JOURNAL OF BACTERIOLOGY, Aug. 1994, p. 4518–4526
0021-9193/94/$04.00+0
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Vol. 176, No. 15

Interaction between the Acceptor End of tRNA and the T Box Stimulates Antitermination in the *Bacillus subtilis tyrS* Gene: a New Role for the Discriminator Base

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Received 23 January 1994/Accepted 18 May 1994

The *Bacillus subtilis tyrS* gene is a member of a group of gram-positive aminoacyl-tRNA synthetase and amino acid biosynthesis genes which are regulated by transcription antitermination. Each gene in the group is specifically induced by limitation for the appropriate amino acid. This response is mediated by interaction of the cognate tRNA with the mRNA leader region to promote formation of an antiterminator structure. The tRNA interacts with the leader by codon-anticodon pairing at a position designated the specifier sequence which is upstream of the antiterminator. In this study, an additional site of possible contact between the tRNA and the leader was identified through covariation of leader mRNA and tRNA sequences. Mutations in the acceptor end of $tRNA^{Tyr}$ could suppress mutations in the side bulge of the antiterminator, in a pattern consistent with base pairing. This base pairing may thereby directly affect the formation and/or function of the antiterminator. The discriminator position of the tRNA, an important identity determinant for a number of tRNAs, including $tRNA^{Tyr}$, was shown to act as a second specificity determinant for assuring response to the appropriate tRNA. Furthermore, overproduction of an unchargeable variant of $tRNA^{Tyr}$ resulted in antitermination in the absence of limitation for tyrosine, supporting the proposal that uncharged tRNA is the effector in this system.

Most of the aminoacyl-tRNA synthetase genes identified to date in *Bacillus subtilis* are regulated by transcription antitermination (17, 22, 24, 35). All of these genes exhibit very strong conservation of a set of primary-sequence and secondary-structure elements in their mRNA leader regions, upstream of the start of the coding region (17, 18). These elements, which include three stem-loop structures, a highly conserved 14-bp sequence designated the T box, and a factor-independent transcriptional terminator (Fig. 1), are also found in certain amino acid biosynthesis genes and tRNA synthetase genes in *Bacillus* and other gram-positive species, including *B. subtilis ilv-leu* and *cysE-cysS*, *Lactobacillus casei valS* and *trp*, *Lactococcus lactis trp* and *his*, and *Brevibacterium lactofermentum* (and *Corynebacterium glutamicum*) *argS-lysA*, suggesting that this mechanism is widespread in gram-positive bacteria (18, 22). For each gene that has been examined, readthrough of the leader region terminator is induced by limitation for the appropriate amino acid and not by general amino acid starvation (5, 12, 13, 24, 35). Analysis of the mechanism for the specific response of each gene to the cognate amino acid has been a major focus of attention in our laboratory.

We noted that each leader region structure contains a triplet sequence corresponding to a codon specifying the appropriate amino acid, displayed at a specific position within a side bulge of the first stem-loop structure (17). Alteration of this triplet in the *tyrS* leader from a UAC tyrosine codon to a UUC phenylalanine codon was sufficient to switch the specificity of the response to amino acid limitation; this triplet was designated the specifier sequence (17). Conversion of this triplet to a UAA or UAG nonsense codon resulted in an uninducible phenotype which was suppressed by introduction of a mutant suppressor tRNA capable of recognizing these nonsense codons. Furthermore, the response was now generated by starvation for the amino acid specified by the suppressor tRNA, indicating that tRNA is likely to be the effector which interacts with the specifier sequence (17). These results suggested that interaction of the leader with the appropriate tRNA is necessary for antitermination; since antitermination is induced by amino acid limitation, under conditions where uncharged tRNA would accumulate, the implication was that uncharged tRNA activates antitermination. It is also possible that the charged cognate tRNA can interfere with the ability of uncharged tRNA to interact with the leader so that the critical parameter is the balance between charged and uncharged tRNA.

Interaction of uncharged tRNA with the leader was suggested to cause or stabilize formation of an alternate antiterminator structure in the mRNA, which prevents terminator formation (17). All of the leaders can form similar antiterminator structures by base pairing of a portion of the T box with a conserved sequence on the 5′ side of the terminators (Fig. 1B). Since the proposed antiterminator structures are in all cases predicted to be of lower stability than the terminators, it was postulated that the role of the tRNA is to promote antiterminator formation.

In recent years, a number of genes in *B. subtilis* have been shown to be controlled at the level of transcription termination. These systems include the *sac* genes (4), where readthrough is directed by a specific antiterminator protein, and the *trp* operon, where the TRAP protein directs termination (10). Systems in which the ribosome controls transcription termination by translation of a leader open reading frame, like the *Escherichia coli trp* operon (28), have not yet been identified in *B. subtilis*, although the *L. lactis ilv-leu* operon (9) is a likely candidate. Use of a tRNA to regulate transcription

