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Analysis of Deamidation of Small, Acid-Soluble Spore Proteins from *Bacillus subtilis* In Vitro and In Vivo

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Deamidation of one specific asparagine residue in an α/β-type small, acid-soluble spore protein (SASP) of *Bacillus subtilis* took place readily in vitro (time for 50% deamidation [$t_{1/2}$], ~1 h at 70°C), and the deamidated SASP no longer bound to DNA effectively. However, DNA binding protected against this deamidation in vitro. A mutant α/β-type SASP in which the reactive asparagine was changed to aspartate also failed to bind to DNA in vitro, and this protein did not restore UV radiation and heat resistance to spores lacking the majority of their α/β-type SASP. When expressed in *Escherichia coli*, where it is bound to DNA, the α/β-type SASP deamidated with a $t_{1/2}$ of 2 to 3 h at 95°C. However, the α/β-type SASP was extremely resistant to deamidation within spores ($t_{1/2}$, >50 h at 95°C). A γ-type SASP of *B. subtilis* also deamidated readily in vitro ($t_{1/2}$ for one net deamidation, ~1 h at 70°C), but this protein (which is not associated with DNA) deamidated fairly readily in spores ($t_{1/2}$, ~1 h at 95°C). Total spore core protein also deamidated in vivo, although the rate was two- to threefold slower than that of deamidation of total protein in heated vegetative cells. These data indicate that protein deamidation is slowed significantly in spores, presumably due to the spore's environment. However, α/β-type SASP are even more strongly protected against deamidation in vivo, presumably by their binding to spore DNA. Thus, not only do α/β-type SASP protect spore DNA from damage; DNA also protects α/β-type SASP.

Protein deamidation is a nonenzymatic process that contributes to loss of protein function (35, 40, 41). Deamidation occurs most readily at asparagine residues preceding small residues such as glycine, serine, and alanine. Under mild conditions (37°C, pH 7.4), model peptides containing NG, NS, or NA sequences deamidate with half-lives of approximately 1, 8, and 20 days, respectively (35). However, the deamidation rates for these sequences within structured proteins are much slower (41). Asparagine deamidation in proteins takes place through an intramolecular nucleophilic attack by the amide backbone nitrogen of the residue at the carboxyl side of the asparagine on the γ-carbonyl carbon of the asparagine side chain, releasing ammonia and ultimately forming isoaspartate and aspartate residues through the hydrolysis of a cyclic succinimidyl intermediate (35, 40). L-Isoaspartate residues constitute 60 to 70% of the deamidation products and have been postulated to introduce kinks into the peptide backbone and thereby disrupt protein structure and function (4). Conversion of asparagine to L-aspartate may also alter protein function (4, 41).

Although protein deamidation can occur under physiological conditions (11), growing cells presumably minimize the effects of this protein damage through protein turnover, such that isoaspartate does not normally accumulate to high levels in cellular proteins. In contrast, spores of *Bacillus* and *Clostridium* species are metabolically inert; consequently, spore proteins synthesized during sporulation are present throughout spore dormancy (31, 32). In addition, bacterial spores are able to survive conditions (5 h at 100°C for *Bacillus stearothermophilus*) that could result in a large degree of protein deamidation (26). *Bacillus subtilis* and *B. stearothermophilus* also appear to lack the enzyme protein isoaspartyl methyltransferase, which has been implicated in the repair of asparagine deamidation in proteins (4, 15). As a consequence of these properties, bacterial spores could be an interesting system in which to study protein deamidation. Our interest in this process was further piqued by the realization that a family of highly abundant spore proteins, the α/β-type small, acid-soluble spore proteins (SASP), contain an NG sequence that is tremendously conserved throughout evolution (5, 31).

The α/β-type SASP of *Bacillus* and *Clostridium* species are nonspecific DNA binding proteins that are expressed only within the forespore compartment of the developing sporangium, and they comprise 5 to 10% of the total spore protein (5, 31). In most species, there are two major α/β-type SASP along with four to seven additional, minor α/β-type SASP which are expressed at lower levels. The primary sequences of all α/β-type SASP are very highly conserved both within and between species (5, 31). These proteins saturate the spore chromosome, alter the chemical and photochemical reactivity of the DNA, and are major or significant determinants of spore resistance to heat, UV radiation, and oxidizing agents (19, 32). Indeed, loss of α/β-type SASP function is extremely detrimental to long-term spore survival. In addition to α/β-type SASP, spores of *Bacillus* species also contain a single γ-type SASP (31, 32). This protein is also present only in spores and at high levels (5 to 10% of total spore protein), but its primary sequence is not similar to that of α/β-type SASP. However, SASP-γ of *B. subtilis* does have two NS sequences and one NA sequence (31). In further contrast to α/β-type SASP, SASP-γ of *B. subtilis* does not bind to DNA in vitro or in vivo, and its only known function is to be degraded and supply amino acids during spore germination (9, 13, 31, 32). In this communication, we report the results of studies on the deamidation of α/β- and γ-type SASP in vivo and in vitro as well as analyses of the deamidation of vegetative cell and spore core protein.

MATERIALS AND METHODS

Bacteria and plasmids used, and conditions for growth and heat treatment. The bacterial strains and plasmids used in these studies are listed in Table 1. All *B. subtilis* strains are derivatives of strain 168. *B. subtilis* was transformed as

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TABLE 1. Bacterial strains and plasmids used in these studies

Bacterial strain or plasmid	Characteristics	Source or reference
Bacteria		
B. subtilis		
PS356	Δ*sspA* Δ*sspB trpC2*	19
PS482	Δ*sspA* Δ*sspB* Δ*sspE trpC2* Cm^r	13
PS832	trp^+ revertant of strain 168	Laboratory stock
PS1450	Δ*sspA* Δ*sspB* (pSspC^{wt}) Km^r	39
PS1465	Δ*sspA* Δ*sspB* (pSspC^{G52A}) Km^r	39
PS2437	Δ*sspA* Δ*sspB* Δ*sspE* (pSspC^{wt}) Cm^r Km^r	This work
PS2438	Δ*sspA* Δ*sspB* Δ*sspE* (pSspC^{G52A}) Cm^r Km^r	This work
PS2526	Δ*sspA* Δ*sspB* (pSspC^{N48D}) Km^r	This work
E. coli		
PS708	JM107 (pPS708) Amp^r	27
PS1342	JM107 (pPS1342) Amp^r	39
BL21(DE3)	DE3 lysogen expressing T7 RNA polymerase under the control of the *lac* promoter	37
Plasmids		
pDG148	Amp^r Km^r	36
pET3	T7 promoter Amp^r	37
pPS708	pDG148 with a 0.6-kb fragment containing *sspC*	27
pPS1342	pDG148 with a 0.6-kb fragment containing the gene encoding SspC^{G52A}	39
pPS1451	pUC19/pUB110 hybrid plasmid with a 0.6-kb fragment containing *sspC* under the control of the *sspB* promoter	39
pPS2461	pET3 with a 0.3-kb fragment containing *sspC*	This work
pPS2474	pET3 with a 0.3-kb fragment containing the gene encoding SspC^{N48D}	This work
pSspC	pUB110 with *sspC* under the control of the *sspB* promoter	39
pSspC^{G52A}	pUB110 with *sspC*^{G52A} under the control of the *sspB* promoter	39
pSspC^{N48D}	pUB110 with *sspC*^{N48D} under the control of the *sspB* promoter	This work

previously described (3), and transformants were selected by their resistance to kanamycin (10 μg/ml). *B. subtilis* was routinely grown and sporulated at 37°C in 2× SG medium (12), and spores were purified either by high salt-lysozyme treatment (PS2437 and PS2438) or by sonication and distilled-water washing (all other strains) as described previously (21). All spores used were free (>98%) from sporulating cells and cell debris.

