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# The role of *Bacillus anthracis* germinant receptors in germination and virulence

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## Summary

**Nutrient-dependent germination of *Bacillus anthracis* spores is stimulated when receptors located in the inner membrane detect combinations of amino acid and purine nucleoside germinants. *B. anthracis* produces five distinct germinant receptors, GerH, GerK, GerL, GerS and GerX. Otherwise isogenic mutant strains expressing only one of these receptors were created and tested for germination and virulence. The GerH receptor was necessary and sufficient for wild-type levels of germination with inosine-containing germinants in the absence of other receptors. GerK and GerL were sufficient for germination in 50 mM L-alanine. When mutants were inoculated intratracheally, any receptor, except for GerX, was sufficient to allow for a fully virulent infection. In contrast, when inoculated subcutaneously only the GerH receptor was able to facilitate a fully virulent infection. These results suggest that route of infection determines germinant receptor requirements. A mutant lacking all five germinant receptors was also attenuated and exhibited a severe germination defect *in vitro*. Together, these data give us a greater understanding of the earliest moments of germination, and provide a more detailed picture of the signals required to stimulate this process.**

## Introduction

The Gram-positive bacterium *Bacillus anthracis* exists in two morphologically distinct forms, the metabolically active bacillus and the dormant spore. Bacterial spores form in response to nutrient depletion to protect the organism from environmental stresses such as heat, desiccation, chemicals and radiation (Nicholson *et al.*, 2000; Piggot and Hilbert, 2004). Spore formation enables *B. anthracis* to survive dormant in the environment for years, until it comes in contact with an appropriate mammalian host, germinates and outgrows, resulting in the disease anthrax (Dixon *et al.*, 1999). Although dormancy can last for an extensive period of time, germination is a very rapid process, allowing *B. anthracis* to multiply and spread quickly (Dixon *et al.*, 1999).

Anthrax can present in a variety of ways, depending upon route of infection. The most severe of these presentations is inhalational anthrax. Upon entering the lungs of a host, spores are taken up into phagocytes, where they germinate and are transported to the mediastinal lymph nodes (Guidi-Rontani *et al.*, 1999a). Here, they can disseminate systemically and cause disease, and oftentimes, death. Other forms of the disease also exist, including cutaneous and gastrointestinal anthrax (Dixon *et al.*, 1999).

The onset of germination is dependent upon appropriate germinant/germinant receptor interactions (Dixon *et al.*, 1999). For *B. anthracis*, nutrient germinants are primarily amino acids and purine nucleosides, which interact with their specific receptors located in the inner membrane of the spore (Ireland and Hanna, 2002; Weiner *et al.*, 2003; Fisher and Hanna, 2005). Alanine or inosine typically serve as primary germinants, with a separate amino acid functioning as a cogerminant. L-alanine can trigger germination of spores by itself, although only at very high concentrations (Ireland and Hanna, 2002). Inosine is the more potent of the primary germinants, stimulating a greater germination response at a lower concentration than L-alanine. Inosine can be paired with a variety of L-amino acids, including L-histidine, L-serine, L-valine, L-tryptophan and L-methionine, as well as with the primary germinant L-alanine. Inosine alone, however, is not sufficient to germinate *B. anthracis* spores (Weiner *et al.*, 2003).

After receptors recognize their specific germinants, the cascade of germination events is initiated. Stores of dipicolinic acid and its associated calcium ions (Ca-DPA) are released from the spore core, allowing for water to flow back into the core and begin rehydration (Moir, 2003). Activation of lytic enzymes that hydrolyse peptidoglycan in the spore cortex then leads to further rehydration of the

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spore (Setlow *et al.*, 2001). RNA, protein and DNA synthesis then resumes in the outgrowth phase, resulting in a vegetative bacillus. This entire process is very rapid, occurring within minutes under optimal conditions.

The five germinant receptors of *B. anthracis* are encoded by the tricistronic operons, *gerH*, *gerK*, *gerL* and *gerS*, located on the chromosome, and *gerX*, located on the pXO1 virulence plasmid (Read *et al.*, 2003). Two additional receptor-like operons exist, named *gerA* and *gerY,* but these contain frameshift mutations, and were shown to not play a role in germination (Fisher and Hanna, 2005). The five functional germinant receptors have been characterized previously using single receptor operon mutations (Guidi-Rontani *et al.*, 1999b; Ireland and Hanna, 2002; Weiner *et al.*, 2003; Fisher and Hanna, 2005). In this study we have used markerless deletions to create isogenic quadruple mutants lacking all but one germinant receptor, as well as a null strain lacking all five functional germinant receptors. These mutants, as well as isogenic single receptor mutants, were used to further elucidate the roles of these receptors during germination *in vitro*. The receptor mutant strains were assayed for virulence in mice in order to correlate our *in vitro* data with conditions *in vivo*.

## Results

### In vitro *characterization of germinant receptor mutants*

Mutants in which individual germinant receptor operons were disrupted have been studied previously (Guidi-Rontani *et al.*, 1999b; Ireland and Hanna, 2002; Weiner *et al.*, 2003; Fisher and Hanna, 2005). However, the use of antibiotic resistance markers in these strains prevented the subsequent isolation of strains containing more than a single deletion. To examine the role of the individual germinant receptors more thoroughly, we created markerless deletion mutants targeting each operon in our parental 34F2 strain. The three genes for each tricistronic germinant receptor operon were removed, resulting in strains lacking one receptor operon each; these were named Δ*gerH*, Δ*gerK*, Δ*gerL*, Δ*gerS* and Δ*gerX*, respectively. A schematic of a typical germinant receptor operon and our deletion scheme is depicted in Fig. 1. This mutagenesis method allowed the deletion of additional germinant receptor operons in these strains, eventually leading to the creation of five distinct quadruple mutants, each containing only a single germinant receptor operon. These were named *gerH*$^+$, *gerK*$^+$, *gerL*$^+$, *gerS*$^+$ and *gerX*$^+$, to denote the remaining functional germinant receptor operon. For example, the *gerH*$^+$ strain retained the *gerH* operon but contained the deleted forms of the operons *gerK*, *gerL, gerS* and *gerX*. Additionally, a mutant was constructed that lacked all five germinant receptors (*ger*$_{null}$). Creation of these mutant strains allowed us to directly assay the function of each individual receptor in the absence of the other four, and alleviated complications from the potential cooperativity between different germinant receptor proteins described in other *Bacillus* species (Atluri *et al.*, 2006).

