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Production of Diarrheal Enterotoxins and Other Potential Virulence Factors by Veterinary Isolates of *Bacillus* Species Associated with Nongastrointestinal Infections

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With the exceptions of *Bacillus cereus* and *Bacillus anthracis*, *Bacillus* species are generally perceived to be inconsequential. However, the relevance of other *Bacillus* species as food poisoning organisms and etiological agents in nongastrointestinal infections is being increasingly recognized. Eleven *Bacillus* species isolated from veterinary samples associated with severe nongastrointestinal infections were assessed for the presence and expression of diarrheagenic enterotoxins and other potential virulence factors. PCR studies revealed the presence of DNA sequences encoding hemolysin BL (HBL) enterotoxin complex and *B. cereus* enterotoxin T (BceT) in five *B. cereus* strains and in *Bacillus coagulans* NB11. Enterotoxin HBL was also harbored by *Bacillus polymyxa* NB6. After 18 h of growth in brain heart infusion broth, all seven *Bacillus* isolates carrying genes encoding enterotoxin HBL produced this toxin. Cell-free supernatant fluids from all 11 *Bacillus* isolates demonstrated cytotoxicity toward human HEp-2 cells; only one *Bacillus licheniformis* strain adhered to this test cell line, and none of the *Bacillus* isolates were invasive. This study constitutes the first demonstration that *Bacillus* spp. associated with serious nongastrointestinal infections in animals may harbor and express diarrheagenic enterotoxins traditionally linked to toxigenic *B. cereus*.

Members of the *Bacillus* genus are ubiquitous soil microorganisms that frequently contaminate foods (21, 22, 26). With the exceptions of *Bacillus anthracis* and *Bacillus cereus*, *Bacillus* species are generally perceived to be inconsequential and of little clinical significance (8). A number of food poisoning incidents can be attributed to *B. cereus*, and this bacterium is known to cause a variety of nongastrointestinal diseases as well as two different types of food poisoning (for reviews, see references 12, 19, 21, and 22), which are characterized by either diarrhea or emesis. The diarrheal type is attributed to heat-labile enterotoxins, namely, the hemolysin BL (HBL) and non-hemolytic enterotoxin protein complexes, and to a *B. cereus*-related enterotoxic protein T (BceT). In *B. cereus* strain F837/76, the three HBL components are encoded by an operon containing *hblC*, *hblD*, and *hblA*, in that order, which respectively encode two lytic components designated L_2 and L_1 and a binding component designated B (30). We recently reported on the abilities of different clinical and food isolates of *B. cereus* and other *Bacillus* spp. to express diarrheal enterotoxins HBL and BceT after growth in reconstituted infant milk formulae (26). Previous research has also shown that 14 different *Bacillus* species isolated from raw milk and from the farm environment may have the potential to produce diarrheal enterotoxins (4). The emetic type is caused by a heat-stable dodecadepsideptide, cereulide (21).

The relevance of other *Bacillus* species as food poisoning organisms and as etiological agents in nongastrointestinal infections, including local, deep-tissue, and systemic infections, is being increasingly recognized (8). Nongastrointestinal infections have been seen primarily in individuals who are intravenous drug abusers or immunocompromised as a consequence of infection with human immunodeficiency virus, chemotherapy, or malignancy (4, 28). *B. cereus* strains isolated from nongastrointestinal infections have shown the ability to synthesize many virulence factors, including necrotizing exotoxin-like hemolysins, phospholipases, collagenases, and proteases (8).

Due to their endospore-forming abilities, these bacteria tolerate adverse conditions better than most bacterial enteropathogens do and may proliferate in a wide range of environments, including processed and untreated foods (22, 26). Raw milk is frequently contaminated by *Bacillus* endospores that originate from bedding, fodder, dung, soil, improperly cleaned milking utensils, and the surrounding environment (35). Contamination of raw milk may also occur through infection of a cow's udder, and serious herd outbreaks of bovine mastitis have been previously attributed to members of this genus (17, 35). *Bacillus* spp. have also been implicated as causative agents of mastitis and abortions in other animals, including cows (17), pigs (18), horses (23), water buffalo (10), and dromedary camels (37).

