and if collection to culture is less than 24 hours. Mentioned previously, Bordet-Gengou, Regan-Lowe and charcoal only agar are commonly used for culture. Although culture is the gold standard for the detection of *Bordetella* spp., sensitively is very low at 2-60%, and visible growth on laboratory agar can take from 5-10 days [109]. It is recommended that samples are taken from patients at earlier stages in the disease to increase detection [8].

1.9.2. Serology

ELISA techniques include measuring increasing antibodies titres to specific *B. pertussis* antigens such as PT, FHA, PRN, and FIM in serum. However, FHA and PRN antibodies are not specific to *B. pertussis* and can be found following other *Bordetella* infections [1]. Additionally, increased concentrations of the FHA antibody can cross-react to epitopes from

other bacteria such as non-encapsulated *C. pneumoniae, H. influenzae*, and *M. pneumonia* mimicking a *B. pertussis* infection resulting in false-positives [1,8].

The immunoglobulins IgG, IgA, and IgM increase in serum after a natural infection with *B. pertussis,* while immunisation in children induce IgG and IgM antibodies [1].

IgG and IgA antibodies to PT measured using ELISA has shown the greatest sensitivity and specificity and indicate an infection in adolescents and adults. Of the two type types of immunoglobulins, IgA antibodies to PT indicate a recent infection. However, younger children develop a less consistent IgA antibody response. Interestingly, infants under 3 months old may fail to develop antibodies at measurable concentrations, despite being culture positive. IgG responses are the most consistent antibody response [1].

A major concern to the use of serology for detecting *B. pertussis* infections is a delay in receiving the appropriate acute-phase specimen. If the specimen is not taken from patients in the acute-phase, increases in antibody titre may have already occurred and become missed [1]. Additionally, the diagnostic cut off values dictate the sensitivity and specificity of the assay, however if an antibody value is near the cut-off values, this patient may be misdiagnosed [8]. If the sample is taken at the correct time during the infection, this method of detection has been suggested to be superior to both culture and PCR [8].

1.9.3. Polymerase chain reaction (PCR)

A much more rapid method in comparison to culture includes PCR, which is suitable for the early stages of infection and is highly sensitive. Due to the increased sensitivity of this method, it has been suggested to be preferential to detect *B. pertussis* in adults with a chronic cough [8].

Sample collection and preparation, primer or target selection and amplification conditions and detection are key factors involved in the diagnosis of *B. pertussis* using PCR [1]. There are many inhibitory factors present in the fibre of swabs, therefore only Dracon swabs are suitable for collection of PCR specimens [1].

Specific target regions for *B. pertussis* have been defined and are in use in clinical and academic laboratories. These targets include repeat elements and insertion sequences (IS). Target regions will be discussed in greater detail in the Section 1.9.4.1 [110].

PCR measures the accumulated PCR product at the end of each cycle and can be used to indicate the presence or absence of the target gene, however it does not discern between live and dead *B. pertussis* cells. Additionally, traditional PCR is not truly quantitative and is more so semi-quantitative if band intensities on a gel are compared to standards with known concentrations. To overcome both concerns, qPCR can be employed [1].

1.9.4. Real-time PCR detection (qPCR)

Real-time, or quantitative PCR detection (qPCR) is also a rapid and highly sensitive technique, however it is quantitative. It can be used to measure PCR amplification as it occurs allowing results to be obtained within hours. However, like traditional PCR, it is unable to discriminate between live and dead cells. To overcome this problem, the culture-independent qPCR can be combined with the intercalating photoactive dye propidium monoazide (PMA), which will be discussed in Chapter 3 [111].

1.9.4.1. (q)PCR target genes

Multiple targets that have been used for the clinical diagnosis of pertussis include *cyaA*, *prn*, *por*, *recA*, and *ptx*-Pr. It is for this reason that the standardisation of both PCR and qPCR across clinical laboratories for the detection *B. pertussis* has been a challenge. Despite this, the target in routine use is the insertion sequence *IS481* [109].

This multi-copy insertion sequence is a highly sensitive target with approximately 250 copies in the *B. pertussis* genome [112]. However, it is not a specific target due to the cross-reactivity of *IS481* with are other species of *Bordetella* that infect humans, namely *B. bronchiseptica* and *B. holmesii* [109]. Therefore, utilising *IS481* alone to detect *B. pertussis* in clinical samples has resulted in the false positive diagnosis of pertussis.

Multiple target sequences have been used in clinical laboratories to discern from other *Bordetella* species and increase the specificity with the qPCR assay. These targets include *B. pertussis* specific targets *BP0283,* which encodes for a putative thiolase protein and *BP0485,* which encodes a probable acylphosphatase (pseudogene). Both of these genes are found as a single copy on the *B. pertussis* genome and show sequence divergence from *B. bronchiseptica* and *B. parapertussis* [113].

Furthermore, *IS1001* and BH*recA* present in *B. parapertussis* and *B. holmesii*, respectively can be used to rule out coinfections or symptoms caused by another *Bordetella* spp. *IS1001* can also be found in *B. bronchiseptica.* Both of these targets are not present in *B. pertussis* [114–117].

The determination of using a single target or multiple targets in a multi-plex PCR will dictate the sensitivity and specificity of the qPCR assay. In addition, amplifying longer DNA sequences can also increase the probability of at least one dye binding event occurring in the target amplicon [111,118,119].

The use of qPCR to enumerate live *B. pertussis* during the Human Challenge Model will be explored in Chapter 3.

1.10.   Aims and Objectives of PhD

B. pertussis is a highly fastidious and slow-growing organism that is the causative agent of pertussis. Despite many years of research and global vaccination programs beginning in the 1940s, cases of pertussis have increased in recent years in numerous countries. The reason for the resurgence is not known, however it has created a need to better understand the growth of *B. pertussis*. *B. pertussis'* growth was observed under three conditions:

     a. *In vivo* during colonisation
     b. *In vitro* during phenotypic modulation
     c. In response to the antibiotic ampicillin

Therefore, this thesis will be divided in three parts to investigate the growth dynamics and variability of *B. pertussis* in six different strains, BP536, B1917, UK48, UK71, B2973 (B184), and B1878 (B204). These strains represent geographically distinct strains sampled from different time periods over a span of greater than 50 years and were used to ensure that any observations made using one strain were applicable to the *B. pertussis* population in general.

In part one, there was a requirement to monitor the *in vivo* colonisation of healthy adult subjects inoculated with *B. pertussis* BP1917 during the human challenge model in real-time. The development of this model was designed to allow researchers to validate animal model findings, observe the early stages of infection, and identify immune responses to infection and vaccination in an effort to explore correlates of immunity in humans [120]. The rapid enumeration will ensure the safety of participants and allow for treatment prior to the onset of symptoms. Therefore, the aim of this part of the research project is to:

1. Design and optimise a qPCR-based molecular assay that will distinguish between live and dead cells in real-time
2. Enumerate live and dead *B. pertussis* from human challenge model samples

Additional aims for part one include:

3. Assess the utility of this assay for additional strains of *B. pertussis* to enumerate live and dead cells in suspensions
4. Extend the use of this assay to measure the viability of *B. pertussis* during *in vitro* growth
5. Investigate the total number, live, and dead cells taken up by macrophages during an infection

In part two, the BvgAS Two-component system of *B. pertussis* during *in vitro* growth will be investigated. This system is responsible for the activation of nearly all the virulence factors in

B. pertussis and is made up of a sensor kinase (BvgS) and a response regulator (BvgA). Virulence activated genes (*vags*) are expressed when BvgS is active and BvgA is phosphorylated. This is also referred to as the Bvg$^+$ phase. Virulence-repressed genes (*vrgs*) are induced in the Bvg$^-$ phase when BvgS is inactive and BvgA is not phosphorylated [121]. Entry into these Bvg phases is modulated by changing growth conditions and mutations amongst the BvgS have been shown to affect sensitivity to modulation [2]. To validate these findings, the aims of this part of the project are therefore to:

1. Create reporter constructs specific for each Bvg phase
2. Determine the sensitivity to modulation amongst different strains
3. Investigate mutations in the BvgAS of *B. pertussis* strains and assess the functional implications of these mutations'

Finally, part three will investigate an earlier finding in the Preston Lab where ampicillin resistance was observed in broth grown cultures in comparison to ampicillin sensitive plate cultures. Ampicillin is a β-lactam antibiotic that inhibits the growth of *B. pertussis* by preventing cell wall synthesis. It is not currently employed to treat *B. pertussis* infections, however, to better understand antibiotic resistance mechanisms of *B. pertussis,* the aims of this section of the project are to:

1. Confirm the ampicillin resistance found previously in liquid broth cultures
2. Evaluate the ampicillin resistance of additional strains in both plate and broth cultures
3. Investigate the mechanisms of ampicillin resistance using LC/MS and gene knock-outs to study the enzymatic degradation of ampicillin, and gene expression analyses to determine changes in the cell wall

Taken together, the variability of *B. pertussis* growth will be observed utilising a variety of tools *in vivo* and *in vitro* in order to illustrate the dynamic nature of *B. pertussis* growth amongst strains. The work will confirm the importance of studying the growth of *B. pertussis* in order to better understand how to protect ourselves against it. This information supports future work to improve the course of treatment and aid in uncovering new methods of prevention, potentially saving lives.

1.11.   Outline of presented research

The main aims of this thesis have been detailed in Section 1.10, where a qPCR assay designed to enumerate live and dead *B. pertussis* during the human challenge model will be described in Chapter 3, reporter constructs created to determine sensitivity to modulation in different strains of *B. pertussis* in Chapter 4, and mechanisms of ampicillin resistance detailed in Chapter 5. Chapter 6 consolidates and discusses the findings presented in the previous chapters and reflects on the significance of this work for the future.

Chapter 2
Methods and Materials
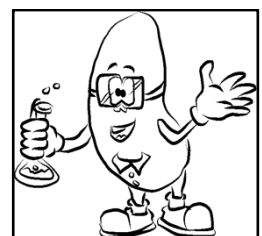
2. Methods and Materials

2.1. Bacterial strains

The six different strains of *B. pertussis* used in all chapters of this thesis include *B. pertussis* strain B1917, which is a wild-type strain considered representative of currently circulating *B. pertussis* and was the strain used for the Human Challenge Model described in Section 1.7 [99]. *B. pertussis* strain B204 (B1878) and B184 (B2973) were both derived from the Netherlands and can be located with the following NCBI Genbank accession numbers: NZ_CSNV00000000 and NZ_CSRZ00000000, respectively [99]. Both B1917 and B1878 were discovered in the Netherlands in the year 2000, while B2973 was collected in 1988 [99].

B. pertussis strains UK48 and UK71 were discovered in the UK in 2012 from Kingston and Cardiff, respectively [122]. Both strains can be identified with European Nucleotide Archive Accession numbers: ERS176875 and ERS227772, respectively [122]. UK48 contains a duplicated region of motility related-genes in its genome, while UK71 was similar to UK48 differing only by 6 SNPs within its core genome, but did not carry this duplication [123,124]. Both strains are part of the PERISCOPE strain panel [125].

Finally, the streptomycin-resistant *B. pertussis* strain BP536 was derived from Tohama 1, a strain of *B. pertussis* isolated in the 1950s from Japan [126].

The extended spectrum β-lactamase *Proteus mirabilis* (JN40 PM), used as a positive control for β-lactamase production, contained *ampR* in the chromosome.

Escherichia coli Nissle pUC19 EC, was also used as a positive control for β-lactamase production and contained a plasmid with ampicillin resistance.

The NEB® High efficiency 5α competent *E. coli* strain (New England Biolabs, Hitchin, UK) was used for vector transformations and the *E. coli* strain ST18 was used as a donor strain for conjugations [127]. *E. coli* ST18 contains a *hemA* deletion preventing tetrapyrrole biosynthesis. Through the use of 5-aminolevulinic aid (5ALA) in the media, this mutation can be complemented allowing counterselection of *E. coli* ST18 strains containing the promoter-probe insert on the plasmid pBBR1KANGW [127]. Coupled with kanamycin, colonies containing this vector can be further selected and conjugated into *B. pertussis* strains [127].

2.2. Culture conditions

E. coli was cultured in both Lennox Luria- Bertani (LB) agar and broth (Sigma, Gillingham, UK) for 18 hours at 37°C. Both *P. mirabilis* (JN40 PM) and *E. coli* Nissle pUC19 EC were grown in LB medium with ampicillin (Table 7).

B. pertussis strains were cultured on charcoal agar (Thermo Fisher Scientific, Oxoid™, Basingstoke, UK) at 37°C for 3 days for routine culture.

Liquid cultures of *B. pertussis* were grown in Stainer-Scholte (SS) broth (Table 5).

Table 5. Stainer-Scholte broth recipe (autoclave portion)

FW	Concentration (mM)	Component	1 L
187.7	**57**	**L-glutamic Acid (Na salt)**	10.70
115.1	**2.1**	**L-proline**	0.24
58.44	**43**	**NaCl**	2.5
136.1	**31**	**KH_2PO_4**	0.5
74.56	**2.7**	**KCl**	0.2
203.3	**0.49**	**$MgCl_2.6H_2O$**	0.1
147.0	**0.18**	**$CaCl_2.2H_2O$**	0.0265
121.14	**50**	**Tris base**	6.1

These components were dissolved in 90% of final volume and the pH adjusted to 7.6 with NaOH or HCl prior to being made up to the final volume.

10 ml of *100 x supplements* were added per 1 L broth, just before use and made with the recipe found in Table 6.

Table 6. Stainer- Scholte broth recipe (supplements 100x)

Temperature (°C)	FW	Concentration in 1x	Component	10 ml
4	121.2	0.33 mM	**L-Cysteine**	0.04
Room Temperature	278.0	36 μM	**$FeSO_4.7H_2O$**	0.01
Room Temperature	123.1	33 μM	**Niacin**	0.004
4	307.3	0.49 mM	**Glutathione**	0.15
Room Temperature	176.1	2.3 mM	**Ascorbic acid**	0.40

Supplements were made up to the final volume with H_2O, filter-sterilised and stored at -20°C until ready to use.

Overnight cultures of *B. pertussis* were made using plate grown cultures resuspended in SS broth with supplement at a starting OD_{600} of 0.15. 20ml cultures were grown in 100ml flasks and incubated for 16-20 hours at 37°C with shaking. Kanamycin was added for reporter

strains grown overnight and ampicillin was added to cultures for RNA isolation and Reverse Phase Chromatography/ Mass Spectrometry.

The Bvg$^+$, Bvg^i, Bvg$^-$ reporter constructs were cultured on charcoal agar for 2 days at 37°C or in SS broth as overnight cultures using different concentrations of MgSO$_4$ described in Chapter 4. Both plate and broth grown cultures were grown with kanamycin.

The concentrations of antibiotics used for all experiments can be found in Table 7.

Table 7. Antibiotics and concentrations used for all experiments

Antibiotic	Concentration Used (µg/ml)	Experiment
Kanamycin	50	Reporter Constructs (Chapter 4)
Ampicillin	100 (*E. coli*)	Strains containing YFP and RFP on a plasmid (Chapter 4)
	100 (*P. mirabilis* (JN40 PM) *and E. coli* Nissle pUC19 EC - plate culture)	RPC/MS (Chapter 5)
	10 (*P. mirabilis* (JN40 PM), *E. coli* Nissle pUC19 EC, *B. pertussis*-overnights)	
	2.5, 5, 10, 20, 100 (*B. pertussis*)	Amp MIC (Chapter 5)
Gentamycin	5 (*E. coli*), 30 (*B. pertussis*)	Knock-out strains (Chapter 5)
Polymyxin B	100 (macrophages)	Macrophage infection (Chapter 3)

2.3. Primers and vectors

Table 8 represents the primers used in this thesis. *BsaI* restriction recognition sites and cloning-specific overhangs were introduced for use in Golden Gate cloning. *BsaI* restriction sites are indicated in bold within Table 8.

Table 8. Primers

PMA-qPCR assay (Chapter 3) [113]			
Primer	**Sequence**	**Product**	**Additional Details**
IS481F	ATCAAGCACCGCTTTACCC	*IS481* left region of gene	
IS481R	TTGGGAGTTCTGGTAGGTGTG	*IS481* right region of gene	
IS481_FAM_B HQ1	AATGGCAAGGCCGAACGCTTCA	IS481 Probe	Labelled with FAM and Black Hole Quencher
Construction of BvgAS Reporter Strains (Chapter 4)			
Primer	**Sequence***	**Product**	**Reporter protein**
GFPGGF	AAAA**GGTCTC**CATGAGTAAAGGAG AAGAAC	short half-life *gfp* right region of gene	Short half-life GFP: *gfp* [AAV]
GFPGGR	AAAA**GGTCTC**GAACTTTAAACTGCT GCAGCGTAG	short half-life *gfp* left region of gene	

GFPGGR_LONG	AAAA**GGTCTC**GAACTCTATTTGTATAGTTCATCCATGC	regular half-life *gfp* left region of gene	Regular half-life GFP
YFPGGF	AAAA**GGTCTC**TATGGATTCAATAGAAAAGG	regular half-life *yfp* right region of gene	Regular half-life YFP: pYFP_Star
YFPGGR_NEW	AAAA**GGTCTC**GAACTTTACTTGTACAGCTCGTCC	regular half-life *yfp* left region of gene	
mKateGGF	AAAA**GGTCTC**CGACTTTTACCGCGGGCTTT	regular half-life *rfp* right region of gene	Regular half-life RFP: pBS1C mkate2
mkateGGR	AAAA**GGTCTC**GAACTGGTAGCGACCGGCGCTCACAT	regular half-life *rfp* left region of gene	
Bvg plus (+)			
BP2924Prom_GGF	AAAA**GGTCTC**TCGAGAGGTCCGAGATTATAAACAGC	*BP2924* right region of promoter	Short half-life GFP
BP2924Prom_GGR	AAAA**GGTCTC**CTCATGAATGAACTCCGTAAAG	*BP2924* left region of promoter	
PrnPromGGF	AAAA**GGTCTC**TCGAGAACAGGCACCCTGGCCTGC	*BP1054 (prn)* right region of promoter	
PrnPromGGR	AAAA**GGTCTC**CTCATTGGATGCCAGGTGGAGAG	*BP1054 (prn)* left region of promoter	
BP1879GGF	AAAA**GGTCTC**TCGAGAATTCCGACCAGCGAAGTG	*BP1879 (fhaB)* right region of promoter	
BP1879GGR	AAAA**GGTCTC**CTCATAGATAAGAAGAATATGCTT	*BP1879 (fhaB)* left region of promoter	
BP1119GGF	AAAA**GGTCTC**TCGAGACGCTGATGCGCCGGCCCG	*BP1119 (fim2)* right region of promoter	
BP1119GGR	AAAA**GGTCTC**CTCATAGGGCGTCTTGGCGCCAGA	*BP1119 (fim2)* left region of promoter	
PTPromGGF	AAAA**GGTCTC**TCGAGACAGCGCAGCCCTCCAACGC	*BP3783 (ptxA)* right region of promoter	Regular and short half-life GFP
PTPromGGR	AAAA**GGTCTC**CTCATCCCGTCTTCCCCTCTGCG	*BP3783 (ptxA)* left region of promoter	
BP2936GGF	AAAA**GGTCTC**TCGAGAGCGGCCATCCCCGCGACG	*BP2936* right region of promoter	
BP2936GGR	AAAA**GGTCTC**CTCATATTGCTCCGCGAAAAGTGG	*BP2936* left region of promoter	
YFP_BP2936GGR	AAAA**GGTCTC**CCCATTTGCTCCGCGAAAAGTGG	*BP2936* left region of promoter	Regular half-life YFP
Bvg intermediate (i)			
BP1879GGF	AAAA**GGTCTC**TCGAGAATTCCGACCAGCGAAGTG	*BP1879 (fhaB)* left region of promoter	Short half-life GFP
BP1879GGR	AAAA**GGTCTC**CTCATAGATAAGAAGAATATGCTT	*BP1879 (fhaB)* right region of promoter	
BP0682PromGGF	AAAA**GGTCTC**TCGAGACGCATCCGCCTTCGCCTG	*BP0682* left region of promoter	
BP0682PromGGR	AAAA**GGTCTC**CTCATCTGTATTCCCCTTGTGGCC	*BP0682* right region of promoter	
mkateBP0682GGF	AAAA**GGTCTC**TCGAGCATCTTTCCGCCGCTGCC	*BP0682* left region of promoter	regular half-life RFP
mkateBP0682GGR	AAAA**GGTCTC**CAGTCGACTTTGGCTAGAAAACTACGG	*BP0682* right region of promoter	
mkate1879GGF	AAAA**GGTCTC**TCGAGGGCGAATCTCAGTACAGGGT	*BP1879 (fhaB)* left region of promoter	
mkate1879GGR	AAAA**GGTCTC**CAGTCAAACCTCAGCACTTCGTCAG	*BP1879 (fhaB)* right region of promoter	
mkateBipAGGF	AAAA**GGTCTC**TCGAGCTATCCCGGCCTGTTCGAC	*BP1112 (bipA)* left region of promoter	
mkateBipAGGR	AAAA**GGTCTC**CAGTCCCAATGACCAGACAACACGG	*BP1112 (bipA)* right region of promoter	
Bvg minus (-)			

KpsMPromGGF	AAAA**GGTCTC**TCGAGAGGCGGCCTTGCAAGTGTG	*BP1623 (kpsM)* left region of promoter	Short half-life GFP
KpsMPromGGR	AAAA**GGTCTC**CTCATACCTGGGCTCCCGATGCC	*BP1623 (kpsM)* right region of promoter	
BP1620GGF	AAAA**GGTCTC**TCGAGAGGCGGCCTTGCAAGTGTG	*BP1620* left region of promoter	
BP1620GGR	AAAA**GGTCTC**CTCATAACCTGGGCTCCCGATGC	*BP1620* right region of promoter	
BP2782GGF	AAAA**GGTCTC**TCGAGAAGACGCCTTGCAGGCTGT	*BP2782* left region of promoter	
BP2782GGR	AAAA**GGTCTC**CTCATAGGGCAATCTCCAAAGTGA	*BP2782* right region of promoter	
BP3517PromGGF	AAAA**GGTCTC**TCGAGAGACGGGGTTCCTTCCAGATG	*BP3517* left region of promoter	
BP3517PromGGR	AAAA**GGTCTC**CTCATAGCGAATCTCCCACTTGG	*BP3517* right region of promoter	
YFP_VrgxGGF	AAAA**GGTCTC**TCGAGCCAGGCTGAGCGACGAAG	*vrgX* left region of promoter	Regular half-life YFP
YFP_VrgxGGR	AAAA**GGTCTC**CCCATGGTCGACACTCCTGTTGATGA	*vrgX* right region of promoter	
YFP_BP2782GGR	AAAA**GGTCTC**CCCATGGGCAATCTCCAAAGTGA	*BP2782* right region of promoter	
YFP_KpsMGGF	AAAA**GGTCTC**TCGAGACTTGTGTAAGGCCAGGTCAG	*BP1623 (kpsM)* left region of promoter	
YFP_KpsMGGR	AAAA**GGTCTC**CCCATACCTGGGCTCCCGATGCCTGC	*BP1623 (kpsM)* right region of promoter	
YFP_KpsMGGR_New2	AAAA**GGTCTC**CCCATTATCAAAGACCGATCACCCG	*BP1623 (kpsM)* larger intergenic right region of promoter	
YFP_flaAGGF	AAAA**GGTCTC**TCGAGCAAGGCAAGTTTAGGGGCG	*BP0996 (flaA)* left region of promoter	
YFP_flaAGGR	AAAA**GGTCTC**CCCATTAGAAACGGGGCAGGGACAT	*BP0996 (flaA)* right region of promoter	
YFP_flaAGGF_S	AAAA**GGTCTC**TCGAGTACCAGAACTCCCAACACCG	*BP0996 (flaA_S)* shorter intergenic left region of promoter	
YFP_flaAGGR_S	AAAA**GGTCTC**CCCATGCAACCAGCGACAAGTAGTT	*BP0996 (flaA_S)* shorter intergenic right region of promoter	
Constitutively Expressed (+, i, -)			
BP0840GGF	AAAA**GGTCTC**TCGAGACCGGCCGGGCTCCTTGAG	*BP0840* left region of promoter	Short half-life GFP
BP0840GGR	AAAA**GGTCTC**CTCATATTAAGAAATCTCCGTTGA	*BP0840* right region of promoter	Regular half-life GFP
YFP_BP0840GGR	AAAA**GGTCTC**ACCATTTAAGAAATCTCCGTTGA	*BP0840* right region of promoter	Regular half-life YFP
Ampicillin Resistance Mechanisms (Chapter 5)			
Penicillin binding protein Expression Analysis using qPCR			
Primer	**Sequence***	**Product**	
BP0102RT_F	AACAAGATCACCCAGCCCAA	*BP0102* left region	
BP0102 RT_R	CGTACAGCTTGACCGTATCG	*BP0102* right region	
BP0905RT_F	GGCGAGTACTACTTCAGCGA	*BP0905* left region	
BP0905RT_R	AATCGGTGGAATGGGAGGTT	*BP0905* right region	
BP1051RT_F	CTGGTGGTGCAGGAGATCAA	*BP1051* left region	
BP1051RT_R	GCTCGGTCAGGAATTCGGT	*BP1051* right region	
BP1545RT_F	CAAGAACGAAAACGTGGTGC	*BP1545* left region	

BP1545RT_R	GGTTTTCCGAGGACATCAGC	*BP1545* right region	
BP2754RT_F	CCATTCGGAAAGCCTGCTG	*BP2745* left region	
BP2754RT_R	GCCCAAAATCAAGCTGAGGT	*BP2745* right region	
BP3028RT_F	GTGGTGATCGACGTGCATAC	*BP3028* left region	
BP3028RT_R	CTGCCCTGGTATTGAAAGCG	*BP3028* right region	
BP3655RT_F	AGCTCACCAAGGACCAGATC	*BP3655* left region	
BP3655RT_R	GTAGCCCAGCGAATACATGC	*BP3655* right region	
BP1061RT_F	CATCGTGACAGTGAGCATGG	*BP1061* left region	
BP1061RT_R	GAGGAATCCGGTCGCATTG	*BP1061* right region	
BP3214RT_F	AAAGCTGTTCGAGATGCAGG	*BP3214* left region	
BP3214RT_R	AATACGGGGTGTTCTCGACC	*BP3214* right region	
BP3268RT_F	ACTTCTCTGTCTGTCCCTGC	*BP3268* left region	
BP3268RT_R	GTCGTCGCTCTCCCAGTT	*BP3268* right region	
Knockout of putative β-lactamase genes			
Primer	**Sequence***	**Product**	
BP3130GGF_ FLANKA	AAAA**GGTCTC**TCGAGCTGGGCATC GAACCGTTC	*BP3130* left flanking region	
BP3130GGR_ FLANKA	AAAA**GGTCTC**CATGTGGTGGGCTC CTTCCACTTAT	*BP3130* left flanking region	
BP3130GGF_ FLANKB	AAAA**GGTCTC**CACATGTGGGAGAC ATCGACTACCA	*BP3130* right flanking region	
BP3130GGR_ FLANKB	AAAA**GGTCTC**GAACTTTTCCAGGT CCAGCAGCTT	*BP3130* right flanking region	
BP0442GGF_ FLANKA	AAAA**GGTCTC**TCGAGGCACTTACA CCTTTAGCGGG	*BP0442* left flanking region	
BP0442GGR_ FLANKA	AAAA**GGTCTC**CATGTTGCCTCTCC CTGTATTGGCT	*BP0442* left flanking region	
BP0442GGF_ FLANKB	AAAA**GGTCTC**CACATCGCTGTATTT CGAAGGCAA	*BP0442* right flanking region	
BP0442GGR_ FLANKB	AAAA**GGTCTC**GAACTTTCACCGAC ATCCACCCC	*BP0442* right flanking region	
3130_flanking_ cpcr_F	CGTATTTCTGTCCGAGCTGC	*BP3130* left flanking region	
3130_flanking_ cpcr_R	CCCACCACGATCACACAGT	*BP3130* right flanking region	
0442_flanking_ cpcr_F	TCCAGGTAGACATGAGCGTT	*BP0442* left flanking region	
0442_flanking_ cpcr_R	ACCCATCTCCAGACTCAACC	*BP0442* right flanking region	

Table 9 represents the vectors used. pCR^{TM}8/GW (Invitrogen, California, USA) was modified to include the Golden Gate insert resulting in the vector pCR8GW:GG [128–130]. pBBR1KANGW was derived from the pBBR1 plasmid isolated from *B. bronchiseptica* S87 [131]. The Gateway^{TM} Vector Conversion System (Invitrogen, Loughborough, UK) was used to convert this vector into a Gateway® destination vector through the ligation of the Gateway (GW) cassette into the MCS of pBBR1KAN [132].

The suicide vector pSS4940GW was provided by Scott Stibitz. It was modified for use in Golden Gate cloning by the addition of a Golden Gate cloning cassette.

pPROBE-NT and pPROBE-gfp [AAV] contained regular and short half-life *gfp* genes, respectively, and were therefore used to amplify the promoterless *gfp* genes during the construction of reporter strains [133].

Promoterless *yfp* and *rfp* were amplified from the pYFP_Star plasmid and pBS1C-mRFP vector, respectively [134,135].

Table 9. Vectors

Vector	Selection	Source
pCR8GW:GG	Spectinomycin	Preston lab stocks
pBBR1KANGW	Kanamycin	Preston lab stocks
pSS4940GW	Gentamycin	Gift from S. Stibitz, USDA
pPROBE-NT	Kanamycin	Preston lab stocks
pYFP_Star	Ampicillin	Gifted from the Denham Lab
pBS1C-mRFP	Ampicillin	Gifted from the Denham Lab

Reporter constructs conjugated into the six different strains of *B. pertussis* contained a *B. pertussis* promoter and promoterless reporter gene on a pBBR1KAN plasmid. These constructs can be found in Table 10.

Table 10. Reporter strains

Bvg plus (Bvg⁺)			
Gene	**Reporter Protein**	**Nomenclature**	**Comments**
2924	Short half-life GFP	*2924GFP_S*pBBRK	
1054 (prn)		*prnGFP_S*pBBRK	
2936		*2936GFP_S*pBBRK	
1879 (fhaB)		*1879GFP_S*pBBRK	
3783 (ptxA)		*ptxAGFP_S*pBBRK	
1119 (fim2)		*1119GFP_S*pBBRK	
2936	Regular half-life GFP	*2936GFP*pBBRK	
3783 (ptxA)		*ptxAGFP*pBBRK	
2924	Regular half-life YFP	*2924YFP*pBBRK	
2936		*2936YFP*pBBRK	
3783 (ptxA)		*ptxAYFP*pBBRK	
3783 (ptxA)	Regular half-life RFP	*ptxARFP*pBBRK	
Bvg intermediate (Bvg⁻)			
Gene	**Reporter Protein**	**Nomenclature**	**Comments**
1879 (fhaB)	Short half-life GFP	*1879GFP_S*pBBRK	
1112 (bipA)		*bipAGFP_S*pBBRK	
1878 (bvgA)		*1878GFP_S*pBBRK	
0682		*0682GFP_S*pBBRK	
0682	Regular half-life RFP	*0682RFP*pBBRK	
1879		*1879RFP*pBBRK	
bipA		b*ipARFP*pBBRK	
0682	Regular half-life YFP	*0682YFP*pBBRK	

Bvg minus (Bvg⁻)			
Gene	**Reporter Protein**	**Nomenclature**	**Comments**
1623 (kpsM)	Short half-life GFP	*kpsMGFP_Sp*BBRK	
1620		*1620GFP_Sp*BBRK	
2782		*2782GFP_Sp*BBRK	
3517		*3517GFP_Sp*BBRK	
0996 (flaA)		*flaAGFP_Sp*BBRK	
vrgX		*vrgXGFP_Sp*BBRK	
vrgX	Regular half-life YFP	*vrgXYFP*pBBRK	
2782		*2782YFP*pBBRK	
1623 (kpsM)		*kpsMYFP*pBBRK	
1623 (kpsM_2)		*kpsM_2YFP*pBBRK	Larger intergenic region for *kpsM* promoter
0996 (flaA)		*flaAYFP*pBBRK	
0996 (flaA_S)		*flaA_SYFP*pBBRK	Shorter intergenic region for *0996* promoter
3517		*3517YFP*pBBRK	
3783 (ptxA)		*ptxAYFP*pBBRK	
1054 (prn)		*prnYFP*pBBRK	
Constitutively Expressed (in all phases)			
Gene	**Reporter Protein**	**Nomenclature**	**Comments**
0840	Short half-life GFP	*0840GFP_Sp*BBRK	
	Regular half-life GFP	*0840GFP*pBBRK	
	Regular half-life YFP	*0840YFP*pBBRK	

2.4. CFU viable counts

Samples were enumerated using culture by creating 10-fold serial dilutions of bacterial suspensions in PBS. 100 µl of the 10^{-5}, 10^{-6}, and 10^{-7} dilutions were spread onto charcoal agar and incubated for 5 days at 37°C. The number of colonies were counted, and the CFU/ml were calculated using the following equation:

Equation 1. CFU/ml

CFU/ml　　　=　　　$\dfrac{\text{Number of colonies x dilution factor}}{\text{Volume of suspension plated (ml)}}$

2.5. Genomic DNA Isolation

Genomic DNA (gDNA) was isolated using the GenElute Bacteria Genomic DNA Kit (Sigma-Aldrich, Dorset, UK) according to the manufacturer's instructions and eluted with 200µl of

elution buffer. gDNA was purified from THP-1 cells and human challenge model samples using the QIAmp DNA mini and blood extraction kit (QIAgen, Manchester, UK) as per the manufacturer's protocol. gDNA was quantified using a Qubit 1.0 fluorometer (Invitrogen, Loughborough, UK) according to the manufacturer's instructions.

2.6. Polymerase Chain Reaction (PCR)

PCR was performed using Q5® High-fidelity 2X Master Mix (New England Biolabs, Hitchin, UK) containing Q5® High-fidelity DNA Polymerase. Reactions were assembled on ice as per manufactures instructions (Table 11):

Table 11. Q5® High-fidelity 2X Master Mix reaction setup

Component	Reaction Volume (µl)	Final Concentration
Q5 High-Fidelity 2X Master Mix	25	1 X
10 µM Forward Primer	2.5	0.5 µM
10 µM Reverse Primer	2.5	0.5 µM
Template DNA	Variable	<1000 ng
Nuclease-Free Water	to 50	

Template DNA was either 1ng- 1µg of genomic DNA (gDNA) or 1pg- 1ng of plasmid DNA (pDNA). DNA was extracted using a kit or by the colony lysis method where a pipette tip was touched to colonies growing on agar and immediately resuspended in 100 µl of DNAse-free water. This suspension was heated to 95°C for 15 minutes and centrifuged at 16,000 x g for 10 minutes. 1-4 µl of supernatant was used as a DNA template in a 50 µl reaction.

Reactions were gently mixed and transferred to a preheated thermocycler. Routine PCR was performed as per manufacturer's instructions (Table 12):

Table 12. Q5® High-Fidelity 2X Master Mix thermocycling conditions

STEP	TEMPERATURE	TIME
Initial Denaturation	98°C	30 seconds
25–35 Cycles	98°C	5–10 seconds
	*50–72°C	10–30 seconds
	72°C	20–30 seconds/kb
Final Extension	72°C	2 minutes
Hold	4–10°C	

*Annealing temperatures were determined using NEB's Tm Calculator https://tmcalculator.neb.com/#!/main

2.7. Golden gate reaction

Golden gate reactions were performed using 100 ng of the vector pCR8GW:GG mixed with equimolar amounts of purified inserts. These components were combined with 1.5 µl 10X NEB Cutsmart Buffer, 1.5µl ATP (10 mM solution), 1µl of BsaI- HF, and 1µl of High concentration NEB T4 Ligase (2 million units/ mL) (New England Biolabs, Hitchin, UK). The assembly reaction occurred in the thermocycler as follows (Table 13) [130]:

Table 13. Golden Gate thermocycling conditions

STEP	TEMPERATURE (°C)	TIME
25 cycles	37	3 minutes
	16	4 minutes
1 cycle	50	5 minutes
	80	5 minutes

1µl of this reaction was transformed into NEB® 5-α Competent *E. coli* as per manufacturer's instructions.

2.8. Gateway™ reaction

This cloning method moves DNA sequences between vector systems. In this case, the entry clone was pCR8GW:GG contained the insert to be moved into the destination vector pBBR1KANGW for the construction of reporter strains, and PSS4940GW to knock-out putative β-lactamase genes [136]. The manufacturer's instructions were used to complete the Gateway™ reaction (Invitrogen, Loughborough, UK) and includes the addition of the following components at room temperature: 1-7 µl of the entry clone (50-150 ng), 1 µl destination vector, and TE buffer (pH of 8.0) to 8 µl. 2 µl of thawed LR Clonase™ II enzyme mix (Invitrogen, Loughborough, UK) was added to the reaction and vortexed/ microcentrifuged briefly. Unlike the manufacturer's instruction, this reaction was incubated at room temperature overnight with 1 µl of Proteinase K solution added and incubated at 37°C for 10 minutes to terminate the reaction.

5 µl of this reaction was transformed into the *E. coli* strain ST18 following the manufacturers' instructions for NEB® 5-α Competent *E. coli* (New England Biolabs, Hitchin, UK).

2.9. Construction of reporter strains

Reporter strains were made by conjugating promoter-probe vectors into the six strains of *B. pertussis* mentioned earlier. Promoter-probe vectors or reporter constructs were made using PCR to amplify promoters from *B. pertussis* genes that were highly expressed during each

phenotypic phase, as well as amplifying promoterless reporter genes from plasmid DNA. These genes include regular and short half-life GFP from pPROBE-NT and pPROBE-gfp [AAV], respectively, YFP from the pYFP_Star plasmid, and RFP from pBS1C-RFP vector [133–135].

The promoters were ligated ahead of these promoterless reporter genes and into the pCR8GW:GG plasmid (Invitrogen, Loughborough, UK) using golden gate assembly. pCR8GW:GG (Invitrogen, Loughborough, UK) containing the promoter-probe insert was transformed into High-Efficiency NEB® 5α *E. coli* (New England Biolabs, Hitchin, UK). An EcoR1 (New England Biolabs, Hitchin, UK) digest as well as sequencing were performed to confirm the presence of the insert prior to the extraction of plasmid DNA using the Monarch® Plasmid Miniprep Kit (New England Biolabs, Hitchin, UK). The Gateway™ reaction (Invitrogen, Loughborough, UK) allowed for this insert to be moved to pBBR1KANGW and transformed into *E. coli* ST18.

2.10. Conjugations

Biparental conjugation enabled the transfer of reporter constructs from *E. coli* ST18 into six *B. pertussis* strains, as well as facilitate knocking out putative β-lactamase genes.

B. pertussis was cultured routinely on agar, while donor *E. coli* ST18 was grown overnight in 5 ml of LB broth with kanamycin and 50 µg/ml ALA. 3 ml of *E. coli* ST18 were pelleted and resuspended in 800 µl of sterile PBS in Tube 1. Equal quantities of *B. pertussis* were also harvested in 800 µl of sterile PBS in Tube 2. 400 µl of both these suspensions, *B. pertussis* and *E. coli,* were combined in Tube 3. All three tubes were centrifuged at 13,000 x g for 1 minute. 50-100 µl of the supernatant was discarded and the pellets were resuspended in the remaining volume of PBS. This thick bacterial suspension was transferred to charcoal agar with 10 mM of $MgCl_2$ as a droplet in the centre of the plate. These plates were incubated at 37°C for 5-7 hours or until the liquid from the drop was absorbed into the agar. After this time, bacteria were harvested and resuspended into 800 µl of PBS and used to make serial dilutions. 80 µl of each undiluted and diluted suspension were plated on charcoal agar with 50 µg/ml of kanamycin for the conjugation of pBBR1KANGW containing the promoter-probe insert.

2.11.   The preparation of heat-killed bacterial cell suspensions

Plate-grown B1917 were resuspended in PBS (Thermo Fisher Scientific, Oxoid™, Basingstoke, UK) to an OD_{600} = 1.0 (approximately 10^9 cfu/ml). To optimise heat killing, 1 ml aliquots were heat-killed at 80°C for 1, 3 and 6 minutes in a pre-heated heat block. Aliquots were placed on ice immediately after incubation. Bacterial death was confirmed by the absence of growth after streaking 10µl of suspension onto charcoal agar plates and incubating at 37°C for 5 days. To ascertain the integrity of heat-killed cells, samples were subjected to flow cytometry (FACSCantoII, BD UK Ltd, Wokingham, U.K.). A detergent-lysed sample acted as a positive control for lysis and a sample containing live cells was a positive control for cell integrity.

2.12.   The preparation of THP-1 cells THP-1

THP-1 (ATCC® TIB-202™) cells were maintained in RPMI 1640 medium (Thermo Fisher Scientific, Gibco™, Loughborough, UK), fetal bovine serum (10%) (Thermo Fisher Scientific, Gibco™, Loughborough, UK), 1% streptomycin, penicillin and glutamine (Thermo Fisher Scientific, Loughborough, UK) as per standard methods. Heat-killed THP-1 cells were prepared by incubating cell suspensions at 10^5 cells/ml at 80°C for 6 minutes in a pre-heated heat block.

2.13.   Optimisation of PMA treatment conditions

PMA Dye, 20 mM in H_2O (Cambridge BioSciences, Cambridge, UK), was stored at -20°C in the dark, thawed on ice and added to 2 ml clear centrifuge tubes containing 200µl of cell suspensions to a final concentration of 20µM, 30µM, or 50µM. PMA-free samples served as controls for each condition tested. Tubes were covered with aluminium foil and shaken on an orbital shaker for 5, 10, 20, 30 or 70 minutes. Samples were then exposed to light using the PMA-Lite LED Photolysis Device (Cambridge BioSciences, Cambridge, UK) for 5, 10, 20, 30 or 40 minutes. Samples were pelleted using the Heraeus Pico 17 Centrifuge at 2000 x g (Thermo Fisher Scientific, Loughborough, UK) for 10 minutes at room temperature prior to DNA isolation. Non-PMA treated controls allow for the total number of *B. pertussis* cells to be enumerated. The number of viable *B. pertussis* cells calculated from PMA-treated samples can be subtracted from the total number of *B. pertussis* cells to provide the number of dead cells in a sample.

2.14.   RNA isolation and DNase treatment

RNA was harvested from both plate and broth cultures. Plate cultures were resuspended in 20 ml of PBS to an OD_{600} of 1.0.

Starter cultures grown up to an OD_{600} of 0.6 were used to inoculate a secondary overnight culture with an initial OD_{600} of 0.15. This culture was incubated until a final OD of 0.8- 1.0 was reached representing the mid log phase. It was from these samples that broth cultures were harvested.

The cultures were centrifuged at 3220 x g for 5 minutes with the supernatant discarded and pellet resuspended in 750 µl of TRI Reagent™ Solution (Invitrogen, California, US). The sample was centrifuged at 16,000 x g for 1 minute at room temperature. The supernatant was transferred to an Eppendorf tube with 200 µl of chloroform added. The tube was shaken vigorously for 15 seconds, incubated at room temperature for 15 minutes, and centrifuged at 4°C for 15 minutes at 12,000 x g. The clear upper aqueous phase was transferred to a new Eppendorf and 500 µl of isopropanol was added, mixed and allowed to precipitate at room temperature for 10 minutes. This tube was centrifuged at 4°C for 10 minutes at 12,000 x g. The supernatant was removed, 1.5 ml of 75% ethanol added, and the sample was centrifuged at 4°C for 5 minutes at 7,500 x g. The supernatant was removed, and the pellet was air dried for 15 minutes. 90 µl of nuclease free water was added to resuspend the sediment. The sample was incubated for 5 minutes at 65°C. DNA was removed from the RNA preparation using TURBO DNA-*free*™ DNase Treatment (Invitrogen, Loughborough, UK) as per the manufacturer's instructions using a two-step incubation. Using the Qiagen RNeasy Mini Kit (Qiagen, Manchester, UK), 350 µl buffer RLT was added to the sample, mixed, and mixed again after the addition of 250 µl of 100% ethanol. The sample as applied to the RNeasy columns and the manufacturer's protocol was followed. PCR was used to rule out gDNA contamination and the Qubit 3.0 fluorometer (Invitrogen, Loughborough, UK) was used to determine the RNA concentration prior to cDNA synthesis.

2.15.   cDNA synthesis

Up to 1 µg of template RNA was used to synthesise cDNA as per the Quick Protocol NEB #M0368 for first strand cDNA synthesis. Components were thawed prior to being combined and included up to 1 µg of template RNA, 2 µl of Random Primer Mix (60 µM), 4 µl of 5X ProtoScript II buffer, 2 µl of 0.1 M DTT, 1 µl of both ProtoScript II RT (200 U/µl) and 10 mM dNTP, 0.2 µl of RNase Inhibitor (40U/µl) and nuclease free water to bring the total to 20 µl (New England Biolabs, Hitchin, UK). This reaction was incubated at 25°C for 5 minutes, followed by a 1-hour incubation at 42°C. The enzyme was inactivated by incubating for 20

minutes at 65°C. The final volume of the cDNA product was brought up to 50 μl with nuclease-free water.

2.16. Quantitative PCR

qPCR was utilised to measure the gene expression levels of penicillin binding proteins during growth of *B. pertussis* with and without ampicillin in both plate and broth grown cultures. The final reaction volume was 25μl comprising of 12.5μl SYBR green PCR Master Mix (Applied Biosystems, UK), 7.3μl of DNAse free water, 0.1μl of both 400nM forward and reverse primer, and 5μl of template cDNA. cDNA was diluted in a 1 in 5 dilution with nuclease free water, and amplifications were performed using primers mentioned in Table 8. For the amplification of *BP905* and *BP3268*, 6.55μl of DNAse free water was used instead in addition to 0.75μl of 100% DMSO resulting in a final concentration of 3% DMSO for this reaction.

qPCR using SYBR Green was performed using StepOne Plus RT PCR System (Applied Biosystem, USA). The thermocycling parameters are described in Table 14.

Table 14. Sybr Green thermocycling conditions for qPCR

STEP	TEMPERATURE	TIME
Initial Denaturation	95°C	10 minutes
40 Cycles	95°C	15 s
	60°C	60 s
Melt Curve Analysis	95°C	15 s
	60°C	60 s

qPCR was also used to enumerate *B. pertussis* during the optimisation of the PMA-qPCR assay and was performed using a fluorogenic probe (Eurofins, Ebersberg, Germany) targeting the insertion sequence *IS481*. The reaction volume was 20μl comprising of 2μl of 1x Taqman Gene Expression Mastermix (Applied Biosystems, Loughborough, UK), 2μl of 900nM stocks of each primer, 2μl of 150nM stock of probe, 2μl of nuclease-free water (Thermo Fisher Scientific, Invitrogen ™, Loughborough, UK) and 2μl of template sample. The reactions were run using the StepOne Plus RT PCR System (Thermo Fisher Scientific, Loughborough, UK) using the cycling parameters found in Table 15. The sequence of primers and probe can be found in Table 8 and were described previously [113].

Table 15. TaqMan thermocycling conditions for qPCR

STEP	TEMP	TIME
Step 1 **Holding Stage**	50°C	2 minutes
Step 2 **40 Cycles**	95°C	10 minutes
	95°C	15 s
	60°C	60 s

2.17. Establishing linearity using standard curves for qPCR

A 10-fold serial dilution of gDNA in nuclease- free water was assayed by qPCR as described previously. A standard curve was automatically generated using StepOnePlus™ Software v2.3 to establish the linearity of the assay and to allow for the absolute quantification of unknowns.

2.18. Calculating copy number from Ct values/ DNA concentration

The genome copy number equivalent to the amount of template in a qPCR reaction was calculated using the formula:

Equation 2. Copy number

$$\text{copy number} = \frac{50 \times (\text{amount of template in ng} \times 6.022 \times 10^{23})}{\text{length of genome in bp} \times 1 \times 10^9 \times 650}$$

The 50x accounts for the 200 µl volume of extracted gDNA divided by 4 µl of gDNA used in the qPCR reaction. 650 Daltons denotes the average mass of a base pair (bp) and the number of molecules of the template/ gram can be calculated using Avogadro's number, 6.022×10^{23} molecules/mole. The genome of B1917 is 4,102,186 bp [99].
The genome of BP536, UK48, and the mean of all classical *B. pertussis* closed genomes available on RefSeq as of March 2019 was 4.1 Mb [112,123,126].

2.19. Preparation of bacterial and THP-1 cell suspensions

To evaluate if eukaryotic cells interfere with the enumeration of live *B. pertussis* cells using qPCR, 10^3 live *B. pertussis* B1917 were combined with THP-1 gDNA equivalent to 10843, 8414, 5385, 3446, 2804, 2316, 1868, 1503, 1251, 1023, 875, 746, 671, 507, 366, 275, 141, or 29 cells. A sample without THP-1 DNA served as a control.

To evaluate the possible sequestration of PMA by eukaryotic DNA, 10^6 heat-killed *B. pertussis* B1917 were combined with either 100,000 heat-killed THP-1 cells, 100,000 live THP-1 cells or without THP-1 cells and were then treated with the selected PMA treatment. Non PMA-treated samples were run in parallel.

To determine if eukaryotic cells interfered with the action of PMA on dead bacterial cells, 100,000 live THP-1 cells were combined with different ratios of viable *B. pertussis* cells and heat-killed *B. pertussis* cells (final bacterial concentration was 10^6 CFU/ml) in a clear Eppendorf tube, total volume 200µl. These samples were then subjected to the selected PMA treatment. A non-PMA treated control was included. gDNA was extracted from each sample and used for qPCR.

2.20.   Macrophage differentiation and infection

To determine the total number of *B. pertussis* cells taken up by macrophages during an infection, THP-1 (ATCC® TIB-202™) monocytes were first differentiated into macrophages. This differentiation was induced by adding 12 µl of 100 ng ml^{-1} phorbol-12-myristate-13-acetate (Fisher Scientific, Loughborough, UK) to 12 ml of approximately 10^6 THP1 cells/ml in an adherent 24 well plate containing RPMI 1640 plus 10% (w/v) of FBS. Cells were incubated for 24 hours at 37°C to enable differentiation before infection.

THP-1 (ATCC® TIB-202™) cells were infected with B1917 *B. pertussis* suspended in RPMI 1640 plus 10% (w/v) of FBS. 500 µl of a viable suspension of B1917 were used to infect 2 ml of human monocyte derived macrophages in suspension in order to obtain a multiplicity of infection (MOI) of 250 bacteria/macrophage. The MOI was calculated using 1 OD_{600} live suspension of B1917 cells containing approximately 10^9 CFU/ml and approximately 1 x 10^6 macrophages/ml in the following equation:

Equation 3. Multiplicity of Infection (MOI)

MOI   = $\dfrac{\text{Volume of } \textit{B. pertussis} \text{ suspension (ml) x (1 x } 10^9 \text{ CFU/ml)}}{\text{Volume of macrophage suspension (ml) x (1x } 10^6 \text{ cells/ml)}}$

   =   0.5 ml x (1 x 10^9 CFU/ml) / 2 ml x (1 x 10^6 cells/ml)

   =   5 x 10^8/2 x 10^6

   =   250 Bacteria/ Macrophage

Plates were centrifuged for 5 minutes at 640 x g to aid bacterial interaction with eukaryotic cells and incubated for 1, 3, 24, 48 and 72 hours at 37°C with 5% CO_2.

After incubation, non-adherent bacteria were removed with three washes of RPMI 1640 plus 10% (w/v) of FBS and fresh RPMI 1640 plus 10% (w/v) of FBS and 100 µg/ml polymyxin B (Sigma-Aldrich, Dorset, United Kingdom) was added to all the wells and incubated for 1 hour at 37°C to kill extracellular bacteria.

Infected macrophages were washed three times with PBS to ensure any remaining bacteria in the extracellular medium was washed away. The final wash from each time interval was cultured to assess the bactericidal efficacy of polymyxin B to kill all *B. pertussis* in the extracellular medium not taken up by the macrophage. The goal of the use of the antibiotic polymyxin B was to ensure counts reflect intracellular bacteria only once macrophages are lysed.

Cells were soaked for 10-15 minutes in PBS to promote macrophages-cell detachment prior to harvest. A scraper was used in addition to a pipette containing additional PBS to gently scrape, mix and aspirate dislodged macrophages with care taken to avoid creating bubbles and damage to cells. The viability of macrophages was checked using trypan-blue dye exclusion.

Afterwards, macrophages were permeabilized to enable the release of intracellular *B. pertussis* during a 30-minute incubation at 37°C in a PBS solution containing 0.1% (w/v) saponin (Sigma-Aldrich, Dorset, United Kingdom).

Lysed macrophages samples from each time interval were taken and split into two aliquots, with one treated with propidium monoazide (PMA), the other was not treated and used as a non-PMA control. *B. pertussis* from both samples were enumerated using qPCR and culture. Heat-killed bacterial cell suspensions were used as a 'dead' control for qPCR made by heating *B. pertussis* at 80°C for 6 minutes and confirmed by plating. The number of cells in the 1 OD_{600} suspension of *B. pertussis* enumeration was confirmed by plating serial dilutions in concert with performing qPCR. This sample was also used as a 'live' control.

2.21.   Statistical analysis

Unpaired T tests, corrected for multiple comparisons, and two-way ANOVA using the Holm-Sidak method and Dunnett's multiple comparison test was carried out using Prism 8 for macOS Version 8.2.1 to evaluate statistical significance. One-way ANOVA and Dunnett's

multiple comparisons test, with a single pooled variance was also used. A p value of <0.05 was defined as statistically significant and is indicated by asterisks.

Robust statistics were used when determining the fluorescence of reporter constructs using flow cytometry to increase the accuracy of performance parameters, reduce the need to use strict gating strategies around the populations of interest and minimised the impact of outlying events on statistical estimates and measurements [137].
The median, which is the robust equivalent of the mean, is the statistical centre of the population and represents the 50th percentile of the values.
The robust standard deviation (rSD) is a measure of variance and represents the dispersion of individual data points from the median of the population. The rSD was calculated as follows:

Equation 4. Robust Standard Deviation (rSD)

$rSD=$ (Median of $\{|X_i - Median_x|\}$) × 1.4826

Where X represents individual data points and 1.4826 represents a constant factor allowing the robust value to be adjusted to the equivalent of a normal population distribution.
The robust coefficient of variation (%rCV) was calculated as follows:

Equation 5. Robust Coefficient of Variation

$\%rCV=$ $\dfrac{rSD}{Median_x}$ x 100%

2.22.   Ethics

Samples from volunteers participating in the human challenge model study were obtained in accordance with the provisions of the Declaration of Helsinki (1996) and the International Conference on Harmonization Guidelines for Good Clinical Practice. This study is registered with ClinicalTrials.gov: NCT03751514, was reviewed and approved by the South Central – Oxford A Research Ethics Committee (REC reference: 17/SC/0006, 24 February 2017) and the UK Health Research Authority (IRAS project ID: 219496, 1 March 2017). The protocol has been published previously, with details written and oral consent received from human participants [23]. It can be found on www.periscope-project.eu.

2.23.   Relative quantification of gene expression

This method was used to determine the change in expression of genes encoding for penicillin binding proteins in *B. pertussis* BP536 during different growth conditions relative to the housekeeping gene *recA*. *recA* is constitutively expressed in all Bvg phases of growth and promotes both DNA repair and recombination [138]. The $2^{-\Delta\Delta C_T}$ method, described by Livak and Schmittgen, was used to calculate the relative changes in gene expression using data obtained from real-time quantitative PCR experiments [139].

The average C_T was calculated for both the target genes and the housekeeping gene *recA* in all growth conditions. These growth conditions included broth cultures with and without $MgSO_4$ and ampicillin, as well as plate cultures with and without $MgSO_4$. The target genes were the genes encoding penicillin binding proteins *(BP0102, BP0905, BP1051, BP1545, B2754, BP3028, BP3655, BP1061, BP3214, BP3268)*. This data was used to calculate the ΔC_T in the following equation:

Equation 6. ΔC_T

$\Delta C_T=$ Average target gene C_T - Average Housekeeping gene C_T

Target genes were normalised to housekeeping gene *recA* using the previous equation.

The relative quantification of the target was calculated using the following $\Delta\Delta C_T$ equation:

Equation 7. ΔΔC_T

$\Delta\Delta C_T=$ sample ΔC_T - average control group ΔC_T

In this analysis, different groups acted as the sample group and the control group in an effort to maximise comparisons. For example, when comparing the expression of *BP0102* in broth cultures with $MgSO_4$ to broth cultures without $MgSO_4$, the sample group was broth cultures with $MgSO_4$, while the control group was broth cultures without $MgSO_4$.

The fold change in gene expression normalised to a housekeeping gene and relative to a control was calculated using the $2^{-\Delta\Delta C_T}$ method in the following equation:

Equation 8. $2^{-\Delta\Delta C_T}$

$2^{-\Delta\Delta C_T}=$ $2^{-(\text{sample }\Delta C_T - \text{ average control group }\Delta C_T)}$

2.24.   Fluorescence microscopy

The NIKON C1 Confocal Microscope (Nikon U.K. Ltd., Surbiton, UK) and the companion software EZ-C1 FreeViewer was used to image reporter strains grown overnight. UV was used to detect fluorescence. The vector only control was used as a negative fluorescence control, and *B. bronchiseptica* RBpagprom2b was used as a positive fluorescence control [140].

2.25.   Flow cytometry

Fluorescence was detected in plate grown suspensions and overnight cultures of reporter strains. These samples had an OD_{600} of 1.0, were fixed in 0.5% paraformaldehyde (Sigma-Aldrich, Gillingham, UK) and sent to Public Health England for flow cytometry. Once there, samples were analysed on the Beckman Coulter CytoFLEX S V4-B4-R3-12 (Beckman Coulter Life Sciences, Indianapolis, US). The filter for the fluorescein isothiocyanate (FITC) channel was used and had an excitation and emission of 525/40 nm in the blue/green spectrum. The filter for the Phycoerythrin (PE-A) channel was also used and had an excitation and emission of 585/42 nm in the green/yellow spectrum.

2.26.   Microplate reader detection of fluorescence

Fluorescence from the reporter strains was also detected using the Clariostar Monochromator Microplate Reader (BMG LABTECH, Offenburg, Germany) and Synergy H1 Microplate Reader (BioTek, Swindon, United Kingdom). The Clariostar monochromator was used to select the most sensitive reporter protein for the creation of reporter constructs, while the BioTek was used for the majority of fluorescent reads.

200 µl of plate growth resuspended in PBS and overnight cultures, both at an OD_{600} of 1.0 were inoculated into a 96-well plate PS uClear black TC plate, flat bottom (Greiner Bio-one, Kennebunk, ME, USA). End-point reads were taken at 37°C.

The fluorescence intensity was detected using the bottom optics for both microplate readers with the excitation and emission spectrum for the reporter proteins analysed in Table 16.

Table 16. Reporter proteins excitation and emission spectra

Reporter Protein	Excitation λ (nm)	Emission λ (nm)
GFP	479[a]/ 445[b]	520[a]/ 510[b]
YFP	500[a]/ 514[b]	541[a]/ 580[b]
RFP	588[a]/ 505[b]	633[a]/ 558[b]

[a] Spectra used with the Synergy H1 Microplate reader
[b] Spectra used with the Clariostar monochromator

2.27.   Growth Assay

Overnight cultures of *B. pertussis* were diluted to an OD_{600} of 0.15 using fresh SS broth, supplement, modulator and different concentrations ampicillin added. 200 µl of this suspension was added to clear, sterile, round-bottom 96-well plate (Corning, Flintshire, United Kingdom).

Blanks comprising of SS broth, supplement, modulator and antibiotics were added to the plates. All outside wells contained 200µl of water to prevent evaporation of the cultures. Reads were performed using FLUOstar Omega Microplate Reader (BMG LABTECH, Offenburg, Germany), with plates incubated at 37°C. The plate was shaken using the double orbital motion at 500 rpm before absorbance readings at 600 nm were taken every hour.

2.28.   Measurement of minimal inhibitory concentration (MIC) of ampicillin

Epsilometer tests or E-tests (Biomerieux, Basingstoke, United Kingdom) were used to quantify the susceptibility of plate-grown *B. pertussis* to ampicillin. Ampicillin E-test strips were taken out of the -20°C freezer for at least 30 minutes, while plate grown *B. pertussis* were resuspended in PBS to an OD_{600} of 0.1. The manufacturer's guidelines were followed for the application of the E-test strips. The bacterial suspensions were inoculated on charcoal agar with and without 50 mM $MgSO_4$ using a sterile swab streaked out over the entire surface of the agar plate by rotating the plate approximately 60° to allow for confluent growth during incubation at 37°C. The lid of the plate was left off for 15 minutes to allow for drying of excess liquid prior to the application of the E-test strips using a sterile tweezer. Plates were incubated for routine culture and the MIC was determined by reading the reading scale where the pointed end of the ellipse of bacterial growth intersected with the strip [141].

2.29.   Reverse Phase Chromatography/ Mass Spectrometry (RPC/MS)

Reverse Phase Chromatography (RPC) separate proteins based on hydrophobicity, while Mass spectrometry (MS) captures these ions and sorts them based on their mass-to-charge ratio (m/z). This tool was used to determine if ampicillin is being degraded in *B. pertussis* broth cultures. The time it takes for the compounds to be separated through a column is called the Retention time (RT). This value was determined for ampicillin and the ampicillin degradation product. The chemical formula of ampicillin and the ampicillin degradation product (ampilloic acid) was $C_{16}H_{19}N_3O_4S$ and $C_{15}H_{21}N_3O_3S$, respectively.

The Agilent QTOF 6545 (Agilent, Santa Clara, California) was used to perform the LC-MS analyses. This instrument contained a Jetstream ESI spray source coupled to an Agilent 1260 Infinity II Quat pump HPLC with 1260 autosampler, column oven compartment and variable wavelength detector (VWD).

The MS method was set-up and complete as per the Instrument Specialist's instructions: The MS was operated in positive ionization mode with the gas temperature at 250°C, the drying gas at 12 L/min and the nebulizer gas at 45 psi (3.10 bar). The sheath gas temperature and flow were set to 350°C and 12 L/min, respectively. The MS was calibrated using reference calibrant introduced from the independent ESI reference sprayer.

The VCap, Fragmentor and Skimmer was set to 3500, 125 and 45 respectively.

Chromatographic separation of a 5 µL sample injection was performed on a InfinityLab Poroshell 120 EC-C18 (3.0 x 50 mm, 2.7 µm) column using H20 (Merck, LC-MS grade) with 0.1 % formic acid (FA, Fluka) v/v and methanol (MeOH, VWR, HiPerSolv) with 0.1 % FA v/v as mobile phase A and B, respectively.

The column was operated at flow rate of 0.5 mL/min at 50°C starting with 5 % mobile phase B for 0.5 min, thereafter the gradient was started for 2 min to a final 100% B, held at 100% B for 1 min then returned to 5% B for 3.9 min in a total 7.5 min run time.

The variable wavelength detector (VWD) was set to collect 254 and 320 nm wavelengths at 2.5 Hz.

Data was processed using the Quantitative Analysis (for Q-TOF) version 10.1 software.

A standard curve was created to establish the linear range of detection for ampicillin. Standards were created using a two-fold dilution series of ampicillin in SS broth. *B. pertussis* BP536 was incubated at 37°C with and without ampicillin and 50 mM $MgSO_4$ for up to 23 hours. SS and supplement were combined with 10 µg/ml ampicillin and used as the negative degradation control. 5 and 30 mg of lyophilised β-lactamase from *Enterobacter cloacae* Type IV (Sigma- Aldrich, Dorset, UK) dissolved into 10 ml of SS broth with ampicillin to give a final concentration of 0.1-0.3 and 0.6-1.6 mg/ml, respectively, was used as the positive degradation control. *P. mirabilis* (JN40) and *E. coli* Nissle pUC19 EC were also used as a positive degradation control. All samples were incubated for a total of 23 hours with samples collected at 0, 6, and 23 hours, and spun down at 16,000 x g for 5 minutes.

At least 700 µl of the supernatant was removed for analysis and transferred to vials specific for the 6545 QTof LC/MS System (Agilent, Santa Clara, California). Water and SS samples were run as blanks between samples to prevent carryover.

A value known as the *'Response factor'*, which is a ratio between the signal produced by an analyte and the quantity of that analyte can provide an estimate for the concentration of the compound in the sample.

2.30.   Tandem Mass spectrometry (MS/MS)

Tandem Mass spectrometry (MS/MS) was used to further fragment compounds by using a second stage of mass spectrometry allowing further details regarding the structure and composition of the molecule to be obtained.

The collision energy was set to 20 and 40 eV in which 106.0673, 160.0455, 174.0604 m/z ions were used to qualify the ampicillin degradation peak.

2.31.   Determining putative β-lactamase genes in *B. pertussis*

Putative β-lactamase genes in *B. pertussis* were determined by performing a tblastn with all 2761 β-lactamase proteins in the NCBI database compared to the *B. pertussis* BP536 genome.

2.32.   Knocking out the putative β-lactamase genes

Knock-out mutants were created by amplifying the flanking regions around the putative β-lactamase gene with the golden gate overhangs. These regions were referred to as Flank A and Flank B. For both knock out mutants, Flank A and Flank B were ligated into pC8/GW:GG using Golden Gate Assembly and transformed into 5α *E. coli*. Plasmid DNA was extracted and digested, as well as sequenced to confirm the absence of genes in the inserts. Inserts were moved into the suicide vector pSS4940GW using a Gateway™ Reaction and transformed into *E. coli* ST18. At this stage, pSS4940GW in *E. coli* ST18 was ready for conjugation into *B. pertussis* and selection for recombination events.

2.33.   Selections for recombination events to knock out putative β-lactamase genes.

The protocol for biparental conjugation was modified for the conjugation of pSS4940 in *E. coli* ST18 into *B. pertussis,* where the thick bacterial suspension was centrally placed on charcoal agar with 10 mM $MgCl_2$, and 50 mM $MgSO_4$ prior to incubation for 5-7 hours. After incubation, resuspension, and serially diluting the resuspension, the suspensions were

plated onto selective charcoal agar containing 50 mM MgSO$_4$ and gentamycin. It was at this point that the first recombination event occurred resulting in single crossovers where the pSS4940 plasmid was integrated onto the chromosome of *B. pertussis.* These cells were selected for using gentamycin. However, both the mutant and wild-type alleles were present in the cell. MgSO$_4$ was used to grow strains in the Bvg⁻ phase preventing the *ptx* promoter, that regulates *I-Sce*I gene expression, from being activated. Colonies were passaged twice and stored at -80°C in 20% glycerol.

To select four double crossovers, the frozen clones were cultured on charcoal agar. Growing clones in the absence of MgSO$_4$ allowed for the *ptx* promoter ahead of the I-*Sce*I gene to be activated resulting in a second recombination event where the wild-type allele was excised, or the wild-type genotype was reverted back to. Colony PCR was used to confirm knock-out mutants.

Chapter 3

Results

Monitoring growth of *B. pertussis in vivo* during colonisation using the PMA-qPCR assay
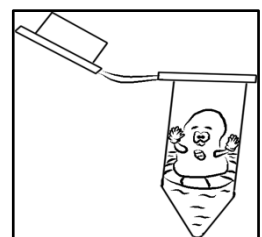
3. Monitoring growth of *B. pertussis in vivo* during colonisation using the PMA-qPCR assay

3.1. Rationale

In order to inform future vaccine improvement by identifying immune correlates of protection, a human challenge model of *B. pertussis* colonisation has been developed. Accurate measurement of colonisation status in this model has required the development of a modified qPCR-based assay to enumerate *B. pertussis* in samples that distinguishes between viable and dead bacteria.

In the first part of this chapter, I report the development of this assay and its performance in the quantification of *B. pertussis* B1917 from human challenge model samples.

Through treatment of samples with the intercalating dye, propidium monoazide (PMA), PCR-mediated amplification of DNA from dead cells is inhibited, allowing viable cells to be distinguished from dead cells [118,142–145].

The use of PMA involves an initial incubation of samples with PMA in darkness, during which it diffuses into dead cells, followed by light activation of PMA that permanently modifies the genomic DNA (gDNA) of dead cells, preventing it from acting as a template in PCR.

In the second part of this chapter, I will explore the utility for this assay to detect and quantitatively enumerate viable *B. pertussis* taken up by macrophages over time.

Macrophages are key immune cells that become engaged during an infection and have the ability to recognise and remove microbes and bacterial products [146].

Lamberti *et al.* and Valdez *et al.* have both speculated that *B. pertussis* may survive and replicate inside of macrophages due to the presence of an 'intracellular phase' that allows them to adapt to the intracellular environment, evade microbicidal effector mechanisms and therefore enables their persistence. However, the mechanism behind this hypothesised phase remains unclear [147,148].

Previously, live cells remaining in the macrophages over time have been evaluated quantitatively using culture and observed qualitatively with fluorescence microscopy [147].

By using the PMA-qPCR assay, it will provide the first quantitative measurements for the total number of cells taken up by the macrophages, as well as allow for the enumeration of live and dead intracellular *B. pertussis* cells over time.

Taken together, there are many uses for the PMA-qPCR assay in both diagnostic and research labs where a quantitative measure of both *B. pertussis* number and viability is required.

3.2. Introduction

Culture is a highly specific method of detection for *B. pertussis* [5]. However, growth on this media can take over one week and offers low sensitivity [109]. To ensure the safety of volunteers in the human challenge, faster detection is required for colonisation of participants to be monitored in real-time. Real-time PCR detection (qPCR) provides this speed and is also highly sensitive. To ensure that only live cells are being enumerated, qPCR in combination with PMA was optimised for *B. pertussis* [111].

3.2.1. The action of propidium monoazide (PMA)

PMA is a selective, photoreactive dye that can be used to distinguish viable microbes in the presence of dead bacteria. PMA has been determined to be more selective than ethidium monoazide (EMA) for the ability to selectively penetrate dead cells and suppressing their signal with minimal dye uptake by intact live cells [111]. PMA has a higher positive charge than EMA, which could explain the higher selectivity of PMA for dead cells [149]. PMA derives from propidium iodide (PI), which is used to stain dead cells in microscopy and flow cytometry, however it was modified to contain an azide group [150]. Despite this modification, PMA maintains much of the same characteristics of PI [151]. It has a high affinity for DNA and is a membrane-impermeable dye that is not able to penetrate the intact cell membrane of viable cells, but is able to diffuse across the compromised membrane of dead cells and intercalate into their DNA upon photoactivation [152].

Photoactivation of PMA is induced by exposure to light emitted by either a thermally stable LED or UV light source. Upon exposure to light, the photoreactive azido group on the PMA dye is converted into highly reactive nitrene radicals. Nitrogen-carbon covalent bonds are formed between the nitrene radical and hydrocarbons in the dsDNA of dead cells. This cross-linking results in structural changes to the nucleotide angle and permanent modification of DNA in dead cells (Figure 7) [111].
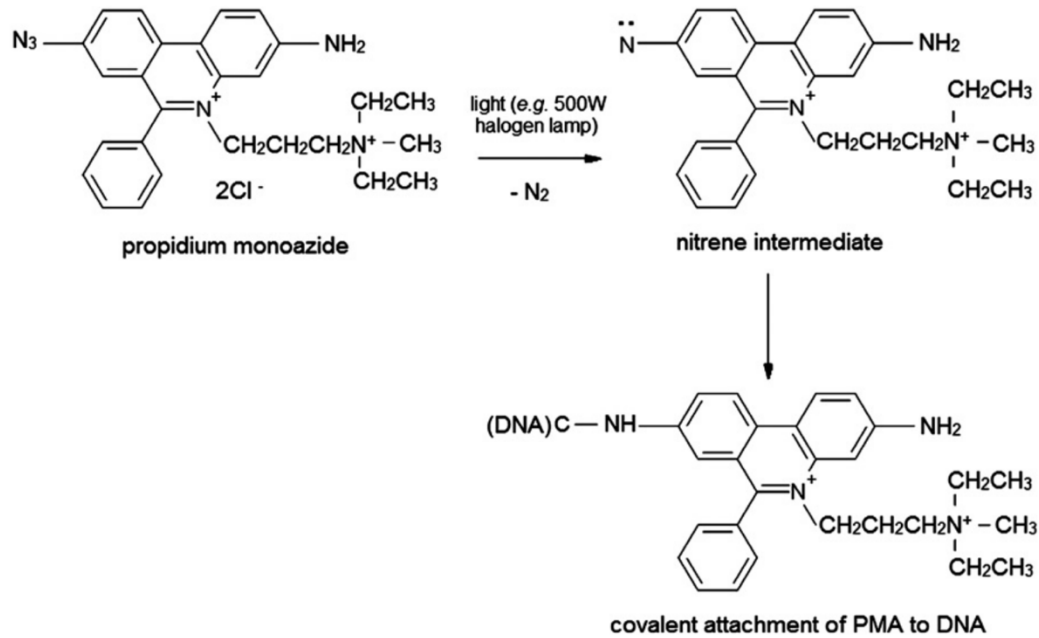
Figure 7. Proposed mechanism of action by PMA.

Following photoactivation, carbon-nitrogen bonds are formed between the recently converted nitrene intermediates from PMA and the double-stranded DNA in dead cells preventing the amplification of dead cell DNA during qPCR (Reproduced from Biotium product information http://www.biotium.com/) [150].

Excess photoactivated PMA is quenched and inactivated by water molecules resulting in the production of hydroxylamine. This molecule is unable to form covalent bonds with DNA preventing it from modifying the DNA from intact viable cells during DNA extraction [111,149].

During qPCR, the amplification of the modified DNA of dead cells is inhibited, allowing only the amplification of DNA from viable cells. This proposed mechanism of action for PMA can be found in Figure 7 [150]. Therefore, PMA dye is used to pre-treat samples within mixed populations of live and dead cells, prior to gDNA extraction and in combination with qPCR to quantify viable cells only (Figure 8) [151].
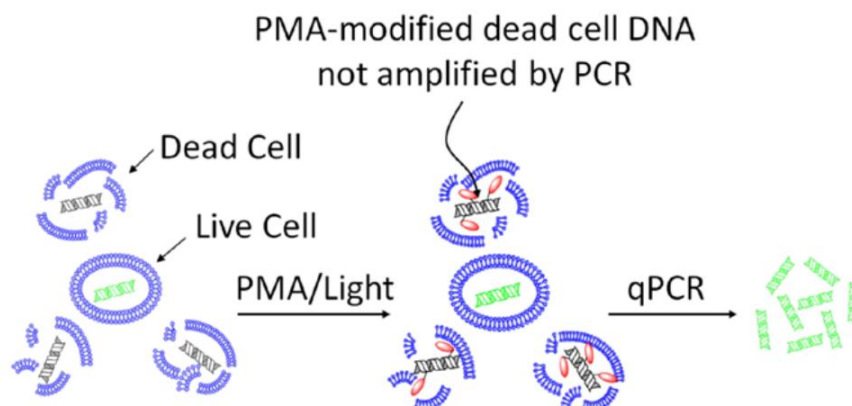
Figure 8. Principle of PMA-qPCR to quantify viable bacteria.

The use of PMA involves an initial incubation of samples with PMA in darkness, during which, it diffuses into dead cell. Followed by light activation, PMA permanently modifies the genomic DNA (gDNA) of dead cells, preventing it from acting as a template in PCR and only allowing the quantification of live cells (Reproduced from Biotium product information http://www.biotium.com/) [153–155].

This assay can generate results within one day and is of particular use for fastidious or slowing growing bacteria that take prolonged periods of time to culture and as a result, cell survival is difficult to estimate for these organisms [151]. *B. pertussis* fits this description, and the use of this assay will speed up the enumeration of viable cells to just a few hours and will enable researchers to determine whether participants in the challenge are actively colonised during the challenge or effectively cleared of the infection post-challenge.

Standardised conditions for this highly sensitive and specific assay have been successfully optimised in previous studies to distinguish between live and dead bacterial cells. These studies include samples of different origins including multi-species oral biofilms, fungi, spores, lyophilised bacterial samples, viruses, and protozoa [111,118,156–161,142–145,149,151,152,155].

To optimise PMA-qPCR for *B. pertussis*, cell density, sample matrix, PMA concentration, exposure time in darkness and light, incubation and PCR targets need to be considered.

3.2.2. The concentration of propidium monoazide (PMA)

The concentration of PMA is critical to this assay and needs to be optimised specifically for each sample type. This is because PMA has the potential for cytotoxicity if its concentration is not ideal. Concentrations for PMA have ranged from 6 µM to treat the yeast *S. cerevisiae* to greater than 100 µM to treat *Bacteroides* without cytotoxic effect and enough suppression

of dead cell amplification signal. Complicated matrices or high cell turbidity, such as those that may be present in human challenge volunteer samples may warrant an increase in the PMA concentration in order to surpass these conditions and prevent false positives during enumeration. Complexity of the sample matrix can suppress the PCR signal from dead cells resulting in an overestimation of live cells. Turbidity as a result of inorganic or organic compounds, such as epithelial cells, can interfere with chemical adsorption of the PMA, as well as prevent light penetration during photoactivation [111].

The concentration of PMA can also dictate the incubation time and temperature. For example, using low dye concentrations will allow for more flexible incubation periods. Previous studies have found that five minutes was sufficient to allow PMA to enter the membrane of compromised dead bacterial cells and intercalate into their DNA despite varying concentrations of PMA and complicated matrices [111,143,169–172,152,162–168]. In organisms with low dye penetration rates such as *Mycobacterium*, repeated dye treatments were found to work more effectively [149].

The mechanism for the uptake for PMA is not yet defined, however studies have indicated that temperature affects membrane permeability. As a result of PMA having a lesser effect on the membrane permeability of intact cells in comparison to EMA, most studies have used room temperature during incubation with PMA [111].

3.2.3. Photoactivation conditions

For photoactivation, the selection of the light source and light exposure time is of equal importance to the other experimental parameters of the PMA-qPCR assay. However, with the advent of the Biotium PMA-Lite LED photolysis device, photo-crosslinking can be carried out in a more standardized manner, limiting experimental variation. Vials can be positioned evenly with maximal illumination from all sides. The emission of the blue LEDS of this device is approximately 465 nm, which enables the activation of PMA at 465 nm. Additionally an internal fan is used to lower the temperature produced by the lights to room temperature [153].

The duration of light exposure of dye treated samples should enable nucleic acid-bound dye to be activated and excess dye to be inactivated so it does not enter intact cells during DNA extraction. The times typically reported for this parameter range from 2 to 20 minutes depending on the light source, distance of the sample from the light, turbidity of the sample and the sample matrix.

The final parameter for this assay is to identify highly sensitive and specific target genes for the identification of *B. pertussis* during qPCR. This has been discussed in Chapter 1. The

target selected for use during the optimisation of this assay is the highly sensitive target of the insertion sequence *IS481* [109].

Taken collectively, these parameters need to be considered during the optimisation of the PMA-qPCR assay for use during the Human Challenge Model.

3.2.4. Application of PMA-qPCR assay

3.2.4.1. Determining intracellular viability of *B. pertussis* in macrophages

The utility of this assay outside of the challenge will also be explored in this this chapter, specifically the total number of *B. pertussis* cells taken up by macrophages during infection, as well as the proportion of live and dead cells intracellularly.

Recent evidence has suggested that *B. pertussis* is a facultative intracellular bacterium with the ability to survive and replicate intracellularly in macrophages [146–148]. Macrophages are effector cells for bacterial killing. During pathogenic invasion, macrophages remove microbes through phagocytosis preventing the growth and survival of pathogens [146]. However, infected host cells may also promote bacterial survival [146].

Persistence within the host may also be as a result of adaptations by *B. pertussis* to the intramacrophage environment and the presence of an 'intracellular phase' [146–148]. This 'intracellular phase' appears to have enabled *B. pertussis* to evade phagosome-lysosome fusion, survive inside non-acidic compartments in human macrophages, and replicate after 48 hours [148].

Intracellular viability was previously determined using fluorescent in situ hybridization as well as culture, however the PMA-qPCR assay can also be used for this purpose.

3.3. Results

3.3.1. Optimisation of PMA- qPCR assay in *B. pertussis*

3.3.1.1. *qPCR performed using the target IS481 provides a limit of detection of 2 B. pertussis cells*

IS481 is often used as the target for qPCR detection of *B. pertussis* as it is present at ~250 copies per cell in *B. pertussis*, providing great sensitivity. To develop a PMA-qPCR assay, the sensitivity of qPCR for detection of *B. pertussis* was tested over a range of template gDNA concentrations. A linear relationship between Ct value and template concentration was observed over the range of 2 to approximately 2.26×10^6 B1917 genomes for qPCR

using TaqMan internal probe-based detection (Figure 9). Ct values greater than 35 were considered to be a negative reaction for TaqMan probe detection. Thus, the assay is able to detect *B. pertussis* gDNA equivalent to very few bacterial cells and is linear over a wide range of *B. pertussis* concentrations with the TaqMan probe detection.
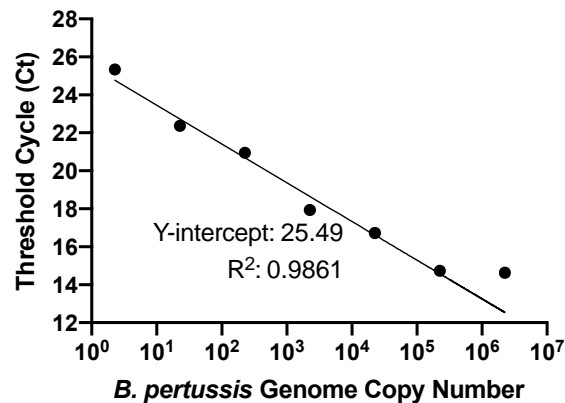
Figure 9. Standard curve of Ct value versus template concentration.

Template DNA concentration is expressed as B1917 genome copy number. The linearity of the standard curve made using TaqMan probe detection was determined to be from 2 to approximately 2.26×10^6 B1917 genomes for qPCR.

3.3.1.2.   Heat-killing B. pertussis at 80°C for 6 minutes maintained the integrity of cells

The ability of PMA to inhibit PCR-amplification from dead *B. pertussis* was tested using heat-killed *B. pertussis* B1917. It was envisaged that clinical samples may contain dead, but intact, *B. pertussis.* Heat-killing may cause cell lysis which would not mimic intact dead cells. Thus, the integrity of cells following heat killing was assessed using flow cytometry (Figure 10).
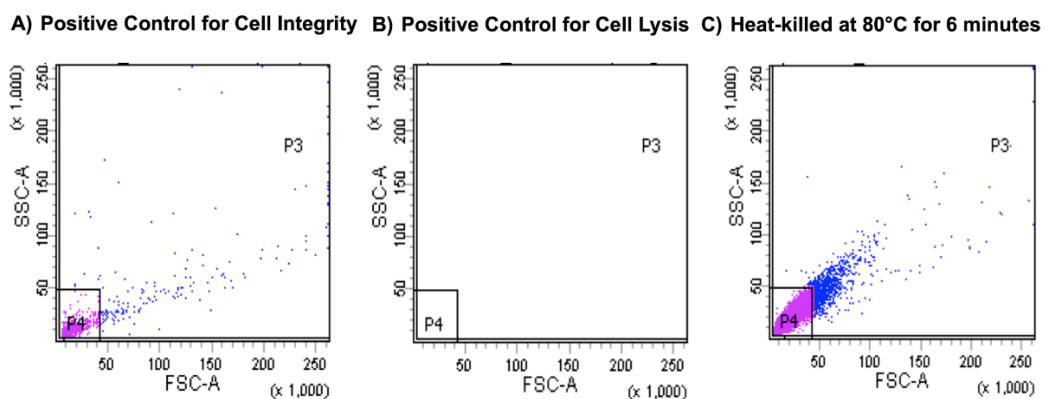
Figure 10. The effect of heat killing on the integrity of B. pertussis cells.

A) Positive control for cell integrity – a suspension of live *B. pertussis*; B) Positive control for cell lysis – detergent lysed *B. pertussis*; C) Heat-killed *B. pertussis* suspension. The heat-killed *B. pertussis* suspension incubated for 6 minutes at 80°C displayed similar scatter as the live cell suspension. No particles were detected in a suspension of detergent-lysed *B. pertussis*. Therefore, cells remained intact in the heat-killed *B. pertussis* suspension incubated for 6 minutes at 80°C when compared to the positive cell integrity control.

Incubation of *B. pertussis* suspensions at 80°C for 6 minutes appeared to result in 100% killing with cells remaining intact. Therefore, the heat-killing condition of 80°C for 6 minutes was used for future work.

It is important to note that this experiment was not able to directly detect lysed cells in heat-killed samples, rather it provided an indication of lysed verses intact cells simply based on size. Therefore, lysis in heat-killed samples was possible, but could not be detected in this experiment.

3.3.1.3.   Optimisation of PMA treatment

The effect of PMA concentration on the inhibition of PCR amplification from dead *B. pertussis* B1917 was tested. Incubation of heat-killed cells with 50 µM of PMA resulted in a 97.42 % reduction in PCR signal compared to that generated from untreated samples. Lower levels of PMA also resulted in very similar levels of inhibition (Figure 11).
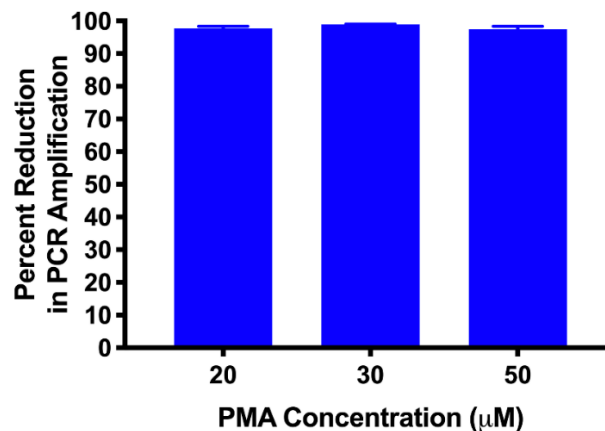
Figure 11. The effect of PMA concentration on the PCR amplification signal from heat-killed cells.

Treatment of samples with either 20µM, 30µM, or 50µM of PMA produced a >97% reduction in the PCR amplification signal compared to untreated samples. Error bars represent standard deviations from two biological replicates performed in technical triplicates.

The optimal conditions for photo-activation of PMA were determined. Incubation under dark conditions for 10 minutes followed by light activation for between 5 and 30 minutes resulted in greater than 99% reduction in PCR signal from dead cells compared to untreated controls. Five minutes of light activation following 10 minutes of darkness resulted in 99.64% reduction in detection of *B. pertussis* DNA (Figure 12).
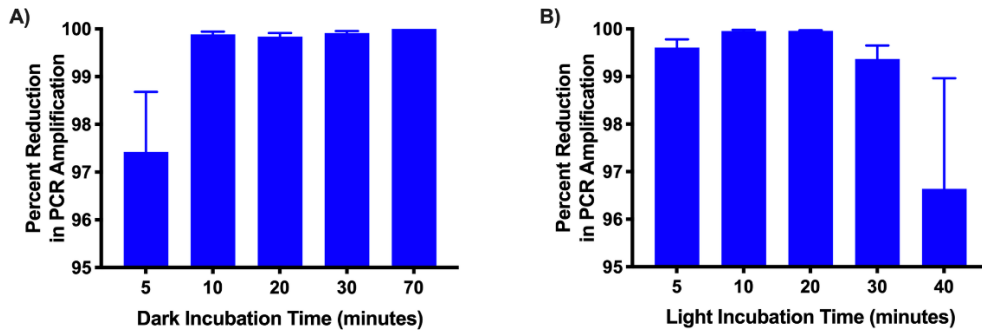
Figure 12. The effect of dark and light exposure times on PMA-inhibition of PCR amplification.

A) Dark incubation B) Light Incubation. PMA and untreated heat-killed suspensions were incubated for different times in the dark followed by exposure to 5 minutes of light. 10 minutes or longer of incubation in the dark produced a >99% reduction in the PCR amplification signal. Optimal light incubation periods were determined by incubating untreated and PMA treated heat-killed suspensions in the dark for 10 minutes followed by light exposure for multiple time periods. Incubating PMA treated heat-killed samples under light for periods of 5, 10, 20, and 30 minutes produced a 99% or greater reduction in the PCR amplification signal. Five minutes was selected as the standard light incubation period. Error bars represent standard deviation from two biological replicates. The experiment was repeated with the same result.

From these optimisations, standard conditions of 50µM PMA and incubation in the dark for 10 minutes followed by light activation for 5 minutes were selected as the minimal incubation times that achieved high levels of inhibition. Even though 20µM PMA inhibited PCR amplification from dead cells, 50µM PMA was selected as the concentration to use in the assay, as clinical samples will contain cells other than *B. pertussis* that may sequester PMA, requiring an excess for consistent inhibition of PCR signal from dead *B. pertussis.* These conditions were tested in four independent assays. An average of 94.15% reduction in PCR signal was observed compared to untreated controls (Figure 13).
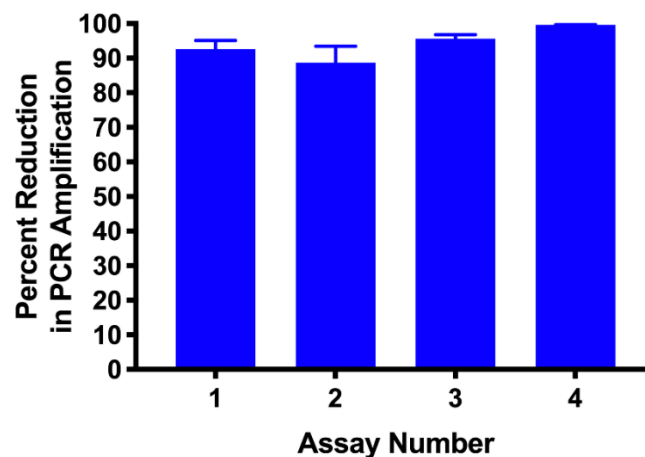
Figure 13. Selected assay conditions gave reproducible inhibition of PCR signal from dead cells.

Heat-killed samples were treated with 50 µM of PMA and incubated in the dark for 10 minutes followed by 5 minutes of light activation. A 94.15% reduction in the PCR amplification signal was observed. Error bars represent standard deviations from five biological replicates.

3.3.1.4. The effect of exogenous cells on detection and PMA-mediated inhibition

Clinical samples are likely to contain cells other than *B. pertussis*, including eukaryotic cells that contain very large amounts of DNA compared to *B. pertussis* cells. Eukaryotic cells may interfere with the PMA-mediated inhibition of amplification from dead *B. pertussis* preventing distinguishing between live and dead *B. pertussis*. To test this, varying amounts of gDNA from THP-1 cells were combined with a constant amount of *B. pertussis* B1917 gDNA, and Ct values were determined and compared to samples containing *B. pertussis* B1917 gDNA only. No effect of THP-1 gDNA on detection of *B. pertussis* was observed up to an equivalent of approximately 3446 THP-1 cells per assay (Figure 14).
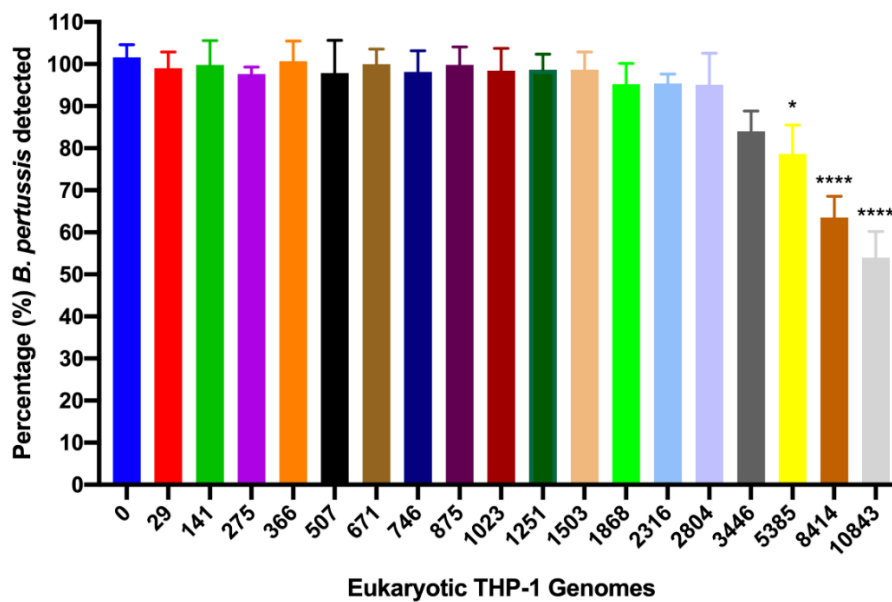
Figure 14. Effect of eukaryotic gDNA on the detection of B. pertussis.

Varying amounts of gDNA from THP-1 cells were combined with gDNA equivalent to 10^3 *B. pertussis* cells. No interference in detection of *B. pertussis* was observed up to the equivalent of 3446 THP-1 cells, after which the sensitivity of detection was reduced when compared to viable *B. pertussis* detected in the presence of 0 THP-1 cells. *: $p < 0.05$, determined by one-way ANOVA and Dunnett's multiple comparisons test, with a single pooled variance. Error bars represent standard deviations from three biological replicates.

