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Molecular Dissection of Mutations in the *Bacillus subtilis* Spore Photoproduct Lyase Gene Which Affect Repair of Spore DNA Damage Caused by UV Radiation

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In response to UV irradiation, *Bacillus subtilis* spore DNA accumulates the unique thymine dimer 5-thyminyl-5,6-dihydrothymine, or spore photoproduct (SP). SP is broken down into monomers during spore germination by the product of the *spl* gene which has been proposed to encode the enzyme SP lyase. The wild-type *spl* gene was cloned by complementation of a mutation designated *spl-1*; the putative *spl* gene product is a 40-kDa protein whose deduced amino acid sequence contains regions homologous to DNA photolyases. During phenotypic characterization of *spl* subclones using transformation crosses between the cloned wild-type *spl* gene and an *spl-1* mutant recipient, in addition to the expected transformant classes exhibiting UV-resistant (type I) and UV-sensitive (type III) spores, an additional recombinant class was observed (called type II), spores of which exhibited slower germination kinetics following UV irradiation. The results suggested that the *spl-1* allele consisted of at least two separable mutations. The DNA region which could rescue the *spl-1* allele was localized to a 511-bp region within the *spl* coding sequence; this region was amplified from the *spl-1* mutant chromosome by PCR and sequenced. The region contained two amino acid substitutions, an Arg replacing Gly-168 (G168R) and an Asp replacing Gly-242 (G242D) in the deduced SP lyase sequence, as well as 18 silent mutations. PCR amplification of chromosomal DNA from a selected type II recombinant and sequence analysis of the amplification product confirmed that recombination had indeed occurred between codons 168 and 242 and further localized the point of crossover by using the 18 silent mutations as molecular markers throughout the region. By in vitro mutagenesis, alleles of *spl* containing all combinations of single and double amino acid substitutions were introduced into the cloned wild-type *spl* gene. When integrated into the *B. subtilis* chromosome at the *amyE* locus, it was observed that although both amino acid substitutions contribute to the *spl-1* phenotype, the G168R mutation exerted a much greater effect than did the G242D mutation.

The high resistance of bacterial endospores to the lethal and mutagenic effects of UV radiation can in large part be explained as resulting from the unique photochemistry of spore DNA and the subsequent efficient and error-free repair of this DNA damage during spore germination (reviewed in references 19 to 22). Research in this area over the past 30 years has demonstrated that DNA from UV-irradiated dormant spores accumulates a novel spore photoproduct (SP) instead of cyclobutyl pyrimidine dimers (5); the structure of SP has been determined to be the thymine dimer 5-thyminyl-5,6-dihydrothymine (27). In the best-characterized example, *Bacillus subtilis*, SP repair occurs during germination of UV-irradiated spores via two major routes, the general nucleotide excision repair (*uvr*) pathway and a putative SP-specific enzyme called SP lyase (10, 12, 13), encoded by a gene designated *spl* (6, 10). SP lyase appears to act by direct in situ monomerization of SP in DNA to two thymines (12, 13, 26, 28). Taken together, the *uvr* and *spl* pathways probably account for most of the repair activity which removes UV damage from DNA during spore germination, as it has been observed that mutant strains of *B. subtilis* lacking both repair pathways produce spores which are even more sensitive to UV radiation than vegetative cells of the same strain (10, 11).

The SP lyase repair system was originally defined by a mutation now called *spl-1* (6, 10). To better understand *spl*-mediated DNA repair, the wild-type *spl* gene from *B. subtilis* 168 was recently cloned by a transformation technique using integrative plasmids (30), by the ability of the wild-type *spl* gene to restore UV resistance to spores of a *B. subtilis* recipient strain containing both the *uvrA42* and *spl-1* mutations (6). The physical organization of the *spl* locus was deduced by nucleotide sequence analysis of the region, revealing that the *spl* gene potentially encodes a 40-kDa protein (6). Transformation experiments using integrative plasmids, in which subfragments of the wild-type *spl* gene were tested for the ability to rescue the *spl-1* mutation, localized the *spl-1* allele to a 511-bp sequence between a *Bcl*I restriction site at coordinate 1373 and an *Sph*I site at coordinate 1884 of the published sequence (6). These coordinates correspond to a region spanning amino acids 149 to 320 in the carboxyl half of the deduced SP lyase amino acid sequence (6). Computer-assisted comparisons performed between the deduced amino acid sequence of SP lyase and other DNA repair proteins revealed that a subset of the carboxyl half of SP lyase (amino acids 170 to 250) shares sequence homology with the DNA photolyases from a number of prokaryotes and lower eukaryotes, suggesting that these two classes of enzymes may have descended from a common ancestral protein (6). This finding is interesting in light of the similar function of these two classes of repair proteins (i.e., in situ breakdown of

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TABLE 1. Bacterial strains used in this study

Strain (original code)	Genotype or phenotype	Source or reference[a]
Bacillus subtilis		
168	*trpC2*	Laboratory stock
1A345 (UVS-42)	*metC14 sul thyA1 thyB1 trpC2 uvrA42*	BGSC (10)
1A489 (UVSSP-42-1)	*metC14 spl-1 sul thyA1 thyB1 trpC2 uvrA42*	BGSC (10)
WN47	*metC14 sul thyA1 thyB1 trpC2 uvrA42 spl*$^+$; type I; Cm^r	pWN42^{tf}1A489; type I transformant
WN48	*metC14 sul thyA1 thyB1 trpC2 uvrA42 spl*$^+$; type II; Cm^r	pWN42^{tf}1A489; type II transformant
WN49	*metC14 sul thyA1 thyB1 trpC2 uvrA42 spl*; type III; Cm^r	pWN42^{tf}1A489; type III transformant
WN319	Same as 1A489; Cm^r; pDG364 at *amyE*	This study
WN322	Same as 1A489; Cm^r; pWN318 at *amyE*	This study
WN329	Same as 1A489; Cm^r; pWN325 at *amyE*	This study
WN330	Same as 1A489; Cm^r; pWN326 at *amyE*	This study
WN331	Same as 1A489; Cm^r; pWN327 at *amyE*	This study
Escherichia coli		
JM83	*ara* Δ(*lac-proAB*) *rpsL* φ80 *lacZ*Δ*M15*	Laboratory stock (29)
JM109	*endA1 recA1 gyrA96 thi hsdR17* (r_K^- m_K^+) *relA1 supE44* λ$^-$ Δ(*lac-proAB*) [F′ *traD36 proA*$^+$*B*$^+$ *lacI*^{q}*Z*Δ*M15*]	Laboratory stock

[a] BGSC, *Bacillus* Genetic Stock Center; tf, transformation.

thymine dimers into monomers), although important differences exist between the two proteins in (i) their distinct substrate specificities and (ii) the fact that SP lyase is not dependent on visible light for its activity.

