Characterization of *Clostridium perfringens* Spores That Lack SpoVA Proteins and Dipicolinic Acid[∇]

Daniel Paredes-Sabja,¹ Barbara Setlow,³ Peter Setlow,³ and Mahfuzur R. Sarker^{1,2*}

Department of Biomedical Sciences, College of Veterinary Medicine,¹ and Department of Microbiology, College of Science,² Oregon State University, Corvallis, Oregon 97331, and Department of Molecular, Microbial and Structural Biology, University of Connecticut Health Center, Farmington, Connecticut 06030³

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Spores of *Clostridium perfringens* possess high heat resistance, and when these spores germinate and return to active growth, they can cause gastrointestinal disease. Work with *Bacillus subtilis* has shown that the spore's dipicolinic acid (DPA) level can markedly influence both spore germination and resistance and that the proteins encoded by the *spoVA* operon are essential for DPA uptake by the developing spore during sporulation. We now find that proteins encoded by the *spoVA* operon are also essential for the uptake of Ca^{2+} and DPA into the developing spore during *C. perfringens* sporulation. Spores of a *spoVA* mutant had little, if any, Ca^{2+} and DPA, and their core water content was approximately twofold higher than that of wild-type spores. These DPA-less spores did not germinate spontaneously, as DPA-less *B. subtilis* spores do. Indeed, wild-type and *spoVA* *C. perfringens* spores germinated similarly with a mixture of L-asparagine and KCl (AK), KCl alone, or a 1:1 chelate of Ca^{2+} and DPA (Ca-DPA). However, the viability of *C. perfringens* *spoVA* spores was 20-fold lower than the viability of wild-type spores. Decoated wild-type and *spoVA* spores exhibited little, if any, germination with AK, KCl, or exogenous Ca-DPA, and their colony-forming efficiency was 10^3 - to 10^4 -fold lower than that of intact spores. However, lysozyme treatment rescued these decoated spores. Although the levels of DNA-protective α/β -type, small, acid-soluble spore proteins in *spoVA* spores were similar to those in wild-type spores, *spoVA* spores exhibited markedly lower resistance to moist heat, formaldehyde, HCl, hydrogen peroxide, nitrous acid, and UV radiation than wild-type spores did. In sum, these results suggest the following. (i) SpoVA proteins are essential for Ca-DPA uptake by developing spores during *C. perfringens* sporulation. (ii) SpoVA proteins and Ca-DPA release are not required for *C. perfringens* spore germination. (iii) A low spore core water content is essential for full resistance of *C. perfringens* spores to moist heat, UV radiation, and chemicals.

Clostridium perfringens food poisoning is caused mainly by enterotoxigenic type A isolates that have the ability to form metabolically dormant spores that are extremely resistant to heat, radiation, and toxic chemicals (45, 49, 50, 55). These highly resistant spores can survive traditional methods for cooking meat and poultry products as well as other processing treatments used in the food industry. The surviving spores can then go through germination and outgrowth, generating the vegetative cells that cause disease (30).

Spores of *Bacillus* species also have the extreme resistance of *C. perfringens* spores. The factors involved in *Bacillus subtilis* spore resistance include the following: (i) relatively impermeable spore inner membrane; (ii) low water content of the spore core; (iii) high levels of pyridine-2,6-dicarboxylic acid (dipicolinic acid [DPA]) in the spore core and the type and amount of cations chelated by DPA; and (iv) the saturation of spore DNA with α/β -type small, acid-soluble spore proteins (SASP) (63, 64). Studies have shown that α/β -type SASP are also an important factor in *C. perfringens* spore resistance to moist heat, UV radiation, and some chemicals (45, 49, 50), while small changes in the water content of the cores of these spores alter resistance to moist heat and nitrous acid (46).

Spore germination has been well studied in *B. subtilis* (34, 62) and can be initiated by a variety of compounds (termed germinants) including amino acids and some other nutrients and nutrient mixtures, a 1:1 chelate of Ca^{2+} and DPA (Ca-DPA), cationic surfactants, and hydrostatic pressure (41, 44). The germinant receptors, located in the spore's inner membrane (18, 43), sense nutrients present in the environment and stimulate the release of monovalent cations (H^+ , Na^+ , and K^+), divalent cations (Ca^{2+} , Mg^{2+} , and Mn^{2+}), and the spore core's large depot (~25% of core weight [dry weight]) of DPA (62) present in a 1:1 chelate with divalent cations, predominantly Ca^{2+} (Ca-DPA) (35, 36, 59, 61). Ca-DPA release is crucial to spore progression from germination into outgrowth because of the following. (i) Water replaces Ca-DPA, thus elevating the spore core water content. (ii) Ca-DPA triggers the initiation of hydrolysis of the spore's peptidoglycan (PG) cortex by the cortex-lytic enzyme (CLE) CwlJ that is probably directly activated by Ca-DPA (42). Cortex hydrolysis then allows the core to expand and take up even more water to the level found in vegetative cells. This latter event restores protein movement and enzyme activity in the spore core and allows resumption of energy metabolism and macromolecular synthesis (9, 62).

Release of Ca-DPA during *B. subtilis* spore germination and DPA uptake during sporulation into the developing spore from the mother cell, the site of DPA synthesis, require the SpoVA proteins that may be components of some type of gated channel in the spore's inner membrane (70, 72). In *B. subtilis*, the

