Toxin-Producing Ability among *Bacillus* spp. Outside the *Bacillus cereus* Group

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A total of 333 *Bacillus* spp. isolated from foods, water, and food plants were examined for the production of possible enterotoxins and emetic toxins using a cytotoxicity assay on Vero cells, the boar spermatozoa motility assay, and a liquid chromatography-mass spectrometry method. Eight strains produced detectable toxins; six strains were cytotoxic, three strains produced putative emetic toxins (different in size from cereulide), and one strain produced both cytotoxin(s) and putative emetic toxin(s). The toxin-producing strains could be assigned to four different species, *B. subtilis*, *B. mojavensis*, *B. pumilus*, or *B. fusiformis*, by using a polyphasic approach including biochemical, chemotaxonomic, and DNA-based analyses. Four of the strains produced cytotoxins that were concentrated by ammonium sulfate followed by dialysis, and two strains produced cytotoxins that were not concentrated by such a treatment. Two cultures maintained full cytotoxic activity, two cultures reduced their activity, and two cultures lost their activity after boiling. The two most cytotoxic strains (both *B. mojavensis*) were tested for toxin production at different temperatures. One of these strains produced cytotoxin at growth temperatures ranging from 25 to 42°C, and no reduction in activity was observed even after 24 h of growth at 42°C. The strains that produced putative emetic toxins were tested for the influence of time and temperature on the toxin production. It was shown that they produced putative emetic toxin faster or just as fast at 30 as at 22°C. None of the cytotoxic strains produced *B. cereus*-like enterotoxins as tested by PCR or by immunological methods.

The genus *Bacillus* consists of a heterogenic group of gram-positive, endospore-forming, rod-shaped, facultative anaerobic bacteria that are widely distributed in nature. Due to their endospore-forming abilities, these bacteria tolerate adverse conditions better than the nonsporulating bacterial entero-pathogens and may proliferate in a wide range of environments including water and processed and untreated foods.

Bacillus cereus is the most important cause of food poisoning from this group due to its ability to produce enterotoxins and emetic toxin. The diarrheal type of food-borne illness is caused by three different heat-labile enterotoxins produced during vegetative growth of *B. cereus* in the small intestine after ingestion (9). The emetic type is due to the production of a heat-stable emetic toxin (cereulide) during growth in foods under various conditions (9). Other *Bacillus* species have generally been considered of little significance in food poisoning incidents (26), but their ability to produce both enterotoxins and emetic toxin has been increasingly recognized. It is mainly strains of *B. subtilis*, *B. pumilus*, and *B. licheniformis* that have been linked to incidents of food-borne illness (12, 20, 27), and it is believed that the enterotoxins produced by *Bacillus* spp. other than *B. cereus* are proteins transcribed from genes that are similar to those of *B. cereus* enterotoxins (21, 25, 26).

In this study, we have examined 333 *Bacillus* strains (outside the *B. cereus* group) from various sources related to foods for cytotoxicity and production of putative emetic toxins. For the first time, toxin-producing *Bacillus* spp. strains not belonging to the *B. cereus* group were classified using several methods, giving definite species identification.

MATERIALS AND METHODS

Bacterial strains. A total of 333 *Bacillus* strains outside the *Bacillus cereus* group were isolated from various foods, water sources, and a food plant. The samples were seeded onto blood agar plates and grown at 30°C. The collected strains were stored in Microbank tubes (Pro-lab Diagnostics, Richmond Hill, Ontario, Canada) at −70°C until use. A total of 122 strains were isolated from different types of lightly heat-treated (sous vide) food products (kindly provided by J. T. Rosnes, Norconserv), "ready-to-eat" pizza, raw material from the food industry, and food samples sent to our reference laboratory at the Norwegian School of Veterinary Science which were suspected to be involved in food poisoning. We isolated 59 strains from spices, 42 strains from water, and 100 strains from tap water from different parts of Norway. All strains sporulated on blood agar after 2 to 5 days of growth.

