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Macrophage-Enhanced Germination of *Bacillus anthracis* Endospores Requires *gerS*

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Germination of *Bacillus anthracis* Sterne and plasmidless Δ-Sterne endospores was dramatically enhanced in RAW264.7 macrophage-like cells, while germination of nonpathogenic *Bacillus* endospores was not. Elimination of *gerS*, a germinant receptor locus, caused a complete loss of cell-enhanced germination, implicating *gerS* in the breaking of endospore dormancy in vivo.

The endospores of *Bacillus anthracis* ensure its prolonged persistence in the environment and are the infectious particles during anthrax infections. Upon entering the body, endospores are engulfed by regional phagocytes, germinate, and begin a vegetative, often lethal, growth cycle (3, 7, 10, 12, 14, 16). Work with nonpathogenic *Bacillus subtilis* and *Bacillus megaterium* endospores indicates that small molecules from the environment specifically bind to germinant receptors, encoded by tricistronic operons and localized to the plasma membrane of the endospore (5). Receptor occupancy results in rapid hydration, breakdown of spore structures, and bacilli outgrowth (5). The chemical germinant profiles vary among *Bacillus* species. *B. anthracis* endospores are capable of rapid germination and outgrowth in certain animal and macrophage (Mϕ) culture models (2, 9, 12, 16, 21, 22,), but they do not germinate or outgrow effectively in rats (10). In vivo studies indicate that endospores are rapidly (within minutes) associated with regional Mϕ, with no obvious involvement of polymorphonuclear cells (9, 16, 24). During inhalation anthrax, endospores germinate while associated with alveolar Mϕ traveling en route to regional lymph nodes (9, 10, 12, 16). Since *B. anthracis* endospores interact with phagocytic cells during early infection, we examined these initial interactions in cultured RAW 264.7 Mϕ cultures.

The parental strain of *B. anthracis* used for this experimentation is Sterne 34F2, containing one of the two virulence plasmids, toxin plasmid pXO1. The strain Δ-Sterne 34F2 is a plasmidless variant of Sterne 34F2, derived by temperature curing pXO1. Construction and growth of *B. anthracis* Δ*gerS* strains is described for Δ-Sterne 34F2, and an analogous allelic-exchange procedure was used for the Sterne 34F2 construct (14). *B. subtilis* and *B. megaterium* were manipulated and stored according to accepted protocols. *B. anthracis* endospores were prepared and stored as described elsewhere (14). The murine peritoneal cell line RAW 264.7 (ATCC TIB-71) used in this study maintains most, if not all, markers and functions common to primary Mϕ (13). Mϕ were grown at 37°C (humidified, 5% CO_2) in minimal essential media (MEM; Gibco-BRL) supplemented with 10% (vol/vol) animal sera. Sera used included fetal bovine (Gibco-BRL), equine (HyClone), rabbit, porcine, goat, and rat (Sigma). Mϕ were grown to 80% confluence, washed twice with fresh media, recovered by scraping, and suspended in fresh media at a density of 10^6 Mϕ/ml. The degree of endospore germination and germination kinetics calculations were obtained through measurements of release of ^{45}Ca from prelabeled endospores as recently described (14). ^{45}Ca-labeled endospores were mixed at the indicated multiplicities of infection (MOIs) and incubated at room temperature, and the percent germination was scored (14). Endospore uptake measurement was as described in previous studies (2).

The behavior of *B. anthracis* endospores in a controlled cell culture microenvironment was investigated as a model to study early germination events. First, virtually no germination of *B. anthracis* endospores occurred in MEM culture medium alone or MEM supplemented with horse, rat, porcine, or rabbit serum. Only limited germination was observed in MEM with fetal bovine serum, while goat serum permitted robust germination of endospores (Table 1). These data are in agreement with findings indicating that serum is not the sole germination factor in susceptible animals, nor the sole reason for animal resistance (10, 18, 23). Second, *B. anthracis* endospores also

TABLE 1. Rate and Extent of *B. anthracis* Sterne 34F2 germination in MEM with animal sera

Serum or additive[a]	Max. germination rate (% germination/min)[b,c]	Total germination (%)[c] in 1 h
BHI	17.7 ± 4.0	99.2 ± 2.3
Water	<0.1	2.0 ± 0.3
Rat serum	<0.1	4.7 ± 0.5
Horse serum	<0.1	5.4 ± 0.5
Rabbit serum	<0.1	4.9 ± 0.3
Goat serum	8.3 ± 1.2	89.4 ± 0.9
Fetal bovine serum	4.0 ± 1.0	36.6 ± 1.8
Pig serum	<0.1	9.6 ± 0.8

[a] All serum or additives are at 10% (vol/vol) with MEM (Gibco-BRL).
[b] Rates marked as <0.1 showed no significant germination in 60 min.
[c] Values reported are the average and one standard deviation for three independent replicates on each of two independent endospore samples.

