Macrophage-Mediated Germination of *Bacillus anthracis* Endospores Requires the *gerH* Operon

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The *gerHABC* operon of *Bacillus anthracis*, encoding a *gerA*-like family member of germinant sensors, was shown to be required for endospore germination in the presence of macrophages and in macrophage-conditioned media. The loss of the germination phenotype in macrophage cultures of *B. anthracis* *gerH*-null endospores was restored by complementation *in trans* with a wild-type copy of *gerH* expressed under the control of its own promoter. Using endospores from both the parental strain *B. anthracis* Sterne and an isogenic *gerH*-null strain, we partially characterized germinants secreted by macrophages into the extracellular medium.

Bacillus anthracis endospores can persist for long periods in soil but, upon entering a host, germinate quickly into vegetative bacilli that grow and cause disease (4). Endospores germinate inside of or proximal to phagocytes. These cells are believed to be *de facto* sites of germination and contribute to the establishment of *B. anthracis* infection, as phagocytes are the primary means of bacterial access to the body in cases of inhalation anthrax (6, 8, 10, 20). Several recent studies examined the fate of *B. anthracis* endospores in macrophages (M ϕ). Guidi-Rontani et al. showed that murine alveolar phagocytes ingest endospores that then germinate and grow into vegetative bacilli but do not replicate (6). Welkos et al. observed intact endospores and recently germinated nascent bacilli within phagosomes in M ϕ that are subsequently destroyed by these immune cells (26, 27, 28). Work by Dixon et al. indicated that newly vegetative bacilli escape from the phagocytic vesicles of the M ϕ where they can replicate freely in the host cell cytoplasm and release themselves from the M ϕ (3). Though there are important differences among these researchers' observations of the ultimate fates of phagocytosed endospores, it is generally agreed that germinant sensors recognize and bind small-molecule germinants and initiate the germination cascade (17). These sensors belong to the GerA family of germinant receptors, which are best characterized in *Bacillus subtilis* but which have also been studied in other *Bacillus* spp. (2, 5, 15, 16, 18, 19, 29, 30).

Six tricistronic *gerA*-like chromosomal loci (*gerAA*, *gerAB*, *gerAC*) plus the *gerX* locus carried on pXO1 exist in *B. anthracis*; these loci encode germinant recognition proteins (6, 10, 11, 25). The *gerS* operon in *B. anthracis* mediates germination in response to aromatic ring structures and is required for germination in the presence of cultured M ϕ (11, 12). The *gerH* operon also mediates germination in response to aromatic ring structures and germination triggered by inosine (Ino) in combination with amino acids (25). The germinants recognized by

the *gerX* operon on pXO1 have not yet been identified, although Δ Sterne strains lacking pXO1 do not exhibit any gross germination defects *in vitro* (11, 12). Endospores from a *gerX*-null strain, identified by Guidi-Rontani et al., germinates slightly less well than do Sterne endospores in the presence of M ϕ (6, 12). One *ger* locus has homology to the *gerL* locus of *Bacillus cereus* (alanine response) (1), and another has homology to the *gerA* locus of *B. subtilis* (alanine response) (15, 29) but has frameshift mutations in the *b* and *c* moieties. The remaining two loci have yet to be investigated.

In the host, germination of *B. anthracis* endospores constitutes the first stage of establishment during infection. It is essential for *B. anthracis* to recognize an appropriate host environment in order for germination to occur and for infection to proceed. The *gerHABC* locus is required for germination with the following combinations of cogerminants: Ino-His, Ino-Met, Ino-Phe, Ino-Tyr, and Ino-Val, which collectively are referred to as the Ino-His germination response pathway (25). The previous findings that adenosine acts as a cogerminant with L-alanine and is important for the germination of *B. anthracis* both *in vitro* and in the rat peritoneal cavity further implicate purines in the establishment of anthrax (7, 9, 24). We report here that *gerH* is required for *B. anthracis* endospore germination in the presence of cultured M ϕ , and we identify M ϕ -secreted germinants that may trigger this response.

