J. Microbiol. Biotechnol. (2013), **23**(1), 15–21
http://dx.doi.org/10.4014/jmb.1206.06028
First published online November 20, 2012
pISSN 1017-7825 eISSN 1738-8872

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

"A La Recherche" of Functions for the Spore Protein SASP-E from *Bacillus subtilis*

Ruzal, Sandra M., Patricia L. Bustos, and Carmen Sánchez-Rivas*

Universidad de Buenos Aires, Facultad Ciencias Exactas y Naturales, Departamento de Química Biológica, Ciudad Universitaria Pabellón II, 4º piso, C1428EGA Buenos Aires, Argentina

Received: June 12, 2012 / Revised: August 26, 2012 / Accepted: August 28, 2012

We previously observed that *Bacillus subtilis* spores from *sspE* mutants presented a lower germination capacity in media containing high salt concentrations (0.9 M NaCl). This deficiency was attributed to the absence of SASP-E (gamma-type small-acid-soluble protein), rich in osmocompatible amino acids released by degradation. Herein we observed that, in addition, this mutant spore presented a reduced capacity to use L-alanine as germinant (L-ala pathway), required longer times to germinate in calcium dipicolinate (Ca^{2+}-DPA), but germinated well in asparagine, glucose, fructose, and potassium chloride (AGFK pathway). Moreover, mild sonic treatment of mutant spores partially recovered their germination capacity in L-ala. Spore qualities were also altered, since sporulating colonies from the *sspE* mutant showed a pale brownish color, a higher adherence to agar plates, and lower auto-fluorescence, properties related to their spore coat content. Furthermore, biochemical analysis showed a reduced partition in hexadecane and a higher content of Ca^{2+}-DPA when compared with its isogenic wild-type control. Coat protein preparations showed a different electrophoretic pattern, in particular when detected with antibodies against CotG and CotE. The complementation with a wild-type *sspE* gene in a plasmid allowed for recovering the wild-type coat phenotype. This is the first report of a direct involvement of SASP-E in the spore coat assembly during the differentiation program of sporulation.

Keywords: *Bacillus subtilis*, spores, SASP-E, germination, coat

Members of the genera *Bacillus* are prepared to resist adverse environmental conditions because of their ability to form spores and to start growing whenever conditions become adequate. The particular composition and structure of spores are the support for such adaptation.

Spores from *Bacillus subtilis* are essentially composed of a dehydrated cytoplasm (the core) surrounded by two membranes of opposite orientation that contain a thick layer of a modified peptidoglycan structure, the cortex, and an outer protein shell, the coat, which is composed of about 50 keratin-like proteins. The core contains the DNA as a supercoiled nucleoid maintained by the presence of small-acid-soluble proteins of α/β type (SASP-αβ), and free proteins, among which the most abundant is SASP-γ or SASP-E, and two important biochemical components: calcium dipicolinate (Ca^{2+}-DPA) and 3-phosphoglycerate (3-PGA) [23, 31].

In the presence of germinants (nutrients, chemical compounds, or physical factors), an irreversible process of germination takes place. Among nutrients, the two best-known are amino acids and carbohydrates, which determine the two main pathways of germination: L-ala (L-alanine) and AGFK (asparagine, glucose, fructose, and potassium ions), respectively [23, 24, 26]. Each of these pathways involves a different specific Ger receptor located in the inner membrane [12, 25]. Once the germinant reaches its receptor (GerA for L-alanine and GerB for AGFK), ions (Na^+, K^+, and Ca^{++}) are released while water enters; the Ca^{2+}-DPA stored in the core is then released outside the spore, while nucleotides and 3-PGA are retained [31]. Core expansion allows enzymatic activation, the most relevant being the cortex hydrolases and intracellular proteases. Cortex hydrolases allow breaking down of the peptidoglycan chains of the cortex. The intracellular SASPs proteases release SASP-α/β from the nucleoid, ensuring the start of the replication–transcription machinery. The amino acids obtained from SASPs proteolysis are used to begin protein synthesis. Small molecules such as 3-PGA stored in the core bring the energy necessary for starting protein synthesis

**Corresponding author*
Phone: +54 11 4576 33 42; Fax: +54 11 4576 33 42;
E-mail: sanchez@qb.fcen.uba.ar

and outgrowth. Finally, extracellular nutrients (carbon source and amino acids) are incorporated and vegetative growth resumes [16]. Therefore, two main events are taking place; one involving release or degradation of some component of the spore, referred to as the germination step, and the other involving biosynthesis or polymerization that is considered the outgrowth step.

Several reports have shown that *ssp* mutants present defects in both germination and outgrowth [9], in particular on plates containing high salt concentrations [29, 32]. On the other hand, spores from *B. thuringiensis* and *B. sphaericus*, containing low levels of SASPs without a *bona fide* SASP-E, show reduced germination on hypertonic plates, but the introduction of a *sspE* gene from *B. subtilis* allows to recover partially their germination capacity on these same plates [3, 4].