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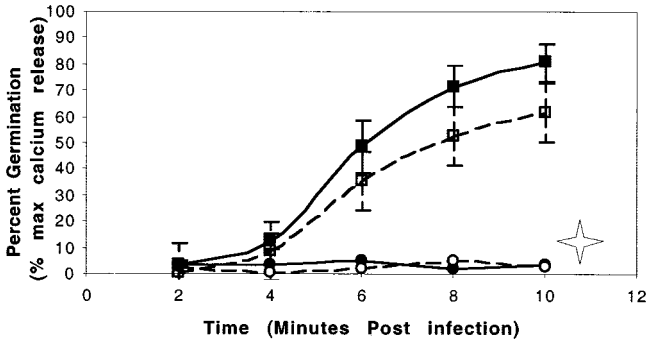

FIG. 1. Germination kinetics for *B. anthracis* endospores in Mϕ cultures. *gerS,* but not *gerX*, is required for Mϕ-induced germination. Mϕ were infected (MOI of 10:1) with *B. anthracis* strains Sterne (*gerS*$^+$, *gerX*$^+$) ■, Sterne Δ*gerS* (*gerS*-deficient, *gerX*$^+$) ●, Δ-Sterne (*gerS*$^+$, *gerX*-deficient) □, or Δ-Sterne Δ*gerS* (*gerS* and *gerX* deficient) ○. Germination was monitored by release of ^{45}Ca from prelabeled endospores. Both Δ*gerS* mutant strains (circles) did not germinate out to 4 h, and only limited germination was seen after 16 h, while the Δ*gerX* mutant containing wild-type *gerS* (open squares) was capable of significant Mϕ germination. Endospores recovered from Mϕ 16 h postinfection (open star symbol) were capable of fully germinating on BHI agar plates, indicating no loss of viability (see text). All cultures were in MEM with 10% (vol/vol) horse serum. The mean of triplicate experiments of each of two endospore preparations with one standard deviation is shown (standard deviations in mutant *gerS* samples were too small to depict).

rapidly and efficiently associated with RAW 264.7 Mϕ-like cells (10, 18, 23). By microscopic visualization and plating assays, >90% of endospores, independent of species or MOI (up to 20:1 endospore/Mϕ ratio), were found Mϕ associated within 10 min (data not shown).

A dramatic increase in germination was seen when anthrax endospores were exposed to Mϕ cultures, increasing from nearly 0 to 80% in the presence of Mϕ in 10 min (Fig. 1). This increase in germination was independent of input MOI (up to 20:1), of species of animal sera used, or of heat inactivation of sera. Additional requirements for both serum and, to a lesser extent, MEM were also seen, as germination increases were not as robust when either was substituted with phosphate-buffered saline (Table 2). The possibility that endospore uptake by phagocytes could be mimicked by other conditions and cell types was also addressed. Endospores separated physically from Mϕ cultures by use of Transwell membranes still germinated (35 to 50% in 1 h), indicating that neither phagocytosis nor direct contact with the Mϕ is an absolute requirement for the germination event but that these cells condition the medium to become germination competent, likely by contributing required germinant molecules, perhaps including some known to stimulate through the *gerS*-dependent pathway in vitro (14). NIH 3T3 fibroblasts were also competent to induce germination (30 to 40% in 1 h), indicating the possibility that non-Mϕ cells can also supply germinants. Although germination of *B. anthracis* endospores in various tissue culture systems remains a formal possibility, all prior work indicates that Mϕ are the de facto site for germination within the animal host (3, 7, 9, 12), and direct observations in animal studies are required before assigning a role for other cells in vivo.

TABLE 3. Germination of *B. anthracis, B. subtilis,* and *B. megaterium* in Mϕ cell culture

Bacterial species and strain	% Germination in 30 min[a]			
	MEM + horse serum[b]	Macrophage culture[c]	Water	BHI
B. anthracis Sterne 34F2	2.0 ± 0.3	75.0 ± 2.0	1.0 ± 0.4	100 ± 0.3
B. subtilis BD170	7.0 ± 1.0	7.0 ± 2.0	2.0 ± 1.0	95.0 ± 2.0
B. megaterium QMD1551	45.0 ± 3.0	47.0 ± 4.0	3.0 ± 2.0	100.0 ± 2.0

[a] Values reported are the experimental mean, and one standard deviation, for three independent replicates each of two independent endospore samples.
[b] Horse serum is at 10% (vol/vol).
[c] MEM, 10% (vol/vol) horse serum, and 10^5 Mϕ.

We next examined whether the effect of Mϕ on germination was unique to *B. anthracis,* as it was possible that host-associated germination of endospores might be a general response common to many *Bacillus* species, pathogen and nonpathogen alike. To test this, the nonpathogenic species *B. subtilis* and *B. megaterium* were compared to *B. anthracis* (Table 3). Both *B. anthracis* and *B. subtilis* exhibited extremely limited baseline germination in complete culture medium in the absence of Mϕ. *B. subtilis* and *B. megaterium* endospores showed no increase in germination in the presence of Mϕ, even at extended times, while *B. anthracis* showed a large and rapid increase in germination. The background response of *B. megaterium* to medium alone is likely due to the proline in MEM, a powerful *B. megaterium* germinant (5). Therefore, while a limited germination can occur with all three *Bacillus* species tested, only the pathogen *B. anthracis* exhibited a rapid host cell-specific response.

