

gerT, a Newly Discovered Germination Gene under the Control of the Sporulation Transcription Factor σ^K in *Bacillus subtilis*[∇]

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We report the identification of a gene, herein designated *gerT* (formerly *yoZR*), that is involved in germination by spores of *Bacillus subtilis*. The *gerT* gene is induced late in sporulation under the positive control of the transcription factor σ^K and under the negative control of the DNA-binding protein GerE. The *gerT* gene product (GerT) is a component of the spore coat, and its incorporation into the coat takes place in two stages. GerT initially assembles into foci, which then spread around the developing spore in a process that is dependent on the morphogenetic protein CotE. Mutant spores lacking GerT respond poorly to multiple germinants and are impaired at an early stage of germination.

Bacillus subtilis is a gram-positive soil bacterium that is capable of metamorphosing into a dormant cell known as the spore (or more properly, the endospore) (14, 23). Under conditions of nutrient availability, *B. subtilis* cells are metabolically active and grow and divide by binary fission. When nutrients become scarce, however, the cells initiate the process of sporulation and undergo a single round of asymmetric division to form two distinct cell types: the mother cell and the forespore, the prospective spore. The septal membranes dividing the two cells then migrate around the forespore in a process known as engulfment. Ultimately, the forespore is pinched off as a protoplast in the mother-cell cytoplasm, surrounded by a double membrane. Protective layers are then assembled around the forespore: a peptidoglycan cortex between the two membranes and a proteinaceous shell, the coat, around the outer membrane. The coat, which consists of an inner coat and an outer coat, is a complex structure that is composed of more than 50 proteins (2, 4, 11–13). Once the spore is fully mature, the mother cell lyses and releases the spore, which can survive for long periods of time in a dormant, metabolically inactive state, protected from harsh environmental conditions. However, the spore is equipped to monitor its environment and rapidly resumes vegetative growth when exposed to nutrients in a process known as germination (20, 29).

Gene expression during sporulation is governed by the ordered appearance of a series of RNA polymerase sigma factors and DNA-binding proteins (32). The last sigma factor to appear is σ^K , which acts in the mother cell and is responsible for the transcription of approximately 89 single-gene transcription units and operons (5). Many of these σ^K -controlled genes contribute to spore formation, to the formation of the spore coat, and to the formation of a spore that is competent to germinate efficiently. One of the genes under σ^K control, *gerE*, encodes the terminal transcription factor in the sporulation regulatory cascade. The *gerE* gene encodes a DNA-binding

protein that is both an activator and a repressor. GerE acts in conjunction with σ^K -containing RNA polymerase to turn on the expression of the final class of sporulation genes. The appearance of GerE also switches off the expression of some genes that had been activated by σ^K .

Here we report the identification of a member of the σ^K regulon, *yoZR*, that is involved in spore germination. The *yoZR* gene, which we henceforth refer to as *gerT*, is a newly identified member of the σ^K regulon that had been overlooked in earlier transcriptional profiling experiments (5, 30). As in the case of certain other members of the regulon, *gerT* is switched on by σ^K and then switched off by the appearance of the DNA-binding protein GerE (5). The *gerT* gene product is herein shown to be a component of the coat that is incorporated into the protein shell that surrounds the spore in a two-step process. Evidence indicates that spores lacking GerT, which respond poorly to a variety of germinants, are impaired at an early stage of germination.

MATERIALS AND METHODS

Growth conditions. Strains were grown in Luria-Bertani medium (LB) at 37°C unless otherwise noted. The following antibiotics were included when appropriate: chloramphenicol (5 μ g/ml), erythromycin plus lincomycin (1 μ g/ml and 25 μ g/ml, respectively), spectinomycin (100 μ g/ml), ampicillin (100 μ g/ml), kanamycin (5 μ g/ml; also used to select for neomycin resistance), and tetracycline (10 μ g/ml).

Strain and plasmid construction. Strains and plasmids used for this study are listed in Table 1. *B. subtilis* strains were derived from the wild-type strain PY79 (38) or, for the strains used in Fig. 4, the wild-type strain PS832 (gift of Peter Setlow). *B. subtilis* competent cells were prepared by the one-step method previously described (37). All PCR products utilized in plasmid construction were amplified from PY79 chromosomal DNA; primers used for the generation of these and other PCR products are listed in Table 2. All plasmids were propagated in *Escherichia coli* strain DH5 α , with the exception that the integration plasmid pCF170 (see below) was passaged through the *recA*⁺ *E. coli* host TG1 prior to being used for transformation of *B. subtilis*.

To generate the translational *gerT-lacZ* reporter gene (pCF173), a PCR fragment containing the first 6 codons of *gerT* and approximately 325 bp of upstream sequence was amplified using the oligonucleotide primers oCF120 and oCF121, digested with BamHI/SalI, and ligated into BamHI/SalI-digested pDG1728 (7). Transformation of PY79 with pCF173 yielded CF174 (*amyE::gerT-lacZ spc*). To generate CF183 (*spoIVCBA::erm amyE::gerT-lacZ spc*) and CF184 (*gerE Δ ::erm amyE::gerT-lacZ spc*), PE451 (gift of Patrick Stragier) and PE454 (5), respectively, were transformed to spectinomycin resistance with chromosomal DNA from CF174.

