Virulence and Evolutionary Ecology in the Entomopathogen *Bacillus thuringiensis*

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// Abstract

Bacillus thuringiensis is an entomopathogen in the *Bacillus cereus* species group, and has been used as a biopesticide for over 50 years. Despite extensive use of *B. thuringiensis*, there remain questions over its specific ecology compared to other members of the *B. cereus* group which poses problems for its continued applied use. Tying entomopathogenic ecology to a specific clade within the *B. cereus* group will limit confusion between *B. thuringiensis* used in agriculture and more harmful strains. Better understanding of *B. thuringiensis* ecology can also be used to combat resistance in pest species through selective passaging.

The ecology of *B. thuringiensis* was explored through competitions in *Plutella xylostella* (diamondback moth) larvae, which showed clade 2 *B. thuringiensis* have improved fitness in insects compared to clade 1 strains. Additionally, growth rates were compared *in vitro*, giving different thermal profiles for the two clades. Growth media preference was assessed for *B. cereus* group species with all favouring protein media over soil-based ones.

Selective passaging explored the effects of relatedness and host background on virulence evolution. For relatedness, *B. thuringiensis* subsp. *aizawai* was passaged for five rounds in *P. xylostella* larvae with none, one or two bottlenecking events. These treatments failed to produce any increase in virulence. In the second, *B. thuringiensis* subsp. *entomocidus* was passaged either in Cry1Ac-resistant, Cry1Ac-susceptible, alternating rounds of each or coevolved *P. xylostella*, with all containing a mutagenesis step with ethyl methanesulfonate. Virulence increased in the resistant and coevolved treatments, confirming that resistance is best overcome by passaging in harder-to-kill hosts.

The ecological and genetic distinctiveness of clade 2 *B. thuringiensis* suggests the species should be reclassified to solely this clade, which will limit safety concerns. Selective passaging can improve the virulence of strains, even if the underlying interactions are unknown; it can also provide insight into virulence evolution which would be lost when improving only at the protein level.

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List of Contents

Introduction

Overview **9**

History of the use of *B. thuringiensis* **10**

Biology of Cry Toxins **12**

Mode of Infection **16**

Resistance **18**

Ecology and Phylogeny **19**

Virulence **21**

Kin Selection & Public Goods **23**

Toxin Improvement **24**

Aims **27**

I: Ecology of *Bacillus thuringiensis*

Abstract **29**

Introduction **30**

Methodology **37**

Results **44**

Discussion **55**

II: Competition & Frequency Dependence in *Bacillus thuringiensis* Infections

Abstract **63**

Introduction **64**

Methodology **71**

Results **77**

Discussion **87**

III: Selective Passaging of *Bacillus thuringiensis* while Controlling for Within-host Relatedness

Abstract **95**

Introduction **96**

Methodology **103**

Results **108**
Discussion **115**

IV: Selective Passaging of *Bacillus thuringiensis* in Multiple Host Backgrounds
Abstract **123**
Introduction **124**
Methodology **132**
Results **139**
Discussion **145**

Discussion
Overview **155**
Safety & Regulation of *Bacillus thuringiensis* **156**
Passaging versus Protein Evolution **157**
Bacillus weihenstephanensis **162**
Population structure in Experimental Evolution **164**
Concluding Remarks **167**

Bibliography **169**

List of Figures & Tables

I: Ecology of *Bacillus thuringiensis*

Introduction

Figure 1.1 **33**

Methodology

Table 1.a **37**

Table 1.b **40**

Results

Figure 1.2 **45**

Figure 1.3 **46**

Figure 1.4 **47**

Figure 1.5 **49**

Figure 1.6 **50**

Table 1.c **50**

Figure 1.7 **51**

Table 1.d **51**

Figure 1.8 **52**

Table 1.e **54**

II: Competition & Frequency Dependence in *Bacillus thuringiensis* Infections

Introduction

Figure 2.1 **68**

Methodology

Table 2.a **72**

Results

Figure 2.2 **78**

Figure 2.3 **79**

Figure 2.4 **80**

Figure 2.5 **81**

Figure 2.6 **83**

Figure 2.7 **84**

Figure 2.8 **85**

Figure 2.9 **86**

III: Selective Passaging of *Bacillus thuringiensis* while Controlling for Within-host Relatedness

Methodology

Figure 3.1 **105**

Results

Figure 3.2 **109**

Figure 3.3 **110**

Figure 3.4 **111**

Figure 3.5 **112**

Figure 3.6 **113**

Figure 3.7 **114**

IV: Selective Passaging of *Bacillus thuringiensis* in Multiple Host Backgrounds

Methodology

Table 4.a **134**

Figure 4.1 **135**

Results

Figure 4.2 **140**

Figure 4.3 **141**

Figure 4.4 **143**

Figure 4.5 **144**

Introduction

Overview

Bacillus thuringiensis (Berliner 1911) is a Gram-positive, endospore-forming motile bacterium and is part of the *Bacillus cereus* species group in the Bacillaceae family (Turnbull 1996, Liu, Lai et al. 2015). As with another species in the *B. cereus* group – *Bacillus anthracis*, the causative agent of anthrax (Zwick, Joseph et al. 2012) – *B. thuringiensis* has been distinguished from *B. cereus sensu stricto* through specific virulence factors it produces and associated epidemiology. In particular, *B. thuringiensis* is characterised by the presence of crystalline δ-endotoxins (Cry toxins) within the parasporal body. While *B. anthracis* is a mammalian pathogen (Zwick, Joseph et al. 2012), *B. thuringiensis* infections are limited to invertebrates (Aronson and Shai 2001). Entomocidal activity has been demonstrated against a number of insect Orders (Aronson and Shai 2001), especially Lepidoptera, Coleoptera and some Diptera (de Almeida Melo, Soccol et al. 2016), while some isolates have activity against nematodes and gastropods (Rae, Iatsenko et al. 2010, Palma, Munoz et al. 2014).

The entomocidal abilities of *B. thuringiensis* have led to it being adapted for use as a biopesticide, with genes for its Cry toxins also inserted into transgenic crops. At least 15 pest species of moth have since evolved resistance to particular commercialised toxins (Tabashnik and Carriere 2017) and this has meant there are ongoing attempts to produce improved or novel strains and toxins to overcome this problem (Park, Abdullah et al. 2009, Deist, Rausch et al. 2014, Rausch, Chougule et al. 2016). As explored here, selection experiments may provide a robust way to produce toxins that can overcome resistance. Along with these clear applied aspects of studying *B. thuringiensis*, the large variety of strains and toxins, combined with the availability of resistant host populations, make *B. thuringiensis* a viable candidate for exploring the mechanistic aspects of virulence and its associated traits, as well as the

evolutionary ecology shaping investment in various virulence factors (Raymond, West et al. 2012, Zhou, Slamti et al. 2014).

Experimental evolution provides one of the most powerful tools for examining how ecological factors affect evolution of virulence in *B. thuringiensis* and other pathogens (Kawecki, Lenski et al. 2012, Rafaluk, Jansen et al. 2015). In this thesis the effects of within-host relatedness and mutation rates on *B. thuringiensis* virulence evolution are explored through selective passaging. Treatments that appear to increase the virulence can then be incorporated into future passages with the ultimate aim of identifying a selection protocol for reliably producing isolates with restored mortality rates in initially resistant hosts. Achieving this will require a clearer understanding of the growth conditions favoured by *B. thuringiensis,* in part because any isolates of interest need to be viable *in vitro* to produce the quantities needed for applied work. This will also give greater clarity over the *B. cereus* group's ecology and classification, which may be required to avoid future regulatory concerns. The within-host interactions between commercialised *B. thuringiensis* strains also will be characterised to better understand how mixed infections affect strain fitness and to inform the design of relatedness treatments in the selective passages.

History of the use of *B. thuringiensis*

B. thuringiensis was originally isolated from diseased *Bombyx mori* silkworms in Japan in 1901 by Shigetane (Roh, Choi et al. 2007). It was fully described by Berliner in 1911 after being isolated again, in this instance from the Mediterranean flour moth *Anagasta kuehniella*, and given the species name *thuringiensis* after the East German city of Thuringen where the moths were captured (Berliner 1911). As a result of their entomocidal abilities, *B. thuringiensis* strains were subsequently adapted for use in agriculture as biopesticides, either through the use of spores or purified toxins. This value as a biocontrol agent was first recognised in France in 1938, with the first commercial product launched under the name Sporeine (Lambert and Peferoen 1992), though this was preceded elsewhere by non-commercial production

targeted at curbing the European corn borer moth, *Ostrinia nubilalis* (Lambert and Peferoen 1992). Subsequently, *B. thuringiensis* pesticides were registered in the United States in 1961 (Sanahuja, Banakar et al. 2011). Despite this long period of use *B. thuringiensis*-based pesticides still only make up 2% of total global pesticide use (Adang, Crickmore et al. 2014). There are several potential reasons: weak persistence on the surface of crops (Sanchis, Gohar et al. 1999); high costs relative to chemical alternatives but also because of the emergence of resistance to common products in some pest species, particularly in South East Asia (Gong, Wang et al. 2010, Zhang, Zhang et al. 2016, Tabashnik and Carriere 2017).

Biopesticide use preceded knowledge of precisely how *B. thuringiensis* acted to kill the pests it was employed against – it was not until 1956 that Angus discerned that crystalline toxins provided the entomocidal activity and they were located within the parasporal body (Angus 1956), having been produced during sporulation. Toxin identification was followed in the 1980s by the discovery that these crystal toxins were encoded on genes typically located on plasmids rather than in the main bacterial chromosome. Cloning and characterisation of these genes in the reference strain *B. thuringiensis* subsp. kurstaki HD1 (BGSC 4D1) was carried out around this time by Schnepf and Whiteley (1981). At the same time the viability of transferring genes into plants to produce transgenic crops was being successfully demonstrated, with virus-resistant tobacco first introduced commercially in 1992 (James 1998) and in 1996 the first transgenic plant (potato, *Solanum tuberosum*) containing a *B. thuringiensis*-sourced toxin gene was approved for sale in the USA (James 1998). As knowledge of genetic modification techniques has improved *B. thuringiensis* genes have been incorporated into a growing range of plants to produce more pest-resistant transgenic crops, with two thirds of the USA maize planted in 2012 containing such genes (Tabashnik, Brevault et al. 2013). By 2003 80% of biopesticides were *B. thuringiensis*-based (Whalon and Wingerd 2003) and *B. thuringiensis*-facilitated products are now used on 75% of arable and forested lands in North America (Bailey, Boyetchko et al. 2010). Increasing use has led to increasing resistance – in the last 20 years resistance to *B. thuringiensis* products has emerged in multiple pests, including the diamondback moth, *Plutella xylostella* (Zhang, Zhang et al. 2016, Tabashnik and Carriere 2017).

P. xylostella is a member of the lepidopteran family Plutellidae and a global pest of cruciferous crops (Miyata, Kawai et al. 1982), with control and crop losses together costing approximately $5 billion per annum (Furlong, Wright et al. 2013). They are a constant pest in South East Asia (Gu 2009) while they also threaten harvests in more temperate northern regions, where colder winters prevent them from becoming established year-round (Honda, Miyahara et al. 1992). The moth's ancestral range has not yet been pinpointed though both Mediterranean and South African origins have been proposed (Kfir 1998). *P. xylostella,* in conjunction with *B. thuringiensis*, represents an ideal study system for both practical considerations and applied reasons. Under lab conditions generation time is two weeks at 24°C and, combined with their small size (<1.5cm) and high fecundity, this allows large numbers to be reared with little effort. This means that any bioassays or other *in vivo* experiments carried out using larvae can be done with larger sample sizes across multiple treatments. In the field *P. xylostella* was the first insect species to show resistance to Cry1Ac-based biopesticides with resistance to the DiPel® (Valent BioSciences LLC) product appearing in the first half of the 1990s (Tabashnik, Cushing et al. 1990, de Almeida Melo, Soccol et al. 2016).

Biology of Cry Toxins

The crystalline toxins underlying the value of *B. thuringiensis* to agriculture can be split into Cry (crystal) and Cyt (cytolytic) toxins and the former have been the primary focus of biotechnological applications with *B. thuringiensis* so far (Kim, Roh et al. 2008, Gong, Wang et al. 2010). Cry toxins also provided the basis for identifying *B. thuringiensis* (Priest, Barker et al. 2004, Rasko, Altherr et al. 2005) and there are currently over 300 recognised Cry toxins in 75 classes, as maintained in the *Bacillus thuringiensis* toxin nomenclature full list of δ-endotoxins (Berry and Crickmore 2017, Crickmore, Baum et al. 2018). To be recognised as a Cry toxin on the database a protein must be sufficiently similar to existing Cry toxins and/or be a protein with entomocidal activity that can be isolated from crystal inclusions in a *B. thuringiensis* isolate. This does mean that if a protein is significantly similar to

an existing Cry toxin it can also be categorised as one even if its action or target are unknown (Crickmore, Baum et al. 2018). Within the currently defined *B. thuringiensis* species there are hundreds of named strains (Aronson and Shai 2001) which have mainly been identified and initially grouped together based on serotyping, but this is being replaced by genome sequencing and associated methods such as multi-locus sequence typing (Maiden, van Rensburg et al. 2013). Individual strains may carry multiple different Cry toxins from the same or different toxin groups, and these toxins determine which hosts a strain is able to successfully infect. The host range of any particular strain is generally restricted to a number of species within an Order, with some exceptions spanning Diptera and Lepidoptera (Zheng, Gao et al. 2017). However, across all the toxins and strains, a wide range of host species can be infected. This means that a new *B. thuringiensis* isolate potentially can be screened against various pest species to see which it produces high mortality in, while a specific pest can be bioassayed with a large variety of *B. thuringiensis* strains to find one that can be used against it. This combination of high toxicity, specificity and diversity is the core reason that *B. thuringiensis* has been so highly utilised for insect pest control, as it enables individual pest species to be targeted without causing mortality in the wider invertebrate fauna (particularly natural enemies and pollinators). In addition to the Cyt and characteristic Cry toxins *B. thuringiensis* strains can produce other virulence factors, including over 150 vegetative insecticidal proteins (Zheng, Gao et al. 2017), which are secreted during vegetative growth. Production of additional virulence factors is regulated through quorum sensing, with the transcription factor PlcR controlling production of stationary phase virulence factors and Rap controlling sporulation (Slamti, Perchat et al. 2014, Zhou, Slamti et al. 2014). The importance of quorum sensing to virulence factor production suggests that virulence may be strongly affected by within-host densities and population structure.

The many currently catalogued Cry toxins can be divided into different groups based on their levels of amino acid sequence similarity. Taking the commonly commercialised toxin Cry1Ac as an example, each character in the name represents a particular level of amino acid sequence identity. If two toxins have the same characters in the first three ranks (requiring a fourth rank to distinguish them) then their sequences are at least 95% identical; differing

primary ranks mean a similarity of less than 45% (Crickmore, Zeigler et al. 1998, de Maagd, Bravo et al. 2001). The catalogue currently lists Cry toxins labelled from Cry1Aa1 to Cry75Aa3 with the majority being 3-domain Cry toxins (Berry and Crickmore 2017, Crickmore, Baum et al. 2018). By contrast, there are only three subgroups of 40 Cyt toxins, and the third only contains a single toxin, Cyt3Aa1 (Crickmore, Baum et al. 2018).

In Cry toxins the majority of groupings share 5 conserved regions, with a smaller number of other groups lacking one or more of them. These regions were originally identified by Hofte and Whiteley (1989) and together the five appear to correspond to a 3-domain structure so that all toxins with these five conserved regions also possess a 3-domain structure. The first toxin to have its structure discerned was Cry3Aa in 1991, via X-ray crystallography (Li, Carroll et al. 1991, Berry and Crickmore 2017). The 3-domain proteins can be split into domains I, II and III. Domain I is thought to primarily play a role in pore formation, while domain II has a greater role in specificity and binding (particularly through its exposed surface loops) and domain III potentially has functions in both binding and insertion of toxins into the host cell membrane (Xu, Wang et al. 2014, Berry and Crickmore 2017).

In the 3-domain proteins the domains can be linked back to the five conserved regions, with the first region corresponding to the centre of domain I. Regions 2 and 3 are found at the joins of domains I-II and II-III, respectively. The final two regions are both in domain III, with 4 in the centre and 5 at the end (Schnepf, Crickmore et al. 1998) and the majority of categorised Cry toxins are thought to have this 3-domain form (Bravo, Gomez et al. 2013). However, Cry protoxins range from only 369 to 1344 amino acid residues in length, with the shorter ones lacking some or all of the five normally conserved regions (Adang, Crickmore et al. 2014). Some of this can be attributed to the varied length of the C-terminal extension which is cleaved off in the gut to leave the active toxin and is thought to play a role in crystal formation (de Maagd, Bravo et al. 2001).

Cry toxins were initially classified based on their entomocidal activities, i.e. toxins that were active against closely related host species were considered to be closely related themselves (Hofte and Whiteley 1989). Sequence data have replaced this functional classification, although sequence similarity can still

predict range of host activity to some extent. Sequence data may include the cleaved protoxin region which is unlikely to affect host-binding specificity (Bravo 1997, de Maagd, Bravo et al. 2001). Likewise, the three different domains of most Cry toxins are not all involved in binding to receptors (Schnepf, Crickmore et al. 1998) so it may be necessary to limit the sequence any tree is built from before comparisons with older entomocidal-based classifications. Indeed the old host-based classification is close to the trees constructed for domains I and II, though there are exceptions e.g., Cry9Aa toxins show sequence similarity to other Cry9 ones in the cleaved protoxin region and domain III instead (Bravo, Gill et al. 2007). While the similarity between host-based trees and domain II is unsurprising given its role in receptor binding, domain I's similarity may be a result of different host groups possessing different gut conditions: lepidopteran-targeting 3-domain toxins appear to favour arginine over lysine in domain II, but not those affecting the more acidic guts of Coleoptera (Grochulski, Masson et al. 1995).

While domains I and II appear to have mainly coevolved, the distinct tree generated by domain III may help to explain the great variety of 3-domain Cry toxins (Bravo 1997, Bravo, Gill et al. 2007). There is clear evidence of homologous recombination between Cry toxin genes facilitating the swapping of domain III (Bosch, Schipper et al. 1994, de Maagd, Weemen-Hendriks et al. 2000). This is evident when examining the Cry1C and Cry1E toxins – in each group, the I and II domains are almost identical yet some Cry1C domain IIIs are far more similar to Cry1E ones than their supposed Cry1C neighbours (Bravo 1997). This shuffling of domains increases the number of combinations, giving greater toxin variety and activity against more host species. This has been demonstrated in the laboratory, with domain swapping between Cry1Ac and Cry1Fa toxins producing ones that are effective against a greater host range than either of the initial toxins (Malvar and Gilmer 1998, de Maagd, Bravo et al. 2001).

Mode of Infection

The structure of 3-domain Cry toxins ultimately allows them to trigger the breakdown of a host's midgut lining, allowing *B. thuringiensis* cells to invade and proliferate in the haemolymph. In lepidopterans the alkaline conditions of the pro-gut cause the crystals to dissolve, after which protoxins are cleaved by gut enzymes to give active toxin (Hofmann, Luthy et al. 1988, Aronson and Shai 2001). Activated 3-domain toxins then bind to receptors on microvilli in membranes of cells lining the midgut (de Maagd, Bravo et al. 2001, Bravo, Gill et al. 2005). Binding to the receptors ultimately triggers cell death, though there are at least two potential routes for this (de Almeida Melo, Soccol et al. 2016). Cell death results in the midgut wall breaking down which allows mixing of gut contents, including *B. thuringiensis*, with the haemolymph where the bacteria can proliferate vegetatively. Host death can result solely from sufficient damage to the midgut, as shown by the existence of toxin-only biopesticides and transgenic crops. Mortality is increased with the presence of vegetative cells, which can cause septicaemia as they reproduce; other bacteria present in the gut becoming pathogenic when given access to the haemocoel might also contribute to the success of toxin-only control methods (Raymond, Johnston et al. 2009). While any particular part of the infection process could contribute to host specificity, it is thought this is principally achieved through binding to host-specific receptors (Berry and Crickmore 2017). Lepidopteran larvae can respond to infection with melanisation of infected tissue (Rahman, Roberts et al. 2004), which results in *B. thuringiensis* cadavers having a characteristic black appearance that is easily distinguishable in laboratory conditions.

As described, the overall process of *B. thuringiensis* infection is well characterised. However, the exact way 3-domain Cry toxins trigger cell death is not fully resolved. The better established classic model is that sequential binding and pore formation in the plasma membrane lead to osmotic shock and cell death (Bravo, Gill et al. 2007, de Almeida Melo, Soccol et al. 2016). The cleaved Cry toxin may bind to cadherin, N-aminopeptidase or other receptors which causes a conformational change in the toxin, as well as allowing further toxin proteins to bind and form pores (Dorsch, Candas et al. 2002, Jimenez-Juarez, Munoz-Garay et al. 2008). Other membrane proteins have also been

linked to Cry toxin binding, including ABC type-C reporters (abcc2) (Gahan, Pauchet et al. 2010, Baxter, Badenes-Perez et al. 2011, Park, Gonzalez-Martinez et al. 2014) and alkaline phosphatase (Jurat-Fuentes and Adang 2004, Guo, Kang et al. 2015). The proliferation of pores in the plasma membrane causes it to break down and the resulting loss of control over osmotic gradients results in cell death (de Almeida Melo, Soccol et al. 2016).

The alternative mechanism for cell death still has toxins binding to surface receptors and generating pores, but includes an additional signalling pathway where binding to cell-surface receptors triggers a change in cell metabolism which is the ultimate cause of cell death (Zhang, Candas et al. 2006, de Almeida Melo, Soccol et al. 2016). This mechanism partly has been proposed to explain alternate forms of Cry toxin resistance which have been observed. It is clear that changing membrane receptor shape can produce resistance, as toxins can no longer bind to form pores, and this is compatible with the classical model of toxin action. In addition, preventing expression of the receptors also generates resistance, while restoring either cadherin or N-aminopeptidase can be enough to rescue susceptibility (Schwartz, Lu et al. 1997). However, there is evidence of resistance occurring without any change to surface receptors, possibly implying that pores are still being formed through toxin-receptor binding but not ultimately causing cell death and a breakdown in midgut epithelium (Vachon, Laprade et al. 2012); while receptor binding does confer specificity it cannot necessarily predict susceptibility (Adang, Crickmore et al. 2014). This could mean that resistance has evolved to an alternate trigger for cell death, hence the potential requirement for a different pathway. However, as cells that are riddled with pores could be expected to undergo apoptosis anyway, it may be difficult to fully confirm this distinct pathway for Cry toxin action.

Resistance

Against a susceptible host, ingestion of only a few *B. thuringiensis* spores can be sufficient for host death (Cornforth, Matthews et al. 2015). However, one of the reasons that *B. thuringiensis* has not become a panacea for agricultural pests is that high selection pressure rapidly leads to resistance e.g., through modification or loss of the cadherin-like or abcc2 receptors with the latter being the likely mechanism in *P. xylostella* (Baxter, Zhao et al. 2005, Baxter, Badenes-Perez et al. 2011). *B. thuringiensis* has been widely deployed against *P. xylostella*, particularly in South East Asia. Here the climate, as well as being favourable for crop growing, allows at least 12 *P. xylostella* generations a year (Koshihara and Yamada 1981). The short generational turn-over and large population sizes mean that resistance in *P. xylostella* can emerge rapidly and it was the first species to show resistance to *B. thuringiensis* products in the field (Tabashnik, Cushing et al. 1990).

While resistance can appear through heritable behaviour such as avoiding high levels of *B. thuringiensis* (Aronson and Shai 2001, Schulenburg and Muller 2004), it appears primarily to be a result of shape changes to, or deletion of, receptors to which the toxin binds (de Almeida Melo, Soccol et al. 2016). Within *P. xylostella*, Park was able to identify 52 genes that coded for cadherins or highly similar proteins, but only absence of the midgut-expressed one reduced toxin susceptibility (Park, Herrero et al. 2015). However, Cry toxins can still cause mortality in *P. xylostella* carrying the same cadherin mutations which confer resistance in the bollworms *Pectinophora gossypiella* and *Helicoverpa armigera* (Morin, Biggs et al. 2003, Xu, Yu et al. 2005, Baxter, Badenes-Perez et al. 2011). Instead, based on genetic mapping it is likely that resistance for the *Plutella*-targeting Cry1Ac toxin is actually linked to the *abcc2* gene (Baxter, Badenes-Perez et al. 2011); transforming *Drosophila* cells to express moth abcc2 receptors but not cadherins also renders them susceptible to Cry1Ac (Stevens, Song et al. 2017). As changes to a receptor's structure could be the result of a single nucleotide polymorphism, large host populations and short generations mean that such a mutation is highly likely to occur and quickly sweep to fixation once it does. This appears to be the case in the field,

as resistance to Cry toxin-based transgenic crops is far more widespread than it was a decade ago (Tabashnik and Carriere 2017). While resistance management techniques like refugia can help limit the emergence of resistance (Tabashnik, Gassmann et al. 2008), it is unlikely that only a small pool of applicable *B. thuringiensis* strains and toxins will be sufficient in the future.

Ecology and Phylogeny

The ability of *B. thuringiensis* to kill susceptible invertebrates through its Cry toxins is well established but there is still some debate about whether growth in a host is essential to the life-cycle of *B. thuringiensis*, i.e. if it is an obligate pathogen (Jensen, Hansen et al. 2003, Raymond, Wyres et al. 2010). This has remained an issue because of the phylogeny of *B. thuringiensis* and its ubiquity in the environment. As *B. thuringiensis* can be isolated readily from soils and leaves alongside *B. cereus*, while cadavers are rarely encountered, it has been suggested that entomocidal activity is incidental and most vegetative *B. thuringiensis* growth happens in the wider environment (Jensen, Hansen et al. 2003, Hendriksen 2016). However, this has been countered by the fact that much of the *B. thuringiensis* in the soil may be there as spores or associated with nematodes (Ruan, Crickmore et al. 2015). While some strains do possess Cry toxins that are nematocidal (Schulte, Makus et al. 2010, Kho, Bellier et al. 2011) strains may also rely on nematodes to move them through soil and into contact with suitable hosts. This could occur through direct nematode-insect contact or by moving spores to plants where they are more likely to be consumed by potential hosts (Ruan, Crickmore et al. 2015), as it appears that *B. thuringiensis* spores can be preferentially moved to seedlings from the soil, though the mechanism is currently unclear (Monnerat, Soares et al. 2009, Raymond, Wyres et al. 2010).

There is also confusion over whether *B. thuringiensis* should be considered separate from *B. cereus sensu stricto*. The morphological similarities of *B. thuringiensis* to *B. cereus* made it easy to identify the two as sister species in a pre-genomic world, with Cry toxin possession a clear marker

to separate them. In turn, serological responses to H-flagellin proteins were used to group together different *B. thuringiensis* isolates (Lecadet, Frachon et al. 1999). There were exceptions to the apparently neat classification of *B. thuringiensis*, with some isolates lacking crystals but still responding to *B. thuringiensis*-specific serotyping (Lecadet, Frachon et al. 1999). The fact that only plasmid-encoded Cry toxins were needed for identification meant that *B. thuringiensis* and *B. cereus* strains could be otherwise identical at the morphological level (Helgason, Okstad et al. 2000, Priest, Barker et al. 2004). As Cry plasmids could be gained and lost through horizontal transfer (Meric, Mageiros et al. 2018) then the clear distinction between the two species appeared to break down.

The revolution in genome-wide sequencing has fully removed the *B. thuringiensis-cereus* divide as it was previously understood. Full genome sequencing of all available isolates in the *B. cereus* group showed that *B. thuringiensis* does not form a single clade either as a sister to or within *B. cereus* – instead *B. thuringiensis* strains are distributed through the *B. cereus* group (Priest, Barker et al. 2004, Didelot, Barker et al. 2009). Notably, the other species thought to be closely related, *B. anthracis*, maintains its monophyletic grouping when scrutinised with genome sequencing. The rest of the *B. cereus* group splits into clades, starting from work with sequence types based off seven alleles (Priest, Barker et al. 2004): Clade 1, containing the *B. anthracis* sub-clade and the majority of *B. cereus*, clade 2 with the majority of *B. thuringiensis* and clade 3 mainly containing *Bacillus weihenstephanensis* and *Bacillus mycoides*. As *B. thuringiensis* strains are found in clade 1 and 2, the existing species is polyphyletic so an alternative is required. There are two solutions, with the simplest being to discard *B. thuringiensis* as a species altogether and note which *B. cereus* strains possess Cry toxins. The second is to examine further the distribution of *B. thuringiensis* in the *B. cereus* clades and look for other factors that might distinguish them. If *B. thuringiensis* is an entomopathogen and *B. cereus* primarily a soil saprophyte (Raymond, Davis et al. 2007, Didelot, Barker et al. 2009) it may be that clades' ability to grow in these environments could be linked to separate species. Maintaining the *B. thuringiensis* species in some form may be useful if this is the case – *B.*

anthracis remains a separate species because its divergence from *B. cereus* lines up with important epidemiological differences.

From a commercial standpoint, how *B. thuringiensis* is classified has implications for its regulation and use. Clade 2 contains the currently commercialised strains such as *kurstaki* and *aizawai* (Zheng, Gao et al. 2017) and these could be a starting point for experimental evolution to produce or identify novel toxins or strains, as attempted here. The existing classification of *B. thuringiensis* means it includes strains in clade 1 which have products that bind to *B. anthracis* antigens and others that are closely related to *B. cereus* strains causing emetic food poisoning (Cachat, Barker et al. 2008, Didelot, Barker et al. 2009). In particular, the cereulide toxins that cause emetic food poisoning are also plasmid-borne so there are fears in the European Food Safety Authority that commercial *B. thuringiensis* strains could become human pathogens through horizontal transfer (EFSA 2016). However, this ignores the fact that the clade 2 strains used on crops are not closely related to the clade 1 emetic strains. Clade 2 having a distinct ecology as an obligate insect pathogen would also mean there are no opportunities for horizontal exchange of cereulide toxins and its strains would lack other genes for successful infections in humans.

Virulence

Virulence in pathogens generally can be seen as the fitness cost to the host of infection, although difficulties in measuring this accurately mean it is often reframed as the pathogen-induced mortality rate (Bull 1994). Earlier work considered the extent to which a pathogen's virulence might change as it adapted to a host e.g., myxomatosis (*myxoma* virus) becoming progressively less virulent after its initial release into Australian rabbit populations (Kerr 2012). This thinking forms part of the virulence-transmission trade-off theory (Frank 1996, Levin 1996, Galvani 2003). For myxomatosis this explained transmission increasing at the expense of virulence: if viral mortality takes longer there is increased chance of transmission to other hosts, so less virulent strains should

become more prevalent over time. However, the trade-off also suggests that pathogens can become more virulent given appropriate conditions. If a pathogen reproduces slowly (low virulence) it may not reach sufficient densities for successful transmission e.g., blood pathogen levels might be too low to ensure uptake by a vector, or the infection might be cleared by the host immune system before transmission occurs (Frank 1996). Increased virulence would therefore be selected for to increase transmission (Frank 1996) e.g., attenuated vaccines regaining pathogenicity (Bull 1994). This trade-off can be seen in *B. thuringiensis* with spore numbers highest in cadavers with an intermediate infection length (Raymond, Ellis et al. 2009).

Transmission potential is not the only factor affecting the virulence of pathogens. There is increasing evidence that mixed-strain infections may be the rule rather than the exception (Gilbert, Plebanski et al. 1998, Petney and Andrews 1998, Lord, Barnard et al. 1999), even down to the level of varied plasmid carriage in the same genotype (Medaney, Ellis et al. 2016). Mixed-strain infections can occur either as super- or co-infections (Nowak and May 1994, May and Nowak 1995): in the former, at any one point there is a dominant strain in the infection and any strains subsequently colonising the host either replace it or are out-competed, with only one strain transmitted; in co-infections multiple strains persist at the same time and potentially all can be transmitted (Alizon and van Baalen 2008). Multiple pathogen genotypes add another factor to any virulence-transmission trade-off considerations. A host is a finite resource so the faster a strain grows the more of the host it can utilise at the expense of competing strains. Therefore within-host competition would favour fast growing, more virulent strains and increase overall virulence under the established trade-off theory of virulence (Nowak and May 1994, Frank 1996, Gandon, Jansen et al. 2001). Single-strain infections would instead favour more prudent strains where virulence remains low enough to ensure transmission before host death. When tested there is evidence both for (de Roode, Pansini et al. 2005, Bell, De Roode et al. 2006) and against (Turner and Chao 1999) more virulent strains being favoured under experimental co-infections (Alizon and van Baalen 2008). However, this view of virulence does not fully consider how bacteria interact with each other in an infection.

Kin Selection & Public Goods

In the last 25 years there has been ever increasing evidence that bacteria are highly social, in the sense that there is communication and pooling of resources between coexisting cells (Brown and Johnstone 2001, Griffin, West et al. 2004, Harrison, Browning et al. 2006). Many bacteria secrete extra-cellular products which are 'public goods' – while an individual secretes them, any local bacteria may benefit from their action. This is a clear example of kin selection, where such sharing is favoured as long as the recipients share the same secretory genes and so have a higher level of relatedness than average (Rumbaugh, Trivedi et al. 2012). Pathogenic bacteria produce many secretory products including those for sequestering resources from the host, such as iron-gathering siderophores, or triggering cell death as Cry toxins do (Griffin, West et al. 2004, Deng, Slamti et al. 2015). Bacteria can also secrete quorum-sensing molecules for the purpose of determining population density. Bacteria that share the same quorum-sensing mechanism will be more closely related, favouring the secretion of pubic goods and allowing group-level control of their production (Leggett, Brown et al. 2014); quorum sensing systems oversee both secretion of virulence factors and sporulation in *B. thuringiensis* (Slamti, Perchat et al. 2014). When this system is considered in a host it is clear that close relatedness may favour higher virulence, if virulence relies more on production of public goods rather than growth rate (Frank 1996). When bacteria in a host are closely related production of public goods required for growth can be maintained; at low relatedness non-relatives will benefit from public goods so they are not produced, limiting growth and virulence. This goes further than altruistic public goods as bacteria can also show spiteful behaviour to eliminate non-relatives (Gardner, West et al. 2004), most commonly via secreted bacteriocins and antibiotics (Riley and Gordon 1999). Clearly, if bacteria limit each other's growth through spite it will reduce overall virulence, both by initially reducing bacterial load and by diverting resources away from growth into anti-competition measures (Gardner, West et al. 2004).

The social aspect of Cry toxin production provides a possible explanation for why the majority of *cry* genes are maintained on plasmids rather than in the

bacterial chromosome. If *B. thuringiensis* is an obligate pathogen, with Cry toxins crucial to its life cycle, it might be expected that these genes would be in the core genome rather than on elements that could be lost more easily. However, if *cry* genes can easily be transferred to other bacteria it may help maintain higher levels of genetic relatedness, preventing Cry-null cheats that avoid toxin production costs from emerging. This social transfer explanation can be countered by arguing that cheat plasmids might then be favoured in a population that exchanged Cry plasmids. This may be limited in the case of *B. thuringiensis* and other pathogens, as Leggett, Brown et al. (2014) argue the population structuring provided by hosts means that horizontal gene transfer rates will affect levels of relatedness in different parts of the genome. Plasmids which are readily transferred will be transferred into neighbours and their products will only benefit neighbouring cells that now also carry the plasmid. This is supported by work in *Escherichia coli* showing that genes which were more likely to be horizontally transferred were also more likely to encode secreted products (Nogueira, Rankin et al. 2009), while larger Cry plasmids have higher transfer rates within-strain than outside (Meric, Mageiros et al. 2018).

Toxin Improvement

Resistance to *B. thuringiensis* products and toxins is now a global concern (Jorgensen, Aktipis et al. 2018). To overcome it a combination of effective toxins and prudent management of their use e.g., strategies involving refugia, will be needed (Tabashnik, Carriere et al. 2003). As existing toxins become less effective one solution is to find or create new ones. Novel toxins may be identified in strains newly isolated from the environment (Roh, Choi et al. 2007) although they may then only be tested for activity against a few pest species (van Frankenhuyzen 2009). Alternatively, as more strains are sequenced it is possible to search through them for toxin or toxin-like genes that have been overlooked so far (Ye, Zhu et al. 2012). To generate novel toxins site-directed mutagenesis has been used to alter existing ones which have limited activity against a pest (Kim, Roh et al. 2008, Deist, Rausch et al. 2014),

or regions of the toxin have been removed to restore activity (Soberon, Pardo-Lopez et al. 2007). Altered toxins can then be screened via bioassays to see whether entomocidal activity has been increased, eventually producing a toxin with restored effectiveness. If there are multiple Cry toxins that produce low levels of mortality in a pest, it may be possible to combine them in a transformed strain where their effects synergise to produce sufficiently high mortality, perhaps because the toxins target different receptors (Emiliano Canton, Zanicthe Reyes et al. 2011, Raymond, Wright et al. 2013). The same idea of mix-and-match can also be applied at the Cry protein domain level. In 3-domain Cry proteins, exchanging binding domains of a toxin which is effective in one species for one with low mortality in another may produce a toxin which is now sufficiently effective in the second species (Deist, Rausch et al. 2014).

In the above methods any novel toxins generated then need to be assayed against the target pests. Depending on the techniques used and initial number of proteins there may be a large library of toxins produced, all needing their entomocidal activities determined. Assaying this many products can be labourious and time-consuming, even if it may now be possible to automate much of it, and there is no guarantee that any effective toxin will be identified. These problems can potentially be circumvented by directed experimental evolution. In its simplest form this means exposing a resistant pest population to intermediate doses, recovering spores from pests that die and then growing them up to infect further resistant pests. This should mean that only bacteria generating higher mortalities are selected and brought forward for the next round of infection. Ideally, such increases in mortality would result from changes to the Cry toxins, though it may be the case that toxin production or other virulence factors are upregulated instead. If resistance appears through conformational changes to a toxin receptor to prevent binding (Ayra-Pardo, Raymond et al. 2015) a complementary conformational change in the toxin could be sufficient to restore entomocidal activity. Rounds of selection could then bring to prominence any sequence variation that restores toxin effectiveness. However, it could be that a restorative mutation is not already present within the population, or it might require multiple mutations to reach the desired effectiveness. In either case, there is insufficient variation present and waiting for it to emerge may exceed an appropriate timescale.

A lack of variation could be solved by combining mutagenesis and directed experimental evolution. Mutagenesis should greatly increase variation within a *B. thuringiensis* population, while combining it with selection limits the need to isolate many different bacteria or toxins and bioassay them all. Sufficient rounds of selection could result in bacterial lineages that have insecticidal activity orders of magnitude greater than the ancestral strain (Ebert 1998). Beyond this theoretical basis there are multiple variables that can be explored in an attempt to maximise the success of the process e.g., how many rounds of selection and mutagenesis are required or how should bacteria be recovered and processed between rounds? Afterwards sequence comparisons with the ancestor can determine whether any increase in virulence is the result of changes in *cry* genes or the result of some other change such as up-regulation of toxin production. While a strain producing more toxin could be useful as a biopesticide, it would be less so when looking for new *cry* genes to trial in transgenic crops.

A selection protocol as outlined above assumes that any bacteria isolated between rounds will be from the lineages of interest, but this is by no means a certainty. As the mutagenesis process creates high variation within the infecting population there should effectively be a co-infection within the host. Biopesticide applications of *B. thuringiensis* could also result in mixed infections, with sprayed spores being consumed alongside naturally-occurring spores already on crops. Competition experiments show that infecting hosts with multiple strains of *B. thuringiensis* can result in all strains being recovered from cadavers, meaning that co-infection generally occurs rather than super-infection (Raymond, Davis et al. 2007). Therefore infecting a host with a pool of non-clonal bacteria (as with the combination of mutagenesis and selection) also results in a co-infection. Recovery of multiple strains may partly be a product of the speed of infection as mortality in *P. xylostella* second-instar larvae occurs within four days of infection.

Trade-offs have already been raised in regards to mortality versus transmission, but there are others to consider. Cry toxins are public goods and require energy that could otherwise be spent on reproduction, so there is a selective advantage to losing Cry toxin production when producers are common (Raymond, West et al. 2012). As highlighted this can lead to a ‘tragedy of the

commons' situation where, if there are sufficient cheats, insufficient Cry toxin is produced to establish a successful infection. Even if there are no Cry-null cheats, variation in the insecticidal activity of toxins for different clonal groups might correspond with variation in haemolymph growth or sporulation rate. This is a potential stumbling block when trying to select for increased entomocidal activity. While toxins alone are sufficient to cause mortality via breakdown of the midgut lining, the ensuing septicaemia from bacteria growth in the haemocoel also contributes (Johnston and Crickmore 2009). While cadavers may contain clones with high insecticidal activity there may be others present which lack effective toxins but are able to sporulate more rapidly, increasing their proportion of the population for the next round. Conversely, while co-infections are possible, the dynamics of competition between clones within cadavers (van Leeuwen, O'Neill et al. 2015) suggests that low relatedness may not be a barrier to experimental evolution of increased virulence. This is encouraging as high relatedness may favour the cooperation necessary for higher virulence, but in turn it could limit the variation required for selection to increase virulence further. In any case, experiments can address this issue and explore whether this may or may not be a problem.

Aims

Exploring the outcomes of within host-competition for *B. thuringiensis* strains will aid understanding of the trade-offs between toxin production and competitive ability. While this knowledge should be valuable in its own right it will also help to inform conditions when attempting selective passages to produce improved entomocidal activity. Most obviously, there is a wide body of existing work suggesting that the level of relatedness between pathogens in a host is central to the production of public goods and growth (Brown, Hochberg et al. 2002, Griffin, West et al. 2004, Garbutt, Bonsall et al. 2011, Deng, Slamti et al. 2015). As Cry toxins are public goods and high growth rates correspond to septicaemia then both principal components of *B. thuringiensis* entomocidal activity should be linked to within-host relatedness. Levels of relatedness in an infection can be varied by infecting with a single clone, multiple clones of the

same strain or multiple distinct strains. Different strains carry different Cry toxin plasmids so mixed infections could also provide an opportunity to shuffle plasmids, potentially giving rise to new Cry toxin combinations (Zheng, Gao et al. 2017). Likewise, bringing different Cry toxin genes together may give novel domain combinations through recombination and domain shuffling (Bosch, Schipper et al. 1994, de Maagd, Weemen-Hendriks et al. 2000). If mixed infections persist as co-infections then the door is open to explore how competition during selection can affect attempts to select for high entomocidal activity during rounds of experimental evolution. Levels of relatedness could be further controlled by altering the levels of spatial structuring in a host, which is most easily achieved by altering the number of spores in an infective dose.

The effects of relatedness are best explored before adding variables such as mutagens to the selection process. Use of mutagens will increase variation within the infection and correspondingly decrease relatedness. If relatedness does interact with artificial selection for greater insecticidal activity, then this needs to be considered and accounted for in any subsequent selection experiments that also include mutagens.

Growth experiments with strains from *B. cereus* group clades 1, 2 and 3 and competitions between *B. thuringiensis* strains in clades 1 and 2 will aim to highlight the differences in ecology between the clades, including specialisation of clade 2 *B. thuringiensis* as insect pathogens. Any ecological differences identified will reflect the genetic separation of clade 2 from the majority of *B. cereus* strains in clade 1. This may appear a distinct issue from factors affecting the evolution of *B. thuringiensis* virulence and Cry toxin improvement, but the two are interlinked biologically and commercially. That there is still confusion over whether *B. thuringiensis* is an obligate pathogen or whether *B. cereus* is only a generalist soil saprophyte shows the ecology of this group is not fully understood. It would be unwise to carry out selection experiments without a better understanding of which growth conditions are most appropriate or how factors such as sporulation might interact with experimental conditions and trait targeted for selection. Confirming the specialisation and separation of clade 2 strains may also temper current unease about *B. thuringiensis* spore use on food crops.

I: Ecology of *Bacillus thuringiensis*

Abstract

Phylogenetic distinctions and taxonomy are extremely important for applied microbiology, as names are expected to summarise biological attributes and therefore hazard. The *Bacillus cereus* group encompasses a wide range of environmental and pathogenic isolates, including the aetiological agents of anthrax and lethal food poisoning, as well as the entomopathogen *Bacillus thuringiensis,* which is widely exploited in insect pest management. Characterising the niches of different members of this group can identify the ecological factors that might affect the prevalence of aetiological agents of disease. In addition the extent of the ecological distinctiveness of the different members is extremely important in informing the potential hazards involved in exploiting member species such as *B. thuringiensis.*

Here the hypothesis that *B. thuringiensis* constitutes a distinctive and ecologically specialised group of invertebrate pathogens was tested. In addition, whether *B. cereus* and the predominantly environmental species *Bacillus weihenstephanensis* are better adapted to exploit soil or plant derived nutrients than these putative pathogens was tested. In contrast to some previous work, all species could only grow vigorously in protein-rich media. Importantly, phylogenetic clade, in addition to nominal species, was an important predictor of growth in insects and of thermal profiles in growth rate and productivity, although anthracis clade isolates did not show the expected ability to grow at 37°C, which is characteristic of vertebrate pathogens. However, these data are consistent with the hypothesis that *B. cereus* isolates have evolved from a necromenic (cadaver-specialised) ancestor.

Characterising species solely on the basis of secondary metabolism, especially when these traits are encoded on mobile elements can mean that species names are poor predictors of broad biological traits. In contrast data-rich phylogenetic analyses, based on readily available genomes, are more likely to give informative and useful species designations.

