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Properties of *Bacillus subtilis* Small, Acid-Soluble Spore Proteins with Changes in the Sequence Recognized by Their Specific Protease

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α/β-type small, acid-soluble proteins (SASP) of dormant spores of *Bacillus subtilis* bind to DNA and increase its resistance to a variety of damaging agents both in vivo and in vitro. When spores germinate, degradation of α/β-type SASP is rapidly initiated by a sequence-specific protease, which is termed GPR. Three mutations have been introduced into the *B. subtilis sspC* gene, which codes for the wild-type α/β-type SASP $SspC^{wt}$; all three mutations change residues in the highly conserved sequence recognized by GPR. In one mutant protein ($SspC^{V}$), residue 33 (Ser) was changed to Val; in the second ($SspC^{DL}$), residues 30 and 31 (Glu and Ile) were changed to Asp and Leu, respectively; and in the third mutant protein ($SspC^{DLV}$), residues 30, 31, and 33 were changed to Asp, Leu, and Val. All three mutant proteins were rapidly degraded by GPR during spore germination, and $SspC^{DL}$ and $SspC^{DLV}$ were degraded by GPR in vitro at rates 8 to 9% of that for $SspC^{wt}$, although not exclusively at the single site cleaved by GPR in $SspC^{wt}$. These results indicate (i) that the sequence specificity of GPR is broader than originally imagined and (ii) that GPR can cleave the sequence in $SspC^{DLV}$. Since the latter sequence is identical to that cleaved during the proteolytic activation of GPR, this result further supports an autoprocessing model for GPR activation during sporulation. The properties of these mutant proteins were also examined, both in vivo in *B. subtilis* spores and in *Escherichia coli* and in vitro with purified protein. $SspC^{V}$ interacted with DNA similarly to $SspC^{wt}$ in vivo, restoring UV and heat resistance to spores lacking major α/β-type SASP to the same extent as $SspC^{wt}$. In contrast, $SspC^{DL}$ had much less effect on DNA properties in vivo and bound strongly only to poly(dG) · poly(dC) in vitro; $SspC^{DLV}$ exhibited only weak binding to poly(dG) · poly(dC) in vitro. These results confirm the importance of the conserved primary sequence of α/β-type SASP in the binding of these proteins to spore DNA and alteration of DNA properties and show further that the GPR recognition region in α/β-type SASP plays some role in DNA binding.

Approximately 10 to 20% of the proteins in dormant spores of *Bacillus* species form a group of small, acid-soluble proteins (SASP), which are synthesized in the developing spore midway in sporulation and degraded early in spore germination, thus providing amino acids for protein synthesis (20, 22). These proteins are of two types. The α/β-type SASP are nonspecific DNA binding proteins involved in various aspects of spore resistance (2, 10, 17, 21) and are encoded by a multigene family, and their amino acid sequences have been highly conserved throughout evolution (20, 22). The γ-type SASP have no known function other than to provide amino acids by their degradation during spore germination and are encoded by a single gene, and their amino acid sequences are not nearly as conserved throughout evolution as are those of α/β-type SASP (20, 22).

Degradation of both types of SASP is initiated during spore germination by a sequence-specific endoprotease, termed GPR, which cleaves once in α/β-type SASP and at two or three places in γ-type SASP (Fig. 1) (6, 20). GPR is also synthesized in the developing spore only during sporulation and as an inactive precursor which is termed P_{46} (14, 20). This precursor is processed to an active enzyme (termed P_{41}) about 2 h later in sporulation by a single proteolytic cleavage, removing 15 or 16 amino-terminal residues (depending on the species) (14). The sequence around the site cleaved in the $P_{46} \rightarrow P_{41}$ conversion is similar but not identical to that cleaved in SASP by GPR (14) (Fig. 1). This observation, as well as a variety of other data, has led to the suggestion that P_{46} autoprocesses to P_{41} (4, 14). However, the $P_{46} \rightarrow P_{41}$ cleavage site sequence is not identical to the highly conserved sequence in GPR cleavage sites in SASP (4, 14) (Fig. 1). Consequently, it seemed worthwhile to directly prove that GPR can cleave proteins at sequences identical to that in the $P_{46} \rightarrow P_{41}$ cleavage site. Thus, we have generated mutant α/β-type SASP in which the GPR cleavage site has been changed to that of the $P_{46} \rightarrow P_{41}$ cleavage site and have assessed the abilities of these mutant proteins to serve as GPR substrates. In addition, since previous work (24) has shown that alteration of residues highly conserved in α/β-type SASP greatly decreases the binding of these proteins to DNA, we have analyzed the mutant proteins generated in the present study for their ability to bind DNA in vivo and in vitro.

MATERIALS AND METHODS

Bacteria and plasmids used and isolation of DNA. The bacterial strains and plasmids used in this work are listed in Table 1. *Escherichia coli* strains were routinely grown at 37°C in 2× YT medium (4). *Bacillus subtilis* strains were grown and sporulated at 37°C in 2× SG medium (3), and spores were purified and germinated as described previously (14). Antibiotics were added to the following final concentrations: ampicillin, 50 μg/ml; and kanamycin, 10 μg/ml. Plasmid DNA was isolated from *E. coli* or *B. subtilis* cells as previously described (1, 11). Poly(dG) · poly(dC) was purchased from Sigma.

