Chapter 2
The Biology, Ecology and Taxonomy of *Bacillus thuringiensis* and Related Bacteria

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Abstract *Bacillus thuringiensis* produces a range of specialized virulence factors that enable it to infect invertebrate hosts. Despite the level of interest in this species, there have been a number of controversies and disagreements regarding its ecological niche, how it kills its hosts and benefits from the production of Cry toxins and whether *B. thuringiensis* constitutes a real species that is a distinct member of the *Bacillus cereus* group. Hypotheses arguing that *Bt* is a soil saprophyte, a gut or plant commensal or a specialized pathogen are critically evaluated. Evidence supporting the specialized pathogen hypothesis includes proteomic and genomic studies revealing adaptations to lyse cells and exploit peptide-rich resources. *Bt* infects insects and reproduces effectively in the field without obvious epizootics and uses plants to vector inocula from soil to the phylloplane. *Bt* Cry toxins, and other virulence factors, can be treated as cooperative public goods. Cooperative production of virulence factors has implications for dose-response curves and understanding which ecological factors can select for the maintenance of virulence. Finally, the taxonomy of *Bt* and the phylogeny of the *B. cereus* group are discussed. The genetic and ecological variation within the *B. cereus* group is substantial and argues against lumping all members of this clade into one species; a revised nomenclature of the group is suggested that includes restricting the use of *B. thuringiensis* to a single clade that contains the vast majority of invertebrate-adapted isolates and revising the use of the *cereus* and *anthracis* epithets.

Keywords Evolution of virulence • Phylogeny • Transmission

Bacillus thuringiensis is defined as a member of the broader *Bacillus cereus* that is capable of producing crystalline inclusion bodies. The *B. cereus* group contains a diverse array of pathogenic strains. *Bacillus cereus* sensu stricto can be a causative agent of two forms of human food poisoning: emetic (associated with the toxin cereulide) and diarrhoeal (associated with a broad range of enterotoxins) (Stenfors

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Arnesen et al. 2008). The *B. cereus* group also contains *Bacillus anthracis*, the aetiological agent of anthrax, which is predominantly a disease of vertebrate herbivores (Turnbull 2002). Without doubt *B. thuringiensis* (*Bt*) remains the single most important bacterial species in insect pest management. Its utility derives from the large quantities of proteinaceous toxins that form crystalline parasporal inclusions, termed the Cry (crystal) and Cyt (cytolytic) proteins (Schnepf et al. 1998). As described elsewhere in this book, the vast majority of commercially viable genetically modified (GM) insect-resistant crops express one or more Cry toxins. The total planted area of Cry toxin expressing crops exceeded 20 million hectares in 2015 (James 2015). Moreover, spore and toxin *Bt* formulations are also the most successful organic microbial pesticide, with target hosts ranging from mosquitoes to lepidopteran pests of agriculture, horticulture and forestry (Glare and O'Callaghan 2000). Nearly 100 years of research has meant that the diversity, structure and mode of action of *Bt* Cry toxins have been intensively studied.

However, despite this long period of interest the biology, phylogeny and ecology of the bacterium have been the subject of much controversy and disagreement. Controversies that have dogged our understanding of this bacterium include whether or not it is a specialized invertebrate pathogen (Jensen et al. 2003; Raymond et al. 2008a, 2010a), how it manages to benefit from the production of highly costly Cry toxins (Martin and Travers 1989), whether it can infect vertebrates (Siegel 2001; Federici and Siegel 2007) and whether *Bt* should be regarded as a species or a mere plasmid carrying subtype of *Bacillus cereus* (Helgason et al. 2000; Didelot et al. 2009). One highly cited studied has even claimed that *Bt* requires gut bacteria to assist in killing hosts, although the results of that study have not proved repeatable by other groups and were caused by confounding the removal of gut bacteria with the use of broad-spectrum antibiotics (Broderick et al. 2006; Johnston and Crickmore 2009; Raymond et al. 2009; van Frankenhuyzen et al. 2010). These disagreements have implications outside of the purely academic sphere. As this book goes to press, the European Union regulators are debating whether the safety regulation of *Bt* microbial products needs to be tightened. This revision of its status as one of the safest pest control products on the market was prompted by a controversial review arguing that *Bt* is biologically and ecologically indistinguishable from *B. cereus* (EFSA 2016). In addition to safety issues, understanding the details of pathogenicity and the infection process and how *Bt* benefits from the production of its toxins may help inform strategies for both the discovery of new strains and the improvement of existing products.

This chapter will critically examine some of the controversies surrounding the biology of this important microbe and address some of the recent advances in our understanding of the fundamental biology of *Bt.* In particular, the evidence base to support the view that *Bt* is a specialized invertebrate pathogen is now substantially stronger than it was at the time of the last review on this topic (Raymond et al. 2010a). A caveat here is that *Bt* as a species is in need of taxonomic revision. Key developments in this area covered in this review include fundamental field ecology experiments demonstrating benefits of Cry toxins for bacterial reproduction and fitness (Raymond et al. 2010b, 2012); better genomic and phylogenetic data on *Bacillus* including an understanding of biological and ecological variation across distinct clades (Cardazzo et al. 2008;

Guinebretière et al. 2008; Didelot et al. 2009; Alcaraz et al. 2010; Raymond et al. 2010b; Raymond and Bonsall 2013), application of evolutionary theory to explain how selection for high virulence is maintained in *Bt* (Schulte et al. 2010; Raymond et al. 2012; Zhou et al. 2014; Cornforth et al. 2015; Deng et al. 2015; van Leeuwen et al. 2015), an appreciation of the prevalence of *Bt* strains pathogenic to nematodes (Ruan et al. 2015) and an increased understanding of the relationship of *Bt* with plants (Monnerat et al. 2009; Raymond et al. 2010b; Vidal-Quist et al. 2013).

