

The *Clostridium perfringens* Germinant Receptor Protein GerKC Is Located in the Spore Inner Membrane and Is Crucial for Spore Germination

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The Gram-positive, anaerobic, spore-forming bacterium *Clostridium perfringens* causes a variety of diseases in both humans and animals, and spore germination is thought to be the first stage of *C. perfringens* infection. Previous studies have indicated that the germinant receptor (GR) proteins encoded by the bicistronic *gerKA-gerKC* operon as well as the proteins encoded by the *gerKB* and *gerAA* genes are required for normal germination of *C. perfringens* spores. We now report the individual role of these GR proteins by analyzing the germination of strains carrying mutations in *gerKA*, *gerKC*, or both *gerKB* and *gerAA*. Western blot analysis was also used to determine the location and numbers of GerKC proteins in spores. Conclusions from this work include the following: (i) *gerKC* mutant spores germinate extremely poorly with KCl, L-asparagine, a mixture of asparagine and KCl, or NaP₃; (ii) *gerKC* spores germinate significantly more slowly than wild-type and other GR mutant spores with a 1:1 chelate of Ca²⁺ and dipicolinic acid and very slightly more slowly with dodecylamine; (iii) the germination defects in *gerKC* spores are largely restored by expressing the wild-type *gerKA-gerKC* operon in *trans*; (iv) GerKC is required for the spores' viability, almost certainly because of the *gerKC* spores' poor germination; and (v) GerKC is located in the spores' inner membrane, with ~250 molecules/spore. Collectively, these results indicate that GerKC is the main GR protein required for nutrient and nonnutrient germination of spores of *C. perfringens* food-poisoning isolates.

Clostridium perfringens is a Gram-positive, anaerobic, spore-forming pathogenic bacterium causing gastrointestinal (GI) diseases in humans and animals (1–3). The most important type that causes *C. perfringens*-associated food poisoning (FP) in humans is *C. perfringens* type A, and this illness is the third most commonly reported food-borne disease in the United States (1, 4). *C. perfringens* spores are resistant to many environmental stresses and remain dormant in the environment for a long period of time (5, 6). Once conditions are favorable, they can break their dormancy and initiate germination in response to a variety of compounds (called germinants), including amino acids, a mixture of a 1:1 chelate of Ca²⁺ and pyridine-2,6-dicarboxylic acid (dipicolinic acid [DPA]), and other nonnutrient compounds like dodecylamine, a cationic surfactant (7, 8).

In *Bacillus subtilis* spores, binding of specific nutrient germinants to their cognate germinant receptors (GRs) (7, 9–11) located in the spore's inner membrane triggers the following events: (i) release of monovalent cations, (ii) release of the spore core's large depot (~20% of core dry weight) of DPA, and (iii) hydrolysis of the spore's peptidoglycan (PG) cortex by one or more spore cortex lytic enzymes that allows water uptake into the core to the level found in vegetative cells and then resumption of metabolism; spores also lose most of their resistance properties upon completion of germination (12, 13). *B. subtilis* spores have three major GRs, GerA, GerB, and GerK, each encoded by tricistronic operons that are expressed only in the developing forespore late in sporulation (10, 14). Loss of the A, B, or C cistron in these tricistronic GR operons leads to a loss of function of that GR (10, 15). Several studies (11, 16, 17) have shown that *B. subtilis* GerAA, GerAC, GerBA, and GerKA are located in the spore's inner membrane,

with much of these proteins exposed on the outer surface of the inner membrane (18).

In *C. perfringens*, the organization of GR genes is different from that in *B. subtilis* (8, 19). There is no tricistronic *gerA*-like operon but rather a monocistronic *gerAA* located rather far from a *gerK* locus. The *gerK* locus includes a monocistronic *gerKB* in an orientation opposite of that of a bicistronic *gerKA-gerKC* (8). Previous work has shown that *gerKA* and/or *gerKC* is required for normal germination of *C. perfringens* spores with germinants such as KCl, L-asparagine, or an L-asparagine–KCl mixture termed AK (8). However, the *gerAA* and *gerKB* genes have at most only a minor role in normal spore germination with KCl (8, 19). In this study, we have evaluated the role of the GerKA and GerKC proteins in the germination of *C. perfringens* spores. The work has demonstrated that GerKC is the most important GR protein in the germination of *C. perfringens* spores and, further, that GerKC is located in the spores' inner membrane, with at least some of the protein being found on this membrane's outer surface.

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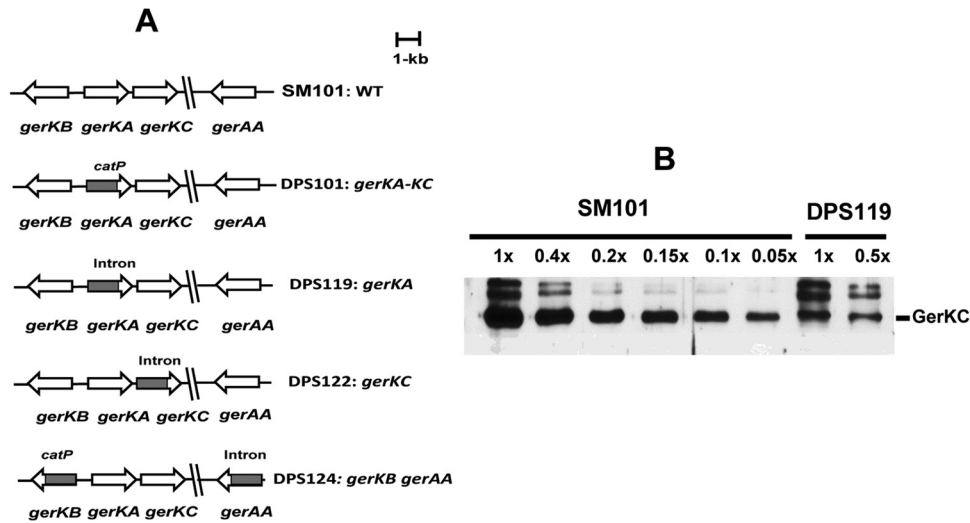


FIG 1 *C. perfringens* GR mutants. (A) Arrangement of GR genes in various *C. perfringens* mutant strains. (B) Analysis of GerKC levels in total lysates of *C. perfringens* SM101 (wild-type [WT]) and DPS119 ($\Delta gerKA$) spores. Spores were decoated and lysed, and various amounts of protein in the total lysates from the spores of the two strains were run on SDS-polyacrylamide gels, followed by Western blotting using GerKC antibody, as described in Materials and Methods. The intensity of the GerKC band in the DPS119 lysate was compared to the intensities of bands given by various amounts of GerKC in the SM101 lysate (1 \times corresponds to the same amount of spores, $\sim 1.5 \times 10^7$, in the two fractions). Analysis of the various band intensities by ImageJ gave a value for the GerKC level in DPS119 spores that was 12% of that in SM101 spores. The two bands above the GerKC band are likely bands that reacted nonspecifically, since their intensities were greatly increased relative to the intensity of the GerKC band in the DPS119 spores.

