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The *Bacillus cereus* Group: *Bacillus* species with Pathogenic Potential

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Abstract

The “*Bacillus cereus* group” includes several *Bacillus* species with closely related phylogeny. The most well-studied members of the group, *Bacillus anthracis*, *B. cereus*, and *B. thuringiensis* are known for their pathogenic potential. Here we present the historical rationale for speciation and discuss shared and unique features of these bacteria. Aspects of cell morphology and physiology, and genome sequence similarity and gene synteny support close evolutionary relationships for these three species. For many strains, distinct differences in virulence factor synthesis provide facile means for species assignment. *B. anthracis* is the causative agent of anthrax. Some *B. cereus* strains are commonly recognized as food poisoning agents, but strains can also cause localized wound and eye infections as well as systemic disease. Certain *B. thuringiensis* strains are entomopathogens and have been commercialized for use as biopesticides, while some strains have been reported to cause infection in immunocompromised individuals. In this chapter we compare and contrast *B. anthracis*, *B. cereus*, and *B. thuringiensis*, including ecology, cell structure and development, virulence attributes, gene regulation and genetic exchange systems, and experimental models of disease.

I. TAXONOMY- HISTORICAL AND SOCIO-ECONOMICAL ASPECTS

The microorganisms constituting the *Bacillus cereus* group are gram-positive low GC% bacteria belonging to the phylum Firmicutes. The group of spore-forming, aerobic, facultative anaerobic, rod-shaped bacteria is comprised at least eight closely related species: *B. anthracis*, *B. cereus*, *B. thuringiensis*, *B. mycoides*, *B. pseudomycoides*, *B. weihenstephanensis*, *B. cytotoxicus*, and *B. toyonensis* (1). With the exception of *B. cytotoxicus*, which is the most divergent of the group with a chromosome of 4.085 Mb (2),

the genomes of the *B. cereus* group species are highly conserved, with sizes of 5.2- to 5.5-Mb and very similar 16S rRNA gene sequences. The definition of these species and the distribution of the strains within them have been based on phenotypes and clinical and economic significance, and mainly associated with plasmid content (3, 4). *B. anthracis* harbors two plasmids, pXO1 (182 kb) and pXO2 (95 kb) carrying the structural genes for the toxin proteins and the biosynthetic genes for capsule formation, respectively (5). The emetic *B. cereus* strains harbor plasmid pCER270 encoding enzymatic components required for the nonribosomal biosynthesis of the toxin cereulide (6, 7). *B. thuringiensis* harbors several plasmids encoding a large variety of the insecticidal toxins Cry and Cyt, that form the parasporal crystal characteristic of the species (8–10). The other five species, are discriminated by morphological or physiological traits. *B. mycoides* and *B. pseudomycoides* have typical rhizoid growth (11, 12). *B. weihenstephanensis* strains are psychrotolerant. Like the mostly mesophilic members of the *B. cereus* group, the optimal growth temperature for *B. weihenstephanensis* is between 25 and 35°C, but the species is distinguished by its ability to grow at temperatures as low as 4°C (13). On the other hand, *B. cytotoxicus* strains are thermotolerant and can grow up to 50°C (14). Finally, *B. toyonensis* was characterized for probiotic properties and is used as such in animal feed (15).

Speciation based on the plasmid content and on morphologic or certain physiological features is confusing and can result in subjective conclusions. A recent genomic analysis of 224 strains shows that the *B. cereus* group can be divided in 5 major clades, which do not match the eight species described above (see figure 1). This lack of congruence between genomic-based phylogenetic analysis and specific phenotypes leads to the conclusion that plasmids carrying toxin genes such as the pXO plasmids of *B. anthracis* and the *cry* plasmids of *B. thuringiensis* cannot serve as signatures for species identification (1). As proposed by Helgason and colleagues (16) and by Rasko and coworkers (4), it is more useful to consider the *B. cereus* group as a unique species comprised of extremely diverse strains whose properties differ due to plasmid content or gene expression associated with key regulatory genes. For example, the global transcription regulator PlcR is active in *B. cereus* and *B. thuringiensis*, but inactive in *B. anthracis*, abolishing the expression of a major regulon in that species (17, 18).

While it is arguably appropriate to refer to members of this highly-related group of bacteria as “*B. cereus* subsp. *cereus*, *B. cereus* subsp. *anthracis*, *B. cereus* subsp. *thuringiensis*”, etc. In keeping with current practice, we will refer to the individual subspecies by their traditional names. We focus here on *B. anthracis*, *B. cereus* and *B. thuringiensis* because of the abundance of research related to their clinical and socio-economical importance. We first present some unique and overlapping traits of these bacteria.

Bacillus anthracis

B. anthracis, a major scourge of humanity, was first characterized by R. Koch and L. Pasteur as the etiological agent of anthrax. Their investigations of this bacterium in the late nineteenth century represented early breakthroughs in microbiology and vaccination. Koch’s postulates, criteria designed to establish specific causal relationships between microbes and

diseases, were based on studies of *B. anthracis*. In addition, an attenuated strain of *B. anthracis* served as the first live bacterial vaccine (19, 20).

Historically, strains harboring pXO1 and/or pXO2 have been designated as *B. anthracis*. However, recent reports of closely-related species harboring similar plasmids indicate that speciation can be complex. *B. cereus* strains that harbor pXO1- and pXO2-like plasmids, named *B. cereus* Biovar *anthracis*, have been isolated as the causative agents of anthrax-like infections in primates in Cameroon and Côte d'Ivoire (21, 22). Similar *B. cereus* strains that produce the anthrax toxins were identified as the etiological agents of an anthrax-like cutaneous infection and anthrax-like respiratory infections in metalworkers (23–27). The well-studied strain, *B. cereus* strain G9241, isolated from a welder with a severe anthrax-like respiratory illness, contains two virulence plasmids, pBCXO1 and pBC210 (also known as pBC218), as well as pBClin29, a linear plasmid that harbors cryptic prophage genes. The plasmid pBCXO1 has high similarity to pXO1 and contains the toxin genes *pagA*, *lef*, and *cya*, encoding toxin proteins with amino acid sequences of 96% or greater identity to their counterparts in *B. anthracis* (24). Plasmid pBC210 encodes a toxin protein paralogue, PA2, and the novel ADP-ribosyltransferase, certhraX (24, 28–30). Each plasmid contains genes required for synthesis of a unique polysaccharide capsule; the enzymes needed for hyaluronic acid (HA) capsule production are encoded by the *hasACB* operon on pBCXO1, and the proteins required for elaboration of a putative tetrasaccharide capsule are encoded by the *bps* locus on pBC210 (24, 31). Interestingly, although *B. cereus* G9241 and *B. anthracis* Ames both produce capsule(s) and the anthrax toxin proteins, the virulence of G9241 in rabbits is more similar to that of *B. anthracis* Sterne strain 34F2 (pXO2-) that produces the toxins but not capsule (32).

Bacillus cereus

B. cereus, was first isolated from air in a cow-shed and was characterized as a highly motile bacterium, generally appearing as single cells but occasionally forming longer threads, and causing rapid liquefaction of gelatin (33). In the second half of the twentieth century, *B. cereus* was recognized as a common food contaminant responsible for two types of poisonings. More recently *B. cereus* has been increasingly recognized as an aetiological agent of localized wound and eye infections as well as systemic infections (34, 35).

In the 1950s, Hauge showed that consumption of *B. cereus*-contaminated vanilla sauce, can cause diarrhea (36). Since Hauge's remarkable discovery, two protein toxin complexes (Nhe and Hbl) and a single protein toxin (CytK) have been described, which are generally thought to contribute to the diarrheal type of food poisoning (37). More recently, several exoproteins with enzymatic activities, such as proteases or phospholipases, have been discussed as putative virulence factors, contributing to the diarrheal syndrome (38, 39). Notably, these diarrhea-associated known and putative virulence factors are encoded by chromosome genes. In contrast, genes encoding the biosynthetic machinery for production of the depsipeptide toxin cereulide, which is the causative agent of the emetic type of *B. cereus*-mediated food poisoning, are on the 270-kb megaplasmid pCER270 (35). In contrast to the enterotoxin genes, which are broadly distributed among the different members of the *B. cereus* group and show a high degree of molecular diversity (40), the cereulide synthetase

gene are restricted to genetically closely-related strains and show rather low molecular diversity (41, 42). The ecological and evolutionary mechanisms driving the diversification processes of *B. cereus* virulence is hitherto poorly understood.

Bacillus thuringiensis

B. thuringiensis was first isolated in 1901 in Japan from infected silkworm larvae (*Bombyx mori*). The bacterium was identified as the causal agent of the sotto disease (sudden-collapse disease), a lethal infection of this economically important insect (43). Subsequently, Berliner isolated the bacterium from the cadaver of flour moth larvae (*Ephestia kuehniella*) collected in a mill in Thuringia (44), and named it *Bacillus thuringiensis*, after the German province.

Based on its entomopathogen properties, *B. thuringiensis* was rapidly commercialized and used as a biopesticide for pest control. The insecticidal activity spectrum of the bacterium was initially thought to be restricted to a few lepidopteran species. However, in 1977 discovery of the *israelensis* strain of *B. thuringiensis* which is active against mosquitoes (45), accelerated a search for additional strains active against several species of insects and other invertebrates. Today, a large number of strains have been shown to have activity against coleopteran insects or nematodes (46, 47). *B. thuringiensis*-based biopesticides comprise approximately 75% of the global bioinsecticide market, representing about 4% of the global insecticides (48). Several thousand *B. thuringiensis* strains have been shown to form crystalline Cry protein inclusions during sporulation, which in many cases are key to the insecticidal or nematocidal activity of the bacteria. However, not all *B. thuringiensis* isolates producing Cry proteins have been found to be toxic. It is possible that some Cry proteins are inactive or have unidentified target hosts.

All the insecticidal toxin genes (*cry*, *cyt* and *vip*) of *B. thuringiensis* are located on large plasmids (9, 49, 50). Generally these plasmids are conjugative (51), and the *cry* genes are frequently found in the vicinity of various mobile genetic elements, such as the transposon Tn4430 and the insertion sequences IS231 and IS232 (8, 52, 53). The localization of the insecticidal toxin genes on elements associated with horizontal gene transfer is likely responsible for the great diversity of these genes and their multiplicity within the *B. thuringiensis* strains.

