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Identification and Characterization of the *gerH* Operon of *Bacillus anthracis* Endospores: a Differential Role for Purine Nucleosides in Germination

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We identified a tri-cistronic operon, *gerH*, in *Bacillus anthracis* that is important for endospore germination triggered by two distinct germination response pathways termed inosine-His and purine-Ala. Together, the two pathways allow *B. anthracis* endospores a broader recognition of purines and amino acids that may be important for host-mediated germination.

Bacterial endospores are metabolically inactive and are capable of surviving extended periods of time under harsh environmental conditions but germinate rapidly in the presence of small molecules termed germinants (2). Work by Hachisuka demonstrated that the addition of exogenous adenosine and L-alanine was required for in vivo germination of *Bacillus anthracis* in the rat peritoneal cavity, thus establishing nucleosides as potentially contributing to in vivo germination (5). Germination is characterized by the hydration of the core and the breakup of the endospore cortex, though the molecular mechanisms underlying these activities remain undetermined (2, 9). Endospore germination results in the expulsion of Ca^{2+} and dipicolinic acid and the initiation of metabolic activity (11). Nutrient-triggered endospore germination is facilitated by *ger* operons, which are believed to encode germinant sensor proteins (9).

Identification and characterization of *gerH* in *B. anthracis*. In *Bacillus cereus*, *gerI* (*gerIABC*) is necessary for the triggering of germination by inosine and the disruption of *gerIA* or *gerIB* abolishes inosine-triggered germination (1). A BLAST search of the *B. anthracis* genome (http://www.tigr.org) with *gerIA*, *gerIB*, and *gerIC* from *B. cereus* (GenBank accession number AF067645) identified homologs in *B. anthracis* referred to here as *gerHA*, *-B*, and *-C* (open reading frames 02625, 02624, and 02623, respectively). The putative proteins encoded by *gerHA*, *gerHB*, and *gerHC* have 78, 92, and 89% amino acid identity, respectively, to their *B. cereus* GerI homologs. Unlike with *B. cereus*, inosine alone does not trigger *B. anthracis* endospore germination but acts as a potent cogerminant with several amino acids (Table 1) (1, 6).

To determine a role for *gerH* in *B. anthracis* germination, a *gerHA*-null strain was constructed from the Sterne 34F2 ($pXO1^+$, $pXO2^-$) strain by using forward and reverse primers with 5′ *Xma*I restriction sites (5′-TCCCCCCGGGCAAGAA GGTTTTGTAGAGGA-3′, 5′-TCCCCCCGGGGATTGCAT AGGCTTTTTAAC-3′) to PCR amplify *gerHA* DNA (1.7 kb), which was cloned into pUC19 (New England Biolabs) and maintained in *Escherichia coli* XL1 Blue (American Type Culture Collection). A central section of *gerHA* (Δ*gerHA*) was deleted and replaced by an erythromycin cassette, which had been amplified from pDG641 (*Bacillus* Genetic Stock Center), at the *gerHA* *Cla*I restriction site with appropriate primers (5′-CCATCGATGGGCGGTGTAGATGTTGATGA-3′, 5′-C CATCGATGGACATGCTACACCTCCGGATA-3′) (3). The resulting construct was transferred into the gram-positive shuttle vector pKSV7, creating pKSV7:Δ*gerHA*:*Erm*, and maintained in *E. coli* GM272 (*Bacillus* Genetic Stock Center) (10). Electroporation of *B. anthracis* Sterne 34F2 was performed with polyethylene glycol-precipitated plasmid DNA (8). Transformants were plated on selective medium, and *B. anthracis* Sterne *gerHA*-null strains were obtained via allelic exchange, after curing of the plasmid vector containing wild-type *gerHA* (8). The identity of the null construct was confirmed by PCR and by Southern blotting.

