A new regulatory circuit in ribosomal protein operons: S2-mediated control of the *rpsB-tsf* expression in vivo

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ABSTRACT

Autogenous regulation is a general strategy of balancing ribosomal protein synthesis in bacteria. Control mechanisms have been studied in detail for most of ribosomal protein operons, except for *rpsB-tsf* encoding essential r-protein S2 and elongation factor Ts, where even the promoter has remained unknown. By using single-copy translational fusions with the chromosomal *lacZ* gene and Western-blot analysis, we demonstrate here that S2 serves as a negative regulator of both *rpsB* and *tsf* expression in vivo, acting at a single target within the *rpsB* 5′-untranslated region (5′-UTR). As determined by primer extension, transcription of the *Escherichia coli rpsB-tsf* operon starts 162 nucleotides upstream of the *rpsB* initiation codon at a single promoter TGTGGTATAAA belonging to the extended −10 promoter class. Both the promoter signature and the 5′-UTR structure of the *rpsB* gene appear to be highly conserved in γ-proteobacteria. Deletion analysis of the *rpsB* 5′-UTR within *rpsB′-′lacZ* fusions has revealed that an operator region involved in the S2 autoregulation comprises conserved structural elements located upstream of the *rpsB* ribosome binding site. The S2-mediated autogenous control is impaired in *rpsB* mutants and, more surprisingly, in the *rpsA* mutant producing decreased amounts of truncated r-protein S1 (*rpsA*::IS*10*), indicating that S2 might act as a repressor in cooperation with S1.

Keywords: ***rpsB-tsf*** **operon; extended −10 promoter; translation initiation region; autogenous regulation; ribosomal proteins S1 and S2; elongation factor Ts**

INTRODUCTION

In bacteria, ribosome biosynthesis is governed by transcriptional and translational regulatory mechanisms that provide a balanced and coordinated production of individual ribosomal components. Transcription of rRNA responds to nutritional cues, while production of ribosomal proteins (r-proteins) is tightly linked to the rRNA level by the feedback inhibition mechanism known as autogenous control. Most of r-protein operons encode a regulatory r-protein that directly binds 16S or 23S rRNA during ribosome assembly, but if synthesized in excess relative to its target on rRNA, serves as an operon-specific translational repressor by binding to its own mRNA to prevent further translation (for reviews, see Nomura et al. 1984; Zengel and Lindahl 1994; Nomura 1999). In the 30S ribosomal subunit, only r-proteins S1 and S2 present exceptional cases, as they do not recognize naked 16S rRNA and participate in the 30S assembly at the very late step (Culver 2003), but nevertheless possess the potential to act as specific autogenous regulators. However, while the autoregulation of a key mRNA-binding protein S1 has been corroborated both in vitro and in vivo by various approaches (Skouv et al. 1990; Boni et al. 2000, 2001), still little is known about how the synthesis of essential r-protein S2 might be controlled.

Ribosomal protein S2 is highly conserved in all forms of life (its counterparts are referred to as S0 in yeast and SA in higher eukaryotes) and is essential for the translational machinery in all prokaryotes, eukaryotes, mitochondria, and chloroplasts (Ardini et al. 1998; Wilson and Nierhaus 2005). Moreover, concurrent with the appearance of the extracellular matrix in higher eukaryotes, S2 (SA) acquired an additional extraribosomal function of laminin-binding receptor during evolution (Ardini et al. 1998). At the same time, a functional role of S2 in ribosomal translational activity remains unclear. Recent observations suggest that in prokaryotes, S2 might be involved in protecting and stabilizing the Shine–Dalgarno (SD) helix docked in the "chamber" between the head and the platform of the 30S subunit (Kaminishi et al. 2007) as well as in binding the SD

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Article published online ahead of print. Article and publication date are at http://www.rnajournal.org/cgi/doi/10.1261/rna.1099108.

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duplex at the post-initiation step (Yusupova et al. 2006). However, these data cannot explain an essential role of S2 in translational systems that follow the prokaryotic scenario but do not use the SD interaction during translation initiation, as is the case of the smallest γ-bacterial endosymbiont *Carsonella ruddii* (Nakabachi et al. 2006) or mammalian mitochondria (Koc et al. 2001), where the 3′-tail of a small ribosomal subunit RNA is naturally deleted. Thus, essential functions of a highly conserved protein S2 in protein synthesis in all cells are still waiting for a rational explanation.

According to the high-resolution structure of the small subunit from *Thermus thermophilus*, S2 within 30S has an elongated bidomain structure α_2, $\alpha_1\beta_5\alpha_3$, and contacts with two rather distant 16S rRNA regions involving helices 26 in the body and 35–37 in the head of the subunit (Wimberly et al. 2000; Brodersen et al. 2002). This suggests that, in spite of being one of the latest proteins in 30S assembly (Mizushima and Nomura 1970; Culver 2003), S2 is capable of 16S rRNA binding, but the binding surface of RNA must be preformed by the earlier r-proteins. Incorporation of S2 is absolutely necessary for binding r-protein S1 that accomplishes the assembly of the 30S subunit capable of recruiting mRNA in *Escherichia coli* and most likely in other S1-dependent translational systems (Bollen et al. 1979; Moll et al. 2002). Interestingly, a stoichiometric S1–S2 complex was copurified with RNA-polymerase and a global regulator Hfq from *E. coli* stationary-phase cultures (Sukhodolets and Garges 2003), implying that S1 and S2 are capable of interacting with each other even outside the ribosome. Other known protein–protein interactions of S2 in a cell include a chaperonin GroEL required for initial folding and conformational maintenance of S2 in vivo (Houry et al. 1999) and the ATP-dependent Lon protease that specifically degrades S2 in the presence of inorganic polyphosphate, providing an important source of amino acids for cell survival during starvation (Kuroda et al. 2001; Nishii et al. 2005).

In eubacteria, S2 is encoded in the *rpsB-tsf* operon that also codes for translation elongation factor Ts. It is logical to surmise that like in the case of other r-protein operons, production of these essential components of translational machinery might also be coordinated with the overall ribosome synthesis via the feedback mechanism. The S2 capability to modulate the *rpsB-tsf* expression has never been assayed, and the only argument in favor of the S2 autoregulation came from the observation that expression of extra copies of the plasmid-borne *rpsB* gene did not augment the S2 level in a cell (An et al. 1981; Bendiak and Friesen 1981). Furthermore, the promoter(s) responsible for the *rpsB-tsf* transcription in *E. coli* has so far remained unknown.

The main goal of this work was to study the regulatory mechanisms that govern *rpsB-tsf* expression in vivo. We determined the length of the *rpsB* 5′-untranslated (5′-UTR) region and located the *rpsB* promoter that appeared to belong to the extended −10 promoter class. Phylogenetic analysis revealed that both the promoter signature and the *rpsB* 5′-UTR structure are highly conserved in γ-proteobacteria. By using single-copy translational fusions with the chromosomal *lacZ* gene and Western-blot analysis, we show that S2 functions as a negative regulator of both its own and *tsf* expression in vivo, acting at a single target within the *rpsB* 5′-UTR. Surprisingly, the S2 repressor activity was found decreased in the *rpsA*::IS*10* mutant (*ssyF29*) producing a subnormal amount of truncated S1 (Boni et al. 2000), thus implying that S2 may cooperate with S1 in regulating the *rpsB-tsf* expression. Possible control mechanisms are discussed.

RESULTS

S2 negatively regulates its own gene expression in vivo

One of the approaches to examine autogenous regulation in vivo is monitoring expression of the corresponding translational fusion with the reporter (usually *lacZ*) gene under normal versus augmented or, on the contrary, reduced synthesis of a presumable regulator. This technique has been successfully used for dissecting the *rpsA* (Boni et al. 2000, 2001), *rpsO* (Mathy et al. 2004, and references therein), *thrS* (Sacerdot et al. 1998), *rnc* (Matsunaga et al. 1996), and L20 (Guillier et al. 2002, 2005) autoregulatory circuits. To maintain the dose of the reporter gene unaltered under different growth conditions, single-copy reporter constructs are preferable. Guided by this strategy, we assessed the capability of S2 to regulate its own gene expression.

