

Effect of a Small, Acid-Soluble Spore Protein from *Clostridium perfringens* on the Resistance Properties of *Bacillus subtilis* Spores[∇]

Juan Francisco Leyva-Illades,¹ Barbara Setlow,¹ Mahfuzur R. Sarker,^{2,3} and Peter Setlow^{1*}

Department of Molecular, Microbial and Structural Biology, University of Connecticut Health Center, Farmington, Connecticut 06030-3305,¹ and Department of Biomedical Sciences, College of Veterinary Medicine,² and Department of Microbiology,³ Oregon State University, Corvallis, Oregon 97331

Received 25 July 2007/Accepted 22 August 2007

α/β -type small, acid-soluble spore proteins (SASP) are essential for the resistance of DNA in spores of *Bacillus* species to damage. An α/β -type SASP, Ssp2, from *Clostridium perfringens* was expressed at significant levels in *B. subtilis* spores lacking one or both major α/β -type SASP (α^- and $\alpha^- \beta^-$ strains, respectively). Ssp2 restored some of the resistance of $\alpha^- \beta^-$ spores to UV and nitrous acid and of α^- spores to dry heat. Ssp2 also restored much of the resistance of α^- spores to nitrous acid and restored full resistance of α^- spores to UV and moist heat. These results further indicate the interchangeability of α/β -type SASP in DNA protection in spores.

Spores of *Bacillus* species are much more resistant to a variety of environmental stress factors, including heat, radiation, desiccation, and toxic chemicals, than are their corresponding growing cells (6, 13). Many factors are responsible for spore resistance properties, including the spore coats, the relatively impermeable spore inner membrane, and the spore core's low water content and high levels of dipicolinic acid and associated divalent cations (13). However, the major factor involved in protection of spore DNA against damage is a group of novel proteins, termed the α/β -type small, acid-soluble spore proteins (SASP) (13, 14). These proteins saturate spore DNA and alter both its physical structure and its chemical and photochemical reactivity. The latter changes protect DNA from heat and many genotoxic chemicals and cause a change in spore DNA photochemistry that is the major reason for spore resistance to UV light.

The α/β -type SASP are encoded by a multigene family of three to seven members in *Bacillus* species, and the sequences of these proteins are well conserved both within and across species (14). While the many α/β -type SASP in any one species are present in spores at quite different levels, studies in *Bacillus* spores and in vitro have shown that all α/β -type SASP, whether normally expressed at high or low levels, have similar effects on DNA if present at high enough levels (4, 17).

Spores of *Clostridium* species also contain α/β -type SASP, and these proteins appear at approximately the same time in sporulation as they do in *B. subtilis* (11). While the sequences of the *C. perfringens* α/β -type SASP differ somewhat from those of *Bacillus* species, many features of the sequences of these proteins are conserved in both species (14). Indeed, several purified clostridial α/β -type SASP have effects on DNA's UV photochemistry in vitro similar to those of purified α/β -type SASP from *Bacillus* species (2, 7). In addition, recent work has

indicated that α/β -type SASP are very important in the resistance of *C. perfringens* spores to UV radiation and moist heat, since *C. perfringens* spores with reduced levels of α/β -type SASP have reduced resistance to these two agents (10, 11). However, α/β -type SASP may not be important in the resistance of *C. perfringens* spores to dry heat (10), in contrast to results with *B. subtilis* spores (13, 14). Dry heat kills *B. subtilis* spores in large part by DNA damage, and *B. subtilis* α/β -type SASP protect DNA from damage both in vitro and in spores (7, 12, 13). Consequently, the lack of an effect of decreased α/β -type SASP levels on the dry-heat resistance of *C. perfringens* spores suggested that the *C. perfringens* proteins may have different effects on DNA properties than do the comparable proteins from spores of *Bacillus* species. To more closely assess the functions of *C. perfringens* α/β -type SASP, we expressed a *C. perfringens* α/β -type SASP at high levels in spores of *B. subtilis* strains PS356 and PS260 (termed $\alpha^- \beta^-$ and α^- , respectively) (3). In the latter two strains, either both *sspA* and *sspB*, which encode ~85% of *B. subtilis* α/β -type SASP (SASP- α and - β), or only *sspA*, encoding SASP- α , which comprises ~55% of wild-type spores' α/β -type SASP, had been inactivated (3). The resistance properties of the spores of these strains were then compared to those of wild-type, $\alpha^- \beta^-$, and α^- *B. subtilis* spores, as well as α^- and $\alpha^- \beta^-$ *B. subtilis* spores expressing high levels of SspC (strain PS1450 [17]), a *B. subtilis* α/β -type SASP normally present at only low levels. Wild-type and $\alpha^- \beta^-$ spores used for comparative purposes were from strains PS533 (wild-type) and PS578 ($\alpha^- \beta^-$), which each also carry plasmid pUB110, encoding resistance to kanamycin (Km) (10 $\mu\text{g}/\text{ml}$) (12).

