Chapter 2
Genomics of *Bacillus* Species

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2.1 The Genus *Bacillus*

Members of the genus *Bacillus* are rod-shaped spore-forming bacteria belonging to the Firmicutes, the low G+C gram-positive bacteria. The *Bacillus* genus was first described and classified by Ferdinand Cohn in Cohn (1872), and *Bacillus subtilis* was defined as the type species (Soule, 1932). The genus is large, encompassing more than 60 species with a great genetic diversity (Priest, 1993) (Fig. 2.1), most of which are considered non-pathogenic. *Bacillus* species may be divided into five or six groups (groups I–VI), based on 16S rRNA phylogeny or phenotypic features, respectively (Priest, 1993).

The *Bacillus* genus includes a range of species of human interest. This is mostly due to either (1) the use of the bacteria in industrial applications, such as for example in the making of biotechnological products (insect toxins, peptide antibiotics, enzymes for detergents, etc.) (Priest, 1993); (2) the employment of the spore as a model system for studying bacterial cellular differentiation, and its resistance to decontaminating agents or treatments; or (3) the role of certain *Bacillus* species in causing human disease. The latter interest can be followed back to the late nineteenth century and the studies of Louis Pasteur, using heat-attenuated cells of *Bacillus anthracis* as the first anti-bacterial vaccine, and Robert Koch, elucidating the role of a specific microorganism (*B. anthracis*) in causing a specific disease (anthrax).

2.2 Pathogenicity of *Bacillus* Species

Several Bacilli may be linked to opportunistic infections, e.g. in post-surgical wounds, cancer patients, or immunocompromised individuals. Pathogenicity among *Bacillus* spp. is however mainly a feature of organisms belonging to the *B. cereus* group, a subgroup of the *B. subtilis* group (group II) within the *Bacillus* genus

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M. Wiedmann, W. Zhang (eds.), *Genomics of Foodborne Bacterial Pathogens*,
Food Microbiology and Food Safety, DOI 10.1007/978-1-4419-7686-4_2,

Fig. 2.1 Phylogeny of species within the *Bacillus* genus, based on 16S rRNA sequence data. The *B. cereus* group members are *boxed* in *pink*. The *horizontal bar* indicates a genetic distance of 0.02

(Fig. 2.1), and which are commonly found in the environment (reviewed by Drobniewski, 1993). In line with this, although *Bacillus licheniformis, Bacillus pumilus*, and *B. mojavensis* have all been implicated in food poisoning incidents (Salkinoja-Salonen et al., 1999; Nieminen et al., 2007; Apetroaie-Constantin et al.,

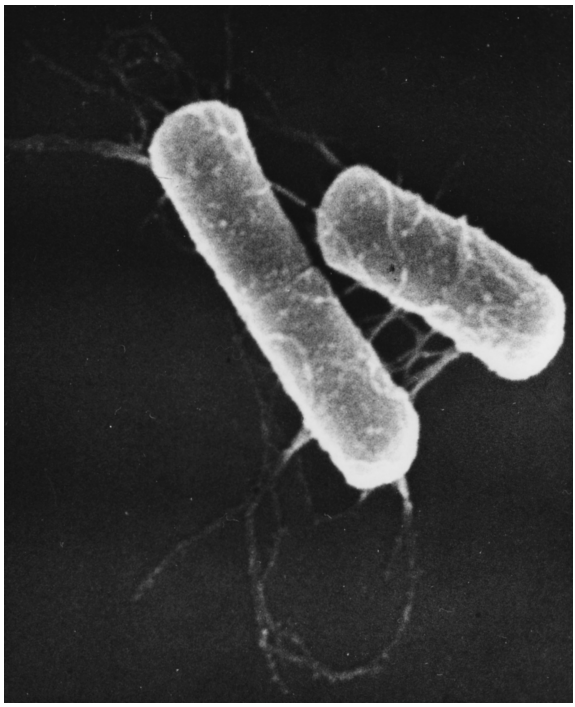

Fig. 2.2 Scanning electron micrograph (Hitachi HHS/2R) of *B. cereus* ATCC 10987

2009), the majority of reported cases of *Bacillus* food poisoning are caused by *B. cereus* and its close relatives (Fig. 2.2). In this chapter, we will therefore discuss the genomics of *Bacillus cereus* group bacteria in relation to their roles as etiological agents of two food poisoning syndromes.

2.3 The *Bacillus cereus* Group

The *B. cereus* group (*B. cereus sensu lato*) includes six approved species: *B. anthracis, B. cereus* (*sensu stricto*)*, B. thuringiensis*, *B. mycoides, B. pseudomycoides,* and *B. weihenstephanensis.* In addition, a remote cluster of three thermophilic strains has been identified within the group (Lund et al., 2000; Fagerlund et al., 2007; Auger et al., 2008). This cluster has been suggested as a new species: *B. cytotoxicus* (or *B. cytoxis*) (Fig. 2.3) (Lapidus et al., 2008). The phylogeny of the *B. cereus* group has been mapped extensively by various methods, including multilocus enzyme electrophoresis (MLEE; Helgason et al., 1998, 2000a, c), amplified fragment length polymorphism (AFLP; Keim et al., 1997a; Jackson et al., 1999; Ticknor et al., 2001; Hill et al., 2004; Mignot et al., 2004), and by what is currently considered the gold standard for such studies, multilocus sequence typing (MLST; Helgason et al., 2004; Barker et al., 2005; Tourasse et al., 2006). Altogether five MLST schemes exist for the group, based largely on non-overlapping genes and strain sets. Strains from all five schemes have however been integrated into one phylogeny using supertree methodology (Tourasse and

