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ORIGINAL ARTICLE

Metabolic capacities and toxigenic potential as key drivers of *Bacillus cereus* ubiquity and adaptation

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Abstract *Bacillus cereus* is ubiquitous and is commonly found in a wide range of environments, including food. In this study, we analyzed 114 foodborne *B. cereus* strains isolated mainly from starchy and dairy products in order to investigate their phenotypic diversity (API system), antimicrobial resistance and toxigenic profiles (*hblA*, *nheA*, *hlyII*, *cereolysin* O, *cytK2*, *cytK1* and *EM1* genes). All isolates were confirmed as *B. cereus* using their 16–23S ribosomal DNA intergenic transcribed spacer (ITS) signature, and were shown to be Gram-positive, catalase and caseinase positive, hemolytic (97 %), and positive for lecithin hydrolysis and motility (97 and 87 %, respectively). PCR detection of *B. cereus*-specific toxin genes revealed occurrence rates of 100 % for *cereolysin* O, 98 % for *nheA*, 74 % for *cytk2*, 52 % for *hblA*, 28 % for *hlyII*, and the absence of *cytK1*. Only two strains (2 %), isolated from intestine of boar and pheasant, carried the emetic toxin genetic determinants (*ces*). The antimicrobial susceptibility of isolates was tested towards 15 different antimicrobial agents. We detected susceptibility of all strains to most antibiotics, intermediate resistance to clindamycin, and resistance to β-lactam antibiotics with 83 % of the resistant isolates producing β-lactamase enzyme. This large phenotypic diversity, combined with the toxigenic traits and antibiotic resistance, emphasize the high potential risk of food poisoning of *B. cereus* isolates. Additionally, a clear correlation between the metabolic features and the origin of isolation was shown. Most starchy isolates were able to hydrolyze starch while dairy strains were not able to produce amylases. Overall, our results reveal that metabolic flexibility and toxigenic potential represent the main drivers for *B. cereus* ubiquity and adaptation in a given ecological niche.

Keywords *Bacillus cereus* · Origin of isolation · Phenotypic diversity · Starch hydrolysis · Adaptation · Toxigenic potential

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Introduction

Bacillus cereus is a constant problem due to its ubiquitous occurrence, nutritional versatility, ability to form endospores and to grow over a broad temperature range (Setlow and Setlow 1994). It is commonly found in a wide range of environments including foods (Bartoszewicz et al. 2008). *Bacillus cereus* causes two kinds of food poisoning: diarrheal and emetic syndromes (Drobniewski 1993). The emetic syndrome is characterized by nausea and vomiting, starting 1–5 h after ingestion and is associated mostly with farinaceous foods such as cooked rice (Valéro et al. 2002). This emetic type of gastrointestinal disease is caused by the cereulide, a dodecadepsipeptide structurally related to valinomycin (Finlay et al. 2002). It has been shown that the genetic determinants of this toxin are located on a 270 kb megaplasmid (Hoton et al. 2005). This toxin results from non-ribosomal peptide synthesis (NRPS) performed by the cereulide synthetase (*ces*) gene cluster (Ehling-Schulz et al. 2006).

The diarrhoeal syndrome is characterized by abdominal pain, profuse watery diarrhoea and rectal tenesmus, which occur between 8 and 16 h after ingestion of *B. cereus*-contaminated food, generally of a proteinaceous nature (Notermans and Batt 1998). At least three different enterotoxins seem to be involved

in this syndrome: hemolytic toxin (Hbl), non-hemolytic toxin (Nhe) (McKillip 2000) and cytotoxin K (CytK) (Lund et al. 2000). These enterotoxins are encoded chromosomally and are produced during vegetative growth of *B. cereus* in the small intestine. Expression of these three enterotoxins is regulated positively by the pleiotropic PlcR/PapR quorum-sensing system (Agaisse et al. 1999; Slamti and Lereclus 2002; Gohar et al. 2002). Both Nhe and Hbl are three-component toxins while CytK is a single-component toxin that belongs to the family of β-barrel pore-forming toxins. Two forms of CytK have been characterized, namely CytK1 and CytK2. The amino acids sequence of CytK1 (the original and particularly cytotoxic CytK variant) displays 89 % identity with CytK2. The strain NVH391/98, where CytK was first identified (CytK1), was shown to be rather peculiar since it represents a cluster of thermophilic strains among the *B. cereus* group and was suggested to be recognized as a novel species "*B. cytotoxicus*" (Auger et al. 2008; Guinebretière et al. 2008, 2013; Lapidus et al. 2008). Another single-component toxin of *B. cereus*, namely HlyII (hemolysin II), which belongs to the family of oligometric β-barrel channel-forming toxins, is hemolytic and cytotoxic towards human cell lines (Andreva et al. 2007).

Bacillus cereus is ubiquitous in highly diverse ecological niches and often encountered in the food matrix. Indeed, *B. cereus* is able to multiply from biotic or abiotic soil fractions (Vilain et al. 2006) to the human gastro-intestinal tract, passing through food environments. This ecological flexibility is made possible by its metabolic versatility and is, in general, due to its genotypic and phenotypic diversity (Chaves et al. 2011; Chon et al. 2012). The species is known as panmictic, displaying a wide diversity of virulence factors (Helgason et al. 2004), growth behavior and survival characteristics (Choma et al. 2000). To face changing and sometimes hostile environments, *B. cereus* has to develop adaptive strategies by elaborating different physiological responses depending on the stress and habitat type encountered (Carlin et al. 2009).

The present study was undertaken to assess the occurrence of *B. cereus* in different food matrices (e.g., dairy and starchy products) and to elucidate their phenotypic, metabolic and toxicological profiles in correlation with their ecological features. The study was based on the investigation of phenotypic traits using the API system, biochemical tests and antibiotic resistance, and the use of PCR analysis to detect virulence-related genes and the presence of emetic strains and/or high virulent strains of *B. cereus* (*B. cytotoxicus*).

Materials and methods

Isolation of *B. cereus* strains

A total of 114 *B. cereus* strains was isolated from dairy, starchy and other food products according to the AFNOR method (AFNOR 1996) (Table 1). In brief, 10 g food was homogenized in a Stomacher with 90 mL sterile peptone water. Next 0.1 mL of the suspension was spread on the selective polymixin, mannitol, egg yolk, phenol red agar medium (MYP), containing 100 mg L^{-1} polymixin B sulphate and 100 mL L^{-1} egg yolk (Mossel et al. 1967). The plates were incubated at 30 °C for 24 h and colonies displaying a pink or purple color with an irregular edge surrounded by a white area were considered as positive. The isolates were confirmed subsequently as *B. cereus* by Gram staining, shape and biochemical tests. Cultures were then streaked in tryptone soybean agar (TSA) slants and stored refrigerated as pure cultures. Strain suspensions were maintained at −80 °C in culture medium supplemented with 20 % glycerol.

