The *rpsD* Gene, Encoding Ribosomal Protein S4, Is Autogenously Regulated in *Bacillus subtilis*

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Although the mechanisms for regulation of ribosomal protein gene expression have been established for gram-negative bacteria such as *Escherichia coli*, the regulation of these genes in gram-positive bacteria such as *Bacillus subtilis* has not yet been characterized. In this study, the *B. subtilis rpsD* gene, encoding ribosomal protein S4, was found to be subject to autogenous control. In *E. coli*, *rpsD* is located in the α operon, and S4 acts as the translational regulator for α operon expression, binding to a target site in the α operon mRNA. The target site for repression of *B. subtilis rpsD* by protein S4 was localized by deletion and oligonucleotide-directed mutagenesis to the leader region of the monocistronic *rpsD* gene. The *B. subtilis rpsD* leader exhibits little sequence homology to the *E. coli* α operon leader but may be able to form a pseudoknot-like structure similar to that found in *E. coli*.

The regulation of ribosomal protein biosynthesis has been characterized in depth in the gram-negative bacterium *Escherichia coli* (for a review, see reference 15) and has been found in a number of cases to involve a negative feedback mechanism in which certain ribosomal proteins act as autogenous repressors, controlling the expression of the operon in which they are encoded (4, 29). The regulatory ribosomal proteins are all proteins which bind to rRNA early in ribosomal assembly; when rRNA levels are low, the regulatory protein binds instead to a specific target site in the ribosomal protein operon mRNA. Binding of the repressor protein to the mRNA results in turn-off of expression of the operon. The mechanism of inhibition varies in different operons and can include inhibition of translation initiation, targeting of the mRNA for degradation, and transcription attenuation. This regulatory system permits stoichiometric synthesis of ribosomal proteins and maintenance of the balance of ribosomal protein and rRNA levels.

Although some information has been collected about the organization of ribosomal protein genes in bacterial species other than *E. coli*, very little is known about the regulation of expression of these genes. Analysis of the mechanisms controlling the expression of ribosomal protein genes in evolutionary divergent systems will provide valuable information about the biological significance of these regulatory mechanisms. *Bacillus subtilis* is a gram-positive, spore-forming soil bacterium which is evolutionarily distant from *E. coli* (time of divergence estimated at 10⁸ years [12]). Given the availability of tools for genetic analysis and the information that is already known about the organization of ribosomal determinants, *B. subtilis* is highly suitable for such a study.

A large number of ribosomal protein genes have been identified in *B. subtilis*; in general, their organizational patterns are similar to those found in *E. coli*. The most intriguing difference in the organization of ribosomal protein genes between *B. subtilis* and *E. coli* is the location of the *rpsD* gene, encoding protein S4. In *E. coli*, *rpsD* is the third gene in the α operon, which also contains *rpsM*, *rpsK*, and *rpoA* (ab subunit of RNA polymerase), and *rpQ* (1). Protein S4, encoded by *rpsD*, acts as the translational repressor controlling α operon expression (14). In *B. subtilis*, the organization of the α operon is similar except that *rpsD* is located at position 263° on the 360° chromosomal map (9), distant from the remainder of the α operon genes, which are at 12° (3). The *B. subtilis rpsD* gene has been cloned, and its predicted product is 50% identical to *E. coli* S4 in amino acid sequence (8). Transcriptional analysis indicated that *rpsD* is monocistronic and that the coding region is preceded by a leader region 182 bases in length. It is therefore of interest to determine whether the difference in gene organization in these species also reflects a difference in gene regulation. This study presents an initial examination of the mechanisms regulating *rpsD* expression in *B. subtilis* and to our knowledge represents the first analysis of ribosomal protein gene regulation in a gram-positive bacterium. We find that *rpsD* is autogenously regulated in *B. subtilis* and that the target site for regulation is the leader region preceding the *rpsD* coding sequence.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** The bacterial strains used are listed in Table 1. Cultures of *B. subtilis* and *E. coli* were routinely grown in L broth; for propagation of bacteriophage λ, MgSO₄ was added to a final concentration of 10 mM. Derivatives of bacteriophage M13 were propagated in strain JM103. Plasmids were routinely propagated in strain DH5α. Phage λRZ5 (21) was obtained from C. Turnbough. Ampicillin was used at 50 μg/ml for high-copy-number plasmids and at 10 μg/ml for λ lysogens; kanamycin was used at 25 μg/ml for *E. coli* strains carrying derivatives of plasmid pLG338; chloramphenicol and neomycin were used at 5 μg/ml for *B. subtilis* strains containing single-copy insertions; erythromycin and lincomycin were used at 1 and 25 μg/ml, respectively, for selection for the Tn917-derived *erm* gene; chloramphenicol and erythromycin were used at 0.1 μg/ml for induction of the *cat* and *erm* genes, respectively. All antibiotics were purchased from Sigma Chemical Co. β-Galactosidase production was detected by the addition of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyra-
noside; Gold Biotechnologies) to solid media at a concentration of 40 µg/ml. Induction of the lac promoter was carried out by the addition of IPTG (isopropyl-β-D-thiogalactoside; Sigma Chemical Co.) to a final concentration of 1 mM. Amylase production was tested qualitatively on solid media containing potato starch (1%, w/vol) by halo production after staining with KI-I₂ solution.

