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Role of GerKB in Germination and Outgrowth of *Clostridium perfringens* Spores▽†

Daniel Paredes-Sabja,[1] Peter Setlow,[3] and Mahfuzur R. Sarker[1,2]*

Department of Biomedical Sciences[1] and Department of Microbiology,[2] Oregon State University, Corvallis, Oregon 97331, and Department of Molecular, Microbial and Structural Biology, University of Connecticut Health Center, Farmington, Connecticut 06030[3]

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Previous work indicated that *Clostridium perfringens gerKA gerKC* spores germinate significantly, suggesting that *gerKB* also has a role in *C. perfringens* spore germination. We now find that (i) *gerKB* was expressed only during sporulation, likely in the forespore; (ii) *gerKB* spores germinated like wild-type spores with nonnutrient germinants and with high concentrations of nutrients but more slowly with low nutrient concentrations; and (iii) *gerKB* spores had lower colony-forming efficiency and slower outgrowth than wild-type spores. These results suggest that GerKB plays an auxiliary role in spore germination under some conditions and is required for normal spore viability and outgrowth.

Spores of *Bacillus* and *Clostridium* species can break dormancy upon sensing a variety of compounds (termed germinants), including amino acids, nutrient mixtures, a 1:1 chelate of Ca^{2+} and pyridine-2,6-dicarboxylic acid (dipicolinic acid [DPA]), and cationic surfactants such as dodecylamine (20). Nutrient germinants are sensed by their cognate receptors, located in the spore's inner membrane (6), which are composed of proteins belonging to the GerA family (10, 11). In *Bacillus subtilis*, three tricistronic operons (*gerA*, *gerB*, and *gerK*) expressed uniquely during sporulation in the developing forespore each encode the three major germinant receptors, with different receptors responding to a different spectrum of nutrient germinants (5, 9, 20). Null mutations in any cistron in a *gerA* family operon inactivate the function of the respective receptor (9, 11). In contrast, *Clostridium perfringens*, a gram-positive, spore-forming, anaerobic pathogenic bacterium, has no tricistronic *gerA*-like operon but only a monocistronic *gerAA* that is far from a *gerK* locus. This locus contains a bicistronic *gerKA-gerKC* operon and a monocistronic *gerKB* upstream of and in the opposite orientation to *gerKA-gerKC* (Fig. 1A) (16). GerAA has an auxiliary role in the germination of *C. perfringens* spores at low germinant concentrations, while GerKA and/or GerKC are required for L-asparagine germination and have partial roles in germination with KCl and a mixture of KCl and L-asparagine (AK) (16). In contrast to the situation with *B. subtilis*, where germinant receptors play no role in Ca-DPA germination (12, 13), GerKA and/or GerKC is required for Ca-DPA germination (16). The partial requirement for GerKA and/or GerKC in *C. perfringens* spore germination by KCl and AK suggests that the upstream gene product, GerKB, might also have some role in KCl and AK germination of *C. perfringens* spores. Therefore, in this study we have investigated the role of GerKB in the germination and outgrowth of *C. perfringens* spores.