To determine the specific germinant recognized by each receptor, *in vitro* germination profiles were generated for each receptor mutant using a variety of germinant mixtures defined previously (Ireland and Hanna, 2002; Weiner *et al.*, 2003; Fisher and Hanna, 2005). Germinant mixtures used in this study included either the amino acid L-alanine or the purine nucleoside inosine as the 'primary' germinant, and an additional amino acid as a 'cogerminant.' Cogerminants used included L-tryptophan, L-histidine, L-serine, L-valine or L-methionine, and a mixture of both primary germinants (L-alanine/inosine) as well. L-alanine was also tested in the absence of a cogerminant because it has previously been shown to function without a cogerminant. However, this response required a much higher concentration of L-alanine (50 mM) than was necessary when it was paired with a cogerminant (Fisher and Hanna, 2005). Where possible, two separate assays were performed to quantify the germination of each mutant in the individual germination mixtures. One assay measured the hallmark loss of heat resistance associated with the earliest moments of germination (Paidhungat and Setlow, 2002). The other assay measured the decreased optical density (OD) associated with a germinating spore suspension (Paidhungat and Setlow, 2002). Both assays are described in detail in the *Experimental procedures* section.

### *Spores lacking all germinant receptors*

As predicted, the spores from the strain lacking all five germinant receptors (*ger*$_{null}$) exhibited a severe germination defect, even when plated on the rich medium brain heart infusion agar (BHI). In order to quantify the severity of this defect, colony forming efficiency was determined. Colony forming efficiency was defined as the number of colony-forming units (cfu) formed, when compared with the number of phase-bright spore particles plated. Spores were titered by visual counting via haemacytometer, then diluted and plated on BHI to measure cfu. Wild-type spores had a colony forming efficiency of approximately one cfu per spore particle plated (Fig. 2). In contrast, the *ger*$_{null}$ strain had a reduced colony forming efficiency of one cfu per 1000 spore particles plated. The low level of germination and outgrowth seen with this mutant is similar to that seen in an analogous receptor deficient strain of *Bacillus subtilis* (Paidhungat and Setlow, 2000). When tested more directly for germination, none of the previously defined germinant combinations were able to germinate these spores, as measured by decrease in $OD_{600}$ (Table 1). The

