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Structure and Regulation of Expression of the *Bacillus subtilis* Valyl-tRNA Synthetase Gene

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We have sequenced the valyl-tRNA synthetase gene (*valS*) of *Bacillus subtilis* and found an open reading frame coding for a protein of 880 amino acids with a molar mass of 101,749. The predicted amino acid sequence shares strong similarity with the valyl-tRNA synthetases from *Bacillus stearothermophilus*, *Lactobacillus casei*, and *Escherichia coli*. Extracts of *B. subtilis* strains overexpressing the *valS* gene on a plasmid have increased valyl-tRNA aminoacylation activity. Northern analysis shows that *valS* is cotranscribed with the *folC* gene (encoding folyl-polyglutamate synthetase) lying downstream. The 300-bp 5′ noncoding region of the gene contains the characteristic regulatory elements, T box, "specifier codon" (GUC), and *rho*-independant transcription terminator of a gene family in gram-positive bacteria that encodes many aminoacyl-tRNA synthetases and some amino acid biosynthetic enzymes and that is regulated by tRNA-mediated antitermination. We have shown that *valS* expression is induced by valine limitation and that the specificity of induction can be switched to threonine by changing the GUC (Val) specifier triplet to ACC (Thr). Overexpression of *valS* from a recombinant plasmid leads to autorepression of a *valS-lacZ* transcriptional fusion. Like induction by valine starvation, autoregulation of *valS* depends on the presence of the GUC specifier codon. Disruption of the *valS* gene was not lethal, suggesting the existence of a second gene, as is the case for both the *thrS* and the *tyrS* genes.

The aminoacyl-tRNA synthetases (aaRS) catalyze the covalent attachment of amino acids to their cognate tRNAs, a reaction crucial for the accuracy of protein synthesis. For the most part, there is only one aaRS for each amino acid species in bacteria, although several exceptions are known. The presence of two very similar lysyl-tRNA synthetases represents the singular exception in *Escherichia coli* (21, 22, 26), where the tRNA synthetases for all 20 amino acids have been cloned (12). The situation is different in gram-positive organisms. On the one hand, they lack a glutaminyl-tRNA synthetase (43), and on the other hand, there are two distinct threonyl-tRNA synthetase genes (*thrS* and *thrZ* [32]) and two tyrosyl-tRNA synthetase genes (*tyrS* and *tyrZ* [9, 20]) in *Bacillus subtilis* and two histidyl-tRNA synthetase genes in *Lactococcus lactis* (36). Chances are that other duplicate genes will be identified with further progress in the various genome-sequencing projects. We have previously shown that the normally silent *thrZ* gene is induced during threonine starvation or by reducing the intracellular concentration of the housekeeping synthetase, ThrS (33).

In contrast to *E. coli*, in which the mechanisms for aaRS gene regulation are as disparate as the number of genes studied (for a review, see references 12 and 34), most of the *B. subtilis* genes isolated appear to be regulated by a common mechanism. Of the 15 tRNA synthetase genes cloned and sequenced in *B. subtilis* (for a review, see references 4 and 34), all but the asparaginyl (*asnS* [2])-, glutamyl (*gltX* [7])-, lysyl (*lysS* [31])-, and methionyl (*metS* [31])-tRNA synthetase genes share common sequence and structural motifs in the leader regions upstream of the translation initiation site (14). Their leader regions are about 300 bp long, and each contains a transcriptional terminator immediately preceded by a 14-nucleotide consensus sequence known as the T box (19, 20, 33). This configuration is found not just in the aaRS genes but also in several of the amino acid biosynthetic operons in *Bacillus* spp. and other gram-positive organisms (13, 34). The leader region of the *thrZ* gene extends over 800 bases and comprises three such tandem domains (33).

For several genes of this family, it has been shown that they are specifically induced by starvation for their cognate amino acid via a mechanism involving transcriptional antitermination. This is the case in the *tyrS* (14), *pheS* (35), and *thrS* and *thrZ* genes (33) and the *ilv-leu* operon (11). *thrS* and *thrZ* are also autorepressed by overproduction of the synthetases themselves (8, 33).

Base pairing between part of the conserved T-box sequence and an equally conserved sequence in the 5′ half of the terminator stem can lead to the formation of an alternative, and mutually exclusive, structure called the antiterminator (14, 33). Studying the *tyrS* system, Grundy and colleagues first provided evidence that the uncharged tRNA can stabilize the formation of the antiterminator structure by interacting with two sites in the leader mRNA (14, 15). The first is between the anticodon loop of the uncharged tRNA and a "specifier codon" that is likely to be bulged out of a large stem-loop structure found in the 5′ half of the leader RNAs of this gene family. The second proposed interaction occurs through base pairing between the NCCA-3′ acceptor end of the uncharged tRNA (including the discriminator base) and the perfectly complementary -UGGN′- sequence in the T box, which is bulged out in the antiterminator conformation (14, 15). Several mutational studies have now been carried out in different systems, and they basically confirm the importance of the specifier codon for the specificity of induction during cognate amino acid starvation (14, 28, 35). Changing the identity of the specifier codon has, in many cases, permitted a switch in the identity of the regulatory amino acid.

