Divergence in environmental adaptation between terrestrial clades of the *Bacillus cereus* group

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1 Divergence in environmental adaptation between terrestrial clades of the
2 *Bacillus cereus* group

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Running head –Ecological specialization in *B. cereus*

Keywords: bacterial taxonomy; *Bacillus thuringiensis*; environment dependent fitness; niche differentiation; psychrotolerance

Abstract

Bacteria in the *Bacillus cereus* group encompasses diverse niches and include causative agents of anthrax and food poisoning, specialized invertebrate pathogens and psychrotolerant species. Understanding whether natural selection has led to divergence between phylogenetic clades can help understanding the processes shaping speciation, and has important implications for understanding the distribution and biological safety of this group.

We tested whether the three most common terrestrial species in this group (*B. cereus*, *Bacillus thuringiensis* and *Bacillus mycoides*) are ecologically specialized in terms of resource use, thermal adaptation and fitness in different environmental conditions. All isolates tested grew vigorously in protein rich media and insect cadavers, but exploitation of soil or plant derived nutrients was similarly weak for all species. Psychrotolerance rather than clade predicted growth patterns in artificial media, while psychrotolerant isolates could outcompete *B. thuringiensis* in insect cadavers at low temperature. Nevertheless, for *B. thuringiensis* and *B. mycoides*, clade and taxonomic species were important predictors of growth and relative fitness in live insect infections.

The common ecological niche in these terrestrial *B. cereus* species is the ability to exploit protein rich resources such as cadavers. However, selection has led to different phylogenetic groups developing different strategies for accessing this resource. Thus, clades, as well as traditional taxonomic phenotypes, predict biologically important traits.

INTRODUCTION

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The *Bacillus cereus* group has radiated to exploit a taxonomically very broad range of hosts that includes insects, nematodes and vertebrates (Priest, et al. 2004, Raymond and Bonsall 2013, Stenfors Arnesen, et al. 2008). It contains one of the most widely exploited bacteria in agriculture, *Bacillus thuringiensis* (*Bt*), as well as the causative agents of anthrax (*B. anthracis*) and food poisoning (*B. cereus*) (Stenfors Arnesen, et al. 2008). The ability of *Bt* to infect and kill insects has given it an unrivalled status as a microbial insecticide. However, there are at least eight further species in the group, including *Bacillus mycoides*, which has been recently re-defined to incorporate *Bacillus weihenstephanensis* (Liu, et al. 2018) (Liu, et al. 2015a, Papazisi, et al. 2011). Currently, the most widely accepted taxonomic definitions of species in this group are based on morphology and biochemistry. Thus, *Bt* is defined by its production of crystalline inclusion bodies, while *B. anthracis* (*Ba*) is recognised by its possession of a capsule, among other traits (Logan and De Vos 2009), and *B. mycoides* (*Bm*) is predominantly defined by its psychrotolerance (growth below 7°C) (Lechner, et al. 1998, Liu, et al. 2018). One complication of these definitions is that some of these traits, especially the insecticidal Cry proteins of *Bt*, are encoded on integrons and are potentially horizontally mobile (Zheng, et al. 2017), while *B. cereus sensu stricto* (*Bc*) is defined by the absence of these characteristics as much as by any one particular trait.

The nature and extent of differentiation in ecology and host range across the *B. cereus* group has been intensely debated, and is tied up with discussion of its safety and its taxonomic division into species (Helgason, et al. 2000, Hendriksen, et al. 2006, Jensen, et al. 2003, Raymond and Federici 2017, Raymond, et al. 2010a, Raymond, et al. 2010b). For example, it has been argued that there are no real differences between *Bt* and *Bc* and that both share the ability to cause diarrhoea in humans; if true regulation on the use of *Bt* control pests on food crops should reflect this (EFSA 2016). However, a substantial weight of evidence indicates that entomopathogenic *Bt* cannot infect humans, and that this species is ecologically specialized on invertebrates (Raymond 2017, Raymond and Federici 2017, Raymond, et al. 2010a, Raymond, et al. 2010b) suggesting that continued horticultural applications of *Bt* impose minimal risks.

The use of diverse sequencing and taxonomic approaches has led to numerous suggestions for revising the taxonomy of *Bc* and its relatives. These range from lumping the entire group into single species (Helgason, et al. 2000, Tourasse, et al. 2011) to splitting the group into 19-20 species (Liu, et al. 2015b). However, many phylogenetic studies emphasize that the majority of isolates fall into the three robust clades originally defined by multi-locus sequence typing (MLST). These are: *B. anthracis*, emetic *B. cereus*, and *Ba* relatives – Clade 1; entomocidal *Bt* and *Bc* relatives - Clade 2; and *B. mycoides* - Clade 3 (Cardazzo, et al. 2008, Didelot, et al. 2009, Guinebretière, et al. 2008, Méric, et al. 2018, Priest, et al. 2004, Raymond, et al. 2010b, Sorokin, et al. 2006, Vassileva, et al. 2006, Vilas-Boas, et al. 2002). These three clades are also well supported as species using average nucleotide identity (ANI) (Zheng, et al. 2017). However, there is now good justification for splitting clade 1 into psychrotolerant (*Bacillus weidmannii* and allies) and mesophilic (*Ba* and its relatives) clades, sometimes referred to as genetic groups II and III respectively (Drewnowska, et al. 2020, Guinebretière, et al. 2008, Miller, et al. 2016).