Phenotypic characterization: biochemical and enzymatic tests and psychrotrophy

Biochemical tests based on the API 50CHB gallery were used to investigate the fermentation of 49 sugars, and the API 20E gallery was used for 12 additional tests (ONPG, ADH, LDC, ODC, CIT, H2S, URE, TDA, IND, VP, GEL and NIT) (Biomérieux, Marcy-l'Etoile, France) according to the manufacturer's instructions. All strains were cultured on TSA at 30 °C for 24 h and suspended in each medium used as inoculates for the biochemical tests. Biochemical kits inoculated with suspended medium were incubated at 30 °C for 24 h. The biochemical profiles obtained from the API 50CHB and API 20E were analyzed using APILAB Plus software. Catalase and oxydase activities were also determined, the hemolytic activity in blood agar medium was determined as described by (Pruss et al. 1999), and lecithinase, caseinase and motility were evaluated. The overall results (18 characters) of the phenotypic characterization were statistically analyzed using the Jaccard coefficient and unweighted pair-group method with arithmetic mean (UPGMA) aggregation method, by multivariate statistical package (MVSP) software to construct a dendrogram. To determine the growth of *B. cereus* at a psychrotrophic temperature, all strains were inoculated onto nutrient agar followed by incubation at 7 °C up to 21 days. Isolates giving visual colonies at 7 °C within this time frame were considered to be psychrotrophic.

DNA extraction and PCR analysis

Total DNA was extracted using a modification of the Kalman's method (Kalman et al. 1993; Cherif et al. 2003). DNA samples were stored at −20 °C until further use. In order to confirm the phenotypic identification of the isolates, the 16–23S rRNA internal transcribed spacers (ITS) were amplified by PCR using the primers S-D-Bact-1494-a-S-20 and S-D-Bact-0035-a-A-15 (Jensen et al. 1993) as described previously (Cherif et al. 2003).

Table 1 Origin of *Bacillus cereus* isolates (n=114)

Origin	Number of isolates
Dairy products	37
Ricotta	25
Milk	7
Butter	2
Leben	3
Starchy products	49
Rice	43
Pasta	5
Potato salad	1
Other (spices, animal intestine, honey)	28
Total	114

Virulence-related genes of *B. cereus* were amplified using the primers listed in Table 2. This includes *nheA*, *hblA*, *cytk2*, *cereolysin O* and *hlyII* as described (Guinebretière et al. 2002; Yang et al. 2005), Other high virulence-related genes were also detected: the *EM1* gene specific for the emetic toxin gene (Ehling-Schulz et al. 2004) and *cytk1* gene for the detection of high virulent food poisoning strain (Guinebretière et al. 2006).

The PCR reactions were performed using a thermocycler (Applied Biosystems, Foster City, CA) in 25 μL reaction volumes using 1 μL DNA extract, 1x *Taq* polymerase buffer, 2 mM final concentration of $MgCl_2$, 0.2 mM for each dNTP, 1 μM for each primer and 1 U *Taq* DNA polymerase. For the cereulide genetic determinants the PCR protocol started with a denaturation step for 15 min at 95 °C, followed by 30 cycles of 30 s at 95 °C, 30 s at 60 °C and 60 s at 72 °C, with a final elongation step at 72 °C for 5 min (Ehling-Schulz et al. 2004). PCR reaction condition for *cytk1* and *cytk2* genes consisted of an initial denaturation step at 94 °C for 5 min followed by 30 cycles of 95 °C for 15 s, 57 °C for 30 s and 72 °C for 30 s ended with a final extension at 72 °C for 7 min (Guinebretière et al. 2006). PCR amplification of *nheA*, *cereolysin O*, *hblA and hlyII*, and the 16–23S rRNA ITS, was performed with an initial denaturation step at 94 °C for 5 min followed by 30 cycles of 94 °C for 15 s, 55 °C for 45 s and 72 °C for 2 min completed with a final extension at 72 °C for 10 min (Hansen and Hendriksen 2001). The PCR products were then analyzed on a 1 or 2 % agarose gel containing ethidium bromide (0.5 μg mL^{-1}).

Antibiotic resistance

The Kirby-Bauer disk diffusion method (Drew et al. 1972) was used to evaluate the antibiotic susceptibility patterns of *B. cereus* isolates. All strains were grown in Mueller Hinton agar (Biokar Diagnostics, Beauvais, France) in the presence of various antimicrobial impregnated filter paper disks and incubated for 18 h at 37 °C. The used disks (Liofilchem, Roseto degli Abruzzi, Italy) and antibiotic concentration were as follows: amoxicillin-clavulanic acid (30 μg), ampicillin (10 μg), penicillin (10 U), cefotaxim (30 μg), oxacillin (1 μg), cefoxithin (30 μg), vancomycin (30 μg), ciprofloxacin (5 μg), gentamicin (10 μg), rifampicin (5 μg), amikacin (30 μg), tetracycline (30 μg), clindamicin (2 μg), erythromycin (15 μg) and streptomycin (10 μg). All isolates were classified as susceptible, intermediate susceptible, or resistant according to standard CLSI guidelines (Clinical and Laboratory Standards Institute 2010).

Results

Isolation and phenotypic characterization of foodborne *Bacillus cereus*

A total of 114 presumptive *B. cereus* were isolated on MYP (mannitol egg yolk polymixine) selective medium mainly from starchy and dairy products with the following percentages: starchy products (rice, pasta, potato salad, n=49; 433 %), dairy products (ricotta, milk, butter, and leben, n= 37; 32 %) and other products (spices, animal intestine, honey, n=28; 24 %; Table 1). All isolates were confirmed as Gram-positive, catalase positive, caseinase positive and oxydase negative, non psychrotrophic (unable to grow at 7 °C after 21 days) and 97 % of the isolates were shown to be hemolytic on blood agar with 74 % β-hemolytic and 26 % α-hemolytic. From the 114 *B. cereus* isolates, 111 (97 %) and 99 (87 %) strains were positive for lecithinase and motility tests, respectively. These isolates were further characterized and identified using the API 50CH/20E phenotypic system and APILAB Plus software. Most of the isolates belonged to the profile *B. cereus* 1 (65 %) and 33 strains (29 %) presented the API profile *B. cereus* 2. Five strains (4 %: RC11, RC33, S1, F1, BC922) showed a profile intermediate between *B. anthracis* and *B. cereus* 2, and two strains (RC35 and R35) were assigned to an intermediate profile between *B. megaterium* and *B. cereus* 2. The result of API 50CHB and API 20E characterization combined with other classic biochemical tests (motility, hemolysis and lecithinase) confirmed the identification of 100 % of the isolates as *B. cereus*. To further confirm this identification, all isolates were subjected to ITS-PCR targeting the 16–23S rRNA internal transcribed spacer (ITS). PCR results revealed identical patterns for all isolates, comprising two bands of 260 and 500 bp, which corresponds to the standard profile observed for the *B. cereus* group species (Wunschel et al. 1994).