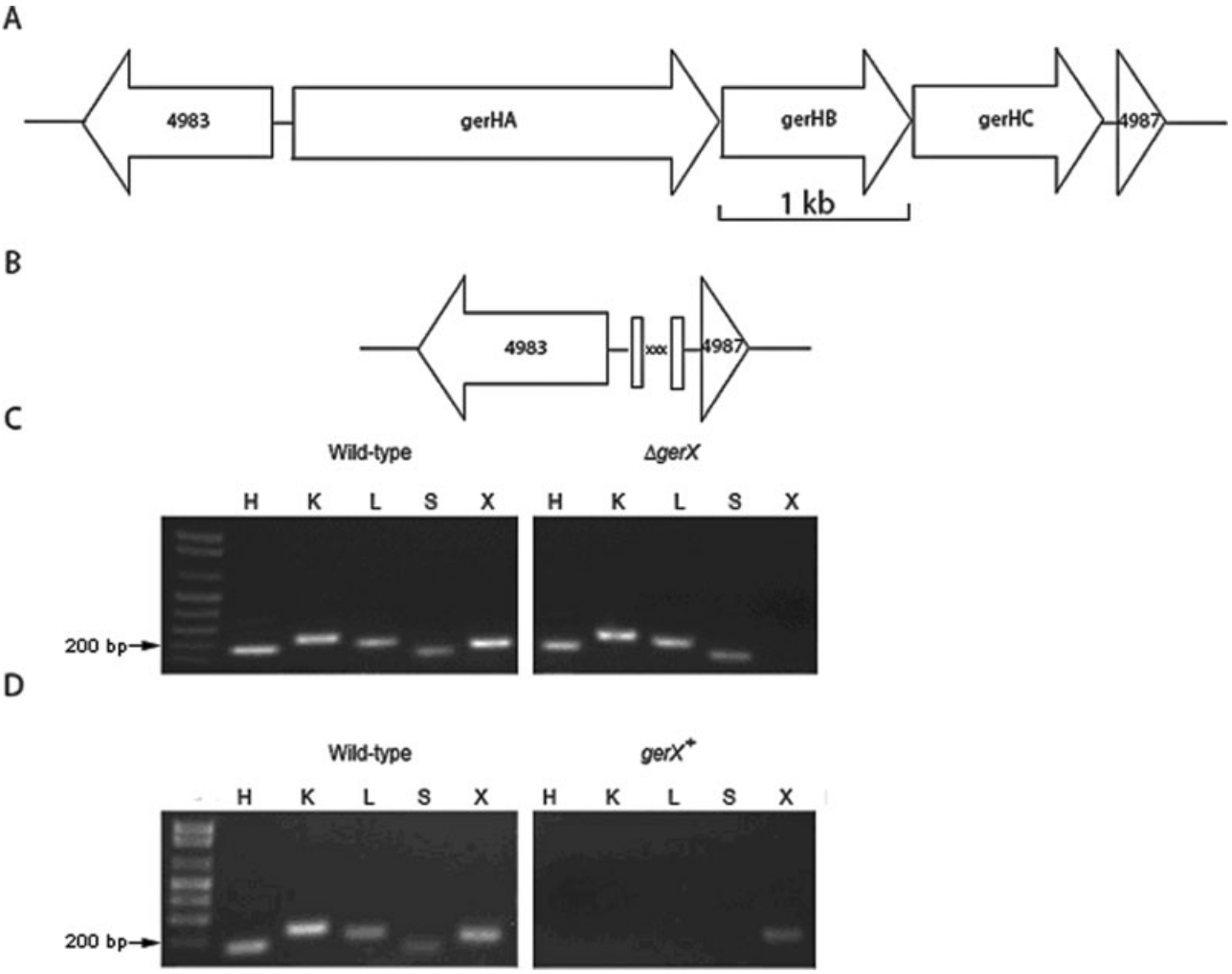


**Fig. 1.** Germinant receptor operon organization and mutagenesis.
A. Gene map of the *gerH* operon. The other *ger* operons discussed in this work have the same gene order, with the exception of *gerX,* in which the *gerXB* and *gerXA* genes are transposed.
B. Gene map of the Δ*gerH* allele. The mutant allele was designed as a markerless deletion of nearly the entire operon. Non-native sequences are denoted by 'X' (see *Experimental procedures*). This general scheme was used to construct each of the *ger* operon deletions.
C. Verification of the Δ*gerX* operon genotype. PCR was performed using primers for each locus that amplified approximately 200 bp of the first gene of each receptor operon. Letters denote the germinant receptor being tested. Additional PCR reactions were performed with primers flanking the loci, to verify the deleted allele (data not shown).
D. Expression of germinant receptor operons in the *gerX*$^+$ strain. Reverse transcription PCR was performed using the same primers as in Fig. 1C.

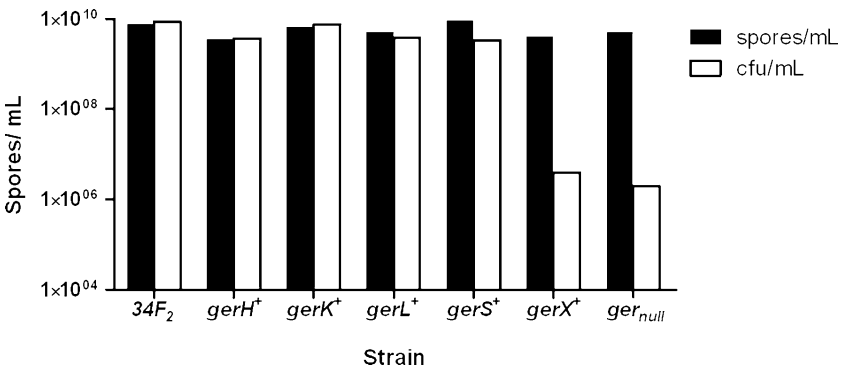


**Fig. 2.** Colony forming efficiency of germinant receptor mutants. Colony forming efficiency is reported as the number of spore particles per ml, as determined visually by haemacytometer, or via cfu when plated. Data shown are the average of two independent experiments with independent spore preparations.

 

**Table 1.** Decrease in optical density of strains expressing only one germinant receptor after exposure to germinants.

| | Mean percentage loss of $OD_{600}$ after 30 min[a] | | | | | | |
|---|---|---|---|---|---|---|---|
| Germinant[b] | $34F_2$ | *gerH*$^+$ | *gerK*$^+$ | *gerL*$^+$ | *gerS*$^+$ | *gerX*$^+$ | $ger_{null}$ |
| PBS, pH 7.4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| L-ala (50 mM) | 61 | 19 | 60 | 44 | 30 | 6 | 2 |
| L-ala/inosine | 66 | 72 | 9 | 49 | 30 | 11 | 6 |
| L-ala/L-trp | 34 | 17 | 22 | 19 | 16 | 15 | 3 |
| Inosine/L-trp | 66 | 57 | 8 | 8 | 16 | 8 | 8 |
| Inosine/L-his | 40 | 60 | 4 | 5 | 5 | 9 | 9 |
| Inosine/L-met | 72 | 58 | 9 | 0 | 2 | 7 | 3 |
| Inosine/L-ser | 72 | 60 | 4 | 38 | 21 | 11 | 1 |
| Inosine/L-val | 65 | 55 | 9 | 8 | 4 | 6 | 9 |

**a.** Results are the average of four experiments with two independent spore preparations. Standard error of the mean was ≤10% of the mean in all instances.
**b.** Germinant concentrations were 0.5 mM L-alanine, 1 mM inosine, 5 mM L-tryptophan and 50 mM of other amino acids. ~65% decrease in $OD_{600}$ represents ~100% germination.

$ger_{null}$ strain was not tested in our heat sensitivity assay, as its defect in colony forming efficiency precluded direct comparisons between it and the wild-type strain.

### *GerX*

The strain lacking only GerX (Δ*gerX*) was tested via heat sensitivity and scored for a germination deficiency in our panel of germinants. Although PCR analysis of genomic DNA confirmed that our Δ*gerX* mutant had the correct genotype (Fig. 1C), we could not detect any germination defect for Δ*gerX* in response to any germinant mix (data not shown). Like the $ger_{null}$ strain, spores of the *gerX*$^+$ strain showed a germination defect when plated on BHI. Indeed this quadruple mutant, expressing only the *gerX* germinant receptor operon, behaved identically to $ger_{null}$ in all *in vitro* assays tested (Fig. 2, Table 1). RNA expression of the five receptor operons was verified in the *gerX*$^+$ strain in order to ensure that *gerX* was, indeed, expressed (Fig. 1D).