It was possible that the presence of other cells would interfere with the PMA-mediated inhibition of PCR signal from dead *B. pertussis.* Thus, the effect of heat-killed or live THP-1 cells on PMA-mediated inhibition of PCR amplification from heat-killed *B. pertussis* was tested. A 99.94% reduction in PCR signal was observed indicating that THP-1 cells did not prevent PMA-mediated inhibition of PCR signal from dead *B. pertussis* (Figure 15).
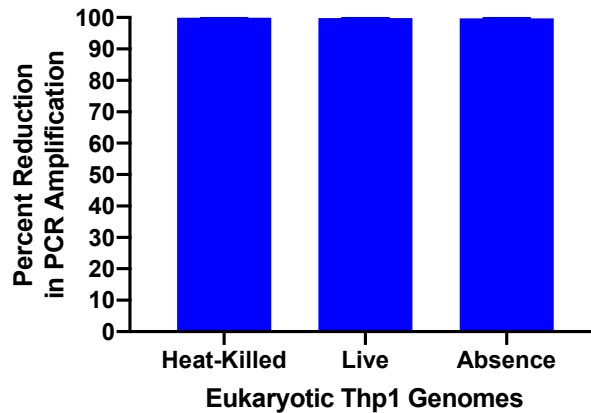
Figure 15. Effect of eukaryotic THP-1 gDNA on the qPCR enumeration of dead B. pertussis.

gDNA from 10^6 heat-killed *B. pertussis* were combined with either 10^5 heat-killed THP-1 cells, 10^5 live THP-1 cells or a no THP-1 cell control and treated with PMA. Non-PMA-treated samples were run in parallel. The presence of live or dead THP-1 cells did not interfere with the action of PMA on dead *B. pertussis* cells allowing the PCR amplification signal to be reduced by >99%. Error bars represent standard deviations from three biological replicates.

To test the assay's ability to distinguish between viable and dead *B. pertussis,* in the presence of other cells, a constant number of THP-1 cells were combined with different ratios of heat-killed and viable *B. pertussis* B1917 cells. The reduction in PCR signal was proportional to the number of heat-killed cells in each suspension demonstrating that the assay was able to distinguish viable from dead *B. pertussis*, even in the presence of human cells (Figure 16).
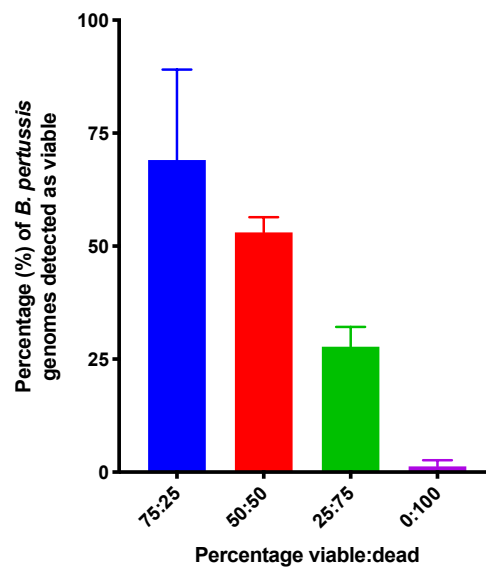
Figure 16. Viable B. pertussis cells enumerated from PMA treated samples in the presence of eukaryotic cells.

100,000 THP-1 cells were combined with suspensions of different ratios of heat-killed and viable *B. pertussis* cells. The assay accurately distinguished viable from dead *B. pertussis* in each suspension. Error bars represent standard deviations from three biological replicates.

Collectively, these studies revealed that the THP-1 cells did not interfere with the PMA-mediated inhibition of PCR signal from dead *B. pertussis* or prevent the accurate enumeration of viable *B. pertussis* cells.

3.3.1.5.   Measuring the viability of B. pertussis during in vitro growth.

During development of the assay, it was observed that PMA treatment of live *B. pertussis* suspensions used as controls consistently reduced the PCR signal compared to untreated samples. This suggested that *B. pertussis* colonies taken from plate grown cultures contains both live and dead bacteria. To investigate this, and to determine the proportion of live to dead *B. pertussis* in plate grown cultures over time, suspensions of cells were made of *B. pertussis* B1917 grown on plates for either 3, 4, 5 or 8 days. The suspensions were treated with PMA and qPCR performed. *B. pertussis* is relatively slow growing and many protocols for plate growth involve incubation for 72 hours to achieve visible colonies. The percentage of PCR signal observed at this time was compared to untreated controls and showed that the viability of *B. pertussis* at 72 hours was only 89% (Figure 17). Interestingly, although colony size continued to increase between days 3 and 5, percentage viability decreased to 24%. Further incubation resulted in further loss in viability. Thus, when using plate grown *B. pertussis* in assays, suspensions will be a mixture of live and dead bacteria, and enumeration of *B. pertussis* by plating serial dilutions of a suspension and counting the resulting CFU's will not be a measure of the total number of cells in the suspension.
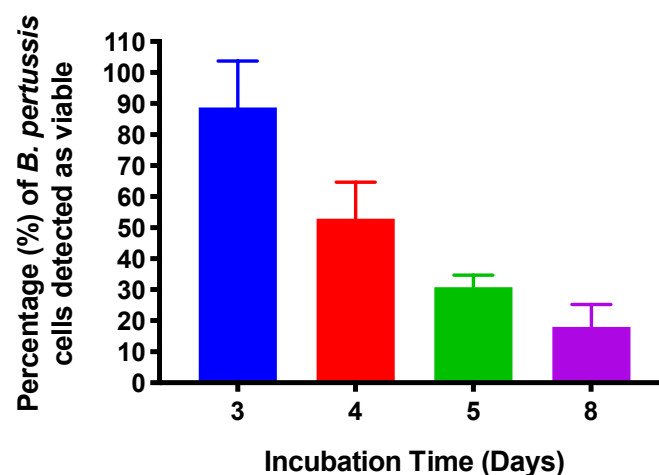
Figure 17. The viability of B. pertussis decreases during growth on agar plates.

The viability of *B. pertussis* growing on agar plates was measured over time. Viability decreased as the incubation time increased with only 24% of cells being viable after 5 days of incubation. Error bars represent standard deviations from three biological replicates.

3.3.1.6. Use of the assay to enumerate live and dead B. pertussis from human challenge model samples

The assay was developed in order to provide a method for monitoring the colonisation status of participants in a novel human challenge model of *B. pertussis* colonisation. During development of this model, a group of participants were inoculated with 10^5 CFU of *B. pertussis* B1917 and daily samples were taken over a 14-day period to monitor colonisation, including nasosorption fluids, pernasal swabs, throat swabs, and nasopharyngeal washes [173].

Here, post-challenge samples were tested by PMA-qPCR and by culture and are shown in Figure 18. Samples were split and one portion was treated with PMA. *B. pertussis* were enumerated using qPCR from PMA-treated and untreated samples.
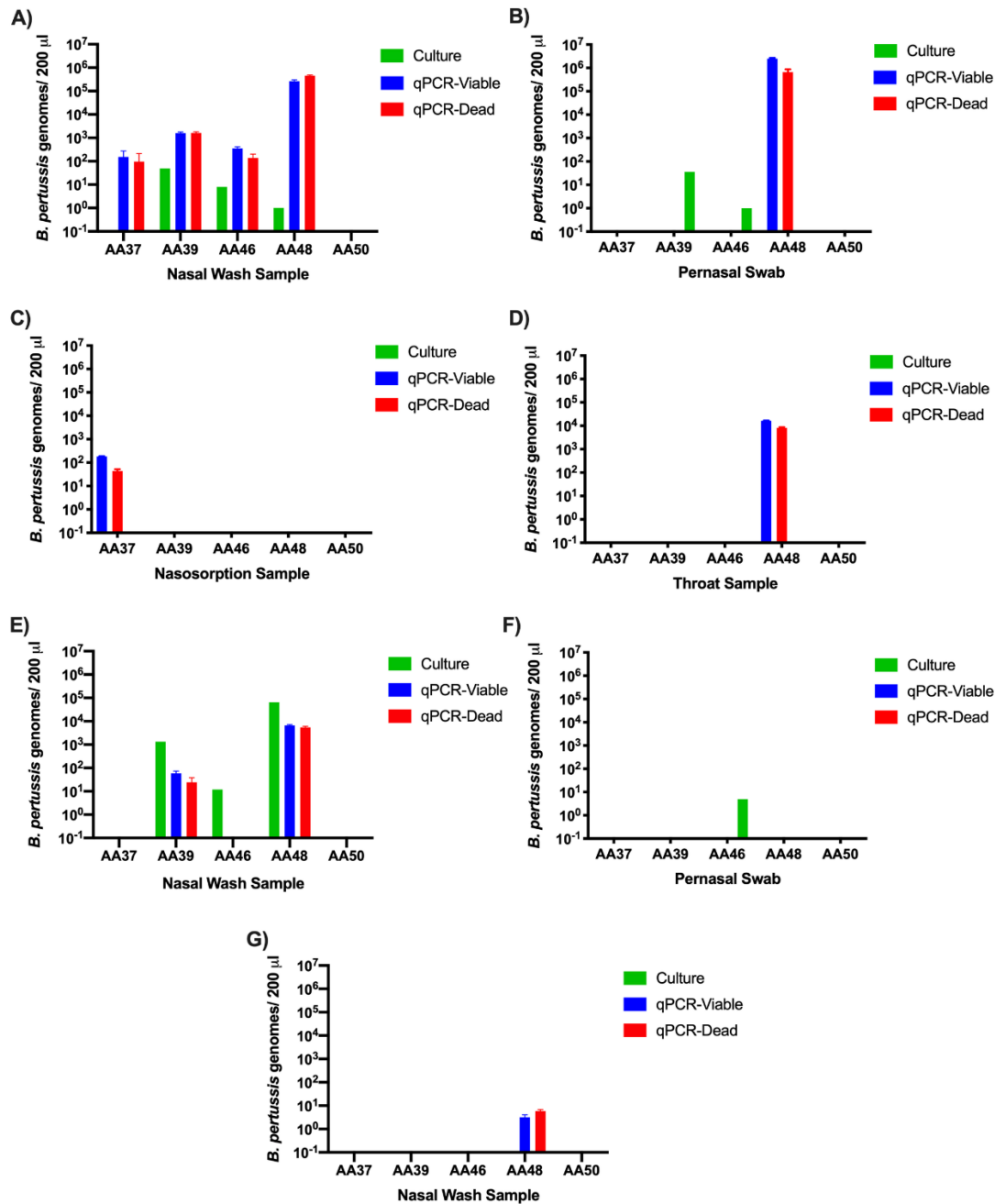
Figure 18. PMA-qPCR detected viable B. pertussis from human challenge model samples within hours compared to culture.

Viable and dead *B. pertussis* were enumerated in samples from 5 volunteers in the human challenge model, collected Day 9 (A-D), Day 11 (E-F) and Day 16 (G) after inoculation, from the sample type indicated. Two hundred μl of samples were processed. Day 16 samples are taken two days after volunteers started azithromycin treatment to clear infection. Values below the lower limit of detection were considered undetectable and given a value of 0.

Using culture, 3 out of 5 participants were determined to be colonised [173]. By PMA-qPCR, 4 out of 5 volunteers were deemed to carry viable *B. pertussis*, with detection from nasal washes, pernasal swabs, nasosorption and throat samples. Nasal washes from Day 11 samples also had detectable viable *B. pertussis* in 2 of the 5 volunteers using PMA-qPCR

and in a third volunteer using culture (Figure 18E). Pernasal samples from Day 11 revealed detectable *B. pertussis* in a single volunteer using culture, that was not detected using PMA-qPCR (Figure 18F). PMA-qPCR revealed samples contained both viable and dead *B. pertussis*, in approximately equal numbers. Interestingly, on Day 16 of sampling, two days after volunteers started azithromycin treatment to eradicate the infection, all but one volunteer was negative for detectable *B. pertussis* cells. In this volunteer, the PMA-qPCR assay was able to detect low levels of viable and dead *B. pertussis*, with a higher proportion of dead genomes detected compared to viable genomes, however these low levels of *B. pertussis* were undetectable by culture (Figure 18G). Nasosorptions from this cohort of volunteers were all culture negative.

Taken together, these results illustrate an increased sensitivity in using the PMA-qPCR assay compared to culture, with the exception of pernasal swabs, where two out of five volunteers had detectable levels of *B. pertussis* using culture rather than PMA- qPCR. However, these volunteers were detected as positive for *B. pertussis* in nasal washes using PMA-qPCR. Additionally, the PMA-qPCR assay was able to detect positive volunteers in nasosorptions and throat samples at day 9 and nasal wash samples at day 16, while culture was not.

3.3.1.7. Confirming utility of PMA-qPCR assay by enumerating five additional strains of B. pertussis

To confirm the utility of this assay with other strains outside of the human challenge model, qPCR was performed on gDNA extracted from PMA and non-PMA treated suspensions from the following strains: BP536, UK48, UK71, B204 (B1878), B184 (B2973). B1917 was also enumerated as a control (Figure 19). These suspensions contained *B. pertussis* cells taken from three-day old plate cultures resuspended in PBS to an $OD_{600}=1.0$. The suspensions were plated onto agar to confirm the enumeration obtained by the PMA-qPCR assay. The recovery of viable *B. pertussis* cells from both PMA-qPCR and culture were comparable for all strains, confirming the utility of this assay as a reliable method for enumeration, however, there was a significant increase in the recovery of viable cells from B1917 and BP536 using PMA-qPCR compared to culture. This was likely due to increased sensitivity of the PMA-qPCR assay in comparison to plate culture. DNA copy numbers were calculated using the mean genome size of 4.1 Mb.
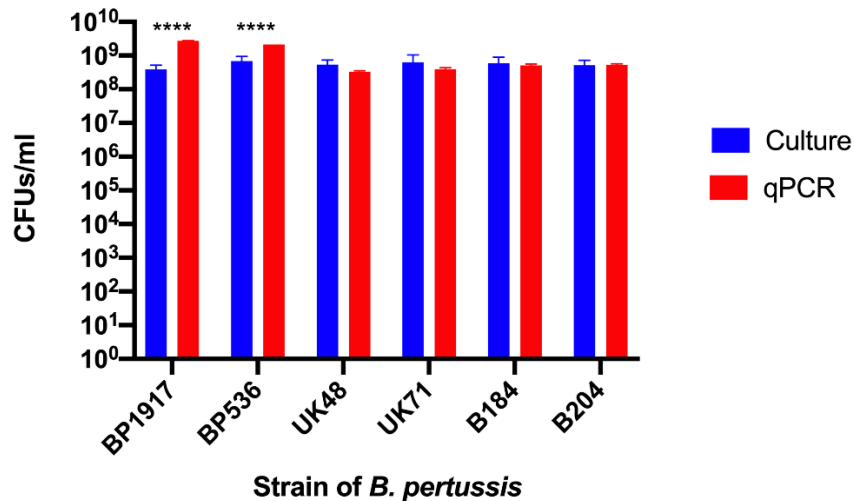
Figure 19. Enumeration of six strains of B. pertussis with both culture and PMA-qPCR producing comparable values.

The exceptions were B1917 and BP536 for which a higher number of viable *B. pertussis* cells were detected using the PMA- qPCR assay. Error bars represent standard deviations from three biological replicates.

3.3.2. Detection of the total number of *B. pertussis* taken up by THP-1 derived macrophages over time using the PMA-qPCR assay

In the second section, I highlight one particular use of the PMA-qPCR assay—the detection of the total number of *B. pertussis* taken up by macrophages during an infection. This quantitative measurement has not yet been possible to detect using traditional culture methods or any other method.

3.3.2.1. Investigating the total number of B. pertussis cells taken up by the THP-1 derived macrophages using PMA-qPCR.

To determine the total number of *B. pertussis* cells taken up by macrophages during an infection, I completed a macrophage uptake assay where human monocyte derived macrophages were infected with a multiplicity of infection (MOI) of 250 bacteria/macrophage for 1, 3, 24, 48, 72 hours. During this assay, the extracellular medium was also sampled prior to macrophage lysis and the release of intracellular *B. pertussis* detected. I took samples from each time interval, split the samples into two aliquots and treated the first aliquot with PMA alongside a non-PMA control and performed qPCR (Figure 20). Additionally, I enumerated the second aliquot using plate culture (Figure 21).
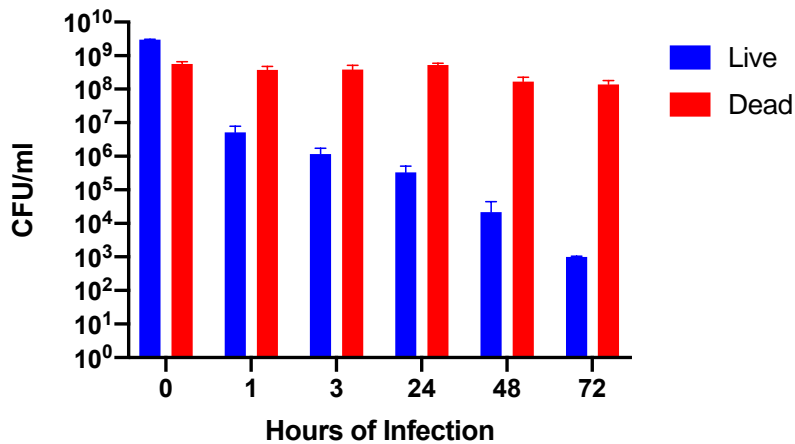
Figure 20. Viable B. pertussis cells were detected in macrophages for up to 72 hours after infection using PMA-qPCR.

The viability of *B. pertussis* decreased over time resulting in approximately 10^3 CFU/ml after 72 hours in a lysate containing 10^6 macrophages/ml. However, there was a higher proportion of dead *B. pertussis* cells compared to viable cells. Error bars represent standard deviations from four biological replicates.

The total number of *B. pertussis* cells taken up by 10^6 macrophages/ml during the infection at 0, 1, 3, 24, 48, and 72 hours was approximately 10^9, 10^8, 10^8, 10^8, 10^8, and 10^8 CFU/ml, respectively. Interestingly, the total number of viable intracellular *B. pertussis* decreased over time of the infection and at 0, 1, 3, 24, and 48 hours, the number of viable *B. pertussis* cells was approximately 10^9, 10^7, 10^6, 10^5, 10^4 CFU/ml, respectively. Time 0 represents the number of *B. pertussis* in the initial inoculum.

Interestingly, there were still viable *B. pertussis* cells present at 72 hours of approximately 10^3 CFU/ml. There was a higher proportion of dead cells compared to live cells throughout the course of the infection emphasizing the killing activity of macrophages over time. I was not able to confirm whether or not *B. pertussis* was replicating in macrophages, rather the pathogen appeared to be dying, albeit slowly.

3.3.2.2. *Approximately 10^2 CFU/ml viable B. pertussis cells were detected in macrophages 24 hours after infection using culture.*

To determine the number of viable *B. pertussis* cells from the macrophage uptake assay using culture, the *B. pertussis* cells from second aliquot was enumerated by culture. In comparison to the PMA- qPCR data, the enumeration from culture revealed far fewer viable cells over time. There was a steep decrease in the number of viable cells in macrophages after 1 hour of infection from 10^9 CFU/ml to 10^4 CFU/ml. At 3 hours, there were approximately 10^4 CFU/ml detected and at 24 hours there were 10^2 CFU/ml viable cells detected. After 24 hours there were no detectable cells enumerated with culture, whereas there were 10^4 CFU/ml viable cells enumerated with PMA-qPCR (Figure 21). Possible reasons for this difference can be found in the discussion below.
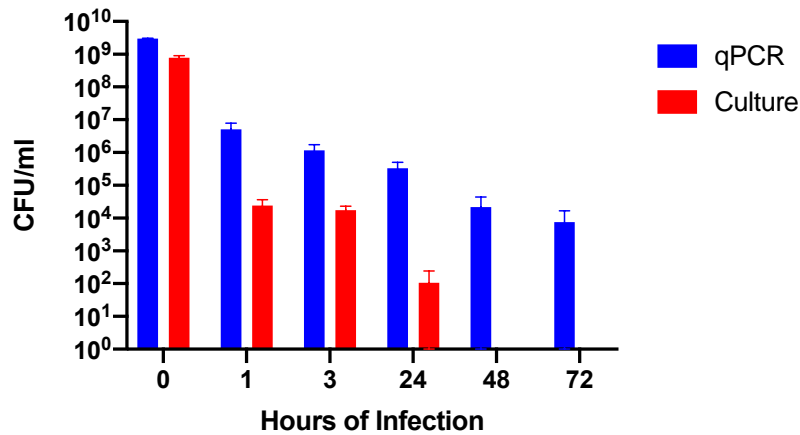
Figure 21. Viable B. pertussis cells were detected using both qPCR and culture from infected macrophages.

Viable *B. pertussis* cells were detected in macrophages for up to 72 hours after infection using PMA-qPCR compared to 24 hours using culture, where approximately 10^2 CFU/ml were detected. Beyond 24 hours of infection there were no viable *B. pertussis* cells detected with culture. Error bars represent standard deviations from four biological replicates.

3.3.2.3. *B. pertussis cells survived in the extracellular medium for up to 3 hours after infection despite treatment with polymyxin B.*

To ensure that the *B. pertussis* enumerated in the macrophage uptake assay came from the intracellular compartment of macrophages alone, extracellular *B. pertussis* were killed with the antibiotic polymyxin B and the extracellular medium was cultured to assess the bactericidal efficacy of polymyxin B to kill *B. pertussis* (Figure 22).
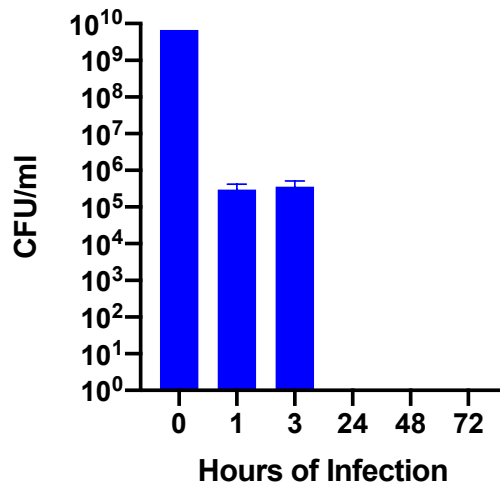
Figure 22. The extracellular medium contained >10,000 CFU/ml of B. pertussis 3 hours after infection, despite treatment with polymyxin B.

Error bars represent standard deviations from four biological replicates.

Extracellular medium cultured 1-hour and 3-hour post-infection revealed 10^5 CFU/ml *B. pertussis*, despite treatment with polymyxin B. However, beyond three hours, there were no

detectable cells cultured. Therefore, the enumeration of intracellular macrophages infected for less than three hours were falsely elevated. The reason for this has been explored below, however from these results it is possible to deduce that the intracellular enumeration of *B. pertussis* three hours post-infection was accurate.

3.4. Discussion

Ordinarily, the detection and quantification of viable *B. pertussis* is achieved through culture on laboratory agar. However, the relatively slow growth rate of *B. pertussis* means that the growth of countable colonies can take between 72 – 120 hours. The development of a human challenge model for *B. pertussis* as part of the PERISCOPE project requires that enumeration of viable *B. pertussis* be achieved in a much shorter time than this, in order to be able follow colonisation closely.

In addition, simple enumeration of viable bacteria within a sample doesn't provide the complete picture. In many scenarios, such as measuring bacterial load in an infection model, it is of great interest to know the total bacterial number, as understanding the dynamics of bacterial growth that involves both cell division and cell death is very important. Thus, while traditional qPCR provides a faster detection method for *B. pertussis* than culture, the modification of a qPCR assay with the introduction of PMA treatment of samples reported here enables both fast detection of *B. pertussis* and the ability to distinguish viable from dead cells.

Here, I demonstrate that PMA inhibits PCR-mediated amplification from dead *B. pertussis* and that inhibition of signal from dead cells occurs even in the presence of high numbers of eukaryotic cells. This may be important for the detection of *B. pertussis* from complex samples that contain a mix of cell types as seen in the human challenge model. Samples obtained from volunteers that were identified as positive for *B. pertussis* by culture, were also detected in our initial test of the PMA-qPCR assay. The same volunteers were identified as being negative for *B. pertussis* by both qPCR and culture at Day 16, with the exception of a single sample that had low levels of *B. pertussis* identified only by qPCR. This result demonstrates the high sensitivity of this assay to detect very low levels of viable and dead *B. pertussis.* Further optimisation studies to determine the exact point in which there is loss in sensitivity when amplifying fewer than 10^3 *B. pertussis* cells in the presence of THP1 cells would further support the results obtained from the human challenge model. Interestingly, the PMA-qPCR assay detected approximately equal numbers of viable and dead *B. pertussis,* demonstrating its use to enumerate total bacteria rather than only viable cells. The

full results of the human challenge model are published elsewhere [173]. Here I demonstrate that the PMA-qPCR assay allowed for a determination of colonisation status within hours of obtaining the samples compared to days when using culture.

To confirm that the assay can be used with strains other than B1917, I tested five additional *B. pertussis* strains. Approximately 250 copies of IS481 were found in all isolates of *B. pertussis*, however, the exact number of copies differs amongst strains within a narrow range [113]. The number of copies range from 236-272 among the closed genome sequences available for *B. pertussis*. Thus, this will create some error when performing absolute quantification of strains for which the copy number is not known, but this error is not large (<10%).

The use of *IS481* as a target means that there is the chance of cross-reactivity of *IS481* with *B. holmesii* and *B. bronchiseptica,* although *B. holmesii* is rarely recovered from nasopharyngeal samples [115]. However, here, this assay was specifically designed to support the human challenge model, in which there is confidence that volunteers are not colonised by *Bordetella* other than the administered *B. pertussis* B1917. Therefore, using a single qPCR target of *IS481* to detect these bacteria is appropriate. However, to extend the use of this assay and to increase specificity, the single copy targets *BP0283* or *BP0485*, which are commonly used in many diagnostic labs, should be considered [113]. These regions show sequence divergence from *B. bronchiseptica* and *B. parapertussis*, enabling *B. pertussis*-specific amplification.
Additional targets can also be used to rule in *Bordetella* spp. such as IS1001 as a high copy target to screen for the presence of *B. parapertussis* and *B. bronchiseptica,* and a low copy target such as BH*recA* to confirm the presence of *B. holmesii* [115–117]. These changes will reduce false positives and result in a more sensitive and specific assay for the accurate diagnosis of *B. pertussis*, as well as help rule out coinfections or pertussis symptoms caused by other *Bordetella* spp.

Throughout this chapter, there appeared to be a discrepancy between the enumeration of *B. pertussis* using plate culture in comparison to qPCR where qPCR appeared to be over-estimating viable cells or plate culture appeared to be under-estimating viable cells. The potential over-estimation of viable cells using qPCR may be a result of the increased sensitivity of the qPCR assay or PMA may be inhibited by the conditions of the assay. However, this assay has been fully optimised for the enumeration of *B. pertussis*; therefore, it is unlikely that PMA is inhibited from entering dead cells. A potential reason why the plate culture enumeration may be under-estimating viable cells may be due to the slow-growing

nature of *B. pertussis* requiring more days for growth than the five days used for routine culture for CFU viable counts. Additionally, it is well established that plate culture is a low-sensitivity method for enumeration of viable cells. Both of these reasons appear to be the likely cause for the discrepancy between the enumeration of *B. pertussis* using the PMA-qPCR assay in comparison to plate culture. However, as these sources of error have not been explored further during the duration of this chapter, it is not possible to evaluate which enumeration result is correct and therefore requires further work.

Although optimised for use in the human challenge model, the use of the PMA-qPCR assay has been extended to wider applications. One such utility is to evaluate the total number of *B. pertussis* cells taken up during a macrophage uptake assay. Typically, this information would be impossible to detect using traditional culture, as culture would only be able to enumerate the number of viable cells and completely exclude the number of dead cells. However, the number of dead cells provides critical information about the bactericidal action of macrophages. Previous literature have suggested that macrophages kill the majority of *B. pertussis* immediately upon infection, with a small proportion surviving and replicating in a non-acidic intracellular compartment after 48 hours [148,174]. My observations revealed a majority of bacterial cells dying within the first hour, however, I was unable to confirm the replication of *B. pertussis* after 48 hours, rather viable *B. pertussis* cells were slowly dying over time. It is possible that replication is occurring at a slower rate than cell death, however it could not be determined. Additionally, replication could be occurring past 72 hours and if repeated this should be taken into consideration.

One of the pitfalls of this experiment centered around the use of polymyxin B. Immediately after infecting macrophages with *B. pertussis,* 100 µg/ml of polymyxin B was used to kill extracellular *B. pertussis*. However, viable extracellular *B. pertussis* remained for up to three hours during the infection. A second round of 5 µg/ml of polymyxin B could have been used during the infection at 1, and ≥45 hours to ensure extracellular bacteria was killed prior to macrophage lysis [146,148].

These results support the complex interplay between *B. pertussis* and the host cell and with the PMA-qPCR assay, cell survival and cell death of *B. pertussis* inside macrophages was explored. Importantly, this is the first assay that allows the total number of cells taken up by macrophages over time to determined. To further investigate the growth dynamics of intracellular *B. pertussis*, the PMA-qPCR assay could be used with additional strains such as the five additional *B. pertussis* mentioned previously—BP536, UK48, UK71, B184, and B204.

Finally, the PMA-qPCR assay could also be used in a range of research and industrial settings or in diagnostic laboratories, where ascertaining if *B. pertussis* is viable or dead will facilitate whether to pursue culture as a means to obtaining a live culture for characterisation.

Chapter 4

Results

Utilising reporter constructs to determine the sensitivity to modulation of six strains of *B. pertussis in vitro*
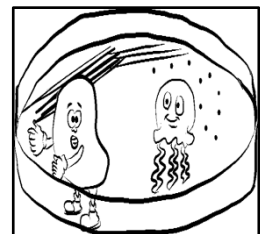
4. Utilising reporter constructs to determine the sensitivity to modulation of different strains of *B. pertussis in vitro*

4.1. Rationale

The BvgAS Two Component System is a global regulator of gene expression conserved in the *Bordetella* spp. This system utilises signal transduction to control the differential regulation of genes in response to environmental signals resulting in three distinct Bvg phases: 1) Bvg^+ (virulent), 2) Bvg^i (intermediate), and 3) Bvg^- (avirulent) [60,64].

Differences in the Bvg activity amongst strains have been hypothesised to affect growth characteristics. In fact, the Preston Lab has identified variances in the final biomass and growth rate between strains. To further explore these growth differences, reporter constructs were created to monitor the Bvg phase adopted by different strains of *B. pertussis* during growth.

The building and testing of the reporter constructs specific for each Bvg phase in different strains of *B. pertussis* will be detailed in the first part of this two part results section. The second part explores the sensitivity of the different reporter strains to the chemical modulator $MgSO_4$ and concludes with a genotypic comparison between the BvgAS of different strains of *B. pertussis,* with the functional implications of mutations' assessed.

4.2. Introduction

4.2.1. Molecular diversity in the BvgAS and functional implications

To better understand the variability of the BvgAS amongst *Bordetella* strains, Herrou *et al.* compared the molecular diversity of the *bvgS* and the *bvgA* amongst three closely related *Bordetella* species, *B. pertussis*, *B. bronchiseptica*, and *B. parapertussis*. It was found that BvgA of *Bordetella* is highly conserved in contrast to BvgS, which differs amongst species. While some species are in the process of diversification, BvgS of *B. pertussis* exhibits low levels of diversity having undergone recent selective sweeps and appearing to reach equilibrium in their human host. Of the low-level changes, the periplasmic domains— specifically the external surfaces of the VFT domains have evolved the most [2]. The conservation of the cavities of the VFT is important to transmit signals where in the absence

of a ligand binding to the cavities VFT domain, BvgS is in the activated state. However, the activity of BvgS can be decreased in the presence of modulators and the sensitivity to modulators is proposed to be dependent on efficient signal transduction between the VFT1 and VFT2 domains with VFT2 playing a larger role when it comes to sensitivity to modulation. The linker B segment, which joins the PAS domain with the His-kinase domain also shares this function. Within the linker B segment, *B. pertussis* Tohama 1 lies the non-conservative substitution of glutamic acid to lysine at position 705 ($E_{705}K$) resulting in the increased insensitivity to negative modulating signals in the BvgS of this strain. This mutation, although the exception among *B. pertussis*, *B. bronchiseptica* and *B. parapertussis* isolates illustrates that the linkers involved in phosphorylation are not under strong selective pressures [2].

The Bvg minus phase has been shown to be important to allow *B. bronchiseptica* to survive outside of the host, however, the importance of entry into this phase and down-modulation of virulence factor expression is less clear for *B. pertussis*. Therefore, responsiveness to modulation of BvgS of *B. pertussis in vivo* remains to be elucidated [2].

4.2.2.   Reporter constructs to monitor sensitivity to modulation during growth

Our lab has identified differences in growth characteristics between *B. pertussis* strains such as the final biomass and growth rate (unpublished). The Bvg phase is also known to affect growth. In order to determine if different strains are at different points along the Bvg phase spectrum when grown in the lab, as well as determine if the differences in Bvg phases are responsible for the different growth patterns, transcriptional fusions were used. The use of transcriptional fusions will also allow for a greater understanding of the sensitivity to modulation for *B. pertussis*.

Transcriptional fusions or reporter constructs have long been used to understand gene regulation and gene expression. Promoter-probe vectors facilitate the construction of these fusions and the common motif of these constructs contain a promoterless reporter gene located downstream restriction sites. The reporter gene encodes for an assayable protein such as green, yellow or red fluorescent protein known as GFP, YFP and RFP, respectively. Ligated upstream of the reporter gene is a known promoter sequence. The expression of the reporter gene is under the control of the cloned promoter and can be measured using various approaches and under various growth conditions. This set-up allows fluorescence generated, under regulation of the promoter sequence, to be used as a proxy for promoter activity.

Promoter-probe vectors should be functional in multiple taxa, highly sensitive to allow the detection of promoters with weak activity, and highly stable *in vivo* in the absence of antibiotic selection [133].

A common problem with this system is the presence of high background levels of reporter gene expression preventing promoters with weak to moderate transcriptional signals from being detected. This can happen when transcription initiates upstream of the promoter fusion. To reduce this interference, transcriptional terminators can be introduced upstream of the multicloning site (MCS). Interestingly a single copy of the terminator can reduce noise by 94% and increase the overall sensitivity of the vector. It then makes sense that four tandem copies will reduce the background fluorescence further with 97% inhibition [133]. To achieve detectable levels of fluorescence the sensitivity of the reporter gene must also be taken into consideration, especially when fused to weakly transcribed promoters or when placed in low-copy number plasmids.

Finally, recombination events such as the upstream terminator binding with the downstream terminator resulting in the loss of the promoter-probe cassette should be assessed in transcriptional fusions before assaying for reporter gene activity [133].

4.2.2.1. pBBR1 Plasmid

An example of a promoter-probe vector that is highly functional in many taxa includes the pBBR1 plasmid. pBBR1 plasmid is a 2.6 kb plasmid isolated from *B. bronchiseptica* S87 and is known for containing a broad-host-range origin of replication and is small in size [131]. These broad-host-range cloning vectors are stable and able to replicate in a diverse array of species that include *E. coli, B. pertussis*, *Vibrio cholerae, Rhizobium meliloti, and Pseudomonas putida*. This plasmid can be transferred into these hosts via transformation or conjugation and contain a *lacZα* fragment from pBluescript II-KS and two open reading frames: 1) Rep, involved in plasmid replication, and 2) Mob, involved in mobilisation. pBBR1 is also speculated to have a high copy number in *B. pertussis*. A diagram of pBBR1 is shown in Figure 23 [131].
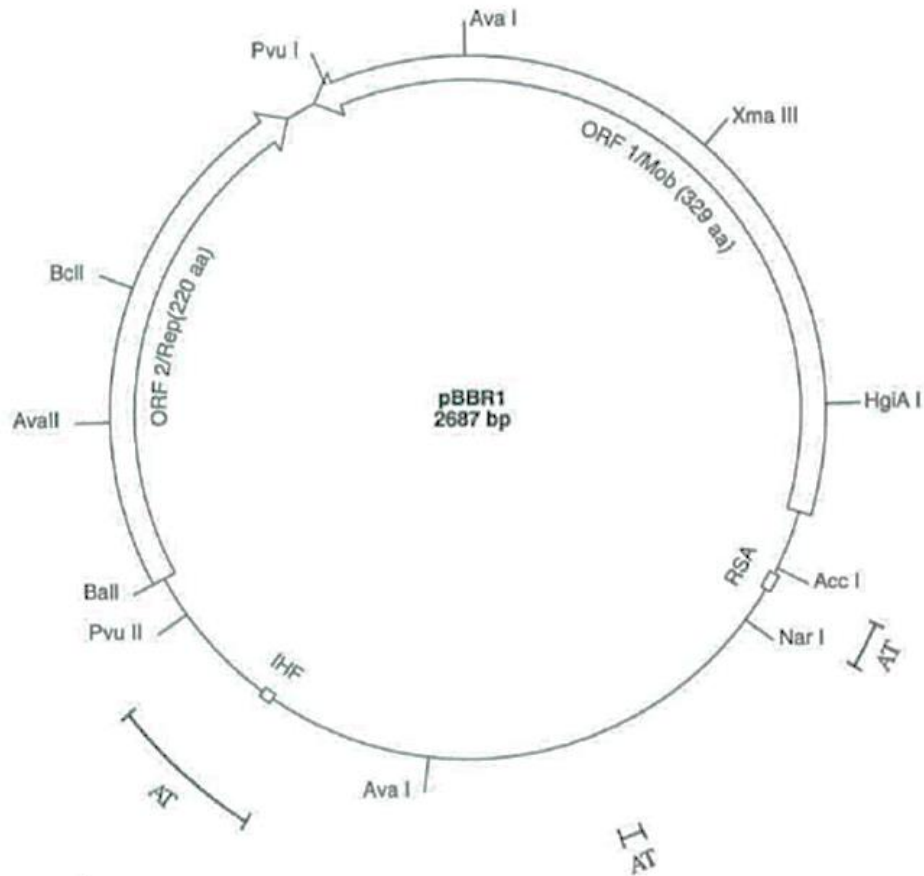
Figure 23. Plasmid map of pBBR1.

Restriction sites are indicated on this plasmid map, as well as A+T-rich regions indicated by 'AT'. Open arrows represent the two open reading frame Rep and Mob indicated by ORFI2/Rep and ORFI/Mob, respectively [131].

Other plasmids deriving from pBBR1 include pBBR1MCS, pBBR1MCS-2, pBBR1MCS-3, pBBR1MCS-4 and pBBR1MCS-5 conferring resistance to chloramphenicol (Cm), kanamycin (Km), tetracycline (Tc), ampicillin (Ap), and gentamicin (Gm), respectively (Figure 24) [133].

A)

ORF frame 3
ORF frame 3
NcoI (3715)
CAT/CamR (variant)
AatII (4513)
NarI (935)
ORF frame 3
ORF frame 2

pBBR1MCS
4707 bp

lac_promoter
M13_pUC_rev_primer
M13_reverse_primer
T3_promoter
KpnI (3321)
ApaI (3315)
pBluescriptKS_primer
XhoI (3302)
SalI (3296)
ClaI (3288)
HindIII (3281)
EcoRV (3277)
PstI (3267)
SmaI (3259)
XmaI (3257)
BamHI (3251)
SpeI (3245)
pBluescriptSK_primer
XbaI (3239)
SacII (3226)
SacI (3219)
T7_promoter
M13_forward20_primer
M13_pUC_fwd_primer
lacZ_a

AgeI (2752)

Figure 24. pBBR1MCS and four antibiotic resistant derivatives.

A) pBBR1MCS, B) pBBR1MCS-2, C) pBBR1MCS-3, D) pBBR1MCS-4, and E) pBBR1MCS-5 [175,176].

The pBB1MCS plasmid confers chloramphenicol resistance (Cm^R) and is a broad-host plasmid containing 16 unique cloning sites in the *lacZα* gene, is 4.7 kb in size, and has been moderately stable *in vitro* (>10 days) and *in vivo* in mice (>4 weeks) without antibiotic selection [175,176].

The pBBR1KAN vector was made in the Preston Lab and derives from the pBBR1MCS plasmid and includes an APH (aminoglycoside phosphotransferase gene) sequence insertion in the CAT (chloramphenicol acetyltransferase) or Cm cassette in the pBBR1MCS.

The APH cassette confers resistance to the aminoglycoside kanamycin and is used as an antibiotic marker [177].

This plasmid is of particular use in *B. pertussis* because this organism is not naturally resistant to kanamycin [133,178]. pBBRKAN was used to make all reporter constructs in this chapter.

4.2.2.2. Reporter Genes

The reporter genes for *gfp* were found on pBBR1-derived plasmids. pPROBE-NT was used to amplify the promoterless regular half-life *gfp* gene and pPROBE-gfp [AAV] was used to amplify the promoterless short half-life *gfp* gene. Both genes were used during the construction of reporter strains in this chapter [133].

In addition to the reporter gene *gfp*, *lacZ* may also be a useful alternative. However, some species naturally produce β-galactosidase, preventing the use of this probe in these organisms, resulting in it being less usable in comparison to *gfp*.

InaZ, on the other hand, produces ice nucleation and is highly sensitive allowing for the detection of weaker promoters. *gfp* is a less sensitive reporter in comparison to *InaZ*, however the construction of unstable *gfp* or short half-life variants has extended its use for real-time studies of gene regulation *in situ*. This is because wild-type *gfp* is a highly stable protein, with a half-life of greater than 24 hours extending over many bacterial generations. It should be noted though that this long half-life makes monitoring decreasing gene expression levels complicated, therefore a shorter half-life *gfp* variant would be more suitable.

4.2.2.2.1. Short half-life variants of *gfp*

In order to create short-half-life variants of *gfp*, wild-type *gfp* from pPROBE-NT was modified to include an AANDENYALAA tag at the C-terminal end. This reduced the half-life of the protein from >24 hours to approximately 1 hour in *E. coli*. Three different alleles have derived from this tag and include Gfp [LVA], Gfp [AAV], Gfp [ASV] with the final three codons of the tag altered and indicated in brackets. In *E. coli*, the half-lives of the LVA allele was 40 mins, while the AAV and ASV allele were 60 and 110 minutes, respectively [133].

4.2.2.3. *In vivo example of promoter-probe vectors in B. pertussis*

Transcriptional fusions have been utilised to study *B. pertussis* colonisation in the baboon model. Bacterial colonisation and the kinetics of colonisation during the course of disease has been difficult to observe thus far, however utilising a GFP-expressing *B. pertussis* strain derived from B1917 has made this possible creating a better understanding of pertussis

pathogenesis. This strain consisted of the pBBPG vector, derived from pBBR1MCS plasmid and carrying the *gfp*, under the control of the strong and constitutively expressed porin (Ppor) gene promoter (Figure 25) [179].
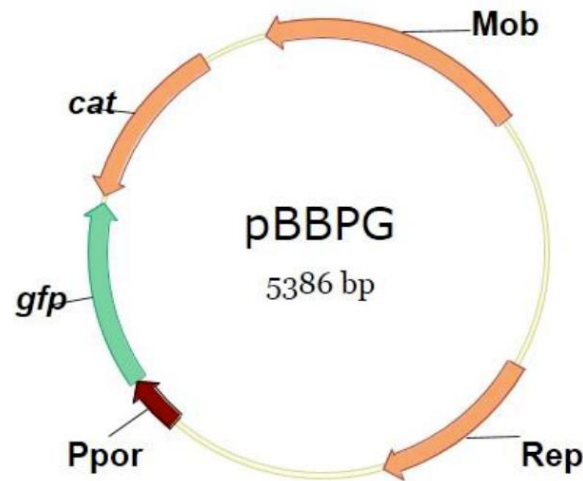
Figure 25. Plasmid map for pBBPG vector.

pBBPG is a GFP expressing vector used for transformation into *B. pertussis* B1917. This plasmid includes a plasmid replication of origin (Rep), mobility gene locus (Mob), chloramphenicol resistance gene (cat), green fluorescent protein (GFP) and porin promoter from *B. pertussis* (Ppor) [179]**.**

This reporter containing strain was used to monitor bacteria-host interactions in the lower airways of infected animals and visualise fluorescent bacterial localisation. *In vivo* imaging procedures and probe-based confocal laser endomicroscopy (pCLE) were used to assay fluorescence allowing colonisation and the susceptibility to infection and transmission to be assessed [179].

In vitro, B. pertussis B1917- GFP was highly fluorescent when grown on chloramphenicol-containing Bordet-Gengou (BG) plates. When grown in liquid culture and compared to wild-type B1917, the growth rate was comparable during the exponential phase. *In vivo*, the B1917-GFP strain colonised the upper respiratory tract of juvenile baboons and was able to induce infection with the classical symptoms of pertussis. Progressive bacterial colonisation of the trachea was detected in the first two weeks of infection in these animals using pCLE. A symptomatic infection was induced by B1917-GFP, B1917 and D420 alike in these 39-month-old baboons, regardless of the production of GFP illustrating that GFP did not affect clinical symptoms in a significant way. D420 is an American strain that is genetically similar to European isolate B1917.

As well, transmission was not impeded by the addition of the reporter gene to B1917, with naïve juvenile baboons co-housed in the same cage unit with juvenile baboons challenged with B1917-GFP and becoming colonised.

This study illustrates the effective use of promoter-probe vectors made with *gfp* in *B. pertussis* to detect bacterial colonisation and infection *in vivo*. Mentioned previously, a major drawback to using this system *in vivo* is the absence of selective pressure resulting in the steady loss of plasmid-mediated fluorescence limiting the length of this study to just two weeks. To circumvent this and allow long-term *B. pertussis* colonisation to be imaged, *gfp* can be cloned onto the *B. pertussis* genome to be maintained over generations [180].

The BvgAS two-component system regulates virulence gene expression, but also plays a role in phenotypic modulation. To better understand what phase the bacteria is in *in vivo* during an infection, such as in the case of the baboon model or the human challenge model, *B. pertussis* strains containing promoter-probe vectors or reporter constructs under the control of promoters of genes highly expressed in each Bvg phase could be used. The construction of these reporter constructs was the aim of the first part of this chapter and will enable the growth dynamics and sensitivity to modulation of different strains of *B. pertussis* to be studied.

4.3. Results

4.3.1. Creating reporter constructs to indicate the Bvg phase of *B. pertussis*

Reporter strains were created by conjugating vectors containing Bvg-regulated promoters ahead of promoterless fluorescent protein coding sequences into different strains of *B. pertussis*. The fluorescence levels produced by these strains were taken to indicate the activity of the Bvg-regulated promoters, and thus the Bvg phase of the strains.

To determine the viability of this system, the fluorescence of these reporter constructs was tested using a variety of tools.

4.3.1.1. GFP, YFP, and RFP were selected as reporter proteins for reporter constructs

To determine useable fluorescent reporters for this system, reporter constructs were created using the highly expressed Bvg plus regulated promoter, pertussis toxin (*ptxP),* ligated

ahead of the promoterless short half-life variant of *gfp,* and regular half-life variant of *yfp*, *rfp* (mKate) and *cfp* on the plasmid pBBR1KAN. Figure 26 shows the fluorescence signal of these reporter constructs in *B. pertussis* strain BP536, where the background fluorescence (noise) obtained from the pBBR1KAN vector only was subtracted from the fluorescent signal from the reporter construct.
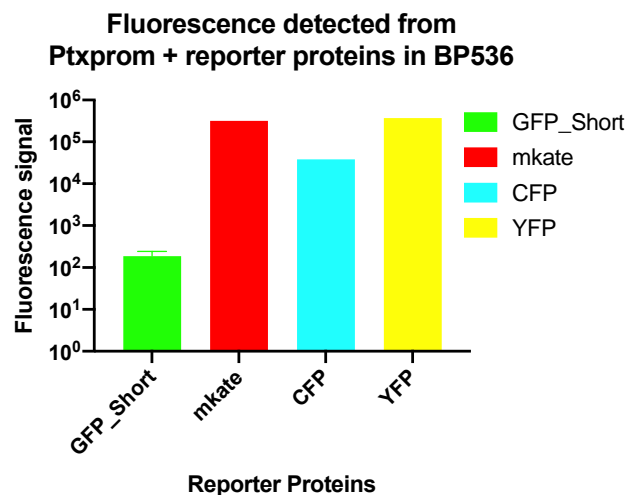
Figure 26. The most sensitive reporter proteins are YFP and RFP.

Under the control of the *ptx* promoter, the highest levels of fluorescence determined were red and yellow indicating that RFP and YFP were highly sensitive reporter proteins, while the short half-life variant of GFP and the regular half-life CFP were less so. This data represents two biological repeats.

YFP and RFP were selected for future work and determined to be the most sensitive reporter proteins with a fluorescence signal of 372089 and 317550, respectively. The short half-life GFP variant and CFP were not as sensitive with a fluorescent signal of 186 and 38419, respectively. CFP was abandoned for use in futher experiments and the short half-life GFP was deemed unsuitable for use with genes expressed at low levels. However, the use of the regular half-life variant of GFP was to be investigated instead in future experiments in order to determine if it was more sensitive than the short half-life variant.

4.3.1.2. Promoters from ptxA, bipA, and vrgX genes were selected as Bvg regulated promoters

To determine usable promoters for the construction of reporter constructs, promoters of genes highly expressed in each Bvg phases were selected. Expression levels were elucidated from RNA sequencing (RNA-seq) completed previously in the Preston Group (unpublished).

Table 17 displays the promoters chosen for each Bvg phase. The fold change represents the difference in gene expression between Bvg phases. The Bvg minus gene, *vrgX*, was

selected based on a recent annotation of the BP536 genome identifying this gene as being highly expressed in the Bvg minus phase [181].

Table 17. Promoters of genes selected for use in reporter constructs.

Bvg⁺		
Gene	**Product**	**Fold Change (Expression in Bvg⁺ compared to Bvg⁻)**
2924	Putative exported protein	183.7
1054 (prn)	Pertactin precursor	97.4
2936	Putative exported protein	70.4
1879 (fhaB)	Filamentous hemagglutinin/ adhesin	70.8
3783 (ptxA)	Pertussis toxin subunit 1 precursor	44.2
1119 (fim2)	Serotype 2 fimbrial subunit precursor	30.2
Bvgⁱ		
Gene	**Product**	**Fold Change (Expression in Bvgⁱ compared to Bvg⁺)**
1879 (fhaB)	Filamentous hemagglutinin/adhesin	67.3
1112 (bipA)	Putative outer membrane ligand binding protein	31.0
1878 (bvgA)	Virulence factors transcription regulator	3.5
0682	Putative exported protein	2.4
Bvg⁻		
Gene	**Product**	**Fold Change (Expression in Bvg⁻ compared to Bvg⁺)**
1623 (kpsM)	Putative polysialic acid transport protein	119.1
1620	Putative glycosyl transferase	46.4
2782	Lipoprotein	45.7
3517	Putative membrane protein	19.6
0996 (flaA)	Flagellin	0.9
vrgX	Virulence repressed protein	Not available
Constitutively expressed		
Gene	**Function**	**Fold Change**
0840	Outer membrane porin protein precursor	**Expression in Bvg⁺ compared to Bvg⁻** 1.2
		Expression in Bvgⁱ compared to Bvg⁺ 1.2
		Expression in Bvg⁻ compared to Bvg⁺ 0.8

The selected promoters were used to make reporter constructs with the short half-life variant of *gfp* and the regular half-life variants of *gfp, rfp* and *yfp*. The nomenclature for each reporter construct made and analysed in this chapter can be found in Table 18.

Table 18. Nomenclature for reporter constructs.

Bvg ⁺			
Gene	**Reporter Protein**	**Nomenclature**	**Comments**
2924	Short half-life GFP	*2924GFP_S*pBBRK	
1054 (prn)		*prnGFP_S*pBBRK	
2936		*2936GFP_S*pBBRK	
1879 (fhaB)		*1879GFP_S*pBBRK	
3783 (ptxA)		*ptxAGFP_S*pBBRK	
1119 (fim2)		*1119GFP_S*pBBRK	
2936	Regular half-life GFP	*2936GFP*pBBRK	
3783 (ptxA)		*ptxAGFP*pBBRK	
2924	Regular half-life YFP	*2924YFP*pBBRK	
2936		*2936YFP*pBBRK	
3783 (ptxA)		*ptxAYFP*pBBRK	
3783 (ptxA)	Regular half-life RFP	*ptxARFP*pBBRK	
Bvgⁱ			
Gene	**Reporter Protein**	**Nomenclature**	**Comments**
1879 (fhaB)	Short half-life GFP	*1879GFP_S*pBBRK	
1112 (bipA)		*bipAGFP_S*pBBRK	
1878 (bvgA)		*1878GFP_S*pBBRK	
0682		*0682GFP_S*pBBRK	
0682	Regular half-life RFP	*0682RFP*pBBRK	
1879		*1879RFP*pBBRK	
bipA		b*ipARFP*pBBRK	
0682	Regular half-life YFP	*0682YFP*pBBRK	
Bvg⁻			
Gene	**Reporter Protein**	**Nomenclature**	**Comments**
1623 (kpsM)	Short half-life GFP	*kpsMGFP_S*pBBRK	
1620		*1620GFP_S*pBBRK	
2782		*2782GFP_S*pBBRK	
3517		*3517GFP_S*pBBRK	
0996 (flaA)		*flaAGFP_S*pBBRK	
vrgX		*vrgXGFP_S*pBBRK	
vrgX	Regular half-life YFP	*vrgXYFP*pBBRK	
2782		*2782YFP*pBBRK	
1623 (kpsM)		*kpsMYFP*pBBRK	
1623 (kpsM_2)		*kpsM_2YFP*pBBRK	Larger intergenic region for *kpsM* promoter
0996 (flaA)		*flaAYFP*pBBRK	
0996 (flaA_S)		*flaA_SYFP*pBBRK	Shorter intergenic region for *0996* promoter
3517		*3517YFP*pBBRK	
3783 (ptxA)		*ptxAYFP*pBBRK	
1054 (prn)		*prnYFP*pBBRK	
Constitutively Expressed			
Gene	**Reporter Protein**	**Nomenclature**	**Comments**
0840	Short half-life GFP	*0840GFP_S*pBBRK	
	Regular half-life GFP	*0840GFP*pBBRK	
	Regular half-life YFP	*0840YFP*pBBRK	

4.3.1.3. Testing reporter constructs for fluorescence

Fluorescent microscopy, flow cytometry, and measurement of fluorescence in a plate reader were all used to determine the fluorescence of *B. pertussis* carrying the reporter constructs, grown in modulated and unmodulated conditions.

4.3.1.4. Fluorescence microscopy

Figure 27 shows a selection of images taken of the strain BP536 containing reporter constructs made with short half-life *gfp*. These cultures were grown in either Bvg plus or minus conditions (indicated below each image) to qualitatively assess fluorescence.

A)

B)

Bvgi: *BP1878* (*bvgA*)	
X40 Bvg- Clone 1 2018-10-04	X40 Bvg+ Clone 1 2018-10-13

C)

Bvg-: *BP1620*	Bvg-: *BP2782*
X40 Bvg- Clone 1 2018-10-13	X60 Bvg- Clone 1 2018-10-02

D)

Constitutively expressed: *BP0840*	
X40 Bvg+ Clone 1 2018-09-27	X40 Bvg- Clone 3 2018-10-02

E)

Positive Control*: B. bronchiseptica* RBpagprom2b	
X40 Bvg+ Clone 1 2018-10-13	X40 Bvg+ Clone 2 2018-10-13

F)

Negative Control: Empty Vector in *B. pertussis* BP536	
X40 Bvg+ Clone 1 2018-09-27	X40 Bvg- Clone 2 2018-10-04

Figure 27. Fluorescent images of Bvg reporter constructs made with the short half-life variant of GFP in B. pertussis BP536.

A) Bvg plus reporter constructs grown in Bvg plus conditions, B) Bvgi reporter constructs grown in both Bvg plus and minus conditions, C) Bvg minus reporter constructs grown in Bvg minus conditions, D) Constitutively expressed reporter construct 0*840GFP*_SpBBRK grown in both Bvg plus and minus conditions, E) Positive fluorescence control *B. bronchiseptica* RBpagprom2b grown in Bvg plus conditions, and F) Negative control consisting of an empty vector in *B. pertussis* grown in both Bvg plus and minus conditions. The reporter constructs *2782GFP*_SpBBRK, *ptxAGFP*_SpBBRK and *0840GFP*_SpBBRK provided varying levels of observable fluorescence when grown in Bvg minus, plus and in both Bvg minus and plus conditions, respectively, and are outlined with a red box. Biological repeats were used to validate these results.

The only reporter constructs that provided detectable fluorescence using microscopy were: *2782GFP_S*pBBRK grown in the Bvg minus phase, *ptxAGFP_S*pBBRK grown in the Bvg plus phase, and *0840GFP_S*pBBRK grown in both Bvg plus and minus conditions. Interestingly when qualitatively evaluating the number of fluorescent cells in *ptxAGFP_S*pBBRK, the majority of bacterial cells appeared to be fluorescent, while for *0840GFP_S*pBBRK only 75% of bacterial cells fluoresced. For *2782GFP_S*pBBRK, approximately 25% of bacterial cells fluoresced.

These results illustrate promising preliminary results where the reporter constructs *2782GFP_S*pBBRK grown in the Bvg minus phase and *ptxAGFP_S*pBBRK grown in the Bvg plus phase were suitable for use in future work. This includes confirming the fluorescence observed with fluorescence microscopy with flow cytometry and the microplate plate reader. These two methods were also used to test reporter constructs for fluorescence in all phases including the Bvg intermediate phase, which was not tested for using fluorescence microscopy.

4.3.1.5. Flow cytometry

To confirm the fluorescence signal obtained from the Bvg reporter constructs observed under the microscope, I sent samples to Public Health England to be analysed by flow cytometry.

The amount of fluorescence and the number of fluorescent cells can be determined using flow cytometry.

Figure 28 represents flow cytometry data obtained from comparing promoter activity and half-life variants of *2936* and *gfp,* respectively. Reporter constructs were evaluated in BP536 grown in the absence of $MgSO_4$. The reporter construct with the promoter *BP0840* was used as a positive fluorescence control because this gene is constitutively expressed in all Bvg phases. Additionally, the empty vector was used as a negative control to illustrate an absence of fluorescence. The x axis of the histogram represents the fluorescence intensity produced by the fluorescent proteins in the sample and is directly proportional to the promoter activity, while the y axis represents the number of cells expressing the fluorescent protein in the sample.
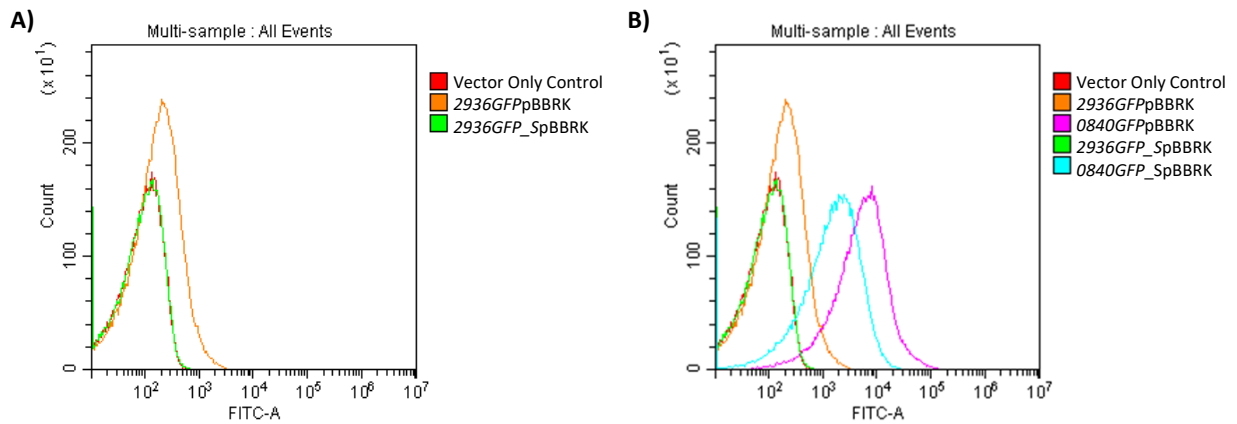
Figure 28. Comparison of fluorescence from reporter constructs containing different promoters and GFP variants in BP536.

A) Overlay of the reporter constructs *2936GFP*pBBR and *2936GFP*_SpBBR compared to the pBBR1KAN vector only control, and B) Overlay of the reporter constructs *2936GFP*pBBR and *2936GFP*_SpBBR compared to *0840GFP*pBBR, *0840GFP*_SpBBR and the pBBR1KAN vector only control. The fluorescence intensity of reporter constructs made with the *2936* promoter was higher with the regular half-life GFP variant compared to the short half-life variant. This data represents two biological duplicates.

These samples were overlayed in Figure 28A illustrating an increase in fluorescence intensity for reporter constructs made with *BP2936* and the regular half-life *gfp* compared to the constructs made with the short half-life *gfp* in comparison to the fluorescence from the vector only control. This increase in fluorescence intensity using regular half-life *gfp* compared to short half-life *gfp* was echoed in reporter constructs made with the *BP0840* promoter (Figure 28B).

These results provide support for the use of the *BP2936* promoter for the creation of a Bvg plus reporter construct. Additionally, regular half-life GFP has been shown to be a sensitive reporter protein for future work.

Additional reporter constructs were created using the fluorescent proteins YFP and RFP in *B. pertussis* strains BP536 and B1917. For the majority of samples, the cell population was relatively uniform, however some samples had a spectrum of variation that was slightly more complex. Figure 29 represents the dot plot of the reporter construct of *kpsM_2YFP*pBBRK in BP1917 grown in both Bvg plus and minus conditions. In Figure 29A, the diagonal smear in the Bvg plus sample had a higher forward scatter (FSc) and side scatter (SSc) profile compared to Bvg minus grown sample in Figure 29B, indicating larger and more complex cells, respectively. This is likely due to the increase in clumping of cells observed in Bvg plus grown samples. The diagonal smear in these figures represents fluorescence from multiple cells but have inaccurately been represented as single events in these figures. To correct this problem and obtain accurate fluorescence for single cells, the gating strategy was

revised. Figure 29C reflects the revised gating strategy in the Bvg plus dot plots to reflect where the majority of events lie in Bvg minus dot plots (Figure 29D).
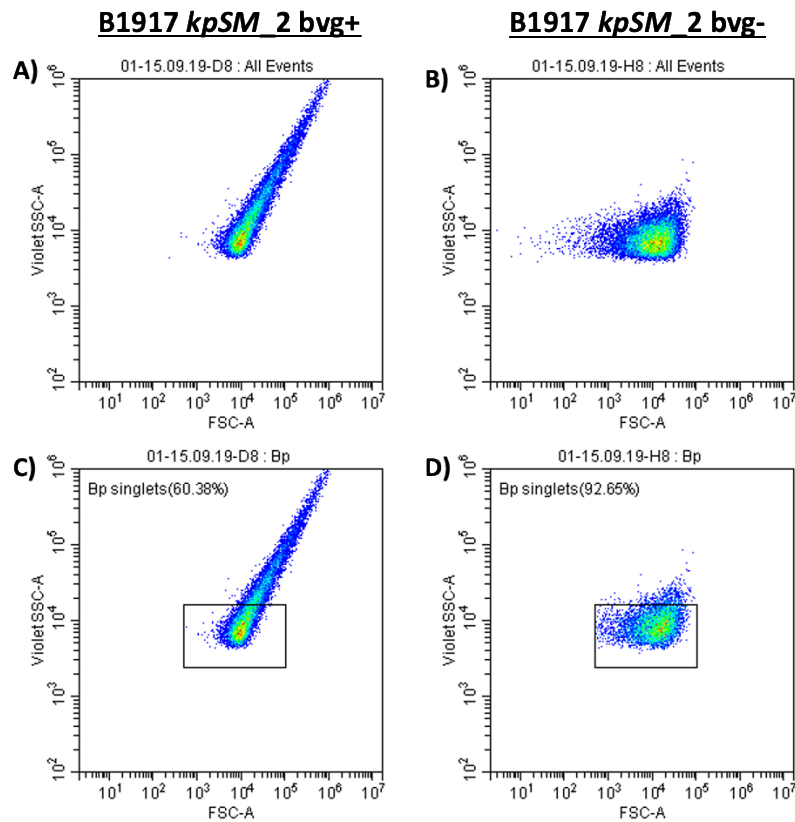
Figure 29. Revised gating strategy for reporter constructs with a spectrum of variation indicated with diagonal smear.

A) Spectrum of variation *kpsM_2YFP*pBBRK in BP1917 with diagonal smear in Bvg plus conditions, B) Spectrum of variation for *kpsM_2YFP*pBBRK in BP1917 without diagonal smear in Bvg minus conditions, C) Gating around majority of events for *kpsM_2YFP*pBBRK in BP1917 in Bvg plus conditions, and D) Gating around majority of events for *kpsM_2YFP*pBBRK in BP1917 in Bvg minus conditions. Due to clumping observed in Bvg plus grown samples, a diagonal smear was produced. Fluorescence in these samples represents duplets and more, rather than single events. To correct for clumping, the gating strategy was revised to focus on the majority of events allowing the fluorescence of singlets to be captured.

In addition to the revised gating strategy, the robust coefficient of variation (%rCV) was employed to each sample in order to minimise the impact of outliers on performance parameters.

Figure 30 illustrates the fluorescence intensity of the reporter constructs *2782YFP*pBBRK, *ptxAYFP*pBBRK, *vrgXYFP*pBBRK, *0996YFP*pBBRK, and *0996_SYFP*pBBRK compared to the pBBR1KAN vector control in BP536 grown in Bvg plus and minus conditions. Figure 31 represents the fluorescence intensity of these reporter constructs and vector control in B1917 grown in the same conditions.
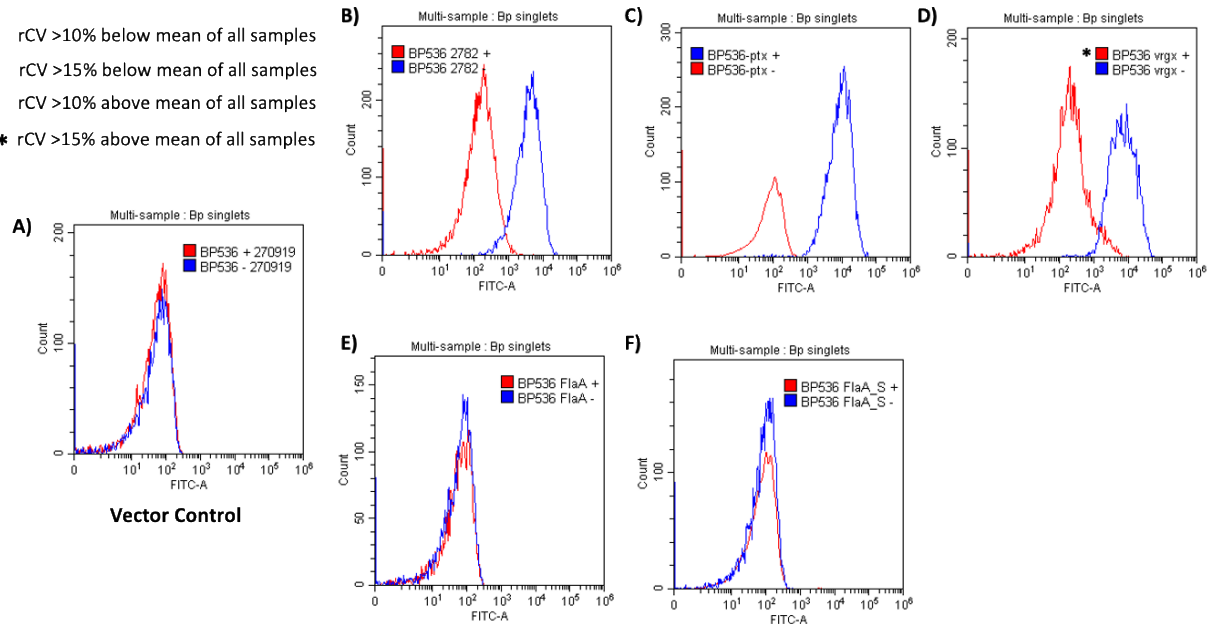
Figure 30. Fluorescence intensity from reporter constructs made with YFP in BP536 grown in both Bvg plus and minus conditions.

A) pBBR1KAN vector only control, B) *2782YFP*pBBRK, C) *ptxAYFP*pBBRK, D) *vrgXYFP*pBBRK, E) *0996YFP*pBBRK, and F) *0996_SYFP*pBBRK. *ptxAYFP*pBBRK grown in Bvg plus conditions, and both *2782YFP*pBBRK and *vrgXYFP*pBBRK grown in Bvg minus conditions produced fluorescence above the background fluorescence from the pBBR1KAN vector control. The data represents two biological duplicates.
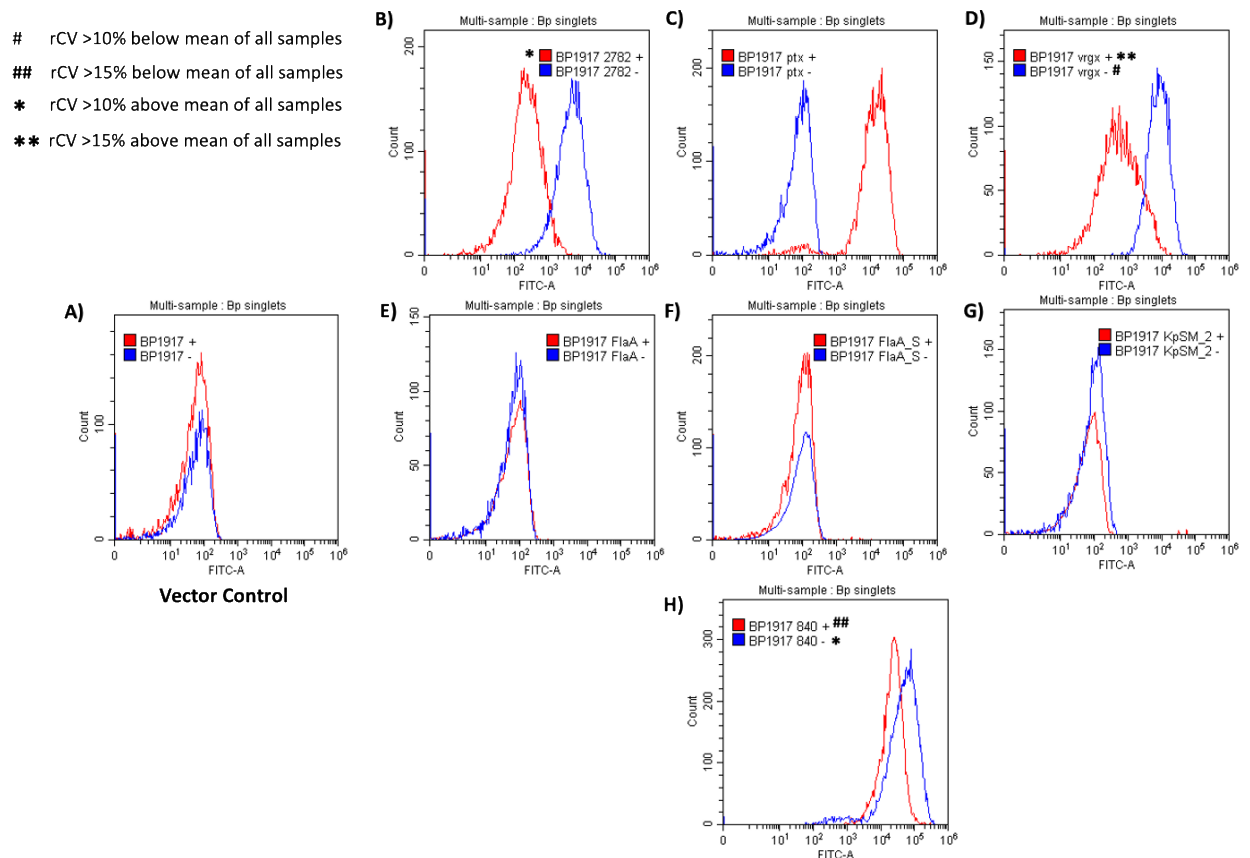
Figure 31. Fluorescence intensity from reporter constructs made with YFP in B1917 grown in both Bvg plus and minus conditions.

A) pBBR1KAN vector only control, B) *2782YFP*pBBRK, C) *ptxAYFP*pBBRK, D) *vrgXYFP*pBBRK, E) *0996YFP*pBBRK, F) *0996_SYFP*pBBRK, G) *kpsM_2YFP*pBBRK and H) *0840YFP*pBBRK. *ptxAYFP*pBBRK and *0840YFP*pBBRK grown in Bvg plus conditions and *2782YFP*pBBRK, *vrgXYFP*pBBRK, and *0840YFP*pBBRK grown in Bvg minus conditions produced fluorescence above the background fluorescence from the pBBR1KAN vector control. The data represents two biological duplicates.

The reporter constructs *2782YFP*pBBRK and *vrgXYFP*pBBRK grown in Bvg minus conditions and *ptxAYFP*pBBRK grown in Bvg plus conditions were selected as useable reporter constructs for future work because they provided a higher fluorescence intensity compared to the pBBR1KAN vector only control in both BP536 and B1917. In B1917, *2782YFP*pBBRK grown in Bvg plus conditions had a %rCV> 10% above the mean of all samples in Bvg plus conditions supporting the complexity of analysing samples with a tendency to clump using flow cytometry. This was echoed with the reporter construct *vrgXYFP*pBBRK in BP536 and BP1917, which had a %rCV>10% and %rCV>15%, respectively. Interestingly, in Bvg minus conditions, the %rCV>15% of *vrgXYFP*pBBRK was below the mean of all samples.

*0840YFP*pBBRK was selected as a control for fluorescence when grown in both Bvg plus and minus conditions, However, the reporter constructs *kpsM_2YFP*pBBRK, *0996YFP*pBBRK, and *0996_SYFP*pBBRK grown in both Bvg conditions were not selected

as usable reporter constructs for future work because they did not fluoresce above the pBBR1KAN vector control.

Figure 32 illustrates the fluorescence intensity of the reporter constructs *0682RFP*pBBRK, *1879RFP*pBBRK, *bipARFP*pBBRK, *ptxARFP*pBBRK in BP536. Figure 33 illustrates the fluorescence intensity of these reporter constructs in B1917 except *ptxARFP*pBBRK. All samples were grown in both Bvg plus and intermediate conditions.
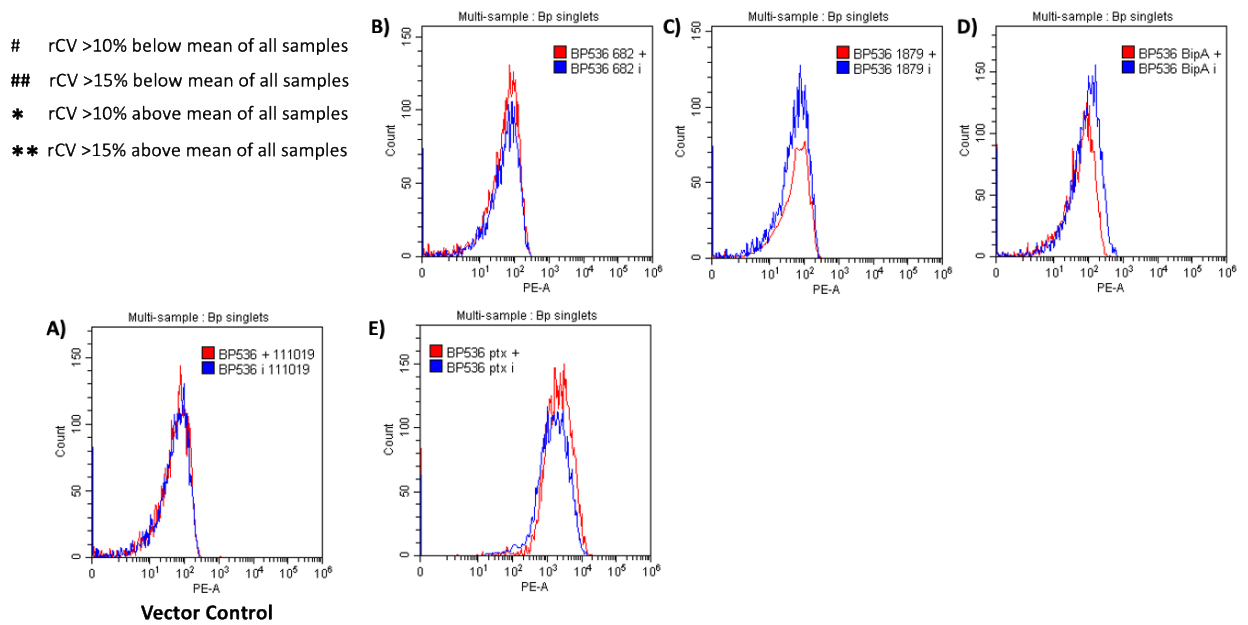
Figure 32. Fluorescence intensity from reporter constructs made with RFP grown in Bvg plus and intermediate conditions in BP536.

A) pBBR1KAN vector control, B) *0682RFP*pBBRK, C) *1879RFP*pBBRK, D) *bipARFP*pBBRK, and E) *ptxARFP*pBBRK. *ptxARFP*pBBRK grown in Bvg plus conditions and *bipARFP*pBBRK grown in Bvg intermediate conditions produced fluorescence above the background fluorescence from the pBBR1KAN vector control. The data represents two biological duplicates.
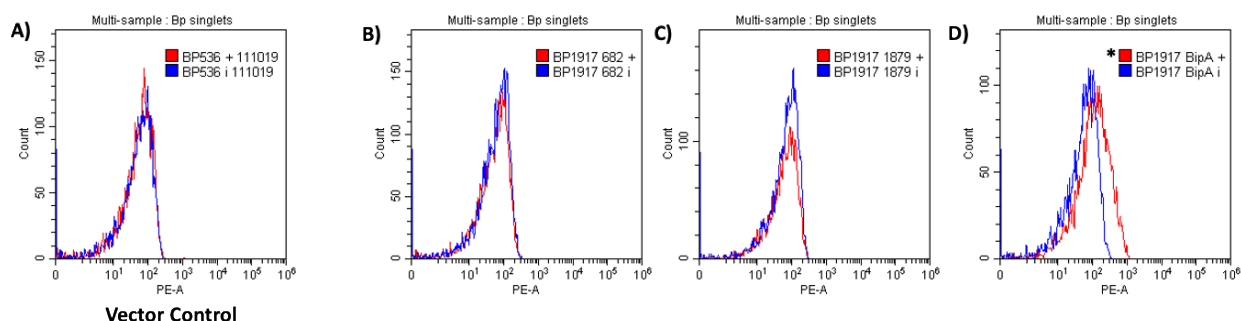
Figure 33. Fluorescence intensity from reporter constructs made with RFP grown in Bvg plus and intermediate conditions in B1917.

A) pBBR1KAN vector only control, B) *0682RFP*pBBRK, C) *1879RFP*pBBRK, and D) *bipARFP*pBBRK. *ptxARFP*pBBRK and *bipARFP*pBBRK grown in Bvg plus conditions produced fluorescence above the background fluorescence from the pBBR1KAN vector control in BP536. The data represents two biological duplicates.

In *B. pertussis* strains BP536 and B1917, the reporter constructs *0682RFP*pBBRK and *1879RFP*pBBRK did not fluoresce and were therefore unusable for the Bvg intermediate phase.

The reporter construct *ptxARFP*pBBRK in BP536 provided a high level of fluorescence when grown in Bvg plus conditions, echoing the fluorescence previously observed with reporter constructs made with the *ptxA* promoter ahead of yellow and green fluorescent proteins. In Bvg intermediate conditions, however, *ptxARFP*pBBRK provided slightly less fluorescence than in Bvg plus conditions. This was to be expected based on the known Bvg dependence of Pertussis toxin expression and supported by RNA-seq data [52].

Also expected, *bipARFP*pBBRK provided a slightly higher level of fluorescence intensity when grown in Bvg intermediate conditions compared to Bvg plus conditions in BP536. Interestingly, *bipARFP*pBBRK in B1917 produced a higher level of fluorescence when grown in Bvg plus conditions compared to Bvg intermediate conditions. Despite this discrepancy, these observations confirmed the use of *bipARFP*pBBRK to investigate entry of *B. pertussis* into the Bvg intermediate phase. Additionally, the %rCV of *bipARFP*pBBRK grown in Bvg plus samples was >10% above the mean of all samples.

In the next section, the sensitivity of selected Bvg reporter constructs to modulation among different strains was investigated (Figure 36- Figure 37).

The flow cytometry data representing the fluorescence intensity from reporter constructs made with GFP, YFP and RFP in both BP536 and B1917 was summarised in Figure 34. The

majority of samples fell within +/- 10% of the mean rCV and had similar variation based on this arbitrary cut-off.
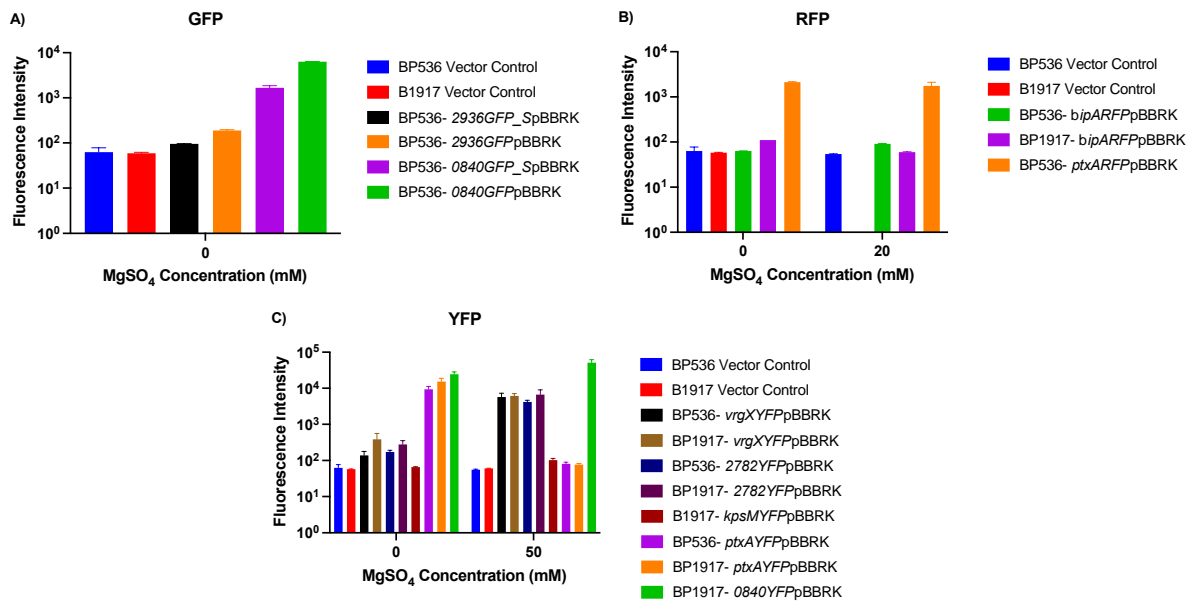
Figure 34. A summary of usable Bvg promoters determined by flow cytometry.

A) GFP, B) RFP, and C) YFP. The usable promoters for investigating entry into Bvg phenotypic phases are as follows: *ptxA* and BP*2936* in the Bvg plus phase, *bipA* in the Bvg intermediate phase and *2782*, and *vrgX* in the Bvg minus phase. The *0840* promoter was used as a positive fluorescence control for all Bvg phases because it is constitutively expressed. This data represents two biological repeats.

Based on the flow cytometry observations, the following promoters can be used to investigate entry into the Bvg phenotypic phases: Bvg plus— *ptxA* and *2936,* Bvg intermediate—*bipA*, and Bvg minus— *2782*, and *vrgX.* The promoter *0840* was selected as the positive control for fluorescence in all Bvg phases.

4.3.1.6.   Fluorescence microplate reader

To determine the fluorescence intensity of additional reporter constructs not analysed using flow cytometry or fluorescence microscopy and to confirm the fluorescence levels observed previously, the fluorescence microplate reader was employed.

Figure 35 provides a summary of these results illustrating the fluorescence of reporter constructs in *B. pertussis* BP536 and B1917.
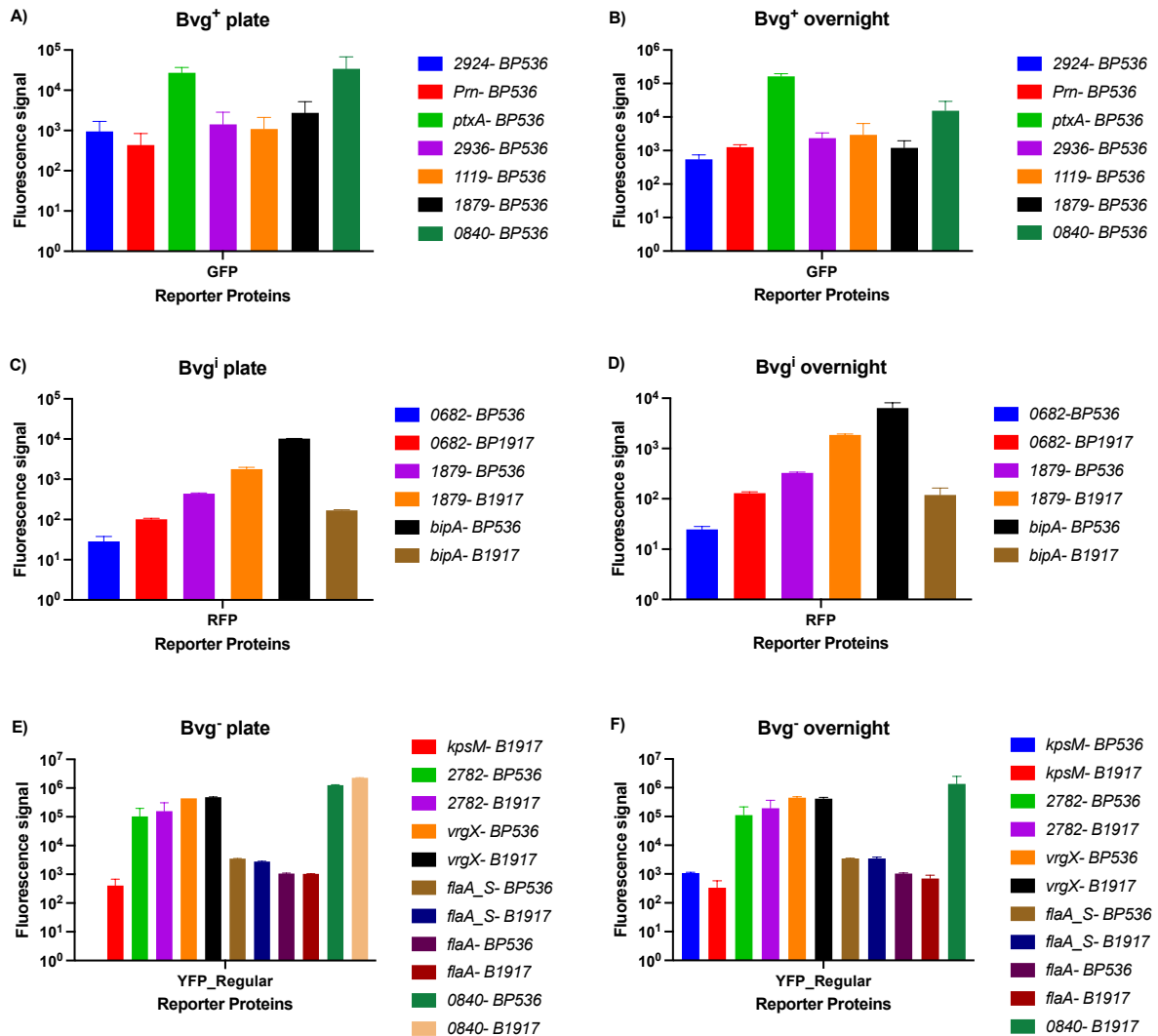
Figure 35. Fluorescence detected from reporter constructs in broth and plate grown cultures of B. pertussis BP536 and B1917.

A) Plate cultures of reporter constructs made with GFP in BP536, B) Overnight cultures of reporter constructs made with GFP in BP536, C) Plate cultures of reporter constructs made with RFP in both BP536 and B1917, D) Overnight culture of reporter constructs made with RFP in both BP536 and B1917, E) Plate cultures of reporter constructs made with YFP in both BP536 and B1917, and F) Overnight culture of reporter constructs made with YFP in both BP536 and B1917. Based on plate and broth cultures, *ptxA, bipA,* and *vrgX* were confirmed as usable promoters in reporter constructs to determine entry into the Bvg plus, intermediate and minus phase, respectively.

The reporter constructs that provided the most fluorescence in the Bvg plus, intermediate and minus phase were *ptxAGFP*pBBRK, b*ipARFP*pBBRK, and *vrgXYFP*pBBRK, respectively (Figure 35). Reporter constructs made with the promoter *0840* were also confirmed to be highly fluorescent in both Bvg plus and minus conditions. Reporter constructs *ptxAYFP*pBBRK and *2936YFP*pBBRK, not represented in Figure 35, also provided fluorescence above the background noise when grown as overnight cultures in Bvg plus conditions.

Interestingly, reporter constructs *ptxAYFP*pBBRK had detectable levels of fluorescence in both the Bvg plus and minus phase despite being designed to observe fluorescence in the Bvg plus phase only, however, the fluorescence detected in the Bvg minus phase was approximately 80% less than the fluorescence detected in Bvg plus conditions. Therefore, reporter constructs made with the *ptxA* promoter were confirmed to be usable to determine entry into the Bvg plus phase.

*2936YFP*pBBRK was designed to determine entry into the Bvg plus phase, however, when grown in Bvg minus conditions provided comparable fluorescence to levels produced in Bvg plus conditions. Therefore promoter *2936* was unusable as a Bvg plus regulated promoter because the reporter construct containing this promoter did not provide clear and distinct fluorescent levels between Bvg phases.

The plate reader results for the reporter construct b*ipARFP*pBBRK grown in both B1917 and BP536 confirmed earlier flow cytometry results where, in Bvg intermediate conditions, there was an increase in fluorescence of this reporter construct in BP536 compared to a 98% reduction in fluorescence when in B1917. In Bvg plus conditions, the reverse was observed where there was a 75% increase in the fluorescence of b*ipARFP*pBBRK in B1917 compared to B536.

In Bvg minus conditions, both *2782YFP*pBBRK and *vrgXYFP*pBBRK provided a high fluorescent signal in both strains of *B. pertussis* tested, BP536 and B1917, with a 95% more fluorescence when grown in Bvg minus conditions compared to Bvg plus conditions. However, *vrgXYFP*pBBRK was three times more fluorescent than *2782YFP*pBBRK and nearly 500 times more fluorescent than *kpsMGFP*pBBRK in both strains of *B. pertussis* and was therefore used in future experiments.

Armed with fluorescent data from the fluorescent microplate reader, fluorescent microscope and flow cytometry for multiple reporter constructs made with GFP, RFP and YFP, the use of *ptxA* as a usable Bvg plus promoter, *bipA* as a useable Bvg intermediate promoter, *vrgX* as a useable Bvg minus promoter, and *0840* as a promoter that is constitutively expressed in all Bvg phases was confirmed.

4.3.2. Investigating the sensitivity of reporter constructs to chemical modulation in six different *B. pertussis* strains

This section will explore the sensitivity of the Bvg reporter constructs, *ptxAGFP*pBBRK, *bipARFP*pBBRK, and *vrgXYFP*pBBRK, to the chemical modulator $MgSO_4$ in six different

strains of *B. pertussis*. Any observable differences in phenotype were compared to the genotype of these six strains.

4.3.2.1. The sensitivity of six different B. pertussis strains to the chemical modulator MgSO₄ were variable

To investigate the sensitivity of *B. pertussis* to chemical modulation, different concentrations of MgSO$_4$ were used to modulate *B. pertussis* containing reporter constructs into the Bvg plus, intermediate and minus phase.

The reporter constructs were conjugated into the following four additional strains of *B. pertussis*: UK48, UK71, B184 and B204. Entry into different Bvg phases was determined in these four strains as well as BP536 and B1917.

Figure 36 represents reporter constructs grown in 10 mM increments of MgSO$_4$. In Figure 37, the increments were reduced to 4 mM to hone-in on the exact concentration of MgSO$_4$ required for maximal expression of Bvg-regulated genes.
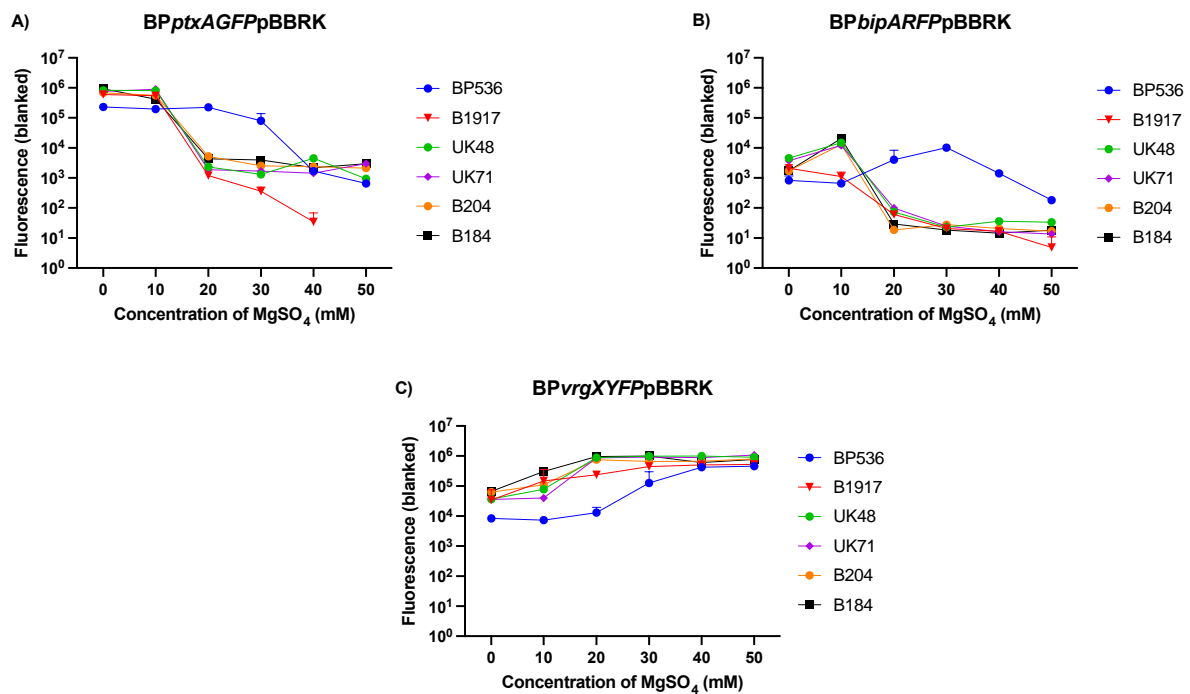
Figure 36. Reporter constructs in six strains of B. pertussis grown in 10 mM increments of MgSO₄.

