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Expression of Both *Bacillus subtilis* Threonyl-tRNA Synthetase Genes Is Autogenously Regulated

NATHALIE GENDRON, HARALD PUTZER,* AND MARIANNE GRUNBERG-MANAGO

Service de Biochimie, Centre National de la Recherche Scientifique URA 1139, Institut de Biologie Physico-Chimique, 75005 Paris, France

Received 3 August 1993/Accepted 2 November 1993

The "housekeeping" threonyl-tRNA synthetase gene (*thrS*) of *Bacillus subtilis* is shown to be transcribed in vivo and in vitro from a single promoter. In vitro, 85% of all messages transcribed from the *thrS* promoter are terminated at a strong factor-independent terminator localized upstream of the *thrS* Shine-Dalgarno sequence, within the 305-nucleotide-long leader region. Overexpression of *thrS* represses transcriptional and translational *thrS-lacZ* fusions to a similar extent, suggesting that *thrS* is autoregulated at the transcriptional level. We show that autogenous control does not act at the level of transcription initiation but involves antitermination of the transcription mechanism. *thrZ*, the second threonyl-tRNA synthetase gene, is also autogenously regulated. However, the ability of the ThrS synthetase to repress *thrS* as well as *thrZ* expression is much greater than that of the ThrZ synthetase.

In *Bacillus* species, most of the known aminoacyl-tRNA synthetase genes seem to be regulated by a similar mechanism involving antitermination of transcription. This assumption is based on the presence of strongly conserved structural elements in their regulatory regions (12, 14, 22; for a review, see reference 23); an 18-nucleotide (nt) consensus sequence called the T-box, followed by a stem-and-loop structure upstream of the Shine-Dalgarno sequence. Upstream leader sequences comprise domains of conserved secondary structure, with a codon specific for the appropriate amino acid at the identical position in each sequence. In the case of *tyrS*, this codon has been shown to be sufficient for the specificity of derepression following tyrosine starvation (12). Of the 12 aminoacyl-tRNA synthetase genes cloned and sequenced so far, only 3 do not present the conserved elements in their leader region: *Bacillus stearothermophilus metS* (17), *Bacillus subtilis metS* (which nevertheless contains a stem-loop structure upstream of the Shine-Dalgarno sequence [18]), and *B. subtilis gltX* (4). Furthermore, certain amino acid biosynthesis operons in *B. subtilis*, *ilv-leu* (10) and *cysE-cysS* (4), and in *Lactobacillus* species, the *trpDCFBA* operon of *Lactobacillus casei* (19) and the *trpEGDCFBA* and *his* operons of *Lactococcus lactis* (2, 7), also carry these conserved elements. This situation is in contrast to that in *Escherichia coli*, for which there is no evidence for a general mechanism of regulation (11, 23).

Two independent genes, *thrS* and *thrZ*, encode two isozymes of threonyl-tRNA synthetase, ThrS and ThrZ, respectively, in *B. subtilis* (21). Expression of both genes has been shown to be regulated by antitermination of transcription, but only *thrS* is expressed under normal growth conditions (22). Disruption of *thrS* or a decrease in the intracellular ThrS concentration induces *thrZ* expression. Both *thrS* and *thrZ* are derepressed under starvation conditions for the cognate amino acid but not for other amino acids, such as tryptophan or arginine (22). Destruction of the T-box leads to an uninducible phenotype and a dramatic drop (>60-fold) in the basal level of expression (22). The T-box element has been shown to be essential for derepression of *B. subtilis tyrS* after tyrosine starvation (14). The terminator structure itself and the T-box are functionally related. Destruction of the T-box has no effect on *thrS* expression in the absence of a functional terminator (22).

Here we show that expression of the *B. subtilis thrS* gene influences not only *thrZ* expression but also the expression of its own gene. Our data suggest that *thrS* is autogenously controlled at the transcriptional level, involving an antitermination mechanism. Similarly, expression of the *thrZ* gene is also autoregulated. However, in contrast to ThrS, overexpression of ThrZ influences *thrS* expression only slightly.

MATERIALS AND METHODS

Strains. *E. coli* JM109 [*recA1 endA1 gyrA86 thi hsdR17 supE44 relA1* λ⁻ Δ(*lac-proAB*) F′ (*traD36 proAB lacI*^{q} *lacZ*ΔM15)] was used for plasmid construction except when concatemer plasmids were needed for *B. subtilis* transformation. In that case, *E. coli* JM101 [*thi supE44* Δ(*lac-proAB*) F′ (*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15)] was used. *E. coli* RZ1032 (*thi-1 supE44 relA1 spoT1 dut-1 ung-1 zbd*::Tn*10*) and helper phage M13KO7 were used to prepare single-stranded plasmid DNA templates for site-directed mutagenesis (15). The *B. subtilis* strains used are listed in Table 1.

Transformation of strains. *E. coli* and *B. subtilis* cells were transformed as described before (6, 13). *E. coli* plasmid transformants were selected on LB plates supplemented with ampicillin (100 μg/ml). The antibiotics used for selection of chromosomal plasmid integrants of *B. subtilis* were added at 5 μg/ml for chloramphenicol and for kanamycin, and those used for selection of replicative plasmids were added at 20 μg/ml for tetracycline and 0.5 μg/ml for phleomycin.

Plasmid constructions. For pHM2, the *Sal*I site at position 5164 of pDG268 (1) was deleted by *Sal*I partial digestion, Klenow filling, religation, and transformation. Clones lacking this site were selected by double digestion. This treatment leaves a unique *Sal*I site at bp 3725 in *lacZ* for the construction of protein fusions.

For pHMS2, a 0.9-kb *Dra*I-*Eco*RI fragment of plasmid P33 (20) containing the *thrS* leader and 535 bp of the structural gene was inserted into the Bluescript KS+ vector (Stratagene) digested with *Sma*I and *Eco*RI.

* Corresponding author. Mailing address: Institut de Biologie Physico-Chimique, 13, rue Pierre et Marie Curie, 75005 Paris, France. Phone: 33-1-43298226. Fax: 33-1-40468331. Electronic mail address: putzer@ibpc.fr.

