Occurrence and pathogenic potential of *Bacillus cereus* group bacteria in a sandy loam

Niels Bohse Hendriksen*, Bjarne Munk Hansen and Jens Efsen Johansen
*Department of Environmental Chemistry and Microbiology, National Environmental Research Institute, Frederiksborgvej 399, P.O. Box 358, DK-4000, Roskilde, Denmark; *Author for correspondence (e-mail: nbh@dmu.dk; phone: +45-46301372)*

Abstract

The major part (94%) of the *Bacillus cereus*-like isolates from a Danish sandy loam are psychrotolerant *Bacillus weihenstephanensis* according to their ability to grow at temperatures below 7 °C and/or two PCR-based methods, while the remaining 6% are *B. cereus*. The *Bacillus mycoides*-like isolates could also be divided into psychrotolerant and mesophilic isolates. The psychrotolerant isolates of *B. mycoides* could be discriminated from the mesophilic by the two PCR-based methods used to characterize *B. weihenstephanensis*. It is likely that the mesophilic *B. mycoides* strains are synonymous with *Bacillus pseudomycoides*, while psychrotolerant *B. weihenstephanensis*, like *B. mycoides*, are *B. mycoides* senso stricto. *B. cereus* is known to produce a number of factors, which are involved in its ability to cause gastrointestinal and somatic diseases. All the *B. cereus*-like and *B. mycoides* like isolates from the sandy loam were investigated by PCR for the presence of 12 genes encoding toxins. Genes for the enterotoxins (hemolysin BL and nonhemolytic enterotoxin) and the two of the enzymes (cereolysin AB) were present in the major part of the isolates, while genes for phospolipase C and hemolysin III were present in fewer isolates, especially among *B. mycoides* like isolates. Genes for cytotoxin K and the hemolysin II were only present in isolates affiliated to *B. cereus*. Most of the mesophilic *B. mycoides* isolates did not possess the genes for the nonhemolytic enterotoxin and the cereolysin AB. The presence of multiple genes coding for virulence factors in all the isolates from the *B. cereus* group suggests that all the isolates from the sandy loam are potential pathogens.

Introduction

The *Bacillus cereus* group bacteria are ubiquitous organisms commonly occurring in soil, feed and food. They encompass at least four species: *Bacillus cereus*, *Bacillus anthracis*, *Bacillus mycoides* and *Bacillus thuringiensis*. *B. cereus* is motile, hemolytic and produces lecithinase. It causes food-borne gastrointestinal and somatic diseases and is known to be an important food-spoiling organism. *B. anthracis* is non-motile, non-hemolytic and a weak lecithinase producer. It causes anthrax and can be used as a biological weapon. *B. thuringiensis* produces parasporal crystalline bodies containing plasmid encoded δ-endotoxins and is used for microbiological control of insect larvae. *B. mycoides* is non-motile and has a rhizoid growth on agar plates. Recently, two new species, *Bacillus*

pseudomycoides, which has a specific fatty acid composition, and a psychrotolerant species, *Bacillus weihenstephanensis*, have been described (Lechner et al. 1998; Nakamura 1998). Two PCR-based methods have been developed to discriminate mesophilic (*B. cereus*) isolates from psychrotolerant (*B. weihenstephanensis*) (Francis et al. 1998; von Stetten et al. 1998). The first is based on the amplification of a segment of a cold-shock protein gene *cspA* in psychrotolerant isolates. The second method takes advantage of specific sequence differences between psychrotolerant and mesophilic isolates in the 16S rDNA gene sequence. The taxonomy of the *B. cereus* group is controversial and it has been suggested that the closely related species all should be grouped as members of one species (Helgason et al. 2000).

Bacillus cereus is known to produce a number of toxins, which are involved in its ability to cause gastro-intestinal and somatic diseases. These factors include at least one emetic toxin, two enterotoxins, one cytotoxin, two hemolysins and three enzymes involved in the degradation of phospholipids. The emetic toxin is a cyclic dodecadepsipeptide named cereulide (Agata et al. 1995a), produced by non-ribosomal protein synthetases (Ehling-Schulz et al. 2005). The two enterotoxins, hemolysin BL (HBL) and nonhemolytic enterotoxin (NHE), are both three protein components complexes. HBL contains a binding component B, and two lytic components L_1 and L_2 encoded by *hblA*, *hblC* and *hblD* respectively (Heinrichs et al. 1993; Ryan et al. 1997). NHE also contains two lytic element NheA and NheB, similar to L_2 and L_1, encoded by *nheA* and *nheB*, and an unknown protein (encoded by *nheC*), that probably encodes for a protein similar to the B protein of HBL (Granum et al. 1999). The cytotoxin K is similar to the *β*-toxin of *Clostridium perfringens*, and is encoded by the sequenced gene *cytK* (Lund et al. 2000). The two hemolysins, hemolysin II and III, are single gene products encoded by *hlyII* and *hlyIII*, respectively (Baida and Kuzmin 1995; Baida et al. 1999). Hemolysin II is a structural and functional homolog of the pore-forming staphylococcal α-hemolysin (Miles et al. 2002). The phospholipases are a phosphatidylinositol-specific phospholipase C, a phosphatidylcholine-preferring phospholipase C and a sphingomyelinase (Kuppe

