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Molecular Cloning and Characterization of the *Bacillus subtilis* Spore Photoproduct Lyase (*spl*) Gene, Which Is Involved in Repair of UV Radiation-Induced DNA Damage during Spore Germination

PATRICIA FAJARDO-CAVAZOS, CRESCENCIO SALAZAR, AND WAYNE L. NICHOLSON*

Department of Microbiology and Immunology, Texas College of Osteopathic Medicine, 3500 Camp Bowie Boulevard, Fort Worth, Texas 76107

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Upon UV irradiation, *Bacillus subtilis* spore DNA accumulates the novel thymine dimer 5-thyminyl-5,6-dihydrothymine. Spores can repair this "spore photoproduct" (SP) upon germination either by the *uvr*-mediated general excision repair pathway or by the SP-specific *spl* pathway, which involves in situ monomerization of SP to two thymines by an enzyme named SP lyase. Mutants lacking both repair pathways produce spores that are extremely sensitive to UV. For cloning DNA that can repair a mutation in the *spl* pathway called *spl-1*, a library of *Eco*RI fragments of chromosomal DNA from *B. subtilis* 168 was constructed in integrative plasmid pJH101 and introduced by transformation into a mutant *B. subtilis* strain that carries both the *uvrA42* and *spl-1* mutations, and transformants whose spores exhibited UV resistance were selected by UV irradiation. With a combination of genetic and physical mapping techniques, the DNA responsible for the restoration of UV resistance was shown to be present on a 2.3-kb *Eco*RI-*Hin*dIII fragment that was mapped to a new locus in the *metC-pyrD* region of the *B. subtilis* chromosome immediately downstream from the *pstI* gene. The *spl* coding sequence was localized on the cloned fragment by analysis of in vitro-generated deletions and by nucleotide sequencing. The *spl* nucleotide sequence contains an open reading frame capable of encoding a 40-kDa polypeptide that shows regional amino acid sequence homology to DNA photolyases from a number of bacteria and fungi.

A notable property of bacterial spores is their ability to survive for extended periods under conditions that are lethal for vegetative bacterial cells (18). Even though spores are dormant and metabolically inactive, they are nonetheless capable of sensing the return of favorable growth conditions, to which they respond within minutes by germinating and resuming vegetative growth (14). Unlike actively metabolizing cells, however, spores are faced with a unique problem during dormancy in that they are incapable of immediately repairing damage inflicted upon their DNA by UV irradiation. The duration of dormancy cannot be predicted beforehand, and DNA damage to spores in the environment may accumulate over years of UV exposure. It is thus imperative to the survival of dormant spores that cumulative DNA damage be corrected rapidly and efficiently very early during germination, before gene expression or DNA replication can be reactivated.

To date, spore UV resistance (UV^r) has best been characterized for the gram-positive bacterium *Bacillus subtilis*, spores of which are 1 to 2 orders of magnitude more resistant to killing by UV than are vegetative cells (reviewed in references 50, 53, and 54). Our current understanding is that the high UV^r of *B. subtilis* spores results from a combination of two coupled phenomena. (i) Upon UV irradiation, dormant spores accumulate a spore-specific UV photoproduct (9) resulting from spore DNA being in an A-like conformation (reviewed in references 53 and 54). This "spore photoproduct" (SP) has been deduced to be the novel thymine dimer 5-thyminyl-5,6-dihydrothymine (64). (ii) Germinating *B. subtilis* spores repair SP by using two major repair pathways. In addition to SP repair via the general nucleotide excision repair pathway mediated by the *uvr* genes (31), *B. subtilis* spores possess a unique DNA repair system (previously known as *ssp* but here referred to as *spl*, for SP lyase) dedicated to the accurate repair of SP during germination (30, 33, 34). Both the *uvr* and *spl* repair pathways must be inactivated by mutation for spores to exhibit extreme UV sensitivity (UV^s) (30, 32). Existing evidence suggests that SP removal during spore germination is due to the in situ monomerization of SP to two thymines (33, 34, 65), but little else is known concerning this novel DNA repair system.

As a first step towards studying the molecular details of *spl*-mediated repair of SP in *B. subtilis*, we report the cloning and characterization of DNA from *B. subtilis* 168 that can repair a mutation in the *spl* system, its localization on the *B. subtilis* genetic and physical maps, and its nucleotide sequence.

(A preliminary account of these results was presented at the 11th International Spores Conference, Woods Hole, Mass., 9 to 13 May 1992 [10a].)

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. All *B. subtilis* and *Escherichia coli* strains used in this study are listed in Table 1. Plasmids and cloned fragments of *B. subtilis* DNA are described in Table 2. Media used were Difco sporulation medium (DSM; 48), Luria-Bertani medium (28), and Spizizen minimal medium (56). Auxotrophic requirements were each added to Spizizen minimal medium to a final concentration of 50 μg/ml. When appropriate, antibiotics were added to media at the following final concentrations: chloramphenicol, 3 μg/ml; and ampicillin, 50 μg/ml. Cells were incubated at 37°C unless otherwise indicated. Cells were grown in liquid media with vigorous aeration, and

* Corresponding author.

TABLE 1. Bacterial strains used in this study

Strain	Genotype or phenotype	Source or reference[a]
B. subtilis		
168	*trpC2*	Laboratory stock
168T+	Prototroph	Trp+ revertant of 168
1A5	*glyB133 metC3 tre-12 trpC2*	BGSC
1A237	*fruB22 ura-3*	BGSC
1A345	*metC14 sul thyA1 thyB1 trpC2 uvrA42*	BGSC
1A488	*metC14 spl-1 sul thyA1 thyB1 trpC2*	BGSC
1A489	*metC14 spl-1 sul thyA1 thyB1 trpC2 uvrA42*	BGSC
KS115	*cysA14 hisA1 leuA8 metC3 trpC2*	K. Sandman
R12	Prototroph; Cm^r	This study
1S59	*spoIIF96 trpC2*	BGSC
WN59	*spoIIF96 trpC2* Cm^r	R12→1S59 (tf)
WN97	*spl*::Cm^r	pWN95→168T+ (tf)
WN109	*metC14 sul thyA1 thyB1 trpC2 uvrA42 spl*::Cm^r	WN97→1A345 (td)
WN110	*metC14 spl-1 sul thyA1 thyB1 trpC2 spl*::Cm^r	WN97→1A488 (td)
WN111	*metC14 spl-1 sul thyA1 thyB1 trpC2 uvrA42 spl*::Cm^r	WN97→1A489 (td)
E. coli		
HB101	F$^-$ *hsdS20 recA13 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 supE44* λ$^-$	Laboratory stock (5)
JM83	*ara* Δ(*lac-proAB*) *rpsL* φ80 *lacZ*Δ*M15*	Laboratory stock (67)
GM161	F$^-$ *thr-1 leuB6 dam-4 thi-1 hsdS1 lacY1 tonA21* λ$^-$ *supE44*	T. Romeo (3)

[a] tf, transformation; td, transduction.

