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Co-ordinate expression of the two threonyl-tRNA synthetase genes in *Bacillus subtilis*: control by transcriptional antitermination involving a conserved regulatory sequence

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In *Bacillus subtilis*, two genes, *thrS* and *thrZ*, encode distinct threonyl-tRNA synthetase enzymes. Normally, only the *thrS* gene is expressed. Here we show that either gene, *thrS* or *thrZ*, is sufficient for normal cell growth and sporulation. Reducing the intracellular ThrS protein concentration induces *thrZ* expression in a dose-compensatory manner. Starvation for threonine simultaneously induces *thrZ* and stimulates *thrS* expression. The 5′-leader sequences of *thrS* and *thrZ* contain, respectively, one and three transcription terminators preceded by a conserved sequence. We show that this sequence is essential for the regulation of *thrS* via a transcriptional antitermination mechanism. We propose that both genes, *thrS* and *thrZ*, are regulated by the same mechanism such that the additional regulatory domains present before *thrZ* account for its non-expression. In contrast to *Escherichia coli*, structurally similar regulatory domains, i.e. the consensus sequence preceeding a terminator structure, are found in the leader regions of most aminoacyl-tRNA synthetase genes of Gram-positive bacteria. This suggests that they are regulated by a common mechanism.

Key words: antitermination/*Bacillus subtilis*/threonyl-tRNA synthetase

Introduction

The Gram-positive organism *Bacillus subtilis* has two genes both encoding a functional threonyl-tRNA synthetase (*thrS* and *thrZ*, replacing the former designations *thrSv* and *thrS2*). The two proteins share only 51% identical residues which makes them almost as distinct from each other as they are from the corresponding *Escherichia coli* enzyme (42 and 47%) or the human threonyl-tRNA synthetase (37 and 42%). During vegetative growth of *B.subtilis*, only the *thrS* gene is expressed (Putzer *et al.*, 1990). In eukaryotes, multiple gene families of proteins are frequently encountered. However, only a few examples are known in prokaryotic organisms where proteins encoded by independent genes appear to have exactly the same function. In addition to the system described here, this is the case for the *E.coli* enzymes aspartokinase-homoserine dehydrogenase (*thrA* and *metL* genes, Ferrara *et al.*, 1984), ornithine carbamoyltransferase (*argF* and *argI*, Van Vliet *et al.*, 1984), elongation factor EF-Tu (*tufA* and *tufB*, Jaskunas *et al.*, 1975), glycerol-3-phosphate dehydrogenase (*glpACB* and *glpD*, Kuritzkes *et al.*, 1984; Iuchi *et al.*, 1990), lysyl-tRNA synthetase (*lysS* and *lysU*, Hirshfield *et al.*, 1981, 1984; Lévêque *et al.*, 1991) and very recently *B.subtilis* tyrosyl-tRNA synthetase (Glaser *et al.*, 1990; Henkin *et al.*, 1992). In the first four cases both genes are always expressed, the expression of the *glp* operons being under anaerobic/aerobic control. The *E.coli lysU* gene encodes a weakly expressed lysyl-tRNA synthetase whose expression is stimulated under certain physiological conditions like anaerobic growth (Lévêque *et al.*, 1991). In this paper we demonstrate that the expression of the *B.subtilis thrS* and *thrZ* genes is tightly co-ordinated and that a reduction in the intracellular ThrS protein concentration stimulates *thrZ* expression in a dose-compensatory manner. Both genes appear to be regulated by transcriptional antitermination involving structural elements conserved not only between *thrS* and *thrZ* but in nearly all known aminoacyl-tRNA synthetases in Gram-positive organisms.

Results

Inactivation of thrS activates thrZ

We have described previously the presence in *B.subtilis* of two *thrS* genes (now called *thrS* and *thrZ*) of which only the *thrS* gene is expressed during vegetative growth (Putzer *et al.*, 1990). Since threonyl-tRNA synthetase is essential for cell growth, inactivation of the *thrS* gene was expected to be lethal. However, Campbell type insertion of two integrative plasmids pHMS7 and pHMS8 (Figure 1A), where the first reconstitutes an entire *thrS* gene and the second disrupts the gene, gave rise to the same number of colonies. pHMS8 recombinants carrying an interrupted *thrS* transcriptional unit were further analysed for threonyl-tRNA synthetase expression by Western blot using anti-*E.coli* ThrRS antibodies. The results are shown in Figure 1B. pHMS8 integrants are characterized by the total absence of ThrS protein while the expression of ThrZ is induced which presumably permits cell survival (Figure 1B, lane 2). On the other hand, interruption of the *thrZ* gene does not alter the wild type expression pattern (Figure 1B, lane 4).

Simultaneous inactivation of thrS and thrZ is lethal

Although either gene, *thrS* or *thrZ*, can sustain cell growth, the simultaneous interruption of both synthetase genes should be lethal. We tested this assumption by making use of co-transformation (congression) as outlined in Figure 2. A *B.subtilis* strain (HP 1) with an insertionally inactivated *thrZ* gene pHMZ1, conferring chloramphenicol (Cm) resistance was transformed with chromosomal DNA from strains carrying a *thrS* insertion [conferring erythromycin inducible erythromycin/lincomycin (Mls) resistance] which either regenerates an intact *thrS* transcriptional unit (pHMS7 integrant) or disrupts the gene (pHMS8 integrant, for details see Materials and methods). Thus, Mls-resistant transformants should only arise with $thrS^+$ DNA unless, at high DNA concentrations, the disrupted *thrZ* gene is

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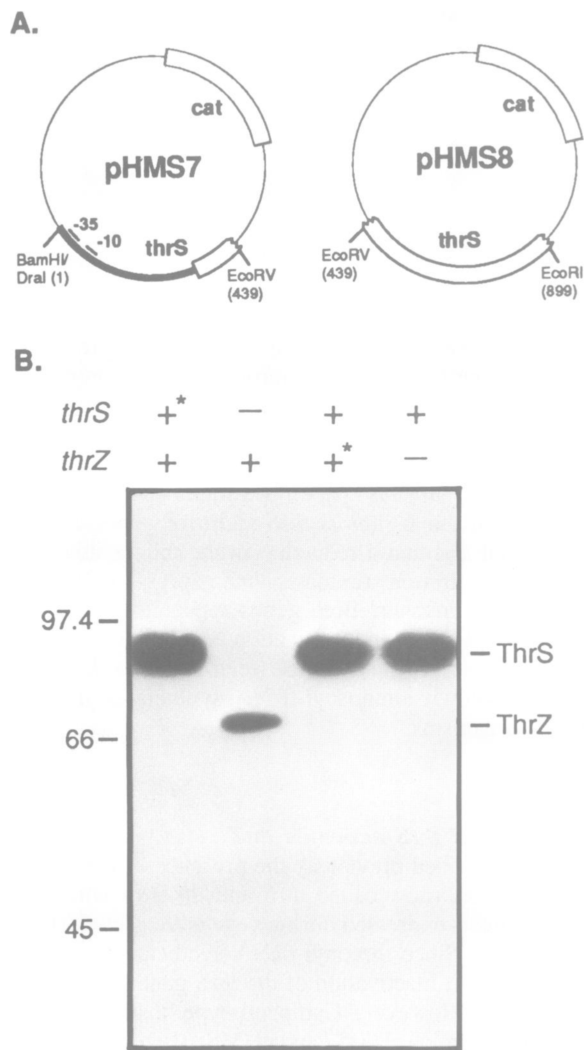