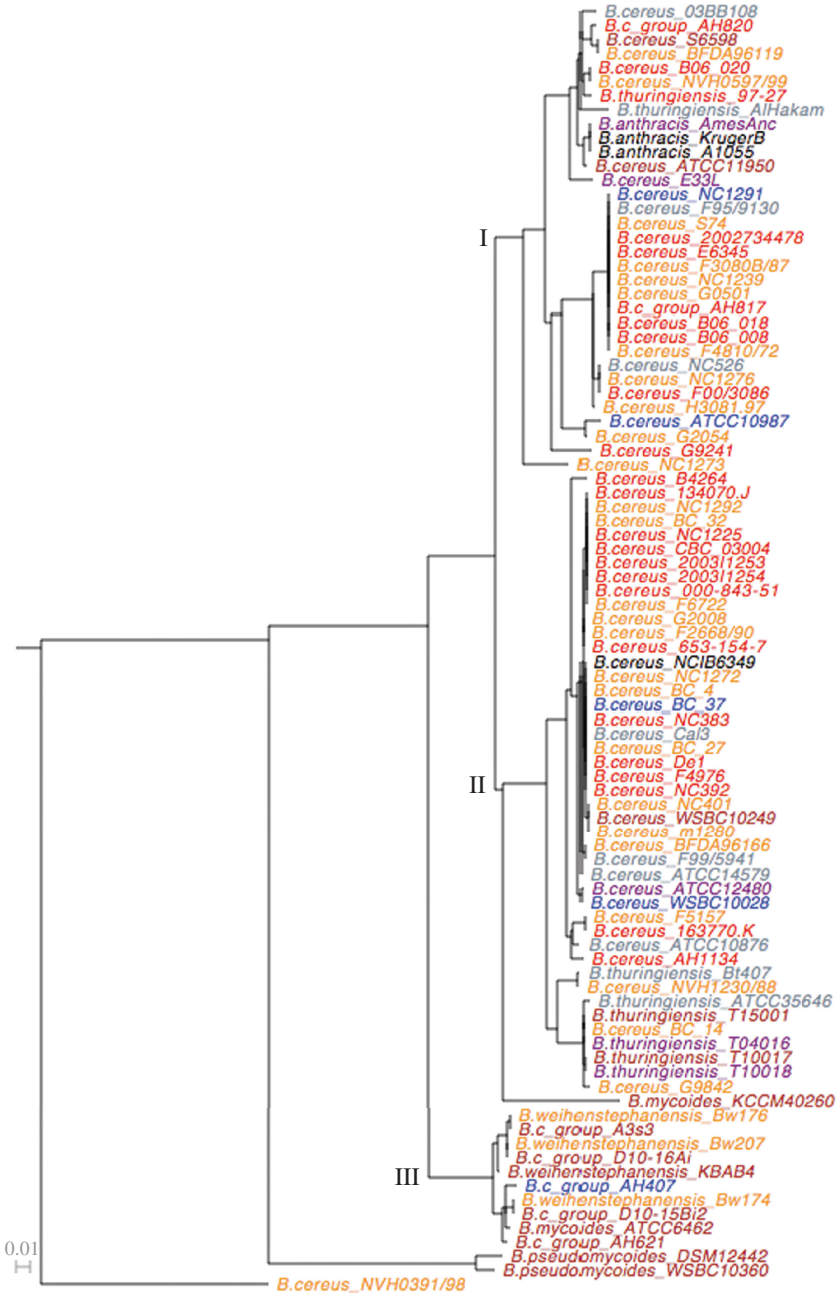

Fig. 2.3 Molecular phylogeny of *Bacillus cereus* group bacteria as analyzed by multilocus sequence typing (MLST), employing supertree technology. The species displayed form a subtree of 91 isolates extracted from the supertree of 1,400 isolates available in the SuperCAT database provided at the University of Oslo typing website (http://mlstoslo.uio.no/). The supertree is based on a

Kolstø, 2008), resulting in the hitherto most comprehensive view of the *B. cereus* group population, encompassing more than 1,400 isolates (SuperCAT database; http://mlstoslo.uio.no). By MLST analysis, the *B. cereus* group population can be grouped into at least three main clusters of isolates (Fig. 2.3); cluster I contains *B. anthracis* and related *B. cereus* and *B. thuringiensis* strains and carries mostly isolates of clinical origin; cluster II harbours *B. cereus* and *B. thuringiensis* strains from a variety of sources including food poisoning events; while cluster III contains *B. weihenstephanensis* and *B. mycoides* isolates.

2.3.1 B. cereus, B. anthracis, *and* B. thuringiensis

The three most frequently studied species within the *B. cereus* group are *B. anthracis, B. thuringiensis,* and *B. cereus. B. cereus* is a common bacterium of the soil and can colonize invertebrate guts as a symbiont, an environment which has been suggested to be its natural habitat (Margulis et al., 1998; Jensen et al., 2003). It is however a frequent cause of human food poisoning, as well as various opportunistic and nosocomial infections, e.g. in the immunocompromised or following trauma to the eye (reviewed by Drobniewski, 1993; Kotiranta et al., 2000; Bottone, 2010). *B. cereus* can cause two types of food poisoning syndromes, namely, the emetic syndrome, due to the synthesis of cereulide, a small, heat-stable non-ribosomally synthesized dodecadepsipeptide, and the diarrhoeal syndrome, caused by enterotoxins (Drobniewski, 1993; Stenfors Arnesen et al., 2008). Other potential virulence factors include secreted phospholipases, haemolysins, proteases, and other degradative enzymes. These proteins are extracellular virulence factors, and their expression is under the control of the global pleiotropic transcriptional regulator PlcR (Agaisse et al., 1999; Økstad et al., 1999b). Genes encoding the proteinaceous virulence factors, enterotoxins included, are located on the chromosome, while the genes responsible for synthesis of the emetic toxin are located on a large (270 kb) plasmid, pCER270 (Hoton et al., 2005; Ehling-Schulz et al., 2006).

B. thuringiensis is also frequent in soil, is an entomopathogenic bacterium, and is the most commonly used commercial biopesticide worldwide (Soberon et al., 2007). Its identification and classification are based on the production of insecticidal proteinaceous toxin crystals (Cry and Cyt proteins) during sporulation (Schnepf et al., 1998; Aronson, 2002), a feature recognized by microscopy. The Cry and Cyt toxins are of different classes and exhibit variable specificities towards the larvae of different classes of insects (reviewed in Whiteley and Schnepf, 1986; Schnepf et al., 1998).

Fig. 2.3 (continued) combination of sequence data from all five published MLST schemes available for the *B. cereus* group (Tourasse and Kolstø, 2008). Three main phylogenetic clusters of the *B. cereus* group population are indicated by *Roman numerals*, and strains are *coloured* by source of isolation, following their representation in SuperCAT (*red*, human; *purple*, animal; *dark brown*, soil; *orange*, food; *blue*, dairy; *grey*, other)

B. anthracis is a highly monomorphic species within the *B. cereus* group, showing very little genetic variation (Fig. 2.3; Keim et al., 1997b; Van Ert et al., 2007). In the environment, the bacterium primarily exists as a highly stable, dormant spore in the soil. Nevertheless, it has been claimed that the organism can grow and persist outside the host, in the rhizosphere of plants (Saile and Koehler, 2006). *B. anthracis* is the cause of anthrax, primarily a disease of herbivores, but may also cause isolated cases of infections in man. In several regions of the world, including parts of Africa and Asia, *B. anthracis* is endemic or hyperendemic, while being sporadic in Australia and the United States (http://www.vetmed.lsu.edu/whocc/mp_world.htm). Anthrax takes three forms: cutaneous, gastro-intestinal, or inhalational (reviewed in Mock and Fouet, 2001). While the cutaneous form is easily treatable with antimicrobials, the gastro-intestinal and inhalational forms of the disease are more severe, as has been demonstrated by human deaths following the ingestion of meat from animals that died from anthrax disease (reviewed by Beatty et al., 2003), as well as in the bioterror attacks against the United States in fall of 2001, when letters containing *B. anthracis* spores were distributed through the US Postal Service, killing five people (Jernigan et al., 2001). Anthrax disease is primarily caused by two virulence traits: (1) the synthesis of two A–B type toxins from three toxin components, namely, lethal toxin (LT) being composed of protective antigen (PA) and lethal factor (LF), and edema toxin (ET) which is composed of PA and edema factor (EF) (Mock and Fouet, 2001; Mock and Mignot, 2003; Young and Collier, 2007), and (2) the presence of a poly-γ-D-glutamic acid (polyglutamate) capsule which is important for *B. anthracis* survival in a host, as it helps the bacterium evade the host immune system by protecting vegetative cells from phagocytotic killing during infection (Preisz, 1909; Drysdale et al., 2005; Candela and Fouet, 2006; Richter et al., 2009).