The *C. perfringens* gene used for this work was *ssp2* from *C. perfringens* strain 13. The amino acid sequence of the encoded protein is identical to that of Ssp2 from *C. perfringens* SM101, but the gene in strain 13 has a likely strong transcription terminator just downstream of the translation stop codon (5, 15). We chose the *ssp2* gene for this work because it is likely expressed at the highest level of all *ssp* genes in *C. perfringens* (10, 11). The *ssp2* gene was PCR amplified from *C. perfringens*

* Corresponding author. Mailing address: Department of Molecular, Microbial and Structural Biology, University of Connecticut Health Center, Farmington, CT 06030-3305. Phone: (860) 679-2607. Fax: (860) 679-3408. E-mail: setlow@nso2.uconn.edu.

[∇] Published ahead of print on 31 August 2007.

strain 13 DNA using the upstream primer 5'-CCCCGGGGAATAACTAAGGAGGAATGAA-3' and the downstream primer 5'-CCCCGGGGAATATAAAATGAGTTTAATGGG-3', each containing extra 5' residues, including a SmaI cleavage site (underlined). The resultant ~435-bp PCR fragment was cloned in plasmid pCR-TOPO (Invitrogen, Carlsbad, CA) in *Escherichia coli*, giving plasmid pPS4046. The insert in this plasmid was sequenced to confirm that the PCR product had the DNA sequence of *C. perfringens* strain 13 DNA. The insert was removed from plasmid pPS4046 by digestion with SmaI (sites within original PCR primers) and cloned in the HpaI site of plasmid pPS4047 in *E. coli* DH5 α . Plasmid pPS4047 was derived from plasmid pPS1393 (17) by digestion with HpaI, removal of a 130-bp fragment that contains a region of the *B. subtilis* *sspB* gene beginning just after its promoter and ending midway in its coding sequence, and ligation and transformation of the remaining plasmid backbone into *E. coli* DH5 α . A plasmid termed pPS4048, carrying the *C. perfringens* *ssp2* gene in the HpaI site of plasmid pPS4047, was identified by restriction enzyme digestion, and the orientation of the *ssp2* coding sequence just downstream of the strong forespore-specific promoter of the *B. subtilis* *sspB* gene was confirmed by digestion with PvuII. Plasmid pPS4048 was cut with BamHI to remove the pUC19 sequence in this hybrid plasmid derived from pPS1393 (17), ligated, and transformed into *B. subtilis* strain PS356 ($\alpha^- \beta^-$) (3) with selection for Km^r, giving strain PS4049 ($\alpha^- \beta^-$ Ssp2). Plasmids from strains PS1450 ($\alpha^- \beta^-$ SspC) and PS4049 were used to transform strain PS260 (α^- and resistant to 3 μ g/ml chloramphenicol) (3) to Km^r, giving strains PS4051 (α^- Ssp2) and PS4052 (α^- SspC), respectively, in which the *ssp2* or *sspC* gene is present on a plasmid and just downstream of the *sspB* promoter.

