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Antimicrobial production by *Pectobacterium carotovorum* subspecies *brasiliensis* and its role in competitive fitness of the potato pathogen

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A Dissertation
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of the requirements for the Degree of
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Abigail Durrant

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Antimicrobial production by *Pectobacterium carotovorum* subspecies *brasiliensis* and its role in competitive fitness of the potato pathogen

Abigail Durrant

Pectobacterium carotovorum subspecies *brasiliensis* (*P. c.* subsp. *brasiliensis*), a member of the soft rot Erwinias (SREs), was first described as the causative agent of a stem disease in potato called blackleg. Blackleg describes the blackening, wilting and necrosis of potato stem tissue. Initially detected in Brazil, *P. c.* subsp. *brasiliensis* subsequently emerged as a pathogen in temperate regions, although the mechanisms that contributed to its emergence are unknown.

A second SRE pathogen, *Dickeya solani,* also emerged as an aggressive potato pathogen in Europe. *Dickeya solani* successfully displaced the previously dominant blackleg causing pathogens, such as *P. atrosepticum*. Comparative genomic studies, using the genome of *D. solani* plus other SRE genomes such as *Pectobacterium,* identified some *D. solani* specific genes. Three of these loci were identified as novel non-ribosomal synthetase/polyketide synthatase (NRPS/PKS) genes, which all encoded previously unknown products. It was predicted that the combination of these novel gene clusters provided the adaptive advantage, which enabled *D. solani* to successfully emerge as a pathogen.

The genome of a *P. c.* subsp. *brasiliensis* strain isolated from infected potato plants in New Zealand, *P. c.* subsp. *brasiliensis* ICMP 19477, was recently sequenced. The bacterium was found to encode many genes associated with antimicrobial production, including bacteriocin and carbapenem synthesis, as well as a putative novel NRPS locus. A number of the identified loci were not present in the genomes of other SREs. One of these antimicrobial clusters, or a combination of these clusters, may be an important mechanism in the emergence of *P. c.* subsp. *brasiliensis.* However, the ecological significance of antimicrobial molecules is not understood.

It has previously been reported that, *P. c.* subsp. *brasiliensis* PBR1692, is antagonistic to *P. atrosepticum* SCRI1043 *in vitro* (Marquez-Villavicencio et al., 2011). However, *in planta* significance of this

interaction appeared minimal during co-inoculation studies in potato stems. *Pectobacterium betavasculorum,* was also reported to inhibit the growth of other *Pectobacterium* species when co-inoculated in potato tubers.

This study found that *P. c.* subsp. *brasiliensis* ICMP 19477 outcompetes *P. atrosepticum* SCRI1043 in both *in vitro* plate and *in planta* competition assays*,* when co-inoculated in potato tubers. However, this was not observed in *in vitro* liquid competition assays. This suggested that the antagonistic effect of *P. c.* subsp. *brasiliensis* ICMP 19477 on *P. atrosepticum* SCRI1043 only occurred in structured environments.

Functional studies identified that *P. c.* subsp. *brasiliensis* ICMP 19477 produces a secreted antimicrobial molecule at late exponential / early stationary phase. A random transposon (Tn*5*) mutant library of *P. c.* subsp. *brasiliensis* ICMP 19477 identified three mutants, within the genes *carR*, *slyA* and *carI*, which were unable to inhibit the growth of *P. atrosepticum* SCRI1043 *in vitro.* These mutated genes are known to be involved in carbapenem regulation in *P. c.* subsp. *carotovorum.* Furthermore, these mutants also lost the competitive advantage against *P. atrosepticum* SCRI1043 when co-inoculated in potato tubers. This evidence suggested that a carbapenem molecule, produced by *P. c.* subsp. *brasiliensis* ICMP 19477, enhances the competitive fitness of the bacterium *in planta.*

Overall, this study provided novel insights into the ecological significance of antimicrobial production by plant pathogens, thereby, identifying possible mechanisms for pathogen emergence.

Keywords: Soft rot Erwinias, *Pectobacterium,* emerging pathogen, antimicrobial

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'Twenty years from now you will be more disappointed by the things you didn't do than by the ones you did do. So throw off the bowlines. Sail away from the safe harbour. Catch the trade winds in your sails. Explore. Dream. Discover'. H. Jackson Brown Jr.

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List of abbreviations

°C- degrees Celsius

µg- microgram

µl- microliter

µM- micromolar

Amp- Ampicillin

BLAST- Basic local alignment search tool

bp- Base pairs

CDI- Contact dependent inhibition

cDNA- Complementary deoxyribonucleic acid

CDS- Coding sequence

CFU- Colony forming units

Chl- Chloramphenicol

CI-Competitive index

cm- Centimetres

Ctv- carotovoricin

d- Day

ddH$_2$O- Double distilled water

DNA- Deoxyribonucleic acid

dNTP- Deoxynucleotide triphosphate

dpi- Days post inoculation

E. coli- *Escherichia coli*

ESS- *Escherichia coli* supersensitive

EDTA- Ethylene diamine tetra acetic acid

F- Forward

gDNA- Genomic deoxyribonucleic acid

g- grams

GFP- Green fluorescence protein

GI- Genomic island

h- Hour

HAI- Horizontally acquired island

His- Hexahistidine

hpi- Hours post inoculation

IPTG- Isopropyl β-D-1-thiogalactopyranoside

Kb- Kilobase pairs

Km- Kanamycin

l- Litre

LB- Luria-Bertani broth

LBA- Luria-Bertani agar

LC-MS- Liquid chromatography- mass spectrometry

mg- milligram

Milli-Q- Filter sterilised ddH$_2$O

min- Minute

M- Molar

ml- millilitre

mM- millimolar

MM- M9 minimal media

MMA- M9 minimal media agar

mRNA- Messenger ribonucleic acid

MS- Mass spectrometry

***N*-AHL-** *N*-acyl homoserine lactone

ng- Nano gram

nm- Nanometre

NRP- Non-ribosomal protein

NRPS- Non-ribosomal protein synthetase

OD-optical density

OHHL- N-(3-oxohexanoyl)-L-homoserine lactone

ORF- Open reading frame

oriT- Origin of transfer

P. c.* subsp. *brasiliensis- *Pectobacterium carotovorum* subspecies *brasiliensis*

***P. c.* subsp. *carotovorum* -** *Pectobacterium carotovorum* subspecies *caratovorum*

PCR- Polymerase chain reaction

PCWDEs-Plant cell wall degrading enzymes

Pel-Pectin lyase

PKS-Polyketide synthetase

PSI-Pounds per square inch

pv.- pathovar

QS-Quorum sensing

R- Reverse

RBS- Ribosome binding site

Rif- Rifampicin

RNA- Ribonucleic acid

mRNA- Messenger ribonucleic acid

rRNA- ribosomal ribonucleic acid

RPM-Revolutions per minute

RT- Reverse transcription

RT-PCR-Reverse transcription polymerase

chain reaction

sec- second

sp.- Species

SREs-Soft rot Erwinias

Str- Streptomycin

subsp.- subspecies

T3SS-Type three secretion system

TAE- Tris-acetate-EDTA

TE-Tris-EDTA

Tet- Tetracycline

TMHs- Transmembrane helices

Tn*5*- Transposon

UV-ultra violet

V- volts

v/v- volume/volume ratio

WT- Wild type

w/v- weight/volume ratio

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Chapter 1

Introduction

1.1 Background

Crop loss due to weeds, pests and pathogens places pressure on the production of food for human consumption. Studies on potato crops, the world's most produced non-grain food commodity (http://www.fao.org/potato-2008/en/aboutiyp/index.html), show that 40% of the worldwide crop is lost each year. Pathogens account for 14% of this loss (Oerke, 2006). The soft-rot erwinias (SREs) are considered important pathogens that influence productivity of the potato industry, causing crop damage both in the field and post-harvest. In 1980, it was estimated that the total global economic loss due to these bacteria was US$50-100 million annually (Pérombelon and Kelman, 1980). Within New Zealand the importance of the potato crop is such that more arable land is used for potato production than for any other vegetable. The total export and retail value of the New Zealand potato industry is valued at NZ$570 million annually (http://www.plantandfood.co.nz/growingfutures/case-studies/potatoes-for-boiling-crisping-chipping). As there are currently no effective chemical controls for management of diseases caused by SREs and the resistance of commercially available cultivars is considered low, disease control is limited to preventative methods such as seed grading (Lebecka and Zimnoch-Guzowska, 2004). A greater understanding of the interactions between host and pathogen during disease development could therefore improve breeding of cultivars for resistance and consequently improve the productivity and security of both the global and New Zealand potato crop.

1.2 The genus *Erwinia*

The SREs are necrotrophic, gram negative, rod shaped, facultative anaerobic bacteria. They are pathogenic to many plants, including both crop and ornamental species, and are distinct from other plant pathogens as they produce a wide range of plant cell wall degrading enzymes (PCWDEs) (Pérombelon and Kelman, 1980). They are members of the *Enterobacteriaceae,* which includes human pathogens such as *Escherichia coli* and *Salmonella* as well as other plant pathogens such as *Erwinia amylovora,* the causative agent of fire blight of apple. The genus *Erwinia* was initially proposed in 1917 (Winslow et al., 1917) to encompass all plant pathogenic *Enterobacteriaceae*, regardless of their degree of relatedness to the other members within the family (Perombelon, 1992). Based on 16S rDNA sequence analysis, the genus was subsequently divided into five groups; the 'true' *Erwinia* (Hauben et

al., 1998), the SRE (Young et al., 1996) *Pantoea* (Gavini et al., 1989), *Enterobacter* (Brenner et al., 1986) and *Brenneria* (Hauben et al., 1998). More recent analysis led to the reclassification of the SREs into *Pectobacterium* (Hauben et al., 1998) and *Dickeya* (Samson et al., 2005) following DNA-DNA hybridisation and 16S rDNA sequence analysis as well as serological and numerical taxonomy. This reclassification also resulted in elevation of three of the *E. carotovorum* subspecies to species level: (*Pectobacterium atrosepticum, P. wasabiae* and *P. betavasculorum* (synonyms; *E. carotovorum* subsp. *atroseptica, E. carotovorum* subsp. *wasabiae*, and *E. carotovorum* subsp. *betavasculorum*, respectively)) (Gardan et al., 2003).

Genomic approaches have further enhanced the classification of SREs. For example, *P. wasabiae* SCC3193 was reclassified by genome and proteome comparison with all sequenced SRE genomes (Nykyri et al., 2012). It was originally classified as *P. c.* subsp. *carotovorum*, following assessment of its virulence on potatoes, the fatty acid composition, the range of PCWDEs produced as well as other biochemical characteristics (Pirhonen et al., 1988). This was important as until this classification *P. wasabiae* was present in Europe but was not identified (Nykyri et al., 2012). Similarly, pectinolytic bacteria isolated from diseased potato plants exhibiting blackleg and slow wilt symptoms in Europe and Israel were also identified using genome-based average nucleotide identity analysis and DNA–DNA hybridization as being representative of a novel species, for which the name *Dickeya solani* sp. nov. was proposed (Van Der Wolf et al., 2014). Initially, they had been classified as belonging to the genus *Dickeya*, previously the *P. chrysanthemi* complex (*E. chrysanthemi*) on the basis of production of a PCR product with the pelADE primers, 16S rRNA gene sequence analysis, fatty acid methyl esterase analysis, the production of phosphatases and the ability to produce indole and acids from α-methylglucoside (Nasser et al., 1999; Laurila et al., 2008). Reclassification of these isolates into a distinct species was important for reasons of pest management and food security.

1.3 Host ranges and ecology

It was initially considered that different SRE species inhabited distinct host ranges and geographical ranges largely as a result of their capacity to cause disease at different temperatures (Pérombelon and Kelman, 1980; Pérombelon, 1992). For example, *P. atrosepticum* was believed to have a very narrow host range, infecting mainly potato in temperate regions whilst *Dickeya* were considered broad host-range pathogens infecting plants in tropical and subtropical regions. However, it has now been shown that many of the SREs have a global distribution, consistent with very little difference in their growth temperatures *in vitro* (Du Raan et al., 2016).

The major SREs associated with crop losses in potato are *P. atrosepticum, P. c.* subsp. *carotovorum* and *Dickeya*, although other *Pectobacterium* species have also been shown to infect this host. *Pectobacterium betavasculorum,* for example, causes tuber soft rot as well as vascular necrosis of sugar beet and has been isolated from sunflower, potato and artichoke (Thomson et al., 1981; Gardan et al., 2003). *Pectobacterium wasabiae,* which was originally thought to have a host range restricted to Japanese horseradish (Goto and Matsumoto, 1987), was also found to cause rot of various vegetables worldwide including potato (Pitman et al., 2009; Nykyri et al., 2012; Moleleki et al., 2013; Waleron et al., 2013). *Pectobacterium carotovorum* subsp. *carotovorum* has a broad host range including potato, celery and capsicum. It is associated with disease in both temperate and subtropical regions (Pérombelon and Kelman, 1980; Kado, 2006). On potato, *P. c.* subsp. *carotovorum* is usually associated with soft rot of tubers. However a recently identified subspecies of this bacterium, *P. c. subsp. brasiliensis,* was shown to be associated with potato blackleg in Brazil (Duarte et al., 2004). Blackleg is characterised by blackening and wilting of the potato stem, which also causes stunting of the plant. It was subsequently detected in South Africa (van der Merwe et al., 2010), North America (De Boer et al., 2012) and New Zealand (Panda et al., 2012) suggesting it has a global impact on potato production.

Pectobacterium atrosepticum infects a very narrow host range, which appears to be mainly limited to potato in temperate regions; although some strains were reported to cause sunflower rot in Turkey (Bastas et al., 2009). *Pectobacterium atrosepticum* is primarily known to cause blackleg disease of potato crops but infection can also result in soft rot of tubers especially in storage (Pérombelon, 1992; Perombelon, 2002). Although the identified host range for this bacteria is limited, it appears that *P. atrosepticum* may have an increased ability to survive in soils or the rhizosphere, due to the presence of nitrogen fixation genes within the genome (Bell et al., 2004). It therefore may have a different lifestyle on other plants. For example, *P. atrosepticum* has been isolated from the rhizosphere of cucumbers grown in glasshouses without the presence of disease symptoms (Butler, 1978). Such genes have not been identified in other SREs (Bell et al., 2004). The survival of both *P. c.* subsp. *carotovorum* and *P. atrosepticum* in soils appears to rely on the presence of contaminating plant material. Under these conditions, they are both able to persist for long periods of time within the fields and are able to overwinter under these conditions (Voronkevich, 1960; Burr and Schroth, 1977). In non-contaminated soil, the bacteria are only able to survive for one week to six months, depending on temperature and water availability (Anilkumar and Chakravarti, 1970). *Pectobacterium carotovorum* subsp. *carotovorum* is able to out-survive *P. atrosepticum* in soil isolated from Scotland (Pérombelon, 1972). In cabbage fields in both Japan and Taiwan, *P. c.* subsp. *carotovorum* is

considered to be present in soils below detection levels as bacteria are easily isolated from plants grown in these fields (Kikumoto and Sakamoto, 1970; Mew et al., 1976).

The *Dickeya* (formally *E. chrysanthemi*) are classified into eight species (Samson et al., 2005), although unique bacteria have been identified which may represent new species and are therefore not classified into one of these subspecies (Samson et al., 2005; Van Der Wolf et al., 2014). The disease symptoms caused by these bacteria are almost indistinguishable from those produced by *Pectobacterium.* However, *Dickeya* infection can be determined by a higher optimal growth temperature and the ability to cause disease at lower inoculum levels (Toth et al., 2011). In Europe, *D. dianthicola* and the newly identified *D. solani* are prevalent potato pathogens, although other species are known to infect potato crops in more tropical regions. *Dickeya solani* is now considered a major threat to the potato production industry (Toth et al., 2011).

1.4 Conditions required for disease

The SREs have been described as 'opportunistic' pathogens, as they enter potato plants through natural openings of the tuber surface (lenticels), or via wound sites. Following multiplication within the mother tuber, the bacteria are able to spread either to the stem, causing blackleg, or to the progeny tubers (Pérombelon and Kelman, 1980).

The environmental factors impacting on SRE disease development are not fully understood. However, certain environmental and nutritional conditions have been identified that enhance disease progression. For example, high water level, high nitrogen content and low calcium or magnesium ion concentrations (McGuire and Kelman, 1986; Bain et al., 1996; Lambert et al., 2005; Cho et al., 2013). Ion levels influence both plant and bacterial behaviour, therefore a certain balance is required for disease progression. At high calcium ion concentrations the plant cell walls within the potato tubers are strengthened (Barras et al., 1994), however calcium is also required for the enzymic activity of the pectinases produced by the bacteria. Similarly, nitrates promote growth of the plant, but also enhance the anaerobic respiration of SREs (Smid et al., 1993).

The SREs have been isolated from apparently disease free plants and tubers, indicating an ability to remain latent until conditions are favourable for disease development (Pérombelon, 1972; Hayward, 1974), when sufficient free water becomes available, anaerobic conditions develop and the optimal growth temperature is reached. The nature of the latent period is still not understood (Pérombelon and Kelman, 1980; Perombelon, 2002). Recent evidence suggests that the bacteria may have different

stages in the plant. A motile stage is used to invade the tissue, but cell density dependent sensing is required to establish sessile colonies that initiate release of pectolytic enzymes for the acquisition of nutrients from the plant (and consequently disease symptoms) (Moleleki et al., 2016).

1.5 Virulence determinants

A variety of virulence determinants have been identified in SREs that enhance the ability of these pathogens to colonise potato plants. These include PCWDEs, secretion systems, phytotoxins and iron scavengers. These are co-ordinately expressed as a consequence of a complex regulatory system to ensure virulence factors are not expressed when they are not required.

1.5.1 Quorum sensing

Quorum sensing (QS) regulates the expression of many virulence factors in SREs. Quorum sensing is a genetic regulatory system in bacteria that allows the bacteria to control the expression of certain genes in a population density dependent manner (Whitehead et al., 2001). Population density is detected by the production of a small diffusible signalling molecules of the *N*-acyl homoserine lactone family (*N*-AHL). The *N*-AHL molecules are constitutively expressed by the bacteria. When the bacterial population reaches a critical level, the *N*-AHLs also reach a high enough concentration to initiate transcription of virulence genes (Whitehead et al., 2001).

Some bacteria produce multiple QS signalling molecules. For example, *Pseudomonas aeruginosa* produces both *N*-(3-oxododecanoyl) homoserine lactone and *N*-butyryl homoserine lactone (Singh et al., 2000; Wu et al., 2000). However, in both *P. c.* subsp. *carotovorum* and *P. atrosepticum* only one QS molecule, *N*-(3-oxohexanoyl) homoserine lactone (OHHL), is produced (Andersson et al., 2000). The OHHL molecule is constitutively expressed within the population and when sufficient OHHL is present within the population, expression of many secondary metabolite genes, including those encoding the PCWDEs, are activated (Perombelon, 2002). Other novel pathogenicity determinants are also under QS control such as Nip, which induces necrosis in plants (Pemberton et al., 2005) and Svx, the function of which is unknown (Corbett et al., 2005).

Although QS regulates expression of key virulence factors, a *P. atrosepticum* strain modified so that it could not produce OHHL was not affected in its ability to colonise the host (Smadja et al., 2004). The strain showed very low expression of the PCWDEs, therefore, it was concluded that QS was vital for disease progression, but not for plant colonisation (Smadja et al., 2004). More recent data indicates that QS regulates the colonisation of potato xylem tissue by *P. c.* subsp. *brasiliensis* PBR1692 (Moleleki

et al., 2016). *Pectobacterium carotovorum* subsp. *brasiliensis* PBR1692 was observed to accumulate within xylem tissue when inoculated *in planta.* However, a QS defective mutant was restricted to intercellular spaces and was unable to colonise the xylem (Moleleki et al., 2016). These data suggest that QS regulates the invasion of xylem tissue by blackleg-causing pathogens.

In *P. atrosepticum,* microarray analysis found that 26% of the genome was under QS control. This showed that the regulatory system was a more important genetic regulatory system than previously thought (Liu et al., 2008).The QS regulated genes were identified and included secretion systems, regulators and systems involved in subverting the plants defences (Liu et al., 2008). Therefore, the QS system is a global regulator of *P. atrosepticum* pathogenicity and plays a greater role than as the activating system of the PCWDEs.

1.5.2 Plant cell wall degrading enzymes

The main pathogenicity determinants of the SREs are the PCWDEs, which include the cellulases, proteases and pectinases that are co-ordinately produced during infection. The pectinases are of most significance to the pathogen, with a variety of these enzymes secreted to degrade the α-1,4-glycosidic linkage in pectate (Barras et al., 1994; Toth et al., 2003). Their release leads to extensive tissue maceration and degradation of cellular components in the primary and secondary cell walls as well as in the middle lamella. The resulting products are used as a nutrient source for the bacteria. This enzymatic degradation enhances the ability of *Pectobacterium* to colonize and penetrate the host (Collmer and Keen, 1986; Barras et al., 1994). *Pectobacterium carotovorum* subsp. *carotovorum* mutants defective in extracellular enzyme production are non-pathogenic (Pirhonen et al., 1991), confirming PCWDEs are essential for disease development. Two secretion systems have been identified that are required for the transport of the PCWDEs. Proteases are transported in a one-step mechanism by the type I secretion system, which appears to play a relatively small role in bacterial pathogenicity (Delepelaire and Wandersman, 1990). Cellulases and pectinases are transported in a sec dependent, two-step process by a type II secretion system called the OUT complex. This system is required for disease progression (Andro et al., 1984; Thurn and Chatterjee, 1985; Murata et al., 1990).

1.5.3 Secretion systems

Genes encoding a type III secretion system (T3SS) have been identified in *P. atrosepticum* (Bell et al., 2004), *P. c.* subsp. *carotovorum* (Rantakari et al., 2001) and *Dickeya* (Ham et al., 1998). In other

bacteria, this system is used to secrete effector and helper proteins directly into the host cell, which alter cellular processes such as host defence systems (Rosqvist et al., 1994). In *P. atrosepticum*, mutants in the helper (*hrpN*) and effector (*dspE/A)* genes have reduced virulence, indicating a role for this system in pathogenicity (Holeva et al., 2004). Similar results have also been observed in *Dickeya* (Bauer et al., 1994; Yang et al., 2002) and *P. c.* subsp. *carotovorum* (Rantakari et al., 2001) using susceptible hosts, such as *Arabidopsis* and African violet. However, pathogenic *Pectobacterium* lacking a functional T3SS have been isolated from infected potato crops (Kim et al., 2009; Pitman et al., 2009), suggesting that this system may not be essential for pathogenicity of all *Pectobacterium* strains or at least for pathogenicity in potato tubers.

Pectobacterium also encode other well characterised secretion systems such as the type II secretion system (T2SS), also named the OUT system, which exports PCWDEs fromm the cell during infection of the host plant (Johnson et al., 2006). A two-partner type V secretion system (T5SS), which is associated with cell to cell adherence and microbial competition (Charkowski et al., 2012), has also been identified in *Pectobacterium*. In both *E. coli* and *Dickeya*, the T5SS is known to function in contact dependent inhibition (Aoki et al., 2010).

1.5.4 Iron acquisition

Iron is a vital nutritional factor that is required for cellular signalling or as a cofactor for various proteins including enzymes. The low-iron environment found in host organisms, especially plants, means that pathogens require iron uptake systems in order to colonise and initiate disease (Expert, 1999). The iron acquisition systems of the SREs have been studied extensively. *Dickeya* encodes the siderophores chrysobactin and achromobactin, which are required for full virulence and systemic infection by the bacteria (Expert and Toussaint, 1985; Enard et al., 1988). *Pectobacterium carotovorum* subsp. *carotovorum* also produces two siderophores, chrysobactin and aerobactin, although neither appear to be essential for tuber or stem rot disease of potatoes by the bacterium (Bull et al., 1996). It is, however, possible that these bacteria utilize another iron acquisition system, such as a heme or ferric citrate transporter, or that an iron acquisition mechanism is only required in the stem where iron limitation may be greater (Expert, 1999). The availability of iron, as well as the ability of the pathogen to acquire iron within the host, is an important host-pathogen interaction during disease. For example, it has been demonstrated that in *Arabidopsis* plants starved of iron, *D. dadantii* were less pathogenic and the host showed greater levels of resistance (Kieu et al., 2012).

Iron accumulation can be important for the protection of plant pathogens against the 'oxidative burst'; a defence system initiated by the host involving the production of large amounts of reactive oxygen species following invasion by a pathogen (Bolwell and Wojtaszek, 1997). In *Dickeya*, the incorporation of iron into Fe-S clusters has been linked to oxidative stress resistance. Proteins encoded by the *suf* operon have been shown to be involved in the metabolism of iron and possibly in the assembly of Fe-S clusters. Bacteria with mutations in *suf* genes show reduced growth under oxidative conditions. These mutants are also less virulent when inoculated into host plants (Nachin et al., 2001). Little is known about iron uptake in *P. atrosepticum*. Genes encoding for achromobactin uptake and transport and enterobactin synthesis are present in the genome, but their synthesis by the bacteria has not been confirmed experimentally (Bell et al., 2004). Although enterobactin has not previously been identified in SREs, it is a well characterized system in other enterobacteriaceae such as *E. coli,* where it functions as a high affinity iron scavenger (Raymond et al., 2003).

1.5.5 Toxins

The availability of the genome sequence of *P. atrosepticum* SCRI1043 (*E. carotovorum* subsp. *atrosepticum* SCRI1043) has revealed the presence of genes with homology to the *cfa* genes of *Pseudomonas syringae* (Bell et al., 2004), which encode the synthetase for the polyketide portion (coronafacic acid) of the phytotoxin coronatine. Coronatine is an important virulence factor for some pathovars of the bacterial pathogen *P. syringae* (Bender et al., 1999a). Coronatine acts as a molecular mimic of 12-oxo-phytodienoic acid, a precursor of jasmonic acid (Weiler et al., 1994), therefore stimulating the jasmonate response pathway (Zhao et al., 2003). Subsequently, it represses the salicylic acid plant defence pathway, which would otherwise promote a plant response to the bacterium (Uppalapati et al., 2007). Although the genes encoding the synthesis of the second component of coronatine (coronamic acid) are absent from the *P. atrosepticum* genome, mutations in the *cfa6* and *cfa7* genes cause a reduction in virulence (Bell et al., 2004). Such a reduction indicates that the *P. atrosepticum cfa* genes have a role in virulence and that this may result from its interaction with a different conjugate.

1.6 Antimicrobial molecules

The virulence determinants described above contribute to the interactions between the SREs and their plant host, but under field conditions, these bacteria do not exist in isolation. For example, different

Pectobacterium species have been isolated from the same potato fields in the U.S.A (Gross et al., 1991) and multiple strains have been isolated from the same disease lesions (Yap et al., 2004). In mixed microbial populations, many interactions are likely to occur between the bacteria. For example, *P. c.* subsp. *brasiliensis* appears to out-compete *P. atrosepticum,* as *P. atrosepticum* does not occur if *P. c.* subsp. *brasiliensis* is present on potato (Duarte et al., 2004). This may be why only four of the 89 isolates collected from potato in New Zealand were *P. atrosepticum* even though the climate is temperate and considered more suitable for *P. atrosepticum* (Pitman et al., 2008). Instead, 18 isolates were *P. c.* subsp. *brasiliensis* (Pitman et al., 2008).

Genome comparisons have revealed that SREs encode a variety of genes likely to encode antimicrobial molecules. Carbapenem synthesis genes have been identified in strains of *P. c.* subsp. *carotovorum* (Bycroft et al., 1988). Bacteriocins such as carotovoricin (Nguyen et al., 1999) and carocin (Chuang et al., 2007) have also been described, along with phenazine genes (Mavrodi et al., 2010). Phenazine synthesis genes are also found in the genome of *P. atrosepticum* (Bell et al., 2004). This suggests that antimicrobial molecules play an important role in the lifestyle of SREs. Antimicrobial production is likely particularly important in interactions with other organisms both on and off the plant, but the ecological significance of antimicrobial production in SREs has not been proven.

1.6.1 Carbapenems

Carbapenems are a member of the β-lactam antibiotic family, which includes many medically important antibiotics. It has been estimated that 50% of globally used antibiotics are of the β-lactam family (Elander, 2003; Coulthurst et al., 2005; Schmidt, 2011). Carbapenemsare described as having a broad spectrum of activity, compared to the bacteriocin antimicrobials, and a structure that is relatively resistant to degradation by β-lactamases (Ratcliffe and Albers-Schonberg, 1982). The target of action of β-lactams is the cell wall of the recipient bacteria. They act by inhibiting the activity of transpeptidase enzymes (Waxman and Strominger, 1983) or penicillin binding proteins (Sauvage et al., 2008). This prevents crosslinking of peptidoglycan during cell wall biosynthesis.

β-lactams are produced by a range of bacteria, both gram positive and negative. Members of the genus *Streptomyces* produce complex molecules such as thienamycin (a β-lactam) (Kahan et al., 1979). Research into the synthesis and regulation of this molecule has been hindered by the growth rate of the producers and by the complexity of the related pathways (Williamson et al., 1985). A simple β-lactam, carbapenem, however, is produced by some strains of *Serratia* and *Pectobacterium*. This molecule is (5R)-carbapen-2-em-3-carboxylic acid (Parker et al., 1982; Bycroft et al., 1988). The fast

growth rate and the relative ease of genetic manipulation in these producer strains has enabled genetic and molecular studies (Bainton et al., 1992).

1.6.1.1 Carbapenem Biosynthesis

The genes encoding carbapenem have been identified as a gene operon of eight genes; *carABCDEFGH* (McGowan et al., 1996). In *P. c.* subsp. *carotovorum*, the *carABCDE* genes function in carbapenem biosynthesis, however, only the *carA*, *carB* and *carC* genes have been shown to be vital for the biosynthesis of the carbapenem molecule (McGowan et al., 1997; Li et al., 2000; Clifton et al., 2003; Sleeman and Schofield, 2004). The *carF* and *carG* genes encode an intrinsic carbapenem resistance system, which protects the producing cell. The *carH* gene has an unknown function (McGowan et al., 1997) (Figure 1.1).

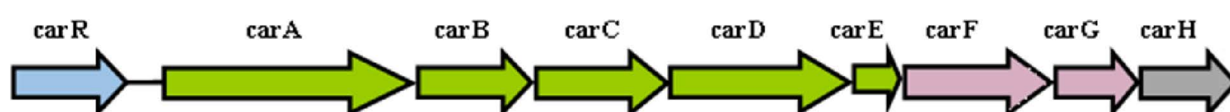


Figure 1.1. The gene cluster of *P. c.* subsp. *carotovorum* responsible for carbapenem production. The functions of these genes are listed in Table 1.1. The cluster contains a regulator (blue), five synthesis genes (green), two resistance encoding genes (pink) and a gene of unknown function (grey).

Table 1.1. The function of the proteins encoded by the carbapenem synthesis cluster of *P. c.* subsp. *carotovorum*. (Adapted from Hamed et al., (2013).

Gene	Function of gene product
carR	Transcriptional regulator (LuxR type) (McGowan et al., 1995)
carA	β-Lactam synthesis (Miller et al., 2003)
carB	t-CMP production (Gerratana et al., 2004; Sleeman and Schofield, 2004)
carC	Carbapenem synthesis (Clifton et al., 2003)
carD	Proline dehydrogenase (McGowan et al., 1996)
carE	2Fe-2S ferrodoxin

carF	Resistance system (McGowan et al., 1997)
carG	Resistance system (McGowan et al., 1997)
carH	Unknown

The structure of the carbapenem molecule produced by *P. c.* subsp. *carotovorum* has been determined as (5R)-carbapen-2-em-3-carboxylic acid (Parker et al., 1982). Isolation of this simple molecule has enabled the study of carbapenem biosynthesis, due to the simpler process compared to C6 molecules. (5R)-carbapen-2-em-3-carboxylic acid is processed from proline via the intermediates (3S,5S) and (3S,5R)-carbapenam-3-carboxylic acid (Bycroft et al., 1987; Bycroft and Chhabra, 1989) by CarC (Stapon et al., 2003) (Figure 1.2).

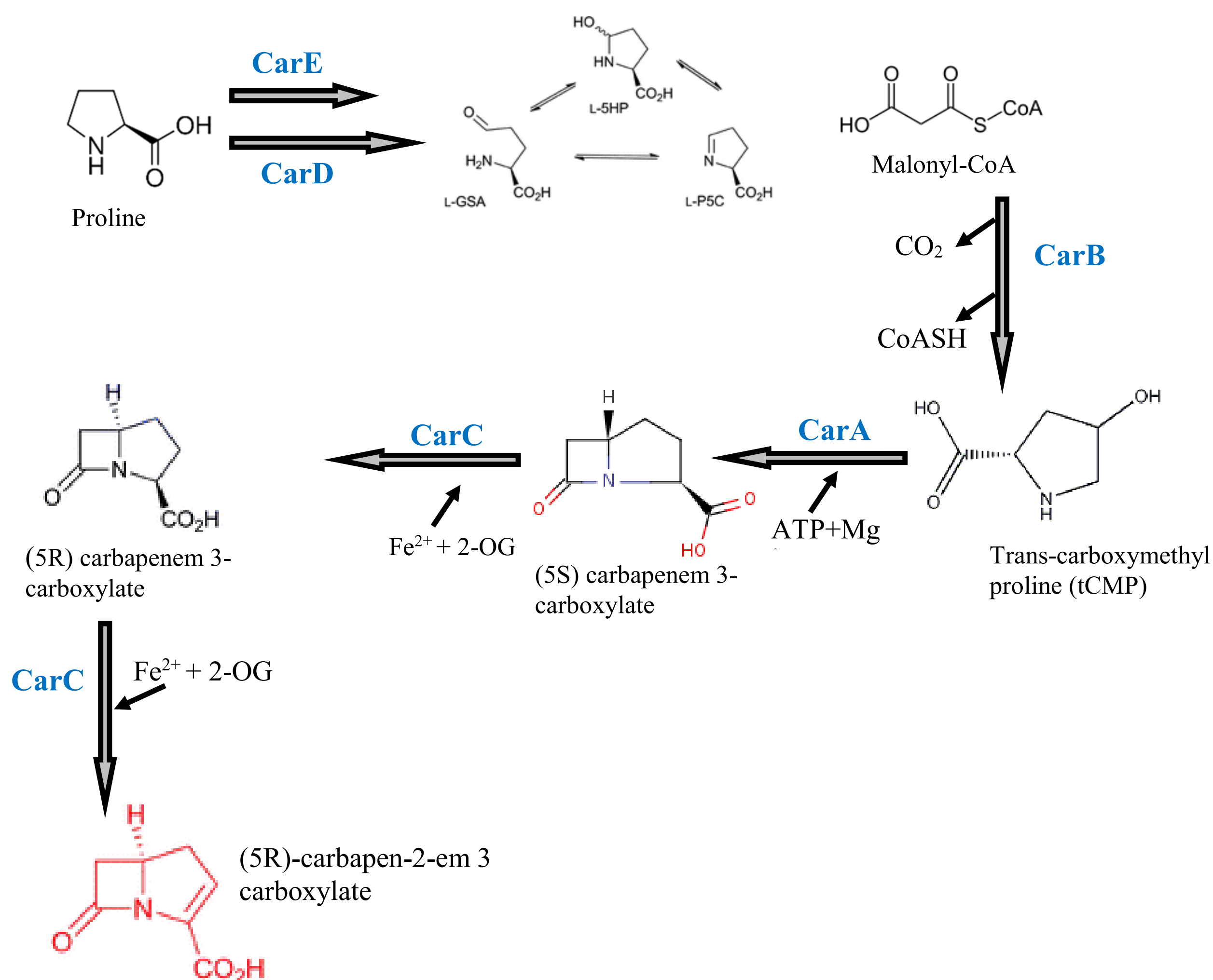


Figure 1.2. A schematic of the biosynthesis pathway producing the carbapenem product, in *P. c.* subsp. *carotovorum*, (5R)-carbapen-2-em 3-carboxylate. The roles of each synthetase gene in the pathway are indicated. Adapted from (Hamed et al., 2013).

1.6.1.2 Regulation

Regulation of the *carA-H* operon in *P. c.* subsp. *carotovorum* is complicated, as its transcription is modulated by many genetic and physiological factors. Upstream of the biosynthesis cluster is a LuxR-type transcriptional regulator encoding gene, called *carR*. LuxR-type regulators respond to low molecular weight signal molecules, such as OHHL (see Section 1.5.1 on QS). CarR is no exception, responding to OHHL to activate transcription of the *car* operon when population size increases at late exponential or early stationary phase (McGowan et al., 1995). The OHHL molecule is produced by an OHHL synthetase called CarI which is unlinked to *carR* (McGowan and Salmond, 1999). The concentration of OHHL decreases during stationary phase, likely due to changes in buffer conditions (Byers et al., 2002). This reduction in OHHL concentration corresponds to the ceasing of carbapenem production (McGowan et al., 2005).

Physiological factors also modulate carbapenem production by affecting QS. The available carbon source alters the level of the *carI* transcript at the transcriptional level. In particular, the presence of glycerol in the growth media significantly reduces the level of *carI* transcription in comparison with media containing glucose (McGowan et al., 2005). Growth temperature also influences carbapenem production. For example, the OHHL concentration present in the growth media of *P. c.* subsp. *carotovorum* ATTn10 was reduced when cultures were grown at 37°C rather than 30°C. The OHHL concentration achieved at 37°C was subsequently found to be below the level required to initiate *carA-H* transcription (McGowan et al., 2005). It is thought that an increase in temperature negatively affects *carI* transcription, however an additional mechanism is also likely to be involved in the reduced expression of the *car* operon (McGowan et al., 2005). Finally, an oxygen limitation causes a reduction in *carA* transcription. Reduced transcription appears to result from directly affecting the transcription of the *car* operon (McGowan et al., 2005), however it may also be associated with a negative impact on *carI* transcription (McGowan et al., unpublished data).

Quorum sensing-independent mechanisms also modulate carbapenem gene expression. For example, constitutive expression of a promoter within the *carA-H* operon ensures the continuous transcription of the *carF* and *car*G genes. The promoter is expressed even in a Δ*carR* mutant (McGowan et al., 2005). The promoter region was mapped to a site within *carD*, 571 bp upstream of *carE*. It is therefore thought that this promoter initiates transcription of *carEFGH*. The individual functions of *carE* and

carH are not understood. Therefore, the main purpose of this promoter is to ensure constant synthesis of the novel carbapenem resistance mechanism encoded by *carEF*, to provide 'self-resistance' to the carbapenem produced by the bacteria (McGowan et al., 2005). (For more information on CarF and CarG mediated resistance see Section 1.6.1.3).

Transcription of the *car* operon in *P. c.* subsp. *carotovorum* is also controlled by Hor*,* a regulator belonging to the SlyA/MarR-type family of transcriptional regulators (Thomson et al., 1997). Transcription of the *hor* gene is controlled differently in carbapenem-producing and non-producing *P. c.* subsp. *carotovorum* strains. *Pectobacterium carotovorum* subsp. *carotovorum* SCC3193 lacks carbapenem synthesis genes. In this strain, *hor* transcription is moderated by ExpR, which responds to OHHL. At low population levels when OHHL is not present at the critical level, ExpR binds to the *hor* promoter region preventing transcription (Sjöblom et al., 2008). However, in the carbapenem-producing strain *P. c.* subsp. *carotovorum* ATTn10, the Hor regulatory system operates concurrently with the QS system as a QS-independent network (McGowan et al., 2005).

The mechanisms by which different members of the SlyA/MarR family modulate gene expression varies between regulators. A conserved mechanism among the regulators, however, is competition for binding sites with other binding proteins (Ellison and Miller, 2006). The regulators SlyA (*Salmonella*), PecS (*Dickeya*) and RovA (*Yerssinia*) all act as de-repressors of gene transcription by competing for promoter binding sites with the histone-like protein H-NS (Heroven et al., 2004). Activation of gene expression by this mechanism is considered the primary function of RovA (Stapleton et al., 2002). In contrast, PecS of *Dickeya* both represses and activates gene transcription. The regulator has been shown to negatively affect the transcription of pectate lyase and cellulase genes by competing for binding sites with cAMP, the cofactor of the CRP-cAMP transcriptional activator complex (Rouanet et al., 1999). By preventing the binding of transcriptional repressors, PecS acts to positively affect the transcription of polygalacturonase genes (Nasser et al., 1999; Hugouvieux-Cotte-Pattat et al., 2002). The regulator SlyA, from *Salmonella*, has also been shown to prevent the binding of RNA polymerase at the *slyA* promoter site, repressing its own transcription (Stapleton et al., 2002).

SlyA/MarR-type regulators are involved in antibiotic resistance and the production of antimicrobial molecules (i.e. *Rap* in *S. marcescens*) (George and Levy, 1983; Thomson et al., 1997; Srikumar et al., 1998; Fineran et al., 2005). Such regulators are also involved in initiating transcription of virulence genes. For example, the MarR-type regulator Hor from *P. c.* subsp. *carotovorum,* regulates the production of exoenzymes as well as antimicrobial production (Thomson et al., 1997).

The carbapenem regulatory network is summarised in Figure 1.3.

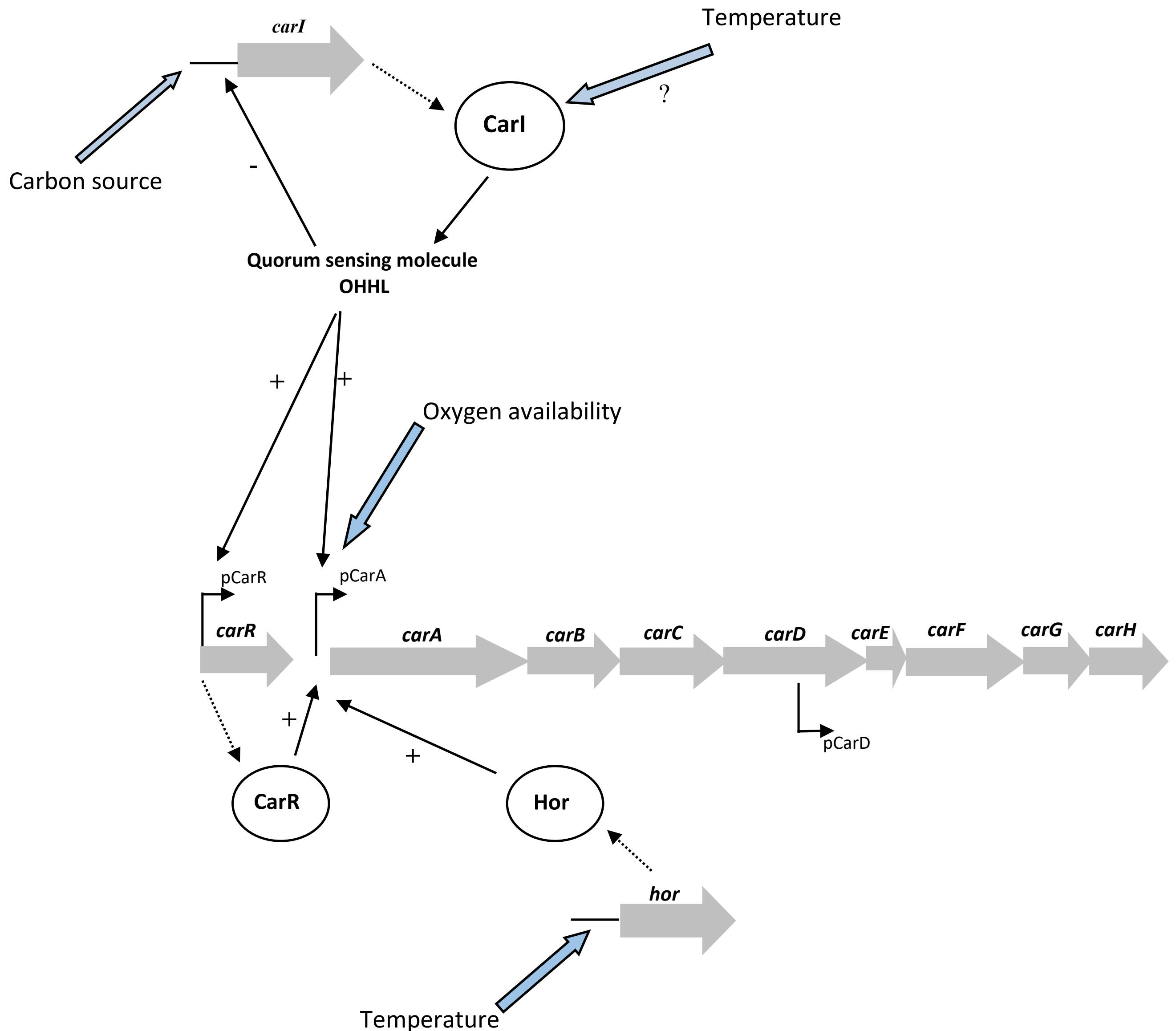


Figure 1.3. Regulation of the carbapenem gene operon in *P. c.* subsp. *carotovorum.* Many genetic networks and physiological factors modulate the transcription of the *carA-H* operon.

Genes are represented as grey arrows. Transcription of a gene is represented as a dashed arrow, the resultant gene product is shown in a red circle. Promoters within the carbapenem synthesis operon are shown as black arrows above or below the in which the promoter sequence is found. Physiological factors are shown as blue arrows and are positioned to show which genes they influence at the transcriptional level. The QS molecule N-(3-oxohexanoyl)-L-homoserine lactone is designated as OHHL. The influence of the regulatory gene products, as well as OHHL, on the level of *carA-H* operon transcription is shown by the black arrows. A positive influence is designated as +, whereas a negative influence is designated as -.

1.6.1.3 Novel carbapenem resistance mechanism

While investigating the genetic basis of carbapenem production by *P. c.* subsp. *carotovorum,* an *E. coli* strain supersensitive to β-lactams (ESS) (Hirvas et al., 1997), was transformed with a plasmid containing *carR, carI* and *carA-H* (McGowan et al., 1996). The transformed strain produced a low level of carbapenem and showed an increased resistance to the carbapenem. These data suggested that the carbapenem cluster contained both synthesis and resistance genes. As *carA-E* had previously been identified as involved in carbapenem synthesis, the *carFGH* genes were studied to ascertain their roles in resistance. An *E. coli* strain, that was supersensitive to β-lactams, was subsequently transformed with a plasmid containing only the genes *carF-H*, which resulted in increased resistance of the transformed ESS strain to the carbapenem produced by *P. c.* subsp. *carotovorum* (McGowan et al., 1996). Deletion experiments confirmed that both the *carF* and *carG* genes were required for resistance whereas *carH* was not involved (McGowan et al., 1996). The *carF* and *carG* genes did not confer resistance to other carbapenems or β-lactams, however, which suggested that they are required for self-resistance to the carbapenem synthesised by the *car* gene cluster (McGowan et al., 1997). The mechanism of resistance is unknown. The structure of CarG is not a homologue of any identified antimicrobial resistance genes (Tichy et al., 2014). Therefore, this intrinsic carbapenem resistance mechanism appears to be novel compared to other identified resistance systems.

Other mechanisms, such as the over expression of efflux pumps and mutations that alter the function of porins, enhance resistance to carbapenems in gram negative bacteria (Limansky et al., 2002; Mena et al., 2006; Rodríguez-Martínez et al., 2009).

Carbapenems have been predicted to provide a competitive fitness advantage to *P. c.* subsp. *carotovorum* strains within the host plant (Axelrood et al., 1988; Marquez-Villavicencio et al., 2011). However, this remains to be confirmed.

1.6.2 Bacteriocins

Bacteriocins are antimicrobial molecules produced by both gram negative and gram positive bacteria. The term bacteriocin describes many different antimicrobial molecules (Klaenhammer, 1988). Bacteriocins are unique when compared to other common antimicrobials, as they tend to have a narrow host range and are usually toxic to bacterial species that are related to the bacteriocin-producing bacterium (Tagg et al., 1976).

1.6.2.1 Colicins

Colicins are a type of bacteriocin that was first described in certain strains of *E. coli*. However, they are also produced by other bacteria, such as *Shigella*, *Serratia* and *Citrobacter* (Šmarda and Oravec, 1993; Guasch et al., 1995; Riley and Wertz, 2002). In *E. coli*, colicin is produced by a cluster of three genes; the toxin, immunity and lysis genes, which are carried on a plasmid (Bazaeal and Helinski, 1968). Expression of the genes and production of colicin are both mediated by the SOS regulon and therefore associated with stress experienced by the bacterial cell (Salles et al., 1987). The SOS regulon is comprised of genes involved in DNA repair, as well as mutagenesis following increased DNA damage (Radman, 1975). Colicin acts on sensitive cells by both pore-forming (Braun et al., 1994) and nuclease activity (Nomura, 1963). For example, colicin D inhibits a colicin D sensitive strain by affecting RNA and protein synthesis (Timmis and Hedges, 1972). Colicins bind to the sensitive cell via receptor domains within the proteins (Gouaux, 1997).

1.6.2.2 Carotovoricin

Pectobacterium carotovorum subsp. *carotovorum* produces a bacteriocin named carotovoricin, which was first described in strain Er (Itoh et al., 1978). Carotovoricin is produced at the same time as the PCWDE Pel (Itoh et al., 1980; Nguyen et al., 1999; Nguyen et al., 2001). Therefore, carotovoricin may act to exclude competing bacteria from the environment following the release of nutrients by the PCWDEs. However, the production of carotovoricin is regulated differently to the synthesis of Pel. Synthesis of both molecules is temperature dependent, with maximal Pel production occurring at 30 °C whereas production of carotovoricin is greatest at 23 °C and decreases dramatically at temperatures above 26 °C (Nguyen et al., 2002).

The carotovoricin molecule acts on sensitive cells by degrading phospholipids of the bacterial cell wall (Itoh et al., 1980). The carotovoricin genes are comprised of four transcriptional units which encode 21 genes. These genes encode phage tail-like proteins, a phage sheath, core and fibre proteins as well as ferrodoxin and lysis proteins (Yamada et al., 2006).

1.6.2.3 Carocins

The first carocin-type molecule to be identified in *P. c.* subsp. *carotovorum* was a low molecular weight bacteriocin, called carocin S1 (Chuang et al., 2007). This molecule was found to contain both killing and immunity domains. Glucose and lactose induce carocin S1 production (Chuang et al., 2007). More

recently, carocin S2 (Chan et al., 2011) and carocin D (Roh et al., 2010) have been identified as bacteriocins produced by *P. c.* subsp. *carotovorum* F-rif-18 and *P. c.* subsp. *carotovorum*-21, respectively. Carocin S2 has nuclease activity against *P. c.* subsp. *carotovorum* SP33. Unlike other bacteriocins, carocin S2 production is induced following DNA damage by ultra violet (UV) radiation but not following treatment with mitomycin C, a chemotherapeutic agent that induces DNA crosslinks (Chan et al., 2011). Carocin D is active against another P. c. subsp. carotovorum strain, *P. c.* subsp. *carotovorum*-3. The genes encoding carocin D were identified to be similar to the *E. coli colicinDK* (killing) and *colicinDI* (immunity) genes (Roh et al., 2010).

1.6.2.4 Regulation of bacteriocins

Bacteriocin production occurs when the bacterial cells are under stress (Herschman and Helinski, 1967; Cotter et al., 2005). For example, under growth culture conditions, bacteriocin production is increased when available oxygen is limited (Eraso and Weinstock, 1992), at high temperatures (Kennedy, 1971; Cavard, 1995) and during growth at stationary phase (Eraso et al., 1996). With this in mind, the antibiotic mytomycin C (Iijima, 1962; Itoh et al., 1980) and UV radiation (Lwoff et al., 1952; Itoh et al., 1980) were used to induce DNA damage and bacteriocin production.

As with carbapenems, the exact ecological role of the bacteriocins within bacterial populations has not been established, but they are most likely ‘anti-competitor’ molecules; either allowing the producing bacteria to invade an established population or prevent invasion by other bacteria (Riley and Wertz, 2002).

1.6.3 Non-ribosomal peptides

Non-ribosomal peptides (NRPs) display a wide range of functions and have been shown to contribute to virulence and *in vitro* fitness of plant pathogens. *Pseudomonas syringae* p.v *syringae*, for example, produces the phytotoxin syringomycin. Syringomycin not only promotes disease progression on the host plant, it also inhibits the growth of many other organisms including bacteria and fungi (Sinden et al., 1971). Other NRPs include the siderophore enterobactin, an important virulence determinant in *Dickeya* (Expert, 1999), and thaxtomin A, a toxin from the potato pathogen *Streptomyces acidiscabies* (Healy et al., 2000*)*. An unnamed orange pigment has also been associated with virulence of *P. c.* subsp. *carotovorum* in host plants (Williamson et al., 2010). It has been proposed that the emergence of *D. solani* as a pathogen is related to the presence of multiple NRP encoding genes not encoded by

other organisms found in a similar environment, such as *Pectobacterium* and other *Dickeya* (Garlant et al., 2013).

1.6.3.1 Non-ribosomal peptide biosynthesis

All NRPs are synthesised independently of the ribosomes and messenger ribonucleic acid (mRNA) on large, modular, multi-enzyme complexes called non-ribosomal peptide synthetases (NRPSs). These peptides contain distinct features compared to ribosomal peptides; commonly having a macrocyclic or branched macrocyclic structure, containing non-proteinogenic amino acids (Schwarzer et al., 2003). Non-ribosomal peptide synthetases are polypeptide chains or single polypeptides that are organised into modules. Modules are areas of the polypeptide chain that makes up the NRPS, which incorporate amino acids into the final product. These modules are sub-divided into domains that act as the active sites of the synthetase (Stachelhaus and Marahiel, 1995). The minimal domains in an NRPS, which are necessary for synthesis of an NRP are an adenylation (A) domain, a thiolation (T) domain (also called the peptidyl carrier protein) and a condensation (C) domain. Synthesis begins with substrate recognition and activation by the A-domain (Figure 1.4). This results in the incorporation of a particular amino acid, as A domains are specific to an amino acid residues. The activated amino acid is then transferred to the T-domain, where a thioester bond is formed. The C-domain subsequently forms a peptide bond between the amino acids on the T-domain on the same module as well as on the proceeding module. One module, therefore, results in the incorporation of a single amino acid. A thioesterase (TE) domain may be present on the final module of an NRPS, which releases the product from the synthetase. An epimerization domain may also be present, which initiates epimerisation of a target residue. Epimerisation refers to the alteration of the residue configuration, which results in stereo-isomers. Epimerisation does not occur in all cases. The number and type of modification domains also varies greatly between NRPSs.

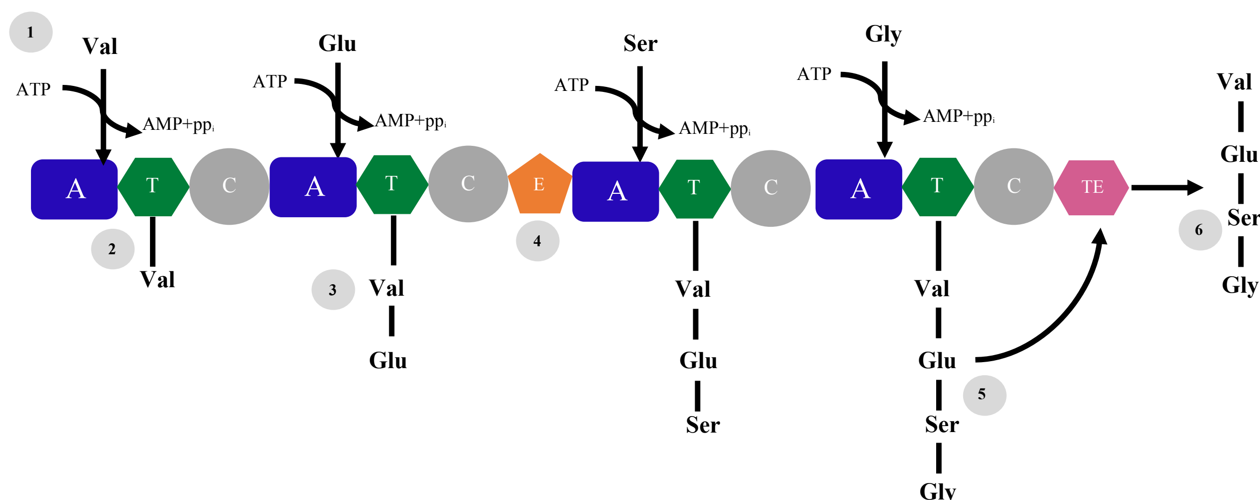


Figure 1.4. A representation of the mechanism of non-ribosomal peptide synthesis.

1: Activation of the amino acid substrate by the adenylation (A) domain. 2: The amino acid is then transferred to the thiolation (T) domain where it is held by a phosphopantetheine prosthetic group. 3: Condensation of the bound amino acid by the condensation (C) domain. 4: Possible modification of the amino acid, by the epimerization (E) domain. 5: Transesterification of the peptide chain from the final T domain to the thioesterase (TE) domain. 6: Release of the peptide product by the TE domain, either by hydrolysis or macrocyclization (Strieker et al., 2010). Amino acids are given as examples of a grown peptide chain. Standard amino acid abbreviations are given; val (valine), glu (glutamate), ser (serine), gly (glycine).

1.6.3.2 Non-ribosomal peptide synthesis regulation

Many bacterial NRPS genes are organised into operons. The genus *Bacilli* provides a number of examples, including the synthetase genes for the antimicrobials ituin (Tsuge et al., 2001) and mycosubtilin (Duitman et al., 1999). Furthermore, the NRP indigoidine, a blue pigment molecule linked to oxidative stress resistance in *Dickeya*, is also produced by genes which are organised in an operon (Reverchon et al., 2002). Therefore, NRPS operons are spread across bacterial species and represent synthetases of different types of molecules.

Environmental factors, particularly nutrient availability, have been described as important regulatory factors in the production of many NRPs. For example, the production of syringomycin by *P. syringae* is enhanced by increased levels of inorganic phosphate in *in vitro* media (Gross, 1985). Production was

further enhanced by phenolic glycosides, i.e. arbutin, which are encountered by the bacterium in host plants (Bender et al., 1999b). *In planta* conditions also influence the production of the thaxtomin A toxin by the potato pathogen *Streptomyces scabies.* Increased glucose and nitrogen concentrations, encountered following tissue maceration, reduce thaxtomin A production as the toxin is no longer required (Babcock et al., 1993).

Two component regulator systems which encode a transmembrane and response regulator component have been identified as important genetic regulators of NRPS clusters. GacS/GacA in *P. syringae* regulate syringomycin production (Bender et al., 1999b) and PecM/PecS regulate the dye molecule indigoidine in *Dickeya* (Rouanet and Nasser, 2001). The pleotrophic regulator DegQ also regulates the production of the antimicrobial molecule plipastatin in *B. subtilis* (Tsuge et al., 1999).

1.6.4 The ecological importance of antimicrobial molecules

The exact ecological function of antimicrobials is not understood (Price-Whelan et al., 2006). However, it is thought that within diverse ecological bacterial populations they confer an advantage to the producer when in competition for nutrients and resources (Sogin et al., 2006). This appears to be the case for *E. coli*, as the ability to produce bacteriocins in this bacterium is under positive selection even though there is almost certainly a large metabolic cost associated with antimicrobial synthesis (Reeves, 1972; Chao and Levin, 1981; Riley and Wertz, 2002).

Competition is described as being either 'scrabble competition', the hasty use of available nutrients to exclude them from use by other organisms or 'contest competition', which involves the deployment of direct, hostile mechanisms to reduce the success of competing organisms (Nicholson, 1954). Antimicrobials have been predicted to be involved in contest competition, although more recently their ecological competitive importance has been questioned. Indeed, given that antimicrobials are likely to be at sub-inhibitory concentrations in the environment, it has been suggested that they may act as intraspecies signalling molecules (Davies et al., 2006; Yim et al., 2007). For example, they may act as an indicator for other bacteria to change their metabolic functions (Price-Whelan et al., 2006).

Within nature, colicin producing, sensitive and resistant *E. coli* coexist within a population. Diversity studies have shown that resistant strains are most common within an *E. coli* population (50-98%) compared to producing strains (10 - 50%) (Gordon et al., 1998). Only a small sensitive population was present in these populations. A multi-generation study conducted using *E. coli* found in mouse intestines demonstrated that producers tend to lose the ability to produce the antimicrobial molecule,

in favour of gaining resistance (Gordon et al., 1998). This is likely due to the cost of producing the antimicrobial, as well as the relative ease of movement of resistance genes via horizontal gene transfer (Czárán et al., 2002). Furthermore, it has been described that the virulence potential of an organism is maximal when not competing with other organisms (i.e. not producing large amounts of antimicrobials) (Gardner et al., 2004). It would, therefore, be most beneficial for pathogens such as *Pectobacterium* to gain resistance to antimicrobials produced by competitors and focus resources on production of virulence determinants.

The difficulty of investigating bacterial interactions within the environment has led to the development of models describing these interactions. These models are based on laboratory empirical studies, as well as *in silico* and statistical analysis of bacteriocin biology. Initially it was predicted that bacteria produced antimicrobial molecules in order to out-compete other microbes for access to nutrients under limited nutrient conditions (Chao and Levin, 1981; Ivanovska and Hardwick, 2005).

A study by Wolch-Salamon et al, (1998), which studied the interaction of yeast grown in nutrient rich conditions reached a different conclusion. They demonstrated that for *Saccharomyces cerevisiae* producing the toxin K1, toxin production was only beneficial under nutrient rich conditions. High nutrient levels were required to outweigh the metabolic costs associated with toxin production and to allow invasion into the toxin sensitive population (Wloch-Salamon et al., 2008). It is now theorised that the production of antimicrobial molecules is advantageous for the producer when invading new ecological niches, rather than for scavenging nutrients (Brown et al., 2009).

The success of antimicrobial producing organisms is also dependent on the nature of the growth conditions. For example, in an unstructured environment, such as liquid media, the producers need to be more numerous than the sensitive cells if they are to successfully outcompete the sensitive cells (Durrett and Levin, 1997). This was thought to be due to insufficient toxin production by the producers to have a great enough affect to compensate the cost of production (Durrett and Levin, 1997). However, in a structured environment it is advantageous to produce an antimicrobial molecule even when the producer is rare (Chao and Levin, 1981; Durrett and Levin, 1997; Gardner et al., 2004).

Models have also been produced to understand the occurrence of antimicrobial producer, sensitive and resistant strains (Frank, 1994). These models have been confirmed via serial-transfer studies (Riley and Gordon, 1999). Initially, a microbial population is considered to be 'sensitive'. Without the presence of an antimicrobial molecule in the environment, this phenotype is most appropriate due to its low metabolic load. If a producer strain then enters the population, it will out-compete the sensitive cells (Durrett and Levin, 1997; Riley and Wertz, 2002). Over time, mutations will occur within the

sensitive population, which will render them resistant to the antimicrobial. These mutations include alterations to the cell surface, or transporters, and acquisition of resistance plasmids (Smarda, 1992; Feldgarden and Riley, 1998). These resistance characteristics will increase in frequency among the population until resistant cells displace the producers (Tan and Riley, 1996). It is then no longer advantageous to expend the energy required to produce the antimicrobial molecule. Furthermore, without the stress of the toxin being present, reversions back to the sensitive phenotype will occur as this is the least energy-intensive state. Therefore, under this scenario a crash in the antimicrobial-producing population is expected. This is a simplified scenario of the population dynamics that may occur. For example, the resistant population may be displaced by a strain producing another antimicrobial to which it does not have resistance (Feldgarden and Riley, 1998; James et al., 2013). In this case, the dominant producer may also be replaced (Tan and Riley, 1997; Riley, 1998). Therefore, the production of an antimicrobial molecule by a bacterium will only be advantageous for a limited amount of time. This is possibly what occurs when a new pathogen emerges, displacing other established pathogens.

1.7 Thesis context

Studies conducted to understand the variation of colicins produced by different strains of *E. coli* found that 25 different colicin molecules were produced in a single population (Riley and Gordon, 1999). As colicins have a very specific spectrum of activity, this variation was proposed to provide evidence for the extent of intraspecies competition that occurs within populations. The diversity of antimicrobials encoded within SRE genomes also suggests a high degree of competition is experienced by these bacteria. This may be a result of the diverse lifestyles and niches for SREs. For example, SREs are found in plants and on their surfaces, in water and in insects (Pérombelon, 1972; Pérombelon and Kelman, 1980; McCarter-Zorner et al., 1984; Basset et al., 2003). Several *Pectobacterium* species have been isolated from the same field location (Gross et al., 1991) or even from the same diseased potato stem tissue (De Boer et al., 2012). Therefore, competitive interactions are likely to occur between the bacteria.

Under these circumstances, different competition mechanisms may be required for survival in these different environments (Pérombelon and Kelman, 1980; Quinn et al., 1980).

The emergence of different enterobacterial pathogens has been noted on potato in recent times. For example, *D. solani* recently emerged as a pathogen in Europe (Toth et al., 2011). Genomic studies identified novel secondary metabolite genes, which were specific to this SRE (Garlant et al., 2013). It

was predicted that the unique combination of these novel genes may have enabled this bacterium to replace established blackleg pathogens such as *P. atrosepticum*, which previously dominated on potato in Europe (Garlant et al., 2013). Supporting this theory, it has previously been shown that *P. c.* subsp. *betavasculorum* is able to inhibit the growth of some *P. c.* subsp. *carotovorum* strains both *in vitro* and in tubers (Axelrood et al., 1988) suggesting that the antimicrobial activity of *P. c.* subsp. *betavasculorum* is important during colonisation of the host plant.

Pectobacterium carotovorum subsp. *brasiliensis* has also increased in prevalence on potatoes in recent years (Duarte et al., 2004; van der Merwe et al., 2010; Leite et al., 2014; Werra et al., 2015). Genome comparisons of two closely related *P. c.* subsp. *brasiliensis* strains, *P. c.* subsp. *brasiliensis* ICMP 19477 and *P. c.* subsp. *brasiliensis* PBR1692 (Panda et al., 2015b), have identified a variety of antimicrobial biosynthetic clusters in the genomes of these SREs (Table 1.2). It is possible that one, or a combination of these, has contributed to the emergence of *P. c.* subsp. *brasiliensis* at the expense of related SREs.

Three genes have been identified within the horizontally acquired island (HAI) PbN1-GI38 of *P. c.* subsp. *brasiliensis* ICMP 19477 that may be involved in bacteriocin production and are unique to *P. c.* subsp. *brasiliensis* (Panda et al., 2015b). Interestingly, Marquez-Villavicencio et al., (2011) observed that *P. c.* subsp. *brasiliensis* PBR1692 has antimicrobial activity against *P. atrosepticum* SCRI1043 *in vitro*. This activity was predicted to be a consequence of the production of a carbapenem, although the ecological significance of carbapenem production appeared to be minor. No competitive advantage was observed by the producer in co-inoculation studies between *P. c.* subsp. *brasiliensis* PBR1692 and *P. atrosepticum* SCRI1043 in potato stems (Marquez-Villavicencio et al., 2011).

Genes for carbapenem synthesis are present in the genome of *P. c.* subsp. *brasiliensis* PBR1692 (Glasner et al., 2008) and in *P. c.* subsp. *brasiliensis* ICMP 19477. Considering the population dynamics of antimicrobial producers in ecological niches, it is likely that the production of an antimicrobial molecule by *Pectobacterium* subsp. would be more advantageous for the colonisation of potato tubers, rather than the stems. Tubers provide a higher nutrient and more structured environment compared to stems, providing conditions more conducive to domination by producer strains (Chao and Levin, 1981; Wloch-Salamon et al., 2008). Thus, the experiments in stems may have overlooked the most likely niche in which antimicrobial activity provides a competitive advantage.

Table 1.2. Genomic islands in *P. c.* subsp. *brasiliensis* ICMP 19477 harbouring antimicrobial biosynthetic clusters.

The H-values calculated from comparisons with the genomes of *P. c.* subsp. *brasiliensis* PBR1692, *P. atrosepticum* SCRI1043, *P. c.* subsp. *carotovorum* ICMP 5702 and *P.c.* subsp. *carotovorum* WPP14 are given. An H-value of >0.8 indicates that the ORF from *P. c.* subsp. *brasiliensis* ICMP19477, has an indistinguishable homologue in the genome being compared. Table adapted from Preetinanda Panda., (2014) doctoral thesis.

***P. c.* subsp. *brasiliensis* ICMP 19477 Genomic Island**	**CDSs**	**Genomic coordinates**	**Antimicrobial molecule**	**Similarity of Genomic Islands in *Pectobacterium* (H-Value)**			
				P. c. subsp. brasiliensis PBR1692	*P. atrosepticum* SCRI1043	*P. c.* subsp. carotovorum ICMP 5702	*P. c.* subsp. carotovorum PC1
PbN1-GI20	KCO_06120-t17277	1525440-1587140	Non-ribosomal peptide	0.4	0.3	0.1	0.1
PbN1-GI25	KCO_16177-KCO_16067	1823757-1840265	Carotovoricin	1.0	0.3	0.8	0.8
PbN1-GI31	KCO_14567-KCO_14527	2180859-2189122	Phenazine	0.8	0.7	0.7	0.8
PbN1-GI38	KCO_12557-KCO_12472	2601719-2635218	Colicin-like	0.8	0.4	0.7	0.6
PbN1-GI65	KCO_04647-KCO_04687	4699476-4706891	Carbapenem	1.0	0.0	0.8	0.0

1.8 Aims

This study aimed to examine whether antimicrobial production contributes to the ecological fitness of *P. c.* subsp. *brasiliensis* on potato, by enabling the bacterium to out-compete closely-related pathogens such as *P. atrosepticum*. These bacteria are both responsible for blackleg (Pérombelon, 1992; Duarte et al., 2004), yet their entry is likely to occur via wounds or lenticels on the tuber. Thus, they are likely to inhabit the same ecological niches (at least in potato crops).

To address this question, i). the *in vitro* antimicrobial activity of *P. c.* subsp. *brasiliensis* was confirmed by testing a representative *P. c.* subsp. *brasiliensis* strain (ICMP 19477) (Panda et al., 2015b) against other *Pectobacterium* such as *P. atrosepticum* SCRI1043; ii). *P. c.* subsp. *brasiliensis* ICMP 19477 was then studied in the host potato plant (stems and tubers), to determine whether greater competitive fitness was observed for *P. c.* subsp. *brasiliensis* ICMP 19477 *in planta*; iii) Functional studies were performed in order to identify the genes and pathways involved in the competition phenotype of *P. c.* subsp. *brasiliensis* ICMP 19477. Furthermore, mutants reduced in the competition phenotype were compared to the *P. c.* subsp. *brasiliensis* ICMP 19477 WT, in order to determine if the *in vitro* antimicrobial activity was associated with the *in planta* fitness.

