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Research Paper

Prevalence and Diversity of the Thermotolerant Bacterium *Bacillus cytotoxicus* among Dried Food Products

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ABSTRACT

Bacillus cytotoxicus, a member of the *Bacillus cereus* group, is a thermotolerant species originally reported from a lethal foodborne infection in France in 1998. The strain NVH391-98, isolated from this outbreak, produces cytotoxin K1, a potential cytotoxic enterotoxin. However, the habitat and diversity of *B. cytotoxicus* isolates so far have been poorly explored. The objective of this study was to assess the prevalence of this bacterium in different food products (mainly dried) and to estimate its diversity. Among the 210 samples analyzed, all potato flakes contained the bacterium at low concentrations ($\leq 10^2$ CFU/g). However, prepared and kept at room temperature for 2 days, the puree contained ca. 10^5 CFU/g *B. cytotoxicus*. Besides potato flakes, some samples of millet flour, salted potato chips, and soups also contained *B. cytotoxicus*. From these samples, 55 thermotolerant *B. cytotoxicus* isolates were obtained. When classified into six distinct random amplified polymorphism DNA patterns, they showed the existence of 11 distinct plasmid profiles. Although most isolates (including the reference strains NVH391-98 and NVH883-00) contained no detectable plasmid, some displayed one to three plasmids with sizes from ca. 8 to 90 kb. It also emerged from this study that a single food sample could contain *B. cytotoxicus* isolates with different genetic profiles.

HIGHLIGHTS

- *Bacillus cytotoxicus* was found in all tested potato flakes but at loads lower than 10^2 CFU/g.
- *B. cytotoxicus* was observed in other potato-containing products and in millet flour.
- *B. cytotoxicus* isolates ($n = 57$) fell into six RAPD patterns and 11 plasmid profiles.
- A large proportion of *B. cytotoxicus* isolates contained small and/or large plasmids.

Key words: *Bacillus cereus*; *Bacillus cytotoxicus*; Cytotoxin K1; Plasmid; Potato flakes

Author: Per journal style, we did not italicize sensu lato or sensu stricto (or their abbreviations).

Bacillus cytotoxicus is the thermotolerant member of *Bacillus cereus* sensu lato (B. cereus s.l. *(4, 17)*). *B. cereus* s.l., also called the *B. cereus* group, is a cluster of closely related species: *B. anthracis, B. thuringiensis, B. mycoides, B. pseudomycoides, B. weihenstephanensis, B. cereus* sensu stricto (s.s.), and *B. cytotoxicus (40)*. This family contains not only psychrotolerant, mesophilic, and thermotolerant strains *(19)* but also pathogens (for mammals and insects) and probiotic strains *(14, 22)*. In particular, *B. anthracis* is a pathogen of mammals that causes anthrax *(25, 35),* while *B. thuringiensis* is an entomopathogen used in agriculture, as environmental-friendly biopesticide, or as a bioinsecticide to control mosquito-borne diseases such as malaria or yellow fever *(14, 27). B. mycoides* and *B. pseudomycoides* are characterized by the rhizoidal aspect of their colonies *(36, 37)*. Several new members of the *B. cereus* group have been proposed, including *B. gaemokensis (28), B. toyonensis (26), B. bingmayongensis (31), B. wiedmannii (34),* and nine new species *(32)*. Although *B. weihenstephanensis* was long regarded as the sole psychrotolerant species of the *B. cereus* group because of its ability to grow below 7°C *(30),* several strains of *B. mycoides* and *B. wiedmannii* have been classified in this psychrotolerant cluster *(34, 39)*.

Some *B. cereus* s.s. strains can cause two types of foodborne diseases, with either an emetic or a diarrheic syndrome *(11, 40)*. The emetic syndrome is characterized by nausea and vomiting. It results from cereulide, a dodecadepsipeptide produced in the food that is heat stable and resistant to acid pH and gastric enzymes *(1, 9, 10)*. The diarrheic syndrome is thought to be caused by one or a combination of potential heat-labile enterotoxins and enzymes, presumably produced in the intestine by *B. cereus* isolates *(38)*. This syndrome is characterized by abdominal pain and diarrhea *(29)*. In addition to these foodborne diseases, some *B. cereus* s.s. strains have been implicated in human infections such as severe eye infection (endophthal-

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TABLE 1. *Prevalence of* B. cytotoxicus *in food samples*

