Properties of *Bacillus megaterium* and *Bacillus subtilis* Mutants Which Lack the Protease That Degrades Small, Acid-Soluble Proteins during Spore Germination

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Received 15 October 1991/Accepted 11 November 1991

During germination of spores of *Bacillus* species the degradation of the spore's pool of small, acid-soluble proteins (SASP) is initiated by a protease termed GPR, the product of the *gpr* gene. *Bacillus megaterium* and *B. subtilis* mutants with an inactivated *gpr* gene grew, sporulated, and triggered spore germination as did *gpr*⁺ strains. However, SASP degradation was very slow during germination of *gpr* mutant spores, and in rich media the time taken for spores to return to vegetative growth (defined as outgrowth) was much longer in *gpr* than in *gpr*⁺ spores. Not surprisingly, *gpr* spores had much lower rates of RNA and protein synthesis during outgrowth than did *gpr*⁺ spores, although both types of spores had similar levels of ATP. The rapid decrease in the number of negative supertwists in plasmid DNA seen during germination of *gpr*⁺ spores was also much slower in *gpr* spores. Additionally, UV irradiation of *gpr* *B. subtilis* spores early in germination generated significant amounts of spore photoproduct and only small amounts of thymine dimers (TT); in contrast UV irradiation of germinated *gpr*⁺ spores generated almost no spore photoproduct and three to four times more TT. Consequently, germinated *gpr* spores were more UV resistant than germinated *gpr*⁺ spores. Strikingly, the slow outgrowth phenotype of *B. subtilis* *gpr* spores was suppressed by the absence of major α/β -type SASP. These data suggest that (i) α/β -type SASP remain bound to much, although not all, of the chromosome in germinated *gpr* spores; (ii) the α/β -type SASP bound to the chromosome in *gpr* spores alter this DNA's topology and UV photochemistry; and (iii) the presence of α/β -type SASP on the chromosome is detrimental to normal spore outgrowth.

Approximately 10 to 20% of the protein of dormant spores of *Bacillus* species is degraded to free amino acids in the first minutes of spore germination (22). These amino acids can support much of the protein synthesis early in the development of the germinating and outgrowing spore (22). The proteins degraded in this process are a group of small, acid-soluble proteins (SASP) of two types, α/β and γ (22). There are multiple α/β -type SASP in spores of *Bacillus* species, and the amino acid sequences of these proteins have been highly conserved both within and across species (22). The α/β -type SASP are associated with spore DNA and play a key role in the resistance of spores to UV light (22, 23). *Bacillus* spores have only one γ -type SASP, and the primary sequence of this protein has not been as highly conserved in evolution as has those of α/β -type SASP. γ -Type SASP are not associated with DNA in vivo and have no known function other than generating amino acids by their degradation during spore germination.

The great majority, if not all, SASP degradation during spore germination is initiated by a single protease, which has been termed GPR (8, 9, 22). This enzyme is an endoprotease specific for a pentapeptide sequence found once or twice in all SASP. GPR is initially synthesized during sporulation as a 46-kDa precursor (termed P₄₆) which associates into tetramers but is enzymatically inactive. Later in sporulation P₄₆ is processed to a 41-kDa polypeptide (termed P₄₁) which also forms tetramers and is inactive in vivo but is active in vitro; P₄₁ is stored in the dormant spore. During spore germination

P₄₁ is processed to a 40-kDa form (termed P₄₀) which then acts to initiate SASP degradation in vivo. We have long thought it would be of interest to analyze the effects of lack of GPR on spore germination and outgrowth, in particular because there is evidence that a high level of α/β -type SASP in growing cells is deleterious (16). We had isolated and analyzed some GPR mutants in *B. megaterium* a number of years ago (15), but it now appears likely that these were missense mutants which retained significant enzyme activity in vivo. However, the recent cloning and sequencing of the gene coding for GPR (termed *gpr*) from both *B. megaterium* and *B. subtilis* (28) has made it possible to construct null mutations in *gpr*. In this communication we report the phenotypic effects of such null mutations.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth and production of spores. The bacterial strains and plasmids used in this work are listed in Table 1. *B. megaterium* and *B. subtilis* strains were routinely grown and sporulated in supplemented nutrient broth (SNB) or 2× SG medium (4), respectively, and spores were purified as previously described (4, 10, 24). Antibiotics were added to media for growth of resistant strains at the following concentrations: chloramphenicol, 3 µg/ml; kanamycin, 10 µg/ml. *B. subtilis* spores labeled in DNA with [*methyl*-³H]thymidine were prepared as previously described (17). Cells of *B. subtilis* were made competent for transformation and transformed with plasmids, and transformants were selected as previously described (10, 12).

