

SleC Is Essential for Cortex Peptidoglycan Hydrolysis during Germination of Spores of the Pathogenic Bacterium *Clostridium perfringens*^{∇†}

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Clostridial spore germination requires degradation of the spore's peptidoglycan (PG) cortex by cortex-lytic enzymes (CLEs), and two *Clostridium perfringens* CLEs, SleC and SleM, degrade cortex PG in vitro. We now find that only SleC is essential for cortex hydrolysis and viability of *C. perfringens* spores. *C. perfringens* *sleC* spores did not germinate completely with nutrients, KCl, or a 1:1 chelate of Ca²⁺ and dipicolinic acid (Ca-DPA), and the colony-forming efficiency of *sleC* spores was 10³-fold lower than that of wild-type spores. However, *sleC* spores incubated with various germinants released most of their DPA, although slower than wild-type or *sleM* spores, and DPA release from *sleC sleM* spores was very slow. In contrast, germination and viability of *sleM* spores were similar to that of wild-type spores, although *sleC sleM* spores had 10⁵-fold-lower viability. These results allow the following conclusions about *C. perfringens* spore germination: (i) SleC is essential for cortex hydrolysis; (ii) although SleM can degrade cortex PG in vitro, this enzyme is not essential; (iii) action of SleC alone or with SleM can accelerate DPA release; and (iv) Ca-DPA does not trigger spore germination by activation of CLEs.

Clostridium perfringens is a gram-positive, spore-forming anaerobic bacterium and a significant cause of histotoxic and gastrointestinal diseases in humans and animals (19, 20). *C. perfringens* isolates are classified into five types, A through E, based on their ability to produce alpha-, beta-, epsilon- and iota-toxin (20). A small percentage (<5%) of type A isolates produce the *C. perfringens* enterotoxin and cause type A food poisoning and non-food-borne gastrointestinal diseases (19). *C. perfringens* isolates also can form metabolically dormant spores that are resistant to many stress factors and can survive for long periods in the environment. Under favorable conditions these surviving spores can germinate, outgrow, return to vegetative growth, and then release toxins and cause disease (20).

Bacterial spores initiate germination when they sense compounds termed germinants, including nutrients, a 1:1 chelate of Ca²⁺ and pyridine-2,6-dicarboxylic acid (dipicolinic acid [DPA]) (Ca-DPA) and cationic surfactants (27, 30, 42). In spores of *Bacillus subtilis* and related species, nutrient germinants are sensed by receptors located in the spore's inner membrane, with subsequent initiation of biophysical events including release of monovalent cations (H⁺, Na⁺, and K⁺), and the spore core's large depot of DPA present as a 1:1 chelate with divalent cations, predominantly Ca²⁺ (42). Hydrolysis of the spore's peptidoglycan (PG) cortex is a later event in germination, and in *B. subtilis* is triggered at least in part by Ca-DPA release (29, 42). Cortex PG hydrolysis is

essential for completion of spore germination, since this removes a physical constraint allowing the core to expand and take up water to the level found in vegetative cells (33). This full hydration now allows enzyme activity in the spore core leading to initiation of energy metabolism, macromolecular synthesis and spore outgrowth (9, 42).

In *Bacillus* spores the PG cortex comprises ≥80% of spore PG, with the remainder in the nascent germ cell wall that becomes the cell wall of the outgrowing spore (21). Cortex PG generally has three structural differences from germ cell wall and vegetative cell PG, as studied best in *B. subtilis* (2, 34, 35). (i) Although about one-fourth of cortex *N*-acetylmuramic acid (NAM) residues are substituted with short peptides, essentially all NAM residues in germ cell wall and vegetative cell PG carry these short peptides. As a consequence cortex PG is less highly cross-linked than germ cell wall or vegetative PG. (ii) About one-fourth of NAM residues in cortex PG carry a single L-alanine residue, a modification not found in germ cell wall or vegetative cell PG, although this modification is not present in *C. perfringens* cortex PG (26). (iii) Approximately every second muramic acid residue in cortex PG has been converted to muramic- δ -lactam, a modification again not found in germ cell wall or vegetative cell PG (47, 48). Muramic- δ -lactam appears to be the recognition element for cortex-lytic enzymes (CLEs) that hydrolyze the cortex during spore germination but do not act on germ cell wall PG (17, 42).

In *B. subtilis* and *Bacillus megaterium* there are two redundant CLEs that degrade cortex PG during germination, SleB and CwlJ, as well as a third enzyme SleL (YaaH) that by itself is not sufficient for cortex hydrolysis during germination, although it may contribute to the hydrolysis in some way (9, 16, 17, 42). SleB is synthesized in the developing forespore and is located primarily at the inner edge of the cortex in a mature form possessing lytic transglycosylase activity (4, 8), but the

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mechanism for regulation of SleB activity is unclear. In contrast to SleB, CwlJ is synthesized in the mother cell and is located primarily at the outer edge of the spore cortex (3, 8, 13). While CwlJ appears to be synthesized in an active form, the specificity of this enzyme has not been determined. However, CwlJ is activated during germination by Ca-DPA, either released from the spore core or supplied exogenously (17, 42).

Work with *C. perfringens* strain S40 has identified two CLEs, SleC and SleM, as potentially involved in cortex PG hydrolysis during spore germination. The *sleC* and *sleM* genes are present in the genomes of all sequenced *C. perfringens* strains (23, 43), and homologues are also present in other *Clostridium* species (5, 24, 39). SleC is synthesized in the mother cell compartment of the sporulating cell as a precursor with four domains, and the N-terminal preredion and the C-terminal pro-region are removed during sporulation. SleC exists in the spore as the inactive zymogen, pro-SleC, consisting of an N-terminal pro-sequence and the mature active enzyme, and pro-SleC is converted to the active enzyme early in spore germination through removal of the pro-region by germination-specific proteases (22, 25, 45). However, it is not clear what regulates germination-specific protease activity. Active SleC is a bifunctional enzyme with lytic transglycosylase and *N*-acetylmuramoyl-L-alanine amidase activity on cross-linked peptide moieties in the cortex (15), and the purified enzyme is active on the cortex in decoated spores (15). SleM is also synthesized in the mother cell compartment during sporulation but as the mature enzyme and has *N*-acetylmuramidase activity. SleM appears to degrade only SleC-modified cortex PG and has no activity on decoated spores (7). Both SleC and SleM appear to be located on the outer edge of the spore cortex and are removed when spores are decoated (7).

While significant knowledge has been obtained on the properties of SleC and SleM in vitro, the actual role of these enzymes during *C. perfringens* spore germination has not been established. Consequently, in the current study we have constructed *sleC*, *sleM*, and *sleC sleM* mutant strains of *C. perfringens* and used these strains to determine the roles of SleC and SleM during spore germination.

