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Bacillus subtilis Mutants with Alterations in Ribosomal Protein S4

TINA M. HENKIN,[1]* GLENN H. CHAMBLISS,[2] AND FRANK J. GRUNDY[1]

Department of Biochemistry and Molecular Biology, Louisiana State University Medical Center in Shreveport, Shreveport, Louisiana 71130,[1] and Department of Bacteriology, University of Wisconsin, Madison, Wisconsin 53706[2]

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Two mutants with different alterations in the electrophoretic mobility of ribosomal protein S4 were isolated as spore-plus revertants of a streptomycin-resistant, spore-minus strain of *Bacillus subtilis*. The mutations causing the S4 alterations, designated *rpsD1* and *rpsD2*, were located between the *argGH* and *aroG* genes, at 263° on the *B. subtilis* chromosome, distant from the major ribosomal protein gene cluster at 12°. The mutant *rpsD* alleles were isolated by hybridization using a wild-type *rpsD* probe, and their DNA sequences were determined. The two mutants contained alterations at the same position within the S4-coding sequence, in a region containing a 12-bp tandem duplication; the *rpsD1* allele corresponded to an additional copy of this repeated segment, resulting in the insertion of four amino acids, whereas the *rpsD2* allele corresponded to deletion of one copy of this segment, resulting in the loss of four amino acids. The effects of these mutations, alone and in combination with streptomycin resistance mutations, on growth, sporulation, and streptomycin resistance were analyzed.

A powerful strategy in characterization of the structure and function of the bacterial ribosome is the isolation and analysis of mutants with alterations in ribosomal components. In previous studies, we reported the isolation and characterization of a streptomycin-resistant (Str^r), spore-minus (Spo^-) mutant of *Bacillus subtilis* (4, 14) and second-site revertants in which the Spo^- phenotype was suppressed. Several of these revertants exhibited changes in the electrophoretic mobility of individual ribosomal proteins (13). The original Str^r Spo^- strain contains two mutations, one of which, designated *rpsL2*, affects the 30S subunit ribosomal protein S12; the second, designated *strR*, affects an unidentified 30S subunit component (14). One revertant strain contained an additional mutation that causes an alteration in the electrophoretic mobility of ribosomal protein S4. This mutation, designated *rpsD1*, maps to position 263° on the *B. subtilis* chromosome, between *argGH* (260°) and *aroG* (264°; 23), distant from the major cluster of ribosomal protein genes at 12° (15). Although the *rpsD1* mutation was presumed to be a lesion in the structural gene encoding ribosomal protein S4, it was also possible that the mutation affected a gene involved in the posttranslational modification of S4.

In this study, a second mutant with a different alteration in the electrophoretic mobility of protein S4 was isolated by using a similar selection procedure. Since this mutation presumably causes a different structural alteration in protein S4, analysis of the mutations at the primary structural level would provide strong evidence as to whether they affect the S4 structural gene. We report (i) the isolation and characterization of the second S4 mutant and (ii) the cloning and analysis of both mutant alleles. The results presented show that both mutations do in fact represent alterations in *rpsD*, the gene encoding S4, and provide information about the role of S4 in the ribosome. The effects of the *rpsD* mutations on growth, streptomycin resistance, and sporulation were also examined.

* Corresponding author.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *B. subtilis* strains used are listed in Table 1. Media used for growth of *B. subtilis* were nutrient sporulation medium (NSM) (26); twofold-concentrated NSM (2× NSM), for liquid cultures; and Spizizen minimal medium (2), supplemented with 0.1% glucose and the appropriate auxotrophic requirements at 20 μg/ml. Streptomycin sulfate was purchased from Sigma Chemical Co. and was used at 500 μg/ml for highly resistant strains and 50 μg/ml for strains with low resistance. Growth was measured with a Klett-Summerson colorimeter (no. 66 red filter).

Growth characteristics. Doubling times, frequency of sporulation, and resistance to streptomycin in vivo were determined in 2× NSM as described previously (14). Sporulation frequency was defined as (heat-resistance CFU per milliliter/total CFU per milliliter) × 100%. The streptomycin MIC 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. Spo^+ revertants of strain SRB15T^+ were selected as previously described (13). Isolation of chromosomal DNA, preparation of competent *B. subtilis* cells, and bacteriophage PBS-1-mediated generalized transduction were carried out as previously described (15). All strain constructions involving mutations affecting ribosomal proteins were confirmed by polyacrylamide gel electrophoresis of isolated ribosomal proteins. Recombination index transformation experiments (5, 18) used saturating DNA concentrations and recombination between *rpsD* alleles was normalized to that of an unlinked auxotrophic marker (*metB* or *lys-3*).

