JOURNAL OF BACTERIOLOGY, Dec. 2005, p. 8055–8062 Vol. 187, No. 23
0021-9193/05/$08.00+0 doi:10.1128/JB.187.23.8055–8062.2005

Characterization of *Bacillus anthracis* Germinant Receptors In Vitro

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Received 29 June 2005/Accepted 12 September 2005

Bacillus anthracis **begins its infectious cycle as a metabolically dormant cell type, the endospore. Upon entry into a host, endospores rapidly differentiate into vegetative bacilli through the process of germination, thus initiating anthrax. Elucidation of the signals that trigger germination and the receptors that recognize them is critical to understanding the pathogenesis of *B. anthracis*. Individual mutants deficient in each of the seven putative germinant receptor-encoding loci were constructed via temperature-dependent, plasmid insertion mutagenesis and used to correlate these receptors with known germinant molecules. These analyses showed that the GerK and GerL receptors are jointly required for the alanine germination pathway and also are individually required for recognition of either proline and methionine (GerK) or serine and valine (GerL) as cogerminants in combination with inosine. The germinant specificity of GerS was refined from a previous study in a nonisogenic background since it was required only for germination in response to aromatic amino acid cogerminants. The *gerA* and *gerY* loci were found to be dispensable for recognition of all known germinant molecules. In addition, we show that the promoter of each putative germinant receptor operon, except that of the *gerA* locus, is active during sporulation. A current model of *B. anthracis* endospore germination is presented.**

Bacillus anthracis, like many *Bacillus* and *Clostridium* species, is capable of differentiation between two distinct cellular morphologies: the vegetative cell and the endospore. The endospore is metabolically dormant and provides the cell with the ability to withstand environmental conditions disadvantageous for vegetative life (28). *B. anthracis* endospores are thought to be the sole infectious cell morphotype, and their inoculation into a suitable host organism initiates the development of anthrax disease. Germination of the endospore, which is the regulated resumption of metabolic activity within the bacterial cell, is the earliest pathogenic event after endospore entry into the body (5, 6, 10, 11).

Germination has been well studied in a variety of endospore-forming species, and much is known regarding the specific signals that initiate the process (21). The signaling molecules vary widely among species, but in general small molecule nutrients, termed germinants, are recognized by receptors located within the inner endospore membrane (2, 12, 13, 22, 24, 25, 27). Activation of germinant receptors initiates a series of complex biophysical processes, which subsequently activate intracellular proteases and extracellular hydrolases that facilitate cellular differentiation to the vegetative form (28). The mechanism(s) of signal transduction from germinant receptors to downstream degradative enzymes remains poorly understood.

In *B. anthracis*, five distinct germination pathways have been recognized (16, 31). The alanine germination pathway (Ala) requires only the presence of L-alanine in concentrations above 30 mM, which are thought to be considerably higher than the concentration of this nutrient freely available in the host. At lower, physiologically relevant concentrations, L-alanine can cooperate with L-proline, comprising the alanine and proline (AP) response, or with either L-histidine, L-tyrosine, or L-tryptophan to make up the aromatic amino acid-enhanced alanine (AEA) pathway. Purine ribonucleosides have been noted to be of particular importance to germination of some *Bacillus* endospores (1, 2, 24). In *B. anthracis*, these nutrients must be in combination with a second cogerminant in order to trigger endospore germination (16). Inosine is the most potent purine cogerminant and is able to combine with several amino acids to comprise the amino acid and inosine-dependent (AAID) responses. AAID-1 includes the binary combination of inosine and either L-alanine, L-serine, L-valine, L-methionine, or L-proline, whereas in AAID-2 inosine pairs with L-histidine, L-tyrosine, L-tryptophan, or L-phenylalanine (16).

The genetic loci that encode germinant receptors have been identified and are typified by the *gerA* locus of *Bacillus subtilis* (21, 28), which functions in a simple germination response by sensing the presence of L-alanine. These loci are tricistronic operons expressed in the developing forespore during sporulation in a sigma G-dependent manner (3, 7), and homologous operons are found throughout the *Bacillus* and *Clostridium* genera (20, 26). Mutations in members of the *gerA* family of operons have been shown to cause loss of germination responses to specific germinant molecules (1, 2, 12, 16, 22, 23, 31). Seven *gerA*-type loci have been identified within the recently sequenced genome of *B. anthracis* (26). Six of the identified operons, *gerA*, *gerH*, *gerK*, *gerL*, *gerS*, and *gerY*, are located on the chromosome, while the seventh, *gerX*, is found within a pathogenicity island on the pX01 virulence plasmid (9). Two of the chromosomal operons, *gerS* and *gerH*, have been characterized previously by our group and are important, in combination, in response to inosine or alanine plus aromatic compounds (15, 16, 30, 31). The *gerX* locus has been shown to play a role in germination in vivo and in virulence in an animal model of infection (9). Here we use temperature-dependent,

