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Putative Virulence Factor Expression by Clinical and Food Isolates of *Bacillus* spp. after Growth in Reconstituted Infant Milk Formulae

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Forty-seven strains representing 14 different *Bacillus* species isolated from clinical and food samples were grown in reconstituted infant milk formulae (IMF) and subsequently assessed for adherence to, invasion of, and cytotoxicity toward HEp-2 and Caco-2 cells. Cell-free supernatant fluids from 38 strains (81%) were shown to be cytotoxic, 43 strains (91%) adhered to the test cell lines, and 23 strains (49%) demonstrated various levels of invasion. Of the 21 *Bacillus cereus* strains examined, 5 (24%) were invasive. A larger percentage of clinically derived *Bacillus* species (20%) than of similar species tested from the food environment were invasive. Increased invasion occurred after growth of selected *Bacillus* species in reconstituted IMF containing glucose. While PCR primer studies revealed that many different *Bacillus* species contained DNA sequences encoding the hemolysin BL (HBL) enterotoxin complex and *B. cereus* enterotoxin T, not all of these isolates expressed these diarrheagenic genes after growth in reconstituted IMF. Of the 47 *Bacillus* isolates examined, 3 isolates of *B. cereus* and 1 isolate of *B. subtilis* produced the HBL enterotoxin after 18 h of growth in brain heart infusion broth. However, eight isolates belonging to the species *B. cereus*, *B. licheniformis*, *B. circulans*, and *B. megaterium* were found to produce this enterotoxin after growth in reconstituted IMF when assessed with the *B. cereus* enterotoxin (diarrheal type) reversed passive latex agglutination (RPLA) kit. It is concluded that several *Bacillus* species occurring occasionally in clinical specimens and food samples are of potential medical significance due to the expression of putative virulence factors.

The bacterial genus *Bacillus* comprises a very large and diverse group whose members are found ubiquitously (2, 10, 22). With the exception of *Bacillus anthracis* and *B. cereus*, other *Bacillus* species are generally perceived as inconsequential and of little clinical significance (8). Due to the endospore-forming ability of members of this genus, these bacteria tolerate adverse conditions better than most bacterial enteropathogens. They occur frequently in hospital foods (21) and domestically prepared foods (22).

Most food poisoning incidents attributed to *Bacillus* species are associated with *B. cereus*; this bacterium is known to cause a variety of nongastrointestinal diseases as well as two different types of food poisoning (for reviews, see references 7, 10, 13, and 14), which are characterized by either diarrhea or emesis. The diarrheal type is attributed to heat-labile enterotoxins that cause cytotoxicity, fluid accumulation in the ligated ileal loop of experimental animals, and dermonecrosis and is lethal for mice (14, 17). Two protein complexes from *B. cereus* strains, hemolysin BL (HBL) and nonhemolytic enterotoxin (NHE), and an enterotoxic protein, enterotoxin T (BceT), with these diarrheagenic properties have been previously characterized (3, 11, 14, 25). The emetic type is caused by a heat-stable dodecadepsipeptide, cereulide (14). The relevance of other *Bacillus* species as food poisoning organisms is being increasingly recognized, with recent epidemiological evidence linking *B. licheniformis*, *B. subtilis*, *B. pumilus*, and *B. thuringiensis* with incidents of food-borne illness (8). Previous research has shown that a variety of different *Bacillus* species isolated from the dairy environment may have the potential to produce diarrheal enterotoxins (2). Beattie and Williams (2) recently showed that supernatant fluids from isolates of *B. thuringiensis*, *B. circulans*, *B. licheniformis*, *B. lentus*, *B. laterosporus*, and *B. mycoides* reacted positively with the commercially available *Bacillus* diarrheal enterotoxin (BDE) visual immunoassay (Tecra VIA; International Bioproducts Inc., Redmond, Wash.) and the *B. cereus* enterotoxin (diarrheal type) reversed passive latex agglutination (RPLA) kit (Oxoid Ltd., Basingstoke, England).

Many members of the genus *Bacillus* have also been shown to be the etiological agents in local, deep-tissue, and systemic infections (8, 15). Nongastrointestinal infections have been primarily seen in individuals who are intravenous drug abusers or immunocompromised as a consequence of human immunodeficiency virus infection, chemotherapy, or malignancy (2). Due to the marked involvement of the liver and spleen with a brevity of gastrointestinal symptoms, the possibility that these systemic infections may have resulted from bacterial translocation from the gastrointestinal tract has been raised (8). While *Bacillus* spp. have been associated with human illnesses, the virulence status of many members of this genus has yet to be defined.

We report on the ability of different clinical and food isolates of *Bacillus* to adhere to, invade, and produce a cytotoxic effect in human HEp-2 and Caco-2 epithelial cells after growth in commercially produced baby foods. We also report that the ability of selected *Bacillus* isolates to express diarrheal enterotoxins HBL and BceT after growth in baby foods was influenced by the nutritional compositions of the products.

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TABLE 1. Bacterial strains used