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TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Genotype or phenotype	Source or reference
Strains		
<i>B. megaterium</i>		
QMB1551	<i>gpr</i> ⁺	Hillel Levinson
PS1551	<i>gpr</i> ::pJH101 Cm ^r <i>gpr</i>	pPS434→QMB1551
<i>B. subtilis</i>		
PS533	$\alpha^+\beta^+$ pUB110 <i>gpr</i> ⁺ Km ^r	12
PS578	$\alpha^-\beta^-$ pUB110 <i>gpr</i> ⁺ Km ^r	12
PS930	$\alpha^+\beta^+$ <i>amyE</i> ::ptrpBG-1 Cm ^r <i>gpr</i> ⁺	28
PS1029	$\alpha^+\beta^+$ <i>gpr</i> ::pJF751 Cm ^r <i>gpr</i>	28
PS1590	$\alpha^+\beta^+$ <i>gpr</i> ::pJF751 pUB110 Cm ^r <i>gpr</i> Km ^r	pPS1027→PS533
PS1621	$\alpha^-\beta^-$ <i>gpr</i> ::pJF751 pUB110 Cm ^r <i>gpr</i> Km ^r	pPS1027→PS578
Plasmids		
pPS494	0.7-kb <i>Hae</i> III fragment from within <i>B. megaterium gpr</i> coding sequence, carrying <i>Eco</i> RI linkers and cloned in the <i>Eco</i> RI sites of pJH101 (Amp ^r Cm ^r)	28
pPS1027	pJF751 with internal region of <i>B. subtilis gpr</i> coding sequence in frame with <i>lacZ</i> (Amp ^r Cm ^r)	28
pUB110	Km ^r	28

Spore germination and measurement of ATP, macromolecular synthesis, and SASP. Spores of *B. megaterium* (5 to 15 mg [dry weight]/ml of water) were germinated after a heat shock at 60°C for 15 min; *B. subtilis* spores (5 to 15 mg [dry weight]/ml of water) were heat shocked at 70°C for 30 min. After cooling in ice, spores were germinated at 0.5 to 1 mg/ml and either at 30°C (*B. megaterium*) or 37°C (*B. subtilis*). Germination media used were as follows: 2× SG (4) plus 4 mM L-alanine, 2× YT (11) plus 4 mM L-alanine, SNB (23), or Spizizen's minimal medium (26) plus 4 mM L-alanine and 50 µg of tryptophan per ml. Appropriate antibiotics were added as noted above.

Germination media were supplemented with [5-³H]uridine (50 µM; 5 µCi/ml) or [³H]leucine (100 µM; 33 µCi/ml) for measurement of RNA and protein synthesis, respectively. Aliquots (0.25 ml) of culture were added to 2 ml of 5% trichloroacetic acid; after 30 min at 4°C, acid-insoluble material was collected on a glass-fiber filter, and the filter was washed and counted in a toluene-based scintillation fluid as previously described (16). ATP was extracted from samples of germinating spores (2 ml) with 8 ml of boiling *n*-propanol, and extracts were assayed for ATP as previously described (25).

For measurement of SASP levels during spore germination, samples of culture equivalent to 15 mg (dry weight) of initial dormant spores were harvested by centrifugation (5 min; 8,000 × *g*), the pellet was resuspended in 1.5 ml of cold water, centrifuged in a microcentrifuge, and lyophilized. SASP were extracted after dry breakage in a dental amalgamator (Wig-L-Bug) with glass beads as the abrasive; eight 1-min periods of shaking sufficed for breakage of both dormant and germinated spores. The dry powder was extracted with 1.5 ml of cold 3% acetic acid for 30 min at 4°C, the sample was centrifuged, and the pellet was reextracted with 1 ml of 3% acetic acid. Both supernatant fluids were pooled and lyophilized, and the dry residue was dissolved in 200 µl of 8 M urea-acid gel diluent as previously described (4). Samples from the equivalent of 1.5 mg of dormant spores were run on acrylamide gel electrophoresis at a low pH to separate SASP, and the proteins on the gel were transferred to nitrocellulose, and the paper was baked overnight at 80°C (4). SASP on the nitrocellulose were then detected with antisera against *B. megaterium* SASP-A and -C (α/β -type

SASP), *B. megaterium* SASP-B (γ -type SASP), *B. subtilis* SASP α plus β , or *B. subtilis* SASP- γ (4, 10).

Other analytical methods. The spore protease was extracted from dry-ruptured spores, run on sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, proteins were transferred to nitrocellulose, and proteins were detected with antiserum against *B. megaterium* GPR as previously described (9). The latter antiserum had been adsorbed to remove much (although not all) of the contaminating antibodies to DnaK (28).

Plasmid was extracted from dormant *B. subtilis* spores after the latter were made lysozyme sensitive by spore coat removal (12). Plasmid was extracted from *B. subtilis* spores germinated for 30 min in 2× YT plus 4 mM L-alanine by using direct lysozyme extraction (12). In one case dormant spores were first decoated and then germinated, since plasmid recoveries from *gpr* spores early in germination were otherwise low. After plasmids were purified by CsCl gradient centrifugation, their average number of supertwists (τ_{av}) was determined as previously described (12).