MATERIALS AND METHODS

Bacterial strains and plasmids. The *C. perfringens* strains and plasmids used in this study are described in Fig. 1 and Table S1 in the supplemental material.

Construction of a *gerKC* mutant. Construction of a *gerKC* mutant of *C. perfringens* strain SM101 was based on the modified group II intron (the ClosTron) using the ClosTron website to retarget a suitable insertion at bp 468/469 from the start codon of *gerKC*. PCR was performed on SM101 DNA to retarget the intron by using *gerKC*-specific primers CPP779, CPP780, and CPP781 (see Table S2 in the supplemental material) and the LtrBasEBS2 universal primer (CGAAATTAGAACTTGCGTTCAGTAAAC) provided with the Targetron gene knockout system (Sigma-Aldrich Corporation, St. Louis, MO). An ~ 350 -bp BsrGI-HindIII fragment retargeted to position 468/469 in the sense orientation of *gerKC* was cloned between the BsrGI and HindIII sites of the pJIR3566 vector (20), giving plasmid pDP276. Plasmid pDP276 was electroporated (21) into *C. perfringens* strain SM101, and erythromycin (Em)-resistant (Em^r) transformants were selected on brain heart infusion (BHI) agar plates supplemented with 30 μ g/ml Em. Em^r transformants were screened for insertion of the Targetron by PCR using *gerKC*-specific primers CPP440 and CPP443 (see Table S2 in the supplemental material). To cure the Em^r-coding vector, one candidate Targetron-carrying clone was subcultured three times in nonselective fluid thioglycolate (FTG) broth and plated onto BHI agar, and single colonies were patched onto BHI agar with or without Em, giving strain DPS122.

Construction of a *gerKA* mutant. A *C. perfringens* SM101 derivative with an intron inserted into the *gerKA* gene was constructed as follows. To target the *L1.LtrB* intron to *gerKA*, the intron sequence in plasmid pJIR3566 was modified using the InGex intron prediction program (Sigma-Aldrich), and an insertion site at bp 90/91 (score, 10.5) from the start codon was chosen. PCR was performed on SM101 DNA to retarget the intron using *gerKA*-specific primers CPP776, CPP777, and CPP778 (see Table S2 in the supplemental material) and the LtrBasEBS2 universal primer (CGAAATTAGAACTTGCGTTCAGTAAAC) provided with the Targetron gene knockout system (Sigma-Aldrich). An ~ 350 -bp BsrGI-HindIII fragment was retargeted to *gerKA* and was cloned between the BsrGI and HindIII sites in pJIR3566, giving plasmid pDP300. Plasmid

pDP300 was electroporated into *C. perfringens* strain SM101, and the *gerKA* mutant strain was isolated essentially as described above for the *gerKC* mutant.

Construction of a *gerKB gerAA* double mutant. A mutation in the *gerAA* gene of a previously isolated *gerKB* mutant strain, DPS108 (*gerKB::catP*) (19), was generated by inserting a Targetron in the antisense strand of *gerAA* between bp 123 and 124 downstream of the *gerAA* start codon, as previously described (8). Briefly, the *gerAA* Targetron containing plasmid pDP13 carrying the Targetron insertion between bp 123 and 124 was introduced into DPS108 by electroporation, and clones were identified with an insertion of the intron into *gerAA*, giving a *C. perfringens gerKB::catP gerAA::intron* double mutant strain, DPS124.

Construction of a *gerKC* strain complemented with wild-type *gerKA-gerKC*. To construct a *gerKC* strain complemented with wild-type *gerKA-gerKC*, we first constructed a shuttle plasmid carrying the wild-type *gerKA-gerKC* operon. An ~ 3.2 -kb KpnI-SalI fragment carrying the *gerKA-gerKC* operon with its own promoter and terminator was excised from plasmid pDP10 (8) by digestion with KpnI and SalI and then ligated between the KpnI and SalI sites of the *Escherichia coli-C. perfringens* shuttle plasmid pJIR750 that encodes chloramphenicol resistance (Cm^r) (22), giving plasmid pSB18. The recombinant plasmid pSB18 was introduced into *C. perfringens gerKC* mutant strain DPS122 by electroporation (21) and Cm^r Em^r transformants were selected. The presence of plasmid pSB18 in strain DPS122(pSB18) was confirmed by PCR and Southern blot analyses (data not shown).

Spore preparation. Starter *C. perfringens* cultures were prepared by inoculating 0.1 ml of cooked meat stock culture into 10 ml FTG broth (Difco) and incubating at 37°C, as described previously (23). Overnight FTG-grown *C. perfringens* cultures (0.4 ml) were inoculated into 10 ml fresh FTG broth and incubated at 37°C for 8 to 12 h. To prepare sporulating cultures, 0.2 ml of FTG-grown culture was transferred into 10 ml Duncan-Strong broth (24) and incubated at 37°C for 24 h. Spore formation was confirmed by phase-contrast microscopy. Scaling up of this procedure was needed for large-scale spore preparation. For purification, spore suspensions were washed with sterile distilled water until they were >98% free of vegetative cells, germinated spores, and debris. Purified

spores were resuspended in sterile distilled water at an optical density at 600 nm (OD_{600}) of ~ 6 and stored at -80°C .