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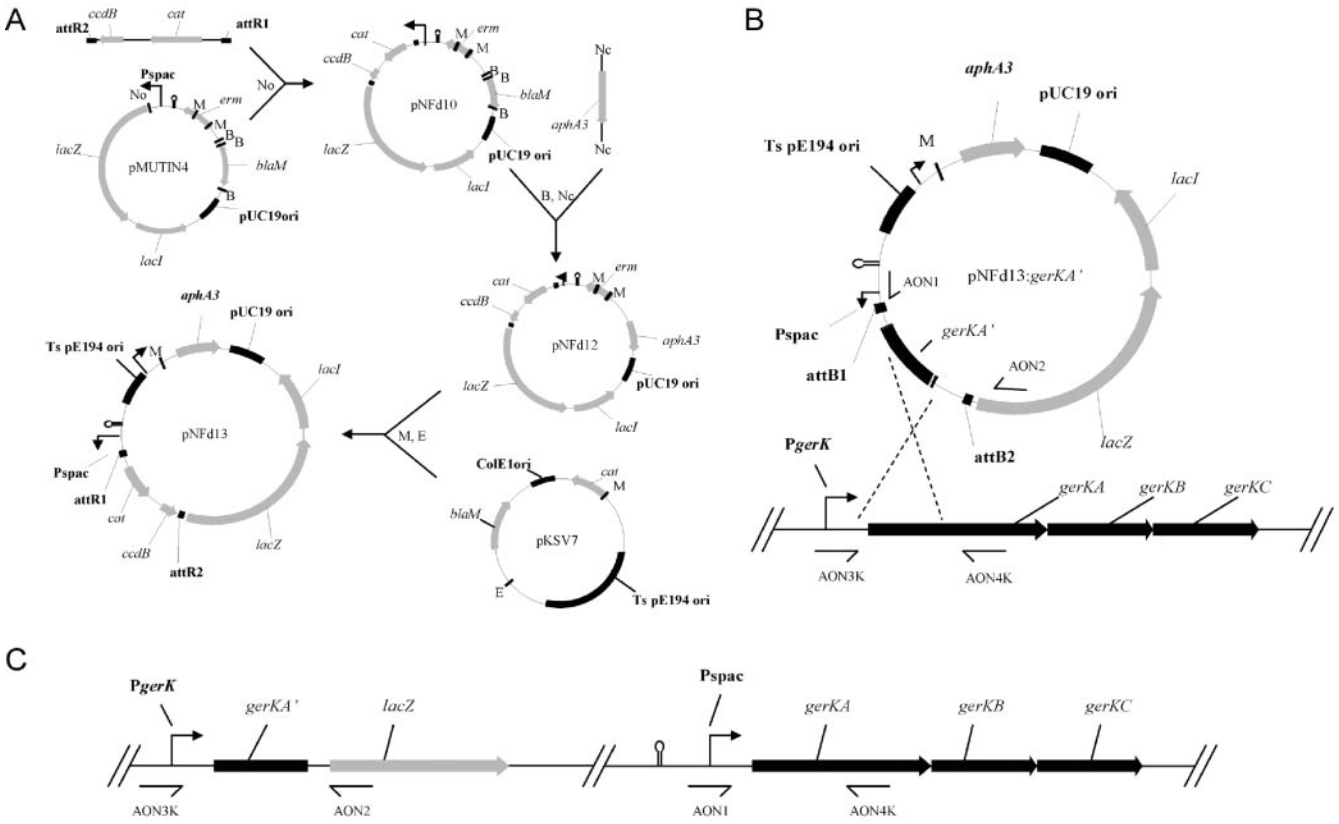

FIG. 1. Construction (A) and use (B and C) of pNFd13 for temperature-dependent, plasmid-insertion mutagenesis. Construction is described in detail in the text. No, NotI; B, BspHI; Nc, NcoI; M, MfeI; E, EcoRI. Note the strong transcriptional terminator upstream from Pspac in pMUTIN4 is maintained in pNFd13. The relevant genomic structure of *B. anthracis* carrying a pNFd13 derivative for mutation of the *gerK* locus is shown at 30°C (B) and at 39°C (C). Analysis primer-binding sites are indicated by single-sided arrows and are denoted AON1, AON2, AON3K, and AON4K.

plasmid insertion mutagenesis to determine the effect of mutation in any single germinant receptor locus on the in vitro germination profile of *B. anthracis* endospores as part of an effort to understand the mechanism through which this pathogen senses the desired host environment.

MATERIALS AND METHODS

Bacterial strains and antibiotics. The *B. anthracis* Sterne 34F2 strain was cultured on brain heart infusion (BHI; Difco) broth or solid media containing 15 g of agar per liter. *Escherichia coli* DB3.1 was used for cloning and propagation of plasmids containing the Gateway counter selectable marker *ccdB*. Otherwise, *E. coli* DH10B was used. Both strains were maintained on LB broth or plates. Plasmids were maintained by the addition of 50 μg of kanamycin sulfate/ml, 100 μg of ampicillin/ml, or 10 μg of tetracycline/ml, as appropriate. In order to prepare mutant and parental endospores, cells were revived from −80°C glycerol stocks by incubating overnight on BHI agar plates at 39°C. A single colony was then inoculated into 3 ml of BHI broth containing 0.5% glycerol. This culture was incubated 8 to 12 h at 39°C before back-dilution into 75 ml of modified G medium (31) without antibiotics (mutant precultures contained 50 μg of kanamycin sulfate/ml to maintain pNFd13). Cultures were then incubated at 39°C for 4 days. Endospores were collected by centrifugation, any residual vegetative cells were killed by incubation at 65°C for 30 min, and the pellets were washed extensively in deionized, distilled water to remove all cellular debris. Endospores were stored in deionized, distilled water at room temperature prior to germination analysis. For complementation studies, 10 mM IPTG (isopropyl-β-D-thiogalactopyranoside; Sigma) was added to sporulation cultures at the time of back-dilution into modified G medium since this resulted in greater levels of complementation than did addition later during growth or at the onset of sporulation (not shown). Maintaining cultures at 39°C had no deleterious effect on growth, and all mutants exhibited rates of sporulation similar to the parental strain, as judged by the acquisition of endospore heat resistance (not shown).