To determine if *gerKB* is expressed during sporulation, 485 bp upstream of the *gerKB* coding sequence, including DNA between *gerKB* and *gerKA*, was PCR amplified with primer pair CPP389/CPP391, which had SalI and PstI cleavage sites, respectively (see Table S2 in the supplemental material). The PCR fragment was cloned between SalI and PstI cleavage sites in plasmid pMRS127 (17) to create a *gerKB-gusA* fusion in plasmid pDP84 (see Table S1 in the supplemental material). This plasmid was introduced into *C. perfringens* SM101 by electroporation (3), and Em^r transformants were selected. The SM101 transformant carrying plasmid pDP84 was grown in TGY vegetative growth medium (3% Trypticase soy, 2% glucose, 1% yeast extract, 0.1% L-cysteine) (7) and in Duncan-Strong (DS) (4) sporulation medium and assayed for β-glucuronidase (GUS) activity as described previously (23). Vegetative cultures of strain SM101 carrying plasmid pMRS127 (empty vector) or pDP84 (*gerKB-gusA*) exhibited no significant GUS activity, and strain SM101 grown in DS medium also exhibited no significant GUS activity (Fig. 1B and data not shown). However, GUS activity was observed in sporulating cultures of SM101(pDP84) (Fig. 1B), indicating that a sporulation-specific promoter is located upstream of *gerKB*. The expression of the *gerKB-gusA* fusion began ~3 h after induction of sporulation and reached a maximum after ~6 h of sporulation (Fig. 1B). The decrease in GUS activity observed after ~6 h is consistent with the GerKB-GusA protein being packaged into the dormant spore where it cannot be easily assayed and thus with *gerKB* being expressed in the forespore compartment of the sporulating cell (8). These results confirm that, as with the *gerKA-gerKC* operon (16), *gerKB* is also expressed only during sporulation.

To investigate the role of GerKB in *C. perfringens* spore germination, we constructed a *gerKB* mutant strain (DPS108) as described previously (14–16). A 2,203-bp DNA fragment carrying 2,080 bp upstream of and 123 bp from the N-terminal coding region of *gerKB* was PCR amplified using primers

* Corresponding author. Mailing address: Department of Biomedical Sciences, College of Veterinary Medicine, Oregon State University, 216 Dryden Hall, Corvallis, OR 97331. Phone: (541) 737-6918. Fax: (541) 737-2730. E-mail: sarkerm@oregonstate.edu.

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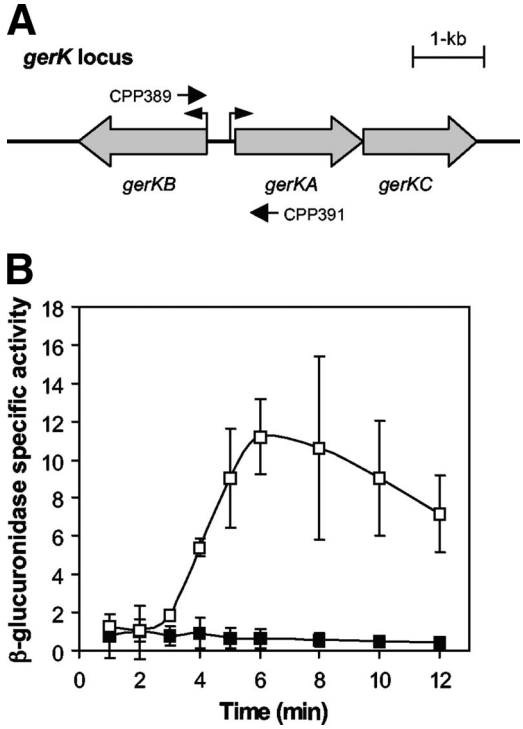

FIG. 1. Arrangement and expression of *gerKB* in *C. perfringens* SM101. (A) The arrangement of the *gerK* locus in *C. perfringens* SM101 is shown, and the locations of the primers used to amplify the upstream regions of the *gerKB* gene and the putative promoters of *gerKB* and *gerKA* are indicated. The *gerKB* promoter was predicted to be within the intergenic regions between *gerKB* and the *gerK* operon. (B) GUS specific activities from the *gerKB-gusA* fusion in strain SM101(pDP84) grown in TGY vegetative (filled squares) and DS sporulation (open squares) media were determined as described in the text. Data represent averages from three independent experiments with the error bars representing standard deviations, and time zero denotes the time of inoculation of cells into either TGY or DS medium.