Bacterial species	Isolate	Provider	Reference no.	Source	Associated disease
Bacillus cereus	KD4	GRI	85895.F	Human blood	Sepsis (burn unit)
	KD5	GRI	20191.V	Human blood	Sepsis (special care baby unit)
	KD6	GRI	33247.K	Human blood	Sepsis
	KD10	GRI	45221.W	Human blood	Sepsis (general medicine)
	KD11	GRI	59180.X	Human blood	Sepsis (general medicine)
	KD12	GRI	32465.X	Human blood	Sepsis (orthopedic unit)
	Et	NCTC	11145	Stool	Diarrheal food poisoning
	Em	NCTC	11143	Vomit	Emetic food poisoning
	DC1	GRI	SU112/99	Enteral feed	None reported
	DC2	GRI	SU113/99	Enteral feed	Diarrheal food poisoning
	NR11	Rowan et al. (22)	SU11/95	IMF	None reported
	NR14	YH	SU14/95	Pasteurized milk	None reported
	NR30	Rowan et al. (22)	SU30/95	Enteral feed	None reported
	NR42	Rowan et al. (22)	SU42/95	IMF	None reported
	NR46	Rowan et al. (22)	SU46/95	IMF	None reported
	NR50	Rowan et al. (22)	SU50/95	IMF	None reported
	NR53	Rowan et al. (22)	SU53/95	Enteral feed	None reported
	NR62	Rowan et al. (22)	SU62/96	IMF	None reported
	NR91	Rowan et al. (22)	SU91/96	UHT milk[a]	None reported
	NR93	Rowan et al. (22)	SU93/96	IMF	None reported
	B-4ac		DSM4384	Pea soup	Diarrheal food poisoning
Bacillus licheniformis	KD1	GRI	102469.S	Human blood	Sepsis
	KD8	GRI	33263.Y	Human blood	Sepsis
	KD17	PHLS	R2512/98	Knee fluid	Septic arthritis
	H6	HDRI		Dairy environment	None reported
Bacillus subtilis	KD19	PHLS	R422/98	Human blood	Sepsis
	NR 106	Rowan et al. (22)	SU106/96	IMF	None reported
	H7	HDRI		Dairy environment	None reported
Bacillus brevis	H4	HDRI		Dairy environment	None reported
	NR83	Rowan et al. (22)	SU83/96	UHT milk	None reported
Bacillus megaterium	KD16	PHLS	B17/97	Finger	Pyrexia and sepsis
	NR73	Rowan et al. (22)	SU73/95	IMF	None reported
Bacillus circulans	KD9	GRI	79491.N	Human blood	Sepsis
	KD15	PHLS	R7106/97	Human blood	Lymphoma
	H3	HDRI		Dairy environment	None reported
Bacillus firmus	KD2	GRI	88773.T	Human blood	Sepsis
	KD7	GRI	88022.Q	Human blood	Sepsis
Bacillus sphaericus	KD18	PHLS	R3794/98	Human blood	Sepsis
	NR66	Rowan et al. (22)	SU66/95	Pasteurized milk	None reported
Bacillus pumilus	KD14	PHLS	R7106/97	Necrotic tissue aspirate	Spreading fasciitis
	NR103	Rowan et al. (22)	SU103/95	Enteral feed	None reported
Bacillus mycoides	H1	HDRI		Dairy environment	None reported
	NR98	YH	SU98/96	Pasteurized milk	None reported
Bacillus thuringiensis	KD3	GRI	78420.S	Human blood	Sepsis
Bacillus polymyxa	H2	HDRI		Dairy environment	None reported
Bacillus lentus	H5	HDRI		Dairy environment	None reported
Bacillus coagulans	KD13		SU114/99	IMF	None reported
Listeria monocytogenes	NR1	NCTC	11994	Cerebrospinal fluid	Adult meningitis

[a] UHT, ultra-high-temperature treated.

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MATERIALS AND METHODS

Bacterial strains and growth media. The *Bacillus* strains (Table 1) used in this study were, if not otherwise indicated, obtained from the Department of Bacteriology, Glasgow Royal Infirmary, Glasgow, Scotland (GRI); from Yorkhill Hospital, Glasgow, Scotland (YH); from the Food Safety Microbiology Laboratory, Central Public Health Laboratory, Colindale, United Kingdom (PHLS); from the Hannah Dairy Research Institute, Ayr, Scotland (HDRI); and from the National Collection of Type Cultures, Central Public Health Laboratory, Colindale, United Kingdom (NCTC). Other *Bacillus* strains were isolated from reconstituted infant milk formulae (IMF), pasteurized milk, enteral feeds, and high-energy buildup foods. *Listeria monocytogenes* NCTC 11994 was used as a positive control for bacterial adherence and invasion studies. The identity of each *Bacillus* isolate was confirmed by performing a sequence of characteristic morphological and physiological tests as described previously (22) and by use of

miniaturized biochemical API 50CHB and API 20E galleries (bioMérieux, Marcy l'Etiole, France).

Bacillus cells were grown in brain heart infusion (BHI) broth (Oxoid) or in reconstituted IMF (tyndallized to sterility; three consecutive days of steaming for 30 min) at 37°C with agitation. The reconstituted IMF used in this study were Farley's Follow-On milk (designated IMF 1; containing maltodextrin), SMA WYSOY (designated IMF 2; containing glucose), and SMA Gold and SMA White (designated IMF 3 and IMF 4, respectively; containing neither glucose nor maltodextrin). IMF were used as test food matrices in this study, as they are frequently contaminated with acceptably low numbers of *Bacillus* spores and have supported the growth of these organisms (22). For adherence and invasion assays, the bacteria were harvested after 5 and 18 h of growth and were washed three times by centrifugation (MSE Centaur 1) at 3,000 rpm for 10 min each time in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Life Technologies Ltd., Paisley, Scotland). The bacteria were stored at -70°C (Microbank System, Pro-Lab Diagnostics, Ontario, Canada) to prevent the loss of virulence characteristics.

Adherence and invasion assays. The ability of *Bacillus* test strains to adhere to and invade HEp-2 and Caco-2 cells was determined by previously described procedures (19), with minor modifications. HEp-2 and Caco-2 cell monolayers were grown overnight in a 5% CO_2 atmosphere at 37°C in DMEM supplemented with 10% fetal calf serum (FCS; Gibco BRL) in 24-well tissue culture plates seeded with approximately 10^5 cells per well. Prior to assays, the monolayers were washed three times with DMEM, inoculated with 1 ml of bacterial culture (in DMEM with 10% FCS) in triplicate, and incubated for 2 h at 37°C in a 5% CO_2 atmosphere. After incubation, the monolayers were washed three times with DMEM to remove any nonadherent cells, and then 1 ml of DMEM containing 10% FCS was added to each well of one of the test plates and incubated for 2 h. For the invasion assays, 1 ml of DMEM containing 10% FCS and 100 μg of gentamicin ml^{-1} was added to each well of the other 24-well plate and incubated for 2 h. The monolayers were then washed three times with DMEM, and the tissue culture cells were lysed with 1 ml of 1% Triton X-100 (vol/vol in distilled water) for 5 min at 37°C. Samples (0.1 ml) of lysate from each tissue culture plate were serially diluted in 0.9 ml of sterile distilled water, with subsequent enumeration by plating of 20 μl of appropriate 10-fold dilutions on BHI agar plates.