et al. 1989; Lechner et al. 1989). The two last mentioned enzymes constitute a functional cytolytic determinant termed cereolysin AB (Gilmore et al. 1989). The genes (*piplc, pcplc* and *sph*) encoding these three phospholipases have been cloned and sequenced (Kuppe et al. 1989; Lechner et al. 1989). Further two enterotoxins, enterotoxin T (Agata et al. 1995b) and enterotoxin FM (Asano et al. 1997) have been reported. However, there is no evidence that they can cause food-borne illness (Granum 2001) and enterotoxin T is considered a cloning artifact (Hansen et al. 2003).

The ability to produce the emetic toxin is restricted to a few serotypes of *B. cereus* notably serotype 1, which is unable to degrade starch (Ehling-Schulz et al. 2004). Almost all tested *B. cereus* isolates possess the genes for NHE (Hansen and Hendriksen 2001) and they have also been identified in most *B. thuringiensis, B. weihenstephanensis* and *B. anthracis* isolates tested (Hansen and Hendriksen 2001; Stenfors et al. 2002)). About 60% of the tested *B. cereus* isolates contain genes for HBL, and at least one of the genes of this operon has been identified in *B. thuringiensis, B. mycoides, B. pseudomycoides, B. weihenstephanensis* and *B. anthracis* isolates (Ryan et al. 1997; Pruss et al. 1999b; Hansen and Hendriksen 2001). The hemolysin II has been shown to be present in about 30% of the *B. cereus* isolates analyzed and 90% of the *B. thuringiensis* isolates, while the hemolysin III gene has been identified in a few *B. cereus* and *B. thuringiensis* isolates (Budarina et al. 1994; Hansen and Hendriksen 1998). The phospholipases seem to be widely distributed within the entire *B. cereus* group. The gene for the sphingomyelinase has been identified in all *B. cereus, B. thuringiensis, B. mycoides* and *B. anthracis* isolates investigated (Hsieh et al. 1999), and the two other phospholipases have been identified in all *B. cereus* and *B. thuringiensis* isolates investigated (Damgaard et al. 1996; Hansen and Hendriksen 1998).

The objectives of this study were to (i) to identify, enumerate and characterize *B. cereus* group bacteria in a sandy loam; (ii) to discriminate between mesophilic and psychrotolerant isolates; and (iii) to detect by PCR the *hblA, hblC* and *hblD* genes of the HBL complex; the *nheA,*

nheB and nheC genes of the NHE complex; and the *cytK, hlyII, hlyIII, piplc, pcplc* and *sph* genes in isolates from this soil.

Material and methods

Six surface soil samples (0–5 cm depth, diameter 2.1 cm) were taken 26 November 2001 within one square meter from an organic grown curly kale field (*Brasica olearacea acephala*) at Møn, Teglværksvej 50, Stege, Denmark. The field is a sandy loam (pH 7.0). Each of the soil samples was gently mixed in a plastic bag by hand. To a 2.5 g sub-sample of the mixed soil was added 25 ml demineralized water. The soil samples were shaken for 5 min by a multi-wrist shaker (Lab-line, speed 5). Ten ml of the suspension was afterwards heat-treated in a water bath (35 min at 65 °C). Ten-fold serial dilutions of the suspension were plated on T3 sporulation agar (Travers et al. 1987) and incubated for 20–24 h at 30 °C. Colonies having a rugose, ice-crystal like appearance and a diameter >1 mm was counted as *B. cereus*-like colonies. Distinctive rhizoid colonies were counted as *B. mycoides*-like. A total of 409 *B. cereus* like and 449 *B. mycoides* like colonies were counted and isolated and subcultured on T3-agar. Among these isolates 96 of each type were randomly selected for further characterization.

The isolates were examined for their ability to produce parasporal inclusion bodies (crystals) in the sporangium by phase-contrast microscopy after growth to sporulation on T3-agar for three days. The ability to hydrolyze starch and lecithin was studied on starch agar (Farrar and Reboli 1991) and *B. cereus* selective agar base supplemented with Egg Yolk Emulsion (Oxoid), respectively. Hemolysis was studied on blood agar base (Oxoid) supplemented with defibrinated sheep blood. Motility of the cells was examined by the method of Harmon (1982). Growth at 6° and 42 °C was studied on T3-agar plates in duplicate. The plates were incubated at the two temperatures for 28 and 4 days, respectively. The plates were inspected regularly for growth. Isolates showing no growth at the selected temperature were checked for their ability to grow at 30 °C.

