The *Bacillus cereus* GerN and GerT Protein Homologs Have Distinct Roles in Spore Germination and Outgrowth, Respectively[▽]

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The GerT protein of *Bacillus cereus* shares 74% amino acid identity with its homolog GerN. The latter is a Na^+/H^+-K^+ antiporter that is required for normal spore germination in inosine. The germination properties of single and double mutants of *B. cereus* ATCC 10876 reveal that unlike GerN, which is required for all germination responses that involve the GerI germinant receptor, the GerT protein does not have a significant role in germination, although it is required for the residual GerI-mediated inosine germination response of a *gerN* mutant. In contrast, GerT has a significant role in outgrowth; *gerT* mutant spores do not outgrow efficiently under alkaline conditions and outgrow more slowly than the wild type in the presence of high NaCl concentrations. The GerT protein in *B. cereus* therefore contributes to the success of spore outgrowth from the germinated state during alkaline or Na^+ stress.

Bacillus cereus ATCC 10876 spores germinate in inosine or in L-alanine as sole germinants (5). They require both GerI and GerQ germinant receptors for germination in inosine as the sole germinant, whereas the GerL receptor is responsible for most of the response to L-alanine as the sole germinant, with a smaller contribution from the GerI receptor (3). The GerN protein is needed for successful inosine germination, as spores of a *gerN* mutant of *B. cereus* ATCC 10876 demonstrate a slow and abnormally ordered germination response in inosine as the sole germinant (24). The residual germination was very asynchronous, and some spores appeared to become phase dark before losing heat resistance (24). This GerN protein is a homolog of a widely distributed family of cation transporters (9) and has been demonstrated to mediate Na^+/H^+ and Na^+/H^+-K^+ antiport activity when expressed at a low level in *Escherichia coli* (21). Such GerN-mediated antiport activity is therefore apparently required for the germination response mediated by the GerI germinant receptor in *B. cereus*. The role of such an ion transport protein in germination remains unclear, especially as other germinant receptors in the same strain do not require a functional GerN protein; for example, the *gerL*-dependent alanine response is almost identical to that of the wild type in a *gerN* mutant (24). Both GerN and a closely related homolog are encoded in *B. cereus* ATCC 14579 (13) and in other members of the family, including *Bacillus anthracis* (17) and *Bacillus thuringiensis* (16). Both genes appear to be monocistronic, and their coding sequences are widely separated on the genome. We have called this second *gerN*-like gene *gerT* (19). An entirely different (spore coat) protein in *B. subtilis* has recently been called *gerT* (7), but that gene does not have a homolog in *B. cereus*, and as *B subtilis* does not carry an equivalent homolog of the *B. cereus gerN* or *gerT* gene, the nomenclature will not overlap. We describe here the spore germination and outgrowth phenotypes of mutants of *B. cereus* ATCC 10876 carrying an insertionally inactivated *gerT* gene. Resulting phenotypes suggest that the GerN and GerT proteins have distinct roles in germination and outgrowth.

MATERIALS AND METHODS

Bacterial growth, media, and strains. *Escherichia coli* cloning strain TG1 and *B. cereus* ATCC 10876-derived strains were routinely cultured in L broth (Difco Bacto tryptone at 10 g liter^{-1}, Difco yeast extract at 10 g liter^{-1}, and NaCl at 5 g liter^{-1}, pH 7.2) or on L agar containing the appropriate antibiotics (for *E. coli*, ampicillin at 50 μg ml^{-1} and chloramphenicol at 30 μg ml^{-1}; for *B. cereus*, erythromycin and lincomycin at 1 and 25 μg ml^{-1}, respectively, and kanamycin at 50 μg ml^{-1}). Spores of *B. cereus* were prepared in CCY medium, harvested, and washed at least 10 times in water, as described previously (5). Oxoid nutrient broth (NB; pH 7.4) was used for outgrowth experiments.