To date, very little is known concerning the mechanism by which SP functions. It is reasonable to assume, however, that understanding of this novel enzyme may be guided in part by using the well-characterized example of DNA photolyase (16) as a paradigm. The observations cited above suggest that the *spl-1* mutation may affect amino acids which are conserved in both classes of proteins, perhaps lending a clue to common amino acid residues used by SP lyase and photolyases for DNA binding or for catalysis. In this communication we report on the results of experiments designed to ascertain the molecular nature of the *spl-1* mutation.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The *B. subtilis* and *Escherichia coli* strains used in this study are listed in Table 1. Plasmids and cloned DNA fragments used are described in Table 2; where appropriate, specific details of plasmid construction are given in the text. Media used were Difco sporulation medium (DSM; 18), 2×SG medium (7), Luria-Bertani medium (9), and Spizizen minimal medium (25). Auxotrophic requirements were each added to Spizizen minimal medium to a final concentration of 50 μg/ml, or 100 μg/ml for thymidine. When appropriate, antibiotics were added to media at the following final concentrations: chloramphenicol, 3 μg/ml; ampicillin, 50 or 125 μg/ml; and tetracycline, 15 μg/ml. All cell incubations were at 37°C. Cells were grown in liquid media with vigorous aeration, and optical density was monitored with a Klett-Summerson colorimeter fitted with the no. 66 (red) filter.

Spore preparation and germination. Spores were produced by incubation of *B. subtilis* strains for 48 h in liquid or on solid DSM containing the appropriate selective antibiotic and were purified and heat shocked as previously described (6, 14). Spores were germinated in liquid 2×SG medium containing 1 mM L-alanine as described previously (14, 26), and germination was monitored spectrophotometrically with a Klett-Summerson colorimeter. Germination of UV-irradiated spores on DSM plates was performed as described previously (6).

UV irradiation of spores. Irradiation of spores with 254-nm-wavelength UV and determination of survival were accomplished essentially as described previously (6, 14). Lamp output at 254 nm was determined by a UVX radiometer (UV Products, San Gabriel, Calif.); UV dosage is reported in units of joules per square meter.

Molecular biology techniques. Large- and small-scale extractions of chromosomal DNA from *B. subtilis* (4) and plasmid DNA from *E. coli* (2) were accom-

TABLE 2. Plasmids used in this study

Plasmid	Description	Source or reference[a]
pUC19	Multisite *E. coli* cloning vector	29
pBGSC6	Integrational plasmid derived from pUC19 and pC194	BGSC
pALTER-1	In vitro mutagenesis vector	Promega
pDG364	*amyE* integration vector	BGSC (4)
pWN42	1.77-kb *Pst*I-*Sph*I fragment of ORF-*spl* from strain 168 (nt 117–1884) cloned in pBGSC6	6
pWN121	511-bp *Bcl*I-*Sph*I PCR fragment of *spl-1* allele (nt 1373–1884) from 1A489 cloned in pUC19	This study
pWN135	511-bp *Bcl*I-*Sph*I PCR fragment of *spl* gene (nt 1373–1884) from 1A345 cloned in pUC19	This study
pWN124	511-bp *Bcl*I-*Sph*I PCR fragment of *spl* gene (nt 1373–1884) from strain 168 cloned in pUC19	This study
pWN148	511-bp *Bcl*I-*Sph*I PCR fragment of complete copy of *spl* gene (nt 1373–1884) from WN48 cloned in pUC19	This study
pWN153	511-bp *Bcl*I-*Sph*I PCR fragment of truncated *spl* gene from WN48 cloned in pUC19; lacks *Hin*dIII site polymorphism	This study
pWN154	Same as pWN153 but contains *Hin*dIII site polymorphism	This study
pWN160	1.75-kb *Eco*RI-*Hin*dIII fragment (from nt 527–2276) of ORF-*spl* cloned in pALTER-1	This study
pWN177	Same as pWN160 but G168R in *spl*	This study
pWN180	Same as pWN160 but G242D in *spl*	This study
pWN181	Same as pWN160 but G168R G242D in *spl*	This study
pWN318	1.75-kb *Eco*RI-*Hin*dIII fragment from pWN160 cloned in pDG364	This study
pWN325	1.75-kb *Eco*RI-*Hin*dIII fragment from pWN177 cloned in pDG364	This study
pWN326	1.75-kb *Eco*RI-*Hin*dIII fragment from pWN180 cloned in pDG364	This study
pWN327	1.75-kb *Eco*RI-*Hin*dIII fragment from pWN181 cloned in pDG364	This study

[a] BGSC, *Bacillus* Genetic Stock Center.

plished by published techniques. Plasmid DNA was further purified by equilibrium gradient ultracentrifugation with cesium chloride-ethidium bromide (15). Standard techniques were used throughout for enzymatic manipulations and agarose gel electrophoretic analyses of DNA (15). Nucleic acid sequencing by dideoxynucleotide chain termination (17) 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 6% polyacrylamide sequencing gels (8).

Genetic techniques. Preparation of competent *E. coli* or *B. subtilis* cells and their transformation with plasmid or chromosomal DNA have been described in detail elsewhere (3, 4, 6, 15).

PCR and cloning of amplified products. Amplification of the region of the *spl* coding sequence between the *Bcl*I site at coordinate 1373 and the *Sph*I site at coordinate 1884 was accomplished with the synthetic oligonucleotide primers 5′-CAGACATTGTAGGAATTGATCATC-3′ (primer 1, containing the *Bcl*I site underlined) and 5′-CAAGTGCCTCTCGAAGTGCATGCT-3′ (primer 2, containing the *Sph*I site underlined). Amplification of the truncated *spl* gene copy present in strain WN48 was accomplished with primer 1 and the pUC19 reverse sequencing primer (New England Biolabs, Beverly, Mass.) (see Fig. 3 for details). In all cases, amplification of target sequences was performed on 0.3 μg of the appropriate chromosomal DNA in a Perkin-Elmer Cetus thermal cycler with Vent DNA polymerase (New England Biolabs) according to the manufacturer's recommendations.

PCR products were digested with *Bcl*I and *Sph*I and were cloned into *Bam*HI-*Sph*I-cleaved plasmid pUC19 for nucleotide sequencing (see Table 2 for specific plasmids generated). Sequencing was performed on two independent plasmid isolates carrying each cloned PCR product; in no cases were sequence discrepancies found as a result of the amplification process.