TABLE 2. Plasmids used in this study

Plasmid	Description	Source or reference
pJH101	Integrational plasmid derived from pBR322 and pC194	13
pUC18	Multisite *E. coli* cloning vector	67
pUC19	Multisite *E. coli* cloning vector	67
pBGSC6	Integrational plasmid derived from pUC19 and pC194	BGSC
pWN35	pJH101 with 2.3-kb *Eco*RI-*Hin*dIII *spl*-containing fragment	This study (see Fig. 1)
pWN36	pJH101 with 7.0-kb *Eco*RI-*Sal*I *spl*-containing fragment	This study (see Fig. 1)
pWN95	pBGSC6 with 286-bp *Eco*RI-*Bcl*I fragment from Δ5 to *Bcl*I within *spl*	This study (see Fig. 3)

optical density was monitored with a Klett-Summerson colorimeter fitted with a no. 66 (red) filter.

Molecular biology techniques. Large- and small-scale extractions of chromosomal DNA from *B. subtilis* (8) and plasmid DNA from *E. coli* (4) were accomplished by published techniques. Plasmid DNA was further purified by equilibrium gradient ultracentrifugation with cesium chloride-ethidium bromide (25). Standard techniques were used throughout for enzymatic manipulations, agarose gel electrophoresis, and Southern blot analysis of DNA (25, 55). Radioactively labelled DNA probes were prepared by the oligonucleotide labelling technique with 5′-[α-thio]dATP (12). Subclones were created for nucleotide sequencing in plasmid pUC18 or pUC19 (67) or pBGSC6 (*Bacillus* Genetic Stock Center, Columbus, Ohio [BGSC]) either by use of appropriate restriction endonucleases or by unidirectional exonuclease III-mung bean nuclease digestion of cloned DNA fragments (19).

Nucleic acid sequencing by dideoxynucleotide chain termination (47) was performed with the Sequenase version 2.0 kit (United States Biochemical Corp., Cleveland, Ohio), and sequencing products were analyzed by autoradiography after electrophoresis through either 6% polyacrylamide sequencing gels (27) or 5% Long Ranger gels (AT Biochemicals, Malvern, Pa.).

Genetic techniques. Preparation of competent *E. coli* or *B. subtilis* cells and their transformation with plasmid or chromosomal DNA have been described (6, 25). Generalized transduction with *B. subtilis* phage PBS-1 and analysis of recombinants were accomplished by standard procedures (8).

UV irradiation of spores. Suspensions of heat-resistant (80°C, 15 min) spores were diluted to 10^6 CFU/ml in 10 ml of irradiation buffer (10 mM potassium phosphate [pH 7.4], 150 mM NaCl), the dilutions were placed in a 6-cm-diameter plastic petri dish with the lid removed, and the dish was placed on a rotating platform and irradiated from above with a shortwave UV lamp (maximum output at 254 nm; UV Products, San Gabriel, Calif.). The UV-treated suspension was diluted 10-fold serially in irradiation buffer, the dilutions were plated on solid DSM containing the appropriate selective antibiotic, and the surviving fraction of the spores was quantitated by counting colonies after overnight incubation at 37°C (37). Lamp output was determined by use of a ferric oxalate chemical actinometer (49).

Alternatively, when the UV^r or UV^s of large numbers of transformants was to be determined, a qualitative method was used. Transformants were placed in a grid pattern on selective DSM plates and incubated for 2 days. Single colonies were removed from the plates by cutting out each colony on a block of agar with a sterile scalpel, and each colony was resuspended in 1 ml of irradiation buffer. After heat shock (80°C, 15 min), the suspensions were diluted 1:50 in irradiation buffer and 10-μl spots were pipetted in a grid pattern on the bottom of a sterile plastic petri dish. The drops were irradiated with shortwave UV to a dose of 60 J/m^2, and a 1-μl aliquot was removed from each drop and spotted in an identical grid pattern on solid DSM. After overnight incubation, spots containing spores of Uvr$^-$ Spl$^-$ double-mutant strains (e.g., strain 1A489 or double-mutant transformants) failed to form colonies, whereas spots containing spores of either Uvr$^-$ Spl$^+$ or Uvr$^+$ Spl$^-$ single-mutant strains or wild-type strains yielded several small colonies arising from the surviving spores.

Nucleotide sequence accession number. The sequence data reported in this article will appear in the GenBank nucleotide sequence data library under accession number L08809.

RESULTS

Cloning of the *spl* gene in plasmid pJH101. The initial cloning strategy used a strain of *B. subtilis*, 1A489, that carries mutations in the excision repair pathway (*uvrA42*) and the SP-specific pathway (*spl-1*) (30; Table 1). This strain

produces very UV-sensitive spores, and it was previously demonstrated that transformation of 1A489 with DNA extracted from wild-type *B. subtilis* strains results in transformants that produce UV^r spores by virtue of incorporation into their genome of either the wild-type *uvrA*$^+$ or the wild-type *spl*$^+$ gene (30). In preliminary experiments, it was found that *Eco*RI fragments of genomic DNA from *B. subtilis* 168 also efficiently transformed strain 1A489 to the ability to produce UV^r spores. Therefore, chromosomal DNA extracted from strain 168 was cleaved with *Eco*RI and ligated with *Eco*RI-cleaved plasmid pJH101, an integrative plasmid that is unable to replicate in *B. subtilis* but that carries a Cm^r marker selectable in *B. subtilis* (13). The ligation mixture was introduced by transformation into competent cells of strain 1A489, and the transformation mixtures were plated on DSM plates containing chloramphenicol. The Cm^r transformants obtained were allowed to sporulate by incubation for 48 h, the spores from approximately 10,000 Cm^r colonies were washed from the plates with sterile irradiation buffer, and cells that had not produced spores were killed by heating of the suspension at 80°C for 15 min. The suspension was then diluted to 10^6 spores per ml and subjected to 254-nm UV at a final dose of 60 J/m^2. (In calibration experiments, it was determined that under these conditions, approximately 50% of wild-type spores survive, but the survival of spores of strain 1A489 is decreased by at least 5 orders of magnitude; data not shown.) Twenty-four survivors from this UV treatment were streak purified and sporulated individually in liquid DSM containing chloramphenicol, and their UV survival curves were determined. Thirteen of the 24 Cm^r transformants produced spores with survival kinetics similar to those of spores produced by wild-type or single-mutant strains and were characterized further. For testing for close genetic linkage between the Cm^r marker of pJH101 and the UV^r phenotype, chromosomal DNA isolated from the 13 transformants was introduced by transformation into competent cells of strain 1A489, again with selection for Cm^r and screening for UV^r spores. Close genetic linkage was detected in 11 of the 13 strains; the Cm^r and UV^r phenotypes of the other 2 transformants were readily separable by transformation, indicating that these transformants probably arose by congression during the transformation of the initial ligation mixture into 1A489 (data not shown).