Spores (15 to 16 mg [dry weight]/ml) were routinely heated in 5 ml of distilled water. In some experiments, the spore core pH was first elevated by incubating spores (3 to 4 mg [dry weight]/ml) in 0.2 M Tris-HCl (pH 8.6)–20 mM $(NH_4)_2SO_4$ for 18 h at 4°C. Previous work has indicated that this treatment elevates the core pH of *Bacillus megaterium* spores to ~7.3 (38). These spores were heated as described above but in 0.2 M Tris-HCl (pH 8.6)–20 mM $(NH_4)SO_4$. Killing of spores by heat and UV radiation was measured as described previously (23).

Escherichia coli strains were grown at 37°C in 2× YT medium (16 g of tryptone, 10 g of yeast extract, and 5 g of NaCl per liter of distilled H_2O) with ampicillin (50 μg/ml). *E. coli* PS708 and PS1342 were grown to an optical density at 600 nm of 1.5, at which time isopropyl-β-D-thiogalactopyranoside (IPTG) was added to 0.5 mM to induce synthesis of α/β-type SASP; the cells were harvested by centrifugation after 2 h of further incubation, the cell pellet was resuspended in 2 ml of 50 mM Tris-HCl (pH 7.4)–150 mM NaCl, and the suspension was quickly brought to 95°C by the addition of 8 ml of preheated buffer. *B. subtilis* vegetative cells were grown and heated similarly but without IPTG induction.

SASP purification. *E. coli* BL21(DE3), containing plasmid pPS2461 or pPS2474 with the genes encoding SspC or SspC^{N48D}, respectively, under the control of a phage T7 promoter, was grown to an optical density at 600 nm of 1.5, adjusted to 0.5 mM IPTG, and incubated for 1 h. Cells were harvested, washed once with 0.15 M NaCl, frozen, and lyophilized. Cells (2 to 4 g [dry weight]) were broken by 2 min of dry rupture (100 mg [dry weight] at a time) in a dental amalgamator (Wig-L-Bug) with glass beads (150 mg) as the abrasive; SASP were extracted twice with 50 ml of ice-cold 3% acetic acid–30 mM HCl. SspC^{G52A} and an α/β-type SASP from *Clostridium bifermentans* (the *C. bifermentans* gene coding for SASP-C was cloned into plasmid pET3 for high-level expression [2]) were extracted from *E. coli* similarly. The acid extract was dialyzed in Spectrapor 3 tubing at 4°C against 1% acetic acid (three changes of 10 liters), and the dialyzed material was run through a DEAE-cellulose column preequilibrated in 1% acetic acid. The flowthrough fractions were lyophilized, and the dry residue was dissolved in 5 to 10 ml of 8 M urea–10 mM Tris-maleate (pH 5.5). The dissolved protein was adsorbed to a CM-cellulose column (200 ml) preequilibrated in 10 mM Tris-maleate (pH 5.5), and proteins were eluted with a linear salt gradient (600 ml) of 0 to 400 mM NaCl in 10 mM Tris-maleate (pH 5.5). Appropriate fractions were pooled, lyophilized, redissolved in distilled water, and dialyzed against 10 mM sodium phosphate (pH 7.5).

SspC, SspC^{G52A}, and SASP-γ were purified from heated cells or from heated and unheated spores essentially as described above, with the following modifications: lyophilized cells or spores (100 to 150 mg [dry weight]) were broken by either 2 min (cells) or 8 min (spores) of dry rupture and extracted with 3 ml of 3% acetic acid–30 mM HCl, and proteins were purified on a 20 ml CM-cellulose column in 10 mM Tris-maleate (pH 5.5) with a linear salt gradient (60 ml) of 0 to 400 mM NaCl. Deamidated SspC and SASP-γ were found to copurify with native SspC and SASP-γ, respectively. All SASP analyzed were ≥90% pure as assessed by polyacrylamide gel electrophoresis at low pH followed by staining with Coomassie blue (25).

Extraction of spore and cell proteins. Spores were decoated in freshly prepared 50 mM Tris-HCl (pH 8.0)–1% sodium dodecyl sulfate–8 M urea–50 mM dithiothreitol (DTT)–10 mM EDTA for 90 min at 37°C and then washed five times with 10 mM potassium phosphate (pH 7.0)–10 mM EDTA–150 mM NaCl (21, 30). Decoated spores were lysed with lysozyme (16 μg/ml) in wash buffer adjusted to 0.3 mM in phenylmethylsulfonyl fluoride. DNA was precipitated with 1% streptomycin sulfate, and the supernatant fluid was dialyzed in Spectrapor 4 tubing at 4°C against 2 mM potassium phosphate (pH 7.0)–0.05 mM phenylmethylsulfonyl fluoride–0.1 mM EDTA. The dialyzed protein was frozen, lyophilized, and dissolved in distilled water at 1 to 5 mg/ml. SASP are not retained during this procedure.

Lyophilized *B. subtilis* cells (150 mg [dry weight]) were broken by 2 min of dry rupture in a dental amalgamator with glass beads (150 mg) as the abrasive. Total protein was extracted twice with 0.75 ml of freshly prepared 8 M urea, and the two extracts were pooled prior to analyses.

SASP deamidation and analysis. Purified SASP (0.5 mg/ml) were deamidated in 25 mM sodium phosphate (pH 7.5)–5 mM DTT. SspC-poly(dG) · poly(dC) (Sigma) complexes were formed at a 2:1 (wt/wt) protein/DNA ratio with 0.5 mg of SspC per ml in 25 mM sodium phosphate (pH 7.5)–5 mM DTT for 20 min at 22°C. SspC was purified from this complex by the addition of solid urea to 8 M followed by passage over a DEAE-cellulose column equilibrated with 3% acetic acid. The flowthrough fractions were lyophilized, dissolved in 8 M urea, and dialyzed against 10 mM sodium phosphate (pH 7.5) prior to use.