Isolation and analysis of GPR and SASP. P_{41}, the active form of *Bacillus megaterium* GPR, was purified from *E. coli*

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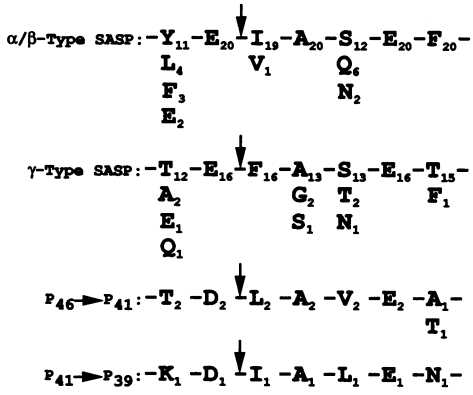

FIG. 1. GPR cleavage sites in α/β- and γ-type SASP, as well as processing sites in GPR. Amino acids are given in the one-letter code, with the subscript noting the number of times a residue is found at that position; cleavage sites are denoted by the vertical arrows. Data for α/β-type SASP are from references 6, 20, and 22 and include 20 GPR cleavage sites in proteins from *Bacillus aminovorans*, *Bacillus cereus*, *B. megaterium*, *Bacillus stearothermophilus*, *B. subtilis*, *Sporosarcina halophila*, *Sporosarcina ureae*, and *Thermoactinomyces thalpophilus*. Data for γ-type SASP are from references 6, 12, 20, and 22 and include 17 GPR cleavage sites in proteins from *B. aminovorans*, *B. cereus*, *Bacillus firmus*, *B. megaterium*, *B. stearothermophilus*, *B. subtilis*, *S. ureae*, and *T. thalpophilus*. Data for the $P_{46}\rightarrow P_{41}$ cleavage sites are from reference 14 and are from *B. megaterium* and *B. subtilis* GPR. The $P_{41}\rightarrow P_{39}$ cleavage site is from reference 4 and is only for *B. megaterium* GPR.

expressing this form of the protease and assayed as described previously (4). Synthesis of $SspC^{wt}$ as well as SspC variants was induced by the addition of isopropyl-β-D-galactopyranoside (IPTG; 0.5 mM) to *E. coli* strains (generally grown to an optical density at 600 nm of 0.3) carrying the appropriate *sspC* genes in plasmid pDG148 as described previously (9, 24). The *E. coli* cells were harvested 2.5 h after the addition of IPTG by centrifugation (10 min; 8,000 × *g*), and the cell pellets were frozen and lyophilized. The dry cells (up to 100 mg) were broken by rupturing for 2 min in a dental amalgamator (Wig-L-Bug) with glass beads (~100 mg) as the abrasive. SASP were extracted from the dry powder with cold 3% acetic acid–30 mM HCl as described previously (9). In experiments for SASP analysis from small (20-ml) aliquots of cells, the acid extract was evaporated to dryness in a Speed-Vac, redissolved in 1 ml of water, frozen, and lyophilized; the final dry residue was dissolved in 100 to 200 μl of 8 M urea plus 50 to 100 μl of acid diluent; and aliquots were analyzed by polyacrylamide gel electrophoresis at low pH as described previously (24). For purification of SspC variants, cells were harvested from 10 liters of IPTG-induced culture (with IPTG added at an optical density at 600 nm of 0.5), the acid extract was dialyzed exhaustively against 1% acetic acid in Spectrapor-3 tubing and passed through a column of DEAE-cellulose in 1% acetic acid, and the run-through fraction was lyophilized (9). The dry residue was dissolved in ~100 ml of 8 M urea–5 mM Tris-maleate (pH 7.0), and this material was passed through a carboxymethyl cellulose column equilibrated in 5 mM Tris-maleate (pH 7.0). SspC ran through this column; however, much interfering protein was removed. The flowthrough fractions were collected, adjusted to pH 5.5, diluted ~2-fold with 8 M urea, and applied to a carboxymethyl cellulose column in 5 mM Tris-maleate (pH 5.5). SspC adsorbed to this column and was eluted with a salt gradient as described previously (9).

In vitro mutagenesis of the *sspC* gene. As described previously (24), the 0.6-kb *Hin*dIII fragment containing the *sspC* gene was cloned in the *Hin*dIII site of plasmid pTZ19U, with the amino-terminal coding sequence adjacent to the universal primer site. Oligonucleotide-directed mutagenesis was carried out as described previously (24) with two different oligonucleotides designed to change residues around the GPR cleavage site. The oligonucleotides used (with changes from the wild-type sequence underlined) were 5′-GAAACTTGACCTAGC TGTTGAGT-3′ (oligonucleotide 1) and 5′-GAAACTTGAA/CA/CTAGCTGTTGAGT-3′ (oligonucleotide 2). These oligonucleotides are from residues 81 to 103 of the coding sequence of the *sspC* gene and change amino acid 30 (Glu) to Asp, amino acid 31 (Ile) to Leu, and amino acid 33 (Ser) to Val (1) (Fig. 2). Mutant plasmids were identified by DNA sequence analysis, with oligonucleotide 2 generating the mutant with the Val alteration alone. Oligonucleotide 1 was used to isolate the Asp-Leu mutation; a second mutagenesis reaction with oligo-