MATERIALS AND METHODS

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

Construction of *gusA* fusion plasmids and β -glucuronidase assay. DNA fragments (300 to 400 bp) upstream of *sleC* and *sleM* from *C. perfringens* SM101, which included the 52- and 74-bp intergenic regions between *sleC* and *csxB* and between *sleM* and CPR1312, respectively, that most likely contain these genes' promoters, as shown for *C. perfringens* strain S40 (18), were PCR amplified using the primer pairs CPP399/PPP388 and CPP396/PPP398. The forward and reverse primers (see Table S2 in the supplemental material) had SalI and PstI cleavage sites, respectively. These PCR fragments were digested with SalI and PstI and cloned between SalI and PstI sites in pMRS127 to create *sleC*- and *sleM-gusA* fusions, giving plasmids pDP86 and pDP87 (see Table S1 in the supplemental material). These plasmids were introduced by electroporation (10) into *C. perfringens* SM101, and erythromycin-resistant (50 μ g/ml) transformants were selected. Transformants carrying plasmids with the *sleC-gusA* and *sleM-gusA* fusions were grown vegetatively in TGY medium (3% Trypticase soy, 2% glucose, 1% yeast extract, 0.1% L-cysteine) (14) and in Duncan-Strong (DS) (11) sporulation medium, and assayed for β -glucuronidase (GUS) activity as described previously (49). GUS specific activity was expressed in Miller units that were calculated as described previously (36).

Construction of a *C. perfringens sleC* deletion mutant. To isolate a derivative of *C. perfringens* SM101 with a deletion of *sleC*, a Δ *sleC* suicide vector was con-

structed as follows. A 2,235-bp DNA fragment carrying 114 bp from the N-terminal coding region and 2,121 bp upstream of *sleC* was PCR amplified by using the primer pair CPP357/PPP365 (forward and reverse primers [see Table S2 in the supplemental material] had KpnI and BamHI cleavage sites at the 5' ends, respectively). A 2,087-bp DNA fragment carrying 242 bp from the C-terminal coding region and 1,845 bp downstream of *sleC* was PCR amplified using the primer pair CPP359/PPP364 (forward and reverse primers [see Table S2 in the supplemental material] had PstI and XhoI cleavage sites at the 5' ends, respectively). These PCR fragments were cloned into pCR-XL-TOPO (Invitrogen, Carlsbad, CA), yielding plasmids pDP60 and pDP62. An \sim 2.2-kb KpnI-BamHI fragment from pDP60 was cloned in pDP25 (see Table S1 in the supplemental material) giving plasmid pDP61, and an \sim 2.1-kb PstI-XhoI fragment from pDP62 was cloned into pDP61 (see Table S1 in the supplemental material) giving pDP63. Finally, an \sim 5.5-kb KpnI-XhoI fragment from pDP63 was cloned into pMRS104 (see Table S1 in the supplemental material), yielding plasmid pDP64, which cannot replicate in *C. perfringens*. Plasmid pDP64 was introduced into *C. perfringens* SM101 by electroporation, and a chloramphenicol-resistant (Cm^r ; 20 μ g/ml) *sleC* mutant was isolated as described previously (38). The identity of the *sleC* strain DPS107 was confirmed by PCR and Southern blot analyses (data not shown).

Construction of a *C. perfringens sleM* deletion mutant. To isolate a derivative of *C. perfringens* SM101 with a deletion of *sleM*, a Δ *sleM* suicide vector was constructed as follows. A 1,006-bp DNA fragment carrying 61 bp from the N-terminal coding region and 945 bp upstream of *sleM* was PCR amplified using the primer pair CPP401/PPP403 (forward and reverse primers [see Table S2 in the supplemental material] had KpnI and SpeI cleavage sites at the 5' ends, respectively). A 1,436-bp DNA fragment carrying 225-bp from the C-terminal coding region and 1,211 bp downstream of *sleM* was PCR amplified using the primer pair CPP404/PPP400 (forward and reverse primers [see Table S2 in the supplemental material] had PstI and XhoI cleavage sites at the 5' ends, respectively). These PCR fragments were cloned into pCR-XL-TOPO, yielding plasmids pDP91 and pDP92. An \sim 1.0-kb KpnI-SpeI fragment from pDP91 was cloned in pDP25 (see Table S1 in the supplemental material), yielding plasmid pDP93, and an \sim 1.4-kb PstI-XhoI fragment from pDP92 was cloned into pDP93 (see Table S1 in the supplemental material), yielding plasmid pDP94. Finally, an \sim 3.7-kb KpnI-XhoI fragment from pDP94 was cloned into pMRS104 (see Table S1 in the supplemental material), yielding pDP95, which cannot replicate in *C. perfringens*. Plasmid pDP95 was introduced into *C. perfringens* SM101 by electroporation, and a Cm^r *sleM* mutant was isolated as described previously (38). The identity of the *sleM* strain DPS109 was confirmed by PCR and Southern blot analyses (data not shown).

Construction of a *sleC sleM* double mutant. To isolate a derivative of *C. perfringens* SM101 with deletions of both *sleC* and *sleM*, a Δ *sleC* suicide vector encoding tetracycline resistance (2 μ g/ml) was constructed as follows. A 3.2-kb BamHI-PstI fragment carrying *tetM* from pDP35 was cloned into pDP64 (see Table S1 in the supplemental material), yielding plasmid pDP65. Next, a \sim 1.1-kb SmaI fragment carrying *ermB* from pJIR599 was cloned into pDP65 (see Table S1 in the supplemental material), yielding pDP66, which cannot replicate in *C. perfringens*. Plasmid pDP66 was introduced into *C. perfringens* DPS109 by electroporation, and a Cm^r tetracycline-resistant *sleC sleM* mutant was isolated as described previously (38). The identity of the *sleC sleM* strain DPS110 was confirmed by PCR and Southern blot analyses (data not shown).

Construction of a Δ *sleC* strain complemented with *sleC*. To construct a *sleC* strain complemented with wild-type *sleC*, a suicide-complementing plasmid targeted to the *plc* locus was constructed as follows. A 1,704-bp PCR fragment carrying 1,590 bp upstream and 114 bp of the N-terminal coding region of *plc* was PCR amplified using the primer pair CPP507/PPP511 (forward and reverse primers [see Table S2 in the supplemental material] had SacI and KpnI cleavage sites at the 5' ends, respectively). A 1,264-bp PCR fragment carrying 309 bp from the C-terminal coding region and 955 bp downstream of *plc* was PCR amplified using the primer pair CPP516/PPP510 (forward and reverse primers [see Table S2 in the supplemental material] had SalI and SphI cleavage sites at the 5' ends, respectively). These PCR fragments were cloned into pCR-XL-TOPO (Invitrogen), yielding the plasmids pDP126 and pDP127 (see Table S1 in the supplemental material). An \sim 1.7-kb SacI-KpnI fragment from pDP126 was cloned into pMRS104 (see Table S1 in the supplemental material) giving plasmid pDP128, and an \sim 1.3-kb SalI-SphI fragment from pDP127 was cloned into pDP128 (Table S1), yielding plasmid pDP129. Next, an \sim 1.9-kb PCR fragment (including 437 bp upstream of *sleC* and the *sleC* coding region) was PCR amplified with Phusion High-Fidelity DNA polymerase (New England Biolabs, Inc.) with the primer pair CPP482/PPP488 (forward and reverse primers [see Table S2 in the supplemental material] had KpnI and SalI cleavage sites at the 5' ends, respectively), and cloned into Zero-Blunt-TOPO (Table S1) giving pDP115. As shown