The UV resistance of spores during germination was determined as previously described (18), and survivors were enumerated by plating on 2× YT medium plates plus the appropriate antibiotic as noted above. Samples ($\sim 3 \times 10^5$ cpm) of [³H]thymidine-labeled *B. subtilis* dormant spores or spores germinated in 2× YT plus 4 mM L-alanine as described above were irradiated with UV light primarily at 254 nm, and the dosages given were determined as previously described (17). Irradiated spores or unirradiated control samples were centrifuged, and the pellet was lyophilized. The dry spores were then broken, DNA purified, and hydrolyzed; photoproducts were separated by descending paper chromatography; and the various photoproducts were quantitated by counting in a scintillation counter as previously described (17).

RESULTS

Construction of *gpr* mutants and proof of their identity. The *gpr* genes from *B. megaterium* and *B. subtilis* were previously mapped (27, 28) by cloning a fragment of the *gpr* gene in the integrable plasmid pJH101 (2), allowing the resulting recombinant plasmid to integrate into the *Bacillus* chromo-

FIG. 1. GPR analysis in *B. megaterium* and *B. subtilis* strains. Samples of extract from ~20 mg of spores of the following: (A) lane 1, *B. megaterium* QMB1551 (*gpr*⁺); lane 2, *B. megaterium* PS1551 (*gpr*⁻); (B) lane 1, *B. subtilis* PS930 (*gpr*⁺); lane 2, *B. subtilis* PS1029 (*gpr*⁻). The samples were separated by electrophoresis on SDS-polyacrylamide gels, proteins were transferred to nitrocellulose, and GPR antigen was detected with antiserum against *B. megaterium* GPR. Arrow labeled a, migration position of DnaK, a protein to which there are antibodies in the anti-GPR antiserum because DnaK was a contaminant in the GPR used as the original immunogen (9, 28); arrow labeled b, the position of the P₄₁ form of GPR from *B. megaterium* and the analogous band from *B. subtilis* extracts.

some, and then mapping the plasmid-carried chloramphenicol resistance marker. Since the *gpr* fragments cloned into pJH101 were entirely within the coding sequence, integration of these recombinant plasmids into the chromosome by a Campbell-like mechanism should disrupt the *gpr* gene. Southern blot analysis of chromosomal DNA from these integrants showed that the *gpr* coding sequence was indeed disrupted (27, 28). The putative *gpr* strains of both species grew and sporulated normally (27, 28; data not shown). Therefore, we felt it was prudent to confirm that GPR was absent from the spores of both *gpr* mutants. Consequently, Western immunoblot analyses were carried out on extracts from *gpr*⁺ and *gpr* spores of both species (Fig. 1) (6, 7). As seen previously (9) the major GPR antigen in *gpr*⁺ *B. megaterium* spores was P₄₁ (Fig. 1A, lane 1). In contrast, P₄₁ was absent from *gpr* *B. megaterium* spores and no new immunoreactive bands appeared (Fig. 1A, lane 2). *B. subtilis* *gpr*⁺ spores also gave a band at 41 kDa which reacted with the antiserum against *B. megaterium* GPR; again this band (as well as a slightly larger band which may be P₄₆) was absent from *gpr* *B. subtilis* spores (Fig. 1B, lanes 1 and 2). Thus, it appears that GPR is absent from spores carrying plasmids interrupting the *gpr* gene.

Germination, outgrowth, and SASP degradation. In addition to identical growth and sporulation of *gpr* and *gpr*⁺ strains, mutant and wild-type spores of both species initiated spore germination with similar kinetics (Fig. 2 and 3). This was shown by the similar rate of fall in optical density beginning immediately after spores were mixed with germinant (Fig. 2 and 3). However, in a rich medium the optical density of *gpr* spore cultures began to increase later and the rate of increase was initially lower than that of *gpr*⁺ spores, indicating that outgrowth of *gpr* spores was slower than that of *gpr*⁺ spores (Fig. 2 and 3). Similar results were obtained with three different preparations of *gpr*⁺ and *gpr* spores of

FIG. 2. Germination and outgrowth of *B. subtilis* spores in a rich medium. Dormant spores of various *B. subtilis* strains were heat shocked and then germinated in a rich medium (2× YT plus 4 mM L-alanine) with chloramphenicol (3 µg/ml) as described in Materials and Methods. Symbols: ○, PS930 (*gpr*⁺); ●, PS1029 (*gpr*⁻); ▲, PS1621 (αβ⁻ *gpr*⁺). Spores of strain PS578 (αβ⁻ *gpr*⁺) gave the same curve (data not shown) as that for strain PS1621.

each species, including strains PS533 and PS1590 (data not shown). Although outgrowth was slower for αβ⁺ *gpr* spores in a rich medium, both *gpr* and *gpr*⁺ spores had identical viability. However, spores of PS930 (*gpr*⁺) gave readily visible colonies after ~16 h at 37°C on 2× YT medium plates, while spores of PS1029 (*gpr*⁻) required 24 to 30 h to obtain maximum colony counts (data not shown). In contrast to the results with rich media, the rates of outgrowth of *gpr* and *gpr*⁺ spores were less different in a poor medium for both *B. megaterium* (data not shown) and *B. subtilis* (Fig. 4) than they were in a rich medium (Fig. 2 and 3). Analysis of SASP levels in *gpr* and *gpr*⁺ spores during germination in a rich medium showed that SASP degradation in *gpr*⁺ spores was >90% complete within 5 min for *B. megaterium* or 15 min for *B. subtilis*, as found previously (7, 15) (Fig. 5A and B). In contrast, noticeable SASP loss was not observed until 90 min after the start of germination of *gpr* spores (Fig. 5A and B). Degradation of both α/β-type (A and C in *B. megaterium* and α and β in *B. subtilis*) and γ-type SASP (B in *B. megaterium* and γ in *B. subtilis*) was slowed in the *gpr* mutants (Fig. 5A and B).