TABLE 1. *B. subtilis* strains

Strain	Relevant genotype	Integrated plasmid construct(s)	Source or reference
1A2	Prototroph		BGSC[a]
HP18	*amyE*::*thrS'-lacZ*	pHMS11	22
HP20	*amyE*::*thrZ'-lacZ thrS*::*erm*	pHMZ9, M13tgS10	22
HP25	*amyE*::*thrS'-'lacZ*	pHMS12	This study
HP29	*amyE*::*thrZ'-lacZ* Δ*thrS*::*kan*	pHMZ9, pHMS15	22
HP45	*amyE*::*thrS'* (*Eco*NI)-*lacZ*	pHMS21	22
HP47	Δ*thrS*::*kan*	pHMS15	This study
HP79	*amyE*::*thrS'-lacZ* Δ*thrS*::*kan*	pHMS11, pHMS15	This study
HP124	*amyE*::*thrS'* (*Afl*III)	pHMS30	This study

[a] *Bacillus* Genetic Stock Center, Columbus, Ohio.

For pHMS12, a *thrS-lacZ* translational fusion was constructed by inserting a *thrS Bam*HI-*Cla*I (position 142 of the *thrS* structural gene, treated with mung bean nuclease to produce a blunt end) fragment from pHMS2 into pHM2 digested with *Bam*HI and *Sal*I (blunted by mung bean nuclease). The correct reading frame was confirmed by DNA sequencing (24).

Plasmid pHMS15 is based on a recombinant pUC18 plasmid carrying a 3-kb *Eco*RV fragment which contains the *B. subtilis thrS* gene downstream of nt 75 of the structural gene. A 930-bp *Stu*I fragment, internal to *thrS*, was replaced with a kanamycin resistance cartridge from transposon Tn*903* to give pHMS15. It was used to disrupt the *B. subtilis thrS* chromosomal copy after integration by double-crossing-over recombination.

A 0.4-kb *Cla*I fragment was deleted from plasmid pHMS2 to give pHMS17. This deletion comprises all *thrS* sequences downstream of the *Cla*I site (ordinate 507 in reference 21). The truncated insert contains the *thrS* leader and 142 bp of the structural gene.

For pHMS23, a 0.44-kb *Xba*I (filled in with Klenow fragment)-*Eco*RV fragment from pHMS2 was cloned in the shuttle vector pHM3 (22) digested with *Sma*I. The selected plasmid contains the *thrS* regulatory region in the opposite orientation to *lacZ*.

For pHMS30, an 80-bp *Bam*HI-*Afl*III (filled in with Klenow fragment, ordinate 77 in reference 21) fragment from pHMS2 (containing the promoter and encoding only 20 bp of the *thrS* mRNA) was cloned in the *lacZ* fusion vector pHM2 digested with *Eco*RI (filled in with Klenow fragment) and *Bam*HI.

For pHMZ8, a 1-kb *Nhe*I-*Hin*dIII fragment from pHMZ6 (22) containing the entire *thrZ* regulatory region and 135 nt of the structural gene was subcloned in the shuttle vector pHM3 (22) digested by *Xba*I and *Hin*dIII, in the opposite orientation with respect to *lacZ*.

For pHMZ10, the entire *thrZ* gene (from 80 bp downstream of the *thrZ* promoter) on a 4.0-kb *Bgl*II fragment was cloned in plasmid pTZ19R (USB) digested by *Bam*HI, in the same orientation as *lacZ*. A spontaneous deletion occurred from 150 bp downstream of the *thrZ* stop codon to the pTZ19R polylinker.

Nested deletions were made in plasmid pHMZ10 for sequence purposes as described before (31). Clone pHMZ20, deleted to nt 521 of the *thrZ* sequence (22), lacks part of the *thrZ* regulatory region.

For pHMZ23, the entire *thrZ* gene was subcloned from the mutated pHMZ20 (see below) as an *Xba*I-*Nsi*I fragment in pDG148 (28) fully digested by *Xba*I and incompletely digested by *Pst*I. Since one of the two *Pst*I sites is situated in the β-lactamase gene, insertion in the right polylinker *Pst*I site was selected on LB plates with ampicillin. Expression of *thrZ* from pHMZ23 is dependent on the addition of isopropylthiogalactopyranoside (IPTG) to the growth medium.

Site-directed mutagenesis. Site-directed mutagenesis was performed on a single-stranded DNA template by the method of Kunkel (15). The synthetic oligonucleotide G(823)TGTTT TTTTCTAGACAACAATGAATAAGGAG(854) (the numbering corresponds to the *thrZ* sequence shown in reference 22) was used to create an *Xba*I site in pHMZ20 16 nt upstream of the Shine-Dalgarno sequence of *thrZ*.

DNA sequencing. Double-stranded recombinant plasmid DNA was used as the template in dideoxy chain termination sequencing reactions (24, 25).

Isolation of *B. subtilis* chromosomal DNA and total cellular RNA. *B. subtilis* chromosomal DNA and total cellular RNA were isolated as described previously (22).

Transcription in vitro. The method described by Grandoni et al. (10) was used for in vitro transcription. Purified fragments of plasmid pHMS17 were used as templates. Purified *B. subtilis* RNA polymerase (sigma A) was a gift of Stewart Legrice (Institut Pasteur, Paris, France). Reaction mixtures (20 μl) containing a 0.2 mM concentration (each) ATP, CTP, GTP, and UTP, 10 μCi of [α-^{32}P]UTP (Amersham), 0.5 μg of purified *B. subtilis* RNA polymerase (sigma A), 60 ng of template, and 8 U of RNasin (Promega) in 40 mM Tris-Cl (pH 8.0)–50 mM KCl–4 mM $MgCl_2$–1 mM dithiothreitol–50 μg of bovine serum albumin per ml. Samples were incubated for 30 min at 30°C, and then 160 μl of water, 20 μl of sodium acetate (pH 5.2), and 20 μl of 10% sodium dodecyl sulfate (SDS) were added. The RNA was extracted with water-equilibrated phenol, precipitated, washed, dried, suspended in 10 μl of formamide loading buffer, and analyzed on a denaturing 5% polyacrylamide gel. Transcription in vitro was also performed under the same conditions with the addition of purified *B. subtilis* ThrS (0.15 μM; isolation of the protein will be described elsewhere), ATP (50 μM), and threonine (50 μM) in the following combinations: ThrS alone; ThrS, ATP, and threonine; ThrS and ATP; and ATP and threonine.