Although the *B. cereus* group in general is phylogenetically heterogeneous, strains of the same species, as well as of different species, may be very closely related and phylogenetically intermixed, when employing genetic markers at the chromosomal level (Fig. 2.3; a more complete representation of the *B. cereus* group phylogeny can be found at http://mlstoslo.uio.no). Bacteria belonging to the *B. cereus* group generally exhibit complex genomes; different strains may carry plasmids in variable numbers (1–>12) and sizes (2–600 kb; frequently >80 kb) (reviewed in Kolstø et al., 2009), some of which are conjugative or mobilizable and can host a number of different IS elements. Typically, strains also contain bacteriophages which may be integrated in the chromosome as prophages or which may replicate as independent linear elements (Carlson et al., 1994a; Rasko et al., 2005; Verheust et al., 2005; Sozhamannan et al., 2006; Lapidus et al., 2008). The traditional species distinctions of *B. anthracis* and *B. thuringiensis* were largely based on their different pathogenic specificities towards vertebrates and insect larvae, respectively. Interestingly, however, the genes coding for the typical species-specific virulence properties of both *B. anthracis* and *B. thuringiensis* are plasmid-borne, a fact unknown at the time of species designation; in *B. thuringiensis* the crystal protein toxins (Cry or Cyt) causing pathogenicity to insects are almost exclusively encoded by genes present on plasmids of various sizes and often associated with IS

elements (Schnepf et al., 1998). Similarly, in *B. anthracis* two large plasmids, pXO1 (182 kb) and pXO2 (95 kb), are necessary for full *B. anthracis* virulence (Mock and Fouet, 2001; Passalacqua and Bergman, 2006). pXO1 carries the genes coding for the anthrax toxin components (*pag, lef,* and *cya*), all located within a 44.8 kb pathogenicity island (PAI; Okinaka et al., 1999b) which also encodes the transcriptional activator AtxA and the repressor PagR that regulate the expression of the toxin genes (Uchida et al., 1993; Dai et al., 1995; Mignot et al., 2003; Fouet and Mock, 2006). pXO2 encodes the other major *B. anthracis* virulence factor, the poly-γ-D-glutamic acid (polyglutamate) capsule, in a five-gene operon (*capBCADE*). Capsule expression is activated by the transcriptional regulators AcpA and AcpB, and the capsule operon and its regulator genes are all located in a 35 kb PAI (Pannucci et al., 2002b; Van der Auwera et al., 2005). Expression of *acpA* and *acpB* (located on pXO2) is under the control of AtxA (encoded on pXO1) (Fouet and Mock, 2006; Perego and Hoch, 2008); thus cross-talk occurs between the two virulence plasmids.

The phylogenetic intermixing of strains of different species, in particular *B. cereus* and *B. thuringiensis* isolates (Fig. 2.3; http://mlstoslo.uio.no), and the fact that the main phenotypical traits classically used to define each of the *B. cereus* group species are carried by plasmids, including the insect toxicity of *B. thuringiensis* and human pathogenicity of *B. anthracis*, have led to disputes regarding the species definitions within the group (Helgason et al., 2000b; Rasko et al., 2005). Hitherto, no species-specific property outside the plasmid-borne *cry* and *cyt* genes, has been identified for *B. thuringiensis*, and *B. thuringiensis* strains can carry the same chromosomally encoded virulence genes as *B. cereus*, including genes for enterotoxins, phospholipases, haemolysins, and proteases (Han et al., 2006; Scarano et al., 2009). These genes may in fact be important for the virulence of the bacterium, following its entry into the insect larvae haemocol (Fedhila et al., 2002, 2003, 2004, 2006). Thus, a *B. thuringiensis* strain that has lost the *cry*- or *cyt*-containing plasmids will be indistinguishable from *B. cereus* and will be identified as such. Therefore, although the current nomenclature is kept, largely based on the well-established differences observed in pathogenicity profiles of the two species towards insects, the two bacterial species are indistinguishable when a chromosomal phylogeny is reconstructed based on a sufficient number of isolates (http://mlstoslo.uio.no). *B. cereus* and *B. thuringiensis* have therefore been suggested to constitute one species in genetic terms (Helgason et al., 2000c).

Members of the *B. cereus* group are found in various habitats in the environment, including different types of soils, plant leaves, the rhizosphere, the intestinal tract of soil invertebrates, as well as man-made settings such as food production factories and hospital environments (Jensen et al., 2003), where they may constitute reservoirs for disease. The ubiquitous presence of several *B. cereus* group members in a great variety of natural habitats, combined with the ability to survive in nutrient-poor and otherwise hostile environments due to spore-forming abilities, contributes to their role as common polluter organisms (Drobniewski, 1993). The presence of *B. cereus* in these habitats allows the organism to easily spread to different foods, including milk and milk products (via the udders of grazing cows), as well as rice and other carbohydrate-rich foodstuffs. From these locations it may cause gastro-intestinal disease.

2.3.2 Bacillus mycoides, Bacillus pseudomycoides, B. weihenstephanensis, *and* "Bacillus cytotoxicus"

The three remaining species of the *B. cereus* group, *Bacillus mycoides, Bacillus pseudomycoides,* and *B. weihenstephanensis* have been studied less extensively. *B. mycoides* and *B. pseudomycoides* are characterized based on one morphological property – rhizoidal growth on solid medium. Phylogenetically, *B. mycoides* strains are widely distributed within the *B. cereus* group population, while *B. pseudomycoides* may appear to be limited to a remote cluster (Fig. 2.3). Only a very low number of *B. pseudomycoides* strains have been identified, however, and there is a need for more strains to be mapped by molecular phylogeny before firm conclusions about a possible clonal distribution pattern for this species can be reached. Although both *B. mycoides and B. pseudomycoides* are generally regarded as non-pathogenic, certain strains of *B. mycoides* have been linked to cases of food-borne disease (McIntyre et al., 2008).

