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Roles of DacB and Spm Proteins in *Clostridium perfringens* Spore Resistance to Moist Heat, Chemicals, and UV Radiation[▽]

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Clostridium perfringens **food poisoning is caused mainly by enterotoxigenic type A isolates that typically possess high spore heat resistance. Previous studies have shown that α/β-type small, acid-soluble proteins (SASP) play a major role in the resistance of *Bacillus subtilis* and *C. perfringens* spores to moist heat, UV radiation, and some chemicals. Additional major factors in *B. subtilis* spore resistance are the spore's core water content and cortex peptidoglycan (PG) structure, with the latter properties modulated by the *spm* and *dacB* gene products and the sporulation temperature. In the current work, we have shown that the *spm* and *dacB* genes are expressed only during *C. perfringens* sporulation and have examined the effects of *spm* and *dacB* mutations and sporulation temperature on spore core water content and spore resistance to moist heat, UV radiation, and a number of chemicals. The results of these analyses indicate that for *C. perfringens* SM101 (i) core water content and, probably, cortex PG structure have little if any role in spore resistance to UV and formaldehyde, presumably because these spores' DNA is saturated with α/β-type SASP; (ii) spore resistance to moist heat and nitrous acid is determined to a large extent by core water content and, probably, cortex structure; (iii) core water content and cortex PG cross-linking play little or no role in spore resistance to hydrogen peroxide; (iv) spore core water content decreases with higher sporulation temperatures, resulting in spores that are more resistant to moist heat; and (v) factors in addition to SpmAB, DacB, and sporulation temperature play roles in determining spore core water content and thus, spore resistance to moist heat.**

Clostridium perfringens is a gram-positive, spore-forming, anaerobic bacterium that causes both gastrointenstinal and histotoxic diseases in humans and animals (19). *C. perfringens* food poisoning is caused mainly by enterotoxigenic type A isolates that produce spores that are highly resistant to heat and other environmental stress factors (32, 33, 36). These resistant spores can survive traditional cooking methods for meat and poultry products, as well as other processing treatments used in the food industry; the surviving spores will germinate and outgrow during improper cooling, and the resultant cells can then cause disease (2). Extensive work with *Bacillus subtilis* spores (23, 41, 42) has shown that factors involved in spore resistance include (i) the spore coats, (ii) the cortex peptidoglycan (PG) structure, (iii) the relatively impermeable spore inner membrane, (iv) the spore core's low water content, (v) the high levels of pyridine-2,6-dicarboxylic acid (DPA) in the spore core and the type and amount of cations chelated by DPA, and (vi) the saturation of spore DNA with α/β-type small, acid-soluble spore proteins (SASP). The most-important factors in the moist-heat resistance of *B. subtilis* spores are the cortex PG structure, the low core water content, the levels of DPA and associated cations in the core, and the α/β-type SASP (44). However, to date only the role of α/β-type SASP in *C. perfringens* spore resistance to moist heat, UV radiation, and some chemicals has been studied (26, 32, 33).

The spore cortex is composed of PG, with a structure similar to that of vegetative PG but with some cortex-specific modifications, as well as a lower degree of peptide cross-links (31, 50). The degree of PG cross-linking is determined by the low-molecular-weight penicillin-binding proteins (PBPs) (28) whose D,D-carboxypeptidase activity removes the terminal D-alanine of the PG's peptide side chain, preventing this side chain from serving as a donor in the formation of peptide cross-links. The *B. subtilis* genome encodes six low-molecular-weight PBPs, two of which, *dacB* and *dacF*, are expressed only during sporulation in the developing forespore under the control of the RNA polymerase sigma factors σ^E and σ^F, respectively (37, 45). DacB and, to a much-lesser extent, DacF determine the degree of cortex PG cross-linking (28), and the degree of cross-linking determines the amount of PG flexibility, with a loosely cross-linked PG exhibiting the greatest flexibility (25). Interestingly, *B. subtilis* spores lacking *dacB* have the same core water content but a much-lower moist-heat resistance than wild-type spores (28). In *B. subtilis*, *dacB* is the first gene in a three-gene operon with two genes (*spmA* and *spmB*) encoding proteins required for normal core dehydration during spore formation (Fig. 1A) (29). Mutations in either *spmA* or *spmB* result in *B. subtilis* spores with increased core water content and decreased resistance to moist heat (29, 30). The core water content of spores of *Bacillus* species is also affected by the sporulation temperature, with higher temperatures giving spores that have less core water and are more

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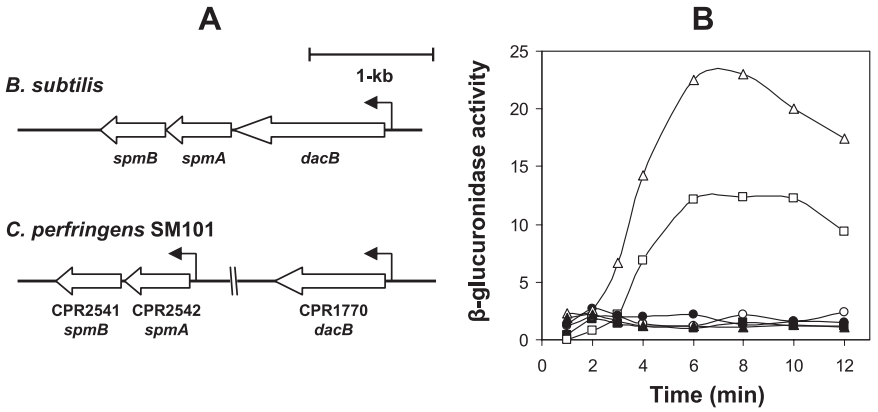

