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Genetic Analysis of a Streptomycin-Resistant Oligosporogenous *Bacillus subtilis* Mutant

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Strain SRB15T$^+$, a streptomycin-resistant, oligosporogenous mutant of *Bacillus subtilis*, contains two mutations, *fun* and *strR*. These mutations were mapped by PBS-1 mediated transduction and by transformation to two different sites in the *cysA*-linked region of the *B. subtilis* chromosome. The *fun* mutation mapped very close to *rpsL1*, a classic *strA* mutation, whereas *strR* mapped to a site distal to *rpsE*. The effects of these mutations on growth, sporulation, and streptomycin resistance in vivo and in vitro were determined. The *fun* mutation gave a different phenotype than did the *rpsL1* mutation and caused altered migration of a ribosomal protein which was identified as S12, the protein encoded by *rpsL*. It therefore appears that *fun* is an allele of the *rpsL* gene.

Mutants resistant to antibiotics which affect the bacterial ribosome have been extremely valuable in the study of ribosome structure and function. In *Escherichia coli*, resistance to streptomycin has been especially interesting because of the pleiotropic phenotype exhibited by many streptomycin-resistant (Str^r) mutants. Effects on growth rate (43), catabolite repression (13), suppression or readthrough of nonsense mutations (40, 42), peptide chain elongation rate (15, 43), and misreading (11) have been observed. Str^r mutants of *Bacillus subtilis* have also been isolated, and effects on growth, misreading, and endospore formation have been noted (5, 30, 39).

Ribosomal Str^r mutants in *E. coli* contain an alteration in the *rpsL* gene, which codes for 30S subunit protein S12 (33). In *B. subtilis*, Str^r mutants have been isolated which contain an altered 30S ribosomal protein identified by polyacrylamide gel electrophoresis and column chromatography (37). Although no difference was observed in migration in the two-dimensional polyacrylamide gel system for which a nomenclature is available (32), the modified protein was identified as *B. subtilis* protein S12 by coelectrophoresis of the purified protein with the S12 spot on two-dimensional gels (25). These mutations, originally named *strA*, have now been designated *rpsL* (21), in accordance with the nomenclature used for *E. coli* (24). The probable correspondence between 30S proteins of *B. subtilis* and *E. coli* has been established using protein sequence data (22), and *B. subtilis* protein S12 is structurally related to *E. coli* protein S12.

In *B. subtilis*, the *rpsL* gene is located in the classic "ribosomal region" of the chromosome (16, 18). Other mutations conferring an Str^r phenotype have been isolated and mapped to different sites on the chromosome (39), but these mutations were not clearly demonstrated to directly affect the ribosome. A Str^r, oligosporogenous (Spo$^-$) mutant was isolated in our laboratory, and it was demonstrated that ribosomes from this strain are resistant to high concentrations of streptomycin (5) and that this resistance is associated with the 30S ribosomal subunit (20). This strain contains two mutations: *fun*, which confers an alteration in a 30S ribosomal protein and resistance to low levels of streptomycin, and *strR*, which in conjunction with *fun* confers resistance to high concentrations of streptomycin (20). In this paper we report the mapping of the *fun* and *strR* mutations and compare these mutations to *rpsL1*, a classic streptomycin resistance mutation in *B. subtilis*.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. The *B. subtilis* strains used in this study are listed in Table 1. Media used were: NSM, nutrient sporulation medium (35); 2× NSM, twofold-concentrated NSM for liquid media; A3 medium (antibiotic medium no. 3, Difco Laboratories); Spizizen minimal medium (38) supplemented with 0.1% glucose and the appropriate auxotrophic requirements at 20 μg/ml; and PBAB (peptone blood agar base, GIBCO Diagnostics). Davis salts used for dilutions contained 0.7% K_2HPO_4, 0.3% KH_2PO_4, 0.1% $(NH_4)_2SO_4$, and 0.005% $MgSO_4 \cdot 7H_2O$. Streptomycin sulfate, erythromycin, and rifampin were purchased from Sigma Chemical Co. Spectinomycin was a gift from the Upjohn Co. Growth was measured with a Klett-Summerson colorimeter with the No. 66 red filter or with a Bausch and Lomb Spectronic 20 spectrophotometer. All growth unless otherwise indicated was at 37°C.

Growth characteristics. Doubling times, frequency of sporulation, and in vivo resistance to streptomycin were determined in 2× NSM as described previously (20). Sporulation frequency was defined as: (heat-resistant CFU per milliliter/total CFU per milliliter) × 100%. The minimum inhibitory streptomycin concentration was defined as the concentration of streptomycin required to reduce growth to less than 50% of the level obtained in the absence of streptomycin.

Genetic techniques. DNA isolation, transformation, transduction, and selection for recombinants were done as described previously (T. M. Henkin and G. H. Chambliss, Mol. Gen. Genet., in press). Antibiotic concentrations used were: spectinomycin (spc), 200 μg/ml; rifampin (rif), 5 μg/ml; erythromycin (ery), 2 μg/ml; and streptomycin (str), 500 μg/ml for selection for high levels of streptomycin resistance and 50 μg/ml for screening for mutations conferring resistance to low levels of streptomycin. Temperature-resistant (Ts^r) recombinants were selected by plating on PBAB and incubating at 37°C for 3 h, then shifting to 52°C.