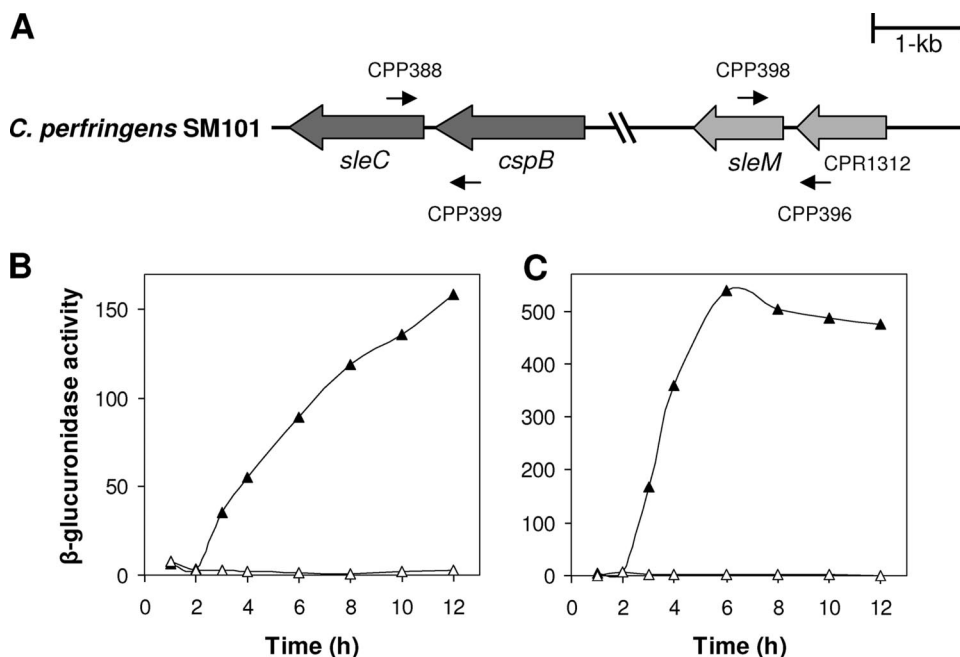


FIG. 1. (A to C) Arrangement and expression of *sle* genes in *C. perfringens* SM101. (A) The arrangement of *sleC* and *sleM* in *C. perfringens* SM101 is shown, and the locations of the primers used to amplify the upstream regions of each gene are indicated. The *sleC* and *sleM* promoters were predicted to be within the intergenic regions between *sleC* and *cspB* and between *sleM* and CPR1312, respectively (18). (B and C) GUS specific activities from *sleC-gusA* (B) and *sleM-gusA* (C) fusions in *C. perfringens* SM101 grown in TGY vegetative (Δ) and DS sporulation (\blacktriangle) media were determined as described in Materials and Methods. The data represent averages of three independent experiments, and time zero denotes the time of inoculation of cells into either TGY or DS medium.

by assays of GUS activity (Fig. 1B), the 437-bp region upstream of *sleC* contained a strong promoter, as expected (18). Finally, an ~1.9-kb *KpnI-SalI* fragment from pDP115 was cloned into pDP129, yielding pDP138 (see Table S1 in the supplemental material), which cannot replicate in *C. perfringens*. Plasmid pDP138 was introduced into the *C. perfringens* Δ *sleC* strain DPS107 by electroporation (10), and erythromycin-resistant Cm^r transformants were selected. The presence of plasmid pDP138 in DPS107(pDP138) was confirmed by PCR and Southern blot analyses (data not shown).

Spore preparation and purification. Starter cultures (10 ml) of *C. perfringens* isolates were prepared by overnight growth at 37°C in fluid thioglycolate broth (Difco) as described previously (14). *C. perfringens* sporulating cultures were prepared by inoculating 0.2 ml of a fluid thioglycolate starter culture into 10 ml of DS sporulation medium (11), followed by incubation for 24 h at 37°C, and the presence of spores was confirmed by phase-contrast microscopy. Large amounts of spores were prepared by scaling up the latter procedure, as described previously (30). Spore preparations were cleaned by repeated centrifugation and washing with sterile distilled water until spore suspensions were >99% free of sporulating cells, cell debris, and germinated spores and then suspended in distilled water at a final optical density at 600 nm (OD_{600}) of ~6 and stored at -20°C.

Decoating treatment. Spores at an OD_{600} of 20 were decoated in 1 ml of 50 mM Tris-HCl (pH 8.0)-8 M urea-1% (wt/vol) sodium dodecyl sulfate-50 mM dithiothreitol for 90 min at 37°C, and the spores were washed three times with 150 mM NaCl and twice with water (32). This extraction procedure did not kill the spores, as determined on brain heart infusion (BHI) agar plates (see below) containing 1 μ g of lysozyme/ml.

Assessment of the colony-forming efficiency of spores. Untreated and decoated spores at an OD_{600} of 1.0 were heat activated at 80°C for 10 min, aliquots of various dilutions were plated on BHI agar with or without lysozyme (1 μ g/ml) in the plates, and the plates were incubated at 37°C anaerobically for 24 h. The colonies were counted, and the results were expressed as CFU/ml/ OD_{600} .

Spore germination. Spore suspensions were heat activated at 80°C for 10 min, cooled in water at an ambient temperature for 5 min, and incubated at 40°C for 10 min as described previously (30). Germination of spores with an OD_{600} of 1 with BHI broth, Ca-DPA (50 mM $CaCl_2$, 50 mM DPA adjusted to pH 8.0 with Tris-HCl), or KCl (100 mM KCl-25 mM sodium phosphate buffer [pH 7.0]) (30) was routinely measured by monitoring the OD_{600} of spore cultures (Smartspec

3000 spectrophotometer; Bio-Rad Laboratories, Hercules, CA), which falls ~60% upon complete germination of wild-type spores. The levels of germination were also confirmed by phase-contrast microscopy. All reported values are averages of two experiments performed on at least two independent spore preparations, and individual values varied by <15% from the average values shown.

DPA release. DPA release from spores during nutrient and nonnutrient germination was measured by heat activating (80°C, 10 min) a spore suspension (OD_{600} of 1.5), cooling, and incubation in BHI broth or in 25 mM sodium phosphate buffer (pH 7.0) alone or with 100 mM KCl (pH 7.0) or in 25 mM Tris-HCl (pH 8.0) alone or with 50 mM Ca-DPA adjusted to pH 8.0 with Tris-HCl at 40°C for 1 or 18 h. A 1-ml aliquot was centrifuged in a microcentrifuge (13,200 rpm, 2 min), and the spore pellet was washed four times with 1 ml of distilled water. Control experiments were done for each experiment and reveal that losses of spores due to these multiple centrifugations were <10% of the initial amount, and appropriate corrections for such losses were made accordingly. The residual spore DPA content was determined by boiling the samples for 60 min, cooling them on ice for 5 min, centrifugation at 13,200 rpm in a microcentrifuge for 5 min, and measuring the OD_{270} of the supernatant fluid as described previously (6, 41). The DPA content of the initial dormant spores was measured by boiling an aliquot (1 ml) for 60 min, centrifugation at 13,200 rpm in a microcentrifuge for 5 min, and measuring the OD_{270} of the supernatant fluid as described previously (6, 30). In *B. subtilis* spores, $\geq 85\%$ of the material absorbing at 270 nm is DPA (1, 6). To confirm that the material from *C. perfringens* spores that absorbed at 270 nm was indeed DPA, total DPA and DPA released upon germination from *C. perfringens* spores were also measured by a colorimetric assay in parallel with control assays with pure DPA (37). Comparison of the measurements by OD_{270} and the colorimetric assay indicated that ~90% of the material absorbing at 270 nm was indeed DPA (data not shown).