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Reference, source, or construction
<i>B. subtilis</i> strains		
PY79 derivatives		
PY79	Wild type; prototrophic	38
AHB83	<i>amyE::Phyerspank-luxABCDE spc</i>	pAH60 → PY79
CF111	$\Delta 1::tet$	This study
CF152	$\Delta 2::erm$	This study
CF164	<i>gerTΔ::spc</i> ($\Delta 3$)	This study
CF167	<i>gerTΔ::spc; amyE::gerT cm</i>	pCF165 → CF164
CF172	<i>gerTΩpCF170(gerT-gfp) spc</i>	pCF170 → PY79
CF174	<i>amyE::gerT-lacZ spc</i>	pCF173 → PY79
CF177	<i>cotEΔ::cm; gerTΩpCF170(gerT-gfp) spc</i>	CF172 → RL322
CF178	<i>spoIVAΔ::neo; gerTΩpCF170(gerT-gfp) spc</i>	CF172 → RL1397
CF183	<i>spoIVCBΔ::erm; amyE::gerT-lacZ spc</i>	CF174 → PE451
CF184	<i>gerEΔ::cm; amyE::gerT-lacZ spc</i>	CF174 → PE454
CF203	<i>gerTΔ::erm</i> ($\Delta 3$)	This study
CF205	<i>gerTΔ::erm; amyE::Phyerspank-luxABCDE spc</i>	pAH60 → CF203
PE451	<i>spoIVCBΔ::erm</i>	Gift of P. Stragier
PE454	<i>gerEΔ::cm</i>	5
RL322	<i>cotEΔ::cm</i>	39
RL1397	<i>spoIVAΔ::neo</i>	3
PS832 derivatives		
PS832	Wild type; prototrophic	Gift of P. Setlow
CF224	<i>gerAΔ::erm</i>	This study
CF280	<i>gerTΔ::erm</i>	CF203 → PS832
CF292	<i>gerTΔ::erm; amyE::gerT cm</i>	CF167 → CF280
<i>E. coli</i> strain DH5 α	Cloning host	Laboratory stock
<i>E. coli</i> strain TG1	Cloning host	Laboratory stock
Plasmids		
pAH52	Contains <i>erm</i> antibiotic cassette	This study
pAH60	pDR111 with <i>luxABCDE</i> operon from pSB2025	This study
pCF165	pDG364 with <i>gerT</i> and upstream sequence	This study
pCF170	pCVO119 with fragment of <i>gerT</i> coding gene bases 113–471	This study
pCF173	pDG1728 with first 6 codons of <i>gerT</i> and upstream sequence	This study
pCVO119	Allows for Campbell integration of translational fusions to <i>gfp</i>	33
pDG364	Permits insertion into <i>amyE</i> locus	7
pDG646	Contains <i>erm</i> antibiotic cassette	7
pDG1514	Contains <i>tet</i> antibiotic cassette	7
pDG1726	Contains <i>spc</i> antibiotic cassette	7
pDG1728	Permits translational fusion to <i>lacZ</i> (<i>spc</i>)	7
pDR111	Contains <i>Phyerspank</i> promoter for insertion into <i>amyE</i>	Gift of D. Rudner
pSB2025	Contains <i>luxABCDE</i> operon from <i>Photobacterium luminescens</i>	24

To create a strain expressing *gerT-gfp*, the oligonucleotide primers oCF116 and oCF117 were used to amplify nucleotides 113 to 471 of the *gerT* coding sequence, omitting the stop codon after base 471. This fragment was digested with BamHI/XhoI and ligated with BamHI/XhoI-digested pCVO119 (33), yielding an in-frame fusion of the *gerT* coding fragment to *gfp*, pCF170. Single-recombination integration of pCF170 into PY79 yielded CF172 [*gerTΩpCF170(gerT-gfp) spc*]. The *gerT-gfp* fusion was judged to be functional based on the normal germination of CF172-derived spores. CF177 [*cotEΔ::cm gerTΩpCF170(gerT-gfp) spc*] and CF178 [*spoIVAΔ::neo gerTΩpCF170(gerT-gfp) spc*] were generated by transformation of RL322 and RL1397, respectively (3, 39) to spectinomycin resistance with chromosomal DNA from CF172.

We used the long-flanking homology PCR method (34) for creating deletion/insertion mutants using the following primers: oCF23, oCF24, oCF25, and oCF26 for $\Delta 1::tet$ (CF111), oCF94, oCF95, oCF96, and oCF97 for $\Delta 2::erm$ (CF152), oCF108, oCF109, oCF110, and oCF111 for *gerTΔ::spc* ($\Delta 3$) (CF164); oCF300, oCF301, oCF302, and oCF303 for *gerTΔ::erm* ($\Delta 3$) (CF203); and oCF310, oCF311, oCF312, and oCF313 for *gerAΔ::erm* (CF224). pDG1726 was used as the source for the spectinomycin cassette, pDG1514 for the tetracycline cassette, pDG646 for the erythromycin cassette for CF152 ($\Delta 2::erm$), and pAH52 [constructed by subcloning the BamHI/ClaI fragment from pDG646 into pBlue-script KS(+)] for the erythromycin cassette for CF203 (*gerTΔ::erm*) (7). Strain PY79 was directly transformed with long-flanking homology PCR products with selection for the appropriate antibiotic resistance. Mutants were confirmed by

PCR. Chromosomal DNA from CF203 was used to transform PS832 to make CF280 (*gerTΔ::erm*).

To generate a strain expressing *gerT* at the *amyE* locus, the *gerT* coding sequence and approximately 230 bp of upstream sequence were amplified with primers oCF112 and oCF113, digested with EcoRI/BamHI, and ligated with EcoRI/BamHI-digested pDG364 (7) to create pCF165. Transformation of CF164 with pCF165 yielded CF167 (*gerTΔ::spc amyE::gerT cm*). CF292 (*gerTΔ::erm amyE::gerT cm*) was constructed by transforming CF280 to chloramphenicol resistance with chromosomal DNA from CF167.

To construct pAH60, a SpeI/SalI fragment containing the *luxABCDE* luciferase-encoding operon from *Photobacterium luminescens* (optimized for expression in low-G+C gram-positive bacteria) was liberated from pSB2025 (24) and ligated into NheI/SalI-digested pDR111 (gift of David Rudner). The resulting *Phyerspank* promoter fusion to *luxABCDE* permits high-level, constitutive expression of bacterial luciferase in the presence of the inducer isopropyl β -D-thiogalactopyranoside. PY79 and CF203 were transformed with pAH60 to create AHB83 (*amyE::Phyerspank-luxABCDE spc*) and CF205 (*gerTΔ::erm amyE::Phyerspank-luxABCDE spc*), respectively.

