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Thiostrepton-resistant mutants exhibit relaxed synthesis of RNA

(*Bacillus subtilis*/guanine nucleotides/bryamycin)

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ABSTRACT **Spontaneous mutants of *Bacillus subtilis* resistant to thiostrepton (*tsp*) exhibit relaxed synthesis of RNA when starved for required amino acids. Intact cells of *tsp* mutants cannot synthesize the regulatory nucleotides, ppGpp and pppGpp, after amino acid deprivation. Because ribosomes isolated from spontaneous revertants to thiostrepton sensitivity and from wild-type stringent strains can synthesize (p)ppGpp whereas ribosomes isolated from *tsp* strains cannot synthesize these regulatory nucleotides in the presence of stringent factor, it appears that the lesion is expressed at the level of the ribosome. Genetic mapping, via three-factor transformational crosses, has shown that *tsp* is closely linked to *rif*, in the order *cysA14, tsp, rif-1, strA*. The phenotype of the *tsp* mutants indicates that they are of the *relC* type. Their map position indicates that they are different from a previously described *B. subtilis rel* mutation. Ribosomes from the latter strain can synthesize (p)ppGpp in cell-free extracts.**

Bacillus subtilis is normally sensitive to thiostrepton (also known as bryamycin), an antibiotic that inhibits several ribosomal functions, including initiation, elongation factor Tu- and G-dependent reactions, and termination (1). Spontaneous mutations to thiostrepton resistance (*tsp*) have been isolated in *B. subtilis*, and ribosomes isolated from *tsp* strains are resistant to the antibiotic (2). Resistance to thiostrepton is expressed at the level of the 50S subunit, as shown by GTP binding and protein synthesis in cell-free extracts (3, 4). Immunochemical analysis and two-dimensional gel electrophoresis have shown that the 50S protein BS L11 of *B. subtilis* is immunologically related to *Escherichia coli* L11 and is missing from the ribosomes of *tsp* strains (unpublished data). Ribosomal protein L11 is altered in *E. coli relC* mutants, which are unable to synthesize the regulatory nucleotides ppGpp and pppGpp when starved for amino acids (5, 6). In addition, ribosomes from *relC* strains do not synthesize ppGpp and pppGpp in extracts (6). Because *tsp* mutants seem to lack protein BS L11, it was possible that *tsp* mutants, derived from stringent *B. subtilis* parental strains, would exhibit a relaxed phenotype in response to amino acid starvation and that ribosomes derived from *tsp* strains, like ribosomes isolated from *relC* mutants, would be unable to synthesize ppGpp and pppGpp in extracts. This report examines these predictions.

EXPERIMENTAL PROCEDURES

Genetic Methods. Media, transforming DNA, and competent cells were prepared as described (7). Selection for antibiotic resistance (8) and DNA-mediated transformation (7) were as described.

Labeling of Intact Cells. *B. subtilis* strains IS75 and IS127 (Table 1) were grown at 37°C in low phosphate medium containing the following ingredients in g/liter unless otherwise specified: NaCl, 3.0; KCl, 0.5; $(NH_4)_2SO_4$ (anhydrous), 2.0; $MgSO_4 \cdot 7H_2O$, 0.2; sodium citrate, 1.0; glucose, 5; Tris·HCl (pH 7.5), 25 mM; and NaH_2PO_4, 0.5 mM. The medium contained 19 natural amino acids without glutamine at 10 μg/ml each, except for leucine, histidine, and methionine, which were present at 50 μg/ml.

For double-labeling experiments, portions of the culture were removed from the low phosphate growth medium at a density of approximately 3×10^8 cells per ml. The samples were filtered under sterile conditions, washed with low phosphate medium lacking the three required amino acids, and resuspended in an equal volume of the medium in the absence of leucine, methionine, and histidine. For labeling, 20 μl of a solution containing 125 μCi of [5-^{3}H]uridine per ml (250 μg/ml) and 6 μCi of [*U*-^{14}C]phenylalanine per ml (2.5 μg/ml) was added to 1.0-ml cell samples in low phosphate medium without the required amino acids while duplicate cultures received 50 μg each per ml of leucine, histidine, and methionine in addition to the labeled substrate. The cells were incubated at 37°C with shaking, and 0.1-ml portions were removed at intervals. Trichloroacetic acid-insoluble radioactivity was determined as described (9).