Spores of *B. subtilis* strains with and without various SASP sporulated with similar efficiencies at 37°C on 2 \times Schaeffer's glucose medium agar plates, as determined by phase-contrast microscopy (data not shown), and spores were harvested, purified, and stored as described elsewhere (8, 9). All spore preparations used in this work were free ($\geq 98\%$) of growing or sporulating cells or germinated spores, as determined by phase-contrast microscopy. Spores (~6 mg, dry weight) were dry ruptured, SASP were extracted with 1 ml and then 0.5 ml ice-cold 0.1 mM HCl, and both supernatant fluids were pooled, dialyzed in 3,500-molecular-weight-cutoff dialysis tubing against 1% acetic acid, and lyophilized as described elsewhere (8). The dry residue was dissolved in 30 μ l 8 M urea, 15 μ l acid gel diluent was added, aliquots from ~1.3 mg (dry weight) of spores were subjected to polyacrylamide gel electrophoresis at low pH, and the gel was stained with Coomassie blue as described elsewhere (8). Spores of strain PS4049 ($\alpha^- \beta^-$ Ssp2) did not contain SASP- α and - β as expected but did contain an additional SASP migrating at the position expected for Ssp2 (Fig. 1A, compare lanes 1, 2, and 3; also data not shown). Extract from 0.7 mg of PS4049 spores was also subjected to polyacrylamide gel electrophoresis at low pH, the proteins on the gel were transferred to polyvinylidene difluoride paper (Immobilon-P; Millipore Corporation, Billerica, MA), the paper was lightly stained with Coomassie blue, and the putative Ssp2 band was cut out and subjected to automated protein sequence analysis. The first eight amino acids of the major protein in this band were Ser-Gln-His-Leu-Val-Pro-Glu-Ala

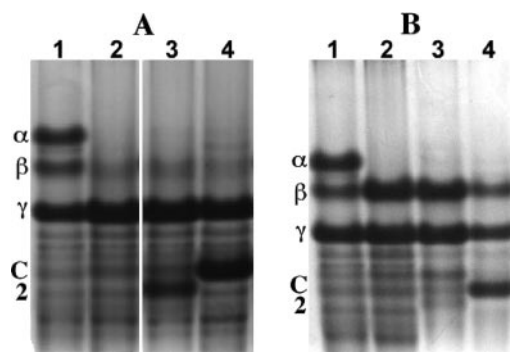


FIG. 1. Acid-gel electrophoresis of SASP extracts from spores of various strains. Spores were prepared, purified, and extracted; the extracts were processed; aliquots were subjected to acid-gel electrophoresis; and the gels were stained, as described in the text. The extracts run in the various lanes were from spores of the following strains: (A) lane 1, PS533 (wild-type); lane 2, PS578 ($\alpha^- \beta^-$); lane 3, PS4049 ($\alpha^- \beta^-$ Ssp2); and lane 4, PS1450 ($\alpha^- \beta^-$ SspC); (B) lane 1, PS533 (wild type); lane 2, PS260 (α^-); lane 3, PS4051 (α^- Ssp2); and lane 4, PS4052 (α^- SspC). α , β , C, and 2, migration positions of SASP- α , SASP- β , SspC, and Ssp2, respectively; γ , migration position of the single γ -type SASP in *B. subtilis* spores. This last protein is very different from α/β -type SASP and plays no role in spore resistance properties (6, 13). The lanes in panel A are from the same gel, but several intervening lanes have been removed for clarity, and a white space was left to indicate where lanes were removed; the lanes in panel B are also from the same gel and were run adjacent to each other.

(data not shown), as expected for Ssp2 with its amino-terminal methionine removed posttranslationally as is the rule in α/β -type SASP (14; also, see below). While PS4049 spores contained Ssp2, the level of this protein appeared to be slightly lower than that of either SASP- α plus SASP- β in wild-type spores or SspC in PS1450 ($\alpha^- \beta^-$ SspC) spores (Fig. 1A, compare lanes 1, 3, and 4), although the relative staining efficiencies of Ssp2, SASP- α , SASP- β , and SspC on polyacrylamide gels are not known.

Analysis of the resistance of PS4049 ($\alpha^- \beta^-$ Ssp2) spores to various agents determined on plates containing the appropriate antibiotics as described previously (4, 10, 16) indicated that Ssp2 restored significant UV and nitrous acid resistance to $\alpha^- \beta^-$ spores, although not as much as did SspC (Fig. 2A and B). However, only a minimal amount of moist-heat resistance was restored to $\alpha^- \beta^-$ spores by expression of SspC or Ssp2 (Fig. 2C), and neither protein restored any dry-heat resistance to $\alpha^- \beta^-$ spores (data not shown). All of these resistance results were obtained in two separate experiments with two sets of spore preparations.