The role of the discriminator base in stabilizing the interaction between the acceptor end of the uncharged tRNA and the T box has been studied for *tyrS* (15), *pheS* (35), and *thrS* (35). These reports show that while this interaction is important, the

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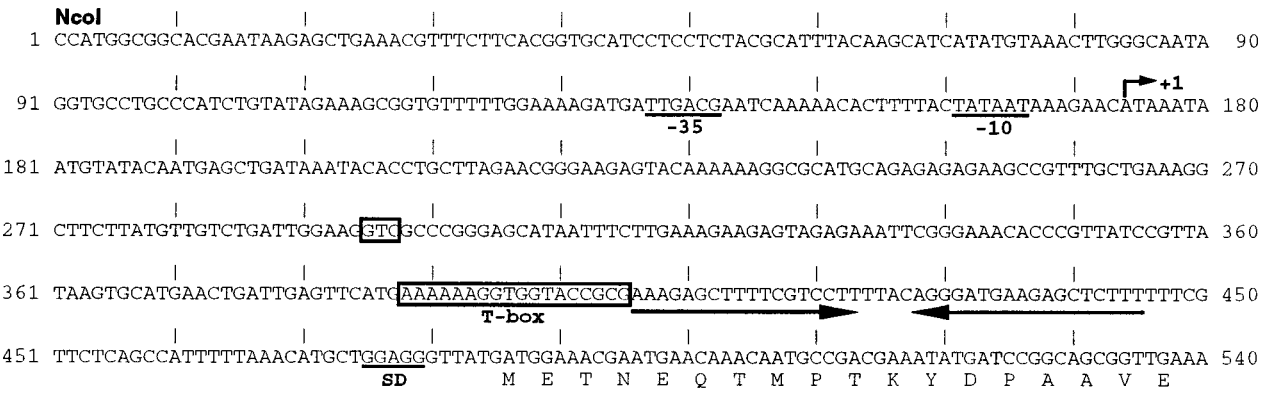

FIG. 1. Nucleotide sequence of the 5′ noncoding region of the *B. subtilis valS* gene. The consensus promoter sequences (−35 and −10 regions) are underlined. The bent arrow indicates the +1 transcription start point. The deduced Shine-Dalgarno-type sequence (SD) is underlined. Converging arrows indicate a potential Rho-independent transcription terminator. The specifier codon (GTC) and the T-box consensus sequence are boxed. The sequence of the whole gene has been deposited in the GenBank/EMBL databases.

sometimes ambiguous results obtained with different mutants suggest that other points of interaction between the tRNA and mRNA, and possibly protein factors, are involved in regulation.

An additional level of regulatory complexity was recently introduced with the discovery that the leader mRNA of *thrS* and at least five other members of this gene family is cleaved just upstream of the transcription terminator in vivo (5). The processed *thrS* transcript is significantly more stable than the full-length mRNA and is the predominant form under threonine starvation conditions. Even though processing can occur in the absence of the tRNA-leader interaction, its contribution to overall induction levels following threonine starvation is substantial (5).

One of the reasons we have studied the expression of the *valS* gene is to find out whether the different aspects of regulation described above apply to other genes of this family or if, on the contrary, some of the regulatory mechanisms are confined to specific genes. For example, all the genes cited above are induced by tRNA-mediated antitermination, but autoregulation has thus far been described only for *thrS* and *thrZ* expression. The only other gene tested in this respect, *pheS*, although induced by phenylalanine starvation, was not repressed by overproduction of phenylalanyl-tRNA synthetase.

In this report, we describe the identification, sequencing and characterization of the *valS* gene. We analyze its transcription pattern and the importance of the specifier codon and the T box for the specificity of induction by valine starvation. Furthermore, we provide evidence that *valS*, like *thrS*/*thrZ* but unlike *pheS*, is autoregulated.