β -Galactosidase assays. Cells were grown in hydrolyzed casein growth medium and induced to sporulate by transfer to Sterlini-Mandelstam sporulation medium as described previously (9, 31). One-milliliter aliquots of sporulating cells were harvested by centrifugation at intervals, and pellets were stored at

TABLE 2. Primers used in this study

Primer	Sequence (5' to 3') ^a
oCF23.....	cccatattggccagggtgctcttt
oCF24.....	gaacaacctgcaccattgcaagactgggattccgcgtaaatacgaat
oCF25.....	ttgatctttttataacaggaattcgacggctttgtcctttgctgctt
oCF26.....	gccatggtgacagccatattga
oCF94.....	ccgccttaaatgttactacgggat
oCF95.....	attatgtcttttgcgcagtcggcaacgattgggctacggactgtcta
oCF96.....	catctaatgttgggggtgccgtaataaaaaaacggcctgcatcga
oCF97.....	aaaatccaagagcactgataca
oCF108.....	tgaaaacgtaaacagagcagcgc
oCF109.....	acatgtattaccgaacgaaaatcgacggacaaaatagatagcccacc
oCF110.....	atfttagaaaacaataaaccttgcacatcgcgaaaaaagtaaacctcc
oCF111.....	ttatagaattatcgccactcggg
oCF112.....	aggagGAATTCatcgggggatacctcggcttcta
oCF113.....	ctctGGATCCatcacgatgagcgaataatcaagacg
oCF116.....	aggagGGATCCtgtcggcagtgattatgcggctc
oCF117.....	ctctCTCGAGtcggatgatttctacctcggacat
oCF120.....	aggagGGATCCctcctcctgtttttcagagacg
oCF121.....	ctctGTCGACctgttccactcaaacattgtcttc
oCF300.....	tgaaaacgtaaacagagcagcgc
oCF301.....	caattgcctatagtgagtcgacgacaaaatagatagcccacc
oCF302.....	ccagctttgttccctttagtgagtcacccgaaaaaagtaaaccttcc
oCF303.....	ttatagaattatcgccactcggg
oCF310.....	cttccgaacggtccagcatgtgaa
oCF311.....	caattgcctatagtgagtcgttctgtttgtccaatgaggtcacc
oCF312.....	ccagctttgttccctttagtgagactcggaaacgaaagggcgc
oCF313.....	gcagccgattgatattgagtggg

^a Bold capital letters indicate restriction enzyme sites included in primer.

–80°C until further analysis. β -Galactosidase activity was measured as previously described (9).

Microscopy. Cells were induced to sporulate by growth in liquid Schaeffer's sporulation medium (27). The onset of sporulation was considered to be the last time point after the end of exponential phase. At various time points thereafter, cells were collected, immobilized using poly-L-lysine-treated coverslips, and observed by fluorescence microscopy as previously described (6).

Spore purification. Spores were prepared as described previously (8), with a few modifications. In brief, cells were induced to sporulate in 100 ml of Schaeffer's sporulation (DS) medium for 72 h at 37°C. Spores and other cells/cellular debris were collected by centrifugation and washed twice with distilled water. Next, the pellet was resuspended in 10 ml TE80 (10 mM Tris, 1 mM EDTA, pH 8.0) and 1 mg/ml lysozyme and incubated for 1 h at 37°C. Sodium dodecyl sulfate was then added (2 ml of 10% stock), and incubation was continued for 20 min. The pellet was washed twice with 0.01% Tween 20 and twice with distilled water and finally stored in water at 4°C. All centrifugations were performed at 3,500 × g. Spore preparations routinely contained ≥95% phase-bright spores. All strains required for a single experiment were prepared together to ensure uniformity of growth and preparation conditions.

After the experiments shown in Fig. 3 were complete, it was observed that the spore preparation method described above had begun to yield preparations containing a significant proportion of phase-dark spores. Therefore, we wished to verify our results and continue with further experiments using spores prepared using a different method. In an alternative spore preparation protocol, cells are induced to sporulate on 2× SG medium agar (9) plates for 6 days at 37°C. Spores are then harvested from the plate surface and purified by sonication and repeated washes with cold water (9). Due to poor sporulation of PY79-derived strains on 2× SG agar, we built the required strains in the PS832 strain background, which sporulates efficiently on 2× SG. Spores purified from these new strains using the 2× SG method were used for the experiments shown in Fig. 4. In addition, the new strains were subjected to the loss-of-optical-density germination assay and yielded results virtually identical to those shown in Fig. 3A.

Assays for sporulation and germination. Sporulation efficiency and the heat resistance of spores were measured as the fraction of total CFU that survive incubation at 80°C for 20 min. Lysozyme resistance of spores was measured as the fraction of total CFU that survive lysozyme treatment (125 µg/ml) (9).

Tests for germination using 2,3,5-triphenyltetrazolium chloride overlay were carried out as previously described on DS medium (9), with the exception that heat treatment was performed in a 65°C oven for 3 h.

Pure spore preparations were routinely heat activated at 80°C for 20 min prior

to germination assays. Unless otherwise stated, germination of pure spore preparations was initiated by the addition of L-alanine at a final concentration of 10 mM. For germination in AGFK, D-glucose, D-fructose, KCl, and L-asparagine were added to a final concentration (each) of 10 mM. The standard germination assays for loss of optical density and dipicolinic acid (DPA) release during germination were performed as previously described (9). To assess spore germination in response to Ca²⁺-DPA, purified spores were tested for loss of heat resistance at various time points after the addition of 60 mM Ca²⁺-DPA (or 10 mM L-alanine as a control) (9).