### *GerS*

The GerS receptor was sufficient for germination in rich media, as the strain that expressed only this receptor (*gerS*$^+$) exhibited a colony forming efficiency similar to the wild-type level of one cfu for every spore plated (Fig. 2). In addition, the *gerS*$^+$ strain exhibited some level of germination from many of the germinant mixes tested (Tables 1 and 2), although none as high as wild-type levels. Previous studies have implicated GerS in germination with inosine, with L-histidine or L-tryptophan as cogerminants (Ireland and Hanna, 2002; Fisher and Hanna, 2005), and our Δ*gerS* mutant, missing only *gerS*, mimicked these results. However, the *gerS*$^+$ strain, missing all *ger* operons other than *gerS*, was unable to germinate to

**Table 2.** Loss of heat resistance of strains expressing only one germinant receptor after exposure to germinants.

| | Mean percentage loss of heat resistance after 30 min[a] | | | | |
|---|---|---|---|---|---|
| Germinant[b] | $34F_2$ | *gerH*$^+$ | *gerK*$^+$ | *gerL*$^+$ | *gerS*$^+$ |
| PBS, pH 7.4 | 0 | 0 | 0 | 0 | 0 |
| L-alanine (50 mM) | 86 | 43 | 53 | 22 | 50 |
| L-ala/L-trp | 63 | 52 | 7 | 35 | 9 |
| L-ala/inosine | 100 | 99 | 11 | 32 | 51 |
| Inosine/L-trp | 76 | 84 | 16 | 19 | 11 |
| Inosine/L-his | 52 | 45 | 21 | 21 | 9 |
| Inosine/L-met | 78 | 60 | 21 | 12 | 31 |
| Inosine/L-ser | 100 | 100 | 22 | 25 | 37 |
| Inosine/L-val | 95 | 91 | 37 | 20 | 40 |

**a.** Results are the average of four experiments with two independent spore preparations. Standard error of the mean was ≤ 10% of the mean in all instances.
**b.** Germinant concentrations were 0.5 mM L-alanine, 1 mM inosine, 5 mM L-tryptophan and 50 mM of other amino acids.

 

wild-type levels in the presence of either of these cogerminants (Tables 1 and 2).

### GerK and GerL

As with the strain containing only GerS, strains that had either GerK or GerL as the only germinant receptor (*gerK*$^+$ and *gerL*$^+$ respectively) had colony forming efficiencies identical to wild type, suggesting that they could recognize some germinant combination present in rich media. Earlier work implicated a role for GerK and GerL in response to L-alanine (Fisher and Hanna, 2005). Indeed, the *gerK*$^+$ strain responded strongly when L-alanine was the sole germinant, nearly as well as wild type with respect to loss of heat resistance, and indistinguishable from wild type as scored by drop in absorbance. The *gerL*$^+$ strain also exhibited an ability to respond to 50 mM L-alanine, when measured by drop in absorbance (Table 1), although it appeared less sufficient when measured by a loss in heat resistance (Table 2).

A role has also been suggested for GerK in response to L-methionine when inosine was the primary germinant, and for GerL in response to L-serine or L-valine with inosine as the primary germinant (Fisher and Hanna, 2005). When expressed as the sole germinant receptor, roles for these proteins appeared less specific. As measured by heat sensitivity, a *gerL*$^+$ strain appeared to respond weakly to all germinants tested, with the possible exception of inosine paired with methionine (Table 2). When germination was measured by a loss of absorbance at 600 nm, this same strain exhibited some greater discrimination, failing to germinate well with inosine as the primary germinant, with the exception of the case where serine or alanine was the cogerminant. However, none of these responses were as strong as that of wild type (Table 1). The *gerK*$^+$ strain appeared less promiscuous than *gerL*$^+$ when germination was assessed by loss of absorbance at 600 nm, failing to respond well to any germinant combination (Table 1). When measured by a loss of heat resistance, however, this strain exhibited a low-level response to inosine paired with the cogerminants L-histidine, L-methionine, L-serine and L-valine (Table 2).

### GerH

Previous reports have shown that GerH is important for purine nucleoside-mediated germination (Weiner *et al.*, 2003). Indeed, our Δ*gerH* mutant, missing *gerH* but maintaining all other *ger* operons, had a severe germination defect in the presence of all germinant mixes containing inosine (data not shown), consistent with the previous report (Weiner *et al.*, 2003). The *gerH*$^+$ strain, expressing only the GerH receptor, exhibited wild-type colony forming efficiency (Fig. 2). The germination profile of the *gerH*$^+$ mutant mirrored wild-type spores when inosine was used as the primary germinant in either assay used (Tables 1 and 2). It should be noted, however, that although GerH can trigger germination without the need for other receptors, it requires both inosine and a cogerminant to do so, as neither the wild type nor *gerH*$^+$ spores could respond to inosine alone at any concentration tested (data not shown). None of the other quadruple mutants were able to germinate to a similar level in the germinant mixtures containing inosine as the primary germinant. Together, these data suggest that not only is GerH required for the inosine-dependent responses, but that it is also able to trigger a full response in the absence of all other receptors.

### Virulence of germinant receptor mutants in mice

As the spore's ability to germinate upon entering a potential host is a requirement in the establishment of disease, we sought to determine the roles of the germinant receptors in a murine model of anthrax infection. Two separate sites of inoculation were used, intratracheal injection to simulate an inhalational method of infection, and subcutaneous injection that bypasses uptake through the lungs. Six-week-old female DBA/2J mice were used, due to their susceptibility to the 34F2 Sterne strain of *B. anthracis* (Sterne, 1939; Harvill *et al.*, 2005). Results from these infections, and how disruption of individual germinant receptors impacted virulence, are described below.

As mentioned above, both the $ger_{null}$ and *gerX*$^+$ strains exhibited a severe, and nearly identical, germination defect when compared with wild-type spores. When either of these strains was inoculated into mice intratracheally, a strong attenuation was observed, with both strains causing only a single fatal infection through the duration of the experiment (Fig. 3). To further investigate the severity

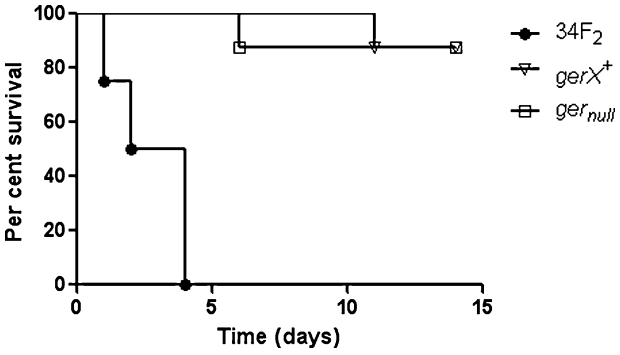


**Fig. 3.** Survival curves of germinant receptor mutants in mice after intratracheal inoculation. DBA/2J mice were inoculated intratracheally with either the parent or mutant strain at a dose of $1.5 \times 10^6$ spores/mouse. The initial group size was 8, and the percentage of each group surviving is shown over time. Survival curves of the *gerX*$^+$ and $ger_{null}$ strains were statistically significant when compared with wild type, with $P = 0.0001$, as calculated by the log-rank (Mantel-Cox) test.