Locating *spl* on the *B. subtilis* chromosome. Because donor strain 168 carries both the *uvrA*$^+$ and the *spl*$^+$ genes, in principle it was possible that the transformation of strain 1A489 with DNA extracted from strain 168 would result in the production of both Uvr$^+$ Spl$^-$ and Uvr$^-$ Spl$^+$ transformants, either of which can produce UV^r spores (30). The location of the *spl* gene on the *B. subtilis* genetic map was unknown, but the *uvrA* gene had previously been localized at 305° on the *B. subtilis* genetic map (41), approximately 85% linked by phage PBS-1 transduction to the histidine auxotrophic marker *hisA* (31). To determine whether the Cm^r marker harbored by the UV^r transformants was linked to the *uvrA* gene, chromosomal DNA isolated from the 11 UV^r transformants described above was introduced into competent cells of prototrophic strain 168T$^+$, with selection for Cm^r, and then phage PBS-1-generalized transducing lysates prepared from the prototrophic Cm^r transformants were introduced into strain KS115 (Table 1), with selection for His$^+$ transductants and screening for cotransduction of the Cm^r marker. None of the donor strains demonstrated transductional linkage between *hisA* and the Cm^r marker, indicating that the Cm^r marker in the original transformants was not closely associated with the *uvrA* gene (data not shown).

A PBS-1 lysate prepared from one of the prototrophic Cm^r transformants, strain R12 (Table 1), was next transduced into strain KS115, with selection for Cys$^+$, Leu$^+$, Met$^+$, or Trp$^+$ transductants and screening for linkage to the Cm^r marker. Genetic linkage (approximately 30%) was detected between the *metC3* marker of KS115 and the Cm^r marker (data not shown). Three-factor transductional crosses placed the Cm^r marker of strain R12 between *pyrD* and *metC* on the *B. subtilis* genetic map, very tightly linked to sporulation mutation *spoIIF96* (Table 3). (The *spoIIF* locus is identical to the *spoIIJ* or *kinA* locus in *B. subtilis* [2, 29a, 40]). Analysis of the three-factor cross data resulted in the construction of a map consistent with the order *pyrD-fruB*-(*spoIIF*-Cm^r)-*metC* (Table 3). The phenotype of the transductants and their localization to a previously undescribed position on the *B. subtilis* chromosome led us to the working hypothesis that the Cm^r marker of pJH101 had integrated adjacent to the *spl* locus in the R12 transformant.

Cloning of *spl* in *E. coli*. It was reasoned that in Cm^r UV^r strains such as R12, the putative *spl*$^+$ gene is situated adjacent to plasmid pJH101 vector sequences and can therefore be cloned from the *B. subtilis* chromosome by digestion of strain R12 DNA with an appropriate restriction enzyme, other than *Eco*RI, followed by recircularization of the vector and adjacent chromosomal DNA sequences and recovery of the clone by transformation into *E. coli* (70). Separate aliquots of chromosomal DNA isolated from strain R12 were therefore digested with restriction endonuclease *Bam*HI, *Hin*dIII, *Pst*I, or *Sal*I (each of which cleaves plasmid pJH101 only once; 13), and the digested DNAs were subjected to ligation under conditions favoring intramolecular circularization. The ligation products were introduced into competent cells of *E. coli* HB101, and Cm^r transformants were selected. The *Bam*HI- and *Pst*I-digested and circularized DNAs failed to yield transformants; however, Cm^r transformants were obtained with *Hin*dIII- and *Sal*I-digested and circularized DNAs, resulting in plasmid clones pWN35 and pWN36, respectively (Fig. 1). It was determined by restriction analysis that the 2.3-kb *Eco*RI-*Hin*dIII fragment carried by plasmid pWN35 was a subset of the 7.0-kb *Eco*RI-*Sal*I insert carried on plasmid pWN36 (Fig. 1).

Plasmids pWN35 and pWN36 were propagated in *E. coli* and subsequently reintroduced into *B. subtilis*, in which they could integrate into the chromosome at the location homologous to that of the cloned insert. When introduced by transformation into competent cells of either strain 1A488 (*spl-1*) or 1A489 (*uvrA42 spl-1*), the 2.3-kb insert present on plasmid pWN35 was able to restore a UV^r phenotype to spores of the Cm^r transformants (Fig. 2). Transductional mapping of the Cm^r marker of integrants of plasmid pWN35 or of its subclones confirmed that integration had occurred in the *metC-pyrD* region of the *B. subtilis* chromosome (data not shown). Thus, it is highly probable that wild-type DNA repairing the *spl-1* mutation had been cloned and that the cloned region corresponded at least in part to the *spl*$^+$ locus. Southern blot analysis of chromosomal DNA isolated from wild-type strain 168, using as a probe the insert DNA from pWN35, confirmed that the restriction map of the insert in plasmid pWN35 (Fig. 1) matched the chromosomal arrangement of restriction sites (data not shown). This result indicated that the insert in pWN35 contained an intact fragment of *B. subtilis* chromosomal DNA that had not suffered rearrangements or ligation artifacts during cloning.

Functional localization of *spl*. The position of the *spl* gene

TABLE 3. Mapping of the *spl* gene by three-factor PBS-1 transductional crosses[a]

Recipient genotype (strain)	Selected phenotype	Recombinant class for the following marker[b]:					No. of recombinants	Suggested order
		metC	Cm^r	*spoIIF*	*fruB*	*pyrD*		
fruB22 pyrD (1A237)	Cm^r		1	1		1	3	
			1	1		0	83	
			1	0		1	0	Cm^r-*spoIIF-pyrD*
			1	0		0	8	
	Ura$^+$		1	1		1	14	
			0	1		1	3	
			1	0		1	1	*pyrD-spoIIF*-Cm^r
			0	0		1	182	
	Ura$^+$		1		1	1	14	
			0		1	1	36	
			1		0	1	1	*pyrD-fruB*-Cm^r
			0		0	1	149	
	Ura$^+$			1	1	1	16	
				0	1	1	34	
				1	0	1	1	*pyrD-fruB-spoIIF*
				0	0	1	149	
metC3 (1A5)	Met$^+$	1	1	1			41	
		1	1	0			10	
		1	0	1			5	*metC*-Cm^r-*spoIIF*
		1	0	0			141	

[a] The donor genotype (strain) was *spl*::Cm^r *spoIIF96* (WN59).
[b] Donor and recipient markers are denoted by 1 and 0, respectively.

on the 2.3-kb *Eco*RI-*Hin*dIII fragment present in pWN35 was determined by testing of the functional properties of subclones of this fragment. Subclones were generated by restriction endonuclease subcloning and/or exonuclease III-mung bean nuclease deletion, followed by insertion into the multiple cloning site of plasmid pBGSC6 (Table 2 and Fig. 3). The subclones generated were tested for (i) the ability to restore a UV^r (i.e., Spl$^+$) phenotype to spores of Cm^r transformants of strain 1A489 (*uvrA42 spl-1*) or (ii) the ability to transform strain 1A345 (*uvrA42*) to produce spores with a UV^s (i.e., Uvr$^-$ Spl$^-$) phenotype (Fig. 3). The results of the subcloning experiments located the boundaries of the *spl* gene between deletion 3 and deletion 4 on the leftward end and between the *Sph*I and *Hin*dIII sites on the rightward end of the 2.3-kb *Eco*RI-*Hin*dIII fragment (Fig. 3). In addition, the *spl-1* mutation itself was localized between the *Bcl*I site at bp 1373 and the *Sph*I site at bp 1884 (Fig. 3).

