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Screening food for *Bacillus cereus* toxins using whole genome sequencing

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1. Introduction

Bacillus cereus is a Gram-positive, rod-shaped, spore-forming bacterium that is commonly found in soil (Kotiranta et al., 2000; Schoeni and Wong, 2005) and food (Kramer, 1989; Arnesen et al., 2008). *B. cereus* spores can persist in harsh, desiccate conditions and can survive the pasteurization process (Drobniewski, 1993). As such, *B. cereus* is among the top pathogens for causing foodborne illness in the US (Scallan et al., 2011). Food generally contaminated with *B. cereus* include dry food products and cooked rice that have been time-temperature abused, allowing for growth of *B. cereus*. *B. cereus* foodborne illness causes severe nausea, vomiting, and diarrhea and is usually self-limiting (Tallent, 2012), although some serious illnesses and deaths have been reported (Mahler et al., 1997; Lund et al., 2000; Dierick et al., 2005). *B. cereus* produces toxins, which result in two distinct foodborne diseases. One disease, known as diarrheal disease, is characterized by abdominal cramps and watery or profuse diarrhea with an onset of 6–15 h (Kramer, 1989). The second disease is known as the emetic disease, and is characterized by nausea, vomiting, and abdominal cramps with a more rapid onset of 30 min to 6 h (Ehling-Schulz et al., 2004a,b). This disease is sometimes mistaken for Staphylococcal food poisoning because of the similar symptoms and disease onset (Seo and Bohach, 2007). In both disease manifestations, symptoms generally clear within 24 h.

B. cereus diarrheal disease results from production of hemolysin BL (Hbl), nonhemolytic enterotoxin (Nhe), or cytotoxin (CytK) in the gut (Beecher and Macmillan, 1991; Lund and Granum, 1996; Lund et al., 2000). Hbl and Nhe are both three-component protein toxins that require all three components to exhibit biological activity (Beecher and Macmillan, 1991; Beecher et al., 1995; Granum et al., 1999; Lindback et al., 2004). Hbl consists of three proteins L2, L1, and B which are encoded by the operon *hblCDA* (Beecher and Macmillan, 1991; Heinrichs et al., 1993; Ryan et al., 1997; Lindback et al., 1999). It has been shown that Hbl can bind erythrocytes and lyse them by forming a transmembrane pore and disrupting osmotic equilibrium (Beecher and Wong, 1997). In addition, Hbl has been shown to have cytotoxic activities and contribute to vascular permeability of various cell types including vero cells and retinal tissue (Beecher et al., 1995; Granum and Lund, 1997). Nhe is also three-component toxin complex that is composed of NheA, NheB, and NheC, all encoded by the *nheABC* operon (Granum, O'Sullivan et al., 1999). Like Hbl, Nhe can also cause cell death through osmotic lysis and transmembrane pore formation (Fagerlund et al., 2008). Additionally, Nhe has hemolytic activity towards erythrocytes from several mammalian species (Fagerlund et al., 2008). Both Hbl and Nhe are pore-forming toxins and can cause intestinal fluid secretion; however, how the three components interact and which components are responsible for pore formation is not well understood (Arnesen et al., 2008). In contrast to the three-component toxins Hbl and Nhe, CytK is a single-component protein toxin that is a member of the β-barrel pore-forming family (Lund et al., 2000). There are two forms, CytK1 or CytK2, with CytK1 exhibiting greater toxicity to human cells (Fagerlund et al., 2004). CytK is a water-soluble monomer that forms a transmembrane pore in cell membranes (Bhakdi and Tranum-Jensen, 1991). CytK is dermo-necrotic, cytotoxic, and also possesses hemolytic activities similar to Hbl and Nhe (Lund et al., 2000). Generally, Hbl, Nhe, and CytK diarrheal toxins are not directly detectable from food due to the instability of these proteins.