We report the detection of diarrheagenic enterotoxins HBL and BceT (traditionally harbored by toxigenic *B. cereus*) in a number of veterinary isolates of *Bacillus* spp. that were associated with serious nongastrointestinal infections in animals. We also report on the ability of these *Bacillus* isolates to be cytotoxic towards human HEp-2 cells and to express HBL enterotoxins. All experiments were performed in triplicate, with averages and standard errors of results shown. Differences in bacterial adherence, invasion, and cytotoxicity were exam-

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TABLE 1. Bacterial strains used

Bacterial species	Isolate	Provider	Reference no.	Isolate source	Gross lesion(s)	Associated disease
B. cereus	NB3	SAC	C20593	Bovine milk	None recorded	Bovine mastitis
	NB23	SAC	PC181	FSC, placenta, foetal lung, and liver	None recorded	Bovine abortion
	NB27	SAC	C175	Bovine milk	None found	Bovine mastitis
	NB35	SAC	S20247	Ovine FSC	None found	Ovine abortion
	NB40	SAC	S20254	Ovine FSC	None recorded	Ovine abortion
	NB50	SAC	AYRS31375	Lamb brain	—[b]	
	NB51	SAC	AURM31973	Caprine milk	None recorded	Caprine mastitis
	SU52	NCTC	11145	Human stool	—	Diarrheal food poisoning[a]
B. licheniformis	NB42	SAC	S20282	Ovine FSC	None found	Ovine abortion
	NB14	SAC	C205	Bovine placenta, FSC, fotal lung and liver	None recorded	Bovine abortion
B. polymyxa	NB6	SAC	C83	Bovine fotal lung	None recorded	Bovine abortion
B. coagulans	NB11	SAC	C70	Bovine FSC, fotal lung and liver	None recorded	Bovine abortion
L. monocytogenes	SU1	NCTC	11994	Cerebrospinal fluid	—	Adult meningitis[a]
E. coli	HB101	ATCC	33694	Laboratory K-strain	—	None

[a] Associated with disease in humans only.
[b] —, not known.

ined in HEp-2 cells at 95 or 99.9% confidence intervals by using analysis of variance (one-way or balanced models) with Minitab (State College, Pa.) software (release 11).

Isolation and identification of *Bacillus* species associated with serious nongastrointestinal infections in animals. The *Bacillus* strains used in this study (Table 1) were obtained from the diagnostic laboratories of the Scottish Agricultural College (SAC), Veterinary Science Division, Scotland; the American Type Culture Collection (ATCC), Manassas, Va.; and the National Collection of Type Cultures (NCTC), Public Health Laboratory Service, Colindale, United Kingdom. Eleven members of the genus *Bacillus* comprising seven *B. cereus* strains, two *Bacillus licheniformis* strains, one *Bacillus polymyxa* strain, and one *Bacillus coagulans* strain were isolated from veterinary samples associated with serious nongastrointestinal infections (Table 1); of these veterinary samples, 25-g amounts from the placenta, fetal stomach contents (FSC), and fetal brain, lung, and liver were aseptically transferred to Todd-Hewitt broth (Oxoid, Basingstoke, United Kingdom) for 24 h at 37°C. These particular organs and materials are routinely examined in cases of animal abortions. Samples were transferred directly and after the aforementioned overnight enrichment onto 7% (vol/vol) horse erythrocyte-blood agar plates (Oxoid) and were incubated for 48 h at 37°C in a 5% CO_2 atmosphere (CO_2 incubator; LEEC Ltd., Nottingham, England). The diseases associated with the veterinary *Bacillus* isolates ranged from severe infections, such as induction of fetal abortions in sheep and cattle (e.g., from isolates *B. cereus* NB35, *B. licheniformis* NB42, *B. polymyxa* NB6, and *B. coagulans* NB11), to the less serious mastitis (e.g., from *B. cereus* NB3) (Table 1).

The identity of each *Bacillus* isolate was confirmed by performing a sequence of characteristic morphological and physiological tests described previously (29) and by using miniaturized biochemical API 50 CHB and API 20E galleries (bioMérieux, Marcy l'Etoile, France). All *Bacillus* isolates exhibited characteristic morphological, physiological, and biochemical properties to the species level. The bacteria were stored at −70°C (Microbank System; Pro-Lab Diagnostics, Richmond Hill, Ontario, Canada) to prevent loss of virulence characteristics.