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

Measurement of other virulence factors. All the isolates were tested for lecithinase (phosphatidylinositol-specific phospholipase C) activity after overnight growth on nutrient agar supplemented with 8% egg yolk (Oxoid) and by overlaying 1% L-*d*-phosphatidylinositol substrate (Sigma) in 0.7% agarose on overnight cultures of the bacteria on L agar plates. Lecithinase-positive strains produced a halo of precipitation (insoluble diacylglycerol) around the bacterial colonies. Production of catalase was assayed for by using an ID Color Catalase testing kit (bioMérieux). The ability of the isolates to induce hemolysis of 7% horse erythrocytes on blood agar was examined. In addition, hemolysis of 10% horse erythrocytes in BHI broth by the isolates was determined spectrophotometrically at 640 nm. The presence of diarrheal enterotoxin was measured using the *B. cereus* enterotoxin (diarrheal type) RPLA kit according to the manufacturer's instructions.

Screening of *Bacillus* spp. for the presence of *bceT*, *hblA*, *hblC*, and *hblD* enterotoxin DNA sequences by PCR. Chromosomal DNA was isolated from the test *Bacillus* spp. by a previously described procedure (4). The DNA sequences of the diarrheagenic genes *bceT* (1), *hblC* and *hblA* (23), and *hblD* (11) were used to design primers that would amplify segments of the genes, if present, in a selection of the above-mentioned test *Bacillus* strains. Amplification was carried out with a DNA thermal cycler for 36 cycles of 30 s at 94°C; 1 min at 54°C, 60°C, 62°C, and 65°C for the *hblD*, *bceT*, *hblC*, and *hblA* genes, respectively; and 1 min at 72°C. PCR products of 429, 617, 399, and 873 bp were detected when the following pairs of oligonucleotide primers were used, respectively: HBLD-N (5′-AATCAAGAGCTGTCACGAAT-3′) and HBLD-C (5′-CACCAATTGACCATGCTAAT-3′), BCET6-N (5′-CATATGAAAGAGTTAGTTTCA-3′) and BCET5-C (5′-CGGATGAGGTGAGAAATGAAC-3′), HBLA-N (5′-GCTAATGTAGTTTCACCTAGCAAC-3′) and HBLA-C (5′-AATCATGCCACTGCGTGGACATATAA-3′), and HBLC-N (5′-AATAGGTACAGATGGAACAGG-3′) and HBLC-C (5′-GGCTTTCATCAGGTCATACTC-3′).

Statistical analysis. All studies were performed in triplicate, and averages and standard errors were determined. Differences in bacterial adherence, invasion, and cytotoxicity were examined with human epithelial HEp-2 and Caco-2 cells at the 95 or 99.9% confidence interval using analysis of variance (one-way or balanced model) with Minitab software, release 11 (Minitab Inc., State College, Pa.).

RESULTS

Forty-seven *Bacillus* isolates representing 14 different species from clinical and food environments were used in this study (Table 1). The human diseases associated with the clinical *Bacillus* isolates ranged from severe infections, such as spreading fasciitis (e.g., *B. pumilus* KD14), to less serious foodborne illnesses.

Confirmation of the identity of some of the *Bacillus* isolates to species level was problematic, as they showed atypical characteristics for a number of the physiological and biochemical tests. For instance, the clinical isolates *B. licheniformis* KD1 and KD8 and *B. pumilus* KD14 produced lecithinase, yet these species were previously reported to be unable to produce this phospholipase. Interestingly, *B. licheniformis* KD8 produced lecithinase at 37°C but not at the lower culture temperature of 30°C. All *Bacillus* species tested were shown to be catalase positive and, with the exception of *B. firmus*, were also shown to be hemolytic. Irrespective of the source of the organism, the commercially available API 50CHB system was unable to differentiate between *B. licheniformis* and the closely related *B. subtilis*.

Ability of *Bacillus* test isolates to adhere to, invade, and produce cytotoxic effects in epithelial cells after growth in laboratory-based culture media. The ability of the *Bacillus* test isolates to adhere to, invade, and produce cytotoxic effects in Caco-2 and HEp-2 cells was assessed after 18 h of growth in BHI broth. While the results showed that there were species-to-species variations in the levels of cytotoxicity produced, the culture supernatant fluids from 38 *Bacillus* isolates (81%, representing all 14 *Bacillus* species) had cytotoxic effects in both epithelial cell lines (Table 2). Clinical *Bacillus* isolates were significantly more cytotoxic to HEp-2 cells ($P < 0.05$); the mean toxicities of 19 clinical and food *Bacillus* isolates were 70.7% ± 12% and 34.8% ± 24%, respectively (Table 2). While both cell lines were susceptible to the culture supernatant fluids from many *Bacillus* isolates, some *Bacillus* spp. were cytotoxic to one cell line only. Other species, such as *B. megaterium* NR73, *B. subtilis* NR106, *B. cereus* DC1, and *B. mycoides* H1, were shown to be noncytotoxic. Separate heat and trypsin treatments of culture supernatant fluids either reduced or eliminated toxicity in HEp-2 and Caco-2 cells, suggesting that

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TABLE 2. Adhesion, invasion, and cytotoxic abilities of *Bacillus* species for HEp-2 and Caco-2 cells after bacterial cultivation in BHI broth at 37°C for 18 h[a]