Preparation of ribosomes. Washed 70S ribosomes were prepared as described previously (20). A rapid small-scale ribosome extraction procedure was employed for screening

TABLE 1. Bacterial strains

Strain	Genotype	Origin (reference)
168T$^+$	prototroph	P. Schaeffer
BR151	*lys-3 metB10 trpC2*	G. Wilson
BR151T$^+$	*lys-3 metB10*	(20)
SRB151T$^+$	*lys-3 metB10 fun strR*	(20)
BRfun	*lys-3 fun*	(20)
IS26	*thr5 trpC rpsL1 rpsE*	I. Smith
BR151T$^+$strA	*lys-3 metB10 rpsL1*	Str^r transformant of BR151T$^+$; DNA from IS26
BRfunstrA	*lys-3 fun rpsL1*	Str^r transformant of BRfun; DNA from BR151T$^+$ strA
BR151T$^+$spc	*lys-3 metB10 rpsE*	Spc^r transformant of BR151T$^+$; DNA from IS26
BRfunstrAspc	*lys-3 fun rpsL1 rpsE*	Spc^r transformant of BRfunstrA; DNA from BR151T$^+$spc
BRfunspc	*lys-3 fun rpsE*	Spc^r transformant of BRfun; DNA from BR151T$^+$spc
1S45	*trpC spoVD156 rpoB2*	Bacillus Genetic Stock Center
BR151T$^+$strArif	*lys-3 metB10 rpsL1*	Rif^r transformant of BR151T$^+$strA; DNA from 1S45
BRfunrif	*lys-3 fun rpoB2*	Rif^r transformant of BRfun; DNA from 1S45
QB944	*purA16 cysA14 trpC2*	F. Kunst; (12)
QB944fun	*cysA14 trpC2 fun*	Transformant of QB944; DNA from BRfun
1A88	*trpC2 sacS32 rplV1*	Bacillus Genetic Stock Center
QB944ery	*purA16 cysA14 trpC2 rplV1*	Ery^r transformant of QB944; DNA from 1A88
IS4	*rpsE efg-10 (ts-1)*	I. Smith; (14)
SRBc	*lys-3 cysA14 fun strR*	Cys$^-$ transformant of SRB15T$^+$, by congression; DNA from QB944
SRBspc	*lys-3 metB10 fun strR rpsE*	Spc^r transformant of SRB15T$^+$; DNA from BR151T$^+$spc
TR21	*lys-3 metB10 fun strR rpr-21 (tr21)*	(20)
TR21c	*lys-3 cysA14 fun strR rpr-21*	Cys$^-$ transformant of TR21, by congression; DNA from QB944
BR21	*lys-3 rpr-21*	Cys$^+$Str^s transductant of TR21c; BR151T$^+$ as donor
BR21strR	*lys-3 strR rpr-21*	Cys$^+$ transductant of TR21c; BR151T$^+$ as donor
BR21strRspcR	*lys-3 strR rpsE rpr-21*	Spc^r transformant of BR21strR; DNA from BR151T$^+$spc
BR21c	*cysA14 rpr-21*	Cys$^-$ transformant of BR21, by congression; DNA from QB944

of transformants and transductants (Henkin and Chambliss, in press). Ribosomal subunits were prepared according to Cannon and Bott (6), with the modifications described in Henkin et al. (20).

Polyacrylamide gel electrophoresis. The procedure of Adoutte-Panvier et al. (1) was employed for one-dimensional sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, with minor modifications (20). Gels contained a 12 to 20% linear gradient of acrylamide and 0.2% SDS (specially purified; BDH). Molecular weight standards were from Pharmacia Fine Chemicals, Inc.

For two-dimensional polyacrylamide gel electrophoresis, ribosomal proteins were extracted with acetic acid and precipitated with acetone (2). The gel techniques of Howard and Traut (23) were used to examine basic 70S ribosomal proteins.

Polyacrylamide gel electrophoresis of ribosomal proteins purified by gel electrophoresis was performed as described by Cleveland et al. (10) by using protein spots containing ca. 10 μg of protein excised from two-dimensional gels stained with Coomassie blue G250. Gel spots were equilibrated in 5 ml of buffer containing 0.125 M Tris-hydrochloride (pH 6.8), 0.1% SDS, and 1 mM EDTA for 30 min at room temperature. One-dimensional SDS-polyacrylamide gradient gels (1.5 mm thick) were prepared as above, with 1 mM EDTA in the stacking gel solution and electrode buffer and with longer stacking gels and wider wells (5 mm). Gel pieces were inserted into the wells with a spatula; more even bands were obtained when the gel pieces were broken into small fragments in the well. Electrophoresis was performed as described above, and the gels were stained and destained as usual. Either freshly stained and destained gels or gels which had been vacuum dried onto filter paper were suitable sources of protein for this procedure. In the case of dried gels, excised spots were rehydrated in distilled water for 15 min, after which they were easily lifted from the filter paper backing. The gel pieces were then treated as described above.