II. ECOLOGY – ENVIRONMENTS AND PATHOGENICITY

The *B. cereus* group species are endemic soil bacteria that occupy diverse ecological habitats as depicted in figure 2 (41, 54). Due to the formation of heat-, UV-, acid- and desiccation-resistant endospores, the bacteria can persist in a dormant state, making it difficult to elucidate their primary ecological niches. In addition to soil, the species have been isolated from fresh and stored foods, invertebrates, and plants (55–60). Some reports suggest that the *B. cereus* group species do not germinate and grow in non-sterile soils unless a nutrient source, such as decomposing plants, insects or animals is present (61–64). Reports of the isolation of *B. cereus* associated with plants may even point toward an endophytic lifestyle as an alternative niche for *B. cereus* (65, 66). Due to their ubiquitous presence these organisms are hard to control and can enter food production and processing at various points (Fig. 2). Certain *B. cereus* strains have been reported to promote plant growth and to

suppress plant diseases (67, 68). Recently, it was reported that the *B. cereus* AR156 strain induces systemic resistance to *Pseudomonas syringae* pv. tomato in *Arabidopsis thaliana* (69), suggesting that plant rhizospheres represent a natural niche for *B. cereus*.

Genome and physiology studies reveal that unlike true soil microorganisms, bacteria of the *B. cereus* group have few genes involved in the degradation of carbohydrate polymers. Conversely, these species contain a wide arsenal of genes encoding extracellular factors such as degradative enzymes (phospholipases, proteases, and chitinases), cytotoxic proteins (haemolysins, enterotoxins, and cytotoxins) and cell surface proteins (70, 71). These factors are potentially involved in the virulence and propagation of the bacteria in an animal host, thus conferring an obvious pathogenic lifestyle to the species (72). Outside of their hosts, the *B. cereus* group species are generally considered to reside as spores. Nevertheless, it is likely that the bacteria undergo some cycling of saprophytic growth, sporulation, and germination in the soil. Epidemiological studies of clinical and non-clinical isolates suggest loss and horizontal movement of plasmids (22, 73–75).

The ecology of *B. thuringiensis* remains unclear despite multiple investigations (54, 76), and *B. cereus* ecology is even less explored (66). Studies of *B. anthracis* ecology have been fueled by the severity and worldwide occurrence of anthrax disease. *B. anthracis* can infect all mammals, some birds, and possibly even reptiles. The most severely affected parts of the world are central Asia and western areas of Africa. An “anthrax belt” exists from Turkey to Pakistan. In North America, anthrax is endemic in northern Alberta and the Northwest Territories. In the United States, sporadic outbreaks of anthrax occur in northwest Mississippi/southeast Arkansas and western Texas. The disease is also endemic throughout Mexico, Central America, and in many South American countries. “Anthrax Seasons” are characterized by hot-dry weather which stresses animals and reduces their innate resistance to infection. Data from studies of central Asia support seasonal outbreaks with the largest number of cases occurring in late summer (77, 78).

Prevalence of *B. anthracis* spores in the soil is affected by mean annual temperature, annual precipitation, elevation, amount of vegetation, soil moisture content, and soil pH (77, 78). Multiplication of *B. anthracis* outside of the host has been controversial. Concentrations of spores following disease outbreaks are thought to result from rains that pool spores in low-lying areas, as opposed to bacterial proliferation. Decomposition of infected animals at anthrax carcass sites leads to increased numbers of vegetative cells, but ultimately the numbers decrease as cells sporulate or die (79). Some studies suggest that *B. anthracis* competes poorly against indigenous bacteria, however germination and amplification of anthrax spores by soil-dwelling amoebas and in the context of the plant rhizosphere have been reported (63, 80).

Humans and scavenging animals acquire gastrointestinal anthrax by ingesting spores in meat from infected animals, while wild and domestic herbivores ingest or inhale *B. anthracis* spores from the soil while grazing (81). Acquisition of spores from water appears to be less common (82). The infectious dose and severity of disease vary widely in different hosts. Domestic livestock, including sheep and cattle, and wild herbivores, such as bison, elephants, hippopotami, and kudu, are particularly susceptible to infection (83).

Inhalation anthrax in humans, also known as wool-sorter's disease, was once common among textile workers who inhaled spores in dust generated from contaminated wool and animal hides. Today, most naturally-acquired human cases of anthrax are cutaneous infections resulting from contact with dead infected animals or their products. Intentional dissemination of *B. anthracis* spores occurred in the United States in 2001. Eleven inhalation and eleven cutaneous cases of anthrax in humans were traced to four envelopes containing *B. anthracis* spores in powder-form. The envelopes were sent to different locations via the U.S. Postal Service and most of the patients were either mail-handlers or were exposed at worksites where contaminated letters were processed or received. Others are thought to have been exposed to cross-contaminated mail (84). The tragic event demonstrated the apparent ease with which the organism can be dispersed and solidified the placement of *B. anthracis* on the list of biothreat agents.

***Bacillus anthracis* the anthrax pathogen**

Systemic anthrax, generally resulting from inhalation or ingestion of *B. anthracis* spores, has a high fatality rate. When *B. anthracis* spores are inhaled, alveolar macrophages and dendritic cells transport the organism to the proximal lymph nodes. Following spore germination, vegetative cells produce the anti-phagocytic capsule and the anthrax toxin proteins, virulence factors which are critical for initiation of systemic disease (85, 86). In humans, bacteremia begins after an asymptomatic incubation period of 1–6 days. Following the onset of bacteremia, patients can experience flu-like symptoms for 1–5 days prior to an acute disease stage that lasts 1–2 days. Ultimately, death results from respiratory failure, sepsis, and shock (84) (87).

B. anthracis virulence is attributed primarily to production of anthrax toxin and capsule. The toxin structural genes and capsule biosynthesis genes are encoded by plasmids pXO1 and pXO2 respectively, and both plasmids are required for full virulence in immunocompetent hosts. The tripartite anthrax toxin, which is comprised of three secreted proteins, protective antigen (PA), edema factor (EF), and lethal factor (LF) has been the focus of investigations of anthrax disease. PA facilitates entry of EF and LF into host cells. EF is a calmodulin-dependent adenylyl cyclase and LF is a zinc protease. Together the toxins disable innate and adaptive immune systems of the host to induce lethal disease (88, 89).

The poly- γ -D-glutamic acid (PGA) capsule of *B. anthracis*, provides protection of the bacterium from phagocytosis, as is true for many bacterial capsules which are more commonly made of polysaccharide. High concentrations of capsule released from *B. anthracis* cells during infection is important also for host-pathogen interactions (90–93). The PGA capsule has been shown to augment LT-mediated cytotoxicity and induce production of inflammatory cytokines and nitric oxide, which likely contribute to systemic inflammation and sepsis at the terminal stage of infection (94–97).

In addition to toxin and capsule, other secreted and non-secreted factors have been reported to affect host-pathogen interactions and affect host pathology (98, 99). Among these are extracellular proteases that interfere with the immune response by cleaving antimicrobial peptides (100). The proteases NprB/Npr599 (also designated NprA in *B. thuringiensis* and *B. cereus*) and InhA1 can degrade host tissues, resulting in increasing barrier permeability

(101–103). Crossing of the brain barrier is considered to be key to the extensive brain pathology that has been reported for human disease (104).

***Bacillus cereus* and gastrointestinal disease**

B. cereus pathogenicity is based on a panoply of virulence factors, which are far from understood, contributing to a range of human disease. *B. cereus* can cause two types of food poisoning, which are primarily characterized by diarrhea and emesis (37, 105, 106). The diarrheal syndrome has been linked to different enterotoxins, thought to be produced after outgrowth of spores, taken up with contaminated foods, and active in the small intestine. The emetic syndrome is the result of an intoxication caused by the single depsipeptide toxin cereulide, pre-formed in food. The genes encoding the cereulide synthetase toxin genes are primarily found in a distinct subgroup of *B. cereus* while the enterotoxin genes are broadly distributed among the members of the *B. cereus* group (41, 107). Usually these gastrointestinal (GI)-related diseases are self-limiting but more severe forms require hospitalization, and occasionally fatalities have been reported (58, 108, 109). Intoxication related to cereulide has been linked to severe clinical manifestation, such as acute liver failures and acute encephalopathy (110). Due to the high biological activity of the cereulide toxin, occasionally liver transplantations are necessary as a life saving measure (111, 112). Generally, the pathogenic potential of *B. cereus* strains is highly variable and ranges from strains that show no cytotoxic activity in cell culture assays to strains that are highly cytotoxic (113, 114). In addition to intrinsic strain-associated differences in pathogenic potential, virulence is affected by environmental factors (58, 115, 116).

Apart from its food poisoning potential, *B. cereus* is increasingly recognized as an opportunistic pathogen that can cause local as well as systemic infections. Immunocompromised people and neonates are of special risk for hospital acquired *B. cereus* infections, such as infections of the central nervous system, endocarditis, respiratory and urinary tract infections, wound infections and septicemia (34). Outside of nosocomial infections, *B. cereus* has been linked to eye infections, such as endophthalmitis and keratitis (117). Furthermore, *B. cereus* can cause severe mammary gland infections in ruminants (118, 119). Virulence factors contributing to infections in ruminants and to most non-GI related human infections are unknown.

***Bacillus thuringiensis* infection of invertebrates**

In addition to virulence genes common to the *B. cereus* group and to specific insecticidal toxin genes, the *B. thuringiensis* genome contains several genes encoding chitinases, suggesting a close relationship between this bacterium and the insects. Many well-studied *B. thuringiensis* strains were isolated from insects (44–46) and large screens of natural environments have shown that insects were more fruitful sources of the bacterium than soil samples (120). About 40% of the invertebrates tested were found to harbor *B. thuringiensis* spores. While insects appear to be the preferred target of *B. thuringiensis*, the bacterium can propagate in other invertebrates (121, 122). Nematode-*B. thuringiensis* association is highly probable in soil and other natural environments, and several *B. thuringiensis* strains harbor *cry* genes specifically active against nematodes (123). When nutrients are available, *B. thuringiensis* might be a multi-host pathogen (124) with two pathogenic lifestyles: one

resulting from its specific toxins (Cry or Vip) encoded by plasmid genes and targeting insects, nematodes, and other invertebrates (49, 125), and one non-specific, depending on various chromosomal factors providing opportunistic properties. These non-specific factors may allow the bacteria to propagate and eventually to kill various organisms if they are weakened by any motive, for example by Cry toxins in the case of insects (126). This second pathogenic lifestyle may be shared with *B. cereus* which possesses the same chromosome-encoded virulence factors (70). In this point of view, the Cry toxins of *B. thuringiensis* are “public goods” which can act cooperatively and thus profit cheater bacteria as *B. cereus* (127).

The genome of *B. thuringiensis* contains a 41-gene regulon devoted to its saprophitic or necrotrophic lifestyle (128). This regulon, which includes genes encoding various degradative enzymes, is activated by the quorum sensing regulator NprR and allows *B. thuringiensis* to survive in the insect cadaver. The NprR regulon is also transcribed in all other *B. cereus* group species, thus suggesting that the bacteria of this group have similar saprophitic properties (129). Altogether, these pathogenic and saprophytic functions are highly efficient adaptive traits that allow the species to grow and survive in various natural conditions and niches.