FIG. 1. Organization of *dacB* and *spmAB* genes in *B. subtilis* and *C. perfringens*, and expression of these genes in *C. perfringens*. (A) Schematic representation of the genomic organization of *dacB* and *spmAB* in *B. subtilis* (15) and *C. perfringens* (22). (B) Expression of *spmA-gusA* (squares), *spmB-gusA* (circles), and *dacB-gusA* (triangles) fusions in *C. perfringens* wild-type SM101 grown in TGY vegetative (filled symbols) and DS sporulation (open symbols) media. GUS activity (in Miller units) was calculated as described in Materials and Methods. Data represent the averages of the results of three independent experiments, and time zero denotes the time of inoculation of cells into either TGY or DS medium.

moist-heat resistant (21, 30). An increased core water content is also correlated with increased sensitivity of *B. subtilis* spores to some chemicals (21, 30). In this study, through the construction of mutations in *spmA*, *spmB*, and *dacB* and by preparation of spores at different temperatures, we have investigated the roles of cortex PG structure and core water content in the resistance of spores of a pathogenic *C. perfringens* isolate to moist heat, chemicals, and UV radiation.

MATERIALS AND METHODS

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

Construction of *gusA* fusion plasmids and GUS assay. The expression of the *C. perfringens spmA*, *spmB*, and *dacB* genes was examined by fusing a large amount of DNA upstream of each gene to *Escherichia coli gusA* in pMRS127, an *E. coli-C. perfringens* shuttle vector (33). Briefly, 300- to 400-bp DNA fragments upstream of *spmA*, *spmB*, and *dacB* from *C. perfringens* SM101 were PCR amplified by using primers CPP376/CPP373, CPP374/CPP377, and CPP375/CPP378 (forward and reverse primers had SalI and PstI cleavage sites, respectively) and cloned into pCR-XL-TOPO (Invitrogen, Carlsbad, CA) to create pDP70, pDP71, and pDP72, respectively (Tables 1 and 2). The SalI-PstI fragments from pDP70, pDP71, and pDP72 were cloned between the SalI and PstI sites in pMRS127 to create *spmA-gusA*, *spmB-gusA*, and *dacB-gusA* fusion constructs in pDP73, pDP74, and pDP75, respectively. These plasmids were introduced into *C. perfringens* SM101 by electroporation (7), and erythromycin-resistant (Em^r; 50 μg/ml) transformants were selected. The SM101 transformants carrying the plasmids with the *spmA-gusA*, *spmB-gusA*, and *dacB-gusA* fusions were grown in TGY (3% trypticase soy, 2% glucose, 1% yeast extract, 0.1% L-cysteine) vegetative (14) and Duncan-Strong (DS) sporulation (8) medium and assayed for β-glucuronidase (GUS) activity as described previously (11, 33).

Construction of a *C. perfringens spmAB* mutant. To isolate a derivative of *C. perfringens* SM101 with an insertion of *catP* giving chloramphenicol resistance (Cm^r; 20 μg/ml) in the *spmAB* operon, an *spmAB* mutator plasmid was constructed as follows. A 3,157-bp fragment carrying the *spmAB* operon plus 1,196 bp upstream of *spmA* and 851 bp downstream of *spmB* was PCR amplified by using primers CPP220/CPP221 (Table 2). The ~3.2-kb PCR fragment was cloned into pCR-XL-TOPO (Invitrogen) in *E. coli*, giving plasmid pMS1. This plasmid was digested with BglII, which cuts only once within the *spmA* open reading frame (ORF); the ends were filled; and the ~1.3-kb SmaI-NaeI *catP* gene from plasmid pJIR418 (46) was inserted, giving plasmid pMS2. This plasmid was digested with KpnI and XhoI, and the ~4.5-kb KpnI-XhoI fragment was cloned into pMRS104 digested with KpnI and SalI to create pMS3. This latter plasmid contains an inactivated *spm* operon, and since it contains no *C. perfringens* origin of replication, cannot replicate in this host. Plasmid pMS3 was introduced into *C. perfringens* strain SM101 by electroporation (7), and an *spmAB* mutant, strain NM101, was selected by allelic exchange as described previously (35). The replacement of wild-type *spmA* with the mutant allele in strain NM101 and the loss of the plasmid from this strain were confirmed by PCR and Southern blot analyses (data not shown).

Construction of a *C. perfringens dacB* mutant. To isolate a derivative of *C. perfringens* SM101 with an insertion of *catP* in the *dacB* ORF, a *dacB* mutator plasmid was constructed as follows. A 3,405-bp fragment carrying the *dacB* ORF plus 1,330 bp upstream and 1,199 bp downstream was PCR amplified with primers CPP222/CPP223 (Table 2). The ~3.4-kb PCR fragment was cloned into pCR-XL-TOPO (Invitrogen) in *E. coli*, giving plasmid pMS4. This plasmid was digested with NdeI, which cuts only once within the *dacB* ORF; the ends were filled; and the ~1.3-kb SmaI-NaeI *catP* gene from plasmid pJIR418 (46) was inserted, giving plasmid pNM13. This plasmid was digested with KpnI and XhoI, and the ~4.7-kb KpnI-XhoI fragment was cloned into pMRS104 digested with KpnI and SalI to give plasmid pNM14. This latter plasmid contains an inactivated *dacB* gene, and since it contains no *C. perfringens* origin of replication, cannot replicate in this host. Plasmid pNM14 was introduced into *C. perfringens* strain SM101 by electroporation (7), and a *dacB* mutant, strain NM102, was selected by allelic exchange as described previously (35). The expected genomic structure in the *dacB* region of strain NM102 was confirmed by PCR and Southern blot analyses (data not shown).

