Antisense-RNA-Mediated Decreased Synthesis of Small, Acid-Soluble Spore Proteins Leads to Decreased Resistance of *Clostridium perfringens* Spores to Moist Heat and UV Radiation[▽]

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Previous work has suggested that a group of α/β -type small, acid-soluble spore proteins (SASP) is involved in the resistance of *Clostridium perfringens* spores to moist heat. However, this suggestion is based on the analysis of *C. perfringens* spores lacking only one of the three genes encoding α/β -type SASP in this organism. We have now used antisense RNA to decrease levels of α/β -type SASP in *C. perfringens* spores by ~90%. These spores had significantly reduced resistance to both moist heat and UV radiation but not to dry heat. These results clearly demonstrate the important role of α/β -type SASP in the resistance of *C. perfringens* spores.

Clostridium perfringens is a gram-positive, spore-forming, anaerobic bacterium that causes both histotoxic and gastrointestinal (GI) diseases in humans and animals (8, 10). *C. perfringens* isolates are classified into one of five types, types A through E (10, 11), based upon their ability to produce the four major lethal toxins, the alpha-, beta-, epsilon- and iota-toxins. The major lethal toxins, however, are not the only medically important toxins; some *C. perfringens* isolates (mostly those belonging to type A) produce *C. perfringens* enterotoxin (CPE). The CPE-producing type A isolates are important human GI pathogens, causing *C. perfringens* type A food poisoning as well as non-food-borne GI diseases (10, 19). In addition to producing CPE, *C. perfringens* food poisoning isolates have the ability to form spores that are extremely resistant to heat and other environmental stress factors (20), facilitating spore survival in primary food vehicles (e.g., meat and poultry products) for *C. perfringens* type A food poisoning (10).

The molecular basis for the resistance of *C. perfringens* spores to heat and other environmental stress factors remains unknown. However, the α/β -type small, acid-soluble spore proteins (SASP) play a major role in the resistance of *Bacillus subtilis* spores to heat, UV radiation, and peroxides (24). Previous studies (1, 2) have shown that the genomes of *Clostridium* species contain multiple genes (termed *ssp*) encoding α/β -type SASP, with three genes (*ssp1*, *ssp2*, and *ssp3*) in *C. perfringens* (13, 25). However, in contrast to *Bacillus* species, *Clostridium* species do not contain genes encoding the γ -type SASP (1). Evidence obtained recently has suggested that α/β -type SASP plays a role in the resistance of *C. perfringens* spores to moist heat, as spores of a strain with an *ssp3* deletion had slightly lower moist heat resistance than wild-type spores, and this defect was eliminated by complementing the *ssp3* mutant with

wild-type *ssp3* (18). However, the link between α/β -type SASP levels and spore heat resistance was tentative because only one of the three *ssp* genes in *C. perfringens* was deleted.

In order to fully explore the relationship between levels of α/β -type SASP and *C. perfringens* spore resistance, it would be necessary to generate a *C. perfringens* strain lacking all three *ssp* genes. However, since no technique is currently available to introduce multiple knockout mutations in a single *C. perfringens* strain, we have used an antisense RNA (asRNA) strategy to decrease the synthesis of α/β -type SASP in *C. perfringens*. asRNA molecules act by hybridizing with complementary mRNAs, and the RNA duplexes are then destroyed (3, 16), leading to the down-regulation of the expression of the targeted gene. asRNA strategies have been successfully used to down-regulate the production of enzymes in *Clostridium acetobutylicum* (3, 26, 27). Since the coding regions of the three *C. perfringens* *ssp* genes are very homologous (~90% identity), we have used *ssp2* asRNA to decrease the synthesis of α/β -type SASP in *C. perfringens*. Analysis of the resultant spores with low levels of α/β -type SASP provided clear evidence that these proteins play a major role in the resistance of *C. perfringens* spores to moist heat and UV radiation but not to dry heat.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and spore preparations. The bacterial strains and the plasmids used in this work are listed in Table 1. *C. perfringens* starter cultures (10 ml) were prepared by growth at 37°C in fluid thioglycolate broth (FTG) (Difco) overnight as described previously (7). For selecting *C. perfringens* transformants carrying recombinant plasmids, cultures were spread onto brain heart infusion (BHI) agar plates containing erythromycin (50 µg/ml) and incubated anaerobically at 37°C. Sporulating cultures of *C. perfringens* were prepared by inoculating 0.2 ml of an FTG starter culture into 10 ml of Duncan-Strong sporulation medium (7), which was incubated for 24 h at 37°C. The presence of spores in Duncan-Strong medium cultures was confirmed by phase-contrast microscopy. For purification of spores, the sporulating cultures were sonicated to kill the remaining vegetative cells, and the resultant spore suspension was cleared of debris by repeated washing with sterile distilled water until the spores were >95% pure as determined by microscopy. Purified spores (10⁶ to 10⁷ spores/ml) were routinely treated for 20 min at 75°C before use to inactivate contaminating vegetative and sporulating cells. Spores of *B. subtilis*