Ability of *Bacillus* veterinary strains to adhere to, invade, and produce a cytotoxic effect on epithelial cells. The ability of *Bacillus* test strains to adhere to and invade HEp-2 cells was determined after 18 h of growth in brain heart infusion (BHI) broth by previously described procedures (25), with minor modifications. HEp-2 monolayers were grown for 24 h in a 5% CO_2 atmosphere (CO_2 incubator; LEEC Ltd.) at 37°C in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS) (Gibco Life Sciences, Paisley, Scotland) in 24-well tissue culture plates (Scientific Lab Supplies, Nottingham, England) seeded with approximately 10^5 cells per well. Prior to assay, the monolayers were washed three times with DMEM and the monolayers were inoculated with 1 ml of bacterial culture containing ~10^7 CFU ml^{-1} (in DMEM with 10% FCS) in triplicate followed by a 2-h incubation at 37°C in a 5% CO_2 atmosphere. After incubation, the monolayers were washed three times with DMEM to remove any nonadherent cells and then 1 ml of DMEM containing 10% FCS was added to each well of one of the test plates, which were incubated for 2 h. For the invasion assays, 1 ml of DMEM containing 10% FCS and 100 μg of gentamicin ml^{-1} (Gibco) was added to each well of the other 24-well plate, which was similarly incubated for 2 h. The monolayers were then washed three times with DMEM, and the tissue culture cells were lysed with 1 ml of 1% (vol/vol) Triton X-100 in distilled water (Sigma, Dorset, England) for 5 min at 37°C. Samples (0.1 ml) of lysate from each tissue culture plate were serially diluted in 0.9 ml of sterile distilled water, with subsequent enumeration by plating 20 μl of appropriate 10-fold dilutions on BHI agar plates. *Listeria monocytogenes* NCTC 11994 and *Escherichia coli* ATCC 33694 were used as positive and negative control strains, respectively, in tests of adherence to and invasion of HEp-2 cells.

Results showed that 10 (91%) of 11 *Bacillus* spp. isolated from the veterinary environment were unable to adhere to or invade HEp-2 cells. Only *B. licheniformis* NB14 adhered to

TABLE 2. Potential virulence factor expression by *Bacillus* spp. isolated from veterinary and clinical samples

Bacterial species	Isolate	Toxin concn detected (ng/ml)[a]	% Cytotoxic activity[b]			Detection of the indicated diarrheagenic genes by PCR[d]				% of bacteria showing[c]:	
			Normal	HT	TT	*hblA*	*hblC*	*hblD*	*bceT*	Adherence	Invasion
B. cereus	NB3	—	85 ± 4	15	10 ± 1	−	−	−	−	−	−
	NB23	≥128	77 ± 6	20 ± 6	26 ± 6	+	+	+	+	−	−
	NB27	—	82 ± 5	—	—	−	−	−	+	−	−
	NB35	64	88 ± 3	16 ± 1	24 ± 6	+	+	+	+	−	−
	NB40	≥128	78 ± 3	18 ± 2	10	+	+	+	+	−	−
	NB50	≥128	89 ± 1	22 ± 3	—	+	+	+	+	−	−
	NB51	64	93	13 ± 1	11 ± 2	+	+	+	+	−	−
	SU52	≥128	85 ± 2	12 ± 1	28 ± 6	+	+	+	+	0.03 ± 0.005	−
B. licheniformis	NB42	—	39 ± 5	9 ± 2	—	−	−	−	−	−	−
	NB14	—	66 ± 3	12 ± 2	22 ± 6	−	−	−	−	2.8 ± 0.22	−
B. polymyxa	NB6	16	88 ± 4	13 ± 1	18 ± 1	+	+	+	−	−	−
B. coagulans	NB11	64	89 ± 6	10 ± 3	5 ± 1	+	+	+	+	−	−
L. monocytogenes	SU1	—	82 ± 5	14 ± 3	24 ± 5	−	−	−	−	3.88 ± 0.37	1.15 ± 0.23
E. coli	HB101	—	—	—	—	−	−	−	−	−	−

[a] Detection of diarrheal enterotoxin in test media by the RPLA test. —, no diarrheal enterotoxin detected.
[b] Supernatant fluids were untreated (Normal), heated (HT), or trypsin treated (TT) prior to assessment of toxicity to HEp-2 cells. Values shown are means ± standard errors for results from triplicate trials where samples were examined three times. —, no effect.
[c] Values are reported as means ± standard errors. −, no adherence or invasion.
[d] +, gene was detected; −, gene was not detected.