Northern (RNA blot) analysis. Routinely, 10 μg of total cellular RNA was separated on formaldehyde-containing gels essentially as described by Lehrach et al. (16) and transferred to a nylon membrane (Amersham Hybond N) in 10× SSC (1× SSC is 0.15 M NaCl, 0.015 M sodium citrate) with a vacuum blotter (Hoefer). Hybridization was performed in 50% formamide–5× SSC–5× Denhardt's solution–0.3% SDS–200 μg of denatured herring sperm DNA per ml at 45°C overnight with probes radiolabeled with ^{32}P by random priming (9). Blots were washed three times for 15 min each in 50% formamide–5× SSC–0.3% SDS at 45°C and rinsed in 2× SSC before autoradiographic exposure.

Reverse transcription. The technique of Uzan et al. (29) was used for reverse transcription. Ten micrograms of *B. subtilis* total RNA and 2 pmol of ^{32}P-labeled specific oligonucleotide in 5 μl of RT buffer (50 mM Tris-Cl [pH 8.3], 10 mM $MgCl_2$, 80 mM KCl) were heated at 60°C for 3 min and then placed in ethanol-dry ice. After thawing on ice, 5 μl of a mixture containing a 2 mM concentration (each) dATP, dCTP, dGTP, and dTTP and 8 mM dithiothreitol in RT buffer and 1 U of avian myeloblastosis virus reverse transcriptase (Boehringer) were added. Samples were incubated at 37 or 48°C for 30 min, precipitated, washed, dried, suspended in 5 μl of formamide loading buffer-TE (1:1), and run on a denaturing 5% polyacrylamide gel.

β-Galactosidase assay. The β-galactosidase activity of *lacZ* fusions was measured as described before (22).

Western blots (immunoblots). *B. subtilis* ThrS and ThrZ proteins were immunodetected as described before (21). An-

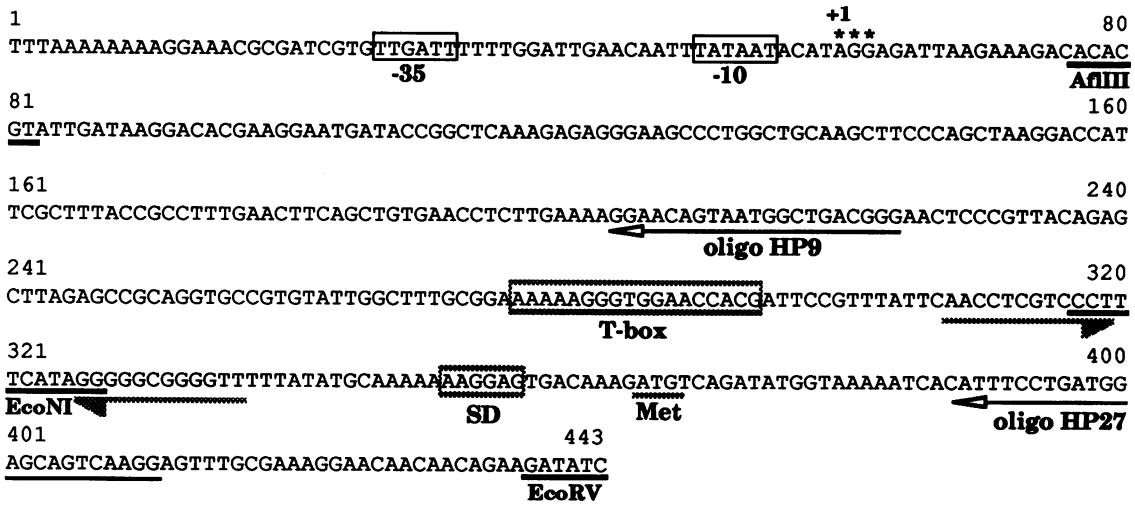

FIG. 1. Sequence and organization of the *thrS* regulatory region. Restriction sites used for cloning are heavily underlined. The primers used for primer extension experiments (HP9 and HP27) are shown by open-headed arrows. The 5′ ends of the *thrS* mRNA found by primer extension with oligonucleotide HP9 are shown by asterisks. The terminator stem-loop structure is indicated by the shaded half-arrows. The locations of the T-box and the putative Shine-Dalgarno site (SD) are indicated.

tibodies against *E. coli* ThrRS were a generous gift of J. Dondon. Under the conditions used, these antibodies recognize *B. subtilis* ThrS about twice as well as ThrZ (as judged from serial dilutions of ThrS- and ThrZ-overproducing *E. coli* strains). The blots were analyzed in a PhosphorImager (Molecular Dynamics) for quantification of radioactivity in specific bands.

Computer analysis. For general sequence analysis, we used the facilities of the Centre Interuniversitaire d'Informatique à Orientation Biomédicale, Paris, France (8). RNA secondary structures with minimum free energy were identified by the program described by Zuker (32).

RESULTS

Mapping of the *thrS* promoter. The 5′ end of the *thrS* mRNA produced in vivo was mapped by reverse transcription of *B. subtilis* 1A2 total RNA with a specific oligonucleotide (HP9 in Fig. 1) as the primer. The reaction was performed at 37 and 48°C to discriminate between signals caused by secondary structures and genuine 5′ ends. The control sequence was determined on plasmid pHMS17 as the template with the same primer. Only one signal was found at both temperatures, localized immediately downstream of a putative sigma A promoter sequence (Fig. 2). Reverse transcription was also performed with an oligonucleotide (HP27 in Fig. 1) located within the *thrS* structural gene. No additional signals other than the genuine 5′ end were detected, with the exception of an artifactual signal caused by a premature stop of the reverse transcriptase at the stem-and-loop structure (data not shown).