MATERIALS AND METHODS

Bacterial strains, plasmids, and cloning. *B. anthracis* Sterne 34F2 (containing plasmid pXO1 but not plasmid pXO2) and the isogenic *gerH*-null strain were cultured for vegetative growth in or on brain heart infusion (BHI) broth (Difco) or agar at 37°C unless otherwise specified (25). The gram-positive shuttle vector pKSV7 (the kind gift of N. Freitag, Seattle Biomedical Research Institute) was maintained in *Escherichia coli* (21). The *gerHA*-null strain of *B. anthracis* was complemented by cloning the full-length *gerH*, including the upstream sequence, from *B. anthracis* into the gram-positive shuttle vector pKSV7, which was maintained in *E. coli* by using the following forward and reverse primers containing *Pst*I and *Hind*III restriction sites, respectively: 5'-AACTGCAGTTTTCGGATT AATTGATTTTCGGCCC-3' and 5'-CCCAAGCTTGGGAGAGGAGTTCAAT CATTTCATTCATCA-3'.

Plasmid pKSV7:*gerH* was passaged through *E. coli* GM272 (Bacillus Genetic Stock Center) and purified with a Plasmid Maxi Kit (Qiagen). DNA was precipitated with polyethylene glycol overnight at 4°C (30% [wt/vol] polyethylene glycol 8000 in 1.6 M NaCl), recovered by centrifugation at 10,000 $\times$ g for 20 min, and

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washed two times with 70% ice-cold ethanol. The pellet was resuspended in 1 ml of Tris-EDTA buffer. Electroporation of *B. anthracis* Sterne 34F2 was performed according to the protocol previously described by Koehler et al. (14). Cells were plated on BHI agar with 10 µg of chloramphenicol/ml and 1 µg of erythromycin/ml and incubated at 30°C for 36 h. Transformant colonies were picked and inoculated into 4 ml of BHI agar with chloramphenicol and erythromycin (10 and 1 µg/ml, respectively) and incubated for 8 h at 30°C with gentle shaking. The presence of the *B. anthracis gerH*-null strain carrying pKSV7:*gerH* was confirmed by PCR with primers for both wild-type and *gerHA*-null copies of *gerH*.

Endospore preparation and radiolabeling. *B. anthracis* Sterne endospores were radiolabeled by sporulating the bacilli in modified G medium [0.2% yeast extract, 0.0025% CaCl₂ dihydrate, 0.05% K₂HPO₄, 0.02% MgSO₄ heptahydrate, 0.005% MnSO₄ quatrhydrate, 0.0005% ZnSO₄ dihydrate, 0.0005% CuSO₄ pentahydrate, 0.00005% FeSO₄ heptahydrate, 0.2% (NH₄)₂SO₄] with the addition of 20 µCi of ⁴⁵CaCl₂ per 25 ml (21 mCi/mg; ICN Chemicals) as described previously (13, 25). Radiolabeling does not affect any biological activity associated with endospores (11, 12, 25). Endospore preparations were heated at 65°C for 30 min to kill vegetative bacilli and then washed 10 times with sterile H₂O to remove vegetative debris. Endospore preparations were determined to be of >99% purity with no detectable vegetative bacilli or debris via phase-contrast microscopy.