CPP369 and CPP367, which had XhoI and BamHI cleavage sites at the 5′ ends of the forward and reverse primers, respectively (see Table S2 in the supplemental material). A 1,329-bp fragment carrying 134 bp from the C-terminal and 1,195 bp downstream of the coding region of *gerKB* was PCR amplified using primers CPP371 and CPP370, which had BamHI and KpnI cleavage sites at the 5′ ends of the forward and reverse primers, respectively (see Table S2 in the supplemental material). These PCR fragments were cloned into plasmid pCR-XL-TOPO, giving plasmids pDP67 and pDP69, respectively (see Table S1 in the supplemental material). An ~2.2-kb BamHI-XhoI fragment from pDP67 was cloned into pDP1 (pCR-XL-TOPO carrying an internal fragment of *gerAA*), giving plasmid pDP68, and an ~1.4-kb KpnI-BamHI fragment from pDP69 was cloned in pDP68, giving pDP73 (see Table S1 in the supplemental material). The latter plasmid was digested with BamHI, the ends were filled, and an ~1.3-kb NaeI-SmaI fragment carrying *catP* from pJIR418 (1) was inserted, giving plasmid pDP74. Finally, an ~4.8-kb KpnI-XhoI fragment from pDP74 (see Table S1 in the supplemental material) was cloned between the KpnI and SalI sites of pMRS104, giving pDP75, which cannot replicate in *C. perfringens*. Plasmid pDP75 was introduced into *C. perfringens* SM101 by electroporation (3), and the *gerKB* deletion strain DPS108 was isolated as described previously (18). The presence of the *gerKB* deletion in strain DPS108 was confirmed by PCR and Southern blot analyses (data not shown). Strain DPS108 gave ~70% sporulating cells in DS sporulation medium, similar to results with the wild-type strain, SM101 (data not shown).

Having obtained evidence for successful construction of the *gerKB* mutant, we compared the germinations of heat-activated (80°C; 10 min) *gerKB* and wild-type spores as previously described (16). Both the *gerKB* and wild-type spores germinated identically and nearly completely in 60 min at 40°C in brain heart infusion (BHI) broth as determined by the fall in optical density at 600 nm (OD_{600}) of germinating cultures and phase-contrast microscopy (data not shown). This result suggests that GerKB plays no essential role in spore germination in rich medium. The role of GerKB in *C. perfringens* spore germination was also assessed with individual germinants identified previously (16). Heat-activated wild-type and *gerKB* spores germinated similarly with high (100 mM) concentrations of KCl, L-asparagine, and AK, all in 25 mM sodium phosphate (pH 7.0), and in 50 mM Ca-DPA adjusted to pH 8.0 with Tris base (Fig. 2A to D). These results were also confirmed by phase-contrast microscopy (data not shown). However, with lower (10 to 20 mM) concentrations of KCl, L-asparagine, and AK, *gerKB* spore germination was very slightly (Fig. 2A) to significantly (Fig. 2B and C) slower than that of wild-type spores. These results suggest that while GerKB is not essential for germination with high concentrations of KCl, L-asparagine, or AK, it plays a significant role in germination with low L-asparagine and AK concentrations and, further, that GerKB is not required for Ca-DPA germination. This latter finding is similar to the situation with *B. subtilis* spores where germinant receptors play no role in Ca-DPA germination (19, 20). However, in *C. perfringens* spores, GerKA and/or GerKC do play a significant role in Ca-DPA germination (16).

Bacterial spores can also germinate with dodecylamine, a cationic surfactant (19). In *B. subtilis* spores, dodecylamine induces germination most likely by opening channels composed, at least in part, of SpoVA proteins (22), allowing release of the spores' Ca-DPA (19). Spores of *B. subtilis* lacking all three functional germinant receptors release DPA, as do wild-type spores, upon incubation with dodecylamine (19), while *C. perfringens* spores lacking GerKA-GerKC incubated with dodecylamine release DPA slower than wild-type spores (16). However, when *C. perfringens gerKB* spores at an OD_{600} of 1.5 were incubated with 1 mM dodecylamine in Tris-HCl (pH 7.4) at 60°C (2, 16), *gerKB* spores released their DPA slightly faster than wild-type spores (Fig. 3) when DPA release was measured as described previously (16). These results suggest that GerKB has no role in dodecylamine germination.