The dendrogram generated from phenotypic patterns represented in Fig. 1 showed four groups (A, B, C and D). Groups A and B included ten isolates, originating mainly from ricotta (90 %) and characterized by their negative response to starch hydrolysis (90 %) and their incapacity to ferment either salicin (100 %) or esculin (60 %). Group C was composed of 73 isolates, dominated largely by strains from starchy products

Table 2 Nucleotide sequences, locations and references of the *B. cereus* virulence-related gene PCR primers

Primer set and reference	Primer name[a] and sequence (5′→3′)	Target gene	Expected size (bp)
Hemolysin A (cereolysinO) (Raddadi et al. 2006)	FHEMAF : CGTGAGAAAGCAAAACGC FHEMAR : TCAACAGAAACTGGAGAATGAT)	*hlyA*	725
Hemolysin II (Fagerlund et al. 2004)	FHLY II : GATTCTAAAGGAACTGTAG RHLY II : GGTTATCAAGAGTAACTTG	*hlyII*	868
Non hemolytic enterotoxin (NHE) (Hansen and Hendriksen 2001)	nheA 344S : TACGCTAAGGAGGGGCA nheA 843A : GTTTTTATTGCTTCATCGGCT	*nheA*	499
S-D-Bact-1494-a-S-20 (Jensen et al. 1993) S-D-Bact-0035-a-A-15 (Jensen et al. 1993)	1494ADNr 16S: GTCGTAACAAGGTAGCCGTA 35 ADNr 23S: CAAGGCATCCACCGT	ITS 16–23S rDNA profiles	
Hemolysin BL (HBL) (Hansen and Hendriksen 2001)	HBLA1: GTGCAGATGTTGATGCCGAT HBLA2: ATGCCACTGCGTGGACATAT	*hblA*	319
EM1 (Ehling-Schulz et al. 2004)	EM1F: GACAAGAGAAATTTCTACGAGCAAGTACAAT EM1R: GCAGCCTTCCAATTACTCCTTCTGCCACAGT	Cereulide synthetase (*ces*) gene cluster	635
Cytotoxin K2 (Guinebretière et al. 2006)	CytK2R: GTG IAG CCT GGA CGA AGT TGG CytK2 F: CAA TCC CTG GCG CTA GTG CA	*CytK2*	585
Cytotoxin K1 (Guinebretière et al. 2006)	CytK1 F: CAA TTC CAG GGG CAA GTG TC CytK1 R: CCT CGT GCA TCT GTT TCA TGA G	*CytK1*	426

[a] Primers used to amplify *B. cereus* virulence-related genes and ITS rRNA regions, with the corresponding references; *F*, forward primer; *R*, reverse primer

and spices. These isolates showed positive starch hydrolysis activity (99 %) and were able to ferment salicin (73 %). Group D was formed by 31 strains isolated mostly from dairy products. Isolates of this cluster were shown to be unable to degrade starch (100 %) and positive for salicin fermentation (64 %). Interestingly, from the eight isolates of the whole collection able to ferment lactose, six belong to group D, indicating that lactose degradation may be associated with dairy isolates.

Virulence-related genes and toxicological potential of the *B. cereus* isolates

Using standard primers specific to the virulence-related genes of *B. cereus* (Table 2), all the PCR reactions generated fragments of the expected sizes. *Cereolysin O* was the most common gene and was detected in all the isolates. The *nheA* gene encoding protein A of the non-hemolytic enterotoxin Nhe, was found in 112 strains (98 %). With regard to hemolytic activities, the *hblA* gene encoding binding component B, which is one of three proteins composing the Hbl complex, was detected in 59 strains (52 %). The *hlyII* gene encoding hemolysin II was detected in 32 strains (28 %). For the *cyt K* gene, 84 strains were positive (74 %) using the general PCR primers (Guinebretière et al. 2002) and all were assigned to the *cytK2* variant by specific primers (Yang et al. 2005). Only two isolates, S1 and F1, were positive for the emetic toxin gene EM1. These strains were isolated from intestine of boar and pheasant, respectively.

Combining these overall results, 11 different toxigenic patterns were identified with 2 patterns being strain-specific (LB2 and CER6 showing the X and XI patterns, respectively). Pattern IX was specific to the two emetic isolates (S1 and F1, Table 3). Three patterns were shown to be predominant. Pattern I contained 34 isolates (30 %) positive for the *nheA* and the *cytK2* genes, and pattern II (n=34, 30 %) included strains also positive for the *nheA* and *cytK2* genes in addition to the *hblA* gene (hemolytic enterotoxin). Eleven strains (10 %) were positive for all the toxin genes except *EM1* and formed toxigenic pattern III (Table 3). Five other toxin gene patterns were shown by a limited number of isolates. This includes pattern IV shown by eight isolates positive for *nheA* and *hlyII*; pattern V with six isolates positive for *nheA*, *cytK2* and *hlyII*; and patterns VI, VII and IIX with isolates positive for *nheA* and variable for *cytK*, *hblA* and *hlyII* genes. No clear correlation was observed between the predominant toxigenic patterns and isolation origin.