Fig. 1. (**A**) Schematic representation of plasmids pHMS7 and pHMS8 used for integration at the *thrS* chromosomal locus. The *thrS* regulatory region is represented by a thick black line, the consensus promoter sequences (−35 and −10) are indicated (see Materials and methods for details). (**B**) Western blot analysis of *B.subtilis* threonyl-tRNA synthetase expression. Total cellular proteins from *B.subtilis* wild type, *thrS* mutant and *thrZ* mutant strains were fractionated on a 10% SDS−polyacrylamide gel, transferred to nitrocellulose and probed with polyclonal anti-*E.coli* ThrRS antibodies. The characteristic migration properties of ThrS and ThrZ have previously been analysed (Putzer *et al.*, 1990). +: the corresponding gene is of wild type and functional (but not necessarily expressed). +*: a chloramphenicol resistance marker has been integrated via Campbell type insertion at the corresponding chromosomal locus. The constructs used for insertion (pHMS7 for *thrS*, pHMZ4 for *thrZ*, see Materials and methods) do not interrupt the transcriptional units of *thrS* and *thrZ*, respectively. −: the genes concerned have been disrupted via Campbell type insertion (pHMS8 for *thrS*, pHMZ1 for *thrZ*).

simultaneously exchanged for a wild type gene copy by co-transformation. In that case an inactivation of *thrS* (by transformation with DNA from the *thrS* mutant strain) would not be lethal, since cell survival would be assured by the *thrZ* gene. Besides the fact that double transformants occur only at low frequency (1−5% of total transformants), they can easily be identified by their sensitivity to chloramphenicol due to the loss of the *thrZ* insertion. The results are summarized in Table I. Transformants with DNA from the

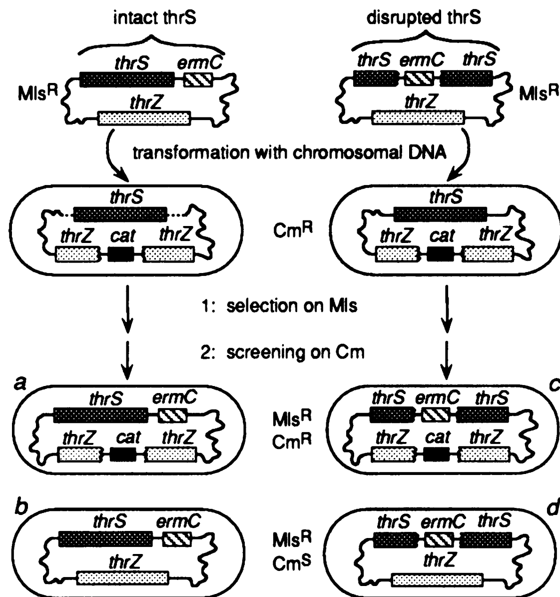

Fig. 2. Simultaneous inactivation of *thrS* and *thrZ* is lethal. Schematic diagram outlining the strategy of the congression experiment. A *thrZ* disruption strain (chloramphenicol resistant) was transformed with varying amounts of chromosomal DNA from a $thrS^+$ and a $thrS^-$ strain (confering Mls resistance). Mls resistant transformants were screened for their sensitivity to chloramphenicol indicating the restoration of a functional *thrZ* gene due to co-transformation (b and d).

Table I. Statistical evaluation of the congression experiment shown in Figure 2

Chromosomal DNA		*thrZ*::*cat* transformants		
		Mls^R	Cm^R	Cm^S
50 μg	$thrS^+$::*erm*	477	459	18
	thrS::*erm*	10	0	10
5 μg	$thrS^+$::*erm*	48	47	1
	thrS::*erm*	1	0	1
0.5 μg	$thrS^+$::*erm*	12	11	1
	thrS::*erm*	0	0	0
0.05 μg	$thrS^+$::*erm*	1	1	0
	thrS::*erm*	0	0	0

thrS strain are obtained only at high DNA concentrations and they are without exception chloramphenicol sensitive. Thus, as expected, at least one of the two genes, *thrS* or *thrZ* has to remain functional in order to support growth.

thrZ is probably transcribed from a σ^A-type promoter

The *thrS* gene is transcribed from a σ^A promoter (Putzer *et al.*, 1990 and manuscript in preparation). In order to identify the sequences necessary for *thrZ* transcription, fragments containing the N-terminal part of *thrZ* and differing amounts of upstream DNA were tested *in vivo* for their ability to promote *thrZ* transcription.

The different fragments were cloned into an integrational plasmid which was subsequently used for a Campbell insertion at the *thrZ* locus of a *thrS* mutant strain. Since cell survival of a *thrS* mutant strain depends on *thrZ* expression, only those fragments including the elements essential for a functional *thrZ* transcription unit will give rise to colonies after integration.

Using pHMZ2 (an integrative plasmid containing a

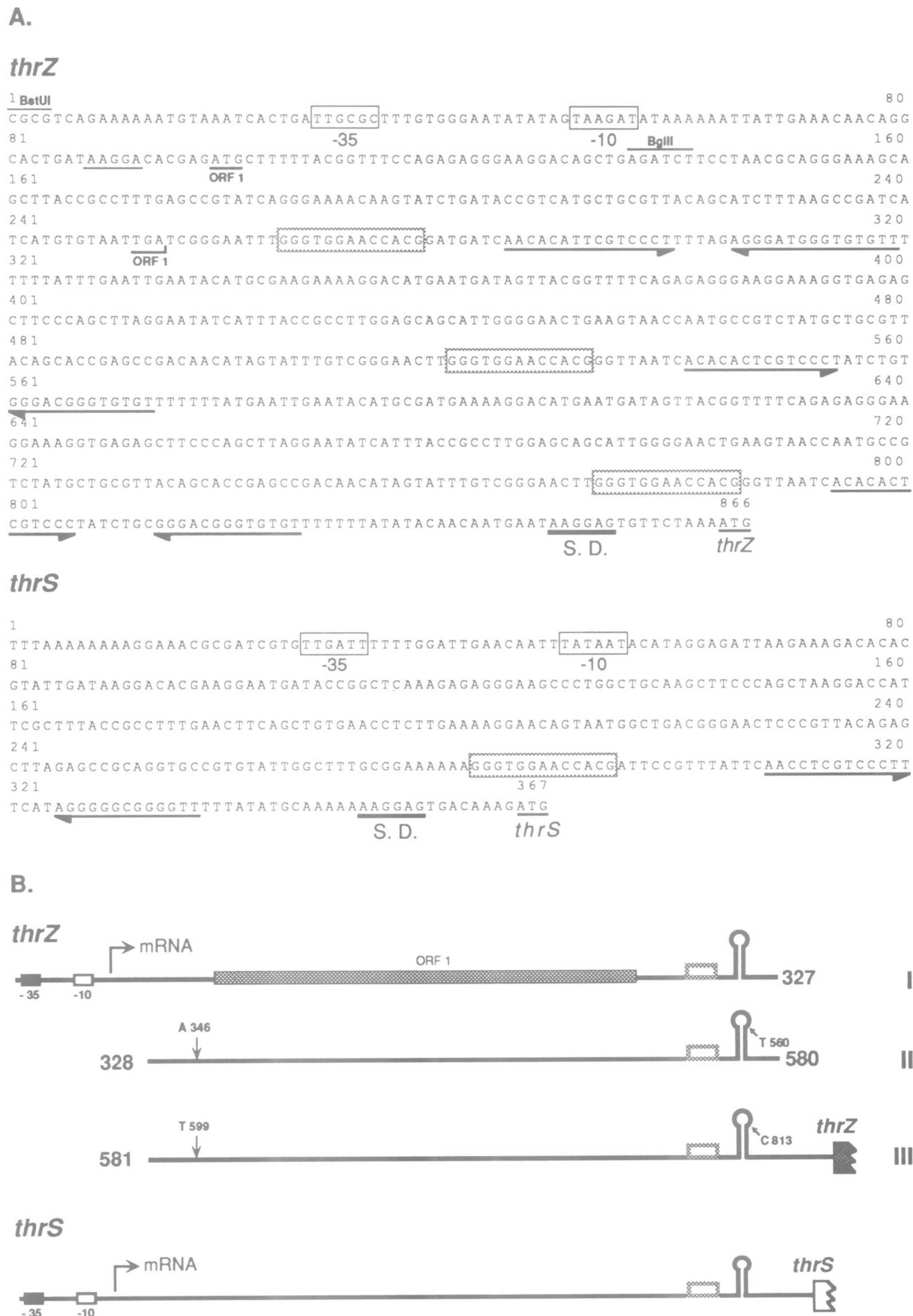

Fig. 3. Schematic representation of the *thrZ* and *thrS* leader sequences and their structural elements. (**A**) Nucleotide sequence of the *thrZ* and *thrS* leader regions. Promoter consensus sequences (−35 and −10) are boxed. The putative Shine–Dalgarno sequences (S.D.) and initiation codons are underlined. Short ORFs are indicated by their start and stop codons. The inverted arrows represent the stems of putative transcription terminators. A 13 bp sequence stretch conserved upstream of the terminator structures in the *thrZ* and *thrS* leaders is indicated by hatched boxes. (**B**) Schematic diagram of the *thrZ* and *thrS* leader regions. In the case of *thrZ* the leader is structured into three possibly functional domains (I, II and III). The only two nucleotide changes between *thrZ* domains II and III are indicated by arrows.