2.1 Competing Hypotheses Regarding the Fundamental Ecology of Bt

How did these controversies arise? For spore-forming bacteria, a common source of confusion is that viable material can be readily isolated from habitats that are largely unsuitable for growth. *Bt* is readily isolated from soil and plants (Delucca et al. 1981; Martin and Travers 1989; Smith and Couche 1991; Kaur and Singh 2000; Hendriksen et al. 2006). Another source of confusion is the fact that *Bt* strains have a variable ability to grow and sporulate within particular insect species, despite often having high pathogenicity (Prasertphon et al. 1973; Suzuki et al. 2004). Moreover, disease outbreaks or epizootics are very rare in the field (Porcar and Caballero 2000) although they occur readily in grain stores and in insect culture in the laboratory (Burges and Hurst 1977; Delucca et al. 1982; Itova-Aoyolo 1995; Federici and Siegel 2007). Effective transmission of *Bt* between larvae has also been difficult to demonstrate experimentally (Takatsuka and Kunimi 1998) and can require a high density of hosts and/or cannibalism (Knell et al. 1998).

Although Bt is readily recovered from the environment, an early influential paper reported a lack of correlation between host abundance and the abundance of entomopathogenic *Bt* (Martin and Travers 1989). The combination of high prevalence, but the difficulty in observing transmission, has led to a wide speculation on the ecological niche of *Bt.* It has been suggested that *Bt* is a soil micro-organism with incidental insecticidal activity (Martin and Travers 1989), that *Bt* is part of the phylloplane microbiota and has evolved to provide symbiotic protection against insect attack (Smith and Couche 1991; Elliot et al. 2000) or that *Bt* may be part of the commensal gut microbiota of many insects without causing overt disease (Jensen et al. 2003). Many of these ideas are persistent although several have been tested and failed to gain support in a number of studies.

2.2 *Bt* Grows Poorly in Soil and Is Poorly Adapted to the Nutritional Resources Prevalent in Soils

To begin with *Bt* is not a soil bacterium, in the conventional sense, as it has a very poor ability to grow in unamended soil (West et al. 1984, 1985; Yara et al. 1997). Growth in autoclaved soil, which both removes saprophytic competitors and release

additional nutrients, is not particularly convincing evidence for having a saprophytic niche. For *B. cereus* sensu stricto, which can be chromosomally very similar to entomopathogenic *Bt* (see discussion below), growth in sterile filtered media made of soluble soil nutrients is also questionable support for saprophytism (Vilain et al. 2006). In the field, *Bt* populations decline slowly in soil over several years (Addison 1993; Eskils and Lovgren 1997; Hendriksen and Carstensen 2013). In one case, the evidence suggests that cycles of germination, growth and sporulation occur seasonally in soil, although this is against a background of reducing population size, and this observational study could not preclude the contribution of insect mortality (Hendriksen and Carstensen 2013).

One of the most convincing lines of evidence against *Bt* or *B. cereus s.s.* being soil saprophytes comes from comparative genomics (Alcaraz et al. 2010). Typical members of the genus *Bacillus* (e.g. *B. subtilis* group species) have a large number of genes involved in processing carbohydrates, particularly complex carbohydrates that derive from plants (Wipat and Harwood 1999; Alcaraz et al. 2010). Processing of complex plant carbohydrates is a key adaptation for saprophytes and plant commensals (Badri et al. 2013). *Bt* and *Bc* are unusual in that they possess relatively few carbohydrate-processing genes and lack the capacity to use many simple sugars such as mannose, arabinose, and in some cases, sucrose (Rasko et al. 2005; Alcaraz et al. 2010). In contrast, bacteria in the *B. cereus* group are rich in enzymes involved in peptide and amino-acid processing in comparison to members of the *B. subtilis* group (Read et al. 2003; Rasko et al. 2005; Alcaraz et al. 2010). For example, *B. cereus* and *B. anthracis* possess six amino-acid efflux systems, which prevent the accumulation of amino acids intracellularly to levels that can inhibit growth (Read et al. 2003). In addition, a substantial proportion of the secretome of *Bt* and *B. cereus* (70% of secreted stationary phase proteins) is composed of proteases or other enzymes/virulence factors with putative roles in cell lysis and disrupting membranes (Gohar et al. 2005). In short, the *B. cereus* group is dominated by meat-eaters. Any readily culturable bacterium with a large genome and a flexible metabolism will be able to grow when nutrients are provided, so some germination and growth in the soil may be possible. Nevertheless, the balance of evidence indicates that *Bt* is not well adapted to soil conditions and is poor at competing in this environment.