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

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Reference or source
<i>C. perfringens</i> strains		
SM101	Electroporatable derivative of food poisoning type A isolate, NCTC8798; carries a chromosomal <i>cpe</i>	74
DPS104	<i>spoVA</i> mutant (<i>spoVA::tetM</i>) derivative of SM101	This study
SM101(pDP51)	Wild-type strain carrying <i>spoVA-gusA</i> fusion	This study
SM102(pDP79)	Wild-type strain carrying <i>spoVAD-gusA</i> fusion	This study
SM101(pDP80)	Wild-type strain carrying <i>spoVAE-gusA</i> fusion	This study
DP104(pDP54)	<i>spoVA</i> mutant complemented with wild-type <i>spoVA</i>	This study
Plasmids		
pJIR418	<i>C. perfringens</i> / <i>E. coli</i> shuttle vector; Cm ^r and Em ^r	66
pMRS127	<i>C. perfringens</i> / <i>E. coli</i> shuttle vector carrying a promoterless <i>gusA</i> ; Em ^r	49
pJIR599	pBluescript II carrying erythromycin resistance gene (<i>ermB</i>); Em ^r	2
pJIR750	<i>C. perfringens</i> / <i>E. coli</i> shuttle vector; Cm ^r	3
pJIR1886	pBluescript II carrying tetracycline resistance gene (<i>tetM</i>); Tet ^r	J. I. Rood
pDP1	<i>gerAA</i> internal fragment (~800 bp) cloned in EcoRI site of pCR-XL-TOPO	D. Paredes-Sabja
pDP31	~1.0-kb PCR fragment containing 730 bp upstream and 270 bp of the N-terminus-coding region of <i>spoVAC</i> in pCR-XL-TOPO	This study
pDP32	~1.4-kb PCR fragment containing 324 bp of the C-terminus-coding region and 1,054-bp downstream of <i>spoVAE</i> in pCR-XL-TOPO	This study
pDP33	~1.0-kb KpnI-SpeI fragment from pDP31 cloned between KpnI/SpeI sites of pDP1	This study
pDP34	~1.4-kb PstI-XhoI fragment from pDP32 cloned between PstI/XhoI sites of pDP33	This study
pDP35	~3.2-kb EcoRI fragment carrying <i>tetM</i> from pJIR1886 cloned in the EcoRI site of pDP34	This study
pDP45	~1.1-kb SmaI fragment carrying <i>ermB</i> from pJIR599 cloned in the SmaI site of pDP35	This study
pDP51	~393-bp PCR amplified upstream region of <i>spoVAC</i> cloned in pMRS127	This study
pDP53	~2.5-kb PCR fragment containing <i>spoVA</i> operon and upstream region cloned in pCR-XL-TOPO	This study
pDP54	~2.5-kb KpnI-SalI fragment containing the <i>spoVA</i> operon and upstream region from pDP53 in pJIR750	This study
pDP79	~378 bp of PCR-amplified upstream region of <i>spoVAD</i> cloned in pMRS127	This study
pDP80	~351 bp of PCR-amplified upstream region of <i>spoVAE</i> cloned in pMRS127	This study

hexacistronic *spoVA* operon encodes the SpoVA proteins (24), and most, if not all, of these are likely to be membrane proteins as shown directly for SpoVAD proteins (71). The *spoVA* operon is transcribed exclusively during *B. subtilis* sporulation in the developing forespore just prior to DPA uptake (68, 70). *B. subtilis* strains with deletions in the *spoVA* operon initiate sporulation, but the *spoVA* spores are extremely unstable and lyse during sporulation, probably because of their lack of DPA (68). *B. subtilis* spores that lack DPA due to inactivation of the DPA synthase are also unstable and lyse during sporulation (68). The reason for the instability of DPA-less *B. subtilis* spores is not clear but may be because the second of two redundant CLEs, SleB, is spontaneously activated in spores that lack DPA (68).

Clostridium species also contain a *spoVA* operon, although this operon is only tricistronic, containing *spoVAC*, *spoVAD*, and *spoVAE* (4, 5, 38, 56), suggesting that not all the SpoVA proteins in the spores of *Bacillus* species are essential for DPA movement during sporulation and spore germination in *Clostridium* species. To study the roles of SpoVA proteins and DPA levels in the properties of spores of *Clostridium* species, *C. perfringens* was used to examine the expression of the *spoVA* operon during growth and sporulation and to construct a *spoVA* deletion mutation. The properties of the resultant *spoVA* spores, in particular their Ca-DPA level, resistance, and germination, were then examined to help elucidate the roles of SpoVA proteins and DPA in *C. perfringens* spore properties. Surprisingly, while *C. perfringens* *spoVA* spores lacked DPA, these spores were stable and germinated relatively normally. However, these *spoVA* spores had significantly higher core

water content and exhibited markedly decreased resistance to moist heat, UV radiation, and a number of chemicals than wild-type spores did.

MATERIALS AND METHODS

Bacterial strains and plasmids. The *C. perfringens* strains and plasmids used in this study are described in Table 1.

Construction of *gusA* fusion plasmids and GUS assay. Expression of *C. perfringens* *spoVAC*, *spoVAD*, and *spoVAE* was examined by fusing DNA upstream of each gene to *Escherichia coli gusA* in pMRS127, an *E. coli-C. perfringens* shuttle vector (49). Briefly, 300- to 400-bp DNA fragments upstream of *spoVAC*, *spoVAD*, or *spoVAE* from *C. perfringens* SM101 were PCR amplified using primers CPP274/CPP275, CPP382/CPP381, and CPP387/CPP384 (the forward and reverse primers had SalI and PstI cleavage sites, respectively) (Table 2). These PCR fragments were digested with SalI and PstI and cloned between the SalI and PstI cleavage sites in pMRS127 to create *spoVAC-gusA*, *spoVAD-gusA*, and *spoVAE-gusA* fusion constructs, giving plasmids pDP51, pDP79, and pDP80, respectively (Table 1). These plasmids were introduced into *C. perfringens* SM101 by electroporation (10), and erythromycin-resistant (Em^r) (50 µg/ml) transformants were selected. The transformants carrying the plasmid with the *spoVAC-gusA*, *spoVAD-gusA*, or *spoVAE-gusA* fusion were grown vegetatively in TGY medium (3% Trypticase soy, 2% glucose, 1% yeast extract, 0.1% L-cysteine) (22), sporulated in Duncan-Strong (DS) (11) medium, and assayed for β-glucuronidase (GUS) activity as described previously (17, 49). GUS specific activity was expressed in Miller units and calculated as described previously (21).

Construction of a *C. perfringens* *spoVA* deletion mutant. To isolate a derivative of *C. perfringens* SM101 with a deletion of the entire *spoVA* operon, a *ΔspoVA* suicide vector was constructed as follows. A 1,001-bp DNA fragment carrying 270 bp from the N-terminal coding region and 731 bp upstream of *spoVAC* was PCR amplified using primers CPP288/CPP289 (Table 2), which had KpnI and SpeI cleavage sites at the 5' ends of the forward and reverse primers, respectively. A 1,378-bp fragment carrying 324 bp from the C-terminal coding region and 1,054 bp downstream of *spoVAE* was PCR amplified using primers CPP290/CPP291 (Table 2), which had PstI and XhoI cleavage sites at the 5' ends of the forward and reverse primers, respectively (Table 2). These PCR fragments were cloned

TABLE 2. Primers used in this study

Primer name	Primer sequence ^a	Positions ^b	Genes	Use ^c
CPP288	GGGTACCCCTATGAGGATATATTTTCAGATTGGG	−731 to −706	<i>spoVAC</i>	MP
CPP289	GACTAGTCTGCTCCAGCCTTAGCGGC	+252 to +270	<i>spoVAC</i>	MP
CPP290	GCTGCAGCGGTGCAATCTGTGTAATAGGACAG	+42 to +66	<i>spoVAE</i>	MP
CPP291	CCTCGAGGCTTGTGCAGACAGTCCTAAGC	+1399 to +1420	<i>spoVAE</i>	MP
CPP341	GGGTACCCGATGAAGGAACGCCGTT	−393 to −375	<i>spoVAC</i>	CP
CPP347	GCGTCGACGTACAATAAAAAAGCTGTACCT	+521 to +544	<i>spoVAE</i>	CP
CPP274	GCGTCGACGATGAAGGAACGCCGTT	−393 to −375	<i>spoVAC</i>	GUS
CPP275	GCTGCAGAGCTATATTAATCACCTTTTCTATT	−27 to −1	<i>spoVAC</i>	GUS
CPP382	GCAGCGTCGACGTAGGTGGATTAATTTGTGTAATAGG	−371 to −346	<i>spoVAD</i>	GUS
CPP381	GACGCTGCAGTACTCATTCTTATCACCCAC	−14 to +7	<i>spoVAD</i>	GUS
CPP387	GCAGCGTCGACAAAGAGTATGGATATGATATTAGTAAGG	−335 to −307	<i>spoVAE</i>	GUS
CPP384	GACGCTGCAGCTTGCAAAATTCTCATCTCCTCATTATT	−12 to +16	<i>spoVAE</i>	GUS

^a Restriction sites are marked by underlining.