Nucleotide sequence of the *spl* gene. With the sequencing strategy outlined in Fig. 4A, the complete nucleotide sequence of the 2.3-kb *Eco*RI-*Hin*dIII *spl*-containing fragment was determined (Fig. 4B). Examination of the nucleotide sequence revealed the 3′ end of a partial open reading frame (ORF), extending into the 2.3-kb fragment from beyond the *Eco*RI site, and then two complete ORFs, the first potentially encoding a small polypeptide of 9 kDa and the second potentially encoding a protein of nearly 40 kDa (Fig. 4B). The 5′ end of this second large complete ORF was situated between exonuclease III-mung bean nuclease deletions 3 and 4; the 3′ end of this ORF was situated between the unique *Sph*I and *Hin*dIII sites. Because the ends of this large ORF (Fig. 4B) matched the boundaries of *spl* delimited by the functional mapping experiments (Fig. 3), this large ORF likely encodes the *spl* gene (Fig. 3 and 4B; see below).

The nucleotide sequence and deduced amino acid sequence for each ORF were used to search the nucleic acid and protein sequence data bases contained in the University of Wisconsin Genetics Computer Group software package (including GenBank version 74.0, EMBL version 32.0, Swiss-Prot version 23.0, Protein Information Resource [PIR] nucleic acid version 36.0, and PIR protein version 33.0). The search failed to identify sequences with significant homology to either the genes or the deduced proteins potentially encoded by the putative *spl* gene or the small ORF preceding *spl*. However, a direct pairwise comparison between the deduced Spl amino acid sequence and the amino acid sequences of DNA photolyases from a number of microorganisms by use of the sequence comparison program FASTA (39) revealed that these sequences share local amino acid homologies in their carboxy-terminal halves (see Fig. 7 and Discussion).

A search of the data base by use of the deduced amino acid sequence for the upstream partial ORF revealed that this deduced protein sequence displayed a high degree of homology to the sequences of the enzyme I component of the phosphoenolpyruvate:phosphotransferase (PTS) systems of *Staphylococcus carnosus* (22), *Salmonella typhimurium* (7, 24, 35), and *Escherichia coli* (44). In *B. subtilis*, enzyme I of the PTS is encoded by *ptsI*, the second gene in the *ptsHI* operon (17). Thus, the small ORF (that encoding the 9-kDa protein) and the *spl* gene are located immediately downstream from the *ptsI* gene, and the three genes are oriented in the same direction (Fig. 4B).

By comparison of the restriction map of the cloned DNA region including *spl* (Fig. 1) with published restriction maps of the analogous region of the *B. subtilis* chromosome, including the *ptsG* gene, the *ptsHI* operon (16, 17, 62), and the *spoIIF* (also known as *spoIIJ* and *kinA*) locus (2, 29a, 40), it was possible to determine the position of the *spl* locus

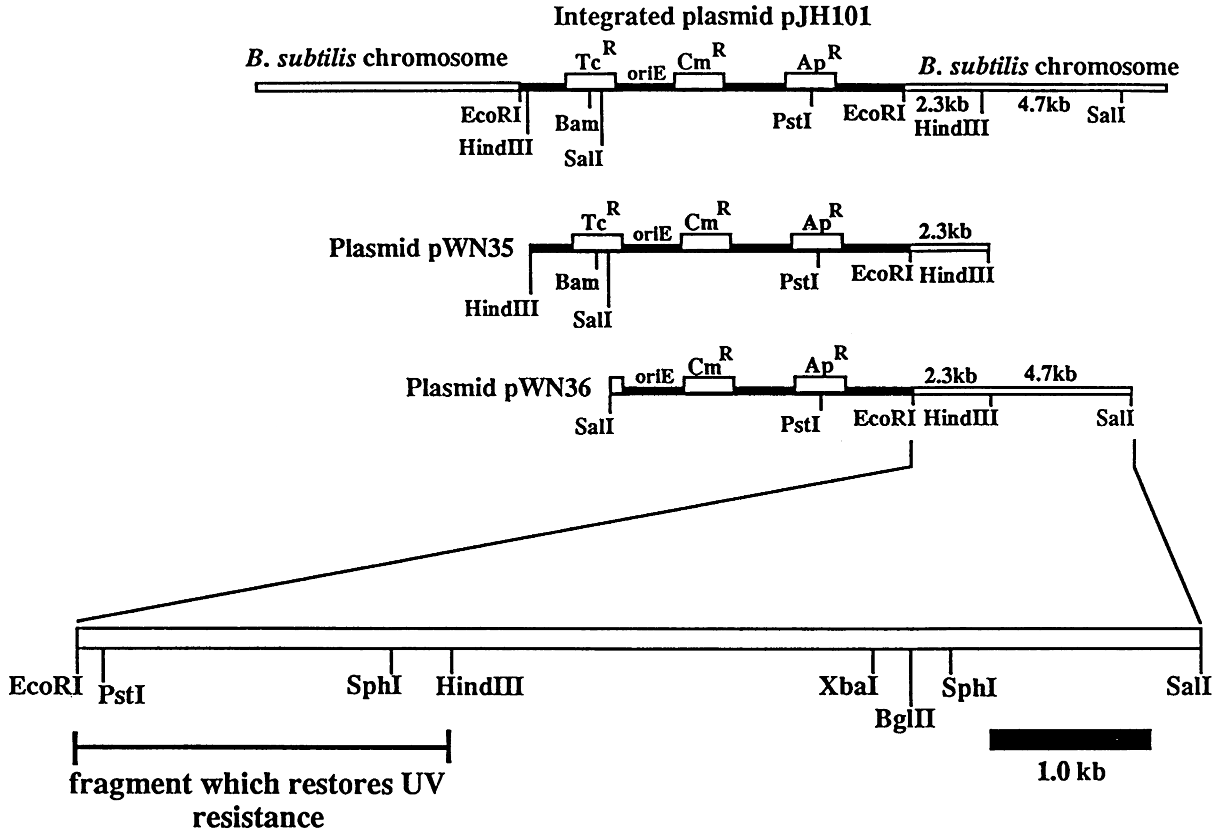

FIG. 1. Restriction endonuclease cleavage maps of the site at which plasmid pJH101 is integrated into the *B. subtilis* chromosome (top line), plasmid pWN35 (second line), and plasmid pWN36 (third line). The bottom line is an expanded map of the insert present in pWN36. Solid bars, plasmid pJH101 sequences; open bars, *B. subtilis* sequences.

on the *B. subtilis* physical map (1) and to construct a restriction map spanning approximately 17 kb of this region (Fig. 5).