pNFd13 construction. In order to create the temperature-dependent disruption plasmid, a Gateway conversion cassette (Invitrogen) was inserted into the multiple cloning site of pMUTIN4 followed by exchange of the *erm* and *blaM* resistance determinants with a kanamycin resistance marker and the insertion of the temperature-dependent gram-positive origin of replication (Fig. 1). All enzymes were purchased from New England Biolabs, unless otherwise stated, and used according to the manufacturer's directions. pMUTIN4 was linearized at the unique NotI site, in the multiple cloning site, and the ends were made blunt by digestion with mung bean nuclease and dephosphorylated with shrimp alkaline phosphatase (Roche). Quick Ligase was then used to insert the Gateway vector conversion cassette B (Invitrogen) and create pNFd10. pNFd10 was digested with BspHI and ligated to an *aphA3* (kanamycin resistance) fragment amplified from pDG783 and digested with NcoI, to create pNFd12. pNFd12 was digested with MfeI, dephosphorylated, and ligated to an EcoRI-MfeI fragment from pKSV7 containing the temperature-dependent, gram-positive origin of replication. The resulting plasmid was denoted pNFd13. In order to accommodate a Gateway destination plasmid carrying a kanamycin resistance marker, pDONRtet was constructed by digesting pDONR201 with BspHI and ligation of a similarly digested amplicon from pDG1515 containing a tetracycline resistance marker.

Disruption of germinant receptor operons by temperature-dependent, plasmid-insertion mutagenesis. Genomic DNA was harvested from *B. anthracis* 34F2 by using the DNeasy protocol (QIAGEN). In cases where DNA was isolated from endospores, one half-hour of germination in LB broth at 37°C preceded the isolation protocol. Fragments approximately 0.5 kb in size, from ca. positions −15 to 485 with respect to the translational start sites of $gerA_A$, $gerK_A$, $gerH_A$, $gerL_A$, $gerS_A$, $gerX_B$, and $gerY_A$, including the Shine-Dalgarno sequences, were amplified from *B. anthracis* 34F2 genomic DNA by using primers designed to incorporate a 5′ attB1 sequence and a 3′ attB2 sequence for entry into the

Gateway cloning system (Invitrogen). Primer sequences will be provided upon request. Each resulting amplicon was transferred to pDONRtet by using standard BP reaction conditions (Invitrogen), followed by transformation of chemically competent DH10B *E. coli* and selection on LB plates containing 10 μg of tetracycline/ml. Cloned amplicons observed to be free from PCR-generated mutations were transferred to pNFd13 by using standard LR reaction conditions (Invitrogen), again followed by transformation of chemically competent DH10B *E. coli* and selection on LB plates containing 50 μg of kanamycin sulfate/ml. The resulting plasmids, in which the negative selectable Gateway cassette was replaced with the cloned amplicons, were passed through the *dam dcm E. coli* host INV110 (Invitrogen) prior to electroporation into *B. anthracis* as described previously (18) with the following modifications; LB medium with the addition of 1% glucose was used to prepare competent cells, the culture was incubated with vigorous aeration, and cells were harvested at an optical density of between 0.2 and 0.4. Transformants were recovered at 30°C on LB plates containing 1% glucose and 5 μg of kanamycin sulfate/ml. Individual transformant colonies were then suspended in BHI broth and serial dilutions were plated on BHI agar plates containing 50 μg of kanamycin sulfate/ml. After overnight incubation at 30°C, single colonies were used to inoculate 3 ml of BHI broth containing 50 μg of kanamycin sulfate/ml. This culture was incubated at 30°C until stationary phase was reached, at which time 2 ml of the culture was collected for storage (−80°C glycerol stock), and total DNA was isolated from the remaining 1 ml. Isolated DNA was used to transform *E. coli* DH10B to kanamycin resistance in order to verify the presence of pNFd13.

Integration of the plasmid into the targeted locus was accomplished by revival of the appropriate −80°C glycerol stock on BHI agar plates containing 50 μg of kanamycin sulfate/ml and overnight incubation at 30°C. A single colony was then suspended and incubated overnight at 30°C in 2 ml of BHI broth containing 50 μg of kanamycin sulfate/ml. The next day, the culture was back-diluted 1:100 into the same medium (50 ml, total volume) and incubated at 30°C with vigorous aeration until an optical density of 0.2 to 0.3 was reached, at which point the culture was shifted to 39°C with continued shaking until the optical density reached 0.9. Serial dilutions were then plated on BHI agar containing 50 μg of kanamycin sulfate/ml and incubated at 39°C to obtain colonies in which pNFd13 had integrated into the cloned locus by homologous recombination. Individual colonies (two for each target) were then suspended in 1 ml of BHI and serial dilutions were plated as described above. The serial dilution procedure was repeated twice more to ensure the isolation of a clonal population for each mutant strain. After the third round of enrichment, 3 ml of BHI broth containing 50 μg of kanamycin sulfate/ml was inoculated with a single colony. After the culture reached stationary phase, total DNA was isolated from 1 ml of the culture for genomic characterization ensuring the expected genotype, and the remaining 2 ml was collected by centrifugation and suspended in 1 ml of 50% glycerol for storage at −80°C. Resulting plasmid-insertion mutants were designated GERAd, GERHd, GERKd, GERLd, GERSd, GERXd, and GERYd.