 

**Table 3.** The $LD_{50}$ values of germinant receptor mutants.

| Strain | $LD_{50}$ value[a] | |
|---|---|---|
| | IT | SubQ |
| 34F2 | $1.50 \times 10^4$ | $1.03 \times 10^4$ |
| Δ*gerH* | ND | $3.52 \times 10^4$ |
| *gerS*$^+$ | ND | $3.07 \times 10^5$ |
| *gerX*$^+$ | $1.06 \times 10^7$ | $3.50 \times 10^6$ |
| *ger*$_{null}$ | $1.20 \times 10^7$ | $5.00 \times 10^6$ |

**a.** $LD_{50}$ values were calculated via the methods of Reed and Muench (1938) and were based on survival data from three groups of mice ($n = 8$) at three different doses of each of the strains assayed.
ND, not determined; IT, intratracheal inoculation; SubQ, subcutaneous inoculation.

of this attenuation, $LD_{50}$ values were determined for the wild type, *gerX*$^+$ and *ger*$_{null}$ strains. Both mutant strains were greater than 700-fold attenuated when compared with wild type (Table 3).

Mice were also inoculated with mutant or wild-type spores subcutaneously by receiving injections in the scruff of their necks. Animals receiving wild-type spores showed a median time to death of 3 days (Fig. 4A). Similar to the survival patterns seen above via intratracheal infections, the *gerX*$^+$ and *ger*$_{null}$ strains were highly attenuated, with $LD_{50}$ values about 400-fold greater than wild type (Table 3). It was previously reported that a Δ*gerX* mutant was attenuated in a subcutaneous infection (Guidi-Rontani *et al.*, 1999b). Our findings contradict that report, as our Δ*gerX* strain appeared just as lethal as wild type (Fig. 4A).

In the *in vitro* studies, the GerS receptor was found to be important, but not sufficient, for germination in the presence of the cogerminants L-histidine (in inosine) or L-tryptophan (in either primary germinant) (Tables 1 and 2, data not shown). In fact, the *gerS*$^+$ strain, containing only the GerS receptor, was unable to germinate fully in any of the germinant mixes tested (Tables 1 and 2). It was somewhat surprising that the *gerS*$^+$ strain scored as virulent as wild-type spores when inoculated intratracheally, with all mice succumbing to infection by day 4 (data not shown). Likewise, each of the *gerH*$^+$, *gerK*$^+$ and *gerL*$^+$ strains were sufficient to cause fully virulent infections (data not shown). Together, these data suggest that none of these receptors are absolutely required for initiation of disease via an intratracheal route of infection, so long as one of them is present. Because of the level of virulence seen with each of these quadruple mutants, none of the single receptor mutants were tested.

In contrast, equivalent levels of virulence were not observed when these strains were inoculated subcutaneously (Fig. 4B). With this route of infection the $LD_{50}$ value of the *gerS*$^+$ strain was 30-fold higher than wild type (Table 3). When the single receptor mutant Δ*gerS* was inoculated subcutaneously, it showed no attenuation when compared with wild type, suggesting that it was not necessary at this site of infection (data not shown). Collectively, these data indicate that the ability of the GerS receptor to stimulate germination, and thereby cause disease, is dependent upon the site of inoculation. This also suggests that this receptor's specific ligand was not present at sufficient concentration to stimulate germination at the site of subcutaneous inoculation.

The *gerL*$^+$ strain showed moderate attenuation when inoculated subcutaneously, suggesting that this receptor may be sufficient to trigger some germination at this site of infection, but not enough to cause a fully virulent infection (Fig. 4B). Likewise, the *gerK*$^+$ strain also exhibited a slight attenuation when injected subcutaneously, with a delay in median time to death (5 days), as compared with wild-type strain (3 days). In contrast, the *gerH*$^+$ strain more closely resembled wild-type spores in terms of lethality, as