B. weihenstephanensis is a psychrotolerant species in the *B. cereus* group and carries specific 16S rRNA and *cspA* (major cold-shock protein) signatures (Thorsen et al., 2006). Psychrotolerant *B. cereus* group members however do not necessarily belong to *B. weihenstephanensis*, as psychrotolerant strains of *B. cereus* also exist (Stenfors and Granum, 2001). *B. weihenstephanensis* may produce enterotoxins even at refrigerator temperatures (Baron et al., 2007), and some *B. weihenstephanensis* strains can produce emetic toxin (cereulide) (Thorsen et al., 2006), further emphasizing the food-borne intoxication potential of this species.

The food poisoning strain *B. cereus* NVH391-98 was originally isolated from cases of severe gastroenteritis linked to an outbreak in an elderly home in France, in which three people were killed (Lund et al., 2000). Molecular typing identified this strain as a remote member of the *B. cereus* group (Fagerlund et al., 2007), and following the discovery of two novel *B. cereus* group strains forming a phylogenetic cluster together with the NVH391-98 strain (Auger et al., 2008), the new species name, *B. cytotoxicus*, was proposed (Lapidus et al., 2008). The species designation however remains to be formally approved. These isolates are all thermotolerant, but the degree of CytK and Nhe enterotoxin production, as well as cytotoxicity, was variable between the strains in the cluster; while *B. cereus* NVH391-98 produced high levels of the CytK cytotoxin, *B. cereus* 883/00 produced little or no CytK and Nhe enterotoxin, and was not cytotoxic to Vero cells (Fagerlund et al., 2007).

2.4 Genome Sequencing of the *Bacillus cereus* Group

The first *B. cereus* group genome project, that of the *B. anthracis* Ames Porton model strain, was initiated in 2000 at the Institute for Genomic Research (TIGR) and published in 2003 (Read et al., 2003a). The bioterror events during the fall of 2001, involving the distribution of letters containing *B. anthracis* spores via the US Postal System, have however spurred the sequencing of a multitude of *B. cereus* group strains during the past decade. Following the sequencing of the bioterror attack strain *B. anthracis* Ames Florida (Read et al., 2002) and the first *B. cereus*

strain (ATCC 14579, type strain; Ivanova et al., 2003), 105 additional genomes have been or are being sequenced (Entrez genome project: http://www.ncbi.nlm.nih.gov/genomeprj; Genomes OnLine database: www.genomesonline.org), providing an unprecedented reservoir for doing comparative genomics. The genome sequences cover all six approved species within the group, in addition to the *B. cereus* NVH391-98 strain from the remote "*Bacillus cytotoxicus*" cluster. In one of the latest genome sequencing projects involving *B. cereus* group isolates, more than 50 strains have been sequenced to draft stage using next-generation sequencing technology. Strains were selected so as to provide the best possible coverage of the group phylogeny (Timothy D. Read, personal communication), as mapped by MLST (http://mlstoslo.uio.no). Among the 108 strains that have been or are being sequenced (per 20 May 2010; http://www.ncbi.nlm.nih.gov/genomeprj; www.genomesonline.org) (Kolstø et al., 2009), there are eight isolates that were linked to cases of food-borne disease or were isolated from foodstuffs (Table 2.1).

Table 2.1 Sequenced *B. cereus* group isolates linked to food-borne disease or isolated from contaminated food. Genome project and source information were obtained from the NCBI Entrez Genome Project database (http://www.ncbi.nlm.nih.gov/genomeprj)

Isolate/strain	Source	Genome publication
B. cereus ATCC 10987	Spoiled cheese, Canada, 1930	Rasko et al. (2004)
B. cereus NVH 0597/99	Isolated from spice mix, believed to be the cause of a food poisoning outbreak in Norway, 1999	Not published
B. cereus m1293	Isolated from cream cheese	Not published
B. cereus m1550	Isolated from uncooked chicken	Not published
B. cereus MM3	Isolated from food	Not published
B. cereus F4810/72 (AH187)	Emetic food poisoning outbreak, 1972 (emetic type strain)	Not published
B. cereus H3081.97	Emetic strain	Not published
B. cereus NVH391/98 ("*B. cytotoxicus*")	Outbreak of severe gastroenteritis, elderly home, France, 1998	Lapidus et al. (2008)

2.4.1 The B. cereus *Group from a Genomic Perspective*

Prior to 2000, only sporadic genome sequence data had been produced from *B. cereus* group organisms. The *B. anthracis* virulence plasmids, pXO1 and pXO2, were sequenced in 1999 (Okinaka et al., 1999a, b), while more than 100 kb of random genome sequence data was produced from *B. cereus* ATCC 10987 and ATCC 14579 (type strain) in the late 1990s (Økstad et al., 1999a; b). Today, the *B. cereus* group is one of the groups of closely related bacteria with the highest number of sequenced genomes. The first genomes sequenced from the group, those of *B. anthracis* Ames and *B. cereus* strains ATCC 14579 and ATCC 10987, were all closed and serve as reference genomes for later projects. Although the current

trend of sequencing genomes to high-quality draft format also applies to the current *B. cereus* group projects, altogether 17 genomes have now been closed, showing that chromosome sizes for *B. cereus* group members are in the range 5.2–5.4 Mb (with the exception of *B. cereus* NVH391–98 which is 4.1 Mb), confirming previous pulsed-field gel electrophoresis-based estimations (Carlson et al., 1992; Carlson and Kolstø, 1993; Carlson et al., 1996; Lovgren et al., 2002). Chromosomes have a GC content of around 35.3–35.4%, and strains typically carry a large number of rRNA operons (11 and 13 for *B. anthracis* Ames Porton and *B. cereus* ATCC 14579, respectively). A comparison of *B. anthracis* Ames to 19 *B. cereus* and *B. thuringiensis* isolates by comparative genome hybridization (CGH) revealed that the 2-Mb region surrounding the chromosomal replication terminus contained a significantly higher proportion of strain-specific genes and was clearly of higher plasticity than the rest of the chromosome (Read et al., 2003a), suggesting that gene mobility events are probably more frequent in the terminus region. This is in line with studies in other bacterial species, describing the region surrounding the replication terminus as a high-plasticity region (Suyama and Bork, 2001).