Introduction

The *Bacillus* genus contains Gram-positive, aerobic, spore-forming bacteria (Maughan and Van der Auwera 2011, Liu, Lai et al. 2015). Many clinically and economically important bacteria are in the *Bacillus cereus* species group, including: *Bacillus cereus sensu stricto* (strains of which can cause severe emetic food poisoning), *Bacillus thuringiensis* and *Bacillus anthracis* (anthrax) (Papazisi, Rasko et al. 2011). There are up to eight further species in the group, including *Bacillus weihenstephanensis* (Papazisi, Rasko et al. 2011, Liu, Lai et al. 2015). Prior to the sequencing age these species were defined based on their morphology and biochemistry, as were many other bacteria (Gupta and Maiden 2001, Priest, Barker et al. 2004, Papazisi, Rasko et al. 2011, Zheng, Gao et al. 2017). In principal *B. thuringiensis* is defined by its production of crystalline delta-endotoxins (Cry toxins) (Aronson and Shai 2001, Vilas-Boas, Peruca et al. 2007) and associated mortality in insects, while *B. anthracis* is recognised by its possession of pXO plasmids and associated capsule and toxin, and anthrax epidemiology in mammals, among other traits (Okinaka, Pearson et al. 2006, Kolsto, Tourasse et al. 2009). *B. weihenstephanensis* is defined by its psychrotolerance (growth below 7°C but not above 43°C) (Soufiane and Cote 2010) while *B. cereus* is as much defined by the absence of these characteristics as it is by any one particular trait.

As the main identifiers of *B. thuringiensis* and *B. anthracis* are plasmid-borne, arguments have been made that chromosomally these bacteria are indistinguishable from *B. cereus* (Helgason, Okstad et al. 2000). Indeed curing them of their plasmids makes them morphologically identical to *B. cereus* (Helgason, Okstad et al. 2000), although subsequent phylogenetic analyses based on DNA sequences rather than band mobilities have not repeated the detailed findings of the Helgason paper (Priest, Barker et al. 2004). The question then becomes whether there is anything within the genomes or ecology of these species that sufficiently distinguishes them, or if they should all be considered the same. This has led to some disagreement over the identities and relatedness of *B. cereus* group species (Liu, Lai et al. 2015). However, conflating *B. cereus* and *thuringiensis* has important implications for food safety

regulation because *B. thuringiensis* is in regular use on food crops while many strains of *B. cereus* can cause food poisoning, with those carrying cereulide toxin plasmids potentially being the most severe (Dierick, Van Coillie et al. 2005, Arnesen, Fagerlund et al. 2008).

What defines a species is not only a concern within *Bacillus*, but throughout the prokaryote phyla (Rossello-Mora 2003). In eukaryotes Mayr's biological species concept is sufficiently robust to demark species and is based on there being barriers to genetic exchange between organisms in different species (Mayr 1944, Cohan 2002). This was constructed with sexual reproduction in mind, but can be adapted to fit a hypothetical bacterial species. In a fully clonal population any single lineage could be construed as a species as there is complete reproductive isolation. However, the increasing volume of sequence data has made apparent that the majority of bacteria do exchange genetic material, whether directly or not (Ochman, Lawrence et al. 2000, Ochman, Lerat et al. 2005). This gives a way of labelling bacterial species in line with the biological concept, as one could argue that bacteria can only exchange genetic material with sufficiently close relatives i.e. within the same species. The *B. cereus* group shows reasonably high levels of recombination – more than Gram-positive *Staphylococcus aureus*, although recombination contributes less genetic change than point mutations but has a greater effect on the genome (Didelot, Barker et al. 2009). These recombination events appear to be mainly intra-clade with incorporation of material from outside the group rarer except in clade 1 (Didelot, Barker et al. 2009). Sequence data also show that genetic distance between exchangers can vary greatly across bacteria (Didelot and Maiden 2010), so this cannot be a universal method for splitting bacterial species as is.

In general, bacterial species are now defined on overall genetic similarity. This started with DNA-DNA hybridisation in the 1960s, with >70% similarity placing isolates in the same species (Richter and Rossello-Mora 2009, Peak, Duncan et al. 2011). More efficient modern techniques have used comparisons *in silico*, focusing on the similarity of single, highly conserved genes, such as 16S rRNA (>97%) (Cohan 2002) or comparing across whole genomes via average nucleotide identity (ANI; with >95% corresponding to the >70% required by DNA-DNA hybridisation) (Richter and Rossello-Mora 2009, Chan, Halachev

et al. 2012). Sticking rigidly to these barriers may not be a viable solution either – returning to *Bacillus*, the currently defined species within the *B. cereus* group cannot always be separated along these lines. Early sequencing efforts suggested that together they share at least 99% 16S rRNA sequence similarity (Ash, Farrow et al. 1991, Bavykin, Mikhailovich et al. 2008) which would group them together. However, subsequent efforts using the more comprehensive techniques like ANI give sufficient separation between *B. cereus* group clades for them to be maintained as distinct species: <92% ANI between isolates of clades 1 and 2, but >95% within clade 2 (Zheng, Gao et al. 2017).

The methodologies using a wider amount of sequence information agree on their revised broad phylogenetic structure of the *B. cereus* species group (figure 1.1): *B. anthracis* strains fall neatly into their own clonal grouping, as an offshoot of a larger *B. cereus sensu stricto*-heavy clade (clade 1 or 'anthracis' group); this leaves a clade containing the majority of *B. thuringiensis* isolates (clade 2 or 'thuringiensis' group) and a primarily *B. weihenstephanensis* clade (clade 3 or 'weihenstephanensis') (Priest, Barker et al. 2004, Didelot, Barker et al. 2009, Zheng, Gao et al. 2017). Note that these three clades are sexually isolated to a degree (Didelot, Barker et al. 2009). The main difficulty principally comes from some strains (as currently labelled) not falling into the appropriate/expected clades e.g., some *B. thuringiensis* strains group with the majority of *B. cereus* strains in clade 1 (figure 1.1) (Didelot, Barker et al. 2009). This disorder has partly led to safety concerns being raised about *B. thuringiensis,* both in the past (Bavykin, Mikhailovich et al. 2008) and recently by the European Food Safety Authority (EFSA 2016). This is primarily because some Cry toxin-possessing strains in clade 1 are closely related to enteric *B. cereus* strains that can cause emetic food poisoning and may themselves be capable of causing enteric diarrhoea (EFSA 2016), while strains carrying Cry toxins isolated from burn wound infections and bacteraemia have also raised concerns (Damgaard, Granum et al. 1997, Han, Xie et al. 2006, Kuroki, Kawakami et al. 2009). Returning to sequence-based phylogeny of the *B. cereus* group, these fears appear misguided as all the highly insecticidal strains of *B. thuringiensis* (i.e. the ones used for agricultural pest control) are found in clade 2, making them genetically distant from the potentially troubling strains mentioned above and from *B. anthracis* (Han, Xie et al. 2006).

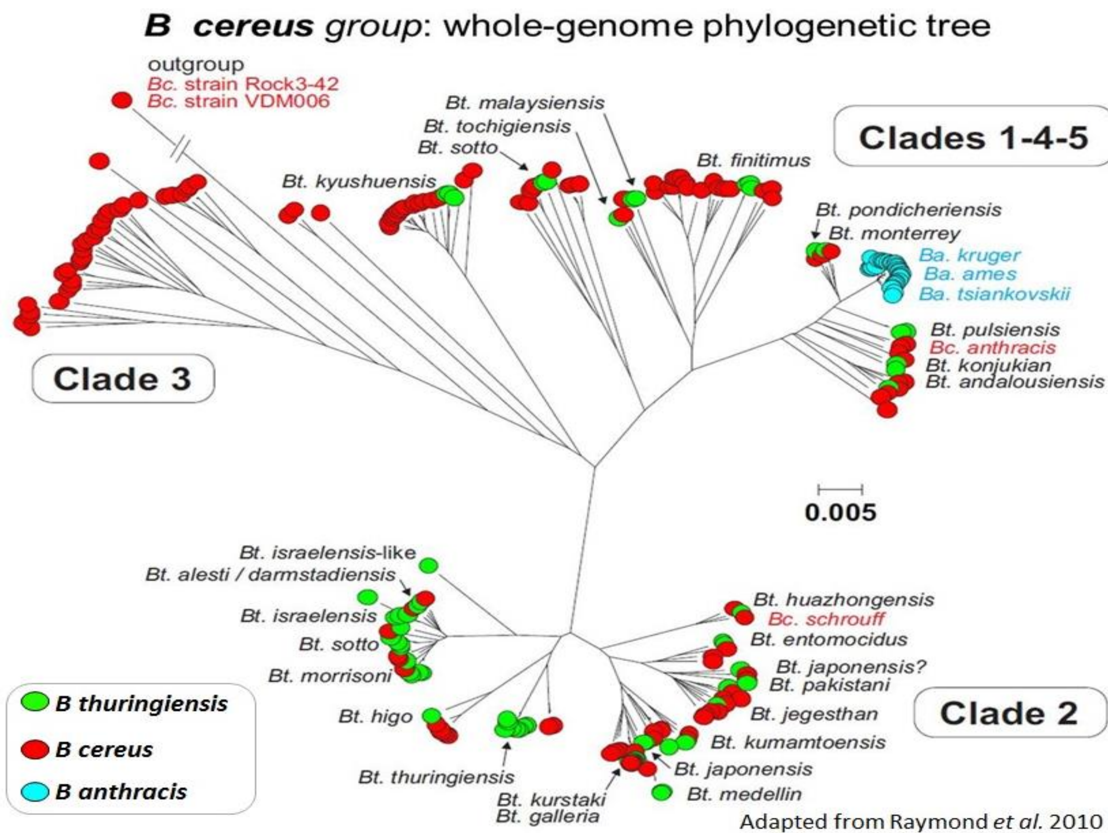

Figure 1.1: Clade relationships within the *B. cereus* species group. *B. thuringiensis* strains are highlighted in green, showing that while the majority are in clade 2, some are found in clade 1 (the anthracis group).

As an alternative to the strict divisions expected by the biological species concept and the problems distinct names appear to have caused, Cohan proposed the idea of 'ecotypes' for prokaryotes (Cohan 2002). These ecotypes consist of populations of bacteria that, while they might not meet DNA-DNA hybridisation requirements, do occupy distinct niches. Even if the clades within the *B. cereus* group were not sufficiently far enough apart, then their different niches (e.g., *B. thuringiensis* being a specialised insect pathogen) should be enough to separate them as distinct ecotypes. Others have also proposed that phenotype still inform species when hybridisation limits are not fully met (Logan, Berge et al. 2009). However, the status of *B. thuringiensis* as an obligate pathogen has also been questioned in the past (Broderick, Raffa et al. 2006) – in part from supposed lack of differentiation from *B. cereus* (Helgason, Okstad et al. 2000). However, as mentioned improved sequence comparison techniques across more data are now providing clearer support for distinct

clades (Priest, Barker et al. 2004, Didelot, Barker et al. 2009, Raymond, Wyres et al. 2010). Any genetic differences between clades that do exist should be as a result of the requirements of their different ecologies, as only around 80% of chromosomal genes occur in both (Hendriksen 2016).

Whether or not an ecotype-based solution is appropriate to solve the classification of *B. thuringiensis* and *B. cereus*, there is still disagreement over what constitutes the principal niche of *B. cereus*. If *B. cereus* can be found in a wide variety of possible niches (pathogen, commensal, symbiote, saprophyte or necrotroph) (Jensen, Hansen et al. 2003, Hendriksen 2016), then the entomocidal role of *B. thuringiensis* could more easily be seen as just another niche. In this instance *B. thuringiensis* would only be another ecotype and possibly no more worthy of individual species designation than any other. Genome comparisons show a distinct clade 2 containing the majority (and most highly insecticidal) of the *B. thuringiensis* strains (Zheng, Gao et al. 2017) while other clades in the *B. cereus* group can be broadly linked to particular habitats based on their most likely source of isolation (Raymond, Wyres et al. 2010). While many of the *B. cereus* strains in clade 1 carry genes permitting a pathogenic lifestyle (such as emetic cereulides) and can be isolated from infections (Ehling-Schulz, Fricker et al. 2004, Arnesen, Fagerlund et al. 2008), the majority of other *B. cereus* group strains are isolated from soil (Raymond, Wyres et al. 2010). Following this there is growing evidence that the source of isolation will predict the clade of the strain. It could be that human pathogenic strains of *B. cereus* are on the first steps of the path taken by *B. thuringiensis* and *B. anthracis* which is supported by the clonal grouping of most emetic strains (Didelot, Barker et al. 2009). Association with a particular virulence factor opens up a pathogenic niche in a host and over time specialisation leads to a distinct pathogenic clade (Daffonchio, Raddadi et al. 2006) – the highly clonal nature of *B. anthracis* might suggest that it is less far down this route than clade 2 *B. thuringiensis* (Priest, Barker et al. 2004). Loguercio and Argolo-Filho (2015) suggest that human action, through the proliferation of agriculture and associated crops, may have favoured the emergence of entomocidal *B. thuringiensis*.

B. thuringiensis can be easily isolated from soil (Nicholson 2002), which could support it being able to grow there and has previously been raised as a

point against its status as an obligate pathogen. However, this overlooks a defining feature of *Bacillus* – they can form highly resistant endospores (Alcaraz, Moreno-Hagelsieb et al. 2010, Liu, Lai et al. 2015). It is therefore unsurprising that *B. thuringiensis* can be isolated from soil even if it does not proliferate there; this presents another aspect of its ecology that could be explored. *B. thuringiensis* in the soil needs to be ingested by an insect to successfully reproduce, but any host is most likely herbivorous as most are lepidopteran or coleopteran larvae. It would therefore be highly advantageous to *B. thuringiensis* if it could be selectively recruited from soil to leaves via plants. In turn this scenario could be selected for in plants as it would provide an additional defence against insect herbivory and the potential for some level of symbiosis (Smith and Couche 1991, Elliot, Sabelis et al. 2000, Aronson and Shai 2001). Comparisons between B*. thuringiensis*, *B. cereus* and *B. weihenstephanensis* in greenhouse experiments with cabbage have shown that *B. thuringiensis* is selectively recruited to leaves at a higher rate than the others, though all three can be recruited (Raymond, Wyres et al. 2010).

If *B. thuringiensis* is a specialised insect pathogen then it should also possess a suite of genes that better facilitate this lifestyle, compared to *B. cereus*. The obvious example would be the Cry toxins, along with other virulence factors such as vegetative insecticidal proteins. However, these characteristics contributed to the existing classification knot because of their propensity for being plasmid-borne and so potentially lost or transferred through the group (Ceuppens, Boon et al. 2013, Liu, Lai et al. 2015). Cry toxins break down the midgut epithelium allowing bacteria to enter the haemocoel. There must therefore be other genes that have been selected to allow/increase vegetative growth in the haemolymph, through sequestering and processing the available metabolites while also defending against or avoiding the host immune system (Fedhila, Daou et al. 2006) e.g., genes involved in kurstakin production, a peptide required for cadaver growth (Dubois, Faegri et al. 2012). Conversely, strains currently labelled *B. thuringiensis* in clade 1 may have only recently acquired their Cry toxins, with the rest of their genes the same as in sister *B. cereus* strains. This would means they are less able to grow in an insect host.

A previous argument against possession of any insect-specific gene suite was that much of the mortality from *B. thuringiensis* infections was actually

a result of other gut bacteria becoming pathogenic once Cry toxins allowed access to the haemolymph (Broderick, Raffa et al. 2006). This was purportedly shown by feeding gypsy moth (*Lymantria dispar*) larvae an antibiotic mixture to clear the gut and then unsuccessfully producing mortality with *B. thuringiensis* (Broderick, Raffa et al. 2006). However, this ignored the fact that antibiotics persist in larval tissue after feeding, preventing subsequent *B. thuringiensis* growth and inhibiting pathogenicity (Raymond, Johnston et al. 2009); *B. thuringiensis* is also capable of producing septicaemia alone after direct injection into the haemocoel (Johnston and Crickmore 2009). If Cry toxins were the sole contributors to *B. thuringiensis* pathogenicity this might instead suggest that *B. thuringiensis* would do as well when grown in non-insect conditions more common to the rest of the *B. cereus* species group (Hendriksen 2016).

Adaptation to within-insect growth can be explored experimentally by comparing *in vivo* growth between clade 1 and 2 strains, with the expectation that clade 2 strains will be fitter. However, this is complicated by the fact that different strains will potentially have different Cry toxin suites – any apparent fitness differences might only be a result of whether or not an infection can be established, rather than actual differences in haemolymph growth. This can be mitigated by carrying out all infections alongside a common competitor *B. thuringiensis* strain that has highly effective toxins against the host (diamondback moth, *Plutella xylostella*). Doing so ensures that all treatment strains can successfully access the haemolymph and means that relative fitness of strains can be used to assess their adaptation to growth in insect hosts. The degree of host specialisation should go hand in hand with different temperature preferences across the *B. cereus* species group. If *B. thuringiensis* is an insect pathogen, *B. cereus* primarily necromenic and *B. weihenstephanensis* is soil-based then different temperature growth profiles should be expected for each clade. Guinebretière, Thompson et al. (2008) have already argued that “modification of temperature tolerance” has helped shape the evolution of the *B. cereus* species group here it is tied to further ecological specialisation.

Methodology

B. cereus growth comparisons across potential niche-simulating media

Three *B. cereus*, five Cry-null *B. thuringiensis* and seven *B. weihenstephanensis* strains (table 1.a) were streaked from -80°C stocks, single colonies picked and grown overnight in 5ml 2$^{\%}$ LB at 30°C with 150rpm shaking. The *B. thuringiensis* strains used were Cry-null strains to give a fair growth comparison with *B. cereus* and *B. weihenstephanensis* strains that do not have the potential metabolic handicap of toxin production. Overnight cultures were then diluted to 10^{-3} in saline (0.85$^{\%}$ NaCl), and 10µl added to 200µl of growth medium in three wells of a 96-well plate for each strain, with well order randomised. Six different media were used, with one plate for each. Plates were incubated in a plate reader (Thermo Fisher Scientific Inc. Varioskan Flash) at 26°C for 24 hours with OD_{600} readings taken every 2mins with shaking before each reading.

***Bacillus* species**	**Strain / isolate**	**Source**
B. cereus	*B. cereus* type-strain	ATCC 11778
B. cereus	(wild-type isolate)	BGSC 6A4
B. cereus	enterotoxic	NCTC 11145
B. thuringiensis	*israelensis* Cry-	BGSC 4Q7
B. thuringiensis	*sotto* Cry-	BGSC 4E5
B. thuringiensis	*kurstaki* Cry-	BGSC 4D7
B. thuringiensis	*thuringiensis* Cry-	BGSC 4A12
B. thuringiensis	*kurstaki* Cry-	BGSC 4D22
B. weihenstephanensis	ST 353	Raymond
B. weihenstephanensis	ST 417	Raymond
B. weihenstephanensis	(wild-type isolate)	BGSC 6A50
B. weihenstephanensis	(wild-type isolate)	BGSC 6A21
B. weihenstephanensis	*vazensis*	BGSC 4CE1
B. weihenstephanensis	*navarrensis*	BGSC 4BM1
B. weihenstephanensis	(wild-type isolate)	BGSC 6A46

Table 1.a: *Bacillus* species and strains used in media growth experiment. *B. weihenstephanensis* strains *vazensis* and *navarrensis* are listed as *B. thuringiensis* on the BGSC but should be reclassified (Soufiane and Cote 2010); non-catalogued strains from Raymond, Wyres et al. (2010)

Brain heart infusion (BHI; Honeywell International Inc. Fluka) was used as it is a protein rich medium and so represents a controlled artificial simulation of cadaver. SESOM (soil-extracted soluble organic matter) is a soil-simulating medium (Vilain, Luo et al. 2006) while TREM (tomato root extract medium) artificially replicates the nutrients around plant roots (Meyer and Abdallah 1978, Lugtenberg, Kravchenko et al. 1999). SESOM and TREM were produced in both base and sugar-enriched forms, and a control version of SESOM was also used with the same buffer and sugar enrichment, but no soil – MOPS (3-(N-morpholino)propanesulfonic acid) (Vilain, Luo et al. 2006). These media were used as artificial representations of the three proposed niches for *B. cereus*: cadavers, the soil and plant roots, with V_{max} and productivity calculated from the resulting 24 hour growth data. MOPS medium was used to contrast with SESOM media, as the only difference was mixing with soil in SESOM. The MOPS, SESOM and TREM media were produced as follows:

- **MOPS enriched:** 100mg glucose, 1mg casein hydrolysate and 1mg yeast extract added to 500ml of 10mM pH7 3-(N-morpholino) propanesulfonic acid
- **SESOM base:** 100g commercial loam-based topsoil (Asda Stores Ltd.) mixed in 500ml of 10mM pH7 MOPS via shaking for one hour at 200rpm and filter sterilised with 0.2µm filters
- **SESOM enriched:** 100mg glucose, 1mg casein hydrolysate and 1mg yeast extract added to 1l SESOM base
- **TREM base:** 6g K_2HPO_4, 3g KH_2PO_4 and 1g $(NH_4)_2SO_4$ dissolved in 1l H2O and autoclaved, then 1ml of filter-sterilised 20$^{\%}$ $MgSO_4$ added followed by 2.5ml of filter-sterilised stock trace elements solution (0.61g $MnSO_4$, 0.1g $ZnSO_4.7H_2O$, 1.27g H_3BO_3, 0.4g $Na_2MoO_4.2H_2O$, 0.04g $CuSO_4$ and 13.5g Fe_2 EDTA in 1l H_2O)
- **TREM enriched:** 50ml of filter-sterilised “root exudate” solution (1g citric acid, 0.5g succinic acid, 0.02g malic acid, 0.01g glucose, 0.02g fructose, 0.02g thiamine and 0.02g biotin in 1l H_2O) added to TREM base

Production of *B. thuringiensis* spores and marked strains

A marked *B. thuringiensis* clone was produced for use as the standard competitor in all competition experiments. *B. thuringiensis* subsp. *kurstaki* 7.1.o was transformed with the plasmid pHT315 carrying *DsRed* and *tetR* for tetracycline resistance (Zhou, Slamti et al. 2014). To produce electrocompetent cells for transformation the strain was cultured overnight at 30°C with 150rpm shaking in 2% LB broth. 5ml of overnight culture was then added to 500ml pre-warmed 2% LB in a conical flask and incubated for four hours at 30°C with 150rpm shaking. After four hours, 67ml of 50% glycine was added to give 3% in the culture, and the culture incubated for an additional hour. Culture was transferred to eight 50ml pre-chilled Falcon tubes and these left on ice for 5mins. Tubes were centrifuged at 3,200*g* and 4°C for 12mins. Tubes were put on ice while supernatant was poured off. Cell pellets were gently re-suspended with 10ml ice-cold buffer 'F' (272mM sucrose, 0.5 mM $MgCl_2$, 0.5mM K_2HPO_4 and 0.5mM KH_2PO_4 at pH 7.2) (Peng, Luo et al. 2009). This was repeated two further times, with final resuspension in 500µl of buffer 'F' and transferred to a pre-chilled Eppendorf. 100µl of electrocompetent cells were added to a pre-chilled 0.1cm^3 cuvette with 2µl plasmid DNA and electroporated at 1.8kV (MicroPulser, Bio-Rad Laboratories Inc.). The cells were then transferred to 1ml of pre-warmed 2% LB and incubated at 30°C for one hour. After one hour, 100µl of culture was spread onto a 10µg/ml tetracycline 2% LB agar plate and 900µl onto another and incubated at 30°C overnight. Colonies produced were re-streaked on tetracycline plates and viewed under UV light to confirm plasmid uptake, and the resulting strain named *Btk* pHT315-*DsRed-tetR*.

B. thuringiensis strains from clade 2 (*n* = 11) and clade 1 (*n* = 6) (table 1.b) were assessed in thermal profiling and competition experiment. To produce spores each strain was cultured on *B. cereus*-selective agar (Oxoid Ltd.) at 30°C for six days. After this, spores for each strain were suspended in 700µl saline, centrifuged at 6,200*g* for 2mins and then the supernatant removed. This was repeated three times then spores were re-suspended in 1ml saline with 0.05% Triton X surfactant. All dilutions were carried out in saline with Triton surfactant, unless stated otherwise.

Clade	Strain / isolate	Source
1	*finitimus*	BGSC 4B2
1	*kyushuensis*	BGSC 4U1
1	*monterrey*	BGSC 4AJ1
1	*pulsiensis*	BGSC 4CC1
1	*roskildiensis*	BGSC 4BG1
1	*sotto*	NRRL B-18679
2	*alesti*	BGSC 4C3
2	*darmstadiensis*	BGSC 4M3
2	*entomocidus*	BGSC 4I4
2	*israelensis*	BGSC 4Q1
2	*jegathesan*	NRRL B-23141
2	*kumamtoensis*	BGSC 4W1
2	*kurstaki* 7.1.o	Raymond
2	*morrisoni*	BGSC 4AA1
2	*sotto*	BGSC 4E3
2	*tenebrionis*	BGSC 4AA1
2	*thuringiensis*	BGSC 4A3
2	*thuringiensis*	BGSC 4A5

Table 1.b: *Bacillus thuringiensis* subsp. used in competition temperature growth experiments. Strains are split by clade based on analyses in Meric, Mageiros et al. (2018); *kurstaki* 7.1.o from Raymond, Wyres et al. (2010).

Thermal profiling

The growth rates of the 17 strains were assessed across a temperature range. The strains were grown overnight in 5ml $2^{\%}$ LB at 30°C with 150rpm shaking and then diluted 1,000-fold. 10µl of each strain culture was added to three wells of 200µl $2^{\%}$ LB in a 96-well plate with wells randomly ordered on each plate for 10 plates. Preliminary work in just LB showed that strains would not grow at 50°C and had limited growth at 10°C. OD_{600} readings were taken every 2mins and plates shaken before each reading. Plates were grown for 24 hours at 14, 17, 20, 23, 26, 29, 32, 35 and 38°C in both $2^{\%}$ LB and $2^{\%}$ BHI with OD_{600} readings taken every 2mins and plates shaken before each reading. V_{max} and productivity were calculated from the resulting growth curve data.

B. thuringiensis competition experiments

Spore preparations were enumerated by diluting, plating and counting on 2% LB agar plates after overnight incubation at 30°C. Spores were diluted to a final concentration of 600cfu/µl in sterile H_2O. *Btk* pHT315-*DsRed-tetR* was used as a standard competitor and to ensure that bacteria can pass into the haemocoel of larvae even if the treatment strain lacked effective toxins for the experimental host. *Btk* pHT315-*DsRed-tetR* spore suspensions were diluted to 200cfu/µl. Final suspensions were re-checked before mixing to produce a 300:100 ratio of experimental strains to *Btk* pHT315-*DsRed-tetR*.

In vivo competition

Plutella xylostella larvae (VLSS population – Cry1Ac-susceptible) were raised from surface-sterilised eggs on sterile insect diet until late second-instar (Zhou, Alphey et al. 2018). Eggs were sterilised for 30s in 10% bleach solution, then drained and twice washed with sterile H_2O, then dried for 15mins in a laminar flow hood. Diet was 90g of dry mix (44.3g wheatgerm, 18.4g casein, 0.1g inositol, 1.5g sodium propionate, 5.9g Wesson salts, 1.5g sorbic acid, 0.9g cholesterol, 1.5g methyl-4-hydroxybenzoate, 0.3g choline chloride, 3g locust bean gum, 1.6 cellulose, 1.6g fructose and 9.4g agar) with 420ml demineralised water. After autoclaving, 2g of Vanderzant vitamin mix and 1.8g ascorbic acid were each dissolved in 30ml sterile H_2O, filter-sterilised and added to cooled autoclaved diet. Diet was poured into 12oz plastic pots (Vegware Ltd.) to 15mm depth for insect rearing or 50mm Petris to 5mm depth for experiments and allowed to set in a laminar flow hood. Quarters of diet in eight 50mm Petri dishes were coated with 100µl of a competition treatment and dried in a laminar flow hood. Ten late second-instar larvae were added to each Petri for 80 per treatment. The Petri dishes were then incubated at 22°C for five days. After, 48 cadavers from each treatment were transferred to sterile 1ml microtubes and 10µl of sterile H_2O added to each. The microtubes were sealed and incubated at 30°C for seven days to ensure sporulation. 690µl sterile H_2O and sterile 5mm steel ball bearings were then added to microtubes to lyse cadavers via a tissue lyser (Qiagen N.V.), shaking at 1,350rpm for 4.5mins. The tubes were then pasteurised in a 65°C water bath for 25mins and afterwards diluted in 10-

fold series to 10^{-6}, and 10^{-3} to 10^{-6} dilutions plated out on plain and 10μg/ml tetracycline $2^{\%}$ LB agar plates for colony counts.

In vitro competition

These used the same 3:1 treatment mixtures as the *in vivo* competitions. Here, 10μl of each mixture was added to eight wells of 1ml HCO sporulation medium in 24-well plates. HCO sporulation medium was adapted from Lecadet, Blondel et al. (1980) and made by adding 7g casein hydrolysate and 6.8g KH_2PO_4 to 1l demineralised H_2O, adjusting pH to 7.2 with 2M NaOH and autoclaving. After cooling, 1ml $20^{\%}$ $MgSO_4.7H_2O$, 1ml $2^{\%}$ $Fe_2(SO_4)_3$, 2.2ml $1^{\%}$ $ZnSO_4.H_2O$, 2.2ml $1^{\%}$ $CaCl_2.2H_2O$, 440μl $0.5^{\%}$ $MnSO_4.4H_2O$, 15ml of $20^{\%}$ glucose (from sterile stocks) and two tablets of Polymyxin B were added (50,000 IU per tablet, Oxoid Ltd.). The plates were sealed and incubated at 30°C with 150rpm shaking for four days to ensure sporulation. Spores were then enumerated by diluting plating and culture plating on LB agar without antibiotics and on plates containing 10μg/ml tetracycline.

Statistical analysis

All bacterial growth experiment (thermal profile and media comparisons) were initially analysed in the same manner using RStudio 1.1.456 (based on R 3.2.5) (R Core Team 2016, RStudio Team 2016). OD_{600} data from 24 hour growth had growth curve models fitted individually for every plate well, using 4-parameter logistic models for growth (Crawley 2013). For wells that would not fit this (e.g., those with no/limited growth) a linear model was used. Fitted models provided V_{max} (the maximum growth rate) and productivity values for each well. Overall growth curve plots for each clade or species were produced by fitting secondary 4-parameter logistic models for all wells of a given set of conditions. V_{max} was the maximum slope of the fitted curve. Production was calculated by integrating the model and subtracting the initial media OD_{600} value; the initial media value was taken as the first fitted value. V_{max} and productivity were analysed via generalised linear models (GLMs).

For competitions, cfu/µl values were used to calculate Malthusian fitnesses for competitor and treatment strains and these were combined to produce relative fitness for the treatment strain in each competition as follows, then $\log_e$ transformed and analysed using GLMs:

$$\boldsymbol{Malthusian\ fitness = log_2 \frac{final\ cfu/\mu l}{inoculant\ cfu/\mu l}}$$

$$\boldsymbol{Relative\ fitness_a = \frac{Malthusian\ fitness_a}{Malthusian\ fitness_b}}$$

Results

B. cereus group growth comparisons across potential niche-simulating media (figure 1.2)

Whether different nominal species within the *B. cereus* species group would have enhanced growth (figure 1.3) or productivity (figure 1.4) on media that would reflect specialization on protein-rich resources (hosts or cadavers), plant-derived nutrients and soil-derived nutrients was tested. Comparison across media showed vastly enhanced growth rates and productivity in protein-rich BHI compared to soil nutrient (SESOM) and plant nutrient (TREM) artificial media. *B. cereus* showed significantly faster growth in the protein-rich BHI, but this was not reflected in the soil and root media. Both species and growth media had strong main effects on V_{max} ($F_{2, 629} = 29.3$, $p < 6.56x10^{-13}$; $F_{6, 629} = 497$ $p < 2x10^{-16}$). Additionally, the media treatment used could be simplified down to either BHI or not without losing any explanatory power. There was also a significant interaction effect between species and whether or not the growth medium was BHI ($F_{2, 644} = 45.6$, $p < 2x10^{-16}$) and such an interaction implies that there is some degree of specialisation in the species for or against particular media, with *B. cereus* more strongly favouring BHI. For productivity, only whether or not the medium was BHI had a significant effect ($F_{1, 648} = 2940$, $p < 2x10^{-16}$), with no interactions being significant.

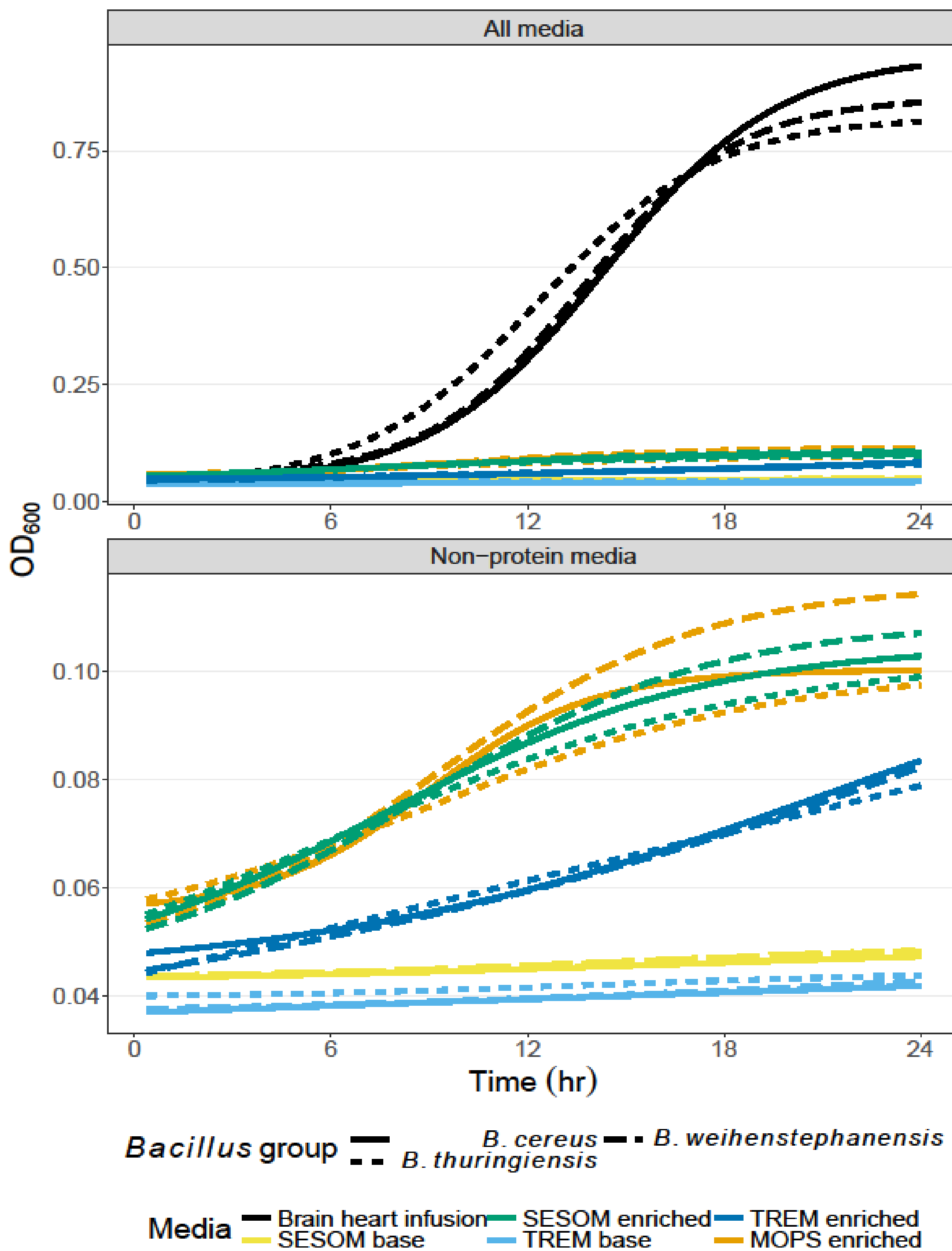

Figure 1.2: OD_{600} 24 hour growth of of *B. cereus*, *B. thuringiensis* and *B. weihenstephanensis* strains (table 1.a) in protein-based (BHI), soil-simulating (SESOM) and plant root exudate-simulating media (TREM). The lower graph excludes BHI protein media to clarify differences between the others.

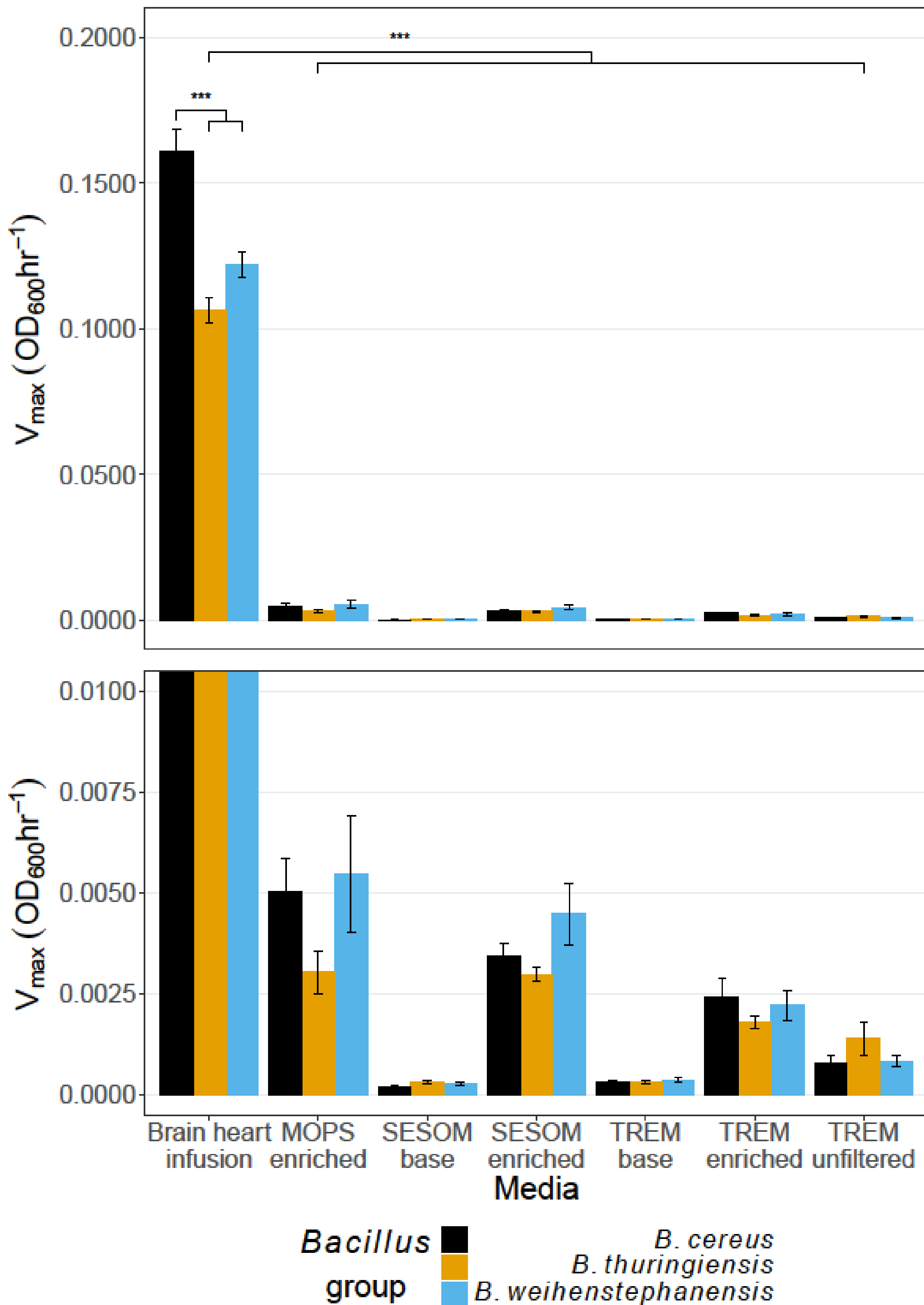

Figure 1.3: V_{max} growth rate (OD_{600} increase per hour) of *B. cereus*, *B. thuringiensis* and *B. weihenstephanensis* strains (table 1.a) in protein-based media (BHI), soil-simulating media (SESOM) and plant root exudate-simulating media (TREM). The lower graph shows the same data magnified. NS = not significant, *** = $p < 0.001$.

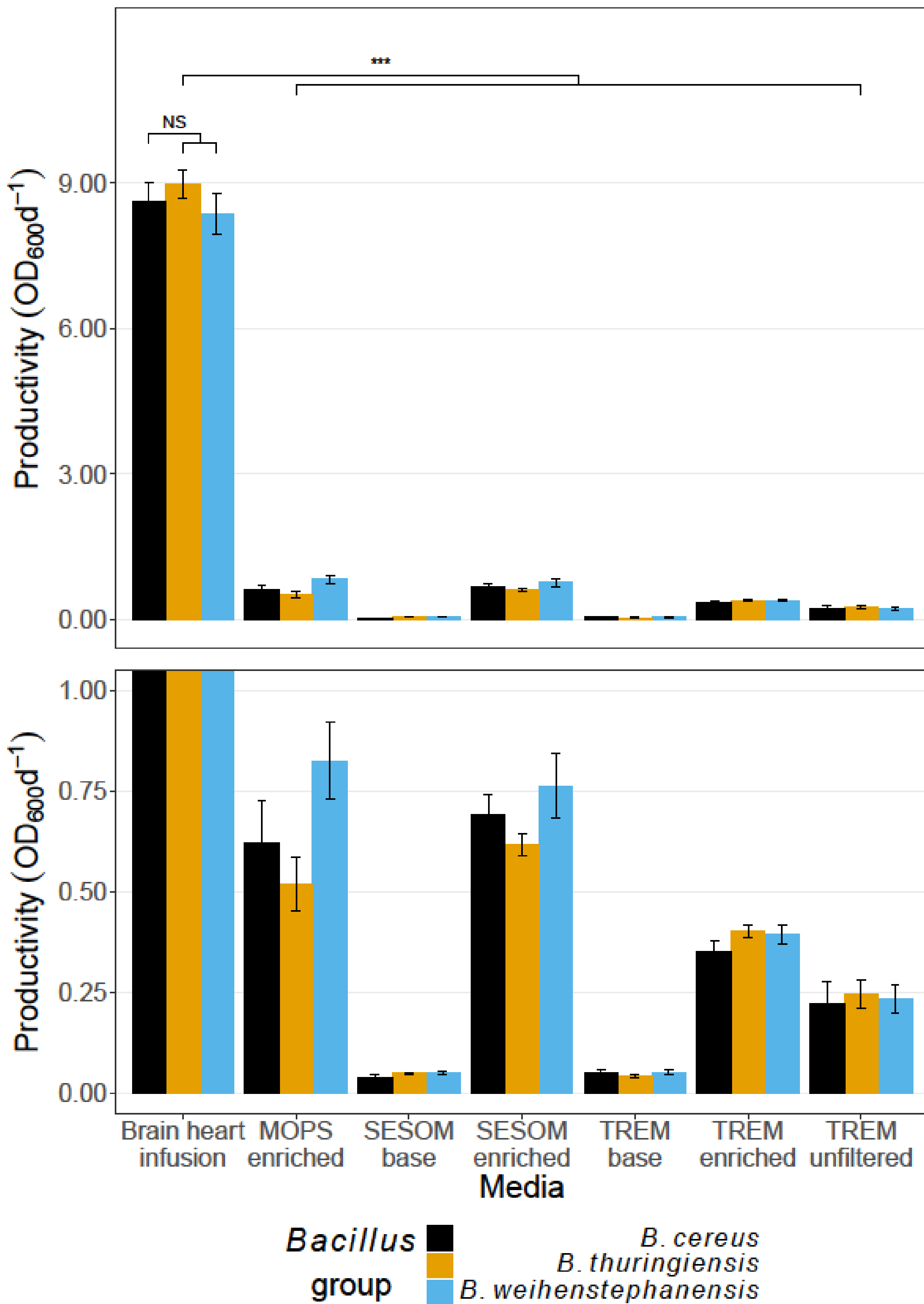

Figure 1.4: Productivity (cumulative OD_{600} over 24 hours) of *B. cereus*, *B. thuringiensis* and *B. weihenstephanensis* strains (table 1.a) in protein-based media (BHI), soil-simulating media (SESOM) and plant root exudate-simulating media (TREM). The lower graph shows the same data magnified. NS = not significant, *** = $p < 0.001$.

Thermal profiling of *B. thuringiensis* clades (figure 1.5)

The two clades of *B. thuringiensis* show distinct growth preferences across the temperature range and two growth media used. The growth experiment in LB and BHI at 14 to 38°C with 3°C intervals was analysed fully by fitting models at the clade level in each medium (tables 1.c and 1.d).

For productivity (figure 1.6, table 1.c) the effect of temperature on each clade could be fitted for graphs using a cubic, and a general additive model with 6 d.f. for smoothing was used to compare the two clades in each medium. In both media, clade 2 had lower productivity than clade 1 (BHI: $F_{1, 532} = 7.53$, $p = 6.27 \times 10^{-3}$; LB: $F_{1, 529} = 24.4$, $p = 1.07 \times 10^{-6}$), as can be seen in figure 1.6 from the lower curves and data points of clade 2.