Isolation of *Bacillus* spp. from foods, raw materials, and water. Ten grams of samples was transferred to 90 g of peptone water and homogenized for 2 min using a Stomacher. One-half milliliter of 10^{-2} and 10^{-3} dilutions of the samples was transferred to bovine blood agar. From the ready-to-eat pizzas, samples were picked from the center and the edge of the pizza. For the lightly heat-treated food products and the spices, the samples were homogenized for 2 min using a Stomacher lab blender. A 10^{-1} dilution was heat treated for 10 min at 75°C before a 10^{-2} dilution of this heat-treated solution was embedded in plate count agar. The industrial samples from the food industry were collected from various parts of the pizza production line by swabs moistened with peptone water and transferred directly to bovine blood agar plates. All samples were incubated both aerobically and anaerobically at 37°C for 24 h.

After a 24-h incubation of the different samples, the bovine blood agar plates were examined. Presumptive *Bacillus* spp. were identified as isolates that showed morphological resemblance to *Bacillus* spp. Colonies producing hemolysis were identified by examination of the blood agar plates from the bottom side. Presumptive *Bacillus* colonies were transferred to blood agar plates and incubated at 37°C for 24 h. Isolates were kept if they were gram-positive rods (<1 to 1 μm by 2 to 8 μm) with oval, central-to-lateral spores after growth for 5 to 7 days. Strains

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TABLE 1. PCR primers used for detection of _B. cereus_-like enterotoxin genes

Primer name	Sequence	Positions[a]	Product size (bp)	Target
L2F	AGAAACTCAACAAGAAAACATGG	1278–1300	444	_hblC_
L2R	TTGCGCAGTTGCCACATTAG	1719–1700		
0045F	GTTAGGATCACAATCACCGC	430–449	264	_nheA_
4091R	CCATATGCATTTGTAAAATCTG	711–689		
517	CGGTTCATCTGTTGCGACAGC	2180–2200	335	_nheB_
8368	GATCCCATTGTGTACCATTGG	2512–2492		
4924F	GCGATTGATCAAAAGGATAG	3206–3225	411	_nheC_
1141R	CGACTTCTGCTTGTGCTCCTG	3614–3594		
FC	GTAACTTTCATTTGATGATC	1946–1964	505	_cytK_
RC	GAATACATAAATAATTGGT	2451–2433		

[a] According to the sequences given for the following GenBank accession numbers: U63928 (_hbl_), Y19005 (_nhe_), and AJ277962 (_cytK_).

identified as _B. cereus_ (18) were not collected. From the water samples, the _Bacillus_ spp. were isolated from 1, 10, and 100 ml of pasteurized samples (80°C for 30 min) by membrane filtration using 0.45-μm-pore membrane filters (Millipore HA). After membrane filtration of the samples, the membrane filters were placed onto bovine blood agar plates and incubated aerobically at 30°C for 24 h. All colonies growing on the membrane filters were regarded as presumptive _Bacillus_ spp.

The strains that were toxin producers (by methods described below) were tested for anaerobic growth after 24 h at 30°C on bovine blood agar plates that were preincubated anaerobically for 1 h before use (Microbiologie Anaerocult A; Merck). These strains were also grown on _B. cereus_ selective agar at 30°C for 24 h to rule out possible _B. cereus_.

Typing methods used for the toxin-producing strains. The strains that were found to be toxin producers (by methods described below) were initially typed by API CH50 and API 20E (bioMèrieux, Marcy l'ètoile, France) according to the manufacturer's instructions. These strains were also characterized by use of 16S rRNA partial sequence analysis, riboprinting, enterobacterial repetitive intergenic consensus (ERIC)- and BOX-PCR, and examination for the presence of _meso_-α,ε-diaminopimelic acid. Strain numbers 31, 75, and 162 were additionally characterized by use of partial _gyrA_ gene sequence analysis (6).

For phylogenetic analysis, genomic DNA was extracted from bacteria by methods described below for detection of enterotoxin genes by PCR, and the primer pair 27f (5′-GAGTTTGATCCTGGCTCAG-3′) and 1385r (5′-CGGTGTGTR CAAGGCCC-3′) was used for amplification of the 16S rRNA gene (14). PCR amplification was done as described previously by Pukall et al. (22), and the first 530 bp of the amplicons was sequenced by using a dye-labeled dideoxy terminator cycle sequencing quick-start kit and the Ceq8000 genetic analysis system from Beckman Coulter. The sequences were manually aligned and compared with published sequences from the Deutsche Sammlung von Mikroorganismen und Zellkulturen 16S rRNA database, including sequences available from the Ribosomal Data Project (16) and EMBL.