While taxonomic species definitions are intensely debated, the readily resolved MLST clades in the *Bc* group may be actually be more useful for issues such as predicting biological safety (Guinebretière, et al. 2008). If selection and adaptation has driven genetic divergence then the genetic distance between these major clades should predict important biological traits, horizontal gene transfer notwithstanding. For instance *Bt* can cause rare opportunistic infections of humans (Hernandez, et al. 1998); however these putative *Bt* have been associated with sequence types that are much more closely related to *Ba* (in clade 1) than to the clade 2 genotypes used in pest management (Raymond 2017, Raymond and Federici 2017). Some have argued that *Bt* may have been responsible for a large number of unrecognized cases of food poisoning in humans (EFSA 2016, Stenfors Arnesen, et al. 2008). However, this hypothesis is not supported by multi-locus sequence typing (MLST) of clinical isolates: bacteria isolated from humans have genotypes that are distinct from the typical entomopathogenic Cry toxin-expressing strains. In addition the common *Bt* genotypes sit in a different clade to the most dangerous emetic food-poisoning *Bc* genotypes, which are also in clade 1 (Raymond and Federici 2017, Raymond and Federici 2018). Overall, we would predict that

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3 92 phylogenetic clade, in addition to species designations, is likely to determine key ecological attributes
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5 93 (Cardazzo, et al. 2008, Guinebretière, et al. 2010, Raymond and Bonsall 2013).
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8 94 The main aim of this study is to test whether genetically differentiated clades in the *B. cereus*
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10 95 group predict fitness and eco-physiological traits across the group. Despite the importance of
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12 96 ecological traits in determining safety there has been little experimental work to test for ecological
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14 97 differences between clades or formal taxonomic species except in terms of temperature tolerance,
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16 98 biogeography and degree of pathogenicity (Drewnowska, et al. 2020, Guinebretière, et al. 2008,
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18 99 Guinebretière, et al. 2010, Raymond, et al. 2010b). The secondary aim of this work is to clarify the
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21 100 biological differences between the different clades and taxonomic species across the *B. cereus* group.
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23 101 Although the *B. cereus* group now includes a number of recently described species, especially from
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25 102 marine species environments (Liu, et al. 2017, Miller, et al. 2016) this study was restricted to the most
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27 103 commonly isolated terrestrial species. To date, a wide range of ecological roles has been proposed for
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29 104 different members and encompasses soil, plant and animal associated niches (Guinebretière, et al.
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31 105 2008, Halverson, et al. 1993, Hendriksen and Carstensen 2013, Raymond 2017, Ruan, et al. 2015,
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33 106 Vidal-Quist, et al. 2013, Vilain, et al. 2006). These have led to some specific predictions that
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35 107 informed our choice of media and experimental conditions. We favour the hypothesis that the
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37 108 common ecology of the *B. cereus* group is the ability to efficiently exploit vertebrate and/or
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39 109 invertebrate cadavers (Ruan, et al. 2015), while distinct clades or species may have additional
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41 110 adaptations as invertebrate pathogens (Raymond, et al. 2010a, Raymond, et al. 2010b) or different
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43 111 thermal profiles that allow exploitation of different hosts (Guinebretière, et al. 2008). Plant associated
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45 112 niches have been proposed for *Ba* and individual *Bc* isolates (Ganz, et al. 2014, Halverson, et al.
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47 113 1993), but broader comparisons across the group suggest that *Bm* and its relatives (Clade 3) are
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49 114 potentially better able to colonize roots (Vidal-Quist, et al. 2013). In order to test these ideas we
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51 115 characterized a collection of *B. cereus* group strains from three species (*Bt*, *Bc*, *Bm*), clarified their
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53 116 phylogenetic and taxonomic status and assessed their ability to exploit media simulating plant root,
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55 117 animal and soil niches. For *Bm* and *Bt*, we also characterized whether membership of distinct clades
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118 would predict fitness or growth patterns at different temperatures or in different environmental
119 conditions including insect hosts and artificial media.

120 MATERIALS AND METHODS

121 Strains

122 Isolates of *B. cereus s. stricto*, *Bt* and *Bm* strains used in these experiments are listed in Table

123 1. The presence and absence Cry inclusion bodies in *B. thuringiensis* was confirmed via Coomassie
124 Blue staining and examination under oil immersion microscopy in an earlier study (Méric, et al.
125 2018). In order to determine whether strains were formally *B. mycoides* we tested for
126 psychrotolerance for all strains in two ways. First, we tested for the ability to grow at 10°C on
127 Lysogeny Broth (LB) over 48 hours (broad psychrotolerance); the second method used the more strict
128 definition of psychrotolerance used by Lechner, et al. (1998) which was assessing growth at 7°C in
129 liquid PC medium (with shaking) over 7 days. We identified three broadly psychrotolerant isolates
130 that were previously been identified as *B. weihenstephanensis* and would now be classed as *B.*
131 *mycoides* (Soufiane and Côté 2010). However, these isolate could not grow at 7°C in this study; we
132 initially retained the *Bm* species designation for these three isolates but have highlighted the
133 implications of this below. Spores for competition experiments were produced on *B. cereus*-selective
134 agar (Oxoid Ltd.), washed, pasteurized and diluted as described previously (Zhou, et al. 2014).

135 Phylogenetic reconstruction

136 All strains listed in Table 1 had MLST profiles that were listed in the pubmlst.org database (Jolley, et
137 al. 2018). A small number of strains had duplicated and conflicting profiles. For these ambiguous
138 strains we confirmed the appropriate sequence type using the *pta* and *gmk* primers from the *B. cereus*
139 group scheme using methods described previously (Raymond, et al. 2010b). In order to create a
140 phylogeny that would allow us to place experimental strains with major clades we extracted sequence
141 profiles from the PubMLST database for each sequence type from the experimental strains and for
142 175 additional profiles. These included the MLST sequences of several type strains, such as *Ba*

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Ames strain, *Bt entomocidus*, and *Bm* WSBC 10364. All extra sequences came from the first 200 sequence types in the database. All sequences were extracted in a concatenated format. The concatenated sequences were aligned in PubMLST using the MAFFT algorithm (Pais, et al. 2014) and a maximum-likelihood tree was created using IQ-Tree (Minh, et al. 2020) and visualised in gg-tree (Yu, et al. 2016).