For DNA preparation, bacteria were plated on Luria-Bertani (LB) agar and incubated overnight at 30 °C. An amount of bacteria corresponding to

a colony 1–2 mm in diameter was transferred to 200 µl of Tris–EDTA buffer. Bacteria were lysed by incubation at 102 °C for 10 min, and debris was removed by centrifugation at $15,000 \times g$ for 3 min. The DNA containing supernatant was transferred to a new microfuge tube and stored at 4 °C. The primer sets used in this study are shown in Table 1. PCR detection of *hblA, hblC, hblD, nheA, nheB nheC, sph, piplc, pcplc, hlyI* and *hlyIII* was performed essentially as described elsewhere (Hansen et al. 1998). One microliter of DNA extract was amplified with 0.5 U of *Taq* polymerase (Roche, Mannheim, Germany) in a 25-µl reaction mixture using 30 cycles of denaturation at 94 °C for 15 s, annealing at 55 °C for 45 s, and extinction at 72 °C for 2 min. For *cytK* the denaturation temperature was raised to 95 °C. The multiplex PCR procedures for the affiliation of the bacteria to the *B. cereus*-group and as being psychrotolerant or mesophilic, for the detection of the genes for cold-shock proteins and for the anthrax-plasmids pXO1 and pXO2 was performed as described elsewhere (Cheun et al. 2001; Francis et al. 1998; Hansen et al. 2001; von Stetten et al. 1998)). The RAPD-PCR with the primers OPA9 was performed as described by Hansen et al. (1998) PCR analysis of the 16S-23S rRNA gene (rDNA) spacer region with the L1-G1 primer set (Willumsen et al. 2005) was used as a control of DNA quality and for the procedure. PCR products were analyzed by 1.5% agarose gel electrophoresis, using MW VI (Roche) as a molecular weight marker.

Results

Bacillus cereus- and *B. mycoides*-like colony forming units (CFU) constituted $1.16 \pm 0.16 \times 10^5/$ g and $1.79 \pm 0.33 \times 10^5/g$, respectively in the soil. The affiliation of 192 randomly chosen *B. cereus*-like and *B. mycoides*-like isolates (96 each) to the *B. cereus* group by colony-morphology was confirmed by two independent group specific PCR-assays based on the 16S rDNA-23S rDNA spacer region and the 16S rDNA genes. Furthermore, these isolates were analyzed for production of parasporal crystalline bodies by microscopy and for the occurrence of the *B. anthracis* specific plasmids pXO1 and pXO2 by PCR. No isolates produced crystalline bodies nor harbored pXO1or

Table 1. Nucleotide sequences and affiliations of primers used in this study.