In vitro mutagenesis of *spl* and insertion of mutated *spl* genes at the *amyE* locus. A 1.75-kbp *Eco*RI-*Hin*dIII fragment containing the ORF-*spl* operon extending from coordinates 527 to 2276 of the published sequence (6) was removed from plasmid pWN151 and inserted into *Eco*RI-*Hin*dIII-cleaved plasmid pALTER-1 (Promega, Madison, Wis.) to create plasmid pWN160. In vitro mutagenesis was performed with the Altered Sites kit (Promega) as recommended by the manufacturer, using the following oligonucleotide primers: 5′-AGTGATCTCAGAAAGCTCC-3′ to create mutation G168R (an Arg replacing Gly-168) and 5′-TTCATGAAGACTGGGAAGA-3′ to create mutation G242D (an Asp replacing Gly-242); both mutagenic primers were used simultaneously to construct the G168R G242D double mutant. Plasmids carrying the desired mutations were identified by nucleotide sequencing and designated plasmids pWN177, pWN180, and pWN181 (Table 2). The wild-type and mutant inserts were then removed from the appropriate mutagenesis vectors by digestion with *Eco*RI and *Hin*dIII and were inserted into *Eco*RI-*Hin*dIII-cleaved plasmid pDG364, resulting in plasmids pWN318 (wild-type *spl*), pWN325 (G168R *spl*), pWN326 (G242D *spl*), and pWN327 (G168R G242D *spl*) (Table 2). Engineered alleles of *spl* cloned in plasmid pDG364 were integrated into the *B. subtilis* chromosome at the *amyE* locus as follows: plasmid DNA (10 μg) was linearized by digestion with *Pst*I and introduced by transformation into competent cells of strain 1A489. Cm^r transformants were screened for plasmid integration at the *amyE* locus by picking candidates onto solid Luria-Bertani medium containing 3 μg of chloramphenicol per ml and 1% (wt/vol) soluble starch. After overnight incubation, the plates were stained with iodine vapor to identify Amy$^-$ colonies which lacked a clear halo of starch hydrolysis. The resulting strains are listed in Table 1.

RESULTS

The *spl-1* allele contains two separable mutations. In a previous communication (6), it was reported that the wild-type *B. subtilis spl* gene was contained on a 2.3-kb *Eco*RI-*Hin*dIII fragment of DNA cloned from *B. subtilis* 168 on the basis of the ability of this restriction fragment to restore the ability to produce UV^r spores to strains harboring the mutant *spl-1* allele (10). The *spl* coding sequence was further localized to the rightward half of the 2.3-kb *Eco*RI-*Hin*dIII fragment (6) as a result of transformation experiments in which subclones of this fragment, cloned in the integrational plasmid pBGSC6, were tested for the ability to correct the *spl-1* mutation after transformation into strain 1A489, a mutant *B. subtilis* strain which lacks both the *uvr* and *spl* pathways and which produces UV^s spores (10; Table 1). By this technique, insert DNA which could correct the *spl-1* mutation was localized to a 511-bp region between a *Bcl*I site at nucleotide 1373 and an *Sph*I site at nucleotide 1884 (6).

When integrative plasmid pWN42, containing the cloned *spl* gene from coordinates 117 to 1884 (Table 2), was used as donor DNA for transformation into strain 1A489, 47% of the total Cm^r transformants obtained from the cross formed spores which were UV^r, while 53% of the Cm^r transformants produced UV^s spores (Table 3). Further analysis of transformants obtained from this cross revealed that the UV^r recombinant class could be further subdivided into two separate classes, visually distinguishable by the rate at which surviving spores from a standardized UV treatment (30 J/m^2) (6) could germinate and form colonies when plated and incubated on solid DSM supplemented with chloramphenicol (Table 3). These two types of UV^r transformants were designated type I (fast-germinating) and type II (slowly germinating) transformants; UV^s transformants were designated type III (Table 3). The results suggested to us that the *spl-1* mutant allele may be complex, consisting of at least two mutations separable by recombination, and the results further suggested that an intragenic recombination event occurring between these two hypothetical mutations during plasmid integration via Campbell recombination (3a) gave rise to type II recombinants, which presumably synthesize a hybrid SP lyase with lowered activity. This conclusion is supported by the results of an elegant series of experiments performed by Munakata and Rupert (12), who showed that the lag time between germination and outgrowth of UV-irradiated spores was a function of both the UV dose (hence the amount of SP produced in spore DNA) and the genetic capacity of the strain tested to eliminate SP from DNA.

TABLE 3. Properties of three classes of Cm^r transformants[a]

Cm^r transformant class	% of total transformants	LD_{90}[b] (J/m^2) of spores	Germination after UV treatment[c]
Type I (UV^r)	8.8	106.5	Fast
Type II (UV^r)	38.2	113.1	Slow
Type III (UV^s)	53.0	9.2	No survivors

[a] Donor DNA was plasmid pWN42; recipient was strain 1A489. See text for details.

[b] LD_{90}, UV dose required to kill 90% of the population (calculated from data of Fig. 1).

[c] Spores received a UV dose of 30 J/m^2 and were germinated on solid DSM as described in Materials and Methods.

In addition to the above-mentioned UV-induced lag in spore outgrowth, genetic differences in DNA repair capacity are also manifested in spores by their characteristic UV dose-dependent inactivation kinetics (6, 10, 12, 13). Therefore, the notion that type I, II, and III transformants differ in SP lyase activity was first tested by characterization of their spore UV resistance properties. Type I and II strains WN47 and WN48 produced UV^r spores which exhibited essentially indistinguishable inactivation kinetics over 3 orders of magnitude, while spores of type III strain WN49 produced UV^s spores which had inactivation kinetics very similar to those of spores of the original recipient in the cross, strain 1A489 (Fig. 1 and Table 3) (6, 10). Furthermore, UV-irradiated spores of type I and II strains WN47 and WN48 did not exhibit a significant difference in outgrowth delay when germinated in liquid medium, whereas outgrowth of UV-irradiated spores of type III transformant strain WN49 was completely abolished (data not shown). Therefore, it appeared that the differences in the germination kinetics of UV-irradiated spores of type I and II transformants, originally observed on solid media immediately after transformation had occurred (Table 3), could not be reproduced after prolonged cultivation of strains WN47 and WN48 and production of spores of these two strains in liquid media (Fig. 1 and data not shown). The data further suggested that the identical inactivation kinetics of spores of these two strains reflected

FIG. 1. UV resistance of spores of transformant strains WN47 (type I; circles), WN48 (type II; triangles), and WN49 (type III; squares). Spores were prepared and UV irradiated and survival was determined as described in Materials and Methods. The datum points represent averages of two independent determinations.