For labeling with ^{32}P, *B. subtilis* strains IS75 and IS127 were grown and starved for the essential amino acids as described for the double-labeling experiments. $NaH_2{}^{32}PO_4$ (200 Ci/mol) was present throughout the initial growth of the cultures at 0.5 mM. Subsequent resuspension and growth was performed, with the same ^{32}P concentration, in the presence and absence of the required amino acids. At intervals, samples of 50 μl were removed and chilled, and 2 μl of 90% formic acid was added. The samples were centrifuged in an Eppendorf microcentrifuge for 5 min. The supernatants were spotted onto polyethyleneimine thin-layer plastic plates (Merck) with GDP, GTP, ppGpp, and pppGpp standards. The thin-layer chromatograms were developed with 1.5 M KH_2PO_4; the resulting spots were visualized by scanning with UV light and autoradiography. The pppGpp and pppGpp spots were cut out and their radioactivity was determined by liquid scintillation.

Assays in Cell-Free Extracts. Cells, cell extracts, ribosomes, and supernatant fractions were prepared as described (9). In some experiments noted in the text, 30,000 × *g* supernatant fractions (S-30) were prepared in the presence of phenylmethylsulfonyl chloride and hemoglobin-Sepharose as described by Nakayama *et al.* (12) to inhibit and remove endogenous proteases. Poly(U)-dependent polyphenylalanine synthesis was done as described, but in 50-μl reaction volumes (9). In extracts, (p)ppGpp synthesis was assayed in reaction mixtures containing the following components in a final volume of 50 μl: 40 mM Tris·HCl (pH 7.9), 20 mM magnesium acetate, 4 mM dithiothreitol, 4 mM ATP, 0.4 mM [α-^{32}P]GTP (2.5×10^4

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Abbreviation: *tsp*, gene for thiostrepton resistance.

Table 1. Strains

Strain	Parent	Genotype	Origin
IS1		*trpC2, thr-5*	Ref. 7
IS3		*cysA14*	Ref. 8
IS6		*cysA14, trpC2, thr-5*	Ref. 9
IS56		*trpC2, lys, rel*	Ref. 10
IS58		*trpC2, lys*	Ref. 9
IS75		met_{am}, $hisB_{am}$, *leu*	Ref. 11
IS77	IS1	*rif-1, strA, trpC2, thr-5*	Sequential transformation (*rif, str*) of IS1
IS127	IS75	*tsp-6**, met_{am}, $hisB_{am}$, *leu*	Tsp^r transduction, IS134 × IS75
IS128	IS1	*tsp-6*, trpC2, thr-5*	Tsp^r transformation, IS134 × IS1
IS134	IS3	*tsp-6*, cysA14*	Ref. 8
IS138	IS3	*tsp-4, cysA14*	Ref. 4
IS140	IS77	*rif-1, strA, tsp-6, trpC2, thr-5*	Tsp^r transformation, IS128 × IS77
IS143	IS138	*cysA14*	Ref. 4, spontaneous revertant

* *tsp* is the abbreviation for the mutation to thiostrepton resistance, replacing *thi*, which was used previously (4). This avoids confusion with the *E. coli thi* (thiamine) marker. *tsp-6* was formerly known as *bry-2* (4, 8). The change was for uniformity of nomenclature.

cpm/nmol), 30 μg of tRNA, 5 μg of poly(U), and 3 μg of purified *E. coli* stringent factor kindly provided by Jose Sy (Rockefeller University). Ribosomes were added as indicated in the legend to Table 3. Assays were carried out for 60 min at 30°C. Reactions were terminated by chilling and addition of 2 μl of 90% formic acid. The (p)ppGpp synthesized was assayed by thin-layer chromatography as described above.

Gel Electrophoresis. Ribosomal proteins were prepared from S-30 fractions and purified 70S ribosomes by extraction with 66% (vol/vol) acetic acid/0.1 M $MgCl_2$ (13). Two-dimensional gel electrophoresis was performed as described (13), but 1-mm-thick gels were used for the second dimension with the slab-gel apparatus described by Studier (14).