Food products[a] (no. of samples tested)	No. of positive samples
Potato flakes (20, including 1 from Mali)	20
Moroccan chorba and harira instant soups (5)	3
Crunchy instant soup (2)	2
Salted potato chips (3)	2
Potato peelings (6)	1
Improved millet flour for children (13, from Mali)	1
Dried products (42)	0
Atiéke (1), coriander (1), crackers (1), crispy thins (snack biscuits) (1), curcuma (5), dehydrated aloe vera (1), dried pancakes (1), fine crystallized sugar (1), gary (1), hop (1), instant mushroom soup (1), instant noodles (1), instant pea soup (1), instant skimmed milk (1), instant vegetable soups (5), maple syrup powder (1), Mexican fajita spice mix (1), milk substitute powdered (2), mixed spices for noodles (1), mushroom (1), parsley (1), potato flour (1), potato slices (1), powdered milk (2), rice cake (1), sauce chasseur (1), soluble coffee substitute (1), vanilla flavor pudding (1), vegetable bouillon cube (1), vegetable soup (1), wheat semolina (1), yam meal (1)	
Dried products from West Africa (110)	0
Adja broth powder (1), bogonin (8), cassava flour (1), chicken broth powder (1), corn flour (2), couscous (1), datou (condiment) (4), degué (8), dehydrated mango (1), dolly broth powder (1), dried ginger (5), dried gumbo (1), dried néré (1), dried onion (2), fish powder (4), flour for animals (1), instant skimmed milk (1), jujube powder (1), kokonte lafu (dried cassava) (1), millet flour (4), onion powder (1), peanut powder (6), sesame seeds (3), sira mougou (baobab leaf powder) (12), soumbala (fermented condiment) (14), spices (22), wheat flour (3)	
Not dried products (5)	0
Frozen potato puree (1), Gnocchi (1), Mashed potato (from fresh potato) (1), Sweet potato peelings (2)	
Not dried products from West Africa (4)	0
Date juice (zeguene) (1), honey (1), peanut paste (1), tamarind juice (1)	
Total (210)	29

[a] Unless otherwise indicated, the samples originated from Belgium.

mitis), pneumonia, fulminant sepsis, and wound infections *(5)*.

NVH391-98, the type strain of *B. cytotoxicus,* was originally isolated from an episode of severe and fatal diarrhea for three people in France in 1998 *(33)*. It produces a necrotic and hemolytic toxin called cytotoxin K (CytK). Two CytK variants have since been described, CytK1 and CytK2 *(13),* and their genes are present in 5 and 42%, respectively, of the *B. cereus* group members *(16)*. CytK1 was described as more cytotoxic than the CytK2 variants present in the other *B. cereus* s.l. strains. It was also reported that the high cytotoxicity of the *B. cytotoxicus* type strain resulted not only from the important toxicity of its CytK1 but also from the high expression level of its *cytK1* gene *(6)*. However, NVH883-00, another strain of *B. cytotoxicus* isolated from spice, weakly expressed the *cytK1* gene and was found to be noncytotoxic *(12)*. Moreover, a study suggested that the cytotoxicity of *B. cytotoxicus* strains might have been overestimated in the past *(21)*.

B. cytotoxicus isolates are relatively rare and form a solid genetic cluster (phylogenetic group VII), distinct from the other species of the *B. cereus* group *(12, 17)*. This rarity may be partly because the official routine method used for the isolation of *B. cereus* makes it almost impossible to distinguish among the seven species *(40)*. However, after preenrichment at 50°C, several *B. cytotoxicus* have been isolated from potato products *(8, 21)*.

B. cytotoxicus, like other bacilli, is able to form spores highly resistant to harsh environmental conditions. It is therefore difficult to eliminate them with conventional cleaning and disinfection methods. They can easily contaminate food products and may represent a risk to the consumer health. Although some *B. cereus* group members are soil dwellers *(24),* the ecological niche of *B. cytotoxicus* is not clearly established and no study has hitherto focused on the diversity of this potentially dangerous species. This study aimed to study the foodborne habitat and genetic diversity of *B. cytotoxicus,* mainly among dried food products.

MATERIALS AND METHODS

Isolation and enumeration of thermotolerant *B. cytotoxicus*. A total of 210 samples were explored to assess the prevalence of *B. cytotoxicus* in food products. They were purchased in either Belgium or West Africa (Ghana, Mali, or Burkina Faso) (Table 1). *B. cytotoxicus* is a spore-forming bacterium and can contaminate dehydrated food products; therefore, most tested samples were dried products. Concerning the potato flakes, six distinct brands from five stores were sampled.

Next, 10 g of food product was thoroughly mixed with 90 mL of buffered peptone water (Bio-Rad, France) in a stomacher bag. For the isolation of *B. cytotoxicus* from the food samples, a bag was incubated at 50°C for 24 h for preenrichment *(8)*. The next day, serial dilutions were made in Ringer solution and 100 µL of each dilution was spread on Luria-Bertani (LB) agar and incubated at 37°C for 24 h. Five characteristic colonies (large, gray, flat, and generally, with an irregular border) of *B. cereus*–like bacteria were

selected, streaked on a new LB agar, and incubated at 50°C for 24 h. For the enumeration experiments, serial dilutions of the food products (recovered in buffered peptone water, as indicated earlier, but without enrichment) were made from 10^{-2} to 10^{-7} in Ringer solution, and 100 µL of each dilution was spread on mannitol egg yolk polymixin agar and incubated 24 h at 37°C.

Estimation of *B. cytotoxicus* load in mimetic household-cooked potato puree. To determine the load of *B. cytotoxicus* in cooked potato puree kept at household conditions, a puree of potato flakes was prepared according to the manufacturer's procedure and kept at room temperature (20°C) for 48 h. The number of *B. cytotoxicus* was determined at 0, 8, 24, and 48 h after the puree preparation as described earlier for the enumeration, except that the bacteria were incubated at 50°C for 24 h.

Genetic diversity and PCR detection of virulence genetic determinants. To assess the bacterial diversity, two to five isolates were selected from each positive sample. In total, 55 *B. cytotoxicus* isolates were retained from diverse products. The reference strains *B. cytotoxicus* NVH391-98 *(33)* and *B. cytotoxicus* NVH883-00 *(18)* were used as positive controls for the *cytK1* gene and as reference in the genetic diversity analyses.