Antibiotic susceptibility

All strains were susceptible to gentamicin, ciprofloxacin, streptomycin, vancomycin, amikacin and rifampicin, whereas 110 isolates (97 %) were susceptible to tetracycline and 100

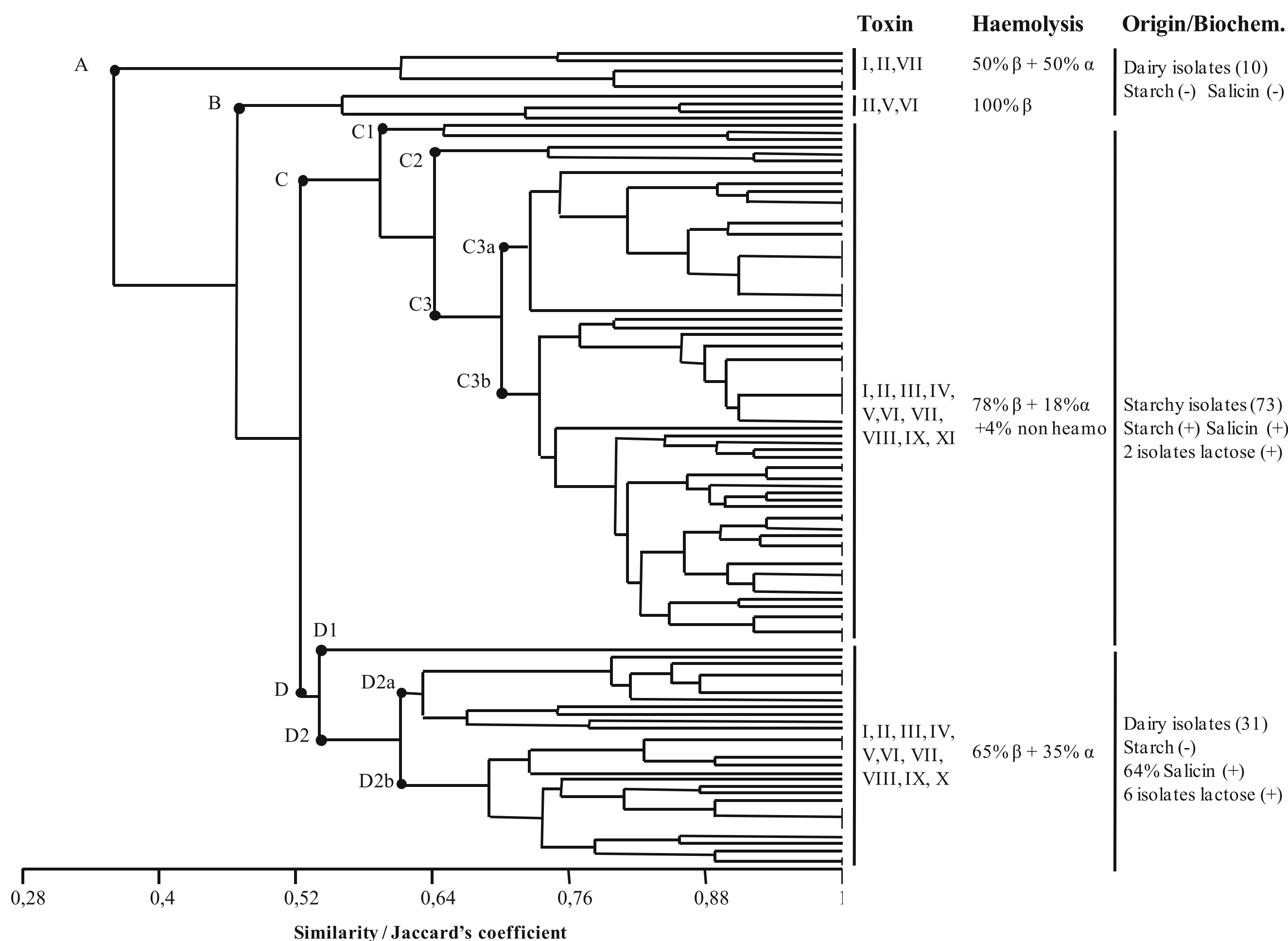

Fig. 1 The unweighted pair-group method with arithmetic mean (UPGMA) dendrogram of foodborne *Bacillus cereus* isolates based on phenotypic traits analysis (API and biochemical tests). *Toxin* Toxic profile (from I to XI); *Hemolysis* hemolytic trait (β, α or non hemolytic); *Origin/Biochem.* origin of isolates (dairy or starchy) and biochemical tests salicin, starch hydrolysis and lactose fermentation. *A* RC35, RC34, RC20, RC14, R35, RC12; *B* RC30, RC32, RC17, RC9; *C1* PMD, PMB, PMA; *C2* IM1, LB3, R16; *C3a* BC847, MK1, R19, R25, R28, R27, R18, CO1, ML3, C3, CER5, PMC, ML2, COR2, COR1, R30, R39, R24, R20, R17, PA1; *C3b* C2, R13, R3, A1, R8,R34, R9, R7, BC848, BC87, P3, SRG1, CUM1, R6, P1, RC31, R41, BC117, BC134, R33, C4, R15, R10, CER3, CER6, R26, PA2, R23, R22, R21, MY1, BR2, R12, R11, PA3, R38, R36, R31, RC19, C1, R29, R14, R5, R4, RC27, RC7; *D1* RC4; *D2a* RC4, RC33, R42, BC922, F1, S1, RC15, RC11, P2, RC10, RC22; *D2b* RC6, L5, L4, L3, ML1, R37, RC18, LB2, L2, R32, R2, LB1, RC26, RC25, RC3, L1, R40, A2, BR1, RC2

strains (88 %) to erythromycin. Intermediate resistance was recorded for clindamycin (100 %) and for the remaining isolates for erythromycine (12 %) and tetracycline (3 %) (Table 4). Resistance profiles were recorded for the overall strain collection towards amoxicillin-clavulanic acid, penicillin, cefotaxim, oxacillin, cefoxithin and ampicillin. For the latter, β-lactamase production was tested using the cefinase test, which showed that 83 % of the isolates were positive. Antimicrobial resistance patterns found were not associated with the origin of isolation or type of food.

Discussion

The present study phenotypically characterized 114 *B. cereus* strains, isolated mainly from starchy and dairy products, and evaluated their toxigenic potential and antimicrobial resistance. The assignment of the isolates to *B. cereus* species was based on the API system (50 CHB and 20E), biochemical tests and ITS-PCR analysis. With this latter approach, we detected a uniform ITS profile for all isolates, confirming their identification as belonging to the *B. cereus* group. The 16–23S rRNA spacer region remains a very valuable tool for PCR-based typing and bacterial identification. However, the method does not allow clear discrimination between *B. cereus*, *Bacillus thuringiensis* and *Bacillus anthracis*, which are still considered as very closely related species forming a unique taxonomic unit and distinguished only through their phenotypic and virulence specificities (Wunschel et al. 1994; Cherif et al. 2003). This taxonomic ambiguity is not resolved by the application of phenotypic and biochemical characterization. Indeed, the results obtained from the API characterization