Chemicals. $NaH^{32}PO_4$ was obtained from Amersham/Searle; all other isotopes were purchased from New England Nuclear Corp. All nucleotides were from P-L Laboratories. All other reagents were obtained as described (4, 9).

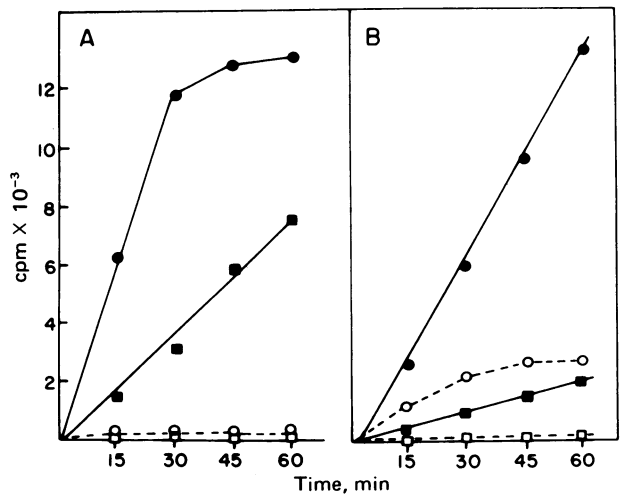

FIG. 1. Effect of amino acid starvation on RNA and protein synthesis of intact cells. IS75 and IS127 were grown in minimal medium containing the required amino acids, leucine, methionine, and histidine. At zero time, samples were filtered and resuspended in fresh medium containing [^{3}H]uridine and [^{14}C]phenylalanine with or without the required amino acids. At intervals, samples were removed and radioactivity in the various fractions was determined. RNA (●) and protein (■) synthesis in the presence of amino acids; RNA (○) and protein (□) synthesis in the absence of amino acids. (*A*) IS75, the thiostrepton-sensitive parent; (*B*) IS127, *tsp*.

RESULTS

Physiology of *tsp* Mutants. *B. subtilis* IS75, a *met, leu,* and *his* auxotroph, and IS127, an isogenic *tsp* derivative, were grown in minimal low phosphate medium containing leucine, methionine, and histidine. Then each was starved for the three required amino acids in the presence of [^{3}H]uridine and [^{14}C]phenylalanine (Fig. 1). The wild-type strain exhibited the typical stringent response, showing a complete cessation of total RNA and protein synthesis. The *tsp* mutant initially synthesized approximately 50% as much RNA in the absence of amino acids as it did in their presence. Similar results were obtained in high phosphate medium supplemented with yeast extract (data not shown). The ratio of uridine to phenylalanine incorporated in the presence of amino acids was higher in the mutant strain than in the wild type. In six experiments the ratio of uridine to phenylalanine incorporation of the *tsp* mutant (IS127) was double that of the parental strain (IS75) at all time points. IS127, grown in minimal or in rich medium, has a generation time 50% greater than that of IS75. In addition, IS127, like all *tsp* mutants, is asporogenous at all temperatures and does not grow at 53°C. Wild-type *B. subtilis* strains, including IS75, grow normally at this temperature.

During amino acid starvation of IS75 and IS127, ppGpp formation was measured (Table 2). The results demonstrate that the *tsp* mutant (IS127) did not synthesize more pppGpp and ppGpp after amino acid starvation, whereas the parental strain (IS75) did. Thus, the *tsp* mutant (IS127) resembles a relaxed

Table 2. Synthesis of ppGpp and pppGpp in cells

Min after amino acid starvation	IS75 (parental), cpm	IS127 (*tsp-6*), cpm
0	4,841	4036
15	12,237	4832
30	17,572	3420
60	16,860	3920

IS75 and IS127 were grown in low phosphate medium containing $NaH_2{}^{32}PO_4$. At time zero, cell samples were filtered and resuspended in medium containing $NaH_2{}^{32}PO_4$ without or with the required amino acids leucine, methionine, and histidine. At intervals, portions were removed, and intracellular nucleotides were extracted by formic acid and analyzed by polyethyleneimine thin-layer chromatography. In the absence of amino acid starvation, parental (IS75) and *tsp* mutant (IS127) yielded 5444 and 3343 cpm, respectively, for total ppGpp and pppGpp synthesis at 15 min after resuspension into medium containing amino acids.

