Characteristics of enterotoxin distribution, hemolysis, lecithinase, and starch hydrolysis of *Bacillus cereus* isolated from infant formulas and ready-to-eat foods

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ABSTRACT

Bacillus cereus is a ubiquitous environmental microbe implicated as a main cause of food poisoning with various symptoms, depending on the strain type and the isolation source. In this study, the potential virulence factors and biochemical properties of *B. cereus* isolated from infant formulas and ready-to-eat (RTE) foods were analyzed and compared. A total of 347 *B. cereus* strains were isolated and identified from 687 infant food formulas and RTE food samples. All the isolates had one or more enterotoxin genes, and one-half of the strains had all 3 enterotoxin genes (*hbl*, *nhe*, and *cytK*) that are involved in food poisoning in humans. Here, all the 3 genes were detected in 50% of the *B. cereus* isolates from RTE foods and only 14% of the isolates were identified from infant formulas. The latter harbored low *cytK* and *bceT*, and very low *hbl* genes. Most *B. cereus* isolates possessed the hemolysis gene, but not the *ces* gene. The infant formula isolates showed stronger hemolysis activity than the other isolates. In addition, 26% of the total isolates showed low lecithinase activities and 10% showed high lecithinase activities. A greater number of isolates from the infant formula showed high lecithinase activity than those from the RTE foods. Approximately 83% of the isolates were positive and 17% were negative for starch hydrolysis. Over 90% of the RTE food isolates and only 35% of the infant formula isolates were positive for starch hydrolysis. However, all the strains possessed *nhe*, but their harboring patterns of *hbl* and *cytK* were significantly different. Most starch-hydrolyzing strains possessed *hbl*, but only 23% nonstarch-hydrolyzing isolates possessed this gene. Moreover, very low nonstarch hydrolyzing strains harbored *cytK*. Most nonstarch-hydrolyzing isolates showed high lecithinase and strong hemolysis activities, and very low *hbl* and *cytK* harboring. In summary, most infant formula isolates showed stronger hemolysis and higher lecithinase activities with lower

frequency of harboring *hbl* and *cytK* and lower starch hydrolysis compared with RTE food isolates.

Key words: *Bacillus cereus*, enterotoxin gene, lecithinase activity, starch hydrolysis, infant formula

INTRODUCTION

Bacillus cereus is widely distributed in nature as a soil microorganism, and consumption of food contaminated with $>10^5$ cells/g of *B. cereus* may cause food poisoning. In particular, *B. cereus* strains form spores that facilitate their survival for a long period in dry foods even after heat treatment, owing to the thermal resistance offered by the spore form, thereby easily causing food poisoning (Goepfert et al., 1972; Kramer and Gilbert, 1989).

The clinical manifestations of *B. cereus* food poisoning are known to be caused by activities of exotoxins, lecithinase, protease, and hemolysis (Turnbull et al., 1979). In particular, *B. cereus* strains produce diverse types of toxins that cause food poisoning of the diarrheal or emetic type (Agata et al., 1995; Granum et al., 1996; Lund and Granum, 1997; Ombui et al., 1997; Schoeni and Wong, 2005). *Bacillus cereus* strains produce at least 5 different enterotoxins, excluding the emetic toxin. Hemolysin BL (HBL) and nonhemolytic enterotoxin (Nhe) are composed of 3 proteins, whereas EntFM, CytK, and BceT are composed of only one protein each (Granum and Lund, 1997; Hansen et al., 2003). The HBL, Nhe, and CytK toxins are implicated in food poisoning in humans, whereas HBL and Nhe toxins have been implicated in exerting cytotoxic and hemolytic activities. The CytK toxin was reported to cause severe symptoms and bloody diarrhea in a recent outbreak (Lund et al., 2000).

The second type of *B. cereus* toxin discovered in the 1970s was associated with the consumption of fried rice. This toxin caused food poisoning of the emetic type and was referred to as cereulide; it was a small, cyclic, heat-stable peptide (Marahiel et al., 1997). Because of the lack of a convenient method for detecting the emetic toxin, the role of *B. cereus* strains as the causative agents in emetic food poisoning remains underestimated.