To verify the activity of this predicted promoter, transcription in vitro was performed with two different fragments of pHMS17 as templates: a 510-bp *Bam*HI-*Cla*I fragment and a 440-bp *Bam*HI-*Eco*RV fragment (Fig. 3A). The lengths of the transcripts were evaluated by comparison to DNA size standards. The size of the runoff fragment produced by initiation at the putative *thrS* promoter should be 450 nt (*Cla*I) or 380 nt (*Eco*RV). As shown in Fig. 3B, the predicted *thrS* promoter is effectively recognized by the *B. subtilis* RNA polymerase, since transcripts of the expected size are present. A 280-nt-long transcript was found with both templates, and its size corresponds to termination of transcription in the run of uridines following the stem-and-loop structure (Fig. 1 and 3). Quantification of the different signals with a PhosphorImager revealed that 85% of the total *thrS* transcripts terminate at this secondary structure, indicating that it actually functions as a

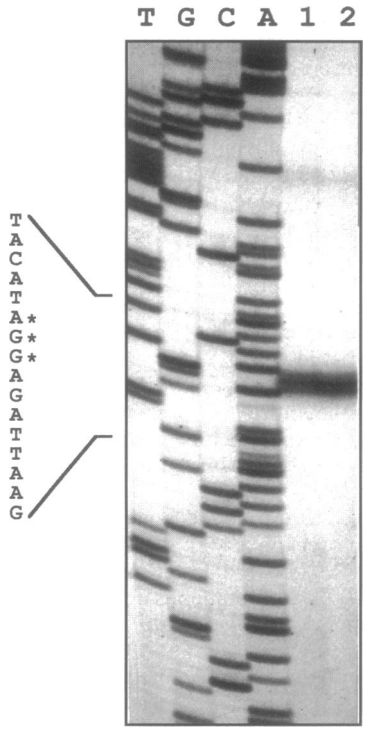

FIG. 2. Primer extension of the in vivo-produced *thrS* mRNA. *B. subtilis* 1A2 total RNA was used as the template for primer extension with primer HP9. Lane 1, reaction at 37°C; lane 2, reaction at 48°C. The control sequence was performed with pHMS17 as the template and oligonucleotide HP9 as the primer. Asterisks indicate nucleotides of the control sequence corresponding to the 5′ ends of the *thrS* mRNA found by primer extension (see Fig. 1).

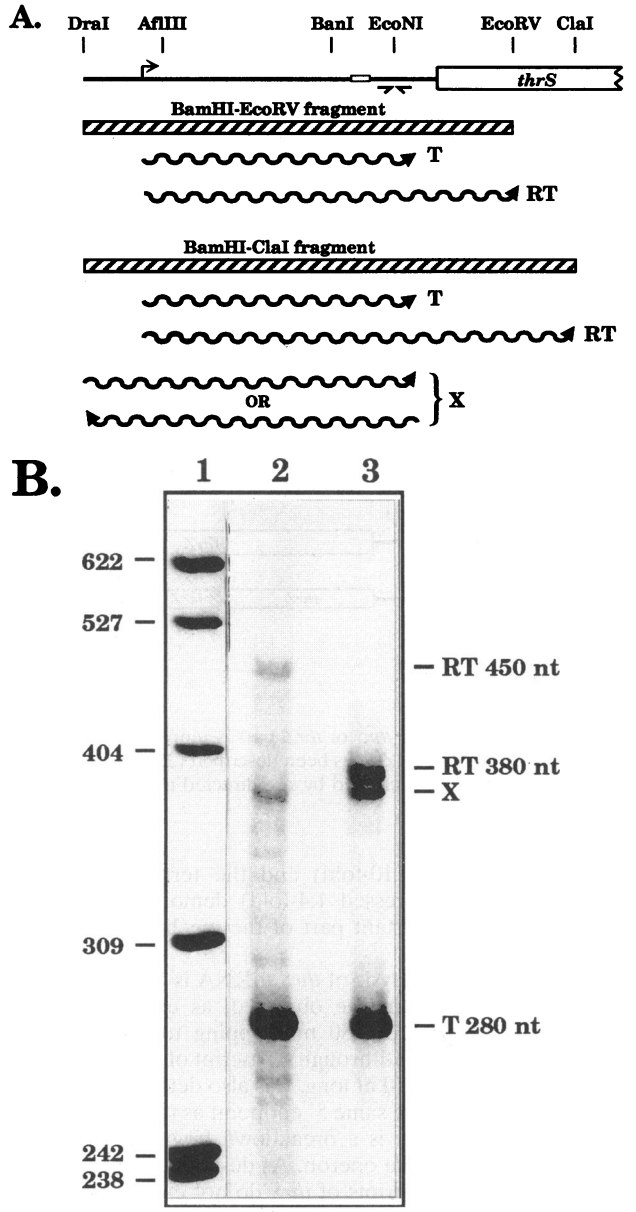

FIG. 3. Transcription in vitro. A 510-bp-long *Bam*HI-*Cla*I fragment and a 440-bp-long *Bam*HI-*Eco*RV fragment from pHMS17 (see Materials and Methods) were used as templates. Transcription in vitro was performed with purified *B. subtilis* σ^A RNA polymerase, and the transcripts were labeled by including [^{32}P]UTP in the reaction mix. (A) Schematic representation of the transcripts obtained. The templates are represented by hatched boxes, and the RNA is represented by curvy arrows. (B) Lane 1, *Msp*I-digested pBR322 size standards (in nucleotides). Lane 2, transcription reaction on the *Bam*HI-*Cla*I fragment. Lane 3, transcription reaction on the *Bam*HI-*Eco*RV fragment. RT, runoff transcripts; T, terminated transcript; X, contaminating band (see text).

factor-independent terminator of transcription. An additional band of approximately 360 nt was detected in both reactions. Since it was observed with both templates, it cannot be due to a second initiation site downstream of the identified promoter, and no secondary structure was found to explain a stop of the polymerase about 20 nt upstream of the *Eco*RV site. Its size is compatible with a transcript initiating at the *Bam*HI end and ending at the terminator. Alternatively, this band could be caused by transcription initiation on the complementary strand, resulting in a fragment terminated at the *Bam*HI site. However, S1 mapping analysis of *thrS* transcripts did not detect a corresponding mRNA species, indicating that this additional transcript observed in vitro is most likely an artifact and does not exist in vivo (data not shown).

