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Germination of *Bacillus cereus* Spores Is Induced by Germinants from Differentiated Caco-2 Cells, a Human Cell Line Mimicking the Epithelial Cells of the Small Intestine▽

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Spores of 11 enterotoxigenic strains of *Bacillus cereus* isolated from foods and humans adhered with similar efficiencies to Caco-2 cells, whereas subsequent germination triggering was observed with only 8 of these strains. Notably, Hep-2 cells did not trigger germination, while spores of all strains displayed similar germination efficiencies in brain heart infusion broth.

The food-borne *Bacillus cereus* diarrheal syndrome is caused by enterotoxins produced during growth of the microorganism in the small intestine. When *B. cereus* spores are ingested, germination and subsequent outgrowth are essential steps in the onset of the diarrheal syndrome. Germination is a complicated process involving the action of germination receptors in the dormant spores and specific germinants. Spores lacking receptors are strongly impaired in their response to germinants (2, 4, 10). We aimed to investigate adhesion of *B. cereus* spores to differentiated Caco-2 cells (human colorectal adenocarcinoma cells) and assess the germination-inducing capacity of these cells and/or their released compounds.

The *B. cereus* food and human isolates used for these investigations are described in Table 1. Production and storage of spores were described previously (11). Culturing and differentiation of Caco-2 cells in 12-well plates using Dulbecco's modified Eagle medium (DMEM) (Gibco catalog no. 42430) supplemented with fetal calf serum and antibiotics and culturing of Hep-2 cells (human laryngeal epidermoid carcinoma cells) using minimal essential medium with Hanks' salts (MEM) (Gibco catalog no. 21575) supplemented with fetal calf serum and antibiotics were carried out using prescribed procedures (3, 8). Adhesion-invasion experiments with Caco-2 cells and Hep-2 cells were carried out in 12-well plates. Spores, washed with DMEM or MEM (depending on the cell type), were added to the cells in the plates and incubated. After 1 h the plates were washed with DMEM or MEM and either treated with 1% Triton X-100 in phosphate-buffered saline or incubated with DMEM or MEM for an additional 1 h and subsequently treated with Triton X-100. The 1-h procedure was used to establish adhesion and invasion; the 2-h procedure was used to investigate germination. After treatment with Triton X-100, the total and spore counts were determined as previously described (11). Similar adhesion-invasion and germination experiments were carried out in cell-free plates. Confocal laser scanning microscopy was used to assess invasion after staining with propidium iodide according to the manufacturer's instructions.

Spores from all 11 strains were found to adhere to differentiated Caco-2 cells (Fig. 1). The numbers of adhered *B. cereus* cells were plotted against the number of *B. cereus* cells added, and as the correlation coefficient (R^2) of the trend line is 0.947, we concluded that adhesion is nonspecific instead of through the action of specific adhesins like p104, which is involved in adhesion of *Listeria monocytogenes* to Caco-2 cells (7). The adhesion efficiency was approximately 1% for spores of all 11 strains. In experiments with selected strains 1 and 11, adhesion of spores to Hep-2 cells was observed as well (data not shown). Previously, Andersson et al. found that 4 of 10 strains adhered to differentiated Caco-2 cells (1). The differences in adhesion efficiency between their investigation and our investigation may be due to differences in the detection techniques; whereas

TABLE 1. Origin of enterotoxigenic *B. cereus* strains

Strain	Designation	Origin	Source
1	9901672	RIVM[a]	Human feces from healthy individual
2	9901909-1	RIVM[a]	Human feces from healthy individual
3	9902169-2	RIVM[a]	Human feces from case associated with diarrheal food-borne disease
4	9902187-1	RIVM[a]	Human feces from healthy individual
5	9903040-1	RIVM[a]	Human feces from healthy individual
6	9903295-4	RIVM[a]	Human feces from case associated with diarrheal food-borne disease
7	TZ 415	INRA[b]	Cooked chilled food[f]
8	Z 4222	INRA[b]	Cooked chilled food[f]
9	NCTC 11145	NCTC[c]	Food poisoning (diarrheal food-borne disease)
10	DSM 4384	DSMZ[d]	Food poisoning (diarrheal food-borne disease)
11	1230-88	NVH[e]	Human feces from case involved in diarrheal food-borne disease

[a] Strain from a RIVM project on the incidence and pathogenesis of gastroenteritis.
[b] INRA, Institut National de la Recherche Agronomique, Avignon, France.
[c] NCTC, National Collection of Type Cultures.
[d] DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen.
[e] NVH, Norwegian School of Veterinary Science.
[f] See reference 3.