Deamidation reaction products were analyzed by reverse-phase high-performance liquid chromatography (HPLC) after trypsin digestion. SspC (~40 μg) was digested with trypsin (5 μg; Worthington) in 200 mM NH_4HCO_3–1 M urea–10 mM $CaCl_2$ for 15 to 18 h at 22°C, and digests were injected onto a Waters μBondapak C_{18} column. After the column was washed for 5 min with a solution containing 98% buffer A and 2% buffer B, peptides were eluted at a flow

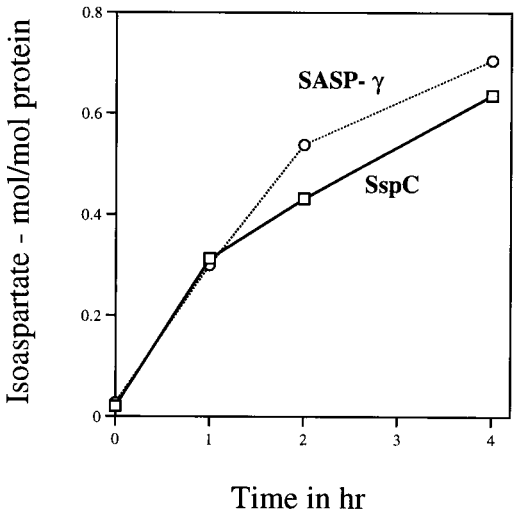

FIG. 1. Kinetics of isoaspartate formation in SASP in vitro. Purified protein (0.5 mg/ml) was heated at 70°C in 25 mM sodium phosphate (pH 7.5)–5 mM DTT, and isoaspartate and total protein were quantitated as described in Materials and Methods.

rate of 1 ml/min with a linear gradient of buffer B as follows: 5 to 60 min, 2 to 32% buffer B; and 60 to 85 min, 32 to 98% buffer B. Buffer A was 0.06% aqueous trifluoroacetic acid, and buffer B was 0.052% trifluoroacetic acid in 80% acetonitrile. Peptides were detected by their UV absorbance at 214 nm and collected for amino acid analysis and determination of isoaspartate content.

Isoaspartate residues were quantitated by using the Isoquant protein deamidation detection kit (Promega) according to the manufacturer's instructions, except that proteins (100 to 200 pmol) and peptides (50 pmol) were methylated at 30°C for 1 or 2 h, respectively. All isoaspartate levels are averages of at least duplicate determinations. Levels of peptides and proteins were quantitated by amino acid analysis following acid hydrolysis (5) or by the Lowry method (16).

PCR amplification and site-directed mutagenesis. The SspC mutant in which the asparagine residue at position 48 has been replaced by an aspartic acid (the N48D mutant) was generated by using the Transformer site-directed mutagenesis kit (Clontech) according to the manufacturer's instructions. Plasmid pPS1451, a hybrid plasmid derived from pUC19 and pUB110, contains a 0.6-kb *Hin*dIII fragment carrying *sspC* under the control of the strong, forespore-specific *sspB* promoter (39). Phosphorylated oligonucleotide primers that were complementary, except for mismatches (underlined nucleotides), to the *Pst*I site of pUC19 (5′-GCATGCCTATAGGTCGACTC) and the S^{45} to G^{52} coding region of *sspC* (5′-ACCAACTGAACCGTCTGCACGAG) (8), were used to synthesize a mutagenized plasmid lacking the *Pst*I site and with an aspartate replacing asparagine 48. Mutagenized plasmids were propagated by transformation into a mismatch-repair-deficient *E. coli* strain (*mutS*::Tn*10* Tet^r) with selection for ampicillin resistance. Mutagenized plasmids were selected from parental plasmids by multiple rounds of transformation, plasmid isolation, *Pst*I digestion, and retransformation of *E. coli* JM83. The identity of the mutagenized gene encoding $SspC^{N48D}$ was confirmed by DNA sequencing. Plasmid $pSspC^{N48D}$ was released from the parental plasmid by digestion with *Bam*HI (39), religated, and used to transform *B. subtilis* to kanamycin resistance.

Primers were designed to PCR amplify a 318-bp fragment containing the *sspC* gene (including its ribosome binding site and transcription terminator) with Vent polymerase. The upstream primer, SSPC1 (5′-ACGTGGATCCAGGAGATGAATAAGATGG), and downstream primer, SSPC2 (5′-ACGTGGATCCACCATTAGTTCTGTATGG), both contained *Bam*HI sites and 5′ flanking sequences (underlined residues) for cloning purposes. The PCR fragment was cloned into plasmid pET3, generating plasmid pPS2461. The N48D mutagenic oligonucleotide (see above), in conjunction with primers SSPC1 and SSPC2, was used to generate a 318-bp fragment containing the gene encoding $SspC^{N48D}$ by the PCR megaprimer method (1). This PCR fragment was cloned into plasmid pET3, generating plasmid pPS2474. Analysis of plasmids pPS2461 and pPS2474 by digestion with *Bsm*AI confirmed that the *sspC* genes were under the control of the T7 promoter, and DNA sequence analysis confirmed that both genes had the expected sequence.

DNase protection assays. The DNA binding activity of SspC was assessed by its ability to protect linearized plasmid pUC19 from DNase I digestion. SspC (0.6 to 1.2 mg/ml) was complexed with *Eco*RI-linearized pUC19 (0.12 mg/ml) in 10 mM sodium phosphate (pH 7.5)–3 mM $MgCl_2$ for 10 min at 22°C. DNase I was added to a concentration of 0.04 mg/ml, and the solution was incubated at 37°C for 15 min. Digestion was arrested by adjusting the solution to 1% sodium dodecyl sulfate–20 mM EDTA; this was followed by ethanol precipitation of the DNA and its analysis by 1% agarose gel electrophoresis (29).

RESULTS

SASP deamidation in vitro. The α/β-SASP chosen for most of this work was SspC, a minor α/β-type SASP from *B. subtilis* which contains the NG sequence conserved in all *Bacillus* species α/β-type SASP analyzed to date (8, 31, 32). SspC was chosen because it is readily overexpressed in *E. coli*, binds well to DNA in vitro, and has effects on spore resistance in vivo (29, 39). In addition, the interaction of SspC with DNA has been well characterized, and several variants have been purified and their interactions with DNA have been studied in vivo and in vitro (29, 39). Purified SspC typically contained only small amounts of isoaspartate, and this level rose significantly upon heat treatment (Fig. 1). The rate of isoaspartate formation in SspC at 70°C and pH 7.5 corresponded to a time for 50% deamidation ($t_{1/2}$) of approximately 1 h for the deamidation of one residue (recall that 60 to 70% of deamidation products are L-isoaspartate). This rate of isoaspartate formation is in good agreement with rates observed in vitro with peptides containing NG sequences (11). Incubation for longer than 4 h led to further accumulation of isoaspartate, presumably due to other minor sites of deamidation (data not shown). Analysis of the deamidation of a purified α/β-type SASP from *C. bifermentans* (which also contains the conserved NG sequence) gave a rate of deamidation similar to that seen for SspC (data not shown).