Germination assays. Germination assays measured the amount of ⁴⁵Ca released from endospores and were performed at a concentration of 10⁶ endospores/ml of germinant solution, unless otherwise noted. The total release of calcium and dipicolinic acid from the core of the endospore signaled its irreversible commitment to germinate (22). Measurement of the amount of free ⁴⁵Ca in a mixture of prelabeled spores and germinant solution, relative to the total amount of ⁴⁵Ca in a sample, resulted in a quantifiable and directly proportional measure of germination (11, 12, 23). *B. anthracis* endospores were mixed with germinant solutions at room temperature (19 to 21°C) or at 37°C. At the time points indicated (see Fig. 2), 400 µl of the mixture was removed and pressed through a 0.2-µm-pore-size Nylon Acrodisc syringe filter (Gelman Laboratory), which allowed the passage of free ⁴⁵Ca and the retention of any ⁴⁵Ca still associated with intact spores. The filtrate was mixed with 3 ml of BioSafe II liquid scintillation fluid and measured in a scintillation counter (Beckman Instruments) with a ⁴⁵Ca window for 30 s. Samples were run in triplicate with three independent endospore preparations. The percentage of calcium released (percent germination) was calculated by the following formula: $Y = [(X - C)/(M - C)] \times 100$, where *Y* is the percentage of ⁴⁵Ca released, *X* is the radioactivity detected (in counts per minute) in the filtered sample at a given time, *C* is the radioactivity detected in a filtered sample at time zero (background radiation, usually under 100 cpm), and *M* is the radioactivity detected in an unfiltered sample of the germination mixture. The specific activity of typical endospore preparations was 4,000 to 6,000 cpm/10⁶ spores.

Macrophage cell culture. RAW 264.7 murine (ATCC TIB-71) Mφ-like cells and J774A.1 (ATCC TIB-67) Mφ-like cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (Gibco BRL) at 37°C with 5% CO₂ in a humidified incubator. For germination assays, Mφ were washed twice with prewarmed serum-free minimal essential medium (MEM) or Hanks balanced salt solution (Hanks), resuspended in MEM or Hanks, and counted with a hemocytometer (Hausser Scientific). For cytochalasin D (Sigma) assays, Mφs were washed twice with MEM plus cytochalasin D (2 µg/ml) prior to being resuspended in fresh MEM plus cytochalasin D (2 µg/ml) with a final Mφ concentration of 2 × 10⁶ cells per ml. Conditioned medium was obtained at desired times by incubating Mφ in appropriate experimental media in six-well tissue culture plates (Costar) at 37°C with 5% CO₂.

RESULTS

***B. anthracis gerH* is required for Mφ-related endospore germination.** Differences between the germination of parental endospores and that of *gerH*-null endospores in Mφ cultures establish the degree of importance of *gerH* in cell-associated germination. *B. anthracis* Sterne parental endospores germinated completely in 60 min in RAW 264.7 Mφ cultures but not in MEM alone (Fig. 1). Conversely, *gerHA*-null endospores did not germinate in either Mφ culture or in MEM alone (Fig. 1A). Germination of the null strain in Mφ culture was rescued by complementation with wild-type *gerHABC* expressed under

A.

B.

FIG. 1. *B. anthracis* Sterne endospores require *gerH* for germination in macrophage cultures. (A) RAW 264.7 Mφ were infected with ⁴⁵Ca-radiolabeled *B. anthracis* Sterne 34F2 (black bars), the *gerH*-null strain carrying pKSV7:*gerH* (open bars), and *gerH*-null endospores (grey bars). (B) Germination experiments were performed with ⁴⁵Ca-radiolabeled 34F2 endospores in cultures of J774A.1 (grey bars) and RAW 264.7 (black bars) Mφ that were incubated in MEM with or without the addition of cytochalasin D. For all experiments, Mφ were infected with endospores and incubated in MEM at 37°C with 5% CO₂ for 60 min. After 60 min, the percentage of endospore germination was calculated as described in the text. Each experiment was performed in triplicate with at least two separate preparations of labeled endospores, and results are reported with an error of ±1 standard deviation of the mean.

the control of its endogenous promoter from plasmid pKSV7 (Fig. 1A).

Treatment of Mφ cultures with cytochalasin D prior to in vitro infections with endospores inhibits phagocytosis and facilitates the study of extracellular germination in the presence of Mφ. Cytochalasin D inhibits actin polymerization and, therefore, any actin-dependent vesicle transport in eukaryotic cells (including phagocytosis). In studies with cytochalasin D, the extracellular germination of endospores suggested that Mφ secrete some germinants by actin-independent mechanisms. These studies were restricted to extracellular germination because no endospores were taken up by Mφ under these exper-

imental conditions. Findings indicate that parental endospores germinated completely and equally in cultures of both J774A.1 and RAW 264.7 M ϕ , with or without cytochalasin D (Fig. 1B). As predicted, no germination occurred in MEM alone, with or without cytochalasin D (Fig. 1B). Since MEM did not trigger germination of *B. anthracis* endospores, germination resulted from the conditioning of the MEM by M ϕ . Because cytochalasin D broadly inhibits actin polymerization, we conclude that the conditioning of the extracellular medium by the M ϕ occurred via actin-independent pathways.