^b The nucleotide position numbering begins from the first codon and refers to the relevant position within the respective gene sequence.

^c MP, construction of mutator plasmid; CP, construction of complemented plasmid; GUS, construction of *gusA* fusion plasmid.

into plasmid pCR-XL-TOPO, giving plasmids pDP31 and pDP32. An ~1.0-kb KpnI-SpeI fragment from pDP31 was cloned into pDP1 (Table 1), giving plasmid pDP33, and an ~1.4-kb PstI-XhoI fragment from pDP32 was cloned in pDP33, giving pDP34. Next, an ~3.2-kb EcoRI fragment carrying the tetracycline resistance gene (*tetM*) from pJIR1886 (Table 1) was cloned into the EcoRI site of pDP34, giving pDP35. Finally, an ~1.1-kb SmaI fragment carrying the erythromycin resistance gene (*ermB*) from pJIR599 (Table 1) was cloned into a unique SmaI site in pDP35, giving pDP45 which cannot replicate in *C. perfringens*. Plasmid pDP45 was introduced into *C. perfringens* SM101 by electroporation (10), and the *C. perfringens* *spoVA* deletion strain DPS104 was isolated by allelic exchange (54). The presence of the *spoVA* deletion in strain DPS104 (Fig. 1A) was confirmed by PCR and Southern blot analyses (data not shown).

Construction of a *spoVA*-complemented strain. An ~2.5-kb fragment containing *spoVAC*, *spoVAD*, and *spoVAE* plus 393 bp upstream of *spoVAC* was PCR amplified using primers CPP341/PPP347 (Table 2) and cloned into pCR-XL-TOPO (Invitrogen), giving pDP53. The ~2.5-kb KpnI-SalI fragment of pDP53 was cloned between the KpnI and SalI sites of plasmid pJIR750 (3) to create the *spoVA*-complementing plasmid, pDP54. Plasmid pDP54 was introduced into *C. perfringens* strain DPS104 by electroporation (10), and chloramphenicol-resistant (*Cm^r*) transformants were selected. The presence of plasmid pDP54 in *C. perfringens* DPS104(pDP54) was confirmed by PCR (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 (FTG) (Difco) as described previously (22). Sporulating cultures of *C. perfringens* were prepared by inoculating 0.2 ml of an FTG starter culture into 10 ml of DS

sporulation medium (11), and this culture was incubated for 24 h at 37°C to form spores as confirmed by phase-contrast microscopy. Large amounts of spores were prepared by scaling up the latter procedure. Spore preparations were cleaned by repeated centrifugation and washing with sterile distilled water until the spores were >99% free of sporulating cells, cell debris, and germinated spores, suspended in distilled water to a final optical density at 600 nm (OD₆₀₀) of ~6, and stored at −20°C (47).

Measurement of spore properties. For most analyses of spore Ca²⁺ and DPA content, spore coats were extracted to remove material that might interfere with the quantitation of either the amount of spores being assayed or the amount of DPA. Spore coats were extracted from a spore suspension with an OD₆₀₀ of ~20 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 (treatment 1), and the spores were washed three times with 150 mM NaCl and twice with water (48). Note that this extraction procedure did not kill spores as determined on plates containing lysozyme (see Results). An aliquot of decoated spores at an OD₆₀₀ of 12 was incubated at 100°C for 60 min, the sample was cooled on ice and centrifuged for 5 min, and Ca²⁺ and DPA were measured in the supernatant fluid as described previously (51, 73). While most analyses used decoated spores, similar results were obtained when spores were not initially decoated.

For determination of spore core densities (wet weight) by equilibrium density gradient centrifugation (27), decoated spores were suspended in 100 µl of 30% Histodenz (Nycodenz) (Sigma, St. Louis, MO), incubated for 60 min on ice, loaded on the top of 2-ml step gradients of 51 to 70% (for *spoVA* spores) or 61 to 80% (for wild-type spores) Histodenz in ultraclear centrifuge tubes, and the

FIG. 1. Arrangement of the *spoVA* operon in *B. subtilis* and in *C. perfringens* strains and expression of the *spoVA* operon during *C. perfringens* sporulation. (A) The arrangement of the *spoVA* operons of the various organisms is shown with the location of the known (*B. subtilis*) and putative (*C. perfringens*) promoters indicated by the leftward pointing arrows. (B) The GUS specific activity from the *spoVAC-gusA* fusion (open squares), *spoVAD-gusA* fusion (open diamonds), or *spoVAE-gusA* fusion (open triangles) and the level of DPA accumulated (filled circles) in *C. perfringens* SM101 grown in sporulation medium were determined as described in Materials and Methods.

tubes were centrifuged for 45 min at 14,000 rpm and 20°C in a swinging bucket rotor in a Beckman TL-100 ultracentrifuge (27).

Spore germination assays. Spore suspensions were heat activated at 80°C for 10 min, cooled in water at ambient temperature for 5 min, and subsequently incubated at 40°C for 10 min as described previously (47). Spore germination with nutrients or Ca-DPA (50 mM CaCl₂ and 50 mM DPA adjusted to pH 8.0 with Tris base) was routinely measured by monitoring the OD₆₀₀ of spore cultures (Smartspec 3000 spectrophotometer; Bio-Rad Laboratories, Hercules, CA), which falls ~60% upon complete germination of wild-type spores, and the levels of germination were confirmed by phase-contrast microscopy. However, upon complete germination of *spoVA* spores, the OD₆₀₀ fell by only ~40% due to the lower refractive index of *spoVA* spores. The extent of spore germination was calculated by measuring the decrease in OD₆₀₀ and expressed as a percentage of the initial OD₆₀₀. All values reported are averages of two experiments performed on at least two independent spore preparations, and individual values varied by less than 10% from the average values shown.

Decoating treatments. Spores (1 ml at an OD₆₀₀ of 20) were decoated either by treatment 1 as described above or in 1 ml of 0.1 M sodium borate (pH 10)–2% 2-mercaptoethanol for 60 min at 37°C (32) (treatment 2). Decoated spores were washed at least nine times with sterile distilled water before use.