SASP-E represents the most abundant uniformly distributed spore protein [11] and its content in glutamate/glutamine (osmoprotectant amino acids) is particularly high (30%), probably accounting for the low germination and outgrowth on hyperosmotic plates of SASP-E-deficient strains. However, this would not explain our observation of a reduced germination in isotonic media with L-alanine as the germinant. In order to further investigate the role and influence of the SASP-E protein during germination and the sporulation process, spores prepared from a *sspE* mutant and its wild-type isogenic strain were analyzed for their capacity to respond to different germinants, analyzing in particular the first germination step. Additionally, several biochemical spore parameters, like hydrophobic partition index, DPA, and coat contents, were determined and compared. Complementation of the *sspE* null strain with a wild-type gene was also performed to provide confirmation of SASP-E's role.

Materials and Methods

Strains

The *Bacillus subtilis* strains used are the wild-type YB886 and its isogenic *sspE* mutant (8E; *sspE*::Cm^R). [29]. The *gerA* mutant 1G7 was obtained from the *Bacillus* Genetic Stock Center. They were cultured in LB or sporulation medium [30].

Plasmid pPS623 (Km^R) containing the *sspE* gene in the pUB110 plasmid [20] was introduced in 8E competent cells selecting both *sspE*::Cm^R null mutation and the pPS623 plasmid. Plasmid presence was confirmed by agarose gel electrophoresis.

Preparation of Spores

Spores were obtained from sporulation agar medium (D) incubated 72 h at 37°C. Spores were recovered with ice-cold 1 M NaCl and washing 5 times in ice-cold double-distilled water. In this way, about 99% of the suspensions were spores, as determined by phase-contrast microscopy. Pellets were conserved at −20°C.

Calcofluor (fluorescent brightener 28; Sigma) was used to verify differences in its access to the external coat of spores and it was added into the D medium at 1 mg/ml. Photographs from plates were taken under visible light (Dplates) or UV light after 15 s exposition (DCalcofluor), using a CCD camera of Fuji LAS1000 and Image Gauge 3.122 software (Fuji Film).

Autofluorescence was examined by fluorescence microscopy (Carl Zeiss) of dormant spores in saline phosphate buffer with excitation at 488 nm and emission at 505 nm, and quantified by flow cytometry in saline phosphate buffer at an optical density of 0.4–4 at 600 nm (OD_{600}) and analyzed on a FACS Calibur flow cytometer (BD Biosciences) using a 530 nm (FITC) bandpass filter. Results were plotted as intensity of fluorescence against light side scatter or level of fluorescence against the number of events counted.

Germinations with Nutrients

Heat-activated spores (10 min at 70°C) were analyzed for germination as indicated [33]. With our spore preparations, the maximum decrease in OD_{550} of the wild-type strain was around 55% and was taken as 100%.

The L-alanine germinant was as follows: 10 mM L-alanine, 10 mM Tris-HCl, pH 7.6, 10 mM KCl; the AGFK was 10 mM Tris-HCl, pH 7.6, 10 mM KCl, 10 mM glucose, 10 mM L-asparagine, and 10 mM fructose. Complete germination was in LB2X, 10 mM glucose, and 10 mM fructose (LB+GF).

When indicated, mild sonic treatment was done for 15 s to spores before starting germination.

The Ca^{2+}-DPA germinant contained 60 mM Ca^{2+}-DPA [27].

DPA Content and Hydrophobic Partition Index of Spores

The DPA content was determined according to Rotman and Fields [28] from 10 DO units (5 mg dry weight, 4.10^9 CFU) of spores. The hydrophobic index (HI) was determined as described by Johnson *et al*. [14] using hexadecane as the organic solvent.

Coat Preparations

The procedure used for extraction was according to Henriques *et al*. [10] using identical amounts of spores. Two DO units of spores were loaded in 12.5% SDS-PAGE, stained with Coomassie Brilliant Blue [17]. For Western blot analysis, gels were electrotransferred with a Semi-dry Blotter (Amersham Biosciences) to PVDF membranes (Macherey-Nagel, Germany), soaked with a polyclonal anti-coat antibodies diluted 1:1,000 to 1:10,000 depending on the antibody, and visualized with biotin-conjugated anti-rabbit followed by streptavidin-HRP conjugate (Pierce). Chemiluminescence was detected with ECL substrate (Sigma). Images were acquired with a Fuji LAS1000 digitalizer.