Table 3. Cell-free synthesis of pppGpp and ppGpp

Source of ribosomes	*tsp* phenotype	nmol (p)ppGpp synthesized
Exp. 1		
IS75	*tsp*$^+$	10.6
IS127	*tsp-6*	0.9
IS127 (300 μg)	*tsp-6*	0.7
Exp. 2		
IS3	*tsp*$^+$	6.4
IS6	*tsp*$^+$	6.4
IS138	*tsp-4*	0.7
IS143	*tsp*$^+$	6.2
Exp. 3		
IS56	*tsp*$^+$	4.9
IS58	*tsp*$^+$	4.9

Each reaction mixture, containing 150 μg of ribosomes, except where indicated, from the above strains, was incubated with purified *E. coli* stringent factor for 60 min at 30°C. The data are corrected for values obtained in reaction mixtures lacking ribosomes.

mutant. Accordingly, we then investigated the nature of the *tsp* lesion in cell-free extracts.

Cell-Free Studies. Salt-washed ribosomes from IS127 and IS75 were incubated with stringent factor from *E. coli* and [α-^{32}P]GTP. Formation of ppGpp and pppGpp was measured (Table 3, Exp. 1). Ribosomes from two other *tsp*$^+$ strains (IS3 and IS6), a *tsp* mutant (IS138) isogenic with IS3, and a spontaneous revertant to thiostrepton sensitivity (IS143) derived from IS138 were also examined for their ability to synthesize the regulatory nucleotides (Table 3, Exp. 2). In all cases, ribosomes from parental and revertant strains (*tsp*$^+$) synthesized approximately 10 times the amount of pppGpp and ppGpp as did the *tsp* ribosomes.

Ribosomes from other *tsp* strains are active in cell-free protein synthesis (2, 4). We thus tested the ability of ribosomes from strains IS75 and IS127 to synthesize polyphenylalanine in the presence of poly(U) (Table 4). The *tsp* ribosomes had approximately 70% of the wild-type protein-synthetic activity and were more resistant to thiostrepton, confirming earlier experiments with other *tsp* strains (2, 4).

Attempts to Localize BS L11 in *tsp* Mutants. All spontaneous *tsp* mutants thus far examined show an absence of 50S ribosomal protein BS L11 when ribosomes are analyzed both electrophoretically and immunologically (unpublished data). In addition, 20 other ethyl methanesulfonate- and nitrosoguanidine-induced *tsp* mutants are missing BS L11 from their ribosomes, as observed on two-dimensional gels (unpublished data).

Table 4. Polyphenylalanine synthesis *in vitro**

Source of ribosomes	Thiostrepton, μg/ml 0	1	5	10
IS75	28.7 (100)	16.1 (56)	1.9 (6)	0.5 (2)
IS127	20.4 (100)	16.5 (81)	8.2 (40)	6.5 (32)

Each reaction mixture contained 45 μg of ribosomes from the strains indicated as well as 15 μg of S-100 protein isolated from IS6. Phenylalanine incorporation dependent on poly(U) was determined as described (9). Data in parentheses refer to percentage of residual activity in the presence of thiostrepton.

* Values are pmol of phenylalanine incorporated.

B. subtilis produces large quantities of extra- and intracellular proteases (12). The absence of BS L11 from ribosomes could be due to the release of a mutated BS L11 from the ribosome during cell lysis and ribosome purification with subsequent proteolytic degradation of the free protein. In *E. coli*, L11 can be removed from 50S ribosomes by mild salt or ethanol washing, indicating that it is not tightly bound (5, 15, 16). To test this possibility, we used a wild-type strain, IS1, and its isogenic *tsp-6* derivative, IS128, to prepare S-30 and ribosome fractions, but with the protease inhibitor, phenylmethylsulfonyl chloride, present throughout cell washing, lysis, and ribosome purification. In addition, cells were lysed in the presence of hemoglobin-Sepharose, which binds all measurable proteases in *B. subtilis* (12). Ribosomal proteins, prepared from S-30 and from 70S ribosomes, were analyzed by two-dimensional gel electrophoresis (Fig. 2). Ribosomal protein BS L11 was observed in S-30 and in 70S ribosomes of IS1 but was not seen in the corresponding fractions of IS128. This observation suggests that BS L11 is not released during cell lysis and is not present in *tsp* cells. These results confirm unpublished observations that demonstrate the absence of BS L11 in *tsp* mutants.