Because the difference in outgrowth between *gpr* and *gpr*⁺ spores was maximal in rich media and minimal in a poor medium, this suggested that it was the presence of SASP in the germinated *gpr* spores which was slowing outgrowth. The SASP most likely to be having this effect were α/β-type SASP, since these can block growth in *Escherichia coli* and inhibit many reactions involving DNA in vitro (16, 20). In contrast, γ-type SASP have no effect in *E. coli*, and do not interact with DNA in vivo or in vitro (14, 18). In order to test this possibility directly, we introduced the *gpr* mutation into a *B. subtilis* strain lacking the two major α/β-type SASP, α and β (αβ⁻ strain). As predicted, spores of the αβ⁻ *gpr*

FIG. 3. Germination and outgrowth of *B. megaterium* spores in a rich medium. Dormant spores of *B. megaterium* strains were heat shocked and germinated in SNB medium as described in Materials and Methods. Symbols: ○, strain QMB1551 (*gpr*⁺); ●, PS1551 (*gpr*).

strain proceeded through outgrowth in a rich medium at a rate close to that of $\alpha^+\beta^+$ *gpr*⁺ spores and identical to that of $\alpha^-\beta^+$ *gpr*⁺ spores (Fig. 2; data not shown).

Analysis of RNA and protein synthesis during outgrowth in a rich medium showed that macromolecular synthesis was much slower with *gpr* spores (Fig. 6). This is not surprising given the slower outgrowth of *gpr* spores under these conditions. However, ATP analyses in spores germinated 30 min showed that there was no significant difference in ATP levels in cultures of germinated *gpr* and *gpr*⁺ spores (85 and 75 pmol/ml, respectively, in cultures analogous to those in Fig. 6 [data not shown]). Consequently, the reason for slow macromolecular synthesis in *gpr* spores during germination and outgrowth is not a defect in energy metabolism. Note that in the experiments measuring macromolecular synthesis it appeared that protein synthesis began before RNA synthesis during germination and outgrowth, in contrast to previous results (25). However, this apparent discrepancy is due to the use of less than optimal conditions for measuring RNA synthesis because of the rich medium used in these experiments.

Properties of DNA in germinated *gpr* spores. The data given above suggest that the slow outgrowth of *gpr* spores in rich media is due to slowed RNA synthesis, with this in turn due to the continued presence of α/β -type SASP in these spores. Since previous work has shown that α/β -type SASP are bound to DNA *in vivo* (3, 16), it seemed likely that this would also be the case in germinated *gpr* spores. If so, then this binding should have effects on the properties of DNA in germinated *gpr* spores in addition to slowed transcription. One obvious property to test is plasmid DNA supercoiling which is extremely high in dormant spores, primarily be-

FIG. 4. Germination and outgrowth of *B. subtilis* spores in a minimal medium. Dormant spores were heat shocked and germinated in Spizizen's minimal medium plus 4 mM L-alanine, 50 μ g of tryptophan per ml and 3 μ g of chloramphenicol per ml. Symbols: ○, PS930 (*gpr*⁺); ●, PS1029 (*gpr*).

cause of α/β -type SASP binding, but decreases rapidly in the first minutes of spore germination (12). As found previously the average number of supertwists (τ_{av}) in plasmid pUB110 fell ~25% (from -46 to -34) by 30 min of germination of *gpr*⁺ spores (12) (Fig. 7, lanes 1 and 2). In contrast, the τ_{av} value of this plasmid fell <5% in *gpr* spores after 30 min of germination (Fig. 7, lanes 3 and 4). However, plasmid pUB110 from dormant spores of both *gpr*⁺ and *gpr* strains had similar τ_{av} values (Fig. 7, lanes 1 and 3).

We also monitored the transient UV resistance during germination of *gpr* and *gpr*⁺ spores, since this has been suggested to be a reflection of a conformational change in DNA early in germination, a conformational change dependent on α/β -type SASP (18, 23). With both species *gpr* and *gpr*⁺ spores exhibited nearly identical transient UV resistance during germination (Fig. 8; data not shown). However, this analysis only detects the extremely UV-resistant spores which transiently exist early in spore germination; even late in germination the survivors of the huge UV dose used in analysis of transient UV resistance are those spores triggering germination well after addition of germinant (18). Consequently, it was possible that the total population of germinated *gpr* spores might be somewhat more UV resistant than that of germinated *gpr*⁺ spores. Indeed, this was the case (Fig. 9). Similar results were obtained when 30-min germinated spores of strains PS930 (*gpr*⁺) and PS1029 (*gpr*) were compared (data not shown).