Gene (phenotype)	Primer	Primer sequences (5′–3′)*	Position (5′–3′)	Product size	Sequence reference (accession number)	Primer reference
hblA (B)	HBLA 1 HBLA 2	GTGCAGATGTTGATGCCGAT ATGCCACTGCGTGGACATAT	671–690 990–971	320	Heinrichs et al. (1993) (L20441)	Hansen and Hendriksen (2001)
hblC (L2)	L2A L2B	AATGGTCATCGGAACTCTAT CTCGCTGTTCTGCTGTTAAT	1448–1467 2197–2178	750	Ryan et al. (1997) (U63928)	Hansen and Hendriksen (2001)
hblD (L1)	L1A L1B	AATCAAGAGAGCTGTCACGAAT CACCAATTGACCATGCTAAT	2854–2873 3283–3264	430	Ryan et al. (1997) (U63928)	Hansen and Hendriksen(2001)
nheA	nheA 344 S nheA 843 A	TACGCTAAGGAGGGGCA GTTTTTATTGCTTCATCGGCT	344–360 843–823	500	Granum et al. (1999) (Y19005)	Hansen and Hendriksen (2001)
nheB	nheB 1500 S nheB 2269 A	CTATCAGCACTTATGGCAG ACTCCTAGCGGTGTTCC	1500–1518 2269–2253	770	Granum et al. (1999) (Y19005)	Hansen and Hendriksen (2001)
nheC	nheC 2820 S nheC 3401 A	CGGTAGTGATTGCTGGG CAGCATTCGTACTTGCCAA	2820–2836 3401–3383	582	Granum et al. (1999) (Y19005)	Hansen and Hendriksen (2001)
pcpI (cerA)	CERA 1 CERA 2	ACTGAGTTAGAGAACGGTAT CGCTTACCTGTCATTGGTGT	447–466 982–963	536	Gilmore et al. (1989) (M24149)	This study
sph (cerB)	CERB 1 CERB 2	TCGTAGTAGTGGAAGCGAAT AGTCGCTGTATGTCCAGTAT	1446–1465 1902–1883	457	Gilmore et al. (1989) (M24149)	This study
cytK	CK-F-1859 CK-R-2668	ACAGATATCGGKCAAAATGC TCCAACCCAGTTWSCAGTTTC	1859–1878 2668–2649	810	Lund et al., 2000 (AJ277962)	Guinebretiere et al. (2002)
piplc	phosC 1 phosC 2	CGCTATCAAATGGACCATGG GGACTATTCCATGCTGTACC	712–731 1280–1261	569	Lechner et al. (1989) (X14178)	Hansen et al. (1998)
hlyII	BcHlyII-S BcHlyII-A	AGAAGGAGGTGGCTGTCTGTA TTCTTTCCAAGCAAAGCTAC	251–270 785–766	535	Baida et al. (1999) (U94743)	This study
hlyIII	BCHEM 1 BCHEM 3	AATGACACGAATGACACAAT ACGATTATGAGCCATCCCAT	344–363 787–768	444	Baida and Kuzmin (1995) (X84058)	This study
cspA (psychrotrophic signature)	BcAPF1 BcAPR1	GAGGAAATAATTATGACAGTT CTTYTTGGCCTTCTTCTAA	–**	284	–	Francis et al. (1998)
CspF (mesophilic and psychrotrophic signature)	BcFF2 BcAPR1	GAGATTTAAATGAGCTGTAA CTTYTTGGCCTTCTTCTAA	–**	160	–	Francis et al. (1998)
16S rDNA (psycrotolerant 16S rDNA signature)	bc-uf bc-pr	CAAGGCTGAAACTCAAAGGA GAGAAGCTCTATCTCTAGA	–**	130	–	Von Stetten et al. (1999)
16S rDNA (mesophilic 16S rDNA signature)	bc-mf bc-ur	ATAACATTTTGAACCGCATG CTTCATCACTCACGCGGC	–**	250	–	Von Stetten et al. (1999)

16S rDNA (B. cereus group specific signature)	S-S-Bc-200-a-S-18	TCGAAATTGAAAGGCGGC	200–217		Hansen et al. (2001)
	S-S-Bc-470-a-A-18	GGTGCCAGCTTATTCAAC	487–470	288	
	S-*-Univ-518-b-S-18	CAGCAGCCGCGGTAATAC	518–535	993	Bavykin et al. (1999, unpublished) (AF176322)
	S-*-Univ-1492-b-A-19	GGTTACCTTGTTACGACTT	1510–1492		
16S–23S rDNA ITS	ITS-16S-1392-S-15	GNACACACCGCCCGT	–**	–	Willumsen et al. (2005)
	ITS-23S-206-r-A-21	NCTTAGATGTTTCAGTTCVCY			
	OPA 9	GGGTAACGCC	–**	–	Operon Technologies, Inc., Alameda, CA

*N = A/G/C/T; V = A/C/G; K = G/T; W = A/T; S = G/C; M = A/C; R = A/G; Y = C/T.
**Primer binding position not specified in the reference.

pXO2. Thus *B. thuringiensis* and *B. anthracis* were not represented in our samples.

All these isolates were separated into psychrotolerant or mesophilic types by their ability to grow at 6 °C and at 42 °C and by the *cspA* and *16S rDNA* signatures (Table 2). Of the 96 *B. cereus*-like isolates, 84 grew at 6 °C but not at 42 °C. Among the remaining 12 *B. cereus*-like isolates, six did not grow at 6 °C and 42 °C, although they possessed the *cspA* and 16S rDNA pattern characteristic for *B. weihenstephanensis*. The remaining six *B. cereus*-like isolates are mesophilic *B. cereus* due to the 16S rDNA and *cspA* PCR amplification patterns. Of these, four isolates grew at 42 °C but not at 6 °C and are therefore strictly mesophilic. Eighty-four of the *B. cereus*-like isolates had growth characteristics and genetic signatures identical with the definition of the species *B. weihenstephanensis*. In addition, six isolates had genetic signatures identical with *B. weihenstephanensis*. Hence, these 90 isolates were affiliated to *B. weihenstephanensis* and the other six to *B. cereus*.

Of the 96 *B. mycoides*-like isolates, 67 grew at 6 °C but not at 42 °C. Of the remaining 29 *B. mycoides*-like isolates, 10 were strictly mesophilic as evidenced by growth at 42 °C. We designated these ten isolates as mesophilic *B. mycoides*. The remaining 19 *B. mycoides*-like isolates all had the *cspA* and 16S rDNA PCR signature characteristic of *B. weihenstephanensis* and they were not able to grow at 42 °C. These 19 isolates, which differ from *B. weihenstephanensis* by their inability to grow at 6 °C, together with the 67 strains able to grow at 6 °C were collectively designated "*B. weihenstephanensis*-like *B. mycoides*".