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype or phenotype	Source or reference
E. coli		
JM107		Bethesda Research Laboratories
UT481	*lacI*^{q}	M. Deutscher
B. subtilis		
PS356	Δ*sspA sspB trpC2*	7
PS832	*trp*$^+$ revertant of strain 168	Laboratory stock
Plasmids		
pDG148	Amp^r Km^r	23
pPS708	pDG148 with 0.6-kb fragment carrying *sspC*^{wt}; Amp^r	24
pPS1231	pTZ19U with 0.6-kb fragment carrying *sspC*^{wt}; Amp^r	24
pPS1450	pPS1916 with *sspC*^{wt}, but with pUC sequence removed; Km^r	24
pPS1916	Hybrid plasmid between pUBB and pUC19 with 0.13-kb *Hpa*I fragment carrying amino-terminal-coding region of *sspB* deleted; Amp^r Km^r	24
pPS1986	pDG148 with *sspC*^{V}; Amp^r	This work
pPS1987	pDG148 with *sspC*^{DL}; Amp^r	This work
pPS1988	pDG148 with *sspC*^{DLV}; Amp^r	This work
pPS1993	pPS1916 with *sspC*^{V}, but with pUC sequence removed; Km^r	This work
pPS1994	pPS1916 with *sspC*^{DL}, but with pUC sequence removed; Km^r	This work
pPS1995	pPS1916 with *sspC*^{DLV}, but with pUC sequence removed; Km^r	This work

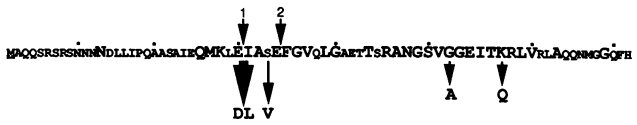

FIG. 2. Amino acid sequence of $SspC^{wt}$ and residues altered by mutation. The amino acid sequence of $SspC^{wt}$ is from reference 1 and is given in the one-letter code; dots above residues denote every 10th amino acid. Residues conserved in ≥85% of all known α/β-type SASP of *Bacillus* species (6, 20) are in larger letters. The initiating methionine residue which is removed posttranslationally (1) is underlined. The site of GPR cleavage in $SspC^{wt}$ is denoted by the arrow labeled 1 above the sequence; the major site of GPR cleavage of $SspC^{DL}$ and $SspC^{DLV}$ found in this work is denoted by the arrow labeled 2 above the sequence. Residues altered in this work are denoted by the longer vertical arrows below the sequence; the residue or residues substituted are at the ends of the arrows. Residues altered in previous work (24) are denoted by the shorter arrows. The variants with Ala instead of Gly and Gln instead of Lys are termed $SspC^{A}$ and $SspC^{Q}$, respectively.

nucleotide 1 and the *sspC* gene carrying the Asp-Leu mutation as a template generated the Asp-Leu-Val mutant. The appropriate mutant *Hin*dIII fragments were then ligated with *Hin*dIII-cut plasmid pDG148, and the mix was used to transform *E. coli* UT481 to ampicillin resistance. This strain was used because it has a high level of *lac* repressor, which prevents *sspC* transcription from the *spac* promoter of plasmid pDG148. We used this strategy because synthesis of $SspC^{wt}$ can kill *E. coli* (16). Plasmids from appropriate clones were identified by colony hybridization and were digested with *Eco*RV; plasmids giving 0.7-, 1.2-, and 7-kb fragments were identified as having the *sspC* gene under the control of the *spac* promoter of pDG148 (1, 24). These plasmids were used to transform *E. coli* JM107 (which has a low wild-type level of *lac* repressor) the day before SASP expression was to be measured.

For expression of SspC variants in *B. subtilis*, the ends of the 0.6-kb *Hin*dIII fragment containing a mutant *sspC* gene were filled in with the large fragment of *E. coli* DNA polymerase, the fragment was ligated with *Hpa*I-cut pPS1916, and the mix was used to transform *E. coli* JM83 to ampicillin resistance. Clones carrying inserts were identified by colony hybridization, and plasmids in which the *sspC* gene is just downstream of the plasmid's *sspB* promoter were identified as giving 0.7-, 3.5-, and 4.2-kb fragments upon digestion with *Eco*RV and *Hin*dIII. These plasmids were digested with *Bam*HI to remove pUC19 sequences (24), religated, and used to transform *B. subtilis* to kanamycin resistance. The resulting plasmids carry the *sspC* gene under the control of the *sspB* gene's promoter and thus express SspC only in the forespore at the appropriate time in sporulation (24). These plasmids are also present in high copy number, giving multiple copies of *sspC* in developing spores (24).

Analytical procedures. Analysis of the heat resistance and UV resistance of cleaned spores was as previously described (8, 24). Measurement of the ability of purified SspC variants to provide DNase I resistance to *Eco*RI-linearized plasmid pUC19 or poly(dG)·poly(dC) was carried out essentially as described previously (19).