2.3 *Bt* Is Not a Commensal Bacterium

The idea that *Bt* might be able to reproduce as a commensal originated from observations of the occurrence of vegetative cells in the midguts of soil invertebrates (Hendriksen and Hansen 2002), rather than from experimental work. *Bt* can grow and germinate vegetatively in the insect midgut in the process of lethal infections, although the number of cells can be very few in some hosts (Chiang et al. 1986; Zhou et al. 2014). *B. cereus* group strains have also been regularly recovered from the guts of Lepidoptera and other invertebrates, and the specialized ‘Arthromitus’

form of *B. cereus* appears to have adaptations specifically for attachment to the midgut (Margulis et al. 1998; Jung and Kim 2006; Raymond et al. 2008b). However, an explicit experimental test of the commensal hypothesis using *Bt kurstaki* and larvae of the diamondback moth, *Plutella xylostella*, showed that *Bt* does not replicate in sublethal infections and cannot be transmitted vertically from female to eggs (Raymond et al. 2008a). In fact, this study showed that survival of spores in the midgut is lowest when ingested *Bt* strains carry Cry toxins with suitable receptors in the insect midgut (Raymond et al. 2008a), the inference here being that germination of spores is increased in susceptible hosts but that without a successful lethal infection, vegetative cells pass out through the hindgut via peristalsis. Growth in the gut prior to invasion of the haemocoel is ecologically very different, first because the action of the Cry toxins paralyses the gut (Endo and Nishiitsutsujiuwo 1980), ensuring that germinating material remains in the digestive tract, and second because pore formation leads to leakage of haemolymph into the gut, which increases nutrient supply (Heimpel and Angus 1959). The available evidence is therefore that *Bt* is an 'obligate killer', a pathogen that requires host death for reproduction (Ebert and Weisser 1997).

2.4 Transmission and the Relationship of *Bt* with Plants

While we have an expectation that *Bt* has to kill invertebrates in order to reproduce, we also have a pathogen that rarely causes disease outbreaks. For *Bt* strains attacking herbivorous hosts, one way of reconciling these facts is to consider that *Bt* transmission may not typically occur directly from cadaver to larva but may use plants as vectors. High concentrations of spores and toxins can deter feeding (Knell et al. 1998), and concentration of cultivable spores above 10^3 per cm^2 is rare on plant tissue in the field (Maduell et al. 2002; Collier et al. 2005; Raymond et al. 2010b). Thus the majority of naturalistic infections may be initiated from relatively low doses. Instead of being randomly distributed onto plants by rain splash, bacteria in soil may invade plant tissue through the xylem (Monnerat et al. 2009). Experimental inoculation of sterilized seeds with *Bt* spores shows that this bacterium is capable of colonizing plants endophytically directly from the soil (Bizzarri and Bishop 2008; Monnerat et al. 2009; Raymond et al. 2010b). Colonization from soil is sufficient to ensure that bacteria are present on growing leaf material at doses high enough to kill insects (Bizzarri and Bishop 2008; Monnerat et al. 2009); precise analysis of dose-response curves shows that doses in the region of ten spores can cause detectable levels of mortality in susceptible hosts such as *P. xylostella* (Cornforth et al. 2015).

Moreover, the ability to colonize plants is not universally distributed across the *B. cereus* group (Vidal-Quist et al. 2013); strains of *Bacillus weihenstephanensis*, which have a presumed niche in the plant rhizosphere, are poor leaf colonists, while a range of pathogenic isolates of *Bt kurstaki* ST8 are efficient leaf colonizers (Raymond et al. 2010b). While *Bt* can be readily recovered from plant surfaces, this does not necessarily mean that *Bt* is a specialist epiphyte using the plant exudates as

a primary resource for growth. In comparison with many bacterial epiphytes, *Bt* proliferates weakly on the leaf surface, sporulates readily and persists quite well when humidity is low (Maduell et al. 2008). This weak epiphytic proliferation suggests that colonization of plant material takes place in order for these bacteria to reach a habitat where infection of hosts is likely to take place. While there is no particular evidence to indicate that plants are maintaining *Bt* populations as symbiotic bodyguards, *Bt* can colonize leaf tissue and evades plant immunity in roots (Vidal-Quist et al. 2013) indicating the presence of adaptations that allow *Bt* to use plants to effectively vector bacteria from the main spore reservoir in the soil to tissues where hosts are likely to be feeding.