Resource specialization across the *B. cereus* group

Given the major impact of Cry protein production on growth and productivity (Raymond, et al. 2012), we used Cry-null plasmid cured *Bt* strains for the resource specialization experiments study to give a fair growth comparison with *Bc* and *Bm* (see isolates with asterisks in Table 1). To test for resource specialization across this species we measured growth and productivity across six different media selected to represent animal, soil and plant resources. Brain heart infusion (BHI; Honeywell International Inc. Fluka) was selected as protein rich media to represent growth within a host or cadaver; SESOM (soil-extracted soluble organic matter) is a soil-simulating media previously used to culture *B. cereus* (Vilain, et al. 2006); while TREM (tomato root extract medium) replicates the nutrients around plant roots (Lugtenberg, et al. 1999).

SESOM media was produced in three forms: as a base form that used soil as the only carbon source; in an enriched form (with soil and additional carbon) and as a control media with enrichment but no soil. Base SESOM was produced as per Vilain, et al. (2006) by mixing 100g commercial loam-based topsoil (Asda Stores Ltd.) in 500ml of 10mM pH7 MOPS 3-(N-morpholino)propanesulfonic acid) buffer via shaking for one hour at 200rpm and then filter sterilising with 0.2µm filters. The media was checked for contamination by plating out 100µl on 2% LB agar plates and incubating overnight. For enriched SESOM, 100mg glucose, 1mg casein hydrolysate and 1mg yeast extract was added to 1l base SESOM. The ‘MOPs enriched’ control version of SESOM used the same buffer and sugar enrichment but no soil (Vilain, et al. 2006). Base TREM is salt buffer and contains no carbon source and was included as a control, while enriched TREM contains amino acids as well as simple

and complex sugars (Lugtenberg, et al. 1999). Enriched TREM resulted in an opaque broth: this was used in a filtered form (filtered through Whatman No 1 paper) and an unfiltered form.

Growth rate and productivity were assessed using a plate reader (Thermo Fisher Scientific Inc. Varioskan Flash). Standard pre-culture conditions were overnight growth in 5ml 2% LB at 30°C with 150rpm shaking from glycerol stocks. Overnight cultures were then diluted 1,000-fold in sterile saline (0.45% NaCl), and 10µl of this diluent added to 200µl of growth medium in 96-well plates. Growth of each strain was recorded across six wells in two plates at 26°C for 24 hours. OD₆₀₀ readings were taken every 2mins with shaking before each reading. Each plate used distinct media treatments.

Clade dependent variation in thermal profiles and fitness in *Bt*

Here we specifically sought to test whether *Bt* isolates from clades 1 and 2 were ecologically divergent and had distinct thermal profiles, or varying fitness in different environmental conditions. Since clade 1 *Bc* isolates are much more commonly associated with vertebrate hosts (Raymond and Bonsall 2013) we predicted that clade 1 *Bt* strains would grow better than clade 2 at higher temperatures (*ca.* 37°C). Similarly, since clade 2 appears to hold the majority of specialized insect pathogens and bacteria with insect associated virulence factors (Méric, et al. 2018, Raymond and Bonsall 2013, Zheng, et al. 2017) we predict that this clade would have higher fitness in an insect host, but lower fitness in simple media where investment in virulence would impose costs.

In order to carry out thermal profiling the growth rate and productivity of 17 Cry positive isolates were assessed in across a temperature range in 96-well plates, as described above. The experimental temperature range was 14, 17, 20, 23, 26, 29, 32, 35 and 38°C in both 2% LB and 2% BHI; all growth measurements were replicated in three wells. V_{max} and productivity were calculated from the resulting growth curve data, as described below. *Bt* strains from clade 2 (*n* = 12) and clade 1 (*n* = 5) (Table 1) were assessed in thermal profiling and competition experiments.

We conducted competition experiments in sporulation media and insects to measure relative fitness of isolates in different contexts. Sporulation media (HCO) (Lecadet, et al. 1980) was used in preference to nutrient rich BHI or LB, where sporulation is inefficient, as it allows a measure of relative fitness from spore to vegetative growth to spore in a manner that is comparable to growth in cadavers. A marked *Bt* clone was produced for use as the standard competitor in all competition experiments: *Bt kurstaki* isolate 7.1.o was transformed with the plasmid pHT315 carrying a red fluorescent marker (*DsRed*) and tetracycline resistance (*tet^R*) (Zhou, et al. 2014). Bacteria were transformed by electroporation following Peng, et al. (2009) and the resulting strain named *Btk* pHT315-*DsRed-tet^R*. Since not all strains in our panel are capable of infecting and killing our host the Cry toxins produced by this marked competitor ensured disintegration of the midgut and invasion of the host by all competing strains (Raymond, et al. 2007). Note that fitness *in vivo* in this study assessed the ability to replicate in hosts post-invasion, but not infectivity or pathogenicity.

In vivo competition experiments used diamondback moth, *Plutella xylostella*, larvae from the VLSS population, reared on artificial diet as described previously (Zhou, et al. 2018), except that all experimental infections used surface sterilized eggs produced by a 30s wash in 2% bleach solution, followed by two washes in sterile H₂O. Mixtures for competition assays were prepared by diluting *Btk* pHT315-*DsRed-tet^R* spore suspensions to 200cfu/μl and mixing to produce a 3:1 ratio of experimental strains to *Btk* pHT315-*DsRed-tet^R*. Late 2nd-instar insects were infected on sterile diet in 50mm Petri dishes, the resulting cadavers were incubated until sporulation, pasteurized and homogenized as described previously (Matthews, et al. 2019, Zhou, et al. 2014). Insect homogenates were dilution plated onto antibiotic-free and 10μg/ml tetracycline 2% LB agar plates. Colony counts on tetracycline plates gave cfu/μl for the *Btk* pHT315-*DsRed-tet^R* competitor. These values were subtracted from those obtained from plain LB plates to give cfu/ml values for marked and unmarked competitors. If *tet^R* counts exceeded those on LB, the reproductive rate of the antibiotic-susceptible competitor was inferred to be zero.