B. cereus emetic disease results from ingestion of pre-formed cereulide (Ces) toxin in food (Arnesen et al., 2008). Ces is a cyclic dodecadepsipeptide that is encoded by the non-ribosomal peptide synthase *ces* gene cluster (Ehling-Schulz et al., 2004a,b). The mechanism of action of Ces is not well understood, although animal feeding experiments suggest its mechanism is receptor-mediated (Arnesen et al., 2008). Some biological effects of this toxin include; action as a cation ionopore

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inhibiting mitochondrial activity by inhibiting fatty acid oxidation (Mahler et al., 1997; Mikkola et al., 1999), degeneration of hepatocytes (Yokoyama et al., 1999), cellular damage (Shinagawa et al., 1996), and inhibition of natural killer cells in the immune system (Paananen et al., 2002). Ces can be directly detected from food because it is resistant to heat, acid, and proteolysis (Agata et al., 1995).

Existing detection methods for *B. cereus* toxins focuses on the three cytotoxins Hbl, Nhe, CytK and Ces. Two antibody-based detection kits for Nhe and Hbl are currently commercially available (Arnesen et al., 2008). The BCET-RPLA kit (Oxoid Ltd., UK) is a semi-quantitative assay using reversed antibody agglutination. It detects the L2 component of Hbl (Beecher and Wong, 1994). Previously, the TECRA-BDE kit (3M Tecra, St. Paul, MN), a double sandwich enzyme immunoassay detected the NheA component of Nhe (Beecher and Wong, 1994); however, the Tecra has since been discontinued. Currently, there is also the Duopath Cereus Enterotoxin lateral flow device (EMD Millipore, Darmstadt, Germany) that detects both NheB and the L2 component of Hbl using monoclonal antibodies (Krause et al., 2010; Tallent et al., 2015). Neither of these antibody-based kits confirm functional or biological activity of either toxin, because only one component of each three-component toxin is detected. Other nonspecific detection methods using animal and tissue culture assays have also been employed for detection of Hbl and Nhe (Arnesen et al., 2008). Like Hbl and Nhe, Ces is detectable using various activity assays including, monkey feeding tests (Melling et al., 1976), variations of cell culture assays using HEp-2 cells (Hughes et al., 1988; Agata et al., 1994; Sakurai et al., 1994; Finlay et al., 1999), and a boar sperm motility assay (Andersson et al. 1998, 2004; Hoornstra et al., 2003). Although these are activity-based assays, the only method to specifically and semi-quantitatively detect Ces is a LC-MS/MS assay (Haggblom et al., 2002; Shaheen et al., 2006; Biesta-Peters et al., 2010; Tallent et al., 2017).

In addition to the toxin detection assays previously mentioned, various multiplex PCR assays for detection of toxin genes has been developed (Mantynen and Lindstrom, 1998; Hansen et al., 2001; Guinebretiere et al., 2002; Ehling-Schulz et al., 2004a,b; Yang et al., 2005; Fricker et al., 2007; Ngamwongsatit et al., 2008; Ueda et al., 2013). The utility of these multiplex PCR assays to identify *B. cereus* toxins however is questioned because of the wide distribution of genes across *B. cereus* strains and the fact that the number of genes detected by PCR is limited. In this study, we propose to use whole genome sequencing to expand the number of detectable genes that are present in food contaminated with *B. cereus*. Additionally, use of a computational tool like BTyper will assist in data analysis of these sequenced strains. Development of this whole genome sequencing method and pipeline for detection of toxin genes will be a more powerful and detailed primary screening method than the current PCR-based methods and will provide more information on *B. cereus* toxins and other virulence genes that may be of concern.

2. Materials and methods

2.1. Bacterial strains and culture conditions

The 50 inclusivity strains (*Bacillus cereus* group) and 30 exclusivity strains including members from *Escherichia coli, Staphylococcus aureus, Citrobacter sp., Enterobacter sp., Salmonella sp., Bacillus amyloliquifaciens, Bacillus subtilis, Bacillus licheniformis, Acinetobacter sp., Listeria sp., and Pseudomonas sp*. used in this study were preserved in nutrient broth with glycerol and maintained at −80 °C. All strains were streaked twice onto tryptic soy agar (TSA) and incubated at 30 °C or 37 °C. Isolated colonies were transferred to brain heart infusion broth (pH 7.4) containing 0.1% glucose (BHIG) (Difco, Franklin Lakes, NJ), and incubated overnight with shaking at 30 °C or 37 °C.