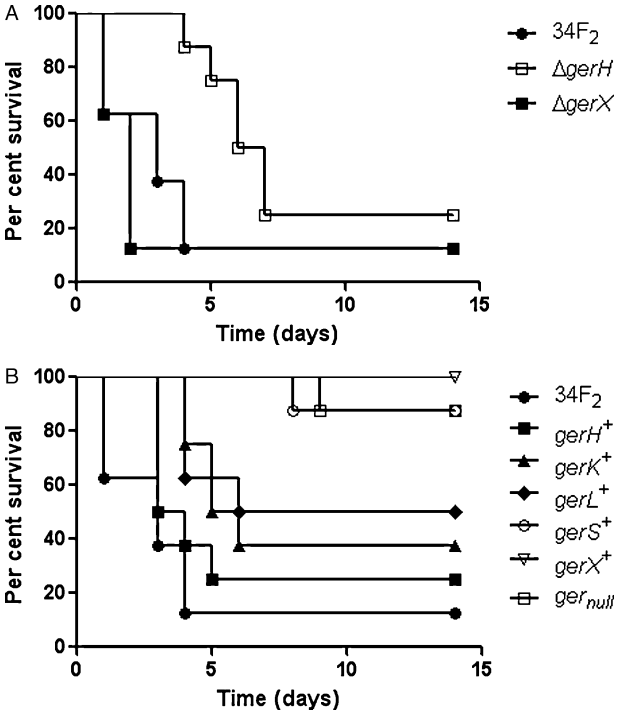


**Fig. 4.** Survival curves of germinant receptor mutants in mice after subcutaneous inoculation. DBA/2J mice were inoculated subcutaneously with either the parent or mutant strain at a dose of $4 \times 10^5$ spores/mouse. The initial group size was 8, and the percentage of each group surviving is shown over time.
A. Survival curves of the single receptor mutants. The survival curve of the Δ*gerH* strain was statistically significant when compared with wild type, with $P = 0.033$, as calculated by the log-rank (Mantel-Cox) test.
B. Survival curves of the quadruple receptor mutants. Survival curves of the *gerK*$^+$ and *gerL*$^+$ strains were statistically significant when compared with wild type, with $P = 0.026$ and $P = 0.018$ respectively. The survival curves of *gerS*$^+$, *gerX*$^+$ and *ger*$_{null}$ strains were also statistically significant when compared with wild type, with $P = 0.001$ for each.


**Fig. 5.** Germination phenotypes of the ger receptor mutants in response to whole mammalian blood. Wild-type or mutant spores were incubated in whole mouse blood at 37°C for 30 min, and germination was measured as a loss in heat resistance. 'Single mutants' (dark bars) denotes the Δ*ger* strains (missing only one germinant receptor). 'Quadruple mutants' (white bars) denotes the *ger*$^+$ strains (expressing only one germinant receptor). Data presented are mean values of four independent experiments with two different spore preparations. Error bars represent ± standard error of the mean.

well as median time to death. These data suggested that the GerH receptor alone is sufficient for the spore to be able to respond and germinate at this site of infection. Indeed, the Δ*gerH* strain exhibited a delay in median time to death (6 days) when compared with the wild-type strain (Fig. 4A). In contrast, the other single receptor mutants, Δ*gerK,* Δ*gerL,* Δ*gerS* and Δ*gerX,* were all as virulent as wild type, suggesting that these receptors were not absolutely necessary for germination, and ultimately virulence, in this route of infection (Fig. 4A, data not shown). Together, these data suggested that the GerH receptor is the primary receptor required for germination and establishment of disease in a subcutaneous route of infection.

### *Germination of receptor mutants in blood*

The observation that the GerH receptor was required for full, rapid disease progression via a subcutaneous route of infection led us to speculate that the GerH receptor was fully capable of responding to its germinants at the site of inoculation, in the absence of other receptor family members. As spores inoculated subcutaneously likely come in direct contact with blood, we tested whether the GerH receptor was responding specifically to factors contained within blood. To test this, *in vitro* germination experiments were performed using whole mouse blood as the source of germinants. Heat sensitivity was used as a measure of germination. After 30 min in mouse blood, 54% of wild-type spores became heat-sensitive (Fig. 5). Likewise, the single receptor mutants that maintained a functional copy of *gerH*, specifically Δ*gerK*, Δ*gerL*, Δ*gerS* and Δ*gerX,* all germinated as efficiently as wild-type spores. In contrast, the Δ*gerH* strain, which maintains all other *ger* receptor family members, was unable to germinate to any significant level after 30 min. Furthermore, the quadruple mutants *gerK*$^+$, *gerL*$^+$ and *gerS*$^+$ were unable to germinate to a substantial degree. This finding was in contrast to the *gerH*$^+$ mutant that germinated nearly as well as wild-type spores, suggesting that not only is the GerH receptor necessary for germination in blood, but that it is also sufficient in the absence of all other receptors. This assay was repeated in the presence of other mammalian bloods including sheep, rabbit and cow. The trend that the GerH receptor was both necessary and sufficient for germination was consistent in all mammalian bloods tested (data not shown).

## Discussion

*Bacillus anthracis* may have evolved a variety of germinant receptors in order to optimize its germinant recognition capabilities, thereby increasing chances of efficient germination and survival in various niches. This is exemplified by the *in vivo* data suggesting that route of infection dictates which germinant receptor is stimulated. The wild-type level of virulence seen upon intratracheal challenges of the strains expressing only GerH, GerK, GerL or GerS implies that the presence of any one of these germinant receptors is sufficient to cause disease in the intratracheal mouse model of infection. This suggests that the site of germination after an intratracheal inoculation may contain many discrete germinant signals, and is able therefore to fulfil the broad specificities of these different receptors (Ireland and Hanna, 2002; Weiner *et al*., 2003; Fisher and Hanna, 2005). In contrast, only the *gerH*$^+$ strain (expressing only the GerH receptor) retained wild-type level virulence when inoculated subcutaneously (Fig. 4B). This suggests that during a subcutaneous inoculation the GerH receptor is more essential for germination and disease. Additional support for the centrality of GerH comes from data showing that the Δ*gerH* strain was the only strain of the single mutants to exhibit any significant attenuation. It should be noted that the delay in the survival curve seen with this mutant did not substantially impact its $LD_{50}$ value (Table 3). The delayed appearance of disease was likely due to germination triggered by one of the other four germinant receptors still present in the mutant; Perhaps, as the Δ*gerH* strain travelled through the body, it eventually found a site rich in a germinant that either the GerK, GerL, GerS or GerX receptors recognized, stimulating germination, and

ultimately resulting in disease. These data support the notion that different routes of infection require different germinant receptors for efficient, rapid germination and full virulence. They also suggest that a specific ligand at the site of a subcutaneous inoculation is able to rapidly, and specifically, trigger germination in a GerH receptor-dependent manner. The data from spores germinated in blood *in vitro* further support this hypothesis, as efficient germination, in all mammalian blood types tested, was dependent on the presence of GerH (Fig. 5). Therefore, the GerH receptor is likely triggered specifically by a component in the blood in the mouse model, resulting in the virulence seen in our mouse experiments. This may also be true of other species, as suggested by the *in vitro* testing of the additional mammalian bloods, although this remains to be tested. Although the blood component that stimulates germination via the GerH receptor has yet to be identified, possible candidates may be purine nucleoside-related, as evidence in this study, and others, have implicated GerH in purine nucleoside based germination (Weiner *et al.*, 2003).