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ed. Emetic toxin-producing *B. cereus* has been reported to neither produce diarrheal toxin nor hydrolyze starch (Ehling-Schulz et al., 2005; Pirhonen et al., 2005).

Besides the enterotoxins and emetic toxin, *B. cereus* produces several other virulence factors that damage the cell membrane. Several types of lecithinase, sphingomyelinase, and cereolysin are known to possess thiol group-activated hemolysis activity and have been associated with outbreaks (Brillard and Lereclus, 2007). Lecithinase secreted by *B. cereus* is similar to the α -toxin of *Clostridium perfringens* and has weak toxicity (Beecher et al., 2000). Lecithinase may play a synergistic role in dissolving red blood cells with sphingomyelinase.

Although *B. cereus* is known to contaminate a variety of foods as a soil microorganism, diarrheal poisoning has not been frequently reported. In addition, in the reported cases, the ability to cause diarrheal outbreaks has been attributed to several different types of strains (Guinebreière et al., 2002). According to the type of disease characterized in each country, emetic poisoning has been reported more than 10 times as frequently as diarrhea in Japan, but diarrheal poisoning has been reported mostly in Europe and North America. The difference in the occurrences of diarrheal poisoning in different countries depends on the differences in food consumption habits (Rhodehamel and Harmon, 1995; Granum and Baird-Parker, 2000). It is therefore necessary to study the profiles of these virulence factors because they may differ in *B. cereus* toxicity according to the type of contaminated food considered.

Recently, due to changes in dietary habits, the production of ready-to-eat (RTE) foods, refrigerated foods, and retort pouch foods has also increased. Although RTE foods offer the advantage of saving cooking time, the lack of heating and cooking processes involved in their consumption makes them vulnerable to contamination by food-poisoning bacteria. After first identifying *B. cereus* as a foodborne pathogen in Norway in 1949, its outbreaks have been reported to be related to the consumption of fried rice, pasta, cooked vegetables, salad, and sprouts in several countries (Lindqvist et al., 2000; Sewell and Farber, 2001; Sivapalasingam et al., 2004). Infant formula is a milk-based nonsterile food product fed to infants for whom breastfeeding is difficult and for infants with incompletely developed immune systems. A possibility of contamination of infant formula by opportunistic pathogens always exists, albeit with a low probability. The opportunistic pathogens in infant formula are classified into categories A, B, and C, with reference to the Food and Agricultural Organization (FAO)/World Health Organization (WHO) report (FAO/WHO, 2004). In fact, it has been reported that milk and milk-based products have

a very high prevalence of *B. cereus* (Reyes et al., 2007; Bartoszewicz et al., 2008; Hwang et al., 2008). There is a paucity of reports regarding *B. cereus* (category C pathogen) contamination of infant formula; therefore, it is important to analyze the relative hazard of a *B. cereus*-contaminated infant formula.

Therefore, in this study, the characteristics of the virulence factors and the biochemical characteristics of *B. cereus* from infant formula and RTE foods were analyzed.

MATERIALS AND METHODS

Isolation Samples

A total of 687 samples of powdered infant formula and RTE foods such as produce, cup-drinks sold by street vendors, instant cereals (*Sunsik*), sushi, and steamed rice rolled in *Gim* (*Gimbap*) in the market were collected for isolating *B. cereus*. The samples were collected during 4 intermittent survey research projects from the 2002 to 2007. Ninety-nine powdered infant formulas were reinforced with starch, oil, minerals, and vitamins in addition to the main cow milk component for infants aged <1 yr. These formulas were purchased from different production batches of various brands from local manufacturers as well as imported brands available at the time of collection. In addition, 193 samples of fresh food items such as salad, sprouts, fruits, and vegetables in RTE form were purchased from local markets. Moreover, 234 samples of complex foods such as *Sunsik*, *Gimbap*, and sushi purchased from local markets, and 161 cup-drinks were purchased at vending machines in the streets.