2.2. Food preparation

Food included infant formula, white chocolate pancake mix, whey powder, mashed potato mix, gravy, and cooked rice purchased from local markets. Dehydrated products (formula, pancake, whey, and potato) were rehydrated in Butterfield's Phosphate Buffered Dilution Water (BPB), gravy was diluted 1:10 in BPB, and cooked rice was homogenized 1:10 in BPB prior to inoculation and incubation. Food portions were inoculated with a 1:10 dilution of cells and incubated at 30 °C for 24 h. Each portion was tested in duplicate and inoculated with cells or BPB as a control.

The Duopath® cereus enterotoxins (EMD Millipore, Darmstadt, Germany) assay is a lateral flow device (LFD) that uses monoclonal antibodies for the detection NheB and the L_2 component of Hbl with a reported detection limit of 6 ng ml^{-1} and 20 ng ml^{-1}, respectively (Krause et al., 2010).

Bacterial cells: Six model strains were used in this study. Three *B. cereus* strains (A14579, F4227A, TJL-16, 905–9) were used as inclusivity strains and one *S. aureus* strain (963–3) and one *B. licheniformis* (F1383), were used as exclusivity strains. Strains were inoculated as previously described in the bacterial strains and culture conditions. For food inoculation, cells were pelleted at 4000 *x g* for 15 min. The supernatant was discarded, and the cells were washed twice with BPB and finally resuspended in 10 mLs of BPB. Each food sample (10 g) was added and mixed with 90 ml BPB heated at 75 °C for 30 min, transferred (9 ml) to conical tubes. This heat-treated food sample was spiked with1ml of resuspended culture, incubated at 30 °C for 24 h prior to processing.

2.3. DNA extraction

DNA was extracted from overnight cultures in BHIG or food portions. For cultures, DNA was isolated after a 30-min pre-treatment of lysozyme at 35 °C then extraction using the DNeasy Power Soil kit (Qiagen, Germantown, MD) following the manufacturer's instructions. For food portions, DNA was isolated using the DNeasy mericon Food Kit (Qiagen, Germantown, MD) following the manufacturer's instructions.

2.3.1. Polymerase chain reaction (PCR)

Primers for PCR were purchased from Integrated DNA Technologies (IDT) (Coralville, IA) (Ehling-Schulz et al., 2004a,b; Ngamwongsatit et al., 2008) and used with conditions previously described (Tallent et al., 2015). A single 50 µl reaction tube contained HotStar DNA Polymerase (Qiagen, Germantown, MD) (1 unit DNA polymerase, 1.5 mM $MgCl_2$ and 200 µ mol l^{-1} each dNTP), four primer sets (Table 1) with final concentrations as follows: 0.2 µ mol l^{-1} of CesF1 and CesR2; 1 µ mol l^{-1} of HD2F and HA4R; 0.3 µ mol l^{-1} of NA2F and NB1R; 0.4 µ mol l^{-1} CKF2 and CFR5 and DNA extracted from cell pellets. The running conditions included 30 cycles: 95 °C/30 s, 49 °C/30 s, 72 °C 1 min with a final annealing step at 72 °C/2 min. Amplified products were resolved by electrophoresis using Agilent TapeStation 4200 (Santa Clara, CA).

2.3.2. Whole genome sequencing (WGS)

DNA for whole genome sequencing was diluted to 1 ng in Ultrapure DNase/RNase-Free Distilled Water (Invitrogen, Carlsbad, CA). Library preparation was performed per manufacturer's instructions using Illumina Nextera XT Library Prep kit (San Diego, CA). All sequencing was performed on Illumina MiSeq desktop sequencers using the 500-cycle MiSeq Reagent V2 Kits (San Diego, CA).

2.4. Genome assembly and BTyper analysis

Genome assemblies of raw FASTQ files were performed using SPAdes. All FASTA files were analyzed through the BTyper computational tool as published in (Carroll et al., 2017). Genes were called

Table 1
Primers used for toxin gene targets.