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        BclI      leu thr his thr leu lys arg ala ile glu his phe 162
168    T GAT CAT CTG ACA CAC ACG CTG AAG CGC GCC ATT GAA CAT TTT 1414
1A345  - --- --- --- --- --- --- --- --- --- --- --- --- --- ---
1A489  - --- --- --- --- --- --- --- --- --- --- --- --- --- ---

         gly gln ser asp leu|gly|lys leu arg phe val thr lys phe 176
168      GGC CAA AGT GAT CTC|GGA|AAG CTC CGA TTT GTA ACG AAA TTT 1456
1A345    --A --- --- --C ---|---|--- --- A-- --- --- --- --- --C
1A489    --A --- --- --C ---|A--|--- --- A-- --- --- --- --- --C
                            |arg|

         his his val asp his leu leu asp ala lys his asn gly lys 190
168      CAT CAT GTC GAT CAC CTA TTA GAC GCA AAG CAT AAC GGG AAA 1498
1A345    --- --- --- --- --- --G --- --T --- --- --- --- --- ---
1A489    --- --- --- --- --- --G --- --T --- --- --- --- --- ---

         thr arg phe arg phe ser ile asn ala asp tyr val ile lys 204
168      ACG AGA TTC AGA TTC AGT ATT AAT GCC GAC TAT GTG ATT AAA 1540
1A345    --- --- --T --G --- --- --- --- --- --- --- --- --- --G
1A489    --- --- --T --G --- --- --- --- --- --- --- --- --- --G

         asn phe glu pro gly thr ser pro leu asp lys arg ile glu 218
168      AAC TTT GAG CCG GGA ACT TCA CCT CTT GAT AAG CGG ATA GAA 1582
1A345    --- --- --- --- --- --C --- --- --- --- --- --- --- ---
1A489    --- --- --- --- --- --C --- --- --- --- --- --- --- ---

         ala ala val lys val ala lys ala gly tyr pro leu gly phe 232
168      GCG GCA GTA AAA GTT GCA AAA GCA GGC TAC CCG CTA GGC TTT 1624
1A345    --- --- --- --- --- --- --- --- --A --- --- --- --- ---
1A489    --- --- --- --- --- --- --- --- --A --- --- --- --- ---

         ile val ala pro ile tyr ile his glu|gly|trp glu glu gly 246
168      ATT GTT GCT CCG ATT TAT ATT CAT GAA|GGC|TGG GAA GAA GGA 1666
1A345    --- --- --G --- --- --- --- --- ---|-A-|--- --- --- ---
1A489    --- --- --G --- --- --- --- --- ---|-A-|--- --- --- ---
                                            |asp|

         tyr arg his leu phe glu lys leu asp ala ala leu pro gln 260
168      TAC AGA CAT CTG TTT GAA AAG CTA GAT GCT GCT TTG CCG CAG 1708
1A345    --- --- --- --- --- ---(--- --T)--- --- --- --- --- ---
1A489    --- --- --- --- --- ---(--- --T)--- --- --- --- --- ---
                                (HindIII)

         asp val arg his asp ile thr phe glu leu ile gln his arg 274
168      GAC GTT AGA CAT GAC ATT ACG TTT GAA TTA ATT CAA CAC CGT 1750
1A345    --- --C --- --- --- --- --- --- --- --- --- --G --- ---
1A489    --- --C --- --- --- --- --- --- --- --- --- --G --- ---

         phe thr lys pro ala lys arg val ile glu lys asn tyr pro 288
168      TTT ACA AAA CCG GCC AAA CGA GTG ATA GAG AAA AAT TAT CCG 1792
1A345    --- --- --- --- --- --- --- --- --- --- --G --- --C ---
1A489    --- --- --- --- --- --- --- --- --- --- --G --- --C ---

         lys thr lys leu glu leu asp glu glu lys arg arg tyr lys 302
168      AAG ACG AAG CTC GAA TTA GAT GAA GAA AAG CGC CGT TAT AAA 1834
1A345    --- --- --- --- --- --- --- --- --- --- --T --- --- ---
1A489    --- --- --- --- --- --- --- --- --- --- --T --- --- ---

         trp gly arg tyr gly ile gly lys tyr ile tyr gln lys asp 316
168      TGG GGC CGT TAC GGG ATC GGA AAA TAT ATT TAT CAG AAA GAT 1876
1A345    --- --- --- --- --- --- --- --- --- --- --- --- --- ---
1A489    --- --- --- --- --- --- --- --- --- --- --- --- --- ---