***thrS* is autogenously controlled at the transcriptional level.** To test whether *thrS* expression was controlled by its own product, which is the case for the *E. coli thrS* gene, we constructed two *thrS-lacZ* fusions, an operon fusion (pHMS11 [22]) (Fig. 4) and a protein fusion (pHMS12; see Materials and Methods and Fig. 4). These fusions were integrated at the *amyE* locus on the *B. subtilis* chromosome by double cross-over recombination, resulting in a single copy of the fusion per cell. Integration at the *amyE* locus is indicated by an *amy* phenotype on LB-starch plates. The β-galactosidase activities of these fusions were measured in strains overexpressing ThrS from the recombinant multicopy plasmid pHMS3 and, as a control, in strains containing pHM3, the cloning vector. The results are presented in Table 2. ThrS was overexpressed in strains HP18 (containing the operon fusion) and HP25 (containing the protein fusion) 9.8- and 5.0-fold, respectively, as measured by immunoblotting (Fig. 5). These levels of ThrS overproduction resulted in a 10.7-fold decrease in β-galactosidase activity in HP18 and a 7.3-fold decrease in HP25. ThrS overexpression represses the operon fusion as well as the protein fusion to a similar extent, suggesting that the control of *thrS* expression occurs at the transcriptional level and not, as in *E. coli*, at the translational level (5, 26, 27).

The *thrS*-overexpressing plasmid pHMS3 contains the entire *thrS* transcriptional unit. It is thus possible that the decrease in β-galactosidase activity in ThrS-overexpressing strains is caused by the excess of leader mRNA in the cell titrating a putative activator factor involved in the antitermination mechanism of *thrS*. To test this hypothesis, we constructed a plasmid, plasmid pHMS23 (see Materials and Methods), carrying only the *thrS* regulatory region in the shuttle vector pHM3 (the same plasmid used to overexpress ThrS). pHMS23 was used to transform strain HP18 containing the *thrS-lacZ* operon fusion. As shown in Table 2, overexpression of the regulatory region alone did not repress *thrS-lacZ* fusion expression, and thus the high copy number of the *thrS* regulatory region is not sufficient to account for the decrease in β-galactosidase activity obtained when ThrS is overexpressed.

We have shown previously that ThrS overexpression practically abolishes expression of a *thrZ-lacZ* transcriptional fusion (pHMZ9 [22]) (Fig. 4) in a *thrS* mutant strain (see also Table 2). Here, we also examined the effect of overexpression of the *thrS* regulatory region on *thrZ-lacZ* fusion expression. The results are presented in Table 2 and show that overexpression of the *thrS* regulatory region alone cannot account for the complete shutoff of *thrZ* expression obtained when ThrS is overproduced. Nevertheless, we observed a twofold repression of the *thrZ-lacZ* fusion. This might actually be caused by titration of a putative antiterminator factor (see Discussion). As for the *thrS-lacZ* fusion, it is the overexpression of the ThrS synthetase that is responsible for the efficient repression of the *thrZ-lacZ* fusion. However, *thrZ* is much more sensitive to repression by ThrS overexpression (greater than 200-fold repression) than is *thrS* (10-fold repression at the most).

Control of *thrS* expression involves antitermination. A *thrS* promoter-*lacZ* fusion lacking the downstream regulatory elements (pHMS30; see Materials and Methods and Fig. 4) was constructed, and β-galactosidase activity was measured in the

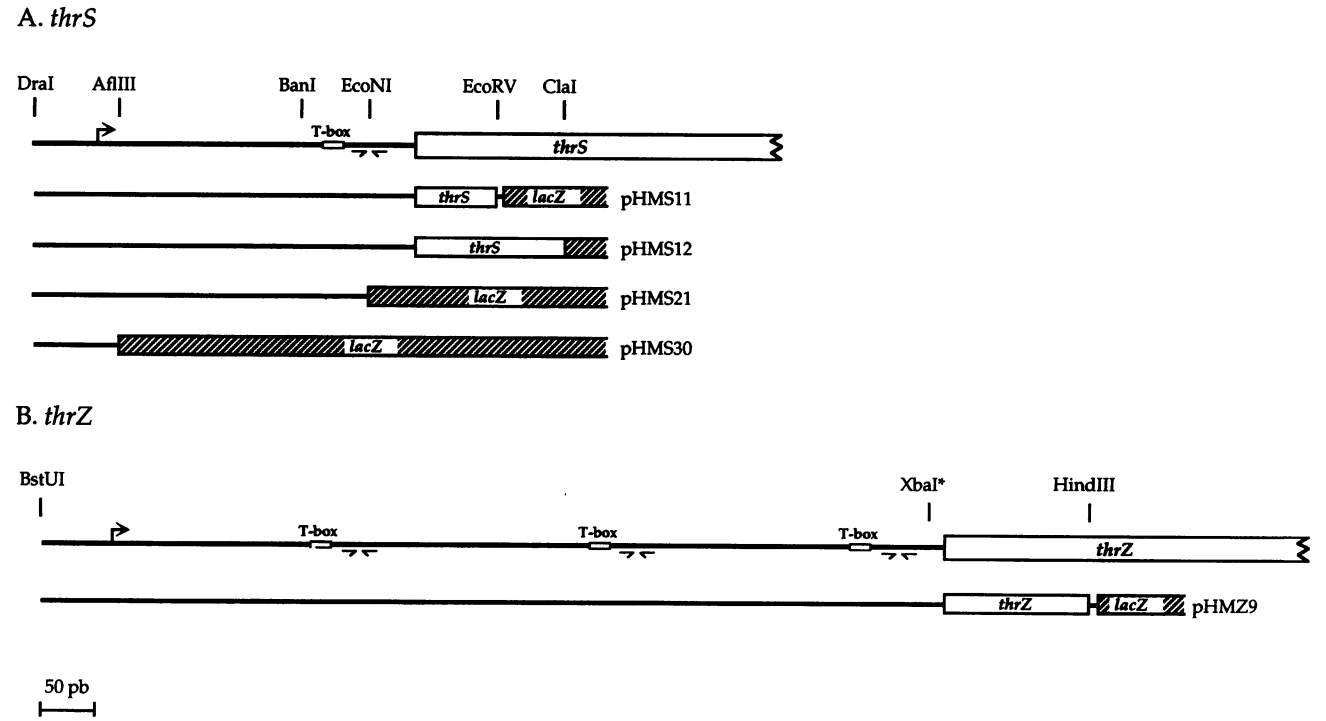

FIG. 4. Fusions between *thrS* or *thrZ* and *lacZ*. (A) *thrS-lacZ* fusions; (B) *thrZ-lacZ* fusions. The *thrS* or *thrZ* part is represented by an open box labeled with the name of the gene, and *lacZ* is shown as a hatched box. The construction of pHMZ9 has been described (22). Inverted arrows represent factor-independent terminators. The T-box is shown as an open box. The *Xba*I* site was created by site-directed mutagenesis.