Isolation and Identification of *B. cereus*

Isolation and identification of *B. cereus* were carried out according to the conventional and modified method of Food Code established by the Korea Food and Drug Administration. The samples were homogenized with sterile buffered peptone water (Oxoid, Basingstoke, UK) and inoculated on mannitol-egg yolk-polymyxin B agar (MYP; Difco, Detroit, MI); the inoculated media were incubated at 36°C for 24 h. Following incubation, pink colonies with opaque halos were selected and subcultured at 36°C for 24 h on tryptic soy agar (TSA; Difco). The isolates grown on TSA were further confirmed on MYP agar and subjected to PCR using the primer pair BC1/BC2r, and classified as part of the *B. cereus* group (Yamada et al., 1999). *Bacillus cereus* strains were distinguished from *Bacillus thuringiensis* strains by PCR with the BT1/BT2r primer pair targeting the *gyrB* gene (Yamada et al., 1999). Finally, once

more the isolates were confirmed as *B. cereus* by PCR with the K3/K5 primer pair targeting the *cry* gene on the basis that *B. cereus* does not produce crystal toxin, whereas *B. thuringiensis* does (Kuo and Chak, 1996). *Bacillus cereus* KCCM 40935, *B. cereus* KCTC 1094, *B. thuringiensis* KCCM 11428, and *B. thuringiensis* KCTC 3452 type strains were used as a reference enterotoxin control for PCR. To prepare DNA template, cells were collected from 1 loop of colony on TSA incubated for 24 h. The pellet was washed twice with 1 mL of sterile distilled water after centrifugation at $9,810 \times g$ for 5 min at 4°C and boiled for 5 min. The supernatants were used as DNA templates. Biochemical tests were performed after sufficiently activating *B. cereus* on TSA. The biochemical tests, including Gram staining, glucose fermentation, Voges-Proskauer reaction, nitrate reduction, and motility, were performed as per the Food and Drug Administration Bacterial Analytical Manual methods (Rhodehamel and Harmon, 1995).

Detection of Exotoxin Genes

The following *B. cereus* exotoxin genes were detected and confirmed by PCR: HBL, Nhe, cytotoxin K, enterotoxin T, enterotoxin FM, and emetic toxin genes. To detect the *hbl* gene, PCR was performed with the HBLC-N/HBLC-C primer pair targeting the *hblC* component gene. The *hblC*-nonharboring *B. cereus* were further subjected to PCR with the HBLD-N/HBLD-C and HBLA1/HBLA2 primers targeting *hblD* and *hblA* component genes, respectively (Rowan et al., 2003). To detect *nhe*, PCR was performed with the *nhe344S/nheB843A* primer pair targeting the *nheA* component gene. When *nheA* was not detected, the NBF/NBR and NCF/NCR primers were used to target *nheB* and *nheC* (Ghelardi et al., 2002; Guinebretière et al., 2002). To detect *cytK*, PCR was performed with the F2/R7 primer pair targeting *cytK* (Lund et al., 2000). The ETF/ETR primer pair was used to detect *becT* of enterotoxin T, and the ENTA/ENTB primer was used to detect *entFM* of enterotoxin FM (Asano et al., 1997; Ghelardi et al., 2002). For detecting the emetic toxin gene, PCR was performed with the CesF1/CesR2 primer pair targeting *ces* (Ehling-Schulz et al., 2005).

Hemolysis and Lecithinase Activity Analyses

Bacillus cereus was incubated for 24 h at 36°C on TSA with sheep blood. After incubation, the diameter of the hemolytic zone formed was measured to determine the extent of hemolysis by using calipers (Anyi Instrument, Guilin, China). For determining the lecithinase activity, *B. cereus* was inoculated in *B. cereus*-selective MYP agar and incubated for 21 h at 36°C, followed by mea-

surement of the length of the opaque halo formed on the media.

Starch Hydrolysis

To confirm the starch hydrolysis activity, *B. cereus* isolates were grown on nutrient agar (Oxoid) containing 1% soluble starch and incubated at 30°C for 24 h. Hydrolysis was observed as formation of a colorless zone around a *B. cereus* colony after flooding the plate with Gram's iodine, followed by measurement of the length of the zone using calipers.