Ribosome preparation and polyacrylamide gel electrophoresis. Ribosomes were isolated from exponential-growth-phase cultures as previously described (15). One-dimensional sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed as described by Adoutte-Panvier et al. (1), using a 12 to 20% linear gradient of acrylamide and 0.2% SDS (BDH). Molecular weight standards were from Pharmacia Fine Chemicals. For two-dimensional polyacrylamide gel electrophoresis, ribosomal pro-

TABLE 1. Bacterial strains

Strain	Genotype	Origin or reference
BR151T$^+$	*lys-3 metB10*	16
SRB15T$^+$	*lys-3 metB10 strR rpsL2*	16
TR21	*lys-3 metB10 strR rpsL2 rpsD1*	16
TR3A	*lys-3 metB10 strR rpsL2 rpsD2*	Spo$^+$ revertant of SRB15T$^+$
TR21c	*lys-3 cysA14 strR rpsL2 rpsD1*	17
TR3Ac	*lys-3 cysA14 strR rpsL2 rpsD2*	Cys$^-$ transformant of TR21, by congression; DNA from QB944
BRfun	*lys-3 rpsL2*	16
BR21	*lys-3 rpsD1*	17
BR3A	*lys-3 rpsD2*	Cys$^+$ Str^s transductant of TR3Ac; BR151T$^+$ as donor
BR21fun	*lys-3 rpsD1 rpsL2*	Cys$^+$ Str^s transductant of TR21c; BRfun as donor
BR3Afun	*lys-3 rpsD2 rpsL2*	Cys$^+$ Str^s transductant of TR3Ac; BRfun as donor
BR21strR	*lys-3 rpsD1 strR*	17
BR3AstrR	*lys-3 rpsD2 strR*	Cys$^+$ transductant of TR21c; BR151T$^+$ as donor
QB944	*cysA14 purA16 trpC2*	F. Kunst
1A92	*arg(GH)2 aroG932 bioB141 sacA321*	Bacillus Genetic Stock Center

teins were extracted with acetic acid, precipitated with acetone, electrophoresed, and stained with Coomassie blue as described previously (15).

Cloning techniques. Restriction endonucleases and DNA-modifying enzymes were purchased from New England BioLabs and used as instructed by the manufacturer. Techniques for cloning in bacteriophage M13 vectors were as described by Messing (21). *B. subtilis* chromosomal DNA was digested to completion with *Sau*3A1, and the 1.0-kb-size fraction was isolated after electrophoresis through low-melting-temperature agarose (SeaPlaque GTG; FMC Bioproducts) and inserted into bacteriophage M13mp18 replicative-form DNA digested with *Bam*HI. Plaque lifts onto nitrocellulose filters (BA85; Schleicher & Schuell) were carried out under nondenaturing conditions for single-stranded DNA transfer (32). Hybridization was done as previously described, using a 5'-end-labeled oligonucleotide designed according to the wild-type *rpsD* gene sequence (12). Recombinant phage M13 DNA was prepared (21), and DNA sequencing was carried out by using Sequenase (United States Biochemical) as described by the manufacturer; an M13 primer (New England BioLabs) and synthetic oligonucleotide primers (The DNA Factory) were used.

RESULTS

Isolation of a mutant with a new alteration in protein S4. Initial characterization of mutants isolated as Spo$^+$ revertants of a Str^r Spo$^-$ mutant of *B. subtilis*, strain SRB15T$^+$, showed that several retained the streptomycin resistance mutations of the parent strain but had acquired one of several additional mutations; in some cases, these additional mutations resulted in alterations in the electrophoretic mobility of individual ribosomal proteins (13). One of these mutations, initially designated *rpr-21* (14) and now renamed *rpsD1*, results in an alteration of protein S4; this mutation was mapped to position 263° on the *B. subtilis* chromosome (15). Other revertants contained an alteration in ribosomal protein S17 or BL18.

In this study, additional revertants were isolated and characterized according to previously described methods (13). Of 90 revertants examined, 33 exhibited ribosomal protein alterations detectable in one-dimensionsal SDS-polyacrylamide gels. Of these, 6 showed an alteration in protein S4 indistinguishable from that previously described, and 15 showed a different alteration in protein S4. Three mutants had an alteration in protein S5, whereas the remainder with detectable alterations appeared identical to those previously observed in proteins S17 and BL18 and were not characterized further.