To obtain the bacterial DNA, a fresh overnight colony on LB agar was suspended in 20 µL of deionized water. Total DNA extractions were performed using the Wizard Genomic DNA purification kit (Promega) from overnight cultures (LB, 30°C, 120 rpm). The primers used in this study (Supplemental Table S1) were cytK1F and cytK1R for the search of the gene *cytK1,* and EMIF and EMIR for the detection of the cereulide *crs2* genetic determinant (cereulide synthetase 2 gene). OPA9 and OPA10 *(23)* were used for random amplified polymorphism DNA (RAPD) typing. The PCR detection of the *cytK1* gene and the cereulide genetic determinants was performed as previously described *(7)*. For the RAPD experiments, the PCR mix consisted of 9.7 µL of demineralized water, 4.0 µL of buffer (Promega, Madison, WI), 2.0 µL of deoxynucleoside triphosphate, 2.0 µL of OPA primers, 1.2 µL of $MgCl_2$, 0.1 µL of *Taq* polymerase (Promega, Madison, WI), and 1.0 µL of template DNA. The following cycle was performed: 94°C (5 min); 45 cycles of 94°C (5 min), 32°C (1 min), and 72°C (2 min); 72°C (7 min); and 10°C (infinitely). Migration of the PCR amplicons of the virulence genetic determinants and RAPD were carried out on a 0.8 and 1.2% gel agarose (Santa Cruz Biotechnology, Dallas, TX), respectively, in Tris-acetate-EDTA buffer with 1 µg/mL ethidium bromide for 30 min at 100 V. Photographs of the gels were taken with the ChemiDoc MP Imaging System (Bio-Rad).

Extraction and visualization of plasmids. To further explore the genomic diversity of the isolates, gel electrophoreses of plasmid preparations were performed as previously described *(2, 15)*. Bacteria were grown in 7 mL of brain heart infusion (BHI) broth for 15 h at 30°C with shaking (120 rpm). A 2-mL volume of cells was centrifuged (8,000 × *g* for 7 min), and pellets were carefully resuspended in 100 µL of E buffer (15% sucrose, 40 mM Tris-hydroxide, 2 mM EDTA, pH 7.9). Then, 200 mL of lysing solution (3% SDS, 50 mM Tris, pH 12.5) was added. The lysates were heated at 60°C for 60 min, followed by the addition of 20 mL of proteinase K (20 mg/mL). The solutions were gently inverted and incubated at 37°C for 60 min. One mL of phenol–chloroform–isoamyl alcohol (25:24:1) was added, and the solutions were inverted carefully several times. After centrifugation (8,000 × *g* for 7 min), the upper aqueous layer was subjected to 25 h of agarose electrophoresis (80 V, 4°C). DNA was stained in 1 µg/mL ethidium bromide in Milli-Q water for 20 min and destained in Milli-Q water up to 7 days (at 4°C). Plasmid sizes were estimated by comparing with the reference plasmids of *B. thuringiensis* serovar *israelensis* AND508 *(3)*.

RESULTS

Isolation of *B. cytotoxicus* from food products. *B. cytotoxicus* isolates have been reported to be relatively rare in food products, with the notable exception of potato flakes. To investigate the source of this bacterium in food, and more specifically in dried food products, 210 samples were analyzed for the presence of the thermotolerant *B. cytotoxicus*. The samples originated from Belgium (82) and from West African countries (Ghana, Mali, or Burkina Faso) (128) and mostly constituted dried products (195 of 210, ca. 93%). As shown in Table 1, the bacterium was isolated from 29 samples, represented by six types of foods: 20 of 20 potato flakes, 3 of 5 Moroccan soups, 2 of 2 crunchy instant soups, 2 of 3 salted potato chips, 1 of 6 potato peelings, and 1 of 13 improved millet flour for children. All of these food products contained potatoes, with the exception of the improved millet flour, for which no potato was indicated by the producer. No thermotolerant *B. cytotoxicus* could be isolated from the other 181 samples, although some samples (12) contained or were derived from potatoes. In addition, the bacterium was not detected in 142 samples of dried food products either from Belgium or from West African countries (where direct contamination of food products by the environment is more likely).

Note: You had two instances of 1 of 6 potato peelings; we deleted one
Re: OK

Three to five isolates were obtained from each positive sample and tested by PCR for the presence of the *cytk1* gene and the cereulide genetic determinants. From the 114 *B. cytotoxicus* isolates tested, all were positive for *cytk1* and negative for the cereulide (data not shown).

RAPD pattern classification. To assess the diversity of the different *B. cytotoxicus* isolates, RAPD analysis was performed on 55 isolates, together with two reference strains. Using the OPA9 and OPA10 primers (Fig. 1), six distinct profiles (A to F) were obtained (Table 2). The reference strains NVH883-00 and NVH391-98 were classified in profiles A and B, respectively. As shown in Table 2, the first three profiles (A, B, and C) contained 22, 15, and 10 isolates, respectively, and constituted the largest part (47 of 57, or ca. 82%) of all tested isolates. While the isolates derived from potato-related products were distributed in all RAPD patterns, those from the Moroccan instant soups were limited to profile D. Only two isolates from potato flakes were classified in the latter profile. Profiles E and F contained only one isolate each.