PCR (PCR Master Mix; Promega) was used to assess the genomic structure of each strain. Primer binding sites are shown in Fig. 1 (sequences provided upon request). Primers AON1 and AON2 are common to all strains and adhere to sequences within pNFd13, upstream of P_{spac} and within *lacZ*, respectively. Primers AON3 and AON4 are unique to each targeted locus and adhere to sequences outside of the region of homology cloned into pNFd13, within the promoter region and first cistron of each operon, respectively. Four PCRs, along with the pertinent control reactions, were conducted to characterize the location of integration in each mutant. Reaction 1, a 30-cycle reaction with AON3 and AON4, was used to verify the disruption of the original chromosomal structure. Since integration of pNFd13 at the cloned locus results in a loss of the expected amplicon from the parental genome, a control reaction was carried out in parallel in which 5 ng of parental DNA (2% of total template) was added to the reaction to ensure the negative result was specific to the strain analyzed. Reactions 2 and 3, 25 cycles with AON1 and AON3 or AON2 and AON4, respectively, were performed to amplify the left and right sides of the expected integration structure. Reaction 4, a 30-cycle reaction with AON1 and AON2, was used to verify the absence of replicating plasmid. In order to verify the fidelity of this reaction, 5 ng of DNA isolated from the original 30°C clone (before integration) was added to a parallel reaction. It is important to note that the reaction four amplicon should be detected from strains in which a double integration event has occurred either at the targeted locus or elsewhere on the chromosome. Back-transformation of isolated DNA into *E. coli* DH10B was also used to verify the absence of replicating plasmid. After the integration protocol described above for each disruption, reactions 1 and 4 showed no product (with positive control reactions), and reactions 2 and 3 produced readily detectable amplicons of the expected size. Since passage at high temperature has the potential to cure the pX01 plasmid at low frequencies, primers AON3X and AON4X were used to verify the presence of pX01 in the parental, GERAd, GERHd, GERKd, GERLd, GERSd, and GERYd strains. After integration, all manipulations were conducted at 39°C to maintain the desired genomic structure, which was verified after each endospore preparation prior to phenotypic evaluation.

Expression analysis. In order to monitor expression levels from the promoter of each putative *ger* operon, cultures were prepared for sporulation as described above. We collected 1-ml aliquots from each culture throughout the growth cycle and monitored promoter activity by assaying for β-galactosidase activity as described previously (4) using toluene for cell disruption.

Germination analysis. After heat activation (20 min at 65°C) of endospores, germination was analyzed at 37°C by loss of optical density as described previously (12) except that phosphate-buffered saline (PBS; pH 7.4; Gibco) was used as the base germination buffer. Since buffer constituents can have major affects on germination rates, PBS is used in the present study for the following reasons: its approximation of physiological fluids in terms of osmolarity and buffering capacity, the potential for included sodium ions to enhance otherwise weak germination responses (8, 31), and the commercial availability of consistently formulated lots. Endospores and experimental germinants were individually suspended at a 2× concentration in PBS and then mixed in a 1:1 ratio to initiate the germination reaction. Germination is presented as percent decrease in optical density at 600 nm (OD_{600}) of the test mixture versus time. A decrease in the optical density equal to ~60% correlated to a loss of heat resistance in >99% of the culture for all conditions tested, and the relationship between decreases in optical density and loss of heat resistance was linear for decreases in optical density of between 10 and 50% (not shown).

RESULTS

Disruption of putative germinant receptors by temperature-dependent, plasmid insertion mutagenesis. In order to assess the physiological role of each of the seven putative germinant receptors encoded within the *B. anthracis* genome, temperature-dependent, plasmid insertion mutagenesis was used to individually disrupt the transcription of each operon by the native promoter and to allow controlled isogenic complementation via IPTG-induced transcription from the plasmid-encoded P_{spac} promoter (Fig. 1). Similar approaches have been successful in other gram-positive organisms, including *B. subtilis*, where recent efforts have used plasmid insertion mutagenesis by pMUTIN4, the parent plasmid of pNFd13, to assign functions to a large number of uncharacterized genes (17). Other researchers have recently reported the successful use of pMUTIN4 for mutation of the *gerR* germinant receptor operon within *Bacillus cereus* 14579, a close genetic relative of *B. anthracis* (12). Perhaps because the regions of homology between the chromosome and plasmid are considerably smaller than those used by Hornstra et al. (12), initial efforts to use unaltered pMUTIN4 within *B. anthracis* were unsuccessful (not shown). Therefore, in addition to making the plasmid compatible with the Gateway system for rapid subcloning, the *erm* selection marker was replaced with a *kan* cassette from pDG783 and the temperature-dependent gram-positive origin of replication from pKSV7 was inserted. With the resulting plasmid, pNFd13, it was possible to achieve consistent transformation of *B. anthracis* at the permissive temperature of 30°C and to select for specific integration into the chromosome by Campbell-type single-crossover recombination with the targeted locus at the nonpermissive temperature of 39°C (Fig. 1).

Each of the seven *gerA*-type operons encoded within the *B. anthracis* genome was successfully disrupted by using this approach (Table 1). The chromosomal structure of the resulting mutants was monitored by PCR after each preparation of endospores since control experiments showed this technique to be extremely sensitive in detecting undesirable plasmid exci-

TABLE 1. Strains used in this study

Strain	Relevant genotype	GenBank no. of target	Source or reference
Parental			
34F2	pX01$^+$ pX02$^-$		Laboratory stock
Derivatives			
GERAd	*gerA*::pNFd13	AAT55237.1	This work
GERHd	*gerH*::pNFd13	AAT56925.1	This work
GERKd	*gerK*::pNFd13	AAT52930.1	This work
GERLd	*gerL*::pNFd13	AAT53003.1	This work
GERSd	*gerS*::pNFd13	AAP27387.1[a]	This work
GERXd	*gerX*::pNFd13	AAM26101.1[b]	This work
GERYd	*gerY*::pNFd13	AAT53054.1	This work
ΔgerH_A	*ger*H_A::*erm*		31
Δ34F2	pX01$^-$ (*gerX*$^-$)		Laboratory stock
Δ34F2 *gerS*	pX01$^-$ (*gerX*$^-$) *ger*S_A::pT7Blue *erm*		16

[a] GenBank sequence from *B. anthracis* Ames.
[b] GenBank sequence from *B. anthracis* A2012.

sion events, even if they occur in <1% of the endospore population (not shown). Although plasmid excision events are extremely rare, these did occasionally take place when mutants were sporulated without the presence of antibiotic, as assessed by PCR amplification of the parental locus (reaction one) or replicating plasmid (reaction four or positive back-transformation) and, in these instances, that endospore sample was discarded.