The *B. cereus* strains are listed in Table 1. The *E. coli* K-12 strains used for attempts at complementation of ion transport defects were the Na^+/H^+ antiporter-deficient strain KNabc (*chaA nhaA nhaB*) (15) and the potassium uptake-deficient strain TK2420 (Δ*kdpABC trkD1* Δ*trkA*) (11), as already used in the study of *gerN* (21). Strain KNabc is defective in sodium efflux and consequently is unable to grow at high concentrations of Na^+ (>75 mM) but will grow in a modified low-sodium medium, LBK (11), which contains 10 mM NaCl and 50 mM KCl. To test for Na^+ sensitivity, colonies of KNabc/pAS1 and KNabc/pGEM3zf+ were grown for 24 h at 37°C in 5 ml of LBK plus chloramphenicol, and the culture was then diluted 250-fold into LBK, in which the NaCl concentration was varied, and grown for 16 h at 37°C.

Cloning and sequencing of *gerT* from *B. cereus* ATCC 10876. PCR primers based on the *B. anthracis* genome sequence data around BA0819 (*gerT*) were used to amplify the regions flanking the *gerT* gene of *B. cereus*, using high-fidelity *Taq* Extend (Boehringer Mannheim). The resulting *B. cereus*-derived PCR products were sequenced, and primers were designed for cloning *B. cereus gerT* without its promoter region, as a 1.1-kb fragment. These were APS11 (CTG<u>G TCGAC</u>TAAAGGAGGAGCAGATGCTAT) and APS12 (TTC<u>GAGCTC</u>CTA TCTATACAAAATATTTC), incorporating (underlined) SalI and SacI sites, respectively, at their ends. Following restriction enzyme digestion, the PCR product was ligated into SalI- and SacI-digested pGEM3zf+, with *gerT* in the reverse orientation relative to the vector's *lac* promoter but downstream of the T7 promoter. *E. coli* TG1 (a strain lacking T7 polymerase) was transformed, yielding plasmid pAS1 for sequencing and complementation studies.

The monocistronic *gerT* locus encodes a 375-amino-acid GerT protein, with 99% amino acid identity to the equivalent protein in *B. anthracis*. A potential ribosome binding site is appropriately located upstream of the putative *gerT* start codon, and the *gerT* stop codon is followed by a potential rho-independent terminator.

Construction of *gerT*-null mutants of *B. cereus*. Plasmid vector pSMUT lacks an origin of replication for *B. cereus* but carries a ColE1 replicon and a β-lac-

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TABLE 1. *Bacillus cereus* strains used

Strain	Relevant genotype/antibiotic resistance marker(s)	Source or reference
ATCC10876 UM20.1	*trp-1* Str^r	3
AM1314	Tn*917*-LTV1::*gerIA5* Ery^r *trp-1* Str^r	3
AM1311	Tn*917*-LTV1::*gerQA2*Ery^r *trp-1* Str^r	3
AM1316	Tn*917*-LTV1::*gerLA1* Ery^r *trp-1* Str^r	3
AM1421	*gerN17*::pMUTIN4 Ery^r *trp-1* Str^r	23
AM1631	*gerT*::pSMUT Kan^r *trp-1* Str^r	This work
AM1632	*gerN17*::pMUTIN4 *gerT*::pSMUT Kan^r Ery^r *trp-1* Str^r	This work

tamase gene for amplification and selection, respectively, in *E. coli*. It is a derivative of pMUTIN4 (25), in which the *lacZ* gene has been removed and the erythromycin/lincomycin resistance gene replaced by a kanamycin resistance cassette.