Critically, a manipulative field experiment has shown that populations of *Bt kurstaki* in the environment increase in the presence of larval hosts, without the existence of obvious epizootics (Raymond et al. 2010b). The increase in total numbers of bacteria in the *B. cereus* group and in the proportion of strains carrying Cry toxins was detectable in the top 1 cm of soil but not on leaf tissue. Experimental cages that excluded Lepidoptera larvae showed no such increase. Application of biopesticides to this experiment (DiPel WP) resulted in a very transient increase in *Bt* density on leaf tissue, while adding hosts to experimental cages had a more substantial impact on *Bt* density in the long term (Raymond et al. 2010b). This work corroborates earlier observational studies reporting increases in the abundance of dipteran herbivores can increase the prevalence of *Bt* strains pathogenic to Diptera (Damgaard et al. 1998). These data also support the indirect transmission hypothesis. An increase in *Bt* density in the top layer of soil suggests that infected and paralysed insects quickly fall off plants into leaf litter, from which spores may then enter soil and persist long enough to be taken up into plants endophytically where they may sporulate and produce Cry toxins (Raymond et al. 2010a, b). Notably this proposed life cycle does not require adaptations for efficient attachment of spore and crystals or efficient persistence of Cry toxin in soil, relevant because there do not seem to be any specific adaptations for spore crystal attachment for *Bt kurstaki* and many other strains (Deng et al. 2015). Roughly 50% of *Bt* colonizing plants from soil were located endophytically (Raymond et al. 2010b) so both spores and crystals may be located in stomata or co-localized within plant tissue. This life cycle is similar to the one proposed for *B. anthracis*. Anthrax spores must persist for long periods before finding a new host; infections in ungulates are typically acquired orally when animals consume contaminated plant material, allowing bacteria to enter the host through abrasions in the mouth (Dragon and Rennie 1995). The selective advantages of an association with plants may explain the ability of *B. anthracis* to persist in the rhizosphere (Saile and Koehler 2006) and why *B. anthracis* may be able to promote the growth of some plant species (Ganz et al. 2014). In contrast, direct cadaver host transmission may be more important for nematode-infecting *Bt* strains (Ruan et al. 2015), while *Bt israelensis* spores and toxins are efficiently concentrated by aquatic filter feeding in blackflies and mosquitoes (Lacey et al. 1978; Charles and de Barjac 1981), so that direct transmission of spores in aquatic habitats is very plausible.

2.5 *Bt* as a Specialized Pathogen

To paraphrase the old adage, if it looks like a pathogen, kills insects like a pathogen and reproduces like a pathogen, it probably is a pathogen. In addition to the production of Cry toxins, *Bt* has numerous adaptations associated with being an efficient specialized invertebrate pathogen (Raymond et al. 2010a). To summarize, *Bt* has to be able to disrupt or pass through the peritrophic membrane surrounding the gut, cross the midgut epithelium, evade host immunity and suppress competition from bacteria in the gut. Adaptations to survive on a peptide-rich diet have been discussed above; these are complemented by the secretion of iron-scavenging siderophores. *Bt* can produce a range of additional virulence factors such as Vip and Cyt toxins that enable it to cross the midgut epithelium (Yu et al. 1997; Perez et al. 2005; Bravo et al. 2007). The PlcR-papR quorum-sensing system, possibly active at the level of the microcolony very early in infection, coordinates the release of a large array of enterotoxins, proteases and phospholipases with a role in assisting invasion from the midgut (Salamitou et al. 2000; Gohar et al. 2002, 2008; Slamti et al. 2014; Zhou et al. 2014). Immune inhibitors such as the InhA1 and InhA3 metalloproteases may help evade haemocytes and break down antimicrobial peptides (Ramarao and Lereclus 2005; Guillemet et al. 2010). A second later acting quorum-sensing system, NprR, helps coordinate efficient resource use and sporulation during proliferation in late infection in the cadaver and activates degradative enzymes such as lipases, proteases and chitinases (Dubois et al. 2012, 2013; Slamti et al. 2014). While a number of the above systems might be general virulence factors which occur commonly in both invertebrate and vertebrate pathogens, the mass of evidence certainly points to a pathogenic lifestyle, albeit one in which plants may be exploited as vectors. Since *Bt* populations can increase in response to the presence of insect hosts (Ohba and Aratake 1994; Damgaard et al. 1998; Raymond et al. 2010b), a simple explanation for why we might not always see a correlation between the presence of insects and *Bt* abundance in the field is that *Bt* spores are persistent, readily dispersed (Damgaard et al. 1997), and the availability of hosts transient. *Bt*, as we discuss below, is genetically heterogeneous, and given its prevalence in the environment and the concomitant selection pressure imposed on hosts, we might not expect all bacterial genotypes to do equally well in all invertebrate species (Schulte et al. 2010). Adaptations to overcome resistance in one particular genetic background can also trade off and reduce efficacy in other host genetic backgrounds (Soberon et al. 2007). As we have described previously, experiments using biopesticide-derived strains might also underestimate the transmission and replication potential of *Bt* relative to freshly isolated wild-type strains (Raymond et al. 2010b, 2013).

2.6 Virulence, Cooperation and How Investment in Cry Toxins Is Maintained in the Field

Although we are now better informed as to how *Bt* populations can benefit from the presence of invertebrates hosts, the magnitude of the cost of investment in Cry toxins, up to 25% of dry weight at sporulation (Agaisse and Lereclus 1995), is difficult to comprehend. The persistence of this high-cost investment is harder to understand when it is appreciated that the Cry toxins must be solubilized in the insect midgut before being activated and able to bind to receptors (Schnepf et al. 1998). Thus, these metabolically costly products are not even privately available to benefit of the bacteria that produce them. Cry toxins would be described by economists as 'public goods', a term now widely employed by evolutionary biologists (Sachs et al. 2004; West et al. 2007a). For example, *Bt* spores may coexist with *B. cereus* spores on the leaf surface (Collier et al. 2005; Raymond et al. 2010b). If *B. cereus* spores are ingested with *Bt*, then in some cases the *B. cereus* strain can exploit the action of Cry toxins by invading the host haemolymph, where they can outcompete *Bt* by virtue of the fact that they do not have to invest in Cry toxin production in the cadaver (Raymond et al. 2008b). Similar results have been observed in experiments competing *B. anthracis* (essentially a Cry-null *B. cereus* biovar) against a Cry5B expressing *Bt* strain in nematodes (Kho et al. 2011). Note that this ability to outcompete *Bt* in the host does not extend to all members of the *B. cereus* group, such as the more saprophytic *B. weihenstephanensis* (B. Raymond unpublished data). However, if we cure *Bt* strains of the plasmids carrying Cry toxin genes, these approximately isogenic strains can outcompete Cry producers in the cadaver (Raymond et al. 2012).