We first constructed single-copy (chromosomal) *rpsB′-′lacZ* translational fusions and a plasmid expressing the *rpsB* gene as a source of S2 in *trans* (Fig. 1A). To create the plasmid, the *E. coli* chromosomal region encompassing the *rpsB* structural gene and its 5′- and 3′-flanks was amplified by PCR and then cloned into pACYC184. Taking into account that in *E. coli* translational starts of *rpsB* and of the preceding *map* gene (transcribed in opposite direction) are separated by 367 base pairs (bp), and corresponding promoters remained undetected, we arbitrarily chose the 208-bp region in front of the *rpsB* start codon for this construct. The *rpsB* and *tsf* genes in the operon are separated by a long (258 bp) intergenic region comprising a perfect inverted repeat followed by a run of T residues, a so-called attenuator/terminator (Merino and Yanofsky 2005) where about two-thirds of transcripts terminate, generating a monocistronic *rpsB* mRNA, while the remainder read through the attenuator until the major terminator downstream of *tsf* (An et al. 1981). This intergenic attenuator was included in our plasmid construct to terminate the *rpsB* transcription (Fig. 1A). The resulting

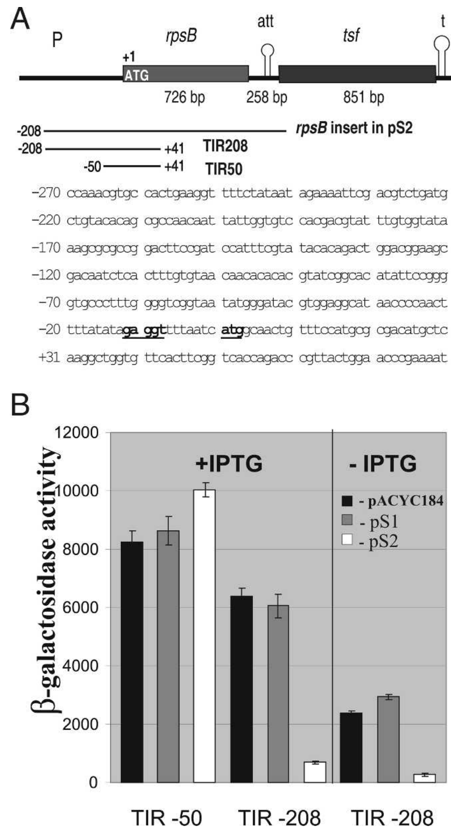

FIGURE 1. S2 serves as a negative regulator of the *rpsB* expression. (*A*) Structure of the *E. coli rpsB-tsf* operon. (P) Promoter; (att) intergenic attenuator; (t) terminator. Regions amplified by PCR for constructing the plasmid pS2 and *rpsB*′-′*lacZ* fusions are shown by lines. (*Bottom*) The sequence of the 5′-UTR and beginning of the *rpsB* coding part; the Shine-Dalgarno element and the start codon are in bold underlined. (*B*) Effects of S2 in *trans* on *rpsB* TIR50 and TIR208 activities in the β-galactosidase assay. Average of three independent assays and standard deviations are shown. An empty vector pACYC184 and its derivative pS1 were used as specificity controls.

plasmid, here referred to as pS2, complemented the temperature-sensitive *rpsB1* allele (Bollen et al. 1979), indicating that it produces biologically active S2 (data not shown), and therefore the 208-bp region in front of the *rpsB* coding frame comprises the promoter. In a wild-type strain, pS2 slowed down the growth rate more than two-fold. The harmful effect of S2 expressing plasmids was noted earlier (Bendiak and Friesen 1981) but still remains to be explained.

Next, we created the *rpsB*′-′*lacZ* chromosomal translational fusions, one of which comprised the same 208-nucleotide (nt) 5′-extension as in pS2 and the beginning of the *rpsB* coding part, while another had a much shorter 5′-UTR of 50 nt in length (Fig. 1A). The corresponding *rpsB* regions were amplified by PCR and inserted behind the *lac*-promoter/operator region of pEMBLΔ46 in-frame with the *lacZ* coding sequence (Dreyfus 1988), generating plasmids pES2TIR208 and pES2TIR50. The *rpsB*′-′*lacZ* fusions were next transferred onto the *lac* locus of the *E. coli* chromosome by homologous recombination as described earlier (Dreyfus 1988; Boni et al. 2000), generating strains LAB-rpsBTIR208::lacZ and LABrpsBTIR50::lacZ. In the first one, the β-galactosidase synthesis is under transcriptional control of both the *lac* promoter/operator and the *rpsB* promoter and governed by the full-length *rpsB* translation initiation region (TIR) at the translation level. The truncated TIR50 in the second construct comprises all conventional elements necessary for ribosome binding, but presumably not the *rpsB* promoter (Fig. 1A).

We then tested the *rpsB*′-′*lacZ* expression for the S2-mediated regulation by measuring the effect of pS2 on the β-galactosidase steady-state synthesis. An empty vector, pACYC184, its derivative pS1 expressing the *rpsA* gene coding for S1 (Skouv et al. 1990; Boni et al. 2000), and the isogenic strain bearing the *rpsA*′-′*lacZ* fusion autoregulated by S1 (Boni et al. 2000, 2001) were used to provide specificity controls. The drop in the β-galactosidase activity of the *rpsB*TIR208-*lacZ* construct in the presence of pS2 was about one order of magnitude compared with pACYC184 and pS1 controls, demonstrating the capability of S2 to function as a negative regulator of its own gene expression (Fig. 1B). The shorter TIR50 governed efficient expression unaffected by the presence of pS2. The latter fact, as well as the absence of the pS2-mediated inhibition of the noncognate *rpsA*′-′*lacZ* fusion (data not shown), provide evidence that the observed S2-mediated repression is specific and requires the region upstream of the position –50 relative to the start codon. Noteworthy, the TIR208 construct, but not TIR50, governed considerable and regulated β-galactosidase synthesis in the absence of *lac*-promoter induction (Fig. 1B), indicating the presence of the *rpsB* promoter(s) in the region between positions –50 and –208.

The single *E. coli rpsB* promoter belongs to the "extended −10" class

Most of the known *E. coli* promoters have been located by identifying transcriptional start sites (Hershberg et al. 2001). To locate the *rpsB* promoter(s), 5′-ends of the *rpsB* in vivo transcripts were mapped by primer extension, using total RNA isolated from exponential *E. coli* cultures and 5′-^{32}P-labeled primers complementary to the beginning of the *rpsB* coding region. Only one extension product with the 5′-terminus corresponding to position –162 (relative to the A+1 in the initiator codon) was observed in sequencing gels, indicating the presence of a single promoter responsible for the *rpsB* expression (Fig. 2).