To assay for luciferase activity during germination, the AHB83 (*amyE::Phypherspank-luxABCDE spc*) and CF205 (*gerTΔ::sem amyE::Phypherspank-luxABCDE spc*) strains were induced to sporulate in DS medium supplemented with 1 mM isopropyl β-D-1-thiogalactopyranoside, which induces expression of the *Phypherspank* promoter to high levels. Resulting spores were purified as described above. Spore suspensions were diluted to an optical density at 600 nm of 2 in 50 mM KPO₄, pH 7.5, and the luciferase substrate decanal (Sigma) was added to a final concentration of 0.02%. This spore mixture was arrayed in a 96-well plate format, and germination was induced by addition of germinant to each well. Luminescence was read using a Perkin-Elmer TopCount NXT microplate scintillation and luminescence counter kept in a 30°C room. Readings were acquired with a 1-s integration time per well every 5 min for 7 h.

RESULTS

Discovery of a germination gene. During the course of systematically inactivating genes under the control of σ^K , we discovered that a deletion (ΔI) that eliminated most of the regulon member *yoyB* resulted in the production of spores that were defective in germination (data not shown, but see below). Because the ΔI mutation extended from 37 bp downstream of the start codon for *yoyB* into the overlapping downstream open reading frame *yoyC* (ending 27 bp upstream of the annotated stop codon for *yoyC*), it was possible that either *yoyB* or *yoyC* (or both) was needed for proper germination (Fig. 1A) (18, 19). Next, we built a deletion ($\Delta 2$) that was fully contained within the *yoyB* coding sequence and removed only the region of overlap between *yoyB* and *yoyC*. To our surprise, the $\Delta 2$ mutation caused no measurable defect in germination, a finding that suggested that neither open reading frame was needed for proper germination.

Just downstream of, and in convergent orientation to, *yoyB* is a third open reading frame, *yozR*, which also overlaps with *yoyC*. The ΔI mutation, which had caused a germination phenotype, but not $\Delta 2$ extended into the 3' region of *yozR*. To investigate whether inactivation of *yozR* was responsible for the germination phenotype, we built a third deletion ($\Delta 3$) that eliminated the 5' region of *yozR* but not the region of overlap between *yozR* and *yoyC*. Spores produced by cells harboring $\Delta 3$ exhibited a germination defect similar to that observed with ΔI . We conclude from this that *yozR* (but evidently not *yoyB* or *yoyC*) is a germination gene. Finally, and in confirmation of this conclusion, the germination defect caused by either ΔI or $\Delta 3$ was reversed in complementation experiments in which DNA that contained *yozR* and only the region of overlap with *yoyC* was inserted into the chromosome at the *amyE* locus. The *yozR* gene is henceforth referred to as *gerT*.

In light of these findings and for the following additional reasons, we believe that *yoyC* is not a functional open reading frame (Fig. 1C). First, all but 40 bp of the 231-bp coding sequence annotated as *yoyC* overlaps either *gerT* or *yoyB* (18, 19). Second, the region directly upstream of the annotated *yoyC* start codon does not contain a sequence that significantly resembles a ribosome binding site for *B. subtilis* (16). Third, an in-frame fusion of the gene for green fluorescent protein

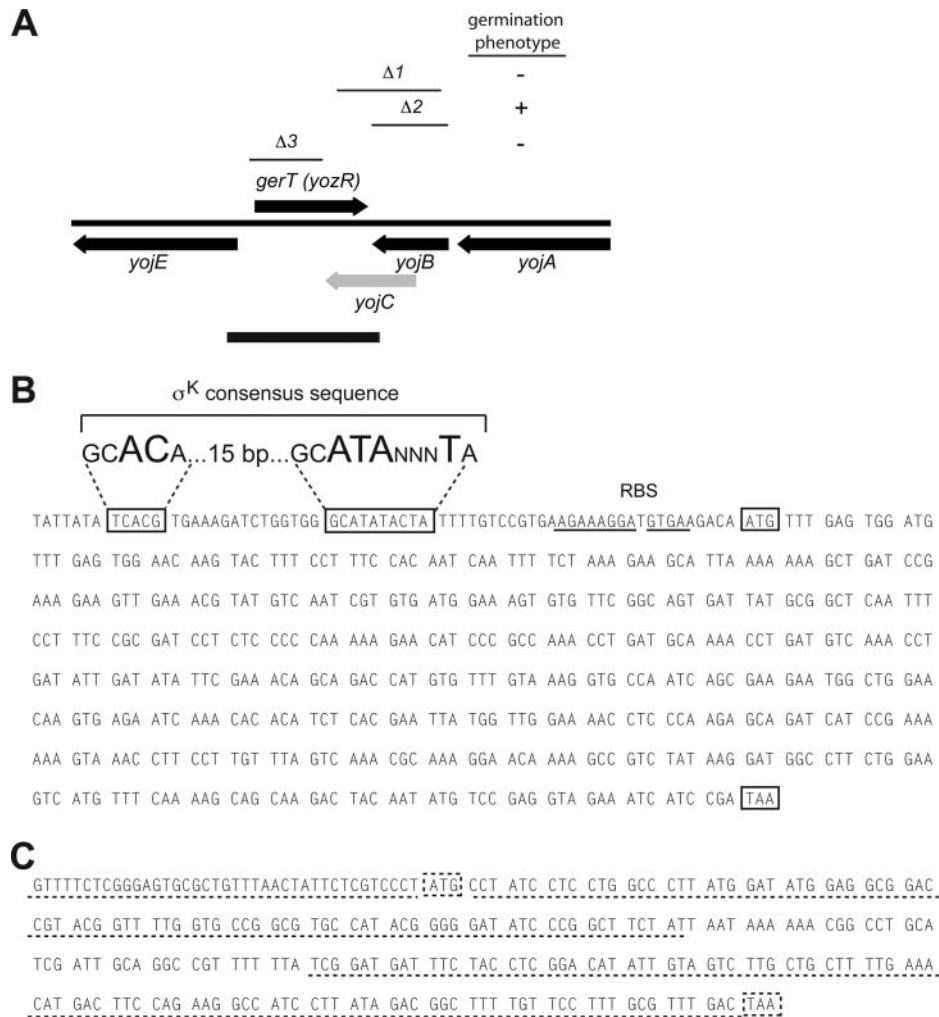


FIG. 1. The *gerT* chromosomal region. (A) *gerT* is located in a convergent orientation with *yojB* and overlaps the hypothetical open reading frame *yojC*. Regions deleted in $\Delta 1$, $\Delta 2$, and $\Delta 3$ and the germination phenotypes of these deletion mutants are depicted. The black bar indicates the region used to complement *gerT* at the *amyE* locus. (B) The *gerT* coding gene and promoter regions are depicted. The putative -10 and -35 regions are boxed, and the consensus σ^K binding sequences and conserved spacing are shown above. Highly conserved residues are shown in large, bold letters. Perfect matches to the ideal ribosome binding site (RBS) are underlined. The boxed residues ATG and TAA are the projected start and stop codons for *gerT*. (C) The hypothetical *yojC* coding region is shown. Dotted underlining represents regions of overlap with the *gerT* and *yojB* coding sequences. Annotated start and stop codons are boxed.