III. CELL STRUCTURE AND DEVELOPMENT

Vegetative cell and spore structures do not vary greatly among the *B. cereus* group species. Generally vegetative cells appear in chains, with the square ends of the cells fitting closely together. Variations in chain length occur as a normal aspect of growth in different media and environments (34, 130). Interestingly, *B. anthracis* vegetative cell chains in infected tissues are generally shorter than those formed during growth *in vitro*. Chaining of *B. anthracis* cells impedes invasion of host cells and is associated with escape from phagocytic clearance (131). Spores of the three species also have similar morphology. Spore size and morphology can vary between strains of the same species, but most strains produce spores with diameters of approximately 1 μ m that are surrounded by a loose-fitting outer layer termed the exosporium (Fig. 3). Fine features of spores can be altered in different growth and sporulation conditions.

Vegetative cell envelope

The surface of *B. anthracis*, including secondary cell wall polysaccharides (SCWPs), the S (surface)-layer, and capsule, has been the most well studied of the *B. cereus* group species. The *B. anthracis* SCWP is comprised of trisaccharide repeats [$\rightarrow$ 4)- β -ManNAc-(1 $\rightarrow$ 4)- β -GlcNAc-(1 $\rightarrow$ 6)- α -GlcNAc-(1 $\rightarrow$)] with α -Gal and β -Gal substitutions tethered to the peptidoglycan by acid-labile phosphodiester bonds. SCWP is essential for cell growth, shape, and division. Although the enzymes for SCWP synthesis have not been fully characterized, two proteins, WpaA and WpaB, have been proposed as glycosyltransferases that act to assemble the SCWP polymers prior to attachment to the peptidoglycan. The *B. anthracis* SCWP is critical for maintenance of cell shape and for full virulence. Depletion of the glycosyltransferases results in irregularly shaped non-viable cells (132). In addition, a mutant lacking a UDP-glucose 4-epimerase required for SCWP galactosylation exhibits

reduced encapsulation and decreased virulence in a murine model for anthrax (133). Recent investigations of *B. cereus* isolates causing anthrax-like disease indicate SCWP with structure similar to that of *B. anthracis*, but with an additional α -Gal substitution at O3 of ManNAc (134).

In *B. anthracis* and some pathogenic *B. cereus* strains, S-layer proteins have been shown to attach to the cell wall via noncovalent interactions with SCWPs (135). The S-layers are paracrystalline arrays of protein, that serve as protective barriers and as scaffolds for housekeeping enzymes and virulence factors (136). Major S-layer proteins of *B. anthracis* are Sap (for surface associated protein) and EA1 (for extractable antigen 1). Fully assembled and acetylated SCWP is important for assembly of some S-layer proteins. Mutants deleted for putative acetyltransferases are unable to deposit the the S-layer associated murein hydrolase BsIO at cell division septa and have altered chain length (137).

Finally, a key distinguishing feature of *B. anthracis* is its capsule. The *B. anthracis* capsule is comprised of gamma-linked poly-D-glutamic acid (PGA). As is true for typical polysaccharide capsules, the *B. anthracis* capsule protects cells from phagocytosis during infection. In addition, the *B. anthracis* capsule renders the bacterium somewhat resistant to mammalian proteases, and is a poor immunogen (138, 139). Immunoassay for PGA using monoclonal antibodies has revealed that PGA is shed into the blood during infection of mice and non-human primates (91, 140, 141), likely due to the activity of a capsule depolymerizing enzyme, CapD, that is co-expressed with the capsule biosynthesis operon. The capsule degradation products are thought to inhibit the innate immune response through targeting of cytokine pathways (142).

The PGA capsule is also produced by some but not all *B. cereus* isolates that cause anthrax-like disease. These strains harbor plasmids that allow strain-specific differences in synthesis of the PGA capsule, a hyaluronic acid capsule, and a tetrasaccharide capsule. Results of virulence studies to assess the role(s) of the *B. cereus* capsules are somewhat contradictory and incomplete (24) (31) (75) (22). Further investigations are needed to determine the specific functions of these capsules.

Spore architecture

The architecture of the *B. cereus* group species spore is comparable to the spores of other *Bacillus* species. The innermost compartment of the spore contains the DNA. The DNA is housed with cations and pyridine-2,6-dicarboxylic acid in a 1:1 chelate with divalent calcium ions, which serve to maintain the spore's dormant state and resistance to DNA-damaging agents. Germinant receptors in the inner membrane initiate the signal transduction pathway that leads to outgrowth of the spore and the vegetative cell state (143). Germinants of the *B. cereus* group species can be a subset of sugars, amino acids and ribonucleosides, as well as other small molecules, depending on the species (144). A shell of peptidoglycan containing two sublayers, the cortex and the germ cell wall, surrounds the inner membrane. The cortex constricts the core's volume to retain the relatively dry state of the core. At outgrowth of the spore, the germ cell wall becomes the nascent cell wall of the vegetative cell. Surrounding the cortex is the spore coat containing approximately 70 proteins arranged in layers to form a somewhat flexible structure (143).

The exosporium, an additional layer surrounding the spore coat of some *Bacillus* species, has been most well studied in *B. cereus* and *B. anthracis* (145), can respond to changes in spore hydration. The exosporium is comprised of a crystalline basal layer overlaid by a “hairy nap”. The composition and assembly process of the exosporium are poorly understood. BclA and related proteins form the hairy nap. Among the approximately 20 proteins of the basal layer is ExsY, which can self-assemble into structures resembling the intact basal layer and thus appears to be a key player in exosporium formation (146). The exosporium excludes large molecules. It also provides resistance to specific chemicals *in vitro* and to nitric oxide in the context of the macrophage (147) (148) (149). Removal of the exosporium from *B. anthracis* spores does not impede virulence in several animal models (150–152). However, the exosporium protein BclA binds macrophage receptors to mediate interactions with epithelial cells and extracellular matrix proteins (153, 154), suggesting that a role for the exosporium in pathogenesis (145).

Germination Dynamics and Signals

The overall process of germination, in which spores transition to metabolically active vegetative cells, is highly conserved in the *Bacillus* genus, but the specific signals and associated receptors vary among species. *B. anthracis* germinates in response to alanine or purine ribonucleotides, with one or more additional amino acids acting as co-germinants (155–157). Many germinant receptors have been described in the inner membrane of *Bacillus* species. Notably, a unique set of germination genes, the *gerX* operon, is located on pXO1 of *B. anthracis*. Mutants missing the operon are altered for germination *in vivo* (158, 159). Once *Bacillus* spores germinate, spore cortex lytic enzymes degrade the cortex peptidoglycan allowing core rehydration can cell outgrowth (160). *B. anthracis* differs from other species examined in that it contains an additional spore coat lytic enzyme which appears to work synergistically with the common enzymes (161).

Germination in the Host

Multiple routes of infection with *B. anthracis* spores lead to disease, suggesting that germination can be triggered by signals in different host niches. Studies of *B. anthracis* germination *in vivo* have yielded intriguing results. Inhalation anthrax models have shown that phagocytes and dendritic cells phagocytose spores and transport the bacteria to the lymph nodes (162, 163). Spores and vegetative cells appear in phagocytic cells, but it is unclear whether spores germinate before or after phagocytosis. Moreover, some strains germinate quickly in the lung, while others remain ungerminated for multiple days (164, 165), suggesting that germinants plus additional unknown factors influence development *in vivo*. Finally, germination occurs in cell-free macrophage-conditioned media, indicating that phagocytes are not required for *B. anthracis* spore germination (157).

Studies of *B. thuringiensis* germination include experiments simulating conditions encountered in the insect midgut. The pH of the lepidopteran midgut is 10, and pretreatment of *B. thuringiensis* spores with larval midgut fluid or with an alkaline buffer stimulates germination (166, 167). This alkaline activation of spores requires plasmid-encoded germination genes (168). Cry proteins associated with the spore surface allow *B. thuringiensis* spores to bind brush border membrane vesicles prepared from lepidopteran

insects, and the binding has been proposed to be responsible for stimulation of alkali-activated germination (169). It should be noted however, that the spore surface – Cry protein association may simply result from concomitant synthesis, rather than a specific interaction. Finally, germination of *B. thuringiensis* spores in the intestine of nematodes has also been reported (122).

Sporulation

The sporulation signal cascade has been well-studied in *Bacillus subtilis*, and the pathways and regulators of sporulation in the *B. cereus* group species appear to be similar to those of *B. subtilis*. Nevertheless, variations among species have evolved with specific niches. *B. anthracis* does not sporulate during infection. In the wild, spores (which are the infectious form of the bacterium) only form when the blood of an infected carcass is exposed to the environment (78). The lack of sporulation in the mammalian host appears to result from a specific configuration of the phosphorylation cascade in this species (170) (171) (172). In contrast, it has been shown that a part of the *B. thuringiensis* population sporulates during infection in insect larvae, and this process is under the control of the bifunctional regulator NprR (173, 174). The Rap phosphatases negatively control sporulation by stimulating a component of the phosphorelay involved in the phosphorylation of Spo0A, the master regulator of sporulation initiation (175). Rap activity is controlled by the Phr signaling peptides through a quorum sensing system. In *B. anthracis* and *B. thuringiensis*, plasmids harbor *rap-phr* genes involved in sporulation (176). These plasmid-borne Rap-Phr systems may provide advantages for adaptation of these pathogen bacteria to their respective ecological niches.

IV. TOXINS

Toxins, the first bacterial virulence factors to be described (177), represent the major drivers of pathogenicity within the *B. cereus* group. A variety of chromosome- and plasmid-encoded toxins, some shared among the species, and some species-specific, have been described. The species-specific toxins, namely the *B. anthracis* tripartite anthrax toxin complex and the *B. thuringiensis* insecticidal Cry Toxins, as well as the depsipeptide toxin cereulide of the emetic type of *B. cereus*, are encoded on plasmids, while many other *B. cereus* group toxins are encoded by the chromosome. Some *B. anthracis* and *B. cereus* isolates harbor duplicated and/or closely-related toxin genes (35, 178), and *B. thuringiensis* strains can harbor several *cry* genes.

B. anthracis toxins

Anthrax toxin has been a major focus of anthrax research since the initial discovery of the toxigenic potential of *B. anthracis* culture supernates. *B. anthracis* secretes three proteins, protective antigen (PA), lethal factor (LF), and edema factor (EF), known collectively as anthrax toxin. Purified toxin proteins in binary combinations have distinct effects when injected into experimental animals. Lethal toxin (LeTx), a mixture of LF and PA, causes death while edema toxin (EdTx), a mixture of EF and PA, produces edema. PA (83 kD) mediates binding and translocation of the enzymatic proteins LF (90 kD) and EF (89 kD) into host cells.