For measuring DPA release during dodecylamine germination, spores (OD_{600} of 1.5) were incubated at 60°C with 1 mM dodecylamine in 25 mM Tris-HCl (pH 7.4). Aliquots (1 ml) of germinating cultures were centrifuged for 3 min at 13,200 rpm in a microcentrifuge, and DPA in the supernatant fluid was measured by monitoring the OD_{270} as described previously (6, 30). The initial DPA content in dormant spores was measured similarly. No significant DPA release was observed when spores were incubated in 25 mM Tris-HCl (pH 7.4) at 60°C for 1 h (data not shown).

Hexosamine release. The release of hexosamine-containing fragments of cortex PG into the germination medium was measured by germinating heat-activated spores at an OD_{600} of 25 in 100 mM KCl and 10 mM Tris-HCl (pH 7.4). After incubation for 2 h at 40°C, samples (1 ml) were centrifuged, and analyses of hexosamine in the supernatant fluid were carried out as described previously (12, 31). Analyses of hexosamine-containing material in dormant spores were carried out similarly.

RESULTS

Evaluation of expression of *sle* genes in *C. perfringens* SM101. When the *C. perfringens* SM101 (23) genome was subjected to BLASTP analyses to identify genes encoding CLEs, two open reading frames (CPR2566 and CPR1311) encoding proteins with high identity (90 to 94%) to CLEs from *C. perfringens* strain S40 were identified. CPR2566 is predicted to encode a 438-amino-acid protein with 90% identity to SleC from *C. perfringens* S40 (Fig. 1A). CPR1311 is predicted to encode a 200-amino-acid protein with 94% identity to SleM from *C. perfringens* S40 (Fig. 1A). Both the *sleC* and the *sleM* genes appear likely to be monocistronic (18).

To assess whether the *C. perfringens* SM101 *sle* genes are expressed during sporulation, DNA upstream of each *sle* gene's coding sequence, including the intergenic regions between the *sle* genes and the preceding gene (Fig. 1A), which most likely contain these genes' promoters as shown for *C. perfringens* strain S40 (18), was fused to *Escherichia coli gusA*, and the GUS activity was measured after introducing the various fusions into *C. perfringens* SM101. No significant GUS activity was observed in vegetative cultures of strain SM101 carrying *sleC-gusA* or *sleM-gusA* (Fig. 1B and C). However, sporulating cultures of strains carrying these *gusA* fusions exhibited significant GUS activity (Fig. 1B and C), indicating that a sporulation-specific promoter is located upstream of each *sle* gene. Expression of GUS from the *sleC-gusA* fusion began ~2 h after induction of sporulation, and the GUS specific activity increased until 12 h (Fig. 1B). Expression of GUS from the *sleM-gusA* fusion also began ~2 h after the induction of sporulation and reached a maximum specific activity ~6 h after the induction of sporulation (Fig. 1C). Collectively, these results agree with previous work on the expression of the *sleC* and *sleM* genes in *C. perfringens* S40 (18) and indicate that SleC and SleM could be involved in cortex hydrolysis during *C. perfringens* spore germination.

Effect of *sleC* and *sleM* mutations on spore germination in BHI broth and colony formation of spores on BHI plates. Several in vitro studies (7, 15, 45) indicate that *C. perfringens* SleC and SleM can specifically hydrolyze intact spore cortex or cortex fragments. To establish the roles of these CLEs during *C. perfringens* spore germination, we constructed strains carrying deletions of *sleC* (strain DPS107), *sleM* (strain DPS109), and both *sleC* and *sleM* (strain DPS110) (see Table S1 in the supplemental material and also Fig. 2A), and we examined the germination of spores of these strains in BHI broth (Fig. 2B). The decrease in OD_{600} of the *sleM* spores in BHI broth was significantly ($P < 0.001$) faster and reached a lower level than that of wild-type spores during the first 30 min of incubation for reasons that are not clear, but spores of both strains germinated to a similar extent after 60 min (Fig. 2B). Phase-contrast microscopy of these spores also showed similar levels (~80%) of phase-dark spores (which is indicative of the com-

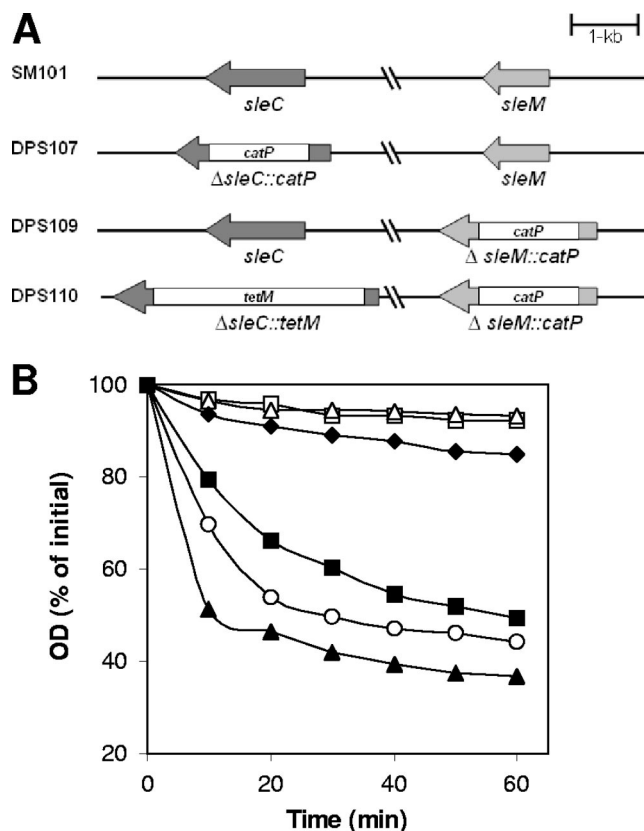


FIG. 2. (A and B) *C. perfringens* *sle* deletion mutants and germination of their spores in BHI broth. (A) Arrangement of *sleC* and *sleM* in various *C. perfringens* deletion mutant strains. (B) Spores of *C. perfringens* strains SM101 (wild-type) (■), DPS107 (*sleC*) (△), DPS109 (*sleM*) (○), DPS110 (*sleC sleM*) (□), and DPS107(pDP138) (*sleC* strain complemented with wild-type *sleC*) (▲) were incubated at 40°C in BHI broth, and the OD_{600} was measured as described in Materials and Methods. Spores of *C. perfringens* strains SM101 (wild-type) (◆) and DPS107 (*sleC*) (▲) were incubated at 40°C in 25 mM sodium phosphate buffer (pH 7.0), and the OD_{600} was measured as described in Materials and Methods. There was essentially no decrease in the OD_{600} for the *sleC* spores incubated in buffer alone (data not shown).