**Bacteriophage and plasmids.** A 1.0-kb Sau3A1 fragment containing the entire rpsD gene, including sequences 134 bp upstream of the transcription start site and 59 bp downstream of the transcription terminator (Fig. 1A), was isolated from phage ARc2-1 (8) and inserted into bacteriophage M13 mp18 digested with BamH1, generating M13-C1. A HindIII synthetic oligonucleotide linker (New England BioLabs, Beverly, Mass.) was inserted at the Smal site upstream of the rpsD insert, generating M13-C1H. A neo gene cassette was isolated as a 1.3-kb EcoRI fragment from plasmid pBEST502 (13), obtained from P. Zuber, and inserted into the EcoRI site of M13-C1H, generating M13-C1neo. Similar constructs containing the Pst-1 leader mutation, designated M13-Pneo, and the Pst-1 leader mutation plus the rpsD2 allele of S4, designated M13-3APneo, were also generated.

Plasmid pTMH115, which was used to direct integration of a small segment of phage M13 DNA into the amyE locus of the *B. subtilis* genome, was derived from plasmid pDEB844 (2), obtained from A. L. Sonenshein; pDEB844 is a derivative of the *amy* insertion vector pTRPBG1 (22). The 4.4-kb EcoRI-BamH1 fragment of pDEB844, containing *glt* sequences, was replaced with the 0.65-kb EcoRI-BglII fragment of M13 mp18. This plasmid also carries a *cat* gene selectable in *B. subtilis* and two segments of the *amyE* gene. Plasmid pTMH123 is unable to replicate in *B. subtilis*, so selection for *Cm* transformants results in isolates in which plasmid sequences have integrated at the *amyE* locus by homologous recombination; *Cm* *Amy*⁻ transformants arise by replacement of the central portion of the chromosomal *amyE* gene with the *cat* and M13 sequences.

Plasmid pTMH115 is a derivative of plasmid pLG338, a low-copy-number *E. coli* plasmid carrying the pSC101 replicon and a selectable kanamycin resistance gene (24). A 0.75-kb TaqI-PstI fragment containing *rpsD* sequences from the *tacl* site at position +123 relative to the transcription start point (60 bp upstream from the AUG translational start codon) to the Sau3A1 site past the end of the S4 coding region was inserted into plasmid pGEM7Z(+) (Promega) between the ClaI and NsiI sites. A 205-bp EcoRI fragment containing the *lacUV5* promoter variant, which gives a fourfold increase in expression over the *lacUV5* promoter in *E. coli* (11), was isolated from plasmid pTMH35 and inserted upstream of *rpsD*; the orientation of the promoter fragment was tested by using an asymmetric *PvuII* site within the *lacUV5* fragment. The *lac*-*rpsD* construct was removed as an *XhoI*- *SalI* fragment and inserted into *SalI*-digested pLG338, generating plasmid pTMH115. In this construct, the *rpsD* promoter and leader region have been replaced by the lac promoter, so that expression of *rpsD* is now under *lac* control.