The electrophoretic mobility of protein S4 in the new class of mutants was markedly different from that in the original S4 mutants. The original *rpsD1* mutation caused an apparent increase in the molecular weight of protein S4 on SDS-polyacrylamide gels (Fig. 1); in two-dimensional polyacrylamide gel electrophoresis (Figure 2), the S4 protein exhibited a more basic charge. The new S4 mutation, designated *rpsD2*, caused an apparent decrease in molecular weight in SDS-polyacrylamide gel electrophoresis (Fig. 1) and a decrease in charge (Fig. 2).

Genetic analysis of the *rpsD2* mutation. The *rpsD1* mutation was mapped to a position near 263°, between the *argGH* (formerly known as *argA*) and *aroG* loci (15). Similar genetic analyses performed with the *rpsD2* mutation gave the same gene order, i.e., *argGH-rpsD2-aroG* (data not shown). In addition, the linkages obtained were very similar to those obtained with *rpsD1* (20% cotransformation with *argGH*, 70% cotransformation with *aroG*). It therefore appeared that the *rpsD1* and *rpsD2* mutations were located very close to each other.

Recombination index experiments (5, 18) were used to measure the recombination frequency between the two mutant alleles. No wild-type recombinants generated by recombination between *rpsD1* and *rpsD2* were detected among 4,000 transformants under conditions in which the wild-type allele gave 0.5% recombination. The calculated recombination index was therefore <0.05, where a recombination index of less than 0.1 indicates that two mutations are in the same or contiguous genes (5). These results indicate that the two mutations very likely represent alterations in the same gene. The identification of two mutations in one locus causing markedly different alterations in protein S4 strongly suggests that the mutations lie in the structural gene for protein S4, *rpsD*.

Sequence analysis of the *rpsD1* and *rpsD2* mutations. Clones containing a 1.0-kb *Sau*3A1 fragment of chromosomal DNA from the mutant strains inserted in phage M13mp18 were identified by hybridization, using an oligonucleotide probe derived from the wild-type *rpsD* sequence (12). DNA sequence analysis showed that the *rpsD1* allele had a small duplication at nucleotide positions 222 to 233 (Fig. 3), resulting in the insertion of 12 bp in the DNA sequence and four amino acids (Ala-Gly-Lys-Leu) in the gene product. This change would therefore explain the observed increase in molecular weight and basic charge (due to the extra lysine residue). DNA sequence analysis of the *rpsD2* allele revealed a deletion of 12 bp at precisely the same position as the insertion in *rpsD1*, resulting in the loss of four amino acids (Ala-Gly-Lys-Leu) from the protein. This change is also consistent with the observed behavior of the mutant protein in polyacrylamide gel electrophoresis (reduction in molecular weight and loss of one basic residue). Inspection

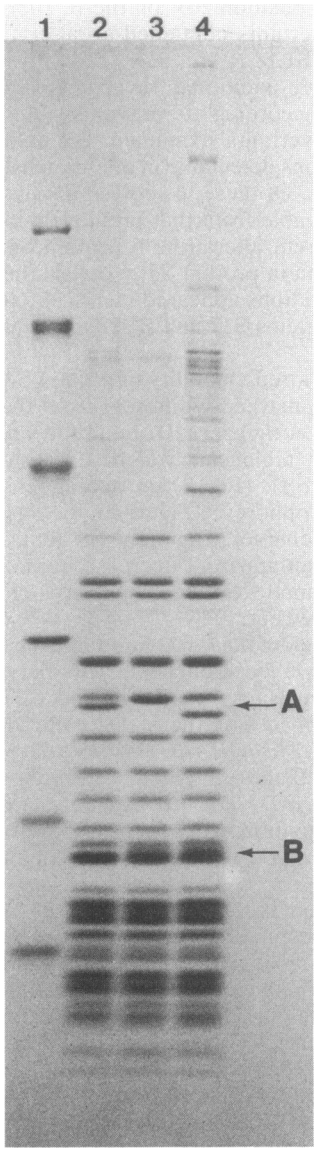

FIG. 1. One-dimensional SDS-polyacrylamede gel electrophoresis of 70S ribosomal proteins. Molecular size standards in lane 1 are 94, 67, 43, 30, 20.1, and 14.4 kDa. Arrow A indicates protein S4 (altered by the *rpsD1* and *rpsD2* mutations); arrow B indicates protein S12 (altered by the *rpsL2* mutation). Lane 2, BR151T$^+$ (wild-type); lane 3, TR21 (*rpsD1 rpsL2 strR*); lane 3, TR3A (*rpsD2 rpsL2 strR*).