pletion of spore germination) after 60 min of incubation in BHI broth, with this number rising to $\geq 99\%$ after 18 h (data not shown). However, only ~10 and 20% of wild-type spores had become phase dark after incubation in phosphate buffer alone for 1 and 18 h, respectively (data not shown). In contrast to wild-type and *sleM* spores, *sleC* and *sleC sleM* spores exhibited only a small decrease in OD_{600} after 60 min of incubation in BHI broth (Fig. 2B), and this decrease was similar to that observed with *sleC sleM* spores incubated in phosphate buffer alone (data not shown). These results strongly suggest that SleC, but not SleM, is essential for hydrolysis of the PG cortex during *C. perfringens* spore germination and thus the large decrease in the OD_{600} during germination.

Phase-contrast microscopy further indicated that ~50 and 30% of the *sleC* and *sleC sleM* spores, respectively, had become phase gray after 1 h of incubation in BHI broth, a finding indicative of some decrease in the spore core's refractive index, while after 18 h, ~80 and 60% of the *sleC* and *sleC sleM* spores, respectively, had become phase gray (data not shown). How-

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

Strain/genotype	Spore titer (CFU/ml/OD ₆₀₀) on ^b :	
	BHI	BHI + Lyz ^c
SM101/wild-type	4.0×10^7	8.9×10^7
DPS107/ <i>sleC</i>	1.4×10^4	3.9×10^7
DPS107(pDP138)/ <i>sleC</i> carrying wild-type <i>sleC</i>	2.7×10^7	ND
DPS109/ <i>sleM</i>	3.4×10^7	ND
DPS110/ <i>sleM sleC</i>	1.6×10^2	6.6×10^7

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

^b Titers are the average of CFU/ml/OD₆₀₀ determined in three experiments, and the variance was <25%.

^c Spores were decoated and plated on BHI plates containing 1 μg of lysozyme (Lyz)/ml. ND, not determined.

ever, ~30 and 20% of the *sleC* and *sleC sleM* spores, respectively, had become phase gray after 1 h of incubation in phosphate buffer alone, and these values increased to ~60% for both *sleC* and *sleC sleM* spores after 18 h. These data suggest that SleM may be important in conjunction with SleC in some way in effecting a fall in the spore core's refractive index during germination, perhaps by facilitating DPA release (see below).

The severe germination defects of *sleC* and *sleC sleM* spores suggested that the colony-forming efficiency of these spores might be lower than that of wild-type spores, since cortex hydrolysis is essential for the resumption of vegetative growth. No significant difference in colony-forming efficiency was observed between wild-type and *sleM* spores on BHI agar plates (Table 1). In contrast, *sleC* spores exhibited ~10³-fold-lower colony formation efficiency than did wild-type spores, and the colony-forming efficiency of *sleC sleM* spores was ~10⁵- and 10²-fold lower than that of the wild-type and *sleC* spores, respectively (Table 1). However, the colony-forming efficiencies of the *sleC* and *sleC sleM* spores were restored to wild-type levels when the spores were decoated and plated on BHI agar containing 1 μg of lysozyme/ml. Thus, the *sleC* and *sleC sleM* spores were completely viable but just unable to complete cortex hydrolysis and germination. These results (i) further support the essential role of SleC in cortex hydrolysis during

the germination and outgrowth of *C. perfringens* spores and (ii) suggest that only in the absence of SleC does SleM have a role in the germination of *C. perfringens* spores, and even then it has only a secondary role.

The appearance of at least the great majority of colonies from *sleC* and *sleC sleM* spores was not due to genetic reversion, since PCR analyses did not detect wild-type *sleC* and *sleM* genes in colonies obtained from *sleC* and *sleC sleM* spores after 24 h (data not shown). To test whether longer incubation of *sleC* and *sleC sleM* spores on BHI agar plates at 37°C would lead to increased colony formation efficiency, plates were incubated for up to 7 days, and colonies were counted every 24 h. As expected, >99% of the total colonies from wild-type spores appeared after only 24 h (data not shown). Surprisingly, when plates with *sleC* and *sleC sleM* spores were incubated for longer periods, 10- to 100-fold-higher colony counts appeared (Fig. 3A). However, these additional colonies only appeared surrounding colonies that arose during the first 24 h of incubation (Fig. 3B), and no new isolated colonies appeared (data not shown). While the cause of this behavior of the *sleC* and *sleC sleM* spores is not completely clear, it could be due to the diffusion of some cell wall hydrolase released by vegetative cells in the initial colony, with this hydrolase then degrading the cortex of a small population of *sleC* spores that have a defective coat. Indeed, the maximum number of colonies that appeared from *sleC* and *sleC sleM* spores during the 7 days of incubation was 10- to 100-fold lower than from wild-type spores (data not shown), suggesting that only a small percentage of *C. perfringens* spore populations gave rise to these late-appearing colonies. In addition, *sleC* spore viability went up only ~10-fold in 48 h, whereas the *sleC sleM* spores required ~5 days to reach their maximum level. Perhaps cortex hydrolysis by a cell wall hydrolase from vegetative cells on defective spores is accelerated by SleM.

DPA release by *C. perfringens* spores germinated with BHI broth. The germination and viability defects noted above with *sleC* and *sleC sleM* spores suggest that these spores cannot complete the germination process because they cannot degrade cortex PG. However, since many of the *sleC* and *sleC*

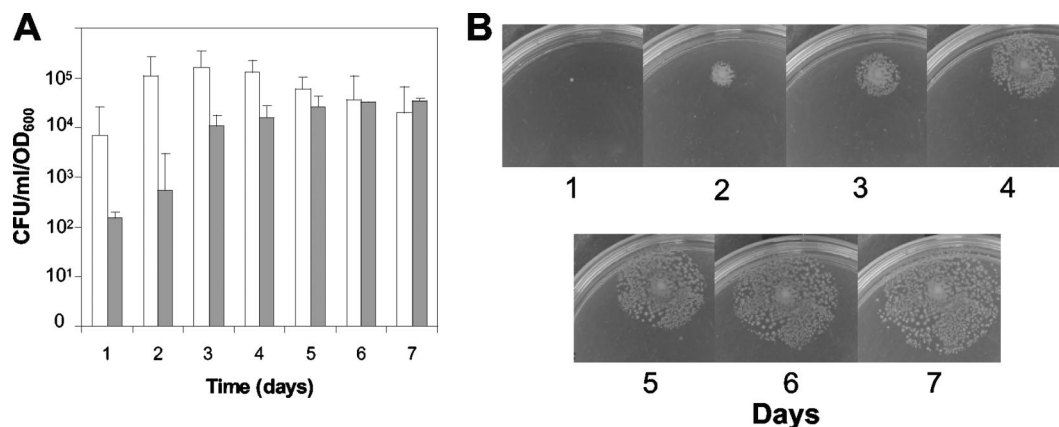


FIG. 3. (A and B) Germination of *C. perfringens* spores over long periods on BHI agar plates. (A) Spores of *C. perfringens* strains DPS107 (*sleC*) (□) and DPS110 (*sleC sleM*) (■) were applied to BHI plates that were incubated at 37°C for 7 days, and the total number of colonies were counted every 24 h and expressed as CFU. (B) The plates described in panel A were photographed every 24 h. Similar results in the experiments in panels A and B were obtained with two different batches of spores, and error bars indicate the standard deviations.