Spore germination. *C. perfringens* spore germination was done as previously described (8, 25). Briefly, heat-activated (heated at 80°C for 10 min and then cooled on ice) spore suspensions (OD_{600} , ~ 1) were incubated with KCl (100 mM KCl in 25 mM Tris-HCl buffer, pH 7.0), L-asparagine (100 mM L-asparagine in 25 mM Tris-HCl buffer, pH 7.0), L-asparagine plus KCl (100 mM L-asparagine–100 mM KCl in 25 mM Tris-HCl buffer, pH 7.0), NaPO_4 buffer (100 mM NaPO_4 buffer, pH 6.0), or Ca-DPA (50 mM CaCl_2 , 50 mM DPA, adjusted to pH 8.0 with Tris-HCl) at 40°C , and spore germination was measured by monitoring the OD_{600} of germinating spores in a Synergy Mx multimode microplate reader (BioTek Instruments Inc., Winooski, VT). Levels of spore germination were also routinely confirmed at the end of the experiments by phase-contrast microscopy. The extent of spore germination was expressed as a percentage of the initial OD_{600} , with a decrease of 60% seen with $\sim 100\%$ spore germination. All values reported are averages of three experiments performed with at least three independent spore preparations.

For measuring DPA release during KCl-triggered germination, heat-activated spores (OD_{600} , 1.5) were incubated at 40°C in 100 mM KCl in 25 mM Tris-HCl buffer (pH 7.0). DPA release during dodecylamine-triggered germination was measured by incubating heat-activated spore suspensions (OD_{600} , 1.5) in 1 mM dodecylamine in 25 mM Tris-HCl (pH 7.4) at 60°C . Aliquots (1 ml) of spores whose germination was triggered by KCl or dodecylamine were centrifuged for 3 min at 13,000 rpm in a microcentrifuge, and the DPA in the supernatant was measured by monitoring the OD at 270 nm as previously described (8, 26, 27). Initial DPA levels in dormant spores were measured by boiling 1-ml aliquots for 60 min and centrifugation for 3 min at 13,000 rpm, and the DPA content of the supernatant was measured at 270 nm (8, 26, 27).

Preparation of the spore IM fraction. The spore inner membrane (IM) was prepared as described previously (16, 18, 28–30). Spores (~ 3 ml) at an OD_{600} of 25 were first decoated by incubation at 70°C for 2 h in 0.1 M NaOH, 0.1 M NaCl, 0.5% SDS, 0.1 M dithiothreitol (DTT) and washed 10 times with water, as described previously (31). The washed decoated spores of wild-type strain SM101 and strain DPS122 were suspended at an OD_{600} of 50 to 70 in 0.5 ml TEP buffer (50 mM Tris-HCl [pH 7.4], 5 mM EDTA, 1 mM phenylmethanesulfonyl fluoride [PMSF]) with 1 mg lysozyme, 1 μg each of RNase A and DNase I, and 20 μg MgCl_2 , as described previously (16, 18, 29, 32). The spore suspension was then incubated for 5 min at 37°C . After cooling on ice for 20 min, suspensions were briefly sonicated with 100 mg glass beads, examined microscopically for lysis, and centrifuged for 5 min in a microcentrifuge at maximum speed, and the supernatant was saved. The pellet was resuspended in 0.5 ml TEP buffer and centrifuged again as described above, the supernatants were pooled, and the pellet was also saved. The pooled supernatant was centrifuged at $100,000 \times g$ for 1 h at 4°C in an ultracentrifuge with a TLS 100.2 rotor, giving a soluble fraction (S100) and the IM pellet fraction (P100), which was suspended in 40 to 80 μl of TEP buffer containing 1% Triton X-100.

GerKC expression and purification and antibody production. The *gerKC* gene was amplified by PCR using genomic DNA from *C. perfringens* strain SM101 as the template and cloned into a modified pET15b expression vector containing a tobacco etch virus (TEV) protease cleavage site between the N-terminal His₆ tag and the target gene. The identity of the resultant plasmid was verified by DNA sequencing. The GerKC protein (residues 28 to 374) was overexpressed in *Escherichia coli* BL21 cells and purified by Ni^{2+} -nitrilotriacetic acid affinity chromatography, followed by TEV protease cleavage of the His₆ tag and by anion exchange and gel filtration chromatography (SD200; GE Healthcare, Piscataway, NJ). Purified protein (~ 2 mg) was used to raise polyclonal antibodies in two New Zealand White female rabbits (Pacific Immunology Group, Ramona, CA). Anti-GerKC antibodies were detected by Western blotting in a blood sample collected 2 months after the initial injection, at which time the animals were exsanguinated. The specificity of the antiserum was confirmed by Western blotting using purified GerKC protein (see Results).

Animals were used by Pacific Immunology only to prepare antibody in their facility (NIH Animal Welfare Assurance number A41820-01; USDA license 93-R-283). Their facility adheres to the standards and regulations under the Animal Welfare Act and requires that any exceptions to the Act be approved by the IACUC.

Western blot analysis. Western blot analyses were carried out following SDS-polyacrylamide (12%) gel electrophoresis (SDS-PAGE) and transfer of proteins to polyvinylidene difluoride (PVDF) membranes as described previously (13, 18, 32). The GerKC protein was detected by using polyclonal rabbit antisera (1:1,000) against GerKC and goat anti-rabbit IgG–horseradish peroxidase (HRP) conjugate (1:10,000). The activity of HRP was detected using a PicoMax sensitive chemiluminescent HRP substrate (Rockland Immunochemicals, Gilbertsville, PA) with a chemiluminescence detection system (Molecular imagDoc XRS+ system; Bio-Rad, Hercules, CA).

Determination of GerKC level in spores. Dormant spores (~ 20 mg) of *C. perfringens* strain SM101 (wild type) were decoated, treated with lysozyme, disrupted with glass beads and sonication ($\sim 8.4 \times 10^9$ spores were disrupted), and then centrifuged at $14,000 \times g$ for 10 min as described above. The pellet from this initial centrifugation was saved and termed the pellet fraction. The supernatant fraction was further centrifuged as described above, giving inner membrane pellet and supernatant fractions, and various percentages of the inner membrane and initial pellet fractions along with known quantities of purified GerKC were subjected to Western blot analysis using anti-GerKC serum. The intensities of the resulting bands were quantitated using ImageJ software, and the average total number of GerKC molecules per spore was calculated (29, 32).