Previous work (16) found that *C. perfringens* spores lacking GerKA-GerKC had lower viability than wild-type spores on rich medium plates, and it was thus of interest to determine *gerKB* spore viability, which was measured as previously described (14, 16). Strikingly, the colony-forming ability of *gerKB* spores was ~7-fold lower ($P < 0.01$) than that of wild-type spores after 24 h on BHI plates (Table 1), and no additional colonies appeared when plates were incubated for up to 3 days (data not shown). The colony-forming ability of spores lacking GerKA and GerKC determined in parallel was ~12-fold lower

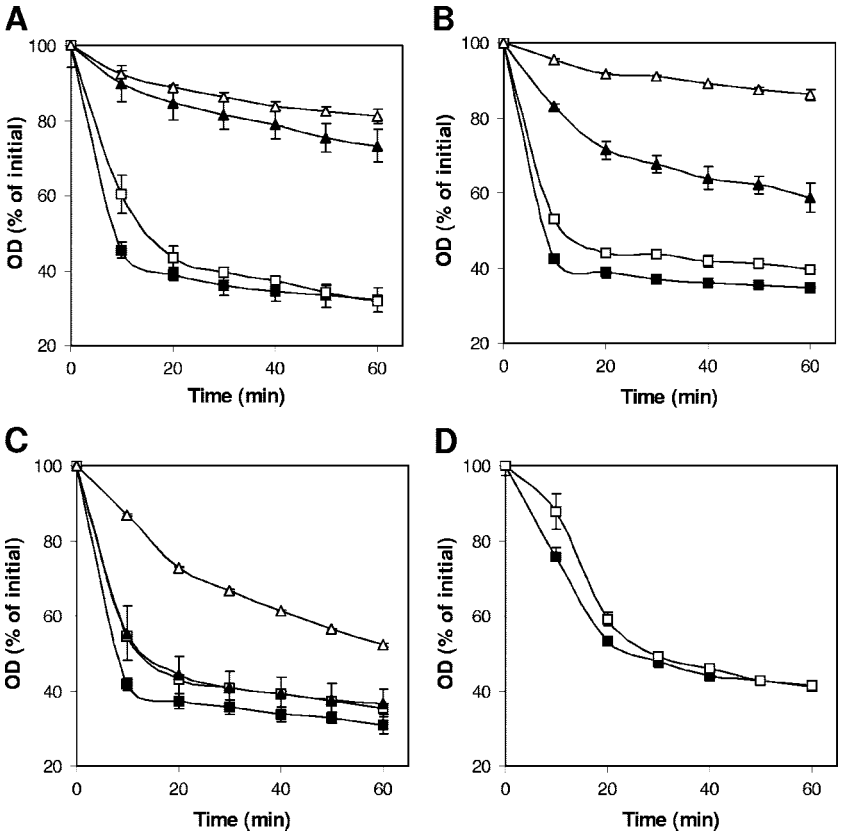

FIG. 2. Germination of spores of *C. perfringens* strains with various germinants. Heat-activated spores of strains SM101 (wild type) (filled symbols) and DPS108 (*gerKB*) (open symbols) were incubated at an OD_{600} of 1 at 40°C with high (squares) and low (triangles) germinant concentrations of 100 and 10 mM KCl (A), 100 and 20 mM L-asparagine (B), 100 and 10 mM AK (C), and 50 mM Ca-DPA (D) as described in the text, and at various times the OD_{600} was measured. No significant germination was observed when heat-activated spores of SM101 and DPS108 were incubated for 60 min at 40°C in 25 mM sodium phosphate buffer (pH 7.0) (data not shown). The data shown are averages from duplicate determinations with two different spore preparations, and error bars represent standard deviations.

than that of wild-type spores (Table 1). Phase-contrast microscopy of *C. perfringens* spores incubated in BHI broth for 24 h under aerobic conditions to prevent vegetative cell growth indicated that >90% of wild-type spores not only had germinated but had also released the nascent vegetative cell, while >85% of *gerKA gerKC* and *gerKB* spores remained as only phase-dark germinated spores with no evidence of nascent cell release (data not shown), as found previously with *gerKA gerKC* spores (16). The fact that >85% of *gerKB* spores germinated in BHI medium in 24 h but most of these germinated spores did not progress further in development strongly suggests that GerKB is needed for normal spore outgrowth (and see below) as well as for normal spore germination.