PA is a non-enzymatic but critical component of LeTx and EdTX because it mediates toxin binding to host cells and facilitates translocation of LF and EF into cells. PA binds to one of two related proteins, capillary morphogenesis protein 2 (CMG2 or ANTRX2) and tumor endothelial marker 8 (TEM8 or ANTRX1), which are found on cells of many different tissues. CMG-2 is of most importance for pathogenesis, while TEM-8 has a more minor role (179, 180). In the model for anthrax toxin entry, furin and possibly additional host associated proteases, cleave PA into two fragments, PA63 (63 kDa) and PA20 (20 kDa) (89). The receptor-bound carboxyterminal 63- kDa fragment (PA63) oligomerizes to form a ring-shaped structure comprised of 7 or 8 PA63 molecules. One to three molecules of LF and/or EF bind to the PA ring and the complex is endocytosed by clathrin-coated vesicles. Endosome acidification induces a conformational change in the ring such that it converts from a “prepore” to a pore (channel) in the membrane, facilitating translocation of EF and LF across the membrane (181).

LF, a zinc-dependent metalloproteinase is the central effector of shock and death from systemic anthrax. Initial investigations of LF activity revealed that the protein cleaves mitogen-activated protein kinase kinases (MAPKKs) disrupting multiple signaling pathways and causing broad downstream effects on cell physiology (182). More recent reports show that low levels of LeTx inhibit the glucocorticoid receptor, an important component of host defense against inflammation. LF targets the inflammasome sensor NLRP1, causing activation of the caspase-1 dependent cell death signaling pathway (183). Finally, murine susceptibility to LT has been associated with genetic polymorphisms in the *Ltx* gene on mouse chromosome 11. The *Ltx* gene encodes a kinesin-like motor protein, Kif1C, but the relationship between LT and Kif1C is not clear (184).

EF, is a calmodulin-dependent and Ca²⁺-dependent adenylyl cyclase (185). As is true for other bacterial toxins that elevate cAMP levels, disruption of normal second-messenger signaling has many effects on host cell physiology. EdTx weakens antibacterial responses of phagocytes, impairing apoptosis, motility, and release of inflammatory cytokines. Human neutrophils treated with the toxin have reduced phagocytic and oxidative burst abilities (186).

A comprehensive model for the molecular basis of anthrax toxin effects on mammalian hosts is still evolving, but a wealth of current information reveals that together, the toxin components severely impinge immune functions of susceptible hosts, which are key to the outcome of *B. anthracis* infection (89, 187, 188).

AnthrolysinO is a cholesterol-dependent cytolysin with properties similar to pore-forming toxins of other Gram-positive pathogens (189). Initially discovered as a hemolysin (190), ALO has lytic activity against phagocytes (191), decreases the barrier function of human polarized epithelial cells (192), and appears to play a role in gut epithelial disruption during *B. anthracis* infection (193). Expression of the *alo* gene is highly responsive to *in vitro* growth conditions (190). During infection, *alo* transcripts are present in newly germinated bacteria after phagocytosis of the spores by mouse macrophages (194) (195). Deletion of *alo* with phospholipase genes attenuates virulence and reduces growth and survival in

phagocytes, suggesting a role for the toxin in destabilization of the phagocytosing membrane (196).

***Bacillus cereus* toxins**

Various *B. cereus* exotoxins have been associated with pathogenicity but the actual contribution and importance of these toxins for GI and non-GI related diseases is largely unknown. So far, four *B. cereus* exotoxins have been clearly linked to food-poisoning. Three pore-forming enterotoxins, Nhe, a three-component non-hemolytic enterotoxin, Hbl, a three-component hemolytic enterotoxin, and the single protein cytotoxin CytK, are thought to provoke diarrhea after toxin-coinfection with *B. cereus* spore contaminated food (37). Hbl enterotoxic activity has been demonstrated *in vivo* on rabbit intestine (197), but direct *in vivo* proof for Nhe and CytK activity is lacking. Nhe was identified from a strain isolated from a foodborne outbreak in Norway (198) and CytK was originally isolated from a *B. cereus* strain that was responsible for a severe foodborne outbreak in which several people developed bloody diarrhea and three elderly people died (199). Enterotoxins may act synergistically with each other and other virulence factors, making it difficult to predict the overall enterotoxic potential of a given strain (39, 106, 200–204). Nhe, Hbl, and CytK show cytotoxic activity on Vero and Caco2 cells as well as on other human cells. Different cell lines show distinct susceptibilities to the different toxins (205), suggesting different modes of toxin action. Typically, symptoms occur after 6 to 12 hours and are self-limiting. However, in rare cases severity of symptoms requires hospitalization and fatalities among neonates and immunocompromised patients, have been reported (108, 109, 199).

The fourth toxin, the so called cereulide, is linked to the emetic type of *B. cereus* food poisoning (206). Cereulide is a depsipetide toxin, composed of alternating α -amino and α -hydroxy acids (D-O-Leu-D-Ala-L-O-Val-L-Val)₃ (Fig. 4), that is structurally related to the potassium ionophore valinomycin (206). It is produced by the cereulide peptide synthetase Ces (6, 207, 208) (42), which represents a novel type of non-ribosomal peptide synthetases (NRPS) (209) (6, 207, 208). Cereulide is usually preformed in food and the onset of symptoms occurs between 15 minutes and 6 hours after uptake of contaminated food (105). In the past, quantification of cereulide has been hampered by the lack of appropriate methods (58). The recently developed ISO method (EN ISO 18465), based on a stable isotope mass spectrometry-based dilution assay (MS- SIDA) (210), will allow accurate cereulide quantitation in the future. Recently, 18 different cereulide isoforms have been described with cytotoxic potential ranging from non-toxic to 10-fold more toxic than the classical cereulide (211). Cereulide is not inactivated during stomach passage in the host because it is resistant to cleavage by pepsin and trypsin (105, 212). Due to its hepatotoxic activity, cereulide can cause rhabdomyolysis, liver damage and severe multi-organ failure (110, 112). Recently cereulide was linked to the induction of diabetes by causing beta cell dysfunctions (213).

***Bacillus thuringiensis* toxins**

Except for the cereulide toxin, *B. thuringiensis* strains generally produce the same toxins as *B. cereus* (214). The phenotype distinguishing *B. thuringiensis* from the other species of the *B. cereus* group is the production of a crystal inclusion during sporulation (Fig. 3). This

crystal generally appears beside the spore in the mother cell compartment of the bacteria (215); but other situations have been reported in a few strains including a crystal between the exosporium and the spore coat (216), and production of spores and crystals in two distinct subpopulations of bacterial cells (217). The crystal inclusion can account for up to 25% of the dry weight of *B. thuringiensis* cells, which means that a massive production of proteins should occur during sporulation or stationary phase. This massive protein production results from several mechanisms including efficient transcription regulation, an exceptionally high stability of *cry* gene mRNA, and the crystallization of the proteins (218). In most of the *B. thuringiensis* strains the expression of the *cry* genes depends on sporulation specific sigma factors, Sigma E and Sigma K, which are functional in the mother cell compartment during a large period of the sporulation process. Some exceptions have been observed. For example, the *cry3* genes encoding coleopteran-active toxins are expressed during stationary phase independently of the sporulation sigma factors (218).

The crystal inclusions are mainly composed by two types of proteins, the Cry and the Cyt proteins, also called δ -endotoxins (49) (219). Various combinations of toxins can be found on the crystal depending on the *B. thuringiensis* strain. The commercial strain *kurstaki* HD1 Dipel® contains 5 *cry* genes (*cry1Aa*, *cry1Ab*, *cry1Ac*, *cry2Aa* and *cry2Ab*) all encoding proteins active against lepidopteran larvae (220). The strain *israelensis* contains 4 *cry* genes (*cry4Aa*, *cry4Ba*, *cry10Aa*, *cry11Aa*) and 2 *cyt* genes (*cyt1Aa* and *cyt2Ba*) all encoding proteins toxic for the mosquito larvae (9). The Cry proteins are distributed into 74 distinct classes, with less than 45% identity between them (http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/.) They are pore-forming proteins whose activity requires the binding to specific receptors on the surface of midgut cells (194). The Cyt toxins are cytotoxins specifically active against dipteran larvae (199); they have also a synergistic action by serving as a receptor for dipteran-active Cry proteins (221).

In addition to the Cry proteins forming parasporal inclusions, some *B. thuringiensis* strains secrete insecticidal proteins: the Sip (secreted insecticidal protein) and Vip (vegetative insecticidal proteins) proteins. The Sip1A is active against coleopteran insects and was isolated from a *B. thuringiensis* strain harboring a coleopteran-specific *cry3* gene (207). The toxin Vip3A is specifically toxic against lepidopteran insects and is generally produced by strains also producing Cry1A lepidopteran-active toxins (209). The insecticidal activity of the exported proteins Vip and Sip and their association with Cry proteins showing related activity spectrum suggest that the larvicidal activity of the Cry proteins is reinforced by the secretion of Sip and Vip proteins, thus increasing the entomopathogenicity of the *B. thuringiensis* cells multiplying in the infected insect larvae.

Common secreted enzymes of the *B. cereus* group species.

Phospholipases, sphingomyelinases, hemolysins, proteinases and peptidases likely represent additional virulence factors of the *B. cereus* group species (39, 106, 202–204). In a murine model for anthrax, protease inhibitors enhance the positive effect of antibiotics, suggesting that one or more proteases contribute to anthrax disease (222).

InhA1 (Immune Inhibitor A) is a particularly well-studied protease that has homologues in all three species. *B. anthracis*, *B. cereus*, and *B. thuringiensis* each carry multiple apparent

inhA genes with transcription of each gene subject to different regulation (223, 224). Autolytically processed active InhA1 of *B. anthracis* cleaves multiple host proteins and other proteins in the *B. anthracis* secretome (225, 226). A *B. anthracis inhA1*-null mutant is attenuated in a murine model for anthrax (227). The reduced virulence of the mutant likely results from its broad activity on multiple targets, ultimately facilitating bacterial dissemination. InhA1 disturbs the host coagulation machinery by cleaving von Willebrand factor and its regulator ADAMTS13 (102). The enzyme also cleaves zonula occluden-1 resulting in disruption of the blood-brain barrier (103). Degradation of hemoglobin by InhA in the bloodstream has been proposed to allow *B. anthracis* access to essential amino acids required for proliferation in the blood (227). In *B. cereus* and *B. thuringiensis*, deletion of the three *inhA* genes strongly affects the virulence (202). InhA1 is required for efficient escape from macrophages (228).

V. MODELLING HOST INTERACTIONS Animal Models for Anthrax

Multiple animal models differing in host species, bacterial strain, and delivery route are used to investigate *B. anthracis* – host interactions. The significant variability among laboratory animals in susceptibility to *B. anthracis* infection makes modeling human anthrax particularly challenging. Non-human primates are considered superior models for studies of potential therapeutics and vaccines, but high cost and ethical considerations limit their use. Rabbits serve as good models because the disease pathology is similar to that observed in non-human primates and clinical human cases of anthrax. Mice and guinea pig models are used frequently because of experimental ease and moderate cost. For most animal studies, the spore form of the bacterium is used as the infectious agent, modelling natural infection. Methods for pulmonary delivery of spores include intranasal inoculation, aerosol exposure, and tracheal injection. Spores can also be introduced subcutaneously. To model late state disease vegetative cells are inoculated directly into the bloodstream, bypassing the requirement for *in vivo* germination of spores {Welkos, 2015 #754}.