In vitro protein synthesis. Cell extracts were prepared as described in Chambliss et al. (9). Initiation factors and high-speed supernatant were from strain BR151. The in vitro protein synthesis assay measured incorporation of [^{14}C]phenylalanine (14 cpm/pmol) into hot trichloroacetic acid-insoluble material, with phage SPO1 RNA as template (29).

RESULTS

Growth characteristics of strains. The effects of the streptomycin resistance mutations on growth, sporulation frequency, and streptomycin resistance in vivo are shown in Table 2. Strains containing either the *fun* mutation or the *rpsL1* mutation grew more slowly than did the wild-type strain, but their growth rates were not as severely reduced as was that of strain SRB15T$^+$, which contains *fun* and *strR*. The *fun* and *rpsL1* mutations also exerted a slight inhibitory effect on sporulation, but this effect was much less pronounced than that observed in strain SRB15T$^+$, in which sporulation was reduced to 1% of the wild-type level. Strain BRfunstrA, which contains both *fun* and *rpsL1*, showed a greater reduction in growth rate and sporulation than was observed with either mutation alone, but the sporulation frequency is still 15-fold higher than that of strain SRB15T$^+$.

The *fun* mutation confers resistance to low concentrations of streptomycin (100 μg/ml). When the *rpsL1* mutation, which causes resistance to high levels of streptomycin (>1000 μg/ml), was combined with the *fun* mutation, the

resulting strain (BRfunstrA) exhibited an intermediate resistance phenotype (475 μg/ml). The *strR* mutation in conjunction with the *fun* mutation (strain SRB15T$^+$) showed a much stronger resistance to streptomycin (>1000 μg/ml) than was seen in strain BRfunstrA.

In vitro streptomycin resistance of 70S ribosomes. Purified 70S ribosomes were tested for the ability to translate phage SPO1 mRNA in vitro in the presence of streptomycin (Fig. 1). Activity was expressed relative to that obtained in the absence of streptomycin for each strain to correct for differences between ribosome preparations. Ribosomes from strain BR151T$^+$ were very sensitive to streptomycin; at 5 μg/ml, activity was reduced to 50% of the activity in the absence of streptomycin. Ribosomes from strains SRB15T$^+$, BR151T$^+$strA, and BRfunstrA were all highly resistant, whereas BRfun ribosomes gave an intermediate level of resistance, as was observed in vivo. The reduced resistance of strain BRfunstrA as compared with strains BR151T$^+$strA and SRB15T$^+$ observed in vivo was not detected at the streptomycin concentrations tested in vitro, which were lower than the highest concentrations tested in vivo; higher concentrations of streptomycin caused a slight stimulation of incorporation in vitro by all ribosome preparations, possibly because of the strong cationic nature of streptomycin or perhaps due to increased misincorporation induced by streptomycin.

Genetic mapping of the streptomycin resistance mutations of strain SRB15T$^+$. The map locations of the *strR* and *fun* mutations were compared with that of a standard *strA* allele, *rpsL1*, obtained from I. Smith. Three-factor transduction crosses were used to locate mutations to a general region, and three-factor transformation crosses were used for more precise determinations of map location. The location of the *rpsL1* mutation on the genetic map is well established (16, 18), and our mapping data with this mutation agree with the literature data. The order of markers in this region is: *purA cysA rpoB rpsL efg-10 rplV rpsE* (36). Mutations in the *rpoB* gene cause resistance to rifampin (Rif^r); mutations in *rplV* cause erythromycin resistance (Ery^r); mutations in *rpsE* cause spectinomycin resistance (Spc^r); and the *efg-10* mutation cause elongation factor temperature sensitivity (14). As expected, we found that the *rpsL1* mutation mapped between *rpoB* and *efg-10* (Fig. 2).

Parallel PBS-1 transduction crosses with donors containing *rpsL1* or *fun* indicated that *fun* mapped to the right of *cysA* relative to *purA* in the classic ribosomal region of the *B. subtilis* chromosome (16, 18), as does *rpsL1* (data not shown). The recombination frequency was too low in these crosses to position the mutations relative to *rpsE*. Three-factor transformation crosses were therefore employed, and data from representative crosses are shown in Table 3. Reciprocal crosses gave similar results for gene order and percent linkage (data not shown). The transduction and transformation data were used to generate a genetic map (Fig. 2). Both *rpsL1* and *fun* mapped between *rpoB* and *efg-10*.

TABLE 2. Growth characteristics of strains

Strain	Genotype	Doubling time (min)[a]	Sporulation frequency (%)[b]	Streptomycin MIC (μg/ml)[c]
BR151T$^+$	wild type	45	88	6
SRB15T$^+$	*fun strR*	84	0.9	>1000
BRfun	*fun*	66	20	100
BR151T$^+$strA	*rpsL1*	57	37	>1000
BRfunstrA	*fun rpsL1*	74	13	475

[a] Growth in 2 × NSM.
[b] (Heat-resistant CFU/total CFU) × 100% in 2 × NSM.
[c] Concentration of streptomycin which inhibited growth to 50% of growth in the absence of drug. MIC, minimal inhibitory concentration.