For V_{max} (figure 1.7, table 1.d) only linear models provided an accurate fit for each clade, though when compared they showed that V_{max} was not affected by clade in either media (BHI: $F_{1, 536} = 2.69$, $p = 0.102$; LB: $F_{1, 533} = 2.10$, $p = 0.148$). However, there was an interaction between clade and temperature in LB ($F_{1, 533} = 31.2$, $p = 3.64 \times 10^{-8}$), which reflects the alternate slope gradient signs for the two clades. The negative relationship between temperature and V_{max} for clade 1 does not reflect the pattern seen in productivity; while linear models may fit the V_{max} data here, they will not reflect growth rates across larger temperature ranges, so appear a less good measure of growth than productivity here.

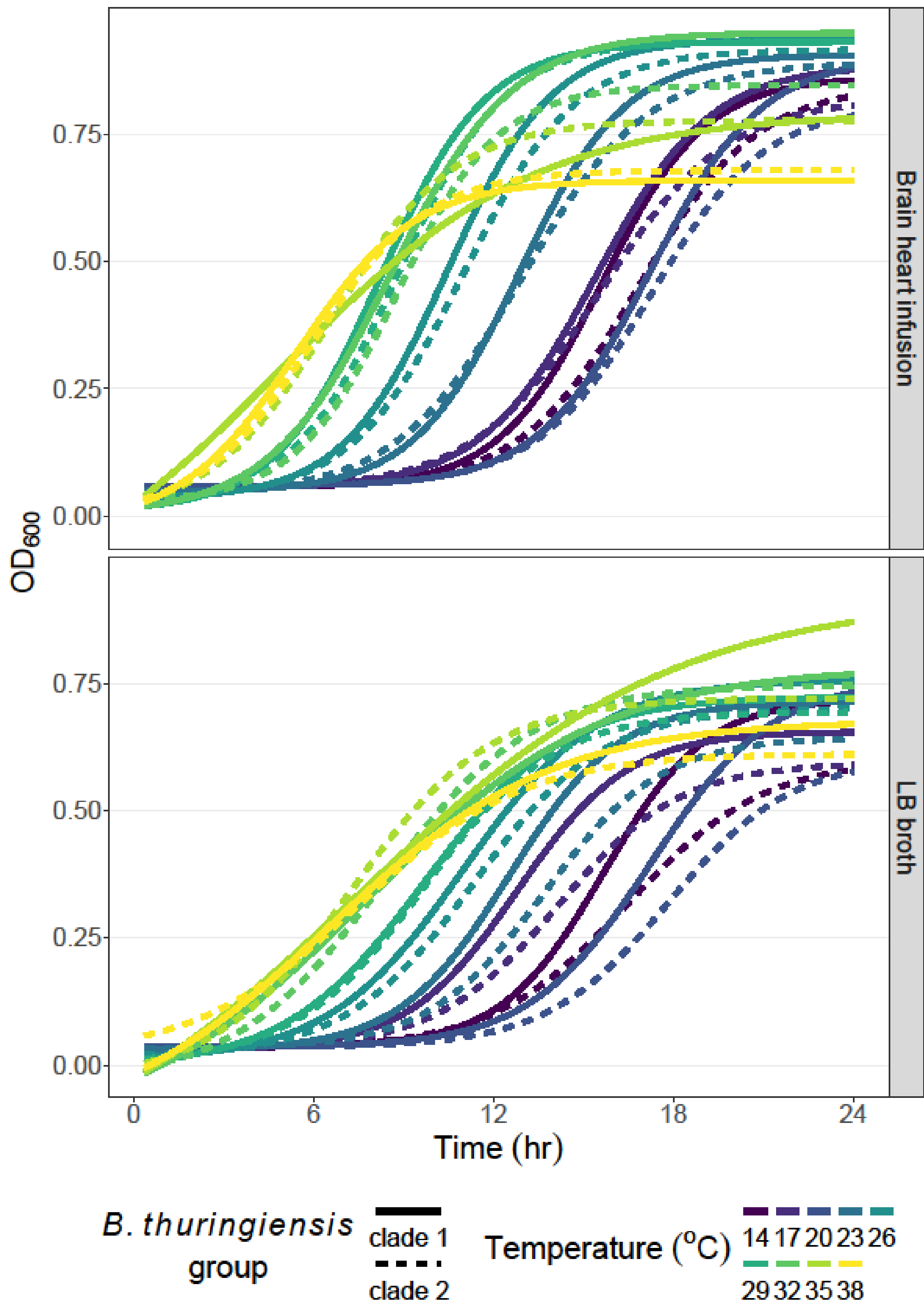

Figure 1.5: 24 hour growth in terms of OD_{600} of *B. thuringiensis* strains (table 1.b) across a temperature range, grouped by clade, in BHI and LB media.

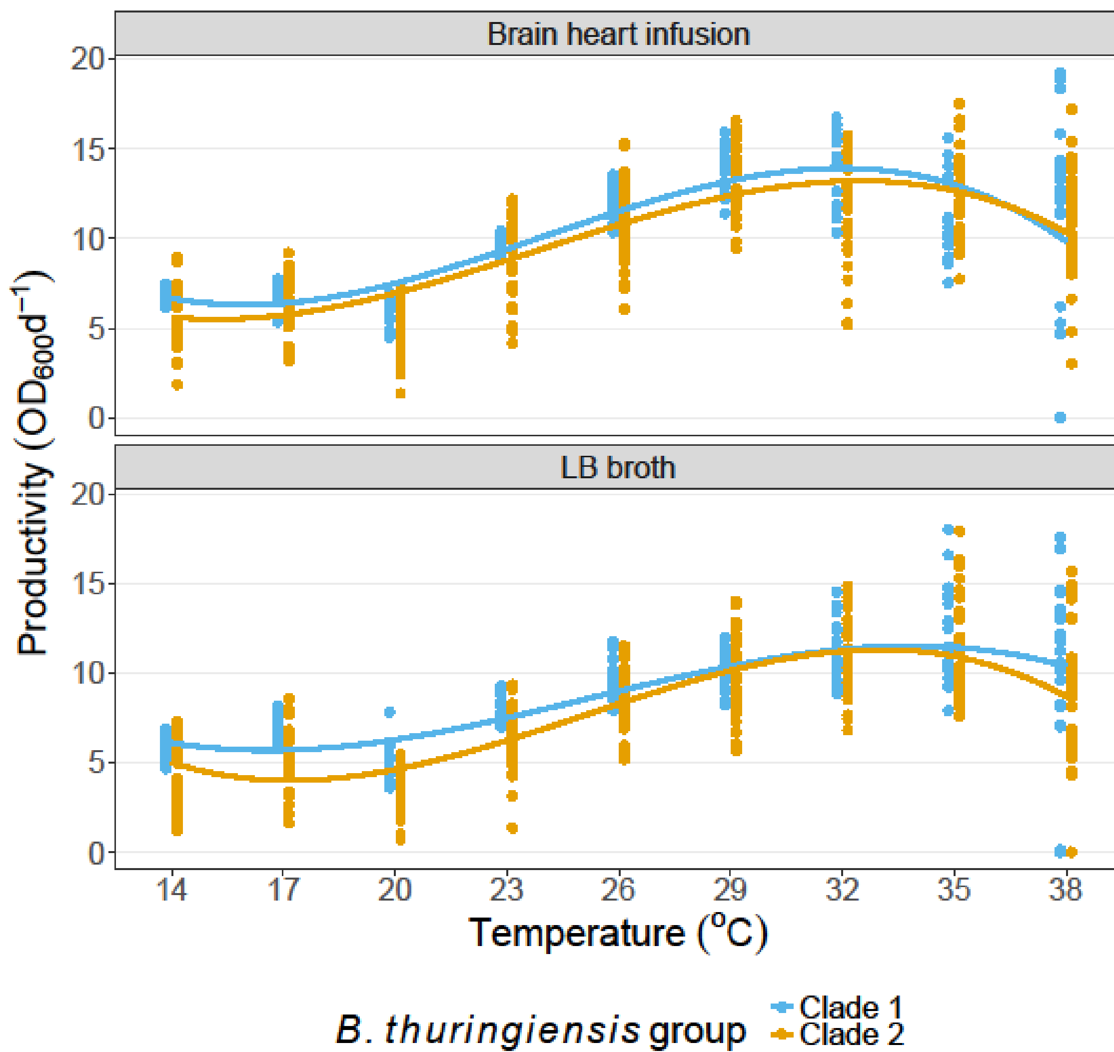

Figure 1.6: Productivity (in terms of cumulative OD_{600} over 24 hours) of *B. thuringiensis* strains (table 1.b) across a temperature range, grouped by clade, in BHI and LB media.

Media	Clade	Model	R^2	F	p
BHI	1	Cubic	0.547	74.4 (3, 185 d.f.)	$< 2.2 \times 10^{-16}$
BHI	2	Cubic	0.593	168 (3, 347 d.f.)	$< 2.2 \times 10^{-16}$
LB	1	Cubic	0.496	59.9 (3, 183 d.f.)	$< 2.2 \times 10^{-16}$
LB	2	Cubic	0.567	151 (3, 346 d.f.)	$< 2.2 \times 10^{-16}$

Table 1.c: Graph fits for *B. thuringiensis* clade 1 and 2 productivities across temperature, as shown in figure 1.6.

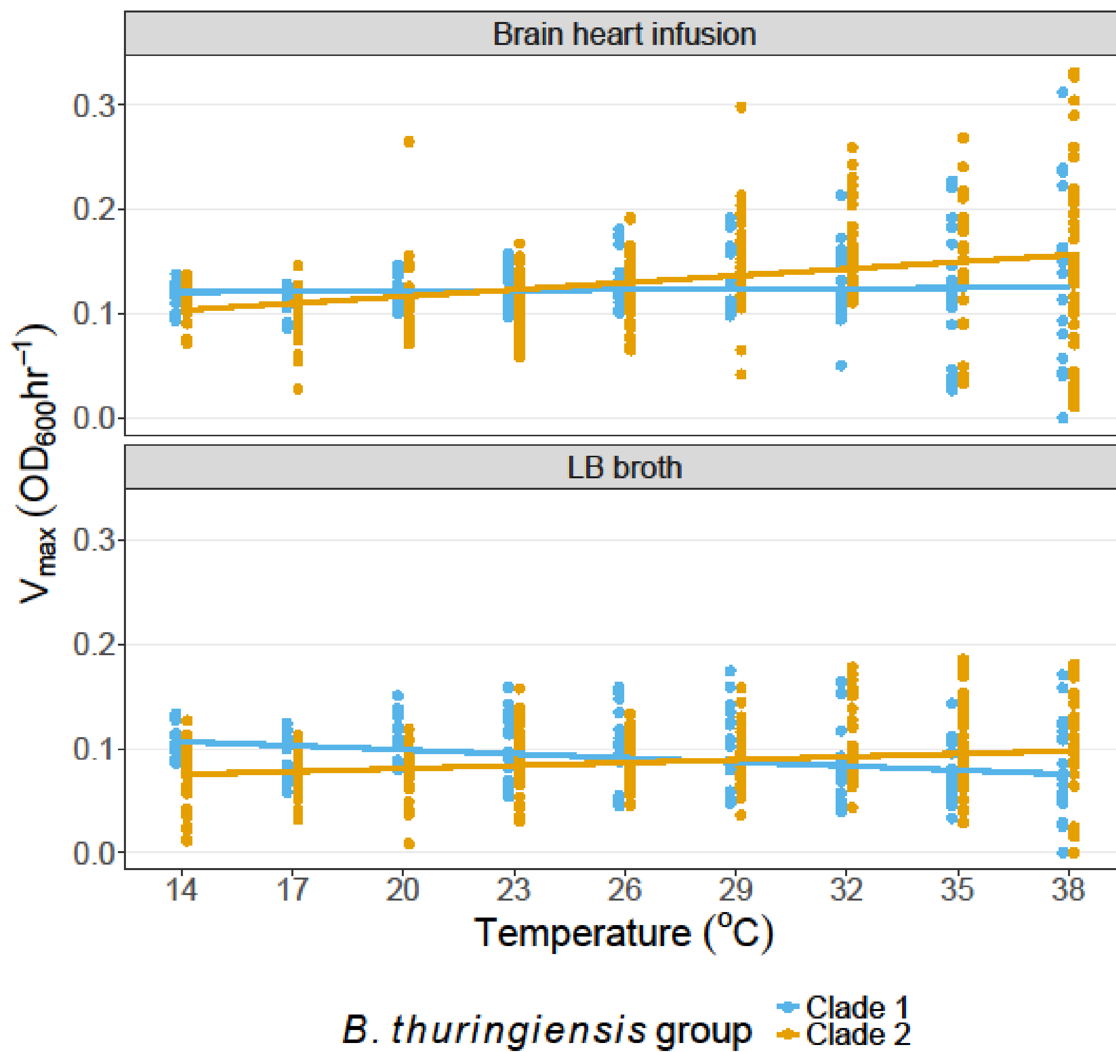

Figure 1.7: V_{max} growth rates (in terms of OD_{600} increase per hour) of *B. thuringiensis* strains (table 1.b) across temperature range, grouped by clade, in BHI and LB media.

Media	Clade	Model	R^2	F	p
BHI	1	Linear	0.001	0.192 (1, 187 d.f.)	0.662
BHI	2	Linear	0.119	47.2 (1, 349 d.f.)	3.02×10^{-11}
LB	1	Linear	0.085	17.3 (1, 185 d.f.)	5.00×10^{-5}
LB	2	Linear	0.042	15.2 (1, 348 d.f.)	1.14×10^{-4}

Table 1.d: Model fits for *B. thuringiensis* clade 1 and 2 V_{max} across temperature, as shown in figure 1.7.

B. thuringiensis in vivo competitions (figure 1.8)

All larvae died in every treatment. Colony counts on the 2% 10µg/ml tetracycline LB agar plates gave cfu/µl for the *Btk* pHT315-*DsRed-tetR* competitor. These values were subtracted from those obtained from plain 2% LB plates to give cfu/ml values for the clade 1 and 2 treatment strains, from which Malthusian and relative fitness were calculated. If a negative cfu/µl value was obtained this was taken as zero.

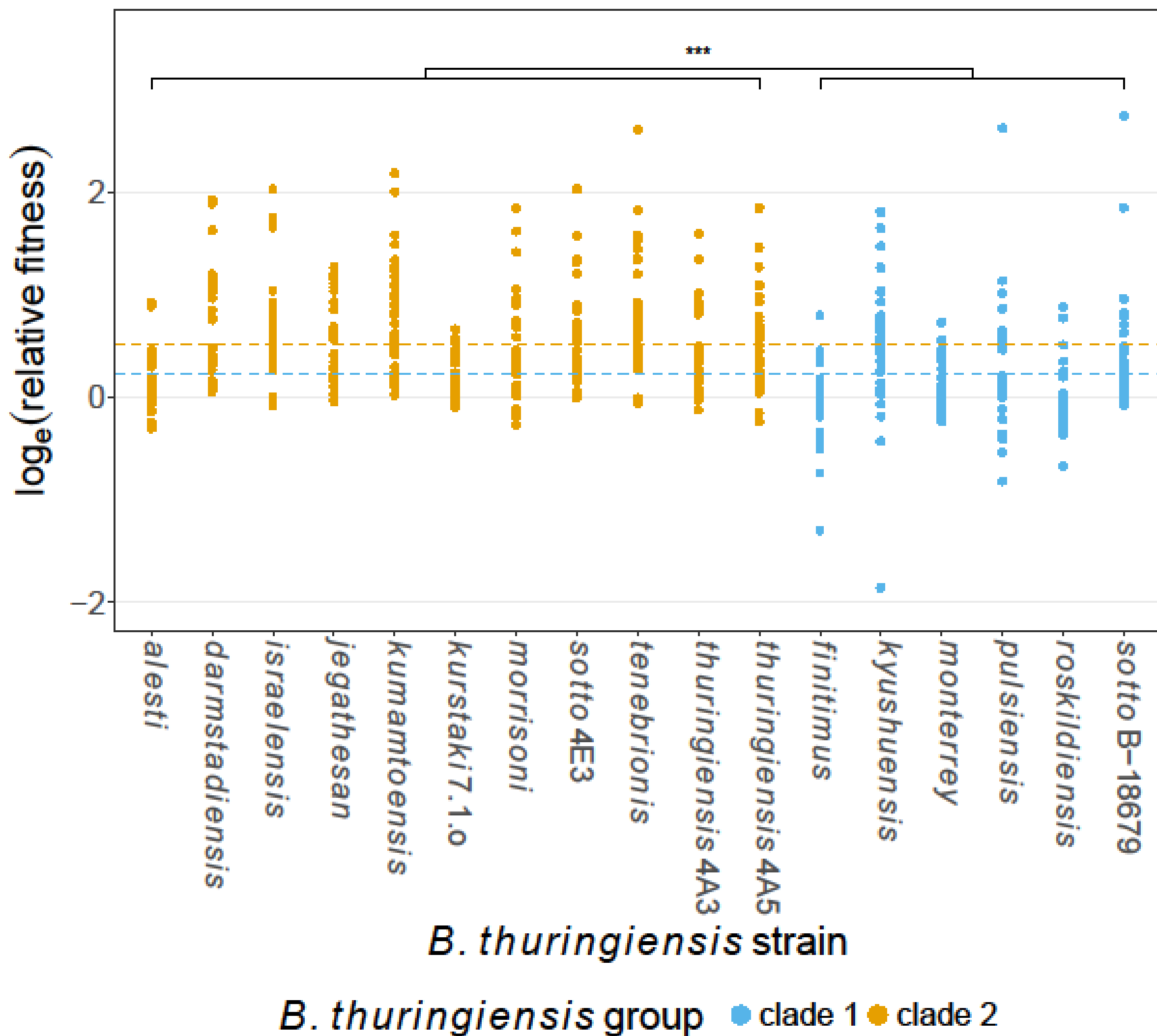

Figure 1.8: Graph showing the $\log_e$ relative fitness of *B. thuringiensis* strains (table 1.b) in competitions *in vivo* against *DsRed*- and *tetR*-tagged *B. thuringiensis* subsp. *kurstaki* competitor in Cry1Ac-susceptible *P. xylostella* larvae. All relative fitnesses are from 3:1 competitions, with data points as values for each replicate and dashed lines means for each clade. *** = $p < 0.001$.

Relative fitnesses were grouped according to treatment strain clade and $\log_e$ transformed to account for the asymmetric nature of relative fitness data (as it is bound by 0). ANOVA of strain $\log_e$ relative fitnesses by clade showed that clade 2 strains had a significantly higher relative fitness than clade 1 strains ($F_{1, 647} = 59.9$, $p = 3.77 \times 10^{-14}$) – clade 2 strains had an overall mean $\log_e$ relative fitness of 0.510 compared to 0.223 in clade 1.

One of the treatment strains used was an untransformed wildtype of *Btk* pHT315-*DsRed-tetR*. As expected, the *kurstaki* isolate did not have a $\log_e$ relative fitness significantly different from 0 (i.e. equally as fit as *Btk* pHT315-*DsRed-tetR*; $F_{16, 647} = 13.0$, $t = -0.255$, $p = 0.799$). This also shows that the marker plasmid inserted into the standard competitor does not noticeably affect its fitness under these experimental conditions.

B. thuringiensis in vitro competitions (table 1.e)

Overall, clade 2 *B. thuringiensis* strains were less fit in HCO sporulation broth than their clade 1 counterparts. The *in vitro* competitions could not be analysed via relative fitness, as in eight of the competitions only one of the two strains could be recovered on plates after incubation. In competitions where only *Btk* pHT315-*DsRed-tetR* could be recovered the cultures were noticeably red in colour after incubation. Relative fitness is meaningless with only a single strain present, so competitions were assessed solely on which of the treatment or competitor strains was more common or the only survivor when plated out. The more common/surviving strain was therefore taken to be the fitter of the two competitors, as shown in table 1.e. These data produced a contingency table of count data and analysis by Fisher's exact test showed that clade 1 strains were significantly more likely to persist in sporulation media at the expense of *Btk* pHT315-*DsRed-tetR* than clade 2 strains were ($n = 152$; odds ratio = 8.28, $p = 7.14\times10^{-8}$). This supports the hypothesis that clade 1 strains are fitter than clade 2 strains *in vitro* in HCO sporulation broth. In contrast to the *in vivo* competitions *Btk* pHT315-*DsRed-tetR* was less fit than its untransformed wildtype in broth ($F_{10, 76} = 28.1$, $t = 3.13$, p 0.00252).

Fitter than competitor *in vitro*		Less fit than competitor *in vitro*	
Strain	**Clade**	**Strain**	**Clade**
pulsiensis	**1**	*sotto* B-18679	**1**
kyushuensis	**1**	*alesti*	**2**
roskildiensis	**1**	*tenebrionis*	**2**
finitimus	**1**	*kurstaki* 7.1.o	**2**
monterrey	**1**	*jegathesan*	**2**
israelensis	**2**	*morrisoni*	**2**
kumamtoensis	**2**	*darmstadiensis*	**2**
sotto 4E3	**2**	*thuringiensis* 4A3	**2**
thuringiensis 4A5	**2**		

Table 1.e: Fitness of *B. thuringiensis* strains (table 1.b) by clade when competed against the *Btk* pHT315-*DsRed-tetR* competitor *in vitro* in HCO sporulation media. Relative fitness values could not be obtained in competitions where only one strain persisted to the end. Fitter strains either gave higher relative fitness values than the competitor, or persisted at the expense of the competitor, with the opposite the case for less fit strains. All fitnesses are from 3:1 competitions.

Discussion

The three Bacillus cereus-group species all grew significantly faster with significantly higher production in Brain and Heart infusion. Within the presumed B. cereus and thuringiensis species the phylogenetic clade of strains significantly affected overall in vivo growth in P. xylostella, with clade 2 strains being relatively fitter, along with their temperature-dependent growth rate and productivity. Unexpectedly, anthracis clade strains showed decreased ability to grow at 37°C, despite this being the characteristic growth temperature of vertebrate pathogens. Overall these data also support the hypothesis that B. cereus isolates evolved from necromenic (cadaver-specialised) ancestors.

The comparisons in growth across distinct media types show that the *B. cereus* group require protein, which is unsurprising as they carry many protease genes but few for processing carbohydrates, particularly when compared to soil-dwelling *Bacillus subtilis* (Rasko, Altherr et al. 2005, Han, Xie et al. 2006, Alcaraz, Moreno-Hagelsieb et al. 2010). Enriching soil-simulating media with sugars did not significantly improve growth and there was no clear difference between SESOM and TREM. These findings contrast those by Vilain, Luo et al. (2006) that showed clear differentiation between successful growth in SESOM and no growth in MOPS. This previous study also showed growth in base SESOM and no lag phase with vegetative cells in enriched SESOM while there is in LB. This was not found here – there was no growth after 24 hours in unenriched SESOM. The enriched SESOM produced very limited growth, but this was best modelled as a shallow (s-shaped) 4-parameter growth curve, implying there is a lag phase. Overall this shows that soil or plant nutrient simulating media whether simulating just soil (SESOM) or the root-soil interface (TREM) do not provide favourable environments for growth. Previous experiments have also suggested this, with *B. thuringiensis* cells only able to sporulate but not grow when added to soil (Yara, Kunimi et al. 1997).

There are differences between *Bacillus* species, with *B. weihenstephanensis* growing best and *B. thuringiensis* worst in both MOPS and SESOM enriched media, while *B. cereus* grew best in BHI. It is not surprising

that *B. weihenstephanensis* grew most in soil media as it has been characterised by its psychrotolerance and isolation solely from soil, plant material and food (Lechner, Mayr et al. 1998, Sorokin, Candelon et al. 2006, Raymond, Wyres et al. 2010) although its poorer growth in TREM appears to run counter to a possible association with plant roots (Dutta, Rani et al. 2013, Vidal-Quist, Rogers et al. 2013). In contrast, *B. thuringiensis* does not appear to reproduce in soil (Vilas-Boas, Vilas-Boas et al. 2000). BHI is produced from bovine and porcine organs, and so represents a protein rich resource such an invertebrate or vertebrate host or carcass. As *B. cereus* can be isolated from mammals (Arnesen, Fagerlund et al. 2008), this improved growth relative to *B. weihenstephanensis* is not surprising. In line with this *B. anthracis* is more specialised as a mammalian pathogen and shows an increased growth rate compared to *B. cereus* across multiple strains in BHI (De Siano, Padhi et al. 2006). Overall then, this study found no support for the general hypothesis that *B. cereus* is a soil saprophyte (Jensen, Hansen et al. 2003, Priest, Barker et al. 2004, Vilain, Luo et al. 2006) although individual isolates may of course vary in their propensity to grow in different media; while some differences were found, the magnitude of effect sizes tended to be small.

The thermal profile of different species or clades can also be informative as to niche. If clade 1 bacteria are mammalian pathogens, then these strains would be expected to grow efficiently at 37°C. The fact that growth of both clade 1 and clade 2 *B. thuringiensis* tends to decline as temperatures exceed 29°C suggested that mammals are not key hosts for these strains. Previous work has shown that there are two relatively deep branches in clade 1 (Guinebretiere, Thompson et al. 2008). There appears to be different thermal profiles across these two sub-clades, those more closely related to *B. anthracis* grow across a higher temperature range (15-45°C), consistent with the ability to infect mammals, while those in the other sub-branch (equivalent to *PanC* genetic group 2) have a lower temperature profile (10-40°C) consistent with the majority of the isolates in this study. Guinebretière also confirms the psychrotrophic nature of *B. weihenstephanensis*, with its ability to grow at low temperatures and separation in clade 3 supporting a distinct niche for it as well (Guinebretiere, Thompson et al. 2008).

This feeds back to the differences in temperature preference of clades 1 and 2. If *B. cereus* is found in/around mammalian cadavers, it may be that these cadavers reach different temperatures to insect ones. A rat is roughly 10^4 times larger than a *Plutella* larva and so may retain sufficient heat from decomposition that its temperature remains above ambient. If clade 1 *B. thuringiensis* strains are *B. cereus* strains that have recently acquired Cry plasmids they may be better adapted to growth in mammalian cadavers, reflected by their poorer relative fitness in insects and differing optimal temperatures. The different temperature profiles favoured by *B. cereus sensu stricto* and *B. anthracis* could be indicative of a necromenic lifestyle for *B. cereus*. This means that it colonises mammalian cadavers rather than creating them from live hosts, which may explain why it lacks the 37°C favoured growth temperature of *B. anthracis*. Additionally, some 'virulence factors' in *B. cereus* such as cereulide may originally have been antimicrobials (Tempelaars, Rodrigues et al. 2011), perhaps for limiting competition in cadavers, but have subsequently permitted opportunistic or accidental pathogenicity in humans.

The temperature growth experiments focussing on just the clade 1 and 2 *B. thuringiensis* strains also showed differences in favoured growth conditions between the selected strains. However, it is difficult to directly relate the two clades' differences in V_{max} and production to the ecological roles that they may occupy. *B. thuringiensis* spores can be isolated from soil globally (Martin and Travers 1989, Bizzarri and Bishop 2008, Ruan, Crickmore et al. 2015), so the differing responses might simply reflect where strains have been isolated. However, the supported hypothesis here is that clade 2 *B. thuringiensis* are obligate pathogens with in-soil growth not significant to the life cycle. If *B. thuringiensis* is a specialised insect pathogen then this still does not necessarily explain temperature growth differences. Insects are endothermic and small so internal temperature is likely similar to ambient temperature, so again the geographic range of *B. thuringiensis* might account for differing growth rates. However, the results obtained would require the two clades to have distinct ranges, which is not the case, although the ratio of *B. cereus* to *thuringiensis* isolated from soil can vary by climate (Hendriksen 2016). It is therefore difficult to give a satisfying conclusion why strains in the two clades might favour different temperatures based solely on growth curve data, beyond the point that

different temperature preferences should reflect adaptation to different niches. Across the whole *B. cereus* group distinct clades within do appear to favour different temperature ranges, most obviously the psychrotrophic *B. weihenstephanensis* (Guinebretiere, Thompson et al. 2008).

The competition experiments better highlight the hypothesised differences between the clade 1 and 2 *B. thuringiensis* strains. Clade 2 strains have higher relative fitness on average *in vivo* while the reverse is true *in vitro*. If fitness relative to *Btk* pHT315-*DsRed-tetR* is an accurate representation of absolute fitness, then it is clear that clade 2 *B. thuringiensis* strains are better able to grow and sporulate in an insect host than clade 1 strains and poorer at this in a non-insect environment. There are a few possible explanations for this result: the clade 2 strains grow faster outright, they are better able to supress competitors or they can avoid any immune response. If suppression were the cause then this should be the same *in vitro* as bacteriocins would still limit competition (Riley and Gordon 1999), but this was not the case. It could be argued that *in vitro* clade 2 strains lack access to nutrients required for bacteriocin production and so can no longer suppress the faster growing competitor. Either way, making better use of growth medium resources for other products is just as clear an indicator of adaptation to that environment as absolute growth is. The same argument can be made if immune escape is the reason for the improved competitive ability *in vivo* – having an advantage dependent on avoiding specific host responses would also be a clear indication of adaptation to that host. While *P. xylostella* larvae were used throughout the *in vivo* work, they are unlikely to be natural hosts of all strains used. Toxins from *kurstaki*, *aizawai* and *berliner* strains have all been employed against lepidopteran pests successfully (Iqbal, Verkerk et al. 1996), but *israelensis* carries toxins that make it effective against dipterans, in particular mosquitoes (Ankarloo, Caugant et al. 2000). This suggests that if strains were competed in their preferred host species then fitness differences compared to clade 1 might be greater.

The improved growth and separation of clade 2 from the rest of the *B. cereus* group strongly support the hypothesis that Cry toxins and the entomopathogen lifestyle emerged together and diversified as putative *B. thuringiensis* came into contact with more insect species. If *B. thuringiensis*

were only an opportunistic pathogen, then Cry toxins should have some function in environments other than the insect gut (Brown, Cornforth et al. 2012) such as aiding plant colonisation, but this is not the case (Bizzarri and Bishop 2008). However, while some toxins have been identified that bind to non-insect cells and some have no currently identified insecticidal activity (Mizuki, Park et al. 2000, Ruan, Crickmore et al. 2015), there is no general secondary function, which further supports specialisation by *B. thuringiensis*. This formative association with Cry toxins then allowed for adaptation elsewhere in the genome to an entomopathogen lifestyle and necrotrophic exploitation of resulting cadavers (Dubois, Faegri et al. 2012). Clade 1 strains appear to lack such adaptations and so may have acquired Cry toxins only recently as it is clear they can be transferred from *B. thuringiensis* to *cereus* (Ceuppens, Boon et al. 2013, Palma, Munoz et al. 2014). Some of the confusion over the abundance of *B. thuringiensis* in soil could be because of its associations with nematodes, which can be hosts or provide access to insects and are plentiful in soil (Loguercio and Argolo-Filho 2015, Ruan, Crickmore et al. 2015). *B. thuringiensis* shows specialisation even within herbivorous insects – while lepidopterans and coleopterans are common hosts along with some dipterans, there are few toxins that target hemipterans (Chougule and Bonning 2012, Palma, Munoz et al. 2014). This is unlikely to be because of a lack of searching as hemipterans are also important crop pests; instead *B. thuringiensis* may not be sufficiently exposed to hemipterans to evolve toxins against them. While lepidopteran larvae consume leaf tissue carrying spores, hemipterans instead pierce to access phloem sap, bypassing spores on the leaf. Plants may recruit *B. thuringiensis* to defend against such herbivory (Elliot, Sabelis et al. 2000, Bizzarri and Bishop 2008); an experiment was attempted to explore any differences in this between the two clades (in the manner of Raymond, Wyres et al. (2010)), but this failed. The lack of bug-targeting toxins has meant expanding use of *B. thuringiensis*-based transgenic crops has increased problems with hemipteran pests, such as mirid outbreaks in China (Lu, Wu et al. 2010, Chougule and Bonning 2012).

Realigning *B. thuringiensis* to solely clade 2 to reflect its distinct ecology should also remove confusion over links between clade 1 strains of *B. thuringiensis* and more harmful strains. *B. thuringiensis* subsp. *monterrey* is

more closely related to *B. anthracis* than to other clade 1 strains used here and produces a capsule than binds to *B. anthracis* antigens (Cachat, Barker et al. 2008). This may appear to be of little concern as *monterrey* is harmless to humans while *B. anthracis* is clearly not, but there are also related harmful *B. cereus* strains in clade 1 involved in food poisonings, particularly those carrying cereulide toxins. These links have fuelled the European Food Safety Authority's concerns about *B. thuringiensis* on food crops as a safe product for human consumption, as in their view commercialised strains from clade 2 could possess human virulence factors like their 'close' relatives (EFSA 2016).

The apparent safety of *B. thuringiensis* matters because it is such an important part of agriculture and may become more so with food production needing to increase at least 70% by 2050 (Godfray, Beddington et al. 2010). Already, *B. thuringiensis* or its genes are used on three quarters of North American arable land (Bailey, Boyetchko et al. 2010). Spore-based biopesticides provide a defence against specific pests without harming other species, which is more of a concern than ever – e.g., neonicotinoids and bees (Henry, Beguin et al. 2012) – and allows for fewer other insecticides to the benefits of natural predators (Lu, Wu et al. 2012). Their provenance also means they can be used on organic produce, which have more limited options for precise pest control. This safety argument does not directly impact transgenic crops (which are far more important agriculturally than *B. thuringiensis* biopesticides) but any discussions about the safety of *B. thuringiensis* could alter public perception of anything linked, further harming support for transgenic crops. If resources are committed towards engineering improved strains and Cry toxins then this needs to be done knowing that they will not become hostage to potential new regulation (Maughan and Van der Auwera 2011). *B. thuringiensis* has been used and therefore consumed since the mid-20th century (Lambert and Peferoen 1992, Sanahuja, Banakar et al. 2011) and yet the only potential black mark against it has been a recent food poisoning incident in Germany that gave the EFSA cold feet (EFSA 2016, Raymond and Federici 2017). However, this case was more likely caused by *B. cereus* also isolated from the contaminated food (Raymond and Federici 2017). The EFSA support their wariness by arguing that all *B. thuringiensis* and *cereus* are equally closely related so the former could easily become a human

pathogen, as it is already highly related to one. As shown here however, the clade 2 strains of *B. thuringiensis* used as biopesticides have clearly adapted to a distinct niche and are genetically separate from the more dangerous forms of *B. cereus.* As *B. thuringiensis* has adapted to an entomopathogenic lifestyle opportunities for recombination with *B. cereus* will have fallen, greatly limiting the likelihood of human virulence factors such as cereulide genes passing between them (Didelot and Maiden 2010).

The genetic and ecological distinction between clade 2 and the rest of the *B. cereus* family appears sufficient to maintain use of the *B. thuringiensis* species name, provided it is realigned to only clade 2. Sequence data make it clear that *B. thuringiensis* cannot persist just as a name for bacteria that possess Cry toxins within the *B. cereus* group, and that plasmids as a whole should not be relied on for *Bacillus* taxonomy (Liu, Lai et al. 2015). The supposed value in the existing system is that it identifies insect pathogens (Priest, Barker et al. 2004), except that all of the commercially viable biopesticide strains are already located in clade 2 and as shown here clade 1 strains are not as well adapted to growth within insect hosts (Zheng, Peng et al. 2013). It would therefore be better to align the name with the single clade that does contain clear, successful entomopathogen strains and that appears to be the origin of this lifestyle. This distinction between the clades already exists informally with clade 2 being referred to as the thuringiensis group and clade 1 the anthracis group as it also contains the *B. anthracis* sub-clade (Sorokin, Candelon et al. 2006, Raymond, Wyres et al. 2010, Zheng, Gao et al. 2017). An approach that combines both phylogenetic and ecological or phenotypic information to determine species could be used throughout the prokaryotes. The work here has shown that defining solely by phenotype can lead to disordered relationships. However splitting species solely on the basis of a particular level of genetic distance seems foolish – ultimately, species are defined and catalogued because it is beneficial to us to do so; not considering phenotypic differences in bacterial species would waste much of this benefit.

The combination of distinct genetic grouping and correspondingly distinct niche supports the previously suggested rearrangement of the *B. cereus* species group so that *B. thuringiensis* is limited to clade 2 and *B. cereus* excludes these strains, independent of possession or lack of Cry toxins. As

discussed, this would provide a clearer picture to regulators, but more importantly it would represent the reality of the group, unlike the current classification. The existing one was built using identification methods that have been superseded by sequencing, and an argument for maintaining it was that it simplified regulation of new products. The current concerns of the EFSA have removed this benefit of the existing system, so there is little reason not to take advantage of improved knowledge of the *B. cereus* species group by updating its classifications.

II: Competition & Frequency Dependence in *Bacillus thuringiensis* Infections

Abstract

Competition between co-infecting strains is known to strongly affect the virulence of infections. Classical virulence theory states that strain competition should result in a race to consume the most resources, increasing virulence to the host. However, the role of public goods in infections suggests that high virulence would be favoured by cooperation rather than antagonism. Competition is then expected to be highest between distantly related strains with a corresponding decrease in cooperation. Shared public goods include many virulence factors, like the crystal (Cry) endotoxins of the entomopathogen *Bacillus thuringiensis*. *B. thuringiensis* infections are established from the insect midgut so spore distribution may affect infectivity. Increased infectivity may correspond to improved competitive ability, although the public nature of Cry toxins could also allow less infective strains access to the haemolymph.

The insecticidal activity of three well-characterised *B. thuringiensis* subsp. (*aizawai*, *entomocidus* and *kurstaki*) was confirmed in Cry1Ac-resistant and -susceptible *Plutella xylostella* larvae, pre- and post-transformation with marker plasmids. As expected, the strains had differing activities, with *aizawai* the most lethal to resistant larvae and *kurstaki* to susceptible larvae. In order to test the hypotheses that virulence predicts competitive ability and infection composition is influenced by midgut spatial structuring, competitions were carried out in resistant larvae with *B. thuringiensis* subsp. *aizawai* or *kurstaki* against *entomocidus*; these were done at multiple densities and frequencies.

Gut spatial structuring does not appear to affect infection composition for *B. thuringiensis* in *P. xylostella*. However, *B. thuringiensis* fitness outcomes in co-infections appear to be generally frequency dependent within-host, as has been shown previously. Since increased infectivity does not provide a competitive advantage within-host, selection for increased virulence must rely fully on between-host competition, i.e. selection at larger spatial scales, while co-infections are unlikely to select for increased virulence in this system.

Introduction

Competition has always been at the centre of ideas regarding how natural selection operates. Conflict over resources is readily apparent down to a microscopic level, including with bacteria, where antibiotics are the best known example of antagonistic competition (Linares, Gustafsson et al. 2006). Many infections may consist of multiple strains of a particular pathogen (Gilbert, Plebanski et al. 1998, Lord, Barnard et al. 1999, Read and Taylor 2001, Alizon and van Baalen 2008), as clearly has been shown with malarial infections (de Roode, Pansini et al. 2005, Bell, De Roode et al. 2006). In mixed infections competition is expected between strains and may strongly affect infection intensity and outcome, whether they take the form of super- or co-infections. For the former, one strain completely outcompetes and replaces the other so that only it remains in the host to be transmitted (Alizon and van Baalen 2008). In co-infections multiple genotypes persist or invade simultaneously, although the conditions for long-term coexistence can be quite restrictive (Alizon and van Baalen 2008). Under classical models of virulence, competitive ability is linked to the harm done to a host, so that mixed infections may select for an increase in virulence (in terms of "disease-induced mortality of the host") (Frank 1996, Gandon, Jansen et al. 2001, Day 2002). This has been demonstrated experimentally in some cases (Ebert and Mangin 1997, Davies, Fairbrother et al. 2002, de Roode, Pansini et al. 2005) although Bell, De Roode et al. (2006) suggest that there is no "logical necessity" for virulence and competitive ability to be linked.

In contrast, when virulence is cooperative and depends upon the secretion of public goods e.g., siderophores or the crystalline δ-endotoxins of *Bacillus thuringiensis*, competition within-host in mixed infections will decrease relatedness and promote conflict and/or cheating (Massey, Buckling et al. 2004). Thus mixed infections are predicted to favour cheaters and select for lower investment in virulence factors (Brown, Hochberg et al. 2002). Experimentally, decreasing relatedness appears to favour reduced investment in costly virulence factors (Griffin, West et al. 2004, Harrison, Browning et al. 2006).

In ecological terms, competition between strains that produce antagonistic compounds (antibiotics, bacteriocins) can also reduce virulence (Massey, Buckling et al. 2004) and there is plenty of experimental evidence showing a decrease in virulence as more strains infect the host (Turner and Chao 1999, Brown and Johnstone 2001, Gower and Webster 2005, Harrison, Browning et al. 2006). Unlike antibiotics, which are principally employed against more distantly-related organisms, bacteriocins are targeted more at conspecifics or other closely related bacteria (Riley and Wertz 2002). While an antibiotic producer may be protected from it through lack of the specific pathway targeted, bacteriocins require specific antidotes e.g., *Escherichia coli* producing colicins and manufacturing corresponding immunity proteins (Riley and Gordon 1999). The antidote gene is typically linked to the bacteriocin one so that another immune bacterium is likely to share both genes, making it more closely related than the surrounding susceptible population (Riley and Wertz 2002, Garbutt, Bonsall et al. 2011). Producing bacteriocins is costly (Dobson, Cotter et al. 2012) so increased antagonistic interactions are likely to come at the cost of reduced investment in growth or virulence.

The dominant, and obligate, virulence factors of *B. thuringiensis* are the crystalline δ-endotoxins (Cry toxins) and these are known to behave as public goods within the insect midgut (Raymond, West et al. 2012). Cry toxin production also has high metabolic costs (Agaisse and Lereclus 1995) which gives a clear opportunity for exploitation by cheats. However, unlike many public goods that are secreted during vegetative growth and provide near-immediate benefit, Cry toxins produced at the end of one infectious cycle during sporulation (Deng, Peng et al. 2014, Deng, Slamti et al. 2015) are not active until ingestion by the next host. Furthermore, Cry toxins require no carriage of a receptor molecule or similar – once released and activated in the insect midgut their action is independent of the bacteria. Instead they cause perforations in the gut epithelial cells' microvilli (Bravo, Gill et al. 2005), giving *B. thuringiensis* access to haemolymph nutrients (Schnepf, Crickmore et al. 1998). The separate action means that Cry toxins do not control access to perforations – entry to the haemolymph is unrestricted (Raymond, Davis et al. 2007). A consequence of this is that cheat *B. thuringiensis* that produce no Cry toxins could still pass into the haemocoel. As with cheats in other systems the Cry-

null strains would then be expected to either outgrow their honest counterparts or sporulate faster without the burden of toxin production (Raymond, Davis et al. 2007). The possibility of cheats may help to explain why Cry toxin genes remain on plasmids rather than integrated into the chromosome, as horizontal transfer could restore them to Cry-null strains (Nogueira, Rankin et al. 2009, Mc Ginty, Rankin et al. 2011, Leggett, Brown et al. 2014).

Given the high cost of Cry toxin production, mixed infections between Cry null cheats and toxin producers should give a strong competitive advantage to toxin cheats (Raymond, Davis et al. 2007, Raymond, West et al. 2012). However, there are a great breadth of Cry toxins and *B. thuringiensis* strains (Aronson and Shai 2001), each with a limited host range against which they are effective (de Maagd, Bravo et al. 2001). The outcome of competitive interactions between two strains with similar levels of investment in different Cry toxins, but very different infectivity in a host, is harder to predict. If competing strains are investing in antagonists such as bacteriocins the most common strain may have a competitive advantage and produce positive frequency dependent selection (Levin 1988, Gardner, West et al. 2004). Antagonism aside, social evolution theory can predict the conditions favouring investment in public goods. Spatial structure in populations, for instance, is known to be fundamental for the operation of kin selection (Taylor 1992, Griffin, West et al. 2004). For example, when competition is purely local organisms will be competing with their relatives, undermining the benefits of cooperation. Put another way, the existence of spatially distinct groups allows the operation of global group level competition and so that investment in traits that benefit group can pay dividends (Frank 1998). Virulence factors do not necessarily move freely through hosts. Indeed spatial structure in the insect midgut is believed to account for the low fitness of null quorum sensing (QS) mutants of *B. thuringiensis* (Zhou, Slamti et al. 2014), although QS signals and QS-regulated traits behave as public goods in other experimental systems (Brown and Johnstone 2001, Diggle, Griffin et al. 2007).

Therefore it could be predicted that conditions favouring spatial structure in the midgut (figure 2.1) might provide an increased selective advantage to more infective strains, i.e. strains producing Cry toxins which more effectively perforate the midgut of their host. For instance, relatively few spores can be

sufficient to cause mortality in a susceptible host (Cornforth, Matthews et al. 2015); the fewer spores ingested then the fewer toxins will be released into the midgut with correspondingly fewer perforations in the epithelium. Spatial variation in toxin concentration is therefore more likely to occur if toxin levels are low and if toxins are released by relatively few spores. As well as low absolute density, a low frequency of spores in a mixed infection might also lead to local release of toxins and provide an advantage to a more infectious strain. Notably, some Cry toxins appear to be bound to the spore surface and may trigger spore germination through binding to midgut receptors (Du and Nickerson 1996); if this also triggers pore formation it could limit epithelial breakdown to a spore's immediate area when there are fewer solubilised toxins.

This issue has important fundamental and applied implications. First, how does selection for increased infectivity operate when virulence is based on public goods? Is it entirely dependent on group-level competition, i.e. differences in infectivity between near clonal infections in different hosts or populations of hosts; or does within host competition play a role in selection for infectivity at some level? The answer to this fundamental question has important implications for how one might design artificial selection regimes that seek to increase the infectivity of social pathogens such as *B. thuringiensis*, the focus of a substantial part of this thesis. Should some degree of within-host competition be facilitated or will relying entirely on group level selection be sufficient?