MATERIALS AND METHODS

Bacterial strains and culture and transformation conditions. All *B. subtilis* strains used in this study are derivatives of the prototrophic strain 168 (BGSC 1A2) or the auxotrophic strain BGSC 1A232 (*ilvD4 trpD2*), containing *valS-lacZ* fusions integrated into the *amy* locus. Strains were grown in M9 minimal medium (29) supplemented with 0.5 mM Trp, 3 mM Ile, 3 mM Leu, 3 mM Val, and trace elements (17). For valine starvation experiments, cells were grown as just described but in the presence of only 0.6 mM Val and harvested for β-galactosidase measurements 2 h after the end of logarithmic growth. Threonine starvation was achieved by the addition of 600 μg of DL-threonine hydroxamate per ml to a M9 medium culture at an optical density at 600 nm of 0.3 to 0.4 (prototrophic strain), which still allowed logarithmic growth. Cells were harvested 2 h later.

Plasmid manipulations were performed in *E. coli* JM109 [*recA1 endA1 gyrA86 thi hsdR17 supE44 relA* λ⁻ Δ(*lac-proAB*), F′(*traD36 proAB lacI*^{q} *lacZ*Δ*M15*)]. *E. coli* KE89 (F⁻ *endA1 hsdR1 hsdM*$^+$ *supE44 thi-1 pcnB*) served as a host for overexpression studies with the *valS*-containing plasmid pHMV11, since this high-copy-number plasmid could not be stably maintained in a *pcnB*$^+$ strain. Concatemeric plasmids for transformation of *B. subtilis* were isolated from *E. coli* JM101 [*thi supE44* Δ(*lac-proAB*) F′(*traD36 proAB*$^+$ *lacI*^{q} *lacZ*Δ*M15*)].

E. coli cells were transformed by electroporation (37), and *B. subtilis* cells were transformed as described elsewhere (25). *E. coli* transformants were selected on LB plates supplemented with 100 μg of ampicillin per ml, and *B. subtilis* transformants were selected on LB plates with 4 μg of chloramphenicol (integrative plasmids) or 10 μg of tetracycline (replicative plasmids) per ml.

Plasmid constructions. Plasmid pDG1129 was a generous gift from P. Stragier. It was constructed by insertion of a 3.15-kb *Nco*I-*Xba*I chromosomal DNA fragment containing the *valS* gene into the vector pMTL22 (3), which was cut with the same enzymes.

For pHMV4, a 1-kb *Bgl*II-*Hin*dIII fragment (coordinates 1 to 978 of the *valS* sequence) from pDG1129 was inserted into plasmid pTZ18R (USB) cut with *Bam*HI and *Hin*dIII.

For pHMV8, the 1-kb insert of pHMV4 was excised as an *Eco*RI-*Hin*dIII fragment and cloned into plasmid pHM2 (8) cut with *Eco*RI and *Hin*dIII.

For pHMV11, the 3.15-kb insert of pDG1129 containing the entire *valS* gene was excised as an *Nsi*I-*Xba*I fragment and inserted into the shuttle vector pHM3 (33) cut with *Pst*I and *Xba*I.

For pHMV12, an internal 1.5-kb *Hin*dIII fragment of *valS* was inserted into the integrative vector pDG641 (16) cut with *Hin*dIII.

For pHMV13, the 1-kb insert of pHMV4 was mutated at two sites: the GUC triplet (coordinates 295 to 297 in Fig. 1) was altered to ACC, and the -TGGT- sequence of the T box (coordinates 396 to 399 in Fig. 1) was changed to -TGGA-. The mutated fragment was excised with *Eco*RI and *Hin*dIII and inserted into pHM2 cut with *Eco*RI and *Hin*dIII.

For pHMV14, the 1-kb insert of pHMV4 where the GUC specifier codon has been mutated to UAA was excised with *Eco*RI and *Hin*dIII and inserted into pHM2 cut with *Eco*RI and *Hin*dIII.

For pHMV15, the 1-kb insert of pHMV4 where the GUC specifier codon has been mutated to ACC was inserted as an *Eco*RI-*Hin*dIII fragment into pHM2 cut with *Eco*RI and *Hin*dIII.

DNA manipulations. The 3.15-kb insert of plasmid pDG1129, containing the entire *valS* gene, was subcloned as three separate fragments in the multicopy plasmids pTZ18R and pTZ19R (USB) for sequencing. The double-stranded recombinant DNAs were used as templates in dideoxy chain termination sequencing reactions (38), using the universal and reverse primers as well as specific synthetic oligonucleotides for the central regions of the cloned fragments.

Site-directed mutagenesis was performed on a single-stranded DNA template by the method of Kunkel et al. (24). Mutations in the *valS* leader were generally introduced on plasmid pHMV4 before being transferred to the *lacZ* fusion vector pHM2. Oligonucleotides used for mutagenesis extended 12 to 15 nucleotides on either side of the mutation site, and sequences are available on request.