Experiments were conducted to compare the roles of the chromosomal locus *gerS* to those of the plasmid-encoded *gerX* locus with respect to the rapid germination seen in Mϕ cultures. The *gerS* locus was recently shown to act as a *B. anthracis*

TABLE 2. Role of Mϕ, serum, culture medium, and the *gerS* and *gerX* loci in *B. anthracis* germination kinetics

B. anthracis strain (genotype)	Maximum germination rate (% germination/min)[a] for germination condition:			
	+ MEM,[b] + serum, −Mϕ	+ MEM, − serum, + Mϕ	− MEM, + serum, + Mϕ	+ MEM, + serum, + Mϕ
Sterne (*gerS*$^+$ *gerX*$^+$)	<0.1	0.7 ± 0.2	1.4 ± 0.5	11.0 ± 2.0
Δ-Sterne (*gerS*$^+$, *gerX* deficient)	<0.1	0.5 ± 0.3	1.2 ± 0.5	9.0 ± 1.1
Sterne ΔgerS (*gerS* deficient, *gerX*$^+$)	<0.1	<0.1	<0.1	<0.1
Δ-Sterne ΔgerS (*gerS* and *gerX* deficient)	<0.1	<0.1	<0.1	<0.1

[a] Values reported are the experimental mean, and one standard deviation, for three independent replicates each of two independent endospore samples.
[b] + or − symbols indicate presence or absence of MEM, 10% (vol/vol) horse serum, and 10^5 Mϕ. Equivalent volumes of phosphate-buffered saline were used to replace the missing ingredient.

germinant receptor recognizing aromatic ring-like germinant signals (14). No germinants are yet known to be associated with *gerX* receptor activity. The Δ*gerS* mutant was severely attenuated in Mϕ germination. Compared to parental cells, Δ*gerS* mutants were incapable of germination in Mϕ (Fig. 1 and Table 2), with a severely limited germination at 16 h (<4% [data not shown]). Endospore strains that did not readily germinate in Mϕ did not lose viability after 16 h and were fully able to germinate when removed from cultures and plated on brain heart infusion (BHI) agar plates (Fig. 1). Collectively, these results indicate that *gerS* is important for cell-associated germination and suggest a role for aromatic amino acids and purines as potential host-supplied germinants (14). In contrast, *B. anthracis* strains not containing the pXO1-encoded germinant receptor operon *gerX* (Δ-Sterne strains) showed only a minor defect in Mϕ-associated germination extents or rates (Fig. 1 and Table 2). This *gerX* observation differs with earlier reports by Guidi-Rontani et al. (7–8) in that we found only a slight germination defect in pXO1$^-$ (*gerX*-deficient) strains. Our assay conditions, however, diverge in three important ways from the previous works. First, gentamicin was not used in our assays, as gentamicin has a severe inhibitory effect on the germination and outgrowth of *Bacillus* endospores, even at low concentrations, and gentamicin can be internalized or concentrated into the phagolysosomes of Mϕ (4, 11, 15, 19, 20). Second, all assays were done in the presence of serum, while the earlier work utilized serum-free conditions that we found severely limited endospore uptake and slowed germination rates. Third, our germination assay is based on the release of radioactive calcium ions from prelabeled endospores (14, 17), allowing direct measurement of germination without the need for survival of the newly vegetative bacilli.

Data presented here indicate that the *B. anthracis* endospore responds dramatically to the microenvironment of eukaryotic cells, reaching >80% germination after as little as 10 min coincubation with Mϕ. This is consistent with these phagocytes serving as the relevant site of germination during infections (2–3, 7, 9–10, 12, 16, 18, 22–24). Furthermore, host cell-associated germination was not seen with nonpathogenic *Bacillus* species, implying that the pathogen *B. anthracis* has evolved specialized abilities to sense its entry into a host and initiate vegetative growth. These data and previous work also support a model where in vivo germination is controlled by combinations of a number of discrete germinant signals (1) and multiple receptors, including *gerS* encoded on the chromosome and perhaps, to a lesser degree, the *gerX* encoded on pXO1 (8). However, since *B. anthracis* strains (Δ-Sterne) cured of all plasmids also exhibit significant germination in the presence of Mϕ (Fig. 1 and Table 2), our data contradict the notion that host-dependent germination is strictly controlled by elements on the virulence plasmid pXO1. Additionally, since defined chemical triggers for the *gerS* germination locus are also known (14), we hypothesize that these molecules may be utilized as signals for germination in vivo. Work presented here supports a model where rapid and strong germination responses greatly influence the early stages of Mϕ-endospore interactions. The combinations of multiple host-supplied germinant molecules, recognized by multiple endospore *ger* receptors, are likely requirements for a successful infection. Knowledge of the specific germinant molecules and their receptors allows further mechanistic studies of the germination process and for the development of compounds useful for blocking, or stimulating, the germination state of the anthrax endospore.

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