Results

Germination with Specific Nutrients

In order to investigate the germination capacity of the *sspE* mutant, spores from the wild-type (YB886) and mutant (8E) strains were allowed to germinate using the two main pathways for nutrients, L-Ala and AGFK. Germinations were performed and measured by the decrease in OD_{550}. Maximum decrease of OD_{550} occurred after 1 h incubation. Using L-alanine as the germinant, the WT spores showed 45% to 55% decreases of OD, which correspond essentially

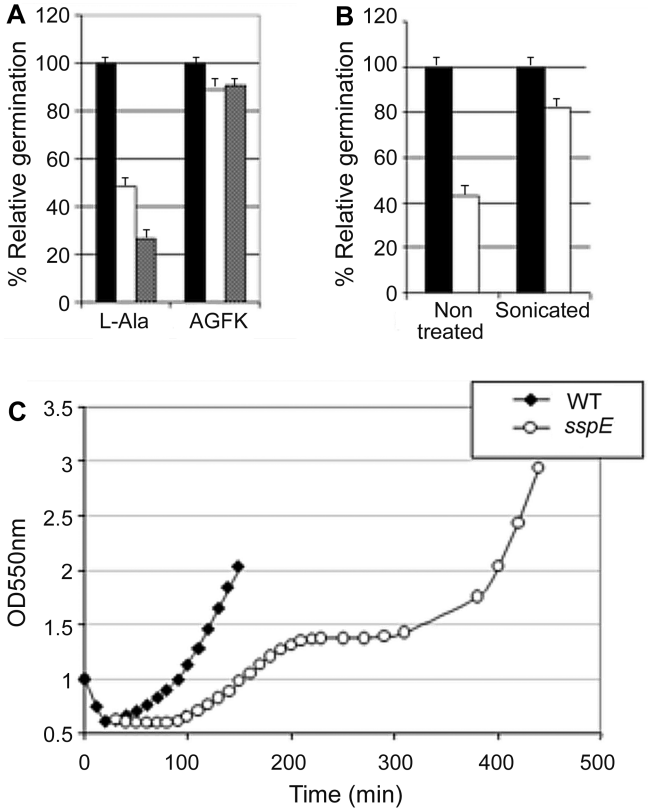

Fig. 1. Comparison of the germination capacities in L-alanine or AGFK, with or without sonic treatment.
(**A**) Spores were germinated in either L-alanine or AGFK. Relative germination was calculated as ΔOD_{550} mutant $\times 100/\Delta OD_{550}$ WT and reported. Data are representative of several experiments. (**B**) Spores, sonicated or not, were germinated in L-alanine. Black columns, YB886 (WT); white columns, 8E (*sspE*); hatched column for 1A (*gerA*) spores. (**C**) Heat-activated spores were diluted at an initial $OD_{550} \sim 0.5$ in LB Glucose Fructose, incubated at 37°C, and OD_{550} measured at intervals. Black squares represent the YB886 (WT) control; empty circles, the *sspE* mutant (8E).

to the germination step I, and this value was taken as 100%. However, during this same period, less than 20% decrease of OD was observed for 8E spores. In contrast, in the AGFK condition, the same 8E spores behaved as the wild type, and the presence of carbon sources allowed to pursue germination until growth resumed (Fig. 1A). This result indicates that once germination begun, the following steps were not impaired in the 8E mutant in this condition and only the access of the L-ala germinant was impaired. In order to investigate if the access of germinant was impaired, spores were treated with mild sonication. Whereas sonication showed no difference in the germination efficiency of the wild-type spores, the germination capacity of mutant spores (8E) was recovered, as shown in Fig. 1B. This result suggests that the most external envelope of the spore would be less permeable.

Ca^{2+}-DPA, an important component of the spores, added exogenously triggered germination. As shown in Table 1, in the AGFK condition, the 8E spores were delayed in germination: 78% compared with 98% of wild-type spores geminated after 45 min incubation, but at 90 min both spores reached similar values. Once again, a defect in the capacity for the germinant to reach its receptor was observed.

In order to evaluate both germination and outgrowth, spores were incubated in LB medium containing glucose and fructose. In this medium a combined pathway of germination takes place. For WT spores, a clear two-step curve accounting for germination and outgrowth was observed. The *sspE* mutant spores presented a longer period of germination that could be due to its inability to use both germination pathways in this medium. For the outgrowth, a diauxic curve was observed, revealing that the absence of SASP-E led to a lower provision of amino acids necessary for starting biosynthetic processes. It should be remarked that once the vegetative stage was reached, allowing the up-take of the amino acids from the medium, both strains grew at the same rate (Fig. 1C).

Biochemical Analysis of Spores

The autofluorescence and pigmentation of dormant *B. subtilis* spores and the binding of dyes to them have been attributed to the coat proteins. In fact, sporulating plates from the *sspE* mutant presented a pale brown color (Fig. 3A), and higher adherence to the surface of agar plates that resulted in a more difficult condition for recovering cells from plates when compared with the WT control. When the fluorescence brightener calcofluor, a dye that is known to bind to glycans, was used, the *sspE* mutant strain showed an increased fluorescence in comparison with the wild-type control, probably reflecting differences in the accessibility of the dye as a result of a modified coat structure, as discussed below (Fig. 2A). The autofluorescence

Table 1. Germination in Ca^{2+}-DPA.