HEp-2 cells (Table 2). These findings contrasted markedly with those of previous infectivity studies, where 43 (91%) of the 47 strains isolated from clinical or food samples adhered to HEp-2 or Caco-2 cells and 23 (49%) demonstrated various levels of invasion (26). Other researchers have also reported that *Bacillus* spp. may occasionally cause mastitis or abortions in animals (10, 16, 33, 37). Recent evidence suggests that many members of the *Bacillus* genus may also be the causes of serious systemic diseases, such as septicemia, endocarditis, peritonitis, ophthalmitis, liver failure, and meningitis in humans (8, 20, 31).

Assessment of cytotoxicity was based on a method described previously (6) for measuring total cellular metabolic activity by using the tetrazolium salt 3-(4,5,-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma), with some minor modifications. HEp-2 monolayers were grown overnight at 37°C in a 5% CO_2 atmosphere in DMEM supplemented with 10% FCS in 96-well microplates seeded with approximately 5×10^4 cells per well. Bacterial cultures were grown for 18 h as described above, and 0.1-ml samples were filter sterilized (0.2-μm-pore-size membranes; Sarstedt, Nümbrecht, Germany) and added in triplicate to the test plates immediately, after heating of the supernatant at 95°C for 10 min, or after enzymatic treatment with 0.1% trypsin. Positive and negative assay controls were 1% Triton X-100 (Sigma) and phosphate-buffered saline, respectively. Tissue culture monolayers containing the bacterial culture supernatants were incubated overnight at 37°C in a 5% CO_2 atmosphere, followed by the addition of phosphate-buffered saline containing 0.5% MTT (Sigma) to each well for 4 h at 37°C. The suspensions in the wells were then removed, and the formazan product was solubilized by the addition of 100 μl of 0.04 M HCl in dimethyl sulfoxide (Sigma, Poole, United Kingdom). The contents of the plates were measured spectrophotometrically at 540 nm in a microplate reader (Labsystems EMS reader). The toxic effect of the cell-free bacterial culture supernatant on the HEp-2 cell line was calculated from the following equation: [(1 − optical density of test sample)/(optical density of negative control)] × 100. Cytotoxic effects produced in HEp-2 cells were also confirmed by light microscopy.

While results showed that there were species-to-species variations in the levels of cytotoxicity produced, the culture supernatant fluids from all 11 *Bacillus* isolates were cytotoxic for this epithelial cell line (Table 2). Six *Bacillus* isolates exhibited levels of toxicity (85.5% ± 5% [mean ± standard deviation]) that were similar to or greater than those produced by clinical isolates of *B. cereus* SU52 and *L. monocytogenes* SU1, which are associated with gastrointestinal and nongastrointestinal infections in humans, respectively (Table 2). A marked variation in the range of toxicity levels exhibited by *Bacillus* isolates associated with more serious veterinary infections, such as ovine and bovine abortions, was apparent (Table 2). Separate heat and trypsin treatments of culture supernatant fluids either reduced or eliminated toxicity in HEp-2 cells, which suggests that the cytotoxic activity was attributed to the proteinaceous fractions of the culture supernatants (Table 2). Cell-free culture supernatants from all 11 *Bacillus* species examined for cytotoxicity in this study showed toxicity in HEp-2 cells. The variation in toxicity levels observed was similar ($P < 0.05$) to that reported previously for cell-free supernatant fluids from 38 strains representing 14 different *Bacillus* species from clinical and food samples (26).