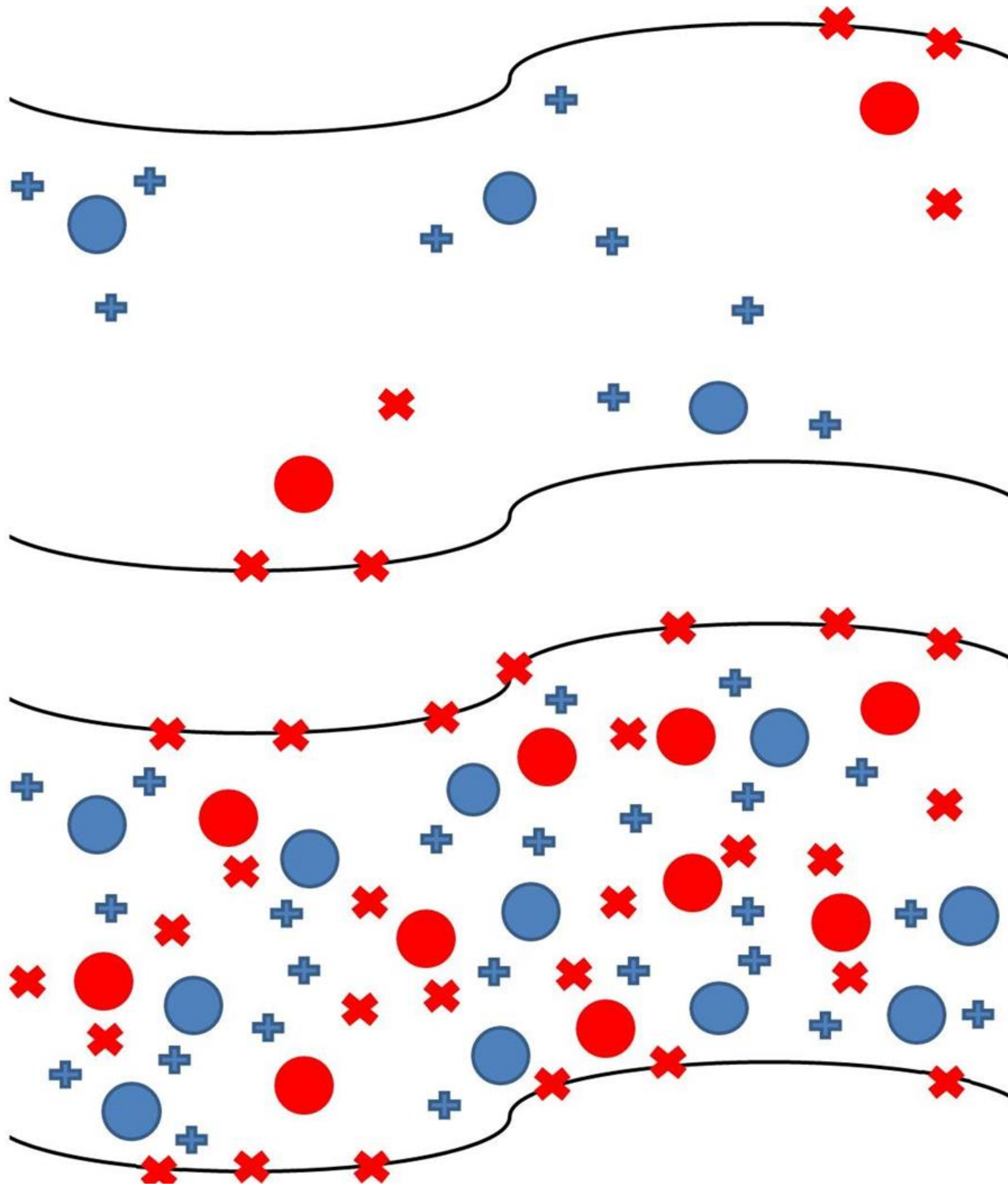

Figure 2.1: Comparison between high spatial structure (top) and low spatial structure (bottom) in the midgut. With high spatial structure (low spore density), toxins that can bind and perforate the midgut may only be in the vicinity of associated *B. thuringiensis* spores. With low spatial structure (high spore density) toxins are distributed throughout the midgut, allowing all spores access to the haemolymph.

While *B. thuringiensis* populations show strong spatial structuring on plants (Collier, Elliot et al. 2005, Raymond, West et al. 2012) the distribution of spores throughout the environment suggests mixed infections might be

somewhat common, although less frequent than clonal infections. The ability of *B. thuringiensis* strains to produce bacteriocins supports the occurrence of intraspecific within-host competition (Cherif, Rezgui et al. 2008, Raymond, Johnston et al. 2010) and within-host competition is likely to affect other aspects of the biology of this important biocontrol organism. Although the rarity of *B. thuringiensis* epizootics in nature makes observing competition directly in the field difficult (Porcar and Caballero 2000), experimental mixed infections can be established easily (Raymond, Davis et al. 2007, Raymond, Wright et al. 2013). The work here tested experimentally whether more infectious strains in mixed infections have an increased selective advantage in conditions favouring spatial structure within the gut (low dose, low frequency). Since infectivity of strains varies with insect genotype, whether the relative fitness of stains in standardised mixes varies in insects with differing susceptibility to a critical commercialised Cry toxin was also tested.

Initial work was done to assess the insecticidal activity of commonly available, well characterised strains in Cry1Ac-susceptible and -resistant *Plutella xylostella* larvae populations. *B. thuringiensis* strains *kurstaki*, *entomocidus* and *aizawai* have all been used for insect control e.g., XenTari® biopesticide containing *aizawai* ABTS-1857 (Valent BioSciences LLC). *B. thuringiensis* subsp. *kurstaki* has been used as a biopesticide principally because its Cry1Ac toxin causes high levels of mortality in a number of lepidopteran pest species (Tabashnik, Carriere et al. 2003). As resistance to Cry1Ac has emerged, *aizawai* has also been used as its Cry1C toxins still produce sufficient mortality in many of these populations (Liu, Tabashnik et al. 2001). However *entomocidus*, which also lacks Cry1Ac but carries Cry1C toxins (Crickmore, Baum et al. 2018), has found less widespread use as a commercial biopesticide. Bioassays of these strains in Cry1Ac-resistant and -susceptible *P. xylostella* were carried out to confirm they each cause different levels of mortality in these insect populations. Based on previous work *kurstaki* was expected to produce the highest virulence in Cry1Ac-susceptible larvae and *aizawai* the highest in Cry1Ac-resistant ones. This had the unexpected effect of identifying the *aizawai* isolate used initially as a contaminant in this formulation. In order to confirm the identity of new isolates from XenTari® as *aizawai* a protocol based around restriction fragment length polymorphisms

(RFLP) was developed in order to distinguish between serotypes. Once correctly identified, *aizawai*, *kurstaki* and *entomocidus* were all transformed with marker plasmids. Initial competitions were predicted to show that the two types of marker plasmid do not impose differing fitness costs.

As discussed above, varying the levels of spatial structuring in the gut should help to highlight potential links between infectivity and competitive ability. Strains were expected to show positive frequency dependence, as higher relatedness during growth in the haemolymph should favour increased public goods production to aid growth. In particular, it was expected that as spatial structure increased (through lower density and frequency), the relative fitness of the more infective strain should increase.

In addition to the commercially available strains, competition experiments also investigated two experimentally evolved *kurstaki* mutants produced in previous experiments (as part of the same overall project) by Dimitriu, Crickmore et al. (unpub. data). These had been generated from *kurstaki* 7.1.o wild-type strain put through selective passaging for higher virulence in Cry1Ac-resistant *P. xylostella* larvae. The two evolved mutants showed higher mortality in Cry1Ac-resistant larvae but did not persist in subsequent selection rounds. It is therefore likely that the altered or increased toxin production necessary to overcome resistance here carries a strong fitness cost. When competed directly with their *kurstaki* ancestor outside of a selection protocol they were therefore expected to be less fit across all spore density and frequency treatments.

Beyond the immediate aims of the work here, the information gained will also inform protocol designs for subsequent selection experiments to evolve more virulent strains – in particular how the different strains perform under competition here may affect their use. Mixed infections could allow for increased horizontal transfer and recombination of *cry* genes, which appears to be the process that has produced such a diversity of Cry toxins (Vilas-Boas, Vilas-Boas et al. 1998, de Maagd, Bravo et al. 2001). However, if the strain targeted for improvement is a poor competitor it may be difficult to facilitate increased recombination and therefore variation through mixed infections.

Methodology

Producing marked strains

Three well-characterised *B. thuringiensis* strains from three serotypes were used in this study (table 2.a): *kurstaki* 7.1.o, *aizawai* and *entomocidus*. Each was initially transformed to give two marked versions, one with plasmid pHT315 carrying *gfp* and *eryR* for erythromycin resistance and one with pHT315 carrying *DsRed* and *tetR* for tetracycline resistance (Zhou, Slamti et al. 2014). To produce electrocompetent cells for transformation the strains were cultured overnight at 30°C with 150rpm shaking in 2$^{\%}$ LB broth. 5ml of overnight culture was then added to 500ml pre-warmed 2$^{\%}$ LB in a conical flask and incubated for four hours at 30°C with 150rpm shaking. After four hours, 67ml of 50$^{\%}$ glycine was added to give 3$^{\%}$ in culture and the culture incubated for an additional hour. Culture was transferred to eight 50ml pre-chilled Falcon tubes and these left on ice for 5mins. Tubes were centrifuged at 3,200*g* and 4°C for 12mins. Tubes were kept on ice while supernatant was poured off. Cell pellets were gently re-suspended with 10ml ice-cold buffer 'F' (272mM sucrose, 0.5mM $MgCl_2$, 0.5mM K_2HPO_4 and 0.5mM KH_2PO_4 at pH 7.2) (Peng, Luo et al. 2009). This was repeated two further times, with final resuspension in 500µl of buffer 'F' and transferred to a pre-chilled Eppendorf. 100µl of electrocompetent cells were added to a pre-chilled 0.1cm^3 cuvette with 2µl plasmid DNA and electroporated at 1.8kV (MicroPulser, Bio-Rad Laboratories Inc.). The cells were then transferred to 1ml of pre-warmed 2$^{\%}$ LB and incubated at 30°C for one hour. After one hour, 100µl of culture was spread onto a 2$^{\%}$ LB agar plate containing the appropriate selective antibiotic and 900µl onto another and plates incubated at 30°C overnight. Colonies were re-streaked on selective plates and viewed under UV light to confirm plasmid uptake.

***Bacillus* species**	**Strain / isolate**	**Source**
B. thuringiensis	*kurstaki* 7.1.o	Raymond
B. thuringiensis	*kurstaki* 7.1.o C1 mutant	Dimitriu
B. thuringiensis	*kurstaki* 7.1.o C2 mutant	Dimitriu
B. thuringiensis	*aizawai* (contaminant)	BGSC 4J4
B. thuringiensis	*aizawai* X1 (confirmed)	XenTari®
B. thuringiensis	*aizawai* X6 (contaminant)	XenTari®
B. thuringiensis	*entomocidus*	BGSC 4I4

Table 2.a: *B. thuringiensis* strains used during competition experiments. C1 and C2 from Dimitriu, Crickmore et al. (unpub. data) – mutants derived from *kurstaki* 7.1.o (Raymond, Wyres et al. 2010) via separate passage experiments; X1 was isolated from XenTari® (Valent BioSciences LLC) product and confirmed as *aizawai* via RFLP of *hag* flagellin gene.

Initial bioassays

Each strain used was streaked on plain 2% LB agar plates and incubated overnight at 30°C, then a colony of each spread on *Bacillus cereus*-selective agar (Oxoid Ltd.) and incubated for six days at 30°C to produce spores. Spores of each marked strain were suspended in 700µl saline (0.85% NaCl), centrifuged at 6,200*g* for 2mins and supernatant removed. This was repeated three times then spores re-suspended in 1ml saline with 0.05% Triton X surfactant. Spores of each marked strain were diluted to 10^{-6}, and 10^{-6} dilutions plated out on 20µg/ml tetracycline or erythromycin 2% LB agar plates to check counts. Inocula were then made from the dilutions as specified in each of the individual bioassays below. This was also done with the original untransformed, wild-type strains.

Experiments used diamondback moth, *Plutella xylostella,* larvae that were either susceptible (VLSS population) or resistant (FR population) to Cry1Ac toxin (Zhou, Alphey et al. 2018). Insects were raised from surface-sterilised eggs on sterile insect diet until late second-instar. Eggs were sterilised for 30s in 10% bleach solution, then drained and twice washed with sterile H_2O then dried for 15mins in a laminar flow hood. Diet was 90g of dry mix (44.3g wheatgerm, 18.4g casein, 0.1g inositol, 1.5g Sodium propionate,

5.9g Wesson salts, 1.5g sorbic acid, 0.9g cholesterol, 1.5g methyl-4-hydroxybenzoate, 0.3g choline chloride, 3g locust bean gum, 1.6 cellulose, 1.6g fructose and 9.4g agar) with 420ml demineralised water. After autoclaving, 2g of Vanderzant vitamin mixture for insects and 1.8g ascorbic acid were each dissolved in 30ml sterile H_2O, sterilised via 0.2µm cellulose filter and added to the cooled autoclaved diet mix. Diet was poured into 12oz plastic pots (Vegware Ltd.) to a depth of 15mm for insect rearing or into 50mm Petris to a depth of 5mm for experiments and allowed to set in a laminar flow hood. Individual quarters of sterile diet in five 50mm Petri dishes were coated with 100µl of spore inoculant and dried in a laminar flow hood. Ten larvae were added to each Petri dish, giving 50 larvae per strain dilution. In all bioassays a control was also used with only sterile H_2O added to diet. Insects fed on inoculated diet at 22°C for five days. After five days the assay was scored for the number of live larvae and melanised cadavers to give mortality for each strain (transformed and wild-type) across all doses.

Preliminary work showed that the available stock isolate of *aizawai* was unable to kill Cry1Ac-resistant larvae any more effectively than *kurstaki*. Therefore a bioassay on resistant larvae was carried out using *kurstaki*, the ineffective *aizawai* and spores grown from XenTari® commercial product (Valent BioSciences LLC) which is marketed as containing *aizawai*. Spore suspensions of 37, 111, 333, 1,000, 3,000 and 9,000cfu/µl were used with *aizawai* and XenTari®, and 111, 333, 1,000, 3,000, 9,000 and 27,000cfu/µl for *kurstaki*.

A second bioassay was then carried out in both resistant and susceptible larvae, using *kurstaki*, *entomocidus* and two isolates from XenTari® picked from individual colonies, named X1 and X6. This was done over the same ranges as before, with all strains at 37-9,000cfu/µl except *kurstaki* at 111-27,000cfu/µl.

A final bioassay was carried out using wild-type and *DsRed*- and *gfp*-transformed versions of both *kurstaki* and X1 in both resistant and susceptible larvae. Again, X1 was used at 37-9,000cfu/µl and *kurstaki* at 111-27,000cfu/µl. This was done to assess the effects of transformation and additional plasmid carriage on mortality, and to confirm that any difference in mortality between X1 and *kurstaki* found in the previous bioassay persisted.

RFLP confirmation of *aizawai*

Isolates from XenTari® commercial spore mix suspected of being *B. thuringiensis aizawai* were grown overnight on 2% LB agar at 30°C, then a colony picked and used to inoculate 5ml 2% LB for overnight growth at 30°C with 150rpm shaking. 70µl of culture and 70µl of 10mM Tris pH 8.0 were boiled for 10mins then centrifuged at 2,000*g* for 4.5mins. Supernatant was then used in PCR to amplify the *B. thuringiensis hag* gene for flagellin, with the primers Bthag-F1 (5'-AGTACATGCGCCAAAACCAAG) and Bthag-R2 (5'-TAACTCAAATGGCTTATTGT) (Xu and Cote 2006). The PCR program used had 5mins initial denaturing at 94°C then 30 cycles of 15s at 95°C, 30s at 56°C and 60s at 72°C with an additional final extension at 72°C for 5mins. PCR products were then digested separately with TaqαI (65°C) and EcoRV (37°C) restriction enzymes (New England Biolabs Inc.) for one hour and deactivated by incubation at 80°C for 20mins. The restriction products were run in 1.5% agarose gel at 100V for 45mins against a non-restricted sample and 2-log ladder. The number of distinct restriction fragments and their lengths were compared against expected patterns from *kurstaki* 7.1.o and *aizawai* restrictions *in silico* using Geneious® R8 (Biomatters Ltd.).

In vivo competitions to investigate spatial structuring

Competition experiments with the *gfp*-tagged *kurstaki* and *aizawai* X1 tested whether these bacteria showed density or frequency dependent fitness against *DsRed*-tagged *entomocidus* in Cry1Ac-resistant larvae. Since *entomocidus* is substantially more infective than *kurstaki* in this genetic background (see Results, figure 2.3), low frequency and low doses were expected to favour this strain in competition with *kurstaki.* In contrast, *aizawai* and *entomocidus* impose similar mortality and differences in infectivity would not be expected to drive frequency or density dependent fitness in this interaction.

Spore suspensions for each strain were counted via diluting in series and plating out on selective 2% LB agar plates and then adjusted to 27,000cfu/µl. Spores were combined in 5:5, 9:1 and 1:9 ratios, keeping the 27,000cfu/µl

overall concentrations. These mixes were also used to produce 10-fold and 100-fold dilutions in the same ratios. Larvae were inoculated as with the single-strain bioassays, with the number of larvae used adjusted based on bioassay mortalities to give an expected minimum of 48 cadavers per treatment.

After five days, up to 48 cadavers were collected for each treatment and placed into sterile 1ml microtubes with 10µl sterile H_2O, sealed and then incubated at 30°C for five days to ensure sporulation. After five days 690µl sterile H_2O was added with a sterilised 5mm stainless steel ball bearing. Tubes were shaken for 4.5mins at 1,350rpm in a tissue lyser (Qiagen N.V.), and then pasteurised at 65°C for 25mins in a water bath. Samples were diluted to 10^{-4} and dilutions 10^{-1} to 10^{-4} plated out onto erythromycin and tetracycline 20µg/ml 2% LB agar plates. Plates were incubated overnight at 30°C and then colonies counted. Competitions were also carried out only in 50:50 ratios at 27,000cfu/µl between all three strains with both *gfp*- and *DsRed*-tagged versions in Cry1Ac-resistant and -susceptible larvae in the same manner.

Competitions using the evolved strains of *kurstaki* (labelled C1 and C2 and also carrying pHT315-*gfp-eryR*) were carried out under the same protocol, using the same ratios and total spore concentrations. The same competition protocol was also carried out between combinations of transformed strains, but all at 50:50 ratios and 27,000cfu/µl to avoid problems with insufficient mortality.

For all competitions colony counts on the 2% 20µg/ml tetracycline LB agar plates gave cfu/µl for the *DsRed-tetR*-transformed competitor and those on 2% 20µg/ml erythromycin gave cfu/µl for the *gfp-eryR*-transformed one.

Statistical analysis

For bioassay analysis larvae were recorded as either alive or dead, allowing mortality to be analysed via GLMs with binomial errors, as per Raymond, Wright et al. (2013). To account for over-dispersion with a standard binomial distribution a quasibinomial one was then used. This was tested using the F-statistic at the overall level against a significance level of $p < 0.01$, given the noisiness of bioassay data, and using the t-statistic for within-treatment

tests. Bioassay mortality data were analysed in RStudio 1.1.456 (based on R 3.2.5; R Core Team 2016, RStudio Team 2016).

For competitions, final spore cfu/µl values were used to calculate Malthusian fitnesses for both competitor strains, which were then used to calculate relative fitness as follows (Lenski, Rose et al. 1991):

$$\boldsymbol{Malthusian\ fitness = log_2 \frac{final\ cfu/\mu l}{inoculant\ cfu/\mu l}}$$

$$\boldsymbol{Relative\ fitness_a = \frac{Malthusian\ fitness_a}{Malthusian\ fitness_b}}$$

Results

Initial Bioassays

Preliminary work suggested that the stock isolate labelled as *B. thuringiensis aizawai* did not kill more Cry1Ac-resistant larvae than *kurstaki*. This runs counter to the expected insecticidal activity of *aizawai* as unlike *kurstaki* it should possess Cry1Ca toxins that permit mortality in the FR Cry1Ac-resistant larval population used (Crickmore, Baum et al. 2018).

The first full bioassay (figure 2.2) using XenTari® spores along with *kurstaki* and defective *aizawai* confirmed that XenTari® produced significantly higher mortality in FR larvae than either of the other two strains. Spore doses were $\log_e$ transformed and the mortality data analysed via GLM with quasibinomial errors, as with all subsequent bioassays. The different strains produced significantly different mortalities ($F_{2, 26} = 23.2$, $p = 2.48 \times 10^{-6}$) along with a significant interaction between strain and dose ($F_{2, 24} = 18.0$, $p = 1.67 \times 10^{-5}$).

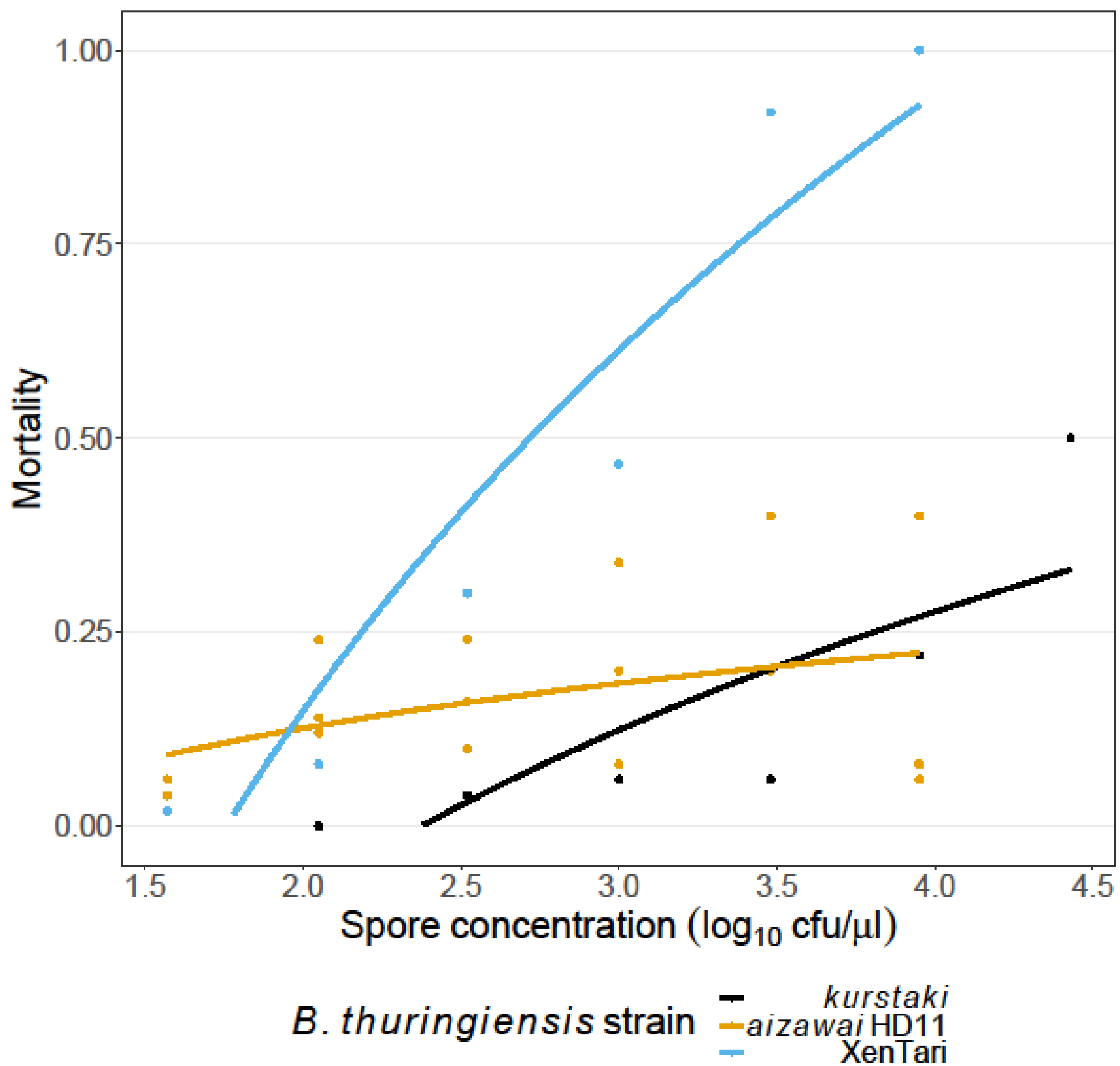

Figure 2.2: Bioassay mortalities of *B. thuringiensis* strains *kurstaki* and *aizawai* with XenTari® product in Cry1Ac-resistant *P. xylostella* larvae.

Plating out the XenTari® and picking colonies produced isolates, two of which (X1 and X6) were used in the larger second bioassay (figure 2.3) in Cry1Ac-resistant and susceptible larvae alongside *kurstaki* and *entomocidus*. This bioassay confirmed that the X1 isolate produced higher mortality in the Cry1Ac-resisant larvae, while the X6 isolate gave low mortalities at all spore levels ($F_{3, 43} = 42.3$, $p = 3.07 \times 10^{-11}$). As expected, the larval type also strongly affected strain mortality ($F_{1, 42} = 405$, $p < 2.20 \times 10^{-16}$).

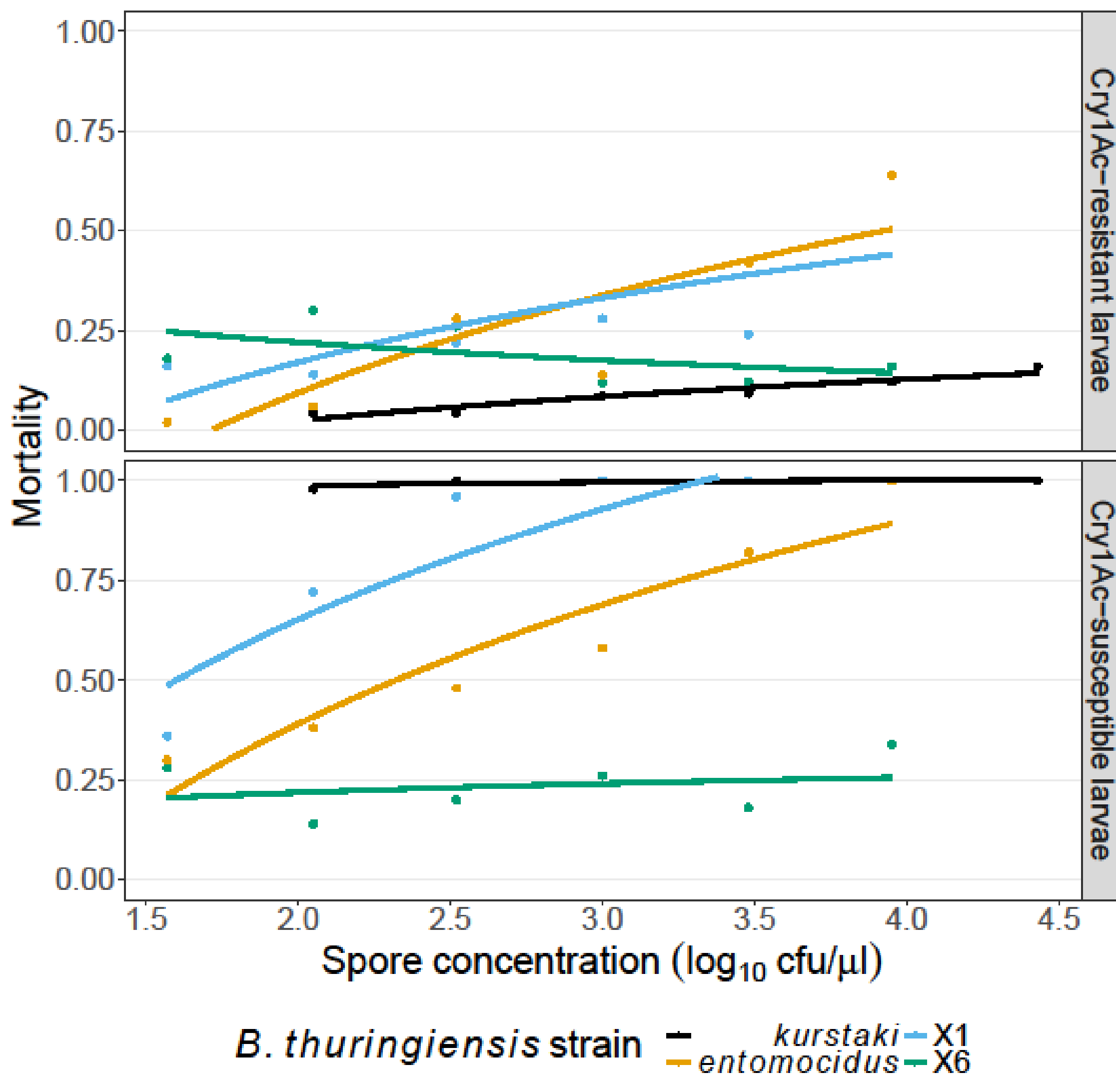

Figure 2.3: Bioassay mortalities of *B. thuringiensis* strains *kurstaki* and *entomocidus* with XenTari® product isolates X1 and X6 in Cry1Ac-resistant and -susceptible *P. xylostella* larvae.

Identifying *aizawai* via RFLP (figure 2.4)

The identity of isolate X1 as *aizawai* was confirmed via RFLP analysis of the *hag* gene for *B. thuringiensis* flagellin in X1 and *kurstaki* 7.1.o. Initial PCR using Bthag-F1 and -R2 primers produced PCR products between 700 and 800bp in length based on comparison with 2-log ladder. Based on existing sequence data, *kurstaki* 7.1.o is expected to give a product of 753bp with Bthag primers and *aizawai* 723bp. Restriction with EcoRV of X1 produced a single fragment the same length as the original PCR product as predicted. The restriction with TaqαI was expected to produce three fragments but only two

could clearly be resolved, perhaps because the third fragment was only expected to be 66bp. The *kurstaki* restriction with EcoRV was expected to produce three fragments, but again the third fragment was not clearly visible, while the TaqαI restriction produced two as expected. Subsequent repeating of RFLP on X1 after transformation with *DsRed* and *gfp* plasmids confirmed the same pattern with the short EcoRV 66bp fragment partially visible. Using RFLP based on the *B. thuringiensis hag* gene appears to be a simple way of distinguishing between previously sequenced strains of *B. thuringiensis*, provided that convenient distinguishing restriction sites are available. RFLP also suggested that the supposed *aizawai* isolate initially bioassayed was contaminated with *kurstaki*.

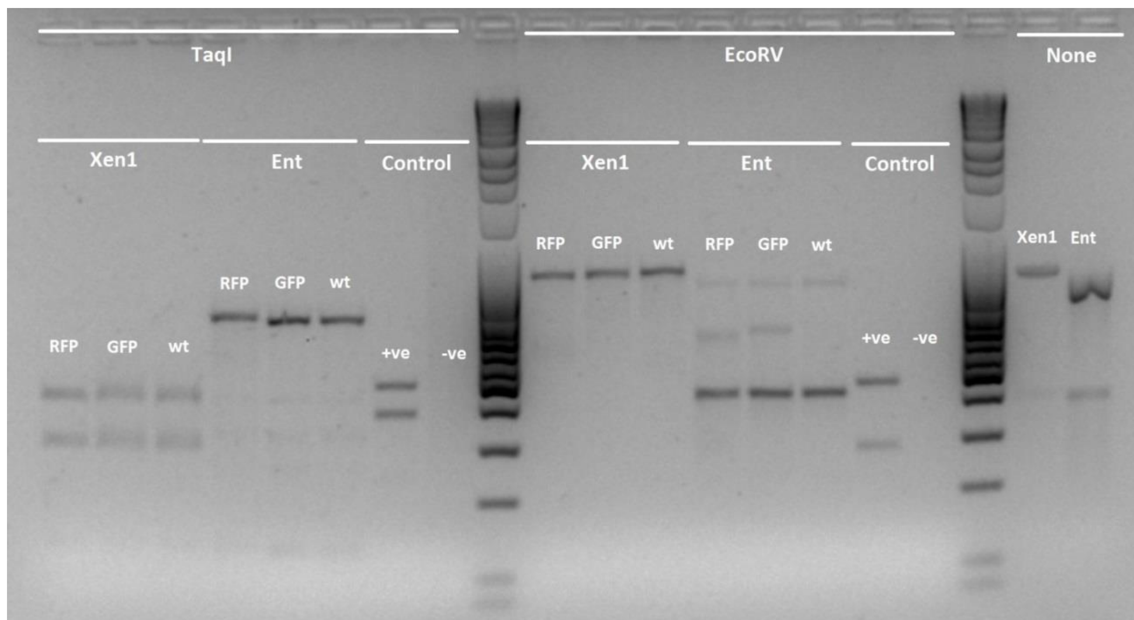

Figure 2.4: RFLP demonstration, here with *entomocidus* (Ent) and *aizawai* X1 (Xen1), showing distinct band patterns for each strain with the restriction enzymes TaqI and EcoRV. This also confirmed that contamination did not occur during transformation with marker plasmids (RFP and GFP) through comparison with untransformed wild-types (wt). Positive control (+ve) was *kurstaki* 7.1.o.

Bioassays with transformed strains (figure 2.5)

B. thuringiensis strains *kurstaki* 7.1.o, *entomocidus* and *aizawai* X1 were all successfully transformed with the *DsRed-tetR-* and *gfp-eryR*-carrying pHT315 plasmids. Bioassays in both Cry1Ac-resistant and -susceptible larvae with *aizawai* X1 showed that it still maintained its increased mortality in Cry1Ac-resistant larvae compared to *kurstaki* ($F_{1,\ 54}$ = 36.8, p = 3.76 x 10^{-7}) and that there was no significant difference between original wild-types and the transformed strains ($F_{2,\ 52}$ = 1.76, p = 0.185).

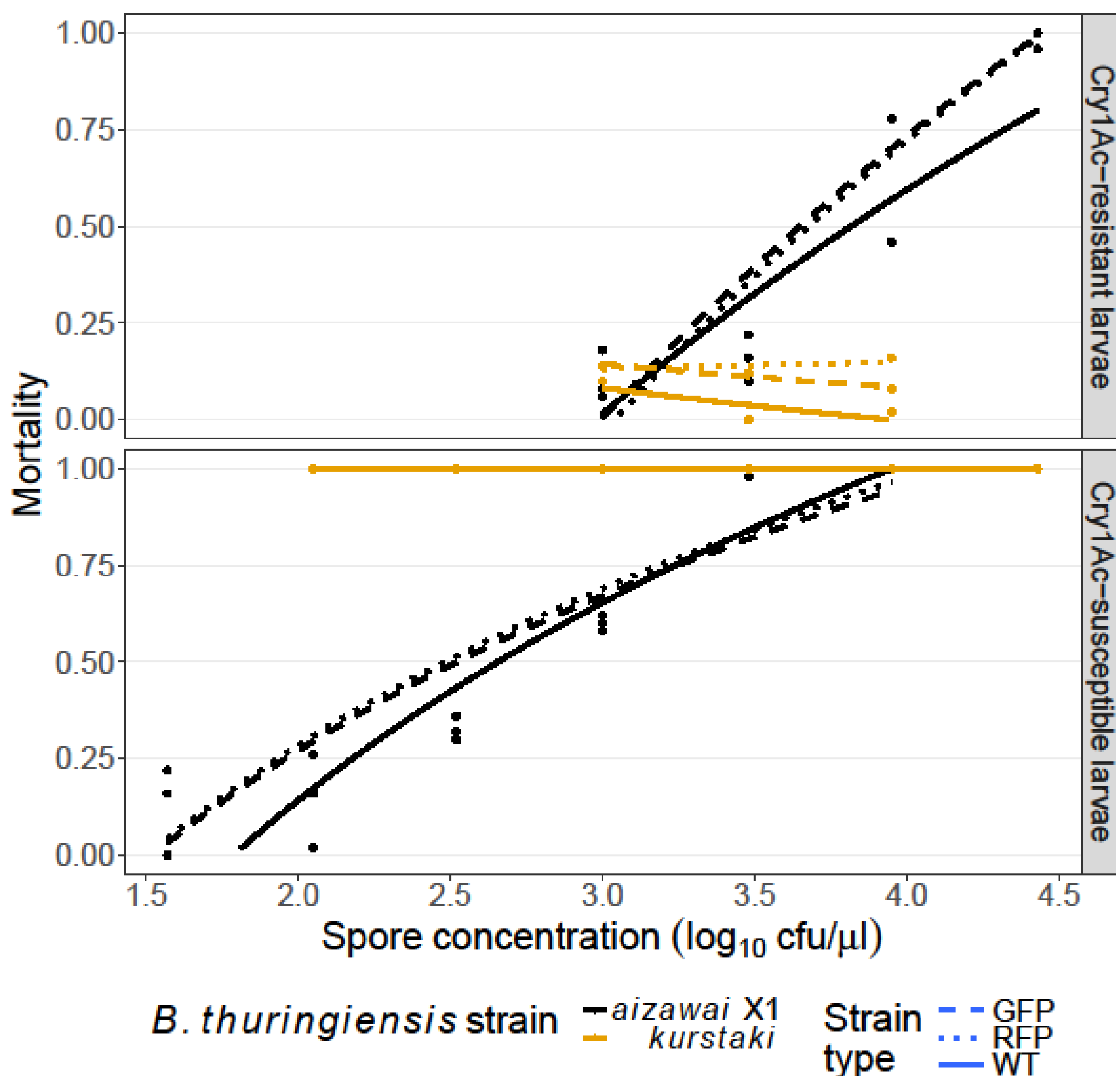

Figure 2.5: Bioassay mortalities of *B. thuringiensis* strains *kurstaki* and *aizawai* X1 in Cry1Ac-resistant and -susceptible *P. xylostella* larvae. Mortalities are split between wildtype (WT) and transformed with the two marker plasmids (GFP and RFP).

Effect of marker pHT315 plasmids

Strains carrying the *DsRed-tetR*- and *gfp-eryR* pHT315 plasmids did not have significantly different relative fitnesses *in vivo* ($F_{1, 253} = 5.34$, $p = 0.0217$). Costs of the plasmids versus untransformed wild-types were not considered as experiments only used transformed strains.

Full competitions *in vivo*; *kurstaki* and *aizawai* versus *entomocidus* (figure 2.6)

In the *kurstaki* versus *entomocidus* competitions, neither the competitions at 270cfu/µl total nor the ones at 99:1 *kurstaki* to *entomocidus* in Cry1Ac-resistant larvae produced sufficient mortality to proceed with calculating relative fitness. For this reason these treatments also were not used in the *aizawai* X1 versus *entomocidus* competitions. The number of larvae used initially meant that it was not considered practical to repeat the competition experiments with a large enough number of larvae to produce sufficient cadavers.

There was no evidence for density dependent fitness in these experiments. Overall concentration of the competition mixture used did not affect the relative fitness of the strains in either competition and could be excluded from the model ($F_{1, 278} = 1.12$ $p = 0.291$), as was the case with all interaction terms including it.

Overall, proportion affected *entomocidus* relative fitness ($F_{1, 279} = 12.8$, $p = 4.01 \times 10^{-4}$), and in particular there was a strong interaction between proportion and strain ($F_{1, 279} = 35.1$, $p = 9.37 \times 10^{-9}$); this fitness being unchanging in the *kurstaki* versus *entomocidus* competition but negative frequency dependent in the *aizawai* X1 versus *entomocidus* one. The competing strain itself also significantly affected the relative fitness of *entomocidus* ($F_{1, 279} = 39.1$, $p = 1.51 \times 10^{-9}$), which again is unsurprising as the change in slope mentioned means that *entomocidus* is fitter when rare against *aizawai* X1, but is always less fit against *kurstaki* in Cry1Ac-resistant larvae.

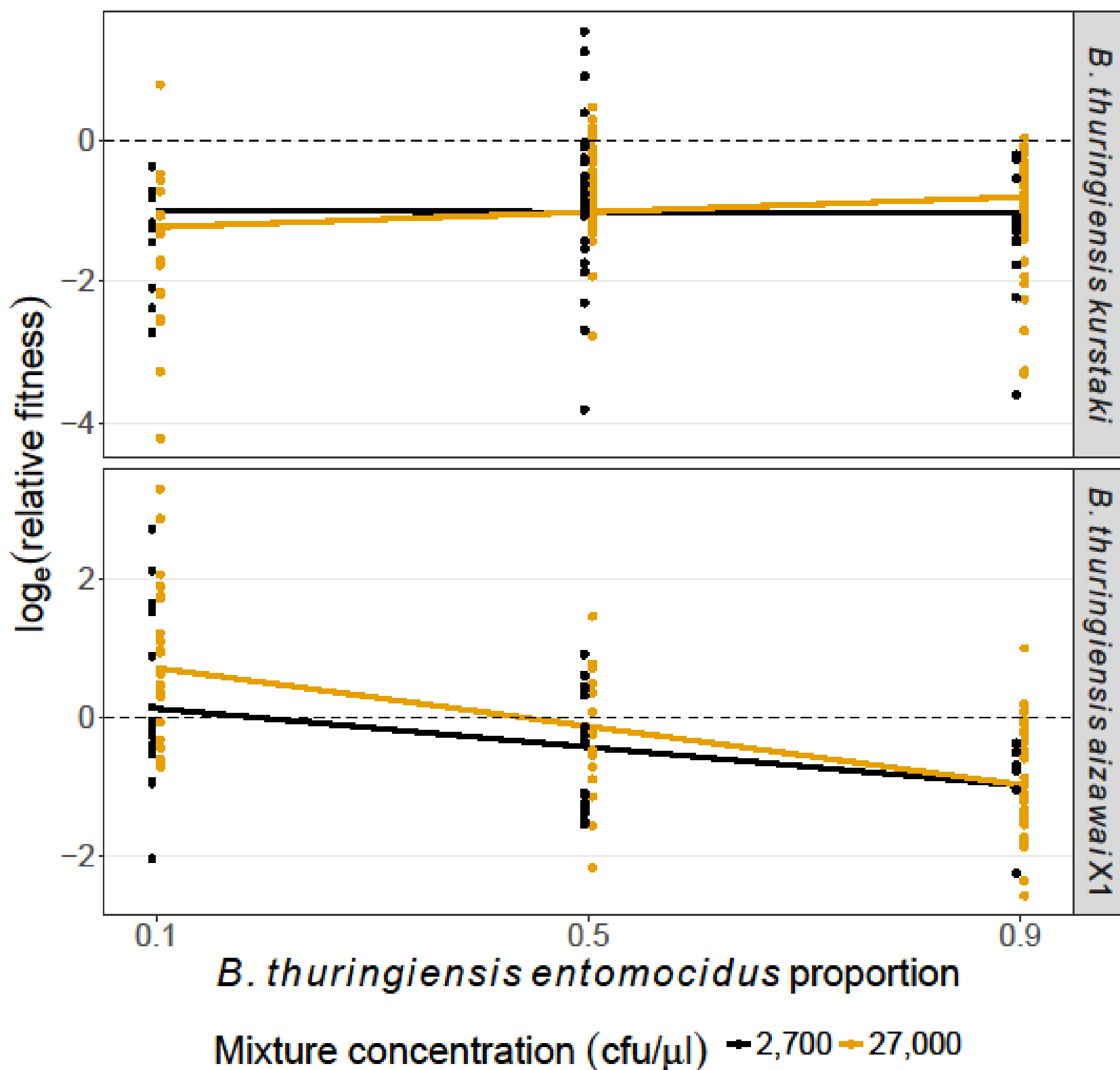

Figure 2.6: Relative fitness of *B. thuringiensis entomocidus* for competitions against *kurstaki* and *aizawai* X1 in Cry1Ac-resistant *P. xylostella* larvae. Relative fitness is shown at two different total spore densities (2,700 and 27,000 cfu/µl) for three frequencies of *entomocidus* relative to the competitor, where a 0.1 proportion of *entomocidus* corresponds to a 10:90 ratio of *entomocidus* to competitor.

50:50 competitions in both host backgrounds (figure 2.7)

Data were split by the two competitor strains (*aizawai* X1 and *kurstaki*) to compare across host genotype. For both sets of competitor, fitness overall did not differ between Cry1Ac-susceptible and –resistant larvae ($F_{1, 143}$ = 0.177, p = 0.186; $F_{1, 111}$ = 0.123, p = 0.727). As well as not having significantly different effects on fitness (described previously) there was no reason to expect an

interaction effect between the pHT315 marker plasmids and host background. In the *entomocidus* competitions the strain had slightly lower relative fitness against *kurstaki* than *aizawai* ($F_{1, 143} = 9.12$, $p = 2.99 \times 10^{-3}$).