Thus, of the $1.16 \pm 0.16 \times 10^5$/g *B. cereus*-like isolates found in the soil 93.7% were *B. weihenstephanensis* and 6.3% *B. cereus*. Of the $1.79 \pm 0.33 \times 10^5$/g soil *B. mycoides*-like isolates, 89.6% are *B. weihenstephanensis*-like *B. mycoides*, and 10.4% are mesophilic *B. mycoides*.

This classification of the isolates was further evidenced by RAPD fingerprinting with the primer OPA9 (results not shown). The six *B. cereus* isolates showed three different patterns represented by 1, 2 and 3 isolates respectively, while the 90 *B. weihenstephanensis* isolates showed 21 different patterns, each represented by 1–14 isolates. None of the three *B. cereus* patterns were identical with any of the *B. weihenstephanensis* patterns. The six *B. weihenstephanensis* isolates not able to grow at

Table 2. Designation of *B. cereus*-like and *B. mycoides*-like isolates into groups according to their ability to grow at 6 ° and 42 °C and the presence of the gene for a coldshock protein (*cspA*) and a 16S rDNA signature for psychrotolerant strains.

Designated name	Number of isolates	Growth at 6 °C	Growth at 42 °C	Bw − *cspA*	Bw 16S rDNA
B. cereus like					
B. cereus	2	–	–	–	–
Strict mesophilic *B. cereus*	4	–	+	–	–
B. weihenstephanensis	84	+	–	+	+
B. weihenstephanensis	6	–	–	+	+
B. mycoides like					
Strict mesophilic *B. mycoides*	10	–	+	–	–
B. weihenstephanensis-like *B. mycoides*	19	–	–	+	+
B. weihenstephanensis-like *B. mycoides*	67	+	–	+	+

6 °C produced five different patterns, of which only one strain had a pattern different from other *B. weihenstephanensis* patterns. The ten strictly mesophilic *B. mycoides* isolates all produced the same RAPD pattern with OPA9. This pattern differs from the nine patterns produced by the 86 *B. weihenstephanensis*-like *B. mycoides*. The *B. weihenstephanensis*-like *B. mycoides* not able to grow at 6 °C produce RAPD patterns which were indistinguishable from patterns produced by some *B. weihenstephanensis*-like *B. mycoides* able to grow at 6 °C.

All 192 isolates were further analyzed for four key phenotypic characters: motility, hemolysis and ability to degrade lecithin and starch (Table 3). The ability to degrade lecithin was widespread among the *B. cereus* and *B. weihenstephanensis* isolates (66% and 98%, respectively), whilst fewer mesophilic and *B. weihenstephanensis*-like *B. mycoides* isolates had this ability (40% and 28%, respectively). Most *B. weihenstephanensis* (93%) and mesophilic *B. mycoides* (100%) were hemolytic, while this property was restricted to 83% of the *B. cereus* and 79% of the *B. weihenstephanensis*-like *B. mycoides* isolates. Many non-motile isolates occurred within the collection, notably among the *B. mycoides* isolates with only 6% of the *B. weihenstephanensis*-like *B. mycoides* isolates being mobile, while 50 and 59% of the *B. cereus* and *B. weihenstephanensis* respectively were mobile. All the isolates except for two *B. weihenstephanensis* isolates were able to degrade starch.

The PCR genetic analysis for genes potentially involved in pathogenesis is presented in Table 4. The presence of the *hblA, hblC* and *hblD* genes encoding the HBL complex, the *nheA, nheB* and *nheC* genes encoding the NHE complex, and the *cytK, hlyII, hlyIII, piplc, pcpl* and *sph* genes encoding other virulence factors were detected by PCR in the 90 *B. weihenstephanensis*, 6 *B. cereus*, 86 *B. weihenstephanensis*-like *B. mycoides* and 10 mesophilic *B. mycoides* isolates. The genes occurred in 32 different combinations in *B. weihenstephanensis* and 22 combinations in the *B. weihenstephanensis*-like *B. mycoides* isolates. Collection curves for these two groups of isolates revealed that these combinations represent the major part of variation occurring in the soil (Figure 1). The combination of genes occurring most often among *B. weihenstephanensis* harbored 11 isolates, whilst eight isolates were unique. Among the *B. weihenstephanensis*-like *B. mycoides* 34 isolates were identical, while four were unique. In both *B. cereus* and the mesophilic *B. mycoides* three different combinations of genes were identified.

Table 3. Phenotypic characteristics of the four groups of *B. cereus*-group isolates identified in this study.