         glu      SphI
168      GAA GAG CAT GC                                          1887
1A345    --- --- --- --
1A489    --- --- --- --
```

FIG. 2. Comparison of the nucleotide sequence of *spl* from *B. subtilis* 168 with the nucleotide sequences obtained from strain 1A489 (*spl-1*) and its immediate parental strain 1A345. Dashes denote identical bases. Above the strain 168 sequence is the deduced amino acid sequence of the strain 168 SP lyase. Amino acid sequence differences at codons 168 and 242 are denoted below the sequences and contained within boxes. The *Hin*dIII restriction site polymorphism present in strains 1A345 and 1A489 is contained within parentheses. Coordinates are taken from reference 6.

their similar time lags between germination and outgrowth of UV-irradiated spores, consistent with previous findings (12). We reasoned that investigation into the molecular nature of the *spl-1* mutation could provide clues to explain the above observations.

PCR amplification and nucleotide sequence of the *spl-1*-containing region. Because wild-type DNA sequences able to correct the *spl-1* allele had previously been localized to a 511-bp region of the cloned *spl* gene between nucleotides (nt) 1373 and 1884 (6), it was predicted that analyzing the nucleotide sequence in this region from an *spl-1* mutant would yield insights into the exact nature of the putative mutation(s) involved. PCR primers which spanned coordinates 1373 to 1884 were used to amplify this region from chromosomal DNA isolated from strain 1A489, and the nucleotide sequence of this region was determined (Fig. 2).

Comparison of the nucleotide sequences between the wild-type strain 168 and the *spl-1* mutant strain 1A489 revealed the presence of 20 separate nucleotide differences distributed throughout the 511-bp region between nt 1373 to 1884 (Fig. 2). When the nucleotide sequence obtained from strain 1A489 was converted to amino acid sequence, it was observed that 18 of the 20 nucleotide differences observed are silent, i.e., do not change the deduced SP lyase amino acid sequence. Two of the nucleotide changes did however, result in changes in the deduced SP lyase amino acid sequence in the *spl-1* mutant; a G-to-A nucleotide transition at nt 1430 resulted in arginine (R) replacing glycine (G) at codon 168, and another G-to-A nucleotide transition at nt 1653 resulted in an aspartic acid (D) replacement of glycine at codon 242 (Fig. 2), consistent with the notion that the *spl-1* allele consists of two distinct mutations separable by recombination.

The high degree of nucleotide sequence divergence in this limited region (20 mismatches of 511 bases, or nearly 4%) prompted us to perform several experiments to assure that the differences observed were not experimental artifacts. First, the inserts contained in two independently isolated plasmid clones representative of pWN121 were sequenced to assure that any departures observed in *spl-1* from the wild-type *spl* nucleotide sequence were due to differences in the strains and not due to artifacts arising during PCR amplification; the sequences of these two clones were identical to one another (data not shown). Second, the same two PCR primers (primers 1 and 2) were used to amplify the homologous region from wild-type strain 168, and two independently isolated plasmid clones derived from strain 168 were sequenced. The nucleotide sequences of these two clones, typified by plasmid pWN124 (Table 2), were also identical to one another and furthermore perfectly matched the wild-type *spl* sequence in this region, which was previously obtained from the complete *spl* gene cloned from strain 168 (6). Third, analysis of the nucleotide sequence of the *spl-1* allele from strain 1A489 revealed that one of the silent mutations, an A-to-T change at nt 1690, was predicted to create a *Hin*dIII restriction cleavage site at nt 1686 (Fig. 2). The presence of this predicted *Hin*dIII restriction site polymorphism in the *spl-1* sequence was confirmed by restriction analysis of plasmid pWN121 (Fig. 2; also data not shown).

Because of the considerable sequence divergence between wild-type strain 168 and *spl-1* strain 1A489 (Fig. 2), it seemed

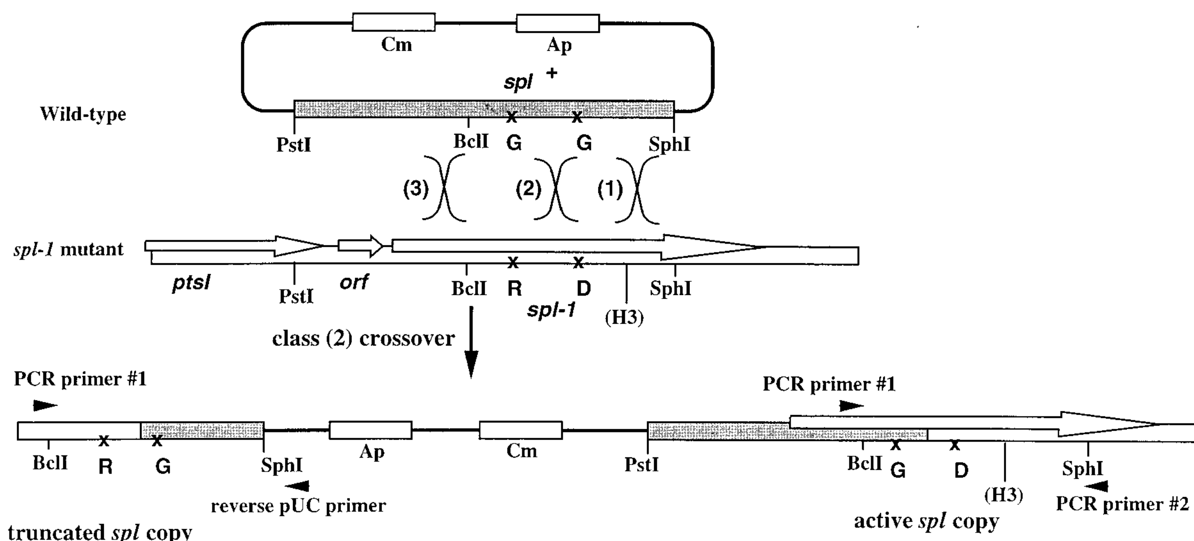

FIG. 3. Schematic representation of hypothetical integration events leading to type I, II, or III transformants. Transformation of plasmid pWN42 containing the wild-type *spl* insert (shaded bar) into recipient strain 1A489 (*spl-1*) is expected to result in plasmid integration resulting from Campbell recombination occurring at position (1), (2), or (3) with respect to codons 168 and 242 (G, glycine; R, arginine; D, aspartic acid) which would lead to type I, II, or III transformants, respectively. Note that each class of transformant carries a truncated (inactive) upstream copy of *spl* and a complete (active) downstream copy of *spl*. Depicted at the bottom of the figure is the chromosomal arrangement predicted from a crossover at position (2), resulting in a type II transformant such as strain WN48. Shown are the combinations of PCR primers used to amplify the indicated regions from the intact *spl* copy (primers 1 and 2) and from the truncated *spl* copy (primer 1 and the reverse pUC sequencing primer). The position of the *Hin*dIII restriction polymorphism (H3) predicted from the sequence of strain 1A489 is indicated.

reasonable that additional valuable information about the region from nt 1373 to 1884 could be gained by sequence analysis of this area amplified from chromosomal DNA of the immediate parental strain of 1A489, strain 1A345 (Table 1). Sequence analysis revealed that the region from nt 1373 to 1884 amplified from strain 1A345 (spl^+) was completely identical to the *spl-1* region from strain 1A489, with the exception of a G residue at nt 1430, as in wild-type strain 168 (Fig. 2). Interestingly, this results in the presence of a wild-type glycine at codon 168 in strain 1A345. Therefore, it appeared from this analysis that amino acid G168 might be a residue of some importance in determining SP lyase activity. Furthermore, the results confirmed that the nucleotide sequence differences seen in the region from nt 1373 to 1884 between strains 168, 1A345, and 1A489 are truly due to strain differences and not due to artifacts introduced during PCR amplification.

Molecular characterization of the *spl* genes from type II transformant WN48. The observation of two amino acid differences in the region from nt 1373 to 1884 between the wild-type and *spl-1* mutant SP lyase amino acid sequences (Fig. 2) was consistent with the prediction that the *spl-1* allele consisted of two separable mutations. On the basis of the above observations, the following model was developed to explain how transformants of type I, II, or III could arise when plasmid pWN42 integrated at the *spl-1* locus of recipient strain 1A489 (Fig. 3). Integration of plasmid pWN42 into the *B. subtilis* chromosome by Campbell recombination would be expected to generate two copies of *spl* separated by vector DNA: an upstream inactive copy truncated at the *Sph*I site at nt 1884 and a downstream complete (and active) copy (Fig. 3). It was postulated that integration by a crossover event occurring downstream from codon 242 would generate a type I transformant, which would be predicted to contain the wild-type G168 and G242 codons in the complete downstream copy of *spl*, and which would be expected to lead to strain 168-type SP lyase activity (Fig. 3). A crossover event occurring upstream from codon 168 would result in a type III transformant, which would be predicted to contain the R168 and D242 codons in the complete *spl* copy (Fig. 3), as found previously in the *spl-1* recipient strain 1A489. Finally, a crossover event occurring between codons 168 and 242 would be predicted to give rise to a type II transformant (such as strain WN48), in which the active downstream copy of *spl* would contain G168 as in the wild type but D242 as in the *spl-1* mutant (Fig. 3). (Coincidently, this arrangement is also found in "wild-type" strain 1A345, the parent from which the *spl-1* mutant strain 1A489 was derived; Fig. 2.) The resulting SP lyase encoded by this type II transformant would presumably be less active than that of a type I transformant, thus accounting for the slower germination kinetics originally observed in UV-irradiated spores of type II strains such as WN48 (Table 3).

We reasoned that this model could be tested by direct analysis of the nucleotide sequence of the two copies of *spl* contained in the chromosome of type II transformant WN48. To accomplish this, we took advantage of the fact that during integration of plasmid pWN42 into the chromosomal *spl* locus of strain 1A489, a tandem duplication of *spl* occurred, creating an upstream truncated (and inactive) copy of *spl* and a downstream complete (and active) *spl* copy (Fig. 3). In principle, the 511-bp region spanning nt 1373 to 1884 could be selectively amplified from the intact *spl* copy of strain WN48 by using PCR primers 1 and 2, and the corresponding region of the truncated *spl* copy could be selectively amplified by using PCR primer 1 and the reverse pUC19 sequencing primer (Fig. 3). Sequencing of these amplification products would test the prediction of the model that the upstream truncated *spl* copy should carry R168 and G242 and that the downstream intact copy of *spl* should carry G168 and D242 (Fig. 3). In addition, it was predicted that the location of the crossover point used during integration of plasmid pWN42 into the 1A489 chromosome could be pinpointed quite accurately by using the additional 18 silent mutations distributed along the region between nt 1373 and 1884 as molecular markers (Fig. 2).

Using PCR primers 1 and 2, we amplified the 511-bp region

from the downstream intact *spl* copy of strain WN48 and sequenced the cloned PCR products from two independent plasmids represented by pWN148 (Table 2). Nucleotide sequence analysis of the region revealed that the sequence from nt 1373 to 1607 corresponded to the wild-type *spl* sequence, including G168, and the region spanning nt 1608 to 1884 corresponded to the *spl-1* mutant sequence, including D242 and the *Hin*dIII site polymorphism (Fig. 4). The crossover junction was localized to a 49-bp region between nt 1559 and 1609 (Fig. 4). These observations were entirely consistent with the proposed model for how a type II transformant could be generated (Fig. 3).

We expected that nucleotide sequence analysis of the upstream truncated copy of *spl* present in strain WN48 would reveal that recombination was symmetrical, as envisioned in Fig. 3. To test this prediction, we amplified, cloned, and sequenced the analogous region spanning nt 1373 to 1884 from the upstream truncated *spl* copy of strain WN48. During screening of putative clones, we observed that 6 of 12 candidates lacked the *Hin*dIII restriction site polymorphism at nt 1686 of the insert, as expected (Fig. 3), but the other 6 candidates contained this *Hin*dIII site (data not shown).

To resolve this apparent discrepancy, we sequenced two representatives of each class of plasmid, typified by plasmid pWN153 (lacking the *Hin*dIII site, as expected) and pWN154 (carrying the *Hin*dIII site) (Table 2). Nucleotide sequence analysis of the 511-bp region spanning nt 1373 to 1884 from the upstream truncated *spl* copy present in plasmid pWN153 revealed that nucleotide sequence organization in this clone was exactly symmetrical to the sequence observed from the intact downstream copy of *spl* derived from plasmid pWN148 (Fig. 4) and was in perfect agreement with the structure predicted for a type II recombinant (Fig. 3). In contrast, nucleotide sequence analysis of the insert present in plasmid pWN154, which unexpectedly contained the *Hin*dIII restriction site polymorphism, revealed a DNA sequence organization very different from either the upstream or downstream copies of *spl* (Fig. 4). This cloned amplification product exhibited *spl-1*-type sequence from nt 1373 to 1824, including both the R168 and D242 codons and the *Hin*dIII site polymorphism, and wild-type sequence from nt 1825 to 1884 (Fig. 4); furthermore, the crossover point was found in a 35-bp region between nt 1789 and 1825, i.e., in a completely different location from the crossover junction determined by analyses of plasmids pWN148 and pWN153 (Fig. 4). From these observations, we concluded that the aberrant structure of the region from nt 1373 to 1884 represented in plasmid pWN154 arose as a result of a subsequent recombination event occurring between the two tandemly repeated copies of *spl* during propagation of transformant strain WN48. The above observations imply that populations of spores of type I, II, and III transformant strains WN47, WN48, and WN49 are heterogeneous with respect to their nucleotide sequence at the active *spl* locus as a result of recombinational shuffling occurring between the two tandem *spl* sequences during growth and sporulation. This recombinational shuffling during strain propagation may have been sufficient to obscure the subtle differences in both spore UV resistance (Fig. 1) and germination of spores after UV treatment (data not shown) which were originally observed between type I and II transformants immediately following their establishment (Table 3).

Integration and expression of *spl* point mutants at the *amyE* locus. On the basis of the above observations, it was possible that the inability to detect differences either in the UV resistance (Fig. 1) or germination kinetics of UV-irradiated spores between populations of type I and II transformants is due to a leveling effect resulting from multiple rounds of recombination