Some isolates from the same food sample had different profiles (e.g., E19.1 and E19.3 that originated from the same potato flake sample displayed profiles C and A, respectively). Conversely, isolates pertaining to the same RAPD pattern originated from distinct origins (e.g., the 15 isolates with the B profile were isolated from 14 samples of five distinct origins: potato flakes, potato peeling, salted potato chips, millet flour, and vegetable puree) (Table 2).

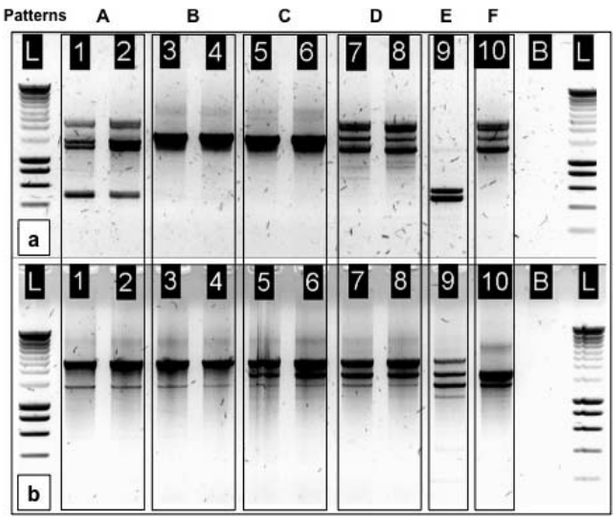

FIGURE 1. *RAPD patterns of* B. cytotoxicus *isolates using OPA9 (a, top panel) and OPA10 primers (b, bottom panel). L, molecular weight markers (linear DNA fragments from 200 bp to 10 kb). Lanes 1 to 10, NVH883-00, E28.3, NVH391-98, E3.1, E5.1, E26.1, SM1.1, SM2.8, E17.4, and PDT2.12. B, Negative control. The six patterns are shown: A (lanes 1 and 2), B (lanes 3 and 4), C (lanes 5 and 6), D (lanes 7 and 8), E (lane 9), and F (lane 10).*

We changed 11 to 10 because there are 10 lanes and there are 10 entitities listed. OK? Re: OK.

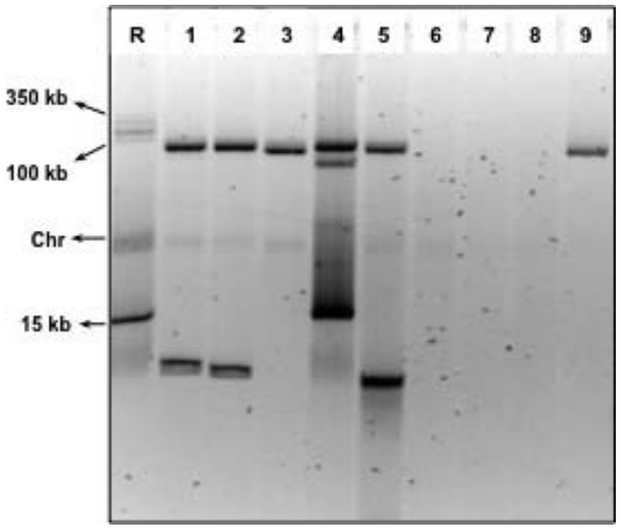

FIGURE 2. *Plasmid profiles of 11 isolates from different RAPD patterns. Chr, chromosome; R, reference profile from* B. thuringiensis *serovar* israelensis *strain AND508, which contains four large plasmids (100 to 350 kb) and a small linear 15-kb molecule. Lanes 1 to 9, E3.1, E4.1, E6.1, E8.1, E26.3, PDT1.6, PDT2.13, PDT3.5, and E6.3.*

Plasmid profiling. To discriminate the 55 isolated *B. cytotoxicus* isolates, their plasmid profiles were analyzed using a gel electrophoresis technique designed for both small and large plasmids *(3)*. Based on the number and estimated sizes of the plasmids, 11 distinct profiles were observed (Table 3). Some of these plasmid profiles are illustrated in Figure 2.

Although ca. 40% of the isolates (23 of 57, including the two reference strains) did not contain detectable plasmid (plasmid profile PP1), at least three large (ca. 70, 80, and 90 kb) and four small (ca. 8, 10, 12, and 15 kb) extrachromosomal molecules could be observed in various combinations in most *B. cytotoxicus* isolates. Isolates from different samples and RAPD patterns showed the same plasmid profiles, while some isolates from a single food product presented distinct patterns of large plasmids. For instance, isolates E28.2 and E28.5 were from the same potato flake sample (and displayed the same RAPD pattern A) but exhibited two different plasmid profiles (PP6 and PP1, respectively; Tables 2 and 3). The same is true for the SM3.11 and SM3.12 samples from the Moroccan instant soup sharing the RAPD pattern D yet showing two distinct plasmid patterns (PP9 and PP2, respectively). Conversely, isolates E6.1 and E6.3 were from the same potato flake sample and fell into different RAPD patterns (B and C, respectively) but showed the same plasmid profile (PP6).