Others have also shown that MTT can be used to assess the cytotoxic effect of culture supernatant fluids of *Bacillus* species isolated from raw milk (4, 9). Only live eukaryotic cells are recognized by this assay because the tetrazolium ring of MTT is cleaved in the mitochondria of metabolically active cells. By using this assay, Beattie and Williams (4) showed that some isolates of *Bacillus circulans*, *Bacillus laterosporus*, *Bacillus lentus*, *B. licheniformis*, *Bacillus mycoides*, *Bacillus subtilis*, *B. cereus*, and *Bacillus thuringiensis* were toxigenic to Chinese hamster ovary cells. Tetrazolium salts have also been used to

assess the cytotoxicities of other pathogens, such as *Mannheimia haemolytica* biotype A serotype 1 leukotoxin (7) and the cytotoxin of *Campylobacter jejuni* (6), and they were recently used to investigate *B. cereus* toxicity (36). Finlay et al. (9) advocated the use of MTT, as the currently used HEp-2 cell vacuolation assay for *Bacillus* emetic toxin is laborious, subjective, and unreliable.

Detection of diarrheagenic enterotoxins by PCR and measurement of other potential virulence factors. *B. cereus* NCTC 11145 was used as a positive control strain in assays for the presence of HBL and BceT enterotoxins, as shown previously (26). *L. monocytogenes* NCTC 11994 and *E. coli* ATCC 33694 were used as negative control strains in assays for the presence of genes encoding HBL and BceT enterotoxins. Chromosomal DNA was isolated from the test *Bacillus* spp. by use of a previously described procedure (26). The DNA sequences encompassing the diarrheagenic genes *bceT* (1), *hblC* and *hblA* (30), and *hblD* (13) were used to design primers that would amplify segments of the genes, if present, in a selection of the above-mentioned test *Bacillus* strains. Amplification was carried out in a DNA thermal cycler for 36 cycles of 30s at 94°C; 1 min at 54°C, 58°C, 62°C, and 63°C for *hblD*, *bceT*, *hblC*, and *hblA* genes, respectively; and 1 min at 72°C. PCR products of 439, 428, 399, and 873 bp were detected when the following pairs of oligonucleotide primers were used, respectively: HBLD-N (5′-AATCAAGAGCTGTCACGAAT-3′) and HBLD-C (5′-CACCA ATTGACCATGCTAAT-3′), BCET-N (5′-TTACATTACCAGGACGTGCTT-3′) and BCET-C (5′-T GTTTGTGATTGTAATTCAGG-3′), HBLC-N (5′-AATAG GTACAGATGGAACAGG-3′) and HBLC-C (5′-GGCTTTC ATCAGGTCATACTC-3′), and HBLA-N (5′-GCTAATG TAGTTTCACCTAGCAAC-3′) and HBLA-C (5′-AATCAT GCCACTGCGTG GACATATAA-3′). Findings from PCR primer studies revealed that seven veterinary *Bacillus* isolates contained *hblA*, *hblC*, and *hblD* genes that encode the tripartite HBL enterotoxin complex traditionally harbored by toxigenic *B. cereus* (Table 2). With the exception of *B. polymyxa* NB6, all *Bacillus* isolates encoding the HBL enterotoxin were also shown to have the *bceT* gene that encodes the BceT enterotoxin (Table 2). None of the other *Bacillus* species contained diarrheagenic enterotoxin genes (Table 2).

Analysis of cell-free culture supernatants from all seven veterinary *Bacillus* isolates displaying positive signals for all three HBL toxin genes tested positive by the *B. cereus* enterotoxin (diarrheal type) reversed passive latex agglutination (RPLA) assay that is specific for the L_2 component of the HBL complex (Table 2). The presence of diarrheal HBL enterotoxin was measured by using the RPLA kit according to the manufacturer's instructions (Oxoid). This finding suggests that after 18 h of growth in BHI broth, these other *Bacillus* isolates produced protein toxins that were very similar to those of *B. cereus* and that these species may have presented a potential hazard if they had entered the food chain. The level of enterotoxin HBL produced by these particular *Bacillus* spp. ranged from 16 to $\geq$128 ng ml^{-1}; the reported sensitivity of the RPLA test is 2 ng ml^{-1} (5). There was good agreement between carriage of *hblA*, *hblC*, and *hblD* genes and detection of secreted HBL enterotoxin in culture supernatants (Table 2).