```
            BclI    leu thr his thr leu lys arg ala ile glu his phe 1622
w.t.     T GAT CAT CTG ACA CAC ACG CTG AAG CGC GCC ATT GAA CAT TTT 1414
48-Dn    - --- --- --- --- --- --- --- --- --- --- --- --- --- ---
48-Up    - --- --- --- --- --- --- --- --- --- --- --- --- --- ---
48-Up*   - --- --- --- --- --- --- --- --- --- --- --- --- --- ---
spl-1    - --- --- --- --- --- --- --- --- --- --- --- --- --- ---

           gly gln ser asp leu gly lys leu arg phe val thr lys phe 176
w.t.       GGC CAA AGT GAT CTC GGA AAG CTC CGA TTT GTA ACG AAA TTT 1456
48-Dn      --- --- --- --- --- --- --- --- --- --- --- --- --- ---
48-Up      --A --- --- --C --- A-- --- --- A-- --- --- --- --- --C
48-Up*     --A --- --- --C --- A-- --- --- A-- --- --- --- --- --C
spl-1      --A --- --- --C --- A-- --- --- A-- --- --- --- --- --C
                               arg

           his his val asp his leu leu asp ala lys his asn gly lys 190
w.t.       CAT CAT GTC GAT CAC CTA TTA GAC GCA AAG CAT AAC GGG AAA 1498
48-Dn      --- --- --- --- --- --- --- --- --- --- --- --- --- ---
48-Up      --- --- --- --- --- --G --- --T --- --- --- --- --- ---
48-Up*     --- --- --- --- --- --G --- --T --- --- --- --- --- ---
spl-1      --- --- --- --- --- --G --- --T --- --- --- --- --- ---

           thr arg phe arg phe ser ile asn ala asp tyr val ile lys 204
w.t.       ACG AGA TTC AGA TTC AGT ATT AAT GCC GAC TAT GTG ATT AAA 1540
48-Dn      --- --- --- --- --- --- --- --- --- --- --- --- --- ---
48-Up      --- --- --T --G --- --- --- --- --- --- --- --- --- --G
48-Up*     --- --- --T --G --- --- --- --- --- --- --- --- --- --G
spl-1      --- --- --T --G --- --- --- --- --- --- --- --- --- --G

           asn phe glu pro gly thr ser pro leu asp lys arg ile glu 218
w.t.       AAC TTT GAG CCG GGA ACT TCA CCT CTT GAT AAG CGG ATA GAA 1582
48-Dn      --- --- --- --- --- --- *** *** *** *** *** *** *** ***
48-Up      --- --- --- --- --- --C *** *** *** *** *** *** *** ***
48-Up*     --- --- --- --- --- --C --- --- --- --- --- --- --- ---
spl-1      --- --- --- --- --- --C --- --- --- --- --- --- --- ---

           ala ala val lys val ala lys ala gly tyr pro leu gly phe 232
w.t.       GCG GCA GTA AAA GTT GCA AAA GCA GGC TAC CCG CTA GGC TTT 1624
48-Dn      *** *** *** *** *** *** *** *** **A --- --- --- --- ---
48-Up      *** *** *** *** *** *** *** *** **- --- --- --- --- ---
48-Up*     --- --- --- --- --- --- --- --- --A --- --- --- --- ---
spl-1      --- --- --- --- --- --- --- --- --A --- --- --- --- ---

