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

Matthew A. Weiner and Philip C. Hanna*

Department of Microbiology and Immunology, University of Michigan Medical School, Ann Arbor, Michigan

Received 31 January 2003/Returned for modification 25 March 2003/Accepted 11 April 2003

The gerHABC operon of *Bacillus anthracis*, encoding a *ger*-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.

*Corresponding author. Mailing address: Department of Microbiology and Immunology, University of Michigan Medical School, 5641 Med. Sci. II, Box 0620, Ann Arbor, MI 48104. Phone: (734) 615-3706. Fax: (734) 764-3562. E-mail: pchanna@umich.edu.

*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 (Mφ). 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 Mφ 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 Mφ where they can replicate freely in the host cell cytoplasm and release themselves from the Mφ (3). Though there are important differences among these researchers’ observations of the ultimate fates of phagocyted 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 *ger*-like chromosomal loci (*gerA*, *gerB*, *gerC*) 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 Mφ (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 ΔSterne 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 Mφ (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 n 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 Mφ, and we identify Mφ-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 pKS7V (the kind gift of N. Freitag, Seattle Biomedical Research Institute) was maintained in *Escherichia coli* (21). The gerH-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 pKS7V, which was maintained in E. coli by using the following forward and reverse primers containing PstI and HindIII restriction sites, respectively: 5′-AAGGTACCAATGGTATCTTTGCAGATTATTAGTTTGCCCGCCGCC-3′ and 5′-CTCTATCGGACGGAGGAGTTGATCATTTCTCATTCA-3′. Plasmid pKS7V/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 × g for 20 min, and...
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 Kochler et al. (14). Cells were plated on BHI agar with 10 μg of chloramphenicol/ml and 1 μg of erythromycin/cm² 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 gerH-null copies of gerH.

**Endospore preparation and radiolabeling.** B. anthracis Sterne endospores were prepared 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 quaterhydrate, 0.0005% ZnSO₄ dihydrate, 0.0005% CaSO₄ pentahydrate, 0.00005% FeSO₄ heptahydrate, 0.2% (NH₄)₂SO₄) with the addition of 20 μCi of ⁴⁵CaCl₂ per 25 ml (21 mCi/g; 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 and incubated at 30°C for 36 h. Transformant colonies were picked and plated on BHI agar with 10°Eagle’s modiﬁed G medium [0.2% yeast extract, 0.00005% FeSO₄ heptahydrate, 0.2% (NH₄)₂SO₄] with the addition of 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.

**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, gerH-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 the control of its endogenous promoter from plasmid pKSV7 (Fig. 1A).

Treatment of Mφ cultures with cytochalasin D prior to infection 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 experi-
imental conditions. Findings indicate that parental endospores germinated completely and equally in cultures of both J774A.1 and RAW 264.7 Mø, 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 Mφ. Because cytochalasin D broadly inhibits actin polymerization, we conclude that the conditioning of the extracellular medium by the Mφ occurred via actin-independent pathways.

Complete conditioning of the extracellular medium is dependent on both Mφ cell density and time. The manner by which Mφ condition their extracellular environment with germinants was investigated by using different concentrations of Mφ and by performing a kinetic analysis of endospore germination in Mφ cultures. Germination experiments with 10⁶ parental endospores/ml and RAW 264.7 Mø densities ranging from 0.1 × 10⁶ to 3 × 10⁸/ml revealed a concentration-dependent germination response. A minimum of 0.4 × 10⁶ Mφ/ml was required to trigger germination above baseline in 60 min (Fig. 2A). A Mφ density of 3 × 10⁶/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 Mφ cultures.

An investigation of the kinetics of germination in Mφ cultures revealed a time-dependent conditioning of the extracellular medium. If a low, initially subgerminal, density of Mφ acquires with time the ability to trigger germination of endospores, then it is possible that Mφ secrete active germinants into the medium. We found that an initially subgerminal Mφ cell density of 0.2 × 10⁶/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 Mφ density of 0.2 × 10⁶/ml indicated that germination above baseline begins after 2 h and reaches 93% after 5 h of incubation. In cultures with a Mφ density of 2 × 10⁷/ml, the total percentage of germination was unchanged when the multiplicity of infection (MOI) was increased from 0.5 (10⁶ 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 Mφ condition media steadily over time, that higher densities of Mφ 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 Mφ 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...
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 MΦ-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 MΦ 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 MΦ. 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 MΦ 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 MΦ 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 MΦ cultures and in MΦ-conditioned medium demonstrates that the germination operon gerH is required for MΦ-mediated germination. MΦ 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 Mø cultures and in Mø-conditioned medium. Mø 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 Mø 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 Mø 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 Mø 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 Mø. 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 Mø phagosomes (3, 26, 27, 28). Welkos et al. reported a Mø 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 Mø. 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 Mø, 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 gerH, the germinaton operon gerSAC is also required for Mø-related germination (12). The addition of d-alanine, a potent inhibitor of the Ala germination response, also inhibits germination in Mø cultures (data not shown) and implicates Ala in Mø-mediated germination. In other studies, the disruption of the pXO1-carried gerX partially attenuates endospore germination in Mø (6), although ΔSterne endospores lacking pXO1 respond to all known germinants and are fully capable of germinating in Mø 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 Mø-related germination, this path is likely to be only one of a variety of germination response pathways contributing to Mø-related germination. Therefore, the term “Mø-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 Mø and not by any other source.

**ACKNOWLEDGMENTS**

We thank B. Thomason, S. Cendrowski, B. Heffernan, N. Fisher, J. Crossno, and N. Bergman for their comments on this work.

This work was supported in part by NIH grants AI-08649 and AI-40644 and by ONR grants N00014-00-1-0422, N00014-01-1-1044, and N00014-02-1-0061.

**REFERENCES**


27. Welkos, S., S. Little, A. Friedlander, D. Fritz, and P. Fellows, 2001. The role of antibodies to *Bacillus anthracis* and anthrax toxin components in inhibiting
the early stages of infection by anthrax spores. Microbiology 147:1677–1685.