RESULTS AND DISCUSSION

Isolation and Identification of Foodborne *B. cereus*

In total, 507 *B. cereus* group isolates were isolated from 687 samples of various food items by culturing on selective MYP medium, followed by identification by biochemical tests and PCR with the BC1/Bc2r primer pair. Of the 507 isolates, 165 were isolated from 99 powdered infant formulas, 144 from fresh foods, and 198 from complex foods (Table 1).

Biochemical tests were performed to differentiate the *B. cereus* group as per Bacterial Analytical Manual methods, such as lecithinase activity, motility, nitrate reduction, Voges-Proskauer, β -hemolysis, and anaerobic utilization of glucose. The biochemical characteristics of the isolates varied widely, except that all showed positive reactions for lecithinase activity and anaerobic utilization of glucose (data not shown). In particular, approximately 80% of the total isolates showed motility and 93% showed β -hemolysis. Typical *B. cereus* showed positive reactions for all of the above-mentioned tests (Rhodehamel and Harmon, 1995). It has been also reported that *B. cereus* and *B. thuringiensis* of the *B. cereus* group can be distinguished by hemolytic activity, motility in a selective medium, and crystal toxin protein from *Bacillus mycoides* and *Bacillus anthracis* (Lechner et al., 1998; Nakamura, 1998; Granum and Baird-Parker, 2000). However, several of the *B. cereus* isolates in this study showed atypical biochemical characteristics not reported earlier. Therefore, it was vital to perform PCR targeting specific genes for the *B. cereus* group to differentiate and identify the isolates.

To distinguish *B. cereus* from *B. thuringiensis* with similar phenotypic and genotypic characteristics, all 507 isolates of the *B. cereus* group were subjected to PCR with the BT1/BT2r primer pair targeting *gyrB*, which finally identified 379 *B. cereus* strains. The selected *B. cereus* strains were subjected to PCR with the K3/K5 primer pair targeting *cry* because it is expressed as crystal toxin by *B. thuringiensis* only. From among the

Table 1. Isolation and identification of *Bacillus cereus* from infant formula and ready-to-eat (RTE) food collected from the local area

Food sample (no. of samples)	No. of <i>B. cereus</i> group isolates	No. of <i>Bacillus</i> strains identified with <i>gyrB</i> and <i>cry</i> PCR primers		
		<i>B. cereus</i>	<i>B. thuringiensis</i>	Others
Infant formulas, powdered (99)	165	69	96	0
RTE, fresh foods (193)	144	115	28	1
Salad (25)	38	23	15	0
Sprout (65)	63	59	4	0
Produce (43)	43	33	9	1
RTE, complex foods (395)	198	163	28	7
Street-vendor cup drink (161)	56	45	7	4
<i>Sunsik</i> , powdered grain (94)	86	69	14	3
Sushi (105)	29	27	2	0
<i>Gimbap</i> (35)	27	22	5	0
Total (687)	507	347	152	8

379 *B. cereus* strains tested, 32 strains were confirmed to be *B. thuringiensis* harboring *cry*. Finally, 347 *B. cereus*, 152 *B. thuringiensis*, and 8 *Bacillus* spp. were identified from among 507 isolates belonging to the *B. cereus* group. Thus, the isolation ratio of 347 *B. cereus* strains from 687 food samples was 50%. The prevalence of the isolates from the food items of dried milk product, rice, ground roasted coffee, and German infant formula was similar to this ratio reported here (Becker et al., 1994; Shaheen et al., 2006; Reyes et al., 2007; Ankolekar et al., 2009; Chaves et al., 2012). However, the prevalence of the isolates for the weaning cereal was found to be less than the reported value (Kunene et al., 1999). In this study, 80% of the *B. cereus* group isolated from RTE foods were *B. cereus*, but only 40% of the *B. cereus* group from infant formulas were *B. cereus*.