           ile val ala pro ile tyr ile his glu gly trp glu glu gly 246
w.t.       ATT GTT GCT CCG ATT TAT ATT CAT GAA GGC TGG GAA GAA GGA 1666
48-Dn      --- --- --G --- --- --- --- --- --- -A- --- --- --- ---
48-Up      --- --- --- --- --- --- --- --- --- --- --- --- --- ---
48-Up*     --- --- --G --- --- --- --- --- --- -A- --- --- --- ---
spl-1      --- --- --G --- --- --- --- --- --- -A- --- --- --- ---
                                               asp
                                  (HindIII)
           tyr arg his leu phe glu lys leu asp ala ala leu pro gln 260
w.t.       TAC AGA CAT CTG TTT GAA AAG CTA GAT GCT GCT TTG CCG CAG 1708
48-Dn      --- --- --- --- --- ---(--- --T)--- --- --- --- --- ---
48-Up      --- --- --- --- --- --- --- --- --- --- --- --- --- ---
48-Up*     --- --- --- --- --- ---(--- --T)--- --- --- --- --- ---
spl-1      --- --- --- --- --- ---(--- --T)--- --- --- --- --- ---

           asp val arg his asp ile thr phe glu leu ile gln his arg 274
w.t.       GAC GTT AGA CAT GAC ATT ACG TTT GAA TTA ATT CAA CAC CGT 1750
48-Dn      --- --C --- --- --- --- --- --- --- --- --- --G --- ---
48-Up      --- --- --- --- --- --- --- --- --- --- --- --- --- ---
48-Up*     --- --C --- --- --- --- --- --- --- --- --- --G --- ---
spl-1      --- --C --- --- --- --- --- --- --- --- --- --G --- ---

           phe thr lys pro ala lys arg val ile glu lys asn tyr pro 288
w.t.       TTT ACA AAA CCG GCC AAA CGA GTG ATA GAG AAA AAT TAT CCG 1792
48-Dn      --- --- --- --- --- --- --- --- --- --- --G --- --C ---
48-Up      --- --- --- --- --- --- --- --- --- --- --- --- --- ---
48-Up*     --- --- --- --- --- --- --- --- --- --- --G --- --C ###
spl-1      --- --- --- --- --- --- --- --- --- --- --G --- --C ---

           lys thr lys leu glu leu asp glu glu lys arg arg tyr lys 302
w.t.       AAG ACG AAG CTC GAA TTA GAT GAA GAA AAG CGC CGT TAT AAA 1834
48-Dn      --- --- --- --- --- --- --- --- --- --- --T --- --- ---
48-Up      --- --- --- --- --- --- --- --- --- --- --- --- --- ---
48-Up*     ### ### ### ### ### ### ### ### ### ### ##- --- --- ---
spl-1      --- --- --- --- --- --- --- --- --- --- --T --- --- ---

           trp gly arg tyr gly ile gly lys tyr ile tyr gln lys asp 316
w.t.       TGG GGC CGT TAC GGG ATC GGA AAA TAT ATT TAT CAG AAA GAT 1876
48-Dn      --- --- --- --- --- --- --- --- --- --- --- --- --- ---
48-Up      --- --- --- --- --- --- --- --- --- --- --- --- --- ---
48-Up*     --- --- --- --- --- --- --- --- --- --- --- --- --- ---
spl-1      --- --- --- --- --- --- --- --- --- --- --- --- --- ---