of the DNA sequence in the region of the insertion-deletion revealed a 12-bp tandem repeat, with 10-of-12-bp identity. A likely mechanism for the generation of both mutant alleles is by slipped-strand mispairing during DNA replication (10). The proportion of deletion versus duplication mutants obtained may reflect the frequency with which the template or nascent strand slipped or may have resulted from a bias in the selection procedure.

No other differences in the DNA sequence between the wild-type and mutant alleles were found except for a deletion of 1 bp in a region of *rpsD2* upstream of the S4-coding sequence, within the leader sequence (12). This residue is located in an unpaired region, according to structural models of the leader region.

Growth characteristics of strains containing *rpsD* mutations. The *rpsD1* and *rpsD2* mutations result in the duplication or deletion of four amino acids within a region of the protein showing high conservation with the *E. coli* S4 protein (12). This region of the protein has been shown to be essential for binding of protein S4 to 16S rRNA in *Escherichia coli* (6, 7). It was therefore of interest to examine the effect of the *rpsD* mutations on the growth properties of the cell. The effects of the *rpsD* mutations on streptomycin resistance and sporulation were also examined, since the mutations were isolated as Spo$^+$ suppressors of the Str^r Spo$^-$ strain SRB15T$^+$, which contains two mutations (*rpsL2* and *strR*) involved in streptomycin resistance.

Cells of strain SRB15T$^+$ grew slowly and sporulated at 1% of the level observed in cultures of the wild-type strain (BR151T$^+$) and were resistant to high concentrations of streptomycin (>1,000 μg/ml) (Table 2). The *rpsL2* mutation alone (strain BRfun) conferred resistance to low concentrations of streptomycin (30 μg/ml) and had a moderate effect on growth and sporulation. The effects of the *strR* mutation alone could not be determined, since it was not possible to construct strains of this type, presumably because the *strR* mutation is lethal in the absence of a compensatory mutation (14). Introduction of *rpsD* mutations into strain SRB15T$^+$ (yielding strains TR21 and TR3A, containing *rpsD1* and *rpsD2*, respectively) resulted in restoration of both rapid growth and sporulation and partially reduced streptomycin resistance. Strains containing either *rpsD* mutation alone (BR21 for *rpsD1*, BR3A for *rpsD2*) closely resembled the wild-type strain in growth and sporulation. Growth in defined media was also unaffected (data not shown). These strains reproducibly exhibited a slight hypersensitivity to streptomycin. This phenotype in *E. coli* has been associated with alterations in protein S4 (25), but the effect in strains BR21 and BR3A was very slight. Thus, in general, the alterations in S4 resulting from these mutations did not have a major effect on *B. subtilis* viability, at least under the conditions tested.

Comparison of strains SRB15T$^+$ (*rpsL2 strR*), BR21strR (*rpsD1 strR*), and BR3AstrR (*rpsD2 strR*) indicates that the *rpsD* mutations were much more effective than the *rpsL2* mutation in suppressing the negative effects of the *strR* mutation, with the *rpsD2* mutation restoring growth to the wild-type rate. Resistance to streptomycin was very much reduced relative to that of SRB15T$^+$, suggesting that the resistance exhibited by SRB15T$^+$ is the result of an interaction between the two streptomycin resistance mutations, *rpsL2* and *strR*. An alternative possibility is that the *rpsD* mutations exert a strong negative effect on streptomycin resistance; however, these mutations had a much smaller effect on the resistance conferred by the *rpsL2* mutation (strains BR21fun and BR3Afun) and little effect in a strain containing *rpsL1* (27), a classical streptomycin resistance mutation conferring an alteration in ribosomal protein S12 but with no effect on sporulation (data not shown).