fragment starting at the *Bgl*II site, nucleotide 139 in Figure 3A) for insertion is lethal, while integration of a fragment extending to the *Bst*UI site (nucleotide 3 in Figure 3A, pHMZ6) gives rise to normally growing colonies. Both Northern and Western analyses showed that *thrZ* was expressed (data not shown). A transcriptional signal is thus localized to the first 140 bp of the sequence shown in Figure 3A (i.e. 860−720 bp upstream of *thrZ*). Inspection of this region reveals a σ^A consensus promoter sequence (see Figure 3A). This is consistent with the fact that *thrZ* is

expressed well during vegetative growth in the absence of a functional *thrS* gene but it does not explain why *thrZ* expression occurs only in a *thrS*$^-$ context.

The thrZ 5'-noncoding region contains extensive repetitive sequences

The *thrZ* mRNA 5'-noncoding region extends over >800 bp. Within this region, three very stable stem–loop structures ($\Delta G = -19.6$, -21.3 and -21.6 kcal/mol) followed by a stretch of T residues which probably correspond to factor-independent transcription terminators can be found (shown by arrows in Figure 3A). Moreover, the sequences preceding these secondary structures show extensive homology. The stem–loop structures located at the 3'-end of the repetitive sequences led us to consider the *thrZ* noncoding region as three functional domains (shown schematically in Figure 3B). Domains II and III are practically identical. Over a stretch of 252 bp only 2 bp are changed, one of them in the loop of the terminator structure (see Figure 3B). In addition, domain I is 77% homologous to domains II and III. Moreover, each of the three *thrZ* upstream domains displays 65% homology to the corresponding *thrS* 5'-noncoding region. Domain I of the *thrZ* regulatory region differs from domains II and III as well as the corresponding *thrS* region by the fact that it contains an open reading frame (52 amino acids) encoding five threonine residues (*thrZ* ORF1 in Figure 3B).

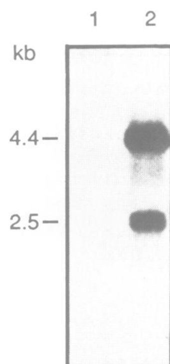

Fig. 4. Expression of *thrZ* is induced at the transcriptional level. Total RNA (10 μg) of an exponentially growing *B.subtilis* wild type (*thrS*$^+$) strain, lane 1, and a *thrS* mutant strain, lane 2, were analysed by Northern blot as described in Materials and methods. Both lanes were probed with a ^{32}P-labelled *thrZ* specific 0.85 kb *Eco*RI fragment (ordinates 1355–2209 in Putzer *et al.*, 1990).

Induction of thrZ expression occurs at the transcriptional level

The results obtained by Western analysis (Figure 1B) showed that synthesis of ThrZ protein was induced in the absence of a functional *thrS* gene. We performed Northern analysis of mRNA from *thrS*$^+$ and *thrS* mutant strains using the *thrZ* specific C-terminal 0.85 kb *Eco*RI fragment (ordinates 1359–2213 in Putzer *et al.*, 1990) as a probe. No *thrZ* mRNA was detected in a *thrS*$^+$ strain (see Figure 4, lane 1). However, inactivation of *thrS* causes the accumulation of two *thrZ* mRNA species of 4.4 kb and 2.5 kb (Figure 4, lane 2). A hybridization probe containing the *thrZ* promoter region and N-terminal sequences also detected both mRNA species (see Figure 6) while a probe comprising

Table II. Effect of *thrS* disruption on the expression of *thrZ–lacZ* fusions

Strain	Relevant genotype	β-Galactosidase specific activity (U/mg)
HP14	*thrS*$^+$::*erm*, *thrZ*$^+$::(*thrZ–lacZ* = pHMZ7)	<0.3
HP15	*thrS*::*erm*, *thrZ*$^+$::(*thrZ–lacZ* = pHMZ7)	210
HP19	*thrS*$^+$::*erm*, *amyE*::(*thrZ'–lacZ* = pHMZ9)	<0.3
HP20	*thrS*::*erm*, *amyE*::(*thrZ'–lacZ* = pHMZ9)	99

Table III. Induction of *thrZ–lacZ* expression in a *thrS* mutant strain can be reversed

A. By expressing *thrS* from a multicopy plasmid

Strain	Relevant genotype	Genes expressed from multicopy plasmid	β-Galactosidase specific activity (U/mg)
HP12	*thrS*::*erm*, *amyE*::(*thrZ'–lacZ* = pHMZ9)	–	96.5
		thrS	<0.3

B. By transducing the *thrS* mutant to a *thrS*$^+$ phenotype

Strain	Initial genotype	β-Galactosidase specific activity (U/mg)	Genotype after transduction	β-Galactosidase specific activity (U/mg)
HP29	Δ*thrS*::*kan* *amyE*::(*thrZ'–lacZ* = pHMZ9)	73	(i) *thrS*::*erm* *amyE*::(*thrZ'–lacZ*, *cat*)	71
			(ii) *thrS*$^+$::*erm* *amyE*::(*thrZ'–lacZ*, *cat*)	<0.3

sequences immediately upstream of the *thrZ* promoter detected neither the 2.5 kb nor the 4.4 kb transcript (data not shown). This indicates that both transcripts originate from the *thrZ* promoter. The shorter transcript is sufficient to cover the entire *thrZ* gene and possibly terminates at a secondary structure immediately downstream of the *thrZ* structural gene (ordinates 2274–2292 in Putzer *et al.*, 1990). The detection of the 4.4 kb transcript suggests that *thrZ* might be part of an operon extending downstream of *thrZ*. Induction of *thrZ* expression, caused by a disruption of the *thrS* gene, is thus likely to occur at the transcriptional level.

Induction of thrZ expression is fully reversible

To facilitate analysis of *thrZ* expression, we constructed two transcriptional fusions between *thrZ* and the *E.coli lacZ* gene. One was integrated at the *thrZ* chromosomal locus without interrupting the *thrZ* transcriptional unit (pHMZ7). Hence, β-galactosidase synthesis is under control of the *thrZ* promoter in its wild type context. In the second gene fusion, recombined into the *amy* locus, *lacZ* expression is dependent on the *thrZ* promoter fragment shown in Figure 3A (pHMZ9, see Materials and methods for details). Synthesis of β-galactosidase from both fusions was observed only upon inactivation of *thrS* (see Table II) and coincided with the appearance of *thrZ* mRNA (compare Figure 4, lane 2). The comparable specific activities obtained with the two gene fusions indicate that the cloned promoter fragment (shown in Figure 3A) contains the principal (if not all) regulatory elements necessary for *thrZ* expression. The simultaneous activation of *thrZ* transcription (as judged by Northern blotting) and of β-galactosidase synthesis from the *thrZ–lacZ* fusion *in trans* demonstrates that induction of *thrZ* expression is a true regulatory phenomenon and not due, for example, to a mutation in the *thrZ* promoter permitting *thrZ* expression and hence cell survival in the *thrS*$^-$ background.