1.9 Hypothesis

It was hypothesised that *P. c.* subsp. *brasiliensis* ICMP 19477 demonstrates a competitive advantage over *P. atrosepticum* SCRI1043, both *in vitro* and in the host plant, due to the production of one or more antimicrobial molecules. It was further predicted that the competitive phenotype, conferring increased fitness to *P. c.* subsp. *brasiliensis* ICMP 19477, is a mechanism associated with the emergence of *P. c.* subsp. *brasiliensis* ICMP 19477 as a recent pathogen.

Chapter 2

Materials and Methods

2.1 Methods

2.1.1 Bioinformatic analysis

Bioinformatics tools were used to identify antimicrobial biosynthetic clusters and to predict the antimicrobial products encoded by these clusters (Table 2.1). These bioinformatic tools were also used for analysis of the genes disrupted by transposon mutagenesis (Sections 2.1.18 and 2.1.19). Similarities to other known genes and proteins were established by an alignment search tool using the NCBI BLAST algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Altschul et al., 1990). Comparative genome analysis was represented using Easyfig (Sullivan et al., 2011).

Table 2.1. The bioinformatics analysis tools used to analyse the antimicrobial biosynthesis gene clusters in *P. c.* subsp. *brasiliensis* ICMP 19477.

Name	Description	URL/Reference
Non-ribosomal peptide synthetase specific tools		
PKS/NRPS Analysis Web-server	Blast server to identify homologues within the database plus domain organisation	http://nrps.igs.umaryland.edu/nrps/ (Bachmann and Ravel, 2009)
NRPS-PKS	Prediction of domain organisation and substrate specificity	URL no longer available (Ansari et al., 2004)
Antibiotics and secondary metabolites analysis shell (antiSMASH)	Identifies, annotates and analyses secondary metabolite producing loci. Also predicts the structure of the resulting product.	http://antismash.secondarymetabolites.org/ (Medema et al., 2011)
NRPSpredictor2	Predicts A domain specificity and the resulting amino acid substrate	http://nrps.informatik.unituebingen.de/ (Rottig et al., 2011)

Name	Description	URL/Reference
General tools used for analysis of antibiotic synthesis clusters		
Pfam 26.0	Identification of protein family similarities within the protein family sequence database	http://pfam.xfam.org/ (Finn et al., 2010)
HMMER	Identification of protein homologs and protein sequence alignments	http://hmmer.janelia.org (Finn et al., 2011)
BLAST-conserved domains	Compares sequence with databases to infer functional relationships.	http://www.ncbi.nlm.nih.gov/blast/Blast.cgi (Altschul et al., 1990)
InterProScan 5	Blasts sequence against the InterPro database of protein signatures to identify the domain structure.	http://www.ebi.ac.uk/Tools/pfa/iprscan (Jones et al., 2014)
I-TASSER server (Version 4.4)	Predicts the structure and function of protein sequences	http://zhanglab.ccmb.med.umich.edu/I-TASSER/ (Zhang, 2008)
PredictProtein (Version 1.0.88)	Secondary structure and structural annotations of protein sequences.	https://www.predictprotein.org/ (Rost et al., 2004)
Raptor X	Protein secondary and tertiary structure prediction	http://raptorx.uchicago.edu/ (Källberg et al., 2012)

2.1.2 Chemicals and Media

All chemicals used in this study were of an analytical grade. The recipes for chemical solutions and media are given in Appendix A. Solutions were routinely prepared using double distilled water (ddH$_2$O) unless otherwise indicated. All chemical solutions and media were sterilized by autoclaving at 121°C (20 psi) for 20 min. Where appropriate, the pH of the solutions and media were measured at room temperature.

2.1.3 Bacterial strains and plasmids

Bacterial strains and plasmids are listed in Table 2.2.

Table 2.2. Bacterial strains and plasmids used in this study.

Antibiotic resistances are represented as follows: Km, Kanamycin; Rif, Rifampicin; Str, streptomycin; Amp, ampicillin; Chl, chloramphenicol; Tet, tetracycline.

Bacterial Strain	Description/Genotype	Source/Reference	Antibiotic Resistance
P. atrosepticum			
SCRI1043	Wild Type	JHI, UK	N/A
SCRI1043 R	Spontaneous genetic mutation conferring resistance to rifampicin	This study	Rif
SCRI1043 K	SCRI1043 ECA0522Ar1::mTn*5-gusA-pgfp*::Km^{R}	(Holeva et al., 2004; Vanga et al., 2012)	Km
P. carotovorum* subsp. *carotovorum			
ICMP 5702	Wild Type	(Panda et al., 2015a)	N/A
P. carotovorum* subsp. *brasiliensis			
ICMP 19477	Wild Type	(Pitman et al., 2008)	N/A
ICMP 19477 R	Spontaneous genetic mutation conferring resistance to rifampicin	This study	Rif
ICMP 19477 K	mTn5-gusA-gfp::Km^{R}	This study	Km
ICMP 19477 *carR*$^{-}$	*carR*::mTn*5-gusA-gfp*::Km^{R}	This study	Km
ICMP 19477 *carR*$^{-}$::pTRB32oriT*carR*	ICMP19477 *carR*$^{-}$ containing pTRB32oriT containing a 738 bp fragment, encompassing KCO_04647.	This study	Chl, Km
ICMP 19477 *carR*$^{-}$::pTRB32oriT	ICMP19477 *carR*$^{-}$ containing an empty copy of pTRB32oriT	This study	Chl, Km
ICMP 19477 *carI*$^{-}$	carI::mTn*5-gusA-gfp*::Km^{R}	This study	Km
ICMP 19477 *slyA*$^{-}$	slyA::mTn*5-gusA-gfp*::Km^{R}	This study	Km
ICMP 19477 *slyA*$^{-}$::pTRB32oriT*slyA*	ICMP19477 *slyA*$^{-}$ pTRB32oriT containing a 436 bp fragment, encompassing of KCO_21137.	This study	Chl, Km
ICMP 19477 *slyA*$^{-}$::pTRB32oriT	ICMP19477 *slyA*$^{-}$ containing an empty copy of pTRB32oriT	This study	Chl, Km
ICMP 19477 *abc*$^{-}$	ICMP19477 carrying a single crossover mutation in ABC, integrated with pK18mobsacB.	(Preetinanda Panda, 2014; Doctoral thesis)	Chl, Km
ICMP 19477 *nrps1*$^{-}$	ICMP19477 carrying a double crossover mutation in *nrps*1 integrated with pK18mobsacB.	(Preetinanda Panda, 2014; Doctoral thesis)	Chl, Km

ICMP 19477 *sim*⁻	ICMP19477 carrying a single crossover mutation in *sim* integrated with pK18mobsacB.	(Preetinanda Panda, 2014; Doctoral thesis)	Chl, Km
PBR1692	Wild Type	(Duarte et al., 2004)	N/A
***E. coli* strains**			
ESS	β-lactam supersensitive indicator strain	(Bainton et al., 1992)	N/A
S17-1λpir (mTn5-gus-pgfp21)	S17-1λpir carrying the Tn*5* 'cassette' mTn*5-gus-pgfp21* in pUT. Provides conjugal function in bacterial crosses	(Sandra Vinovsky, private communication)	Amp, Km
TOP10	F-mcrA Δ(mrr-hsdRMS-mcrBC) Φ80ΔLacZΔ M15 Δ lacX74 recA1 araD139 Δ(araleu) 7697 galU galK rspL (Str^R) endA1 nupG	Life Technologies	Str
HH26 pNJ500	Strain used for mobilisation in conjugal transfer	(Grinter, 1983)	Tet
***Chromobacterium* strains**			
C. violacein CV026	Mini-Tn*5* transposon mutant negative for violacein-pigment production. Biosenser strain for N-acyl homoserine lactone production.	(Latifi et al., 1995)	Km
Plasmids			
pTRB32oriT (pPF259)	Derivative of pQE80-L (Expression vector for native or N-terminal hexahistidine proteins), containing an origin of transfer RP4 oriT.	(M. McNeil, unpublished)	Chl
pTRB32oriT::carR	pTRB32oriT containing a 738 bp fragment, encompassing KCO_04647.	This study	Chl
pTRB32oriT::slyA	pTRB32oriT containing a 436 bp fragment, encompassing of KCO_21137.	This study	Chl
pK18mobsacB	Vector used for the delivery of knockout gene constructs	(Schäfer et al., 1994)	Km
pFAJ1819	pUT derivative, mini Tn*5* transposon vector containing mTn*5-gus-pgfp21*	(Xi et al., 1999)	Amp, Km

2.1.4 Growth conditions, selection and long term storage of bacteria

All *Pectobacterium* and *E. coli* strains were routinely grown on Luria-Bertani agar (LBA). Where appropriate, minimal M9 media containing glucose (MM) was also used. *Pectobacterium* species were grown at 28 °C, *E. coli* was grown at 37 °C and *Chromobacterium violacein* CV026 was grown at 25 °C. For agar plate cultures, *Pectobacterium* and *E. coli* were incubated for 24 h and *C. violacein* CV026 was incubated for 48 h.

To prepare liquid cultures, a single colony was selected from a pre-grown agar plate and inoculated into 5 ml LB medium and incubated for ≥ 16 h. Liquid cultures of *Pectobacterium* were shaken at 180 rpm, *E. coli* at 250 rpm and *C. violacein* at 150 rpm. Where required, antibiotics were added to the growth medium to a final concentration of: ampicillin (Amp) (100 µg/ml), kanamycin (Km) (50 µg/ml), chloramphenicol (Chl) (25 µg/ml), tetracycline (Tet) (15 µg/ml) and rifampicin (Rif) (100 µg/ml). Isopropyl β-D-1 thiogalactopyranoside (IPTG) was also added to the growth media where required, to a final concentration of 20 µg/ml.

Spontaneous Rif resistant mutants of *P. c.* subsp. *brasiliensis* ICMP 19477 (*P. c.* subsp. *brasiliensis* R) and *P. atrosepticum* SCRI1043 (*P. atrosepticum* R) were prepared by growth in LB, plus Rif, overnight at various Rif concentrations (20 µg/ml, 30 µg/ml, 50 µg/ml and 75 µg/ml). Resistant cultures were streaked onto LBA containing Rif at the required concentration and grown overnight. The resultant colonies were repeatedly grown at increasing concentrations of Rif until they were resistant to the required concentration (100 µg/ml).

For long term storage of all bacteria, a 500 µl aliquot of a bacterial culture grown overnight in 5 ml LB culture was added to 500 µl of 40% glycerol and frozen at -80 °C. For short term storage, bacteria were kept on agar plates at 4 °C for up to four weeks. *C. violacein* CV026 was stored at 25 °C for up to two weeks, as the strain would not survive at 4 °C.

2.1.5 Isolation of genomic DNA from bacterial cultures grown *in vitro*

The DNeasy blood and tissue kit (Qiagen) was used to isolate genomic DNA from bacterial broth cultures as per the manufacturer's instructions.

2.1.6 Isolation of plasmid DNA from bacterial cultures grown *in vitro*

The Presto ™ Mini Plasmid Kit (Geneaid) was used for the isolation of high copy number plasmids. Plasmid isolation was carried out as per the manufacturer's instructions.

2.1.7 Isolation of total RNA from bacterial cultures grown *in vitro*

The SV Total RNA Isolation system (Promega) was used to isolate total RNA, using a modified method to that described by the manufacturer. The bacterial cells were harvested from the broth by centrifugation at 4,000 rpm for 10 min at 4 °C. All subsequent centrifugations were also carried out at 4 °C, unless otherwise stated. The pellet was resuspended in 100 µl of TE buffer containing 1.0 mg/ml of lysozyme, by inversion of the tube until the pellet was completely resuspended. The lysis mixture was then incubated at room temperature for 5 min. Three hundred and fifty µl of RNA dilution buffer was then added. This was mixed by vortexing and then centrifuged at full speed for 10 min. The supernatant was transferred to a fresh tube, 1 ml of ice cold ethanol was added, and then the mixture was incubated at -80 °C for 60 min. The sample was subsequently centrifuged at full speed for 5 min and the supernatant discarded. The pellet was then washed with 600 µl of wash buffer, and air dried. The pellet was resuspended in 60 µl RNase free water. For DNase treatment, two volumes of ice cold ethanol were added and mixed gently. The suspension was transferred to a Promega spin column and centrifuged at full speed for 30 sec. A previously prepared DNase mix, supplied by the manufacturer, was applied to the column and incubated at room temperature for 15 min. Two hundred µl of DNase stop solution was then added and centrifuged at full speed for 30 sec. The column was washed with 600 µl of wash solution and the RNA was eluted with RNase free water. If necessary, the DNase treatment was repeated.

2.1.8 Determination of nucleic acid concentration and purity

The concentration of isolated DNA or RNA was measured using a NanoDrop 3.0.0 (Thermo Scientific). The concentration of the nucleic acid was measured using the spectrophotometer by absorbance at 260 and 280 nm. The ratio of the 260/280 nm absorbance was used to determine the nucleic acid purity. A ratio of ~1.8 was accepted as pure DNA and a ratio of ~2 as pure RNA. A lower 260/280 nm ratio may have indicated contamination by phenol or protein which absorb at 280 nm. A second

measure of nucleic acid purity was given by the 260/230 nm ratio. The values representing pure nucleic acids were higher than the 260/280 nm ratio and were generally between 2-2.2. A lower 260/230 nm ratio may have been due to presence of carbohydrates, phenol or guanidine which absorb at 230 nm. Nucleic acid quantification was also confirmed using gel electrophoresis with appropriate DNA standards (Section 2.1.9).

2.1.9 Agarose gel electrophoresis

Nucleic acids were separated based on size by gel electrophoresis using 1- 2% (w/v) agarose. Agarose gels were prepared in 50 ml 10 x TAE buffer. RedSafe™ (iNtRON Biotechnologies) was added to the agarose (1 µl per 100 ml agarose), prior to pouring the agarose into a casting tray. Genomic DNA, polymerase chain reaction (PCR) products or reverse transcription-PCR **(**RT-PCR) products (1 - 4 µl) were pre-mixed with 1 µl 6 × bromophenol loading dye and loaded into the gel after the agarose had solidified. For DNA samples, 5 µl of a DNA size standard (HyperLadder I, IV or V, Bioline UK) was run alongside the samples to measure the size of the separated fragments. For separation of RNA molecules, 3 µl RNA was added to 6 µl formamide plus 1 µl 6 × loading dye. This mix was loaded onto the gel alongside 4 µl of RiboRuler high range ready-to-use RNA Ladder (200-6000 bp, Fermentas). This mix was then added to the gel. After the samples had been loaded, the gel was routinely run at 100 V for 45 min before visualisation of bands under a UV transmitter.

2.1.10 PCR and RT-PCR

PCR was routinely performed using FastStart *Taq* DNA polymerase (Roche). Standard reactions were carried out in a total volume of 50 µl with each reaction containing, 5 µl 10 × PCR buffer, 1 µl 10 mM dNTPs, 5 µl 10 µM forward primer, 5 µl 10 µM reverse primer, 0.4 µl FastStart *Taq* DNA polymerase and 0.1-250 ng of template DNA. PCRs were made up to 50 µl with PCR grade water. PCR reactions were run on an iCycler PCR machine (Bio-Rad Laboratories). PCR conditions generally were: 95 °C for 4 min, followed by 30 cycles of 95 °C for 30 sec, 30 sec at a primer-specific temperature (see Table 2.3) and 70 °C for 3 min. A final elongation step was performed at 72 °C for 7 min. A negative control, containing no template DNA was included for all reactions. A non-RT PCR control was included for RT-PCR reactions.

Geneious 7.0.6 (Biomatters Ltd) available from (http://www.geneious.com/), was used to design primers using the *P. c.* subsp. *brasiliensis* ICMP 19477 reference genome. The primers used in this study are listed in Table 2.3.

Table 2.3. Primers used in this study.

Primer Name	Product Size (bp)	Primer Sequence (5' to 3')	Annealing temperature (°C)
Primers used for operon studies			
Pbr β-lac/ABC F	797	AGCGATCTGGAGTGGACATTC	60
Pbr β-lac/ABC R		ATCGCCCTTTCTACCTGTTCG	60
Pbr ABC/NRPS F	539	TGCCAGGACAGTCAATATGGC	60
Pbr ABC/NRPS R		CAAGCCAATCTGAACACGGTG	60
NRPS/NRPSsmall F	502	TGAGCGCCAATTTATCGAGTTTG	60
NRPS/NRPSsmall R		GCGACATGACCATTTCTTTGAAC	60
Pbr NRPS/tRNA F	866	TCATGACGCGAGACAAGCAT	60
Pbr NRPS/tRNA R		GGTTCGAGTCCAGTCAGAGG	60
KCO_12557/12552 F	1249	CTCCCCGCTGGAAATTAAAGC	60
KCO_12557/12552 R		GCTTGGCTGATTGCTATAATCCA	60
KCO_12552/12547 F	533	TTCCCATCGATTGACAACCGA	60
KCO_12552/12547 R		GCCGGTGGAGTTCGATGATTA	60
KCO_12547/12542 F	648	GCCACCATCAACGGTCATTG	60
KCO_12547/12542 R		CGGTCGCCTTCTTCGATCATA	60
CarR/CarA F	490	CCCTGTGAGCCTGTAGTGATG	60
CarR/CarA R		GAACAGCAACGCTAACAGCTC	60
CarA/CarB F	506	CGAAGGTTCCTCCGTCAATCA	60
CarA/CarB R		TGGAAACCCATACCAATCGCA	60
CarB/CarC F	626	TAAGCATGGTATCGGGTGCTC	60
CarB/CarC R		TCCAGTTTCAACGTATCGCGA	60
CarC/CarD F	539	CCAGGCATTCTTCCAGGAACT	60
CarC/CarD R		CCATTGCCTTATCGAACTGCG	60
CarD/CarE F	668	ATGCGTGAATTTAACCGCGAG	60
CarD/CarE R		GCGATAAGGCAATTCGACACC	60
CarE/CarF F	540	GGTGTCGAATTGCCTTATCGC	60
CarE/CarF R		CGAAAATCAGTGCATCCCACC	60
CarF/CarG F	554	AAGGCCATACGTGAGAATCCG	60
CarF/CarG R		CAAACTGGGCAACAACGACAT	60
CarG/CarH F	574	GCATTACTTTCTGGTGACGGC	60
CarG/CarH R		CCACCGTCAGTTCCTGATTCA	60

Primer Name	Product Size (bp)	Primer Sequence (5’ to 3’)	Annealing temperature (°C)
CarR/Asp II F	505	AGGCATATCGGTTATCGCCTG	60
CarR/Asp II R		GTGGATTCTACAACCCGCAGA	60
Primers used for screening the transposon mutant library			
Pbr NZ F	1714	TCGAACCAGAATGACCCCATG	60
Pbr NZ R		GTCTCAATAACGTGTGGACATAAT	60
Kan F	790	TTGAACAAGATGGATTGCACGC	60
Kan R		CAGAAGAACTCGTCAAGAAGGC	60
Tn5 R	N/A	ATCCTCCTTAGCTAGTCAGG	60
BF9_Tn5	N/A	CTAAGAGCCAAGAATCAGGATG	60
FH1_Tn5	N/A	GTTAGAGATATTTGATGTAAATC	60
IH1_Tn5	N/A	TGATTGGCTCTGCTGATTCGG	60
Primers used for complementation studies			
CarR_complF	783	GCG*GGTACC*GTCGGTAAGAGAGGGTAATATGGA[1]	60
CarR_complR		GTC*AAGCTT*TCCTCCCTATTTAGCAAGCATT[2]	60
SlyA_complF	436	GCG*AAGCTT*TTCTCCCTCTGCGTAACCCA[2]	60
SlyA_complR		GTC*GGTACC*GCGTGCTAACAATAAGGAGAGG[1]	60
pTRB32oriT F	N/A	GCTTTGTGAGCGGATAACAA	61
pTRB32oriT R	N/A	CAAGCTAGCTTGGCGAGATT	61

Enzyme restriction sites are included where required. These sites are written in italics; [1]GGTACC *Kpn*I and [2]AAGCTT *Hind*III.

2.1.11 Restriction endonuclease digestion of DNA

Polymerase chain reaction products or plasmid DNA were routinely digested with restriction endonucleases from New England Biolabs (NEB). Digestion reactions were carried out as per the manufacturer's instructions, using the following components for a single reaction: 1 µg DNA, 5 µl (1×) 10 × NEBuffer, 10 units restriction enzyme and ddH_2O up to 50 µl.

Reactions were incubated at the appropriate temperature (37 °C for most enzymes). Those including PCR products were incubated for ≥1 h whilst those using plasmid DNA were incubated overnight. Following incubation, enzyme reactions were terminated by adding 5 µl 5 × loading dye to the reaction (supplied by NEB).

2.1.12 Purification of PCR products and plasmid DNA following restriction digestion

Digested PCR products and plasmid DNA and were purified using the Wizard® SV gel and PCR clean-up system (Promega), as per the manufacturer's instructions.

2.1.13 Ligation of restriction enzyme-digested PCR products and plasmid DNA

Following restriction enzyme digestion, PCR products were ligated into plasmid vectors using T4 DNA Ligase (Promega). A 3:1 ratio of insert to vector DNA was routinely used, and the amount of insert DNA required was calculated using the following equation:

$$ng\ of\ insert = \frac{ng\ of\ vector\ \times kb\ size\ of\ insert}{kb\ size\ of\ vector} \times \frac{molar\ ratio\ of\ insert}{vector}$$

A standard ligation reaction was carried out in a total volume of 20 µl with each reaction containing 100 ng of vector DNA, the calculated amount of insert DNA, 5 µl of 2 × Rapid Ligation Buffer and 3u (Weiss units) of T4 DNA Ligase. The reaction was made up to 20 µl with ddH_2O. The reaction was mixed gently by pipetting, and then incubated at room temperature for 1 h or at 4 °C overnight. The ligation mix was then kept on ice, or at -80 °C for longer storage, until transformation of recipient cells.

2.1.14 Sanger DNA sequencing of PCR products and cloned DNA

Polymerase chain reaction fragments or plasmid constructs were sequenced by Sanger sequencing (Lincoln University), using an ABI Prism® 3130 Genetic Analyzer, with a 16 capillary 50 cm array installed and using Performance Optimized Polymer 7 (POP7) (Applied Biosystems®). Sequence reactions contained 0.5 µl 2.5 × Big dye terminator, 2 µl 5 × sequence buffer, 1 µl 5 µM primer, template DNA (amount depends on amplicon size) and 10 µl PCR grade water to. Sequencing reactions were carried out in a Thermocycler ABI19600 (Applied Biosystems®). Conditions were generally 96 °C for 1 min, followed by 25 cycles of 96 °C 10 sec, 50 °C 5 sec and 60 °C 2 min. Post sequence reaction clean-up was performed using the Agencourt CleanSEQ+ sequence reaction clean-up system (Beckman Coulter).

DNAMAN (.seq) files were downloaded to Geneious 7.0.6 (Biomatters Ltd, http://www.geneious.com/). The sequence was compared to the *P. c.* subsp. *brasiliensis* ICMP19477 genome, using BLAST analysis Geneious 7.0.6 (Biomatters Ltd, http://www.geneious.com/). Any mismatches were also identified.

2.1.15 cDNA synthesis

Reverse transcription (RT) was performed using a SuperScript VILO cDNA synthesis kit (Invitrogen) as described by the manufacturer. Briefly, up to 2.5 µg of RNA was added to 4 µl 5 × VILO reaction mixture and 2 µl 10 × SuperScript Enzyme Mix. The reaction was made up to a final volume of 20 µl with PCR grade. The RNA was converted to cDNA in an iCycler PCR machine (Bio-Rad Laboratories) using one cycle of 10 min incubation at 25 °C, 60 min at 42 °C and the 5 min at 85 °C to terminate the reaction. The resulting cDNA was stored at -20 °C.

2.1.16 Transformation of *E. coli*

One Shot® TOP10 chemically competent cells (Table 2.2) were used routinely for transformation as part of genetic manipulations such as cloning. Transformation was performed as per the manufacturer's instructions. Briefly, a vial of One Shot® TOP10 chemically competent cells was defrosted on ice and 2 µl of the transformation reaction was added to the vial. The transformation mix was mixed gently and incubated on ice of 30 min. The cells were then heat shocked by incubation at 42 °C for 30 sec followed by transfer to ice. Two hundred and fifty µl of super optimal broth with catabolite repression (SOC) media, pre-warmed to room temperature, was added to the cells. Cells were incubated at 37 °C, 250 rpm for 1 h. One hundred - 200 µl was then spread onto pre-warmed

LBA plates containing appropriate antibiotics. The plates were incubated overnight at 37 °C and colonies were subsequently selected for further analysis. Colonies were grown overnight in 5 ml LB containing appropriate antibiotics. Plasmids were extracted to confirm the authenticity of the transformants, as described in Section 2.1.6. The presence of the plasmid insert was confirmed by PCR (Section 2.1.10), using insert-specific and plasmid-specific primers and DNA sequencing (Section 2.1.14). For sequencing of inserts, gene and plasmid-specific primers were used. Primers are listed in Table 2.3.

2.1.17 Bacterial conjugation

Conjugation of pTRB32oriT and pFAJ1819 was achieved using triparental mating, which involved the *E. coli* donor strain containing the cloned vector, the recipient *P. c.* subsp. *brasiliensis* ICMP 19477 and the *E. coli* helper strain HH6, containing pNJ5000 (Table 2.2). For each bacterial strain, overnight cultures were prepared in 5 ml LB media with appropriate antibiotics (see Section 2.1.4). Cells from 1 ml of each culture were collected by centrifugation at 12,000 rpm for 2 min. The supernatant was discarded and the cells were washed in 750 µl LB containing no antibiotics. The wash was then repeated twice with 300 µl LB, in order to remove all antibiotics from the cell suspensions. One hundred µl of each bacterial culture was subsequently combined in one eppendorf tube and the mixture centrifuged at 13,000 rpm for 1 min. Most of the supernatant was removed and the cells were re-suspended in the remaining supernatant. The resulting conjugation mix was dotted onto an LBA plate and incubated overnight at 28 °C. The next day, the conjugation mix was scraped with a wire loop from the surface of the plate and streaked onto MMA containing appropriate antibiotics. The plates were then incubated at 28°C for 2-4 d before growing colonies were re-streaked onto the same media and incubated for a further 2-4 d. The antibiotic resistance of the colonies was subsequently confirmed, and the presence of the conjugated plasmid established by PCR using insert-specific primers (Section 2.1.10, Table 2.3). The integrity of the plasmid was confirmed further by Sanger sequencing of the insert using insert-specific and plasmid-specific primers (Section 2.1.14; Table 2.3).

2.1.18 Transposon mutagenesis

A *P. c.* subsp. *brasiliensis* ICMP 19477 Tn*5* transposon library was constructed using a method adapted from Holeva et al., (2004). The number of transposon mutants required to generate a one-fold genome coverage was calculated as being equal to the number of genes encoded within the *P. c.* subsp. *brasiliensis* ICMP 19477 genome (Holeva et al., 2004). *Pectobacterium carotovorum* subsp.

brasiliensis ICMP 19477 encodes 4,519 genes (Panda et al., 2015b), therefore 4,520 mutants were produced.

Pectobacterium carotovorum subsp. *brasiliensis* ICMP 19477 was subjected to transposon mutagenesis by conjugation (Section 2.1.17) with *E. coli* S17-1λpir containing pFAJ1819, which houses the Tn*5-gus-gfp21* construct (Xi et al., 1999). The pUT plasmid backbone of pFAJ1819, contains an Amp resistance cassette and the insert contains a Km resistance cassette. Therefore, the desired transconjugates were *P. c.* subsp. *brasiliensis* ICMP 19477 colonies that were Km resistant and Amp sensitive. Transconjugants were stored at -80 °C (Section 2.1.4).

2.1.19 Identification of transposon insertion sites

Whole genome re-sequencing was conducted for each to transposon mutant of *P. c.* subsp. *brasiliensis* ICMP 19477 that showed a phenotype of interest, to identify the insertion site location. In particular, Illumina sequencing (2 x 101 bp fragments) was performed on a Hiseq2000 by Macrogen. Raw reads were downloaded as FASTQ files. The raw read files were then imported into Geneious 7.0.6 (Biomatters Ltd), along with the *P. c.* subsp. *brasiliensis* ICMP 19477 genome sequence (accession number ALIU01000000) and the pFAJ1819 sequence (accession number HQ328084) (Xi et al., 1999), from which the transposon sequence was extracted. Raw reads were aligned to the sequence for the transposon and the sequences at the boundary between the transposon and the bacterial genome were identified. Nucleotide sequences at these boundaries were used to search for identical sequences within the *P. c.* subsp. *brasiliensis* ICMP 19477 genome using BLAST searching in Geneious 7.0.6 (Biomatters Ltd). Mapping the reads to this reference enabled the location of the insertion site within the genome. To confirm the Tn*5* insertion sites identified by genome sequencing, PCR primers were designed (Section 2.1.11) to amplify the boundary between the transposon and the bacterial genome in conjunction with the Tn*5* primer Tn5R (Holeva et al., 2004). All primers used for the identification of Tn*5* insertion sites are provided in Table 2.3. Polymerase chain reaction amplicons were subsequently sequenced by Sanger sequencing for verification (Section 2.1.14). The coordinates for the mutants, used in further studies in this thesis, are given in Table 2.4.

Table 2.4. ***Pectobacterium carotovorum*** **subsp.** ***brasiliensis*** **(*Pbr*) ICMP 19477 transposon mutants used in this study.**

Name of Tn*5* knock-out mutant	Knock-out gene ID	Gene name	Gene location in *P. c.* subsp. *brasiliensis* ICMP19477	Insertion Location	Insertion orientation
Pbr ICMP19477 *carR*⁻	KCO_04647	*carR*	4699476- 4700210	4699984	3′-5′
Pbr ICMP19477 *slyA*⁻	KCO_21137	*slyA*	2744146-2744400	2744469	3′-5′
Pbr ICMP19477 *carI*⁻	KCO_03547	*carI*	4446565-447215	4446676	3′-5′

2.1.20 Complementation studies

The plasmid pTRB32oriT (Table 2.2; for map see Appendix A) was used for complementation studies. pTRB32oriT, a derivative of the expression plasmid pQE-80L (Matthew McNeil; unpublished), was derived by replacing the Amp resistance gene present in pQE-80L (Qiagen) with a Chl resistance gene. The use of a plasmid with the Chl resistance cassette was more appropriate for this study, as a β-lactamase gene prevents complementation (if the antimicrobial product is a carbapenem). The pTRB32oriT plasmid requires the addition of IPTG in order to induce the expression of cloned fragments. The pQE-80L plasmid and its derivative also cause the insertion of an N-terminal hexahistidine (His) tag to the cloned protein. This can be avoided by inserting the PCR product into the *EcoR*I restriction site of pQE-80L. However, the Chl resistance gene of pTRB32oriT contains an *EcoR*I restriction site, consequently this enzyme was not suitable for use. Therefore, the resultant products contained a His tag. The presence of an oriT also allowed for the plasmid to be mobilised into the *P. c.* subsp. *brasiliensis* ICMP 19477 recipient host via conjugation.

Target DNA fragments were amplified from *P. c.* subsp. *brasiliensis* ICMP 19477 by PCR (Section 2.1.10) using primers with a *Kpn*I site included in the forward primer (5′ end) and a *Hind*III restriction site included in the reverse (3′) primer (Table 2.3). The primers were also designed to amplify the native ribosome binding site (RBS) region.

All PCR amplicons were cloned (Sections 2.1.11, 2.1.12, 2.1.13) into pTRB32oriT using the restriction sites described above, and *P. c.* subsp. *brasiliensis* ICMP 19477 transposon mutants were subsequently

transformed with these constructs by conjugation (Section 2.1.17). The authenticity of transconjugants was confirmed by PCR (Section 2.1.10) and sequencing (Section 2.1.14).

2.1.21 Antagonism assay

Inhibition of *P. atrosepticum* SCRI1043 growth by *P. c.* subsp. *brasiliensis* ICMP 19477 was initially determined using an *in vitro* antagonism assay (Marquez-Villavicencio et al., 2011). The assays were conducted by streaking a colony of *P. c.* subsp. *brasiliensis* ICMP 19477 grown on LBA overnight, onto an LBA plate seeded with *P. atrosepticum* SCRI1043. The *P. atrosepticum* SCRI1043 lawn was prepared using a method adapted from the *E. coli* supersensitive strain (ESS) antagonism assay (Peter Fineran, personal communication). Briefly, 20 µl of *P. atrosepticum* SCRI1043 was added to 20 ml of pre-cooled LBA and poured into an agar plate. The plate was air dried for 5 min and then streaked with *P c.* subsp. *brasiliensis* ICMP 19477 using a fresh colony. *P. c.* subsp. *brasiliensis* PBR1692 and *P. c.* subsp. *carotovorum* ICMP 5702 were also prepared and tested in the same way for comparative purposes (data not shown). Plates were incubated at 28 °C overnight and inhibition of *P. atrosepticum* SCRI1043 was measured by the presence of a zone of inhibition around the *P. c.* subsp. *brasiliensis* or *P c.* subsp. *carotovorum* colony. Three plates were prepared for each strain and the experiment was repeated twice. For ESS assays, 20 µL of ESS bacterial culture grown over night, was added to 20 ml precooled LBA and overlaid on an LBA plate. The assay was then conducted as described above.

2.1.22 Measuring growth of bacteria on plates

In order to determine if antibiotic resistance tagging, or the insertion of a transposon cassette, affected bacterial growth the growth was assessed compared to the WT. Bacterial cultures were inoculated into 5 ml LB (with antibiotics where appropriate) and incubated at 28 °C overnight with shaking at 180 rpm. The cultures were washed twice with fresh LB, to remove any antibiotics. Three replicates were prepared by inoculating 20 µl of bacterial culture (optical density (OD_{600}) 0.2, equivalent to 10^4 CFU/ml), onto MMA plates. One aliquot was prepared for each sample time point. The plates were incubated at 28 °C for 48 h. Triplicate samples were taken at 6, 12, 24, 32 and 48 hours post inoculation (hpi) to measure CFUs.

For sampling, bacterial cultures were scraped from plates using a sterile inoculation loop and re-suspended in 1 ml ddH_2O. The CFUs were quantified by plating a series of 10-fold dilutions onto MMA, supplemented with antibiotics when appropriate. The plates were incubated at 28 °C until colonies

were visible and could be counted at 48 h. Counts were recorded when CFUs were between 50 and 200 per plate. Colony counts were then converted to CFUs. Statistical analysis was conducted as described in Section 2.1.25.

2.1.23 Growth of bacterial cultures in *in vitro* liquid cultures

Triplicate overnight cultures of the test bacteria were prepared as described in Section 2.1.22. Three replicate flasks were prepared by inoculating 50 µl of bacterial culture (OD_{600} 0.2, equivalent to 10^4 CFU/ml), into 50 ml MM, in a 250 ml flask. The flasks were incubated at 28 °C for 48 h. Triplicate samples were taken at 6, 12, 16, 24, 32 and 48 hpi. At each time point, two 1 ml samples were removed from each flask. One sample was used to measure the OD_{600} of the culture and a 10-fold dilution series was created using the second sample. The dilutions were plated onto MMA plates, containing antibiotics where appropriate. The plates were incubated at 28 °C for 48 h, and then counted as described in Section 2.1.22. Finally, the number of colonies observed was converted to CFU/ml. Statistical analysis was conducted as described in Section 2.1.25.

2.1.24 Growth of bacterial cultures in potato tubers

Inoculation and sampling conditions were adapted from Bhanupratap Vanga, 2013 (Doctoral thesis).

Triplicate overnight cultures of the test bacteria were prepared as described in Section 2.1.23. Prior to inoculation, potato tubers were soaked in 5% bleach for 15 min, rinsed in tap water and allowed to air dry. To inoculate the tubers, uniform holes were made using a cork borer (5 mm diameter and approx. 10 mm deep). Ten µl of a 10^4 CFU/ml bacterial suspension was inoculated into the hole in each tuber, the tuber plug was replaced, and the inoculation site was sealed with petroleum jelly. Five tubers were inoculated with each strain, for each sampling time (see below). The tubers were then placed in sealed plastic boxes lined with damp paper towels (to retain humidity) and incubated in a growth chamber at 20 °C, for up to 5 d. Tissue samples were taken from tubers at either 1, 2, 3, 4 and 5 days post inoculation (dpi). For sampling, inoculated tubers were cut in half and the macerated tissue from each tuber was scraped into 50 ml sterile water. The macerated tissue from each tuber was then agitated, briefly, by inverting the tube and then left at room temperature for 15 min. This ensured that the bacterial cells entered the water in which the potato tissue was suspended. The starch was removed from the bacterial suspension by centrifugation at 1,000 rpm for 2 min. For dilution plating, a 1 ml sample of the tuber extract was taken and used to produce a 10-fold dilution series. Each dilution was plated onto an MMA plate, containing appropriate antibiotics where necessary. The

plates were incubated at 28 °C for 48 h. The number of colonies observed was then converted to CFUs. Statistical analysis was conducted as described in Section 2.1.25.

2.1.25 Statistical analysis of growth data *in vitro* and *in planta*

The CFU/ml values, for *in vitro* liquid growth assays, were estimated as (counted colonies × $10^{\text{dilution factor}}$) × 100. The CFUs for tuber and *in vitro* plate growth assays were estimated as (counted colonies × $10^{\text{dilution factor}}$). The mean CFUs were calculated on the $\log_{10}$ scale and then back-transformed.

Due to sampling methods of the *in vitro* plate and *in vitro* liquid experiments, each flask or plate was effectively sampled repeatedly. As a consequence, an analysis of data from more than one time point could not be carried out without making adjustments for this repeated sampling. These adjustments were considered technically complex for count data (Ruth Butler; personal communication). Instead, separate analyses were carried out for each sampling time. For tuber experiments, as samples taken at different time points were from independent tubers, no tuber was repeatedly sampled. Therefore, no adjustments were required and data for all tubers was analysed in a single analysis.

All data, following any required adjustments, were analysed using a Poisson generalised linear model with a logarithmic link (GLM, McCullagh and Nelder, 1989). The CFU counted were analysed, and the analysis included an adjustment of -log(Dilution×100) as an 'offset' which corrects the counted CFU to CFU/ml in the results (McCullagh and Nelder, 1989). Within the analysis, the overall difference in CFU between the strains/types and, for tuber experiments, the difference between times and the interaction between times and types, were tested with an F-test as part of the analysis of deviance. Results were presented as mean CFU/ml or CFUs and associated 95% confidence limits. These were obtained on the logarithmic scale, and back-transformed.

All analyses were carried out with GenStat (GenStat Committee, 2014).

2.1.26 *In vitro* competition on agar plates

In vitro plate competition assays were performed as described by Anderson et al., (2012). The bacteria were inoculated into 5 ml LB (with antibiotics where appropriate) and were incubated at 28 °C overnight with shaking at 180 rpm. The cultures were washed twice with fresh LB, to remove any antibiotics. To measure the impact of co-inoculation on growth of bacterial strains, MMA plates were then inoculated with either an individual *P. c.* subsp. *brasiliensis* ICMP 19477 antibiotic-tagged strain

(e.g. *P. c.* subsp. *brasiliensis* R; Table 2.2), a *P. atrosepticum* SCRI1043 antibiotic-tagged strain (*P. atrosepticum* K; Table 2.2) or a mixture of both (at a 1:1 ratio) for 48 h. For singularly inoculated strains, three replicates were prepared by inoculating 20 µl of bacterial culture (OD_{600} 0.2, equivalent to 10^4 CFU/ml), onto MMA plates. One aliquot was prepared for each sample time point. For competition assays, 10 µl of each strain was aliquoted onto triplicate plates. The plates were incubated at 28 °C for 48 h. Triplicate samples were taken at 6, 12, 24, 32 and 48 hpi. For sampling, bacterial cultures were scraped from plates using a sterile inoculation loop and re-suspended in 1 ml ddH_2O. The CFUs were quantified by plating a series of 10-fold dilutions onto MMA, supplemented with antibiotics when appropriate. Singularly inoculated cultures were plated onto MMA containing the required antibiotic (e.g. Rif for *Pbr* R). For competition cultures, aliquots were inoculated onto non-selective MMA to quantify the total number of cells. Aliquots were also plated onto MMA supplemented with either Rif or Km to determine the CFUs of the individual marker strains present in the competition culture. The plates were incubated at 28 °C until colonies were visible and could be counted at 48 h. Counts were recorded when CFUs were between 50 and 200 per plate. Colony counts were then converted to CFUs. Two independent experiments were conducted for each competition assay. Data was analysed graphically and by calculating the competitive index (see below).

To confirm that the results of the competition assays were not due to the mutations resulting from creation of the antibiotic resistant strains needed for these assays, a second series of competition assays were performed using strains tagged with the alternative antibiotic resistance marker (e.g. If *P. c.* subsp. *brasiliensis* R and *P. atrosepticum* K were used in the first experiments, *P. c.* subsp. *brasiliensis* K and *P. atrosepticum* R were used in reciprocal experiments).

For each experiment, the competitive index (CI) of each bacterial strain was calculated for each sampling time as per Anderson et al., (2012). The CI was the ratio of one strain (e.g. *P. c.* subsp. *brasiliensis* K) to the second (e.g. *P. atrosepticum* R) at sample time (X) compared to the ratio at time 0, as described for the Malthusian parameter calculation (Lenski et al., 1991). The Malthusian parameter is used as a calculation of relative fitness between microbes.

The calculation used was:

$$CI = \frac{ratio\ of\ strain\ 1 : strain\ 2\ at\ time\ X}{ratio\ of\ strain\ 1 : strain\ 2\ at\ time\ 0}$$

A value of zero represented no competition between the two strains in a co-inoculation. A Log CI of less than zero indicated that the second strain (e.g. *P. atrosepticum* SCRI1043) outcompetes the first (e.g. *P. c.* subsp. *brasiliensis* ICMP 19477) and a value greater than zero shows that strain 1 was able

to out compete strain 2. Slight competition occurred at a value of 0-1 and strong competition was represented by values above 1 (Auerbuch et al., 2001; Anderson et al., 2012).

2.1.27 *In vitro* competition in liquid cultures

Triplicate test bacterial cultures were inoculated into 5 ml LB (with antibiotics where appropriate) and were incubated at 28°C overnight with shaking at 180 rpm. The cultures were washed twice with fresh LB, to remove any antibiotics. To measure the impact of co-inoculation on growth of bacterial strains, flasks were inoculated with either an individual *P. c.* subsp. *brasiliensis* ICMP 19477 antibiotic-tagged strain (e.g. *P. c.* subsp. *brasiliensis* R; Table 2.2), a *P. atrosepticum* SCRI1043 antibiotic tagged strain (*P. atrosepticum* K; Table 2.2) or a mixture of both (at a 1:1 ratio) for 48 h. For singularly inoculated strains, three replicate flasks were prepared by inoculating 50 µl of bacterial culture (OD_{600} 0.2, equivalent to 10^4 CFU/ml), into 50 ml MM, in a 250 ml flask. For competition assays, 25 µl of each strain was inoculated into triplicate flasks. The plates were incubated at 28°C for 48 h. Triplicate samples were taken at 6, 12, 16, 24, 32 and 48 hpi. At each time point two 1 ml samples were removed from each flask. One sample was used to measure the OD_{600} of the culture and a 10-fold dilution series was created using the second sample. The dilutions were plated onto MMA plates, containing antibiotics where appropriate (as described in Section 21.26). The flasks were incubated at 28 °C for 48 h, and then counted as described above. Finally, the number of colonies observed was converted to CFU/ml and the CI for each bacterial strain was calculated for each sampling time (as described in Section 2.1.26).

2.1.28 *In planta* competition assays in potato tubers

In planta competition assays were performed in potato tubers ('Ilam Hardy') as described by (Axelrood et al., 1988). Inoculation and sampling conditions were adapted from Bhanupratap Vanga, 2013 (Doctoral thesis).

Bacterial cultures and potato tubers were prepared as described in Section 2.1.24. Tissue samples were taken from tubers at either 12 hpi, 1, 2, 3, 4 and 5 dpi. For sampling, inoculated tubers were cut in half and the macerated tissue from each tuber was scraped into 50 ml sterile water. The macerated tissue from each tuber was then agitated, briefly, by inverting the tube and then left at room temperature for 15 min. This ensured that the bacterial cells entered the water in which the potato tissue was suspended. The starch was removed from the bacterial suspension by centrifugation at

1,000 rpm for 2 min. For dilution plating, a 1 ml sample of the tuber extract was taken and used to produce a dilution series (as described in Section 2.1.26). Each dilution was plated onto a MMA plate, containing appropriate antibiotics where necessary. The plates were incubated at 28 °C for 48 h. The number of colonies observed was then converted to CFUs as described in Section 2.1.26. Finally, for each bacterial strain in co-inoculated tubers, the CI was calculated for each sampling time (as described in Section 2.1.26).

2.1.29 *In vitro* assay to confirm secretion of the antimicrobial

In vitro assays were performed to confirm secretion of the antimicrobial and establish the timing of its expression during growth of the producer bacterium. Firstly, bacterial cultures were grown overnight (Section 2.1.4), and the bacterial cells were pelleted by centrifugation at 13,000 rpm for 5 min. The supernatant was then removed and the OD_{600} of the bacterial culture was adjusted to 1 with fresh LB. One ml of the bacterial suspension was then added to 50 ml LB in a 250 ml conical flask and the culture was grown at 28 °C, with shaking at 180 rpm. One ml aliquots were subsequently removed every hour, for at least 12 h and the bacterial growth was estimated by measuring the OD_{600} of the sample. A further 1 ml sample was also removed and assayed for carbapenem production.

To measure carbapenem activity, each 1 ml of culture medium was pelleted by centrifugation at 13,000 rpm for 5 minutes at 4 °C. The supernatant was then filtered using a 0.22 µm filter (Millipore) and stored on ice in a 1.5 ml eppendorf tube until the assay was set up (the same day). The carbapenem assay plate was set up by adding 100 µl of ESS (Table 2.2) to 100 ml of 0.75% molten, cooled LBA. The agar was then poured over a bioassay LBA petri dish and left to solidify. Wells were cut into the solidified plate using a sterilised cork borer. The wells were then filled with the sterile culture supernatant (approx. 200 µl). A negative control of non-inoculated media was included. The plates were incubated at 28 °C for 24 h and the diameter of any zones of inhibition were measured. The areas of the zones were then calculated and carbapenem production expressed as halo size (cm^2/OD_{600}).

2.1.30 Assessing Pectobacterium for *N*-AHL production using a *Chromobacterium violacein* CV026-based bioassay

Bacteria were assayed for the production of an *N*-AHL using a *C. violacein* CV026-based bioassay (McClean et al., 1997). Firstly, bacterial cultures were grown overnight (Section 2.1.4) and the bacterial

cells were pelleted by centrifugation at 13,000 rpm for 5 min. The bacterial growth and sample preparation for this assay was conducted as described in Section 2.1.29. The samples prepared for the *N*-AHL production assay were stored at -20 °C until the assay plate was set up.

To make the *N*-AHL bioassay plates, 2 ml of the CV026 culture, grown overnight, was added to 100 ml 0.75% molten LBA. The suspension was poured into a bioassay plate and left to solidify. A cork borer was then used to cut wells into the agar, the wells were filled with sterile-filtered culture supernatant (approx. 200 µl), and the plates were incubated at 25 °C for 48 h. The plates were subsequently scored for an area of purple pigmentation around the wells, indicative of AHL production (cm^2/OD_{600}). Un-inoculated media was used as a negative control.

2.1.31 Chemical complementation by addition of exogenous OHHL

To assess whether production of carbapenem by *P. c.* subsp. *brasiliensis carI*⁻ could be complemented by supplying exogenous OHHL, chemical complementation assays were performed as per McGowan et al., (1995). Initially, overnight triplicate cultures were prepared in 5 ml LB (Section 2.1.4) for the *P. c.* subsp. *brasiliensis carI*⁻ strain and ESS (Table 2.2). A 5 µl aliquot from each of the *P. c.* subsp. *brasiliensis carI*⁻ cultures was then spotted onto an LBA plate, which was prepared with a fresh top lawn seeded with ESS (as per Section 2.1.21). To test for chemical complementation, 5 µl of 1 mM OHHL (Peter Fineran, University of Otago) was also added to three further aliquots of the *P. c.* subsp. *brasiliensis carI*⁻ mutant cultures, and the samples were spotted onto LBA plates inoculated with the indicator (as above). Plates were then incubated overnight at 28 °C and the presence of zones of inhibition indicative of carbapenem production was recorded. *P. c.* subsp. *brasiliensis* ICMP 19477 was included as a positive control, *P. c.* subsp. *brasiliensis carR*⁻ + OHHL was included as a negative control.

2.1.32 Mass spectrometry and liquid chromatography-mass spectrometry

Antagonism assay plates were prepared as described in Section 2.1.21, *P. atrosepticum* SCRI1043 was used as the bacterial lawn. Plates were also prepared containing only MMA, as well as plates containing only MMA seeded with *P. atrosepticum* SCRI1043 to act as controls. Six plates of each were prepared, which would be combined to form two samples of each treatment. For sample preparation, agar was cut from the zones of inhibition, the agar from three plates were combined for one sample. Agar was also cut form the control plates. The agar was then blended into small pieces, to increase the surface agar of the agar for methanol extraction. Methanol was then added, sufficient to cover the agar, and the suspension blended further until fully suspended in the methanol. The agar and

methanol suspension was then transferred to 50 ml Falcon tubes and centrifuged for 10 min at 10,000 rpm, in order to remove the agar. The supernatant was transferred to a fresh 50 ml Falcon tube. More methanol was added to the agar remaining in the tube, sufficient to cover the agar, and centrifuged again for 10 min at 10,000 rpm. The sample was then placed under nitrogen conditions, to prevent oxidation of compounds present. The methanol was then evaporated from the samples. Once all of the methanol had been removed, the samples were then frozen in liquid nitrogen and concentrated by freeze drying overnight. The resulting pellet then underwent further methanol extraction to ensure all of the metabolites were dissolved in a liquid phase. This was concentrated on a speedy-vac and then resuspended in 50 µL, 5 % AcN. This was then used for liquid chromatography-mass spectrometry (LC-MS) and tandem mass spectrometry (MS-MS) analysis using TripleTOF 6600 (Sciex). Mass spectrometry and LC-MS was conducted at The University of Auckland under the supervision of Prof. David Greenwood (Plant and Food Research).

For both methods, peaks of interest were identified manually and by analysis using PEAKS (Bioinformatics Solutions Inc.) and SEIVE^{TM} software (Thermo Scientific).

Appendix A

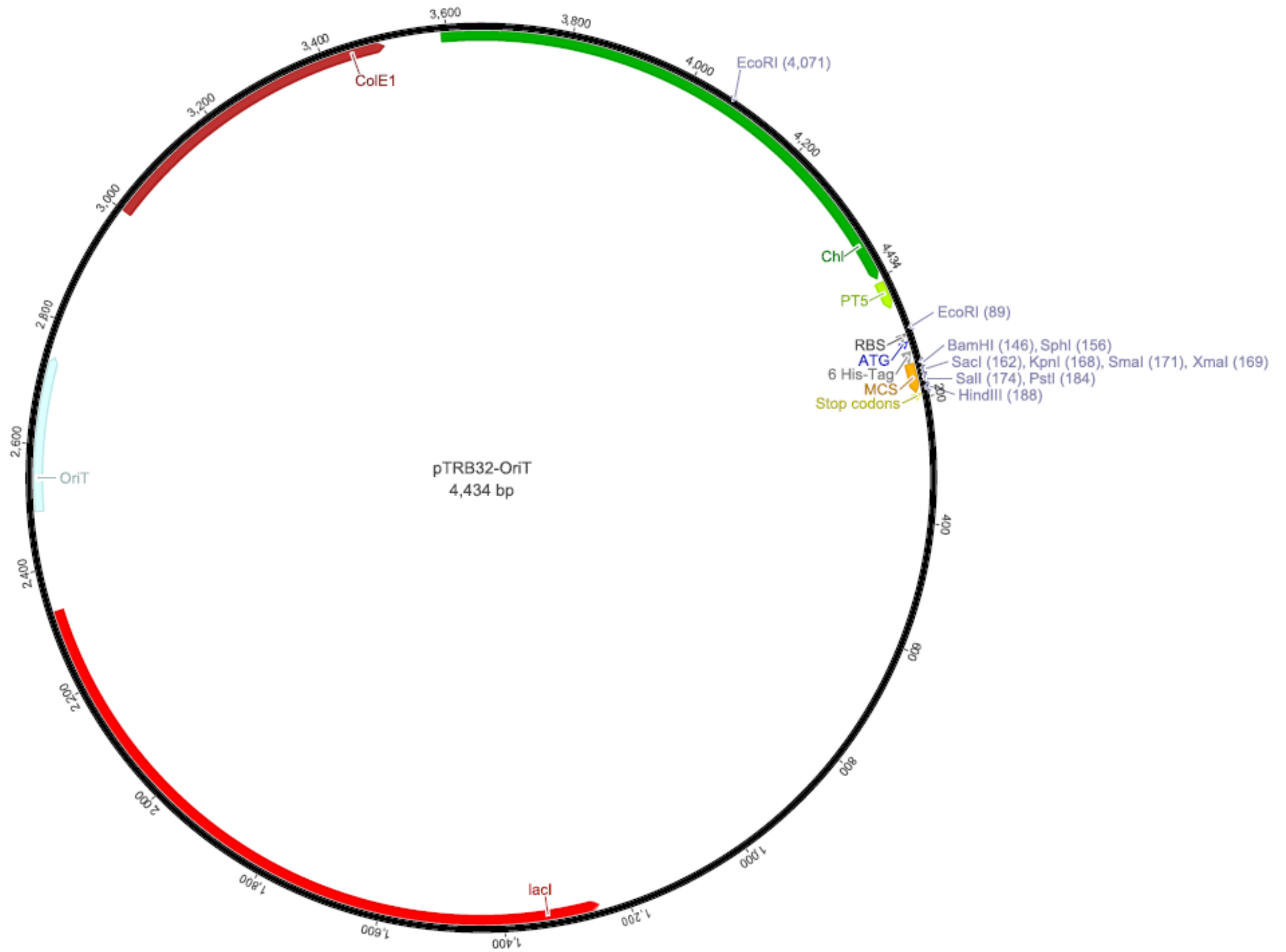
A.1 Media used in this study

Media	Ingredient	Amount
Luria-Bertani (LB) medium	Tryptone	10 g
	Yeast extract	5 g
	Sodium chloride	10 g
	ddH_2O	Up to 1 l
M9 minimal glucose medium (MM)	5 x M9 salts	56.4 g
	ddH_2O	Up to 1 l
	Sterilise by autoclaving. Add 200 ml to 700 ml ddH_2O.	
	Add:	
	20 % (v/v) glucose	20 ml
	1 M $MgSO_4$	2 ml
	1 M $CaCl_2$	ml
	ddH_2O	up to 1 l
LB agar	Tryptone	10 g
	Yeast extract	5 g
	Sodium chloride	10 g
	Agar	6.25g
	ddH_2O	Up to 1 l
MM agar (MMA)	Agar	15 g
	ddH_2O	Up to 1 l
	Sterilise by autoclaving. Add:	
	5 x M9 salt solution sterilised by autoclaving	200ml
	20 % (v/v) glucose	20 ml
	1 M $MgSO_4$	2 ml
	1 M $CaCl_2$	0.1 ml
Super optimal broth with catabolite repression **(**S.O.C) media	Tryptone	20 g
	Yeast extract	5 g
	1 M NaCl	10 ml
	1 M KCl	2.5 ml
	ddH_2O	Up to 970 ml
	sterilised by autoclaving	
	Then add sterile:	
	1 M $MgCl_2 \cdot 6H_2O$	10 ml
	1 M $MgSO_4 \cdot 7H_2O$	10 ml
	1 M Glucose	10 ml

A.2 Chemicals used in this study

Chemical	Ingredients	Amount
1 M $CaCl_2$	$CaCl_2$	147 g
	ddH_2O	Up to 1 l
	sterilise by autoclaving	
1 M KCl	KCl	74.55 g
	ddH_2O	Up to 1 l
	sterilise by autoclaving	
1 M $MgCl_2 \cdot 6H_2O$	$MgCl_2 \cdot 6H_2O$	203 g
	ddH_2O	Up to 1 l
	sterilise by autoclaving	
1 M $MgSO_4 \cdot 7H_2O$	$MgSO_4 \cdot 7H_2O$	246.5 g
	ddH_2O	Up to 1 l
	sterilise by autoclaving	
1 M NaCl	NaCl	58.4 g
	ddH_2O	Up to 1 l
	sterilise by autoclaving	
50× TAE buffer stock solution	Tris-Acetate	242 g
	0.5 M Na_2EDTA	100 ml
	Glacial acetic acid	57.1 ml
	RO water	Up to 1 l
10× TAE buffer working solution	50× TAE buffer stock solution	40 ml
	RO water	Up to 2 l
Bromophenol blue tracking dye (6x)	Glycerol	30 ml
	Bromophenol blue	0.01 g
	ddH_2O	Up to 50 ml
Kanamycin stock solution (50 mg/ml)*	Kanamycin sulphate	0.5 g
	ddH_2O	10 ml
Ampicillin stock solution (50 mg/ml)*	Sodium ampicillin	0.5 g
	ddH_2O	10 ml
Tetracycline stock solution (10 mg/ml)*	Tetracycline	g
	70 % ethanol	10 ml
Rifampicin stock solution (50 mg/ml)*	Rifampicin	0.5 g
	100 % methanol	10 ml
Chloramphenicol stock solution (34 mg/ml)*	Chloramphenicol	0.34 g
	100 % ethanol	10 ml
IPTG (20 mg/ml)	IPTG	200 mg
	ddH_2O	10 ml
	Filter sterilised (0.22 µm syringe filter), aliquots stored at -20 °C.	

A.3 Map of plasmid pTRB32oriT



Chapter 3

Competition between *Pectobacterium carotovorum* subsp. *brasiliensis* ICMP 19477 and *Pectobacterium atrosepticum* SCRI1043

3.1 Introduction

In the environment, bacterial species rarely exist in isolation. Instead, they form mixed populations in which a multitude of interactions occur between individuals (Gross et al., 1991). Interactions are likely to include those associated with competition, for which two types have been identified. The first, called contact dependent inhibition (CDI) (Aoki et al., 2005), requires contact between a producer of an antagonistic molecule and a bacterium that is sensitive to the molecule. The second is based on the production of one or more diffusible antagonistic molecules by a producer that can have an impact on sensitive organisms without direct contact (McGowan et al., 2005).

Contact dependent inhibition was initially described in *E. coli* (Aoki et al., 2005). In this bacterium, a large surface protein that functioned as a toxin (encoded by the *cdiA* gene), was shown to be transported into a sensitive cell on contact with a receptor at the cell surface, resulting in the killing of the sensitive cell (Aoki et al., 2005). The toxin is secreted from the producing cell, via a β-barrel protein encoded by *cdiB* (Henderson et al., 2004; Mazar and Cotter, 2007; Hayes et al., 2010). A third gene, *cdiI*, encoded an immunity protein that protected the producing cell from the effects of the toxin (Aoki et al., 2005). Contact dependent inhibition toxins act on sensitive cells via DNase or tRNase activity or by dissipating the proton motive force (Aoki et al., 2009; Aoki et al., 2010; Poole et al., 2011).

Contact dependent inhibition toxins are widespread among gram-negative bacteria and have been identified in plant pathogens where they aid in colonization of their host (Aoki et al., 2011). For example it was observed that *virA* gene disruption mutants of *E. chrysanthemi* (*Dickeya*) EC16 were defective in growth on different host plants (Collmer, 1998; Rojas et al., 2004). The *virA* gene was predicted to encode an immunity protein, of the CdiI type, which when disrupted caused auto-inhibition of the producer cell leading to reduced growth (Rojas et al., 2004). From these results it was predicted that the CDI locus was expressed when the bacteria colonized the host plant. This hypothesis was supported by work in *D. dadantii* 3937, which showed that the bacterium up-regulated one of its two CDI systems when grown on chicory plants (Aoki et al., 2010).

Other antimicrobial systems have been described where an antibiotic secondary metabolite is produced and secreted by the bacterium (Cascales et al., 2007). These metabolic products are

produced without the requirement for the producer to be in direct contact with the sensitive competitor (McGowan et al., 2005). Many examples of these secreted molecules are known, including those belonging to the β-lactam family (Coulthurst et al., 2005). Carbapenems are well-studied broad spectrum β-lactam molecule produced by the *Enterobacteriaceae* family members *Pectobacterium* and *Serratia* (Parker et al., 1982; Williamson et al., 1985; Bycroft et al., 1987), whereas thienamycin, a more complex molecule, is produced by gram positive *Streptomyces* (Kahan et al., 1979).

The carbapenem produced by both *Pectobacterium* and *Serratia,* (5R)-carbapen-2-em-3-carboxylic acid (Bycroft et al., 1987), is encoded by a gene operon of eight genes annotated as *carA-H* (McGowan et al., 1997). The operon includes genes vital for carbapenem biosynthesis (*carABCDE*) (McGowan et al., 1997; Li et al., 2000) and genes encoding a novel, intrinsic carbapenem resistance mechanism (McGowan et al., 1997). Carbapenems kill sensitive cells by targeting the cell wall. They inhibit the activity of transpeptidase enzymes (Waxman and Strominger, 1983) or penicillin binding proteins (Sauvage et al., 2008), therefore weakening the cell wall by preventing crosslinking of peptidoglycan during cell wall biosynthesis.

Carbapenems have been predicted to be involved in competition between *Pectobacterium* species. Firstly, it was observed that *P. betavasculorum* was able to inhibit sensitive *P. c.* subsp. *carotovorum* strains when co-inoculated *in vitro* as well as in potato tubers (Axelrood et al., 1988). Secondly, it was found that carbapenem encoding *P. c.* subsp. *brasiliensis* PBR1692 strains were able to inhibit the growth of *P. atrosepticum* SCRI1043 in *in vitro* antagonism assays (Marquez-Villavicencio et al., 2011). It therefore appears that carbapenem production may be an important competition mechanism for *Pectobacterium* species, utilised during colonisation of the host plant.

Bacteriocins are also secreted antimicrobial molecules produced by many bacteria (Klaenhammer, 1988; Cascales et al., 2007). Production occurs when the bacteria are under stress (Herschman and Helinski, 1967; Cotter et al., 2005), for example at low oxygen levels (Eraso and Weinstock, 1992) or high temperatures (Cavard, 1995). Therefore, conditions encountered by the bacteria in competition, such as nutrient limitation, induce bacteriocin production.

The production of bacteriocins was predicted to be involved in bacterial competition within the rhizosphere (Dowling and Broughton, 1986). Interestingly, it was observed that bacteriocin negative transposon mutants were not reduced in competition compared to the producing strains (Dowling and Broughton, 1986; Parret et al., 2003). Therefore, much remains unknown about the ecological significance of bacteriocins. However, as bacteriocin synthesis genes are abundant within plant

pathogens, they must play an important role within the bacterias' ecological niche (Hu and Young, 1998; Holtsmark et al., 2008).

More recently it has been proposed that antimicrobial molecules act as signalling molecules in the environment (Yim et al., 2007). Antimicrobial molecules present in the environment are likely to be at sub-inhibitory levels and therefore are unlikely to function in competition. Instead, they may be produced as a method of interspecies signalling in response to changing conditions (Davies et al., 2006; Yim et al., 2007). For example, they may act as an indicator for other bacteria to alter their metabolic functions (Price-Whelan et al., 2006).

The involvement of CDI and secreted toxins in competition of plant and soil associated bacteria has also been examined in potential biocontrol agents (Raaijmakers et al., 2002). In these studies, some of the bacterial species studied have even been found to produce multiple antibiotic molecules. For example, *Bacillus cereus* UW85 (Handelsman and Stabb, 1996) and *Pseudomonas fluorescens* CHAO (Keel et al., 1990; Bender et al., 1999a) produce both pyrrolnitrin (Chernin et al., 1996) and phenazines (Weller, 1983; Gutterson et al., 1986; Rosales et al., 1995). It may be, therefore, that competition in the native ecological niche is complex and involve the production of multiple molecules, or the employment of different mechanism in order to be successful. As yet, the ecological significance of these molecules remains poorly understood.

Ecological studies were difficult to conduct (Thomashow et al., 1997), firstly due to the parameters that were regarded to constrain production *in situ* such as the complex abiotic and biotic conditions encountered in soils and plants environments (Thomashow et al., 1997). The chemical stability of the molecules under such conditions, or the binding of the antimicrobial molecule to organic matter were also considered potentially constraining factors that were not understood (Thomashow et al., 1997). Secondly, methods for detection of bacteria producing these compounds in the environment were poor (Gottlieb, 1976; Williams and Vickers, 1986). The development of reporter gene techniques to monitor gene expression *in situ* (Loper et al., 1997; Lindow and Brandl, 2003) as well as improvements to thin layer chromatography (TLC) and high pressure liquid chromatography (HPLC) methods, however, enabled the production of antibiotics in soil and around plants to be monitored and even quantified (Thomashow et al., 1997). A number of antibiotics produced by biocontrol agents have now been detected as being produced *in situ*, including phenazine-1-carboxylic acid, herbicolin A and surfactin (Thomashow et al., 1997). This however does not address the question of whether these molecules are responsible for the biocontrol activity of certain bacteria. This is a hard problem to address as parameters such as the time of antibiotic production needs to be taken in to account; which

can be difficult to measure *in situ.* Furthermore, the quantification of low levels of antimicrobials remains a challenge (Raaijmakers et al., 2002).

Epiphytic communities of *Pseudomonas,* found on plant leaves, also provide an ecological example of bacterial competition (Lindow and Brandl, 2003). It was observed that the compositions of epiphytic bacterial communities were affected by antagonistic bacteria colonising the leaf at certain times during development (Lindow and Brandl, 2003). An example of this is the biocontrol of plant frost injuries caused by *P. syringae* ICE^{+} (ice nucleation activity positive) bacteria (Lindow et al., 1982). It was found that plant frost injuries could be prevented by the competitive exclusion of ICE^{+} bacteria by ICE^{-} *P. syringae* (Lindow, 1995; Lindow et al., 1996). Successful biocontrol was achieved when at-risk plants were inoculated with ICE^{-} bacteria, before ICE^{+} *P. syringae could* establish. The ICE^{-} bacteria were able to displace the ICE^{+} bacteria via competition for resources such as carbon (Wilson and Lindow, 1993; Wilson and Lindow, 1994). Competition by the secretion of antimicrobial molecules was found to be uncommon on leaf surfaces (Lindow, 1988).

Despite the studies on biocontrol agents, the ecological significance of antimicrobial production by plant pathogens and the involvement of these antimicrobials in driving the evolution of plant pathogen populations has not been investigated. This is despite recent suggestions that the production of antimicrobials might have influenced the emergence of pathogens such as *D. solani*, which has seemingly displaced closely related SREs in potato paddocks in some parts of the world (Garlant et al., 2013). Similarly, *P. c.* subsp. *brasiliensis*, a more recently identified pathogen of potato, produces an unknown antimicrobial that has *in vitro* activity against *P. atrosepticum* SCRI1043 (Marquez-Villavicencio et al., 2011). The ecological significance of this activity remains unclear, however, as co-inoculation assays demonstrated that production of this molecule did not provide a competitive advantage to the producer when the two blackleg-causing pathogens were co-inoculated into potato stems (Marquez-Villavicencio et al., 2011).

In this chapter, the role of antimicrobial production in competitive fitness of a pathogen was studied in order to begin to understand how pathogen populations might evolve. In particular, the antimicrobial production of *P. c.* subsp. *brasiliensis* was re-examined using the New Zealand strain ICMP 19477 (Panda et al., 2015b) as it was hypothesized that the production of an antimicrobial with activity against *P. atrosepticum* would provide enhanced fitness in the structured environment of the tuber rather than in the stem (Section 1.6.4). Focusing on the interactions between these pathogens in the tuber also seemed more ecologically relevant, as the tuber is the more likely point of entry for both species and therefore the location where competition might first take place.

Once these assays had been performed, bioinformatics tools were used to identify the likely antimicrobial biosynthetic clusters encoded within the genome of *P. c.* subsp. *brasiliensis* ICMP 19477, which might be responsible for the activity against *P. atrosepticum* SCRI1043. *P. c.* subsp. *brasiliensis* ICMP 19477 encodes many putative antimicrobial synthesis genes, a number of which are common to *P. c.* subsp. *brasiliensis* ICMP 19477 and PBR1692, but not found in other SREs (Preetinanda Panda, 2014). One or more of these clusters, likely to have been acquired through lateral gene transfer, was predicted to be responsible for the antimicrobial phenotype observed during the experiments described in this chapter.

3.2 Results

3.2.1 Optimisation of antagonism assay

In initial experiments, the optimal conditions to investigate antimicrobial production by *P. c.* subsp. *brasiliensis* ICMP 19477 were considered. Initially, *P. c. subsp. brasiliensis* ICMP 19477 was applied to the test plates by inoculating 10 µl of overnight culture, as described for the antagonism assays performed by (McGowan et al., 1996). Test plates were seeded with *P. atrosepticum* SCRI1043, which is sensitive to the antimicrobial produced by *P. c.* subsp. *brasiliensis* ICMP19477. This method of application, however, produced variable zones of inhibition (data not shown). Instead, a second method described by (Marquez-Villavicencio et al., 2011), where a *P. c.* subsp. *brasiliensis* colony was streaked onto the test plate from a culture plate, produced more reproducible zones of inhibition (data not shown). Finally, it was found that applying the *P. c.* subsp. *brasiliensis* ICMP 19477 colony as a high concentration of cells (in a dot), rather than diluting the cells by streaking, produced the optimal zones of inhibition for continuing these assays (data not shown).