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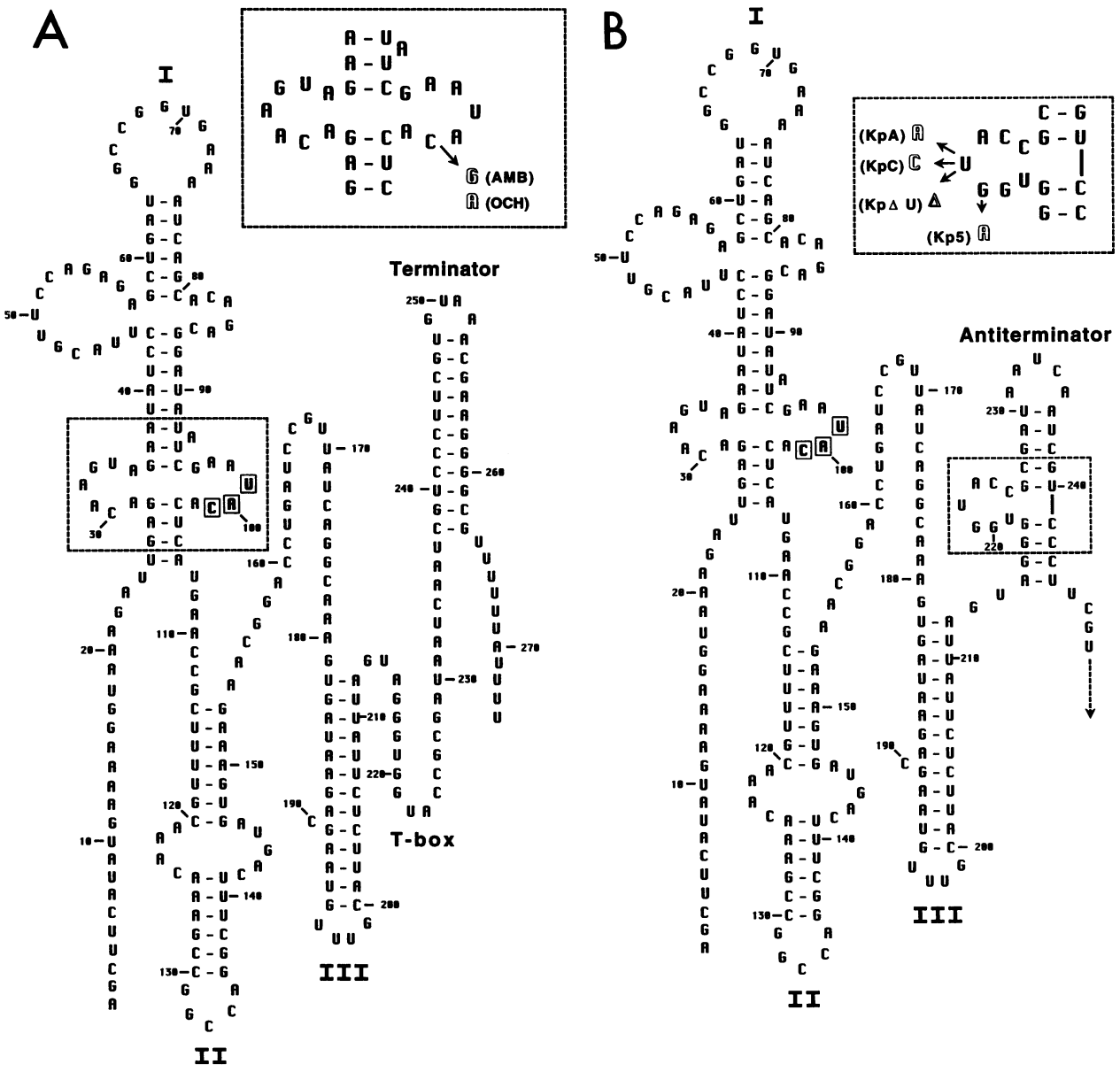


FIG. 1. Structural model of the *B. subtilis tyrS* leader. Numbers indicate positions relative to the transcription start point. Roman numerals indicate stem-loops I, II, and III. The boxed residues are the UAC specifier sequence. (A) Terminator form. The inset is an enlargement of the specifier region, showing the UAG (TyrAMB) and UAA (TyrOCH) specifier mutations. (B) Antiterminator form. The inset is an enlargement of the side-bulge region of the antiterminator, showing the mutations tested in this study. The dashed arrow indicates continued transcription.

antitermination is a novel mechanism found to date only in gram-positive bacteria. The tRNA synthetase genes in *E. coli*, in contrast, are regulated by a variety of mechanisms (15).

The goal of the current study was to determine if there are leader region elements in addition to the specifier sequence which contribute to the specificity of the amino acid limitation response and to search for secondary positions of tRNA-mRNA interaction. In addition, the effect of expression of tRNA variants on antitermination was explored. We show that expression of unchargeable tRNAs capable of interacting with *tyrS* leader region variants can induce antitermination in the absence of amino acid limitation. This effect was magnified with *tyrS* leader variants which can no longer interact with wild-type tyrosyl-tRNA ($tRNA^{Tyr}$), suggesting that competition between charged and uncharged tRNA may affect the efficiency of antitermination.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *B. subtilis* host strains used in this study were TH217 (*tyrA1 trpC2 thr-5*) (24), for testing of *tyrS-lacZ* fusions, and BR151MA (*lys-3 trpC2*) (16), for testing of *rpsD*(Am)-*lacZ* fusions. *E. coli* JM103 (*endA1 supE44 sbcBC thi-1 rpsL* Δ*lac-pro*/F' *traD36 lacI*^{q}ZΔM15 *proAB*) (30) was used for propagation of phage M13 isolates, and strain DH5α [φ80d*lacZ*ΔM15 *endA1 recA1 hsdR17* (r_K^- m_K^+) *thi-1 gyrA96 relA1* Δ(*lacZYA-argF*)U169; Bethesda Research Laboratories] was used for plasmid prop-

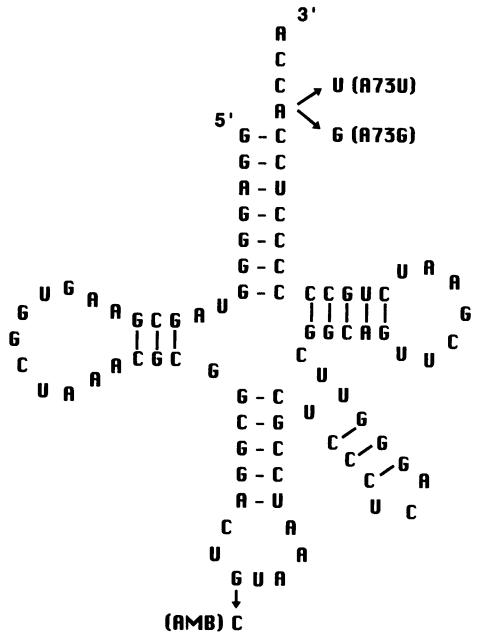


FIG. 2. Cloverleaf model of *B. subtilis* $tRNA^{Tyr}$. Arrows indicate the G-to-C substitution in the anticodon, to generate an amber suppressor variant ($tRNA^{Tyr}$AMB), and the A73U and A73G mutations at position 73.

agation. Cells were routinely propagated at 37°C in Luria-Bertani (LB) medium (31) or tryptose blood agar base (Difco) for *B. subtilis*. For amino acid limitation experiments, cells were grown in Spizizen minimal medium (1) with glucose as the carbon source and appropriate amino acids at 50 μg/ml (amino acid excess) or 5 μg/ml (amino acid limitation). Antibiotics were used at the following concentrations: ampicillin, 50 μg/ml; chloramphenicol, 5 μg/ml for selection and 0.1 μg/ml for induction; neomycin, 5 μg/ml.