The fact that primarily one asparagine residue underwent rapid deamidation in α/β-type SASP strongly suggested that this residue was asparagine 48 (N^{48}), which is followed by a glycine residue. To confirm this, we analyzed tryptic peptides from heated and unheated SspC by HPLC (Fig. 2). Four of the seven predicted tryptic peptides of SspC were readily identified on these chromatograms. The remaining three tryptic peptides are small and do not contain asparagine or aspartate residues (8). Although some minor changes are apparent, for only one

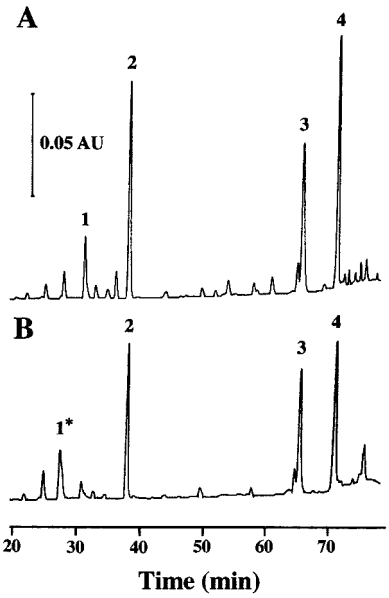

FIG. 2. Analysis of tryptic peptides from unheated (A) and heated (B) SspC. SspC and heat-treated SspC (4 h, 70°C [Fig. 1]) were digested and the products were resolved by HPLC as described in Materials and Methods. The SspC peptides identified are as follows: 1 and 1*, A^{47} to K^{57}; 2, L^{62} to H^{72}; 3, L^{29} to R^{46}; and 4, S^{9} to K^{28}.

TABLE 2. Effect of heat treatment on levels of isoaspartate in SspC and its tryptic peptides[a]

Sample analyzed[b]	Isoaspartate level (mol/mol of protein or peptide) in:	
	Unheated SspC	Heated SspC
Intact protein	0.01	0.56
Tryptic peptide 1 (A^{47}–K^{57})[c]	0.08	
Tryptic peptide 1* (A^{47}–K^{57})[c]		0.51
Tryptic peptide 2 (L^{62}–H^{72})	0.01	0.06
Tryptic peptide 3 (L^{29}–R^{46})	<0.01	<0.01
Tryptic peptide 4 (S^{9}–K^{28})	0.01	0.05

[a] SspC was digested with trypsin before or after heat treatment (4 h at 70°C), peptides were isolated by HPLC, and isoaspartate levels were determined in the intact protein and isolated tryptic peptides as described in Materials and Methods.

[b] The tryptic peptides are numbered as in Fig. 2.

[c] The levels of peptides 1 and 1* were quantitated by amino acid analysis. All other peptides were assumed to have been recovered with approximately the same yield.

asparagine-containing tryptic peptide (termed peptide 1 or 1*) did the elution pattern change appreciably as a result of heat treatment, and peptides 1 and 1* were both identified as A^{47} to K^{57} by amino acid analysis. The A^{47} to K^{57} peptide contains only one asparagine residue, which is part of the labile NG sequence. In order to positively identify N^{48} as the major site of deamidation, tryptic peptides from heat-treated and untreated protein were analyzed for isoaspartate (Table 2). As expected, the great majority of the isoaspartate in heat-treated SspC was found in the A^{47} to K^{57} peptide. Note that the level of isoaspartate in peptide 1* was approximately 0.5 mol/mol of protein and not 1.0 mol/mol of protein. This suggests that the HPLC column used to purify the tryptic peptides did not resolve L-aspartate- and L-isoaspartate-containing deamidation products. The sites of deamidation in the heated α/β-type SASP from *C. bifermentans* were similarly identified as the conserved NG sequence (major deamidation product) and an NS sequence located near the carboxy terminus (minor deamidation product) (data not shown). This latter NS sequence is not conserved in α/β-type SASP and is not found in SspC. It should be noted that the enzyme used in the commercial isoaspartate detection system has different affinities for the various isoaspartate-containing peptide substrates, and therefore the extent of deamidation at some minor sites may be underestimated.

SASP-γ from *B. subtilis* also deamidated readily in vitro at 70°C (Fig. 1). Although SASP-γ does not contain NG sequences, it does have two NS sequence and one NA sequence (31), all of which can deamidate moderately rapidly, although not as rapidly as an NG sequence (35, 40). The $t_{1/2}$ for one net deamidation in SASP-γ was approximately 1 h at 70°C (Fig. 1).

Loss of function of SspC deamidated in vitro and $SspC^{N48D}$. As mentioned above, the primary sequences of α/β-type SASP have been tremendously conserved throughout evolution (31). This sequence conservation includes the NG sequence that is most susceptible to deamidation. The asparagine residue in this sequence has not been changed to an aspartate in any wild-type α/β-type SASP of *Bacillus* species, implying that deamidation of this residue might destroy α/β-type SASP function. Indeed, the NG sequence is in the region of α/β-type SASP thought to be directly involved in DNA binding (24). To test the functionality of deamidated SspC in vitro, DNase protection provided by SspC that had been deamidated to different degrees was tested. As found previously (29), untreated SspC provided significant DNase protection to plasmid pUC19 (Fig. 3A, lane 3). However, SspC heated for 4 h at 70°C gave no DNase protection (Fig. 3A, lane 6). Analysis of the DNase protection provided by SspC heated for intermediate periods of time (Fig. 3A, lanes 4 and 5) gave the results expected based on the degree of deamidation in these samples as determined by isoaspartate quantitation (data not shown). These results suggest that conversion of the conserved asparagine residue in the NG sequence to either aspartate or isoaspartate destroys or at least greatly decreases the ability of α/β-type SASP to either bind to DNA at all or bind to DNA productively.

To demonstrate conclusively that the loss of DNA binding of SspC was due to N^{48} deamidation and not some other change in SspC, we analyzed the properties of an SspC variant in which N^{48} had been changed to aspartate. The HPLC profile of a tryptic digest of purified $SspC^{N48D}$ was essentially identical to that of SspC deamidated in vitro (data not shown), suggesting that heat-induced changes in SspC were confined to deamidation at N^{48}. Most importantly, unheated $SspC^{N48D}$ failed to protect linearized pUC19 from DNase at protein-to-DNA ratios of 5:1 and 10:1 (wt/wt) (Fig. 3B; compare lanes 1 and 2 to lanes 3 and 4). Thus, $SspC^{N48D}$ behaved analogously to deamidated SspC, and deamidation at N^{48} is sufficient to eliminate high-affinity binding between α/β-type SASP and DNA in vitro.

Function of $SspC^{N48D}$ in vivo. Because neither deamidated SspC nor $SspC^{N48D}$ exhibited binding to DNA in vitro, we wanted to assess the function of $SspC^{N48D}$ (and by analogy, all deamidated α/β-type SASP) in vivo. Consequently, the gene encoding $SspC^{N48D}$ in plasmid pUB110 under the control of a strong forespore-specific promoter was introduced into *B. subtilis* PS356, which lacks the *sspA* and *sspB* genes that encode the major α/β-type SASP (α and β, respectively) of *B. subtilis*.