The cell density of the sensitive strain (*P. atrosepticum* SCRI1043), inoculated into the lawn prior to conducting the antagonism assays was also important for reproducible production of zones of inhibition. Indeed, it was found that adding too many *P. atrosepticum* SCRI1043 cells (>10^6/ml cells) to the agar, meant that clear zones of inhibition were not produced. Finally, it was shown that adding 20 µl of overnight *P. atrosepticum* SCRI1043culture to 20 ml LBA produced sufficient growth for the zones to be clear, but not an over-grown lawn that meant zones of inhibition were weak. This method was consistent with that described by McGowan et al., (1995), when using an *E. coli* test strain as the bacterial lawn in antimicrobial assays.

3.2.2 *Pectobacterium carotovorum* subsp. *brasiliensis* ICMP 19477 inhibits the growth of *Pectobacterium atrosepticum* SCRI1043

The optimised plate assays (as described in Section 2.1.21) showed that like *P. c. subsp. brasiliensis* PBR1692, *P. c. subsp. brasiliensis* ICMP 19477 inhibited the growth of *P. atrosepticum* SCRI1043 *in vitro* (Figure 3.1). The inhibition of *P. atrosepticum* SCRI1043 growth by *P. c. subsp. brasiliensis* PBR1692 had previously been described by Marquez-Villavicencio et al., (2011). The described assay was also conducted using *P. c. subsp. carotovorum* ICMP 5702 as the test strain. This bacterial strain showed no zone of inhibition when inoculated onto a *P. atrosepticum* SCRI1043 lawn (data not shown). *Pectobacterium carotovorum* subsp. *brasiliensis* ICMP 19477 was also unable to inhibit the growth of *P. c.* subsp. *carotovorum* ICMP 5702, when the latter was used as the lawn bacteria (data not shown). Therefore, *P. c. subsp. brasiliensis* specific inhibition of *P. atrosepticum* SCRI1043 was observed.

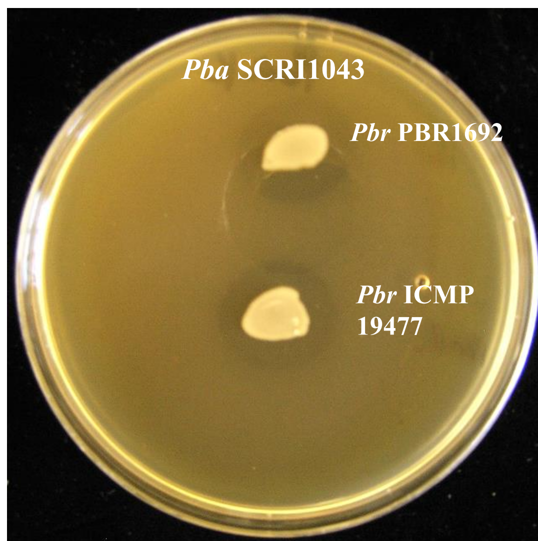


Figure 3.1. *In vitro* growth inhibition of *P. atrosepticum* (*Pba*) SCRI1043 by *P. c.* subsp. *brasiliensis* (*Pbr*) ICMP 19477 and *P.c.* subsp. *brasiliensis* (*Pbr*) PBR1692.

Plates pre-seeded with a lawn of *P. atrosepticum* SCRI1043 were incubated at 28°C for 24 h after inoculation with the producer strain. A positive result was indicated by the presence of a zone of inhibition around the *P. c. subsp. brasiliensis* colony.

3.2.3 Strains of *Pectobacterium carotovorum* subsp. *brasiliensis* ICMP 19477 and *Pectobacterium atrosepticum* SCRI1043 tagged with an antibiotic resistance gene retain the wild type phenotypes relating to antibiotic production and sensitivity *in vitro*

To study the interactions between *P. c. subsp. brasiliensis* ICMP 19477 and *P. atrosepticum* SCRI1043 further using *in vitro* and *in planta* competition assays, antibiotic resistant strains were produced (as described in Section 2.1.4, Table 2.2). Firstly, spontaneous Rif mutants were created for both *P. c. subsp. brasiliensis* ICMP 19477 (*P. c. subsp. brasiliensis* R) and *P. atrosepticum* SCRI1043 (*P. atrosepticum* R). Rifampicin resistance is conferred by a mutation in *rpoB,* the gene encoding the β subunit of the RNA polymerase (Heep et al., 2000; Vogler et al., 2002). Spontaneous Rif resistant mutants of a *P. atrosepticum* strain have successfully been used in *in vitro* and *in planta* studies without any observed difference in phenotype compared to the wild-type (WT) (Cronin et al., 1997; Vanga et al., 2012). Genetic mutants of *P. c. subsp. brasiliensis* ICMP 19477 and *P. atrosepticum* SCRI1043, resistant to Km (*P. c. subsp. brasiliensis* K and *P. atrosepticum* K) were also generated for co-inoculations. Similar Km resistant-tagged strains have previously been used successfully in *in vitro* and *in planta* assays (Vanga et al., 2012). Both the *P. c. subsp. brasiliensis* ICMP 19477 K and *P. c. subsp. brasiliensis* ICMP 19477 R strains retained the producer phenotype, whilst the *P. atrosepticum* SCRI1043 K and *P. atrosepticum* SCRI1043 R strains remained sensitive to the producer (Figure 3.2). Similar sized zones of inhibition were generated using these strains, indicating that they were suitable for use in future antagonism assays.

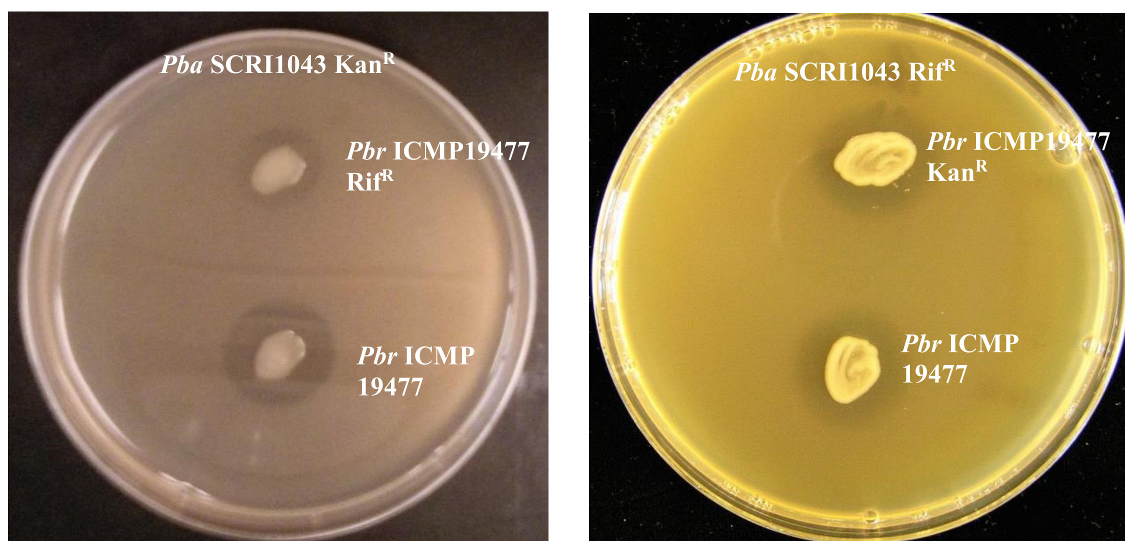


Figure 3.2. *In vitro* growth inhibition of *P. atrosepticum* (*Pba*) SCRI1043 by *P. c.* subsp. *brasiliensis* (*Pbr*) ICMP 19477 was unaffected by tagging the strains with antibiotic resistance.

Antagonism assays were conducted using *P. c. subsp. brasiliensis* ICMP 19477 R or *P. c. subsp. brasiliensis* ICMP 19477 K as the producer strain and *P. atrosepticum* SCRI1043 K or *P. atrosepticum* SCRI1043 R as the sensitive strains in assays described in Section 2.1.21. Assay plates were incubated at 28°C for 24 h, with zones of inhibition around the *P. c. subsp. brasiliensis* ICMP 19477 colonies indicative of antimicrobial production.

The growth of the antibiotic resistant mutants was also compared to the WTs on solid agar plates (Section 2.1.22) and in liquid cultures (Section 2.1.23), to confirm that the tagged strains were representative of the WTs *in vitro*. On solid agar there were no significant differences in growth between the *P. c.* subsp. *brasiliensis* ICMP 19477 mutants and the WT ($p > 0.22$, F-test), with the exception of 24, 32 and 72 h ($p = 0.073$, 0.007 and 0.001 respectively, F-test) (Figure 3.3 A). There were similar results for the *P. atrosepticum* SCRI1043 strains, as the CFUs did not vary significantly between the strains at most time points ($p > 0.4$, F-test). There was, however, a significant variation at 48 h ($p = 0.032$, F-test) (Figure 3.3 B). Regardless of the statistical differentiation between the strains at these few time points on solid media, the overall differences did not appear to be biologically relevant, confirming that the tagged strains were suitable for use in future competition assays performed on solid plates. Furthermore, the growth curves for both species showed that both bacterial species reached mid-stationary phase after 48 h and there was little increase in CFUs after

this time. Therefore, subsequent growth assays were terminated at 48 h. A second independent experiment confirmed the results above.

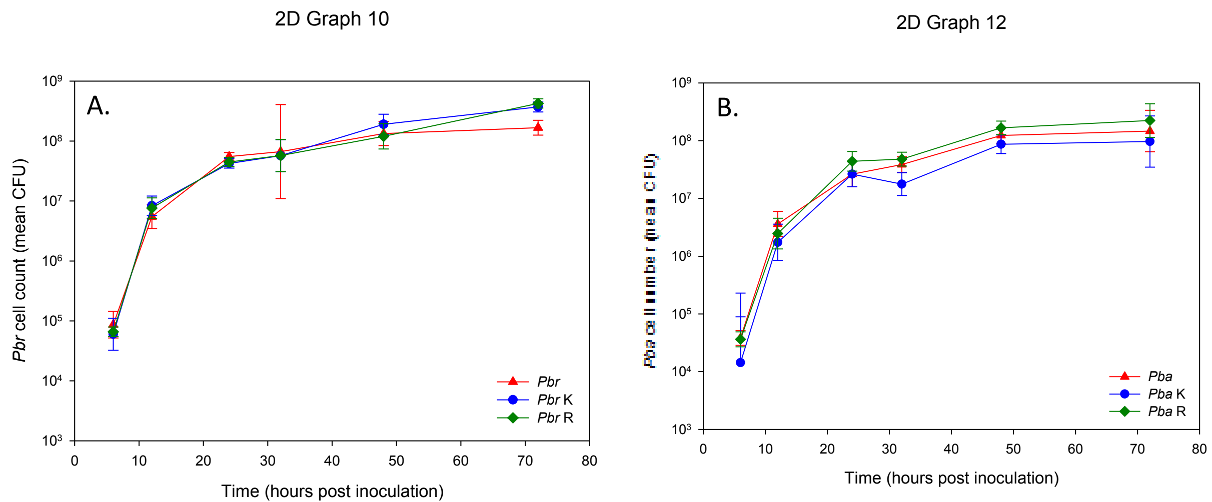


Figure 3.3. Growth of *P. c.* subsp. *brasiliensis* (*Pbr*) ICMP 19477 (A) and *P. atrosepticum* (*Pba*) SCRI1043 (B) on solid agar plates was not affected by tagging them with Km and Rif resistance. Each graph shows the growth (mean CFUs) of the WT as well as the antibiotic tagged strains over 72 h post inoculation with 10^4 CFUs/ml of each bacteria (as described in section 2.1.22). *Pectobacterium caratovorum subsp. brasiliensis* ICMP 19477 Km resistant (*Pbr* K); *P. c. subsp. brasiliensis* ICMP 19477 Rif resistant (*Pba* R); *P. atrosepticum* SCRI1043 Km resistant (*Pba* K); *P. atrosepticum* SCRI1043 Rif resistant (*Pba* R). The mean CFUs represent the mean over three test plates. Error bars are 95% confidence limits.

In liquid cultures, the CFUs for the marked *P. c.* subsp. *brasiliensis* ICMP 19477 strains did not differ significantly from those of the WT at 6, 12 and 16 h ($p = 0.093$, 0.118 for K and R, F-test). However at 24 h, the CFUs for both marked strains were significantly lower than for the WT ($p = 0.013$ and 0.021 for the Km and Rif resistant strains respectively, F-test). At 32 and 48 h, numbers for *P. c. subsp. brasiliensis* K were again similar to the *P. c.* subsp. *brasiliensis* ICMP19477 WT ($p = 0.113$ and 0.553, F-test), but those for *P. c. subsp. brasiliensis* R were statistically significantly lower ($p < 0.001$ and 0.007, F-test) (Figure 3.4 A). For *P. atrosepticum* SCRI1043*,* significant differences were only observed between the strains at 12 and 32 h. The CFUs for the two marked strains were significantly lower than for the *P. atrosepticum* WT strain ($p < 0.001$ at 12 h, and $p = 0.010$ for *P. atrosepticum* K, $p = 0.002$ for *P. atrosepticum* R F-test) (Figures 3.4 B). As for the growth experiments on solid media, although

statistically significant differences were observed at a couple of time points, overall there appeared to be no major biological differences in the growth of the WTs and the relevant tagged strains

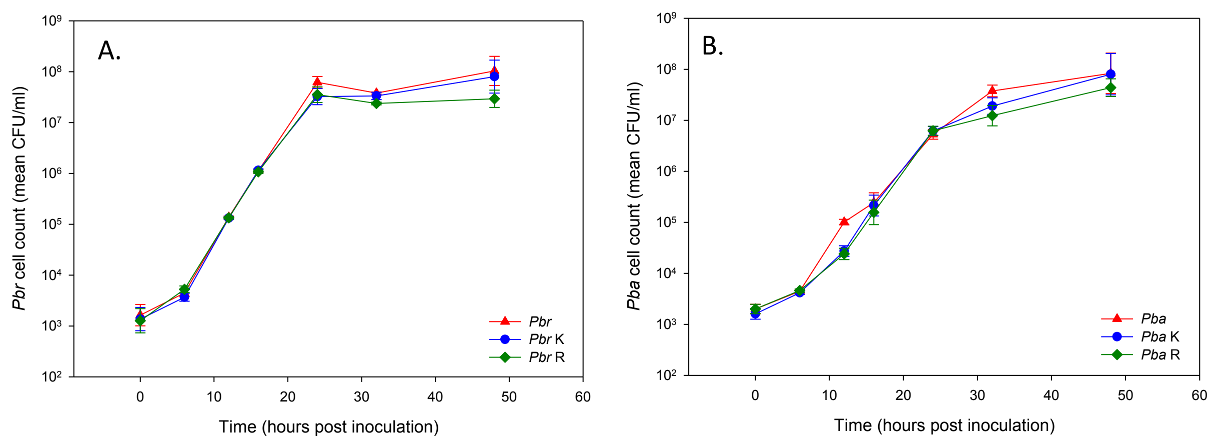


Figure 3.4. Growth of *P. c.* subsp. *brasiliensis* (*Pbr*) ICMP 19477 (A) and *P. atrosepticum* (*Pba*) SCRI1043 (B) and in liquid cultures was not affected by tagging them with Km and Rif resistance. Each graph shows the growth (mean CFUs) of the WT as well as the antibiotic tagged strains over 48 h post inoculation with 10^4 CFUs/ml of each bacteria (as described in section 2.1.23). *P. c. subsp. brasiliensis* ICMP 19477 Km resistant (*Pbr* K); *P. c. subsp. brasiliensis* ICMP 19477 Rif resistant (*Pbr* R); *P. atrosepticum* SCRI1043 Km resistant (*Pbr* K); *P. atrosepticum* SCRI1043 Rif resistant (*Pba* R). The mean CFUs represent the mean over three test plates. Error bars are 95% confidence limits.

3.2.4 *Pectobacterium carotovorum* subsp. *brasiliensis* ICMP 19477 reduces the growth of *Pectobacterium atrosepticum* SCRI1043 when co-inoculated in solid plate competition assays, but not in *in vitro* liquid competition assays

Solid plate competition assays (Section 2.1.13) were conducted to examine whether co-inoculation of solid agar plates with *P. c.* subsp. *brasiliensis* ICMP 19477 and *P. atrosepticum* SCRI1043 resulted in reduced growth of the latter. By 12 h (mid to late exponential phase), the growth of *P. atrosepticum* SCRI1043 was slightly reduced when co-inoculated with *P. c.* subsp. *brasiliensis* ICMP 19477 compared to growth of the bacterium when cultured in isolation, regardless of which antibiotic resistance determinant was used to tag the strains. Furthermore, there continued to be a general trend for reduced growth of *P. atrosepticum* when co-inoculated with *P. c.* subsp. *brasiliensis* ICMP 19477 until late into stationary phase, although the reduction in cell counts was only small ($p < 0.001$ 12, 24, 32

and 48 h. At 24 h, only *P. atrosepticum* R was significantly reduced in growth, F-test; Figure 3.5 A). In contrast, the growth of the *P. c.* subsp. *brasiliensis* ICMP 19477 strains was not reduced when co-inoculated with *P. atrosepticum* SCRI1043 regardless of the antibiotic resistance marker used to tag the strains (p = 0.215, except at 48 h where *P. c.* subsp. *brasiliensis* K inoculated with *P. atrosepticum* R was lower p < 0.001, F-test; Figure 3.5 B). These data indicated that *P. c. subsp. brasiliensis* ICMP 19477 had a small, but significant impact on the growth of *P. atrosepticum* SCRI1043 in the structured environment of the plate assays. These results were consistent in a second independent experiment (Appendix B).

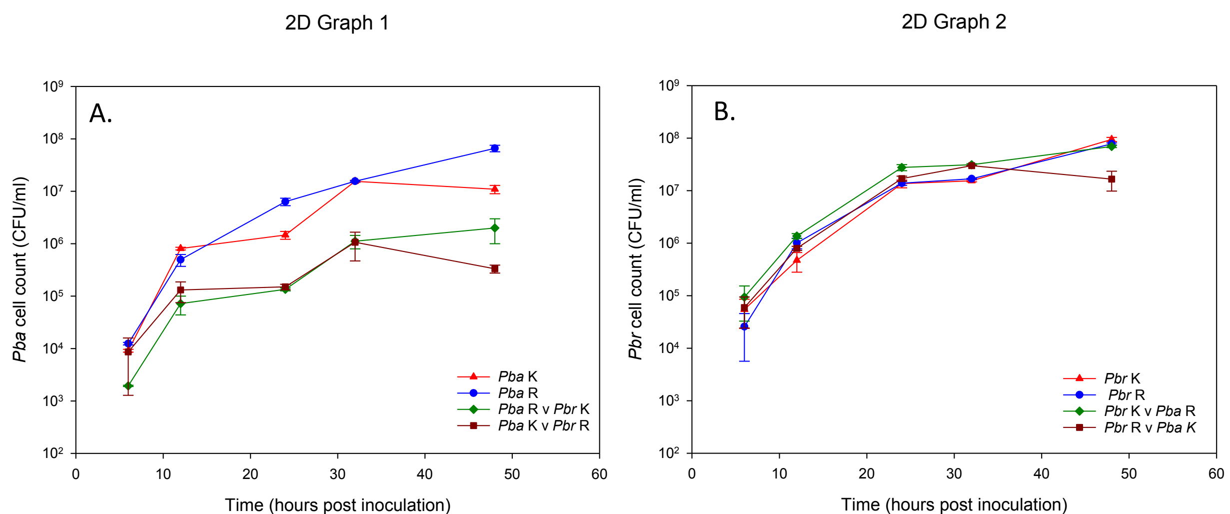


Figure 3.5. The effect of co-inoculation with *P. c.* subsp. *brasiliensis* (*Pbr*) ICMP 19477 on growth of *P. atrosepticum* (*Pba*) SCRI1043 on solid agar plates (MMA).

A: A graph showing the growth (mean CFUs) of *P. atrosepticum* SCRI1043 Km (*Pba* K) and Rif resistant (*Pba* R) resistant strains over a 48 h period at 28°C when inoculated onto MMA in isolation or when co-inoculated with *P. c. subsp. brasiliensis* ICMP 19477 tagged with either a Km (*Pbr* K) or Rif (*Pbr* R) resistance determinant (at a 1:1 ratio). **B**: A graph showing the growth (mean CFUs) of *Pbr* K or *Pbr* R when cultured in isolation on solid agar plates or in co-inoculations with *Pba* K or *Pba* R (at a 1:1 ratio). For each graph, the mean CFUs were calculated for each treatment from triplicate assays (solid line), error bars represent the standard deviation.

The effect of *P. c. subsp. brasiliensis* ICMP 19477 on *P. atrosepticum* SCRI1043 growth in co-inoculated *in vitro* liquid (MM) cultures was also investigated using the experiments described in Section 2.1.27. In these experiments, unlike those conducted on solid media, the growth of *P. atrosepticum* SCRI1043

upon co-inoculation with *P. c.* subsp. *brasiliensis* ICMP 19477 was not reduced when compared to growth of the bacterium in pure culture, at any sampling time during the 48 h experiment ($p > 0.1$, F-test) (Figure 3.6 A). The growth of *P. c. subsp. brasiliensis* ICMP 19477 was not affected ($p > 0.4$, F-test) when co-inoculated with *P. atrosepticum* SCRI1043 either (Figure 3.6 B), indicating that neither bacterial strain had an impact on the other under these conditions. The results were consistent in a second, independently conducted experiment (Appendix B).

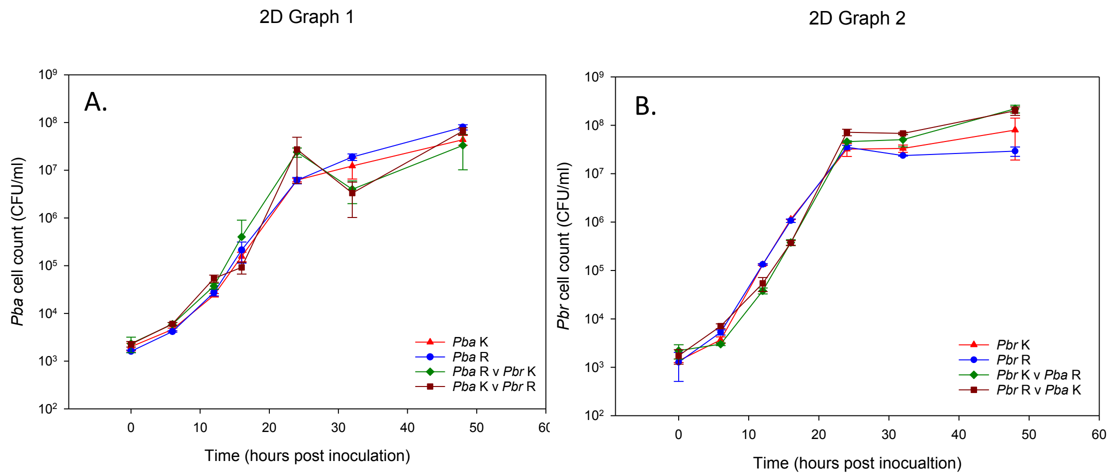


Figure 3.6. The effect of co-inoculation with *P. c.* subsp. *brasiliensis* (*Pbr*) ICMP 19477 on growth of *P. atrosepticum* (*Pba*) SCRI1043 in liquid cultures (MM).

A: A graph showing the growth (mean CFUs) of *P. atrosepticum* SCRI1043 Km (*Pba* K) and Rif (*Pba* R) resistant strains over a 48 h period at 28°C when inoculated into MM in isolation or in media co-inoculated with *P. c. subsp. brasiliensis* ICMP 19477 tagged with either a Km (*Pbr* K) or Rif (*Pbr* R) resistance determinant (at a 1:1 ratio). **B**: A graph showing the growth (mean CFUs) of *Pbr* K or *Pbr* R when cultured in isolation in liquid cultures or in co-inoculations with *Pba* K or *Pba* R (at a 1:1 ratio). For each graph, the mean CFUs were calculated for each treatment from triplicate assays (solid line), error bars represent the standard deviation.

3.2.5 Antimicrobial tagged strains of *Pectobacteirum carotovorum subsp. brasiliensis* ICMP 19477 and *Pectobacterium atrosepticum* SCRI1043 are not altered in growth compared to the wild types when grown in potato tubers

The results of the *in vitro* assays showed that *P. c. subsp. brasiliensis* ICMP 19477 reduced the growth of *P. atrosepticum* SCRI1043 when co-inoculated with the bacterium in the structured environment of the solid plate assay, but not in the less structured environment of a liquid culture. As a consequence, the effect of this bacterium on its close relative was studied *in planta* using co-inoculation experiments in potato tubers (as described in Section 2.1.28) rather than in potato stems. Potato tubers provide a more structured environment than plant stems, which were used in similar experiments conducted by Axelrood et al., (1988).

Prior to these experiments, the growth of the strains of *P. c. subsp. brasiliensis* ICMP 19477 and *P. atrosepticum* SCRI1043 tagged with either the Km or Rif resistance determinant were compared with that of the WT strains in potato tubers of the susceptible cultivar 'Ilam Hardy' to ensure that the modifications to these strains had not affected their growth under these conditions (Section 2.1.24). Statistically, the CFUs varied between times and between the strains (e.g. $p < 0.001$ for the *P. c.* subsp. *brasiliensis* strain and time main effects in all treatments, F-test), and the pattern of change with time varied between the strains (e.g. for the *P. c.* subsp. *brasiliensis* strain by time interaction, $p < 0.001$, Figure 3.7). However, the majority of the differences between the growth of the WT strains and the antibiotic resistance tagged derivatives were observed at the early- to mid-exponential phase (at 1-2 dpi) and could be accounted for by the slightly lower cell counts for the strains tagged with Rif. Secondary metabolites, including antimicrobial molecules, tend to be produced at late exponential to early stationary phase. As growth of the tagged *P. c.* subsp. *brasiliensis* strains was not reduced compared to the WT at this growth stage, it was considered that their use in competition assays would not alter the competition observed between the *P. c.* subsp. *brasiliensis* and *P. atrosepticum* SCRI1043. There was also greater variation in the data for the tagged strains of both species in tubers than was observed on agar plates, likely due to the variable physiology of the tubers encountered by the pathogens. For example, tuber weight and age affected colonisation and growth of potato tubers by *P. atrosepticum* and *P. c.* subsp. *brasiliensis* (Marquez-Villavicencio et al., 2011). Thus, it was decided that these strains did not show any dramatic loss in growth in tubers upon being tagged with either antibiotic resistance determinant.

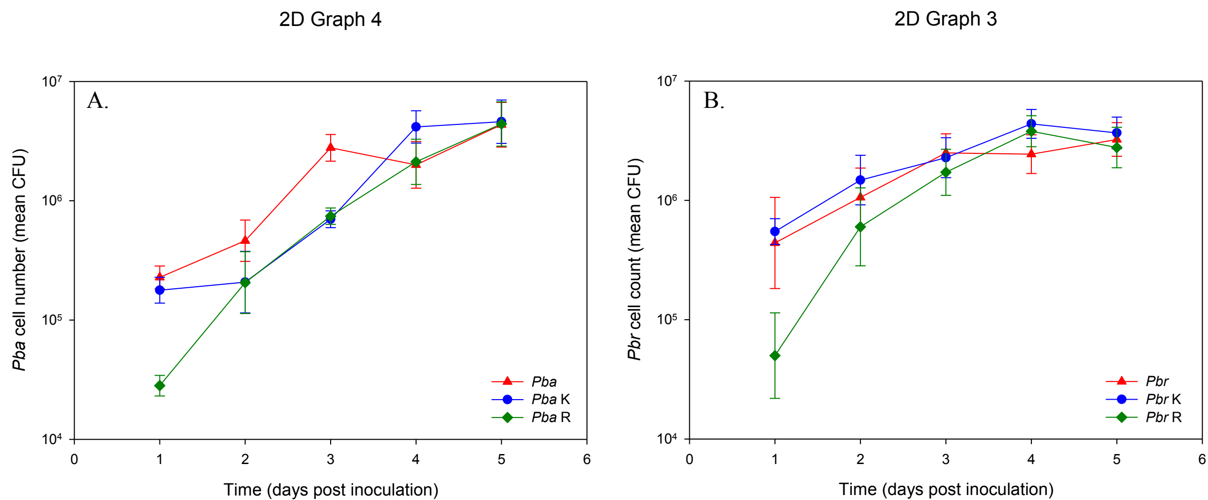


Figure 3.7. Growth of *P. atrosepticum* (*Pba*) SCRI1043 (A) and *P. c.* subsp. *brasiliensis* (*Pbr*) ICMP 19477 (B) was not affected by tagging them with Km and Rif resistance potato tubers ('Ilam Hardy'). Each graph shows the growth (mean CFUs) of the WT as well as the antibiotic tagged strains over 72 h post inoculation with 10^4 CFUs/ml of each bacteria (as described in section 2.1.22). *P. c. subsp. brasiliensis* ICMP 19477 Km resistant (*Pbr* K); *P. c. subsp. brasiliensis* ICMP 19477 Rif resistant (*Pbr* R); *P. atrosepticum* SCRI1043 Km resistant (*Pba* K); *P. atrosepticum* SCRI1043 Rif resistant (*Pba* R). The mean CFUs represent the mean over three test plates. Error bars are 95% confidence limits.

3.2.6 The growth of *Pectobacterium atrosepticum* SCRI1043 is reduced when co-inoculated with *Pectobacterium carotovorum subsp. brasiliensis* ICMP 19477 in potato tubers

The effect of *P. c. subsp. brasiliensis* ICMP 19477 on growth of *P. atrosepticum* SCRI1043 in tubers was subsequently tested using co-inoculation experiments (Section 3.2.5).

At all sampling times, the mean CFU values for the *P. atrosepticum* SCRI1043 strains (both *P. atrosepticum* K and *P. atrosepticum* R) were lower in tubers co-inoculated with *P. c. subsp. brasiliensis* ICMP 19477 when compared to those in which the modified *P. atrosepticum* SCRI1043 strains were inoculated alone ($p < 0.001$, F-test) (Figure 3.8 A). In contrast, the mean CFUs for neither the Km nor the Rif resistant strains of *P. c. subsp. brasiliensis* ICMP 19477 were affected by co-inoculation with *P. atrosepticum* SCRI1043 ($p > 0.22$, F-test; Figure 3.8 B). These results were consistent in a second, independently conducted experiment, using tubers sourced at a different time of year (Appendix B). This demonstrated that the physiology of the potato did not affect the competition between the

bacteria. It has previously been described that the age of potato tubers can affect their susceptibility to tuber soft rot (Marquez-Villavicencio et al., 2011). All competition assays conducted in potato tubers will be confirmed in this manner throughout the study.

Therefore suggesting that *P. c. subsp. brasiliensis* ICMP 19477 inhibits the growth of *P. atrosepticum* SCRI1043 in tubers, and that the reduction in growth is greater in potato tubers than on solid agar plates.

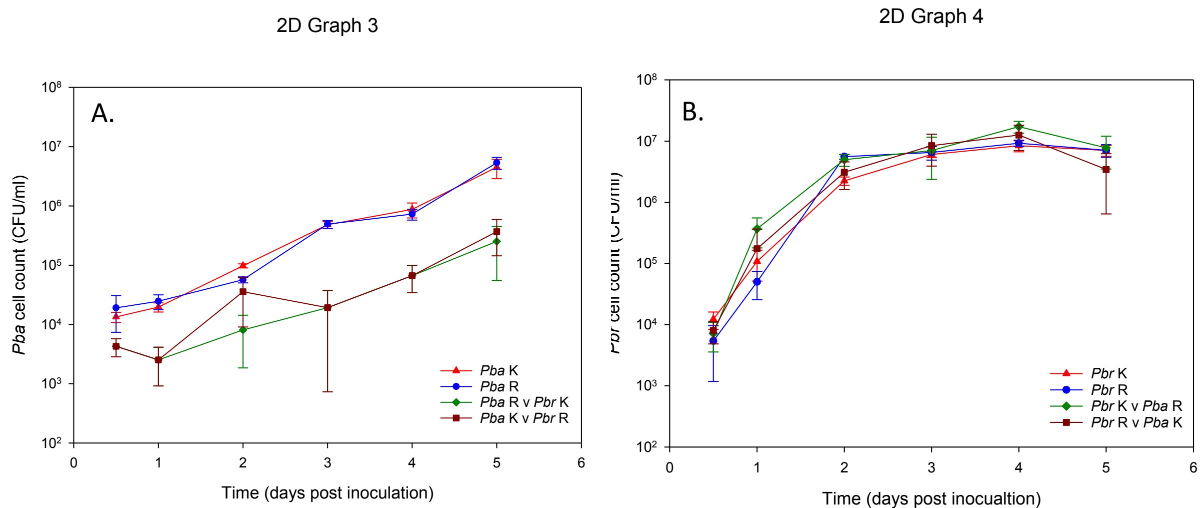


Figure 3.8. The effect of co-inoculation with *P. c.* subsp. *brasiliensis* (*Pbr*) ICMP 19477 on growth of *P. atrosepticum* (*Pba*) SCRI1043 in potato tubers ('Ilam Hardy').

A: A graph showing the growth (mean CFUs) of *P. atrosepticum* SCRI1043 Km (*Pba* K) and Rif (*Pba* R) resistant strains over a 48 h period at 28°C when inoculated into MM in isolation or in media co-inoculated with *P. c. subsp. brasiliensis* ICMP 19477 tagged with either a Km (*Pbr* K) or Rif (*Pbr* R) resistance determinant (at a 1:1 ratio). **B**: A graph showing the growth (mean CFUs) of *Pbr* K or *Pbr* R when cultured in isolation in potato tubers or in co-inoculations with *Pba* K or *Pba* R (at a 1:1 ratio). For each graph, the mean CFUs were calculated for each treatment from triplicate assays (solid line), error bars represent the standard deviation.

3.2.7 Identifying the antimicrobial molecule produced by *Pectobacterium carotovorum* subsp. *brasiliensis* ICMP 19477 via mass spectrometry techniques

Isolation of the antimicrobial molecule produced by *P. c.* subsp. *brasiliensis* ICMP 19477, from agar plates, was attempted using MS and LC-MS (David Greenwood, Plant and Food Research Auckland). The samples were prepared as described in Section 2.1.33. The extraction method used was chosen to extract multiple molecules as the exact antimicrobial was unknown.

Analysis of the MS and LC-MS data identified only one protein present in the agar extracted from a zone of inhibition produced by *P. c.* subsp. *brasiliensis* ICMP 19477, that was not present in MMA or an MMA plate seeded with a *P. atrosepticum* SCRI1043 lawn (data not shown). This protein was identified to be a 50S ribosomal protein (ID = 90%). No putative antimicrobial molecule was identified.

The failure to identify a putative antimicrobial using MS and LC-MS led to an analysis of the antimicrobial encoding genes identified within the *P. c.* subsp. *brasiliensis* ICMP 19477 genome, as an alternative method to identify potential targets responsible for the antagonism of *P. atrosepticum* SCRI1043.

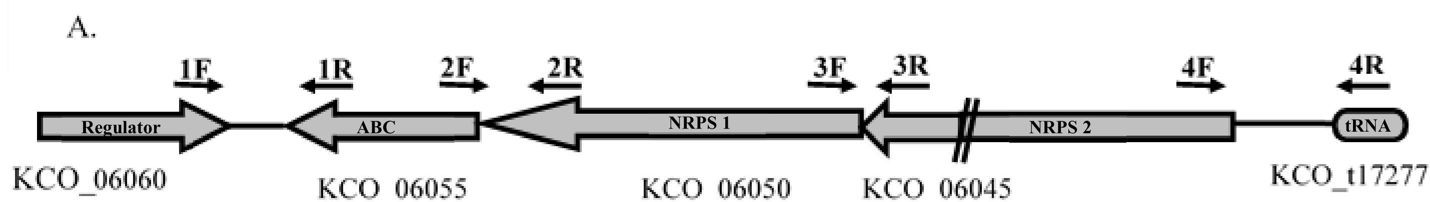
3.2.8 Horizontally acquired island PbN1-GI20 harbours a novel NRP biosynthetic gene cluster

Comparative analyses of the *P. c. subsp. brasiliensis* ICMP 19477 genome with that of other SREs revealed that horizontally acquired island (HAI) PbN1-GI20 (Figure 3.9) harbours a biosynthetic cluster predicted to encode a putative NRP (Preetinanda Panda, 2014). This HAI is similar to HA16 (H-value: 0.4), an HAI in the genome of *P. atrosepticum* SCRI1043 (Bell et al., 2004) as well as regions in the genomes of *P. c.* subsp. *carotovorum* WPP14 (H-value: 0.4) and *P. c.* subsp. *brasiliensis* PBR1692 (H-value: 0.4) (Table 1.2). H-values refer to the similarity of ORFs. An H-value of >0.8 indicates that the ORF in *P. c.* subsp. *brasiliensis* ICMP19477 is indistinguishable homologue in the genome to which it is being compared. However, NRP biosynthetic clusters are only present in *P. c.* subsp. *brasiliensis* ICMP 19477 and HAI6 (Preetinanda Panda, 2014).

PbN1-GI20 possesses 15 CDSs, including those predicted to encode two NRPSs and an ABC transporter thought to be involved in the synthesis and transport of the NRP (Figure 3.9). The island also has genes encoding a β-lactamase, as well as a transcriptional regulator.

Figure 3.9. Gene structure of a horizontally acquired island, PbN1-GI20, of *P. c.* subsp. *brasiliensis* ICMP 19477 that contains the putative NRPS genes. The NRPS cluster includes KCO_06055 (ABC transporter), KCO_06050, (NRPS 1) and KCO_06045/KCO_17262 (NRPS 2). The no entry sign represents a contig break within the NRPS2 gene.

Bioinformatics predicted that the CDSs encoding the two NRPS genes and the ABC transporter were transcribed as an operon (data not shown). To test this, a series of PCR primers were designed to amplify products by rt-PCR that bridged the three genes, plus those either side (Figure 3.13 A; Table 2.3). Amplicons of 539 bp and 502 bp were generated using primers 2F and 2R and 3F and 3R using cDNA from *P. c.* subsp. *brasiliensis* ICMP 19477 whereas rt-PCR with primers 1F and 1R and 4F and 4R failed to produce products (Figures 4.5 B). In contrast, amplicons of the correct size were generated by PCR using all primer pairs when DNA was used as a template. These data indicated that the ABC transporter gene and the two NRPS genes were transcribed as a single mRNA, indicative of being an operon. The failure to amplify products by rt-PCR with primers targeted to the regions spanning either side of these three genes showed that the adjacent genes are not part of the operon. The operon structure of the genes within the NRPS cluster supports the prediction that they synthesise a single NRP, which is transported out of the bacterial cell via the ABC transporter gene product.



1F- β-lac/ABC F

1R- β-lac/ABC R

2F- Pbr ABC/NRPS F

2R- Pbr ABC/NRPS R

3F-NRPS/NRPSsmall F

3R-NRPS/NRPSsmall R

4F- Pbr NRPS/tRNA F

4F- Pbr NRPS/tRNA F

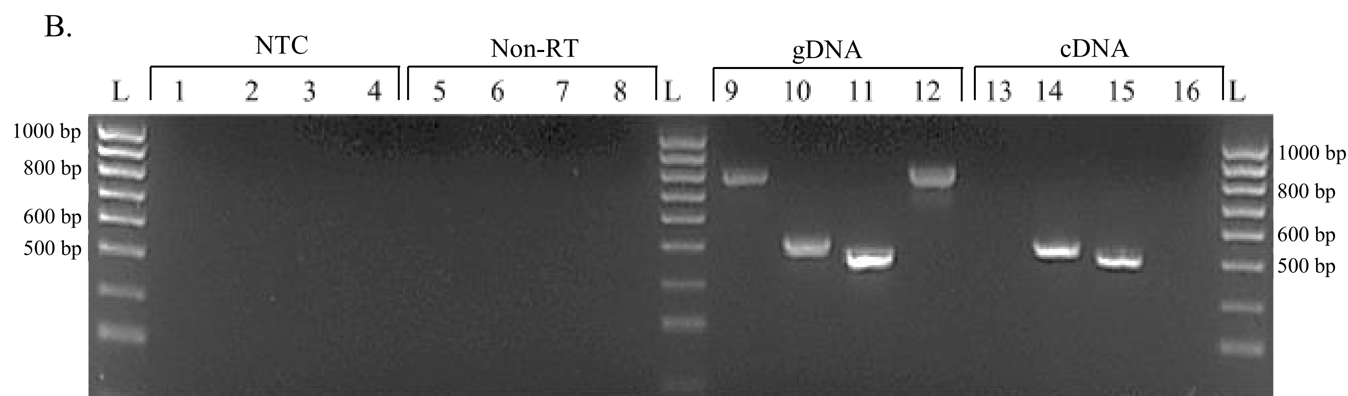


Figure 3.10. The operon structure of the NRP biosynthetic cluster in *P. c.* subsp. *brasiliensis* ICMP 19477.

A: Genetic structure of the NRP biosynthetic cluster and the primer sites for amplification of fragments to define its operon structure. Each gene in the NRPS cluster is represented by a grey arrow. The locus tag/gene name is provided below the grey arrow. Primer binding sites are indicated by black arrows. Primer names are defined as 1F to 4R. Full primer names are however, given in the legend. **B**: PCR amplicons obtained from RT-PCR assays define the operon structure for the NRPS cluster. L, DNA ladder; Lane 1, primer pair 1 Non-template control (NTC); Lane 2, primer pair 2 NTC; Lane 3, primer pair 3 NTC; Lane 4, primer pair 4 NTC; Lanes 5-8, non-RT controls; L, DNA ladder; Lane 9, β-lactamase/ABC DNA; Lane 10, ABC/NRPS DNA; Lane 11, NRPS/NRPS small DNA; Lane 12, NRPS/tRNA DNA, Lane 13, β-lactamase/ABC cDNA; Lane 14, ABC/NRPS cDNA; Lane 15, NRPS/NRPS small cDNA; Lane 16, NRPS/tRNA cDNA; L, DNA ladder.

The region within *P. c.* subsp. *brasiliensis* PBR1692 did not encode the NRPS biosynthetic cluster, although genes outside of the NRPS region are conserved. For example, the first three genes within PbN1-GI20, KCO_06110, KCO_06110 and KCO_06105, are also present in the same region of *P. c.* subsp. *brasiliensis* PBR1692 (ID = 99.7%, 97.3% and 99.7% respectively). Therefore, the NRP product is not produced by *P. c.* subsp. *brasiliensis* PBR1692. The NRPS island is also not encoded by *P. carotovorum* subsp. *carotovorum* PC1.

A nucleotide alignment of HAI6 and PbN1-GI20 (Figure 3.10) also revealed that *P. atrosepticum* SCRI1043 harboured two NRPS genes and an ABC transporter gene with similarity to those in PbN1-GI20, although the remainder of HAI6 appeared to be quite distinct. In particular, the NRPS1 genes had 88.6% pairwise identity, the NRPS2 genes 88.9% and the ABC transporter genes 90%. The remaining genes within the islands were variable, including the putative transcriptional regulators (ECA1482 and KCO_06065) initially predicted to regulate the NRPS genes due to being up-stream of the conserved NRPS clusters. In fact, a BLASTn comparison of the two genes did not identify any pairwise similarity between the two species.

It is worth noting that the nucleotide sequence for the *P. c.* subsp. *brasiliensis* ICMP 19477 NRPS cluster contained a contig break between KCO_06045 and KCO_17262 (Figure 3.9), which was unable to be closed due to the highly repetitive nature of the DNA (Preetinanda Panda, 2014). Owing to the conservation in the remaining NRPS DNA sequences in HAI6 and PbN1-G120, however, it was predicted that the gene organisation and NRPS structure was the same for both species and that KCO_06045 and KCO_17262 in *P. c.* subsp. *brasiliensis* ICMP 19477 should be considered a single CDS encoding the second NRPS gene in *P. c.* subsp. *brasiliensis* ICMP 19477. This suggests that the NRPS cluster may be a separate mobile genetic element, which is present in *P. atrosepticum* SCRI1043 but not *P. c.* subsp. *brasiliensis* PBR1692 or *P. c.* subsp. *carotovorum* PC1.

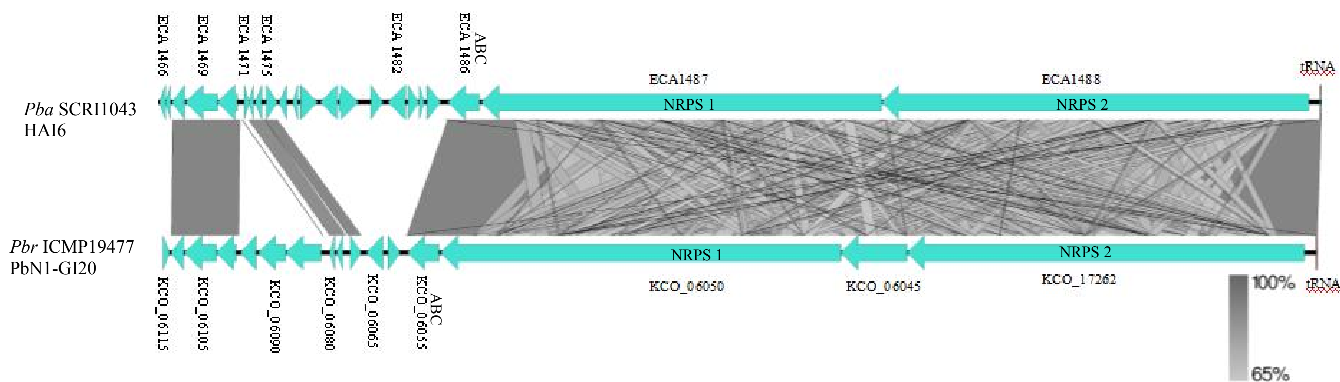


Figure 3.11. The NRPS and ABC transporter genes are conserved between *P. atrosepticum* (*Pba*) SCRI1043 HAI6 (top) and *P. c.* subsp. *brasiliensis* (*Pbr*) ICMP 19477 PbN1-GI20 (bottom).
The NRPS is conserved between the two bacteria. Due to the highly repetitive sequences present in the NRPS genes, the BLAST comparisons are not well represented for those genes. The region outside of the NRPS gene cluster is different in ICMP 19477 compared to SCRI1043. This suggests that these genes are harboured on distinct islands in these SREs.

Despite the contig gap discussed above, the domain organisation of the two *nrps* genes (KCO_06050-KCO_06045/17262) in *P. c.* subsp. *brasiliensis* ICMP 19477 was predicted using several software packages (Table 2.1) (data not shown), to establish the likely structure of the NRP (Table 3.1). Combining the results from these packages, the *nrps* genes were predicted to encode 13 A domains (Figure 3.11), which suggested that the NRP was comprised of 13 amino acids. The domain organisation of the genes also suggested that the NRP has a cyclic structure as the two thioesterase domains present at the C terminus, plus the lack of a typical initiation module (one lacking a condensation domain), are typical features of NRPSs encoding cyclic products (Gross et al., 2007).

The function of the NRP was subsequently predicted by comparing the A domains of the putative NRPSs with those of NRPs with known function using NRPSpredictor2 and antiSMASH (Medema et al., 2011; Rottig et al., 2011) (Table 2.1) These software showed that the A domains of the *P. c.* subsp. *brasiliensis* ICMP 19477 NRPSs had similarity to the A-domain organisation of the syringomycin encoding NRPS (E = 0.028) as well as the NRPSs for antibiotics such as fengycin (E = 0.006) and siderophores (i.e. pyoverdin E = 0.023), suggesting that the NRP might function as a plant toxin or as an antimicrobial.

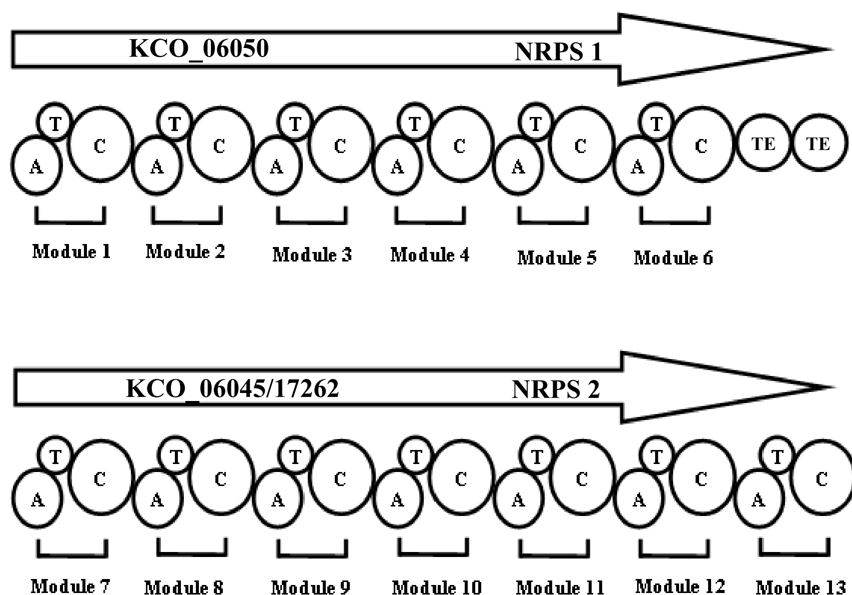


Figure 3.12. The domain and module organisation of the NRPS genes in *P. c.* subsp. *brasiliensis* ICMP 19477 (KCO_06050 and KCO_06045/1726).

Each module has a condensation (C), adenylation (A) and thiolation, (T) domain. Two thioesterase (TE) domains were present following the 6th module. The TE domain catalyses the hydrolysis of an ester bond to form an alcohol and acid group. The TE domain can also initiate the release of water to force product release via intramolecular cyclisation.

The amino acid sequence and secondary structure of the NRP encoded by ICMP 19477 was predicted by analysis of the A domains using antiSMASH (Medema et al., 2011) to predict the mass range for the product (for use in the mass spectroscopy analysis described in Section 3.3.8). It was also used to predict if unusual amino acids were likely to be included in the final product, as these would also need to be considered in MS and LC-MS analysis. The antiSMASH program combines three methods of A domain substrate specificity prediction to determine the consensus NRP product sequence. The three methods used are: the Stachelhaus specificity code (Stachelhaus et al., 1999), the Minowa model (Minowa et al., 2007) and the NRPSpredictor2 support vector machine (Rausch et al., 2005; Rottig et al., 2011). The information from the three tools predicted that the consensus sequence of the putative NRP was comprised of 13 amino acids (Figures 3.12) with an approximate mass of 1495 Da (based on using an average amino acid mass of 115 Da in ribosomal peptides (Promega 2010)). The predicted mass of the NRP was also calculated using the average mass of monomers incorporated in to the NRPs listed in the NORINE database (Caboche et al., 2008). The average monomer has a mass of 118.75 Da

(Kersten et al., 2011), therefore the predicted mass of the NRP was calculated at 1543.75 Da. A number of amino acids did not fit the consensus sequence, however, with only eight of the predicted 13 recognized as standard amino acids. The amino acid sequences encoded by the NRPs in *P. atrosepticum* SCRI1043 and *P. c.* subsp. *brasiliensis* ICMP 19477 were subsequently compared to ascertain whether the differences in nucleotide similarities corresponded to the production of NRPs with different amino acid sequences. Of the predicted amino acids, only four of the eight that were identified are similar between the *P. atrosepticum* SCRI1043 and *P. c.* subsp. *brasiliensis* ICMP 19477 product. This difference may be sufficient to confer a different function to the products of the genes from the two different species.

Surprisingly, given previous analysis showed that a cyclic product was generated, antiSMASH predicted that a non-cyclic product was generated by the NRPSs (Figure 3.13).

The analysis of the NRPS genes was conducted using multiple bioinformatics tools. Such tools are based on statistical methods and therefore the results produced have limitations. The identification of secondary metabolite encoding genes such as NRPSs are based on profile hidden Markov models based on multiple sequence alignments using defined NRPS motifs. This model is accurate to predict the gene clusters, as the models are validated against known sequence data in order to assess their ability to identify clusters in new sequence data. The antiSMASH model for example was able to identify 97% of secondary metabolite gene clusters from the literature and annotated 96.7% of those exactly (Boddy, 2014). However, the prediction of the encoded substrates by the A domains present in an NRPS are less precise and shows variation between different methods (Table 3.1). Therefore, the predicted substrates that form the NRP and the resulting predicted structure should be used only as a rough prediction of the actual structure.

Table 3.1. Different methods used to predict NRPS A domain specificity show variation in their predictions.

The table summarises the three methods used by antiSMASH (Medema et al., 2011), for the prediction of the NRP sequence encoded by *P. c.* subsp. *brasiliensis* ICMP 19477. The consensus sequence based on the results of the three methods is also shown. The amino acids predicted to form the NRP sequence is based on the order of predicted amino acids encoded by each of the 13 A domains predicted to be present in the NRPS genes of both species. Standard amino abbreviations are used (The DDBJ/ENA/ycosubt feature table definition, Version 10.5, 2015). bht- β-hydroxy-tyrosine, iva-isovaleric acid.

A domain	NRPS2Predictor SVM	Stachelhaus code	Minowa	Consensus
1	thr	thr	thr	thr
2	tyr,bht	tyr	phe	-
3	ser	ser	ser	ser
4	hydrophobic aromatic	trp	trp	trp
5	val, leu, ile, abu, iva	his	ala	-
6	ser	ser	ser	ser
7	gly	gly	gly	gly
8	hydrophilic	glu	orn	-
9	asn	asn	asn	asn
10	ser	ser	ser	ser
11	-	tyr	tyr	tyr
12	asp, asn, glu, gln, aad	glu	arg	-
13	asp, asn, glu, gln, aad	glu	orn	-

The limitations of the predicted encoded substrates are due to many factors relating to the statistical models used to form the predictions. Firstly, the programs used rely on available sequence data which may be limited. This is required in order to build and train the prediction models. This will improve as more data becomes available, however a novel system that does not follow the observed patterns may be missed or mis-predicted. Secondly, the programs utilise the colinearity rule to predict the final amino acid sequence of the NRP. This means that the order of the A domains within the NRPS are predicted to be the same sequence as the final product. This is usually the case for NRPs, however there are exceptions, for example those synthesised by the non-linear or iterative system (Mootz et al., 2002).

The ability to predict the amino acid encoded by an A domain is based on the 'specificity codes' identified (Stachelhaus et al., 1999). These codes are experimentally defined and are available for many proteogenic amino acids. However, the specificity codes for some are undefined, for example

lysine and others are very variable such as for alanine (Table 3.2, (Stachelhaus et al., 1999). Furthermore, very few specificity codes have been identified for unusual amino acids, such as homoserine, which are sometimes present in NRPs. Adenylation domains that encode aromatic amino acids are also hard to predict due to the promiscuity of these sequences. Therefore, there are limitations to the prediction methods used to predict an NRP product from an NRPS sequence.

The final NRP structure is difficult to predict as it is defined not only by the A domain sequence but also the interactions between the amino acids. This is taken into account using the Minowa method, which is included in the range of programs used to predict the structure by antiSMASH. The Minowa method is considered the most robust method for structural prediction of NRPs (Boddy, 2014). Statistically, the structural prediction is not considered accurate as it involves combining individual predictions of the amino acid interactions as well as the sequence predictions. Therefore, the structural predictions should only be considered to provide the number of monomers incorporated in to the NRP in order to calculate a mass range for MS analysis of the NRP. Furthermore, it may be used to identify the potential inclusion of unusual amino acids within the product.

Table 3.2. The consensus specificity sequences for the recognition of amino acid substrates by A domains.

As with the ribosomal codon code, the specificity sequences are degenerate, which is indicated by the number in brackets after the amino acid encoded. The variable positions within a sequence are shown in red and the overall similarity of the signature sequences is shown. Standard amino acid abbreviations are used, plus Dab, 2,3-diamino butyric acid; Dhb, 2,3-dihydroxy benzoic acid; Sal, salicylate; Phg, L-phenylglycine; hPhg, 4-hydroxy-L-phenylglycine; Dht, dehydrothreonine. Adapted from (Stachelhaus et al., 1999).

	Position in active site										
Substrate	235	236	239	278	299	301	322	330	331	517	**Similarity**
Aad	E	P	R	N	I	V	E	F	V	K	94%
Ala	D	L	L	F	G	I	A	V	L	K	55%
Asn	D	L	T	K	L	G	E	V	G	K	90%
Asp	D	L	T	K	V	G	H	I	G	K	100%
Cys (1)	D	H	E	S	D	V	G	I	T	K	96%
Cys (2)	D	L	Y	N	L	S	L	I	W	K	88%
Dab	D	L	E	H	N	T	T	V	S	K	100%
Dhb/Sal	P	L	P	A	Q	G	V	V	N	K	83%
Gln	D	A	Q	D	L	G	V	V	D	K	100%
Glu (1)	D	A	W	H	F	G	G	V	D	K	95%
Glu (2)	D	A	K	D	L	G	V	V	D	K	95%
Ile (1)	D	G	F	F	L	G	V	V	Y	K	92%
Ile (2)	D	A	F	F	Y	G	I	T	F	K	100%
Leu (1)	D	A	W	F	L	G	N	V	V	K	99%
Leu (2)	D	A	W	L	Y	G	A	V	M	K	100%
Leu (3)	D	G	A	Y	T	G	E	V	V	K	100%
Leu (4)	D	A	F	M	L	G	M	V	F	K	97%
Orn (1)	D	M	E	N	L	G	L	I	N	K	100%
Orn (2)	D	V	G	E	I	G	S	I	D	K	98%
Phe	D	A	W	T	I	A	A	V	C	K	88%
Phg/hPhg	D	I	F	L	L	G	L	L	C	K	80%
Pro	D	V	Q	L	I	A	H	V	V	k	87%
Ser	D	V	W	H	L	S	L	I	D	K	90%
Thr/Dht	D	F	W	N	I	G	M	V	H	K	91%
Tyr (1)	D	G	T	I	T	A	E	V	A	K	100%
Tyr (2)	D	A	L	V	T	G	A	V	V	K	80%

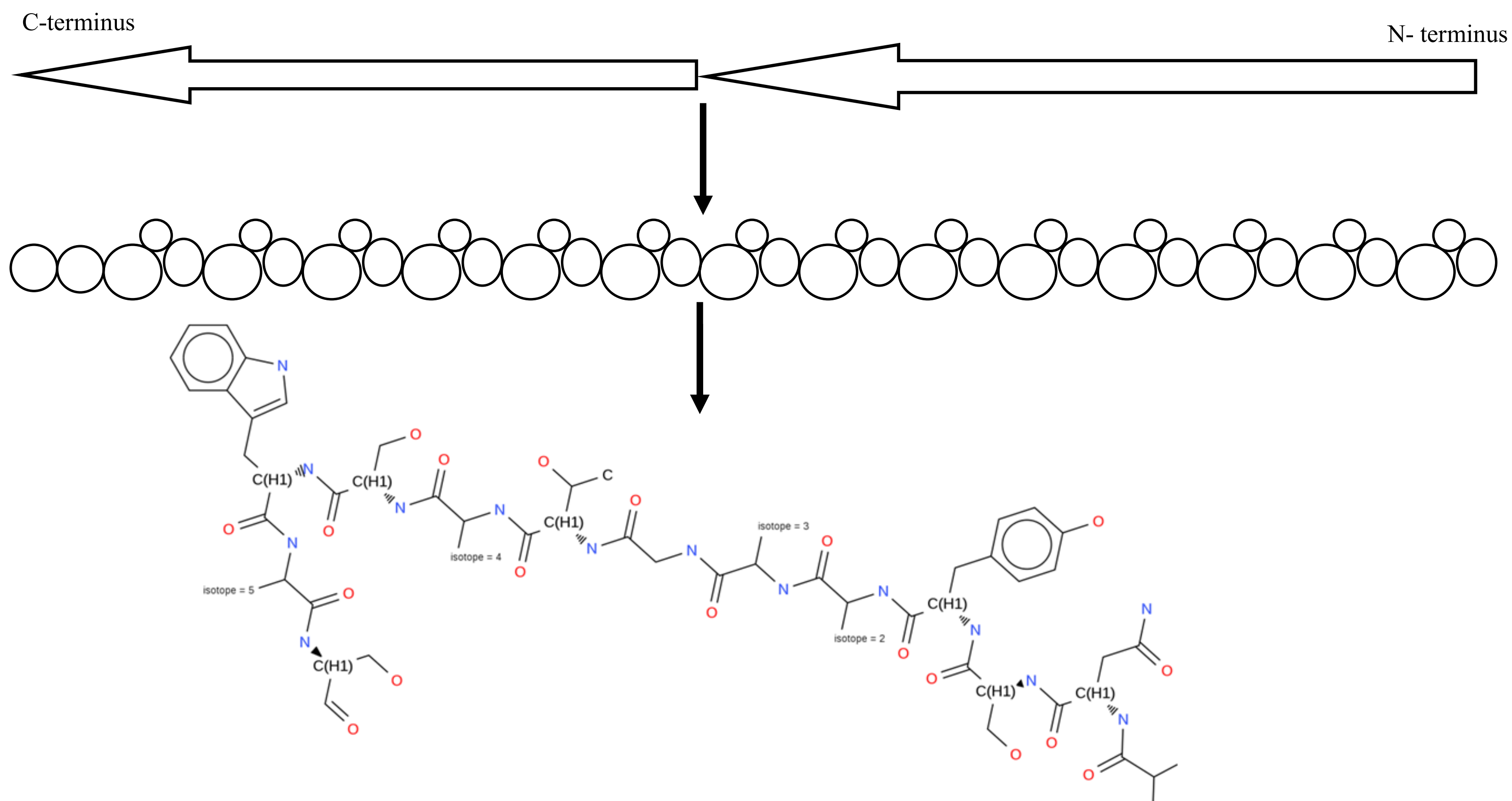


Figure 3.13. The predicted NRP product in *P. c.* subsp. *brasiliensis* ICMP 19477.

The consensus sequence for the NRP primary structure, was predicted using the prediction tools PKS/NRPS analysis webserver (Bachman and Ravel, 2009), NRPS-PKS (Ansari et al., 2004) and NRPSpredictor2 (Rottig et al., 2011). The secondary structure was predicted using antiSMASH (Medema et al., 2011). The primary structure is written in standard amino acid three letter abbreviations, nrp refers to an amino acid that was unable to be predicted as a consensus between the different methods could not be established.

3.2.9 PbN1-GI25 harbours a putative carotovoricin-like biosynthetic cluster

PbN1-GI25 was shown to have similarity to regions in *P. c.* subsp. *brasiliensis* PBR1692 (H-value: 1.0), in *P. atrosepticum* SCRI1043 (H-value: 0.3), *P. c.* subsp. *carotovorum* ICMP 5702 (H-value: 0.8) and PCI (H-value: 0.8). PbN1-GI25 harbours 21 CDSs, which are predicted to encode the bacteriocin carotovoricin (Ctv) (Figure 3.14).

The CDSs predicted to be involved in Ctv production include KCO_16172, which likely encodes a lytic transglycosylase with similarity to *P. c.* subsp. *carotovorum* Pcc21 (ID = 100%, E = 1e-146) predicted by BLASTx analysis. This enzyme acts on the peptidoglycan components of bacterial cell walls, in a similar mechanism to lysozyme. KCO_16142 has greatest nucleotide similarity to a tail protein from *P. c.* subsp. *carotovorum* (ID = 99%, E = 0). KCO_16137 and KCO_16122 were also predicted to encode phage tail proteins from *P. c.* subsp. *carotovorum* (ID = 100%, E = 1e-125 and ID = 99%, E = 0). The KCO_16102 gene product was identified as having greatest similarity to a phage base plate assembly protein V (E = 4.95e-20). Finally, KCO_16077 showed greatest similarity to a tail protein encoded by *P. c.* subsp. *brasiliensis* PBR1692 (ID = 90%, E = 0).

The Ctv biosynthetic cluster of *P. c.* subsp. *brasiliensis* ICMP 19477 was compared to the *P. c.* subsp. *brasiliensis* type strain PBR1692. The 21 Ctv CDSs of *P. c.* subsp. *brasiliensis* PBR1692 (H-value: 1.0), in *P. atrosepticum* SCRI1043 (H-value: 0.3), *P. c.* subsp. *carotovorum* ICMP 5702 showed 97% nucleotide similarity to the Ctv cluster of *P. c.* subsp. *brasiliensis* ICMP 19477.

Other phage-related genes were identified in this island, which are present within the Ctv loci of other SREs (Itoh et al., 1978; Itoh et al., 1980). The CDS KCO_16142 encodes a protein that has similarity to a phage tail sheath protein (ID = 99%, E = 0), KCO_16137 encodes a protein with similarity to a phage tail tube family protein (E = 1.01e-56), KCO_16132 encodes a protein similar to a ferrodoxin of *P. c.* subsp. *carotovorum* (ID = 81%, E = 2e-52) and KCO_16122 encodes a protein similar to phage tail tape measure protein (ID = 99%, E = 0).

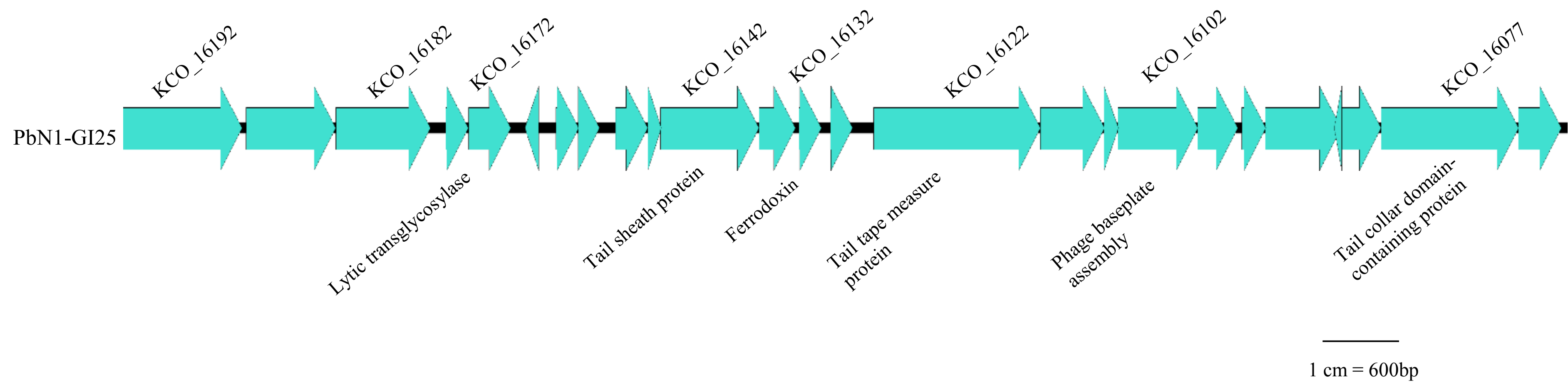


Figure 3.14. A schematic diagram of the structure and organisation of PbN1-G125 in *P. c.* subsp. *brasiliensis* ICMP 19477.

Blue arrows represent the predicted CDSs. The orientation of the CDS is shown by the direction of the arrow. CDS identifiers or putative functions of the encoded proteins are provided for each CDS.

The promoter regions (P_0-P_3) for these units have been determined (Yamada et al., 2006) (Figure 3.15 B). BLASTn analysis of PbN1-GI25 identified regions similar to P_0, P_1, P_2 and P_3 (Figure 3.15 A). The P_0 region was found to be similar to the promoter for the gene KCO_16177 (97%). The region similar to the *E. coli* -35 region was mapped to a region 51 bp from the start codon of KCO_16177 (1823701-1823705). P_1 corresponded to the promoter region of KCO_16152 (96%), the -35 region was 87 bp from the gene start codon (1826125-1826130). The P_2 region was found to be located upstream of KCO_16127 (97%), the -35 region was 48 bp from the gene start codon (1829275-1829280), while P_3 was located within the gene KCO_16117 (97%). Regulators of pectolycin and carotovoricin production (Liu et al., 1994) were also detected in *P. c.* subsp. *brasiliensis* ICMP 19477. For example, KCO_14692 encodes a protein with similarity to RdgB, a Mor family transcriptional regulator (ID = 97%, E = 2e-75). The product of *rdgB* binds directly to the P_0-P_3 motifs in the ctv biosynthetic cluster (Yamada et al., 2008). KCO_14687 shows similarity to the RdgA transcriptional regulator (ID = 99%, E = 1e-175).

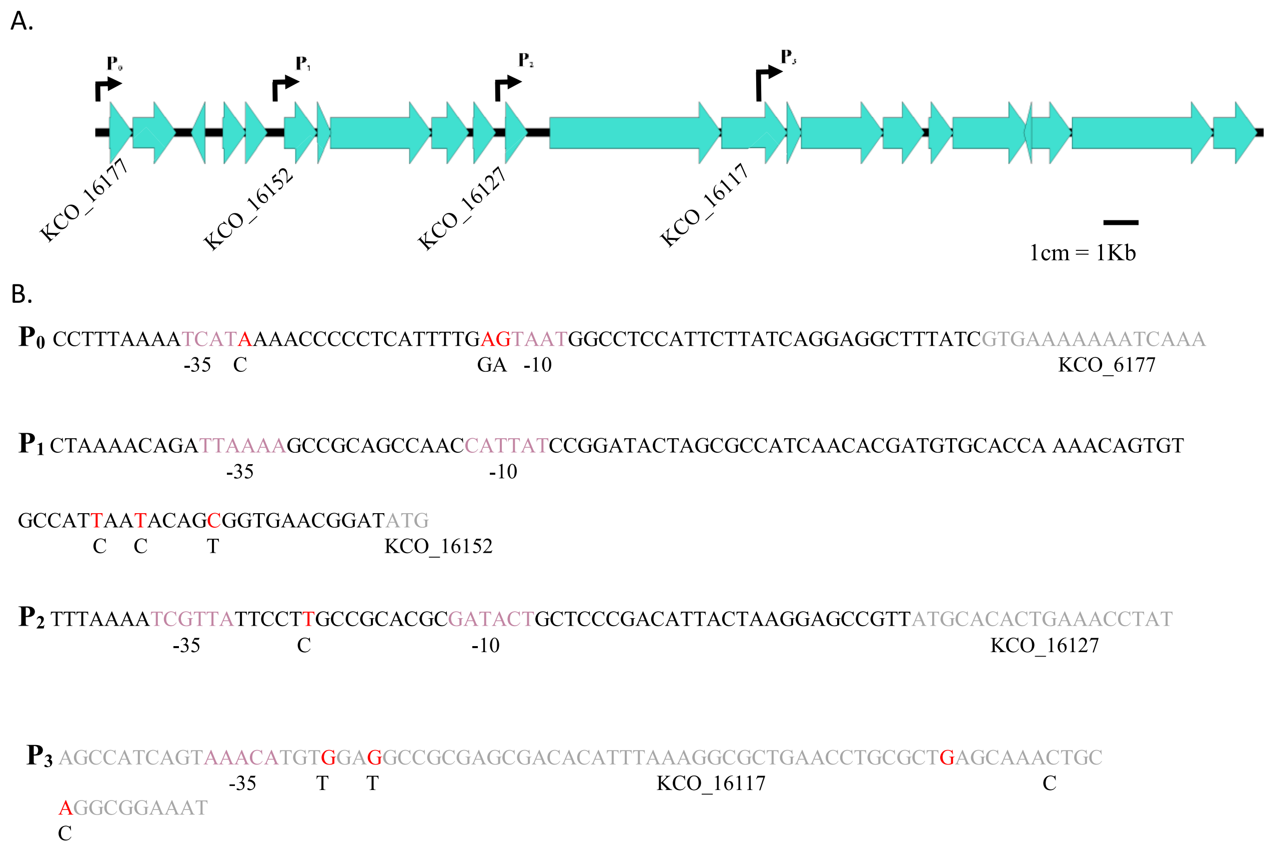


Figure 3.15. The predicted structure of the carotovoricin biosynthetic cluster in *P. c.* subsp. *brasiliensis* ICMP 19477.