Analysis of the UV photoproducts generated in [³H]thymidine-labeled germinated spores gave results consistent with the differences in UV resistance noted above (Table 2). UV irradiation of dormant *gpr*⁺ or *gpr* spores gave only

FIG. 5. Levels of SASP in dormant and germinated spores. (A) SASP were extracted from *B. megaterium* dormant spores (lanes 1 and 6) or spores germinated 5 min (lanes 2 and 7), 15 min (lanes 3 and 8), 40 min (lanes 4 and 9), or 90 min (lanes 5 and 10) from strains QMB1551 (*gpr*⁺) (lanes 1 to 5) or PS1551 (*gpr*) (lanes 6 to 10). Samples from equal amounts of spores (equivalent to 1 mg of dormant spores) were run on two polyacrylamide gels at a low pH, SASP were transferred to nitrocellulose, and antigen on one paper was detected with antiserum against SASP-A and -C, while antiserum against SASP-B was used to detect proteins on the second paper. The lettered horizontal arrows denote the migration position of SASP-A or -C (which comigrate) or SASP-B. (B) SASP were extracted from *B. subtilis* dormant spores (lanes 1 and 5) or spores germinated 15 min (lanes 2 and 6), 60 min (lanes 3 and 7), or 90 min (lanes 4 and 8) from strains PS930 (*gpr*⁺) (lanes 1 to 4) or PS1029 (*gpr*) (lanes 5 to 8). Samples were analyzed as described above with antisera against either *B. subtilis* SASP- α plus - β or SASP- γ . The lettered horizontal arrows denote the migration positions of SASP- α , - β , and - γ .

spore photoproduct (SP), as found previously (17). However, thymine dimer (TT) was the major photoproduct from *gpr*⁺ spores at the earliest time in germination; presumably much of the SP produced in the *gpr*⁺ spores is from those which have not yet germinated (Table 2). In contrast, much less TT was produced in germinated *gpr* spores, even after 100 min of germination, and much more SP was produced (Table 2). Analysis of the yield of various photoproducts as a function of the incident UV fluence showed that SP formation in germinated *gpr* spores required >20 times more fluence than did SP formation in dormant spores or TT formation in germinated spores (Table 3). We have shown previously that the yields of TT in growing cells and SP in dormant spores per incident fluence are similar when the linear portions of dose-response curves are compared (14,

FIG. 6. Macromolecular synthesis during germination and outgrowth of *gpr*⁺ and *gpr* *B. subtilis* strains. Dormant *B. subtilis* spores were heat shocked and germinated in 2× YT medium plus 4 mM L-alanine and chloramphenicol (3 μg/ml), and protein or RNA synthesis was measured as described in Materials and Methods. The data presented are the picomoles of culture of [³H]leucine (protein) or [³H]uridine (RNA) incorporated into acid-insoluble material per milliliter of culture. Symbols: ○, PS930 (*gpr*⁺), protein synthesis; △, PS930 (*gpr*⁺), RNA synthesis; ●, PS1029 (*gpr*), protein synthesis; ▲, PS1029 (*gpr*), RNA synthesis.

17). Indeed, the yields of SP in dormant spores and TT in germinated spores as a function of incident fluence as shown in Table 3 may be similar, but this analysis is complicated by the near saturation of TT formation at the lowest dose used. However, it is clear that TT formation in germinated spores and SP formation in dormant spores requires much less UV fluence than SP formation in germinated *gpr* spores.

DISCUSSION

Since degradation of both α/β - and γ -type SASP is extremely slow during germination of *gpr* mutant spores, GPR must initiate the great majority of degradation of both types of SASP, as was suggested previously (15, 21). Some *B. megaterium* *gpr* mutants isolated a number of years ago by assay of GPR enzyme activity in spores retain significant degradation of α/β -type SASP during spore germination (15). However, these mutants retain wild-type levels of GPR antigen (9), and it seems likely that these mutants retained significant GPR activity in vivo, though not in vitro. In contrast, the *gpr* mutants analyzed in this work retain no detectable GPR antigen.

The major phenotype of the *gpr* mutants of both *B. megaterium* and *B. subtilis* is a slow return to vegetative growth upon spore germination in a rich medium. Since the triggering of spore germination appears normal in *gpr* mutants, their slow return to vegetative growth is because of slow outgrowth which appears due at least in part to slowed transcription during this period of development. Spores in which major SASP are absent even though GPR is present (i.e., $\alpha^- \beta^-$ *gpr*⁺) also exhibit slower outgrowth than wild-type spores (6, 10). However, with $\alpha^- \beta^-$ *gpr*⁺ spores

FIG. 7. Analysis of supercoiling of plasmid pUB110 in dormant and germinated *gpr*⁺ and *gpr* spores. Plasmid pUB110 was isolated and purified from dormant (lanes 1 and 3) or 30-min germinated (lanes 2 and 4) spores of strains PS533 (*gpr*⁺) (lanes 1 and 2) or PS1590 (*gpr*) (lanes 3 and 4), and aliquots (~1 µg) were analyzed by electrophoresis on 1.4% agarose gels containing 4 µg of chloroquine per ml and then by staining with ethidium bromide. The numbered horizontal arrows give the average number of negative supertwists (τ_{av}) of plasmid pUB110 migrating at that position.