Plasmid pTMH123 was constructed by insertion of the 480-bp HindIII fragment from M13-C1H, containing the promoter and amino-terminal portion of *rpsD*, into plasmid pZΔ329 (29a), to generate an *rpsD-lacZ* translational fusion. We generated similar constructs containing various leader region point mutations, obtained by oligonucleotide-directed mutagenesis, and deletions, constructed as derivatives of point mutants containing new *XbaI* restriction sites. The sites of the sequence alterations are shown in Fig. 2.

Fusions were introduced into phage XRZ5 (21) by homologous recombination between the phage, which contains portions of pBR322 and *lacZ* sequences, and plasmid pTMH123 (wild-type leader) and its derivatives pTMH131

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source or reference</th>
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<tbody>
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<td><strong>B. subtilis</strong></td>
<td></td>
<td></td>
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<tr>
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<td>lys-3 metB10 trpC2 amyE</td>
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<tr>
<td>168</td>
<td>trpC2</td>
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<td>Met⁺ <em>Amy</em>⁻ transformant of BR151; 168 DNA</td>
</tr>
<tr>
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<td>BR3AMA</td>
<td>rpsD2</td>
<td>Met⁺ <em>Amy</em>⁻ transformant of BR3A; 168 DNA</td>
</tr>
<tr>
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<td>Cm⁺ <em>Amy</em>⁻ transformant of BR151MA; pDEB13 DNA</td>
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<td>P. Zuber; 30</td>
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<td>ZB449</td>
<td>trpC2 pheA1 abrB703; SPβ cured</td>
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<td>DH5α</td>
<td>Δ800lacZΔM15 endA1 recA1 supE44 hsdR17 (rK⁻ mK⁺) thi-1 gyrA96 relA1 Δ(lacZYA-argF)U169</td>
<td>Bethesda Research Laboratories</td>
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(Pst-1 leader mutation) and pTMH137 (leader deletion ΔXba3-2). Lysogens of strain JM109 (recA lacP°) were isolated which produced blue colonies on media containing X-Gal and which were resistant to ampicillin (10 μg/ml). Plasmid pTMH115 (Plac1015-rpsD) was introduced into the lysogens, with selection for kanamycin resistance.

**Strain constructions.** *B. subtilis* strains overproducing S4 were constructed by integration of M13-Pneo into the amyE locus of strain BR151MA (rpsD°) or BR3AMA (rpsD2). A small segment of M13 DNA was first inserted into the amyE gene, by transformation with plasmid pTMH13 with selection for chloramphenicol resistance and screening for loss of amylase production, to provide a region of homology for integration of M13; strains containing the M13-rpsD insertion were then isolated by selection for the Neo° determinant in M13-rpsD (Fig. 1B). Strain BR151C1 contains an extra copy of a wild-type rpsD gene, and strain BR151P1 contains the Pst-1 leader region mutation in the extra rpsD copy. Strain BR3AP1 is similar to BR151P1 except that it has the rpsD2 allele of S4 both in the normal chromosomal locus and in the extra copy integrated at the amyE locus.

The rpsD-lacZ fusions contained in plasmid pZA329 were introduced by transformation into *B. subtilis* ZB307A. This strain contains a derivative of specialized transducing phage SPβ which permits integration of the plasmid by selection for the plasmid-borne Cm° determinant (30). Phage carrying the fusion were purified by passage through strain ZB449 (SPβ cured) and were then introduced into strains BR151C1 and BR151P1 by selection for the phage-borne MLS° determinant.

**DNA manipulations.** Restriction endonucleases and DNA-modifying enzymes were purchased from New England BioLabs and used as described by the manufacturer. Dideoxynucleotide sequencing was carried out by using Sequenase (United States Biochemical) and single-stranded M13 or double-stranded plasmid templates. *B. subtilis* chromosomal DNA isolation and transformation were done as previously described (9).

**Oligonucleotide-directed mutagenesis.** M13 template DNA preparation and mutagenesis reactions were done as described by Kunkel (16). Synthetic oligonucleotides were purchased from Oligos, Etc. (Guilford, Conn.). Mutants were screened for acquisition of new restriction sites, and sequence alterations were confirmed by DNA sequencing.