	Motility	Lecithinase	Amylase	Hemolysin
B. cereus (*n* = 6)	3	4	6	5
B. weihenstephanensis (*n* = 90)	53	88	88	84
B. mycoides (mesophilic) (*n* = 10)	0	4	10	10
B. weihenstephanensis like *B. mycoides* (psychrotolerant) (*n* = 86)	5	24	86	68

Table 4. Presence of genes associated with pathogenesis in the four different types of *B. cereus*-group bacteria identified in this study.

Gene product:	Hemolysin BL			Nonhemolytic enterotoxin			Cytotoxin K	Cereolysin AB		Phospholipase C	Hemolysin II	Hemolysin III
Gene:	*hblA*	*hblC*	*hblD*	*nheA*	*nheB*	*nheC*	*cytK*	*pcpl*	*sph*	*piplc*	*hlyII*	*hlyIII*
B. cereus (6 isolates)	6 (100)	6 (100)	5 (83)	6 (100)	6 (100)	4 (67)	4 (67)	4 (67)	5 (83)	5 (83)	3 (50)	5 (83)
B. weihenstephanensis (90 isolates)	79 (88)	90 (100)	79 (88)	79 (88)	52 (58)	28 (31)	0 (0)	89 (99)	90 (100)	44 (49)	0 (0)	19 (21)
B. mycoides mesophilic (10 isolates)	9 (90)	10 (100)	8 (80)	1 (10)	0 (0)	0 (0)	0 (0)	1 (10)	1 (10)	1 (10)	0 (0)	9 (90)
B. mycoides-like *weihenstephanensis* (86 isolates)	77 (90)	85 (99)	86 (100)	86 (100)	47 (55)	18 (21)	0 (0)	79 (92)	67 (78)	11 (13)	0 (0)	12 (14)

The values given are numbers of isolates containing the specific gene deduced by PCR-analysis. The numbers in brackets are these numbers given as a percentage of total number of isolates present in each group.
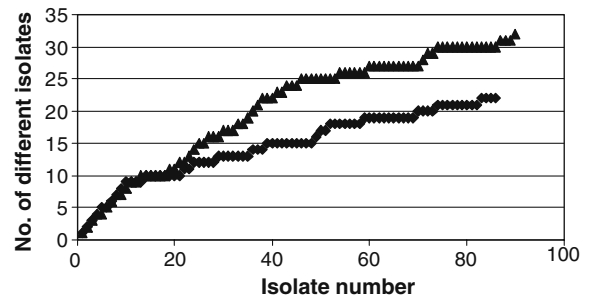

Figure 1. Collection curves for *B. weihenstephanensis* isolates (▲) and *B. weihenstephanensis*-like *B. mycoides* isolates (■) based on the presence or absence of 12 genes involved in pathogenesis.

All *B. weihenstephanensis* and *B. weihenstephanensis*-like *B. mycoides* isolates possessed at least one of the genes encoding the enterotoxic HBL complex (Table 4); *hblC* was present in all isolates except one *B. weihenstephanensis*-like *B. mycoides* isolate, whilst *hblA* and *hblD* were present in between 87.8% and 97.7% of the isolates. All 86 *B. weihenstephanensis* like *B. mycoides* possessed the *nheA* gene of the non-hemolytic enterotoxin complex, while only 79 of the *B. weihenstephanensis* isolates possessed this gene. The frequency of isolates of *B. weihenstephanensis* and *B. weihenstephanensis*-like *B. mycoides* possessing *nheB* constituted only 65.8% and 55.6% of the isolates possessing *nheA*, respectively. Even fewer isolates possessed *nheC*, namely 35.4% and 20.0%, respectively. Three *B. weihenstephanensis* isolates did not possess any of these three genes. The genes *pcplc* and *sph*, encoding the two-component cereolysin AB, are present in all *B. weihenstephanensis* isolates except for one. These two genes were present in fewer *B. weihenstephanensis*-like *B. mycoides* isolates, namely 91.9% and 77.9% of the isolates, respectively. *Piplc* and *hlyIII* were present in 48.9% and 21.1% of the *B. weihenstephanensis* isolates and in 12.8% and 14.0% of the *B. weihenstephanensis*-like *B. mycoides* isolates. Neither *B. weihenstephanensis* nor *B. weihenstephanensis*-like *B. mycoides* isolates possessed *cytK* nor *hlyII*.