These results contrasted markedly with findings from a previous study, in which only 4 (22%) of 18 *Bacillus* isolates from the clinical and food environments harboring all three *hbl* genes produced HBL enterotoxin in cultured BHI broth (26). However, a further eight isolates belonging to *B. cereus*, *B. licheniformis*, *B. circulans*, and *Bacillus megaterium* were found to produce HBL enterotoxin after growth in reconstituted baby food, which suggests that many *Bacillus* isolates that have the necessary diarrheagenic genes require a specific environmental signal(s) for transcriptional activation. Environmental signals have been shown previously to modulate virulence factor expression in *B. cereus* and in other bacterial enteropathogens (24, 27). Schoeni and Wong (32) have previously reported that HBL enterotoxin was secreted by more than 200 tested *B. cereus*, *B. thuringiensis*, and *B. mycoides* strains. Beattie and Williams (4) showed that the supernatant fluids from isolates of *B. thuringiensis*, *B. circulans*, *B. licheniformis*, *B. lentus*, *B. laterosporus*, and *B. mycoides* reacted positively with both the *Bacillus* diarrheal enterotoxin visual immunoassay (BDE; Tecra) or RPLA immunoassays. *B. subtilis*, *B. licheniformis*, *Bacillus pumilus*, and *B. thuringiensis* have been previously implicated in outbreaks of foodborne disease (15, 31).

It has been shown that some *B. cereus* strains may contain multiple copies of the *hbl* genes that arose from duplication of a single gene (32). *hbl* has been mapped to a portion of the *B. cereus* chromosome that exhibits greater variability than do other regions and this variable region is sometimes located on large extrachromosomal DNA fragments that appear to be stable but might also prove to be large mobile plasmids (32). Interestingly, evidence of chromosomal gene mobilization and transfer of plasmids coding for δ-endotoxin among strains of the well-characterized insect pathogen *B. thuringiensis* and *B. cereus* have been provided previously (2, 11). Many survival trait genes, such as those for virulence factors and antibiotic resistance, are located on plasmids (16). *B. anthracis*, belonging to the *B. cereus* group and thus a close relative of *B. cereus* and *B. thuringiensis* (and the causative agent of anthrax) (3), has its crucial virulence factors located on two plasmids, and when one or both are lost the *B. anthracis* becomes avirulent (34). The aforementioned finding, combined with the high degree of phylogenetic relatedness among members of this genus (14), possibly explains why many different *Bacillus* species isolated from the veterinary, clinical, and food environments carry similar enterotoxins traditionally harbored by toxigenic *B. cereus*.

All *Bacillus* veterinary isolates were also tested for lecithinase (phosphatidylinositol-specific phospholipase C) activity after overnight growth on nutrient agar supplemented with 8% egg yolk (Oxoid) and by overlaying 1% L-*d*-phosphatidylinositol substrate (Sigma) in 0.7% agarose on overnight cultures of the bacteria on L agar plates. Lecithinase-positive strains produced a halo of precipitation (the insoluble diacylglycerol) around the bacterial colonies. Production of catalase was assayed by using an ID Color Catalase testing kit (bioMérieux). The ability to induce hemolysis in a 7% concentration of horse erythrocytes (blood agar) was examined. Results showed that only *B. cereus* strains produced lecithinase, whereas beta-hemolysis and catalase activities were exhibited by all *Bacillus* isolates.

In summary, this study constitutes the first demonstration that isolates of many *Bacillus* spp., from samples associated with serious nongastrointestinal infections in animals, may carry diarrheagenic enterotoxin genes traditionally harbored

by toxigenic *B. cereus*. We also have shown that these veterinary *Bacillus* isolates are cytotoxic towards HEp-2 cells and have expressed HBL enterotoxins. While the roles of diarrheagenic enterotoxins and other potential virulence factors in the aforementioned animal infections have yet to be elucidated, this study has demonstrated that a variety of *Bacillus* spp. isolated from the veterinary environment may contain pathogenicity traits that may enhance their fitness for survival and to elicit disease.