Table 3 Summary of the toxigenic potential of the *B. cereus* isolates

Isolates	No. of isolates	Pattern	*nheA*	*cytK2*	*hblA*	*hlyII*	*ces*
RC10, RC11, RC12, RC26, L2, L3, L4, L5, R12, R18, R19, R22, R23,R24, R28, R27, R5, R17, R25, R26, R20, R29, R40, C3, COR1, PMB, A2, R37, R4, R10, BC117, P2, R32, R35	34	I	+	+	−	−	−
RC4, RC9, RC14, RC15, RC17, RC18, RC20, RC22, RC27, My1, PA1, PA3, R16, R30, C2, COR2, R11, R13, R14, R15, R21, R31, PMD, SRG1, R39, CUM1, R41, PMA BC134, CER5, ML1, ML2, ML3,CER3	34	II	+	+	+	−	−
RC3, RC6, RC31, BR2, CO1, PA2, C4, R33, R38, R36 BC847	11	III	+	+	+	+	−
RC19, RC25, R2, R8, R7, R9, PMC, A1	8	IV	+	−	−	+	−
RC32, L1, LB3 MK, R34, IM1	6	V	+	+	−	+	−
RC2, RC7, RC30, LB1, R3 BC848	6	VI	+	−	+	−	−
RC33, RC34, RC35, P1, C1, BC87	6	VII	+	−	+	+	−
BR1, R6, P3, BC922, R42	5	VIII	+	−	−	−	−
S1, F1	2	IX	+	−	−	−	+
LB2	1	X	−	−	+	−	−
CER6	1	XI	−	−	+	+	−
Total	114		112	91	59	35	2
Occurrence of virulence gene (%)			98	74	52	28	2

assigned five isolates, including the two emetic isolates, to intermediate profiles between *B. cereus* and *B. anthracis*. The overall results of the biochemical and phenotypic tests highlighted the huge diversity in the *B. cereus* collection and confirmed the inadequacy of the API system for identification purposes compared to molecular techniques (Ettoumi et al. 2009; Martínez-Blanch et al. 2011). Interestingly, this method, which is based on enzymatic potential was shown to be rather useful for correlating groups of isolates to their origin. The "*B. cereus* 1" API profile was recorded in strains isolated mainly from starchy products (86 %) whereas 76 % of the isolates originated from dairy products were affiliated to the "*B. cereus* 2" API profile. Furthermore, in the generated dendrogram (Fig. 1), the majority of dairy isolates (78 %), including six out of the eight lactose-positive strains, were located in groups A, B and D and corresponded to strains unable to hydrolyze starch. In contrast, the majority of starchy isolates (82 %) were located in group C corresponding to strains able to hydrolyze salicin and starch. These results indicate that the adaptation and the colonization of a given food matrix, or ecological niche, depends on the phenotypic and enzymatic traits that the bacterium displays. In our study, *B. cereus* expressing amylase genes clearly thrive in starchy products while those able to ferment lactose were encountered in dairy products.

The inability to hydrolyze starch and ferment salicin are considered as key traits for distinguishing emetic strains of *B. cereus* (Apetroaie et al. 2005; Ehling-Schulz et al. 2005). Indeed, we noted that the API biochemical profiles of the two emetic *B. cereus* isolates in our collection were characterized by their incapacity to hydrolyze starch or to ferment salicin. However, 36 % of the non-emetic strains showed these emetic-like features, suggesting that these traits could not discriminate emetic *B. cereus* strains. This is in agreement with the findings of Ankolekar et al. (2009) and Chon et al. (2012) who reported that starch hydrolysis phenotype alone is not useful for discrimination because 18 % (in both studies) of non-emetic strains were negative for the reaction and gave emetic-like phenotypes.

Table 4 Antibiotic susceptibility of *B. cereus* isolates. *VA* Vancomycin, *GM* gentamicin, *CIP* ciprofloxacin, *AK* amikacin, *S* streptomycin, *RD* rifampicin, *TET* tetracycline, *E* erythromycin, *CD* clindamicin, *CTX* cefotaxim, *OX* oxacillin, *P* penicillin, *AMP* ampicillin, *AMC* amoxicillin-clavulanic acid, *FOX* cefoxithin

Antibiotic	Amount (μg) on disc	Resistant isolates (%)	Intermediate isolates (%)	Susceptible isolates (%)
VA	30	0	0	100
GM	10	0	0	100
CIP	5	0	0	100
AK	30	0	0	100
S	10	0	0	100
RD	5	0	0	100
TET	30	0	3	97
E	15	0	12	88
CD	2	0	100	0
CTX	30	100	0	0
OX	1	100	0	0
P	10 Units	100	0	0
AMP	10	100	0	0
AMC	30	100	0	0
FOX	30	100	0	0

By using standard biochemical tests, we report the high occurrence of lecithinase activity (97 %) and motility (81 %), which is in agreement with the findings of Kim et al. (2010b), who showed that 100 % of their isolates were positive for these tests, and with the results of Chon et al. (2012), demonstrating that 100 % and 89 % of the strains were positive for motility and lecithinase, respectively.

Hemolysis was shown to be a widely spread characteristic, with 97 % of the strains being hemolytic. Several authors reported similar percentages, such as Kim et al. (2010b) with 93 % hemolytic, Chon et al. (2012) with 89 %, Chaves et al. (2011) with 92 % and Abriouel et al. (2007), who reported 100 % of their strains to be hemolytic. There is no coherence between the hemolytic phenotype and the hemolysis gene content of the isolates. The three non-hemolytic strains in our collection amplify with at least one of the hemolysin primers targeting *hblA*, *hlyII* or *cereolysin O*. This could be due to the non-expression of these genes or their non-functionality because of silencing mutations or deletion of transcriptional regulators, as suggested in previous studies (Van der Voort et al. 2008).

Besides the large diversity among the *B. cereus* isolates collection as revealed by phenotypic tests, several strains exhibited identical phenotypes but showed diverse virulence-related genes content, indicating their intraspecific genetic diversity; for instance BC847 and MK1, C4 and R15, BR2 and R12, and R14 and R5 isolates. In total, 11 distinct toxigenic profiles were detected based on seven toxin genes with no clear correlation with isolation origin except for emetic strains retrieved exclusively in animal intestines. The relative frequencies of these genes were in agreement with several reports in literature. A high occurrence was observed for *cereolysin O* (100 %), *nheA* (98 %) and *cytK2* (74 %)—frequencies very comparable to the reported results of many authors with *B. cereus* collections from diverse origins (Hansen and Hendriksen 2001; Ankolekar et al. 2009; Swiecicka et al. 2006; Chon et al. 2012; Ouoba et al. 2008; Godic Torkar and Seme 2009). Conversely, the *hblA* and *hlyII* genes were shown to be less frequent (52 and 28 %, respectively). Hence, the genes of the HBL complex are generally less common than those of the NHE complex (Al-Khatib et al. 2007; Granum 2007; Moravek et al. 2006), while *hlyII* exhibits a relatively low frequency (Shadrin et al. 2007). The remaining two virulence-related genes screened in this study (*cytK1* and *EM1*) are known to be present only rarely in *B. cereus*. The *cytk1* gene was not detected in any of the 114 isolates. According to Guinebretière et al. (2010), strains harboring this gene belong to a distant phylogenetic group that may represent a novel subspecies designed as *B. cytotoxicus*. The absence or very low frequency of these *cytK1* positive strains was further described by Carlin et al. (2009) and Lapidus et al. (2008). Although *cytK2* is not as toxic as *cytK1*, its potential to cause disease cannot be underestimated (Fagerlund et al. 2010).

