Role of Germinant Receptors in Caco-2 Cell-Initiated Germination of *Bacillus cereus* ATCC 14579 Endospores[▽]

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Spores obtained from *Bacillus cereus* ATCC 14579 and mutant strains lacking each of seven germinant receptor operons were exposed to differentiated Caco-2 cells and monitored for germination. Spores of the *gerI* and *gerL* mutants showed a reduced germination response, pointing to a role for these receptors in Caco-2-induced germination.

Bacillus cereus is a gram-positive, rod-shaped, spore-forming food pathogen (4, 5). Frequent occurrence of this organism in the soil is the source of its easy dissemination into the food chain via raw agricultural products (9, 13). During consumption of contaminated food, spores and/or vegetative cells are ingested, and the spores are able to efficiently pass through the acidic environment of the human stomach (2), after which they enter the small intestine. The diarrheal type of food-borne infection caused by enterotoxin-producing vegetative *B. cereus* cells in the small intestine (6, 13) might be due to consumed spores germinating in the small intestine. Germination of *Bacillus* spores is generally initiated by specific signaling molecules, called germinants, which activate the germinant receptors located in the inner membrane of the spore (10, 11, 14). *B. cereus* ATCC 14579 contains seven different germinant receptors (7, 8). Here we investigate the role of these seven different germinant receptors in initiation of germination after interaction of *B. cereus* ATCC 14579 spores with differentiated Caco-2 cells, which mimic the epithelial layer of the small intestine (1).

Germination after interaction with differentiated Caco-2 cells. Spores of the *B. cereus* ATCC 14579 wild-type and *ger* mutant strains (Table 1) were obtained by growth and sporulation in defined medium and purified as described previously (3); prior to use in germination experiments, the 4-week-old spores were washed and heat activated (8). Subsequently, the spores were incubated at 37°C in Dulbecco's modified Eagle medium (DMEM) with and without 10 mM L-alanine and 1 mM inosine. Germination was measured by determining the decrease in absorbance, signifying the transition of phase-bright, nongerminated spores into phase-dark, germinated spores. No germination is reflected by 100% optical density at 600 nm, whereas 50% indicates >99% germination, as was confirmed by phase-contrast microscopy. No germination of

spores (94.1% remaining of initial optical density at 600 nm) was observed after incubation for 1 h in DMEM (Fig. 1), indicating a lack of germination induction by this medium. In contrast, spores incubated at 37°C in DMEM supplemented with 10 mM L-alanine and 1 mM inosine showed efficient germination. No significant differences were observed between the strains in these germination experiments (Student's *t* test; $P > 0.05$), and these strains are therefore presented as one line (Fig. 1). Microscopic analysis indeed showed <5% nongerminated spores after incubation for 60 min in this medium for all strains (data not shown). Efficient germination in supplemented DMEM shows that spores of all strains are capable of germination in this culture medium, provided that additional germinants are present. Hereafter, the spores were incubated at 37°C for 1 h in wells containing DMEM and Caco-2 cells (15). The Caco-2 cells, obtained from ATCC (HTB-37), were seeded at a concentration of 10^6 cells/ml in 12-well plates and cultured and differentiated as described previously (12). For germination experiments, spores were inoculated at a concentration of $\sim 5 \times 10^6$ spores/ml, of which the average population that adhered to Caco-2 cells was 1.5% (data not shown). Microscopic analysis of nonadhered spores revealed only nongerminated spores after 1 h of incubation in the wells containing DMEM and Caco-2 cells (data not shown). Therefore, data analysis focused on the germination of spores adhered to Caco-2 cells. The data presented are average values for three independent germination experiments. For every experiment, independent Caco-2 cell batches (passages 40, 41, and 42) were used, and three wells were used per strain. For the first and third experiments, one spore batch was used, and for the second experiments, a second independent spore batch was used. After 1 h incubation and subsequent washing, the Caco-2 cells with attached *B. cereus* spores/cells were harvested by removal and lysis of the Caco-2 cells from the culture well (15). This lysate contains the *B. cereus* population adhered to Caco-2 cells, consisting of heat-resistant nongerminated spores, heat-sensitive germinated spores, and vegetative cells formed within the 1-h incubation period. The total CFU of the *B. cereus* population in the lysate was determined by enumeration on brain heart infusion agar (Difco), while the lysate was incu-

TABLE 1. Genetic and germination characteristics of *B. cereus* strains used in this study