Plasmids and bacteriophage. The *rpsD*(Am)-*lacZ* translational fusion (19) and *tyrS-lacZ* transcriptional fusion (24) were integrated into specialized transducing phage SPβ as described previously; chloramphenicol was used to select for maintenance of the prophage. Restriction endonucleases and DNA-modifying enzymes were purchased from New England BioLabs and used as described by the manufacturer. Mutations in the *tyrS-lacZ* fusion were generated by oligonucleotide-directed mutagenesis as described by Kunkel (26). Oligonucleotides were synthesized on an Applied Biosystems synthesizer or were purchased from Genosys Technologies (Woodlands, Tex.). The PCR (Perkin-Elmer Cetus) was used to isolate the $tRNA^{Tyr}$ gene from the *trnD* gene cluster; this region was subcloned into phage M13 mp19 as described previously (19), and mutations in the 3′ acceptor end of the tRNA (Fig. 2) were introduced by substitutions in the PCR primer. The mutation in the anticodon was generated by oligonucleotide-directed mutagenesis. Combinations of anticodon and discriminator base mutations in the tRNA were generated by cleavage with *Bst*BI, a site for which is located between these regions in $tRNA^{Tyr}$, and joining segments from the appropriate single mutants. The TyrAMB-KpA and TyrAMB-KpC double mutants of *tyrS-lacZ* were generated by cleavage of TyrAMB, TyrKpA, and TyrKpC single mutant fusions with *Bgl*I, a site for which is located at position +128 of the *tyrS* leader, and joining the upstream segment from TyrAMB with the downstream segments from TyrKpA or TyrKpC. All mutations were verified by DNA sequencing using Sequenase DNA polymerase (United States Biochemicals). Wild-type and mutant $tRNA^{Tyr}$ genes were inserted into plasmid pDG148 (39), which encodes neomycin resistance, so that expression of the tRNA was under control of the IPTG-inducible P_{spac} promoter. *B. subtilis* transformation was carried out as described previously (23).

β-Galactosidase assays. For amino acid limitation experiments, strains which were auxotrophs for the amino acid to be tested were grown in minimal medium containing all required amino acids until the mid-exponential growth phase. The cultures were split, and the cells were collected by centrifugation and resuspended in medium containing all required amino acids (50 μg/ml) or with low amounts (5 μg/ml) of the amino acid to be tested. Cells were harvested after 3 h of growth, and β-galactosidase activity was measured in toluene-treated cells as described by Miller (31). Inducible expression of tRNA synthesis under P_{spac} control was carried out during growth in LB medium containing chloramphenicol and neomycin. Cultures were grown to the early logarithmic growth phase, split, and diluted twofold in the same medium in the presence or absence of IPTG (isopropyl-β-D-thiogalactopyranoside). Cells were harvested after 2 h, and β-galactosidase activity was measured.

RESULTS

Search for leader region specificity determinants in addition to the specifier sequence. All of the genes in the T box family contain an antiterminator structure in their leader regions which is formed by base pairing of a portion of the T box sequence with a conserved region on the 5′ side of the terminator (17). In all cases, the same portion of the T box is present in a side bulge of the antiterminator. Most of this loop sequence (5′-UGGNACC-3′) is absolutely conserved, while the N represents a variable position. We noted that the conserved UGG sequence of this loop could potentially base pair with the CCA sequence which is always present at the 3′ acceptor end of tRNA; furthermore, the next base of the T box, which varies, was found to covary with the tRNA base which precedes the CCA (Fig. 3). This position of tRNA, base 73, is designated the discriminator base (3) and is an important identity determinant for a number of tRNAs, including $tRNA^{Tyr}$ (27, 37, 38).

In each case, the variable position of the T box (5′-UGGN-3′) is complementary to the discriminator base (5′-NCCA-3′) of the tRNA species with the appropriate anticodon to interact with the specifier sequence for that gene: *tyrS* has U in the T box (as do *pheS*, *leuS*, *ilv-leu*, *valS*, and *tyrZ*), and $tRNA^{Tyr}$ has A at base 73 (as do $tRNA^{Phe}$, $tRNA^{Leu}$, and $tRNA^{Val}$); *thrS*, *thrZ*, and *cysE-cysS* (6) have A in the T box, and $tRNA^{Thr}$ (with a GGU anticodon to interact with the ACC specifier) and $tRNA^{Cys}$ have U at base 73; *trpS* (*B. stearothermophilus*) has C in the T box, and $tRNA^{Trp}$ has G at base 73 (*B. subtilis trpS* has U at this position of the T box (2), so that a G-U pair is predicted). G73 is a major identity determinant for $tRNA^{Trp}$ (43), while for $tRNA^{Thr}$ the identity of position 73 is not important (21); in *E. coli*, it is always A, while in *B. subtilis*, only the variant of $tRNA^{Thr}$ with a GGU anticodon (to match the ACC specifiers of *thrS* and *thrZ*) has U at position 73 (41). The *L. lactis his* operon leader has A in the T box, while base 73 of $tRNA^{His}$ is C in *B. subtilis*; however, $tRNA^{His}$ is unusual in that base 73 is base paired so that the unpaired acceptor end is only 3 bases in length for this tRNA (14). The observation of intermolecular covariance suggested that the variable position in the antiterminator loop was likely to be important and that