Pilot studies using the pNFd13 system in *B. subtilis*, in which the *gerA* locus was targeted, demonstrated the practicality of temperature-dependent, plasmid insertion mutagenesis for analysis of germinant receptor-related phenotypes. The resulting mutant showed a complete and specific loss of alanine-induced germination, as judged by loss of optical density and heat resistance, which was restored by sporulation in the presence of IPTG (not shown). Disruption of *gerH* in the present study serves the same function in *B. anthracis* since the GERHd mutant phenotype is identical to that reported when the *gerH* operon was mutated by allelic exchange (31). In addition to serving as a control strain for temperature-dependent, plasmid insertion mutagenesis, this finding corroborates the previously noted phenotypes associated with mutation to *gerH*.

In addition to providing phenotypic analysis, the temperature-dependent, plasmid insertion mutagenesis approach was useful in providing information regarding activity level of the promoters of each germinant receptor locus, as well as isogenic complementation of the null phenotype by the addition of the P_{spac} inducer, IPTG. In all cases where plasmid-insertion mutagenesis resulted in an altered germination phenotype, sporulation of the mutant in the presence of 10 mM IPTG resulted in partial or complete complementation of the mutant phenotype, confirming the link between the targeted locus and the observed phenotype (Table 2).

Expression levels of the putative *B. anthracis* germinant receptors. One benefit of temperature-dependent, plasmid insertion mutagenesis is that integration of pNFd13 into each germinant receptor locus creates a transcriptional fusion between the native promoter and the *lacZ* reporter gene. In order to assess the level of transcription of each germinant receptor operon, promoter activity was monitored in mutant strain cultures by assaying β-galactosidase activity throughout the growth cycle. As expected, very low, background levels of β-galactosidase activity were observed in cultures lacking pNFd13 and in the described disruption strain cultures prior to the onset of sporulation (Fig. 2). β-Galactosidase levels peaked between 5.5 and 6.0 h postinoculation in cultures of the GERHd, GERKd, GERSd, GERXd, and GERYd mutants but remained at background levels in the GERAd mutant (Fig. 2). The *gerH*, *gerK*, *gerL*, *gerS*, and *gerX* promoters seem to exhibit similar levels of

TABLE 2. In vitro germination

Germinant[a] (pathway)	Mean % decrease in OD_{600} ± SD in 30 min for[b]:						
	34F2	GERKd		GERLd		GERSd	
	−	−	+	−	+	−	+
Buffer[c]	1.9 ± 1.8	2.3 ± 1.9	2.7 ± 2.1	1.7 ± 1.2	2.3 ± 2.5	2.5 ± 2.0	1.8 ± 2.5
L-Ala	2.5 ± 1.7	2.9 ± 3.6	12.3 ± 4.2	3.2 ± 1.6	9.5 ± 2.1	2.8 ± 1.7	2.2 ± 0.8
L-Ala (50 mM) (Ala)	54.2 ± 4.5	23.3 ± 2.8	59.2 ± 7.5	35.2 ± 1.7	63.8 ± 1.8	52.4 ± 2.5	ND
L-Ala L-Pro (AP)	42.5 ± 3.0	5.6 ± 1.5	47.1 ± 3.8	18.3 ± 2.2	48.6 ± 3.9	47.5 ± 2.1	ND
L-Ala L-Trp[d] (5 mM; AEA)	40.1 ± 6.2	32.6 ± 1.3	37.1 ± 1.1	3.4 ± 1.0	20.4 ± 2.7	17.9 ± 3.0	34.6 ± 2.6
L-Ala inosine* (AAID-1Al)	60.2 ± 3.8	59.3 ± 1.4	ND	44.4 ± 3.9	61 ± 1.7	56.0 ± 1.2	ND
Inosine* L-Trp[d] (5 mM; AAID-2)	42.0 ± 1.4	43.5 ± 3.1	ND	44.9 ± 3.7	ND	22.3 ± 1.4	41.4 ± 1.6
Inosine* L-His (AAID-2)	44.2 ± 3.3	44.0 ± 2.1	ND	37.6 ± 2.4	ND	13.7 ± 3.4	33.8 ± 1.2
Inosine* L-Pro (AAID-1)	50.4 ± 2.9	5.7 ± 1.2	56.5 ± 7.1	55.8 ± 1.2	ND	52.1 ± 3.9	ND
Inosine* L-Met (AAID-1)	54.8 ± 5.4	1.57 ± 2.2	60.0 ± 6.1	53.6 ± 2.2	ND	55.4 ± 3.7	ND
Inosine* L-Ser (AAID-1)	46.7 ± 4.3	62.7 ± 1.2	ND	19.7 ± 5.2	40.9 ± 1.3	61.1 ± 1.2	ND
Inosine* L-Val (AAID-1)	45.3 ± 1.6	50.7 ± 2.8	ND	11.4 ± 2.0	29.1 ± 2.1	41.2 ± 3.2	ND

[a] L-Alanine is at 0.5 mM, inosine is at 1 mM, and all other amino acids are at 50 mM unless otherwise noted. *, Similar results were seen with adenosine.
[b] Results are the average of triplicate experiments with duplicate endospore preparations with one standard deviation. The germination profiles of GERAd, GERXd, and GERYd are identical to parental 34F2. The germination profile of GERHd is identical to one already published (31) and is omitted for clarity. − or +, Without or with 10 mM IPTG added to the sporulation culture at the time of inoculation, respectively; this had no effect on germination of the parental strain (omitted for clarity). ND, not determined.
[c] PBS (pH 7.2).
[d] Similar results were seen with L-tyrosine and L-phenylalanine.