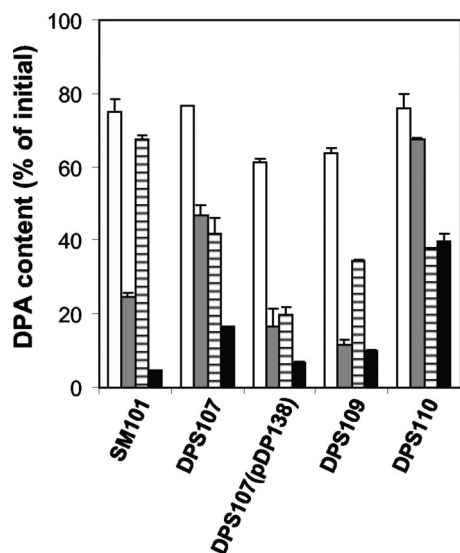


FIG. 4. DPA release during the germination of *C. perfringens* spores with BHI broth. Heat-activated spores of strains SM101 (wild-type), DPS107 (*sleC*), DPS107(pDP138) (*sleC* strain complemented with wild-type *sleC*), DPS109 (*sleM*), and DPS110 (*sleC sleM*) were incubated at 40°C in BHI broth and, after 1 h (□) and 18 h (■), DPA release was measured as described in Materials and Methods. *C. perfringens* spores from various strains were also incubated in 25 mM sodium phosphate buffer (pH 7.0), and after 1 h (□) or 18 h (■) the DPA release was measured as described in Materials and Methods. The data represent the average of two independent experiments with two different spore preparations, and error bars indicate the standard deviations.

sleM spores incubated in BHI broth became phase gray in the phase-contrast microscope, this suggested that there was a decrease in these spores' core refractive index, perhaps because of DPA release and its replacement by water. As expected, wild-type and *sleM* spores released most of their DPA after 1 h of germination in BHI broth (Fig. 4), with slightly more released from the *sleM* spores, a finding consistent with the slightly more rapid germination of these spores, as measured by the decrease in OD₆₀₀ (Fig. 2B), although after 18 h, both *sleM* and wild-type spores had released ≥90% of their DPA (Fig. 4). In contrast, *sleC* spores released only ~50 and 85% of their DPA after 1 and 18 h of germination in BHI broth, respectively (Fig. 4). Although *sleC sleM* spores released DPA during incubation in BHI broth, the amount released was significantly less than that from wild-type, *sleC*, and *sleM* spores incubated similarly and was only slightly higher than the amount of DPA released from *sleC sleM* spores incubated in buffer alone (Fig. 4). Collectively, these results suggest that DPA release during *C. perfringens* spore germination does take place during germination of *sleC* spores in BHI broth but is significantly slowed in the absence of cortex hydrolysis, especially in the absence of both SleC and SleM. These results further suggest that SleM might have some role in DPA release but is clearly not sufficient for cortex hydrolysis.

Complementation of *sleC* mutant with wild-type *sleC*. To be certain that the phenotypes observed for *sleC* spores were due directly to deletion of *sleC* and not to any possible secondary effects due to strain construction, we constructed a suicide plasmid pDP138, carrying a mutated allele of the alpha-toxin

gene (*plc*) in which a wild-type copy of *sleC* plus its promoter region (see Materials and Methods and Fig. 1) was inserted. The *plc* locus was chosen for insertion of the wild-type *sleC* into the chromosome of the *sleC* strain by a homologous single recombination event, assuming that *plc* disruption would not affect spore germination. Plasmid pDP138 was introduced into the *sleC* strain DPS107, and PCR with DNA from one DPS107(pDP138) transformant yielded products from wild-type *plc*, Δ *sleC*, and the Δ *plc::sleC* allele, consistent with wild-type *sleC* in pDP138 being integrated into the *plc* locus by a single crossover event (data not shown). Spores of strain DPS107(pDP138) germinated like wild-type spores in BHI broth, as assessed by either a decrease in OD₆₀₀ (Fig. 2B) or phase-contrast microscopy (data not shown). The low colony formation efficiency of *sleC* spores was also restored to that of wild-type spores when the *sleC* strain was complemented with wild-type *sleC* [strain DPS107(pDP138)] (Fig. 2B and Table 1). The slow DPA release from *sleC* spores during germination in BHI broth was also restored to the faster rate seen with wild-type spores in the complemented strain (Fig. 4). These results thus confirm that the phenotypes of *sleC* spores are due exclusively to deletion of *sleC*.

Effect of *sleC* and *sleM* mutations on *C. perfringens* spore germination with KCl. To gain further understanding of the roles of SleC and SleM in *C. perfringens* spore germination, wild-type, *sleC*, *sleM*, and *sleC sleM* spores were incubated with the germinant KCl (30). As expected, *sleM* spores germinated like wild-type spores with KCl, when germination was assessed by the decrease in OD₆₀₀ (Fig. 5A). In contrast, *sleC* and *sleC sleM* spores showed only a minimal decrease in OD₆₀₀ upon incubation with KCl (Fig. 5A). The small decrease in OD₆₀₀ observed for the latter spores was even lower than that of wild-type spores incubated in sodium phosphate buffer (Fig. 5A). Phase-contrast microscopy indicated that ~60 and ~30% of the *sleC* and *sleC sleM* spores, respectively, incubated in 100 mM KCl had become phase gray after 1 h, whereas ~10% of the wild-type spores incubated for 1 h in sodium phosphate buffer had become phase dark. This suggest that while the OD₆₀₀ decrease of wild-type spores incubated in buffer alone was because a small percentage of the spores germinated spontaneously, thus becoming phase dark, the OD₆₀₀ decrease of *sleC* and *sleC sleM* spores incubated in KCl was due to the release of DPA by a fraction of the spore population, thus slightly reducing these spores' refractive index.

As expected, there were no significant differences in DPA release during KCl germination between *sleM* and wild-type spores, since the majority of these spores' DPA was released in 1 h (Fig. 5B). The *sleC* spores also released DPA during incubation in KCl, and while this release was faster than that for spores incubated in buffer alone, it was significantly slower than for wild-type or *sleM* spores incubated in KCl (Fig. 4 and 5B). DPA release from *sleC sleM* spores incubated in KCl was even slower and no faster than from *sleC sleM* spores incubated in buffer alone (Fig. 4 and 5B).