Strains	% Germinated spores after 45 min incubation in Ca^{2+}-DPA	% Germinated spores after 90 min incubation in Ca^{2+}-DPA
WT (YB886)	94.4	98.2
sspE (8E)	78.0	98.6

Heat-activated spores were incubated either in water (control) or in 60 mM Ca^{2+}-DPA for 45 and 90 min at room temperature and heated for 10 min at 70°C to select non-germinated spores. Serial dilutions and plating on LB allowed determining spores as CFU/ml. Those obtained after incubation in DPA determined the total of non-germinated spores.

Results represent one of at least three independent experiments using independent spore preparations. Percent standard deviation was less than 5%.

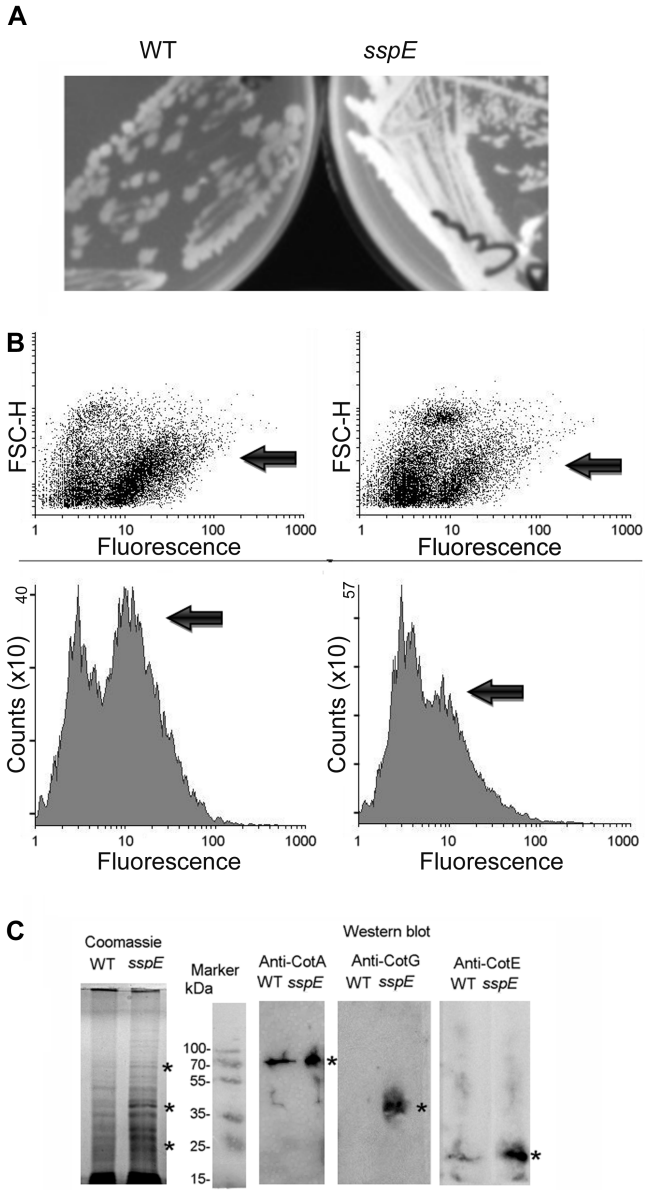

Fig. 2. Differences between *B. subtilis* WT (YB886) and an *sspE* (8E) mutant.
(**A**) Cultures on sporulation medium containing calcofluor dye photographed under UV. (**B**) Autofluorescence of spores was evaluated by flow cytometry to determine the level of fluorescence, and plotted as intensity of fluorescence against light side scatter (upper plot) or level of fluorescence against the number of events counted (lower plot). (**C**) Coomassie stain of SDS-PAGE gels and Western blot (WB) analysis revealed with anti-Cot antisera of coats from spores. Results showed one of at least three independent assays. Molecular weight marker migration is indicated. Asterisks indicate the bands that significantly changed.

of spores has been usually linked to their coat structure [7, 19]. For this reason, dormant spores were visualized for autofluorescence by flow cytometry. As shown in Fig. 2B, decreased autofluorescence was observed for the *sspE* mutant spores.

Table 2. Hydrophobicity index and DPA content of spores.

Strains	Partition in hexadecane (%)[a]	DPA content[b]
WT (YB886)	32.0 ± 1.1	78 ± 10
sspE (8E)	18.7 ± 1.4	116 ± 5

[a]Spores suspended in PUM buffer were mixed with hexadecane and % partition was calculated as indicated in Materials and Methods.
[b]DPA content was determined with 10 U of OD_{550} of spores (5 mg dry weight, 4×10^9 CFU) in 1 ml and treated as indicated in Materials and Methods. Results are expressed in μg/4×10^9 CFU/ml.
Results represent one of at least three independent experiments using independent spore preparations.