Disruption of *spl* by integrational mutagenesis. Examination of the nucleotide sequence of the cloned 2.3-kb *Eco*RI-*Hin*dIII insert indicated that the position of the third ORF on the fragment (Fig. 4B) corresponded well with the limits of the cloned *spl* gene localized by integration experiments involving subclones of the fragment (Fig. 3). On the basis of analysis of the nucleotide sequence, it was predicted that a restriction fragment extending from deletion 5 at nucleotide 1087 to the *Bcl*I site at nucleotide 1373 would be contained completely within the coding sequence of the putative *spl* ORF (Fig. 4). This 286-bp fragment was cloned into plasmid pBGSC6, resulting in plasmid pWN95 (Table 2). Plasmid pWN95 was introduced by transformation into competent cells of strain 168T$^+$, with selection for Cm^r and resulting in strain WN97 (Table 1). A PBS-1 transducing lysate prepared from strain WN97 was used to transduce strains 1A345, 1A488, and 1A489 to Met$^+$, and Met$^+$ transductants were screened for cotransduction of the Cm^r marker of integrated plasmid pWN95, resulting in strains WN109, WN110, and WN111, respectively (Table 1). In each case, the transductional linkage between *metC14* and the Cm^r marker of pWN95 was similar to that observed during the previously described transductional mapping experiments (Table 3 and data not shown).

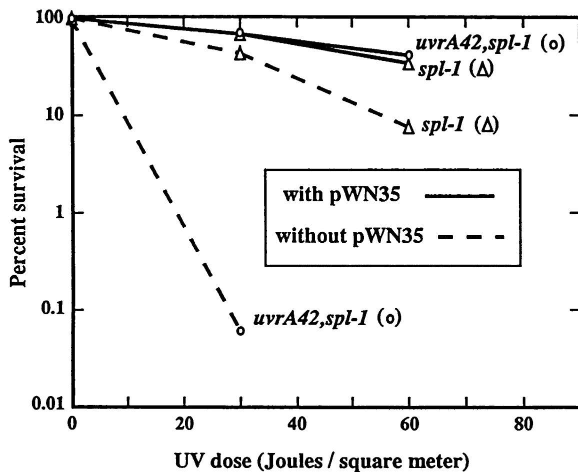

FIG. 2. UV^r of spores of strains 1A488 (*spl-1*) and 1A489 (*uvrA42 spl-1*) without and with integrated plasmid pWN35. Spores were prepared and UV irradiated and survival was quantitated as described in Materials and Methods.

If the insert contained in plasmid pWN95 indeed originated from within the *spl* coding sequence, then its integration into the *B. subtilis* chromosome by Campbell-type recombination should result in the disruption of the *spl* gene. To test this notion, we subjected spores of strains WN97, WN109, WN110, and WN111 to 254-nm UV at a number of doses and calculated the UV dose that killed 90% of the

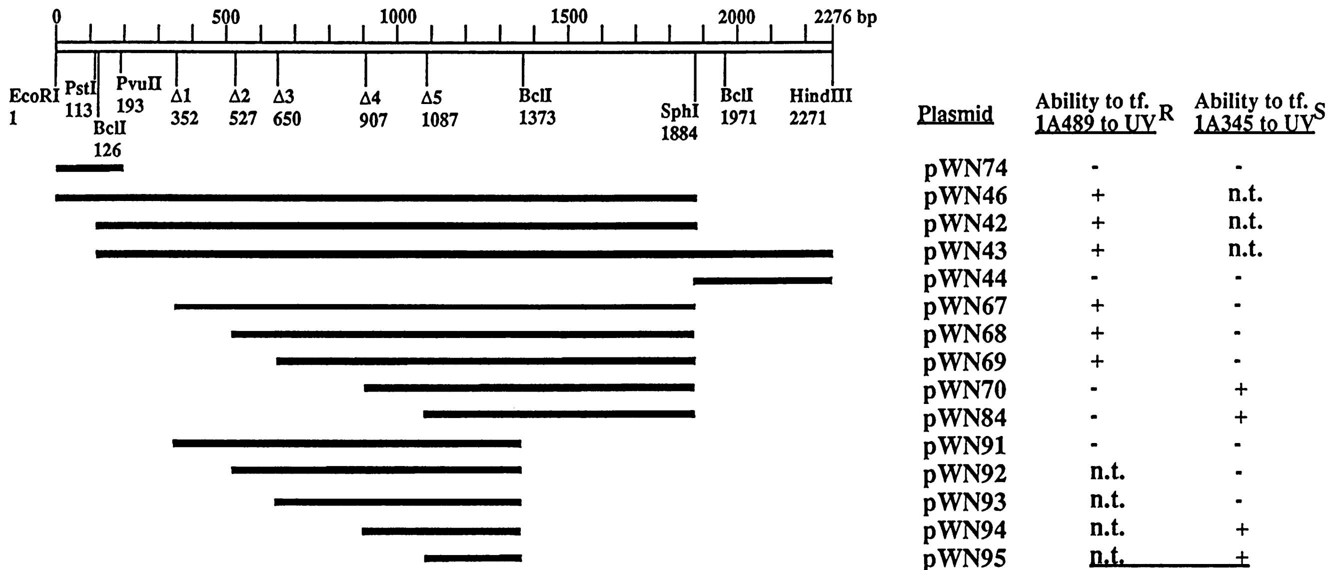

FIG. 3. Deletion analysis for the localization of *spl* on the 2.3-kb *Eco*RI-*Hin*dIII fragment of plasmid pWN35. The open bar represents the 2.3-kb *Eco*RI-*Hin*dIII fragment, including coordinates in base pairs. Beneath the open bar are the positions of restriction sites and deletion endpoints. The dark bars denote subfragments cloned in pBGSC6 to create the indicated plasmids. tf., transform; n.t., not tested.

population (the LD_{90}) for each strain. These LD_{90}s were compared with the LD_{90}s determined for the respective parental strains (Fig. 6). Integration of pWN95 resulted in a decrease in the LD_{90}s for spores of strains 168T$^+$ (wild type) and 1A345 (*uvrA42*) to the levels characteristic for strains 1A488 (*spl-1*) and 1A489 (*uvrA42 spl-1*), respectively (Fig. 6). In contrast, integration of pWN95 into strains 1A488 and 1A489, both of which already carry the *spl-1* mutation, did not significantly alter the UV^r properties of the resulting strains (Fig. 6). The data in Fig. 6 are entirely consistent with the insert contained in pWN95 having originated from within the *spl* gene.

DISCUSSION

The *spl* genetic locus was originally identified some 25 years ago (32) because of a mutation called *spl-1* which, when present in conjunction with a mutation in the *uvr* repair system, renders *B. subtilis* spores extremely UV^s (30, 31). This communication describes the identification and characterization of DNA cloned from *B. subtilis* 168 that can repair the *spl-1* mutation, its localization on the *B. subtilis* chromosome, its nucleotide sequence, and disruption of the *spl* gene by integrational mutagenesis with an internal portion of the cloned DNA. With the cloning of DNA that repairs the *spl-1* mutation, it is now possible to probe several aspects of this interesting but scantily characterized DNA repair system.