If this relationship in the expression of the two threonyl-tRNA synthetase genes actually represents a biologically relevant mechanism, the reintroduction of a functional *thrS* gene should shut off *thrZ* expression. Two methods were employed to reintroduce a functional *thrS* gene: either the entire *thrS* transcriptional unit was provided *in trans* on a multicopy plasmid, or the mutated chromosomal *thrS* copy was exchanged for a wild type gene by PBS1 transduction (see Materials and methods). In both cases *thrS* expression was checked by Western analysis (data not shown) and found to be either identical (gene exchange) or 3-fold higher (from multicopy plasmid) when compared with wild type levels. The effect on *thrZ* expression was monitored by the β-galactosidase activity from a transcriptional *thrZ–lacZ* gene fusion integrated at *amy*. The results are summarized in Table III and demonstrate that the induction of *thrZ* expression is fully reversed when a functional *thrS* gene is provided.

Gradual reduction of ThrS protein stimulates thrZ expression in a dose-compensatory manner

The results described above show an all or none effect for *thrZ* expression which is induced when the *thrS* gene is inactivated. In order to test the sensitivity of this regulatory mechanism, we constructed a strain in which *thrS* is under control of the inducible P_{Spac} promoter, thus rendering its expression IPTG dependent. Transcription of *thrZ* was monitored by a *thrZ–lacZ* fusion and the actual synthesis of the *thrZ*-encoded threonyl-tRNA synthetase was analysed in parallel by Western blotting. The results, summarized in Figure 5, indicate clearly that the expression of *thrS* and *thrZ* are tightly co-ordinated. Any reduction of ThrS synthesis (due to a decrease in the IPTG concentration) is compensated by a proportional stimulation of *thrZ* expression. This shows that we are dealing with a very finely tuned regulatory mechanism since the normal intracellular concentration of *thrS*-encoded threonyl-tRNA synthetase is just sufficient to keep the *thrZ* gene shut off. This two gene system thus seems to have been designed in such a way that, under normal nutrient conditions, one component alone (i.e. the *thrS* gene) can provide the $tRNA^{Thr}$ charging capacity required for optimal growth.

Starvation for threonine induces thrZ and stimulates thrS expression

Since the function of *thrS* is to supply $Thr\text{-}tRNA^{Thr}$ for protein biosynthesis, it seemed possible that the level of charged $tRNA^{Thr}$ was affecting *thrZ* expression. If this was the case then starvation for threonine should have a similar effect on *thrZ* expression to that of reducing the ThrS protein level (and thus the $tRNA^{Thr}$ charging capacity). Effectively, a *thrZ–lacZ* fusion (integrated *in trans* at the *amy* locus) was induced specifically by starvation for threonine in a threonine auxotroph. The specificity of this regulatory response was confirmed by starvation for tryptophan and

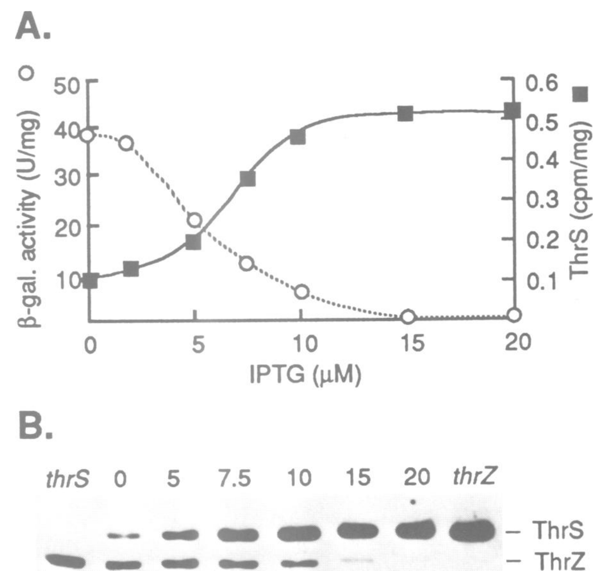

Fig. 5. Expression of a *thrZ–lacZ* fusion under ThrS limiting conditions. The *thrS* gene was put under control of the P_{Spac} promoter by integrating plasmid pHMS16 (see Materials and methods) on the chromosome, thus rendering its expression IPTG dependent. (**A**) ThrS protein synthesis was stimulated by adding increasing amounts of IPTG to the medium and quantified by counting the radioactivity (^{125}I) contained in the specific immunoblot signals shown in part B of the figure. Expression of *thrZ* was monitored by measuring the β-galactosidase activity of a transcriptional *thrZ–lacZ* fusion (pHMZ9, see Materials and methods). All samples were taken from exponentially growing cultures (OD_{600} ~ 1). (**B**) Western analysis of *thrS* and *thrZ* expression from cultures grown in the presence of different IPTG concentrations (see Materials and methods). The samples analysed were taken from the same cultures than those described in part (A) of the figure.

arginine (by adding arginine hydroxamate, a competitive inhibitor for Arg-tRNA synthetase). In neither case did we observe activation of the *thrZ–lacZ* fusion (Table IVA).

As described above, despite the significant difference in length between the *thrS* and *thrZ* 5'-noncoding regions, they share similar structural elements. In order to investigate if they were involved in a common regulatory mechanism, we measured the levels of ThrS and ThrZ in total cell extracts of the threonine auxotrophic strain for threonine. In addition to the induced synthesis of the ThrZ protein, we observed a 4- to 5-fold higher cellular ThrS concentration than in exponentially growing cells (data not shown). This result was confirmed by measuring the β-galactosidase activity from a transcriptional *thrS–lacZ* fusion. We observed 8.5-fold more β-galactosidase activity when cells were grown into premature stationary phase due to limiting amounts of threonine than in cells starved for tryptophan (Table IVB). Thus *thrS* and *thrZ*, despite the very different expression patterns (i.e. normally only *thrS* is expressed) exhibit a very similar regulatory response.

Regulation of thrZ expression involves transcriptional antitermination

The expression of *thrZ* leads to the accumulation of two specific mRNA species (Figure 4) probably transcribed from a σ^A type promoter (Figure 3A). In order to test if induction of *thrZ* expression is due to *de novo* transcriptional initiation, we constructed a *thrZ–lacZ* operon fusion (pHMZ11) in which the *lacZ* gene was under control of the 139 bp *Bst*UI–*Bgl*II fragment (Figure 3A) containing the σ^A consensus promoter sequence. By choosing this short fragment we could monitor transcription from the *thrZ* promoter without interference of the multiple secondary structures present in the *thrZ* leader. The fusion was integrated at the *amy* locus in a *thrS*$^+$ and a *thrS* mutant strain. The β-galactosidase activity data are shown in Table V and demonstrate that initiation of *thrZ* transcription is constitutive and thus not significantly involved in the regulation of *thrZ* expression. Since under normal conditions (*thrS*$^+$), mRNA corresponding to the *thrZ* structural gene is not observed (Figure 4), it is probable that the extensive secondary structures within the *thrZ* leader normally terminate *thrZ* transcription. There is thus a need for an antitermination mechanism in order to allow transcription to proceed through the putative transcription terminators into the structural gene (Figure 3B).