presence of the ThrS-overexpressing vector pHMS3. As shown in Table 3, ThrS overexpression does not affect the β-galactosidase activity of this fusion. Thus, the promoter and sequences immediately adjacent to it are by themselves not sufficient for control by ThrS, suggesting that regulation does not occur at the level of initiation of transcription. One of the characteristic features of the *thrS* leader region is the terminator structure located immediately upstream of the structural gene. In order to examine the importance of this terminator for *thrS* autoregulation, we constructed a transcriptional *thrS-lacZ* fusion lacking the *lacZ* proximal half of the terminator stem (pHMS21 [22]) (Fig. 4). Expression of this fusion is only slightly repressed by ThrS overexpression (Table 3). The significant difference in repression of a wild-type fusion (pHMS11 [22], repressed more than 10-fold) and the terminator deletion fusion (pHMS21, repressed 1.4-fold) demonstrates that the terminator is an important part of the mechanism regulating *thrS* expression.

A Northern blot analysis of *thrS* mRNA is presented in Fig. 6. In a wild-type strain, we observed, as expected, a short terminated transcript of 280 nt mapping to the terminator structure and a long readthrough transcript of 2,300 nt. A short band, approximately 170 nt long, was also detected. We do not know whether it has the same 5′ endpoint as the other mRNAs analyzed or whether it is a breakdown fragment that comes from a distal part of the operon. As described above, in vitro, 85% of the total transcripts of *thrS* do not extend beyond the terminator (Fig. 3B). In vivo, *thrS* transcription is also partially

TABLE 2. Effect of overproduction of ThrS or *thrS* leader on *thrS-lacZ* and *thrZ-lacZ* fusions

Fusion type	Strain	Integrated plasmid	Multicopy plasmid	Insert[a]	β-Galactosidase sp act (U/mg)	Repression factor (fold)	ThrS overproduction[b] (fold)
			pHM3	Control	135		
thrS-lacZ	HP18	pHMS11 (*thrS* operon fusion)					
	HP25	pHMS12 (*thrS* protein fusion)	pHMS3	*thrS*	13	10.4	9.8
			pHM3	Control	1,073		
	HP18	PHMS11 (*thrS* operon fusion)	pHMS3	*thrS*	147	7.3	5.0
			pHM3	Control	135		
thrZ-lacZ	HP20	pHMZ9 (*thrZ* operon fusion)	pHMS23	*thrS* leader	129	1.05	
			pHM3	Control	58		
	HP20	pHMZ9 (*thrZ* operon fusion)	pHMS3	*thrS*	<0.3	>200	
			pHM3	Control	58		
			pHMS23	*thrS* leader	29	2.0	

[a] Fragment cloned in vector pHM3 for overexpression in *B. subtilis*.
[b] Measured by Western blot (see Materials and Methods).

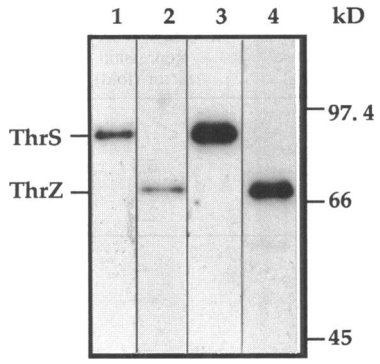

FIG. 5. Overexpression of ThrS and ThrZ. *B. subtilis* total-cell extracts (5 μg) were electrophoresed on SDS-polyacrylamide gel electrophoresis, and ThrS and ThrZ expression was quantified by Western blotting (see Materials and Methods) with antibodies directed against *E. coli* ThrRS. Lane 1, strain HP18 plus pHM3 (vector without insert [22]). Lane 2, strain HP47 plus pDG148 (vector without insert [28]). Lane 3, strain HP18 plus pHMS3 (overexpresses ThrS [22]). Lane 4, strain HP47 plus pHMZ23 (grown in the presence of 0.5 mM IPTG to induce *thrZ* transcription; see text).

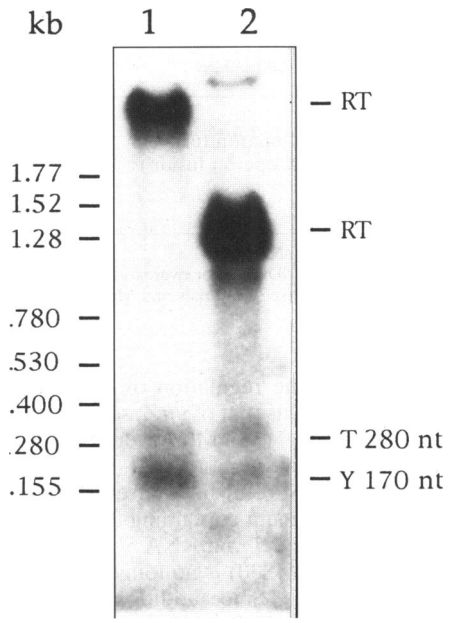

FIG. 6. Northern blot of *thrS* mRNA. Total RNA (10 μg) from *B. subtilis* was hybridized with a ^{32}P-radiolabeled *thrS*-specific probe (*Dra*I-*Eco*RI fragment, coordinates 3 to 899 of reference 21) obtained by random priming (9). It covers the regulatory region and 535 bp of the coding sequence. Lane 1, *B. subtilis* wild-type strain 1A2; lane 2, *B. subtilis thrS* mutant strain HP47. RT, runoff transcript; T, terminated transcript.

terminated at the terminator but much less efficiently, as shown in Fig. 6, lane 1. In a strain in which *thrS* was disrupted by the insertion of a kanamycin resistance cassette, the ratio of readthrough (1,300 nt; caused by the presence of the Km^r cassette) to terminated transcripts is greatly increased (Fig. 6, lane 2). This corresponds well to the β-galactosidase activity data for a wild-type *thrS-lacZ* fusion (pHMS11 [22]) in which expression is stimulated fourfold in the absence of a functional *thrS* gene (688 versus 163 U/mg of protein). Derepression of *thrS* thus occurs via transcriptional antitermination. The great difference in readthrough of the terminator in vitro and in vivo strongly suggests the existence of an antitermination factor involved in the derepression of *thrS*.