The identified 5′-position of the *rpsB* in vivo transcript represents most likely a real transcriptional start site (TSS). The TATAAA sequence situated at a proper distance upstream of this point almost perfectly matches the consensus –10 promoter element recognized by σ^{70} subunit

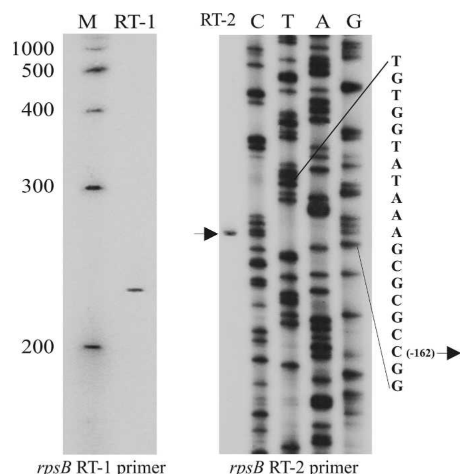

FIGURE 2. Mapping the *rpsB* promoter by primer extension. The products of reverse transcription on total RNA with rpsB_RT1 primer were run along with 5′-^{32}P-labeled ssDNA markers (M). To map the 5′-end of the single *rpsB* transcript, both primer extension on total RNA and pES2TIR208 sequencing were done with the rpsB_RT2 primer.

of RNA polymerase, except for A at the position –7 relative the TSS in place of a highly conserved T. The 5′-TGTG(G)-extension of this –10 hexamer characterizes the *E. coli rpsB* promoter as a member of the minor class of extended –10 promoters (TRTGn-promoters, where R=purine), which exhibit decreased dependence on the –35 promoter region (Kumar et al. 1993; Barne et al. 1997; Burr et al. 2000). The TRTG extension has been shown to ensure a substantial promoter strength due to additional contacts with the region 2.5/3.0 of σ70 (Barne et al. 1997; Burr et al. 2000; Mitchell et al. 2003; Young et al. 2004), stabilizing the transcription initiation open complex (Voskuil and Chambliss 2002). The sequence GCGCGC between the extended *rpsB* promoter and the TSS identified is reminiscent of a GC-rich "discriminator" typical for promoters under growth-rate and stringent control (Pemberton et al. 2000; Haugen et al. 2006, and references therein). Remarkably, analysis of nucleotide sequences upstream of the *rpsB* start codon in fully sequenced bacterial genomes revealed that a combination of the extended −10 promoter with the GC-rich discriminator in front of *rpsB* is highly conserved in γ-proteobacteria, even in distant families (Table 1).

Phylogenetic conservation of the *rpsB* regulatory elements

Conservation of the extended –10 promoter TRTGRTAT AAA specific for the γ-proteobacterial *rpsB-tsf* operons (Table 1) enables comparing *rpsB* 5′-UTRs from diverse species. Although both the primary structure and the length of *rpsB* mRNA leaders appeared to vary significantly, computer modeling (Zuker 2003) revealed striking conservation of their fold (Fig. 3). The *rpsB* leader structure predicted by Mfold consists of two irregular double-stranded regions, left hand (LH) and right hand (RH), separated by a loosely structured (or unstructured, as in *Haemophilus*) central part (central region [CR]). As a rule, the helical regions LH and RH are interrupted by small bulges or internal loops, some of which are highly conserved even in distant species (Fig. 3, encircled). Several specific sequence elements are also remarkably conserved, indicating their potential importance for *rpsB* expression and/or regulation (Table 2). Thus, in most γ-proteobacterial families, the *rpsB* start codon (always AUG) is preceded by a 4–5-nt SD sequence embedded in a AU-rich context, and further upstream there resides a stretch of C residues and an SD-like GUGGAGG sequence, both universally conserved (Table 2). Due to these specific conserved features of the *rpsB* 5′-UTRs we noticed errors in annotation

TABLE 1. Conservation of the extended –10 *rpsB* promoter signature among γ-proteobacteria

Bacteria	Predicted extended *rpsB* promoter, discriminator, and transcription start[a]	Length of 5′-UTR (nt)
Enterobacteriaceae		
Escherichia coli	TGTGGTATAAAGCGCGC**C**	162
Salmonella thyphimurium	TGTGGTATAAAGCGCGC**C**	167
Yersenia pestis	TATGATATAAAGCGCGC**C**	234
Erwinia carotovora	TATGGTATAAAGCGCGC**C**	181
Vibrionaceae		
Vibrio cholerae	TATGATATAAAGCGCGC**C**	158
Photobacterium profundum	TATGGTATAAAGCGCGC**C**	192
Pasteurellaceae		
Pasteurella multocida	TATGGTATAAAGCACCC**C**	145
Haemophilus somnus	TGTGGTATAAAGTGCGA**C**	134
Haemophilus influenzae	TATGGTATAAATCGCCC**C**	131
Pseudoalteromonadaceae		
Pseudoalteromonas haloplanktis	TATGGTATAAAGCGCGC**C**	179
Alteromonadaceae		
Saccharophagus degradans	TGTGGTATAAAGCGCGC**C**	172
Pseudomonadaceae		
Pseudomonas aeruginosa	TATGGTATAAAGCGCCC**C**	126
Pseudomonas putida	TGTGGTATAAAATGCGC**C**	136
Shewanellaceae		
Shewanella oneidensis	TATGGTATAAAGCGCGC**C**	218
Shewanella denitrificans	TATGATATAAAGCGCGC**C**	334
Consensus	TRTGRTATAAA-GC-rich-**C**	

[a]Promoter regions were deduced from the *rpsB* sequences available at the NCBI Entrez Gene searching database. Presumable transcription starts are in bold.

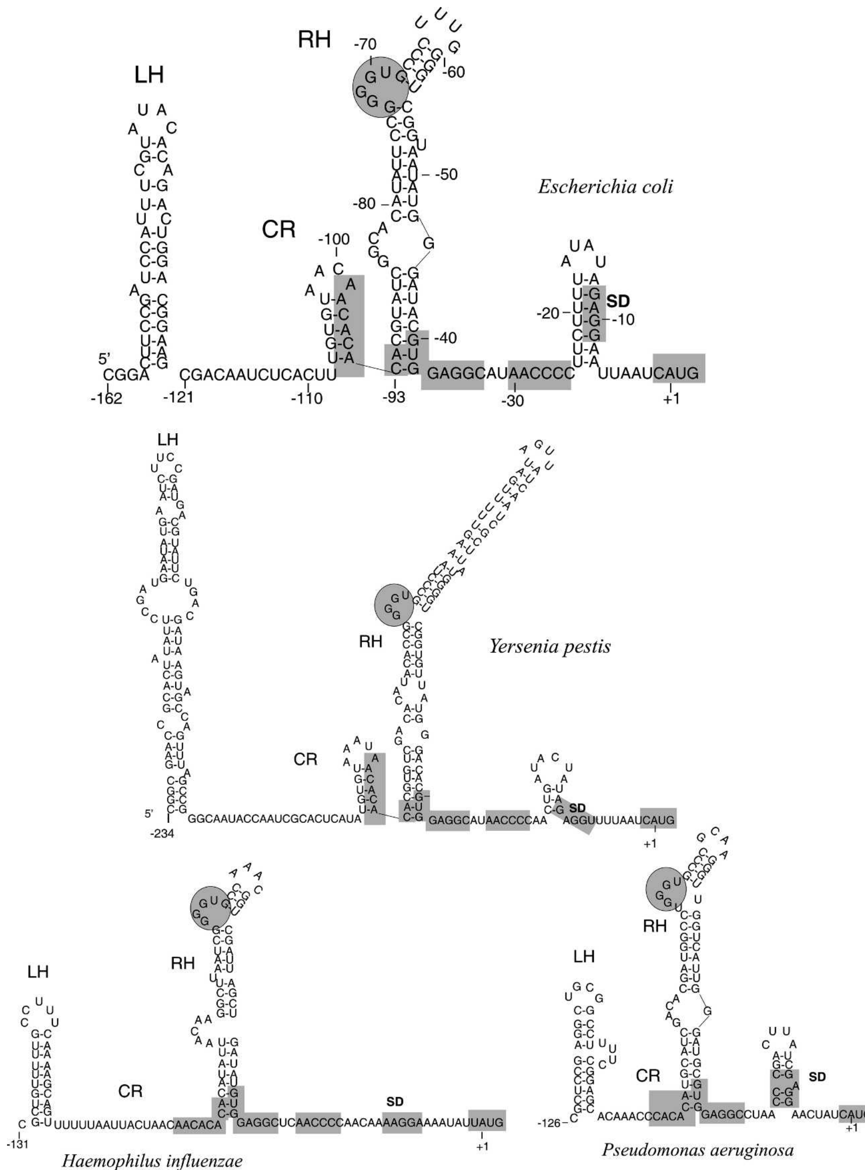

FIGURE 3. Phylogenetic conservation of the *rpsB* 5′-UTR fold in γ-proteobacteria revealed by Mfold program. (LH, RH) Conserved stem–loop structures designated "left hand" and "right hand," (CR) central loosely structured region. In *E. coli*, CR conventionally covers −121 to −93 positions relative to the AUG codon. Conserved elements within 5′-UTRs are shadowed, conserved GGGU-bulges in the *top* part of RH are encircled.

of the *rpsB* start codon in a number of sequenced genomes, e.g., in *E. coli* CFT073 and UTI89, *Haemophilus influenzae* 86-028NP and Rd KW20 (GenBank accession nos. AE014075, CP000243, CP000057, and L42023, respectively).