TABLE 2. *Distribution of the* B. cytotoxicus *isolates among the six RAPD patterns*[a]

	RAPD patterns						
	A	B	C	D	E	F	Total
B. cytotoxicus isolates	E4.2, E4.4, E7.2, E8.1, E18.4, E19.3, E26.3, E27.3, E28.2, E28.3, E28.5, E29.2, NVH883-00,[b] PDT1.3, PDT1.6, PDT2.1, PDT2.6, PDT2.13, PDT3.4, PDT3.5, PDT3.8, TC1.2	E3.1, E4.1, E6.1, E9.4, E17.1, E25.1, E29.3, EM2, MB1.1, NVH391-98,[b] PDT1.1, PL1.1, PL1.2, PPM1.1, TC2.1	E5.1, E6.3, E7.1, E9.2, E19.1, E25.5, E26.1, EM3, PDT1.7, RSG1.3	E8.2, E18.1, RSG2.3, SM1.1, SM1.4, SM2.8, SM3.11, SM3.12	E17.4	PDT2.12	
No. of isolates	22	15	10	8	1	1	57

[a] E, EM, PPM, and PDT, potato flakes; PL, potato peelings; MB, millet flour for children from Mali; SM, instant Moroccan soup; TC, salted potato chips; RSG, crunchy instant soup.
[b] Reference strains.

Number was deleted from the footnote because it is clearly stated in the table.

TABLE 3. *Distribution of isolates among the profiles*[a]

Plasmid profile (no. of isolates)	Plasmid no. and estimated sizes	Isolates	Products	RAPD pattern
PP1 (23)	No visible plasmid	E4.4, E7.2, E18.4, E19.3, E28.5, E29.2, NVH883-00, PDT1.3, PDT1.6, PDT2.13, PDT3.5	Potato flakes	A
		E17.1, EM2, NVH391-98, PDT1.1, PPM1.1		B
		E9.2, E25.5		C
		E18.1		D
		PL1.1	Potato peelings	B
		TC1.2	Salted potato chips	A
		MB1.1	Millet flour	B
		RSG2.3	Crunchy instant soup	D
PP2 (5)	1 small, ~10 kb	E25.1	Potato flakes	B
		E17.4		E
		SM1.4, SM2.8, SM3.12	Instant Moroccan soup	D
PP3 (1)	2 small, ~12 and ~15 kb	RSG1.3	Crunchy instant soup	C
PP4 (1)	1 large, ~70 kb	PDT2.12	Potato flakes	F
PP5 (2)	1 large, ~80 kb, and 2 small, ~8 and ~10 kb	E7.1, E26.1	Potato flakes	C
PP6 (7)	1 large, ~90 kb	E4.2, E28.2, PDT2.1, PDT3.8	Potato flakes	A
		E6.1		B
		PL1.2	Potato peelings	B
		E6.3	Potato flakes	C
PP7 (8)	1 large, ~90 kb, and 1 small, ~10 kb	E26.3	Potato flakes	A
		E3.1, E4.1, E29.3	Potato flakes	B
		E5.1, E19.1		C
		E8.2		D
		TC2.1	Salted potato chips	B
PP8 (4)	1 large, ~90 kb, and 1 small, ~15 kb	E27.3, E28.3	Potato flakes	A
		EM3, PDT1.7		C
PP9 (2)	1 large, ~80 kb, and 1 small, ~10 kb	SM1.1, SM3.11	Instant Moroccan soup	D
PP10 (1)	2 large, ~80 and ~90 kb, and 1 small, ~15 kb	E8.1	Potato flakes	A
PP11 (3)	2 large, ~80 and ~90 kb	PDT2.6, PDT3.4	Potato flakes	A
		E9.4		B

[a] $n = 57$ isolates.

Beside PP1 (no detectable plasmid), the three most abundant plasmid patterns were PP7 (8), PP6 (7), and PP2 (5), totaling 20 of the 57 isolates. Four patterns (PP3, PP4, PP5, and PP10) were observed in single isolates. In addition, the combination of six RAPD patterns and 11 plasmid profiles suggested the existence of at least 23 distinct genomic profiles among the 57 tested isolates (Table 2).

Enumeration of *B. cytotoxicus* in potato puree kept at room temperature. As indicated earlier, most positive samples originated from potato flakes. The load of thermotolerant *B. cytotoxicus* was therefore estimated in potato flakes from five distinct brands. The results revealed that they all contained less than 10^2 CFU/g *B. cytotoxicus*. However, considering that in everyday life cooked puree could be mishandled and kept at room temperature for several hours, enumeration of *B. cytotoxicus* was done in potato puree, cooked as indicated by the manufacturers, and kept at 20°C. After 8, 24, and 48 h of incubation, the concentrations of thermotolerant *B. cytotoxicus* reached 3.5 ± 0.5, 4.5 ± 1.2, and 5.7 ± 1.9 log CFU/g (mean ± standard deviation), respectively. Longer incubation periods resulted in spoiled and smelly purees that would have prevented any likely consumption.