Using *in vitro* competition we tested whether clade differences in competitive fitness were particular to invertebrates or reflected general difference in growth. If exploitation of hosts requires a

range of secreted virulence factors (Dubois, et al. 2012, Zhou, et al. 2014), we hypothesized that adaptations associated with insect pathogenicity would incur costs in simple liquid media. This experiment used the same 3:1 treatment mixtures as the *in vivo* competitions. Here, 10µl of each mixture was added to eight wells of 1ml sporulation medium (HCO) in 24-well plates (Lecadet, et al. 1980) amended with two tablets of Polymyxin B (50,000 IU per tablet, Oxoid Ltd.) per litre. Plates were sealed and incubated at 30°C with 150rpm shaking for four days to ensure sporulation. Spores were enumerated by dilution plating onto antibiotic-free and 10µg/ml tetracycline 2% LB agar plates as above.

Clade dependent variation in fitness in *Bm*

Growth at cool temperatures, or psychrotolerance, is the defining trait of *Bm* (Lechner, et al. 1998). Results of the resource profiling experiments suggested that *Bm* will exploit similar resources to *Bt* (eg insect cadavers), so we hypothesized that variation in temperature will determine fitness in competition with a mesophilic competitor. In addition, while psychrotolerance predominates in clade 3 of the *Bc* group, it can be found in other clades and is present in isolates with *B. mycoides* morphology (Drewnowska and Swiecicka 2013, Soufiane and Cote 2013). As above, we aimed to test whether clade predicted variation in fitness among the broadly psychrotolerant isolates in this study. Experiments used all six broadly psychrotolerant *Bm* strains (Table 1), which were competed against the standard marked *Btk* pht315-*DsRed-Rtet* strain as above. Relative fitness was assessed *in vivo* and *in vitro*, as above, at two temperatures: 15 and 30°C.

Statistical analysis

All bacterial growth experiments (thermal profile and media comparisons) were analysed in the same manner. OD₆₀₀ data from 24 hour growth had growth curve models fitted individually for every plate well, using 4-parameter logistic regressions (Crawley 2007). For wells that would not fit this model, typically those with no or very limited growth, a linear model provided the best fit. The

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choice to use 4-parameter logistic regressions was determined via ANOVA comparison between different models, combined with model AIC values. Fitted models provided $V_{\max}$ (the maximum exponential growth rate) and productivity values for each well. $V_{\max}$ was the maximum slope of the fitted curve, while productivity was calculated by integrating the model and subtracting the initial media OD₆₀₀ value. The initial value in experimental media was taken as the first fitted value.

In relative fitness experiments the Malthusian parameter was calculated as the log₂ ratio of the final / initial spore concentration and relative fitness of strain A with respect to B as the Malthusian parameter of strain A divided by that of B. Relative fitness was log transformed prior to analysis. Statistical analysis of fitness, productivity and $V_{\max}$ used generalized linear mixed models with strain fitted as a random effect using the package *lme4* in RStudio 1.1.456 (R-Development-Core-Team 2016); significance tests used likelihood ratio tests. Graphical analyses were used to check for model assumptions (normality of errors, homoscedasticity). *In vitro* competition data were analysed with Fisher’s exact test when relative fitness could not be calculated.

RESULTS

Phylogenetic resolution

All sequence types were allocated to one of three major clades. Note that in previous analyses of whole genome data two *Bt* isolates were allocated to the ‘minor’ clades 4 and 5 (Méric, et al. 2018). Nevertheless, on the basis of the housekeeping genes used in this study they could be confidently allocated to clade 2 and 1 respectively (Table 1, Figure 1). For a subset of strains that we sequenced there were small differences in sequence between listed profiles and our sequence data. Nevertheless, we were able to assign identities to the level of clonal complex and thus to clade (Table 1). This level of taxonomic resolution was adequate for this study, which was the exploration of patterns at the level of the major clade.

Allocation of strains to clades is broadly consistent with previous studies, the one exception to this rule is the status of the isolates associated with serotypes *asturiensis*, *vazensis* and *navarrensis* (Table

1). These isolates have been variously described as *Bm* or *Bt*, have two sequence types in the MLST database and have different phylogenetic positions in earlier studies (Soufiane, et al. 2013, Soufiane and Côté 2010, Zheng, et al. 2017).

Resource specialization across the *B. cereus* group

We tested whether different species within the *B. cereus* group would have enhanced growth or productivity on media that would reflect specialization on protein rich resources (hosts or cadavers), plant-derived nutrients and soil-derived nutrients. 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 (Figure 2A-B, Figure S1). Additionally, all media treatments could be simplified to two treatments: BHI and ‘all other media’ without losing any explanatory power in statistical models (productivity: Likelihood ratio test $df = 15$, $\chi^2 = 13.1$, $P = 0.60$, Figure 2; growth rate V_{max} Likelihood ratio test $df = 15$, $\chi^2 = 1.90$, $P > 0.99$, Figure 3). There were strong interactions between media type and species, with *Bm* being more productive than the other two species on the protein-rich media (Likelihood ratio test (LRT): $df = 2$, $\chi^2 = 121$, $P < 0.0001$, Figure 2A), while *Bt* had the lowest maximal growth rate on protein-rich media (LRT $df = 2$, $\chi^2 = 73.8$, $P < 0.0001$, Figure 2B).

Using these data we also investigated whether the clade could explain the variation in growth rate and productivity associated with each strain better than phenotypically defined species. These definitions were different primarily because isolates with broad psychrotolerance (ability to grow at 10°C) were found in both clades 2 and 3 (Table 1, Figure 1), while strict psychrotolerance was only found in clade 3. We used the Akaike Information Criterion (AIC) to compare mixed glms with a standard fixed effects structure (containing media X group interactions); models with the lowest AIC values were interpreted as providing the best way of explaining this variation. In analysis of both growth rate and productivity the species designations in Table 1, which defined *Bm* on the basis of broad psychrotolerance, gave the lowest AIC values (productivity AIC 2505, V_{max} AIC -2838). The next best models were those that separated *Bm* from *Bc* on the basis of strict psychrotolerance

(productivity AIC 2604, Vmax AIC -2838); while models using clade gave the highest AIC values (productivity AIC 2626, Vmax AIC -2789). For these data therefore, phenotypes were of more importance than clade in determining growth profiles.