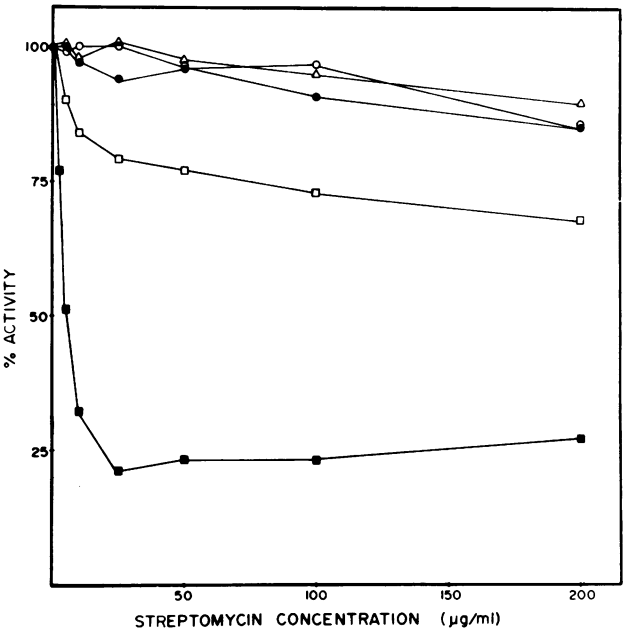

FIG. 1. In vitro streptomycin resistance of 70S ribosomes. Incorporation of [^{14}C]phenylalanine into acid-insoluble material was measured, with phage SPO1 RNA as the template. Percent activity is relative to activity in the absence of streptomycin, which was ca. 300 pmol above endogenous activity for all ribosome preparations. Stimulation by template addition was 10-fold. Symbols: ■, strain BR151T$^+$; ○, strain SRB15T$^+$; □, strain BRfun; △, strain BR151T$^+$strA; ●, strain BRfunstrA.

Comparison mapping of *rpsL1* and *fun*. Although the three-factor cross data suggested that *rpsL1* and *fun* were tightly linked, the relative positions of the two mutations could not yet be determined. To this end, a strain was constructed

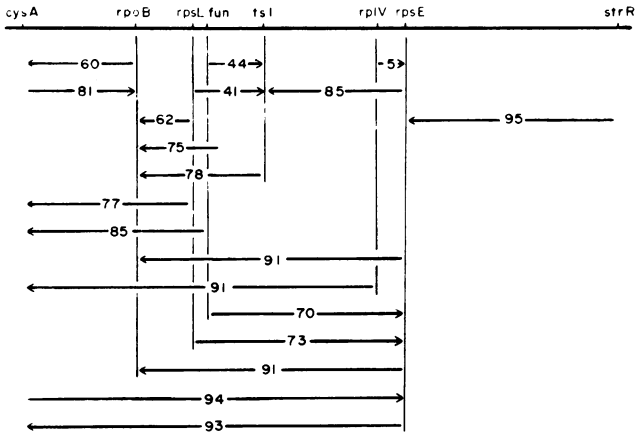

FIG. 2. Transformation map. Numbers are 100 minus the percentage of cotransformation. Arrows point to the selected markers. *tsl* = *efg-10*.

TABLE 3. Three-factor transformation crosses for mapping of *fun* and *rpsL1*

Donor genotype (strain)	Recipient genotype (strain)	Selected phenotype	Recombinant classes			No. of recombinants	Probable order
rpsL1 rpsE (IS26)	*cysA* (QB944)	Cys$^+$	*cysA*	*rpsL1*	*rpsE*		
			1	1	1	11	*cysA rpsL1 rpsE*
			1	0	1	8	
			1	1	0	38	
			1	0	0	170	
fun rpsE (BRfunspc)	*cysA* (QB944)	Cys$^+$	*cysA*	*fun*	*rpsE*		
			1	1	1	4	*cysA fun rpsE*
			1	0	1	9	
			1	1	0	19	
			1	0	0	196	
rpsL1 rpoB (BR151T$^+$strArif)	*cysA* (QB944)	Cys$^+$	*cysA*	*rpoB*	*rpsL1*		
			1	1	1	32	*cysA rpoB fun*
			1	0	1	11	
			1	1	0	49	
			1	0	0	134	
rpsL1 (IS26)	*cysA rplV1* (QB944ery)	Cys$^+$	*cysA*	*rpsL1*	*rplV1*		
			1	1	1	10	*cysA rpsL1 rplV1*
			1	0	1	14	
			1	1	0	27	
			1	0	0	177	
fun (BRfunspc)	*cysA rplV1* (QB944ery)	Cys$^+$	*cysA*	*fun*	*rplV1*		
			1	1	1	10	*cysA fun rplV1*
			1	0	1	9	
			1	1	0	24	
			1	0	0	185	
rpsL1 rpoB (BR151T$^+$strArif)	*efg-10* (IS4)	Ts^r	*efg-10*	*rpsL1*	*rpoB*		
			1	1	1	25	*efg-10 rpsL1 rpoB*
			1	0	1	6	
			1	1	0	39	
			1	0	0	39	
fun rpoB (BRfunrif)	*efg-10* (IS4)	Ts^r	*efg-10*	*fun*	*rpoB*		
			1	1	1	21	*efg-10 fun rpoB*
			1	0	1	2	
			1	1	0	42	
			1	0	0	48	