(GFP) to the putative *yojC* coding sequence was not measurably expressed as judged by fluorescence microscopy.

In contrast to the case of *yojC*, the start codon for *gerT* is preceded at an appropriate distance by a sequence (AGAAAGGATGTGA) that exhibits extensive complementarity to the 3' terminal region of 16S rRNA, being complementary at 12 out of 13 positions in a row (Fig. 1B) (16). Also, as presented below, in-frame fusions of the gene for β -galactosidase or GFP to the *gerT* coding sequence were expressed, as judged by the production of β -galactosidase activity and GFP, respectively. Thus, the *gerT-yojB* region of the chromosome likely consists of just two adjacent genes in convergent orientation.

***gerT* is under the control of σ^K and GerE.** We wondered whether, like *yojB*, *gerT* is under the control of σ^K . A clue that *gerT* is under σ^K control came from the observation that approximately 30 bp upstream of the start codon is a partial match (tCACg, representing a three-out-of-five match [lower-

case letters indicate deviation from the consensus]) to the consensus " -10 " sequence (GCACA) and 15 bp further upstream is a perfect match (GCATATACTA) to the consensus " -35 " sequence (GCATANNNNTA) for promoters recognized by RNA polymerase containing σ^K (Fig. 1B) (5).

To investigate the expression and regulation of *gerT*, we created a translational fusion to *lacZ* in which the reporter gene was fused in-frame to the first six codons of *gerT*. The fusion included the putative promoter and ribosome binding site for *gerT*. The results in Fig. 2 show that the accumulation of β -galactosidase from the translational fusion increased starting at about 4 h after the onset of sporulation (when σ^K is known to be active) and in a manner that was dependent upon σ^K .

Among the genes turned on by σ^K is *gerE*, which encodes a DNA-binding protein that is required for the activation of a subset of genes in the σ^K regulon and up-regulates or down-

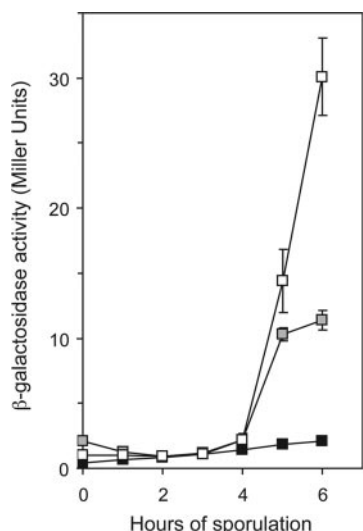


FIG. 2. *gerT* is turned on late during sporulation under the control of σ^K and is repressed by GerE. Accumulation of β -galactosidase was measured in strains harboring *lacZ* fused to the first six codons of *gerT* and upstream regulatory regions. Samples were collected at the indicated times after suspension of growing cultures in Sterlini-Mandelshtam medium. Expression of *gerT-lacZ* was monitored in the wild type (gray boxes; strain CF174) and in *spoIVCB* (part of the coding gene for σ^K ; black boxes; strain CF183) and *gerE* (white boxes; strain CF184) mutant cells.

regulates many other genes under σ^K control (5). We therefore wondered whether the expression of *gerT* was influenced by GerE. The results show that in a mutant lacking *gerE*, β -galactosidase expressed from the *gerT* promoter accumulated starting at 4 h of sporulation, as in the wild type, but increased to a level three times as high as the wild-type level by 6 h of sporulation. Therefore, *gerT* expression is under the negative control of GerE.

***gerT* is a germination gene.** Spores from a *gerT* mutant (containing *gerT* $\Delta 3$) exhibited no measurable defect in heat resistance or in resistance to treatment with lysozyme. In addition, the mutant spores appeared normal under phase-contrast microscopy (data not shown). However, purified spores of the *gerT* mutant were impaired in their ability to reduce 2,3,5-triphenyltetrazolium chloride, which is diagnostic of a defect in germination (data not shown).

To further investigate the *gerT* germination defect, we tested purified spores in an assay for loss of optical density at 600 nm (Fig. 3A, left panel). Wild-type spores have a relatively dehydrated core and are phase bright but darken as they germinate and rehydrate. As this occurs, a suspension of spores loses optical density. We observed that wild-type spores lost about 50 to 60% of their optical density within 90 min of the addition of the germinant L-alanine. The majority of this decrease occurred within 30 min after the addition of germinant. In contrast, *gerT* spores lost a maximum of 25% of their optical density during germination. When observed under the microscope at the end of incubation with germinant, most wild-type spores had turned phase dark. However, only about one in three *gerT* spores was phase dark, while the rest remained phase bright. As noted earlier, when a wild-type copy of *gerT*