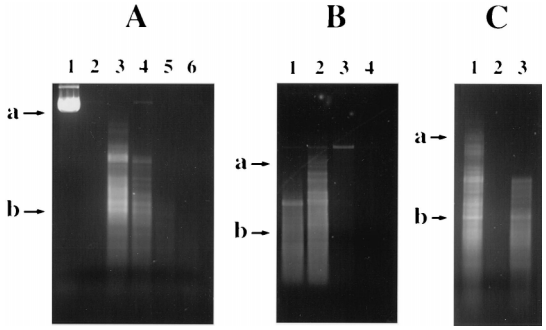

FIG. 3. Ability of various SspC samples to provide DNase protection to plasmid pUC19. SspC and $SspC^{N48D}$ were purified, treated, complexed with linearized pUC19, and DNase treated, and DNA was isolated and analyzed by agarose gel electrophoresis as described in Materials and Methods. The arrows labeled a and b denote the migration positions of 2.3- and 0.56-kb size markers, respectively. (A) Effect of heat treatment on binding of SspC to DNA. Samples run in the various lanes are as follows: lane 1, no DNase treatment; lane 2, no SspC; lane 3, plus unheated SspC (10:1 protein/DNA ratio); lane 4, plus SspC heated 1 h at 70°C (10:1 protein/DNA ratio); lane 5, plus SspC heated 2 h at 70°C (10:1 protein/DNA ratio); and lane 6, plus SspC heated 4 h at 70°C (10:1 protein/DNA ratio). (B) Lack of DNase protection by $SspC^{N48D}$. The samples run in the various lanes are as follows: lane 1, plus SspC (5:1 protein/DNA ratio); lane 2, plus SspC (10:1 protein/DNA ratio); lane 3, plus $SspC^{N48D}$ (5:1 protein/DNA ratio); and lane 4, plus $SspC^{N48D}$ (10:1 protein/DNA ratio). The band at ~2.7 kb in lane 3 is probably a microdrop from the incubation that was not exposed to DNase. (C) Binding to poly(dG) · poly(dC) protects SspC against deamidation. SspC was heated at 70°C for 4 h with and without poly(dG) · poly(dC) as described in Materials and Methods. The SspC was then purified and tested for its ability to provide DNase protection at a 10:1 (wt/wt) protein/DNA ratio. The samples in the various lanes are as follows: lane 1, plus unheated SspC; lane 2, plus SspC heated without poly(dG) · poly(dC); and lane 3, plus SspC heated with poly(dG) · poly(dC).

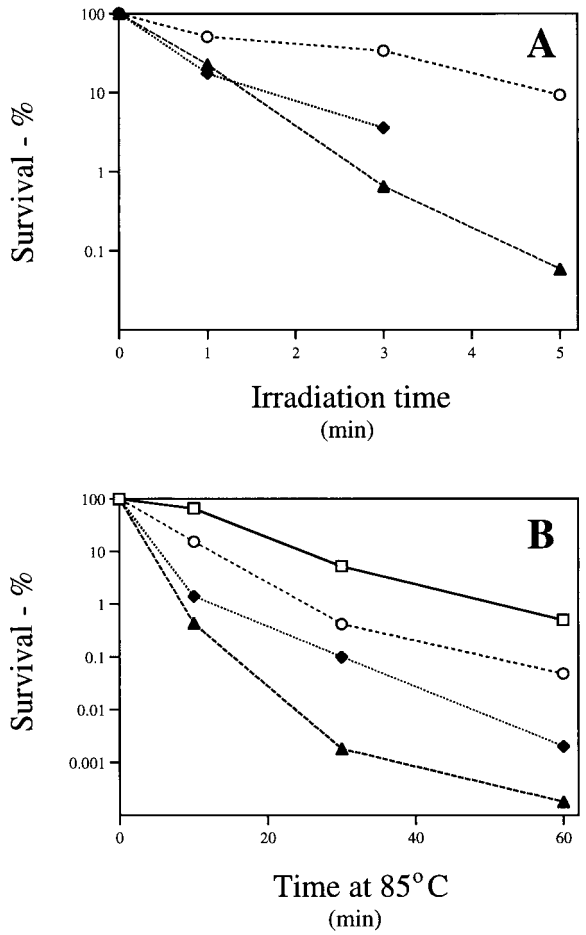

FIG. 4. UV and heat resistance of wild-type spores and $\alpha^-\beta^-$ spores with and without SspC or $SspC^{N48D}$. Spores of various strains were prepared and spore UV (A) and heat (B) resistance was analyzed as described in Materials and Methods. Strain PS832 (wild type) was not used in the UV resistance study because little or no killing of this strain occurs under the conditions used. The symbols used for the various strains are as follows: PS832 (wild type), □; PS356 ($\alpha^-\beta^-$), ◆; PS1450 ($\alpha^-\beta^-$ overexpressing SspC), ○; and PS2526 ($\alpha^-\beta^-$ overexpressing $SspC^{N48D}$), ▲.

Previous work has shown that this plasmid results in production of high levels of its encoded α/β-type SASP in the mature spore (39); indeed, analysis of spores of strain PS356 carrying $pSspC^{N48D}$ showed levels of $SspC^{N48D}$ which were equivalent to those expressed from pSspC in this strain (data not shown). Spores of strain PS356 (termed $\alpha^-\beta^-$) are significantly more sensitive to heat and UV radiation than are wild-type spores, while high SspC levels restore significant heat and UV resistance to otherwise $\alpha^-\beta^-$ spores (19, 39) (Fig. 4). However, $\alpha^-\beta^-$ spores with high levels of $SspC^{N48D}$ exhibited resistance to heat and UV radiation that was similar to or even less than that of $\alpha^-\beta^-$ spores (Fig. 4). These results suggest that $SspC^{N48D}$ and, by analogy, all α/β-type SASP deamidated at the residue equivalent to N^{48} of SspC do not bind productively to DNA in vivo.

Inhibition of deamidation with DNA. The results given above indicate that an α/β-type SASP with aspartate replacing the conserved asparagine in the NG sequence does not bind DNA in vivo or in vitro. Since deamidation of this asparagine residue has a $t_{1/2}$ in vitro of ~1 h at 70°C, one might predict that spores of *Bacillus* species would be sensitive to incubation for a few h at 70°C, but this is not the case (26). Therefore, α/β-type SASP must be protected against deamidation in spores. Preliminary nuclear magnetic resonance and circular dichroism spectroscopic evidence suggests that SspC alone has little, if any, secondary structure in solution (14, 20). However, SspC acquires significant secondary structure upon binding to DNA (20), and it also becomes protease resistant (28). This acquisition of secondary structure might preclude the formation of the peptide backbone conformation required for asparagine deamidation, especially since the NG sequence is likely to be in the region of the protein involved in DNA binding (7, 24). To determine if SspC undergoes deamidation while bound to DNA, complexes of SspC and poly(dG) · poly(dC) were formed with excess DNA (see Materials and Methods) and then heated at pH 7.5 and 70°C. Poly(dG) · poly(dC) was used for this experiment because it is the DNA to which SspC binds with the highest affinity (29). Analysis of SspC purified from this protein-DNA complex heated for 4 h gave 0.35 mol of isoaspartate/mol of protein compared to 0.63 mol of isoaspartate/mol of protein in SspC heated in the absence of DNA. Thus, SspC from the protein-DNA complex deamidated at approximately one-fourth the rate ($t_{1/2}$, ~4 h) of SspC by itself. In addition, SspC purified from the heated protein-DNA complex retained a large degree of DNA binding activity as measured by DNase protection (Fig. 3C, lane 3), when compared to SspC heated in the absence of DNA (Fig. 3C, lane 2).