Exotoxin Gene Distribution of *B. cereus*

To determine the distribution of exotoxins, 347 *B. cereus* isolates were analyzed for 1 emetic toxin gene, *ces*, and 5 enterotoxin genes, *hbl*, *nhe*, *cytK*, *bceT*, and *entFM*, by PCR with the primers listed in Table 2. It has been reported that some isolates may cause poisoning with only 1 or 2 genes from among *hbl* or *nhe* component genes (Hansen and Hendriksen, 2001; Ngamwongsatit et al., 2008). Therefore, the presence of any one of the *hblC*, *hblD*, and *hblB* component genes coding for lytic component L₂, L₁, and binding component B of HBL toxin was considered positive for *hbl*. In addition, the presence of any one of the *nheA*, *nheB*, and *nheC* component genes coding for Nhe toxin was considered positive for *nhe*. Those were due to the reports that some isolates might cause poisoning with only 1 or 2 genes among *hbl* component genes or *nhe* component genes (Hansen and Hendriksen, 2001; Ngamwongsatit et al., 2008).

Among the total 347 *B. cereus* isolates, 290 (84%) harbored *hbl* (Table 3). More than 95% of the strains isolated from RTE foods possessed *hbl* and approximately 30% of isolates from infant formulas possessed *hbl*. Some previous studies have reported that 60 to 90% of the strains isolated from various food items harbored *hbl* (Ankolekar et al., 2009; Chaves et al., 2012; Chon et al., 2012). Here, although the RTE food isolates complied with these earlier reports, unpredictably, only 30% of the infant formula isolates possessed *hbl*. In addition, the isolates from all food samples harbored *nhe*. These results are consistent with the available results of *B. cereus* harboring *nhe* (Guinebrerière et al., 2002; Ankolekar et al., 2009; Chaves et al., 2012; Chon et al., 2012). The CytK toxin is known to induce diarrhea similarly to the HBL and Nhe toxins (Lund and Granum, 1997). Among the isolates in this study, 167 strains (48%) possessed *cytK*, but its prevalence was low compared with that of other enterotoxin genes; this observation is consistent with those of earlier studies reporting 45 to 80% isolates from other foods harboring *cytK* (Chon et al., 2012; Lee et al., 2012). However, the prevalence of *cytK* in the infant formula isolates was less than one-half of that in the RTE food isolates. Similar to other studies, in this study, 277 (80%) and 323 (93%) of isolates possessed the genes of enterotoxin T and enterotoxin FM, respectively, that may have the same biological activities as HBL and Nhe, although evidence for their contribution to food poisoning is unclear. Among the infant formula isolates, the *entFM* harboring ratio was similar to that reported previously, but the *bceT* harboring ratio was 36% and thus very low. However, the emetic toxin gene causing emetic syndrome was not detected in any of the 347 *B. cereus* strains tested (data not shown). Thus, the distribution pattern of the 5 enterotoxin genes of *B. cereus* from RTE foods was similar to those reported earlier. How-

Table 2. Oligonucleotide primers for detecting members of the *Bacillus cereus* group

Target gene	Primer	Primer sequence (5'→3')	Product size (bp)	Reference
<i>gyrB</i>	BC1	ATTGGTGACACCGATCAAACA	365	Yamada et al. (1999)
	BC2r	TCATACGTATGGATGTTATTC		
<i>gyrB</i>	BT1	ATCGGTGATACAGATAAGACT	368	Yamada et al. (1999)
	BT2r	CCTTCATACGTATGAATATTATTT		
<i>cry</i>	K3	GCTGTGACACGAAGGATATAGCCAC	1,600–1,700	Kuo and Chak (1996)
	K5	AGGACCAGGATTTACAGGAGG		
<i>hblA</i>	HblA1	GCTAATGTAGTTTCACCTGTAGCAAC	873	Rowan et al. (2003)
	HblA2	AATCATGCCACTGCGTGACATATAA		
<i>hblC</i>	HBLC-N	AATAGGTACAGATGGAACAGG	399	Rowan et al. (2003)
	HBLC-C	GGCTTTCATCAGGTCATACTC		
<i>hblD</i>	HBLD-N	AATCAAGAGCTGTACAGTAAT	439	Rowan et al. (2003)
	HBLD-C	CACCAATTGACCATGCTAAT		
<i>nheA</i>	nheA344S	TACGCTAAGGAGGGGCA	499	Ghelardi et al. (2002)
	nheB843A	GTTTTTATTGCTTCATCGGCT		
<i>nheB</i>	NBF	TTTAGTAGTGGATCTGTACGC	743	Guinebretière et al. (2002)
	NBR	TTAATGTTTCGTTAATCCTGC		
<i>nheC</i>	NCF	TGGATTCCAAGATGTAACG	683	Guinebretière et al. (2002)
	NCR	ATTACGACTTCTGCTTGTGC		
<i>bceT</i>	ETF	TTACATTACCAGGACGTGCTT	428	Ghelardi et al. (2002)
	ETR	TGTTGTGATTGTAATTTTCAGG		
<i>entFM</i>	ENTA	ATGAAAAAAGTAATTTGCAGG	1,269	Asano et al. (1997)
	ENTB	TTAGTATGCTTTTGTGTAACC		
<i>cytK</i>	F2	AACAGATATCGGTCAAAATGC	623	Lund et al. (2000)
	R7	CGTGCATCTGTTTCATGAGG		
<i>ces</i>	CesF1	GGTGACACATTATCATATAAGGTG	1,271	Ehling-Schluz et al. (2005)
	CesR2	GTAAGCGAACCTGTCTGTAAACAACA		