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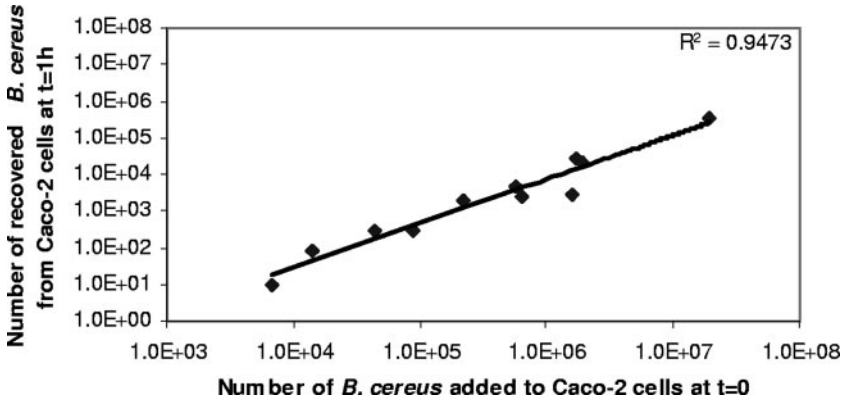

FIG. 1. Correlation between numbers of *B. cereus* cells added to Caco-2 cells at time zero (t=0) and recovered total numbers of *B. cereus* cells adhered to Caco-2 cells after 1 h of incubation (t=1h). Each dot represents the results for one strain.

Andersson et al. used microscopy to assess adhesion, we used the more sensitive culture method.

Subsequently, we investigated germination induction upon adhesion to Caco-2 cells with all 11 *B. cereus* strains (Table 2). In these experiments, total and spore counts were assessed both in the starting spore suspension and after the 2-h incubation with Caco-2 cells. After incubation with differentiated Caco-2 cells, differences in germination capacity between the strains became apparent, as reflected in the ratios of total counts to spore counts (Table 2). Control experiments were carried out in the absence of Caco-2 cells, and they revealed a lack of germination capacity. For all strains, germination indices were calculated by dividing the germination ratio in the absence of Caco-2 cells by the ratio in the presence of Caco-2 cells. A cutoff value of 2.5 was used for germination; this value is clearly higher than the average ratio (1.5) found upon determination of the total and spore counts of spore suspensions. Eight of the 11 strains had a germination index higher than 2.5 (range, 3.0 to 90), indicating that Caco-2 cells induced germination. Similar results were not obtained when the experiments were carried out with Hep-2 cells; the total and spore counts remained approximately the same after the 2-h incubation, indicating that no Hep-2 cell-induced germination had occurred. This shows that germination triggering is not a common trait of epithelial cell lines. Notably, spores from all 11 strains showed similar germination efficiencies in brain heart infusion broth (data not shown). No statistical analyses were carried out to investigate the correlation between germination induction and the origin of the strains, as the numbers of strains per subgroup (healthy individual, food-borne disease, and food) were too small to justify such analyses.

Using spores from strain 11, we started to characterize the Caco-2 cell-produced germination-inducing compound(s). Supernatant from Caco-2 cells induced germination efficiently, as indicated by the low spore counts compared to the total counts, while supernatant from Caco-2 cells that had been preincubated for 1 h with *B. cereus* spores did not. This indicates that the germinant released by Caco-2 cells can be bound (absorbed) and/or inactivated (degraded) by spores. In addition, heating for 5 min at 100°C and addition of proteolytic enzymes like those present in porcine pancreatin (Merck) had no effect on the germination-inducing capacity of the Caco-2 cell-produced compound(s) (Fig. 2).