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REFERENCES

1. **Agata, N., M. Ohta, Y. Arakawa, and M. Mori.** 1995. The *bceT* gene of *Bacillus cereus* encodes an enterotoxic protein. Microbiology **141:**983–988.
2. **Aronson, A. I., and W. Beckman.** 1987. Transfer of chromosomal genes and plasmids in *Bacillus thuringiensis.* Appl. Environ. Microbiol. **53:**1525–1530.
3. **Ash, C., J. A. E. Farrow, M. Dorsch, E. Stackebrandt, and M. D. Collins.** 1991. Comparative analysis of *Bacillus anthracis*, *Bacillus cereus*, and related species on the basis of reverse transcriptase sequencing of 16S rRNA. Int. J. Syst. Bacteriol. **41:**343–346.
4. **Beattie, S. H., and A. G. Williams.** 1999. Detection of toxigenic strains of *Bacillus cereus* and other *Bacillus* spp. with an improved cytotoxicity assay. Lett. Appl. Microbiol. **28:**221–225.
5. **Becker, H., G. Schaller, W. von Wiese, and G. Terplan.** 1994. *Bacillus cereus* in infant foods and dried milk products. Int. J. Food Microbiol. **23:**1–15.
6. **Coote, J. G., and T. Arian.** 1996. A rapid, colorimetric assay for cytotoxin activity in *Campylobacter jejuni*. FEMS Immunol. Med. Microbiol. **13:**65–70.
7. **Craig, F. F., R. Dalgleish, and A. D. Sutherland.** 1990. A colorimetric, microplate assay for the leucotoxin of *Pasteurella haemolytica*. Vet. Microbiol. **22:**309–340.
8. **Drobniewski, F. A.** 1993. *Bacillus cereus* and related species. Clin. Microbiol. Rev. **6:**324–338.
9. **Finlay, W. J. J., N. A. Logan, and A. D. Sutherland.** 1999. Semiautomated metabolic staining assay for *Bacillus cereus* emetic toxin. Appl. Environ. Microbiol. **65:**1811–1812.
10. **Galiero, G., and E. De Carlo.** 1998. Abortion in water buffalo (*Bubalus bubalis*) associated with *Bacillus licheniformis*. Vet. Rec. **143:**640–641.
11. **Gonzalez, J. M., B. J. Brown, and B. C. Carlton.** 1982. Transfer of *Bacillus thuringiensis* plasmids coding for δ-enterotoxin among strains of *B. thuringiensis* and *B. cereus*. Proc. Natl. Acad. Sci. USA **79:**6951–6955.
12. **Granum, P. E., and T. Lund.** 1997. *Bacillus cereus* and its food poisoning toxins. FEMS Microbiol. Lett. **157:**223–228.
13. **Hansen, B. M., and N. B. Hendriksen.** 2001. Detection of enterotoxic *Bacillus cereus* and *Bacillus thuringiensis* strains by PCR analysis. Appl. Environ. Microbiol. **67:**185–189.
14. **Helgason, E., O. A. Økstad, D. A. Caugant, H. A. Johansen, A. Fouet, M. Mock, I. Hegna, and A.-B. Kolstø.** 2000. *Bacillus anthracis*, *Bacillus cereus*, and *Bacillus thuringiensis*–one species on the basis of genetic evidence. Appl. Environ. Microbiol. **66:**2627–2630.
15. **Jackson, S. G., R. B. Goodbrand, R. Ahemd, and S. Kasatiya.** 1995. *Bacillus cereus* and *Bacillus thuringiensis* isolated in a gastroenteritis outbreak investigation. Lett. Appl. Microbiol. **21:**103–105.
16. **Kado, C. I.** 1998. Origin and evolution of plasmids. Antonie Leeuwenhoek **73:**117–126.
17. **Kirkbride, C. A.** Bacterial agents detected in a 10-year study of bovine abortions and stillbirths. J. Vet. Diagn. Investig. **5:**64–68.
18. **Kirkbride, C. A., and J. Collins.** 1996. Porcine abortion caused by *Bacillus* spp. 1986. J. Am. Vet. Med. Assoc. **188:**1060–1061.
19. **Kotiranta, A., K. Lounatmaa, and M. Haapasalo.** 2000. Epidemiology and pathogenesis of *Bacillus cereus* infections. Microbes Infect. **2:**189–198.