ever, interestingly, the infant formula isolates harbored lower *cytK* and *bceT*, and very low *hbl*.

All the isolates in this study possessed one or more of the enterotoxin genes, and 43% of the total isolates possessed all 5 diarrheal enterotoxin genes. In particular, 46% of the total isolates possessed all 3 enterotoxin genes, *hbl*, *nhe*, and *cytK*, which reportedly cause food poisoning in humans. Here, 50% of the RTE food isolates possessed the 3 genes, whereas only 14% of the infant formula isolates possessed these genes. Very low *B. cereus* poisoning has been reported in milk and dairy products, unlike the actual prevalence of *B. cereus*, probably because milk is not suitable for toxin production (Becker et al., 1994; Reyes et al., 2007). Productions of HBL and NHE protein for some isolates were confirmed using commercial toxin detection kit (data not shown). However, the genes might not

be expressed in some culture conditions (Rowan and Anderson, 1997). This study thus suggests that the low outbreak of *B. cereus* poisoning due to infant formula consumption can be attributed to *B. cereus* containing fewer enterotoxin genes.

Characteristics of Hemolysis and Lecithinase for *B. cereus* Isolates

In total, 93 of the 347 isolates (27%) showed weak or no hemolysis and 62 (18%) showed strong hemolysis (Table 4). The infant formula isolates showed a stronger level of hemolysis than the other isolates. It has been reported that 95% of the 133 *B. cereus* isolates from honey demonstrated hemolysis activity (López and Alippi, 2007), and the isolates causing food poisoning showed very strong hemolysis (Stec, 1990; Pirhonen

Table 3. Distribution of enterotoxin genes of *Bacillus cereus* isolates from infant formulas and ready-to-eat (RTE) foods

Source of isolation (no. of isolates)	No. (%) of <i>B. cereus</i> coding the gene(s) at their sources						
	<i>hbl</i>	<i>nhe</i>	<i>cytK</i>	<i>bceT</i>	<i>entFM</i>	3 genes ¹	5 genes ²
Infant formulas (69)	21 (30)	68 (99)	14 (20)	25 (36)	65 (94)	10 (14)	10 (14)
RTE, fresh foods (115)	114 (99)	115 (100)	74 (64)	107 (93)	107 (93)	71 (62)	64 (56)
RTE, complex foods (163)	155 (95)	163 (100)	79 (48)	145 (89)	151 (93)	77 (47)	74 (45)
Total (347)	290 (84)	346 (100)	167 (48)	277 (80)	323 (93)	158 (46)	148 (43)

¹Three genes = *hbl* + *nhe* + *cytK*.

²Five genes = *hbl* + *nhe* + *cytK* + *bceT* + *entFM*.