RNA manipulations. Total cellular RNA was isolated as described previously (33). Reverse transcriptase assays were carried out with 15 μg of total RNA and about 1 pmol of 5′-end-labeled oligonucleotide (sequence complementary to positions 479 to 498 in Fig. 1). The RNA and oligonucleotide were heated together at 65° for 5 min and then frozen in a mixture of dry ice and ethanol and allowed to thaw on ice. Reactions contained 2 U of avian myeloblastosis virus reverse transcriptase (Eurogentec) and were allowed to run for 30 min at 48°C.

Northern analysis of total cellular RNA was performed as described elsewhere (33). A radiolabeled 1.5-kb *Hin*dIII fragment of the *valS* structural gene (see Fig. 5) was used as a *valS*-specific probe. The *folC* probe was amplified by PCR from chromosomal DNA (positions 73 to 1096 of the structural gene; see Fig. 5).

β-Galactosidase and aminoacylation assays. The β-galactosidase activity of *lacZ* fusions was measured as described previously (33).

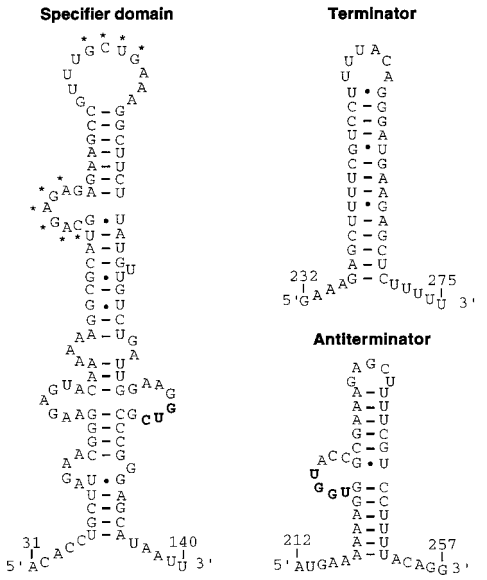

FIG. 2. Putative secondary structures of the specifier domain, the antiterminator, and the terminator of the *B. subtilis valS* leader. The GUC triplet and the -UGGU- sequence in the antiterminator that are believed to interact with the 3′ end of the Val-tRNA^{GAC} are in boldface type. Other conserved sequences (14) are marked by asterisks.

For in vitro aminoacylation measurements, *B. subtilis* or *E. coli* cells harboring recombinant plasmids containing *valS* or the vector alone were grown in LB broth to an optical density at 600 nm of ~1. Cells were harvested and washed with Z buffer without β-mercaptoethanol (29). After suspension in 500 μl of buffer A (10 mM Tris-HCl [pH 7.4], 10% glycerol, 1 mM dithiothreitol), samples were sonicated and clarified by centrifugation. The 100-μl aminoacylation reaction was carried out at 37°C in a reaction mixture containing 50 mM Tris-HCl (pH 7.5), 15 mM $MgCl_2$, 15 mM β-mercaptoethanol, 10 mM ATP, 50 μM L-^{14}C-valine at 200 cpm/pmol, 1 mM dithiothreitol, 120 μg of total *E. coli* tRNA, and various amounts of cellular extract. The nucleic acids were precipitated by trichloroacetic acid and filtered out on GFC filters (Whatman), and the radioactivity retained on the filters was measured by scintillation counting.

Computer analysis. Sequence comparisons were done with the help of the programs BestFit and PileUp of the University of Wisconsin Genetics Computer Group.

Nucleotide sequence accession number. The nucleotide sequence of the *valS* gene has been deposited in the GenBank/EMBL databases under the accession number X77239.

RESULTS

Identification of the *valS* gene. The putative *B. subtilis valS* gene encoding valyl-tRNA synthetase (ValS) had previously been identified by a homology search of a sequence upstream of the *folC* gene (encoding folyl-polyglutamate synthetase) that comprises the C-terminal 56 amino acids of a truncated open reading frame (27). Plasmid pDG1129 was constructed by P. Stragier (unpublished data) and carries a 3.15-kb fragment containing the chromosomal region immediately upstream of *folC*, including the sequence described above. We sequenced this 3.15-kb fragment and found it to contain the entire *valS* transcriptional unit. A 540-bp segment of the 5′ end of this sequence contains the *valS* leader region and is shown in Fig. 1. An open reading frame encoding a protein of 880 amino acids was identified between positions 486 and 3125 (data not shown). The deduced protein sequence shows strong similarity to the valyl-tRNA synthetases of *Bacillus stearothermophilus* (1), *Lactobacillus casei* (40), and *E. coli* (18). Sequence alignment of the four known prokaryotic synthetases shows that, as expected, the *B. subtilis* ValS is more closely related to its homologs from the gram-positive organisms *B. stearothermophilus* and *Lactobacillus casei* (89% similarity and 80% identity, and 75% similarity and 61% identity, respectively) than to the *E. coli* enzyme (67% similarity and 46% identity). The N-terminal two-thirds of the protein is well conserved between all four organisms. This part of the protein contains the catalytic core (23, 41), including the signature sequences HIGH and KMSKS of the Rossmann nucleotide binding fold in class I aaRS (6). However, the *E. coli* synthetase contains some quite extensive insertions in this part of the protein that are found in none of the other three synthetases, possibly reflecting species-related differences between gram-positive and gram-negative organisms. The *L. casei* enzyme has a 19-amino-acid N-terminal extension compared to the two *Bacillus* synthetases. The C-terminal third of the four ValS proteins is more divergent and emphasizes the close evolutionary distance between the two *Bacillus* species. The supposition initially advanced by Wetzel (42), that valyl-, isoleucyl-, leucyl-, and methionyl-tRNA synthetase are all members of a subfamily within the aaRS, is supported by aligning the sequences of these proteins from different origins (see Discussion).