Bacterial species	Isolate	% of the following property for the indicated cells:					
		Cell death		Adherence		Invasion	
		HEp-2	Caco-2	HEp-2	Caco-2	HEp-2	Caco-2
B. licheniformis	KD1	67 ± 5	75 ± 2	2.5 ± 0.25	0.23 ± 0.04	0.02 ± 0.005	0.20 ± 0.03
	KD8	70 ± 6	24 ± 2	0.78 ± 0.04	0.44 ± 0.1	0.02	0.06 ± 0.01
	KD17	68 ± 1	62 ± 10	0.28 ± 0.08	2.7 ± 0.35	0.05 ± 0.001	0.03
	H6	20 ± 2	36 ± 3	0.86 ± 0.15	1.15 ± 0.06	0.03 ± 0.01	0.09 ± 0.02
B. subtilis	KD19	49 ± 10	72 ± 5	0.96 ± 0.08	0.08 ± 0.01	0.02	0.03 ± 0.01
	H7	85 ± 8	62 ± 4	0.63 ± 0.2	0.35 ± 0.05	—	—
	NR106	—	—	—	—	—	—
B. brevis	NR83	83 ± 6	63 ± 5	—	—	—	—
	H4	29 ± 10	15 ± 3	0.12 ± 0.02	0.19 ± 0.14	0.03	0.02
B. megaterium	KD16	56 ± 13	35 ± 4	0.45 ± 0.1	0.43 ± 0.09	0.1 ± 0.01	0.16 ± 0.03
	NR73	—	—	4.0 ± 0.25	0.13 ± 0.04	2.1 ± 0.12	0.09 ± 0.01
B. circulans	H3	26 ± 2	5 ± 1	0.07	0.16 ± 0.02	0.05 ± 0.01	0.05 ± 0.01
	KD15	72 ± 2	45 ± 5	0.56 ± 0.1	0.53 ± 0.1	0.13 ± 0.02	0.01
	KD9	57 ± 6	89 ± 2	0.06 ± 0.01	0.03 ± 0.01	0.07 ± 0.01	0.04 ± 0.01
B. firmus	KD2	80 ± 11	42 ± 5	0.97 ± 0.22	1.07 ± 0.3	—	0.86 ± 0.05
	KD7	63 ± 6	71 ± 5	2.73 ± 1.1	0.63 ± 0.06	—	—
B. cereus	KD4	76 ± 5	66 ± 3	0.38 ± 0.05	—	0.02 ± 0.002	—
	KD5	62 ± 11	84 ± 9	0.02 ± 0.005	0.17 ± 0.03	0.005	0.02
	KD6	69 ± 8	32 ± 1	—	—	—	—
	KD10	83 ± 7	91 ± 7	1.43 ± 0.17	0.02 ± 0.01	—	—
	KD11	86 ± 5	83 ± 5	0.03 ± 0.01	0.40 ± 0.04	—	—
	KD12	85 ± 2	72 ± 6	3.4 ± 0.3	0.26 ± 0.07	—	—
	NR11	65 ± 3	71 ± 10	0.15 ± 0.04	0.22 ± 0.04	—	—
	NR14	63 ± 5	49 ± 3	1.5 ± 0.16	0.9 ± 0.09	—	—
	NR30	67 ± 5	63 ± 2	0.09 ± 0.03	0.03	—	—
	NR42	45 ± 7	71 ± 5	1.0 ± 0.15	1.3 ± 0.11	—	—
	NR50	86 ± 5	80 ± 5	0.46 ± 0.04	0.51 ± 0.07	—	—
	NR53	63 ± 1	83 ± 4	—	—	—	—
	DC1	—	—	1.11 ± 0.23	0.90 ± 0.16	0.32 ± 0.06	0.19 ± 0.03
	DC2	5 ± 1	—	0.93 ± 0.31	1.4 ± 0.36	0.36 ± 0.1	0.22 ± 0.01
	Et	85 ± 2	85 ± 3	0.03 ± 0.005	0.04 ± 0.01	—	—
	Em	83 ± 4	75 ± 1	0.2 ± 0.06	0.01	—	0.04 ± 0.01
B. sphaericus	KD18	61 ± 9	52 ± 5	2.7 ± 0.3	2.67 ± 0.5	0.15 ± 0.02	—
B. pumilus	KD14	48 ± 10	50 ± 4	1.13 ± 0.14	3.72 ± 0.34	0.02	0.03
	NR103	12 ± 3	—	0.15 ± 0.06	0.23 ± 0.05	—	—
B. mycoides	H1	—	—	1.19 ± 0.2	1.16 ± 0.2	0.07 ± 0.01	0.1
B. thuringiensis	KD3	66 ± 11	41 ± 5	3.3 ± 0.3	4.72 ± 0.72	0.13 ± 0.03	0.83 ± 0.11
B. polymyxa	H2	59 ± 4	79 ± 3	0.02 ± 0.01	0.05 ± 0.01	0.2 ± 0.04	0.13 ± 0.02
B. lentus	H5	11 ± 2	17 ± 4	1.14 ± 0.41	0.8 ± 0.15	0.2 ± 0.1	0.09
B. coagulans	KD13	15 ± 3	—	1.97 ± 0.2	1.15 ± 0.3	—	—
L. monocytogenes	NR1	72 ± 5	82 ± 9	3.88 ± 0.37	3.45 ± 0.2	1.15 ± 0.23	1.2 ± 0.04

[a] Sterilized BHI broth was used as a control and had no cytotoxic influence (—) on the cell lines. Values shown are representative of triplicate samples from two separate trials and are reported as means and standard errors.

the cytotoxic activity was attributable to the proteinaceous fraction of the culture supernatant fluids (Table 3).