The relatively low levels of Ssp2 in *B. subtilis* $\alpha^- \beta^-$ spores might well be the reason for the minimal restoration of spore resistance properties by this protein. To further examine the ability of Ssp2 to provide resistance to *B. subtilis* spores, we expressed either this protein or SspC in spores of a *B. subtilis* strain lacking only SASP- α , the major *B. subtilis* α/β -type SASP (3). Previous work has shown that loss of SASP- α alone results in loss of much of the wild-type spore's resistance to UV and moist heat, although not as much as is lost in $\alpha^- \beta^-$ spores (4). More importantly, the UV and moist-heat resistance of α^- spores is more readily restored to wild-type levels by normally minor α/β -type SASP than is the resistance to these agents of

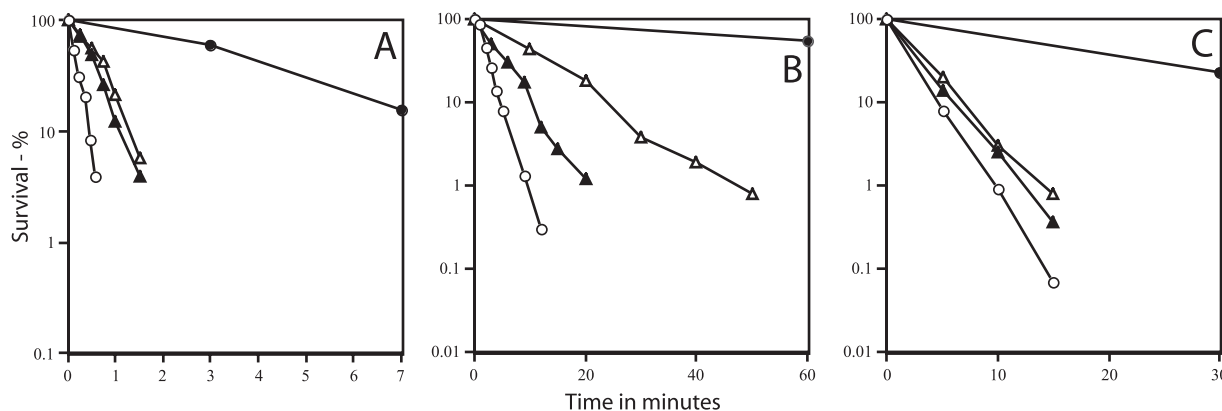


FIG. 2. Resistance of wild-type spores and $\alpha^- \beta^-$ spores of various strains to UV radiation (A), nitrous acid (B), and moist heat (C). Spores of various strains were prepared and purified and their survival after various treatments was measured as described in the text. The variability in survival values in experiments was $\pm 20\%$. Symbols: ●, PS533 (wild type); ○, PS578 ($\alpha^- \beta^-$); △, PS1450 ($\alpha^- \beta^-$ SspC); and ▲, PS4049 ($\alpha^- \beta^-$ Ssp2).

$\alpha^- \beta^-$ spores (4). As expected, spores of all α^- strains lacked SASP- α but retained SASP- β and - γ (Fig. 1B, compare lanes 1 and 2). The level of SspC in the α^- spores relative to that of SASP- γ was also similar to that in spores with an $\alpha^- \beta^-$ background (Fig. 1A and B, compare lanes 4). While Ssp2 was expressed in α^- spores, the level of this protein relative to

SASP- γ was significantly lower than in $\alpha^- \beta^-$ spores (Fig. 1A and B, compare lanes 3). The reason for the difference in levels of Ssp2 in α^- and $\alpha^- \beta^-$ spores is not completely clear, but it may be due to repression of Ssp2 synthesis by SASP- β in α^- spores. As seen previously (3, 4), levels of SASP- β increased significantly in α^- spores compared to levels in wild-type