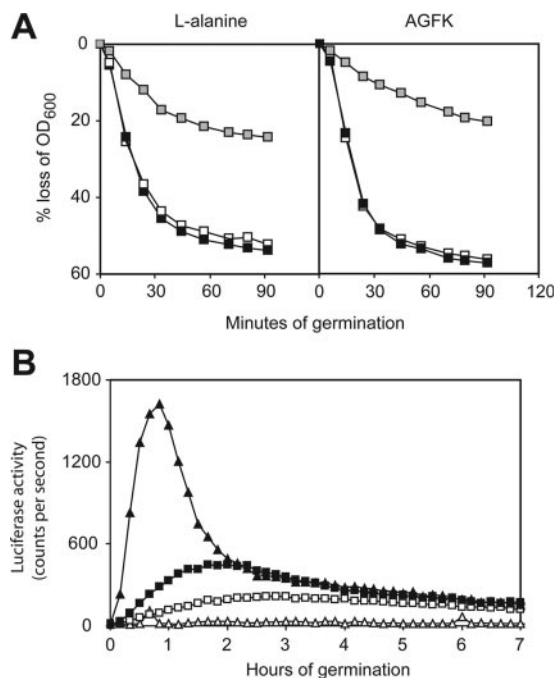


FIG. 3. GerT is required for efficient germination. (A) Purified spores of the wild type (white boxes; strain PY79), from a *gerT* mutant (containing *gerT* $\Delta 3$) (gray boxes; strain CF164), and from a strain with a mutated *gerT* gene and containing a copy of wild-type *gerT* at the *amyE* locus (black boxes; strain CF167) were germinated with either L-alanine (left panel) or AGFK (right panel). Optical density at 600 nm (OD_{600}) was measured as it decreased over time. (B) Wild-type (triangles; strain AHB83) and *gerT* mutant (squares; strain CF205) strains were induced to express the genes of the *Photobacterium luminescens* luciferase operon during sporulation, and spores were purified. L-Alanine (black) or water (white) was then added to spore suspensions, and luciferase activity was monitored over time using a luminescence counter.

was present at the *amyE* locus, the germination defect of cells harboring the *gerT* $\Delta 3$ mutation was repaired.

When plated on solid LB medium, the *gerT* mutant spores yielded approximately as many colonies as did wild-type spores. Yet under the conditions used for Fig. 3, mutant spores were impaired in the loss of optical density and only 30% of the spores turned phase dark. We have no explanation for this apparent contradiction. Evidently, the mutant spores can eventually germinate with high efficiency on solid LB medium.

As another test of germination, we sought to measure the ability of *gerT* mutant spores to reactivate luciferase enzyme that had been packaged into the spore during sporulation upon exposure to germinant. To this end, we purified wild-type and *gerT* mutant spores engineered to contain the bacterial enzyme luciferase, which is known to be inactive in the dormant spore and to rapidly reactivate as germination occurs (10). More specifically, we cloned the *Photobacterium luminescens* luciferase operon downstream of an inducible promoter and inserted this construct into the *amyE* locus in wild-type and *gerT* mutant cells. The operon encodes luciferase and contains genes responsible for the production of the luciferase cofactor reduced riboflavin phosphate. The resulting strains were sporulated in the presence of the inducer, thereby permitting incorporation of luciferase into the developing forespore. (While luciferase

was also produced in the mother cell during sporulation of these strains, this pool of enzyme was likely eliminated during the stringent spore purification procedure. Furthermore, any possible residual luciferase associated with the exterior of purified spores would have been inactive due to the absence of the reduced riboflavin phosphate cofactor.) For wild-type spores that had been induced to produce luciferase during sporulation, we found that luciferase activity began almost immediately upon the addition of germinant and peaked at about 1 h postaddition (Fig. 3B). The activity then decreased rapidly until about 2.5 h after addition, finally tapering off over the next several hours. In contrast, *gerT* spores that had been similarly induced to produce luciferase exhibited a much less dramatic peak of activity, reaching a maximum at about half the height of the wild-type peak at about 2 h after addition of germinant and tapering off slowly for the remainder of the experiment. Surprisingly, *gerT* mutant spores exhibited some luciferase activity even when L-alanine was not added (about half the level of activity of spores that had been induced to germinate). The cause of this activity is unknown, and germination without the addition of germinant was not seen in the wild type in this assay or with *gerT* spores in other types of germination assays.

Spores with mutations in certain germination genes, such as the operon encoding the *gerA* receptor for L-alanine, are defective in response to one germinant but can germinate normally in response to another nutrient germinant (17). To see if this was true for *gerT*, we tested mutant spores for the loss of optical density after addition of the nutrient germinant cocktail AGFK (containing L-asparagine, D-glucose, D-fructose, and potassium) (Fig. 3A, right panel). Wild-type spores again lost approximately 60% of their optical density within 90 min of germinant addition. *gerT* mutant spores did not respond efficiently to the germinant, again losing only 20% of their optical density over the course of the assay. Thus, *gerT* spores are defective in responding to either L-alanine or AGFK, and hence the germination defect is not germinant specific.

***gerT* is needed early in germination.** The process of germination can be divided into three stages. The first stage, germination I, begins when nutrient germinant binds to its cognate germinant receptor in the inner membrane of the spore. This triggers the release of ions and DPA from the spore core; DPA, in a chelate with calcium, composes up to 10% of the dry weight of the spore. At this stage, at least some of the spore's heat resistance is also lost. As Ca^{2+} -DPA and ions are excreted, some water comes into the core; but the core does not expand due to the peptidoglycan cortex that forms a shell between the inner and outer membranes surrounding the core. Germination II requires activation of the cortex lytic enzymes (CLEs) that degrade the cortex to allow full hydration and expansion of the core. This hydration appears to be associated with the loss of phase brightness (and optical density) that occurs during this stage. Finally, metabolism, cell division, and DNA, RNA, and protein synthesis begin during the final stage of germination, outgrowth (20, 28).