Forty-three *Bacillus* isolates (91%) encompassing all 14 species adhered to both cell lines, with 23 isolates (46%) demonstrating various levels of invasion (Table 2). While *B. megaterium* NR73, *B. cereus* KD12, and *B. thuringiensis* KD3 showed levels of adherence similar to that achieved by *L. monocytogenes* NR1, these *Bacillus* isolates were shown to be less invasive ($P < 0.05$) (Table 2). With the exception of *B. coagulans*, isolates of the other 13 different *Bacillus* species were capable of invading epithelial cells. *B. cereus* KD6 and NR53 and *B. subtilis* NR106 were incapable of adhering to these cell lines under the test conditions. Of the 21 *B. cereus* isolates examined, 5 (24%) were invasive. Twenty percent more clinical isolates than similar types of *Bacillus* species tested from the food environment were invasive (Table 2).

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TABLE 3. Effect of heat or trypsin treatment on cytotoxicity of cell-free supernatant fluids from selected *Bacillus* species for HEp-2 and Caco-2 epithelial cells[a]

Bacterial species	Isolate	% of the following cells dead after the indicated treatment:					
		HEp-2			Caco-2		
		Normal	HT	TT	Normal	HT	TT
B. cereus	KD4	76 ± 5	13 ± 1	11 ± 3	66 ± 3	10 ± 1	16 ± 3
	KD5	62 ± 11	—	5 ± 1	84 ± 9	80 ± 8	10 ± 2
	KD10	83 ± 7	46 ± 3	22 ± 3	91 ± 7	60 ± 5	—
	KD11	86 ± 5	35 ± 3	10 ± 1	83 ± 5	—	1
	KD12	85 ± 2	6	8 ± 1	72 ± 6	—	—
	Et	85 ± 2	57 ± 5	3 ± 1	85 ± 7	2	—
	Em	83 ± 4	12 ± 3	—	75 ± 1	—	—
	NR30	67 ± 5	15 ± 1	—	63 ± 2	15 ± 5	—
	NR53	63 ± 1	22 ± 2	—	83 ± 4	4	—
	NR11	65 ± 3	—	—	71 ± 10	—	—
	B4ac	65 ± 10	15	—	75 ± 7	28 ± 3	1
	DC1	—	—	—	—	—	—
	DC2	5 ± 1	—	—	—	—	—
B. licheniformis	KD1	67 ± 5	—	—	75 ± 2	—	—
	KD8	70 ± 6	8 ± 1	—	24 ± 2	—	—
	KD17	68 ± 1	5	1	62 ± 10	—	—
	H6	20 ± 2	3	—	36 ± 3	—	—
B. subtilis	KD19	49 ± 49	27 ± 3	1	72 ± 5	22 ± 2	—
	H7	85 ± 8	5 ± 1	—	62 ± 4	—	—
B. megaterium	KD16	56 ± 13	20 ± 5	3	35 ± 4	—	—
	NR73	—	—	—	—	—	—
B. sphaericus	KD18	61 ± 9	5 ± 2	1	52 ± 5	15 ± 3	—
B. thuringiensis	KD3	66 ± 11	1	—	41 ± 5	—	—
B. coagulans	KD13	15 ± 3	35 ± 6	9 ± 3	—	—	2
B. circulans	KD9	57 ± 6	5	—	89 ± 2	—	—
	KD15	72 ± 2	35 ± 2	2	45 ± 5	—	—
	H3	26 ± 2	—	—	5 ± 1	—	—
B. firmus	KD2	80 ± 11	—	—	42 ± 5	—	—
	KD7	63 ± 6	—	—	71 ± 5	—	—
B. brevis	H4	29 ± 10	—	—	15 ± 3	—	—
B. lentus	H5	11 ± 2	3	—	17 ± 4	—	—
B. mycoides	H1	—	—	—	5	—	—
B. pumilus	KD14	48 ± 10	54 ± 3	12 ± 1	50 ± 4	—	—
B. polymyxa	H2	59 ± 4	5 ± 1	—	79 ± 3	—	—

[a] Supernatant fluids were untreated (Normal), heated (HT), or trypsin treated (TT) prior to assessment of toxicity to HEp-2 and CaCo-2 cells. Values shown are means and standard errors from triplicate trials where samples were examined in triplicate. —, no effect.

Putative virulence factor expression by *Bacillus* species after growth in reconstituted IMF. The cytotoxicity, adherence, and invasion potentials of a selection of *Bacillus* species were assessed after 18 h of growth at 37°C in a variety of commonly used reconstituted IMF (Table 4). All of the *Bacillus* isolates tested were capable of growth in a variety of reconstituted IMF that differed in nutritional compositions. IMF 1 contained the starch derivative maltodextrin and lactose, IMF 2 contained glucose syrup and lactose, and IMF 3 and IMF 4 contained lactose. The results showed that the cell-free culture supernatants from many of the selected *Bacillus* isolates produced various levels of cytotoxicity in HEp-2 cells (Table 4). While some *Bacillus* species showed similar levels of toxicity after growth in all four IMF and in BHI broth (such as *B. cereus* KD4 and *B. megaterium* KD16), most *Bacillus* species varied considerably ($P < 0.05$) in their ability to elicit cytotoxicity in HEp-2 cells. Some *Bacillus* species (such as *B. licheniformis* KD1, *B. circulans* KD9, and *B. thuringiensis* KD3) were cytotoxic after growth in BHI broth but not after growth in certain IMF (Table 4). Not all of the IMF used for growth of the cytotoxigenic *Bacillus* species resulted in toxicity in HEp-2 cells.