To test this assumption, we performed a Northern analysis of promoter proximal *thrZ* transcripts from a strain expressing *thrZ* (i.e. a *thrS* mutant strain) and from a wild type strain where *thrZ* is not expressed. The hybridization probe was the 1 kb *Bst*UI–*Hin*dIII fragment covering the entire *thrZ* regulatory region (Figure 3A) and 137 bp of the *thrZ* structural gene. Short *thrZ* specific transcripts are observed in both strains (Figure 6) and clearly demonstrate that transcription of *thrZ* is indeed initiated in a constitutive manner, even in the wild type strain where no ThrZ protein can be detected under normal growth conditions. The lengths of the short transcripts (250, 550 and 750 nucleotides) correspond well to transcripts that would be generated by transcriptional termination at the terminator structures of domains I, II and III, respectively (Figure 3B). The difference between a strain expressing *thrZ* and one in which no ThrZ protein is synthesized thus resides in the difference in efficiency with which RNA polymerase transcribes through the multiple terminator structures.

Northern analysis and S1-nuclease mapping of *thrS*

Table IV. Induction of a *thrZ–lacZ* fusion and derepression of a *thrS–lacZ* fusion due to threonine starvation

Strain	Genotype	Tryptophan (mM)	Threonine (mM)	Arginine hydroxamate	β-Galactosidase specific activity (U/mg)
A. *thrZ–lacZ*					
HP37	*trp, thr*	1	2		<0.3
	amyE::(*thrZ'–lacZ* = pHMZ9)	0.004	2		<0.3
		1	0.15		79
HP13	*amyE*::(*thrZ'–lacZ* = pHMZ9)	–	–	–	<0.3
		–	–	+ (2 μM)	0.5
B. *thrS–lacZ*					
HP39	*trp, thr*	1	2		37
	amyE::(*thrS'–lacZ* = pHMS11)	0.004	2		16 (38*)
		1	0.15		134 (36*)
HP18	*amyE*::(*thrS'–lacZ* = pHMS11)	–	–	–	38
		–	–	+ (2 μM)	37

The limiting concentrations of amino acids used were adjusted in order to grow cells into premature stationary at OD_{600} of ~0.5. Samples were taken 3 h after the end of logarithmic growth. Control values (in the presence of non-limiting amino acid concentrations) were taken at an OD_{600} of 0.5.
*Denotes values from samples taken from the same culture during exponential growth.

Table V. Initiation of *thrZ* transcription does not depend on the presence or absence of a functional *thrS* gene

Strain	Relevant genotype	β-Galactosidase specific activity (U/mg)
HP43	*thrS*$^+$::*erm*, *amyE*::(*thrZ'–lacZ* = pHMZ11)	229
HP44	*thrS*::*erm*, *amyE*::(*thrZ'–lacZ* = pHMZ11)	280

expression in a wild type strain similarly detect a small truncated transcript of ~280 bp, presumably generated by premature transcriptional termination at the single terminator structure of the *thrS* leader, as well as the full length transcript of 2.3 kb (data not shown).

A highly conserved consensus sequence is involved in antitermination of thrS

The striking parallel between the presence of truncated promoter proximal transcripts for both *thrS* and *thrZ* and the fact that both genes are induced during threonine starvation suggested that the structural homologies observed between the *thrS* and *thrZ* regulatory sequences also reflect a functional homology.

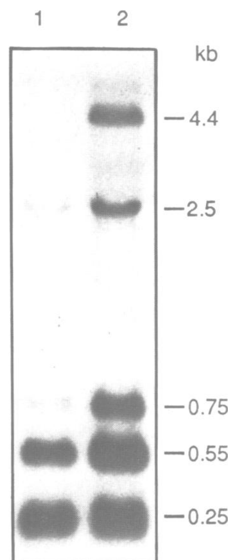

Fig. 6. Expression of *thrZ* involves transcriptional antitermination. Total RNA (10 μg) of an exponentially growing *B.subtilis* wild type (*thrS*$^+$) strain, lane 1, and a *thrS* mutant strain, lane 2, were analysed by Northern blotting as described in Materials and methods. The 1 kb *Bst*UI−*Hin*dIII fragment (covering the entire *thrZ* regulatory region, see text) was used as a hybridization probe.

As described above, the 5′-untranslated regions of *thrS* and *thrZ* (domains I, II and III) exhibit extensive overall sequence homologies. In addition, we noticed an extremely well conserved sequence located just upstream of each of the four transcription terminators. This proposed consensus sequence is 13 nucleotides long (shown by hatched boxes in Figure 3A).

To assess the functional significance of this consensus sequence on the expression of *thrS*, where the presence of only one terminator structure should lead to a more clear-cut result, we constructed a transcriptional *thrS−lacZ* fusion where six nucleotides of the conserved motif (−GGG-TGG−, *thrS*Δ28) were deleted by oligonucleotide-directed mutagenesis (pHMS20Δ28, see Materials and methods). Expression of β-galactosidase from this gene fusion was measured and compared with an analogous wild type fusion. As shown in Table VIA the 6 bp deletion leads to a dramatic decrease (60-fold) in β-galactosidase activity. On the other hand, the same deletion does not have a significant effect on the expression of a *thrS−lacZ* fusion when the *lacZ* proximal half of the *thrS* terminator has been deleted (Table VIA). This conserved sequence is thus an essential part of an antitermination mechanism which permits expression downstream of the terminator. Under wild type conditions, >50% of the initiated *thrS* transcripts extend into the structural gene while the Δ28 mutation causes >99% premature termination. Moreover, the mutated *thrS−lacZ* fusion has become insensitive to derepression due to threonine starvation (Table VIB).

Discussion

Antitermination of thrS and thrZ transcription involves common structural elements

We have shown that the *thrZ* gene encoding an alternative threonyl-tRNA synthetase in *B.subtilis* is normally not expressed and that its expression is induced when that of *thrS* is reduced. Both genes, *thrS* and *thrZ*, are induced by threonine starvation. Northern analysis identified truncated transcripts mapping to terminator structures located upstream

Table VI. Effect of the thrSΔ28 mutation on the β-galactosidase activity of *thrS−lacZ* fusions

A.

Strain	*thrS−lacZ* fusion	Diagram of *thrS* region cloned	β-Galactosidase specific activity (U/mg)
HP18	pHMS11		151
HP41	pHMS20Δ28	-6	2.5
HP45	pHMS21		289
HP46	pHMS21Δ28	-6	220

B.

Strain	Genotype	Threonine (mM)	β-Galactosidase specific activity (U/mg)
HP39	*trp, thr*	2	37
	thrS′−lacZ = pHMS11	0.15	134
HP42	*trp, thr*	2	<0.3
	*thrS*Δ28′−*lacZ* = pHMS20Δ28	0.15	<0.3

of both genes and provided evidence that induction of *thrS* and *thrZ* expression results from extension of the observed truncated transcripts into the respective structural genes. Consistent with the idea that this is due to a transcriptional antitermination mechanism, we have identified a strongly conserved consensus sequence upstream of each terminator which is crucial for the synthesis of read through full-length *thrS* transcripts. In fact, a deletion of six nucleotides within the described consensus sequence results in a dramatic reduction in expression of a transcriptional *lacZ* fusion and a complete loss of response to threonine starvation. The same deletion does not have a significant influence on *thrS* expression in the absence of a functional terminator structure, showing that the consensus sequence is directly involved in antitermination.