	Gene target	Sequence (5′ to 3′)	Product size (bp)	Primer concentration (μM)	Reference
CesF1 CesR2	*ces*	GGTGACACATTATCATATAAGGTG GTAAGCGAACCTGTCTGTAACAACA	1271	0.2	Ehling-Schulz
HD2F HA4R	*hblD-hblA*	GTAAATTAIGATGAICAATTTC AGAATAGGCATTCATAGATT	1091	0.1	Ehling-Schulz
NA2F NB1R	*nheA-nheB*	AAGCIGCTCTTCGIATTC ITIGTTGAAATAAGCTGTGG	766	0.3	Ehling-Schulz
CKF2 CFR5	*cytK*	ACAGATATCGGICAAAATGC CAAGTIACTTGACCOGTTGC	421	0.4	Ehling-Schulz
FHblC RHblC	*hblC*	CCTATCAATACTCTCGCAA TTTCCTTTGTTTATACGTCGC	695	0.4	Ngamwongsatit
FHD RHD2	*hblD*	GAAACAGGGTCTCATATTCT CTGCATCTTTATGAATATCA	1018	0.4	Ngamwongsatit
FHblA RHblA	*hblA*	GCAAAATCCTATGAATCGGTA GCATCTGTTCGTAATGTTTT	884	0.4	Ngamwongsatit
F2NheA RNheA	*nheA*	TAAGGAGGGGCAAAACAGAAG TGAATGCGAAGAGCTGCTGCTTC	759	0.2	Ngamwongsatit
F2NheB RNheB	*nheB*	CAAGCTCCAGTTCATGCGG GATCCCATTGTGTACCATTG	935	0.2	Ngamwongsatit
FNheC R2NheC	*nheC*	ACATCCTTTTGCAGCAGAAC CCACCAGCAATGACCATATC	618	0.2	Ngamwongsatit

positive if coverage was greater than 90%.

3. Results

3.1. Whole genome sequencing was a better predictor of Duopath Hbl expression than PCR

Fifty inclusivity strains of *Bacillus cereus*, including isolates from food, environmental, and clinical sources were sequenced using the Illumina MiSeq desktop sequencer in duplicate. These sequences were analyzed using the BTyper computational tool developed by Carroll et al. (2017). To determine which strains had the potential for production of diarrheal toxins, *hblABCD* genes were analyzed (Table 2). To confirm that the strains with Hbl genes produced this toxin, the Duopath lateral flow device, which detects the HblC protein, was used for toxin detection (Table 2). Duopath detected HblC in 29 of the 50 strains (Table 2) tested in this study. Whole genome sequencing accurately predicted Hbl production in 50 of the 50 strains (100%) when comparing to the Duopath device. To compare to the conventional PCR method, we also performed PCR on all 50 strains. PCR accurately predicted 43 of the 50 strains (86%). In two strains, B6A3 (#5 - Table 2) and 769-2 (#50 - Table 2), PCR produced a negative results while WGS and Duopath showed a positive result for Hbl. These false negatives could have been a result of undigested DNA in the PCR reaction or genetic mutations in the *hbl* genes of these strains. Additionally, PCR predicted positive results for F180WPB (#32 – Table 2), F1261 (#37 – Table 2), F1265 (#38 – Table 2), 495A (#45 – Table 2), and 144-1 (#47 – Table 2), while WGS and Duopath were negative. These discrepancies are likely a result of false positives due to PCR reagents or carry over commonly associated with the highly sensitive amplification PCR process. Overall, whole genome sequencing predicted Hbl production more precisely compared to the conventional PCR method.

3.2. Whole genome sequencing predicted Nhe expression by Duopath better than PCR

B. cereus also produces non-hemolytic enterotoxin (Nhe) that can contribute to diarrheal disease. To predict *nheABC* gene production and subsequent production of NheB, we performed PCR, whole genome sequencing, and the Duopath lateral flow device (Table 2). Duopath detected NheB in 48 of the 50 strains (Table 2) tested in this study. PCR accurately predicted Nhe production for 48 out of 50 strains (96%) when comparing to the Duopath results. In one case, PCR was negative for F3998A (#19- Table 2), when WGS and Duopath were positive for Nhe. This false negative is potentially due to undigested DNA or mutations in the Nhe genes for this strain. On the other hand, whole genome sequencing predicted 49 out of the 50 strains (98%) for Nhe production when comparing to the Duopath lateral flow device. In strain F4047A (#22- Table 2), both PCR and WGS were positive for Nhe, but Duopath was negative. This discrepancy could have been a result of gene expression, but no protein production. This discrepancy highlights the importance of a screening both the gene expression and protein production potential of an organism. Generally, whole genome sequencing predicted Nhe production by Duopath slightly better than PCR.