The emetic genetic determinant EM1 was detected in only the two strains S1and F1 isolated from animal intestines. This very low occurrence, or absence, of emetic phenotype was previously reported for *B. cereus* collections from diverse origins (Svensson et al. 2006; Altayar and Sutherland 2006; Chon et al. 2012) and particularly in food-related isolates (Ouoba et al. 2008; Ankolekar et al. 2009; Chaves et al. 2011). The rationale behind the selection of boar and pheasant for the isolation of *B. cereus* was that the digestive tract of small mammals can be a reservoir of cereulide-producing strains (Hoton et al. 2009), which is confirmed in the present study. As described in other works, these emetic isolates also harbored putative enterotoxin genes, which could reinforce their toxigenic potential (Kim et al. 2010c; Chon et al. 2012). The virulence similarities of these isolates confirm the clonal nature of the emetic phenotype. This *B. cereus* pathogen variant is in fact considered as a single evolutionary lineage of closely related strains (Pirttijärvi et al. 1999; Ehling-Schulz et al. 2005).

Together with this toxigenic potential, our isolates were shown to possess antibiotic resistance traits, particularly to β-lactam antibiotics. This natural resistance, based on the production of β-lactamase, is well known and described in other studies (Andrews and Wise 2002; Park et al. 2009). However, the cefinase test results revealed that 17 % of the β-lactam resistance may be mediated by mechanisms other than enzymatic inactivation by β-lactamases. The susceptibility of all isolates to gentamicin, ciprofloxacin, streptomycin, vancomycin, amikacin, rifampicin and the intermediate resistance of some isolates to tetracycline and erythromycin, is in agreement with previous studies (Jensen et al. 2001; Roy et al. 2007; Banerjee et al. 2011). There were no differences between resistance rates of isolates from different food matrix origins nor were there reported differences between food and clinical isolates (Godic Torkar and Seme 2009). Finally, we noted that 100 % of the isolates showed intermediate resistance for clindamicin, which represents a relatively high proportion compared to previous works such as that of Ankolekar et al. (2009), Kim et al. (2011) and Chon et al. (2012). This intermediate resistance to clindamicin, and for some isolates toward erythromycin (12 %) or tetracycline (3 %), indicates the possible emergence of new multi-drug resistance patterns in foodborne *B. cereus* strains, which may constitute a sanitary risk.

In this study, we investigated the toxin profiles, phenotypic and metabolic patterns, and antibiotic resistance profile of *B. cereus* isolates from different food matrices (mainly starchy and dairy products). Our overall results indicate the huge intraspecific diversity of the foodborne collection. Most *B. cereus* isolates harbored the genetic determinants of several potential enterotoxins. Furthermore, *B. cereus* isolates demonstrated natural antibiotic resistance, indicating potential food poisoning and sanitary risks. The observed phenotypic

diversity was correlated clearly to isolation origin, particularly for starch hydrolysis. These findings reinforce the postulate that metabolic flexibility and toxigenic potential represent the main drivers for *B. cereus* ubiquity and adaptation in a given ecological niche, particularly in food.

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References

Abriouel H, Ben ON, Lucas LR, Martinez CM, Ortega E, Galvez A (2007) Differentiation and characterization by molecular techniques of *Bacillus cereus* group isolates from poto poto and degue, two traditional cereal-based fermented foods of Burkina Faso and Republic of Congo. J Food Protect 70:1165–1173

AFNOR (1996) NF ISO 7932. Microbiologie. Directives générales pour le dénombrement de *Bacillus cereus* Méthode par comptage des colonies à 30°C In: Analyse Microbiologique. Tome 1. Méthodes Horizontales. Contrôle de la Qualité des Produits Alimentaires. pp. 209–217. Paris La Défense, Association Française de Normalisation

Agaisse H, Gominet M, Okstad OA, Kolstø AB, Lereclus D (1999) PlcR is a pleiotropic regulator of extracellular virulence factor gene expression in *Bacillus thuringiensis*. Mol Microbiol 32:1043–1053

Al-Khatib M, Khyami-Horani BE, Shehabi A (2007) Incidence and characterization of diarrheal enterotoxins of fecal Bacillus cereus isolates associated with diarrhea. Diagn Microbiol Infect Dis 59: 383–387

Altayar M, Sutherland AD (2006) *Bacillus cereus* is common in the environment but emetic toxin producing isolates are rare. J Appl Microbiol 100:7–14

Andreeva ZI, Nesterenko VF, Fomkina MG, Ternovsky VI, Suzina NE, Bakulina AY, Solonin AS, Sineva EV (2007). The properties of *Bacillus cereus* hemolysin II pores depend on environmental conditions. Biochim Biophys Acta 1768:253–263

Andrews JM, Wise R (2002) Susceptibility testing of Bacillus species. J Antimicrob Chemother 49:1040–1042

Ankolekar C, Rahmati T, Labbé RG (2009) Detection of toxigenic *Bacillus cereus* and *Bacillus thuringiensis* spores in US rice. Int J Food Microbiol 128:460–466

Apetroaie C, Adersson MA, Spröer C, Tsitko I, Shaheen R, Jääskeläinen EL, Wijnands LM, Heikkilä R, Salkinoja-Salonen MS (2005) Cereulide-producing strains of *Bacillus cereus* show diversity. Arch Microbiol 184:141–151

Auger S, Galleron N, Bidnenko E, Ehrlich SD, Lapidus A, Sorokin A (2008) The genetically remote pathogenic strain NVH391-98 of the *Bacillus cereus* group is representative of a cluster of thermophilic strains. Appl Environ Microbiol 74:1276–1280

Banerjee M, Nair GB, Ramamurthy T (2011) Phenotypic and genetic characterization of *Bacillus cereus* isolated from the acute diarrhoeal patients. Indian J Med Res 133:88–95

Bartoszewicz M, Hansen BM, Swiecicka I (2008) The members of the *Bacillus cereus* group are commonly present contaminants of fresh and heat-treated milk. Food Microbiol 25:588–596