Prior to the sequencing of the first representatives of the *B. cereus* group members, many scientists in the field had viewed *B. anthracis* as more fundamentally different from the other species in the group, based on several phenotypic character differences such as β-lactamase sensitivity, lack of haemolytic activity, and sensitivity to γ-phage. Strikingly, however, comparison of *B. cereus* ATCC 14579 to the draft sequence of *B. anthracis* Florida (A2012), and of *B. anthracis* Ames Porton to the 19 *B. cereus* and *B. thuringiensis* strains by comparative genome hybridization (CGH), revealed that only four regions of the chromosome were unique to *B. anthracis* and that these regions corresponded to four prophages (Ivanova et al., 2003; Read et al., 2003a). Thus, no candidates for unique chromosomal genes of importance to *B. anthracis* virulence could be identified. Most of the toxicity specifically linked to anthrax disease thus seems to be linked to the pXO1 and pXO2 plasmids, although mechanisms of cross-talk between the two plasmids and between plasmids and chromosome seem to be of importance (Uchida et al., 1997; Mignot et al., 2003; Chitlaru et al., 2006).

In addition to the four prophages, one other principle difference between *B. anthracis* and the other *B. cereus* group members is the characteristic nonsense mutation in the global pleiotropic transcriptional regulator of extracellular virulence factors, PlcR, which renders the protein non-functional (Agaisse et al., 1999; Kolstø et al., 2009). In most *B. cereus* and *B. thuringiensis* strains, PlcR is active and important for expression of a range of chromosomally encoded virulence factors, including phospholipases, proteases, haemolysins, and enterotoxin. PlcR is however clearly not necessary for *B. anthracis* virulence (reviewed in Kolstø et al., 2009).

Given the fact that plasmid-borne factors are key elements for the toxicity of *B. thuringiensis* towards insect larvae and for the virulence of *B. anthracis* towards vertebrates, plasmid content and distribution has been a topic of considerable interest to the scientific community studying *B. cereus* group organisms. In general, *B. cereus* group strains are well known for having the potential for harbouring one or more, often several, plasmids in the cell at the same time (reviewed in Kolstø

et al., 2009). The discovery of pXO1 plasmid-like sequences in a large proportion of 19 *B. cereus* and *B. thuringiensis* strains tested for pXO1 gene markers by CGH (Read et al., 2003a) was at the time striking and indicated that plasmids with similarity to pXO1 could potentially exist in species other than *B. anthracis*. This was later confirmed by sequencing of *B. cereus* ATCC 10987 (Rasko et al., 2004) and by Panucci and co-workers, who screened a large number of *B. cereus* group strains for pXO1 gene markers by PCR (Pannucci et al., 2002a). Orthologs to the genes making up the pXO1 PAI containing the anthrax toxin genes and their regulators, as well as the *gerX* locus essential for germination within host macrophages, were however generally not present in the non-*B. anthracis* strains. pXO2-like sequences seemed to be occurring less frequently in *B. cereus* group species other than *B. anthracis* (Pannucci et al., 2002b; Read et al., 2003a).

Many chromosomal features have been identified as being common among *B. cereus* group isolates, unifying the various *B. cereus* group bacteria and emphasizing their relatedness; chromosomes are generally in the same size range for sequenced isolates and are to a large extent syntenic, with common orthologous genes being organized in a conserved order (Ivanova et al., 2003; Rasko et al., 2004). Also, a core gene set has been identified, counting 3,000 ± 200 genes (Lapidus et al., 2008) out of the in excess of 5,000 genes that make up a typical *B. cereus* group chromosome (excluding *B. cereus* NVH391-98, which has an unusually small genome; Lapidus et al., 2008). Furthermore, each strain characteristically has in the order of 400–800 strain-specific genes (Lapidus et al., 2008), which may potentially be involved in niche adaptation processes, and which are contributing to a fairly large *B. cereus* group pan-genome (20–25,000 genes estimated in Lapidus et al., 2008). Another unifying feature for the *B. cereus* group bacteria is the presence of several ubiquitous interspersed repeat elements in the size range 100–400 bp, many of which seem to be unique to the group (Tourasse et al., 2006). These repeats, named *bcr1–bcr18*, are non-protein coding, but some seem to be expressed at the RNA level and are predicted to constitute non-autonomous mobile elements belonging to the class 'miniature inverted-repeat transposable elements' (MITEs) (Økstad et al., 2004; Tourasse et al., 2006; Klevan et al., 2007). Although no specific function has yet been assigned to any of the *bcr* repeats, MITEs are known in other bacteria to be involved in a variety of processes, including regulation of transcription and mRNA degradation, DNA methylation, integration host factor (IHF) binding, and creation of novel gene loci, to mention some (Delihas, 2008). Phylogenetic studies of the *B. cereus* group had already prior to the genomics era shown that closely related strains of *B. cereus* and *B. anthracis* exist, as do close strains of *B. cereus* and *B. thuringiensis*, and that the two latter species are intermixed phylogenetically based on chromosomal markers (Carlson et al., 1994b; Helgason et al., 2000c), leading to the suggestion that *B. cereus* and *B. thuringiensis* belong to the same species in purely genetic terms (Helgason et al., 2000c). Also, the idea has been presented that *B. anthracis* is in reality an over-sampled *B. cereus* (Rasko et al., 2005), which is highly monomorphic and genetically constrained. For the moment, however, species designations are kept, given the fundamental differences in pathogenicity profiles between the different species in the group.

2.4.2 *Phages of the* B. cereus *Group*

For several important pathogenic bacteria, including the ethiological agents of cholera, diphtheria, and enterohaemorrhagic diarrhoeas, bacteriophages are important vectors for the transfer of virulence factors. In general, phages constitute important sources of gene flow in bacteria. *B. cereus* group bacteria can host a range of phages; however, carriage of specific toxin genes has not yet been observed in these elements; while *B. anthracis* ubiquitously carries the four unique prophages (lambda01–lambda04) in its chromosome, *B. cereus* ATCC 10987 carries three chromosomal prophages, while the *B. cereus* type strain, ATCC 14579, has three prophages integrated in its chromosome and in addition carries a linear extrachromosomal phage-like element, pBClin15 (Carlson et al., 1992; Ivanova et al., 2003). Similarly, *B. thuringiensis* 97–27 and *B. cereus* E33L (formerly known as zebra killer, ZK) carry a variety of phages in their chromosome and plasmid(s) (Han et al., 2006). The phages generally exhibit a host range which is limited to the species from which they have been isolated. However, *B. cereus* strains that are very similar to *B. anthracis*, such as *B. cereus* ATCC 4342, may be susceptible to *B. anthracis* phages, such as *Gamma* and *Cherry* (reviewed in Rasko et al., 2005).

2.5 Genome Dynamics Related to Food-Borne Disease

B. cereus can cause food-borne disease by two mechanisms – either by intoxication, following the ingestion of foodstuffs containing pre-formed emetic toxin (cereulide) produced by an emetic strain contaminating a food matrix environment, or by gastro-intestinal infection of vegetative *B. cereus* strains thought to form one or more enterotoxins in the intestine, following the ingestion of spores or viable cells with food or milk products (reviewed in Stenfors Arnesen et al., 2008).