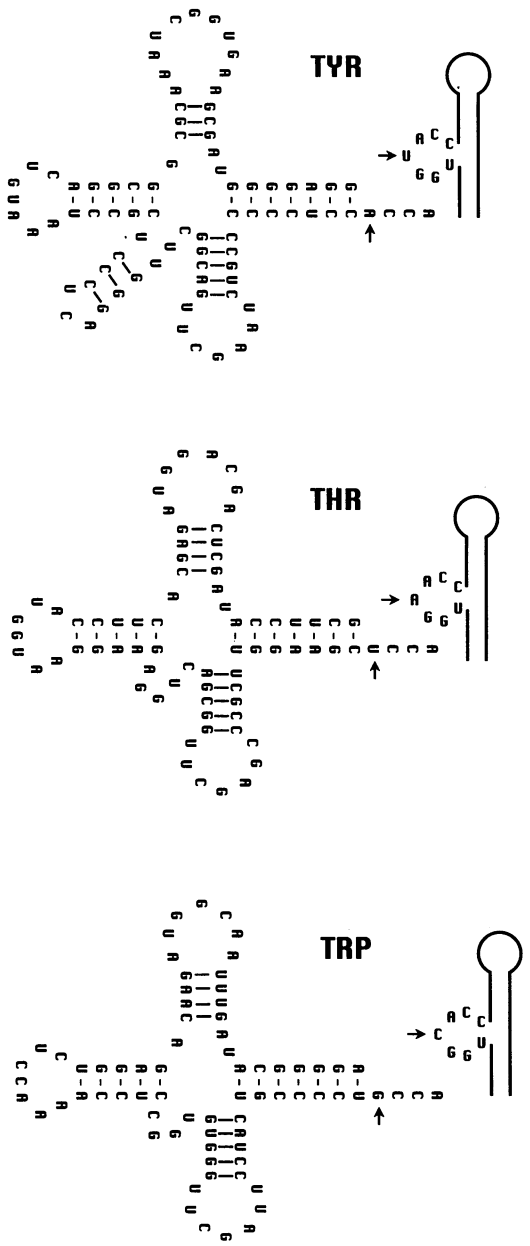


FIG. 3. Intermolecular covariance between the variable position of the T box and the discriminator position of the cognate tRNAs. Horizontal arrows indicate the variable position of the side-bulge portion of the antiterminators for *B. subtilis tyrS* (or *tyrZ*) and *thrS* (or *thrZ*) and *B. stearothermophilus trpS*; *B. subtilis trpS* contains a U at this position. Vertical arrows indicate the discriminator position of the tRNAs. The tRNA sequence data are from Wawrousek and Hansen ($tRNA^{Thr}$ [41]) and Wawrousek et al. ($tRNA^{Tyr}$ and $tRNA^{Trp}$ [42]).

there might be a second position of interaction between the leader antiterminator and the acceptor end of the tRNA.

Mutations in the antiterminator. Deletion of 6 bases in the 7-base antiterminator side bulge (TyrΔKpn mutation) was previously shown to greatly reduce antitermination (24); since this deletion is not predicted to prevent formation of the helical portion of the antiterminator, it appears that the side-bulge region is important. A single G-to-A base substitution at a conserved position (TyrKp5 mutation) (Fig. 1B) was

TABLE 1. Effect of T box mutations on *tyrS-lacZ* expression

tyrS-lacZ fusion	T box sequence[a]	Specifier sequence	β-Galactosidase activity[b]			Induction by tyrosine limitation[c]
			LB medium	TWY	TWy	
Wild type	UGGUACCG	UAC	43	8.6	106	12
ΔKpn	U------G	UAC	8.5	1.7	1.2	0.71
Kp5	UGAUACCG	UAC	0.24	0.28	0.24	0.86
KpΔU	UGG-ACCG	UAC	0.29	0.28	0.24	0.86
KpA	UGGAACCG	UAC	4.1	0.96	7.0	7.3
KpC	UGGCACCG	UAC	52	3.6	37	10
AMB	UGGUACCG	UAG	6.9	14	13	0.92
AMB-KpA	UGGAACCG	UAG	0.71	0.55	0.54	0.92
AMB-KpC	UGGCACCG	UAG	10	2.6	2.0	0.77

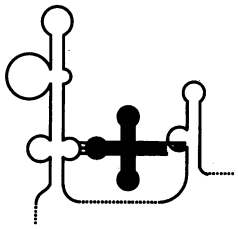
[a] Mutations in the T box sequence are indicated by underlined bases (substitutions) or dashes (deletions).

[b] β-Galactosidase activity is in Miller units (31). Cells were grown in LB medium or in minimal medium with excess threonine, tryptophan, and tyrosine (TWY) or excess threonine and tryptophan and limiting tyrosine (TWy).

[c] Induction is the ratio of units obtained with limiting tyrosine (TWy) to units obtained with excess tyrosine (TWY).

therefore tested and was also found to block antitermination (Table 1), indicating that this region is very sensitive to mutational alteration. Deletion of the next base (the variable position, TyrKpΔU mutation) also gave very low activity. In rich medium, the single-base TyrKpΔU and TyrKp5 mutations exhibited lower expression than was observed with deletion of the entire *Kpn*I site (TyrΔKpn mutation), suggesting that removal of the side bulge is less damaging than an altered sequence, under these conditions. Removal of 6 of 7 bases of the side bulge in TyrΔKpn is predicted to result in a more stable helix, which may partially compensate for the disruption of any sequences in the side bulge which are involved in stabilizing tRNA-antiterminator interactions. Alteration of the variable position of the side bulge from U to A (TyrKpA mutation) resulted in a 10-fold drop in expression in minimal medium; the mutant retained induction in response to tyrosine limitation, but the level of expression was 10-fold lower than that of the wild-type fusion with either excess or limiting tyrosine. This suggests that $tRNA^{Tyr}$ is still able to interact with the TyrKpA mutant leader but that this interaction is much less efficient. Conversion of this position from U to C (TyrKpC mutation) resulted in a threefold drop in expression relative to the wild-type fusion in minimal medium and had no effect in rich medium. These data indicate that although this position of the side bulge is variable in different leaders, the sequence at this position is important in that the A normally found at this position in the *thrS*, *thrZ*, and *cysE-cysS* genes is not tolerated in *tyrS*. The TyrKpΔU and TyrKpA mutants both contain the sequence UGGA in the side-bulge region, but the TyrKpA mutant retains some level of inducibility, while TyrKpΔU is completely uninducible; this suggests that the size and/or sequence of positions 5 to 7 of the side bulge are crucial.