In addition to DPA release, a second major event in spore germination is the hydrolysis of the spore's PG cortex and release of PG fragments into the medium. The hydrolysis of the spore's PG cortex during germination can thus be monitored by measuring the release of hexosamine-containing material into the medium (33, 44). As expected, wild-type and

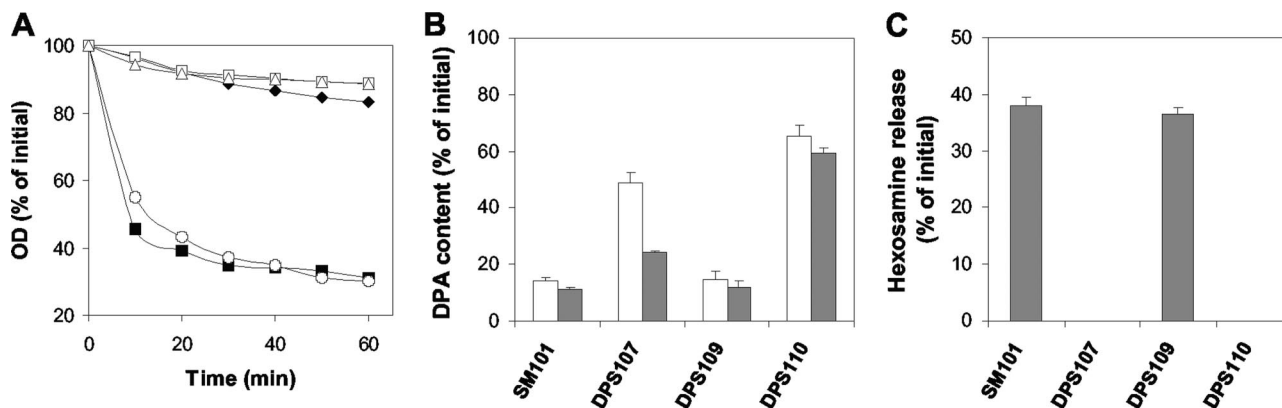


FIG. 5. (A to C) Germination of spores of *C. perfringens* strains with KCl. (A) Heat-activated *C. perfringens* spores of strains SM101 (wild-type) (■), DPS107 (*sleC*) (△), DPS109 (*sleM*) (○), and DPS110 (*sleC sleM*) (□) were germinated with KCl, and the OD₆₀₀ was measured as described in Materials and Methods. Heat-activated spores of strain SM101 (wild-type) (◆) were also incubated in 25 mM sodium phosphate buffer (pH 7.0) alone, and the OD₆₀₀ was measured. (B) DPA release during *C. perfringens* spore germination with KCl. Heat-activated spores of *C. perfringens* strains were germinated with KCl, and after 1 h (□) and 18 h (▣) the DPA content of the spores was measured as described in Materials and Methods. DPA release of *C. perfringens* spores from various strains in 25 mM sodium phosphate buffer (pH 7.0) was as shown in Fig. 4. (C) Release of hexosamine-containing material during *C. perfringens* spore germination with KCl. Heat-activated spores of *C. perfringens* strains were germinated with KCl and, after 2 h, the hexosamine-containing material released into the medium was measured as described in Materials and Methods. Values for hexosamine-containing material released are expressed relative to the amount of hexosamine in dormant spores that was defined as 100%. The data represent the average of two independent experiments with two different spore preparations, and error bars indicate the standard deviations.

sleM spores released similar amounts of hexosamine into the medium after 2 h of germination with KCl: 35 to 40% of total spore hexosamine (Fig. 5C). However, no detectable release of hexosamine-containing material was observed from *sleC* and *sleC sleM* spores after a similar incubation (Fig. 5C). These results are similar to those found with germinating wild-type and *cwlJ sleB* spores of *B. subtilis* and *B. megaterium* (40, 41).

Ca-DPA germination of *C. perfringens* spores. In addition to nutrients, spores of *Bacillus* and *Clostridium* species are germinated by exogenous Ca-DPA (30, 42). Ca-DPA triggers the germination of *B. megaterium* and *B. subtilis* spores by activation of the CLE CwlJ (28, 41). To determine whether a *C. perfringens* CLE is also activated by Ca-DPA, the Ca-DPA germination of wild-type, *sleC*, *sleM*, and *sleC sleM* spores was

examined (Fig. 6). As expected, *sleC* and *sleC sleM* spores did not exhibit the large decrease in OD₆₀₀ in Ca-DPA germination seen with wild-type and *sleM* spores (Fig. 6A), and no significant decrease in OD₆₀₀ was observed when spores of these four strains were incubated in 25 mM Tris-HCl (pH 8.0) (data not shown). However, phase-contrast microscopy found that after 1 h of incubation with Ca-DPA, ~50 to 30% of the *sleC* and *sleC sleM* spores, respectively, had become phase gray, whereas only ~20% of *sleC* and *sleC sleM* spores had become phase gray after 1 h of incubation with 25 mM Tris-HCl (pH 8.0) (data not shown); only ~10% of wild-type spores had become phase dark after 1 h with 25 mM Tris-HCl (pH 8.0) (data not shown).

Measurements of DPA release (Fig. 6B) showed that, as

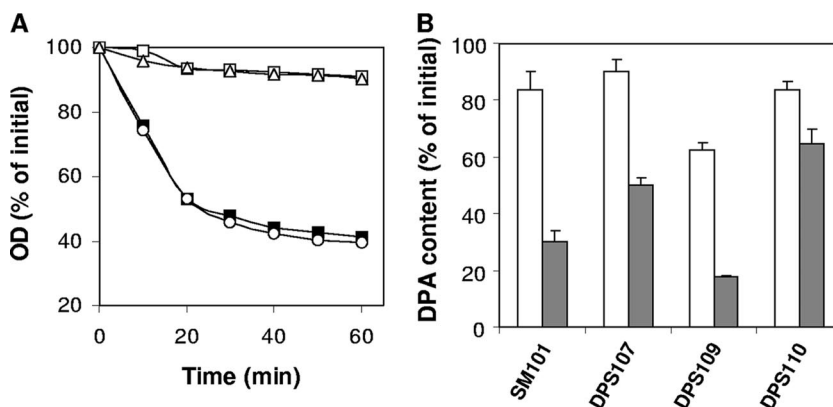


FIG. 6. (A and B) Germination of *C. perfringens* spores with Ca-DPA. (A) Heat-activated *C. perfringens* spores of strains SM101 (wild-type) (■), DPS107 (*sleC*) (△), DPS109 (*sleM*) (○), and DPS110 (*sleC sleM*) (□) were germinated with 50 mM Ca-DPA, and the OD₆₀₀ was measured as described in Materials and Methods. (B) Heat-activated spores of *C. perfringens* strains were germinated with 50 mM Ca-DPA (▣) and 25 mM Tris-HCl buffer (pH 8.0) (□) for 1 h, and the DPA remaining in the spores was measured as described in Materials and Methods. The data represent the average of two independent experiments with two different spore preparations, and error bars indicate the standard deviations.