The conceptual problem of cooperation, i.e. producing goods or investing in behaviour that is beneficial to groups but costly to individuals, may be unfamiliar to many in invertebrate pathology, but has been a long-standing subject of interest to evolutionary biologists (Hamilton 1964a; West et al. 2007a). Cooperation is broadly defined in evolutionary biology as a behaviour that provides a benefit to another individual (Hamilton 1964a; West et al. 2007a). While there are many different forms of cooperation, the most conceptually challenging form to explain is altruism, in which the cost to individuals exceeds the direct benefit to that individual (West et al. 2006). Altruism presents a challenge to evolutionary theory since this form of cooperation can be exploited by 'cheats', individuals that freeload on the cooperative behaviours of others and do not cooperate or cooperate less than expected in return. Cheats, such as our Cry toxin-null mutants, are expected to have higher fitness than cooperators in mixed populations, especially when the frequency of cooperators is high and there are abundant available public goods (Griffin et al. 2004; Ross-Gillespie et al. 2007; Raymond et al. 2012). Over the last two decades, the idea that microbes engage in cooperative and altruistic behaviours has been widely developed. These altruistic behaviours, usually based on the secretion or release of extracellular factors, can have a wide range of functions including biofilm formation, nutrient acquisition, quorum sensing, host-cell lysis, antimicrobial activity and

immune evasion (West and Buckling 2003; Brockhurst et al. 2006; Diggle et al. 2007; West et al. 2007a). Examples of microbes altruistically laying down their lives or sacrificing all future reproduction for their group mates include the autolytic self-destruction of colicin-producing *Escherichia coli* (Cascales et al. 2007) and the non-reproductive role of stalk forming cells in fruiting bodies of microbes that serve to increase the height of dispersal of their reproductive colleagues (Velicer et al. 2000; Strassmann and Queller 2011).

The conditions that can lead to the persistence of altruistic behaviours, such as the production of Cry toxins, are now relatively well understood (Hamilton 1964a, b; Frank 1998, 2010; West et al. 2007a, b). In simple terms, individuals should tend to show altruistic behaviour towards their relatives and individuals also likely to share genes for cooperation; for bacteria this generally means clonemates (Hamilton 1964a, b; Frank 1998). Secondly, there should be enough spatial structure to facilitate competition between groups of individuals (Taylor 1992; Griffin et al. 2004). In other words, if some groups contain high levels of cooperators, individuals in this group should have an improved access to resources compared to groups with a lower level of investment in cooperation. While this may seem a relatively abstract point, for pathogens such as *Bt*, the implications are clear: groups of bacteria with higher levels of investment in Cry toxins are going to be better at establishing infections in hosts, everything else being equal (Raymond et al. 2012). For *Bt* many of the predictions made for the evolution of cooperation seem to hold true for the production of Cry toxins. While *Bt*- and *B. cereus*-like strains can be found in the field, in general *Bt* persists in patches that show a high level of clonality, particularly in places on plants where *Bt* is likely to be eaten by insects (Raymond et al. 2012). While cheater mutants do well when virulent *Bt* has high density and Cry toxin producers are at a high frequency, Cry toxin producers have an advantage at low population density and when Cry toxin production is rare (Raymond et al. 2012). Thus, even in the presence of high levels of competition from social cheaters, Cry toxin producers can invade and have high fitness in the field, once given the presence of selective pressure from hosts. These results could provide another explanation for the relative rarity of *Bt* epizootics: as the density of Cry producers increases, the invasion of non-pathogenic cheaters is more likely and could curtail the spread of disease. Environments in which the invasion of cheating *B. cereus* strains is less likely (grain bins, insect culture in the laboratory) are precisely those environments in which *Bt* seems best able to produce epizootics (Burges and Hurst 1977; Delucca et al. 1982; Itova-Aoyolo 1995; Federici and Siegel 2007). Bottlenecks occurring in the colonization of plants probably play a large part in the near-clonal population structure we see in the field. However, structured and near-clonal populations of *Bt* can emerge simply through the process of invading hosts (van Leeuwen et al. 2015). Not only do *Bt* populations pass through a tight population bottleneck when colonizing the gut (Zhou et al. 2014), but strong competitive interactions based on how quickly competing genotypes invade the host can also limit genetic diversity in the cadaver and increase clonality (van Leeuwen et al. 2015).