Carlin F, Brillard J, Broussole V, Clavel T, Duport C, Jobin M, Guinebretière M-H, Auger S, Sorokine A, Nguyen-The C (2009) Adaptation of *Bacillus cereus*, an ubiquitous worldwide-distributed foodborne pathogen, to a changing environment. Food Res Int 43: 1885–1894

Chaves JQ, Pires ES, Vivoni AM (2011) Genetic diversity, antimicrobial resistance and toxigenic profiles of *Bacillus cereus* isolated from food in Brazil over three decades. Int J Food Microbiol 147:12–16

Cherif A, Borin S, Rizzi A, Ouzari H, Boudabous A, Daffonchio D (2003) *Bacillus anthracis* diverges from related clades of the *Bacillus cereus* group in 16–23S ribosomal DNA intergenic transcribed spacers containing tRNA genes. Appl Environ Microbiol 69: 33–40

Choma C, Guinebretière MH, Carlin F, Schmitt P, Velge P, Granum PE, Nguyen-The C (2000) Prevalence, characterization and growth of *Bacillus cereus* in commercial cooked chilled foods containing vegetables. J Appl Microbiol 88:617–625

Chon JW, Kim JH, Lee SJ, Hyeon JY, Seo KH (2012) Toxin profile, antibiotic resistance, and phenotypic and molecular characterization of *Bacillus cereus* in Sunsik. Food Microbiol 32:217–222

Clinical and Laboratory Standards Institute (2010) Performance standards for antimicrobial susceptibility testing. Twentieth informational supplement. Document M100S20, CLSI. Wayne, PA

Drew WL, Barry AL, O'Toole R, Sherris JC (1972) Reliability of the Kirby-Bauer disc diffusion method for detecting methicillin-resistant strains of *Staphylococcus aureus*. Appl Microbiol 24:240–247

Drobniewski FA (1993) *Bacillus cereus* and related species. Clin Microbiol Rev 6:324–338

Ehling-Schulz M, Fricker M, Scherer S (2004) *Bacillus cereus*, the causative agent of an emetic type of food-borne illness. Mol Nutr Food Res 48:479–487

Ehling-Schulz M, Svensson B, Guinbretiere MH, Lindbäck T, Andersson M, Schulz A, Fricker M, Christiansson A, Granum PE, Märtlbauer E, Nguyen-The C, Salkinoja-Salonen M, Scherer S (2005) Emetic toxin formation of *Bacillus cereus* is restricted to a single evolutionary lineage of closely related strains. Microbiology 151:183–197

Ehling-schulz M, Fricker M, Grallert H, Rieck P, Wagner M, Scherer S (2006) Cereulide synthetase gene cluster from emetic *Bacillus cereus*: structure and location on a mega virulence plasmid related to *Bacillus anthracis* toxin plasmid pXO1. BMC Microbiol 6:20

Ettoumi B, Raddadi N, Borin S, Daffonchio D, Boudabous A, Cherif A (2009) Diversity and phylogeny of culturable spore-forming Bacilli isolated from marine sediments. J Basic Microbiol 49:S13–S23

Fagerlund A, Ween O, Lund T, Hardy SP, Granum PE (2004) Genetic and functional analysis of the cytK family of genes in *Bacillus cereus*. Microbiology 150:2689–2697

Fagerlund A, Lindbäck T, Granum PE (2010) *Bacillus cereus* cytotoxins Hbl, Nhe and CytK are secreted via the Sec translocation pathway. BMC Microbiol 10:304

Finlay WJJ, Logan NA, Sutherland AD (2002) *Bacillus cereus* emetic toxin production in cooked rice. Food Microbiol 19:431–439

Godic Torkar K, Seme K (2009) Antimicrobial susceptibility, beta-lactamase and enterotoxin production in *Bacillus cereus* isolates from clinical and food samples. Folia Microbiol 54:233–238

Gohar M, Okstad OA, Gilois N, Sanchis V, Kolstø AB, Lereclus D (2002) Two-dimensional electrophoresis analysis of the extracellular proteome of *Bacillus cereus* reveals the importance of the PlcR regulon. Proteomics 2:784–791

Granum PE (2007) *Bacillus cereus*. In: Doyle M, Beuchat L (eds) Food microbiology, fundamentals and frontiers, 3rd edn. ASM, Washington, pp 445–456

Guinebretière MH, Broussolle V, Nguyen-The C (2002) Enterotoxigenic profiles of food-poisoning and food-borne *Bacillus cereus* strains. J Clin Microbiol 40:3053–3056

Guinebretière MH, Fagerlund A, Granum PE, Nguyen-The C (2006) Rapid discrimination of cytK-1 and cytK-2 genes in *Bacillus cereus* strains by a novel duplex PCR system. FEMS Microbiol Lett 259: 74–80

Guinebretière MH, Thompson FL, Sorokin A, Normand P, Dawyndt P, Ehling-Schulz M, Svensson B, Sanchis V, Nguyen-The C, Heyndrickx M, De Vos P (2008) Ecological diversification in the *Bacillus cereus* group. Environ Microbiol 10:851–865

Guinebretière MH, Velge P, Couvert O, Carlin F, Debuyser ML, Nguyen-The C (2010) The food poisoning power of *Bacillus cereus* group strains varies according to phylogenetic affiliation (groups I–VII), not to species affiliation. J Clin Microbiol 48:1388–1391

Guinebretière MH, Auger S, Galleron N, Contzen M, De Sarrau B, De Buyser ML, Lamberet G, Fagerlund A, Granum PE, Lereclus D, De Vos P, Nguyen-The C, Sorokin A (2013) *Bacillus cytotoxicus* sp. nov. is a novel thermotolerant species of the *Bacillus cereus* Group occasionally associated with food poisoning. Int J Syst Evol Microbiol 63:31–40

Hansen BM, Hendriksen NB (2001) Detection of enterotoxic *Bacillus cereus* and *Bacillus thuringiensis* strains by PCR analysis. Appl Environ Microbiol 67:185–189

Helgason E, Tourasse NJ, Meisal R, Caugant DA, Kolstø AB (2004) Multilocus sequence typing scheme for bacteria of the *Bacillus cereus* group. Appl Environ Microbiol 70:191–201

Hoton FM, Andrup L, Swiecicka I, Mahillon J (2005) The cereulide genetic determinants of emetic *Bacillus cereus* are plasmid-borne. Microbiology 151:2121–2124

Hoton FM, Fornelos N, N'Guessan E, Hu X, Swiecicka I, Dierick K, Jääskeläinen E, Salkinoja-Salonen M, Mahillon J (2009) Family portrait of *Bacillus cereus* and *Bacillus weihenstephanensis* cereulide-producing strains. Environ Microbiol Reprod 1:177–183