The defects displayed by *gerT* spores in the assay for loss of optical density (which tests for loss of phase brightness) and the tetrazolium overlay assay (which tests for renewed metabolism) suggest that the mutant is impaired at germination I or II. In addition, it has been shown that luciferase activity in the

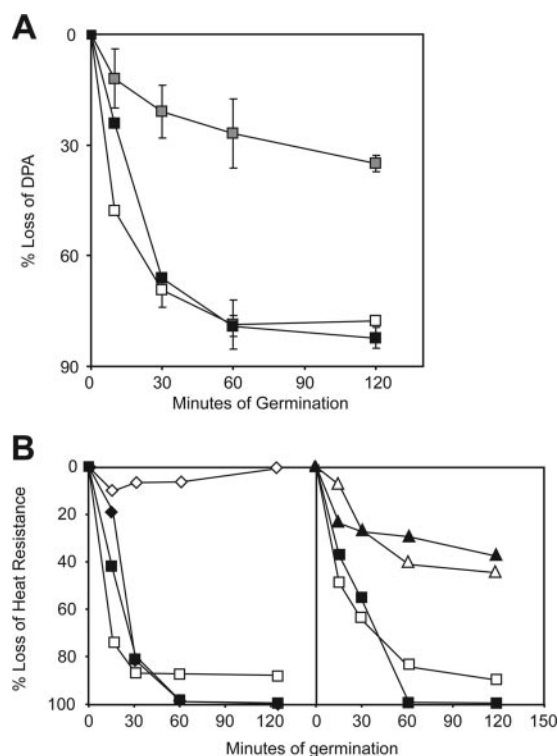


FIG. 4. *gerT* mutant spores are impaired in release of DPA during germination and in responding to DPA as a germinant. (A) Purified spores of the wild type (black; strain PS832), a *gerT* mutant (gray; strain CF280), and a *gerT* mutant harboring a copy of *gerT* at the *amyE* locus (white; strain CF292) were germinated with L-alanine. Samples were collected by centrifugation at the indicated times and assayed for DPA. (B) Wild-type (squares; strain PS832), *gerA* mutant (diamonds, left-hand panel; strain CF224), and *gerT* mutant (triangles, right-hand panel; strain CF280) spores were germinated in L-alanine (white) or Ca^{2+} -DPA (black). Samples were collected at the indicated times and then tested for heat resistance.

assay described above does not commence until at least some cortex hydrolysis has begun (perhaps due to a lack of core water during germination I) (28); as such, the inability of *gerT* mutant spores to fully reactivate luciferase also indicates a block at germination II or earlier. To test whether *gerT* spores are impaired at germination I, we measured the release of DPA from *gerT* spores during germination (Fig. 4A). Wild-type spores released 80 to 90% of their depot of DPA within 60 min after addition of germinant. In contrast, *gerT* spores released only about 30% of their core DPA even after 2 h of germination. Thus, it seems that *gerT* spores are impaired at the earliest stage of germination.

Other mutant strains that are blocked in the initiation of germination I can be rescued for germination by the nonnutrient germinant Ca^{2+} -DPA (22). In wild-type spores, it is thought that the release of endogenous Ca^{2+} -DPA during germination I triggers activation of the CLE CwlJ. This CLE can be artificially activated when Ca^{2+} -DPA is added as an exogenous germinant, allowing the spore to bypass the events of germination I and to initiate degradation of the cortex and the events of germination II (21). *gerA* spores, which are mutant for the L-alanine receptor, are unable to germinate in response to L-alanine (Fig. 4B, left panel) (22). However, they

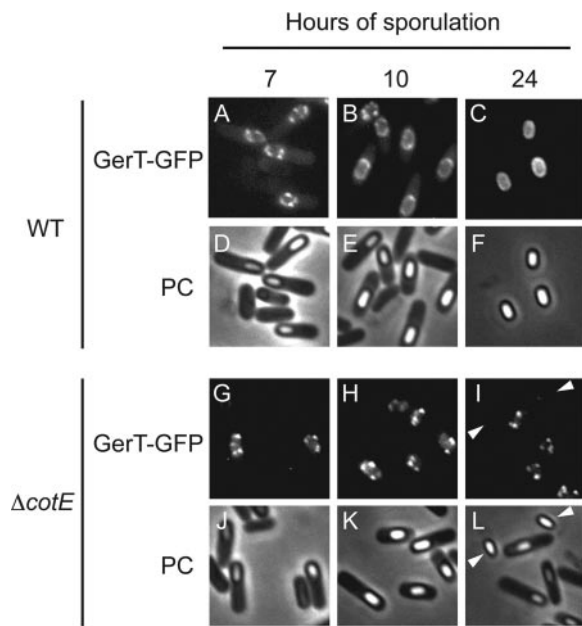


FIG. 5. GerT-GFP localizes to the coat in a two-step process. Wild-type (A to F; strain CF172) and *cotE* mutant cells (G to L; strain CF177) harboring *gerT-gfp* were grown in Difco sporulation medium. The cells were visualized by phase-contrast microscopy (PC), and localization of GerT-GFP was visualized by fluorescence microscopy at the indicated times after the start of sporulation. White arrowheads in panels I and L indicate spores that have been released from the mother cells.

germinate normally when Ca^{2+} -DPA is added. To test whether the *gerT* defect could be similarly bypassed, we added either L-alanine or Ca^{2+} -DPA to spores and observed the loss of heat resistance that occurs during germination. In L-alanine, wild-type spores lost 80 to 90% of their heat resistance within 60 min of germinant addition. *gerT* spores lost only about 40% of their resistance in the same amount of time (Fig. 4B, right panel). When Ca^{2+} -DPA was used as the germinant, wild-type germination improved, allowing close to 100% loss of resistance. Surprisingly, we observed that *gerT* spores lost only 30 to 40% of resistance in response to Ca^{2+} -DPA as a germinant. Thus, it appears that *gerT* spores are impaired in progressing through germination I, but the defect caused by the *gerT* mutation cannot be rescued with Ca^{2+} -DPA. This could indicate that GerT is required independently for both germination I and II or that the passage of small molecules through the coat (i.e., germinants and Ca^{2+} -DPA) is impaired in the absence of GerT.