DISCUSSION

Alterations in proteins S4, S5, S17, and BL18 can suppress the Spo$^-$ phenotype of Str^r Spo$^-$ mutants of *B. subtilis* (13; this work; the equivalence of ribosomal proteins of *B. subtilis* and *E. coli* has been established for 30S proteins [16] but not for all 50S proteins). Two classes of S4 mutants result in different electrophoretic mobilities of protein S4. In this report, we describe a detailed characterization of the two S4 mutants. We have demonstrated that the *rpsD1* and

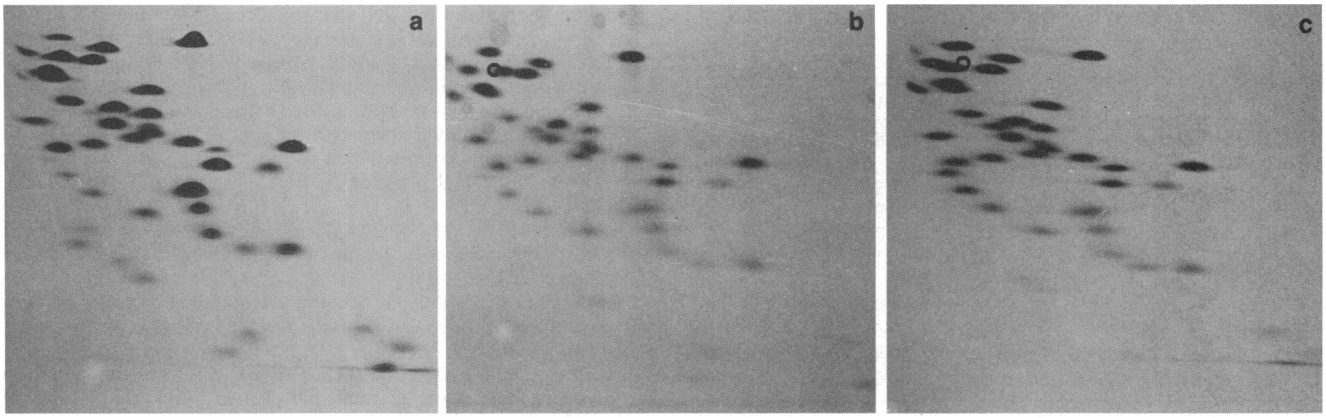

FIG. 2. Two-dimensional polyacrylamide gel electrophoresis of basic 70S ribosomal proteins. (a) BR151T$^+$ (wild type); (b) TR21 (*rpsD1 rpsL2 strR*); (c) TR3A (*rpsD2 rpsL2 strR*). Circles in panels b and c indicate the wild-type position of protein S4.

rpsD2 mutations both involve lesions in the structural gene for protein S4.

The two mutant *rpsD* alleles represent changes at the same position of the protein, within a region homologous to that defined as the 16S rRNA-binding domain in *E. coli* S4 (residues 47 to 103; 6, 7). The two mutant alleles have inverse changes in this region of the protein; the *rpsD1* mutation results in the insertion of four amino acids, whereas the *rpsD2* mutation results in the deletion of four amino acids. It is somewhat surprising that mutations resulting in such different protein alterations are so similar in their effects on suppression of the Spo$^-$ phenotype of strain SRB15T$^+$ and the lethal effect of the *strR* mutation. One interpretation of these results is that the region of the protein altered in both mutants represents a key region for the interaction of protein S4 with the ribosomal structure altered by the *strR* mutation. The wild-type S4 protein does not permit the formation of functional ribosomes containing the *strR*-altered component; mutations of S4 which increase, or decrease, the size or charge of this domain restore ribosomal function in the presence of the *strR* mutation. The interaction between wild-type S4 and the *strR*-altered component could be tighter than the normal interaction, and alterations in this region of the protein may allow an enhanced binding arrangement. Further analysis of the *strR* mutation is necessary for a better understanding of the interactions of these components in the *B. subtilis* ribosome.

Since the *rpsD* mutants were originally isolated on the basis of their effect on the sporulation of strain SRB15T$^+$, this effect was examined in more detail, using strains with various combinations of the *rpsL2*, *strR*, and *rpsD* mutations. The *rpsD* mutations ameliorated both the growth and sporulation defects caused by the *strR* mutation. These mutations in an otherwise wild-type background resulted in little effect on cell viability, at least under the conditions tested. Apparently, certain changes in the structure in this region of the S4 protein can be tolerated without dramatic effect.