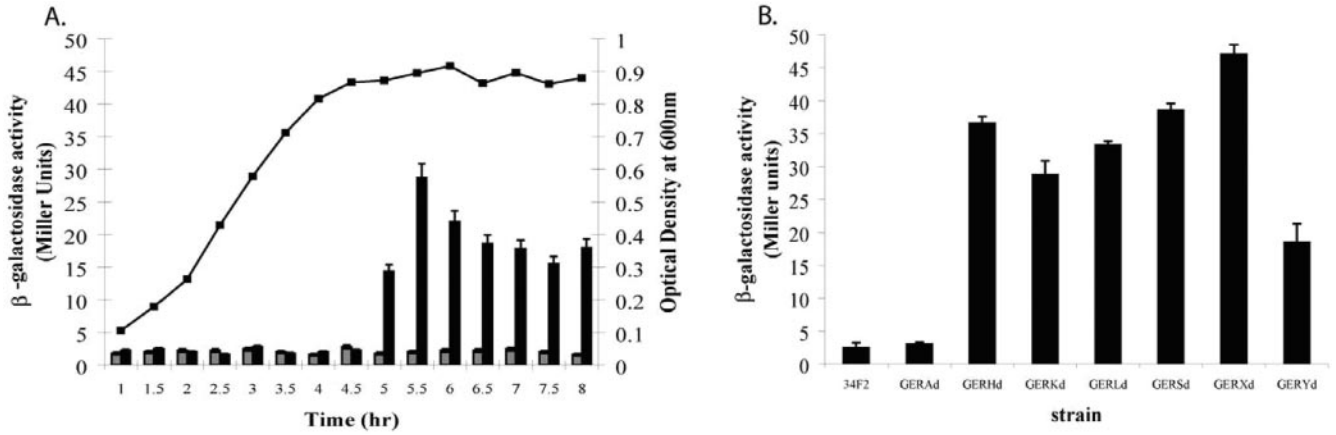

FIG. 2. Expression analysis of the *gerA*-type germinant receptor operons of *B. anthracis*. β-Galactosidase activity from each promoter-*lacZ* fusion was monitored as described in the text for cultures of *B. anthracis* 34F2 (parental, ▨) or plasmid-insertion mutant strains (■) throughout vegetative growth and endospore formation (A) or at the time of optimum expression (B). For clarity, only the parental and GERKd expression profiles are shown in panel A. The GERAd profile was indistinguishable from the parental strain, whereas all other mutant strains exhibited expression profiles very similar to that of GERKd. The time of optimum β-galactosidase activity occurred at either 5.5 h (GERHd, GERKd, and GERSd) or 6.0 h (GERLd, GERXd, and GERYd) postinoculation. The growth rates of all mutants were indistinguishable from the parental strain, thus a single curve is shown. β-Galactosidase values are the average of two independent experiments performed in triplicate with the standard deviation shown.

activity during sporulation, whereas the *gerY* promoter was about half as active (Fig. 2). These results are consistent with mRNA expression patterns seen in previous microarray experiments (19) but allow for better semiquantitative comparison between the respective levels of transcription from each receptor locus.

Role of the *gerA*, *gerX*, and *gerY* loci. The *gerA* and *gerY* loci were not expected to contribute to the germination capabilities of *B. anthracis* endospores because the *gerAA*, *gerYB*, and *gerYC* open reading frames include frameshift mutations (26). However, given the available evidence for functional interactions between different germinant receptors or the individual protein components thereof (14), the contribution of *gerA* and *gerY* to endospore germination was evaluated. Disruption of either locus had no effect on endospore germination profiles under any conditions tested (not shown). Combined with an absence of expression from the *gerA* promoter and the presence of multiple mutations within the coding regions of these loci, this evidence indicates that *gerA* and *gerY* may not encode functional germinant receptors. The presence of nonfunctional germinant receptors does not appear to be a unique occurrence, since the *B. subtilis* genome contains two *gerA*-type operons that do not contribute to the known germination responses of that organism (23). However, it should be noted that the receptors in question may be expressed during sporulation under conditions other than those examined and that the receptors may recognize currently unknown germinant molecules.

All known in vitro germination pathways are utilized by *B. anthracis* Δ34F2 endospores (16), which lack the pX01 virulence plasmid and thus the *gerX* germinant receptor locus. Similarly, the GERXd mutant showed germination profiles identical to the parental strain (not shown). This receptor is expressed during sporulation (Fig. 2), but its cognate ligand remains unknown.

The GerK and GerL receptors are responsive to alanine. In order to identify the particular germinants recognized by each germinant receptor, the responses of parental and mutant endospores to defined germinant combinations were monitored. L-Alanine, albeit at nonphysiologically high levels, is the only germinant identified to date that is capable of initiating germination of *B. anthracis* endospores without the addition of a cogerminant molecule. Early studies of the saturation kinetics the Ala response suggested that it likely involved multiple receptors (16), and here we report that disruption of either *gerK* or *gerL* impaired germination in response to 50 mM L-alanine (Table 2).

The germination response observed when *B. anthracis* endospores are incubated with 50 mM L-alanine is comprised of two phases: an initial rapid phase, which usually results in germination of ca. 80% of the endospore population within 10 min, and a second residual phase where the remaining population completes germination within 30 min (Fig. 3). Interestingly, mutation to either the *gerK* or *gerL* operons resulted in the loss of only one phase of this germination response. GERLd endospores appear able to complete phase 1 of this phenotype but are unable to complete phase 2. The opposite is true for endospores lacking GerK, which only begin to germinate after prolonged incubation. These data suggest that although the specificities of GerL and GerK overlap, both receptors contribute individual components to the overall germination profile seen in response to high concentrations of L-alanine.

Lower, more physiological levels of L-alanine (~1 mM) contribute to the AP, AEA, and AAID-1 germination pathways (16). Disruption of *gerL* leads to a defect in germination in each case where L-alanine serves as a cogerminant (Table 2). However, the requirement is only absolute when aromatic amino acids serve as the cogerminant (AEA pathway). Absence of the GerK receptor abrogated the germination response only when L-proline was the cogerminant (AP pathway) and had no effect when aromatic amino acids or ribonucleosides were used. These observations further the idea that although GerK and GerL both sense L-alanine, they do so differently, perhaps while in complex with separate cogerminant receptors.