extremely slow outgrowth relative to that of $\alpha^+\beta^+$ *gpr*⁺ spores is seen in a poor medium, while outgrowth is only slightly slower than that of $\alpha^+\beta^+$ *gpr*⁺ spores in a rich medium (6, 10). This is consistent with the role for these of

FIG. 8. Transient elevated UV resistance of *gpr*⁺ and *gpr* spores during spore germination. Dormant *B. subtilis* spores were heat shocked and germinated in 2× YT medium plus 4 mM L-alanine. At various times during germination, aliquots were diluted 75-fold in cold 25 mM NaPO₄ (pH 7.2)–0.15 M NaCl and UV irradiated for 30 s with a total dose of 400 J/m², and aliquots were plated on 2× YT medium agar plates containing chloramphenicol (3 µg/ml) for enumeration of survivors. Symbols: ○, PS930 (*gpr*⁺); ●, PS1029 (*gpr*).

FIG. 9. UV resistance of germinated *gpr* and *gpr*⁺ spores. Dormant spores were heat shocked and germinated for 30 min in 2× YT medium plus 4 mM L-alanine and chloramphenicol (3 µg/ml) and kanamycin (10 µg/ml). Aliquots were then diluted 1:100 in cold 25 mM KPO₄ (pH 7.5)–0.15 M NaCl and UV irradiated at a dose rate of 50 J/m² · min, and survivors were determined. Symbols: ○, PS533 (*gpr*⁺); ●, PS1590 (*gpr*).

SASP in supplying amino acids for protein synthesis early in spore germination and outgrowth (22). In contrast, the slower outgrowth of *gpr* spores relative to that of *gpr*⁺ spores is most obvious in rich media and is less noticeable (although still present) in a poor medium. Thus, the defect in *gpr* spore outgrowth is not because of a decreased rate of generation of amino acids for protein synthesis. Rather the defect is related to the continued presence of SASP, in particular α/β -type SASP, in outgrowing *gpr* spores. This is shown by the suppression of the *gpr* spore's outgrowth defect in rich medium by concomitant absence of SASP- α and - β . That α/β -type (but not γ -type) SASP should inhibit cell growth is consistent with the effect of these proteins on *E. coli* in vivo (16). This growth inhibition is most likely because of binding of α/β -type SASP to the chromosomal DNA with attendant decreased transcription of a number of genes, as has been found both in vivo and in vitro (16, 20). Presumably, the α/β -type SASP are slowly degraded during

TABLE 2. UV photoproducts from *gpr*⁺ and *gpr* spores^a

Photo-product	% of total thymine after the following germination times:							
	None (dormant)		13 min		30 min		100 min	
	<i>gpr</i> ⁺	<i>gpr</i>	<i>gpr</i> ⁺ (10) ^b	<i>gpr</i> (9)	<i>gpr</i> ⁺ (6)	<i>gpr</i> (3)	<i>gpr</i> ⁺ (1)	<i>gpr</i> (0)
TT	0.2	0.2	5.6	0.8	6.9	1.5	6.7	4.3
SP	20.6	20.3	1.9	5.8	0.9	4.9	0.6	2.3

^a Dormant or germinated spores of strains PS930 (*gpr*⁺) or PS1029 (*gpr*) were irradiated with 15 kJ/m² of UV light, DNA was isolated, and photoproducts were quantitated as described in Materials and Methods.

^b Values in parentheses are the percentage of dormant spores remaining at these germination times as determined by examination in a phase-contrast microscope.

TABLE 3. Production of TT and SP in dormant and germinated spores as a function of UV fluence^a

Spores irradiated	% of total thymine at the following UV fluence (kJ/m ²):					
	1		7		30	
	TT	SP	TT	SP	TT	SP
Dormant <i>gpr</i> (PS1029)	0.1 ^b	5.1	0.2 ^b	16.0	0.2 ^b	20.0
40-min germinated <i>gpr</i> ⁺ (PS930)	5.1	0.1 ^c	6.7	0.3 ^c	6.6	1.0 ^c
40-min germinated <i>gpr</i> (PS1029)	2.0	0.3 ^c	2.4	1.2 ^c	2.6	4.5 ^c

^a [³H]thymidine-labeled dormant or germinated *B. subtilis* spores were UV irradiated, and DNA was isolated and photoproducts were quantitated as described in Materials and Methods.

^b The TT in the irradiated dormant spore preparations may well come from the small percentage of contaminating germinated spores or vegetative cells.

^c These values have been corrected for the amount of SP generated from the small percentage of dormant spores in the 40-min germinated spore preparations. These latter values were PS930, 6%, and PS1029, 3%.

outgrowth of *gpr* spores, either by a dormant spore protease other than GPR or by one synthesized slowly during outgrowth; this slow SASP degradation then eventually allows rapid growth of the *gpr* strains. The fact that the time taken for outgrowth of *gpr* spores in a poor medium is closer to that of *gpr*⁺ spores is then a reflection of the longer time taken for spore outgrowth in such a medium, sufficient time such that slow SASP degradation no longer has such an enormous effect on the rate of outgrowth.