Content of DPA and HI Index

Ca^{2+}-DPA is an important compound of the spores. It is synthesized in the mother cell but is taken up into the forespore at the end of sporulation. When the content of spores was analyzed, *sspE* mutant spores contained an increased amount of this compound (Table 2).

Coats are the outermost envelope and must contribute to the hydrophobic state of spores. The partition efficiency in hexodecane has been used as indicative of this property [14]. As shown in Table 2, the defect in SASP-E in spores showed a lower hydrophobic index (HI) than its WT control.

Coats Analysis

Studies from several laboratories reveal that the coat proteins are organized into a complex interaction network that display the morphogenetic coat protein CotE at the inner coat–outer coat interface and is responsible for the assembly of the outer coat protein, CotG, which in turn, directs CotB deposition [5, 11, 13, 22]. Most of our results suggested that the outermost envelope (spore coat) of the *sspE* mutant would be impaired. In fact spore-coat extracts analyzed by SDS-PAGE showed differences in the intensity of protein bands between the WT and *sspE* strains (Fig. 2C). Western blot analyses, using antibodies against the external coat proteins CotA, CotE, and CotG of total coat proteins obtained from the WT and *sspE* mutant, were performed to visualize possible differences predicted by our previous results. Significant differences were observed for CotA and CotE, and a particularly higher level of CotG protein was observed in preparations of the *sspE* mutant (Fig. 2C).

Complementation Analysis

Since the defect in germination we are reporting for the *sspE* mutant seems to be related to an altered spore coat, we have to rule out the possibility of secondary mutations in coat genes. Complementation experiments performed with the wild-type cloned *sspE* gene showed that a WT phenotype was restored when we evaluated pigmentation and adherence to the agar surface (Fig. 3A). The coat

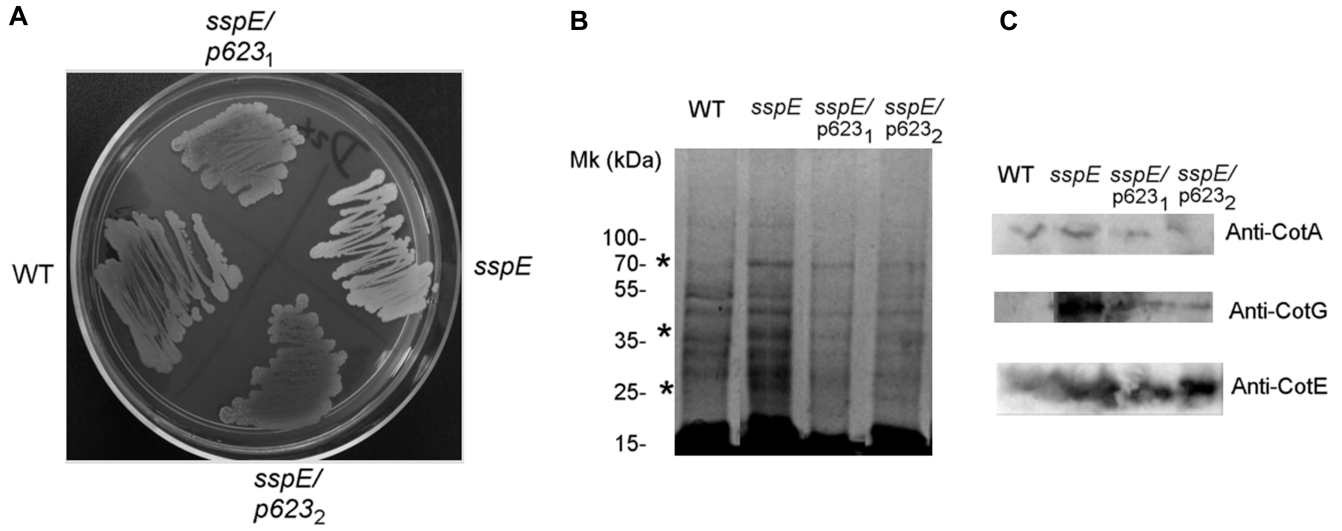

Fig. 3. Complementation with SASP-E.
(**A**) Pigment production of WT (YB886), *sspE* (8E), and clones 1 and 2 (8E/pPS623: plasmid complemented with WT copy of *sspE*). (**B**) Coomassie stain used as a loading control. Asterisks indicate the bands that significantly changed. (**C**) WB revealed with anti-Cot antisera of the same samples indicated in **B**. Results shown are one of at least three independent assays. Mk, molecular weight markers.

proteins from two complementing clones were analyzed by SDS-PAGE and Western blot and compared with *sspE* mutant and WT. As shown in Fig. 3B and 3C, the content of CotA, CotE, and CotG were similar to that of the WT strain, confirming that complementation with intact *sspE* copy rebuilt the coat and decreased the defects of the *sspE* mutant. However, these clones showed a severe instability, as we have already reported, when a high level of expression takes places with high copy number plasmids [29].