Table 4. Distribution of *Bacillus cereus* showing hemolysis isolated from infant formulas and ready-to-eat (RTE) foods

Source of isolation (no. of isolates)	No. (%) of <i>B. cereus</i> showing hemolysis ¹		
	Strong	Intermediate	Weak/none
Infant formulas (69)	21 (30)	37 (54)	11 (16)
RTE, fresh foods (115)	20 (17)	60 (52)	34 (30)
RTE, complex foods (163)	21 (13)	95 (58)	48 (29)
Total (347)	62 (18)	192 (55)	93 (27)

¹The hemolysis activity was measured as the length of the zone from the edge of a colony as follows: >3-mm length = strong; 1.5–3-mm length = intermediate; and <1.5-mm length = weak/none after 24-h incubation in sheep blood agar.

et al., 2005). In general, most *B. cereus* isolated from various food items show hemolysis. According to another report, all 60 of the 222 *B. cereus* isolates showing strong hemolysis did not produce emetic toxin, but 77 isolates showing weak or no hemolysis produced cereulide (Andersson et al., 2004). However, most of the newly isolated *B. cereus* in this study showed hemolytic activity and did not possess *ces*. The hemolytic activity of infant formula isolates was found to be a little stronger than that of the other isolates.

In total, 91 of the 347 *B. cereus* isolates (26%) showed low lecithinase activities, and 35 isolates (10%) showed high lecithinase activities (Table 5). Among the isolates showing low activity, almost all of the strains were isolated from RTE fresh foods and complex foods and only 4 strains were isolated from infant formula, constituting 6% of all isolates from the infant formulas. In contrast, among the isolates showing high lecithinase activity, 18 were isolated from RTE foods and 17 were obtained from infant formula, amounting to 25% of all isolates from infant formulas. Thus, the ratio of isolates showing high lecithinase activity was higher for infant formulas than for other foods. *Bacillus cereus* from milk-based products such as infant formula seemed to have higher lecithinase activity because they could use the lecithin component of the milk fat globule for nutrition. In Table 6, 43% of the isolates with high lecithinase activity showed strong hemolytic activity

and 47% of the isolates with low lecithinase activity showed weak or no hemolytic activity. These results suggest that lecithinase may somehow be involved in hemolysis, even if it is not strictly correlated, as also observed by other studies (Stec, 1990; Beecher et al., 2000). In fact, lecithinase has been suggested as a putative virulence factor in systemic infection with enterotoxin (Drobniewski, 1993). Isolates with low lecithinase activity were also found to harbor *hbl* and *cytK* at about a 2-times greater frequency than isolates with high lecithinase activities. Thus, the infant formula isolates showed stronger hemolysis and higher lecithinase activities with lower frequency of harboring of *hbl* and *cytK* compared with the other isolates.

Starch Hydrolysis and Enterotoxin Gene Profile of the Isolates

The starch hydrolysis characteristics of the isolates were also analyzed as they have been proposed as an indicator that characterizes diarrheal and emetic poisoning by *B. cereus*. In total, 287 of the 347 isolates (83%) were positive for starch hydrolysis and 60 isolates (17%) were negative for starch hydrolysis in Table 7. More than 90% of the RTE food isolates were positive for starch hydrolysis; however, only 35% of the infant formula isolates were positive for starch hydrolysis. In addition, approximately 45 *B. cereus* isolates

Table 5. Distribution of *Bacillus cereus* showing lecithinase activity from infant formulas and ready-to-eat (RTE) foods

Source of isolation (no. of isolates)	No. (%) of <i>B. cereus</i> producing lecithinase on MYP ¹ agar		
	High activity ²	Intermediate activity	Low activity
Infant formulas (69)	17 (25)	48 (69)	4 (6)
RTE, fresh foods (115)	8 (7)	73 (63)	34 (30)
RTE, complex foods (163)	10 (5)	100 (61)	53 (33)
Total (347)	35 (10)	221 (64)	91 (26)

¹MYP = mannitol-egg yolk-polymyxin B.

²The activity levels were measured as the length of the lecithinase halo zone from the edge of a colony as follows: high activity ≥ 3.5 mm; 1.5 mm ≤ intermediate activity <3.5 mm; low activity <1.5 mm.