We identified a potential σA-type promoter (Fig. 1) with a spacing of 17 bp and a near-consensus sequence (TTGACA and TATAAT for *B. subtilis* σ^A- and *E. coli* σ^{70}-type promoters) ~300 nucleotides upstream of the start codon. Its functionality has been confirmed, as described below. The roughly 300-bp leader contains all of the regulatory elements necessary

TABLE 1. ValS activity in total cell extracts of *valS*-overexpressing *E. coli* and *B. subtilis* cells[a]

Bacterial strain	ValS activity (pmol of charged tRNA^{Val}/μg of total protein) in:		Overexpression (fold)
	Vector (pHM3)	Vector + *valS*[b]	
E. coli KE89	1.1	6.5	6
B. subtilis SSB184[c]	11	27	2.5

[a] All data are average values from three independent experiments.
[b] pDG1129 in *E. coli*; pHMV11 in *B. subtilis*.
[c] SSB184 is the *B. subtilis* wild-type strain 1A2 (BGSC) containing the *valS-lacZ* fusion HMV8.

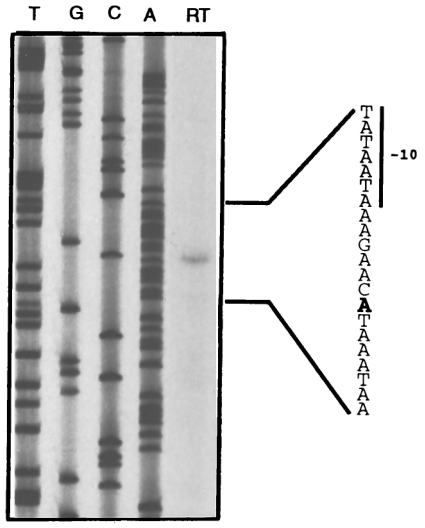

FIG. 3. Primer extension analysis of *B. subtilis valS* mRNA. Total RNA of a *B. subtilis* wild-type strain was reverse transcribed with a primer complementary to nucleotides 479 to 498 in Fig. 1. The same oligonucleotide was used for the sequencing reaction with plasmid pDG1129 as a template. RT, reverse transcription.

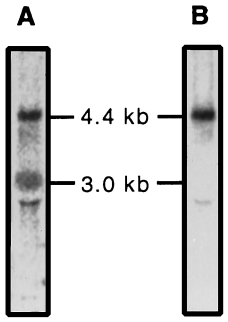

FIG. 4. Northern blot analysis of *valS* transcripts. (A) A radiolabeled 1.5-kb *Hin*dIII fragment of the *valS* structural gene (Fig. 5) was used to probe total RNA extracted from a *B. subtilis* wild-type strain. (B) The same blot was stripped of the *valS* probe and rehybridized with a *folC* PCR probe (positions 73 to 1096 of the structural gene; Fig. 5). The sizes of the two mRNA species were estimated using the BRL 0.24- to 9.5-kb RNA molecular weight marker.

to assign it to the family of genes regulated by tRNA-mediated antitermination (Fig. 1 and 2): a Rho-independent transcription terminator which is preceded by the T-box consensus sequence upstream of the structural gene and a highly structured specifier domain in the 5′ half of the leader which contains the potential GUC specifier codon. We have analyzed the importance of these elements for *valS* regulation (see below).