A: A schematic diagram of the putative carotovoricin gene cluster with CDSs represented by blue arrows and the CDS identifiers written below for key genes. The locations of the four promoter regions are designated as black arrows. **B**: A comparison of the nucleotide sequences of the promoter regions in *P. c.* subsp. *brasiliensis* ICMP 19477 and *P. c.* subsp. *carotovorum* Er, (Yamada et al., 2006). The sequence is provided for the regions in *P. c.* subsp. *brasiliensis* ICMP 19477, with the red letters representing where the *P. c.* subsp. *brasiliensis* ICMP 19477 sequence differed from the *P. c.* subsp. *carotovorum* Er sequence. The *P. c.* subsp. *carotovorum* Er nucleotide sequence (where different) is given in black underneath. The -35 and -10 promoter regions are shaded in purple while the CDS sequences are given in grey. The downstream *P. c.* subsp. *brasiliensis* ICMP 19477 CDS identifier is given below the nucleotide sequence where appropriate.

Although similar regions were identified in other *Pectobacterium*, the CDSs involved in carotovoricin production in *P. c.* subsp. *brasiliensis* ICMP 19477 were also identified in *P. c.* subsp. *carotovorum* ICMP 5702 and PC1. The CDSs in these strains were found to be highly similar, but distinct. For example, *P. c.* subsp. *carotovorum* ICMP 5702 showed on average 91% nucleotide identity to carotovoricin biosynthesis proteins encoded on PbN1-G125 and 90% nucleotide identity in *P. c.* subsp. *carotovorum* PC1. Genes similar to KCO_16177 and KCO_16157 were identified in *P. atrosepticum* SCRI1043 (ID = 89%), the other 16 Ctv CDSs were not present. Carotovoricins have been identified in many *P. c.* subsp. *carotovorum* strains, the structure of the antimicrobial molecule varying between strains (Itoh et al., 1980).

3.2.10 PbN1-GI31 contains a putative phenazine biosynthesis gene cluster

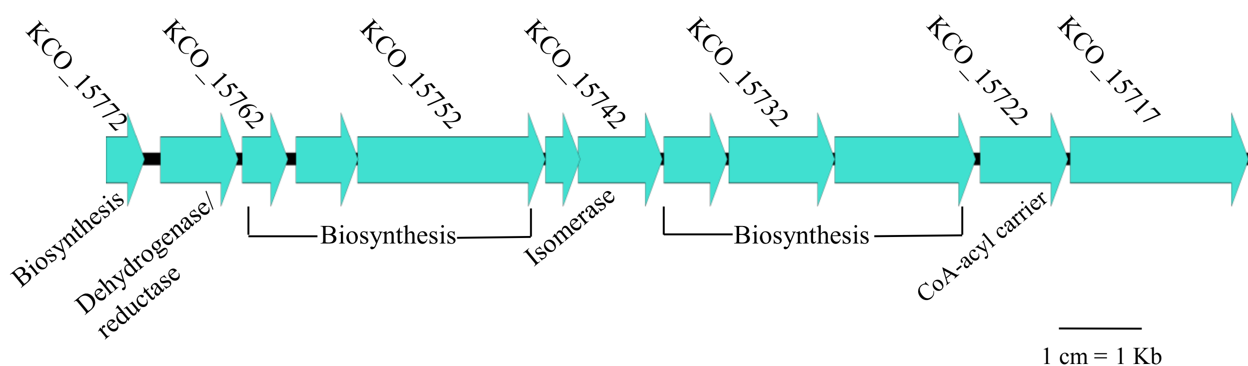


Figure 3.16. A schematic diagram of the phenazine biosynthesis cluster PbN1-GI31. The blue arrows represent genes.

The *P. c.* subsp. *brasiliensis* ICMP 19477 gene numbers are given above and the putative gene functions are written below.

PbN1-GI31 has similarity to regions in all the *Pectobacterium* strains compared in Table 1.2 (H-value: of 0.7-0.8). PbN1-GI31 contains twelve genes that form a putative phenazine biosynthesis cluster (Figure 3.16). Of the twelve genes, seven were found to encode proteins with greatest similarity to phenazine antibiotic synthesis proteins by BLASTx analysis. KCO_15772, the start of PbN1-GI31 is the first biosynthesis gene, which encodes a protein similar to other phenazine biosynthesis genes in

Pectobacterium (ID = 100%, E = 6e-87). KCO_15762, KCO_15757, KCO_15752, KCO_15737, KCO_15732, KCO_15727 also showed greatest similarity to *Pectobacterium* phenazine biosynthesis genes (E = 8e-110, E = 5e-152, E = 0, E = 2e-155, E = 0 and E = 0). KCO_15767, the second gene in the HAI encodes a short chain dehydrogenase/reductase (ID = 99%, E=0). These enzymes catalyse NAD(P)(H) dependent oxidation/reduction reactions. KCO_15742 is predicted to be similar to a 2,3-dihydro-3-hydroxyanthranilate isomerase of *P. c.* subsp. *carotovorum* Pcc21(ID = 100%, E = 0). This enzyme catalyses an isomerase reaction associated with phenazine synthesis. KCO_15722 showed greatest similarity to a CoA-acyl carrier protein (ID = 100%, E = 0). Two genes, KCO_15745 and KCO_15717, were predicted to be hypothetical proteins (ID = 100%, E = 4e-77 and ID = 100%, E = 0).

3.2.11 PbN1-GI38 encodes a putative bacteriocin biosynthetic cluster

PbN1-GI38 harbours a cluster of genes predicted to encode a bacteriocin (Figure 3.17). Similar islands were identified in all strains (Table 1.2) with H-values of between 0.8 and 0.4. Further examination of these regions, however, showed that only *P. c.* subsp. *brasiliensis* PBR1692 had the two CDSs predicted to be involved in production of the bacteriocin-like molecule. Therefore, the bacteriocin cluster was also present in *P. c.* subsp. *brasiliensis* PBR1692 and ICMP 19477, but absent from other SRE genomes (Preetinanda Panda, 2014).

Other genes within PbNI-GI38 were found to be associated with amino-acid transport, metabolism and regulation.

The putative bacteriocin biosynthetic CDS identified in PbN1-G138 (KCO_12557) BLASTn analysis of KCO_12557 showed the gene had greatest similarity to a gene from *P. c.* subsp. *carotovorum* Pcc21 (ID = 99%, E = 0). The BLASTx analysis identified that the protein gene product showed greatest similarity to a ferrodoxin of *P. c.* subsp. *carotovorum* (ID = 100%, E = 0). The BLASTn of KCO_12552 showed greatest similarity to the carocin D gene of *P. c.* subsp. *carotovorum* Pcc21 (ID = 97 %, E = 6e-102), while BLASTx showed that the protein had similarity to the immunity proteins belonging to the colicin superfamily (and to the colicin D immunity protein of *P. c.* subsp. *carotovorum* in particular (ID = 100%, E = 6e-56). This suggested that KCO_12552 may encode the immunity protein for the bacteriocin produced by *P. c.* subsp. *brasiliensis* ICMP 19477. The BLASTn of KCO_12547 showed greatest similarity to carocin synthesis genes of *P. c.* subsp. *carotovorum* PCC21 (ID = 94%, E = 0), while BLASTx showed specific hits to pyocin superfamily (ID = 100%, E = 0) colicin D synthesis protein of *P. c.* subsp. *carotovorum* Pcc21 (ID = 93%, E = 0).

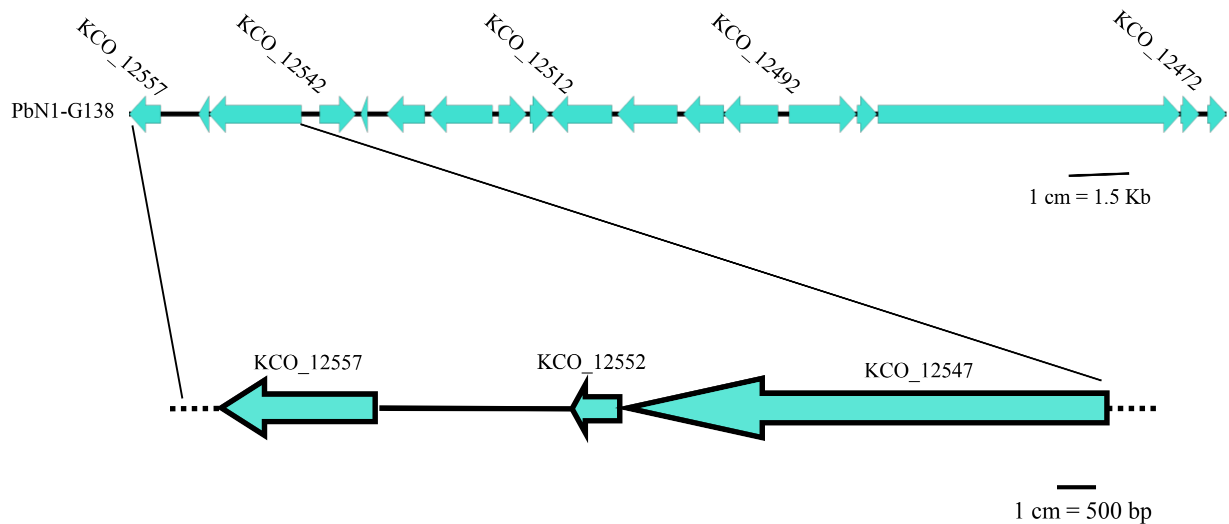


Figure 3.17. Horizontally acquired island PbN1-GI38 encodes a putative bacteriocin biosynthetic cluster.

The cluster is only present in the *P. c.* subsp. *brasiliensis* strains ICMP 19477 and PBR1692. Blue arrows represent CDSs in HAI PbN1-GI38. The three bacteriocin-related CDSs are enlarged (scale: 1cm = 500bp).

The order of the CDSs within the bacteriocin cluster was indicative of an '*E.* coli-type' CDI locus (transporter, toxin, immunity encoding genes). The predicted toxin encoding gene (KCO_12547) was therefore analysed to see if it contained any signatures suggesting that it was involved in CDI. The '*E.* coli-type' CDI toxin encoding genes usually have a variable C-terminal sequence, which is specific to the particular gene, which is separated from the more conserved N-terminal sequence (DUF638 domain, Pfam PF04829) after a valine-glutamate-asparagine-asparagine (VENN) motif (Aoki et al., 2010). There was no such VENN motif within the translated sequence of KCO_12547, suggesting that this gene does not encode a bacteriocin involved in CDI, but a diffusible antimicrobial molecule instead.

The predicted tertiary structures of the KCO_12552 and KCO_12547 gene CDS products were also predicted using I-TASSER (Zhang et al., 2008) and PredictProtein (Rost et al., 2004). The KCO_12552 product (Figure 3.18 A), was predicted to be made up of four alpha-helices similar to proteins that act in bacterial immunity (p = 1.51e-8). The KCO_12547 product (Figure 3.18 B) was predicted to be made up of 36% alpha-helices and 50% turns, a protein similarity was detected. The cellular localization of the gene products was also predicted using Raptor X (Källberg et al., 2012). The product of KCO_12547

was predicted to be secreted from the bacterial cell (p = 1.76e-9), whereas the products from KCO_12552 were predicted to be localized to the cytoplasm (p = 0.3).

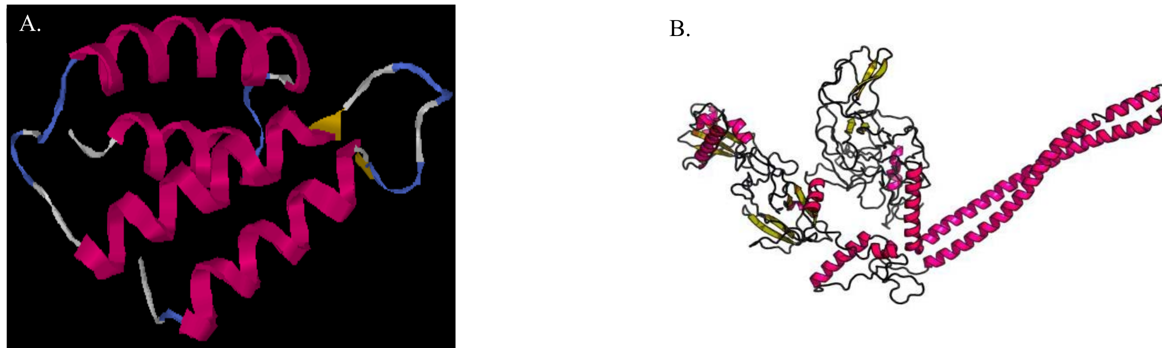


Figure 3.18. The predicted tertiary structure of the gene products encoded by the *P. c.* subsp. *brasiliensis* bacteriocin cluster.

A: KCO_12552 CDS product (p = 1.82e-10), which was similar to a bacterial immunity protein (p = 1.51e-8) (I-TASSER). **B**: The predicted tertiary structure of the KCO_12547 gene product (c-score=1.11) (PredictProtein). The C score refers to the confidence of the predicted structure. The value typically ranges from -5 to 2, with a value of 2 representing the most confident prediction.

Informatics predicted that the CDSs encoding the bacteriocin synthesis and immunity genes were transcribed as an operon (data not shown). To confirm this, a series of PCR primers were designed to amplify products by rt-PCR that bridged the two genes, plus those either side (Figure 3.19 A; Table 2.3). An amplicon of 553 bp was generated using primers 2F and 2R and using cDNA from *P. c.* subsp. *brasiliensis* ICMP 19477 whereas rt-PCR with primers 1F and 1R and 3F and 3R failed to produce products (Figures 4.19 B). In contrast, amplicons of the correct size were generated by PCR using all primer pairs when DNA was used as a template. These data indicated that the biosynthesis gene and the immunity protein are transcribed as a single mRNA, indicative of being an operon. The failure to amplify products by rt-PCR with primers targeted to the regions spanning either side of these three genes showed that the adjacent genes are not part of the operon. The operon structure of the genes within the NRPS cluster supports the prediction that they synthesise a single NRP, which is transported out of the bacterial cell via the ABC transporter gene product.

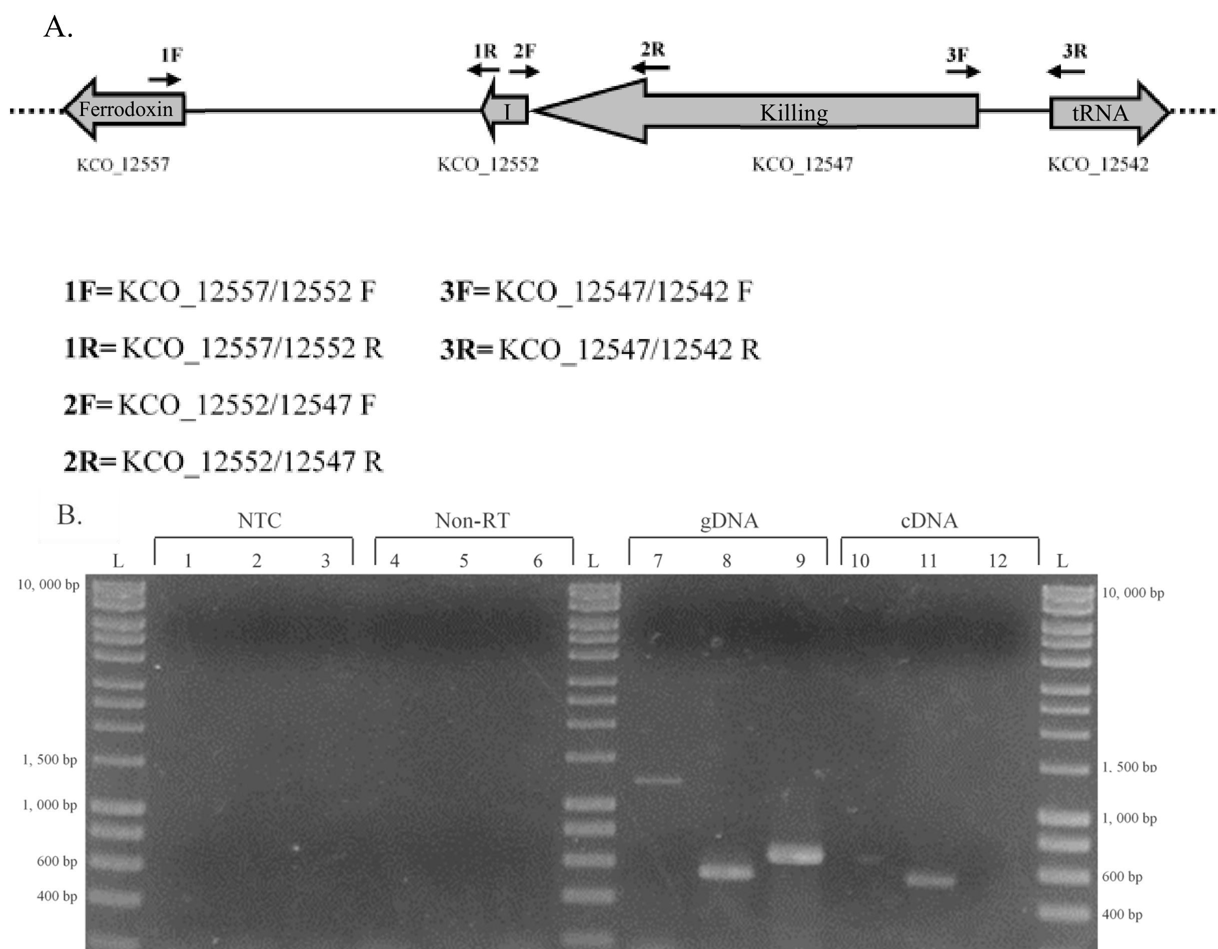


Figure 3.19. The operon structure of the bacteriocin biosynthetic cluster in *P. c.* subsp. *brasiliensis* ICMP 19477.

A: Genetic structure of the bacteriocin biosynthetic cluster and the primer sites for amplification of fragments to define its operon structure. Each gene in the bacteriocin cluster is represented by a grey arrow. The locus tag/gene name is provided below the grey arrow. Primer binding sites are indicated by black arrows. Primer names are defined as 1F to 3R. Full primer names are however, given in the legend. **B**: PCR amplicons obtained from RT-PCR assays define the operon structure for the bacteriocin cluster. L, DNA ladder; Lane 1, primer pair 1 Non-template control (NTC); Lane 2, primer pair 2 NTC; Lane 3, primer pair 3 NTC; Lanes 4-6, non-RT controls; L, DNA ladder; Lane 7, KCO_12557/KCO_12552 DNA; Lane 8, KCO_12552/12547; Lane 9, KCO_12547/12542; Lane 10, KCO_12557/KCO_12552 DNA, Lane 11, KCO_12552/12547 cDNA; Lane 12, KCO_12547/12542 cDNA; L, DNA ladder.

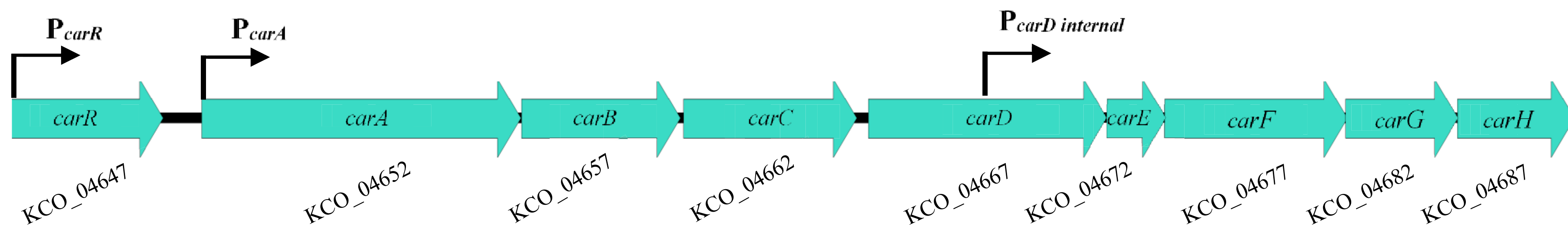
The two CDSs predicted to be involved in bacteriocin production in ICMP 19477 were compared to those in the *P. c.* subsp. *brasiliensis* type strain, PBR1692. KCO_12552 (immunity protein) had 100% identity to PcarbP_02845 and KCO_12547 (carocin synthesis) has 99.6% to PcarbP_02843 in *P. c.* subsp. *brasiliensis* PBR1692.

3.2.12 *Pectobacterium carotovorum* subsp. *brasiliensis* ICMP 19477 encodes a carbapenem biosynthetic cluster on PbN1-GI65

PbN1-GI65 harbours a putative carbapenem synthesis cluster (Figure 3.20 A). Carbapenem synthesis clusters were also detected in *P. c.* subsp. *brasiliensis* PBR1692 (H-value: 1.0) and *P. c.* subsp. *carotovorum* ICMP 5702 (H-value: 0.8), but not in the remaining SRE (Table 1.2).

In *P. c.* subsp. *brasiliensis* ICMP 19477, the carbapenem biosynthetic cluster contained 9 CDSs (Figure 3.20 A). BLASTn analysis showed that the first, KCO_04647, had greatest similarity to *carR* from *P. c.* subsp. *carotovorum* ATCC 39048 (ID = 98%). CarR acts as the regulator for the carbapenem synthesis genes in *P. c.* subsp. *carotovorum* (McGowan et al., 1995). It is a LuxR type transcriptional regulator, which responds to the QS signalling molecule N-(3-oxohexanoyl)-L-homoserine (OHHL) produced by the QS activator CarI. The next gene within the cluster (KCO_04652) had greatest similarity to *carA* from *P. c.* subsp. *carotovorum* ATCC 39048 (ID = 98%) while KCO_04657, KCO_04662, KCO_04667 and KCO_04672 had greatest similarity to the *carB*, *carC*, *carD* and *carE* cluster from *P. c.* subsp. *carotovorum* ATCC39048, respectively (ID = 98%). These genes are directly involved in synthesis of the carbapenem (McGowan et al., 2005). Genes KCO_04677 and KCO_04682 had greatest similarity to the *carF* and *carG* CDSs of *P. c*. subsp. *carotovorum* (ID = 89% and ID = 98%). These genes encode a carbapenem resistance mechanism (McGowan et al., 1997), which protects the producing cell from being killed by its own carbapenem. The ninth gene in the cluster (KCO_04687) had greatest similarity to the *carH* CDS of *P. c.* subsp. *carotovorum* ATCC 39048 (ID = 96%), a gene of unknown function (McGowan et al., 1997). BLASTx analysis confirmed these results (data not shown). Comparison of the putative *carA*-*carH* genes in *P. c.* subsp. *brasiliensis* ICMP 19477 with the type strain *P. c.* subsp. *brasiliensis* PBR1692, showed they had 98% nucleotide identity suggesting that the same carbapenem was produced by both pathogens. Comparison with the cluster from *P. c.* subsp. *carotovorum* ICMP 5702, however, demonstrated that the two gene clusters were distinct, with on average only 87.8% pairwise identity (E = 0). An analysis of the promoter regions within the cluster confirmed that those in *P. c.* subsp. *brasiliensis* ICMP 19477 were highly similar to those characterised in *P. c.* subsp.

carotovorum ATTn10 (McGowan et al., 2005) (Figure 3.20 B). The *E. coli* type -35 region of P_{carR} was mapped to 139 bp from the start codon of *carR* upstream of *carR* (4335827-4834200), the -35 region of P_{carA} was found to be 82 bp upstream from the start codon of *carA* (4699476-4706891). The promoter $P_{carDinternal}$ is located within the *carD* gene.



B.

$\mathbf{P}_{carR}$

ATGTTGACGGATTGTATCTTAATTGATAACTTTCGGCCTTGTTAAATTCTAGTGATTATATCTGGTGTAACTAAAAATAATTATATCTACTCTCTGGGAAAAA
GTTGATCTTTTAATCTTTGAGCAAAGTCGGTAAGAGAGGGTAATATG

$\mathbf{P}_{carA}$

~~AC~~GGTTAATATTTACCTTTGCCTTTTCTATGATACGTGACGAATTCAAACGTTGTTTTCTCTCATGTTGTTATTAAGGTAAGGGTTATTACTGTG

$\mathbf{P}_{carD\ internal}$

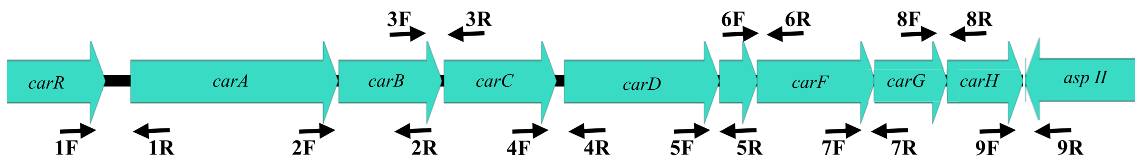
GTCGTGATTAAACCGTCGTCGCTGGGGAGTGTTGCCGTGTATGCCCGGCAAAGTGAAGGGCTCGCGCTGGATGAGGCATTCGCCAGCGCATGGTCACGCATC
GTTACGCG

Figure 3.20. Horizontally acquired island PbNI-GI65 encodes a putative carbapenem biosynthesis cluster.

A: A schematic diagram of the putative carbapenem biosynthetic cluster of *P. c.* subsp. *brasiliensis* ICMP 19477. The genes are represented as blue arrows. Gene names, based on similarities to the *P. c.* subsp. *carotovorum* genome, are given for the genes. The location of the promoter regions are designated as black arrows. **B**: The nucleotide sequences spanning the promoter regions of *carR, carA* and one internal to *carD*. The red letters represent where the *P. c.* subsp. *brasiliensis* ICMP 19477 nucleotide sequence differs from that of the *P. c.* subsp. *carotovorum* ATTn10 sequence. The regions similar to the *E. coli* -35 regions are shaded in purple and those similar to the -10 regions are shaded in blue. The grey shading represents the sequence that is found within a gene. Crossed out nucleotides are those not present in *P. c.* subsp. *brasiliensis* ICMP19477 (represents deletion event).

In *P. c.* subsp. *carotovorum, carA-H* form a transcriptional operon (McGowan et al., 1996). To determine if this is also the case in *P. c.* subsp. *brasiliensis* ICMP 19477, primers were designed to amplify products from cDNA that bridged the gaps between the CDSs, including those predicted to be outside the operon (Figure 3.21 A). As expected, PCR amplicons of 506 bp, 626 bp, 539 bp, 668 bp, 540 bp, 554 bp and 574 bp were generated for primer pairs 3F and 3R, 4F and 4R, 5F and 5R, 6F and 6R, 7F and 7R, 8F and 8R, 9F and 8R when cDNA was the template (Figure 3.21 B) whereas no products were amplified using primers spanning CDSs *carR* and *carA* or *carH* and *aspII*. Yet, appropriately sized PCR amplicons were generated for all primers when DNA was used as the template. Therefore it was concluded that in *P. c.* subsp. *brasiliensis* ICMP 19477 the *carA-H* genes formed a transcriptional operon.

A.

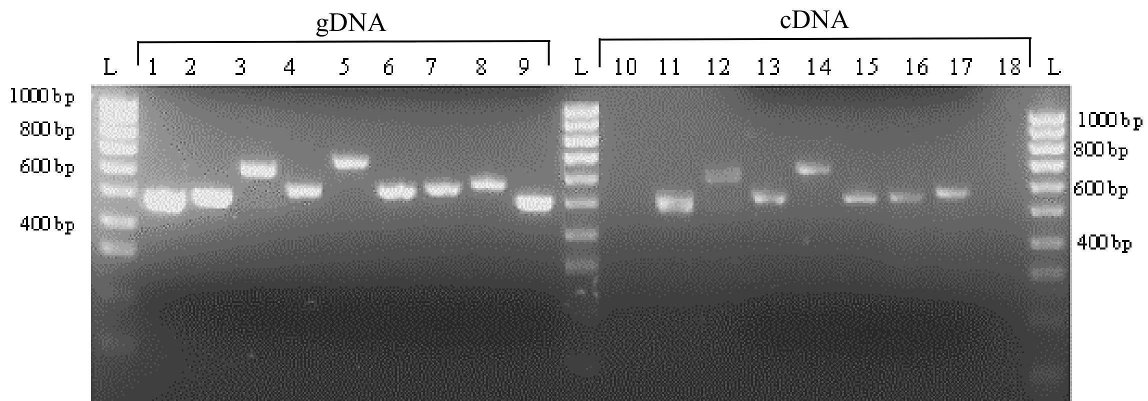


1F= CarR/CarA F
1R= CarR/CarA R
2F= CarA/CarB F
2R= CarA/CarB R
3F= CarB/CarC F
3R= CarB/CarC R

4F= CarC/CarD F
4R= CarC/CarD R
5F= CarD/CarE F
5R= CarD/CarE R
6F= CarE/CarF F
6R= CarE/CarF R

7F= CarF/CarG F
7R= CarF/CarG R
8F= CarG/CarH F
8R= CarG/CarH R
9F= CarH/Asp II F
9R= CarH/Asp II R

B.



C.

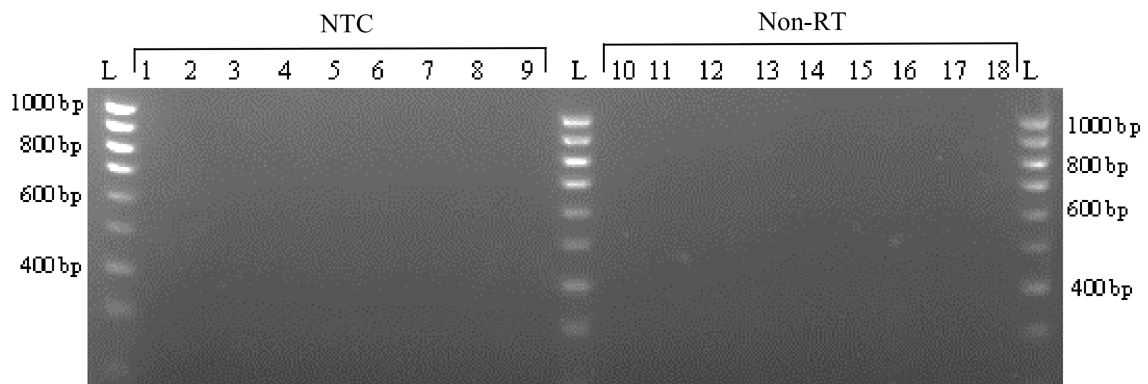


Figure 3.21. The operon structure of the carbapenem biosynthesis gene cluster in *P. c.* subsp. *brasiliensis* ICMP 19477.

A: Primers were designed to amplify fragments spanning the gaps between the *car* genes, in order to assess the operon structure of this cluster. Primer binding sites are indicated by arrows. **B**: PCR amplicons obtained from RT-PCR assays as indicative of an operon structure of the carbapenem cluster. L, DNA ladder; Lane 1, primer pair 1 DNA; Lane 2, primer pair 2 DNA; Lane 3, primer pair 3 DNA; Lane 4, primer pair 4 DNA; Lane 5, primer pair 5 DNA; Lane 6, primer pair 6 DNA; Lane 7, primer pair 7 DNA; Lane 8, primer pair 8 DNA; Lane 9, primer pair 9 DNA; L, DNA ladder; Lane 10, primer pair 1 cDNA; Lane 11, primer pair 2 cDNA; Lane 12, primer pair 3 cDNA; Lane 13, primer pair 4 cDNA; Lane 14, primer pair 5 cDNA; Lane 15, primer pair 6 cDNA; Lane 16, primer pair 7 cDNA; Lane 17 primer pair 8 cDNA; Lane 18 primer pair 9 cDNA; L, DNA ladder. **C**: Negative controls for PCR and RT-PCR reactions. L, DNA ladder; Lane 1, primer pair 1 NTC; Lane 2, primer pair 2 NTC; Lane 3, primer pair 3 NTC; Lane 4, primer pair 4 NTC; Lane 5, primer pair 5 NTC; Lane 6, primer pair 6 NTC; Lane 7, primer pair 7; NTC, Lane 8, primer pair 8, NTC; Lane 9, primer pair 9 NTC; L, DNA ladder; Lane 10, primer pair 1 non-RT; Lane 11, primer pair 2 non-RT; Lane 12, primer pair 3 non-RT; Lane 13, primer pair 4 non-RT; Lane 14, primer pair 5 non-RT; Lane 15, primer pair 6 non-RT; Lane 16, primer pair 7 non-RT; Lane 17, primer pair 8 non-RT; Lane 18, primer pair 9 non-RT; L, DNA ladder.

Other CDSs were also identified in the genome of *P. c.* subsp. *brasiliensis* ICMP 19477 that could encode a β-lactam. Unlike the previously described cluster, however, these CDSs were not part of defined HAIs or gene islets. For example, BLASTx analysis identified that KCO_01587 had greatest similarity to a large carbamoylphosphate synthase subunit of *P. c.* subsp. *carotovorum* Pcc21 (ID = 99%, E = 0). This subunit catalyses the ATP-dependent synthesis of carbamoyl phosphate from glutamine. The neighbouring CDS, KCO_01582, was similar to a small carbamoyl phosphate synthase subunit of *P. c.* subsp. *carotovorum* Pcc21 (ID = 99%, E = 0). Cryptic carbapenem clusters, that are not transcribed and do not produce a product, are widespread in *P. c.* subsp. *carotovorum* (Holden et al., 1998).

3.3 Discussion

Pectobacterium carotovorum subsp. *brasiliensis* PBR1692 was previously shown to inhibit the growth of *P. atrosepticum* SCRI1043 and *P. c.* subsp. *carotovorum* WPP14 *in vitro*, although no ecological benefit was identified in co-inoculation studies in potato stems (Marquez-Villavicencio et al., 2011). Other strains of *P. c.* subsp. *brasiliensis* were not able to inhibit the growth of *P. atrosepticum* SCRI1043, however. This led to the belief that the antimicrobial activity observed was highly strain-specific and potentially not of ecological importance (Marquez-Villavicencio et al., 2011). In this chapter, antagonism assays confirmed that the New Zealand isolate of *P. c.* subsp. *brasiliensis,* ICMP 19477, was also able to generate a zone of inhibition on a lawn of *P. atrosepticum* SCRI1043 in plate assays. This indicated that the capacity to suppress growth of *P. atrosepticum* might be a more generic characteristic of *P. c.* subsp. *brasiliensis* than first thought. This finding concurs with models of bacterial communities, as well as serial transfer studies, which concluded that antimicrobial sensitive, producer and resistant strains are present within a community (Frank, 1994; Riley and Gordon, 1999). Initially, the community is thought to be made up of all 'sensitive' strains, as there is no selection pressure from an antimicrobial molecule. If a producer then enters the community it is able to outcompete the sensitive strain (Durrett and Levin, 1997; Riley and Wertz, 2002). Overtime, however, mutations occur within the sensitive population that render them resistant to the antimicrobial produced by the producer (Smarda, 1992; Feldgarden and Riley, 1998). Thereby resistant, sensitive and producer bacteria are then present within the same community. Over time the advantageous, resistant phenotype will increase within the sensitive population and eventually predominate over the producer, as the resistant phenotype is less energy intensive (Tan and Riley, 1996). The producer will still be present at a low level in the community. Under less selective pressure from the antimicrobial molecule, some resistant strains will revert back to the sensitive phenotype, as this has the least metabolic cost (Feldgarden and Riley, 1998).

In antagonism assays between *P. c.* subsp. *brasiliensis* ICMP 19477 and *P. atrosepticum* SCRI1043, the presence of a zone of inhibition also suggested that the antimicrobial molecule responsible for inhibition of *P. atrosepticum* SCRI1043 was a diffusible molecule, rather than being involved in CDI.

Co-inoculation experiments on solid media subsequently showed a small, but significant effect of *P. c.* subsp. *brasiliensis* ICMP 19477 on the growth of *P. atrosepticum* SCRI1043, reducing cell counts of the latter especially during mid/late exponential phase (Section 3.2.4). Secondary metabolites such as antimicrobial molecules are usually produced in the later phases of growth (McGowan et al., 1995; Ruiz et al., 2010). Similar experiments in liquid media showed no impact of *P. c.* subsp. *brasiliensis* ICMP 19477 on the growth of *P. atrosepticum* SCRI1043 in co-inoculated cultures however, suggesting

that the suppression of growth observed *in vitro* was restricted to the more structured environment of the solid media plates. These results confirmed the initial hypothesis, and supported the idea that an antimicrobial might be involved in the reduction of *P. atrosepticum* SCRI1043 growth (as suggested by the zones of inhibition caused by *P. c.* subsp. *brasiliensis* ICMP 19477 on the sensitive SRE). In other studies, *in vitro* data has shown that producers of antimicrobial molecules gain their greatest competitive advantage (against competing and sensitive strains) under conditions of a more structured environment as the antimicrobial molecule reaches a high enough concentration to be effective, even if the producer is rare (Chao and Levin, 1981; Durrett and Levin, 1997; Gardner et al., 2004).

Greater inhibition of *P. atrosepticum* SCRI1043 populations was observed in potato tubers than in *in vitro* plate assays. This could be due to *P. c.* subsp. *brasiliensis* ICMP 19477 producing virulence factors that enable the bacterium to colonise the tuber more quickly than *P. atrosepticum* SCRI1043, leading to competitive exclusion of its competitor. It has previously been described that *P. c.* subsp. *brasiliensis* is a more aggressive pathogen *in vivo* than *P. atrosepticum* (Marquez-Villavicencio et al., 2011). Genomic comparisons between *P. atrosepticum* SCRI1043, *P. c.* subsp. *brasiliensis* PBR1692 and *P. c.* subsp. *carotovorum* WPP14 identified genes within PBR1692 and WPP14, which encoded putative enzymes associated with the utilisation of plant cell wall degradation products. Homologous genes were not found within the genome of SCRI1043 (Glasner et al., 2008). These genes included a putative permease enzyme that may import digested polymer, a putative polysaccharide deacetylase and a putative Asp/Glu racemase, which may degrade the digested polymers (Glasner et al., 2008).

Given that *P. c.* subsp. *brasiliensis* ICMP 19477 and *P. atrosepticum* SCRI1043 would be in competition with one another and potentially with other organisms in the tuber, the production of one or more antimicrobials by *P. c.* subsp. *brasiliensis* ICMP 19477 may also lead to the suppression of *P. atrosepticum* SCRI1043 by *P. c.* subsp. *brasiliensis* ICMP 19477. This idea has been poorly explored in plant pathology, with the major focus of research on the benefit of virulence factors that influence the interaction of the pathogen with the plant host. In fact, reviewing the current literature failed to provide any evidence that antimicrobial production has been shown to provide a competitive advantage to a bacterial pathogen in its ecological niche (in this case the tuber, where infection first takes place). Antimicroial competition between *P. c.* subsp. *carotovorum* in potato tubers has previously been predicted by Axelrood et al., (1988), however the observed competition was not confirmed to be due to the production of an antimicrobial molecule. More recently, Garlant et al., (2013) proposed that *D. solani* may have emerged as a result of producing a novel array of NRPs, which may function as antimicrobials. Again, functional data to support this theory was not provided.

The production of antimicrobials by plant pathogens such as *P. c.* subsp. *brasiliensis* ICMP 19477 has not been extensively studied, but it is possible that conditions in the tuber lead to increased production of these molecules. However, it was found that production of the phenazine molecule by *P. atrosepticum* SCRI1043, was not induced in the presence of potato tuber extract (Mattinen et al., 2008).

It is known that high levels of some nutrients, including sugars, interfere with the production of secondary metabolites including antimicrobial molecules (Demain, 1989). For example, when high levels of a rapidly used carbon source are present, there is little secondary metabolite production (Demain, 1989). It is not until the preferred carbon source is depleted and the 'second best' nutrient source is utilised, that secondary metabolites are produced (Demain, 1989; Ruiz et al., 2010). Other nutrients such as high levels of inorganic phosphate repress carbapenem production in *Serratia* (Whitehead et al., 2001). Consequently nutrient starvation in the host plant may increase the level of antibiotic production by the bacteria when compared to *in vitro* conditions.

Bioinformatic analysis identified multiple putative antimicrobial clusters within the genome of *P. c.* subsp. *brasiliensis.* It was predicted that one or more of these clusters encodes the antimicrobial molecule that confers a competitive advantage to *P. c.* subsp. *brasiliensis. Pectobacterium carotovorum* subsp. *brasiliensis* ICMP 19477 harbours a variety of horizontally acquired elements, many of which encode putative antimicrobials (Preetinanda Panda, 2014). The antimicrobials encoded vary in type, but include putative NRPs, bacteriocins and carbapenems. A closer examination of the biosynthetic clusters responsible for the production and transport of these molecules demonstrated that the bacteriocin were highly similar in the two *P. c.* subsp. *brasiliensis* strains compared, but were only distantly related or absent in other strains that were unable to inhibit *P. atrosepticum* SCRI1043. For example, the carbapenem cluster in *P. c.* subsp. *brasiliensis* ICMP 19477 is conserved between the *P. c.* subsp. *brasiliensis* strains. A carbapenem cluster is also present in *P. c.* subsp. *carotovorum* ICMP 5702, however the nucleotide identity showed that this cluster was divergent from the *P. c.* subsp. *brasiliensis* cluster; the influence of diversity on the activity of the related molecules is unknown.

Non-ribosomal peptides can be involved in interactions between pathogens and their plant hosts as well as in microbial interactions (Sinden et al., 1971). The NRPS cluster in *P. c.* subsp. *brasiliensis* ICMP 19477 was of initial focus due to its potential role as a phytotoxin, but data not described here showed that inactivation of the NRPS cluster did not influence the capacity of *P. c.* subsp. *brasiliensis* ICMP 19477 to infect potato plants (Preetinanda Panda, 2014). Thus it was deemed highly likely that this molecule was involved in microbial interactions. Furthermore, the NRP biosynthetic cluster was distinct in all strains where it was detected, producing molecules that were related but different. As

the influence of amino acid differences on function or specificity was unknown, a role for the NRP in competition between *P. c.* subsp. *brasiliensis* ICMP 19477 and *P. atrosepticum* SCRI1043 remained a possibility.

The genetic organisation of the genes within the NRPS cluster suggested that the three genes were transcribed as an operon. An operon structure was also predicted as horizontally acquired genes of related function tend to cluster together to increase their chance of transmission (Lawrence, 1999). Operon structures are also seen in other NRPS gene clusters including many within the *Bacillus* species including those for the antifungal compounds iturin A and mycosubtilin (Duitman et al., 1999; Tsuge et al., 2001). Furthermore, two of the three genes involved in the production of the pigment indigoidine in *Dickeya* are also transcribed together (Reverchon et al., 2002). Interestingly, the two synthetase genes which encode syringomycin in *P. syringae,* are individually transcribed (Guenzi et al., 1998).

Carotovoricin was first described as a proteinaceous, narrow spectrum bacteriocin produced by *P. c.* subsp. *carotovorum* (Lysak, 1979). The activity of carotovoricin has previously been described against other *P. c.* subsp. *carotovorum* strains, such as EC-2P7, 645Ar and NA8 (Nguyen et al., 2001; Jabeen et al., 2014), activity against *P. atrosepticum* has not been described. This is consistent with the description that bacteriocins are only active against closely related species (Klaenhammer, 1988). The specific activity against *P. c.* subsp. *carotovorum* strains, as well as the fact that *P. c.* subsp. *carotovorum* ICMP 5702 also encodes the Ctv CDSs but does not inhibit the growth of *P. atrosepticum* SCRI1043, suggests that carotovoricin is not responsible for the activity of ICMP 19477 against SCRI1043.

Phenazines are produced by many host-associated bacteria (Pierson III and Pierson, 2010). The production of phenazines has been associated with the persistence of the producing bacteria within the environment, even without the presence of competitors (Kobayashi and Tagawa, 2004; Price-Whelan et al., 2006; Hassett et al., 2009). Phenazine biosynthesis genes have been identified in both *P. c.* subsp. *brasiliensis* ICMP 19477 (Panda et al., 2015b) and *P. atrosepticum* SCRI1043 (Bell et al., 2004). The two phenazine synthesis clusters showed high nucleotide similarity (93%). This high similarity suggests that the two phenazine molecules will also be highly similar. It is therefore unlikely that the phenazine produced by *P. c.* subsp. *brasiliensis* ICMP 19477 is active against *P. atrosepticum* SCRI043. However, it is not known how the differences in nucleotide identity would influence the specificity of the phenazine molecule.

The *P. c.* subsp. *brasiliensis* strains were found to encode a bacteriocin synthesis cluster that was absent from the genomes of *P. c.* subsp. *carotovorum* ICMP 5702, Pcc21 and *P. atrosepticum* SCRI1043. The absence of the genes from the other SRE genomes, suggested that the bacteriocin is likely involved in the inhibition of *P. atrosepticum* by *P. c.* subsp. *brasiliensis* ICMP 19477. However, bacteriocins produced by *P. c.* subsp. *carotovorum* strains, such as carocin D, have only been described to have activity against other *P. c.* subsp. *carotovorum* strains (Roh et al., 2010). However, some studies focusing on bacteriocin activity have described that they may be active against more distant bacterial species and even viruses (Wachsman et al., 1999; Todorov et al., 2010).

Carbapenem antimicrobial molecules are described as having a broad spectrum of activity (Ratcliffe and Albers-Schonberg, 1982). They have also been predicted to be responsible for increased fitness of the *Pectobacterium* that encode them, both *in vitro* and *in planta* (Axelrood et al., 1988; Marquez-Villavicencio et al., 2011)*;* however, these predictions have not been confirmed genetically or functionally. Therefore the carbapenem encoded within the genome of *P. c.* subsp. *brasiliensis* ICMP 19477 may also be predicted to function in the inhibition of *P. atrosepticum* SCRI1043 *in vitro* and in potato tubers. The *P. c.* subsp. *brasiliensis* type strain PBR1692, which also inhibits *P. atrosepticum* SCRI1043 *in vitro,* encodes a carbapenem cluster with 98% nucleotide identity to ICMP 19477. However, *P. c.* subsp. *carotovorum* ICMP 5702, which is not antagonistic towards *P. atrosepticum* SCRI1043 *in vitro,* also encodes a carbapenem synthesis cluster. This cluster was found to have 87.8% nucleotide identity to the carbapenem cluster of *P. c.* subsp. *brasiliensis* ICMP 19477. This difference may be sufficient to produce carbapenem molecules with different specificities.

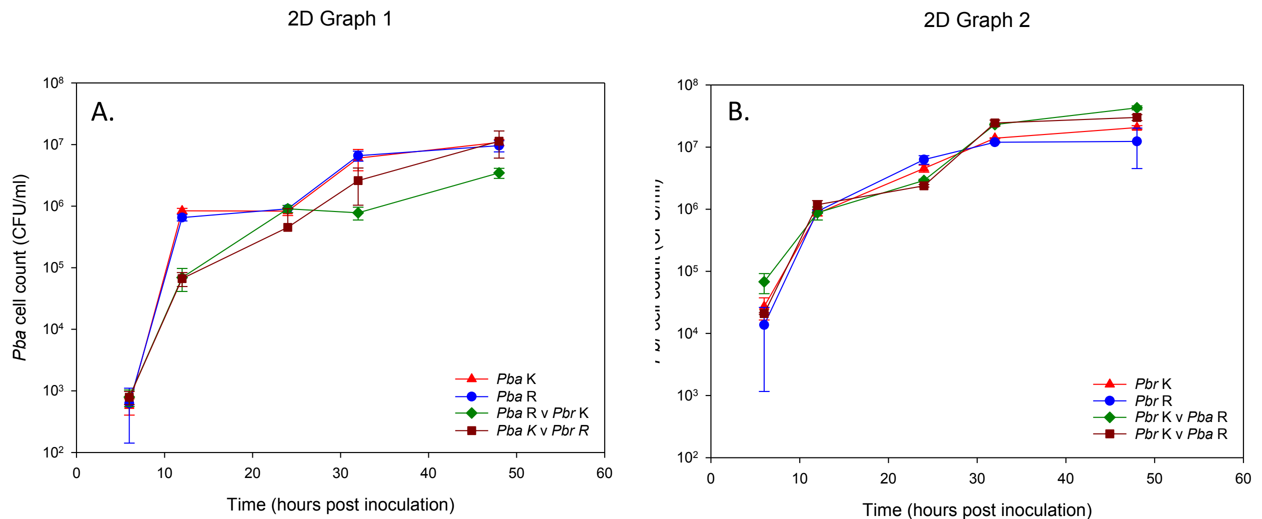
To establish whether one or more of these antimicrobials had activity against *P. atrosepticum* SCRI1043 and was responsible for the inhibition of this bacterium in competition assays *in vitro* and in tubers, attempts were made to purify the molecule(s) using MS techniques. However, no potential antimicrobial molecules were detected, possibly for a number of reasons. Firstly, it is difficult to successfully identify an unknown antimicrobial molecule as the purification methods for each type of molecule are very specific (Hayashi et al., 2014). In this study a general methanol extraction method was used, as this is suitable for most carboxylic acid molecules (David Greenwood, personal communication). However, this may not be suitable for the molecule produced by *P. c.* subsp. *brasiliensis* ICMP 19477. Secondly, it may be that the molecule is unstable, or unstable under the extraction conditions used. For example the simple carbapenem molecule SQ 27,860, produced by *Serratia* and *Erwinia*, has been reported as particularly unstable above temperatures of 5°C (Wagman and Cooper., 1988). Finally, even though triplicate samples were used in order to increase the amount of the antimicrobial present in each sample, the concentration may not have been high enough. It is

often reported that antimicrobial encoding genes are over expressed via an *E. coli* expression system, to produce sufficient antimicrobial product for purification (Trauger and Walsh, 2000; Owston and Serpersu, 2002).

As attempts to purify the antimicrobial were unsuccessful, a functional genetics approach was required to inactivate one or more of the antimicrobials produced by *P. c.* subsp. *brasiliensis* ICMP 19477. This approach was used successfully to identify the carocin D synthesis genes in *P. c.* subsp. *carotovorum* Pcc21 (Roh et al., 2010). Chapter 4 describes the functional genetics experiments that enabled the identification of the biosynthetic cluster responsible for the antagonism of *P. atrosepticum* SCRI1043 by *P. c.* subsp. *brasiliensis* ICMP 19477 both *in vitro* and in potato tubers.

Appendix B

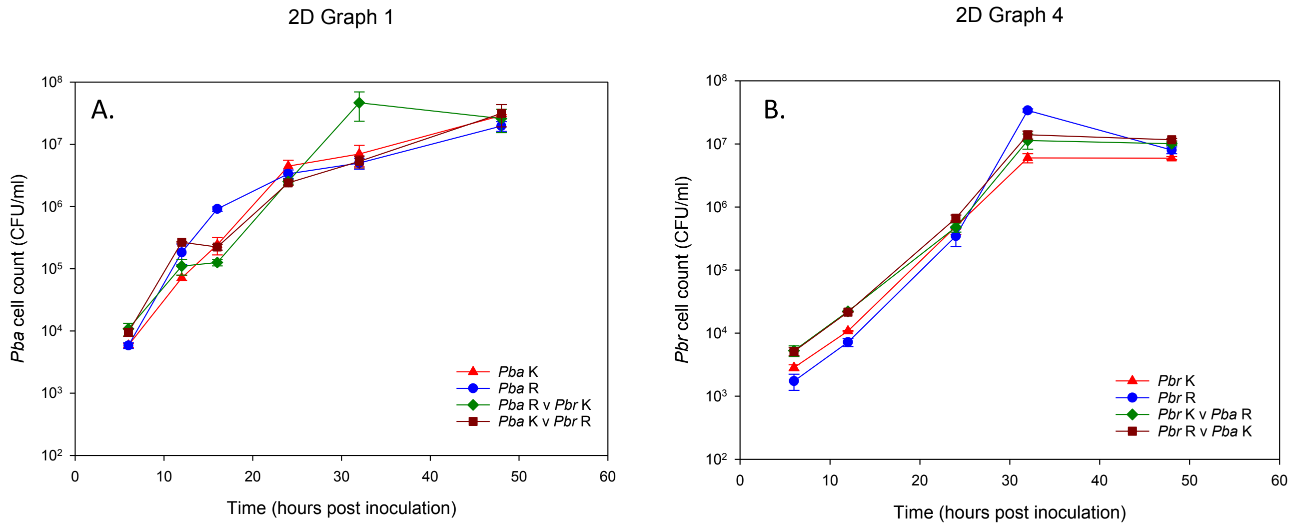
B.1 *In vitro* competition graphs



Second, independently conducted experiment, demonstrating the effect of co-inoculation with *P. c.* subsp. *brasiliensis* (*Pbr*) ICMP 19477 on growth of *P. atrosepticum* (*Pba*) SCRI1043 on solid agar plates (MMA).

A: A graph showing the growth (mean CFUs) of *P. atrosepticum* SCRI1043 Km (*Pba* K) and Rif (*Pba* R) resistant strains over a 48 h period at 28°C when inoculated onto MMA in isolation or when co-inoculated with *P. c. subsp. brasiliensis* ICMP19477 tagged with either a Km (*Pbr* K) or Rif (*Pbr* R) resistance determinant (at a 1:1 ratio). **B**: A graph showing the growth (mean CFUs) of *Pbr* K or *Pbr* R when cultured in isolation on solid agar plates or in co-inoculations with *Pba* K or *Pba* R (at a 1:1 ratio). For each graph, the mean CFUs were calculated for each treatment from triplicate assays (solid line), error bars represent the standard deviation.

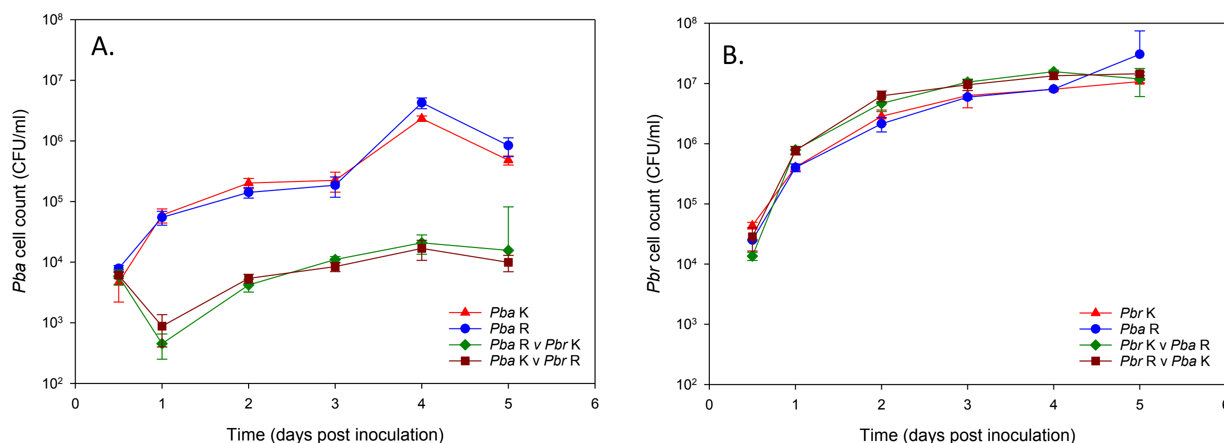
B.2 Liquid culture competition graphs



Second, independently conducted experiment, demonstrating the effect of co-inoculation with *P. c.* subsp. *brasiliensis* (*Pbr*) ICMP 19477 on growth of *P. atrosepticum* (*Pba*) SCRI1043 in liquid cultures (MM).

A: A graph showing the growth (mean CFUs) of *P. atrosepticum* SCRI1043 Km (*Pba* K) and Rif (*Pba* R) resistant strains over a 48 h period at 28°C when inoculated into MM in isolation or in media co-inoculated with *P. c. subsp. brasiliensis* ICMP 19477 tagged with either a Km (*Pbr* K) or Rif (*Pbr* R) resistance determinant (at a 1:1 ratio). **B**: A graph showing the growth (mean CFUs) of *Pbr* K or *Pbr* R when cultured in isolation in liquid cultures or in co-inoculations with *Pba* K or *Pba* R (at a 1:1 ratio). For each graph, the mean CFUs were calculated for each treatment from triplicate assays (solid line), error bars represent the standard deviation.

B.3 Tuber competition graphs



Second, independently conducted experiment, using potato tubers sourced at a different time of year, demonstrating the effect of co-inoculation with *P. c.* subsp. *brasiliensis* (*Pbr*) ICMP 19477 on growth of *P. atrosepticum* (*Pba*) SCRI1043 in potato tubers ('Ilam Hardy').

A: A graph showing the growth (mean CFUs) of *P. atrosepticum* SCRI1043 Km (*Pba* K) and Rif (*Pba* R) resistant strains over a 48 h period at 28°C when inoculated into MM in isolation or in media co-inoculated with *P. c. subsp. brasiliensis* ICMP 19477 tagged with either a Kn (*Pbr* K) or Rif (*Pbr* R) resistance determinant (at a 1:1 ratio). **B**: A graph showing the growth (mean CFUs) of *Pbr* K or *Pbr* R when cultured in isolation in potato tubers or in co-inoculations with *Pba* K or *Pba* R (at a 1:1 ratio). For each graph, the mean CFUs were calculated for each treatment from triplicate assays (solid line), error bars represent the standard deviation.

Chapter 4

Carbapenem production is required for suppression of *Pectobacterium atrosepticum* SCRI1043 by *Pectobacterium carotovorum* subsp. *brasiliensis* ICMP 19477 in co-inoculated tubers

4.1 Introduction

Bioinformatic analyses of the *P. c.* subsp. *brasiliensis* ICMP 19477 genome identified a variety of putative antimicrobial clusters potentially conferring both antimicrobial activity against *P. atrosepticum* SCRI1043 and competitive fitness to this pathogen. Of particular interest was an NRP biosynthetic cluster found only in *P. c.* subsp. *brasiliensis* ICMP 19477 (Section 3.2.8). Non-ribosomal peptides contain features distinct from ribosomally synthesised peptides, forming macrolytic or branched macrolytic structures containing a combination of up to 600 identified non-proteinogenic residues (Caboche et al., 2010). Residues can include *N*-methylated or *D*-structured amino acids (Mootz et al., 2002). The structural diversity provided by this array of residues confers diverse chemical and pharmaceutical activities, which enable the producers to adapt to their environmental niches. For example, NRPs are known to act as siderophores, biosurfactants and antibiotics (Schwarzer et al., 2003; Giessen and Marahiel, 2012). In the plant-pathogenic bacteria *Pseudomonas syringae*, the NRP syringomycin not only acts as a phytotoxic molecule that enhances virulence, it also acts as a broad spectrum antimicrobial and biosurfactant molecule (Backman and DeVay, 1971; Sinden et al., 1971; Bender et al., 1999b). Furthermore, *Dickeya* produces a blue pigmented NRP, indigoidine. Indigoidine defective mutants are less aggressive than the WT, possibly due to decreased resistance to oxidative stress (Reverchon et al., 2002).

Dickeya solani has emerged as an aggressive pathogen in Europe, replacing previously dominant *Pectobacterium* and *Dickeya* species. It also exhibits increased resistance against inhibition from other bacteria within the ecological niche (Czajkowski et al., 2013). *Dickeya solani*-specific NRPS/PKS gene clusters have been identified through genome analysis, which are not present in other *Dickeya* or *Pectobacterium* (Garlant et al., 2013). It is predicted that the presence of these novel NRPS/PKS clusters, or the novel combination of such clusters, have enhanced *D. solani's* adaptation to its environment and enabled its emergence (Garlant et al., 2013). Similarly, the NRPS cluster harboured

in the genome of *P. c.* subsp. *brasiliensis* ICMP 19477 may have contributed to the recent emergence of this pathogen (Duarte et al., 2004).

The bacteriocin harboured on PbN1-GI38 might also contribute to the competitive fitness of *P. c.* subsp. *brasiliensis* ICMP 19477 in potato. Genomic studies previously showed that bacteriocin synthesis genes are abundant in plant pathogens and are likely to play an important role in bacterial interactions (Hu and Young, 1998; Holtsmark et al., 2008). As bacteriocin production is under tight regulation, however, the production of many bacteriocins went unknown prior to the age of genome sequencing (Brurberg et al., 1997). Bacteriocin production has been associated with competition between bacteria within the rhizosphere (Parret et al., 2003). For example, bacteriocin production has been implicated in competition in plant nodules by *Rhizobium* strains. Interestingly, transposon mutants that were unable to produce a bacteriocin were more competitive than the antibiotic producer (Dowling and Broughton, 1986). These results emphasise that much remains unknown about the function of bacteriocins produced by plant-associated bacteria.

The carbapenem encoded by *P. c.* subsp. *brasiliensis* ICMP 19477 may also be involved in the inhibition of *P. atrosepticum* SCRI1043 and its enhanced fitness in potato tubers. Certainly, carbapenem production was previously considered important in the recent emergence of the pathogen overseas (Marquez-Villavicencio et al., 2011). Carbapenems are encoded by various SREs , although many are potentially functionally cryptic (Gardan et al., 2003). Cryptic clusters lack a functional CarR homologue and are therefore not transcribed (Holden et al., 1998). This suggests that carbapenem production may be required under very specific conditions and that their functions in many strains are now redundant. This provides evidence for the argument that antimicrobial molecules do not confer a significant long term advantage.

The QS regulatory network ensures that in SREs, carbapenem synthesis is simultaneous with PCWDE production (Bainton et al., 1992; Jones et al., 1993a). Therefore, synthesis of the antimicrobial is dependent on a high bacterial population density and not the presence of a competing organism (McGowan et al., 2005). As carbapenem production is concurrent with the release of nutrients into the local environment, it suggests that the antimicrobial functions to defend the producer's ecological niche rather than to invade an established population of competing organisms.

The study of antimicrobial production and function requires the creation of mutants that do not produce the molecule. A transposon mutant library is a commonly utilised method in such studies (Dowling and Broughton, 1986; Roh et al., 2010), as this method enables the production and screening of a large number of mutants for changes in activity. This approach was used successfully to identify

the carocin D synthesis genes in *P. c.* subsp. *carotovorum* Pcc21 (Roh et al., 2010). A transposon mutant library has also successfully been created in the SRE *P. atrosepticum* SCRI1043 (Holeva et al., 2004).

In this chapter, *P. c.* subsp. *brasiliensis* ICMP 19477 mutants developed using a combination of allelic exchange mutagenesis and random transposition were screened for changes in their activity against *P. atrosepticum* SCRI1043 and an associated loss of fitness in competition assays. NRP-related mutants generated by allelic exchange were previously produced by Preetinanda Panda (Unpublished doctoral thesis). In her study, however, she described significant difficulties in the production of such mutants, possibly due to the presence of two restriction modification systems (Preetinanda Panda, 2014). Thus, new mutants were generated using a transposon mutagenesis approach. This also seemed appropriate given several candidate loci remained of interest after bioinformatics analysis had been performed.

4.2 Results

4.2.1 The non-ribosomal peptide of *Pectobacterium carotovorum* subsp. *brasiliensis* ICMP 19477 is not involved in the inhibition of *Pectobacterium atrosepticum* SCRI1043

Mutants previously created within the *P. c.* subsp. *brasiliensis* ICMP 19477 NRPS biosynthetic cluster (Preetinanda Panda, 2014) were screened for their inhibition of *P. atrosepticum* SCRI1043. In particular, a double crossover mutant of *nrps1* (KCO_06050) and a single crossover mutant of the ABC transporter (KCO_06055) (Table 2.2) were tested to determine whether these genes were involved in the production of the antimicrobial molecule active against *P. atrosepticum* SCRI1043. The mutants were tested using the antagonism assay (Section 2.1.21). It was believed that a single crossover mutant, although still carrying a wild-type copy of the gene, would be impaired in synthesis or transport of the NRP due to the operon structure of this biosynthetic cluster (Section 3.2.8). A third mutant, *P. c.* subsp. *brasiliensis SIM*$^{-}$ (KCO_20372) (Table 2.2), which harboured a mutation in a gene outside the NRP biosynthetic cluster, was tested to ensure that the process of mutagenesis had not impaired the production of the antimicrobial.

In these assays, the *nrps1* mutant (*P. c.* subsp. *brasiliensis nrps*$^{-}$) produced a clear zone of inhibition (Figure 4.1), which was reduced in size compared to the WT. This indicated a possible contribution by the NRP to the antibiotic affect, although it is unlikely to be the main antibiotic producing mechanism. Contradicting this result, however, the *abc* mutant (*P. c.* subsp. *brasiliensis abc*$^{-}$) repeatedly failed to inhibit the growth of *P. atrosepticum* SCRI1043 (Figure 4.1). *Pectobacterium carotovorum* subsp. *brasiliensis sim*$^{-}$ produced zones of inhibition similar to those of the WT, confirming that the process of mutagenesis was not responsible for the change in phenotype of the *abc* mutant. These results suggested that the ABC transporter may act independently of the other NRP-related genes, to transport the antimicrobial synthesized by another gene cluster out of the cell.

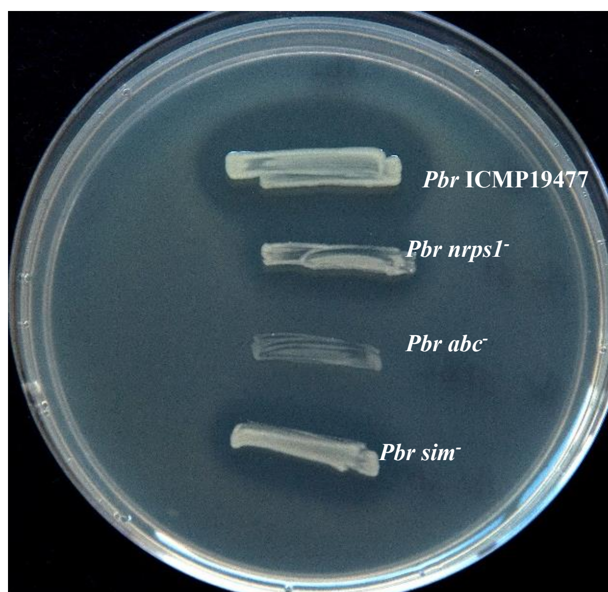


Figure 4.1. ***In vitro*** **growth inhibition of** ***P. atrosepticum*** **(*Pba*) SCRI1043 by** ***P. c.*** **subsp.** ***brasiliensis*** **(*Pbr*) ICMP 19477,** ***Pbr nrps1***$^{-}$ **(non-ribosomal peptide synthetase mutant),** ***Pbr abc***$^{-}$ **(ABC transporter mutant) and** ***Pbr sim***$^{-}$ **(sugar isomerase mutant).**

Plates pre-seeded with a lawn of *Pba* SCRI1043 were incubated at 28 °C for 24 h after inoculation with the producer strain (Section 2.1.21). A positive result was indicated by the presence of a zone of inhibition around the *Pbr* colony.

4.2.2 *Pectobacterium carotovorum* subsp. *brasiliensis* ICMP 19477 produces a diffusible antimicrobial molecule in late exponential/ early stationary phase likely to be a β-lactam.

As inactivation of *nrps1* did not abolish inhibition of *P. atrosepticum* SCRI1043, *P. c.* subsp. *brasiliensis* ICMP 19477 was plated onto an *E. coli* strain (ESS), which is supersensitive to β-lactam antimicrobials, to examine whether the carbapenem produced by this bacterium might be responsible (Bainton et al., 1992). In these assays (Section 2.1.21, data not shown), *P. c.* subsp. *brasiliensis* ICMP 19477 inhibited the growth of ESS indicating that *P. c.* subsp. *brasiliensis* ICMP 19477 does produce a β-lactam (likely to be the carbapenem), as predicted by bioinformatics (Section 3.2.12).

A supernatant antagonism assay (Section 2.1.30), using ESS (Table 2.2) as the bacterial lawn, also confirmed that *P. c.* subsp. *brasiliensis* ICMP 19477 produced a β-lactam (likely the carbapenem) at

late exponential / early stationary phase, 7 hpi (Figure 4.2). A reduction in the zone size between 11 and 12 hpi, however, suggested a reduction in antimicrobial activity later in stationary phase. This profile corresponded with the production of a carbapenem by *P. c.* subsp. *brasiliensis* ICMP 19477.