Analysis of cell-free culture supernatants for HBL diarrheal enterotoxin production using the commercially available RPLA test system revealed four toxin producers after growth in BHI broth (i.e., three isolates of *B. cereus* and one isolate of *B. subtilis*) (Table 4). However, RPLA analysis of culture supernatants from IMF samples revealed a further eight *Bacillus* isolates, belonging to *B. cereus*, *B. licheniformis*, *B. subtilis*, and

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TABLE 4. Screening of *Bacillus* strains for DNA sequences containing genes associated with the HBL complex and *B. cereus* BceT

Bacterial species	Isolate	Cytotoxicity (% cell death) in the following medium[a]:					Detection of the following diarrheagenic gene by PCR[b]:			
		BHI broth	IMF 1	IMF 2	IMF 3	IMF 4	*bceT*	*hblC*	*hblA*	*hblD*
B. cereus	KD4	76 ± 5	**80 ± 5**	**83 ± 12**	**52 ± 5**	**50 ± 10**	−	+	+	+
	KD5	62 ± 11	40 ± 3	**72 ± 7**	55 ± 3	47 ± 8	−	+	+	+
	KD10	83 ± 7	—	**38 ± 3**	53 ± 1	56 ± 3	+	+	+	+
	KD11	86 ± 5	66 ± 7	—	75 ± 6	88 ± 3	−	+	+	+
	NR11	**85 ± 2**	**71 ± 9**	**38 ± 3**	54 ± 5	17 ± 1	+	+	+	+
	NR14	63 ± 5	56 ± 7	74 ± 5	51 ± 3	62 ± 2	−	+	+	+
	NR42	45 ± 7	40 ± 5	**55 ± 5**	61 ± 4	56 ± 3	−	+	+	+
	NR50	86 ± 5	81 ± 4	75 ± 8	69 ± 4	61 ± 2	+	+	+	+
	NR93	**81 ± 4**	**62 ± 5**	**73 ± 5**	54 ± 8	66 ± 8	+	+	+	+
	B-4ac	**75 ± 9**	**82 ± 3**	**64 ± 2**	62 ± 5	55 ± 6	+	+	+	+
	Em	83 ± 4	45 ± 3	59 ± 5	62 ± 3	64 ± 4	−	−	−	−
B. licheniformis	KD1	67 ± 5	41 ± 5	29	—	—	+	+	+	+
	KD8	70 ± 6	**64 ± 2**	**45 ± 3**	55 ± 3	22 ± 3	+	+	+	+
	KD17	68 ± 1	57 ± 6	63 ± 2	60 ± 3	73 ± 8	−	−	−	
	H6	20 ± 2	**65 ± 3**	**52 ± 5**	66 ± 9	68 ± 1	−	+	+	+
B. subtilis	KD19	**49 ± 10**	**37 ± 3**	**30 ± 1**	—	—	+	+	+	+
	H7	85 ± 8	77 ± 8	26 ± 4	70 ± 1	82 ± 3	−	−	−	−
B. pumilus	KD14	48 ± 10	53 ± 10	73 ± 5	43 ± 4	56 ± 1	−	−	−	−
B. circulans	KD9	57 ± 6	26 ± 3	—	29 ± 2	28 ± 1	−	+	+	+
	KD15	72 ± 2	5 ± 1	**41 ± 3**	45 ± 1	38	−	+	+	+
	H3	26 ± 2	47 ± 5	21 ± 4	8 ± 5	16 ± 3	+	−	−	−
B. thuringiensis	KD3	66 ± 11	55 ± 5	73 ± 8	—	—	+	−	−	−
B. megaterium	KD16	56 ± 13	**44 ± 5**	**38 ± 3**	42 ± 3	43 ± 2	+	+	+	+
	NR73	—	54 ± 6	43 ± 6	59 ± 3	65 ± 5	−	−	−	−
B. sphaericus	KD18	61 ± 13	38 ± 5	61 ± 5	64 ± 5	40 ± 5	−	−	+	+

[a] Values are reported as means and standard errors. —, no effect. Samples that tested positive for diarrheal toxin production in the RPLA test kit are shown in bold.
[b] +, gene was detected; −, gene was not detected.

B. megaterium, that were capable of producing HBL enterotoxin. With the exception of *B. cereus* KD4, all other *Bacillus* species tested were incapable of producing HBL enterotoxin in IMF products that contained lactose as the sole carbon source (Table 4). Reconstituted IMF 1, containing maltodextrin (a derivative of starch hydrolysis), and IMF 2, containing glucose, permitted a larger number of *Bacillus* species to produce diarrheal enterotoxin (Table 4). *B. licheniformis*, a known maltodextrin utilizer, was capable of HBL enterotoxin production in both IMF 1 and IMF 2. However, only *B. cereus* KD4 produced enterotoxin in IMF 1, suggesting that not all diarrheagenic *B. cereus* isolates have the appropriate genetic material to utilize maltodextrin for toxin production or that these bacteria have the necessary genes but require a specific environmental signal(s) for transcriptional activation.

Molecular analysis of a selection of different *Bacillus* species revealed that all diarrheagenic enterotoxin producers had the *hblA*, *hblC*, and *hblD* genes, which encode the HBL toxin complex (Table 4). Some of these diarrheagenic *Bacillus* isolates were also shown to have the *bceT* gene, which is associated with *B. cereus* BceT (Table 4). Some *Bacillus* isolates did not produce diarrheal toxins even though they were shown to contain DNA sequences for the above-mentioned diarrheagenic genes, while other *Bacillus* isolates were devoid of these enterotoxin-encoding genes (Table 4). There was good agreement between PCR analysis of *Bacillus* isolates for the diarrheagenic *hblA*, *hblC*, and *hblD* genes and detection of secreted HBL enterotoxin in culture supernatants by the RPLA test system (Table 4). Hansen and Hendriksen (11) detected genes for HBL, NHE, and BceT in 22 *B. cereus* and 41 *B. thuringiensis* strains by PCR (11). At least one gene for the protein complexes HBL and NHE was detected in all of the *B. thuringiensis* strains, while six *B. cereus* strains were devoid of all three HBL genes, three lacked at least two of the three NHE genes, and one lacked all three. The researchers did not mention whether the *B. cereus* or *B. thuringiensis* strains tested were obtained from clinical or food environments.