The *valS* gene product can charge tRNA^{Val} in vitro. In order to prove the identity of the sequenced gene, we overexpressed it in both *E. coli* and *B. subtilis* and measured an increase in tRNA^{Val} aminoacylation activity in cell extracts in vitro. For overexpression in *E. coli*, we transformed plasmid pDG1129 into the *pcnB* strain KE89, since this high-copy-number recombinant plasmid could not be stably maintained in a *pcnB*$^+$ strain. The *valS* gene was transferred to *B. subtilis* by transformation with plasmid pHMV11, constructed by inserting the 3.15-kb insert of pDG1129 into the shuttle vector pHM3. The aminoacylation activities found in the various cell extracts are given in Table 1. The 6- and 2.5-fold increases in activity in *E. coli* and *B. subtilis* cells, respectively, that were harboring the recombinant plasmids clearly show that the cloned gene encodes a functional valyl-tRNA synthetase. The difference in increase in absolute ValS activity between *E. coli* and *B. subtilis* harboring plasmids pDG1129 and pHMV11, respectively, could reflect a lower plasmid copy number in *E. coli* (*pcnB*) than in *B. subtilis* or a lower expression of the heterologous *B. subtilis* *valS* gene in *E. coli*. The 2.5-fold increase in ValS activity observed in *B. subtilis* also serves as a reference value for the autoregulation studies described below.

Mapping of *valS* transcripts. The transcription start site of *valS* was determined by primer extension analysis with an oligonucleotide complementary to nucleotides 479 to 498 in Fig. 1. Reverse transcription reactions identified a single band corresponding to a transcription start point at position 174 (Fig. 3), which is consistent with the proposed promoter.

Northern analysis of *valS* transcripts during exponential growth, using a 1.5-kb *valS* internal *Hin*dIII fragment as a probe (see Fig. 5), revealed two major transcripts of 3 and 4.4 kb (Fig. 4A). Some larger RNAs appear to be carried along in front of the 23S rRNA to give a weak additional signal. A probe specific for the *folC* gene located immediately downstream of *valS* (Fig. 5) also hybridizes to the 4.4-kb transcript but does not hybridize to the 3-kb mRNA (Fig. 4B). Thus, we believe that the 3-kb mRNA species corresponds to the *valS* mRNA and results from transcription termination at the Rho-independent terminator located in the short intergenic region between *valS* and *folC* (Fig. 5) (30, 39) and that the 4.4-kb transcript is a polycistronic mRNA comprising both the *valS* and the *folC* genes.

A GUC triplet confers the specificity of *valS* induction. The expression of the wild-type *valS* gene and that of various leader mutants was studied with the help of *lacZ* transcriptional fusions integrated in single copy at the *amy* locus of a wild-type strain or a strain auxotrophic for valine. The wild-type *valS-lacZ* fusion (HMV8) was induced almost threefold by starvation for valine. It is noteworthy that efficient valine starvation could be achieved only by adding excess leucine to the medium, despite the fact that the strain used (1A232) is not a leucine auxotroph (see Discussion). To test the relevance of the GUC triplet (Fig. 1 and 2) to *valS* induction during valine starvation, we measured β-galactosidase activity in fusions where the amino acid identity of this triplet had been changed. HMV15 has the GUC specifier codon replaced by an ACC triplet, the threonine codon which confers specificity of *thrS* induction. HMV13 contains a mutation in the T box (TGGT → TGGA) in addition to the GUC → ACC mutation to retain base pairing with the discriminator base (U) of the Thr-tRNA^{GGU} isoacceptor. HMV14 has a TAA stop codon in place of the wild-type GUC triplet. The results are summarized in Table 2. Changing the GUC (Val) to an ACC (Thr) triplet causes loss of induction by valine starvation and renders expression inducible by threonine starvation (3.4-fold). At the same time, the basal level of expression decreases more than 10-fold. Adaptation of the T-box sequence to better accommodate the interaction of the *valS* leader with the Thr-tRNA^{GGU} isoacceptor restores the basal level of expression to wild-type levels but, paradoxically, causes a near loss of induction by threonine starvation (Table 2). Replacing the GUC specifier codon with TAA (stop codon) renders the *valS* gene uninducible.

***valS* expression is autoregulated.** We previously showed that expression of *thrS* and *thrZ*, but not *pheS*, is autoregulated in a specifier-codon-dependent manner. In order to analyze whether autorepression is confined to the *thrS*/*thrZ* system or represents a more widespread phenomenon, we introduced the recombinant ValS overproducing plasmid, pHMV11, into a

TABLE 2. Effect of specifier codon and T-box mutations on induction of *valS-lacZ* expression

valS-lacZ fusion[a]	Specifier codon	T-box sequence	β-Galactosidase activity (U/mg)[b]				
			Complete medium	Valine starvation	Induction	Threonine starvation	Induction
HMV8 (wt)	GUC (Val)	-UGGU-	22	56	2.6×	21	0.9×
HMV15	ACC (Thr)	-UGGU-	1.6	0.6	0.4×	5.4	3.4×
HMV13	ACC (Thr)	-UGGA-	15	5	0.3×	18	1.2×
HMV14	UAA (stop)	-UGGU-	0.9	0.5	0.6×	ND	ND

[a] These fusions were measured in a strain auxotrophic for valine, strain 1A96 (see Materials and Methods). wt, wild type.
[b] ND, not done. All data represent average values from at least three independent experiments.