The presence of five distinct receptors, coupled with the possibility of beneficial cooperation between the receptors or, potentially, even the receptor components, may add the additional benefit of vastly increasing the potential array of germinants the spore could respond to. The inability of strains expressing only GerK, GerL or GerS to fully germinate in their previously characterized germinants, with the exception of L-alanine-specific germination in the *gerK*$^+$ and *gerL*$^+$ strains, suggests that there is at least some level of cooperativity between receptors of *B. anthracis*, and that the presence of more than one receptor family member is typically required for a full, robust response to certain germinants. Cooperativity among these various receptors has been speculated to play a role in germinant detection in *B. anthracis* (Fisher and Hanna, 2005).

The finding that the *gerH*$^+$ strain could germinate to wild-type levels in inosine-based germinants independent of all other receptors, however, suggests that not all receptors absolutely need cooperative partners in order to germinate (Tables 1 and 2). It appears that the GerH receptor, in the absence of all others, can recognize and respond to not only inosine, but also the amino acid cogerminants required for inosine-dependent germination. Whether this involves individual GerH receptors binding discrete germinant species and interacting in some beneficial manner, or an individual receptor molecule binding two or more germinant species simultaneously remains unclear. While it is tempting to speculate that the GerH receptor can interact with both inosine and its cogerminants, our data do not preclude that possibility that an additional spore component could be contributing to this germination response.

The *in vitro* and *in vivo* data from the strain lacking all of its germinant receptors (*ger*$_{null}$) further enforce the importance of a germinant detection system for germination and virulence. A *B. subtilis* strain lacking all known germinant receptors exhibited a decrease in colony forming efficiency similar to the defect seen with our *ger*$_{null}$ strain (Paidhungat and Setlow, 2000). The residual growth seen with the *B. subtilis* mutant was attributed to a small percentage of these spores that were still able to germinate, based on a low level of 'spontaneous' germination over time. This is likely the case with our *ger*$_{null}$ strain as well. The 1000-fold decrease in colony forming efficiency translated into a 500–800-fold decrease in virulence in our mouse model, depending on route of infection (Figs 3 and 4B). The low level of virulence seen with the *ger*$_{null}$ strain was likely due to the same spontaneous germination events described *in vitro*, whereby a small fraction of receptor-deficient spores germinate after inoculation into the host, independent of the germinant receptors described in this work. Together, these data suggest that germinant receptors are an essential component to efficient, productive germination that directly contributes to virulence in our mouse model.

Curiously, the *gerX*$^+$ mutant (expressing only the *gerX* receptor operon) exhibited the same phenotypes as the *ger*$_{null}$ mutant in colony forming efficiency, germination and virulence. This observation was somewhat surprising, as a previous study implicated the GerX receptor in germination with L-alanine (Hu *et al.*, 2007). Additionally, the attenuation *in vivo* seen with our *gerX*$^+$ strain disagrees with a previous report implicating GerX in virulence (Guidi-Rontani *et al.*, 1999b), but several experimental differences between our study and the previous study may partially explain the disparity between our results. These include different parental *B. anthracis* strains, differences in mouse strains, mutagenesis methods and inoculation sites. That the *ger*$_{null}$ and *gerX*$^+$ strains behaved identically in all *in vitro* and *in vivo* conditions tested suggests that the GerX receptor, when present by itself in the spore, is not capable of promoting germination under the conditions tested. It is possible, and even likely, that all *B. anthracis* germinant receptors, including GerX, have additional germinant specificities not tested in this work. Although we were not able to determine a role for GerX in this study, several possible functions may include GerX contributing to a cooperative germination response with one or more of the other receptors. Another possibility is that GerX is important for germination in other host species yet to be tested. Further work is needed to better understand what, if any, role this receptor is playing, as its presence on a virulence plasmid leads to speculation of its importance during infection.

The ultimate goal of this study was to provide a more complete characterization of germinant/germinant receptor specificities in *B. anthracis.* Using our set of mutants allowed us to better characterize the receptor–ligand

 

**Table 4.** *Bacillus anthracis* strains used in this study.

| Strain | Mutant name | Relevant characteristics[a] | Reference |
|---|---|---|---|
| $34F_2$ | – | Wild-type (pXO1$^+$, pXO2$^-$) | Sterne (1939) |
| SL110 | Δ*gerH* | $34F_2$, Δ*gerH* | This work |
| SL111 | Δ*gerK* | $34F_2$, Δ*gerK* | This work |
| SL112 | Δ*gerL* | $34F_2$, Δ*gerL* | This work |
| SL113 | Δ*gerS* | $34F_2$, Δ*gerS* | This work |
| SL114 | Δ*gerX* | $34F_2$, Δ*gerX* | This work |
| SL115 | *gerH*$^+$ | $34F_2$, Δ*gerK* Δ*gerL* Δ*gerS* Δ*gerX* | This work |
| SL116 | *gerK*$^+$ | $34F_2$, Δ*gerH* Δ*gerL* Δ*gerS* Δ*gerX* | This work |
| SL117 | *gerL*$^+$ | $34F_2$, Δ*gerH* Δ*gerK* Δ*gerS* Δ*gerX* | This work |
| SL118 | *gerS*$^+$ | $34F_2$, Δ*gerH* Δ*gerK* Δ*gerL* Δ*gerX* | This work |
| SL119 | *gerX*$^+$ | $34F_2$, Δ*gerH* Δ*gerK* Δ*gerL* Δ*gerS* | This work |
| SL120 | $ger_{null}$ | $34F_2$, Δ*gerH* Δ*gerK* Δ*gerL* Δ*gerS* Δ*gerX* | This work |

**a.** Mutants alleles consist of markerless deletions of nearly the entire tricistronic germinant receptor operon (see *Experimental procedures*).

interactions necessary for germination in certain germinants. Additionally, we were able to determine which receptors were sufficient for germination, *in vitro* and *in vivo*, independent of any other kind of germinant receptor. The diversity of germinant/receptor interactions may play an important role in the ability of *B. anthracis* to exploit subtle chemical differences presented at a variety of anatomical sites within a host, allowing for the multiple routes by which disease can be established. Gaining a better understanding of how spores sense germinants in their environment, and what those germinant specificities are, may aid in the development of therapeutics, and allow for a better understanding of disease progression.