Deamidation of SASP in vivo. The observation that DNA binding protects SspC from deamidation in vitro suggested that a similar phenomenon could be occurring within the spore. To address this question, isoaspartate levels were measured in SspC purified from spores heated for 2 h at 95°C. Although these conditions kill >99% of *B. subtilis* spores, *B. stearothermophilus* spores are able to withstand this treatment with minimal killing (26). To facilitate SspC purification, the *B. subtilis* strain used for this experiment lacks SASP-α, -β, and -γ. The SspC purified from unheated spores contained no detectable isoaspartate, and SspC from heated spores contained only a minute amount of isoaspartate (Table 3). To determine if the protection from deamidation in vivo was due to the binding of SspC to spore DNA, identical experiments were performed with spores overexpressing a mutant form of SspC, $SspC^{G52A}$. This protein does not protect linear plasmids from DNase digestion in vitro and fails to restore heat and UV resistance in $\alpha^-\beta^-$ spores (39). $SspC^{G52A}$ from unheated spores contained a minute amount of isoaspartate, and this value rose only slightly in heated spores (Table 3). While this result might suggest that it is not DNA binding that protects SspC against deamidation in vivo, $SspC^{G52A}$ does bind to DNA with an attendant change in $SspC^{G52A}$ structure as determined by circular dichroism spectroscopy (14, 20). However, this binding does not appreciably alter the DNA properties (20). Presumably, $SspC^{G52A}$ still associates with DNA in vivo, resulting in protection of $SspC^{G52A}$ from deamidation, although spore DNA properties are not affected by this mutant protein.

Although the above data are consistent with binding of SspC to DNA being the major reason for the protection of α/β-type SASP against deamidation in spores, the environment in the spore is very different from the conditions we used in vitro. In an attempt to distinguish between DNA binding and the spore environment as the reason for protection against SspC deamidation, we analyzed the deamidation of SspC in *E. coli* expressing this protein, as previous work has shown that SspC (and presumably $SspC^{G52A}$) is associated with *E. coli* DNA in vivo (27). While SspC and $SspC^{G52A}$ from unheated cells had very little isoaspartate, there was a significant amount of isoaspartate in these proteins from heated cells (Table 3). Although the estimated $t_{1/2}$ values at 95°C for the deamidation of these

TABLE 3. Levels of isoaspartate in SASP from cells and spores before and after heat treatment[a]

Sample treated (phenotype)	Treatment	Protein analyzed	Isoaspartate level (mol/mol of protein)
B. subtilis			
Spores of PS2437 ($\alpha^-\beta^-\gamma^-$ expressing SspC)	None	SspC	<0.01
	2 h, 95°C	SspC	0.01
Spores of PS2438 ($\alpha^-\beta^-\gamma^-$ expressing $SspC^{G52A}$)	None	$SspC^{G52A}$	0.01
	2 h, 95°C	$SspC^{G52A}$	0.04
Spores of PS356 ($\alpha^-\beta^-$)	None	SASP-γ	0.02
	2 h, 95°C	SASP-γ	0.58
E. coli			
PS708 (expressing SspC)	None	SspC	0.03
	1 h, 95°C	SspC	0.12
PS1342 (expressing $SspC^{G52A}$)	None	$SspC^{G52A}$	0.03
	1 h, 95°C	$SspC^{G52A}$	0.17

[a] Spores or cells of various strains were isolated, heated, and disrupted; then SASP was purified, and isoaspartate and protein were analyzed as described in Materials and Methods.

proteins in cells were much higher than those for these proteins in vitro, they were significantly lower (especially for wild-type SspC) than the values for spores (Table 4).

As a further assessment of the relative roles of DNA binding and spore environment in the prevention of SspC deamidation, we also examined deamidation of *B. subtilis* SASP-γ in spores, as SASP-γ has little secondary structure in solution (33) and is not associated with DNA in vivo (9). In addition, the two sequences of SASP-γ most prone to deamidation (NS sequences) are located near the N terminus and at the extreme C terminus of this protein (31), suggesting that higher-order protein structure would be less likely to affect the rates of deamidation of these sequences. In contrast to results with SspC and $SspC^{G52A}$, isoaspartate levels in SASP-γ increased substantially when $\alpha^-\beta^-$ spores were heated, with an estimated $t_{1/2}$ at 95°C of ~1 h (Table 3). For reasons that are not clear, the latter rate was about threefold lower in wild-type spores (data not shown).

An additional variable that might affect protein deamidation rates in vivo is pH, and the core pH of *B. subtilis* spores has been estimated at ~6.8 (18). Since protein deamidation is a base-catalyzed reaction (40), it was possible that rates of protein deamidation might increase at an elevated pH. This has been confirmed in vitro with SspC, which retained its DNA binding ability after being heated for 1 h at 80°C at pH 6.5 (data not shown). However, soaking of spores at an elevated pH with a permeant weak base (NH_3), a treatment that raised the core pH of *B. megaterium* spores to ~7.3 (38), had no significant effect on the rate of SspC, $SspC^{G52A}$, or SASP-γ deamidation in vivo (data not shown).

TABLE 4. Estimates of SASP deamidation rates in vivo and in vitro[a]

SASP analyzed	Deamidation rates ($t_{1/2}$ values)[b]		
	In vitro[c]	In vivo	
		B. subtilis spores	*E. coli* cells
SspC	1 h, 70°C (6 min, 95°C)	>50 h, 95°C	3 h, 95°C
$SspC^{G52A}$	1 h, 70°C (6 min, 95°C)	15 h, 95°C	2 h, 95°C
SASP-γ	1 h, 70°C (6 min, 95°C)	1 h, 95°C	ND[d]

[a] Spores and cells were heated as described in Materials and Methods.
[b] $t_{1/2}$ values are for one net deamidation and are the amounts of time needed to accumulate 0.3 mol of isoaspartate/mol of protein. Values were either taken from Fig. 1 or were estimated from data in Table 3 assuming that deamidation is a first-order reaction.
[c] Values in parentheses were calculated assuming an activation energy for the deamidation reaction of 21.7 kcal/mol for NG, NS, and NA sequences (11).
[d] ND, not done.

TABLE 5. Levels of isoaspartate in total protein from *B. subtilis* spores and cells before and after heat treatment[a]

Sample treated (phenotype)	Treatment	Protein analyzed	Isoaspartate level (pmol/μg of protein)[b]
Cells of PS832 (wild type)	None	Total	0.4
	1 h, 95°C	Total	8.4
Spores of PS356 ($\alpha^-\beta^-$)	None	Soluble core[c]	0.6
	1 h, 90°C	Soluble core[c]	1.3
Spores of PS832 (wild type)	None	Soluble core[c]	0.6
	1 h, 90°C	Soluble core[c]	1.3
	1 h, 95°C	Soluble core[c]	3.6

[a] Cells and spores were isolated, heated, and disrupted; then protein was isolated and isoaspartate was analyzed as described in Materials and Methods.
[b] Assuming one labile asparagine residue per 100 residues, this value would be ~60 pmol/μg at complete deamidation.
[c] This sample did not include SASP, as they are lost during protein isolation.