2.5.1 *Enterotoxins*

The identity of the proteins conferring the enterotoxic activity inherent to *B. cereus* is still controversial. The three cytotoxins haemolysin BL (Hbl, three-component toxin), non-haemolytic enterotoxin (Nhe, three-component toxin), and cytotoxin K (CytK, single-component toxin of the β-barrel pore-forming toxin family) are however generally considered to be the causes of the *B. cereus* diarrhoeal syndrome (Beecher and Macmillan, 1991; Lund and Granum, 1996; Lund et al., 2000) and are specified by genes carried on the chromosome. In addition to the well-established Hbl, Nhe, and CytK cytotoxins, several additional candidate proteins have been suggested as potential contributors to the enterotoxic activity of *B. cereus*, including haemolysin II and haemolysin III (Baida and Kuzmin, 1995; Baida et al., 1999), EntFM (Shinagawa et al., 1991; Asano et al., 1997; Tran et al., 2010), phospholipases C (Kuppe et al., 1989), cereolysin O (Kreft et al., 1983), and InhA2 (Fedhila

et al., 2003). Again, these proteins are chromosomally encoded, and the synthesis of several of them is, like Hbl, Nhe, and CytK, subject to regulation by PlcR (Gohar et al., 2008). Most likely, the toxins can act synergistically to cause *B. cereus* food-borne disease (Stenfors Arnesen et al., 2008).

Nhe, originally characterized following a large food poisoning outbreak in Norway in 1995 (Lund and Granum, 1996), is the most commonly found enterotoxin gene complex and is probably ubiquitous in *B. cereus* group bacteria (reviewed in Stenfors Arnesen et al., 2008). The complex, encoded by the *nheA, nheB,* and *nheC* genes (Granum et al., 1999), is, with one recently discovered exception, encoded chromosomally. In *B. weihenstephanensis* KBAB4, however, a second copy of the *nhe* locus is hosted on a 400 kb plasmid, pBWB401 (Lapidus et al., 2008). The identity of the *nhe* locus between strains is, with the exception of the NVH391-98 strain which is phylogenetically remote, generally around 90%, but is approaching 100% within clonal clusters such as the *B. anthracis* cluster and the emetic clusters. The plasmid-borne *nhe* locus in *B. weihenstephanensis* KBAB4 is however only around 58% identical to the chromosomal *nhe* copies. Whether this locus gives rise to a functional enterotoxin is at present unknown. The maximal cytotoxic activity of Nhe towards Vero cells was obtained when the ratio between the Nhe components was 10:10:1 (NheA, NheB, NheC; Lindback et al., 2004), and the enterotoxic activity of Nhe has recently been explained (Fagerlund et al., 2008); exposure of plasma membrane to Nhe leads to rapid membrane lysis, and Nhe has been shown to form pores in lipid bilayers, leading to colloidosmotic lysis. Indeed, Nhe has more recently been shown, in spite of its name, to exhibit haemolytic activity towards erythrocytes from several mammalian species (Fagerlund et al., 2008).

The Hbl complex constitutes another pore-forming enterotoxin in the *B. cereus* group and exhibits haemolytic activity towards erythrocytes from several animal species. It is encoded by the *hblC, hblD,* and *hblA* genes, encoding components L2, L1, and B, respectively. In addition to the three structural genes, the most common variant of the *hbl* locus carries a fourth gene, *hblB*, downstream of *hblCDA*. *hblB*, which has probably originated by duplication of a large part of *hblA* and fusion to an ORF in the 3′ end (Økstad et al., 1999b), is however most probably a pseudogene, since it has not been shown to be transcribed to a detectable level and the *hblCDA* transcript seems to terminate within *hblB* (Lindback et al., 1999). Other variants of the *hbl* locus do however exist: in *B. cereus* 03BB108 and *B. weihenstephanensis* KBAB4 an *hbl* locus exists which consists of the *hblCDA* genes only. Like *B. cereus* MGBC145 (Beecher and Wong, 2000), the 03BB108 strain also carries a second *hbl* locus, which in the latter strain is of the *hblCDA* type. The *hbl* locus is less frequently present in *B. cereus* group strains compared to *nhe* and was identified in approximately 60% of strains in a PCR screening procedure (Pruss et al., 1999).

The *hblCDAB* operon is located on the chromosome, in a conserved location between strains, and is part of a 17.7 kb genome insertion bordered on one side of a degenerate IS*Rso11* transposase fragment which has been suggested to have been acquired as a mobile element (Han et al., 2006). The insertion also contains

genes encoding germination proteins and a transcriptional regulator (*trrA*) and putative histidine kinase that could have the potential for forming a two-component system (Økstad et al., 1999b). In contrast, the *hblCDA* operon seems to exhibit a less conserved genomic localization between strains; in *B. weihenstephanensis* KBAB4 the locus is chromosomally encoded and is flanked by genes encoding a putative β-lactamase and an S-layer domain protein, respectively. In the *B. cereus* 03BB108 draft genome sequence (GenBank entry: ABDM00000000), however, the corresponding *hblCDA* genes are flanked by sequences with similarity to the *B. anthracis* pXO1 plasmid, possibly indicating that the 03BB108 *hblCDA* genes are plasmid-borne (reviewed in Stenfors Arnesen et al., 2008).

The Hbl and Nhe proteins are related in sequence and have probably arisen by several gene duplication events from a common ancestor locus. The crystal structure of HblB has been solved and shows high structural similarity to ClyA (also known as HlyE or SheA), a pore-forming cytolysin from *Escherichia coli, Salmonella enterica* serovars Typhi and Paratyphi, and *Shigella flexneri* (Oscarsson et al., 1996; Wallace et al., 2000; Oscarsson et al., 2002). The HblB structure consists of five α-helix bundles wrapped around each other in a left-handed supercoil and a hydrophobic β-hairpin flanked by two short α-helices (PDB entry 2nrj; Fagerlund et al., 2008). Interestingly, NheB and NheC exhibit sufficient sequence similarity to HblB to allow modelling of their structures based on the HblB crystal structure, and although the toxin components exhibit a very limited primary sequence identity, their conserved structure suggests that the Hbl/Nhe family and the ClyA family constitute a new superfamily of toxins (Fagerlund et al., 2008).

Although much knowledge regarding the structure, function, and genetic organization of the Nhe and Hbl enterotoxins has accumulated over the past two decades, still much remains to be discovered. Any potential effect of the heterogeneity in genome organization and duplication of the *nhe* and *hbl* loci in different *B. cereus* group strains remains to be solved. Furthermore, no host cell receptor has yet been identified neither for Hbl nor for Nhe; while all three Hbl components can bind to the surface of erythrocytes (Beecher and Wong, 1997), out of the three Nhe components only NheB and NheC has been shown to exhibit surface binding to Vero cells (Lindback et al., 2004; Lindbäck et al., 2010). Even though both Nhe and Hbl appear to constitute tri-partite pore-forming toxins, details of the molecular interaction of the various protein components of each complex are yet to be resolved, as is the degree to which oligomerization occurs during formation of the transmembrane pore in the plasma membrane of target cells (reviewed in Stenfors Arnesen et al., 2008).