containing both of these mutations. In constructing this strain, DNA from strain BR151T$^+$strA was used to transform strain BRfun, with selection for resistance to 500 μg/ml streptomycin (to which strain BRfun is sensitive). Most of the transformants obtained exhibited the large colony morphology and wild-type sporulation characteristic of strain BR151T$^+$strA, but a few grew slowly and sporulated less well, as was characteristic of strain BRfun. When ribosomes were isolated from these transformants and screened on one-dimensional SDS-polyacrylamide gels for the *fun*-altered ribosomal protein, it was found that the slow-growing Str^r transformants did contain this protein alteration, whereas the fast-growing Str^r transformants did not. A strain obtained in this way was designated strain BRfunstrA and used in three-factor transformation crosses with *cysA* as the outside marker (Table 4). A derivative of this strain contain-

TABLE 4. Three-factor transformation crosses to order *fun* and *rpsL1*

Donor genotype (strain)	Recipient genotype (strain)	Selected phenotype	Recombinant classes			No. of recombinants	Probable order
fun rpsL1 (BRfunstrA)	*cysA* (QB944)	Cys$^+$	*cysA*	*rpsL1*	*fun*		
			1	1	1	65	*cysA rpsL1 fun*
			1	0	1	1	
			1	1	0	14	
			1	0	0	223	
fun rpsL1 rpsE (BRfunstrAspc)	*cysA* (QB944)	Spc^r	*rpsE*	*fun*	*rpsL1*		
			1	1	1	100	*rpsE fun rpsL1*
			1	0	1	1	
			1	1	0	22	
			1	0	0	274	

ing the *rpsE* mutation was also constructed, so that three-factor crosses with *rpsE* as the outside marker were also possible (Table 4). These data, in conjunction with that described earlier, established the gene order as *cysA rpoB rpsL1 fun efg-10 rplV rpsE*.

Recombination between *rpsL* and *fun*. Since *rpsL1* and *fun* appeared to be very tightly linked, we used the recombination index method (8, 28) to measure the recombination between the two markers, independent of their linkage to any outside marker. In these transformation experiments, saturating DNA concentrations were used, and integration of the marker of interest (*rpsL1* or *fun*) was normalized to that of an unlinked auxotrophic marker (*metB* or *lys-3*). Recombination between the two alleles was indicated by the frequency with which wild-type (Str^s) recombinants were obtained when the donor contained *rpsL1* or *fun* and the recipient contained the opposite allele, relative to that frequency when the donor was wild type (Str^s). The recombination index between *rpsL1* and *fun* was 0.052 when the donor contained *rpsL1* and the recipient contained *fun* and 0.090 in the reciprocal cross (Table 5). No Str^s recombinants were obtained in 1,014 transformants when both donor and recipient contained *rpsL1*. For unlinked markers, the recombination index should be 1.0. Using a set of mutations in the *trpB* gene, Carlton (8) found that two mutations in the same gene generally give a recombination index of less than 0.1. Our data therefore indicate that *rpsL1* and *fun* are in the same or contiguous genes.

Mapping of the *strR* mutation. To map the *strR* mutation of strain $SRB15T^+$, we attempted to move the *strR* mutation to a genetic background which did not contain the *fun* mutation. DNA isolated from strain $SRB15T^+$ was used to transform a wild-type recipient to resistance to 500 μg of streptomycin per ml. However, Str^r transformants were obtained only when high concentrations of transforming DNA were used, and all Str^r transformants obtained were found to contain the altered 30S ribosomal protein associated with the *fun* mutation, suggesting that resistance to high levels of streptomycin requires both *fun* and *strR* or that the *strR* mutation is lethal in the absence of a compensating mutation such as *fun*.

Therefore, preliminary mapping experiments were performed in which both donor and recipient carried the *fun* mutation. Transduction crosses with a Spc^r derivative of strain $SRB15T^+$ as the donor and strain QB944fun (*cysA fun*) as the recipient indicated that the *strR* mutation was located in the ribosomal region but was distal to *rpsE* at a site distinct from *rpsL* (Table 6). From these crosses, the linkage of *strR* to *cysA* was found to be ca. 36%. To construct a fun^+ derivative of strain $SRB15T^+$, we decided to take advantage of the fact that *fun* is 98% linked to *cysA* by transduction, whereas *strR* was only 36% linked to *cysA* and distal to *fun*. To do this, we constructed a derivative of strain $SRB15T^+$ containing the *cysA* marker and transduced it to Cys^+ with transducing lysates grown on strain $BR151T^+$, the wild-type strain. Expected recombinant classes and their phenotypes were fun^+ $strR^+$ (Str^s), *fun strR* (Str^r), *fun* $strR^+$ ($Str^{+/-}$; resistant to 50 μg of streptomycin per ml) and fun^+ *strR* (phenotype unknown). Ribosomal proteins were isolated from recombinants and screened for the *fun*-associated alteration on one-dimensional SDS-polyacrylamide gels to insure that the *fun* allele was scored correctly. Since the *strR* mutation does not confer an alteration in a ribosomal protein detectable on polyacrylamide gels (20), this mutation could not be screened directly.