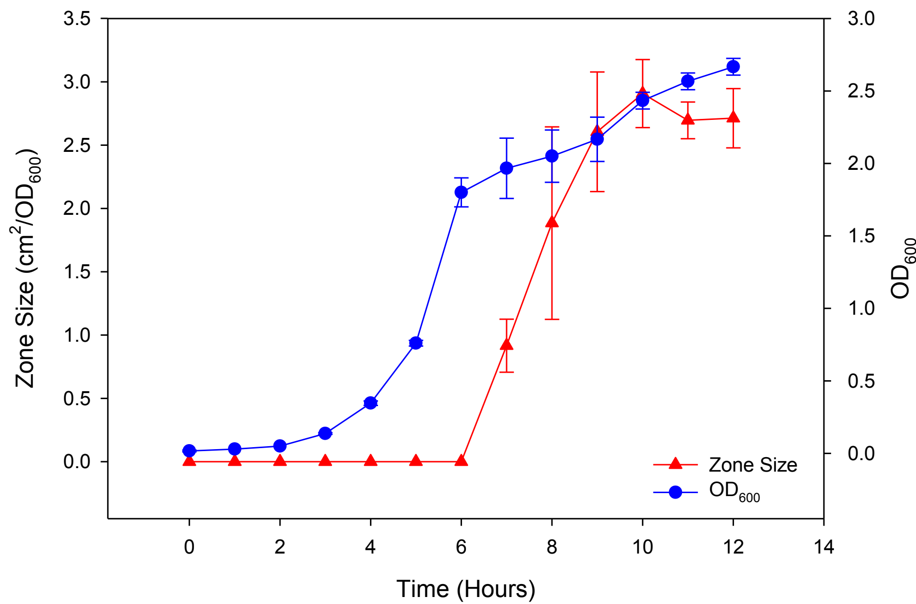


Figure 4.2. *Pectobacterium carotovorum* subsp. *brasiliensis* ICMP 19477 produces a secreted antimicrobial molecule at late exponential/early stationary phase.

Cell-free, growth culture supernatant was prepared from *P. c.* subsp. *brasiliensis* ICMP 19477 cultures, grown in LB media, every hour for 12 h. The supernatant samples were inoculated onto agar plates seeded with the β-lactam super-sensitive *E. coli* (ESS) (as described in Section 2.1.29). After 24 h incubation at 28 °C, the diameter of the resulting zones of inhibition were measured and the zone area (cm^2) calculated. The zone size was calculated from the zone area (cm^2)/OD_{600} to adjust for the growth effect on zone size.

4.2.3 Activity against the *E. coli* super-sensitive strain proves that the ABC transporter of the NRPS cluster is not involved in antimicrobial production by *Pectobacterium carotovorum* subsp. *brasiliensis* ICMP 19477

The assay using the ESS strain was subsequently used to determine if the *P. c.* subsp. *brasiliensis abc*$^{-}$ transporter mutant was unable to inhibit the growth of the bacterium, which would indicate a role in transporting the β-lactam produced by *P. c.* subsp. *brasiliensis* ICMP 19477. In this assay, the ABC transporter mutant produced a zone of inhibition (Figure 4.3), suggesting that the transporter was not involved in the transport of the β-lactam.

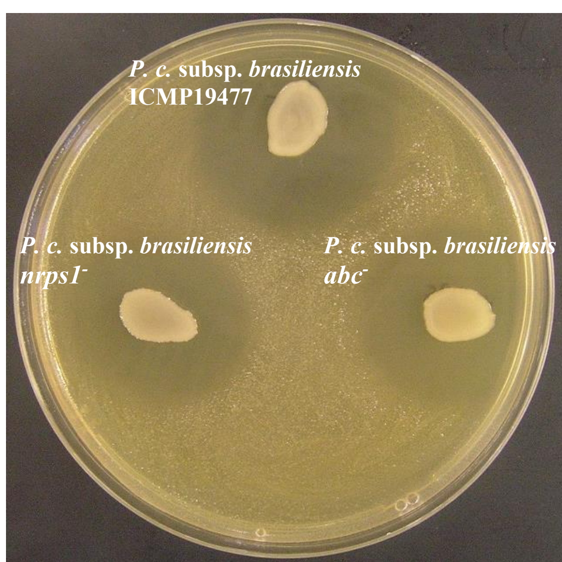


Figure 4.3. *In vitro* growth inhibition of *E. coli* supersensitive stain ESS by *P. c.* subsp. *brasiliensis* (*Pbr*) ICMP 19477, the non-ribosomal peptide synthetase mutant (*Pbr nrps1*$^{-}$) and the *ABC* mutant (*Pbr abc*$^{-}$).

Plates pre-seeded with a lawn of ESS were incubated at 28 °C for 24 h after inoculation with the producer strains (as described in Section 2.1.21). A positive result was indicated by the presence of a zone of inhibition around the *P. c.* subsp. *brasiliensis* colony.

4.2.4 The ABC transporter mutant probably has a non–inhibitor phenotype due to impaired growth

In the antagonism assays, it was observed that the ABC transporter mutant streaked onto the plates produced smaller, seemingly less healthy colonies suggesting the strain may be impaired in growth on *P. atrosepticum* SCRI1043 (Figure 4.1). This was explored by measuring the growth of the mutant in liquid cultures (Section 2.1.23, 2.1.25) compared to the WT. This experiment demonstrated that the mutant was not able to grow as well as the *P. c.* subsp. *brasiliensis* ICMP 19477 WT, the CFU/ml values for (*P. c.* subsp. *brasiliensis ABC*⁻) proving lower than the WT at all sampling times ($p < 0.001$) (Figure 4.4). Results were consistent in a second independently conducted experiment (data not shown), confirming that the reduced growth of the *P. c.* subsp. *brasiliensis abc*⁻ mutant was likely responsible for its inability to inhibit the growth of *P. atrosepticum* SCRI1043 in the original plate assays.

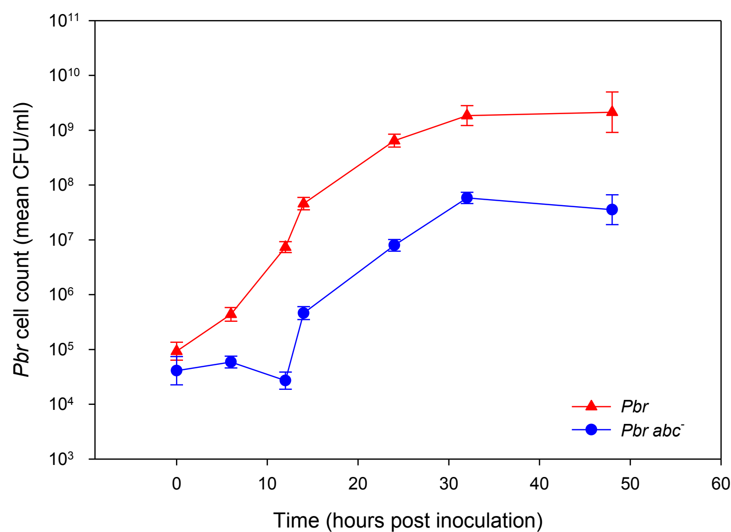


Figure 4.4. Growth of the *P. c.* subsp. *brasiliensis* (*Pbr*) *ABC* transporter mutant (*P. c.* subsp. *brasiliensis abc*⁻) was reduced in liquid cultures, compared to the growth of *P. c.* subsp. *brasiliensis* ICMP 19477 (WT).

The graph shows the growth (mean CFUs) of the WT as well as the ABC transporter mutant over 48 h post inoculation with 10^4 CFUs/ml of each bacteria (as described in Section 2.1.23). The mean CFUs represent the mean over three test cultures. Error bars are 95% confidence limits.

4.2.5 Tn*5* mutagenesis was used to create additional *Pectobacterium carotovorum* subsp. *brasiliensis* ICMP 19477 mutants that did not inhibit the growth of *Pectobacterium atrosepticum* SCRI1043

As functional analysis confirmed that the NRPS cluster (KCO_06055-06045/17262) was not involved in the production of the targeted antimicrobial molecule in *P. c.* subsp. *brasiliensis* ICMP 19477 (Figure 4.3) and the assays using ESS had provided evidence that *P. c.* subsp. *brasiliensis* ICMP 19477 could produce a β-lactam, it was hypothesised that the carbapenem predicted to be produced by the gene cluster on PbN1-G165 was responsible for the inhibition of *P. atrosepticum* SCRI1043 both *in vitro* and in potato tubers. The results did not rule out the involvement of other antimicrobial molecules, however, so to ensure all antimicrobial molecules involved in the activity against *P. atrosepticum* SCRI1043 could be identified, mutants of *P. c.* subsp. *brasiliensis* ICMP 19477 were created via random transposon mutagenesis and screened for loss of activity against *P. atrosepticum* SCRI1043. Transposon mutagenesis was also used instead of allelic exchange (as used to create the NRP mutants) as allelic exchange had previously proven exceptionally difficult in *P. c*. subsp. *brasiliensis* ICMP 19477, largely resulting in single crossover events that did not disrupt the targeted gene.

4.2.5.1 Optimisation and screening of the transposon mutant library identifies mutants unable to generate a zone of inhibition.

Tn*5* mutagenesis was initially conducted using a method adapted from that previously used for *P. c.* subsp. *carotovorum* Pcc11 and *P. atrosepticum* (Holeva et al., 2004). The protocol advised that the incubation time between the conjugation strains could be a minimum of 6 h, or overnight for 16 h. Conjugations were set up and the colonies incubated at 28 °C for 6 h, 16 h or 40 h. The protocol was then completed as described in Sections 2.1.17 and 2.1.18. Using this method, more transconjugants (Amp sensitive and Km resistant, indicating loss of the transposon plasmid and insertion of the Tn*5* cassette) were produced after conjugation for 16 h than after incubation for six or 40 h. Thus, a 16 h conjugation was used for all further experiments to generate the library of Tn*5* mutants.

To confirm the authenticity of each transconjugant, two rounds of replica plating on Amp and Km were carried out. On the second round of replica plating, however, it was observed that many mutants remained resistant to Amp. This suggested that the plasmid had not been cured and the transposon had not incorporated into the genome via a double crossover event. The Amp resistant mutants were confirmed as *P. c.* subsp. *brasiliensis* ICMP 19477 and not *E. coli* by PCR (Section 2.1.10), so in order to

initiate a double crossover, the transconjugates were plated onto LBA containing no antibiotics and incubated at 28 °C overnight. The plates were then stored at 4°C for up to three days and replica plated to determine Km resistance and Amp sensitivity (a process repeated if necessary), the full protocol is given in Sections 2.1.17 and 2.1.18. It was observed that through replica plating, the number of Amp sensitive transconjugates increased, but 50% of those picked remained Amp resistant. Thus, a Tn*5* library of only 1,500 Amp sensitive, Km resistant transconjugates was produced using this method. The reasons for this unexpected retention of the delivery vector for Tn*5* are discussed in Section 4.3

For the production of the remaining mutants required for the library, the transconjugates were grown on non-selective MMA, following the initial growth on MMA containing Km. It was anticipated this would initiate double crossover recombination events and cure the plasmid at a greater frequency. Using this method, a library of 4,520 mutants was generated, which represents a one-fold coverage of the genome (Holeva et al., 2004). The Amp sensitive, Km resistant mutants were confirmed to be *P. c.* subsp. *brasiliensis* ICMP 19477 by PCR (Section 2.1.10) using primers Pbr NZ F and Pbr NZ R, which amplified from *P. c.* subsp. *brasiliensis* ICMP 19477 but not *E. coli* (Table 2.3).

A screen (Section 2.1.21) of the library of Tn*5* mutants for transconjugants unable to generate zones of inhibition on *P. c.* subsp. *brasiliensis* ICMP 19477 identified 42 putative 'non-inhibitors'.

4.2.5.2 Identification of the Tn*5* insertion sites

The transposon insertion sites for 18 of the 42 putative non-inhibitors were identified by genome sequencing followed by sequence analysis (Section 2.1.19). Twelve were shown to have retained the plasmid as well as the transposon, indicative of a single cross-over, despite being sensitive to ampicillin (Figure 4.5 A). As a result these were not analysed further.

Six of the mutants sequenced were found to have been generated via a true transposition event, containing a single copy of the transposon within the genome and no evidence of the delivery vector (Figure 4.5 B). The insertion sites for each of the six mutants were identified within the sequence data. One of these mutants showed poor read coverage, meaning the insertion site identified was unreliable. Therefore, this mutant was excluded from any further analysis.

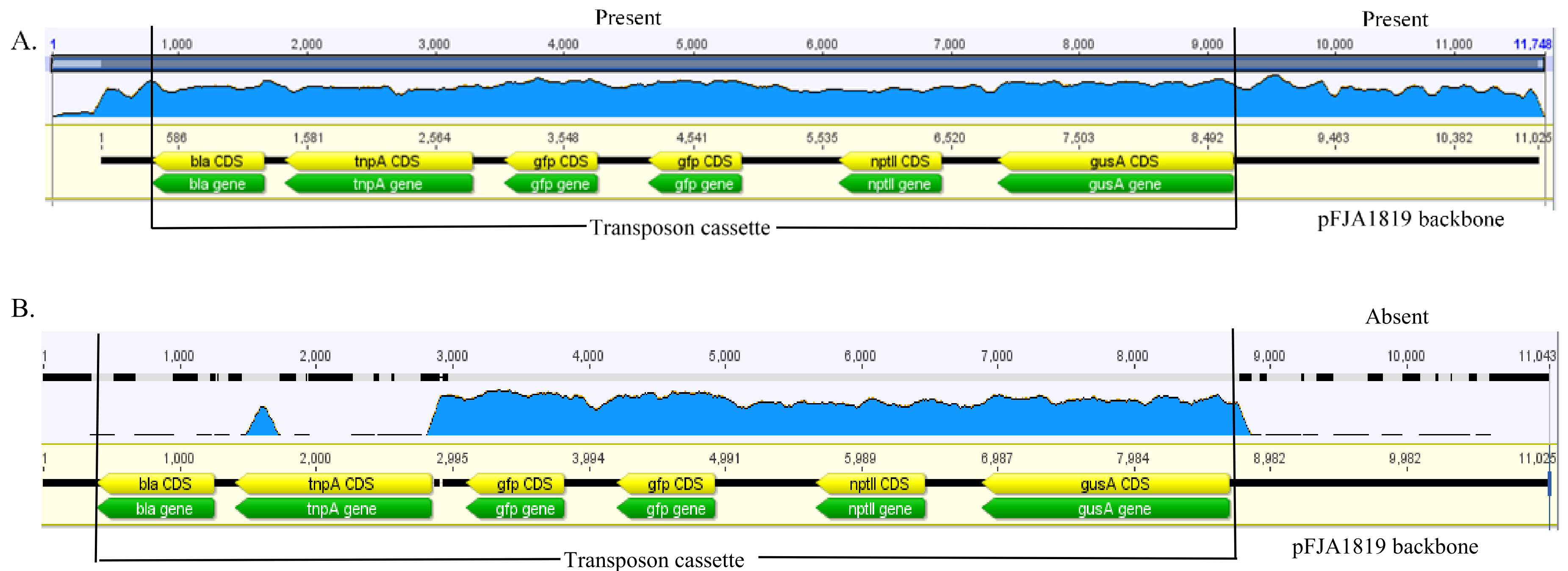


Figure 4.5. Retention of the delivery vector pFJA1819 in the genome of *P. c.* subsp. *brasiliensis* ICMP 19477 transposon mutants.

A: Sequencing reads mapped to the entire plasmid sequence in many of the identified non-producers *P. c.* subsp. *brasiliensis* ICMP19477. **B**: An example of the read pattern when only the transposon had inserted into the genome of *P. c.* subsp. *brasiliensis* ICMP 19477. In both outputs, generated in Geneious 7.0.6 (Biomatters Ltd), the singular read coverage from the mutant genome sequence is represented as blue peaks. The plasmid backbone is shown as a black line and the genes and CDSs represented as green and yellow arrows. The CDSs are labelled as *bla* (ampicillin resistance gene), *tnpA* (transposase), *gfp* (green fluorescence protein marker), *nptII* (kanamycin resistance gene), *gusA* (β-glucuronidase reporter gene)

4.2.6 *Pectobacterium carotovorum* subsp. *brasiliensis* ICMP 19477 mutants unable to inhibit *Pectobacterium atrosepticum* SCRI1043 contained Tn*5* insertions within carbapenem associated genes

The Tn*5* cassette had inserted into CDSs associated with carbapenem production in three of the five mutants analysed using genome sequencing. For example, BLASTn analysis of the DNA sequence flanking the Tn*5* cassette against the genome sequence for *P. c.* subsp. *brasiliensis* ICMP 19477 (Section 2.1.1) showed that the mutant annotated as BF9 was disrupted in the CDS KCO_04647 (Table 2.4). Comparison of KCO_04647 with the nucleotide database in Genbank using BLASTn (Section 2.1.1) showed that it was most similar to *carR* from *P. c.* subsp. *carotovorum* ATCC39048 (ID = 98%, E = 0). CarR is a member of the LuxR type transcriptional regulator family (McGowan et al., 1995), which is located directly upstream of the carbapenem biosynthetic cluster and regulates carbapenem synthesis (Section 1.6.2.2) (McGowan et al., 1995). BF9 was renamed *P. c.* subsp. *brasiliensis carR*$^{-}$, reflecting the insertion site of Tn*5* in this mutant.

In the Tn*5* mutant initially identified as NPFH1, the transposon had inserted into the CDS KCO_03547 (Table 2.4). The protein encoded by KCO_03547 showed greatest similarity to *carI* of *P. c.* subsp. *carotovorum* ATCC39048 (ID = 99%, E = 3e-156,) when compared with the Genbank database using BLASTx (Section 2.1.1). CarI is a LuxI type regulator, which synthesises *N*-AHLs, which are involved in quorum sensing regulation of carbapenem production as well as other cellular processes (Fuqua et al., 1994). As a result, NPFH1 was renamed *P. c.* subsp. *brasiliensis carI*$^{-}$.

The third mutant of interest, IH1, contained a transposon insertion within KCO_21137 (Table 2.4). The protein encoded by this CDS showed greatest similarity to the transcriptional regulator SlyA of *P. c.* subsp. *carotovorum* (E = 3e-98, ID = 100%), when comparing its sequence to the Genbank database using BLASTx (Section 2.1.1). The SlyA/MarR transcriptional regulators have been shown to regulate many processes including carbapenem production (Thomson et al., 1997; Ellison and Miller, 2006). This mutant was renamed *P. c.* subsp. *brasiliensis slyA*$^{-}$.

In the remaining two mutants, the CDSs disrupted by the insertion of the transposon, were seemingly in insertions unrelated to carbapenem production. For example, in NPEA3, Tn*5* was inserted into KCO_20642. This CDS had no similarities to known genes in the Genbank database, and encodes a protein with greatest similarity to a hypothetical protein from *Pseudomonas aeruginosa* (ID = 76%, E = 7e-153). This mutant was known as *P. c.* subsp. *brasiliensis hypo*$^{-}$. Furthermore, mutant LE3 contained a Tn*5* insertion within KCO_11260, which encoded a protein with greatest similarity to a

potential membrane protein of *P. c.* subsp. *carotovorum* (ID = 100%, E = 0). The protein belongs to a superfamily of bacterial proteins of unknown function, with only a few being described as membrane proteins (E = 1.15e-45). The mutant was known as *P. c.* subsp. *brasiliensis mp⁻*.

4.2.7 *Pectobacterium carotovorum* subsp. *brasiliensis* ICMP 19477 mutants harbouring Tn*5* insertions within carbapenem-associated genes were unable to inhibit *Eschericia coli* super-sensitive

The five mutants described above were screened for their capacity to inhibit ESS (Section 2.1.21). This screen was not used when initially selecting the mutants, as the focus of the study was to identify mutants of *P. c.* subsp. *brasiliensis* ICMP 19477 that could not inhibit the growth of *P. atrosepticum* SCRI1043. Instead, the ESS screen was used to support the evidence above, which indicated that genes associated with carbapenem biosynthesis had been inactivated and that production of this β-lactam was probably disabled (or significantly reduced) in at least three of the mutants. The screen revealed that the *carR, carI* and *slyA* mutants were unable to inhibit the growth of the ESS (Figure 4.6 C, E, F). In contrast, the hypothetical protein mutant *P. c.* subsp. *brasiliensis hypo⁻* and the membrane protein mutant *P. c.* subsp. *brasiliensis mp⁻* still produced a zone of inhibition on ESS (Figure 4.6 B and D). This indicated that the *carR, carI* and *slyA* mutants were unable to produce a β-lactam. Furthermore, given these mutants were also unable to inhibit the growth of *P. atrosepticum* SCRI1043, it was considered the production of this β-lactam was required for inhibition of both ESS and *P. atrosepticum* SCRI1043. The two remaining mutants did not appear to be required for production of the carbapenem, although the reduced zone of inhibition produced by *P. c.* subsp. *brasiliensis mp⁻* suggested it may have some more subtle role in production or secretion of the β–lactam.

To confirm the insertion sites for Tn*5* in the three mutants of interest, *P. c.* subsp. *brasiliensis carR⁻, carI⁻* and *slyA⁻,* PCR was conducted using the Tn*5* primer and a primer designed to bind upstream/downstream of the putative insertion site (Table 2.3). The sequences confirming the insertion sites within these mutants are given in Appendix C, the insertion sites are listed in Table 2.4.

The *carR, slyA* and *carI* mutants were subsequently tested in a supernatant antagonism time curve (Section 2.1.30). All mutants failed to produce a zone of inhibition at any sampling time during the assay (data not shown) whereas the WT showed the same production at late exponential – early stationary phase as in previous experiments (data not shown). These results confirmed that antibiotic production had been inactivated in these mutants.

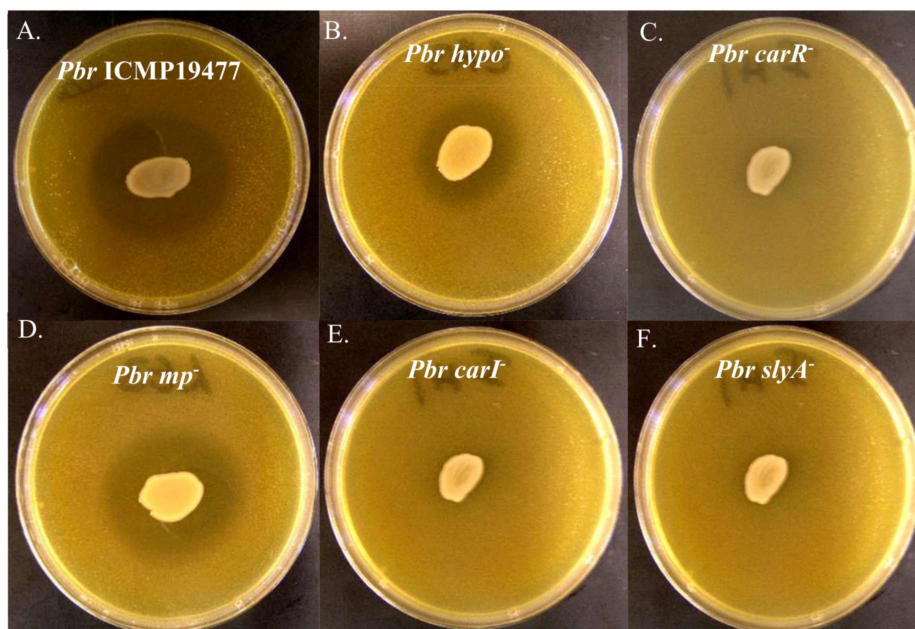


Figure 4.6. *In vitro* growth inhibition of *E. coli* supersensitive strain (ESS) by Tn*5* mutants of *P. c.* subsp. *brasiliensis* (*Pbr*) ICMP 19477.

A: Colonies of *Pbr* ICMP 19477, as well as the five transposon mutants that were unable to inhibit the growth of *P. atrosepticum* (*Pba*) SCRI1043) were streaked onto LBA plates seeded with ESS (as described in Section 2.1.21). The plates were incubated at 28 °C for 24 h and observed for the presence of zones of inhibition. The transposon mutants tested were known to contain insertions within genes encoding the following proteins: **B:** KCO_20642, hypothetical protein; **C:** KCO_04647, CarR transcriptional regulator of carbapenem synthesis; **D:** KCO_11260, membrane protein; **E:** KCO_03547, QS homoserine-lactone synthetase; KCO_21137, **F:** SlyA secondary metabolite transcriptional regulator.

4.2.8 Structural prediction for CarR, CarI and SlyA in *Pectobacterium carotovorum* subsp. *brasiliensis* ICMP 19477

To further understand the possible function of CarR, CarI and SlyA proteins, the secondary structures were predicted using ProteinPredict (Rost et al., 2004) (Version 1.0.88), I-TASSER (Zhang, 2008) (Version 1.0.88) and RaptorX (Källberg et al., 2012) (Table 2.1). These programs provide structural annotations, as well as predictions of secondary and tertiary structures as well as functional predictions.

The *carR* gene was predicted by RaptorX to encode a protein that comprised 45% helices, 11% β-sheets and 42% coils. Coils refers to regions of the secondary structure that do not form recognised secondary structures such as helices, sheets and turns (Kabach and Sander, 1983). 11% of the structure was predicted to be disordered. The structure was predicted to contain nine helices, between the amino acid residues 2-15, 42-50, 58-65, 85-91, 125-154, 171-181, 186-193, 197-211 and 216-225. A helix-turn-helix motif was also predicted between the amino acid residues 197-211. ProteinPredict analysis also identified dinucleotide binding regions within the protein structure (c-score 0.02). The c-score refers to the confidence of the predicted structure, with the value typically ranging from -5 to 2 (with a value of 2 representing the most confident prediction). This was confirmed by I-TASSER, which predicted an 18 residue binding site, predicted to bind OHHL (c-score 0.58). Two nucleic acid binding regions, of nine and seven residues respectively were also predicted (c-score 0.08, c-score 0.03). These features, plus the relatively high helices content and helix-turn-helix motif, were consistent with a putative DNA binding protein. Furthermore, a high proportion of coils was predicted. This suggests that the protein is flexible and is able to bind other molecules (Smith et al., 1996). This would be consisted with a quorum sensing receptor, which binds OHHL as well as DNA.

RaptorX analysis predicted that the *slyA*-type gene was predicted to encode a protein with a secondary structure comprising of 63% helices, 7% β-sheet and 28% coils. It was predicted that there were six helices between the amino acid residues 5-24, 31-41, 49-56, 60-72, 97-114 and 120-142. The high proportion of helices predicted to form the protein is indicative of a DNA-binding protein (Chirgadze et al., 2009). It is also predicted that the protein contains a helix-turn-helix motif between the amino acid residues 59-95, which is also a signature motif of DNA binding proteins. The I-TASSER program predicted that the protein contained a cluster of 15 amino acid residues that form a nucleic acid binding site (c-score 0.57). Binding site analysis, using the RaptorX binding site analysis feature, confirmed that there are six regions present with affinity for nucleic acid bases ($p = 1.43e\text{-}04$). No binding sites for OHHL were found. This concurs with the findings of McGowan et al., (2005), that the Hor regulatory network is not regulated by QS but acts concurrently in the carbapenem producing strain *P. c.* subsp. *carotovorum* ATTn10. The protein structure showed greatest similarity and alignment to the SlyA transcriptional regulator (c-score 0.32).

Finally, ProteinPredict predicted that the *carI* gene was predicted to encode a protein made up of four helices and seven strands. RaptorX predicted the helices to be located between the amino acid residues 14-32, 78-88, 118-133 and 148-153. The secondary structure was predicted to comprise 23% helices, 26% β-sheets and 50% coils. The relatively low level of helices suggests that the protein is not involved in DNA binding. Furthermore, the high proportion of coils suggests that the protein is flexible

and is able to bind intermediates (Smith et al., 1996). The cellular location was determined to be within the cytoplasm (c-score 0.41). This predicted structure, as well as the resultant characteristics are consistent with a synthetase molecule.

Figure 4.7 shows the predicted tertiary structures of the encoded proteins, predicted by I-TASSER server (Zhang, 2008; Roy et al., 2010; Yang et al., 2015).

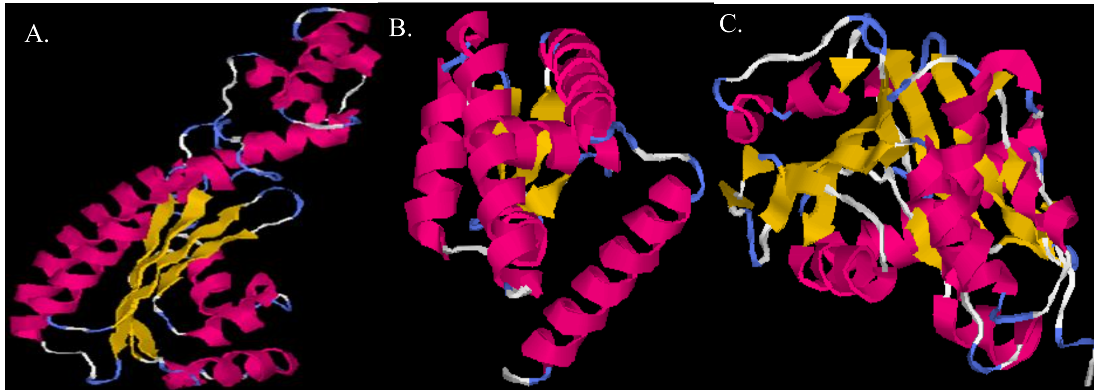


Figure 4.7. Structural predictions for the proteins encoded by the *carR, slyA* and *carI* genes disrupted by transposon mutagenesis.
A: KCO_04647 (*carR*, c-score 0.02). The predicted structure contains a large proportion of helices indicative of a DNA binding protein. **B**: KCO_21137 (*slyA*, c-score 0.32). The predicted structure has a helix-turn-helix motif, which is common amongst DNA binding proteins. **C**: KCO_20642 (*carI*, c-score 0.41). This structure contains a large proportion of coil motifs, indicating a flexible molecule able to bind intermediate molecules. The C score refers to the confidence of the predicted structure. The value typically ranges from -5 to 2, with a value of 2 representing the most confident prediction.

4.2.9 Growth of the non-inhibiting transposon mutants *in vitro* and *in planta*

To examine the role of *slyA, carR* and *carI* in the competitive fitness of the bacteria, competition assays were performed in which the capacity of the mutants to inhibit growth of *P. atrosepticum* SCRI1043 was compared to that of the WT in co-inoculations both *in vitro* and in tubers. Before competition assays were conducted however, the growth of the non-inhibiting mutants was compared to that of the WT to ensure that transposon mutagenesis had not affected the growth of the mutants. *In vitro* growth assays, conducted as described in Section 2.1.22, showed that growth of the *carR, slyA* and *carI* mutants was not significantly different to the growth of the WT ($p < 0.22$, F-test) on solid agar

plates (Figure 4.8). The only variation was at 32 hpi, when the average cell count for the WT was lower than for the other strains ($p < 0.001$, F-test). This was considered an anomaly due to error in plating, especially as a second independent experiment showed no effect of the Tn*5* insertions on growth of the mutants (data not shown).

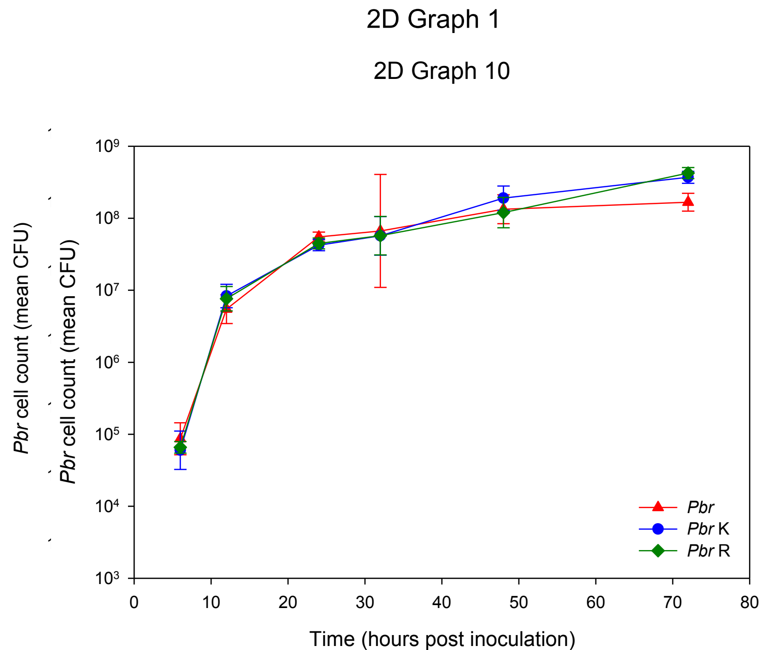


Figure 4.8. Growth of *P. c.* subsp. *brasiliensis* (*Pbr*) ICMP 19477 transposon mutants on solid agar plates compared to WT.

The graph shows the growth (mean CFUs) of the WT as well as the Km antibiotic tagged strain (*Pbr* K) and the Tn*5* mutants of interest: *Pbr carR*⁻, *Pbr slyA*⁻ and *Pbr carI*⁻. The assay was conducted over 48 h post inoculation with 10^4 CFUs/ml of each bacteria used as initial inoculum (as described in Section 2.1.22). The mean CFUs represent the mean over three independent test plates. Error bars are 95% confidence limits.

A growth curve of the same strains conducted in potato tubers (Section 2.1.24). Over the course of the assay, the mutants were not reduced in growth compared to the WT (Figure 4.9). Results were consistent in a second independently conducted experiment, except the drop in growth rate of *P. c.* subsp. *brasiliensis carI*⁻ at 4 dpi was not seen (data not shown). Therefore, this is unlikely to be a reproducible observation.

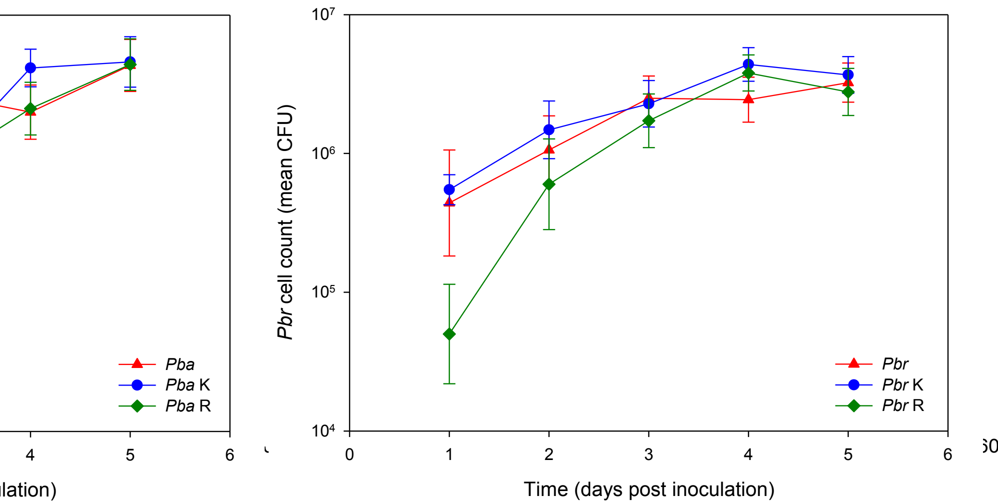


Figure 4.9. Growth of *P. c.* subsp. *brasiliensis* (*Pbr*) ICMP 19477 transposon mutants in potato tubers ('Ilam Hardy').

The graph shows the growth (mean CFUs) of the WT as well as the kanamycin antibiotic-tagged strain (*Pbr* K) and the Tn*5* mutants of interest: *Pbr carR*$^-$, *Pbr slyA*$^-$ and *Pbr carI*$^-$. The assay was conducted over a 48 h period with 10^4 CFUs/ml of each bacteria used as an initial inoculum (as described in Section 2.1.24). The mean CFUs represent the mean over three independent test plates. Error bars are 95% confidence limits.

4.2.10 Inactivation of *carR* results in reduced inhibition of *Pectobacterium atrosepticum* SCRI1043 in co-inoculation assays on solid media

As the growth of the mutants did not appear to be affected by the process of mutagenesis per se, co-inoculation assays were performed with the mutants on solid agar plates (Section 2.1.26). These assays demonstrated that when the *carR* mutant was co-inoculated with *P. atrosepticum* SCRI1043, the growth of *P. atrosepticum* SCRI1043 was similar to its growth when cultured alone and the cell density reached levels higher than when the bacterium was co-inoculated with the WT *P. c.* subsp. *brasiliensis* ICMP 19477 strain (Figure 4.10 A). The growth of the *P. c.* subsp. brasiliensis ICMP 19477 strains was not affected when co-inoculated with *P. atrosepticum* SCRI1043, however (Figure 4.10 B). This indicated that inactivation of *carR* resulted in reduced inhibition of *P. atrosepticum* SCRI1043 by

P. c. subsp. brasiliensis ICMP 19477 under these conditions. Results were consistent in a second independently conducted experiment (Appendix C).

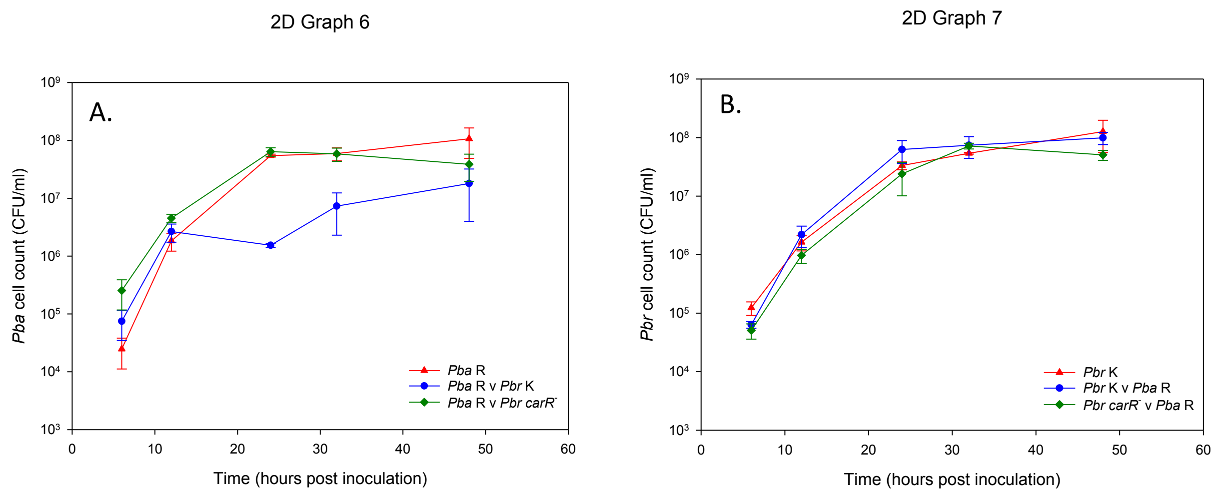


Figure 4.10. The effect of co-inoculation with *P. c.* subsp. *brasiliensis* (*Pbr*) ICMP 19477 and *Pbr carR*⁻ on growth of *P. atrosepticum* (*Pba*) SCRI1043 on solid agar plates (MMA).

A: A graph showing the growth (mean CFUs) of the *Pba* SCRI1043 rifampicin (*Pba* R) resistant strain over a 48 h period at 28°C when inoculated onto MMA in isolation or when co-inoculated with *Pbr* ICMP 19477 tagged with the Km resistance determinant (*Pbr* K), or the *carR* mutant at a 1:1 ratio. **B**: A graph showing the growth (mean CFUs) of *Pbr* strains when cultured in isolation on solid agar plates or in co-inoculations with *Pba* R. For each graph, the mean CFUs were calculated for each treatment from triplicate assays (solid line error bars represent the standard deviation.

The CI of the *P. c.* subsp. *brasiliensis* ICMP 19477 strains was subsequently calculated (Section 2.1.29) to quantify the impact of inactivating *carR* on the competitive fitness of *P. c.* subsp. *brasiliensis* ICMP 19477. Twenty four hpi, when *P. c.* subsp. *brasiliensis* strains were reaching late exponential phase, the CI for the WT rose from approximately 1.0 to 2.0 indicative of strong competitive fitness of this bacterium compared to *P. atrosepticum* SCRI1043. In contrast, *P. c.* subsp. *brasiliensis carR*⁻ showed only weak competitive fitness compared to *P. atrosepticum* SCRI1043, with the CI of the *carR* mutant reaching only 0.5 during late exponential, early stationary phase (Appendix C). Results were consistent in a second independently conducted experiment (data not shown). These results are consistent with the production of the carbapenem having an involvement in the greater competitive fitness of *P. c.* subsp. *brasiliensis* ICMP 19477 on solid agar plates.

4.2.11 Inactivation of *carR* results in reduced inhibition of *Pectobacterium atrosepticum* SCRI1043 in co-inoculation assays in potato tubers

In similar competition assays in potato tubers (Section 2.1.28), the growth of *P. atrosepticum* SCRI1043 was greatly reduced when co-inoculated with *P. c.* subsp. *brasiliensis* K in potato tubers (Figure 5.12 A). However when co-inoculated with the *carR* mutant, the growth of *P. atrosepticum* SCRI1043 was similar to its growth when inoculated into tubers in isolation. The growth of *P. c.* subsp. *brasiliensis* ICMP 19477 strains was not affected by co-inoculation with *P. atrosepticum* SCRI1043 (Figure 5.12 B). Results were consistent in a second independently conducted experiment (Appendix C), confirming that inactivation of *carR* resulted in reduced inhibition of its competitor in co-inoculated potato tubers as well as on solid agar plates (although the impact was far more dramatic in potato tubers). The CI of the mutant was also reduced dramatically from that of WT in co-inoculations in tubers, dropping from between 4.0 and 5.0 for the WT to 1.0–2.5 for the *carR* mutant (Appendix C).

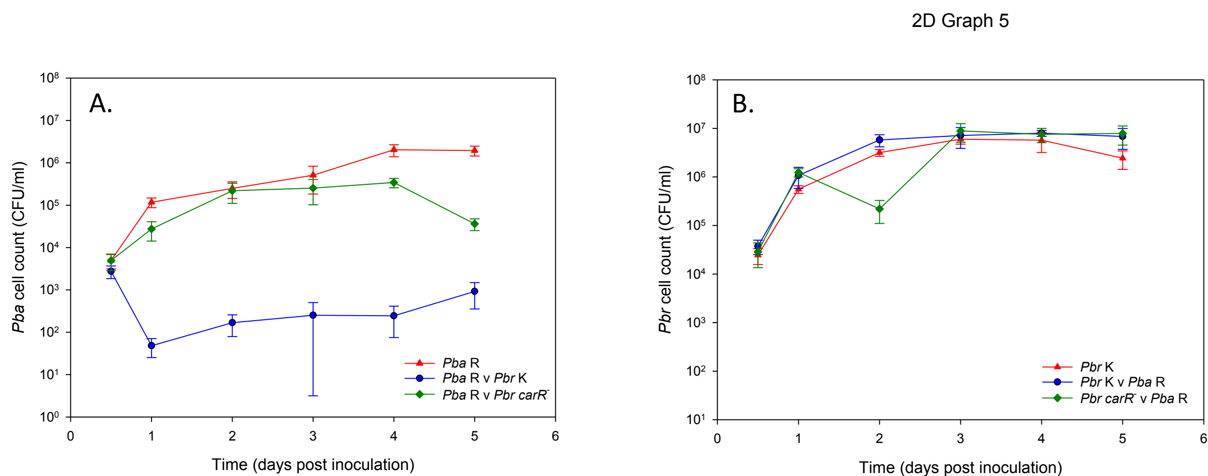


Figure 4.11. The effect of co-inoculation with *P. c.* subsp. *brasiliensis* (*Pbr*) ICMP 19477 and *Pbr carR*⁻ on growth of *P. atrosepticum* (*Pba*) SCRI1043 in potato tubers ('Ilam Hardy').

A: A graph showing the growth (mean CFUs) of *Pba* SCRI1043 Rif (*Pba* R) resistant strain over a 48 h period at 28°C when inoculated into potato tubers in isolation or co-inoculated with *Pbr* ICMP19477 tagged with Km resistance (*Pbr* K), or the *carR* mutant *Pbr carR*⁻ (at a 1:1 ratio). **B**: A graph showing the growth (mean CFUs) of *Pbr* K when cultured in isolation in to potato tubers or in co-inoculations with *Pba* R as well as *Pbr carR*⁻ co-inoculated with *Pba* R (at a 1:1 ratio). For each graph, the mean CFUs were calculated for each treatment from triplicate assays (solid line), error bars represent the standard deviation.

4.2.12 Inactivation of *carR* does not result in reduced inhibition of *Pectobacterium atrosepticum* SCRI1043 in liquid cultures

To verify the effects of inactivating *carR* on competitive fitness of *P. c.* subsp. *brasiliensis* ICMP 19477 with the results of co-inoculation experiments with the WT (as discussed in Chapter 3), competition assays were repeated in liquid cultures (Section 2.1.27). These experiments had previously demonstrated that *P. c.* subsp. *brasiliensis* ICMP 19477 could not inhibit the growth of *P. atrosepticum* SCRI1043 (Section 3.2.4). This was confirmed in these assays and demonstrated that the disruption of *carR* had not impact on the interaction between *P. c.* subsp. *brasiliensis* ICMP 19477 and *P. atrosepticum* SCRI1043 under these conditions (Figure 4.12 A). The growth of the *P. c.* subsp. *brasiliensis* ICMP 19477 strains was not affected when co-inoculated with *P. atrosepticum* SCRI1043 (Figure 4.14 B). The CI of neither strain reached greater than 1.0, indicative of little or no greater competitive fitness of *P. c.* subsp. *brasiliensis* ICMP 19477 over *P. atrosepticum* SCRI1043 in liquid cultures (Appendix C). These results indicate that the competition mechanism, the carbapenem molecule proposed to be regulated by CarR, is not responsible for the weak competition between *P. c.* subsp. *brasiliensis* ICMP 19477 and *P. atrosepticum* SCRI1043 in liquid assays.

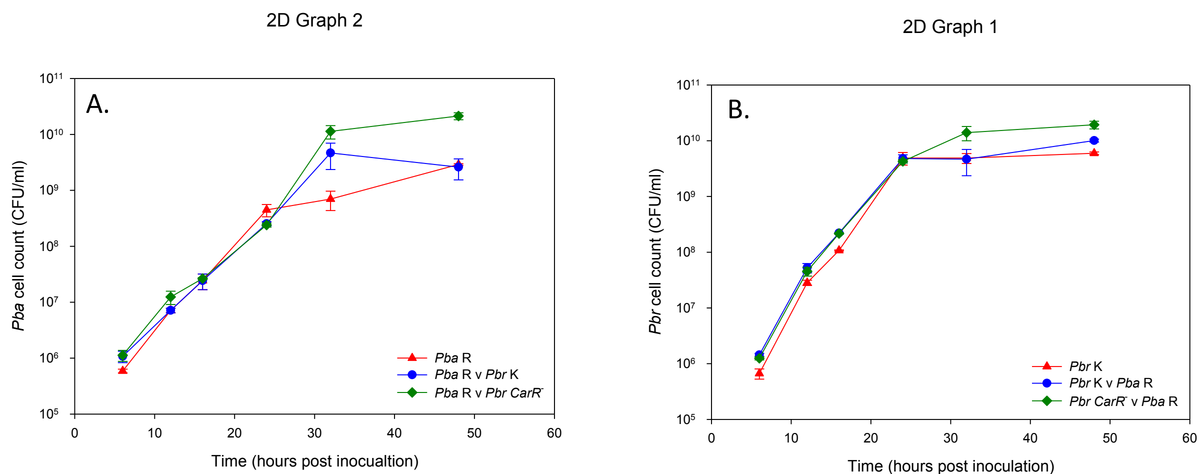


Figure 4.12. The effect of co-inoculation with *P. c.* subsp. *brasiliensis* (*Pbr*) ICMP 19477 and *Pbr carR*⁻ on growth of *P. atrosepticum* (*Pba*) SCRI1043 in liquid cultures (MM).

A: A graph showing the growth (mean CFUs) of the *Pba* SCRI1043 Rif (*Pba* R) resistant strain over a 48 h period at 28°C when inoculated into MM in isolation or when co-inoculated with *Pbr* ICMP 19477 tagged with Km resistance (*Pbr* K) or the *carR*⁻ mutant *Pbr carR*⁻ (at a 1:1 ratio). **B**: A graph showing the growth (mean CFUs) of *Pbr* K when cultured in isolation on solid agar plates or in co-inoculations with *Pba* R as well as *Pbr carR*⁻ co-inoculated with *Pba* R (at a 1:1 ratio). For each graph, the mean CFUs

were calculated for each treatment from triplicate assays (solid line), error bars represent the standard deviation.

4.2.13 Complementation of the *Pectobacterium carotovorum* subsp. *brasiliensis* ICMP 19477 *carR* mutant, with a copy of the WT gene restored production of the antimicrobial molecule

Complementation studies (Section 2.1.20) were conducted to confirm the involvement of *carR* in the competitive fitness of *P. c.* subsp. *brasiliensis* ICMP 19477. First, a derivative of pTRB32oriT was constructed containing a 783 bp PCR fragment encompassing the *carR* gene (Sections 2.1.10-2.1.14, 2.1.16). This recombinant plasmid was named pTRB32oriTcarR. The fragment containing the *carR* gene was amplified using primers carR_compl F and carR_compl R (Table 2.3). The plasmid was then transferred to the *carR*$^-$ mutant via conjugation (Section 2.1.17) and the presence of the appropriate plasmid was confirmed in transconjugants by PCR (Section 2.1.10), using construct-specific primers. An amplicon of 783 bp was also produced in all selected transconjugants using carR_compl F and carR_compl R (Table 2.3). The transconjugates were confirmed as *P. c.* subsp. *brasiliensis* ICMP 19477, using primers Pbr NZ F and Pbr NZ R, which amplify a product within *P. c.* subsp. *brasiliensis* ICMP 19477 but not the *E. coli* strains used in the conjugation (Figure 4.15). The authenticity of the plasmids was further confirmed by sequencing of the plasmid inserts using construct and plasmid-specific primers (Table 2.3, Section 2.1.14). The construct sequence is given in Appendix C. The resultant transformants were designated *P. c.* subsp. *brasiliensis carR*$^-$*::pTRB32oriTcarR* (Table 2.2).

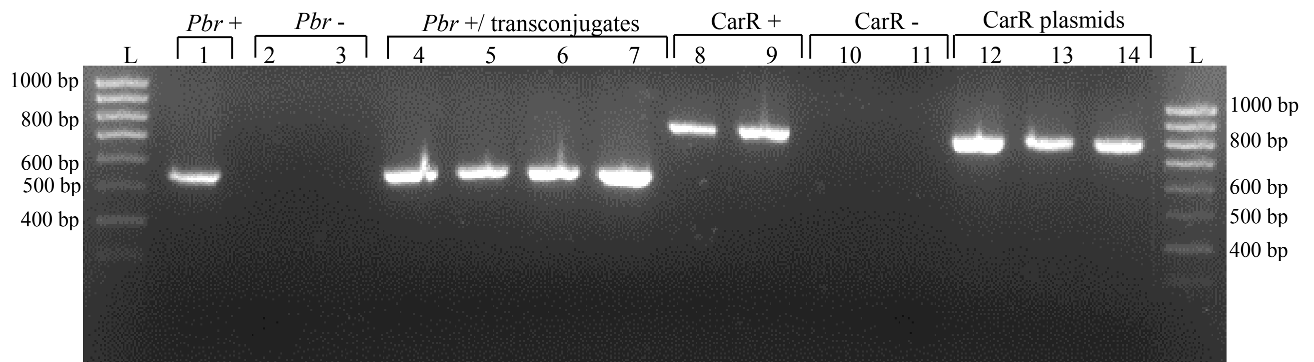


Figure 4.13. PCR amplicons obtained from transconjugants of *P. c.* subsp. *brasiliensis* (Pbr) *carR*$^{-}$ to verify their authenticity prior to complementation studies.

L, DNA ladder (Hyper ladder 100 bp, Bioline); Lane 1, *Pbr* NZ primer pair positive control (*Pbr* ICMP 19477 gDNA); Lane 2, *Pbr* NZ pair primer negative control (*E. coli* donor gDNA) Lane 3, *Pbr* NZ primer pair negative control (*E. coli* helper gDNA); Lane 4, *Pbr* NZ primer pair positive control (*Pbr* ICMP 19477 *carR*$^{-}$ recipient strain); Lanes 5-7, *Pbr* NZ primer pair conjugates 1-3; Lane 8, carR primer pair positive control (*Pbr* ICMP 19477 gDNA); Lane 9, carR primer pair positive control (donor plasmid); Lane 10, carR primer pair negative control (helper plasmid), Lane 11, carR primer pair negative control (*Pbr* ICMP19477 *carR*$^{-}$ recipient strain plasmid extraction); Lanes 12-14 carR primer pair conjugates gDNA 1-3; L, DNA ladder. Primers are listed in Table 2.2.

Complementation assays were subsequently performed using *P. c.* subsp. *brasiliensis carR*$^{-}$ *::pTRB32oriTcarR* by adding IPTG to the LBA plates on which *P. c.* subsp. *brasiliensis carR*$^{-}$ *::pTRB32oriTcarR* was grown overnight prior to performing the antagonism assay (Section 2.2.4). Induction resulted in full complementation of the WT phenotype, as both *P. c.* subsp. *brasiliensis carR*$^{-}$ *::pTRB32oriTcarR* and the WT produced zones of inhibition when plated onto lawns of *P. atrosepticum* SCRI1043. Visually, it appeared that the complemented stain produced a larger zone than the *P. c.* subsp. *brasiliensis* ICMP 19477 WT (Figure 4.16). In contrast, the *P. c.* subsp. *brasiliensis carR*$^{-}$ and a derivative of the mutant containing an empty copy of the pTRB32oriT plasmid, *P. c.* subsp. *brasiliensis carR*$^{-}$*::pTRB32oriT* (Table 2.2), did not inhibit the growth of *P. atrosepticum* SCRI1043. These results confirmed that *carR* is involved in the inhibition of *P. atrosepticum* SCRI1043 in antagonism assays.

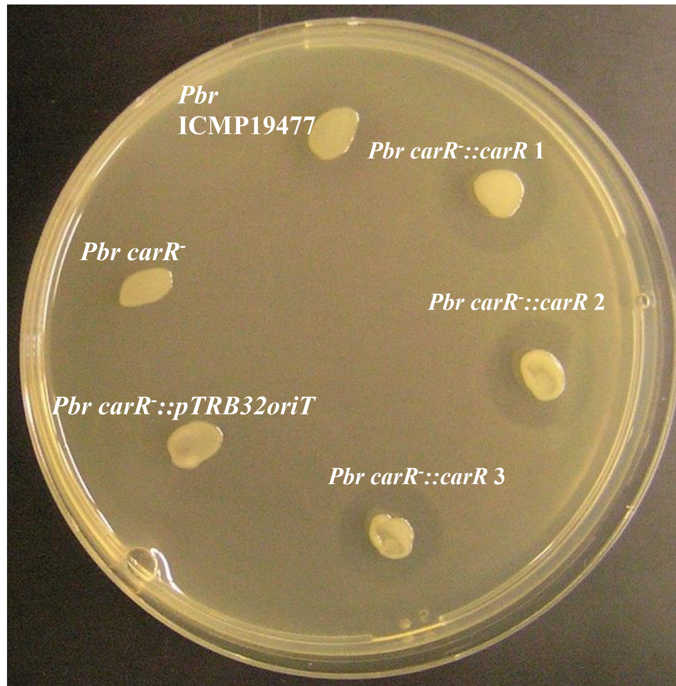


Figure 4.14. Complementation of the *P. c.* subsp. *brasiliensis* (*Pbr*) *carR*⁻ mutant with a plasmid containing a copy of the WT *carR* gene restores inhibition of *P. atrosepticum* (*Pba*) SCRI1043. Colonies of the test strains were streaked onto a lawn of *Pba* SCRI1043, seeded in LB agar (LBA). Plates were incubated at 28 °C for 24 h and then scored for visible zones of inhibition (Section 2.1.21). The complemented strain, *Pbr carR⁻::pTRB32oriTcarR,* was grown on LBA containing IPTG overnight, prior to the assay. IPTG was required for induction of the plasmid construct. As a negative control, a *Pbr carR*⁻ mutant, containing an empty copy of pTRB32oriT (*Pbr carR::pTRB32oriT*), was also included. This strain was also grown on LBA containing IPTG prior to the assay.

4.2.14 The growth of *Pectobacterium carotovorum* subsp. *brasiliensis carR⁻ ::pTRB32oriTcarR* on solid agar plates is not reduced compared to WT

Prior to co-inoculation studies using *P. c.* subsp. *brasiliensis carR⁻::*pTRB32oriTcarR, growth assays were conducted (Section 2.2.22). On solid agar plates throughout the assay there were little or no differences between the WT and recombinant strains (Figure 4.17).

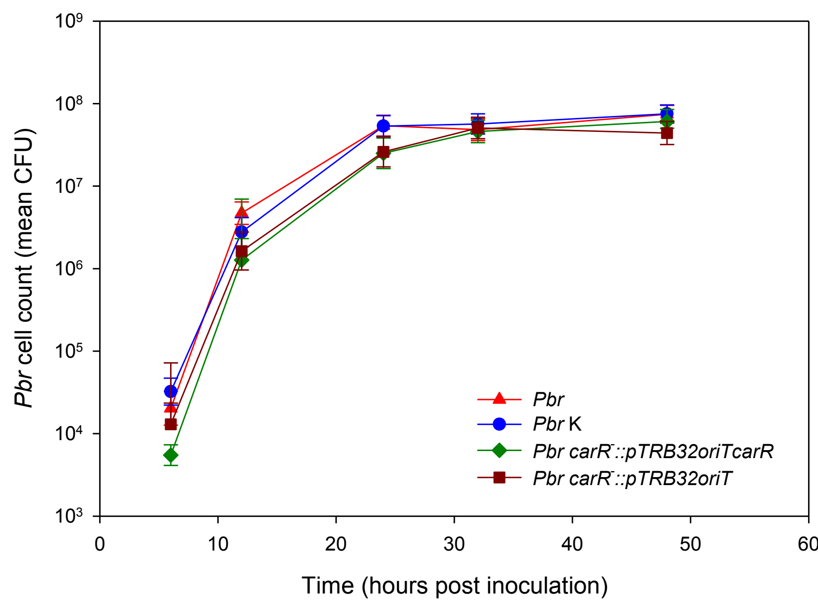


Figure 4.15. The growth of the *P. c.* subsp. *brasiliensis* (*Pbr*) ICMP 19477 Km resistant tagged mutant (*Pbr* K), the *carR* complemented strain *Pbr carR⁻::pTRB32oriTcarR* and the complementation plasmid control strain *Pbr carR⁻::pTRB32oriT* strain compared to the *P. c.* subsp. *brasiliensis* ICMP 19477 WT on solid agar plates.

Growth assays were conducted on MMA, over 48 h. The mean CFU values of three plates are given. Error bars represent 95% confidence limits.

4.2.15 *Pectobacterium carotovorum* subsp. *brasiliensis carR⁻::pTRB32oriTcarR* is restored in the WT competition phenotype in *in vitro* competition assays

Competition assays were conducted as described in Section 2.1.26 using overnight cultures prepared with the addition of IPTG (Section 2.4). Furthermore, IPTG was also added to the MMA plates used directly for the competition assays. In these assays, the growth of *P. atrosepticum* SCRI1043 was not greatly reduced when co-inoculated with *P. c.* subsp. *brasiliensis carR*$^-$ or *P. c.* subsp. *brasiliensis carR*$^-$ *::pTRB32oriT* when compared to the singularly inoculated *P. atrosepticum* R culture. In contrast, the growth of *P. atrosepticum* SCRI1043 co-inoculated with *P. c.* subsp. *brasiliensis carR*$^-$*::pTRB32oriTcarR* or *P. c.* subsp. *brasiliensis* K was reduced significantly compared to the singularly inoculated *P. atrosepticum* R (Figure 4.18 A). Growth of the *P. c.* subsp. *brasiliensis* ICMP 19477 strains was not negatively impacted when co-inoculated with *P. atrosepticum* SCRI1043 (Figure 4.18 B). Results were consistent in a second, independently conducted experiment (Appendix C). The CI calculated for *P. c.* subsp. *brasiliensis carR*$^-$*::pTRB32oriTcarR* reached between 2.0 and 3.5 between late exponential and early stationary, higher than that of the WT (1.5 -2.5), whereas the *carR* mutant and the derivative containing *pTRB32oriT* remained low (between 0 and 1.2) (Appendix C). These results showed that with expression of the WT *carR* gene *in trans*, the competitive fitness of *P. c.* subsp. *brasiliensis carR*$^-$ was restored to WT levels upon co-inoculation with *P. atrosepticum* SCRI1043. The restoration of the WT phenotype in *P. c.* subsp. *brasiliensis carR*$^-$*::pTRB32oriTcarR* confirmed that *carR* was not only involved in the production of a carbapenem with activity against *P. atrosepticum* SCRI1043, but production of this antimicrobial contributed strongly to the competitive fitness of the bacterium in co-inoculation experiments. Due to the requirement for induction of the plasmid with IPTG, the complemented strain could not be used in tuber assays.

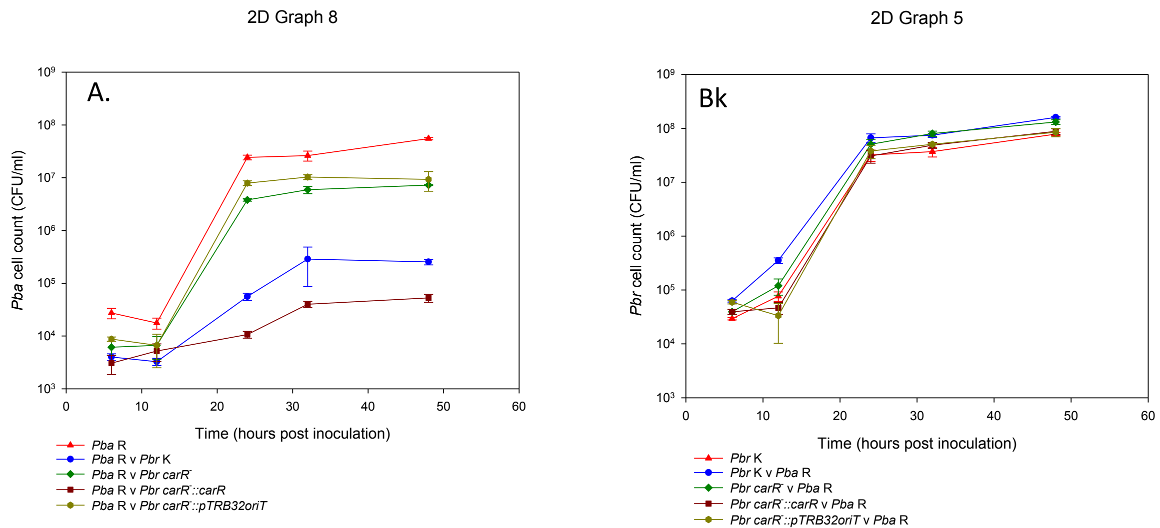


Figure 4.16. The effect of co-inoculation with *P. c.* subsp. *brasiliensis* (*Pbr*) ICMP 19477 *carR*⁻ complemented strain *Pbr carR⁻::pTRB32oriTslyA* on growth of *P. atrosepticum* (*Pba*) SCRI1043 on solid agar plates (MM).

A: A graph showing the growth (mean CFUs) of the *Pba* SCRI1043 rifampicin (*Pba* R) resistant strain over a 48 h period at 28 °C when inoculated onto MMA in isolation or co-inoculated with *Pbr* ICMP19477 tagged with the Km resistance determinant (*Pbr* K), *Pbr carR⁻::pTRB32oriTcarR* or *Pbr carR⁻ ::pTRB32oriT* containing an empty copy of the pTRB32oriT plasmid (at a 1:1 ratio). **B**: A graph showing the growth (mean CFUs) of *Pbr* K when cultured in isolation on solid agar plates as well as the *Pbr* strains in co-inoculation with *Pba* R (at a 1:1 ratio). For each graph, the mean CFUs were calculated for each treatment from triplicate assays (solid line), error bars represent the standard deviation.

4.3 Discussion

Pectobacterium carotovorum subsp. *brasiliensis* ICMP 19477 produces a carbapenem that inhibits growth of *P. atrosepticum* SCRI1043 and confers greater competitive fitness to the producer in co-inoculations with its close relative both *in vitro* and in potato tubers. Carbapenems are produced by a plethora of enterobacteriacae including the plant pathogens *Serratia* and *Erwinia* (Parker et al., 1982). The soil dwelling bacteria *Streptomyces* also produce a wide range of carbapenem molecules. For example, thienamycin, a complex carbapenem with a broad spectrum of activity produced by *Streptomyces* MA4297 (Kahan et al., 1979), olivanic acid (Brown et al., 1979) and carpetimycins (Nakayama et al., 1980). Carbapenems have previously been shown to have activity against other microbes and have been utilised in medicine to combat human infection by *Pseudomonas aeruginosa, Staphylococcus aureus* and *Klebsiella pneumoniae* (Nakayama et al., 1980). Yet despite their widespread distribution, the ecological importance of these antimicrobials to plant pathogens has had only limited attention. This study demonstrates that for *P. c.* subsp. *brasiliensis* ICMP 19477, the production of a carbapenem can provide a competitive advantage in its host plant, enabling the bacterium to inhibit the growth of potential competitors in potato tubers.

The importance of carbapenem production to *P. c.* subsp. *brasiliensis* ICMP 19477 was demonstrated by screening a transposon library for loss of activity against *P. atrosepticum* SCRI1043. This screen showed that an insertion in *carR* resulted in complete loss of inhibition against the sensitive strain in an antagonism assay. Subsequent complementation of the *carR* mutation by expressing the WT *carR* gene *in trans*, also restored the ability of *P. c.* subsp. *brasiliensis* ICMP 19477 to inhibit the growth of *P. atrosepticum* SCRI1043 (Figure 4.17). This reaffirmed that *carR*, and therefore carbapenem production, was required for inhibition of *P. atrosepticum* SCRI1043 as *carR* is located immediately upstream of the carbapenem biosynthetic cluster and has been shown to specifically regulate carbapenem production in *P. c.* subsp. *carotovorum* (McGowan et al., 1995).

Activation of the carbapenem operon by CarR occurs when CarR binds to the promoter upstream of *carA* (P_{carA}) that is located in the intrinsic region between *carR* and *carA* (McGowan et al., 2005). The binding of CarR and the resultant transcription of the carbapenem operon occurs when OHHL is present (Welch et al., 2000).

The results of the screen on *P. atrosepticum* SCRI1043 were supported by screening the mutants in a second assay on ESS, which is super-sensitive to β-lactams such as carbapenems. In this assay, *P. c.* subsp. *brasiliensis* ICMP 19477 generated zones of inhibition, but the *carR* mutant did not. As the carbapenem molecule encoded on HAI PbN1-GI65 is the only β-lactam encoded by *P. c.* subsp.

brasiliensis ICMP 19477, the inability of the *carR* mutant to inhibit ESS reaffirmed that *carR* was involved in production of carbapenem and that this antimicrobial was responsible for the inhibition of *P. atrosepticum* SCRI1043.

The experiments using ESS also showed that the production of carbapenem occurred primarily between the late exponential and early stationary phases. This was consistent with the findings of McGowan et al., (1995), who had shown that carbapenem production occurs at this time, which is concurrent with a large bacterial population density. The production of carbapenem so late in growth of the bacterial population raised questions as to the role of this antimicrobial in interactions between the producer and the sensitive strain. Carbapenem production is independent of the presence of a sensitive strain and requires a high population density (McGowan et al., 2005). Therefore, it is likely that the production of this antimicrobial would function to defend the producers established population, rather than to invade a population of the sensitive bacteria. Moreover, the PCWDEs of *Pectobacterium* also occur at late exponential and early stationary phase, when a high population density is reached (Liu et al., 2008). Therefore, production of the carbapenem would enable the bacteria to defend the nutrients released from the infected plant tissue.

The requirement for high bacterial growth in order to initiate carbapenem production is consistent with the observation that *P. c.* subsp. *brasiliensis abc*$^-$ is unable to inhibit *P. atrosepticum* SCRI1043 in *in vitro* antagonism assays. In growth analysis, it was found that *P. c.* subsp. *brasiliensis abc*$^-$ was reduced in growth compared to *P. c.* subsp. *brasiliensis* ICMP 19477 WT (Figure 4.4). Therefore, *P. c.* subsp. *brasiliensis abc*$^-$ may not reach the growth phase in which the antimicrobial is produced. Alternatively, *P. atrosepticum* SCRI1043 may be able to reach a high enough population density, before the mutant has reached the growth stage in which the antimicrobial molecule is produced, so that the antimicrobial is not effective. This suggests that the antagonism assay using *P. atrosepticum* SCRI1043 is not a very sensitive screen for the production of the carbapenem by *P. c.* subsp. *brasiliensis* ICMP 19477, as it is able to resolve the production of the molecule by slow growing strains.

The function of *carR* in inhibition of *P. atrosepticum* SCRI1043 did not necessitate a role in the competitive fitness of *P. c.* subsp. *brasiliensis* ICMP 19477, as observed on agar plates and in tubers in Chapter 3. Co-inoculation experiments with the *carR* mutant subsequently demonstrated the potential ecological benefit of carbapenem production however, as the competitive index of this strain was dramatically reduced compared to the WT in mixed populations. Complementation also resulted in reversion of the mutant phenotype to that of WT, although the CI values for *P. c.* subsp. *brasiliensis carR*$^-$*::pTRB32oriTcarR* were higher than those of the WT (Figure 4.20). The higher CI in *P. c.* subsp.

brasiliensis carR⁻::pTRB32oriTcarR was probably due to the over-expression of *carR* as a result of induction of the re-introduced *carR* gene using IPTG.

Although disruption of *carR* eliminated antagonism on agar plates the competitive fitness of *P. c.* subsp. *brasiliensis* ICMP 19477 was not abolished entirely in either plate assays or in potato tuber competition assays. For example the CI remained 0.5 *in vitro* and 2.0 in potato tubers. This indicated that the production of the antimicrobial was not solely responsible for enabling *P. c.* subsp. *brasiliensis* ICMP 19477 to out-compete *P. atrosepticum* SCRI1043. Genomic analysis of *P. c.* subsp. *brasiliensis* ICMP 19477 has identified the presence of various genes or gene clusters that might contribute to the success of this bacterium such as those predicted to facilitate utilisation of sucrose (Panda et al., 2015b). Furthermore, genomic comparisons between *P. atrosepticum* SCRI1043, *P. c.* subsp. *brasiliensis* PBR1692 and *P. c.* subsp. *carotovorum* WPP14 identified genes which encoded putative enzymes associated with the utilisation of plant cell wall degradation products, which were found in *P. c.* subsp. *brasiliensis* PBR1692 and *P. c.* subsp. *carotovorum* WPP14 but not *P. atrosepticum* SCRI1043 (Glasner et al., 2008). For example, a putative permease enzyme that may import digested polymer, a putative polysaccharide deacetylase and a putative Asp/Glu racemase, which may degrade the digested polymers (Glasner et al., 2008). Thus, it may be that *P. c.* subsp. *brasiliensis* ICMP 19477 is able to utilize a number of nutrients more successfully than *P. atrosepticum* SCRI1043. *Pectobacterium carotovorum* subsp. *brasiliensis* ICMP 19477 may also produce other secondary antimicrobial molecules that contribute to fitness that were not detected using our pipeline for identification, which relied on antagonism assays rather than testing for any other contribution to fitness (i.e. NRPs can act as phytotoxins, not just antimicrobials).