Thus, both the promoter signature and the 5′-UTR structure of *rpsB* appeared to be conserved in γ-proteobacteria. To find out which is essential for the S2-mediated control, we created two additional *rpsB′-′lacZ* constructs, PrpsBTIR41 and PlacTIR162 (Fig. 4). In the first one, the full-length *rpsB* 5′-UTR was truncated from –162 to –41 positions relative to the initiation codon, so that transcription from the *rpsB* extended promoter started with C–41. In the second one, transcription from a *lac*-promoter started with the authentic A (to keep the specificity of transcription starts for each promoter), immediately followed by the complete *rpsB* TIR (–162 to +41). Because the major *lac*-operator was deleted, the latter construct displayed constitutive expression without induction. The β-galactosidase assay unambiguously demonstrated that the mRNA region upstream of the conventional ribosome binding site, but not the extended promoter, was essential for the *rpsB* autoregulation (Fig. 4).

Deletion analysis of the *rpsB-lacZ* translational fusions

To identify sequence/structure *cis*-acting elements essential for the S2-mediated control, we introduced a series of 5′-truncations and internal deletions into the full-length *rpsB* 5′-UTR within pES2TIR208 (see above) and then created corresponding *rpsB′-′lacZ* chromosomal constructs to measure effects of the mutations on the expression level and inhibition in the presence of pS2. The results are summarized in Table 3. Analysis of β-galactosidase activities in 5′-truncated constructs revealed that the hairpin LH is dispensable, whereas both CR and RH regions are essential for the autocontrol. For internal deletions we chose the conserved features of the *rpsB* TIR and obtained prominent effects on both the expression level and the S2-mediated regulation (Table 3). In particular, deletions of a small G-rich bulge in the upper part of RH, an SD-like sequence upstream of the C-stretch, and the region including the C-stretch all augmented the translation activity of the *rpsB* TIR, simultaneously abrogating autogenous control (Table 3). This implies that within the *rpsB* TIR these universally conserved elements act as negative regulators implicated in autogenous control. A significant increase in the TIR activity and the loss of inhibition by pS2 was also obtained when the conserved G-rich bulge in RH was deleted from the *rpsB* leader of *Pseudomonas aeruginosa*, a γ-proteobacterium very distant from *E. coli* (data not shown). This indicates that the *rpsB* regulation displays higher extent of conservation within γ-proteobacteria than regulation of *rpsA* or S10 operons (Allen et al. 1999; Tchufistova et al.

TABLE 2. Conservation of primary structures of the γ-bacterial *rpsB* mRNAs immediately upstream of the start codon

Bacteria	Sequence[a]	Start codon
Enterobacteriaceae		
E. coli and *Shigellas*	GUGGAGGCAUAACCCCAACUUUUAUAUA**GAGGU**UUUAAUC	**AUG**
Salmonella enterica	GUGGAGGCAUAACCCCAACUUAAUCUAUA**GAGGU**UUUAAAUC	**AUG**
Y. pestis	GUGGAGGCAUAACCCCAACUGAUACUAUA**GAGGU**UUUAAUC	**AUG**
Ervinia carotovara	GUGGAGGCAUAACCCCAUACUUAAUUAAUA**GAGGU**AAUC	**AUG**
Vibrionaceae		
Vibrio cholerae	G UGGAGGCCUAACCCCAUA**GAGG**AUUUUAAA	**AUG**
(V. parahaemolyticus, V. vulnificus)		
Photobacterium profundum	GU GGACGCCUAACCCCAUA**GAGG**AAUUAUUCA	**AUG**
Pasteurellaceae		
Pasteurella multocida[b]	***GUG***GAGGCUAAACCCCAAUUAA**AAGGA**AAUAUUAUU	**AUG**
Haemophilus influenzae[b]	***GUG***GAGGCUCAACCCCAACAA**AA GGA**AAAUAUU	**AUG**
Haemophilus somnus	GUGGAGGCUAAACCCCAAACAA**AAGGA**AAAUUAUCUU	**AUG**
Pseudoalteromonadaceae		
P. haloplanktis	GUGGAGGCCUAACCCUAAACAACUAUA**GAGG**AUCUAUAAA	**AUG**
P. atlantica	AUGGAGGCCUAACCCCAUAU**UAAGGA**AACUAAUAUA	**AUG**
Alteromonadaceae		
Saccarophagus degradans	AUGGAGGCCUAACCCGUAAAAC**AGGA**AUACUAAU	**AUG**
Marinobacter aquaeoli	GUGGAGGACUAACCCGAAGCUAAA**AGGU**AAAUAUC	**AUG**
Pseudomonadaceae		
P. aeruginosa	GUGGAGGCCUAACCCGACUUAUC**GAGG**AACUAUC	**AUG**
P. putida	GUGG AGGCCCAACCCGACUUAUC**AAGGA**ACUAUC	**AUG**
Shewanellaceae		
Sh. oneidensis	GUGGAGGUUCUAACCCCCUAAAUUUUA**AGGU**AAUAGAA	**AUG**
Sh. denitrificans	GUGGAGGUCUAACCCCUUAAUUA**AGGU**AUUAAA	**AUG**
Shewanella sp. MR-4	GUGGAGGUCUAACCCCCUAAAUUUUA**AGGU**AAUAGAA	**AUG**
Idiomarinaceae		
Idiomarina loihiensis	AUG GAGGCUUAACCCCCUAAA**AAGGA**AAAUAUU	**AUG**

[a]Sequences of the 5′-UTR adjacent to the AUG start codon of the *rpsB* genes were found in NCBI Entrez Gene searching database. Initiation codons and SD elements (predicted by their complementarity to the 3′-terminal sequence of 16S rRNA) are in bold; conserved C-stretches and SD-like GUGGAGG sequences upstream of SD are underlined.
[b]***GUG*** in bold italic was erroneously annotated as a start codon of the *rpsB* gene of *Pasteurella multocida* (AE004439), *Haemophilus influenzae* strains Rd KW20 (L42023) and 86-028NP (CP000057), but not in *Haemophilus somnus* (CP000436), where the respective GUG is out-frame relative to the *rpsB* coding sequence.

2003), where the leader structures involved in autocontrol in *E. coli* and *P. aeruginosa* differ entirely.

S2 down-regulates the EF-Ts synthesis by acting at a single target within the *rpsB* 5′-UTR

Earlier works (An et al. 1981; Bendiak and Friesen 1981) failed to identify an additional promoter in front of the *E. coli tsf* gene. Thus, EF-Ts is most likely synthesized from a bicistronic *rpsB-tsf* mRNA, and thereby its synthesis may be controlled by S2. The effect of S2 in *trans* on the EF-Ts steady-state synthesis was studied by Western blotting using polyclonal rabbit antibodies raised against purified Ts (anti-Ts). Significant reduction of the intracellular Ts amount in the presence of pS2, but not pS1, indicated that S2 had a specific negative impact on the Ts synthesis (Fig. 5A). In addition, consistent with the autoregulation circuitry, increase in the *rpsB* gene copies in the presence of pS2 did not proportionally augment the S2 level in a cell (Fig. 5A). Together with the above results obtained with the *rpsB′-′lacZ* fusions, this suggests that both *rpsB* and *tsf* expression are under S2-mediated negative control. Since Ts is a GDP/GTP exchange factor for EF-Tu, and hence an essential player in translation, inhibition of its synthesis is likely one of the major factors causing the growth defect in the presence of pS2.