Table 6. Relationship of lecithinase-producing *Bacillus cereus* from infant formulas and ready-to-eat (RTE) foods showing hemolysis and harboring enterotoxin genes *hbl*, *nhe*, and *cytK*

Virulence factor	No. (%) of lecithinase-producing <i>B. cereus</i>	
	High (n = 35)	Low (n = 88)
Hemolysis		
Strong	15 (43)	5 (6)
Intermediate	18 (51)	42 (48)
Weak/none	2 (6)	41 (47)
Enterotoxin gene		
<i>hbl</i>	19 (54)	84 (95)
<i>nhe</i>	35 (99)	88 (100)
<i>cytK</i>	8 (23)	57 (65)

(65%) from infant formula samples were negative for starch hydrolysis, which is twice as many as the number of positive strains. The strains negative for starch hydrolysis did not possess *ces*. The starch hydrolysis test has been proposed as an indicator to identify the emetic type of *B. cereus* (Ehling-Schulz et al., 2005; Pirhonen et al., 2005). However, some studies have reported that the characteristic of starch hydrolysis is not directly related to the production of emetic toxin (Rahmati and Labbe, 2008; Ankolekar et al., 2009). According to these results, the starch hydrolysis activity did not seem to be related directly to the secretion of emetic toxin.

Nonstarch-hydrolyzing *B. cereus* strains were analyzed to assess the relationship of starch hydrolysis with other virulence factors in Table 8. The 287 strains positive for starch hydrolysis showed high to low lecithinase activity. Thirty percent of the starch-hydrolyzing isolates showed low lecithinase activity. In clear contrast, all of the 60 nonstarch-hydrolyzing strains showed more than the intermediate level of lecithinase activity. Therefore, nonstarch-hydrolyzing

Table 7. Starch hydrolysis of *Bacillus cereus* isolated from infant formulas and ready-to-eat (RTE) foods on nutrient agar containing starch

Source of isolation (no. of <i>B. cereus</i>)	No. (%) of starch-hydrolyzing <i>B. cereus</i> isolates	
	Hydrolysis	Nonhydrolysis
Infant formulas (69)	24 (35)	45 (65)
RTE, fresh foods (115)	113 (98)	2 (2)
RTE, complex foods (163)	150 (92)	13 (8)
Total (347)	287 (83)	60 (17)

strains had a tendency to have the lecithinase enzyme. Here all the strains showing the high lecithinase activity in the group of nonstarch hydrolysis were isolated from infant formula (data not shown). However, no significant correlation was observed between hemolysis and starch hydrolysis. Regarding enterotoxin genes, all isolates harbored *nhe*, but the harboring pattern for *hbl* and *cytK* was significantly different. In particular, only 23% of the nonstarch-hydrolyzing strains possessed *hbl*, whereas most of the starch-hydrolyzing strains possessed the gene. In addition, very few of the nonstarch-hydrolyzing strains harbored *cytK*. *Bacillus cereus* harboring enterotoxin genes was reported to stimulate the production of diarrheal enterotoxins by using hydrolysates such as glucose and maltodextrin from starch hydrolysis (Rowan and Anderson, 1997). Therefore, we can infer that starch hydrolysis for *B. cereus* is required for good expression of the enterotoxin genes such as *hbl* and *cytK*.

In conclusion, nonstarch-hydrolyzing *B. cereus* isolates were presented in infant formula at a high frequency, and these isolates showed high lecithinase activity and a low frequency of *hbl* and *cytK*, suggesting that these genes might not be required in a milk-based environment.

Table 8. Relationship of starch-hydrolyzing *Bacillus cereus* from powdered infant formulas and ready-to-eat (RTE) foods showing lecithinase and hemolysis activities and harboring enterotoxin genes *hbl*, *nhe*, and *cytK*

Virulence factor	No. (%) of starch-hydrolyzing <i>B. cereus</i> isolates	
	Hydrolysis (n = 287)	Nonhydrolysis (n = 60)
Lecithinase activity		
High	21 (7)	14 (23)
Intermediate	178 (62)	46 (77)
Low	88 (31)	0
Hemolysis		
Strong	50 (17)	12 (20)
Intermediate	152 (53)	39 (65)
Weak/none	85 (30)	9 (15)
Enterotoxin gene		
<i>hbl</i>	276 (96)	14 (23)
<i>nhe</i>	287 (100)	60 (100)
<i>cytK</i>	166 (58)	3 (5)

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