3.3. ces and cytK2 gene prediction were comparable between whole genome sequencing and PCR

CytK and Ces are two other *B. cereus* enterotoxins of concern. CytK is a diarrheal toxin like Hbl and Nhe, but there is currently no commercialized antibody-based assay for this toxin. Ces, the emetic toxin, can be detected using a LC-MS/MS method. The BTyper computational tool identifies these toxins as part of its virulence gene profile. The presence *cytK* and *ces* genes were compared, using PCR and whole genome sequencing on all 50 inclusivity strains. Sequencing revealed the presence of *cytK2* using our strain collection. PCR and sequencing results for CytK agreed for 44 out of the 50 strains (88%) and 45 out of 50 strains (90%) for prediction of Ces (Table 3). These results show similar predictive power of PCR and whole genome sequencing concerning these two toxins.

3.4. Whole genome sequencing did not predict Hbl, Nhe, ces or CytK for any exclusivity strain

Food products can be contaminated by numerous other bacterial species besides *B. cereus*. For this reason, in addition to *B. cereus* strains, thirty exclusivity strains including isolates from *Escherichia coli, Staphylococcus aureus, Citrobacter sp., Enterobacter sp., Salmonella sp., Bacillus amyloliquifaciens, Bacillus subtilis, Bacillus licheniformis,*

Table 2
Inclusivity strains Hbl and Nhe profiles by PCR, WGS, and Duopath.

	Strain	Location	Source	Date of collection	Hbl			Nhe		
					PCR	WGS	Duopath	PCR	WGS	Duopath
1	A11778	Unknown	Unknown	Unknown	–	–	–	+	+	+
2	A14579	Unknown	Air in cow shed	1887	+	+	+	+	+	+
3	B6A1	Unknown	Unknown	Unknown	+	+	+	+	+	+
4	B6A2	Unknown	Unknown	Unknown	+	+	+	+	+	+
5	B6A3	Unknown	Unknown	Unknown	–	+	+	+	+	+
6	B6A4	Unknown	Unknown	Unknown	+	+	+	+	+	+
7	B6A8	France	Unknown	Unknown	+	+	+	+	+	+
8	B6A10	Canada	Unknown	Unknown	+	+	+	+	+	+
9	B6A15	Canada	Cheese Spoilage	1930	–	–	–	+	+	+
10	B6A17	Canada	Soil	Unknown	+	+	+	+	+	+
11	B6A18	Unknown	Unknown	Unknown	–	–	–	+	+	+
12	B6A25	Unknown	Unknown	Unknown	–	–	–	–	+	–
13	B6G1	Unknown	Unknown	Unknown	+	+	+	+	+	+
14	B6G2	Unknown	Unknown	Unknown	+	+	+	+	+	+
15	B6E1	Unknown	Soil	Unknown	+	+	+	+	+	+
16	B6G3	Unknown	Unknown	Unknown	+	+	+	+	+	+
17	B6A9	Unknown	Unknown	Unknown	–	–	–	+	+	+
18	F3995A	Norwalk	Patient	5/1/2009	+	+	+	+	+	+
19	F3998A	Norwalk	Patient	4/30/2009	+	+	+	–	+	+
20	F4014A	Norwalk	Patient	4/30/2009	+	+	+	+	+	+
21	399–1	Sarasota, FL	Pancreatin	2010	+	+	+	+	+	+
22	F4047A	Norwalk	Patient	5/7/2009	+	+	+	+	+	–
23	F4225A	Bridgeport	Mac and Cheese	6/11/2009	–	–	–	+	+	+
24	F4226A	Bridgeport	Mac and Cheese	6/11/2009	+	+	+	+	+	+
25	F4228A	Bridgeport	Mac and Cheese	6/11/2009	–	–	–	+	+	+
26	F4229A	Bridgeport	Mac and Cheese	6/11/2009	–	–	–	+	+	+
27	F4230A	Bridgeport	Mac and Cheese	6/11/2009	–	–	–	+	+	+
28	F13061	Unknown	Unknown	Unknown	–	–	–	+	+	+
29	FM77	Unknown	Unknown	Unknown	–	+	–	+	+	+
30	FTJL14	Unknown	Sweet and Sour Pork	Unknown	–	–	–	+	+	+
31	F196668	Unknown	Unknown	Unknown	+	+	+	+	+	+
32	F180WPB	Unknown	Unknown	Unknown	+	–	–	+	+	+
33	F4227A	Bridgeport	Mac and Cheese	6/11/2009	–	–	–	+	+	+
34	401–1	Sarasota, FL	Pancreatin	2010	–	–	–	+	+	+
35	F60006	Unknown	Unknown	Unknown	–	–	–	+	+	+
36	F1259	Unknown	Unknown	Unknown	+	+	+	+	+	+
37	F1261	Unknown	Unknown	Unknown	+	–	–	+	+	+
38	F1263	Unknown	Unknown	Unknown	+	–	–	+	+	+
39	F1265	Unknown	Unknown	Unknown	+	+	+	+	+	+
40	F1396	Unknown	Unknown	Unknown	–	–	–	+	+	+
41	F3A	Unknown	Unknown	Unknown	+	+	+	+	+	+
42	F26	Unknown	Unknown	Unknown	+	+	+	+	+	+
43	F96	Unknown	Unknown	Unknown	+	+	+	+	+	+
44	F4A	Unknown	Unknown	Unknown	+	+	+	+	+	+
45	495A	Wisconsin	Infant cereal	2010	+	–	–	+	+	+
46	F59	Unknown	Unknown	Unknown	+	+	+	+	+	+
47	144–1	California	Strawberry Filled Cake	2014	+	–	–	+	+	+
48	TJL16	Unknown	Unknown	Unknown	+	+	+	+	+	+
49	905–9	Unknown	Infant Formula	Unknown	+	+	+	+	+	+
50	769–2	Sarasota, FL	Pancreatin	2010	–	+	+	+	+	+