Construction of complemented strains. An ~1.7-kb fragment containing the *spmA* and *spmB* ORFs plus 460 bp upstream of *spmA* was PCR amplified by using primers CPP216/CPP217 (Table 2) and cloned into pCR-XL-TOPO (Invitrogen), giving pDP23. The ~1.7-kb KpnI-XhoI fragment of pDP23 was cloned between the KpnI and SalI sites of plasmid pJIR751 (1) to create the *spmAB*-complementing plasmid pDP24. A 3,405-bp fragment carrying the *dacB* ORF plus 1,330 bp upstream and 1,199 bp downstream was PCR amplified by using primers CPP222/CPP223 (Table 2) and cloned into pCR-XL-TOPO (Invitrogen), giving pMS4. The ~3.4-kb KpnI-XhoI fragment of pMS4 was then cloned between the KpnI and SalI sites of plasmid pJIR751 (1) to create the *dacB*-complementing plasmid pDP52. Plasmids pDP24 and pDP52 were introduced into *C. perfringens* strains NM101 and pNM102, respectively, by electroporation (7), and Em^r transformants were selected. The presence of plasmids pDP24 and pDP52 in NM101(pDP24) and NM102(pDP52), respectively, was confirmed by PCR (data not shown).

Spore preparation and purification. Starter cultures (10 ml) of *C. perfringens* strains were prepared by overnight growth at 37°C in fluid thioglycolate broth (FTG) (Difco) as described previously (14). Sporulating cultures of *C. perfringens* were prepared by inoculating 0.2 ml of an FTG starter culture into 10 ml of DS sporulation medium (8), and this culture was incubated for 24 h at 37°C to form spores as confirmed by phase-contrast microscopy. Spore preparations 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 at a final optical density at 600 nm (OD_{600}) of ~6; and stored at −20°C (27).

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic	Source/reference
C. perfringens strains		
SM101	Electroporatable derivative of type A food-poisoning isolate NCTC8798; carries a chromosomal *cpe*	51
NM101	*spmAB*::*catP*	This study
NM102	*dacB*::*catP*	This study
NM101(pDP24)	*spmAB* mutant expressing wild-type *spmAB*	This study
NM102(pDP52)	*dacB* mutant expressing wild-type *dacB*	This study
SM101(pDP73)	Wild-type strain carrying *spmA-gusA* fusion	This study
SM101(pDP74)	Wild-type strain carrying *spmB-gusA* fusion	This study
SM101(pDP75)	Wild-type strain carrying *dacB-gusA* fusion	This study
Plasmids		
pJIR418	*C. perfringens*/*E. coli* shuttle vector; Cm^r Em^r	46
pMRS127	*C. perfringens*/*E. coli* shuttle vector which encodes erythromycin resistance (Em^r), and has a promoterless *gusA*	33
pJIR751	*C. perfringens*/*E. coli* shuttle vector; Em^r	1
pMRS104	Carries no origin of replication for *C. perfringens*; Em^r	12
pMS1	~3.1-kb PCR fragment containing *spmAB* operon in pCR-XL-TOPO	This study
pMS2	~1.3-kb NaeI-SmaI *catP* fragment from pJIR418 cloned into the BglII site in the *spmA* ORF in pMS1	This study
pMS3	~4.4-kb KpnI-XhoI fragment from pMS2 in pMRS104	This study
pMS4	~3.4-kb PCR fragment containing *dacB* ORF in pCR-XL-TOPO	This study
pNM13	~1.3-kb NaeI-SmaI *catP* fragment from pJIR418 cloned into the NdeI site in the *dacB* ORF in pMS4	This study
pNM14	~4.7-kb KpnI-XhoI fragment from pNM13 cloned into pMRS104	This study
pDP23	~1.7-kb PCR fragment containing *spmAB* operon and upstream region in pCR-XL-TOPO	This study
pDP24	~1.7-kb KpnI-XhoI fragment containing *spmAB* operon and its upstream region between the KpnI-SalI sites in pJIR751	This study
pDP52	~3.2-kb KpnI-XhoI fragment containing *dacB* and its upstream region from pMS4 between the KpnI-SalI sites in pJIR751	This study
pDP70	526-bp PCR fragment containing upstream region of *spmA* cloned into pCR-XL-TOPO	This study
pDP71	432-bp PCR fragment containing upstream region of *spmB* cloned into pCR-XL-TOPO	This study
pDP72	489-bp PCR fragment containing upstream region of *dacB* cloned into pCR-XL-TOPO	This study
pDP73	526-bp SalI-PstI fragment carrying *spmA* promoter region cloned into pMRS127 to create an *spmA-gusA* fusion construct	This study
pDP74	432-bp SalI-PstI fragment carrying *spmB* promoter region cloned into pMRS127 to create an *spmB-gusA* fusion construct	This study
pDP75	489-bp SalI-PstI fragment carrying *dacB* promoter region cloned into pMRS127 to create a *dacB-gusA* fusion construct	This study

Measurement of spore properties. Spore core DPA content was determined by incubating 1 ml of spores at an OD_{600} of 6 in a water bath at 100°C for 60 min, cooling the sample on ice, centrifuging for 5 min, and measuring the DPA in the supernatant fluid as described previously (34). For the determination of spore core wet densities by equilibrium density gradient centrifugation (17), spore coats were first removed by the extraction of 3 to 5 mg dry weight of spores with 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 then spores were washed three times with 150 mM NaCl and twice with water (17, 30). Decoated spores were suspended in 100 μl of 30% Histidenz (Nycodenz) (Sigma, St. Louis, MO), incubated for 60 min on ice, and loaded into the top of a 2-ml linear gradient of 51 to 70% Histidenz in ultraclear tubes, and the tubes centrifuged for 45 min at 11,200 × g and 20°C in a swinging bucket rotor in a Beckman TL-100 ultracentrifuge. Spore core water content was calculated according to the formula $y = -0.00254x + 1.460$ (17), where y is the spore core wet density and x is the core water content in g per 100 g of wet protoplast (core).