Jensen MA, Webster JA, Straus N (1993) Rapid identification of bacteria on the basis of polymerase chain reaction—amplified ribosomal DNA spacer polymorphisms. Appl Environ Microbiol 59:945–952

Jensen LB, Baloda S, Boye M, Arestrup FM (2001) Antimicrobial resistance among Pseudomonas spp. and *Bacillus cereus* group isolated from Danish agricultural soil. Environ Int 26:581–587

Kalman S, Kiehne LK, Libs LJ, Yamamota T (1993) Cloning of novel CrylC-Type gene from a strain of *Bacillus thuringiensis* subsp. Galleriae. Appl Environ Microbiol 59:1131–1137

Kim JB, Kim JM, Kim CH, Seo KS, Park YB, Choi NJ, Oh DH (2010) Emetic toxin producing *Bacillus cereus* Korean isolates contain genes encoding diarrheal-related enterotoxins. Int J Food Microbiol 144:182–186

Kim JB, Park JS, Kim MS, Hong SC, Park JH, Oh DH (2011) Genetic diversity of emetic toxin producing *Bacillus cereus* Korean strains. Int J Food Microbiol 150:66–72

Lapidus A, Goltsman E, Auger S, Galleron N, Ségurens B, Dossat C, Land ML, Broussolle V, Brillard J, Guinebretiere MH, Sanchis V, Nguen-The C, Lereclus D, Richardson P, Wincker P, Weissenbach J, Ehrlich SD, Sorokin A (2008) Extending the *Bacillus cereus* group genomics to putative food-borne pathogens of different toxicity. Chem Biol Interact 171:236–249

Lund T, Debuyser ML, Granum PE (2000) A new cytotoxin from *Bacillus cereus* that may cause necrotic enteritis. Mol Microbiol 38:254–261

Martínez-Blanch JF, Sánchez G, Garay E, Aznar R (2011) Evaluation of phenotypic and PCR-based approaches for routine analysis of *Bacillus cereus* group foodborne isolates. Antonie Van Leeuwenhoek 99:697–709

McKillip JL (2000) Prevalence and expression of enterotoxins in *Bacillus cereus* and other *Bacillus* spp., a literature review. Antonie Van Leeuwenhoek 77:393–399

Moravek M, Dietrich R, Buerk C, Broussole V, Guinebretière MH, Granum PE, Nguyen-The C, Märtlbauer E (2006) Determination of the toxin potential of *Bacillus cereus* isolates by quantitative enterotoxin analysis. FEMS Microbiol Lett 257:293–298

Mossel DAA, Koopman MJ, Jongerius E (1967) Enumeration of *Bacillus cereus* in foods. Appl Microbiol 15:650–653

Notermans S, Batt CA (1998) A risk assessment approach for food-borne *Bacillus cereus* and its toxins. Symp Ser Soc Appl Microbiol 27: 51S–61S

Ouoba LI, Thorsen L, Varnam AH (2008) Enterotoxins and emetic toxins production by *Bacillus cereus* and other species of Bacillus isolated from Soumbala and Bikalga, African alkaline fermented food condiments. Int J Food Microbiol 124:224–230

Park YB, Kim JB, Shin SW, Kim JC, Cho SH, Lee BK, Ahn J, Kim JM, Oh DH (2009) Prevalence, genetic diversity, and antibiotic susceptibility of *Bacillus cereus* strains isolated from rice and cereals collected in Korea. J Food Protect 72:612–617

Pirttijärvi TS, Andersson MA, Scoging AC, Salkinoja-Salonen MS (1999) Evaluation of methods for recognizing strains of the *Bacillus cereus* group with food poisoning potential among industrial and environmental contaminants. Syst Appl Microbiol 2:133–144

Pruss BM, Dietrich R, Nibler B, Martlbauer E, Scherer S (1999) The hemolytic enterotoxin HBL is broadly distributed among species of the *Bacillus cereus* group. Appl Environ Microbiol 65:5436–5442

Roy A, Moktan B, Sarkar PK (2007) Characteristics of *Bacillus cereus* strains from legume-based Indian fermented foods. Food Control 18:1555–1564

Setlow B, Setlow P (1994) Heat inactivation of *Bacillus subtilis* spores lacking small, acid-soluble spore proteins is accompanied by generation of abasic sites in spore DNA. J Bacteriol 176:2111–2113

Shadrin AM, Shapyrina EV, Siunov AV, Severinov KV, Solonin AS (2007) *Bacillus cereus* pore-forming toxins hemolysin II and cytotoxin K: polymorphism and distribution of genes among representatives of the cereus group. Mikrobiologiia 76:462–470

Slamti L, Lereclus D (2002) A cell-cell signaling peptide activates the PlcR virulence regulon in bacteria of the *Bacillus cereus* group. EMBO J 21:4550–4559

Svensson B, Monthán A, Shaheen R, Andersson M, Salkinoja-Salonen M, Christiansson A (2006) Occurrence of emetic toxin producing *Bacillus cereus* in the dairy production chain. Int Dairy J 16:740–749

Swiecicka L, Van der Aurera GA, Mahillon J (2006) Hemolytic and non-hemolytic enterotoxin genes are broadly distributed among *Bacillus thuringiensis* isolated from wild mammals. Microb Ecol 52:203–212

Valero M, Hernández-Herrero LA, Fernández PS, Salmerón MC (2002) Characterization of *Bacillus cereus* isolates from fresh vegetables and refrigerated minimally processed foods by biochemical and physiological tests. Food Microbiology 19:491–499

Van der Voort M, Kuipers OP, Buist G, De Vos WM, Abee T (2008) Assessment of CcpA-mediated catabolite control of gene expression in *Bacillus cereus* ATCC 14579. BMC Microbiol 8:62. doi:10.1186/1471-2180-8-62

Vilain S, Luo Y, Hildreth MB, Brozel VS (2006) Analysis of the life cycle of the soil saprophyte *Bacillus cereus* in liquid soil extract and in soil. Appl Environ Microbiol 72:4970–4977

Wunschel D, Fox KF, Black GE, Fox A (1994) Discrimination among the *B. cereus* group, in comparison to *B. subtilis*, by structural carbohydrate profiles and ribosomal RNA spacer region PCR. Syst Appl Microbiol 17:625–635

Yang IC, Shih DY, Huang TP, Huang YP, Wang JY, Pan TM (2005) Establishment of a novel multiplex PCR assay and detection of toxigenic strains of the species in the *Bacillus cereus* group. J Food Protect 68:2123–2130