Isolation of the *spl* gene by use of integrational plasmid cloning resulted in its localization by physical and genetic mapping on the *B. subtilis* chromosome between the previously described genetic markers *ptsI* and *spoIIF* (*spoIIJ* or *kinA*), immediately downstream from a small ORF between *ptsI* and *spl* (Fig. 4). Inactivation of the putative *spl* gene by use of an integrational plasmid containing an insert derived wholly from within the *spl* coding sequence resulted in the

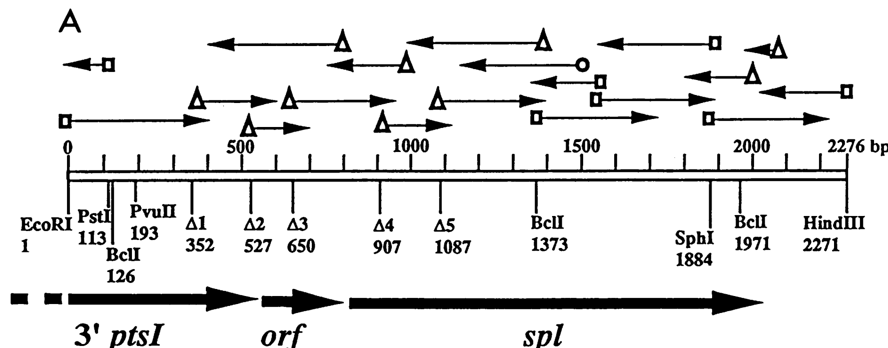

FIG. 4. (A) Strategy for nucleotide sequencing of the 2.3-kb *Eco*RI-*Hin*dIII fragment. Above the restriction map of the fragment (open bar) are denoted the direction and extent (thin arrows) of the nucleotide sequence determined from subclones generated by restriction digestion (□) or exonuclease III-mung bean nuclease deletion (△) or primed by a synthetic oligonucleotide (○). The thick arrows (bottom) denote the approximate locations and extents of the ORFs found on the 2.3-kb fragment. (B) Nucleotide sequence of the 2.3-kb *Eco*RI-*Hin*dIII fragment (top lines) and deduced amino acid sequences for the ORFs found (bottom lines). rbs, putative ribosome binding site. >>><<<, inverted repeat sequences denoting putative transcription termination sequences.

B

```
      EcoRI   3' end of ptsI
1     GAATTCAAAGAAGCAAAAGCGATCCTTCTTGAAGAGAAAGAAAAGCTCGTAAAAGCGGGACAGGCTGTATCTGACGATATTGAAGTCGGAATGATGGTTG
      E  F  K  E  A  K  A  I  L  L  E  E  K  E  K  L  V  K  A  G  Q  A  V  S  D  D  I  E  V  G  M  M  V  E
                  PstI       BclI                                                                 PvuII
101   AGATTCCGTCAACTGCAGTCATCGCTGATCAGTTTGCTAAAGAGGTTGATTTCTTCAGTATCGGAACAAACGATTTGATTCAATACACAATGGCAGCTGA
        I  P  S  T  A  V  I  A  D  Q  F  A  K  E  V  D  F  F  S  I  G  T  N  D  L  I  Q  Y  T  M  A  A  D

201   CCGTATGAATGAACGTGTATCTTACCTGTATCAGCCATACAACCCGGCAATCCTTCGCTTAATTACACTGGTAATTGAAGCAGCACACAAAGAAGGAAAA
       R  M  N  E  R  V  S  Y  L  Y  Q  P  Y  N  P  A  I  L  R  L  I  T  L  V  I  E  A  A  H  K  E  G  K
                                                                     Δ1
301   TGGGTTGGCATGTGCGGAGAAATGGCAGGAGACGAGATTGCGATTCCGATCCTTCTCGGCTTAGGCTTAGATGAGTTCTCAATGAGCGCAACGTCTATCC
      W  V  G  M  C  G  E  M  A  G  D  E  I  A  I  P  I  L  L  G  L  G  L  D  E  F  S  M  S  A  T  S  I  L

401   TTCCGGCAAGAACACAAATCAGCAAATTGTCTAAACAAGAAGCTGAGTCATTCAAAGAGAAAATCTTATCTATGAGCACGACAGAAGAAGTTGTCGCGTT
        P  A  R  T  Q  I  S  K  L  S  K  Q  E  A  E  S  F  K  E  K  I  L  S  M  S  T  T  E  E  V  V  A  F
                                      Δ2
501   CGTAAAAGAAACATTCAAGTAATGTACAAAAACCAGACGGCCTCCGGCCTGTCTGGTTTTTTCATAAGTAAGGGTATAGAAGGACACAATAACATGGCT
       V  K  E  T  F  K  *           >>>>>>>>>    <<<<<<<<<<
              orf                          Δ3
601   AGGAGGGATAGTATGTCAAACCAATTTAACGCAGGAGATACTGTTTATGTGATCTACAGAAATCCGCACGCCGCCAATGTAGCCCACATAAAGGAAGCCG
       rbs        M  S  N  Q  F  N  A  G  D  T  V  Y  V  I  Y  R  N  P  H  A  A  N  V  A  H  I  K  E  A  E

701   AAATTGTGCACCATCCATACCATGAAGGCGAGCTTTCCCTGTTTATTTATGAAACCTATCATCCATTCGCAGAGGACGATGCTGTGTTTGCAAGCTATGA
        I  V  H  H  P  Y  H  E  G  E  L  S  L  F  I  Y  E  T  Y  H  P  F  A  E  D  D  A  V  F  A  S  Y  E

801   AGAAGCTAAATCGCTTTACAAAGAACTGTTTGATATTGATCCGTATGAGTAACAAGGAAATCATTACAACTCATATCCTTTCCGCCTAGTGAGAAAAGTA
       E  A  K  S  L  Y  K  E  L  F  D  I  D  P  Y  E  *
         Δ4                        spl
901   ACGTTAGTAAAGGAAAAGGATGTGGCATCATGCAGAACCCATTTGTTCCGCAGCTTGTGTATATAGAACCGAGGGCGCTGGAATATCCGCTGGGCCAAGA
                      rbs          M  Q  N  P  F  V  P  Q  L  V  Y  I  E  P  R  A  L  E  Y  P  L  G  Q  E
                                                                                            Δ5
1001  ATTACAAGATAAATTTGAGAATATGGGTATTGAAATCAGAGAAACGACATCCCACAATCAGGTGCGTAATATCCCTGGGAAAAATCATCTTCAGCAATAT
       L  Q  D  K  F  E  N  M  G  I  E  I  R  E  T  T  S  H  N  Q  V  R  N  I  P  G  K  N  H  L  Q  Q  Y

1101  CGCAATGCGAAATCAACTTTAGTTATCGGTGTCCGGAAAACATTAAAGTTTGATTCATCCAAACCCTCGGCCGAATATGCCATTCCGTTTGCAACAGGCT
       R  N  A  K  S  T  L  V  I  G  V  R  K  T  L  K  F  D  S  S  K  P  S  A  E  Y  A  I  P  F  A  T  G  C

1201  GCATGGGGCATTGTCATTACTGCTACCTGCAAACAACCATGGGATCAAAGCCGTATATCAGAACTTATGTAAACGTCGAAGAGATTTTAGATCAGGCAGA
        M  G  H  C  H  Y  C  Y  L  Q  T  T  M  G  S  K  P  Y  I  R  T  Y  V  N  V  E  E  I  L  D  Q  A  D
                                                                                 BclI
1301  TAAGTATATGAAGGAGCGCGCACCAGAGTTCACAAGGTTCGAAGCATCATGTACGTCAGACATTGTAGGAATTGATCATCTGACACACACGCTGAAGCGC
       K  Y  M  K  E  R  A  P  E  F  T  R  F  E  A  S  C  T  S  D  I  V  G  I  D  H  L  T  H  T  L  K  R

1401  GCCATTGAACATTTTGGCCAAAGTGATCTCGGAAAGCTCCGATTTGTAACGAAATTTCATCATGTCGATCACCTATTAGACGCAAAGCATAACGGGAAAA
       A  I  E  H  F  G  Q  S  D  L  G  K  L  R  F  V  T  K  F  H  H  V  D  H  L  L  D  A  K  H  N  G  K  T

1501  CGAGATTCAGATTCAGTATTAATGCCGACTATGTGATTAAAAACTTTGAGCCGGGAACTTCACCTCTTGATAAGCGGATAGAAGCGGCAGTAAAAGTTGC
        R  F  R  F  S  I  N  A  D  Y  V  I  K  N  F  E  P  G  T  S  P  L  D  K  R  I  E  A  A  V  K  V  A

1601  AAAAGCAGGCTACCCGCTAGGCTTTATTGTTGCTCCGATTTATATTCATGAAGGCTGGGAAGAAGGATACAGACATCTGTTTGAAAAGCTAGATGCTGCT
       K  A  G  Y  P  L  G  F  I  V  A  P  I  Y  I  H  E  G  W  E  E  G  Y  R  H  L  F  E  K  L  D  A  A

1701  TTGCCGCAGGACGTTAGACATGACATTACGTTTGAATTAATTCAACACCGTTTTACAAAACCGGCCAAACGAGTGATAGAGAAAAATTATCCGAAGACGA
       L  P  Q  D  V  R  H  D  I  T  F  E  L  I  Q  H  R  F  T  K  P  A  K  R  V  I  E  K  N  Y  P  K  T  K
                                                                                             SphI
1801  AGCTCGAATTAGATGAAGAAAAGCGCCGTTATAAATGGGGCCGTTACGGGATCGGAAAATATATTTATCAGAAAGATGAAGAGCATGCACTTCGAGAGGC
        L  E  L  D  E  E  K  R  R  Y  K  W  G  R  Y  G  I  G  K  Y  I  Y  Q  K  D  E  E  H  A  L  R  E  A
                                                                                   BclI
1901  ACTTGAATCCTATATTGATACCTTTTTCCCTAACGCAAAAATTGAATATTTCACTTAAACGGGCTGTTGTGATCAACAGCTCGCTTTTTTATAAAAAACA
        L  E  S  Y  I  D  T  F  F  P  N  A  K  I  E  Y  F  T  *  >> >>>>>>>     <<<<<<< <<

2001  TTTTTCCATTCCATCAAAACGCCGTAATTCAATGATTCTTTATCCTCCAAATAATTGCTGACTACACGAATAGGTGAACGCCCGCAACGGAGGAGAAAGG

2101  ACAGCACAGGGTCGTTTTTCCTGCCTTCCATCACATCTTCACGTACTGAACAGCGCTGATCTCGTGTGCGTATTTATAATACAAAGGAATGCGCCGCCGC
                                                                                      HindIII
2201  CGAGAAGGCGTTCCAGTCTTTTATCAACAGTCAGCTCATACATCGTATTCATCAGCCATTTTCCGATTCCAAGCTT  2276
```