**Hybridization analysis.** Dot blot hybridization was used to confirm that the insertions of M13-Pneo and M13-C1neo...
were in single copy. Parallel dot blots of serials of chromosomal DNA were probed with nick-translated pTMH115 plasmid DNA. Nick translation was carried out using a kit purchased from Bethesda Research Laboratories.

Polycrylamide gel electrophoresis. Ribosomal proteins were isolated from strains BR151 and TR3a as previously described (9). Extracts from strains containing insertions of M13-P1neo and M13-Clneo were prepared from 5 ml of exponentially growing cultures. Cells were harvested, and protoplasts were prepared and lysed by using Brij 56 as previously described (9). Extracts were mixed with sodium dodecyl sulfate (SDS) sample buffer and were boiled for 5 min prior to electrophoresis on a 12% SDS-polycrylamide gel. Molecular weight markers were from Sigma. Gels were stained with Coomassie blue R-250. Overproduction of S4 protein was estimated by laser densitometry, using an LKB Ultrascan XL system.

β-Galactosidase assays. Cells were grown to mid-logarithmic growth phase in L broth containing the appropriate antibiotics. Samples (1 ml) were harvested, permeabilized with toluene, and assayed as described by Miller (18). For *E. coli* strains containing λRZ5 derivatives and plasmid pTMH115, early logarithmic cultures were split and IPTG (1 mM) was added to one of the cultures; samples were taken immediately before and at 15-min intervals after IPTG addition.

RESULTS

Effect of S4 overproduction on expression of *rpsD-lacZ* fusions. To facilitate analysis of the regulation of *rpsD* expression, in-frame *rpsD-lacZ* translational fusions were constructed in plasmid pZA329 by using a DNA fragment containing the promoter, leader, and amino-terminal 57 codons of *rpsD*. Wild-type and leader region mutant fusions were crossed into the bacteriophage λRZ5 genome by homologous recombination, and specialized transducing lysates were prepared and used to construct lysogens of *E. coli* JM109. Plasmid pTMH115, a low-copy-number plasmid containing the *B. subtilis* S4-coding region under the control of a *lac* promoter derivative, was introduced into the fusion-bearing strains, and β-galactosidase production was measured in the presence and absence of IPTG, an inducer of the *lac* promoter. As shown in Fig. 3, induction of S4 synthesis by addition of IPTG resulted in the turn-off of β-galactosidase synthesis by a wild-type *rpsD-lacZ* fusion; under these conditions, growth was affected only slightly by addition of IPTG (data not shown). These results suggest that *rpsD* is subject to autogenous regulation.

A fusion containing a deletion of nucleotides 30 to 128 of the leader (ΔXba3-2) was also tested. In this case, addition of IPTG did not result in repression of β-galactosidase synthesis. These results demonstrate that leader region sequences are necessary for repression of *rpsD* expression by S4 overproduction and indicate that the leader is likely to be the target for repression. In addition, this result shows that the turn-off of β-galactosidase observed with a wild-type fusion was not the consequence of a nonspecific negative effect of S4 overproduction on β-galactosidase production. The ΔXba3-2 fusion consistently gave higher levels of β-galactosidase activity; a similar increase in activity was found when the fusions were assayed in *B. subtilis* (see below). A third fusion, containing the Pst-1 mutation, gave results identical to those shown for ΔXba3-2 (data not shown), indicating that the sequences altered in the Pst-1 mutant are important for regulation. The Pst-1 mutation changes three residues in a region noted to be conserved between the leader sequence and the putative binding site for S4 protein on *B. subtilis* 16S rRNA (8; see below).