DISCUSSION

In the present work, we showed that spores from an *sspE* mutant behaved defective in germination, particularly when the germinant is L-alanine but not AGFK. This result suggests that the interaction, germinant–receptor, necessary for activating germination by the GerA pathway is defective (Fig. 1). In fact both germination and outgrowth were impaired in this mutant, as shown in Fig. 1C in LB-glucose-fructose. In this medium, germination was through both AGFK and L-ala pathways, but the defect in the use of the aminoacid germinant by the mutant led to a delayed outgrowth. This diauxic-like behavior must reflect the absence of the SASP-E, the main provider of amino acids in this period. The germination deficiency observed in L-alanine together with the recovery after a mild sonic treatment of spores, and the increased time needed for Ca^{2+}- DPA to induce germination of the mutant, led us to implicate the outermost coat layer structure and composition for this behavior.

Sporulating colonies of the *sspE* mutant showed differences in color, adherence, and fluorescence compared with the WT control (Fig. 2A and 3). These differences together with those of hydrophobicity index, DPA content (Table 2), and increased fluorescence in the presence of calcofluor, probably due to an increased accessibility of the dye to the peptidoglycan cortex, all provide evidences for a modified outermost coat layer of this mutant's spores.

Coat analysis by SDS-PAGE and Western blot with antibodies against coat proteins allowed visualization of differences in the coat pattern, in particular reflected by a higher CotE and CotG content of the mutant spores (Fig. 2B, 2C, and 3). In fact, the CotG protein was reported to interfere with the location of other Cot proteins [22], probably explaining the differences observed in the Coomassie staining (Fig. 2C and 3B). We are not yet able to attribute a cause for the different level of Cot proteins observed between the WT and *sspE* mutant, but we suggest either a direct relationship of SASP-E for localizing coat proteins, or a side effect that results in external Cot proteins to be more accessible to extraction procedures. It would be worthwhile to remark that several groups have described other small proteins involved in the assembly of the coat and cortex [24, 25] and SASP-E may well be another of this kind of function.

We have shown that complementation with the WT *sspE* gene restored the WT osmoresistant phenotype on germination plates [3, 4, 29]. Using the same construction, we observed that spores from the complemented mutant restored a WT coat proteins profile, suggesting that the coat defects of the *sspE* mutant does not involve a

secondary mutation and that the lack of SASP-E itself is responsible for this behavior.

To this end, we would be questioning how a defect in a protein synthesized in the forespore compartment (stage III of sporulation under sigma G transcription) could affect the envelope composition. It should be worthwhile to remark that SASP-E has also been detected in coat preparations from proteomic analyses, where it was among the most abundant proteins present [Henriques, A.O., personal communication, 18] and localized in part in the periphery of the forespore including the external cortex [8].

Another peculiarity is that *sspE* spores behaved similarly to those of *gerA* regarding their germination capacity in L-alanine (Fig. 1A). The sequence analysis of GerA receptors from several species allows us to identify particularities within the sequence that should help to understand the behavior observed. In particular, the germination receptor GerAA from *B. subtilis* belongs to the same family of proteins as GerIA from *B. cereus.* However, GerIA presents a 170 aa stretch at the N-terminal position that is absent in GerAA. Surprisingly, this 170 aa sequence presented similarities with SASP-E: in the 84 amino acids sequence of SASP-E protein, five glutamine-rich domains are found that showed high identity with those described in the N-terminal sequence of *B. cereus* GerIA protein: 13 QQVRKQNQQ, 41 QQVRKQNQQS, 52 QQGQ, 67 QQVRQQNQ, and 78 QNKQQN, where numbers indicate positions in SASP-E. These repetitions are relatable to Q-linker domains, a class of interdomain linkers found in bacteria, which constitute flexible linkers that have a role in the assembly of macromolecular arrays [36]. In *B. cereus*, Clements and Moir [2] postulated that these domains found in GerIA would be necessary for the protection and correct location of this protein near the inner membrane of spores. Testing several putative Q-rich domain deletion mutants of SASP-E regarding their influence in the sporulation program is a future aim, and would be a way to determine if SASP-E might function as a Q-linker domain able to protect and escort different proteins during their transit to the membrane. It is interesting to remark that suggestions for additional functions of γ-type SASP besides that of an amino acid reservoir was also postulated from the sequence analysis of γ-type SASP in the order Bacillales [34]. In accordance with this analysis, we have also shown that *Bacillus thuringiensis* SASP-E proteins have a very low Q content [4], correlated with an increased osmosensitive phenotype [3], comparable to the one observed for the *sspE* mutant in *B. subtilis* [29].