Clade dependent variation in thermal profiles in *Bt*

In this set of experiments, we explored if there were any differences in thermal profiles between Cry expressing *Bt* isolates in clades 1 and 2 (Table 1). In particular, if *Bt* strains in clade 1 are associated with vertebrate hosts then we expected higher growth and performance at higher temperatures (>30 °C). We measured growth profiles in both LB and BHI media from 14 to 38°C (Figure S2), however data at 38 °C were particularly variable and were excluded from statistical analyses to maintain homoscedasticity.

The temperature dependent response of growth for each clade was fitted using cubic mixed models. Although there were no simple differences between clades in productivity overall (LRT $df=1$, $\chi^2 = 1.62$, $P=0.20$), there was a trend suggesting the clades did have near significant different thermal profiles (clade X temperature interaction LRT $df=1$, $\chi^2 = 2.90$, $P=0.089$) (Figure 3A). Different thermal profiles in productivity were apparent with the LB data alone (clade X temperature interaction LRT $df=1$, $\chi^2 = 8.55$, $P=0.0035$; Figure 3A). Contrary to our hypothesis regarding a higher temperature/vertebrate niche for mesophilic clade 1, this group of strains had higher productivity at lower temperatures in LB, while peak growth was below 35 °C for both clades (Figure 3; supplementary figure S2). Media also affected productivity (LRT $df=1$, $\chi^2 = 175$, $P < 0.0001$) but did not interact with temperature (LRT $df=1$, $\chi^2 = 1.6$, $P=0.20$).

Clade 1 also had higher maximal growth rates (V_{max}) at lower temperatures than clade 2 (clade X temperature interaction LRT $df=1$, $\chi^2 = 96.1$, $P < 0.0001$; clade X temperature² interaction LRT $df=1$, $\chi^2 = 27.9$, $P < 0.0001$ Figure 3B), suggesting different profiles are not driven by productivity growth rate trade-offs but by adaptation to different temperatures. Differences between clades in growth rate were marginally reduced in LB as opposed to BHI (LRT clade X media

interaction $df=1$, $\chi^2=6.8$, $P=0.009$) and media also affected slopes of growth against temperature slightly (LRT temperature X media interaction $df=1$, $\chi^2=13.6$, $P=0.0002$).

Clade dependent variation in fitness in Bt

Here we tested whether the clade association of diverse *Bt* strains predicted varying fitness *in vivo* and *in vitro* in competition with a standard marked competitor *Btk* pHT315-*DsRed-tet^R*. As we predicted clade 2, which contains most of the genotypes pathogenic to insects, had higher competitive fitness in cadavers than clade 1 (LRT main effect of clade $df=1$, $\chi^2=10.3$, $P=0.0016$, Figure 4). As a control we used an untransformed wildtype of *Btk* pHT315-*DsRed-tet^R* namely *Bt kurstaki* 7.1.o. This isolate had a relative fitness estimate that was slightly higher than its marked counterpart *Btk* pHT315-*DsRed-tet^R* (mean 1.15, 95% confidence intervals 1.09-1.21) indicating a modest cost for the carriage of the marker plasmid.

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. Relative fitness is meaningless with only a single strain present, so each replicate competition was assessed solely on a binary outcome: which competitor strain was dominant when plated out. The dominant strain was therefore taken to be the fitter of the two competitors, as shown in Table 2. These data produced a contingency table and analysis by Fisher's exact test showed that clade 1 strains were significantly fitter in sporulation media than clade 2 strains ($n=152$; odds ratio = 6.03, $P<0.0001$).

B. mycoides temperature dependent fitness

In vitro, all the psychrotolerant isolates had higher fitness at 15°C than at 30°C as predicted (LRT, temperature main effect $df=1$, $\chi^2=111$, $P<0.0001$, Figure 5). Fitness (relative to mesophilic *Bt*) is >1 at 15°C indicating these strains could outcompete *Bt* at low temperatures. However, there was a substantial heterogeneity associated with clade (and therefore degree of psychrotolerance). Strictly psychrotolerant isolates from clade 3 were less fit than their mesophilic *Bt* competitor at 30 °C but could outcompete *Bt* at 15°C; temperature was less important for the fitness of the clade 2 isolates

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(LRT temperature X media interaction $df = 1$, $\chi^2 = 111$, $P < 0.0001$) and these strains broadly had the same fitness as the mesophilic competitor throughout (Figure 5). This pattern was largely repeated in the *in vivo* experiment: there was a strong clade X temperature interaction (LRT temperature X media interaction $df = 1$, $\chi^2 = 8.58$, $P = 0.0034$; Figure 5) in which clade 3 isolates outcompete the mesophilic *Bt* at low temperatures but not at 30°C. There was not an overall significant main effect of temperature on relative fitness in cadavers ($\chi^2 = 0.0025$, $P = 0.96$).

Discussion

While microbial ecologists commonly infer that microbes are adapted to different habitats from associational data or sequences (Raymond and Bonsall 2013, Sheppard, et al. 2011, Von Stetten, et al. 1999), in this study we experimentally characterized how fitness varied in different environments. The experiments designed to mimic growth in animal, plant and soil habitats were very informative: there was no evidence to suggest that different species or clades had specialized to a substantial degree on diverse food sources. Protein rich media was clearly a favoured resource for all isolates in this study. *Bm* and *Bc* isolates had faster growth in protein-rich BHI than *Bt*, while previous work has shown that *Ba* has increased growth rate compared to multiple strains of *Bc* (De Siano, et al. 2006). Psychrotolerant isolates (however defined), also had higher productivity in BHI than other species. These growth data support genomic analyses emphasizing that *Bc* and its relatives are well adapted to exploit protein-rich resources (Rasko, et al. 2005, Read, et al. 2003). *Ba*, for instance, carries a large number of peptide-binding proteins, proteases, peptidases and amino acid transporters and efflux systems consistent with adaptation to protein-rich animal resources (Read, et al. 2003).