**S4 overproduction in *B. subtilis***. Constructs containing *rpsD* coding sequences under control of the P*lac*1015 promoter did not result in high-level overproduction of S4 in *B. subtilis*, although this promoter variant gives a 29-fold increase in expression over the *lacUV5* promoter in *B. subtilis* (11; data not shown). We therefore constructed a strain containing an extra copy of the *rpsD* gene, under control of its own strong promoter, integrated at the amylE locus. Three variants were constructed: BR151C1, which contains an
extra copy of rpsD with a wild-type leader sequence; BR151P1, which contains an extra copy of rpsD in which the leader region contains the Pst-1 mutation; and BR3AP1, which is similar to BR151P1 except that it contains the rpsD2 allele of rpsD, which has a four-amino-acid deletion in the S4 coding sequence (10), both at the normal chromosomal locus for rpsD and in the copy integrated at amyE. These strains were constructed by initial insertion of a small segment of M13 DNA into the amyE locus, to generate strains BR151M13 and BR3AM13, followed by integration of M13 replicative-form DNA containing the appropriate rpsD allele as well as a neo gene cassette selectable in single copy. Integration into the amy locus was confirmed by testing for linkage between neo and amy by transformation. It was predicted on the basis of the fusion results described above that expression of the extra copy of rpsD containing the Pst-1 leader mutation would be independent of autogenous regulation and would therefore result in overproduction of S4 protein.

This system was set up in such a way that integration of the M13 by Campbell recombination would result in the presence of repeated sequences flanking the rpsD and neo sequences; these repeated sequences could potentially be exploited for generation of amplified copies of the integrated rpsD gene by selection for increased resistance to neomycin. However, amplified strains exhibited decreased growth rate, so single-copy insertion strains, as determined by neomycin resistance levels and dot blot hybridization analysis (data not shown), were used for all subsequent studies.

S4 overproduction was monitored by SDS-polyacrylamide gel electrophoresis of cell extracts. The change in migration between the S4 products of the wild-type and rpsD2 alleles was used as a marker to indicate the position of S4 protein (Fig. 4, lanes 2, 3, 8, and 9). Extracts from strains BR151M13 (lane 4) and BR151C1 (lane 5) showed identical protein profiles, indicating no overproduction of protein S4. This was the expected result, since the extra copy of rpsD in strain BR151C1 contained an intact leader sequence and was therefore expected to be subject to autogenous control. In contrast, extracts of strain BR151P1 (lane 6) exhibited a substantial increase in the intensity of a band corresponding to the position occupied by protein S4. This increase was estimated by laser densitometry to be approximately threefold. The identity of the overproduced band was confirmed by using strain BR3AP1, which differs from BR151P1 only in the presence of the rpsD2 allele, resulting in a change in migration of protein S4. Extracts of strain BR3AP1 (lane 7) exhibited overproduction of a protein comigrating with the rpsD2-encoded form of S4, indicating that the migration of the overproduced protein was dependent on the specific rpsD allele used and was in fact protein S4. The observed threecold increase in S4 protein is likely to represent a much greater increase in the free pool of S4 in the cell, since under normal conditions the major proportion of the ribosomal protein in the cell is assembled into ribosomes (6).

Expression of rpsD-lacZ fusions in B. subtilis. Single-copy
translational rpsD-lacZ fusions were introduced into normal or S4-overproducing strains of B. subtilis, after integration into transducing phage SPβ, and β-galactosidase production was measured (Table 2). A fusion carrying a wild-type rpsD leader region (SPβ::pTMH123) was found to be 2.2-fold repressed in the S4-overproducing strain, BR151P1, compared with strain BR151C1. This level of repression is an underestimate, since the fusion is likely to be repressed to some degree in strain BR151C1, in which S4 was not significantly overproduced. Deletion of the central portion of the leader region (ΔXba1-2, SPβ::pTMH137) resulted in increased expression in strain BR151C1 and loss of repression in strain BR151P1. The increased expression in the nonoverproducing strain could result from more efficient expression of the mutant fusion construct, independent of regulation; however, the loss of sensitivity to S4 overproduction indicates that the deleted region is necessary for repression by S4 protein. These results are in agreement with the data presented above for response of rpsD expression to S4 overproduction in E. coli.

The Xba-1 (SPβ::pTMH125), Xba-2 (SPβ::pTMH127), and Pst-1 (SPβ::pTMH131) point mutations in the rpsD leader region all gave results similar to that observed for ΔXba1-2 (SPβ::pTMH137), indicating that the mutations altered in these mutations play a role in repression of rpsD expression by protein S4. The Xba-3 mutation (SPβ::pTMH133) resulted in increased expression in strain BR151C1, but expression was still sensitive to S4 overproduction; this finding suggests that while the sequence alteration in this mutation affects expression of rpsD, regulation by S4 is intact. A second leader region deletion (ΔXba1-2, SPβ::pTMH129) resulted in reduced expression of the fusion in strain BR151C1 and loss of sensitivity to S4 overproduction. The most likely explanation for this result is that this mutation results in decreased efficiency of expression, by a reduction in access of the translational initiation site to the ribosome or a reduction in mRNA stability, and disruption of regulation by protein S4.