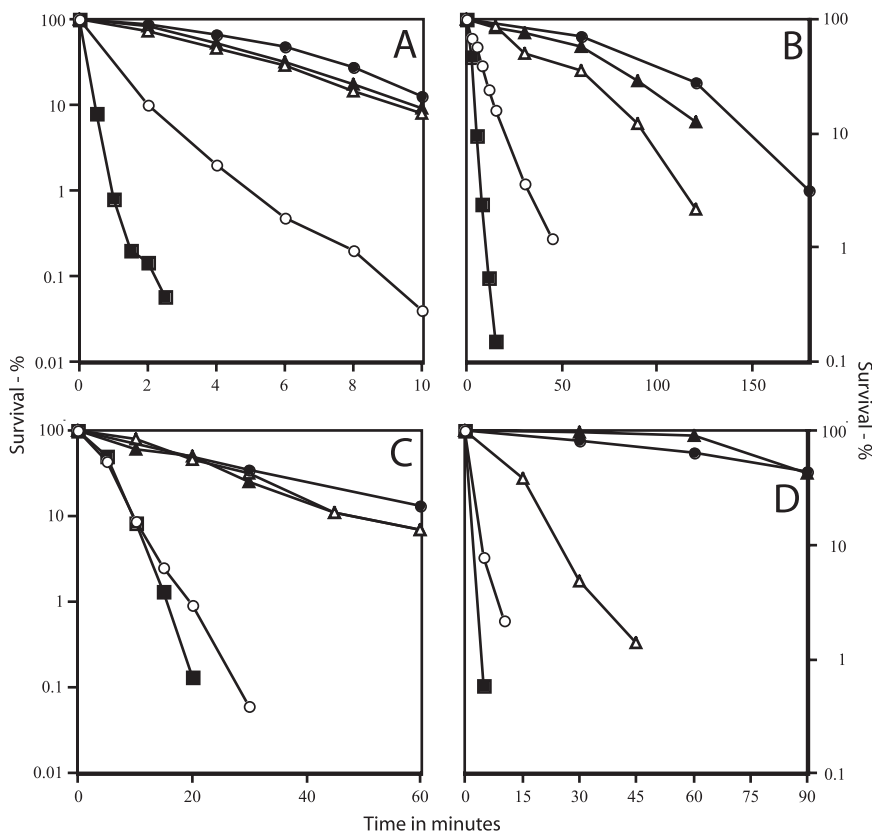


FIG. 3. Resistance of wild-type spores and α^- spores of various strains to UV radiation (A), nitrous acid (B), moist heat (C), and dry heat (D). Spores were prepared and purified and their survival after various treatments was measured as described in the text. The variability in survival values in these experiments was $\pm 15\%$. Symbols: ●, PS533 (wild type); ○, PS260 (α^-); ▲, PS4052 (α^- SspC); △, PS4051 (α^- Ssp2); and ■, PS578 ($\alpha^- \beta^-$).

- of spores of *Bacillus subtilis* which lack dipicolinic acid. *J. Bacteriol.* **182**:5505–5512.
10. **Raju, D., P. Setlow, and M. R. Sarker.** 2007. 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. *Appl. Environ. Microbiol.* **73**:2048–2053.
 11. **Raju, D., M. Waters, P. Setlow, and M. R. Sarker.** 2006. Investigating the role of small, acid-soluble spore proteins (SASPs) in the resistance of *Clostridium perfringens* spores to heat. *BMC Microbiol.* **6**:50.
 12. **Setlow, B., and P. Setlow.** 1996. Role of DNA repair in *Bacillus subtilis* spore resistance. *J. Bacteriol.* **178**:3486–3495.
 13. **Setlow, P.** 2006. Spores of *Bacillus subtilis*: their resistance to radiation, heat and chemicals. *J. Appl. Microbiol.* **101**:514–525.
 14. **Setlow, P.** 2007. I will survive: DNA protection in bacterial spores. *Trends Microbiol.* **15**:172–180.
 15. **Shimizu, T., K. Ohtani, H. Hirakawa, K. Ohshima, A. Yamashita, T. Shiba, N. Ogasawara, M. Hattori, S. Kuhara, and H. Hayashi.** 2002. Complete genome sequence of *Clostridium perfringens*, an anaerobic flesh-eater. *Proc. Natl. Acad. Sci. USA* **99**:996–1001.
 16. **Tennen, R., B. Setlow, K. L. Davis, C. A. Loshon, and P. Setlow.** 2000. Mechanisms of killing of spores of *Bacillus subtilis* by iodine, glutaraldehyde and nitrous acid. *J. Appl. Microbiol.* **89**:330–338.
 17. **Tovar-Rojo, F., and P. Setlow.** 1991. Analysis of the effects of mutant small, acid-soluble spore proteins from *Bacillus subtilis* on DNA in vivo and in vitro. *J. Bacteriol.* **173**:4827–4835.
 18. **Wilson, A. C., H. Ochman, and E. M. Prager.** 1987. Molecular time scale for evolution. *Trends Genet.* **3**:241–247.