Analysis of the rate of GPR cleavage of purified SspC was carried out with 10 mM Tris-acetate (pH 7.0)–2 mM $CaCl_2$ with 1 mg of SspC per ml. Incubations (100-μl mixtures) at 37°C were begun by the addition of purified GPR (P_{41}), aliquots (10 μl) were taken at various times and frozen, 10 μl of 8 M urea plus 10 μl of acid diluent was added, and aliquots (10 to 20 μl) were analyzed by polyacrylamide gel electrophoresis at low pH (24). In some experiments, samples digested to completion were run on gel electrophoresis as described above, transferred to polyvinylidene difluoride paper (Immobilon), and stained lightly with Coomassie brilliant blue R, and the bands were subjected to automated protein sequence analysis as described previously (4).

RESULTS

Construction of mutant *sspC* genes. The *ssp* gene that we chose to mutate was the *B. subtilis sspC* gene (1). This gene codes for an α/β-type SASP present in *B. subtilis* spores, although normally not at a very high level (1, 24). However, we have been able to obtain high-level expression of the *sspC* gene in both *B. subtilis* and *E. coli*, with the latter achievement allowing facile purification of large amounts of SspC (9). Analysis of $SspC^{wt}$ function, both in vitro and in vivo, has shown that the binding and effects of this protein on DNA are essentially indistinguishable from those of other α/β-type SASP (2, 9, 10, 19). We chose three amino acid residues in the region of the GPR cleavage site in $SspC^{wt}$ for mutagenesis (Fig. 2). One mutation changed Ser-33 to a Val (Fig. 2; long thin arrow). This mutant gene was termed $sspC^{V}$, and its product was termed $SspC^{V}$. While a number of other residues have been found at this position in α/β- and γ-type SASP of *Bacillus* species, a large hydrophobic residue has not been found here (Fig. 1). The second mutation changed Glu-30 and Ile-31 to Asp and Leu, respectively (Fig. 2; long thick arrow). This mutant gene was termed $sspC^{DL}$, and its product was termed $SspC^{DL}$. The position comparable to Glu-30 in $SspC^{wt}$ is always a Glu in α/β- and γ-type SASP; the position comparable to Ile-31 in $SspC^{wt}$ is an Ile in 19 of the 20 α/β-type SASP of *Bacillus* species that have been analyzed, with Val in one protein (Fig. 1). However, this position is occupied by Phe in all γ-type SASP that have been analyzed (Fig. 1). The third mutation changed residues 30, 31, and 33 to Asp, Leu, and Val, respectively. This mutant gene was termed $sspC^{DLV}$, and its product was termed $SspC^{DLV}$. The pentapeptide region around the GPR cleavage site in $SspC^{DLV}$ (-DLAVE-) is identical to that cleaved by GPR when it is processed from the P_{46} zymogen to active P_{41} and is similar to a second cleavage site at which P_{41} is cleaved further to the smaller P_{39} (4) (Fig. 1). The $P_{41} \rightarrow P_{39}$ cleavage has been shown to be a self-cleavage reaction (4).

Expression of mutant *sspC* genes in *E. coli* and *B. subtilis*. As found previously (9), we were able to obtain synthesis of all three SspC variants in *E. coli* by expression of the mutant *sspC* genes under control of the *lac* promoter (data not shown). All three SspC variants were expressed to significant levels (data not shown), and this facilitated purification of $SspC^{DL}$ and $SspC^{DLV}$ to greater than 85% homogeneity. Amino acid or protein sequence analysis of the purified proteins confirmed that the mutations had caused the desired amino acid changes (data not shown). For reasons that are unclear, we were unable to purify $SspC^{V}$, since this variant consistently adsorbed irreversibly to carboxymethyl cellulose columns.

We also overexpressed the SspC variants in *B. subtilis* from a multicopy plasmid under control of the strong forespore-specific *sspB* gene promoter. Previous work has shown that this results in high levels of $SspC^{wt}$ and SspC variants in spores (16, 24). Indeed, analysis of SASP levels in spores expressing $SspC^{wt}$ and the variants generated in this work showed that all spores accumulated the same amount of SspC, which was 1.5 to 2 times that of γ-type SASP (data not shown).

Cleavage of SspC variants by GPR. The major reason for generating the SspC variants described in this work was to