The differences in Caco-2 cell-induced germination efficiency between the strains may be due to differences in germination receptor profiles and/or differences in expression levels of the receptors (4). In *B. cereus* ATCC 14579, seven putative *ger* operons have been identified; these operons may equip the

TABLE 2. Spore counts, total counts, ratios of spore count to total count at 2 h for adhered *B. cereus* spores in the presence and absence of Caco-2 cells, and germination indices

Strain	In the presence of Caco-2 cells			In the absence of Caco-2 cells			Germination index[b]
	Total count	Spore count	Spore %[a]	Total count	Spore count	Spore %[a]	
1	1.3E + 02	5.0E + 00	3.8	5.6E + 02	2.7E + 02	48.2	12.7
2	3.2E + 03	7.0E + 02	21.9	1.1E + 04	2.3E + 03	20.9	1.0
3	1.5E + 05	1.7E + 05	113.3	3.2E + 04	2.3E + 04	71.9	0.6
4	5.4E + 03	8.0E + 02	14.8	6.0E + 03	3.0E + 03	50.0	3.4
5	2.8E + 04	5.1E + 03	18.2	8.5E + 04	9.3E + 04	109.4	6.0
6	5.0E + 04	5.3E + 03	10.6	1.3E + 05	1.2E + 05	92.3	8.7
7	8.6E + 03	4.0E + 02	5.7	2.9E + 04	1.5E + 04	51.7	9.1
8	9.6E + 03	9.5E + 02	9.9	2.6E + 04	8.7E + 03	33.5	3.4
9	1.7E + 02	9.0E + 01	52.9	3.0E + 01	3.0E + 01	100.0	1.9
10	5.4E + 03	1.9E + 02	3.5	4.5E + 03	4.7E + 02	10.4	3.0
11	6.4E + 04	3.1E + 02	0.5	7.8E + 04	3.4E + 04	43.6	90.0

[a] Spore % = (spore count/total count) × 100.
[b] Germination index = percentage in experiment without Caco-2 cells/percentage in experiment with Caco-2 cells.

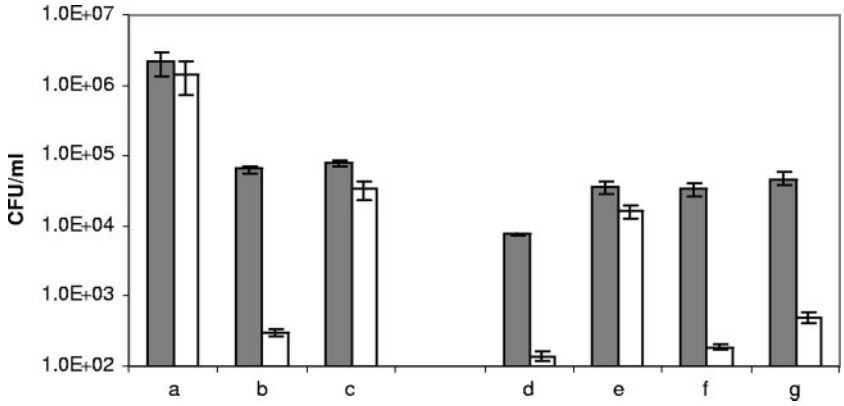

FIG. 2. Influence of various factors on *B. cereus* strain 11 spore germination-inducing capacity of Caco-2 cell culture supernatant. The gray bars indicate total counts, and the open bars indicate spore counts. Bars a, total counts and spore counts of strain 11 used in the experiments at time zero; bars b and c, counts of adhered *B. cereus* after 1 h of incubation with (bars b) and without (bars c) Caco-2 cells; bars d and e, total and spore counts of adhered *B. cereus* after 1 h of incubation with Caco-2 cell culture supernatant (bars d) and after 1 h of incubation with supernatant preincubated with *B. cereus* strain 11 spores (bars e); bars f and g, total and spore counts after incubation for 1 h with heat-treated (bars f) and pancreatin-treated (bars g) Caco-2 cell culture supernatant.

spore with seven functional germination receptors, although the number may vary for other *B. cereus* strains (6). A range of germinants have been identified, including L-alanine, L-phenylalanine, L-glutamine, inosine, and mixtures of L-asparagine, glucose, fructose, and K^+ (2, 5, 9). All these compounds are, like the Caco-2 cell-produced germinant(s), insensitive to heat and protease activity. The precise identity of the Caco-2 cell-produced germinant(s) remains to be elucidated, as does the role of specific germination receptors in the pathogenicity of enterotoxigenic *B. cereus*.

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