Primers APS7 (CGTGAATTCGGTAAGTTAATTGTTGGTTA) and APS8 (AACGGATCCCAATAACAATCGGCTGTGAA) were used to PCR amplify a 1.0-kb internal fragment of *gerT* from 108 bp downstream of the start of the *gerT* open reading frame to 76 bp before the stop codon of *gerT*. This PCR fragment was digested with (underlined) EcoRI and BamHI, respectively, and ligated with EcoRI- and BamHI-digested pSMUT, yielding plasmid pAS3. *B. cereus* was electroporated with 2.5 μg of pAS3 DNA, and transformants were selected on nutrient agar containing kanamycin. Integration into the chromosome by a single crossover within the region of homology interrupts the *gerT* gene so that it encodes a protein truncated by 25 amino acids from the C terminus. The disruption of the *gerT* gene and expected novel junction fragments were confirmed by PCR in a transformant named AM1631 (*gerT1*::pSMUT).

Construction of a *gerN gerT* double mutant. The *gerN* mutant used in this study was strain AM1421 (*gerN17*::pMUTIN4), in which the *gerN* gene was insertionally inactivated by integration of plasmid pMNAP, derived from pMUTIN4 by cloning of an internal fragment of the *gerN* gene, spanning bases 365 to 813 of the open reading frame; integration of this plasmid results in C-terminal truncation of GerN by 120 amino acids. The inactivated gene was checked by Southern blotting (23). This *gerN17*::pMUTIN4 mutation was introduced into strain AM1631 (*gerT1*::pSMUT) by generalized transduction with CP51ts, as described previously (5). Transductants were selected for resistance to erythromycin and lincomycin and screened for retention of the kanamycin resistance marker. Colony PCR confirmed the presence of the mutations in both genes. One transductant was retained and named AM1632 (*gerN17 gerT1*).

Germination experiments. Spore germination used washed spores, heat activated for 30 min at 70°C in H$_2$O, and conditions were as described previously (3), unless otherwise stated. Optical density at 490 nm (OD$_{490}$) was measured at intervals on a Wallac Victor plate reader. For amino acid-enhanced L-alanine germination, germination was initiated by a combination of a subgerminal concentration of L-alanine (20 μM) plus histidine, proline, or tryptophan at 10 mM or tyrosine at 1 mM. The buffer conditions were those optimal for L-alanine as the sole germinant: Tris-HCl (10 mM), pH 8.9, with NH$_4$Cl (50 mM), and germination at 30°C. Spores were preincubated in the alanine racemase inhibitor *O*-carbamyl-D-serine (5 μg/ml) in germination buffer for 5 min before addition of germinants to prevent any conversion of L-alanine to its competitive inhibitor D-alanine. For amino acid-enhanced inosine germination, the germination buffer was Tris-HCl (10 mM), pH 8.0, NaCl (10 mM), and spores were germinated at 37°C; inosine was used in this experiment at a just-subgerminal concentration (40 μM), and the same amino acid adjuncts were added as described above.

Nucleotide sequence accession number. The fully overlapped sequence of the *gerT* region has been submitted to GenBank (accession number EU789572).

RESULTS

GerT complements the Na$^+$ sensitivity of an *E. coli* mutant. Cloning the *gerN* gene into *E. coli* strains with deficient Na$^+$/H$^+$ antiporter activity and K$^+$ uptake (KNabc and TK2420, respectively) was reported to complement their respective compromised phenotypes (21). A similar test was carried out for the homologous *gerT* gene (Fig. 1). Introduction of the vector plasmid pGEM3zf+ did not improve the growth of
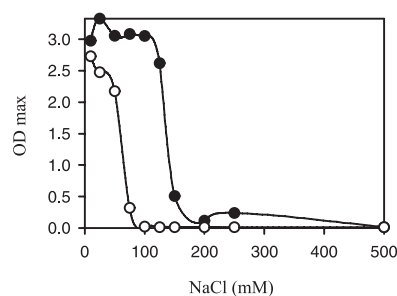