Measurement of spore resistance. The resistance of *C. perfringens* spores to moist heat was determined as previously described (33, 36). Briefly, 10 ml of

TABLE 2. Primers used in this study

Primer name	Primer sequence[a]	Position[b]	Gene	Use(s)[c]
CPP216	ATTAGGTTTAACAGGAGTTTGG	−460 to −438	*spmAB*	CP
CPP217	CTGGAATATATTGTATTATTTCTGTA	+1190 to +1216	*spmAB*	CP
CPP220	CTGCAGTAATGGTGCTTGGA	−1196 to −1176	*spmAB*	MP
CPP221	AGCTCTTGGATGTGGTGAAAA	+1940 to +1961	*spmAB*	MP
CPP222	TTACTCCCTGCGAAGTAAGAAT	−1330 to −1308	*dacB*	MP, CP
CPP223	TTTCTAGGATCACTTTGCACCT	+2053 to +2075	*dacB*	MP, CP
CPP376	GCGTCGACATTAGGTTTAACAGGAGTTTGG	−460 to −438	*spmA*	GUS
CPP373	GCTGCAGGCTAAAAATCAATCCTAAGGC	+30 to +51	*spmA*	GUS
CPP374	GCGTCGACGGAGAGTTCTCATAGTGAC	−351 to −332	*spmB*	GUS
CPP377	GCTGCAGTCCCTTAAACATTCCATAGACT	+44 to +66	*spmB*	GUS
CPP375	GCGTCGACATAATGGTAAGGTTAGATGGAG	−386 to −364	*dacB*	GUS
CPP378	GCTGCAGTACAACTTTCCTTTCCAAGAAC	+66 to +88	*dacB*	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] GUS, construction of plasmid for GUS assays; CP, construction of complementing plasmid; MP, construction of mutator plasmid.

24-hr-grown DS medium cultures of *C. perfringens* strains were heat treated at 75°C for 20 min to kill vegetative cells. An aliquot of each heat-treated DS medium culture was serially diluted in phosphate-buffered saline (140 mM NaCl–25 mM Na_2HPO_4 [pH 7.0]), plated on brain heart infusion (BHI) agar, and incubated anaerobically for 24 h at 37°C to determine initial CFU/ml; these values were routinely 10^6 to 10^7. 75°C-treated DS medium cultures were then immediately heated at 100°C for various times (20, 40, and 60 min), and aliquots of appropriate dilutions were plated and incubated anaerobically for 24 h at 37°C as described above. Plots of CFU/ml versus time at 100°C were used to determine decimal reduction times ($D_{100°}$ values), which are the time cultures need to be kept at 100°C to get a 90% reduction in CFU/ml. All experiments measuring spore moist-heat resistance were repeated at least three times.

The resistance of *C. perfringens* spores to chemicals was determined as previously described (26). Briefly, spore suspensions at an OD_{600} of ~1 (~4×10^7 spores/ml) were (i) treated with 2 M hydrogen peroxide (Mallinckrodt Baker, Inc., Phillipsburg, NJ) at room temperature (20°C), and aliquots were neutralized with catalase (Sigma) as described previously (40); (ii) treated with 300 mM HCl at room temperature, and aliquots were diluted 100-fold in 25 mM in Na_2HPO_4 (pH 7.0); (iii) treated with 400 mM $NaNO_2$–400 mM Na acetate (pH 4.5) at room temperature, and aliquots were diluted 10-fold in 500 mM Na_2HPO_4 (pH 8.5); or (iv) treated with 25 g liter^{-1} 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. For the analysis of spore killing, untreated and treated spores were serially diluted in phosphate-buffered saline and plated on BHI agar and the plates were incubated anaerobically for 24 h at 37°C.

The UV resistance of *C. perfringens* spores was determined as described previously (30, 32). Briefly, purified *C. perfringens* spores at an OD_{600} of 2 (~8×10^7 spores/ml) were diluted 100-fold in 25 mM Na_2HPO_4 buffer (pH 6.8) and UV irradiated at 254 nm with a UVGL-25 Mineralight lamp (UVP, Inc., Upland, CA) for various times (0.5, 1.0, 2.5, and 5.0 min). Appropriate dilutions were spread onto BHI plates and incubated as described above prior to the assessment of colony formation.

Statistical analyses. Student's *t* test was used for statistical analyses.

RESULTS

Identification of putative *C. perfringens dacB* and *spmAB* genes. In *B. subtilis*, a tricistronic operon (Fig. 1A) encodes proteins involved in determining both the degree of cortex PG cross-linking (DacB) and the spore's core water content (SpmA and SpmB); all three genes are expressed only during sporulation (29, 30). When we scanned the *C. perfringens* SM101 genome (22) by BLASTP analysis, three ORFs (CPR1770, CPR2541, and CPR2542) with high similarity to *B. subtilis dacB*, *spmA*, and *spmB* were found (Fig. 1A). However, these genes are organized differently in *C. perfringens* (Fig. 1A), with CPR1770 being monocistronic and located ~800 kb upstream of a bicistronic operon containing CPR2541 and CPR2542 (Fig. 1A). This genetic organization is not unique to *C. perfringens*, as *dacB* is also monocistronic and located hundreds of kb from *spmAB* in the *Clostridium acetobutylicum*, *Clostridium difficile*, *Clostridium novyi*, and *Clostridium tetani* genomes (3, 4, 24, 38).

Analysis of the amino acid sequence of *C. perfringens* CPR1770 revealed a high similarity (62%) with *B. subtilis* DacB (47). Despite the absence of 114 residues from the carboxy terminus of CPR1770 present in *B. subtilis* DacB, four conserved regions typical of PBPs are present in the *C. perfringens* protein (data not shown). Near the amino terminus, a highly hydrophobic region probably facilitates anchoring the protein to the outer membrane during sporulation (9). The other well-conserved regions are a catalytic serine domain (S-X-X-K), an S-X-N domain typical of type A β-lactamases (48), and a K-T-G sequence that is essential for the tertiary structure of the active site (13).