Several reports have clearly established the presence of channels between the forespore and the mother cell [1, 21, 35]. These channels may explain the crossing at both sides of DPA (during its synthesis in the mother cell and final location inside the spore cytoplasm). These or other channels would be operative in both directions, enabling small proteins such as SASP-E to play a chaperon like role in both compartments, explaining their presence in coat preparations [18].

As a corollary, the function of SASP-E in spores is certainly not only restricted to be the source of amino acids for starting outgrowth, a function that the abundant SASPα/β family of proteins also supplied. The results presented here suggest rather that SASP-E may be involved in the correct chronology of the sporulation program itself, either by participating in the communication between forespore and mother cell or by migrating into the mother cell compartment and stabilizing coat proteins. Either hypothesis involves the participation of SASP-E in the building of the outermost envelope of spores, the coat layer. This would be an attractive role for this small protein, for which despite being the most abundant in the spores, until now, no clear role has been assigned.

Although a great deal of work needs to be done to address the type of interaction predicted for SASP-E and the spore proteins, this is the first report of a direct involvement of SASP-E in the sporulation process.

Acknowledgments

We would like to thank Dr. A. O. Henriques for sending the antibodies against coats. This work was supported by research grants from the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) and the Universidad de Buenos Aires (UBA), from Argentina. PLB was supported by a student fellowship of UBA. CSR and SMR are career investigators of CONICET.

References

1. Camp, A. H. and R. Losick. 2008. A novel pathway of intercellular signaling in *Bacillus subtilis* involves a protein with similarity to a component of type III secretion channels. *Mol. Microbiol*. **6:** 402–417.
2. Clements, M. O. and A. Moir. 1998. Role of the *gerI* operon of *Bacillus cereus* 569 in the response of spores to germinants. *J. Bacteriol.* **180:** 6729–6735.
3. Cucchi, A. and C. Sanchez-Rivas. 1995. *ssp* Genes and spore osmotolerance in *Bacillus thuringiensis israelensis* and *Bacillus sphaericus*. *Curr. Microbiol.* **31:** 228–233.
4. Cucchi, A. and C. Sanchez-Rivas. 1998. SASP (small, acid-soluble spore proteins) and spore properties in *Bacillus thuringiensis israelensis* and *Bacillus sphaericus*. *Curr. Microbiol.* **36:** 220–225.
5. Driks, A. 2002. Proteins of the spore core and coat, in *Bacillus subtilis* and its closest relatives, pp. 527–536. *Bacillus subtilis and its Closest Relatives*. ASM Press, Washington, DC. A. L. Sonenshein, J. A. Hoch, and R. Losick (eds.).