Acinetobacter sp., Listeria sp., and Pseudomonas sp., were used to determine if BTyper could distinguish between species that are related or commonly found in similar food products. No diarrheal or emetic toxins were found in any of the exclusivity strains tested by whole genome sequencing (Table 4). The PCR data for the exclusivity strains was published in a previous study (Tallent et al., 2015) and was used for comparison of the WGS data.

3.5. Whole genome sequencing from food in single and mixed cultures can predict toxin production

To determine if foods contaminated with *B. cereus* or model exclusivity strains (*S. aureus and B. licheniformis*) could be identified through sequencing directly from foods, we spiked in on average 1×10^8 CFUs/ml of bacterial cells into six model food products commonly contaminated with *B. cereus*. These foods included; dehydrated infant formula, pancake mix, whey powder, and potatoes; condensed gravy, and cooked rice. Food products were spiked with dilutions of bacterial cells and incubated at 30 °C for 24 h to allow time for equilibration of cells within the matrix. DNA was extracted and sequenced. BTyper analysis of DNA from the food products showed identical profiles for *nhe* and *hbl* in all food products across each parent strain. Foods that were positive for Nhe or Hbl were also tested with the Duopath lateral flow device to confirm production of Nhe or Hbl proteins (Table 5).

Furthermore, to determine if toxin genes could be visualized in mixed cultures in food, we inoculated food with one model *B. cereus* strain (A14579) and two related strains (*S. aureus* 963–9 and *B. licheniformis* F1383). In general, BTyper analysis of mixed species cultures in food was able to accurately identify toxin genes (Table 5). The only difference between single strain and mixed strain food extracts was *hbl* in pancake mix and one replicate of whey powder, where BTyper did not detect *hbl* in the mixed cultures (Table 5). Overall, this computational tool can be used to detect enterotoxigenic strains directly from

Table 3
PCR and WGS comparison of *ces* and *cytK* toxin genes in inclusivity strains.