Amplification of repetitive elements (BOX and ERIC) was carried out with the primer systems published previously by Versalovic et al. (33). PCR amplification was performed with a TC 480 DNA thermal cycler (Perkin-Elmer) with the following cycles: an initial denaturation step for 2 min at 95°C and 31 cycles of 3 s at 94°C, 30 s at 92°C, 1 min at 40°C, and 8 min at 65°C. PCR products were visualized by agarose gel electrophoresis using 1.5% agarose in 1× Tris-acetate-EDTA buffer.

Hydrolysates of whole cells were examined for the presence of _meso_-α,ε-diaminopimelic acid by thin-layer chromatography based on previously described methods (23, 31).

Riboprinting was performed with the automated RiboPrinter microbial characterization system (DuPont Qualicon) according to the manufacturer's instructions. The patterns were generated using the restriction enzyme EcoRI.

For partial _gyrA_ gene sequence analysis, total DNA was extracted from strains B31, B75, and B162 by methods described below for detection of enterotoxin genes by PCR. The partial _gyrA_ fragments, corresponding to _B. subtilis gyrA_ numbering positions 43 to 1065 (13), were PCR amplified using two oligonucleotide primers: p-gyrA-f (5′-CAG TCA GGA AAT CGC TAC GTC CTT-′3) and p-gyrA-r (5′-CAA GGT AAT GCT CCA GGC ATT GCT-′3) (24). The resultant amplicons were purified using a QIAquick PCR purification kit (QIAGEN) and sequenced in both directions by Lark Technologies, Inc. The same primers were used for the sequencing reactions.

The resultant partial _gyrA_ sequences were assembled and aligned by use of Contig Express.

The _gyrA_ sequences of _B. subtilis_ subsp. _subtilis_ strain 168 (GenBank accession

number Z99104 [13]), _B. subtilis_ subsp. _spizizenii_ (accession number AF272020 [6]), and _B. mojavensis_ (accession number AF272019 [6]) were obtained from the GenBank database.

Bases 115 to 894 in the _gyrA_ genes of the strains above were compared with the partial _gyrA_ gene sequences obtained from strains B31, B75, and B162. The values for percent identity of the DNA sequences were obtained by using the Smith-Waterman algorithm for local alignments (30).

Vero cell cytotoxicity assay. The ability of the isolated _Bacillus_ strains to produce enterotoxins was determined after growth of the cells in brain heart infusion broth with 1% glucose at 32°C for 6 h before the extracellular proteins were separated from the cells by centrifugation at room temperature for 5 min at 16,100 × _g_. The supernatants were stored at −20°C until testing. Toxicity was determined by using 100 μl of supernatant to cause swelling, rounding, and disseminating of the Vero cell layer examined microscopically after 1, 3, and 5 h of incubation with 5% CO_2 and 37°C and compared to a positive control supernatant (from _B. cereus_ NVH 1230–88 with the addition of 3, 10, 30, and 100 μl to four different wells). Positive strains from the subjective Vero cell assay were tested by the Vero cell assay (inhibition of the protein synthesis) by using radioactivity according to the method described by Sandvig and Olsnes (28) with the positive control described above. Strains that caused <20% inhibition of protein synthesis of the Vero cells were tested after a 10-fold concentration of the supernatants, using 80% ammonium sulfate (15). Strains that showed an increased toxicity in the Vero cell assay (>20% inhibition of protein synthesis) after the concentration were considered cytotoxin positive. The culture supernatant fluids of the cytotoxin-positive strains were also heat treated for 10 min in a boiling water bath and tested in the Vero cell assay to determine heat stability properties. All experiments were performed in duplicate and repeated three times.