2.7 Bt as a Useful Model in Pathogen Evolutionary Ecology

Cooperation and social evolution have broad implication for understanding the ecology and evolution of a range of pathogens and of *Bt* in particular (Raymond and Bonsall 2013). While cheating and social conflict are strongly in evidence in competition between Cry producers and non-producers, similar conflicts may exist for the production of the other major groups of *Bt* virulence factors: those regulate by the PlcR-papR quorum-sensing system (Zhou et al. 2014). Here, competition between wild-type bacteria and signal-null or signal-blind mutants shows the frequency and density-dependent fitness characteristic of social interactions (Zhou et al. 2014). However null PlcR or papR mutants are not effective cheats in that they do not have higher fitness than wild-type strains when in competition in the cadaver (Zhou et al. 2014). Here, the group-level competition required to stabilize cooperation seems to occur at the level of microcolony within the insect gut, so that patches of microbes with increased investment in quorum-regulated virulence factors are better able to invade the host and potential cheaters fare less well (Zhou et al. 2014).

Cooperation and cheating models may also be relevant for understanding the evolution and maintenance of virulence in other invertebrate parasites. Entomopathogenic nematodes and their bacterial symbionts also rely on a wide range of secreted virulence factors (Forst et al. 1997; Ffrench-Constant and Bowen 2000; Daborn et al. 2002; Eleftherianos et al. 2007). Entomopathogenic strains with high virulence can be hard to maintain without serial propagation in hosts, a process that can lead to attenuation or 'deterioration' (Wang and Grewal 2002; Bai et al. 2005; Bilgrami et al. 2006). Notably, attenuated nematodes, which have reduced ability to infect hosts, have shown reduced expression of secreted proteases with putative roles in suppression of host immunity and tissue invasion (Simões et al. 2000; Adhikari et al. 2009). One hypothesis that could explain the loss of virulence during host passage is that conditions in the laboratory (high doses) could favour cheater mutants with reduced virulence. A test of this hypothesis using experimental evolution showed that conditions that would be expected to favour cheats led to rapid loss of virulence, while low-dose serial propagation regimes maintained a high level of virulence (Shapiro-Ilan and Raymond 2016).

More broadly, understanding that investment in virulence may be a cooperative group-level activity can affect how we might model or understand dose-response mortality curves (Cornforth et al. 2015). Traditionally dose response has been understood to be the result of multiple infectious agents acting individually, each with an independent probability of causing infection – the independent action hypothesis (Haas 1983). This hypothesis has a number of important applications in terms of modelling disease risk, especially for those that extrapolate dose response from limited data (Haas 1983; Haas et al. 1997; Jones et al. 2009). This model of infection has also been widely applied in invertebrate pathology, for instance, in constructing quantitative methods to examine the existence of synergistic interactions in mixed infections or between *Bt* toxins (Tabashnik 1992). However, experiments with *Bt* provide one of the first direct tests of the independent action theory

and do not find good evidence to support its main assumptions (Cornforth et al. 2015). In essence, the mortality/dose-response curves of *Bt* toxin spore mixtures and Cry toxins are too threshold-like to be explained by independent action (Cornforth et al. 2015). Threshold-like dose-response curves, i.e. relatively more efficient infections when pathogens are at high doses, might be expected to drive a greater reliance on cooperation and collective action (Raymond and Bonsall 2013).

2.8 Phylogeny and Relationship to *Bacillus cereus* and Wider Group

The final controversy addressed here is that of the identity of *Bt* as a species. Since *Bt* is currently defined on the basis of the expression of Cry toxin parasporal inclusions, and because the genes responsible for these proteins are typically located on conjugative plasmids (Gonzalez et al. 1982; Gonzalez and Carlton 1984; Vilas-Bôas et al. 2008), it is not surprising that the taxonomy of *Bt* is unconventional if not downright messy. If we define phylogenetic clades based on chromosomal genes, at least two well-defined groups contain *Bt* isolates, giving us the problem of polyphyly (Fig. 2.1). In addition, both of these clades are comprised of both *Bt* and *B. cereus* making *Bt* also paraphyletic (Priest et al. 2004; Cardazzo et al. 2008; Didelot et al. 2009; Raymond et al. 2010b; Raymond and Bonsall 2013). While these facts are not in doubt, disagreement remains on the issue of what exactly we should do about it. One view is that the entire *B. cereus* group containing *Bt*, *B. cereus s.s.*, *B. anthracis*, *B. mycoides*, *and B. weihenstephanensis* should be treated as one species (Helgason et al. 2000; Tourasse et al. 2006), while a recent whole-genome sequencing paper suggested breaking up the group into 19 or 20 species (Liu et al. 2015). Alternative options include leaving things as they are or taking a more moderate view in terms of splitting the group.

While it is going to be difficult to untie this particular Gordian knot, there are a number of convincing arguments against lumping the entire group under one species name. First, only the early protein electrophoresis methods have supported the view that the *B. cereus* group is genetically homogeneous (Helgason et al. 2000). All subsequent phylogenies based on chromosomal sequencing, particularly multilocus sequencing typing and whole-genome methods, have shown that there are several well-supported genetically distinct clades in the *B. cereus* group (Vilas-Boas et al. 2002; Priest et al. 2004; Sorokin et al. 2006; Vassileva et al. 2006; Cardazzo et al. 2008; Guinebretière et al. 2008; Didelot et al. 2009; Raymond et al. 2010b). Analyses of the patterns of horizontal gene transfer suggest that there are at least three major clades and that most recombination occurs within rather than between clades, making these groups something akin to 'biological species' (Didelot et al. 2009). In addition, there is abundant evidence for substantial ecological differentiation between clades, either in terms of their ability to colonize plants (Raymond et al. 2010b; Vidal-Quist et al. 2013), their carriage of virulence factors such as enterotoxins (Cardazzo et al. 2008), the risks they pose to vertebrates (Cardazzo et al. 2008; Guinebretière et al. 2010; Raymond and Bonsall 2013) or their metabolic and