A caveat is that *Bc* and its relatives have large genomes and are capable of reproduction of diverse carbohydrate resources (Stenfors Arnesen, et al. 2008), while experimental results may be dependent on specific choice of media. It is therefore not surprising that there is some evidence for replication in the plant rhizosphere (Dutta, et al. 2013, Ganz, et al. 2014, Halverson, et al. 1993, Vidal-Quist, et al. 2013). However, the balance of growth and genomic evidence suggests that plant-

associated growth may be a minor niche, and perhaps analogous to the use of plants as vectors for accessing animal hosts (Ganz, et al. 2014, Raymond, et al. 2010b). In this study there was no support for the hypothesis that *Bm* may be particularly adapted to grow on nutrients simulating the plant rhizosphere. We also did not replicate previous work showing that *Bc* could grow on soluble soil nutrients (Vilain, et al. 2006): none of the isolates in this study could grow on soil simulating media. While nutrient availability in these media will vary depending on the organic content of the local soil used in media preparation, these data suggest that replication in soil is not robust. Our results agree with the older literature showing that growth of *Bc* or *Bt* in soil requires nutrient amendment or autoclaving of soil preparations (Saleh, et al. 1970, West, et al. 1985, Yara, et al. 1997).

We predicted that clade as well as species designation would be an important predictor of eco-physiological traits. This was apparent in terms of how clade predicted the thermal profiles of *Bt*, the relative fitness of *Bt* and the temperature dependent fitness of psychrotolerant isolates. As noted previously, isolates in clade 2 produce more insect active toxins than isolates from other clades (Raymond and Federici 2017, Zheng, et al. 2017) and this study now shows that clade 2 isolates are better adapted to exploit insect cadavers, at least at 30°C. In contrast, the hosts of many mesophilic clade 1 *Bt* strains are unknown and these strains tend to produce few virulence factors with activity in insects (Zheng, et al. 2017). The reduced fitness of clade 2 in broth, relative to clade 1, is consistent with the interpretation that adaptation to insect hosts involves the expression of costly virulence factors, in addition to Cry toxins (Zhou, et al. 2014). One explanation for the existence of *Bt* strains in clade 1 is that they have recently acquired the integrons responsible for Cry toxin production. Certainly, a number of clade 1 strains that were originally designated as *Bt* e.g., *Bt brasiliensis* and *Bt konkukian* subsequently appeared to lack Cry inclusion bodies or Cry toxin genes (Méric, et al. 2018, Van der Auwera, et al. 2008), suggesting unstable Cry expression or misidentification.

We did find evidence for small differences in the thermal profiles of clade 1 and clade 2 strains, importantly they were in the opposite directions to our predictions, namely that mesophilic clade 1 (genetic group III) would be better able to replicate at 37°C (Guinebretière, et al. 2008). While clade 1 strains could grow at 37°C, peak growth and replication occurred between 30 and 32°C.

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3 398 Optimal temperatures for key metabolic processes typically exceed those normally encountered by
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5 399 organisms (Padfield, et al. 2016). These data are therefore not consistent with clade 1 microbes
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7 400 sharing a vertebrate pathogen niche, or even routinely encountering temperatures above 30°C. The
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9 401 combination of adaptation to protein-rich resources and mesophilic temperatures < 37°C suggest that
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11 402 *B. cereus* group bacteria share the ability to grow in cadavers, rather than live vertebrate hosts. Given
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13 403 the slightly cooler thermal profile of clade 1 *Bt* isolates it is possible that virulence factors are
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15 404 targeting soil dwelling invertebrates such as nematodes rather than the largely above-ground
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17 405 herbivorous insects that make up the hosts for clade 2 isolates.
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21 406 There was clear support for a thermally-defined niche for psychrotolerant *Bm*, although clade
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23 407 and the definition of psychrotolerance were very important here. We found three isolates previously
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25 408 described as *Bm* (Soufiane and Côté 2010) were able to grow at 10°C , unlike most *Bt* strains, but not
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27 409 able to grow at 7°C as per the original definition of *Bm* (Lechner, et al. 1998). They also had two
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29 410 contrasting MLST profiles studies and were located in a different clade here than to previous studies
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31 411 (Soufiane, et al. 2013, Soufiane and Côté 2010, Zheng, et al. 2017) suggesting different laboratories
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33 412 have been working with multiple genotypes with the same name. Irrespective of nomenclature, clade
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35 413 was associated with thermal profiles and predicted temperature-dependent fitness very effectively.
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37 414 The strictly psychrotolerant strains could outcompete *Bt* at 15°C but not at 30°C- and behave as
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39 415 ‘cheats’ by outcompeting *Bt* in insects killed by Cry toxins at lower temperatures (Raymond, et al.
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41 416 2008, Raymond, et al. 2012). Thus, it is a mistake to think of psychrotolerant species such as *Bm* as
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43 417 simply being tolerant of cool temperatures: they are also better adapted to grow at the cool
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49 419 The ecological and taxonomic status of the *Bc* group reflects broader problems in
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51 420 microbiology in terms of how we define species, and how we integrate the modern abundance of
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53 421 sequence data with traditional taxonomic practice. Classical definitions based on biochemistry or
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55 422 accessible morphological traits are still prevalent (Gupta and Maiden 2001, Papazisi, et al. 2011).
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57 423 However, there are a broad range of molecular methods that can complement traditional practice
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59 424 ranging from the use of single, highly conserved genes, such as 16s rRNA (Cohan 2002) to comparing
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genetic distance across whole genomes via measures such as ANI and digital DNA-DNA hybridisation (Chan, et al. 2012, Richter and Rossello-Mora 2009). In the *B. cereus* group, classical methods and different sequence-based approaches lead to very different conclusions (Liu, et al. 2015b, Zheng, et al. 2017). One danger of blindly applying similarity-based methods is that resulting definitions may be a poor means of predicting important biological differences, or that they over-split clades based on neutral genetic divergence and produce uninformative species designations.