It is interesting that the leader regions of most Gram-positive aminoacyl-tRNA synthetase genes sequenced to date contain a sequence homologous to the 13 bp consensus described here located upstream of a putative terminator structure in the same relative position (immediately upstream of the ribosome binding site). Henkin *et al.* (1992) have recently noted that this consensus sequence is present in the leaders of the synthetase genes *pheST* (Brakhage *et al.*, 1990), *trpS* (Chow and Wong, 1988), *tyrS* (Henkin *et al.*, 1992) and *tyrZ* (Glaser *et al.*, 1990) as well as in the *ilv–leu* biosynthetic operon (Grandoni *et al.*, 1992) in *B.subtilis* and *tyrS* (Waye and Winter 1986) and *trpS* (Barstow *et al.*, 1986) in *Bacillus stearothermophilus*. It is not found in the *B.stearothermophilus metS* gene (Mechulum *et al.*, 1991). In *B.subtilis* we also identified this consensus sequence in the leader of the cyE–cyS operon gene (Breton, 1991) but we did not find it or a putative terminator in the leader of the glutamyl-tRNA synthetase gene *gltX* (Breton, 1991). The following general consensus sequence can be deduced: -aAnnnGgGTGGn-ACCrCG- (where n = any nucleotide, r = purine nucleotide, lower case letters = present in at least 11/12). It is tempting to speculate that this strong conservation of structural features could reflect a co-ordinate regulation of these genes. One possible mechanism of co-ordinate regulation could be the so called 'stringent' response to amino acid starvation. However, our results show clearly that the *thrS* and *thrZ* genes are both very specifically regulated by starvation for threonine. Inducing the stringent response with arginine hydroxamate or starving the cells for tryptophan had no effect on *thrS*/*thrZ* expression. This specificity of regulation is also observed for expression of the *B.subtilis tyrS* gene (Henkin *et al.*, 1992). Since we did not test all amino acids we cannot rule out the possibility of a regulatory response involving a restricted number of specific amino acids.

The *thrZ* leader region contains the same structural features outlined above but in triplicate and extends over >800 bp, which is unusually long for a prokaryotic mRNA. It can be divided into three domains (see Figure 3B) of which domains II and III are practically identical. Each of these three segments not only corresponds in size to the *thrS* leader but also contains the elements shown to be involved in transcriptional antitermination of *thrS*, i.e. the terminator structure and the upstream consensus sequence. Domain I nevertheless differs from domains II and III as well as the *thrS* leader in that it contains an open reading frame (52 amino acids) encoding five threonine residues (*thrZ* ORF1 in Figure 3B). This arrangement is reminiscent of the transcriptional attenuators regulating several biosynthetic operons in prokaryotes. However, we have no evidence that ORF1 is translated. It is not preceded by a good translational initiation signal (for a Gram-positive organism) and, since four out of five threonine codons are located in the N-terminal half of the polypeptide, it is difficult to envisage how a ribosome stalling on them would affect formation of the terminator at least 80 nucleotides downstream. Moreover, such a mechanism cannot be invoked to explain anti-termination at *thrZ* terminators II and III.

How is the expression of thrS and thrZ co-ordinated?

Initiation of *thrZ* transcription is constitutive and *thrZ* expression thus seems to be regulated exclusively by the degree of premature transcriptional termination. Our results show that the expression of *thrZ* is induced under two conditions: when the intracellular threonyl-tRNA synthetase concentration (dependent on *thrS* expression) is decreased below its normal physiological level or when the cell is starved for threonine. Reducing ThrS synthesis leads to a reduction in the level of charged $tRNA^{Thr}$. On the other hand, during threonine starvation, efficient $tRNA^{Thr}$ charging is impaired due to the physical absence of one of the components. Since the result common to both cases is a reduction in Thr-$tRNA^{Thr}$ pool size, this could be the real effector of *thrZ* and *thrS* induction. Alternatively, the effector could be the intracellular concentration of free threonine if the biosynthesis of threonine was regulated by the $tRNA^{Thr}$ pool. A transcriptional attenuation mechanism common to many biosynthetic operons in prokaryotes would permit such a coupling but no such regulatory mechanism seems to be involved in the regulation of the biosynthetic *thr* genes in *B.subtilis* (Parsot and Cohen, 1988).

If Thr-$tRNA^{Thr}$ is the effector molecule, how is the Thr-$tRNA^{Thr}$ pool sensed? A candidate would be the *thrS*-encoded synthetase itself. However, the *thrZ* gene is activated in the absence of ThrS, implying that ThrS could only play the role of a repressor. In this model, repression would be eliminated due to a complexing of ThrS with uncharged $tRNA^{Thr}$. The fact that a deletion within the regulatory region practically abolishes β-galactosidase synthesis from a transcriptional *thrS–lacZ* fusion rather makes this sequence a site of action for a positively acting element. We thus favour the idea that *thrS* as well as *thrZ* expression is regulated by a *trans*-acting antitermination protein. The observation that a similar structural arrangement is well conserved in the leader sequences of almost all known Gram-positive aminoacyl-tRNA synthetase genes is reminiscent of the regulation observed for the *B.subtilis sac* genes implicated in sucrose utilization (Steinmetz *et al.*, 1988; Crutz *et al.*, 1990; Débarbouillé *et al.*, 1990), and the *B.subtilis* and *E.coli bgl* operons which are involved in the utilization of aromatic β-glucosides (Zukowski *et al.*, 1988; Houman *et al.*, 1990). All these genes appear to be specifically regulated by antitermination via similar regulatory proteins recognizing a well conserved recognition sequence overlapping a transcriptional terminator. The *trp* operon in *B.subtilis* is regulated by a *trans*-acting factor (encoded by *mtrA* and *mtrB*) promoting transcription termination in the presence of tryptophan by binding to a

recognition sequence overlapping with an antitermination structure, thereby favouring formation of the terminator structure (Kuroda *et al.*, 1988; Gollnick *et al.*, 1990).

In fact, in all four cases (*thrS* leader and *thrZ* domains I, II and III) reasonably stable alternative secondary structures overlapping the terminator stem–loop can be formed ($\Delta G = -6.1$ to -12.1 kcal/mol). All include the consensus sequence as an integral structural part. We thus believe that a putative activator recognizes not necessarily the consensus sequence as such but rather a specific secondary structure (the antiterminator) including this sequence. In this case the activator would stabilize the alternative secondary structures in order to favour antitermination.

The potential antiterminator structures could also function as targets for an endonuclease making the downstream parts of the leader vulnerable to rapid degradation. The consensus sequence included in the secondary structure would then be the target for a protein protecting the mRNA from degradation. However, this regulation by an mRNA turnover mechanism seems unlikely: a *thrS–lacZ* fusion where the *lacZ* proximal half of the terminator has been deleted still has the potential to form the alternative secondary structure but its expression is not reduced by the Δ28 deletion which almost eliminates expression in the presence of the intact terminator (see Table VIA). The role that the ThrS protein, $tRNA^{Thr}$ (charged or uncharged) or free threonine might play in the regulation of the *thrS/thrZ* genes is currently under investigation.

If, as our data suggest, the structural homologies observed between the *thrS* and *thrZ* leader regions also reflect a functional homology, then we are dealing with an intriguing phenomenon in the sense that the cell is using one regulatory unit to obtain completely different, but nevertheless complementary, expression patterns of two genes with identical functions.

The presence of a single regulatory element upstream of *thrS* permits constitutive but modulatable expression under normal growth conditions. The *thrZ* gene is normally not expressed simply because two regulatory units, corresponding to domains II and III which effectively terminate transcripts extending beyond terminator I, have been added. Upon a specific stimulus, e.g. threonine starvation, the cell responds by increasing the cellular concentration of threonyl-tRNA synthetase. This is achieved by favouring transcriptional antitermination in a similar fashion for *thrS* as well as *thrZ*. Thereby *thrS* expression is stimulated and *thrZ* expression induced.

What is the role of thrZ?

Our results show that the *thrZ*-encoded threonyl-tRNA synthetase can effectively replace the ThrS protein in promoting vegetative growth. In fact, cells expressing only *thrS* or *thrZ* (due to *thrS* disruption) grow equally well at 30, 37 and 42°C (data not shown). Thus the major enzymatic activities of both proteins seem to be quite similar.

In response to conditions of nutrient limitation, *B.subtilis* enters a developmental process that culminates in the formation of a dormant cell type known as the endospore. Since numerous genes are specifically expressed in the differentiating cell, we tested if *thrZ* is a developmentally regulated gene. Using a *thrS* and a *thrZ* mutant strain, respectively, we found that each of the two genes (*thrS* or *thrZ*) by itself is sufficient for *B.subtilis* to conclude an entire biological cycle including sporulation and germination. The sporulation efficiency at 37°C and 42°C was identical in both strains: 5×10^8 spores/ml. We also measured β-galactosidase levels from a *thrZ–lacZ* fusion during development. No activity above background could be detected throughout the entire sporulation process (data not shown). Since *thrZ* by itself is able to promote sporulation in a $thrS^-$ context, we conclude that its expression is neither required nor induced during sporulation. However, we cannot yet rule out the possibility of a prespore specific expression of *thrZ* during the last stages of the sporulation process (after stage IV), when β-galactosidase assays were not possible due to the physical resistance of the prespore to the mechanical disruption procedure applied.