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TABLE 1. Bacterial strains and plasmids

Bacterial strain or plasmid	Relevant characteristic(s)	Reference or source
Bacterial strains		
SM101	Electroporatable derivative of food poisoning type A isolate, NCTC8798; carries a chromosomal <i>cpe</i>	29
DR101	<i>ssp3</i> derivative of SM101	18
SM101(pSG22)	Wild-type strain carrying control plasmid	This study
DR101(pSG22)	<i>ssp3</i> mutant carrying control plasmid	This study
SM101(pDR81)	Wild-type strain expressing <i>ssp2</i> asRNA	This study
DR101(pDR81)	<i>ssp3</i> mutant expressing <i>ssp2</i> asRNA	This study
Plasmids		
pSG22	<i>ssp2</i> promoter cloned in pMRS127; Em ^r	18
pDR80	165-bp <i>ssp2</i> fragment in pCR-XL-TOPO	This study
pDR81	165-bp PstI-HindIII fragment from pDR80 cloned in pSG22; Em ^r	This study
pDR13	<i>ssp2</i> plus 1.4 kb each of its upstream and downstream sequence cloned into pCR-XL-TOPO	18

strain JH42 were prepared by growth for ~72 h at 37°C on nutrient agar plates, and the spores were purified as described previously (14, 21).

Construction of plasmid pDR81 and *C. perfringens* strains. The amino acid sequences of the *C. perfringens* α/β -type SASP Ssp1, Ssp2, and Ssp3 are ~85% identical, and the nucleotide sequences also exhibit high sequence identity (Fig. 1A and B). A 165-bp fragment within the *ssp2* coding sequence was PCR amplified using plasmid pDR13 (18) as a template with primers CPP194 (5'-AAG CTTATGTCACAACATTTAGTACCAGAA-3') and CPP195 (5'-CTGCAGTT CTACCATCTTTTAACCATTTTC-3'), which had HindIII and PstI cleavage sites (underlined residues) at the 5' ends of the forward and reverse primers, respectively (Fig. 1B). The nucleotide sequence of this 165-bp *ssp2* fragment was 97% identical to that in the comparable region in *ssp3* and 86% identical to the sequence of this region in *ssp1* (Fig. 1B). Because of this latter sequence homology, it is possible that the *ssp2* asRNA not only could form RNA duplexes and cause the degradation of *ssp2* mRNA but could also cause the degradation of *ssp1* and *ssp3* mRNAs as well. Indeed, studies of *C. acetobutylicum* showed the efficient down-regulation of a targeted gene using asRNA molecules that were only 87% and 96% complementary to the targeted mRNA (3). The amplified *ssp2* DNA fragment was cloned into plasmid pCR-XL-TOPO using a PCR-XL-TOPO cloning kit (Invitrogen, Carlsbad, CA) to create plasmid pDR80. The 165-bp HindIII-PstI *ssp2* fragment from plasmid pDR80 was cloned between the HindIII and PstI sites of plasmid pSG22 (18) in the antisense orientation to the *ssp2* promoter region present in this construct, giving the *ssp2* asRNA plasmid pDR81 (Fig. 1B).