The *carR* mutation conferred reduced fitness to *P. c.* subsp. *brasiliensis* ICMP 19477 when the bacterium was co-inoculated with *P. atrosepticum* SCRI1043 in solid plate and potato tuber assays (Sections 4.2.10 and 4.2.11), however it did not reduce its fitness in liquid cultures (Section 4.2.12). This finding was consistent with the results of co-inoculation experiments in Chapter Three, which showed that inhibition of *P. atrosepticum* SCRI1043 and the competitive advantage of *P. c.* subsp. *brasiliensis* ICMP 19477 was limited to the structured environments of plate and potato tubers (Sections 3.2.4 and 3.2.6). Structured environments have been predicted to be the conditions under which antimicrobial producing strains are dominant, as the antimicrobial molecule is active even if produced at a low concentration (Chao and Levin, 1981; Durrett and Levin, 1997; Gardner et al., 2004).

Although the *carR* mutant showed reduced fitness in both plate and tuber assays, there was a greater difference in the CI values of the WT and *carR⁻* mutant *in planta* than *in vitro*. On solid agar plates, the *P. c.* subsp. *brasiliensis* ICMP 19477 WT achieved CI values of 1.0-2.0 when in co-inoculations with *P.*

atrosepticum SCRI1043 whereas the *P. c.* subsp. *brasiliensis carR*$^{-}$ mutant only reached CI values of ~0.5. In tubers, however, the CI for *P. c.* subsp. *brasiliensis* WT was between 4.0 and 5.0 whereas the CI for the *P. c.* subsp. *brasiliensis carR*$^{-}$ mutant only reached 1.0-2.0. Co-inoculation experiments in Chapter three also showed that *P. c.* subsp. *brasiliensis* ICMP 19477 had greater competitive fitness in tubers than on solid agar plates. This may be due to the increased nutrients available within potato tubers or the type of nutrients available, as such environmental factors are known to influence carbapenem production (McGowan et al., 2005) as discussed in Section 3.3.

Antagonism assays demonstrated that contrary to the initial hypothesis of this thesis, the NRPS cluster harboured by *P. c.* subsp. *brasiliensis* ICMP 19477 was not involved in the antagonism of *P. atrosepticum* SCRI1043 (Figure 4.3). Reaching this conclusion proved difficult however, given that although a mutant in *NRPS1* was still able to produce a zone of inhibition on *P. atrosepticum* SCRI1043, the *ABC* transporter mutant failed to inhibit the growth of *P. atrosepticum* SCRI1043 (Figure 4.1). From these initial experiments, it was proposed that either the mutation in *NRPS1* had not inactivated the NRP or that the ABC transporter had been 'hijacked' for transport of another antimicrobial produced by *P. c.* subsp. *brasiliensis* ICMP 19477. Some antimicrobial synthesis clusters, such as the carbapenem synthesis cluster, do not have associated transporters. Therefore, such molecules must be transported from the producing cell via an unlinked transporter. For example, members of the multi-facilitator transporter (MFS) superfamily actively transport many substrates such as amino acids and drugs and are often unlinked to biosynthesis cluster (Marger and Saier, 1993).

Subsequent assays on ESS clarified that the *abc* transporter mutant was able to inhibit ESS (Figure 4.3). In conjunction with the data for the carbapenem mutants, this confirmed that the NRP and its associated transporter were unlikely to be involved in antagonism of *P. c.* subsp. *brasiliensis* ICMP 19477. Furthermore, growth analysis established that the *abc* transporter mutant had reduced growth compared to WT *in vitro* (Figure 4.4). As initial development of the antagonism assay had shown inoculum density was important in the suppression of *P. atrosepticum* SCRI1043, *P. c.* subsp. *brasiliensis abc* probably could not reach sufficient population density for the antimicrobial to be produced at a high enough concentration to inhibit the growth of *P. atrosepticum* SCRI1043. Alternatively, *P. atrosepticum* SCRI1043 may have reached a sufficiently high population density before the mutant could produce the antimicrobial, enabling the sensitive strain to overcome the antimicrobial effect of the molecule produced by *P. c.* subsp. *brasiliensis* ICMP 19477.

The production of a carbapenem molecule by *P. c.* subsp. *brasiliensis* ICMP 19477, which is active against a competing organism within its ecological niche, is likely to have contributed to the emergence of this pathogen. In recent years, *P. c.* subsp. *brasiliensis* has increased in prevalence on

potato plants (Duarte et al., 2004; van der Merwe et al., 2010; Leite et al., 2014; Werra et al., 2015). This is consistent with population studies of bacterial communities, which predicted that if an antimicrobial producing strain enters a population it will soon increase in prevalence. This is due to the ability to out-compete the established 'sensitive' population (Durrett and Levin, 1997; Riley and Wertz, 2002). Moreover, the production of a carbapenem molecule by both *P. c.* subsp. *brasiliensis* ICMP 19477 and PBR1692 suggests recent emergence. Over time antimicrobial producers tend to lose this ability in favour of a resistant phenotype, as antimicrobial production expends large amounts of energy and resources (Tan and Riley, 1996). This has been confirmed by serial transfer studies (Frank, 1994).

Following the ESS screen of the transposon mutants, it was identified that as well as the three non-inhibiting mutants, one mutant (*P. c.* subsp. *brasiliensis mp*$^-$) produced a reduced zone of inhibition. This suggests that this gene has a subtle influence in the production or secretion of the carbapenem molecule. This mutant would be of interest in future studies to investigate the more subtle inhibition interactions between *P. c.* subsp. *brasiliensis* ICMP 19477 and *P. atrosepticum* SCRI1043.

Although relevant mutants were identified using our transposon library, the development of this library proved more difficult than expected from previous literature on the use of Tn*5* libraries in SRE (Holeva et al., 2004; Roh et al., 2010). In particular, the delivery vector (pFJA1819) proved difficult to cure from the transposants, and a number of mutants thought to have lost the plasmid due to their sensitivity to ampicillin were shown to have the plasmid integrated within the genome (Figure 4.5). Retention of the plasmid by a large number of mutants may explain the larger than expected number (42) of non-inhibiting mutants, as a similar study in *P. c.* subsp. *carotovorum* Pcc21 identified only five non-producer mutants from a similarly sized transposon library (Roh et al., 2010). Firstly, retention of the plasmid was likely to increase the metabolic load on the mutant, possibly affecting secondary metabolism (resulting in reduced production of the antimicrobial and a non-producing phenotype). Secondly, as the plasmid backbone contains an Amp resistance gene, retention of the plasmid may have caused loss of the antibiotic phenotype due to degradation of the antimicrobial molecule produced by *P. c.* subsp. *brasiliensis* ICMP 19477 (i.e. the β-lactamase activity of the Amp genes would degrade the predicted carbapenem molecule).

It has previously been described that the genetic manipulation of *P. c.* subsp. *brasiliensis* ICMP 19477, in order to produce targeted deletion mutants, was difficult (Preetinanda Panda, 2014). This difficulty was predicted to result from the presence of two restriction modification systems within the genome of *P. c.* subsp. *brasiliensis* ICMP 19477 (Preetinanda Panda, 2014), which encode the restriction modification system. Multiple restriction modification systems were also predicted to be encoded by

D. solani (Garlant et al., 2013). Perhaps future studies requiring directed mutagenesis of *P. c.* subsp. *brasiliensis* ICMP 19477 might benefit from utilising a restriction modification system inhibitor such as the Type One™ Restriction Inhibitor (Epicentre Biotechnologies). The inhibitor is a phage-derived protein 'Ocr' (Walkinshaw et al., 2002), which blocks the binding site of the type I restriction modification system and therefore prevents cleavage of the transformed DNA. Alternatively, a different transposon mutagenesis approach might be considered. For example, Roh et al., (2011) utilised a method that did not require a transposon plasmid. Rather a transposon and transposase enzyme complex was directly electroporated into the *P. c.* subsp. *carotovorum* cells. Such constructs are available commercially such as the Ez-Tn*5*™<R6Kγori/Kan-2>Tnp system (Epicentre Biotechnologies). This system has the R6Kγ origin of replication, an antibiotic resistance marker for selection (i.e. Km resistance) and a transposase that is activated within the bacterial cell encoded on a transpososome (Goryshin et al., 2000; Hoffman et al., 2000). Following electroporation of the construct into the recipient cells and selection on appropriate antibiotics, only cells that had integrated the transposon would grow, avoiding the possibility that the plasmid backbone would be retained through concatenation.

As described in Section 3.2.13, efforts to identify the antimicrobial molecule produced by *P. c.* subsp. *brasiliensis* ICMP 19477 failed to identify any antibiotic molecules. This finding can be understood following identification of the antimicrobial as a carbapenem. The carbapenem molecule produced by *Serratia* and *Pectobacterium,* SQ 27,860, is a highly unstable molecule (Parker et al., 1982). During initial attempts to identify the carbapenem it was noted that freezing or concentrating of the bacterial broth culture destroyed the molecule. Furthermore, it could only be stored at 5°C for a few days. For longer storage, the molecule needed to be adsorbed onto charcoal and stored at -90°C (Parker et al., 1982). The molecule could then be eluted form the charcoal matrix using acetone:water (7:3, pH 7.7) and further purified by ion-exchange chromatography (Parker et al., 1982). As standard purification methods proved unsuccessful due to the instability of the carbapenem molecule, it is most appropriate to convert the molecule to a ρ-nitrobenzyl ester before purification (Parker et al., 1982).

The carbapenem biosynthetic cluster is also encoded by *P. c.* subsp. *brasiliensis* PBR1692, which was also shown to have activity against *P. atrosepticum* SCRI1043. This confirms the predictions of (Marquez-Villavicencio et al., 2011), who suggested that the inhibition of *P. atrosepticum* SCRI1043 by *P. c.* subsp. *brasiliensis* PBR1692 was due to the production of a carbapenem. In the study it was observed that *P. c.* subsp. *brasiliensis* PBR1692 was able to inhibit *P. atrosepticum* SCRI1043 *in vitro,* but not when co-inoculated in potato stems (Marquez-Villavicencio et al., 2011). This is consistent with the findings that *P. c.* subsp. *brasiliensis* ICMP 19477 is able to inhibit the growth of *P.*

atrosepticum SCRI1043 in structured environments, but not in unstructured conditions such as in liquid cultures or potato stems. It has also been described that *P. betavasculorum* was able to inhibit the growth of certain *P. c.* subsp. *carotovorum* strains on antagonism assays and in potato tubers (Axelrood et al., 1988). This was also predicted to be due to the production of a carbapenem by *P. betavasculorum* (Axelrood et al., 1988). As these results are consistent with those obtained for *P. c.* subsp. *brasiliensis* ICMP 19477, it appears that this represents another ecological example of the function of carbapenem molecules.

Chapter 5 describes the involvement of the *carI* and *slyA* genes in the competitiveness of functional genetics experiments that enabled the identification of *P. c.* subsp. *brasiliensis* ICMP 19477 against *P. atrosepticum* SCRI1043 *in vitro* and *in planta*. The involvement of these genes is confirmed by chemical complementation with OHHL, for *carI* and genetic complementation for *slyA.*

Appendix C

C.1 Tn*5* flanking sequences

Nucleotide sequences of DNA flanking the Tn*5* insertions, as amplified using a gene-specific primer and Tn*5*R (as described in Section 2.1.19). For each Tn*5* mutant, the corresponding region in the genome of *P. c.* subsp. *brasiliensis* ICMP 19477 amplified by PCR, are given as co-ordinates above the sequence. Tn*5* sequences are highlighted by red colour. Tn*5*R primer sequences are underlined.

***carR::*Tn*5* (4699985-4700210)**

ATGGATCATGAAATCCATTCCTTTATCAAAAGGAAGCTGAAAGGAGTCGGTGATGTATGGTTTTCTTATTTTAT
GATGAGTAAAAACTCTACCAGCCAACCTTATATTATTTCGAATTATCCCGAAGCATGGATGAAGGAGTATATA
AAAAAAGAGATGTTTCTGAGTGATCCTATCATTGTTGCCTCATTAGCTCGGATCACGCCGTTTTCTTGGGATGA
TAATGATCTTGTGACGCTAAGAGCCAAGAATCAGGATGTTTTTATTTCTTCCGTGCAGCACGATATAAGTTCAG
GTTATACCTTTGTTTTGCACGACCATGATAATAATGTGGCGACACTGAGTATAGCGAATCACCTGGAAGATGC
GAATTTCGAAAAATGTATGAAGAATCATGAAAATGATTTGCAGATGTTACTTGTGAATGTACATGAAAAAGTG
ATGGCATATCAGCGTGCTATCAACGTTCAAGATAACCCCCGACTCAGAATATGTGTTCACGCCGGCGTATGCG
GGACTGATCGATTCCTCCTA

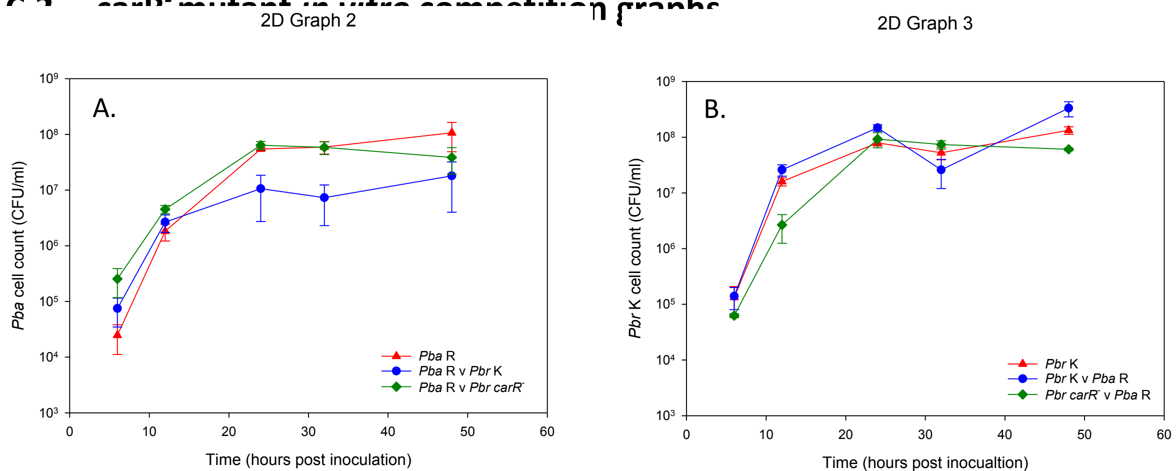
***slyA::*Tn*5* (2744146-2744469)**

CTACGCTTGATTCTCATGTAATGCCAATATATTTTTCTCAAGACGCGAAACCAGCAGCGCTAATTCATCAACCT
GCTCCGGCGTAATACCAAATAAGACTTCACTACGTGTATGGCTAATTACACCATTGACTGCTTGTATGATTGGC
TCTGCTGATTCGGTCAGCATAATACGTTTTGCCCGACGATCGTGCGCGCAAACGTGGCGAGTGATTAACCCTT
TTTCCTCAAGCTGATCCAGTGTTCGGACTAATGAGGGTTGCTCAATACCTATCGCTTTGGCAAGTTGAATCTGC
GACTGCCCAGGGGGTAGATGGTATATGTGACTCAGAATATGTGTTCACGCCGGCGTATGCGGGACTGATCGA
TTCCTCCTA

*carI::*Tn*5* (4446562-4446675)

ATGTTAGAGATATTTGATGTAAATCACACCTTGTTGTCAGAAACGAAATCAGAAGAGCTATTTACCCTCAGAA
AAGAGACGTTTAAAGATCGACTGAATTGGGCCGTGCAAGACTCAGAATATGTGTTCACGCCGGCGTATGCGG
GACTGATCGATTCCTCCTA

C.3 carR⁻ mutant *in vitro* competition graphs



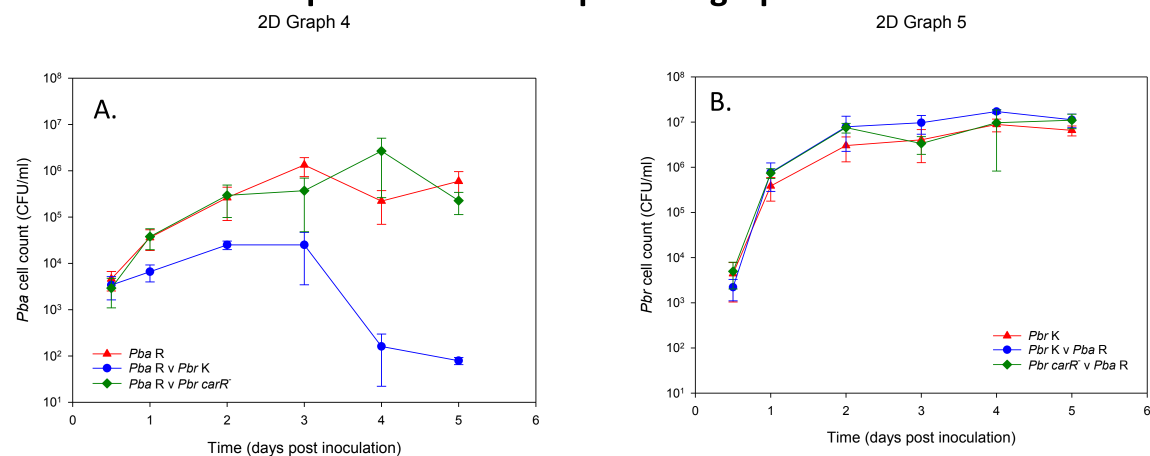
Second, independently conducted experiment, demonstrating the effect of co-inoculation with *P. c.* subsp. *brasiliensis* (*Pbr*) ICMP 19477 and *P. c.* subsp. *brasiliensis carR*⁻ (*Pbr carR*⁻) on growth of *P. atrosepticum* (*Pba*) SCRI1043 on solid agar plates (MMA).

A: A graph showing the growth (mean CFUs) of *P. atrosepticum* SCRI1043 Rif (*Pba* R) resistant strain over a 48 h period at 28°C when inoculated into MM in isolation or co-inoculated with *P. c.* subsp. *brasiliensis* ICMP 19477 tagged with Km resistance (*Pbr* K), or the *carR* mutant *Pbr carR*⁻ (at a 1:1 ratio). **B**: A graph showing the growth (mean CFUs) of *Pbr* K when cultured in isolation on solid agar plates or in co-inoculations with *Pba* R as well as *Pbr carR*⁻ co-inoculated with *Pba* R (at a 1:1 ratio). For each graph, the mean CFUs were calculated for each treatment from triplicate assays (solid line), error bars represent the standard deviation.

C.3 Competitive index of carR$^-$ mutant *in vitro* competition assay

	Pbr **K v *Pba* R**		***Pbr carR***$^-$ **v *Pba* R**	
Time (hours)	Log CI	SD	Log CI	SD
6	0.7	0.2	-0.2	0.3
12	1.4	0.2	0.1	0.2
24	2.1	0.6	0.5	0.1
32	1.0	0.2	0.5	0.1
48	1.9	0.8	0.6	0.3

C.4 *carR*$^-$ mutant potato tuber competition graphs



Second, independently conducted experiment, using potato tubers sourced at a different time of year, demonstrating the effect of co-inoculation with *P. c.* subsp. *brasiliensis* (*Pbr*) ICMP 19477 and *P. c.* subsp. *brasiliensis carR*$^-$ (*Pbr carR*$^-$) on growth of *P. atrosepticum* (*Pba*) SCRI1043 in potato tubers ('Ilam Hardy').

A: A graph showing the growth (mean CFUs) of *Pba* SCRI1043 rifampicin (*Pba* R) resistant strain over a 48 h period at 28 °C when inoculated into potato tubers in isolation or co-inoculated with *P. c.* subsp. *brasiliensis* ICMP 19477 tagged with Kn resistance (*Pbr* K), or the *carR* mutant *Pbr carR*$^-$ (at a 1:1 ratio). **B**: A graph showing the growth (mean CFUs) of *Pbr* K when cultured in isolation in to potato tubers or in co-inoculations with *Pba* R as well as *Pbr carR*$^-$ co-inoculated with *Pba* R (at a 1:1 ratio). For each graph, the mean CFUs were calculated for each treatment from triplicate assays (solid line), error bars represent the standard deviation.

C.5 Competitive index of the *carR*$^{-}$ mutant in *in planta* competition assays

	***Pbr* K v *Pba* R**		***Pbr carR*$^{-}$ v *Pba* R**	
Time (days)	Log CI	SD	Log CI	SD
0.5	1.4	0.2	0.8	0.3
1	4.6	0.3	1.8	0.3
2	4.8	0.4	1.6	0.4
3	4.8	0.6	1.8	0.5
4	4.8	0.4	1.4	0.1
5	4.1	0.5	2.4	0.3

C.6 Competitive index of the *carR*$^{-}$ mutant in liquid *in vitro* competition assays

	***Pbr* K v *Pba* R**		***Pbr carR*$^{-}$ v *Pba* R**	
Time (hours)	Log CI	SD	Log CI	SD
6	0.2	0.1	0.4	0.1
12	0.4	0.1	0.9	0.1
16	1.3	0.04	1.2	0.03
24	1.4	0.1	1.6	0.06
32	0.5	0.4	0.6	0.2
48	0.9	0.2	0.1	0.1

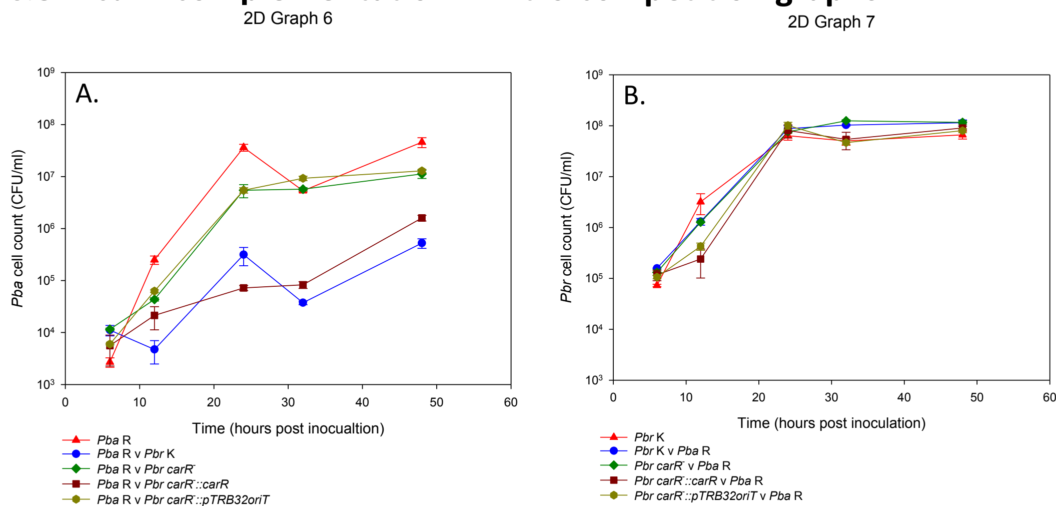
C.7 *carR* complementation sequence

Nucleotide sequence of KCO_04647 (*carR*), following cloning into pTRB32oriT (Section 4.2.10). Insert-specific primers are underlined and restriction sites are written in bold type. The sequence shown in italics represents vector sequence. Nucleotides shown in blue represent mis-matches between the construct and the *P. c.* subsp. *brasiliensis* ICMP 19477 gene.

GCTTTGTGAGCGGATAACAATTATAATAGATTCAATTGTGAGCGGATAACAATTTCACACAGAATTCATTAAAG
*AGGAGAAATTAACTATGAGAGGATCGCATCACCATCACCATCACGGATCCGCATGCGAGCTC*GCG**GGTACC**GT
CGGTAAGAGAGGGTAATATGGAATGGATCATGAAATCCATTCCTTTATCAAAAGGAAGCTGAAAGGAGTCGG
TGATGTATGGTTTTCTTATTTTATGATGAGTAAAAACTCTACCAGCCAACCTTATATTATTTCGAATTATCCCGA
AGCATGGATGAAGGAGTATATAAAAAAAGAGATGTTTCTGAGTGATCCTATCATTGTTGCCTCATTAGCTCGG
ATCACGCCGTTTTCTTGGGATGATAATGATCTTGTGACGCTAAGAGCCAAGAATCAGGATGTTTTTATTTCTTC
CGTGCAGCACGATATAAGTTCAGGTTATACCTTTGTTTTGCACGACCATGATAATAATGTGGCGACACTGAGT

ATAGCGAATCACCTGGAAGATGCGAATTTCGAAAAATGTATGAAGAATCATGAAAATGATTTGCAGATGTTAC
TTGTGAATGTACATGAAAAAGTGATGGCATATCAGCGTGCTATCAACGGTCATAATAACCCCCCCGATAATTC
AAGAAATGCCTTACTCTCTCCGCGTGAAACCGAAGTGCTTTTCCTGGTTAGTAGTGGACGAACTTACAAAGAG
GTTTCTCGTATATTAGGTATTAGTGAGGTCACCGTTAAGTTCCACATTAACAACTCAGTCCGTAAATTGGATGT
TATCAATTCCCGCCATGCTATAACTAAAGCACTTGAGTTAAATCTTTTCCATTCCCCCTGTGAGCCTGTAGTGAT
GAAGCATATGGACGCCCGTTAGAATGCTTGCTAAATAGGGAGGA**AAGCTT**GAC*AATTAGCTGAGCTTGGACT*
CCTGTTGATAGATCCAGTAATGACCTCAGAACTCCATCTGGATTTGTTCAGAACGCTCGGTTGCCGCCGGGCGT
TTTTTATTGGTGAGAATC

C.8 *carR* complementation *in vitro* competition graphs



Second, independently conducted experiment, demonstrating the effect of co-inoculation with *P. c.* subsp. *brasiliensis* (*Pbr*) ICMP 19477 *carR*⁻ complemented strain (*Pbr carR⁻::pTRB32oriTcarR*) on growth of *P. atrosepticum* (*Pba*) SCRI1043 on solid agar plates (MMA).

A: A graph showing the growth (mean CFUs) of *P. atrosepticum* SCRI1043 Rif (*Pba* R) resistant strain over a 48 h period at 28°C when inoculated into MM in isolation or co-inoculated with *P. c.* subsp. *brasiliensis* ICMP 19477 tagged with Km resistance (*Pbr* K), or the *carR* mutant *Pbr carR⁻*, gene complemented strain *Pbr carR⁻::pTRB32oriTslyA* or the control strain *Pbr carR⁻::pTRB32oriT* containing an empty copy of the pTRB32oriT plasmid (at a 1:1 ratio). **B**: A graph showing the growth (mean CFUs) of *Pbr* K when cultured in isolation on solid agar plates as well as the *Pbr* strains in co-inoculation with *Pba* R (at a 1:1 ratio). For each graph, the mean CFUs were calculated for each treatment from triplicate assays (solid line), error bars represent the standard deviation.

C.9 Competitive index of the *carR* complemented strain *in vitro*

	***Pbr* K v *Pba* R**		***Pbr carR⁻* v *Pba* R**		***Pbr carR::carR⁻* v *Pba* R**		***Pbr carR⁻::pTRB32oriT* v *Pba* R**	
Time (hours)	Log CI	SD	Log CI	SD	Log CI	SD	Log CI	SD
6	1.1	0.1	0.9	0.1	1.6	0.3	0.9	0.04
12	2.4	0.1	1.3	0.03	1.3	0.3	0.4	0.1
24	2.4	0.2	1.0	0.1	3.3	0.1	0.9	0.04
32	2.4	0.1	1.1	0.01	3.1	0.1	0.3	0.05
48	1.3	0.1	0.8	0.05	2.0	0.1	0.4	0.04

Chapter 5

Carbapenem production in *Pectobacterium carotovorum* subsp. *brasiliensis* ICMP 19477 is regulated by *carI* and *slyA*

5.1 Introduction

Carbapenem production is under complex regulation in *P. c.* subsp. *carotovorum*. One mechanism of regulation involves QS (McGowan and Salmond, 1999). Quorum sensing is a well understood mechanism of gene regulation within bacteria, which enables the rational expression of genes in a population density dependent manner (Whitehead et al., 2001). Cell density is detected by the production of a small diffusible signalling molecule known as an *N*-AHL.

In *P. atrosepticum*, the QS locus encoding the *N*-AHL is designated *expI*/*expR* (Miller and Bassler, 2001; Liu et al., 2008). The *expI* gene is a *luxI*-type gene that encodes a *N*-AHL synthase, which produces the *N*-AHL *N*-(3-oxohexanoyl) homoserine lactone (OHHL) (Andersson et al., 2000). The *luxI*-type genes are named differently in *P. c.* subsp. *carotovorum* strains. For instance, it is designated *carI* in the carbapenem-producing strain *P. c.* subsp. *carotovorum* ATCC 39048 (Swift et al., 2001). In *P. c.* subsp. *carotovorum* EC153 and *P. c.* subsp. *carotovorum* 71, the *luxI*-type gene is named *ahlI* (Chatterjee et al., 2005; Cui et al., 2006).

The other gene within the QS loci, *expR*, encodes a LuxR type transcriptional-activator (Andersson et al., 2000; Chatterjee et al., 2000). In *P. c.* subsp. *carotovorum* SCC319, this was found to function in the control of PCWDE production, via an interaction with the global RNA binding protein RsmA (Cui et al., 2005). It was found that ExpR is not a classical LuxR type regulator as it is the ExpR protein, rather than the ExpR-*N*-AHL complex, which activates *rsmA* transcription (Chatterjee et al., 2005; Cui et al., 2006). In carbapenem-producing strains of *P. c.* subsp. *carotovorum* such as SCC319, another LuxR-type transcriptional activator is involved in the regulation of carbapenem biosynthesis (McGowan et al., 1995). The CarR regulator specifically activates transcription of the *carA-H* operon as the CarR-*N*-AHL complex specifically binds to DNA upstream of *carA* (McGowan et al., 1995; Holden et al., 1998; Welch et al., 2000). CarR does not play a role in the control of PCWDE production (Whitehead et al., 2002).

Production of PCWDEs is under QS control, with biosynthesis limited to conditions when the pathogen reaches such numbers as to overcome the plant's defences (Jones et al., 1993b; Perombelon, 2002). The coordinated regulation of PCWDE production and carbapenem biosynthesis via QS suggests that

the antimicrobial production acts to protect the nutrients released from the host from competing organisms. In addition to the PCWDEs, QS controls many secondary metabolite processes, including those related to virulence. It was demonstrated that in *P. atrosepticum,* QS regulated secretions systems, regulatory pathways and mechanisms for subverting the plants defences (Liu et al., 2008). Quorum sensing has also been associated with the colonization of xylem tissue by *P. c.* subsp. *brasiliensis* PBR1692 (Moleleki et al., 2016).

Quorum sensing regulation of carbapenem production control has been observed in other enterobacteria such as *Serratia marcescens* (Thomson et al., 2000) and in the human pathogen *P. aeruginosa* (Miller and Bassler, 2001). In these bacteria, an *expI*- type and *carR*-type gene have been implicated as the primary QS signalling systems involved in regulating production of carbapenems. In *S. marcescens* ATCC 39006 the *expI*-type gene is designated *smaI,* which is associated with the LuxR-type transcriptional regulator encoding gene *smaR* (Thomson et al., 2000). This locus has been identified as being involved in the regulation of other secondary metabolites in *S. marcescens* ATCC 39006, as well as carbapenem production, such as the tripyrrole antibiotic prodigiosin

Regulatory mechanisms are also known to function in conjunction with QS to regulate carbapenem production. Of particular interest are the members of the MarR/SlyA family, which influence the production of secondary metabolites in various organisms (Ellison and Miller, 2006). SlyA of *Salmonella typhimurium* was the first example of such a regulator, which was also found to modulate virulence (Libby et al., 1994; Ludwig et al., 1995). The MarR/SlyA family also includes Rap (*S. marcescens*) (Thomson et al., 1997), Hor (*P. c.* subsp. *carotovorum*) (Thomson et al., 1997), PecS (*Dickeya*) (Reverchon et al., 1994) and RovA (*Yersinia pseudotuberculosis*) (Nagel et al., 2001). In all these examples, the regulators control expression of genes related to pathogenesis or those that respond to stress (Reverchon et al., 1994; Nagel et al., 2001). Of particular interest are the *rap* and *hor* genes that control carbapenem production in *Serratia* and *P. c.* subsp. *carotovorum* respectively (Thomson et al., 1997).

The MarR/SlyA protein family are structurally diverse, although their mechanism of regulation is conserved. They act at the transcriptional level by competing with repressors or activators for access to binding sites, within the promoter regions of the genes under their control, enabling transcription of target genes to be activated or repressed (Ellison and Miller, 2006). For example, Hor activates carbapenem production in *P. c.* subsp. *carotovorum* by directly activating transcription of the biosynthesis genes (Thomson et al., 1997). The regulatory network involved in carbapenem production is summarised in Figure 1.3.

SlyA-type regulators have often been described as members of complex, hierarchical regulatory networks. In *P. c.* subsp. *carotovorum*, the expression of Hor is repressed by ExpR at low population densities i.e. the OHHL signalling molecule is not produced. Transcription is then activated via a QS-dependent global regulator at high population numbers (Sjöblom et al., 2008). In *Yersinia* and *Salmonella* SlyA is controlled by the two component regulatory system PhoP/PhoQ, which is not under QS control. Termination of *slyA* transcription is controlled via auto repression by the *slyA* transcript (Miller et al., 1989).

In Chapter Four, carbapenem production was proven to be involved in the antagonism of *P. atrosepticum* SCRI1043 by *P. c.* subsp. *carotovorum* ICMP 19477 in bioassays. Carbapenem production was also important for the competitive fitness of the producer in mixed populations in potato tubers. Two further *Tn5* mutants impaired in carbapenem production, with transposon insertions in *carI* and *slyA*-type gene were identified. Furthermore, a *carI* gene as well as multiple *expR* homologues including a *carR* homologue have been identified within the genome of *P. c.* subsp. *carotovorum* ICMP 19477 (Panda et al., 2015b), this study). Given the role of *carI* and *slyA* in regulating carbapenem production in *P. c.* subsp. *carotovorum*, it was proposed that these regulators were also involved in the production of the carbapenem molecule by *P. c.* subsp. *brasiliensis* ICMP 19477. To confirm this hypothesis we characterised the *carI* and *slyA* mutants for their role in carbapenem production.

5.2 Results

5.2.1 OHHL production by *Pectobacterium carotovorum* subsp. *brasiliensis* ICMP19477 was abolished in the *carI* mutant

As described in Chapter 4, *carI* was predicted to encode a QS synthetase that synthesises the QS molecule OHHL. Thus, *N*-AHL production assays (Section 2.1.30) were performed to confirm that OHHL production had been abolished in *P. c.* subsp. *brasiliensis carI*$^{-}$ by insertion of the Tn*5* cassette into *carI*. The *N*-AHL production bioassay utilises the production of violacein, a purple pigment, by *Chromobacterium violacein* (Ballantine et al., 1958; Lichstein and van de Sand, 1945). The production of violacein was found to be under QS control (Throup et al., 1995). A transposon mutant (*C. violacein* CV026) was produced that was defective in both violacein and *N*-AHL production (Katifi et al., 1993; Throup et al., 1995). Crossfeeding experiments with the parental *C. violacein* strain and CV026 restored violacein production in the transposon mutant (Throup et al., 1995). Therefore, the bioassay functions as *N*-AHLs produced by the test bacteria (i.e. *P. c.* subsp. *brasiliensis* ICMP 19477) restore violacein production and the coloured zones can be measured as an indication of *N*-AHL production by the test bacteria. Mutants unable to produce the *N*-AHL molecule (i.e. *carI*$^{-}$*)* do not restore violacein production and therefore no coloured zones are visible.

The *carI* mutant failed to produce coloured zones on the indicator strain *Chromobacterium violaceum* CV026 whereas the WT produced increasingly large zones from approximately 7 hpi (Figure 5.1). Production of the zones at this time corresponded to *P. c.* subsp. *brasiliensis* ICMP 19477 reaching higher cell densities during the transition from exponential phase into stationary phase (Figure 4.2). The failure of *P. c.* subsp. *brasiliensis carI*$^{-}$ to produce a coloured zone was indicative that OHHL production had been abolished in this strain. As the *carI* mutant had also failed to produce a zone of inhibition on *P. atrosepticum* SCRI1043, these data indicated that the QS molecule was required by *P. c.* subsp. *brasiliensis* ICMP 19477 for the production of the carbapenem, and consequently for antagonism of *P. atrosepticum* SCRI1043. In the same assays, both the *carR*$^{-}$ and the *slyA*$^{-}$ mutants produced coloured zones similar to those of the WT on the indicator strain *C. violaceum* CV026, indicative that these strains were producing OHHL.

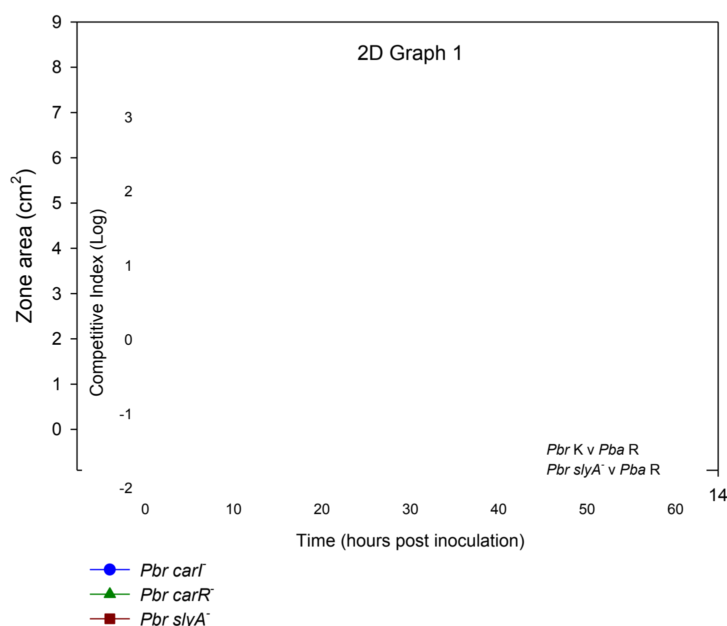


Figure 5.1. Production of the quorum sensing molecule *N*-3-(oxohexanoyl)-L-homoserine lactone (OHHL) by *P. c.* subsp. *brasiliensis* (*Pbr*) ICMP19477 and the Tn*5* mutants *Pbr carI*$^-$, *Pbr carR*$^-$ and *Pbr slyA*$^-$.

Cell-free culture supernatants were prepared throughout growth of each strain in LB and the supernatants were dotted onto bioassay plates containing a lawn of the indicator strain *Chromobacterium violaceum* CV026. The production of OHHL was determined by the presence of coloured halos on the lawn. The sizes of the coloured zones were measured and the area plotted against time. The errors represented are standard deviations (some error bars are hidden behind the graph symbols).

5.2.2 Inactivation of *carI* results in reduced inhibition of *Pectobacterium atrosepticum* SCRI1043 in co-inoculation assays on solid media

Solid plate competition assays (Section 2.1.26) showed that the growth of *P. atrosepticum* SCRI1043 when co-inoculated with the *carI* mutant was similar to the growth of *P. atrosepticum* SCRI1043 when it was cultured in isolation. In contrast, the growth of *P. atrosepticum* SCRI1043 was significantly reduced during the late exponential and early stationary phases in co-inoculations with *P. c.* subsp. *brasiliensis* ICMP 19477 (Figure 5.2 A). As with previous experiments, *P. c.* subsp. *brasiliensis* ICMP 19477 appeared unaffected by co-inoculation with *P. atrosepticum* SCRI1043. (Figure 5.2 B). Data was consistent in a second, independently conducted experiment (Appendix D).

The CI was subsequently calculated for each of the strains (Section 2.1.26). These calculations showed strong competition against *P. atrosepticum* SCRI1043 by *P. c.* subsp. *brasiliensis* ICMP19477, with the Log CI of the WT reaching between 1.0 and 2.0 during the late exponential and early stationary phases. The *carI*⁻ mutant showed only weak competition, with Log CI values for this strain never reaching more than 0.5 (Appendix D). The data were consistent with those in a second independently conducted experiment (data not shown). These results confirmed that inactivation of *carI* reduced inhibition of *P. atrosepticum* SCRI1043 by *P. c.* subsp. *brasiliensis* ICMP 19477 in co-inoculation assays on solid media.

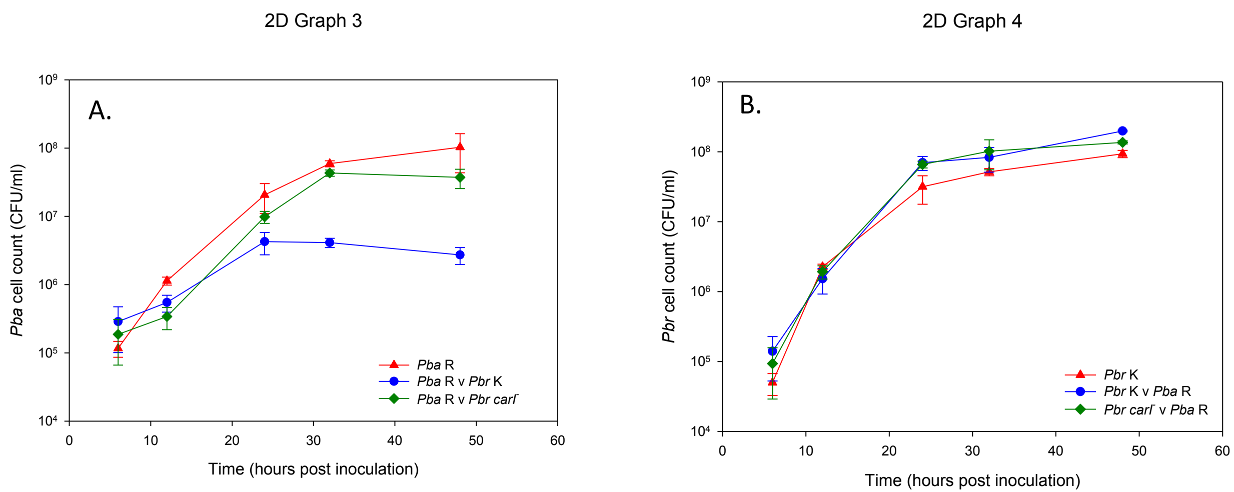


Figure 5.2. The effect of co-inoculation with *P.c.* subsp. *brasiliensis* (*Pbr*) ICMP 19477 and *Pbr carI*⁻ on growth of *P. atrosepticum* (*Pba*) SCRI1043 on solid agar plates (MMA).

A: A graph showing the growth (mean CFUs) of the *Pba* SCRI1043 Rif resistant strain (*Pba* R) over a 48 h period at 28°C when inoculated onto MMA in isolation or co-inoculated with *P. c.* subsp. *brasiliensis* ICMP 19477 tagged with Km resistance (*Pbr* K) or the *carI* mutant *Pbr carI*⁻ at a 1:1 ratio. **B**: A graph showing the growth (mean CFUs) of *Pbr* K when cultured in isolation on solid agar plates or in co-inoculations with *Pba* R as well as the growth of *Pbr carI*⁻ co-inoculated with *Pba* R (at a 1:1 ratio). For each graph, the mean CFUs were calculated for each treatment from triplicate assays (solid line), error bars represent the standard deviation.

5.2.3 Inactivation of *carI* reduces inhibition of *Pectobacterium atrosepticum* SCRI1043 in co-inoculation assays in potato tubers

Growth of *P. atrosepticum* SCRI1043 was reduced only slightly when the bacterium was co-inoculated into potato ('Ilam Hardy') tubers (Section 2.1.28) with *P. c.* subsp. *brasiliensis carI*$^-$ relative to its growth upon inoculation into tubers in isolation (Figure 5.3 A). In contrast, co-inoculation of *P. atrosepticum* SCRI1043 with *P. c.* subsp. *brasiliensis* ICMP 19477 resulted in a dramatic inhibition in growth of the sensitive strain (Figure 5.3 B). In fact, at most sampling times, the CFU values of *P. atrosepticum* SCRI1043 were at least 100 times greater when inoculated with *P. c.* subsp. *brasiliensis carI*$^-$ than when inoculated with WT. The growth of *P. c.* subsp. *brasiliensis* ICMP 19477 was not affected by co-inoculation with *P. atrosepticum* SCRI1043. The results were consistent with those collected in a second independent experiment using potatoes sourced at a different time of year (Appendix D). This demonstrated that the physiology of the potato did not affect the competition between the bacteria. It has previously been described that the age of potato tubers can affect their susceptibility to tuber soft rot (Marquez-Villavicencio et al., 2011).

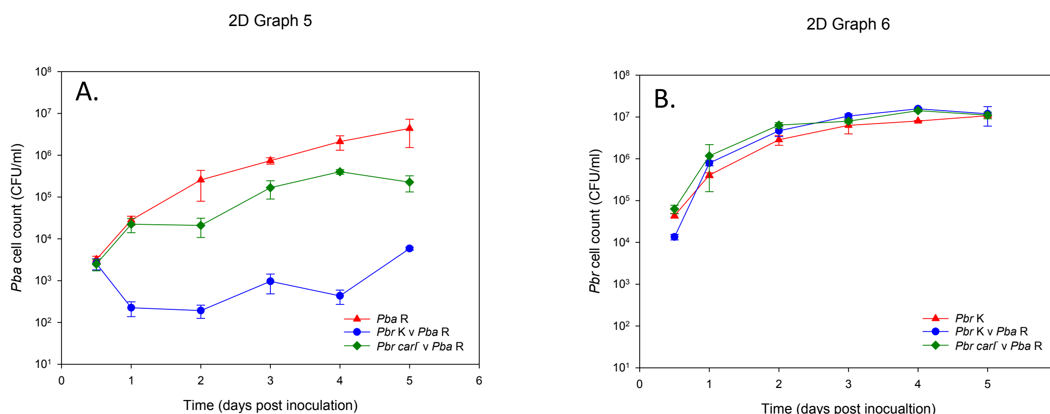


Figure 5.3. The effect on growth of *P. atrosepticum* (*Pba*) SCRI1043 of co-inoculation with *P. c.* subsp. *brasiliensis* (*Pbr*) ICMP 19477 and *Pbr carI*$^-$ in potato tubers ('Ilam Hardy').

A: A graph showing the growth (mean CFUs) of the *Pba* SCRI1043 Rif resistant strain (*Pba* R) over a 48 h period at 28°C when inoculated into potato tubers in isolation or co-inoculated with *Pbr* ICMP19477 tagged with Km resistance (*Pbr* K) or the *carI* mutant *Pbr carI*$^-$ (at a 1:1 ratio). B: A graph showing the growth (mean CFUs) of *Pbr* K when cultured in isolation in potato tubers or in co-inoculations with *Pba* R as well as growth of *Pbr carI*$^-$ co-inoculated with *Pba* R (at a 1:1 ratio). For each graph, the mean CFUs were calculated for each treatment from triplicate assays (solid line), error bars represent the standard deviation.

The CI was calculated for each of the *P. c.* subsp. *brasiliensis* strains (Section 2.1.26). These calculations showed very strong competition against *P. atrosepticum* SCRI1043 by *P. c.* subsp. *brasiliensis* ICMP19477, with the Log CI of the WT reaching between 3 and 5 during the late exponential and early stationary phases. The *carI*⁻ mutant showed markedly weaker competition than the WT, with Log CI values for this strain reaching between 1 and 2 (Appendix D). The CI values at no point reached zero, however (which would indicate no competition between the bacteria).

5.2.4 Addition of exogenous OHHL to *Pectobacterium carotovorum* subsp. *brasiliensis carI*⁻ mutant restores its ability to produce the carbapenem molecule

Chemical complementation of the *P. c.* subsp. *brasiliensis carI*⁻ mutant was undertaken with exogenous OHHL rather than by constructing a derivative of the mutant expressing the WT gene, as the *carI* mutant was defective in OHHL production. In these experiments (Section 2.1.31), addition of OHHL to the *P. c.* subsp. *brasiliensis carI*⁻ mutant resulted in the production of zones of inhibition similar in size to the WT when the bacterium was cultured on a lawn of the indicator strain ESS (Figure 5.4). In contrast, the *carI*⁻ mutant failed to produce zones of inhibition. The restoration of the WT phenotype by addition of exogenous OHHL confirmed the role of this quorum sensing molecule (as encoded by *carI*) in regulating the production of carbapenem, which is required for antagonism of *P. atrosepticum* SCRI1043 by *P. c.* subsp. *brasiliensis* ICMP 19477.

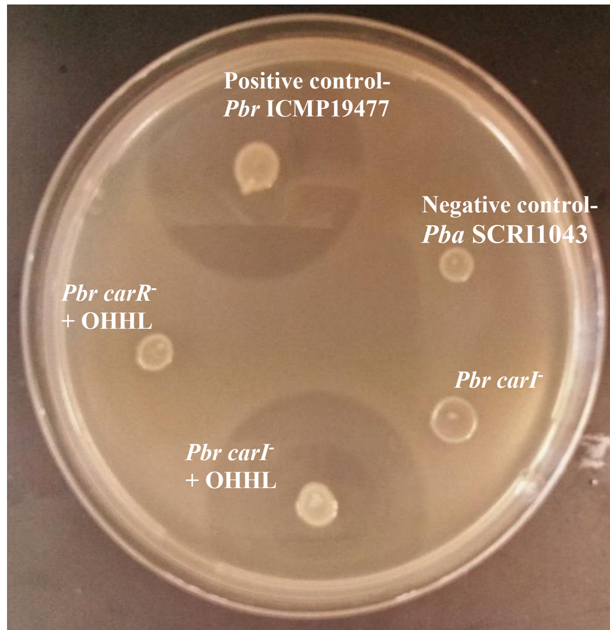


Figure 5.4. Addition of exogenous OHHL to the *P. c.* subsp. *brasiliensis* (*Pbr*) *carI*- mutant restored its capacity to inhibit growth of ESS, an *E. coli* strain super sensitive to carbapenems.

Assays were conducted as described in Section 2.1.31. Plates were incubated at 28 °C for 24 h and scored for zones of inhibition. *Pba* SCRI1043 and *Pbr carI*- were inoculated onto plates as negative controls.

5.2.5 Inactivation of *slyA* results in reduced inhibition of *Pectobacterium atrosepticum* SCRI1043 in co-inoculation assays on solid media

Competition assays on solid media (Section 2.1.26) were conducted using the *P. c.* subsp. *brasiliensis* *slyA*$^{-}$ mutant identified in Chapter Four. In these assays, during the exponential phase of growth the *slyA* mutant showed a similar inhibition of *P. atrosepticum* SCRI1043 to the WT *P. c.* subsp. *brasiliensis* strain in co-inoculations (Figure 5.5 A). Inhibition of *P. atrosepticum* SCRI1043 in late exponential to stationary phase was greater, however, when the sensitive strain was co-inoculated with the WT rather than with the *slyA* mutant. These data suggested that *slyA* was contributing to the inhibition of *P. atrosepticum* SCRI1043 in co-inoculations on solid media during the later stages of growth. Graphical representation of the CFU values over the course of the assay demonstrated that both the *P. c.* subsp. *brasiliensis* *slyA*$^{-}$ mutant and the WT were not affected by co-inoculation with *P. atrosepticum* SCRI1043 (Figure 5.5 B).

The Log CI was calculated for each *P. c.* subsp. *brasiliensis* ICMP 19477 strain in the competition assays (Section 2.1.26). From 24-48 hpi, strong competition was evident when the WT was co-inoculated with *P. atrosepticum* SCRI1043 (Log CI was 1 - 2). At all sampling times during the experiment, weak or no competition was evident when *P. atrosepticum* SCRI1043 was co-inoculated with the *slyA* mutant (Log CI<0.5) (Appendix D). This suggested that the SlyA regulator, along with CarI and CarR, regulates production of the carbapenem responsible for the greater fitness of *P. c.* subsp. *brasiliensis* ICMP 19477 when in competition with *P. atrosepticum* SCRI1043. This hypothesis was confirmed by similar data obtained in a second independently conducted experiment (Appendix D).

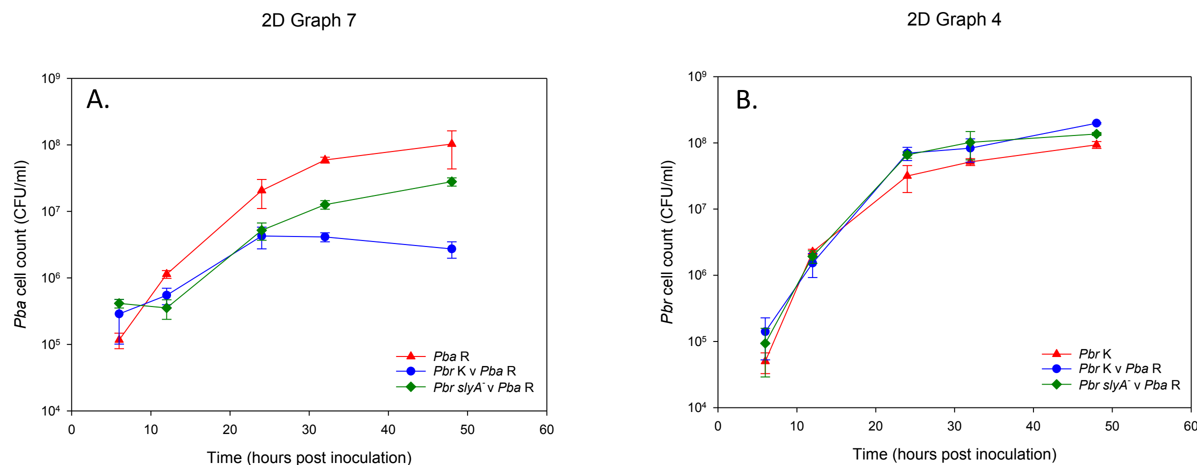


Figure 5.5. The effect of co-inoculation with *P. c.* subsp. *brasiliensis* (*Pbr*) ICMP 19477 and *P. c.* subsp. *brasiliensis* (*Pbr slyA*$^-$) on growth of *P. atrosepticum* (*Pba*) SCRI1043 on solid agar plates (MMA).
A: A graph showing the growth (mean CFUs) of the Rif resistant strain (*Pba* R) over a 48 h period at 28°C when inoculated onto MMA in isolation or when co-inoculated with *Pbr* ICMP 19477 tagged with Km resistance (*Pbr* K) or the *slyA* mutant *Pbr slyA*$^-$ (at a 1:1 ratio). **B**: A graph showing the growth (mean CFUs) of *Pbr* K when cultured in isolation on solid agar plates or in co-inoculations with *Pba* R as well as the growth of *Pbr slyA*$^-$ co-inoculated with *Pba* R (at a 1:1 ratio). For each graph, the mean CFUs were calculated for each treatment from triplicate assays (solid line), error bars represent the standard deviation.

5.2.6 Inactivation of *slyA* results in reduced inhibition of *Pectobacterium atrosepticum* SCRI1043 in co-inoculation assays in potato tubers

Tuber competition assays (Section 2.1.28) were conducted to determine the role of SlyA under conditions of mixed infection in tubers. In these experiments the growth of *P. atrosepticum* SCRI1043 was significantly greater when co-inoculated with the *slyA* mutant than with the WT (Figure 5.6 A). The CFU values did not, however, reach the values for *P. atrosepticum* SCRI1043 when this strain was grown in isolation. *P. c.* subsp. *brasiliensis slyA*$^-$ was not altered when co-inoculated with *P. atrosepticum* SCRI1043 (Figure 5.6 B). Similar results were obtained in a second, independently conducted experiment (Appendix D).

The CI was also calculated (Section 2.1.26). These calculations showed strong competition between *P. c.* subsp. *brasiliensis* ICMP 19477 and *P. atrosepticum* SCRI1043 (Log CI 3-4). Much weaker competition was evident for the *P. c.* subsp. *brasiliensis slyA*⁻ mutant (Log CI 1-2) (Appendix D).

These results were similar to that observed for *P. c.* subsp. *brasiliensis carR*⁻ and *P. c.* subsp. *brasiliensis carI*⁻ (Sections 4.2.8 and 5.2.3).

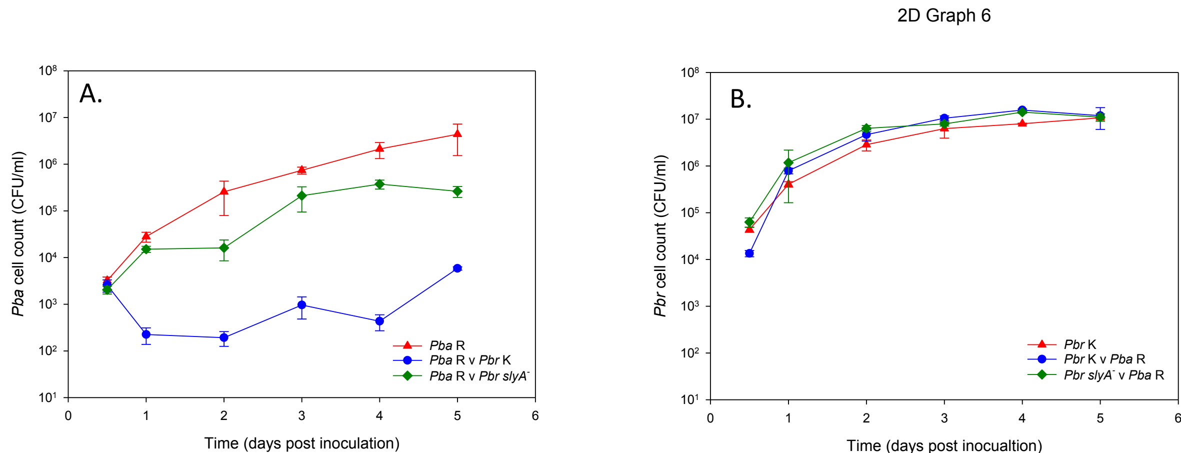


Figure 5.6. The effect of co-inoculation with *P. c.* subsp. *brasiliensis* (*Pbr*) ICMP 19477 and *P. c.* subsp. *brasiliensis* (*Pbr slyA*⁻) on growth of *P. atrosepticum* (*Pba*) SCRI1043 in potato tubers ('Ilam Hardy'). **A**: A graph showing the growth (mean CFUs) of the *Pba* SCRI1043 Rif resistant strain (*Pba* R) over a 48 h period at 28°C when inoculated into potato tubers in isolation or when co-inoculated with *Pbr* ICMP 19477 tagged with the Km resistance cassette (*Pbr* K) or the *slyA* mutant *Pbr slyA*⁻ (at a 1:1 ratio). **B**: A graph showing the growth (mean CFUs) of *Pbr* K when cultured in isolation in potato tubers or in co-inoculations with *Pba* R as well as *Pbr carI*⁻ co-inoculated with *Pba* R (at a 1:1 ratio). For each graph, the mean CFUs were calculated for each treatment from triplicate assays (solid line), error bars represent the standard deviation.

5.2.7 Complementation of *slyA* restored production of the carbapenem

Complementation studies were performed to confirm the role of SlyA. First, a derivative of pTRB32oriT containing a PCR fragment including *slyA* from *P. c.* subsp. *brasiliensis* ICMP 19477 was constructed (Sections 2.1.10-14 and Section 2.1.16) and the resulting plasmid (pTRB32oriT*slyA*) was introduced into *P. c.* subsp. *brasiliensis slyA*$^{-}$ via conjugation (Section 2.1.17). The presence of (pTRB32oriT*slyA*) in the transconjugants was confirmed by PCR using the SlyA_compl F and SlyA_compl R primers (Section 2.1.10) (Figure 5.7). To ensure the integrity of the inserted gene, the PCR amplicon was then sequenced (Section 2.1.14). Sequencing confirmed that the nucleotide sequence was identical to that of the WT gene from *P. c.* subsp. *brasiliensis* ICMP 19477 (Appendix D).

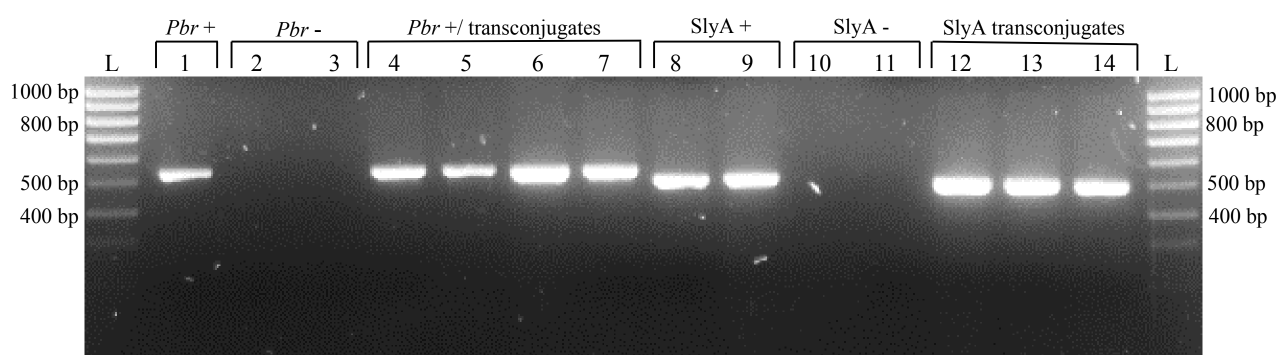


Figure 5.7. PCR amplicons obtained from PCR reactions using genomic DNA (gDNA) and plasmid DNA from the helper, donor, and recipient strains as well as transconjugants after conjugation of *P. c.* subsp. *brasiliensis* (*Pbr*) *slyA*$^{-}$ with the *slyA* gene.

Lanes 1-7 were PCR products obtained using Pbr-specific primers Pbr NZ F and Pbr NZ R (Table 2.3). Lanes 8 -14 were PCR products obtained using *slyA*-specific primers SlyA_compl F and SlyA_compl R (Table 2.3). L, DNA ladder (Hyper ladder 100 bp, Bioline); lane 1 & 8, positive control *Pbr* ICMP19477 gDNA; lane 2 & 9, negative control *E. coli* donor gDNA; lane 3 & 10, negative control *E. coli* helper gDNA; lane 4 & 11, *Pbr slyA*$^{-}$ recipient strain; lanes 5-7 & 12-14, *Pbr slyA*$^{-}$ transconjugants 1-3; Primers used are listed in Table 2.3.

To determine if the introduction of the WT copy of *slyA in trans* restored carbapenem production in the *slyA* mutant, the transconjugant *P. c.* subsp. *brasiliensis slyA*$^{-}$*::pTRB32oriTslyA* was used in an antagonism assay as previously described. As for the *carR* complementation (Section 4.2.10), IPTG was included in the growth plates (Section 2.1.4) to induce *slyA* expression from the plasmid. Induction with IPTG resulted in zones of inhibition being produced by *P. c.* subsp. *brasiliensis slyA*$^{-}$ *::pTRB32oriTslyA* whereas a transconjugant carrying the parent plasmid *pTRB32oriT* failed to produce

any visible zone (as did the *slyA* mutant) (Figure 5.8). The antagonism of *P. atrosepticum* SCRI1043 by the transconjugant carrying a functional *slyA* gene *in trans* confirmed that production of carbapenem by *P. c.* subsp. *brasiliensis* ICMP 19477 requires the SlyA regulator.

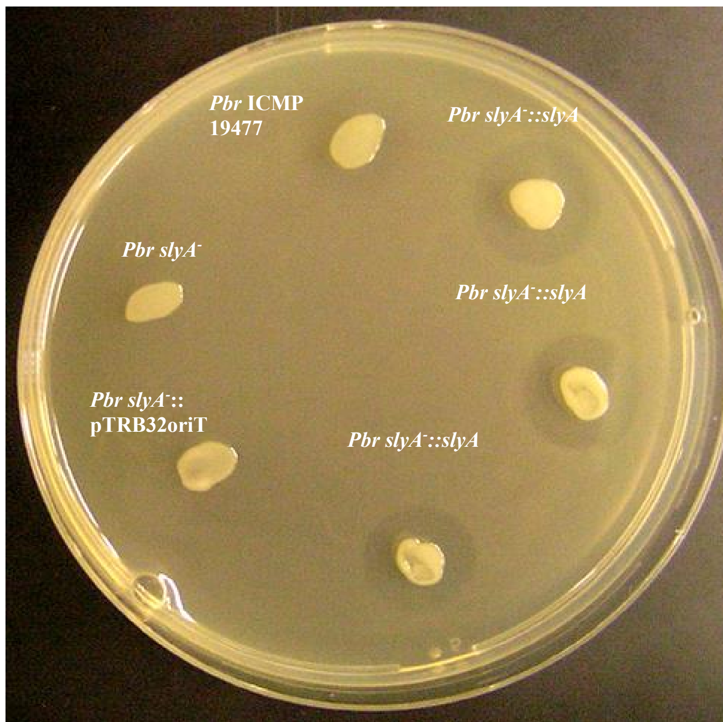


Figure 5.8. *P. c.* subsp. *brasiliensis* (*Pbr*) *slyA*⁻ containing a copy of the WT *slyA* gene, (*Pbr slyA*⁻ *::pTRB32oriTslyA*) restored the ability of the mutant to produce a zone of inhibition on *P. atrosepticum* SCRI1043 in antagonism assays.

The complemented strain, *Pbr slyA⁻::pTRB32oriTslyA* is a transconjugant of the *slyA* mutant carrying a functional copy of the gene *in trans*. As a negative control, a *Pbr slyA*⁻ mutant containing an empty copy of pTRB32oriT (*Pbr slyA⁻::pTRB32oriT*) was included. Antagonism assays were conducted as described in Section 2.1.21. Assay plates were incubated at 28 °C for 24 h, with zones of inhibition around the *Pbr* colonies indicative of antimicrobial production.

The growth of *P. c.* subsp. *brasiliensis slyA⁻::pTRB32oriTslyA* and *P. c.* subsp. *brasiliensis slyA⁻ ::pTRB32oriT* strains were subsequently compared with the growth of the WT and the *slyA* mutant on agar plates (Section 2.1.22), prior to assessing the strains in competition assays. The CFU values obtained at each sampling time indicated that *P. c.* subsp. *brasiliensis slyA⁻::pTRB32oriTslyA* was not

reduced in growth compared to the *P. c.* subsp. *brasiliensis* ICMP 19477 WT or the Km resistant strain. (Figure 5.9). The growth of the control strain, *P. c.* subsp. *brasiliensis slyA$^-$::pTRB32oriT*, did appear to be very slightly reduced compared to the other strains, although formal analysis of this data suggested any observed differences were insignificant, especially after 32 hpi (p = 0.168, F-test).

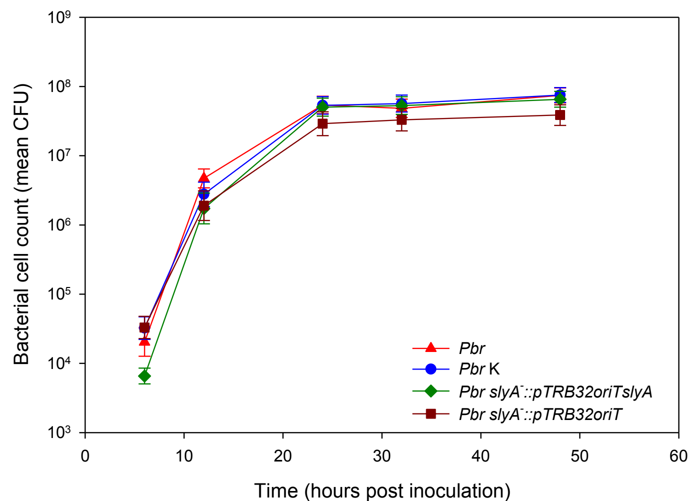


Figure 5.9. Growth of *P. c.* subsp. *brasiliensis* (*Pbr*) *slyA$^-$::pTRB32oriTslyA* and *Pbr slyA$^-$::pTRB32oriT* on solid agar plates was similar to that of the *slyA* mutant.

The graph shows the growth (mean CFUs) of the *Pbr* WT as well as the Km resistant tagged strain *Pbr* ICMP 19477 (*Pbr* K) plus *Pbr slyA$^-$::pTRB32oriTslyA* and *Pbr slyA$^-$::pTRB32oriT* over 48 h post inoculation with 10^4 CFUs/ml of each bacteria (as described in Section 2.1.22). The mean CFUs represent the mean over three test plates. Error bars are 95% confidence limits.

As *P. c.* subsp. *brasiliensis carR$^-$::pTRB32oriTcarR* and *P. c.* subsp. *brasiliensis slyA$^-$::pTRB32oriT* were not significantly affected in growth compared to the *slyA* mutant and the WT, the impacts of these strains on growth of *P. atrosepticum* SCRI1043 were compared in competition assays on solid media (supplemented with IPTG where necessary). In these assays, the growth of *P. atrosepticum* SCRI1043 was reduced when co-inoculated with *P. c.* subsp. *brasiliensis slyA$^-$::pTRB32oriTslyA* + IPTG compared to its growth in isolation or in the presence of either the *slyA* mutant or *P. c.* subsp. *brasiliensis slyA$^-$::pTRB32oriT*, especially during the late exponential and early stationary phases (Figure 5.10 A). Indeed, *P. c.* subsp. *brasiliensis slyA$^-$::pTRB32oriTslyA*, when induced with the addition of IPTG, was able to inhibit the growth of *P. atrosepticum* SCRI1043 to a greater extent than even the WT. The growth of the *P. c.* subsp. *brasiliensis* ICMP 19477 strains was not affected when co-inoculated with *P.*

atrosepticum SCRI1043 (Figure 5.10 B). Results were consistent in a second, independently conducted experiment (Appendix D).