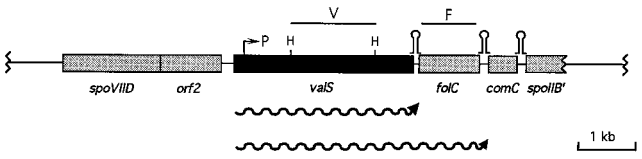

FIG. 5. Chromosomal neighborhood of the *B. subtilis valS* gene (39). The *valS* promoter and potential Rho-independent transcription terminators are indicated. Wavy arrows symbolize the mRNA species observed by Northern analysis (Fig. 4). The lines labeled V and F above the *valS* and *folC* genes indicate the sizes and positions of the fragments used as probes in Northern analysis (Fig. 4). H, *Hin*dIII.

strain carrying the wild-type *valS-lacZ* fusion (HMV8). As shown in Table 3, a 2.5-fold increase in *valS* activity was sufficient to repress the activity of the *valS-lacZ* fusion over 5-fold. Thus, expression of *valS* appears to be extremely sensitive to variations in the intracellular concentration of the synthetase. While a direct role for the synthetase in *valS* regulation cannot be ruled out at present, it appears more likely that autoregulation occurs by altering the ratio of charged to uncharged valyl-tRNA. Due to the extremely low levels of β-galactosidase expression in the stop codon mutant fusion (HMV14, Table 2) and the GUC → ACC mutant fusion (HMV15, Table 2), we could not test them for autoregulation. Therefore, we analyzed the importance of the specifier codon for autorepression in the double mutant HMV13 fusion (specifier codon and T box adapted to match the Thr-tRNA^{GGU} isoacceptor; Table 3), which has a higher basal level of expression (Table 2). The double mutation led to a loss of autoregulation (1.7-fold repression), underlining the importance of these two sites of tRNA-mRNA interaction for this type of regulation. Although no repression was observed with a 10-fold overproduction of ThrS (Table 3), this is perhaps not surprising given that the HMV13 fusion is also not inducible by threonine starvation (see above).

DISCUSSION

The three valyl-tRNA synthetases from the gram-positive organisms *B. stearothermophilus* (1), *Lactobacillus casei* (40), and *B. subtilis* are very similar and more compact than their *E. coli* counterpart (18), which contains some extensive insertions in the amino-terminal two-thirds of the protein. Comparison of the *B. subtilis* ValS sequence with other branched-chain aaRS proteins in bacteria revealed surprisingly strong similarities between *B. subtilis* ValS and the following synthetases (expressed in percentage per unit length, similarity and identity): *B. subtilis* MetS, 49.2 and 26.2%; *E. coli* MetS, 45.6 and 21.4%; *E. coli* IleS 49.5 and 26.3%; *B. subtilis* LeuS, 50.9 and 25.2%; and *E. coli* LeuS, 54.3 and 29.8%. It is interesting that similarities between heterologous synthetases are not necessarily higher when they originate from the same organism (*B. subtilis* ValS is 50.9% similar to *B. subtilis* LeuS but 54.3% similar to *E. coli* LeuS), implying that the common ancestor of the branched-chain aaRS probably existed before the separation of bacteria in a gram-positive and gram-negative kingdom.

Northern blot analysis revealed the presence of two transcripts (3 and 4.4 kb) containing the *valS* gene. The 4.4-kb transcript also hybridized to a *folC*-specific probe, indicating that both genes are likely to be cotranscribed on a polycistronic mRNA originating at the *valS* promoter. This is also the predicted length of a transcript extending from the *valS* promoter to the transcription terminator immediately downstream of *folC*. The presence of roughly equal amounts of the two mRNAs indicates that the *valS* terminator is only about 50% efficient. Overexposure of the Northern blot revealed only very low quantities of transcripts extending beyond the *folC* transcription terminator. This is consistent with the finding that expression of *comC*, the gene lying downstream of *folC* (Fig. 5), is induced only during late competence (30).

We attempted to inactivate the *valS* gene on the chromosome and found this not be lethal. Disruption of *valS* in the survivors was confirmed by Southern blotting (data not shown) and suggests that a second functional gene with valine-tRNA synthetase activity exists in *B. subtilis*, as is the case for the threonyl- and tyrosyl-tRNA synthetases.