CytK is a 34 kDa single-component protein toxin of the β-barrel pore-forming toxin family. It was first identified following a gastroenteritis outbreak in a French nursing home in 1998, in which *B. cereus* NVH391-98 ("*B. cytotoxicus*") was, as already mentioned, identified as the agent causing the disease. In the course of the outbreak, several patients presented with bloody diarrhoea, and three elderly people died (Lund et al., 2000). CytK was originally identified as the prime virulence factor, given its necrotic, haemolytic, and enterotoxic effects, and since no Hbl or Nhe was apparently present in the NVH391-98 strain (Lund et al., 2000). Later, however, the *nhe* genes have been identified in the *B. cereus* NVH391-98 strain,

although with a lower sequence identity to Nhe from other *B. cereus* group strains (Fagerlund et al., 2007). CytK was originally described to be divided into two gene families in different *B. cereus* group isolates, CytK-1 and CytK-2, where CytK-1 corresponded to the variant identified in *B. cereus* NVH391-98, which was thought to represent a particularly toxic variant of the protein. Later the sequence divergence of the CytK protein (CytK-1) from this strain has, as is the case for Nhe, instead been shown to reflect the general phylogenetic divergence of this strain from the rest of the *B. cereus* group. The potent cytotoxicity of the *B. cereus* NVH391-98 strain is probably rather due to an exceptionally high CytK expression level in this isolate (Brillard and Lereclus, 2004).

It is important to note that the genes coding for the enterotoxin components and degradative enzymes are often found in strains of several species within the *B. cereus* group, *B. thuringiensis* and *B. anthracis* included, suggesting that *B. cereus* group organisms in general may have the potential to be pathogenic (Read et al., 2003b; Han et al., 2006; Hendriksen et al., 2006; McIntyre et al., 2008; Stenfors Arnesen et al., 2008). Using the term "non-pathogenic strain" for environmental isolates of *B. cereus* that have not been linked to disease therefore does not seem appropriate, as it is impossible to know whether these isolates could cause infection in man, given the right dose and setting. *B. anthracis* is however different from the other species in the group, in carrying a nonsense mutation in PlcR which makes the protein non-functional (Agaisse et al., 1999). Therefore, *B. anthracis* encodes a very limited extracellular proteome compared to the other *B. cereus* group species (Gohar et al., 2005), and the chromosomal virulence factors belonging to the PlcR regulon (Gohar et al., 2008) are not synthesized, enterotoxins included. *B. anthracis* virulence was not increased in a mouse infection model (intranasal) by transfer of a functional *plcR* gene back into host cells, indicating that the pathogenicity of *B. anthracis* is not dependent on the chromosomal virulence factors expressed as part of the PlcR regulon in other *B. cereus* group bacteria.

2.5.2 Emetic toxin

The emetic toxin, cereulide, is a small (1.2. kDa) non-ribosomally synthesized dodecadepsipeptide, produced by a non-ribosomal peptide synthetase system (*ces*) found in emetic *B. cereus* strains (Ehling-Schulz et al., 2005a). The toxin is heat-stable, acid and protease resistant and can be pre-formed in foodstuffs contaminated with an emetic strain of *B. cereus*, leading to emesis within 0.5–6 h post ingestion (reviewed in Stenfors Arnesen et al., 2008), and occasionally more severe intoxications such as liver failure and death (Mahler et al., 1997; Dierick et al., 2005). Expression of the toxin is known to be induced towards the end of logarithmic growth, reaching a maximum during the early stationary growth phase (reviewed in Stenfors Arnesen et al., 2008). Although the mechanisms regulating emetic toxin production are not well characterized, its expression is affected by

factors like oxygen, pH, and temperature and is regulated by the transitional state regulator AbrB, but not by PlcR (Lucking et al., 2009). Among the sequenced strains from the *B. cereus* group are two emetic isolates, including the reference emetic strain *B. cereus* F4810/72, isolated in 1972 from human vomit following an emetic food poisoning outbreak.

The peptide synthetase responsible for cereulide synthesis is encoded by a 24 kb gene cluster (*ces*) on a 208 kb plasmid, pCER270, with similarity to *B. anthracis* pXO1 and other pXO1-like plasmids (Hoton et al., 2005; Ehling-Schulz et al., 2006; Rasko et al., 2007). The cluster is comprised of seven genes, including typical non-ribosomal peptide synthetase (NRPS) genes such as a phosphopantetheinyl transferase and genes encoding modules for the activation and incorporation of monomers in the growing peptide chain. Also, a potential hydrolase and an ABC transporter are encoded by the *ces* cluster. Interestingly, the pPER270 plasmid is similar to pBC10987 from *B. cereus* ATCC 10987, isolated from spoiled cheese in Canada in 1930 (Herron, 1930; Rasko et al., 2004), and pPER272, a plasmid hosted by *B. cereus* strains AH820 and AH818 isolated from the periodontal pocket and root canal, respectively, in patients with periodontal disease (Helgason et al., 2000a; Rasko et al., 2007). The pXO1 pathogenicity island encoding the anthrax toxin genes and associated regulators is however missing both in pCER270 and in pPER272 and is replaced by a 77 kb insertion in pCER270 which is bordered by transposase and resolvase genes that could potentially have been involved in the insertion of the region (Helgason et al., 2000a; Rasko et al., 2007).

Cereulide production has been mapped to two phylogenetically separated clonal clusters within the *B. cereus* group population, mostly consisting of *B. cereus* isolates (Ehling-Schulz et al., 2005b; Vassileva et al., 2007). However, specific *B. weihenstephanensis* strains have also been found that are capable of forming emetic toxin, even at temperatures as low as 8°C (Thorsen et al., 2006). Given that the genetic determinants of emetic toxin production are plasmid-borne, this implies that acquisition of a plasmid encoding emetic toxin production has probably occurred more than once during evolution and that pCER270 may be subject to lateral transfer between strains.