A) *ptxAGFP*pBBRK, B) *bipARFP*pBBRK, and C) *vrgXYFP*pBBRK. 0 mM of MgSO$_4$ produced the maximal fluorescence for *ptxAGFP*pBBRK in all strains. For the reporter construct *bipARFP*pBBRK, maximal fluorescence occurred when grown in 30 mM of MgSO$_4$ for BP536, and in 10 mM for the rest of the strains. Maximal fluorescence for *vrgXYFP*pBBRK was observed when grown in 40-50 mM of MgSO$_4$ for BP536, 30-50 mM of MgSO$_4$ for B1917, and 20-50 mM of MgSO$_4$ for UK48, UK71, B184 and B204.
Data represents biological triplicates.
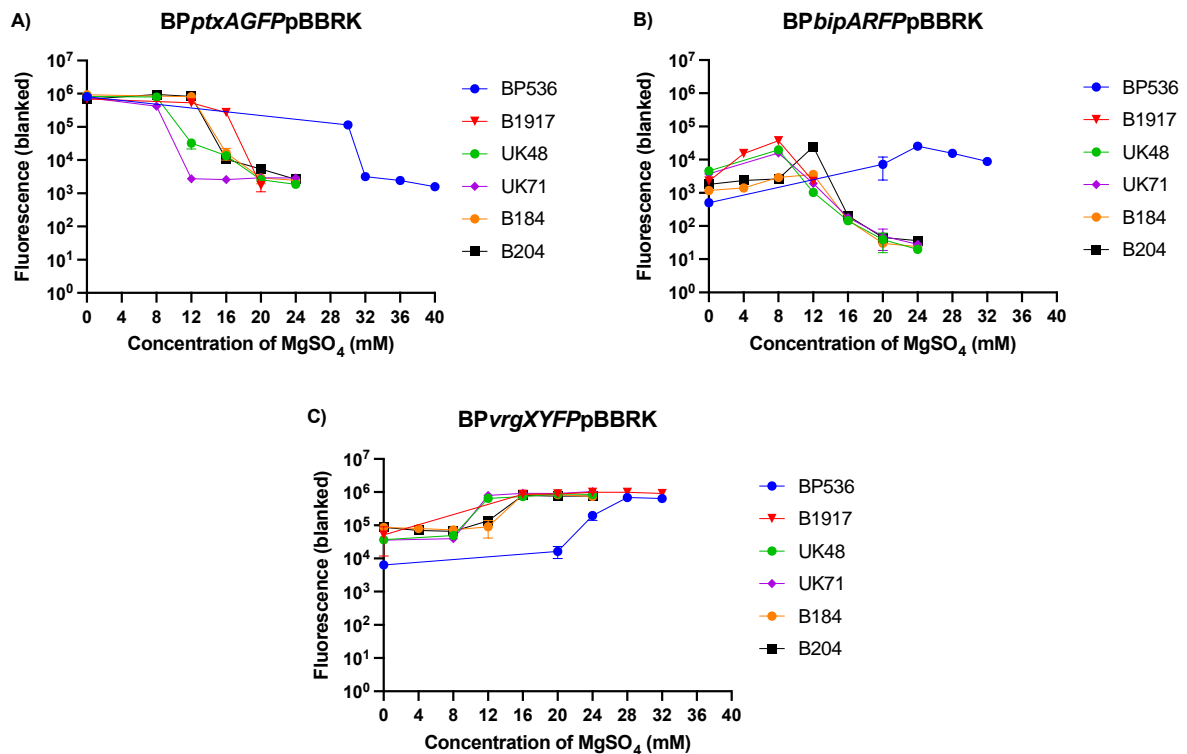
Figure 37. Reporter constructs in six strains of B. pertussis grown in 4 mM increments of MgSO₄.

A) *ptxAGFP*pBBRK, B) *bipARFP*pBBRK, and C) *vrgXYFP*pBBRK. The reporter constructs were grown with 4 mM increments of $MgSO_4$. 0 mM of $MgSO_4$ resulting in the reporter construct *ptxAGFP*pBBRK producing the most fluorescence in all strains of *B. pertussis* tested. Maximal fluorescence was achieved using 24, 12, and 8 mM of $MgSO_4$ for the reporter construct b*ipARFP*pBBRK in BP536, both B184 and B204, and the remaining three strains of B1917, UK48, UK71, respectively. Growing *vrgXYFP*pBBRK in in 28-50 mM of $MgSO_4$ for BP536, 16-50 mM of $MgSO_4$ for B1917, B184 and B204, and 12-50 mM of $MgSO_4$ for UK48 and UK71 yielded the most fluorescence. Data represents biological triplicates.

These experiments revealed that although BP536 appears to enter the Bvg phase in unmodulated conditions, similarly to all of the other strains tested here, there are marked differences in the $MgSO_4$ concentration required for entry into the other two Bvg phases. Specifically, a higher concentration of 24 mM and 28 mM of $MgSO_4$ was required for entry into the Bvg intermediate and minus phase, respectively. There was reduced activity of the Bvg plus phase reporter construct, *ptxAGFP*pBBRK, at approximately 30 mM $MgSO_4$. However, this reduction is likely to have occurred earlier, at approximately 24 mM $MgSO_4$, but was not tested for. Coincidently, as the fluorescence of *ptxAGFP*pBBRK was reduced, there was increased activity of the Bvg intermediate reporter construct, b*ipARFP*pBBRK. At 28 mM of $MgSO_4$, there was an immediate decline of fluorescence for this Bvg intermediate reporter, and increased activity of the Bvg minus reporter *vrgXYFP*pBBRK that was sustained until 50 mM of $MgSO_4$.

Similar trends in fluorescent activity were observed when these reporter constructs were grown in other strains as well, where modulating conditions that triggered the exit of the Bvg plus phase, activated entry into Bvg intermediate phase, and the concentration of $MgSO_4$ that prompted the exit from the Bvg intermediate phase stimulated entry into the Bvg minus phase.

With the entry of strains B1917, B184, B204, UK48, and UK71 into the Bvg plus phase at 0 mM of $MgSO_4$, a drop-off in fluorescence occurred at 16 mM of $MgSO_4$ for B1917, B184 and B204, and 12 mM of $MgSO_4$ for UK48 and UK71.

There was peak activity of b*ipARFP*pBBRK at 8mM of $MgSO_4$ for B1917, UK48 and UK71. For strains B184 and B204, maximum fluorescence occurred at 12 mM of $MgSO_4$ for this Bvg intermediate reporter construct. Again, illustrating the exit from the Bvg plus phase and entry into the Bvg intermediate phase. Additionally, the use of the Bvg intermediate reporter construct illustrated the variability in growth dynamics between strains as the peak fluorescence for some of the strains occurred at different concentrations of modulation.

There was an immediate decline of fluorescent activity from the reporter construct b*ipARFP*pBBRK at 16 mM of $MgSO_4$ for the strains B184 and B204. For the other three strains, B1917, UK48 and UK71, this decline occurred at 12 mM of $MgSO_4$. It was at these concentrations where maximal fluorescence occurred for the reporter construct *vrgXYFP*pBBRK, with the exception of B1917, where peak activity occurred at 16 mM of $MgSO_4$. These results also indicate the exit from the Bvg intermediate phase and entry into the Bvg minus phase. Peak fluorescence was sustained, and activity monitored at every incremental increase of 4 mM $MgSO_4$ until the end of the experiment at 50 mM of $MgSO_4$.

When comparing the sensitivity to modulation amongst strains, maximum fluorescence for each reporter construct in BP536 occurred at a higher concentration of $MgSO_4$.

These results provide an explanation and support earlier observations where there were discrepancies between the peak fluorescence of b*ipARFP*pBBRK for BP536 compared to BP1917. At 20 mM of $MgSO_4$ b*ipARFP*pBBRK was highly fluorescent in BP536, but less so in BP1917, whereas in the absence of modulation, b*ipARFP*pBBRK was highly expressed in BP1917 and less so in B536. Entry into the Bvg intermediate phase occurred with different amounts of modulation for both BP1917 and BP536 exhibiting phenotypic variability in growth dynamics amongst strains. B184, B204, UK71 and UK48 responded similarly to the modulator $MgSO_4$ as BP1917 where the entry and exit into each phase occurred at similar concentrations.

This experiment provides valuable insight into the sensitivity of different strains of *B. pertussis* to the chemical modulator $MgSO_4$ and reinforces the use of *ptxA*, *bipA*, and *vrgX* promoters to demonstrate entry into the Bvg plus, intermediate and minus phase.

4.3.2.2. *Single nucleotide polymorphisms revealed in the BvgS of the five B. pertussis strains compared to BP536*

To better understand the phenotypic variability among strains, the genotype of the following six strains: BP536, B1917, UK48, UK71, B184, and B204 was investigated. Specifically, sequence alignments of the bvgS, bvgA and bvgR genes between these strains were performed. There were no single nucleotide polymorphisms (SNP) identified in bvgA and bvgR, however in the bvgS there was a SNP at nucleotide position 2113 bp where there was a base pair substitution of an A in BP536 to a G in B1917, UK48, UK71, and B204 resulting in an amino acid substitution from lysine to glutamic acid ($K_{705}E$). Interestingly, there were two SNPs in the bvgS of B184 compared to BP536 at nucleotide positions 2113 bp (amino acid position 705) and 2080 bp (amino acid position 694) resulting in the $K_{705}E$ substitution and an isoleucine to valine ($I_{694}V$) substitution, respectively. Both SNPs involved a base pair substitution of an A in BP536 to a G in B184 (Figure 38).

A)

```
Query  2041  GACTCGCTGGGCGAACTCAAGGGCATCATCGGCGGCTGGATCGACATCACCGAACGCGCC  2100
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2041  GACTCGCTGGGCGAACTCAAGGGCATCATCGGCGGCTGGATCGACATCACCGAACGCGCC  2100

Query  2101  GAGCTGCTGCGCAAGCTGCACGACGCCAAGGAAAGCGCCGACGCCGCCAACCGGGCCAAG  2160
             |||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2101  GAGCTGCTGCGCGAGCTGCACGACGCCAAGGAAAGCGCCGACGCCGCCAACCGGGCCAAG  2160

Query  2161  ACCACGTTCCTGGCAACGATGAGCCACGAGATCCGCACGCCGATGAACGCGATCATCGGC  2220
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2161  ACCACGTTCCTGGCAACGATGAGCCACGAGATCCGCACGCCGATGAACGCGATCATCGGC  2220
```

B)

```
Query  1981  CGCGATGTCACGCTGCACGGCCGCACCCGCCATGTCTACCAGTGGACGATTCCGTACGGC  2040
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4779  CGCGATGTCACGCTGCACGGCCGCACCCGCCATGTCTACCAGTGGACGATTCCGTACGGC  4720

Query  2041  GACTCGCTGGGCGAACTCAAGGGCATCATCGGCGGCTGGATCGACATCACCGAACGCGCC  2100
             |||||||||||||||||||||||||||||||||||||||||| |||||||||||||||||
Sbjct  4719  GACTCGCTGGGCGAACTCAAGGGCATCATCGGCGGCTGGGTCGACATCACCGAACGCGCC  4660

Query  2101  GAGCTGCTGCGCAAGCTGCACGACGCCAAGGAAAGCGCCGACGCCGCCAACCGGGCCAAG  2160
             |||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4659  GAGCTGCTGCGCGAGCTGCACGACGCCAAGGAAAGCGCCGACGCCGCCAACCGGGCCAAG  4600

Query  2161  ACCACGTTCCTGGCAACGATGAGCCACGAGATCCGCACGCCGATGAACGCGATCATCGGC  2220
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4599  ACCACGTTCCTGGCAACGATGAGCCACGAGATCCGCACGCCGATGAACGCGATCATCGGC  4540
```

Figure 38. Sequence alignment of the bvgS in five strains of B. pertussis (bottom strain) compared to the reference strain BP536 (top strain).

A) SNP identified in B1917, UK48, UK71, and B204, and B) Two SNPs identified in B184. *bvgS* has a length of 3717 bp and in UK48, UK71, B184, B204, and B184 compared to BP536, there was a base pair substitution of an A to a G at nucleotide position 2113 bp, substituting the amino acid lysine to glutamic acid ($K_{705}E$). There was an additional base pair substitution of an A to a G at nucleotide position 2080 bp observed in B184 compared to BP536, substituting the amino acid isoleucine to valine ($I_{694}V$). SNPs have been highlighted in yellow and the region where the SNP can be found outlined in red squares.

The effects of the amino acid substitution of lysine to glutamic acid ($K_{705}E$) in strains B1917, UK48, UK71, B204 and B184 may have resulted in the divergence in $MgSO_4$ sensitivity observed compared to BP536. However, the presence of the additional amino acid substitution of isoleucine to valine ($I_{694}V$) found only in the *bvgS* of B184 did not seem to yield any obvious additional phenotypic differences in B184 compared to the other strains and appeared to be a neutral substitution.

The entry and exit of *B. pertussis* into the Bvg phases have been explored in this chapter through the use of reporter constructs.

4.4. Discussion

The conservation of *BvgAS* in the *Bordetella* spp. indicates that it has an important role in the infectious cycle, however responsiveness to modulation of the BvgS across different

strains is less understood. The resultant gene expression as a result of modulation during growth also remains to be elucidated.

Previous experiments within the Preston group have explored the use of qPCR to monitor Bvg-regulated gene expression in real time throughout the growth cycle, however this yielded minimal results for maximal effort. A more efficient way to understand phenotypic differences amongst strains is to use the promoter activity of Bvg-regulated genes to create fluorescence producing reporter constructs.

Promoters were carefully selected from genes that were differentially expressed in each Bvg phase and had high levels of expression according to RNA-seq data. As well, reporter proteins were selected to provide fluorescence above the background fluorescence. Despite using these criteria to make reporter constructs, several reporter constructs provided comparable levels of fluorescence in multiple phases, illustrating non-specificity and prevented their use as Bvg phase specific reporter constructs. Others did not provide fluorescence above the background noise or provided low levels of fluorescence. This could be due to the entire regulatory site not being included in the 'promoter' region selected or the fluorescence signal from the reporter protein being too low and therefore indiscernible from the background fluorescence, demonstrating a low sensitivity.

This was addressed for the genes *flaA* and *kpsM*, where reporter constructs were made with a shorter (*flaA_S*) and larger intragenic region (*kpsM_2*) for the promoter, respectively. Surprisingly, the latter resulted in slightly higher levels of fluorescent activity.

Ultimately, the final reporter constructs were made with promoters that resulted in the highest levels of fluorescence paired with regular half-life GFP, RFP and YFP. Regular half-life variants of reporter proteins were selected for the construction of reporter constructs because they were more sensitive than short half-life variants and provided a higher fluorescent signal. The caveat to using reporter proteins that are highly stable and maintain their fluorescence for long periods of time is that the exit from Bvg phases cannot be observed in growth assays conducted in real-time. However, this system does allow for the entry into Bvg phases to be detected during the growth cycle. To capture when *B. pertussis* strains exit Bvg phases, short half-life variants with a higher fluorescent signal need to be employed or an alternative system used. An example of an alternative system is reporter constructs made with firefly luciferase, which has a half-life of approximately three hours in mammalian cells and produces luminescence rather than fluorescence [182].

Each reporter construct made was analysed for fluorescence using fluorescence microscopy, flow cytometry and the fluorescent microplate reader. Interestingly, when

viewing the reporter construct *0840GFP*pBBRK and *2782GFP*pBBRK in BP536 under the microscope only 75% of cells fluoresced. This observation was also seen in the flow cytometry data where there were two peaks in the histogram, one large and one very small for the reporter construct *ptxAYFP*pBBRK in BP1917 grown in Bvg plus conditions and *0840YFP*pBBRK in BP1917 grown in Bvg minus conditions. The small peak represented a small number of cells in the sample that were not fluorescent. A simple reason for why some of the cells in the sample may not be fluorescent is that they are dead cells or are debris. A fixable viability dye to discern between live and dead bacteria, as well as debris will determine this. A more interesting explanation for the two peaks is as a result of modulation, where some cells are switching Bvg phases and losing fluorescence, while others retain fluorescence. However, this explanation can only apply to promoters that are not constitutively expressed. A final explanation is that the cells may no longer contain the reporter construct and therefore cannot fluoresce. The only certainty regarding this observation is that these samples, which were resuspended plate cultures in PBS, contained a non-uniform population. In the future, it would be interesting to sample broth cultures for microscopy and flow cytometry in order to determine if the fluorescence is more homogeneous in broth cultures.

The Bvg-regulated promoters used in reporter constructs *ptxAGFP*pBBRK, b*ipARFP*pBBRK, and *vrgXYFP*pBBRK had a high differential expression in the Bvg plus, intermediate and minus phase, respectively, and produced levels of fluorescence that were greater than the background fluorescence. When used to compare the phenotypic differences in six different strains of *B. pertussis,* it was revealed that the *B. pertussis* strain BP536 entered the Bvg intermediate and minus phase at a higher concentration of $MgSO_4$ compared to B1917, UK48, UK71, B184 and B204 and was therefore less sensitive to modulation. The phenotypic differences between strains could possibly be explained by looking at the genotype. Compared to BP536, the *bvgS* of B1917, UK48, UK71, B184 and B204 contained an amino acid substitution of lysine to glutamic acid ($K_{705}E$) at amino acid position 705. This substitution has been noted previously by Herrou *et al.* and has been shown to result in an increased insensitivity to modulation [2].

This has been confirmed in this chapter and contributes to the phenotypic differences and growth variability observed between BP536 and the other five strains. These results suggest that lysine at amino acid position 705 is not particularly advantageous, as well, suggest that BP536 or Tohama I is not representative of currently circulating strains and therefore no longer suitable as a wild-type strain.

To further endorse the phenotypic effects of this amino acid substitution, mutant strains of B1917, UK48, UK71, B184 and B204 containing the gene knock-in *bvgS* from BP536 and reporter constructs can be tested to determine their sensitivity to modulation. If the sensitivity to modulation for the mutants reverts to that of BP536 in the Bvg intermediate and minus phase, this will provide support for the amino acid substitution as a cause for the phenotypic variation observed between the five strains of *B. pertussis* tested here compared to BP536.

These phenotypic differences observed have been limited to six strains in this chapter, however the SNPs and associated amino acid substitutions are likely to be more far reaching. It would be of interest to create a phylogenetic tree based on SNPs observed in the *bvgS* of the global population of *B. pertussis.* This will provide valuable information regarding the spread of *bvgS* polymorphisms. To support polymorphisms detected in the phylogenetic tree, reporter constructs can be introduced into representative strains from each *bvgS* clade and their sensitivity to $MgSO_4$ tested.

There is a gap in the literature in understanding the responsiveness to modulation of BvgS of *B. pertussis in vivo* at 37°C and the reporter constructs created here can be used for this very purpose. However, addressing this gap has proven to be more difficult than expected. The determination of entry and exit into the Bvg phases using the reporter strains grown at 37°C is dependent on the half-life of the reporter proteins used, specifically when determining the exit from Bvg phases. However, obtaining the half-life of regular half-life variants of GFP and YFP has been inconsistent thus far, and the half-life of RFP has not yet been obtained. Therefore, the method used to terminate promoter activity may require improvement in order to allow half-life data to be acquired.

An additional way to explore sensitivity to modulation during growth could be to create a strain containing reporter constructs for all three Bvg phases on a single plasmid. This would allow the fluorescence from a single growth curve to be observed and enable the control for as many factors as possible. However, using the current reporter constructs to make this 'super plasmid' is not currently possible because YFP and GFP contain an overlapping excitation and emission spectra making it difficult to differentiate between Bvg phases and will inevitably interfere with the interpretation of growth cycle data. Therefore, one of these reporter proteins will need to be replaced with a reporter protein with a different spectrum. A possible candidate is CFP, as it produced fairly high levels of fluorescence in the initial experiments. A concern with using this plasmid in the future is that the three reporter constructs on a single plasmid may stress the bacteria once introduced due to its size.

The current reporter constructs created on three separate plasmids seems suitable to use for the questions I have asked thus far.

To summarise, the highly conserved BvgAS Two-component system of the *Bordetella* spp. is responsible for the activation and repression of virulence factors resulting in three Bvg phases. Entry into these phases is modulated by growth conditions.

Within this chapter, the BvgAS system was successfully utilised to illustrate growth variability amongst strains from the pre- and post-vaccination era by determining the *in vitro* sensitivity to modulation of these strains.

I successfully created fluorescent reporter constructs for all three Bvg phases and validated the work of Herrou *et al.* where the the pre-vaccination reference strain *B. pertussis* BP536 was studied in the Bvg plus phase only [2].

This versatile tool was also utilised for the post-vaccination strains B1917, UK48, UK71, B184 and B204 yielding three genotypic profiles resulting in two unique phenotypic profiles. The first genotypic profile was seen in BP536, where a lysine residue in position 705 within the BvgS led to the decreased sensitivity to modulation in all three Bvg phases—the first phenotypic profile. The second phenotypic profile, where there was an increase in sensitivity to modulation in comparison to BP536 was observed in B1917, UK48, UK71, and B204 when a glutamic acid residue was substituted in position 705 in the BvgS instead ($K_{705}E$)— yielding the second genotypic profile. The third and final genotypic profile was seen in B184 and resulted from a lysine residue at position 705 and a valine residue in position 694 in the BvgS that was not seen in the other five strains tested. Interestingly, this amino acid substitution did not result in a new phenotype, rather this change reverted to the phenotypic profile seen in B1917, UK48, UK71, and B204 which contained an isoleucine residue in position 694.

The results from this chapter are promising and suggest that reporter constructs can be used as a tool to better understand the growth dynamics of *B.* pertussis *in vitro* as illustrated in this chapter, but also *in vivo,* and the future experiments highlighted in this discussion will strengthen the use of this tool for future work. Additionally, this work provides further details regarding the physiology and biology of *B. pertussis* and will allow for a better understanding of host-pathogen interactions.

Chapter 5

Results

Exploring the mechanism of ampicillin resistance

observed in broth grown *B. pertussis* strains
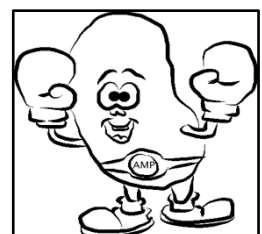
5. Exploring the mechanism of ampicillin resistance observed in broth grown *B. pertussis* strains

5.1. Rationale

This final chapter originates from an earlier observation made with *B. pertussis* strain BP536, where there was a three-fold increase in ampicillin sensitivity in plate grown cultures in the Bvg minus phase compared to Bvg plus phase cultures. Surprisingly in broth grown cultures, the opposite was true, where Bvg minus cultures were resistant to even higher levels of ampicillin compared to Bvg plus cultures. Both Bvg plus and Bvg minus broth cultures grew in high levels of ampicillin. These observations suggest fundamental differences in growth characteristics between Bvg phases, and between plate versus broth growth.

This chapter has been divided into three sections and will investigate the mechanism of ampicillin resistance observed in broth grown cultures in comparison to plate cultures. The first section will confirm the observations seen previously regarding the ampicillin susceptibility in *B. pertussis* plate and broth grown cultures in BP536, as well as determine ampicillin resistance in five other strains.

The second section investigates the potential destruction or enzymatic modification of the antibiotic by *B. pertussis* through the production of a β-lactamase enzyme that inactivates ampicillin leading to resistance.

The third and final section explores the expression levels of penicillin binding proteins as a means to prevent ampicillin from interacting with the cell.

Cumulatively, this chapter aims to identify the mechanisms underpinning the differential ampicillin resistance observed when growing BP536 in different growth conditions.

5.2. Introduction

5.2.1. Ampicillin resistance mechanisms

Resistance to ampicillin can be mediated by the enzymatic modification of the antibiotic, decreased affinity of the antibiotic for its target, altering the target binding site, or bypassing the target, efflux systems preventing this antibiotic from accumulating in the bacterial cell,

and decreased cell wall permeability. Three of these mechanisms have been illustrated in Figure 39.
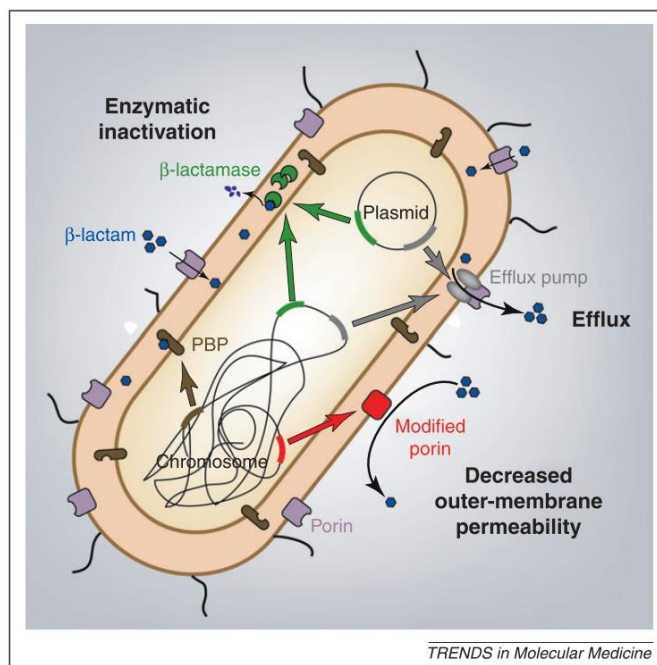
Figure 39. Mechanisms of β-lactam resistance.

Three primary mechanisms of β-lactam resistance have been previously identified for Gram-negative bacteria and include enzymatic inactivation of β-lactam antibiotics through the production of hydrolytic enzymes against β-lactam molecules referred to as β-lactamases, efflux of β-lactamases to outside the cell using an efflux pump, and a reduction in outer-membrane permeability through the loss of porin expression or production of modified porins [89].

As discussed in Chapter 1, the mechanism of action of ampicillin is to inhibit bacterial growth by inhibiting cell wall synthesis. However widespread resistance to ampicillin and other β-lactams occurs via β-lactamases [91,183].

5.2.2. Destruction or Enzymatic Modification of ampicillin

β-lactamases are ancient enzymes that initially emerged from environmental sources, perhaps to protect that bacterium from naturally occurring β-lactams. To date there are almost 2,800 different β-lactamase proteins. These enzymes are used to detoxify and destroy the drugs by hydrolysing the amide bond in the β-lactam ring, producing ineffective products and inhibiting antibiotic action [88,91].

β-lactamases can be divided into four groups depending on their activity profiles: 1) penicillinases, 2) cephalosporinases, 3) extended-spectrum β-lactamases (ESBL), and 4) carbapenemases. Of these groups, ESBLs can inactivate all β-lactams except carbapenems. Fortunately, the β-lactamase inhibitor clavulanic acid can inhibit the activity of

ESBLs reducing the clinical impact of these enzymes [91]. Cephalosporinases, on the other hand, preferentially inactivate cephalosporins and aminopenicillins, but do not affect penicillins. Penicillinases inactivate penicillins, however, are not able to degrade aztreonam, carbapenems or cephalosporins [89].

β-lactamases can be further refined into two broad categories according to their mechanism of hydrolysis. The first category includes β-lactamases that hydrolyse β-lactam rings by forming an acyl enzyme with an active-site-serine. The second category utilises one or two catalytically functional zinc ions in the active sites of metallo-β-lactamases (MBLs) to perform a hydrolytic reaction [91,184].

Lastly, β-lactamases can be characterised into four molecular classes: Classes A, B, C, and D. Classes A, C and D β-lactamases catalyse the hydrolysis of β-lactams via a serine-bound acyl intermediate, while Class B enzymes catalyse β-lactams by directly attacking a hydroxide ion in the active site of the β-lactam that is stabilized by zinc [88,184].

Figure 40 depicts 17 functional groups using the hydrolysis of β-lactam substrates and inhibitor profiles.

β-Lactamases

	Serine				Metallo (Zn^{2+})
Characteristic active site	Serine				Metallo (Zn^{2+})
Molecular Class	A		C	D	B
Functional group	2		1	2d	3

Major functional subgroups	2a	2b	2be	2br	2c	2f	1	1e	2d	2de	2df	3a	3b
Known substrates	P	P Cp	P,Cp E,M	P	P	P,Cp, Cb,E,M	Cp	Cp,E	P	P,E M	P,Cb	P,Cp, E, Cb	Cb
Inhibitor profile AV	+	+	+	+	+	+	+	+	+	+	+	-	-
CA	+	+	+	-	+/-	+/-	-	-	+/-	+/-	-	-	-
EDTA	-	-	-	-	-	-	-	-	-	-	-	+	+
Representative enzymes or enzyme family	PC1	TEM-1 SHV-1	CTX-M ESBLs (TEM,SHV)	IRT SHV-10	CARB-1	KPC SME	AmpC CMY	GC1	OXA-1 OXA-10	OXA-11 OXA-15	OXA-23 OXA-48	IMP,VIM NDM	CphA

Figure 40. Classification of β-lactamases based on molecular and functional relationships.

β-lactamases can be divided into different groups depending on their active site, molecular class, functional group, substrates and inhibitor profiles [91].

Inhibitors of β-lactamases possess the characteristic β-lactam ring, seen in β-lactam antibiotics, which allows them to act as a suicide substrate and bind covalently to the active

sites of bacterial β-lactamases, despite weak intrinsic antimicrobial activity. The following β-lactamase inhibitors derive from β-lactams: clavulanic acid, tazobactam and sulbactam (Figure 41).

β-lactamase inhibitors

Clavulanic acid　　　　Sulbactam　　　　Tazobactam　　　　NXL-104

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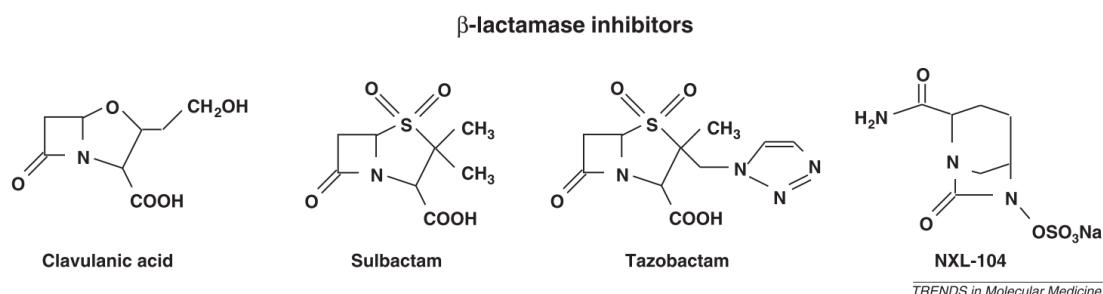
Figure 41. Chemical structure of four main β-lactamase inhibitors clinically available.

Clavulanic acid, sulbactam, tazobactam and NXL-104 are represented in this figure [89].

5.2.2.1. Detection of β-lactamases in Bordetella spp.

In the *Bordetella* spp., β-lactam resistance has been observed in *B. bronchiseptica* and has been attributed to the presence of β-lactamases that hydrolyse oxacillin and penicillin, as well as the intrinsic resistance to β-lactam antibiotics due to low membrane permeability to cephalosporins. [185–188].

One such β-lactamase is encoded by *B. bronchiseptica* bla_{BOR-1}, which was detected from a human isolate that had an MIC for ampicillin of 8 mg/L [186,188].

BOR-1 is a narrow-spectrum enzyme that is inhibited by clavulanic acid and shares 42-45% amino acid identity with the plasmid mediated β-lactamase TEM-1, as well as the β-lactamase L-2 from *Stentotrophomas maltophilia*, PenA from *Burkholderia cepacia,* FAR-1 for *Nocardia farcinica*, AST-1 from *Norcardia asteroids,* YENT from *Yersinia entercolitica*, and SHV-1 from *Klebsiella pneumoniae.* Like L-2, AST-1, FAR-1 and YENT, BOR-1 can hydrolyse ampicillin as well as other penicillins [186]. Interestingly, this gene was also detected in *B. parapertussis,* but not in *B. pertussis* [186].

To further investigate β-lactamase resistance phenotypes in *Bordetella* spp., the minimal inhibitory concentration (MIC) of *B. bronchiseptica* DOR was compared to *B. parapertussis* CIP 63.33. *B. bronchiseptica* DOR is a clinical isolate recovered from France in April 2004 that shares 99% genome identity with *B. bronchiseptica* RB50, while *B. parapertussis* CIP 63.33 possesses the gene bla_{BOR-1} that is identical to that of *B. bronchiseptica* RB50 except for four non-synomomous mutations.

Despite this similarity, *B. bronchiseptica* DOR was resistant to broad-spectrum cephalosporins*,* while *B. parapertussis* CIP 63.33 was resistant to only cefoxitin [186].

β-lactam resistance in *B. bronchiseptica* has been echoed in many studies including Kadlec *et al.* where 349 porcine isolates of *B. bronchiseptica* were determined to have extremely high MICs for ampicillin, cefalotin and ceftiofur. Resistance to ampicillin was overcome through the use of clavulanic acid [185,189].

Plasmids carrying a class 1 integron with a bla_{OXA-2} cassette were identified in eight isolates of *B. bronchiseptica*. Class 1 integrons harbour additional antibiotic resistance genes that are co-expressed from a single promoter and contribute to multi-drug resistance [89]. This class 1 integron was also identified in a plasmid-free isolate. The gene bla_{OXA-2} encodes the β-lactamase OXA-2 and is responsible for the hydrolysis of oxacillin and has been identified in *Salmonella typhimurium*, *Pseudomonas aeruginosa* and *K. pneumoniae*. This enzyme has similar action to the OXA-10 β-lactamase and other class D β-lactamases [188].

In a second study in 2007, Kadlec *et al.* revealed that out of nineteen pig *B. bronchiseptica* isolates, only nine carried the class 1 integron with a bla_{OXA-2} gene cassette with eight of these isolates carrying this integron on a plasmid designated as pKBB282 [188]. When pKBB282 was conjugated into other isolates of *B. bronchiseptica,* it conferred resistance to ampicillin producing an MIC of 128 mg/L. As a result of being located on a conjugative plasmid in *B. bronchiseptica*, the bla_{OXA-2} gene can be transferred to other species [188].

Due to the low number of isolates carrying the bla_{OXA-2} gene, the bla_{BOR-1} resistance gene which was detected in all nineteen isolates of *B. bronchiseptica* in this study has been implicated as the sole molecular mechanism underlying the ampicillin resistance phenotype observed in this study [188].

The *B. bronchiseptica*- specific CLSI recommended breakpoint for resistance to an antimicrobial agent was ≥2 μg/ml for ampicillin and was used in the 2015 study conducted by Prüller *et al.* [190]. 107 *B. bronchiseptica* isolates from different farms in Germany were deemed resistant to ampicillin and contained the β-lactamase bla_{BOR-1}. An additional 43 isolates obtained from companion animals in Germany and other European countries also demonstrated elevated MICs for β-lactam antibiotics. Using PCR assays bla_{BOR-1} (n=147) , bla_{OXA-2} (n=4), *strA* and *strB* (n=17), *sul1* (n=10), *sul2* (n=73), *dfrA7* (n=3), *tet*(A) (n=8) were detected among the 150 isolates [190]. Utilising Southern blotting allowed researchers of this study to determine the possible localisation of resistance genes on plasmids. bla_{OXA-2}, *sul1*, *sul2* and *tet*(A) were localised exclusively on plasmids, whereas fourteen of seventeen occurrences of *strA* and *strB* were on plasmids [190].

Collectively these studies represent β-lactamase enzymes as a resistance mechanism against β-lactams, specifically ampicillin, in the *Bordetella* spp. The β-lactam resistance genes *bla*$_{BOR-1}$, and *bla*$_{OXA-2}$, amongst others, represent the underlying molecular mechanism of the β-lactam resistance phenotype seen in *Bordetella* spp. Probably, their widespread occurrence among *Bordetella* is due to them being carried on plasmids.

Antimicrobial resistance is rarely as a result of one mechanism, rather attributed to multiple mechanisms. Multiple mechanisms of antibiotic resistance can also be found in the same strain such as β-lactamase enzymes found in strains with decreased permeability or increased efflux contributing to even greater clinical resistance [91]. The production of β-lactamases may also act in synergy with changes to target affinity [78,183]. However, in the absence of enzymatic degradation, modifications to the target can still occur.

5.2.3. Altered binding targets

5.2.3.1. Mechanism of action

Bacteria can also acquire β-lactam resistance by modifying binding target sites. These targets play a vital role in microbial survival and growth and are absent from mammalian cells or different enough structurally in its composition to allow for selective inhibition. Taken together, the perfect binding target for β-lactam antibiotics are transpeptidases or PBPs. These enzymes are involved in the synthesis and assembly of the peptidoglycan component of the bacterial cell wall [191].

Despite the interference of β-lactam antibiotics on vital cellular functions, bacteria have evolved multiple ways to thwart their action. One such way is the modification of PBPs preventing antibiotics from interacting with the cell and often results when the sequence of target transpeptidases are altered by spontaneous mutations or recombination. The outcome is a reduced binding affinity to β-lactams. Additionally, changes to target site can occur when resistances genes are acquired from other organisms through genetic exchange. This includes conjugation, transduction, or transformation [191].

5.2.3.2. Altered transpeptidase examples

A prominent example of altered binding targets can be seen in *Enterococci*. There are six PBPs in *E. faecium*, however only PBPs 4 and 5 are relevant target enzymes of β-lactam antibiotics. The overproduction of low-affinity PBP5 results in the reduction of penicillin binding capability and highly resistant strains. The high level of resistance was also

associated with mutations in PBP5. Resistant PBP5 contains a peptide loop on one side that is more rigid than that of sensitive PBPs, as well as a hydrophobic valine residue resulting in low affinity PBPs and reduced accessibility for β-lactams, respectively [191–195].

In *Proteus mirabilis,* the resistance to the β-lactam imipenem also resulted from a decreased affinity for PBPs*,* specifically PBP2. However, additional factors may contribute to antibiotic resistance of *P. mirabilis* such as decreased permeability and efflux [191,196,197].

The expression of foreign PBPs can also result in resistance to β-lactam antibiotics, as seen in the case of methicillin-resistant *Staphylococcus aureus* (MRSA), where the *mecA* gene was acquired from another species of *Staphylococcus. mecA* encodes the protein PBP2a, which is an altered penicillin binding protein involved in the assembly of the peptidoglycan layer of the bacterial cell wall [191,198–203].

This layer is composed of a series of glycan chains with N-acetyl muramique (NAM)/ N-acetyl glucosamine (NAG) residues. Attached to the NAM residue is a stem peptide. Glycan chains in the peptidoglycan are linked together on L-lys residue (position 3) on one stem peptide to D-ALA residue (position 4) of the other. The extension of these glycan chains is called transglycosylation. Transpeptidation reactions or cross-linking occurs in the cytoplasmic membrane and is catalysed by PBPs. When β-lactams are present, transpeptidation is inhibited because these antibiotics resemble the terminal D-alanyl-D-alanine bond of the stem peptide in the transpeptidation domain of PBPs. In the absence of cross-linking the peptidoglycan, the cell wall is weakened, cytoplasmic contents are released and the cell dies [191,198].

There are four PBPs in *S. aureus*, PBP1-4, that vary in composition and order of genes encoded in the element, however only PBP2 has transpeptidase domains that are involved in both transpeptidation and transglycosylation. Despite possessing common structural motifs for penicillin binding, PBP2a lacks the transglycosylation domain of PBP2. In the presence of β-lactams antibiotics such as methicillin, transpeptidase activity can still occur with PBP2a, however, there is a marked reduction in the degree of cross-linking of the glycan chains in the peptidoglycan layer. This is because the acylation of the serine in PBP2a is reduced by 3-4 orders of magnitude compared to β-lactam-sensitive PBP enzymes resulting in PBP2a having a lower affinity for β-lactams. Also, PBP2a does not bind to the beta-lactam ring in beta-lactams in comparison to PBP2 and other DD-transpeptidases. The cross-linking that does occur as a result of the PBP2a is enough to ensure the survival of the bacterial cell by enabling cell wall synthesis and allowing bacterial replication to still occur.

Therefore, the presence of PBP2a results in the resistance to methicillin and other β-lactam antibiotics provides support that acquired targets for binding can promote resistance to β-lactams [191,199–204].

PBP2a is regulated and kept at a low level, however mutations in regulatory genes can enhance synthesis resulting in a takeover of cross-linking reactions in the PBPs of *S. aureus*. The synthesis of PBP2a is regulated by MecI and MecR1 proteins, which are highly homologous to the proteins BlaR1 and BlaI in the *blaZ* system. These *blaZ* regulatory genes are carried on a plasmid and when present can also regulate the synthesis of PBP2a. Interestingly, the *blaZ* gene in this system is also a responsible for the expression of the staphylococcal β-lactamase [204].

β-lactam resistance can be achieved through several different mechanisms working independently or in synergy, however resistance is rarely the result of a single mechanism. Bacteria can also reduce entry of the antibiotic into the cell by reducing the number of entry channels or porins, restricting uptake and lowering the overall permeability of the outer membrane. Bacteria can also actively expel the antibiotic out of the cell through efflux. The AcrAB efflux pumps will not be investigated within the scope of thesis as a result of the lowered activity of the efflux pump in *B. pertussis* noted in Chapter 1.

The more probable mechanisms of enzymatic degradation through the production of β-lactamases, and a reduction in the penicillin binding protein affinity preventing the drug from interacting with these targets will be explored. This will allow for a better understanding of antibiotic resistance mechanisms in *B. pertussis* [78].

5.3. Results

5.3.1. Ampicillin sensitivity is remarkably different between plate and broth grown cultures

This section reproduces the initial ampicillin susceptibility profile of BP536 previously observed by other members of the Preston Lab, as well the ampicillin susceptibility profile of *B. pertussis* B1917, UK48, UK71, B184 and B204 in both plate and broth grown cultures was completed here as well.

5.3.1.1. Plate cultures of B. pertussis BP536 are sensitive to ampicillin compared to broth cultures (Bvg+/-)

The minimal inhibitory concentration (MIC) of BP536 on solid media was 0.094 µg/ml in the Bvg⁺ phase and 0.032 µg/ml in the Bvg⁻ phase. Other strains also show a similar pattern of high ampicillin sensitivity on solid media, where B1917, UK48, UK71, B184, B204 had an MIC of <0.064 and below in Bvg⁺ and <0.016 and below in Bvg⁻. These results provide evidence of increased sensitivity in Bvg⁻ in comparison to Bvg⁺, however it is difficult to obtain clean cut off values using e-test strips at that very low level of resistance.

5.3.1.2. Broth cultures grown with ampicillin

Different levels of ampicillin sensitivity were observed for the same strains grown in broth medium (Figure 42). Cultures were grown in the presence of ampicillin from the beginning of growth (Figure 42A and Figure 42C) and during growth, at 19 hours (Figure 42B and Figure 42D).
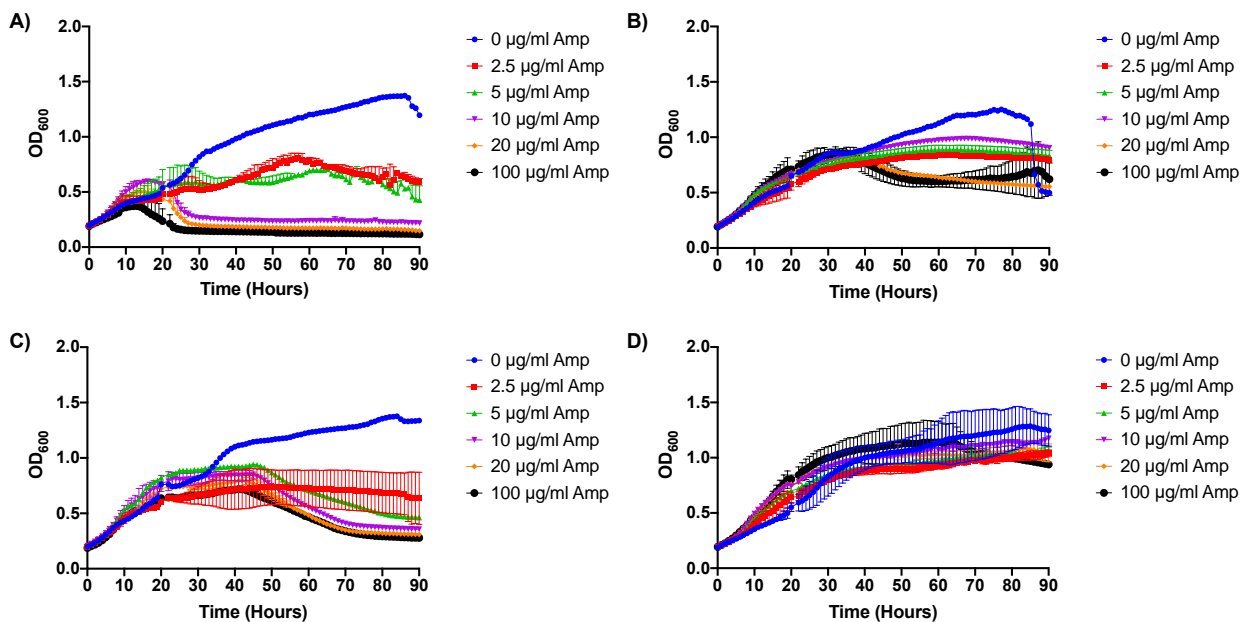
Figure 42. Broth grown cultures of B. pertussis BP536 in Bvg plus and minus conditions.

A) Ampicillin added at 0 hours in Bvg plus conditions. B) Ampicillin added at 19 hours in Bvg plus conditions. C) Ampicillin added at 0 hours in Bvg minus conditions. D) Ampicillin added at 19 hours in Bvg minus conditions. The growth of *B. pertussis* BP536 was inhibited by >5 µg/ml of ampicillin in Bvg plus conditions when ampicillin was added at the start of growth. In Bvg minus grown cultures ampicillin did not inhibit initial growth, but cultures did enter the stationary phase at approximately 20 hours of growth. The addition of ampicillin to either Bvg plus or minus grown cultures at timepoint 19 hours also did not inhibit growth. Data represents biological duplicates.

The ampicillin susceptibility of BP536 broth cultures was vastly different from that of plate cultures, where ampicillin had a minimal inhibitory effect on broth cultures. Therefore, broth cultures illustrated ampicillin resistance and plate cultures did not. However, the ampicillin susceptibility in both broth and plate cultures were Bvg dependent.

In Bvg minus conditions, BP536 was able to grow in the presence of ampicillin at the same growth rate as the control strain grown in the absence of ampicillin for approximately 20 hours, demonstrating resistance. After this time, growth was inhibited, and the strains were in the stationary phase for approximately 20 hours until a drop-off in growth occurred, with the exception of cultures grown in the presence of 2.5 μg/ml of ampicillin.

Bvg plus grown strains were inhibited by >5 μg/ml of ampicillin after approximately 10 hours of growth.

Cultures appeared to be in the lag stage of growth for <5 hours when grown in both Bvg phases, while the time spent in the log stage was the highest in cultures grown in the absence of ampicillin of >80 hours. With the exception of Bvg plus cultures grown with 2.5 and 5 μg/ml of ampicillin, which had a log time of >50 hours as well, cultures grown in the presence of ampicillin in both phases had a log phase of approximately 20 hours and less.

As mentioned, ampicillin was also added after 19 hours of growth to both Bvg plus and minus cultures revealing an inhibition of growth after 40 hours, after which strains entered the stationary phase for the duration of growth.

Compared to the ampicillin sensitive plate grown cultures, there was still a substantial increase in ampicillin resistance in BP536 broth cultures illustrating a variability in the growth dynamics of this strain when using different media.

The ampicillin susceptibility of broth cultures was also evaluated for B1917, UK48, UK71, B184 and B204 in order to determine if the ampicillin resistance observed in broth cultures of BP536 is found in other strains (Figure 43).

Ampicillin was added at the start of growth for all of the growth curves below. Additionally, each growth curve below has a different scale on the y axis for OD_{600} enabling minute changes in growth to be better observed.
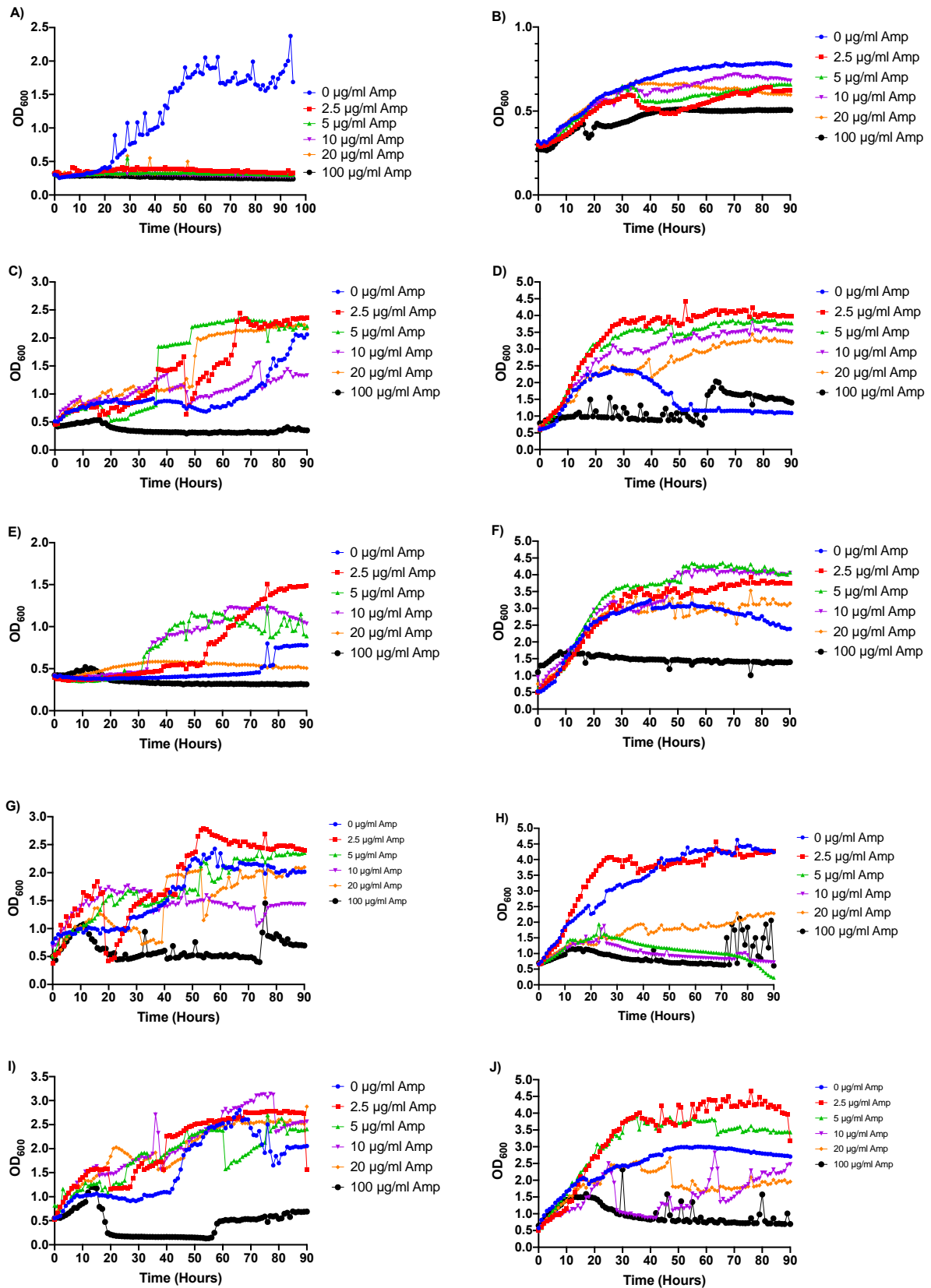
Figure 43. Broth cultures from B1917, UK48, UK71, B184 and B204 grown in Bvg conditions.

A), C), E), G), I) represent growth in Bvg plus conditions, while B), D), F), H) represent grown in Bvg minus conditions. A) and B) represent B1917, C) and D) represent UK48, E) and F) represent UK71, G) and H) represent B184, and I) and J) represent B204. Data represents one biological repeat; however, this experiment was repeated six times with variable results as a result of poor growth.

Bvg minus cultures of BP536 and B1917 appeared to be most resistant to ampicillin in comparison to the other strains tested. With the exception of cultures grown with 100 µg/ml of ampicillin, the UK strains grown in Bvg minus conditions appeared more resistant to ampicillin than the Dutch strains, B184 and B204, grown in the same condition.

Ampicillin resistance appeared to be Bvg dependent in all strains, where there was greater resistance in Bvg minus strains in comparison to Bvg plus strains for all strains except for B184 and B204. In B184 and B204, ampicillin resistance was greater in Bvg plus strains, rather than Bvg minus strains.

B1917, UK48 and UK71 exhibited poor growth in Bvg plus conditions making the interpretation of these cultures difficult. The cause of this poor growth is explored in Chapter 6; however, it did make interpreting these results difficult.

Bvg minus broth grown cultures of B1917 appeared to be resistant to ampicillin, entering the stationary phase early at approximately 30-40 hours of growth. This ampicillin susceptibility profile was comparable to the susceptibility profile observed in Bvg minus cultures of BP536. The average time these cultures spent in the lag phase was 3-5 hours and in the absence of ampicillin spent 70 hours in the log phase. The log phase was reduced to approximately 20 hours and less for cultures grown in the presence of ampicillin (Figure 43B).
Bvg plus cultures of B1917 were far more susceptible to ampicillin in comparison to BP536 with complete inhibition to ampicillin observed in Bvg plus cultures of B1917 grown in the presence of ampicillin in comparison to the no ampicillin control. This control had a lag phase of >20 hours, and log phase of >70 hours (Figure 43A).

Increased ampicillin concentration resulted in decreased incubation time in the lag phase for cultures grown in both Bvg conditions for UK48 cultures. The no ampicillin control in UK48 Bvg plus broth cultures appeared to be in the lag phase until 55 hours of growth prior to entering the log phase. Cultures grown in 2.5, and 5 µg/ml of ampicillin had a lag time of <20 hours, and cultures grown in 10 and 20 µg/ml of ampicillin had a lag time of <2 hours (Figure 43C). The lag time in the Bvg minus phase on average was also much shorter in comparison at approximately 5 hours for 0, 2.5, and 10 µg/ml of ampicillin, and <2 hours for 10, 20 µg/ml of ampicillin (Figure 43D).
Increased ampicillin concentration resulted in increased incubation time in the log phase for cultures grown in both Bvg conditions. In Bvg minus cultures, the time spent in the log phase in the absence of ampicillin was 20 hours. Cultures grown in 2.5 µg/ml of ampicillin was also

in the log phase for 20 hours, this increased to 30 hours for 5 µg/ml of ampicillin, 40 hours for 10 µg/ml of ampicillin and 60 hours for 20 µg/ml of ampicillin (Figure 43D). Similar to BP536, Bvg minus cultures of UK48 were resistant to most concentrations of ampicillin. However, the UK48 no ampicillin control did not grow as well in comparison to the BP536 control in both Bvg conditions. Unlike BP536, UK48 cultures grown in the Bvg plus condition were very variable. Although there appeared to be a reduction of growth in the presence of 10 µg/ml of ampicillin, growth was restored using 20 µg/ml of ampicillin, and then inhibited at 100 µg/ml. Growth was also inhibited in the presence of 10 µg/ml of ampicillin for BP536 cultures grown in Bvg plus conditions.

Like UK48, there were difficulties in culturing UK71 in Bvg plus conditions. The no ampicillin control had a long lag phase of >70 hours. Strains grown in 2.5, 5, and 10 µg/ml of ampicillin grew better with the average time spent in the lag phase being 40 hours and the average time spent in the log phase being approximately 25-30 hours in comparison to the no ampicillin control of 40 hours (Figure 43E). Similar to BP536, UK71 cultures grown in the Bvg plus phase experienced reduced growth in the presence of >10 µg/ml of ampicillin. Bvg minus cultures grew much better than Bvg plus cultures, with lag times of <10 hours for all concentrations of ampicillin used and log times of approximately 30 hours for cultures growth with 0, 2.5, 5, 10, and 20 µg/ml of ampicillin prior to entering the stationary phase for the duration of growth (Figure 43F).

Despite B184 cultures growing more robustly in comparison to the UK strains, growth of Bvg plus cultures was very sporadic (Figure 43G). For example, growth was reduced in Bvg plus cultures grown with 2.5 of ampicillin at 15 hours and increased at 20 hours. This decrease was echoed in cultures grown with 20 µg/ml of ampicillin where there was a reduction of growth at 15 hours and then an increase at 40 hours. Also, Bvg plus cultures grown with 10 µg/ml of ampicillin entered the stationary phase after 20 hours of growth and growth of cultures with 100 µg/ml of ampicillin was inhibited after 20 hours of growth. This inhibition of growth was also observed in Bvg minus cultures, as well there appeared to be abnormal growth in both Bvg conditions after 70 hours of growth.

The incubation time in the lag phase for Bvg plus cultures grown with 0 µg/ml was >25 hours, and 30 hours in the log phase. Cultures grown in 5 ug/ml of ampicillin spent >60 hours in the log phase. For the remaining Bvg plus cultures grown with 5, 10, 20 and 100 µg/ml of ampicillin, the lag phase was <5 hours. This short lag phase was echoed in cultures grown in Bvg minus conditions, regardless of the concentration of ampicillin used. Interestingly, in Bvg minus cultures with >2.5 µg/ml of ampicillin, growth was reduced after approximately 20 hours (Figure 43H). The log time of Bvg minus cultures grown in the

absence of ampicillin was >60 hours and approximately 20-40 hours for cultures grown in 2.5 µg/ml of ampicillin to 5, and 10, 20 µg/ml of ampicillin.

Comparing the growth curves of B184 to BP536 revealed a dissimilar ampicillin susceptibility profile for both Bvg conditions. Where there was a clear reduction of growth at >5 µg/ml of ampicillin for Bvg plus cultures of BP536, the reduction of growth was less clear in Bvg plus cultures of B184. This divergence was also seen in Bvg minus cultures of B184 where there was a reduction or inhibition of growth using >2.5 µg/ml of ampicillin, in BP536 cultures grown with and without ampicillin were able to grow and enter the stationary phase.
These results revealed that in Bvg plus conditions, B184 was more resistant to ampicillin in comparison to BP536. In Bvg minus conditions however, B184 was less resistant than BP536, B1917, UK48 and UK71.

B204 also grew better than the UK strains, however the no ampicillin control in Bvg plus conditions was in the lag phase for 40 hours before entry into the 25-hour log phase. This lag phase was unusual compared to the rest of the growth curves grown in both Bvg phases, which were approximately 10 hours or less. The remaining Bvg plus cultures remained in the log phase for approximately 60 hours (Figure 43I). Cultures grown with 100 µg/ml of ampicillin were inhibited after 10 hours of growth in both Bvg phases.
Interestingly, in Bvg minus cultures >5 µg/ml of ampicillin had reduced growth (Figure 43J).
This is unlike BP536, where all cultures in the Bvg minus phase resulted in growth and entry into the stationary phase. Growth of Bvg minus cultures in 10 µg/ml of ampicillin resulted in a drop off of growth at 30 hours, after which there was a steady increase in growth until the end of the growth curve at 90 hours. The reason for this was unknown. Finally, the log phase in Bvg minus cultures was approximately 40 hours.
All of these results indicate a greater resistance to ampicillin in Bvg minus BP536 cultures in comparison to Bvg minus B204 cultures.

These observations indicate a marked difference in ampicillin susceptibility between plate and broth grown cultures for the six strains of *B. pertussis* tested here, with broth grown cultures exhibiting much more resistance to ampicillin than their plate grown counterparts. These results validate earlier observations from the Preston Lab and also reveal a Bvg dependency in ampicillin resistance. Similar to BP536, where Bvg minus cultures was more resistant to ampicillin than Bvg plus cultures, Bvg minus cultures of B1917, UK48, and UK17 also had greater ampicillin resistance in Bvg minus cultures.

In order to understand these results and the resistance to ampicillin observed in broth cultures, the mechanism responsible for this phenomenon was explored in the following sections.

5.3.2. Investigating the enzymatic modification of ampicillin by *B. pertussis* during broth growth

The first mechanism that was examined was the possible enzymatic destruction of ampicillin by β-lactamases and the production of this enzyme in Bvg minus broth conditions. If *B. pertussis* BP536 produces β-lactamase in a Bvg dependent manner, then the high levels of ampicillin resistance observed in Bvg minus broth cultures can be explained.

To investigate the production of a putative β-lactamase enzyme(s) by *B. pertussis* BP536, ampicillin was incubated with broth cultures of BP536. Possible degradation of ampicillin was determined using Reverse Phase Chromatography/ Mass Spectrometry (RPC/MS), and was used to indicate β-lactamase production.

When a sample is run for RPC/MS, base peak chromatograms are produced for both the ampicillin and ampicillin degradation peak revealing when both compounds were eluted from the chromatography column and detected using mass spectrometry. This is referred to as the 'Retention Time' and is the time from injection to detection. Figure 44 depicts the base peak chromatograms for the positive degradation control, *Proteus mirabilis* (JN40), where the ampicillin and ampicillin degradation peaks can be found in Figure 44A and Figure 44B, respectively.

A)

B)
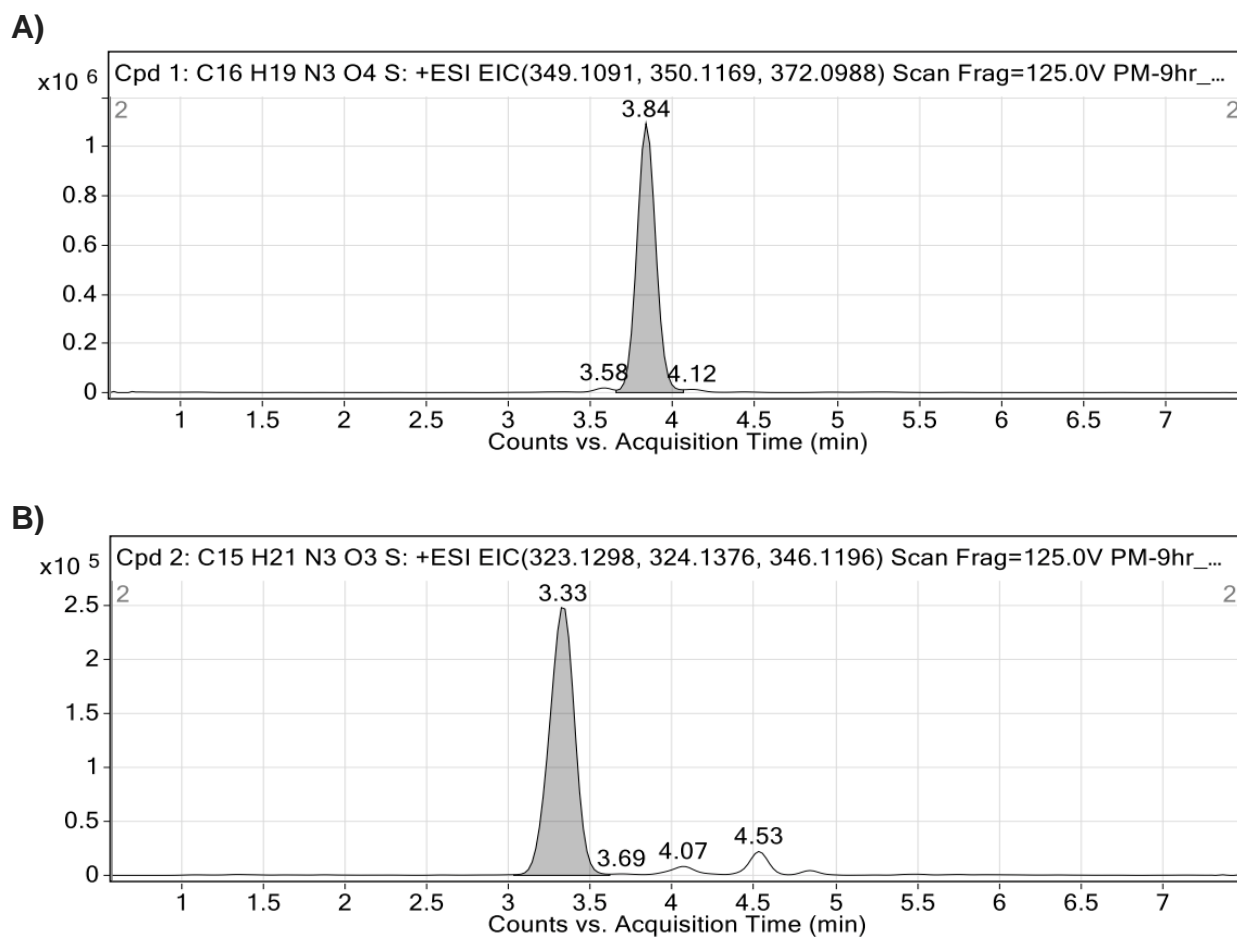
Figure 44. Base peak chromatograms of the positive degradation control.

A) Ampicillin, B) Ampicillin Degradation Product. *P. mirabilis* (JN40) was cultured for 6 hours in SS with 10 µg/ml of ampicillin revealing a peak at 3.84 minute for ampicillin and at 3.33 minutes for the ampicillin degradation product.

The retention time for ampicillin was determined to be 3.84 minutes (Figure 44A) and 3.33 minutes for ampicillin degradation products (Figure 44B).

5.3.2.1. Determining the analytical range for ampicillin using Reverse Phase/ Mass Spectrometry (RPC/MS)

The retention time was used to obtain the 'response factor' for the analytes examined in these experiments. The response factor is a ratio between the response of the detector to that analyte and the quantity of that analyte. The peak area obtained from the chromatograms above at the specified retention times indicate the response to that compound.

This information was used to create a 6-point calibration curve allowing the linearity of this assay to be established for ampicillin (Figure 45). Standards were created using a two-fold dilution series of ampicillin in Stainer- Scholte broth.

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$$Y = 7345579*X + 3245499$$
$$R^2 = 0.9414$$
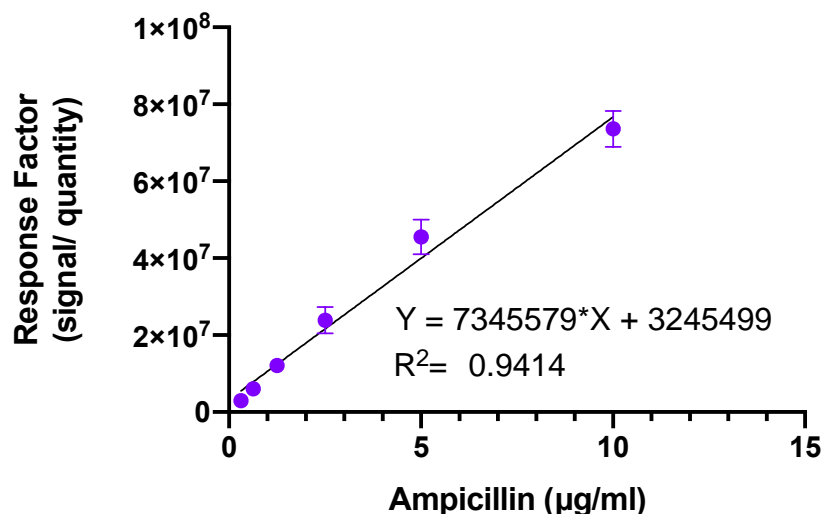
Figure 45. Linearity of RPC/MS assay for ampicillin.

The linearity of the assay was 0.3125- 10 μg/ml of ampicillin. Data represents six biological replicates.

The linearity of the assay was 0.3125- 10 μg/ml of ampicillin. This range was appropriate to detect the degradation product of interest because in the following experiments, the upper limit was not exceeded.

A calibration curve could not be generated to determine the concentration of the ampicillin degradation product in samples with unknown quantities, however the response factor was used as an estimate for the concentration in these samples.

5.3.2.2. Determination of ampicillin degradation product in broth grown cultures using RPC/MS

The ampicillin degradation product was measured in broth cultures of *B. pertussis* BP536 grown in both Bvg conditions with 10 μg/ml of ampicillin. The negative and positive degradation control for this experiment was SS broth incubated with ampicillin, and β-lactamase powder incubated with ampicillin, respectively.

All controls and samples were incubated for up to 23 hours and were assayed at 0, 6 and 23 hours of incubation (Figure 46).
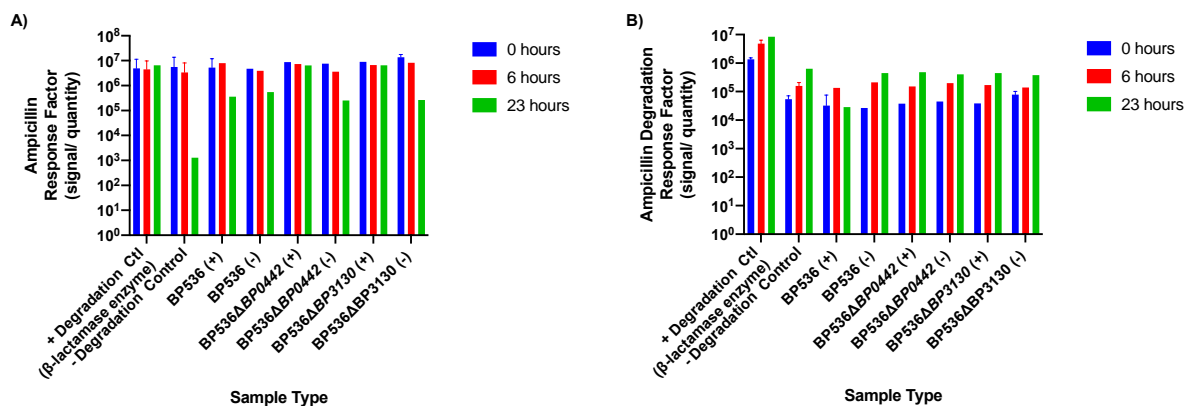
Figure 46. Ampicillin degradation by B. pertussis BP536 in broth grown cultures investigated using RPC/MS.

A) Ampicillin, and B) Ampicillin degradation product. Ampicillin and ampicillin degradation products were detected in 0, 6 and 23-hour samples of *B. pertussis* BP536 samples at comparable levels to the negative degradation control indicating that ampicillin was not degraded by *B. pertussis*. Data represents three biological repeats.

After 6 hours of incubation, there was a greater decrease in the ampicillin detected for the positive degradation control compared to *B. pertussis* BP536 samples and the negative degradation control. Ampicillin levels continued to decrease as the incubation time was increased; this was evident when cultures were assayed 23 hours.

There was a significant decrease in the 23-hour ampicillin level for the negative degradation control that can be attributed to fluctuation between biological repeats.

As expected, there was a resultant increase in the ampicillin degradation product observed as the incubation time increased with the greatest levels detected in the positive degradation control compared to *B. pertussis* BP536 samples and the negative degradation control. This difference was statistically significant, while the difference in the levels of ampicillin degradation product observed between the negative degradation control and *B. pertussis* samples was not statistically significant.

Taken together, these results suggest that ampicillin was not degraded by *B. pertussis* and that *B. pertussis* does not produce a β-lactamase enzyme given the similar levels of ampicillin and ampicillin degradation product obtained by *B. pertussis* compared to the negative degradation control over time.

To ensure that the product assigned as the ampicillin degradation product was correct, it was characterised using Tandem Mass spectrometry (MS/MS). This method further fragments compounds using a second stage of mass spectrometry providing details regarding the structure and composition of the molecule. The spectra of the supernatants of the β-lactamase-producing *E. coli* Nissle pUC19 EC revealed decreased intensities of the molecular peak of ampicillin and the corresponding adducts. Additional peaks at 367.9,

389.9, 411.8, and 324.0 Da appeared to correspond to the hydrolysed form of ampicillin, its sodium adducts, and the hydrolysed, decarboxylated form of ampicillin, respectively (Figure 47 and Figure 48).
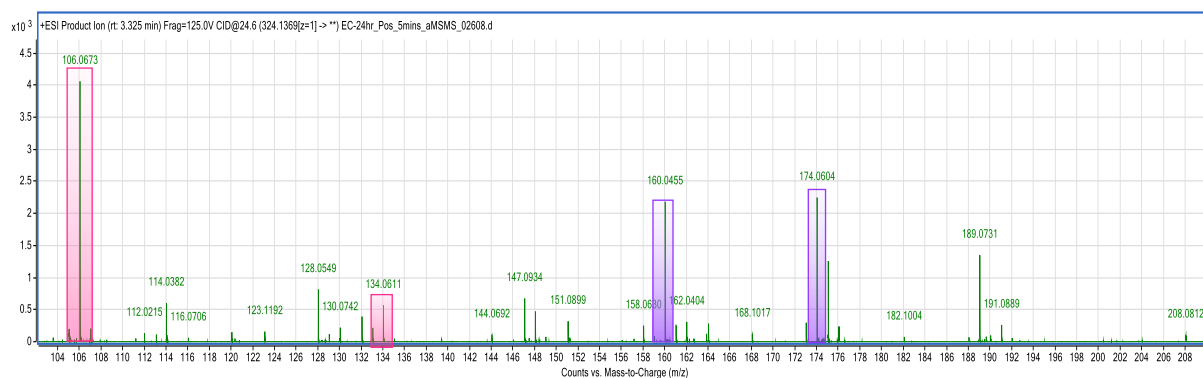
Figure 47. Mass Spectrum of ampicillin and associated degradation product and the relative abundance of each ion.

Distribution of ions from the ampicillin degradation product by mass ($C_{15}H_{21}N_3O_3S$, 324.1376 *m/z*). There were four major product ions for this precursor: 106.0673, 134.0611, 160.0455, 174.0604 *m/z.*

The distribution of ions from the ampicillin degradation product suggests a β-lactam ring of ampicillin is being modified potentially due to the hydrolysis (Figure 48).

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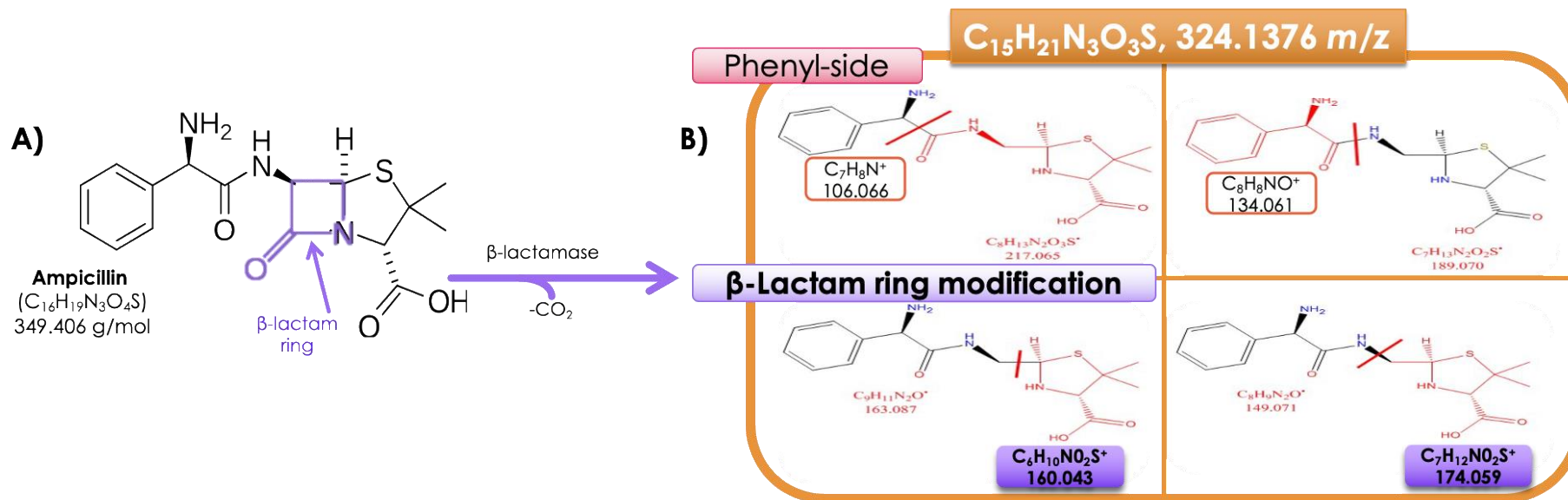
Figure 48. Distribution of ions from the ampicillin degradation product by mass (C15H21N3O3S, 324.1376 m/z).

A) The chemical structure of ampicillin ($C_{16}H_{19}N_3O_4S$) B) There were four major product ions for this precursor. 106.06 *m/z* ($C_7H_6N^+$) and 134.06 *m/z* ($C_8H_8NO^+$) are suggested to be located on the phenyl-side of the molecule, while 160.0455 ($C_6H_{10}NO_2S^+$), $C_7H_{12}NO_2S^+$ (174.0604 *m/z*) *are* potential hydrolysis products produced by β-lactam ring modifications.

Therefore, the MS/MS results confirmed the use of RPC/MS to detect the ampicillin degradation product. It also revealed that the hydrolysis observed in the samples during the RPC/MS analysis was either as a result of the presence of a β-lactamase enzyme produced by *B. pertussis* or hydrolysis from the water component of SS media. It was established that the ampicillin degradation observed in the BP536 samples were not statistically significant, therefore the latter is likely the case. Additionally, there were high levels of degradation observed in the negative degradation control containing SS only, therefore hydrolysis from the water in this media was concluded to be the culprit.

5.3.2.3. Investigating the function of putative β-lactamase genes in B. pertussis BP536

While performing the RPC/MS experiments, putative β-lactamase proteins in *B. pertussis* were also investigated simultaneously to explore the possible production of a β-lactamase in *B. pertussis* BP536.

This experiment began with a tblastn of all β-lactamase proteins in the NCBI database compared to the *B. pertussis* BP536 genome. The hit table from this search can be found in Appendix B. This led to the identification of seven putative metallo-β-lactamase encoding genes in *B. pertussis* represented in Table 19, where the percent identity represents regions of genes found in the *B. pertussis* genome. These regions are represented in the nucleotide range column in Table 19.

Table 19. Homology of putative β-lactamase encoding genes in B. pertussis BP536 compared to known β-lactamase proteins from the NCBI database.

Gene	Percent Identity	Nucleotide Range
BP0442	55.56	445688 to 445741
BP0220	38.30	230844 to 230716
BP3130	30.30	3335419 to 3335601
BP2609	29.07	2762220 to 2761963
BP1113	27.87	1171449 to 1171625
BP0333	26.76	337153 to 336956
BP0230	21.33	238039 to 237815

The genes *BP3130* and *BP0442* were selected to determine if these genes encode for a β-lactamase because they had a percent identity of >30%.

Genes that had homology to *BP3130* include cyclases, putative cyclases, metallo-beta-lactamase domains and MBL fold metallo-hydrolases. These genes were from *Streptomyces cavourensis*, different species of *Bordetella* including *B. parapertussis*, *B. bronchiseptica*, *B. holmesii, Bordetella avium, Bordetella ansorpii, Bordetella hinzii, Bordetella petrii, Bordetella*

trematum, and many species of *Achromobacter* such as *Achromobacter anxifer, Achromobacter pulmonis, Achromobacter insolitus,* and *Achromobacter xylosoxidans.* For *BP0442,* homologous genes include Zn-dependent hydrolases, metallo-beta-lactamase family proteins, and MLB fold metallo-hydrolase from *Streptomyces cavourensis, many species of Achromobacter, B. petrii* and *B. bronchiseptica.*

Critical domains were not identified in these homologous genes.

Both *BP3130* and *BP0442* were deleted from the strain BP536 producing the mutants: BP536Δ*BP3130* and BP536Δ*BP0442.*

The ampicillin susceptibility of these knockouts was tested on solid and liquid media and illustrated in Table 20 and Figure 49-Figure 50, respectively.

Table 20 represents the ampicillin sensitivity of wildtype (WT) BP536 compared to BP536Δ*BP3130* and BP536Δ*BP0442* in plate grown cultures.

Table 20. Minimal inhibitory concentrations of WT B. pertussis BP536, BP536ΔBP3130, and BP536ΔBP0442.

Strains	Bvg⁺ (µg/ml)	Bvg⁻ (µg/ml)
BP536	0.094	0.032
BP536Δ*BP0442*	0.064	0.064
BP536Δ*BP3130*	0.064	0.032

All three samples were sensitive to ampicillin when grown on solid media. Data represents the average of two biological repeats.

Knock-out strains, BP536Δ*BP3130* and BP536Δ*BP0442*, were sensitive to ampicillin when grown on solid media. These results replicate the ampicillin susceptibility of WT BP536 in plate grown cultures and were expected because the proposed production of β-lactamase is assumed to be exclusive to broth grown cultures.

Figure 49 and Figure 50 illustrates the ampicillin susceptibility of these knockout strains in broth grown cultures. If the genes *BP3130* and *BP0442* are responsible for the production of β-lactamase, when knocked out of BP536 the knockout mutants would be more sensitive to ampicillin than WT BP536.
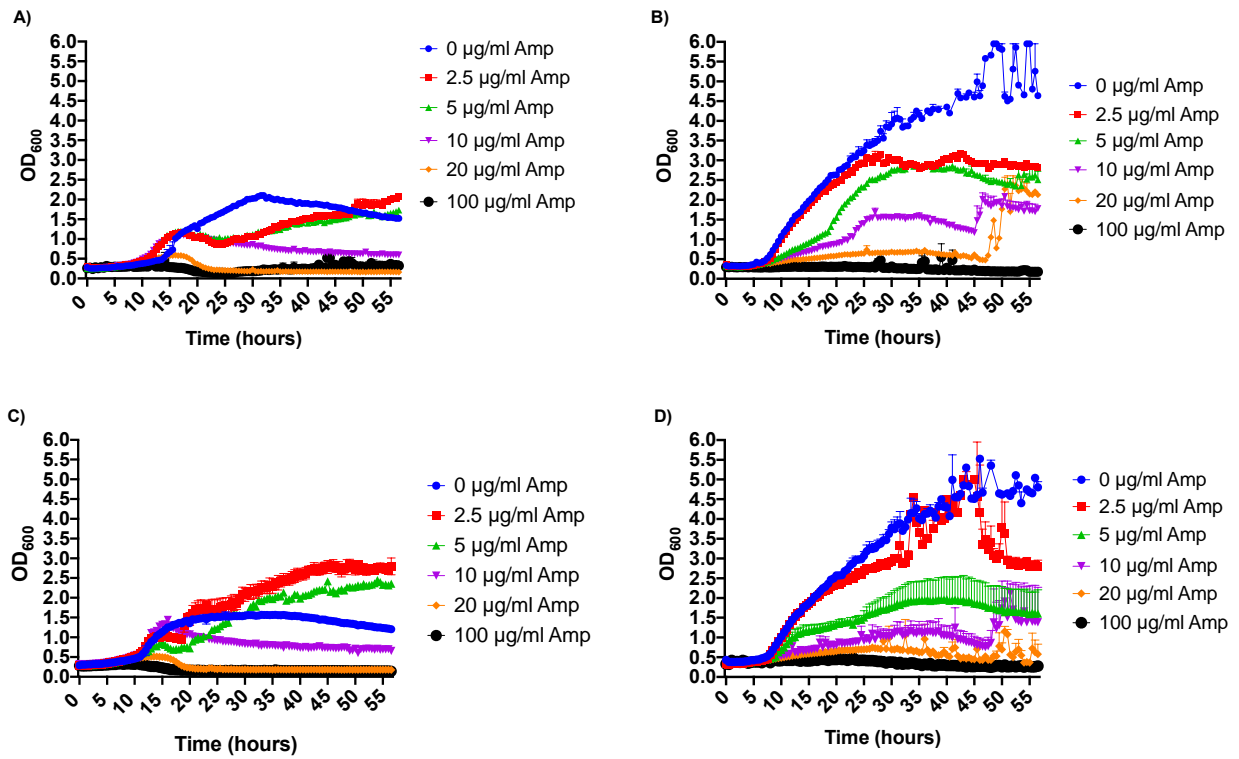
Figure 49. Ampicillin susceptibility for broth grown knockout strains BP536ΔBP3130.

A) BP536 in Bvg plus conditions, B) BP536 in Bvg minus conditions, C) BP536ΔBP3130 in Bvg plus conditions, and D) BP536ΔBP3130 in Bvg minus conditions. In comparison to BP536 grown in Bvg plus conditions, BP536ΔBP3130 grown in the same conditions was also inhibited by >20 μg/ml of ampicillin. In Bvg minus condition, growth of BP536ΔBP3130 was reduced by >2.5 μg/ml of ampicillin and inhibited by >20 μg/ml of ampicillin. However, the control for this experiment conflicted with the original BP536 Bvg minus cultures observed previously, where BP536 strains grown in Bvg minus conditions were resistant to ampicillin. The BP536 Bvg minus control here had reduced growth at >5 μg/ml. Data represents two biological repeats.
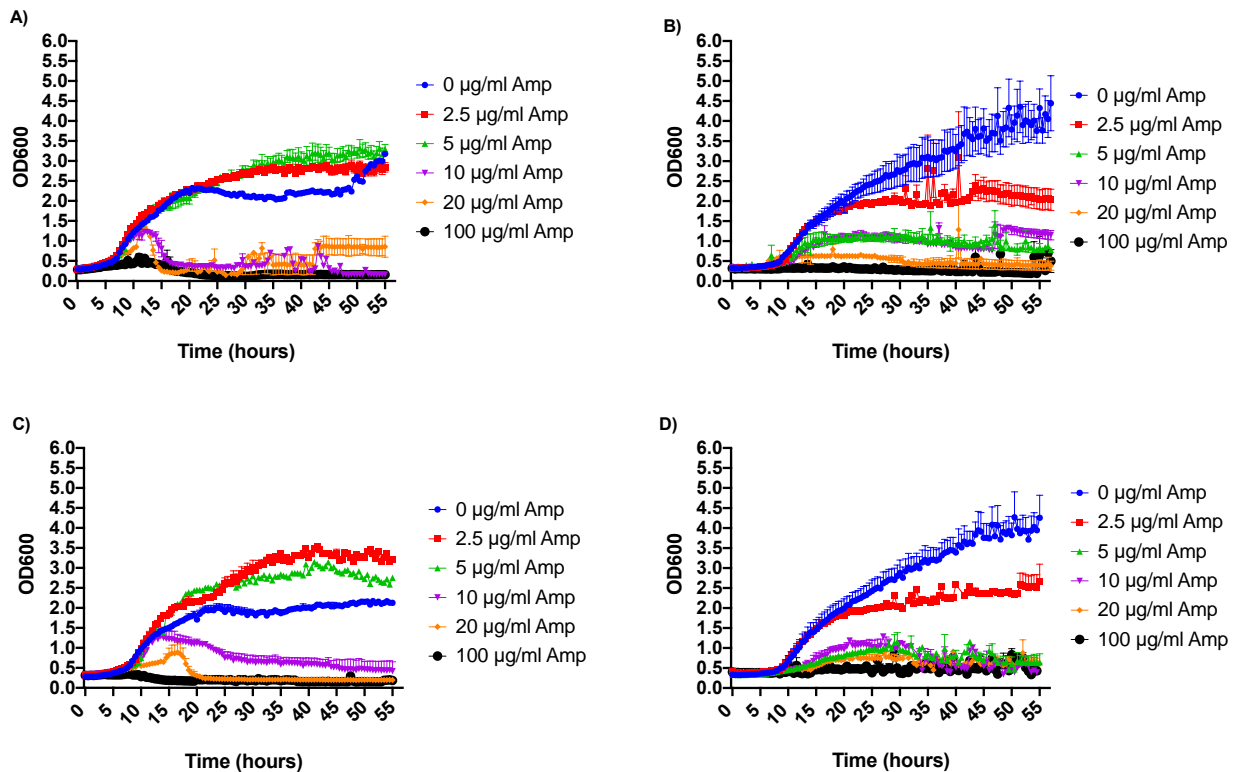
Figure 50. Ampicillin susceptibility for broth grown knockout strains BP536ΔBP0442.

A) BP536 in Bvg plus conditions, B) BP536 in Bvg minus conditions, C) BP536ΔBP0442 in Bvg plus conditions, and D) BP536ΔBP0442 in Bvg minus conditions. BP536ΔBP0442 grown in both Bvg plus and minus conditions were inhibited by >20 µg/ml of ampicillin, however growth in Bvg minus conditions appeared to be reduced after the addition of all concentrations of ampicillin. The growth of this knockout compared to the BP536 control strain in this experiment were comparable and showed the same susceptibility to ampicillin. However, the control for this experiment conflicted with the original BP536 Bvg minus cultures observed previously, where BP536 strains grown in Bvg minus conditions were resistant to ampicillin. Data represents two biological repeats

The knockout strains were grown in broth cultures in both Bvg conditions alongside a WT BP536 control in the same conditions.

The growth of both knockouts was inhibited by >20 µg/ml of ampicillin when grown in Bvg plus and minus conditions and reduced in the presence of low concentrations of ampicillin. In Bvg plus conditions, >5 µg/ml of ampicillin resulted in a reduction of growth for both knockouts. In Bvg minus conditions, >2.5 µg/ml of ampicillin for BP536ΔBP3130 and ≥2.5 µg/ml for BP536ΔBP0442 resulted in a growth reduction.

Compared to WT BP536 in the previous experiments (Figure 42) and previous work completed in the Preston group, ampicillin sensitivity appeared to be restored in broth cultures grown in Bvg minus conditions for BP536ΔBP3130 and BP536ΔBP0442, however compared to the WT BP536 controls completed with this experiment ampicillin sensitivity appeared the same. The WT BP536 controls in this experiment were not resistant to ampicillin when grown in Bvg minus conditions. These experiments were repeated four times in total with similar results (data not shown).

The system does not appear to be reproducible, therefore it was not possible to conclude whether or not the knocked-out genes supported the production of β-lactamase by *B. pertussis*.

These specific genes may work in conjunction with other genes in regard to promoting ampicillin resistance in Bvg minus broth grown cultures. Therefore, these results led to the investigation of additional genes in order to determine their role in the ampicillin susceptibility of plate grown and broth grown cultures.

The next and final section will focus on the increased expression of genes encoding for penicillin binding proteins as a mechanism for ampicillin resistance.

5.3.3. Does *B. pertussis* increase expression of penicillin binding proteins in response to different growth conditions?

This section will explore the increased expression of genes encoding penicillin binding proteins in broth grown *B. pertussis* BP536 as a mechanism to partially circumvent the action of ampicillin in the bacterial cell wall allowing some PBPs to be available for transpeptidation and transglycosylation. This will enable enough cell wall synthesis to occur allowing the bacteria to survive in broth cultures grown in the presence of ampicillin. These candidate penicillin binding proteins have been described previously and are highlighted in Table 21 [205].

Table 21. Penicillin- binding proteins and murein lytic transglycosylases of B. pertussis

Gene	Protein name	Function
BP0102	DacC	D-ala-D-ala carboxypeptidases
BP1051	DacB	
BP1545	DacC2	
BP1061	MltE	Murein lytic transglycosylase*s*
BP3214	MltD	
BP3268	MltA	
BP3028	FtsI	Cell division transpeptidase
BP0905	MrcA	DD-transpeptidases
BP3655	Pbp	
BP2754	Pbp1C	

Gene expression levels for the ten PBPs grown in different media and growth conditions were determined in order to provide a smaller set of genes to evaluate in future experiments.

5.3.3.1. Screening ten genes encoding penicillin binding proteins in B. pertussis BP536 for increased expression in Bvg minus broth cultures

To determine the gene expression levels of the ten genes encoding for PBPs, WT BP536 was grown in plate and broth grown cultures in both Bvg conditions with and without ampicillin. RNA was extracted from each sample and used as a single-stranded template by reverse transcriptase to make cDNA. cDNA was then used as a template for the qPCR reaction.

To establish the linearity of the RT-qPCR assay to screen for increased expression of genes encoding PBPs, primer pairs for each gene were made and used to create standard curves (Figure 51).