Immunoelectron microscopic analysis of both developing forespores of *B. subtilis* as well as *E. coli* in which α/β -type SASP synthesis is induced has shown that α/β -type SASP are associated with the nucleoid in vivo (3, 16). While we have not shown this directly with germinated *gpr* spores, we feel a significant amount of α/β -type SASP must be associated with the nucleoid of germinated *gpr* spores, because the effect of the *gpr* mutation is to maintain the plasmid supercoiling and UV photochemistry generally as it is in dormant spores, in which we know these properties are due to direct α/β -type SASP-DNA interaction (3, 12, 17). However, we have no proof that all α/β -type SASP in germinated *gpr* spores are nucleoid associated. Indeed, there is reason to suspect (see below) that some regions of the chromosome are free of α/β -type SASP in germinated *gpr* spores. Our initial hypothesis concerning the fate of undegraded α/β -type SASP in germinated *gpr* spores was that they would all remain on the chromosome (23). If this were the case then we expected that the transient high UV resistance seen during germination of *gpr*⁺ spores (18) would last much longer in *gpr* spores. Clearly, this was not the case, as the number of survivors of the high UV dose used to detect transient UV resistance was essentially the same during the germination of both *gpr* and *gpr*⁺ spores. Analysis of the thymidine-derived photoproducts from germinated *gpr* spores indicated why there was no increased transient UV resistance, since at the earliest time in germination tested UV irradiation of *gpr* spores produced some TT. In contrast, the transient UV-resistant state during germination is characterized by an extremely low yield of all UV photoproducts, including no TT production (23). However, after 30 min of germination, the *gpr* spore population as a whole was more UV resistant than were comparable *gpr*⁺ spores. This enhanced UV resistance of germinated *gpr* spores appears to be due to decreased TT production in these spores, as well as the decreased production of both TT and SP at a given UV dose. We believe the enhanced UV resistance and decreased

TT production in germinated *gpr* spores are due to association of α/β -type SASP with much of the chromosome, since this has been shown to reduce TT formation and increase SP production both in vivo and in vitro (14, 16). Since UV irradiation of DNA saturated with α/β -type SASP in vitro gives no TT, some of the DNA in *gpr* spores early in germination may well be free from SASP (see below). Presumably, it is this fraction of SASP-free DNA in which TT are produced in *gpr* spores and from which transcription allows an eventual return to vegetative growth. Indeed, it is possible that only those α/β -type SASP not bound to DNA are good substrates for GPR attack early in germination, since in vitro GPR does not act on α/β -type SASP bound to DNA (19). The SP yield per incident UV fluence in germinated *gpr* spores was very similar to that found in *E. coli* and with an α/β -type SASP-DNA complex in vitro (14, 16, 17). However, the yield of SP per incident UV fluence in germinated *gpr* spores was >20-fold lower than that in dormant spores (*gpr*⁺ or *gpr*). As suggested previously (14, 23), this indicates that the high yield of SP per incident fluence in dormant spores is due to some factor(s) in addition to binding of α/β -type SASP. While the identity of this factor(s) has not been established, one possibility is the huge depot of dipicolinic acid present in dormant spores which is excreted in the first minutes of spore germination (14).

Previous work has shown that spores have sufficient α/β -type SASP to saturate the DNA and that UV irradiation in vitro of a saturated α/β -type SASP/DNA complex produces no TT (13, 14, 22). Thus, we were somewhat surprised by the production of significant amounts of TT upon UV irradiation of *gpr* spores early in germination, as this suggested the presence of significant amounts of SASP-free DNA even though there had been no apparent loss of total α/β -type SASP. While it is of course possible that a small amount of α/β -type SASP had been lost in these germinated *gpr* spores, it is also possible that some had simply dissociated from the DNA early in spore germination. Early in spore germination there is a significant increase in spore core volume because of an influx of water (5), as well as an increase in mobile ions in the spore core (1), perhaps indicative of an increased apparent ionic strength. Since binding of α/β -type SASP to some DNA, in particular AT-rich regions, is not tremendously strong (20), it is possible that both dilution and increased ionic strength could result in dissociation of some α/β -type SASP from the chromosome simply by mass action. Indeed, this dissociation may be essential for GPR cleavage of α/β -type SASP during germination of *gpr*⁺ spores, since GPR is inactive on α/β -type SASP bound to DNA (19). Presumably, as free α/β -type SASP are degraded, either by GPR or another protease, more protein dissociates from the DNA, until the entire chromosome is SASP free.

ACKNOWLEDGMENTS

This work was supported by grants from the Army Research Office and the National Institutes of Health (GM 19698). Partial support for J.-L.S.-S. was provided by the Consejo Nacional de Ciencia y Tecnologia.