FIG. 4—*Continued.*

creation of mutant strains that behaved as would be predicted if they were *spl* mutants (Fig. 6). Indeed, spores of these *spl* gene disruption mutants demonstrated slightly more UV^s than spores of strains harboring the *spl-1* mutant allele, suggesting that some residual SP repair activity may persist in the *spl-1* mutant. Identifying the nature of the *spl-1* mutant allele may yield useful insights into features of the Spl protein important for its structure and activity.

Examination of the nucleotide sequence of and surrounding the *spl* gene revealed several features of interest. Both the *spl* gene and the small ORF upstream from *spl* are preceded by sequences that demonstrate good homology to *B. subtilis* ribosome binding sites (Fig. 4B; 29). In addition, both the *ptsI* and the *spl* genes are followed by sequences that have dyad symmetry and that resemble rho-independent transcription termination sequences (Fig. 4B; 43). Interestingly, a

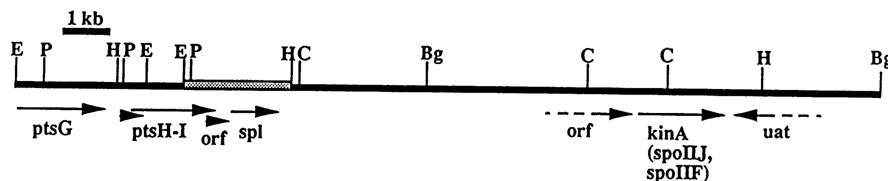

FIG. 5. Partial restriction map of the *B. subtilis* chromosome in the vicinity of the *spl* gene, as determined from data in this study and data taken from references 2, 16, 17, 40, and 62. The shaded bar indicates the 2.3-kb *Eco*RI-*Hin*dIII fragment characterized in this study. Abbreviations: Bg, *Bgl*II; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I.

strong transcription termination signal is apparently absent between the small ORF and *spl* (Fig. 4B), suggesting that these two genes may be part of the same transcription unit.