Pragmatically, it would be desirable if species designations gave some indication as to the important biological and ecological traits of their members. In the *B. cereus* group this study and others have shown that the major phylogenetic clades, rather than existing taxonomic species, tend to better predict the characteristics of strains (Guinebretière, et al. 2008, Guinebretière, et al. 2010, Raymond, et al. 2013, Vidal-Quist, et al. 2013, Zheng, et al. 2017). One reason for clade being a robust predictor of traits in the *B. cereus* group is that multiple independent studies, using a range of methods, have readily identified the core clades. Importantly, clades are separated by long branch-lengths, relative to divergence within clades, so that each group represents a cluster within which horizontal gene transfer may help to maintain some integrity (Didelot, et al. 2009). This principle of robustness is difficult to encapsulate in distance methods that seek to devise one-dimensional objective criteria for bacterial species boundaries. Inevitably, different methods for taxonomy and phylogenetic reconstruction will compete, and a pragmatic approach is not to place too much weight on individual studies.

The issue of how we define species has broad ramifications (Rosselló-Móra 2003). In higher organisms, where species and subspecies can have a legal protected status, species definitions can lead to intensive debate and have a range of implications for practical conservation. For microbiology, some of the important consequences of delimiting species occur when microbes are exploited in industry or agriculture. Since species are generally associated with a particular safety class (Health 2007), how we define species can determine how regulators perceive the risks and can determine how and if particular groups can be exploited (EFSA 2016). In other words, we expect

species labels to be informative. It follows that ecological differentiation, as well as simple genetic distance, should be incorporated into how we organize microbial species.

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624 transgenic insects in resistance management-A test in experimental mesocosms. 2018;**11**:
625 727-38.

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3 628 Figure 1: The maximum likelihood phylogeny of 200 *Bacillus cereus sensu lato* isolates used to
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5 629 confirm the clade associations of all isolates in this study. The phylogenetic tree was constructed
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7 630 using concatenated sequences from the 7 genes used in the *Bacillus cereus* MLST scheme (Jolley et
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9 631 al, 2010). Labelled strains include all strains used in experiments and listed in Table 1. For
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11 632 orientation, type strains such as *Ba* Ames strain, *Bt entomocidus*, and *B. mycoides* (formerly
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13 633 *weihenstephanensis*) WSBC 1036 and other isolates of note have also been labelled. Where isolates
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15 634 share the same sequence type multiple identifiers have been provided, e.g. BGSC 4Q7/4Q1 represents
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17 635 BGSC 4Q7 and BGSC 4Q1. Species labels were extracted from the MLST database, the literature
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19 636 (Priest et al, 2004; Drewnowska & Swiecicka, 2013; Méric et al, 2018) or defined from experimental
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21 637 tests of psychrotolerance for this study; those listed as NA do not have a confirmed species status.
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26 639 Figure 2: Growth of *B. cereus*, Cry plasmid cured *B. thuringiensis* and *B. mycoides* strains in protein-
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28 640 based media (BHI), soil-simulating media (SESOM) and plant root exudate-simulating media
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30 641 (TREM). Graphs show **A** productivity (cumulative OD₆₀₀ over 24 hours), and **B** maximal growth rate
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32 642 (Vmax), the highest estimate of slope of change in OD₆₀₀ over a 24 hour growth period. Data are the
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34 643 means (±SEs) for all isolates in each species. *Post-hoc* comparisons between species are shown via
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36 644 the black parentheses, *** P < 0.001. This experiment used isolates with asterisks listed in Table 1.
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41 646 Figure 3: Clade base variation in thermally dependent growth profiles of Cry positive *B. thuringiensis*
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43 647 isolates in BHI and LB media. Data points are the means of three wells measured for each isolate.
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45 648 Lines are models fitted for each clade. Graphs show **A** productivity (cumulative OD₆₀₀ over 24
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47 649 hours), and **B** maximal growth rate (Vmax), the highest estimate of slope of change in OD₆₀₀ over a
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49 650 24 hour growth period.
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52 651
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54 652 Figure 4: Effect of clade of origin on relative fitness of *B. thuringiensis* isolates in infections of *P.*
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56 653 *xylostella* larvae. Fitness was measure in competition against a standard labelled competitor (*DsRed*-
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58 654 and *tetR*-tagged *B. thuringiensis* subsp. *kurstaki*). Box plots show medians, the first and third quartiles
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(the boxes), while the whiskers extend to the data points no further than 1.5 the interquartile range, other data point are plotted as outliers. The dashed lines represent the mean fitness for each clade. *Post-hoc* comparisons between species are shown via the black parentheses: *** $P < 0.001$. Note that a relative fitness of 1 indicates equal fitness of the experimental isolate and the standard *Bt* competitor.

Figure 5: Effect of clade and temperature dependent on the relative fitness of psychrotolerant *B. cereus* isolates (putative *B. mycoides*) in *P. xylostella* larvae and in HCO sporulation medium. Fitness was measured using competition against a standard labelled competitor (*DsRed*- and *tetR*-tagged *B. thuringiensis* subsp. *kurstaki*). Box plots show medians, the first and third quartiles (the boxes), while the whiskers extend to the data points no further than 1.5 the interquartile range, other data point are plotted as outliers. Note that a relative fitness of 1 indicates equal fitness of the experimental isolate and the standard *Bt* competitor. All clade 3 isolates are strictly psychotolerant and can grow at 7°C, clade 2 isolates could grow at 10°C but not 7°C.