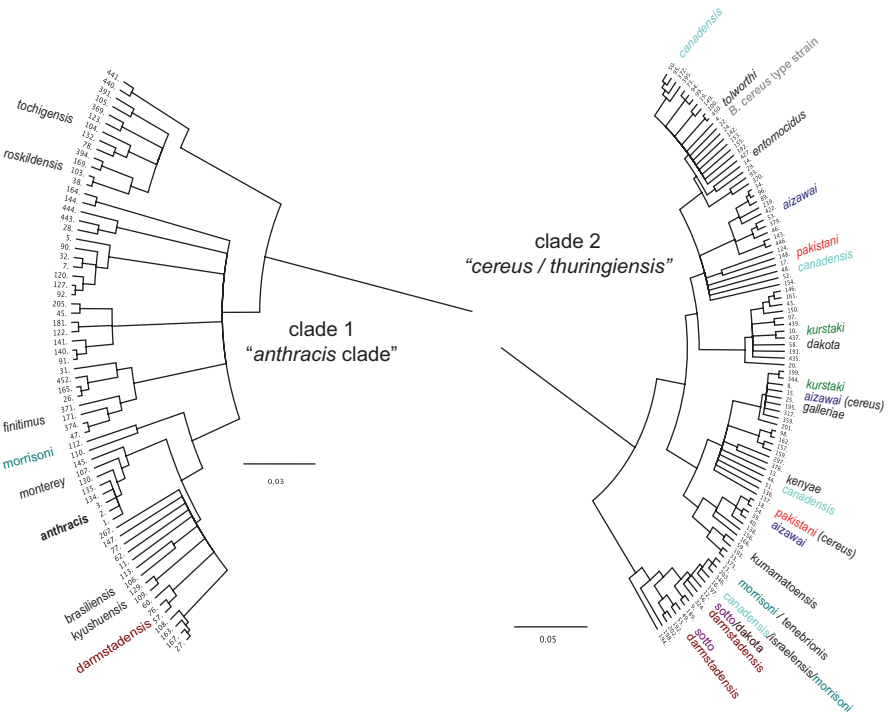

Fig. 2.1 The distribution of Cry-producing strains in two major clades of the *B. cereus* group. The tree is redrawn from the MLST study in Raymond et al. (2010b) using sequences from seven housekeeping genes and data from PubMLST (https://pubmlst.org/). Serovar names have been colour coded to indicate how widely distributed they are across clades

growth characteristics (Guinebretière et al. 2008). Analysis of the surface layer protein *csaB* gene, which is important in eliciting host immune responses, also indicates that host-specific factors may have driven diversification between clades (Zheng et al. 2013). Lumping clades, therefore, seems to be poorly justified on the basis of genetics, biology, ecology and reproductive isolation.

Attributing particular clades to particular species names could be relatively straightforward in some cases. For examples, strains in the clade containing *B. anthracis* are far more likely to have been isolated from acute vertebrate infections (Raymond and Bonsall 2013). *B. anthracis*, for historical reasons, applies to a clone specialized on ungulates, with a handful of SNPs to distinguish isolates (Keim et al. 2009). The justification for retaining this name for a tiny subset of the genetic diversity of *B. cereus s.l.* seems poor when there are other strains with a similar niche and which possess the key virulence plasmids of *B. anthracis* (Hoffmaster et al. 2004). All the potentially lethal emetic strains of *B. cereus* are also situated within the *anthracis* clade (Raymond and Bonsall 2013). A convenient albeit potentially unpopular revision would be to retain *anthracis* as a specific epithet for the whole *anthracis* group and as subspecies specific epithet for strains currently classed as *anthracis.*

While Cry toxin-expressing strains can be found within the 'anthrax clade', they have an elevated ability to cause infection in vertebrates (Hernandez et al. 1998), and most of them (*konkukian, brasiliensis, monterrey, pulsiensis, roskildiensis, tochigiensis*) (Fig. 2.1) have no known invertebrate host. *Bt roskildiensis,* despite being isolated in Denmark, has some activity against termites (Castilhos-Fortes et al. 2002), and one strain of *Bt kyushuensis* has some activity against mosquitoes (Ragni et al. 1996). The antisera standard for *Bt brasiliensis* (BGSC 4AY1/T39001) does not appear to actually produce clear inclusion bodies (B. Raymond unpubl. dat.), while the characterization of *Bt morrisoni* (biovar *san diego*) as ST 112 and therefore a member of the anthrax clade (Kim et al. 2005) is almost certainly an error. The *san diego* biovar is expected to be biological and genetically similar to biovar. *tenebrionis.* which is consistently and clearly related to the other entomopathogenic *Bt* strains (Raymond and Bonsall 2013). *Bt finitimus* HD3 (BGSC 4B2) is one of very few Cry-producing strains in the anthrax clade (Didelot et al. 2009) with an association with insects, as it was isolated from the lepidopteran *Malacosoma disstria* (Zeigler 1999). In short, the Cry-producing strains in the *anthracis* clade are a mixed bag of isolates with poorly characterized biology and host range that may be further confounded by misclassification and contamination. We could withdraw the name *thuringiensis* from this group without any great loss (Fig. 2.1); in fact redefining them as *B. anthracis* could help emphasize that any strain in this group is likely to be far too dangerous to ever be produced as a biopesticide. This one major revision would at least resolve the problem of polyphyly for *B. thuringiensis.*