FIG. 1. Effect of different Na$^+$ concentrations on the growth of *E. coli* strain KNabc upon introduction of the *gerT* gene of *B. cereus*. The ODs of cultures were measured after 16 h of growth at 37°C. The precise growth conditions are described in Materials and Methods. Symbols: ○, KNabc/pGEM3zf+, a strain containing the plasmid vector only; ●, KNabc/pAS1, containing the *gerT* gene cloned in pGEM3zf+.

the KNabc strain, which was unable to grow in concentrations of NaCl over 75 mM; in contrast, KNabc/pAS1, containing a *gerT* gene cloned without an efficient promoter upstream, as had been done for *gerN* previously, was able to grow in NaCl concentrations up to 150 mM. These data suggest that GerT provides some additional capacity for Na$^+$ efflux. Attempts to introduce pAS1 into the K$^+$ transport-deficient *E. coli* strain TK2420 (10) were unsuccessful, so no predictions can be made about the potential for K$^+$ transport by the GerT protein.

Spore germination in single and double mutants in response to single germinants. Water-washed, heat-activated spores of mutants carrying insertionally inactivated *gerN* and *gerT* genes, individually and in combination, were used in germination assays (Fig. 2). Germination was measured as the fall in OD of spore suspensions after exposure to inosine. Spores of both the wild type and the *gerT* mutant germinated rapidly at 0.1 mM
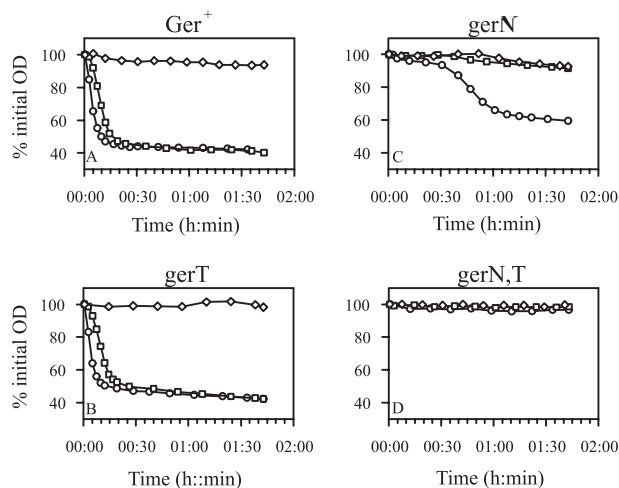

FIG. 2. Effect of *gerN* and *gerT* mutations on the germination response to inosine as the sole germinant, measured by fall in OD of spore suspensions. Spore suspensions were incubated in 10 mM Tris-HCl, pH 8.0, 10 mM NaCl at 37°C over a range of inosine concentrations. (A) Strain *B. cereus* ATCC 10876 (*ger$^+$*); (B) AM1631 (*gerT1*::pSMUT); (C) AM1421 (*gerN17*::pMUTIN4); (D) AM1632 (*gerN17*::pMUTIN4 *gerT1*::pSMUT). Symbols represent different inosine concentrations as follows: ◇, no inosine; □, 100 μM; ○, 1 mM. The data for 10 μM inosine are superimposed on the "no inosine" line.

and 1 mM inosine. As previously reported (24), the *gerN* mutant spores have a severe germination defect in inosine but still show some residual response in high (1 mM) inosine concentrations; this residual response is dependent on GerT, as it is absent in a *gerN gerT* double mutant. GerT is therefore not required for germination in inosine, provided that GerN protein is available, but it can provide a partially functional substitute for GerN if the GerN is missing from the spore. Germination in L-alanine under optimal conditions in the mutants was essentially identical to that in the wild type (data not shown); under these conditions, the receptor involved is GerL (3); therefore, neither GerN nor GerT is required for germination involving the GerL receptor.