Plasmid pDR81 was introduced by electroporation (19) into the wild-type food poisoning *C. perfringens* strain SM101 and an isogenic strain, DR101, with a deletion of *ssp3* (18), and erythromycin-resistant (Em^r) (50 μ g/ml) transformants were selected. SM101 and DR101 transformants were designated strains SM101(pDR81) and DR101(pDR81), respectively. Plasmid pSG22 was also electroporated into strains SM101 and DR101 with selection for Em^r, giving strains SM101(pSG22) and DR101(pSG22), respectively.

SASP extraction and Western blotting. *C. perfringens* SASP was extracted from 50 mg (dry weight) of disrupted purified spores with dilute acid, the extracts were processed (1, 2, 18) and lyophilized, the dry residue was dissolved in 25 μ l of 8 M urea, and 10- μ l aliquots were run on polyacrylamide gels at low pH as described previously (14). The gel was stained with Coomassie brilliant blue (Bio-Rad Laboratories, Hercules, CA), or proteins on the gel were transferred onto a nitrocellulose membrane. The resultant Western blot was probed with antiserum against a *B. subtilis* α/β -type SASP termed SspC (1, 14), and the blot was developed for chemiluminescence detection (Pierce, Rockford, IL) to identify immunoreactive species. 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/ni-image/>), and values are given as mean band intensities.

Measurement of spore resistance. The resistance of *C. perfringens* spores to moist heat was determined as described previously (18, 20). Briefly, spores of *C. perfringens*, prepared and purified as described above, were serially diluted in FTG medium, plated onto BHI agar, and incubated anaerobically for 24 h at 37°C to determine the initial CFU/ml in the spore suspension. The initial

CFU/ml (after heat treatment at 75°C for 20 min to inactivate growing and sporulating cells) was 10^6 to 10^7 . The spore suspension was then heated at 100°C for various times, aliquots of appropriate dilutions were plated, and the numbers of colonies from spores that survived the 100°C treatment were determined as described above. Plots of CFU/ml versus time at 100°C then allowed the determination of decimal reduction times (*D* values), the times that a culture had to be kept at a given temperature to obtain a 90% reduction in CFU/ml.

The resistance of *C. perfringens* spores to dry heat was determined as described previously for spores of *B. subtilis* (17). Spores of *C. perfringens*, prepared as described above, were diluted to a concentration of ~ 10^6 spores/ml. One hundred microliters of this spore suspension was lyophilized in sterile glass tubes, and the lyophilized spores were heated in an oil bath at 120°C. At various times, tubes were removed from the oil bath and cooled, the spores were suspended in 1 ml sterile distilled water and sonicated for 30 s to disperse the spores, appropriate dilutions were plated onto BHI agar plates incubated as described above, and colonies were counted after 24 to 48 h. Using this method with *B. subtilis* spores, we obtained *D* values similar to those reported previously (17) (see Results).

The UV resistance of *C. perfringens* spores was also determined as described previously for *B. subtilis* spores (9, 14). *C. perfringens* spores, prepared as described above, were diluted 100-fold in 25 mM potassium phosphate buffer (pH 6.8) and UV irradiated at 254 nm with a UVGL-25 Mineralight lamp (UVP Inc., Upland, CA) for various times. Dilutions from various samples were spread onto BHI plates, and the plates were incubated as described above prior to assessments of colony formation.

RESULTS

α/β -Type SASP levels in spores of *C. perfringens* strains.

Initial work showed that the sporulation efficiencies of *C. perfringens* strains SM101 and DR101 that contained either plasmid pDR81 or pSG22 were similar (data not shown). An SASP extract from disrupted spores of strain SM101(pSG22) run on polyacrylamide gels at low pH gave only a tight group of protein bands, one of which was a major one (Fig. 2A), as seen previously (1, 18). A group of bands migrating similarly but of slightly lower intensity was also obtained with an extract from spores of strain DR101(pSG22) (Fig. 2A). Strikingly, the intensities of all the bands from SASP extracts on polyacrylamide gels at low pH, and, thus, total α/β -type SASP levels, were much lower in extracts from spores of strains SM101(pDR81) and DR101(pDR81) (Fig. 2A), and this was confirmed by densitometric analysis of gels from three independent experiments (Fig. 2C). The decreased α/β -type SASP levels in spores of strains expressing *ssp2* asRNA were confirmed by Western