Germinant surveys with an L-alanine–amino acid combination versus an inosine-amino acid combination. *B. anthracis* Sterne and *gerHA*-null endospores were preradiolabeled with ^{45}Ca as described previously but with modified G medium (6, 7). Parental and *gerHA*-null strains exhibited similar vegetative growth kinetics (data not shown). The percentage of germination was measured as the percentage of ^{45}Ca released from spores relative to the total amount of ^{45}Ca contained in a sample (6, 12). Next, the binary combinations of germinants known to trigger germination in *B. anthracis* were tested to compare profiles of wild-type and *gerHA*-null strain phenotypes (Tables 1 and 2) in MES (morpholineethanesulfonic acid) buffer at pH 8.0 by using 10^6 endospores/ml of germinant solution. Slight differences between the Sterne germination profiles reported here and those for ΔSterne ($pXO1^-$, $pXO2^-$) reported previously may result from strain differences or from slight variations in experimental conditions (4, 6). In our studies, MES buffer was used to minimize germination enhancement by monovalent ions. The *gerH* locus was required for germination with inosine-His, inosine-Met, inosine-Phe, inosine-Tyr, inosine-Val, and Ala-Tyr (Tables 1 and 2). The loss of Ala-Tyr-triggered germination in *gerHA*-null spores indicated that *gerH* also influenced a non-nucleoside-dependent

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TABLE 1. Germination of *B. anthracis* Sterne 34F2 endospores in a subgerminal concentration of L-alanine plus L-amino acids

Germinant(s)[a]	% Germination in 60 min[b]	
	Parental (34F2) strain	Δ*gerHA* strain
Alanine	0.7 ± 0.2	1.0 ± 0.1
Alanine-His	21.5 ± 3.4	15.2 ± 8.8
Alanine-Pro	38.4 ± 2.8	29.2 ± 0.0
Alanine-Trp	2.2 ± 1.8	0.8 ± 0.5
Alanine-Tyr	7.1 ± 0.8	0.2 ± 1.5

[a] All amino acids were L-isomers. Alanine was at 1 mM, as were Trp and Tyr. His and Pro were at 100 mM. Alanine concentrations of 1 mM and below are subgerminal.

[b] Values are the averages of results of duplicate experiments with two independent preparations. The experimental error is 1 standard deviation from the mean.

germination pathway (Table 1). Therefore, *B. anthracis gerH* facilitates germination via an inosine-amino acid pathway and, to a lesser extent, via an Ala–aromatic-amino-acid pathway. The presence of an aromatic ring structure is required for *gerH*-mediated germination, and Tyr cannot substitute for inosine with any amino acids other than Ala (6).

Germinant studies indicate the testing of purine promiscuity with L-alanine but not with histidine. The degree to which purines could be substituted for each other with an amino acid cogerminant was determined for *gerHA*-null and Sterne endospores. Purine cogerminants (adenosine, guanosine, ATP, GTP, ITP) were substituted for inosine with 1 mM Ala in parental and *gerHA*-null strains. Each triggered germination to similar levels, with the sole exception of GTP plus Ala, with which the *gerHA*-null endospores responded more dramatically than did parental endospores (Fig. 1A). It is possible that a nonproductive interaction that interferes with GTP-Ala-triggered germination in parental, but not *gerHA*-null, endospores occurs between GTP and GerH proteins.

The requirement of inosine for purine-His-triggered germination is absolute. Replacing inosine with any other purine in combination with His (10 mM) resulted in a total loss of germination (Fig. 1B). The ability of the *gerHA*-null strain to germinate via the purine-alanine pathway shows that *gerH* is not required for an inosine-based response to a subgerminal concentration of alanine. These data demonstrate, for the first time, differential responses to purines of *B. anthracis* endospores depending on the amino acid supplied as a cogerminant. The purine-Ala pathway exhibited a higher degree of purine promiscuity than the inosine-His pathway. The purines recognized by the purine-Ala pathway and the amino acids recognized by the inosine-His pathway allow a broad recognition of germinants that likely helps *B. anthracis* endospores recognize varieties of hospitable environments in which to germinate.