Subcellular localization of GerT. In light of its role in germination and its synthesis in the mother cell, we wondered whether GerT is a component of the spore coat, the protective proteinaceous layer that surrounds the outer membrane of the spore. To investigate this, we created a strain bearing a functional, in-frame fusion of the coding sequence for GerT to that of GFP. As seen in Fig. 5C, GerT-GFP localized in an oval pattern around the outside of the mature spore, a localization pattern similar to that of known coat proteins (36). Proper localization of all known coat proteins is dependent on SpoIVA, a protein that composes the basement layer of the spore coat (26). In a *spoIVA* mutant, GerT-GFP did not local-

ize to the forespore, and in fact little if any GFP fluorescence was observed during sporulation (data not shown). It therefore seems likely that GerT is a coat protein, and we surmise that the GerT-GFP fusion is unstable in the absence of proper, SpoIVA-directed coat assembly.

The coat is composed of two primary layers, the inner and outer coat. Formation of the outer coat is dependent on the coat morphogenetic protein CotE, while the inner coat forms independently of this protein (39). To determine whether GerT is a part of the inner or outer coat, we introduced the GerT-GFP fusion into a strain with a mutation in *cotE*. Surprisingly, GerT-GFP localization to the spore coat showed a partial, but not complete, dependence on CotE. Rather than localizing in a ring around the forespore in the absence of CotE, as would be expected for an inner coat protein, or being diffuse in the mother cell, as expected for an outer coat protein, GerT-GFP formed bright foci associated with the forespore as spores developed (Fig. 5G to I). However, when spores were finally released from the mother cell, the GFP fluorescence associated with the spores could no longer be observed (Fig. 5I). Therefore, it appears that CotE is required for the proper pattern of GerT localization during sporulation.

Upon close observation of fields of sporulating wild-type cells bearing the GerT-GFP fusion, it was possible to observe the occasional individual sporangium with a pattern of GFP foci around the forespore similar to that seen in the *cotE* mutant. In each case, these sporangia appeared to be at an earlier stage of sporulation than the neighboring cells, as the forespores appeared slightly less phase bright. To investigate whether these foci represent a normal stage in GerT localization as development progresses, we observed GerT-GFP localization over time. As seen in Fig. 5A, most wild-type cells had foci of GFP associated with the forespore after 7 h of sporulation. By 10 and 24 h of sporulation, however, GerT-GFP had spread out into a ring in most cells, surrounding the forespore roughly evenly (Fig. 5B and C). In contrast, GerT-GFP localized as forespore-associated foci in *cotE* mutant sporangia throughout sporulation, the immature spores losing their associated green fluorescence only after mother-cell lysis (Fig. 5G to I). Therefore, it appears that GerT is incorporated into the coat in a two-step process. The germination protein first assembles into foci associated with the forespore in a manner independent of CotE. Next, in a second step that depends on CotE, GerT molecules spread around the developing spore to form a stable contiguous shell.

DISCUSSION

We have discovered an additional member of the σ^K regulon and have shown that it is involved in the process of germination. The *gerT* gene was missed in previous studies of the regulon (5, 30), which consists of approximately 89 single-gene and multigene transcription units. We note that *gerT* is weakly expressed, and hence it might not have been detected in the transcriptional profiling analysis of Steil et al. (30). In the case of Eichenberger et al. (5), the oligonucleotide spot on the microarray corresponding to *gerT* was missing due to a printing error. Nevertheless, the identification of *gerT* as an additional regulon member reinforces the possibility that the current list

of sporulation-controlled genes is not complete (35). A subset of σ^K -controlled genes is additionally subject to repression by the DNA-binding protein GerE, whose synthesis is itself under σ^K control (5, 35). This kind of regulatory circuit is known as an incoherent feed-forward loop and is believed to cause genes to be expressed in a pulse (15). We therefore surmise that *gerT* is switched on by σ^K and then switched off as GerE accumulates.

Multiple lines of evidence indicated that GerT plays a role in germination. Among these were the following: (i) impaired response to two different nutrient germinants (L-alanine and the cocktail AGFK) and the nonnutrient germinant Ca^{2+} -DPA, which activates the cortex lytic enzyme CwlJ (21); (ii) impaired reduction of the chromogenic dye 2,3,5-triphenyl-tetrazolium chloride; (iii) impaired loss of optical density; (iv) impaired activation of luciferase that had been packaged into spores; and (v) impaired release of Ca^{2+} -DPA. Impaired release of Ca^{2+} -DPA is usually diagnostic of a block at germination I. Interestingly, unlike the germination-I-blocked mutant *gerA*, which is rescued by the addition of Ca^{2+} -DPA, addition of this compound to *gerT* mutant spores did not restore efficient germination.

The use of a functional, in-frame fusion of GerT to GFP revealed that the germination protein is a component of the spore coat. The coat is a complex macromolecular structure that is composed of 50 or more proteins (11). Interestingly, coat proteins exhibit dynamic patterns of subcellular localization (33). For example, the coat protein YutH (as visualized with a GFP fusion) initially forms a focus, which then grows into a ring. The ring, in turn, expands into a cap that spreads around the developing spore. As we have seen, GerT-GFP also forms foci that grow into a shell that surrounds the entire forespore. Interestingly, the spreading step is dependent on the coat protein CotE, which is needed for the assembly of the outer layer of the coat. Perhaps CotE or some outer coat protein whose localization depends on CotE forms a platform that allows the focus of GerT to spread into a shell.

Although many coat proteins are dispensable for spore formation, a significant number of coat proteins are needed for proper germination (1, 21, 25, 29). For example, the hexagenic *gerP* operon is believed to encode coat components (or proteins needed for proper coat assembly) that allow small-molecule germinants to pass through the coat and reach the germination receptors located in the membrane beneath it (17). Interestingly, like *gerT*, *gerP* is under the positive control of σ^K and the negative control of GerE (1). Hence, both transcription units are likely to exhibit a pulse of expression at a similar time late in sporulation. Conceivably, GerT and the GerP proteins contribute to a common feature of the coat that allows it to be permeable to small molecules.

In summary, we have identified an additional gene involved in germination. The *gerT* gene encodes a component of the coat that is produced under the positive control of σ^K and the negative control of GerE. GerT is incorporated into the coat in a dynamic, two-step process that depends on the morphogenetic protein CotE. GerT seems to be needed at the earliest stage (I) of germination.

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