***thrZ* expression is also autogenously controlled.** As shown previously, *thrZ* expression is regulated by antitermination of transcription, in much the same way as *thrS* (22). We wondered whether *thrZ* expression was also autogenously controlled. To verify this, a plasmid overexpressing ThrZ and lacking the entire *thrZ* regulatory region was constructed (pHMZ23). In this plasmid, *thrZ* expression is under the control of an IPTG-inducible promoter (P_{spac}). We used 0.5 mM IPTG to obtain about the same level of overexpression as for ThrS (Tables 2 and 4; Fig. 5). The overproduction levels of ThrZ synthetase are given relative to the intracellular concentration of ThrZ in a *thrS* mutant strain which has the same growth rate as a wild-type strain expressing only *thrS*. The activity of a *thrZ-lacZ* fusion (pHMZ9) in the *thrS* mutant strain was measured in the presence of the *thrZ*-overexpressing plasmid pHMZ23 and, as a control, of the cloning vector alone (pDG148). As shown in Table 4, ThrZ repressed *thrZ-lacZ* fusion activity 6.3-fold. *thrZ* is thus autogenously regulated. We also measured the effect of ThrZ overproduction on a *thrS-lacZ* fusion (pHMS11 [22]). ThrZ also repressed the *thrS-lacZ* fusion but only 2.1-fold, which is much less efficient than the repression observed with ThrS (10.7-fold). Thus, the ThrZ synthetase does resemble the ThrS protein in its regulatory action, but its capacity to repress both *thrS* and *thrZ* is significantly lower than that of ThrS.

DISCUSSION

We have shown in this work that the two genes, *thrS* and *thrZ*, each encoding a functional threonyl-tRNA synthetase in *B. subtilis* are autogenously regulated. This is reminiscent of the situation in *E. coli*, in which the *thrS* gene is also regulated by its own product. The *E. coli* synthetase can bind to a tRNA^{Thr}-like structure in the *thrS* leader region, thereby preventing the ribosome from binding to its translational initiation site (5, 26, 27). The control of expression thus occurs at the level of translation.

TABLE 3. ThrS overproduction does not affect a *thrS* promoter-*lacZ* fusion and a *thrS-lacZ* fusion without its terminator

Strain	Integrated plasmid	Multicopy plasmid	Insert[a]	β-Galactosidase sp act (U/mg)	Repression factor (fold)
HP124	pHMS30 (*thrS* promoter fusion)	pHM3	Control	577	
HP45	pHMS21 (*thrS* without terminator)	pHMS3	*thrS*	557	1.02
		pHM3	Control	238	
		pHMS3	*thrS*	171	1.39

[a] Fragment cloned in vector pHM3 for overexpression in *B. subtilis*.

TABLE 4. Effect of ThrZ overproduction on *thrS-lacZ* and *thrZ-lacZ* transcriptional fusions

Strain[a]	Integrated plasmid	Multicopy plasmid	Insert[b]	β-Galactosidase sp act (U/mg)	Repression factor (fold)	ThrZ overproduction[c] (fold)
		pDG148	Control	25		
HP30	pHMZ9 (*thrZ* operon fusion)					
HP79	pHMS11 (*thrS* operon fusion)	pHMZ23	*thrZ*	4	6.3	11
		pDG148	Control	500		
		pHMZ23	*thrZ*	230	2.1	12

[a] All cultures contained 0.5 mM IPTG.
[b] Fragment cloned in vector pDG148 for overexpression in *B. subtilis*.
[c] Measured by Western blot (see Materials and Methods).

On the other hand, the regulation of expression of the *B. subtilis thrS* gene (as well as that of *thrZ*; see below) takes place at the transcriptional level (22). Moreover, it is not achieved by modulating transcription initiation but rather by transcriptional antitermination. Several lines of evidence support this conclusion. When a fragment containing the promoter and the first 20 bp of the identified *thrS* mRNA 5′ end is fused to *lacZ*, the resulting fusion (pHMS30) is no longer repressed by ThrS overexpression (Table 3). Since additional downstream promoters may be involved in *thrS* regulation, we also verified that *thrS* mRNA transcription is actually initiated at a single σ^A-type promoter 305 bp upstream of the coding sequence, at least during vegetative growth. No additional promoters could be detected in primer extension experiments with an oligonucleotide that hybridized to the structural part of the *thrS* mRNA. Moreover, *B. subtilis* σ^A RNA polymerase can recognize this promoter in vitro (see Fig. 3), and the 5′ end of the synthetic transcript corresponds to that of the *thrS* mRNA synthesized in vivo (Fig. 2). This also demonstrates that the identified mRNA 5′ end is actually generated by transcription initiation and is not due to mRNA processing.

Transcriptional and translational *thrS-lacZ* fusions are repressed to a similar degree when ThrS is overexpressed in *trans* (Table 2). The ratio of *thrS* readthrough to terminated transcripts increases greatly in a *thrS* deletion strain (Fig. 6). This corresponds well to the β-galactosidase activity data for a transcriptional *thrS-lacZ* fusion (pHMS11), the expression of which is stimulated fourfold in the absence of a functional *thrS* gene. The same stimulation is also observed with a translational *thrS-lacZ* fusion (data not shown).

We have shown previously that the *B. subtilis thrZ* gene is usually silent and is expressed only under two conditions: when the cells are starved for threonine and when *thrS* expression is reduced below its normal level (22). Induction of *thrZ* is achieved by transcriptional antitermination involving the three terminator structures present in its leader sequence. We also showed that the 18-bp consensus sequence (T-box) preceding the three *thrZ* terminators as well as the single *thrS* terminator is crucial for efficient readthrough of the terminator. The very strong conservation of these structural regulatory elements in both genes suggested that the two genes are regulated by the same mechanism. The nonexpression of *thrZ* under normal growth conditions is most likely due to the fact that the control elements are present as a triplet (22).

The experiments presented here corroborate this hypothesis. In fact, not only do both synthetases regulate their own synthesis, but each of them is capable of repressing the other gene, *thrS* or *thrZ*, when overproduced. This strongly indicates that the two synthetase genes are subject to the same control mechanism. We have overexpressed both genes to the same or a very similar degree in order to compare their influence on *thrS* and *thrZ* regulation. The overexpression levels indicated are based on the enzyme levels in a wild-type strain (for ThrS) and a *thrS* mutant strain (for ThrZ). Since the wild-type and *thrS* deletion strains grow equally well (22), these values actually represent the overproduction of both synthetases with respect to ThrS and ThrZ levels, allowing identical growth rates.