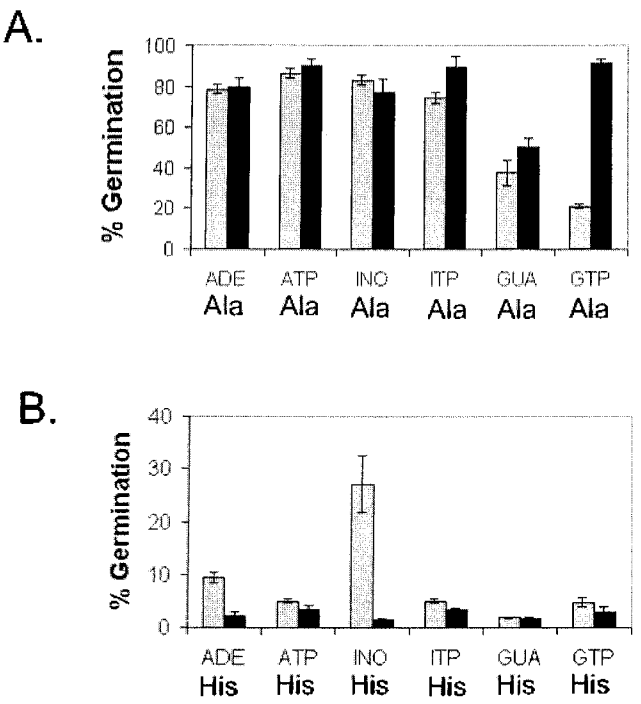

FIG. 1. *B. anthracis* Sterne (parental strain; grey bars) and *gerHA*-null (black bars) endospore germination responses to a subgerminal concentration of alanine (A) and histidine (B) with purine nucleosides and nucleoside triphosphates. We used a subgerminal concentration of L-alanine (1 mM) (A) and a 10 mM concentration of histidine (B) with a 1 mM concentration of adenosine (ADE), inosine (INO), guanosine (GUA), ATP, ITP, or GTP. Purines and amino acids alone at the concentration used stimulated no endospore germination. Percent germination was calculated at 90 min as described in the text. Experiments were performed at pH 8 with 10 mM MES. Each experiment was performed in triplicate with three independent endospore preparations. Experimental error was calculated as 1 standard deviation from the mean.

TABLE 2. Germination of *B. anthracis* Sterne 34F2 in inosine plus L-amino acids

Germinant(s)[a]	% Germination in 60 min[b]	
	Parental (34F2) strain	Δ*gerHA* strain
Inosine	0	0
Inosine-Ala	85.0 ± 7.5	76.9 ± 6.6
Inosine-Cys	0.5 ± 1.2	0.6 ± 1.0
Inosine-His	51.9 ± 2.1	0.3 ± 0.4
Inosine-Met	17.4 ± 1.4	0.3 ± 0.9
Inosine-Phe	23.6 ± 6.3	1.2 ± 0.2
Inosine-Pro	5.9 ± 0.8	0.3 ± 0.7
Inosine-Ser	9.6 ± 1.4	2.3 ± 1.7
Inosine-Trp	1.1 ± 1.3	0.1 ± 0.3
Inosine-Tyr	16.2 ± 2.9	0.6 ± 0.4
Inosine-Val	8.5 ± 0.9	1.0 ± 0.4

[a] All amino acids were L-isomers at a concentration of 100 mM, except tyrosine and tryptophan, which were at 1 mM. Amino acids alone, in the absence of inosine, did not stimulate germination.

[b] Values are the averages of results of duplicate experiments with two independent preparations. The experimental error is 1 standard deviation from the mean.

Recently, the *gerS* operon in *B. anthracis* was characterized and was found to mediate the germination of *B. anthracis* endospores by germinants containing aromatic ring structures (6). Disruption of the *gerS* locus results in germinant profiles similar to that of the *gerH*-null strain. A functional relationship may also exist between *gerS* and *gerH*, though that relationship remains undefined. If the two loci are redundant, the loss of one should not abolish germination in response to a pair of cogerminants. Alternatively, it is possible that GerH and GerS

are functionally redundant and that together they provide a critical number of germinant sensors required to facilitate germination.

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