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relative fitness

Bt group

Clade 1

Clade 2

alesti darmstadtensis israeliensis jegathesan kumamotoensis kurstaki7.1.0 kyushuensis morrisoni sotto 4E3 tenebrionis thuringiensis 4A3 thuringiensis 4A5 finitimus monterrey pulvisensis roskildiensis sotto B-18679

B. thuringiensis strain

ScholarOne Support 1-434/9641100

Table 1: *Bacillus cereus* group isolates used in this study. Strains have been allocated into clades (1= ‘anthracis’ clade; 2 = thuringiensis / diarrhoeal cereus clade , 3 = mycoides clade) based on analyses in Meric, Mageiros et al. (2018); Raymond et al (2010b) or Zheng et al (2017). Note that Cry null “thuringiensis” strains were all produced by curing strains of Cry toxin plasmids, see BGSC Bacillus Genetic Stock Centre catalogue for details. Where sequences types (STs) are described they correspond to the multi-locus sequence typing scheme hosted by <https://pubmlst.org/> and described in Priest et al 2004. The BR source indicates strains collected or described in Raymond et al 2010b. ID codes marked with asterisks * were used in the media experiments described in figures 2 and supplementary figures S1 and S2. Psychrotolerance was assessed in all putative *B. mycoides* strains by the ability to grow in LB at 10°C for 48 hours and growth at 7°C in PC medium over 7 days as per the methods of Lechner et al (1998). † Note that two strains supplied as *B. mycoides* (*ex. weihenstephanensis*) were treated as *B. cereus* in our analysis as they showed none of the traits of this species.

Species	Clade	Serotype / sequence type (ST)	Phenotype / psychro.	ID code / source
<i>cereus</i>	2	ST34	Cry null	ATCC 11778 *
<i>cereus</i>	2	ST33	Cry null	NRRL-569 BGSC 6A3 *
<i>cereus</i>	1	ST441	Cry null	NCTC 11145 *
<i>thruringiensis</i>	2	<i>israelensis</i> ST16	Cry null	BGSC 4Q7 *
<i>thruringiensis</i>	2	<i>sotto</i> ST12	Cry null	BGSC 4E5 *
<i>thruringiensis</i>	2	<i>kurstaki</i> ST8	Cry null	BGSC 4D7 *
<i>thruringiensis</i>	2	<i>thuringiensis</i> ST10	Cry null	Bt 407 / BGSC 4A12 *
<i>thruringiensis</i>	2	<i>kurstaki</i> ST8	Cry null	BGSC 4D22 *
<i>thruringiensis</i>	1	<i>finitimus</i> ST171	Cry	BGSC 4B2
<i>thruringiensis</i>	2 (4)	<i>kyushuensis</i> ST227	Cry	BGSC 4U1
<i>thruringiensis</i>	1	<i>monterrey</i> ST107	Cry	BGSC 4AJ1
<i>thruringiensis</i>	1	<i>pulsiensis</i> ST110	Cry	BGSC 4CC1
<i>thruringiensis</i>	1	<i>roskildensis</i> ST103	Cry	BGSC 4BG1

<i>thruringiensis</i>	1 (5)	<i>sotto</i> ST1461	Cry	NRRL B-18679
<i>thruringiensis</i>	2	<i>alesti</i> ST12	Cry	BGSC 4C3
<i>thruringiensis</i>	2	<i>darmstadiensis</i> ST56	Cry	BGSC 4M3
<i>thruringiensis</i>	2	<i>israelensis</i> ST16	Cry	BGSC 4Q1
<i>thruringiensis</i>	2	<i>jegathesan</i> ST1462	Cry	NRRL B-23141
<i>thruringiensis</i>	2	<i>kumamtoensis</i> ST59	Cry	BGSC 4W1
<i>thruringiensis</i>	2	<i>kurstaki</i> ST8	Cry	BR 7.1.o
<i>thruringiensis</i>	2	<i>morrisoni</i> ST257	Cry	PG14 BGSC 4K4
<i>thruringiensis</i>	2	<i>sotto</i> ST12	Cry	BGSC 4E3
<i>thruringiensis</i>	2	<i>tenebrionis</i> ST23	Cry	BGSC 4AA1
<i>thruringiensis</i>	2	<i>thuringiensis</i> ST10	Cry	BGSC 4A3
<i>thruringiensis</i>	2	<i>thuringiensis</i> ST10	Cry	BGSC 4A5
<i>mycoides</i>	2	<i>asturiensis</i> ST138	10°C	BGSC 4BQ1
<i>mycoides</i>	2	<i>vazensis</i> ST220 / ST142 complex	10°C	BGSC 4CE1 *
<i>mycoides</i>	2	<i>navarrensis</i> ST18 complex	10°C	BGSC 4BM1 *
<i>mycoides</i> > <i>cereus</i> †	3	ST 196 complex	Not psychro	BGSC 6A50 *
<i>mycoides</i> > <i>cereus</i> †	2	ST1127 / ST18 complex	Not psychro	BGSC 6A21 *
<i>mycoides</i>	3	ST 196 complex	7°C	BGSC 6A46 *
<i>mycoides</i>	3	soil isolate ST417	7°C	BR D10 2ai *
<i>mycoides</i>	3	soil isolate ST353	7°C	BR D096i *

Table 2. Effect of clade of origin on fitness of *B. thuringiensis* isolates in HCO sporulation media. All isolates were competed with a standard labelled *B. thuringiensis* subsp. *kurstaki* competitor (Btk pHT315 *DsRed tet^R*) over four days, ie until sporulation. Quantitative estimates of relative fitness were not possible since single isolates predominated at the end of competition experiments; qualitative comparisons of fitness were made depending on which isolate persisted until sporulation.

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

Supplementary Figure S1: Grow of of *B. cereus*, Cry plasmid cured *B. thuringiensis* and *B. mycoides* strains in protein-based media (BHI), soil-simulating media (SESOM) and plant root exudate-simulating media (TREM). Growth is measured from absorbance OD₆₀₀ in microtitre plates over 24 hours. The lower graph shows the same data magnified. Lines are the fitted models for each species. This experiment used isolates with asterisks listed in Table 1.

Supplementary Figure S2: Growth of Cry expressing *B. thuringiensis* from two clades in protein-based media (BHI) and LB at different temperatures. Growth is measured from absorbance at OD₆₀₀ in microtitre plates over 24 hours.