If the GerI receptor is required for a response to combinations of germinants, GerN is also required. Although most members of the *B. cereus* family germinate in L-alanine and inosine, either individually or in combination, the range of germinant receptors encoded by different strains is variable, as is consequently the detailed germination behavior. Unlike *B. cereus* ATCC 14579 (12), which has a slightly different complement of germinant receptors, *B. cereus* ATCC 10876 germinates in L-alanine plus aromatic amino acids in a fashion generally similar to that for the so-called AEA, or aromatic-enhanced alanine, response of *B. anthracis* (8). In ATCC 10876, a subgerminal concentration of L-alanine (20 μM) is effective in combination with tryptophan or tyrosine, although there is no response with histidine or proline (Fig. 3A). Spores of *gerI*, *gerL*, or *gerN* mutants all fail to germinate in the alanine-plus-tryptophan or tyrosine combinations, but *gerQ* and *gerT* mutants germinate like the wild type (reference 19 and data not shown). These data demonstrate a requirement for both GerL and GerI germinant receptors, consistent with previous observations for the equivalent response in *B anthracis* Sterne (8). The third receptor required in *B. anthracis*, GerS, is also encoded in *B. cereus* ATCC 10876 (2) but has not been mutated to test its function. Another germinant combination effective in *B. anthracis* is inosine plus amino acids. In *B. cereus* ATCC 10876, subgerminal (40 μM) inosine with either tryptophan or histidine proves an effective germinant combination (Fig. 3B). As is the case for inosine as the sole germinant, both GerI and GerQ receptors and GerN are required here, but there is no requirement for the GerL alanine receptor or for GerT (Fig. 3C). These data altogether suggest that in every case when the GerI receptor is required, so also is GerN; the GerT protein does not compensate functionally for GerN in these circumstances.

GerT has a role in outgrowth in high salt concentrations and at alkaline pH. As *gerN* and *gerT* mutants germinate normally in L-alanine, it is possible to test for a role for these genes in spore outgrowth. Spores were germinated in L-alanine in Tris-HCl buffer, harvested by centrifugation, and resuspended in NB to allow outgrowth. Spores of the parent strain and the *gerN* and *gerT* mutants all outgrew at similar rates in NB (Fig. 4B). In NB adjusted to pH 9.5 with NaOH, the wild type and *gerN* mutant spores outgrew at the same rate after lags of ca. 30 and 60 min, respectively, but a clear defect was observed for the *gerT* mutant spores, which did not outgrow significantly within the period of the experiment. The *gerT* mutation also reduced the ability of spores to outgrow in NB at pH 7.4 in the presence of additional 0.7 M NaCl (Fig. 4C);
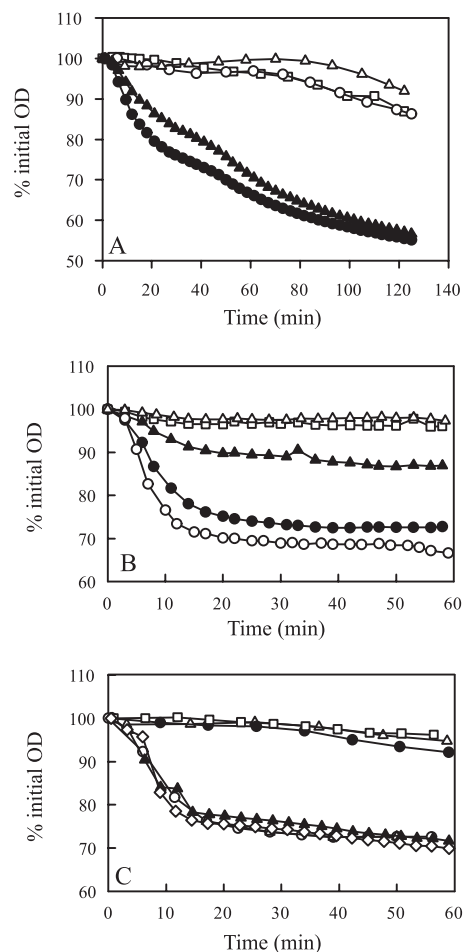