The first gene in the bicistronic operon, CPR2542 (Fig. 1A), encodes a 192-amino-acid-residue protein that is highly similar to *B. subtilis* SpmA (62%), with an estimated molecular mass of 20.4 kDa and four putative transmembrane alpha-helical domains (TMH). The second gene, CPR2541 (Fig. 1A), encodes a protein that is highly similar to *B. subtilis* SpmB (62%), with 172 residues, a molecular mass of 18.8 kDa, and five TMH. The high number of TMH in both putative *C. perfringens* Spm proteins suggests that they may be anchored in either of the forespore membranes as suggested for *B. subtilis* DacB (28).

Evaluation of the expression of *C. perfringens dacB*, *spmA*, and *spmB*. To assess whether the *C. perfringens spm* and *dacB* genes are expressed during sporulation, 300 to 400 bp upstream of each gene's coding sequence, containing the putative promoter region of each gene, was fused to *E. coli gusA* and GUS activity was measured after the fusions were introduced into *C. perfringens* SM101. When vegetative cells of strain SM101 carrying pDP73 (*spmA-gusA*), pDP74 (*spmB-gusA*), and pDP75 (*dacB-gusA*) were assayed for GUS activity, no significant expression of *spmA*, *spmB*, or *dacB* was observed (Fig. 1B). However, sporulating cultures carrying *spmA-gusA* and *dacB-gusA* exhibited significant GUS activity, although no GUS activity was detected in sporulating cultures carrying *spmB-gusA* (Fig. 1B), consistent with *spmB* being the second gene in an operon with *spmA*. The expression of *spmA-gusA* began ~2 h after the initiation of sporulation and reached a maximum specific activity ~4 to 6 h later (Fig. 1B). The expression of *dacB-gusA* also began ~2 h after the induction of sporulation and reached a maximum specific activity 4 to 8 h later (Fig. 1B). Collectively, these results suggest that the *C. perfringens spm* and *dacB* genes are expressed only during sporulation and led us to hypothesize that their products might be involved in resistance of *C. perfringens* spores, as they are in spores of *B. subtilis* (28).

Effect of *spmAB* mutation on spore properties. Studies with *B. subtilis* indicate that the spore core water content is determined during sporulation at least in part by the SpmA and SpmB proteins (29, 30). To assess whether SpmA and -B have any role in *C. perfringens* spore core water content and thus, perhaps, spore moist-heat resistance, we constructed an insertion mutation in *spmA*, giving strain NM101. Since no *spmB-gusA* activity was detected in sporulating SM101 cells (Fig. 1B), it is most likely that disruption of *spmA* has a polar effect on the downstream *spmB* and thus, strain NM101 in effect carries both *spmA* and *spmB* mutations. As expected, the moist-heat resistance of NM101 spores was significantly lower ($P < 0.01$) than that of the wild-type spores, as the D_{100} value for NM101 spores was twofold lower than for SM101 spores (Fig. 2; Table 3). Although the levels of DPA in NM101 and SM101 spores were similar, the core wet density of NM101 spores was significantly lower than that of SM101 spores and thus, NM101 spores have a higher core water content (Table 3). The moist-heat resistance and core water content of NM101 spores were restored to wild-type levels when strain NM101 was complemented with wild-type *spmAB* (Fig. 2; Table 3). These results suggest that SpmAB proteins are involved in the determination of *C. perfringens* spore core water content and further indicate that *C. perfringens* spores with higher water content have lower

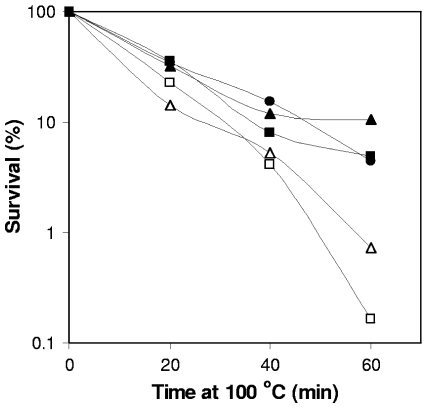

FIG. 2. Thermal-death curves of spores of various *C. perfringens* strains. Spores of strains SM101 (wild type; filled circles), NM101 (*spmAB*; open squares), NM101(pDP24) (*spmAB* strain complemented with wild-type *spmAB*; filled squares), NM102 (*dacB*; open triangles), and NM102(pDP53) (*dacB* strain complemented with wild-type *dacB*; filled triangles) were heated at 100°C for various times, and spore survival was determined as described in Materials and Methods. The data are the results from a representative experiment.

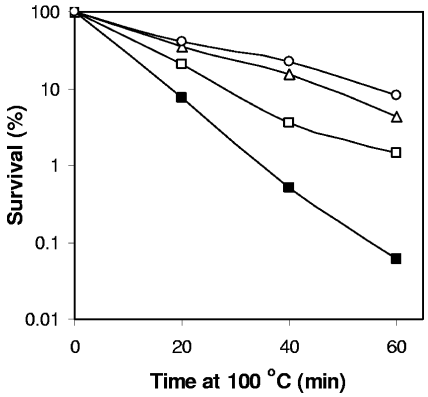

FIG. 3. Effect of sporulation temperature on thermal-death curves of *C. perfringens* spores. SM101 (wild type) spores were prepared at 26°C (filled squares), 32°C (open squares), 37°C (open triangles), and 42°C (open circles) and heated at 100°C for various times, and spore survival was determined as described in Materials and Methods. The data are the results from a representative experiment.

moist-heat resistance, in agreement with the results of studies with *B. subtilis* spores (30).