All six *B. cereus* isolates possessed *hblA* and *hblC* and five of them also contained *hblD* (Table 4). Similarly, all ten of the mesophilic *B. mycoides* isolates possessed *hblC* and 9 and 8 isolates possessed *hblA* and *hblD*, respectively. Between four and six of the *B. cereus* isolates possessed the genes *nheA*, *nheB*, *nheC*, *cytK*, *pcplc*,

sph, piplc and hlyIII. This is in contrast to the mesophilic *B. mycoides* isolates where only one strain contained the *nheA, pcplc, sph, piplc* genes, whilst these strains lacked the remaining genes, except *hlyIII*, which was present in 9 isolates. *HlyII* was present in three *B. cereus* isolates and missing from the mesophilic *B. mycoides* isolates; these three *B. cereus* isolates also harbored *cytK*.

Comparison between the psychrotolerant and mesophilic isolates (Table 4) revealed that the main difference between the *B. cereus* and the *B. weihenstephanensis* isolates was that *cytK* and *hblII* were detected in the *B. cereus* isolates exclusively. The main differences between *B. weihenstephanensis*-like *B. mycoides* and mesophilic *B. mycoides* was that the genes *nheB* and *nheC* were not detected in the mesophilic *B. mycoides*, whilst the occurrence of *nheA, pcplc, sph* and *piplc* was limited to one mesophilic *B. mycoides* isolate.

Discussion

The density of *B. cereus*-like bacteria detected in the loamy sand, i.e. approximately 10^5 cells/g, was just within the densities (10^2–10^5) reported from agricultural soils in Sweden and the Netherlands (Tegiffel et al. 1995; Christiansson et al. 1999), but a factor 10 below the number found in a German soil (von Stetten et al. 1999). No comparable data on the density of *B. mycoides*-like bacteria in soils were found in the literature. *B. thuringiensis* generally constitutes only 0–3% of *B. cereus*-like bacteria isolated from soil (Glare and O'Callaghan 2000), while *B. anthracis* is very rare (Pepper and Gentry 2002). Thus our inability to detect any *B. thuringiensis* or *B. anthracis* among the isolates is consistent with these reports.

The *B. cereus*-like bacteria isolated from the soil could be divided into *B. cereus* and *B. weihenstephanensis* on the basis of genotypic and phenotypic characteristics, as described by Francis et al. (1998) and von Stetten et al. (1999). We found the frequency of *B. cereus* and *B. weihenstephanensis* in the Danish soil to be 6.3% and 93.7%, respectively. Thus the two species coexist in this temperate soil, as also found in two temperate soils in Germany (von Stetten et al. 1999). However in these soils the ratio between the two species was about 1 to 1 (von Stetten et al. 1999). Von Stetten et al. (1999)

found that 16% of their isolates had either a psychrotolerant genotype but a mesophilic phenotype (i.e. no growth below 7 °C) or a mesophilic genotype but a psychrotolerant phenotype. Among the Danish isolates, only psychrotolerant genotypes with a mesophilic phenotype were identified, with a frequency of 6.3%.

As well the *B. cereus*-like bacteria, the *B. mycoides*-like could be divided into *B. weihenstephanensis*-like and mesophilic isolates on the basis of their ability to grow at temperatures below 7 °C and genotypic characteristics. At the genotypic level 89.6% of the *B. mycoides*-like bacteria were identical with *B. weihenstephanensis* and 10.4% identical with *B. cereus*. Of the former, 77.9% were able to grow at temperatures below 7 °C. Francis et al. (1998) found three mesophilic and three psychrotolerant isolates of *B. mycoides* from milk and soil, respectively. All three psychrotolerant isolates harbored the *cspA* gene. Von Stetten et al. (1998) found 100% correlation between the 16S rDNA signature for psychrotolerant and mesophilic isolates of the *B. cereus* group. Their analysis included 33 *B. mycoides* isolates, of which at least two were mesophilic. Bell and Friedman (1994) characterized a soil population of *B. mycoides* by standard metabolic tests, multilocus enzyme electrophoresis, RFLP and hybridization techniques. Their results with the molecular assays indicated that the *B. mycoides* isolates could be separated into two different groups but they did not provide any information about minimal growth temperature of the different isolates. Further, Nakamura and Jackson (1995) concluded that the species *B. mycoides* is composed of two genetically distinct groups which were subsequently established as two species, *B. mycoides* senso stricto and *B. pseudomycoides* (Nakamura 1998). Evidence for this division of the species has also been provided by sequence analysis of 16SrRNA, 23S rRNA and gyrB genes (Bavykin et al. 2004). The two species are not distinguishable by physiological and morphological characteristics but are clearly separable based on fatty acid composition (Nakamura 1998). However, the type strain of *B. mycoides* is psychrotolerant, harbors the *cspA* gene (Lechner et al. 1998) and has the 16S rDNA signature for psychrotolerant isolates (Pruss et al. 1999a), while the minimal growth temperature of *B. pseudomycoides* is indicated to be 15 °C (Nakamura 1998). Thus, it seems

likely that of the strains identified herein, the *B. weihenstephanensis*-like *B. mycoides* are *B. mycoides* senso stricto whilst the mesophilic *B. mycoides* are *B. pseudomycoides*. Further evidence for this is a close relationship between *B. weihenstephanensis* and psychrotolerant *B. mycoides* deduced by RAPD and sequence analysis (Lechner et al. 1998; Bavykin et al. 2004). Further clarification of these relationships will be dependent on fatty acid composition analysis, which is in progress.