Complete conditioning of the extracellular medium is dependent on both M ϕ cell density and time. The manner by which M ϕ condition their extracellular environment with germinants was investigated by using different concentrations of M ϕ and by performing a kinetic analysis of endospore germination in M ϕ cultures. Germination experiments with 10^6 parental endospores/ml and RAW 264.7 M ϕ densities ranging from 0.1×10^6 to 3×10^6 /ml revealed a concentration-dependent germination response. A minimum of 0.4×10^6 M ϕ /ml was required to trigger germination above baseline in 60 min (Fig. 2A). A M ϕ density of 3×10^6 /ml triggered the germination of 93% of the parental endospores in 60 min (Fig. 2A). After several hours, the majority of endospores could be observed floating freely in the extracellular medium, as endospores do not settle out of solution over the time course studied without centrifugation (data not shown). Thus, these experiments included extracellular germination, even without the addition of cytochalasin D to the M ϕ cultures.

An investigation of the kinetics of germination in M ϕ cultures revealed a time-dependent conditioning of the extracellular medium. If a low, initially subgerminal, density of M ϕ acquires with time the ability to trigger germination of endospores, then it is possible that M ϕ secrete active germinants into the medium. We found that an initially subgerminal M ϕ cell density of 0.2×10^6 /ml (Fig. 2A) became capable of conditioning the medium with time (Fig. 2B). Additionally, a time course of germination with parental endospores determined with a M ϕ density of 0.2×10^6 /ml indicated that germination above baseline begins after 2 h and reaches 93% after 5 h of incubation. In cultures with a M ϕ density of 2×10^6 /ml, the total percentage of germination was unchanged when the multiplicity of infection (MOI) was increased from 0.5 (10^6 endospores/ml) to 10 and decreased only slightly at an MOI of 50 (Fig. 2C). This finding indicates that there are abundant levels of germinants in conditioned medium and that endospore density does not significantly affect germination at the concentrations tested. Together, the data in Fig. 2 demonstrated that M ϕ condition media steadily over time, that higher densities of M ϕ will condition media faster than lower densities, and that the ability of endospores to germinate in conditioned media does not relate to the MOI used in the experiment.

Evidence that M ϕ secrete a purine required for *gerH*-mediated germination. Our initial report (25) on *gerH* characterized the profile of the binary pairs of cogerminant molecules that trigger in vitro germination of the *gerH*-null strain relative to that of the parental Sterne strain of *B. anthracis*. That report described the Ino-His germination response of *B. anthracis*, which requires *gerH*, in which neither adenosine nor guanosine can substitute for Ino (25). In contrast, Ino can be replaced by adenosine and guanosine in the Ino-Ala germination response

FIG. 2. Effects of M ϕ density, time, and MOI on the germination of *B. anthracis* Sterne. (A) Effect of M ϕ cell density. RAW 264.7 M ϕ (0 to 3×10^6 /ml) in MEM were added to wells of a 24-well tissue culture plate and infected with 10^6 endospores in a final volume of 1 ml. The plates were incubated at 37°C with 5% CO₂ for 60 min. After 60 min, the percentage of endospore germination was calculated as described in the text. (B) Time course. A subgerminal concentration of RAW 264.7 M ϕ (0.2×10^6 /ml) was infected at an MOI of 5:1 with Sterne endospores to a final endospore concentration of 10^6 /ml (black squares). The M ϕ were incubated at 37°C with 5% CO₂, and measurements of the percentage of germination of radiolabeled endospores were taken hourly for 5 h. Germination assays performed with MEM alone served as a negative control (white squares). (C) Effect of MOI. The effect of the MOI on endospore germination was measured by using RAW 264.7 M ϕ (2×10^6 /ml) and different concentrations of endospores. The M ϕ were incubated at 37°C with 5% CO₂ for 60 min. After 60 min, the percentage of endospore germination was calculated as described in the text. Each experiment was performed in triplicate with at least two separate preparations of labeled endospores, and results are reported with an error of ± 1 standard deviation of the mean.