DPA release. DPA release from untreated or decoated spores during nutrient germination was measured by heat activating a spore suspension (OD₆₀₀ of 1.5) as described above, cooling, and incubating with 100 mM AK (100 mM L-asparagine and 100 mM KCl) in 25 mM sodium phosphate buffer (pH 7.0) at 40°C for 60 min. For measuring DPA release during dodecylamine germination, untreated or decoated spores (OD₆₀₀ 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 2 min at 8,000 rpm in a microcentrifuge, and DPA in the supernatant fluid was measured by monitoring the OD₂₇₀ as described previously (6, 47). The total DPA content of wild-type spores was measured by boiling an aliquot (1 ml) for 60 min, centrifuging at 8,000 rpm in a microcentrifuge for 15 min, and measuring the OD₂₇₀ of the supernatant fluid as described previously (6, 47). In *B. subtilis* spores, ≥85% of the material absorbing at 270 nm is DPA (1, 6).

Assessment of colony-forming efficiency of spores. To assess the colony-forming efficiency of untreated and decoated spores, spores at an OD₆₀₀ of 1 were heat activated at 80°C for 10 min, aliquots of various dilutions were plated on brain heart infusion (BHI) agar with or without lysozyme (1 µg/ml) in the plates, the plates were incubated at 37°C anaerobically for 24 h, and colonies were counted.

SASP extraction. Extraction of SASP from *C. perfringens* spores and their analysis by polyacrylamide gel electrophoresis at low pH were as described previously (49, 50). Briefly, SASP were extracted from 40 mg (dry weight) of disrupted spores with dilute acid, and the extracts were processed and lyophilized. The dry residue was dissolved in 30 µl of 8 M urea, 5-µl aliquots were run on polyacrylamide gels at low pH, and the gels were stained with Coomassie brilliant blue (Bio-Rad Laboratories, Hercules, CA), all as described previously (50). Analysis of relative band intensities on stained gels by densitometry was performed using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at <http://rsb.info.nih.gov/nih-image/>).

Spore resistance. The resistance of *C. perfringens* spores to moist heat was measured as described previously (49, 55). Briefly, cultures of *C. perfringens* strains that had been grown on DS medium for 24 h were heat treated at 75°C for 20 min to kill vegetative cells. The number of initial CFU was determined by serially diluting heat-treated spore cultures grown in DS medium in phosphate-buffered saline (140 mM NaCl–25 mM sodium phosphate buffer [pH 7.0]), plating on BHI agar, and anaerobic incubation for 24 h at 37°C. The spore cultures grown in DS medium and treated at 75°C were then heated at 94 or 100°C for various times, aliquots of appropriate dilutions were plated, and the plates were incubated anaerobically for 24 h at 37°C as described above. Plots of CFU/ml versus time at 94 or 100°C were used to determine decimal reduction values ($D_{94^\circ\text{C}}$ or $D_{100^\circ\text{C}}$ values), which are the times cultures need to be kept at 94 or 100°C to achieve 90% reduction in CFU/ml.

C. perfringens spore resistance to UV radiation was determined as described previously (48, 50). Briefly, purified spores at an OD₆₀₀ of 2 were diluted 100-fold in 25 mM sodium phosphate buffer (pH 6.8) and UV irradiated at 254 nm with a UVGL-25 Mineralight lamp (UVP Inc., Upland, CA) for various times. Appropriate dilutions were spread onto BHI plates and incubated as described above prior to assessment of colony formation.

The resistance of *C. perfringens* spores to chemicals was determined as described previously (45). Briefly, purified spores at an OD₆₀₀ of ~1 were treated as follows. (i) The spores were treated with 2 M hydrogen peroxide (Mallinck-

rodt Baker Inc., Phillipsburg, NJ) at room temperature, and aliquots were neutralized with catalase (Sigma) as described previously (57). (ii) The spores were treated with 300 mM HCl at room temperature, and aliquots were diluted 100-fold in 25 mM sodium phosphate buffer (pH 7.0). (iii) The spores were treated with 400 mM NaNO₂–400 mM Na acetate buffer (pH 4.5) at room temperature, and aliquots were diluted 10-fold in 500 mM sodium phosphate buffer (pH 8.5). (iv) The spores were treated with 25 g/liter formaldehyde (Sigma) at 30°C, and aliquots were diluted 10-fold in 400 mM glycine (pH 7.0) and incubated for 20 min at room temperature prior to analysis. Determination of spore killing was as described above.

RESULTS

Identification of the putative *spoVA* operon in *C. perfringens*.

The SpoVA proteins are encoded by six open reading frames (ORFs) in a hexacistronic operon in *B. subtilis* (24). In contrast, only three ORFs (CPR2017, CPR2018, and CPR2019) encoding proteins with high similarity (65 to 73%) to *B. subtilis* SpoVA proteins were found in the *C. perfringens* SM101 genome (37) (Fig. 1A). These ORFs are annotated as *spoVAC* (CPR2019), *spoVAD* (CPR2018), and *spoVAE* (CPR2017) and form a putative tricistronic operon. This organization of the *spoVA* operon is not unique to *C. perfringens*, as it is conserved among *Clostridium* species (4, 5, 38, 56). The first gene in the putative tricistronic operon, *spoVAC* (CPR2019), is predicted to encode a 155-residue protein with four transmembrane alpha-helical segments (TMS). The *spoVAD* gene (CPR2018) is predicted to encode a protein of 339 amino acid residues with no TMS; perhaps this is a peripheral inner membrane protein that interacts with other inner membrane proteins. The *spoVAE* gene (CPR2017) is predicted to encode a 121-residue protein with four TMS.

Expression of *C. perfringens* *spoVA* genes. To assess whether the putative *C. perfringens* *spoVA* genes are expressed uniquely during sporulation and comprise a tricistronic operon, DNA upstream of each gene was fused to *E. coli* *gusA*, and GUS activity was measured after introduction of the various fusions into *C. perfringens* SM101. No significant GUS activity was observed in vegetative cultures carrying plasmid pDP51 with a *spoVAC-gusA* fusion (data not shown). However, a sporulating culture carrying plasmid pDP51 (*spoVAC-gusA*) exhibited significant GUS activity (Fig. 1B), indicating that a sporulation-specific promoter is located upstream of *spoVAC*. Expression of GUS from the *spoVAC-gusA* fusion began ~3 h after induction of sporulation, and GUS specific activity reached a maximum 8 to 10 h after initiation of sporulation and then fell somewhat (Fig. 1B). The decrease in GUS specific activity late in sporulation is consistent with *spoVA* being packaged into the spore where it cannot be easily assayed (29). Phase bright spores became visible ~6 h after induction of sporulation (data not shown), when ~50% of the spore's maximum DPA level had been accumulated, and DPA accumulation lagged ~1 h behind expression of the *spoVA* operon (Fig. 1B). Thus, *spoVAC* and probably *spoVA* operon transcription and accumulation of the SpoVA proteins precede DPA accumulation by the developing spore, as is also the case in *B. subtilis* (29).