**DISCUSSION**

The goal of this study is the analysis of the regulatory mechanism governing expression of the rpsD gene, encoding ribosomal protein S4, in B. subtilis. While S4 acts as the translational regulator for α operon expression in E. coli, rpsD is not located in the α operon in B. subtilis, suggesting that regulation of rpsD and α expression may differ in the two systems. We have previously shown that rpsD in B. subtilis is monocistronic (8) and that the coding sequence is preceded by a leader region exhibiting interesting structural similarities to the putative binding site for S4 on 16S rRNA (23, 27). We also find similarities to the binding site for E. coli S4 in the α operon mRNA, at the start of the coding sequence for protein S13 (25, 26). In this study, we show that rpsD is subject to autogenous control in B. subtilis, as in E. coli, and that the mRNA leader region is the target site for repression by S4 protein.

Overexpression of protein S4, generated by induction of a P-lac construct in E. coli or by insertion into the B. subtilis chromosome of an extra copy of rpsD containing a derepressing leader mutation, resulted in repression of a wild-type rpsD-lacZ fusion. Similar results were obtained when regulation was tested in B. subtilis and E. coli, indicating that there are no obvious fundamental differences in the operation of this regulatory system in the two organisms. Certain mutations in the rpsD leader region, including deletion and point mutations, resulted in loss of repression by S4. These results indicate that the leader region sequence is important in regulation by S4.

Computer analysis of the rpsD leader sequence indicated that this sequence has the potential to fold into a variety of different secondary structural patterns; possible models include pseudoknot structures, similar in general structure, although not in primary sequence, to the proposed S4 binding site in the E. coli α operon mRNA leader. One such model is shown in Fig. 5A. Of particular interest is the conservation of sequence in the region at 127 to 130 of the B. subtilis rpsD leader and 104 to 107 of the E. coli α operon sequence. This single-stranded region of the E. coli α leader has been shown to be important for S4 binding, since alterations which disrupt helical regions surrounding this area, or which specifically alter the A residue at position 106, result in loss of S4 binding and derepression of α operon expression (26). The Xba-2 mutation, which resulted in derepression of B. subtilis rpsD, alters three nucleotides in this region, including the residue analogous to A106 of the E. coli α leader. Both structures also include a helical region immediately following the translation initiation codon.

Protein S4 is one of the initial proteins binding to 16S RNA during ribosomal assembly, and it is postulated that competition between binding to 16S rRNA and to the regulatory target site on the α mRNA is a key feature of the regulatory system in E. coli (5). The target site for S4 binding on 16S rRNA in E. coli has been well characterized (23, 27) and has been proposed to involve helices 437 to 497 and 500 to 545. The equivalent region is highly conserved in B. subtilis 16S rRNA (Fig. 5B; 7). There is very little primary sequence homology between the binding sites for E. coli S4 protein on E. coli 16S rRNA and on the E. coli α operon leader (26), with the only conserved feature of note located in the region near the above-mentioned A106 of the α leader. In contrast, there are two regions of conservation between the B. subtilis rpsD leader and the putative S4 binding site on B. subtilis 16S rRNA. The first region is at leader positions 70 to 78 and 16S rRNA positions 504 to 512. The Pst-1 mutation, which resulted in overproduction of S4 protein in B. subtilis and loss of repression of an rpsD-lacZ fusion by S4 overproduction, alters three of the residues conserved between the leader and 16S rRNA. These results suggest that the sequence in this region is important for rpsD regulation and that the conservation in sequence with 16S rRNA may be significant. The region near the Pst-1 mutation was also found to be conserved in the rpsD leader region.

**TABLE 2. β-Galactosidase activities of rpsD-lacZ fusions**

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<thead>
<tr>
<th>SPβ-carried rpsD-lacZ fusion</th>
<th>Leader region mutation</th>
<th>β-Galactosidase sp act* (U)</th>
<th>Repression ratio*</th>
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<tr>
<td>pTMH123 Wild type</td>
<td></td>
<td>730</td>
<td>330</td>
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<tr>
<td>pTMH125 Xba-1</td>
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* Determined according to Miller (18).