2.6 Potential for Causing Food-Borne Disease – A General Feature of the *B. cereus* Group?

B. thuringiensis is an insect pathogen; however, isolates may also have the potential to act as opportunistic pathogens in humans and animals, possibly causing tissue necrosis, pulmonary infections, or food poisoning (Hernandez et al., 1999; Ghelardi et al., 2007; McIntyre et al., 2008). In line with this, the sequencing of *B. thuringiensis* strains Al-Hakam, 97–27 (*var.* konkukian) and ATCC 35646 (*var.* israelensis), has shown that *B. thuringiensis* strains can carry the same chromosomal virulence factors that are typical to *B. cereus* (i.e. enterotoxins, haemolysins, phospholipases,

proteases (Han et al., 2006), as can other species in the *B. cereus* group. With what frequency human infections by *B. thuringiensis* actually occur is however at present unknown, since *B. cereus* group strains isolated from human infections (food poisoning included) are most often not tested for the presence of crystal toxin genes or for the production of such toxins. Also, it is plausible that *B. thuringiensis*, during an infection in man, may lose the plasmid encoding its entomopathogenic properties, as these plasmids are often less stably maintained at 37°C than at lower growth temperatures. This would make the isolate practically indistinguishable from *B. cereus*. In fact, the ability to cause the *B. cereus* diarrhoeal syndrome may be an inherent feature of all *B. cereus* group species carrying a functional PlcR regulator gene. Indeed, *B. thuringiensis* was identified in food or clinical samples from four outbreaks, and *B. mycoides* was identified in one outbreak, sampled in British Columbia, Canada, in the period 1991–2005 (McIntyre et al., 2008). Seemingly, contrary to the emetic isolates which group into two clusters phylogenetically, isolates causing the diarrhoeal syndrome are located throughout the *B. cereus* group phylogeny and may frequently share identical molecular typing data (based on chromosomal markers) with environmental strains isolated from soil or plants (Fig. 2.3; Tourasse et al., in press; http://mlstoslo.uio.no). Held together with the fact that genes encoding the non-haemolytic enterotoxin complex (Nhe) are found ubiquitously among *B. cereus* group organisms and that *B. cereus* group species other than *B. cereus sensu stricto* have been characterized as the cause of gastroenteritis (McIntyre et al., 2008), this emphasizes the opportunistic nature of *B. cereus* group bacteria and the potential for other *B. cereus* group species to cause food-borne disease. Also, notably, it is not merely the presence or the absence of toxin and other virulence genes that determines toxicity. Gene expression levels can clearly be highly variable between isolates, exemplified by *B. cereus* NVH391-98, which as mentioned is highly toxic, and has been shown to synthesize higher levels of the *cytK* mRNA compared to other *B. cereus* group strains (Brillard and Lereclus, 2004).

2.7 Future Perspectives – Importance of Plasmids to the Biology of *B. cereus* Group Bacteria

Plasmids are key elements in the encoding of several of the phenotypes characteristic of each species in the *B. cereus* group, including *B. thuringiensis* entomopathogenicity and *B. anthracis* virulence. With the discovery of *B. cereus* strains which are able to cause severe disease symptoms resembling those of anthrax and encode the anthrax toxins from variants of the pXO1 plasmid as well as producing a capsule, the principle differences separating these species are getting increasingly blurry. Such strains include *B. cereus* G9241, which was isolated from the sputum and blood of a patient with life-threatening pneumonia, and carries a 191 kb plasmid (pBCX01) with 99.6% identity to pXO1 in regions shared between the two plasmids, as well as a second 218 kb plasmid, pBC218, which has a gene cluster encoding a polysaccharide capsule (Hoffmaster et al., 2004). Perhaps even more

striking, during recent years *B. cereus* strains causing anthrax-like disease in great apes have been isolated in Côte d'Ivoire and Cameroon (Leendertz et al., 2004; Klee et al., 2006; Leendertz et al., 2006). These strains (*B. cereus* CI and CA, respectively) carry the *B. anthracis* toxin and capsule genes on plasmids of sizes corresponding to pXO1 and pXO2 and have a frameshift mutation in the *plcR* gene, however in a different position than the nonsense mutation universally found in *B. anthracis* strains (Klee et al., 2006). The mutation would produce a PlcR protein with a modified C-terminus, a part of the protein known to be involved in the specific interaction with PapR, its cognate peptide pheromone, which is necessary for activation of the PlcR regulon (Slamti and Lereclus, 2002, 2005; Bouillaut et al., 2008). It is therefore conceivable that as is the case in *B. anthracis*, expression of the PlcR regulon could be abolished in the CI and CA strains (Klee et al., 2006), and functional experiments performed in the CI strain point in this direction (Klee et al., 2010). Further studies to investigate whether other *B. cereus* strains capable of synthesizing anthrax toxins and capsule exist and may occur more frequently than previously known would seem justified.

By comparative analysis to *B. cereus* strains, it is known that very few genes are specific to *B. anthracis* (Read et al., 2003a). Taken together, it is thus apparent that what principally separates *B. anthracis* from *B. cereus* is the following: (1) being located to the *B. anthracis* cluster phylogenetically, (2) the presence of the four unique prophages (lambda01–lambda04) in the *B. anthracis* chromosome, and (3) the unique nonsense mutation in *plcR*, which is found only in *B. anthracis* (reviewed in Kolstø et al., 2009). *B. cereus* strains may harbour a range of plasmids of various sizes and families, many of which are poorly characterized, as well as the pXO1- and pXO2-like plasmids carrying anthrax toxin and capsule synthesis genes mentioned above, which was previously thought to be a specific and unique feature to *B. anthracis*. There is to date, a lack of knowledge of what features *B. cereus* and *B. thuringiensis* plasmids encode, such as novel putative virulence genes or genes potentially involved in the adaptation to specific niches. *B. cereus* plasmids can be mobile, or capable of mobilizing other plasmids in the group, e.g. pXO14 which is efficient in mobilizing pXO1 and pXO2 (Reddy et al., 1987). It is however not known to what extent horizontal transfer of pXO1 and pXO2 plasmids may occur in the *B. cereus* population. Given the fact that newly emerging pathogens (CI/CA strains) seem to arise from transfer of such plasmids, that *B. cereus* strains encoding alternative capsules (G9241) and possibly other virulence traits from plasmid elements exist, and that knowledge of plasmid diversity in the group is rather limiting, a systematic sequencing approach targeting *B. cereus* group plasmids seems warranted. Finally, it should be kept in mind that a considerable fraction of the annotated genes in *B. cereus* group genomes are still categorized as hypothetical or conserved hypothetical genes of unknown function and that these genes are represented both on the chromosome and on the plasmids. What contributions proteins encoded by these genes make to *B. cereus* group biology is still enigmatic.

Acknowledgements The authors wish to thank Dr. Nicolas Tourasse for performing the phylogenetic analyses for Figs. 2.1 and 2.3.

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http://www.springer.com/978-1-4419-7685-7

Genomics of Foodborne Bacterial Pathogens
Wiedmann, M.; Zhang, W. (Eds.)
2011, XII, 524 p., Hardcover
ISBN: 978-1-4419-7685-7