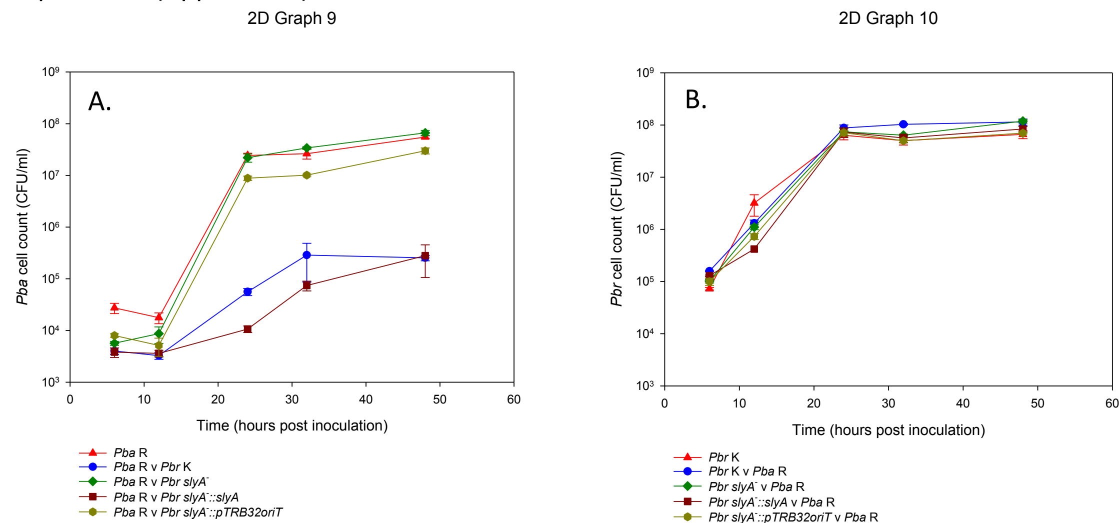


Figure 5.10. Growth of *P. atrosepticum* (*Pba*) SCRI1043 on solid agar plates (MMA) is reduced upon co-inoculation with *Pbr slyA*⁻*::pTRB32oriTslyA*.

A: A graph showing the growth (mean CFUs) of the *Pba* SCRI1043 Rif resistant strain (*Pba* R) over a 48 h period at 28°C when inoculated onto MMA in isolation or when co-inoculated with *Pbr* ICMP 19477 tagged with Km resistance (*Pbr* K) or the *slyA* mutant (*Pbr slyA*⁻), the transconjugant *Pbr slyA*⁻ *::pTRB32oriTslyA* or the control strain *Pbr slyA*⁻*::pTRB32oriT* (containing the pTRB32oriT plasmid) (at a 1:1 ratio). **B**: A graph showing the growth (mean CFUs) of *Pbr* K when cultured in isolation on solid agar plates as well as the various *Pbr* strains in co-inoculation with *Pba* R (at a 1:1 ratio). For each graph, the mean CFUs were calculated for each treatment from triplicate assays (solid line), error bars represent the standard deviation.

The Log CI values (Section 2.1.26) calculated for the *P. c.* subsp. *brasiliensis* ICMP 19477 strains from their growth in the competition experiments confirmed that *P. c.* subsp. *brasiliensis slyA*⁻ *::pTRB32oriTslyA* strongly out-competes *P. atrosepticum* SCRI1043, the Log CI reaching 2.0-3.0 during the late exponential and early stationary phases. As expected from the growth analyses, these CI values were greater than WT, which reached 2.0-2.5 in the same period. In contrast, weak competition was calculated for both *P. c.* subsp. *brasiliensis slyA*⁻ and *P. c.* subsp. *brasiliensis slyA*⁻*::pTRB32oriT* with Log CI rarely breaching 1.0 (Appendix D). A second, independently conducted experiment confirmed these data (data not shown).

As for the *carR* mutant, complementation of the *slyA* mutant required exogenous IPTG. Thus, experiments to confirm complementation in tubers were not performed. Nevertheless, the

restoration of carbapenem production in *P. c.* subsp. *brasiliensis slyA⁻::pTRB32oriTslyA* and its fitness in co-inoculations on solid media was considered sufficient evidence to demonstrate the role of *slyA* in these processes.

5.2.8 The *Pectobacterium carotovorum* subsp. *brasiliensis carI⁻* mutant is complemented by *Pectobacterium carotovorum* subsp. *brasiliensis carR⁻* and *Pectobacterium carotovorum* subsp. *brasiliensis slyA⁻*

To define the hierarchy in the quorum sensing regulation of carbapenem production in *P. c.* subsp. *brasiliensis* ICMP 19477, cross-feeding assays were conducted as described in Section 2.1.21, using the *carI* and *slyA* mutants as well as the *carR* mutant. These assays showed that the supernatants from cultures of the *slyA* and *carR* mutants enabled the *carI* mutant to generate zones of inhibition when grown on a lawn of *E. coli* ESS (Figure 5.11 A and B). *P. c.* subsp. *brasiliensis carR⁻* and *P. c.* subsp. *brasiliensis slyA⁻* could not complement one another, however (Figure 5.11 C).

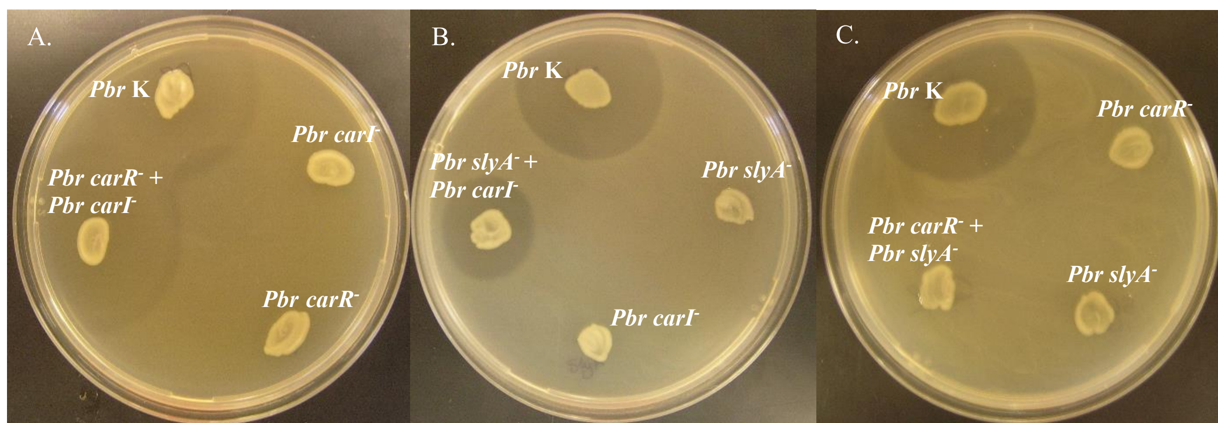


Figure 5.11. ***P. c.* subsp. *brasiliensis* (*Pbr*) *carI⁻* is complemented by cross-feeding with *Pbr carR⁻* and *Pbr slyA⁻*.**

Assays were conducted as described in Section 2.1.21. The plates were incubated for 24 h at 28°C and scored for zones of inhibition. *Pbr* ICMP 19477 Km resistant (*Pbr* K) was included as a positive control. Mutants inoculated without the supernatant were used as negative controls.

5.3 Discussion

In Chapter Four, mutants with a Tn*5* insertion in *carI* and *slyA* were identified, which were unable to cause zones of inhibition on *P. atrosepticum* SCRI1043. In this Chapter, the role of both these genes in inhibition of *P. atrosepticum* SCRI1043 was confirmed. The mutants were successfully complemented, either by chemical complementation using OHHL (for the *carI* mutant) or by introducing a wild-type copy of the gene into the mutant (for the *slyA* mutant). The successful complementation of *carI* demonstrated the role of OHHL and QS in production of the carbapenem (encoded byPbN1-GI65) in *P. c.* subsp. *brasiliensis* ICMP 19477. This was consistent with regulation of carbapenem production by QS in *P. c.* subsp. *carotovorum* (McGowan et al., 1995; McGowan et al., 2005).

The *C. violacein* CV06 bioassay (Section 2.1.30) used to confirm that the *P. c.* subsp. *brasiliensis carI*$^-$ mutant did not produce OHHL also showed that the *carR* and *slyA* mutants produced this QS molecule (Figure 5.1). These data indicated that the inability to produce carbapenem by *P. c.* subsp. *brasiliensis carR*$^-$ (as described in Chapter Four) and *P. c.* subsp. *brasiliensis slyA*$^-$ was likely due to the disruption of a pathway not controlled by QS or because they could not respond to the QS molecule. This was consistent with the role of CarR further down the QS regulatory cascade from *carI* in *P. c.* subsp. *carotovorum* (McGowan et al., 2005) and the concurrent role of SlyA-type regulators within carbapenem production (McGowan et al., 2005).

The ability of the *slyA* mutant to antagonise *P. atrosepticum* SCRI1043 *in vitro* was restored when a copy of the *P. c.* subsp. *brasiliensis* WT *slyA* gene was expressed *in trans* (*P. c.* subsp. *brasiliensis slyA*$^-$ *::pTRB32oriTslyA*) (Section 5.2.7). As previously described, the involvement of SlyA/MarR type transcriptional regulators in the production of carbapenems and other secondary metabolites is well understood in *P. c.* subsp. *carotovorum* (Thomson et al., 1997; McGowan et al., 2005). A *slyA*-type gene is also involved in the production of a carbapenem in *Photorhabdus luminescens,* however the carbapenem synthesis genes of this species are highly divergent from those of *P. c.* subsp. *carotovorum* (and *Serratia*) (Derzelle et al., 2002). This demonstrates that *slyA* regulation of carbapenem production is ubiquitous in enterobacteria producing these antimicrobials.

Cross-feeding assays (Section 5.2.8) confirmed that *slyA* regulation of carbapenem production in *P. c.* subsp. *brasiliensis* ICMP 19477 does not act through regulation of *carR* (or vice versa), as the *slyA* and *carR* mutants did not complement one another (Figure 5.11). In carbapenem producing strains of *P. c.* subsp. *carotovorum*, the SlyA type regulator (Hor) operates in conjunction with the QS system (McGowan et al., 2005). The exact role of Hor in carbapenem synthesis in unclear, however it appears to act via inducing transcription of *carA* (McGowan et al., 2005). The *hor* gene was found to be

expressed at late exponential to late stationary phase and its transcription was not induced by the presence of OHHL. Therefore it was concluded that the SlyA-type regulatory network is a secondary network required for *carA-H* transcription (McGowan et al., 2005). Interestingly, it was found that partial carbapenem synthesis could be restored in a *hor* mutant by the addition of unnaturally high levels of OHHL. This suggests that Hor regulation is able to be circumvented under certain conditions (McGowan et al., 2005). The regulatory network of carbapenem production within *P. c.* subsp. *brasiliensis* ICMP 19477 is summarised in Figure 5.12.

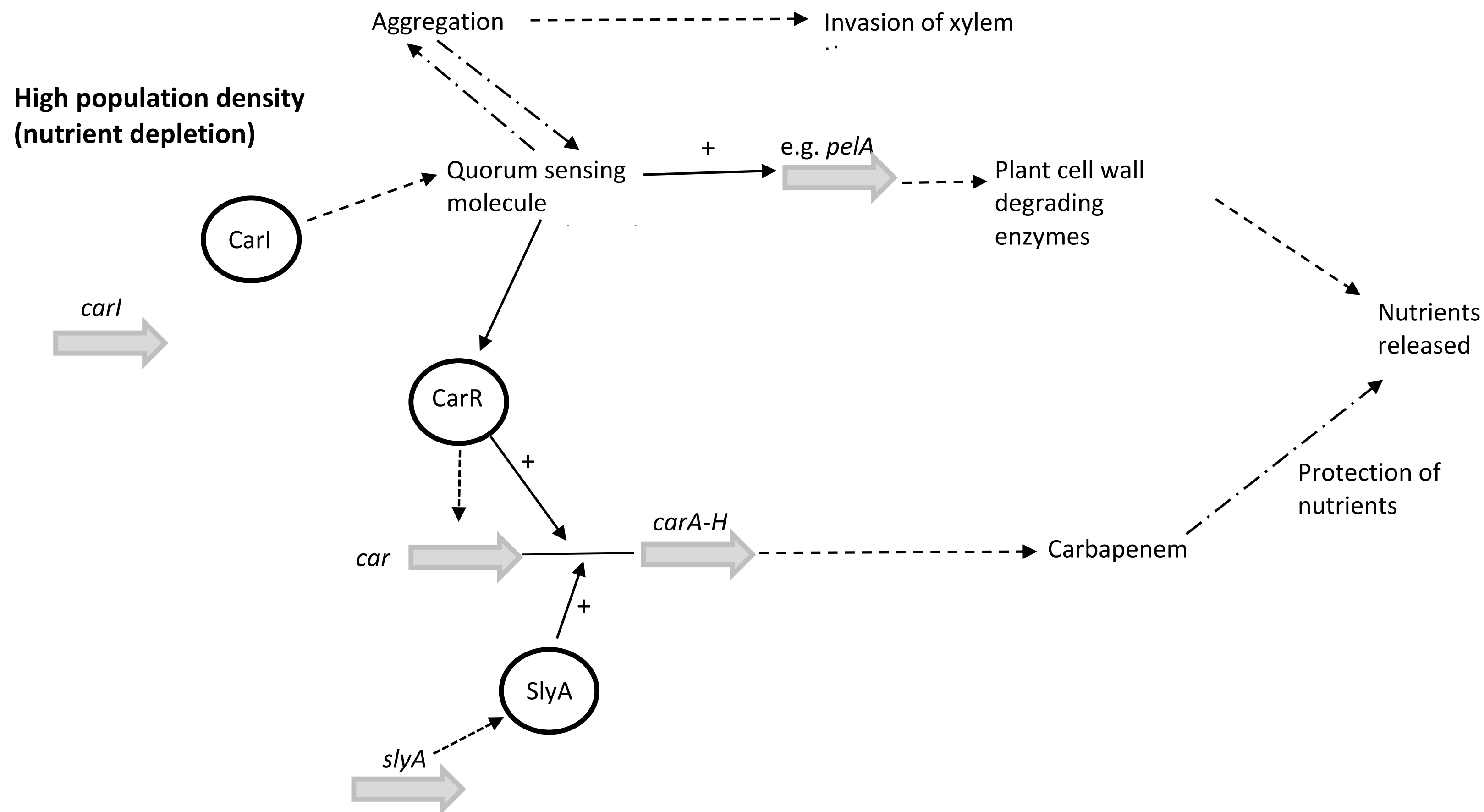


Figure 5.12: The quorum sensing and SlyA-type regulatory network in *P. c.* subsp. *brasiliensis* ICMP 19477. These systems regulate carbapenem production as well as other processes within the bacteria. Genes are represented as grey arrows, transcription of a gene is represented as a dotted arrow and the resultant gene product is given within a circle. The QS molecule *N*-(3-oxohexanoyl)-L-homoserine lactone is designated as OHHL. The influence of the regulatory genes products, as well as OHHL, on the level of gene transcription is shown by the black arrows; a positive influence is designated as +. Resultant products or effects following gene transcription are represented with dashed arrows. Predicted associations are represented as dotted and dashed arrows.

Inactivation of *carI* and *slyA* reduced the CI of *P. c.* subsp. *brasiliensis* ICMP 19477 both on agar plates and in potato tubers (Sections 5.2.5 and 5.2.6). As described previously for the *carR* mutant, the difference in the competitive fitness of the *carI* and *slyA* mutants and the WT was greatest *in planta* (Section 4.3). This may be due to an increased advantage afforded to a bacteria that is able to protect the nutrients released from the host tissue, under the nutrient limited conditions of the tuber. Therefore, antimicrobial production would be selected for under these conditions and confer an adaptive advantage within the ecological niche.

Although carbapenem production, and hence its regulation, were important to competitive fitness of *P. c.* subsp. *brasiliensis* ICMP 19477, when either *carI* or *slyA* was inactivated the competitive advantage of *P. c.* subsp. *brasiliensis* ICMP 19477 was not abolished entirely (sections 5.2.7 and 5.2.8). This was consistent with the results of fitness assays using the *carR* mutant, reiterating the role of other unknown factors in the competitive fitness of the carbapenem producer (Sections 4.2.10 and 4.2.11). Given that CarI is the major QS regulator and SlyA is transcribed at high cell densities, any unknown factor would probably be regulated independently of cell density. This makes it unlikely that another antimicrobial produced by *P. c.* subsp. *brasiliensis* ICMP 19477 confers fitness. Instead, the competitive advantage of *P. c.* subsp. *brasiliensis* ICMP 19477 might be due to the capacity of the bacterium to utilise certain nutrients more efficiently than *P. atrosepticum* SCRI1043 (as discussed in Section 4.3). The impact of differences in nutrient utilisation by these two bacteria would certainly be more apparent in the plant, where the greatest fitness advantage to *P. c.* subsp. *brasiliensis* ICMP 19477 was observed.

Finally, the reduced carbapenem production and associated ecological fitness of the *carI* and *slyA* mutants was not unexpected. Quorum sensing modulates the co-ordinated production of many important components of the pathogen's arsenal for colonising host plants and for competing with other microbes (Mäe et al., 2001). Quorum sensing alone differentially regulates 26% of the *P. atrosepticum* genome during infection of potato (Liu et al., 2008). This co-ordination makes sense when considering the ecology of the pathogen. In *P. c.* subsp. *carotovorum*, quorum sensing regulates the expression of PCWDEs ensuring their production when cell density is high (Pirhonen et al., 1991; Pierson III et al., 1998). The expression of antimicrobials such as carbapenems under similar circumstances ensures that they are synthesised at the same time as the large amount of nutrients are released into the surrounding environment. Thus, as previously predicted for *P. c.* subsp. *carotovorum* (Whitehead et al., 2002; Coulthurst et al., 2005), co-ordinated production of the PCWDEs

and carbapenem enables *P. c.* subsp. *brasiliensis* ICMP 19477 to defend nutrients made available during infection of the host (rather than as a mechanism to invade an already established population).

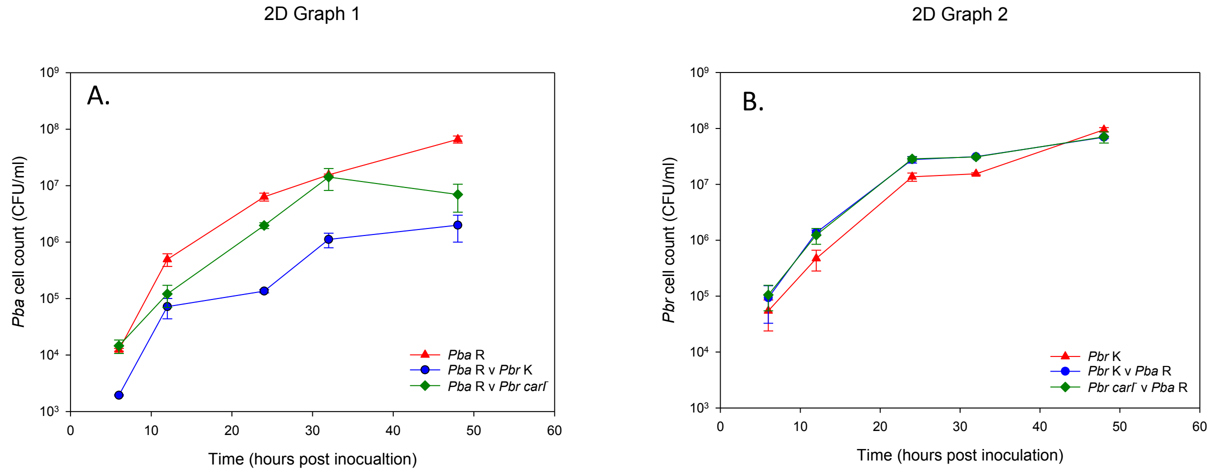
The coordinated, large scale release of the antimicrobial molecule may also be a method of avoiding the development of antimicrobial resistance within the 'sensitive' population. If the carbapenem was released by the bacteria throughout growth, sub-inhibitory levels would be circulating in the environment, possibly enabling tolerance mechanisms to develop (Hibbing et al., 2010).

Results of this study are consistent with findings that demonstrated that in *P. c.* subsp. *carotovorum*, QS is a specific, intra-specific communication system (Fuqua and Greenberg, 2002; Lazdunski et al., 2004; Pappas et al., 2004). *Pectobacterium atrosepticum* SCRI1043 also harbours *carI* (known as *expI*) (Barnard and Salmond, 2007), which is involved in QS regulation of numerous genes including those involved in production of PCWDEs and other traits relating to pathogenicity (Liu et al., 2008). Thus, the identification of a *carI* mutant in *P. c.* subsp. *brasiliensis* ICMP 19477 using the antagonism assays suggested that the OHHL produced by *P. atrosepticum* was not recognised by *P. c.* subsp. *brasiliensis* (otherwise the mutant would have been complemented by the OHHL produced by *P. atrosepticum* SCRI1043). The lack of cross-recognition was consistent with observations in *P. c.* subsp. *carotovorum*, which showed that different strains produce one of three *N*-AHL molecules (Brader et al., 2005). These molecules differ in the length of their acyl chain (Brader et al., 2005). The QS regulators in these strains only recognise *N*-AHL molecules with the same acyl chain length (Brader et al., 2005).

The concept of *N*-AHL molecules forming intra-specific communication systems is distinct from observations in *Pseudomonas*. For example, in *P. aureofaciens*, the production of the antimicrobial phenazine is controlled by QS, via the *phzI* and *phzR* genes (Wood and Pierson, 1996). A mutation in the *phzI* gene results in loss of phenazine production due to the bacteria's inability to produce the QS auto-inducer molecule. Phenazine production, however, was restored when the *phzI* mutant was grown in the presence of other bacteria in its ecological niche. This suggested that *P. aureofaciens* was able to utilise different QS molecules to activate the production of phenazine (Pierson III et al., 1995; Wood and Pierson, 1996). In future, it would be interesting to examine whether the OHHL produced by *P. c.* subsp. *carotovorum* ICMP 5702 could restore the production of carbapenem in *P. c.* subsp. *brasiliensis* ICMP 19477, especially as this strain harbours a carbapenem biosynthetic cluster of its own. This would help to define whether the specificity of OHHL production extends to *P. c.* subsp. *brasiliensis* and other carbapenem producing strains.

Appendix D

D.1 *carI*$^-$ *in vitro* competition graphs



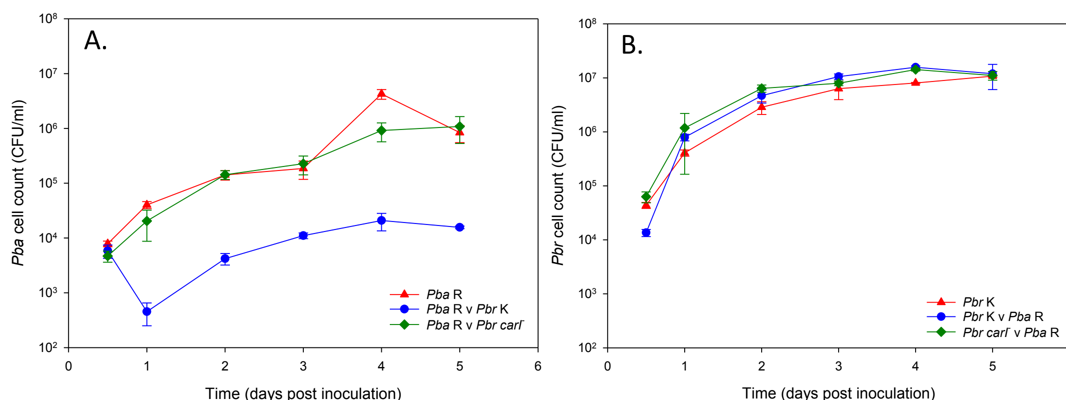
Second, independently conducted experiment demonstrating the effect of co-inoculation with *P.c.* subsp. *brasiliensis* (*Pbr*) ICMP19477 and *P. c.* subsp. *brasiliensis carI*$^-$ (*Pbr carI*$^-$) on growth of *P. atrosepticum* (*Pba*) SCRI1043 on solid agar plates (MMA).

A: A graph showing the growth (mean CFUs) of *Pba* SCRI1043 Rif resistant strain (*Pba* R) over a 48 h period at 28 °C when inoculated onto MMA in isolation or co-inoculated with *Pbr* ICMP 19477 tagged with Km resistance (*Pbr* K), or the *carI* mutant *Pbr carI*$^-$ (at a 1:1 ratio). **B**: A graph showing the growth (mean CFUs) of *Pbr* K when cultured in isolation on solid agar plates or in co-inoculations with *Pba* R as well as *Pbr carI*$^-$ co-inoculated with *Pba* R (at a 1:1 ratio). For each graph, the mean CFUs were calculated for each treatment from triplicate assays (solid line), the error bars represent the standard deviation.

D.2 Competitive index of *carI*$^-$ *in vitro* competition assay

	***Pbr* K v *Pba* R**		***Pbr carI*$^-$ v *Pba* R**	
Time (hours)	Log CI	SD	Log CI	SD
6	1.4	0.4	0.5	0.3
12	1.1	0.2	0.7	0.3
24	2.1	0.03	0.8	0.06
32	0.9	0.5	0.7	0.3
48	1.4	0.2	0.7	0.3

D.3 *carI*$^{-}$ *mutant potato tuber competition graphs*



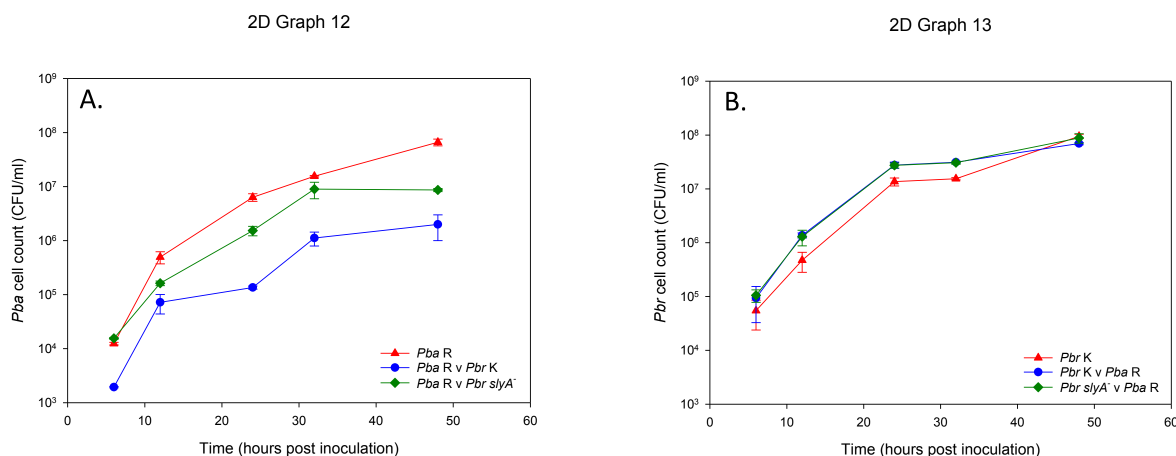
Second, independently conducted experiment, using potato tubers sourced at a different time of year, demonstrating the effect of co-inoculation with *P. c.* subsp. *brasiliensis* (*Pbr*) ICMP19477 and *P. c.* subsp. *brasiliensis carI*$^{-}$ (*Pbr carI*$^{-}$) on growth of *P. atrosepticum* (*Pba*) SCRI1043 in potato tubers ('Ilam Hardy').

A: A graph showing the growth (mean CFUs) of *Pba* SCRI1043 Rif resistant strain (*Pba* R) over a 48 h period at 28 °C when inoculated into potato tubers in isolation or co-inoculated with *Pbr* ICMP19477 tagged with Km resistance (*Pbr* K), or the *carI* mutant *Pbr carI*$^{-}$ (at a 1:1 ratio). **B**: A graph showing the growth (mean CFUs) of *Pbr* K when cultured in isolation in potato tubers or in co-inoculations with *Pba* R as well as *Pbr carI*$^{-}$ co-inoculated with *Pba* R (at a 1:1 ratio). For each graph, the mean CFUs were calculated for each treatment from triplicate assays (solid line), the error bars represent the standard deviation).

D.4 Competitive index of *carI*$^{-}$ *in planta* competition assay

	***Pbr* K v *Pba* R**		***Pbr carI*$^{-}$ v *Pba* R**	
Time (days)	Log CI	SD	Log CI	SD
0.5	0.7	0.2	0.5	0.2
1	3.6	0.2	1.0	0.2
2	4.5	0.2	2.0	0.5
3	4.4	0.2	1.5	0.3
4	4.6	0.1	1.2	0.1
5	3.5	0.1	1.4	0.2

D.5 *slyA*$^-$ *in vitro* competition graphs



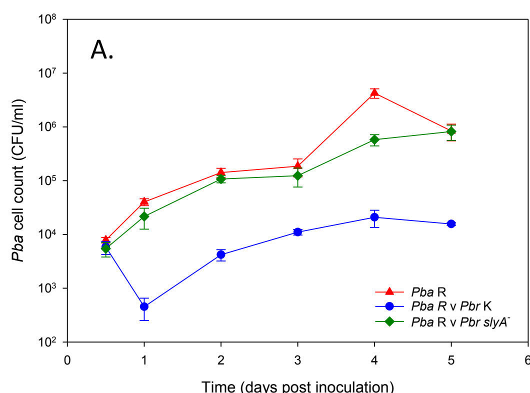
Second, independently conducted experiment demonstrating the effect of co-inoculation with *P. c.* subsp. *brasiliensis* (*Pbr*) ICMP19477 and *P. c.* subsp. *brasiliensis* (*Pbr slyA*$^-$) on growth of *P. atrosepticum* (*Pba*) SCRI1043 on solid agar plates (MMA).

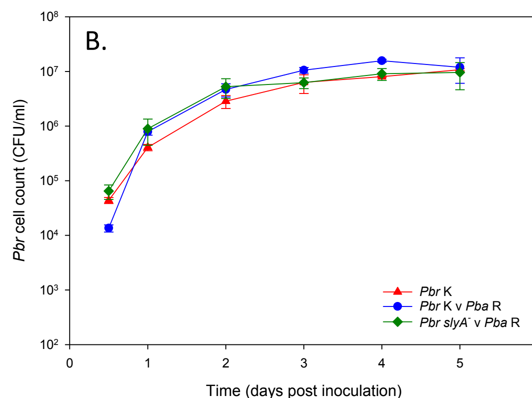
A: A graph showing the growth (mean CFUs) of *Pba* SCRI1043 Rif resistant strain (*Pba* R) over a 48 h period at 28 °C when inoculated onto MM in isolation or co-inoculated with *Pbr* ICMP 19477 tagged with Km resistance (*Pbr* K), or the *slyA* mutant *Pbr slyA*$^-$ (at a 1:1 ratio). **B**: A graph showing the growth (mean CFUs) of *Pbr* K when cultured in isolation on solid agar plates or in co-inoculations with *Pba* R as well as *Pbr slyA*$^-$ co-inoculated with *Pba* R (at a 1:1 ratio). For each graph, the mean CFUs were calculated for each treatment from triplicate assays (solid line), the error bars represent the standard deviation.

D.6 Competitive index of *slyA*$^-$ in *in vitro* competition assay

	***Pbr* K v *Pba* R**		***Pbr slyA*$^-$ v *Pba* R**	
Time (hours)	Log CI	SD	Log CI	SD
6	1.4	0.4	0.6	0.1
12	1.1	0.2	0.7	0.1
24	2.1	0.03	1.1	0.1
32	0.9	0.5	0.4	0.2
48	1.4	0.2	0.8	0.1

D.7 *slyA*⁻ mutant potato tuber competition graphs





Second, independently conducted experiment, using potato tubers sourced at a different time of year, demonstrating the effect of co-inoculation with *P. c.* subsp. *brasiliensis* (*Pbr*) ICMP19477 and *P. c.* subsp. *brasiliensis* (*Pbr slyA*⁻) on growth of *P. atrosepticum* (*Pba*) SCRI1043 in potato tubers ('Ilam Hardy').

A: A graph showing the growth (mean CFUs) of *Pba* SCRI1043 Rif resistant strain (*Pba* R) over a 48 h period at 28 °C when inoculated into potato tubers in isolation or co-inoculated with *Pbr* ICMP 19477 tagged with Km resistance (*Pbr* K), or the *slyA* mutant *Pbr slyA*⁻ (at a 1:1 ratio). **B**: A graph showing the growth (mean CFUs) of *Pbr* K when cultured in isolation in potato tubers or in co-inoculations with *Pba* R as well as *Pbr carI*⁻ co-inoculated with *Pba* R (at a 1:1 ratio). For each graph, the mean CFUs were calculated for each treatment from triplicate assays (solid line), the error bars represent the standard deviation.

D.8 Competitive index of *slyA*⁻ in *in planta* competition assays

	***Pbr* K v *Pba* R**		***Pbr slyA*⁻ v *Pba* R**	
Time (days)	Log CI	SD	Log CI	SD
0.5	0.7	0.2	0.6	0.2
1	3.6	0.2	1.3	0.3
2	4.5	0.2	2.1	0.3
3	4.4	0.2	1.2	0.4
4	4.6	0.1	1.0	0.1
5	3.5	0.1	1.1	0.1

D.9 *slyA* complementation sequence

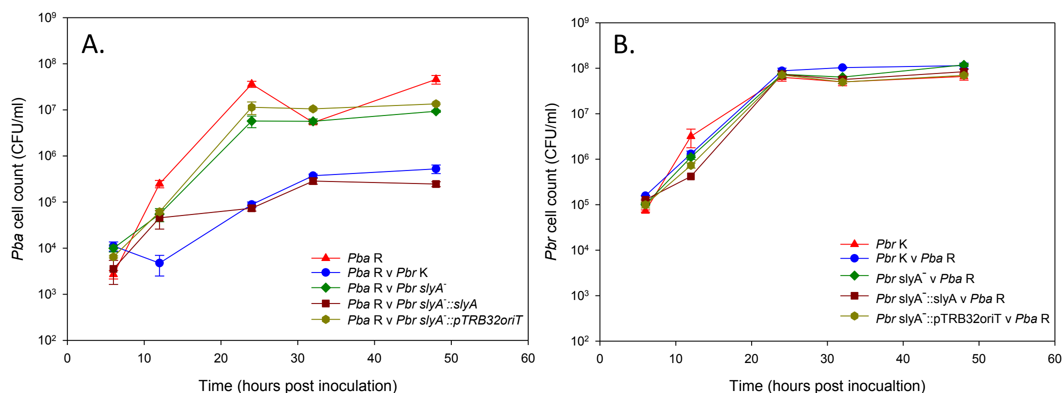
Nucleotide sequence of KCO_21137 (*slyA*), following cloning into pTRB32oriT (Section 5.2.9). Insert-specific primers are underlined and restriction sites are written in bold type. The sequence shown in italics represent vector sequence.

GCTTTGTGAGCGGATAACAATTATAATAGATTCAATTGTGAGCGGATAACAATTTCACACAGAATTCATTAAAG
*AGGAGAAATTAACTATGAGAGGATCGCATCACCATCACCATCACGGATCCGCATGCGAGCTC*GCGGTC**GGTAC**
CGCGTGCTAACAATAAGGAGAGGATGGAATTGCCATTAGGATCTGATTTAGCCCGTCTGGTGCGCGTATGGC
GTGCGCTGGTCGATCATCGATTAAAACCACTTGAACTGACTCAGACGCATTGGGTCACGTTGCATAACATATA
CCATCTACCCCCTGGGCAGTCGCAGATTCAACTTGCCAAAGCGATAGGTATTGAGCAACCCTCATTAGTCCGA
ACACTGGATCAGCTTGAGGAAAAAGGGTTAATCACTCGCCACGTTTGCGCGCACGATCGTCGGGCAAAACGT
ATTATGCTGACCGAATCAGCAGAGCCAATCATACAAGCAGTCAATGGTGTAATTAGCCATACACGTAGTGAAG
TCTTATTTGGTATTACGCCGGAGCAGGTTGATGAATTAGCGCTGCTGGTTTCGCGTCTTGAGAAAAATATATTG
GCATTACATGAGAATCAAGCGTAGTGGGTTACGCAGAGGGAGAA**AAGCTT**CGC*AATTAGCTGAGCTTGGACT*
CCTGTTGATAGATCCAGTAATGACCTCAGAACTCCATCTGGATTTGTTCAGAACGCTCGGTTGCCGCCGGGCGT
TTTTTATTGGTGAGAATC

D.10 Competitive index of the *slyA* complemented strain in vitro competition assays

	***Pbr* K v *Pba* R**		***Pbr slyA*$^{-}$ v *Pba* R**		***Pbr slyA::slyA*$^{-}$ v *Pba* R**		***Pbr slyA^{-}::pTRB32oriT* v *Pba* R**	
Time (hours)	Log CI	SD	Log CI	SD	Log CI	SD	Log CI	SD
6	1.1	0.1	0.8	0.1	2.0	0.3	1.4	0.1
12	2.4	0.1	1.1	0.1	1.3	0.2	1.3	0.1
24	2.4	0.2	0.9	0.2	3.3	0.1	1.1	0.2
32	2.4	0.1	0.9	0.04	2.6	0.1	0.9	0.05
48	1.3	0.1	0.9	0.01	1.9	0.05	1.0	0.02

D.11 *slyA* complementation *in vitro* competition graphs



Second, independently conducted experiment demonstrating the effect of co-inoculation with *P. brasiliensis* (*Pbr*) ICMP 19477 *slyA*⁻ complemented strain (*Pbr slyA⁻::pTRB32oriTslyA*) on growth of *P. atrosepticum* (*Pba*) SCRI1043 on solid agar plates (MMA).

A: A graph showing the growth (mean CFUs) of *Pba* SCRI1043 Rif resistant strain (*Pba* R) over a 48 h period at 28 °C when inoculated into MM in isolation or co-inoculated with *Pbr* ICMP19477 tagged with Km resistance (*Pbr* K), or the *slyA* mutant *Pbr slyA⁻*, gene complemented strain *Pbr slyA⁻ ::pTRB32oriTslyA* or the control strain *Pbr slyA⁻::pTRB32oriT* containing an empty cop of the pTRB32oriT plasmid (at a 1:1 ratio). **B**: A graph showing the growth (mean CFUs) of *Pbr* K when cultured in isolation on solid agar plates as well as the *Pbr* strains in co-inoculation with *Pba* R (at a 1:1 ratio). For each graph, the mean CFUs were calculated for each treatment from triplicate assays (solid line), the error bars represent the standard deviation.

Chapter 6

Final discussion

The *P. c.* subsp. *brasiliensis* strain, PBR1692, has been shown to inhibit the growth of *P. atrosepticum* SCRI1043 *in vitro* (Marquez-Villavicencio et al., 2011). This inhibition was not observed in potato stems and was dependent on the *P. c.* subsp. *brasiliensis* strain tested (Marquez-Villavicencio et al., 2011). It was predicted that this observation was due to the production of a carbapenem by *P. c.* subsp. *brasiliensis* strain PBR1692 (Marquez-Villavicencio et al., 2011), however this was not proven. A New Zealand *P. c.* subsp. *brasiliensis* strain, ICMP 19477, was isolated in recent years (Panda et al., 2012), which was found to encode multiple antimicrobial synthesis genes on HAIs (Panda et al., 2015b). Competition assays established that *P. c.* subsp. *brasiliensis* ICMP 19477 was able to inhibit *P. atrosepticum in vitro* as well as in potato tubers (Sections 3.2.4 and 3.2.6). This showed that *P. c.* subsp. *brasiliensis* ICMP 19477 had a competitive advantage over *P. atrosepticum* SCRI1043 within the host plant. This led to the hypothesis that the production of an antimicrobial molecule by *P. c.* subsp. *brasiliensis* gave it a competitive advantage within its ecological niche and aided in the emergence of *P. c.* subsp. *brasiliensis* as a pathogen.

Interestingly, *P. c.* subsp. *carotovorum* ICMP 5702, which also encodes a carbapenem synthesis cluster, was unable to inhibit the growth of *P. atrosepticum* SCRI1043, however it was resistant to *P. c.* subsp. *brasiliensis* ICMP 19477. Bioinformatic techniques were used to identify antimicrobial genes likely to be responsible for the competitive fitness of *P. c.* subsp. *brasiliensis* ICMP 19477.

The carbapenem molecule, encoded on HAI PbN1-GI20, was identified as a potential antimicrobial gene cluster involved in the competitive fitness of *P. c.* subsp. *brasiliensis* ICMP 19477 (Section 3.2.12). A carbapenem cluster with high nucleotide similarity was also identified in *P. c.* subsp. *brasiliensis* PBR1692 (ID = 98%). Therefore, it did not appear that inhibition of bacteria via production of the carbapenem molecule was as strain specific as previously described (Marquez-Villavicencio et al., 2011). The carbapenem synthesis cluster of *P. c.* subsp. *carotovorum* ICMP 5702 was less similar to ICMP 19477 than PBR1692 (ID = 87.8%). As *P. c.* subsp. *carotovorum* ICMP 5702 did not inhibit the growth of *P. atrosepticum* SCRI1043, it suggests that this level of nucleotide difference is sufficient to cause the difference in specificity of the molecule.

The differences in nucleotide identity of the carbapenem synthesis clusters, as well as the specificity of the resultant molecules, produced by the two SREs suggests that a *P. c.* subsp. *carotovorum* and *P. c.* subsp. *brasiliensis* carbapenem cluster diverged. It may be that the HAI containing the carbapenem

cluster was obtained before the two sub-species diverged and it was after this event that the differences between the carbapenem genes occurred. However, it may represent two different insertion events. However, as *P. c.* subsp. *carotovorum* ICMP 5702 has retained the carbapenem synthesis cluster, so one assumes it must provide an advantageous function for the organism. Possibly it is active against other organisms or other *Pectobacterium* strains.

Antagonism assays identified that there was variation between *Pectobacterium* species and subspecies, in relation to their reaction to the carbapenem molecule. *P. c.* subsp. *brasiliensis* ICMP 19477 can be considered a producer, *P. atrosepticum* SCRI1043 is sensitive and *P. c.* subsp. *carotovorum* ICMP 5702 is resistant. This variation within the bacterial community, is concurrent with *in silico* population dynamics studies that have been confirmed by serial transfer experiments (Frank, 1994; Tan and Riley, 1996; Durrett and Levin, 1997; Riley and Gordon, 1999; Riley and Wertz, 2002). These predicted that the initial predominant population is sensitive to antimicrobial molecules (*P. atrosepticum* SCRI1043), as this is the most energy efficient state if not confronted by antibiotics. Therefore, the emergence of a producer strain such as *P. c.* subsp. *brasiliensis* ICMP 19477, sees the producer out-compete the sensitive population. This occurs over time and is more evident as the producer increases in numbers, as has been seen for *P. c.* subsp. *brasiliensis* on potatoes (Duarte et al., 2004; van der Merwe et al., 2010; Leite et al., 2014; Werra et al., 2015). The carbapenem synthesis cluster is likely to be maintained in *P. c.* subsp. *brasiliensis* as it provides a competitive advantage within the ecological niche.

The *P. c.* subsp. *carotovorum* ICMP 5702, also has a carbapenem cluster but was not antagonistic towards *P. atrosepticum* SCRI1043. It may be that over time *P. atrosepticum* has gained resistance to the carbapenem produced by *P. c.* subsp. *carotovorum* ICMP 5702, which is predicted based on the models of population antimicrobial dynamics. For example, efflux pumps or porins may be expressed in *P. atrosepticum* SCRI1043 that are able to express the molecule produced by *P. c.* subsp. *carotovorum* ICMP 5702, but do not confer resistance to the apparently different carbapenem molecule produced by *P. c.* subsp. *brasiliensis* (Limansky et al., 2002; Mena et al., 2006; Rodríguez-Martínez et al., 2009). It may also be the case that the carbapenem cluster of *P. c.* subsp. *carotovorum* ICMP 5702 is not active and therefore no carbapenem product is produced.

Pectobacterium carotovorum subsp. *carotovorum* ICMP 5702 was also found to be resistant to the carbapenem molecule produced by *P. c.* subsp. *brasiliensis* ICMP 19477 (Section 3.2.2). As previously discussed, it is likely that the two bacteria produce carbapenem molecules of different specificities. Therefore, *P. c.* subsp. *carotovorum* ICMP 5702 is likely to encode a β-lactamase active against this molecule as the intrinsic resistance mechanism of *P. c.* subsp. *carotovorum* ICMP 5702, encoded by

carF and *carG,* is unlikely to provide resistance to the carbapenem produced by *P. c.* subsp. *brasiliensis* ICMP 19477 (McGowan et al., 1997).

Pectobacterium carotovorum subsp. *carotovorum* strains have also been identified that do not produce a carbapenem molecule, but encode 'cryptic' carbapenem clusters that lack a functional CarR homologue; these clusters are widespread in *P. c.* subsp. *carotovorum* strains (Holden et al., 1998). This suggests that the production of the carbapenem has been lost by these strains, due to the metabolic cost of producing the molecule and the possible resistance of *P. atrosepticum* to carbapenems produced by *P. c.* subsp. *carotovorum.* Therefore, production of a carbapenem by *P. c.* subsp. *brasiliensis* ICMP 19477 and PBR1692, may only provide a short term competitive advantage, before *P. atrosepticum* SCRI1043 develops a resistance mechanism.

The presence of resistant, producer, non-producer and sensitive strains within a bacterial population would produce variation in the population of *Pectobacterium* found in the environment. It would also account for the observation that inhibition of sensitive *P. atrosepticum* and *P. c.* subsp. *carotovorum* strains, was strain dependent (Marquez-Villavicencio et al., 2011).

An understanding of the flux of the phenotypes observed for the different SREs studied would provide an insight into the evolution and selection pressures associated with carbapenem production within the ecological niche. Long-term studies into the community dynamics within the potato tuber, such as the studies conducted on *E .coli* found in mice (Riley and Gordon, 1999), could be utilised to study such interactions. For example, *P. atrosepticum* SCRI1043, *P. c.* subsp. *carotovorum* ICMP 5702 and *P. c.* subsp. *brasiliensis* ICMP 19477 could be co-inoculated into potato tubers and serially transferred (Riley and Wertz, 2002) to other tubers over the course of a number of months. In colicin evolution and adaption, changes in the population dynamics were noticeable after seven months (Riley and Gordon, 1999). This would provide an insight into whether the predicted population dynamics of antimicrobial producing bacteria, is relevant for carbapenem producers.

The results of this study are limited to an understanding of how the carbapenem produced by *P. c.* subsp. *brasiliensis* ICMP 19477 influences related bacteria. However clinically available carbapenems have been shown to have activity against a broad spectrum of bacteria, both gram positive and gram negative (Nix et al., 2004; Bassetti et al., 2009; Queenan et al., 2010). More importantly, the carbapenem molecule SQ27,860, produced by *Serratia* and *Erwinia,* was shown to inhibit the growth of the gram positive bacteria *Staphylococcus aureus,* as well as the gram negative *E. coli* and *Enterobacter cloacae* (Parker et al., 1982). Therefore, it is likely that the production of the carbapenem

molecule by *P. c.* subsp. *brasiliensis* ICMP 19477 would have a greater ecological impact than just inhibiting *P. atrosepticum* SCRI1043.

To understand the influence of the carbapenem molecule produced by *P. c.* subsp. *brasiliensis* ICMP 19477 on the wider microbial community in potato tubers, whole community studies need to be considered. Any changes within the bacterial endophytic community could be compared when the *P. c.* subsp. *brasiliensis* ICMP 19477 WT is inoculated compared to when a *carR* mutant is used. The endophytic community would be determined using next generation sequencing. Such techniques have been successfully used to study changes in the potato endophyte community following *P. atrosepticum* infection, over the course of seven months (Kõiv et al., 2015). The activity and impact of the carbapenem produced by *P. c.* subsp. *brasiliensis* ICMP 19477 in the soil environment should also be considered.

The genome of *P. c.* subsp. *brasiliensis* ICMP 19477 encodes multiple antimicrobial synthesis genes (summarised in Figure 6.1). The competitive fitness of *P. c.* subsp. *brasiliensis* ICMP 19477 was not abolished in any of the carbapenem associated transposon mutants. Therefore, it is likely that one or more of these molecules are active within the ecological niche and responsible for this observation. The identified antimicrobials are both broad spectrum molecules, such as carbapenem and phenazine as well as those that target closely related species, such as the bacteriocin. Significant effort is therefore given to inhibiting closely related bacterial strains, which are most likely to be found within the same ecological niche or host plant of the producer. This suggests an ecological significance associated with antimicrobial production.

Many studies relating to plant pathogens focus on the virulence determinants produced by these organisms. However, with the identification of multiple HAIs dedicated to antimicrobial synthesis in *P. c.* subsp. *brasiliensis* ICMP 19477, this suggests that microbe-microbe interactions are also important for these plant pathogens.

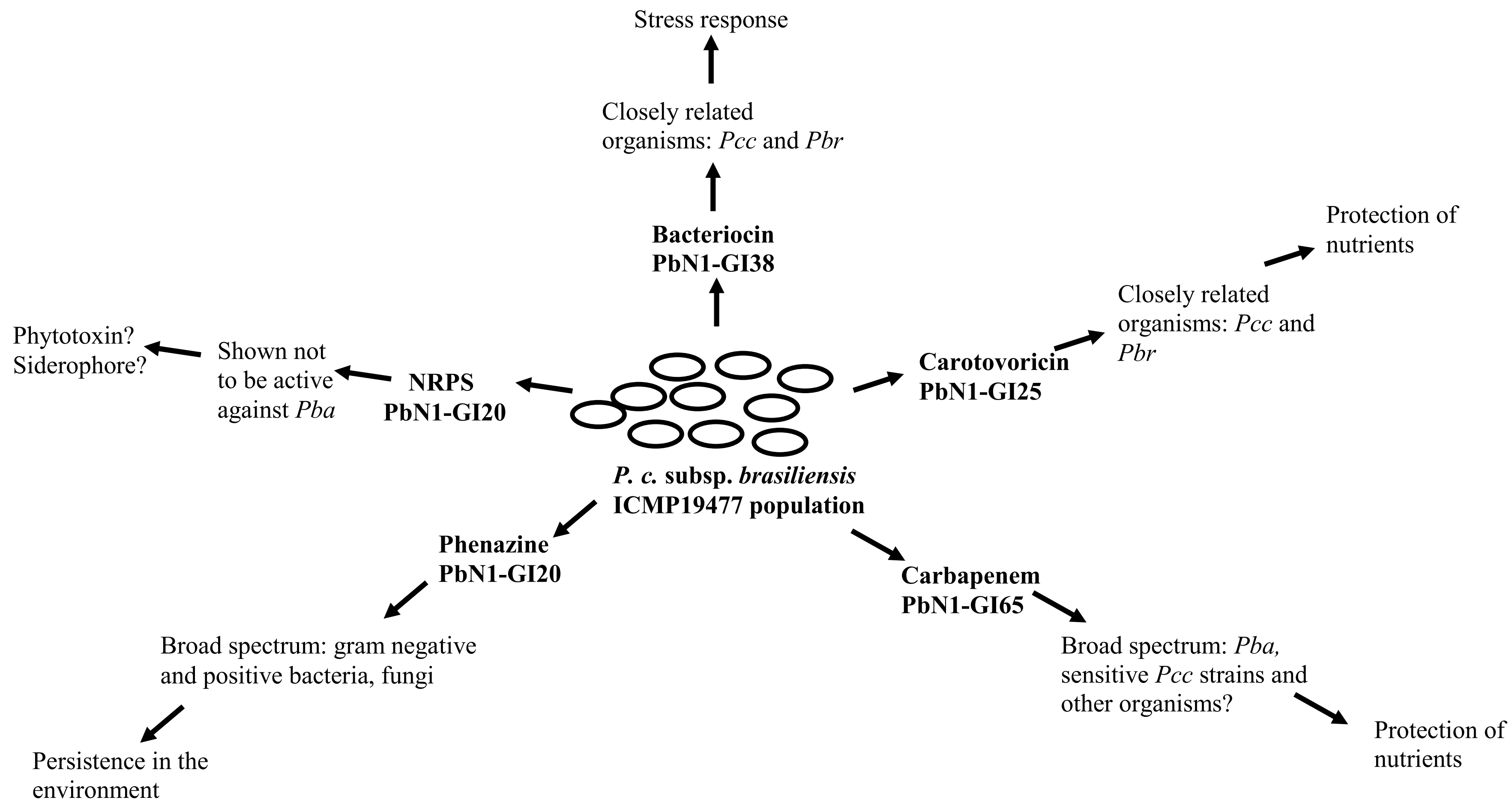


Figure 6.1. Proposed network of antimicrobial molecules produced by *P. c.* subsp. *brasiliensis* (*Pbr*) ICMP19477.

The HAI location, target organisms and function in potato tubers are given. The organisms likely to be inhibited by the produced antimicrobial molecule are also described; *P. atrosepticum* (*Pba*), *P. c.* subsp. *carotovorum* (*Pcc*).

Further evidence that the antimicrobial genes of *P. c.* subsp. *brasiliensis* ICMP 19477 provide a competitive advantage is the retention of large gene clusters such as the 21 CDSs of the carotovoricin cluster (Section 3.2.9). This is because a large metabolic cost is associated with the production of antimicrobial molecules (Hibbing et al., 2010), the benefit of antimicrobial production must therefore outweigh the cost of production. When this is no longer the case, and it appears that the antimicrobial no longer provides an advantage, the function is lost, such as the cryptic carbapenem clusters widespread within *P. c.* subsp. *carotovorum* (Holden et al., 1998).

In this study, it was demonstrated that the regulatory network involved in carbapenem production involves the QS and SlyA-type regulatory systems (Chapter 5). These pathways are associated with the regulation of many secondary metabolites including virulence determinants (Perombelon, 2002) (Ellison and Miller, 2006). Therefore, the regulation of carbapenem synthesis is organised in such a way as to minimise the metabolic impact as a specific regulatory pathway is not dedicated to the task. This enables the coordination of resources during the production of many secondary metabolites. The coordination of resources is also demonstrated in carbapenem transport. In *Serratia* and *Pectobacterium,* the carbapenem synthesis clusters are not associated with a dedicated transporter. However, the thienamycin cluster in *Streptomyces cattleya* contains the gene *thnJ,* which is predicted to encode a protein similar to a membrane transport protein in *S. coelicolor* (Núñez et al., 2003). This therefore suggests that the carbapenem molecules are actively transported from the producing cells. Some transporters, particularly members of the MFS superfamily, are not associated with a biosynthetic cluster and transport many different molecules (Marger and Saier, 1993). Therefore, the carbapenem molecule may be exported via such a transporter to avoid the requirement for production of a carbapenem-specific transporter.

The transporter involved in carbapenem transport in *P. c.* subsp. *brasiliensis* ICMP 19477 was not identified in this study. However, a transposon mutant disrupted in a membrane associated gene (*P. c.* subsp. *brasiliensis mp*$^{-}$) showed a reduced zone of inhibition when co-inoculated with *P. atrosepticum* SCRI1043 (Section 4.2.5). Further analysis is required of the gene and the putative gene product, however this mutant may provide an insight into one method of carbapenem transport.

It is also possible that the carbapenem molecule has a secondary function that was not detected in this study. For example, syringomycin produced by *Pseudomonas syringae* pv. *syringae* primarily functions as a phytotoxin required for disease progression within the host (Sinden et al., 1971). However, the molecule also demonstrated antimicrobial activity against a wide range of organisms such as fungi, gram-positive and gram-negative bacteria including other plant pathogens such as

Xanthomonas malvaceanum and *Arobacterium tumefaciens* (Sinden et al., 1971). It may be, therefore, that identified antimicrobial molecules are in fact secondary metabolites with another primary function.

The discussed results are one interpretation of the data. An alternative should be considered when predicting the ecological importance of antimicrobial molecules. It has been proposed that in the environment antimicrobial molecules are present at sub-inhibitory levels. At such concentrations, it is thought that the molecules mainly function as signalling molecules (Davies et al., 2006). Such molecules are considered as a large group of small molecules, which regulate gene expression within a bacterial population and possibly initiate interactions between organisms (Yim et al., 2007). Such molecules have been shown to both increase and decrease gene expression. In group A *Streptococci,* sub-inhibitory levels of protein synthesis has been shown to downregulate virulence genes (Tanaka et al., 2005). Under the same conditions, amioglycosides, also protein synthesis inhibitors, induce biofilm formation (Hoffman et al., 2005). Interestingly, the known QS signalling molecule *N*-(3-oxododecanoyl)-homoserine lactone, has been shown to have antimicrobial activity against gram-positive bacteria (Kaufmann et al., 2005). However, no studies focusing on the signalling potential of antimicrobial molecules have been conducted *in situ,* rather these are laboratory based results.

Considering the different potential functions of antimicrobial molecules, the competition assay methods used in this study are a limitation on the results. For the in-tuber competition assays, bacterial inoculums above natural levels were used. Therefore, the carbapenem is likely to be produced at unnaturally high levels not encountered within the environment. Therefore, the potential function of the molecule, as well as the effect on other organisms that occur *in situ*, may not have been determined. Studies focusing on lower, more natural inoculum levels should therefore be considered. It may be possible to study such interactions using the natural infection of potato tubers by fluorescently tagged bacteria. Furthermore, the transcriptome of the antimicrobial producing bacteria could be studied and compared to a mutant that is unable to produce the antimicrobial molecule. This would determine any alteration in gene transcription that occurs due to production of the antimicrobial molecule.

References

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., and Lipman, D.J. 1990. Basic local alignment search tool. Journal of Molecular Biology 215:403-410.

Anderson, M.S., Garcia, E.C., and Cotter, P.A. 2012. The *Burkholderia bcpAIOB* genes define unique classes of two-partner secretion and contact dependent growth inhibition systems. Public Library of Science Genetics 8:e1002877.

Andersson, R.A., Eriksson, A.R., Heikinheimo, R., Mäe, A., Pirhonen, M., Kõiv, V., Hyytiäinen, H., Tuikkala, A., and Palva, E.T. 2000. Quorum sensing in the plant pathogen *Erwinia carotovora* subsp. *carotovora*: the role of *expR*$_{Ecc}$. Molecular Plant-Microbe Interactions 13:384-393.

Andro, T., Chambost, J.P., Kotoujansky, A., Cattaneo, J., Bertheau, Y., Barras, F., Van Gijsegem, F., and Coleno, A. 1984. Mutants of Erwinia chrysanthemi defective in secretion of pectinase and cellulase. Journal of Bacteriology 160:1199-1203.

Anilkumar, T., and Chakravarti, B. 1970. Factors affecting survival of *Erwinia carotovora*, causal organism of stalk rot of Maize in soil. Acta Phytopathologica Academiae Scientiarum Hungaricae 5:333-340.

Ansari, M.Z., Yadav, G., Gokhale, R.S., and Mohanty, D. 2004. NRPS-PKS: a knowledge-based resource for analysis of NRPS/PKS megasynthases. Nucleic Acids Research 32:W405-413.

Aoki, S., Webb, J., Braaten, B., and Low, D. 2009. Contact-dependent growth inhibition causes reversible metabolic downregulation in *Escherichia coli*. Journal of Bacteriology 191:1777-1786.

Aoki, S.K., Poole, S.J., Hayes, C.S., and Low, D.A. 2011. Toxin on a stick: modular CDI toxin delivery systems play roles in bacterial competition. Virulence 2:356-359.

Aoki, S.K., Pamma, R., Hernday, A.D., Bickham, J.E., Braaten, B.A., and Low, D.A. 2005. Contact-dependent inhibition of growth in *Escherichia coli*. Science 309:1245-1248.

Aoki, S.K., Diner, E.J., de Roodenbeke, C.t.K., Burgess, B.R., Poole, S.J., Braaten, B.A., Jones, A.M., Webb, J.S., Hayes, C.S., and Cotter, P.A. 2010. A widespread family of polymorphic contact-dependent toxin delivery systems in bacteria. Nature 468:439-442.

Auerbuch, V., Lenz, L.L., and Portnoy, D.A. 2001. Development of a competitive index assay to evaluate the virulence of *Listeria monocytogenes actA* mutants during primary and secondary infection of mice. Infection and Immunity 69:5953-5957.

Axelrood, P.E., Rella, M., and Schroth, M.N. 1988. Role of antibiosis in competition of *Erwinia* strains in potato infection courts. Applied and Environmental Microbiology 54:1222-1229.

Babcock, M.J., Eckwall, E.C., and Schottel, J.L. 1993. Production and regulation of potato-scab-inducing phytotoxins by *Streptomyces scabies*. Journal of General Microbiology 139:1579-1586.

Bachmann, B.O., and Ravel, J. 2009. Methods for *In silico* Prediction of Microbial Polyketide and Nonribosomal Peptide Biosynthetic Pathways from DNA Sequence Data. Methods in Enzymology 458:181-217.

Backman, P.A., and DeVay, J.E. 1971. Studies on the mode of action and biogenesis of the phytotoxin syringomycin. Physiological Plant Pathology 1:215-233.

Bain, R., Millard, P., and Perombelon, M. 1996. The resistance of potato plants to *Erwinia carotovora* subsp. *atroseptica* in relation to their calcium and magnesium content. Potato Research 39:185-193.

Bainton, N., Stead, P., Chhabra, S., Bycroft, B., Salmond, G., Stewart, G., and Williams, P. 1992. *N*-(3-oxohexanoyl)-L-homoserine lactone regulates carbapenem antibiotic production in *Erwinia carotovora*. Biochemical Journal 288:997-1004.

Barnard, A.M., and Salmond, G.P. 2007. Quorum sensing in *Erwinia* species. Analytical and Bioanalytical Chemistry 387:415-423.

Barras, F., van Gijsegem, F., and Chatterjee, A.K. 1994. Extracellular enzymes and pathogenesis of soft-rot Erwinia. Annual Review of Phytopathology 32:201-234.

Basset, A., Tzou, P., Lemaitre, B., and Boccard, F. 2003. A single gene that promotes interaction of a phytopathogenic bacterium with its insect vector, *Drosophila melanogaster*. Federation of European Biochemical Societies Reports 4:205-209.

Bassetti, M., Nicolini, L., Esposito, S., Righi, E., and Viscoli, C. 2009. Current status of newer carbapenems. Current Medicinal Chemistry 16:564-575.

Bastas, K.K., Hekimhan, H., Maden, S., and Tör, M. 2009. First report of bacterial stalk and head rot disease caused by *Pectobacterium atrosepticum* on sunflower in Turkey. Plant Disease 93:1352-1352.

Bauer, D.W., Bogdanove, A.J., Beer, S.V., and Collmer, A. 1994. *Erwinia chrysanthemi hrp* genes and their involvement in soft rot pathogenesis and elicitation of the hypersensitive response. Molecular Plant-Microbe Interactions 7:573-581.

Bazaeal, M., and Helinski, D.R. 1968. Circular DNA forms of colicinogenic factors E1, E2 and E3 from *Escherichia coli*. Journal of Molecular Biology 36:185-194.

Bell, K.S., Sebaihia, M., Pritchard, L., Holden, M.T., Hyman, L.J., Holeva, M.C., Thomson, N.R., Bentley, S.D., Churcher, L.J., Mungall, K., Atkin, R., Bason, N., Brooks, K.,

Chillingworth, T., Clark, K., Doggett, J., Fraser, A., Hance, Z., Hauser, H., Jagels, K., Moule, S., Norbertczak, H., Ormond, D., Price, C., Quail, M.A., Sanders, M., Walker, D., Whitehead, S., Salmond, G.P., Birch, P.R., Parkhill, J., and Toth, I.K. 2004. Genome sequence of the enterobacterial phytopathogen *Erwinia carotovora* subsp. *atroseptica* and characterization of virulence factors. Proceedings of the National Academy of Sciences 101:11105-11110.

Bender, C., Rangaswamy, V., and Loper, J. 1999a. Polyketide Production by Plant-Associated *Pseudomonads* Annual Review of Phytopathology 37:175-196.

Bender, C.L., Alarcon-Chaidez, F., and Gross, D.C. 1999b. *Pseudomonas syringae* phytotoxins: Mode of action, regulation, and biosynthesis by peptide and polyketide synthetases. Microbiology and Molecular Biology Reviews 63:266-292.

Boddy, C.N. 2014. Bioinformatics tools for genome mining of polyketide and non-ribosomal peptides. Journal of Industrial Microbiology and Biotechnology 41:443-450.

Bolwell, G.P., and Wojtaszek, P. 1997. Mechanisms for the generation of reactive oxygen species in plant defence–a broad perspective. Physiological and Molecular Plant Pathology 51:347-366.

Brader, G., Sjöblom, S., Hyytiäinen, H., Sims-Huopaniemi, K., and Palva, E.T. 2005. Altering substrate chain length specificity of an acylhomoserine lactone synthase in bacterial communication. Journal of Biological Chemistry 280:10403-10409.

Braun, V., Pilsl, H., and Groß, P. 1994. Colicins: structures, modes of action, transfer through membranes, and evolution. Archives of Microbiology 161:199-206.

Brenner, D.J., McWhorter, A.C., Kai, A., Steigerwalt, A.G., and Farmer, J.J. 1986. *Enterobacter asburiae* sp. nov., a new species found in clinical specimens, and reassignment of *Erwinia dissolvens* and *Erwinia nimipressuralis* to the genus Enterobacter as *Enterobacter dissolvens* comb. nov. and *Enterobacter nimipressuralis* comb. nov. Journal of Clinical Microbiology 23:1114-1120.

Brown, A., Corbett, D., Eglington, A., and Howarth, T. 1979. Structures of olivanic acid derivatives MM22380, MM22381, MM22382 and MM22383; Four new antibiotics isolated from *Streptomyces olivaceus*. The Journal of Antibiotics 32:961-963.

Brown, S.P., Fredrik Inglis, R., and Taddei, F. 2009. Evolutionary ecology of microbial wars: within-host competition and (incidental) virulence. Evolutionary Applications 2:32-39.

Brurberg, M.B., Nes, I.F., and Eijsink, V.G. 1997. Pheromone-induced production of antimicrobial peptides in *Lactobacillus*. Molecular Microbiology 26:347-360.

Bull, C.T., Carnegie, S.R., and Loper, J.E. 1996. Pathogenicity of mutants of *Erwinia carotovora* subsp. *carotovora* deficient in aerobactin and catecholate siderophore production. Phytopathology 86:260-266.

Burr, T.-J., and Schroth, M. 1977. Occurrence of soft-rot *Erwinia* spp. in soil and plant material. Phytopathology 67:1382-1387.

Butler, L. 1978. Naturally occurring soil populations of *Erwinia* sp. in rhizospheres of greenhouse cucumbers and tomatoes. Phytopathology News 12:136-137.

Bycroft, B.W., and Chhabra, S.R. 1989. A chiral synthesis of trans-carbapenam-3-carboxylic acid and the assignment of (3-S, 5-S) configuration to the corresponding product from *Serratia* and *Erwinia* species. Journal of the Chemical Society, Chemical Communications:423-425.

Bycroft, B.W., Maslen, C., Box, S.J., Brown, A.G., and Tyler, J.W. 1987. The isolation and characterisation of (3 R, 5 R)-and (3 S, 5 R)-carbapenam-3-carboxylic acid from *Serratia* and *Erwinia* species and their putative biosynthetic role. Journal of the Chemical Society, Chemical Communications:1623-1625.

Bycroft, B.W., Maslen, C., Box, S.J., Brown, A., and Tyler, J.W. 1988. The biosynthetic implications of acetate and glutamate incorporation into (3R, 5R)-carbapenam-3-carboxylic acid and (5R)-carbapen-2-em-3-carboxylic acid by *Serratia* sp. The Journal of antibiotics 41:1231-1242.

Byers, J.T., Lucas, C., Salmond, G.P., and Welch, M. 2002. Nonenzymatic turnover of an *Erwinia carotovora* quorum-sensing signaling molecule. Journal of Bacteriology 184:1163-1171.

Caboche, S., Leclère, V., Pupin, M., Kucherov, G., and Jacques, P. 2010. Diversity of monomers in nonribosomal peptides: towards the prediction of origin and biological activity. Journal of Bacteriology 192:5143-5150.

Caboche, S., Pupin, M., Leclère, V., Fontaine, A., Jacques, P., and Kucherov, G. 2008. NORINE: a database of nonribosomal peptides. Nucleic Acids Research 36:D326-D331.

Cascales, E., Buchanan, S.K., Duché, D., Kleanthous, C., Lloubes, R., Postle, K., Riley, M., Slatin, S., and Cavard, D. 2007. Colicin biology. Microbiology and Molecular Biology Reviews 71:158-229.

Cavard, D. 1995. Effects of temperature and of heat shock on the expression and action of the colicin A lysis protein. Journal of Bacteriology 177:5189-5192.

Chan, Y.-C., Wu, J.-L., Wu, H.-P., Tzeng, K.-C., and Chuang, D.-Y. 2011. Cloning, purification, and functional characterization of Carocin S2, a ribonuclease bacteriocin produced by Pectobacterium carotovorum. BioMed Central Microbiology 11:99.

Chao, L., and Levin, B.R. 1981. Structured habitats and the evolution of anticompetitor toxins in bacteria. Proceedings of the National Academy of Sciences 78:6324-6328.

Chatterjee, A., Cui, Y., Hasegawa, H., Leigh, N., Dixit, V., and Chatterjee, A.K. 2005. Comparative analysis of two classes of quorum-sensing signaling systems that control production of extracellular proteins and secondary metabolites in *Erwinia carotovora* subspecies. Journal of Bacteriology 187:8026-8038.

Chatterjee, A.K., Dumenyo, C.K., Liu, Y., and Chatterjee, A. 2000. *Erwinia*: genetics of pathogenicity factors. Encyclopedia of Microbiology 2:236-260.

Chernin, L., Brandis, A., Ismailov, Z., and Chet, I. 1996. Pyrrolnitrin production by an *Enterobacter agglomerans* strain with a broad spectrum of antagonistic activity towards fungal and bacterial phytopathogens. Current Microbiology 32:208-212.

Chirgadze, Y.N., Larionova, E., and Ivanov, V. 2009. Novel Recognition Sign of DNA-binding α-Helix in Complexes of Transcription Factors from Different Families with Operator DNA. Journal of Biomolecular Structure and Dynamics 27:83-95.