Detection of enterotoxin genes by PCR. To screen isolates for the presence of one or more enterotoxin genes (similar to those of _B. cereus_), total DNA was extracted by first growing the strains on bovine blood agar plates for 24 h at 30°C and then transferring the strains to brain heart infusion broth (Difco) and growing them for 4 h in a shaking water bath at 30°C before the cells were harvested by centrifugation of 3 ml of supernatant at 16,100 × _g_ for 5 min. The cell pellets were frozen at −20°C overnight. The pellets were resuspended in 495 μl of SET (75 mM NaCl, 25 mM EDTA, 20 mM Tris, pH 7.5), and 50 μl of freshly made 5-mg/ml lysozyme was added before incubation at 37°C for 1 h. Before another incubation at 50°C for 2 h, 50 μl of 10% sodium dodecyl sulfate and 5 μl of 25-mg/ml proteinase K were added. Two hundred microliters of 5.0 M NaCl and 700 μl of chloroform-isoamyl alcohol (24:1) were then added and incubated at room temperature with frequent inversions for 30 min. The sample was centrifuged at 16,100 × _g_ for 30 min, and the aqueous phase was transferred to a fresh tube. The DNA was precipitated with an equal volume of isopropanol by centrifugation at 16,100 × _g_ for 20 min. The precipitate was washed with 70% ethanol and centrifuged at 16,100 × _g_ for 10 min before it was left to dry. The DNA was resuspended in 100 μl of distilled water, and 10 μl of 10-μg/ml RNase solution was added before incubation at 37°C for 30 min. PCR was performed by using a Gene Cycler (Bio-Rad). The PCR mixture consisted of 1 μl of template DNA, 1 μl of each primer (Table 1), 5 μl of 10× PCR buffer, 1 μl of deoxynucleoside triphosphates, 0.5 μl of _Taq_ (DNA polymerase), and 40.5 μl of sterile water per sample. Amplification consisted of an initial denaturation step at 94°C for 3 min followed by 30 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min. A final extension step for 10 min at 72°C followed. The primers used in the PCRs are listed in Table 1. Five microliters of the PCR mixture was analyzed on a 1% agarose gel (Seakem LE agarose) at 80 V for 30 min.

TABLE 2. Toxin-producing *Bacillus* spp. from different sources

Source	Total no. of strains	No. of enterotoxin-producing strains[a]	No. of putative emetic toxin-producing strains[b]	No. of strains that produce both types of toxins
Natural water	42	3	0	0
Tap water	100	1	1	0
Foods	122	1	1	0
Food plant	10	0	0	0
Spices	59	1	1	1
Total	333	6	3	1

[a] Cytotoxicity assay on Vero cells (28) based on duplicate experiments repeated three times.
[b] According to the boar sperm motility assay (4).

Detection of enterotoxins by using two commercial immunoassay kits. Cell-free culture supernatants that showed positive results in the Vero cell assay were also tested by the *B. cereus* enterotoxin (diarrheal type) reversed passive latex agglutination assay (BCET-RPLA TD950; Oxoid, Basingstoke, United Kingdom) that is specific for the L_2 component of the Hemolysin BL complex (Oxoid) and by the Bacillus Diarrheal Enterotoxin Visual Immunoassay (Tecra) that detects the NheA component of the Nhe-toxin complex. Both kits were used according to the manufacturers' instructions.

Screening for putative emetic toxins. The strains to be tested were grown for 5 days at 22 and 30°C. Colony material from the different strains was collected after 1, 3, and 5 days for the strains grown at 30°C and after 2, 3, and 5 days for the strains grown at 22°C. The assay was performed according to the protocol described by Andersson et al. (4). Colony material was dissolved into 200 μl of methanol and cooked in glass tubes for 10 min in a water bath. Afterwards, 50 μl of methanol was added to samples where the methanol had evaporated during the boiling. Ten microliters of the sample was added to 200 μl of boar spermatozoa at 37°C and incubated at 37°C for 10 min. After 10 min, the sample was examined by microscopy. Strains that caused inhibition of the motility of the boar spermatozoa were considered putative emetic toxin positive. For the positive control, the emetic *B. cereus* strain RIVM BC 00062 was used, and for the negative control, 10 μl of methanol was added to the boar spermatozoa.

LC-MS. Two loops of bacterial colony material from strains B31, B174, and B279 grown aerobically on blood agar plates for 5 days at 30°C were collected and dissolved in 1 ml of methanol by vortexing for 20 s. The samples were left in an ultrasonic bath for 5 min before vortexing for another 20 s and centrifugation at $1.5 \times g$ for 5 min. Fifty microliters of this sample was mixed with 800 μl of methanol and 100 μl of 0.5% acetic acid and used for analysis by liquid chromatography-mass spectrometry (LC-MS). The LC-MS instrumentation used in this experiment consisted of a syringe pump (Harvard Apparatus, Inc.) operated at a constant flow of 0.3 ml/h and a single quadrupole mass spectrometer (Applied Biosystems, Ontario, Canada) equipped with a Turbo-Ion Spray ion source. The turbo probe of the interface was maintained at 150°C, and the probe airflow rate was 6 liters/min. The nitrogen nebulizer and curtain gas airflow rates were 5 and 10 liters/min, respectively. The mass spectrometer was set to collect scan-ion data in positive-ion mode from 200 to 1,000 and 1,000 to 2,000 atomic mass units (AMU) and thereafter from 1,020 to 1,065 AMU for strains B31 and B174 and from 1,030 to 1,080 AMU for strain B279. The entrance electrode