Genetic Mapping of *tsp* Mutations. We previously mapped several *tsp* mutations, including *tsp-6*, between *cysA14* and *strA* (4, 17). The *rif* gene, which determines the β subunit of RNA polymerase, also maps between *cysA14* and *strA* (refs. 18 and 19; unpublished experiments). Since the *rif* gene in *E. coli*, the genetic determinant for the β subunit (*rpoB*), is closely linked to *relC* (20), we mapped *tsp-6* relative to *rif-1*, a spontaneous Rif^r mutation isolated in our laboratory. DNA prepared from strain IS140 (*rif-1 tsp-6 strA*) was used to transform strain IS6, with subsequent selection for the various antibiotic resistance markers (Table 5). Analysis of the recombinant classes places *tsp-6* to the left of *rif-1*, and the two-factor distances are illustrated in Fig. 3. Another *rel* mutation has been isolated in *B. subtilis* and characterized in intact cells (10). Preliminary mapping data indicate that this mutation is unlinked to *strA* by transduction, whereas the *tsp* mutation is more than 90% linked to *strA* (4, 17). The unlinked *rel* mutation has not been characterized in extracts, but its phenotype, showing high levels of relaxed RNA synthesis (10), suggests it is of the *relA* type. Ribosomes from strain IS56, bearing this mutation, are able to synthesize (p)ppGpp in the presence of stringent factor to the

Table 5. Three-factor transformation crosses involving thiostrepton resistance

Selected phenotype	Recombinant classes* *tsp-6*	*rif-1*	*strA*	No. of recombinants	Marker order
Tsp^r	1	1	1	6	
	1	1	0	32	*tsp-6, rif-1, strA*
	1	0	1	0	
	1	0	0	62	
Rif^r	1	1	1	0	
	1	1	0	18	*tsp-6, rif-1, strA*
	0	1	1	23	
	0	1	0	44	
Str^r	1	1	1	0	
	0	1	1	25	*tsp-6, rif-1, strA*
	1	0	1	0	
	0	0	1	75	

Donor: IS140 (*tsp-6, rif-1, strA, trpC2, thr-5*).
Recipient: IS6 (*cysA14, trpC2, thr-5*).

* "1" and "0" refer to donor and recipient class, respectively. The order is determined from that arrangement requiring the fewest multiple crossovers.