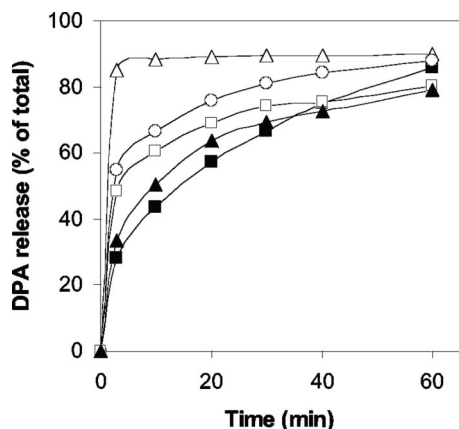


FIG. 7. Dodecylamine germination of *C. perfringens* spores. *C. perfringens* spores of strains SM101 (wild-type) (■), DPS107 (*sleC*) (△), DPS109 (*sleM*) (○), DPS110 (*sleC sleM*) (□), and DPS107(pDP138) (*sleC* strain complemented with wild-type *sleC*) (▲) were germinated with dodecylamine, and at various times the DPA release was measured as described in Materials and Methods.

found with BHI broth and KCl incubations, wild-type and *sleM* spores incubated for 1 h with Ca-DPA released significantly more DPA than did *sleC* spores, although DPA release from the *sleC* spores was much greater than during incubation with buffer alone (Fig. 6B). However, the amount of DPA released from *sleC sleM* spores during incubation with Ca-DPA was only slightly higher than the amount released during incubation in buffer (Fig. 6B). Unfortunately, the precipitation of the exogenous Ca-DPA after 1 h precluded assessment of DPA release over longer times.

Dodecylamine germination of *C. perfringens* spores. In addition to Ca-DPA, KCl, and nutrients, *C. perfringens* spores are also germinated by the cationic surfactant dodecylamine (30). While the precise mechanism of dodecylamine germination is not known, the absence of CLEs does not have any effect on DPA release during the dodecylamine germination of the spores of at least two *Bacillus* species (41, 42). Similarly, wild-type, *sleM*, and *sleC sleM* *C. perfringens* spores and spores of the *sleC* strain complemented with wild-type *sleC* exhibited relatively similar rates and extents of DPA release during dodecylamine germination, while *sleC* spores released their DPA even faster (Fig. 7). Thus, cortex hydrolysis is also not essential for normal DPA release during dodecylamine germination of *C. perfringens* spores.

DISCUSSION

Spores of *Bacillus* species contain three CLEs, with either of two of these enzymes being essential (7, 17, 41, 42). In contrast, SleC is the single essential CLE in *C. perfringens* spores, with SleM playing only an auxiliary role. However, SleM can contribute to cortex hydrolysis during spore germination, as shown by the lower viability of *sleC sleM* spores than *sleC* spores. SleC and SleM are active on cortex PG fragments in vitro, but SleM, unlike SleC, is inactive on the cortex in intact but decanted spores (7, 22). This suggests that SleM, unlike SleC, is normally inactive on cortex PG in intact spores that may be under strain because of the high osmotic pressure of the poorly hydrated

spore core (42). By this reasoning, only SleC would be essential for cortex hydrolysis during spore germination. However, perhaps in a minority of *sleC* spores the cortex is under less stress, and SleM can hydrolyze this "low-stress" cortex. Alternatively, SleM may act in a small percentage of *sleC* spores that have a defective cortex, perhaps due to partial cortex hydrolysis during spore formation by an enzyme that acts on sporulating cell PG to allow release of the dormant spore. Indeed, if a few *sleC* spores have defective coats, a small amount of hydrolytic enzyme action during sporulation on such defective spores might generate spores whose cortex is susceptible to hydrolysis by SleM alone.

Although the loss of SleC eliminated cortex hydrolysis during *C. perfringens* spore germination, *sleC* spores still released their DPA in response to germinants that act through the spore's germinant receptors (30). Spores of *Bacillus* species lacking both essential CLEs also release their DPA during nutrient germination (41, 42). These observations suggest that full cortex hydrolysis is not essential for DPA release during spore germination and that at least the initiation of DPA release likely precedes cortex hydrolysis. However, DPA release from *sleC* spores germinating with BHI broth or KCl was slower than from germinating wild-type or *sleM* spores, with the rate of DPA release from germinating *sleC sleM* spores being even slower and at most only slightly greater than from spores incubated in buffer. This further indicates that while cortex hydrolysis is not essential for the initiation of DPA release during spore germination, cortex hydrolysis can accelerate DPA release. This effect of cortex hydrolysis on the rate of DPA release during germinant receptor-mediated spore germination has also been seen with *B. megaterium* and *B. subtilis* spores in which the loss of CwlJ but not of SleB slows DPA release significantly during nutrient germination (13, 41). Unfortunately, why cortex hydrolysis should accelerate DPA release from spores during germinant receptor-mediated germination and why SleM should contribute to this effect are not clear.

Another notable observation in the present study concerns the effects of the loss of CLEs on Ca-DPA germination of *C. perfringens* spores. With *B. megaterium* and *B. subtilis* spores, Ca-DPA triggers germination by activation of the CLE CwlJ and bypasses the spore's germinant receptors (41, 42). In contrast, Ca-DPA appears to trigger *C. perfringens* spore germination through the GerK germinant receptor (30). This latter finding leads to the prediction that Ca-DPA will trigger DPA release from *C. perfringens* spores lacking SleC, and this is what was observed, even though DPA release during Ca-DPA germination of *sleC* *C. perfringens* spores was slower than from wild-type or *sleM* spores. The similar response of rates of Ca-DPA release during Ca-DPA, BHI broth, and KCl germination to the absence of SleC and SleC plus SleM further suggests that DPA is released by the same mechanism during spore germination triggered by these three agents. Unfortunately, the mechanism for DPA release during spore germination is not known, although it may involve the SpoVA proteins (29, 46).

The final notable finding was that DPA release by *C. perfringens* spores during dodecylamine germination was not slowed by the absence of SleC or SleC and SleM. Indeed, *sleC* spores released their DPA faster during dodecylamine germi-

nation than did wild-type spores. DPA release during dodecylamine germination of *B. megaterium* and *B. subtilis* spores is also not slowed by the absence of these spores' redundant CLEs (41, 42). The mechanism of spore germination by dodecylamine is not known, but the germinant receptors are likely not involved (30, 42). There is also evidence with *B. subtilis* spores that DPA release during dodecylamine germination involves the SpoVA proteins thought to be involved in DPA release during nutrient germination (46). It has been suggested that dodecylamine may activate a DPA channel present in the spore's inner membrane (42, 46), but this has not been proven.

Hydrolysis of cortex PG is the culminating event in bacterial spore germination and is essential for resumption of enzymatic activity in the spore core and eventual vegetative growth (42). As a consequence, cortex hydrolysis is essential for spores of pathogenic organisms such as *C. perfringens* to cause disease. The identification of SleC as the major essential CLE in *C. perfringens* spores thus makes this enzyme of interest for the development of inhibitors, since such compounds would block spore germination and thus the ability of spores to cause disease. Cortex hydrolysis also makes the now fully germinated spore much less resistant to common decontamination procedures. Consequently, a drug that could rapidly activate SleC in spores would also be useful, since this would allow decontamination of germinated *C. perfringens* spores under less harsh conditions than needed for destruction of the more resistant dormant spores.

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