A) Standard Curve- 102

Y-intercept: 22.30
R^2: 0.9508

B) Standard Curve- 1545

Y-intercept: 24.26
R^2: 0.9871

C) Standard Curve- 2754

Y-intercept: 25.50
R^2: 0.9745

D) Standard Curve- 3314

Y-intercept: 23.16
R^2: 0.9531

E) Standard Curve- 1061

Y-intercept: 23.99
R^2: 0.9624

F) Standard Curve- 3655

Y-intercept: 21.21
R^2: 0.9397
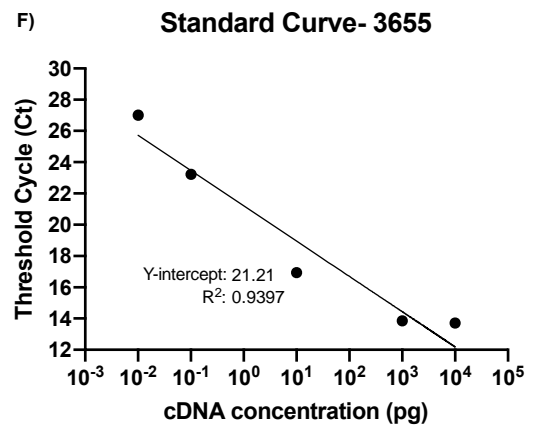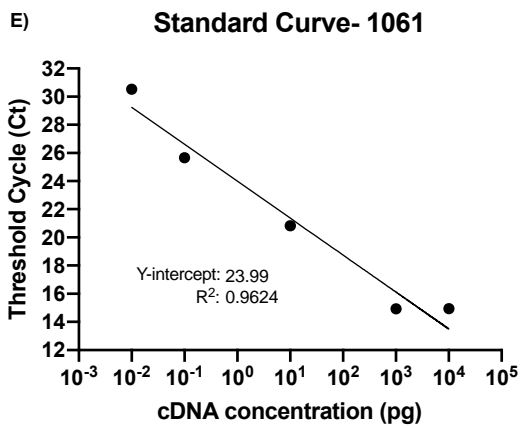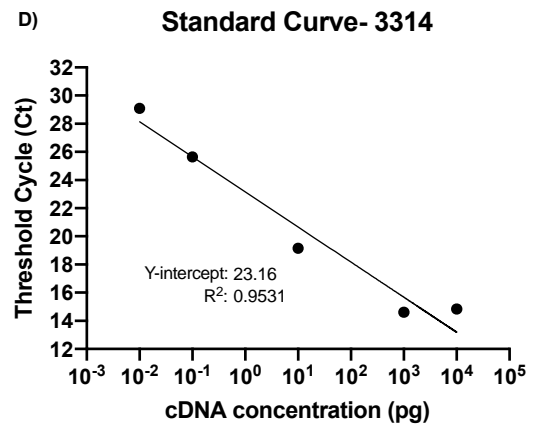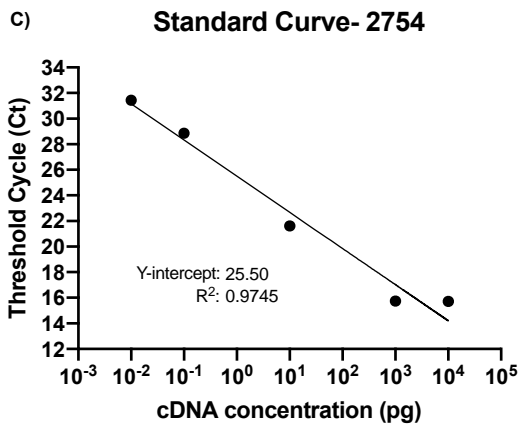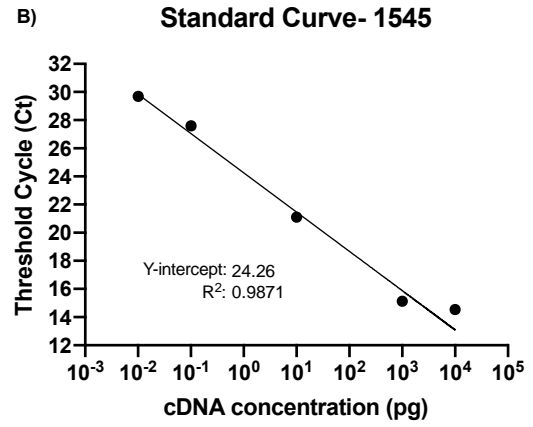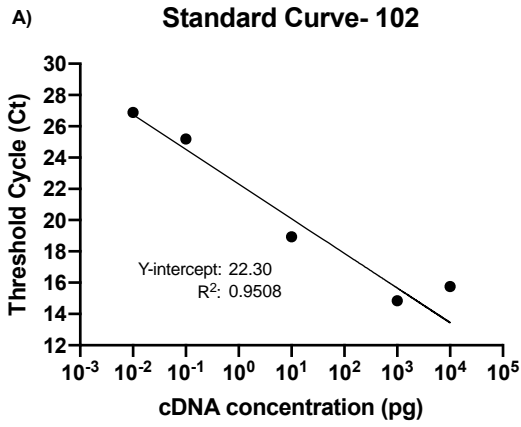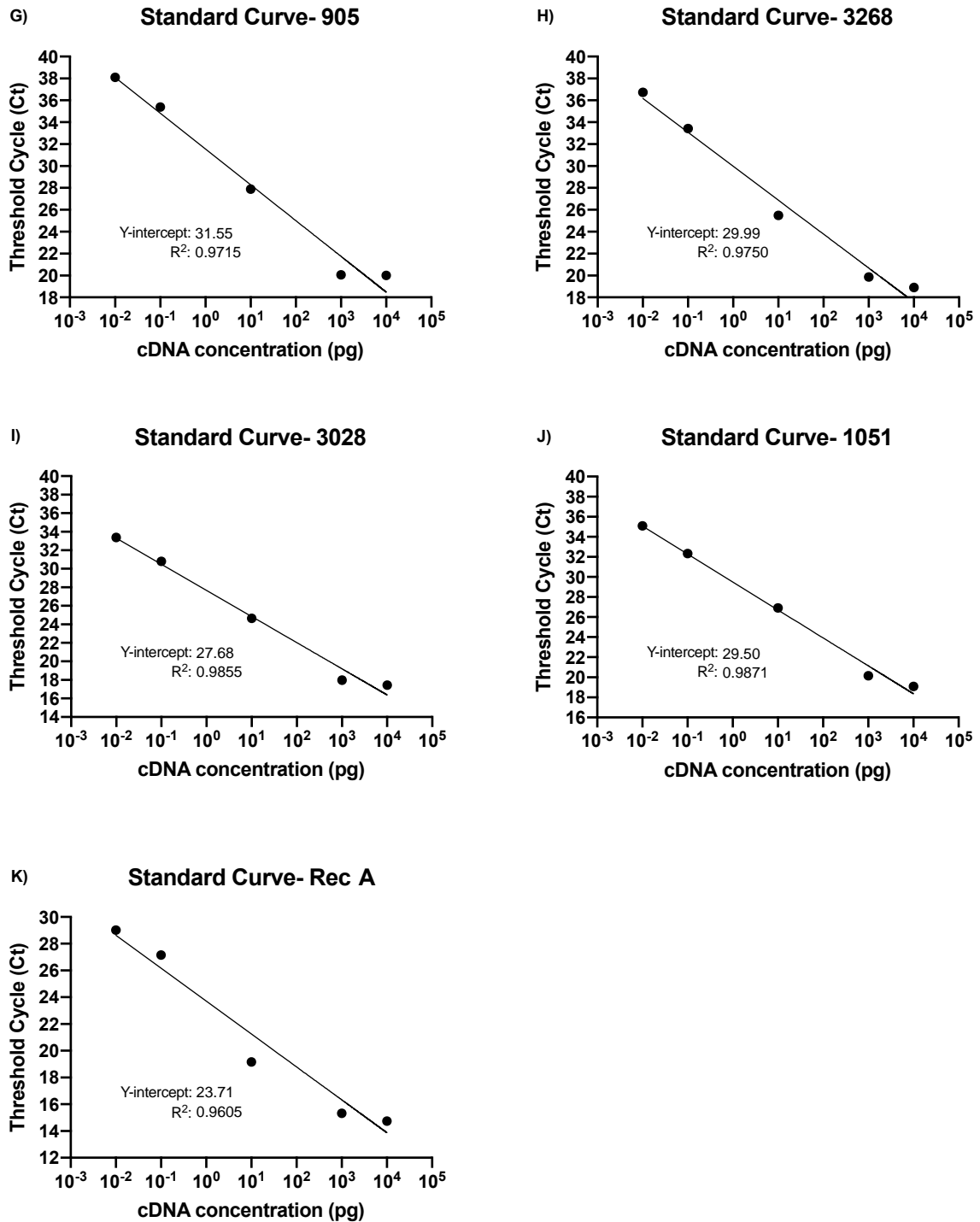
Figure 51. Standard curves for ten genes encoding penicillin binding proteins in B. pertussis BP536.

A) *BP0102*, B) *BP1545*, C) *BP2754*, D) *BP3314*, E) *BP1061*, F) *BP3655*, G) *BP0905*, H) *BP3268*, I) *BP3028*, J) *BP1051*, and K) *RecA*. Each standard curve provided a linear range of 0.01pg- 1000pg of cDNA to be used in the RT-qPCR. Standards were created using pooled cDNA from all samples tested and using a 10-fold serial dilution. Data represents three technical triplicates.

The standard curves for these figures were made using pooled cDNA from all samples

tested, as well the PCR efficiency was approximately 100% for each curve and the

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correlation of coefficient (R^2) was between 0.94-0.98. The linearity of the assay was between 0.01-1000pg of cDNA for the RT-qPCR PBP screen.

The pooled cDNA was used as a representative sample that contained each gene of interest and was used to established linearity of the RT-qPCR assay. A PCR efficiency of 100% indicated that the amount of product was doubling during each cycle. This variable was automatically calculated by the analyser, where the slope of the standard curve was obtained. The R^2 value is the coefficient of correlation of the standard curve and should be approximately 1 in order to signify optimal performance of the primer set.

5.3.3.2. Gene expression analysis revealed three highly expressed PBPs in broth cultures

The levels of gene expression for each of the ten penicillin binding proteins in each sample type was determined using RT-qPCR (Figure 52 and Figure 53). The gene expression of target genes was normalised to the housekeeping gene *RecA* and compared to a control group.
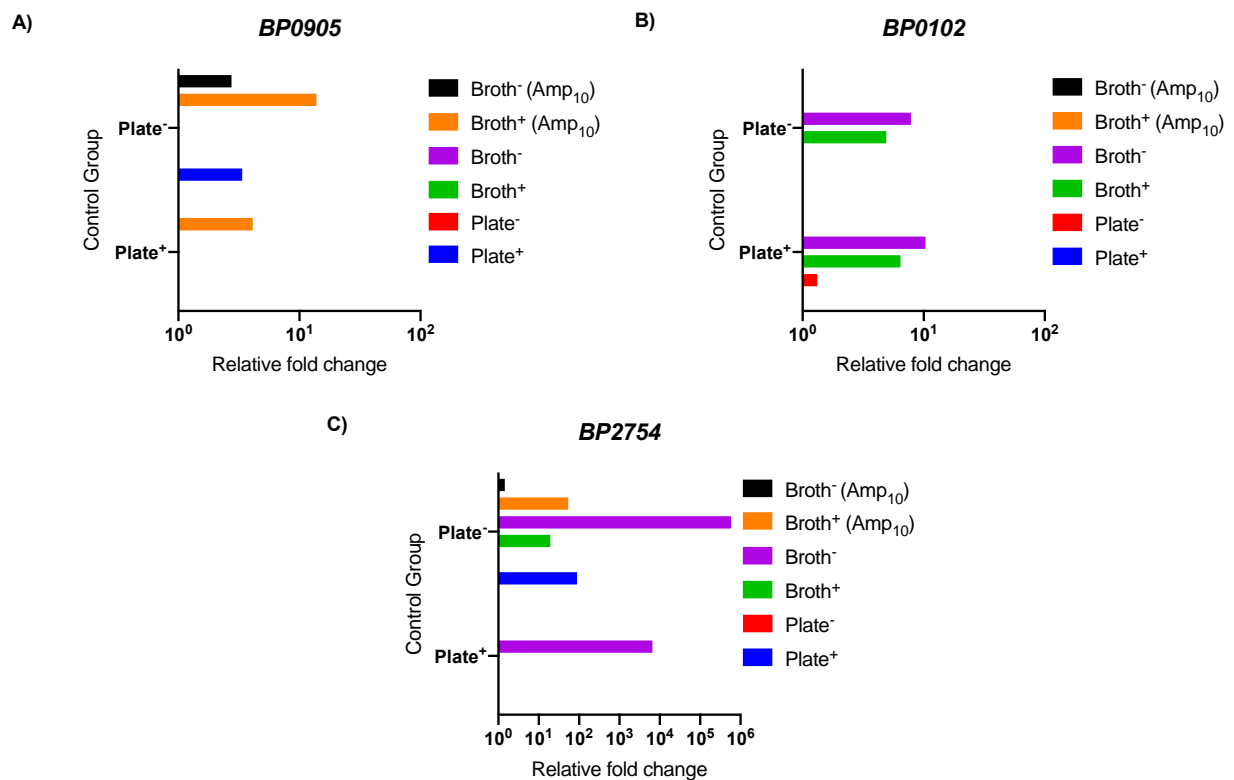
Figure 52. Three genes encoding for PBPs that were highly expressed in broth grown cultures compared to plate grown cultures.

A) *BP0905*, B) *BP0102*, C) *BP2754*. The gene expression levels of these three penicillin binding proteins were higher in broth cultures compared to plate cultures. *BP0905* was the only gene that had higher expression levels in broth cultures with ampicillin, specifically in the Bvg plus phase, compared to cultures grown in other conditions. Only fold change values of >1 were represented here. The data in this figure represents the average of technical triplicates from a single biological experiment.
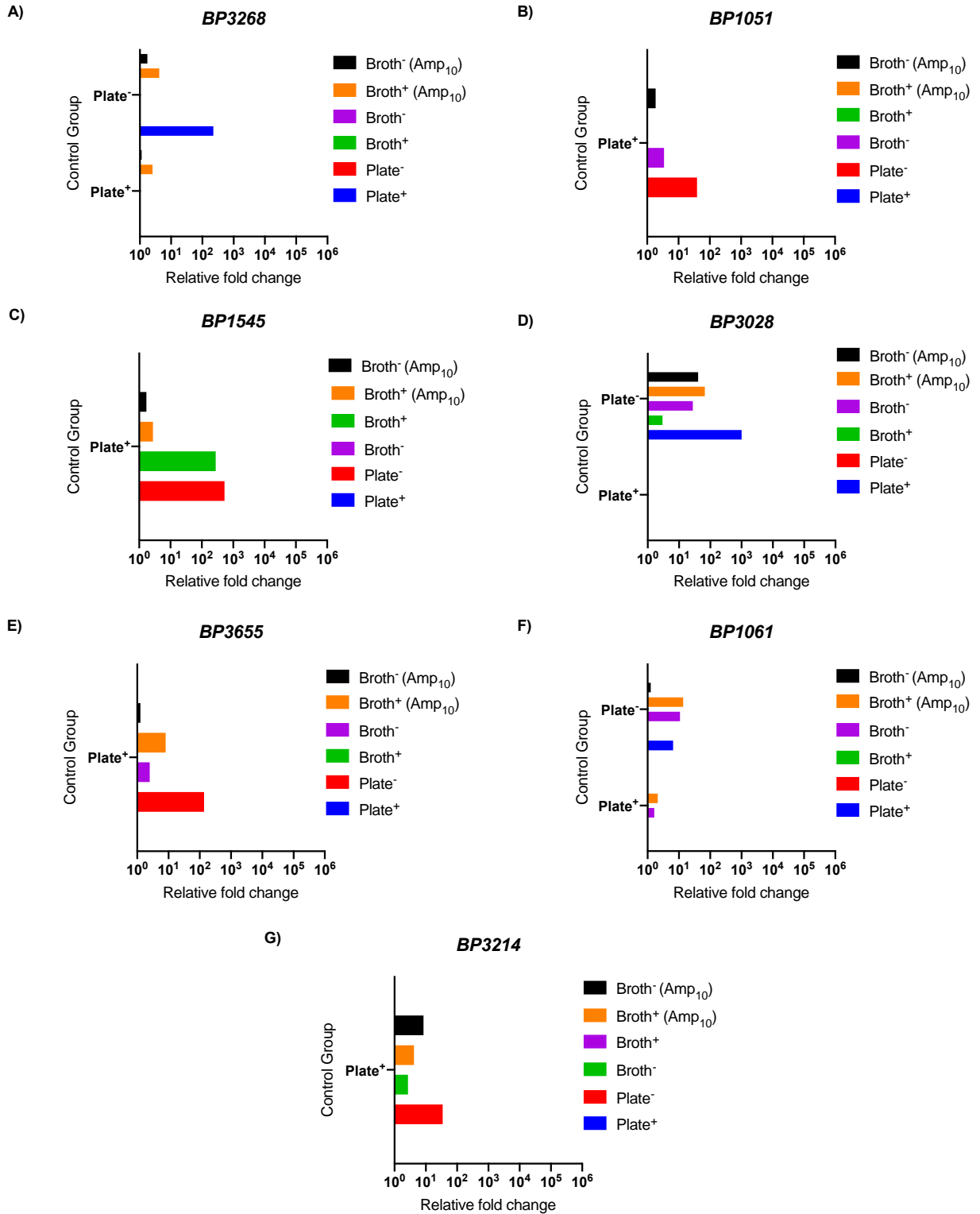
Figure 53. Genes encoding for PBPs that were less expressed in broth grown cultures compared to plate grown cultures.

A) *BP3268*, B) *BP1051*, C) *BP1545*, D) *BP3028*, E) *BP3655*, F) *BP1061*, and G) *BP3214*. The gene expression levels of these penicillin binding proteins were lower in broth cultures compared to plate cultures. Only fold change values of >1 were represented here. The data in this figure represents the average of technical triplicates from a single biological experiment.

The gene expression analysis revealed elevated levels of *BP0905, BP0102,* and *BP2754* in broth cultures compared to plate cultures. All of these genes appeared to be expressed in a Bvg dependent manner with a higher expression of *BP0905* in Bvg plus conditions, and both *BP0102 and BP2754* being highly expressed in Bvg minus conditions.

This work needs to be repeated in order to validate these findings, however based on observations thus far, the increased expression of the three genes that encode for PBPs in broth cultures compared to plate cultures could contribute to the ampicillin resistance mechanisms observed in *B. pertussis* BP536 broth grown cultures.

5.4. Discussion

The origins of this chapter arose from a simple, yet puzzling, observation in the *B. pertussis* strain BP536, where there was a dynamic increase in ampicillin resistance in broth grown cultures compared to plate grown cultures. The mechanism behind the ampicillin resistance observed is unknown and was therefore explored. By investigating the enzymatic modification of the antibiotic and increased expression of periplasmic peptidoglycan transpeptidases, this chapter highlights potential mechanisms preventing ampicillin from interacting with the cell.

The initial observations regarding ampicillin resistance in broth cultures were validated in BP536, as well as investigated for five other strains of *B. pertussis*.
Ampicillin sensitivity was confirmed in plate grown cultures for all six strains tested, however when grown in broth cultures each strain produced different susceptibilities to ampicillin. Unlike BP536, B1917 and the UK strains, where there was greater ampicillin resistance observed in Bvg minus broth cultures in comparison to Bvg plus broth cultures, ampicillin resistance was higher in Bvg plus broth cultures of B184 and B204. Both B184 and B204 were collected in the Netherlands, but years apart, 1988 and 2000, respectively. B1917 was also collected in the Netherlands in the year 2000, but growth was not inhibited in Bvg minus broth cultures, while in B184 and B204, it was. The UK strains were collected in 2012 and BP536 was collected in the 1950's in Japan [122]. Time and location of collection may have influenced the evolution of ampicillin resistance in these strains; however, this was not investigated in this chapter. What was investigated however, was the production of β-lactamase by *B. pertussis*.
Bacteria produce β-lactamases as a source of protection against β-lactams. These enzymes have been instrumental in the resistance against β-lactam antibiotics in many gram negative

bacteria and work by inactivating the antibiotic preventing it from acting on the bacterial cell [91]. The caveat with applying this mechanism to support the initial observations is that *B. pertussis* BP536 must produce and release β-lactamase at higher levels in Bvg minus conditions compared to Bvg plus conditions because of the Bvg dependency of ampicillin resistance noted earlier.

The production of β-lactamase by BP536 was explored using two methods. The first method was also the simplest, where the presence of this enzyme was determined through its action, that is by observing the degradation of ampicillin. In the second method, I knocked out putative β-lactamase genes in BP536 in order to assess if ampicillin sensitivity was restored in Bvg minus broth grown cultures.

Determining the ampicillin degradation product can be a useful tool to assist in the determination of β-lactamase production, however ampicillin appears to decompose in water containing divalent cations such as Ca^{2+} and Mg^{2+}, both of which are found in SS broth [206]. This was due to the hydrolysis of the β-lactam ring in ampicillin when incubated in water. This was evident when measuring the ampicillin degradation product in all samples including the ampicillin degradation negative control, where there were high levels of ampicillin degradation product observed in the negative control and the *B. pertussis* samples. However, neither of these levels were as high as the levels observed in the positive degradation control illustrating the value in utilising the proper controls and statistical tools. Collectively, these results indicate that there were no ampicillin degradation products produced by *B. pertussis* and that it is unlikely that this pathogen produces the β-lactamases enzyme.

There are many other ways in which ampicillin can become degraded, however in order to speculate the presence of a β-lactamase enzyme, hydrolysis of the β-lactam ring must be present in the ampicillin degradation product. It is for this reason that ampilloic acid ($C_{15}H_{21}N_3O_3S$) was selected as an ampicillin degradation product. Additional ampicillin degradation products that also could be used to investigate the production of β-lactamase include 2-hydroxy-3-phenylpyrazine ($C_{10}H_8N_2O$) and ampicillin diketopiperazine ($C_{16}H_{19}N_3O_4S$).

While performing this RPC/MS, I also created the knockout mutants BP536Δ*BP3130* and BP536Δ*BP0442* with the aim of evaluating the function *BP3130* and *BP0442 as* β-lactamase producing genes. Interestingly, ampicillin sensitivity appeared to be restored when these knockouts were grown in Bvg minus conditions compared to the WT BP536 growth in the original experiments. However, in comparison to the WT BP536 controls grown with the

knockout strains, the restoration of ampicillin sensitivity could not be determined because ampicillin resistance was no longer detected in the Bvg minus WT BP536 control.

As a result of inconsistent ampicillin susceptibility profiles of broth grown Bvg minus WT BP536, it was not possible to determine if *BP3130* and *BP0442* were β-lactamase producing genes or play a role in the ampicillin resistance observed in the original observations. These results indicate that growing *B. pertussis* over time in broth cultures is not a reproducible system.

Whether or not *B. pertussis* produces β-lactamase enzymes, inhibition to β-lactam antibiotics can still occur due to multiple mechanisms or genes that work together to promote resistance and retain cellular function in the bacteria. Therefore, the increased expression of penicillin binding proteins was explored and proved to be the most convincing mechanism of ampicillin resistance presented in this chapter thus far.

There was increased expression of the genes *BP0905*, *BP0102,* and *BP2754* in broth cultures compared to plate cultures. These results may indicate broth induced changes in the cell wall of *B. pertussis.*

This work only represents a single experiment because it was incredibly cumbersome to carry-out due the number of genes evaluated, and growth conditions involved. If these results are validated with repeat experiments, illustrating increased expression of genes encoding PBPs in Bvg minus conditions compared to plate cultures, then those genes could be knocked out in BP536 and their affinity for ampicillin assessed.

The purpose of this chapter was not to evaluate new prophylactic treatments for pertussis, but as mentioned, to better understand an interesting observation within the Preston lab where broth grown cultures of BP536 were resistant to ampicillin, while plate grown cultures of BP536 were sensitive to ampicillin.

I successfully validated this observation in BP536, as well in the five other post-vaccination strains and proceeded to take a broad stroke approach to briefly explore two possible mechanisms of antibiotic resistance and to determine which path to take forward in future work.

To summarise, the results from this work included ruling out the production of an ampicillin degradation product, while simultaneously creating successful knockouts of two putative β-lactamase producing genes. Testing the knockouts to determine if ampicillin sensitivity in

broth cultures was restored yielded inconclusive results preventing the production of β-lactamases production as mechanism of resistance from being conclusively ruled out.

When investigating cell wall changes as a mechanism of ampicillin resistance, there was an increase in expression of three genes encoding for penicillin binding proteins in broth grown cultures. This change in expression appeared to be broth induced and may contribute to ampicillin resistance.

Although the Bvg dependency of ampicillin resistance in BP536 makes this work more complex, both the production of β-lactamase enzymes and the increase in penicillin binding proteins require further investigation in order to confirm the mechanisms behind ampicillin resistance in broth cultures of *B. pertussis*.

This work showcases the *in vitro* growth dynamics of plate grown and broth grown cultures of *B. pertussis* in the presence of antibiotics amongst pre- and post- vaccination strains enabling a better understanding of the physiology and biology of *B. pertussis.*

Chapter 6

Conclusions and Future Perspectives
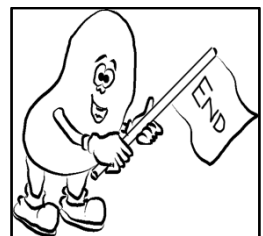
6. Conclusions and Future Perspectives

This chapter consolidates and discusses the findings presented in the previous chapters and reflects on the significance of that work for the future.

6.1. Summary of aims

B. pertussis is the causative agent of pertussis, a vaccine preventable illness currently experiencing a global resurgence. To better cope with the resurgence, there needs to be a greater understanding of how this bacterium grows *in vivo* and *in vitro,* and the growth variability between strains. To summarise, the aim of this thesis was to better understand the growth dynamics of *B. pertussis.* To accomplish this a diverse subset of strains from a variety of regions and time periods were used and compared to the reference *B. pertussis* strain BP536. The additional five strains used include: B1917, UK48, UK71, B2973 (B184), and B1878 (B204). The growth of *B. pertussis in vivo* during colonisation, *in vitro* during phenotypic modulation, and in response to the antibiotic ampicillin, were highlighted in Chapter three, four and five, respectively.

In Chapter three, the *in vivo* colonisation of *B. pertussis* during the Human Challenge Model was determined using the PMA-qPCR assay optimised for *B. pertussis B1917.* The successful use of this assay for the enumeration of live cells from *B. pertussis* strains in general was also illustrated. As well the PMA-qPCR assay was also used to count the total number of live and dead *B. pertussis* cells taken up by macrophages during an *in vitro* infection.

In Chapter four, the *in vitro* sensitivity of *B. pertussis* to phenotypic modulation amongst the different strains was investigated using reporter constructs made specifically for each Bvg phase. The genotypic variation of the BvgAS virulence regulon from these strains were compared and the functional implications investigated.

Finally, in Chapter five, different strains of *B. pertussis* were grown in the presence of ampicillin *in vitro.* The ampicillin susceptibility of these strains was evaluated in both plate and broth cultures and compared to BP536 to validate previous results found in the Preston Lab where broth cultures of BP536 were more resistant to ampicillin than plate cultures. Once validated, the mechanism of ampicillin resistance was explored. Areas for exploration included β-lactamases production, as well as the increased expression of genes encoding penicillin binding proteins.

Taken together, the variability of *B. pertussis* growth was observed utilising a variety of tools *in vivo* and *in vitro* for six different strains providing a better understanding of the growth dynamics of *B. pertussis* in general*.*

6.2. Overview of research presented and significance of findings

6.2.1. PMA-qPCR assay

PMA inhibited PCR-mediated amplification from dead *B. pertussis* B1917 allowing for the enumeration of live cells using qPCR resulting in the successful optimisation of the PMA-qPCR assay for use during the human colonisation study.

The optimised method was shown to be useable for at least five other strains of *B. pertussis*, despite being optimised only for B1917. The qPCR target was the multi-copy insertion sequence IS481 and its copy number is similar in the other five strains used here. Additionally, high numbers of exogenous cells were not found to inhibit the action of PMA demonstrating the high sensitivity of this assay to detect very low levels of viable and dead *B. pertussis.*

The utility of the assay was extended to use on macrophage uptake assays, where the total, live, and dead number of *B. pertussis* B1917 was obtained over time. Paralleling the pre-existing literature, viable *B. pertussis* cells were detected in macrophages 24 hours after infection using culture, however unlike the literature replication within macrophages could not be confirmed using this method.

Collectively, the optimisation of PMA-qPCR assay allowed for the enumeration of *B. pertussis in vivo* during colonisation of human challenge volunteers. The use of the assay was extended to enumerate five additional strains of *B. pertussis* and *B. pertussis* in macrophages.

6.2.2. Sensitivity to modulation amongst different strains

The *BvgAS* virulence regulon is conserved amongst the *Bordetella* spp. and plays a signifcant role in the growth cycle. Previous literature have determined that mutations within *bvgS* affects sensitivity to modulation [2]. These results have been confirmed within this thesis and while the original study completed by Herrou *et al.* determined the functional implications of mutations using both the modulators nicotinate and $MgSO_4$, and the reporter

gene *lacZ* under the control of the pertussis toxin operon, it only represents the sensitivity to modulation of a single strain (BP536). In Chapter 4, sensitivity to modulation was determined for six strains of *B. pertussis* grown in each of the three Bvg phases *in vitro*, using reporter proteins under the control of promoters from genes expressed differentially in each phase.

ptxA and *vrgX* promoters drove high levels of reporter expression in the Bvg plus and Bvg minus phases respectively. Specifically, strains grown in the absence of modulation produced the highest levels of green fluorescence signifying entry into the Bvg plus phase. However, there was a drop-off in green fluorescence from *ptxAGFP*pBBRK that occurred at approximately 30 mM MgSO4 for BP536, 16 mM of MgSO4 for B1917, B184 and B204, and 12 mM of MgSO4 for UK48 and UK71. It was approximately at these concentrations where the levels of *bipA* expression were the highest indicating entry into the Bvg intermediate phase. The decline of red fluorescent activity from the reporter construct *bipARFP*pBBRK at 28 mM of MgSO4 for BP536, 16 mM of MgSO4 for the strains B184 and B204 and 12 mM of MgSO4 for B1917, UK48 and UK71 marked the entry into the Bvg minus phase. It was at these concentrations where maximal fluorescence occurred for the reporter construct *vrgXYFP*pBBRK.

To summarise, B184, B204, UK71, UK48 and B1917 responded similarly to the modulator MgSO$_4$ where the entry and exit into each phase occurred at comparable concentrations, while the entry into both the Bvg intermediate and minus phase utilised a greater concentration of modulator in BP536. Additionally, it was through the use of modulators and reporter constructs that revealed that entry into each phase was triggered by the exit from the previous phase. Finally, the use of the reporter construct *bipARFP*pBBRK in the six different strains truly showcased the phenotypic variability in growth amongst strains and separated this work from previous literature [2].

To explore possible mutations in the *bvgAS* that may be responsible for the phenotypic variability seen amongst the six strains, a sequence alignment between each component of the *bvgAS* in BP536 was compared to the phentotypically similar B1917, UK48, UK71, B184 and B204 revealing no SNPs in *bvgA* and *bvgR*. This was not the case in the *bvgS*, where lysine found at position 705 in both BP536 and B184, was replaced with a glutamic acid in B1917, UK48, UK71 and B204 ($K_{705}E$). Noted previously and confirmed using reporter constructs in Chapter 4, the lysine in position 705 in BP536 appeared to increase resitance to modulation with the concentration of modulation required for entry into the Bvg intermediate phase being higher than the other five strains, which had glutamic acid in this position instead [2]. B184 also had a second a mutation, $I_{694}V$. However, it appears that the

combination of both $K_{705}E$ and the $I_{694}V$ did not increase resistance to modulation during work with reporter constructs, rather appeared to be a neutral substitution. B184 appeared to enter and exit the Bvg phases using similar concentrations of modulator as B1917, UK48, UK71, and B204.

In the work presented in this chapter, the sensitivity to modulation was successfully monitored using reporter constructs in plate grown *B. pertussis* cultures. However, many reporter constructs failed to provide fluorescence above background levels, despite the selection of promoters from differentially and highly expressed Bvg regulated genes from the RNA seq data. This could be as a result of reporter genes influencing certain promoters as seen in the case of the *luxA* gene that contains an intrinsically curved DNA segment within the 5' coding sequence that suppresses the transcription for some promoters [207]. However, this was not explored further in this work.

Interestingly, what emerged from these findings is that the commonly used WT strain BP536 may not be representative to currently circulating strains, as the other five strains were more sensitive to modulation in all Bvg phases and, with the exception of B184, contained the *bvgS* mutation, $K_{705}E$.

6.2.3. Ampicillin resistance mechanisms of *B. pertussis*

Ampicillin is a broad-spectrum β-lactam antibiotic known for inhibiting bacterial cell wall synthesis. Despite not being the treatment of choice for pertussis infections, ampicillin resistance was detected in broth cultures in comparison to plate cultures.

This resistance appeared to be heightened in Bvg minus cultures of BP536, B1917, UK48 and UK71 in comparison to these strains grown in Bvg plus conditions. This Bvg dependency was not observed in B184 or B204.

Mechanisms behind this ampicillin resistance and the Bvg dependency of resistance were investigated with limited success. The two mechanisms include the production of a β-lactamase enzyme and the increased expression of penicillin binding proteins, both of which were hypothesised to limit the action of ampicillin on the cell.

A major hurdle to analysing the results of this chapter was difficulty in obtaining reproducible growth curves in 96-well plates. This hampered my ability to analyse the data from the β-lactamase experiment where the control WT BP536 grown in Bvg minus conditions was sometimes resistant to ampicillin, and at other times was sensitive. Therefore, the restoration of ampicillin sensitivity seen in the growth curves of strains with putative β-lactamase genes knocked-out could have been as a result of inconsistent growth curves or

because β-lactamase enzymes were no longer being produced. Without consistent WT control data, experiments such as the knock-out growth curve data could not be interpreted.

A more promising mechanism of ampicillin resistance was the increase in the expression of genes encoding for penicillin bindings proteins in broth cultures. Changes to the bacterial cell wall, including the increase in penicillin binding proteins, may be induced by liquid culture conditions, rather than ampicillin induced changes. This was noted because ampicillin did not seem to enhance the expression of genes encoding for PBPs in broth cultures. The increased expression of three PBPs as a mechanism of ampicillin resistance requires additional biological repeats prior to evaluating the function of each of these three genes.

Throughout this work, it was evident that water hydrolyses ampicillin. Therefore, ampicillin will become degraded by the water in the SS broth media over time in the growth assays. Ampicillin degradation by the SS broth may even be a contributing factor to the inconsistent growth of *B. pertussis* in broth cultures depending on the rate of ampicillin degradation.

Taken together with the limitations of growing *B. pertussis* in a 96-well plate and the preliminary data from the PBP gene expression analysis, ampicillin resistance in broth and the mechanisms behind this perceived resistance requires further investigation in *B. pertussis.*

As mentioned, a majority of experiments in Chapter 4 and Chapter 5 were hindered by the poor growth of *B. pertussis* in 96-well microplates. In many cases repeat growth curves were not reproducible. Therefore, the limitations of this system were explored further.
A reason for the poor growth could be that bacteria were inoculated into 96-well microplates from starter cultures grown in flasks. This was done to ensure plates were inoculated with actively growing cells to try and overcome the difficulties of multi well plate growth. These difficulties may include increased lag times for bacterial growth in the wells of the microplate. The use of a common glycerol stock made from a broth culture in the log phase can be used to standardise the inoculation of starter cultures. This will allow all starter cultures and broth cultures to be made from the same stock, in the same growth phase, instead of multiple plate cultures at different stages of growth, grown on slightly media prepared at different times with bacteria grown for different amounts of time. This could assist in creating more reproducible growth curves and increase traceability when it comes to troubleshooting issues with growth.

An essential part of these experiments included the detection of the stationary phase during growth. Therefore, shaking of the culture during growth was important to ensure homogenisation and uniformity of bacterial cells in the media, as well to increase oxygen uptake by strict aerobes such as *B. pertussis*. This will prevent cells from clumping together and settling to the bottom, creating an anaerobic environment that will inhibit the growth of *B. pertussis*. I used the double orbital shaking mode with a shaking frequency of 500 rpm, however this may not have been the best mode. The linear, bidirectional shaking has been described as preferential for the analysis of the stationary phase in growth curves [108].

Incubation in the FLUOstar Omega Microplate Reader was completed using temperature-regulated heating plates above and below the plate movement area. This allowed for uniform incubation in each well and a stable air temperature. However, a major source of error was the evaporation of samples in each well during growth at 37°C. This was because culture volumes were very small at 200 µl/ml for each well and a reduction of liquid volume levels greatly skewed the OD_{600} values. Evaporation was particularly evident in wells close to the edge of the plate. Therefore the wells on the edge were filled with water and samples were restricted to rows B-G and columns 2-11 [108]. Additionally, samples were clustered together enabling insulation from the other wells, and a deep cover lid was used to reduce evaporation.

Despite attempting to limit experimental error and increase reproducibility during experiments, inconsistent observations were still observed in both Chapter 4 and 5. Previous literature have suggested that bacteria cultured for greater than 48 hours in 96-well plates will be subjected to evaporation resulting in inconsistent growth curves [108]. I found this to be true for the slow growing *B. pertussis,* that had an average culture time of approximately 60-90 hours in the 96-well plate.

Due to the high number of strains analysed in various growth conditions throughout this thesis, traditional broth culture in flasks with physical measurements taken throughout the duration of growth would have been unmanageable. An alternative approach for future work could be to utilise a SFR Shake Flask Reader (Presens Precision Sensing, Regensburg, Germany) to take continuous reads during growth that can be correlated to the optical density (OD).

6.3. Future Perspectives

In addition to improving the growth of broth cultures mentioned above, there is also other work throughout this thesis that can be improved and built upon.

For the PMA-qPCR assay successfully optimised in Chapter 3, this assay can also be optimised for the other five strains described in this thesis to confirm that the enumeration of these strains using the optimised assay for B1917 was transferable and accurate for other strains. Additionally, to increase the specificity of this assay, the single copy targets *BP0283* or *BP0485*, which are commonly used in many diagnostic labs, could also considered in addition to the multi-copy target *IS481* in a multiplex PCR [113].

To improve upon the use of the PMA-qPCR assay to detect replication of *B. pertussis* in macrophages over time, the total time for infecting the macrophages should be increased to greater than 72 hours if using a MOI of 250. Alternatively, the MOI can be reduced to 120, the value used in previous literature, and the infection repeated for 72 hours using B1917 as well as additional *B. pertussis* strains [174].

The sensitivity to modulation of six strains were successfully evaluated using the reporter constructs in Chapter 4. The next step to this work would be to use these reporter constructs to better understand growth of *B. pertussis* at 37°C in broth cultures, however the half-life of the reporter proteins used needs to be established first.

The half-life of reporter proteins is critical in understanding these growth curves and refers to a reduction in the level of protein by 50%. For the reporters used, fluorescence can be used as a proxy to determine a 50% reduction in the level of protein. In pre-liminary experiments, reporter constructs were grown in conditions where the promoter was downregulated, however inconsistent results for the half-life of GFP, RFP and YFP in *B. pertussis* BP536 were produced. This could be due to the fact the method used to downregulate promoter activity was not suitable to allow for half-life data to be acquired. The current method involved growing strains in the presence or absence of modulation that will allow for downregulation of promoter activity in a 96-well plate, however inconsistent growth has been observed resulting in difficult to interpret results. Perhaps growing broth cultures in a flask and sampling periodically for fluorescence may be a more suitable approach.

Without the half-life of each reporter gene in *B. pertussis*, the exit from each phase during growth cannot be determined if the fluorescent signal from reporter protein stays elevated for a prolonged period. Therefore, short half-life variants of reporter genes should also be explored further to accommodate for the exit from each Bvg phase.

Additionally, improving the broth growth of *B. pertussis* strains in the 96-well microplate strains should be considered prior to evaluating the entry and exit of the strains from each Bvg phase during growth at 37°C. Heptakis may be a useful agent to address this because it sequesters inhibitory compounds found in the SS media during growth.

Ampicillin resistance mechanisms of broth grown cultures were explored in Chapter 5 revealing many areas for additional work.

First, the ampicillin levels throughout the entire duration of broth culture growth needs to be assessed in both Bvg phases. This will reveal whether or not the ampicillin is becoming degraded over time enabling *B. pertussis* to grow. This will also reveal whether or not broth grown *B. pertussis* is truly resistant to ampicillin if ampicillin levels are maintained throughout growth.

If ampicillin levels are maintained during growth, then WT BP536 control growth curves should be re-examined and established in order to evaluate the restoration of ampicillin sensitivity in strains where putative β-lactamase genes have been knocked-out. Additionally, the increase of the three genes encoding for PBPs should be confirmed with a second and third repeat of the gene expression analysis using RNA from broth and plate cultures. If the increases in gene expression are confirmed, then removing these genes via a knock-out would result in a decrease of PBPs able to bind to ampicillin and continue synthesizing the bacterial cell wall resulting in ampicillin sensitivity. If sensitivity to ampicillin is restored as a result of the knock-out, then it can be assumed that these three PBPs contribute to the ampicillin resistance of broth cultures.

This work can also benefit from improving the growth of broth cultures on 96-well plates. Finally, these experiments can be repeated for the other five strains to the confirm any ampicillin resistance mechanisms found using BP536.

6.4. Conclusion

Within the scope of this thesis the variability of growth of *B. pertussis* was explored illustrating the dynamic nature of *B. pertussis* growth, as well as limitations to growing *B. pertussis in vitro*.

Through the optimisation of the quick and highly sensitive PMA-qPCR assay, live and dead *B. pertussis* from different strains were enumerated both *in vivo* and *in vitro* in a research setting, with the ability to use the assay in a range of other settings, including industry and diagnostic laboratories where a quantitative measure of viable *B. pertussis* number is required.

The sensitivity to modulation of the different strains was also successfully evaluated in plate grown strains using reporter constructs that can also be used to determine the Bvg phase of *B. pertussis* during growth in broth cultures.

Finally, ampicillin resistance was observed in all six strains grown in broth *in vitro*. Two mechanisms of resistance were explored that include the increased expression of penicillin binding proteins and production of a β-lactamase enzyme. However, broth cultures were

difficult to grow and the analysis of data from these experiments were challenging to evaluate as a result.

Taken together, this thesis explored and illustrated the variability of growth of *B. pertussis in vivo* and *in vitro* allowing for a better understanding of the biology of this pathogen. As stated in the introduction, pertussis is resurgent in vaccinated populations world-wide. Through the application of these findings, we hope to support future work in the treatment and prevention of pertussis.

References
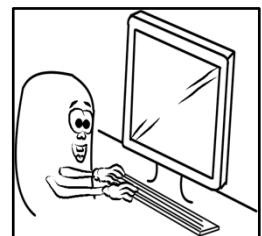
References

1. Mattoo S, Cherry JD. Clinical Manifestations of Respiratory Infections Due to Bordetella pertussis and Other Bordetella Subspecies Molecular Pathogenesis , Epidemiology , and Clinical Manifestations of Respiratory Infections Due to Bordetella pertussis and Other Bordetella Su. Clin Microbiol Rev. 2005;18(2):326–82.

2. Herrou J, Debrie AS, Willery E, Renaud-Mongénie G, Locht C, Mooi F, et al. Molecular evolution of the two-component system BvgAs involved in virulence regulation in Bordetolla. PLoS One. 2009 Sep 14;4(9).

3. Elahi S, Holmstrom J, Gerdts V. The benefits of using diverse animal models for studying pertussis. Trends Microbiol. 2007;15(10):462–8.

4. Ramkissoon S, MacArthur I, Ibrahim M, de Graaf H, Read RC, Preston A. A qPCR assay for Bordetella pertussis cells that enumerates both live and dead bacteria. Ho PL, editor. PLoS One [Internet]. 2020 Apr 30;15(4):e0232334. Available from: https://dx.plos.org/10.1371/journal.pone.0232334

5. Kilgore PE, Salim AM, Zervos MJ, Schmitt H. Pertussis : Microbiology , Disease , Treatment , and Prevention. 2016;29(3):449–86.

6. Paddock CD, Sanden GN, Cherry JD, Gal AA, Langston C, Tatti KM, et al. Pathology and pathogenesis of fatal Bordetella pertussis infection in infants. Clin Infect Dis. 2008;47(3):328–38.

7. Jõgi P, Oona M, Kaart T, Toompere K, Maskina T, Koort I, et al. Pertussis and parapertussis in children and adults with a persistent cough: an observational study. Infection. 2018;46(1):83–91.

8. Birkebæk NH, Kristiansen M, Seefeldt T, Degn J, Møller A, Heron I, et al. Bordetella pertussis and chronic cough in adults. Clin Infect Dis. 1999;29(5):1239–42.

9. Harnden A, Grant C, Harrison T, Perera R, Brueggemann AB, Mayon-White R, et al. Whooping cough in school age children with persistent cough: Prospective cohort study in primary care. Br Med J. 2006;333(7560):174–7.

10. Gilberg S, Njamkepo E, Du Châtelet IP, Partouche H, Gueirard P, Ghasarossian C, et al. Evidence of Bordetella pertussis infection in adults presenting with persistent cough in a French area with very high whole-cell vaccine coverage. J Infect Dis. 2002;186(3):415–8.

11. Strebel P, Nordin J, Edwards K, Hunt J, Besser J, Burns S, et al. Population-based incidence of pertussis among adolescents and adults, Minnesota, 1995-1996. J Infect Dis. 2001;183(9):1353–9.

12. Park S, Lee SH, Seo KH, Shin KC, Park YB, Lee MG, et al. Epidemiological aspects of pertussis among adults and adolescents in a Korean outpatient setting: A multicenter, PCR-based study. J Korean Med Sci. 2014;29(9):1232–9.

13. Dalby T, Harboe ZB, Krogfelt KA. Seroprevalence of pertussis among Danish patients with cough of unknown etiology. Clin Vaccine Immunol. 2010;17(12):2016–23.

14. Teepe J, Broekhuizen BDL, Kretzschmar M, Verheij TJM, Ieven M, Loens K, et al. Prevalence, diagnosis, and disease course of pertussis in adults with acute cough: A prospective, observational study in primary care. Br J Gen Pract. 2015;65(639):e662–7.

15. Senzilet LD, Halperin SA, Spika JS, Alagaratnam M, Morris A, Smith B, et al. Pertussis is a frequent cause of prolonged cough illness in adults and adolescents. Clin Infect Dis. 2001;32(12):1691–7.

16. Sealey KL, Belcher T, Preston A. Bordetella pertussis epidemiology and evolution in the light of pertussis resurgence. Infect Genet Evol. 2016;40:136–43.

17. Hellwig SMM, Rodriguez ME, Berbers G a M, van de Winkel JGJ, Mooi FR. Crucial role of antibodies to pertactin in Bordetella pertussis immunity. J Infect Dis. 2003;188(5):738–42.

18. Warfel JM, Edwards KM. Pertussis vaccines and the challenge of inducing durable immunity. Curr Opin Immunol [Internet]. 2015;35:48–54. Available from:

http://dx.doi.org/10.1016/j.coi.2015.05.008

19. HPA. Confirmed pertussis cases (England and Wales). Heal Prot Rep. 2012;6(43).

20. Higgs R, Higgins SC, Ross PJ, Mills KHG. Immunity to the respiratory pathogen Bordetella pertussis. Mucosal Immunol [Internet]. 2012;5(5):485–500. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22718262

21. Spokes PJ, Quinn HE, McAnulty JM. Review of the 2008–2009 pertussis epidemic in NSW: notifications and hospitalisations. N S W Public Health Bull. 2010;21(8):167.

22. De Melker HE, Schellekens JFP, Neppelenbroek SE, Mooi FR, Rümke HC, Conyn-Van Spaendonck MAE. Reemergence of pertussis in the highly vaccinated population of the Netherlands: Observations on surveillance data. Emerg Infect Dis. 2000;6(4):348–57.

23. Van der Maas NAT, Mooi FR, de Greeff SC, Berbers GAM, Spaendonck MAEC van, de Melker HE. Pertussis in the Netherlands, is the current vaccination strategy sufficient to reduce disease burden in young infants? Vaccine [Internet]. 2013;31(41):4541–7. Available from: http://dx.doi.org/10.1016/j.vaccine.2013.07.060

24. Fisman DN, Tang P, Hauck T, Richardson S, Drews SJ, Low DE, et al. Pertussis resurgence in Toronto, Canada: A population-based study including test-incidence feedback modeling. BMC Public Health [Internet]. 2011;11(1):694. Available from: http://www.biomedcentral.com/1471-2458/11/694

25. Winter K, Harriman K, Zipprich J, Schechter R, Talarico J, Watt J, et al. California Pertussis epidemic, 2010. J Pediatr [Internet]. 2012;161(6):1091–6. Available from: http://dx.doi.org/10.1016/j.jpeds.2012.05.041

26. Acosta AM, DeBolt C, Tasslimi A, Lewis M, Stewart LK, Misegades LK, et al. Tdap Vaccine Effectiveness in Adolescents During the 2012 Washington State Pertussis Epidemic. Pediatrics. 2015;135(6):981–9.

27. Ross PJ, Sutton CE, Higgins S, Allen AC, Walsh K, Misiak A, et al. Relative Contribution of Th1 and Th17 Cells in Adaptive Immunity to Bordetella pertussis: Towards the Rational Design of an Improved Acellular Pertussis Vaccine. PLoS Pathog. 2013;9(4).

28. De Graaf H, Gbesemete D, Gorringe AR, Diavatopoulos DA, Kester KE, Faust SN, et al. Investigating Bordetella pertussis colonisation and immunity: Protocol for an inpatient controlled human infection model. BMJ Open. 2017;7(10):1–9.

29. COTTER PA, MILLER JF. Bordetella. In: Principles of Bacterial Pathogenesis. Elsevier; 2001. p. 619–74.

30. Munoz JJ, Peacock MG. Action of pertussigen (pertussis toxin) on serum IgE and on Fcε receptors on lymphocytes. Cell Immunol. 1990;127(2):327–36.

31. Goldman WE, Cookson BT. Structure and functions of the Bordetella tracheal cytotoxin. Tokai J Exp Clin Med. 1988;13 Suppl:187–91.

32. Cookson BT, Cho HL, Herwaldt LA, Goldman WE. Biological activities and chemical composition of purified tracheal cytotoxin of Bortella pertussis. Infect Immun. 1989;57(7):2223–9.

33. Carbonetti NH. Bordetella pertussis. Curr Opin Infect Dis [Internet]. 2016 Jun;29(3):287–94. Available from: http://journals.lww.com/00001432-201606000-00010

34. Locht C, Keith JM, Science S, Series N, Jun N, Locht C, et al. Pertussis Toxin Gene: Nucleotide Sequence and Genetic Organization Published by: American Association for the Advancement of Science Stable URL: https://www.jstor.org/stable/1697142 REFERENCES Linked references are available on JSTOR for this article: 1986;232(4755):1258–64.

35. Nicosia A, Perugini M, Franzini C, Casagli MC, Borri MG, Antoni G, et al. Cloning and sequencing of the pertussis toxin genes: operon structure and gene duplication. Proc Natl Acad Sci [Internet]. 1986 Jul 1 [cited 2021 Mar 21];83(13):4631–5. Available from: https://www.pnas.org/content/83/13/4631

36. Farizo KM, Cafarella TG, Burns DL. Evidence for a ninth gene, ptlI, in the locus encoding the pertussis toxin secretion system of Bordetella pertussis and formation of

a PtlI-PtlF complex. J Biol Chem [Internet]. 1996;271(49):31643–9. Available from: http://dx.doi.org/10.1074/jbc.271.49.31643

37.   Tamura M, Nogimori K, Murai S, Yajima M, Ito K, Katada T, et al. Subunit Structure of Islet-Activating Protein, Pertussis Toxin, in Conformity with the A-B Model. Biochemistry. 1982;21(22):5516–22.

38.   Ormazábal M, Bartel E, Gaillard ME, Bottero D, Errea A, Zurita ME, et al. Characterization of the key antigenic components of pertussis vaccine based on outer membrane vesicles. Vaccine [Internet]. 2014;32(46):6084–90. Available from: http://dx.doi.org/10.1016/j.vaccine.2014.08.084

39.   Katada T, Tamura M, Ui M. The A protomer of islet-activating protein, pertussis toxin, as an active peptide catalyzing ADP-ribosylation of a membrane protein. Arch Biochem Biophys. 1983 Jul 1;224(1):290–8.

40.   Kaslow HR, Burns usilla L. Pertussis toxin and target eukaryotic cells: binding, entry, and activation. FASEB J. 1992;6(9):2684–90.

41.   Carbonetti NH. Contribution of pertussis toxin to the pathogenesis of pertussis disease. Pathog Dis. 2015;73(8):ftv073.

42.   Roberts M, Fairweather NF, Leininger E, Pickard D, Hewlett EL, Robinson A, et al. Construction and characterization of Bordetella pertussis mutants lacking the vir-regulated P.69 outer membrane protein. Mol Microbiol [Internet]. 1991 Jun;5(6):1393–404. Available from: http://doi.wiley.com/10.1111/j.1365-2958.1991.tb00786.x

43.   Inatsuka CS, Xu Q, Vujkovic-Cvijin I, Wong S, Stibitz S, Miller JF, et al. Pertactin is required for Bordetella species to resist neutrophil-mediated clearance. Infect Immun. 2010;78(7):2901–9.

44.   Cherry JD, Gornbein J, Heininger U, Stehr K. A search for serologic correlates of immunity to Bordetella pertussis cough illnesses. Vaccine. 1998;16(20):1901–6.

45.   Baće A, Zrnić T, Begovac J, Kuzmanović N, Čulig J. Short-term treatment of pertussis with azithromycin in infants and young children. Eur J Clin Microbiol Infect Dis. 1999;18(4):296–8.

46.   Storsaeter J, Hallander HO, Gustafsson L, Olin P. Levels of anti-pertussis antibodies related to protection after household exposure to Bordetella pertussis. Vaccine. 1998;16(20):1907–16.

47.   Mattoo S, Cotter P a, Miller JF. [Frontiers in Bioscience 6, e168-e186, November 1, 2001] MECHANISMS OF. Front Biosci. 2001;6:168–86.

48.   Cotter PA, Yuk MH, Mattoo S, Akerley BJ, Boschwitz J, Relman DA, et al. Filamentous hemagglutinin of Bordetella bronchiseptica is required for efficient establishment of tracheal colonization. Infect Immun. 1998;66(12):5921–9.

49.   Prasad SM, Yin Y, Rodzinski E, Tuomanen EI, Masure HR. Identification of a carbohydrate recognition domain in filamentous hemagglutinin from Bordetella pertussis. Infect Immun. 1993;61(7):2780–5.

50.   Hazenbos WLW, Geuijen CAW, van den Berg BM, Mooi FR, van Furth R. Bordetella pertussis Fimbriae Bind to Human Monocytes via the Minor Fimbria) Subunit FimD. J Infect Dis [Internet]. 1995 Apr 1;171(4):924–9. Available from: https://academic.oup.com/jid/article-lookup/doi/10.1093/infdis/171.4.924

51.   Dupré E, Wohlkonig A, Herrou J, Locht C, Jacob-Dubuisson F, Antoine R. Characterization of the PAS domain in the sensor-kinase BvgS: mechanical role in signal transmission. BMC Microbiol [Internet]. 2013;13(1):172. Available from: http://www.biomedcentral.com/1471-2180/13/172

52.   Melvin JA, Scheller E V, Miller JF, Cotter PA. Bordetella pertussis pathogenesis: current and future challenges. Nat Rev Microbiol [Internet]. 2014;12(4):274–88. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4205565&tool=pmcentrez&rendertype=abstract

53.   Uhl MA, Miller JF. Central role of the BvgS receiver as a phosphorylated intermediate in a complex two-component phosphorelay. J Biol Chem. 1996;271(52):33176–80.

54.   Sobran, Ashley M. and Cotter PA. The BvgS PAS domain: an independent sensory

perception module in the Bordetella BvgAS phosphorelay. 2019;8(5):55.

55. Dupré E, Herrou J, Lensink MF, Wintjens R, Vagin A, Lebedev A, et al. Virulence Regulation with Venus Flytrap Domains: Structure and Function of the Periplasmic Moiety of the Sensor-Kinase BvgS. PLoS Pathog. 2015;11(3):1–21.

56. Herrou J, Bompard C, Wintjens R, Dupré E, Willery E, Villeret V, et al. Periplasmic domain of the sensor-kinase BvgS reveals a new paradigm for the Venus flytrap mechanism. Proc Natl Acad Sci U S A. 2010;107(40):17351–5.

57. Smith AM, Guzmán CA, Walker MJ. The virulence factors of Bordetella pertussis: A matter of control. FEMS Microbiol Rev. 2001;25(3):309–33.

58. Perraud AL, Kimmel B, Weiss V, Gross R. Specificity of the BvgAS and EvgAS phosphorelay is mediated by the C- terminal HPt domains of the sensor proteins. Mol Microbiol. 1998;27(5):875–87.

59. Mishra M, Deora R. Mode of action of the Bordetella BvgA protein: Transcriptional activation and repression of the Bordetella bronchiseptica bipA promoter. J Bacteriol. 2005;187(18):6290–9.

60. Bock A, Gross R. The BvgAS two-component system of Bordetella spp.: a versatile modulator of virulence gene expression. Int J Med Microbiol [Internet]. 2001;291(2):119–30. Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=11437335

61. Veal-Carr WL, Stibitz S. Demonstration of differential virulence gene promoter activation in vivo in Bordetella pertussis using RIVET. Mol Microbiol. 2005;55(3):788–98.

62. Jones AM, Boucher PE, Williams CL, Stibitz S, Cotter PA. Role of BvgA phosphorylation and DNA binding affinity in control of Bvg-mediated phenotypic phase transition in Bordetella pertussis. Mol Microbiol. 2005;58(3):700–13.

63. Hengge R. Principles of c-di-GMP signalling in bacteria. Nat Rev Microbiol. 2009;7(4):263–73.

64. Chen Q, Stibitz S. The BvgASR virulence regulon of Bordetella pertussis. Curr Opin Microbiol [Internet]. 2019;47:74–81. Available from: https://doi.org/10.1016/j.mib.2019.01.002

65. Cotter PA, Jones AM. Phosphorelay control of virulence gene expression in Bordetella. Trends Microbiol. 2003;11(8):367–73.

66. Kinnear SM, Boucher PE, Stibitz S, Carbonetti NH. Analysis of BvgA activation of the pertactin gene promoter in Bordetella pertussis. J Bacteriol. 1999;181(17):5234–41.

67. Goffin P, Slock T, Smessaert V, De Rop P, Dehottay P. A versatile, non genetically modified organism (GMO)-based strategy for controlling low-producer mutants in Bordetella pertussis cultures using antigenic modulation. Biotechnol J. 2015;10(8):1269–80.

68. Cotter PA, Miller JF. BvgAS-mediated signal transduction: Analysis of phase-locked regulatory mutants of Bordetella bronchiseptica in a rabbit model. Infect Immun. 1994;62(8):3381–90.

69. Warfel JM, Zimmerman LI, Merkel TJ. Comparison of three whole-cell pertussis vaccines in the baboon model of pertussis. Clin Vaccine Immunol. 2016;23(1):47–54.

70. Ebell MH, Marchello C, Callahan M. Clinical diagnosis of Bordetella pertussis infection: A systematic review. J Am Board Fam Med. 2017;30(3):308–19.

71. Tiwari T, Murphy, Trudy V, Moran J. Recommended Antimicrobial Agents for the Treatment and Postexposure Prophylaxis of Pertussis. CDC Recomm Reports [Internet]. 2005;54(14):1–16. Available from: https://www.cdc.gov/mmwr/preview/mmwrhtml/rr5414a1.htm

72. Zhanel GG, Dueck M, Hoban DJ, Vercaigne LM, Embil JM, Gin AS, et al. Review of Macrolides and Ketolides. Drugs. 2001;61(4):443–98.

73. Asano K, Tryka E, Cho JS, Keicho N. Macrolide therapy in chronic inflammatory diseases. Mediators Inflamm. 2012;2012:2012–4.

74. Pichichero ME, Hoeger WJ, Casey JR. AZITHROMYCIN FOR THE TREATMENT OF

PERTUSSIS. Pediatr Infect Dis J. 2003;22(9):847–9.

75. de Graaf H, Ibrahim M, Hill AR, Gbesemete D, Vaughan AT, Gorringe A, et al. Controlled Human Infection With Bordetella pertussis Induces Asymptomatic, Immunizing Colonization. Clin Infect Dis. 2020;71(2):403–11.

76. Bass JW, Klenk EL, Kotheimer JB, Linnemann CC, Smith MHD, Mitchell IA, et al. Antimicrobial treatment of pertussis. J Pediatr [Internet]. 1969 Nov;75(5):768–81. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0022347669802994

77. Trollfors B. Effect of erythromycin and amoxycillin on bordetella pertussis in the nasopharynx. Infection [Internet]. 1978 Sep;6(5):228–30. Available from: http://link.springer.com/10.1007/BF01642314

78. Fernández L, Hancock REW. Adaptive and mutational resistance: Role of porins and efflux pumps in drug resistance. Clin Microbiol Rev. 2012;25(4):661–81.

79. Brazas MD, Hancock REW. Using microarray gene signatures to elucidate mechanisms of antibiotic action and resistance. Drug Discov Today. 2005;10(18):1245–52.

80. Guillot S, Descours G, Gillet Y, Etienne J, Floret D, Guiso N. Macrolide-resistant bordetella pertussis infection in newborn girl, France. Emerg Infect Dis. 2012;18(6):966–8.

81. Shahcheraghi F, Lotfi MN, Nikbin VS, Shooraj F, Azizian R, Parzadeh M, et al. The first macrolide-resistant Bordetella pertussis strains isolated from Iranian patients. Jundishapur J Microbiol. 2014;7(6):1–5.

82. Zhang Q, Li M, Wang L, Xin T, He Q. High-resolution melting analysis for the detection of two erythromycin-resistant Bordetella pertussis strains carried by healthy schoolchildren in China. Clin Microbiol Infect. 2013;19(6).

83. Bartkus JM, Juni BA, Ehresmann K, Miller CA, Sanden GN, Cassiday PK, et al. Identification of a mutation associated with erythromycin resistance in Bordetella pertussis: Implications for surveillance of antimicrobial resistance. J Clin Microbiol. 2003;41(3):1167–72.

84. Yamaguchi T, Kawasaki Y, Katsukawa C, Kawahara R, Kawatsu K. The first report of macrolide-resistant bordetella pertussis isolation in Japan. Jpn J Infect Dis. 2020;73(5):361–2.

85. Yao SM, Liaw GJ, Chen YY, Yen MH, Chen YH, Mu JJ, et al. Antimicrobial susceptibility testing of Bordetella pertussis in Taiwan prompted by a case of pertussis in a paediatric patient. J Med Microbiol. 2008;57(12):1577–80.

86. Cassiday PK, Tobin-D'Angelo M, Watson JR, Wu KH, Park MM, Sanden GN. Co-infection with two different strains of Bordetella pertussis in an infant. J Med Microbiol. 2008;57(3):388–91.

87. Ohtsuka M, Kikuchi K, Shimizu K, Takahashi N, Ono Y, Sasaki T, et al. Emergence of quinolone-resistant Bordetella pertussis in Japan. Antimicrob Agents Chemother. 2009;53(7):3147–9.

88. Palzkill T. Metallo-β-lactamase structure and function. Ann N Y Acad Sci. 2013;1277(1):91–104.

89. Nordmann P, Dortet L, Poirel L. Carbapenem resistance in Enterobacteriaceae: Here is the storm! Trends Mol Med [Internet]. 2012;18(5):263–72. Available from: http://dx.doi.org/10.1016/j.molmed.2012.03.003

90. Kaushik D, Mohan M, Borade DM, Swami OC. Ampicillin: Rise fall & resurgence. J Clin Diagnostic Res. 2014;8(5).

91. Bush K. Past and Present Perspectives on β-Lactamases. Antimicrob Agents Chemother [Internet]. 2018 Jul 30;62(10):1–20. Available from: https://aac.asm.org/content/62/10/e01076-18

92. ACRED P, BROWN DM, TURNER DH, WILSON MJ. PHARMACOLOGY AND CHEMOTHERAPY OF AMPICILLIN—A NEW BROAD-SPECTRUM PENICILLIN. Br J Pharmacol Chemother. 1962;18(2):356–69.

93. Niessen WMA. Analysis of antibiotics by liquid chromatography – mass spectrometry. 1998;812:53–75.

94. Burger A. Selective Toxicity. J Med Chem [Internet]. 1966 Mar;9(2):270–270. Available from: https://pubs.acs.org/doi/abs/10.1021/jm00320a046

95. Cherry JD. Pertussis: Challenges Today and for the Future. PLoS Pathog. 2013;9(7):e1003418.

96. Warfel JM, Merkel TJ. Bordetella pertussis infection induces a mucosal IL-17 response and long-lived Th17 and Th1 immune memory cells in nonhuman primates. Mucosal Immunol [Internet]. 2012;6(4):787–96. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23187316%5Cnhttp://dx.doi.org/10.1038/mi.2012.117

97. Warfel JM, Zimmerman LI, Merkel TJ. Acellular pertussis vaccines protect against disease butfail to prevent infection and transmission ina nonhuman primate model. Proc Natl Acad Sci U S A. 2014;111(2):787–92.

98. De Graaf H, Ibrahim M, Hill A, Gbesemete D, Vaughn A, Gorringe A, et al. Controlled human infection with Bordetella pertussis induces asymptomatic, immunising colonisation. Clin Infect Dis. 2019;

99. Bart MJ, Zeddeman A, van der Heide HG, Heuvelman K, van Gent M, Mooi FR. Complete Genome Sequences of Bordetella pertussis Isolates B1917 and B1920, Representing Two Predominant Global Lineages. Genome Announc [Internet]. 2014;2(6):2–3. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25540342

100. Diavatopoulos DA, Mills KHG, Kester KE, Kampmann B, Silerova M, Heininger U, et al. PERISCOPE: road towards effective control of pertussis. Lancet Infect Dis. 2019;19(5):e179–86.

101. Sato Y. Heptakis (2 , 6-0-Dimethyl), 8-Cyclodextrin : a Novel Growth Stimulant for Bordetella pertussis Phase I. 1983;17(5):781–6.

102. Ohtsuka M, Kikuchi K, Shundo K, Okada K, Higashide M, Sunakawa K, et al. Improved Selective Isolation of Bordetella pertussis by Use of Modified Cyclodextrin Solid Medium □. 2009;47(12):4164–7.

103. Rowatt E. The Growth of Bordetella pertussis: a Review. J Gen Microbiol. 1957;17(2):297–326.

104. MacArthur I, Belcher T, King JD, Ramasamy V, Alhammadi M, Preston A. The evolution of *Bordetella pertussis* has selected for mutations of *acr* that lead to sensitivity to hydrophobic molecules and fatty acids. Emerg Microbes Infect [Internet]. 2019 Jan 10;8(1):603–12. Available from: https://www.tandfonline.com/doi/full/10.1080/22221751.2019.1601502

105. Hoppe JE, Schwaderer J. Comparison of Four Charcoal Media for the Isolation of Bordetella pertussis. 1989;27(5):1097–8.

106. Von Koenig CHW, Tacken A, Finger H. Use of supplemented Stainer-Scholte broth for the isolation of Bordetella pertussis from clinical material. J Clin Microbiol. 1988;26(12):2558–60.

107. Stainer DW, Scholte MJ. A simple chemically defined medium for the production of phase I Bordetella pertussis. J Gen Microbiol. 1970;63(2):211–20.

108. Kurokawa M, Ying B. Precise , High-throughput Analysis of Bacterial Growth. 2017;(September):1–7.

109. Tatti KM, Wu KH, Tondella ML, Cassiday PK, Cortese MM, Wilkins PP, et al. Development and evaluation of dual-target real-time polymerase chain reaction assays to detect Bordetella spp. Diagn Microbiol Infect Dis. 2008;61(3):264–72.

110. Reischl U, Lehn N, Sanden GN, Loeffelholz MJ. Real-time PCR assay targeting IS481 of bordetella pertussis and molecular basis for detecting bordetella holmesii. J Clin Microbiol. 2001;39(5):1963–6.

111. Fittipaldi M, Nocker A, Codony F. Progress in understanding preferential detection of live cells using viability dyes in combination with DNA amplification. J Microbiol Methods [Internet]. 2012;91(2):276–89. Available from: http://dx.doi.org/10.1016/j.mimet.2012.08.007

112. Ring N, Abrahams JS, Bagby S, Preston A, MacArthur I. How Genomics Is Changing What We Know About the Evolution and Genome of Bordetella pertussis. Adv Exp

Med Biol. 2019;1183:1–17.

113. Probert WS, Ely J, Schrader K, Atwell J, Nossoff A, Kwan S. Identification and evaluation of new target sequences for specific detection of Bordetella pertussis by real-time PCR. J Clin Microbiol. 2008;46(10):3228–31.

114. Tatti KM, Sparks KN, Boney KO, Tondella ML. Novel multitarget real-time PCR assay for rapid detection of Bordetella species in clinical specimens. J Clin Microbiol. 2011;49(12):4059–66.

115. Martini H, Detemmerman L, Soetens O, Yusuf E, Piérard D. Improving specificity of Bordetella pertussis detection using a four target real-time PCR. PLoS One. 2017;12(4):1–11.

116. Guthrie JL, Robertson A V., Tang P, Jamieson F, Drews SJ. Novel duplex real-time PCR assay detects bordetella holmesii in specimens from patients with pertussis-like symptoms in Ontario, Canada. J Clin Microbiol. 2010;48(4):1435–7.

117. Pittet LF, Emonet S, Schrenzel J, Siegrist CA, Posfay-Barbe KM. Bordetella holmesii: An under-recognised Bordetella species. Lancet Infect Dis. 2014;14(6):510–9.

118. Nkuipou-Kenfack E, Engel H, Fakih S, Nocker A. Improving efficiency of viability-PCR for selective detection of live cells. J Microbiol Methods [Internet]. 2013;93(1):20–4. Available from: http://dx.doi.org/10.1016/j.mimet.2013.01.018

119. Kragh ML, Thykier M, Hansen LT. A long-amplicon quantitative PCR assay with propidium monoazide to enumerate viable Listeria monocytogenes after heat and desiccation treatments. J Food Microbiol [Internet]. 2020;86(March 2019):103310. Available from: https://doi.org/10.1016/j.fm.2019.103310

120. Merkel TJ. Toward a controlled human infection model of pertussis. Clin Infect Dis. 2020;71(2):412–4.

121. Moon K, Bonocora RP, Kim DD, Chen Q, Wade JT, Stibitz S, et al. The BvgAS regulon of Bordetella pertussis. MBio. 2017;8(5):1–15.

122. Sealey KL, Harris SR, Fry NK, Hurst LD, Gorringe AR, Parkhill J, et al. Genomic analysis of isolates from the United Kingdom 2012 pertussis outbreak reveals that vaccine antigen genes are unusually fast evolving. J Infect Dis. 2015;212(2):294–301.

123. Ring N, Abrahams JS, Jain M, Olsen H, Preston A, Bagby S. Resolving the complex Bordetella pertussis genome using barcoded nanopore sequencing. Microb genomics. 2018;4(11).

124. Ring N. Investigating the genome of Bordetella pertussis using long-read sequencing submitted by University of Bath. 2020;

125. Diavatopoulos DA, Mills KHG, Kester KE, Kampmann B, Silerova M, Heininger U, et al. PERISCOPE: road towards effective control of pertussis. Lancet Infect Dis. 2019;19(5):e179–86.

126. Parkhill J, Sebaihia M, Preston A, Murphy LD, Thomson N, Harris DE, et al. Comparative analysis of the genome sequences of bordetella pertussis, bordetella parapertussis and bordetella bronchiseptica. Nat Genet. 2003;35(1):32–40.

127. Thoma S, Schobert M. An improved escherichia coli donor strain for diparental mating. FEMS Microbiol Lett. 2009;294(2):127–32.

128. Potapov V, Ong JL, Kucera RB, Langhorst BW, Bilotti K, Pryor JM, et al. Comprehensive Profiling of Four Base Overhang Ligation Fidelity by T4 DNA Ligase and Application to DNA Assembly. ACS Synth Biol. 2018;7(11):2665–74.

129. Engler C, Kandzia R, Marillonnet S. A one pot, one step, precision cloning method with high throughput capability. PLoS One. 2008;3(11).

130. Engler C, Gruetzner R, Kandzia R, Marillonnet S. Golden gate shuffling: A one-pot DNA shuffling method based on type ils restriction enzymes. PLoS One. 2009;4(5).

131. Antoine R, Locht C. Isolation and molecular characterization of a novel broad-host-range plasmid from Bordetella bronchiseptica with sequence similarities to plasmids from gram-positive organisms. Mol Microbiol [Internet]. 1992 Jul;6(13):1785—1799. Available from: https://doi.org/10.1111/j.1365-2958.1992.tb01351.x

132. Guide U. Gateway ® Vector Conversion System with One Shot ® ccd B Survival ™ 2 T1 R Competent Cells For conversion of any vector of choice into. 2012;(11828).

133. Miller WG, Leveau JH, Lindow SE. Improved gfp and inaZ broad-host-range promoter-probe vectors. Mol Plant Microbe Interact. 2000;13(11):1243–50.

134. Radeck J, Kraft K, Bartels J, Cikovic T, Dürr F, Emenegger J, et al. The Bacillus BioBrick Box: Generation and evaluation of essential genetic building blocks for standardized work with Bacillus subtilis. J Biol Eng. 2013;7(1).

135. Trauth S, Bischofs IB. Ectopic integration vectors for generating fluorescent promoter fusions in Bacillus subtilis with minimal dark noise. PLoS One. 2014;9(5).

136. Invitrogen. Gateway LR Clonase II Enzyme mix - Manual. Life Technol [Internet]. 2011;(11791):3. Available from: https://www.lifetechnologies.com/order/catalog/product/11791100

137. Note T. Robust Statistics in BD FACSDiva ™ Software. 2012;(June).

138. Iv CSR, Kuhl SA. Construction and characterization of Bordetellapertussis RecA-mutants. 1995;33.

139. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. Methods. 2001;25(4):402–8.

140. Preston A, Maxim E, Toland E, Pishko EJ, Harvill ET, Caroff M, et al. Bordetella bronchiseptica PagP is a Bvg-regulated lipid A palmitoyl transferase that is required for persistent colonization of the mouse respiratory tract. Mol Microbiol. 2003;48(3):725–36.

141. Leader T, Mic IN, Technology GS. P / T ETEST ®. p. 0–2.

142. Wu B, Liang W, Kan B. Enumeration of viable non-culturable Vibrio cholerae using propidium monoazide combined with quantitative PCR. J Microbiol Methods [Internet]. 2015;115:147–52. Available from: http://dx.doi.org/10.1016/j.mimet.2015.05.016

143. Nocker A, Cheung CY, Camper AK. Comparison of propidium monoazide with ethidium monoazide for differentiation of live vs. dead bacteria by selective removal of DNA from dead cells. J Microbiol Methods. 2006;67(2):310–20.

144. Taylor MJ, Bentham RH, Ross KE. Limitations of Using Propidium Monoazide with qPCR to Discriminate between Live and Dead Legionella in Biofilm Samples. Microbiol insights [Internet]. 2014;7(7):15–24. Available from: http://www.la-press.com/limitations-of-using-propidium-monoazide-with-qpcr-to-discriminate-bet-article-a4365

145. Ruike W, Higashimori A, Yaguchi J, Li Y -y. Use of real-time PCR with propidium monoazide for enumeration of viable Escherichia coli in anaerobic digestion. Water Sci Technol [Internet]. 2016;74(5):1243–54. Available from: http://wst.iwaponline.com/cgi/doi/10.2166/wst.2016.327

146. Valdez HA, Oviedo JM, Gorgojo JP, Lamberti Y, Rodriguez ME. Bordetella pertussis modulates human macrophage defense gene expression. Pathog Dis. 2016;74(6):1–14.

147. Lamberti YA, Hayes JA, Perez Vidakovics ML, Harvill ET, Rodriguez ME. Intracellular trafficking of Bordetella pertussis in human macrophages. Infect Immun. 2010;78(3):907–13.

148. Lamberti Y, Cafiero JH, Surmann K, Valdez H, Holubova J, Večerek B, et al. Proteome analysis of Bordetella pertussis isolated from human macrophages. J Proteomics. 2016;136:55–67.

149. Kralik P, Nocker A, Pavlik I. Mycobacterium avium subsp. paratuberculosis viability determination using F57 quantitative PCR in combination with propidium monoazide treatment. Int J Food Microbiol [Internet]. 2010;141(SUPPL.):S80–6. Available from: http://dx.doi.org/10.1016/j.ijfoodmicro.2010.03.018

150. van Frankenhuyzen JK, Trevors JT, Lee H, Flemming CA, Habash MB. Molecular pathogen detection in biosolids with a focus on quantitative PCR using propidium monoazide for viable cell enumeration. J Microbiol Methods [Internet]. 2011;87(3):263–72. Available from: http://dx.doi.org/10.1016/j.mimet.2011.09.007

151. Àlvarez G, González M, Isabal S, Blanc V, León R. Method to quantify live and dead cells in multi-species oral biofilm by real-time PCR with propidium monoazide. AMB Express [Internet]. 2013;3(1):1. Available from: http://www.amb-

express.com/content/3/1/1/abstract%5Cnhttp://www.amb-express.com/content/3/1/1%5Cnhttp://www.amb-express.com/content/pdf/2191-0855-3-1.pdf

152. Nocker A, Sossa-Fernandez P, Burr MD, Camper AK. Use of propidium monoazide for live/dead distinction in microbial ecology. Appl Environ Microbiol. 2007;73(16):5111–7.

153. Biotium. PMA-Lite LED Photolysis Device. 2013;1–2.

154. Biotium. PMA dye. 2015;1–2.

155. Xie X, Wang S, Jiang SC, Bahnemann J, Hoffmann MR. Sunlight-Activated Propidium Monoazide Pretreatment for Differentiation of Viable and Dead Bacteria by Quantitative Real-Time Polymerase Chain Reaction. Environ Sci Technol Lett. 2016;3(2):57–61.

156. Li F, Xie G, Zhou B, Yu P, Yu S, Aguilar ZP, et al. Rapid and simultaneous detection of viable Cronobacter sakazakii, Staphylococcus aureus, and Bacillus cereus in infant food products by PMA-mPCR assay with internal amplification control. LWT - Food Sci Technol. 2016;74:176–82.

157. Krüger NJ, Buhler C, Iwobi AN, Huber I, Ellerbroek L, Appel B, et al. "Limits of control" - Crucial parameters for a reliable quantification of viable Campylobacter by Real-Time PCR. PLoS One. 2014;9(2).

158. Tantikachornkiat M, Sakakibara S, Neuner M, Durall DM. The use of propidium monoazide in conjunction with qPCR and Illumina sequencing to identify and quantify live yeasts and bacteria. Int J Food Microbiol [Internet]. 2016;234:53–9. Available from: http://dx.doi.org/10.1016/j.ijfoodmicro.2016.06.031

159. ZhiHong Z, WenTing L, HengYi X, Aguilar ZP, Shah NP, Hua W. Propidium monoazide combined with real-time PCR for selective detection of viable Staphylococcus aureus in milk powder and meat products. J Dairy Sci [Internet]. 2015;98(3):1625–33. Available from: http://www.sciencedirect.com/science/article/pii/S0022030215000107

160. Rawsthorne H, Dock CN, Jaykus LA. Pcr-based method using propidium monoazide to distinguish viable from nonviable Bacillus subtilis spores. Appl Environ Microbiol. 2009;75(9):2936–9.

161. Desneux J, Chemaly M, Pourcher A-M. Experimental design for the optimization of propidium monoazide treatment to quantify viable and non-viable bacteria in piggery effluents. BMC Microbiol [Internet]. 2015;15(1):164. Available from: http://www.biomedcentral.com/1471-2180/15/164

162. Cawthorn DM, Witthuhn RC. Selective PCR detection of viable Enterobacter sakazakii cells utilizing propidium monoazide or ethidium bromide monoazide. J Appl Microbiol. 2008;105(4):1178–85.

163. Varma M, Field R, Stinson M, Rukovets B, Wymer L, Haugland R. Quantitative real-time PCR analysis of total and propidium monoazide-resistant fecal indicator bacteria in wastewater. Water Res [Internet]. 2009;43(19):4790–801. Available from: http://dx.doi.org/10.1016/j.watres.2009.05.031

164. Chen N, Chang C. Rapid quantification of viable legionellae in water and biofilm using ethidium monoazide coupled with real-time quantitative PCR. 2010;109(Cdc 2005):623–34.

165. Chang B, Taguri T, Sugiyama K, Amemura-maekawa J, Kura F, Watanabe H. Comparison of Ethidium Monoazide and Propidium Monoazide for the Selective Detection of Viable Legionella Cells. 2010;4(1):119–23.

166. Graiver DA, Saunders SE, Topliff CL, Kelling CL, Bartelt-hunt SL. Ethidium monoazide does not inhibit RT-PCR amplification of nonviable avian influenza RNA. 2010;164:51–4.

167. Gedalanga PB, Olson BH. Development of a quantitative PCR method to differentiate between viable and nonviable bacteria in environmental water samples. 2009;587–96.

168. Kobayashi H, Oethinger M, Tuohy MJ, Hall GS, Bauer TW. Unsuitable distinction between viable and dead Staphylococcus aureus and Staphylococcus epidermidis by

ethidium bromide monoazide. 2009;48:633–8.

169. Kramer M. Quantification of live and dead probiotic bacteria in lyophilised product by real-time PCR and by flow cytometry. 2009;1137–47.

170. Pisz JM, Lawrence JR, Schafer AN, Siciliano SD. Differentiation of genes extracted from non-viable versus viable micro-organisms in environmental samples using ethidium monoazide bromide. 2007;71:312–8.

171. Soejima T, Schlitt-dittrich F, Yoshida S. Polymerase chain reaction amplification length-dependent ethidium monoazide suppression power for heat-killed cells of Enterobacteriaceae. Anal Biochem [Internet]. 2011;418(1):37–43. Available from: http://dx.doi.org/10.1016/j.ab.2011.06.027

172. Wang S, Levin RE. Discrimination of viable Vibrio vulnificus cells from dead cells in real-time PCR. 2006;64:1–8.

173. De Graaf H, Ibrahim M, Hill AR, Gbesemete D, Vaughan AT, Gorringe A, et al. Controlled human infection with Bordetella pertussis induces asymptomatic, immunising colonisation. Clin Infect Dis [Internet]. 2019 Sep 28; Available from: https://doi.org/10.1093/cid/ciz840

174. Valdez HA, Oviedo JM, Gorgojo JP, Lamberti Y, Rodriguez ME. Bordetella pertussis modulates human macrophage defense gene expression. Carbonetti N, editor. Pathog Dis [Internet]. 2016 Aug;74(6):ftw073. Available from: https://academic.oup.com/femspd/article/2198100/Bordetella-pertussis-modulates-human-macrophage

175. Kovach ME, Elzer PH, Hill DS, Robertson GT, Farris MA, Roop RM, et al. Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. Gene [Internet]. 1995;166(1):175–6. Available from: http://www-ab.informatik.uni-tuebingen.de/toolbox/index.php?view=domainpred

176. Kovach ME, Phillips RW, Elzer PH, Roop RM 2nd, Peterson KM. pBBR1MCS: a broad-host-range cloning vector. Biotechniques. 1994 May;16(5):800–2.

177. Zeng L, Jin S. Is under the Positive Control of Surrogate Regulator HpaA. 2003;47(12):3867–76.

178. Ouahrani-Bettache S, Porte F, Teyssier J, Liautard JP, Köhler S. pBBR1-GFP: A broadhost-range vector for prokaryotic promoter studies. Biotechniques. 1999;26(4):620–2.

179. Naninck T, Coutte L, Mayet C, Contreras V, Locht C, Grand R Le. In vivo imaging of bacterial colonization of the lower respiratory tract in a baboon model of Bordetella pertussis infection and transmission. :1–22.

180. Naninck T, Coutte L, Mayet C, Contreras V, Locht C, Le Grand R, et al. In vivo imaging of bacterial colonization of the lower respiratory tract in a baboon model of Bordetella pertussis infection and transmission. Sci Rep. 2018;8(1):1–22.

181. Chen Q, Lee G, Craig C, Ng V, Carlson PE, Hinton DM, et al. A novel Bvg-repressed promoter causes vrg-like transcription of Fim3 but does not result in the production of serotype 3 fimbriae in Bvg mode bordetella pertussis. J Bacteriol. 2018;200(20).

182. Thompson JF, Hayes LS, Lloyd DB. Modulation of firefly luciferase stability and impact on studies of gene regulation. Gene. 1991;103(2):171–7.

183. Sacco E, Josseaume N, Bouchier C, Hugonnet J, Mainardi J, Rice LB, et al. Mutation Landscape of Acquired Cross-Resistance to Glycopeptide and $_L$-Lactam Antibiotics in Enterococcus faecium. 2015;59(9):5306–15.

184. Wright GD. Bacterial resistance to antibiotics: Enzymatic degradation and modification. Adv Drug Deliv Rev. 2005;57(10):1451–70.

185. Niemann L, Müller P, Brauns J, Nathaus R, Schäkel F, Kipschull K, et al. Antimicrobial susceptibility and genetic relatedness of respiratory tract pathogens in weaner pigs over a 12-month period. Vet Microbiol [Internet]. 2018;219(December 2017):165–70. Available from: https://doi.org/10.1016/j.vetmic.2018.03.030

186. Lartigue MF, Poirel L, Fortineau N, Nordmann P. Chromosome-borne class A BOR-1 β-lactamase of Bordetella bronchiseptica and Bordetella parapertussis. Antimicrob

Agents Chemother. 2005;49(6):2565–7.

187. Burton PJ, Thornsberry C, Yee YC, Watts JL, Yancey RJ. Interpretive criteria for antimicrobial susceptibility testing of ceftiofur against bacteria associated with swine respiratory disease. J Vet Diagnostic Investig. 1996;8(4):464–8.

188. Kadlec K, Wiegand I, Kehrenberg C, Schwarz S. Studies on the mechanisms of β-lactam resistance in Bordetella bronchiseptica. J Antimicrob Chemother. 2007;59(3):396–402.

189. Kadlec K, Kehrenberg C, Wallmann J, Schwarz S. Antimicrobial susceptibility of Bordetella bronchiseptica isolates from porcine respiratory tract infections. Antimicrob Agents Chemother. 2004;48(12):4903–6.

190. Prüller S, Rensch U, Meemken D, Kaspar H, Kopp PA, Klein G, et al. Antimicrobial susceptibility of Bordetella bronchiseptica isolates from swine and companion animals and detection of resistance genes. PLoS One. 2015;10(8):1–14.

191. Lambert PA. Bacterial resistance to antibiotics: Modified target sites. Adv Drug Deliv Rev. 2005;57(10):1471–85.

192. Fontana R, Aldegheri M, Ligozzi M, Lopez H, Sucari A, Satta G. Overproduction of a low-affinity penicillin-binding protein and high- level ampicillin resistance in Enterococcus faecium. Antimicrob Agents Chemother. 1994;38(9):1980–3.

193. Sauvage E, Kerff F, Fonzé E, Herman R, Schoot B, Marquette JP, et al. The 2.4-Å crystal structure of the penicillin-resistant penicillin-binding protein PBP5fm from Enterococcus faecium in complex with benzylpenicillin. Cell Mol Life Sci. 2002;59(7):1223–32.

194. Rybkine T, Mainardi JL, Sougakoff W, Collatz E, Gutmann L. Penicillin-binding protein 5 sequence alterations in clinical isolates of Enterococcus faecium with different levels of β-lactam resistance. J Infect Dis. 1998;178(1):159–63.

195. Chen HY, Williams JD. Penicillin-binding proteins in Streptococcus faecalis and S. faecium. J Med Microbiol. 1987;23(2):141–7.

196. Neuwirth C, Siébor E, Duez JM, Péchinot A, Kazmierczak A. Imipenem resistance in clinical isolates of proteus mirabilis associated with alterations in penicillin-binding proteins. J Antimicrob Chemother. 1995;36(2):335–42.

197. Bellido F, Veuthey C, Blaser J, Banernfeind A, Pechére JC. Novel resistance to imipenem associated with an altered PBP-4 in a pseudomonas aeruginosa clinical isolate. J Antimicrob Chemother. 1990;25(1):57–68.

198. Fogarty LR, Haack SK, Johnson HE, Brennan AK, Isaacs NM, Spencer C. Staphylococcus aureus and methicillin-resistant s. aureus (MRSA) at ambient freshwater beaches. J Water Health. 2015;13(3):680–92.

199. Ito T, Okuma K, Ma XX, Yuzawa H, Hiramatsu K. Insights on antibiotic resistance of Staphylococcus aureus from its whole genome: Genomic island SCC. Drug Resist Updat. 2003;6(1):41–52.

200. Lu WP, Sun Y, Bauer MD, Paule S, Koenigs PM, Kraft WG. Penicillin-binding protein 2a from methicillin-resistant Staphylococcus aureus: Kinetic characterization of its interactions with β-lactams using electrospray mass spectrometry. Biochemistry. 1999;38(20):6537–46.

201. Lim D, Strynadka NCJ. Structural basis for the β-lactam resistance of PBP2a from methicillin-resistant Staphylococcus aureus. Nat Struct Biol. 2002;9(11):870–6.

202. Enright MC, Robinson DA, Randle G, Feil EJ, Grundmann H, Spratt BG. The evolutionary history of methicillin-resistant Staphylococcus aureus (MRSA). Proc Natl Acad Sci U S A. 2002;99(11):7687–92.

203. Enright MC. The evolution of a resistant pathogen - The case of MRSA. Curr Opin Pharmacol. 2003;3(5):474–9.

204. Stapleton PD, Taylor PW. Methicillin Resistance in Staphylococcus Aureus: Mechanisms and Modulation. Sci Prog [Internet]. 2002 Feb 27;85(1):57–72. Available from: https://linkinghub.elsevier.com/retrieve/pii/B9780128135471000170

205. Belcher T, Macarthur I, King JD, Langridge GC, Mayho M, Parkhill J, et al. Fundamental differences in physiology of Bordetella pertussis dependent on the two- -

component system Bvg revealed by gene essentiality studies. 2020;

206. Sy N Van, Harada K, Asayama M, Warisaya M, Dung LH, Sumimura Y, et al. Residues of 2-hydroxy-3-phenylpyrazine, a degradation product of some B-lactam antibiotics, in environmental water in Vietnam. Chemosphere [Internet]. 2017;172:355–62. Available from: http://dx.doi.org/10.1016/j.chemosphere.2016.12.156

207. Forsberg AJ, Pavitt GD, Higgins CF. Use of transcriptional fusions to monitor gene expression: A cautionary tale. J Bacteriol. 1994;176(7):2128–32.

Appendices
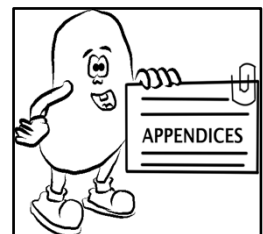
Appendices

Appendix A: Statement of Authorship

This declaration concerns the article entitled:	

A qPCR assay for Bordetella pertussis cells that enumerates both live and dead bacteria

Publication status (tick one)

Draft manuscript		**Submitted**		**In review**		**Accepted**		**Published**	✓

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Candidate's contribution to the paper (provide details, and also indicate as a percentage)	The candidate contributed to / considerably contributed to / predominantly executed the… **Formulation of ideas: 40%** This paper provides support for the Human Challenge Model Study with the original idea conceptualised by members of the Periscope Consortium and fleshed out by the candidate. **Design of methodology: 85%** This paper aims to optimise a method where the candidate designed the majority of the methodology. Established linearity of assay, optimised heat-killing conditions, optimised PMA treatment, measured the effect of exogenous cells on detection and PMA- mediated inhibition, measured viability during *in vitro* growth, enumeration of live and dead *B. pertussis* from human challenge model (HCM) samples, the use of the assay on additional strains. **Experimental work: 90%** All experimental work completed solely by the candidate except for some help to extract genomic DNA from the samples of volunteers from Human Challenge Model at the time of collection. As well, help was also obtained from the Material and Chemical Characterisation Facility (MC2) at the University of Bath to perform flow cytometry. **Presentation of data in journal format: 95%** Produced all figures and wrote the manuscript.

Statement from Candidate	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature.

Signed		**Date**	2021-01-01

Last update: Feb 2019

Appendix A

PLOS ONE

A qPCR assay for *Bordetella pertussis* cells that enumerates both live and dead bacteria

Stacy Ramkissoon [1,2], Iain MacArthur[1,2], Muktar Ibrahim[3,4,5,6], Hans de Graaf[3,4,5,6], Robert C. Read [3,4,5,6], Andrew Preston[1,2]*

1 Milner Centre for Evolution, University of Bath, Bath, United Kingdom, 2 Department of Biology and Biochemistry, University of Bath, Bath, United Kingdom, 3 Faculty of Medicine and Institute for Life Sciences, University of Southampton, Southampton, United Kingdom, 4 School of Clinical Experimental Sciences, University Hospital Southampton NHS Foundation Trust, Southampton General Hospital, University of Southampton, Southampton, United Kingdom, 5 NIHR Clinical Research Facility, University Hospital Southampton NHS Foundation Trust, Southampton General Hospital, University of Southampton, Southampton, United Kingdom, 6 NIHR Southampton Biomedical Research Centre, University Hospital Southampton NHS Foundation Trust, Southampton General Hospital, University of Southampton, Southampton, United Kingdom

* a.preston@bath.ac.uk

Abstract

Bordetella pertussis is the causative agent of whooping cough, commonly referred to as pertussis. Although the incidence of pertussis was reduced through vaccination, during the last thirty years it has returned to high levels in a number of countries. This resurgence has been linked to the switch from the use of whole-cell to acellular vaccines. Protection afforded by acellular vaccines appears to be short-lived compared to that afforded by whole cell vaccines. In order to inform future vaccine improvement by identifying immune correlates of protection, a human challenge model of *B. pertussis* colonisation has been developed. Accurate measurement of colonisation status in this model has required development of a qPCR-based assay to enumerate *B. pertussis* in samples that distinguishes between viable and dead bacteria. Here we report the development of this assay and its performance in the quantification of *B. pertussis* from human challenge model samples. This assay has future utility in diagnostic labs and in research where a quantitative measure of both *B. pertussis* number and viability is required.