It should be recalled that certain aminoacyl-tRNA synthetases are capable of sustaining unusual functions, beyond the normal reaction of aminoacylation of the tRNAs. Several aminoacyl-tRNA synthetases from prokaryotes and eukaryotes catalyse the formation of Ap_4A, in response to heat shock or oxidative stress (Brevet *et al.*, 1989; Wahab and Yang, 1985) and a mitochondrial aminoacyl-tRNA synthetase is involved in the splicing of group I and II introns (Akins and Lambowitz, 1987; Herbert *et al.*, 1988). So far we have no indication for an alternative function of either protein, ThrS or ThrZ.

Comparison with similar two-gene systems

The only similar system for which significant data are available is the gene pair *lysS/lysU* encoding lysyl-tRNA synthetase in *E.coli*. The *lysS* gene is constitutive, while *lysU*, which is normally only very weakly expressed, can be induced under certain physiological conditions, e.g. after heat shock treatment (Neidhardt and VanBogelen, 1981) and during anaerobic growth (Lévêque *et al.*, 1991). However, the expression of *lysS* has no influence on the expression of *lysU*. We have recently analysed the expression pattern of the *B.subtilis tyrZ* gene, which encodes a second tyrosyl-tRNA synthetase (Glaser *et al.*, 1990). In analogy to *thrZ*, we detected no *tyrZ* mRNA under normal growth conditions but survivors growing exclusively on *tyrZ* can be obtained after disruption of *tyrS* (unpublished data, *tyrS* and *tyrZ* containing plasmids were kindly provided by T.Henkin and P.Glaser, respectively). Whether this induction of *tyrZ* is due to a mutational event or represents a true regulatory mechanism, similar to the one described here for *thrS/thrZ*, remains to be analysed. However, an analogy between these two systems might help to reveal the biological role for such gene duplications.

The strong conservation of structural regulatory elements between the Gram-positive aminoacyl-tRNA synthetase genes is in sharp contrast to the situation observed in *E.coli* where each aminoacyl-tRNA synthetase seems to be regulated by its own very specific regulatory mechanism (Grunberg-Manago, 1987). The challenging question now is to see whether the expression of a whole class of essential genes, encoding different aminoacyl-tRNA synthetases, is controlled in a similar way by a unique protein or by a set of regulatory proteins recognizing a conserved structural motif but acting in response to a specific stimulus, e.g. variations in the pool of the individual charged tRNAs.

Materials and methods

Strains and growth conditions

All *B.subtilis* strains are derivatives of the prototrophic strain 168 (BGSC 1A2) and auxotrophic strain BGSC 1A42 (*trpC2, thr-5*). They were grown on TBAB (Difco) agar plates as solid medium and in LB-1% glucose as liquid culture. For sporulation experiments, *B.subtilis* strains were propagated in 2 × SG medium (Schaeffer *et al.*, 1965). Where not specifically indicated, cells were grown at 37°C. Antibiotics for selection of chromosomal plasmid integrants were added at 5 μg/ml for chloramphenicol, 5 μg/ml for kanamycin, 0.5/12.5 μg/ml for erythromycin/lincomycin (Mls) and for selection of replicative plasmids at 20 μg/ml for tetracycline. For starvation experiments, cells were grown in M9 minimal medium (Sambrook *et al.*, 1989) with 1% glucose and varying concentrations of threonine and tryptophan.

E.coli strain JM109 served as the host for plasmid constructions and was grown in LB medium (Miller, 1972) in the presence of the appropriate antibiotics (ampicillin, 100 μg/ml, tetracycline, 20 μg/ml).

Transformation and transduction procedures

E.coli cells were transformed according to Chung *et al.* (1989) and *B.subtilis* cells according to Hardy (1985). PBS1 transduction was performed as described in Haworth and Brown (1973). It was used to replace the disrupted *thrS* gene of the pHMS15 (*kan*) integrant strain HP29 against the intact *thrS* copy of the M13tgS9 (*erm*) integrant strain HP14 by marker exchange.

Isolation of B.subtilis chromosomal DNA

A 100 ml culture was grown to an optical density at 600 nm of 1.5–1.8 in LB containing 1% glucose. Cells were harvested by centrifugation (5000 *g*, 15 min, 4°C) and washed once with 20 ml of ice-cold 0.1 M NaCl, 0.05 M Tris–HCl (pH 8), 5 mM EDTA. The pellet was suspended in 3 ml of 25% sucrose, 50 mM Tris–HCl (pH 8), 5 mM EDTA and, after addition of 2 mg of lysozyme, incubated for 15 min at 37°C. One volume of 1% sodium dodecyl sulfate (SDS), 1 mg/ml proteinase K, 50 mM Tris–HCl (pH 8), 500 mM EDTA was added, and the mixture was incubated for 2 h at 50°C. The DNA was precipitated with 1 volume of isopropanol, spooled on a glass rod, washed with 80% alcohol, dried and resuspended in 3 ml of TE buffer [10 mM Tris–HCl (pH 8), 1 mM EDTA]. After RNase A treatment (30 min, 50°C) the DNA was once more precipitated and resuspended in TE buffer as described above.

DNA sequencing

Double-stranded recombinant plasmid DNAs were used as templates in dideoxy-chain termination sequencing reactions (Sanger *et al.*, 1977) using sequence specific synthetic oligonucleotides as primers.

Plasmid constructions

pHM3. Plasmid pHM3 is a shuttle vector capable of replicating in *E.coli* as well as *B.subtilis* and is composed of the *Bacillus cereus* plasmid pBC16-1 (Kreft *et al.*, 1978) and pTZ19R (USB) ligated at their unique *Eco*RI sites. With the exception of *Eco*RI, all other sites of the pTZ19R multiple cloning site remain unique and can be used for insertions.

pHMS3. Plasmid pHMS3 contains the entire *thrS* transcriptional unit cloned as a *Bam*HI–*Sst*I fragment (nucleotides 1–2614) derived from plasmid pHTv (Putzer *et al.*, 1990) into the corresponding sites of pHM3. pHMS3 expresses *thrS* in *E.coli* and *B.subitlis*.

pHMS7. A 0.44 kb *Bam*HI–*Eco*RV fragment covering the *thrS* regulatory region and N-terminal sequences (ordinates 3–439 in Putzer *et al.*, 1990) was inserted into the respective sites of the integrative plasmid pCP115 (Price and Henner, 1985).

pHMS8. Plasmid pCP115 carrying, in the respective sites, an internal *Eco*RV–*Eco*RI *thrS* fragment (ordinates 440–899 in Putzer *et al.*, 1990).

pHMS15. 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 nucleotide 75 of the structural gene. A 930 bp *Stu*I fragment, internal to *thrS*, has been replaced by 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.

pHMS16. A 583 bp *Eco*NI–*Eco*RI *thrS* fragment containing the N-terminal part of *thrS* (ordinates 321–904 in Putzer *et al.*, 1990) was cloned into the *Sma*I–*Eco*RI sites of pBluescript$^+$ in order to reisolate it as a *Xba*I–*Sal*I fragment. This fragment was then inserted into the *Xba*I (partial digest)–*Sal*I sites of the integrative plasmid pDG648 (provided by P.Stragier) downstream of the P_{Spac} promoter to give pHMS16. Campbell type integration of pHMS16 on the *B.subtilis* chromosome renders *thrS* expression IPTG dependent.