Strain	Characteristics	Observed germinant(s)[a]	Source or reference
B. cereus			
ATCC 14579	Wild-type strain used in this study		BGSC
LH129 R	*gerRC1*::pMUTIN4 Ery[r]	Ala, Cys, Thr, Ino, Ade	7
LH130 Q	*gerQA1*::pMUTIN4 Ery[r]	Ino	8
LH132 G	*gerGA1*::pMUTIN4 Ery[r]	Glu	8
LH140 K	*gerKA1*::pMUTIN4 Ery[r]	ND	8
LH142 L	*gerLB1*::pMUTIN4 Ery	ND	8
LH144 S	*gerSA1*::pMUTIN4 Ery[r]	ND	8
LH148 I	*gerIA1*::pMUTIN4 Ery[r]	Phe, Ino, Ade	8

[a] Amino acids are indicated by their three letter symbols. ND, not determined; no germinant molecule has been identified for this receptor.

bated for 15 min at 70°C prior to plating for determination of the number of nongerminated spores. Furthermore, the results were verified by phase-contrast microscopic observation. For the wild type, the percentage of nongerminated spores present in the lysate was determined to be 9% (Fig. 2), indicating that germination was specifically triggered by Caco-2 cell-derived germinants. For *B. cereus* spores with either a disrupted *gerI* or *gerL* operon, the germination was observed to be significantly less efficient than that of the wild type, as the adhered populations of nongerminated spores for these strains after 1 h of incubation were still 72% and 36%, respectively (Fig. 2). Spores with a disrupted *gerK*, *gerQ*, *gerR*, or *gerS* operon germinated with similar efficiency as the wild type (Fig. 2).

Of all *B. cereus* mutants tested in this study, spores with either a disrupted *gerI* or *gerL* operon showed a reduced germination response to Caco-2 cells, indicating that the germinant receptors encoded by these operons were involved in the germination of spores adhered to the Caco-2 cells. The germinant specificity of the receptors encoded by *gerI* and *gerL* in *B. cereus* ATCC 14579 has been investigated before (8). The GerI receptor was demonstrated to be involved in purine riboside-induced germination and aromatic amino acid-induced germination (8), suggesting that the
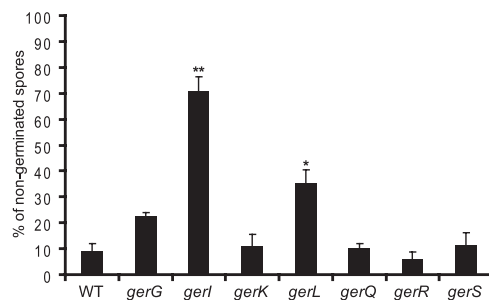
FIG. 2. Percentage of nongerminated spores present in the adhered population of the wild-type and mutant spores after incubation with Caco-2 cells for 1 h. One hundred percent reflects no germination, whereas 0% represents total germination. A one-way analysis of variance with Tukey's multiple comparison test was used to indicate significantly different germination from the wild-type (WT) spores with a *P* value of <0.001 (∗∗) or <0.01 (∗), using averages of the three independent experiments (*n* = 3). Bars represent standard errors of the means.

Caco-2 cells may release a similar compound(s). A previous survey with a range of amino acids and purine ribosides did not result in the identification of germinants recognized by GerL (8). The reduced germination capacity displayed by spores lacking the GerL receptor after incubation with Caco-2 cells indicates the release of one or more yet-to-be-identified germinants by these cells. Wijnands et al. recently observed that the germination-inducing compounds excreted by Caco-2 cells were heat stable and insensitive to proteolytic activity (15), which may point toward small molecules, such as amino acids and purine ribosides. The nature of these Caco-2-derived compounds remains to be elucidated.

In conclusion, our results with spores of the wild-type and germinant receptor mutant strains suggest that germination of *B. cereus* spores can be initiated by specific germinants in the small intestine and point to niche-specific roles for the different germinant receptors in *B. cereus*.

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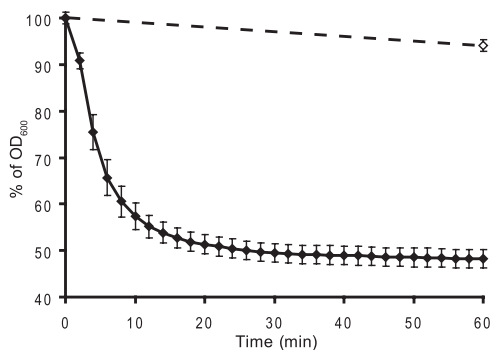
FIG. 1. Germination efficiency of the wild-type and mutant spores in DMEM supplemented with 10 mM L-alanine and 1 mM inosine. Shown are average values from wild-type and mutant spores in DMEM (open diamond) and DMEM supplemented with 10 mM alanine and 1 mM inosine (closed diamonds). Bars represent standard errors of the means. OD$_{600}$, optical density at 600 nm.

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