Possible clues regarding the developmental regulation of the *spl* gene can be inferred from the available data. The observations that *spl*-mediated repair of SP happens very early during spore germination and that it is insensitive to antibiotics that inhibit protein or RNA synthesis (34) imply that the Spl enzyme is synthesized during the previous round of sporulation and is packaged within the dormant spore. While the regulation of *spl* expression is at present unknown, it is reasonable to speculate that it may be similar to that of several genes (e.g., those of the *ssp* and *ger* families) whose products function either in the dormant spore or early during spore germination and whose expression has been demonstrated to be coordinately activated specifically in the forespore compartment during morphological stage III of sporulation (11, 26). These genes have been shown to be members of a regulon sharing common promoter sequences recognized by Eσ^G, the sporulation-specific RNA polymerase containing the σ^G subunit (20, 38, 60; reviewed in reference 52). In addition, the sporulation-specific promoters of the *gpr* and *spoIIIG* genes are transcribed in the forespore at least in part by Eσ^F RNA polymerase (58, 61). Is the *spl* gene transcribed by either (or both) of these RNA polymerases? Examination of the nucleotide sequence preceding *spl* reveals sequences that exhibit some degree of homology to the canonical promoters recognized by Eσ^F and Eσ^G (38, 59). By testing the ability of subclones generated throughout the *spl* region to inactivate the wild-type *spl* gene by integration (Fig. 3), we deduced that the *spl* transcription unit begins between the sequences defined by the endpoints of deletions 3 and 4 (Fig. 3 and 4B). However, the absence of a strong transcription termination signal between the small ORF and *spl* presents the possibility that these two genes are cotranscribed. This possibility can be resolved by identifying and mapping transcripts originating in the *spl* region, experiments which are currently ongoing. Having the gene that repairs the *spl-1* mutation cloned also leads to the interesting question of whether the cloned *spl* gene is sufficient to encode SP repair activity or whether possible other subunits of the enzyme are encoded by additional cistrons (such as the small ORF upstream from *spl*). Again, experiments are currently under way to place the cloned *spl* gene into an expression system and to assay its gene product for SP repair activity.

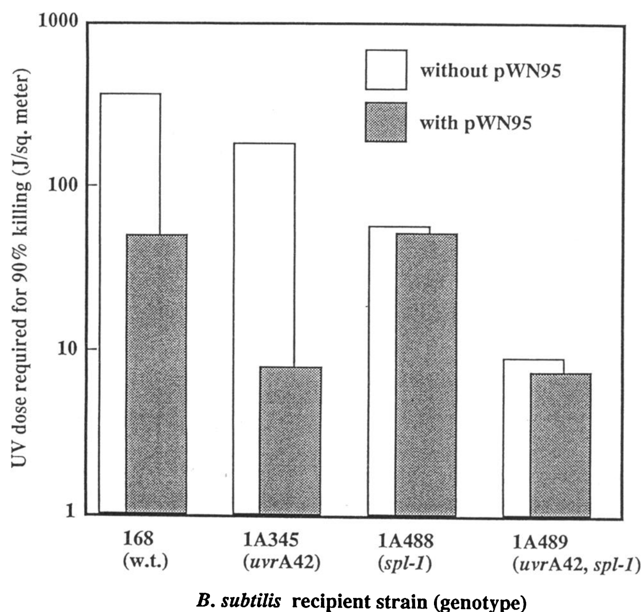

FIG. 6. Disruption of the *spl* gene by integration of plasmid pWN95 into the *B. subtilis* chromosome. w.t., wild type.

Finally, by what mechanism does the Spl protein repair SP? On the basis of early experiments that tested the kinetics of disappearance of SP from UV-irradiated spores of strains of *B. subtilis* defective in SP repair because of mutations in the *uvr* or *spl* pathways, it was concluded that *spl*-mediated repair of SP was through a direct action of the *spl* gene product(s) to monomerize SP in situ to two thymines during spore germination (33, 34, 65). In this aspect, *spl*-mediated repair of SP resembles the photoreactivation of cyclobutane thymine dimers in situ by the enzyme DNA photolyase. A comparison by FASTA analysis (39) of the deduced amino acid sequence of the Spl protein with the amino acid sequences of DNA photolyases from a number of microorganisms revealed a region of sequence homology in the carboxyl-terminal portions of the proteins (Fig. 7), suggesting that these enzymes have descended from a common ancestral protein. *spl*-mediated SP repair differs from photoreactivation, however, in that UV-irradiated spores cannot be photoreactivated, and the Spl protein does not require visible light for activity (10, 57). Although the functional relationship underlying the amino acid sequence similarities (and differences) between Spl and photolyases is at present unknown, in a recent study of the factors determining the active site of the *E. coli* photolyase, it was demonstrated that tryptophan 306 of the *E. coli* enzyme was absolutely essential for its light-dependent activity (23). It is interesting to note that although this tryptophan residue is absolutely conserved throughout the DNA photolyases, it is absent in the Spl protein sequence, being replaced at position 211 by a serine (Fig. 7).

Interestingly, although spore photochemistry and presumably *spl*-mediated repair of SP exist in a large number of spore-forming bacteria (36, 51), it has been reported that

```
N.c.  WPA--GEEEALKRNEKFCDEA-IGKVAERRNIPAMQGTSNL  367
S.c.  CLPDVSEEAALSRLKDFL-GTKSSKYNNEKDMLYLGGTSGL  341
E.c.  FPVE--EKAAIAQLRQFCQ-NGAGEYEQQRDFPAVEGTSRL  238
H.h.  AVPDAGTAAARSLGDAFRESGDIYRYEDRRDYPHEEPTSRL  243
A.n.  FPVEPGETAAIARLQEFCD-RAIADYDPQRNFPAEAGTSGL  243
S.g.  GLARGGEEAGRKLVTSWLNG-PMADYEDGHDDLAGDATSRL  234
       ::  :  |::  |||| :||:: :|:: : || : : : |
B.s.  LRFVTKFHHVDHLLDAKHNGKTRFRFSINADYVIKNFEPGT  210

N.c.  WSYNVDHFHAWTQGRTGFPIIDAAMRQVLSTGYMHNRLRMI  477
S.c.  WENNPVAFEKWCTGNTGIPIVDAIMRKLLYTGYINNRSRMI  454
E.c.  WQSNPAHLQAWQEGKTGYPIVDAAMRQLNSTGWMHNRLRMI  346
H.h.  WRDEPAALQAWRDGETGYPIVDAGMRQLRAEAYMHNRVRMI  353
A.n.  WENREALFTAWTQAQTGYPIVDAAMRQLTETGWMHNRCRMI  353
S.g.  WRSDADEMHAWKSGLTGYPIVDAAMRQLAHEGWMHNRARML  334
         : |  : :| |:||:||||   : :  :|||| ::  :::
B.s.  SPLDKRIEAAVKVAKAGYPLGFIVAPIYIHEGWEEGYRHLF  250
```

FIG. 7. Amino acid sequence comparison for the deduced Spl protein sequence of *B. subtilis* (*B.s.*) and sequences of DNA photolyases from *Neurospora crassa* (*N.c.*; 66), *Saccharomyces cerevisiae* (*S.c.*; 45), *E. coli* (*E.c.*; 46), *Halobacterium halobium* (*H.h.*; 63), *Anacystis nidulans* (*A.n.*; 69), and *Streptomyces griseus* (*S.g.*; 21). Identical amino acids are boxed and denoted by a vertical line. Conserved amino acids (15) are denoted by colons.

photoreactivation is detectable in vegetative cells of certain spore-forming bacilli, such as *B. megaterium* (10) and *B. cereus* (42, 57), but absent from *B. subtilis* (68). It should prove fascinating to explore how and why photolyase and SP lyase have evolved their present related, yet distinct, functions.

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