FIG. 1. Alignments of sequences of Ssp proteins and *ssp* genes and construction of a plasmid containing *ssp2* in the antisense orientation to its promoter. (A) Amino acid sequences of *B. subtilis* SspC and Ssp1, Ssp2, and Ssp3 of *C. perfringens* are shown using the one-letter amino acid code. The symbols : and * below the sequences indicate residues that are conserved in all three *C. perfringens* proteins and residues conserved in the *B. subtilis* and the *C. perfringens* proteins, respectively. The dashes in the SspC sequence indicate the position of a gap that was introduced to maximize the sequence alignment with the *C. perfringens* proteins. (B) Alignment of nucleotide sequences of *C. perfringens ssp* genes. The symbols * and : below the sequences indicate nucleotides that are identical in all three sequences and nucleotides that are identical in *ssp2* and in either *ssp1* or *ssp3*, respectively. The alignment was performed using the ClustalW program and modified with Microsoft PowerPoint. CPP194 and CPP195 indicate forward and reverse primers, respectively, to amplify an internal *ssp2* fragment. (C) Construction of plasmid pDR81 containing the *ssp2* fragment in the antisense direction to the *ssp2* promoter (*ssp2* pro). *oriCP* and *oriEC* indicate origins of replication for *C. perfringens* and *E. coli*, respectively; *rep* indicates the replication gene from pIP404; *ermBP* indicates an Em^r gene; and *lacZ'* is the gene encoding the β -galactosidase α -peptide. The various genes are transcribed towards the arrowheads.

regulated α/β -type SASP production in spores of strains SM101(pDR81) and DR101(pDR81) by ~ 10 -fold.

Heat resistance of spores with different levels of α/β -type SASP. Having shown that spores of strains SM101(pDR81) and DR101(pDR81) had significantly lower levels of α/β -type SASP than the parental wild-type spores, it was of obvious interest to compare the heat resistance of these various spores. Similar to results found previously (18), SM101(pSG22) spores exposed to moist heat had a D at 100°C ($D_{100^\circ\text{C}}$) of 80 min, while DR101 spores lacking only *ssp3* and also containing

FIG. 2. Levels of α/β -type SASP in spores of various *C. perfringens* strains. (A) α/β -Type SASP was extracted from equal amounts of spores (50 mg [dry weight]) of various *C. perfringens* strains, lyophilized, and dissolved in 25 μ l of 8 M urea, 10- μ l aliquots were run on polyacrylamide gels at low pH, and the gel was stained with Coomassie brilliant blue. The spores from which extracts were prepared are SM101(pSG22) (wild-type strain carrying the control plasmid), DR101(pSG22) (an isogenic *ssp3* mutant carrying the control plasmid), SM101(pDR81) (wild-type strain carrying the plasmid expressing *ssp2* asRNA), and DR101(pDR81) (*ssp3* mutant carrying the plasmid expressing *ssp2* asRNA). The arrow indicates the major band that was immunoreactive with *B. subtilis* SspC antiserum. (B) Western blot of samples run on a gel as shown in A. The blot was probed with antiserum against *B. subtilis* SspC and developed for chemiluminescence detection to identify immunoreactive species. (C) Densitometric analysis of the α/β -type SASP band intensities with extracts from spores of various *C. perfringens* strains run on polyacrylamide gels at low pH and stained with Coomassie blue. Densitometric analysis was performed as described in Materials and Methods, and the results are averages of three independent experiments.

plasmid pSG22 were slightly less resistant ($D_{100^\circ\text{C}}$ of 50 min) (Fig. 3), presumably due to the slightly lower α/β -type SASP levels in DR101(pSG22) spores (Fig. 2C). However, the moist heat resistance of spores expressing *ssp2* asRNA was even lower, with $D_{100^\circ\text{C}}$ values of 26 and 13 min for spores of strains

FIG. 3. Thermal death curves of spores of various *C. perfringens* strains. Spores of strains SM101(pSG22) (●) (wild type), DR101(pSG22) (◆) (*ssp3* mutant with the control plasmid), SM101(pDR81) (■) (wild type expressing *ssp2* asRNA), and DR101(pDR81) (▲) (*ssp3* mutant expressing *ssp2* asRNA) were heated at 100°C for various times, and CFU/ml were determined as described in Materials and Methods.