Determination of GerKC susceptibility to modification by an exogenous biotinylation reagent. Biotinylation was performed as previously described (18) with some modifications. Dormant SM101 spores at an OD_{600} of ~ 25 (5 ml) were decoated and washed as described above. Buoyant density gradient centrifugation was performed in 50% Histodenz medium (Sigma Chemical Co., St. Louis, MO) to remove small amounts of germinated spores. The dormant spore pellet from this centrifugation was washed extensively with water, and the resulting decoated dormant spores were suspended in water at an OD_{600} of 100.

Germinated spores were obtained by first heat shocking intact dormant spores (3 ml at an OD_{600} of 50) at 80°C for 10 min. After cooling to 25°C for 5 min, spores were heated to 40°C for 10 min and germinated at an OD_{600} of ~ 1.5 in 50 ml of 100 mM KCl in 25 mM sodium phosphate buffer (pH 7.0) for 2 h, until at least 90% of spores were germinated, as determined by phase-contrast microscopy. Small amounts of dormant spores were not removed from these germinated spore preparations. However, these dormant spores had not been decoated and were thus not lysed by subsequent lysozyme treatment. Consequently, the IM was not isolated from these dormant spores but was isolated only from the spores that had become lysozyme sensitive due to their germination.

Biotinylation of lysyl amino groups in dormant or germinated spore protein was done with 5 ml of spores at an OD_{600} of ~ 15 in 10 mM K-HEPES buffer (pH 7.4) and 150 mM NaCl using 2 mM EZ-Link sulfo-NHS-SS-biotin reagent (Pierce Chemical Co., Rockford, IL) for 1 h at room temperature on a rocker. Unreacted reagent was quenched by addition of 1 ml of 2 M glycine and 1 ml of 1 M Tris-HCl buffer (pH 7.4) and incubation for 30 min at 23°C . All labeled spores were washed twice with 20 ml water.

For analysis of GerKC biotinylation, NeutrAvidin agarose beads (Pierce) were prepared by spinning 100 μl of slurry at $1,000 \times g$ for 1 min at 23°C , discarding the supernatant, and then washing twice with 100 μl TEP buffer containing 1% Triton X-100. Sixty microliters of the IM fraction was added to the resulting bead pellet, and the mixture was shaken on a rocker. After shaking at 23°C for 1 h, the sample was centrifuged at $1,000 \times g$ for 1 min and the supernatant containing unbound proteins () was saved. The resulting bead pellet was washed three times with 100 μl TEP buffer containing 1% Triton X-100, and the washes were also saved. Bound proteins were eluted by incubating the beads with 60 μl Laemmli sample buffer containing 55 mM DTT for 1 h at 23°C . Western blot anal-

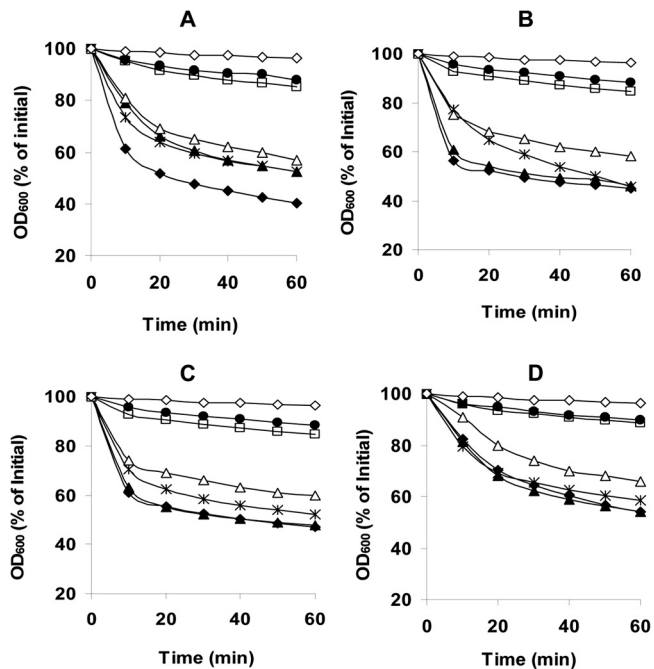


FIG 2 Germination of *C. perfringens* spores with various germinants. Heat-activated spores of strains SM101 (wild type) (◆), DPS101 (*gerKA-KC*) (●), DPS119 (*gerKA*) (▲), DPS122 (*gerKC*) (□), DPS124 (*gerKB gerAA*) (×), and DPS122(pSB18) (*gerKC* mutant complemented with wild-type *gerKA-gerKC*) (△) were incubated at 40°C with KCl (A), L-Asn (B), AK (C), or NaP_i (pH 6.0) (D), and germination was measured by determination of the OD₆₀₀, as described in Materials and Methods. The control germination (◇) was heat activation of spores, which were incubated in 25 mM Tris-HCl buffer (pH 7.0) at 40°C, and no germination difference was observed between spores of SM101 and the GR mutant strains.

yses were performed as described above following SDS-PAGE of equal percentages of the total IM fraction, the flowthrough fraction, and the eluted fraction, all run on the same Western blot and in some cases with various dilutions of the flowthrough or eluted fraction.

Other methods. For determination of the colony-forming efficiencies of spores of various strains, spores at an OD₆₀₀ of 1 (~10⁸ spores/ml) were heat activated as described above, aliquots of dilutions were spread on BHI agar with or without lysozyme (1 μg/ml) and incubated anaerobically at 37°C for 24 h, and colonies were counted.

Student's *t* test was used for specific comparisons between data sets.