Deamidation of total cell protein in vivo. To further evaluate the significance of α/β- and γ-type SASP deamidation in vivo, deamidation in total protein from spores and vegetative cells of *B. subtilis* was also measured. In contrast to α/β- and γ-type SASP, most proteins have ordered structure in the absence of other macromolecules and would presumably deamidate more slowly than short peptides containing asparagine. Although vegetative cells and spores contain different complements of proteins, examination of known protein sequences from *B. subtilis* has shown that the average protein contains a potentially labile asparagine (in NG, NS, or NA sequences) approximately every 100 amino acids. Total protein from unheated cells and that from spores contained similar levels of isoaspartate (Table 5). However, isoaspartate levels increased significantly upon heating of vegetative cells, with spore core proteins showing a smaller increase (Table 5). This result indicates that while the rate of deamidation of total protein is lower in spores than in cells, the decrease is not nearly as dramatic as is the decrease in the deamidation rate of SspC in cells and spores (Table 4).

DISCUSSION

The lability of asparagine residues has been postulated to be involved in protein damage resulting in loss of function and targeting of the damaged protein for degradation (34). This type of protein damage may be a special problem in cells that are quiescent (e.g., plant seeds) or lack transcriptional and translational activity (e.g., erythrocytes) (10). Spores from *Bacillus* and *Clostridium* species are the most extreme example of such a dormant cell, yet they are able to survive in a metabolically dormant state for several decades and possibly longer (6, 32). Because we were unable to detect protein isoaspartyl methyltransferase activity in *B. subtilis* spore extracts (data not shown), it appears that repair of deamidation damage cannot occur during spore germination. Therefore, bacterial spore proteins must presumably resist accumulation of such damage over the period of spore dormancy, as excessive protein damage could lead to defects in spore germination and outgrowth or decreased spore viability. One potentially important target

for this protein damage in spores is α/β-type SASP, which contain a potentially labile NG sequence. Indeed, we have found that deamidated α/β-type SASP have a much lower affinity for DNA in vitro and presumably would not confer UV and heat resistance in vivo, based on the phenotype of spores expressing $SspC^{N48D}$.

In vitro, two different α/β-type SASP deamidated with $t_{1/2}$ values at 70°C of ~1 h (Table 4 and data not shown). The activation energy of asparagine deamidation within NG sequences has been estimated at 21.7 kcal/mol (11). This value predicts a 2.5-fold increase in the reaction rate for every 10°C increase in temperature, or a $t_{1/2}$ of ~6 min at 95°C for deamidation of SspC. However, in spores, SspC underwent essentially no deamidation at 95°C in vivo ($t_{1/2}$, >50 h [Table 4]). Although SspC was refractory to deamidation within spores, this protein did deamidate ($t_{1/2}$, ~3 h [95°C]) within heated *E. coli* cells (Table 4). Thus, an ~30-fold reduction in deamidation rate was observed for SspC expressed in vegetative cells compared to the in vitro rate. $SspC^{G52A}$ also failed to deamidate rapidly in cells (Table 4), suggesting a possible interaction between this protein and DNA that is not strong enough to confer heat and UV resistance to the spore but is sufficient to inhibit deamidation. This suggestion is supported by evidence for significant interaction between $SspC^{G52A}$ and DNA in vitro (14, 20). In contrast to SspC and $SspC^{G52A}$, SASP-γ deamidated fairly readily in vivo. SASP-γ from heated spores deamidated with a $t_{1/2}$ of ~1 h at 95°C, while the estimated in vitro $t_{1/2}$ at 95°C is ~6 min (Table 4). These values correspond to a 10-fold decrease in the deamidation rate in spores. Deamidation of total protein also appeared to be inhibited within spores, with the rate of this reaction being reduced two- to threefold compared to that in vegetative cells (Table 5).

The fact that α/β-type SASP deamidate more slowly within spores is not surprising given that the spore core environment is significantly dehydrated. However, several mechanisms could be responsible for protection from deamidation in vivo. Potentially protective mechanisms include pH, dehydration, and protein conformation. A slightly acidic pH can be protective in vitro (34). However, experimental elevation of spore pH failed to stimulate protein deamidation. This may be due to increased spore stability at a higher pH, such that denaturation of spore proteins is less likely to occur. Conversely, at lower pH values, spores may be less stable and thus protein denaturation may be facilitated, which in turn promotes deamidation. Thus, in pH-elevated spores, a balance may exist in which the increase in the rate of deamidation is counteracted by a decrease in protein denaturation.

The spore environment, possibly the dehydration and extremely high protein concentration, certainly slows deamidation in SASP-γ and total protein from heated spores. However, deamidation was inhibited to a much larger degree in SspC than in either SASP-γ or total protein, suggesting a role for a factor in addition to the spore environment. Protein conformation has long been recognized as a fundamental determinant of the deamidation rate in proteins, as asparagines within highly structured regions of proteins fail to deamidate or do so slowly (7, 17). Consistent with these observations, we found that SspC within a protein-DNA complex deamidates more slowly in vitro than SspC by itself. Furthermore, because α/β-type SASP have a relatively low affinity for DNA in solution, it is reasonable to assume that protein bound to DNA is almost completely resistant to deamidation and that deamidation can occur only while protein is in the off state of the binding equilibrium. Based on the 20- to 30-fold reduction in the deamidation rates of SspC and $SspC^{G52A}$ in heated *E. coli* cells (Table 4), it appears that a large degree of protection of SspC against deamidation is possible outside of the spore core environment. The most attractive explanation for this finding is that inhibition of SspC (and $SspC^{G52A}$) deamidation in *E. coli* is due to an interaction with cell DNA. This interaction has been confirmed previously for SspC (27), although no data exist for $SspC^{G52A}$ in *E. coli*. Unfortunately, we have been unable to express high levels of SASP-γ in *E. coli*, so we have been unable to analyze SASP-γ deamidation in a fully hydrated cell.

These data suggest that the spore core environment or dehydration per se is not as important in inhibiting deamidation in α/β-type SASP as the protein conformation that may be stabilized by the binding of these proteins to DNA in the spore environment. This conclusion is supported by at least one study of dehydration and deamidation in a model peptide (22). Consequently, it appears that binding to spore DNA in vivo protects α/β-type SASP from asparagine deamidation. So in addition to the well-characterized function that α/β-type SASP perform in protecting spore DNA from damage (32), there is a reciprocal process in which spore DNA protects α/β-type SASP from damage.