Sequence and two-dimensional structure analyses of the *valS* leader suggested that this gene is a member of the family of genes in gram-positive organisms that comprises aaRS and amino acid biosynthetic genes regulated by tRNA-mediated antitermination (14). Expression of *valS* was induced by starvation for valine, but this derepression could be observed only when the cells were grown in the presence of excess leucine, despite the fact that the *trpC2 ilvD4* mutant strain used in this study is auxotrophic for tryptophan, isoleucine, and valine but not for leucine. A rationale for this observation may be found in the way the *ilv-leu* biosynthetic operon is regulated. Expression of the *ilv-leu* operon is also likely to be regulated by the level of charged/uncharged Leu-tRNA via tRNA-mediated antitermination (28) and responds to variations in leucine concentration (10). Since the uncharacterized *ilvD4* mutation used here shows a slightly leaky phenotype, we believe that excess leucine further shuts down *ilv-leu* expression, thereby creating conditions whereby valine starvation can occur more efficiently.

The specificity of *valS* induction depends on the identity of the strategically placed GUC (Val) triplet (specifier codon) in the extensive 5′-terminal secondary structure (Fig. 2). Changing the GUC triplet to ACC (Thr) switched the specificity of induction from valine to threonine starvation. However, the GUC → ACC transition leads to a more than 10-fold drop in the basal level of expression, very close to the activity of an uninducible fusion in which the GUC specifier was mutated to a UAA stop codon. Clearly, the Thr-tRNA^{GGU} isoacceptor interacts much less efficiently with the *valS* leader containing the ACC codon than Val-tRNA interacts with the wild-type leader. In order to improve this interaction, we altered the T-box sequence to match the discriminator base of the Thr-

TABLE 3. Effect of *valS* and *thrS* overexpression on wild-type and mutant *valS-lacZ* fusions

valS-lacZ fusion	Specifier codon	T-box sequence	Multicopy plasmid	Insert	β-Galactosidase sp act (U/mg)[a]	Repression factor (fold)	Overexpression of synthetase (fold)
HMV8 (wt)[b]	GUC	-UGGU-	pHM3	Control	39		
			pHMV11	*valS*	7.5	5.2	2.5
HMV13	ACC	-UGGA-	pHM3	Control	44		
			pHMV11	*valS*	26	1.7	2.5

[a] All β-galactosidase activities are average values from at least three independent experiments.
[b] wt, wild type.

$tRNA^{GGU}$ isoacceptor. Indeed, basal expression rose about 10-fold and approached wild-type levels but at the same time became almost uninducible by threonine starvation (Table 2). This phenomenon is difficult to explain. The GUC → ACC transition (HMV15) was sufficient to render the mutated *valS-lacZ* fusion inducible by starvation for threonine, indicating that the Thr-$tRNA^{GGU}$ isoacceptor can, albeit not very efficiently, interact with the *valS* leader and recognize the ACC specifier codon. Permitting the T-box sequence to base pair with the discriminator base of the Thr-$tRNA^{GGU}$ (HMV13) seemed to improve the tRNA-mRNA interaction, as reflected by a 10-fold increase in basal expression. However, if this interaction is indeed so efficient, one would expect this mutant fusion to be highly inducible by threonine starvation, which is clearly not the case. We previously encountered a similar phenomenon when introducing analogous mutations in the *thrS* leader to complement the discriminator base of Phe-tRNA (35).

It seems logical that genes whose expression responds to the ratio of charged to uncharged cognate tRNA would also be affected by the intracellular concentration of their product responsible for charging these tRNAs, but of the two systems studied to date (*thrS*/*thrZ* [8] and *pheS* [35]), only the *thrS* and *thrZ* genes were autoregulated. To see whether this type of regulation represents a more common phenomenon was one of the reasons we tested whether *valS* expression is autoregulated. As shown in this study, a 2.5-fold overexpression of *valS* from a multicopy plasmid led to a more than 5-fold repression of a wild-type *valS-lacZ* fusion. Expression of the *valS* gene is thus more tightly controlled by the intracellular concentration of its product than is the case for *thrS*, where 10-fold overproduction leads to 10-fold repression (8). This could be explained if basal *valS* expression were to depend much more heavily on antitermination mediated by uncharged tRNA than is the case for *thrS*. In minimal medium, expression of a *valS-lacZ* fusion, in which the GUC specifier codon has been changed to a TAA stop codon, drops 20-fold compared to expression of the wild-type fusion (Table 2), while an equivalent change in the *thrS* system leads to only a 2-fold drop in expression (35). While it seems likely that autorepression occurs by altering the charged/uncharged tRNA ratio in the cell, an additional direct interaction of the synthetase with the mRNA cannot yet be excluded.

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