Suppression of leader region mutations by mutant tRNAs. The $tRNA^{Tyr}$ gene was isolated from the *trnD* region by PCR, and the anticodon was changed from 5′-GUA-3′ to 5′-CUA-3′ by oligonucleotide-directed mutagenesis to generate an amber suppressor tRNA. The wild-type and amber suppressor mutant $tRNA^{Tyr}$ genes were then inserted into a multicopy P_{spac} expression plasmid so that expression of the tRNA genes would be inducible by IPTG. Variants of $tRNA^{Tyr}$ with alterations in the anticodon or discriminator position were tested for the ability to suppress the effect of specific *tyrS* leader



tyrS-lacZ Fusion	tRNA^{Tyr}	specifier	anticodon	acceptor	T-box	β – galactosidase - IPTG	β – galactosidase + IPTG	Induction Ratio
WT	WT	UAC	GUA	ACCA	UGGU	69	58	0.84
WT	A73U	UAC	GUA	UCCA	UGGU	34	41	1.2
WT	A73G	UAC	GUA	GCCA	UGGU	26	43	1.7
KpA	WT	UAC	GUA	ACCA	UGGU	2.7	2.4	0.89
KpA	A73U	UAC	GUA	UCCA	UGGU	3.2	23	7.2
KpA	A73G	UAC	GUA	GCCA	UGGU	5.6	14	2.5
AMB	WT	UAG	GUA	ACCA	UGGU	15	16	1.1
AMB-KpA	A73U	UAG	GUA	UCCA	UGGU	0.96	1.3	1.4
AMB	AMB	UAG	CUA	ACCA	UGGU	13	21	1.6
AMB	AMB-A73U	UAG	CUA	UCCA	UGGU	7.1	12	1.7
KpA	AMB-A73U	UAC	CUA	UCCA	UGGU	4.0	4.0	1.0
AMB-KpA	AMB-A73U	UAG	CUA	UCCA	UGGU	0.95	26	27
AMB	AMB-A73G	UAG	CUA	GCCA	UGGU	6.8	24	3.5
AMB-KpA	AMB-A73G	UAG	CUA	GCCA	UGGU	0.71	4.5	6.3
AMB-KpC	AMB-A73U	UAG	CUA	UCCA	UGGC	9.6	25	2.6
AMB-KpC	AMB-A73G	UAG	CUA	GCCA	UGGC	10	40	4.0

FIG. 4. Effect of IPTG-induced synthesis of variants of tRNA^{Tyr} on expression of *tyrS-lacZ* fusions. The unshaded region represents stem I and the antiterminator region of the *tyrS* leader; the dashed line indicates regions of the leader which were omitted for clarity. The shaded cloverleaf structure represents tRNA^{Tyr}, which was inserted downstream of P_{spac} in plasmid pDG148 so that expression was induced by the addition of IPTG. Positions of deviation from the wild-type *tyrS* and tRNA^{Tyr} sequences are boxed. Lines pairing the specifier and anticodon, or acceptor and T box, indicate complementary sequences. Cells were grown in LB medium to the early exponential growth phase, diluted twofold, and divided, and IPTG was added (0, 0.04, 0.2, or 1.0 mM) to induce expression of the plasmid-borne tRNA. Growth was continued for 2 h, and then cells were harvested and assayed for β-galactosidase activity (in Miller units) (31). In each case, induction was maximal at 1.0 mM IPTG, so these data are listed. The induction ratio is the level of β-galactosidase obtained in the presence of IPTG relative to the level of β-galactosidase in the absence of IPTG.

region mutations and to give induction in rich medium, without tyrosine limitation. The addition of extra tyrosine to the growth medium had no effect on the basal-level expression of the wild-type *tyrS-lacZ* fusion, indicating that tyrosine was not limiting under these conditions (20). In these experiments, the wild-type tRNA^{Tyr} gene is present in the cell, and any effect of induction of the plasmid-encoded tRNA is examined against the background effect of the chromosomal tRNA^{Tyr} product. IPTG-induced expression of wild-type tRNA^{Tyr} did not result in induction of a wild-type *tyrS-lacZ* fusion (Fig. 4), presumably because the tRNA was rapidly charged during growth in rich medium. Any excess of this uncharged tRNA would be predicted to also induce expression of the chromosomal *tyrS* gene, resulting in increased levels of tyrosyl-tRNA synthetase (TyrTS) enzyme, and even more efficient charging in the presence of excess tyrosine.

Mutations at the discriminator position of tRNA^{Tyr} have been shown to block charging by TyrTS in vivo and in vitro in *E. coli* (37, 38). This was tested in *B. subtilis* by introducing these alterations into an amber suppressor variant of tRNA^{Tyr} and measuring expression of an *rpsD-lacZ* translational fusion containing an amber mutation previously constructed for use as a reporter of amber suppressor tRNA activity (19). The A73U mutation resulted in a 20-fold drop in amber suppression, while the A73G mutation almost completely abolished amber suppressor activity (Table 2). Since these tRNA variants were functional in induction of antitermination of appropriate fusions (see below), it is unlikely that the mutations drastically affect tRNA structure; the loss of amber suppressor activity is most likely due to poor charging by TyrTS enzyme in vivo. These data also indicate that it is unlikely that another tRNA synthetase can charge these tRNAs in vivo.

Expression of tRNA^{Tyr}A73U also failed to induce the wild-type fusion (Fig. 4) despite the fact that this mutation

TABLE 2. Efficiency of amber suppression by tRNA^{Tyr} variants with discriminator base mutations

tRNA^{Tyr} variant[a]	Acceptor end sequence	IPTG (1.0 mM)	β-Galactosidase activity[b]
AMB	ACCA	–	1.8
		+	92
AMB-A73U	UCCA	–	0.61
		+	4.7
AMB-A73G	GCCA	–	0.47
		+	0.58

[a] Variants of tRNA^{Tyr} with an amber suppressor anticodon and A, C, or G at position 73 were inserted into plasmid pDG148 so that synthesis of the tRNA was under control of the IPTG-inducible P_{spac} promoter.