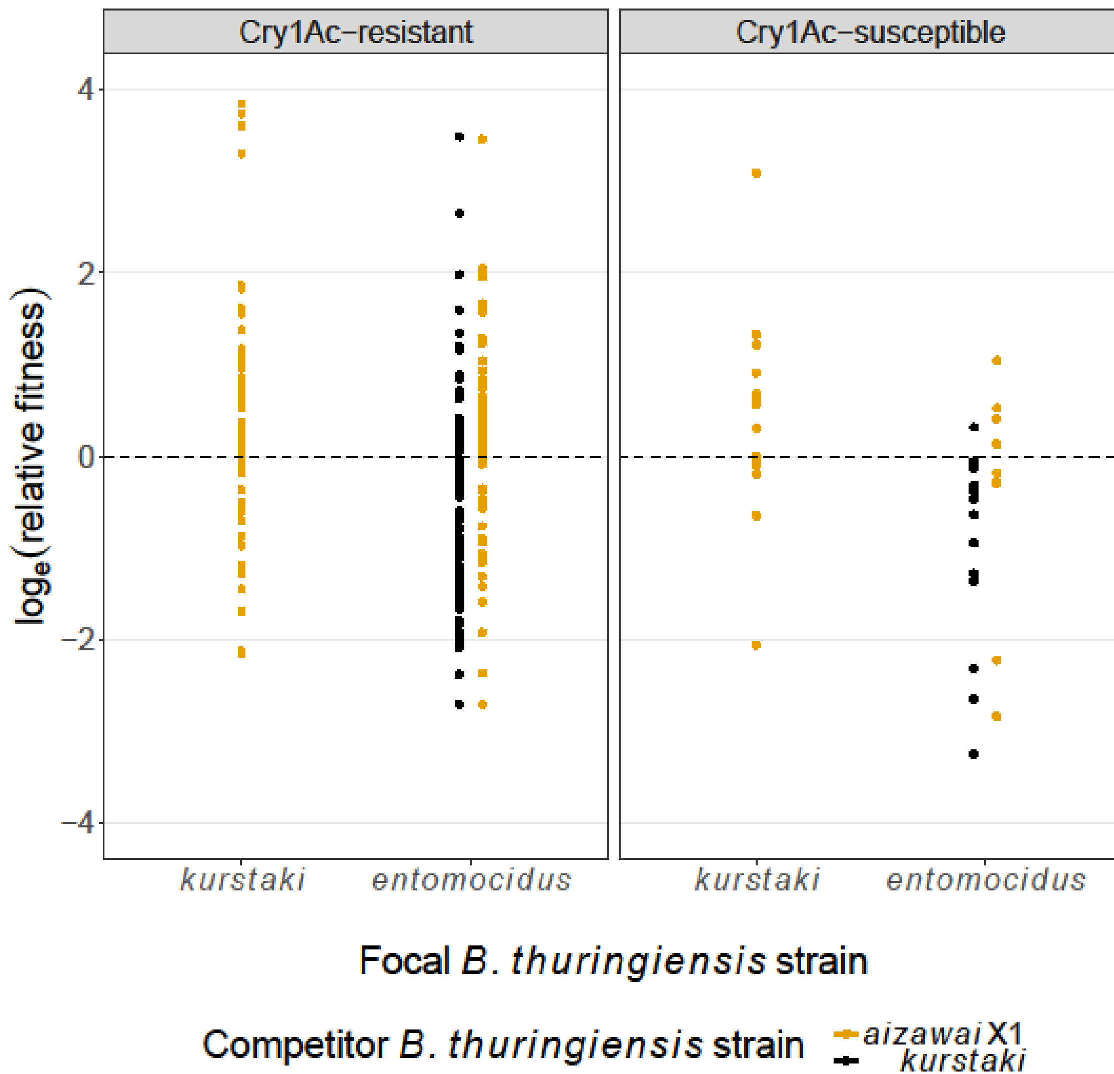

Figure 2.7: Relative fitness of *B. thuringiensis* strains *entomocidus*, *kurstaki* and *aizawai* X1 in competitions against each other in Cry1Ac-resistant and -susceptible *P. xylostella* larvae. All relative fitnesses are from 50:50 competitions at 27,000cfu/μl.

C1 and C2 *kurstaki* mutants

The C1 and C2 mutants produced significantly fewer spores both *in vivo* and *in vitro* than their *kurstaki* ancestor when initial and final spore counts were

combined to give Malthusian fitnesses (figure 2.8). Overall strain type was significant ($F_{2, 275} = 57.4$, $p < 2.2 \times 10^{-16}$), and a Tukey HSD post-hoc test confirmed that both the mutants were less fit than their *kurstaki* ancestor. Simplifying the model down to just *kurstaki* and mutants could not be justified as the two show differing fitness levels in both growth media, with C2 fitter in both. In the competitions against the *kurstaki* ancestor (figure 2.9) sufficient cadavers could only be collected for competitions with C2. There was a clear difference in the fitness of the C2 mutant depending on whether it was in susceptible or resistant larvae and its proportion. This is reflected by the significance of the interaction terms involved, between strain and proportion ($F_{1, 403} = 64.1$, $p = 1.29 \times 10^{-14}$) and larvae and proportion ($F_{1, 402} = 78.9$, $p < 2.20 \times 10^{-16}$). Strain and proportion were significant ($F_{1, 406} = 6.87$, $p = 0.009$; $F_{1, 405} = 186$, $p < 2.20 \times 10^{-16}$) but larval background alone was not ($F_{1, 404} = 4.50$, $p = 0.0345$).

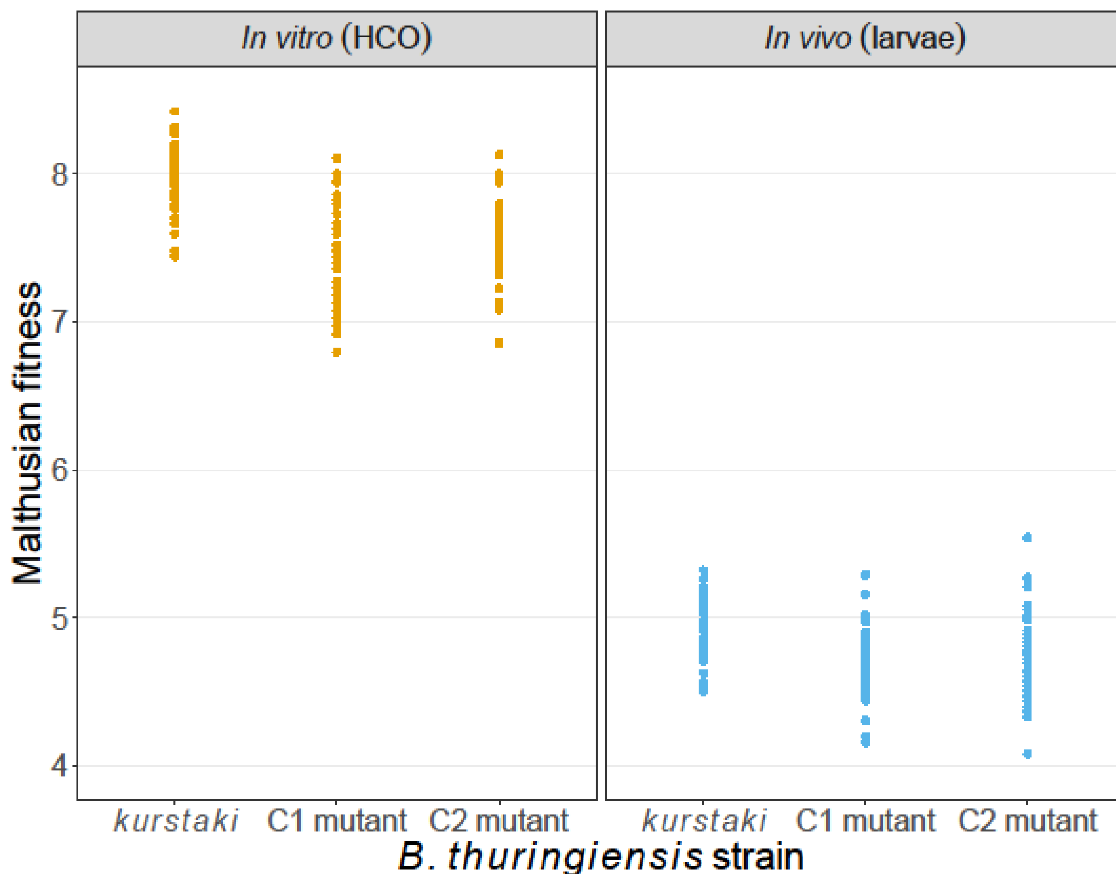

Figure 2.8: Malthusian fitness of *B. thuringiensis kurstaki* C1 and C2 mutants compared to their wild-type *kurstaki* ancestor in HCO sporulation medium and Cry1Ac-susceptible *P. xylostella* larvae.

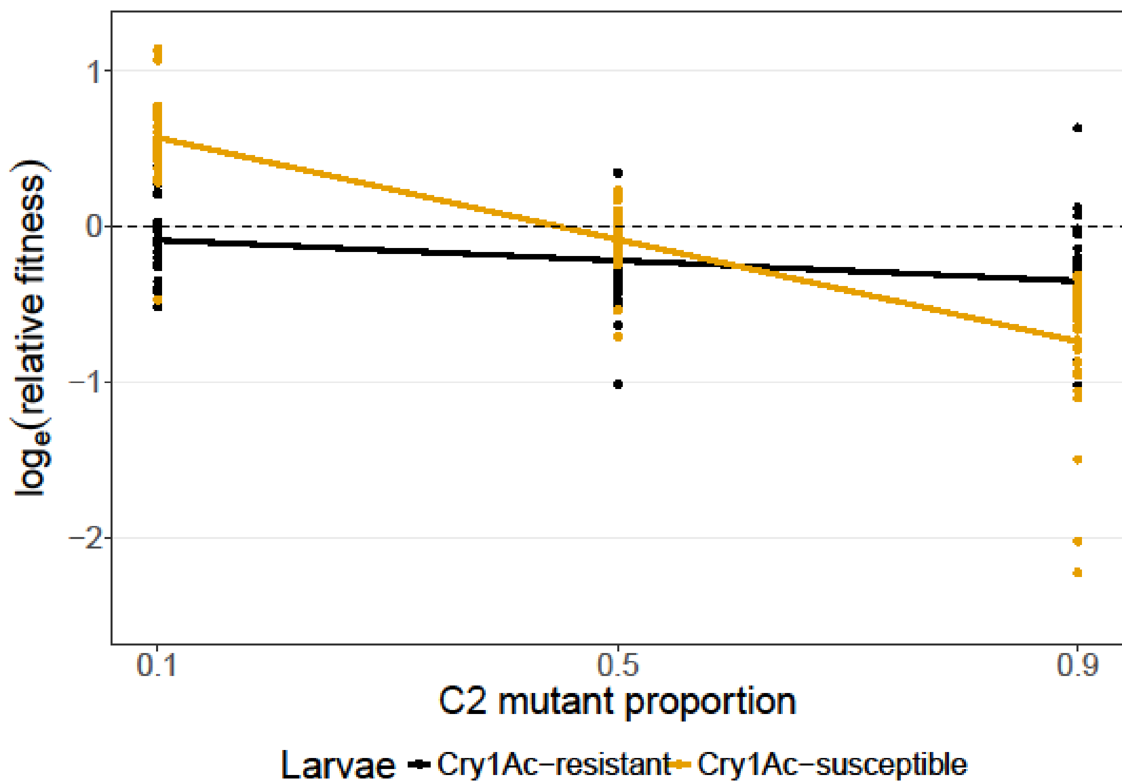

Figure 2.9: Relative fitness of *B. thuringiensis kurstaki* C2 mutant in competition against its wild-type *kurstaki* ancestor in Cry1Ac-resistant and -susceptible *P. xylostella* larvae. Relative fitness is shown at two different total spore densities (2,700 and 27,000 cfu/µl) for three frequencies of C2 relative to its ancestor, where a 0.1 proportion of C2 corresponds to a 10:90 ratio of C2 to ancestor.

Discussion

In line with both preliminary work and existing data, B. thuringiensis aizawai had the highest mortality in Cry1Ac-resistant P. xylostella larvae and kurstaki in Cry1Ac-susceptible ones. Spatial structuring in larval guts at the levels generated here does not significantly affect B. thuringiensis infection composition in P. xylostella larvae. However, the fitness of these B. thuringiensis strains in co-infections shows frequency dependence within the host. As there does not appear to be a within-host competitive advantage conferred by the increased infectivity of a strain, between-host competition should instead be used when selecting for increased virulence, as co-infections do not select for increased virulence in this system.

Competition between pathogens relying on virulence factors which are public goods and act at a distance is largely unexplored. Here, the principal hypothesis that increased infectivity can improve the relative fitness of competing strains in hosts was not supported, even when conditions were created which were expected to increase spatial structure within infections. Nevertheless, host genotype did affect the outcome of some competition experiments. As the host *P. xylostella* genotypes should differ only in their resistance or susceptibility to the Cry1Ac toxin (potentially via loss of abcc2 receptors) (Baxter, Zhao et al. 2005, Baxter, Badenes-Perez et al. 2011) the C1 and C2 results suggest that interactions between distantly-acting toxins and receptors can affect relative fitness of invading social pathogens, though this was not found to be the case with the non-passaged strains. Negative frequency dependent fitness was also observed in interactions between mutants or different serotypes, which for *entomocidus* versus *aizawai* was consistent with social exploitation of virulence factors between strains that differ in competitive ability.

Bioassays confirmed that the *aizawai*, *entomocidus* and *kurstaki* strains used produce differing mortality levels in the Cry1Ac-resistant and susceptible hosts. The *aizawai* and *entomocidus* strains overcome Cry1Ac resistance in the larvae through possession of Cry toxins which *kurstaki* lacks e.g., Cry1C

(Crickmore, Baum et al. 2018); in turn possession of Cry1Ac gives *kurstaki* very high mortality rates in the Cry1Ac-susceptible larvae. Additionally, transforming the strains with marker plasmids carrying fluorescence genes and antibiotic resistance traits did not affect their virulence. The different marker plasmids used also had indistinguishable effects on bacterial relative fitness, confirming that the methods used to distinguish competing strains did not have undue effects on the outcome of experiments and that the experimental set-up was appropriate for testing the key hypotheses.

The 50:50 competitions in larvae at 27,000cfu/µl spore doses should limit any spatial structuring in the insect midgut, with both strains at high density and equal frequency. Lack of structuring should mean that differences in colonies counted at the end of the competition should reflect strain competition during growth and sporulation in the haemolymph, rather than just which strain could access the haemolymph. The results from the 50:50 competitions suggest that the *aizawai* and *kurstaki* strains used here have similar fitness levels within hosts. However, the *entomocidus* tended to be less fit on average than either *aizawai* or *kurstaki*. Recent genomic analyses confirm that *aizawai* and *kurstaki* serotypes are very closely related, while *entomocidus* (BGSC 4I4) is placed on a nearby but separate branch of clade 2 (Zheng, Gao et al. 2017, Meric, Mageiros et al. 2018). In general *entomocidus* is more closely related to a range of typical Cry-null *Bacillus cereus* isolates than *kurstaki* and *aizawai* (Liu, Lai et al. 2015) and is very closely related to the *B. cereus* type strain (ATCC 14579) with which it shares the *entomocidus* serotype (Carlson, Johansen et al. 1996, Xu and Cote 2006). It is therefore plausible that *entomocidus* is less of an invertebrate specialist than the *B. thuringiensis* strains more closely related to *kurstaki* (Raymond and Bonsall 2013, Meric, Mageiros et al. 2018) and the fitness data here support this interpretation.

The *entomocidus* strain used also showed negative frequency dependent fitness in competition with *aizawai.* Since these stains have similar infectivity it is less likely that this frequency dependence is driven by differences in the ability to infect and kill hosts. Various mechanisms can drive negative frequency dependence (Ross-Gillespie, Gardner et al. 2007, Healey, Axelrod et al. 2016), many of which involve social interactions (Rainey and Rainey 2003, Dugatkin, Perlin et al. 2005). For example, significant spatial structuring or a

link between overall cooperation and total population growth can lead to negative frequency dependence for social cheats (Ross-Gillespie, Gardner et al. 2007). In *B. thuringiensis* there is significant spatial structure within the gut in terms of different foci of invasion, and this is sufficient to drive negative frequency dependent fitness of quorum-regulated (PlcR-dependent) virulence mutants (Zhou, Slamti et al. 2014). These virulence factors are believed to be expressed in the gut just prior to invasion of the haemolymph and in *P. xylostella* increase the invasiveness of *B. thuringiensis* infections (Zhou, Slamti et al. 2014). While *B. thuringiensis* produces crystal endotoxins during sporulation for a subsequent infection (Aronson and Shai 2001), the bacteria also produce other virulence factors during vegetative growth in the haemolymph including the vegetative insecticidal proteins (vips) (Estruch, Warren et al. 1996, Ruan, Crickmore et al. 2015). These other virulence factors provide another avenue for cheats to outcompete strains which alone give higher insect mortalities. However, unlike the Cry toxins which allow access to the haemolymph and so are the difference between growth or no growth, vips and other virulence factors may be more likely to modulate growth rates in the haemolymph (Fedhila, Daou et al. 2006), where the majority of vegetative growth is thought to occur. Virulence factors may only cause host damage as a secondary effect to aiding bacterial growth e.g., the cereulide toxins in some *B. cereus* are principally antimicrobial but have the side effect of causing vomiting and food poisoning in humans. Virulence factors secreted during vegetative growth in the haemocoel may have originally evolved to gather nutrients or limit competitors but have the side effect of further damaging the host. They may also be directly employed against the host to release further nutrients or combat the efforts of the host immune system. Even if strains do produce a full suite of Cry toxins they could still cheat by cutting production elsewhere which could still have an effect on mortality when in a single strain infection. It follows that vegetatively produced virulence factors, which increase invasiveness or local reproduction, could account for the negative frequency dependence seen here and also why fitness does not correspond to spore-associated virulence factors. Since *aizawai* is fitter than *entomocidus,* and therefore potentially more invasive, the frequency dependent fitness in these competition experiments could be related to exploitation of vegetative virulence factors produced at a higher rate by *aizawai*.

The negative frequency dependence shown by the C2 mutant is slightly harder to explain. This mutant has a lower fitness than its wild type *kurstaki* ancestor in 50:50 competitions in both insect genetic backgrounds. In the C2 mutant the most significant change to the genome is the deletion of a 328kb region, potentially a pathogenicity island (Hacker and Kaper 2000). It could be that the loss of such a region forces the mutant to rely more on co-infecting strains for a particular aspect of infection biology. Such a split has been found in the *B. thuringiensis* strain LM1212, with some bacteria sporulating without toxins and others producing toxin crystals but failing to sporulate (Deng, Slamti et al. 2015). However, C2 cannot be said to be an effective cheater since it has low fitness in competition. Moreover, C2 has lower productivity in live infections as well as *in vitro.* A link between investment in virulence factors and population growth can help drive frequency dependence, but only because additional opportunities for growth increase the fitness differences between cheaters and producers (Ross-Gillespie, Gardner et al. 2007), and this is not the case in this study. Frequency dependence might explain why subsequent attempts to get C2 to invade an ancestor population from rare through passage rounds (as must have happened during the original selection experiments that produced it) were unsuccessful irrespective of the number of C2 bacteria introduced.

Separate from cheats, strains may carry different Cry toxins that vary in cost or produce differing toxin quantities. Cry toxin inclusions in *B. thuringiensis* spores can take up over a quarter of total volume (Agaisse and Lereclus 1995), representing a heavy energetic investment as proved by the emergence of Cry-null cheats (Raymond, West et al. 2012). If growth rates in the haemocoel do differ there a number of possibilities for this. It could simply be a race to gather resources and convert them into spores, with one strain either requiring less energy or possessing more efficient resource-gathering mechanisms. Strains will also employ bacteriocins with their energetic cost being sufficiently offset by the reduction in competitors. Finally, a particular strain may be better able to evade any immune response at an acceptable metabolic cost (Brown, Inglis et al. 2009, Garbutt, Bonsall et al. 2011), with melanisation the principal response in *P. xylostella* (Cerenius, Lee et al. 2008).

Host background clearly affected the fitness of the C2 mutant with negative frequency dependence being much stronger in the Cry1Ac-susceptible

background. Using relative fitness values gives a clear indication of which strain is fitter in a host as their calculation accounts for any differences in host quality. However, the values do not give any insight into the underlying causes of the fitness differences e.g., is it because a strain grows faster outright (Rafaluk, Jansen et al. 2015) or produces more bacteriocins (Garbutt, Bonsall et al. 2011), though increasing antagonistic competition may be the more likely reason (Raymond, Davis et al. 2007). Growing strains individually allows for comparisons in basic growth rate via Malthusian fitness and it is clear for the C1 and C2 mutants that they produce viable spores at a slower rate than their *kurstaki* ancestor both *in vivo* and *in vitro*. This could account for a lower relative fitness across all competitions, but does not explain the frequency dependent fitness pattern as discussed above. Decreased spatial structure, in terms of mixing of genotypes or diffusion of virulence factors could explain the weaker frequency dependence in the Cry1Ac-resistant background; however this does not fit with our expectation of the slower and less effective disintegration of the midgut in these insects. As for the cause of C1 and C2's loss of growth, it could be a direct consequence of their increased insecticidal activity, a consequence of the loss of particular genes or even a change in the rate of sporulation. If the improved mortality is simply a result of more toxin being produced per spore then the extra energy put towards this would be expected to cause a corresponding decrease in growth and/or sporulation rates as toxin production is known to be highly metabolically costly (Agaisse and Lereclus 1995, Deng, Slamti et al. 2015).

If Cry spores were unevenly distributed in the midgut then it was hypothesised that only those with high infectivity would colonise the haemolymph. Therefore the lack of varying fitness in the *entomocidus* versus *kurstaki* competition suggests toxins and spores do mix through the gut independently. The second competition between *entomocidus* and *aizawai* also does not suggest any spatial structuring, appearing to show that the fitness of *entomocidus* is negative frequency dependent rather than a result of the increasing midgut epithelial breakdown provided by *aizawai* toxins. There are also the other virulence factors released by *B. thuringiensis* during germination in the midgut. While the solubility of Cry toxins may give them a more even

distribution, other factors could still be limited in how far they spread, such as the PlcR-dependent ones previously mentioned (Zhou, Slamti et al. 2014).

The apparent lack of any spatial structuring is not the only result from the competitions. As discussed in reference to the C1 and C2 mutants, growth rate alone is unlikely to account for any differences in fitness. However, considering growth rate in the context of public goods may explain the pattern, with non-producers gaining an advantage when rare (Turner and Chao 1999, Brown, Hochberg et al. 2002). The spatial structuring of clones would then make a difference in public goods-secreting communities. It appears that the frequency dependent fitness of strains, in particular here *entomocidus*, is important with patch structure of growing bacteria in the haemolymph mattering more in deciding spore production.

Together, growth rate and competitive ability of *B. thuringiensis* strains *in vivo* should reflect adaptation to a host. Outright growth, even *in vivo*, will not necessarily be a good predictor of fitness and spore production in co-infections, because of the varied effects of bacteriocins, virulence factors and other secreted products. These also contribute to the negative frequency dependence seen in the competitions, mostly likely in the form of public goods cheats which succeed when rare. The *kurstaki* and *aizawai* strains used are assumed to be well adapted to their lepidopteran hosts, including their competitive ability in-host, while *entomocidus* appears to favour growth mixed with these 'fitter' strains. Their competitive abilities and relative fitness matter in the wider context of the thesis work to improve *B. thuringiensis* insecticidal ability against initially resistant hosts. For example, while *entomocidus* can produce relatively high mortality when infected alone as shown here, its negative frequency dependent fitness suggests it could be a poor strain to use in experiments focusing on high levels of within-host relatedness.

Beyond demonstrating the effects of frequency dependence and absence of gut spatial structuring on *B. thuringiensis* co-infections this work was in part carried out to inform the experimental conditions used in subsequent selection experiments and experimental evolution. Selection of non-microscopic organisms allows enough time between generations that any individual can have its attributes measured with only certain selected individuals

permitted to reproduce. Selecting for higher host mortality in *B. thuringiensis* is instead done at the host level, with many rounds of bacterial proliferation occurring within. There will be a risk that within-host selection pressures counter host-level selection, causing strains with potentially desirable traits to be replaced before they can be isolated. Increasing toxin production is one mechanism for increased virulence, but the metabolic costs this brings might favour cheating with other public goods. As with the C2 mutant this could lead to negative frequency dependence which may limit persistence through the experiment. These considerations mean that selection experiments will require careful planning of selection protocols to produce highly insecticidal, stable *B. thuringiensis* isolates.

The *B. thuringiensis* strains targeted for improvement via selection should ideally be those already used in agriculture. There is existing regulation for these strains, while their effectiveness against different pest species is already established. Generating improved virulence in these strains through selection may not mean that they necessarily remain as viable before. The experiments with the *kurstaki* C2 mutant appear to show that increasing insecticidal activity can come at the cost of growth rate, which is likely linked to the negative frequency dependence it also showed. However, there is no certainty that such a trade-off would occur every time. If virulence were increased through a toxin conformational change giving improved binding to midgut receptors there is no reason why this would necessarily carry a metabolic penalty. In any case, C2 still reached sufficient densities to cause a detectable spike in host mortality, allowing it to be isolated.

Finally, it is worth remembering that if a protocol can produce an isolate with high insecticidal activity the isolate's poor competitive ability may not necessarily matter. *B. thuringiensis* is now primarily used as a source for Cry toxins to engineer into transgenic crops (Tabashnik, Brevault et al. 2013). Provided the increase in insecticidal ability is linked to changes in the toxin itself rather than upregulation of toxin production, the relevant *cry* gene can be removed from the strain, making the strain's lack of within-host competitive ability potentially irrelevant for commercial purposes.

III: Selective Passaging of *Bacillus thuringiensis* while Controlling for Within-host Relatedness

Abstract

Experimental evolution is well established as a method for exploring evolutionary dynamics, including the selective pressures that might affect a pathogen's virulence. At the same time it is being used in biotechnology to produce more efficient enzymes for commercial use. *Bacillus thuringiensis* is well known in biotechnology for its stock of insecticidal crystal (Cry) δ-endotoxins which are widely incorporated into pest-resistant crops, along with using the bacterium itself as a biopesticide. In recent years resistance has emerged in pests including *Plutella xylostella* moths. Experimental evolution of *B. thuringiensis* in its hosts provides a promising avenue for overcoming resistance and maintaining the necessary supply of effective toxins. Social theories of virulence suggest that increased relatedness within infections should promote cooperation, limiting the appearance of virulence factor cheats. From this it is predicted that increasing levels of within-host relatedness in experimental evolution will result in increased levels of virulence.

An assessment of the impact of within-host relatedness on the virulence evolution of *B. thuringiensis* was attempted. *B. thuringiensis* subsp. *aizawai* was selectively passaged through Cry1Ac-susceptible *P. xylostella* under four relatedness treatments: a control passage, a single bottleneck passage, a two bottleneck passage and a coinfection alongside *B. thuringiensis* subsp. *entomocidus*. Initial results suggested that the high within-host relatedness provided by bottlenecking favoured the evolution of more virulent strains. However, after accounting for concerns with the HCO sporulation medium used between *in vivo* passages to increase spore numbers, there was no apparent change in virulence for any treatment compared to the ancestor. The lack of a positive result suggests that under the current selective passage protocol within-host relatedness has no effect on virulence. Moreover, poor response to selection may have resulted from limitations in the supply of mutations, an issue that will be addressed in later work.

Introduction

Experimental evolution uses controlled conditions on an evolving population to explore the underlying evolutionary processes and test predictions about them (Rose and Garland 2009, Kawecki, Lenski et al. 2012, Rafaluk, Jansen et al. 2015). The technique can be a powerful tool to investigate how particular genotypes and phenotypes have arisen and the factors that might shape them subsequently (Elena and Lenski 2003). Experimental evolution was being utilised even as evolutionary theory appeared, with a contemporary of Darwin producing thermophilic protozoa (Dallinger 1878). Experimental evolution is most effective in organisms with short generation times and small sizes, which allow for large populations (Elena and Lenski 2003). Along these lines, experiments in *Drosophila* or bacteria are common e.g., Lenski's *Escherichia coli* long-term evolution experiment or Morgan's early work with *Drosophila* (Lenski, Rose et al. 1991, Elena and Lenski 2003, Blount, Borland et al. 2008, Burke, Dunham et al. 2010); simpler organisms can also be more easily characterised phenotypically and have their underlying genotypes and mutations identified. The increasing availability of next generation sequencing techniques for identification is likely to have contributed to the increasing use of experimental evolution (Van den Bergh, Swings et al. 2018). Short generations and large populations increase the likelihood that phenotypes or genotypes of interest are present or will arise within a practical timeframe (Elena and Lenski 2003). The former can decrease the absolute time that is then required for a genotype to spread through the population, though increasing the size of the experimental population will act in opposition. If the aim is to bring specific variation to fixation then the opposing benefits and costs of population size can be mitigated via introducing direct selection, greatly increasing the chance of phenotype fixation provided there is sufficient heritability (Ebert and Bull 2003).

Virulence defines the pathogenic lifestyle and understanding its evolution has clear applied benefits, most obviously in medicine and agriculture (Casadevall and Pirofski 2001, Pirofski and Casadevall 2012). There are examples of pathogens becoming more or less virulent as they continue to adapt to hosts; both can be seen in the *myxoma* virus, with initial attenuation

after its release into Australian rabbits, followed more recently by the appearance of novel, highly virulent strains (Kerr 2012, Kerr, Cattadori et al. 2017). The same increases and decreases have also been produced through experimental evolution in multiple different systems (Harrison, Browning et al. 2006, Ebert 2008, Racey, Inglis et al. 2010, Masri, Branca et al. 2015, Rafaluk, Jansen et al. 2015). Identifying the underlying principles that direct these changes should allow for better risk assessment and control of emerging zoonotic diseases or improvement in the efficacy of biocontrol. From the end of the 19th century much theoretical work has been done on virulence (Smith 1904, Alizon, Hurford et al. 2009), leading to hypotheses such as virulence-transmission trade-off (Anderson and May 1982). Better understanding of the systems at work (such as the role of cooperation) has led to refined models, but these still may not neatly describe all actual results (Alizon, Hurford et al. 2009). Models that look to predict the evolution of virulence can then be tested through experimental evolution, even if definitions of virulence in theoretical and experimental work may not fully overlap (Cressler, McLeod et al. 2016).

Experimental evolution with pathogens can take the form of serial passage experiments, either *in vitro* or *in vivo*, with some of the earliest carried out by Pasteur (Mendelsohn 2002, Rafaluk, Jansen et al. 2015) for work on the rabies virus. *In vivo* these can be coevolution experiments, or hosts can be taken from the same population for each round, which can be either naïve or have previous experience of the pathogen (one-sided selection) (Rafaluk, Jansen et al. 2015). Hosts are infected with the pathogen(s) of interest and then at the desired point in the infection cycle they are collected from the initial host and used to infect a new susceptible one. In passage experiments the need to apply pathogen selection and/or experimental constraints from working with potentially hazardous organisms mean that transmission is often facilitated by the experimenter (Rafaluk, Jansen et al. 2015). This removes selection pressures that may have been acting on the pathogen to maintain effective transmission (Day 2001). The idea of a transmission-virulence trade-off, derived from theoretical models, is well established in epidemiology and is separate to any effects of multi-strain infections (Alizon and Michalakis 2011). In its basic form, a pathogen needs to reproduce sufficiently to ensure there are enough infectious forms to infect new hosts. Reproducing comes at a cost to

the host though, with the end result potentially being host death. Clearly then, if the pathogen reproduces too rapidly it may risk killing its current host before transmission to new susceptible ones occurs (Frank 1996). This principal has some strong experimental support (Messenger, Molineux et al. 1999, Jensen, Little et al. 2006, de Roode, Yates et al. 2008, Alizon, Hurford et al. 2009, Doumayrou, Avellan et al. 2013). Serial passaging can remove this trade-off so that there should be no limit on how quickly a pathogen can reproduce and correspondingly no limit to how virulent it should become. However for obligate killers such as *B. thuringiensis*, trade-offs for rapid killing usually occur in terms of reduced production of infectious particles, especially if early death curtails host growth (Ben-Ami, Mouton et al. 2008, Raymond, Ellis et al. 2009).

Transmission is commonly thought to rely on either a vector between hosts or on an infected host being in close proximity to susceptible ones. *Bacillus thuringiensis* does not appear to require these mechanisms, instead using spores that can persist near-indefinitely in the environment under favourable conditions – spores can be found at high levels in soil globally (Martin and Travers 1989, Bizzarri and Bishop 2008, Ruan, Crickmore et al. 2015). However, there is evidence that spores can be recruited from the soil to leaves by plants (Raymond, Wyres et al. 2010) and of phoresis with nematodes in soil (Loguercio and Argolo-Filho 2015, Ruan, Crickmore et al. 2015). While there appears to be no direct trade-off between transmission and virulence for *B. thuringiensis*, as it does not require a live host for transmission, there still may be trade-offs between vegetative growth rate and quantity or quality of spores – less robust spores may not persist in the environment sufficiently long for a host to ingest them. Cry toxin production also occurs during spore formation (Hendriksen 2016) and could be involved in similar trade-offs, with more virulent strains producing fewer spores or toxins (Raymond, Ellis et al. 2009). Trade-offs between growth and spore/toxin production may also lead to cheats and selfish loss of toxin production (Turner and Chao 2003, Raymond, West et al. 2012, Deng, Slamti et al. 2015).

There is clear evidence that the virulence-transmission trade-off can be realised in serial passages with end-point strains producing higher mortality than ancestors in hosts, though increasing virulence appears to be favoured in novel hosts versus attenuation in former hosts (Ebert 1998). This evolution

through passage also implies that any initial infection contains variation, or that variation is rapidly generated during the infection (Alizon, de Roode et al. 2013). If this is the case for *B. thuringiensis* then it may provide a viable method for producing more virulent strains that could be commercialised – either directly, or by taking *cry* genes for use in transgenic crops. This could be employed either to overcome emerging resistance in pests such as *Plutella xylostella* (Zhang, Zhang et al. 2016, Tabashnik and Carriere 2017) or potentially to adapt a strain to novel pest species. If passages are to be used for commercial means then then they must be shown to be an efficient and effective way of improving virulence in the passage host. It is therefore prescient to investigate factors within a passage experiment that might favour the evolution of increased virulence. This is important beyond the application as it may also provide further support to particular theoretical views on the evolution of virulence.

For selective passaging to work, there must be variation present in the initial populations. In the case of *B. thuringiensis*, the aim is to select for an increased mortality phenotype with the expectation that this reflects change in underlying toxin genotypes. Variation in 3-domain Cry toxin peptide sequences should result in conformational changes to toxins when cleaved and activated in the insect midgut. This should affect their binding affinity to receptors on cells lining the gut (Park, Herrero et al. 2015, de Almeida Melo, Soccol et al. 2016). Improved binding could cause greater pore formation in the membranes of gut lining cells, resulting in more cell death and increased breakdown of the lining – though this assumes binding is the rate-limiting step in pore formation. This is both sufficient to cause mortality alone and increases the number of bacteria that can pass into the haemocoel, causing septicaemia (Raymond, Johnston et al. 2009).

The majority of Cry toxins are 3-domain (though certain toxins may lack one or more) (Aronson and Shai 2001, Xu, Wang et al. 2014) and it appears that the high diversity of these toxins has been generated partly through domain swapping in corresponding genes (Bosch, Schipper et al. 1994, Zheng, Gao et al. 2017). Such recombination in mixed infections has already been shown as central to the existing diversity of Cry toxins and within-host conjugation rates can be very high (Vilas-Boas, Vilas-Boas et al. 1998, de Maagd, Bravo et al. 2001). Domain swapping could be further facilitated by the ease with which *cry*

genes can be exchanged between strains (bringing them into contact with novel partners to exchange domains with). Horizontal gene transfer rates are high in *Bacillus* (Didelot, Barker et al. 2009) and *cry* genes are primarily plasmid-borne (Meric, Mageiros et al. 2018). Considering this in terms of increasing virulence through selective passaging, it may mean that using multiple strains in the same passage will increase domain shuffling or horizontal gene transfer in general. Shuffling could be of both plasmids (and toxin genes they carry) between bacteria and domain regions between genes, increasing toxin variation for selection to act on.

Allowing for co-infections between two (or more) strains in an infection can affect the overall observed virulence through competition (Frank 1996). Virulence models that take into account public goods (as Cry toxins are) show that as relatedness in an infection decreases, investment in virulence should also decrease (Brown 1999, Brown, Hochberg et al. 2002, Garbutt, Bonsall et al. 2011). Bacteria stop cooperating and instead divert energy to spiteful behaviour, such as the release of bacteriocins, rather than growth. While this could be explored with two distinct strains, their differing toxin suites may confound any potential observed changes. As the stated aim here is to increase virulence then relatedness of the pathogen population should be kept high through the course of the passage, to prevent the emergence of Cry-null mutants or other cheats (Deng, Slamti et al. 2015). The selective nature of the passage does not aid in controlling relatedness, as selection only occurs between (alive and dead) hosts rather than within-host. The only way to control relatedness within-host is to restrict the founding population, though this will not prevent clonal interference between emerging mutants. Restriction already appears to occur at the start of an infection, as most spores in the gut will pass through without proliferating in the haemolymph (Zhou, Slamti et al. 2014, van Leeuwen, O'Neill et al. 2015) and in hosts with high susceptibility to a particular toxin-strain combination, ingesting only a few spores may be sufficient to cause host death (Cornforth, Matthews et al. 2015). *In silico* work also predicts that ingesting small spore numbers should select for more virulent strains (Strauss, Crain et al. 2016). However, this is less useful in an experimental system, particularly when an aim of the passaging is to increase the virulence of strains that currently need to be ingested in larger quantities to be effective. Instead,

after recovering spores from cadavers, taking a single colony and then growing the infective dose up from this for the next round may be sufficient.

Bottlenecking to give a smaller founding population will ensure that high levels of relatedness are maintained (Frank 1996), with shared ancestry also giving similar relatedness over all alleles (Rumbaugh, Trivedi et al. 2012). However, the expected higher virulence of the infection is because of circumstances that favour maintenance of cooperation rather than any novel evolutionary change. Retaining high levels of relatedness will purge cheats from the population as well as genetic variation in general. In any public goods situation cheats are expected to invade a population of producers as relatedness falls (Brown, Hochberg et al. 2002, Garbutt, Bonsall et al. 2011), reinforcing the need for bottlenecking here. Purging cheats though is just an alternate way of saying that variation is being removed from the population, even if it undesirable variation in this case. This means that there needs to be a balance between maintaining relatedness but still allowing variation in pathogen virulence to persist so that higher virulence can then be selected for. Work by Garbutt, Bonsall et al. (2011) showed that mixed infections of *B. thuringiensis* produced isolates that were better at suppressing competitors but gave lower mortality. In serial passaging this appears best achieved by varying the rate of bottlenecking across treatments in search of an optimal level.

Previous work has already shown that for a given passage treatment cadavers from a particular treatment should not have all the spores they produce pooled together into a single group for the next round. Instead, sub-lineages should be maintained through the passage, as explained in the methods. Doing so produced more virulent clones and already confirms that relatedness can affect passage outcome (Dimitriu, Crickmore et al. unpub. data). This may also be because increased structuring in the overall *B. thuringiensis* population can better explore the landscape of mutations that would increase virulence (Nahum, Godfrey-Smith et al. 2015). Bottlenecking should allow for further fine control of the levels of relatedness.

B. thuringiensis has been successful as a biopesticide and tool for producing transgenic crops (Tabashnik, Brevault et al. 2013) because its Cry toxins have narrow target ranges but high diversity across these ranges

(Schnepf, Crickmore et al. 1998). High mortalities place strong selective pressure on evolving resistance, as seen by resistant populations of *P. xylostella* emerging in the field (particularly South East Asia) to all commonly used *B. thuringiensis* toxins (Iqbal, Verkerk et al. 1996, Gong, Wang et al. 2010, Zhang, Zhang et al. 2016). While robust agricultural and pest management practices may reduce the accumulation of resistance and limit the turnover of toxins (Gould 1998, Tabashnik, Brevault et al. 2013), novel ones to overcome resistance will still be required. Resistance to Cry toxins may be a result of changes in binding affinity with receptors in the midgut (Ayra-Pardo, Raymond et al. 2015), although multiple resistance genes have been identified even within the same population of *P. xylostella* (Gonzalez-Cabrera, Herrero et al. 2001). However, the exact mode of resistance that needs to be overcome should not matter when selecting for virulence at the host level.

The work carried out here will attempt to increase the effectiveness of *B. thuringiensis* subsp. *aizawai* in Cry1Ac-susceptible *P. xylostella* larvae through varying the level of relatedness in selective passages. The *aizawai* strain is principally used commercially because it produces higher mortality in pests such as *P. xylostella* that have become resistant to *kurstaki* and its Cry1Ac toxin, or can provide effective control of pests that are not particularly susceptible to the Cry1A toxins e.g., *Spodoptera* (Polanczyk, da Silva et al. 2000). However, *aizawai* toxicity in Cry1Ac-susceptible *P. xylostella* is much less than that of commercially exploited *B. thuringiensis kurstaki* strains (Chapter II, figure 2.3). These passages will attempt to raise its effectiveness against Cry1Ac-susceptible larvae; within-host relatedness can be controlled by isolating a single colony from the previous round and growing it up to the infective dose. The expectation is that the treatments with more bottlenecking will produce more virulent lineages at the passage end. An additional treatment will greatly increase the variation in-host by coinfecting *B. thuringiensis* subsp. *entomocidus* alongside *aizawai*. While this could increase the material available for horizontal transfer, it could also favour the emergence of antagonistic mutants over highly virulent ones.

Methodology

B. thuringiensis subsp. *aizawai* and *entomocidus* marker plasmid transformation

B. thuringiensis subsp. *aizawai* X1 isolated from XenTari® (Valent BioSciences LLC; Chapter II) spore product was transformed with plasmid pHT315 carrying *gfp* and *eryR* for erythromycin resistance and *B. thuringiensis* subsp. *entomocidus* (BGSC 4I4) transformed with pHT315 carrying *DsRed* and *tetR* for tetracycline resistance (Zhou, Slamti et al. 2014), using the methods described in Chapter I. Colonies grown after transformation were re-streaked on selective antibiotic plates and viewed under UV light to confirm plasmid uptake, giving *Bta* pHT315-*gfp-eryR* and *Bte* pHT315-*DsRed-tetR*.

Initial spore production

A single clone of *Bta* pHT315-*gfp-eryR* was isolated and used to inoculate 40 1ml wells of HCO sporulation medium (Lecadet, Blondel et al. 1980) in 24-well plates. The 24-well plates were sealed with tape and incubated for six days at 30°C with 150rpm shaking. After incubation, wells were pooled into four 15ml Falcon tubes and centrifuged at 3,200*g* for 6mins. The supernatant was discarded and pellets re-suspended in 500µl saline (0.85% NaCl) with 0.05% Triton X surfactant and pooled into two Eppendorfs. Pooled spores were pasteurised at 65°C for 25mins in a heat block. Spores were diluted in series to 10^{-7} with saline with 0.05% Triton X surfactant and plated out on 20µg/ml erythromycin 2% LB agar plates and incubated overnight at 30°C to confirm spore counts. The *Bta* pHT315-*gfp-eryR* spores were diluted to 20cfu/µl, which was expected to give approximately 30% mortality based on existing bioassay data, and plated out again to confirm cfu/µl.

Selection treatments & selective passaging (figure 3.1)

Cry1Ac-susceptible *Plutella xylostella* larvae (VLSS population) were divided into four treatments: control treatment, two treatments with population bottlenecks (either after second and fourth passage rounds or only after third)

and coinfection treatment where *aizawai* was infected alongside *Bte* pHT315-*DsRed-tetR*. The treatment with two bottlenecks should maintain the highest within-host relatedness, so the hypothesis is that it will produce the greatest increase in virulence. For the co-infection treatment, *aizawai* and *entomocidus* spores were mixed in ratio 4:1 while maintaining a constant *aizawai* spore density of 20cfu/µl. For each treatment there were four replicates, each with 10 subpopulations of larvae per passage round. For the first passage round a quarter of sterile diet in 50mm Petri dish was coated with 100µl of spore suspension and allowed to dry, then 12 late second-instar larvae added per subpopulation. This meant 120 larvae per replicate and 480 larvae in each treatment round. Subpopulations were incubated at 24°C, and cadavers counted every day. When mortality reached approximately 25% (usually after three days) in subpopulations, cadavers in the five subpopulations with the highest (or earliest if tied) mortality were collected into a 1.5ml Eppendorf using a sterile toothpick and 10µl saline added. Cadavers were then incubated at 30°C for three days. 50µl of saline was added and the cadavers mashed in Eppendorfs using sterile toothpicks. 50µl was then transferred into 1ml of HCO sporulation media with 20µg/ml erythromycin in a 24-well plate, with one well per subpopulation. Plates were sealed with tape and incubated for six days at 30°C with 150rpm shaking.

After incubation, each well had 2x200µl of culture transferred to wells in a 96-well PCR plate (for inoculating the next passage round) and a third 200µl to another PCR plate (for freezing). Plates were centrifuged at 3,200*g* for 15mins and 180µl of supernatant removed. Spores for the next passage round were re-suspended in 80µl saline, and those for freezing were re-suspended in 100µl of saline. The PCR plates for the next round were pasteurised at 65°C for 25mins in a thermocycler and stored at 10°C until use; spores for freezing were stored at -20°C. To produce inocula for the next passage round, the spore stocks were diluted in series using saline down to 10^{-3} and plated out onto selective plates to give colony counts. These counts were then used to adjust dilutions to give the desired inocula. For the co-infection treatment, the erythromycin added to the HCO sporulation media selected out any *Bte* pHT315-*DsRed-tetR* that had persisted through the infection, and so these were added to the inocula fresh from un-passaged stocks for each round of the treatment.