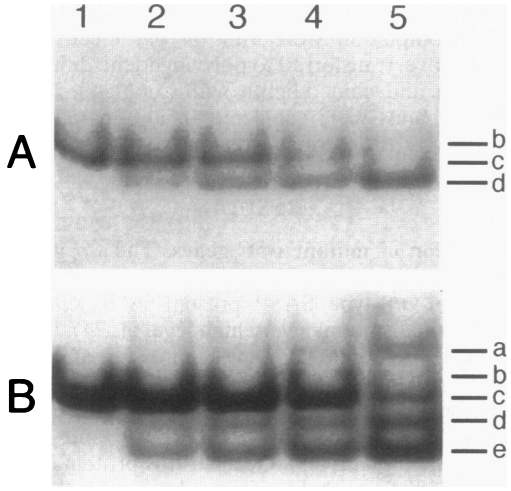

FIG. 3. GPR cleavage of $SspC^{wt}$ and $SspC^{DL}$. Cleavage of $SspC^{wt}$ and $SspC^{DL}$ was carried out as described in Materials and Methods, and products were analyzed by polyacrylamide gel electrophoresis at low pH. The proteins digested were $SspC^{wt}$ (A) and $SspC^{DL}$ (B). The incubation mixtures contained 2,000 U of GPR (A) and 20,000 U of GPR (B) per ml. The incubation times of the samples run in the various lanes were as follows: lane 1, 0 min; lane 2, 5 min; lane 3, 10 min; lane 4, 15 min; and lane 5, 30 min. The marker bars in panels A and B denote the migration positions of the following GPR digestion products of SspC: a, residues 2 to 34; b, residues 2 to 30; c, intact SspC; d, residues 31 to 72; and e, residues 35 to 72. These peptides were identified either by automated protein sequenator analysis as described in Materials and Methods or by their comigration with peptides previously identified by protein sequence analysis. Note that the peptide labeled b is barely visible, since it was rapidly lost upon destaining of gels. However, it was readily visible when peptides were transferred to polyvinylidene difluoride paper prior to staining and subsequent protein sequence analysis.

assess the ability of GPR to cleave these proteins at sites identical to those in processing sites in GPR itself. Analysis of cleavage of the SspC variants in vivo during spore germination indicated that degradation of all three SspC variants was as fast as that of $SspC^{wt}$ (data not shown). However, during spore germination, the initial cleavage by GPR is probably not the rate-limiting event in SASP degradation, since this is likely to be the triggering of germination itself. Furthermore, we cannot analyze the products of GPR cleavage of SspC variants in germinating spores in order to determine the cleavage site. Consequently, we analyzed the kinetics and products of SspC digestion by GPR in vitro with purified proteins. As expected, GPR digestion of $SspC^{wt}$ gave two major peptide products, with the site of cleavage between residues 30 and 31 (Fig. 2, arrow labeled 1; Fig. 3A) (Table 2). In contrast, cleavage of $SspC^{DL}$ and $SspC^{DLV}$ was much slower, and there was clearly more than one cleavage site, since four peptide products were generated (Fig. 3B) (Table 2). Automated protein sequence analysis of these peptides after their transfer to polyvinylidene difluoride paper showed that there were indeed two GPR cleavage sites in $SspC^{DL}$ and $SspC^{DLV}$, one (15 to 20% of the total) between residues 30 and 31, with the major cleavage between residues 34 and 35 (Fig. 2 [arrow labeled 2] and 3B) (Table 2). While the latter cleavage site was unexpected, the sequence around this site does have a number of the features conserved in GPR cleavage sites (see Discussion). Although we were unable to directly analyze GPR cleavage of $SspC^{V}$ in vitro, we note that the rates of GPR cleavage between residues 30 and 31 of $SspC^{DL}$ and $SspC^{DLV}$ were essentially identical (Table 3). Thus, it appears unlikely that substitution of a valine for the usual serine in the fifth position in the cleavage site sequence in a SASP (Fig. 1) slows GPR cleavage significantly.

TABLE 2. GPR cleavage of $SspC^{wt}$ and SspC variants[a]

GPR substrate	Rate of GPR cleavage (% of maximum)[b]	Residues at GPR cleavage sites (%)[c]
$SspC^{wt}$	100	30 and 31 (>93)
$SspC^{DL}$	8	34 and 35 (80); 30 and 31 (20)
$SspC^{DLV}$	9	34 and 35 (85); 30 and 31 (15)

[a] The rate of cleavage of SspC variants by GPR in vitro was measured as described in the legend to Fig. 3 and in Materials and Methods. The site(s) of GPR cleavage was established by automated protein sequence analysis, as described in Materials and Methods, of peptides produced from a digestion that had gone to completion (i.e., Fig. 3A, lane 5).

[b] Values are expressed relative to the rate with $SspC^{wt}$, which was set as 100%.

[c] Residue numbers given are as described in the legend to Fig. 2. Values in parentheses are the percentages of the protein cleaved at these residues as determined by automated protein sequence analysis of samples digested to completion, as described in Materials and Methods.

Effects of SspC variants in *E. coli* and *B. subtilis*. As noted above, significant levels of all three SspC variants could be accumulated in *E. coli*. However, in contrast to the effect of $SspC^{wt}$, which rapidly shuts off *E. coli* growth because of binding to the cell's nucleoid, we obtained somewhat different results with several of the SspC variants (Fig. 4) (16). While induction of $SspC^{V}$ synthesis resulted in significant slowing of *E. coli* growth, $SspC^{DL}$ had less of an effect, and $SspC^{DLV}$ had no apparent effect (Fig. 4). Since similar amounts of all three SspC variants were accumulated in *E. coli* (data not shown), these findings suggested that $SspC^{DL}$ and $SspC^{DLV}$ bound significantly more weakly to DNA than did $SspC^{wt}$. While several other SspC variants bind much more weakly to DNA than $SspC^{wt}$, these variants ($SspC^{A}$ and $SspC^{Q}$) have alterations in the extremely highly conserved more carboxyl-terminal region that is thought to be involved directly in DNA binding (Fig. 2) (13, 24). Thus, residues in the GPR cleavage region may also play some important role in DNA binding.