[b] Strain BR151MA containing an *rpsD-lacZ* translational fusion with a UAG amber mutation in the S4 coding region (19), carried on an SPβ prophage, was grown to the early exponential growth phase. The cell cultures were diluted twofold and divided, and IPTG (1.0 mM) was added to one of the cultures. Growth was continued for 2 h, and then cells were harvested. β-Galactosidase activity is reported in Miller units (31).

renders $tRNA^{Tyr}$ unchargeable by TyrTS; this lack of induction could be due to the mismatch between the discriminator position of the tRNA and the variable position of the antiterminator. Expression of $tRNA^{Tyr}$A73G resulted in a 1.7-fold induction, even in the absence of tyrosine limitation. This tRNA is also predicted to be unchargeable by TyrTS; therefore, IPTG induction should result in accumulation of the uncharged tRNA. The $tRNA^{Tyr}$A73G mutation would result in a G-U pair, rather than the normal A-U, at the discriminator-antiterminator; G-U was predicted to be a functional pairing since sequence data suggest that this pairing exists for *B. subtilis trpS*. The presence of wild-type $tRNA^{Tyr}$ in the cell could be responsible for the inefficient induction of the wild-type fusion by this tRNA if an abundance of charged $tRNA^{Tyr}$ can interfere with binding of uncharged tRNA to the leader.

The TyrKpA mutation, in which the variable position of the antiterminator side bulge is changed from U to A, resulted in low activity (Table 1). IPTG-induced expression of wild-type $tRNA^{Tyr}$ (A-A mismatch at the discriminator-antiterminator) had no effect (Fig. 4), while $tRNA^{Tyr}$A73U (U-A match) conferred a 7.2-fold induction in response to IPTG; $tRNA^{Tyr}$A73G (G-A mismatch) gave 2.5-fold induction by IPTG. These results indicate that overexpression of an unchargeable tRNA can give strong induction of *tyrS* even in the absence of limitation for tyrosine, suggesting that the amino acid limitation effect is mediated through an increase in uncharged tRNA, as proposed. A match at the discriminator position of the tRNA and the antiterminator variable position contributes significantly to the efficiency of the tRNA-leader interaction. The efficient induction of the TyrKpA mutant fusion relative to the wild-type fusion by the mutant tRNAs could be due to reduced interference by wild-type $tRNA^{Tyr}$ in the cell, which interacts less efficiently with the mutant leader.

Fusions with amber or ochre nonsense specifiers. A possible complication in analysis of fusions containing the UAC tyrosine specifier sequence is that wild-type $tRNA^{Tyr}$ present in the cell could interfere with the ability of the plasmid-encoded mutant tRNAs to interact with the leader. A fusion containing a nonsense codon at the specifier position would be less susceptible to interference by chromosomally encoded tRNAs. In previous studies, introduction of a UAG amber or UAA ochre codon at the position of the UAC tyrosine specifier sequence in the *tyrS* leader (Fig. 1) was shown to result in uninducible expression of the *tyrS-lacZ* transcriptional fusion in a wild-type strain (17). Introduction of the *sup-3* allele, which is an ochre suppressor mutant lysyl-tRNA (32), resulted in partial restoration of *tyrS-lacZ* expression in the TyrAMB (UAG specifier) and TyrOCH (UAA specifier) mutants, and expression was now inducible in response to limitation for lysine (17); this result strongly implicated tRNA as a signal necessary for antitermination. However, the 3-fold *sup-3*-mediated response of the mutant fusions to lysine limitation was significantly lower than the 10-fold response of the wild-type fusion to tyrosine limitation, suggesting that the interaction of the $tRNA^{Lys}$ variant with the *tyrS* leader might be less efficient than that of $tRNA^{Tyr}$.

An amber suppressor variant of $tRNA^{Tyr}$ (Fig. 2) was constructed to test whether this tRNA could give more efficient suppression of the TyrAMB mutant. Unlike ochre suppressor tRNAs which can suppress amber (UAG) as well as ochre (UAA) nonsense mutants, amber suppressor tRNAs are unable to suppress ochre nonsense mutants during translation; therefore, this tRNA was predicted to give specific suppression of TyrAMB but not TyrOCH. The amber suppressor tRNA ($tRNA^{Tyr}$AMB) was shown to function efficiently in suppression of the translational block of a *rpsD-lacZ* translational fusion containing an amber codon, indicating that the tRNA expressed from this construct is functional in protein synthesis in vivo and suppression was IPTG dependent (19) (Table 2).

TABLE 3. Effect of IPTG-induced expression of $tRNA^{Tyr}$ variants on TyrAMB and TyrOCH *tyrS-lacZ* fusions

tyrS-lacZ fusion	Plasmid-borne $tRNA^{Tyr}$	IPTG (1 mM)[a]	β-Galactosidase activity[b]	Induction ratio[c]
AMB	Wild type	−	15	1.1
		+	16	
	AMB	−	13	1.6
		+	21	
OCH	Wild type	−	5.3	1.0
		+	5.5	
	AMB	−	4.1	1.0
		+	4.1	

[a] Cell cultures were grown in LB medium to the early logarithmic growth phase, diluted twofold, and divided, and IPTG was added (0, 0.04, 0.2, or 1.0 mM). Growth was continued for 2 h, and then cells were harvested. Maximal induction was obtained with 1.0 mM IPTG, and these data are reported.

[b] β-Galactosidase activity is reported in Miller units (31).

[c] Induction ratio is the level of activity of cells grown in the presence of IPTG relative to that of cells without IPTG.