The results obtained in this cross were anomalous (Table 6), with only three identifiable phenotypic classes of recombinants and higher numbers of Str^s and Str^r recombinants than would be predicted from the linkages of *strR* and *fun* to *cysA* observed previously. Also, all Str^r and $Str^{+/-}$ recombinants screened proved to contain the *fun*-altered protein. One possible explanation for these results is that the fun^+ *strR* recombinants gave a Str^s phenotype and were scored as fun^+ $strR^+$; this would explain the elevated frequency of Str^s recombinants, but it fails to account for the low observed linkage of fun^+ to *cysA* (60% instead of 98%) or the higher than expected frequency of Str^r recombinants (*fun strR*). The fun^+ *strR* recombinants were not scored as Str^r or $Str^{+/-}$ since all recombinants examined from these phenotypic classes contained the *fun*-altered protein, and the elevated frequency of Str^s recombinants is also not explained. An alternative explanation is that fun^+ *strR* recombinants did not survive, in which case one class of recombinants would have been missing, and consequently the frequencies of the surviving classes would appear to be elevated.

To test this hypothesis, we needed to somehow visualize the supposedly missing fun^+ *strR* recombinants by suppressing the putative lethal effect of the *strR* mutation. We therefore introduced the *rpr-21* mutation, which gives an alteration in ribosomal protein S4, suppresses the oligosporogenous phenotype of strain $SRB15T^+$ (20), and maps to a distant site on the chromosome near *aroG* (Henkin and Chambliss, in press). Since this mutation interacts with the *strR* mutation of strain $SRB15T^+$ to somehow alleviate the sporulation defect in this strain, it was considered possible that it might also replace the *fun* mutation in suppressing any lethal effect of the *strR* mutation. The results of this cross (Table 6) indicate that the fun^+ *strR* recombinants were in fact missing from the previous cross but were identifiable in a genetic background containing the *rpr-21* mutation. The fun^+ *strR* and *fun* $strR^+$ recombinants were identified on the basis of colony morphology and examination in one-dimensional SDS gels for the *fun*-altered ribosomal protein. The linkages of *fun* and *strR* to *cysA* and the gene order predicted from three-factor analysis of this cross corresponded to what

TABLE 5. Recombination between *rpsL1* and *fun*

Cross[a]	Donor genotype (strain)	Recipient genotype (strain)	Selected marker	Str^s recombinants	Total recombinants	Frequency
I	*rpsL1* (IS26)	*lys-3 fun* (BRfun)	Lys^+	7	1,819	0.0038
II	$rpsL^+$ ($168T^+$)	*lys-3 fun* (BRfun)	Lys^+	108	1,464	0.0738
III	*fun* (BRfun)	*metB rpsL1* ($BR151T^+$strA)	Met^+	6	1,830	0.0034
IV	fun^+ ($168T^+$)	*metB rpsL1* ($BR151T^+$strA)	Met^+	69	1,837	0.0376

[a] Crosses I and II: recombination index (frequency of Str^s[I]/frequency of Str^s[II]) = 0.052. Crosses III and IV: recombination index (frequency of Str^s [III]/frequency of Str^s[IV]) = 0.090.

TABLE 6. Three-factor transduction crosses for mapping of *strR*

Donor genotype (strain)	Recipient genotype (strain)	Selected phenotype	Recombinant classes			No. of recombinants	Probable order
strR rpsE fun (SRBspc)[a]	*cysA fun* (QB944fun)	Cys$^+$	*cysA*	*rpsE*	*strR*		
			1	1	1	43	*cysA rpsE strR*
			1	0	1	8	
			1	1	0	81	
			1	0	0	9	
strR rpsE rpr-21 (BR21strRspc)[b]	*cysA rpr-21* (BR21c)	Cys$^+$	*cysA*	*rpsE*	*strR*		
			1	1	1	95	*cysA rpsE strR*
			1	0	1	16	
			1	1	0	179	
			1	0	0	13	
strR$^+$ *fun*$^+$ (BR151T$^+$)[c]	*cysA strR fun* (SRBc)	Cys$^+$	*cysA*	*fun*	*strR*		
			1	1	1	58	
			1	0	1	20	
			1	1	0	0?	
			1	0	0	17	
strR$^+$ *fun*$^+$ (BR151T$^+$)[d]	*cysA strR fun rpr-21* (TR21c)	Cys$^+$	*cysA*	*fun*	*strR*		
			1	1	1	37	*cys fun strR*
			1	0	1	15	
			1	1	0	90	
			1	0	0	10	