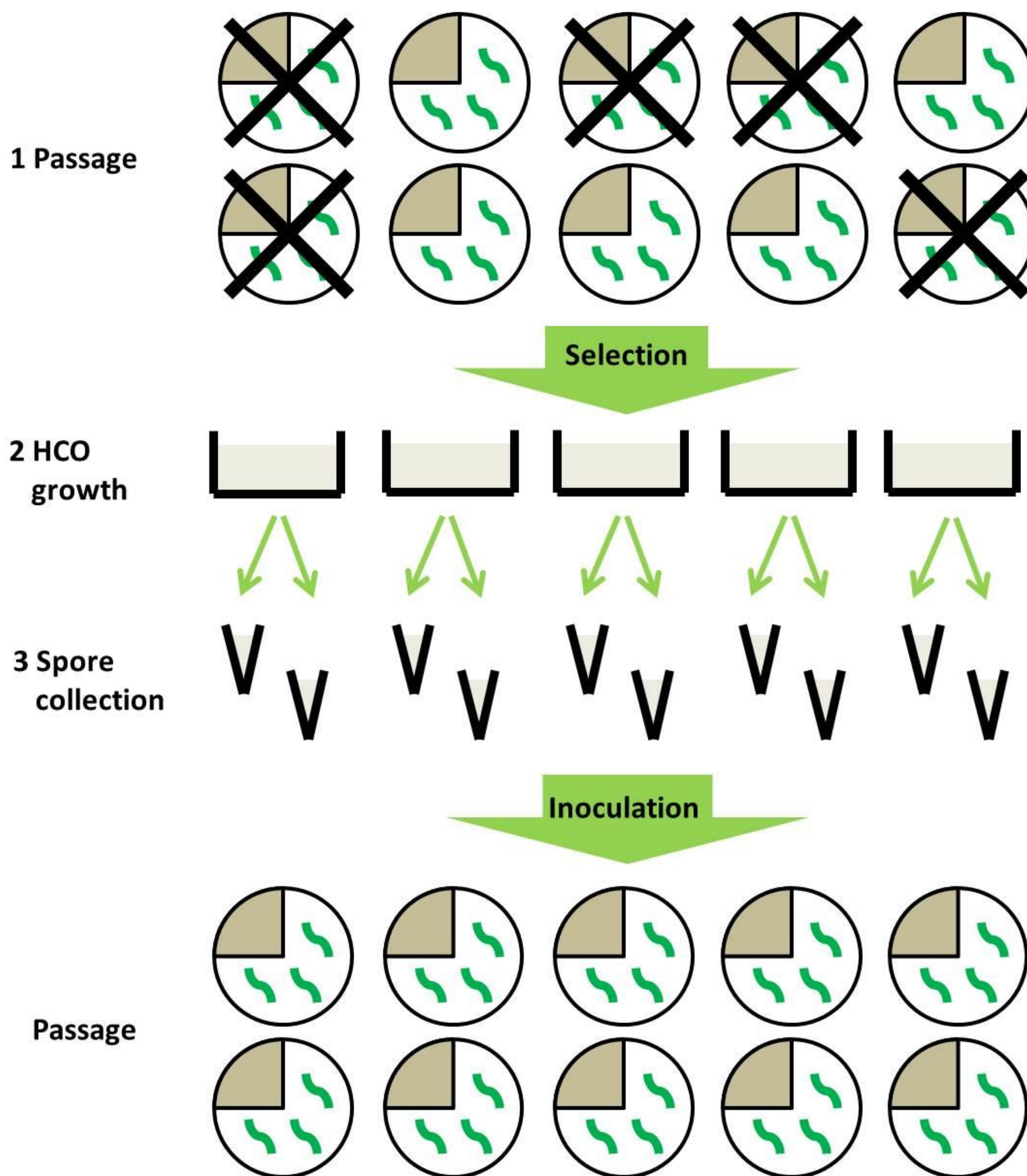

Figure 3.1: Selective passage protocol – core steps between infection rounds.

1 In a treatment replicate, the five subpopulations with the highest mortality are selected and cadavers from each moved to an Eppendorf.
Cadavers, with 10µl saline added, are incubated for three days at 30°C.
50µl of saline added and cadavers mashed using sterile toothpicks.

2 50µl inoculated into 1ml HCO with 20µg/ml erythromycin and incubated at 30°C for six days.

3 2 x 200µl moved into PCR plate wells and centrifuged at 3,200*g* for 15mins.
Spores re-suspended in 80µl saline, diluted in series and plated for counts, then dilutions adjusted for desired inocula.

Bioassays

After the fifth round, for each treatment replicate, spores from the subpopulation with the highest mortality in the fifth round were diluted to 40 and 20cfu/µl; higher dilutions could not be used because of the number of spores collected. These were bioassayed against Cry1Ac-susceptible larvae alongside the ancestor, with 60 larvae per treatment dilution. Spore inocula were added to quarters of sterile diet in 50mm Petris, with 100µl of inoculate per quarter, and then 12 late second-instar larvae added per Petri. These were incubated at 24°C for five days and then mortality scored. Spores collected from subpopulations were also grown up in HCO (as described above) to produce further spores for a repeat bioassay at higher cfu/µl. This bioassay was carried out in the same manner as the previous one, but with spore dilutions at 500, 100 and 20 cfu/µl.

However, complications over spore viability (as covered in Results) meant additional bioassays were needed. Rather than being based on spore counts, they were based solely on dilution factors from spore stocks grown up in HCO from those collected from subpopulations after the final passage round. All spores were diluted in a 3-fold series from a 750x dilution to 182,250x dilution, and these used in further bioassays against Cry1Ac-susceptible larvae as previously described. Actual cfu/µl counts were then also taken for these dilutions.

Finally, spore production in the *aizawai* X1 ancestor and passaged lineages was also assessed. 10µl of each were inoculated into 5ml 2$^{\%}$ LB and incubated overnight at 30°C with 150rpm shaking. Overnight cultures were diluted 10^{-3}-fold and 10µl of each inoculated into 1ml HCO in 24-well plates. These were sealed and incubated for four days at 30°C with 150rpm shaking. Culture samples were taken at two, three and four days and 10µl of each sample was also plated out onto 20µg/ml erythromycin 2$^{\%}$ LB agar and colonies counted. Samples were also taken at the same points and then pasteurised at 65°C for 25mins and then stained and plated out in the same manner.

This was then repeated with growth in both HCO (Lecadet, Blondel et al. 1980) and improved HCO adapted from Lecadet (Chapter I Methodology; with filter-sterilised salts added after initial autoclaving), with 10µl samples taken at

two and three days from both media and then pasteurised at 65°C for 25mins. Pasteurised and unpasteurised samples were stained on glass slides with Giemsa stain and then endospores and cells counted under a confocal microscope at 400x magnification.

Statistical analysis

For bioassay analysis larvae were recorded as either alive or dead, allowing mortality to be considered in terms of GLMs with binomial errors. To account for over-dispersion with a standard binomial distribution a quasibinomial one was then used. This was tested using the F-statistic at the overall level against a significance level of $p < 0.01$, given the noisiness of bioassay data, and using the t-statistic for within-treatment tests. Bioassay mortality data were analysed in RStudio 1.1.456 (based on R 3.2.5; R Core Team 2016, RStudio Team 2016).

Results

Preliminary work showed both that an antibiotic was needed during the HCO growth step to prevent contamination, and erythromycin at 20µg/ml was more effective at preventing it than tetracycline when combined with the corresponding resistance marker in transformed *aizawai* X1. During passaging not all replicates for each treatment could be continued through all five rounds to the end. This was primarily because of problems during the HCO growth step. Of the four treatments used in the passages, only the single bottleneck treatment had all four replicates pass through all five passage rounds. Three replicates reached the end for the two bottleneck treatment, two for the no bottleneck one and only a single replicate for the co-infection passage. This was because the wells in these replicates failed to produce any spores after a particular round and so could not be continued.

The replicates that did continue through to the end of the passage each had all the spores produced from their final subpopulations pooled together and bioassayed. This clearly showed (figure 3.2) that overall passage treatment was highly significant ($F_{4, 16} = 48.9$, $p = 9.02 \times 10^{-9}$), though only the two bottleneck treatment produced lineages that gave significantly higher mortality than the ancestor ($t_{16} = 6.126$, $p = 1.46 \times 10^{-5}$), appearing to confirm the initial prediction that high relatedness matters to virulence. However, a repeat bioassay (figure 3.3), while still showing treatment as significant overall ($F_{4, 27} = 13.8$, $p = 2.94 \times 10^{-6}$), now had only the single bottleneck treatment as producing a significantly different mortality ($t_{27} = 3.01$, $p = 5.66 \times 10^{-3}$) and this was now lower than the ancestor. It was also apparent when preparing spores for the bioassays that the visible quantities of spores in Eppendorfs did not correspond to plate counts they produced when diluted down to produce inocula, with the plate counts being lower than expected. This meant the result above could not be taken at face value. Replicates that appeared to have low spore counts would be diluted far less to reach inoculation levels, and so in fact could contain very high levels of toxin.

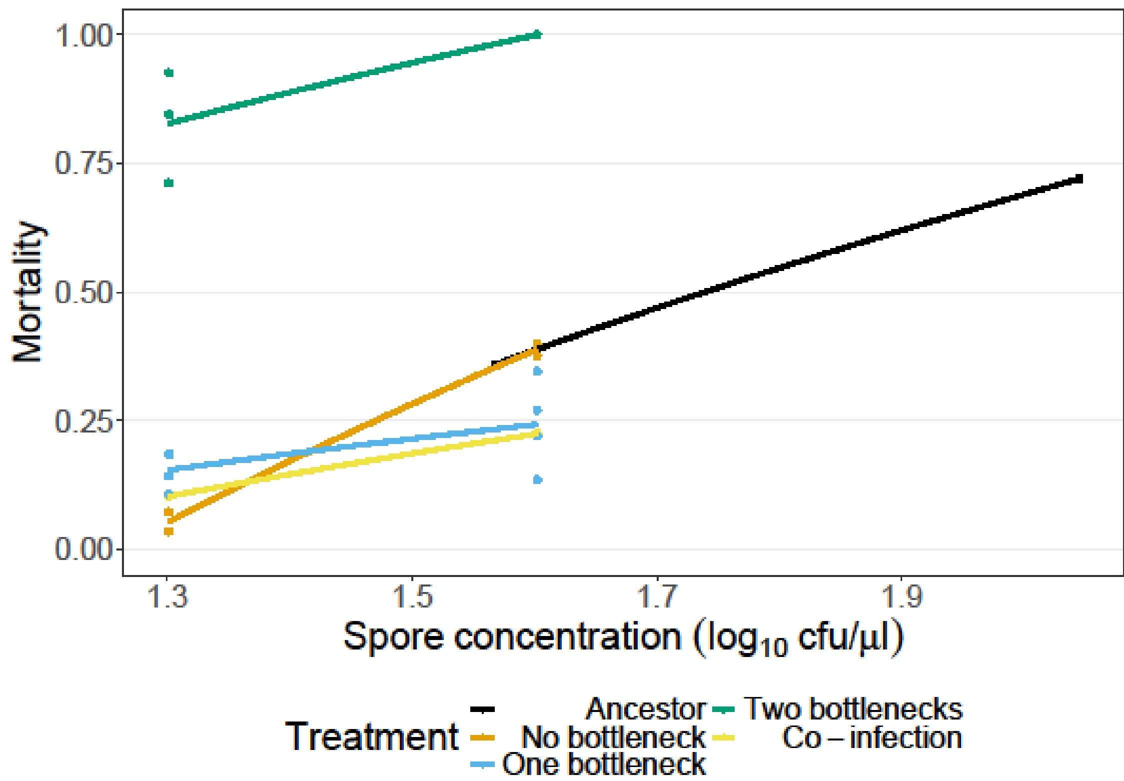

Figure 3.2: Bioassay mortalities of one lineage per passage treatment replicate in Cry1Ac-susceptible *P. xylostella* larvae against the wild-type *aizawai* X1 ancestor.

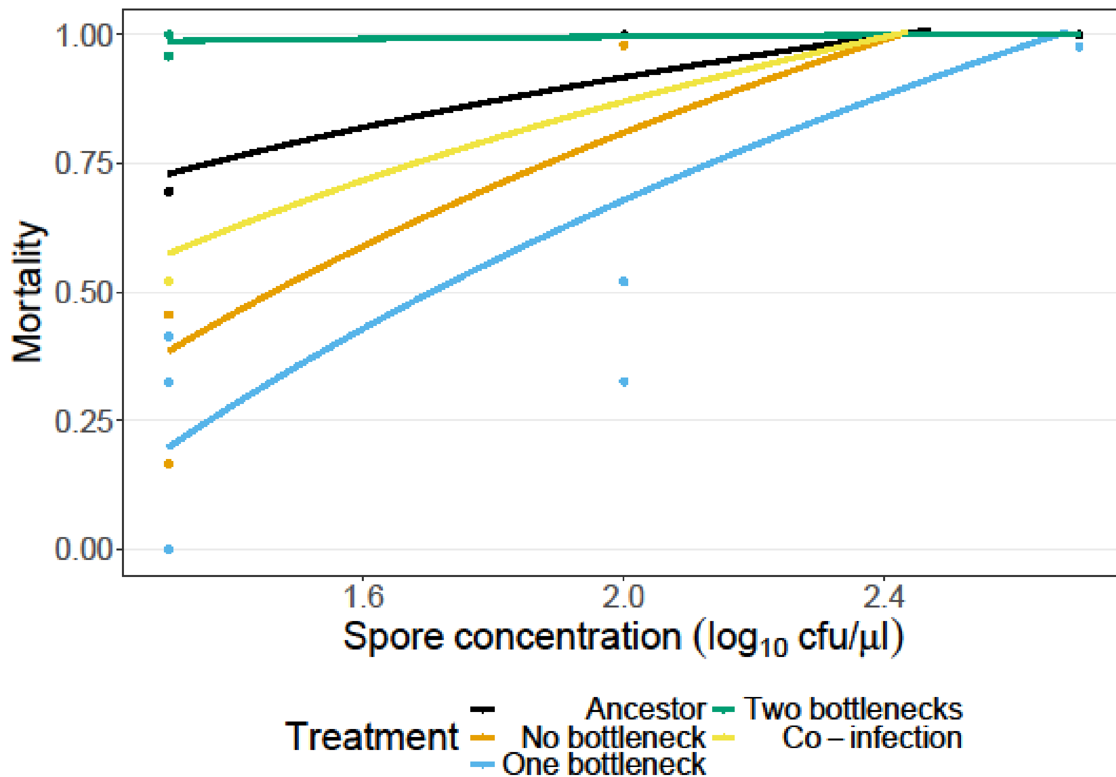

Figure 3.3: Bioassay mortalities of one lineage per passage treatment replicate in Cry1Ac-susceptible *P. xylostella* larvae against the wild-type *aizawai* X1 ancestor. This was a repeat of the bioassay carried out in figure 3.2.

To confirm this a third bioassay was carried out, except rather than using absolute counts replicate pool spores were all diluted by the same factors, and then counts also taken from these dilutions. In this bioassay, whether measuring against dilution factor (figure 3.4) or spore count (figure 3.5) there was no difference in host mortality for the passage treatments compared to the ancestor (for dilutions: $F_{4, 64} = 1.91$, $p = 0.121$; for spore counts: $F_{4, 51} = 0.601$, $p = 0.663$). It therefore seems likely that any differences in the initial bioassay are a result of differences in sporulation and the viability of spores produced. The lack of any difference in mortality between replicate pools meant that no individual isolates were subsequently assessed. Likewise, given the concerns over the mortalities produced at the end it was not considered worthwhile to assess mortality rates mid-passage using the frozen samples.

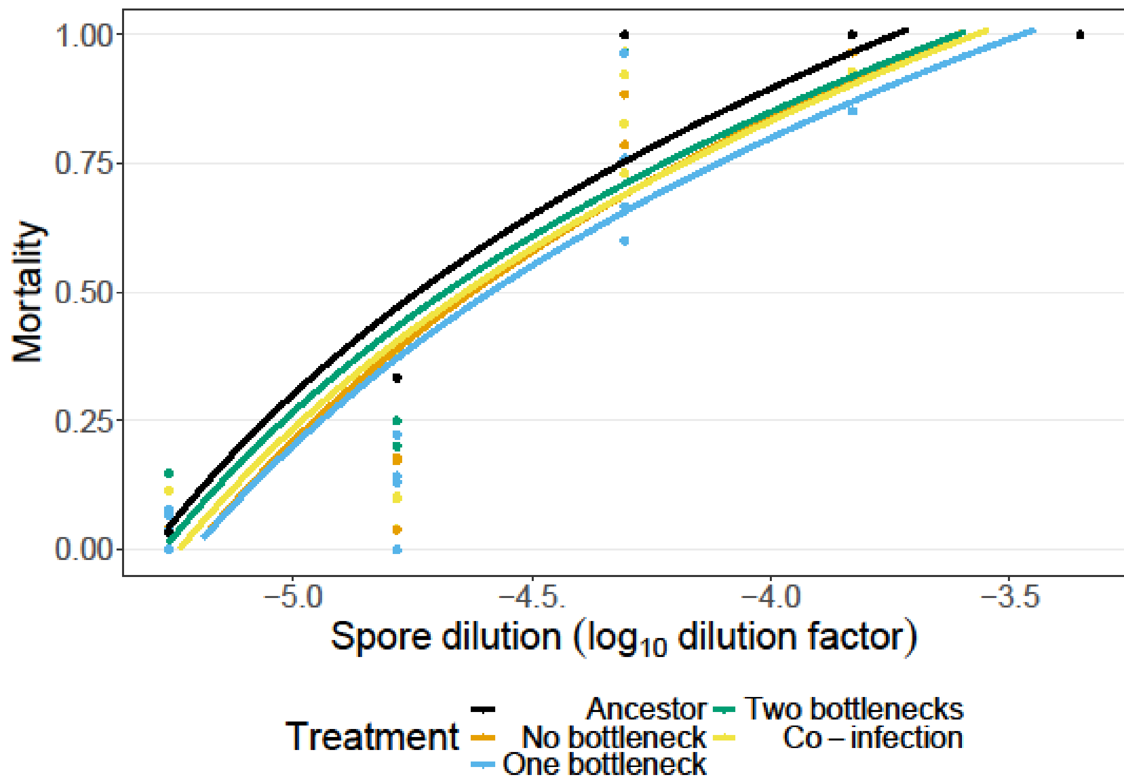

Figure 3.4: Bioassay mortalities of one lineage per passage treatment replicate in Cry1Ac-susceptible *P. xylostella* larvae against the wild-type *aizawai* X1 ancestor. The x-axis uses dilution levels for spores, rather than spore numbers counted, in attempt to account for poor spore germination on initial count plates.

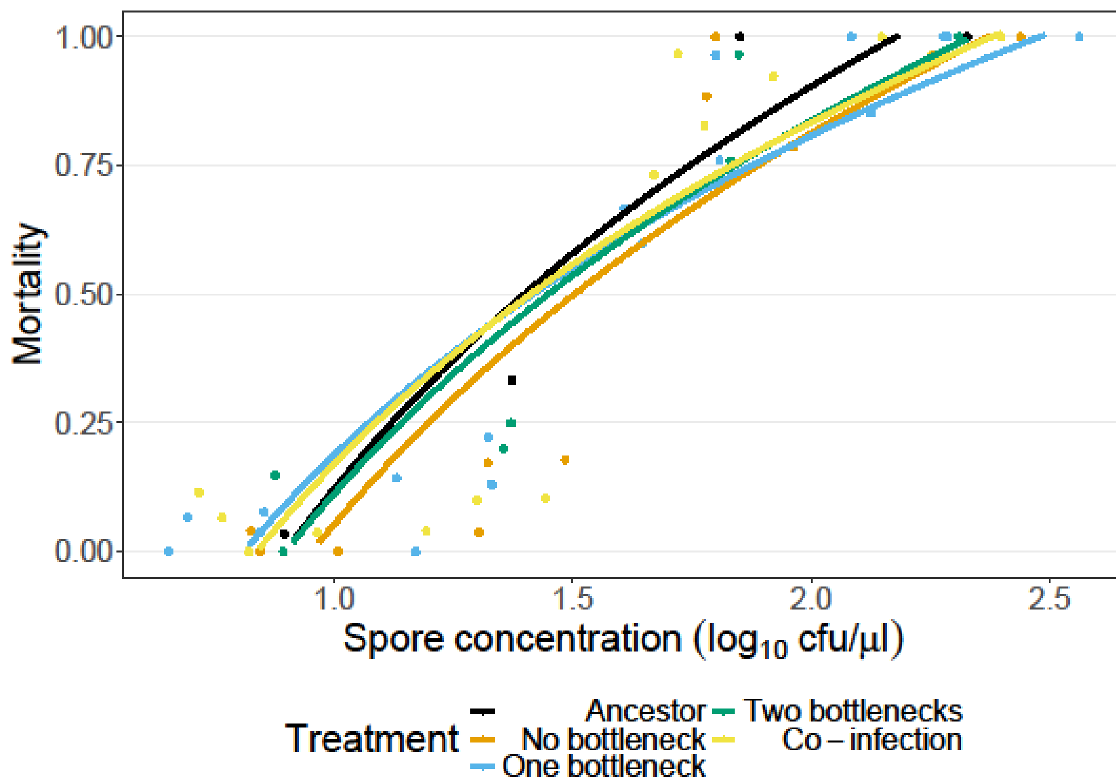

Figure 3.5: Bioassay mortalities of one lineage per passage treatment replicate in Cry1Ac-susceptible *P. xylostella* larvae against the wild-type *aizawai* X1 ancestor. This uses the same data as figure 3.4, but using cfu/µl counts taken from the dilutions.

Testing the sporulation rates of the ancestor and passaged strains in the same HCO formulation (figure 3.6) showed that after four days the ancestor produced more viable spores than any of the treatment strains ($F_{4, 54} = 6.15$, $p = 3.73 \times 10^{-4}$). When vegetative cell and spore counts were compared under microscope with Giemsa staining there was no overall effect of passage treatment on sporulation rates ($F_{4, 230} = 3.38$, $p = 0.0104$), though the single bottleneck and *entomocidus* coinfection treatments did have lower rates than the ancestor ($t_{228} = 3.05$, $p = 2.53 \times 10^{-3}$; $t_{228} = 3.07$, $p = 2.37 \times 10^{-3}$). Unsurprisingly, there was a higher proportion of spores in samples after pasteurisation ($F_{1, 228} = 12.2$, $p = 5.62 \times 10^{-4}$) though not by as much as expected, possibly because vegetative cells killed by pasteurisation were still visible and so counted. HCO sporulation medium type also mattered, though these counts suggest that the original Lecadet version produced a higher

proportion of spores ($F_{1, 229} = 24.6$, $p = 1.41 \times 10^{-6}$), even if this does not reflect experimental experience.

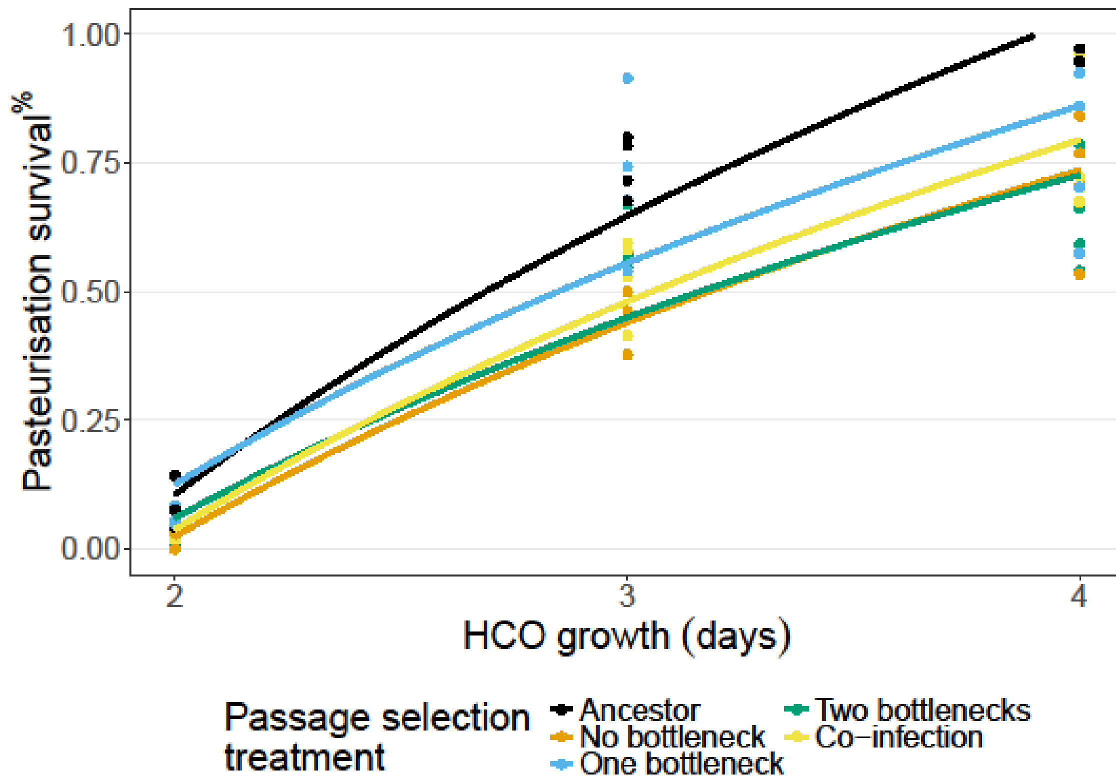

Figure 3.6: Sporulation rates of passage treatments against *aizawai* X1 ancestor. Plate counts were taken from HCO cultures after 2, 3 and 4 days incubation at 30°C, before and after pasteurisation. Pasteurisation survival percentage was calculated from post-pasteurisation counts divided by pre-pasteurisation counts.

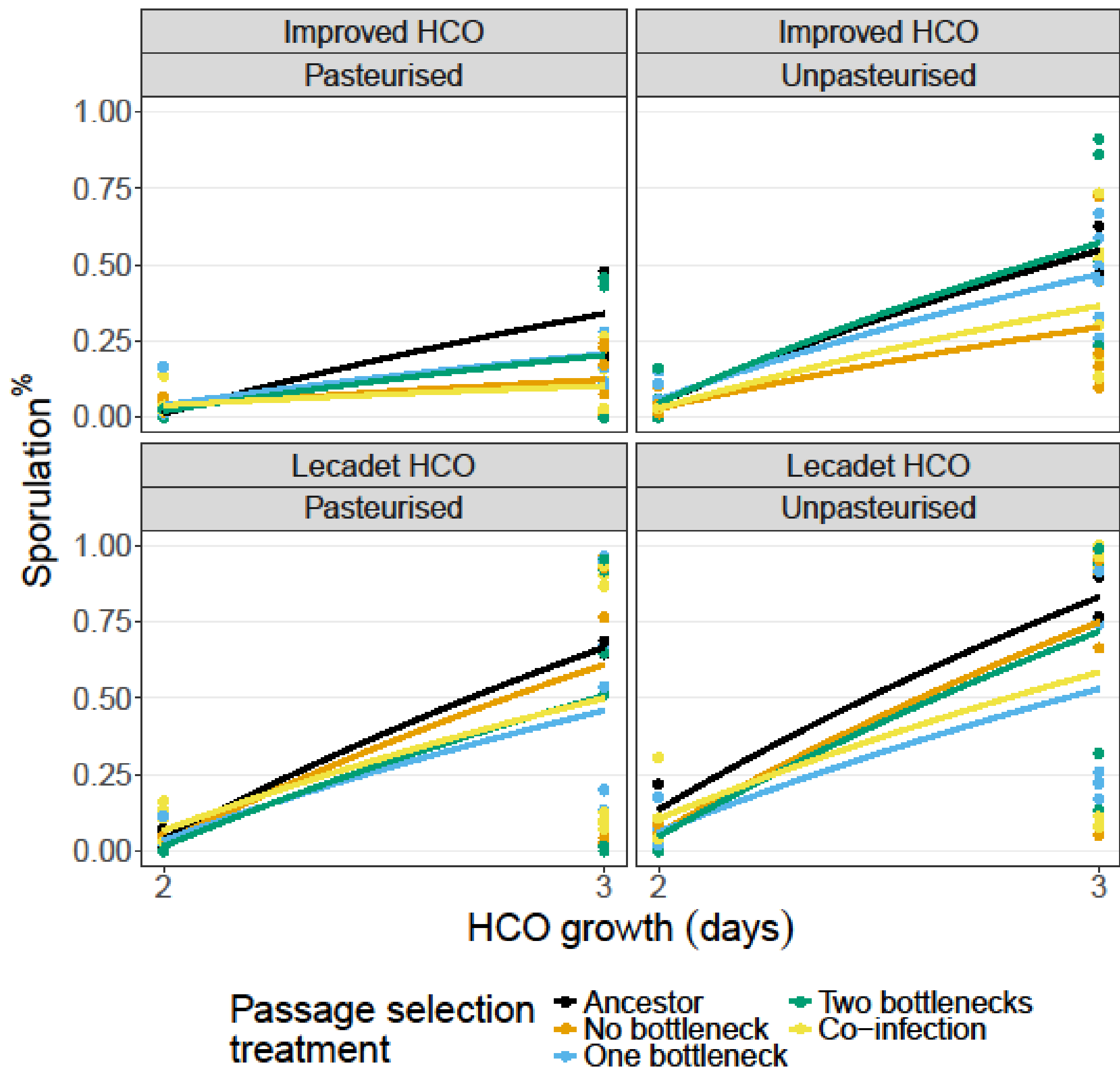

Figure 3.7: Sporulation rates of passage treatments against *aizawai* X1 ancestor. Vegetative cells and spores were counted under microscope with Giemsa staining. Counts were taken from HCO cultures (Lecadet, Blondel et al.'s (1980) original protocol or the improved protocol with filter-sterilised salts added after autoclaving) after 2 and 3 days incubation at 30°C, before and after pasteurisation.

Discussion

Strong population bottlenecking appeared to favour the evolution of more virulent strains, through a reduction in within-host relatedness. However, after conducting further bioassays accounting for sporulation rates caused by the since-changed HCO sporulation medium, no passage treatment produced isolates with significantly different virulences compared to the B. thuringiensis aizawai ancestor. It therefore appears that under the passage protocol used here virulence is not significantly affected by within-host relatedness; this neutral outcome may have resulted from a lack of available variation, which will be addressed in Chapter IV.

For the selective passage treatments to vary within-host relatedness it was expected that the treatment which maintained the highest level of relatedness would produce *B. thuringiensis* subsp. *aizawai* X1 lineages with the highest mortality in Cry1Ac-susceptible *P. xylostella* larvae. Initially it appeared this was the case, with the two bottleneck treatment producing isolates that gave approximately five times higher mortality than the ancestor at the same dose. Producing these mortality results after only five passage rounds would have been a promising success. However, subsequent work with the passaged lineages strongly suggested that any apparently significant results were affected by sporulation success in HCO and spore viability. This likely also explains the problems with losing replicates through the course of the passage because of insufficient spores being produced. Once these were accounted for, it appears that controlling within-host relatedness limits the ability to select for improved virulence, a potential concern noted in the introduction.

There appear to be two possible explanations for the actual results. The first is that passaging had no effect on *aizawai*, and this appears to be the case for its insecticidal abilities. The loss of replicates during sporulation could be down to insufficient bacteria being carried over from cadavers, although this appears unlikely as only a few spores should be need to inoculate the HCO and spore densities in cadavers should easily be sufficient for this (Raymond, Ellis et al. 2009). It is also unlikely that larvae otherwise dead were misidentified as

B. thuringiensis cadavers, as these are easily distinguishable. An alternative is that the passage selection protocol actually selects for something else and this overrides the aim of selecting for increased host mortality, although it has been suggested that in coevolution systems biotic selection pressures should override abiotic ones (Scanlan, Hall et al. 2015).

The most likely other target of (unintended) selection is sporulation. The bacteria spend five days of each passage round growing in HCO sporulation media, which is longer than they spend in live hosts. After growth in HCO the spores are pasteurised which should kill any vegetative cells so there is strong selective pressure to sporulate sufficiently quickly. Any particular lineage in the HCO that does not will be killed during pasteurisation and not infect the next round of hosts nor persist through the passage. Sporulation and Cry toxin production are tied together, with *B. thuringiensis* strains producing the majority of their Cry toxins during sporulation (Aronson and Shai 2001, Deng, Peng et al. 2014): triggering of *cry* genes linked to sporulation is controlled at multiple levels including through transcription by the chromosomal sporulation-specific sigma factors SigK or SigE (Deng, Peng et al. 2014). Increasing investment in one of sporulation or toxin production may come at the other's expense, particularly given the huge investment in producing Cry toxins which may make up 25% of a spore's weight (Agaisse and Lereclus 1995). This is perhaps illustrated by a strain of *B. thuringiensis* that has two forms for a division of labour, one a spore-former without toxins and the other a crystal producer that subsequently lyses to release them (Deng, Slamti et al. 2015). Increasing toxin production could be one way mortality is increased through selection, so also potentially selecting for faster sporulation would appear to counter to this. Pasteurising after five days should allow sufficient time for this pressure to be minor – the *aizawai* ancestor appeared to show more than 95% sporulation after four days. However, this would not necessarily prevent particular cheats from arising in the HCO that grow and sporulate even faster to make up a greater proportion of the spores collected for the next passage round.

Whatever additional selection pressures the HCO growth stage introduces, it seems difficult to exclude it from the passaging protocol. Preliminary work showed there were insufficient spores available to produce required doses and mortality for the next passage round if spores were taken

directly from cadavers. While the larvae used were raised under sterile conditions and passages also set up under them, it was also apparent that antibiotic screening was needed to prevent contamination. Both of these could be achieved by growing up collected spores in HCO, while there is no obvious method that would negate this step and its additional selective pressures on sporulation. Using strains that do not require a large infective dose to negate the HCO step would be reductive – a strain that requires a small dose already will cause high mortality and not need improving through selective passaging.

While increasingly rapid sporulation is perhaps the expected outcome of using an additional growth step it does not appear that this was the main problem with the passage, as sporulation rates appear higher in the *aizawai* X1 ancestor (figure 3.6). HCO is an established sporulation medium for *B. thuringiensis* (Lecadet, Blondel et al. 1980) and, despite the sporulation rates observed under microscope (figure 3.7), comparing these passages and subsequent in-lab work strongly suggested that the Lecadet protocol used here might affect its viability. In particular, the $Fe_2(SO_4)_3$ added before autoclaving did not always remain in solution; adding it and $MgSO_4.7H_2O$ as filter sterilised solutions after autoclaving appeared to improve the viability of spores. While HCO needs to be nutrient poor for starvation to trigger sporulation in *Bacillus* (Narula, Kuchina et al. 2015), the apparent lack of iron cations may have hindered successful sporulation, as adding trace iron sulphate to brewer's yeast extract appears to greatly improve *B. thuringiensis* sporulation rates in it (Saksinchai, Suphantharika et al. 2001). Again, it might be expected that the strains passaged through this HCO would adapt to it, but they all had lower sporulation rates than the ancestor. It is likely that the competing selective pressures of sporulation and host mortality produced this outcome, though it is difficult to unpick exactly why this particular result was produced, or how (if at all) the different levels of relatedness imposed by the passage treatments factor in to it. It also may be the case that Cry toxins attached to the surface of spores may aid in initial germination (Du and Nickerson 1996), so non-ideal sporulation conditions could potentially affect germination through disruption of this mechanism.

Despite the discovered concerns over the HCO sporulation medium used, it seems unlikely based on these results that changing the medium would

drastically affect the mortality of lineages at the passage end. It is not certain how effective trying to control for apparent poor sporulation was, but the results from the secondary bioassays based on dilutions rather than absolute counts suggest that there was no change in mortality. As discussed, sporulation and Cry toxin production are linked but the inability to sporulate or return to vegetative growth does not appear to have affected overall toxin production here. If toxin production was directly linked to viable spore production then the initial bioassay would not have suggested such a high mortality in the double-bottleneck treatment. The small number of viable spores meant it was hardly diluted for bioassays, so if there were large volumes of toxin this would not have been diluted, giving the increased mortality levels observed. In any case there are many other mechanisms through which mortality could be increased depending on the underlying mode of resistance, such as conformational changes to Cry toxins to restore binding efficiency to defective binding sites, or increased production of any other virulence factors a strain might possess such as vegetative insecticidal proteins.

As covered, relatedness should favour higher virulence but it will also limit the availability of variation for selection to act on. Strong bottlenecking may also allow genetic drift rather than selection to drive evolution, which could decrease fitness relative to the desired outcome (Silander, Tenaillon et al. 2007). Variation across the experiment could be improved by increasing the numbers used (or mutation rates, discussed below) – more passage rounds or more larvae used each round, either by increasing the number of replicates or subpopulations within the replicates. However, increasing the number of larvae or passages used without error is not practical for a single experimenter, though automation of experimental evolution is increasing principally to enable larger-scale experiments (Van den Bergh, Swings et al. 2018). Previous selective passages as part of the same overall project suggest that lineages with higher mortalities can be produced within the number of passage rounds used here (Dimitriu, Crickmore et al. unpub. data). Also, this is partly an attempt to find efficient means of overcoming resistance and increasing the number of passage rounds (and time taken) would dilute this aim. This leaves variation as the most practical avenue to explore further. The passages here did include a co-infection treatment with *aizawai* and *entomocidus* that would have increased

variation relative to other treatments, but there may still have been limited within-strain variation so the net effect of having two strains may have been limited. Additionally, *B. thuringiensis* strains produce bacteriocins which could be employed against each other (Raymond, Johnston et al. 2010, Garbutt, Bonsall et al. 2011). Antibiotic screening meant only *aizawai* was recovered after each passage round; this may have selected for *aizawai* that were good competitors e.g., through increased bacteriocin production, so they could be collected for the next round. This also fits with theoretical views that lower relatedness should lead to a breakdown in cooperation and lower virulence (Strauss, Crain et al. 2016). It was hoped the increased opportunities for horizontal exchange between strains with different toxin suites would offset this, but this does not appear to be the case, at least under the experimental conditions employed here.

Infecting with a larger dose would increase the variation within-host but this is of limited practical value. The strains used here were from clonal stocks so initial variation should be minimal. It is desirable that this remains the case as it means any isolates can be compared directly back to the ancestor, both in bioassays and then potentially via sequencing if lineages of note are identified. Also, the starting dose is strongly constrained by the need to produce intermediate mortality in the hosts. If all or none of the hosts die in most of the subpopulations of a replicate then it is impossible to apply selection when collecting spores. Therefore variation needs to be increased for a given population (inoculate) size rather than just increasing overall population size.

If population size cannot be altered, variation can instead be raised by increasing the rate of mutation, which can be achieved potentially either internally or externally. As few as 1-2% of mutations in bacteria are thought to be beneficial, with strongly beneficial ones only a fraction of this (Eyre-Walker and Keightley 2007, Sniegowski and Gerrish 2010), and the work here is only interested in a subset that will increase host mortality. Increasing the mutation rate should therefore increase the rate at which mutations of interest appear, and random mutagenesis has been widely used to improve particular qualities of genes or genomes (Labrou 2010). Working with bacteria rather than higher organisms allows for a greater degree of genetic manipulation, through which the intrinsic mutation rate of the organism can be altered. This can be done for

the whole genome by altering or removing genes involved in the proofing of DNA copying to increase the copy error rate e.g., by silencing the *mutS* mismatch repair gene (Horst, Wu et al. 1999, Packer and Liu 2015). The alternative is to introduce triggers for mutation such as radiation or chemical mutagens. These tend to favour a particular type of mutation depending on the mutagen employed e.g., alkylating agents like ethyl methanesulfonate causing alkylated guanine to be paired with thymine (Sega 1984).

Using a mutagen over a mutator strain appears to have a number of benefits. The mutagen concentration, and therefore mutation levels, can be calibrated and controlled, while it can be introduced for a limited period during the protocol rather than being present throughout. The problems with sporulation in this selective passage suggest that it might be unwise to complicate this step further through adding mutagens to it. Additionally, at the end of the passage any isolates of interest can be used as they are, instead of fixing mutator genes. Potential downsides of using mutagens are they will be harmful to the experimenter and so might place additional safety constraints on experimental procedure, while neither mutation method as described is site-directed. The effects of mutation rate on within-host competition and virulence evolution have been explored in a similar system with *Psedomonas aeruginosa* in *Galleria mellonella* waxmoth larvae, which suggested that increased mutation rates may favour increased virulence (Racey, Inglis et al. 2010); selective passaging could potentially enhance this further.

While increasing variation with mutagens is a potential pathogen-focused avenue, work by Masri, Branca et al. (2015) with *B. thuringiensis* BT-679 in *Caenorhabditis elegans* nematodes suggests that changes could also be made to how the host is used. The *P. xylostella* larvae used here were replaced each passage round from the same naïve lab population which excluded any opportunities for coevolution. While the *myxoma* virus showed that coevolution can lead to both attenuated and highly virulent strains (Kerr, Cattadori et al. 2017), theoretical work suggests that a coevolving pathogen should be under extremely high selective pressure from the correspondingly increasing host resistance (Woolhouse, Webster et al. 2002, Zaman, Meyer et al. 2014). When restored to a naïve host a coevolving pathogen is freed from the resistance mechanisms it has been battling to overcome, giving higher mortality compared

to either it in the coevolved host or the ancestral strain in a naïve host. In the *C. elegans* experiment, BT-679 lineages were more virulent after coevolution than pathogen-only evolution and in particular this corresponded to an increase in toxin plasmid copy number (Masri, Branca et al. 2015). For the passage here including coevolution was considered a treatment too many above looking for effects of within-host relatedness.

A final consideration is whether or not *aizawai* was an appropriate strain to use in this passage experiment. The strain was chosen in part because it is a well-known, commercialised strain and because of its limited effectiveness compared to *kurstaki* against Cry1Ac-susceptible larvae; as part of the overall project a similar passage was already being carried out with *kurstaki* in Cry1Ac-resistant larvae (Dimitriu, Crickmore et al. unpub. data). It may be the case that the current insecticidal activity of *aizawai* in Cry1Ac-susceptible hosts represents a local fitness peak and its toxin suite cannot be made more effective in this host background through selection (Orr 2009). Much of the diversity of Cry toxins in *B. thuringiensis* is thought to have arisen from horizontal transfer between strains to give different toxin suites alongside domain shuffling between toxins to produce new ones with novel effectiveness (Bosch, Schipper et al. 1994, de Maagd, Bravo et al. 2001, Meric, Mageiros et al. 2018). Therefore, even with increasing variation it may not be possible to increase the effectiveness of *aizawai* to the Cry1Ac-susceptible host population used here because its toxin suite is too different. This suggests that future work with this protocol might be more effective at restoring insecticidal activity in strains that have recently encountered resistance, as the desired fitness peak to overcome the resistance mechanism e.g., restoring effective Cry toxin binding, could be within reach.

Experimental evolution should negate the need for mass screening of toxins as a selective regime could produce Cry toxins which are now highly effective against the initially resistant host. Identifying optimal conditions for the production of highly virulent strains should allow for a set protocol where, once obtained, resistant pests immediately can be used in the protocol to produce a strain and ideally a specific Cry toxin that can then be employed successfully in the field. Alternatively, production of Cry toxins could be increased, giving a larger dose for a given number of spores. This can be achieved through

genetic editing such as increasing *cry* gene copy number or expression of P20 helper proteins (Arantes and Lereclus 1991, Shao, Liu et al. 2001), but could also be an outcome of selecting for higher mortality through passaging, rather than specific changes to Cry toxin effectiveness.

The selective passage protocol employed here requires further work. For certain, the concerns over the particular HCO sporulation medium used here need to be resolved before attempting to use it again. Experimental evolution has already been employed successfully for biotechnological aims (Van den Bergh, Swings et al. 2018) and there is no reason why further work with *B. thuringiensis* and selective passaging to overcome resistance will not ultimately be successful. Selective passaging has worked for other pathogens and there is no obvious reason why it should not for *B. thuringiensis* in *P. xylostella*. Maintaining an *in vitro* growth step may still limit the protocol in terms of providing another selective pressure but there does not seem to be a practical alternative when working with *B. thuringiensis*. However, this can also be viewed in a more favourable light as it would be difficult to work with a highly insecticidal strain which was also difficult to culture, particularly if it was being considered for commercial applications.

While the results here are not conclusive, they do suggest that a future attempt to adjust this protocol would be better served by increasing variation rather than attempting to limit it as here. The most obvious way to achieve this is through increasing the mutation rate, though there are multiple ways of achieving this.

IV: Selective Passaging of *Bacillus thuringiensis* in Multiple Host Backgrounds

Abstract

Experimental evolution has helped highlight which factors can increase the rate of evolution. Understanding these will be highly desirable when trying to increase the virulence of biocontrol pathogens, potentially reducing the time and cost. Long-term evolutionary experiments may favour mutators as these can generate beneficial mutations at a higher rate. Coevolution experiments appear to increase the rate of evolution compared to one-sided ones as selective pressure is maintained on both sides. These could be combined through carrying out coevolution experiments with mutagenesis. Biocontrol pathogens may be used against one or multiple host types so can be considered as specialists or generalists. However, generalists may have lower virulence across their range, potentially as a result of antagonistic pleiotropy.

The entomopathogen *Bacillus thuringiensis* is used against lepidopterans and selective passaging could increase its virulence against emerging resistant pests. It was predicted that passaging *B. thuringiensis* in coevolution with *Plutella xylostella* larvae will produce a greater virulence increase than repeated passaging in naïve larvae. Additionally, passaging in single larval genotypes is expected to result in higher virulence than passaging in multiple ones.