Introduction

Whooping cough, or pertussis, is a highly contagious respiratory tract infection of humans caused by the gram-negative coccobacillus *Bordetella pertussis*. Clinical manifestations of pertussis depend on age and immune status of the host and include a low-grade fever, cyanosis, and paroxysmal cough accompanied by a high-pitched "whoop" [1]. Infants aged less than 1 year old present the highest incidence of pertussis and are also at the greatest risk of severe disease and death [2].

The introduction of vaccination in the early 1950s significantly reduced the incidence of pertussis in developed nations, however the number of reports of pertussis has been

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progressively increasing over the last thirty years [3]. For example, in the UK, Public Health England has reported a greater than ten-fold increase in pertussis cases over the eight-year period of 2005–2013 [4]. This rise has been echoed in other countries including Australia, the Netherlands, and the US [5–10].

The reason for this resurgence is not certain, however it has been strongly linked to the switch from using whole-cell vaccines (WCVs) to using acellular vaccines (ACVs). ACV-induced immunity appears to wane more quickly than WCV-induced immunity. In baboons, compared to WCV-induced immunity, ACV-induced immunity protects from disease, but does not prevent colonisation by *B. pertussis* or prevent transmission of the bacteria to other hosts [11,12]. Asymptomatic transmission from colonised carriers to naive contacts could be contributing to the resurgence in the US and UK [13]. In many industrialised countries using ACVs (Denmark, Norway, Netherlands, Finland, Sweden, the United Kingdom, United States of America, Australia, France, and Japan) there has been a dramatic increase in the isolation of *B. pertussis* deficient for the production of the ACV-vaccine antigen pertactin [14–18]. In ACV-immunised hosts pertactin-deficient *B. pertussis* may have a fitness advantage over pertactin-producing isolates, raising concern that the use of ACVs is selecting for vaccine escape strains of *B. pertussis* [19]. These issues have highlighted the need to better understand the detailed differences between WCV and ACV induced immune responses and the immune response to infection, and to identify biomarkers of protective immunity to *B. pertussis* infection. This would aid the evaluation of the efficacy of future *B. pertussis* vaccines that might be needed to combat *B. pertussis* resurgence. To this end, a human challenge model of *B. pertussis* colonisation has been developed as part of the EU-funded PERISCOPE Project [20,21]. In this model it is necessary to be able to monitor the colonisation status of participants at frequent intervals. Current detection methods for *B. pertussis* include culture from nasopharyngeal swabs or other nasopharyngeal samples. However, *B. pertussis* is slow-growing and takes several days to produce visible growth on laboratory agar. A more rapid method would improve safety for human challenge model volunteers. Real-time PCR detection (qPCR) of *B. pertussis* DNA provides identification of *B. pertussis* within hours. Use of the multicopy insertion sequence IS481 as a target makes this assay much more sensitive for the diagnosis of *B. pertussis* infection than culture [22]. However, traditional qPCR assays cannot distinguish between viable and dead bacteria, which is essential to determine whether participants are actively colonised. Enumerating viable bacteria is especially important in the prevention of pertussis and associated symptoms during the challenge, as well as transmission to others post-challenge by ensuring the efficacy of treatment.

Here we report the modification of a standard qPCR assay used for laboratory diagnosis of *B. pertussis*, through treatment of samples with propidium monoazide (PMA) that inhibits PCR-mediated amplification of DNA from dead cells and allows distinguishing of viable from dead cells [23–27]. The use of PMA involves an initial incubation of samples with PMA in darkness, during which it diffuses into dead cells, followed by light activation of PMA that permanently modifies the genomic DNA (gDNA) of dead cells, preventing it from acting as a template in PCR. The optimisation of this assay and its use to enumerate viable and dead *B. pertussis* from human challenge model samples is described. In addition, this assay has wider uses in diagnostic and other research settings where a quantitative measure of viable *B. pertussis* number is required.

Materials and methods

Bacterial strains and culture conditions

B. pertussis strain B1917 is a wild-type strain considered representative of currently circulating *B. pertussis* [28]. The streptomycin-resistant *B. pertussis* strain BP536 was derived from

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Tohama 1 [29]. *B. pertussis* strains UK48 and UK71 can both be identified with European Nucleotide Archive Accession numbers: ERS176875 and ERS227772, respectively. *B. pertussis* strain B204 (B1878) and B184 (B2973) were both derived from the Netherlands and can be located with the following NCBI Genbank accession numbers: NZ_CSNV00000000 and NZ_CSRZ00000000, respectively. All strains were cultured on charcoal agar (ThermoFisher Scientific, Oxoid™, Basingstoke, UK) at 37°C for 3 days for routine culture.

The preparation of heat-killed bacterial cell suspensions

Plate-grown B1917 were resuspended in PBS (ThermoFisher Scientific, Oxoid™, Basingstoke, UK) to an $OD_{600} = 1.0$ (approximately 10^9 cfu/ml). To optimise heat killing, 1 ml aliquots were heat-killed at 80°C for 1, 3 and 6 minutes in a pre-heated heat block. Aliquots were placed on ice immediately after incubation. Bacterial death was confirmed by the absence of growth after streaking 10 μl of suspension onto charcoal agar plates and incubating at 37°C for 5 days. To ascertain the integrity of heat-killed cells, samples were subjected to flow cytometry (FACS-CantoII, BD UK Ltd, Wokingham, U.K.). A detergent-lysed sample acted as a positive control for lysis and a sample containing live cells was a positive control for cell integrity.

The preparation of THP-1 cells

THP-1 (ATCC® TIB-202™) cells were maintained in RPMI 1640 medium (ThermoFisher Scientific, Gibco™, Loughborough, UK), fetal bovine serum (10%) (ThermoFisher Scientific, Gibco™, Loughborough, UK), 1% streptomycin, penicillin and glutamine (ThermoFisher Scientific, Loughborough, UK) as per standard methods. Heat-killed THP-1 cells were prepared by incubating cell suspensions at 10^5 cells/ml at 80°C for 6 minutes in a pre-heated heat block.

Optimisation of PMA treatment conditions

PMA Dye, 20 mM in H_2O (Cambridge BioSciences, Cambridge, UK), was stored at -20°C in the dark, thawed on ice and added to 2 ml clear centrifuge tubes containing 200 μl of cell suspensions to a final concentration of 20 μM, 30 μM, or 50 μM. PMA-free samples served as controls for each condition tested. Tubes were covered with aluminium foil and shaken on an orbital shaker for 5, 10, 20, 30 or 70 minutes. Samples were then exposed to light using the PMA-Lite LED Photolysis Device (Cambridge BioSciences, Cambridge, UK) for 5, 10, 20, 30 or 40 minutes. Samples were pelleted using the Heraeus Pico 17 Centrifuge at 2000xg (ThermoFisher Scientific, Loughborough, UK) for 10 minutes at room temperature prior to DNA isolation. Non-PMA treated controls allow for the total number of *B. pertussis* cells to be enumerated. The number of viable *B. pertussis* cells calculated from PMA-treated samples can be subtracted from the total number of *B. pertussis* cells to provide the number of dead cells in a sample.

Genomic DNA Isolation

Genomic DNA (gDNA) was isolated using the GenElute Bacteria Genomic DNA Kit (Sigma-Aldrich, Dorset, UK) according to the manufacturer's instructions and eluted with 200 μl of elution buffer. gDNA was purified from THP-1 cells and human challenge model samples using the QIAmp DNA mini and blood extraction kit (QIAgen, Manchester, UK) as per the manufacturer's protocol. gDNA was quantified using a Qubit 1.0 fluorometer (Invitrogen, Loughborough, UK) according to the manufacturer's instructions.

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Quantitative PCR

qPCR was performed using a fluorogenic probe (Eurofins, Ebersberg, Germany) targeting insertion sequence IS481. The reaction volume was 20 μl comprising of 2 μl of 1x Taqman Gene Expression Mastermix (Applied Biosystems, Loughborough, UK), 2 μl of 900 nM stocks of each primer, 2 μl of 150 nM stock of probe, 2 μl of nuclease-free water (ThermoFisher Scientific, Invitrogen ™, Loughborough, UK) and 2 μl of template sample. The reactions were run using the StepOne Plus RT PCR System (ThermoFisher Scientific, Loughborough, UK) using the cycling parameters found in Table 1. The sequence of primers and probe were as described previously [30]: forward primer (5' ATCAAGCACCGCTTTACCC 3'), reverse primer (5' TTGGGAGTTCTGGTAGGTGTG 3') and probe (5' AATGGCAAGGCCGAACGCTTCA 3') was labelled with FAM and Black Hole Quencher.

Establishing linerarity using standard curves

A 10-fold serial dilution of gDNA in nuclease- free water was assayed for qPCR as described previously. A standard curve was automatically generated using StepOnePlus™ Software v2.3 to establish the linearity of the assay and to allow for the absolute quantification of unknowns.

Calculating copy number from Ct values/ DNA concentration

The genome copy number equivalent to the amount of template in a qPCR reaction was calculated using the formula: copy number = 50 x (amount of template in ng * 6.022×10^{23}) / (length of genome in bp x 1×10^9 * 650). The 50x accounts for the 200 μl volume of extracted gDNA divided by 4 μl of gDNA used in the qPCR reaction. 650 Daltons denotes the average mass of a base pair (bp) and the number of molecules of the template/ gram can be calculated using Avogadro's number, 6.022×10^{23} molecules/mole. The genome of B1917 is 4,102,186 bp [28]. The genome of BP536, UK48, and the mean of all classical *B. pertussis* closed genomes available on RefSeq as of March 2019 was 4.1 mb [22,29,31].

Protocol deposited on protocols.io

The PMA-qPCR method has been deposited on protocols.io and can be accessed here: http://dx.doi.org/10.17504/protocols.io.bc5niy5e.

Preparation of bacterial and THP-1 cell suspensions

To evaluate if eukaryotic cells interfere with the enumeration of live *B. pertussis* cells using qPCR, 10^3 live *B. pertussis* B1917 were combined with THP-1 gDNA equivalent to 10843, 8414, 5385, 3446, 2804, 2316, 1868, 1503, 1251, 1023, 875, 746, 671, 507, 366, or 275, 141, 29 cells. A sample without THP-1 DNA served as a control.

To evaluate the possible sequestration of PMA by eukaryotic DNA, 10^6 heat-killed *B. pertussis* B1917 were combined with either 100,000 heat-killed THP-1 cells, 100,000 live THP-1

Table 1. TaqMan thermocycling conditions for qPCR.

STEP	TEMP	TIME
Step 1 **Holding Stage**	50˚C	2 minutes
Step 2	95˚C	10 minutes
40 Cycles	95˚C	15 s
	60˚C	60 s

https://doi.org/10.1371/journal.pone.0232334.t001

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cells or without THP-1 cells and were then treated with the selected PMA treatment. Non PMA-treated samples were run in parallel.

To determine if eukaryotic cells interfered with the action of PMA on dead bacterial cells, 100,000 live THP-1 cells were combined with different ratios of viable *B. pertussis* cells and heat-killed *B. pertussis* cells (final bacterial concentration was 10^6 cfu/ml) in a clear Eppendorf tube, total volume 200 µl. These samples were then subjected to the selected PMA treatment. A non-PMA treated control was included. gDNA was extracted from each sample and used for qPCR.

Statistical analysis

Unpaired T tests, corrected for multiple comparisons, and two-way ANOVA using the Holm-Sidak method was carried out using Prism 8 for macOS Version 8.2.1 to evaluate statistical significance. One-way ANOVA and Dunnett's multiple comparisons test, with a single pooled variance was also used. A p value of <0.05 was defined as statistically significant and is indicated by asterisks.

Ethics

Samples from volunteers participating in the human challenge model study were obtained in accordance with the provisions of the Declaration of Helsinki (1996) and the International Conference on Harmonization Guidelines for Good Clinical Practice. This study is registered with ClinicalTrials.gov: NCT03751514, was reviewed and approved by the South Central–Oxford A Research Ethics Committee (REC reference: 17/SC/0006, 24 February 2017) and the UK Health Research Authority (IRAS project ID: 219496, 1 March 2017). The protocol has been published previously and details written and oral consent received from human participants [23]. It can be found on www.periscope-project.eu.

Results

qPCR provides a lower limit of detection of 2 *B. pertussis* cells

IS481 is often used as the target for qPCR detection of *B. pertussis* as it is present at ~250 copies per cell in *B. pertussis*, providing great sensitivity. To develop a PMA-qPCR assay, the sensitivity of qPCR for detection of *B. pertussis* was tested over a range of template gDNA concentrations. A linear relationship between Ct value and template concentration was observed over the range of 2 to approximately 2.42×10^6 B1917 cells for qPCR (Fig 1). Ct values greater than 35 were considered to be a negative reaction. Thus, the assay is able to detect *B. pertussis* gDNA equivalent to very few bacterial cells and is linear over a wide range of *B. pertussis* concentrations.

Heat-killing *B. pertussis* at 80˚C for 6 minutes maintained the integrity of cells

The ability of PMA to inhibit PCR-amplification from dead *B. pertussis* was tested using heat-killed *B. pertussis* B1917. It was envisaged that clinical samples may contain dead, but intact, *B. pertussis*. Heat-killing may cause cell lysis which would not mimic intact dead cells. Thus, the integrity of cells following heat killing was assessed using flow cytometry. Incubation of *B. pertussis* suspensions at 80˚C for 6 minutes resulted in 100% killing, but with cells remaining intact and were the conditions used throughout (Fig 2).

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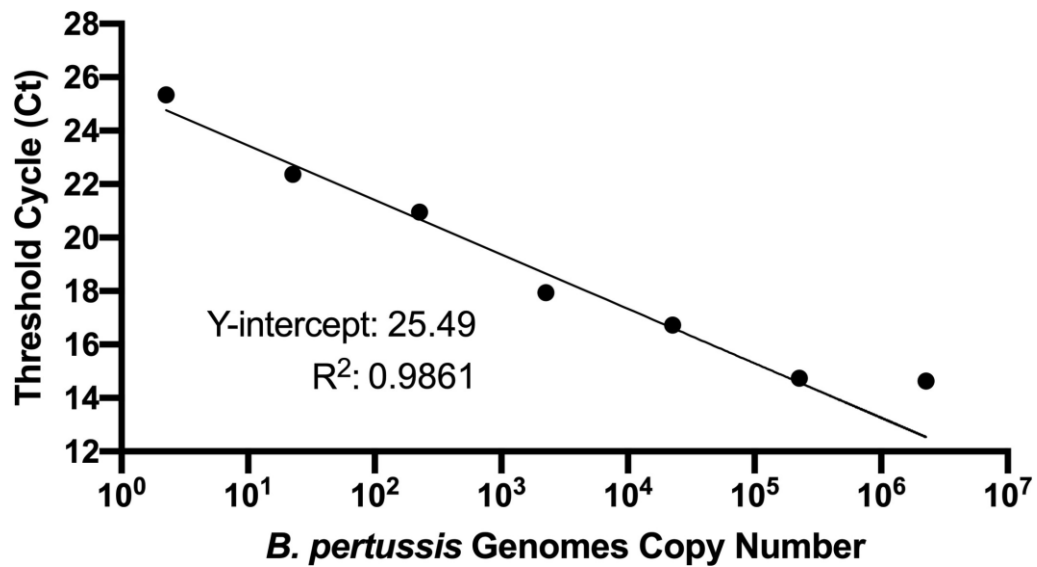
Fig 1. Standard curve of Ct value versus template concentration. Template DNA concentration is expressed as B1917 genome copy number. The linearity was determined to be from 2 to approximately 2.42×10^6 B1917 genomes for qPCR.

Optimisation of PMA treatment

The effect of PMA concentration on inhibition of PCR amplification from dead *B. pertussis* B1917 was tested (Fig 3). Incubation of heat-killed cells with 50μM of PMA resulted in a

Fig 2. The effect of heat killing on the integrity of *B. pertussis* cells. A) Positive control for cell integrity–a suspension of live *B. pertussis*; B) Positive control for cell lysis–detergent lysed *B. pertussis*; C) Heat-killed *B. pertussis* suspension. The heat-killed *B. pertussis* suspension incubated for 6 minutes at 80˚C displayed similar scatter as the live cell suspension. No particles were detected in a suspension of detergent-lysed *B. pertussis*. Therefore, cells remained intact in the heat-killed *B. pertussis* suspension incubated for 6 minutes at 80˚C when compared to the positive cell integrity control.

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97.42% reduction in PCR signal compared to that generated from untreated samples. Lower levels of PMA also resulted in very similar levels of inhibition (Fig 3).

The optimal conditions for photo-activation of PMA were determined. Incubation under dark conditions for 10 minutes followed by light activation for between 5 and 30 minutes resulted in greater than 99% reduction in PCR signal from dead cells compared to untreated controls. Five minutes of light activation following 10 minutes of darkness resulted in 99.64% reduction in detection of *B. pertussis* DNA (Fig 4).

From these optimisations, standard conditions of 50μM PMA and incubation in the dark for 10 minutes followed by light activation for 5 minutes were selected as minimal incubation times that achieved high levels of inhibition. Even though 20μM PMA inhibited PCR amplification from dead cells, 50μM PMA was selected as the concentration to use in the assay, as clinical samples will contain cells other than *B. pertussis* that may sequester PMA, requiring an excess for consistent inhibition of PCR signal from dead *B. pertussis*. These conditions were tested in four independent assays. An average of 94.15% reduction in PCR signal was observed compared to untreated controls (Fig 5).

The effect of exogenous cells on detection and PMA-mediated inhibition

Clinical samples are likely to contain cells other than *B. pertussis*, including eukaryotic cells that contain very large amounts of DNA compared to *B. pertussis* cells. Eukaryotic cells may
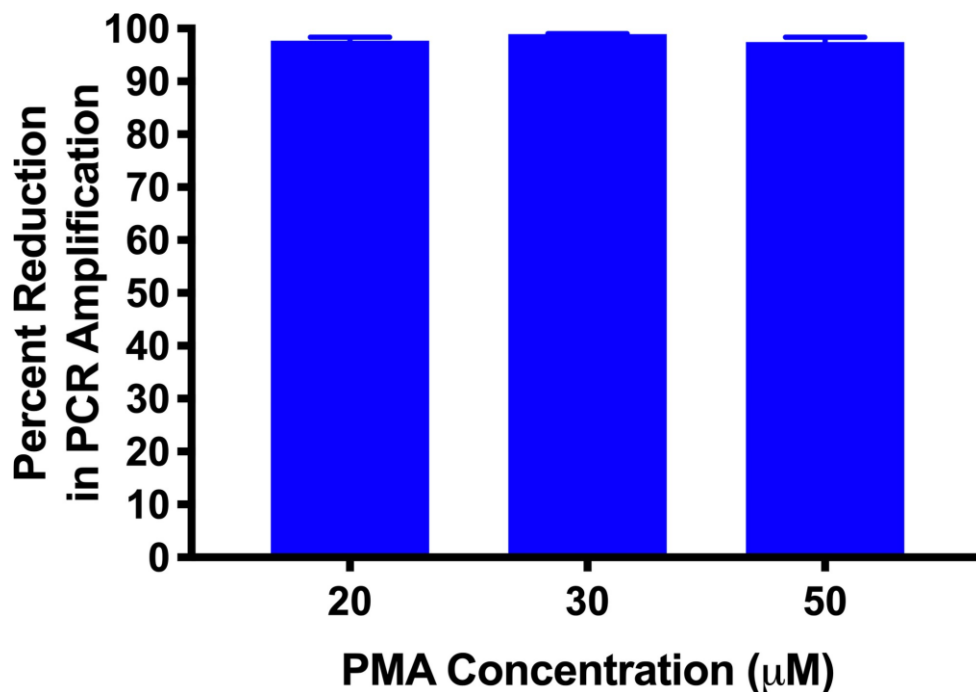
Fig 3. The effect of PMA concentration on the PCR amplification signal from heat-killed cells. Treatment of samples with either 20 μM, 30 μM, or 50 μM of PMA produced a ≥97% reduction in the PCR amplification signal compared to untreated samples. Error bars represent standard deviations from two biological replicates. Data from a representative experiment repeated three times.

https://doi.org/10.1371/journal.pone.0232334.g003

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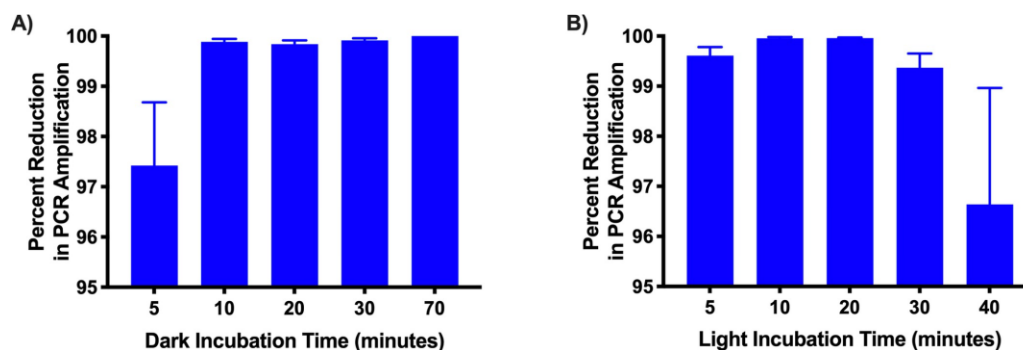
Fig 4. The effect of dark and light exposure times on PMA-inhibition of PCR amplification. A) Dark incubation B) Light Incubation. PMA and untreated heat-killed suspensions were incubated for 10, 20, 30, and 70 minutes in the dark followed by exposure to 5 minutes of light. 10 minutes or longer of incubation in the dark produced a ≥99% reduction in the PCR amplification signal. Optimal light incubation periods were determined by incubating untreated and PMA treated heat-killed suspensions in the dark for 10 minutes followed by light exposure for 5, 10, 20, 30, and 40 minutes. Incubating PMA treated heat-killed samples under light for periods of 5, 10, 20, and 30 minutes produced a 99% or greater reduction in the PCR amplification signal. Five minutes was selected as the standard light incubation period. Error bars represent standard deviation from two biological replicates. The experiment was repeated with the same result.

https://doi.org/10.1371/journal.pone.0232334.g004

interfere with the PMA-mediated inhibition of amplification from dead *B. pertussis* preventing distinguishing between live and dead *B. pertussis*. To test this, varying amounts of gDNA from THP-1 cells were combined with a constant amount of *B. pertussis* B1917 gDNA, and Ct values were determined and compared to samples containing *B. pertussis* B1917 only. No effect of THP-1 gDNA on detection of *B. pertussis* was observed up to an equivalent of approximately 5500 THP-1 cells per assay (Fig 6).

It was possible that the presence of other cells would interfere with the PMA-mediated inhibition of PCR signal from dead *B. pertussis*. Thus, the effect of heat-killed or live THP-1 cells on PMA-mediated inhibition of PCR amplification from heat-killed *B. pertussis* was tested. A 99.94% reduction in PCR signal was observed indicating that THP-1 cells did not prevent PMA-mediated inhibition of PCR signal from dead *B. pertussis* (Fig 7).

To test the assay's ability to distinguish between viable and dead *B. pertussis*, in the presence of other cells, a constant number of THP-1 cells were combined with different ratios of heat-killed and viable *B. pertussis* B1917 cells. The reduction in PCR signal was proportional to the amount of heat-killed cells in each suspension (Fig 8) demonstrating that the assay was able to distinguish viable from dead *B. pertussis*, even in the presence of human cells.

Collectively, these studies revealed that the THP-1 cells did not interfere with the PMA-mediated inhibition of PCR signal from dead *B. pertussis* or prevent the accurate enumeration of viable *B. pertussis* cells.

Measuring the viability of *B. pertussis* during in vitro growth

During development of the assay, it was observed that PMA treatment of live *B. pertussis* suspensions used as controls consistently reduced the PCR signal compared to untreated samples. This suggested that *B. pertussis* colonies taken from plate grown cultures contains both live and dead bacteria. To investigate this, and to determine the proportion of live to dead *B. pertussis* in plate grown cultures over time, suspensions of cells were made of *B. pertussis* B1917 grown on plates for either 3, 4, 5 or 8 days. The suspensions were treated with PMA and qPCR

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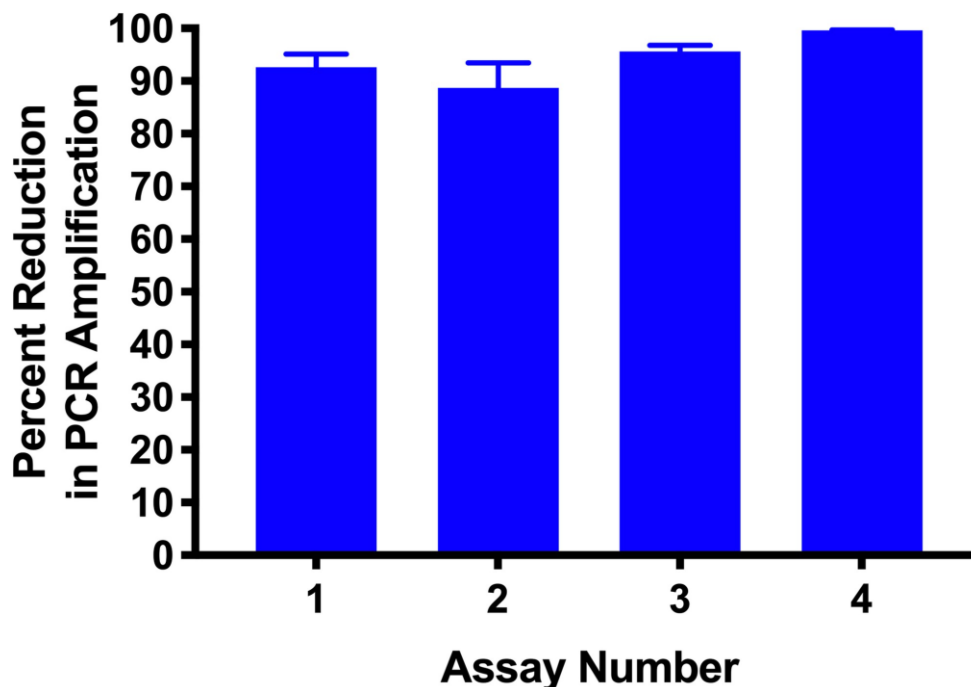
Fig 5. Selected assay conditions gave reproducible inhibition of PCR signal from dead cells. Heat-killed samples were treated with 50 μM of PMA and incubated in the dark for 10 minutes followed by 5 minutes of light activation. A 94.15% reduction in the PCR amplification signal was observed. Error bars represent standard deviations from five biological replicates.

performed. The percentage of PCR signal observed was compared to untreated controls, Fig 9. *B. pertussis* is relatively slow growing and many protocols for plate growth involve incubation for 72 hours to achieve visible colonies. However, at this point *B. pertussis* viability was only 89%. Interestingly, although colony size continued to increase between days 3 and 5, percentage viability decreased to 24%. Further incubation resulted in further loss in viability. Thus, when using plate grown *B. pertussis* in assays, suspensions will be a mixture of live and dead bacteria, and that enumeration of *B. pertussis* by plating serial dilutions of a suspension and counting the resulting CFU's will not be a measure of the total number of cells in the suspension.

Use of the assay to enumerate live and dead *B. pertussis* from human challenge model samples

The assay was developed in order to provide a method for monitoring the colonisation status of participants in a novel human challenge model of *B. pertussis* colonisation. During development of this model, a group of participants were inoculated with 10^5 CFU of *B. pertussis* B1917 and daily samples were taken over a 14-day period to monitor colonisation, including nasosorption fluids, pernasal swabs, throat swabs, and nasopharyngeal washes [21].

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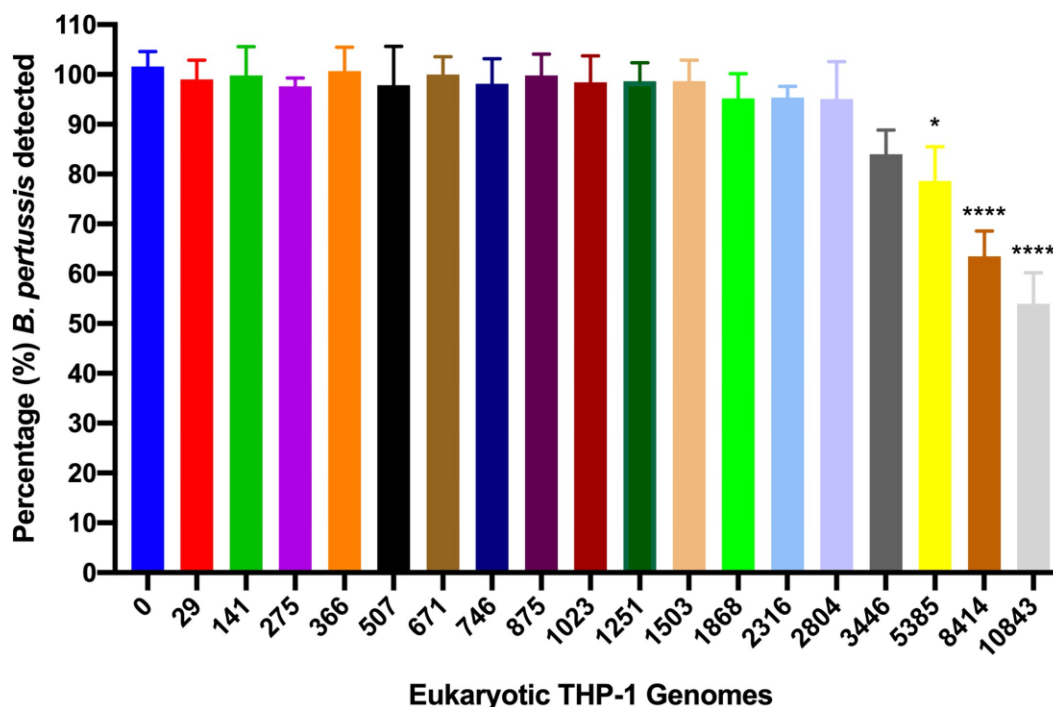
Fig 6. Effect of eukaryotic gDNA on the detection of *B. pertussis*. Varying amounts of gDNA from THP-1 cells were combined with gDNA equivalent to 10^3 *B. pertussis* cells. No interference in detection of *B. pertussis* was observed up to the equivalent of 3446 THP-1 cells, after which the sensitivity of detection was reduced when compared to viable *B. pertussis* detected in the presence of 0 THP-1 cells. *: p<0.05, determined by one-way ANOVA and Dunnett's multiple comparisons test, with a single pooled variance. Error bars represent standard deviations from three biological replicates.

Here, samples from Day 9 post-challenge were tested by PMA-qPCR, and by culture, Fig 10. Samples were split and one portion was treated with PMA. *B. pertussis* were enumerated using qPCR from PMA-treated and untreated samples. Using culture, 3 out of 5 participants were determined to be colonised [21]. By PMA-qPCR, 4 out of 5 volunteers were deemed to carry viable *B. pertussis*, with detection from nasal washes, pernasal swabs, nasosorption and throat samples. Nasal washes from Day 11 samples also had detectable viable *B. pertussis* in 2 of the 5 volunteers using PMA-qPCR and in a third volunteer using culture. (Fig 10E). Pernasal samples from Day 11 revealed detectable *B. pertussis* in a single volunteer using culture, that was not detected using PMA-qPCR (Fig 10F). PMA-qPCR revealed samples contained both viable and dead *B. pertussis*, in approximately equal numbers. Interestingly, on Day 16 of sampling, two days after volunteers started azithromycin treatment to eradicate the infection, all but one volunteer was negative for detectable *B. pertussis* cells. In this volunteer, the PMA-qPCR assay was able to detect low levels of viable and dead *B. pertussis*, with a higher proportion of dead genomes detected compared to viable genomes, however these low levels of *B. pertussis* were undetectable by culture (Fig 10G). Nasosorptions from this cohort of volunteers were all culture-negative.

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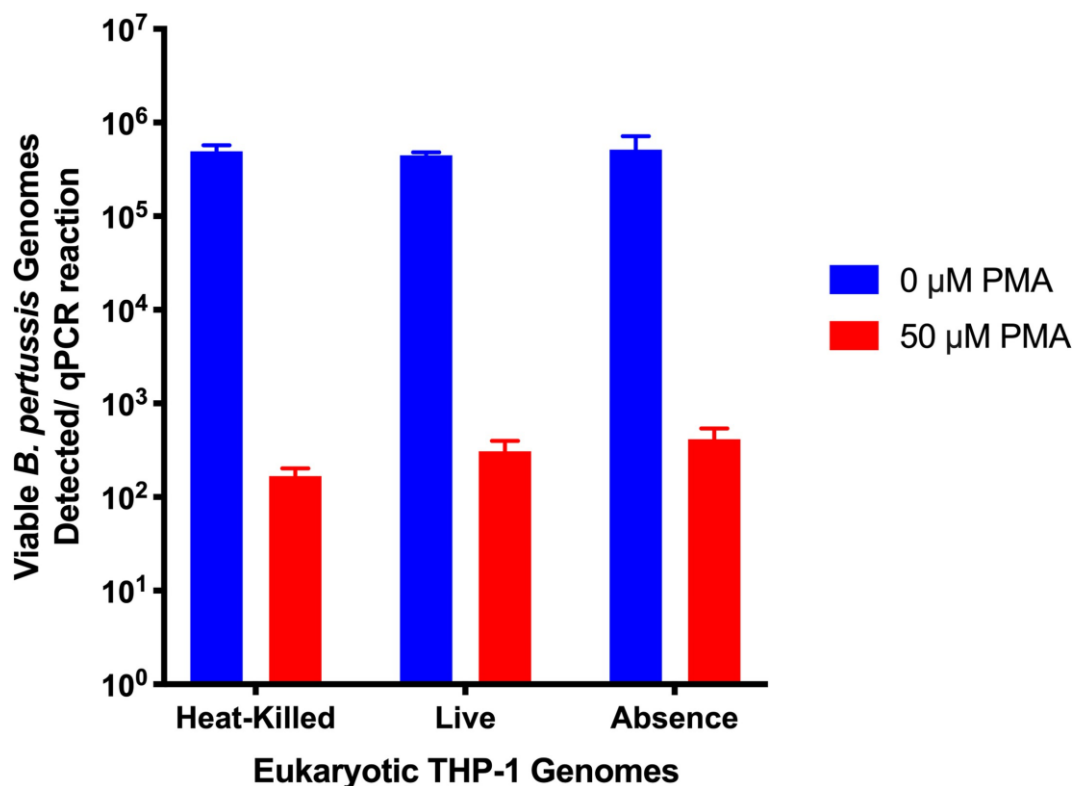
Fig 7. Eukaryotic THP-1 gDNA did not interfere with enumeration of *B. pertussis*. gDNA from 10^6 heat-killed *B. pertussis* were combined with either 10^5 heat-killed THP-1 cells, 10^5 live THP-1 cells or a no THP-1 cell control and treated with PMA. Non PMA-treated samples were run in parallel. The presence of live or dead THP-1 cells did not interfere with the action of PMA on dead *B. pertussis* cells. Error bars represent standard deviations from three biological replicates.

Confirming utility of PMA-qPCR assay by enumerating five additional strains of *B. pertussis*

To confirm the utility of this assay with other strains outside of the human challenge model, qPCR was performed on gDNA extracted from PMA and non-PMA treated suspensions from the following strains: BP536, UK48, UK71, B204 (B1878), B184 (B2973). B1917 was also enumerated as a control (Fig 11). These suspensions contained *B. pertussis* cells taken from three-day old plate cultures resuspended in PBS to an $OD_{600} = 1.0$. The suspensions were plated onto agar to confirm the enumeration obtained by the PMA-qPCR assay. The recovery of viable *B. pertussis* cells from both PMA-qPCR and culture were comparable for all strains, confirming the utility of this assay as a reliable method for enumeration, however, there was a significant increase in the recovery of viable cells from B1917 and BP536 using PMA-qPCR compared to culture. DNA copy numbers were calculated using the mean genome size of 4.1 mb.

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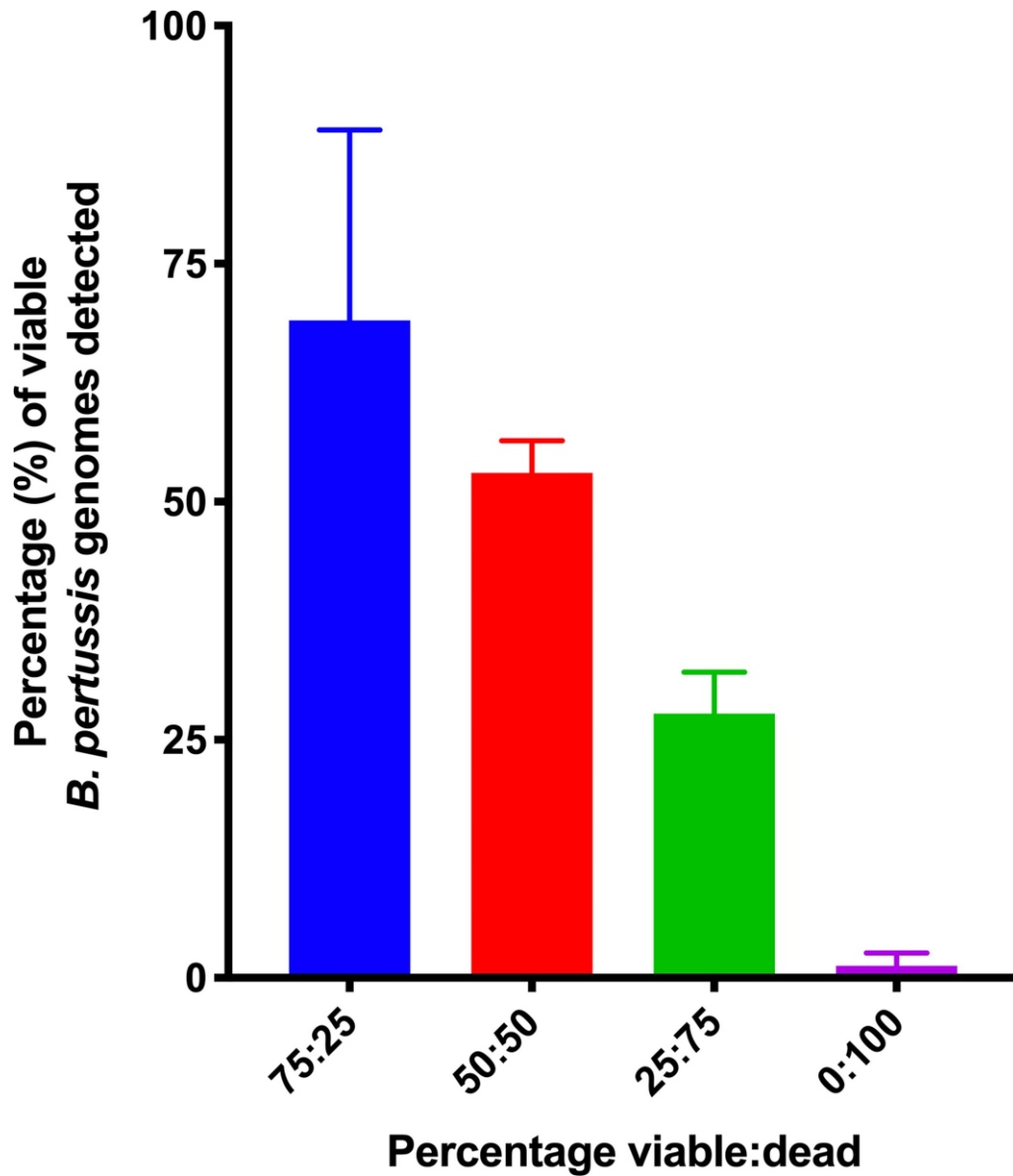
Fig 8. Viable *B. pertussis* cells enumerated from PMA treated samples in the presence of eukaryotic cells. 100,000 THP-1 cells were combined with suspensions of different ratios of heat-killed and viable *B. pertussis* cells. The assay accurately distinguished viable from dead *B. pertussis* in each suspension. Error bars represent standard deviations from three biological replicates.

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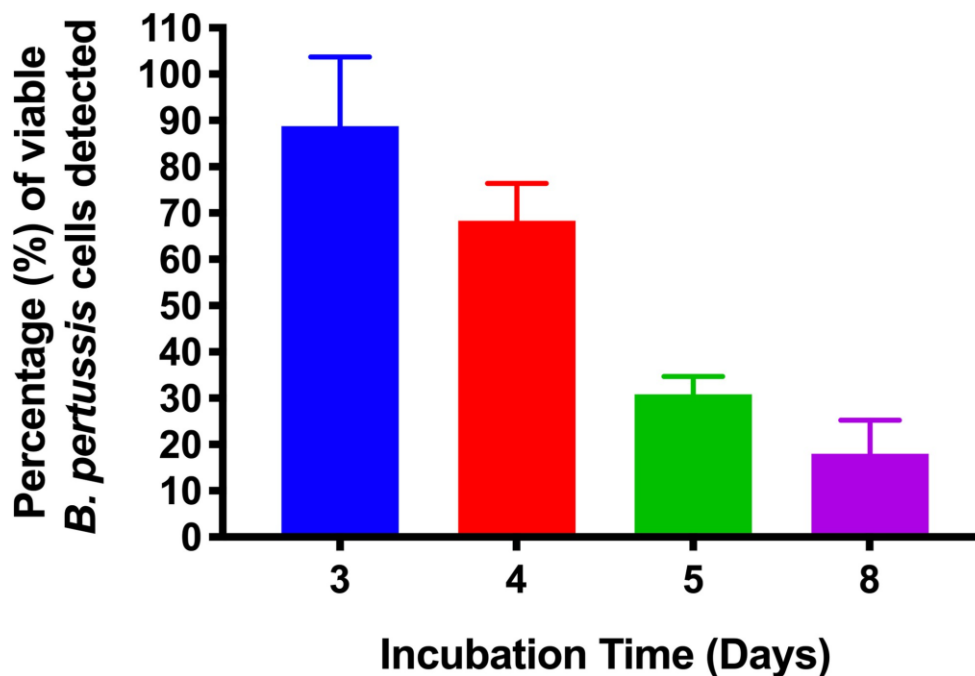
Fig 9. The viability of *B. pertussis* decreases during growth on agar plates. The viability of *B. pertussis* growing on agar plates was measured over time. Viability decreased as the incubation time increased with only 24% of cells being viable after 5 days of incubation. Error bars represent standard deviations from three biological replicates.

Discussion

Ordinarily, the detection and quantification of viable *B. pertussis* is achieved through culture on laboratory agar. However, the relatively slow growth rate of *B. pertussis* means that the growth of countable colonies can take between 72–120 hours. The development of a human challenge model for *B. pertussis* as part of the PERISCOPE project requires that enumeration of viable *B. pertussis* be achieved in a much shorter time than this, in order to be able follow colonisation closely.

In addition, simple enumeration of viable bacteria within a sample doesn't provide the complete picture. In many scenarios, such as measuring bacterial load in an infection model, it is of great interest to know the total bacterial number as understanding the dynamics of bacterial growth that involves both cell division and cell death is very important. Thus, while traditional qPCR provides a faster detection method for *B. pertussis* than culture, the modification of a qPCR assay with the introduction of PMA treatment of samples reported here enables both fast detection of *B. pertussis* and the ability to distinguish viable from dead cells.

Here, we demonstrate that PMA inhibits PCR-mediated amplification from dead *B. pertussis* and that inhibition of signal from dead cells occurs even in the presence of high numbers of eukaryotic cells. This may be important for the detection of *B. pertussis* from complex samples that contain a mix of cell types as seen in the human challenge model. Samples obtained from

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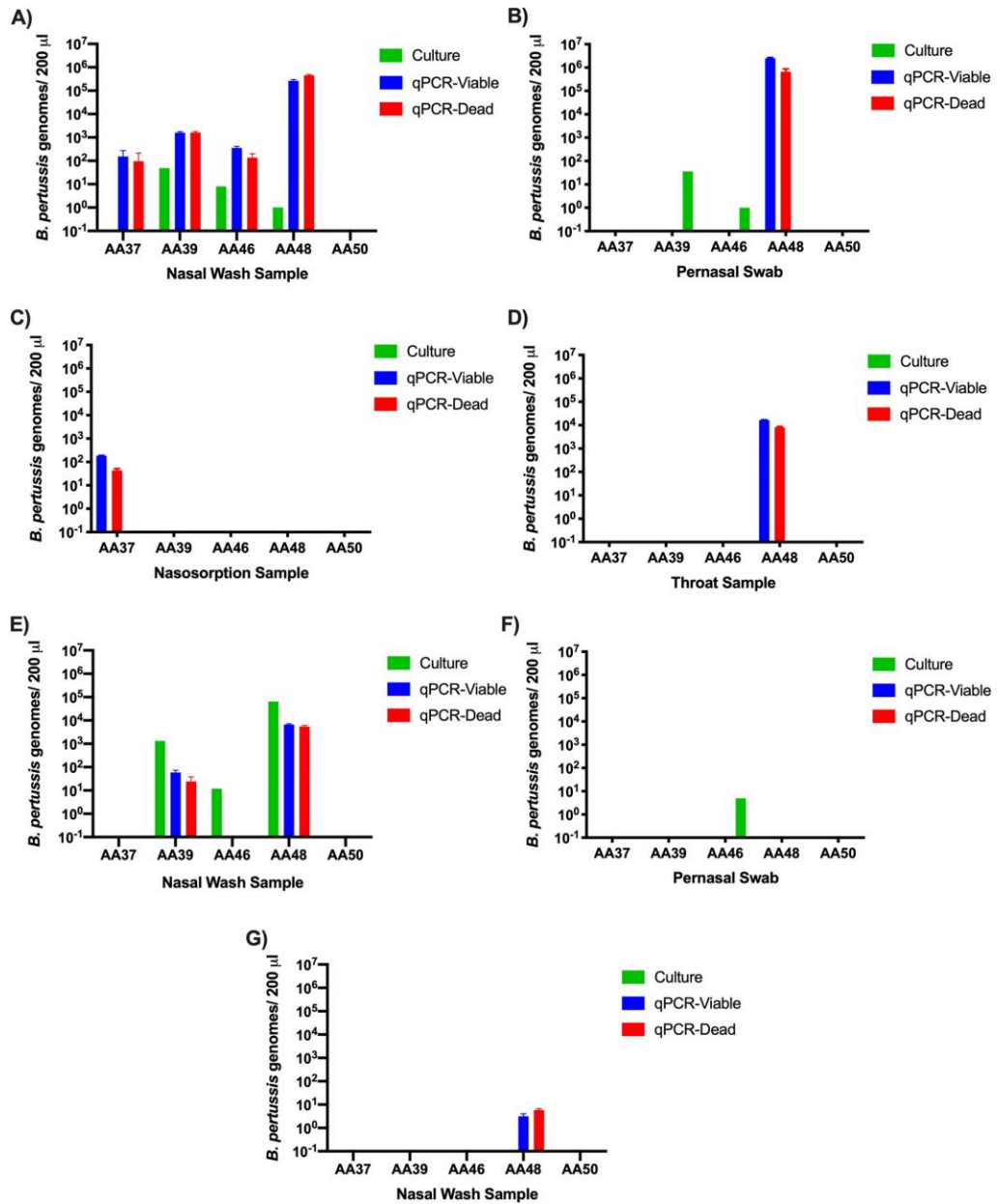
Fig 10. PMA-qPCR detected viable *B. pertussis* from human challenge model samples within hours compared to culture. Viable and dead *B. pertussis* were enumerated in samples from 5 volunteers in the human challenge model, collected Day 9 (A-D), Day 11 (E-F) and Day 16 (G) after inoculation, from the sample type indicated. Two hundred µl of samples were processed. Day 16 samples are taken two days after volunteers started azithromycin treatment to clear infection. Values below the lower limit of detection were considered undetectable and given a value of 0.

volunteers that were identified as positive for *B. pertussis* by culture, were also detected in our initial test of the PMA-qPCR assay. The same volunteers were identified as being negative for *B. pertussis* by both qPCR and culture, with the exception of a single sample that had low levels of *B. pertussis* identified only by qPCR. This result demonstrates the high sensitivity of this assay to detect very low levels of viable and dead *B. pertussis*. Further optimisation studies to determine the exact point in which there is loss in sensitivity when amplifying fewer than 10^3 *B. pertussis* cells in the presence of THP1 cells would further support the results obtained from the human challenge model. Interestingly, PMA-qPCR detected approximately equal numbers of viable and dead *B. pertussis*, demonstrating its use to enumerate total bacteria rather than only viable cells. The full results of the human challenge model are published elsewhere [21]. Here we demonstrate that the PMA-qPCR assay allowed for a determination of colonisation status within hours of obtaining the samples compared to days when using culture.

To confirm that the assay can be used with strains other than B1917, we tested five additional *B. pertussis* strains. Approximately 250 copies of IS481 were found in all isolates of *B. pertussis*, however, the exact number of copies differs amongst strains within a narrow range
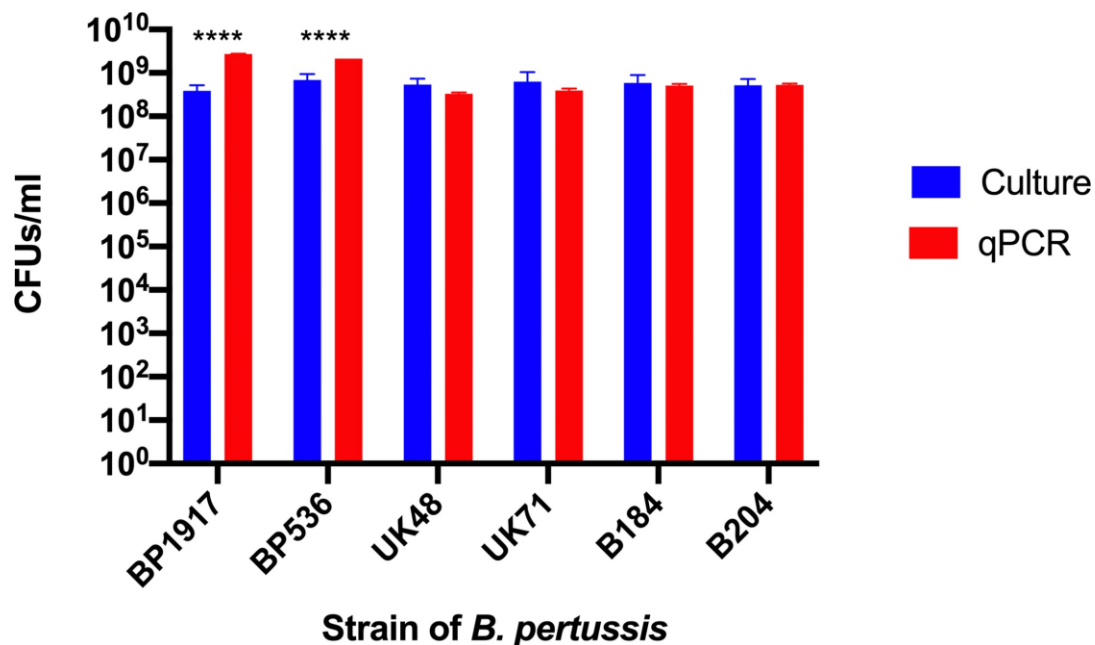
Fig 11. Enumeration of six strains of *B. pertussis* with both culture and PMA-qPCR produced comparable values. The exceptions were B1917 and BP536 for which a higher number of viable *B. pertussis* cells were detected using the PMA- qPCR assay. Error bars represent standard deviations from three biological replicates.

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[30]. The number of copies range from 236–272 among the closed genome sequences available for *B. pertussis*. Thus, this will create some error when performing absolute quantification of strains for which the copy number is not known, but this error is not large (<10%).

The use of IS481 as a target means that there is the chance of cross-reactivity of IS481 with *B. holmseii* and *B. bronchiseptica*, although *B. holmseii* is rarely recovered from nasopharyngeal samples [32]. However, here, this assay was specifically designed to support the human challenge model, therefore using a single qPCR target of IS481 to detect known amounts of *B. pertussis* B1917 that has been administered to volunteers was appropriate. To extend the use of this assay and to increase specificity, species-specific targets, which are commonly used in many diagnostic labs, should be considered. [30]. These changes will reduce false positives and result in a more sensitive and specific assay for the accurate diagnosis of *B. pertussis*, as well as help rule out coinfections or pertussis symptoms caused by other *Bordetella spp*.

The utility of the PMA-qPCR assay has been shown for the human challenge model, but has wider uses. For example, in diagnostic laboratories, where ascertaining if *B. pertussis* is viable or dead will facilitate whether to pursue culture as a means to obtaining a live culture for characterisation. It is also of use in a range of research and industrial settings enabling investigation of the dynamics of *B. pertussis* growth by determining both cell division and cell death.

Supporting information

S1 Table. Standard curve of Ct value versus template concentration.
(XLSX)

S2 Table. The effect of PMA concentration on the PCR amplification signal from heat-killed cells.
(XLSX)

S3 Table. The effect of dark and light exposure times on PMA-inhibition of PCR amplification. A) Dark Incubation B) Light Incubation.
(XLSX)

S4 Table. Selected assay conditions gave reproducible inhibition of PCR signal from dead cells.
(XLSX)

S5 Table. Effect of eukaryotic gDNA on the detection of *B. pertussis*.
(XLSX)

S6 Table. Eukaryotic THP-1 gDNA did not interfere with enumeration of *B. pertussis*.
(XLSX)

S7 Table. Viable *B. pertussis* cells enumerated from PMA treated samples in the presence of eukaryotic cells.
(XLSX)

S8 Table. The viability of *B. pertussis* decreases during growth on agar plates.
(XLSX)

S9 Table. PMA-qPCR detected viable *B. pertussis* from human challenge model samples within hours compared to culture. A) Day 9- Nasal washes B) Day 9- Pernasal Swab C) Day 9- Nasosorption D) Day 9- Throat E) Day 11- Nasal washes F) Day 11- Pernasal swabs G) Day 16- Nasal washes.
(XLSX)

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S10 Table. Enumeration of six strains of *B. pertussis* with both culture and PMA-qPCR produced comparable values.
(XLSX)

Author Contributions

Conceptualization: Iain MacArthur, Hans de Graaf, Robert C. Read, Andrew Preston.

Data curation: Stacy Ramkissoon, Iain MacArthur, Andrew Preston.

Formal analysis: Stacy Ramkissoon, Iain MacArthur, Muktar Ibrahim, Andrew Preston.

Funding acquisition: Andrew Preston.

Investigation: Stacy Ramkissoon, Iain MacArthur, Muktar Ibrahim, Hans de Graaf, Robert C. Read, Andrew Preston.

Methodology: Stacy Ramkissoon, Iain MacArthur, Hans de Graaf, Robert C. Read, Andrew Preston.

Project administration: Hans de Graaf, Robert C. Read, Andrew Preston.

Resources: Muktar Ibrahim, Hans de Graaf, Robert C. Read, Andrew Preston.

Software: Andrew Preston.

Supervision: Iain MacArthur, Andrew Preston.

Validation: Stacy Ramkissoon, Iain MacArthur, Andrew Preston.

Visualization: Stacy Ramkissoon, Iain MacArthur, Andrew Preston.

Writing – original draft: Stacy Ramkissoon, Andrew Preston.

Writing – review & editing: Stacy Ramkissoon, Andrew Preston.

References

1. Mattoo S, Cherry JD. Clinical Manifestations of Respiratory Infections Due to Bordetella pertussis and Other Bordetella Subspecies Molecular Pathogenesis, Epidemiology, and Clinical Manifestations of Respiratory Infections Due to Bordetella pertussis and Other Bordetella Su. Clin Microbiol Rev. 2005; 18(2):326–82. https://doi.org/10.1128/CMR.18.2.326-382.2005 PMID: 15831828

2. Kilgore PE, Salim AM, Zervos MJ, Schmitt H. Pertussis: Microbiology, Disease, Treatment, and Prevention. 2016; 29(3):449–86.

3. Wagner B, Melzer H, Freymüller G, Stumvoll S, Rendi-Wagner P, Paulke-Korinek M, et al. Genetic variation of bordetella pertussis in Austria. PLoS One. 2015; 10(7):1–13.

4. HPA. Confirmed pertussis cases (England and Wales). Heal Prot Rep. 2012; 6(43).

5. Spokes PJ, Quinn HE, McAnulty JM. Review of the 2008–2009 pertussis epidemic in NSW: notifications and hospitalisations. N S W Public Health Bull. 2010; 21(8):167.

6. De Melker HE, Schellekens JFP, Neppelenbroek SE, Mooi FR, Rümke HC, Conyn-Van Spaendonck MAE. Reemergence of pertussis in the highly vaccinated population of the Netherlands: Observations on surveillance data. Emerg Infect Dis. 2000; 6(4):348–57. https://doi.org/10.3201/eid0604.000404 PMID: 10905967

7. Kanojia G, Raeven RHM, van der Maas L, Bindels THE, van Riet E, Metz B, et al. Development of a thermostable spray dried outer membrane vesicle pertussis vaccine for pulmonary immunization. J Control Release [Internet]. 2018; 286(July):167–78. Available from: https://doi.org/10.1016/j.jconrel. 2018.07.035

8. Van der Maas NAT, Mooi FR, de Greeff SC, Berbers GAM, Spaendonck MAEC, de Melker HE. Pertussis in the Netherlands, is the current vaccination strategy sufficient to reduce disease burden in young infants? Vaccine [Internet]. 2013; 31(41):4541–7. Available from: https://doi.org/10.1016/j.vaccine. 2013.07.060 PMID: 23933365

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9. Winter K, Harriman K, Zipprich J, Schechter R, Talarico J, Watt J, et al. California Pertussis epidemic, 2010. J Pediatr [Internet]. 2012; 161(6):1091–6. Available from: https://doi.org/10.1016/j.jpeds.2012.05.041 PMID: 22819634

10. Acosta AM, DeBolt C, Tasslimi A, Lewis M, Stewart LK, Misegades LK, et al. Tdap Vaccine Effectiveness in Adolescents During the 2012 Washington State Pertussis Epidemic. Pediatrics. 2015; 135 (6):981–9. https://doi.org/10.1542/peds.2014-3358 PMID: 25941309

11. Warfel JM, Merkel TJ. Bordetella pertussis infection induces a mucosal IL-17 response and long-lived Th17 and Th1 immune memory cells in nonhuman primates. Mucosal Immunol [Internet]. 2012; 6 (4):787–96. Available from: https://doi.org/10.1038/mi.2012.117 PMID: 23187316

12. Ross PJ, Sutton CE, Higgins S, Allen AC, Walsh K, Misiak A, et al. Relative Contribution of Th1 and Th17 Cells in Adaptive Immunity to Bordetella pertussis: Towards the Rational Design of an Improved Acellular Pertussis Vaccine. PLoS Pathog. 2013; 9(4).

13. Althouse BM, Scarpino S V. Asymptomatic transmission and the resurgence of Bordetella pertussis. BMC Med [Internet]. 2015; 13(1):1–12. Available from: http://dx.doi.org/10.1186/s12916-015-0382-8

14. Zeddeman A, van Gent M, Heuvelman CJ, van der Heide HG, Bart MJ, Advani A, et al. Investigations into the emergence of pertactin-deficient Bordetella pertussis isolates in six European countries, 1996 to 2012. Euro Surveill Bull Eur sur les Mal Transm = Eur Commun Dis Bull. 2014; 19(33):1–11.

15. Bodilis H, Guiso N. Virulence of pertactin-negative Bordetella pertussis isolates from infants, France. Emerg Infect Dis. 2013; 19(3):471–4. https://doi.org/10.3201/1903.121475 PMID: 23621904

16. Otsuka N, Han HJ, Toyoizumi-Ajisaka H, Nakamura Y, Arakawa Y, Shibayama K, et al. Prevalence and genetic characterization of pertactin-deficient bordetella pertussis in Japan. PLoS One. 2012; 7(2):1–7.

17. Lam C, Octavia S, Ricafort L, Sintchenko V, Gilbert GL, Wood N, et al. Rapid increase in pertactin-deficient Bordetella pertussis isolates, Australia. Emerg Infect Dis. 2014; 20(4):626–33. https://doi.org/10.3201/eid2004.131478 PMID: 24655754

18. Pawloski LC, Queenan AM, Cassiday PK, Lynch AS, Harrison MJ, Shang W, et al. Prevalence and molecular characterization of pertactin-deficient Bordetella pertussis in the United States. Clin Vaccine Immunol. 2014; 21(2):119–25. https://doi.org/10.1128/CVI.00717-13 PMID: 24256623

19. Safarchi A, Octavia S, Luu LDW, Tay CY, Sintchenko V, Wood N, et al. Pertactin negative Bordetella pertussis demonstrates higher fitness under vaccine selection pressure in a mixed infection model. Vaccine [Internet]. 2015; 33(46):6277–81. Available from: https://doi.org/10.1016/j.vaccine.2015.09.064 PMID: 26432908

20. Diavatopoulos DA, Mills KHG, Kester KE, Kampmann B, Silerova M, Heininger U, et al. PERISCOPE: road towards effective control of pertussis. Lancet Infect Dis. 2019; 19(5):e179–86. https://doi.org/10.1016/S1473-3099(18)30646-7 PMID: 30503084

21. De Graaf H, Ibrahim M, Hill AR, Gbesemete D, Vaughan AT, Gorringe A, et al. Controlled human infection with Bordetella pertussis induces asymptomatic, immunising colonisation. Clin Infect Dis [Internet]. 2019 Sep 28; Available from: https://doi.org/10.1093/cid/ciz840

22. Ring N, Abrahams JS, Bagby S, Preston A, MacArthur I. How Genomics Is Changing What We Know About the Evolution and Genome of Bordetella pertussis. Adv Exp Med Biol. 2019; 1183:1–17. https://doi.org/10.1007/5584_2019_401 PMID: 31321755

23. Wu B, Liang W, Kan B. Enumeration of viable non-culturable Vibrio cholerae using propidium monoazide combined with quantitative PCR. J Microbiol Methods [Internet]. 2015; 115:147–52. Available from: https://doi.org/10.1016/j.mimet.2015.05.016 PMID: 26001818

24. Nocker A, Cheung CY, Camper AK. Comparison of propidium monoazide with ethidium monoazide for differentiation of live vs. dead bacteria by selective removal of DNA from dead cells. J Microbiol Methods. 2006; 67(2):310–20. https://doi.org/10.1016/j.mimet.2006.04.015 PMID: 16753236

25. Taylor MJ, Bentham RH, Ross KE. Limitations of Using Propidium Monoazide with qPCR to Discriminate between Live and Dead Legionella in Biofilm Samples. Microbiol insights [Internet]. 2014; 7(7):15–24. Available from: http://www.la-press.com/limitations-of-using-propidium-monoazide-with-qpcr-to-discriminate-bet-article-a4365

26. Ruike W, Higashimori A, Yaguchi J, Li Y -y. Use of real-time PCR with propidium monoazide for enumeration of viable Escherichia coli in anaerobic digestion. Water Sci Technol [Internet]. 2016; 74 (5):1243–54. Available from: https://doi.org/10.2166/wst.2016.327 PMID: 27642844

27. Nkuipou-Kenfack E, Engel H, Fakih S, Nocker A. Improving efficiency of viability-PCR for selective detection of live cells. J Microbiol Methods [Internet]. 2013; 93(1):20–4. Available from: https://doi.org/10.1016/j.mimet.2013.01.018 PMID: 23389080

28. Bart MJ, Zeddeman A, van der Heide HG, Heuvelman K, van Gent M, Mooi FR. Complete Genome Sequences of Bordetella pertussis Isolates B1917 and B1920, Representing Two Predominant Global

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Lineages. Genome Announc [Internet]. 2014; 2(6):2–3. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25540342

29. Parkhill J, Sebaihia M, Preston A, Murphy LD, Thomson N, Harris DE, et al. Comparative analysis of the genome sequences of bordetella pertussis, bordetella parapertussis and bordetella bronchiseptica. Nat Genet. 2003; 35(1):32–40. https://doi.org/10.1038/ng1227 PMID: 12910271

30. Probert WS, Ely J, Schrader K, Atwell J, Nossoff A, Kwan S. Identification and evaluation of new target sequences for specific detection of Bordetella pertussis by real-time PCR. J Clin Microbiol. 2008; 46 (10):3228–31. https://doi.org/10.1128/JCM.00386-08 PMID: 18753352

31. Ring N, Abrahams JS, Jain M, Olsen H, Preston A, Bagby S. Resolving the complex Bordetella pertussis genome using barcoded nanopore sequencing. Microb genomics. 2018; 4(11).

32. Martini H, Detemmerman L, Soetens O, Yusuf E, Piérard D. Improving specificity of Bordetella pertussis detection using a four target real-time PCR. PLoS One. 2017; 12(4):1–11.

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Appendix B: Homology of putative β-lactamase encoding genes in *B. pertussis* BP536 compared to known β-lactamase proteins from the NCBI database

Gene	Percent Identity	Nucleotide Range	Nucleotide Range
BP0442	55.56	445688	445741
BP0220	38.3	230844	230716
BP3130	30.3	3335419	3335601
BP2609	29.07	2762220	2761963
BP1113	27.87	1171449	1171625
BP0333	26.76	337153	336956
BP0230	21.33	238039	237815

Appendix B: Homology of putative β-lactamase encoding genes in *B. pertussis* BP536 compared to known β-lactamase proteins from the NCBI database

Function	Gene		Accession	Percent Identity	Identities (total)		Query Start	Query End	Nucleotide start (Range)	Nucleotide End (range)	Length (bp)	Expect	Score (bits) (57)	Positives (%)
	BP2982	WP_122630834.1	NZ_CP039021.1	66.667	21	7	100	120	3173470	3173532	62	5.5	27.3	76.19
		WP_122630834.1	NZ_CP039022.1	66.667	21	7	100	120	3173470	3173532	62	5.5	27.3	76.19
		WP_122630834.1	NC_002929.2	66.667	21	7	100	120	3173470	3173532	62	5.5	27.3	76.19
		WP_122630833.1	NZ_CP039021.1	66.667	21	7	103	123	3173470	3173532	62	3.8	27.7	76.19
		WP_122630833.1	NZ_CP039022.1	66.667	21	7	103	123	3173470	3173532	62	3.8	27.7	76.19
		WP_122630833.1	NC_002929.2	66.667	21	7	103	123	3173470	3173532	62	3.8	27.7	76.19
	BP2376	WP_005087154.1	NZ_CP039021.1	66.667	15	5	16	30	2515872	2515916	44	5.5	27.3	66.67
		WP_005087154.1	NZ_CP039022.1	66.667	15	5	16	30	2515872	2515916	44	5.5	27.3	66.67
		WP_005087154.1	NC_002929.2	66.667	15	5	16	30	2515872	2515916	44	5.5	27.3	66.67
	bfeA	WP_007708339.1	NZ_CP039021.1	64.706	17	6	204	220	3079129	3079179	50	4.8	27.7	76.47
		WP_007708339.1	NZ_CP039022.1	64.706	17	6	204	220	3079129	3079179	50	4.8	27.7	76.47
		WP_007708339.1	NC_002929.2	64.706	17	6	204	220	3079129	3079179	50	4.8	27.7	76.47
	BP0423	WP_028030786.1	NZ_CP039021.1	64.706	17	6	203	219	425168	425218	50	5.7	27.3	82.35
		WP_028030786.1	NZ_CP039022.1	64.706	17	6	203	219	425168	425218	50	5.7	27.3	82.35
		WP_028030786.1	NC_002929.2	64.706	17	6	203	219	425168	425218	50	5.7	27.3	82.35
	fdhA	WP_063862741.1	NZ_CP039021.1	60.87	23	9	52	74	1549572	1549640	68	0.97	29.6	65.22
		WP_063862741.1	NZ_CP039022.1	60.87	23	9	52	74	1549572	1549640	68	0.97	29.6	65.22
		WP_063862741.1	NC_002929.2	60.87	23	9	52	74	1549572	1549640	68	0.97	29.6	65.22
	glgB	WP_063842268.1	NZ_CP039021.1	58.824	17	7	130	146	1399602	1399652	50	9.5	26.9	58.82
		WP_063842268.1	NZ_CP039022.1	58.824	17	7	130	146	1399602	1399652	50	9.5	26.9	58.82
		WP_063842268.1	NC_002929.2	58.824	17	7	130	146	1399602	1399652	50	9.5	26.9	58.82
		WP_063842701.1	NZ_CP039021.1	58.621	29	12	105	133	3424755	3424841	86	2.59E-04	41.2	79.31
		WP_063842701.1	NZ_CP039022.1	58.621	29	12	105	133	3424755	3424841	86	2.59E-04	41.2	79.31

		WP_063842701.1	NC_002929.2	58.621	29	12	105	133	3424755	3424841	86	2.59E-04	41.2	79.31
	BP0128	WP_122630824.1	NZ_CP039021.1	57.895	19	8	40	58	124690	124634	-56	4.9	27.7	63.16
		WP_122630824.1	NZ_CP039022.1	57.895	19	8	40	58	124690	124634	-56	4.9	27.7	63.16
		WP_122630824.1	NC_002929.2	57.895	19	8	40	58	124690	124634	-56	4.9	27.7	63.16
	ugpE	WP_063843238.1	NZ_CP039021.1	56.25	16	7	206	221	1357113	1357160	47	2.4	28.5	81.25
		WP_063843238.1	NZ_CP039022.1	56.25	16	7	206	221	1357113	1357160	47	2.4	28.5	81.25
		WP_063843238.1	NC_002929.2	56.25	16	7	206	221	1357113	1357160	47	2.4	28.5	81.25
	phaD	WP_122630830.1	NZ_CP039021.1	55.556	18	8	231	248	1778843	1778790	-53	9.1	26.6	66.67
		WP_122630830.1	NZ_CP039022.1	55.556	18	8	231	248	1778843	1778790	-53	9.1	26.6	66.67
		WP_122630830.1	NC_002929.2	55.556	18	8	231	248	1778843	1778790	-53	9.1	26.6	66.67
Metallo-beta lactamase superfamily	BP0442	WP_063865175.1	NZ_CP039021.1	55.556	18	8	104	121	445688	445741	53	7.9	26.6	66.67
		WP_063865175.1	NZ_CP039022.1	55.556	18	8	104	121	445688	445741	53	7.9	26.6	66.67
		WP_063865175.1	NC_002929.2	55.556	18	8	104	121	445688	445741	53	7.9	26.6	66.67
		WP_060589395.1	NZ_CP039021.1	55.556	18	8	104	121	445688	445741	53	7.7	26.6	66.67
		WP_060589395.1	NZ_CP039022.1	55.556	18	8	104	121	445688	445741	53	7.7	26.6	66.67
		WP_060589395.1	NC_002929.2	55.556	18	8	104	121	445688	445741	53	7.7	26.6	66.67
		WP_122630828.1	NZ_CP039021.1	55.556	18	8	104	121	445688	445741	53	7.5	26.6	66.67
		WP_122630828.1	NZ_CP039022.1	55.556	18	8	104	121	445688	445741	53	7.5	26.6	66.67
		WP_122630828.1	NC_002929.2	55.556	18	8	104	121	445688	445741	53	7.5	26.6	66.67
		WP_114699283.1	NZ_CP039021.1	55.556	18	8	104	121	445688	445741	53	7.7	26.6	66.67
		WP_114699283.1	NZ_CP039022.1	55.556	18	8	104	121	445688	445741	53	7.7	26.6	66.67
		WP_114699283.1	NC_002929.2	55.556	18	8	104	121	445688	445741	53	7.7	26.6	66.67
		WP_114699277.1	NZ_CP039021.1	55.556	18	8	104	121	445688	445741	53	7.4	26.6	66.67
		WP_114699277.1	NZ_CP039022.1	55.556	18	8	104	121	445688	445741	53	7.4	26.6	66.67
		WP_114699277.1	NC_002929.2	55.556	18	8	104	121	445688	445741	53	7.4	26.6	66.67
		WP_111772163.1	NZ_CP039021.1	55.556	18	8	104	121	445688	445741	53	7.5	26.6	66.67
		WP_111772163.1	NZ_CP039022.1	55.556	18	8	104	121	445688	445741	53	7.5	26.6	66.67

		WP_111772163.1	NC_002929.2	55.556	18	8	104	121	445688	445741	53	7.5	26.6	66.67
		WP_111672914.1	NZ_CP039021.1	55.556	18	8	104	121	445688	445741	53	8	26.6	66.67
		WP_111672914.1	NZ_CP039022.1	55.556	18	8	104	121	445688	445741	53	8	26.6	66.67
		WP_111672914.1	NC_002929.2	55.556	18	8	104	121	445688	445741	53	8	26.6	66.67
		WP_104009846.1	NZ_CP039021.1	55.556	18	8	104	121	445688	445741	53	7.8	26.6	66.67
		WP_104009846.1	NZ_CP039022.1	55.556	18	8	104	121	445688	445741	53	7.8	26.6	66.67
		WP_104009846.1	NC_002929.2	55.556	18	8	104	121	445688	445741	53	7.8	26.6	66.67
		WP_094009807.1	NZ_CP039021.1	55.556	18	8	104	121	445688	445741	53	7.7	26.6	66.67
		WP_094009807.1	NZ_CP039022.1	55.556	18	8	104	121	445688	445741	53	7.7	26.6	66.67
		WP_094009807.1	NC_002929.2	55.556	18	8	104	121	445688	445741	53	7.7	26.6	66.67
		WP_032492008.1	NZ_CP039021.1	55.556	18	8	104	121	445688	445741	53	8.4	26.6	66.67
		WP_032492008.1	NZ_CP039022.1	55.556	18	8	104	121	445688	445741	53	8.4	26.6	66.67
		WP_032492008.1	NC_002929.2	55.556	18	8	104	121	445688	445741	53	8.4	26.6	66.67
		WP_003108247.1	NZ_CP039021.1	55.556	18	8	104	121	445688	445741	53	7.4	26.6	66.67
		WP_003108247.1	NZ_CP039022.1	55.556	18	8	104	121	445688	445741	53	7.4	26.6	66.67
		WP_003108247.1	NC_002929.2	55.556	18	8	104	121	445688	445741	53	7.4	26.6	66.67
		WP_063865181.1	NZ_CP039021.1	55.556	18	8	104	121	445688	445741	53	7.5	26.6	66.67
		WP_063865181.1	NZ_CP039022.1	55.556	18	8	104	121	445688	445741	53	7.5	26.6	66.67
		WP_063865181.1	NC_002929.2	55.556	18	8	104	121	445688	445741	53	7.5	26.6	66.67
		WP_063865201.1	NZ_CP039021.1	55.556	18	8	104	121	445688	445741	53	7.5	26.6	66.67
		WP_063865201.1	NZ_CP039022.1	55.556	18	8	104	121	445688	445741	53	7.5	26.6	66.67
		WP_063865201.1	NC_002929.2	55.556	18	8	104	121	445688	445741	53	7.5	26.6	66.67
		WP_032491390.1	NZ_CP039021.1	55.556	18	8	104	121	445688	445741	53	8.3	26.6	66.67
		WP_032491390.1	NZ_CP039022.1	55.556	18	8	104	121	445688	445741	53	8.3	26.6	66.67
		WP_032491390.1	NC_002929.2	55.556	18	8	104	121	445688	445741	53	8.3	26.6	66.67
		WP_032492472.1	NZ_CP039021.1	55.556	18	8	104	121	445688	445741	53	8.1	26.6	66.67
		WP_032492472.1	NZ_CP039022.1	55.556	18	8	104	121	445688	445741	53	8.1	26.6	66.67
		WP_032492472.1	NC_002929.2	55.556	18	8	104	121	445688	445741	53	8.1	26.6	66.67

		WP_077064888.1	NZ_CP039021.1	55.556	18	8	104	121	445688	445741	53	8.4	26.6	66.67
		WP_077064888.1	NZ_CP039022.1	55.556	18	8	104	121	445688	445741	53	8.4	26.6	66.67
		WP_077064888.1	NC_002929.2	55.556	18	8	104	121	445688	445741	53	8.4	26.6	66.67
		WP_023442721.1	NZ_CP039021.1	55.556	18	8	104	121	445688	445741	53	7.5	26.6	66.67
		WP_023442721.1	NZ_CP039022.1	55.556	18	8	104	121	445688	445741	53	7.5	26.6	66.67
		WP_023442721.1	NC_002929.2	55.556	18	8	104	121	445688	445741	53	7.5	26.6	66.67
		WP_063865187.1	NZ_CP039021.1	55.556	18	8	104	121	445688	445741	53	8.3	26.6	66.67
		WP_063865187.1	NZ_CP039022.1	55.556	18	8	104	121	445688	445741	53	8.3	26.6	66.67
		WP_063865187.1	NC_002929.2	55.556	18	8	104	121	445688	445741	53	8.3	26.6	66.67
		WP_063865186.1	NZ_CP039021.1	55.556	18	8	104	121	445688	445741	53	7.5	26.6	66.67
		WP_063865186.1	NZ_CP039022.1	55.556	18	8	104	121	445688	445741	53	7.5	26.6	66.67
		WP_063865186.1	NC_002929.2	55.556	18	8	104	121	445688	445741	53	7.5	26.6	66.67
		WP_063865185.1	NZ_CP039021.1	55.556	18	8	104	121	445688	445741	53	8.2	26.6	66.67
		WP_063865185.1	NZ_CP039022.1	55.556	18	8	104	121	445688	445741	53	8.2	26.6	66.67
		WP_063865185.1	NC_002929.2	55.556	18	8	104	121	445688	445741	53	8.2	26.6	66.67
		WP_063865203.1	NZ_CP039021.1	55.556	18	8	104	121	445688	445741	53	7.7	26.6	66.67
		WP_063865203.1	NZ_CP039022.1	55.556	18	8	104	121	445688	445741	53	7.7	26.6	66.67
		WP_063865203.1	NC_002929.2	55.556	18	8	104	121	445688	445741	53	7.7	26.6	66.67
		WP_063865202.1	NZ_CP039021.1	55.556	18	8	104	121	445688	445741	53	7.3	26.9	66.67
		WP_063865202.1	NZ_CP039022.1	55.556	18	8	104	121	445688	445741	53	7.3	26.9	66.67
		WP_063865202.1	NC_002929.2	55.556	18	8	104	121	445688	445741	53	7.3	26.9	66.67
		WP_063865200.1	NZ_CP039021.1	55.556	18	8	104	121	445688	445741	53	7.9	26.6	66.67
		WP_063865200.1	NZ_CP039022.1	55.556	18	8	104	121	445688	445741	53	7.9	26.6	66.67
		WP_063865200.1	NC_002929.2	55.556	18	8	104	121	445688	445741	53	7.9	26.6	66.67
		WP_063865199.1	NZ_CP039021.1	55.556	18	8	104	121	445688	445741	53	7.9	26.6	66.67
		WP_063865199.1	NZ_CP039022.1	55.556	18	8	104	121	445688	445741	53	7.9	26.6	66.67
		WP_063865199.1	NC_002929.2	55.556	18	8	104	121	445688	445741	53	7.9	26.6	66.67
		WP_063865198.1	NZ_CP039021.1	55.556	18	8	104	121	445688	445741	53	8.3	26.6	66.67

		WP_063865198.1	NZ_CP039022.1	55.556	18	8	104	121	445688	445741	53	8.3	26.6	66.67
		WP_063865198.1	NC_002929.2	55.556	18	8	104	121	445688	445741	53	8.3	26.6	66.67
		WP_063865196.1	NZ_CP039021.1	55.556	18	8	104	121	445688	445741	53	7	26.9	66.67
		WP_063865196.1	NZ_CP039022.1	55.556	18	8	104	121	445688	445741	53	7	26.9	66.67
		WP_063865196.1	NC_002929.2	55.556	18	8	104	121	445688	445741	53	7	26.9	66.67
		WP_063865195.1	NZ_CP039021.1	55.556	18	8	104	121	445688	445741	53	7.7	26.6	66.67
		WP_063865195.1	NZ_CP039022.1	55.556	18	8	104	121	445688	445741	53	7.7	26.6	66.67
		WP_063865195.1	NC_002929.2	55.556	18	8	104	121	445688	445741	53	7.7	26.6	66.67
		WP_063865194.1	NZ_CP039021.1	55.556	18	8	104	121	445688	445741	53	7.5	26.6	66.67
		WP_063865194.1	NZ_CP039022.1	55.556	18	8	104	121	445688	445741	53	7.5	26.6	66.67
		WP_063865194.1	NC_002929.2	55.556	18	8	104	121	445688	445741	53	7.5	26.6	66.67
		WP_063865193.1	NZ_CP039021.1	55.556	18	8	104	121	445688	445741	53	8.7	26.6	66.67
		WP_063865193.1	NZ_CP039022.1	55.556	18	8	104	121	445688	445741	53	8.7	26.6	66.67
		WP_063865193.1	NC_002929.2	55.556	18	8	104	121	445688	445741	53	8.7	26.6	66.67
		WP_063865192.1	NZ_CP039021.1	55.556	18	8	104	121	445688	445741	53	8.4	26.6	66.67
		WP_063865192.1	NZ_CP039022.1	55.556	18	8	104	121	445688	445741	53	8.4	26.6	66.67
		WP_063865192.1	NC_002929.2	55.556	18	8	104	121	445688	445741	53	8.4	26.6	66.67
		WP_063865191.1	NZ_CP039021.1	55.556	18	8	104	121	445688	445741	53	7.4	26.6	66.67
		WP_063865191.1	NZ_CP039022.1	55.556	18	8	104	121	445688	445741	53	7.4	26.6	66.67
		WP_063865191.1	NC_002929.2	55.556	18	8	104	121	445688	445741	53	7.4	26.6	66.67
		WP_063865190.1	NZ_CP039021.1	55.556	18	8	104	121	445688	445741	53	8.7	26.6	66.67
		WP_063865190.1	NZ_CP039022.1	55.556	18	8	104	121	445688	445741	53	8.7	26.6	66.67
		WP_063865190.1	NC_002929.2	55.556	18	8	104	121	445688	445741	53	8.7	26.6	66.67
		WP_063865189.1	NZ_CP039021.1	55.556	18	8	104	121	445688	445741	53	8.1	26.6	66.67
		WP_063865189.1	NZ_CP039022.1	55.556	18	8	104	121	445688	445741	53	8.1	26.6	66.67
		WP_063865189.1	NC_002929.2	55.556	18	8	104	121	445688	445741	53	8.1	26.6	66.67
		WP_063865188.1	NZ_CP039021.1	55.556	18	8	104	121	445688	445741	53	8.6	26.6	66.67
		WP_063865188.1	NZ_CP039022.1	55.556	18	8	104	121	445688	445741	53	8.6	26.6	66.67

		WP_063865188.1	NC_002929.2	55.556	18	8	104	121	445688	445741	53	8.6	26.6	66.67
		WP_063865184.1	NZ_CP039021.1	55.556	18	8	104	121	445688	445741	53	6.5	26.9	66.67
		WP_063865184.1	NZ_CP039022.1	55.556	18	8	104	121	445688	445741	53	6.5	26.9	66.67
		WP_063865184.1	NC_002929.2	55.556	18	8	104	121	445688	445741	53	6.5	26.9	66.67
		WP_063865183.1	NZ_CP039021.1	55.556	18	8	104	121	445688	445741	53	8.4	26.6	66.67
		WP_063865183.1	NZ_CP039022.1	55.556	18	8	104	121	445688	445741	53	8.4	26.6	66.67
		WP_063865183.1	NC_002929.2	55.556	18	8	104	121	445688	445741	53	8.4	26.6	66.67
		WP_063865182.1	NZ_CP039021.1	55.556	18	8	104	121	445688	445741	53	8.4	26.6	66.67
		WP_063865182.1	NZ_CP039022.1	55.556	18	8	104	121	445688	445741	53	8.4	26.6	66.67
		WP_063865182.1	NC_002929.2	55.556	18	8	104	121	445688	445741	53	8.4	26.6	66.67
		WP_063865180.1	NZ_CP039021.1	55.556	18	8	104	121	445688	445741	53	7.4	26.6	66.67
		WP_063865180.1	NZ_CP039022.1	55.556	18	8	104	121	445688	445741	53	7.4	26.6	66.67
		WP_063865180.1	NC_002929.2	55.556	18	8	104	121	445688	445741	53	7.4	26.6	66.67
		WP_063865179.1	NZ_CP039021.1	55.556	18	8	104	121	445688	445741	53	7.5	26.6	66.67
		WP_063865179.1	NZ_CP039022.1	55.556	18	8	104	121	445688	445741	53	7.5	26.6	66.67
		WP_063865179.1	NC_002929.2	55.556	18	8	104	121	445688	445741	53	7.5	26.6	66.67
		WP_063865178.1	NZ_CP039021.1	55.556	18	8	104	121	445688	445741	53	8.1	26.6	66.67
		WP_063865178.1	NZ_CP039022.1	55.556	18	8	104	121	445688	445741	53	8.1	26.6	66.67
		WP_063865178.1	NC_002929.2	55.556	18	8	104	121	445688	445741	53	8.1	26.6	66.67
		WP_063865177.1	NZ_CP039021.1	55.556	18	8	104	121	445688	445741	53	8.2	26.6	66.67
		WP_063865177.1	NZ_CP039022.1	55.556	18	8	104	121	445688	445741	53	8.2	26.6	66.67
		WP_063865177.1	NC_002929.2	55.556	18	8	104	121	445688	445741	53	8.2	26.6	66.67
		WP_063865176.1	NZ_CP039021.1	55.556	18	8	104	121	445688	445741	53	8.4	26.6	66.67
		WP_063865176.1	NZ_CP039022.1	55.556	18	8	104	121	445688	445741	53	8.4	26.6	66.67
		WP_063865176.1	NC_002929.2	55.556	18	8	104	121	445688	445741	53	8.4	26.6	66.67
		WP_063865174.1	NZ_CP039021.1	55.556	18	8	104	121	445688	445741	53	7.5	26.6	66.67
		WP_063865174.1	NZ_CP039022.1	55.556	18	8	104	121	445688	445741	53	7.5	26.6	66.67
		WP_063865174.1	NC_002929.2	55.556	18	8	104	121	445688	445741	53	7.5	26.6	66.67

		WP_063865173.1	NZ_CP039021.1	55.556	18	8	104	121	445688	445741	53	7.1	26.9	66.67
		WP_063865173.1	NZ_CP039022.1	55.556	18	8	104	121	445688	445741	53	7.1	26.9	66.67
		WP_063865173.1	NC_002929.2	55.556	18	8	104	121	445688	445741	53	7.1	26.9	66.67
		WP_063865172.1	NZ_CP039021.1	55.556	18	8	104	121	445688	445741	53	7.7	26.6	66.67
		WP_063865172.1	NZ_CP039022.1	55.556	18	8	104	121	445688	445741	53	7.7	26.6	66.67
		WP_063865172.1	NC_002929.2	55.556	18	8	104	121	445688	445741	53	7.7	26.6	66.67
		WP_063865171.1	NZ_CP039021.1	55.556	18	8	104	121	445688	445741	53	7.9	26.6	66.67
		WP_063865171.1	NZ_CP039022.1	55.556	18	8	104	121	445688	445741	53	7.9	26.6	66.67
		WP_063865171.1	NC_002929.2	55.556	18	8	104	121	445688	445741	53	7.9	26.6	66.67
		WP_063865170.1	NZ_CP039021.1	55.556	18	8	104	121	445688	445741	53	7.5	26.6	66.67
		WP_063865170.1	NZ_CP039022.1	55.556	18	8	104	121	445688	445741	53	7.5	26.6	66.67
		WP_063865170.1	NC_002929.2	55.556	18	8	104	121	445688	445741	53	7.5	26.6	66.67
		WP_063865169.1	NZ_CP039021.1	55.556	18	8	104	121	445688	445741	53	8.1	26.6	66.67
		WP_063865169.1	NZ_CP039022.1	55.556	18	8	104	121	445688	445741	53	8.1	26.6	66.67
		WP_063865169.1	NC_002929.2	55.556	18	8	104	121	445688	445741	53	8.1	26.6	66.67
		WP_063865167.1	NZ_CP039021.1	55.556	18	8	104	121	445688	445741	53	8.5	26.6	66.67
		WP_063865167.1	NZ_CP039022.1	55.556	18	8	104	121	445688	445741	53	8.5	26.6	66.67
		WP_063865167.1	NC_002929.2	55.556	18	8	104	121	445688	445741	53	8.5	26.6	66.67
		WP_063865166.1	NZ_CP039021.1	55.556	18	8	104	121	445688	445741	53	7.5	26.6	66.67
		WP_063865166.1	NZ_CP039022.1	55.556	18	8	104	121	445688	445741	53	7.5	26.6	66.67
		WP_063865166.1	NC_002929.2	55.556	18	8	104	121	445688	445741	53	7.5	26.6	66.67
		WP_013263789.1	NZ_CP039021.1	55.556	18	8	104	121	445688	445741	53	8.3	26.6	66.67
		WP_013263789.1	NZ_CP039022.1	55.556	18	8	104	121	445688	445741	53	8.3	26.6	66.67
		WP_013263789.1	NC_002929.2	55.556	18	8	104	121	445688	445741	53	8.3	26.6	66.67
		WP_034080264.1	NZ_CP039021.1	55.556	18	8	104	121	445688	445741	53	7.3	26.9	66.67
		WP_034080264.1	NZ_CP039022.1	55.556	18	8	104	121	445688	445741	53	7.3	26.9	66.67
		WP_034080264.1	NC_002929.2	55.556	18	8	104	121	445688	445741	53	7.3	26.9	66.67
		WP_032495187.1	NZ_CP039021.1	55.556	18	8	104	121	445688	445741	53	8.1	26.6	66.67

			WP_032495187.1	NZ_CP039022.1	55.556	18	8	104	121	445688	445741	53	8.1	26.6	66.67
			WP_032495187.1	NC_002929.2	55.556	18	8	104	121	445688	445741	53	8.1	26.6	66.67
		BP0246	WP_063864745.1	NZ_CP039021.1	55.556	18	8	219	236	255051	254998	-53	7	26.9	83.33
			WP_063864745.1	NZ_CP039022.1	55.556	18	8	219	236	255051	254998	-53	7	26.9	83.33
			WP_063864745.1	NC_002929.2	55.556	18	8	219	236	255051	254998	-53	7	26.9	83.33
			WP_032489168.1	NZ_CP039021.1	54.545	22	10	217	238	1335212	1335147	-65	6.9	26.9	59.09
			WP_032489168.1	NZ_CP039022.1	54.545	22	10	217	238	1335212	1335147	-65	6.9	26.9	59.09
			WP_032489168.1	NC_002929.2	54.545	22	10	217	238	1335212	1335147	-65	6.9	26.9	59.09
		argoG	WP_016660101.1	NZ_CP039021.1	52	25	12	90	114	3099530	3099604	74	6.2	26.9	64
			WP_016660101.1	NZ_CP039022.1	52	25	12	90	114	3099530	3099604	74	6.2	26.9	64
			WP_016660101.1	NC_002929.2	52	25	12	90	114	3099530	3099604	74	6.2	26.9	64
			WP_063842703.1	NZ_CP039021.1	51.724	29	14	103	131	3424755	3424841	86	0.01	36.2	72.41
			WP_063842703.1	NZ_CP039022.1	51.724	29	14	103	131	3424755	3424841	86	0.01	36.2	72.41
			WP_063842703.1	NC_002929.2	51.724	29	14	103	131	3424755	3424841	86	0.01	36.2	72.41
		gloB	WP_122630834.1	NZ_CP039021.1	50	28	14	87	114	3424755	3424838	83	0.059	33.5	71.43
			WP_122630834.1	NZ_CP039022.1	50	28	14	87	114	3424755	3424838	83	0.059	33.5	71.43
			WP_122630834.1	NC_002929.2	50	28	14	87	114	3424755	3424838	83	0.059	33.5	71.43
		BP1714	WP_063861776.1	NZ_CP039021.1	50	26	12	206	230	1798587	1798510	-77	6.7	26.9	57.69
			WP_063861776.1	NZ_CP039022.1	50	26	12	206	230	1798587	1798510	-77	6.7	26.9	57.69
			WP_063861776.1	NC_002929.2	50	26	12	206	230	1798587	1798510	-77	6.7	26.9	57.69
			WP_053073497.1	NZ_CP039021.1	50	18	9	205	222	9079	9026	-53	7.8	26.9	72.22
			WP_053073497.1	NZ_CP039022.1	50	18	9	205	222	9079	9026	-53	7.8	26.9	72.22
			WP_053073497.1	NC_002929.2	50	18	9	205	222	9079	9026	-53	7.8	26.9	72.22
			WP_100086555.1	NZ_CP039021.1	50	18	9	205	222	9079	9026	-53	7.3	26.9	72.22
			WP_100086555.1	NZ_CP039022.1	50	18	9	205	222	9079	9026	-53	7.3	26.9	72.22
			WP_100086555.1	NC_002929.2	50	18	9	205	222	9079	9026	-53	7.3	26.9	72.22
			WP_094009816.1	NZ_CP039021.1	50	18	9	205	222	9079	9026	-53	7.8	26.9	72.22
			WP_094009816.1	NZ_CP039022.1	50	18	9	205	222	9079	9026	-53	7.8	26.9	72.22