Cho, H.R., Joung, H.Y., Lim, K.-B., and Kim, K.S. 2013. Effect of calcium and silicate application on pathogenicity of *Erwinia carotovora* subsp. *carotovora* in *Zantedeschia* spp. Horticulture, Environment, and Biotechnology 54:364-371.

Chuang, D.-y., Chien, Y.-c., and Wu, H.-P. 2007. Cloning and expression of the *Erwinia carotovora* subsp. *carotovora* gene encoding the low-molecular-weight bacteriocin carocin S1. Journal of Bacteriology 189:620-626.

Clifton, I.J., Doan, L.X., Sleeman, M.C., Topf, M., Suzuki, H., Wilmouth, R.C., and Schofield, C.J. 2003. Crystal structure of carbapenem synthase (CarC). Journal of Biological Chemistry 278:20843-20850.

Collmer, A. 1998. Determinants of pathogenicity and avirulence in plant pathogenic bacteria. Current Opinion in Plant Biology 1:329-335.

Collmer, A., and Keen, N.T. 1986. The role of pectic enzymes in plant pathogenesis. Annual Review of Phytopathology 24:383-409.

Corbett, M., Virtue, S., Bell, K., Birch, P., Burr, T., Hyman, L., Lilley, K., Poock, S., Toth, I., and Salmond, G. 2005. Identification of a new quorum-sensing-controlled virulence factor in *Erwinia carotovora* subsp. *atroseptica* secreted via the type II targeting pathway. Molecular Plant-Microbe Interactions 18:334-342.

Cotter, P.D., Hill, C., and Ross, R.P. 2005. Bacteriocins: developing innate immunity for food. Nature Reviews Microbiology 3:777-788.

Coulthurst, S.J., Barnard, A.M., and Salmond, G.P. 2005. Regulation and biosynthesis of carbapenem antibiotics in bacteria. Nature Reviews Microbiology 3:295-306.

Cronin, D., Moënne-Loccoz, Y., Fenton, A., Dunne, C., Dowling, D.N., and O'Gara, F. 1997. Ecological interaction of a biocontrol *Pseudomonas fluorescens* strain producing 2, 4-diacetylphloroglucinol with the soft rot potato pathogen *Erwinia carotovora* subsp.

atroseptica. Federation of European Biochemical Societies Microbiology Ecology 23:95-106.

Cui, Y., Chatterjee, A., Hasegawa, H., and Chatterjee, A.K. 2006. *Erwinia carotovora* subspecies produce duplicate variants of ExpR, LuxR homologs that activate rsmA transcription but differ in their interactions with N-acylhomoserine lactone signals. Journal of Bacteriology 188:4715-4726.

Cui, Y., Chatterjee, A., Hasegawa, H., Dixit, V., Leigh, N., and Chatterjee, A.K. 2005. ExpR, a LuxR homolog of *Erwinia carotovora* subsp. *carotovora*, activates transcription of *rsmA*, which specifies a global regulatory RNA-binding protein. Journal of Bacteriology 187:4792-4803.

Czajkowski, R., De Boer, W., Van der Zouwen, P., Kastelein, P., Jafra, S., De Haan, E., Van den Bovenkamp, G., and Van der Wolf, J. 2013. Virulence of '*Dickeya solani*' and *Dickeya dianthicola* biovar-1 and-7 strains on potato (*Solanum tuberosum*). Plant Pathology 62:597-610.

Czárán, T.L., Hoekstra, R.F., and Pagie, L. 2002. Chemical warfare between microbes promotes biodiversity. Proceedings of the National Academy of Sciences 99:786-790.

Davies, J., Spiegelman, G.B., and Yim, G. 2006. The world of subinhibitory antibiotic concentrations. Current Opinion in Microbiology 9:445-453.

De Boer, S.H., Li, X., and Ward, L.J. 2012. *Pectobacterium* spp. associated with bacterial stem rot syndrome of potato in Canada. Phytopathology 102:937-947.

Delepelaire, P., and Wandersman, C. 1990. Protein secretion in gram-negative bacteria. The extracellular metalloprotease B from *Erwinia chrysanthemi* contains a C-terminal secretion signal analogous to that of *Escherichia coli* alpha-hemolysin. Journal of Biological Chemistry 265:17118-17125.

Demain, A.L. 1989. Carbon source regulation of idiolite biosynthesis in *Actinomycetes*. Boca Raton: CRC Press.

Derzelle, S., Duchaud, E., Kunst, F., Danchin, A., and Bertin, P. 2002. Identification, characterization, and regulation of a cluster of genes involved in carbapenem biosynthesis in *Photorhabdus luminescens*. Applied and Environmental Microbiology 68:3780-3789.

Dowling, D., and Broughton, W. 1986. Competition for nodulation of legumes. Annual Reviews in Microbiology 40:131-157.

Du Raan, S., Coutinho, T.A., and Van der Waals, J.E. 2016. Cardinal temperature differences, determined *in vitro*, between closely related species and subspecies of

pectinolytic bacteria responsible for blackleg and soft rot on potatoes. European Journal of Plant Pathology 144:361-369.

Duarte, V., De Boer, S.H., Ward, L.J., and Oliveira, A.M.R. 2004. Characterization of atypical *Erwinia carotovora* strains causing blackleg of potato in Brazil. Journal of Applied Microbiology 96:535-545.

Duitman, E.H., Hamoen, L.W., Rembold, M., Venema, G., Seitz, H., Saenger, W., Bernhard, F., Reinhardt, R., Schmidt, M., and Ullrich, C. 1999. The mycosubtilin synthetase of *Bacillus subtilis* ATCC6633: a multifunctional hybrid between a peptide synthetase, an amino transferase, and a fatty acid synthase. Proceedings of the National Academy of Sciences 96:13294-13299.

Durrett, R., and Levin, S. 1997. Allelopathy in spatially distributed populations. Journal of Theoretical Biology 185:165-171.

Elander, R. 2003. Industrial production of β-lactam antibiotics. Applied Microbiology and Biotechnology 61:385-392.

Ellison, D.W., and Miller, V.L. 2006. Regulation of virulence by members of the MarR/SlyA family. Current Opinion in Microbiology 9:153-159.

Enard, C., Diolez, A., and Expert, D. 1988. Systemic virulence of *Erwinia chrysanthemi* 3937 requires a functional iron assimilation system. Journal of Bacteriology 170:2419-2426.

Eraso, J.M., and Weinstock, G.M. 1992. Anaerobic control of colicin E1 production. Journal of Bacteriology 174:5101-5109.

Eraso, J.M., Chidambaram, M., and Weinstock, G.M. 1996. Increased production of colicin E1 in stationary phase. Journal of Bacteriology 178:1928-1935.

Expert, D. 1999. Withholding and exchanging iron: interactions between *Erwinia* spp. and their plant hosts. Annual Review of Phytopathology 37:307-334.

Expert, D., and Toussaint, A. 1985. Bacteriocin-resistant mutants of *Erwinia chrysanthemi*: possible involvement of iron acquisition in phytopathogenicity. Journal of Bacteriology 163:221-227.

Feldgarden, M., and Riley, M.A. 1998. High levels of colicin resistance in *Escherichia coli*. Evolution:1270-1276.

Fineran, P.C., Slater, H., Everson, L., Hughes, K., and Salmond, G.P. 2005. Biosynthesis of tripyrrole and β-lactam secondary metabolites in *Serratia*: integration of quorum sensing with multiple new regulatory components in the control of prodigiosin and carbapenem antibiotic production. Molecular Microbiology 56:1495-1517.

Finn, R.D., Clements, J., and Eddy, S.R. 2011. HMMER web server: interactive sequence similarity searching. Nucleic Acids Research 39:W29-W37.

Finn, R.D., Mistry, J., Tate, J., Coggill, P., Heger, A., Pollington, J.E., Gavin, O.L., Gunasekaran, P., Ceric, G., and Forslund, K. 2010. The Pfam protein families database. Nucleic Acids Research 38:D211-D222.

Frank, S.A. 1994. Spatial polymorphism of bacteriocins and other allelopathic traits. Evolutionary Ecology 8:369-386.

Fuqua, C., and Greenberg, E.P. 2002. Listening in on bacteria: acyl-homoserine lactone signalling. Nature Reviews Molecular Cell Biology 3:685-695.

Fuqua, W.C., Winans, S.C., and Greenberg, E.P. 1994. Quorum sensing in bacteria: the LuxR-LuxI family of cell density-responsive transcriptional regulators. Journal of Bacteriology 176:269.

Gardan, L., Gouy, C., Christen, R., and Samson, R. 2003. Elevation of three subspecies of *Pectobacterium carotovorum* to species level: *Pectobacterium atrosepticum* sp. nov., *Pectobacterium betavasculorum* sp. nov. and *Pectobacterium wasabiae* sp. nov. International journal of systematic and evolutionary microbiology 53:381-391.

Gardner, A., West, S.A., and Buckling, A. 2004. Bacteriocins, spite and virulence. Proceedings of the Royal Society of London B: Biological Sciences 271:1529-1535.

Garlant, L., Koskinen, P., Rouhiainen, L., Laine, P., Paulin, L., Auvinen, P., Holm, L., and Pirhonen, M. 2013. Genome sequence of *Dickeya solani*, a new soft rot pathogen of potato, suggests its emergence may be related to a novel combination of non-ribosomal peptide/polyketide synthetase clusters. Diversity 5:824-842.

Gavini, F., Mergaert, J., Beji, A., Mielcarek, C., Izard, D., Kersters, K., and De Ley, J. 1989. Transfer of *Enterobacter agglomerans* (Beijerinck 1888) Ewing and Fife 1972 to *Pantoea* gen. nov. as *Pantoea agglomerans* comb. nov. and Description of *Pantoea dispersa* sp. nov. International Journal of Systematic Bacteriology 39:337-345.

GenStat Committee. 2014. The Guide to the GenStat® Command Language (Release 17). VSN International, Hemel Hempsted, Hertfordshire, Uk.

George, A., and Levy, S. 1983. Gene in the major cotransduction gap of the *Escherichia coli* K-12 linkage map required for the expression of chromosomal resistance to tetracycline and other antibiotics. Journal of Bacteriology 155:541-548.

Gerratana, B., Arnett, S.O., Stapon, A., and Townsend, C.A. 2004. Carboxymethylproline synthase from *Pectobacterium carotorova*: a multifaceted member of the crotonase superfamily. Biochemistry 43:15936-15945.

Giessen, T.W., and Marahiel, M.A. 2012. Ribosome-independent biosynthesis of biologically active peptides: Application of synthetic biology to generate structural diversity. Federation of European Biochemical Societies Letters.

Glasner, J.D., Marquez-Villavicencio, M., Kim, H.S., Jahn, C.E., Ma, B., Biehl, B.S., Rissman, A.I., Mole, B., Yi, X., and Yang, C.H. 2008. Niche-specificity and the variable fraction of the *Pectobacterium* pan-genome. Molecular Plant-Microbe Interactions 21:1549-1560.

Gordon, D.M., Riley, M.A., and Pinou, T. 1998. Temporal changes in the frequency of colicinogeny in *Escherichia coli* from house mice. Microbiology 144:2233-2240.

Goryshin, I.Y., Jendrisak, J., Hoffman, L.M., Meis, R., and Reznikoff, W.S. 2000. Insertional transposon mutagenesis by electroporation of released Tn*5* transposition complexes. Nature Biotechnology 18:97-100.

Goto, M., and Matsumoto, K. 1987. *Erwinia carotovora* subsp. *wasabiae* subsp. nov. isolated from diseased rhizomes and fibrous roots of Japanese horseradish (Eutrema wasabi Maxim.). International Journal of Systematic Bacteriology 37:130-135.

Gottlieb, D. 1976. The production and role of antibiotics in soil. The Journal of Antibiotics 29:987-1000.

Gouaux, E. 1997. The long and short of colicin action: the molecular basis for the biological activity of channel-forming colicins. Structure 5:313-317.

Grinter, N.J. 1983. A broad-host-range cloning vector transposable to various replicons. Gene 21:133-143.

Gross, D., Powelson, M., Regner, K., and Radamaker, G. 1991. A bacteriophage-typing system for surveying the diversity and distribution of strains of *Erwinia carotovora* in potato fields. Phytopathology 81:220-226.

Gross, D.C. 1985. Regulation of syringomycin synthesis in *Pseudomonas syringae* pv. *syringae* and defined conditions for its production. Journal of Applied Microbiology 58:167-174.

Gross, H., Stockwell, V.O., Henkels, M.D., Nowak-Thompson, B., Loper, J.E., and Gerwick, W.H. 2007. The genomisotopic approach: a systematic method to isolate products of orphan biosynthetic gene clusters. Chemistry and Biology 14:53-63.

Guasch, J., Enfedaque, J., Ferrer, S., Gargallo, D., and Regue, M. 1995. Bacteriocin 28b, a chromosomally encoded bacteriocin produced by most *Serratia marcescens* biotypes. Research in Microbiology 146:477-483.

Guenzi, E., Galli, G., Grgurina, I., Gross, D.C., and Grandi, G. 1998. Characterization of the syringomycin synthetase gene cluster. Journal of Biological Chemistry 273:32857-32863.

Gutterson, N.I., Layton, T.J., Ziegle, J.S., and Warren, G.J. 1986. Molecular cloning of genetic determinants for inhibition of fungal growth by a fluorescent *Pseudomonad*. Journal of Bacteriology 165:696-703.

Ham, J.H., Bauer, D.W., Fouts, D.E., and Collmer, A. 1998. A cloned *Erwinia chrysanthemi* Hrp (type III protein secretion) system functions in *Escherichia coli* to deliver *Pseudomonas syringae* Avr signals to plant cells and to secrete Avr proteins in culture. Proceedings of the National Academy of Sciences 95:10206-10211.

Hamed, R.B., Gomez-Castellanos, J.R., Henry, L., Ducho, C., McDonough, M.A., and Schofield, C.J. 2013. The enzymes of β-lactam biosynthesis. Natural Product Reports 30:21-107.

Handelsman, J., and Stabb, E.V. 1996. Biocontrol of soilborne plant pathogens. The Plant Cell 8:1855.

Hassett, D.J., Sutton, M.D., Schurr, M.J., Herr, A.B., Caldwell, C.C., and Matu, J.O. 2009. *Pseudomonas aeruginosa* hypoxic or anaerobic biofilm infections within cystic fibrosis airways. Trends in Microbiology 17:130-138.

Hauben, L., Moore, E.R.B., Vauterin, L., Steenackers, M., Mergaert, J., Verdonck, L., and Swings, J. 1998. Phylogenetic Position of Phytopathogens within the *Enterobacteriaceae*. Systematic and Applied Microbiology 21:384-397.

Hayashi, M.A., Bizerra, F.C., and Junior, P.I.D.S. 2014. Antimicrobial compounds from natural sources. Frontiers E-books.

Hayes, C.S., Aoki, S.K., and Low, D.A. 2010. Bacterial contact-dependent delivery systems. Annual Review of Genetics 44:71-90.

Hayward, A.C. 1974. Latent infections by bacteria. Annual Review of Phytopathology 12:87-97.

Healy, F.G., Wach, M., Krasnoff, S.B., Gibson, D.M., and Loria, R. 2000. The *txtAB* genes of the plant pathogen *Streptomyces acidiscabies* encode a peptide synthetase required for phytotoxin thaxtomin A production and pathogenicity. Molecular Microbiology 38:794-804.

Heep, M., Rieger, U., Beck, D., and Lehn, N. 2000. Mutations in the Beginning of the *rpoB* Gene Can Induce Resistance to Rifamycins in both *Helicobacter pylori* and *Mycobacterium tuberculosis*. Antimicrobial Agents and Chemotherapy 44:1075-1077.

Henderson, I.R., Navarro-Garcia, F., Desvaux, M., Fernandez, R.C., and Ala'Aldeen, D. 2004. Type V protein secretion pathway: the autotransporter story. Microbiology and Molecular Biology Reviews 68:692-744.

Heroven, A.K., Nagel, G., Tran, H.J., Parr, S., and Dersch, P. 2004. RovA is autoregulated and antagonizes H-NS-mediated silencing of invasin and *rovA* expression in *Yersinia pseudotuberculosis*. Molecular Microbiology 53:871-888.

Herschman, H.R., and Helinski, D.R. 1967. Comparative study of the events associated with colicin induction. Journal of Bacteriology 94:691-699.

Hibbing, M.E., Fuqua, C., Parsek, M.R., and Peterson, S.B. 2010. Bacterial competition: surviving and thriving in the microbial jungle. Nature Reviews Microbiology 8:15-25.

Hirvas, L., Nurminen, M., Helander, I.M., Vuorio, R., and Vaara, M. 1997. The lipid A biosynthesis deficiency of the *Escherichia coli* antibiotic-supersensitive mutant LH530 is suppressed by a novel locus, ORF195. Microbiology 143:73-81.

Hoffman, L.M., Jendrisak, J.J., Meis, R.J., Goryshin, I.Y., and Reznikoff, W.S. 2000. Transposome insertional mutagenesis and direct sequencing of microbial genomes. Genetica 108:19-24.

Hoffman, L.R., D'Argenio, D.A., MacCoss, M.J., Zhang, Z., Jones, R.A., and Miller, S.I. 2005. Aminoglycoside antibiotics induce bacterial biofilm formation. Nature 436:1171-1175.

Holden, M.T., McGowan, S.J., Bycroft, B.W., Stewart, G.S., Williams, P., and Salmond, G.P. 1998. Cryptic carbapenem antibiotic production genes are widespread in *Erwinia carotovora*: facile trans activation by the CarR transcriptional regulator. Microbiology 144:1495-1508.

Holeva, M.C., Bell, K.S., Hyman, L.J., Avrova, A.O., Whisson, S.C., Birch, P.R.J., and Toth, I.K. 2004. Use of a pooled transposon mutation grid to demonstrate roles in disease development for *Erwinia carotovora* subsp. *atroseptica* putative type III secreted effector (DspE/A) and helper (HrpN) proteins. Molecular Plant-Microbe Interactions 17:943-950.

Holtsmark, I., Eijsink, V.G., and Brurberg, M.B. 2008. Bacteriocins from plant pathogenic bacteria. Federation of European Biochemical Societies Microbiology Letters 280:1-7.

Hu, F., and Young, J. 1998. Biocidal activity in plant pathogenic *Acidovorax*, *Burkholderia*, *Herbaspirillum*, *Ralstonia* and *Xanthomonas* spp. Journal of Applied Microbiology 84:263-271.

Hugouvieux-Cotte-Pattat, N., Shevchik, V.E., and Nasser, W. 2002. PehN, a polygalacturonase homologue with a low hydrolase activity, is coregulated with the other *Erwinia chrysanthemi* polygalacturonases. Journal of Bacteriology 184:2664-2673.

Iijima, T. 1962. Studies on the colicinogenic factor in *Escherichia coli* K12. Induction of colicin production by mitomycin C. Biken Journal 5.

Itoh, Y., Izakil, K., and Takahashi, H. 1978. Purification and characterization of a bacteriocin from *Erwinia carotovora*. The Journal of General and Applied Microbiology 24:27-39.

Itoh, Y., Izaki, K., and Takahashi, H. 1980. Simultaneous synthesis of pectin lyase and carotovoricin induced by mitomycin C, nalidixic acid or ultraviolet light irradiation in *Erwinia carotovora*. Agricultural and Biological Chemistry 44:1135-1140.

Ivanovska, I., and Hardwick, J.M. 2005. Viruses activate a genetically conserved cell death pathway in a unicellular organism. The Journal of Cell Biology 170:391-399.

Jabeen, N., Hussain, M., Khan, J.A., Naz, S.A., Subhan, S.A., and Rasool, S.A. 2014. Bio-physicochemical characterization and applied studies of carotovoricin na5 (crna5) on blb affected rice plants. Pakistan Journal of Botany 46:2249-2256.

James, R., Lazdunski, C., and Pattus, F. 2013. Bacteriocins, microcins and lantibiotics. Springer Science & Business Media.

Jones, P., Binns, D., Chang, H.-Y., Fraser, M., Li, W., McAnulla, C., McWilliam, H., Maslen, J., Mitchell, A., and Nuka, G. 2014. InterProScan 5: genome-scale protein function classification. Bioinformatics 30:1236-1240.

Jones, S., Yu, B., Bainton, N.a., Birdsall, M., Bycroft, B., Chhabra, S., Cox, A., Golby, P., Reeves, P., and Stephens, S. 1993a. The Lux autoinducer regulates the production of exoenzyme virulence determinants in *Erwinia carotovora* and *Pseudomonas aeruginosa*. The European Molecular Biology Organisation Journal 12:2477.

Jones, S., Yu, B., Bainton, N.J., Birdsall, M., Bycroft, B.W., Chhabra, S.R., Cox, A.J., Golby, P., Reeves, P.J., and Stephens, S. 1993b. The Lux autoinducer regulates the production of exoenzyme virulence determinants in *Erwinia carotovora* and *Pseudomonas aeruginosa*. The European Molecular Biology Organisation Journal 12:2477.

Kabach, W., and Sander, C. 1983. A dictionary of protein secondary structure. Biopolymers 22:2577-2637.

Kado, C.I. 2006. *Erwinia* and Related Genera. The Prokaryotes:443-450.

Kahan, J., Kahan, F., Goegelman, R., Currie, S., Jackson, M., Stapley, E., Miller, T., Miller, A., Hendlin, D., and Mochales, S. 1979. Thienamycin, a new. Β-lactam antibiotic. I. Discovery, taxonomy, isolation and physical properties. The Journal of Antibiotics 32:1-12.

Källberg, M., Wang, H., Wang, S., Peng, J., Wang, Z., Lu, H., and Xu, J. 2012. Template-based protein structure modeling using the RaptorX web server. Nature Protocols 7:1511-1522.

Kaufmann, G.F., Sartorio, R., Lee, S.-H., Rogers, C.J., Meijler, M.M., Moss, J.A., Clapham, B., Brogan, A.P., Dickerson, T.J., and Janda, K.D. 2005. Revisiting quorum sensing: discovery of additional chemical and biological functions for 3-oxo-N-acylhomoserine

lactones. Proceedings of the National Academy of Sciences of the United States of America 102:309-314.

Keel, C., Wirthner, P., Oberhänsli, T., Voisard, C., Haas, D., and Défago, G. 1990. Pseudomonads as antagonists of plant pathogens in the rhizosphere: role of the antibiotic 2, 4-diacetylphloroglucinol in the suppression of black root rot of tobacco. Symbiosis (Rehovot) 9:327-341.

Kennedy, C. 1971. Induction of colicin production by high temperature or inhibition of protein synthesis. Journal of Bacteriology 108:10-19.

Kersten, R.D., Yang, Y.L., Xu, Y., Cimermancic, P., Nam, S.J., Fenical, W., Fischbach, M.A., Moore, B.S., and Dorrestein, P.C. 2011. A mass spectrometry-guided genome mining approach for natural product peptidogenomics. Nat Chem Biol 7:794-802.

Kieu, N.A.M.P., Aznar, A., Segond, D., Rigault, M., Simond-CÔTe, E., Kunz, C., Soulie, M.C., Expert, D., and Dellagi, A. 2012. Iron deficiency affects plant defence responses and confers resistance to *Dickeya dadantii* and *Botrytis cinerea*. Molecular Plant Pathology.

Kikumoto, T., and Sakamoto, M. 1970. Ecological studies on the soft-rot bacteria of vegetables. X. The distribution of soft-rot bacteria within the soil aggregates. Annuals of the Phytopathological Society of Japan 36:207-213.

Kim, H.S., Ma, B., Perna, N.T., and Charkowski, A.O. 2009. Phylogeny and virulence of naturally occurring type III secretion system-deficient *Pectobacterium* strains. Applied Environmental Microbiology 75:4539-4549.

Klaenhammer, T.R. 1988. Bacteriocins of lactic acid bacteria. Biochimie 70:337-349.

Kobayashi, K., and Tagawa, S. 2004. Activation of SoxR-dependent transcription in *Pseudomonas aeruginosa*. Journal of Biochemistry 136:607-615.

Kõiv, V., Roosaare, M., Vedler, E., Kivistik, P.A., Toppi, K., Schryer, D.W., Remm, M., Tenson, T., and Mäe, A. 2015. Microbial population dynamics in response to *Pectobacterium atrosepticum* infection in potato tubers. Scientific Reports 5.

Lambert, D., Powelson, M., and Stevenson, W. 2005. Nutritional interactions influencing diseases of potato. American Journal of Potato Research 82:309-319.

Latifi, A., Winson, M.K., Foglino, M., Bycroft, B.W., Stewart, G.S., Lazdunski, A., and Williams, P. 1995. Multiple homologues of LuxR and LuxI control expression of virulence determinants and secondary metabolites through quorum sensing in *Pseudomonas aeruginosa* PAO1. Molecular Microbiology 17:333-343.

Laurila, J., Ahola, V., Lehtinen, A., Joutsjoki, T., Hannukkala, A., Rahkonen, A., and Pirhonen, M. 2008. Characterization of *Dickeya* strains isolated from potato and river water samples in Finland. European Journal of Plant Pathology 122:213-225.

Lawrence, J. 1999. Selfish operons: the evolutionary impact of gene clustering in prokaryotes and eukaryotes. Current Opinion in Genetics & Development 9:642-648.

Lazdunski, A.M., Ventre, I., and Sturgis, J.N. 2004. Regulatory circuits and communication in Gram-negative bacteria. Nature Reviews Microbiology 2:581-592.

Lebecka, R., and Zimnoch-Guzowska, E. 2004. The inheritance of resistance to soft rot (*Erwinia carotovora* subsp. *atroseptica*) in diploid potato families. American Journal of Potato Research 81:395-401.

Leite, L.N., de Haan, E., Krijger, M., Kastelein, P., van der Zouwen, P., van den Bovenkamp, G., Tebaldi, N., and van der Wolf, J. 2014. First report of potato blackleg caused by *Pectobacterium carotovorum* subsp. *brasiliensis* in the Netherlands. New Disease Reports 29:24.

Lenski, R.E., Rose, M.R., Simpson, S.C., and Tadler, S.C. 1991. Long-term experimental evolution in *Escherichia coli*. I. Adaptation and divergence during 2,000 generations. American Naturalist:1315-1341.

Li, R., Stapon, A., Blanchfield, J.T., and Townsend, C.A. 2000. Three unusual reactions mediate carbapenem and carbapenam biosynthesis. Journal of the American Chemical Society 122:9296-9297.

Libby, S.J., Goebel, W., Ludwig, A., Buchmeier, N., Bowe, F., Fang, F.C., Guiney, D.G., Songer, J.G., and Heffron, F. 1994. A cytolysin encoded by *Salmonella* is required for survival within macrophages. Proceedings of the National Academy of Sciences 91:489-493.

Limansky, A.S., Mussi, M.A., and Viale, A.M. 2002. Loss of a 29-kilodalton outer membrane protein in *Acinetobacter baumannii* is associated with imipenem resistance. Journal of Clinical Microbiology 40:4776-4778.

Lindow, S. 1988. Lack of correlation of *in vitro* antibiosis with antagonism of ice nucleation active bacteria on leaf surface by non-ice nucleation active bacteria. Phytopathology 78:444-450.

Lindow, S. 1995. Control of epiphytic ice nucleation-active bacteria for management of plant frost injury. Biological Ice Nucleation and its Applications:239-256.

Lindow, S.E., and Brandl, M.T. 2003. Microbiology of the phyllosphere. Applied and Environmental Microbiology 69:1875-1883.

Lindow, S.E., Arny, D.C., and Upper, C.D. 1982. Bacterial ice nucleation: a factor in frost injury to plants. Plant physiology 70:1084-1089.

Lindow, S.E., McGourty, G., and Elkins, R. 1996. Interactions of antibiotics with *Pseudomonas fluorescens* strain A506 in the control of fire blight and frost injury to pear. Phytopathology 86:841-848.

Liu, H., Coulthurst, S.J., Pritchard, L., Hedley, P.E., Ravensdale, M., Humphris, S., Burr, T., Takle, G., Brurberg, M.B., Birch, P.R., Salmond, G.P., and Toth, I.K. 2008. Quorum sensing coordinates brute force and stealth modes of infection in the plant pathogen *Pectobacterium atrosepticum*. Public Library of Science Pathogens 4:e1000093.

Liu, Y., Chatterjee, A., and Chatterjee, A.K. 1994. Nucleotide sequence, organization and expression of *rdgA* and *rdgB* genes that regulate pectin lyase production in the plant pathogenic bacterium *Erwinia carotovora* subsp. *cartovora* in response to DNA damaging agents. Molecular Microbiology 14:999-1010.

Loper, J.E., Lindow, S.E., Hurst, C., Knudsen, G., McInerney, M., Stetzenbach, L., and Walter, M. 1997. Reporter gene systems useful in evaluating *in situ* gene expression by soil-and plant-associated bacteria. Manual of Environmental Microbiology.:482-492.

Ludwig, A., Tengel, C., Bauer, S., Bubert, A., Benz, R., Mollenkopf, H.-J., and Goebel, W. 1995. SlyA, a regulatory protein from *Salmonella typhimurium*, induces a haemolytic and pore-forming protein in *Escherichia coli*. Molecular and General Genetics MGG 249:474-486.

Lwoff, A., Jacob, F., Ritz, E., and Gage, M. 1952. Induction of bacteriophage production and of a colicine by peroxides, ethyleneimines and halogenated alkylamines. Comptes Rendus Hebdomadaires des Séances de l'Académie des Sciences 234:2308.

Lysak, V. 1979. Some properties of bacteriocins from *Erwinia*. Prikladnaia Biokhimiia I Mikrobiologiia 16:372-376.

Mäe, A., Montesano, M., Koiv, V., and Palva, E.T. 2001. Transgenic plants producing the bacterial pheromone *N*-acyl-homoserine lactone exhibit enhanced resistance to the bacterial phytopathogen *Erwinia carotovora*. Molecular Plant-Microbe Interactions 14:1035-1042.

Marger, M.D., and Saier, M.H. 1993. A major superfamily of transmembrane facilitators that catalyse uniport, symport and antiport. Trends in Biochemical Sciences 18:13-20.

Marquez-Villavicencio, M.d.P., Groves, R.L., and Charkowski, A.O. 2011. Soft Rot Disease Severity Is Affected by Potato Physiology and *Pectobacterium* taxa. Plant Disease 95:232-241.

Mattinen, L., Somervuo, P., Nykyri, J., Nissinen, R., Kouvonen, P., Corthals, G., Auvinen, P., Aittamaa, M., Valkonen, J.P., and Pirhonen, M. 2008. Microarray profiling of host-extract-induced genes and characterization of the type VI secretion cluster in the potato pathogen *Pectobacterium atrosepticum*. Microbiology 154:2387-2396.

Mavrodi, D.V., Peever, T.L., Mavrodi, O.V., Parejko, J.A., Raaijmakers, J.M., Lemanceau, P., Mazurier, S., Heide, L., Blankenfeldt, W., and Weller, D.M. 2010. Diversity and

evolution of the phenazine biosynthesis pathway. Applied and Environmental Microbiology 76:866-879.

Mazar, J., and Cotter, P.A. 2007. New insight into the molecular mechanisms of two-partner secretion. Trends in Microbiology 15:508-515.

McCarter-Zorner, N.J., Franc, G., Harrison, M., Michaud, J.E., Quinn, C., Sells, I., and Graham, D. 1984. Soft rot Erwinia bacteria in surface and underground waters in southern Scotland and in Colorado, United States. Journal of Applied Bacteriology 57:95-105.

McClean, K.H., Winson, M.K., Fish, L., Taylor, A., Chhabra, S.R., Camara, M., Daykin, M., Lamb, J.H., Swift, S., and Bycroft, B.W. 1997. Quorum sensing and *Chromobacterium violaceum*: exploitation of violacein production and inhibition for the detection of *N*-acylhomoserine lactones. Microbiology 143:3703-3711.

McCullagh, P., and Nelder, J.A. 1989. Generalized Linear Models. Chapman & Hall, London.

McGowan, S., and Salmond, G. (1999). Regulation of carbapenem antibiotic and exoenzyme synthesis by chemical signalling. In Symposia-Society For General Microbiology (Cambridge University Press), pp. 161-176.

McGowan, S., Sebaihia, M., Porter, L., Stewart, G., Williams, P., Bycroft, B., and Salmond, G. 1996. Analysis of bacterial carbapenem antibiotic production genes reveals a novel β-lactam biosynthesis pathway. Molecular Microbiology 22:415-426.

McGowan, S., Sebaihia, M., O'leary, S., Hardie, K., Williams, P., Stewart, G., Bycroft, B., and Salmond, G. 1997. Analysis of the carbapenem gene cluster of *Erwinia carotovora*: definition of the antibiotic biosynthetic genes and evidence for a novel β-lactam resistance mechanism. Molecular Microbiology 26:545-556.

McGowan, S., Sebaihia, M., Jones, S., Yu, B., Bainton, N., Chan, P., Bycroft, B., Stewart, G., Williams, P., and Salmond, G. 1995. Carbapenem antibiotic production in *Erwinia carotovora* is regulated by CarR, a homologue of the LuxR transcriptional activator. Microbiology 141:541-550.

McGowan, S.J., Barnard, A.M., Bosgelmez, G., Sebaihia, M., Simpson, N.J., Thomson, N.R., Todd, D.E., Welch, M., Whitehead, N.A., and Salmond, G.P. 2005. Carbapenem antibiotic biosynthesis in *Erwinia carotovora* is regulated by physiological and genetic factors modulating the quorum sensing dependent control pathway. Molecular Microbiology 55:526-545.

McGuire, R.G., and Kelman, A. 1986. Calcium in potato tuber cell walls in relation to tissue maceration by *Erwinia carotovora* pv. *atroseptica*. Phytopathology 76:401-406.

Medema, M.H., Blin, K., Cimermancic, P., de Jager, V., Zakrzewski, P., Fischbach, M.A., Weber, T., Takano, E., and Breitling, R. 2011. antiSMASH: rapid identification,

annotation and analysis of secondary metabolite biosynthesis gene clusters in bacterial and fungal genome sequences. Nucleic Acids Research 39:W339-W346.

Mena, A., Plasencia, V., García, L., Hidalgo, O., Ayestarán, J.I., Alberti, S., Borrell, N., Pérez, J.L., and Oliver, A. 2006. Characterization of a large outbreak by CTX-M-1-producing *Klebsiella pneumoniae* and mechanisms leading to i*n vivo* carbapenem resistance development. Journal of Clinical Microbiology 44:2831-2837.

Mew, T., Ho, W., and Chu, L. 1976. Infectivity and survival of soft-rot bacteria in Chinese cabbage. Phytopathology 66:1325-1327.

Miller, M.B., and Bassler, B.L. 2001. Quorum sensing in bacteria. Annual Reviews in Microbiology 55:165-199.

Miller, M.T., Gerratana, B., Stapon, A., Townsend, C.A., and Rosenzweig, A.C. 2003. Crystal structure of carbapenam synthetase (CarA). Journal of Biological Chemistry 278:40996-41002.

Miller, S.I., Kukral, A.M., and Mekalanos, J.J. 1989. A two-component regulatory system (*phoP phoQ*) controls *Salmonella typhimurium* virulence. Proceedings of the National Academy of Sciences 86:5054-5058.

Minowa, Y., Araki, M., and Kanehisa, M. 2007. Comprehensive analysis of distinctive polyketide and nonribosomal peptide structural motifs encoded in microbial genomes. Journal of Molecular Biology 368:1500-1517.

Moleleki, L., Pretorius, R., Tanui, C., Mosina, G., and Theron, J. 2016. A quorum sensing-defective mutant of *Pectobacterium carotovorum* subsp. *brasiliense* 1692 is attenuated in virulence and unable to occlude xylem tissue of susceptible potato plant stems. Molecular Plant Pathology.

Moleleki, L.N., Onkendi, E.M., Mongae, A., and Kubheka, G.C. 2013. Characterisation of *Pectobacterium wasabiae* causing blackleg and soft rot diseases in South Africa. European Journal of Plant Pathology 135:279-288.

Mootz, H.D., Schwarzer, D., and Marahiel, M.A. 2002. Ways of assembling complex natural products on modular nonribosomal peptide synthetases. Chembiochem 3:490-504.

Murata, H., Fons, M., Chatterjee, A., Collmer, A., and Chatterjee, A. 1990. Characterization of transposon insertion out-mutants of *Erwinia carotovora* subsp. *carotovora* defective in enzyme export and of a DNA segment that complements out mutations in *E. carotovora* subsp. *carotovora*, *E. carotovora* subsp. *atroseptica*, and *Erwinia chrysanthemi*. Journal of Bacteriology 172:2970-2978.

Nachin, L., El Hassouni, M., Loiseau, L., Expert, D., and Barras, F. 2001. SoxR-dependent response to oxidative stress and virulence of *Erwinia chrysanthemi*: the key role of SufC, an orphan ABC ATPase. Molecular Microbiology 39:960-972.

Nagel, G., Lahrz, A., and Dersch, P. 2001. Environmental control of invasin expression in *Yersinia pseudotuberculosis* is mediated by regulation of RovA, a transcriptional activator of the SlyA/Hor family. Molecular Microbiology 41:1249-1269.

Nakayama, M., Iwasaki, A., Kimura, S., Mizoguchi, T., Tanabe, S., Murakami, A., Watanabe, I., Okuchi, M., Itoh, H., and Saino, Y. 1980. Carpetimycins A and B, new beta-lactam antibiotics. The Journal of Antibiotics 33:1388-1390.

Nasser, W., Shevchik, V.E., and Hugouvieux-Cotte-Pattat, N. 1999. Analysis of three clustered polygalacturonase genes in *Erwinia chrysanthemi* 3937 revealed an anti-repressor function for the PecS regulator. Molecular Microbiology 34:641-650.

Nguyen, A.H., Tomita, T., Hirota, M., Sato, T., and Kamio, Y. 1999. A simple purification method and morphology and component analyses for carotovoricin Er, a phage-tail-like bacteriocin from the plant pathogen *Erwinia carotovora* Er. Bioscience, Biotechnology, and Biochemistry 63:1360-1369.

Nguyen, H.A., Kaneko, J., and Kamio, Y. 2002. Temperature-dependent production of carotovoricin Er and pectin lyase in phytopathogenic *Erwinia carotovora* subsp. *carotovora* Er. Bioscience, Biotechnology, and Biochemistry 66:444-447.

Nguyen, H.A., Tomita, T., Hirota, M., Kaneko, J., Hayashi, T., and Kamio, Y. 2001. DNA inversion in the tail fiber gene alters the host range specificity of carotovoricin Er, a phage-tail-like bacteriocin of phytopathogenic *Erwinia carotovora* subsp. *carotovora* Er. Journal of Bacteriology 183:6274-6281.

Nicholson, A.J. 1954. An outline of the dynamics of animal populations. Australian Journal of Zoology 2:9-65.

Nix, D.E., Majumdar, A.K., and DiNubile, M.J. 2004. Pharmacokinetics and pharmacodynamics of ertapenem: an overview for clinicians. Journal of Antimicrobial Chemotherapy 53:ii23-ii28.

Nomura, M. (1963). Mode of action of colicines. In Cold Spring Harbor Symposia on Quantitative Biology (Cold Spring Harbor Laboratory Press), pp. 315-324.

Núñez, L.E., Méndez, C., Braña, A.F., Blanco, G., and Salas, J.A. 2003. The biosynthetic gene cluster for the β-lactam carbapenem thienamycin in *Streptomyces cattleya*. Chemistry and Biology 10:301-311.

Nykyri, J., Niemi, O., Koskinen, P., Nokso-Koivisto, J., Pasanen, M., Broberg, M., Plyusnin, I., Törönen, P., Holm, L., and Pirhonen, M. 2012. Revised Phylogeny and Novel Horizontally Acquired Virulence Determinants of the Model Soft Rot Phytopathogen *Pectobacterium wasabiae* SCC3193. PLoS Pathogens 8:e1003013.

Oerke, E.C. 2006. Crop losses to pests. Journal of Agricultural Science- 144:31.

Owston, M.A., and Serpersu, E.H. 2002. Cloning, overexpression, and purification of aminoglycoside antibiotic 3-acetyltransferase-IIIb: conformational studies with bound substrates. Biochemistry 41:10764-10770.

Panda, P., Fiers, M., Armstrong, K., and Pitman, A.R. 2012. First report of blackleg and soft rot of potato caused by *Pectobacterium carotovorum* subsp. *brasiliensis* in New Zealand. New Disease Reports.

Panda, P., Lu, A., Armstrong, K.F., and Pitman, A.R. 2015a. Draft genome sequence for ICMP 5702, the type strain of *Pectobacterium carotovorum* subsp. *carotovorum* that causes soft rot disease on potato. Genome Announcements 3:e00875-00815.

Panda, P., Fiers, M.W., Lu, A., Armstrong, K.F., and Pitman, A.R. 2015b. Draft Genome Sequences of Three *Pectobacterium* Strains Causing Blackleg of Potato: *P. carotovorum* subsp. *brasiliensis* ICMP 19477, *P. atrosepticum* ICMP 1526, and *P. carotovorum* subsp. *carotovorum* UGC32. Genome Announcements 3:e00874-00815.

Pappas, K.M., Weingart, C.L., and Winans, S.C. 2004. Chemical communication in proteobacteria: biochemical and structural studies of signal synthases and receptors required for intercellular signalling. Molecular Microbiology 53:755-769.

Parker, W.L., Rathmum, M.L., Wells, J.J.S., Trejo, W.H., Principe, P.A., and Sykes, R.B. 1982. SQ 27,860, a simple carbapenem produced by species of *Serratia* and *Erwinia*. The Journal of Antibiotics 35:653-660.

Parret, A.H., Schoofs, G., Proost, P., and De Mot, R. 2003. Plant lectin-like bacteriocin from a rhizosphere-colonizing *Pseudomonas* isolate. Journal of Bacteriology 185:897-908.

Pemberton, C., Whitehead, N., Sebaihia, M., Bell, K., Hyman, L., Harris, S., Matlin, A., Robson, N., Birch, P., and Carr, J. 2005. Novel quorum-sensing-controlled genes in *Erwinia carotovora* subsp. *carotovora*: identification of a fungal elicitor homologue in a soft-rotting bacterium. Molecular Plant-Microbe Interactions 18:343-353.

Perombelon, M.C.M. (1992). The genus *Erwinia* (New York: Springer-Verlag), pp. 2899-2921.

Perombelon, M.C.M. 2002. Potato diseases caused by soft rot erwinias: an overview of pathogenesis. Plant Pathology 51:1-12.

Pérombelon, M.C.M. 1972. The extent and survival of contamination of potato stocks in Scotland by *Erwinia carotovora* var. *carotovora* and *E. carotovora* var. *atroseptica*. Annals of Applied Biology 71:111-117.

Pérombelon, M.C.M. 1992. Potato blackleg: epidemiology, host-pathogen interaction and control. European Journal of Plant Pathology 98:135-146.

Pérombelon, M.C.M., and Kelman, A. 1980. Ecology of the Soft Rot Erwinias. Annual Review of Phytopathology 18:361-387.

Pierson III, L.S., and Pierson, E.A. 2010. Metabolism and function of phenazines in bacteria: impacts on the behavior of bacteria in the environment and biotechnological processes. Applied Microbiology and Biotechnology 86:1659-1670.

Pierson III, L.S., Wood, D.W., and Pierson, E.A. 1998. Homoserine lactone-mediated gene regulation in plant-associated bacteria. Annual Review of Phytopathology 36:207-225.

Pierson III, L.S., Gaffney, T., Lam, S., and Gong, F. 1995. Molecular analysis of genes encoding phenazine biosynthesis in the biological control bacterium *Pseudomonas aureofaciens* 30-84. FEMS Microbiology Letters 134:299-307.

Pirhonen, M., Saarilahti, H., Karlsson, M.B., and Palva, E.T. 1991. Identification of pathogenicity determinants of *Erwinia carotovora* subsp. *carotovora* by transposon mutagenesis. Molecular Plant-Microbe Interactions 4:276-283.

Pirhonen, M., Heino, P., Helander, I., Harju, P., and Palva, E.T. 1988. Bacteriophage T4 resistant mutants of the plant pathogen *Erwinia carotovora*. Microbial Pathogenesis 4:359-367.

Pitman, A.R., Harrow, S.A., and Visnovsky, S.B. 2009. Genetic characterisation of *Pectobacterium wasabiae* causing soft rot disease of potato in New Zealand. European Journal of Plant Pathology 126:423-435.

Pitman, A.R., Wright, P.J., Galbraith, M.D., and Harrow, S.A. 2008. Biochemical and genetic diversity of pectolytic enterobacteria causing soft rot disease of potatoes in New Zealand. Australasian Plant Pathology 37:559-568.

Poole, S.J., Diner, E.J., Aoki, S.K., Braaten, B.A., T'kint De Roodenbeke, C., Low, D.A., and Hayes, C.S. 2011. Identification of functional toxin/immunity genes linked to contact-dependent growth inhibition (CDI) and rearrangement hotspot (Rhs) systems. Public Library of Science Genetics 7:e1002217.

Price-Whelan, A., Dietrich, L.E., and Newman, D.K. 2006. Rethinking 'secondary' metabolism: physiological roles for phenazine antibiotics. Nature Chemical Biology 2:71-78.

Queenan, A.M., Shang, W., Flamm, R., and Bush, K. 2010. Hydrolysis and inhibition profiles of β-lactamases from molecular classes A to D with doripenem, imipenem, and meropenem. Antimicrobial Agents and Chemotherapy 54:565-569.

Quinn, C., Sells, I., and Graham, D. 1980. Soft rot Erwinia bacteria in the atmospheric bacterial aerosol. Journal of Applied Bacteriology 49:175-181.

Raaijmakers, J.M., Vlami, M., and De Souza, J.T. 2002. Antibiotic production by bacterial biocontrol agents. Antonie van Leeuwenhoek 81:537-547.

Radman, M. 1975. SOS repair hypothesis: phenomenology of an inducible DNA repair which is accompanied by mutagenesis. Pages 355-367 in: Molecular Mechanisms for Repair of DNA>.

Rantakari, A., Virtaharju, O., Vähämiko, S., Taira, S., Palva, E.T., Saarilahti, H.T., and Romantschuk, M. 2001. Type III secretion contributes to the pathogenesis of the soft-rot pathogen *Erwinia carotovora*: partial characterization of the *hrp* gene cluster. Molecular Plant-Microbe Interactions 14:962-968.

Ratcliffe, R.W., and Albers-Schonberg, G. 1982. The chemistry of thienamycin and other carbapenem antibiotics. Chemistry and Biology of β-lactam Antibiotics 2:227-313.

Rausch, C., Weber, T., Kohlbacher, O., Wohlleben, W., and Huson, D.H. 2005. Specificity prediction of adenylation domains in nonribosomal peptide synthetases (NRPS) using transductive support vector machines (TSVMs). Nucleic Acids Research 33:5799-5808.

Raymond, K.N., Dertz, E.A., and Kim, S.S. 2003. Enterobactin: an archetype for microbial iron transport. Proceedings of the National Academy of Sciences 100:3584-3588.

Reeves, P. 1972. The Bacteriocins in Nature. Pages 81-86 in: The Bacteriocins> N. S. Iacobellis, Collmer, A., Hutcheson,

Reverchon, S., Nasser, W., and Robert-Baudouy, J. 1994. *pecS*: a locus controlling pectinase, cellulase and blue pigment production in *Erwinia chrysanthemi*. Molecular Microbiology 11:1127-1139.

Reverchon, S., Rouanet, C., Expert, D., and Nasser, W. 2002. Characterization of Indigoidine Biosynthetic Genes in *Erwinia chrysanthemi* and Role of this Blue Pigment in Pathogenicity. Journal of Bacteriology 184:654-665.

Riley, M.A. 1998. Molecular mechanisms of bacteriocin evolution. Annual Review of Genetics 32:255-278.

Riley, M.A., and Gordon, D.M. 1999. The ecological role of bacteriocins in bacterial competition. Trends in Microbiology 7:129-133.

Riley, M.A., and Wertz, J.E. 2002. Bacteriocins: evolution, ecology, and application. Annual Reviews in Microbiology 56:117-137.

Rodríguez-Martínez, J.-M., Poirel, L., and Nordmann, P. 2009. Molecular epidemiology and mechanisms of carbapenem resistance in *Pseudomonas aeruginosa*. Antimicrobial Agents and Chemotherapy 53:4783-4788.

Roh, E., Park, T.-H., Kim, M.-i., Lee, S., Ryu, S., Oh, C.-S., Rhee, S., Kim, D.-H., Park, B.-S., and Heu, S. 2010. Characterization of a new bacteriocin, Carocin D, from

Pectobacterium carotovorum subsp. *carotovorum* Pcc21. Applied and Environmental Microbiology 76:7541-7549.

Rojas, C.M., Ham, J.H., Schechter, L.M., Kim, J.F., Beer, S.V., and Collmer, A. 2004. The *Erwinia chrysanthemi* EC16 *hrp/hrc* gene cluster encodes an active Hrp type III secretion system that is flanked by virulence genes functionally unrelated to the Hrp system. Molecular Plant-Microbe Interactions 17:644-653.

Rosales, A., Thomashow, L., Cook, R., and Mew, T. 1995. Isolation and identification of antifungal metabolites produced by rice-associated antagonistic *Pseudomonas* spp. Phytopathology 85:1028-1032.

Rosqvist, R., Magnusson, K.-E., and Wolf-Watz, H. 1994. Target cell contact triggers expression and polarized transfer of *Yersinia* YopE cytotoxin into mammalian cells. The European Molecular Biology Organisation journal 13:964.

Rost, B., Yachdav, G., and Liu, J. 2004. The PredictProtein server. Nucleic Acids Research 32:W321-W326.

Rottig, M., Medema, M.H., Blin, K., Weber, T., Rausch, C., and Kohlbacher, O. 2011. NRPSpredictor2-a web server for predicting NRPS adenylation domain specificity. Nucleic Acids Research 39:W362-367.

Rouanet, C., and Nasser, W. 2001. The PecM protein of the phytopathogenic bacterium *Erwinia chrysanthemi*, membrane topology and possible involvement in the efflux of the blue pigment indigoidine. Journal of Molecular Microbiology and Biotechnology 3:309-318.

Rouanet, C., Nomura, K., Tsuyumu, S., and Nasser, W. 1999. Regulation of *pelD* and *pelE*, encoding major alkaline pectate lyases in *Erwinia chrysanthemi*: involvement of the main transcriptional factors. Journal of Bacteriology 181:5948-5957.

Roy, A., Kucukural, A., and Zhang, Y. 2010. I-TASSER: a unified platform for automated protein structure and function prediction. Nature Protocols 5:725-738.

Ruiz, B., Chávez, A., Forero, A., García-Huante, Y., Romero, A., Sánchez, M., Rocha, D., Sánchez, B., Rodríguez-Sanoja, R., and Sánchez, S. 2010. Production of microbial secondary metabolites: regulation by the carbon source. Critical Reviews in Microbiology 36:146-167.

Salles, B., Weisemann, J., and Weinstock, G.M. 1987. Temporal control of colicin E1 induction. Journal of Bacteriology 169:5028-5034.

Samson, R., Legendre, J.B., Christen, R., Fischer-Le Saux, M., Achouak, W., and Gardan, L. 2005. Transfer of *Pectobacterium chrysanthemi* (Burkholder et al. 1953) Brenner et al. 1973 and *Brenneria paradisiaca* to the genus *Dickeya* gen. nov. as *Dickeya chrysanthemi* comb. nov. and *Dickeya paradisiaca* comb. nov. and delineation of four

novel species, *Dickeya dadantii* sp. nov., *Dickeya dianthicola* sp. nov., *Dickeya dieffenbachiae* sp. nov. and *Dickeya zeae* sp. nov. International journal of systematic and evolutionary microbiology 55:1415-1427.

Sauvage, E., Kerff, F., Terrak, M., Ayala, J.A., and Charlier, P. 2008. The penicillin-binding proteins: structure and role in peptidoglycan biosynthesis. Federation of European Biochemical Societies Microbiology Reviews 32:234-258.

Schäfer, A., Tauch, A., Jäger, W., Kalinowski, J., Thierbach, G., and Pühler, A. 1994. Small mobilizable multi-purpose cloning vectors derived from the *Escherichia coli* plasmids pK18 and pK19: selection of defined deletions in the chromosome of *Corynebacterium glutamicum*. Gene 145:69-73.

Schmidt, F.-R. 2011. The β-Lactam Antibiotics: Current Situation and Future Prospects in Manufacture and Therapy. Pages 101-121 in: Industrial Applications> N. S. Iacobellis, Collmer, A., Hutcheson,

Schwarzer, D., Finking, R., and Marahiel, M.A. 2003. Nonribosomal peptides: from genes to products. Natural Product Reports 20:275.

Sinden, S.L., DeVay, J.E., and Backman, P.A. 1971. Properties of syringomycin, a wide spectrum antibiotic and phytotoxin produced by *Pseudomonas syringae*, and its role in the bacterial canker disease of peach trees. Physiological Plant Pathology 1:199-213.

Singh, P.K., Schaefer, A.L., Parsek, M.R., Moninger, T.O., Welsh, M.J., and Greenberg, E. 2000. Quorum-sensing signals indicate that cystic fibrosis lungs are infected with bacterial biofilms. Nature 407:762-764.

Sjöblom, S., Harjunpää, H., Brader, G., and Palva, E.T. 2008. A novel plant ferredoxin-like protein and the regulator Hor are quorum-sensing targets in the plant pathogen *Erwinia carotovora*. Molecular Plant-Microbe Interactions 21:967-978.

Sleeman, M.C., and Schofield, C.J. 2004. Carboxymethylproline synthase (CarB), an unusual carbon-carbon bond-forming enzyme of the crotonase superfamily involved in carbapenem biosynthesis. Journal of Biological Chemistry 279:6730-6736.

Smadja, B., Latour, X., Faure, D., Chevalier, S., Dessaux, Y., and Orange, N. 2004. Involvement of N-acylhomoserine lactones throughout plant infection by *Erwinia carotovora* subsp. *atroseptica* (*Pectobacterium atrosepticum*). Molecular Plant-Microbe Interactions 17:1269-1278.

Smarda, J. 1992. Resistance and tolerance of bacteria to E colicins. Pages 493-502 in: Bacteriocins, Microcins and Lantibiotics>

Šmarda, J., and Oravec, C. 1993. *In vitro* and *in vivo* inhibition of blast lymphocytes by colicins. Molecular Microbiology 38:120-121.

Smid, E.J., Jansen, A.H., and Tuijn, C.J. 1993. Anaerobic nitrate respiration by *Erwinia carotovora* subsp. *atroseptica* during potato tuber invasion. Applied and Environmental Microbiology 59:3648-3653.

Smith, L.J., Fiebig, K.M., Schwalbe, H., and Dobson, C.M. 1996. The concept of a random coil: Residual structure in peptides and denatured proteins. Folding and Design 1:R95-R106.

Sogin, M.L., Morrison, H.G., Huber, J.A., Welch, D.M., Huse, S.M., Neal, P.R., Arrieta, J.M., and Herndl, G.J. 2006. Microbial diversity in the deep sea and the underexplored “rare biosphere”. Proceedings of the National Academy of Sciences 103:12115-12120.

Srikumar, R., Kon, T., Gotoh, N., and Poole, K. 1998. Expression of *Pseudomonas aeruginosa* Multidrug Efflux Pumps MexA-MexB-OprM and MexC-MexD-OprJ in a Multidrug-Sensitive *Escherichia coli* Strain. Antimicrobial Agents and Chemotherapy 42:65-71.

Stachelhaus, T., and Marahiel, M.A. 1995. Modular structure of genes encoding multifunctional peptide synthetases required for non-ribosomal peptide synthesis. Federation of European Biochemical Societies Microbiology Letters 125:3-14.

Stachelhaus, T., Mootz, H.D., and Marahiel, M.A. 1999. The specificity-conferring code of adenylation domains in nonribosomal peptide synthetases. Chemistry & Biology 6:493-505.

Stapleton, M.R., Norte, V.A., Read, R.C., and Green, J. 2002. Interaction of the *Salmonella typhimurium* Transcription and Virulence Factor *SlyA* with Target DNA and Identification of Members of the SlyA Regulon. Journal of Biological Chemistry 277:17630-17637.

Stapon, A., Li, R., and Townsend, C.A. 2003. Synthesis of (3 S, 5 R)-Carbapenam-3-carboxylic Acid and Its Role in Carbapenem Biosynthesis and the Stereoinversion Problem. Journal of the American Chemical Society 125:15746-15747.

Strieker, M., Tanović, A., and Marahiel, M.A. 2010. Nonribosomal peptide synthetases: structures and dynamics. Current Opinion in Structural Biology 20:234-240.

Sullivan, M.J., Petty, N.K., and Beatson, S.A. 2011. Easyfig: a genome comparison visualizer. Bioinformatics 27:1009-1010.

Swift, S., Downie, J.A., Whitehead, N.A., Barnard, A.M., Salmond, G.P., and Williams, P. 2001. Quorum sensing as a population-density-dependent determinant of bacterial physiology. Advances in Microbial Physiology 45:199-270.

Tagg, J.R., Dajani, A.S., and Wannamaker, L.W. 1976. Bacteriocins of gram-positive bacteria. Bacteriological Reviews 40:722.

Tan, Y., and Riley, M.A. 1996. Rapid invasion by colicinogenic *Escherichia coli* with novel immunity functions. Microbiology 142:2175-2180.

Tan, Y., and Riley, M.A. 1997. Positive selection and recombination: major molecular mechanisms in colicin diversification. Trends in Ecology & Evolution 12:348-351.

Tanaka, M., Hasegawa, T., Okamoto, A., Torii, K., and Ohta, M. 2005. Effect of antibiotics on group A *Streptococcus* exoprotein production analyzed by two-dimensional gel electrophoresis. Antimicrobial Agents and Chemotherapy 49:88-96.

Thomashow, L.S., Bonsall, R.F., and Weller, D.M. 1997. Antibiotic production by soil and rhizosphere microbes *in situ*. Manual of Environmental Microbiology:493-499.

Thomson, N., Crow, M., McGowan, S., Cox, A., and Salmond, G. 2000. Biosynthesis of carbapenem antibiotic and prodigiosin pigment in *Serratia* is under quorum sensing control. Molecular Microbiology 36:539-556.

Thomson, N., Cox, A., Bycroft, B., Stewart, G., Williams, P., and Salmond, G. 1997. The *rap* and *hor* proteins of *Erwinia, Serratia* and *Yersinia*: a novel subgroup in a growing superfamily of proteins regulating diverse physiological processes in bacterial pathogens. Molecular Microbiology 26:531-544.

Thomson, S.V., Hildebrand, D.C., and Schroth, M.N. 1981. Identification and Nutritional Differentiation of the *Erwinia* Sugar Beet Pathogen from Members of *Erwinia carotovora* and *Erwinia chrysanthemi*. Phytopathology 71:1037-1042.

Thurn, K.K., and Chatterjee, A.K. 1985. Single-site chromosomal Tn5 insertions affect the export of pectolytic and cellulolytic enzymes in *Erwinia chrysanthemi* EC16. Applied and Environmental Microbiology 50:894-898.

Tichy, E., Luisi, B., and Salmond, G. 2014. Crystal Structure of the Carbapenem Intrinsic Resistance Protein CarG. Journal of Molecular Biology 426:1958-1970.

Timmis, K., and Hedges, A.J. 1972. The killing of sensitive cells by colicin D. Biochimica et Biophysica Acta (BBA)-Nucleic Acids and Protein Synthesis 262:200-207.

Todorov, S.D., Wachsman, M., Tomé, E., Dousset, X., Destro, M.T., Dicks, L.M.T., de Melo Franco, B.D.G., Vaz-Velho, M., and Drider, D. 2010. Characterisation of an antiviral pediocin-like bacteriocin produced by *Enterococcus faecium*. Food Microbiology 27:869-879.

Toth, I.K., Bell, K.S., Holeva, M.C., and Birch, P.R.J. 2003. Soft rot erwiniae: from genes to genomes. Molecular Plant Pathology 4:17-30.

Toth, I.K., Van Der Wolf, J.M., Saddler, G., Lojkowska, E., Hélias, V., Pirhonen, M., and Elphinstone, J.G. 2011. *Dickeya* species: an emerging problem for potato production in Europe. Plant Pathology 60:385-399.

Trauger, J.W., and Walsh, C.T. 2000. Heterologous expression in *Escherichia coli* of the first module of the nonribosomal peptide synthetase for chloroeremomycin, a vancomycin-type glycopeptide antibiotic. Proceedings of the National Academy of Sciences 97:3112-3117.

Tsuge, K., Akiyama, T., and Shoda, M. 2001. Cloning, sequencing, and characterization of the iturin A operon. Journal of Bacteriology 183:6265-6273.

Tsuge, K., Ano, T., Hirai, M., Nakamura, Y., and Shoda, M. 1999. The genes degQ, pps, and lpa-8 (sfp) are responsible for conversion of *Bacillus subtilis* 168 to plipastatin production. Antimicrobial Agents and Chemotherapy 43:2183-2192.

Uppalapati, S.R., Ishiga, Y., Wangdi, T., Kunkel, B.N., Anand, A., Mysore, K.S., and Bender, C.L. 2007. The phytotoxin coronatine contributes to pathogen fitness and is required for suppression of salicylic acid accumulation in tomato inoculated with *Pseudomonas syringae* pv. *tomato* DC3000. Molecular Plant-Microbe Interactions 20:955-965.

van der Merwe, J.J., Coutinho, T.A., Korsten, L., and van der Waals, J.E. 2010. *Pectobacterium carotovorum* subsp. *brasiliensis* causing blackleg on potatoes in South Africa. European Journal of Plant Pathology 126:175-185.

Van Der Wolf, J.M., Nijhuis, E.H., Kowalewska, M.J., Saddler, G.S., Parkinson, N., Elphinstone, J.G., Pritchard, L., Toth, I.K., Lojkowska, E., and Potrykus, M. 2014. *Dickeya solani* sp. nov., a pectinolytic plant pathogenic bacterium isolated from potato (*Solanum tuberosum*). International journal of systematic and evolutionary microbiology 64:768-774.

Vanga, B.R., Butler, R.C., Toth, I.K., Ronson, C.W., and Pitman, A.R. 2012. Inactivation of *Pb*Topo IIIβ causes hyper-excision of the Pathogenicity Island HAI2 resulting in reduced virulence of *Pectobacterium atrosepticum*. Molecular Microbiology 84:648-663.

Vogler, A.J., Busch, J.D., Percy-Fine, S., Tipton-Hunton, C., Smith, K.L., and Keim, P. 2002. Molecular analysis of rifampin resistance in *Bacillus anthracis* and *Bacillus cereus*. Antimicrobial Agents and Chemotherapy 46:511-513.

Voronkevich, I. 1960. On the survival in the soil of bacteria of the genus *Erwinia* causal agents of soft rots in plants. Bullrtin of the Mosco Society of Naturalists Biological Series 65:95-105.

Wachsman, M.B., Farı́as, M.a.E., Takeda, E., Sesma, F., de Ruiz Holgado, A.P., de Torres, R.A., and Coto, C.E. 1999. Antiviral activity of enterocin CRL35 against herpesviruses. International Journal of Antimicrobial Agents 12:293-299.

Waleron, M., Waleron, K., and Lojkowska, E. 2013. Occurrence of *Pectobacterium wasabiae* in potato field samples. European Journal of Plant Pathology 137:149-158.

Walkinshaw, M., Taylor, P., Sturrock, S., Atanasiu, C., Berge, T., Henderson, R., Edwardson, J., and Dryden, D. 2002. Structure of Ocr from bacteriophage T7, a protein that mimics B-form DNA. Molecular Cell 9:187-194.

Waxman, D.J., and Strominger, J.L. 1983. Penicillin-binding proteins and the mechanism of action of beta-lactam antibiotics1. Annual Review of Biochemistry 52:825-869.

Weiler, E., Kutchan, T., Gorba, T., Brodschelm, W., Niesel, U., and Bublitz, F. 1994. The *Pseudomonas* phytotoxin coronatine mimics octadecanoid signalling molecules of higher plants. Federation of European Biochemical Societies Letters 345:9-13.

Welch, M., Todd, D.E., Whitehead, N.A., McGowan, S.J., Bycroft, B.W., and Salmond, G.P. 2000. N-acyl homoserine lactone binding to the CarR receptor determines quorum-sensing specificity in *Erwinia*. The European Molecular Biology Organisation Journal 19:631-641.

Weller, D. 1983. Colonization of wheat roots by a fluorescent *Pseudomonad* suppressive to take-all. Phytopathology 73:1548-1553.

Werra, P.d., Bussereau, F., Keiser, A., and Ziegler, D. 2015. First report of potato blackleg caused by *Pectobacterium carotovorum* subsp. *brasiliense* in Switzerland. Plant Disease 99:551-552.

Whitehead, N.A., Barnard, A.M., Slater, H., Simpson, N.J., and Salmond, G.P. 2001. Quorum-sensing in Gram-negative bacteria. Federation of European Biochemical Societies Microbiology Reviews 25:365-404.

Whitehead, N.A., Byers, J.T., Commander, P., Corbett, M.J., Coulthurst, S.J., Everson, L., Harris, A.K., Pemberton, C.L., Simpson, N.J., and Slater, H. 2002. The regulation of virulence in phytopathogenic *Erwinia* species: quorum sensing, antibiotics and ecological considerations. Antonie Van Leeuwenhoek 81:223-231.

Williams, S., and Vickers, J. 1986. The ecology of antibiotic production. Microbial Ecology 12:43-52.

Williamson, J.M., Inamine, E., Wilson, K.E., Douglas, A.W., Liesch, J.M., and Albers-Schönberg, G. 1985. Biosynthesis of the beta-lactam antibiotic, thienamycin, by *Streptomyces cattleya*. Journal of Biological Chemistry 260:4637-4647.

Williamson, N.R., Commander, P.M., and Salmond, G.P. 2010. Quorum sensing-controlled Evr regulates a conserved cryptic pigment biosynthetic cluster and a novel phenomycin-like locus in the plant pathogen, *Pectobacterium carotovorum*. Environmental Microbiology 12:1811-1827.

Wilson, M., and Lindow, S. 1993. Interactions between the biological control agent *Pseudomonas fluorescens* A506 and *Erwinia amylovora* in pear blossoms. Phytopathology 83:117-123.

Wilson, M., and Lindow, S.E. 1994. Ecological similarity and coexistence of epiphytic ice-nucleating (Ice+) *Pseudomonas syringae* strains and a non-ice-nucleating (Ice-) biological control agent. Applied and Environmental Microbiology 60:3128-3137.

Winslow, C.E.A., Broadhurst, J., Buchanan, R.E., Krumwiede Jr, C., Rogers, L.A., and Smith, G.H. 1917. The Families and Genera of the Bacteria: Preliminary Report of the Committee of the Society of American Bacteriologists on Characterization and Classification of Bacterial Types. Journal of Bacteriology 2:505.

Wloch-Salamon, D.M., Gerla, D., Hoekstra, R.F., and de Visser, J.A.G. 2008. Effect of dispersal and nutrient availability on the competitive ability of toxin-producing yeast. Proceedings of the Royal Society of London B: Biological Sciences 275:535-541.

Wood, D.W., and Pierson, L.S. 1996. The *phzI* gene of *Pseudomonas aureofaciens* 30–84 is responsible for the production of a diffusible signal required for phenazine antibiotic production. Gene 168:49-53.

Wu, H., Song, Z., Hentzer, M., Andersen, J.B., Heydorn, A., Mathee, K., Moser, C., Eberl, L., Molin, S., and Høiby, N. 2000. Detection of *N*-acylhomoserine lactones in lung tissues of mice infected with *Pseudomonas aeruginosa*. Microbiology 146:2481-2493.

Xi, C., Lambrecht, M., Vanderleyden, J., and Michiels, J. 1999. Bi-functional *gfp* and *gusA*-containing mini-Tn*5* transposon derivatives for combined gene expression and bacterial localization studies. Journal of Microbiological Methods 35:85-92.

Yamada, K., Kaneko, J., Kamio, Y., and Itoh, Y. 2008. Binding sequences for RdgB, a DNA damage-responsive transcriptional activator, and temperature-dependent expression of bacteriocin and pectin lyase genes in *Pectobacterium carotovorum* subsp. *carotovorum*. Applied and Environmental Microbiology 74:6017-6025.

Yamada, K., Hirota, M., Niimi, Y., Nguyen, H.A., Takahara, Y., Kamio, Y., and Kaneko, J. 2006. Nucleotide sequences and organization of the genes for carotovoricin (Ctv) from *Erwinia carotovora* indicate that Ctv evolved from the same ancestor as *Salmonella typhi* prophage. Bioscience, Biotechnology, and Biochemistry 70:2236-2247.

Yang, C.H., Gavilanes-Ruiz, M., Okinaka, Y., Vedel, R., Berthuy, I., Boccara, M., Chen, J.W.T., Perna, N.T., and Keen, N.T. 2002. *hrp* genes of *Erwinia chrysanthemi* 3937 are important virulence factors. Molecular Plant-Microbe Interactions 15:472-480.

Yang, J., Yan, R., Roy, A., Xu, D., Poisson, J., and Zhang, Y. 2015. The I-TASSER Suite: protein structure and function prediction. Nature Methods 12:7-8.

Yap, M.-N., Barak, J.D., and Charkowski, A.O. 2004. Genomic diversity of *Erwinia carotovora* subsp. *carotovora* and its correlation with virulence. Applied and Environmental Microbiology 70:3013-3023.

Yim, G., Wang, H.H., and FRS, J.D. 2007. Antibiotics as signalling molecules. Philosophical Transactions of the Royal Society B: Biological Sciences 362:1195-1200.

Young, J.M., Saddler, G.S., Takikawa, Y., Boer, S.H., Vauterin, L., Gardan, L., Gvozdyak, R.I., and Stead, D.E. 1996. Names of plant pathogenic bacteria 1864-1995. Review of Plant Pathology 75:721-763.

Zhang, Y. 2008. I-TASSER server for protein 3D structure prediction. BioMed Central Bioinformatics 9:40.

Zhao, Y., Thilmony, R., Bender, C.L., Schaller, A., He, S.Y., and Howe, G.A. 2003. Virulence systems of *Pseudomonas syringae* pv. *tomato* promote bacterial speck disease in tomato by targeting the jasmonate signaling pathway. The Plant Journal 36:485-499.