[a] Cotransduction of *strR* with *cysA*: 50 of 140 (36%).
[b] Cotransduction of *strR* with *cysA*: 111 of 303 (37%).
[c] Cotransduction of *strR*$^+$ with *cysA*: 78 of 95 (82%). Cotransduction of *fun*$^+$ with *cysA*: 58 of 95 (61%).
[d] Cotransduction of *strR*$^+$ with *cysA*: 52 of 152 (34%). Cotransduction of *fun*$^+$ with *cysA*: 100 of 152 (84%).

was observed in the earlier crosses, in which *fun* was present in both donor and recipient for mapping of *strR*. We therefore introduced the *rpr-21* mutation into all strains used in the mapping of the *strR* mutation, so that *strR* could be mapped independent of the presence of the *fun* mutation. The results of these crosses gave the map location indicated in Fig. 2. The *strR* mutation cotransduced with *cysA* with a frequency of 35% (407 of 1,166). Low levels of transductants were obtained in crosses with selection for antibiotic resistance markers; this precluded more precise placement of the *strR* marker, which was too far away from *rpsE* to yield good linkage by transformation (5% cotransformation). The *strR* mutation was unlinked by PBS-1 transduction to *aroI*, the nearest auxotrophic marker distal to *rpsE*.

Polyacrylamide gel electrophoresis of 70S ribosomes. One-dimensional SDS-polyacrylamide gel electrophoresis of 70S ribosomal proteins (Fig. 3) was used to identify the *fun*-altered protein in recombinants in genetic crosses to insure that this allele, normally identified by sporulation phenotype and low-level streptomycin resistance, was properly scored in different genetic backgrounds. Arrow A indicates the *fun*-altered protein, seen in ribosomes isolated from strains BRfun, SRB15T$^+$, and BRfunstrA. No other alterations were seen in ribosomes from strain SRB15T$^+$, which also contains the *strR* mutation. Ribosomes from strain BR151T$^+$strA appeared identical to those from strain BR151T$^+$, the wild-type strain. Arrow B indicates the alteration in protein S4 associated with the *rpr-21* mutation.

Identification of the *fun*-altered protein. Previous studies showed that the *fun* mutation conferred an alteration in a 30S subunit ribosomal protein, detectable on one-dimensional SDS-polyacrylamide gels (20). However, the identity of this protein was not known, since no correlation had been made between bands in the one-dimensional gel system and spots in the two-dimensional system for which a ribosomal protein nomenclature was available (32). We therefore employed a "hybrid" gel system, substituting an SDS-acrylamide gradient gel for the standard urea-acrylamide gel normally used in the second dimension (23). This procedure allowed the identification of a number of bands in the one-dimensional system and was especially useful with 30S subunits, where the overall number of proteins is greatly reduced, and for

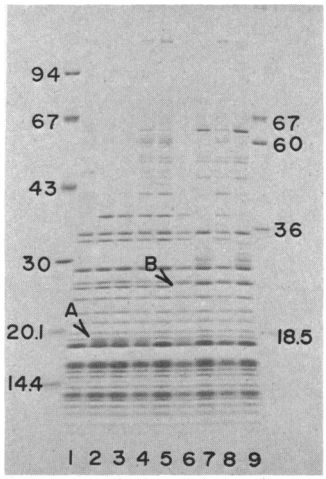

FIG. 3. One-dimensional SDS-polyacrylamide gel electrophoresis of ribosomal proteins. Molecular weight standards are labeled on the outside lanes. Arrows indicate altered ribosomal proteins. Lanes contain ribosomal proteins from strains: BR151T$^+$ (lane 1); SRB15T$^+$ (lane 2); BRfun (lane 3); BRfunstrA (lane 4); BR151T$^+$strA (lane 5); TR21 (lane 6); BR21 (lane 7); BR21strR (lane 8); BR21c (lane 9).

proteins like S12 which show extensive migration in the first dimension of the two-dimensional system and are therefore well separated from the remaining proteins. When the gels of 30S proteins from strains BR151T$^+$ and BRfun were superimposed, all protein spots were matched except for S12, which migrated more slowly in the second (SDS) dimension in gels containing ribosomal proteins from strain BRfun (data not shown). The *fun*-altered ribosomal protein was therefore presumed to be S12.

To confirm this observation, the protein S12 spot excised from two-dimensional polyacrylamide gels of BR151T$^+$ and BRfun ribosomes was used as a source of wild-type and mutant S12 protein. The migration of purified S12 could then be compared with that of the *fun*-altered protein in total 30S ribosomal proteins in one-dimensional SDS-polyacrylamide gels. Lanes 1 and 2 in Fig. 4 contain 30S ribosomal proteins from strains BR151T$^+$ and BRfun. The next two lanes contain protein S12 from strain BR151T$^+$ and BRfun, obtained directly from the S12 spot of two-dimensional polyacrylamide gels of ribosomal proteins from these strains. The BR151T$^+$ and BRfun S12 proteins migrated to the positions of the *fun*-altered protein observed in unfractionated 30S ribosomal proteins of these strains. The S12 band from strain BRfun shows the shift in migration characteristic of the *fun* alteration, confirming that S12 is the *fun*-altered ribosomal protein.