Our data show that the ThrZ synthetase is a remarkably less efficient effector of regulation. ThrZ overproduction represses a *thrZ-lacZ* fusion 6-fold, whereas a similar ThrS overexpression represses the same *thrZ-lacZ* fusion more than 200-fold (compare Tables 2 and 4). Similarly, ThrZ can repress *thrS* expression but only slightly (2-fold [versus 10-fold for ThrS]; Tables 2 and 4). These values imply that under normal physiological conditions (e.g., in the absence of a multicopy plasmid overexpressing ThrZ), the ThrZ synthetase cannot practically influence *thrS* expression but that the intracellular concentration of ThrS is extremely critical for ThrZ synthesis (22). The higher efficiency of ThrS than of ThrZ as a repressor is also reflected by the fact that in a *thrS* deletion strain (depending exclusively on ThrZ synthesis), *thrS* expression (as monitored by a *thrS-lacZ* fusion) is stimulated more than fourfold compared with expression in a wild-type strain.

ThrS can thus quite effectively downregulate its own as well as *thrZ* expression when overproduced. On the other hand, ThrS can be effectively overexpressed (10-fold) from a multicopy plasmid containing the entire *thrS* transcriptional unit (pHMS3; 20 to 30 copies per cell [3]). This indicates a less strict autocontrol than that observed for most ribosomal proteins. However, our data closely resemble those obtained for the *E. coli thrS* gene, which is autogenously regulated at the translational level. In fact, a pBR322-derived multicopy plasmid (30 to 50 copies per cell) carrying the *E. coli thrS* transcriptional unit also overexpresses *thrS* 10-fold (27).

We have examined whether ThrS can interact with the nascent *thrS* mRNA and alter readthrough at the terminator structure. The in vitro transcription experiments showed that addition of purified ThrS with or without threonine and ATP did not affect the ratio of terminated transcripts to runoff transcripts (data not shown). In all cases, 85% of the initiated transcripts were prematurely terminated. Similar results were also obtained in a study on the *B. stearothermophilus tyrS* gene (30). Thus, these data did not provide evidence of a direct interaction of the ThrS protein with its mRNA. On the other hand, they confirm our suggestion (22) that the alternative secondary structure (the antiterminator) involving the T-box element and a second complementary sequence localized in the promoter-proximal half of the terminator stem (23) is not stable enough by itself to allow efficient readthrough of the terminator ($\Delta G = -6.1$ kcal/mol [versus -16.2 kcal/mol for the terminator]). ThrS cannot increase termination above the 85% caused by the terminator structure alone. Moreover, these in vitro experiments demonstrate the need for an anti-

termination factor in order to obtain the expression levels observed in vivo.

Further evidence of an antitermination factor comes from in vivo titration experiments. Overexpression of the *thrS* leader region represses a *thrZ-lacZ* fusion twofold, while a *thrS-lacZ* fusion is not repressed under the same conditions. This can be explained by the presence of three terminators in the *thrZ* leader, compared with one in *thrS*, which renders *thrZ* particularly sensitive to titration of an antitermination factor. Recently, it has been shown for the *B. subtilis* tyrosyl-tRNA synthetase that the specificity of induction following tyrosine starvation probably involves the uncharged cognate tRNA interacting with a loop structure containing a tyrosine codon (12). Since most of the gram-positive aminoacyl-tRNA synthetase genes, including *thrS* and *thrZ*, contain the same structural features (similar codon-bearing loop, T-box, and terminator), the cognate tRNA (the $tRNA^{Thr}ACC$ isoacceptor in the case of *thrS* and *thrZ*) probably plays an important role in the specificity of antitermination of these genes (12). If the uncharged tRNA has a direct role in antitermination of *thrS* and/or *thrZ* transcription, then overexpression of the *thrS* leader could derepress *thrZ* by titrating the uncharged $tRNA^{Thr}ACC$ species and possibly other factors which might be involved in antitermination. So far, the following elements in the regulatory events have been identified: (i) the T-box consensus sequence and the terminator; (ii) the aminoacyl-tRNA synthetase itself; and (iii) a cognate codon triplet in the leader mRNA which could interact with the tRNA anticodon. The aminoacyl-tRNA synthetase and the codon in the mRNA leader, which are related to the cognate tRNA, could thus act to specifically regulate the expression of each aminoacyl-tRNA synthetase gene.

Autogenous control of *thrS* and *thrZ* can be achieved by at least two different mechanisms. When overexpressed, the synthetases could compete with other components of the antitermination mechanism by interacting directly (possibly via the uncharged tRNA, as described above) with the mRNA. Second, the synthetases could regulate their expression more indirectly via the pool of uncharged tRNA. Overexpression of the charging enzymes would thus lead to a decrease in the concentration of uncharged tRNA, possibly a direct positive effector in antitermination. We have shown previously that the wild-type, *thrS* mutant, and *thrZ* mutant strains have identical growth rates, implying that ThrS and ThrZ are equally efficient in tRNA charging. In this case, the different abilities of ThrS and ThrZ to act as a repressor could be due to differences in recognizing regulatory structures on the mRNA. However, we do not know whether ThrS and ThrZ are equally efficient in the charging of both $tRNA^{Thr}$ isoacceptors, of which only the $tRNA^{Thr}ACC$ should have a regulatory role by analogy with the model proposed for *tyrS* (12).

It is difficult to predict whether different catalytic parameters of ThrS and ThrZ for the two $tRNA^{Thr}$ species would be sufficient to explain the different regulatory capacities of ThrS and ThrZ and at the same time have no effect on growth rate. We are currently investigating the mechanism of autocontrol in order to distinguish between a direct or indirect (via tRNA charging) regulatory role for the synthetases. Nevertheless, the existence of different regulatory properties for the two proteins (which have only 51.5% identical residues [21]) seems logical in the sense that the product of an auxiliary gene like *thrZ*, which is synthesized only when there is a need for elevated levels of threonyl-tRNA synthetase activity, should not counteract the purpose of its induction by downregulating too strongly *thrS* or its own expression.

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