B. thuringiensis subsp. *entomocidus* was passaged in either Cry1Ac-resistant *P. xylostella* larvae, Cry1Ac-susceptible ones, alternating rounds of both or a coevolution treatment. All treatments included mutagenesis using ethyl methanesulfonate. After passages, lineages were bioassayed alongside the ancestor in resistant, susceptible and coevolved larvae to assess changes in virulence. Passage treatments increased virulence in coevolved and resistant larvae, and there was no difference between the single and multi-host passage treatments. Together these suggest that, after further refinement, coevolution with mutagenesis may be an effective way to produce more virulent *B. thuringiensis* to target resistant hosts in the field and prevent their spread.

Introduction

Experimental evolution is a powerful tool for investigating how particular conditions might influence the evolutionary path of an organism. Bringing evolution into a laboratory setting means variables apart from the trait or environmental factor of interest can be controlled and evolution directly observed (Elena and Lenski 2003, Kawecki, Lenski et al. 2012, Rafaluk, Jansen et al. 2015, Schlotterer, Kofler et al. 2015). As evolution corresponds to change over generations it is unsurprising that this area has strongly favoured work with bacteria; many generations can be gone through in a manageable time and space as exemplified by Lenski's 30,000+ generation *Escherichia coli* experiment (Cooper and Lenski 2000, Blount, Borland et al. 2008). The study of these experiments has been facilitated by the wider availability of fast, affordable whole-genome sequencing (Ensminger 2013, Van den Bergh, Swings et al. 2018). This allows direct tying of phenotypes to genotypes, along with easier identification and quantification of the many mutants that might appear during experimental culturing (Schlotterer, Kofler et al. 2015).

The variable(s) in an experimental set up may be abiotic or biotic in nature, with biotic systems featuring one or more additional species or genotypes along with the focal one. Biotic experimental systems are appealing most obviously because in the field organisms do not live in isolation, with many of the influences on their evolution coming through interactions with other organisms. Perhaps as a reflection of their importance, adaptation to biotic factors appears to take precedence over abiotic ones in experiments where both are varied (Scanlan, Hall et al. 2015, Van den Bergh, Swings et al. 2018). A clear example would be the interactions between pathogen and host; this is most apparent with the pressure for a host to evolve resistance and the pathogen to overcome this (Schulte, Makus et al. 2011). Better understanding of the factors that affect virulence evolution may aid the control of emerging pathogens, treatment of infectious disease (Pirofski and Casadevall 2012, Rafaluk, Jansen et al. 2015) or (as here) the improvement of biocontrol agents.

Experimental evolution of pathogens has primarily been done through serial passaging (Ebert 1998, Yourth and Schmid-Hempel 2006, Berenos,

Schmid-Hempel et al. 2011), with the pathogen repeatedly collected from one host or population and then inoculated into a fresh one. As transfer between hosts is often facilitated by an experimenter this generally allows the pathogen to evolve higher levels of virulence (Bull and Lauring 2014, Le Clec'h, Dittmer et al. 2017). While this may demonstrate the core truth of the virulence-transmission trade-off hypothesis (Anderson and May 1982, Alizon, Hurford et al. 2009), it gives little further insight into pathogen-host interactions in the real world. Lab populations of pathogens and hosts are often clonal or at least homozygous at loci of interest. This allows for uniformity, removing any potential confounding effects of different genetic backgrounds. However, in the field pathogens may not be infecting a single genotype, but multiple ones or even multiple species (Woolhouse, Taylor et al. 2001). They can therefore be characterised as either specialists or generalists. A generalist may experience an unpredictable supply of a particular host genotype and so is adapted to multiple ones; increased availability of hosts should favour higher virulence (Hellgren, Perez-Tris et al. 2009, Leggett, Buckling et al. 2013). This is effectively another example of the virulence-transmission trade-off – a specialist should delay killing its host to ensure a new suitable host is available (Pfennig 2001).

Conversely, infecting multiple hosts could constrain the virulence of generalists (Garamszegi 2006, Yourth and Schmid-Hempel 2006). A specialist can increase its virulence in one host without these adaptations having negative consequences elsewhere (Leggett, Buckling et al. 2013), and specialism appears to be the normal outcome from passaging (Little, Watt et al. 2006). A generalist may instead experience antagonistic pleiotropy: increasing virulence in one host is kept in check by negative selective pressure against the same adaptation in another (Ensminger 2013, Kerstes and Martin 2014). The contrasting effects of specialism and generalism on virulence can be explored by passaging through single or multiple host backgrounds. High virulence in a generalist would likely be desirable for biopesticide use, so passaging in single or multiple host genotypes may help inform how to achieve this.

The above only considers experimental evolution of the pathogen, with naïve hosts introduced for each passage round from a separate population. Clearly this is a poor approximation of actual pathogen-host systems as in

reality a host should also evolve resistance to its pathogens. Experimental coevolution therefore allows for both sides to adapt during the experiment (Kerstes and Martin 2014). For pathogen-host systems this is achieved by allowing any hosts that survive a passage round to breed, using their offspring as hosts in the following passage round. This can make coevolution experiments more difficult to carry out as host generation time is likely to be significantly longer than the pathogen's, so waiting for the next host generation can extend the time between passage rounds. There also need to be sufficient surviving hosts to produce enough offspring for the next passage round, which may constrain the infective doses used.

When host and pathogen coevolve it is expected that there will be a persistent 'arms race', where any increase in virulence is met by a corresponding increase in resistance and vice versa ad infinitum. Van Valen termed this the Red Queen hypothesis with both agents forever running to stay still, resulting in a higher rate of molecular evolution (Van Valen 1974, Paterson, Vogwill et al. 2010, Scanlan, Hall et al. 2015). A potential outcome of this is that exposing the evolved form of either to the ancestral version of its antagonist should result in improved performance e.g., a coevolved pathogen will have far higher virulence in a naïve host than its own ancestor would (Nidelet and Kaltz 2007, Schulte, Makus et al. 2010). Coevolution leading to increased virulence and resistance has been observed across many pathogen-host systems as well as *in silico* (Buckling and Rainey 2002, Schulte, Makus et al. 2010, Schulte, Makus et al. 2011, Masri, Branca et al. 2015, Strauss, Crain et al. 2016), though the reverse has also been observed in sexual systems (Berenos, Schmid-Hempel et al. 2011). When coevolution is combined with variation in the host and pathogen this is expected to lead to negative frequency dependent selection (Ebert 2008). If a particular host genotype is common, a specialist pathogen for this genotype should be favoured (Hamilton 1980, Ebert 2008). The specialist will in turn lead to selection against the most common host genotype, reducing or removing it. This should continue, cycling between different specialist pathogens, or specialists and generalists.

Any experimental evolution, whether considering host background or coevolution, requires sufficient time and variation for observable change. More time, in the form of additional passage rounds, allows for more mutations and

additional variation in the host or pathogen population. It also allows time for these mutations to become fixed in the population, with the time taken inversely proportional to the benefit (Elena and Lenski 2003). This may be particularly important in clonal populations, which are more common with pathogens, as clonal interference may limit the number of beneficial mutations that can be fixed in the population at any point; more time means more beneficial mutations can build up. However, dramatic changes in virulence can still occur in only a few passage rounds (Woo and Reifman 2014, Rafaluk, Gildenhard et al. 2015, Le Clec'h, Dittmer et al. 2017). *Wolbachia* evolved from being reasonably benign, because of its vertical transmission, to extremely lethal after only a few rounds of experimenter-mediated passaging in an isopod host (Le Clec'h, Dittmer et al. 2017). Importantly, while the number of passage rounds or host generations may be limited there should still be many rounds of pathogen reproduction within each of these. Additionally, the most beneficial mutations are likely to be fixed through selection earlier, resulting in decreasing fitness returns as a passage continues (Khan, Dinh et al. 2011).

If variation is being generated at a low rate then it may be difficult to produce observable change in a smaller number of passage rounds. To increase variation in a shorter passage experiment, a non-clonal founding population could be used (Rafaluk, Gildenhard et al. 2015). This has difficulties as the pathogen of interest may only be kept as a clonal stock and working with a clonal stock makes comparisons back to the ancestor, especially at the genetic level, simpler. Variation can instead be improved by increasing the underlying mutation rate during passages. A raised mutation rate appears to be favoured in long-term experimental evolution with mutators emerging and taking over populations, as mutator genes can hitchhike along with the additional beneficial mutations they have facilitated (Sniegowski, Gerrish et al. 1997, Shaver, Dombrowski et al. 2002, Elena and Lenski 2003). Clearly there is a limit to the variation that can be introduced, as above a certain mutation rate accumulation of deleterious mutations becomes too high and results in extinction (Martin and Gandon 2010, Packer and Liu 2015). Mutation rates can be increased with a mutator strain e.g., with a faulty *mutS* leading to poor mismatch repair (Horst, Wu et al. 1999, Packer and Liu 2015), or by including a chemical mutagen like ethyl methanesulfonate (EMS). EMS ($CH_3SO_3C_2H_5$) is

an alkylating agent that has been used as a mutagen in experiments with *Bacillus cereus* amongst other organisms (Necasek, Pikalek et al. 1967, Sega 1984). It is primarily thought to generate mutations through alkylation of guanine and cytosine residues, causing mismatches with thymine and adenine during DNA copying (Labrou 2010).

Increasing variation does not necessarily solve the problem of producing observable change in a short timeframe. Additional mutations are still constrained by the rate they can spread through the population. In a clonal population there will still be the problem of clonal interference with only the fittest mutation being fixed at any one time – any mutations that are beneficial to a lesser degree may be pushed aside and lost (Gerrish and Lenski 1998, Imhof and Schlotterer 2001). In bacteria approximately 1-2% of mutations may be beneficial, although it is difficult to accurately determine this rate and only a subset will be strongly beneficial (Eyre-Walker and Keightley 2007, Perfeito, Fernandes et al. 2007, Sniegowski and Gerrish 2010, Zhang, Sehgal et al. 2012). However, with a higher mutation rate it is expected beneficial mutations will occur more frequently, so even if one is initially lost through clonal interference it may still occur again before passage end. Alternatively, there is an increased chance multiple beneficial mutations will occur in the same background during fixation, limiting the number lost through clonal interference. Passaging of pathogens with raised mutation rates is expected to produce higher virulence, provided lethal mutagenesis is avoided.

The increase in deleterious mutations could potentially be limited with selective passaging. With pathogens, selection on host mortality can act as a proxy for virulence in most experimental situations (Bull 1994, Cressler, McLeod et al. 2016). Using selective passaging deviates from pure experimental evolution (Kawecki, Lenski et al. 2012) as the ability to evolve outside the desired direction is constrained by selection, though there is not full artificial selection on the pathogen as selection occurs at the host level or higher. This still allows insight into the evolution of virulence as comparisons between treatments should show which conditions favour evolution of the desired virulence level. Selection can also be employed to produce a pathogen for a specific purpose. Attenuated vaccines were generated by repeated passaging of human pathogens in other backgrounds resulting in a loss of human

virulence (Plotkin and Plotkin 2011, Kawecki, Lenski et al. 2012), but perhaps this could have been accelerated by selecting only pathogen lines that had the highest virulence each round. Selective passaging could also be used to overcome emerging resistance in hosts (Kolodny-Hirsch and Van Beek 1997, Berling, Blachere-Lopez et al. 2009).

Previous work (Chapter III) carried out selective passages on *Bacillus thuringiensis* subsp. *aizawai* isolated from XenTari® commercial product (Valent BioSciences LLC) in *Plutella xylostella* moth larvae. These passages had treatments that attempted to vary the level of within-host relatedness, as public goods models of virulence suggest that higher within-host relatedness should favour cooperation and increased virulence (Buckling and Brockhurst 2008, Raymond, West et al. 2012). Relatedness was varied by isolating single colonies between passage rounds and growing spores from these for the next round (bottlenecking) with the number of bottlenecks varied between treatments. The expectation was that frequent bottlenecking would produce lineages with the highest mortality, as these should maintain higher within-host relatedness. However, once bioassayed there were no significant increases in virulence from any treatment, including the control treatment with no bottlenecking. While the *aizawai* strain used did not sporulate reliably during the experiment there was still reasonable evidence to suggest varying relatedness in this way would not produce higher virulence. One explanation was that there was insufficient variation present. Extreme bottlenecking could act against improved virulence as it removes all heterozygosity and instantly fixes a set of mutations in the population, irrespective of whether they are the most beneficial ones currently available (Nei, Maruyama et al. 1975). As covered, variation can be increased through mutagenesis which appears to favour higher virulence in passages (Racey, Inglis et al. 2010). Using a mutagen rather than a mutator means the window during which increased mutation occurs can be controlled, so that mutants with stable phenotypes can be produced at the end of experiments. This seems favourable given the difficulties with the sporulation phase of the previous passage experiment (Chapter III).

The main concern with *B. thuringiensis* as a biocontrol agent, either directly or through its Cry toxins in transgenic crops, is the emergence of

resistance, notably in the South East Asian populations of *P. xylostella* (Zhang, Zhang et al. 2016) as well as *Spodoptera frugiperda* & *Helicoverpa zea* (Tabashnik and Carriere 2017). Selective passaging may provide a route to overcome resistance (Berling, Blachere-Lopez et al. 2009) while also gaining insight into factors favouring increased virulence. Host background, generalism versus specialism and coevolution could all potentially favour increased virulence in *P. xylostella*. *B. thuringiensis* subsp. *entomocidus* is not currently employed as a biocontrol agent and lacks the Cry1Ac toxin commonly used commercially (Crickmore, Baum et al. 2018). It therefore has intermediate virulence in both Cry1Ac-susceptible and -resistant *P. xylostella* larvae (Chapter II, figure 2.3). These two host genotypes provide a convenient system to test the effects of host background on pathogen virulence, through separate passages in the two genotypes. *P. xylostella* resistance to Cry toxins is thought to occur principally through modification of receptors such as abcc2 that act to prevent toxin binding (Baxter, Zhao et al. 2005, Baxter, Badenes-Perez et al. 2011). This means that it is unlikely adaptation to a particular receptor, giving an increase in virulence, should also permit increased binding to a different receptor in a different host genotype. Therefore it is hypothesised that selective passaging should increase virulence in each host genotype, but this increase will not carry over to the other background. There is already evidence that the virulence of *B. thuringiensis* can be increased through passaging in nematodes and that viral *P. xylostella* pathogens can be improved in this manner (Kolodny-Hirsch and Van Beek 1997, Schulte, Makus et al. 2010, Masri, Branca et al. 2015).

The difference between generalism and specialism can be compared between changes in virulence for the above single-host passages and for a two-background passage. For this passage, host background can be alternated between Cry1Ac-suscpetible and -resistant each passage round. Using both backgrounds in each round would be ineffective in a selective protocol as it would likely result in collecting spores only from Cry1Ac-suscpetible cadavers; these have higher mortality for a given *entomocidus* dose (Chapter II, figure 2.3). Selecting for increased virulence in both host backgrounds is predicted to produce an increase in virulence both genetic backgrounds, unlike the single

background passages, but the increase in each background will be less than the corresponding single background passage.

Finally, coevolution effects can be explored by allowing surviving *P. xylostella* larvae to pupate and breed, providing hosts for the next passage round. As explained, coevolution of pathogen and host should push both virulence and resistance higher during passaging. It is therefore hypothesised that coevolved *B. thuringiensis* subsp. *entomocidus* should produce higher virulence than both its ancestor and the other selective passage treatments in naïve hosts. Correspondingly, it should also have higher virulence in the coevolved *P. xylostella* than its ancestor. Similar comparisons between one-sided and coevolution are currently limited outside of bacteria-phage systems (Masri, Branca et al. 2015). All four treatments will be conducted with the same mutagenesis treatment using EMS. While it is hoped that increased variation will aid the passages, the effects of host background and coevolution on producing higher virulence are of more interest. As well as providing insight into which of these factors most favour higher virulence, it is hoped that this work may lead to refined selective passage protocols for producing more virulent strains to aid in controlling emerging resistance in insect pests.

Methodology

Transformation

B. thuringiensis subsp. *entomocidus* (BGSC 4I4) was transformed with pHT315 plasmid carrying *gfp* and *eryR* for erythromycin resistance. Wild-type *entomocidus* from stock was cultured overnight at 30°C with 150rpm shaking in 2% LB broth. 5ml of culture was added to 500ml pre-warmed 2% LB in a conical flask and incubated for four hours at 30°C with 150rpm shaking. 67ml of 50% glycine was added to give 3% in culture and incubated for an additional hour. The culture was transferred to eight 50ml pre-chilled Falcon tubes and these left on ice for 5mins. Tubes were centrifuged at 3,200*g* and 4°C for 12mins. Tubes were then put on ice and supernatant poured off. Cell pellets were gently re-suspended with 10ml ice-cold buffer 'F' (272mM sucrose, 0.5mM $MgCl_2$, 0.5mM K_2HPO_4 and 0.5mM KH_2PO_4 at pH 7.2) (Peng, Luo et al. 2009). This was repeated two further times, final re-suspension done with 500µl of buffer 'F' and transferred to a pre-chilled Eppendorf. 100µl of electrocompetent cells was added to a pre-chilled 0.1cm^3 electroporation cuvette with 2µl plasmid DNA and electroporated at 1.8kV using a MicroPulser Electroporator (Bio-Rad Laboratories, Inc.). The cells were transferred to 1ml pre-warmed 2% LB and incubated at 30°C for one hour. After, 100µl of culture was spread onto 2% LB agar plate containing 10ug/ml erythromycin and 900µl onto another and plates incubated at 30°C overnight. Colonies were re-streaked and viewed under UV light to confirm plasmid uptake, and the resulting transformed strain named *Bte* pHT315-*gfp*-*eryR*.

Spore production

Bte pHT315-*gfp*-*eryR* was inoculated into 40 1ml wells of HCO sporulation medium in 24-well plates. HCO sporulation medium was adapted from Lecadet, Blondel et al. (1980) and produced by adding 7g casein hydrolysate and 6.8g KH_2PO_4 in 1l distilled water and adjusting to pH7.2 with NaOH. After autoclaving 1ml 20% $MgSO_4.7H_2O$, 1ml 2% $Fe_2(SO4)_3$, 2.2ml 1% $ZnSO_4.H2O$, 2.2ml 1% $CaCl_2.2H_2O$, 440µl 0.5% $MnSO_4.4H_2O$ and 15ml 20%

glucose from sterile stocks along with two tablets of Polymyxin B (50,000 IU per tablet, Oxoid Ltd.) and 1ml of 20mg/ml erythromycin stock solution were added. Plates were sealed with tape and incubated for six days at 30°C with 150rpm shaking. After incubation, wells were pooled into four 15ml Falcon tubes and centrifuged at 3,200*g* for 6mins. The supernatant was discarded and spore pellets re-suspended in 500µl saline (0.85$^{\%}$ NaCl) with 0.05$^{\%}$ Triton X surfactant and pooled into two Eppendorfs. These were pasteurised at 65°C for 25mins in a heat block. Spores were then diluted in series to 10^{-7} with saline and Triton, plated out on 20µg/ml erythromycin LB agar plates and incubated overnight at 30°C to confirm counts. Spores were diluted to 400cfu/µl for the susceptible host passage, 2,000cfu/µl for resistant and 800cfu/µl for coevolution; the alternate host passage used the same doses as the susceptible and resistant ones, depending on which larvae were used that round. These were expected to give approximately 30$^{\%}$ mortality based on existing bioassay data, and plated out again to confirm cfu/ml.

Mutagen passaging & treatments (table 4.a)

The selective passage was split into four treatments. In the first *Bte* pHT315-*gfp*-*eryR* was passaged in Cry1Ac-susceptible *Plutella xylostella* larvae. The second treatment was in Cry1Ac-resistant larvae. In the third, host background was alternated in successive passage rounds between Cry1Ac-susceptible and -resistant larvae, with the first round in Cry1Ac-susceptible larvae. For the final treatment an initially mixed population of Cry1Ac-susceptible and -resistant larvae was coevolved with the passaged *Bte* pHT315-*gfp*-*eryR* bacteria. All treatments had five passage rounds with four replicates of ten subpopulations as per the passage protocol in Chapter III, though passage rounds were not carried out concurrently as coevolution passages could only occur when the experimental *P. xylostella* population had bred.

Passage treatment	Description	Prediction
Susceptible hosts	Five rounds of passaging in naïve Cry1Ac-susceptible larvae	Increased virulence relative to ancestor in Cry1Ac-susceptible larvae
Resistant hosts	Five rounds of passaging in naïve Cry1Ac-resistant larvae	Increased virulence relative to ancestor in Cry1Ac-resistant larvae
Alternate hosts	Five rounds of passaging alternating between naïve Cry1Ac-susceptible and -resistant larvae	Increased virulence relative to ancestor in each larvae type, but less so than above treatments
Coevolution	Five rounds of passaging in coevolved partially-Cry1Ac-resistant larvae	Increased virulence relative in coevolved larvae relative to ancestor; increased virulence in ancestor larvae relative to coevolved ones

Table 4.a: Passage treatments, with basic descriptions and principal predictions for each.

In the coevolution treatment each subpopulation of larvae in the first passage round had eight Cry1Ac-susceptible and four -resistant larvae. After cadavers had been collected Petri dishes were returned to the incubator at 24°C and left until all remaining live larvae had pupated. Pupae from all subpopulations across the four coevolution replicates were collected and moved into a single breading cage. Offspring were used in the next passage round in the same manner as naïve larvae in the other treatments.

For all four treatments the passage protocol was carried out as described in Chapter III Methodology, with the addition of a mutagen growth phase as follows (figure 4.1): After cadavers were collected 90μl of 2$^{\%}$ LB broth was added per Eppendorf, cadavers mashed with a sterile toothpick and incubated at 30°C with 150rpm shaking for three hours. After incubation, 10μl of 2$^{\%}$ by volume ethyl methanesulfonate (EMS) mutagen was added and Eppendorfs incubated for a further three hours at 30°C with 150rpm shaking. Eppendorfs were centrifuged at 2,400*g* for 5mins and supernatant removed. The pellet was re-suspended in 50μl saline and 10μl used to inoculate 1ml of HCO sporulation medium, as in the standard passage protocol.

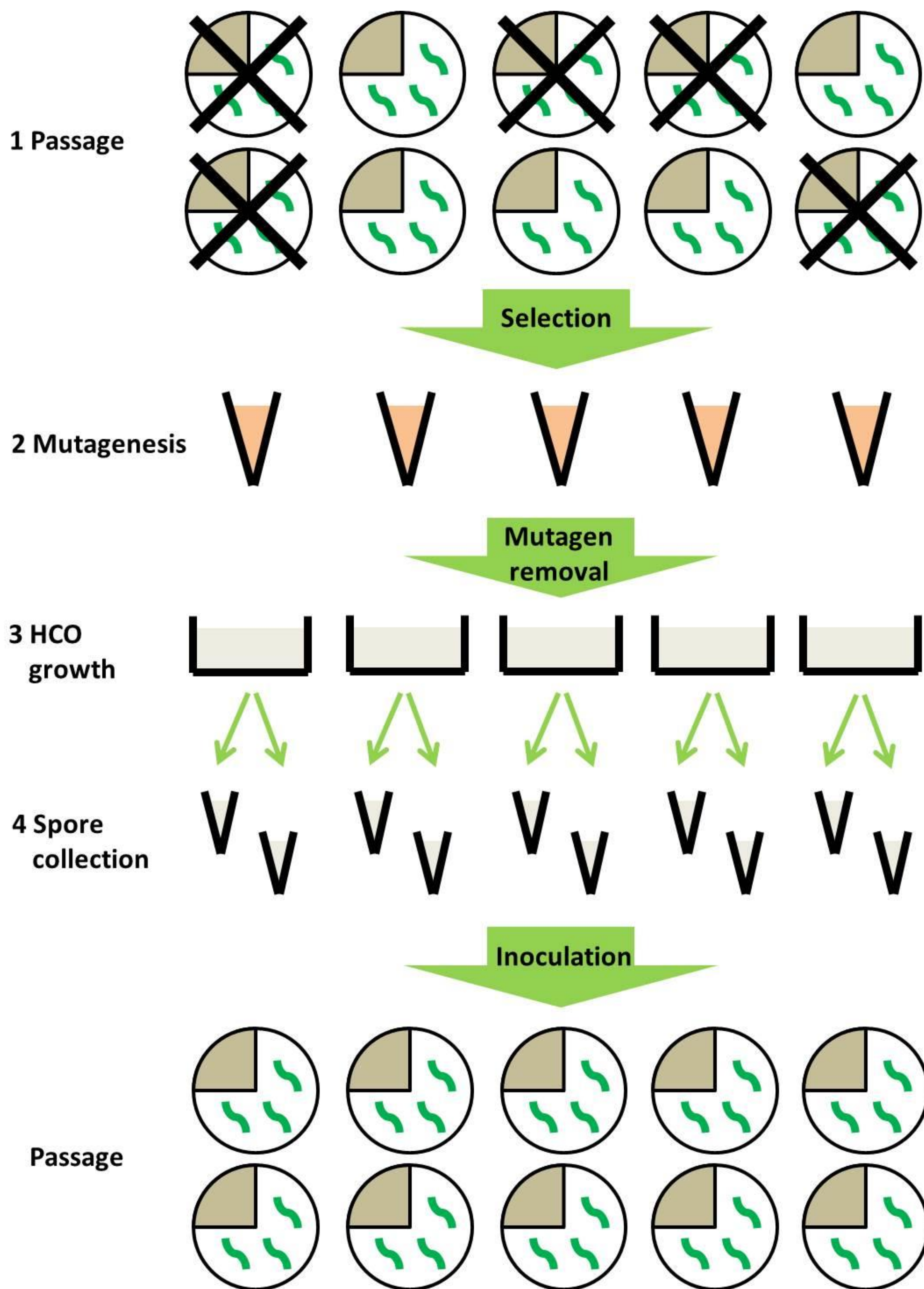

Figure 4.1: Selective mutagen passage protocol – core steps between infection rounds.

1 In a treatment replicate, the five subpopulations with the highest mortality are selected and cadavers from each moved to an Eppendorf.

2 Cadavers are mashed in 90µl 2% LB and incubated for three hours at 30°C; 10µl 2% ethyl methanesulfonate added and incubated for further three hours.

Mutagen removed via centrifuging at 2,400*g* for 5mins, spore pellet re-suspended in 50µl saline.

3 10µl inoculated into 1ml HCO with 20µg/ml erythromycin and incubated at 30°C for six days.

4 2 x 200µl moved into PCR plate wells and centrifuged at 3,200*g* for 15mins.

Spores re-suspended in 80µl saline, diluted in series and plated for counts, then dilutions adjusted for desired inocula.

Bioassays

After passages were completed, bioassays were carried out with passaged lineages against the *Bte* pHT315-*gfp*-*eryR* ancestor. All passage treatment bioassays used both Cry1Ac-susceptible and -resistant larvae. The resistant background and coevolution treatment bioassays were repeated in the coevolved population of *P. xylostella* (offspring of larvae that survived the final passage). The spore concentrations used differed depending on larvae being bioassayed: 40 to 4,000 cfu/µl in Cry1Ac-susceptible bioassays and 400 to 10,000cfu/µl in the Cry1Ac-resistant and coevolved bioassays. For all bioassays inocula were diluted from spore stocks recovered from the HCO growth phase after the final round of passaging. Stocks were diluted to give bioassay inoculants and plated out on 10µg/ml erythromycin 2% LB agar to confirm counts. In the bioassays, for each treatment replicate the spores from the single subpopulation with the highest mortality after the final passage round were used.

DNA extraction & sequencing

After bioassays, CTAB (cetyl trimethylammonium bromide) salt DNA extractions were run on a single pooled subpopulation per treatment replicate (16 in total across the four treatments), and on one isolated clone per treatment

for sequencing, along with the ancestor and one additional clone from the susceptible background treatment (for a total of six). Additionally, any clones identified as giving higher mortality also had DNA extracted. For extraction, pools or clones were cultured overnight for 16 hours in 2% LB broth at 30°C with 150rpm shaking. Cultures were pelleted by centrifuging at 17,000*g* for 10mins and supernatant removed before being re-suspended in 500µl lysis buffer (9.32ml 1mM TE buffer, 100µl 10mg/ml lysozyme, 500µl 10% SDS and 80µl 25mg/ml proteinase k) and incubated at 30°C for 30mins then 55°C for 20mins. 2µl of 100mg/ml RNAse A was added and samples vortexed, spun down and incubated at 30°C for 30mins. 100µl of 5M NaCl and 80µl of 10% CTAB 0.7M NaCl were added, samples vortexed again and incubated at 65°C for 20mins. For chloroform extraction, 700µl of chloroform:isoamyl (24:1) was added and mixed vigorously then centrifuged at 16,200*g* for 6mins and 550µl of the top aqueous phase moved to a fresh Eppendorf. This was repeated three times, except in the second round phenol:chloroform:isoamyl (25:24:1) was used instead. After chloroform extraction 0.6x sample volume of isopropanol was added and incubated at -20°C for 45mins. These were spun down at 4°C and 17,950*g* for 15mins. The supernatant was removed and DNA pellet washed in 250µl chilled 70% ethanol before being centrifuged at 4°C and 17,950*g* for 10mins and then supernatant removed. This was repeated and DNA pellets left to dry at 22°C for 20mins before re-suspension in 100µl 10mM TE buffer and left at 4°C overnight. DNA quality and yield were assessed by running 4µl of each sample with loading dye in a 1.5% agarose gel at 100V for 50mins as well as taking Nanodrop (Thermo Fisher Scientific Inc.) and broad-range Qubit (Thermo Fisher Scientific Inc.) readings of 10-fold dilutions.

The DNA is being sequenced via Illumina sequencing with 16 lineage pools at 200x coverage and six clones at 50x coverage by Exeter Sequencing Service at the University of Exeter. The ancestral *Bte* pHT315-*gfp*-*eryR* was also sent to MicrobesNG at the University of Birmingham, prepared there and sequenced via Illumina short reads with >30x coverage and Oxford Nanopore long reads.

Statistical analysis

In bioassay analysis larvae were recorded as either alive or dead, meaning mortality could be analysed via GLMs with binomial errors. To account for over-dispersion with a standard binomial distribution a quasibinomial one was then used. This was tested using the F-statistic at the overall level against a significance level of $p < 0.01$, given the noisiness of bioassay data, and using the t-statistic for within-treatment tests. Bioassay mortality data were analysed in RStudio 1.1.456 (based on R 3.2.5; R Core Team 2016, RStudio Team 2016).

Results

Mutagen passages

All four replicates in each treatment persisted through all five rounds of passaging (figure 4.2). However three replicates did lose subpopulations because of either insufficient mortality to select from or occasional failures of sporulation. The Cry1Ac-susceptible host background and alternating host background treatments maintained a full set of subpopulations in all replicates. The coevolution treatment lost all but two subpopulations in one replicate, while the Cry1Ac-resistant host background passage lost two subpopulations in two replicates. Figure 4.2 shows the lineages for each treatment replicate. The improved persistence of subpopulations, with no replicates completely failing, suggests that changes made to the production of the HCO sporulation media compared to that used in the Chapter III passages were successful. This was principally achieved by adding the majority of required salts and glucose after initial autoclaving as detailed in the Methodology. This was not distinctly tested, but after moving to the new HCO formulation for general lab work there were no longer cases of completely failed sporulation.

The ancestry through the selective passages of isolates collected after the final passage round is shown in figure 4.2. In all but two treatment replicates (coevolution replicate 3 and resistant host replicate 1) the isolates collected at the end were descended from only two of the initial 10 subpopulations. The figure also shows that in three treatment replicates the number of subpopulations used decreased from ten, primarily because of poor spore growth after using cadavers to inoculate HCO sporulation medium.

Mortality for each treatment passage round was recorded (figure 4.3); however there is limited use in analysing this further, as any change in mortality for a particular treatment round was compensated for in the next by altering spore dose levels, as shown by increases in mean mortality being followed by a decrease in the next passage round. This was to try and maintain a mortality rate of 20-30%, so the selection of subpopulations based on differing mortality could still occur.

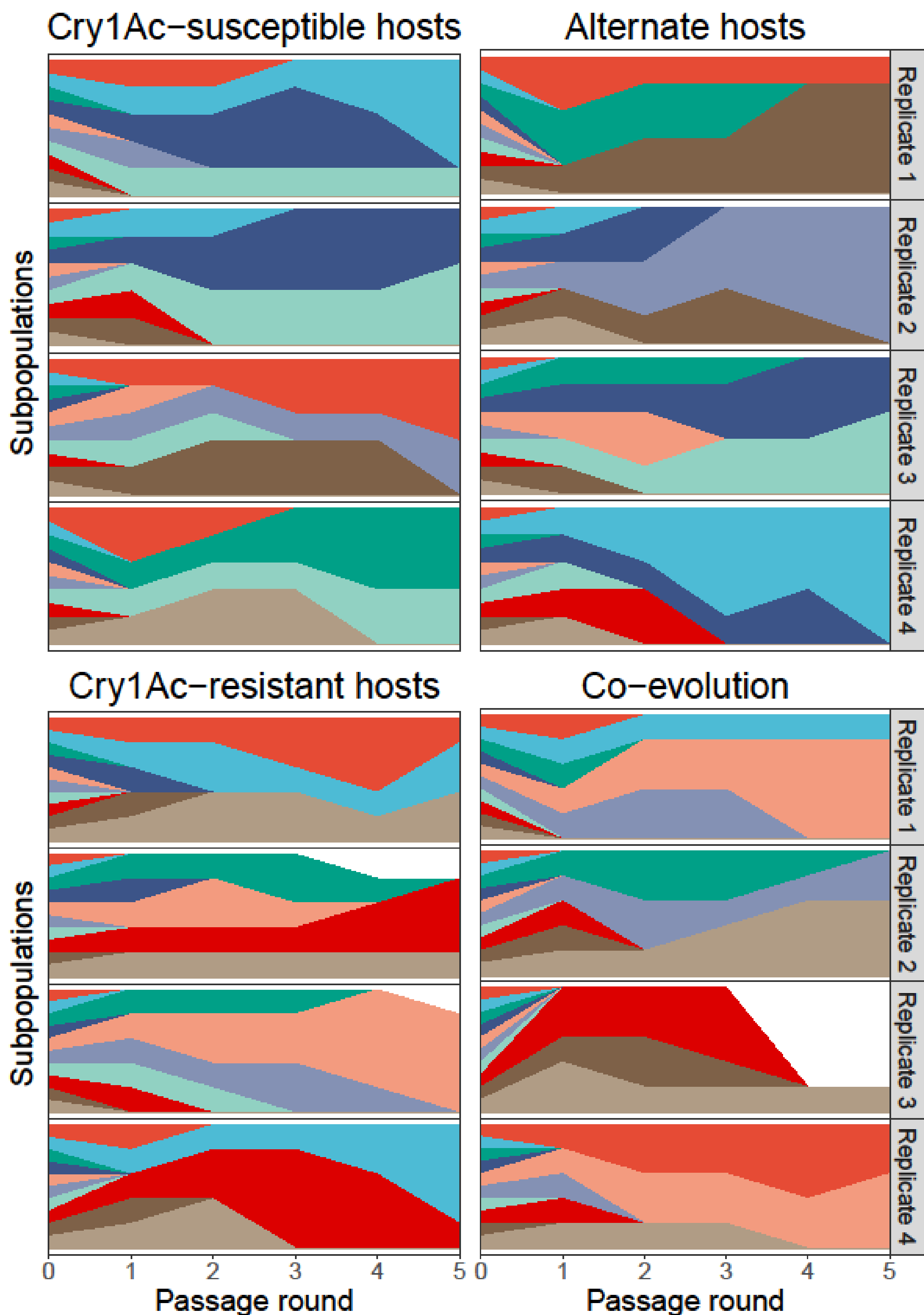

Figure 4.2: Tracking lineages over the five rounds of mutagen selective passaging, showing ancestry of end-passage isolates. Each passage treatment is split into its four replicates, with each initial subpopulation a different colour. White corresponds to fewer than 10 subpopulations in a passage round, as a result of no mortality for selection or insufficient spore growth.

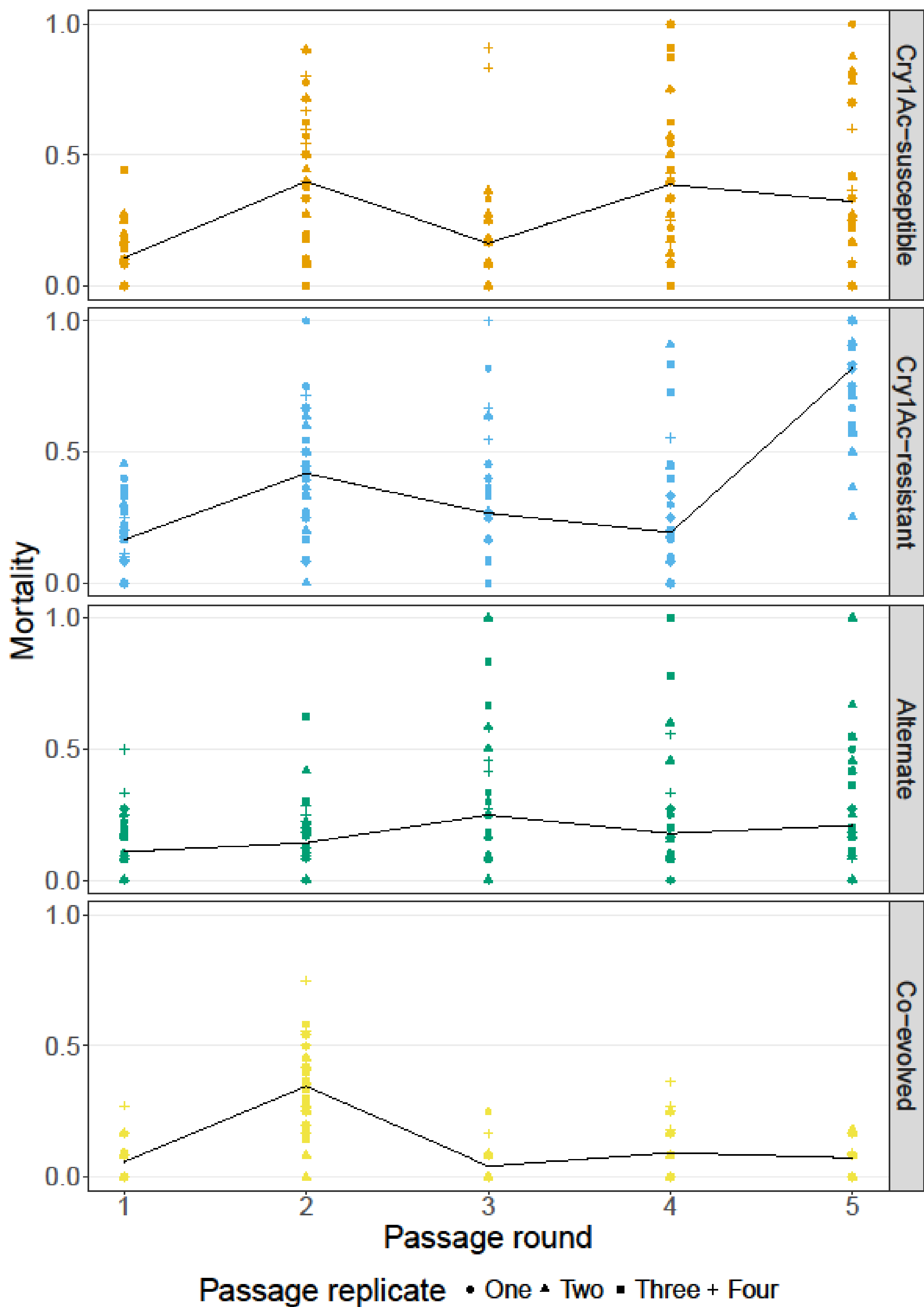

Figure 4.3: Mean *P. xylostella* mortality for each passage round in each treatment. Inocula were varied from round to round to try and maintain a 20-30% mortality rate, so increasing mortality here is not necessarily reflective of an actual increase in the individual spores.

Bioassays (figure 4.4)

There was an overall mortality difference between passage treatments and the ancestor when bioassayed in Cry1Ac-susceptible larvae ($F_{3, 69} = 7.25$, $p = 3.02 \times 10^{-4}$). This result fits with the prediction that passaging in general should increase virulence in naïve hosts. However, this was only caused by a single lineage per treatment diverging from the ancestor in the resistant and susceptible passage treatments ($t_{62} = -2.661$, $p = 9.91 \times 10^{-3}$; $t_{62} = -3.74$, $p = 4.11 \times 10^{-4}$) and the alternating host one ($t_{62} = -8.24$, $p = 1.5 \times 10^{-11}$). This particular alternating host lineage (lineage 3) can clearly be distinguished from all other treatment lineages (figure 4.4, highlighted in figure 4.5). This large increase goes against the initial prediction that generalism should give a lower increase in virulence than passaging in a single host type.

In the Cry1Ac-resistant host, again treatments overall produced higher mortalities than the ancestor ($F_{4, 115} = 11.2$, $p = 1.47 \times 10^{-7}$) and the same was true for the resistant, susceptible and alternating host treatments when considered separately ($t_{103} = -5.24$, $p = 8.56 \times 10^{-7}$; $t_{103} = -2.64$, $p = 9.54 \times 10^{-3}$; $t_{103} = -3.69$, $p = 3.55 \times 10^{-4}$) though the coevolution passage treatment was not significant at the $p = 0.01$ level ($t_{103} = -2.01$, $p = 4.66 \times 10^{-2}$). At the lineage level, the same alternating host lineage that had significantly higher mortality in Cry1Ac-susceptible larvae had significantly lower mortality here ($t_{103} = 4.42$, $p = 2.43 \times 10^{-5}$) (again highlighted in figure 4.5).

When bioassayed with larvae from the coevolved experimental host population, strains from the coevolution and resistant background treatments produced higher mortalities overall than the ancestor ($F_{2, 62} = 10.9$, $p = 1.02 \times 10^{-4}$). All the lineages from the coevolved passages had significantly higher mortality than the ancestor ($t_{56} = -2.96$, $p = 5.23 \times 10^{-3}$), though only two of the four resistant host treatment lineages gave significantly higher mortalities ($t_{56} = -3.37$, $p = 1.35 \times 10^{-3}$; $t_{56} = -4.41$, $p = 4.75 \times 10^{-5}$). The small size of the coevolved host population meant that only lineages from the resistant background and coevolved passage treatments could be assayed against it.

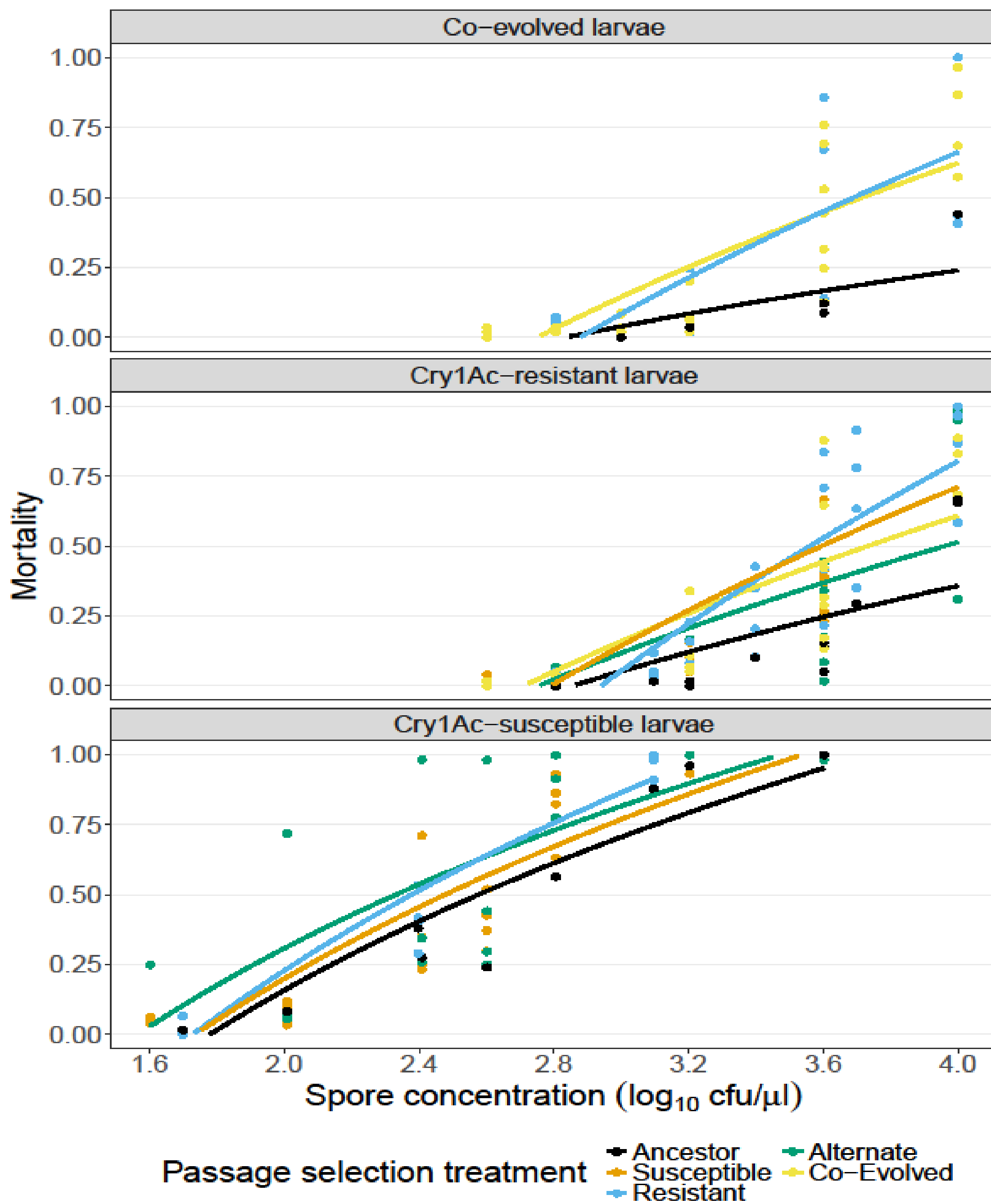

Figure 4.4: Bioassay mortalities of one lineage per passage treatment replicate in Cry1Ac-resistant and -susceptible *P. xylostella* larvae, as well as in the coevolved larvae for the resistant and coevolved passage treatments.