To examine this last possibility further, we analyzed the

TABLE 3. Rates of cleavage of various sequences by GPR in vitro and in vivo[a]

Sequence cleaved[b]	Rate of bond cleavage (μmol/mg of GPR/h)
LE↓IASEF ($SspC^{wt}$ in vitro)	37
LE↓IASEF ($SspC^{wt}$ in vivo)	27
LD↓LASEF ($SspC^{DL}$ in vitro)	0.6
LD↓LAVEF ($SspC^{DLV}$ in vitro)	0.5
SE↓FGVQL ($SspC^{DL}$ in vitro)	2.5
VE↓FGVQL ($SspC^{DLV}$ in vitro)	2.9
TD↓LAVEA/T ($P_{46}\rightarrow P_{41}$ in vivo)	0.01
KD↓IALEN ($P_{41}\rightarrow P_{39}$ in vitro)	0.0013

[a] Rates of SspC cleavage in vitro were calculated from data in Fig. 3 and Table 2, using 2.5×10^5 U/mg as the specific activity of P_{41} (4) and a molecular mass of 40 kDa for the GPR subunit. The rate of $SspC^{wt}$ degradation in vivo is from germinating spores of pPS1450 using a molar ratio of SspC-to-GPR subunits (in grams) in dormant spores of ~100/1 (14, 20, 24). The rate of GPR processing from $P_{46}\rightarrow P_{41}$ in vivo is from data in reference 14, which indicate that this processing reaction takes ~2 h to reach 80% completion during sporulation. The rate of $P_{41}\rightarrow P_{39}$ cleavage in vitro is from reference 4.

[b] The cleavage site is denoted by the vertical arrow. The substrate cleaved and the condition (in vitro or in vivo) are given in parentheses.

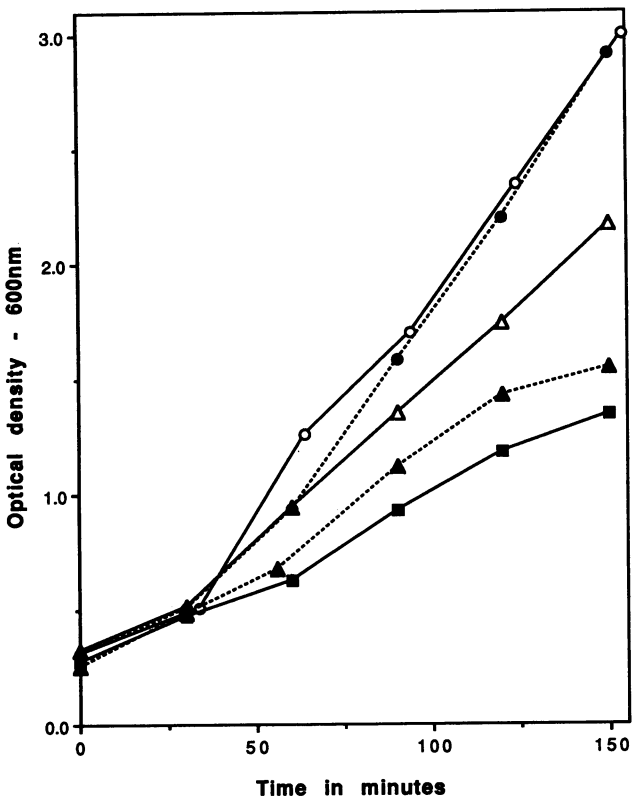

FIG. 4. Growth of *E. coli* expressing wild-type and mutant SspC. *E. coli* JM107 carrying plasmids directing IPTG-dependent expression of SspC variants were grown to an optical density at 600 nm of 0.3, IPTG was added, and growth was monitored. The symbols denoting the plasmids (and the proteins produced) in the strains analyzed are as follows: ○, pDG148; ●, pPS1988 ($SspC^{DLV}$); △, pPS1987 ($SspC^{DL}$); ▲, pPS1986 ($SspC^{V}$); and □, pPS708 ($SspC^{wt}$).