* Ratio of β-galactosidase activity obtained in a normal strain (BR151C1) to that obtained in a strain overproducing S4 protein (BR151P1). A ratio of 1.0 indicates loss of sensitivity of the rpsD-lacZ fusion to repression by S4.

* Host strain.
from *B. stearothermophilus*, which exhibits conservation of a number of structural features of the leader despite variation at the primary sequence level (8a). The second region of homology between the *B. subtilis* rpsD leader and 16S rRNA sequences is at leader positions 123 to 130 and 3′ 16S rRNA positions 463 to 470. The Xba-2 mutation alters two residues in this position and results in loss of autogenous regulation. Additional mutagenesis studies will be necessary to determine the importance of each of these residues in repression by 84.

In the *E. coli* system, it has been clearly demonstrated that addition of protein 84 to an in vitro translation system containing *E. coli* α operon mRNA as a template results in turn-off of synthesis of α-encoded ribosomal proteins (29). It has also been shown that 84 protein binds to the leader region of the *E. coli* α operon mRNA and that binding of 84 to leader mRNA variants in general correlates with repression, although certain leader mutations permit binding without repression (26). The results presented here indicate that *B. subtilis* 84 represses its own synthesis, but it has not yet been determined whether this occurs by direct binding of 84 to the rpsD mRNA leader region, not has it been demonstrated that repression occurs at the translational level, although this is likely to be the case by analogy with the *E. coli* system. The effect of 84 overproduction on expression of the *B. subtilis* α operon has not yet been tested.

Introduction of an extra copy of rpsD containing the Pst-1 leader region mutation into the *B. subtilis* chromosome resulted in a threefold increase in the amount of 84 protein in the cell. Since the majority of ribosomal protein in the cell is normally assembled into ribosomes, this is likely to represent a much greater increase in the free pool of 84 (5). However, it is not known how much of this excess 84 protein is available for regulation, since it could be aggregated or associated with other cellular components. This 3-fold excess in total 84 protein resulted in a 2.2-fold repression of rpsD-lacZ expression in *B. subtilis*. The measured level of repression is likely to be underestimated, since it is probable that the fusion is not fully derepressed in the parent strain lacking 84 overproduction. It may be that higher levels of repression cannot be observed because a major portion of the excess 84 protein is inaccessible. It is also possible that there is a limit to the repressibility of rpsD expression, perhaps because only a portion of the mRNA is in a conformation accessible to S4-mediated repression, while the remainder is always available for expression. A similar twofold repression ratio is also observed in the case of the *E. coli* α operon (26).

Plasmid constructs containing the intact rpsD gene under control of its own promoter, or high-copy-number plasmids containing *P lac*-rpsD constructs, proved to be unstable in *E. coli*, even in strains containing the *lacF* allele. The most likely explanation for this observation is that overproduction of 84 in *E. coli* resulted in turn-off of *E. coli* α operon expression, resulting in unbalanced ribosome synthesis. Preliminary experiments using plasmid pACSPA2 (26), which contains an *E. coli* α-lacZ fusion, indicated that induction of 84 synthesis resulted in repression of β-galactosidase production (8a), suggesting that *B. subtilis* 84 can interact with the *E. coli* α mRNA target site. Further studies are needed to establish this more clearly.

The lack of sequence homology between the *B. subtilis*
and *E. coli* S4 target sites is similar to the divergence of the *B. subtilis* and *E. coli* RNAse P RNA sequences (20). In that case, functionally homologous RNA molecules exhibit little primary sequence homology and only partial structural relatedness. In the case of the *B. subtilis* rpsD and *E. coli* α mRNA leader regions, it appears that the leader RNAs have similar regulatory roles and interact with related S4 proteins, despite considerable structural divergence. Further analysis of the *B. subtilis* rpsD leader structure, by mutagenic and phylogenetic analysis, and characterization of the ability of S4 protein variants to bind to various RNA target sites, will provide valuable information about structural features important in protein-RNA recognition.

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