Greater levels of adherence to and invasion of HEp-2 cells were achieved by a selection of different *Bacillus* species after 18 h of growth at 37°C in IMF 2, containing glucose, than by the same species cultured in BHI broth or in IMF 3 or IMF 4, containing lactose (Fig. 1). Similar findings were observed when this study was repeated at the lower storage temperature of 30°C (data not shown).

DISCUSSION

This study constitutes the first demonstration that isolates of a wide variety of *Bacillus* spp., previously isolated from clinical specimens and food samples, are capable of adhering to, invading, and producing cytotoxic effects in human epithelial cells. Forty-seven *Bacillus* isolates were assessed for these putative virulence factors after growth in laboratory-based culture media and in baby foods. Production of the HBL entero-

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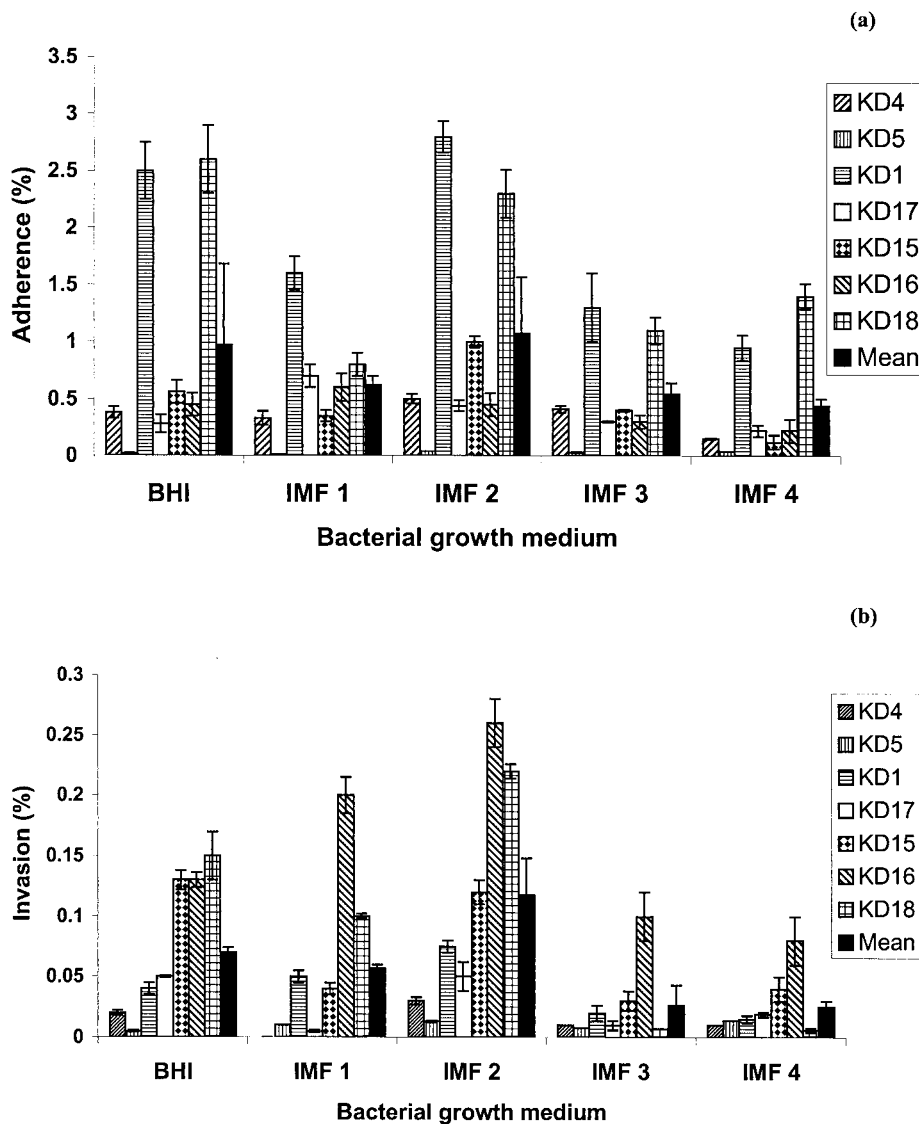

FIG. 1. Ability of selected *Bacillus* isolates to adhere to (a) and invade (b) HEp-2 cells after 18 h of growth in a variety of reconstituted IMF and in BHI broth.

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toxin complex was assessed using the *B. cereus* enterotoxin (diarrheal type) RPLA kit. Detection of DNA sequences encoding HBL enterotoxin and *B. cereus* BceT was achieved by PCR primer analysis.

With the exception of *B. anthracis* and *B. cereus*, which can be identified using rapid molecular and immunological approaches (12, 16), identification of other *Bacillus* isolates to the species level remains arduous, as it largely depends on performing a series of biochemical and physiological tests (8, 21, 22). Identification is further complicated by the fact that many clinical isolates of a species occasionally do not provide characteristic reactions that are considered typical of that species. For instance, during this study, the clinical isolates *B. licheniformis* KD1 and KD8 and *B. pumilus* KD14 were shown to be atypically lecithinase positive. Despite these constraints, all *Bacillus* isolates were identified to the species level. While the miniaturized biochemical API 50CHB assay was used to confirm the identity of each *Bacillus* species, this method was unable to differentiate between *B. licheniformis* and *B. subtilis* (in such instances, additional physiological discriminatory tests were performed).