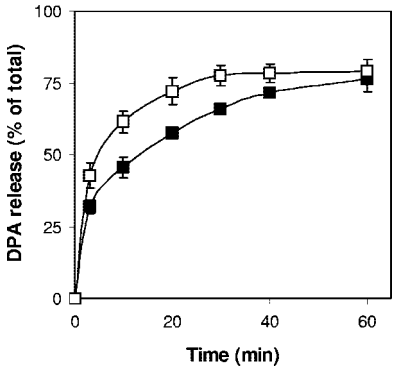

FIG. 3. Germination of spores of *C. perfringens* strains with dodecylamine. Spores of strains SM101 (wild type) (filled squares) and DPS108 (*gerKB*) (open squares) were germinated with dodecylamine, and germination was monitored by measuring DPA release as described in the text. There was no significant DPA release in 60 min by spores incubated similarly but without dodecylamine (data not shown). Error bars represent standard deviations.

TABLE 1. Colony formation by spores of *C. perfringens* strains[a]

Strain (genotype)	Spore titer ($CFU/ml/OD_{600}$)[b]		
	BHI	BHI + Ca-DPA[c]	BHI + Lyz[d]
SM101 (wild type)	3.1×10^7	3.3×10^7	3.9×10^7
DPS101 (*gerKA gerKC*)	2.6×10^6	3.5×10^6	2.0×10^6
DPS108 (*gerKB*)	4.4×10^6	4.2×10^6	8.6×10^6

[a] Heat-activated spores of various strains were plated on BHI agar, and colonies were counted after anaerobic incubation at 37°C for 24 h.

[b] Titers are the average number of $CFU/ml/OD_{600}$ determined in three experiments, and the variance was less than 15%.

[c] Heat-activated spores were preincubated with Ca-DPA as described in the text and plated on BHI plates.

[d] Spores were decoated, heat activated, and plated on BHI agar containing lysozyme (Lyz), and colonies were counted after anaerobic incubation at 37°C for 24 h.

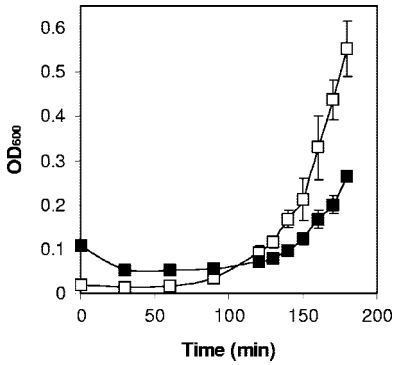

FIG. 4. Outgrowth of spores of *C. perfringens* strains. Heat-activated spores of strains DPS108 (*gerKB*) (filled squares) and SM101 (wild type) (open squares) were incubated anaerobically in TGY broth at an initial OD_{600} of 0.110 and 0.015, respectively, and the OD_{600} of the cultures was measured. Error bars represent standard deviations.

To evaluate whether preincubation with Ca-DPA could rescue apparently inviable *gerKB* spores via activation of GerKA and/or GerKC (16), *C. perfringens* spores of the wild-type and various *gerK* strains were heat activated, cooled, and incubated in 50 mM Ca-DPA (made to pH 8.0 with Tris base) for 20 min at 40°C, plated on BHI medium agar plates with or without lysozyme, and counted after anaerobic incubation at 37°C for 24 h. The preincubation of mutant spores with Ca-DPA gave no significant increase in colony-forming efficiency (Table 1). To test whether spores with a lesion in either *gerKB* or the *gerKA-gerKC* operon could be recovered by digestion of the spore's peptidoglycan cortex, spores of various strains were decoated in 1 ml of 0.1 M sodium borate (pH 10)-2% 2-mercaptoethanol for 60 min at 37°C, washed at least nine times with sterile distilled water (14), and plated on BHI plates containing lysozyme (1 μg/ml). While the viability of *gerKA gerKC* spores remained ~12-fold lower than that of wild-type spores, *gerKB* spores' viability increased slightly but was still ~5-fold lower than that of wild-type spores (Table 1).