RESULTS

***gerKA* mutant spores produce lower levels of GerKC than wild-type spores.** Previous studies (8, 19) suggested that the main GR proteins involved in the germination of *C. perfringens* spores are encoded by the bicistronic *gerKA-gerKC* operon, while the products of *gerKB* and *gerAA* have auxiliary roles. However, those studies were conducted on a *C. perfringens* strain (DPS101) with an intron insertion into the *gerKA* cistron that likely had a polar effect on expression of the downstream *gerKC*. To dissect the role of the GerKA and GerKC GR proteins individually in *C. perfringens* spore germination, we constructed mutants with double *gerAA gerKB* mutations and single *gerKA* or *gerKC* mutations in strain SM101 (Fig. 1A). To evaluate whether insertion of an intron in *gerKA* exerted a polar effect on the downstream *gerKC*, the levels of GerKC in spores of wild-type and *gerKA* mutant strains were compared (Fig. 1B). Western blotting with antisera against GerKC

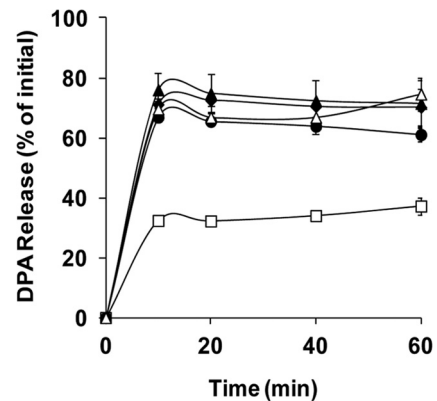


FIG 3 DPA release by spores of *C. perfringens* strains during germination with KCl. Heat-activated spores of strains SM101 (wild-type) (◆), DPS119 (*gerKA*) (▲), DPS122 (*gerKC*) (□), DPS124 (*gerKB gerAA*) (●), and DPS122(pSB18) (*gerKC* mutant complemented with wild-type *gerKA-gerKC*) (△) were germinated with KCl (pH 7.0), and DPA release was measured as described in Materials and Methods. Error bars represent standard deviations.

demonstrated the presence of GerKC in *gerKA* mutant spores. However, quantitative analyses showed that there was only ~12% of the wild-type GerKC level in *gerKA* mutant spores. These results indicate that the *gerKA* mutation did indeed exert a partial polar effect on the downstream *gerKC* gene.

GerKC, but not GerKA, has a major role in *C. perfringens* spore germination. Spores of the double *gerAA gerKB* mutant strain germinated well in the presence of L-Asn, KCl, AK, or NaP_i (Fig. 2A to D), confirming the minor roles played by GerKB and GerAA in spore germination (8, 19) and consistent with GerKA and GerKC being the main GR proteins in spores of *C. perfringens* FP strain SM101.

To dissect the precise roles of GerKA and GerKC in *C. perfringens* spore germination, we constructed a single *gerKA* or *gerKC* mutant in strain SM101 (Fig. 1) and measured the germination of the spores of these various strains. As expected, *gerKA gerKC* spores germinated poorly and to a much lesser extent than wild-type spores with L-Asn, KCl, AK, or NaP_i (Fig. 2A to D). While *gerKA* and wild-type spores germinated similarly, *gerKC* spores germinated much more poorly with these germinants than wild-type and other GR mutant spores ($P < 0.05$) (Fig. 2A to D). Most importantly, the germination defects of *gerKC* spores were largely corrected by complementing the *gerKC* mutant with the wild-type *gerKA-gerKC* operon. Collectively, these results indicate that GerKC is the most important GR protein in *C. perfringens* SM101 spore germination.

After germinants bind their cognate GR, the next easily measurable event in spore germination is the release of DPA from the spore core. Therefore, we also evaluated the effects of mutations in GR protein genes on DPA release during germination with KCl (Fig. 3). As expected, wild-type spores released ~70% of their DPA within the first 10 min of germination with KCl (Fig. 3). Strikingly, the rate and amount of DPA release by *gerKA* spores incubated with KCl were similar to those of wild-type spores, indicating that GerKA is not essential for DPA release during spore germination, at least when GerKB and GerAA are present, and only slightly less DPA release was obtained when *gerAA gerKB* spores were germinated with KCl. In contrast, *gerKC* spores released ~50% less DPA ($P < 0.05$) than wild-type spores during KCl

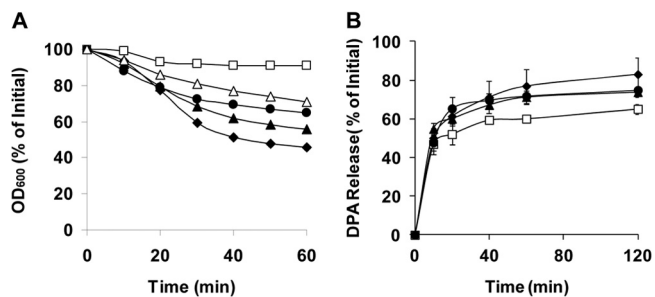


FIG 4 Germination of *C. perfringens* wild-type and mutant spores with Ca-DPA (A) and dodecylamine (B). Heat-activated spores of strains SM101 (wild-type) (♦), DPS119 (*gerKA*) (▲), DPS122 (*gerKC*) (□), DPS124 (*gerKB gerAA*) (●), and DPS122(pSB18) (*gerKC* mutant complemented with wild-type *gerKA-gerKC*) (△) were germinated with Ca-DPA and dodecylamine. For Ca-DPA, changes in OD₆₀₀ were measured as described in Materials and Methods, and for dodecylamine, germination was monitored by measurement of DPA release, as described in Materials and Methods. Error bars represent standard deviations.

germination (Fig. 3). The DPA release defect in *gerKC* spores was also corrected by complementation with a plasmid carrying wild-type *gerKA-gerKC*. These results further indicate that GerKC is the main GR protein involved in *C. perfringens* spore germination.

GerKC is also important in germination of *C. perfringens* spores with Ca-DPA and dodecylamine. Germination of bacterial spores can also be triggered by nonnutrient germinants, such as the cationic surfactant dodecylamine (27) and exogenous Ca-DPA, and in *B. subtilis* spores, germination with these agents does not involve any GR protein (7, 15). It was previously shown that unlike spores of *Bacillus* species, where Ca-DPA triggers spore germination by direct activation of the cortex-lytic enzyme CwlJ (9, 33), in *C. perfringens*, Ca-DPA triggers germination in a GerKA- and/or GerKC-dependent pathway (8). When wild-type, *gerKB gerAA*, and *gerKA* spores were incubated with Ca-DPA, significant germination was observed, as measured by the decrease in the OD₆₀₀ (Fig. 4A). In contrast, there was minimal germination of *gerKC* spores with Ca-DPA (Fig. 4A), and phase-contrast microscopy confirmed that ~95% of these spores remained phase bright (data not shown). The Ca-DPA germination defect of the *gerKC* spores was partially corrected by complementation with wild-type *gerKA-gerKC* (Fig. 4A), indicating that the Ca-DPA germination pathway is dependent on GerKC.