M13tgS9 and M13tgS10. M13tgS9 phage contains a *Sal*I–*Hin*dIII *thrS* fragment derived from plasmid pHTv (ordinates 2103–2628 in Putzer *et al.*, 1990) covering the *thrS* C-terminal end and the *ermC* gene of pE194 (Horinouchi and Weisblum, 1980), both cloned in the polylinker of M13tg130 (Kieny *et al.*, 1983). The *erm* gene was cloned as a *Xba*I–*Sma*I fragment (from pDG641, provided by P.Stragier) into the respective sites of pBluescript$^+$ to give pBSerm. The 526 bp *Sal*I–*Hin*dIII *thrS* fragment was inserted into the corresponding sites of pBSerm and the resulting insert (*erm* plus *thrS* C-terminal end) was cloned as a *Xba*I–*Sal*I fragment in the respective polylinker sites of M13tg130 to give M13tgS9. M13tgS10 was constructed by inserting an internal *Eco*RV–*Eco*RI *thrS* fragment derived from plasmid pHTv (ordinates 440–899 in Putzer *et al.*, 1990) into pBSerm and transferring the hybrid insert as a *Xba*I–*Eco*RV fragment into the corresponding sites of the M13tg130 polylinker.

Transcriptional fusions with thrS. Four different fusions were constructed: for the first one, pHMS11, a 0.44 kb *Dra*I–*Eco*RV fragment covering the *thrS* regulatory region and N-terminal sequences (ordinates 3–439 in Putzer *et al.*, 1990) was inserted into the *Hin*dIII site (filled in using Klenow fragment) upstream of the *lacZ* gene in plasmid pDG268 (Antoniewski *et al.*, 1990). The resulting plasmid, pHMS11, was integrated at the *amy* locus by double crossing over recombination. For the second fusion, pHMS20Δ28, a 6 bp deletion (-GGGTGG-, ordinates 281–286 in Figure 3A) was introduced by site directed mutagenesis into the 0.5 kb *Dra*I–*Cla*I fragment (ordinates 3–507 in Putzer *et al.*, 1990). The mutated fragment was inserted between the *Eco*RI and *Hin*dIII sites upstream of the *lacZ* gene in plasmid pDG268 after having been subcloned in plasmid pMTL23 (Chambers *et al.*, 1988) in order to reisolate it with the corresponding cohesive ends. For the third fusion, pHMS21, a 0.32 kb *Dra*I–*Eco*NI fragment (ordinates 3–321 in Putzer *et al.*, 1990), isolated as an *Eco*RI–*Eco*NI fragment, was inserted between the *Eco*RI and *Bam*HI (filled in) sites upstream of the *lacZ* gene in plasmid pDG268. The fourth fusion pHMS21Δ28 is identical to the third, pHMS21, except that a 6 bp deletion (-GGGTGG-, ordinates 281–286 in Figure 3A) was introduced by site-directed mutagenesis.

pHMZ1. Plasmid pHMZ1 contains a 1.3 kb *Bst*NI (filled in)–*Mlu*I internal *thrZ* fragment (nucleotides 44–1329 of the structural gene) cloned in the *Eco*RV site of pCP115 (Price and Henner, 1985).

pHMZ2. Plasmid pHMZ2 is based on the integrative vector pCP115. It contains a 2.1 kb *Bgl*II–*Mlu*I *thrZ* fragment (starting at nucleotide 139 in Figure 3A) covering 0.8 kb of the promoter region plus 1.3 kb of the N-terminal part of the structural gene. It was isolated as a *Sph*I–*Mlu*I fragment from a pUC18 plasmid carrying the 4 kb *Bgl*II fragment originally isolated from a recombinant λ phage (Putzer *et al.*, 1990).

pHMZ6. Plasmid pHMZ6 is composed of the integrative plasmid pDG271 (Antoniewski *et al.*, 1990) carrying a 1 kb *Bst*UI–*Hin*dIII *thrZ* fragment (nucleotides 3–1004, shown up to nucleotide 866 in Figure 3A) inserted between the *Eco*RV and *Hin*dIII sites.

Transcriptional fusions with thrZ. Three different *lacZ* gene fusions were constructed. For the first one, pHMZ9, the 1 kb *Bst*UI–*Hin*dIII *thrZ* fragment (comprising the entire *thrZ* promoter region, nucleotides 3–1004, shown up to nucleotide 866 in Figure 3A) was isolated as an *Nhe*I–*Hin*dIII fragment from plasmid pHMZ6, cloned into plasmid pUC18 (*Xba*I–*Hin*dIII) and reisolated as an *Eco*RI–*Hin*dIII fragment, which was inserted into the respective sites of the *lacZ* fusion vector pDG268. This construct was integrated at the *amy* locus by double crossing over recombination. For the second *lacZ* fusion, pHMZ7, a 0.85 kb *Eco*RI C-terminal *thrZ* fragment (covering 13 nucleotides downstream of the *thrZ* stop codon, ordinates 1355–2209 in Putzer *et al.*, 1990) was cloned into the *lacZ* fusion plasmid pJM783 (Perego *et al.*, 1988) and integrated by Campbell type insertion at the *thrZ* chromosomal locus. This way the *thrZ* gene was not disrupted and the *lacZ* gene was under control of the *thrZ* promoter in its wild type context. The gene fusion pHMZ11 was constructed by inserting the 139 bp *Bst*UI–*Bgl*II fragment (ordinates 3–139 in Figure 3A), reisolated as an *Eco*RI–*Bgl*II fragment, into the *Eco*RI–*Bam*HI sites of the *lacZ* fusion plasmid pDG268.

Isolation of total cellular RNA

B.subtilis cultures were grown in LB medium to an OD_{600} of ~1, centrifuged (10 min, 4000 *g*, 4°C) and resuspended in 4 ml of ice-cold TE buffer. The cell suspension was added to a tube containing 3.5 g of glass beads, 3 ml phenol/H_2O, 0.5 ml $CHCl_3$, 0.25 ml 10% SDS and vortexed three times for each 1 min with 1 min intervals at 4°C. The phases were separated by centrifugation (10 min, 8000 *g*, 4°C) and the aqueous phase was re-extracted twice with 3 ml phenol/H_2O, 0.5 ml $CHCl_3$ as above. The nucleic acids were precipitated with 0.1 vol LiCl/3 vol EtOH and dissolved in diethylpyrocarbonate-treated water.

Northern blotting

Routinely, 10 μg of total cellular RNA were separated on formaldehyde containing gels essentially as described by Lehrach *et al.* (1977) and transferred to a nylon membrane (Amersham Hybond N) in 10 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate) using a vacuum blotter (Hoefer). Hybridization was performed in 50% formamide, 5 × SSC, 5 × Denhardt's solution, 0.3% SDS, 200 μg/ml of denatured herring sperm DNA at 45°C overnight using probes radiolabelled with ^{32}P by random priming (Feinberg and Vogelstein, 1983). 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 prior to autoradiographic exposure.

Western blotting

Blotting and immunodetection of proteins were performed essentially as described by Putzer *et al.* (1990). Proteins recognized by anti-*E.coli* ThrRS antibodies were visualized either by using [^{125}I]protein A or the enhanced chemiluminescence (ECL) protein detection kit following the manufacturer's instructions (Amersham).

β-Galactosidase assay

1.5 ml samples of culture were harvested, washed with Z buffer (Miller, 1972) and kept at −20°C. After resuspension in 0.5 ml of Z buffer, the samples were sonicated and clarified by centrifugation. β-galactosidase specific activity was measured according to Miller (1972) and expressed as nmol ONPG produced per min per mg of protein.

Computer analysis

For general sequence analysis we used the facilities of the Centre Inter-Universitaire d'Informatique à Orientation Biomédicale, Paris, France (Dessen *et al.*, 1990). RNA secondary structures of minimum free energy were identified using the program described by Zuker (1989).

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