voltages and the position of the ion spray inlet were adjusted to provide optimum intensity for the molecular ions.

Nucleotide sequence accession numbers. The partial *gyrA* nucleotide sequences of strains B31 and B162 were deposited in the GenBank database under accession numbers AY599914 and AY613439.

RESULTS

Isolation of toxin-producing *Bacillus* spp. We collected 333 *Bacillus* spp. outside the *B. cereus* group from different environments, without species identification, as shown in Table 2. Initially, culture supernatants (100 μl) after 6 h of growth in brain heart infusion broth with 1% glucose (at 32°C) were used to test the strains for production of cytotoxins on Vero cells, and a microscope was used to determine whether the strains were cytotoxic. The supernatants that caused visible morphological changes of the Vero cells after 2 to 5 h of incubation (19 strains total) were further tested for cytotoxicity by using an objective method as described previously (28). This method records inhibition of protein synthesis either because of cell death or by specific reduction in the protein synthesis. The supernatants resulting in less than 20% inhibition of protein synthesis were also tested after a 10-fold concentration using ammonium sulfate (80% saturated) and dialysis. All 333 *Bacillus* spp. strains were also tested for production of putative emetic toxins by using the boar spermatozoa motility assay (4). As shown in Table 2, only eight strains were toxic: six strains were cytotoxic and three strains inhibited sperm movement, indicating the presence of emetic toxins. One strain was cytotoxic and produced putative emetic toxin(s). These eight strains were further characterized, and the species were identified by using a polyphasic approach consisting of the API CH50 and API 20E 16S rRNA partial sequence analysis, riboprinting, and repetitive extragenic palindromic sequence PCR (ERIC- and BOX-PCR). Strains B31, B75, and B162 were additionally characterized by partial *gyrA* gene sequence analysis. The species according to the different typing methods are shown in Table 3.

The strains B9 and B318 contained no diaminopimelic acid isomer, whereas the other six strains contained *meso*-α,ε-diaminopimelic acid. This diamino acid is found in the majority of *Bacillus* species. However, the "round-spore-forming group 2 bacilli," e.g., *B. sphaericus* and *B. fusiformis*, are characterized by the occurrence of lysine or ornithine instead of *meso*-α,ε-diaminopimelic acid as the diamino acid of the peptidoglycan (7). In agreement with the absence of *meso*-α,ε-diaminopimelic

TABLE 3. Species identification according to the different typing methods

Strain	Source	Species identification by				
		API 20E/API CH50	16S rRNA sequence analysis	Riboprinting	GyrA sequencing	Species assignment
B9	Water	*B. sphaericus*	*B. fusiformis*	No identification		*B. fusiformis*
B31	Spices	*B. subtilis*	*B. subtilis* subsp. *spizezinii*	*B. mojavensis*	*B. mojavensis*	*B. mojavensis*
B75	Spices	*B. subtilis*	*B. subtilis* subsp. *spizezinii*	*B. mojavensis*	*B. mojavensis*	*B. mojavensis*
B162	Food	*B. subtilis*	*B. mojavensis*	*B. mojavensis*	*B. mojavensis*	*B. mojavensis*
B174	Food	*B. licheniformis*	*B. subtilis* subsp. *subtilis*	*B. subtilis*		*B. subtilis*
B252	Tap water	*B. subtilis*	*B. subtilis* subsp. *subtilis*	*B. subtilis*		*B. subtilis*
B279	Tap water	*B. pumilus*	*B. pumilus*	*B. pumilus*		*B. pumilus*
B318	Water	*B. sphaericus*	*B. fusiformis*	No identification		*B. fusiformis*

TABLE 4. Toxic properties of the emetic toxin-and enterotoxin-producing *Bacillus* spp. strains