Previous work showed that GerKA and/or GerKC is also required for normal spore germination with dodecylamine (8). In the current work, *gerKB gerAA* and *gerKA* spores released almost the same amount of DPA as wild-type spores during germination with dodecylamine (Fig. 4B), suggesting that GerAA, GerKB, and GerKA might have no role in dodecylamine germination. The *gerKC* spores incubated with dodecylamine released slightly less DPA than wild-type spores (Fig. 4B), indicating that GerKC may play a minor role in triggering DPA release during dodecylamine germination.

GerKC is required for normal colony formation in BHI medium. Unlike *B. subtilis*, where spores lacking all GRs retain full viability once these are decoated and rescued in the presence of lysozyme, the absence of *gerKA-gerKC* and *gerKB* does affect the apparent viability of *C. perfringens* spores (8, 19). Therefore, to establish if it is GerKA or GerKC that is involved in *C. perfringens* spores' apparent viability, the colony-forming efficiencies of in-

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

Strain (genotype)	Spore titer (no. of CFU/ml/OD ₆₀₀ unit) ^b	
	BHI	BHI + Lyz ^c
SM101 (wild type)	2.4×10^8	7.1×10^8
DPS 119 (<i>gerKA</i>)	1.7×10^8	5.7×10^8
DPS122 (<i>gerKC</i>)	2.3×10^6	4.2×10^8
DPS124 (<i>gerKB gerAA</i>)	3.8×10^7	6.1×10^8
DPS122(pSB18) (<i>gerKC</i> mutant complemented with wild-type <i>gerKA-gerKC</i>)	1.2×10^8	ND ^d

^a Heat-activated spores of various strains were plated on BHI agar with or without lysozyme, and colonies were counted after anaerobic incubation at 37°C for 24 h, as described in Materials and Methods.

^b Titers are the average number of CFU/ml/OD₆₀₀ unit determined in three experiments, and the variation was less than 15%.

^c Spores were decoated, heat activated, and plated onto BHI agar containing lysozyme (Lyz), and colonies were counted after they were incubated overnight anaerobically at 37°C.

^d ND, not determined.

tact and decoated wild-type and mutant *C. perfringens* spores were assessed by plating spores onto BHI agar and incubating them anaerobically for 24 h at 37°C. There was no significant ($P > 0.05$) difference in the colony formation efficiency of wild-type and *gerKA* spores, while *gerAA gerKB* spores had an ~6-fold lower colony formation efficiency (Table 1). However, intact *gerKC* spores exhibited an ~100-fold lower colony formation efficiency than wild-type and *gerKA* spores (Table 1). Decoating of spores and plating on BHI agar containing lysozyme increased the colony-forming efficiencies of spores of all mutant strains tested, including the *gerKC* spores, to a level approximately equal to that of the wild-type spores (Table 1). Thus, the *gerKC* spores are fully viable, and most simply do not germinate well.

To directly examine the possibility that the lower colony formation efficiency of *gerKC* spores was due to their poor germination in BHI broth, we compared the germination of spores of all four strains in this medium. As expected, *gerKC* spores exhibited significantly ($P < 0.05$) lower germination in BHI broth than wild-type spores, and the germination defect in *gerKC* spores was at least partially restored by complementing the mutant with wild-type *gerKA-gerKC* (Fig. 5). The *gerKA* and *gerKB gerAA* spores also

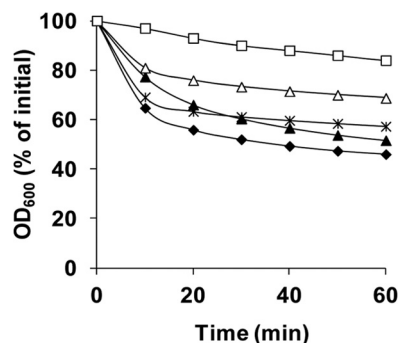


FIG 5 Germination of spores of *C. perfringens* strains in BHI broth. Heat-activated spores of strains SM101 (wild-type) (♦), DPS119 (*gerKA*) (▲), DPS122 (*gerKC*) (□), DPS124 (*gerKB gerAA*) (×), and DPS122(pSB18) (*gerKC* mutant complemented with wild-type *gerKA-gerKC*) (△) were germinated at 40°C in BHI broth, and the OD₆₀₀ was measured as described in Materials and Methods.

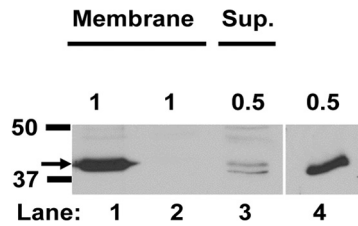


FIG 6 Localization of GerKC in *C. perfringens* spores. Dormant spores of *C. perfringens* strains SM101 (wild type) and DPS122 (*gerKC*) were decoated, disrupted with glass beads and sonication after lysozyme treatment, and centrifuged at $14,000 \times g$ for 10 min, and the supernatant was centrifuged at $100,000 \times g$ for 1 h, as described in Materials and Methods. Aliquots of the P100 pellet IM fractions (lanes 1 and 2) from wild-type and *gerKC* spores and the S100 supernatant (Sup.) fraction from wild-type spores (lane 3) were run on SDA-polyacrylamide gels, followed by Western blotting using GerKC antibody, as described in Materials and Methods. The arrow on the left indicates the size of GerKC, ~ 40 kDa. The amounts of sample run in lanes 1 to 3 are given above the lanes as the percentage of the total IM protein or supernatant fraction protein. The S100 supernatant fraction from *gerKC* spores had no detectable GerKC band (data not shown). Lane 4, 0.5 ng purified GerKC antigen. The numbered lines to the left of the figure denote the migration positions of molecular mass markers (in kDa). All lanes are from the same gel, but an intervening region between lanes 3 and 4 was removed for clarity.