DISCUSSION

B. cytotoxicus is considered a potential foodborne pathogen, because it was originally isolated from an outbreak causing the death of three people *(33)*. Based on genomic data, it was also shown to represent a new thermotolerant species within the *B. cereus* group *(17)*. From the isolation of this first *B. cytotoxicus* strain, few new isolates have been reported *(8, 21)*. The species seems to be rare, because only 5% of a *B. cereus* s.l. collection in France was identified as *B. cytotoxicus (16)*. In the present study, *B. cytotoxicus* was found in only 29 of 210 tested samples,

most of which (20 of 29) were isolated from potato flakes (Table 1). The same low prevalence was observed in both African and non-African products. The high prevalence in flakes is in agreement with what was found by Contzen et al. *(8)* or Heini et al. *(21)* and is related to the only reported fatal case of diarrheal food poisoning involving vegetable puree *(33)*. Similarly, another early reported strain (INRA AF2) was isolated from vegetable puree *(12, 17)*.

Yet, in the present study, thermotolerant *B. cytotoxicus* isolates were not limited to potato flakes but also were recovered from other potato-containing foods that had not been previously reported (e.g., instant soups, salted chips, or potato peelings). The bacterium was also isolated in 1 of 13 samples of "improved millet flour for children," which according to the Malian producer, did not contain potato. This raises the question of the actual origin of all these isolates: Is there a particular environmental source for *B. cytotoxicus,* and/or is the process of food drying preferentially selecting for this thermotolerant bacterium? Along with this questioning, we have explored several potential ecological niches for *B. cytotoxicus,* including a set of human and environmental samples (diarrheal stools from children and dust samples), but none contained the thermotolerant bacterium (data not shown).

Notwithstanding its high prevalence in potato flakes and instant soups, *B. cytotoxicus* was always found at low concentrations ($<10^2$ CFU/g). However, we showed that commercial potato puree, cooked and kept at room temperature (20°C) for 48 h, contained ca. 10^5 CFU/g of *B. cytotoxicus,* a concentration similar to the one found in the vegetable puree that caused the human death in 1998 *(33)*. Even though it has been suggested that the cytotoxicity of *B. cytotoxicus* might have been overestimated *(21),* the presence of the bacterium, even at low doses, should not be neglected, because the innocuity of CytK1 is not yet clearly demonstrated. It is generally accepted that a dose of at least 10^5 CFU/g *B. cereus* is required the diarrheal disease to occur *(11)*. However, cases of diarrhea with less than 10^3 CFU/g have already been recorded *(16)*.

The *cytK1* gene is a characteristic feature and reliable marker of *B. cytotoxicus (20)*. In the present study, all isolates were PCR positive for *cytK1* but negative in the PCR screening of the cereulide genetic determinants. This is consistent with previous studies and reassuring in terms of food safety.

Concerning diversity within the *B. cytotoxicus* species, it has previously been demonstrated that the first three isolated strains (NVH391-98, INRA AF2, and NVH883-00) could be differentiated upon their sensitivity to the temperate phage phBC391A2 *(4)*. Even though they form a phylogenetic cluster far from the other members of the *B. cereus* group *(12),* in the present study, isolates could be classified into six RAPD patterns (Table 2 and Fig. 1) and 11 distinct plasmid profiles (Table 2), giving rise to at least 23 distinct genotypic profiles. At least three large (ca. 70, 80, and 90 kb) and four small (ca. 8, 10, 12, and 15 kb) plasmids could be observed among the isolates. Although these extrachromosomal molecules were absent from the reference strains NVH391-98 and NVH883-00, as well as about 40% of the isolates, it would be interesting to decipher their contribution to the ecology of this thermotolerant bacterium. Further genomic and proteomic investigations of *B. cytotoxicus* should shed light on its potential virulence and should help us to prevent its contribution to foodborne diseases. These approaches are underway.

The aim of this work was to assess the prevalence of *B. cytotoxicus* in food matrices and to investigate the isolate diversity within this species. A high prevalence of *B. cytotoxicus* was observed in potato flakes but at loads lower than 10^2 CFU/g. However, potato puree prepared and kept at room temperature for several hours can become potentially dangerous, with a load in *B. cytotoxicus* as high as 10^5 CFU/g. Nevertheless, thermotolerant *B. cytotoxicus* remains rare in food products and is essentially restricted to potato-containing products. Finally, contrary to what was generally admitted, *B. cytotoxicus* isolates display significant diversity, in particular with regard to their plasmid content. Genome sequencing of isolates representative of *B. cytotoxicus* diversity is in progress.

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SUPPLEMENTAL MATERIAL

Supplemental material associated with this article can be found online at: https://doi.org/10.4315/0362-028X.JFP-19-006.s1