Strain	Species	Hemolysis	Cyt (%)[a]	Cyt 10× (%)[b]	Cyt 100°C (%)[c]	Putative emetic toxin[d]
B9	*B. fusiformis*	Positive	36	33	ND	ND
B31	*B. mojavensis*	ND[e]	69	91	35	Positive, 1,036.7
B75	*B. mojavensis*	ND	73	93	45	ND
B162	*B. mojavensis*	ND	<20	49	45	ND
B174	*B. subtilis*	ND	ND	ND	ND	Positive, 1,036.7
B252	*B. subtilis*	ND	<20	56	64	ND
B279	*B. pumilus*	ND	ND	ND	ND	Positive, 1,072.7
B318	*B. fusiformis*	Positive	33	30	ND	ND

[a] Percent cytotoxicity on Vero cells by using 100 μl of supernatant after 6 h of growth at 32°C.
[b] Percent cytotoxicity on Vero cells by using 100 μl of 10-fold-concentrated supernatant (by ammonium sulfate precipitation and dialysis) as described above.
[c] Percent cytotoxicity on Vero cells by using 100 μl of supernatant after 6 h of growth at 32°C and after boiling for 5 min. For strains B162 and B252, a 10-fold-concentrated extract was used.
[d] As determined by the boar sperm motility test and LC-MS. The number of AMU is given for the main peak in the methanol extract as $[M + H]^+$ for strains B31 and B174 and $[M + Na]^+$ for strain B279.
[e] ND, not detected.

acid, the strains B9 and B318 were identified as either *B. fusiformis* or *B. sphaericus*. By 16S rRNA gene sequencing, strains B9 and B318 were identified as *B. fusiformis* (98.3% sequence similarity). Neither of these two strains was identified by the RiboPrinter system because the patterns of strains B9 and B318 showed similarities below the threshold value for identification of 0.85 to all entries of the identification library. DNA fingerprints obtained from BOX- and ERIC-PCR revealed nearly identical patterns for both strains. Strain B279 was identified as *B. pumilus* and strain B252 was identified as *B. subtilis* by all employed methods. Strains B31, B75, B174, and B162 were identified by the different methods as *B. subtilis*, *B. subtilis* subsp. *spizizenii*, *B. licheniformis*, or *B. mojavensis*.

Differentiation between *B. subtilis* and *B. mojavensis* can be resolved at the species level only by additional methods such as DNA-DNA hybridization (29) or partial *gyrA* gene sequence analysis (6). Strains B31 and B75 were assigned to the same ribogroup by automated ribotyping, and also, the fingerprints derived from repetitive elements were identical for both strains. ERIC and BOX fingerprint patterns obtained from strain 162 were most similar to this group.

To further characterize these three strains (B31, B75, and B162), partial *gyrA* gene sequence analysis was performed. The partial *gyrA* gene sequences obtained for these strains were compared with published *gyrA* gene sequences for *B. subtilis* subsp. *subtilis*, *B. subtilis* subsp. *spizizenii*, and *B. mojavensis*. The partial *gyrA* gene sequences for the three strains were between 97.7 and 98.3% identical to the gene sequence for *B. mojavensis* but only 84.4 to 84.5% equal to the *gyrA* gene sequence for *B. subtilis* subsp. *spizizenii* and 83.1 to 83.2% identical to the partial *gyrA* gene sequence for *B. subtilis* subsp. *subtilis*. Strains B31 and B75 were 100% identical and strain B162 was 99.2% identical to strains B31 and B75. Based on these results, strains B31, B75, and B162 were assigned to the species *B. mojavensis*. The final species assignment based on the performed analysis is given in the last column of Table 3.

All cytotoxin- and putative emetic toxin-producing strains grew anaerobically, and none of them were positive on the *B. cereus* selective medium.