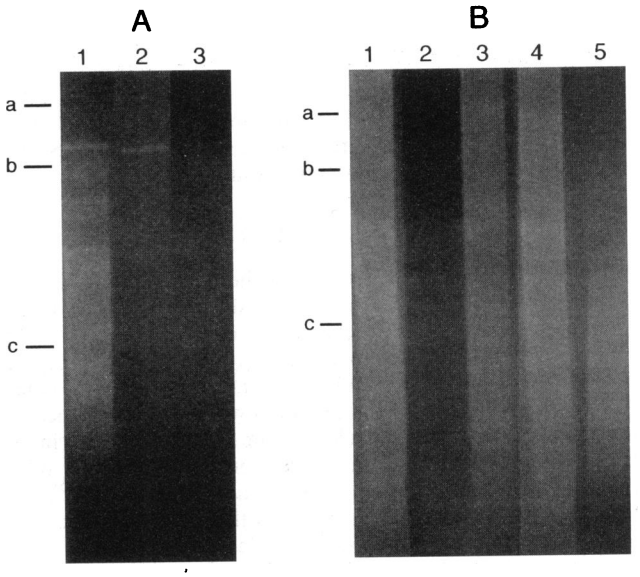

FIG. 5. DNase protection of pUC19 and poly(dG) · poly(dC) by SspC variants. Various amounts of purified SspC variants and 2.5 μg of either *Eco*RI-linearized pUC19 (A) or poly(dG) · poly(dC) (B) were preincubated in a total volume of 25 μl and digested with DNase I (except for lane 1 of panel B), and samples were analyzed by agarose gel electrophoresis as described previously (19). The additions to the incubation mixtures in the various lanes were as follows: (A) lane 1, $SspC^{wt}$ (12 μg); lane 2, $SspC^{DL}$ (30 μg); lane 3, $SspC^{DLV}$ (30 μg); (B) lanes 1 and 2, no additions (in lane 1, there was no DNase treatment); lane 3, $SspC^{wt}$ (12 μg); lane 4, $SspC^{DL}$ (12 μg); and lane 5, $SspC^{DLV}$ (30 μg). In panels A and B, the marker bars a, b, and c denote the migration positions of size markers of 9.3, 2.3, and 0.6 kb, respectively. The small amount of undigested pUC19 in lane 2 of panel A is presumably a minute amount of plasmid that physically escaped contact with the DNase, as observed previously (19).

effect of accumulation of SspC variants in *B. subtilis* spores, which was achieved as described above. The *B. subtilis* strain used for expression of the SspC variants was PS356, which carries large deletions in the two genes (*sspA* and *sspB*) coding for the major *B. subtilis* α/β-type SASP. The spores of this strain (termed $\alpha^-\beta^-$ spores) lack the majority of their α/β-type SASP and are much more sensitive to heat and UV irradiation than are wild-type spores (Table 4) (7, 8). However, much of the heat and UV resistance of $\alpha^-\beta^-$ spores is restored by high-level expression of $SspC^{wt}$, as well as other wild-type α/β-type SASP (Table 4) (2, 21, 24). While high levels of $SspC^{V}$ and $SspC^{DL}$ restored the heat resistance of $\alpha^-\beta^-$ spores as well as did $SspC^{wt}$, $SspC^{DL}$ was much less effective than $SspC^{wt}$ and $SspC^{V}$ in restoring UV resistance (Table 4). $SspC^{DLV}$ restored no UV resistance and little if any heat resistance to $\alpha^-\beta^-$ spores (Table 4).

TABLE 4. Heat and UV resistance of spores with and without various SASP[a]

Strain[b]	% Survival	
	Heat treatment	UV treatment
PS832(pUB110)	10	8
PS356(pUB110)	<0.001	0.02
PS356(pPS1450) [$SspC^{wt}$]	1.5	2
PS356(pPS1993) [$SspC^{V}$]	1.5	3.5
PS356(pPS1994) [$SspC^{DL}$]	3	0.3
PS356(pPS1995) [$SspC^{DLV}$]	0.05	0.02

[a] Spores of various strains were prepared and heat treated (30 min, 85°C) or UV irradiated (~10^3 ergs/mm^2), and survival was determined as described in Materials and Methods.

[b] The SspC accumulated in these strains is noted in brackets.

Effects of purified SspC on DNA in vitro. The results obtained with both *E. coli* and *B. subtilis* were consistent with the suggestion that two of the SspC variants generated in this study, $SspC^{DL}$ and $SspC^{DLV}$, bound significantly more poorly to DNA than did $SspC^{wt}$. To test this suggestion directly, we analyzed the DNase resistance provided to various nucleic acids by these two SspC variants and compared these data with those for $SspC^{wt}$. Previous work has shown that measurement of the DNase resistance provided by an α/β-type SASP gives a good assessment of the strength of the SASP-DNA interaction (19, 24). As found previously (19), $SspC^{wt}$ provided substantial DNase resistance to linear pUC19 at a protein/DNA ratio (wt/wt) of ~5/1 (Fig. 5A, lane 1). This ratio is approximately that needed to saturate this DNA with $SspC^{wt}$ (19). In contrast, $SspC^{DL}$ and $SspC^{DLV}$ gave pUC19 no detectable DNase resistance, even at a protein/DNA ratio of 12/1 (Fig. 5A, lanes 2 and 3). While $SspC^{wt}$ does bind to essentially random sequence DNAs such as pUC19, the DNA bound best by all α/β-type SASP is poly(dG) · poly(dC) (19), as is evidenced by the essentially complete DNase resistance provided to this polynucleotide by a 5/1 ratio of $SspC^{wt}$/DNA (Fig. 5B, lanes 1

to 3). $SspC^{DL}$ also gave complete DNase protection to poly(dG) · poly(dC) at a protein/DNA ratio of ~5/1, while $SspC^{DLV}$ gave some but significantly less protection even at a protein/DNA ratio of 12/1 (Fig. 5B, lanes 4 and 5). These data indicate that $SspC^{DL}$ and, even more so, $SspC^{DLV}$ have reduced DNA binding abilities compared with that of $SspC^{wt}$ but that their DNA binding abilities are not abolished.