Effect of *dacB* mutation on spore properties. Another factor that has been suggested to be important in the moist-heat resistance of *B. subtilis* spores is the cortex PG structure, specifically, the degree of cortex PG cross-linking that is determined primarily by the D,D-carboxypeptidase DacB (29). To test the role of DacB in *C. perfringens* spore resistance, we constructed an insertion mutation in *dacB*, giving strain NM102. As expected, the moist-heat resistance of NM102 spores was significantly lower than that of SM101 spores, although the levels of DPA in both wild-type and *dacB* spores were almost identical (Fig. 2; Table 3). The core wet density of *dacB* spores was also similar to that of SM101 spores (Table 3). Again, the defects in strain NM102 were complemented with wild-type *dacB* [strain NM102(pDP52)] (Fig. 2; Table 3).

TABLE 3. Effects of *spmAB* and *dacB* mutations on *C. perfringens* spore properties

Strain (genotype)	Heat resistance ($D_{100°}$ [min])[a]	Amt of DPA (μg/ml/ OD_{600})[b]	Core wet density (g/ml)[c]	Water content (g/100 g of wet protoplast)[d]
SM101 (wild type)	49.1 ± 6	16.7	1.378	32.4
NM101 (*spmAB*::*catP*)	19.2 ± 3	16.4	1.370	35.4
NM101(pDP24)	45.2 ± 2	ND[e]	1.382	30.7
NM102 (*dacB*::*catP*)	28.8 ± 1	17.2	1.382	30.7
NM102(pDP52)	52.2 ± 7	ND	1.383	30.3

[a] Values are averages ± standard deviations of determinations for three spore preparations.
[b] Values are averages of determinations for three different spore preparations.
[c] Values are averages of determinations for two to four different spore preparations, and the standard deviation was ± 0.001 g/ml.
[d] Spore core water contents were calculated as described in Materials and Methods.
[e] ND, not determined.

Effect of sporulation temperature on *C. perfringens* spore heat resistance and core water content. An additional factor that can significantly influence the core water content and moist-heat resistance of *B. subtilis* spores is the sporulation temperature, with higher sporulation temperatures giving spores with lower core water content and higher moist-heat resistance (10, 21), although how the sporulation temperature affects the core water content and moist-heat resistance of these spores is not known. *C. perfringens* spores of strains SM101 (wild type), NM101 (*spmAB*), and NM102 (*dacB*) were prepared at different sporulation temperatures, and the spore core water content and moist-heat resistance were determined. In agreement with results with *B. subtilis* spores (10, 21), *C. perfringens* wild-type spores prepared at higher temperatures exhibited higher resistance to moist heat and had a lower core water content (Fig. 3; Table 4). This trend was also observed for *spmAB* and *dacB* spores (Table 4). However, there was no obvious quantitative relationship between the precise core water content and the moist-heat resistance of the spores of these different strains (Table 4), suggesting that additional factors besides core water content influence the moist-heat resistance of *C. perfringens* spores and that these unknown factors are in turn influenced in some way by DacB and SpmAB.

Effects of *spmAB* and *dacB* mutations on chemical resistance of *C. perfringens* spores. To gain insight into the roles of SpmAB and DacB in *C. perfringens* spore resistance to treatments other than heat, we also measured the resistance of *spmAB* and *dacB* spores to a variety of chemicals. Previous work indicated that *B. subtilis* spores with an altered cortex and higher core water content have lower hydrogen peroxide resistance than wild-type spores (20, 30). In contrast, *C. perfringens spmAB*, *dacB*, and wild-type spores had similar hydrogen peroxide resistance (Fig. 4A), suggesting that low core water content and a wild-type cortex PG structure have no significant role in *C. perfringens* spore resistance to hydrogen peroxide. This suggestion is consistent with the results of previous work indicating that α/β-type SASP are a major factor in hydrogen peroxide resistance of *B. subtilis* and *C. perfringens* spores (26, 44). The *spmAB*, *dacB*, and wild-type spores also exhibited

TABLE 4. Effect of sporulation temperature on *C. perfringens* spore properties

Strain (genotype)	D_{100° values of spores prepared at[a]:				Water content of spores prepared at[b]:			
	26°C	32°C	37°C[c]	42°C	26°C	32°C	37°C[c]	42°C
SM101 (wild type)	15 ± 3	26 ± 4	49 ± 6	54 ± 4	39.0	38.6	32.4	30.7
NM101 (*spmAB*)	12 ± 2	12 ± 0	19 ± 3	34 ± 2	36.2	36.2	35.4	31.3
NM102 (*dacB*)	8 ± 2	12 ± 2	29 ± 1	71 ± 6	39.0	36.6	30.7	33.3

[a] Spores of various strains were prepared at different sporulation temperatures, and heat resistance was determined and expressed as D_{100° values as described in Materials and Methods. Values are averages ± standard deviations of the results for three spore preparations.

[b] Spore core wet densities were determined for spores of various strains prepared at different temperatures, and core water contents were calculated as described in Materials and Methods. Values presented are averages of determinations for two different spore preparations.

[c] The 37°C data are from Table 1.

similar resistance to HCl (Fig. 4B), a chemical that kills *B. subtilis* spores by somehow disrupting the inner-membrane permeability barrier (39).

The other two chemicals tested, formaldehyde and nitrous acid, kill *B. subtilis* spores at least in large part by DNA damage, and α/β-type SASP are very important in spore resistance to these agents (18, 49). Wild-type and *spmAB C. perfringens* spores exhibited no difference in formaldehyde resistance, although *dacB* spores were slightly more sensitive (Fig. 4C). Surprisingly, *spmAB* and *dacB* spores were much more sensitive ($P < 0.01$) than wild-type spores to nitrous acid (Fig. 4D). However, wild-type levels of nitrous acid resistance were restored to spores of strains NM101 (*spmAB*) and NM102 (*dacB*) when the latter strains were complemented with wild-type *spmAB* and *dacB* genes, respectively (Fig. 4D). These results clearly indicate that in addition to α/β-type SASP (26), core water content and cortex PG structure are also likely to be major determinants in the nitrous acid resistance of *C. perfringens* spores.