In many r-protein polycistronic mRNAs, the repressor binding inhibits translation of the downstream cistrons due to translational coupling (Zengel and Lindahl 1994). Since the *rpsB* and *tsf* cistrons are separated by a long 258-nt untranslated region comprising a number of strong stem–loop structures, the translation coupling between them seems improbable. Downstream from the tandem *rpsB* stop codons there resides a 127-nt enterobacterial repetitive intergenic consensus (ERIC) element of unknown function, which forms a long irregular hairpin due to the presence of inverted repeats (Hulton et al. 1991); further downstream, a set of stem–loops forms the terminator/antiterminator region (Merino and Yanofsky 2005). One cannot completely rule out that S2 might independently down-regulate the *tsf* expression by acting at some of these structural elements. To test this possibility, we constructed

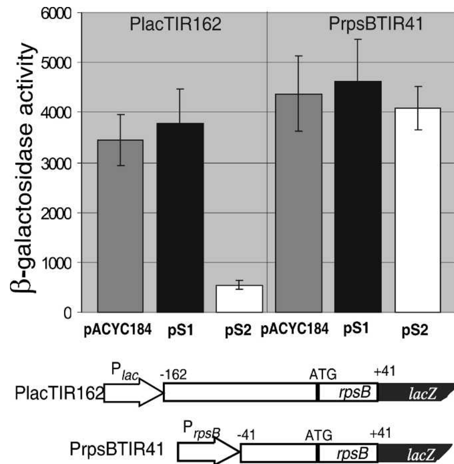

FIGURE 4. The leader part of the *rpsB* mRNA, not the promoter design, is essential for the S2 autoregulation. The β-galactosidase activities in the presence of pACYC184, pS1, and pS2 are shown for the PrpsBTIR41 and PlacTIR162 constructs. PrpsBTIR41 is a derivative of the TIR208 with a deleted promoter-proximal region –162 to –41. In PlacTIR162, transcription from the *lac*-promoter starts from A, which is immediately followed by a complete *rpsB* TIR .

the *tsf′-′lacZ* translational fusion comprising the end of the *rpsB* cistron, the full-length intercistronic region, and the beginning of the *tsf* coding sequence. The β-galactosidase assay clearly showed that this construct is not regulated by S2 in *trans* (Fig. 5B). Furthermore, no detectable expression of *tsf-lacZ* was observed without *lac*-promoter induction, thus confirming the absence of a *tsf*-specific promoter within the *rpsB-tsf* intercistronic region. These results imply that regulation of both *rpsB* and *tsf* cistrons occurs from a single target within the *rpsB* 5′-UTR, and the S2-mediated repression of the *rpsB* translation most probably exerts a polar effect on expression of a downstream *tsf* via impairing transcription–translation coupling within *rpsB*.

Synthesis of S2 and Ts in *rpsB* mutants

The negative control of the *rpsB-tsf* operon by S2 was then confirmed by evaluating effects of *rpsB* mutations on *rpsB* and *tsf* expression. Two previously characterized mutants, *rpsB1*(Ts) and *rpsB11* (*rpsB*::IS*1*), were used in these experiments. The *rpsB1* mutation confers the temperature-sensitive phenotype, i.e., the mutant cannot grow above 40°C and grows much slower than wild-type cells at permissive 37°C (at which the mutant S2 is partially inactivated) because the denaturated S2 is unable to assist in 30S assembly (Bollen et al. 1979). The *rpsB11* mutation is an insertion of a transposable element IS*1* between the *rpsB* stop codons and ERIC, which destabilizes the *rpsB* mRNA, thereby decreasing the intracellular S2 level (Shean and Gottesman 1992). The *rpsB11* cells form tiny colonies on agar plates and grow more than two times slower than *rpsB*$^+$ in liquid medium (data not shown).

The two mutations were P1 transduced in strains bearing *rpsB′-′lacZ* fusions (see Materials and Methods). Western blot analysis revealed that in *rpsB1* the level of EF-Ts is visibly higher than in wild-type (wt) cells, whereas in *rpsB11* it is dramatically reduced (Fig. 6A). The increased Ts level in *rpsB1* indicates the weakening of the S2-mediated repression, which was further confirmed by the β-galactosidase assay. The activity of the unregulated TIR50 construct did not alter in the presence of the mutations, whereas the activity of TIR208 increased about threefold in *rpsB1* and more than sixfold in *rpsB11* (Fig. 6B). These data indicate that normally (in *rpsB*+ background) the *rpsB-tsf* expression is partially repressed by S2 synthesized in excess over ribosomes, and in both mutants this intrinsic autogenous control is impaired. Analogous partial repression in wt cells and derepression caused by mutations in a repressor-encoding gene were previously shown for the *rpsA* (Boni et al. 2000), *rpsO* (Mathy et al.

TABLE 3. Effects of 5′-truncations and internal deletions within the 162-nt *rpsB* 5′-UTR upon the activity of the *rpsB* TIR and its autocontrol by S2

	β-galactosidase activity[b]		
Deletion description[a]	pACYC184	pS2	Repression[c]
Wild-type (162 nt)	6400 ± 250	680 ± 40	9.4
ΔLH (121 nt)	7120 ± 1090	890 ± 180	8.0
ΔLH, CR (93 nt)	7520 ± 1650	8200 ± 1780	0.9
ΔCR, RH (−121 to −41)	2890 ± 240	1850 ± 50	1.56
“Short-RH” (−81 to −48)	25,250 ± 2560	24,160 ± 2500	1.05
ΔGGGU (−72 to −69)	67,700 ± 8500	73,400 ± 6700	0.9
ΔCR (−121 to −93)	30,870 ± 4200	33,300 ± 3500	0.92
ΔGUGGAGG (−40 to −33)	35,700 ± 4000	37,200 ± 6500	0.96
Δ (−33 to −23)	10,600 ± 1500	8900 ± 1100	1.2

[a]The length of the 5′-UTR for 5′-truncations or exact positions of deleted regions are indicated (in parenthesis).
[b]Nanomoles of ONPG hydrolyzed per min and per mg of total soluble protein. Average of three or more independent assays and standard deviations are shown.
[c]Ratio of the β-galactosidase activities in cells carrying empty vector (pACYC184) and its S2-expressing derivative (pS2), respectively.

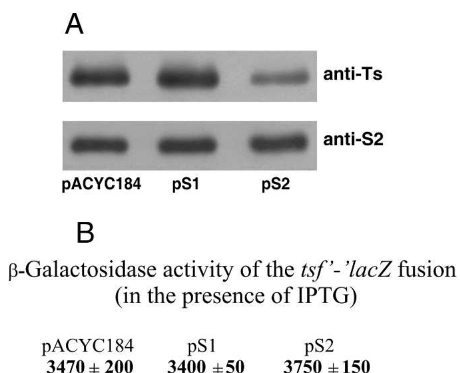

FIGURE 5. Effects of S2 in *trans* on the *tsf* expression as revealed by Western blotting (*A*) and the β-galactosidase assay of the *tsf-lacZ* translational fusion (*B*).

2004), and *rnc* (Matsunaga et al. 1996) autoregulatory circuits. Most likely, in *rpsB1* the mutant S2 lost, in part, its repressor activity at 37°C because of partial denaturation. Consistently, the presence of pS2 makes the TIR208 activity in *rpsB1* indistinguishable from that in *rpsB*+/pS2 cells (Fig. 6B). The absence of a visible increase in the intracellular S2 level in *rpsB1* (Fig. 6A) might be accounted for by proteolytic instability of the denatured S2.

Destabilization of the *rpsB* mRNA in *rpsB11* (Shean and Gottesman 1992), confirmed for our strains (data not shown), causes only a modest decrease in the S2 level (Fig. 6A). By destabilizing the *rpsB* mRNA, the *rpsB11* mutation slows down accumulation of S2 in a cell, so that all synthesized S2 associates with 30S and no superfluous S2 to repress its own mRNA is generated, hence, an apparent increase in the *rpsB* TIR activity (Fig. 6B). The fact that a small amount of mRNA can supply a sufficient amount of the essential r-protein due to abolishing intrinsic autogenous repression emphasizes a vital importance of this control mechanism.