Mice are the most commonly experimental animal for investigations of *B. anthracis*. Inbred mice infected via the parenteral and pulmonary routes develop acute disease, with vegetative cells in multiple tissues. Immunodeficient (C5-) mouse strains such as A/J and DBA/2J are susceptible to relatively low doses (10³–10⁴ CFU) of capsule-deficient (pXO1+pXO2-) Sterne-type strains and are used often for studies of the roles of anthrax toxin *in vivo* and for testing the efficacy of toxin-based therapeutics and vaccines. Exposure of C5- mice to aerosolized Sterne spores results in pathology similar to that observed in rabbit and non-human primate models, with lethal toxin required for dissemination and development of protective immunity (229).

Studies of pathogenesis and host responses related to non-toxin factors have been commonly assessed in infections of immunocompetent mice with non-toxigenic (pXO1-pXO2+) strains. Interestingly, guinea pigs, rabbits, and non-human primates are not susceptible to infection by pXO1-pXO2+ strains (230). It is also notable that mice are not protected from fully virulent (pXO1+ pXO2+) strains by the PA-based vaccine AVA, whereas AVA is fully protective for rabbits and non-human primates, but only partially protective for guinea pigs (231) (232).

Mouse models have revealed interesting aspects of germination and dissemination of *B. anthracis* during infection (233). Carr and coworkers (234) demonstrated functional redundancy among the five known germinant receptors, and McKevitt and coworkers (159) showed that the germination inhibitor D-alanine reduced the infectious dose of spores, suggesting that timing of germination is important for disease. Experiments of Glomski and colleagues (235, 236) using luminescent recombinant strains showed that germination and dissemination from pulmonary, skin, and gastrointestinal entry points does not require transport to lymph nodes, but spore dose and method of delivery can affect timing and location of germination. Their data also revealed a role for capsule in dissemination.

Inbred mice have also been used extensively to explore host response to *B. anthracis* infections. Investigations of host systems important for recognition of bacterial factors and activation of immunity have revealed a model in which *B. anthracis* activates Toll-like receptors and the NOD-like receptor (NLR) inflammasome, resulting in tumor necrosis factor alpha production by a mitogen-activated protein kinase (MAPK) signaling pathway. LeTx suppresses this response by cleaving MAPK kinases. EdTx-mediated increases in cAMP also disrupt cell signaling pathways. In the battle between host and pathogen, the immunosuppressive effects of LeTx are countered by Nlrp1b, the product of a “lethal toxin sensitivity” locus on chromosome 11. In response to lethal toxin, the lethal toxin-sensitive Nlrp1b allele activates caspase-1, inducing pro-inflammatory cytokines that recruit neutrophils and mononuclear cells. Thus, in mice, LeTx-mediated Nlrp1b-dependent activation of caspase-1 is a major factor in control of *B. anthracis* infection (233).

Guinea pigs are keenly susceptible to *B. anthracis* spore infection but not very sensitive to the anthrax toxins. While other animal models show some inconsistencies in terminal loads of bacteria, intramuscular challenge of guinea pigs with a fully virulent (pXO1+ pXO2+) strain leads to specially and temporally consistent infections. Aerosol infection of guinea pigs results in edema and hemorrhage in the spleen, lungs, and lymph nodes, facilitating studies of pathology. Guinea pigs have been used in competitive infection studies employing different strains and mutants. These experiments have revealed roles for biosynthetic genes and surface proteins in disease (233).

Rabbits have been useful for determination of virulence differences in *B. anthracis* strains and mutants, and to examine pathology, which is similar to that observed in humans and non-human primates. Multiple investigations of vaccines and therapeutics have employed rabbit models. Rabbits exhibit a strong immune response to PA and have played a key role in development of correlates of immunity. A quantitative anti-PA IgG enzyme-linked immunosorbent assay and a toxin neutralization assay developed using sera from vaccinated rabbits are used to evaluate the human immune response to PA-based vaccines (237–240).

Nonhuman primates provide a valuable model for anthrax disease because infection with aerosolized spores results in a disease course that most closely matches human infection. Nonhuman primates including rhesus macaques, cynomolgus macaques, African green monkeys, and marmosets represent the only animal models for inhalational anthrax in which anthrax meningitis is consistently observed. Infected nonhuman primates, like humans, also develop bacteremia, lymphadenitis, splenitis, mediastinitis, pneumonia, pleural effusions,

hepatitis, adenitis, and vasculitis. Tissues of diseased animals and humans also show hemorrhage, necrosis, and edema. Sera from infected nonhuman primates have been used to develop biomarkers for early diagnosis of infection. Capsule and toxin, detected using ELISA, chemiluminescence, and mass spectrometry serve as reliable markers.

Because nonhuman primate responses to infection most closely resemble human physiological and immune responses, these animals are best suited for immunogenicity, toxicity, and safety testing of vaccines and therapeutics for anthrax. Nonhuman primates have been used extensively for studies of antibiotic efficiency. Investigations of dose, pharmacokinetics, and time course of antibiotic treatment have shown that antibiotics are only affective against germinating spores and vegetative cells. Long term persistence of spores, and extended time periods to germination mean that complete protection from high doses of aerosolized spores requires prolonged antibiotic treatments over greater than 30 days.

Rats are somewhat resistant to challenge with *B. anthracis* spores, but they are exquisitely sensitive to injection of LeTx. Thus, the Fisher 344 strain has been used as a model system for toxin activity. The rat toxin model was key to determining the role of the NLR sensor Nlrp1 in animal susceptibility to LeTx (241, 242). The model also provides a sensitive means to test efficacy of small-molecule toxin inhibitors, monoclonal antibodies, dominant-negative toxin components, and other potential antitoxin countermeasures (233).

Animal Models for *B. thuringiensis* and *B. cereus* Infection

The opportunistic properties of *B. thuringiensis* and *B. cereus* have been investigated using nasal instillation in mice (243, 244) and intraocular infection in mice and rabbits (245). In all the cases, the results highlight the importance of the PlcR regulon in the pathogenic properties of these bacteria. However, established mammalian models for GI infections caused by *B. cereus* are lacking. It has been hypothesized that rodents are not suitable models for GI-related *B. cereus* infection because their gut physiology does not support germination of spores and subsequent enterotoxin production (Bishop et al., 1999; Wilcks et al., 2006). In a more recent study, Rolny et al., (2014) used intragastric gavage to test the effect of vegetative *B. cereus* cells on the host immune system. They found some modifications in immune cells in the spleen, Peyer's patches, and mesenteric lymph nodes but did not report on any enterotoxin production. Thus, an appropriate mammal model for enterotoxigenic *B. cereus* awaits development.

The emetic activity of the *B. cereus* toxin cereulide, was first shown in a rhesus monkey model (246, 247). Results from a study in *Suncus murinus* suggests that cereulide causes emesis through the 5-HT receptor (206). However, it is still unclear if the *S. murinus* model reflects the physiology of the human gut. Thus, further studies will be needed using model organisms, such as pig, more closely resembling the human gut physiology.

Insect Models for *B. cereus* and *B. thuringiensis* Infection

In insects susceptible to *B. thuringiensis* infection, the first stage of the disease results from a gut paralysis due to the crystal inclusion (248, 249). Cry toxins are responsible for this initial step. The toxins damage the peritrophic membrane and cause lysis of the intestinal

cells by producing an osmotic shock (250–252). Depending on the amount of Cry toxins and on the susceptibility of the insect larvae to a given Cry toxin, the toxemia resulting from toxin ingestion may result in insect death. This toxemia and the paralysis of the gut allow the germination of the spores. Several studies have shown that the presence of *B. thuringiensis* cells synergizes the toxic effect of the crystals (243, 253–257).

As natural targets of *B. thuringiensis*, insect larvae are a valuable model for studying the virulence of these bacteria. Utility of this infection model is increased by using insects that are poorly susceptible to the ingestion of crystals alone. A commonly used insect is the lepidopteran species *Galleria mellonella* (the greater wax moth) because the larvae are killed by the ingestion of *B. thuringiensis* spore-crystal mixtures but only weakly affected by the ingestion of crystals alone (243, 256, 257). This model host has also been used to assess virulence of *B. cereus*. Many other human pathogens are unable to kill *G. mellonella* in this infection model, reflecting the entomopathogenic specificity of these two species (258).

The *G. mellonella* host can be used in two infection methods. In association with Cry toxins to impair the insect defenses, the pathogenicity of *B. thuringiensis* and *B. cereus* can be assayed via the oral route. At the last instar, *G. mellonella* larvae are large enough (2 cm long and 250 mg) to be force-fed, thus allowing the ingestion of defined doses of bacteria (243). Larvae can be raised at various temperatures to mimic different natural conditions, including the mammal environment. This infection model allows determination of LD50, measurement of bacterial survival, and assessment of the infectious process. The behavior of the bacteria in the intestinal environment, including bacterial factors interacting with the intestinal barrier can be investigated (259). *G. mellonella* was used to demonstrate the major role of the PlcR regulon in the virulence of *B. thuringiensis* and *B. cereus* (243). The regulon consists in 45 genes encoding a large number of extracellular virulence factors such as degradative enzymes, hemolysins and cytotoxins (17, 70). The PlcR-regulated metalloproteases InhA2, Bel and ColB were demonstrated to be involved in *B. thuringiensis* virulence (38, 260, 261). In addition to *G. mellonella*, a Nematode oral infection model in association with Cry proteins has also revealed virulence factors of *B. thuringiensis* (262). The ColB protease and another metalloprotease (Bmp1) were found involved in the pathogenicity of *B. thuringiensis* against *Caenorhabditis elegans* (260, 263).

A second mode of insect infection is direct injection of the bacteria into the hemocoelic cavity of the larvae. This model bypasses the intestinal barrier and therefore does not require the crystal proteins. In addition to *G. mellonella*, the silk worm, *Bombyx mori* and several other insect species can be used. Bacteria are injected through the intersegmental membrane between the fourth and the fifth abdominal legs of fourth instar larvae (38). Most insect larvae are highly susceptible to the direct injection of vegetative cells or spores of *B. thuringiensis* and *B. cereus* into the hemocoel. This mode of infection led to identification of multiple virulence factors, including genes associated with (1) iron metabolism and acquisition (264–266), (2) resistance to the host defenses including antimicrobial peptides, phagocytic cells, or nitric stress (202, 267–269), (3) motility (270), and (4) key transcription factors for *B. cereus* pathogenicity (271). The major role of the sphingomyelinase in the pathogenicity of *B. cereus* was also revealed by this method (39), thus confirming the

observation that clinical strains of *B. cereus* overproducing sphingomyelinase were more virulent than environmental strains (203).