Effect of *spmAB* and *dacB* mutations on UV resistance of *C. perfringens* spores. Previous work (42) has shown that with *B. subtilis* spores, binding of α/β-type SASP to spore DNA is the predominant factor in spore resistance to UV radiation, a treatment commonly used to sterilize surfaces in the food

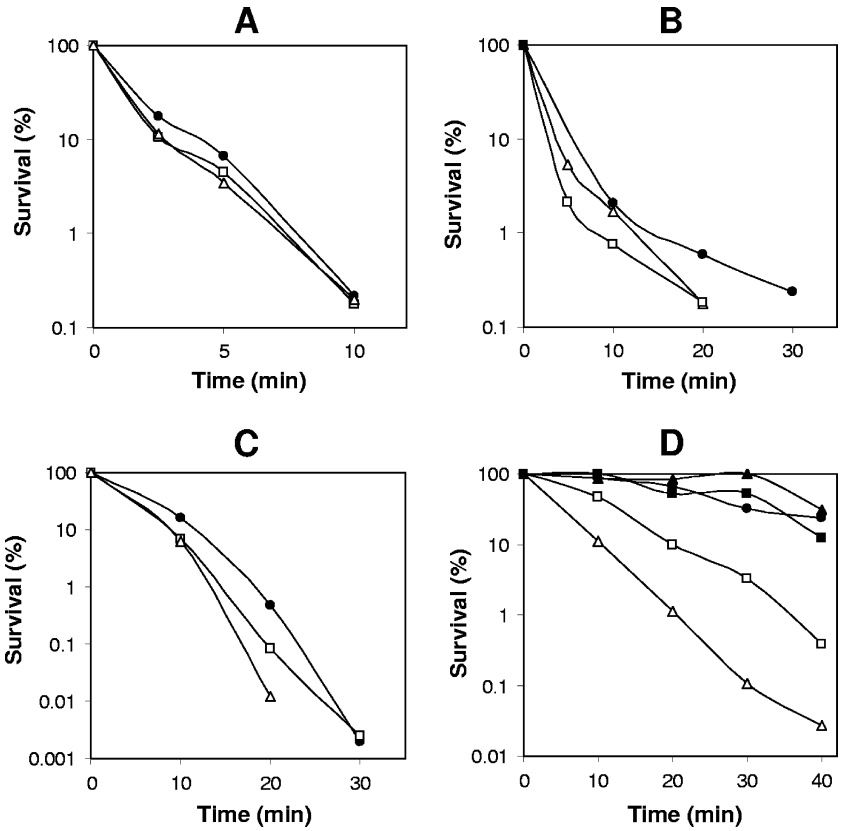

FIG. 4. Resistance of spores of various *C. perfringens* strains to hydrogen peroxide (A), hydrochloride acid (B), formaldehyde (C), and nitrous acid (D). Spores of strains SM101 (wild type; filled circles), NM101 (*spmAB*; open squares), NM101(pDP2) (*spmAB* strain complemented with wild-type *spmAB*; filled squares), NM102 (*dacB* mutant; open triangles), and NM102(pDP53) (*dacB* strain complemented with wild-type *dacB*; filled triangles) were purified, and their survival after various treatments was determined as described in Materials and Methods. The variability in survival values in these experiments was ±15%.

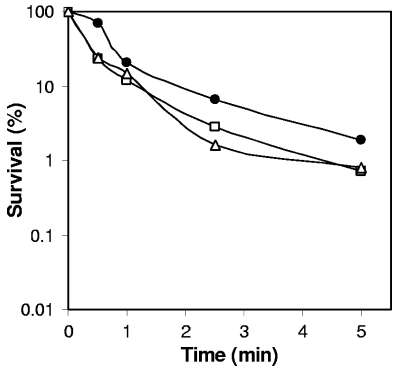

FIG. 5. UV resistance of spores of various *C. perfringens* strains. Spores of strains SM101 (wild type; filled circles), NM101 (*spmAB*; open squares), and NM102 (*dacB*; open triangles) were purified, and their survival after UV treatment was determined as described in Materials and Methods.

industry. *C. perfringens* spores with decreased levels of α/β-type SASP are also more sensitive to UV treatment than the parental wild-type spores (32). Consistent with the predominant role for α/β-type SASP in spore UV resistance, spores of wild-type, *spmAB*, and *dacB C. perfringens* strains showed essentially identical UV resistance (Fig. 5), as was found previously for *B. subtilis* spores lacking SpmAB and DacB (30).

DISCUSSION

Dormant spores of *C. perfringens* type A food poisoning isolates are the causative agent of type A *C. perfringens*-caused food-borne illness. These spores have high heat resistance that favors spore survival during inadequate cooking or holding temperatures (19, 36). Consequently, an understanding of the molecular basis of *C. perfringens* spore resistance is crucial to the development of better strategies for spore inactivation. In this communication, we report a number of observations leading to new conclusions about the mechanisms of *C. perfringens* spore resistance to a variety of agents, an area that has generally received very little study. While many of these conclusions reinforce previous conclusions about the mechanisms of resistance of spores of *Bacillus* species, primarily *B. subtilis*, there are some significant differences in the details of the resistance of spores of *C. perfringens* and *B. subtilis*.