REFERENCES

1. Carstensen, E. L., and R. E. Marquis. 1975. Dielectric and electrochemical properties of bacterial cells, p. 563-571. In P. Gerhardt, R. N. Costilow, and H. L. Sadoff (ed.), *Spores* VI. American Society for Microbiology, Washington, D.C.
2. Ferrari, F. A., A. Nguyen, D. Long, and J. A. Hoch. 1983. Construction and properties of an integrable plasmid for *Bacil-*

- lus subtilis*. J. Bacteriol. 154:1513–1515.
3. 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.
 4. Goldrick, S., and P. Setlow. 1983. Expression of a *Bacillus megaterium* sporulation specific gene in *Bacillus subtilis*. J. Bacteriol. 155:1459–1462.
 5. Gould, G. W. 1969. Germination, p. 397–444. In G. W. Gould, and A. Hurst (ed.), The bacterial spore. Academic Press, Inc., New York.
 6. 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.
 7. Johnson, W. C., and D. J. Tipper. 1981. Acid-soluble spore proteins of *Bacillus subtilis*. J. Bacteriol. 146:972–982.
 8. Loshon, C. A., and P. Setlow. 1982. *Bacillus megaterium* spore protease: purification, radioimmunoassay, and analysis of antigen level and localization during growth, sporulation and spore germination. J. Bacteriol. 150:303–311.
 9. Loshon, C. A., B. M. Swerdlow, and P. Setlow. 1982. *Bacillus megaterium* spore protease: synthesis and processing of precursor forms during sporulation and germination. J. Biol. Chem. 257:10838–10845.
 10. Mason, J. M., and P. Setlow. 1986. Evidence for an essential role for small, acid-soluble, spore proteins in the resistance of *Bacillus subtilis* spores to ultraviolet light. J. Bacteriol. 167:174–178.
 11. Messing, J. 1983. New M13 vectors for cloning. Methods Enzymol. 101:20–78.
 12. Nicholson, W. L., and P. Setlow. 1990. Dramatic increase in the negative superhelicity of plasmid DNA in the forespore compartment of sporulating cells of *Bacillus subtilis*. J. Bacteriol. 172:7–14.
 13. Nicholson, W. L., B. Setlow, and P. Setlow. 1990. Binding of DNA in vitro by a small, acid-soluble spore protein and its effect on DNA topology. J. Bacteriol. 172:6900–6906.
 14. Nicholson, W. L., B. Setlow, and P. Setlow. 1991. Ultraviolet irradiation of DNA complexed with α/β -type small, acid-soluble proteins from spores of *Bacillus* or *Clostridium* species makes spore photoproduct but not thymine dimers. Proc. Natl. Acad. Sci. USA 88:8288–8292.
 15. Postemsky, C. J., S. S. Dignam, and P. Setlow. 1978. Isolation and characterization of *Bacillus megaterium* mutants containing decreased levels of spore protease. J. Bacteriol. 135:841.
 16. 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.
 17. Setlow, B., and P. Setlow. 1987. Thymine containing dimers as well as spore photoproducts are found in ultraviolet-irradiated *Bacillus subtilis* spores that lack small acid-soluble proteins. Proc. Natl. Acad. Sci. USA 84:421–423.
 18. Setlow, B., and P. Setlow. 1988. Absence of transient elevated UV resistance during germination of *Bacillus subtilis* spores lacking small, acid-soluble spore proteins α and β . J. Bacteriol. 170:2858–2859.
 19. Setlow, B., and P. Setlow. Unpublished data.
 20. Setlow, B., D. Sun, and P. Setlow. Submitted for publication.
 21. Setlow, P. 1976. Purification and properties of a specific proteolytic enzyme present in spores of *Bacillus megaterium*. J. Biol. Chem. 251:8168.
 22. Setlow, P. 1988. Small acid-soluble, spore proteins of *Bacillus* species: Structure, synthesis, genetics, function and degradation. Annu. Rev. Microbiol. 42:319–338.
 23. Setlow, P. 1988. Resistance of bacterial spores to ultraviolet light. Comments Mol. Cell. Biophys. 5:253–264.
 24. Setlow, P., and A. Kornberg. 1969. Biochemical studies of bacterial sporulation and germination. XVII. Sulfhydryl and disulfide levels in dormancy and germination. J. Bacteriol. 100:1155.
 25. Setlow, P., and A. Kornberg. 1970. Biochemical studies of bacterial sporulation and germination. XXII. Energy metabolism in early stages of germination of *Bacillus megaterium* spores. J. Biol. Chem. 245:3637.
 26. Spizizen, J. 1958. Transformation of biochemically deficient strains of *Bacillus subtilis* by deoxyribonucleate. Proc. Natl. Acad. Sci. USA 44:1072–1078.
 27. Sussman, M. D., P. S. Vary, C. Hartman, and P. Setlow. 1988. Integration and mapping of *Bacillus megaterium* genes which code for small, acid-soluble spore proteins and their protease. J. Bacteriol. 170:4942–4945.
 28. Sussman, M. D., and P. Setlow. 1991. Cloning, nucleotide sequence, and regulation of the *Bacillus subtilis* *gpr* gene which codes for the protease that initiates degradation of small, acid-soluble, proteins during spore germination. J. Bacteriol. 173:293–300.