FIG. 3. Partial characterization of M ϕ -secreted germinants in Hanks with *B. anthracis* Sterne (black bars) and *gerH*-null endospores (grey bars). (A) Hanks was used alone or was supplemented with 1 mM Ala, 100 mM His, or 100 mM Ino. (B) Hanks was conditioned with 2×10^6 M ϕ /ml for 60 min and then passed through a sterile filter as described in the text. Ala, His, or Ino was added to conditioned Hanks at the concentrations listed above. Radiolabeled endospores were added to a final concentration of 10^6 endospores/ml and were incubated at room temperature for 60 min. After 60 min, the percentage of endospore germination was calculated as described in the text. Each experiment was performed in triplicate with at least two separate preparations of labeled endospores, and the results are reported with an error of ± 1 standard deviation of the mean.

pathway. Therefore, Ino-His and Ino-Ala represent distinct germination pathways (25). Additionally, germination of *B. anthracis* endospores can occur in L-Ala alone at concentrations of 100 mM in water and in L-Ala at concentrations as low as 1 mM in certain salt solutions (11, 25) (Fig. 3A). Our finding that *gerH* is required for germination in M ϕ cultures suggests an important role for the Ino-His germination response pathway. If M ϕ secrete purines or amino acids that participate in the Ino-His germination response pathway, then the addition of a complementary exogenous cogerminant should trigger germination. Because amino acid and nucleoside cogerminant profiles of *B. anthracis* are known, we may then infer the nature of M ϕ -secreted germinants from the identity of the defined cogerminant added to the solution.

To characterize germinants secreted by RAW 264.7 M ϕ , cells were washed thoroughly with Hanks and incubated in fresh Hanks for 3 h to allow M ϕ conditioning of this buffer,

which was then passed through a sterile filter and used in germination assays (as described above). Hanks is a salt solution that does not contain any germinant molecules. Unconditioned Hanks or conditioned Hanks alone did not trigger endospore germination, while the addition of 1 mM L-alanine to either solution did, as expected (Fig. 3). Germination studies of parental and *gerH*-null endospores performed with conditioned Hanks supplemented with histidine or Ino (100 and 1 mM, respectively) identified cogerminants that pair effectively with M ϕ -secreted germinants. Conditioned Hanks, supplemented with His, triggered the germination of parental endospores in 60 min (Fig. 3B), in contrast to unconditioned Hanks with His, which did not (Fig. 3A). His participates in two distinct germination pathways: Ino-His and Ala-His (11, 25). Conditioned Hanks, supplemented with His, did not trigger germination in *gerH*-null endospores (Fig. 3B). *gerH*-null endospores maintain an intact Ala-His germination response but lack the Ino-His germination response (25). We can conclude from these data that Ino is likely to be one of the cogerminants secreted by M ϕ because of the inability of other purines to substitute for Ino in the Ino-His germination pathway (25). Additionally, since purines act as cogerminants for *B. anthracis* endospores only in the presence of specific amino acids (11, 25), germination in Hanks supplemented with Ino would indicate the secretion of at least one amino acid by M ϕ . However, the possibility also exists that the secreted molecules may be novel germinants that have yet to be identified. We report here that conditioned Hanks supplemented with Ino triggered germination in both parental and *gerH*-null endospores, while unconditioned Hanks supplemented with Ino did not (Fig. 3). These data strongly suggest that M ϕ also secrete, along with Ino, at least some amino acids into the extracellular medium.