Some characteristics of the toxins produced. To characterize the cytotoxins, we looked at the activity after concentrating the supernatants 10-fold by using ammonium sulfate followed by dialysis and the heat stability by boiling the supernatants for 10 min (Table 4). For the two strains B162 and B252, a 10-fold-concentrated extract was boiled due to low initial activity in the culture supernatant. For the other strains, we used the nonconcentrated extracts to keep even small molecules that might not be precipitated by ammonium sulfate or that could escape during dialysis. Interestingly, two of the strains produced cytotoxins that were not concentrated by ammonium sulfate followed by dialysis (strains B9 and B318). The remaining strains produced toxins that were concentrated by this treatment. The heat stability test showed that two of the six strains (*B. fusiformis* strains B9 and B318) produced only heat-labile cytotoxins, while four strains (B31, B75, B162, and B252) also produced a heat-stable toxin (Table 4). For two of these strains (B31 and B75), cooking reduced the cytotoxic activity, while the remaining two (B162 and B252) maintained the activity after boiling.

For strains B31 and B75 (*B. mojavensis*), which gave the highest cytotoxicity after concentration of the supernatants, we determined the influence of growth temperature and growth time on cytotoxin production. Strains B31 and B75, although the same species, responded quite differently to the growth conditions. Strain B31 produced cytotoxins after 6 h but not after 2 h at all temperatures. Maximal cytotoxin production was relatively unchanged when growth temperatures ranging from 30 to 42°C were used, and no degradation (or loss of activity) was observed even at 42°C after 24 h of growth. For strain B75, the situation was quite different. Cytotoxin production had already started after 2 h at 25°C and was optimal at 30°C after 24 h. Surprisingly, the cytotoxin seemed to be degraded at 37° after 24 h of growth and was not produced at all at 42°C (data not shown).

Production of putative emetic toxins. The three putative emetic toxin-producing strains were from three different species: *B. mojavensis* (B31), *B. subtilis* (B174), and *B. pumilus* (B279). These three strains were tested for the influence of temperature and time on the putative emetic toxin production. Unlike *B. cereus*, where the optimum temperature for emetic toxin production is in the area between 12 and 22°C (3, 7, 10), our three strains (B31, B174, and B279) produced the putative emetic toxin faster (or just as fast) at 22 than at 30°C. It took longer for the *B. pumilus* strain (B279) to produce a putative emetic toxin at 30°C than it did for the two other strains (data not shown).

The number of AMU of the main peak of the putative emetic toxin methanol extract produced by the three strains was found by LC-MS. The number of AMU recorded for strains B31 (*B. mojavensis*) and B174 (*B. subtilis*) was 1,036.7 $[M + H]^+$, while from strain B279 (*B. pumilus*), the recorded mass was 1,072.7 $[M + Na]^+$ (Table 4).

PCR and immunological detection of *B. cereus*-like cytotoxins. Finally, we looked at the presence of *B. cereus*-like enterotoxins in the cytotoxic strains. None of the strains produced *B. cereus*-like enterotoxins or cytotoxins as shown by immunological methods, and none of them were positive by PCR.

DISCUSSION

Out of 333 *Bacillus* spp. strains isolated from natural water, tap water, different food products, spices, and a food plant, only eight strains were found to be toxin producers. Although this is a low number, it is not surprising. We have isolated a toxin-producing *Bacillus* sp. (except *B. cereus*) from foods believed to be involved in food poisoning only once, through the Norwegian Reference Laboratory. This strain (*B. subtilis* strain B174) was isolated from fried, marinated chicken and suspected of causing food poisoning involving several people. The meal was consumed at 7 p.m., and the symptoms started about 6 h later, with nausea, vomiting, severe stomach pain, headache, and, a little later, diarrhea.

We would also expect that strain B31 (*B. mojavensis*) would be able to cause diarrhea as judged from our experience with toxicity tests for food poisoning with *B. cereus*. Such *B. cereus* strains will usually give similar (or higher) inhibition of protein synthesis of Vero cells (the Norwegian Reference Laboratory). There is no doubt that several *Bacillus* spp. outside the *B. cereus* group can cause food poisoning (12). However, with the exception of an emetic toxin from *B. licheniformis* (17, 27, 32), the toxins from these species have never been purified and characterized, even more than three decades after the toxins were first described from outbreaks in the United Kingdom (12).