Data from these experimental infections clearly demonstrate that *B. thuringiensis* is a true entomopathogenic bacterium (Fig. 5), producing specific toxins that weaken or kill invertebrates and other virulence factors, shared with *other B. cereus* group isolates, that allow bacteria to provoke septicemia and death of the host (72). Moreover, it has been shown that these bacteria harbor a regulon conferring them the ability to survive in the insect cadaver through a pathway independent of the sporulation and corresponding to a necrotrophic lifestyle (128, 174). This regulon is activated during the stationary phase of the bacteria by the NprR-NprX quorum sensing system and consists of 41 genes encoding several degradative enzymes including proteases and chitinases. Although the role of each component is not determined, altogether they should contribute to maximizing the consumption of the nutrients found in the host cadaver. Furthermore, the insect model was successfully employed to demonstrate antibiotic-induced small colony variant (SCVs), a novel persister phenotype of *B. cereus* (272).

Cell culture

Immortalized cell lines are employed for testing the cytotoxic potential of *B. cereus* strains. Hep2 cells are used to check strains for the emetic toxin cereulide while Vero cells are commonly used to check strains for their enterotoxic potential (35). However, more recently it has been reported that different human cell lines show distinct susceptibility for specific enterotoxin, which must be taken into consideration when functional studies on enterotoxins are carried out (205). These results are in line with a previous study from Doll et al. (39), showing that in polarized colon epithelial cells *B. cereus* sphingomyelinase is a strong inducer of epithelial cell death, by synergistically interacting with Nhe, while most of the cytotoxic effect on primary endothelial cells (HUVEC) can be explained by the cytotoxic activity of Nhe (205). Studies carried out on pancreatic beta cells, revealed a detrimental effect of cereulide toxin on these cells, even in low concentrations, rendering it possible that a long-term uptake of cereulide could have some implications in diabetes (213). Since beta cell dysfunction and cell death plays key roles in pathophysiology of diabetes, it will be important to study longtime effects of sub-emetic doses of cereulide in more detail in appropriate eukaryotic cell models.

Furthermore, boar sperms have been used to show the mitochondrial activity of the *B. cereus* emetic toxin cereulide (273) and the enterotoxic activity of Hbl has been demonstrated in a rabbit ligated ileal loop test (197).

In vitro simulation of gastrointestinal transit

Several systems to mimic gastric passage of *B. cereus* have been described (274–276). *B. cereus* spores and to a certain extent vegetative cells, survive in these models, but enterotoxin production has not been detected, making these models suboptimal for studies of *B. cereus* enteropathogenicity. Such systems mimicking the physico-chemical condition from mouth to ileum have been successfully used to study probiotic bacteria (277) but lack

the influence of host factors, which have been shown to be important for enterotoxin production of *B. cereus* (116).

VI. VIRULENCE GENE EXPRESSION

Virulence gene expression within the *B. cereus* group is controlled by complex regulatory circuits. In addition to global transcription regulators, such as CodY and AbrB, that play key roles in the integration of virulence and bacterial metabolism (271, 278, 279), *B. cereus* group-specific regulators exert control of virulence gene expression on transcriptional and posttranscriptional level. The global transcriptional regulator AtxA, encoded by pXO1 of *B. anthracis*, plays a key role in pathogenicity of *B. anthracis* while the chromosome-encoded pleiotropic transcriptional regulator PlcR, is the key regulator in *B. cereus* virulence (70, 280). Most of the virulence factors in *B. cereus* described so far are at least partially under control of PlcR, except cereulide toxin synthesis, which belongs to the Spo0A-AbrB regulon (281). In addition, some reports highlight the importance of post-transcriptional regulation of virulence factor expression, which is largely unexplored (114, 282, 283).

Global Transcription Regulators

CodY, a global transcriptional regulator integrating virulence and metabolism, plays an essential role in regulation and timing of virulence gene expression in the *B. cereus* group. CodY, which is highly conserved in low G+C gram positive bacteria, senses the nutritional and energetic status of a cell by binding to branched chain amino acids (BCAA) and GTP (284, 285). It often exerts control of virulence factors indirectly by acting on downstream regulators (286, 287). Although the majority of CodY-regulated genes are repressed during rapid growth, CodY is a positive regulator of some virulence genes (284, 288). In *B. cereus*, CodY gears the expression of virulence factors by impacting key regulatory circuits (66). CodY directly represses cereulide toxin synthesis in emetic *B. cereus* during early growth stages but acts indirectly on virulence genes belonging to the PlcR regulon by controlling import of the signaling peptide PapR, required for PlcR activity (271, 279). CodY was also reported to be essential for full virulence of *B. anthracis*. Data suggest that CodY exerts indirect positive control on post-translational regulation of AtxA accumulation (282). By acting, either directly or indirectly, on other virulence regulators, CodY adds an additional level of complexity to the regulatory circuits governing metabolic activities and virulence within the *B. cereus* group.

AbrB is a global transition state regulator that links virulence and cell development. AbrB has been extensively characterized in *B. subtilis* but also highly conserved in other Gram-positive bacteria (289, 290). As is true in *B. subtilis*, AbrB is negatively regulated in the *B. cereus* group by the response regulator Spo0A at the onset of transition phase. AbrB has been shown to control the expression of the anthrax toxin genes *pagA*, *lef*, and *cya* in *B. anthracis* as well as the cereulide (*ces*) synthetase genes in emetic *B. cereus*. In *B. thuringiensis*, *abrB* represses expression of *inhA1* during vegetative growth (291). During early exponential growth phase, *abrB*-null mutants synthesize elevated levels of anthrax toxin in *B. anthracis* and cereulide levels in emetic *B. cereus* (281, 292). Thus, it can be

assumed that AbrB plays a pivotal role in the correct timing of toxin expression in *B. anthracis* as well as in emetic *B. cereus*.

Alternative sigma factors have also been implicated in transcription of virulence factors of the *B. cereus* group. The sporulation gene regulator SigH represents a target of the AbrB repressor in *B. subtilis* (293) and it has been postulated that AbrB acts on anthrax toxin gene expression via control of *sigH* (294). However, the *B. subtilis* consensus sequence for recognition by SigH has not been found upstream of the anthrax toxin genes nor upstream of the *ces* genes in emetic *B. cereus* and no direct SigH-dependent toxin promoter activity could be shown (281, 295). Therefore, it is tempting to speculate that SigH is not directly linked to toxin gene regulation in *B. anthracis* or emetic *B. cereus*. SigB, another alternative sigma factor, may be a minor regulator of virulence in *B. anthracis*. Although there is no evidence that SigB affects toxin or capsule gene expression, a *sigB* mutant exhibits reduced virulence in a mouse model for anthrax (296).

RNPP quorum sensors

A main trait of the *B. cereus* group is the unique presence of two transcriptional regulators PlcR and NprR forming two distinct quorum sensing systems in association with their cognate signaling peptides PapR and NprX, respectively (129, 297). In addition to these specific regulators, the bacteria of the *B. cereus* group produce proteins homologous to the Rap phosphatases of *B. subtilis* (175, 298). These quorum sensors define the RNPP family according to the name of the prototypical members Rap, NprR, PlcR and PrgX (299). Quorum sensing systems connect gene expression to cell density, thus coordinating the behavior of the whole bacterial community able to respond to the same signaling peptide. In the insect, it was shown that the sequential activity of RNPP regulators (PlcR, NprR and Rap) controls the physiological stages of *B. thuringiensis* throughout the infectious cycle, from virulence to sporulation (300).

PlcR positively regulates 45 genes, most of which encode exported virulence factors as the enterotoxins Hbl and Nhe, the cytotoxin CytK, the cholesterol-dependent hemolysins and several degradative enzymes (70). As discussed above, PlcR plays a key role in the virulence of *B. thuringiensis* and *B. cereus* in their invertebrate or mammal hosts (70, 72, 245). The *plcR* gene is present in all *B. cereus* group species, but does not always produce a functional protein (301). Notably in *B. anthracis* a nonsense mutation inactivates the PlcR function (17, 18). PlcR is a 34-kDa helix-turn-helix (HTH)-type transcription factor activated upon binding of PapR. This binding induces a drastic conformational change of the dimeric form of PlcR allowing an appropriate positioning of the HTH domains and their binding to the PlcR-boxes located upstream from the – 10 region of the PlcR-regulated promoters (297, 299, 302). Binding of PapR to PlcR is highly specific and polymorphisms exist within the *B. cereus* group. Four specificity groups, or pherotypes, can be distinguished (303, 304).

NprR is also found in all *B. cereus* group species and acts as a transcriptional regulator at the onset of sporulation (128). As indicated above, NprR is a RNPP regulator whose transcriptional activity depends on a signaling peptide (NprX) that switches NprR from a dimeric apo form to a tetrameric form (305). Similarly to PlcR-PapR, the NprR-NprX system is strain specific and seven pherotypes were defined within the *B. cereus* group

(129). NprR-NprX activates the transcription of a regulon including genes encoding degradative enzymes (proteases, lipases and chitinases) and a lipopeptide (kurstakin) involved in biofilm formation (128, 306). Using insect larvae as an infection model, it was shown that the NprR regulon allows bacteria to enter in a necrotrophic lifestyle essential for the survival of the bacilli in the cadavers of infected insects. In the absence of its cognate peptide, the apo form of NprR functions as a phosphatase inhibiting the sporulation process (307). It has been shown that sporulation occurs only in bacteria expressing the NprR regulon and following a necrotrophic lifestyle in the insect cadaver (174). These results are in agreement with the dual function of NprR and indicates that its phosphatase activity should be turned off by NprX to allow the sporulation of the bacteria.

AtxA, the key regulator in *B. anthracis* virulence

AtxA, named for its control of toxin gene expression (308) and encoded by a pXO1 gene, is the master virulence regulator of *B. anthracis*. AtxA positively controls transcript levels of the structural genes for anthrax toxin, *pagA*, *lef*, and *cya*, located on pXO1, the capsule biosynthetic operon *capBCADE* located on pXO2, and multiple other genes on the plasmids and chromosome (5, 280, 309–316). An *atxA*-null *B. anthracis* strain is highly attenuated in a murine model of anthrax disease (316) (317). The *atxA* gene has also been associated with control of *B. anthracis* development. AtxA activates expression of a pXO2-encoded sporulation kinase inhibitor to repress sporulation in conditions in which AtxA is highly expressed and active (318). This activity may be related to the lack of *B. anthracis* sporulation during infection.