In contrast to the results with the *spoVAC-gusA* fusion, there was no detectable GUS activity observed in either vegetative or sporulating cultures carrying plasmids pDP79 (*spoVAD-gusA*) or pDP80 (*spoVAE-gusA*) (Fig. 1B and data not shown). These results indicate that no promoter is present in DNA 300 to 400 bp upstream of *spoVAD* or *spoVAE*, and thus, these genes

TABLE 3. Effects of *spoVA* mutation on *C. perfringens* spore properties

Strain (genotype)	DPA ($\mu\text{g/ml}$ / OD_{600}) ^a	Ca ²⁺ ($\mu\text{g/ml}$ / OD_{600}) ^a	Core density (g [wet weight]/ml) ^b	Water content (g/100 g of protoplast [wet weight]) ^c
SM101 (wild type)	21.7	3.6	1.381	31
DPS104 (<i>spoVA</i>)	<0.27	<0.07	1.303	62

^a Values are averages of determinations on three different spore preparations.

^b Values are averages of determination of two different spore preparations that differed by ≤ 0.003 g/ml.

^c Spore core water content was calculated according to the formula $y = -0.00254x + 1.460$ (27), where y is the spore core density (wet weight) and x is the core water content in grams per 100 g of protoplast (core) weight (wet weight).

almost certainly form a tricistronic operon in *C. perfringens* with *spoVAC* as the first gene in the operon, and these results further indicate that this operon is expressed only during sporulation.

Effect of a *spoVA* mutation on *C. perfringens* spore properties. Previous studies with *B. subtilis* (12–14, 33, 68) have shown that sporulation of strains with null mutations in any of the first five cistrons of the *spoVA* operon gives immature forespores that lyse within the sporangium. Isolation of stable *spoVA* spores in *B. subtilis* requires the elimination of either all three functional nutrient germinant receptors or the CLE, SleB (68). Surprisingly, spores of the *C. perfringens* *spoVA* mutant (strain DPS104) were stable, appeared bright when viewed by phase-contrast microscopy, and were easily purified to give clean spore preparations containing >95% free spores. As expected, the Ca²⁺ and DPA contents of *C. perfringens* *spoVA* spores were negligible compared to those in wild-type spores (Table 3). Sporulation of *C. perfringens* strain DPS104 with DPA present in the sporulation medium at 100 $\mu\text{g/ml}$ also gave spores that contained no DPA (data not shown), although this exogenous DPA level restores nearly wild-type DPA levels to spores of *B. subtilis* strains defective in DPA synthase (12, 13, 33, 40). However, attempts to correct the DPA-less phenotype of *spoVA* spores by complementation with the wild-type *spoVA* operon failed, as the DPA level in *C. perfringens* DPS104 (pDPS54) spores was similar to that of *spoVA* spores (data not shown). Together the latter findings indicate that SpoVA proteins are essential for Ca-DPA uptake during *C. perfringens* sporulation. Interestingly, the molar ratio of Ca²⁺ to DPA in *C. perfringens* wild-type spores was 0.7:1, indicating that DPA may also be forming complexes with other divalent cations, such as Mg²⁺ and Mn²⁺ (Table 3) (16, 35, 36, 59, 61).

As noted above, the *C. perfringens* *spoVA* spores appeared bright when observed by phase-contrast microscopy, suggesting that the cores of these spores were less hydrated than the protoplast of growing cells. However, the *spoVA* spores were less bright than wild-type spores (data not shown), suggesting that the cores of these spores are not as dehydrated as those of wild-type spores. Indeed, the core density (wet weight) of *spoVA* spores was significantly lower than that of wild-type spores (Table 3). The latter difference indicated that the *spoVA* spore core contains almost twice as much water per gram (wet weight) as the wild-type spore core does (Table 3).

In addition to differences in Ca-DPA content and core den-

TABLE 4. Colony formation by spores of *C. perfringens* strains^a

Treatment ^b and strain (genotype)	Spore titer (CFU/ml/OD ₆₀₀) ^c	
	BHI	BHI + Lyz ^d
No treatment		
SM101 (wild type)	3.7×10^7	4.6×10^7
DPS104 (<i>spoVA</i>)	1.9×10^6	2.2×10^6
Treatment 1		
SM101 (wild type)	3.3×10^3	9.8×10^7
DPS104 (<i>spoVA</i>)	2.2×10^3	1.7×10^6
Treatment 2		
SM101 (wild type)	3.6×10^6	3.7×10^7
DPS104 (<i>spoVA</i>)	9.3×10^5	5.7×10^6

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

^b Spores were decoated by treatment 1 or treatment 2.

^c The titers are the averages from three experiments.

^d BHI plates containing 1 $\mu\text{g/ml}$ lysozyme.

sity (wet weight) between wild-type and *spoVA* spores, *spoVA* spores had lower colony-forming efficiency than wild-type spores did. When wild-type and *spoVA* spores were heat activated (80°C, 10 min), plated on BHI agar, and incubated overnight at 37°C under anaerobic conditions, the viability of *spoVA* spores was ~20-fold lower than that of wild-type spores (Table 4). The lower apparent viability of *spoVA* spores was not due to their killing during heat activation, as unheated *spoVA* spores actually gave sixfold-fewer colonies than heat-activated *spoVA* spores (data not shown). The viability of the *spoVA* spores was also not increased on plates containing lysozyme (Table 4).

Effect of a *spoVA* mutation on germination of *C. perfringens* spores. One possible explanation for the lower viability of the *C. perfringens* *spoVA* spores noted above is that Ca-DPA and/or SpoVA proteins are also essential for the germination of *C. perfringens* spores. If this is correct, then *C. perfringens* *spoVA* spores should germinate to a much lower extent than wild-type spores do. However, wild-type and *spoVA* spores germinated similarly in BHI broth through 60 min of incubation (Fig. 2A). Although the decreases in OD₆₀₀ of wild-type and *spoVA* spores incubated aerobically at 40°C in BHI broth for 18 h to inhibit cell growth were 60 and 45%, respectively (data not shown), phase-contrast microscopy revealed that >99% of both the *spoVA* and wild-type spores had become phase dark and thus had germinated fully. Similarly, while *spoVA* spores appeared to germinate to a lesser extent than wild-type spores incubated with AK or Ca-DPA for 60 min when spore germination was monitored by the OD₆₀₀ of the cultures (Fig. 2B and C), phase-contrast microscopy indicated again that >99 and ~90% of both the wild-type and *spoVA* spores incubated with AK and Ca-DPA had germinated fully, respectively (data not shown). To more rigorously quantify this result, *spoVA* and wild-type spores were germinated with 100 mM AK (pH 7.0) at 40°C for 80 min. While >99% of the spores of both strains had become phase dark as determined by phase-contrast microscopy, the OD₆₀₀ of the germinating wild-type and *spoVA* spore cultures had decreased by 62 and 42%, respectively (average of four independent experiments). These results indicate the following. (i) As suggested above, the re-