Cell-free culture supernatants of 90 and 81% of the 47 *Bacillus* isolates examined for cytotoxicity in this study showed toxicity in HEp-2 and Caco-2 epithelial cells, respectively. Others have shown that the tetrazolium salt MTT can be used to assess the cytotoxic effects of culture supernatant fluids of *Bacillus* species isolated from raw milk (2, 9). Only living eukaryotic cells are detected using this assay, as the tetrazolium ring of MTT is cleaved in the mitochondria of metabolically active cells. Beattie and Williams (2) showed that some isolates of *B. circulans*, *B. laterosporus*, *B. lentus*, *B. licheniformis*, *B. mycoides*, *B. subtilis*, *B. cereus*, and *B. thuringiensis* were toxigenic to Chinese hamster ovary (CHO) cells using this MTT assay. Tetrazolium salts have also been used to assess the cytotoxicity of other pathogens, such as *Pasteurella haemolytica* A1 leukotoxin (6) and the cytotoxin of *Campylobacter jejuni* (5), and

they were recently used to investigate *B. cereus* toxicity (26). Findlay et al. (9) advocated the use of MTT, as the currently used HEp-2 cell vacuolation assay for *Bacillus* emetic toxin is laborious, subjective, and unreliable.

This study also showed that greater detection of toxigenic *Bacillus* isolates occurred with different cell lines, as the culture supernatant fluids of some *Bacillus* species did not produce a cytotoxic effect in each of the cell lines investigated. In this study and another study (2), a higher proportion of toxigenic strains was detected by cytotoxicological methods. The apparent lower detection rate with immunological methods (Tecra BDE and Oxoid RPLA diarrheal enterotoxin test kits) is likely to be attributable to their specificity for individual components in the toxin complexes. This study showed that the supernatant fluids from isolates of *B. licheniformis*, *B. subtilis*, *B. circulans*, and *B. megaterium* were positive in the *B. cereus* enterotoxin (diarrheal type) RPLA assay, which is specific for the L_2 component of the HBL complex. This finding indicates that these isolates produced protein toxins that were very similar to those of *B. cereus* and that these species may also present a potential hazard in food products. HBL contains the protein components B (37.5 kDa), L_1 (38.2 kDa), and L_2 (43.5 kDa), and all three components are required to produce maximal biological activity. The toxic activities so far identified for HBL include hemolysis, vascular permeability and necrosis in rabbit skin, fluid accumulation in rabbit ileal loops, toxicity to a number of transformed cell lines, in vitro degradation of explanted rabbit retinal tissue, and in vivo ocular necrosis and inflammation in rabbits (14).

Schoeni and Wong (25) previously reported that HBL was secreted by over 200 *B. cereus*, *B. thuringiensis*, and *B. mycoides* strains tested. Beattie and Williams (2) showed that the supernatant fluids from isolates of *B. thuringiensis*, *B. circulans*, *B. licheniformis*, *B. lentus*, *B. laterosporus*, and *B. mycoides* reacted positively with both the BDE and the RPLA immunoassays. *B. subtilis*, *B. licheniformis*, *B. pumilus*, and *B. thuringiensis* were previously implicated in outbreaks of food-borne disease (13, 24). It has been shown that some *B. cereus* strains may contain multiple copies of the *hbl* genes that arose from duplication of a single gene (25). HBL has been mapped to a portion of the *B. cereus* chromosome that exhibits exceptional variability compared with other regions, and this variable region is sometimes located on large extrachromosomal DNA fragments that appear to be stable but may prove to be large mobile plasmids (25). These findings may explain why many different *Bacillus* species appear to have the *B. cereus*-associated HBL enterotoxin complex.

This study recognizes the existence of lecithinase-positive toxigenic *B. licheniformis* strains isolated from the clinical environment. Interestingly, of the 23 toxin-producing isolates of *B. licheniformis* previously reported, all were incapable of producing lecithinase (24). The production of lecithinase, other phospholipases, proteases, and enterotoxins is recognized as a putative virulence factor that is required by invasive bacterial pathogens to elicit successful systemic infections (8, 19). The identification of lecithinase activity in clinical isolates of *B. licheniformis* is significant (possibly arising from genetic exchanges with the ubiquitous *B. cereus*), as this finding suggests that toxigenic *B. licheniformis* isolates may also acquire the necessary virulence determinants to support infection; lecithinase-positive *B. pumilus* KD14 was associated with a patient who had spreading fasciitis.

While a number of *Bacillus* species have been occasionally associated with gastrointestinal illnesses, recent evidence suggests that many members of this genus may be the cause of serious systemic diseases, such as septicemia, endocarditis, peritonitis, ophthalmitis, liver failure, and meningitis (8, 15, 24). We have shown that 21 *Bacillus* isolates representing 14 different species can invade HEp-2 and Caco-2 epithelial cells. Interestingly, the nutritional composition of the growth medium influenced bacterial virulence factor expression, such that baby foods containing both glucose syrup and lactose supported growth and enhanced levels of invasion in HEp-2 cells. Environmental signals, such as the presence of a readily utilizable carbon source, have been previously shown to modulate virulence factor expression in other bacterial enteropathogens (18, 20). PrfA, the central virulence transcriptional activator in *L. monocytogenes*, is regulated by a variety of environmental cues (18). This study has also provided evidence for expressional cross talk between environmental signals and virulence in *Bacillus* species. For instance, many of the clinical and food isolates of *Bacillus* were shown to contain DNA sequences encoding the HBL complex, yet only certain *Bacillus* isolates expressed the necessary *hblA*, *hblC*, and *hblD* genes to produce this diarrheal toxin after growth in reconstituted IMF products and in BHI broth.

In summary, this study has shown that a variety of different *Bacillus* spp. isolated from clinical specimens and food samples were capable of adhering to, invading, and producing cytotoxic effects in epithelial cells after growth in reconstituted IMF. While many different *Bacillus* isolates were shown to possess DNA sequences associated with the *bceT*, *hblA*, *hblC*, and *hblD* diarrheal enterotoxin genes, the composition of the bacterial culture medium influenced the ability of these *Bacillus* isolates to express these genes. It should be noted that reconstituted IMF were used as test food matrices in this study, as these products are frequently contaminated with acceptably low numbers of *Bacillus* spores (22). Properly reconstituted IMF containing *Bacillus* spp. have not been previously associated with food-borne illness in infants.

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