Paralogous regulators, AcpA and AcpB encoded by pXO2 genes, were first identified as regulators of *capBCADE* (319, 320). AcpA and AcpB do not appear to affect toxin gene expression, but may affect expression of non-capsule genes (280, 321). Data from experiments employing null-mutants reveal that expression of the *atxA*, *acpA*, and *acpB* genes is inter-dependent and suggest that AcpA and AcpB have similar functions. The primary means for AtxA control of *capBCADE* is via positive regulation of *acpA* transcription. The *acpA* gene is located upstream of the *cap* locus and in the opposite orientation. Two apparent transcriptional start sites, one expressed at a low constitutive level and one controlled positively by AtxA, drive expression of *acpA*. AcpA can positively affect transcription of *capBCADE* in the absence of AtxA and AcpB. The *acpB* gene, located downstream of *capBCADE* and in the same orientation, is expressed as a monocistronic transcript initiated from a weak constitutive promoter. AcpB, like AcpA, can positively affect *capBCADE* expression in the absence of the other regulators. A weak transcription terminator located between *capE* and *acpB* results in co-transcription of *acpB* with *capBCADE* in roughly 10% of transcripts, thus forming a positive feed-back loop (322).

AtxA expression is also subject to complex regulation. Transcription of *atxA* is affected by temperature, carbohydrate availability, redox potential, metabolic state, and growth phase (323–326). In addition, CodY controls AtxA protein levels post-transcriptionally by an unknown mechanism (282), and AbrB represses *atxA* expression by binding to the *atxA* promoter region upstream of the P1 transcriptional start site (292, 327). Finally, an unknown additional repressor binds to the *atxA* promoter to negatively regulate expression (317).

AtxA, AcpA, and AcpB belong to an emerging class of regulators termed “PCVRs” (Phosphoenolpyruvate-dependent phosphotransferase system regulation Domain [PRD]-Containing Virulence Regulators) (328, 329). Generally, PCVRs contain nucleic acid-binding domains and PRDs subject to phosphorylation by the phosphotransferase system (330). AtxA crystalizes as a dimer, and distinct folds associated predicted DNA-binding domains and PRDs are apparent in each chain. The model for PRD control of AtxA function is that phosphorylation events at two specific histidines, one in each PRD, have opposing effects on AtxA function. Specific DNA-binding activity of AtxA has not been reported, but phosphorylation of H199 of PRD1 is predicted to affect interaction of AtxA with DNA, while phosphorylation of H379 in PRD2 disrupts AtxA dimerization and abolishes activity (331). Phosphorylation of AcpA and AcpB has not been demonstrated. Although comparisons of the AcpA and AcpB amino acid sequences and structure predictions are highly suggestive of the presence of PRDs, the placement of histidines within the PRDs differ between the three regulators, suggesting large differences in the potential phosphorylation sites (328). If indeed the AcpA and AcpB PRDs control PCVR function, the relationships between phosphorylation and protein structure may differ between the PCVRs, affecting their target specificity and relative activity.

VII. GENETIC TOOLS

I. Natural genetic exchange – intra and inter-species

Historically, facile identification of the *B. cereus* group species has relied upon their plasmid content: plasmids carrying the crystal insecticidal genes of *B. thuringiensis*, the anthrax toxin and capsule genes of *B. anthracis* and more recently *B. cereus* Biovar *anthracis*, and the biosynthetic gene cluster for cereulide synthesis of *B. cereus* (6, 16, 49, 332). However, genetic experiments have revealed that plasmids can move among the strains of the *B. cereus* group by conjugation, and transduction (333–335) (Green et al), thus complicating the speciation of strains belonging to this bacterial group. Horizontal genetic transfer of plasmid and chromosome DNA has likely contributed to the remarkable diversity of the strains composing the *B. cereus* group.

Transducing phage have been characterized for all three *B. cereus* species. The bacteriophage CP-51, isolated from *B. cereus*, was the first generalized transducing phage discovered for the group, and has been the most utilized for genetic manipulation (336). CP-51 and CP-54, a phage with a broader host range than CP-51, also mediate generalized transduction in *B. thuringiensis* (337, 338). They can transduce various plasmids between the *B. cereus* group species and are also useful for intra-species transduction of chromosomal markers (339, 340). CP-51-mediated transduction of pXO2 from *B. anthracis* to *B. cereus* was instrumental in demonstrating that the plasmid was associated with capsule synthesis (341), while the first genome maps of *B. thuringiensis* were established using this phage (337, 338, 342).

Plasmid conjugation in bacteria of the *B. cereus* group was first demonstrated by showing the transfer of plasmid-borne *cry* genes from *B. thuringiensis* strains to *B. cereus* (51). These observations were then extended to other plasmids and species including *B. anthracis* (341, 343). Conjugative plasmids native to *B. thuringiensis* have been shown to transfer to *B.*

anthracis and *B. cereus*, and in some cases to mobilize other non-self-transmissible plasmids. Among these are *B. thuringiensis* plasmids harboring insecticidal toxin genes, such as pXO12 (345) and pHT73 (344). The conjugation experiments performed between *B. anthracis* and *B. thuringiensis* revealed transfer of plasmids by a conjugation process involving the formation of cointegrate structures between a conjugative plasmid (*ie* pXO12) and the nonconjugative plasmid pXO1. These experiments showed that the transposon Tn4430, located in various conjugative plasmids of *B. thuringiensis* (8, 345), mediates the transfer of nonconjugative plasmids by a conjugation process (346). These findings indicate that transposable elements associated to conjugative plasmids may have important adaptive functions by mediating the horizontal transfer of genetic material, including toxin genes, within the *B. cereus* group.

Plasmid transfer among the *B. cereus* group has been reported to occur in various niches including the insect gut (347–349), gnotobiotic rats (350), the plant rhizosphere (63), soil (61), water (351), and dairy products (352, 353), and a growing data base of DNA sequences from clinical and environmental isolates reveals evidence for extensive genetic exchange among the *B. cereus* group. Multiple isolates from soil carry pXO1- and pXO2-like plasmids (24, 353–356). In some cases, clinical isolates from humans and animals have been reported with newly-discovered combinations of potential plasmid-borne virulence genes. Interestingly, pAW63, from a *B. thuringiensis kurstaki* strain, shares a common backbone sequence with pXO2 of *B. anthracis* and with pBT9727 from the pathogenic *B. thuringiensis subspecies konkukian* (357, 358). *B. cereus* strain G9241, isolated from a patient with life-threatening pneumonia, carries a specific protective antigen gene (*pagA*) on plasmid pBCXO1 and a gene on plasmid pBC218 that encodes a polysaccharide capsule (24).

Some reports have also described genetic exchange between *B. subtilis* and members of the *B. cereus* group. Plasmid pLS20 from *B. subtilis subspecies natto* is self-transmissible and facilitates transfer of other plasmids from *B. subtilis* to the *B. cereus* group species (359). Although integrative and conjugative elements (ICEs) have not been reported in the *B. cereus* group, *B. anthracis* has been shown to be an effective recipient of ICEBs1, from *B. subtilis* (360). The presence of *cry* genes encoding insecticidal toxins in *Bacillus popilliae* (361) and *Clostridium bifermentans* (362) strongly suggests an horizontal transfer of *B. thuringiensis* plasmids to other spore-forming Gram-positive bacteria.

Generation of recombinant strains and genetic analysis

B. cereus group members are not naturally transformable and the commercialization of the BioRad's electroporation system in the 1980s years was a crucial milestone in the development of genetic approaches in these bacteria. In 1989, several electroporation procedures were published for the transformation of *B. thuringiensis* and *B. cereus* (363–365), and were followed by procedures functional in *B. anthracis* (366, 367) or in the emetic *B. cereus* strains (41, 368).

Interestingly, it was shown that electrotransformation is significantly improved when plasmids are prepared in DNA methyltransferase-deficient (*dam*) *E. coli* strains or from *B. subtilis* 168, a strain that is not known to possess adenine-methylating activities (369, 370). Plasmids can be also transferred from *Escherichia coli* to the bacteria of the *B. cereus* group

by hetergramic conjugation (371). A second important milestone for the development of genetic studies in the *B. cereus* group was the construction of cloning and expression vectors. This was notably achieved thanks to the use of the pHT1030 replicon which allowed the construction of a series of highly stable vectors (340, 372). Due to the relatively low transformation frequency of the *B. cereus* strains, site-directed mutagenesis and direct allelic exchange is not possible by using linear DNA, and requires the use of shuttle plasmids like pMAD or pRN5101 whose replication in Gram-positive bacteria is thermosensitive (340, 373). More recent systems draw upon methods first developed for other bacteria. They include group II intron targeting (374) and systems using tyrosine site-specific recombinases (T-SSRs) that catalyze cutting and joining reactions between short specific DNA sequences. The systems employing T-SSRs and their cognate target sequences, have been derived from *E. coli* bacteriophage P1 Cre-loxP, *Saccharomyces cerevisiae* Flp-FRT, and a newly discovered IntXO-PSL system from *B. anthracis* plasmid pXO1 (375). A method for conducting I-SceI-mediated allelic exchange in which recombinational loss of a chromosomally-integrated allelic exchange vector is stimulated by creation of a double-stranded break within the vector by the homing endonuclease I-SceI has also been described (376, 377). This system has been used to make in-frame deletions in genetic elements of *B. anthracis* (152, 161, 378), *B. cereus* (379, 380) and *B. thuringiensis* (381).

For random mutagenesis, Tn10- and *mariner*-based mini-transposons have proved useful for the detection of virulence genes or for the studies of genetic systems (382–386). Insertion mutant libraries have also been generated using *Enterococcus faecalis* transposons Tn916 and Tn917. However, insertions of these transposons can be unstable and are sometimes accompanied by local deletions and DNA rearrangements (387, 388).

An *in vivo* expression technology (IVET) was developed for identification of *B. cereus* genes specifically expressed during infection in an insect host (264). This genetic tool allows identification of transient promoter activation by using site-specific recombination. The activity of a promoter cloned upstream from a recombinase gene results in the loss of a resistance marker and in the activation of a new resistance gene. The *in vivo* screening of a *B. cereus* genomic library led to the characterization of genes expressed during infection in the insect *G. mellonella*. One of these genes was found specifically expressed in the hemocoel and required for iron acquisition (265); while a 5-gene cluster, specifically expressed in the larval gut, was essential for glucose-6-phosphate uptake (389).

Measurements of gene expression

Whole transcriptome sequences of *B. anthracis* and *B. cereus* cultured in different growth conditions have been described (390–393). Not surprisingly, the data predict gene functions and reveal multiple transcripts not associated with previous genome annotations. As is true for other bacteria, the advent of RNA Seq and quantitative RT-PCR has lessened the need for recombinant methods to measure gene expression. Nevertheless, for some studies with the bacteria of the *B. cereus* group, expression of specific genes can be readily monitored using transcriptional fusions employing promoterless genes for β -galactosidase and chloramphenicol acetyltransferase (367, 394, 395). Investigations of transcript and protein function are also aided by use of recombinant constructs harboring promoters inducible by

IPTG or xylose pAW285 (223, 271, 297, 396) or by use of recombinant constructs harboring promoters inducible by cold (281).