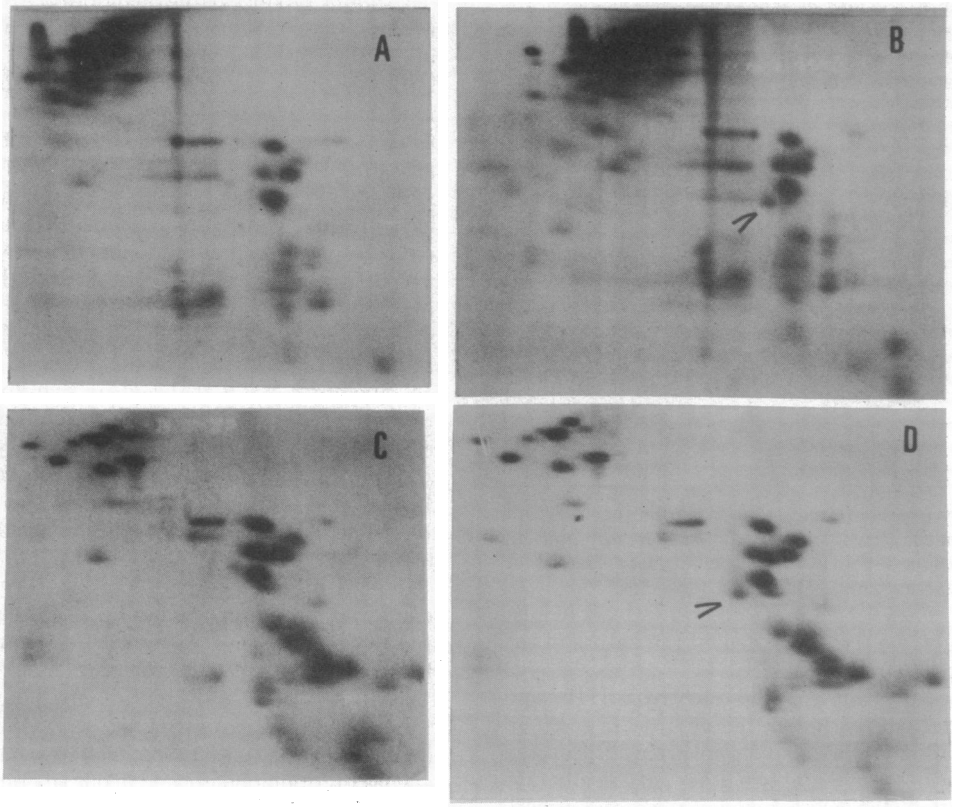

FIG. 2. Two-dimensional gel electrophoresis of ribosomal proteins. S-30 and purified ribosomes from *B. subtilis* strains IS1 and IS128 were purified as described (9) but with the protease inhibitor, phenylmethylsulfonyl chloride (2 mM), present during cell wash and lysis. In addition, hemoglobin-Sepharose was added during cell lysis to eliminate endogenous protease (12). Ribosomal proteins were extracted from the S-30 and 70S ribosomal fractions by extraction with 66% (vol/vol) acetic acid/0.1 M $MgCl_2$. The ribosomal proteins were analyzed electrophoretically on two-dimensional acrylamide gels (13) with approximately 200 μg of ribosomal protein per gel. (*A*) Strain IS127, S-30; (*B*) strain IS1, S-30; (*C*) strain IS127, 70S ribosomes; (*D*) strain IS1, 70S ribosomes. Arrows in *B* and *D* point to 50S ribosomal protein BS L11, which is missing in *A* and *C*.

same extent as its isogenic parent IS58 (Table 3, Exp. 3). This indicates that it is not a *relC* mutation. We are now attempting to isolate stringent factor from IS56 and IS58. This should determine whether the *rel* mutation carried by IS56 is *relA*.

Characterization of Other *tsp* Mutations. We have not analyzed the regulation of RNA synthesis in all *tsp* strains and the sensitive revertants previously described (4) in as much detail as in strains IS75 and IS127. However, we have examined (by autoradiography) RNA synthesis in Tsp^r and Tsp^s colonies growing on agar plates containing [^{14}C]uracil, with and without amino acids. These experiments show that IS135 (*cysA14, tsp-1*), IS136 (*cysA, tsp-2*), IS137 (*cysA14, tsp-3*), IS138 (*cysA14, tsp-4*), and IS139 (*cysA14, tsp-5*) are relaxed (data not shown). The parent IS3 (*cysA14*) and three spontaneous revertants to thiostrepton sensitivity, IS141 (revertant of IS136), IS142 (revertant of IS137), and IS143 (revertant of IS138), are all stringent. Thus, all *tsp* mutants show relaxed control of RNA synthesis.

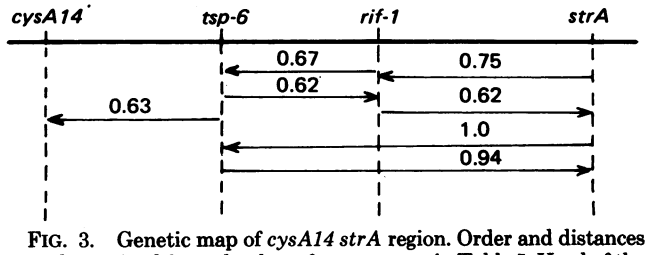

FIG. 3. Genetic map of *cysA14 strA* region. Order and distances were determined from the three-factor crosses in Table 5. Head of the arrow points to the recipient marker of the recombinant class. Numbers represent the fraction of recombination between markers. Data for the recombinational distance between *cysA14* and *tsp-6* were obtained from other crosses (data not shown here).