	Strain	*ces*		*cytK2*	
		PCR	WGS	PCR	WGS
1	A11778	–	–	+	+
2	A14579	–	–	+	+
3	B6A1	–	–	+	+
4	B6A2	–	–	+	+
5	B6A3	–	–	+	+
6	B6A4	–	–	+	+
7	B6A8	–	–	–	–
8	B6A10	–	–	+	+
9	B6A15	–	–	+	+
10	B6A17	–	–	+	+
11	B6A18	–	–	–	–
12	B6A25	–	–	–	–
13	B6G1	–	–	–	–
14	B6G2	–	–	+	+
15	B6E1	–	–	–	–
16	B6G3	–	–	+	+
17	B6A9	–	–	–	–
18	F3995A	–	–	–	+
19	F3998A	–	–	+	+
20	F4014A	+	–	–	–
21	399–1	+	+	+	+
22	F4047A	+	–	+	+
23	F4225A	–	–	+	+
24	F4226A	+	–	–	+
25	F4228A	–	–	–	–
26	F4229A	–	–	–	–
27	F4230A	–	–	+	+
28	F13061	–	–	–	–
29	FM77	+	+	–	–
30	FTJL14	+	+	–	–
31	F196668	–	–	–	+
32	F180WPB	–	–	+	–
33	F4227A	+	+	–	–
34	565401–1	–	–	–	–
35	F60006	+	+	–	–
36	F1259	–	–	+	+
37	F1261	–	–	+	+
38	F1263	–	–	+	–
39	F1265	–	–	+	+
40	F1396	–	–	+	+
41	F3A	–	–	+	+
42	F26	–	–	+	+
43	F96	–	–	+	+
44	F4A	–	–	–	+
45	495A	+	–	+	+
46	F59	–	–	+	+
47	144–1	+	–	+	+
48	TJL16	–	–	+	+
49	905–9	–	–	+	+
50	769–2	–	–	+	+

foods contaminated with *B. cereus* in single and mixed cultures extracted from food.

To demonstrate a detection limit of the whole genome sequencing and BTyper analysis, we diluted DNA extracts from two model strains of *B. cereus* (A14579 and F4227A) from 1 ng to 0.125 ng. Since sequencing requires at least 1 ng of DNA, we prepared a DNA extract mixture substituting the diluted *B. cereus* DNA with *S. aureus* (963–3) DNA. Surprisingly, BTyper analysis was able to detect *B. cereus* DNA down to the lowest level (0.125 ng) with an abundance of *S. aureus* DNA (0.875 ng) (Supplementary Tables 1 and 2). Moreover, the antimicrobial resistant genes that were unique to each strain and present in the mixed cultures could be traced back to their individual strains. Consequently, this demonstrates that whole genome sequencing and BTyper analysis is sensitive enough to detect *B. cereus* toxin producing strains in a mixture or abundance of other strains that may be present in contaminated food.

Table 4
hbl, *nhe*, *ces*, and *cytK* gene profile of exclusivity strains from whole genome sequencing.

Exclusivity Strains			Sequencing			
Strain No.		Genus/species	*hbl*	*nhe*	*ces*	*cytK*
1	1	*Salmonella enterica*	–	–	–	–
2	176	*Bacillus amyloliquifaciens*	–	–	–	–
3	177	*Bacillus amyloliquifaciens*	–	–	–	–
4	178	*Bacillus amyloliquifaciens*	–	–	–	–
5	179	*Bacillus amyloliquifaciens*	–	–	–	–
6	180	*Bacillus amyloliquifaciens*	–	–	–	–
7	181	*Bacillus amyloliquifaciens*	–	–	–	–
8	182	*Bacillus amyloliquifaciens*	–	–	–	–
9	183	*Bacillus amyloliquifaciens*	–	–	–	–
10	249	*Salmonella enterica*	–	–	–	–
11	336	*Salmonella enterica*	–	–	–	–
12	385	*Escherichia coli*	–	–	–	–
13	386	*Escherichia coli*	–	–	–	–
14	387	*Escherichia coli*	–	–	–	–
15	392	*Escherichia coli*	–	–	–	–
16	522	*Salmonella enterica*	–	–	–	–
17	611	*Enterobacter sakazakii*	–	–	–	–
18	872	*Acinetobacter sp.*	–	–	–	–
19	1267	*Bacillus subtilis*	–	–	–	–
20	1268	*Bacillus subtilis*	–	–	–	–
21	1269	*Bacillus subtilis*	–	–	–	–
22	1270	*Bacillus subtilis*	–	–	–	–
23	2136	*Citrobacter freundii*	–	–	–	–
24	2145	*Citrobacter koseri*	–	–	–	–
25	1403	*Listeria monocytogenes*	–	–	–	–
26	963–3	*Staphylococcus aureus*	–	–	–	–
27	963–57	*Staphylococcus aureus*	–	–	–	–
28	963–75	*Staphylococcus aureus*	–	–	–	–
29	F1383	*Bacillus licheniformis*	–	–	–	–
30	ATCC 9027	*Pseudomonas aeruginosa*	–	–	–	–