FIG. 2. Germination of intact and decoated *C. perfringens* wild-type and *spoVA* spores with various germinants. (A to C) Heat-activated intact spores of strains SM101 (wild type) (filled squares) and DPS104 (*spoVA*) (open squares) were incubated at 40°C with BHI broth (A), 100 mM AK (B), or 50 mM Ca-DPA (C), and at various times, the OD₆₀₀ was recorded as described in Materials and Methods. (D to F) Spores were decoated by treatment 1 (squares) or treatment 2 (triangles). Decoated spores of strains SM101 (wild type) (filled symbols) and DPS104 (*spoVA*) (open symbols) were heat activated and incubated with BHI broth (D), 100 mM AK (E), or 50 mM Ca-DPA (F), and at various times, the OD₆₀₀ was recorded as described in Materials and Methods. No significant germination of intact or decoated spores of strain SM101 or DPS104 was observed when heat-activated spores were incubated in 25 mM sodium phosphate buffer (pH 7.0) at 40°C for 60 min.

fractive index of *spoVA* spores, and thus, their OD₆₀₀ is ~30% lower than that of wild-type spores, most likely due to the higher water content in the *spoVA* spore core; consequently, the difference between the OD₆₀₀ of a culture of dormant and fully germinated spores is ~30% greater for wild-type spores. (ii) DPA-less *C. perfringens* spores are able to germinate normally with either nutrients or exogenous Ca-DPA.

Germination of decoated *C. perfringens* spores. The results noted above suggested that cortex hydrolysis during *C. perfringens* spore germination can be independent of both SpoVA proteins and any signaling by Ca-DPA, and thus, perhaps CLEs are not activated directly by Ca-DPA in *C. perfringens*. To begin to examine the roles of CLEs in *C. perfringens* spore germination, we decoated wild-type and *spoVA* spores with treatments that are reported to remove CLEs (32, 42) and examined germination of the decoated spores. While it is still not clear which *C. perfringens* CLEs identified by analyses of enzyme activity on spore cortex peptidoglycan *in vitro* are actually involved in cortex PG degradation during spore germination, it was notable that both wild-type and *spoVA* spores decoated by treatment 1 were unable to germinate and remained phase bright when incubated for 60 min with BHI, AK, or Ca-DPA (Fig. 2D, E, and F and data not shown). Even after an 18-h incubation, >99% of these decoated spores remained phase bright (data not shown). These results are consistent with treatment 1 removing or inactivating all CLEs in *C. per-*

fringens spores, including any putative CLE directly activated by Ca-DPA. Although wild-type and *spoVA* spores decoated with treatment 2 exhibited little germination after incubation at 40°C for 60 min in BHI broth (Fig. 2D), ~20% of the spores had become phase dark after 18 h of incubation (data not shown). In addition, wild-type and *spoVA* spores decoated by treatment 2 did germinate at a very low rate with AK or Ca-DPA (Fig. 2E and F), and ~30% of the spores were phase dark after 18 h of incubation with AK and Ca-DPA (data not shown), consistent with treatment 2 completely removing the CLE SleC but removing only ~90% of SleM from *C. perfringens* spores (23). Interestingly, no DPA was released from the cores of wild-type spores decoated with treatment 1 when these spores were incubated with AK, yet nearly all the spore's DPA was released when these spores were incubated with dodecylamine (Fig. 3), suggesting that perhaps the spore's germinant receptors had been damaged by treatment 1. In contrast, wild-type spores decoated with treatment 2 did release DPA when incubated with AK or dodecylamine (Fig. 3). These results strongly suggest that *spoVA* spores need undamaged germinant receptors and at least one CLE to initiate cortex hydrolysis during spore germination.

Colony-forming efficiency of decoated *C. perfringens* spores. The extremely poor germination of the decoated spores noted above suggested that decoated wild-type and *spoVA* spores might have much lower colony-forming efficiency than intact

FIG. 3. DPA release from decoated *C. perfringens* spores incubated with various germinants. Spores of *C. perfringens* strain SM101 (wild type) were decoated by either treatment 1 (Treat 1) or treatment 2 (Treat 2). Decoated spores were germinated with 100 mM AK and 25 mM sodium phosphate buffer (pH 7.0) for 60 min at 37°C (white bars) or with 1 mM dodecylamine and 25 mM Tris-HCl (pH 7.4) for 60 min at 60°C (gray bars), and DPA release was measured as described in Materials and Methods.

spores do. Indeed, when heat-activated wild-type and *spoVA* spores were plated on BHI agar and incubated for 24 h at 37°C under anaerobic conditions, the colony-forming efficiencies of wild-type and *spoVA* spores decoated by treatment 1 were $\sim 10^4$ - and 10^3 -fold lower, respectively, than those of the corresponding intact spores (Table 4). No additional colonies appeared when plates were incubated for up to 3 days. Wild-type and *spoVA* spores decoated with the milder treatment (treatment 2) exhibited much smaller decreases in colony-forming efficiencies (Table 4). Strikingly, when spores decoated with either treatment were plated on BHI agar containing 1 μ g/ml lysozyme, the colony-forming efficiencies were restored to those of the corresponding intact spores (Table 4). These results indicate that the decoating treatments had not simply killed the spores.

Levels of α/β -type SASP in wild-type and *spoVA* spores. In *B. subtilis*, α/β -type SASP are degraded during the first minute of germination (52, 60) by a germination-specific protease (GPR) specific for a highly conserved sequence found in all SASP (64, 67). *B. subtilis* GPR is synthesized as a zymogen with a molecular mass of ~ 46 kDa (termed P_{46}) beginning at approximately the third hour of sporulation (53) and is autoprocessed to the ~ 41 -kDa active protease (P_{41}) about 2 h later. This latter autoprocessing is triggered synergistically by Ca-DPA accumulation in the forespore and by decreases in the developing forespore's core pH and water content, conditions that preclude attack of P_{41} on its SASP substrates (19, 20, 53). *C. perfringens* also contains a *gpr* gene encoding a protein with high similarity to *B. subtilis* GPR, including the highly conserved $P_{46} \rightarrow P_{41}$ autoprocessing site (53). If P_{46} autoprocessing is regulated in *C. perfringens* as it is in *B. subtilis*, perhaps little, if any, GPR is autoprocessed to P_{41} during sporulation of *C. perfringens* strain DPS104 (*spoVA*). However, if P_{41} is present in *C. perfringens spoVA* spores, perhaps this protease can catalyze some SASP hydrolysis in the more hydrated core of *spoVA* spores. Given the important role played by α/β -type SASP in *C. perfringens* spore resistance (45, 49, 50), it was thus of interest to compare the levels of α/β -type SASP in *C. perfringens spoVA* and wild-type spores. Polyacrylamide gel elec-