DISCUSSION

The major reason for generating the SspC variants used in this work was to determine the ability of GPR to cleave sequences similar or identical to that cleaved when the GPR zymogen P_{46} is processed to active P_{41}. If GPR could carry out cleavage of such a sequence in *trans* (i.e., in a SASP), it would lend further support for the model in which P_{46} autoprocesses to P_{41}. Clearly, GPR can cleave the sequence found in the $P_{46} \rightarrow P_{41}$ cleavage site when this sequence is in a SASP. While the rate of cleavage of the Asp-Leu bond in $SspC^{DL}$ and $SspC^{DLV}$ is 60- to 70-fold lower than that of the Glu-Ile bond in $SspC^{wt}$ (Table 3), the rate of cleavage of this bond in $SspC^{DLV}$ is 50 to 60 times faster than is conversion of P_{46} to P_{41} in vivo (Table 3). Thus, GPR can cleave sequences identical to those in the $P_{46} \rightarrow P_{41}$ cleavage site at rates more than fast enough to be consistent with an autoprocessing model for generation of P_{41}, although admittedly this is an extrapolation from GPR cleavage in *trans* to GPR cleavage in *cis*. Surprisingly, analysis of the GPR cleavage products from $SspC^{DL}$ and $SspC^{DLV}$ indicated that GPR can also cleave a second site in these proteins (Table 3). While cleavage of this second site is slower than cleavage of the Glu-Ile bond in $SspC^{wt}$, it is ~5 times faster than cleavage of the Asp-Leu bond in $SspC^{DL}$ and $SspC^{DLV}$. The sequence around this second cleavage site (Fig. 2, arrow labeled 2) shares three amino acids (Glu-Phe-Gly) found in several GPR cleavage sites in γ-type SASP (Fig. 1), and, as we have shown in this work, a Val residue in the fourth position in the cleavage site sequence probably slows GPR cleavage little if at all. However, the Gln residue in the fifth position of this second cleavage site is unique, since this position is Glu in all other known GPR cleavage sites (Fig. 1). Clearly, the substrate specificity of GPR is broader than was initially imagined, and features in addition to amino acid sequence—possibly, lack of significant secondary structure—may also be important in determining good GPR substrates.

It is, of course, possible that the sequence around the GPR cleavage site is not the major determinant for GPR recognition and that this is instead far removed from the cleavage site itself. Consequently, changes in the cleavage site sequence might only reduce but not abolish GPR cleavage. We believe that this explanation is extremely unlikely for several reasons. First, the SspC variants $SspC^{A}$ and $SspC^{Q}$, which have alterations in the more carboxyl-terminal highly conserved region, are cleaved by GPR in vitro as fast as is $SspC^{wt}$ (18). Second, and most tellingly, we have recently obtained autoprocessing of GPR from $P_{46} \rightarrow P_{41}$ in vitro (5), with the cleavage generating P_{41} taking place at the site cleaved in vivo which is identical to that cleaved in $SspC^{DLV}$ (Fig. 1). Since α/β-type SASP and GPR have no sequence homology other than in this cleavage site, this is strong evidence that it is this sequence which is GPR's primary recognition determinant.

The mutants generated in this work increase to five the number of SspC variants in which residues conserved in essentially all α/β-type SASP have been altered (Fig. 2). It is striking that these SspC variants, with the possible exception of $SspC^{V}$, bind to DNA significantly more poorly than does $SspC^{wt}$. The two SspC variants studied previously ($SspC^{A}$ and $SspC^{Q}$; Fig. 2) had changes in the more carboxy-terminal extremely highly conserved region, which is thought to be involved directly in α/β-type SASP-DNA interaction (13, 24). Indeed, $SspC^{Q}$ binds poorly to DNA in vivo and binds only poly(dG) · poly(dC) in vitro, while $SspC^{A}$ binds no DNAs, either in vivo or in vitro (24). In contrast, the variants studied in the present work had changes in the GPR cleavage region which is in the center of the protein (Fig. 2). Although the precise structure of α/β-type SASP on DNA is not yet known, the lack of good binding of $SspC^{DL}$ and $SspC^{DLV}$ to pUC19 indicates that the GPR cleavage region also has a role in DNA-protein interaction. Since binding of $SspC^{wt}$ to pUC19 is quite cooperative (19), possibly the region around the GPR cleavage site is also involved in SspC-SspC interaction on DNA. While this suggestion is only speculative at present, the continued binding of poly(dG) · poly(dC) to SspC variants with alterations in the GPR cleavage region is consistent with this region being important for SspC-SspC interaction, since binding of $SspC^{wt}$ to poly(dG) · poly(dC) is not very cooperative (19).

As observed previously with other SspC variants (15, 24), we also observed a good correlation between the effects of the SspC variants generated in this work on DNA properties in vivo in *E. coli* and in *B. subtilis*, as well as in vitro. Thus, $SspC^{V}$, which appeared to bind best to *E. coli* DNA in vivo, was also best at restoring the heat and UV resistance of $\alpha^-\beta^-$ spores. Previous work has shown that the increased sensitivity of $\alpha^-\beta^-$ spores to heat and UV radiation compared with that of wild-type spores is due to an increase in DNA damage in $\alpha^-\beta^-$ spores (2, 21). In contrast, $SspC^{DLV}$, which bound most weakly to DNA in vitro, had essentially no effect on the DNA properties of $\alpha^-\beta^-$ spores; $SspC^{DL}$ had intermediate effects on DNA properties in vivo and in vitro, in particular in that heat resistance but not UV resistance was restored to $\alpha^-\beta^-$ spores by $SspC^{DL}$. Previous work has shown that restoration of $\alpha^-\beta^-$ spore heat resistance takes significantly less functional wild-type α/β-type SASP than does restoration of UV resistance (8).

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