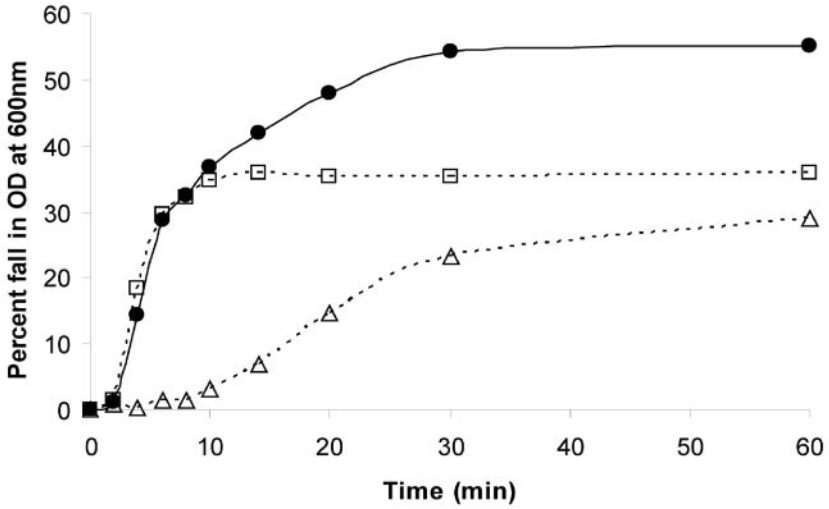

FIG. 3. Germination of parental (●), GERLd (□), and GERKd (△) endospores as monitored by the decrease in optical density of an endospore suspension during incubation with 50 mM L-alanine in PBS (pH 7.2). The results are averages from triplicate experiments on two independent endospore preparations. Standard deviation is <15% of the mean.

GerK and GerL partially diverge in ligand recognition. In addition to showing the defects described above, the GERKd and GERLd mutants are defective for unique subsets of the AAID-1 response. The AAID-2 response is fully functional in both mutants, correlating the AAID-1 defect with the amino acid cogerminant. When proline or methionine is used, there is an absolute requirement for the GerK receptor, but not GerL. However, the cogerminants serine and valine require GerL but not GerK (Table 2). These results show that GerK and GerL exhibit partially overlapping germinant specificities, in that they are both involved in the alanine response, while also maintaining unique amino acid recognition capabilities.

GerS is required for recognition of aromatic amino acids. A previous study (16) showed that the GerS receptor was required for recognition of aromatic compounds important for the AEA, AAID-1, and AAID-2 germination pathways. Without a single germinant (or class of germinants) that is consistently required for those three responses, it was impossible to further define the specificity of the GerS receptor in the original study. However, the present study used a different genetic background (which includes pX01, and thus *gerX*) for the analysis of mutation to *gerS*. The GERSd mutant shows a defect only in the AEA and AAID-2 pathways, which both require aromatic amino acids as cogerminant molecules. The defect can be linked to the aromatic amino acid requirement by the observation that pathways involving alanine or inosine are fully functional in the GERSd mutant when nonaromatic amino acid cogerminants are supplied.

DISCUSSION

Identification of germinant molecules that trigger germination of *B. anthracis* endospores in vitro, and the germinant receptors that sense them, is an important first step toward characterizing the chemical signals detected by this invading bacterium upon entry into a host organism. Recent work has identified a number of germinants to include concentrations of L-alanine greater than 30 mM or various binary combinations of micromolar concentrations of L-alanine, L-proline, L-serine, L-methionine, L-histine, aromatic amino acids, and purine ribonucleosides (16, 31; the present study). Although these surveys have tested many compounds, they are by no means exhaustive, and other germinants are likely to exist. Buffer conditions, especially with respect to ion composition, can have remarkable effects on in vitro bacterial endospore germination. Initial germination studies (16, 31) focused on reaction conditions conducive to the identification of strong in vitro germinants by minimizing monovalent ion composition. In these buffers, certain germinant combinations perform poorly. In the present study, the ionic conditions of PBS are used to enhance these responses so that mutations leading to their loss might be assessed more readily. The mechanism(s) of ion contribution to germination is currently unknown.

We used temperature-dependent, plasmid-insertion mutagenesis here to analyze the effects of mutational loss of any single germinant receptor within the *B. anthracis* genome. Mutation to *gerH* corroborated the previously described (31) role for this receptor and substantiated the mutational approach used in the present study. Mutation of the *gerA* or *gerY* operons, which both contain natural frameshift mutations, had no detectable effect on in vitro germination. Germination profiles of endospores lacking either GerK or GerL indicate that these receptors both recognize L-alanine while also maintaining unique coligand profiles. Mutation to the *gerS* locus specifically inhibited germination in response to aromatic amino acid cogerminants. Comparison between results obtained by loss of GerS alone (the present study) or both GerS and GerX (16; by specific mutation to *gerS* and loss of the pX01 plasmid) may be useful in assigning a putative role for GerX in the AAID-1 response. However, it is not clear whether GerX would function to sense the purine or the amino acid cogerminant in that response. Furthermore, it is reasonable to conclude that germination-specific determinants other than *gerX* may exist on the pX01 plasmid. Thus, a more direct method should be used