FIG. 4. UV survival curves for spores of various *C. perfringens* strains. Spores of strains SM101(pSG22) (wild type) (●), SM101(pDR81) (wild type expressing *ssp2* asRNA) (■), and DR101(pDR81) (*ssp3* mutant expressing *ssp2* asRNA) (▲) were exposed to UV radiation for various times, and CFU/ml were determined as described in Materials and Methods.

SM101(pDR81) and DR101(pDR81), respectively (Fig. 3). The differences in $D_{100^\circ\text{C}}$ values between spores with *ssp2* asRNA and those without *ssp2* asRNA were statistically significant at a P value of <0.001 .

Analysis of the dry heat resistance of spores of the *C. perfringens* wild-type strain showed that these spores had a $D_{120^\circ\text{C}}$ value similar to that found for *B. subtilis* spores both previously (17) and in current work. However, in contrast to the results when moist heat resistance was examined, *C. perfringens* spores with decreased α/β -type SASP levels had the same dry heat resistance as the spores of the wild-type strain. $D_{120^\circ\text{C}}$ values for SM101(pSG22), SM101(pDR81), DR101(pDR81), and *B. subtilis* JH642 were 15.6 ± 1.1 min, 14.0 ± 0.7 min, 15.0 ± 0.2 min, and 16.0 ± 1.0 min, respectively (results are the averages of at least three independent determinations with spores of each strain).

UV resistance of spores with different levels of α/β -type SASP. In addition to an auxiliary role in the heat resistance of *B. subtilis* spores, α/β -type SASP plays an essential role in the resistance of *B. subtilis* spores to UV radiation (24), a treatment commonly used to sterilize surfaces in the food industry. Comparison of the resistances of spores of the various *C. perfringens* strains to UV radiation at 254 nm showed that spores of strains with much lower α/β -type SASP levels, SM101(pDR81) and DR101(pDR81), were significantly less UV resistant than wild-type spores (Fig. 4). However, spores of strain DR101(pSG22) did not show any significant difference in UV resistance compared to spores of strain SM101(pSG22) (data not shown). The large differences in the UV resistances of the spores with normal or low levels of α/β -type SASP were observed in three independent experiments (data not shown).

DISCUSSION

A major conclusion from the results in this communication is that levels of α/β -type SASP are significantly reduced, albeit not completely abolished, in spores of *C. perfringens* strains expressing *ssp2* asRNA. Although asRNA has been used to down-regulate the expression of genes in gram-positive and gram-negative bacteria (6, 12, 16), as well as in *C. acetobutylicum* (3, 26, 27), this is the first report of the down-regulation of targeted genes in *C. perfringens*.

It was surprising that, as seen previously (18), there were at least six bands on polyacrylamide gels of SASP extracts from wild-type *C. perfringens* spores at low pH, since this species has only three *ssp* genes. Since the intensities of these bands were all reduced by the expression of *ssp2* asRNA, the proteins in these bands are almost certainly α/β -type SASP. We do not know why there are more α/β -type SASP bands than *ssp* genes. However, methionine and cysteine residues in these *C. perfringens* proteins could undergo oxidation (5), which would alter protein mobility on polyacrylamide gels. We also do not know which protein band is the product of which *ssp* gene. Given the high degree of sequence homology in the three *C. perfringens* proteins, it would not be surprising that the proteins should migrate similarly on polyacrylamide gels at low pH.

It was also surprising that only the major band in SASP extracts from wild-type spores reacted strongly with the antiserum against *B. subtilis* SspC, as seen previously (18). The finding that the cross-reaction between this antiserum and the *C. perfringens* proteins should be weak is not surprising, since the sequence identity between *B. subtilis* SspC and the *C. perfringens* proteins is only ~35% (Fig. 1A). Perhaps slight variations in the sequences of the different *C. perfringens* α/β -type SASP have a large effect on their reactions with antiserum against *B. subtilis* SspC. Alternatively, lower levels of the *C. perfringens* proteins on Western blots may not react well with the antiserum.