The widespread occurrence of genes encoding virulence factors in *B. cereus* and *B. thuringiensis* is in accordance with other studies on the occurrence of these genes (Hsieh et al. 1999; Hansen and Hendriksen 2001; Guinebretiere et al. 2002) and the full genome sequences of three *B. cereus* strains (Ivanova et al. 2003; Hoffmaster et al. 2004; Rasko et al. 2004). However, knowledge of the occurrence of these genes in *B. weihenstephanensis* and *B. mycoides* is much more restricted. Pruss et al. (1999b), Stenfors and Granum (2001) and Stenfors et al. (2002) found genes encoding the enterotoxins HBL and NHE in 28 of 42 *B. weihenstephanensis* isolates. Mäntynen and Lindstrom (1998) and Hsieh et al. (1999) found the *hblA* gene to be present in 17 of 26 *B. mycoides* isolates. Further, Hsieh et al. (1999) found the gene (*sph*) for sphingomyelinase in three *B. mycoides* isolates. In addition to this, we have here found that *B. weihenstephanensis* strains have genes encoding cereolysin AB, phosphatidylinositol-specific phospholipase C and hemolysin III. *B. mycoides* was found to have the genes for NHE, phosphatidylinositol-specific phospholipase C and hemolysin III. Further, we have here found one mesophilic *B. mycoides* strain that possessed the same virulence genes as psychrotolerant *B. mycoides*, while the other nine *B. mycoides* strains only contained genes for HBL and hemolysin III. Four *B. pseudomycoides* isolates were all found to possess *hblA* (Pruss et al. 1999b). Thus it seems reasonable to conclude that all species in the *B. cereus* group include many isolates which contain genes for the enterotoxins HBL and NHE, hemolysin III, and the phospholipases cereolysin AB and a phosphatidylinositol-specific phosphoplipase C. On the contrary, the occurrence of cytolysin K seems, based on current knowledge, to be restricted to some *B. cereus* isolates and hemolysin II seems to be restricted to some *B. cereus* and *B. thuringiensis* isolates.

The genes encoding for enterotoxin NHE, *nheA*, *nehB* and *nheC*, occurred with a decreasing frequency in the isolates, but as the occurrence of the three genes shows significant association and the functioning of the complex depends on products from all three genes (Granum 2001), it is most likely that polymorphism among the genes, at least partly, was responsible for our inability to detect all three genes in a number of isolates. The observed variation in occurrence of the genes encoding HBL and Cereolysin AB might also be due to sequence polymorphism.

We found the 90 *B. weihenstephanensis* and the 86 psychrotolerant *B. mycoides* isolates to be composed of 32 and 22 different genotypes, respectively, most likely representing most of the variation present in the soil, as evidenced by collection curves. Such high diversity in a single soil has previously only been shown for *B. cereus*, *B. thuringiensis* (Vilas-Boas et al. 2002) and *B. mycoides* (Bell and Friedman 1994) by multilocus enzyme electrophoresis.

Conclusions

The presence of multiple genes coding for virulence factors in all isolates representing the species *B. cereus*, *B. weihenstephanensis*, *B. weihenstephanensis*-like *B. mycoides* and mesophilic *B. mycoides*, in this soil, suggests that all the isolates are potential pathogens. Thus, one gram of this soil harbors $2-3 \times 10^5$ potential pathogenic *B. cereus* group bacteria. As these pathogenic traits are widely present in the isolates from the soil, it is very likely that these traits play an important role in the lifestyle of these bacteria. Ivanova et al. (2003) suggest that the insect intestine could have been the natural habitat for the common ancestor of the *B. cereus* group, as the abundance of proteolytic enzymes, the multiplicity of peptide and amino-acid transporters and the variety of amino-acid degradation pathways indicate that proteins, peptides and amino-acids may be their preferred nutrient source. Furthermore, Jensen et al. (2003) hypothesized that *B. cereus* group bacteria all disclose symbiotic relationships with appropriate invertebrates and Margulis et al. (1998) showed that *B. cereus* group bacteria formed filamentous segmented bacteria in the gut of a number of different arthropods. The functions

of all the virulence factors mentioned are related to interactions with plasma membranes, as pore-formers or enzymes with phospholipid degrading abilities, suggesting that *B. cereus* group bacteria are capable of interacting with plasma membranes and that their activities are associated with cell surfaces.

Acknowledgements

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