## Experimental procedures

### *Strains and culture conditions*

Strains used in this study are listed in Table 4. Strains were cultured in BHI (Difco) broth or solid media containing 15 g agar per litre. Spores were prepared by growth in modified G medium (Kim, 1974) for 3 days at 37°C with shaking. Spores were prepared as previously described (Passalacqua *et al.*, 2006). Spores were stored at room temperature in sterile water and titered by counting phase-bright particles using a haemacytometer (spores ml$^{-1}$) and/or by plate count (cfu ml$^{-1}$), as indicated in the text.

### *Mutant construction*

Each of the mutant strains used in this work were created using allelic exchange, resulting in markerless deletions (Fig. 1). Each mutant allele was designed to contain the first 10 codons of the first gene in the operon (*gerHA, gerKA, gerLA, gerSA* or *gerXB*), a short insert sequence of three stop codons and restriction sites for the restriction endonucleases BamHI and SmaI, followed by the final 10 codons of the last gene in the operon, including the putative stop codon (*gerHC, gerKC, gerLC, gerSC* or *gerXC*). The constructs used to create each mutant were isolated by PCR (primer sequences available upon request). In addition to the mutant allele described above, each PCR product contained approximately 500 bp of DNA sequence homologous to the upstream and downstream region of the *ger* operon, flanked by the recognition sequence for the restriction endonuclease NotI. Each PCR product was cloned into the pCR8/*GW/TOPO* vector (Invitrogen) according to manufacturer's instructions. The DNA sequence of each construct was verified to ensure that no additional mutations were present due to PCR error. The NotI fragment was then cloned into the allelic exchange vector pBKJ258-kan. This vector was identical to the previously described pBKJ258, with the exception that a kanamycin-resistance cassette was exchanged for the original erythromycin cassette (Lee *et al.*, 2007). Allelic exchange was performed essentially as described previously (Janes and Stibitz, 2006). The quadruple mutants and $ger_{null}$ strain were isolated by performing allelic exchange sequentially, knocking out the appropriate *ger* allele. All mutant alleles were verified using PCR, with primers designed to anneal outside of the sequences used for homologous recombination. Additionally, expression of the *gerX* operon was verified via reverse transcription PCR in the *gerX*$^+$ strain, as its phenotype resembled that of a $ger_{null}$ strain *in vitro* and *in vivo*. RNA isolation was performed as previously described (Carlson *et al.*, 2009). The primers to verify the Δ*gerX* mutant, via PCR, and expression in the *gerX*$^+$ strain, via reverse transcription PCR, were specific to approximately 200 bp of the first gene of each receptor operon.

### *Germination assays*

Germination was measured by two assays, loss of heat resistance and decrease in optical density of spore suspensions after exposure to germinants. For the loss of heat resistance experiments, spores were first heat-activated by incubating at 65°C for 20 min. $5 \times 10^3$ spores were mixed with 2 ml of germinant in 1× PBS, pH 7.4 (Gibco). Germinant concentrations were as follows: inosine, 1 mM; L-tryptophan, 5 mM; L-serine, L-valine, L-methionine and L-histidine, 50 mM; L-alanine was at either 50 mM or 0.5 mM, as noted in Tables 1 and 2. Germination assays in blood were performed in whole mouse blood treated with the anticoagulant EDTA (Lampire), defibrinated whole sheep blood (Lampire), defibrinated whole rabbit blood (Lampire) and defibrinated whole

 

cow blood (Hemostat). Samples were vortexed very briefly and 50 µl were plated on a BHI plate. Samples were incubated at 37°C for 30 min. At 30 min, 200 µl aliquots were moved to a fresh tube and heat-treated at 65°C for 20 min, after which 100 µl was plated on BHI. Experiments in blood were started with 10-fold more spores, and then diluted 1:10 in PBS before heat treatment to prevent clotting. Plates were incubated overnight at 37°C and colonies were counted. A sample that was 100% sensitive to a 65°C incubation was considered 100% germinated. Standard error of the mean was calculated as (standard deviation/ $\sqrt{n}$ ).

To assay decrease in optical density, heat-activated spores (see above) were added to 400 µl of germinant, giving a starting $OD_{600}$ of 0.3 using a Genesys 10UV spectrophotometer (Spectronic Unicam, Rochester, NY). The reaction was incubated at 37°C while shaking at 200 r.p.m. for 30 min. After this time the $OD_{600}$ of the germinated spore mixture was measured. It has been previously established that a loss of 60–70% of the starting $OD_{600}$ value corresponds with complete germination (Fisher and Hanna, 2005). In all cases the parental 34F2 Sterne strain was used as the positive control.

### *Colony forming efficiency*

Colony forming efficiencies were determined by comparing the number of spores $ml^{-1}$ with the number of cfu $ml^{-1}$. Spore stocks were titered by counting phase bright particles using an Improved Neubauer haemacytometer. The same spore stock was then diluted and plated, and colonies were counted after an overnight incubation at 37°C.

### *Murine challenges*

Intratracheal infections of 6-week-old female DBA/2J mice (Jackson Laboratories) were performed as previously described (Heffernan *et al.*, 2006). Groups of eight mice were infected with either mutant or wild-type spores at a variety of doses ranging between $1.5 \times 10^3$ and $1.5 \times 10^7$ spores per mouse. Mice were monitored for a period of 14 days. For subcutaneous infections mice were infected in the scruff of the neck with doses ranging between $5 \times 10^3$ and $5 \times 10^6$ spores per mouse. $LD_{50}$ values were calculated using the methods of Reed and Muench (1938). All mouse experiments were performed using protocols approved by the University of Michigan on the Use and Care of Animals.

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