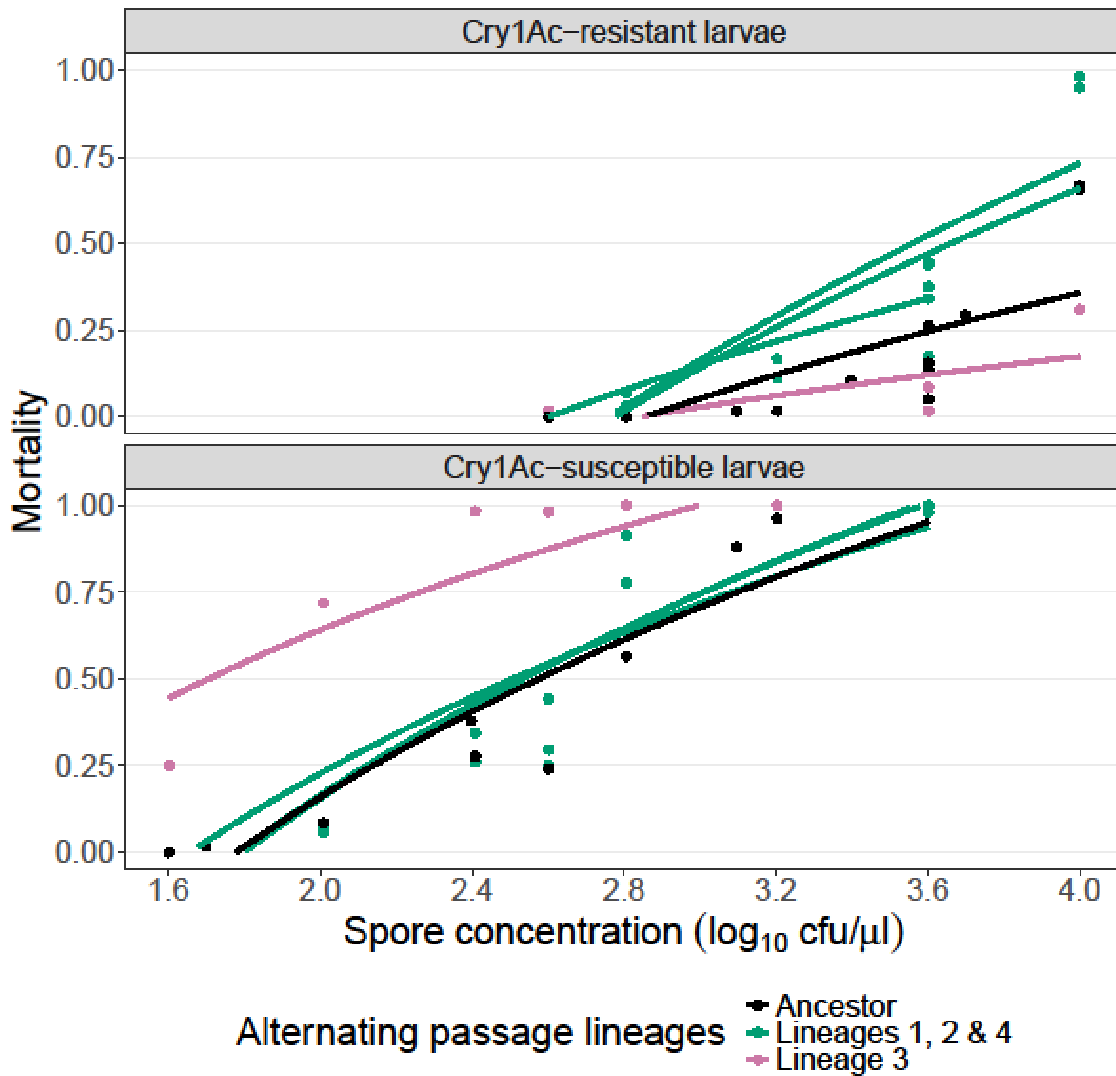

Figure 4.5: Variation between lineages in response to selection in the alternating treatment; bioassay mortalities of Cry1Ac-resistant and -susceptible *P. xylostella* larvae for one lineage per treatment replicate of the alternating host background passage treatment, alongside the *entomocidus* ancestor.

Beyond the overall trends described, two lineages can be highlighted, as mentioned. In the alternating host passage one lineage developed significantly higher virulence in the Cry1Ac-susceptible larvae compared to all others and lower virulence in the resistant host. Additionally, the lineages passaged through Cry1Ac-resistant hosts appeared to produce greater variation in mortality when bioassayed against coevolved and Cry1Ac-resistant hosts, with one lineage having lower virulence in both.

DNA sequencing

DNA was successfully obtained via CTAB salt extraction from each replicate subpopulation used in the bioassays, as well as from a single clone taken from the replicate with the highest mortality. For the alternating host treatment, because one clone gave such a significantly different level of mortality, a second one was also picked from another replicate. The ancestral *Bte* pHT315-*gfp*-*eryR* was successfully sequenced by MicrobesNG, with the DNA extraction done on-site after delivery of the strain to them. Sequencing of the passaged lineages by Exeter Sequencing Service and analysis are ongoing at time of publication.

Discussion

Selective passaging in both coevolved and Cry1Ac-resistant P. xylostella larvae, combined with an EMS mutagen in vitro growth phase, increased virulence in these host populations compared to the B. thuringiensis entomocidus ancestor. However there was not a significant difference when passaging in either single- or alternating-host treatments. This suggests that coevolution aided by mutagenesis may be effective in producing B. thuringiensis strains with increased virulence to resistant hosts, controlling their spread in the field.

When compared to the ancestor *B. thuringiensis* subsp. *entomocidus*, the greatest change in host mortality for passaged lineages was observed in the larval bioassays with lineages from coevolved and resistant treatments in the corresponding *P. xylostella* populations. These results suggest that virulence is most effectively raised when hosts are more difficult to kill; the similarity between resistant and coevolution treatments could be caused by the monogenic nature of Cry1Ac-resistance, allowing rapid fixation in the coevolved host population. This appears to fit with the existing body of work showing increased rates of evolution under experimental coevolution (Adiba, Huet et al. 2010, Schulte, Makus et al. 2010), while the Cry1Ac-resistant hosts may already possess the resistance mechanism the coevolved population is moving towards. In the coevolved hosts increased virulence was in turn countered by the host through increasing resistance. While coevolved *P. xylostella* can be killed by the two passaged lineages there is reduced mortality against the ancestral *entomocidus*, in accordance with the Red Queen hypothesis (Van Valen 1974, Scanlan, Hall et al. 2015). It has been suggested that this ratcheting up should be more apparent in infectivity than virulence (Greischar and Koskella 2007, Hoeksema and Forde 2008), though these are difficult to disentangle in the obligate killer *B. thuringiensis*. Unfortunately there were insufficient coevolved larvae for bioassays of all four treatments, and allowing further post-passage generations to increase numbers would have tempered the selective effects of coevolution. Using all four passage treatments here

might have helped to confirm that increases in virulence were principally independent of a particular lineages passage host background, as discussed below.

An increase in virulence compared to the *entomocidus* ancestor was also seen with the bioassays in Cry1Ac-resistant larvae. As this occurred for lineages from all four passage treatments, including the Cry1Ac-susceptible passage treatment, it suggests that just the act of passaging in the host may be enough to increase virulence (Locher, Witt et al. 2003, Bull and Lauring 2014, Le Clec'h, Dittmer et al. 2017). Increasing virulence is a commonly-found result of serial passaging and is generally explained via the virulence-transmission trade-off, as experimentally ensuring successful transmission removes the need for any prudence on the part of the pathogen (Anderson and May 1982, Ebert 1998). This may be less clear-cut for *B. thuringiensis* as it relies on durable endospores (Raymond, Wyres et al. 2010, Narula, Kuchina et al. 2015) for transmission rather than vectors or direct transmission. However, it has still been shown that in *B. thuringiensis* infections spore production is maximised at an intermediate time to host death, again suggesting a trade-off between spore production and virulence (Raymond, Ellis et al. 2009). The passaging protocol used here also ensured (through pasteurisation) that *entomocidus* lineages needed to maintain successful sporulation to persist through the experiment, preventing sporulation investment from potentially being abandoned for even higher virulence (Rafaluk, Gildenhard et al. 2015).

All passage treatments appear to result in higher mortality in the Cry1Ac-resistant larvae, as do the two treatments with lineages bioassayed against the coevolved larvae. This could suggest a common mechanism for the universal increase in virulence. It appears unlikely this is caused by a common change in Cry toxin binding efficiency – larvae in the susceptible host passage treatment have different cell-surface receptors to the resistant ones (causing their differing mortalities with Cry1Ac) (Gong, Wang et al. 2010, de Almeida Melo, Soccol et al. 2016). An alternate mechanism could be an upregulation in the production of Cry toxins or other virulence factors, which would fit with passaging of any sort increasing virulence. *B. thuringiensis* bacteria may already produce large quantities of Cry toxins, corresponding to up to 25% of spore weight in some strains (Agaisse and Lereclus 1995), so other virulence factors might be more

likely recipients of any increases in secretion. Notably, one of the resistant background passage lineages had reduced virulence in both the coevolved and Cry1Ac-resistant bioassays which could suggest that the increasing resistance in the coevolved larvae is underpinned by the same mechanism as in the Cry1Ac-resistant ones. However, the resistant *P. xylostella* population is thought to gain its resistance through a modified abbcc2 receptor that prevents Cry1Ac toxin binding (Baxter, Zhao et al. 2005, Baxter, Badenes-Perez et al. 2011). *B. thuringiensis* subsp. *entomocidus* lacks Cry1Ac, though the same receptor might still bind other structurally similar toxins carried by *entomocidus* at a lower rate e.g., Cry1Aa (Crickmore, Baum et al. 2018); as Cry1Ac and Cry1Aa both fall into the Cry1A grouping their protein sequences will be at least 78% identical (Crickmore, Zeigler et al. 1998, de Maagd, Bravo et al. 2001). In any case it appears that the highlighted resistant host passage lineage failed to generate or fix the necessary mutation(s) to increase its virulence here.

The other replicate lineage showing distinct virulence levels compared to all others, whether from the same or different passage treatment, was from the alternating host background passage (figure 4.5). This lineage had a much higher mortality in Cry1Ac-susceptible larvae than all other treatments or the *entomocidus* ancestor and a correspondingly lower mortality in Cry1Ac-resistant hosts, suggesting antagonistic pleiotropy for the particular mutation(s) it accrued (Ensminger 2013). This was an unexpected result as this apparent Cry1Ac-susceptible host specialist emerged in the multi-genotype treatment that was predicted to favour generalists (Kassen 2002, Elena, Agudelo-Romero et al. 2009). For the alternating host passage treatment the final passage round was in the Cry1Ac-susceptible background; it could be this specialist mutant was not completely selected out in a round with Cry1Ac-resistant larvae before increasing in number in the final round. There are two possible reasons why only a single lineage contained this apparent Cry1Ac-susceptible specialist. One is that the specialist's lack of virulence in Cry1Ac-resistant larvae could have meant that while the same mutant appeared in other replicates it was selected out in those.

Alternatively, it could be that the observed mutation is particularly rare and only appeared once in the experiment – it appears to confer a large fitness benefit so should be easily fixed in the population (Lenski, Rose et al. 1991).

As this was not the case it suggests that the mutation did not appear in the Cry1Ac-susceptible host passage and so is likely to be rare. Even in larger experiments mutation supply may be a limiting factor. In continuous evolution of *E. coli* it took over 30,000 generations to produce a mutant capable of metabolising citrate as a novel carbon source (Blount, Borland et al. 2008). Given the underlying mutation rate of *E. coli* and population size, there were enough generations to ensure all possible basic mutations should have been experienced (Blount, Borland et al. 2008). Citrate metabolism could therefore depend on stacking of multiple rare mutations or ones that are significantly rarer than simple nucleotide substitutions.

If the mutation for high virulence specialism is rare it may also explain the lack of change in mortality in Cry1Ac-susceptible hosts for all other lineages, as on average others were neither significantly different from each other nor the ancestor. Serial passaging is generally expected to increase pathogen virulence (Ebert 1998, Locher, Witt et al. 2003, Bull and Lauring 2014) which could be achieved through upregulation of virulence factors, though passaging may still lead to increased reproduction at the expense of virulence in some instances (Shapiro-Ilan and Raymond 2016). However, the doses needed for high mortality in Cry1Ac-susceptible hosts may be sufficiently low already that passaging had no observable effect here; roughly 20 times fewer spores of the *entomocidus* ancestor were needed to produce a particular mortality in Cry1Ac-susceptible larvae compared to resistant ones. The only way to produce a large change might instead be through a significant change in the pathways involved which would likely be a rare event. However once such a beneficial mutation appears it should spread rapidly through the experimental population. Cry toxins appear more tolerant of increased mutations only in domain III of their three structural domains (Knight, Broadwell et al. 2004, Shan, Zhang et al. 2011), so if changes in virulence were more linked to the other domains this could contribute to their rarity.

It is not possible to confirm that the inclusion of the EMS mutagen directly improved the virulence outcomes of these selective passages, as this was not specifically tested here. Previous work in the same project suggested that mutator strains evolved higher virulence, while the passages in Chapter III which specifically limited relatedness and variation were not successful.

Increased mutation rates improve the rate at which beneficial mutations are generated (Elena and Lenski 2003) and this set of selective passages did produce lineages with raised virulence. The apparently rare occurrence of greatly increased virulence in the Cry1Ac-susceptible host presents a potential way to confirm the importance of EMS to this selective passage protocol in the future. Knowing that such a mutation and virulence change is possible its occurrence rate could be compared between multiple passages in Cry1Ac-susceptible larvae with and without the EMS mutagen.

The specialist (single host background) and generalist (alternating host background) passage treatments do not appear to have produced significantly different mortality rates overall, as both gave similar results in the Cry1Ac-susceptible and -resistant bioassays, with the exception of the one lineage discussed above. This result goes against the expectation that serial passage in a particular host background should lead to specialisation in it and a greater increase in virulence compared to generalists (Little, Watt et al. 2006, Leggett, Buckling et al. 2013). This might be because an increase in virulence factor production was selected for in both passage treatments, preventing any obvious differentiation. It might be that the two host backgrounds are not sufficiently different to allow for specialisation. The Cry1Ac-susceptible and -resistant larvae should only differ in possession of the Cry1Ac resistance allele (Gong, Wang et al. 2010) and *entomocidus* does not typically possess Cry1Ac (Crickmore, Baum et al. 2018). However, the already highlighted replicate lineage (figure 4.5) appears to refute this as it suggests specialisation through adaptation to the Cry1Ac-susceptible host at the expense of virulence in the resistant one (Little, Watt et al. 2006). Outside of the *entomocidus* strain used in this work there is also apparent host specialisation through *B. thuringiensis* as a whole (Aronson and Shai 2001, de Almeida Melo, Soccol et al. 2016). While this does not appear to occur to the level of individual host species, particular strains only produce high mortality in certain subsets of insect orders e.g., *B. thuringiensis* subsp. *israelensis* specialising in killing mosquitoes (Ankarloo, Caugant et al. 2000), while a strain like *B. thuringiensis* subsp. *kurstaki* is viewed as more of a lepidopteran generalist (making it a favoured strain for biopesticides) (Johnson, Scriber et al. 1995, Park, Herrero et al. 2015).

Although this specificity is principally conferred by Cry toxins and their ability to bind to different cell-surface receptors in the midgut e.g., abcc2 receptors in *Plutella* and cadherins in *Helicoverpa*, it is unlikely to be limited to them (Baxter, Zhao et al. 2005, Xu, Yu et al. 2005, Baxter, Badenes-Perez et al. 2011). If Cry toxin binding restricts particular *B. thuringiensis* strains to certain hosts they might be expected to adapt to haemolymph growing conditions within those hosts, though this would assume haemolymphs and toxin receptors are similarly varied. However it does not appear that the within-host fitness of particular *B. thuringiensis* strains across various insect hosts has been widely explored elsewhere in the literature, though there is earlier work exploring which hosts permit growth (Prasertphon, Areekul et al. 1973, Suzuki, Lereclus et al. 2004). This may in part be because the main point of interest in *B. thuringiensis* is deployment of its Cry toxins in transgenic crops (Tabashnik, Brevault et al. 2013), where growth adaptations of *B. thuringiensis* to different host species likely will be irrelevant.

Comparing genome sequencing data (once available) between the *entomocidus* ancestor and passaged lineages should allow for identification of any SNPs or deletions, with such a step now being promoted as an intrinsic part of any passaging or coevolution experiments (Schlotterer, Kofler et al. 2015). The location of these in the genome may determine whether any of the observed changes in virulence are the result of changes in *cry* gene sequences (and corresponding structural changes in Cry toxins), other known virulence factors or elsewhere. Without any sequence data, the similar changes in virulence across passage treatments observed in the bioassays suggest a more general underlying mechanism such as increased production of Cry toxins or other virulence factors. While toxin-rich strains should still be useful commercially, such changes would not be of value in the larger market of *B. thuringiensis* transgenics. The previously highlighted ‘rare’ changes appear more likely to be linked to Cry toxins and as discussed further experiments with and without mutagenesis could suggest how rare such mutations are in this selective passage system. In either case, it may be that selective passaging aided by mutagenesis is not an effective technique if solely attempting to produce better-binding Cry toxins, as it is not possible to focus selection onto just the Cry toxins.

However, the same principals of increasing variation and selection can also be applied effectively at the protein level of Cry toxins rather than the bacterial level of *B. thuringiensis* (Packer and Liu 2015). In simplest terms this takes the form of generating a varied library from an initial Cry toxin e.g., through error-prone PCR, selecting those variants that produce highest binding rate to the receptor of interest and then iterating the process with those selected. Phage-assisted continuous evolution (PACE) is a refined version of this idea (Esvelt, Carlson et al. 2011) and has been applied to Cry toxin improvement (Badran, Guzov et al. 2016). Successful Cry toxin-receptor binding is required for phage infectivity and proliferation by incorporating Cry toxins into the phage and the receptor into the membrane of *E. coli*, while mutagenesis is provided by faulty polymerase in the host cells. This technique was able to produce a 300-fold increase in mortality for Cry1Ac in Cry1Ac-resistant *Trichoplusia ni* moths after 500 phage generations (Badran and Liu 2015, Badran, Guzov et al. 2016). This system also could easily be adapted to other Cry toxins and/or hosts with known Cry toxin receptors.

The work here with *B. thuringiensis* subsp. *entomocidus* appears to confirm that mutagenesis in combination with selective passaging can increase the virulence of *B. thuringiensis*. Based on previous passage failures, it appears that any subsequent attempts to refine the selective passage protocol or use it to explore other aspects of virulence evolution should continue to use an EMS mutagenesis step. In particular, overall virulence appeared to be most successfully increased when *B. thuringiensis* was selectively passaged in more resistant hosts. This falls in line with existing theories and experimental work on the race between virulence and resistance, and the increasing selective pressures they exert upon each other. Overall the passage treatments and bioassays fit with existing patterns, but show them in a pathogen-host system that has been rarely utilised in this manner, despite its economic importance and relative ease of use; much of the coevolution and passaging work with *B. thuringiensis* so far has been with nematodes (Schulte, Makus et al. 2011, Masri, Branca et al. 2015, Shapiro-Ilan and Raymond 2016). With pest control in mind, it does not appear that even the resistant or coevolution treatment passage sufficiently increased virulence compared to alternatives – a roughly three-fold increase was achieved over a few months here, whilst PACE could

achieve a 300-fold increase in what is assumed to be a shorter timespan given the 500 phage generations it required (Badran, Guzov et al. 2016). While the selective passage protocol could be refined further, the amount of time required for significant increases in virulence would be difficult to reduce to a level that could compete with PACE.

However, even if Cry toxins produced by PACE or similar methods can have their genes transformed back into *B. thuringiensis* to improve biopesticides, full passages may still be viable for increasing the virulence of commercialised strains. Working only with the Cry toxin divorces it from other aspects of *B. thuringiensis* biology that are needed for successful infections and might also be improved through selective passaging. While transgenics dominate the agricultural uses of *B. thuringiensis* and its Cry toxins, spore-based biopesticides will still be required, in particular for organic farming where genetic modification may still be viewed with suspicion (Wickson, Binimelis et al. 2016), leaving a need for alternative non-chemical pest treatments.

Discussion

Overview

Moving to the post-genomic age has made constructing phylogenies or linking phenotypic change over time to underlying mutations common-place. However, a possible disconnect remains between this new wealth of genomic data and organisms' precise modi vivendi. This is most apparent with sequences found in environmental sampling that cannot be tied to culturable organisms, but the same can be true for supposedly far better characterised ones (Rappe and Giovannoni 2003, Ungerer, Johnson et al. 2008). The evolution of genomes also cannot be fully understood without also understanding the ecology and selective pressures that shape them.

The entomopathogen *Bacillus thuringiensis* has been used as commercial biopesticide for over 50 years (Lambert and Peferoen 1992), yet its ecology and that of its sister species in the *Bacillus cereus* group are still not fully understood. This produces problems for ongoing *B. thuringiensis* use, in terms of whether it continues to be viewed as safe (EFSA 2016) and how its effectiveness can be maintained against spreading resistance in pest species (Tabashnik, Brevault et al. 2013, Tabashnik and Carriere 2017). This thesis has shown that the *B. thuringiensis* strains used in agriculture are distinct ecologically as well as genetically from the majority of *B. cereus*. Within hosts it appears that the outcomes of *B. thuringiensis* co-infections are strongly dependent on the relative frequencies of strains, likely because of the shared nature of virulence factors. Experimental evolution can be used to tie ecological interactions to phenotypic (and ultimately genetic) change. The selective passages used here suggest that virulence of *B. thuringiensis* can be best maintained or increased through passaging with resistant hosts; despite predictions to the contrary, high relatedness does not appear to favour high virulence, perhaps because its effects were outweighed by local competition within-host or loss of selectable diversity.

Safety & Regulation of *Bacillus thuringiensis*

Concerns over the safety risks of *B. thuringiensis* ultimately appear borne out of confusion over what this name actually describes and covers. Ideally the *B. thuringiensis* species should be realigned to only bacteria found in clade 2 of the *Bacillus cereus* species group, based on ecological differences between clade 2 and others, combined with the growing reinforcement of genetic divides (Didelot, Barker et al. 2009, Meric, Mageiros et al. 2018). Both areas suggest a clear separation between commercialised *B. thuringiensis* strains and medically significant strains of emetic *B. cereus* or even *B. anthracis*. Even without this growing data, the European Food Safety Authority's (EFSA) newly apparent apprehensions over human consumption of *B. thuringiensis* – "levels of *B. cereus* that can be considered as a risk for consumers are also valid for *B. thuringiensis*" – appear misguided (EFSA 2016, Raymond and Federici 2017). Given the historic and widespread use of *B. thuringiensis* spores on food crops (Lambert and Peferoen 1992, Siegel 2001) it would be expected that any negative effects of their consumption would have been felt, but there have been no definitive cases (Raymond and Federici 2017). The EFSA highlighted a case of food poisoning, but other microbes, including *B. cereus*, are far more plausible culprits (EFSA 2016, Raymond and Federici 2017). Safety concerns may also extend to the Cry toxins, which could be ingested separately depending on biopesticide formulations or within transgenic crops. Certain Cry toxins (e.g., Cry31Aa1) have been identified that can bind to receptors on human cell lines (Mizuki, Park et al. 2000). It is not impossible that Cry toxins exist that can bind to and kill healthy human gut cells, resulting in similar illness to emetic forms of *B. cereus*. Again though, the strains used in agriculture are well characterised along with their Cry toxins. There is clear evidence that consuming Cry toxins or *B. thuringiensis* spores in amounts larger than would be found with crops has no negative effects (Mendelsohn, Kough et al. 2003, Koch, Ward et al. 2015). In all, it seems somewhat misguided for the EFSA to publish concerns over limited data despite the clear opposing evidence.

Worries over *B. thuringiensis* pesticide use and transgenic crops in general, which are already under heavy European regulation (Davison 2010),

have impacts beyond human health. Insect-resistant crops and biopesticides, both markets dominated by *B. thuringiensis*-related products (Bailey, Boyetchko et al. 2010), mean far fewer crops are lost to herbivory, including in neighbouring non-transgenic fields (Cattaneo, Yafuso et al. 2006, Dively, Venugopal et al. 2018). Higher yields in turn mean that smaller areas of land can be used to produce the necessary amounts of food or cotton. Turning land over to farming is a principal cause of habitat loss (Curtis, Slay et al. 2018), so maximising crop production from a given area is highly desirable. *B. thuringiensis* is already a valuable tool for achieving this (Whalon and Wingerd 2003, Tabashnik, Brevault et al. 2013) and its importance is only likely to grow: global food demands are expected to increase until 2050 (Godfray, Beddington et al. 2010) whilst running up against environmental concerns, including opposition to chemical pesticides. Increasing control of *B. thuringiensis* use when there is currently no clear need would therefore prevent full advantage being taken of it in the search for more sustainable food production.

Passaging versus Protein Evolution

Regulations aside, the role of *B. thuringiensis* in a more sustainable future will only be realised if it remains effective at controlling crop pests. Farming management strategies and pyramided transgenics combining multiple Cry toxins are currently used in an attempt to limit the emergence and spread of resistance to existing commercial Cry toxins (Zhao, Cao et al. 2005, Tabashnik, Brevault et al. 2013). However, resistance has readily occurred to common toxins like Cry1Ac in a number of species, in part because of lax enforcement of resistance management (Storer, Kubiszak et al. 2012, Tabashnik and Carriere 2017). It is hoped that emerging resistance can be overcome by finding or generating new *B. thuringiensis* strains and toxins to restore high mortality in resistant pests (Roh, Choi et al. 2007). The growing number of sequenced isolates means that discovering new toxins is increasingly efficient (Ye, Zhu et al. 2012), but this only provides what is already available in nature; it is possible that the most useful Cry toxins have already been identified, while discovery is mainly limited to homologs of existing toxins. In the laboratory, engineering or

directed evolution of proteins provides an alternative means of producing Cry (or other) toxins that might never be found in wild-type isolates e.g., by combining domains from Cry toxins isolated on opposite sides of the globe. Random mutagenesis, domain shuffling or even chimeric combinations using proteins outside the Cry family can all be employed (Bravo, Gomez et al. 2013, Deist, Rausch et al. 2014) e.g., combining Cry3Aa with a chymotrypsin proteolytic site to increase mortality in *Diabrotica virgifera* (corn rootworm) on transgenic maize (Walters, Stacy et al. 2008, Hibbard, Frank et al. 2011).

In particular, directed *in vivo* methods like phage assisted continuous evolution (PACE) can produce rapid increases in mortality over the starting toxin (Esvelt, Carlson et al. 2011, Badran and Liu 2015, Badran, Guzov et al. 2016). These techniques have the potential to rapidly restore toxin effectiveness in resistant pests, but also have limitations as they normally rely on knowledge of the particular toxin-receptor relationship involved. If a potential receptor is not known then it cannot be engineered into the membrane of *Escherichia coli* as required for PACE to work. If Cry resistance does occur through conformational changes to a single receptor, then the receptor could presumably be identified through crosses of susceptible and resistant homozygous hosts. PACE would then be ideal for reshaping toxins to restore their binding and overcome resistance. However, even in major global pests like *Plutella xylostella* the receptors involved in Cry toxin binding have only recently been fully identified e.g., abcc2 for Cry1Ac (Baxter, Badenes-Perez et al. 2011). There are still competing models for how Cry toxins bind to receptors and ultimately trigger cell death (Bravo, Gomez et al. 2013, de Almeida Melo, Soccol et al. 2016). While this may not be crucial to engineering new Cry toxins, as shown by the existing progress with PACE, clearer knowledge might further guide toxin development. Additionally, resistant insects with receptor deletion rather than moderation present additional challenges which may not be easily addressed solely by PACE and similar techniques.

The selective passage technique used and refined here does not require any foreknowledge of toxin-receptor interactions; it only requires a strain that can grow *in vivo* and produce some level of mortality on which to then select individual hosts or subpopulations. This is an important strength compared to protein-level work, though it would appear to come with the trade-off of slower

virulence improvement. This lack of understanding is not just limited to *P. xylostella*; to fully take advantage of PACE would require full characterisation of Cry toxins and potential receptors across many pest species. Additionally, toxins or receptors might be identified that cannot be easily expressed by the phage or *E. coli*. *Plutella* are far from the only insect crop pests and selective passaging could just as easily be applied against other Cry-resistant lepidopteran pests e.g., *Spodoptera* or *Helicoverpa* (Herrero, Bel et al. 2016, Tabashnik and Carriere 2017), or even against coleopterans and dipterans. More broadly, there is no reason why selective passaging could not be applied to pathogens other than *B. thuringiensis*, whether bacterial, fungal or viral. Increasing pathogen mutation rates and passaging with resistant hosts may be the best ways to achieve the desired increases in virulence, and evidence in support of them promoting higher virulence is widespread. Therefore these conditions were predicted to have the same effects on *B. thuringiensis* when selectively passaged in *P. xylostella*. If these conditions are taken to be universal drivers of increased evolutionary rate they should have the same effect when used to improve virulence in other pathogen systems. Fungal pathogens including *Beauveria bassiana* are already used to control aphids or other insects and these could be another target for improvement through selective passaging.

Directed evolution of Cry toxins or other proteins only allows for iterative changes to that particular target's structure. However, Cry toxins are only a part of the virulence factors carried by *B. thuringiensis*. Selective passaging should allow for the mutations that have the greatest beneficial fitness effects to surface – here mutations that increase host mortality. It may be the case that there is little room for improvement via Cry toxins as local fitness peaks have already been reached. Any increases in virulence might instead be achieved through other virulence factors such as vegetative insecticidal proteins, or changing regulation of toxin production. This appears particularly relevant if transforming *B. thuringiensis* with previously modified toxin sequences, as the strain may not produce the desired quantities of toxin, while any toxin mortality could be bolstered by within-host replication and septicaemia. If *B. thuringiensis* spores also rely on attached Cry toxins for germination (Du and Nickerson 1996) it may be that modified toxins can no longer bind in this way,

as this selection pressure would be removed in a protein-only system. While PACE produced more virulent Cry1Ac toxins, they also had reduced stability (Badran, Guzov et al. 2016). These improvements could all be achieved through within-host passaging, with no specific target needed.

Decreasing virulence might also be desirable in certain situations. While there is no current applied need to do this for *B. thuringiensis* or its toxins, it may be the case for other pathogens. PACE and similar protein techniques cannot currently be configured to specifically favour decreased binding, though PACE can be altered to avoid selection of a particular trait (Carlson, Badran et al. 2014), leaving only repeated screening of mutant protein libraries for reduced binding efficiencies. Selective passaging could allow for selection of strains with reduced virulence, which potentially could be useful for attenuated vaccines. For *B. thuringiensis* or other pathogens with shared virulence factors such selection might be more likely to result in cheats than anything else. It would be interesting to see if repeated selection for reduced virulence could reduce *B. thuringiensis* to a more commensal or symbiotic role, as has tentatively been achieved in some other systems (Marchetti, Jauneau et al. 2014, King, Brockhurst et al. 2016), although it lacks a means of attaching to the host (though there is possible evidence for this in *B. cereus* (Margulis, Jorgensen et al. 1998)). This could depend on *B. thuringiensis* maintaining some Cry toxin production to access the haemolymph, but heavily modulating its growth there to prevent septicaemia and host death. However, if sufficient Cry-null cheats were selected for then this resource would be barred and *B. thuringiensis* might lack the means to establish itself against existing commensals in the insect gut.

Away from applied factors, selective passaging has one final, important mark in its favour. Experimental manipulation of proteins may be efficient, but it does not appear to shed light on anything new. In fact, as discussed above, it requires pre-existing knowledge of receptors involved for it to be carried out effectively. A selective passaging system can produce strains with increased mortality while at the same time identifying which conditions favour higher virulence. These findings might reflect conditions that favour higher virulence everywhere, though there is the caveat that this experimental system used artificial selection at the host level rather than allowing unrestricted evolution.

Coevolution should favour increased virulence (Buckling and Rainey 2002, Schulte, Makus et al. 2010, Masri, Branca et al. 2015) and using it in passages appears to have additional outcomes beyond just improving the rate of virulence evolution. Allowing the host population to coevolve should also increase its resistance. In *P. xylostella* this could be caused by a number of things e.g., changing gut conditions (Forcada, Alcacer et al. 1996), but it could also be caused by changes to midgut epithelial receptors. Comparisons with the ancestral host population could then allow for easier identification of the receptor(s) of interest, providing an obvious target to investigate for PACE or similar. If an improved Cry toxin is developed, trying to predict how pest populations might evolve resistance in response to exposure would likely be welcomed. Coevolution passages could then be carried out to determine the possible routes resistance could emerge through, with repeat experiments highlighting the most likely. This in turn might affect toxin use or allow for a head start on developing different toxins for when resistance does emerge in field populations.

In the same manner, selective passaging for decreased virulence could help identify factors that favour this decrease. It could be assumed that these factors will be the opposite of those favouring high virulence e.g., limiting variation, but this might just maintain virulence at its initial level rather than actually decreasing it. While *B. thuringiensis* is a pathogen that has been commercialised there are obviously many others that cause agricultural damage or are medically significant. Understanding the conditions that favour lower virulence during selective passaging could then inform farming or treatment practices in an attempt to replicate any decreases in the field. Similarly, factors that favour host resistance could be explored, while any resistant phenotypes generated could be searched for alleles to transfer into field populations. Alternatively, a wide range of *Bacillus* species are used in commercial microbiology (Schallmey, Singh et al. 2004); if protein secretion is important in their application then passage conditions could inform their selection and improvement.

Bacillus weihenstephanensis

To take full advantage of any evolved strains and toxin proteins produced, the difference between highly insecticidal strains of *B. thuringiensis* and dangerous strains of *B. cereus* needs to be understood. However, the alignment of species within the *B. cereus* group is also important for fundamental reasons. Genetic separation should reflect ecological separation and this appears to be the case for clade 2 *B. thuringiensis* against *B. cereus sensu stricto*, with the former identified as specialised invertebrate pathogens. However, less attention has been paid to the other significant member of the group (in terms of abundance, if not human impact (Raymond, Wyres et al. 2010)): *Bacillus weihenstephanensis*, in clade 3 (Didelot, Barker et al. 2009, Zheng, Gao et al. 2017). While the *B. cereus* group in general is often characterised as being soil dwelling, the majority of isolates from soil may in fact be *B. weihenstephanensis* (Raymond, Wyres et al. 2010). *B. weihenstephanensis* is characterised as being psychrotrophic or psychrotolerant (growing at 7°C but not 43°C) but this gives little indication as to what it actually does while active at colder temperatures (Soufiane and Cote 2010). *B. weihenstephanensis* appears no better able to grow on soil nutrients or simulated root extract than *B. cereus* or *B. thuringiensis*, though these had previously been suggested as potential niches. However, *B. weihenstephanensis* would grow on animal-based protein media, and given that other members of the *B. cereus* species group have niches built around animal protein this is not surprising. Therefore *B. weihenstephanensis* may have a similar or the same niche as the others, except at lower temperatures. For example, it might be that both *B. weihenstephanensis* and *B. thuringiensis* are adapted to growth in insect haemolymph, except across different temperature ranges.

The spatial structuring of *B. thuringiensis* spores on plant leaves suggest that insects generally ingest one strain (Collier, Elliot et al. 2005, Raymond, West et al. 2012), resulting in single-strain infections. Yet *B. thuringiensis* also possesses a wide suite of bacteriocins (Cherif, Rezgui et al. 2008, Raymond, Johnston et al. 2010, Garbutt, Bonsall et al. 2011), meaning it should regularly

be in competition with closely related strains or species. The high likelihood of initially single-strain infections could instead mean that these bacteriocins are employed against *B. weihenstephanensis* attempting to colonise cadavers that have fallen from leaves to the soil. At intermediate temperatures both species can grow, so bacteriocins may be required to drive home any slight advantage one species gains in growth rate from a cadaver's temperature. This appears plausible, and could seemingly be tested in a straightforward manner. Taking the *in vivo* competitions in Chapter I as a template, a marked competitor strain of *B. thuringiensis* could be competed against a set of *B. weihenstephanensis* strains across a range of temperatures. The clear prediction would be that the relative fitness of *B. weihenstephanensis* would be negatively correlated with temperature, and would only be higher than *B. thuringiensis* at lower temperatures e.g., below 10°C.

Notably, a number of strains initially classified as *B. thuringiensis*, including *vazensis* and *navarrensis*, have been proposed for reclassification as *B. weihenstephanensis* (Soufiane and Cote 2010); they are still listed as *B. thuringiensis* in the Bacillus Genetic Stock Centre. It is assumed that they were originally identified based on the possession of Cry toxins, though there is the strong possibility that contamination or similar issues resulted in misclassification. However, they are psychrotolerant and carry the two specific sequences indicative of *B. weihenstephanensis* in the 16S rRNA and *cspA* genes (Mayr, Kaplan et al. 1996, Lechner, Mayr et al. 1998). Furthermore, in full-genome phylogenies they group with the rest of *B. weihenstephanensis* strains in clade 3 (Priest, Barker et al. 2004, Didelot, Barker et al. 2009, Zheng, Gao et al. 2017). If these strains do actually possess Cry toxins it would fit with above hypothesis that *B. weihenstephanensis* has a similar niche to *B. thuringiensis*, but at colder temperatures, hence their possible need for Cry toxins. While it has previously been suggested that Cry toxins might have a role in bacteria-plant associations (Aronson and Shai 2001), the overwhelming evidence is that they are virulence factors that allow for invertebrate infections. There is limited evidence for *B. weihenstephanensis* carrying Cry toxins; alternatively some *B. thuringiensis* strains have been found with cold shock proteins which they could have acquired subsequently (Bartoszewicz, Bideshi et al. 2009), although an exact order of acquisition is difficult to infer without full

genotyping. The consensus is that the majority of known *B. weihenstephanensis* strains do not carry Cry toxins, as otherwise they too would have been previously classified as *B. thuringiensis*, but it does not seem impossible for some to possess them. There could potentially be a similar story to that proposed for '*B. thuringiensis*' strains in clade 1 (Didelot, Barker et al. 2009) with toxins being horizontally transferred into clade 1 *B. cereus* strains more recently. It could be the case that while the majority *B. weihenstephanensis* focus on invertebrate cadavers some have adapted, assisted by Cry toxin acquisition, to a more actively entomopathogenic lifestyle, albeit at lower temperatures than *B. thuringiensis*.

This of course presupposes that most *B. weihenstephanensis* strains do lack Cry toxins. While *B. thuringiensis* was first identified over a century ago, *B. weihenstephanensis* was only described 20 years ago on the basis of two genetic markers common to psychrotolerant *Bacillus* isolates (Lechner, Mayr et al. 1998) and subsequently confirmed in whole-genome phylogenies (Didelot, Barker et al. 2009). As any *Bacillus* spore carrying Cry toxins would have been automatically labelled *B. thuringiensis* this could mean there are further Cry-carrying isolates that when fully sequenced will fall into clade 3. The recent phylogenies for the *Bacillus* species group appear to have only found misclassified *B. thuringiensis* in clade 1 (Didelot, Barker et al. 2009), but this obviously does not rule out any other misidentified clade 3 strains existing among the many *B. thuringiensis* isolates held around the world.

Population structure in Experimental Evolution

The majority of experimental evolution studies have used bacteria evolved in serial transfers or chemostats (Elena and Lenski 2003). These methods ensure a replenishing supply of nutrients and prevent the build-up of potentially harmful waste products. Serial transfer and chemostat systems have provided insights into many aspects of evolution, which have also informed the selective passages carried out here e.g., the impact raised mutation rates have on the accumulation of beneficial or desired mutations (Elena and Lenski 2003).

However, there is a major flaw with such experiments when it comes drawing real-world inferences from them; the very factors that make these long-term bacterial cultures feasible also make them unrealistic. Using a liquid culture means that samples can easily be taken for storage, analysis or to inoculate the next batch; it also means the culture can be easily mixed to ensure food is distributed to all bacteria within, while conditions can be easily adjusted through the addition of chemicals or organisms from other cultures. Mixing also means that the culture will be homogenous throughout. At the within-host level, many (if not all) interactions between bacteria are influenced by community structure and this is likely also the case for all levels of ecological interaction (Stubbendieck, Vargas-Bautista et al. 2016). Patch structure can play an important role in determining the success of particular strategies, most obviously through allowing frequency dependent phenotypes to persist even when rare in the overall population. In a mixed, unstructured environment these phenotypes would be unable to invade from rare, so they would not be expected to emerge in the long-term cultures described.

Frequency-dependent traits in bacteria are common; 30% of *E. coli* strains produce colicins for intraspecific competition (Levin 1988, Gordon and Riley 1999). In an unstructured environment bacteriocin-producing strains are not expected to invade from rare. Once mixed through the culture, the small volume of bacteriocins produced would not remove sufficient competition by non-producers to compensate for any differences in basic growth rate. Conversely, when producers are common susceptible strains should not invade from rare because large quantities of bacteriocin will be spread throughout the mixed culture (Chao and Levin 1981). Structured populations allow frequency-dependent strains to achieve persistence at the local level from where they may spread to further patches, eventually becoming common across the whole population. Structured populations are likely to be common in nature e.g., biofilms only permitting bacteria to interact with their neighbours (Costerton, Lewandowski et al. 1995, Kolter and Greenberg 2006). When bacterial densities are low patchiness should also occur, simply because there are insufficient bacteria to uniformly fill the total available niche. Clearly, frequently or continuously mixed experimental populations would not allow for any

structuring – their advantage is only that they allow larger populations to be maintained over potentially many thousands of generations.

Structuring could be introduced into these systems by removing mixing or growing on a solid matrix, though it may be difficult to maintain structuring during replacement of nutrients e.g., transferring a previously unmixed culture to fresh media. While levels of local and global competition can be controlled in bacterial multi-culture systems (Griffin, West et al. 2004), *in vivo* passaging of pathogens not only allows for a structured population but also better approaches real-world conditions and permits a greater range of selective forces than artificial liquid media in flasks e.g., comparing local and global competition *in vivo* (Shapiro-Ilan and Raymond 2016). Inoculating the initial pathogen population automatically creates patchiness, as pathogens are partitioned between hosts. The overall structure of the passage experiment can then determine how sharply these patches are defined. If infected hosts are in a larger population then pathogens can be transmitted on to susceptible hosts. This would likely generate new patches with a mix of the members of existing ones, as pathogens from multiple hosts may be transmitted to a single new one. Alternatively, hosts could be kept separate, and transmission limited to one-to-one, potentially done by the experimenter. Earlier experiments in this project compared global and local competition (Dimitriu, Crickmore et al. unpub. data). In the former, all *B. thuringiensis* spores collected from a passage round were then grown up together before being used to infect the next round. This was contrasted against splitting hosts into subpopulations and only pooling collected spores within these. Structuring the population into subpopulations removed global competition and produced lineages with higher virulence than found in those exposed to global competition.

Long-term experimental evolution in bacterial cultures has provided support for a number of hypotheses, but it must be remembered that such systems are only approximations of natural ones, and crude ones at that. In an ideal world insect-pathogen passages could be continued for decades too, and while these would not reach 10,000 generations or more in this time, it is conceivable that they could reach hundreds. The *P. xylostella* and *B. thuringiensis* system used here may not be ideal for longer-term experiments; experience suggests it is difficult to ensure cadaver spores are ingested by

subsequent generations without experimenter intervention, while mould is also a concern. However, systems using the red flour beetle *Tribolium castaneum* and microsporidium *Nosema whitei* may be more useful for long-term experiments (Berenos, Schmid-Hempel et al. 2011, Kerstes and Martin 2014). Microsporidian spores can more easily disperse through the experimental environment and flour-based food can be easily replenished. Over the last decade *T. castaneum* has been used in a number of longer coevolution experiments which have provided further evidence in favour of the Red Queen hypothesis, amongst others (Van Valen 1974, Salathe, Kouyos et al. 2008, Kerstes and Martin 2014). While such experiments do not approach the hundreds of generations suggested above, they do reach into the tens, which is more than has been achieved so far with *P. xylostella.* Whatever the system used, efforts should be made carry out longer experiments in a variety of systems more complex than bacterial liquid cultures. Bringing experimental systems closer in line with real-world situations may generate more applied knowledge, but this would come at the expense of the tractability available with liquid bacterial cultures.

Concluding Remarks

This thesis has clear applied potential as it aimed to produce a within-host passaging protocol that could raise the virulence of *B. thuringiensis* strains in the crop pest *P. xylostella*. While there is not yet a finalised protocol, this work has highlighted key areas that should be focused on, namely increasing the mutation rate, targeting resistant hosts and avoiding unnecessary within-host competition. These findings also fit with existing theoretical and experimental work on the evolution of pathogen virulence, but demonstrate them in a novel, economically important pathogen-host system. Along with this, it is hoped that the work demonstrating the differing ecologies of clade 1 and 2 *B. thuringiensis* strains will permit a reconsideration of the classification of *B. thuringiensis*. Ideally this will be to strains only in clade 2, irrespective of their possession of Cry toxins, as classifying in this way best combines the available ecological and genomic data.

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