The ability of the amber suppressor $tRNA^{Tyr}$ to suppress the TyrAMB and TyrOCH specifier sequence mutations was tested in rich medium (LB medium) (Table 3). In the absence of IPTG, expression of the mutant *tyrS-lacZ* fusions was low, as observed previously (17). Addition of IPTG to induce synthesis of the plasmid-borne amber suppressor tRNA resulted in induction of the TyrAMB mutant fusion and had no effect on the TyrOCH fusion. Production of wild-type $tRNA^{Tyr}$ had no effect on expression of either fusion. The ability of the amber suppressor tRNA to specifically suppress the TyrAMB mutation strongly supports the model that codon-anticodon interaction between the leader region specifier sequence and the tRNA is a critical feature in this antitermination system. The modest level of induction of the TyrAMB fusion in response to overexpression of the amber suppressor tRNA is probably due to the fact that this tRNA is efficiently charged during growth in rich medium. These results led us to focus on studies with unchargeable tRNA variants.

TyrAMB-KpA and TyrAMB-KpC double-mutant fusions. Fusions containing both the UAG amber specifier and the KpA antiterminator mutation (TyrAMB-KpA) or the UAG specifier and the KpC antiterminator (TyrAMB-KpC) were constructed since no tRNA capable of efficient interaction with this specifier should be present in the cell and derivatives of $tRNA^{Tyr}$ matching these antiterminators are unchargeable. Expression of the TyrAMB-KpA fusion was very low (Table 1) and was stimulated 27-fold by IPTG-induced expression of a variant of $tRNA^{Tyr}$ with an anticodon matching the UAG specifier and the A73U mutation at the discriminator position ($tRNA^{Tyr}$AMB-A73U) (Fig. 4). A match at the discriminator-antiterminator without a match at the specifier (TyrAMB-KpA fusion with $tRNA^{Tyr}$A73U or TyrKpA fusion with $tRNA^{Tyr}$AMB-A73U) resulted in poor induction, indicating that the interaction between the specifier and anticodon is crucial. The effect of $tRNA^{Tyr}$AMB-A73U on expression of the TyrAMB fusion (U-U mismatch at the discriminator-antiterminator) was only 1.7-fold, while a G-U pair (TyrAMB with $tRNA^{Tyr}$AMB-A73G) resulted in 3.5-fold induction. A G-A mismatch (TyrAMB-KpA with $tRNA^{Tyr}$AMB-A73G) resulted in 6.3-fold

induction; it is not clear why a G-A mismatch should be better than a G-U pair, but this result suggests that there may be unknown structural constraints on this interaction. These data demonstrate that there is likely to be a site of interaction between the acceptor end of the tRNA and the side bulge of the leader region antiterminator since mutations in this region of the leader can be suppressed by appropriate compensatory mutations in the tRNA.

Induction of the TyrAMB-KpA fusion by $tRNA^{Tyr}$AMB-A73U was 27-fold, while induction of the TyrKpA fusion by $tRNA^{Tyr}$A73U was 7.2-fold, despite the fact that both of these pairs of fusions and tRNAs are matched at both the specifier-anticodon and discriminator-antiterminator. The reduced efficiency of induction of the TyrKpA fusion could be due to interference by the chromosomally encoded wild-type $tRNA^{Tyr}$ with the interaction of the plasmid-encoded mutant tRNA with the mutant leader, which contains a normal UAC tyrosine specifier. Introduction of the UAG amber specifier would be predicted to eliminate this interference.

The TyrAMB and TyrAMB-KpC mutant fusions exhibited basal expression in LB medium higher than that of TyrAMB-KpA (Table 1). The reason for this is unknown but could be the presence of a tRNA in the cell which interacts weakly with these leader variants. Expression was not inducible by limitation for tyrosine, indicating that this tRNA is unlikely to be $tRNA^{Tyr}$. Overproduction of $tRNA^{Tyr}$AMB-A73G resulted in 4-fold induction of the TyrAMB-KpC fusion (match at both the specifier and antiterminator-discriminator), while $tRNA^{Tyr}$AMB-A73U (U-C mismatch at the discriminator-antiterminator) resulted in 2.6-fold induction. It is not clear why certain tRNA fusion combinations give better differentiation at the discriminator-antiterminator interaction site. A possible explanation is that fusions such as TyrAMB-KpC, which have higher basal activity, are recognized, albeit inefficiently, by a cellular tRNA which can interfere with the ability of the plasmid-encoded tRNA to induce antitermination. Nevertheless, a consistent pattern is observed: for a given fusion, production of a tRNA which matches at both the specifier and the discriminator-antiterminator always results in maximal induction.

DISCUSSION

Previous studies have suggested that expression of the *B. subtilis tyrS* gene as well as a number of other tRNA synthetase and amino acid biosynthesis genes in gram-positive species is controlled by readthrough of a transcriptional termination site in the mRNA leader region (8, 12, 13, 17, 24, 35). For several genes in this group, including *B. subtilis tyrS* (24), *ilv-leu* (12, 13), and *thrS* and *thrZ* (35), *L. lactis his* and *trp* (5), and *B. lactofermentum argS* (34), expression is induced by limitation for the appropriate amino acid and not by general amino acid starvation. The specificity of the amino acid response was shown for *tyrS* to be dependent on a triplet sequence in the leader region, a UAC tyrosine codon which is necessary for interaction with $tRNA^{Tyr}$, and is mediated through codon-anticodon interaction with the cognate tRNA (17). In this article, we demonstrate the existence of a second site of possible interaction between the leader region antiterminator sequence and the acceptor end of the tRNA. This site was suggested by the complementarity of the two sequences and the intermolecular covariance between the variable position of the antiterminator side bulge and the discriminator position of the cognate tRNAs and was tested by mutational analysis of both the antiterminator and tRNA sequences. The genetic data strongly suggest interactions between tRNA and the leader; biochemical studies will be necessary to prove direct contact.

IPTG-induced expression of plasmid-borne variants of $tRNA^{Tyr}$ resulted in induction of *tyrS-lacZ* expression during growth in rich medium in the absence of limitation for tyrosine. This induction was most strongly dependent upon a match between the tRNA anticodon and the specifier sequence of the *tyrS* leader and was also significantly affected by a match at the discriminator position of the tRNA and the variable position of the antiterminator side bulge.