The determination of which *ssp* gene encodes which α/β -type SASP seen on polyacrylamide gels at low pH is important in determining those *ssp* mRNAs whose levels are decreased by asRNA. Levels of *ssp2* mRNA and perhaps *ssp3* mRNA should be decreased because of the perfect or large degree of sequence complementarity between these mRNAs and *ssp2* asRNA. However, the lower degree of sequence complementarity between *ssp2* asRNA and *ssp1* mRNA might not result in as effective a reduction in levels of *ssp1* mRNA. Since a deletion of *ssp3* did not disproportionately reduce the intensity of individual protein bands on polyacrylamide gels of SASP extracts, Ssp3 is likely not the major α/β -type SASP in *C. perfringens* spores. This is consistent with the low level of expression of an *ssp3-gusA* fusion during sporulation compared to that of *ssp1*- and *ssp2-gusA* fusions (18). The large reduction in total α/β -type SASP levels by the expression of *ssp2*-asRNA is consistent with Ssp2 being a major α/β -type SASP. However, determinations of which Ssp protein is encoded by which *ssp* gene will likely require protein sequence analysis of the bands obtained on polyacrylamide gels of SASP extracts.

A second major conclusion from current work is that a previous suggestion (18) that *C. perfringens* α/β -type SASP plays a significant role in the moist heat resistance of *C. perfringens* spores is correct. Spore moist heat resistance decreased about sixfold when levels of α/β -type SASP in spores decreased ~10-fold ($D_{100^\circ\text{C}}$ value going from 80 min to 13 min). This is similar to the decrease in *B. subtilis* spores' moist heat resistance upon an ~85% reduction in the level of α/β -type SASP (9). However, even with the ~90% reduction of α/β -type SASP levels in spores of strain DR101(pDR81), the $D_{100^\circ\text{C}}$ value for these spores with moist heat (~13 min) (Fig. 3) was slightly higher than the $D_{55^\circ\text{C}}$ value for vegetative cells of *C. perfringens* strain SM101 (~10 min) (20). This suggests that α/β -type SASP is not the only factor in the moist heat

resistance of *C. perfringens* spores. Indeed, the water content in the spore core is the most important factor in the moist heat resistance of spores of *Bacillus* species, with spore moist heat resistance increasing as core water content decreases (4). Among the factors responsible for determining the level of core water in spores of *B. subtilis* are the products of the *dacB*, *spmA*, and *spmB* genes (17). Homologues of these genes are present in the *C. perfringens* genome (13, 25), and it would be interesting to study the role of these homologues in the resistance of *C. perfringens* spores to moist heat.

The third major conclusion is that α/β -type SASP plays a significant role in the resistance of *C. perfringens* spores to UV light. This is consistent with previous work in vitro in which purified *Clostridium bifermentans* α/β -type SASP caused the same changes in the UV photochemistry of DNA as *B. subtilis* α/β -type SASP (15). In *B. subtilis*, all α/β -type SASP appear to be interchangeable in their ability to confer UV resistance to spores (23). This also seems likely to be the case with *C. perfringens*, given the high amino acid sequence homology among the *C. perfringens* α/β -type SASP (Fig. 1A).

The final and perhaps most novel major conclusion from this work is that in contrast to results with *B. subtilis* spores (17, 22), *C. perfringens* α/β -type SASP appears not to have a major role in the resistance of *C. perfringens* spores to dry heat. These results suggest that some other factors, perhaps DNA repair, as shown for *B. subtilis* spores (28), may be of primary importance in the resistance of *C. perfringens* spores to dry heat. Further research to identify factors responsible for *C. perfringens* spore dry heat resistance, as well as to elucidate the mechanism of action of α/β -type SASP, will help in understanding the mechanism of resistance of *C. perfringens* spores to stress factors. This knowledge may well have applied implications in the areas of food safety and food preservation.

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