B. weihenstephanensis was originally defined as a cool-adapted psychrotolerant member of the *B. cereus* group (Lechner et al. 1998). Strains in the *B. weihenstephanensis* clade or clade 3 have almost exclusively isolated from plants and soil (Raymond et al. 2010b; Raymond and Bonsall 2013); this group is depauperate in many enterotoxin genes (Cardazzo et al. 2008), is consistently adapted to low temperatures (Sorokin et al. 2006; Guinebretière et al. 2008) and has a poor ability to grow in insects; evidence that strongly points to this being the most saprophytic clade in the group. Cry-producing strains are typically not found in this group (Raymond et al. 2010b), although psychrotolerant adaptations can be found widely in *B. cereus s.l.* (Stenfors and Granum 2001; Bartoszewicz et al. 2009). Thus, while psychrotolerance should not be seen as sufficient to define a strain as *B. weihenstephanensis* all members of this 'clade 3' could usefully be called *B. weihenstephanensis.* Note that this clade does include isolates of *Bacillus mycoides,* a common saprophytic variant showing hyphal-like colonies on solid media. However, the distinctive *mycoides* phenotype seems to be distributed widely across the group and therefore may be an unreliable species name (Cardazzo et al. 2008; Raymond et al. 2010b; Liu et al. 2015). This is in contrast to *Bacillus pseudomycoides,* which makes for an apparently coherent and distinct lineage (Cardazzo et al. 2008; Guinebretière et al. 2008).

The final major clade was originally named 'clade 2' by the first MLST scheme (Priest et al. 2004) (Fig. 2.1). While this clade is relatively diverse, it contains nearly all the *Bt* isolates that have been well characterized as insect or nematode pathogens and certainly all the well-studied strains (Cardazzo et al. 2008; Didelot et al. 2009; Raymond et al. 2010b; Raymond and Bonsall 2013). Genotypes from this clade tend not be associated with acute vertebrate infections (Raymond and Bonsall

2013), a finding consistent with what we know of very low risk imposed by *Bt* biopesticides for mammals (Siegel 2001; Federici and Siegel 2007) and assessment of clade-level variation in cytotoxicity (Guinebretière et al. 2010). The *B. cereus*-type strain is very closely related to *Bt* serovar *entomocidus* (Federici and Siegel 2007) (Fig. 2.1) and sits within an ecologically diverse sub-clade of this group (Raymond and Bonsall 2013; Liu et al. 2015). Nevertheless, stable exchange of Cry toxin-bearing plasmids seems relatively rare, and the vast majority of genotypes are stably associated with Cry toxin production or not (Raymond et al. 2010b). One taxonomic solution is to retain the designations *cereus* and *thuringiensis* as useful terms and accept that these are paraphyletic species but to restrict the use of these specific epithets for clade 2 bacteria only (Fig. 2.1). The current practice using *B. cereus* as a catch-all species name for any strain with limited characterization – or using the terms *cereus* I, II and III to denote particular clades – is only going to lead to confusion.

2.9 Concluding Remarks

The nature of horizontal gene transfer in bacteria is such that phylogenies of global collections are likely to give a confusing picture. The use of multiple gene or genomic data can lead to better justified clades, but more and more sequencing is not necessarily going to resolve our taxonomic difficulties. More data will almost certainly reveal more isolates with intermediate phenotypes/genotypes and can lead to confusing over-splitting of species (Liu et al. 2015). Nevertheless, at a local scale, *Bt* and *B. cereus* strains are highly differentiated, and groups such as *B. weihenstephanensis* are genetically and ecologically coherent, ensuring that these species names are useful and informative (Vilas-Boas et al. 2002; Raymond et al. 2010b). The assumption that all members of the *B. cereus* group are genetically and ecologically homogeneous can have misleading consequences in terms of assessing safety risks (EFSA 2016) and does not accurately represent what we see in the field. Social interactions may mean that selection for cheating can produce *B. cereus* strains when *Bt* is cured of toxin-producing plasmids; however, this does appear to happen very frequently. Moreover, the production of Cry toxins has profound consequences on the growth and sporulation characteristics of *Bacillus*; it is likely therefore that it will have considerable direct implications for its realized niche. A key barrier for *B. cereus* establishing infections in the vertebrate gut is competition with existing microbes (Ceuppens et al. 2012). The production of Cry toxins substantially weakens the competitive ability of vegetative cells in vivo (Raymond et al. 2007, 2012), and this is likely to make *B. thuringiensis* substantially less fit in the gut of vertebrates, where Cry toxin production is not adaptive. Thus, not only does the production of Cry toxin facilitate the invertebrate pathogenic niche; it may also largely preclude strains from efficiently exploiting vertebrates. As such the designation *B. thuringiensis* remains valuable, and its link to the phenotypic production of Cry toxins is sensible, given the caveats discussed above.

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