FIG. 4. Levels of α/β -type SASP in *C. perfringens* wild-type and *spoVA* spores. Spores of *C. perfringens* strains SM101 (wild type) and DPS104 (*spoVA*) were dry ruptured, SASP were extracted, and extracts were processed and lyophilized. The dry residue was dissolved in 30 μ l of 8 M urea, 5- μ l aliquots were run on polyacrylamide gels at low pH, and the gel was stained as described in Materials and Methods. The arrow indicates the major band reactive with antibody to the *B. subtilis* α/β -type SASP, SspC (50).

trophoresis at low pH of a SASP extract from wild-type and *spoVA* spores gave only a tight group of protein bands (Fig. 4), one of which was a major one, as seen previously (50). The intensities of these bands, and thus total α/β -type SASP levels, were similar in extracts from spores of these two strains (Fig. 4), as was confirmed by densitometric analysis of gels from three independent experiments (data not shown). These results suggest that either little, if any, P_{46} is autoprocessed to P_{41} during sporulation of strain DPS104 (*spoVA*) or the hydration level of the *spoVA* spore core is still low enough to preclude P_{41} activity on α/β -type SASP. It is also possible that the binding of α/β -type SASP to DNA in the *spoVA* spores helps protect these proteins against GPR digestion (19, 20, 53) (but see Discussion).

Moist heat, UV, and chemical resistance of *C. perfringens spoVA* spores. Previous work (49, 50) has shown that binding of α/β -type SASP to DNA is a major factor in *C. perfringens* spore resistance to moist heat and UV radiation and that small changes in spore core water content have significant effects on spore resistance to moist heat and nitrous acid but not UV radiation (46). As expected, *C. perfringens spoVA* spores were very sensitive to moist heat (Fig. 5A and B), with 70- to 80-fold lower $D_{94^\circ\text{C}}$ (5 min; results from three independent experiments) and $D_{100^\circ\text{C}}$ (0.5 min) values than wild-type spores ($D_{94^\circ\text{C}}$, 410 min; $D_{100^\circ\text{C}}$, 45 min). However, the heat resistance of *spoVA* spores could not be restored to wild-type levels by complementation with the wild-type *spoVA* operon, as the heat resistance of *C. perfringens* DPS104(pDP54) spores was similar to that of *spoVA* spores (data not shown). The *spoVA* spores were also significantly more sensitive to UV radiation than wild-type spores were (Fig. 5C).

FIG. 5. Resistance of *C. perfringens* wild-type and *spoVA* spores to moist heat at 94°C (A) or 100°C (B) and to UV radiation (C). Spore cultures grown in DS medium or purified spores of *C. perfringens* strains SM101 (wild type) (filled squares) and DPS104 (*spoVA*) (open squares) were used to measure survival upon various treatments as described in Materials and Methods. The variability of the survival values in these experiments was $\pm 20\%$.

Previous studies (46) have also shown that the resistance of *C. perfringens* mutant spores with slightly higher core water content to hydrogen peroxide and HCl is essentially identical to that of wild-type spores. However, *C. perfringens spoVA*

spores with approximately twofold-higher water content than wild-type spores were more sensitive than wild-type spores not only to nitrous acid (Fig. 6A) but also to HCl (Fig. 6B), formaldehyde (Fig. 6C), and hydrogen peroxide (Fig. 6D).

FIG. 6. Resistance of *C. perfringens* wild-type and *spoVA* spores to nitrous acid (A), HCl (B), formaldehyde (C), and hydrogen peroxide (D). Spores of *C. perfringens* strains SM101 (wild type) (filled squares) and DPS104 (*spoVA*) (open squares) were purified, and their survival upon various treatments was measured as described in Materials and Methods. The variability of the survival values in these experiments was $\pm 15\%$.

DISCUSSION

The work in this communication indicates that *C. perfringens* *spoVA* spores accumulate no Ca-DPA and do not take up exogenous DPA and that the *spoVA* operon is expressed only during *C. perfringens* sporulation. These results are similar to those found with *B. subtilis* (13, 40, 68) and indicate that in *C. perfringens* the *spoVA* operon also encodes sporulation-specific genes essential for the uptake of Ca^{2+} and DPA during spore maturation. Unfortunately, we were unable to restore the DPA-less phenotype of *C. perfringens* *spoVA* spores by complementation with a wild-type *spoVA* operon, although the reason for this failure is not clear. Perhaps an alternative approach, such as introduction of the *C. perfringens* *spoVA* operon into a *B. subtilis* *spoVA* strain and vice versa could be successful. It was, however, most striking that sporulation of the *C. perfringens* *spoVA* strain gave stable Ca-DPA-less spores, since *B. subtilis* *spoVA* strains give only immature spores that lyse during sporulation, probably due to their germination within the sporangium (13, 68). Spores of *B. subtilis* strains that cannot synthesize DPA are also unstable and lyse during sporulation (13, 40). *B. subtilis* spores with significantly lower than normal DPA levels can be isolated, but they exhibit relatively rapid spontaneous germination (28). Thus, *B. subtilis* spores with low or no DPA levels, for whatever reason, spontaneously and rapidly trigger subsequent events in the spore germination process, most notably the hydrolysis of cortex PG. Indeed, the activity of one of the two redundant CLEs, CwlJ, appears to be directly stimulated by Ca-DPA, while the second redundant CLE, SleB, is somehow activated in spores that lack DPA or have low DPA, possibly responding to a change in the strain on cortex PG (42, 68). However, clearly, the CLEs in *C. perfringens* spores are not activated in spores that lack DPA. The reason for the stability of the DPA-less *C. perfringens* *spoVA* spores is unclear and may well reflect significant differences in the signaling mechanisms operating during germination of *B. subtilis* and *C. perfringens* spores, in particular the signaling mechanisms involving Ca-DPA. It will obviously be of great interest in future work to determine whether *C. perfringens* spores also have a CLE whose activity responds directly to DPA or Ca-DPA, although work thus far indicates that Ca-DPA triggers germination of these spores by acting through a nutrient germinant receptor (47).

In addition to the major general conclusions noted above, the current work also strongly indicates that the core water content plays a major role in the resistance of *C. perfringens* spores to moist heat. A recent study (46) found that wild-type *C. perfringens* spores from cultures grown at 42°C had ~20% less core water than spores from cultures grown at 26°C and that $D_{100^\circ\text{C}}$ values for moist heat were approximately fourfold lower in spores at 26°C than in spores at 42°C, both of which had essentially identical DPA levels. In the current work, an approximately twofold-higher core water content in *spoVA* spores was accompanied by an 80-fold-lower $D_{100^\circ\text{C}}$ value. While some of the effects on the resistance of *spoVA* spores to moist heat may be due to the loss of specific protective effects of Ca-DPA, most previous work (15), primarily with spores of *Bacillus* species, has found a strong inverse relationship between a spore's moist heat resistance and its core water content; this also seems to be true for *C. perfringens* spores over a

wide range of core water contents. Presumably, a low core water content is most effective in stabilizing essential proteins in the spore core, and thus, a higher core water core results in an increased rate of inactivation of such proteins during moist heat treatment (8).