exhibited slightly lower germination than wild-type spores (Fig. 5). Phase-contrast microscopy found that $>65\%$ of wild-type and *gerKA* spores, $\sim 50\%$ of *gerKB gerAA* and *gerKC*-complemented spores, but only $\sim 10\%$ of *gerKC* spores were phase dark after 1 h of incubation in BHI broth (data not shown), in agreement with the results obtained by measurements of germination by determination of the OD_{600} (Fig. 5). In addition, when the spores were incubated aerobically for 18 h in BHI broth, $\sim 95\%$ of wild-type, *gerKA*, *gerKB gerAA*, and *gerKC*-complemented spores were phase dark, and $\sim 90\%$ of these phase-dark spores had released the nascent vegetative cell (data not shown). In contrast, only $\sim 65\%$ of *gerKC* spores were phase dark and less than 10% of the phase-dark spores had released the nascent vegetative cell (data not shown), again consistent with the lower colony formation efficiency observed with these spores on plates without lysozyme. Collectively, these results indicate that GerKC, but not GerKA, is essential for normal completion of spore germination and perhaps also outgrowth and, thus, for normal colony formation in BHI medium.

GerKC localizes to the IM of *C. perfringens* spores. The GR proteins of *Bacillus* species have been localized in spores' IM (11, 16, 17). Therefore, since GerKC is essential for germination of *C. perfringens* spores, we attempted to immunolocalize GerKC in spores using polyclonal rabbit antiserum raised against recombinant *C. perfringens* GerKC, and the recombinant GerKC was detected as an ~ 40 -kDa protein by the antiserum (Fig. 6). When spore IM fractions obtained from decoated *C. perfringens* wild-type and *gerKC* spores were separated by SDS-PAGE and subjected to Western blot analysis (Fig. 6), a dominant 40-kDa protein band was detected in the IM fraction from wild-type but not *gerKC* spores. These results clearly indicate that the GerKC localizes to the IM of *C. perfringens* spore.

***C. perfringens* has ~ 250 GerKC molecules per spore.** In bacterial spores, GRs act as environmental sensors that trigger spore germination when conditions are deemed adequate for growth (7, 10, 34). The germination machinery in *B. subtilis* spores appears to amplify the germination signal through increases in the levels of

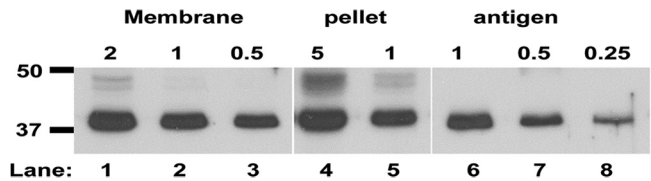


FIG 7 Quantitation of the GerKC level in spores of *C. perfringens* strain SM101. Various amounts of P100 pellet IM fractions (lanes 1 to 3) and pellet fractions of decoated, disrupted spores obtained by centrifugation at $14,000 \times g$ (lanes 4 and 5) and various amounts of purified GerKC antigen (lanes 6 to 8) were run on SDS-polyacrylamide gels, followed by Western blot analysis using anti-GerKC antibody. The amounts of samples run are shown above the lanes as either the percentage of the total membrane protein or pellet fraction protein from 8.4×10^9 spores (lanes 1 to 5) or the amount (in ng) of GerKC antigen (lanes 6 to 8). The numbered lines to the left of the figure denote the migration positions of molecular mass markers (in kDa). All lanes are from the same gel, but lanes 4 and 5 were moved from their original position to the right of the GerKC antigen lanes.

various germination components, with the GRs being present in hundreds of molecules per spore, and with the proteins involved downstream of GRs, the SpoVA proteins that are essential for DPA release in spore germination, being present at 5,000 to 10,000 molecules per spore (16, 17, 28, 32, 35). Consequently, it was of interest to analyze the level of GerKC in *C. perfringens* spores. In order to obtain a complete assessment of GerKC levels in spores, the IM fraction and the pellet fraction obtained by low-speed centrifugation after spore disruption were analyzed for GerKC by Western blotting, and the intensities of the GerKC bands in these fractions were compared to those of purified GerKC (Fig. 7). While $\sim 60\%$ of the GerKC was present in the IM fraction, $\sim 40\%$ was found in the pellet fraction obtained by low-speed centrifugation, presumably reflecting the presence of significant amounts of inner membrane in this fraction as well. The same results were also found for spores of *B. subtilis* (32). In total, *C. perfringens* spores were calculated to have ~ 250 GerKC molecules per spore, although this number may vary significantly between individual spores in the population.

Topology of GerKC in *C. perfringens* spores' IM. Analysis of the amino acid sequence encoded by the *gerKC* gene indicates that GerKC is likely a peripheral membrane lipoprotein. While most of the protein is relatively hydrophilic, it has a hydrophobic N-terminal region that is likely a signal peptide that is followed by a consensus sequence for covalent addition of a diacylglycerol moiety to an N-terminal cysteine residue formed by signal peptide cleavage. A high-resolution structure of a *B. subtilis* GR C protein has been determined and is a relatively globular structure (36). Even more importantly, prediction of the structure of other GR C proteins, including those from *Clostridium* species, suggests that all GR C proteins adopt a similar structure. Given this likely structure of *C. perfringens* GerKC, as well as the expression of *gerKA-gerKC* only in the developing forespore, it is thus most likely that GerKC is a peripheral membrane protein on the outer leaflet of the spore IM.