FIG. 3. Germination in subgerminative concentrations of alanine or inosine in combination with amino acid adjuncts. (A) Germination of spores of *B. cereus* ATCC 10876, the Ger⁺ parental strain, in L-alanine (20 μM) plus other amino acids. Symbols: □, no addition; ○, 10 mM histidine; △, 10 mM proline; ▲, 1 mM tyrosine; ●, 10 mM tryptophan. (B) Germination of spores of *B. cereus* ATCC 10876, the Ger⁺ parental strain, in 40 μM inosine plus amino acids. Symbols are as in panel A: □, no addition; ○, 10 mM histidine; △, 10 mM proline; ▲, 1 mM tyrosine; ●, 10 mM tryptophan. (C) Germination of mutants in inosine (40 μM) plus tryptophan (10 mM). Symbols: ○, *B. cereus* ATCC 10876, the Ger⁺ parental strain; ◇, *gerL*; ▲, *gerT*. These three graphs are superimposed and demonstrate complete germination. The following graphs showed little or no germination and are largely superimposed: ●, *gerQ*; △, *gerI*; □, *gerN*.

there was significant cell lysis and no outgrowth above the initial OD within 2.5 h. Unlike the situation in outgrowth, vegetative cells of these mutants all grew at the same rate as the wild type in NB at pH 9.5 (Fig. 4D). Sensitivity to alkaline pH in the *gerT* mutant is therefore limited to the outgrowing state. No difference was seen in the behavior of mutant and wild-type cells during vegetative growth following salt stress (data not shown). These data suggest that during vegetative growth, but not during outgrowth, alternative sodium efflux systems are likely to be present at a sufficient level for dealing with these environmental stresses, although the nature of such Na⁺ transport systems in the *B. cereus* family has not yet been explored.
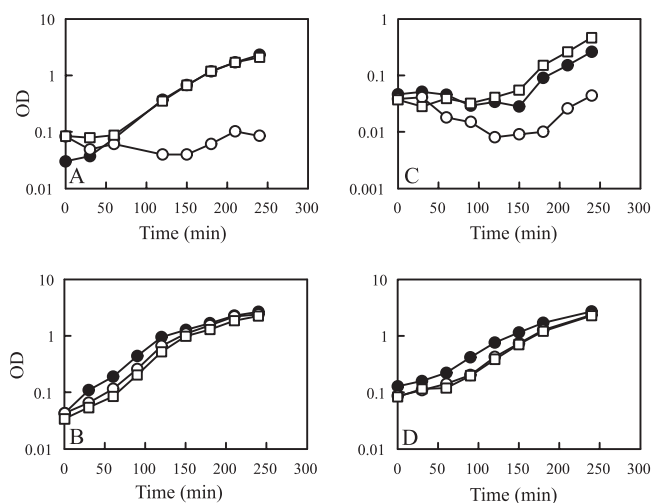
FIG. 4. Effect of *gerT* and *gerN* mutations on spore outgrowth and on vegetative growth. Spores were germinated in L-alanine and then resuspended in fresh medium for outgrowth. ●, wild type; □, *gerN*; ○, *gerT*. (A) Outgrowth in NB adjusted to pH 9.5. (B) Outgrowth in NB. (C) Outgrowth in NB plus 0.7 M NaCl. (D) Vegetative growth in NB, pH 9.5.

DISCUSSION

Based on the phenotypes of *B. cereus* mutants, the related proteins GerN and GerT, both of which are likely to have Na$^+$/H$^+$ antiport activity, have primary roles in germination and outgrowth, respectively, and as neither protein is required for NaCl resistance in vegetative cells, their role appears to be mainly in spore biology. There is some evidence that both *gerN* and *gerT* genes are expressed during sporulation; the *gerN* gene of *B cereus* is specifically sporulation expressed (20) at 4 hours after initiation of sporulation at a very low level; according to *lacZ* fusion data, its expression level is approximately 1/3 the level seen for the *gerI* operon and 1/100 that of *exsA*, which encodes a spore coat morphogenetic protein (1). A *lacZ* fusion to the *gerT* gene of *B. cereus* is not available, but the *gerT* gene of *B. anthracis* (BA 0819) has been reported in microarray data as late-sporulation expressed (14).