Five research papers have recently described non-*B. cereus* toxin-producing strains by different techniques (5, 11, 21, 25, 26). Several of those authors reported *B. cereus*-like enterotoxins from non-*B. cereus* species but without a definite identification of the species or the toxins. None of the *Bacillus* spp. described in this paper produced *B. cereus*-like toxins. It should also be noted that the regulation of transcription of all the enterotoxins or cytotoxins in *B. cereus* are under control of the positive regulator PlcR (19), and if the enterotoxin genes should be acquired from *B. cereus* by other *Bacillus* spp., they would need either a change in the promoter in order to be transcribed or a cotransfer of the regulator gene, which is not likely, as the two genes are on different parts of the genome. As we described initially, only 6 of the 19 strains that caused morphological changes on the Vero cells after 5 h produced what we have identified as specific cytotoxins. From our research on *B. cereus* over many years, we have never seen low-level toxin producers (although detectable after 2 h) that are involved in food poisoning (9). Species identification without using DNA-based techniques may often be incomplete. In earlier papers related to food poisoning and toxin identification for *Bacillus* spp. (except *B. cereus*), species identification has been based mainly on biochemical methods or the identification methods have not been given at all.

Although all the strains isolated from the water sources are Norwegian, many of the foods and all of the spices were imported from various parts of the world. Strain B31 (*B. mojavensis*) was isolated from a mixture of spices used in Norwegian food production. The different spices in the mixture were from Chile, Denmark, France, Holland, India, Indonesia, Spain, Sweden, and the United States. Strain B75 (identical to strain B31 by riboprinting) was isolated from imported basil spices of unknown origin, and strain B162 (*B. subtilis*) was from figs imported from Turkey, so it is likely that the low percentage of toxin-producing strains also reflects the situation in other countries.

Some of the toxins detected in this study seem to be quite small since they are not concentrated to the expected level with 80% saturated ammonium sulfate precipitation followed by dialysis. Although it is possible that some of the active molecules are not proteins (and thus not precipitated), we believe that it is more likely that they are small proteins or peptides (<8 to 10 kDa) that may escape during dialysis. Interestingly, three strains either produce a protein that is relatively heat stable or produce both heat-stable and heat-labile toxins (Table 4). One of these strains (*B. mojavensis* strain B31) is able to produce cytotoxins at temperatures between 25 and 42°C.

None of our six cytotoxin-producing strains made toxins that are similar to the *B. cereus* toxins as reported previously for several *Bacillus* spp. (5, 21, 25, 26). *B. subtilis*, *B. licheniformis*, *B. circulans*, *B. megaterium*, *B. lentimorbis*, *B. amyloliquefaciens*, and *B. lentus* have been reported to contain the *hbl* and *nhe* genes or to produce these proteins (detected by commercial kits for *B. cereus* enterotoxins). Until these species are identified by DNA techniques, we remain skeptical as to the identification of these species.

Emetic ring-formed toxins have been isolated from *B. cereus* (cereulide), *B. licheniformis*, and *B. pumilus* (1, 2, 17, 27) but not from *B. subtilis* or *B. mojavensis*. All such toxins are likely to belong to the same family of ring-formed peptides as the two well-characterized emetic toxins (1, 17). We have shown by LC-MS that three strains, B31 (*B. mojavensis*), B174 (*B. subtilis*), and B279 (*B. pumilus*), produce such peptides of expected size, distinctively different in size from cereulide (1). Interestingly, the putative emetic toxins we have described here are produced better or just as well at 30 as at 22°C. For *B. cereus*, the optimal temperature is much lower, between 12 and 22°C (3, 8, 10), and for *B. licheniformis*, the optimal temperature for emetic toxin production is probably close to 28°C, as this temperature was used for the toxin production when it was first characterized (27). Whether or not putative emetic toxins are real emetic toxins, however, can be verified only through feeding experiments using primates (12).

In conclusion, toxin-producing *Bacillus* spp. seem to be rare among isolates from water, food, and food environments, and none of the toxins detected are similar to the *B. cereus* enterotoxins or to cereulide. However, although rare, *Bacillus* spp. outside the *B. cereus* group might still be involved in food poisoning through foods that are considered safe by the public, such as pizza and pasta dishes (specifically if left overnight without refrigeration). Also, the variety of lightly heated ready-to-eat foods that are supposed to have a shelf life of several

weeks if cooled properly might result in food poisoning through surviving spores that will germinate and multiply. We would like the food industry to be able to detect possible virulent *Bacillus* spp. in food ingredients and in the food production plants. However, this detection requires knowledge of the toxins produced and their genes. We are in the process of purifying and characterizing potential food poisoning toxins from *Bacillus* spp. outside the *B. cereus* group.

ACKNOWLEDGMENTS

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