4. Discussion

Bacillus cereus toxins are common causes of symptoms associated with foodborne illness. Detection of these toxins is paramount to ensuring food safety; however, enumeration and extraction of toxins from food is a laborious and time-consuming process and some toxins are not directly detectable from food. In this study, we showed that whole genome sequencing could be used as a pre-screening method for *B. cereus* toxins in food. Furthermore, this study demonstrates the power of sequencing compared to conventional PCR methods and provides more resolution when acquiring data about a food-contaminating strain.

Traditional PCR methods require DNA extracts from a food that is suspected to result from a *B. cereus* food related illness. Likewise, WGS starts with DNA extracted from the suspicious product and although the run time for WGS is longer than conventional PCR, the resolution that WGS provides is invaluable. Mutated genes can be problematic with PCR applications since the gene may not be amplified or may be an aberrant size and not recognized as the target. WGS also reduces the potential for false positive or negative results as compared to PCR and provides more information about the suspected strain.

Additionally, WGS tools could become a pre-screening method that determines when to pursue a toxin expression protocols such as the mass spectral assay to confirm the presence of cereulide.

The assays supporting the diarrheal enterotoxins are not as robust. The Duopath® detects single components of two different tripartite proteins and not a functional protein. PCR assays have been developed for *cytk1* and *cytk2*, but that is not evidence of protein expression and is a gap in the ability to properly analyze food matrices linked to foodborne illness.

The complexity of different food matrices in addition to multi-species competition could have contributed to the difference in toxin profiles between mixed culture and single culture food spikes. The only discrepancy was seen in the pancake mix and one replicate of whey

Table 5
Toxin gene profile by WGS from representative strains and after extraction from food.

Strain name	Genus/ species	*hbl*							*nhe*						
		Strain(s)	Infant Formula	Pancake	Whey Powder	Potatoes	Gravy	Cooked Rice	Strain(s)	Infant Formula	Pancake	Whey Powder	Potatoes	Gravy	Cooked Rice
A14579	*B. cereus*	+	+	+	+	+	+	+	+	+	+	+	+	+	+
F4227A	*B. cereus*	–	–	–	–	–	–	–	+	+	+	+	+	+	+
TJL16	*B. cereus*	+	+	+	+	+	+	+	+	+	+	+	+	+	+
905–9	*B. cereus*	+	+	+	+	+	+	+	+	+	+	+	+	+	+
963–3	*S. aureus*	–	–	–	–	–	–	–	–	–	–	–	–	–	–
F1383	*B. licheniformis*	–	–	–	–	–	–	–	–	–	–	–	–	–	–
A14579/ 963–3/ F138-3	*B. cereus/S. aureus/B. licheniformis*	+	+	–	+[a]	+	+	+	+	+	+	+	+	+	+

[a] 1 replicate from whey powder was negative for *hbl*.

powder. Both of these food products are labeled “high protein” with whey protein isolate added in the ingredients. Besides polymicrobial competition, these ingredients could have contributed to a difference in growth or toxin production and expression of *B. cereus* in these food products in mixed cultures.

It is important to note that the use of PCR/WGS and a gene expression assay are both necessary to determine if a food is violated with a toxin-producing strain of *B. cereus*, since one alone does not give sufficient determination of the toxin profile or biological activity. Because the current commercially available antibody-based assays only detect one component of the three-component toxins, it is important to have another method to examine the whole toxin profile of a strain and to confirm the results by an expression assay.

The work in this study demonstrates that whole genome sequencing could be used as a pre-screening method for detection of *B. cereus* toxin genes. These results show that DNA can directly be extracted from food commonly contaminated with *B. cereus*. Furthermore, we show that sequencing and BTyper analysis can identify toxin-producing genes in mixed cultures. This is a significant finding because food matrixes are complex and are likely contaminated with polymicrobial populations. Whole genome sequencing provides greater resolution than the conventional PCR method and will be a valuable resource for examining contaminated foods for hazardous toxin genes.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2018.10.008.

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