REFERENCES

1. Agata, N., M. Mori, M. Ohta, S. Suwan, I. Ohtani, and M. Isobe. 1994. A novel dodecadepsipeptide, cereulide, isolated from *Bacillus cereus* causes vacuole formation in HEp-2 cells. *FEMS Microbiol. Lett*. 121:31–34.
2. Andrup, L., K. K. Barfod, G. B. Jensen, and L. Smidt. 2008. Detection of large plasmids from the *Bacillus cereus* group. *Plasmid* 59:139–143.
3. Andrup, L., J. Damgaard, and K. Wassermann. 1993. Mobilization of small plasmids in *Bacillus thuringiensis* subsp. *israelensis* is accompanied by specific aggregation. *J. Bacteriol*. 175:6530–6536.
4. Auger, S., N. Galleron, E. Bidnenko, S. D. Ehrlich, A. Lapidus, and A. Sorokin. 2008. The genetically remote pathogenic strain NVH391-98 of the *Bacillus cereus* group is representative of a cluster of thermophilic strains. *Appl. Environ. Microbiol*. 74:1276–1280.
5. Bottone, E. J. 2010. *Bacillus cereus,* a volatile human pathogen. *Clin. Microbiol. Rev.* 23:382–398.
6. Brillard, J., and D. Lereclus. 2004. Comparison of cytotoxin cytK promoters from *Bacillus cereus* strain ATCC 14579 and from a *B. cereus* food-poisoning strain. *Microbiology* 150:2699–2705.
7. Castiaux, V., L. Laloux, Y. J. Schneider, and J. Mahillon. 2016. Screening of cytotoxic *B. cereus* on differentiated caco-2 cells and in co-culture with mucus-secreting (HT29-MTX) cells. *Toxins (Basel)* 8 (11):320.
8. Contzen, M., M. Hailer, and J. Rau. 2014. Isolation of *Bacillus cytotoxicus* from various commercial potato products. *Int. J. Food Microbiol*. 174:19–22.
9. Dierick, K., E. Van Coillie, I. Swiecicka, G. Meyfroidt, H. Devlieger, A. Meulemans, G. Hoedemaekers, L. Heyndrickx, and J. Mahillon. 2005. Fatal family outbreak of *Bacillus cereus*-associated food poisoning. *J. Clin. Microbiol*. 43:4277–4279.
10. Ehling-Schulz, M., M. Fricker, and S. Scherer. 2004. *Bacillus cereus,* the causative agent of an emetic type of food-borne illness. *Mol. Nutr. Food Res*. 48:479–487.