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REFERENCES

1. **Aiyar, A., and J. Leis.** 1993. Modification of the megaprimer method of PCR mutagenesis: improved amplification of the final product. BioTechniques **14:**366–369.
2. **Alarcon-Hernandez, E., and P. Setlow.** Unpublished results.
3. **Anagnostopoulos, C., and J. Spizizen.** 1961. Requirements for transformation in *Bacillus subtilis*. J. Bacteriol. **81:**741–746.
4. **Brennan, T. V., J. W. Anderson, Z. Jia, E. B. Waygood, and S. Clarke.** 1994. Repair of spontaneously deamidated HPr phosphocarrier protein catalyzed by the L-isoaspartate-(D-aspartate) *O*-methyltransferase. J. Biol. Chem. **269:** 24586–24595.
5. **Cabrera-Martinez, R. M., J. M. Mason, B. Setlow, W. M. Waites, and P. Setlow.** 1989. Purification and amino acid sequence of two small, acid-soluble proteins from *Clostridium bifermentans* spores. FEMS Microbiol. Lett. **52:** 139–143.
6. **Cano, R. J., and M. K. Borucki.** 1995. Revival and identification of bacterial spores in 25- to 40-million-year-old Dominican amber. Science **268:**1060–1064.
7. **Clarke, S.** 1987. Propensity for spontaneous succinimide formation from aspartyl and asparaginyl residues in cellular proteins. Int. J. Pept. Protein Res. **30:**808–821.
8. **Connors, M. J., and P. Setlow.** 1985. Cloning of a small, acid-soluble spore protein gene from *Bacillus subtilis* and determination of its complete nucleotide sequence. J. Bacteriol. **161:**333–339.
9. **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.
10. **Galletti, P., D. Ingrosso, C. Manna, G. Clemente, and V. Zappia.** 1995. Protein damage and methylation-mediated repair in the erythrocyte. Biochem. J. **306:**313–325.
11. **Geiger, T., and S. Clarke.** 1987. Deamidation, isomerization, and racemization at asparaginyl and aspartyl residues in peptides. Succinimide-linked reactions that contribute to protein degradation. J. Biol. Chem. **262:**785–794.
12. **Goldrick, S., and P. Setlow.** 1983. Expression of a *Bacillus megaterium* sporulation-specific gene during sporulation of *Bacillus subtilis*. J. Bacteriol. **155:** 1459–1462.
13. **Hackett, R. H., and P. Setlow.** 1988. Properties of spores of *Bacillus subtilis* strains which lack the major small, acid-soluble protein. J. Bacteriol. **170:** 1403–1404.
14. **Hayes, C. S., and P. Setlow.** 1997. Unpublished results.
15. **Li, C., and S. Clarke.** 1992. Distribution of an L-isoaspartyl protein methyltransferase in eubacteria. J. Bacteriol. **174:**355–361.
16. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193:**265–275.
17. **Lura, R., and V. Schirch.** 1988. Role of peptide conformation in the rate and mechanism of deamidation of asparaginyl residues. Biochemistry **27:**7671–7677.

18. **Magill, N. G., A. E. Cowan, M. A. Leyva-Vazquez, M. Brown, D. E. Koppel, and P. Setlow.** 1996. Analysis of the relationship between the decrease in pH and accumulation of 3-phosphoglyceric acid in developing forespores of *Bacillus* species. J. Bacteriol. **178:**2204–2210.
19. **Mason, J. M., and P. Setlow.** 1986. Essential role of small, acid-soluble spore proteins in resistance of *Bacillus subtilis* spores to UV light. J. Bacteriol. **167:**174–178.
20. **Mohr, S., and P. Setlow.** Unpublished results.
21. **Nicholson, W. L., and P. Setlow.** 1990. Sporulation, germination, and outgrowth, p. 391–450. *In* C. R. Harwood and S. M. Cutting (ed.), Molecular biological methods for *Bacillus*. John Wiley & Sons Ltd., Chichester, England.
22. **Oliyai, C., J. P. Patel, L. Carr, and R. T. Borchardt.** 1994. Solid state chemical instability of an asparaginyl residue in a model hexapeptide. J. Pharm. Sci. Technol. **48:**167–173.
23. **Popham, D. L., S. Sengupta, and P. Setlow.** 1995. Heat, hydrogen peroxide, and UV resistance of *Bacillus subtilis* spores with increased core water content and with or without major DNA-binding proteins. Appl. Environ. Microbiol. **61:**3633–3638.
24. **Rao, H., S. C. Mohr, H. Fairhead, and P. Setlow.** 1992. Synthesis and characterization of a 29-amino acid residue DNA-binding peptide derived from α/β-type small, acid-soluble spore proteins (SASP) of bacteria. FEBS Lett. **305:**115–120.
25. **Reisfield, R. A., V. J. Lewis, and D. E. Williams.** 1962. Disk electrophoresis of basic proteins and peptides on polyacrylamide gels. Nature **195:**281–283.
26. **Russell, A. D.** 1982. The destruction of bacterial spores. Academic Press, New York, N.Y.
27. **Setlow, B., A. R. Hand, and P. Setlow.** 1991. Synthesis of a *Bacillus subtilis* small, acid-soluble spore protein in *Escherichia coli* causes cell DNA to assume some characteristics of spore DNA. J. Bacteriol. **173:**1642–1653.
28. **Setlow, B., and P. Setlow.** 1995. Binding to DNA protects α/β-type, small, acid-soluble spore proteins of *Bacillus* and *Clostridium* species against digestion by their specific protease as well as by other proteases. J. Bacteriol. **177:**4149–4151.
29. **Setlow, B., D. Sun, and P. Setlow.** 1992. Interaction between DNA and α/β-type small, acid-soluble spore proteins: a new class of DNA-binding protein. J. Bacteriol. **174:**2312–2322.
30. **Setlow, P.** 1975. Identification and localization of the major proteins degraded during germination of *Bacillus megaterium* spores. J. Biol. Chem. **250:**8159–8167.
31. **Setlow, P.** 1988. Small, acid-soluble spore proteins of *Bacillus* species: structure, synthesis, genetics, function and degradation. Annu. Rev. Microbiol. **42:**319–338.
32. **Setlow, P.** 1995. Mechanisms for the prevention of damage to DNA in spores of *Bacillus* species. Annu. Rev. Microbiol. **49:**29–54.
33. **Setlow, P.** Unpublished results.
34. **Sharma, S., P. K. Hammen, J. W. Anderson, A. Leung, F. Georges, W. Hengstenberg, R. E. Klevit, and E. B. Waygood.** 1993. Deamidation of HPr, a phosphocarrier protein of the phosphoenolpyruvate:sugar phosphotransferase system, involves asparagine 38 (HPr-1) and asparagine 12 (HPr-2) in isoaspartyl acid formation. J. Biol. Chem. **268:**17695–17704.
35. **Stephenson, R. C., and S. Clarke.** 1989. Succinimide formation from aspartyl and asparaginyl peptides as a model for the spontaneous degradation of proteins. J. Biol. Chem. **264:**6164–6170.
36. **Stragier, P., C. Bonamy, and C. Karmazyn-Campelli.** 1988. Processing of a sporulation sigma factor in *Bacillus subtilis*: how morphological structure could control gene expression. Cell **52:**697–704.
37. **Studier, F. W., and B. A. Moffatt.** 1986. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. J. Mol. Biol. **189:**113–130.
38. **Swerdlow, B. M., B. Setlow, and P. Setlow.** 1981. Levels of H^+ and other monovalent cations in dormant and germinating spores of *Bacillus megaterium*. J. Bacteriol. **148:**20–29.
39. **Tovar-Rojo, F., and P. Setlow.** 1991. Effects of mutant small, acid-soluble spore proteins from *Bacillus subtilis* on DNA in vivo and in vitro. J. Bacteriol. **173:**4827–4835.
40. **Tyler-Cross, R., and V. Schirch.** 1991. Effects of amino acid sequence, buffers, and ionic strength on the rate and mechanism of deamidation of asparagine residues in small peptides. J. Biol. Chem. **266:**22549–22556.
41. **Wright, H. T.** 1991. Nonenzymatic deamidation of asparaginyl and glutaminyl residues in proteins. Crit. Rev. Biochem. Mol. Biol. **26:**1–52.