One straightforward conclusion is that *dacB* and *spmAB* are sporulation genes in both *B. subtilis* and *C. perfringens*, and the products of these genes have very similar effects on spore properties in these two species. In *B. subtilis*, the mutation of *spmA* and *-B* or *spmB* alone results in a significant increase in spore core wet density and an ~eightfold decrease in the $D_{90°}$ value for moist-heat killing (29). The effects of the *spmAB* mutation on *C. perfringens* spores are similar, albeit of lower magnitude (~twofold decrease in the $D_{100°}$). In *B. subtilis*, an in-frame *dacB* mutation has no effect on core water content but does cause an ~fivefold decrease in the $D_{90°}$ value (28, 29). Again, the effects of a *dacB* mutation on *C. perfringens* spores are similar, although the decrease in the $D_{100°}$ value in *dacB C. perfringens* spores is smaller than the effect of a *dacB* mutation on $D_{90°}$ values for *B. subtilis* spores (28–30). A *dacB* mutation also has a large effect on *B. subtilis* spore cortex PG structure, as cortex PG cross-linking increases two- to fourfold, since DacB is by far the major D,D-carboxypeptidase affecting the degree of cross-linking (28). We have not examined the cross-linking of cortex PG in *C. perfringens* wild-type and *dacB* spores but would suggest that by analogy with results in *B. subtilis*, *C. perfringens dacB* spores will also exhibit a significantly higher degree of cortex PG cross-linking than wild-type spores.

A second significant conclusion is that neither the level of core water nor, most likely, the degree of cortex cross-linking plays any role in *C. perfringens* spore UV resistance, as is also the case with *B. subtilis* spores (28, 30). The UV resistance of *B. subtilis* spores is due almost exclusively to the saturation of spore DNA with α/β-type SASP, proteins that are also present in *C. perfringens* spores (42, 44). Presumably, small changes in core water content do not affect α/β-type SASP-DNA binding appreciably and thus do not affect spore UV resistance. Spore core water content and, probably, the degree of cortex PG cross-linking also play no role in *C. perfringens* spore resistance to formaldehyde, as is also the case at least for changes in core water content in *B. subtilis* spores (21). Again, the saturation of spore DNA with α/β-type SASP is a major factor in spore resistance to formaldehyde (26, 44).

A third conclusion is that the lack of appreciable effects of core water content and, probably, the degree of cortex cross-linking on *C. perfringens* spore hydrogen peroxide resistance are consistent with α/β-type SASP binding as a major factor protecting *C. perfringens* spore DNA from this reagent (26). An in-frame *dacB* mutation giving increased cortex PG cross-linking also has only a minimal effect on *B. subtilis* spore resistance to hydrogen peroxide (30). However, *spmAB* mutations decreased *B. subtilis* spore resistance to hydrogen peroxide significantly (30). We do not understand the reason for this difference between the spores of these two species. However, perhaps the α/β-type SASP in *C. perfringens* are more effective in preventing DNA damage due to hydrogen peroxide than in *B. subtilis* spores, such that the effects of changes in core water content on the hydrogen peroxide resistance of *C. perfringens* spores are minimal. Another explanation is that perhaps the structure of the complex between α/β-type SASP and DNA is somewhat different in *B. subtilis* and *C. perfringens* spores and this alters DNA protection against hydrogen peroxide in these two types of spores. While the amino acid sequences of α/β-type SASP from *B. subtilis* and *C. perfringens* are quite similar, they do have a significant difference in the spacing between the two most-highly conserved structural elements (41). Since the structure of DNA bound to a *B. subtilis* α/β-type SASP has now been determined (16), it may be possible to use this structure to model the effects of a *C. perfringens* α/β-type SASP on DNA structure and properties.

A fourth conclusion is that it appears that core water content and, likely, the degree of cortex PG cross-linking play major roles in determining *C. perfringens* spore resistance to nitrous acid. While this may initially seem surprising, since α/β-type SASP-DNA binding is a major factor in *B. subtilis* and *C. perfringens* spore resistance to this agent (26, 41), *B. subtilis* spores made at higher temperatures that have the lowest core water content are significantly more nitrous acid resistant than spores made at low temperatures that have the highest core water content (21). However, the low permeability of the spore's inner membrane is also an important factor in *B. sub-*

tilis spore resistance to nitrous acid, and this membrane's permeability decreases significantly in spores made at higher temperatures (6). It is also possible that there are significant differences in the structure of DNA when saturated with α/β-type SASP from *B. subtilis* and *C. perfringens*, as noted above, and perhaps these differences result in different protection of DNA against nitrous acid in the complexes formed with the proteins of the two species. Clearly, more work is needed with *C. perfringens* spores to identify all factors involved in spore resistance to nitrous acid.

The final and perhaps the most-significant conclusion is that core water content is a major factor in *C. perfringens* spore resistance to moist heat, as is also the case with *B. subtilis* spores (10, 44). Presumably a higher core water content results in more-rapid inactivation of one or more key proteins in the spore core whose loss results in spore death, as recent work has provided strong evidence that it is through core protein inactivation that moist heat kills *B. subtilis* spores (5). The effects of core water content on *C. perfringens* spore moist-heat resistance appear to be relatively independent of the cause of alterations in core water content, whether via *spmAB* mutations or sporulation at different temperatures. However, factors in addition to core water content can be affected by sporulation temperature and the *spmAB* mutations, as the response of *C. perfringens spmAB* and *dacB* spore resistance to sporulation temperature is quantitatively different than that of wild-type spores. Thus, while it is clear that core water content is a major factor in determining *C. perfringens* spore resistance to moist heat, this is clearly not the only factor. As noted above, α/β-type SASP-DNA binding is one additional factor in *C. perfringens* spore moist-heat resistance (32, 33), and it appears likely that the degree of cortex PG cross-linking is an additional factor, as *dacB* spores had lower moist-heat resistance than wild-type spores, although both had similar core water contents. It has been suggested for spores of *B. subtilis* (28) that more-loosely cross-linked PG in the spore cortex may be less able to maintain the spore core's low water content upon heating at elevated temperatures, and we suggest that the same is true with *C. perfringens* spores. However, data on the degree of cross-linking of cortex PG in spores of these various strains is needed before this conclusion can be made definitive.

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