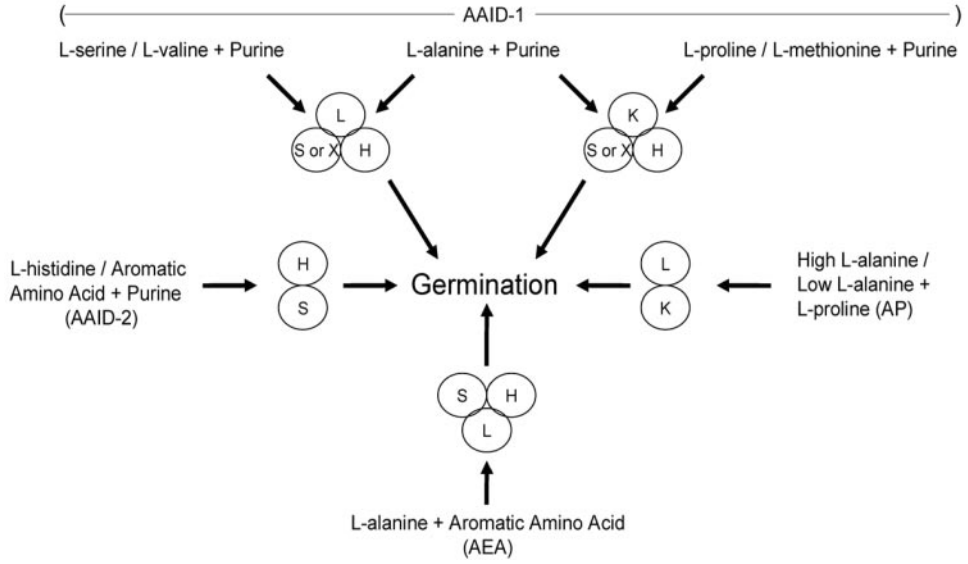

FIG. 4. Current model for *B. anthracis* germination pathways. Receptor designations are placed in particular pathways if their presence is required for its proper function. Either the GerS or GerX receptor, but not both, is required for function of the AAID-1 pathway. The binary germinant combination of 1 mM inosine and 1 mM alanine apparently activates at least two receptor complexes and can tolerate the mutation of any single receptor operon.

for identification of germinants recognized by GerX. Others have shown that loss of GerX results in a 70% decrease in macrophage-associated germination (9), and we have seen a similar effect in the GERXd strain (not shown). Whatever the ligand specificity of this receptor, GerX is functional and appears to play some role in the pathogenesis of *B. anthracis*.

Our findings allow an updated model of the in vitro germination pathways recognized by *B. anthracis* endospores to be presented (Fig. 4). The alanine and AP pathways show a requirement for both the GerK and GerL receptors, with GerK apparently playing a role in sensing both L-alanine and L-proline, whereas GerL is specific to L-alanine in these pathways. The AEA response requires GerL, GerS, and GerH, again with GerL contributing to the L-alanine-sensing capability, while GerS and GerH seem to be jointly required for the recognition of the aromatic amino acid. The AAID-1 pathway also requires GerH, presumably for recognition of the aromatic nucleoside. The initial definition of this pathway (16) included only biochemical parameters and, after this mutational analysis, it is now clear that the AAID-1 pathway can be divided further. GerL is required when either L-serine or L-valine is the cogerminant (AAID-1L), and GerK is required when either L-proline or L-methionine is present (AAID-1K). In addition, the binary combination of L-alanine and inosine tolerates mutation to any single receptor and could therefore be thought of as a separate AAID-1 subgroup. Finally, the AAID-2 pathway requires the GerH and GerS receptors with no detectable contribution from GerK or GerL.

We believe this model of in vitro germination to be relevant to understanding the mechanism(s) through which *B. anthracis* accurately distinguishes between the soil and host environments in order to break dormancy and initiate infection. Thus, it is interesting that diverse sets of purine ribonucleosides, aromatic amino acids, or nonaromatic amino acids can serve as cogerminants, and the present study shows that both the GerK and GerL receptors recognize multiple amino acids. Albeit less dramatic than that seen here, some promiscuity in germinant recognition has been reported previously for the *B. subtilis* alanine germination pathway and may be common to a variety of endospore-forming species (8, 29, 32). The mechanism of such promiscuity is unknown, but it could result from flexible ligand binding sites in these receptors or from alternative pairing of inter-receptor proteins such as that recently reported for the GerAA, GerAB, and GerBC proteins in *B. subtilis* (14). In any case, such observations likely indicate that germinant receptors recognize molecular patterns indicative of an environment conducive to the vegetative life cycle and are not exclusively devoted to the sensing of any single nutrient. We hypothesize that this function may contribute to the broad host range of *B. anthracis* (5) by allowing recognition of one or more classes of compounds that may serve as host-specific signals, despite the possibility that the specific member molecules, and relative amounts thereof, could vary between potential host species.

In addition, each of the known *B. anthracis* germination pathways requires at least two distinct receptors. Cooperation between receptors with different ligand profiles may represent an effective strategy for ensuring endospore dormancy is broken only in environments favorable for growth. The absolute requirement for activation of at least two distinct germinant receptors for efficient in vitro germination of *B. anthracis* endospores may indicate that this pathogen requires multiple signals for accurate recognition of the host environment. The mechanism of germinant receptor cooperativity is unknown and a focus of current research efforts.

This in vitro analysis has identified the individual contribution of each germinant receptor to the known germination phenotypes of *B. anthracis* endospores. In addition, we have seen that germinant receptors are able to recognize multiple compounds within a specific class of nutrients and can thus be

thought of as molecular pattern receptors and that each known germination pathway requires at least two distinct receptors. It is likely that, together, these requirements for germination contribute to the high fidelity with which *B. anthracis* must distinguish the soil environment from a host animal during its complex life cycle.

ACKNOWLEDGMENTS

We thank L. Shetron-Rama and N. Bergman for valuable discussions.

This study was supported in part by HHS contract N266200400059C/N01-AI-40059, by NIH grant AI08649, and by the Great Lakes and the Southeast Regional Centers of Excellence for Biodefense and Emerging Infections.

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