A notable observation made in this work was that *C. perfringens* *spoVA* spores were significantly more sensitive to formaldehyde, hydrogen peroxide, and nitrous acid than were wild-type spores. Previous work has shown that *B. subtilis* spores with or without Ca-DPA have core water contents similar to those found in this work for the corresponding *C. perfringens* spores (40, 68). *B. subtilis* spores that lack DPA are also significantly more sensitive to formaldehyde and hydrogen peroxide than wild-type spores are, although nitrous acid resistance has not been tested (40). *B. subtilis* spore resistance to formaldehyde, hydrogen peroxide, and nitrous acid is due in large part to the protection of spore DNA against these agents by DNA's saturation with α/β -type SASP (63). However, the levels of α/β -type SASP in DPA-less *B. subtilis* spores appear to be essentially identical to those in wild-type spores (40, 68), as was also the case with *C. perfringens* spores. Thus, the decreased resistance of DPA-less *C. perfringens* spores to formaldehyde, hydrogen peroxide, and nitrous acid is not due to decreased levels of α/β -type SASP. Possible explanations for this decreased resistance follow. (i) The increased hydration of the DPA-less spores allows more rapid reaction of reactive chemicals with either DNA saturated with α/β -type SASP or other targets in the spore core, such as proteins. (ii) While the levels of α/β -type SASP in the DPA-less spores are normal, a significant amount of these proteins may not be bound to the DNA because a higher core water content promotes their dissociation, a process known to take place in fully germinated *B. subtilis* spores that have a core water content comparable to that in growing cells (52); this protein-free DNA would then be very sensitive to DNA-damaging chemicals, as is the case in *B. subtilis* and *C. perfringens* spores (45, 49, 50, 63). Analysis of whether hydrogen peroxide kills DPA-less *C. perfringens* spores by DNA damage might resolve this issue, as at least with *B. subtilis* spores, DNA is so well protected by α/β -type SASP that this agent does not kill these spores by DNA damage, although it does cause lethal DNA damage in *B. subtilis* spores that lack most α/β -type SASP (57, 63). However, there have as yet been no detailed studies of the mechanism of the killing of *C. perfringens* spores by hydrogen peroxide or any other agent. The DPA-less *C. perfringens* *spoVA* spores were also more sensitive to HCl than wild-type spores were. HCl resistance of DPA-less *B. subtilis* spores has not been tested, so this is a novel observation. Since the mechanism of *C. perfringens* spore killing by and resistance to HCl are not known, it is difficult to explain this observation, although at least in *B. subtilis* spores, the α/β -type SASP are not involved in HCl resistance (63).

A third notable observation made in this work is that the DPA-less *C. perfringens* *spoVA* spores were markedly more UV sensitive than wild-type spores were. This was not seen previously with *C. perfringens* spores that had 15 to 20% higher core water contents but retained normal DPA levels, so perhaps sensitization to UV requires a much larger increase in core water content, as was also the case for sensitization to hydrogen peroxide (46). The major factor in the UV resistance of *B. subtilis* spores is the saturation of spore DNA with α/β -type

SASP, and this is also important in the UV resistance of *C. perfringens* spores (49, 50, 60, 61, 63). Perhaps a significant amount of the *C. perfringens* α/β -type SASP are dissociated from the DNA in the DPA-less spores as suggested above, thus causing their decreased UV resistance. This does not appear to occur in DPA-less *B. subtilis* spores that have almost identical UV resistance to wild-type spores (40, 68), but perhaps the *C. perfringens* α/β -type SASP have significantly lower affinity for DNA than do the comparable *B. subtilis* proteins. While this has not been studied directly, it has been shown that one *C. perfringens* α/β -type SASP is less effective in restoring resistance properties to α/β -type SASP-deficient *B. subtilis* spores than a *B. subtilis* protein is, although the reason for this difference has not been established (26). In addition, the *C. perfringens* and *B. subtilis* α/β -type SASP exhibit significant differences in primary sequence, in particular in the spacing between two highly conserved regions recently shown to be crucial for the binding to and protection of DNA by a *B. subtilis* protein (25, 64).

One of the more surprising findings in this work was that the colony-forming efficiency of the *C. perfringens* *spoVA* spores was 20-fold lower than that of wild-type spores, although this is not the case with DPA-less *B. subtilis* spores (40, 68). The lower colony-forming efficiency of the *C. perfringens* *spoVA* spores was not due to inefficient spore germination, as these spores germinated as well as wild-type spores did and their viability was not increased by inclusion of lysozyme in plating medium. Thus, the majority of these spores appear to be dead. While we are not certain why this is the case, if some of the chromosome in the DPA-less *C. perfringens* spores is indeed not saturated with α/β -type SASP, as suggested above, perhaps this naked DNA is particularly susceptible to damage caused by endogenous agents. Indeed, while *B. subtilis* spores that lack either DPA or most α/β -type SASP are fully viable, the viability of *B. subtilis* spores that lack both DPA and α/β -type SASP is drastically reduced, and these spores die during sporulation largely due to DNA damage (58). Perhaps analysis of surviving *C. perfringens* *spoVA* spores for evidence of DNA damage, for example mutations, might clarify the reason why so many of these spores appear dead.

It was also notable that decoating of wild-type or *spoVA* *C. perfringens* spores caused a significant reduction in apparent spore viability, with this reduction being greatest for the harsher decoating regimen of treatment 1. However, these decoated spores were not dead, since their viability could be completely restored by inclusion of lysozyme in the plating medium. This result strongly suggests that the decoating treatment has removed the CLEs that degrade the *C. perfringens* cortex PG during spore germination, although this same decoating treatment does not remove all CLEs from *B. subtilis* spores. This suggests that CLEs in *C. perfringens* spores are located only in or adjacent to the outer edge of the cortex. The CLEs SleC and SleM, one of which (SleC) is activated by proteolysis early in germination, have been identified in and purified from *C. perfringens* spores, and their enzymatic activity has been characterized (7, 23, 31, 39, 65, 69). However, the roles of these proteins in vivo during spore germination have not been established, and they show little homology with the CLEs CwlJ and SleB that catalyze cortex hydrolysis during *B. subtilis* spore germination. Analysis of *C. perfringens* spores

with mutations in the *sleC* and *sleM* genes should allow a better understanding of the roles of the proteins encoded by the *sleC* and *sleM* genes in spore germination and how these CLEs interact with germinant receptors and Ca-DPA.

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