           glu       SphI
w.t.       GAA GAG CAT GC                                          1887
48-Dn      --- --- --- --
48-Up      --- --- --- --
48-Up*     --- --- --- --
spl-1      --- --- --- --
```

FIG. 4. Nucleotide sequences of the 511-bp region (nt 1373 to 1884) from the upstream truncated copy (48-Up) and downstream intact copy (48-Dn) of *spl* amplified from type II transformant strain WN48, cloned in plasmids pWN153 and pWN148, respectively. Also included for comparison are the wild-type (w.t.) and *spl-1* sequences of the region. The recombination junction used during integration of plasmid pWN42 into the 1A489 chromosome is denoted by asterisks. Also shown is the sequence derived from plasmid pWN154, an aberrant recombinant detected in the truncated upstream *spl* copy of strain WN48 (48-Up*), which presumably arose during strain propagation (see text for details). The location of the site of recombination in this sequence is denoted by pound signs.

occurring between the tandem *spl* copies present in these strains during propagation. Another factor which may contribute to the final phenotype of type I and II transformants and which adds another level of complication to the interpretation of their phenotypes is that the differences in nucleotide sequence (i.e., the 18 silent changes) which occur between the *spl* genes of strains 168, 1A345, and 1A489 in the region from nt 1373 to 1884 result in alterations in the predicted pattern of codon usage among these three strains; some of these alterations in codon usage (particularly in codons specifying Gly, Arg, or Lys) (23, 24; data not shown) may be dramatic enough to affect the rate of translation of *spl* mRNA, hence the final levels of SP lyase, in spores of type I and II transformants.

Two approaches were taken in order to circumvent the above problems and to assay the in vivo activities of SP lyase encoded by point mutants in a more reliable manner.

(i) By in vitro mutagenesis, *spl* genes were constructed which contained amino acid replacements at codons 168 and 242 in all four possible combinations (G168 and G242 [i.e., strain 168 type]; G168 and G242D [i.e., strains WN48 and 1A345 type]; G168R and G242 [for which no strain existed previously]; and G168R and G242D [i.e., strain 1A489 type]). As the parental *spl* gene for all constructions was the gene cloned from strain 168 (6), all of the *spl* alleles constructed by in vitro mutagenesis contained the 18 silent nucleotide markers characteristic of strain 168.

(ii) All alleles of the *spl* gene constructed by in vitro mutagenesis were integrated at the *amyE* locus of strain 1A489 (at 25° on the *B. subtilis* genetic map [1]), far removed from the *spl* locus at 118° on the *B. subtilis* genetic map (1, 6), to reduce the probability of recombination occurring between the two copies of *spl* during strain propagation.

Integration of plasmid pWN318, carrying the strain 168-type *spl* gene cloned in plasmid pDG364, into the *amyE* locus of strain 1A489 resulted in restoration of UV resistance to spores, whereas integration of the plasmid vector pDG364 alone did not (Fig. 5). Integration of plasmid pWN327, carrying the engineered *spl-1* allele (G168R G242D) at *amyE*, resulted in only a very slight increase in the UV resistance of spores of the resulting strain WN331, consistent with an earlier observation that SP lyase encoded by the *spl-1* mutant allele retains a small amount of residual activity (6). Engineered alleles of *spl* containing single amino acid substitutions integrated at *amyE* were tested for the ability to restore UV resistance to spores of strain 1A489. It was observed that spores of strain WN329, harboring the *spl* allele containing only the G168R amino acid substitution at *amyE*, were UV^s, being very similar in their resistance to UV to spores of the isogenic strain carrying the G168R G242D (i.e., *spl-1*) double mutation integrated at *amyE* (Fig. 5). In contrast, spores of strain WN330, which harbor at *amyE* the engineered *spl* allele containing only the G242D amino acid substitution characteristic of type II strain WN48 and strain 1A345, were slightly more UV sensitive than were spores of strain WN322, which carry the 168-type *spl* allele (Fig. 5). Taken together, the data indicate that amino acid substitutions at both codons 168 and 242 contribute to the UV^s phenotype of spores of strains harboring the *spl-1* allele, but the relative contribution made by the G242D substitution to the *spl-1* phenotype is minor compared with that made by the G168R substitution (Fig. 5). The data demonstrate that utilizing the information gained from molecular analysis of the *spl* locus (Fig. 2 and 4), type I (i.e., strain WN322) and type II (i.e., strain WN330) transformants can be reconstructed by in vitro mutagenesis in a manner that avoids recombinational shuffling; furthermore, spores of the reconstructed type II strain WN330 indeed exhibit a subtle, but demonstrable, decrease in UV resistance (Fig. 5), as was originally detected immediately after establishment of type II strain WN48 (Table 3).

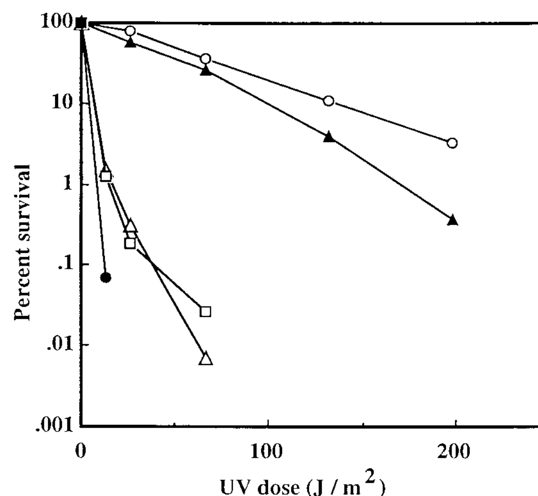

FIG. 5. UV resistance of spores of *B. subtilis* strains carrying various alleles of *spl* integrated at the *amyE* locus. Spores of the indicated strains were irradiated with 254-nm-wavelength UV and spore survival was determined as described in Materials and Methods: WN319 (vector only; solid circles); WN322 (G168/G242; open circles); WN329 (G168R/G242; open triangles); WN330 (G168/G242D; solid triangles); WN331 (G168R/G242D; open squares). The parental background in each case is strain 1A489 (*uvrA42 spl-1*). The datum points represent the averages of two independent determinations.

DISCUSSION

As a first step toward answering the question "what are common structural and functional determinants between SP lyase and DNA photolyases?", this communication describes the molecular analysis of point mutations occurring within the *spl* gene which affect activity of SP lyase in repairing SP during germination of UV-irradiated *B. subtilis* spores. This study was prompted by the observation that there is apparent overlap between the homologous region shared by SP lyase and DNA photolyases, deduced by DNA sequence analysis (6), and the region of the wild-type *spl* gene which can correct the *spl-1* mutation, deduced from transformation analyses (6; this communication). Determination of the primary nucleotide sequence of the region of the *spl* gene affected by the *spl-1* mutation revealed that the *spl-1* allele differed from the prototypic (i.e., strain 168) *spl* allele at 20 discrete positions, two of which (G168R and G242D) changed the deduced SP lyase amino acid sequence (Fig. 2). Subsequent sequence analysis of this region from Munakata's original "wild-type" parental strain, 1A345, revealed that the *spl* gene from this strain also contained the G242D replacement (Fig. 2), suggesting that this amino acid alteration at codon 242 has only a minor effect on SP lyase activity; it is of interest to note that G242 is conserved between SP lyase and DNA photolyases (6).

Differences in the phenotypes imparted to *B. subtilis* spores harboring either the G168 G242 (type I) or the G168 D242 (type II) isoforms of SP lyase were originally detected by a simple plate screening assay of UV-irradiated spores obtained directly from transformant colonies (Table 3), but subsequent quantitative determinations of UV resistance (Fig. 1) and the velocity of outgrowth of germinating spores following UV irradiation (data not shown) failed to reveal substantial differences between spores of types I and II strains WN47 and WN48, carrying either the G168 G242 or G168 D242 isoforms,

respectively. The inconclusive results of these early experiments may have been due to population heterogeneity of spores of type I and II strains arising by recombinational shuffling of tandem *spl* alleles during growth and sporulation; direct molecular evidence in support of this explanation was obtained from the detection by PCR of at least one additional recombination event occurring in the upstream truncated *spl* copy of strain WN48 (Fig. 4). In contrast, the R168 D242 isoform of SP lyase encoded by the *spl-1* allele is severely crippled in its activity, as judged by the UV^s phenotype imparted to spores of strains carrying this mutant enzyme (Fig. 1 and 5) (6, 10, 11).

A question arising from the above observations regarded the relative importance to SP lyase activity of amino acid replacements at codons 168 and 242: is the G168R replacement sufficient in itself to severely lower SP lyase activity, or is activity compromised only by the double replacements of G168R and G242D? The answer to this question could be obtained only by constructing appropriate *spl* alleles which contained all combinations of the G168R and G242D replacements and testing the resulting phenotypes of spores carrying these alleles. It was found that amino acid substitutions at both positions contribute to the final phenotype of the *spl-1* mutation and that the G168R replacement alone exerts a much more profound negative effect on SP lyase activity than does the G242D replacement alone (Fig. 5). Although almost nothing is known of the three-dimensional structure or mechanism of action of SP lyase, the experimental observations of Fig. 5 are consistent with the results of computer-assisted secondary structure analyses indicating that the G168R mutation causes more pronounced changes in the predicted SP lyase secondary structure than does the G242D mutation (data not shown).

It should be noted that, thus far, the in vitro-engineered alleles of *spl* have been integrated into the *B. subtilis* chromosome at the *amyE* locus of strain 1A489, a strain which also carries the R168 D242 isoform of SP lyase encoded from its own *spl-1* locus. Because the subunit composition of SP lyase is unknown, at present it may not be valid to assume that SP lyase is active as a monomer, as are DNA photolyases (16). If SP lyase were in fact active as a homomultimer, the results of Fig. 5 could be confounded by mixing of the subunits encoded by the two *spl* loci, resulting in a heterogeneous population of SP lyases within the germinating spore. To circumvent this potential confounding factor, we are currently constructing a *B. subtilis* host strain which carries a deletion of the entire ORF-*spl* operon. Nevertheless, the preliminary experiments reported in this communication demonstrate that targeted in vitro mutagenesis of *spl* and expression of the mutated alleles at the *amyE* locus have the potential to be powerful tools for the in vivo analysis of structure-function relationships in SP lyase.

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