The dramatic reduction of the Ts level in *rpsB11* (Fig. 6A) results from a polar effect of IS*1* on the *tsf* expression. Transcriptional terminator within IS*1* (Hubner et al. 1987) is supposed to impede synthesis of the bicistronic *rpsB-tsf* mRNA as a source of Ts, because unlike IS*10* (Ciampi et al. 1982) IS*1* does not possess its own outward promoter for transcription of downstream genes, it may only provide the −35 region for endogenous "−10"-like sequences (Prentki et al. 1986). Nevertheless, the *rpsB11* cells are still able to synthesize Ts, though at a reduced level. Two possibilities can be considered: either polarity is not complete or IS*1* is capable of activating a cryptic promoter in the intergenic sequence. In this regard, the fact that the *rpsB11* cells cannot be transformed by pS2 gives a strong argument in favor of the first variant. Indeed, if a small amount of Ts in *rpsB11* is produced from a bicistronic mRNA synthesized due to incompleteness of polarity, it should be further reduced in the presence of pS2 because of resuming S2-mediated repression (see Fig. 5A), making the cell non-viable. To prove this directly, we transformed the *rpsB11* cells with two compatible plasmids, pS2 and pTrc99A-tsf (Karring et al. 2004), expressing the *tsf* gene under the control of the *trc*-promoter. The lethal effect of pS2 was suppressed by the second plasmid that supplied Ts necessary for survival, thus providing strong evidence for this interpretation.

Protein S1 may assist S2 in *rpsB* autoregulation

Surprisingly, the activity of the *rpsB* TIR in the TIR-208 construct appeared to be also augmented in the presence of the *rpsA*::IS*10* (*ssyF29*) mutation (Fig. 7A). This mutation causes production of the reduced amount of truncated protein S1 and confers slow-growth phenotype, because insertion of the IS*10* mobile element within the 3′-terminal part of the *rpsA* gene destabilizes the *rpsA* mRNA (Boni et al. 2000). The observed increase in the TIR activity (by about 50%, compared with the TIR208 activity in wild-type cells) disappeared in the presence of pS1 (Fig. 7A), indicating that the enhancing effect is relevant to the S1 mutation. Moreover, S2 in *trans* did not repress the *rpsB* TIR208 activity in *rpsA*::IS*10* cells to the same extent as in $rpsA^+$ background, implying that the S2 autoregulation requires a normal supply of wild-type S1. To verify if this is

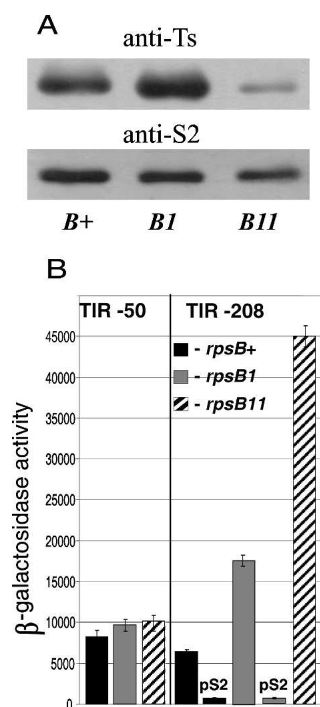

FIGURE 6. Effects of the mutations *rpsB1*(ts) and *rpsB11* (*rpsB*::IS*1*) on the *rpsB-tsf* expression. (*A*) Western blotting to evaluate the S2 and Ts level in the *rpsB* mutants. (*B*) β-Galactosidase activities of the *rpsB′*-*′lacZ* constructs were measured for cells exponentially grown in the presence of pACYC184 for TIR50, or pACYC184 and pS2 for TIR208. The *rpsB11* cells cannot be transformed with pS2.

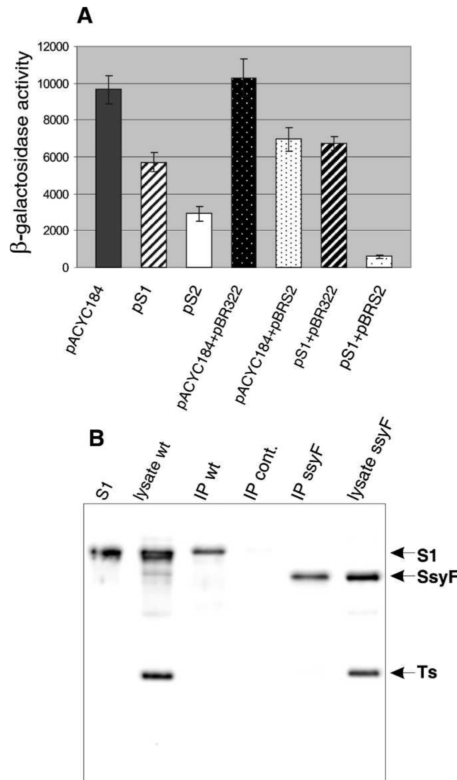

FIGURE 7. The repressor activity of S2 depends on S1 concentration in a cell. (*A*) The reduced repressor activity of S2 in the *ssyF29* (*rpsA*::IS*10*) mutant producing a subnormal amount of truncated S1. The β-galactosidase activities were measured for TIR208 in the presence of pACYC184, pS1, and pS2, and in a biplasmid system where the high repression level can be achieved only when pS1 and pBRS2 (expressing wt S1 and S2) are simultaneously present. (*B*) Immunoprecipitation of S2–S1 complex from wild-type (IP wt) and *ssyF29* cell lysates (IP ssyF) in the presence of polyclonal goat antibodies against S2. Western blotting of 12.5% PAAG revealed with anti-S1 and anti-Ts rabbit polyclonal antibodies (S1) −50 ng of purified S1; (lysate wt and lysate ssyF) 2 μg of total soluble cell proteins from rpsA$^+$ and *ssyF29* exponentially grown cells; (IP cont) a specificity control showing that no S1 is precipitated from wt cell lysate in the presence of goat polyclonal antibodies against ribosomal protein S15.

the case, we cloned the *rpsB* gene into pBR322 (Amp^r) compatible with pS1 (Cm^r), generating a new construct expressing S2 and designated pBRS2. The β-galactosidase assay confirmed the decreased S2-mediated inhibition of the *rpsB*TIR208-*lacZ* expression in the presence of *rpsA*::IS*10* mutation and resuming autogenous regulation in the presence of both pBRS2 and pS1 in a cell (Fig. 7A). These results imply that S1 may assist S2 in regulating the *rpsB* expression, a so-far unknown activity for this multifunctional ribosomal protein. In this regard, several possibilities can be considered. Primary binding of S1 to the *rpsB* 5′-UTR due to its pronounced RNA-binding capacity (Subramanian 1983) could modify the RNA structure and facilitate further binding of S2 to its operator site to inhibit the *rpsB* expression. Alternatively, the preformed complex S1–S2 (both synthesized in excess over ribosomes) may act as a repressor. Interaction of S1 with S2 in the 30S subunit (Moll et al. 2002) and the described purification of a stoichiometric S1–S2 complex (Sukhodolets and Garges 2003) argue in support of the latter variant. Additional arguments were obtained using immunoprecipitation. A substantial amount of S1 was found in a protein fraction coprecipitated with S2 on the Protein A/G PLUS agarose in the presence of highly specific goat antibodies against S2 (Fig. 7B). Further studies (mainly in vitro experiments) are necessary to define the exact mode of cooperation of S2 and S1 in the *rpsB* autoregulation found here.