		WP_094009816.1	NC_002929.2	50	18	9	205	222	9079	9026	-53	7.8	26.9	72.22
		WP_032491237.1	NZ_CP039021.1	50	18	9	205	222	9079	9026	-53	7.9	26.9	72.22
		WP_032491237.1	NZ_CP039022.1	50	18	9	205	222	9079	9026	-53	7.9	26.9	72.22
		WP_032491237.1	NC_002929.2	50	18	9	205	222	9079	9026	-53	7.9	26.9	72.22
		WP_085562405.1	NZ_CP039021.1	50	18	9	205	222	9079	9026	-53	7	26.9	72.22
		WP_085562405.1	NZ_CP039022.1	50	18	9	205	222	9079	9026	-53	7	26.9	72.22
		WP_085562405.1	NC_002929.2	50	18	9	205	222	9079	9026	-53	7	26.9	72.22
		WP_085562404.1	NZ_CP039021.1	50	18	9	205	222	9079	9026	-53	7.6	26.9	72.22
		WP_085562404.1	NZ_CP039022.1	50	18	9	205	222	9079	9026	-53	7.6	26.9	72.22
		WP_085562404.1	NC_002929.2	50	18	9	205	222	9079	9026	-53	7.6	26.9	72.22
		WP_077064887.1	NZ_CP039021.1	50	18	9	205	222	9079	9026	-53	7.8	26.9	72.22
		WP_077064887.1	NZ_CP039022.1	50	18	9	205	222	9079	9026	-53	7.8	26.9	72.22
		WP_077064887.1	NC_002929.2	50	18	9	205	222	9079	9026	-53	7.8	26.9	72.22
		WP_064190970.1	NZ_CP039021.1	50	18	9	205	222	9079	9026	-53	7.8	26.9	72.22
		WP_064190970.1	NZ_CP039022.1	50	18	9	205	222	9079	9026	-53	7.8	26.9	72.22
		WP_064190970.1	NC_002929.2	50	18	9	205	222	9079	9026	-53	7.8	26.9	72.22
		WP_063860571.1	NZ_CP039021.1	50	18	9	205	222	9079	9026	-53	7.8	26.9	72.22
		WP_063860571.1	NZ_CP039022.1	50	18	9	205	222	9079	9026	-53	7.8	26.9	72.22
		WP_063860571.1	NC_002929.2	50	18	9	205	222	9079	9026	-53	7.8	26.9	72.22
		WP_063860570.1	NZ_CP039021.1	50	18	9	205	222	9079	9026	-53	7.4	26.9	72.22
		WP_063860570.1	NZ_CP039022.1	50	18	9	205	222	9079	9026	-53	7.4	26.9	72.22
		WP_063860570.1	NC_002929.2	50	18	9	205	222	9079	9026	-53	7.4	26.9	72.22
		WP_063860569.1	NZ_CP039021.1	50	18	9	205	222	9079	9026	-53	7.8	26.9	72.22
		WP_063860569.1	NZ_CP039022.1	50	18	9	205	222	9079	9026	-53	7.8	26.9	72.22
		WP_063860569.1	NC_002929.2	50	18	9	205	222	9079	9026	-53	7.8	26.9	72.22
		WP_063860568.1	NZ_CP039021.1	50	18	9	205	222	9079	9026	-53	7.2	26.9	72.22
		WP_063860568.1	NZ_CP039022.1	50	18	9	205	222	9079	9026	-53	7.2	26.9	72.22
		WP_063860568.1	NC_002929.2	50	18	9	205	222	9079	9026	-53	7.2	26.9	72.22

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		WP_060571602.1	NZ_CP039021.1	50	18	9	205	222	9079	9026	-53	8	26.9	72.22
		WP_060571602.1	NZ_CP039022.1	50	18	9	205	222	9079	9026	-53	8	26.9	72.22
		WP_060571602.1	NC_002929.2	50	18	9	205	222	9079	9026	-53	8	26.9	72.22
		WP_104531868.1	NZ_CP039021.1	48.387	31	16	54	84	2519817	2519909	92	0.05	34.7	67.74
		WP_104531868.1	NZ_CP039022.1	48.387	31	16	54	84	2519817	2519909	92	0.05	34.7	67.74
		WP_104531868.1	NC_002929.2	48.387	31	16	54	84	2519817	2519909	92	0.05	34.7	67.74
		WP_104531867.1	NZ_CP039021.1	48.387	31	16	54	84	2519817	2519909	92	0.046	34.7	67.74
		WP_104531867.1	NZ_CP039022.1	48.387	31	16	54	84	2519817	2519909	92	0.046	34.7	67.74
		WP_104531867.1	NC_002929.2	48.387	31	16	54	84	2519817	2519909	92	0.046	34.7	67.74
		WP_104531866.1	NZ_CP039021.1	48.387	31	16	54	84	2519817	2519909	92	0.047	34.7	67.74
		WP_104531866.1	NZ_CP039022.1	48.387	31	16	54	84	2519817	2519909	92	0.047	34.7	67.74
		WP_104531866.1	NC_002929.2	48.387	31	16	54	84	2519817	2519909	92	0.047	34.7	67.74
		WP_104531865.1	NZ_CP039021.1	48.387	31	16	54	84	2519817	2519909	92	0.037	35	70.97
		WP_104531865.1	NZ_CP039022.1	48.387	31	16	54	84	2519817	2519909	92	0.037	35	70.97
		WP_104531865.1	NC_002929.2	48.387	31	16	54	84	2519817	2519909	92	0.037	35	70.97
		WP_012698469.1	NZ_CP039021.1	48.387	31	16	54	84	2519817	2519909	92	0.047	34.7	67.74
		WP_012698469.1	NZ_CP039022.1	48.387	31	16	54	84	2519817	2519909	92	0.047	34.7	67.74
		WP_012698469.1	NC_002929.2	48.387	31	16	54	84	2519817	2519909	92	0.047	34.7	67.74
	BP2627	WP_063861498.1	NZ_CP039021.1	47.826	23	12	38	60	2783033	2782965	-68	3.1	28.1	60.87
		WP_063861498.1	NZ_CP039022.1	47.826	23	12	38	60	2783033	2782965	-68	3.1	28.1	60.87
		WP_063861498.1	NC_002929.2	47.826	23	12	38	60	2783033	2782965	-68	3.1	28.1	60.87
		WP_032492430.1	NZ_CP039021.1	47.826	23	12	196	218	897151	897219	68	8.9	26.2	65.22
		WP_032492430.1	NZ_CP039022.1	47.826	23	12	196	218	897151	897219	68	8.9	26.2	65.22
		WP_032492430.1	NC_002929.2	47.826	23	12	196	218	897151	897219	68	8.9	26.2	65.22
	BP0390	WP_109545071.1	NZ_CP039021.1	47.826	23	11	225	247	389544	389609	65	7.8	26.6	60.87
		WP_109545071.1	NZ_CP039022.1	47.826	23	11	225	247	389544	389609	65	7.8	26.6	60.87
		WP_109545071.1	NC_002929.2	47.826	23	11	225	247	389544	389609	65	7.8	26.6	60.87
		WP_063864112.1	NZ_CP039021.1	47.826	23	11	225	247	389544	389609	65	8.1	26.6	60.87

		WP_063864112.1	NZ_CP039022.1	47.826	23	11	225	247	389544	389609	65	8.1	26.6	60.87
		WP_063864112.1	NC_002929.2	47.826	23	11	225	247	389544	389609	65	8.1	26.6	60.87
		WP_063862440.1	NZ_CP039021.1	47.826	23	11	225	247	389544	389609	65	8.3	26.6	60.87
		WP_063862440.1	NZ_CP039022.1	47.826	23	11	225	247	389544	389609	65	8.3	26.6	60.87
		WP_063862440.1	NC_002929.2	47.826	23	11	225	247	389544	389609	65	8.3	26.6	60.87
	proceeding BP0010	WP_050737109.1	NZ_CP039021.1	47.368	19	10	204	222	9082	9026	-56	6.4	27.3	68.42
		WP_050737109.1	NZ_CP039022.1	47.368	19	10	204	222	9082	9026	-56	6.4	27.3	68.42
		WP_050737109.1	NC_002929.2	47.368	19	10	204	222	9082	9026	-56	6.4	27.3	68.42
		WP_063860572.1	NZ_CP039021.1	47.368	19	10	204	222	9082	9026	-56	6.4	26.9	68.42
		WP_063860572.1	NZ_CP039022.1	47.368	19	10	204	222	9082	9026	-56	6.4	26.9	68.42
		WP_063860572.1	NC_002929.2	47.368	19	10	204	222	9082	9026	-56	6.4	26.9	68.42
		WP_122630825.1	NZ_CP039021.1	46.429	28	15	120	147	3424755	3424838	83	0.52	30.8	67.86
		WP_122630825.1	NZ_CP039022.1	46.429	28	15	120	147	3424755	3424838	83	0.52	30.8	67.86
		WP_122630825.1	NC_002929.2	46.429	28	15	120	147	3424755	3424838	83	0.52	30.8	67.86
	BP2303	WP_057689930.1	NZ_CP039021.1	46.429	28	14	6	33	2426807	2426727	-80	1.6	28.9	64.29
		WP_057689930.1	NZ_CP039022.1	46.429	28	14	6	33	2426807	2426727	-80	1.6	28.9	64.29
		WP_057689930.1	NC_002929.2	46.429	28	14	6	33	2426807	2426727	-80	1.6	28.9	64.29
		WP_063864079.1	NZ_CP039021.1	46.429	28	14	6	33	2426807	2426727	-80	1.7	28.9	64.29
		WP_063864079.1	NZ_CP039022.1	46.429	28	14	6	33	2426807	2426727	-80	1.7	28.9	64.29
		WP_063864079.1	NC_002929.2	46.429	28	14	6	33	2426807	2426727	-80	1.7	28.9	64.29
		WP_063862442.1	NZ_CP039021.1	46.429	28	14	6	33	2426807	2426727	-80	1.6	28.9	64.29
		WP_063862442.1	NZ_CP039022.1	46.429	28	14	6	33	2426807	2426727	-80	1.6	28.9	64.29
		WP_063862442.1	NC_002929.2	46.429	28	14	6	33	2426807	2426727	-80	1.6	28.9	64.29
		WP_063862416.1	NZ_CP039021.1	46.429	28	14	6	33	2426807	2426727	-80	1.6	28.9	64.29
		WP_063862416.1	NZ_CP039022.1	46.429	28	14	6	33	2426807	2426727	-80	1.6	28.9	64.29
		WP_063862416.1	NC_002929.2	46.429	28	14	6	33	2426807	2426727	-80	1.6	28.9	64.29
		WP_063861093.1	NZ_CP039021.1	46.429	28	14	6	33	2426807	2426727	-80	1.7	28.9	64.29
		WP_063861093.1	NZ_CP039022.1	46.429	28	14	6	33	2426807	2426727	-80	1.7	28.9	64.29

		WP_063861093.1	NC_002929.2	46.429	28	14	6	33	2426807	2426727	-80	1.7	28.9	64.29
		WP_063861040.1	NZ_CP039021.1	46.429	28	14	6	33	2426807	2426727	-80	1.6	28.9	64.29
		WP_063861040.1	NZ_CP039022.1	46.429	28	14	6	33	2426807	2426727	-80	1.6	28.9	64.29
		WP_063861040.1	NC_002929.2	46.429	28	14	6	33	2426807	2426727	-80	1.6	28.9	64.29
		WP_012754353.1	NZ_CP039021.1	46.429	28	14	6	33	2426807	2426727	-80	1.6	28.9	64.29
		WP_012754353.1	NZ_CP039022.1	46.429	28	14	6	33	2426807	2426727	-80	1.6	28.9	64.29
		WP_012754353.1	NC_002929.2	46.429	28	14	6	33	2426807	2426727	-80	1.6	28.9	64.29
		WP_000713530.1	NZ_CP039021.1	46.429	28	14	6	33	2426807	2426727	-80	1.6	28.9	64.29
		WP_000713530.1	NZ_CP039022.1	46.429	28	14	6	33	2426807	2426727	-80	1.6	28.9	64.29
		WP_000713530.1	NC_002929.2	46.429	28	14	6	33	2426807	2426727	-80	1.6	28.9	64.29
	BP1933	WP_052157330.1	NZ_CP039021.1	46.429	28	15	225	252	2035865	2035948	83	5.5	26.9	57.14
		WP_052157330.1	NZ_CP039022.1	46.429	28	15	225	252	2035865	2035948	83	5.5	26.9	57.14
		WP_052157330.1	NC_002929.2	46.429	28	15	225	252	2035865	2035948	83	5.5	26.9	57.14
	BP0649	WP_063860835.1	NZ_CP039021.1	46.429	28	15	250	277	656057	655974	-83	0.88	30	64.29
		WP_063860835.1	NZ_CP039022.1	46.429	28	15	250	277	656057	655974	-83	0.88	30	64.29
		WP_063860835.1	NC_002929.2	46.429	28	15	250	277	656057	655974	-83	0.88	30	64.29
		WP_063859390.1	NZ_CP039021.1	46.429	28	15	250	277	656057	655974	-83	0.95	29.6	64.29
		WP_063859390.1	NZ_CP039022.1	46.429	28	15	250	277	656057	655974	-83	0.95	29.6	64.29
		WP_063859390.1	NC_002929.2	46.429	28	15	250	277	656057	655974	-83	0.95	29.6	64.29
		WP_063861000.1	NZ_CP039021.1	46.154	26	14	143	168	1625533	1625456	-77	2.7	28.1	61.54
		WP_063861000.1	NZ_CP039022.1	46.154	26	14	143	168	1625533	1625456	-77	2.7	28.1	61.54
		WP_063861000.1	NC_002929.2	46.154	26	14	143	168	1625533	1625456	-77	2.7	28.1	61.54
		WP_063860594.1	NZ_CP039021.1	45.946	37	14	183	214	1525858	1525965	107	2.1	28.5	59.46
		WP_063860594.1	NZ_CP039022.1	45.946	37	14	183	214	1525858	1525965	107	2.1	28.5	59.46
		WP_063860594.1	NC_002929.2	45.946	37	14	183	214	1525858	1525965	107	2.1	28.5	59.46
		WP_060614779.1	NZ_CP039021.1	45.714	35	13	183	212	1525858	1525959	101	5.5	26.9	60
		WP_060614779.1	NZ_CP039022.1	45.714	35	13	183	212	1525858	1525959	101	5.5	26.9	60
		WP_060614779.1	NC_002929.2	45.714	35	13	183	212	1525858	1525959	101	5.5	26.9	60

		WP_015060105.1	NZ_CP039021.1	45.714	35	13	183	212	1525858	1525959	101	5.5	26.9	60
		WP_015060105.1	NZ_CP039022.1	45.714	35	13	183	212	1525858	1525959	101	5.5	26.9	60
		WP_015060105.1	NC_002929.2	45.714	35	13	183	212	1525858	1525959	101	5.5	26.9	60
	BP3443	WP_064511492.1	NZ_CP039021.1	45.161	31	15	164	194	3654770	3654856	86	2.6	28.9	51.61
		WP_064511492.1	NZ_CP039022.1	45.161	31	15	164	194	3654770	3654856	86	2.6	28.9	51.61
		WP_064511492.1	NC_002929.2	45.161	31	15	164	194	3654770	3654856	86	2.6	28.9	51.61
		WP_016809943.1	NZ_CP039021.1	45.161	31	15	164	194	3654770	3654856	86	2.5	28.9	51.61
		WP_016809943.1	NZ_CP039022.1	45.161	31	15	164	194	3654770	3654856	86	2.5	28.9	51.61
		WP_016809943.1	NC_002929.2	45.161	31	15	164	194	3654770	3654856	86	2.5	28.9	51.61
		WP_033147149.1	NZ_CP039021.1	45.161	31	15	164	194	3654770	3654856	86	2.7	28.9	51.61
		WP_033147149.1	NZ_CP039022.1	45.161	31	15	164	194	3654770	3654856	86	2.7	28.9	51.61
		WP_033147149.1	NC_002929.2	45.161	31	15	164	194	3654770	3654856	86	2.7	28.9	51.61
		WP_063860496.1	NZ_CP039021.1	45.161	31	15	164	194	3654770	3654856	86	2.5	28.9	51.61
		WP_063860496.1	NZ_CP039022.1	45.161	31	15	164	194	3654770	3654856	86	2.5	28.9	51.61
		WP_063860496.1	NC_002929.2	45.161	31	15	164	194	3654770	3654856	86	2.5	28.9	51.61
		WP_032495011.1	NZ_CP039021.1	45.161	31	15	164	194	3654770	3654856	86	2.7	28.9	51.61
		WP_032495011.1	NZ_CP039022.1	45.161	31	15	164	194	3654770	3654856	86	2.7	28.9	51.61
		WP_032495011.1	NC_002929.2	45.161	31	15	164	194	3654770	3654856	86	2.7	28.9	51.61
		WP_032489727.1	NZ_CP039021.1	45.161	31	15	164	194	3654770	3654856	86	2.5	28.9	51.61
		WP_032489727.1	NZ_CP039022.1	45.161	31	15	164	194	3654770	3654856	86	2.5	28.9	51.61
		WP_032489727.1	NC_002929.2	45.161	31	15	164	194	3654770	3654856	86	2.5	28.9	51.61
	BP2380	WP_104531869.1	NZ_CP039021.1	45.161	31	17	54	84	2519817	2519909	92	0.062	34.3	67.74
		WP_104531869.1	NZ_CP039022.1	45.161	31	17	54	84	2519817	2519909	92	0.062	34.3	67.74
		WP_104531869.1	NC_002929.2	45.161	31	17	54	84	2519817	2519909	92	0.062	34.3	67.74
	BP0953	WP_070064531.1	NZ_CP039021.1	45.161	31	17	190	220	992572	992664	92	3.6	28.1	51.61
		WP_070064531.1	NZ_CP039022.1	45.161	31	17	190	220	992572	992664	92	3.6	28.1	51.61
		WP_070064531.1	NC_002929.2	45.161	31	17	190	220	992572	992664	92	3.6	28.1	51.61
		WP_063857820.1	NZ_CP039021.1	44.444	27	15	90	116	337021	336941	-80	4.4	27.7	48.15

		WP_063857820.1	NZ_CP039022.1	44.444	27	15	90	116	337021	336941	-80	4.4	27.7	48.15
		WP_063857820.1	NC_002929.2	44.444	27	15	90	116	337021	336941	-80	4.4	27.7	48.15
		WP_015266427.1	NZ_CP039021.1	44	25	14	218	242	897148	897222	74	4.1	27.3	60
		WP_015266427.1	NZ_CP039022.1	44	25	14	218	242	897148	897222	74	4.1	27.3	60
		WP_015266427.1	NC_002929.2	44	25	14	218	242	897148	897222	74	4.1	27.3	60
	preceeding *BP0380*	WP_009821475.1	NZ_CP039021.1	43.902	41	21	180	219	383473	383592	119	8.6	26.9	58.54
		WP_009821475.1	NZ_CP039022.1	43.902	41	21	180	219	383473	383592	119	8.6	26.9	58.54
		WP_009821475.1	NC_002929.2	43.902	41	21	180	219	383473	383592	119	8.6	26.9	58.54
	BP1571	WP_063842717.1	NZ_CP039021.1	43.478	23	13	160	182	1650561	1650493	-68	6.7	26.9	60.87
		WP_063842717.1	NZ_CP039022.1	43.478	23	13	160	182	1650561	1650493	-68	6.7	26.9	60.87
		WP_063842717.1	NC_002929.2	43.478	23	13	160	182	1650561	1650493	-68	6.7	26.9	60.87
		WP_063860608.1	NZ_CP039021.1	43.243	37	15	183	214	1525858	1525965	107	3.7	27.7	59.46
		WP_063860608.1	NZ_CP039022.1	43.243	37	15	183	214	1525858	1525965	107	3.7	27.7	59.46
		WP_063860608.1	NC_002929.2	43.243	37	15	183	214	1525858	1525965	107	3.7	27.7	59.46
		WP_063860614.1	NZ_CP039021.1	43.243	37	15	183	214	1525858	1525965	107	3.6	27.7	59.46
		WP_063860614.1	NZ_CP039022.1	43.243	37	15	183	214	1525858	1525965	107	3.6	27.7	59.46
		WP_063860614.1	NC_002929.2	43.243	37	15	183	214	1525858	1525965	107	3.6	27.7	59.46
		WP_063860612.1	NZ_CP039021.1	43.243	37	15	183	214	1525858	1525965	107	3.2	27.7	59.46
		WP_063860612.1	NZ_CP039022.1	43.243	37	15	183	214	1525858	1525965	107	3.2	27.7	59.46
		WP_063860612.1	NC_002929.2	43.243	37	15	183	214	1525858	1525965	107	3.2	27.7	59.46
		WP_109545053.1	NZ_CP039021.1	42.857	35	14	183	212	1525858	1525959	101	8	26.6	60
		WP_109545053.1	NZ_CP039022.1	42.857	35	14	183	212	1525858	1525959	101	8	26.6	60
		WP_109545053.1	NC_002929.2	42.857	35	14	183	212	1525858	1525959	101	8	26.6	60
		WP_063860619.1	NZ_CP039021.1	42.857	35	14	183	212	1525858	1525959	101	8.3	26.6	60
		WP_063860619.1	NZ_CP039022.1	42.857	35	14	183	212	1525858	1525959	101	8.3	26.6	60
		WP_063860619.1	NC_002929.2	42.857	35	14	183	212	1525858	1525959	101	8.3	26.6	60
		WP_063860606.1	NZ_CP039021.1	42.857	35	14	183	212	1525858	1525959	101	8.5	26.6	60
		WP_063860606.1	NZ_CP039022.1	42.857	35	14	183	212	1525858	1525959	101	8.5	26.6	60

		WP_063860606.1	NC_002929.2	42.857	35	14	183	212	1525858	1525959	101	8.5	26.6	60
	preceeding BP0707	WP_071766402.1	NZ_CP039021.1	42.857	28	16	229	256	722260	722177	-83	8.9	26.6	50
		WP_071766402.1	NZ_CP039022.1	42.857	28	16	229	256	722260	722177	-83	8.9	26.6	50
		WP_071766402.1	NC_002929.2	42.857	28	16	229	256	722260	722177	-83	8.9	26.6	50
	BP0501	WP_003118203.1	NZ_CP039021.1	42.857	28	16	163	190	515048	514965	-83	3.9	28.5	53.57
		WP_003118203.1	NZ_CP039022.1	42.857	28	16	163	190	515048	514965	-83	3.9	28.5	53.57
		WP_003118203.1	NC_002929.2	42.857	28	16	163	190	515048	514965	-83	3.9	28.5	53.57
		WP_122630845.1	NZ_CP039021.1	42.857	28	16	163	190	515048	514965	-83	3.9	28.5	53.57
		WP_122630845.1	NZ_CP039022.1	42.857	28	16	163	190	515048	514965	-83	3.9	28.5	53.57
		WP_122630845.1	NC_002929.2	42.857	28	16	163	190	515048	514965	-83	3.9	28.5	53.57
		WP_070698497.1	NZ_CP039021.1	42.857	28	16	163	190	515048	514965	-83	3.8	28.5	53.57
		WP_070698497.1	NZ_CP039022.1	42.857	28	16	163	190	515048	514965	-83	3.8	28.5	53.57
		WP_070698497.1	NC_002929.2	42.857	28	16	163	190	515048	514965	-83	3.8	28.5	53.57
		WP_111672896.1	NZ_CP039021.1	42.857	28	16	163	190	515048	514965	-83	3.9	28.5	53.57
		WP_111672896.1	NZ_CP039022.1	42.857	28	16	163	190	515048	514965	-83	3.9	28.5	53.57
		WP_111672896.1	NC_002929.2	42.857	28	16	163	190	515048	514965	-83	3.9	28.5	53.57
		WP_111672895.1	NZ_CP039021.1	42.857	28	16	163	190	515048	514965	-83	3.9	28.5	53.57
		WP_111672895.1	NZ_CP039022.1	42.857	28	16	163	190	515048	514965	-83	3.9	28.5	53.57
		WP_111672895.1	NC_002929.2	42.857	28	16	163	190	515048	514965	-83	3.9	28.5	53.57
		WP_109791203.1	NZ_CP039021.1	42.857	28	16	163	190	515048	514965	-83	2.9	28.9	53.57
		WP_109791203.1	NZ_CP039022.1	42.857	28	16	163	190	515048	514965	-83	2.9	28.9	53.57
		WP_109791203.1	NC_002929.2	42.857	28	16	163	190	515048	514965	-83	2.9	28.9	53.57
		WP_109791195.1	NZ_CP039021.1	42.857	28	16	163	190	515048	514965	-83	3.9	28.5	53.57
		WP_109791195.1	NZ_CP039022.1	42.857	28	16	163	190	515048	514965	-83	3.9	28.5	53.57
		WP_109791195.1	NC_002929.2	42.857	28	16	163	190	515048	514965	-83	3.9	28.5	53.57
		WP_109791193.1	NZ_CP039021.1	42.857	28	16	163	190	515048	514965	-83	4	28.5	53.57
		WP_109791193.1	NZ_CP039022.1	42.857	28	16	163	190	515048	514965	-83	4	28.5	53.57
		WP_109791193.1	NC_002929.2	42.857	28	16	163	190	515048	514965	-83	4	28.5	53.57

		WP_109791189.1	NZ_CP039021.1	42.857	28	16	163	190	515048	514965	-83	3.8	28.5	53.57
		WP_109791189.1	NZ_CP039022.1	42.857	28	16	163	190	515048	514965	-83	3.8	28.5	53.57
		WP_109791189.1	NC_002929.2	42.857	28	16	163	190	515048	514965	-83	3.8	28.5	53.57
		WP_109791188.1	NZ_CP039021.1	42.857	28	16	163	190	515048	514965	-83	3.9	28.5	53.57
		WP_109791188.1	NZ_CP039022.1	42.857	28	16	163	190	515048	514965	-83	3.9	28.5	53.57
		WP_109791188.1	NC_002929.2	42.857	28	16	163	190	515048	514965	-83	3.9	28.5	53.57
		WP_104009842.1	NZ_CP039021.1	42.857	28	16	163	190	515048	514965	-83	4.1	28.5	53.57
		WP_104009842.1	NZ_CP039022.1	42.857	28	16	163	190	515048	514965	-83	4.1	28.5	53.57
		WP_104009842.1	NC_002929.2	42.857	28	16	163	190	515048	514965	-83	4.1	28.5	53.57
		WP_034035338.1	NZ_CP039021.1	42.857	28	16	163	190	515048	514965	-83	4	28.5	53.57
		WP_034035338.1	NZ_CP039022.1	42.857	28	16	163	190	515048	514965	-83	4	28.5	53.57
		WP_034035338.1	NC_002929.2	42.857	28	16	163	190	515048	514965	-83	4	28.5	53.57
		WP_061199990.1	NZ_CP039021.1	42.857	28	16	163	190	515048	514965	-83	3.9	28.5	53.57
		WP_061199990.1	NZ_CP039022.1	42.857	28	16	163	190	515048	514965	-83	3.9	28.5	53.57
		WP_061199990.1	NC_002929.2	42.857	28	16	163	190	515048	514965	-83	3.9	28.5	53.57
		WP_058161279.1	NZ_CP039021.1	42.857	28	16	163	190	515048	514965	-83	4	28.5	53.57
		WP_058161279.1	NZ_CP039022.1	42.857	28	16	163	190	515048	514965	-83	4	28.5	53.57
		WP_058161279.1	NC_002929.2	42.857	28	16	163	190	515048	514965	-83	4	28.5	53.57
		WP_087587970.1	NZ_CP039021.1	42.857	28	16	163	190	515048	514965	-83	3.9	28.5	53.57
		WP_087587970.1	NZ_CP039022.1	42.857	28	16	163	190	515048	514965	-83	3.9	28.5	53.57
		WP_087587970.1	NC_002929.2	42.857	28	16	163	190	515048	514965	-83	3.9	28.5	53.57
		WP_087587966.1	NZ_CP039021.1	42.857	28	16	163	190	515048	514965	-83	3.9	28.5	53.57
		WP_087587966.1	NZ_CP039022.1	42.857	28	16	163	190	515048	514965	-83	3.9	28.5	53.57
		WP_087587966.1	NC_002929.2	42.857	28	16	163	190	515048	514965	-83	3.9	28.5	53.57
		WP_087587955.1	NZ_CP039021.1	42.857	28	16	163	190	515048	514965	-83	2.2	29.3	57.14
		WP_087587955.1	NZ_CP039022.1	42.857	28	16	163	190	515048	514965	-83	2.2	29.3	57.14
		WP_087587955.1	NC_002929.2	42.857	28	16	163	190	515048	514965	-83	2.2	29.3	57.14
		WP_087587953.1	NZ_CP039021.1	42.857	28	16	163	190	515048	514965	-83	3.8	28.5	53.57

		WP_087587953.1	NZ_CP039022.1	42.857	28	16	163	190	515048	514965	-83	3.8	28.5	53.57
		WP_087587953.1	NC_002929.2	42.857	28	16	163	190	515048	514965	-83	3.8	28.5	53.57
		WP_065159032.1	NZ_CP039021.1	42.857	28	16	163	190	515048	514965	-83	4	28.5	53.57
		WP_065159032.1	NZ_CP039022.1	42.857	28	16	163	190	515048	514965	-83	4	28.5	53.57
		WP_065159032.1	NC_002929.2	42.857	28	16	163	190	515048	514965	-83	4	28.5	53.57
		WP_023913271.1	NZ_CP039021.1	42.857	28	16	163	190	515048	514965	-83	3.9	28.5	53.57
		WP_023913271.1	NZ_CP039022.1	42.857	28	16	163	190	515048	514965	-83	3.9	28.5	53.57
		WP_023913271.1	NC_002929.2	42.857	28	16	163	190	515048	514965	-83	3.9	28.5	53.57
		WP_064511507.1	NZ_CP039021.1	42.857	28	16	163	190	515048	514965	-83	3.9	28.5	53.57
		WP_064511507.1	NZ_CP039022.1	42.857	28	16	163	190	515048	514965	-83	3.9	28.5	53.57
		WP_064511507.1	NC_002929.2	42.857	28	16	163	190	515048	514965	-83	3.9	28.5	53.57
		WP_064511505.1	NZ_CP039021.1	42.857	28	16	163	190	515048	514965	-83	3.9	28.5	53.57
		WP_064511505.1	NZ_CP039022.1	42.857	28	16	163	190	515048	514965	-83	3.9	28.5	53.57
		WP_064511505.1	NC_002929.2	42.857	28	16	163	190	515048	514965	-83	3.9	28.5	53.57
		WP_064511500.1	NZ_CP039021.1	42.857	28	16	163	190	515048	514965	-83	3.9	28.5	53.57
		WP_064511500.1	NZ_CP039022.1	42.857	28	16	163	190	515048	514965	-83	3.9	28.5	53.57
		WP_064511500.1	NC_002929.2	42.857	28	16	163	190	515048	514965	-83	3.9	28.5	53.57
		WP_064511499.1	NZ_CP039021.1	42.857	28	16	163	190	515048	514965	-83	3.9	28.5	53.57
		WP_064511499.1	NZ_CP039022.1	42.857	28	16	163	190	515048	514965	-83	3.9	28.5	53.57
		WP_064511499.1	NC_002929.2	42.857	28	16	163	190	515048	514965	-83	3.9	28.5	53.57
		WP_064484006.1	NZ_CP039021.1	42.857	28	16	163	190	515048	514965	-83	3.9	28.5	53.57
		WP_064484006.1	NZ_CP039022.1	42.857	28	16	163	190	515048	514965	-83	3.9	28.5	53.57
		WP_064484006.1	NC_002929.2	42.857	28	16	163	190	515048	514965	-83	3.9	28.5	53.57
		WP_063864577.1	NZ_CP039021.1	42.857	28	16	163	190	515048	514965	-83	3.8	28.5	53.57
		WP_063864577.1	NZ_CP039022.1	42.857	28	16	163	190	515048	514965	-83	3.8	28.5	53.57
		WP_063864577.1	NC_002929.2	42.857	28	16	163	190	515048	514965	-83	3.8	28.5	53.57
		WP_063864574.1	NZ_CP039021.1	42.857	28	16	163	190	515048	514965	-83	3.9	28.5	53.57
		WP_063864574.1	NZ_CP039022.1	42.857	28	16	163	190	515048	514965	-83	3.9	28.5	53.57

		WP_063864574.1	NC_002929.2	42.857	28	16	163	190	515048	514965	-83	3.9	28.5	53.57
		WP_063864569.1	NZ_CP039021.1	42.857	28	16	163	190	515048	514965	-83	3.9	28.5	53.57
		WP_063864569.1	NZ_CP039022.1	42.857	28	16	163	190	515048	514965	-83	3.9	28.5	53.57
		WP_063864569.1	NC_002929.2	42.857	28	16	163	190	515048	514965	-83	3.9	28.5	53.57
		WP_016263090.1	NZ_CP039021.1	42.857	28	16	163	190	515048	514965	-83	3.9	28.5	53.57
		WP_016263090.1	NZ_CP039022.1	42.857	28	16	163	190	515048	514965	-83	3.9	28.5	53.57
		WP_016263090.1	NC_002929.2	42.857	28	16	163	190	515048	514965	-83	3.9	28.5	53.57
	BP1548	WP_063864194.1	NZ_CP039021.1	42.308	26	15	143	168	1625533	1625456	-77	1.6	28.9	65.38
		WP_063864194.1	NZ_CP039022.1	42.308	26	15	143	168	1625533	1625456	-77	1.6	28.9	65.38
		WP_063864194.1	NC_002929.2	42.308	26	15	143	168	1625533	1625456	-77	1.6	28.9	65.38
		WP_054428802.1	NZ_CP039021.1	42.308	26	15	143	168	1625533	1625456	-77	1.8	28.9	65.38
		WP_054428802.1	NZ_CP039022.1	42.308	26	15	143	168	1625533	1625456	-77	1.8	28.9	65.38
		WP_054428802.1	NC_002929.2	42.308	26	15	143	168	1625533	1625456	-77	1.8	28.9	65.38
		WP_054505840.1	NZ_CP039021.1	42.308	26	15	143	168	1625533	1625456	-77	4.5	27.3	61.54
		WP_054505840.1	NZ_CP039022.1	42.308	26	15	143	168	1625533	1625456	-77	4.5	27.3	61.54
		WP_054505840.1	NC_002929.2	42.308	26	15	143	168	1625533	1625456	-77	4.5	27.3	61.54
		WP_054448427.1	NZ_CP039021.1	42.308	26	15	143	168	1625533	1625456	-77	4.7	27.3	61.54
		WP_054448427.1	NZ_CP039022.1	42.308	26	15	143	168	1625533	1625456	-77	4.7	27.3	61.54
		WP_054448427.1	NC_002929.2	42.308	26	15	143	168	1625533	1625456	-77	4.7	27.3	61.54
		WP_006384924.1	NZ_CP039021.1	42.308	26	15	143	168	1625533	1625456	-77	4.6	27.3	61.54
		WP_006384924.1	NZ_CP039022.1	42.308	26	15	143	168	1625533	1625456	-77	4.6	27.3	61.54
		WP_006384924.1	NC_002929.2	42.308	26	15	143	168	1625533	1625456	-77	4.6	27.3	61.54
		WP_006391197.1	NZ_CP039021.1	42.308	26	15	143	168	1625533	1625456	-77	1.5	28.9	65.38
		WP_006391197.1	NZ_CP039022.1	42.308	26	15	143	168	1625533	1625456	-77	1.5	28.9	65.38
		WP_006391197.1	NC_002929.2	42.308	26	15	143	168	1625533	1625456	-77	1.5	28.9	65.38
		WP_063864089.1	NZ_CP039021.1	42.308	26	15	143	168	1625533	1625456	-77	4.6	27.3	61.54
		WP_063864089.1	NZ_CP039022.1	42.308	26	15	143	168	1625533	1625456	-77	4.6	27.3	61.54
		WP_063864089.1	NC_002929.2	42.308	26	15	143	168	1625533	1625456	-77	4.6	27.3	61.54

			WP_063864088.1	NZ_CP039021.1	42.308	26	15	143	168	1625533	1625456	-77	1.6	28.9	65.38
			WP_063864088.1	NZ_CP039022.1	42.308	26	15	143	168	1625533	1625456	-77	1.6	28.9	65.38
			WP_063864088.1	NC_002929.2	42.308	26	15	143	168	1625533	1625456	-77	1.6	28.9	65.38
			WP_063864087.1	NZ_CP039021.1	42.308	26	15	143	168	1625533	1625456	-77	1.5	28.9	65.38
			WP_063864087.1	NZ_CP039022.1	42.308	26	15	143	168	1625533	1625456	-77	1.5	28.9	65.38
			WP_063864087.1	NC_002929.2	42.308	26	15	143	168	1625533	1625456	-77	1.5	28.9	65.38
			WP_063862734.1	NZ_CP039021.1	42.308	26	15	143	168	1625533	1625456	-77	1.5	28.9	65.38
			WP_063862734.1	NZ_CP039022.1	42.308	26	15	143	168	1625533	1625456	-77	1.5	28.9	65.38
			WP_063862734.1	NC_002929.2	42.308	26	15	143	168	1625533	1625456	-77	1.5	28.9	65.38
			WP_063860980.1	NZ_CP039021.1	42.308	26	15	143	168	1625533	1625456	-77	4.7	27.3	61.54
			WP_063860980.1	NZ_CP039022.1	42.308	26	15	143	168	1625533	1625456	-77	4.7	27.3	61.54
			WP_063860980.1	NC_002929.2	42.308	26	15	143	168	1625533	1625456	-77	4.7	27.3	61.54
			WP_049058560.1	NZ_CP039021.1	42.308	26	15	143	168	1625533	1625456	-77	4.7	27.3	61.54
			WP_049058560.1	NZ_CP039022.1	42.308	26	15	143	168	1625533	1625456	-77	4.7	27.3	61.54
			WP_049058560.1	NC_002929.2	42.308	26	15	143	168	1625533	1625456	-77	4.7	27.3	61.54
			WP_024069684.1	NZ_CP039021.1	42.308	26	15	143	168	1625533	1625456	-77	4.6	27.3	61.54
			WP_024069684.1	NZ_CP039022.1	42.308	26	15	143	168	1625533	1625456	-77	4.6	27.3	61.54
			WP_024069684.1	NC_002929.2	42.308	26	15	143	168	1625533	1625456	-77	4.6	27.3	61.54
		BP1523	WP_070210118.1	NZ_CP039021.1	42.105	19	11	73	91	1597715	1597771	56	8.1	26.6	73.68
			WP_070210118.1	NZ_CP039022.1	42.105	19	11	73	91	1597715	1597771	56	8.1	26.6	73.68
			WP_070210118.1	NC_002929.2	42.105	19	11	73	91	1597715	1597771	56	8.1	26.6	73.68
			WP_109545116.1	NZ_CP039021.1	42.105	19	11	73	91	1597715	1597771	56	8.8	26.6	73.68
			WP_109545116.1	NZ_CP039022.1	42.105	19	11	73	91	1597715	1597771	56	8.8	26.6	73.68
			WP_109545116.1	NC_002929.2	42.105	19	11	73	91	1597715	1597771	56	8.8	26.6	73.68
			WP_109545115.1	NZ_CP039021.1	42.105	19	11	73	91	1597715	1597771	56	8.6	26.6	73.68
			WP_109545115.1	NZ_CP039022.1	42.105	19	11	73	91	1597715	1597771	56	8.6	26.6	73.68
			WP_109545115.1	NC_002929.2	42.105	19	11	73	91	1597715	1597771	56	8.6	26.6	73.68
			WP_109545114.1	NZ_CP039021.1	42.105	19	11	73	91	1597715	1597771	56	8.2	26.6	73.68

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		WP_109545114.1	NZ_CP039022.1	42.105	19	11	73	91	1597715	1597771	56	8.2	26.6	73.68
		WP_109545114.1	NC_002929.2	42.105	19	11	73	91	1597715	1597771	56	8.2	26.6	73.68
		WP_109545113.1	NZ_CP039021.1	42.105	19	11	73	91	1597715	1597771	56	8.3	26.6	73.68
		WP_109545113.1	NZ_CP039022.1	42.105	19	11	73	91	1597715	1597771	56	8.3	26.6	73.68
		WP_109545113.1	NC_002929.2	42.105	19	11	73	91	1597715	1597771	56	8.3	26.6	73.68
		WP_109545112.1	NZ_CP039021.1	42.105	19	11	73	91	1597715	1597771	56	8.4	26.6	73.68
		WP_109545112.1	NZ_CP039022.1	42.105	19	11	73	91	1597715	1597771	56	8.4	26.6	73.68
		WP_109545112.1	NC_002929.2	42.105	19	11	73	91	1597715	1597771	56	8.4	26.6	73.68
		WP_109545111.1	NZ_CP039021.1	42.105	19	11	73	91	1597715	1597771	56	8.3	26.6	73.68
		WP_109545111.1	NZ_CP039022.1	42.105	19	11	73	91	1597715	1597771	56	8.3	26.6	73.68
		WP_109545111.1	NC_002929.2	42.105	19	11	73	91	1597715	1597771	56	8.3	26.6	73.68
		WP_109545110.1	NZ_CP039021.1	42.105	19	11	73	91	1597715	1597771	56	8.8	26.6	73.68
		WP_109545110.1	NZ_CP039022.1	42.105	19	11	73	91	1597715	1597771	56	8.8	26.6	73.68
		WP_109545110.1	NC_002929.2	42.105	19	11	73	91	1597715	1597771	56	8.8	26.6	73.68
		WP_109545076.1	NZ_CP039021.1	42.105	19	11	73	91	1597715	1597771	56	8.2	26.6	73.68
		WP_109545076.1	NZ_CP039022.1	42.105	19	11	73	91	1597715	1597771	56	8.2	26.6	73.68
		WP_109545076.1	NC_002929.2	42.105	19	11	73	91	1597715	1597771	56	8.2	26.6	73.68
		WP_109545075.1	NZ_CP039021.1	42.105	19	11	73	91	1597715	1597771	56	8.3	26.6	73.68
		WP_109545075.1	NZ_CP039022.1	42.105	19	11	73	91	1597715	1597771	56	8.3	26.6	73.68
		WP_109545075.1	NC_002929.2	42.105	19	11	73	91	1597715	1597771	56	8.3	26.6	73.68
		WP_088303859.1	NZ_CP039021.1	42.105	19	11	73	91	1597715	1597771	56	8.2	26.6	73.68
		WP_088303859.1	NZ_CP039022.1	42.105	19	11	73	91	1597715	1597771	56	8.2	26.6	73.68
		WP_088303859.1	NC_002929.2	42.105	19	11	73	91	1597715	1597771	56	8.2	26.6	73.68
		WP_070233680.1	NZ_CP039021.1	42.105	19	11	73	91	1597715	1597771	56	9	26.6	73.68
		WP_070233680.1	NZ_CP039022.1	42.105	19	11	73	91	1597715	1597771	56	9	26.6	73.68
		WP_070233680.1	NC_002929.2	42.105	19	11	73	91	1597715	1597771	56	9	26.6	73.68
		WP_053872444.1	NZ_CP039021.1	42.105	19	11	73	91	1597715	1597771	56	9.2	26.2	73.68
		WP_053872444.1	NZ_CP039022.1	42.105	19	11	73	91	1597715	1597771	56	9.2	26.2	73.68

		WP_053872444.1	NC_002929.2	42.105	19	11	73	91	1597715	1597771	56	9.2	26.2	73.68
		WP_052858477.1	NZ_CP039021.1	42.105	19	11	73	91	1597715	1597771	56	9.4	26.2	73.68
		WP_052858477.1	NZ_CP039022.1	42.105	19	11	73	91	1597715	1597771	56	9.4	26.2	73.68
		WP_052858477.1	NC_002929.2	42.105	19	11	73	91	1597715	1597771	56	9.4	26.2	73.68
		WP_044779552.1	NZ_CP039021.1	42.105	19	11	73	91	1597715	1597771	56	8.7	26.6	73.68
		WP_044779552.1	NZ_CP039022.1	42.105	19	11	73	91	1597715	1597771	56	8.7	26.6	73.68
		WP_044779552.1	NC_002929.2	42.105	19	11	73	91	1597715	1597771	56	8.7	26.6	73.68
		WP_002934081.1	NZ_CP039021.1	42.105	19	11	73	91	1597715	1597771	56	8.2	26.6	73.68
		WP_002934081.1	NZ_CP039022.1	42.105	19	11	73	91	1597715	1597771	56	8.2	26.6	73.68
		WP_002934081.1	NC_002929.2	42.105	19	11	73	91	1597715	1597771	56	8.2	26.6	73.68
		WP_063864084.1	NZ_CP039021.1	42.105	19	11	73	91	1597715	1597771	56	9.3	26.2	73.68
		WP_063864084.1	NZ_CP039022.1	42.105	19	11	73	91	1597715	1597771	56	9.3	26.2	73.68
		WP_063864084.1	NC_002929.2	42.105	19	11	73	91	1597715	1597771	56	9.3	26.2	73.68
		WP_032489067.1	NZ_CP039021.1	41.935	31	16	164	194	3654770	3654856	86	6.4	27.7	51.61
		WP_032489067.1	NZ_CP039022.1	41.935	31	16	164	194	3654770	3654856	86	6.4	27.7	51.61
		WP_032489067.1	NC_002929.2	41.935	31	16	164	194	3654770	3654856	86	6.4	27.7	51.61
	BP3854	WP_015266427.1	NZ_CP039021.1	41.667	24	14	131	154	4064891	4064962	71	7.8	26.6	58.33
		WP_015266427.1	NZ_CP039022.1	41.667	24	14	131	154	4064891	4064962	71	7.8	26.6	58.33
		WP_015266427.1	NC_002929.2	41.667	24	14	131	154	4064891	4064962	71	7.8	26.6	58.33
	dgkA	WP_011497575.1	NZ_CP039021.1	41.667	36	19	141	176	3701585	3701686	101	3.4	27.7	47.22
		WP_011497575.1	NZ_CP039022.1	41.667	36	19	141	176	3701585	3701686	101	3.4	27.7	47.22
		WP_011497575.1	NC_002929.2	41.667	36	19	141	176	3701585	3701686	101	3.4	27.7	47.22
		WP_088245214.1	NZ_CP039021.1	41.667	36	17	183	214	1525858	1525965	107	0.89	29.6	55.56
		WP_088245214.1	NZ_CP039022.1	41.667	36	17	183	214	1525858	1525965	107	0.89	29.6	55.56
		WP_088245214.1	NC_002929.2	41.667	36	17	183	214	1525858	1525965	107	0.89	29.6	55.56
		WP_063860585.1	NZ_CP039021.1	41.667	36	17	183	214	1525858	1525965	107	0.91	29.6	55.56
		WP_063860585.1	NZ_CP039022.1	41.667	36	17	183	214	1525858	1525965	107	0.91	29.6	55.56
		WP_063860585.1	NC_002929.2	41.667	36	17	183	214	1525858	1525965	107	0.91	29.6	55.56

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		WP_071593226.1	NZ_CP039021.1	41.667	36	17	183	214	1525858	1525965	107	0.93	29.6	55.56
		WP_071593226.1	NZ_CP039022.1	41.667	36	17	183	214	1525858	1525965	107	0.93	29.6	55.56
		WP_071593226.1	NC_002929.2	41.667	36	17	183	214	1525858	1525965	107	0.93	29.6	55.56
		WP_069280710.1	NZ_CP039021.1	41.667	36	17	183	214	1525858	1525965	107	1.7	28.5	55.56
		WP_069280710.1	NZ_CP039022.1	41.667	36	17	183	214	1525858	1525965	107	1.7	28.5	55.56
		WP_069280710.1	NC_002929.2	41.667	36	17	183	214	1525858	1525965	107	1.7	28.5	55.56
		WP_063860617.1	NZ_CP039021.1	41.667	36	17	183	214	1525858	1525965	107	1.5	28.9	55.56
		WP_063860617.1	NZ_CP039022.1	41.667	36	17	183	214	1525858	1525965	107	1.5	28.9	55.56
		WP_063860617.1	NC_002929.2	41.667	36	17	183	214	1525858	1525965	107	1.5	28.9	55.56
	region preceeding *talB*	WP_109791208.1	NZ_CP039021.1	41.176	34	16	183	212	1525858	1525959	101	6.9	26.6	55.88
		WP_109791208.1	NZ_CP039022.1	41.176	34	16	183	212	1525858	1525959	101	6.9	26.6	55.88
		WP_109791208.1	NC_002929.2	41.176	34	16	183	212	1525858	1525959	101	6.9	26.6	55.88
		WP_104009852.1	NZ_CP039021.1	41.176	34	16	183	212	1525858	1525959	101	6.7	26.6	55.88
		WP_104009852.1	NZ_CP039022.1	41.176	34	16	183	212	1525858	1525959	101	6.7	26.6	55.88
		WP_104009852.1	NC_002929.2	41.176	34	16	183	212	1525858	1525959	101	6.7	26.6	55.88
		WP_012695457.1	NZ_CP039021.1	41.176	34	16	183	212	1525858	1525959	101	6.8	26.6	55.88
		WP_012695457.1	NZ_CP039022.1	41.176	34	16	183	212	1525858	1525959	101	6.8	26.6	55.88
		WP_012695457.1	NC_002929.2	41.176	34	16	183	212	1525858	1525959	101	6.8	26.6	55.88
		WP_063848609.1	NZ_CP039021.1	41.176	34	16	183	212	1525858	1525959	101	6.7	26.6	55.88
		WP_063848609.1	NZ_CP039022.1	41.176	34	16	183	212	1525858	1525959	101	6.7	26.6	55.88
		WP_063848609.1	NC_002929.2	41.176	34	16	183	212	1525858	1525959	101	6.7	26.6	55.88
		WP_042862936.1	NZ_CP039021.1	41.176	34	16	183	212	1525858	1525959	101	6.6	26.6	55.88
		WP_042862936.1	NZ_CP039022.1	41.176	34	16	183	212	1525858	1525959	101	6.6	26.6	55.88
		WP_042862936.1	NC_002929.2	41.176	34	16	183	212	1525858	1525959	101	6.6	26.6	55.88
		WP_032491874.1	NZ_CP039021.1	41.176	34	16	183	212	1525858	1525959	101	2.4	28.1	58.82
		WP_032491874.1	NZ_CP039022.1	41.176	34	16	183	212	1525858	1525959	101	2.4	28.1	58.82
		WP_032491874.1	NC_002929.2	41.176	34	16	183	212	1525858	1525959	101	2.4	28.1	58.82

		WP_063860610.1	NZ_CP039021.1	41.176	34	16	183	212	1525858	1525959	101	6.8	26.6	55.88
		WP_063860610.1	NZ_CP039022.1	41.176	34	16	183	212	1525858	1525959	101	6.8	26.6	55.88
		WP_063860610.1	NC_002929.2	41.176	34	16	183	212	1525858	1525959	101	6.8	26.6	55.88
		WP_063860593.1	NZ_CP039021.1	41.176	34	16	183	212	1525858	1525959	101	6.1	26.9	55.88
		WP_063860593.1	NZ_CP039022.1	41.176	34	16	183	212	1525858	1525959	101	6.1	26.9	55.88
		WP_063860593.1	NC_002929.2	41.176	34	16	183	212	1525858	1525959	101	6.1	26.9	55.88
		WP_063860591.1	NZ_CP039021.1	41.176	34	16	183	212	1525858	1525959	101	6.1	26.9	55.88
		WP_063860591.1	NZ_CP039022.1	41.176	34	16	183	212	1525858	1525959	101	6.1	26.9	55.88
		WP_063860591.1	NC_002929.2	41.176	34	16	183	212	1525858	1525959	101	6.1	26.9	55.88
		WP_063860583.1	NZ_CP039021.1	41.176	34	16	183	212	1525858	1525959	101	7.1	26.6	55.88
		WP_063860583.1	NZ_CP039022.1	41.176	34	16	183	212	1525858	1525959	101	7.1	26.6	55.88
		WP_063860583.1	NC_002929.2	41.176	34	16	183	212	1525858	1525959	101	7.1	26.6	55.88
		WP_063860582.1	NZ_CP039021.1	41.176	34	16	183	212	1525858	1525959	101	6.9	26.6	55.88
		WP_063860582.1	NZ_CP039022.1	41.176	34	16	183	212	1525858	1525959	101	6.9	26.6	55.88
		WP_063860582.1	NC_002929.2	41.176	34	16	183	212	1525858	1525959	101	6.9	26.6	55.88
		WP_063860579.1	NZ_CP039021.1	41.176	34	16	183	212	1525858	1525959	101	2.4	28.1	58.82
		WP_063860579.1	NZ_CP039022.1	41.176	34	16	183	212	1525858	1525959	101	2.4	28.1	58.82
		WP_063860579.1	NC_002929.2	41.176	34	16	183	212	1525858	1525959	101	2.4	28.1	58.82
		WP_063860578.1	NZ_CP039021.1	41.176	34	16	183	212	1525858	1525959	101	2.4	28.1	58.82
		WP_063860578.1	NZ_CP039022.1	41.176	34	16	183	212	1525858	1525959	101	2.4	28.1	58.82
		WP_063860578.1	NC_002929.2	41.176	34	16	183	212	1525858	1525959	101	2.4	28.1	58.82
	bscC	WP_063978070.1	NZ_CP039021.1	41.026	39	23	182	220	2365011	2364895	-116	0.32	30.8	51.28
		WP_063978070.1	NZ_CP039022.1	41.026	39	23	182	220	2365011	2364895	-116	0.32	30.8	51.28
		WP_063978070.1	NC_002929.2	41.026	39	23	182	220	2365011	2364895	-116	0.32	30.8	51.28
		WP_104531869.1	NZ_CP039021.1	41.026	39	22	171	209	802703	802590	-113	2.2	29.3	56.41
		WP_104531869.1	NZ_CP039022.1	41.026	39	22	171	209	802703	802590	-113	2.2	29.3	56.41
		WP_104531869.1	NC_002929.2	41.026	39	22	171	209	802703	802590	-113	2.2	29.3	56.41
		WP_104531868.1	NZ_CP039021.1	41.026	39	22	171	209	802703	802590	-113	4	28.5	53.85

		WP_104531868.1	NZ_CP039022.1	41.026	39	22	171	209	802703	802590	-113	4	28.5	53.85
		WP_104531868.1	NC_002929.2	41.026	39	22	171	209	802703	802590	-113	4	28.5	53.85
		WP_104531867.1	NZ_CP039021.1	41.026	39	22	171	209	802703	802590	-113	2	29.3	56.41
		WP_104531867.1	NZ_CP039022.1	41.026	39	22	171	209	802703	802590	-113	2	29.3	56.41
		WP_104531867.1	NC_002929.2	41.026	39	22	171	209	802703	802590	-113	2	29.3	56.41
		WP_104531866.1	NZ_CP039021.1	41.026	39	22	171	209	802703	802590	-113	1.9	29.3	56.41
		WP_104531866.1	NZ_CP039022.1	41.026	39	22	171	209	802703	802590	-113	1.9	29.3	56.41
		WP_104531866.1	NC_002929.2	41.026	39	22	171	209	802703	802590	-113	1.9	29.3	56.41
		WP_081666691.1	NZ_CP039021.1	41.026	39	22	169	207	802703	802590	-113	4	28.5	53.85
		WP_081666691.1	NZ_CP039022.1	41.026	39	22	169	207	802703	802590	-113	4	28.5	53.85
		WP_081666691.1	NC_002929.2	41.026	39	22	169	207	802703	802590	-113	4	28.5	53.85
	dgkA	WP_109791208.1	NZ_CP039021.1	40.909	22	13	156	177	3701621	3701686	65	9.3	26.2	59.09
		WP_109791208.1	NZ_CP039022.1	40.909	22	13	156	177	3701621	3701686	65	9.3	26.2	59.09
		WP_109791208.1	NC_002929.2	40.909	22	13	156	177	3701621	3701686	65	9.3	26.2	59.09
		WP_109791207.1	NZ_CP039021.1	40.909	22	13	156	177	3701621	3701686	65	8.8	26.2	59.09
		WP_109791207.1	NZ_CP039022.1	40.909	22	13	156	177	3701621	3701686	65	8.8	26.2	59.09
		WP_109791207.1	NC_002929.2	40.909	22	13	156	177	3701621	3701686	65	8.8	26.2	59.09
		WP_104009852.1	NZ_CP039021.1	40.909	22	13	156	177	3701621	3701686	65	9.7	26.2	59.09
		WP_104009852.1	NZ_CP039022.1	40.909	22	13	156	177	3701621	3701686	65	9.7	26.2	59.09
		WP_104009852.1	NC_002929.2	40.909	22	13	156	177	3701621	3701686	65	9.7	26.2	59.09
		WP_012695457.1	NZ_CP039021.1	40.909	22	13	156	177	3701621	3701686	65	7.5	26.6	59.09
		WP_012695457.1	NZ_CP039022.1	40.909	22	13	156	177	3701621	3701686	65	7.5	26.6	59.09
		WP_012695457.1	NC_002929.2	40.909	22	13	156	177	3701621	3701686	65	7.5	26.6	59.09
		WP_063848609.1	NZ_CP039021.1	40.909	22	13	156	177	3701621	3701686	65	9.5	26.2	59.09
		WP_063848609.1	NZ_CP039022.1	40.909	22	13	156	177	3701621	3701686	65	9.5	26.2	59.09
		WP_063848609.1	NC_002929.2	40.909	22	13	156	177	3701621	3701686	65	9.5	26.2	59.09
		WP_039819893.1	NZ_CP039021.1	40.909	22	13	156	177	3701621	3701686	65	9.7	26.2	59.09
		WP_039819893.1	NZ_CP039022.1	40.909	22	13	156	177	3701621	3701686	65	9.7	26.2	59.09

		WP_039819893.1	NC_002929.2	40.909	22	13	156	177	3701621	3701686	65	9.7	26.2	59.09
		WP_032491874.1	NZ_CP039021.1	40.909	22	13	156	177	3701621	3701686	65	7.3	26.6	59.09
		WP_032491874.1	NZ_CP039022.1	40.909	22	13	156	177	3701621	3701686	65	7.3	26.6	59.09
		WP_032491874.1	NC_002929.2	40.909	22	13	156	177	3701621	3701686	65	7.3	26.6	59.09
		WP_031943232.1	NZ_CP039021.1	40.909	22	13	156	177	3701621	3701686	65	7.7	26.6	59.09
		WP_031943232.1	NZ_CP039022.1	40.909	22	13	156	177	3701621	3701686	65	7.7	26.6	59.09
		WP_031943232.1	NC_002929.2	40.909	22	13	156	177	3701621	3701686	65	7.7	26.6	59.09
		WP_063860618.1	NZ_CP039021.1	40.909	22	13	156	177	3701621	3701686	65	8.1	26.6	59.09
		WP_063860618.1	NZ_CP039022.1	40.909	22	13	156	177	3701621	3701686	65	8.1	26.6	59.09
		WP_063860618.1	NC_002929.2	40.909	22	13	156	177	3701621	3701686	65	8.1	26.6	59.09
		WP_063860617.1	NZ_CP039021.1	40.909	22	13	156	177	3701621	3701686	65	9.6	26.2	59.09
		WP_063860617.1	NZ_CP039022.1	40.909	22	13	156	177	3701621	3701686	65	9.6	26.2	59.09
		WP_063860617.1	NC_002929.2	40.909	22	13	156	177	3701621	3701686	65	9.6	26.2	59.09
		WP_063860615.1	NZ_CP039021.1	40.909	22	13	156	177	3701621	3701686	65	9.5	26.2	59.09
		WP_063860615.1	NZ_CP039022.1	40.909	22	13	156	177	3701621	3701686	65	9.5	26.2	59.09
		WP_063860615.1	NC_002929.2	40.909	22	13	156	177	3701621	3701686	65	9.5	26.2	59.09
		WP_063860610.1	NZ_CP039021.1	40.909	22	13	156	177	3701621	3701686	65	9.8	26.2	59.09
		WP_063860610.1	NZ_CP039022.1	40.909	22	13	156	177	3701621	3701686	65	9.8	26.2	59.09
		WP_063860610.1	NC_002929.2	40.909	22	13	156	177	3701621	3701686	65	9.8	26.2	59.09
		WP_063860609.1	NZ_CP039021.1	40.909	22	13	156	177	3701621	3701686	65	9.5	26.2	59.09
		WP_063860609.1	NZ_CP039022.1	40.909	22	13	156	177	3701621	3701686	65	9.5	26.2	59.09
		WP_063860609.1	NC_002929.2	40.909	22	13	156	177	3701621	3701686	65	9.5	26.2	59.09
		WP_063860592.1	NZ_CP039021.1	40.909	22	13	156	177	3701621	3701686	65	7.8	26.6	59.09
		WP_063860592.1	NZ_CP039022.1	40.909	22	13	156	177	3701621	3701686	65	7.8	26.6	59.09
		WP_063860592.1	NC_002929.2	40.909	22	13	156	177	3701621	3701686	65	7.8	26.6	59.09
		WP_063860590.1	NZ_CP039021.1	40.909	22	13	156	177	3701621	3701686	65	9.8	26.2	59.09
		WP_063860590.1	NZ_CP039022.1	40.909	22	13	156	177	3701621	3701686	65	9.8	26.2	59.09
		WP_063860590.1	NC_002929.2	40.909	22	13	156	177	3701621	3701686	65	9.8	26.2	59.09

		WP_063860583.1	NZ_CP039021.1	40.909	22	13	156	177	3701621	3701686	65	7.5	26.6	59.09
		WP_063860583.1	NZ_CP039022.1	40.909	22	13	156	177	3701621	3701686	65	7.5	26.6	59.09
		WP_063860583.1	NC_002929.2	40.909	22	13	156	177	3701621	3701686	65	7.5	26.6	59.09
		WP_063860582.1	NZ_CP039021.1	40.909	22	13	156	177	3701621	3701686	65	7.5	26.6	59.09
		WP_063860582.1	NZ_CP039022.1	40.909	22	13	156	177	3701621	3701686	65	7.5	26.6	59.09
		WP_063860582.1	NC_002929.2	40.909	22	13	156	177	3701621	3701686	65	7.5	26.6	59.09
		WP_063860581.1	NZ_CP039021.1	40.909	22	13	156	177	3701621	3701686	65	8.1	26.6	59.09
		WP_063860581.1	NZ_CP039022.1	40.909	22	13	156	177	3701621	3701686	65	8.1	26.6	59.09
		WP_063860581.1	NC_002929.2	40.909	22	13	156	177	3701621	3701686	65	8.1	26.6	59.09
		WP_063860579.1	NZ_CP039021.1	40.909	22	13	156	177	3701621	3701686	65	7.6	26.6	59.09
		WP_063860579.1	NZ_CP039022.1	40.909	22	13	156	177	3701621	3701686	65	7.6	26.6	59.09
		WP_063860579.1	NC_002929.2	40.909	22	13	156	177	3701621	3701686	65	7.6	26.6	59.09
		WP_063860578.1	NZ_CP039021.1	40.909	22	13	156	177	3701621	3701686	65	7.7	26.6	59.09
		WP_063860578.1	NZ_CP039022.1	40.909	22	13	156	177	3701621	3701686	65	7.7	26.6	59.09
		WP_063860578.1	NC_002929.2	40.909	22	13	156	177	3701621	3701686	65	7.7	26.6	59.09
		WP_063860576.1	NZ_CP039021.1	40.909	22	13	156	177	3701621	3701686	65	8.9	26.2	59.09
		WP_063860576.1	NZ_CP039022.1	40.909	22	13	156	177	3701621	3701686	65	8.9	26.2	59.09
		WP_063860576.1	NC_002929.2	40.909	22	13	156	177	3701621	3701686	65	8.9	26.2	59.09
	BP0342	WP_050737109.1	NZ_CP039021.1	40.625	32	18	189	220	344471	344563	92	7.1	26.9	56.25
		WP_050737109.1	NZ_CP039022.1	40.625	32	18	189	220	344471	344563	92	7.1	26.9	56.25
		WP_050737109.1	NC_002929.2	40.625	32	18	189	220	344471	344563	92	7.1	26.9	56.25
	BP3388	WP_109545059.1	NZ_CP039021.1	40	25	15	48	72	3599085	3599159	74	8	26.9	56
		WP_109545059.1	NZ_CP039022.1	40	25	15	48	72	3599085	3599159	74	8	26.9	56
		WP_109545059.1	NC_002929.2	40	25	15	48	72	3599085	3599159	74	8	26.9	56
		WP_063864872.1	NZ_CP039021.1	40	25	15	48	72	3599085	3599159	74	9.7	26.6	60
		WP_063864872.1	NZ_CP039022.1	40	25	15	48	72	3599085	3599159	74	9.7	26.6	60
		WP_063864872.1	NC_002929.2	40	25	15	48	72	3599085	3599159	74	9.7	26.6	60
	gloB	WP_041258349.1	NZ_CP039021.1	40	35	20	82	116	3424722	3424823	101	1.4	28.9	65.71

		WP_041258349.1	NZ_CP039022.1	40	35	20	82	116	3424722	3424823	101	1.4	28.9	65.71
		WP_041258349.1	NC_002929.2	40	35	20	82	116	3424722	3424823	101	1.4	28.9	65.71
	brfB	WP_011013281.1	NZ_CP039021.1	40	40	19	203	242	2130451	2130555	104	2.8	28.1	52.5
		WP_011013281.1	NZ_CP039022.1	40	40	19	203	242	2130451	2130555	104	2.8	28.1	52.5
		WP_011013281.1	NC_002929.2	40	40	19	203	242	2130451	2130555	104	2.8	28.1	52.5
		WP_038813458.1	NZ_CP039021.1	40	25	15	25	49	1814176	1814102	-74	1.9	28.5	52
		WP_038813458.1	NZ_CP039022.1	40	25	15	25	49	1814176	1814102	-74	1.9	28.5	52
		WP_038813458.1	NC_002929.2	40	25	15	25	49	1814176	1814102	-74	1.9	28.5	52
		WP_063860577.1	NZ_CP039021.1	40	35	17	183	213	1525858	1525962	104	6.5	26.9	54.29
		WP_063860577.1	NZ_CP039022.1	40	35	17	183	213	1525858	1525962	104	6.5	26.9	54.29
		WP_063860577.1	NC_002929.2	40	35	17	183	213	1525858	1525962	104	6.5	26.9	54.29
		WP_063860575.1	NZ_CP039021.1	40	35	17	183	213	1525858	1525962	104	7.6	26.6	54.29
		WP_063860575.1	NZ_CP039022.1	40	35	17	183	213	1525858	1525962	104	7.6	26.6	54.29
		WP_063860575.1	NC_002929.2	40	35	17	183	213	1525858	1525962	104	7.6	26.6	54.29
		WP_063842694.1	NZ_CP039021.1	40	35	21	158	192	520970	520866	-104	1.4	29.3	48.57
		WP_063842694.1	NZ_CP039022.1	40	35	21	158	192	520970	520866	-104	1.4	29.3	48.57
		WP_063842694.1	NC_002929.2	40	35	21	158	192	520970	520866	-104	1.4	29.3	48.57
	sphB3	WP_063864194.1	NZ_CP039021.1	39.286	28	17	142	169	1163752	1163669	-83	2.7	28.1	60.71
	sphB3	WP_063864194.1	NZ_CP039022.1	39.286	28	17	142	169	1163752	1163669	-83	2.7	28.1	60.71
		WP_063864194.1	NC_002929.2	39.286	28	17	142	169	1163752	1163669	-83	2.7	28.1	60.71
		WP_054428802.1	NZ_CP039021.1	39.286	28	17	142	169	1163752	1163669	-83	2.5	28.5	60.71
		WP_054428802.1	NZ_CP039022.1	39.286	28	17	142	169	1163752	1163669	-83	2.5	28.5	60.71
		WP_054428802.1	NC_002929.2	39.286	28	17	142	169	1163752	1163669	-83	2.5	28.5	60.71
		WP_006391197.1	NZ_CP039021.1	39.286	28	17	142	169	1163752	1163669	-83	2.7	28.1	60.71
		WP_006391197.1	NZ_CP039022.1	39.286	28	17	142	169	1163752	1163669	-83	2.7	28.1	60.71
		WP_006391197.1	NC_002929.2	39.286	28	17	142	169	1163752	1163669	-83	2.7	28.1	60.71
		WP_063864088.1	NZ_CP039021.1	39.286	28	17	142	169	1163752	1163669	-83	2.5	28.5	60.71
		WP_063864088.1	NZ_CP039022.1	39.286	28	17	142	169	1163752	1163669	-83	2.5	28.5	60.71

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		WP_063864088.1	NC_002929.2	39.286	28	17	142	169	1163752	1163669	-83	2.5	28.5	60.71
		WP_063864087.1	NZ_CP039021.1	39.286	28	17	142	169	1163752	1163669	-83	2.9	28.1	60.71
		WP_063864087.1	NZ_CP039022.1	39.286	28	17	142	169	1163752	1163669	-83	2.9	28.1	60.71
		WP_063864087.1	NC_002929.2	39.286	28	17	142	169	1163752	1163669	-83	2.9	28.1	60.71
		WP_063862734.1	NZ_CP039021.1	39.286	28	17	142	169	1163752	1163669	-83	2.5	28.5	60.71
		WP_063862734.1	NZ_CP039022.1	39.286	28	17	142	169	1163752	1163669	-83	2.5	28.5	60.71
		WP_063862734.1	NC_002929.2	39.286	28	17	142	169	1163752	1163669	-83	2.5	28.5	60.71
		WP_001021788.1	NZ_CP039021.1	39.286	28	17	14	41	561454	561537	83	1.7	28.9	57.14
		WP_001021788.1	NZ_CP039022.1	39.286	28	17	14	41	561454	561537	83	1.7	28.9	57.14
		WP_001021788.1	NC_002929.2	39.286	28	17	14	41	561454	561537	83	1.7	28.9	57.14
		WP_063862765.1	NZ_CP039021.1	39.286	28	17	15	42	561454	561537	83	3.8	27.7	57.14
		WP_063862765.1	NZ_CP039022.1	39.286	28	17	15	42	561454	561537	83	3.8	27.7	57.14
		WP_063862765.1	NC_002929.2	39.286	28	17	15	42	561454	561537	83	3.8	27.7	57.14
	BP0088	WP_122630823.1	NZ_CP039021.1	39.13	23	14	194	216	86645	86713	68	3.6	28.1	56.52
		WP_122630823.1	NZ_CP039022.1	39.13	23	14	194	216	86645	86713	68	3.6	28.1	56.52
		WP_122630823.1	NC_002929.2	39.13	23	14	194	216	86645	86713	68	3.6	28.1	56.52
		WP_011997479.1	NZ_CP039021.1	38.889	36	19	88	120	445634	445741	107	9.5	26.6	58.33
		WP_011997479.1	NZ_CP039022.1	38.889	36	19	88	120	445634	445741	107	9.5	26.6	58.33
		WP_011997479.1	NC_002929.2	38.889	36	19	88	120	445634	445741	107	9.5	26.6	58.33
	BP2487	WP_032072208.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4	27.7	58.06
		WP_032072208.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4	27.7	58.06
		WP_032072208.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4	27.7	58.06
		WP_063864887.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4	27.7	58.06
		WP_063864887.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4	27.7	58.06
		WP_063864887.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4	27.7	58.06
		WP_122630841.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4	27.7	58.06
		WP_122630841.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4	27.7	58.06
		WP_122630841.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4	27.7	58.06

		WP_063864897.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4	27.7	58.06
		WP_063864897.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4	27.7	58.06
		WP_063864897.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4	27.7	58.06
		WP_015379489.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4	27.7	58.06
		WP_015379489.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4	27.7	58.06
		WP_015379489.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4	27.7	58.06
		WP_013279314.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_013279314.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_013279314.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_116786831.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_116786831.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_116786831.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_102607453.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4.4	27.7	58.06
		WP_102607453.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4.4	27.7	58.06
		WP_102607453.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4.4	27.7	58.06
		WP_109791210.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4.3	27.7	58.06
		WP_109791210.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4.3	27.7	58.06
		WP_109791210.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4.3	27.7	58.06
		WP_109791184.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_109791184.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_109791184.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_011645017.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_011645017.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_011645017.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_109545059.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4.4	27.7	58.06
		WP_109545059.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4.4	27.7	58.06
		WP_109545059.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4.4	27.7	58.06
		WP_021561477.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	2.7	28.1	61.29

		WP_021561477.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	2.7	28.1	61.29
		WP_021561477.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	2.7	28.1	61.29
		WP_104009855.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_104009855.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_104009855.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_099156041.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4	27.7	58.06
		WP_099156041.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4	27.7	58.06
		WP_099156041.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4	27.7	58.06
		WP_000027050.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4.2	27.7	58.06
		WP_000027050.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4.2	27.7	58.06
		WP_000027050.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4.2	27.7	58.06
		WP_072186106.1	NZ_CP039021.1	38.71	31	19	223	253	2635545	2635453	-92	6.6	26.9	58.06
		WP_072186106.1	NZ_CP039022.1	38.71	31	19	223	253	2635545	2635453	-92	6.6	26.9	58.06
		WP_072186106.1	NC_002929.2	38.71	31	19	223	253	2635545	2635453	-92	6.6	26.9	58.06
		WP_000027060.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_000027060.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_000027060.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_055314004.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4.6	27.7	58.06
		WP_055314004.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4.6	27.7	58.06
		WP_055314004.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4.6	27.7	58.06
		WP_052944427.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4.3	27.7	58.06
		WP_052944427.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4.3	27.7	58.06
		WP_052944427.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4.3	27.7	58.06
		WP_060615518.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_060615518.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_060615518.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_032492330.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4	27.7	58.06
		WP_032492330.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4	27.7	58.06

		WP_032492330.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4	27.7	58.06
		WP_077064889.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4.7	27.7	58.06
		WP_077064889.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4.7	27.7	58.06
		WP_077064889.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4.7	27.7	58.06
		WP_075985685.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	3.9	27.7	58.06
		WP_075985685.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	3.9	27.7	58.06
		WP_075985685.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	3.9	27.7	58.06
		WP_061158039.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_061158039.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_061158039.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_032494237.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4.3	27.7	58.06
		WP_032494237.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4.3	27.7	58.06
		WP_032494237.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4.3	27.7	58.06
		WP_000027061.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4.8	27.3	58.06
		WP_000027061.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4.8	27.3	58.06
		WP_000027061.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4.8	27.3	58.06
		WP_063864901.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4.3	27.7	58.06
		WP_063864901.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4.3	27.7	58.06
		WP_063864901.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4.3	27.7	58.06
		WP_063864895.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4.6	27.7	58.06
		WP_063864895.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4.6	27.7	58.06
		WP_063864895.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4.6	27.7	58.06
		WP_061659705.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_061659705.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_061659705.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_050190632.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_050190632.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_050190632.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06

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		WP_040197984.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4.3	27.7	58.06
		WP_040197984.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4.3	27.7	58.06
		WP_040197984.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4.3	27.7	58.06
		WP_032490103.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_032490103.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_032490103.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_015365452.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	3.8	27.7	58.06
		WP_015365452.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	3.8	27.7	58.06
		WP_015365452.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	3.8	27.7	58.06
		WP_063864907.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4	27.7	58.06
		WP_063864907.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4	27.7	58.06
		WP_063864907.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4	27.7	58.06
		WP_047028173.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4.2	27.7	58.06
		WP_047028173.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4.2	27.7	58.06
		WP_047028173.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4.2	27.7	58.06
		WP_063864890.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4	27.7	58.06
		WP_063864890.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4	27.7	58.06
		WP_063864890.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4	27.7	58.06
		WP_063864889.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	3.9	27.7	58.06
		WP_063864889.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	3.9	27.7	58.06
		WP_063864889.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	3.9	27.7	58.06
		WP_063864888.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4	27.7	58.06
		WP_063864888.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4	27.7	58.06
		WP_063864888.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4	27.7	58.06
		WP_063865144.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	2.6	28.5	61.29
		WP_063865144.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	2.6	28.5	61.29
		WP_063865144.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	2.6	28.5	61.29
		WP_063865138.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	3.8	27.7	58.06

		WP_063865138.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	3.8	27.7	58.06
		WP_063865138.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	3.8	27.7	58.06
		WP_063865132.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	3.8	27.7	58.06
		WP_063865132.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	3.8	27.7	58.06
		WP_063865132.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	3.8	27.7	58.06
		WP_063865129.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_063865129.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_063865129.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_063865088.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4.7	27.3	58.06
		WP_063865088.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4.7	27.3	58.06
		WP_063865088.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4.7	27.3	58.06
		WP_063865053.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	3.8	27.7	58.06
		WP_063865053.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	3.8	27.7	58.06
		WP_063865053.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	3.8	27.7	58.06
		WP_063865043.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_063865043.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_063865043.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_063865036.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_063865036.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_063865036.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_063865030.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4	27.7	58.06
		WP_063865030.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4	27.7	58.06
		WP_063865030.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4	27.7	58.06
		WP_063865024.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	3.8	27.7	58.06
		WP_063865024.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	3.8	27.7	58.06
		WP_063865024.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	3.8	27.7	58.06
		WP_063865008.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	3.9	27.7	58.06
		WP_063865008.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	3.9	27.7	58.06

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		WP_063865008.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	3.9	27.7	58.06
		WP_063865003.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	3.8	27.7	58.06
		WP_063865003.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	3.8	27.7	58.06
		WP_063865003.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	3.8	27.7	58.06
		WP_063864996.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	2.8	28.1	61.29
		WP_063864996.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	2.8	28.1	61.29
		WP_063864996.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	2.8	28.1	61.29
		WP_063864990.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4	27.7	58.06
		WP_063864990.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4	27.7	58.06
		WP_063864990.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4	27.7	58.06
		WP_063864974.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_063864974.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_063864974.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_063864949.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_063864949.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_063864949.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_063864938.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4	27.7	58.06
		WP_063864938.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4	27.7	58.06
		WP_063864938.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4	27.7	58.06
		WP_063864930.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	8	26.9	58.06
		WP_063864930.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	8	26.9	58.06
		WP_063864930.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	8	26.9	58.06
		WP_063864923.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4.6	27.7	58.06
		WP_063864923.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4.6	27.7	58.06
		WP_063864923.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4.6	27.7	58.06
		WP_063864913.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4.2	27.7	58.06
		WP_063864913.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4.2	27.7	58.06
		WP_063864913.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4.2	27.7	58.06

		WP_063864912.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4.2	27.7	58.06
		WP_063864912.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4.2	27.7	58.06
		WP_063864912.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4.2	27.7	58.06
		WP_063864910.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4	27.7	58.06
		WP_063864910.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4	27.7	58.06
		WP_063864910.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4	27.7	58.06
		WP_063864909.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4.6	27.7	58.06
		WP_063864909.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4.6	27.7	58.06
		WP_063864909.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4.6	27.7	58.06
		WP_063864908.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	3.7	27.7	58.06
		WP_063864908.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	3.7	27.7	58.06
		WP_063864908.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	3.7	27.7	58.06
		WP_063864905.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4	27.7	58.06
		WP_063864905.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4	27.7	58.06
		WP_063864905.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4	27.7	58.06
		WP_063864904.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	3.9	27.7	58.06
		WP_063864904.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	3.9	27.7	58.06
		WP_063864904.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	3.9	27.7	58.06
		WP_063864903.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_063864903.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_063864903.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_063864902.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4.2	27.7	58.06
		WP_063864902.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4.2	27.7	58.06
		WP_063864902.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4.2	27.7	58.06
		WP_063864898.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4	27.7	58.06
		WP_063864898.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4	27.7	58.06
		WP_063864898.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4	27.7	58.06
		WP_063864894.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06

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		WP_063864894.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_063864894.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_063864892.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4	27.7	58.06
		WP_063864892.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4	27.7	58.06
		WP_063864892.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4	27.7	58.06
		WP_063864886.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4.7	27.3	58.06
		WP_063864886.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4.7	27.3	58.06
		WP_063864886.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4.7	27.3	58.06
		WP_063864884.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4.4	27.7	58.06
		WP_063864884.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4.4	27.7	58.06
		WP_063864884.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4.4	27.7	58.06
		WP_063864883.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	3.9	27.7	58.06
		WP_063864883.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	3.9	27.7	58.06
		WP_063864883.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	3.9	27.7	58.06
		WP_063864882.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4.4	27.7	58.06
		WP_063864882.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4.4	27.7	58.06
		WP_063864882.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4.4	27.7	58.06
		WP_063864880.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4.4	27.7	58.06
		WP_063864880.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4.4	27.7	58.06
		WP_063864880.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4.4	27.7	58.06
		WP_063864878.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	3.9	27.7	58.06
		WP_063864878.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	3.9	27.7	58.06
		WP_063864878.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	3.9	27.7	58.06
		WP_063864877.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_063864877.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_063864877.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_063864876.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	3.9	27.7	58.06
		WP_063864876.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	3.9	27.7	58.06

		WP_063864876.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	3.9	27.7	58.06
		WP_063864874.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_063864874.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_063864874.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_063864873.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4.3	27.7	58.06
		WP_063864873.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4.3	27.7	58.06
		WP_063864873.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4.3	27.7	58.06
		WP_063864872.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4.4	27.7	58.06
		WP_063864872.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4.4	27.7	58.06
		WP_063864872.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4.4	27.7	58.06
		WP_063864871.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_063864871.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_063864871.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_063864870.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_063864870.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_063864870.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_063864869.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4.6	27.7	58.06
		WP_063864869.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4.6	27.7	58.06
		WP_063864869.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4.6	27.7	58.06
		WP_063864868.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4.2	27.7	58.06
		WP_063864868.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4.2	27.7	58.06
		WP_063864868.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4.2	27.7	58.06
		WP_063864867.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_063864867.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_063864867.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_063864866.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4.8	27.3	58.06
		WP_063864866.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4.8	27.3	58.06
		WP_063864866.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4.8	27.3	58.06

		WP_063864865.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4.3	27.7	58.06
		WP_063864865.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4.3	27.7	58.06
		WP_063864865.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4.3	27.7	58.06
		WP_063864864.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4.3	27.7	58.06
		WP_063864864.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4.3	27.7	58.06
		WP_063864864.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4.3	27.7	58.06
		WP_063864863.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4.5	27.7	58.06
		WP_063864863.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4.5	27.7	58.06
		WP_063864863.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4.5	27.7	58.06
		WP_063864862.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4	27.7	58.06
		WP_063864862.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4	27.7	58.06
		WP_063864862.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4	27.7	58.06
		WP_063864861.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4	27.7	58.06
		WP_063864861.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4	27.7	58.06
		WP_063864861.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4	27.7	58.06
		WP_063864859.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4.3	27.7	58.06
		WP_063864859.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4.3	27.7	58.06
	BP2487	WP_063864859.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4.3	27.7	58.06
		WP_063864858.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	3.4	28.1	61.29
		WP_063864858.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	3.4	28.1	61.29
		WP_063864858.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	3.4	28.1	61.29
		WP_063864857.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	3.6	27.7	61.29
		WP_063864857.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	3.6	27.7	61.29
		WP_063864857.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	3.6	27.7	61.29
		WP_063864856.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_063864856.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_063864856.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_063864853.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	3.7	27.7	58.06

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			WP_063864853.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	3.7	27.7	58.06
			WP_063864853.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	3.7	27.7	58.06
			WP_063864851.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4.6	27.7	58.06
			WP_063864851.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4.6	27.7	58.06
			WP_063864851.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4.6	27.7	58.06
			WP_063864850.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	3.6	27.7	58.06
			WP_063864850.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	3.6	27.7	58.06
			WP_063864850.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	3.6	27.7	58.06
			WP_063864843.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	3.3	28.1	58.06
			WP_063864843.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	3.3	28.1	58.06
			WP_063864843.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	3.3	28.1	58.06
			WP_063864833.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4.3	27.7	58.06
			WP_063864833.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4.3	27.7	58.06
			WP_063864833.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4.3	27.7	58.06
			WP_063864819.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4.2	27.7	58.06
			WP_063864819.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4.2	27.7	58.06
			WP_063864819.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4.2	27.7	58.06
			WP_063864811.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
			WP_063864811.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
			WP_063864811.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
			WP_063864808.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
			WP_063864808.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
			WP_063864808.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
			WP_063864807.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	2.4	28.5	61.29
			WP_063864807.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	2.4	28.5	61.29
			WP_063864807.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	2.4	28.5	61.29
			WP_063864805.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4.5	27.7	58.06
			WP_063864805.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4.5	27.7	58.06

		WP_063864805.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4.5	27.7	58.06
		WP_063864800.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_063864800.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_063864800.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_063864799.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_063864799.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_063864799.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_063864798.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	3.6	27.7	58.06
		WP_063864798.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	3.6	27.7	58.06
		WP_063864798.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	3.6	27.7	58.06
		WP_063864796.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_063864796.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_063864796.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_063864795.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_063864795.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_063864795.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_063864804.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	3.8	27.7	58.06
		WP_063864804.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	3.8	27.7	58.06
		WP_063864804.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	3.8	27.7	58.06
		WP_055314508.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_055314508.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_055314508.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_038976851.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_038976851.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_038976851.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_048235862.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	3.9	27.7	58.06
		WP_048235862.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	3.9	27.7	58.06
		WP_048235862.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	3.9	27.7	58.06

		WP_042065300.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4.3	27.7	58.06
		WP_042065300.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4.3	27.7	58.06
		WP_042065300.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4.3	27.7	58.06
		WP_032494853.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4.7	27.7	58.06
		WP_032494853.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4.7	27.7	58.06
		WP_032494853.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4.7	27.7	58.06
		WP_032492113.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_032492113.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_032492113.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_032491376.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_032491376.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_032491376.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_032490960.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_032490960.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_032490960.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_032490959.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4.3	27.7	58.06
		WP_032490959.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4.3	27.7	58.06
		WP_032490959.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4.3	27.7	58.06
		WP_032490956.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4.6	27.7	58.06
		WP_032490956.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4.6	27.7	58.06
		WP_032490956.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4.6	27.7	58.06
		WP_032490504.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	5.4	27.3	58.06
		WP_032490504.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	5.4	27.3	58.06
		WP_032490504.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	5.4	27.3	58.06
		WP_032490102.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	3.9	27.7	58.06
		WP_032490102.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	3.9	27.7	58.06
		WP_032490102.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	3.9	27.7	58.06
		WP_032490101.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	5.1	27.3	58.06

		WP_032490101.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	5.1	27.3	58.06
		WP_032490101.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	5.1	27.3	58.06
		WP_032490026.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_032490026.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_032490026.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_032489893.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4.2	27.7	58.06
		WP_032489893.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4.2	27.7	58.06
		WP_032489893.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4.2	27.7	58.06
		WP_032221497.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4	27.7	58.06
		WP_032221497.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4	27.7	58.06
		WP_032221497.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4	27.7	58.06
		WP_021526512.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_021526512.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_021526512.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_015058868.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_015058868.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_015058868.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4.1	27.7	58.06
		WP_015058867.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4	27.7	58.06
		WP_015058867.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4	27.7	58.06
		WP_015058867.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4	27.7	58.06
		WP_000027057.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4	27.7	58.06
		WP_000027057.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4	27.7	58.06
		WP_000027057.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4	27.7	58.06
		WP_021598056.1	NZ_CP039021.1	38.71	31	19	225	255	2635545	2635453	-92	4.6	27.7	58.06
		WP_021598056.1	NZ_CP039022.1	38.71	31	19	225	255	2635545	2635453	-92	4.6	27.7	58.06
		WP_021598056.1	NC_002929.2	38.71	31	19	225	255	2635545	2635453	-92	4.6	27.7	58.06
	BP0219	WP_063842701.1	NZ_CP039021.1	38.71	31	19	255	285	229792	229884	92	1.3	29.6	54.84
		WP_063842701.1	NZ_CP039022.1	38.71	31	19	255	285	229792	229884	92	1.3	29.6	54.84

		WP_063842701.1	NC_002929.2	38.71	31	19	255	285	229792	229884	92	1.3	29.6	54.84
	BP0135	WP_025370306.1	NZ_CP039021.1	38.636	44	22	221	259	133045	132914	-131	8.5	26.9	52.27
		WP_025370306.1	NZ_CP039022.1	38.636	44	22	221	259	133045	132914	-131	8.5	26.9	52.27
		WP_025370306.1	NC_002929.2	38.636	44	22	221	259	133045	132914	-131	8.5	26.9	52.27
		WP_122630835.1	NZ_CP039021.1	38.462	26	16	196	221	897145	897222	77	7.5	26.6	61.54
		WP_122630835.1	NZ_CP039022.1	38.462	26	16	196	221	897145	897222	77	7.5	26.6	61.54
		WP_122630835.1	NC_002929.2	38.462	26	16	196	221	897145	897222	77	7.5	26.6	61.54
	BP0554	WP_063861733.1	NZ_CP039021.1	38.462	26	16	16	41	561460	561537	77	9.2	26.6	57.69
		WP_063861733.1	NZ_CP039022.1	38.462	26	16	16	41	561460	561537	77	9.2	26.6	57.69
		WP_063861733.1	NC_002929.2	38.462	26	16	16	41	561460	561537	77	9.2	26.6	57.69
		WP_063842695.1	NZ_CP039021.1	38.462	26	16	91	116	237904	237827	-77	5.8	27.3	65.38
		WP_063842695.1	NZ_CP039022.1	38.462	26	16	91	116	237904	237827	-77	5.8	27.3	65.38
		WP_063842695.1	NC_002929.2	38.462	26	16	91	116	237904	237827	-77	5.8	27.3	65.38
		WP_063842690.1	NZ_CP039021.1	38.462	26	16	91	116	237904	237827	-77	5.7	27.3	65.38
		WP_063842690.1	NZ_CP039022.1	38.462	26	16	91	116	237904	237827	-77	5.7	27.3	65.38
		WP_063842690.1	NC_002929.2	38.462	26	16	91	116	237904	237827	-77	5.7	27.3	65.38
		WP_046430859.1	NZ_CP039021.1	38.462	26	16	91	116	237904	237827	-77	5.9	27.3	65.38
		WP_046430859.1	NZ_CP039022.1	38.462	26	16	91	116	237904	237827	-77	5.9	27.3	65.38
		WP_046430859.1	NC_002929.2	38.462	26	16	91	116	237904	237827	-77	5.9	27.3	65.38
Metallo-beta-lactamase superfamily	*BP0220*	WP_004862397.1	NZ_CP039021.1	38.298	47	25	190	236	230844	230716	-128	5.1	27.3	48.94
		WP_004862397.1	NZ_CP039022.1	38.298	47	25	190	236	230844	230716	-128	5.1	27.3	48.94
		WP_004862397.1	NC_002929.2	38.298	47	25	190	236	230844	230716	-128	5.1	27.3	48.94
		WP_063860936.1	NZ_CP039021.1	38.298	47	25	190	236	230844	230716	-128	5.2	27.3	48.94
		WP_063860936.1	NZ_CP039022.1	38.298	47	25	190	236	230844	230716	-128	5.2	27.3	48.94
		WP_063860936.1	NC_002929.2	38.298	47	25	190	236	230844	230716	-128	5.2	27.3	48.94
		WP_063860935.1	NZ_CP039021.1	38.298	47	25	190	236	230844	230716	-128	4.7	27.7	48.94
		WP_063860935.1	NZ_CP039022.1	38.298	47	25	190	236	230844	230716	-128	4.7	27.7	48.94

		WP_063860935.1	NC_002929.2	38.298	47	25	190	236	230844	230716	-128	4.7	27.7	48.94
		WP_063860934.1	NZ_CP039021.1	38.298	47	25	190	236	230844	230716	-128	5.2	27.3	48.94
		WP_063860934.1	NZ_CP039022.1	38.298	47	25	190	236	230844	230716	-128	5.2	27.3	48.94
		WP_063860934.1	NC_002929.2	38.298	47	25	190	236	230844	230716	-128	5.2	27.3	48.94
		WP_063860933.1	NZ_CP039021.1	38.298	47	25	190	236	230844	230716	-128	6.5	26.9	48.94
		WP_063860933.1	NZ_CP039022.1	38.298	47	25	190	236	230844	230716	-128	6.5	26.9	48.94
		WP_063860933.1	NC_002929.2	38.298	47	25	190	236	230844	230716	-128	6.5	26.9	48.94
		WP_063860932.1	NZ_CP039021.1	38.298	47	25	190	236	230844	230716	-128	5.1	27.3	48.94
		WP_063860932.1	NZ_CP039022.1	38.298	47	25	190	236	230844	230716	-128	5.1	27.3	48.94
		WP_063860932.1	NC_002929.2	38.298	47	25	190	236	230844	230716	-128	5.1	27.3	48.94
	BP0511	WP_044403015.1	NZ_CP039021.1	38.235	34	21	157	190	520970	520869	-101	0.88	30	52.94
		WP_044403015.1	NZ_CP039022.1	38.235	34	21	157	190	520970	520869	-101	0.88	30	52.94
		WP_044403015.1	NC_002929.2	38.235	34	21	157	190	520970	520869	-101	0.88	30	52.94
	BP1062	WP_004207704.1	NZ_CP039021.1	37.931	29	18	236	264	1107159	1107073	-86	6.8	27.3	55.17
		WP_004207704.1	NZ_CP039022.1	37.931	29	18	236	264	1107159	1107073	-86	6.8	27.3	55.17
		WP_004207704.1	NC_002929.2	37.931	29	18	236	264	1107159	1107073	-86	6.8	27.3	55.17
		WP_007708339.1	NZ_CP039021.1	37.931	29	18	236	264	1107159	1107073	-86	7.3	27.3	55.17
		WP_007708339.1	NZ_CP039022.1	37.931	29	18	236	264	1107159	1107073	-86	7.3	27.3	55.17
		WP_007708339.1	NC_002929.2	37.931	29	18	236	264	1107159	1107073	-86	7.3	27.3	55.17
	BP1643	WP_122630873.1	NZ_CP039021.1	37.838	37	23	16	52	1730807	1730917	110	2.1	28.5	54.05
		WP_122630873.1	NZ_CP039022.1	37.838	37	23	16	52	1730807	1730917	110	2.1	28.5	54.05
		WP_122630873.1	NC_002929.2	37.838	37	23	16	52	1730807	1730917	110	2.1	28.5	54.05
		WP_063864667.1	NZ_CP039021.1	37.838	37	23	16	52	1730807	1730917	110	8.4	26.6	54.05
		WP_063864667.1	NZ_CP039022.1	37.838	37	23	16	52	1730807	1730917	110	8.4	26.6	54.05
		WP_063864667.1	NC_002929.2	37.838	37	23	16	52	1730807	1730917	110	8.4	26.6	54.05
		WP_063842698.1	NZ_CP039021.1	37.838	37	23	161	197	520961	520851	-110	3	28.1	45.95
		WP_063842698.1	NZ_CP039022.1	37.838	37	23	161	197	520961	520851	-110	3	28.1	45.95
		WP_063842698.1	NC_002929.2	37.838	37	23	161	197	520961	520851	-110	3	28.1	45.95