DISCUSSION

The bacterial 50S ribosome modulates the synthesis of (p)ppGpp depending on the binding of charged aminoacyl tRNA or uncharged tRNA to the ribosomal A site in the presence of the correct triplet codon. In the presence of aminoacyl-tRNA, peptide bond formation occurs by transfer of the peptidyl group from peptidyl-tRNA in the P site to aminoacyl-tRNA. In the presence of uncharged tRNA, the product of the *relA* gene, ATP:GTP-3′-pyrophosphotransferase (stringent factor), catalyzes the synthesis of the regulatory nucleotides ppGpp and pppGpp with the ribosome–mRNA–uncharged tRNA complex (21–24). These compounds then act at the transcriptional level to regulate the synthesis of RNA in an operon-specific manner (25): synthesis of rRNA is decreased by these nucleotides, but the syntheses of some mRNAs are stimulated and others are blocked. Ribosomal protein L11 is involved in the stringent response in *E. coli* (6) and, according to the results of this communication, in *B. subtilis* as well. The role of L11 in protein synthesis is unclear; earlier reports, suggesting it was essential for peptidyltransferase activity, have been disproven (refs. 26–28; unpublished data). Clearly, ribosomes from *B. subtilis tsp* strains, devoid of BS L11, can form peptide bonds (Table 4; ref. 4). However, translation of natural mRNA may yield more insight into the role of BS L11 in protein synthesis.

The molecular nature of the *tsp* mutation is unknown. In addition to showing resistance to thiostrepton, *tsp* cells grow

slowly, are temperature sensitive for growth, do not sporulate, and cannot synthesize ppGpp and pppGpp during amino acid starvation. The *tsp* strain IS127 also has a higher ratio of uridine to phenylalanine incorporation than does the isogenic wild type, IS75 (Fig. 1). Whereas this could result from precursor pool variations, it might be due to lower (p)ppGpp levels in the mutant during normal growth.

We have been unable to detect 50S ribosomal protein BS L11 in purified ribosomes of *tsp* cells. It is not certain that BS L11 is missing from the ribosomes, for we have not definitively ruled out the proteolytic degradation of an altered BS L11 during cell lysis and ribosome purification. However, we have shown that when *tsp* cells are exhaustively washed in high salt buffers, which remove proteases, and when protease inhibitors are used during harvesting and cell lysis, BS L11 is not observed either on the ribosomes or in the supernatant (Fig. 2). This suggests that BS L11 is not made in *tsp* strains. We have tried to introduce the amber suppressor mutation *su-1* (11) into IS127 (*tsp-6* his_{am}, $metB_{am}$, *leu*) to see whether *tsp-6* is an amber mutation. If *tsp* were a suppressible amber mutation, one would expect the *su-1* mutation to restore thiostrepton sensitivity and a normal BS L11 while suppressing the his_{am} and met_{am} mutations. We have been unable to create, by either recombination or spontaneous mutation, a *su-1, tsp* strain.

The *B. subtilis* ribosome, as well as synthesizing peptide bonds and aiding in the synthesis of the regulatory nucleotides ppGpp and pppGpp (see above), also helps synthesize the highly phosphorylated nucleotides, pppApp and ppApp, which appear during sporulation (29). It is tempting to speculate that a functional ribosome is necessary for the synthesis of these molecules that trigger sporulation. The *tsp* mutation may prevent their synthesis as well as that of ppGpp and pppGpp since *tsp* strains are asporogenous. We are currently studying the synthesis of the highly phosphorylated guanosine and adenosine nucleotides in *tsp* and wild-type strains to test this hypothesis.

Note Added in Proof. We have recently purified stringent factor from *B. subtilis* IS58 (rel^+), whereas we have not been able to detect this enzyme in IS56 (the isogenic *rel* derivative of IS58). Thus, the latter mutation is of the *relA* class. In addition, we have shown that all *tsp* mutants are protease minus and antibiotic negative, indicating they are *spoO* type sporulation-deficient mutants.

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