By using different cogerminants of *B. anthracis* endospores that trigger germination only when in binary combination with a specific cogerminant, we were able to report here that M ϕ secrete a purine, which is likely Ino, and at least one or more of several candidate amino acids. The decrease of approximately 40% in the germination of *gerH*-null endospores relative to that of the parental endospores in conditioned Hanks with Ino is likely to be due to the absence of the Ino-His germination response pathway controlled by the *gerH* operon (Fig. 3B). The lack of germination in conditioned Hanks alone suggests that the concentrations of the secreted cogerminants are too low under the experimental conditions measured to trigger germination or that the correct binary pairs of cogerminants do not exist in the secreted medium. Because we used endospore germination in conditioned Hanks as our measure of a secreted cogerminant, these findings suggest a functional role for Ino and the other cogerminants *in vivo* that may be sensed by *gerH*-encoded proteins.

DISCUSSION

The absence of germination of *B. anthracis* *gerH*-null endospores in M ϕ cultures and in M ϕ -conditioned medium demonstrates that the germination operon *gerH* is required for M ϕ -mediated germination. M ϕ secrete one or more cogerminants that fulfill the role that Ino plays *in vitro*, with respect to *gerH*-dependent germination, in the presence of His. The *gerH*-dependent Ino-His germination response is absent in *gerH*-null

endospores (25). This absence is likely responsible for the loss of the ability of the mutant to germinate in M ϕ cultures and in M ϕ -conditioned medium. M ϕ may also contribute amino acids to the extracellular medium in sufficiently high concentrations to perform as cogerminants when exogenous Ino is added to the medium. The data reported here argue against the theory that M ϕ condition the extracellular medium by consuming inhibitors of germination because it is highly unlikely that Hanks contains any molecular species that can affect endospore germination.

Several previous studies implicate alveolar M ϕ as the de facto sites of *B. anthracis* endospore germination although, in vitro, other cell types can fulfill this role (3, 6, 20). While *B. anthracis* endospore germination appears to occur inside of M ϕ in vivo, it remains unclear whether this process can occur extracellularly in an animal. The data reported here suggest that *B. anthracis* endospores may be able to germinate extracellularly in vivo in a position proximal to phagocytes prior to their uptake. Endospores can germinate extracellularly in vitro in MEM conditioned by M ϕ . Electron micrographs created by Dixon et al. and the work of Welkos et al. have recently shown both intact endospores and recently germinated endospores within M ϕ phagosomes (3, 26, 27, 28). Welkos et al. reported a M ϕ sporocidal activity associated with RAW 264.7 cells that we did not observe. The *gerH*-null strain may be useful for addressing the specific question of sporocidal activity since these endospores do not germinate in the presence of M ϕ . The ⁴⁵Ca radiolabel is retained by these endospores, which indicates that they are structurally intact, and serves as a marker for viability. These endospores can be recovered and grown on BHI agar. It is possible that the sporocidal activity reported previously is the result of endospores germinating immediately prior to being taken up by the M ϕ , which then kills a nascent vegetative cell and not an intact endospore.

It is becoming increasingly apparent that *B. anthracis* endospore germination is a multifactorial event. Like *gerHABC*, the germination operon *gerSABC* is also required for M ϕ -related germination (12). The addition of D-alanine, a potent inhibitor of the Ala germination response, also inhibits germination in M ϕ cultures (data not shown) and implicates Ala in M ϕ -mediated germination. In other studies, the disruption of the pXO1-carried *gerX* partially attenuates endospore germination in M ϕ (6), although Δ Sterne endospores lacking pXO1 respond to all known germinants and are fully capable of germinating in M ϕ cultures and in mice (11, 26, 27, 28). Collectively, these studies suggest that *B. anthracis* endospores rely on multiple chromosomally encoded *ger* operons to recognize complex patterns of discrete chemical cogerminants at low concentrations. While the Ino-His germination pathway is required for M ϕ -related germination, this path is likely to be only one of a variety of germination response pathways contributing to M ϕ -related germination. Therefore, the term "M ϕ -mediated germination" may refer to germination in the presence of a variety of germinants secreted by host cells in low concentrations rather than to germination in response to germinants provided only by M ϕ and not by any other source.

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