BP2059	WP_011550565.1	NZ_CP039021.1	37.778	45	28	194	238	2178957	2178823	-134	5.4	27.3	51.11
	WP_011550565.1	NZ_CP039022.1	37.778	45	28	194	238	2178957	2178823	-134	5.4	27.3	51.11
	WP_011550565.1	NC_002929.2	37.778	45	28	194	238	2178957	2178823	-134	5.4	27.3	51.11
	WP_080699425.1	NZ_CP039021.1	37.5	32	20	225	256	2635545	2635450	-95	3.9	27.7	56.25
	WP_080699425.1	NZ_CP039022.1	37.5	32	20	225	256	2635545	2635450	-95	3.9	27.7	56.25
	WP_080699425.1	NC_002929.2	37.5	32	20	225	256	2635545	2635450	-95	3.9	27.7	56.25
	WP_102607462.1	NZ_CP039021.1	37.5	40	25	158	197	684757	684876	119	5.3	27.3	47.5
	WP_102607462.1	NZ_CP039022.1	37.5	40	25	158	197	684757	684876	119	5.3	27.3	47.5
	WP_102607462.1	NC_002929.2	37.5	40	25	158	197	684757	684876	119	5.3	27.3	47.5
	WP_063842697.1	NZ_CP039021.1	37.5	40	25	158	197	520970	520851	-119	1.2	29.6	45
	WP_063842697.1	NZ_CP039022.1	37.5	40	25	158	197	520970	520851	-119	1.2	29.6	45
	WP_063842697.1	NC_002929.2	37.5	40	25	158	197	520970	520851	-119	1.2	29.6	45
	WP_063842693.1	NZ_CP039021.1	37.5	40	25	158	197	520970	520851	-119	1.2	29.3	45
	WP_063842693.1	NZ_CP039022.1	37.5	40	25	158	197	520970	520851	-119	1.2	29.3	45
	WP_063842693.1	NC_002929.2	37.5	40	25	158	197	520970	520851	-119	1.2	29.3	45
BP0135	WP_006027381.1	NZ_CP039021.1	37.255	51	27	214	259	133066	132914	-152	6.1	27.3	52.94
	WP_006027381.1	NZ_CP039022.1	37.255	51	27	214	259	133066	132914	-152	6.1	27.3	52.94
	WP_006027381.1	NC_002929.2	37.255	51	27	214	259	133066	132914	-152	6.1	27.3	52.94
spoT	WP_004746565.1	NZ_CP039021.1	37.209	43	27	247	289	1656783	1656911	128	8.5	27.3	48.84
	WP_004746565.1	NZ_CP039022.1	37.209	43	27	247	289	1656783	1656911	128	8.5	27.3	48.84
	WP_004746565.1	NC_002929.2	37.209	43	27	247	289	1656783	1656911	128	8.5	27.3	48.84
	WP_063857818.1	NZ_CP039021.1	37.209	43	27	247	289	1656783	1656911	128	8.6	27.3	48.84
	WP_063857818.1	NZ_CP039022.1	37.209	43	27	247	289	1656783	1656911	128	8.6	27.3	48.84
	WP_063857818.1	NC_002929.2	37.209	43	27	247	289	1656783	1656911	128	8.6	27.3	48.84
	WP_002118772.1	NZ_CP039021.1	37.209	43	27	247	289	1656783	1656911	128	9.6	26.9	51.16
	WP_002118772.1	NZ_CP039022.1	37.209	43	27	247	289	1656783	1656911	128	9.6	26.9	51.16
	WP_002118772.1	NC_002929.2	37.209	43	27	247	289	1656783	1656911	128	9.6	26.9	51.16
	WP_085562421.1	NZ_CP039021.1	37.209	43	26	210	251	290168	290040	-128	0.84	29.6	51.16

		WP_085562421.1	NZ_CP039022.1	37.209	43	26	210	251	290168	290040	-128	0.84	29.6	51.16
		WP_085562421.1	NC_002929.2	37.209	43	26	210	251	290168	290040	-128	0.84	29.6	51.16
	BP2375	WP_063865197.1	NZ_CP039021.1	37.037	27	17	57	83	2514758	2514838	80	9.5	26.6	66.67
		WP_063865197.1	NZ_CP039022.1	37.037	27	17	57	83	2514758	2514838	80	9.5	26.6	66.67
		WP_063865197.1	NC_002929.2	37.037	27	17	57	83	2514758	2514838	80	9.5	26.6	66.67
		WP_063865168.1	NZ_CP039021.1	37.037	27	17	57	83	2514758	2514838	80	9.4	26.6	66.67
		WP_063865168.1	NZ_CP039022.1	37.037	27	17	57	83	2514758	2514838	80	9.4	26.6	66.67
		WP_063865168.1	NC_002929.2	37.037	27	17	57	83	2514758	2514838	80	9.4	26.6	66.67
	BP0393	WP_063864909.1	NZ_CP039021.1	36.842	38	24	220	257	392187	392300	113	9.6	26.6	50
		WP_063864909.1	NZ_CP039022.1	36.842	38	24	220	257	392187	392300	113	9.6	26.6	50
		WP_063864909.1	NC_002929.2	36.842	38	24	220	257	392187	392300	113	9.6	26.6	50
		WP_063864886.1	NZ_CP039021.1	36.842	38	24	220	257	392187	392300	113	9.8	26.6	50
		WP_063864886.1	NZ_CP039022.1	36.842	38	24	220	257	392187	392300	113	9.8	26.6	50
		WP_063864886.1	NC_002929.2	36.842	38	24	220	257	392187	392300	113	9.8	26.6	50
		WP_063864863.1	NZ_CP039021.1	36.842	38	24	220	257	392187	392300	113	9.2	26.6	50
		WP_063864863.1	NZ_CP039022.1	36.842	38	24	220	257	392187	392300	113	9.2	26.6	50
		WP_063864863.1	NC_002929.2	36.842	38	24	220	257	392187	392300	113	9.2	26.6	50
		WP_032490956.1	NZ_CP039021.1	36.842	38	24	220	257	392187	392300	113	9.8	26.6	50
		WP_032490956.1	NZ_CP039022.1	36.842	38	24	220	257	392187	392300	113	9.8	26.6	50
		WP_032490956.1	NC_002929.2	36.842	38	24	220	257	392187	392300	113	9.8	26.6	50
		WP_032490101.1	NZ_CP039021.1	36.842	38	24	220	257	392187	392300	113	9.5	26.6	50
		WP_032490101.1	NZ_CP039022.1	36.842	38	24	220	257	392187	392300	113	9.5	26.6	50
		WP_032490101.1	NC_002929.2	36.842	38	24	220	257	392187	392300	113	9.5	26.6	50
	rpsH	WP_063860622.1	NZ_CP039021.1	36.667	30	19	189	218	3840417	3840506	89	6.8	26.6	53.33
		WP_063860622.1	NZ_CP039022.1	36.667	30	19	189	218	3840417	3840506	89	6.8	26.6	53.33
		WP_063860622.1	NC_002929.2	36.667	30	19	189	218	3840417	3840506	89	6.8	26.6	53.33
		WP_063861776.1	NZ_CP039021.1	36.667	30	19	207	236	2249128	2249217	89	4.8	27.3	50
		WP_063861776.1	NZ_CP039022.1	36.667	30	19	207	236	2249128	2249217	89	4.8	27.3	50

		WP_063861776.1	NC_002929.2	36.667	30	19	207	236	2249128	2249217	89	4.8	27.3	50
		WP_045219048.1	NZ_CP039021.1	36.667	30	19	208	237	2249128	2249217	89	4.5	27.3	50
		WP_045219048.1	NZ_CP039022.1	36.667	30	19	208	237	2249128	2249217	89	4.5	27.3	50
		WP_045219048.1	NC_002929.2	36.667	30	19	208	237	2249128	2249217	89	4.5	27.3	50
	pepN	WP_032488483.1	NZ_CP039021.1	36.667	30	19	159	188	904168	904257	89	6.6	26.9	53.33
		WP_032488483.1	NZ_CP039022.1	36.667	30	19	159	188	904168	904257	89	6.6	26.9	53.33
		WP_032488483.1	NC_002929.2	36.667	30	19	159	188	904168	904257	89	6.6	26.9	53.33
		WP_063842691.1	NZ_CP039021.1	36.667	30	19	88	117	237916	237827	-89	2.2	28.5	63.33
		WP_063842691.1	NZ_CP039022.1	36.667	30	19	88	117	237916	237827	-89	2.2	28.5	63.33
		WP_063842691.1	NC_002929.2	36.667	30	19	88	117	237916	237827	-89	2.2	28.5	63.33
		WP_063843234.1	NZ_CP039021.1	36.667	30	19	163	192	40380	40291	-89	8.5	27.3	56.67
		WP_063843234.1	NZ_CP039022.1	36.667	30	19	163	192	40380	40291	-89	8.5	27.3	56.67
		WP_063843234.1	NC_002929.2	36.667	30	19	163	192	40380	40291	-89	8.5	27.3	56.67
	BP0329	WP_063842948.1	NZ_CP039021.1	36.538	52	20	253	291	332134	331979	-155	6.6	27.3	44.23
		WP_063842948.1	NZ_CP039022.1	36.538	52	20	253	291	332134	331979	-155	6.6	27.3	44.23
		WP_063842948.1	NC_002929.2	36.538	52	20	253	291	332134	331979	-155	6.6	27.3	44.23
		WP_031943232.1	NZ_CP039021.1	36.364	22	14	158	179	2160311	2160246	-65	6.8	26.6	50
		WP_031943232.1	NZ_CP039022.1	36.364	22	14	158	179	2160311	2160246	-65	6.8	26.6	50
		WP_031943232.1	NC_002929.2	36.364	22	14	158	179	2160311	2160246	-65	6.8	26.6	50
		WP_017901052.1	NZ_CP039021.1	36.364	22	14	158	179	2160311	2160246	-65	8.5	26.6	50
		WP_017901052.1	NZ_CP039022.1	36.364	22	14	158	179	2160311	2160246	-65	8.5	26.6	50
		WP_017901052.1	NC_002929.2	36.364	22	14	158	179	2160311	2160246	-65	8.5	26.6	50
		WP_063864722.1	NZ_CP039021.1	36.364	22	14	158	179	2160311	2160246	-65	8.6	26.6	50
		WP_063864722.1	NZ_CP039022.1	36.364	22	14	158	179	2160311	2160246	-65	8.6	26.6	50
		WP_063864722.1	NC_002929.2	36.364	22	14	158	179	2160311	2160246	-65	8.6	26.6	50
		WP_063860592.1	NZ_CP039021.1	36.364	22	14	158	179	2160311	2160246	-65	6.3	26.9	50
		WP_063860592.1	NZ_CP039022.1	36.364	22	14	158	179	2160311	2160246	-65	6.3	26.9	50
		WP_063860592.1	NC_002929.2	36.364	22	14	158	179	2160311	2160246	-65	6.3	26.9	50

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	BP0136	WP_000778180.1	NZ_CP039021.1	36.364	33	21	298	330	134646	134744	98	7.8	27.3	57.58
		WP_000778180.1	NZ_CP039022.1	36.364	33	21	298	330	134646	134744	98	7.8	27.3	57.58
		WP_000778180.1	NC_002929.2	36.364	33	21	298	330	134646	134744	98	7.8	27.3	57.58
		WP_063842695.1	NZ_CP039021.1	36.082	97	56	77	173	3424704	3424976	272	6.38E-05	43.1	48.45
		WP_063842695.1	NZ_CP039022.1	36.082	97	56	77	173	3424704	3424976	272	6.38E-05	43.1	48.45
		WP_063842695.1	NC_002929.2	36.082	97	56	77	173	3424704	3424976	272	6.38E-05	43.1	48.45
	BP1784	WP_052788483.1	NZ_CP039021.1	36	25	16	25	49	1814176	1814102	-74	6.2	26.9	52
		WP_052788483.1	NZ_CP039022.1	36	25	16	25	49	1814176	1814102	-74	6.2	26.9	52
		WP_052788483.1	NC_002929.2	36	25	16	25	49	1814176	1814102	-74	6.2	26.9	52
		WP_001021782.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	8.9	26.6	53.57
		WP_001021782.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	8.9	26.6	53.57
		WP_001021782.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	8.9	26.6	53.57
		WP_001021785.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	8.9	26.6	53.57
		WP_001021785.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	8.9	26.6	53.57
		WP_001021785.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	8.9	26.6	53.57
		WP_122630853.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	8.3	26.6	53.57
		WP_122630853.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	8.3	26.6	53.57
		WP_122630853.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	8.3	26.6	53.57
		WP_122630921.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	7.8	26.6	53.57
		WP_122630921.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	7.8	26.6	53.57
		WP_122630921.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	7.8	26.6	53.57
		WP_122630920.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	9	26.6	53.57
		WP_122630920.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	9	26.6	53.57
		WP_122630920.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	9	26.6	53.57
		WP_122630917.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	8.9	26.6	53.57
		WP_122630917.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	8.9	26.6	53.57
		WP_122630917.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	8.9	26.6	53.57
		WP_122630914.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	8.9	26.6	53.57

		WP_122630914.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	8.9	26.6	53.57
		WP_122630914.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	8.9	26.6	53.57
		WP_122630913.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	8.5	26.6	53.57
		WP_122630913.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	8.5	26.6	53.57
		WP_122630913.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	8.5	26.6	53.57
		WP_122630912.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	8.8	26.6	53.57
		WP_122630912.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	8.8	26.6	53.57
		WP_122630912.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	8.8	26.6	53.57
		WP_122630911.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	8.4	26.6	53.57
		WP_122630911.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	8.4	26.6	53.57
		WP_122630911.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	8.4	26.6	53.57
		WP_122630907.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	8.9	26.6	53.57
		WP_122630907.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	8.9	26.6	53.57
		WP_122630907.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	8.9	26.6	53.57
		WP_122630906.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	8.2	26.6	53.57
		WP_122630906.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	8.2	26.6	53.57
		WP_122630906.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	8.2	26.6	53.57
		WP_122630904.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	8.6	26.6	53.57
		WP_122630904.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	8.6	26.6	53.57
		WP_122630904.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	8.6	26.6	53.57
		WP_122630903.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	8.6	26.6	53.57
		WP_122630903.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	8.6	26.6	53.57
		WP_122630903.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	8.6	26.6	53.57
		WP_122630901.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	9.3	26.6	53.57
		WP_122630901.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	9.3	26.6	53.57
		WP_122630901.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	9.3	26.6	53.57
		WP_122630898.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	8	26.6	53.57
		WP_122630898.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	8	26.6	53.57

		WP_122630898.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	8	26.6	53.57
		WP_122630897.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	8.7	26.6	53.57
		WP_122630897.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	8.7	26.6	53.57
		WP_122630897.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	8.7	26.6	53.57
		WP_122630895.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	8.4	26.6	53.57
		WP_122630895.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	8.4	26.6	53.57
		WP_122630895.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	8.4	26.6	53.57
		WP_122630894.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	8.4	26.6	53.57
		WP_122630894.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	8.4	26.6	53.57
		WP_122630894.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	8.4	26.6	53.57
		WP_122630893.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	9.6	26.6	53.57
		WP_122630893.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	9.6	26.6	53.57
		WP_122630893.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	9.6	26.6	53.57
		WP_122630891.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	8.7	26.6	53.57
		WP_122630891.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	8.7	26.6	53.57
		WP_122630891.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	8.7	26.6	53.57
		WP_122630890.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	8.2	26.6	53.57
		WP_122630890.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	8.2	26.6	53.57
		WP_122630890.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	8.2	26.6	53.57
		WP_122630887.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	8.9	26.6	53.57
		WP_122630887.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	8.9	26.6	53.57
		WP_122630887.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	8.9	26.6	53.57
		WP_122630884.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	8.8	26.6	53.57
		WP_122630884.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	8.8	26.6	53.57
		WP_122630884.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	8.8	26.6	53.57
		WP_122630864.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	8.4	26.6	53.57
		WP_122630864.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	8.4	26.6	53.57
		WP_122630864.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	8.4	26.6	53.57

		WP_032018141.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	8.6	26.6	53.57
		WP_032018141.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	8.6	26.6	53.57
		WP_032018141.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	8.6	26.6	53.57
		WP_016685888.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	8.2	26.6	53.57
		WP_016685888.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	8.2	26.6	53.57
		WP_016685888.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	8.2	26.6	53.57
		WP_001021776.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	8.6	26.6	53.57
		WP_001021776.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	8.6	26.6	53.57
		WP_001021776.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	8.6	26.6	53.57
		WP_085562416.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	5.3	27.3	57.14
		WP_085562416.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	5.3	27.3	57.14
		WP_085562416.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	5.3	27.3	57.14
		WP_085562415.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	8.5	26.6	53.57
		WP_085562415.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	8.5	26.6	53.57
		WP_085562415.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	8.5	26.6	53.57
		WP_085562413.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	9.9	26.6	53.57
		WP_085562413.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	9.9	26.6	53.57
		WP_085562413.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	9.9	26.6	53.57
		WP_085562412.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	8.9	26.6	53.57
		WP_085562412.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	8.9	26.6	53.57
		WP_085562412.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	8.9	26.6	53.57
		WP_085562411.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	9.1	26.6	53.57
		WP_085562411.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	9.1	26.6	53.57
		WP_085562411.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	9.1	26.6	53.57
		WP_085562409.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	9.2	26.6	53.57
		WP_085562409.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	9.2	26.6	53.57
		WP_085562409.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	9.2	26.6	53.57
		WP_085562402.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	9.2	26.6	53.57

		WP_085562402.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	9.2	26.6	53.57
		WP_085562402.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	9.2	26.6	53.57
		WP_057053864.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	8.6	26.6	53.57
		WP_057053864.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	8.6	26.6	53.57
		WP_057053864.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	8.6	26.6	53.57
		WP_068981647.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	8.7	26.6	53.57
		WP_068981647.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	8.7	26.6	53.57
		WP_068981647.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	8.7	26.6	53.57
		WP_068981645.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	8.8	26.6	53.57
		WP_068981645.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	8.8	26.6	53.57
		WP_068981645.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	8.8	26.6	53.57
		WP_068981638.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	8.8	26.6	53.57
		WP_068981638.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	8.8	26.6	53.57
		WP_068981638.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	8.8	26.6	53.57
		WP_068981635.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	9.1	26.6	53.57
		WP_068981635.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	9.1	26.6	53.57
		WP_068981635.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	9.1	26.6	53.57
		WP_065419572.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	8.7	26.6	53.57
		WP_065419572.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	8.7	26.6	53.57
		WP_065419572.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	8.7	26.6	53.57
		WP_063862769.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	9.2	26.6	53.57
		WP_063862769.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	9.2	26.6	53.57
		WP_063862769.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	9.2	26.6	53.57
		WP_063862768.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	8.2	26.6	53.57
		WP_063862768.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	8.2	26.6	53.57
		WP_063862768.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	8.2	26.6	53.57
		WP_063862766.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	8.4	26.6	53.57
		WP_063862766.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	8.4	26.6	53.57

		WP_063862766.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	8.4	26.6	53.57
		WP_063862764.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	8.6	26.6	53.57
		WP_063862764.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	8.6	26.6	53.57
		WP_063862764.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	8.6	26.6	53.57
		WP_063862759.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	8.2	26.6	53.57
		WP_063862759.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	8.2	26.6	53.57
		WP_063862759.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	8.2	26.6	53.57
		WP_063862716.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	8.6	26.6	53.57
		WP_063862716.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	8.6	26.6	53.57
		WP_063862716.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	8.6	26.6	53.57
		WP_063862713.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	8.9	26.6	53.57
		WP_063862713.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	8.9	26.6	53.57
		WP_063862713.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	8.9	26.6	53.57
		WP_063862712.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	9.6	26.6	53.57
		WP_063862712.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	9.6	26.6	53.57
		WP_063862712.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	9.6	26.6	53.57
		WP_063862710.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	9.3	26.6	53.57
		WP_063862710.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	9.3	26.6	53.57
		WP_063862710.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	9.3	26.6	53.57
		WP_063862605.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	8.7	26.6	53.57
		WP_063862605.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	8.7	26.6	53.57
		WP_063862605.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	8.7	26.6	53.57
		WP_063862603.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	8.8	26.6	53.57
		WP_063862603.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	8.8	26.6	53.57
		WP_063862603.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	8.8	26.6	53.57
		WP_063862598.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	8.4	26.6	53.57
		WP_063862598.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	8.4	26.6	53.57
		WP_063862598.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	8.4	26.6	53.57

		WP_063864542.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	7.8	26.6	53.57
		WP_063864542.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	7.8	26.6	53.57
		WP_063864542.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	7.8	26.6	53.57
		WP_063864534.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	8.4	26.6	53.57
		WP_063864534.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	8.4	26.6	53.57
		WP_063864534.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	8.4	26.6	53.57
		WP_063864158.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	8.2	26.6	53.57
		WP_063864158.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	8.2	26.6	53.57
		WP_063864158.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	8.2	26.6	53.57
		WP_063864083.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	8.6	26.6	53.57
		WP_063864083.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	8.6	26.6	53.57
		WP_063864083.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	8.6	26.6	53.57
		WP_063862758.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	7.9	26.6	53.57
		WP_063862758.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	7.9	26.6	53.57
		WP_063862758.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	7.9	26.6	53.57
		WP_063862753.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	8.4	26.6	53.57
		WP_063862753.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	8.4	26.6	53.57
		WP_063862753.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	8.4	26.6	53.57
		WP_063862752.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	9.3	26.6	53.57
		WP_063862752.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	9.3	26.6	53.57
		WP_063862752.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	9.3	26.6	53.57
		WP_063862751.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	6	26.9	53.57
		WP_063862751.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	6	26.9	53.57
		WP_063862751.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	6	26.9	53.57
		WP_063862750.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	8.9	26.6	53.57
		WP_063862750.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	8.9	26.6	53.57
		WP_063862750.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	8.9	26.6	53.57
		WP_063862749.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	8.7	26.6	53.57

		WP_063862749.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	8.7	26.6	53.57
		WP_063862749.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	8.7	26.6	53.57
		WP_063862748.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	8.9	26.6	53.57
		WP_063862748.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	8.9	26.6	53.57
		WP_063862748.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	8.9	26.6	53.57
		WP_063862743.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	8.8	26.6	53.57
		WP_063862743.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	8.8	26.6	53.57
		WP_063862743.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	8.8	26.6	53.57
		WP_063862742.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	8.6	26.6	53.57
		WP_063862742.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	8.6	26.6	53.57
		WP_063862742.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	8.6	26.6	53.57
		WP_063862741.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	8.6	26.6	53.57
		WP_063862741.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	8.6	26.6	53.57
		WP_063862741.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	8.6	26.6	53.57
		WP_063862735.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	8.7	26.6	53.57
		WP_063862735.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	8.7	26.6	53.57
		WP_063862735.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	8.7	26.6	53.57
		WP_063861765.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	8.7	26.6	53.57
		WP_063861765.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	8.7	26.6	53.57
		WP_063861765.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	8.7	26.6	53.57
		WP_063861567.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	8.4	26.6	53.57
		WP_063861567.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	8.4	26.6	53.57
		WP_063861567.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	8.4	26.6	53.57
		WP_063861547.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	6.7	26.9	53.57
		WP_063861547.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	6.7	26.9	53.57
		WP_063861547.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	6.7	26.9	53.57
		WP_063861229.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	9.1	26.6	53.57
		WP_063861229.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	9.1	26.6	53.57

		WP_063861229.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	9.1	26.6	53.57
		WP_063861049.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	8.8	26.6	53.57
		WP_063861049.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	8.8	26.6	53.57
		WP_063861049.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	8.8	26.6	53.57
		WP_063861047.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	9	26.6	53.57
		WP_063861047.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	9	26.6	53.57
		WP_063861047.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	9	26.6	53.57
		WP_063861030.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	9.1	26.6	53.57
		WP_063861030.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	9.1	26.6	53.57
		WP_063861030.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	9.1	26.6	53.57
		WP_002033109.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	8.6	26.6	53.57
		WP_002033109.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	8.6	26.6	53.57
		WP_002033109.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	8.6	26.6	53.57
		WP_057094079.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	9.7	26.6	53.57
		WP_057094079.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	9.7	26.6	53.57
		WP_057094079.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	9.7	26.6	53.57
		WP_049581961.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	8.6	26.6	53.57
		WP_049581961.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	8.6	26.6	53.57
		WP_049581961.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	8.6	26.6	53.57
		WP_049068184.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	8.5	26.6	53.57
		WP_049068184.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	8.5	26.6	53.57
		WP_049068184.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	8.5	26.6	53.57
		WP_039208445.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	8	26.6	53.57
		WP_039208445.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	8	26.6	53.57
		WP_039208445.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	8	26.6	53.57
		WP_038348482.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	8.7	26.6	53.57
		WP_038348482.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	8.7	26.6	53.57
		WP_038348482.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	8.7	26.6	53.57

		WP_038348285.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	8.9	26.6	53.57
		WP_038348285.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	8.9	26.6	53.57
		WP_038348285.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	8.9	26.6	53.57
		WP_032058439.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	9.2	26.6	53.57
		WP_032058439.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	9.2	26.6	53.57
		WP_032058439.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	9.2	26.6	53.57
		WP_032051604.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	9	26.6	53.57
		WP_032051604.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	9	26.6	53.57
		WP_032051604.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	9	26.6	53.57
		WP_032030965.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	8.6	26.6	53.57
		WP_032030965.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	8.6	26.6	53.57
		WP_032030965.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	8.6	26.6	53.57
		WP_031978611.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	8.9	26.6	53.57
		WP_031978611.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	8.9	26.6	53.57
		WP_031978611.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	8.9	26.6	53.57
		WP_031976872.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	9.6	26.6	53.57
		WP_031976872.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	9.6	26.6	53.57
		WP_031976872.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	9.6	26.6	53.57
		WP_031960432.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	9	26.6	53.57
		WP_031960432.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	9	26.6	53.57
		WP_031960432.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	9	26.6	53.57
		WP_029424390.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	8.9	26.6	53.57
		WP_029424390.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	8.9	26.6	53.57
		WP_029424390.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	8.9	26.6	53.57
		WP_024438993.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	8.4	26.6	53.57
		WP_024438993.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	8.4	26.6	53.57
		WP_024438993.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	8.4	26.6	53.57
		WP_024433915.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	8.9	26.6	53.57

		WP_024433915.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	8.9	26.6	53.57
		WP_024433915.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	8.9	26.6	53.57
		WP_017816859.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	8.7	26.6	53.57
		WP_017816859.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	8.7	26.6	53.57
		WP_017816859.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	8.7	26.6	53.57
		WP_005139262.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	9	26.6	53.57
		WP_005139262.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	9	26.6	53.57
		WP_005139262.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	9	26.6	53.57
		WP_005132156.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	8.4	26.6	53.57
		WP_005132156.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	8.4	26.6	53.57
		WP_005132156.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	8.4	26.6	53.57
		WP_005120067.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	8.9	26.6	53.57
		WP_005120067.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	8.9	26.6	53.57
		WP_005120067.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	8.9	26.6	53.57
		WP_002057397.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	8.1	26.6	53.57
		WP_002057397.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	8.1	26.6	53.57
		WP_002057397.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	8.1	26.6	53.57
		WP_001022758.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	8.6	26.6	53.57
		WP_001022758.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	8.6	26.6	53.57
		WP_001022758.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	8.6	26.6	53.57
		WP_001022756.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	7.8	26.6	53.57
		WP_001022756.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	7.8	26.6	53.57
		WP_001022756.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	7.8	26.6	53.57
		WP_001021787.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	8.7	26.6	53.57
		WP_001021787.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	8.7	26.6	53.57
		WP_001021787.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	8.7	26.6	53.57
		WP_001021786.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	9.2	26.6	53.57
		WP_001021786.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	9.2	26.6	53.57

		WP_001021786.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	9.2	26.6	53.57
		WP_001021784.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	8.7	26.6	53.57
		WP_001021784.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	8.7	26.6	53.57
		WP_001021784.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	8.7	26.6	53.57
		WP_001021783.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	8.7	26.6	53.57
		WP_001021783.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	8.7	26.6	53.57
		WP_001021783.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	8.7	26.6	53.57
		WP_001021781.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	9	26.6	53.57
		WP_001021781.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	9	26.6	53.57
		WP_001021781.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	9	26.6	53.57
		WP_001021778.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	8.9	26.6	53.57
		WP_001021778.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	8.9	26.6	53.57
		WP_001021778.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	8.9	26.6	53.57
		WP_001021777.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	9	26.6	53.57
		WP_001021777.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	9	26.6	53.57
		WP_001021777.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	9	26.6	53.57
		WP_001021775.1	NZ_CP039021.1	35.714	28	18	14	41	561454	561537	83	8.6	26.6	53.57
		WP_001021775.1	NZ_CP039022.1	35.714	28	18	14	41	561454	561537	83	8.6	26.6	53.57
		WP_001021775.1	NC_002929.2	35.714	28	18	14	41	561454	561537	83	8.6	26.6	53.57
		WP_104009854.1	NZ_CP039021.1	35.484	31	20	225	255	2635545	2635453	-92	7.6	26.9	58.06
		WP_104009854.1	NZ_CP039022.1	35.484	31	20	225	255	2635545	2635453	-92	7.6	26.9	58.06
		WP_104009854.1	NC_002929.2	35.484	31	20	225	255	2635545	2635453	-92	7.6	26.9	58.06
		WP_063864794.1	NZ_CP039021.1	35.484	31	20	225	255	2635545	2635453	-92	9.9	26.6	58.06
		WP_063864794.1	NZ_CP039022.1	35.484	31	20	225	255	2635545	2635453	-92	9.9	26.6	58.06
		WP_063864794.1	NC_002929.2	35.484	31	20	225	255	2635545	2635453	-92	9.9	26.6	58.06
	BP0651	WP_000778180.1	NZ_CP039021.1	35.417	48	27	206	249	659105	659248	143	2	29.3	54.17
		WP_000778180.1	NZ_CP039022.1	35.417	48	27	206	249	659105	659248	143	2	29.3	54.17
		WP_000778180.1	NC_002929.2	35.417	48	27	206	249	659105	659248	143	2	29.3	54.17

		WP_044403015.1	NZ_CP039021.1	35.385	65	33	53	112	2762208	2762026	-182	0.011	35.8	56.92
		WP_044403015.1	NZ_CP039022.1	35.385	65	33	53	112	2762208	2762026	-182	0.011	35.8	56.92
		WP_044403015.1	NC_002929.2	35.385	65	33	53	112	2762208	2762026	-182	0.011	35.8	56.92
	glnE	WP_063842628.1	NZ_CP039021.1	35.135	37	24	39	75	1330486	1330596	110	1.2	29.3	45.95
		WP_063842628.1	NZ_CP039022.1	35.135	37	24	39	75	1330486	1330596	110	1.2	29.3	45.95
		WP_063842628.1	NC_002929.2	35.135	37	24	39	75	1330486	1330596	110	1.2	29.3	45.95
	pmi	WP_109545072.1	NZ_CP039021.1	35.135	37	24	81	117	931375	931485	110	5.7	26.9	37.84
		WP_109545072.1	NZ_CP039022.1	35.135	37	24	81	117	931375	931485	110	5.7	26.9	37.84
		WP_109545072.1	NC_002929.2	35.135	37	24	81	117	931375	931485	110	5.7	26.9	37.84
		WP_063862739.1	NZ_CP039021.1	35.135	37	24	81	117	931375	931485	110	3.9	27.7	37.84
		WP_063862739.1	NZ_CP039022.1	35.135	37	24	81	117	931375	931485	110	3.9	27.7	37.84
		WP_063862739.1	NC_002929.2	35.135	37	24	81	117	931375	931485	110	3.9	27.7	37.84
		WP_063842699.1	NZ_CP039021.1	35.106	94	39	98	189	3424755	3424976	221	0.002	38.5	44.68
		WP_063842699.1	NZ_CP039022.1	35.106	94	39	98	189	3424755	3424976	221	0.002	38.5	44.68
		WP_063842699.1	NC_002929.2	35.106	94	39	98	189	3424755	3424976	221	0.002	38.5	44.68
		WP_063842698.1	NZ_CP039021.1	35.106	94	39	97	188	3424755	3424976	221	2.94E-05	44.3	47.87
		WP_063842698.1	NZ_CP039022.1	35.106	94	39	97	188	3424755	3424976	221	2.94E-05	44.3	47.87
		WP_063842698.1	NC_002929.2	35.106	94	39	97	188	3424755	3424976	221	2.94E-05	44.3	47.87
		WP_063842696.1	NZ_CP039021.1	35.106	94	39	98	189	3424755	3424976	221	0.002	38.5	44.68
		WP_063842696.1	NZ_CP039022.1	35.106	94	39	98	189	3424755	3424976	221	0.002	38.5	44.68
		WP_063842696.1	NC_002929.2	35.106	94	39	98	189	3424755	3424976	221	0.002	38.5	44.68
		WP_063842691.1	NZ_CP039021.1	35.106	94	39	98	189	3424755	3424976	221	0.001	39.3	44.68
		WP_063842691.1	NZ_CP039022.1	35.106	94	39	98	189	3424755	3424976	221	0.001	39.3	44.68
		WP_063842691.1	NC_002929.2	35.106	94	39	98	189	3424755	3424976	221	0.001	39.3	44.68
		WP_063842689.1	NZ_CP039021.1	35.106	94	39	98	189	3424755	3424976	221	0.002	38.5	44.68
		WP_063842689.1	NZ_CP039022.1	35.106	94	39	98	189	3424755	3424976	221	0.002	38.5	44.68
		WP_063842689.1	NC_002929.2	35.106	94	39	98	189	3424755	3424976	221	0.002	38.5	44.68
		WP_063842688.1	NZ_CP039021.1	35.106	94	39	98	189	3424755	3424976	221	0.002	38.5	44.68

			WP_063842688.1	NZ_CP039022.1	35.106	94	39	98	189	3424755	3424976	221	0.002	38.5	44.68
			WP_063842688.1	NC_002929.2	35.106	94	39	98	189	3424755	3424976	221	0.002	38.5	44.68
			WP_063842669.1	NZ_CP039021.1	35.106	94	39	98	189	3424755	3424976	221	0.002	38.5	44.68
			WP_063842669.1	NZ_CP039022.1	35.106	94	39	98	189	3424755	3424976	221	0.002	38.5	44.68
			WP_063842669.1	NC_002929.2	35.106	94	39	98	189	3424755	3424976	221	0.002	38.5	44.68
			WP_032966056.1	NZ_CP039021.1	35.106	94	39	98	189	3424755	3424976	221	0.002	38.5	44.68
			WP_032966056.1	NZ_CP039022.1	35.106	94	39	98	189	3424755	3424976	221	0.002	38.5	44.68
			WP_032966056.1	NC_002929.2	35.106	94	39	98	189	3424755	3424976	221	0.002	38.5	44.68
			WP_063842690.1	NZ_CP039021.1	35.052	97	57	77	173	3424704	3424976	272	2.58E-04	41.2	47.42
			WP_063842690.1	NZ_CP039022.1	35.052	97	57	77	173	3424704	3424976	272	2.58E-04	41.2	47.42
			WP_063842690.1	NC_002929.2	35.052	97	57	77	173	3424704	3424976	272	2.58E-04	41.2	47.42
			WP_046430859.1	NZ_CP039021.1	35.052	97	57	77	173	3424704	3424976	272	2.49E-04	41.2	47.42
			WP_046430859.1	NZ_CP039022.1	35.052	97	57	77	173	3424704	3424976	272	2.49E-04	41.2	47.42
			WP_046430859.1	NC_002929.2	35.052	97	57	77	173	3424704	3424976	272	2.49E-04	41.2	47.42
			WP_063842690.1	NZ_CP039021.1	35	40	26	158	197	520970	520851	-119	8.5	26.6	42.5
			WP_063842690.1	NZ_CP039022.1	35	40	26	158	197	520970	520851	-119	8.5	26.6	42.5
			WP_063842690.1	NC_002929.2	35	40	26	158	197	520970	520851	-119	8.5	26.6	42.5
			WP_046430859.1	NZ_CP039021.1	35	40	26	158	197	520970	520851	-119	8.8	26.6	42.5
			WP_046430859.1	NZ_CP039022.1	35	40	26	158	197	520970	520851	-119	8.8	26.6	42.5
			WP_046430859.1	NC_002929.2	35	40	26	158	197	520970	520851	-119	8.8	26.6	42.5
			WP_063843234.1	NZ_CP039021.1	34.921	63	33	163	224	3654764	3654931	167	0.28	32	47.62
			WP_063843234.1	NZ_CP039022.1	34.921	63	33	163	224	3654764	3654931	167	0.28	32	47.62
			WP_063843234.1	NC_002929.2	34.921	63	33	163	224	3654764	3654931	167	0.28	32	47.62
		BP0284	WP_001046004.1	NZ_CP039021.1	34.884	43	27	210	251	290168	290040	-128	4	27.7	48.84
			WP_001046004.1	NZ_CP039022.1	34.884	43	27	210	251	290168	290040	-128	4	27.7	48.84
			WP_001046004.1	NC_002929.2	34.884	43	27	210	251	290168	290040	-128	4	27.7	48.84
			WP_063864531.1	NZ_CP039021.1	34.884	43	27	210	251	290168	290040	-128	3	28.1	51.16
			WP_063864531.1	NZ_CP039022.1	34.884	43	27	210	251	290168	290040	-128	3	28.1	51.16

		WP_063864531.1	NC_002929.2	34.884	43	27	210	251	290168	290040	-128	3	28.1	51.16
		WP_063864108.1	NZ_CP039021.1	34.884	43	27	210	251	290168	290040	-128	4.3	27.7	48.84
		WP_063864108.1	NZ_CP039022.1	34.884	43	27	210	251	290168	290040	-128	4.3	27.7	48.84
		WP_063864108.1	NC_002929.2	34.884	43	27	210	251	290168	290040	-128	4.3	27.7	48.84
		WP_063864078.1	NZ_CP039021.1	34.884	43	27	210	251	290168	290040	-128	3.6	27.7	48.84
		WP_063864078.1	NZ_CP039022.1	34.884	43	27	210	251	290168	290040	-128	3.6	27.7	48.84
		WP_063864078.1	NC_002929.2	34.884	43	27	210	251	290168	290040	-128	3.6	27.7	48.84
		WP_063862833.1	NZ_CP039021.1	34.884	43	27	210	251	290168	290040	-128	3.9	27.7	48.84
		WP_063862833.1	NZ_CP039022.1	34.884	43	27	210	251	290168	290040	-128	3.9	27.7	48.84
		WP_063862833.1	NC_002929.2	34.884	43	27	210	251	290168	290040	-128	3.9	27.7	48.84
		WP_063862828.1	NZ_CP039021.1	34.884	43	27	210	251	290168	290040	-128	3.8	27.7	48.84
		WP_063862828.1	NZ_CP039022.1	34.884	43	27	210	251	290168	290040	-128	3.8	27.7	48.84
		WP_063862828.1	NC_002929.2	34.884	43	27	210	251	290168	290040	-128	3.8	27.7	48.84
		WP_063862736.1	NZ_CP039021.1	34.884	43	27	210	251	290168	290040	-128	3.9	27.7	48.84
		WP_063862736.1	NZ_CP039022.1	34.884	43	27	210	251	290168	290040	-128	3.9	27.7	48.84
		WP_063862736.1	NC_002929.2	34.884	43	27	210	251	290168	290040	-128	3.9	27.7	48.84
		WP_063862443.1	NZ_CP039021.1	34.884	43	27	210	251	290168	290040	-128	2.9	28.1	51.16
		WP_063862443.1	NZ_CP039022.1	34.884	43	27	210	251	290168	290040	-128	2.9	28.1	51.16
		WP_063862443.1	NC_002929.2	34.884	43	27	210	251	290168	290040	-128	2.9	28.1	51.16
		WP_063861157.1	NZ_CP039021.1	34.884	43	27	210	251	290168	290040	-128	3.8	27.7	48.84
		WP_063861157.1	NZ_CP039022.1	34.884	43	27	210	251	290168	290040	-128	3.8	27.7	48.84
		WP_063861157.1	NC_002929.2	34.884	43	27	210	251	290168	290040	-128	3.8	27.7	48.84
		WP_063861151.1	NZ_CP039021.1	34.884	43	27	210	251	290168	290040	-128	4	27.7	48.84
		WP_063861151.1	NZ_CP039022.1	34.884	43	27	210	251	290168	290040	-128	4	27.7	48.84
		WP_063861151.1	NC_002929.2	34.884	43	27	210	251	290168	290040	-128	4	27.7	48.84
		WP_063861140.1	NZ_CP039021.1	34.884	43	27	210	251	290168	290040	-128	3.8	27.7	48.84
		WP_063861140.1	NZ_CP039022.1	34.884	43	27	210	251	290168	290040	-128	3.8	27.7	48.84
		WP_063861140.1	NC_002929.2	34.884	43	27	210	251	290168	290040	-128	3.8	27.7	48.84

		WP_063861136.1	NZ_CP039021.1	34.884	43	27	210	251	290168	290040	-128	4.7	27.3	48.84
		WP_063861136.1	NZ_CP039022.1	34.884	43	27	210	251	290168	290040	-128	4.7	27.3	48.84
		WP_063861136.1	NC_002929.2	34.884	43	27	210	251	290168	290040	-128	4.7	27.3	48.84
		WP_063861133.1	NZ_CP039021.1	34.884	43	27	210	251	290168	290040	-128	3.9	27.7	48.84
		WP_063861133.1	NZ_CP039022.1	34.884	43	27	210	251	290168	290040	-128	3.9	27.7	48.84
		WP_063861133.1	NC_002929.2	34.884	43	27	210	251	290168	290040	-128	3.9	27.7	48.84
		WP_063861129.1	NZ_CP039021.1	34.884	43	27	210	251	290168	290040	-128	3.9	27.7	48.84
		WP_063861129.1	NZ_CP039022.1	34.884	43	27	210	251	290168	290040	-128	3.9	27.7	48.84
		WP_063861129.1	NC_002929.2	34.884	43	27	210	251	290168	290040	-128	3.9	27.7	48.84
		WP_063861123.1	NZ_CP039021.1	34.884	43	27	210	251	290168	290040	-128	5.3	27.3	48.84
		WP_063861123.1	NZ_CP039022.1	34.884	43	27	210	251	290168	290040	-128	5.3	27.3	48.84
		WP_063861123.1	NC_002929.2	34.884	43	27	210	251	290168	290040	-128	5.3	27.3	48.84
		WP_063861038.1	NZ_CP039021.1	34.884	43	27	210	251	290168	290040	-128	4.3	27.7	48.84
		WP_063861038.1	NZ_CP039022.1	34.884	43	27	210	251	290168	290040	-128	4.3	27.7	48.84
		WP_063861038.1	NC_002929.2	34.884	43	27	210	251	290168	290040	-128	4.3	27.7	48.84
		WP_005025422.1	NZ_CP039021.1	34.884	43	27	210	251	290168	290040	-128	4.4	27.3	48.84
		WP_005025422.1	NZ_CP039022.1	34.884	43	27	210	251	290168	290040	-128	4.4	27.3	48.84
		WP_005025422.1	NC_002929.2	34.884	43	27	210	251	290168	290040	-128	4.4	27.3	48.84
		WP_012698469.1	NZ_CP039021.1	34.694	49	31	159	207	802739	802596	-143	1.7	29.6	53.06
		WP_012698469.1	NZ_CP039022.1	34.694	49	31	159	207	802739	802596	-143	1.7	29.6	53.06
		WP_012698469.1	NC_002929.2	34.694	49	31	159	207	802739	802596	-143	1.7	29.6	53.06
	BP0671	WP_064808881.1	NZ_CP039021.1	34.694	49	28	158	202	684757	684903	146	4.3	27.7	46.94
		WP_064808881.1	NZ_CP039022.1	34.694	49	28	158	202	684757	684903	146	4.3	27.7	46.94
		WP_064808881.1	NC_002929.2	34.694	49	28	158	202	684757	684903	146	4.3	27.7	46.94
		WP_063842911.1	NZ_CP039021.1	34.615	78	43	150	227	3335395	3335604	209	1.54E-07	50.4	57.69
		WP_063842911.1	NZ_CP039022.1	34.615	78	43	150	227	3335395	3335604	209	1.54E-07	50.4	57.69
		WP_063842911.1	NC_002929.2	34.615	78	43	150	227	3335395	3335604	209	1.54E-07	50.4	57.69
		WP_102607463.1	NZ_CP039021.1	34.615	52	30	158	205	684757	684912	155	3.3	28.1	46.15

		WP_102607463.1	NZ_CP039022.1	34.615	52	30	158	205	684757	684912	155	3.3	28.1	46.15
		WP_102607463.1	NC_002929.2	34.615	52	30	158	205	684757	684912	155	3.3	28.1	46.15
		WP_045219469.1	NZ_CP039021.1	34.615	52	30	158	205	684757	684912	155	3	28.1	46.15
		WP_045219469.1	NZ_CP039022.1	34.615	52	30	158	205	684757	684912	155	3	28.1	46.15
		WP_045219469.1	NC_002929.2	34.615	52	30	158	205	684757	684912	155	3	28.1	46.15
		WP_104009831.1	NZ_CP039021.1	34.545	55	32	56	109	3838096	3838251	155	0.23	32	47.27
		WP_104009831.1	NZ_CP039022.1	34.545	55	32	56	109	3838096	3838251	155	0.23	32	47.27
		WP_104009831.1	NC_002929.2	34.545	55	32	56	109	3838096	3838251	155	0.23	32	47.27
		WP_001100752.1	NZ_CP039021.1	34.545	55	32	56	109	3838096	3838251	155	0.21	32	47.27
		WP_001100752.1	NZ_CP039022.1	34.545	55	32	56	109	3838096	3838251	155	0.21	32	47.27
		WP_001100752.1	NC_002929.2	34.545	55	32	56	109	3838096	3838251	155	0.21	32	47.27
		WP_063864593.1	NZ_CP039021.1	34.545	55	32	56	109	3838096	3838251	155	0.2	32	47.27
		WP_063864593.1	NZ_CP039022.1	34.545	55	32	56	109	3838096	3838251	155	0.2	32	47.27
		WP_063864593.1	NC_002929.2	34.545	55	32	56	109	3838096	3838251	155	0.2	32	47.27
		WP_049637349.1	NZ_CP039021.1	34.545	55	32	56	109	3838096	3838251	155	0.22	32	47.27
		WP_049637349.1	NZ_CP039022.1	34.545	55	32	56	109	3838096	3838251	155	0.22	32	47.27
		WP_049637349.1	NC_002929.2	34.545	55	32	56	109	3838096	3838251	155	0.22	32	47.27
		WP_001100753.1	NZ_CP039021.1	34.545	55	32	56	109	3838096	3838251	155	0.21	32	47.27
		WP_001100753.1	NZ_CP039022.1	34.545	55	32	56	109	3838096	3838251	155	0.21	32	47.27
		WP_001100753.1	NC_002929.2	34.545	55	32	56	109	3838096	3838251	155	0.21	32	47.27
		WP_063864594.1	NZ_CP039021.1	34.545	55	32	56	109	3838096	3838251	155	0.22	32	47.27
		WP_063864594.1	NZ_CP039022.1	34.545	55	32	56	109	3838096	3838251	155	0.22	32	47.27
		WP_063864594.1	NC_002929.2	34.545	55	32	56	109	3838096	3838251	155	0.22	32	47.27
		WP_063864597.1	NZ_CP039021.1	34.545	55	32	56	109	3838096	3838251	155	0.21	32	47.27
		WP_063864597.1	NZ_CP039022.1	34.545	55	32	56	109	3838096	3838251	155	0.21	32	47.27
		WP_063864597.1	NC_002929.2	34.545	55	32	56	109	3838096	3838251	155	0.21	32	47.27
		WP_063864596.1	NZ_CP039021.1	34.545	55	32	56	109	3838096	3838251	155	0.21	32	47.27
		WP_063864596.1	NZ_CP039022.1	34.545	55	32	56	109	3838096	3838251	155	0.21	32	47.27

		WP_063864596.1	NC_002929.2	34.545	55	32	56	109	3838096	3838251	155	0.21	32	47.27
		WP_032495440.1	NZ_CP039021.1	34.545	55	32	56	109	3838096	3838251	155	0.23	32	47.27
		WP_032495440.1	NZ_CP039022.1	34.545	55	32	56	109	3838096	3838251	155	0.23	32	47.27
		WP_032495440.1	NC_002929.2	34.545	55	32	56	109	3838096	3838251	155	0.23	32	47.27
		WP_045219469.1	NZ_CP039021.1	34.483	58	30	109	159	3858259	3858429	170	2.5	28.5	55.17
		WP_045219469.1	NZ_CP039022.1	34.483	58	30	109	159	3858259	3858429	170	2.5	28.5	55.17
		WP_045219469.1	NC_002929.2	34.483	58	30	109	159	3858259	3858429	170	2.5	28.5	55.17
		WP_063842697.1	NZ_CP039021.1	34.375	64	41	54	116	2762208	2762017	-191	0.12	32.7	56.25
		WP_063842697.1	NZ_CP039022.1	34.375	64	41	54	116	2762208	2762017	-191	0.12	32.7	56.25
		WP_063842697.1	NC_002929.2	34.375	64	41	54	116	2762208	2762017	-191	0.12	32.7	56.25
		WP_063842693.1	NZ_CP039021.1	34.375	64	41	54	116	2762208	2762017	-191	0.25	31.6	54.69
		WP_063842693.1	NZ_CP039022.1	34.375	64	41	54	116	2762208	2762017	-191	0.25	31.6	54.69
		WP_063842693.1	NC_002929.2	34.375	64	41	54	116	2762208	2762017	-191	0.25	31.6	54.69
		WP_063842676.1	NZ_CP039021.1	34.375	64	41	55	117	2762208	2762017	-191	0.15	32.3	54.69
		WP_063842676.1	NZ_CP039022.1	34.375	64	41	55	117	2762208	2762017	-191	0.15	32.3	54.69
		WP_063842676.1	NC_002929.2	34.375	64	41	55	117	2762208	2762017	-191	0.15	32.3	54.69
	BP0696	WP_063860799.1	NZ_CP039021.1	34.375	32	21	22	53	711112	711017	-95	3.9	27.7	65.62
		WP_063860799.1	NZ_CP039022.1	34.375	32	21	22	53	711112	711017	-95	3.9	27.7	65.62
		WP_063860799.1	NC_002929.2	34.375	32	21	22	53	711112	711017	-95	3.9	27.7	65.62
		WP_122630835.1	NZ_CP039021.1	34.247	73	43	148	220	3335401	3335604	203	7.26E-06	45.4	50.68
		WP_122630835.1	NZ_CP039022.1	34.247	73	43	148	220	3335401	3335604	203	7.26E-06	45.4	50.68
		WP_122630835.1	NC_002929.2	34.247	73	43	148	220	3335401	3335604	203	7.26E-06	45.4	50.68
	msrB	WP_063860587.1	NZ_CP039021.1	34.211	38	20	152	184	3778592	3778705	113	9.3	26.2	47.37
		WP_063860587.1	NZ_CP039022.1	34.211	38	20	152	184	3778592	3778705	113	9.3	26.2	47.37
		WP_063860587.1	NC_002929.2	34.211	38	20	152	184	3778592	3778705	113	9.3	26.2	47.37
	BP1899	WP_099982802.1	NZ_CP039021.1	34.091	44	27	43	84	2001772	2001641	-131	7.9	26.9	54.55
		WP_099982802.1	NZ_CP039022.1	34.091	44	27	43	84	2001772	2001641	-131	7.9	26.9	54.55
		WP_099982802.1	NC_002929.2	34.091	44	27	43	84	2001772	2001641	-131	7.9	26.9	54.55

		WP_063842704.1	NZ_CP039021.1	34.091	44	27	42	83	2001772	2001641	-131	7	26.9	54.55
		WP_063842704.1	NZ_CP039022.1	34.091	44	27	42	83	2001772	2001641	-131	7	26.9	54.55
		WP_063842704.1	NC_002929.2	34.091	44	27	42	83	2001772	2001641	-131	7	26.9	54.55
		WP_063842694.1	NZ_CP039021.1	34.043	94	40	97	188	3424755	3424976	221	1.80E-04	41.6	46.81
		WP_063842694.1	NZ_CP039022.1	34.043	94	40	97	188	3424755	3424976	221	1.80E-04	41.6	46.81
		WP_063842694.1	NC_002929.2	34.043	94	40	97	188	3424755	3424976	221	1.80E-04	41.6	46.81
		WP_063842676.1	NZ_CP039021.1	34.043	94	40	98	189	3424755	3424976	221	0.002	38.5	44.68
		WP_063842676.1	NZ_CP039022.1	34.043	94	40	98	189	3424755	3424976	221	0.002	38.5	44.68
		WP_063842676.1	NC_002929.2	34.043	94	40	98	189	3424755	3424976	221	0.002	38.5	44.68
		WP_063842696.1	NZ_CP039021.1	33.929	56	36	63	117	2762184	2762017	-167	0.36	31.2	58.93
		WP_063842696.1	NZ_CP039022.1	33.929	56	36	63	117	2762184	2762017	-167	0.36	31.2	58.93
		WP_063842696.1	NC_002929.2	33.929	56	36	63	117	2762184	2762017	-167	0.36	31.2	58.93
		WP_063842691.1	NZ_CP039021.1	33.929	56	36	63	117	2762184	2762017	-167	0.21	32	58.93
		WP_063842691.1	NZ_CP039022.1	33.929	56	36	63	117	2762184	2762017	-167	0.21	32	58.93
		WP_063842691.1	NC_002929.2	33.929	56	36	63	117	2762184	2762017	-167	0.21	32	58.93
	mfd	WP_063857696.1	NZ_CP039021.1	33.898	59	33	217	274	899497	899336	-161	5.6	27.3	45.76
		WP_063857696.1	NZ_CP039022.1	33.898	59	33	217	274	899497	899336	-161	5.6	27.3	45.76
		WP_063857696.1	NC_002929.2	33.898	59	33	217	274	899497	899336	-161	5.6	27.3	45.76
	BP1784	WP_011816299.1	NZ_CP039021.1	33.824	68	44	168	235	1874830	1875030	200	0.48	30.8	45.59
		WP_011816299.1	NZ_CP039022.1	33.824	68	44	168	235	1874830	1875030	200	0.48	30.8	45.59
		WP_011816299.1	NC_002929.2	33.824	68	44	168	235	1874830	1875030	200	0.48	30.8	45.59
		WP_063859390.1	NZ_CP039021.1	33.803	71	41	64	130	268248	268042	-206	4.5	27.7	47.89
		WP_063859390.1	NZ_CP039022.1	33.803	71	41	64	130	268248	268042	-206	4.5	27.7	47.89
		WP_063859390.1	NC_002929.2	33.803	71	41	64	130	268248	268042	-206	4.5	27.7	47.89
		WP_102607463.1	NZ_CP039021.1	33.333	60	32	109	161	3858259	3858435	176	2.3	28.5	53.33
		WP_102607463.1	NZ_CP039022.1	33.333	60	32	109	161	3858259	3858435	176	2.3	28.5	53.33
		WP_102607463.1	NC_002929.2	33.333	60	32	109	161	3858259	3858435	176	2.3	28.5	53.33
	BP3443	WP_042649345.1	NZ_CP039021.1	33.333	57	31	171	227	3654782	3654931	149	3	28.9	43.86

		WP_042649345.1	NZ_CP039022.1	33.333	57	31	171	227	3654782	3654931	149	3	28.9	43.86
		WP_042649345.1	NC_002929.2	33.333	57	31	171	227	3654782	3654931	149	3	28.9	43.86
	_BP3443_	WP_010673861.1	NZ_CP039021.1	33.333	63	34	166	227	3654764	3654931	167	0.36	31.6	50.79
		WP_010673861.1	NZ_CP039022.1	33.333	63	34	166	227	3654764	3654931	167	0.36	31.6	50.79
		WP_010673861.1	NC_002929.2	33.333	63	34	166	227	3654764	3654931	167	0.36	31.6	50.79
		WP_032489888.1	NZ_CP039021.1	33.333	63	34	166	227	3654764	3654931	167	0.21	32.3	50.79
		WP_032489888.1	NZ_CP039022.1	33.333	63	34	166	227	3654764	3654931	167	0.21	32.3	50.79
		WP_032489888.1	NC_002929.2	33.333	63	34	166	227	3654764	3654931	167	0.21	32.3	50.79
		WP_012477380.1	NZ_CP039021.1	33.333	63	34	166	227	3654764	3654931	167	0.2	32.7	50.79
		WP_012477380.1	NZ_CP039022.1	33.333	63	34	166	227	3654764	3654931	167	0.2	32.7	50.79
		WP_012477380.1	NC_002929.2	33.333	63	34	166	227	3654764	3654931	167	0.2	32.7	50.79
		WP_063860847.1	NZ_CP039021.1	33.333	63	34	166	227	3654764	3654931	167	0.33	32	50.79
		WP_063860847.1	NZ_CP039022.1	33.333	63	34	166	227	3654764	3654931	167	0.33	32	50.79
		WP_063860847.1	NC_002929.2	33.333	63	34	166	227	3654764	3654931	167	0.33	32	50.79
		WP_063860846.1	NZ_CP039021.1	33.333	63	34	166	227	3654764	3654931	167	0.36	31.6	50.79
		WP_063860846.1	NZ_CP039022.1	33.333	63	34	166	227	3654764	3654931	167	0.36	31.6	50.79
		WP_063860846.1	NC_002929.2	33.333	63	34	166	227	3654764	3654931	167	0.36	31.6	50.79
		WP_063860845.1	NZ_CP039021.1	33.333	63	34	166	227	3654764	3654931	167	0.33	32	50.79
		WP_063860845.1	NZ_CP039022.1	33.333	63	34	166	227	3654764	3654931	167	0.33	32	50.79
		WP_063860845.1	NC_002929.2	33.333	63	34	166	227	3654764	3654931	167	0.33	32	50.79
		WP_063860844.1	NZ_CP039021.1	33.333	63	34	166	227	3654764	3654931	167	0.35	31.6	50.79
		WP_063860844.1	NZ_CP039022.1	33.333	63	34	166	227	3654764	3654931	167	0.35	31.6	50.79
		WP_063860844.1	NC_002929.2	33.333	63	34	166	227	3654764	3654931	167	0.35	31.6	50.79
		WP_063860843.1	NZ_CP039021.1	33.333	63	34	166	227	3654764	3654931	167	0.37	31.6	50.79
		WP_063860843.1	NZ_CP039022.1	33.333	63	34	166	227	3654764	3654931	167	0.37	31.6	50.79
		WP_063860843.1	NC_002929.2	33.333	63	34	166	227	3654764	3654931	167	0.37	31.6	50.79
		WP_063859897.1	NZ_CP039021.1	33.333	63	34	166	227	3654764	3654931	167	0.2	32.3	50.79
		WP_063859897.1	NZ_CP039022.1	33.333	63	34	166	227	3654764	3654931	167	0.2	32.3	50.79

		WP_063859897.1	NC_002929.2	33.333	63	34	166	227	3654764	3654931	167	0.2	32.3	50.79
		WP_063859826.1	NZ_CP039021.1	33.333	63	34	166	227	3654764	3654931	167	0.22	32.3	50.79
		WP_063859826.1	NZ_CP039022.1	33.333	63	34	166	227	3654764	3654931	167	0.22	32.3	50.79
		WP_063859826.1	NC_002929.2	33.333	63	34	166	227	3654764	3654931	167	0.22	32.3	50.79
		WP_032490177.1	NZ_CP039021.1	33.333	63	34	166	227	3654764	3654931	167	0.2	32.3	50.79
		WP_032490177.1	NZ_CP039022.1	33.333	63	34	166	227	3654764	3654931	167	0.2	32.3	50.79
		WP_032490177.1	NC_002929.2	33.333	63	34	166	227	3654764	3654931	167	0.2	32.3	50.79
	gloB	WP_032492430.1	NZ_CP039021.1	33.333	36	24	71	106	3424698	3424805	107	0.062	33.1	55.56
		WP_032492430.1	NZ_CP039022.1	33.333	36	24	71	106	3424698	3424805	107	0.062	33.1	55.56
		WP_032492430.1	NC_002929.2	33.333	36	24	71	106	3424698	3424805	107	0.062	33.1	55.56
	BP2809	WP_063864722.1	NZ_CP039021.1	33.333	42	27	181	222	2983466	2983344	-122	8.7	26.2	52.38
		WP_063864722.1	NZ_CP039022.1	33.333	42	27	181	222	2983466	2983344	-122	8.7	26.2	52.38
		WP_063864722.1	NC_002929.2	33.333	42	27	181	222	2983466	2983344	-122	8.7	26.2	52.38
	BP2125	WP_102607464.1	NZ_CP039021.1	33.333	30	20	208	237	2249128	2249217	89	8	26.6	50
		WP_102607464.1	NZ_CP039022.1	33.333	30	20	208	237	2249128	2249217	89	8	26.6	50
		WP_102607464.1	NC_002929.2	33.333	30	20	208	237	2249128	2249217	89	8	26.6	50
	BP0780	WP_104531865.1	NZ_CP039021.1	33.333	51	33	159	209	802739	802590	-149	1	30.4	52.94
		WP_104531865.1	NZ_CP039022.1	33.333	51	33	159	209	802739	802590	-149	1	30.4	52.94
		WP_104531865.1	NC_002929.2	33.333	51	33	159	209	802739	802590	-149	1	30.4	52.94
	BP0654	WP_001443153.1	NZ_CP039021.1	33.333	51	20	109	152	662628	662497	-131	6.2	27.7	49.02
		WP_001443153.1	NZ_CP039022.1	33.333	51	20	109	152	662628	662497	-131	6.2	27.7	49.02
		WP_001443153.1	NC_002929.2	33.333	51	20	109	152	662628	662497	-131	6.2	27.7	49.02
		WP_063610930.1	NZ_CP039021.1	33.333	51	20	109	152	662628	662497	-131	6.6	27.7	49.02
		WP_063610930.1	NZ_CP039022.1	33.333	51	20	109	152	662628	662497	-131	6.6	27.7	49.02
		WP_063610930.1	NC_002929.2	33.333	51	20	109	152	662628	662497	-131	6.6	27.7	49.02
		WP_063860263.1	NZ_CP039021.1	33.333	51	20	109	152	662628	662497	-131	6	27.7	49.02
		WP_063860263.1	NZ_CP039022.1	33.333	51	20	109	152	662628	662497	-131	6	27.7	49.02
		WP_063860263.1	NC_002929.2	33.333	51	20	109	152	662628	662497	-131	6	27.7	49.02

		WP_063860256.1	NZ_CP039021.1	33.333	51	20	109	152	662628	662497	-131	6.5	27.7	49.02
		WP_063860256.1	NZ_CP039022.1	33.333	51	20	109	152	662628	662497	-131	6.5	27.7	49.02
		WP_063860256.1	NC_002929.2	33.333	51	20	109	152	662628	662497	-131	6.5	27.7	49.02
		WP_063860248.1	NZ_CP039021.1	33.333	51	20	109	152	662628	662497	-131	6.5	27.7	49.02
		WP_063860248.1	NZ_CP039022.1	33.333	51	20	109	152	662628	662497	-131	6.5	27.7	49.02
		WP_063860248.1	NC_002929.2	33.333	51	20	109	152	662628	662497	-131	6.5	27.7	49.02
		WP_063860233.1	NZ_CP039021.1	33.333	51	20	109	152	662628	662497	-131	6.6	27.7	49.02
		WP_063860233.1	NZ_CP039022.1	33.333	51	20	109	152	662628	662497	-131	6.6	27.7	49.02
		WP_063860233.1	NC_002929.2	33.333	51	20	109	152	662628	662497	-131	6.6	27.7	49.02
		WP_063860228.1	NZ_CP039021.1	33.333	51	20	109	152	662628	662497	-131	6.8	27.7	49.02
		WP_063860228.1	NZ_CP039022.1	33.333	51	20	109	152	662628	662497	-131	6.8	27.7	49.02
		WP_063860228.1	NC_002929.2	33.333	51	20	109	152	662628	662497	-131	6.8	27.7	49.02
		WP_063842627.1	NZ_CP039021.1	33.333	51	20	109	152	662628	662497	-131	6.4	27.7	49.02
		WP_063842627.1	NZ_CP039022.1	33.333	51	20	109	152	662628	662497	-131	6.4	27.7	49.02
		WP_063842627.1	NC_002929.2	33.333	51	20	109	152	662628	662497	-131	6.4	27.7	49.02
		WP_001460207.1	NZ_CP039021.1	33.333	51	20	109	152	662628	662497	-131	6.3	27.7	49.02
		WP_001460207.1	NZ_CP039022.1	33.333	51	20	109	152	662628	662497	-131	6.3	27.7	49.02
		WP_001460207.1	NC_002929.2	33.333	51	20	109	152	662628	662497	-131	6.3	27.7	49.02
		WP_001336292.1	NZ_CP039021.1	33.333	51	20	109	152	662628	662497	-131	6.9	27.3	49.02
		WP_001336292.1	NZ_CP039022.1	33.333	51	20	109	152	662628	662497	-131	6.9	27.3	49.02
		WP_001336292.1	NC_002929.2	33.333	51	20	109	152	662628	662497	-131	6.9	27.3	49.02
	BP0306	WP_065102178.1	NZ_CP039021.1	33.333	63	40	158	218	312155	312343	188	1	30	49.21
		WP_065102178.1	NZ_CP039022.1	33.333	63	40	158	218	312155	312343	188	1	30	49.21
		WP_065102178.1	NC_002929.2	33.333	63	40	158	218	312155	312343	188	1	30	49.21
		WP_063842692.1	NZ_CP039021.1	32.979	94	41	98	189	3424755	3424976	221	0.004	37.4	45.74
		WP_063842692.1	NZ_CP039022.1	32.979	94	41	98	189	3424755	3424976	221	0.004	37.4	45.74
		WP_063842692.1	NC_002929.2	32.979	94	41	98	189	3424755	3424976	221	0.004	37.4	45.74
		WP_063842699.1	NZ_CP039021.1	32.812	64	42	55	117	2762208	2762017	-191	0.62	30.4	56.25

		WP_063842699.1	NZ_CP039022.1	32.812	64	42	55	117	2762208	2762017	-191	0.62	30.4	56.25
		WP_063842699.1	NC_002929.2	32.812	64	42	55	117	2762208	2762017	-191	0.62	30.4	56.25
		WP_063842698.1	NZ_CP039021.1	32.812	64	42	54	116	2762208	2762017	-191	0.38	31.2	56.25
		WP_063842698.1	NZ_CP039022.1	32.812	64	42	54	116	2762208	2762017	-191	0.38	31.2	56.25
		WP_063842698.1	NC_002929.2	32.812	64	42	54	116	2762208	2762017	-191	0.38	31.2	56.25
		WP_063842695.1	NZ_CP039021.1	32.812	64	42	54	116	2762208	2762017	-191	0.73	30	54.69
		WP_063842695.1	NZ_CP039022.1	32.812	64	42	54	116	2762208	2762017	-191	0.73	30	54.69
		WP_063842695.1	NC_002929.2	32.812	64	42	54	116	2762208	2762017	-191	0.73	30	54.69
		WP_063842694.1	NZ_CP039021.1	32.812	64	42	54	116	2762208	2762017	-191	0.24	31.6	56.25
		WP_063842694.1	NZ_CP039022.1	32.812	64	42	54	116	2762208	2762017	-191	0.24	31.6	56.25
		WP_063842694.1	NC_002929.2	32.812	64	42	54	116	2762208	2762017	-191	0.24	31.6	56.25
		WP_063842692.1	NZ_CP039021.1	32.812	64	42	55	117	2762208	2762017	-191	0.37	31.2	56.25
		WP_063842692.1	NZ_CP039022.1	32.812	64	42	55	117	2762208	2762017	-191	0.37	31.2	56.25
		WP_063842692.1	NC_002929.2	32.812	64	42	55	117	2762208	2762017	-191	0.37	31.2	56.25
		WP_063842690.1	NZ_CP039021.1	32.812	64	42	54	116	2762208	2762017	-191	0.91	29.6	54.69
		WP_063842690.1	NZ_CP039022.1	32.812	64	42	54	116	2762208	2762017	-191	0.91	29.6	54.69
		WP_063842690.1	NC_002929.2	32.812	64	42	54	116	2762208	2762017	-191	0.91	29.6	54.69
		WP_063842689.1	NZ_CP039021.1	32.812	64	42	55	117	2762208	2762017	-191	0.37	31.2	56.25
		WP_063842689.1	NZ_CP039022.1	32.812	64	42	55	117	2762208	2762017	-191	0.37	31.2	56.25
		WP_063842689.1	NC_002929.2	32.812	64	42	55	117	2762208	2762017	-191	0.37	31.2	56.25
		WP_063842688.1	NZ_CP039021.1	32.812	64	42	55	117	2762208	2762017	-191	0.37	31.2	56.25
		WP_063842688.1	NZ_CP039022.1	32.812	64	42	55	117	2762208	2762017	-191	0.37	31.2	56.25
		WP_063842688.1	NC_002929.2	32.812	64	42	55	117	2762208	2762017	-191	0.37	31.2	56.25
		WP_063842669.1	NZ_CP039021.1	32.812	64	42	55	117	2762208	2762017	-191	0.38	31.2	56.25
		WP_063842669.1	NZ_CP039022.1	32.812	64	42	55	117	2762208	2762017	-191	0.38	31.2	56.25
		WP_063842669.1	NC_002929.2	32.812	64	42	55	117	2762208	2762017	-191	0.38	31.2	56.25
		WP_046430859.1	NZ_CP039021.1	32.812	64	42	54	116	2762208	2762017	-191	0.7	30	54.69
		WP_046430859.1	NZ_CP039022.1	32.812	64	42	54	116	2762208	2762017	-191	0.7	30	54.69

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		WP_046430859.1	NC_002929.2	32.812	64	42	54	116	2762208	2762017	-191	0.7	30	54.69
		WP_032966056.1	NZ_CP039021.1	32.812	64	42	55	117	2762208	2762017	-191	0.37	31.2	56.25
		WP_032966056.1	NZ_CP039022.1	32.812	64	42	55	117	2762208	2762017	-191	0.37	31.2	56.25
		WP_032966056.1	NC_002929.2	32.812	64	42	55	117	2762208	2762017	-191	0.37	31.2	56.25
		WP_096865225.1	NZ_CP039021.1	32.727	55	33	56	109	3838096	3838251	155	0.49	30.8	47.27
		WP_096865225.1	NZ_CP039022.1	32.727	55	33	56	109	3838096	3838251	155	0.49	30.8	47.27
		WP_096865225.1	NC_002929.2	32.727	55	33	56	109	3838096	3838251	155	0.49	30.8	47.27
		WP_032491907.1	NZ_CP039021.1	32.727	55	33	56	109	3838096	3838251	155	0.086	33.1	50.91
		WP_032491907.1	NZ_CP039022.1	32.727	55	33	56	109	3838096	3838251	155	0.086	33.1	50.91
		WP_032491907.1	NC_002929.2	32.727	55	33	56	109	3838096	3838251	155	0.086	33.1	50.91
	holA	WP_063865147.1	NZ_CP039021.1	32.558	43	27	154	196	2160320	2160198	-122	5.6	26.9	39.53
		WP_063865147.1	NZ_CP039022.1	32.558	43	27	154	196	2160320	2160198	-122	5.6	26.9	39.53
		WP_063865147.1	NC_002929.2	32.558	43	27	154	196	2160320	2160198	-122	5.6	26.9	39.53
	rpsA	WP_063864747.1	NZ_CP039021.1	32.5	40	27	70	109	989660	989779	119	5.4	27.3	47.5
		WP_063864747.1	NZ_CP039022.1	32.5	40	27	70	109	989660	989779	119	5.4	27.3	47.5
		WP_063864747.1	NC_002929.2	32.5	40	27	70	109	989660	989779	119	5.4	27.3	47.5
		WP_063842697.1	NZ_CP039021.1	32.456	114	52	77	188	3424704	3424976	272	1.72E-04	41.6	45.61
		WP_063842697.1	NZ_CP039022.1	32.456	114	52	77	188	3424704	3424976	272	1.72E-04	41.6	45.61
		WP_063842697.1	NC_002929.2	32.456	114	52	77	188	3424704	3424976	272	1.72E-04	41.6	45.61
	ahpC	WP_016141238.1	NZ_CP039021.1	32.432	37	25	21	57	3763350	3763240	-110	1.2	29.3	54.05
		WP_016141238.1	NZ_CP039022.1	32.432	37	25	21	57	3763350	3763240	-110	1.2	29.3	54.05
		WP_016141238.1	NC_002929.2	32.432	37	25	21	57	3763350	3763240	-110	1.2	29.3	54.05
	fimC	WP_000778180.1	NZ_CP039021.1	32.432	37	24	145	180	1981284	1981394	110	9.9	26.9	54.05
		WP_000778180.1	NZ_CP039022.1	32.432	37	24	145	180	1981284	1981394	110	9.9	26.9	54.05
		WP_000778180.1	NC_002929.2	32.432	37	24	145	180	1981284	1981394	110	9.9	26.9	54.05
		WP_063842693.1	NZ_CP039021.1	32.258	124	56	67	188	3424683	3424976	293	1.22E-04	42	45.97
		WP_063842693.1	NZ_CP039022.1	32.258	124	56	67	188	3424683	3424976	293	1.22E-04	42	45.97
		WP_063842693.1	NC_002929.2	32.258	124	56	67	188	3424683	3424976	293	1.22E-04	42	45.97

		WP_011094382.1	NZ_CP039021.1	32.203	59	39	101	158	2762193	2762017	-176	0.028	35	54.24
		WP_011094382.1	NZ_CP039022.1	32.203	59	39	101	158	2762193	2762017	-176	0.028	35	54.24
		WP_011094382.1	NC_002929.2	32.203	59	39	101	158	2762193	2762017	-176	0.028	35	54.24
		WP_109545078.1	NZ_CP039021.1	32	25	17	73	97	1597715	1597789	74	5	27.3	68
		WP_109545078.1	NZ_CP039022.1	32	25	17	73	97	1597715	1597789	74	5	27.3	68
		WP_109545078.1	NC_002929.2	32	25	17	73	97	1597715	1597789	74	5	27.3	68
		WP_081853183.1	NZ_CP039021.1	31.915	94	45	112	186	3424755	3425036	281	0.008	36.6	43.62
		WP_081853183.1	NZ_CP039022.1	31.915	94	45	112	186	3424755	3425036	281	0.008	36.6	43.62
		WP_081853183.1	NC_002929.2	31.915	94	45	112	186	3424755	3425036	281	0.008	36.6	43.62
	BP1219	WP_011339581.1	NZ_CP039021.1	31.818	66	35	147	212	1282645	1282478	-167	1.6	29.6	42.42
		WP_011339581.1	NZ_CP039022.1	31.818	66	35	147	212	1282645	1282478	-167	1.6	29.6	42.42
		WP_011339581.1	NC_002929.2	31.818	66	35	147	212	1282645	1282478	-167	1.6	29.6	42.42
		WP_063860629.1	NZ_CP039021.1	31.818	44	29	124	166	673191	673322	131	2.1	28.1	52.27
		WP_063860629.1	NZ_CP039022.1	31.818	44	29	124	166	673191	673322	131	2.1	28.1	52.27
		WP_063860629.1	NC_002929.2	31.818	44	29	124	166	673191	673322	131	2.1	28.1	52.27
		WP_032492458.1	NZ_CP039021.1	31.746	63	35	166	227	3654764	3654931	167	1.3	30	49.21
		WP_032492458.1	NZ_CP039022.1	31.746	63	35	166	227	3654764	3654931	167	1.3	30	49.21
		WP_032492458.1	NC_002929.2	31.746	63	35	166	227	3654764	3654931	167	1.3	30	49.21
		WP_032492452.1	NZ_CP039021.1	31.746	63	35	166	227	3654764	3654931	167	1.3	30	49.21
		WP_032492452.1	NZ_CP039022.1	31.746	63	35	166	227	3654764	3654931	167	1.3	30	49.21
		WP_032492452.1	NC_002929.2	31.746	63	35	166	227	3654764	3654931	167	1.3	30	49.21
		WP_032488864.1	NZ_CP039021.1	31.746	63	35	166	227	3654764	3654931	167	1.2	30	49.21
		WP_032488864.1	NZ_CP039022.1	31.746	63	35	166	227	3654764	3654931	167	1.2	30	49.21
		WP_032488864.1	NC_002929.2	31.746	63	35	166	227	3654764	3654931	167	1.2	30	49.21
		WP_063860558.1	NZ_CP039021.1	31.707	82	54	48	127	2762208	2761963	-245	0.013	35.8	47.56
		WP_063860558.1	NZ_CP039022.1	31.707	82	54	48	127	2762208	2761963	-245	0.013	35.8	47.56
		WP_063860558.1	NC_002929.2	31.707	82	54	48	127	2762208	2761963	-245	0.013	35.8	47.56
		WP_043942497.1	NZ_CP039021.1	31.707	41	26	161	201	2160305	2160189	-116	3.1	27.7	46.34

		WP_043942497.1	NZ_CP039022.1	31.707	41	26	161	201	2160305	2160189	-116	3.1	27.7	46.34
		WP_043942497.1	NC_002929.2	31.707	41	26	161	201	2160305	2160189	-116	3.1	27.7	46.34
	BP2292	WP_015818996.1	NZ_CP039021.1	31.579	57	33	2	53	2414833	2414666	-167	8.3	26.6	56.14
		WP_015818996.1	NZ_CP039022.1	31.579	57	33	2	53	2414833	2414666	-167	8.3	26.6	56.14
		WP_015818996.1	NC_002929.2	31.579	57	33	2	53	2414833	2414666	-167	8.3	26.6	56.14
		WP_063842692.1	NZ_CP039021.1	31.507	73	39	108	178	1160226	1160417	191	6.5	26.9	42.47
		WP_063842692.1	NZ_CP039022.1	31.507	73	39	108	178	1160226	1160417	191	6.5	26.9	42.47
		WP_063842692.1	NC_002929.2	31.507	73	39	108	178	1160226	1160417	191	6.5	26.9	42.47
	region before *BP2149*	WP_058174497.1	NZ_CP039021.1	31.481	54	37	290	343	2272131	2271970	-161	2.2	29.3	40.74
		WP_058174497.1	NZ_CP039022.1	31.481	54	37	290	343	2272131	2271970	-161	2.2	29.3	40.74
		WP_058174497.1	NC_002929.2	31.481	54	37	290	343	2272131	2271970	-161	2.2	29.3	40.74
		WP_053816671.1	NZ_CP039021.1	31.481	54	37	290	343	2272131	2271970	-161	1.2	30	40.74
		WP_053816671.1	NZ_CP039022.1	31.481	54	37	290	343	2272131	2271970	-161	1.2	30	40.74
		WP_053816671.1	NC_002929.2	31.481	54	37	290	343	2272131	2271970	-161	1.2	30	40.74
		WP_053813381.1	NZ_CP039021.1	31.481	54	37	290	343	2272131	2271970	-161	2.2	29.3	40.74
		WP_053813381.1	NZ_CP039022.1	31.481	54	37	290	343	2272131	2271970	-161	2.2	29.3	40.74
		WP_053813381.1	NC_002929.2	31.481	54	37	290	343	2272131	2271970	-161	2.2	29.3	40.74
		WP_064511510.1	NZ_CP039021.1	31.481	54	37	290	343	2272131	2271970	-161	1.2	30	40.74
		WP_064511510.1	NZ_CP039022.1	31.481	54	37	290	343	2272131	2271970	-161	1.2	30	40.74
		WP_064511510.1	NC_002929.2	31.481	54	37	290	343	2272131	2271970	-161	1.2	30	40.74
		WP_064484005.1	NZ_CP039021.1	31.481	54	37	290	343	2272131	2271970	-161	2.2	29.3	40.74
		WP_064484005.1	NZ_CP039022.1	31.481	54	37	290	343	2272131	2271970	-161	2.2	29.3	40.74
		WP_064484005.1	NC_002929.2	31.481	54	37	290	343	2272131	2271970	-161	2.2	29.3	40.74
		WP_063864582.1	NZ_CP039021.1	31.481	54	37	290	343	2272131	2271970	-161	2.2	29.3	40.74
		WP_063864582.1	NZ_CP039022.1	31.481	54	37	290	343	2272131	2271970	-161	2.2	29.3	40.74
		WP_063864582.1	NC_002929.2	31.481	54	37	290	343	2272131	2271970	-161	2.2	29.3	40.74
		WP_012074328.1	NZ_CP039021.1	31.481	54	37	290	343	2272131	2271970	-161	1.2	30	40.74

			WP_012074328.1	NZ_CP039022.1	31.481	54	37	290	343	2272131	2271970	-161	1.2	30	40.74
			WP_012074328.1	NC_002929.2	31.481	54	37	290	343	2272131	2271970	-161	1.2	30	40.74
			WP_034081664.1	NZ_CP039021.1	31.481	54	37	290	343	2272131	2271970	-161	1.2	30	40.74
			WP_034081664.1	NZ_CP039022.1	31.481	54	37	290	343	2272131	2271970	-161	1.2	30	40.74
			WP_034081664.1	NC_002929.2	31.481	54	37	290	343	2272131	2271970	-161	1.2	30	40.74
			WP_033998317.1	NZ_CP039021.1	31.481	54	37	290	343	2272131	2271970	-161	1.2	30	40.74
			WP_033998317.1	NZ_CP039022.1	31.481	54	37	290	343	2272131	2271970	-161	1.2	30	40.74
			WP_033998317.1	NC_002929.2	31.481	54	37	290	343	2272131	2271970	-161	1.2	30	40.74
		BP1684	WP_063864095.1	NZ_CP039021.1	31.429	35	24	222	256	1768941	1768837	-104	2	28.5	54.29
			WP_063864095.1	NZ_CP039022.1	31.429	35	24	222	256	1768941	1768837	-104	2	28.5	54.29
			WP_063864095.1	NC_002929.2	31.429	35	24	222	256	1768941	1768837	-104	2	28.5	54.29
			WP_063862733.1	NZ_CP039021.1	31.429	35	24	194	228	1235146	1235042	-104	3.5	27.7	42.86
			WP_063862733.1	NZ_CP039022.1	31.429	35	24	194	228	1235146	1235042	-104	3.5	27.7	42.86
			WP_063862733.1	NC_002929.2	31.429	35	24	194	228	1235146	1235042	-104	3.5	27.7	42.86
			WP_063860559.1	NZ_CP039021.1	31.395	86	57	44	127	2762220	2761963	-257	0.015	35.4	46.51
			WP_063860559.1	NZ_CP039022.1	31.395	86	57	44	127	2762220	2761963	-257	0.015	35.4	46.51
			WP_063860559.1	NC_002929.2	31.395	86	57	44	127	2762220	2761963	-257	0.015	35.4	46.51
			WP_063860562.1	NZ_CP039021.1	31.343	67	45	48	113	2762208	2762008	-200	0.23	31.6	47.76
			WP_063860562.1	NZ_CP039022.1	31.343	67	45	48	113	2762208	2762008	-200	0.23	31.6	47.76
			WP_063860562.1	NC_002929.2	31.343	67	45	48	113	2762208	2762008	-200	0.23	31.6	47.76
			WP_063860561.1	NZ_CP039021.1	31.343	67	45	48	113	2762208	2762008	-200	0.24	31.6	47.76
			WP_063860561.1	NZ_CP039022.1	31.343	67	45	48	113	2762208	2762008	-200	0.24	31.6	47.76
			WP_063860561.1	NC_002929.2	31.343	67	45	48	113	2762208	2762008	-200	0.24	31.6	47.76
			WP_063860525.1	NZ_CP039021.1	31.343	67	45	48	113	2762208	2762008	-200	0.23	31.6	47.76
			WP_063860525.1	NZ_CP039022.1	31.343	67	45	48	113	2762208	2762008	-200	0.23	31.6	47.76
			WP_063860525.1	NC_002929.2	31.343	67	45	48	113	2762208	2762008	-200	0.23	31.6	47.76
		gloB	WP_122630835.1	NZ_CP039021.1	31.25	32	22	74	105	3424710	3424805	95	0.35	30.8	56.25
			WP_122630835.1	NZ_CP039022.1	31.25	32	22	74	105	3424710	3424805	95	0.35	30.8	56.25

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		WP_122630835.1	NC_002929.2	31.25	32	22	74	105	3424710	3424805	95	0.35	30.8	56.25
		WP_063842682.1	NZ_CP039021.1	31.25	112	56	83	194	3424704	3424976	272	0.003	37.7	43.75
		WP_063842682.1	NZ_CP039022.1	31.25	112	56	83	194	3424704	3424976	272	0.003	37.7	43.75
		WP_063842682.1	NC_002929.2	31.25	112	56	83	194	3424704	3424976	272	0.003	37.7	43.75
		WP_063842701.1	NZ_CP039021.1	31.25	64	43	62	124	2762208	2762017	-191	4.1	27.7	48.44
		WP_063842701.1	NZ_CP039022.1	31.25	64	43	62	124	2762208	2762017	-191	4.1	27.7	48.44
		WP_063842701.1	NC_002929.2	31.25	64	43	62	124	2762208	2762017	-191	4.1	27.7	48.44
	BP0624	WP_063857696.1	NZ_CP039021.1	31.25	32	22	36	67	632187	632092	-95	7.8	26.9	56.25
		WP_063857696.1	NZ_CP039022.1	31.25	32	22	36	67	632187	632092	-95	7.8	26.9	56.25
		WP_063857696.1	NC_002929.2	31.25	32	22	36	67	632187	632092	-95	7.8	26.9	56.25
		WP_063860467.1	NZ_CP039021.1	31.25	32	22	36	67	632187	632092	-95	6.6	26.9	56.25
		WP_063860467.1	NZ_CP039022.1	31.25	32	22	36	67	632187	632092	-95	6.6	26.9	56.25
		WP_063860467.1	NC_002929.2	31.25	32	22	36	67	632187	632092	-95	6.6	26.9	56.25
		WP_109545066.1	NZ_CP039021.1	31.148	61	35	164	224	3654770	3654931	161	2	29.3	40.98
		WP_109545066.1	NZ_CP039022.1	31.148	61	35	164	224	3654770	3654931	161	2	29.3	40.98
		WP_109545066.1	NC_002929.2	31.148	61	35	164	224	3654770	3654931	161	2	29.3	40.98
		WP_063860495.1	NZ_CP039021.1	31.148	61	35	164	224	3654770	3654931	161	1.6	29.6	40.98
		WP_063860495.1	NZ_CP039022.1	31.148	61	35	164	224	3654770	3654931	161	1.6	29.6	40.98
		WP_063860495.1	NC_002929.2	31.148	61	35	164	224	3654770	3654931	161	1.6	29.6	40.98
		WP_063860494.1	NZ_CP039021.1	31.148	61	35	164	224	3654770	3654931	161	1.7	29.6	40.98
		WP_063860494.1	NZ_CP039022.1	31.148	61	35	164	224	3654770	3654931	161	1.7	29.6	40.98
		WP_063860494.1	NC_002929.2	31.148	61	35	164	224	3654770	3654931	161	1.7	29.6	40.98
		WP_032072107.1	NZ_CP039021.1	31.148	61	35	164	224	3654770	3654931	161	1.8	29.3	40.98
		WP_032072107.1	NZ_CP039022.1	31.148	61	35	164	224	3654770	3654931	161	1.8	29.3	40.98
		WP_032072107.1	NC_002929.2	31.148	61	35	164	224	3654770	3654931	161	1.8	29.3	40.98
	BP2109	WP_062743204.1	NZ_CP039021.1	31.148	61	30	33	81	2232307	2232489	182	4.5	27.7	49.18
		WP_062743204.1	NZ_CP039022.1	31.148	61	30	33	81	2232307	2232489	182	4.5	27.7	49.18
		WP_062743204.1	NC_002929.2	31.148	61	30	33	81	2232307	2232489	182	4.5	27.7	49.18

		WP_063860536.1	NZ_CP039021.1	30.986	71	48	44	113	2762220	2762008	-212	0.21	32	46.48
		WP_063860536.1	NZ_CP039022.1	30.986	71	48	44	113	2762220	2762008	-212	0.21	32	46.48
		WP_063860536.1	NC_002929.2	30.986	71	48	44	113	2762220	2762008	-212	0.21	32	46.48
	BP2340	WP_063842712.1	NZ_CP039021.1	30.986	71	42	71	138	2480202	2480402	200	1.2	29.6	46.48
		WP_063842712.1	NZ_CP039022.1	30.986	71	42	71	138	2480202	2480402	200	1.2	29.6	46.48
		WP_063842712.1	NC_002929.2	30.986	71	42	71	138	2480202	2480402	200	1.2	29.6	46.48
		WP_063864595.1	NZ_CP039021.1	30.909	55	34	56	109	3838096	3838251	155	0.21	32	49.09
		WP_063864595.1	NZ_CP039022.1	30.909	55	34	56	109	3838096	3838251	155	0.21	32	49.09
		WP_063864595.1	NC_002929.2	30.909	55	34	56	109	3838096	3838251	155	0.21	32	49.09
	BP3802	WP_071766622.1	NZ_CP039021.1	30.864	81	31	162	230	4006267	4006064	-203	8.7	26.9	43.21
		WP_071766622.1	NZ_CP039022.1	30.864	81	31	162	230	4006267	4006064	-203	8.7	26.9	43.21
		WP_071766622.1	NC_002929.2	30.864	81	31	162	230	4006267	4006064	-203	8.7	26.9	43.21
		WP_122630896.1	NZ_CP039021.1	30.769	39	27	14	52	561454	561570	116	5.2	27.3	46.15
		WP_122630896.1	NZ_CP039022.1	30.769	39	27	14	52	561454	561570	116	5.2	27.3	46.15
		WP_122630896.1	NC_002929.2	30.769	39	27	14	52	561454	561570	116	5.2	27.3	46.15
	gloB	WP_122630829.1	NZ_CP039021.1	30.702	114	54	91	202	3424704	3424976	272	0.11	32.7	42.98
		WP_122630829.1	NZ_CP039022.1	30.702	114	54	91	202	3424704	3424976	272	0.11	32.7	42.98
		WP_122630829.1	NC_002929.2	30.702	114	54	91	202	3424704	3424976	272	0.11	32.7	42.98
		WP_063862732.1	NZ_CP039021.1	30.556	36	25	194	229	1235146	1235039	-107	3.9	27.7	41.67
		WP_063862732.1	NZ_CP039022.1	30.556	36	25	194	229	1235146	1235039	-107	3.9	27.7	41.67
		WP_063862732.1	NC_002929.2	30.556	36	25	194	229	1235146	1235039	-107	3.9	27.7	41.67
		WP_063860559.1	NZ_CP039021.1	30.556	36	25	79	114	237922	237815	-107	0.42	30.8	52.78
		WP_063860559.1	NZ_CP039022.1	30.556	36	25	79	114	237922	237815	-107	0.42	30.8	52.78
		WP_063860559.1	NC_002929.2	30.556	36	25	79	114	237922	237815	-107	0.42	30.8	52.78
		WP_063860558.1	NZ_CP039021.1	30.556	36	25	79	114	237922	237815	-107	0.42	30.8	52.78
		WP_063860558.1	NZ_CP039022.1	30.556	36	25	79	114	237922	237815	-107	0.42	30.8	52.78
		WP_063860558.1	NC_002929.2	30.556	36	25	79	114	237922	237815	-107	0.42	30.8	52.78
		WP_122630825.1	NZ_CP039021.1	30.508	59	37	109	167	237919	237755	-164	0.26	32	45.76