11. European Food Safety Authority (EFSA). 2005. Opinion of the scientific panel on biological hazards on *Bacillus cereus* and other *Bacillus* spp. in foodstuffs. *EFSA J*. 175:1–48.
12. Fagerlund, A., J. Brillard, R. Furst, M. H. Guinebretière, and P. E. Granum. 2007. Toxin production in a rare and genetically remote cluster of strains of the *Bacillus cereus* group. *BMC Microbiol*. 7:43.
13. Fagerlund, A., O. Ween, T. Lund, S. P. Hardy, and P. E. Granum. 2004. Genetic and functional analysis of the *cytK* family of genes in *Bacillus cereus*. *Microbiology* 150:2689–2697.
14. Federici, B. A. 2005. Insecticidal bacteria: an overwhelming success for invertebrate pathology. *J. Invert*. *Pathol*. 89:30–38.
15. Gillis, A., S. Guo, A. Bolotin, L. Makart, A. Sorokin, and J. Mahillon. 2017. Detection of the cryptic prophage-like molecule pBtic235 in *Bacillus thuringiensis* subsp. *israelensis*. *Res. Microbiol*. 168:319–330.
16. Glasset, B., S. Herbin, L. Guillier, S. Cadel-Six, M. L. Vignaud, J. Grout, S. Pairaud, V. Michel, J. A. Hennekinne, N. Ramarao, and A. Brisabois. 2016. *Bacillus cereus*–induced food-borne outbreaks in France, 2007 to 2014: epidemiology and genetic characterisation. *Euro Surveill*. 21:48.
17. Guinebretière, M. H., S. Auger, N. Galleron, M. Contzen, B. De Sarrau, M. L. De Buyser, G. Lamberet, A. Fagerlund, P. E. Granum, D. Lereclus, P. De Vos, C. Nguyen-The, and A. Sorokin. 2013. *Bacillus cytotoxicus* sp. nov. is a novel thermotolerant species of the *Bacillus cereus* group occasionally associated with food poisoning. *Int. J. Syst. Evol*. *Microbiol*. 63:31–40.
18. Guinebretière, M. H., A. Fagerlund, P. E. Granum, and C. Nguyen-The. 2006. Rapid discrimination of cytK-1 and cytK-2 genes in *Bacillus cereus* strains by a novel duplex PCR system. *FEMS Microbiol. Lett*. 259:74–80.
19. Guinebretière, M. H., F. L. Thompson, A. Sorokin, P. Normand, P. Dawyndt, M. Ehling-Schulz, B. Svensson, V. Sanchis, C. Nguyen-The, M. Heyndrickx, and P. De Vos. 2008. Ecological diversification in the *Bacillus cereus* group. *Environ. Microbiol*. 10:851–865.
20. Guinebretière, M. H., P. Velge, O. Couvert, F. Carlin, M. L. Debuyser, and C. Nguyen-The. 2010. Ability of *Bacillus cereus* group strains to cause food poisoning varies according to phylogenetic affiliation (groups I to VII) rather than species affiliation. *J. Clin. Microbiol*. 48:3388–3391.
21. Heini, N., R. Stephan, M. Ehling-Schulz, and S. Johler. 2018. Characterization of *Bacillus cereus* group isolates from powdered food products. *Int. J. Food Microbiol*. 283:59–64.
22. Hong, H. A., H. Le Duc, and S. M. Cutting. 2005. The use of bacterial spore formers as probiotics. *FEMS Microbiol. Rev*. 29:813–835.
23. Hu, X., B. M. Hansen, J. Eilenberg, N. B. Hendriksen, L. Smidt, Z. Yuan, and G. B. Jensen. 2004. Conjugative transfer, stability and expression of a plasmid encoding a *cry1Ac* gene in *Bacillus cereus* group strains. *FEMS Microbiol. Lett*. 231:45–52.
24. Jensen, G. B., B. M. Hansen, J. Eilenberg, and J. Mahillon. 2003. The hidden lifestyles of *Bacillus cereus* and relatives. *Environ. Microbiol*. 5:631–640.
25. Jernigan, J. A., D. S. Stephens, D. A. Ashford, C. Omenaca, M. S. Topiel, M. Galbraith, M. Tapper, T. L. Fisk, S. Zaki, T. Popovic, R. F. Meyer, C. P. Quinn, S. A. Harper, S. K. Fridkin, J. J. Sejvar, C. W. Shepard, M. McConnell, J. Guarner, W. J. Shieh, J. M. Malecki, J. L. Gerberding, J. M. Hughes, B. A. Perkins, and members of the Anthrax Bioterrorism Investigation Team. 2001. Bioterrorism-related inhalational anthrax: the first 10 cases reported in the United States. *Emerg. Inf. Dis*. 7:933–944.
26. Jiménez, G., M. Urdiain, A. Cifuentes, A. López-López, A. R. Blanch, J. Tamames, P. Kämpfer, A.-B. Kolstø, D. Ramón, J. F. Martínez, F. M. Codoñer, and R. Rosselló-Móra. 2013. Description of *Bacillus toyonensis* sp. nov., a novel species of the *Bacillus cereus* group, and pairwise genome comparisons of the species of the group by means of ANI calculations. *Syst. Appl. Microbiol*. 36:383–391.
27. Jouzani, G. S., E. Valijanian, and R. Sharafi. 2017. *Bacillus thuringiensis*: a successful insecticide with new environmental features and tidings. *Appl. Microbiol. Biotechnol*. 101:2691–2711.
28. Jung, M. Y., W. K. Paek, I. S. Park, J. R. Han, Y. Sin, J. Paek, M. S. Rhee, H. Kim, H. S. Song, and Y. H. Chang. 2010. *Bacillus gaemokensis* sp. nov., isolated from foreshore tidal flat sediment from the Yellow Sea. *J. Microbiol*. 48:867–871.
29. Kramer, J. M., and R. J. Gilbert. 1989. *Bacillus cereus* and other *Bacillus* species, p. 21–70. *In* M. P. Doyle (ed.), Foodborne bacterial pathogens. Marcel Dekker, New York.
30. Lechner, S., R. Mayr, K. P. Francis, B. M. Pruss, T. Kaplan, E. Wiessner-Gunkel, G. S. Stewart, and S. Scherer. 1998. *Bacillus weihenstephanensis* sp. nov. is a new psychrotolerant species of the *Bacillus cereus* group. *Int. J. Syst. Bacteriol*. 48:1373–1382.
31. Liu, B., G. H. Liu, G. P. Hu, C. Sengonca, N. Q. Lin, J. Y. Tang, W. Q. Tang, and Y. Z. Lin. 2014. *Bacillus bingmayongensis* sp. nov., isolated from the pit soil of Emperor Qin's terra-cotta warriors in China. *Anonie Leeuwenhoek* 105:501–510.
32. Liu, Y., J. Du, Q. Lai, R. Zeng, D. Ye, J. Xu, and Z. Shao. 2017. Proposal of nine novel species of the *Bacillus cereus* group. *Int. J. Syst. Evol. Microbiol*. 67:2499–2508.
33. Lund, T., M. L. De Buyser, and P. E. Granum. 2000. A new cytotoxin from *Bacillus cereus* that may cause necrotic enteritis. *Mol. Microbiol*. 38:254–261.
34. Miller, R. A., S. M. Beno, D. J. Kent, L. M. Carroll, N. H. Martin, K. J. Boor, and J. Kovac. 2016. *Bacillus wiedmannii* sp. nov., a psychrotolerant and cytotoxic *Bacillus cereus* group species isolated from dairy foods and dairy environments. *Int. J. Syst. Evol. Microbiol*. 66:4744–4753.
35. Mock, M., and A. Fouet. 2001. Anthrax. *Ann. Rev. Microbiol*. 55:647–671.
36. Nakamura, L. K. 1998. *Bacillus pseudomycoides* sp. nov. *Int. J. Syst. Bacteriol*. 48:1031–1035.
37. Nakamura, L. K., and M. Jackson. 1995. Clarification of the taxonomy of *Bacillus mycoides*. *Int. J. Syst. Evol. Microbiol*. 45:46–49.
38. Senesi, S., and E. Ghelardi. 2010. Production, secretion and biological activity of *Bacillus cereus* enterotoxins. *Toxins (Basel)* 2:1690–1703.
39. Soufiane, B., and J.-C. Coté. 2013. *Bacillus weihenstephanensis* characteristics are present in *Bacillus cereus* and *Bacillus mycoides* strains. *FEMS Microbiol. Lett*. 341:127–137.
40. Stenfors Arnesen, L. P., A. Fagerlund, and P. E. Granum. 2008. From soil to gut: *Bacillus cereus* and its food poisoning toxins. *FEMS Microbiol. Rev*. 32:579–606.