The results given above suggest that GerKB is essential not only for normal spore germination but also for normal spore viability and outgrowth. To further examine if GerKB is involved in spore outgrowth, heat-activated spores of DPS108 (*gerKB*) and SM101 (wild-type) strains were inoculated into 10 ml TGY broth to a final OD_{600} of 0.110 and 0.015 (one-seventh that of the *gerKB* spores), respectively, and incubated anaerobically at 37°C, and at various times the OD_{600} was measured. Although the initial wild-type spores were diluted to one-seventh the OD_{600} of *gerKB* spores to correct for the *gerKB* spores' lower viability, the wild-type spores' outgrowth was much faster than that of the *gerKB* spores (Fig. 4), suggesting that GerKB is essential not only for normal spore germination and viability but also for normal spore outgrowth, since the growth rates of wild-type and *gerKB* cells are identical (data not shown). The difference in rates of outgrowth of wild-type and *gerKB* spores was even greater when the initial spores were at the same starting OD_{600} (data not shown).

The lack of effect of lysozyme on the viability of decoated *gerKB* (or *gerKA gerKC*) spores indicates that the defect in these spores is not the inability to degrade cortex peptidoglycan, since exogenous lysozyme restores viability to decoated *C. perfringens* spores that lack the essential cortex-lytic enzyme SleC (15). Indeed, *gerKB* spores degraded cortex peptidoglycan normally during spore germination with KCl (data not shown). The loss of GerKB (and perhaps GerKA and/or GerKC [16]) also slowed spore outgrowth noticeably. Some of this effect may be due to the low viability of the mutant spores, as the viability defect in these spores could manifest itself in spore outgrowth (and see below). However, when differences in spore viability were corrected for, *gerKB* spores still went through spore outgrowth more slowly than wild-type spores. The latter two findings are again different than the situation with *B. subtilis* spores, as while *B. subtilis* spores lacking known germinant receptors show low apparent viability on nutrient plates, the viability of these spores can be restored to almost that of wild-type spores by preincubation with Ca-DPA (12, 13).

The more novel conclusions from this work concern the role of GerKB in spore germination. GerKB is the only evident *C. perfringens* homolog of B proteins encoded by *gerA* operon homologs, and in *B. subtilis*, loss of the B protein from a GerA-type receptor eliminates the function of that receptor (20). One would therefore predict, based on the *B. subtilis* model, that loss of GerKB would largely eliminate *C. perfringens* spore germination. However, this was certainly not the case. There appear to be a number of possible explanations for the marked difference in the germination behaviors of spores of these two genera. First, the various GerA family proteins in *C. perfringens* spores may be able to function independently of each other, as opposed to the situation with *B. subtilis* spores. Second, it is possible that there are additional *gerA* family genes in the *C. perfringens* genome that encode proteins sufficiently different in sequence such that they are not detected by sequence alignment programs. However, use of the *C. perfringens gerA* family genes as query sequences also does not detect additional *gerA* family members (data not shown). Third, perhaps there is a radically different mechanism than activation of germinant receptors for triggering germination of *C. perfringens* spores. There is of course no evidence for this. However, recent work has identified a novel mechanism for triggering germination of spores of *Bacillus* species that does not involve the germinant receptors (21), and perhaps *C. perfringens* has a novel germination mechanism as well. At present we cannot decide definitively between these possible explanations. However, deletion of all known *gerA* family genes from *C. perfringens* and examination of the germination of these multiply deficient spores would certainly help in deciding between these possibilities.

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