		WP_122630825.1	NZ_CP039022.1	30.508	59	37	109	167	237919	237755	-164	0.26	32	45.76
		WP_122630825.1	NC_002929.2	30.508	59	37	109	167	237919	237755	-164	0.26	32	45.76
		WP_063842629.1	NZ_CP039021.1	30.488	82	55	48	127	2762208	2761963	-245	0.097	33.1	46.34
		WP_063842629.1	NZ_CP039022.1	30.488	82	55	48	127	2762208	2761963	-245	0.097	33.1	46.34
		WP_063842629.1	NC_002929.2	30.488	82	55	48	127	2762208	2761963	-245	0.097	33.1	46.34
	gloB	WP_063860203.1	NZ_CP039021.1	30.435	46	28	59	104	3424680	3424805	125	1.8	28.5	45.65
		WP_063860203.1	NZ_CP039022.1	30.435	46	28	59	104	3424680	3424805	125	1.8	28.5	45.65
		WP_063860203.1	NC_002929.2	30.435	46	28	59	104	3424680	3424805	125	1.8	28.5	45.65
Metallo-beta-lactamase superfamily *BP3130*		WP_063860625.1	NZ_CP039021.1	30.303	66	41	155	220	3335419	3335601	182	3.01E-04	40.4	53.03
		WP_063860625.1	NZ_CP039022.1	30.303	66	41	155	220	3335419	3335601	182	3.01E-04	40.4	53.03
		WP_063860625.1	NC_002929.2	30.303	66	41	155	220	3335419	3335601	182	3.01E-04	40.4	53.03
		WP_063860623.1	NZ_CP039021.1	30.303	66	41	155	220	3335419	3335601	182	2.80E-04	40.4	53.03
		WP_063860623.1	NZ_CP039022.1	30.303	66	41	155	220	3335419	3335601	182	2.80E-04	40.4	53.03
		WP_063860623.1	NC_002929.2	30.303	66	41	155	220	3335419	3335601	182	2.80E-04	40.4	53.03
		WP_034735890.1	NZ_CP039021.1	30.303	66	41	155	220	3335419	3335601	182	2.88E-04	40.4	53.03
		WP_034735890.1	NZ_CP039022.1	30.303	66	41	155	220	3335419	3335601	182	2.88E-04	40.4	53.03
		WP_034735890.1	NC_002929.2	30.303	66	41	155	220	3335419	3335601	182	2.88E-04	40.4	53.03
	BP0232	WP_122634438.1	NZ_CP039021.1	30.108	93	57	35	126	239225	238968	-257	6.8	26.9	39.78
		WP_122634438.1	NZ_CP039022.1	30.108	93	57	35	126	239225	238968	-257	6.8	26.9	39.78
		WP_122634438.1	NC_002929.2	30.108	93	57	35	126	239225	238968	-257	6.8	26.9	39.78
		WP_063864090.1	NZ_CP039021.1	30.108	93	57	35	126	239225	238968	-257	5	27.3	40.86
		WP_063864090.1	NZ_CP039022.1	30.108	93	57	35	126	239225	238968	-257	5	27.3	40.86
		WP_063864090.1	NC_002929.2	30.108	93	57	35	126	239225	238968	-257	5	27.3	40.86
	BP3063	WP_004207704.1	NZ_CP039021.1	30	70	44	185	253	3265922	3266119	197	6.4	27.3	44.29
		WP_004207704.1	NZ_CP039022.1	30	70	44	185	253	3265922	3266119	197	6.4	27.3	44.29
		WP_004207704.1	NC_002929.2	30	70	44	185	253	3265922	3266119	197	6.4	27.3	44.29
	BP1927	WP_063860892.1	NZ_CP039021.1	30	50	33	124	171	2029371	2029520	149	7.3	27.3	46

		WP_063860892.1	NZ_CP039022.1	30	50	33	124	171	2029371	2029520	149	7.3	27.3	46
		WP_063860892.1	NC_002929.2	30	50	33	124	171	2029371	2029520	149	7.3	27.3	46
	BP1155	WP_007688792.1	NZ_CP039021.1	29.87	77	33	198	253	1220667	1220897	230	9.8	26.6	44.16
	BP1155	WP_007688792.1	NZ_CP039022.1	29.87	77	33	198	253	1220667	1220897	230	9.8	26.6	44.16
	BP1155	WP_007688792.1	NC_002929.2	29.87	77	33	198	253	1220667	1220897	230	9.8	26.6	44.16
		WP_059155257.1	NZ_CP039021.1	29.851	67	46	50	115	2762208	2762008	-200	0.86	30	46.27
		WP_059155257.1	NZ_CP039022.1	29.851	67	46	50	115	2762208	2762008	-200	0.86	30	46.27
		WP_059155257.1	NC_002929.2	29.851	67	46	50	115	2762208	2762008	-200	0.86	30	46.27
		WP_063860554.1	NZ_CP039021.1	29.851	67	46	50	115	2762208	2762008	-200	1.9	28.9	46.27
		WP_063860554.1	NZ_CP039022.1	29.851	67	46	50	115	2762208	2762008	-200	1.9	28.9	46.27
		WP_063860554.1	NC_002929.2	29.851	67	46	50	115	2762208	2762008	-200	1.9	28.9	46.27
		WP_063860547.1	NZ_CP039021.1	29.851	67	46	50	115	2762208	2762008	-200	0.86	30	46.27
		WP_063860547.1	NZ_CP039022.1	29.851	67	46	50	115	2762208	2762008	-200	0.86	30	46.27
		WP_063860547.1	NC_002929.2	29.851	67	46	50	115	2762208	2762008	-200	0.86	30	46.27
	BP1715	WP_063865149.1	NZ_CP039021.1	29.787	47	31	95	139	1800449	1800309	-140	1.6	28.9	48.94
		WP_063865149.1	NZ_CP039022.1	29.787	47	31	95	139	1800449	1800309	-140	1.6	28.9	48.94
		WP_063865149.1	NC_002929.2	29.787	47	31	95	139	1800449	1800309	-140	1.6	28.9	48.94
		WP_063857820.1	NZ_CP039021.1	29.63	54	34	71	120	237976	237815	-161	0.25	31.6	50
		WP_063857820.1	NZ_CP039022.1	29.63	54	34	71	120	237976	237815	-161	0.25	31.6	50
		WP_063857820.1	NC_002929.2	29.63	54	34	71	120	237976	237815	-161	0.25	31.6	50
		WP_063860531.1	NZ_CP039021.1	29.268	82	56	50	129	2762208	2761963	-245	0.13	32.7	45.12
		WP_063860531.1	NZ_CP039022.1	29.268	82	56	50	129	2762208	2761963	-245	0.13	32.7	45.12
		WP_063860531.1	NC_002929.2	29.268	82	56	50	129	2762208	2761963	-245	0.13	32.7	45.12
		WP_063860523.1	NZ_CP039021.1	29.268	82	56	48	127	2762208	2761963	-245	0.19	32	45.12
		WP_063860523.1	NZ_CP039022.1	29.268	82	56	48	127	2762208	2761963	-245	0.19	32	45.12
		WP_063860523.1	NC_002929.2	29.268	82	56	48	127	2762208	2761963	-245	0.19	32	45.12
	BP2552	WP_057689930.1	NZ_CP039021.1	29.167	48	33	201	248	2707101	2706961	-140	7.2	26.9	50
		WP_057689930.1	NZ_CP039022.1	29.167	48	33	201	248	2707101	2706961	-140	7.2	26.9	50

		WP_057689930.1	NC_002929.2	29.167	48	33	201	248	2707101	2706961	-140	7.2	26.9	50
		WP_063857820.1	NZ_CP039021.1	29.091	165	85	83	247	3424722	3425120	398	5.49E-05	43.5	43.03
		WP_063857820.1	NZ_CP039022.1	29.091	165	85	83	247	3424722	3425120	398	5.49E-05	43.5	43.03
		WP_063857820.1	NC_002929.2	29.091	165	85	83	247	3424722	3425120	398	5.49E-05	43.5	43.03
	rho	WP_063865165.1	NZ_CP039021.1	29.091	55	34	187	236	1335311	1335147	-164	9.1	26.6	43.64
		WP_063865165.1	NZ_CP039022.1	29.091	55	34	187	236	1335311	1335147	-164	9.1	26.6	43.64
		WP_063865165.1	NC_002929.2	29.091	55	34	187	236	1335311	1335147	-164	9.1	26.6	43.64
		WP_063865164.1	NZ_CP039021.1	29.091	55	34	186	235	1335311	1335147	-164	7.8	26.9	43.64
		WP_063865164.1	NZ_CP039022.1	29.091	55	34	186	235	1335311	1335147	-164	7.8	26.9	43.64
		WP_063865164.1	NC_002929.2	29.091	55	34	186	235	1335311	1335147	-164	7.8	26.9	43.64
		WP_063859390.1	NZ_CP039021.1	29.091	110	59	77	175	237922	237617	-305	0.066	33.5	40.91
		WP_063859390.1	NZ_CP039022.1	29.091	110	59	77	175	237922	237617	-305	0.066	33.5	40.91
		WP_063859390.1	NC_002929.2	29.091	110	59	77	175	237922	237617	-305	0.066	33.5	40.91
		WP_063842701.1	NZ_CP039021.1	29.091	55	35	98	152	237907	237755	-152	0.74	30	50.91
		WP_063842701.1	NZ_CP039022.1	29.091	55	35	98	152	237907	237755	-152	0.74	30	50.91
		WP_063842701.1	NC_002929.2	29.091	55	35	98	152	237907	237755	-152	0.74	30	50.91
Metallo-beta-lactamase superfamily	*BP2609*	WP_049037557.1	NZ_CP039021.1	29.07	86	59	44	127	2762220	2761963	-257	0.23	31.6	44.19
		WP_049037557.1	NZ_CP039022.1	29.07	86	59	44	127	2762220	2761963	-257	0.23	31.6	44.19
		WP_049037557.1	NC_002929.2	29.07	86	59	44	127	2762220	2761963	-257	0.23	31.6	44.19
	BP0035	WP_010673861.1	NZ_CP039021.1	28.846	52	36	166	217	40380	40228	-152	7.6	27.3	48.08
		WP_010673861.1	NZ_CP039022.1	28.846	52	36	166	217	40380	40228	-152	7.6	27.3	48.08
		WP_010673861.1	NC_002929.2	28.846	52	36	166	217	40380	40228	-152	7.6	27.3	48.08
		WP_063860847.1	NZ_CP039021.1	28.846	52	36	166	217	40380	40228	-152	7.6	27.3	48.08
		WP_063860847.1	NZ_CP039022.1	28.846	52	36	166	217	40380	40228	-152	7.6	27.3	48.08
		WP_063860847.1	NC_002929.2	28.846	52	36	166	217	40380	40228	-152	7.6	27.3	48.08
		WP_063860846.1	NZ_CP039021.1	28.846	52	36	166	217	40380	40228	-152	7.7	27.3	48.08
		WP_063860846.1	NZ_CP039022.1	28.846	52	36	166	217	40380	40228	-152	7.7	27.3	48.08

		WP_063860846.1	NC_002929.2	28.846	52	36	166	217	40380	40228	-152	7.7	27.3	48.08
		WP_063860845.1	NZ_CP039021.1	28.846	52	36	166	217	40380	40228	-152	7.6	27.3	48.08
		WP_063860845.1	NZ_CP039022.1	28.846	52	36	166	217	40380	40228	-152	7.6	27.3	48.08
		WP_063860845.1	NC_002929.2	28.846	52	36	166	217	40380	40228	-152	7.6	27.3	48.08
		WP_063860844.1	NZ_CP039021.1	28.846	52	36	166	217	40380	40228	-152	7.7	27.3	48.08
		WP_063860844.1	NZ_CP039022.1	28.846	52	36	166	217	40380	40228	-152	7.7	27.3	48.08
		WP_063860844.1	NC_002929.2	28.846	52	36	166	217	40380	40228	-152	7.7	27.3	48.08
		WP_063860843.1	NZ_CP039021.1	28.846	52	36	166	217	40380	40228	-152	7.8	27.3	48.08
		WP_063860843.1	NZ_CP039022.1	28.846	52	36	166	217	40380	40228	-152	7.8	27.3	48.08
		WP_063860843.1	NC_002929.2	28.846	52	36	166	217	40380	40228	-152	7.8	27.3	48.08
		WP_005245537.1	NZ_CP039021.1	28.571	35	25	194	228	1235146	1235042	-104	9.9	26.6	40
		WP_005245537.1	NZ_CP039022.1	28.571	35	25	194	228	1235146	1235042	-104	9.9	26.6	40
		WP_005245537.1	NC_002929.2	28.571	35	25	194	228	1235146	1235042	-104	9.9	26.6	40
		WP_063860560.1	NZ_CP039021.1	28.358	67	47	48	113	2762208	2762008	-200	1.1	29.6	44.78
		WP_063860560.1	NZ_CP039022.1	28.358	67	47	48	113	2762208	2762008	-200	1.1	29.6	44.78
		WP_063860560.1	NC_002929.2	28.358	67	47	48	113	2762208	2762008	-200	1.1	29.6	44.78
	gloB	WP_081048762.1	NZ_CP039021.1	28.302	53	33	84	136	3424671	3424814	143	1.4	28.9	47.17
		WP_081048762.1	NZ_CP039022.1	28.302	53	33	84	136	3424671	3424814	143	1.4	28.9	47.17
		WP_081048762.1	NC_002929.2	28.302	53	33	84	136	3424671	3424814	143	1.4	28.9	47.17
	BP2428	WP_063978070.1	NZ_CP039021.1	28.261	46	32	87	131	2571520	2571657	137	4.6	27.3	52.17
		WP_063978070.1	NZ_CP039022.1	28.261	46	32	87	131	2571520	2571657	137	4.6	27.3	52.17
		WP_063978070.1	NC_002929.2	28.261	46	32	87	131	2571520	2571657	137	4.6	27.3	52.17
		WP_063860560.1	NZ_CP039021.1	28.235	85	47	51	124	238039	237794	-245	0.028	34.7	47.06
		WP_063860560.1	NZ_CP039022.1	28.235	85	47	51	124	238039	237794	-245	0.028	34.7	47.06
		WP_063860560.1	NC_002929.2	28.235	85	47	51	124	238039	237794	-245	0.028	34.7	47.06
		WP_063859390.1	NZ_CP039021.1	28.205	78	52	49	122	445565	445798	233	1.7	28.9	41.03
		WP_063859390.1	NZ_CP039022.1	28.205	78	52	49	122	445565	445798	233	1.7	28.9	41.03
		WP_063859390.1	NC_002929.2	28.205	78	52	49	122	445565	445798	233	1.7	28.9	41.03

		WP_063860835.1	NZ_CP039021.1	28.182	110	60	77	175	237922	237617	-305	0.17	32.3	40.91
		WP_063860835.1	NZ_CP039022.1	28.182	110	60	77	175	237922	237617	-305	0.17	32.3	40.91
		WP_063860835.1	NC_002929.2	28.182	110	60	77	175	237922	237617	-305	0.17	32.3	40.91
	gloB	WP_011094382.1	NZ_CP039021.1	28.125	96	46	137	230	3424749	3424973	224	0.007	37	44.79
		WP_011094382.1	NZ_CP039022.1	28.125	96	46	137	230	3424749	3424973	224	0.007	37	44.79
		WP_011094382.1	NC_002929.2	28.125	96	46	137	230	3424749	3424973	224	0.007	37	44.79
		WP_122630832.1	NZ_CP039021.1	28.025	157	74	112	264	3424755	3425120	365	0.011	36.2	43.31
		WP_122630832.1	NZ_CP039022.1	28.025	157	74	112	264	3424755	3425120	365	0.011	36.2	43.31
		WP_122630832.1	NC_002929.2	28.025	157	74	112	264	3424755	3425120	365	0.011	36.2	43.31
	BP1973	WP_069280710.1	NZ_CP039021.1	28	50	32	120	169	2081128	2080991	-137	7	26.6	40
		WP_069280710.1	NZ_CP039022.1	28	50	32	120	169	2081128	2080991	-137	7	26.6	40
		WP_069280710.1	NC_002929.2	28	50	32	120	169	2081128	2080991	-137	7	26.6	40
		WP_063860586.1	NZ_CP039021.1	28	50	32	120	169	2081128	2080991	-137	3	27.7	42
		WP_063860586.1	NZ_CP039022.1	28	50	32	120	169	2081128	2080991	-137	3	27.7	42
		WP_063860586.1	NC_002929.2	28	50	32	120	169	2081128	2080991	-137	3	27.7	42
		WP_063860575.1	NZ_CP039021.1	28	50	32	120	169	2081128	2080991	-137	6.8	26.6	40
		WP_063860575.1	NZ_CP039022.1	28	50	32	120	169	2081128	2080991	-137	6.8	26.6	40
		WP_063860575.1	NC_002929.2	28	50	32	120	169	2081128	2080991	-137	6.8	26.6	40
		WP_034866180.1	NZ_CP039021.1	28	75	43	51	114	238039	237815	-224	0.03	34.7	46.67
		WP_034866180.1	NZ_CP039022.1	28	75	43	51	114	238039	237815	-224	0.03	34.7	46.67
		WP_034866180.1	NC_002929.2	28	75	43	51	114	238039	237815	-224	0.03	34.7	46.67
		WP_059155257.1	NZ_CP039021.1	28	75	43	53	116	238039	237815	-224	0.026	34.7	46.67
		WP_059155257.1	NZ_CP039022.1	28	75	43	53	116	238039	237815	-224	0.026	34.7	46.67
		WP_059155257.1	NC_002929.2	28	75	43	53	116	238039	237815	-224	0.026	34.7	46.67
		WP_063860554.1	NZ_CP039021.1	28	75	43	53	116	238039	237815	-224	0.045	33.9	46.67
		WP_063860554.1	NZ_CP039022.1	28	75	43	53	116	238039	237815	-224	0.045	33.9	46.67
		WP_063860554.1	NC_002929.2	28	75	43	53	116	238039	237815	-224	0.045	33.9	46.67
		WP_063860547.1	NZ_CP039021.1	28	75	43	53	116	238039	237815	-224	0.028	34.7	46.67

		WP_063860547.1	NZ_CP039022.1	28	75	43	53	116	238039	237815	-224	0.028	34.7	46.67
		WP_063860547.1	NC_002929.2	28	75	43	53	116	238039	237815	-224	0.028	34.7	46.67
		WP_063860531.1	NZ_CP039021.1	28	75	43	53	116	238039	237815	-224	0.021	35	46.67
		WP_063860531.1	NZ_CP039022.1	28	75	43	53	116	238039	237815	-224	0.021	35	46.67
		WP_063860531.1	NC_002929.2	28	75	43	53	116	238039	237815	-224	0.021	35	46.67
		WP_122630829.1	NZ_CP039021.1	27.941	68	40	68	130	2762208	2762017	-191	0.5	30.8	52.94
		WP_122630829.1	NZ_CP039022.1	27.941	68	40	68	130	2762208	2762017	-191	0.5	30.8	52.94
		WP_122630829.1	NC_002929.2	27.941	68	40	68	130	2762208	2762017	-191	0.5	30.8	52.94
		WP_034866180.1	NZ_CP039021.1	27.907	86	60	44	127	2762220	2761963	-257	0.48	30.8	43.02
		WP_034866180.1	NZ_CP039022.1	27.907	86	60	44	127	2762220	2761963	-257	0.48	30.8	43.02
		WP_034866180.1	NC_002929.2	27.907	86	60	44	127	2762220	2761963	-257	0.48	30.8	43.02
	gloB	WP_071766402.1	NZ_CP039021.1	27.879	165	86	77	239	3424725	3425126	401	2.86E-05	44.3	40.61
		WP_071766402.1	NZ_CP039022.1	27.879	165	86	77	239	3424725	3425126	401	2.86E-05	44.3	40.61
		WP_071766402.1	NC_002929.2	27.879	165	86	77	239	3424725	3425126	401	2.86E-05	44.3	40.61
Metallo-beta-lactamase superfamily	BP1113	WP_011088970.1	NZ_CP039021.1	27.869	61	36	59	113	1171449	1171625	176	8.5	26.9	50.82
		WP_011088970.1	NZ_CP039022.1	27.869	61	36	59	113	1171449	1171625	176	8.5	26.9	50.82
		WP_011088970.1	NC_002929.2	27.869	61	36	59	113	1171449	1171625	176	8.5	26.9	50.82
		WP_063857696.1	NZ_CP039021.1	27.778	90	42	91	178	3424755	3424961	206	0.062	33.5	40
		WP_063857696.1	NZ_CP039022.1	27.778	90	42	91	178	3424755	3424961	206	0.062	33.5	40
		WP_063857696.1	NC_002929.2	27.778	90	42	91	178	3424755	3424961	206	0.062	33.5	40
	BP1168	WP_070064536.1	NZ_CP039021.1	27.778	36	26	194	229	1235146	1235039	-107	8.7	26.6	41.67
		WP_070064536.1	NZ_CP039022.1	27.778	36	26	194	229	1235146	1235039	-107	8.7	26.6	41.67
		WP_070064536.1	NC_002929.2	27.778	36	26	194	229	1235146	1235039	-107	8.7	26.6	41.67
		WP_064483990.1	NZ_CP039021.1	27.778	36	26	194	229	1235146	1235039	-107	9.1	26.6	38.89
		WP_064483990.1	NZ_CP039022.1	27.778	36	26	194	229	1235146	1235039	-107	9.1	26.6	38.89
		WP_064483990.1	NC_002929.2	27.778	36	26	194	229	1235146	1235039	-107	9.1	26.6	38.89
		WP_005268230.1	NZ_CP039021.1	27.778	36	26	194	229	1235146	1235039	-107	9	26.6	41.67

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		WP_005268230.1	NZ_CP039022.1	27.778	36	26	194	229	1235146	1235039	-107	9	26.6	41.67
		WP_005268230.1	NC_002929.2	27.778	36	26	194	229	1235146	1235039	-107	9	26.6	41.67
		WP_004728961.1	NZ_CP039021.1	27.778	36	26	194	229	1235146	1235039	-107	9.9	26.6	38.89
		WP_004728961.1	NZ_CP039022.1	27.778	36	26	194	229	1235146	1235039	-107	9.9	26.6	38.89
		WP_004728961.1	NC_002929.2	27.778	36	26	194	229	1235146	1235039	-107	9.9	26.6	38.89
		WP_122630831.1	NZ_CP039021.1	27.679	112	59	110	221	3424755	3425024	269	0.009	36.2	42.86
		WP_122630831.1	NZ_CP039022.1	27.679	112	59	110	221	3424755	3425024	269	0.009	36.2	42.86
		WP_122630831.1	NC_002929.2	27.679	112	59	110	221	3424755	3425024	269	0.009	36.2	42.86
	proceeding BP0103	WP_063864647.1	NZ_CP039021.1	27.66	47	34	16	62	101821	101961	140	4.4	27.7	44.68
		WP_063864647.1	NZ_CP039022.1	27.66	47	34	16	62	101821	101961	140	4.4	27.7	44.68
		WP_063864647.1	NC_002929.2	27.66	47	34	16	62	101821	101961	140	4.4	27.7	44.68
	BP2767	WP_063842703.1	NZ_CP039021.1	27.473	91	57	84	174	2944126	2944371	245	0.04	34.3	42.86
		WP_063842703.1	NZ_CP039022.1	27.473	91	57	84	174	2944126	2944371	245	0.04	34.3	42.86
		WP_063842703.1	NC_002929.2	27.473	91	57	84	174	2944126	2944371	245	0.04	34.3	42.86
	BP1312	WP_063860495.1	NZ_CP039021.1	27.397	146	76	59	183	1380125	1380535	410	0.075	33.9	37.67
		WP_063860495.1	NZ_CP039022.1	27.397	146	76	59	183	1380125	1380535	410	0.075	33.9	37.67
		WP_063860495.1	NC_002929.2	27.397	146	76	59	183	1380125	1380535	410	0.075	33.9	37.67
		WP_063860494.1	NZ_CP039021.1	27.397	146	76	59	183	1380125	1380535	410	0.077	33.9	37.67
		WP_063860494.1	NZ_CP039022.1	27.397	146	76	59	183	1380125	1380535	410	0.077	33.9	37.67
		WP_063860494.1	NC_002929.2	27.397	146	76	59	183	1380125	1380535	410	0.077	33.9	37.67
		WP_032072107.1	NZ_CP039021.1	27.397	146	76	59	183	1380125	1380535	410	0.071	33.9	37.67
		WP_032072107.1	NZ_CP039022.1	27.397	146	76	59	183	1380125	1380535	410	0.071	33.9	37.67
		WP_032072107.1	NC_002929.2	27.397	146	76	59	183	1380125	1380535	410	0.071	33.9	37.67
	BP3118	WP_034051940.1	NZ_CP039021.1	27.273	66	41	205	270	3323848	3323672	-176	0.39	31.6	40.91
		WP_034051940.1	NZ_CP039022.1	27.273	66	41	205	270	3323848	3323672	-176	0.39	31.6	40.91
		WP_034051940.1	NC_002929.2	27.273	66	41	205	270	3323848	3323672	-176	0.39	31.6	40.91
	BP1983	WP_013255389.1	NZ_CP039021.1	27.273	88	60	3	88	2093677	2093420	-257	2.4	28.5	43.18
		WP_013255389.1	NZ_CP039022.1	27.273	88	60	3	88	2093677	2093420	-257	2.4	28.5	43.18

		WP_013255389.1	NC_002929.2	27.273	88	60	3	88	2093677	2093420	-257	2.4	28.5	43.18
		WP_012695457.1	NZ_CP039021.1	27.23	213	117	17	201	3334957	3335565	608	6.02E-11	60.8	42.72
		WP_012695457.1	NZ_CP039022.1	27.23	213	117	17	201	3334957	3335565	608	6.02E-11	60.8	42.72
		WP_012695457.1	NC_002929.2	27.23	213	117	17	201	3334957	3335565	608	6.02E-11	60.8	42.72
		WP_032491874.1	NZ_CP039021.1	27.23	213	117	17	201	3334957	3335565	608	7.11E-11	60.5	42.72
		WP_032491874.1	NZ_CP039022.1	27.23	213	117	17	201	3334957	3335565	608	7.11E-11	60.5	42.72
		WP_032491874.1	NC_002929.2	27.23	213	117	17	201	3334957	3335565	608	7.11E-11	60.5	42.72
		WP_063860583.1	NZ_CP039021.1	27.23	213	117	17	201	3334957	3335565	608	6.85E-11	60.5	42.72
		WP_063860583.1	NZ_CP039022.1	27.23	213	117	17	201	3334957	3335565	608	6.85E-11	60.5	42.72
		WP_063860583.1	NC_002929.2	27.23	213	117	17	201	3334957	3335565	608	6.85E-11	60.5	42.72
		WP_063860582.1	NZ_CP039021.1	27.23	213	117	17	201	3334957	3335565	608	1.44E-10	59.7	42.25
		WP_063860582.1	NZ_CP039022.1	27.23	213	117	17	201	3334957	3335565	608	1.44E-10	59.7	42.25
		WP_063860582.1	NC_002929.2	27.23	213	117	17	201	3334957	3335565	608	1.44E-10	59.7	42.25
		WP_063860579.1	NZ_CP039021.1	27.23	213	117	17	201	3334957	3335565	608	1.53E-10	59.7	42.25
		WP_063860579.1	NZ_CP039022.1	27.23	213	117	17	201	3334957	3335565	608	1.53E-10	59.7	42.25
		WP_063860579.1	NC_002929.2	27.23	213	117	17	201	3334957	3335565	608	1.53E-10	59.7	42.25
		WP_063860578.1	NZ_CP039021.1	27.23	213	117	17	201	3334957	3335565	608	6.98E-11	60.5	42.72
		WP_063860578.1	NZ_CP039022.1	27.23	213	117	17	201	3334957	3335565	608	6.98E-11	60.5	42.72
		WP_063860578.1	NC_002929.2	27.23	213	117	17	201	3334957	3335565	608	6.98E-11	60.5	42.72
		WP_063842915.1	NZ_CP039021.1	27.197	239	132	12	223	3334933	3335604	671	9.53E-11	60.5	43.93
		WP_063842915.1	NZ_CP039022.1	27.197	239	132	12	223	3334933	3335604	671	9.53E-11	60.5	43.93
		WP_063842915.1	NC_002929.2	27.197	239	132	12	223	3334933	3335604	671	9.53E-11	60.5	43.93
		WP_063860523.1	NZ_CP039021.1	27.059	85	48	51	124	238039	237794	-245	0.086	33.1	45.88
		WP_063860523.1	NZ_CP039022.1	27.059	85	48	51	124	238039	237794	-245	0.086	33.1	45.88
		WP_063860523.1	NC_002929.2	27.059	85	48	51	124	238039	237794	-245	0.086	33.1	45.88
Metallo-beta-lactamase superfamily	*BP0333*	WP_122630825.1	NZ_CP039021.1	26.761	71	39	72	134	337153	336956	-197	4.8	27.7	40.85
		WP_122630825.1	NZ_CP039022.1	26.761	71	39	72	134	337153	336956	-197	4.8	27.7	40.85

		WP_122630825.1	NC_002929.2	26.761	71	39	72	134	337153	336956	-197	4.8	27.7	40.85
		WP_063860592.1	NZ_CP039021.1	26.733	202	114	25	201	3334987	3335565	578	6.86E-14	69.7	44.55
		WP_063860592.1	NZ_CP039022.1	26.733	202	114	25	201	3334987	3335565	578	6.86E-14	69.7	44.55
		WP_063860592.1	NC_002929.2	26.733	202	114	25	201	3334987	3335565	578	6.86E-14	69.7	44.55
		WP_071766619.1	NZ_CP039021.1	26.627	169	95	66	216	3335101	3335574	473	1.52E-08	53.9	49.11
		WP_071766619.1	NZ_CP039022.1	26.627	169	95	66	216	3335101	3335574	473	1.52E-08	53.9	49.11
		WP_071766619.1	NC_002929.2	26.627	169	95	66	216	3335101	3335574	473	1.52E-08	53.9	49.11
		WP_109791207.1	NZ_CP039021.1	26.601	203	113	25	201	3334987	3335565	578	1.49E-06	47.4	41.87
		WP_109791207.1	NZ_CP039022.1	26.601	203	113	25	201	3334987	3335565	578	1.49E-06	47.4	41.87
		WP_109791207.1	NC_002929.2	26.601	203	113	25	201	3334987	3335565	578	1.49E-06	47.4	41.87
		WP_094009805.1	NZ_CP039021.1	26.601	203	113	25	201	3334987	3335565	578	9.05E-11	60.5	42.86
		WP_094009805.1	NZ_CP039022.1	26.601	203	113	25	201	3334987	3335565	578	9.05E-11	60.5	42.86
		WP_094009805.1	NC_002929.2	26.601	203	113	25	201	3334987	3335565	578	9.05E-11	60.5	42.86
		WP_069280710.1	NZ_CP039021.1	26.601	203	113	25	201	3334987	3335565	578	3.93E-10	58.5	42.86
		WP_069280710.1	NZ_CP039022.1	26.601	203	113	25	201	3334987	3335565	578	3.93E-10	58.5	42.86
		WP_069280710.1	NC_002929.2	26.601	203	113	25	201	3334987	3335565	578	3.93E-10	58.5	42.86
		WP_063860618.1	NZ_CP039021.1	26.601	203	113	25	201	3334987	3335565	578	5.63E-10	57.8	42.36
		WP_063860618.1	NZ_CP039022.1	26.601	203	113	25	201	3334987	3335565	578	5.63E-10	57.8	42.36
		WP_063860618.1	NC_002929.2	26.601	203	113	25	201	3334987	3335565	578	5.63E-10	57.8	42.36
		WP_063860581.1	NZ_CP039021.1	26.601	203	113	25	201	3334987	3335565	578	2.50E-10	58.9	42.86
		WP_063860581.1	NZ_CP039022.1	26.601	203	113	25	201	3334987	3335565	578	2.50E-10	58.9	42.86
		WP_063860581.1	NC_002929.2	26.601	203	113	25	201	3334987	3335565	578	2.50E-10	58.9	42.86
		WP_063860576.1	NZ_CP039021.1	26.601	203	113	25	201	3334987	3335565	578	6.41E-07	48.5	42.36
		WP_063860576.1	NZ_CP039022.1	26.601	203	113	25	201	3334987	3335565	578	6.41E-07	48.5	42.36
		WP_063860576.1	NC_002929.2	26.601	203	113	25	201	3334987	3335565	578	6.41E-07	48.5	42.36
		WP_063860575.1	NZ_CP039021.1	26.601	203	113	25	201	3334987	3335565	578	3.42E-10	58.5	42.86
		WP_063860575.1	NZ_CP039022.1	26.601	203	113	25	201	3334987	3335565	578	3.42E-10	58.5	42.86
		WP_063860575.1	NC_002929.2	26.601	203	113	25	201	3334987	3335565	578	3.42E-10	58.5	42.86

		WP_081853183.1	NZ_CP039021.1	26.531	49	36	186	234	1317494	1317348	-146	5.4	27.3	40.82
		WP_081853183.1	NZ_CP039022.1	26.531	49	36	186	234	1317494	1317348	-146	5.4	27.3	40.82
		WP_081853183.1	NC_002929.2	26.531	49	36	186	234	1317494	1317348	-146	5.4	27.3	40.82
		WP_065419570.1	NZ_CP039021.1	26.471	204	112	25	201	3334987	3335565	578	1.70E-08	53.5	43.14
		WP_065419570.1	NZ_CP039022.1	26.471	204	112	25	201	3334987	3335565	578	1.70E-08	53.5	43.14
		WP_065419570.1	NC_002929.2	26.471	204	112	25	201	3334987	3335565	578	1.70E-08	53.5	43.14
		WP_096807443.1	NZ_CP039021.1	26.432	227	127	17	215	3334957	3335601	644	1.08E-09	57	42.73
		WP_096807443.1	NZ_CP039022.1	26.432	227	127	17	215	3334957	3335601	644	1.08E-09	57	42.73
		WP_096807443.1	NC_002929.2	26.432	227	127	17	215	3334957	3335601	644	1.08E-09	57	42.73
		WP_063860594.1	NZ_CP039021.1	26.364	220	124	25	218	3334987	3335610	623	2.14E-10	59.3	42.73
		WP_063860594.1	NZ_CP039022.1	26.364	220	124	25	218	3334987	3335610	623	2.14E-10	59.3	42.73
		WP_063860594.1	NC_002929.2	26.364	220	124	25	218	3334987	3335610	623	2.14E-10	59.3	42.73
	gloB	WP_063864591.1	NZ_CP039021.1	26.316	95	47	90	182	3424752	3424973	221	0.01	36.2	42.11
		WP_063864591.1	NZ_CP039022.1	26.316	95	47	90	182	3424752	3424973	221	0.01	36.2	42.11
		WP_063864591.1	NC_002929.2	26.316	95	47	90	182	3424752	3424973	221	0.01	36.2	42.11
		WP_063860607.1	NZ_CP039021.1	26.316	228	122	17	208	3334957	3335610	653	7.91E-09	54.3	41.67
		WP_063860607.1	NZ_CP039022.1	26.316	228	122	17	208	3334957	3335610	653	7.91E-09	54.3	41.67
		WP_063860607.1	NC_002929.2	26.316	228	122	17	208	3334957	3335610	653	7.91E-09	54.3	41.67
		WP_063860601.1	NZ_CP039021.1	26.316	228	122	17	208	3334957	3335610	653	5.38E-09	55.1	41.67
		WP_063860601.1	NZ_CP039022.1	26.316	228	122	17	208	3334957	3335610	653	5.38E-09	55.1	41.67
		WP_063860601.1	NC_002929.2	26.316	228	122	17	208	3334957	3335610	653	5.38E-09	55.1	41.67
		WP_063860580.1	NZ_CP039021.1	26.316	228	122	17	208	3334957	3335610	653	9.59E-09	54.3	41.67
		WP_063860580.1	NZ_CP039022.1	26.316	228	122	17	208	3334957	3335610	653	9.59E-09	54.3	41.67
		WP_063860580.1	NC_002929.2	26.316	228	122	17	208	3334957	3335610	653	9.59E-09	54.3	41.67
		WP_063860573.1	NZ_CP039021.1	26.316	228	122	17	208	3334957	3335610	653	2.31E-09	56.2	42.11
		WP_063860573.1	NZ_CP039022.1	26.316	228	122	17	208	3334957	3335610	653	2.31E-09	56.2	42.11
		WP_063860573.1	NC_002929.2	26.316	228	122	17	208	3334957	3335610	653	2.31E-09	56.2	42.11
		WP_031943232.1	NZ_CP039021.1	26.238	202	115	25	201	3334987	3335565	578	7.01E-13	66.6	44.06

		WP_031943232.1	NZ_CP039022.1	26.238	202	115	25	201	3334987	3335565	578	7.01E-13	66.6	44.06
		WP_031943232.1	NC_002929.2	26.238	202	115	25	201	3334987	3335565	578	7.01E-13	66.6	44.06
		WP_122630833.1	NZ_CP039021.1	26.19	126	80	70	187	237946	237584	-362	0.11	32.7	38.1
		WP_122630833.1	NZ_CP039022.1	26.19	126	80	70	187	237946	237584	-362	0.11	32.7	38.1
		WP_122630833.1	NC_002929.2	26.19	126	80	70	187	237946	237584	-362	0.11	32.7	38.1
		WP_060614779.1	NZ_CP039021.1	26.108	203	114	25	201	3334987	3335565	578	2.26E-09	56.2	41.87
		WP_060614779.1	NZ_CP039022.1	26.108	203	114	25	201	3334987	3335565	578	2.26E-09	56.2	41.87
		WP_060614779.1	NC_002929.2	26.108	203	114	25	201	3334987	3335565	578	2.26E-09	56.2	41.87
		WP_015060105.1	NZ_CP039021.1	26.108	203	114	25	201	3334987	3335565	578	1.18E-09	57	42.36
		WP_015060105.1	NZ_CP039022.1	26.108	203	114	25	201	3334987	3335565	578	1.18E-09	57	42.36
		WP_015060105.1	NC_002929.2	26.108	203	114	25	201	3334987	3335565	578	1.18E-09	57	42.36
	BP2804	WP_063842862.1	NZ_CP039021.1	26	50	34	132	180	2978798	2978655	-143	8.5	26.6	48
		WP_063842862.1	NZ_CP039022.1	26	50	34	132	180	2978798	2978655	-143	8.5	26.6	48
		WP_063842862.1	NC_002929.2	26	50	34	132	180	2978798	2978655	-143	8.5	26.6	48
	aroB	WP_071593224.1	NZ_CP039021.1	25.974	77	49	228	300	3862954	3863172	218	7	27.3	46.75
		WP_071593224.1	NZ_CP039022.1	25.974	77	49	228	300	3862954	3863172	218	7	27.3	46.75
		WP_071593224.1	NC_002929.2	25.974	77	49	228	300	3862954	3863172	218	7	27.3	46.75
		WP_020996270.1	NZ_CP039021.1	25.974	77	49	228	300	3862954	3863172	218	6.7	27.7	46.75
		WP_020996270.1	NZ_CP039022.1	25.974	77	49	228	300	3862954	3863172	218	6.7	27.7	46.75
		WP_020996270.1	NC_002929.2	25.974	77	49	228	300	3862954	3863172	218	6.7	27.7	46.75
		WP_063859830.1	NZ_CP039021.1	25.974	77	49	228	300	3862954	3863172	218	6.3	27.7	46.75
		WP_063859830.1	NZ_CP039022.1	25.974	77	49	228	300	3862954	3863172	218	6.3	27.7	46.75
		WP_063859830.1	NC_002929.2	25.974	77	49	228	300	3862954	3863172	218	6.3	27.7	46.75
		WP_063860577.1	NZ_CP039021.1	25.822	213	120	17	201	3334957	3335565	608	5.58E-10	57.8	42.25
		WP_063860577.1	NZ_CP039022.1	25.822	213	120	17	201	3334957	3335565	608	5.58E-10	57.8	42.25
		WP_063860577.1	NC_002929.2	25.822	213	120	17	201	3334957	3335565	608	5.58E-10	57.8	42.25
	BP1291	WP_001460207.1	NZ_CP039021.1	25.714	105	53	193	285	1363115	1362840	-275	3.8	28.5	46.67
		WP_001460207.1	NZ_CP039022.1	25.714	105	53	193	285	1363115	1362840	-275	3.8	28.5	46.67

		WP_001460207.1	NC_002929.2	25.714	105	53	193	285	1363115	1362840	-275	3.8	28.5	46.67
		WP_063860615.1	NZ_CP039021.1	25.616	203	115	25	201	3334987	3335565	578	9.79E-10	57	40.89
		WP_063860615.1	NZ_CP039022.1	25.616	203	115	25	201	3334987	3335565	578	9.79E-10	57	40.89
		WP_063860615.1	NC_002929.2	25.616	203	115	25	201	3334987	3335565	578	9.79E-10	57	40.89
		WP_015588753.1	NZ_CP039021.1	25.571	219	118	28	218	3334999	3335604	605	6.72E-10	57.8	42.01
		WP_015588753.1	NZ_CP039022.1	25.571	219	118	28	218	3334999	3335604	605	6.72E-10	57.8	42.01
		WP_015588753.1	NC_002929.2	25.571	219	118	28	218	3334999	3335604	605	6.72E-10	57.8	42.01
		WP_063860522.1	NZ_CP039021.1	25.571	219	118	28	218	3334999	3335604	605	5.05E-10	58.2	42.01
		WP_063860522.1	NZ_CP039022.1	25.571	219	118	28	218	3334999	3335604	605	5.05E-10	58.2	42.01
		WP_063860522.1	NC_002929.2	25.571	219	118	28	218	3334999	3335604	605	5.05E-10	58.2	42.01
		WP_032492430.1	NZ_CP039021.1	25.571	219	123	27	218	3334987	3335604	617	1.67E-13	68.2	42.01
		WP_032492430.1	NZ_CP039022.1	25.571	219	123	27	218	3334987	3335604	617	1.67E-13	68.2	42.01
		WP_032492430.1	NC_002929.2	25.571	219	123	27	218	3334987	3335604	617	1.67E-13	68.2	42.01
		WP_063860608.1	NZ_CP039021.1	25.472	106	67	152	245	3778592	3778909	317	3.3	27.7	39.62
		WP_063860608.1	NZ_CP039022.1	25.472	106	67	152	245	3778592	3778909	317	3.3	27.7	39.62
		WP_063860608.1	NC_002929.2	25.472	106	67	152	245	3778592	3778909	317	3.3	27.7	39.62
		WP_021018609.1	NZ_CP039021.1	25.472	106	67	152	245	3778592	3778909	317	1.6	28.5	39.62
		WP_021018609.1	NZ_CP039022.1	25.472	106	67	152	245	3778592	3778909	317	1.6	28.5	39.62
		WP_021018609.1	NC_002929.2	25.472	106	67	152	245	3778592	3778909	317	1.6	28.5	39.62
		WP_042862936.1	NZ_CP039021.1	25.472	212	122	17	201	3334957	3335565	608	1.55E-09	56.6	42.45
		WP_042862936.1	NZ_CP039022.1	25.472	212	122	17	201	3334957	3335565	608	1.55E-09	56.6	42.45
		WP_042862936.1	NC_002929.2	25.472	212	122	17	201	3334957	3335565	608	1.55E-09	56.6	42.45
		WP_063860593.1	NZ_CP039021.1	25.472	212	122	17	201	3334957	3335565	608	2.03E-09	56.2	42.45
		WP_063860593.1	NZ_CP039022.1	25.472	212	122	17	201	3334957	3335565	608	2.03E-09	56.2	42.45
		WP_063860593.1	NC_002929.2	25.472	212	122	17	201	3334957	3335565	608	2.03E-09	56.2	42.45
	preceeding *BP0814*	WP_063857702.1	NZ_CP039021.1	25.472	106	66	46	140	839260	839571	311	8.4	27.3	41.51
		WP_063857702.1	NZ_CP039022.1	25.472	106	66	46	140	839260	839571	311	8.4	27.3	41.51
		WP_063857702.1	NC_002929.2	25.472	106	66	46	140	839260	839571	311	8.4	27.3	41.51

		WP_063860612.1	NZ_CP039021.1	25.346	217	124	25	215	3334987	3335601	614	1.70E-09	56.6	41.94
		WP_063860612.1	NZ_CP039022.1	25.346	217	124	25	215	3334987	3335601	614	1.70E-09	56.6	41.94
		WP_063860612.1	NC_002929.2	25.346	217	124	25	215	3334987	3335601	614	1.70E-09	56.6	41.94
		WP_049037557.1	NZ_CP039021.1	25.333	75	45	51	114	238039	237815	-224	0.47	30.8	44
		WP_049037557.1	NZ_CP039022.1	25.333	75	45	51	114	238039	237815	-224	0.47	30.8	44
		WP_049037557.1	NC_002929.2	25.333	75	45	51	114	238039	237815	-224	0.47	30.8	44
		WP_063860562.1	NZ_CP039021.1	25.333	75	45	51	114	238039	237815	-224	0.46	30.8	44
		WP_063860562.1	NZ_CP039022.1	25.333	75	45	51	114	238039	237815	-224	0.46	30.8	44
		WP_063860562.1	NC_002929.2	25.333	75	45	51	114	238039	237815	-224	0.46	30.8	44
		WP_063860561.1	NZ_CP039021.1	25.333	75	45	51	114	238039	237815	-224	0.47	30.8	44
		WP_063860561.1	NZ_CP039022.1	25.333	75	45	51	114	238039	237815	-224	0.47	30.8	44
		WP_063860561.1	NC_002929.2	25.333	75	45	51	114	238039	237815	-224	0.47	30.8	44
		WP_063860536.1	NZ_CP039021.1	25.333	75	45	51	114	238039	237815	-224	0.48	30.8	44
		WP_063860536.1	NZ_CP039022.1	25.333	75	45	51	114	238039	237815	-224	0.48	30.8	44
		WP_063860536.1	NC_002929.2	25.333	75	45	51	114	238039	237815	-224	0.48	30.8	44
		WP_063860525.1	NZ_CP039021.1	25.333	75	45	51	114	238039	237815	-224	0.51	30.8	44
		WP_063860525.1	NZ_CP039022.1	25.333	75	45	51	114	238039	237815	-224	0.51	30.8	44
		WP_063860525.1	NC_002929.2	25.333	75	45	51	114	238039	237815	-224	0.51	30.8	44
		WP_063842629.1	NZ_CP039021.1	25.333	75	45	51	114	238039	237815	-224	0.55	30.4	44
		WP_063842629.1	NZ_CP039022.1	25.333	75	45	51	114	238039	237815	-224	0.55	30.4	44
		WP_063842629.1	NC_002929.2	25.333	75	45	51	114	238039	237815	-224	0.55	30.4	44
		WP_063860591.1	NZ_CP039021.1	25.248	202	117	25	201	3334987	3335565	578	1.13E-09	57	42.08
		WP_063860591.1	NZ_CP039022.1	25.248	202	117	25	201	3334987	3335565	578	1.13E-09	57	42.08
		WP_063860591.1	NC_002929.2	25.248	202	117	25	201	3334987	3335565	578	1.13E-09	57	42.08
		WP_122630834.1	NZ_CP039021.1	25.243	103	62	145	232	1317551	1317243	-308	0.58	30.4	35.92
		WP_122630834.1	NZ_CP039022.1	25.243	103	62	145	232	1317551	1317243	-308	0.58	30.4	35.92
		WP_122630834.1	NC_002929.2	25.243	103	62	145	232	1317551	1317243	-308	0.58	30.4	35.92
		WP_063860614.1	NZ_CP039021.1	25.234	107	66	152	245	3778592	3778909	317	1.9	28.5	42.99

		WP_063860614.1	NZ_CP039022.1	25.234	107	66	152	245	3778592	3778909	317	1.9	28.5	42.99
		WP_063860614.1	NC_002929.2	25.234	107	66	152	245	3778592	3778909	317	1.9	28.5	42.99
		WP_122630823.1	NZ_CP039021.1	25.166	151	79	110	258	3424755	3425111	356	0.027	34.7	37.75
		WP_122630823.1	NZ_CP039022.1	25.166	151	79	110	258	3424755	3425111	356	0.027	34.7	37.75
		WP_122630823.1	NC_002929.2	25.166	151	79	110	258	3424755	3425111	356	0.027	34.7	37.75
		WP_063857820.1	NZ_CP039021.1	25.131	191	115	54	224	2762208	2761660	-548	0.005	37	37.7
		WP_063857820.1	NZ_CP039022.1	25.131	191	115	54	224	2762208	2761660	-548	0.005	37	37.7
		WP_063857820.1	NC_002929.2	25.131	191	115	54	224	2762208	2761660	-548	0.005	37	37.7
		WP_109545053.1	NZ_CP039021.1	25.123	203	116	25	201	3334987	3335565	578	2.67E-08	52.8	40.89
		WP_109545053.1	NZ_CP039022.1	25.123	203	116	25	201	3334987	3335565	578	2.67E-08	52.8	40.89
		WP_109545053.1	NC_002929.2	25.123	203	116	25	201	3334987	3335565	578	2.67E-08	52.8	40.89
		WP_039819893.1	NZ_CP039021.1	25.123	203	116	25	201	3334987	3335565	578	1.31E-09	56.6	40.89
		WP_039819893.1	NZ_CP039022.1	25.123	203	116	25	201	3334987	3335565	578	1.31E-09	56.6	40.89
		WP_039819893.1	NC_002929.2	25.123	203	116	25	201	3334987	3335565	578	1.31E-09	56.6	40.89
		WP_063860619.1	NZ_CP039021.1	25.123	203	116	25	201	3334987	3335565	578	7.53E-09	54.7	41.38
		WP_063860619.1	NZ_CP039022.1	25.123	203	116	25	201	3334987	3335565	578	7.53E-09	54.7	41.38
		WP_063860619.1	NC_002929.2	25.123	203	116	25	201	3334987	3335565	578	7.53E-09	54.7	41.38
		WP_063860611.1	NZ_CP039021.1	25.123	203	116	25	201	3334987	3335565	578	7.40E-09	54.7	41.87
		WP_063860611.1	NZ_CP039022.1	25.123	203	116	25	201	3334987	3335565	578	7.40E-09	54.7	41.87
		WP_063860611.1	NC_002929.2	25.123	203	116	25	201	3334987	3335565	578	7.40E-09	54.7	41.87
		WP_063860609.1	NZ_CP039021.1	25.123	203	116	25	201	3334987	3335565	578	3.33E-09	55.5	40.39
		WP_063860609.1	NZ_CP039022.1	25.123	203	116	25	201	3334987	3335565	578	3.33E-09	55.5	40.39
		WP_063860609.1	NC_002929.2	25.123	203	116	25	201	3334987	3335565	578	3.33E-09	55.5	40.39
		WP_063860606.1	NZ_CP039021.1	25.123	203	116	25	201	3334987	3335565	578	1.48E-08	53.5	40.89
		WP_063860606.1	NZ_CP039022.1	25.123	203	116	25	201	3334987	3335565	578	1.48E-08	53.5	40.89
		WP_063860606.1	NC_002929.2	25.123	203	116	25	201	3334987	3335565	578	1.48E-08	53.5	40.89
		WP_063860590.1	NZ_CP039021.1	25.123	203	116	25	201	3334987	3335565	578	8.69E-10	57.4	40.89
		WP_063860590.1	NZ_CP039022.1	25.123	203	116	25	201	3334987	3335565	578	8.69E-10	57.4	40.89

		WP_063860590.1	NC_002929.2	25.123	203	116	25	201	3334987	3335565	578	8.69E-10	57.4	40.89
		WP_063860586.1	NZ_CP039021.1	25.123	203	116	25	201	3334987	3335565	578	5.62E-09	55.1	41.87
		WP_063860586.1	NZ_CP039022.1	25.123	203	116	25	201	3334987	3335565	578	5.62E-09	55.1	41.87
		WP_063860586.1	NC_002929.2	25.123	203	116	25	201	3334987	3335565	578	5.62E-09	55.1	41.87
		WP_032492096.1	NZ_CP039021.1	25	216	126	25	215	3334987	3335601	614	4.19E-09	55.5	43.06
		WP_032492096.1	NZ_CP039022.1	25	216	126	25	215	3334987	3335601	614	4.19E-09	55.5	43.06
		WP_032492096.1	NC_002929.2	25	216	126	25	215	3334987	3335601	614	4.19E-09	55.5	43.06
		WP_114699282.1	NZ_CP039021.1	25	216	126	25	215	3334987	3335601	614	9.05E-09	54.3	42.59
		WP_114699282.1	NZ_CP039022.1	25	216	126	25	215	3334987	3335601	614	9.05E-09	54.3	42.59
		WP_114699282.1	NC_002929.2	25	216	126	25	215	3334987	3335601	614	9.05E-09	54.3	42.59
		WP_063860608.1	NZ_CP039021.1	25	216	126	25	215	3334987	3335601	614	1.46E-08	53.5	41.67
		WP_063860608.1	NZ_CP039022.1	25	216	126	25	215	3334987	3335601	614	1.46E-08	53.5	41.67
		WP_063860608.1	NC_002929.2	25	216	126	25	215	3334987	3335601	614	1.46E-08	53.5	41.67
		WP_063860614.1	NZ_CP039021.1	25	216	126	25	215	3334987	3335601	614	9.77E-09	54.3	41.67
		WP_063860614.1	NZ_CP039022.1	25	216	126	25	215	3334987	3335601	614	9.77E-09	54.3	41.67
		WP_063860614.1	NC_002929.2	25	216	126	25	215	3334987	3335601	614	9.77E-09	54.3	41.67
		WP_063860588.1	NZ_CP039021.1	25	216	126	25	215	3334987	3335601	614	3.89E-09	55.5	43.06
		WP_063860588.1	NZ_CP039022.1	25	216	126	25	215	3334987	3335601	614	3.89E-09	55.5	43.06
		WP_063860588.1	NC_002929.2	25	216	126	25	215	3334987	3335601	614	3.89E-09	55.5	43.06
	BP0334	WP_081853183.1	NZ_CP039021.1	25	148	83	2	131	337354	336941	-413	1.9	28.9	37.84
		WP_081853183.1	NZ_CP039022.1	25	148	83	2	131	337354	336941	-413	1.9	28.9	37.84
		WP_081853183.1	NC_002929.2	25	148	83	2	131	337354	336941	-413	1.9	28.9	37.84
		WP_081048762.1	NZ_CP039021.1	24.87	193	117	80	250	3335044	3335604	560	2.79E-13	68.2	45.08
		WP_081048762.1	NZ_CP039022.1	24.87	193	117	80	250	3335044	3335604	560	2.79E-13	68.2	45.08
		WP_081048762.1	NC_002929.2	24.87	193	117	80	250	3335044	3335604	560	2.79E-13	68.2	45.08
		WP_041449074.1	NZ_CP039021.1	24.783	230	138	33	232	3334945	3335619	674	1.05E-09	57.4	40.43
		WP_041449074.1	NZ_CP039022.1	24.783	230	138	33	232	3334945	3335619	674	1.05E-09	57.4	40.43
		WP_041449074.1	NC_002929.2	24.783	230	138	33	232	3334945	3335619	674	1.05E-09	57.4	40.43

		WP_003159548.1	NZ_CP039021.1	24.752	202	118	25	201	3334987	3335565	578	1.50E-08	53.5	42.57
		WP_003159548.1	NZ_CP039022.1	24.752	202	118	25	201	3334987	3335565	578	1.50E-08	53.5	42.57
		WP_003159548.1	NC_002929.2	24.752	202	118	25	201	3334987	3335565	578	1.50E-08	53.5	42.57
		WP_071593225.1	NZ_CP039021.1	24.752	202	118	25	201	3334987	3335565	578	5.34E-08	52	41.09
		WP_071593225.1	NZ_CP039022.1	24.752	202	118	25	201	3334987	3335565	578	5.34E-08	52	41.09
		WP_071593225.1	NC_002929.2	24.752	202	118	25	201	3334987	3335565	578	5.34E-08	52	41.09
		WP_122630861.1	NZ_CP039021.1	24.752	202	118	25	201	3334987	3335565	578	3.26E-08	52.8	42.08
		WP_122630861.1	NZ_CP039022.1	24.752	202	118	25	201	3334987	3335565	578	3.26E-08	52.8	42.08
		WP_122630861.1	NC_002929.2	24.752	202	118	25	201	3334987	3335565	578	3.26E-08	52.8	42.08
		WP_114699281.1	NZ_CP039021.1	24.752	202	118	25	201	3334987	3335565	578	1.38E-08	53.5	42.57
		WP_114699281.1	NZ_CP039022.1	24.752	202	118	25	201	3334987	3335565	578	1.38E-08	53.5	42.57
		WP_114699281.1	NC_002929.2	24.752	202	118	25	201	3334987	3335565	578	1.38E-08	53.5	42.57
		WP_114699280.1	NZ_CP039021.1	24.752	202	118	25	201	3334987	3335565	578	5.20E-08	52	42.08
		WP_114699280.1	NZ_CP039022.1	24.752	202	118	25	201	3334987	3335565	578	5.20E-08	52	42.08
		WP_114699280.1	NC_002929.2	24.752	202	118	25	201	3334987	3335565	578	5.20E-08	52	42.08
		WP_109791208.1	NZ_CP039021.1	24.752	202	118	25	201	3334987	3335565	578	3.71E-08	52.4	40.1
		WP_109791208.1	NZ_CP039022.1	24.752	202	118	25	201	3334987	3335565	578	3.71E-08	52.4	40.1
		WP_109791208.1	NC_002929.2	24.752	202	118	25	201	3334987	3335565	578	3.71E-08	52.4	40.1
		WP_104009852.1	NZ_CP039021.1	24.752	202	118	25	201	3334987	3335565	578	3.77E-08	52.4	40.1
		WP_104009852.1	NZ_CP039022.1	24.752	202	118	25	201	3334987	3335565	578	3.77E-08	52.4	40.1
		WP_104009852.1	NC_002929.2	24.752	202	118	25	201	3334987	3335565	578	3.77E-08	52.4	40.1
		WP_102607459.1	NZ_CP039021.1	24.752	202	118	25	201	3334987	3335565	578	1.46E-08	53.5	42.57
		WP_102607459.1	NZ_CP039022.1	24.752	202	118	25	201	3334987	3335565	578	1.46E-08	53.5	42.57
		WP_102607459.1	NC_002929.2	24.752	202	118	25	201	3334987	3335565	578	1.46E-08	53.5	42.57
		WP_085562391.1	NZ_CP039021.1	24.752	202	118	25	201	3334987	3335565	578	3.14E-08	52.8	42.08
		WP_085562391.1	NZ_CP039022.1	24.752	202	118	25	201	3334987	3335565	578	3.14E-08	52.8	42.08
		WP_085562391.1	NC_002929.2	24.752	202	118	25	201	3334987	3335565	578	3.14E-08	52.8	42.08
		WP_032490175.1	NZ_CP039021.1	24.752	202	118	25	201	3334987	3335565	578	3.14E-08	52.8	42.08

		WP_032490175.1	NZ_CP039022.1	24.752	202	118	25	201	3334987	3335565	578	3.14E-08	52.8	42.08
		WP_032490175.1	NC_002929.2	24.752	202	118	25	201	3334987	3335565	578	3.14E-08	52.8	42.08
		WP_032492622.1	NZ_CP039021.1	24.752	202	118	25	201	3334987	3335565	578	1.43E-08	53.5	42.57
		WP_032492622.1	NZ_CP039022.1	24.752	202	118	25	201	3334987	3335565	578	1.43E-08	53.5	42.57
		WP_032492622.1	NC_002929.2	24.752	202	118	25	201	3334987	3335565	578	1.43E-08	53.5	42.57
		WP_063848609.1	NZ_CP039021.1	24.752	202	118	25	201	3334987	3335565	578	1.70E-08	53.5	40.59
		WP_063848609.1	NZ_CP039022.1	24.752	202	118	25	201	3334987	3335565	578	1.70E-08	53.5	40.59
		WP_063848609.1	NC_002929.2	24.752	202	118	25	201	3334987	3335565	578	1.70E-08	53.5	40.59
		WP_063860617.1	NZ_CP039021.1	24.752	202	118	25	201	3334987	3335565	578	1.70E-08	53.5	40.59
		WP_063860617.1	NZ_CP039022.1	24.752	202	118	25	201	3334987	3335565	578	1.70E-08	53.5	40.59
		WP_063860617.1	NC_002929.2	24.752	202	118	25	201	3334987	3335565	578	1.70E-08	53.5	40.59
		WP_063860610.1	NZ_CP039021.1	24.752	202	118	25	201	3334987	3335565	578	3.60E-08	52.4	40.1
		WP_063860610.1	NZ_CP039022.1	24.752	202	118	25	201	3334987	3335565	578	3.60E-08	52.4	40.1
		WP_063860610.1	NC_002929.2	24.752	202	118	25	201	3334987	3335565	578	3.60E-08	52.4	40.1
		WP_063860603.1	NZ_CP039021.1	24.752	202	118	25	201	3334987	3335565	578	1.40E-08	53.5	42.57
		WP_063860603.1	NZ_CP039022.1	24.752	202	118	25	201	3334987	3335565	578	1.40E-08	53.5	42.57
		WP_063860603.1	NC_002929.2	24.752	202	118	25	201	3334987	3335565	578	1.40E-08	53.5	42.57
		WP_063860598.1	NZ_CP039021.1	24.752	202	118	25	201	3334987	3335565	578	4.13E-08	52.4	42.08
		WP_063860598.1	NZ_CP039022.1	24.752	202	118	25	201	3334987	3335565	578	4.13E-08	52.4	42.08
		WP_063860598.1	NC_002929.2	24.752	202	118	25	201	3334987	3335565	578	4.13E-08	52.4	42.08
		WP_063860589.1	NZ_CP039021.1	24.752	202	118	25	201	3334987	3335565	578	1.20E-08	53.9	42.57
		WP_063860589.1	NZ_CP039022.1	24.752	202	118	25	201	3334987	3335565	578	1.20E-08	53.9	42.57
		WP_063860589.1	NC_002929.2	24.752	202	118	25	201	3334987	3335565	578	1.20E-08	53.9	42.57
		WP_063860574.1	NZ_CP039021.1	24.752	202	118	25	201	3334987	3335565	578	5.49E-08	52	41.09
		WP_063860574.1	NZ_CP039022.1	24.752	202	118	25	201	3334987	3335565	578	5.49E-08	52	41.09
		WP_063860574.1	NC_002929.2	24.752	202	118	25	201	3334987	3335565	578	5.49E-08	52	41.09
		WP_021018609.1	NZ_CP039021.1	24.752	202	118	25	201	3334987	3335565	578	7.72E-08	51.6	41.09
		WP_021018609.1	NZ_CP039022.1	24.752	202	118	25	201	3334987	3335565	578	7.72E-08	51.6	41.09

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		WP_021018609.1	NC_002929.2	24.752	202	118	25	201	3334987	3335565	578	7.72E-08	51.6	41.09
		WP_116786839.1	NZ_CP039021.1	24.747	198	115	25	197	3334987	3335553	566	2.10E-08	53.1	42.93
		WP_116786839.1	NZ_CP039022.1	24.747	198	115	25	197	3334987	3335553	566	2.10E-08	53.1	42.93
		WP_116786839.1	NC_002929.2	24.747	198	115	25	197	3334987	3335553	566	2.10E-08	53.1	42.93
		WP_060874262.1	NZ_CP039021.1	24.664	223	130	20	218	3334975	3335601	626	8.82E-16	75.1	42.15
		WP_060874262.1	NZ_CP039022.1	24.664	223	130	20	218	3334975	3335601	626	8.82E-16	75.1	42.15
		WP_060874262.1	NC_002929.2	24.664	223	130	20	218	3334975	3335601	626	8.82E-16	75.1	42.15
preceeding *dps*		WP_063860850.1	NZ_CP039021.1	24.638	138	73	39	176	1699449	1699769	320	1.6	29.6	39.13
		WP_063860850.1	NZ_CP039022.1	24.638	138	73	39	176	1699449	1699769	320	1.6	29.6	39.13
		WP_063860850.1	NC_002929.2	24.638	138	73	39	176	1699449	1699769	320	1.6	29.6	39.13
		WP_063860849.1	NZ_CP039021.1	24.638	138	73	39	176	1699449	1699769	320	1.5	29.6	39.13
		WP_063860849.1	NZ_CP039022.1	24.638	138	73	39	176	1699449	1699769	320	1.5	29.6	39.13
		WP_063860849.1	NC_002929.2	24.638	138	73	39	176	1699449	1699769	320	1.5	29.6	39.13
		WP_043155783.1	NZ_CP039021.1	24.638	138	73	39	176	1699449	1699769	320	3.1	28.5	38.41
		WP_043155783.1	NZ_CP039022.1	24.638	138	73	39	176	1699449	1699769	320	3.1	28.5	38.41
		WP_043155783.1	NC_002929.2	24.638	138	73	39	176	1699449	1699769	320	3.1	28.5	38.41
		WP_011094382.1	NZ_CP039021.1	24.638	69	43	103	162	238021	237815	-206	0.38	31.6	49.28
		WP_011094382.1	NZ_CP039022.1	24.638	69	43	103	162	238021	237815	-206	0.38	31.6	49.28
		WP_011094382.1	NC_002929.2	24.638	69	43	103	162	238021	237815	-206	0.38	31.6	49.28
		WP_063860587.1	NZ_CP039021.1	24.631	203	117	25	201	3334987	3335565	578	7.09E-08	51.6	41.87
		WP_063860587.1	NZ_CP039022.1	24.631	203	117	25	201	3334987	3335565	578	7.09E-08	51.6	41.87
		WP_063860587.1	NC_002929.2	24.631	203	117	25	201	3334987	3335565	578	7.09E-08	51.6	41.87
		WP_088245214.1	NZ_CP039021.1	24.623	199	116	28	201	3334996	3335565	569	2.50E-08	52.8	41.71
		WP_088245214.1	NZ_CP039022.1	24.623	199	116	28	201	3334996	3335565	569	2.50E-08	52.8	41.71
		WP_088245214.1	NC_002929.2	24.623	199	116	28	201	3334996	3335565	569	2.50E-08	52.8	41.71
		WP_063860585.1	NZ_CP039021.1	24.623	199	116	28	201	3334996	3335565	569	2.50E-08	52.8	41.71
		WP_063860585.1	NZ_CP039022.1	24.623	199	116	28	201	3334996	3335565	569	2.50E-08	52.8	41.71
		WP_063860585.1	NC_002929.2	24.623	199	116	28	201	3334996	3335565	569	2.50E-08	52.8	41.71

		WP_071593226.1	NZ_CP039021.1	24.623	199	116	28	201	3334996	3335565	569	2.50E-08	52.8	41.71
		WP_071593226.1	NZ_CP039022.1	24.623	199	116	28	201	3334996	3335565	569	2.50E-08	52.8	41.71
		WP_071593226.1	NC_002929.2	24.623	199	116	28	201	3334996	3335565	569	2.50E-08	52.8	41.71
	BP1251	WP_122630833.1	NZ_CP039021.1	24.615	130	80	121	235	1317623	1317243	-380	0.19	32	35.38
		WP_122630833.1	NZ_CP039022.1	24.615	130	80	121	235	1317623	1317243	-380	0.19	32	35.38
		WP_122630833.1	NC_002929.2	24.615	130	80	121	235	1317623	1317243	-380	0.19	32	35.38
		WP_063860584.1	NZ_CP039021.1	24.537	216	127	25	215	3334987	3335601	614	2.57E-08	52.8	42.59
		WP_063860584.1	NZ_CP039022.1	24.537	216	127	25	215	3334987	3335601	614	2.57E-08	52.8	42.59
		WP_063860584.1	NC_002929.2	24.537	216	127	25	215	3334987	3335601	614	2.57E-08	52.8	42.59
		WP_001336292.1	NZ_CP039021.1	24.528	106	53	193	285	1363115	1362840	-275	5.1	28.1	44.34
		WP_001336292.1	NZ_CP039022.1	24.528	106	53	193	285	1363115	1362840	-275	5.1	28.1	44.34
		WP_001336292.1	NC_002929.2	24.528	106	53	193	285	1363115	1362840	-275	5.1	28.1	44.34
		WP_109545042.1	NZ_CP039021.1	24.352	193	120	67	238	3335041	3335604	563	9.52E-09	54.3	45.6
		WP_109545042.1	NZ_CP039022.1	24.352	193	120	67	238	3335041	3335604	563	9.52E-09	54.3	45.6
		WP_109545042.1	NC_002929.2	24.352	193	120	67	238	3335041	3335604	563	9.52E-09	54.3	45.6
		WP_065102288.1	NZ_CP039021.1	24.257	202	119	25	201	3334987	3335565	578	5.20E-08	52	42.57
		WP_065102288.1	NZ_CP039022.1	24.257	202	119	25	201	3334987	3335565	578	5.20E-08	52	42.57
		WP_065102288.1	NC_002929.2	24.257	202	119	25	201	3334987	3335565	578	5.20E-08	52	42.57
		WP_063860616.1	NZ_CP039021.1	24.257	202	119	25	201	3334987	3335565	578	3.09E-08	52.8	42.08
		WP_063860616.1	NZ_CP039022.1	24.257	202	119	25	201	3334987	3335565	578	3.09E-08	52.8	42.08
		WP_063860616.1	NC_002929.2	24.257	202	119	25	201	3334987	3335565	578	3.09E-08	52.8	42.08
		WP_063860613.1	NZ_CP039021.1	24.257	202	119	25	201	3334987	3335565	578	1.19E-07	50.8	42.08
		WP_063860613.1	NZ_CP039022.1	24.257	202	119	25	201	3334987	3335565	578	1.19E-07	50.8	42.08
		WP_063860613.1	NC_002929.2	24.257	202	119	25	201	3334987	3335565	578	1.19E-07	50.8	42.08
	msrB	WP_069280710.1	NZ_CP039021.1	24.242	99	61	92	184	3778433	3778705	272	6.2	26.9	35.35
		WP_069280710.1	NZ_CP039022.1	24.242	99	61	92	184	3778433	3778705	272	6.2	26.9	35.35
		WP_069280710.1	NC_002929.2	24.242	99	61	92	184	3778433	3778705	272	6.2	26.9	35.35
		WP_063860575.1	NZ_CP039021.1	24.242	99	61	92	184	3778433	3778705	272	6.5	26.9	35.35

		WP_063860575.1	NZ_CP039022.1	24.242	99	61	92	184	3778433	3778705	272	6.5	26.9	35.35
		WP_063860575.1	NC_002929.2	24.242	99	61	92	184	3778433	3778705	272	6.5	26.9	35.35
	BP1522	WP_109545109.1	NZ_CP039021.1	24.074	54	34	45	91	1597610	1597771	161	8.9	26.2	48.15
		WP_109545109.1	NZ_CP039022.1	24.074	54	34	45	91	1597610	1597771	161	8.9	26.2	48.15
		WP_109545109.1	NC_002929.2	24.074	54	34	45	91	1597610	1597771	161	8.9	26.2	48.15
		WP_063978070.1	NZ_CP039021.1	23.963	217	123	30	218	3334996	3335604	608	1.39E-08	53.5	41.47
		WP_063978070.1	NZ_CP039022.1	23.963	217	123	30	218	3334996	3335604	608	1.39E-08	53.5	41.47
		WP_063978070.1	NC_002929.2	23.963	217	123	30	218	3334996	3335604	608	1.39E-08	53.5	41.47
		WP_063860611.1	NZ_CP039021.1	23.913	92	49	120	194	2081128	2080865	-263	1.1	29.3	33.7
		WP_063860611.1	NZ_CP039022.1	23.913	92	49	120	194	2081128	2080865	-263	1.1	29.3	33.7
		WP_063860611.1	NC_002929.2	23.913	92	49	120	194	2081128	2080865	-263	1.1	29.3	33.7
		WP_002988050.1	NZ_CP039021.1	23.881	201	117	24	200	3334987	3335553	566	9.30E-13	66.2	41.79
		WP_002988050.1	NZ_CP039022.1	23.881	201	117	24	200	3334987	3335553	566	9.30E-13	66.2	41.79
		WP_002988050.1	NC_002929.2	23.881	201	117	24	200	3334987	3335553	566	9.30E-13	66.2	41.79
	proceeding *BP0706*	WP_016657654.1	NZ_CP039021.1	23.585	106	68	7	109	720871	721158	287	9.9	26.6	39.62
		WP_016657654.1	NZ_CP039022.1	23.585	106	68	7	109	720871	721158	287	9.9	26.6	39.62
		WP_016657654.1	NC_002929.2	23.585	106	68	7	109	720871	721158	287	9.9	26.6	39.62
		WP_063864747.1	NZ_CP039021.1	23.581	229	165	26	252	3334942	3335604	662	7.32E-10	58.2	44.54
		WP_063864747.1	NZ_CP039022.1	23.581	229	165	26	252	3334942	3335604	662	7.32E-10	58.2	44.54
		WP_063864747.1	NC_002929.2	23.581	229	165	26	252	3334942	3335604	662	7.32E-10	58.2	44.54
		WP_063839879.1	NZ_CP039021.1	23.423	222	136	42	241	3334975	3335604	629	2.49E-11	62.4	43.69
		WP_063839879.1	NZ_CP039022.1	23.423	222	136	42	241	3334975	3335604	629	2.49E-11	62.4	43.69
		WP_063839879.1	NC_002929.2	23.423	222	136	42	241	3334975	3335604	629	2.49E-11	62.4	43.69
		WP_000799223.1	NZ_CP039021.1	23.423	222	136	41	240	3334975	3335604	629	6.24E-12	63.9	43.24
		WP_000799223.1	NZ_CP039022.1	23.423	222	136	41	240	3334975	3335604	629	6.24E-12	63.9	43.24
		WP_000799223.1	NC_002929.2	23.423	222	136	41	240	3334975	3335604	629	6.24E-12	63.9	43.24
		WP_063839880.1	NZ_CP039021.1	23.423	222	136	41	240	3334975	3335604	629	6.53E-12	63.9	43.24
		WP_063839880.1	NZ_CP039022.1	23.423	222	136	41	240	3334975	3335604	629	6.53E-12	63.9	43.24

		WP_063839880.1	NC_002929.2	23.423	222	136	41	240	3334975	3335604	629	6.53E-12	63.9	43.24
	lysA	WP_063864500.1	NZ_CP039021.1	23.418	158	96	109	245	3858259	3858720	461	2.2	28.5	38.61
		WP_063864500.1	NZ_CP039022.1	23.418	158	96	109	245	3858259	3858720	461	2.2	28.5	38.61
		WP_063864500.1	NC_002929.2	23.418	158	96	109	245	3858259	3858720	461	2.2	28.5	38.61
		WP_122630831.1	NZ_CP039021.1	23.404	47	33	86	129	237967	237827	-140	4.1	27.7	53.19
		WP_122630831.1	NZ_CP039022.1	23.404	47	33	86	129	237967	237827	-140	4.1	27.7	53.19
		WP_122630831.1	NC_002929.2	23.404	47	33	86	129	237967	237827	-140	4.1	27.7	53.19
	fabG	WP_015818996.1	NZ_CP039021.1	23.305	236	136	12	237	1876195	1875593	-602	4.2	27.3	38.98
		WP_015818996.1	NZ_CP039022.1	23.305	236	136	12	237	1876195	1875593	-602	4.2	27.3	38.98
		WP_015818996.1	NC_002929.2	23.305	236	136	12	237	1876195	1875593	-602	4.2	27.3	38.98
		WP_063860629.1	NZ_CP039021.1	23.256	215	125	31	220	3335002	3335601	599	3.29E-05	43.1	38.14
		WP_063860629.1	NZ_CP039022.1	23.256	215	125	31	220	3335002	3335601	599	3.29E-05	43.1	38.14
		WP_063860629.1	NC_002929.2	23.256	215	125	31	220	3335002	3335601	599	3.29E-05	43.1	38.14
		WP_015818996.1	NZ_CP039021.1	23.182	220	128	26	219	3334990	3335604	614	1.90E-05	43.9	44.09
		WP_015818996.1	NZ_CP039022.1	23.182	220	128	26	219	3334990	3335604	614	1.90E-05	43.9	44.09
		WP_015818996.1	NC_002929.2	23.182	220	128	26	219	3334990	3335604	614	1.90E-05	43.9	44.09
		WP_000799232.1	NZ_CP039021.1	23.171	246	152	20	240	3334903	3335604	701	2.36E-12	65.1	43.09
		WP_000799232.1	NZ_CP039022.1	23.171	246	152	20	240	3334903	3335604	701	2.36E-12	65.1	43.09
		WP_000799232.1	NC_002929.2	23.171	246	152	20	240	3334903	3335604	701	2.36E-12	65.1	43.09
		WP_000799227.1	NZ_CP039021.1	23.171	246	152	20	240	3334903	3335604	701	5.22E-11	61.2	41.87
		WP_000799227.1	NZ_CP039022.1	23.171	246	152	20	240	3334903	3335604	701	5.22E-11	61.2	41.87
		WP_000799227.1	NC_002929.2	23.171	246	152	20	240	3334903	3335604	701	5.22E-11	61.2	41.87
		WP_000778180.1	NZ_CP039021.1	23.158	190	129	70	250	3334972	3335517	545	8.61E-08	52.8	44.74
		WP_000778180.1	NZ_CP039022.1	23.158	190	129	70	250	3334972	3335517	545	8.61E-08	52.8	44.74
		WP_000778180.1	NC_002929.2	23.158	190	129	70	250	3334972	3335517	545	8.61E-08	52.8	44.74
		WP_052157330.1	NZ_CP039021.1	23.043	230	137	34	239	3334966	3335607	641	2.75E-08	53.1	42.61
		WP_052157330.1	NZ_CP039022.1	23.043	230	137	34	239	3334966	3335607	641	2.75E-08	53.1	42.61
		WP_052157330.1	NC_002929.2	23.043	230	137	34	239	3334966	3335607	641	2.75E-08	53.1	42.61

		WP_012261328.1	NZ_CP039021.1	22.973	222	137	42	241	3334975	3335604	629	5.80E-10	58.2	42.79
		WP_012261328.1	NZ_CP039022.1	22.973	222	137	42	241	3334975	3335604	629	5.80E-10	58.2	42.79
		WP_012261328.1	NC_002929.2	22.973	222	137	42	241	3334975	3335604	629	5.80E-10	58.2	42.79
		WP_063860203.1	NZ_CP039021.1	22.936	218	129	27	219	3334993	3335604	611	1.63E-06	47.4	42.2
		WP_063860203.1	NZ_CP039022.1	22.936	218	129	27	219	3334993	3335604	611	1.63E-06	47.4	42.2
		WP_063860203.1	NC_002929.2	22.936	218	129	27	219	3334993	3335604	611	1.63E-06	47.4	42.2
	BP3847	WP_096807440.1	NZ_CP039021.1	22.656	128	76	89	204	4057994	4058344	350	5	27.3	41.41
		WP_096807440.1	NZ_CP039022.1	22.656	128	76	89	204	4057994	4058344	350	5	27.3	41.41
		WP_096807440.1	NC_002929.2	22.656	128	76	89	204	4057994	4058344	350	5	27.3	41.41
		WP_063864722.1	NZ_CP039021.1	22.535	213	126	12	195	3334939	3335547	608	1.35E-05	44.7	38.97
		WP_063864722.1	NZ_CP039022.1	22.535	213	126	12	195	3334939	3335547	608	1.35E-05	44.7	38.97
		WP_063864722.1	NC_002929.2	22.535	213	126	12	195	3334939	3335547	608	1.35E-05	44.7	38.97
		WP_026025287.1	NZ_CP039021.1	22.363	237	139	15	226	3334954	3335604	650	5.92E-06	45.8	42.62
		WP_026025287.1	NZ_CP039022.1	22.363	237	139	15	226	3334954	3335604	650	5.92E-06	45.8	42.62
		WP_026025287.1	NC_002929.2	22.363	237	139	15	226	3334954	3335604	650	5.92E-06	45.8	42.62
	promoter region/ ahead of *BP3130*	WP_017901052.1	NZ_CP039021.1	22.326	215	128	12	197	3334939	3335553	614	7.50E-06	45.4	39.07
		WP_017901052.1	NZ_CP039022.1	22.326	215	128	12	197	3334939	3335553	614	7.50E-06	45.4	39.07
		WP_017901052.1	NC_002929.2	22.326	215	128	12	197	3334939	3335553	614	7.50E-06	45.4	39.07
		WP_006259855.1	NZ_CP039021.1	21.818	220	132	24	218	3334987	3335601	614	1.26E-11	62.8	45
		WP_006259855.1	NZ_CP039022.1	21.818	220	132	24	218	3334987	3335601	614	1.26E-11	62.8	45
		WP_006259855.1	NC_002929.2	21.818	220	132	24	218	3334987	3335601	614	1.26E-11	62.8	45
	BP0351	WP_032488481.1	NZ_CP039022.1	21.739	92	45	124	211	353335	353129	-206	7.6	26.6	38.04
		WP_032488481.1	NC_002929.2	21.739	92	45	124	211	353335	353129	-206	7.6	26.6	38.04
		WP_032488481.1	NZ_CP039021.1	21.739	92	45	124	211	353335	353129	-206	8.1	26.6	38.04
		WP_063864592.1	NZ_CP039021.1	21.675	203	127	23	203	3334987	3335565	578	3.04E-09	55.5	39.41
		WP_063864592.1	NZ_CP039022.1	21.675	203	127	23	203	3334987	3335565	578	3.04E-09	55.5	39.41
		WP_063864592.1	NC_002929.2	21.675	203	127	23	203	3334987	3335565	578	3.04E-09	55.5	39.41

		WP_041258349.1	NZ_CP039021.1	21.659	217	132	30	224	3334996	3335598	602	2.76E-06	46.6	40.09
		WP_041258349.1	NZ_CP039022.1	21.659	217	132	30	224	3334996	3335598	602	2.76E-06	46.6	40.09
		WP_041258349.1	NC_002929.2	21.659	217	132	30	224	3334996	3335598	602	2.76E-06	46.6	40.09
		WP_015266427.1	NZ_CP039021.1	21.545	246	151	21	241	3334918	3335604	686	2.13E-06	47.4	41.46
		WP_015266427.1	NZ_CP039022.1	21.545	246	151	21	241	3334918	3335604	686	2.13E-06	47.4	41.46
		WP_015266427.1	NC_002929.2	21.545	246	151	21	241	3334918	3335604	686	2.13E-06	47.4	41.46
		WP_063860632.1	NZ_CP039021.1	21.491	228	140	18	220	3334960	3335601	641	4.63E-06	45.8	39.04
		WP_063860632.1	NZ_CP039022.1	21.491	228	140	18	220	3334960	3335601	641	4.63E-06	45.8	39.04
		WP_063860632.1	NC_002929.2	21.491	228	140	18	220	3334960	3335601	641	4.63E-06	45.8	39.04
		WP_063860852.1	NZ_CP039021.1	21.364	220	133	24	218	3334987	3335601	614	4.91E-11	61.2	44.55
		WP_063860852.1	NZ_CP039022.1	21.364	220	133	24	218	3334987	3335601	614	4.91E-11	61.2	44.55
		WP_063860852.1	NC_002929.2	21.364	220	133	24	218	3334987	3335601	614	4.91E-11	61.2	44.55
Metallo-beta-lactamase superfamily	*BP0230*	WP_063857696.1	NZ_CP039021.1	21.333	75	48	51	114	238039	237815	-224	8.9	26.6	41.33
		WP_063857696.1	NZ_CP039022.1	21.333	75	48	51	114	238039	237815	-224	8.9	26.6	41.33
		WP_063857696.1	NC_002929.2	21.333	75	48	51	114	238039	237815	-224	8.9	26.6	41.33
		WP_063860627.1	NZ_CP039021.1	21.32	197	124	49	221	3335032	3335601	569	2.84E-06	46.6	38.07
		WP_063860627.1	NZ_CP039022.1	21.32	197	124	49	221	3335032	3335601	569	2.84E-06	46.6	38.07
		WP_063860627.1	NC_002929.2	21.32	197	124	49	221	3335032	3335601	569	2.84E-06	46.6	38.07
		WP_002979282.1	NZ_CP039021.1	21.32	197	124	51	223	3335032	3335601	569	3.83E-06	46.2	41.12
		WP_002979282.1	NZ_CP039022.1	21.32	197	124	51	223	3335032	3335601	569	3.83E-06	46.2	41.12
		WP_002979282.1	NC_002929.2	21.32	197	124	51	223	3335032	3335601	569	3.83E-06	46.2	41.12
		WP_063857828.1	NZ_CP039021.1	20.976	205	126	28	208	3334987	3335565	578	2.18E-08	53.1	39.02
		WP_063857828.1	NZ_CP039022.1	20.976	205	126	28	208	3334987	3335565	578	2.18E-08	53.1	39.02
		WP_063857828.1	NC_002929.2	20.976	205	126	28	208	3334987	3335565	578	2.18E-08	53.1	39.02
		WP_063865209.1	NZ_CP039021.1	20.974	267	163	25	252	445508	446281	773	0.32	30.8	40.07
		WP_063865209.1	NZ_CP039022.1	20.974	267	163	25	252	445508	446281	773	0.32	30.8	40.07
		WP_063865209.1	NC_002929.2	20.974	267	163	25	252	445508	446281	773	0.32	30.8	40.07

		WP_063860630.1	NZ_CP039021.1	20.87	230	142	17	220	3334954	3335601	647	3.17E-05	43.5	40.87
		WP_063860630.1	NZ_CP039022.1	20.87	230	142	17	220	3334954	3335601	647	3.17E-05	43.5	40.87
		WP_063860630.1	NC_002929.2	20.87	230	142	17	220	3334954	3335601	647	3.17E-05	43.5	40.87
		WP_052769157.1	NZ_CP039021.1	20.614	228	142	18	220	3334960	3335601	641	4.64E-04	39.7	37.72
		WP_052769157.1	NZ_CP039022.1	20.614	228	142	18	220	3334960	3335601	641	4.64E-04	39.7	37.72
		WP_052769157.1	NC_002929.2	20.614	228	142	18	220	3334960	3335601	641	4.64E-04	39.7	37.72
		WP_063860621.1	NZ_CP039021.1	20.614	228	142	18	220	3334960	3335601	641	4.47E-04	39.7	37.72
		WP_063860621.1	NZ_CP039022.1	20.614	228	142	18	220	3334960	3335601	641	4.47E-04	39.7	37.72
		WP_063860621.1	NC_002929.2	20.614	228	142	18	220	3334960	3335601	641	4.47E-04	39.7	37.72
		WP_029729112.1	NZ_CP039021.1	20.574	209	130	24	208	3334975	3335565	590	1.26E-07	50.8	38.76
		WP_029729112.1	NZ_CP039022.1	20.574	209	130	24	208	3334975	3335565	590	1.26E-07	50.8	38.76
		WP_029729112.1	NC_002929.2	20.574	209	130	24	208	3334975	3335565	590	1.26E-07	50.8	38.76
		WP_063857823.1	NZ_CP039021.1	20.574	209	130	24	208	3334975	3335565	590	1.35E-07	50.8	38.76
		WP_063857823.1	NZ_CP039022.1	20.574	209	130	24	208	3334975	3335565	590	1.35E-07	50.8	38.76
		WP_063857823.1	NC_002929.2	20.574	209	130	24	208	3334975	3335565	590	1.35E-07	50.8	38.76
		WP_034848068.1	NZ_CP039021.1	20.574	209	130	23	207	3334975	3335565	590	1.50E-07	50.4	38.76
		WP_034848068.1	NZ_CP039022.1	20.574	209	130	23	207	3334975	3335565	590	1.50E-07	50.4	38.76
		WP_034848068.1	NC_002929.2	20.574	209	130	23	207	3334975	3335565	590	1.50E-07	50.4	38.76
		WP_063860631.1	NZ_CP039021.1	20.561	214	132	31	220	3335002	3335601	599	5.80E-04	39.3	36.45
		WP_063860631.1	NZ_CP039022.1	20.561	214	132	31	220	3335002	3335601	599	5.80E-04	39.3	36.45
		WP_063860631.1	NC_002929.2	20.561	214	132	31	220	3335002	3335601	599	5.80E-04	39.3	36.45
		WP_059154943.1	NZ_CP039021.1	20.488	205	127	27	207	3334987	3335565	578	5.60E-08	52	39.02
		WP_059154943.1	NZ_CP039022.1	20.488	205	127	27	207	3334987	3335565	578	5.60E-08	52	39.02
		WP_059154943.1	NC_002929.2	20.488	205	127	27	207	3334987	3335565	578	5.60E-08	52	39.02
		WP_059345632.1	NZ_CP039021.1	20.488	205	127	27	207	3334987	3335565	578	6.71E-08	51.6	39.02
		WP_059345632.1	NZ_CP039022.1	20.488	205	127	27	207	3334987	3335565	578	6.71E-08	51.6	39.02
		WP_059345632.1	NC_002929.2	20.488	205	127	27	207	3334987	3335565	578	6.71E-08	51.6	39.02
		WP_063860620.1	NZ_CP039021.1	20.435	230	143	17	220	3334954	3335601	647	4.30E-05	42.7	40.87

		WP_063860620.1	NZ_CP039022.1	20.435	230	143	17	220	3334954	3335601	647	4.30E-05	42.7	40.87
		WP_063860620.1	NC_002929.2	20.435	230	143	17	220	3334954	3335601	647	4.30E-05	42.7	40.87
		WP_063860622.1	NZ_CP039021.1	20.379	211	130	34	220	3335011	3335601	590	4.30E-04	40	39.34
		WP_063860622.1	NZ_CP039022.1	20.379	211	130	34	220	3335011	3335601	590	4.30E-04	40	39.34
		WP_063860622.1	NC_002929.2	20.379	211	130	34	220	3335011	3335601	590	4.30E-04	40	39.34
		WP_063842622.1	NZ_CP039021.1	20.339	236	151	24	235	3334975	3335643	668	1.12E-07	50.8	37.71
		WP_063842622.1	NZ_CP039022.1	20.339	236	151	24	235	3334975	3335643	668	1.12E-07	50.8	37.71
		WP_063842622.1	NC_002929.2	20.339	236	151	24	235	3334975	3335643	668	1.12E-07	50.8	37.71
		WP_063860628.1	NZ_CP039021.1	20.305	197	126	48	220	3335032	3335601	569	2.98E-05	43.5	40.1
		WP_063860628.1	NZ_CP039022.1	20.305	197	126	48	220	3335032	3335601	569	2.98E-05	43.5	40.1
		WP_063860628.1	NC_002929.2	20.305	197	126	48	220	3335032	3335601	569	2.98E-05	43.5	40.1
		WP_063857826.1	NZ_CP039021.1	20.096	209	131	24	208	3334975	3335565	590	3.87E-07	49.3	38.76
		WP_063857826.1	NZ_CP039022.1	20.096	209	131	24	208	3334975	3335565	590	3.87E-07	49.3	38.76
		WP_063857826.1	NC_002929.2	20.096	209	131	24	208	3334975	3335565	590	3.87E-07	49.3	38.76
		WP_063857822.1	NZ_CP039021.1	20	205	128	27	207	3334987	3335565	578	4.14E-07	49.3	38.54
		WP_063857822.1	NZ_CP039022.1	20	205	128	27	207	3334987	3335565	578	4.14E-07	49.3	38.54
		WP_063857822.1	NC_002929.2	20	205	128	27	207	3334987	3335565	578	4.14E-07	49.3	38.54
		WP_059324801.1	NZ_CP039021.1	20	205	128	27	207	3334987	3335565	578	3.71E-07	49.3	38.54
		WP_059324801.1	NZ_CP039022.1	20	205	128	27	207	3334987	3335565	578	3.71E-07	49.3	38.54
		WP_059324801.1	NC_002929.2	20	205	128	27	207	3334987	3335565	578	3.71E-07	49.3	38.54
		WP_063860626.1	NZ_CP039021.1	19.797	197	127	48	220	3335032	3335601	569	1.11E-04	41.6	41.12
		WP_063860626.1	NZ_CP039022.1	19.797	197	127	48	220	3335032	3335601	569	1.11E-04	41.6	41.12
		WP_063860626.1	NC_002929.2	19.797	197	127	48	220	3335032	3335601	569	1.11E-04	41.6	41.12
		WP_063860624.1	NZ_CP039021.1	19.797	197	127	48	220	3335032	3335601	569	1.54E-04	41.2	41.12
		WP_063860624.1	NZ_CP039022.1	19.797	197	127	48	220	3335032	3335601	569	1.54E-04	41.2	41.12
		WP_063860624.1	NC_002929.2	19.797	197	127	48	220	3335032	3335601	569	1.54E-04	41.2	41.12
		WP_038333643.1	NZ_CP039021.1	19.731	223	137	20	216	3334981	3335601	620	9.08E-05	42	37.67
		WP_038333643.1	NZ_CP039022.1	19.731	223	137	20	216	3334981	3335601	620	9.08E-05	42	37.67

		WP_038333643.1	NC_002929.2	19.731	223	137	20	216	3334981	3335601	620	9.08E-05	42	37.67
		WP_063860216.1	NZ_CP039021.1	19.731	223	137	20	216	3334981	3335601	620	4.92E-05	42.7	37.22
		WP_063860216.1	NZ_CP039022.1	19.731	223	137	20	216	3334981	3335601	620	4.92E-05	42.7	37.22
		WP_063860216.1	NC_002929.2	19.731	223	137	20	216	3334981	3335601	620	4.92E-05	42.7	37.22
		WP_063857829.1	NZ_CP039021.1	19.512	205	129	27	207	3334987	3335565	578	5.34E-07	48.9	38.54
		WP_063857829.1	NZ_CP039022.1	19.512	205	129	27	207	3334987	3335565	578	5.34E-07	48.9	38.54
		WP_063857829.1	NC_002929.2	19.512	205	129	27	207	3334987	3335565	578	5.34E-07	48.9	38.54
		WP_065597326.1	NZ_CP039021.1	19.298	228	139	24	226	3334981	3335604	623	3.77E-04	40	42.98
		WP_065597326.1	NZ_CP039022.1	19.298	228	139	24	226	3334981	3335604	623	3.77E-04	40	42.98
		WP_065597326.1	NC_002929.2	19.298	228	139	24	226	3334981	3335604	623	3.77E-04	40	42.98
		WP_063857825.1	NZ_CP039021.1	19.024	205	130	28	208	3334987	3335565	578	2.83E-05	43.5	39.02
		WP_063857825.1	NZ_CP039022.1	19.024	205	130	28	208	3334987	3335565	578	2.83E-05	43.5	39.02
		WP_063857825.1	NC_002929.2	19.024	205	130	28	208	3334987	3335565	578	2.83E-05	43.5	39.02
		WP_063857824.1	NZ_CP039021.1	19.024	205	130	28	208	3334987	3335565	578	4.28E-05	43.1	38.54
		WP_063857824.1	NZ_CP039022.1	19.024	205	130	28	208	3334987	3335565	578	4.28E-05	43.1	38.54
		WP_063857824.1	NC_002929.2	19.024	205	130	28	208	3334987	3335565	578	4.28E-05	43.1	38.54
		WP_063857827.1	NZ_CP039021.1	19.024	205	130	28	208	3334987	3335565	578	6.81E-05	42.4	38.05
		WP_063857827.1	NZ_CP039022.1	19.024	205	130	28	208	3334987	3335565	578	6.81E-05	42.4	38.05
		WP_063857827.1	NC_002929.2	19.024	205	130	28	208	3334987	3335565	578	6.81E-05	42.4	38.05