Analysis of the topology of GR C proteins by analysis of these proteins' susceptibility to biotinylation by an exogenous reagent has recently shown that these proteins are well biotinylated in intact *B. subtilis* spores, suggesting that GR C proteins are indeed on the IM outer surface (18). Consequently, we also subjected intact dormant and germinated *C. perfringens* spores to the same

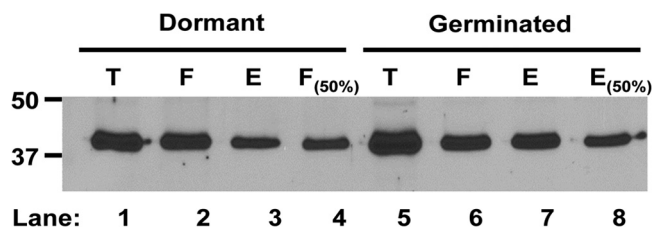


FIG 8 Analysis of the level of biotinylation of GerKC in decoated dormant and germinated spores of *C. perfringens* wild-type strain SM101. Biotinylation was carried out on decoated dormant spores and intact germinated spores as described in Materials and Methods. Spores were disrupted and fractionated, and the levels of various germination proteins in (i) the total IM fraction (T), (ii) the IM fraction that did not adsorb to the NeutrAvidin beads (F), and (iii) the eluate from the NeutrAvidin beads (E) were assayed by Western blotting using anti-GerKC antiserum, as described in Materials and Methods. Aliquots in the T, F, and E samples were from equal amounts of spores. The F_(50%) sample (lane 4) from dormant spores was also the protein that did not adsorb to the NeutrAvidin beads but was from 1/2 the amount of spores as in the other lanes. The E_(50%) sample (lane 8) from germinated spores was also the eluate from the NeutrAvidin beads but was from 1/2 the amount of spores as in the other lanes. Analysis of this Western blot by the program ImageJ indicated that ~33% of GerKC was biotinylated in dormant spores, with ~50% biotinylated in germinated spores.

analysis and evaluated GerKC for biotinylation by Western blotting following affinity purification of biotinylated proteins (Fig. 8). Approximately 33% of *C. perfringens* GerKC was biotinylated in decoated but otherwise intact *C. perfringens* spores, with this value increasing to ~50% in intact germinated spores. This value for dormant spores is almost identical to that found for the biotinylation of two GR C proteins in dormant *B. subtilis* spores, although the same two GR C proteins were 74 to 100% biotinylated in germinated *B. subtilis* spores (18). These results are certainly consistent with the majority of GerKC being located on the outer surface of *C. perfringens* spores' IM, although we cannot be sure that all GerKC is there. We also do not understand why biotinylation of GerKC in dormant and germinated spores is not complete, although this has also been seen with several *B. subtilis* IM proteins, including GR subunits (18).

DISCUSSION

A first major conclusion from this work is that GerKC is the major GR protein in spores of *C. perfringens* food-poisoning strain SM101. Previous work (8) suggested that the products of the bicistronic *gerKA-gerKC* operon were most important in spore germination; however, those studies were done on a *C. perfringens* strain with a mutation on the *gerKA* cistron, which caused a polar effect on the downstream *gerKC*. Here, we show that a Targetron mutant with a mutation in *gerKA* still allows significant germination of *gerKA* spores, suggesting that GerKC plays a major role in this process. In fact, inactivation of *gerKC* in strain SM101 affected spore germination with all known nutrient germinants. However, it is unclear whether GerKC acts as a receptor itself and contains multiple ligand binding sites, is an essential component of a multiple-subunit receptor formed by GerAA, GerKB, and/or GerKA, or even interacts with other unknown proteins. Clearly, further studies are needed to answer these questions.

Another major conclusion is that GerKC is required for full apparent viability of *C. perfringens* spores. The lower colony-forming ability of *gerKC* spores in rich BHI medium compared to that of wild-type and *gerKA* spores was consistent with the signifi-

cantly slower germination of *gerKC* spores in BHI medium and the slower release of nascent vegetative cells than wild-type and *gerKA* spores. The reversion to wild-type levels of colony formation by *gerKC* spores complemented with wild-type *gerKA-gerKC* is further evidence that GerKC is also important in spore viability. However, the lower viability of *gerKC* spores is apparent only since full *gerKC* spore viability was restored when decoated spores were plated in the presence of lysozyme. Perhaps the GerKC protein is involved in the activation of either the cortex lytic enzyme (CLE) SleC to allow cortex hydrolysis or some unknown enzyme that induces the release of nascent vegetative cells from the coat/exosporium. This role of *C. perfringens* GerKC is in contrast to that of *B. subtilis* GRs, where the viability of spores lacking one GR is relatively normal, although spores lacking all GRs do exhibit low apparent viability (15).

A third major conclusion of this work is that GerKC localizes to the inner membrane of *C. perfringens* spores. This finding was not unexpected, since GR proteins of *B. subtilis* have been shown to localize in the spore inner membrane (11, 16, 17). The level of GerKC in *C. perfringens* spores was also just 2- to 4-fold lower than the levels of GR in *B. subtilis* spores. A key difference in the components of the germination machinery between *C. perfringens* and *B. subtilis* spores is that *C. perfringens* spores lack a homologue of the lipoprotein GerD (34), which plays a major role in GR-dependent germination in *B. subtilis* spores (30, 37), perhaps by ensuring that all GRs localize into the germinosome (32).

A minor conclusion from this work is that GerKC is essential for normal DPA release during *C. perfringens* spore germination with many germinants. Previous work (8) suggested that GerKA and/or GerKC is required for normal DPA release with nutrient and nonnutrient germinants. Here, we show that only the absence of GerKC affects DPA release from the spore core during nutrient and nonnutrient germination of *C. perfringens* spores. Further work will be needed to establish the reason for the apparent partial release of DPA during germination of *gerKC C. perfringens* spores. Some possible explanations for these results are that GerKC (i) is required for the correct colocalization or stability of other GR proteins and/or to form a functional GR by itself, with other GR subunits, or with unknown proteins that will efficiently trigger Ca-DPA release; (ii) might be acting as a link between GR subunits (i.e., GerAA, GerKB, and GerKA) and proteins that form the Ca-DPA channel; or (iii) is required for complete DPA release from individual spores during germination, although this seems unlikely, since DPA release from individual germinating spores of both *C. perfringens* and *Bacillus* species is invariably an all-or-none phenomenon (38–40).

In conclusion, the results presented in this work should provide a deeper insight into the mechanism of *C. perfringens* spore germination in which KCl, L-Asn, AK, NaP_i, and the nonnutrient germinants Ca-DPA and dodecylamine act largely or at least in part through the GerKC GR protein. In contrast, GerKA, GerAA, and GerKB play at most auxiliary roles, since deletion of either of these GR proteins has no significant effect on *C. perfringens* spore germination.

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