6. Ebmeier, S. E., I. S. Tan, and K. R. Clapham. 2012. Small proteins link coat and cortex assembly during sporulation in *Bacillus subtilis*. *Mol. Microbiol.* **84:** 682–696.
7. Ferencko, L. and B. Rotman. 2010. Constructing fluorogenic *Bacillus* spores (F-spores) *via* hydrophobic decoration of coat proteins. *PLoS One* **19:** e9283.
8. Francesconi, S. C., T. J. MacAlister, B. Setlow, and P. Setlow. 1988. Immunoelectron microscopic localization of small, acid-soluble spore proteins in sporulating cells of *Bacillus subtilis. J. Bacteriol*. **170:** 5963–5967.
9. Hayes, C. S. and P. Setlow. 2001. An alpha/beta-type, small, acid-soluble spore protein which has very high affinity for DNA prevents outgrowth of *Bacillus subtilis* spores. *J. Bacteriol.* **183:** 2662–2266.
10. Henriques, A. O., L. R. Melsen, and C. P. Moran Jr. 1998. Involvement of superoxide dismutase in spore coat assembly in *Bacillus subtilis*. *J. Bacteriol*. **180:** 2285–2291.
11. Henriques, A. O. and C. P. Moran Jr. 2007. Structure, assembly, and function of the spore surface layers. *Annu. Rev. Microbiol*. **61:** 555–588.
12. Hudson, K. D., B. M. Corfe, E. H. Kemp, I. M. Feavers, P. J. Coote, and A. Moir. 2001. Localization of GerAA and GerAC germination proteins in the *Bacillus subtilis* spore. *J. Bacteriol*. **183:** 4317–4322.
13. Imamura, D., R. Kuwana, H. Takamatsu, and K. Watabe. 2010. Localization of proteins to different layers and regions of *Bacillus subtilis* spore coats. *J. Bacteriol.* **192:** 518–524.
14. Johnson, M. J., D. A. Todd, P. S. Sheperd, and A. Moir. 2006. ExsY and CotY are required for the correct assembly of the exosporium and spore coat of *Bacillus cereus. J. Bacteriol.* **188:** 7905–7913.
15. Kodama, T., T. Matsubayashi, T. Yanagihara, H. Komoto, K. Ara, K. Ozaki, *et al*. 2011. A novel small protein of *Bacillus subtilis* involved in spore germination and spore coat assembly. *Biosci. Biotechnol. Biochem.* **75:** 1119–1128.
16. Keijser, B. J. F., A. T. Beek, H. Rauwerda, F. Schuren, R. Montijn, H. van der Spek, and S. Brul. 2007. Analysis of temporal gene expression during *Bacillus subtilis* spore germination and outgrowth. *J. Bacteriol.* **189:** 3624–3634.
17. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:** 680–685.
18. Lai, E. M., N. D. Phadke, M. T. Kachman, R. Giorno, S. Vazquez, J. A. Vazquez, J. R. Maddock, and A. Driks. 2003. Proteomic analysis of the spore coats of *Bacillus subtilis* and *Bacillus anthracis*. *J. Bacteriol.* **185:** 1443–1454.
19. Magge, A., B. Setlow, A. E. Cowan, and P. Setlow. 2009. Analysis of dye binding by and membrane potential in spores of *Bacillus* species. *J. Appl. Microbiol.* **106:** 814–824.
20. Mason, J. M., R. H. Hackett, and P. Setlow. 1988. Studies on the regulation of expression of genes coding for small acid-soluble proteins of *Bacillus subtilis* spores using *lacZ* gene fusions. *J. Bacteriol.* **170:** 230–244.
21. Meisner, J., X. Wang, M. Serrano, A. O. Henriques, and C. P. Moran Jr. 2008. A channel connecting the mother cell and forespore during bacterial endospore formation. *Proc. Natl. Acad. Sci. USA* **105:** 15100–15105.
22. McKenney, P. T., A. Driks, H. A. Eskandarian, P. Grabowski, J. Guberman, K. H. Wang, *et al*. 2010. A distance-weighted interaction map reveals a previously uncharacterized layer of the *Bacillus subtilis* spore coat. *Curr. Biol.* **20:** 934–938.
23. Moir, A. 2006. How do spores germinate? *J. Appl. Microbiol*. **101:** 526–530.
24. Moir, A., B. M. Corfe, and J. Behravan. 2002. Spore germination. *Cell Mol. Life Sci.* **59:** 403–409.
25. Paidhungat, M. and P. Setlow. 2001. Localization of a germinant receptor protein (GerBA) to the inner membrane of *Bacillus subtilis* spores. *J. Bacteriol.* **183:** 3982–3990.
26. Paidhungat, M. and P. Setlow. 2001. Spore germination and outgrowth in *Bacillus subtilis* and its closest relatives, pp. 537–548. *In* A. L. Sonenshein, J. A. Hoch, and R. Losick (eds.). *Bacillus subtilis and its Closest Relatives*. ASM Press, Washington, DC.
27. Paidhungat, M., K. Ragkousi, and P. Setlow. 2001. Genetic requirements for induction of germination of spores of *Bacillus subtilis* by Ca^{2+}-dipicolinate. *J. Bacteriol.* **183:** 4886–4893.
28. Rotman, Y. and M. L. Fields. 1968. A modified reagent for dipicolinic acid analysis. *Anal. Biochem.* **22:** 168.
29. Ruzal, S. M., A. F. Alice, and C. Sanchez-Rivas. 1994. Osmoresistance of spores from *Bacillus subtilis* and the effect of *ssp* mutations. *Microbiology.* **140:** 2173–2177.
30. Schaeffer, P., J. Miller, and J. Aubert. 1965. Catabolic repression of bacterial sporulation. *Proc. Natl. Acad. Sci. USA* **554:** 701–711.
31. Setlow, B., P. G. Wahome, and P. Setlow. 2008. Release of small molecules during germination of spores of *Bacillus* species. *J. Bacteriol.* **190:** 4759–4763.
32. Tovar-Rojo, F., R. M. Cabrera-Martinez, B. Setlow, and P. Setlow. 2003. Studies on the mechanism of the osmoresistance of spores of *Bacillus subtilis*. *J. Appl. Microbiol.* **95:** 167–179.
33. Vary, J. C. and H. O. Halvorson. 1965. Kinetics of germination of *Bacillus* spores. *J. Bacteriol.* **89:** 1340–1347.
34. Vyas, J., J. Cox, B. Setlow, W. H. Coleman, and P. Setlow. 2011. Extremely variable conservation of γ-type small, acid-soluble proteins from spores of some species in the bacterial order Bacillales. *J. Bacteriol.* **193:** 1884–1892.
35. Vepachedu, V. R. and P. Setlow. 2007. Role of SpoVA proteins in release of dipicolinic acid during germination of *Bacillus subtilis* spores triggered by dodecylamine or lysozyme. *J. Bacteriol.* **189:** 1565–1572.
36. Wootton, J. C. and M. Drummond. 1989. The Q linker: A class of interdomain sequences found in bacterial multidomain regulatory proteins. *Protein Eng.* **2:** 535–543.