Role of GerT. Both GerN and GerT are members of the CPA2 monovalent cation:proton antiporter family of membrane transport proteins (18) and are homologs of the NapA family of proteins. The GerN protein behaves as a Na$^+$/H$^+$-K$^+$ antiporter and is essential for normal GerI receptor function, possibly in the restoration of local ion balance (21). As yet, the transport capabilities of GerT have not been directly measured, but the NaCl and alkali sensitivity of the *B. cereus gerT* mutant during spore outgrowth and the ability of the cloned *gerT* gene to improve the growth of a Na$^+$ transport-defective *E. coli* mutant in complementation studies both suggest a role in Na$^+$ efflux. As described above, there is evidence for *gerT* expression during late sporulation, and GerT must be present in dormant spores, as it is responsible for the phenotypic difference between *gerN* spores and *gerN gerT* double mutant spores in germination at a stage before de novo protein synthesis. Whether preexisting GerT protein in the germinated spore is sufficient for outgrowth or whether in normal cells *gerT* is expressed again during outgrowth is not known.

The GerN and -T proteins are significantly different in sequence and separately encoded in the genome. Consequently, the different roles of GerN and GerT proteins in germination could reflect differences in their ion transport activity or specificity, association with other proteins, or relative levels of protein. There are other reports that precise functions can vary between homologs in the NapA family of transport proteins (27).

The report that led to our initial study of GerN in *B. cereus* was that a related protein, GrmA, is required for germination in *Bacillus megaterium* ATCC 12872 (22). A recent report demonstrates that, in contrast, GrmA is not required for germination in the apparently equivalent *B. megaterium* strain QMB1551 (4). Caution needs to be exercised when different strains of a species are considered, as genomic differences in the spectrum of encoded and functional germinant receptors may be reflected in different germination properties; for example, the germination behaviors of *B. cereus* strains ATCC 14579 and ATCC 10876 show significant differences (12). It is not obvious why, even within a single species, one germinant receptor (GerI) might be strictly dependent on an ion antiporter like GerN, while other receptors are not. The analysis of GerT has not clarified this issue but has highlighted a second role for proteins of this family, this time in outgrowth.

Potential significance of GerT and GerN in the biology of the *B. cereus* family. Considering that it is a naive cell, encountering a new environment, the newly germinated spore will have to deal with environmental stress, for example, by salt or alkali, and it appears that the GerT protein in the *B. cereus* family is one protein that has been recruited to this role. For example, when spores germinate in the alkaline midgut of insects or germinate in alkaline or saline soils, the GerT protein may be an important factor contributing to the resumption of growth.

In an attempt to screen for potential germination defects in *gerT* mutant spores, we studied amino acid-enhanced germination in subgerminal alanine and inosine. This did not reveal any GerT function but demonstrated that GerN is important in any circumstances where the GerI receptor is implicated in germination. In *B. anthracis*, the GerH germinant receptor has been implicated in virulence (26). Apart from sequence differences in the long repeat region near the N termini of GerHA and GerIA, the GerH proteins are almost identical in amino acid sequence to the GerI proteins of *B. cereus* ATCC 10876. Unlike *B. cereus*, *B. anthracis* does not germinate in inosine as the sole germinant; the absence of *gerQ* in the *B. anthracis* genome may explain this failure, so one does not need to invoke a significant difference in function between the GerI and GerH receptors. We predict, therefore, that GerN is likely to be required in *B. anthracis* for GerH receptor function. This is supported by recent evidence (6) that *gerH*, *gerN*, and *gerT* mutants were all enriched in large-scale screens designed to detect defects in sporulation, germination, or outgrowth of *B. anthracis*.

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