The nature of the *strR* mutation is not yet known. No ribosomal protein alteration has been associated with this mutation by either one-dimensional SDS or two-dimensional polyacrylamide gel electrophoresis (13, 14). This of course does not mean that no protein is altered, but that any alteration which does exist does not affect protein migration in either gel system. The resistance phenotype is known to be associated with the 30S ribosomal subunit; subunit mixing experiments showed that the source of the 30S subunit determined resistance to streptomycin in an in vitro translation assay (14). The *strR* mutation maps to the far end of the ribosomal gene cluster near 12°, since it is only 5% linked by transformation to *rpsE*, which is near the end of the *spc* operon. Genes known to be immediately downstream from *spc* are the α operon genes (3, 31), the gene for ribosomal proteins S9 (8) and S20 (22), the attachment site for phage SPO2 (23), *gerD* (33), and three rRNA operons, *rrnI*, *rrnH*, and *rrnG* (17). The low transformation linkage with *rpsE* suggests that the location of *strR* is downstream of α, which is contiguous with *spc*, and probably downstream of the genes for S9 and S20, which show higher linkage to *rpsE*. The *strR* mutation could affect genes known to be located in

```
rpsD1   ...TTGTTTGACAAAGCTGGCAAACTAGCTGGCAAACTAGCTGGTAAACACGGTGAA...
        ... L  F  D  K  A  G  K  L  A  G  K  L  A  G  K  H  G  E ...

wild-type ...TTGTTTGACAAA------GCTGGCAAACTA------GCTGGTAAACACGGTGAA...
          ... L  F  D  K  -  -  A  G  K  L  -  -  A  G  K  H  G  E ...
                                   |                    |
                                  70                   75                   80

rpsD2   ...TTGTTTGACAAA------------------------GCTGGTAAACACGGTGAA...
        ... L  F  D  K  -  -  -  -  -  -  -  -  A  G  K  H  G  E ...
```

FIG. 3. Sequence of the *B. subtilis rpsD* mutants. The region corresponding to nucleotide positions 210 to 251 of the S4-coding region is shown. The 12-bp sequence at positions 222 to 233 (overlined) is the position of the *rpsD1* duplication and the *rpsD2* deletion events. The predicted amino acid sequence of the *rpsD1* and *rpsD2* variants of protein S4 is shown below the DNA sequence. Dashes represent spacers used for sequence alignment.

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$^+$	*rpsL2 strR*	84	0.9	>1,000
BRfun	*rpsL2*	66	20	100
BR21	*rpsD1*	48	95	3
BR3A	*rpsD2*	46	77	3
BR21fun	*rpsD1 rpsL2*	52	11	25
BR3Afun	*rpsD2 rpsL2*	53	6	18
BR21strR	*rpsD1 strR*	65	94	30
BR3AstrR	*rpsD2 strR*	48	51	20
TR21	*rpsD1 rpsL2 strR*	51	89	440
TR3A	*rpsD2 rpsL2 strR*	51	65	300

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

this region or other genes not yet identified. It is possible that *strR* is a mutation in one of the rRNA operons located in this region. Mutations in 16S rRNA that cause streptomycin resistance have been found (11, 20); in general, the resistance phenotype is observed only when the mutant *rrn* operon is overexpressed, since there are 7 copies of *rrn* operons in *E. coli* (9) and 10 in *B. subtilis* (19, 30). If the *strR* mutation is an alteration in one of the *rrn* operons located in this region, then this operon must be expressed at a much higher level than other *rrn* operons.

Recently, dominant lethal mutations at position 530 of *E. coli* 16S rRNA were identified (24). The lethal effect is presumed to occur because ribosomes containing the mutated rRNA compete with wild-type ribosomes in the cell for other components necessary for translation, such as initiation sites on mRNA, by forming nonfunctional initiation complexes. The region of the rRNA altered in these G-530 mutants is located very close to the site that can be mutated to give streptomycin resistance and is within a region known to interact with proteins S4, S5, and S12 (28, 29). The absence of a protein alteration associated with the *B. subtilis strR* mutation, the map location of *strR*, its apparent lethality, and the suppression of its effects by mutations altering proteins S4, S5, and S12 suggest the possibility that the *strR* mutation is similar in type to the *E. coli* G-530 mutation. The slow growth and sporulation defect in strain SRB15T$^+$ may reflect incomplete suppression of *strR* by the *rpsL2* mutation; it is possible that certain mRNA species essential for the initiation of sporulation are present in limited amounts and that formation of nonfunctional translation initiation complexes prevents sporulation in strain SRB15T$^+$. Apparently, the *rpsD* mutations more successfully restore ribosome function. Further analysis of the *strR* mutation and the interactions among the altered ribosomal components will provide valuable information about the role of these components in ribosome function during growth and sporulation.

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