20. **Mahler, H., A. Pasi, J. M. Kramer, P. Schulte, A. C. Scoging, W. Baer, and S. Kraehenbuehl.** 1997. Fulminant liver failure in association with the emetic toxin of *Bacillus cereus*. N. Engl. J. Med. **336:**1142–1148.
21. **McKillip, J. L.** 2000. Prevalence and expression of enterotoxins in *Bacillus cereus* and other *Bacillus* spp., a literature review. Antonie Leeuwenhoek **77:**393–399.
22. **Phelps, R. J., and J. L. McKillip.** 2002. Enterotoxin production in natural isolates of *Bacillaceae* outside the *Bacillus cereus* group. Appl. Environ. Microbiol. **68:**3147–3151.
23. **Ranganatha, H. G., and S. Saki.** 1981. *Bacillus cereus* as a cause of abortion in a mare. Curr. Sci. **50:**458.
24. **Rowan, N. J.** 1999. Evidence that inimical food preservation barriers alter microbial resistance, virulence and cell morphology. Trends Food Sci. Technol. **10:**261–272.
25. **Rowan, N. J., A. A. G. Candlish, A. Bubert, J. G. Anderson, K. Kramer, and J. McLauchlin.** 2000. Virulent rough filaments of *Listeria monocytogenes* from clinical and food samples secreting wild-type levels of cell-free p60 protein. J. Clin. Microbiol. **38:**2643–2648.
26. **Rowan, N. J., K. Deans, J. G. Anderson, C. G. Gemmell, I. S. Hunter, and T. Chaithong.** 2001. Putative virulence factor expression by clinical and food isolates of *Bacillus* spp. after growth in reconstituted infant milk formulae. Appl. Environ. Microbiol. **67:**3873–3881.
27. **Rowan, N. J., and J. G. Anderson.** 1997. Maltodextrin stimulates growth of *Bacillus cereus* and synthesis of diarrheal enterotoxin in infant milk formulae. Appl. Environ. Microbiol. **63:**1182–1184.
28. **Rowan, N. J., and J. G. Anderson.** 1998. Growth and enterotoxin production by diarrhoeagenic *Bacillus cereus* in dietary supplements prepared for hospitalised HIV patients. J. Hosp. Infect. **38:**139–146.
29. **Rowan, N. J., J. G. Anderson, and A. Anderton.** 1997. Bacteriological quality of infant milk formulae examined under a variety of preparation and storage conditions. J. Food Prot. **60:**1089–1094.
30. **Ryan, P. A., J. D. Macmillan, and B. A. Zilinkas.** 1997. Molecular cloning and characterization of the genes encoding the L_1 and L_2 components of hemolysin BL from *Bacillus cereus*. J. Bacteriol. **179:**2551–2556.
31. **Salkinoja-Salonen, M. S., R. Vuorio, M. A. Andersson, P. Kämpfer, M. C. Andersson, T. Honkanen-Buzalski, and A. C. Scoging.** 1999. Toxigenic strains of *Bacillus licheniformis* related to food poisoning. Appl. Environ. Microbiol. **65:**4637–4645.
32. **Schoeni, J. L., and A. C. Wong.** 1999. Heterogeneity observed in the components of hemolysin BL, an enterotoxin produced by *Bacillus cereus*. Int. J. Food Microbiol. **53:**159–167.
33. **Schuh, J., and D. Weinstock.** 1985. Bovine abortion caused by *Bacillus cereus*. J. Am. Vet. Med. Assoc. **187:**1047–1048.
34. **Turnbull, P. C., R. A. Hutson, M. J. Ward, M. N. Jones, C. P. Quinn, N. J. Finnie, C. J. Duggleby, J. M. Kramer, and J. Melling.** 1992. *Bacillus anthracis* but not always anthrax. J. Appl. Bacteriol. **72:**21–28.
35. **Van Heddeghem, A., and G. Vlaemynck.** 1992. Sources of contamination of milk with *Bacillus cereus* on the farm and in the factory. Bull. Int. Dairy Fed. **275:**19–22.
36. **von Dietrich, R., K. Mauersberger, and E. Martlbauer.** 1997. Zur anwendbarkeit des MTT zytotoxizitatstests zum nachwies von *B. cereus* enterotoxin. Arch. Lebensmittelhyg. **48:**73–96.
37. **Wernery, U., and M. Ali.** 1996. *Bacillus cereus* abortion in a nine-year-old dromedary camel–a case report. J. Camel Pract. Res. **3:**153.