DISCUSSION

This work presents new data necessary for correct description of the essential *E. coli rpsB-tsf* operon, including its transcription organization and expression regulation. We report for the first time that transcription of the operon is governed by a single promoter TGTGGTATAAA belonging to the extended –10 promoter class, a rare case among ribosomal protein operons. A GC-rich spacer ("discriminator") separating the *rpsB* promoter from the transcription start site suggests that the *rpsB-tsf* expression may be subjected to the growth rate control and stringent response (Pemberton et al. 2000; Haugen et al. 2006). The design of the *rpsB* promoter region appeared to be highly conserved in γ-proteobacteria. This conservation was supported experimentally. Corresponding DNA regions from *Yersinia pestis*, *H. influenzae*, and *P. aeruginosa* inserted in front of the *E. coli* chromosomal *lacZ* gene indeed possessed promoter activity (will be published elsewhere). It is currently difficult to appreciate the occurrence and conservation of this rare promoter type that combines the extended –10 motif TRTGRTATAAA, a GC-rich discriminator, and a rare C at the transcription start position (Table 1). Some advantages over predominant −35/−10 σ70 promoters may result from the increased tolerance to low temperatures (Phadtare and Severinov 2005, and references therein) and to anti-σ70 proteins similar to T4 AsiA that block interactions of σ70 with the –35 DNA region (Pineda et al. 2004). Further investigation is required to find the rationale for this peculiar design of the *rpsB* promoter and to characterize its regulatory features.

Not only the extended *rpsB* promoter (Table 1), but also a secondary structure of the *rpsB* 5′-UTR (Fig. 3) and several primary sequence elements upstream of the initiator AUG codon (Table 2) are highly conserved in γ subdivision of proteobacteria, thereby facilitating a correct annotation of *rpsB* genes in newly sequenced γ-bacterial genomes. It should be mentioned that a single *rpsB* transcription start experimentally determined here coincides with the annotated 5′-end of a so-called RNA T44, a small 136-nt-long RNA predicted on the basis of RNA structure conservation in the intergenic regions (Rivas et al. 2001; Hershberg et al.

2003). The corresponding gene *t44* (*tff*) as residing in front of the *E. coli rpsB* gene was included in RegulonDB, the reference database of *E. coli* K-12 regulatory network and operon organization (http://regulondb.ccg.unam.mx/). Given that the hypothetical *t44* gene appears to share the promoter with *rpsB* and, in addition, does not possess any reminiscent of transcription terminator, it seems very likely that the reported conservation actually reflects not the presence of a gene for sRNA with still-unknown functions, but the conservation of the *rpsB* 5′-UTR structure involved in the S2 autoregulation (the *rpsB* operator). Phylogenetic conservation among γ-proteobacteria of the hairpin structures implicated in autogenous control is typical for r-protein mRNAs (Allen et al. 1999, 2004; Boni et al. 2001; Tchufistova et al. 2003; Guillier et al. 2005), and the *rpsB* mRNA represents one more prominent example (Fig. 3).

Our findings demonstrate that r-protein S2, like some other r-proteins, has an extraribosomal function in *E. coli* and in related bacteria as a negative effector of expression of the essential *rpsB-tsf* operon. In all regulated r-protein operons, a repressor protein binds to the mRNA, preventing its translation (Zengel and Lindahl 1994). As we show here, the S2-mediated repression similarly operates at the RNA and not at the DNA level (Fig. 4). Both *rpsB* and *tsf* are negatively regulated by S2 from a single operator located within the *rpsB* 5′-UTR. Although the results obtained in vivo do not yet enable a detailed description of the operator site, deletion analysis shows that a large portion of the 5′-UTR is implicated in S2-mediated control, including several highly conserved *cis*-regulatory elements situated upstream of the conventional ribosome binding site (RBS). The fact that autogenous regulation requires a long structured region upstream of the start codon (Table 3) suggests that a simple direct competition between S2 and 30S for RBS binding is unlikely, rather more complex mechanisms (presumably S2-mediated structure rearrangements are inhibitory for 30S initiation complex formation) are involved.

S2 presents an unusual example of a regulatory r-protein, being a tertiary (one of the latest) and not a primary protein in ribosome assembly (Mizushima and Nomura 1970; Culver 2003), i.e., unlike most of the r-protein repressors, S2 is unable to recognize naked rRNA. Most of the primary r-proteins acting as autogenous regulators (e.g., S7, S8, S15, L1, L4, L20) recognize similar targets on free rRNA and on mRNA of its cognate operon (Zengel and Lindahl 1994; Guillier et al. 2002, 2005; Stelzl et al. 2003; Mathy et al. 2004), indicating that the autogenous control is based on molecular mimicry as was originally proposed by Nomura (Nomura et al. 1980). A detailed analysis of the refined crystal structure of the *T. thermophilus* 30S subunit at 3.05Å resolution revealed that S2 is located on the back of 30S and spans the head-body hinge region by forming contacts with 16S rRNA helices H26 in the body and H35–H37 in the head of the subunit (Brodersen et al. 2002). Direct contacts of S2 with a group of helices H35–H37 were also revealed by chemical probing (Powers et al. 1988). Although no visible resemblance exists between 16S RNA elements involved in S2 binding and conserved features within the *rpsB* 5′-UTR, it is too early to conclude that the S2-mediated inhibition is not based on molecular mimicry until the data on exact contacts between S2 as a repressor and its operator become available.

A noteworthy feature of the S2 autoregulation is that S2 alone seems to be unable to effectively repress the *rpsB-lacZ* expression. Indeed, while in wild-type cells the presence of the S2 expressing plasmid pS2 exerts a strong inhibitory effect (Fig. 1B), in the *rpsA*::IS*10* mutant (*ssyF29*, see Boni et al. 2000), which produces a subnormal amount of truncated S1, the repression is obviously reduced (Fig. 7A). Complete restoration of the repression can be obtained after simultaneous transformation of the *ssyF29* cells with compatible plasmids pS1 and pBRS2 (Fig. 7A), suggesting that S2 probably cooperates with S1 in negative regulation of *rpsB*, a unique case for autogeneously controlled r-protein operons. The only case described so far when a complex of r-proteins serves as a translational repressor is the translational inhibition of the *rplJL* operon by a pentameric complex L10(L7/12)$_4$ (Johnsen et al. 1982). However, in this case, both r-proteins are products of the same operon, their pentameric complex is very stable and forms an important functional domain of the 50S ribosomal subunit. As we revealed by immunoprecipitation, S1 and S2 are capable of forming the complex; moreover, S2 coprecipitates also with a truncated variant of S1 from the *ssyF29* mutant (Fig. 7B). This suggests that the reason for the decreased S2 repressor capacity in *ssyF29* is not the C-terminal S1 truncation, but most likely the scanty concentration of S1 in mutant cells. More details about cooperation of S1 and S2 to regulate the *rpsB* expression may be obtained in experiments in vitro that are currently in progress.

As we show here (Fig. 6A), autogenous inhibition of the *rpsB* expression exerts a polar effect on the downstream *tsf* gene. Two plausible reasons for this polarity may be considered. First, the S2 binding to the 5′-UTR promoted by S1 may provoke a rearrangement of the mRNA leader structure inhibitory for translation-initiation complex formation; in this case, translational repression of the *rpsB* mRNA may exert a polar effect on the *tsf* expression via breaking the transcription–translation coupling within *rpsB*. Alternatively, the S2–mRNA complex itself or in concert with other factors may directly stimulate preliminary transcription termination, as in the case of the L4-mediated repression of the S10 operon (Zengel and Lindahl 1994; Stelzl et al. 2003). Future experiments (mainly in vitro) will shed light upon the fine mechanism underlying the S2-mediated negative control of the *rpsB-tsf* expression.