DISCUSSION

Strain SRB15T$^+$, a streptomycin-resistant, oligosporogenous mutant of *B. subtilis* isolated and characterized in our laboratory (5), was found to contain two mutations involved in the streptomycin resistance phenotype. Preliminary results indicated that both these mutations mapped to the *cysA*-linked ribosomal region of the *B. subtilis* chromosome (16, 18), in which *rpsL*, the classic streptomycin resistance gene, is located. We therefore mapped the two mutations of strain SRB15T$^+$ relative to *rpsL1*, a standard allele of the *rpsL* gene, and to other genes in that region for which the map location was already established, to determine if either of these new mutations affecting resistance to streptomycin was located in the *rpsL* gene.

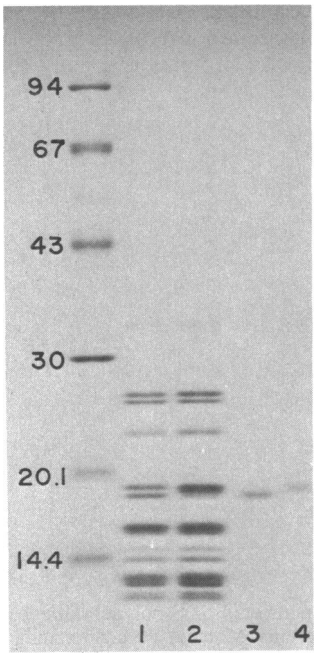

FIG. 4. One-dimensional SDS-polyacrylamide gel electrophoresis of ribosomal protein S12 purified from gel pieces. Lane 1, BR151T$^+$ 30S proteins; lane 2, BRfun 30S proteins; lane 3, BR151T$^+$ S12 protein; lane 4, BRfun S12 protein.

Data from three-factor genetic crosses showed that the *fun* mutation was very tightly linked to *rpsL1*, whereas the *strR* mutation, though in the same region of the chromosome, mapped some distance away, with several known ribosomal genes intervening. The *strR* mutation was located in a region that is not as well characterized as the region near *rpsL*, and fewer markers were available for use in genetic mapping. The location of *strR* is therefore less well defined than the location of *fun*, but *strR* is distal to *rpsE*, although unlinked to *aroI* by PBS-1 transduction.

Our genetic data indicated that the *fun* and *rspL1* mutations were in the same or continuous genes. Since *rpsL1* was known to affect ribosomal protein S12 and the *fun* mutation caused an alteration in the 30S protein in a gel system for which band identities were not yet known, we identified the *fun*-altered band by correlating it with migration in a gel system for which protein identities have been established (32). The *fun*-altered ribosomal protein was found to be S12, suggesting that the *fun* mutation is an allele of the *rpsL* gene.

The *fun* mutation causes a shift in protein S12 in SDS-polyacrylamide gel electrophoresis, whereas the *rpsL1* mutation does not. The two mutations caused different effects on growth and sporulation, and strains containing *rpsL1* were resistant to much higher concentrations of streptomycin in vivo and in the cell-free translation of natural mRNA. It was also possible to construct a strain containing both the *fun* and *rpsL1* mutations, and this strain exhibited a growth and streptomycin resistance phenotype distinct from that seen with either mutation alone. The *fun* mutation is therefore considered to be a new allele of the *rpsL* gene, which we designate *rpsL2*.

The nature of the *strR* mutation and its effect on sporulation is less clear. The mutation maps in a region of the chromosome in which a large number of ribosomal genes are located (16, 18). The high level of streptomycin resistance exhibited by strains carrying *strR* and *fun*, but not *fun* alone, has been shown to be conferred by the 30S ribosomal subunit (20). However, no ribosomal protein alteration has been found to be associated with the *strR* mutation. Studies of the physiological effects of the *strR* mutation have been hampered by our inability to isolate the *strR* mutation in a wild-type background; cells containing this mutation are apparently unable to survive unless the *fun* mutation, which causes an alteration in protein S12, or the *rpr-21* mutation, which causes an alteration in protein S4, is also present. Different ribosomal mutations have been found to interact in effects on translational fidelity (4, 17, 18, 31, 34, 41), the suppression of antibiotic dependence (3, 19, 25, 26), and suppression of conditional and unconditional asporogeny in *B. subtilis* (6, 20). In this case, the *fun* and *rpr-21* mutations appear to suppress the lethal effect of the *strR* mutation. The *fun* mutation apparently does not completely suppress the ill effects of the *strR* mutation, however, since strain SRB15T$^+$, which contains these two mutations, exhibits a much greater defect in sporulation than is observed in strain BRfun, which contains the *fun* mutation alone. Further studies on the effects of these mutations on sporulation in *B. subtilis* and their interaction with other ribosomal protein

mutations which suppress the spore-minus phenotype conferred by *fun* and *strR* (20) are now in progress.

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