Bioluminescent reporter systems, based on *luxABCDE* promoter fusions, for real-time monitoring of gene transcription have been described for *B. anthracis* as well as for *B. cereus*. A *sspBp::lux* plasmid was used for *in vivo* spore germination of *B. anthracis* in mice (164) and a *cesPI::lux* plasmid was successfully employed for monitoring of cereulide synthetase (*ces*) promoter activity in complex environments, such as foods (58, 368). Various fluorescent reporters were used to record gene expression at the single-cell level and to follow the physiological stages of *B. thuringiensis* in different growth conditions including biofilm and infection in insect larvae (174, 397). Furthermore, the *E. coli* / *B. cereus* shuttle vector pAD123 with a promoterless red-shifted green fluorescent protein (GFP) gene (*gfpmut3a*) has been shown to be an effective tool for promoter studies in *B. cereus* (368, 398).

VIII. THERAPEUTICS AND VACCINES

Antibiotic therapy is the primary treatment of anthrax and for severe infections caused by *B. cereus* and *B. thuringiensis*. The *B. cereus* group species have similar antibiotic susceptibility profiles and are susceptible to the majority of front line antibiotics. Historically, penicillin was the antibiotic of choice for treatment of known or suspected anthrax. However, intrinsic or inducible resistance to β -lactam antibiotics has been reported in clinical isolates (399–401). Because *B. cereus* and *B. thuringiensis* infections are relatively rare, are generally treatable with antibiotics, and in the case of *B. cereus* food-poisoning, are self-limiting, vaccines have not been developed for these infections. Anthrax vaccines for humans have been available for decades, however the nature of the vaccine, as described, below provides impetus for continued research in anthrax vaccine development.

Anthrax prophylaxis and treatment

The Centers for Disease Control and Prevention recommends fluoroquinolones, such as ciprofloxacin or doxycycline, as first-line drugs for postexposure prophylaxis and treatment of *B. anthracis* infections. The bactericidal activity and exceptional central nervous system penetration make ciprofloxacin recommended over doxycycline. For treatment of severe systemic and life-threatening disease and for cases with fulminant bacteremia, ciprofloxacin with additional antibiotics, especially clindamycin, is recommended. Clindamycin inhibits protein synthesis, potentially reducing toxin production (402, 403). Treatment with additional antibiotics is also recommended because of reports of ciprofloxacin-resistance generated following serial passage in the presence of increasing concentrations of the antibiotic. Ciprofloxacin-resistance is associated with a point mutation in the *gyrA* gene encoding gyrase A (404, 405).

Long courses of antibiotics are recommended for anthrax prophylaxis and treatment because *B. anthracis* spores have been reported to remain viable up to 60 days in non-human primates (). Recently, the anthrax vaccine AVA was approved by the U.S. Food and Drug Administration for post-exposure prophylaxis in conjunction antimicrobials. The efficacy of antibiotic therapy for anthrax decreases as time between exposure and treatment increases

(406–409) likely due to increasing toxin levels, indicating a need for anti-toxin therapeutics. Toxin inhibitors in development include a variety peptides and antibodies (410, 411).

Anthrax Vaccines

Current human vaccines for anthrax are the U.S.-licensed AVA (anthrax vaccine adsorbed, BioThrax) and U.K.-licensed AVP (anthrax vaccine precipitated). The anthrax toxin protein PA is the major immunogenic component of both vaccines. PA elicits toxin neutralizing antibodies that correlate with protection against anthrax disease in humans and many animal models (412–416). AVA is an aluminum hydroxide-adsorbed, PA-containing supernate from a culture of a toxin-producing (pXO1+) but non-capsulated (pXO2-) strain of *B. anthracis*. The standard AVA vaccination protocol requires five doses administered intramuscularly over 18 months, followed by annual booster injections (417), while a more recently approved regimen requires three doses over six months. AVP is precipitated with aluminum potassium sulfate (Alum) from culture supernates of a different toxinogenic non-capsulated strain. The AVP vaccine is administered as four doses over 32 weeks with annual boosters. Unlike AVA, AVP contains low levels of LF and EF, and some reports indicate that humoral responses to LF and EF generated by AVP may contribute to toxin neutralization (418, 419).

The prolonged immunization regimen with reports of reactogenicity, cold-storage requirement, and four-year half-life make the anthrax vaccines non-optimal for pre-exposure prophylaxis and post-exposure therapy. Thus, various expression systems, immunogens, adjuvants, and delivery vehicles are in development to achieve a higher performing and more user-friendly vaccine. Efforts include investigations of purified recombinant PA preparations, *Lactobacillus*- and *Salmonella*-producing PA for oral delivery, DNA vaccines, and non-PA based vaccines with alternative targets such as the *B. anthracis* capsule, cell wall S-layer proteins, spore components, and other the other toxin proteins EF and LF (420–425).

The veterinary anthrax vaccine is a live spore vaccine developed in 1937 by Max Sterne (426). The strain, 34F2 or “Sterne”, was first isolated as a stable non-capsulated isolate, and was later shown to carry pXO1+ but not pXO2-. Animals injected with the Sterne vaccine develop humoral immunity within 4 weeks, but the immunity is not long-lived and annual revaccination is recommended. Although the vaccine can be effective in livestock populations, in many parts of the world, underreporting, logistics, and limited resources makes implementing vaccination campaigns difficult.

IX. FUTURE DIRECTIONS

Substantial effort has been placed on determination of biomarkers and distinguishing features of the closely related members of the *B. cereus* group. Yet distinctions between the species and between virulence and non-virulent strains of the species is not always clear. A key issue for *B. cereus* (and also for *B. thuringiensis*) is the detection of pathogenic strains responsible for food poisoning resulting in diarrheal syndromes. These syndromes are suspected to be caused by the various cytotoxins and degradative enzymes produced by bacteria under the control of the virulence regulator PlcR. However, most strains produce these factors and are not generally pathogenic, as is likely the case with *B. thuringiensis*

strains that are used as biopesticides. *B. thuringiensis* is defined by the ability to produce a parasporal inclusion made of Cry proteins.

Numerous Cry proteins are insecticidal or nematocidal toxins, but some have no known targets. The search for the presumed targets, whether in arthropods or other organisms, is of scientific and economical interest, and will facilitate increased understanding of the expanded ecology of these bacteria.

Efforts to identify biomarkers specific for *B. anthracis* are a significant part of biodefense research. While such studies have affirmed some novel features of the species, the recent reports of *B. cereus* isolates causing anthrax-like disease suggest that strains of other species may also present a bioweapon threat. Moreover, increased environmental surveillance and expanded genome sequencing are revealing newly-recognized *B. cereus* group isolates with unique physiology, genetics, and virulence capabilities.

The emergence of clinical isolates with novel combinations of plasmids and recombinant forms of well-characterized plasmids suggest an evolving virulence repertoire. It will be of interest to explore the mechanisms by which these changes occur and the frequencies of apparent horizontal gene transfer in non-host environments. What relationships if any exist between plasmid content and specific chromosomes? What happens to the signaling networks controlling gene expression in these atypical isolates? For some *B. cereus* isolates, plasmids can represent up to 20% of the total DNA. With the exception of genes involved in pathogenicity, the adaptive functions of these plasmids are still largely unknown.

Much has been learned about host-pathogen interactions associated with virulent strains of the *B. cereus* group species. What are the core genomic factors of the *B. cereus* group species that are required to cause distinct diseases? Can such factors actually be defined? Do the “virulence” plasmids play roles in the non-host environments of the *B. cereus* group species? To answer these questions, more advances must be made in the area of the ecology of these organisms, their lifestyles outside of their hosts, and their interactions with each other.

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Fig. 1.

The five major phylogenetic clades of *B. cereus* group. Clade I is known as *B. anthracis* clade but also includes emetic *B. cereus* as well as several clinic *B. thuringiensis* isolates. Clade II is known as *B. cereus/B. thuringiensis* clade, which includes most of the commercial used *B. thuringiensis* as well as the *B. cereus* and *B. thuringiensis* type strains. Clade III is known as *B. weihenstephanensis* clade and comprises most of the psychrotolerant isolates of the *B. cereus* group. These clades are based on MLST, AFLP and whole genome sequence data (427–429). The thermotolerant strains belonging to the species *B. cytotoxicus* cluster together in a separate group.

Fig. 2.

Transmission of the *B. cereus* group species from the soil reservoir to humans via food and textile production. Soil and soil-associated organisms including plants, insects, nematodes, and amoebae, serve as the major reservoir for acquisition of spores. The bacteria are transferred to humans through agricultural products including food and animal-associated textiles, entering humans and other mammals through ingestion, inhalation, and breaks in the skin. Illustration credit: Olive E. Morrison.

A

B

Fig. 3.

Thin-section transmission electron micrograph of (A) *B. anthracis* cell after five hours of sporulation (left image) and a mature spore (right image). The forespore inner membrane (FS IM) and nascent exosporium (Ex) (left image), and forespore inner membrane (FS IM), mature exosporium (Ex), coat (Ct), interspace, (Is) and cortex (Cx) (right image) are labeled. *B. thuringiensis* sporulating cell with parasporal crystal and (B) *B. thuringiensis* sporulating cell with parasporal crystal. Cells were sporulated, prepared and imaged as described in Margolis *et al.*, 1993. Image credit: (A) Tyler Boone and Adam Driks.

Fig. 4.
Cereulide, the emetic toxin of *B. cereus*

Fig. 5.

The infectious cycle of *B. thuringiensis* in a susceptible insect larva. Ingestion of spores and crystals is followed by the dissolution of the crystal in the alkaline environment of the midgut. Insect proteases activate Cry proteins. Spores germinate in the paralyzed insect gut. Cry toxins degrade the peritrophic membrane. Bacteria multiply and express PlcR-regulated genes. The intestinal barrier is disrupted and bacteria gain access to the hemocoel. The bacteria resist host defenses and cause a fatal septicemia. The NprR regulon is activated and the bacteria survive in the host cadaver. Finally, spores and vegetative cells are disseminated outside of the host.

Table 1.Exotoxins, the major virulence factors of the *B. cereus* group

Toxin	Toxin type	<i>B. anthracis</i>	<i>B. cereus</i>	<i>B. thuringiensis</i> ²	is
Anthrax toxin (PA, LF, EF)	AB toxin	+	(+) ^a	–	3 4
Emetic toxin (cereulide)	heat stable depsipeptide toxin	–	+	–	5 6 7
Enterotoxins (Nhe, Hbl, CytK)	pore forming toxins	(–) ^b	+	+	8 9 10
Insecticidal toxins (Cry, Cyt, Vip, Sip)	pore forming toxins	–	–	+	11 12 13 14

^aSome rare strains^bnhe genes are present but inactive