MATERIALS AND METHODS

Bacterial strains and plasmids

Genetic constructs harboring single-copy *rpsB′*-*′lacZ* fusions were derivatives of a Lac⁻ENSO strain bearing a short chromosomal deletion encompassing the *lac*-promoter/operator region and the *lacZ* RBS (formerly HfrG6Δ12) (Dreyfus 1988). The *rpsB* fragments comprising 5′-UTR of variable length and the beginning of the coding sequence (Fig. 1) were amplified by PCR on the *E. coli* genomic DNA, using forward primers bearing BamHI site (italicized): −208for (5′-GTG*GGATCC*AACAATTATTGGTGTC) and −50for (5′-TATG*GGATCC*GTGGAGGCATAACC), and a common reverse primer S2TIRrev (5′-CCG*AAGCTT*ACACC AGCCTTGAG) complementary to the *rpsB* coding region +28 to +41 and bearing HindIII site (italicized). The resulting PCR products were treated with BamHI and HindIII and then cloned in-frame with the *lacZ* sequence of pEMBLΔ46 (Dreyfus 1988), generating pES2TIRn plasmids, where n is the *rpsB* 5′-UTR length. Given the absence of the *lacZ* expression in pEMBLΔ46 because of the *lacZ* RBS deletion, proper in-frame clones can be readily identified by white-blue selection and confirmed by sequencing.

Deletion variants of pES2TIR208 were constructed by using *PfuUltra* II fusion HS DNA polymerase (Stratagene) in reverse PCR, with primers flanking the site to be deleted (to amplify the whole plasmid except for the region chosen for deleting). PCR products were purified on agarose gels, then ligated, treated with DpnI, and used to transform DH5α for selecting Lac^+ clones.

All constructs were checked by sequencing and then used for transformation of ENSO. In transformed cells, the *rpsB-lacZ* fusions and the upstream *lac*-promoter/operator region were transferred onto the chromosome via homologous recombination (Dreyfus 1988; Boni et al. 2000), generating recombinant Lac^+ clones designated LABrpsBTIRn::lacZ. The *rpsB1* (Bollen et al. 1979) and *rpsB11* (Shean and Gottesman 1992) alleles were transferred into the strains by P1 transduction. The marker Tn*10* from CAG18436 (Singer et al. 1989) was cotransduced with the *rpsB* mutations to provide selection of the tetracycline-resistant transductants. The *rpsB1* allele in selected clones was further confirmed by its ts phenotype, and the *rpsB11* clones differing from $rpsB^+$ colonies by slow growth on agar plates were checked by PCR for the presence of IS*1*. The *ssyF29* (*rpsA*::IS*10*) allele was P1 transduced as described (Boni et al. 2000).

To create the *tsf′*-*′lacZ* fusion, a chromosomal region encompassing the end of *rpsB*, the intercistronic region, and the beginning of the *tsf* coding frame was amplified by PCR on the *E. coli* DNA with primers rpsBend-for 5′-CGC*GGATCC*GT AGAAGCTGAGTAATAAGGC (bearing BamHI site and overlapping the tandem of the *rpsB* stop codons) and tsfTIR-rev 5′-CATC*AAGCTT*GCGCCAGTACGCT (bearing the HindIII site and complementary to the *tsf* coding sequence +40 to +63 relative to the *tsf* start). The PCR fragment was cloned in pEMBLΔ46/BamHI, HindIII, with further manipulations to create the strain LABtsfTIR::lacZ being exactly the same as described above for the *rpsB-lacZ* fusions.

To build plasmids for S2 expression, the *rpsB* structural gene flanked by the 208-nt 5′-UTR and the *rpsB-tsf* intergenic region including the attenuator was amplified from the *E. coli* genomic DNA using primers 208for (see above) and rpsBrev (5′-CAG*GTC GAC*CTCGGAGATGTGATCTG) bearing SalI site (italicized). The PCR product treated with BamHI and SalI was ligated into respective sites within the *tet*-regions of pACYC184 or pBR322. The resulting plasmids able to complement the temperature-sensitive phenotypes of the *rpsB1* mutant (Bollen et al. 1979) were designated pS2 (pACYC184 derivative) and pBRS2 (pBR322 derivative).

Cell growth and β-galactosidase assay

Cell cultures were grown at 37°C in LB medium supplemented, if necessary, with IPTG (0.2 mM) and appropriate antibiotics and harvested in exponential phase ($OD_{600} \cong 0.4$–0.5). The β-galactosidase-specific activity was measured in clarified cell lysates essentially as described (Tchufistova et al. 2003) and expressed in nmol ONPG (o-nitrophenyl-β-D-galactopiranoside) hydrolyzed per minute per milligram of total soluble cell proteins. Protein concentrations in lysates were determined by Bradford assay (Bio-Rad).

Localization of the *rpsB* promoter by primer extension

E. coli cells were grown at 37°C in LB medium to $OD_{600} = 0.5$. Total RNA was isolated from 6 mL culture by using Aurum Total RNA Mini Kit (Bio-Rad) according to the manufacturer's protocol. Two 5′-^{32}P-labeled primers complementary to the beginning of the *rpsB* coding part were used for primer extension by AMV reverse transcriptase (Promega) on 5−6 mkg of isolated total RNA: rpsB_RT1 5′-GTTCCAGTAACGGGTCTGGTGACC (+49 to +72) and rpsB_RT2 5′-GTCGCGCATGGAAACAGT TGCCATG (–1 to +24). RT products were run on 6% sequencing PAAG along with 5′-^{32}P-labeled ssDNA markers (rpsB_RT1 extension on Fig. 2) or sequencing reactions for pES2TIR208 (Fig. 2, rpsB_RT2 extension).

Western blotting

Total soluble proteins prepared as for the β-galactosidase assay were analyzed in 10% Laemmli gel (5 μg per lane) and transferred onto nitrocellulose membrane (Bio-Rad). The blots were successively revealed with polyclonal rabbit antibodies raised against purified EF-Ts (prepared especially for this work) and with polyclonal goat anti-S2 antibodies (a gift of R. Brimacombe, Max-Planck-Institut, and O. Dontsova, Moscow State University). Secondary anti-rabbit (Bio-Rad) and anti-sheep/goat (Promega) horseradish peroxidase-labeled antibodies were used for visualization with the Immun-Star HRP Chemiluminiscent reagent (Bio-Rad) and Bio-Rad VersaDoc MP4000 image station.

Immunoprecipitation

Immunoprecipitation was carried out according to the protocol of Santa Cruz Biotechnology with minor modifications. Freshly prepared lysates of the exponentially grown *E. coli* cells (100 μg of total soluble proteins), were incubated with 3 μL (about 1 μg) of polyclonal goat anti-S2 antibodies and 10 μL 100 mM PMSF (phenylmethylsulfonyl fluoride) in 1 mL of PBS for 2 h at 4°C using a rotating device. In a control sample, the same amount of polyclonal goat anti-S15 antibodies was used. Then, 20 μL of Protein A/G PLUS-Agarose (Santa Cruz Biotechnology) was added to each tube, and the resulting mix was further incubated under rotation at 4°C overnight. Agarose pellets were collected by

centrifugation at 5000 rpm for 30 sec and supernatants were carefully discarded. The pellets were washed three to four times with PBS, each time repeating the centrifugation step above. After final supernatant discarding, pellets were resuspended in 40 μL of 2x Laemmli sample buffer, boiled for 3 min, and 10 μL of each probe was loaded on 12.5% Laemmli gel and run along with protein markers and 1−2 μg of the initial cell lysate. Immunoblotting was then performed using polyclonal rabbit antibodies against S1 and Ts (the latter to provide specificity control) as primary antibodies and goat-anti-rabbit horseradish peroxidase-labeled secondary antibodies. The Immun-Star HRP Chemiluminiscent reagent (Bio-Rad) and Bio-Rad VersaDoc MP4000 image station were used for visualization.

ACKNOWLEDGMENTS

We thank Max Gottesman for CS509 (*rpsB11*), Isabelle Iost for the *rpsB1* strain, Vyacheslav Kolb for EF-Ts, Charlotte Knudsen for plasmids pTrc99A and pTrc99A-tsf, Elena Svirshevskaya for preparing rabbit polyclonal anti-Ts antibodies, Richard Brimacombe and Olga Dontsova for goat anti-S2 antibodies. We are indebted to Mathias Springer for helpful discussion. This work was supported by RFBR grant 06-04-48353 and partly by the Program of the Presidium of RAS for Basic Research in Molecular and Cellular Biology.

Received March 26, 2008; accepted May 12, 2008.

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