Mutation of the second G of the invariant UGG in the leader to A (TyrKp5 mutation; UGA) resulted in complete loss of expression, indicating that this position is critically important; this mutation is predicted to result in replacement of a G-C base pair with an A-C mismatch. A variant of $tRNA^{Tyr}$ with a compensatory mutation in the CCA sequence (UCA; $tRNA^{Tyr}$C74U) was constructed, but this construct was unable to suppress the TyrKp5 mutation (20). In *E. coli*, certain tRNA mutants with alterations in the CCA acceptor end sequence were shown to be efficiently repaired to CCA in vivo (36), while others were stable (33). Many tRNA genes in *B. subtilis* do not encode the terminal CCA, which must be added posttranscriptionally (14); it appears likely that the CCA addition system is very efficient in *B. subtilis* so that expression of a tRNA gene with the C74U mutation did not result in accumulation of mature tRNA containing this alteration.

The requirement of TyrTS for an A at position 73 of $tRNA^{Tyr}$ is well documented (27, 37, 38); this position is more important than the anticodon for recognition by TyrTS since variants of $tRNA^{Tyr}$ with an altered anticodon are efficient amber suppressor tRNAs in both *E. coli* (*supF*) (11) and *B. subtilis* (19). Introduction of the A73U and A73G substitutions into $tRNA^{Tyr}$AMB greatly reduced the ability of these tRNAs to suppress an amber mutation in vivo in *B. subtilis*, indicating that these tRNAs are not chargeable by any tRNA synthetase in vivo. Introduction of the A73G substitution into the *supF* variant of *E. coli* $tRNA^{Tyr}$, which is equivalent to $tRNA^{Tyr}$AMB-A73G, results in charging by glutaminyl-tRNA synthetase rather than TyrTS (37, 38). This switch in charging specificity apparently does not occur in *B. subtilis*, possibly because there is no separate glutaminyl-tRNA synthetase in *B. subtilis* (glutamyl-tRNA synthetase [GltX] charges both glutamyl and glutaminyl tRNAs with glutamate, which on glutaminyl tRNA is subsequently converted to glutamine) (29).

The initial model for *tyrS* antitermination suggested that interaction of $tRNA^{Tyr}$ with the leader region specifier sequence (at position +100 of the leader) in some way stimulates formation of an antiterminator structure (at position +215). A direct interaction between the acceptor end of the tRNA and the side-bulge portion of the antiterminator could provide the mechanism for this effect. This interaction could also explain the discrimination between uncharged and charged tRNA since an amino acid attached to the 3′ end of charged tRNA could interfere with the interaction of the end of the tRNA with the antiterminator. How interaction of the tRNA and the antiterminator augments readthrough is still not clear. The additional base pairing or contribution of stacking energy by the tRNA may directly shift the balance toward antitermination. Alternatively, the tRNA-T box paired structure may provide a recognition element for factors which promote readthrough of the terminator.

Induction of *tyrS-lacZ* fusions by plasmid-encoded tRNAs in the absence of limitation for tyrosine was most efficient with unchargeable tRNAs and with fusions containing a UAG amber specifier rather than the UAC tyrosine specifier. These observations can be explained by postulating that charged

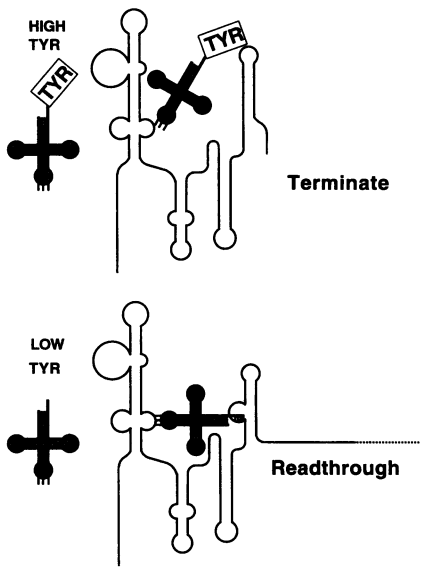


FIG. 5. Model for induction of *tyrS* by uncharged $tRNA^{Tyr}$. The shaded cloverleaf structure represents $tRNA^{Tyr}$, uncharged or charged with tyrosine (box). The upper structure represents the terminator form of the leader. The lower structure represents the antiterminator form of the leader. The two structures are mutually exclusive. The small lines represent base pairing between the anticodon of the tRNA with the specifier sequence of the leader and the acceptor end of the tRNA with the side bulge of the antiterminator. Uncharged $tRNA^{Tyr}$ interacts with the leader region nascent transcript to promote formation of the antiterminator structure, preventing terminator formation. Charged $tRNA^{Tyr}$ is unable to interact appropriately with the leader to promote antitermination and may compete with uncharged tRNA for access to the leader.

$tRNA^{Tyr}$ can inhibit the interaction of uncharged tRNA with the leader, thereby blocking antitermination. This can be envisioned as a competition where both charged and uncharged tRNA can bind at the specifier sequence but only uncharged tRNA can form the second interaction between the acceptor end and the antiterminator (Fig. 5). An excess of charged tRNA capable of interacting at the specifier could therefore block access of uncharged tRNA. Under normal conditions, the gene would therefore respond to the balance of charged and uncharged cognate tRNA rather than to the absolute level of uncharged tRNA. It is also likely that an uncharged tRNA which matches at the specifier, but not at the antiterminator, could interfere.

The role of tRNA in antitermination in the T box genes is strongly supported by the isolation by Garrity and Zahler (7) of *trans*-acting mutants which confer constitutive expression of the *B. subtilis ilv-leu* operon, which normally is induced by limitation for leucine (13). One class of these mutants proved to be in the gene for $tRNA^{Leu}$ with a GAG anticodon, which matches the CUC specifier of *ilv-leu* (18). These mutations are predicted to result in accumulation of uncharged $tRNA^{Leu}$-GAG in the presence of excess leucine and were suppressed by a wild-type copy of this tRNA gene, suggesting that charged $tRNA^{Leu}$GAG could interfere with induction of *ilv-leu* expression by uncharged tRNA (7). This conclusion is in agreement with our results and suggests that the charging ratio may be a critical parameter in vivo. However, it must be kept in mind that the presence in a cell of a chargeable tRNA with the same anticodon as a weakly charged tRNA could avert some unknown signal (e.g., the stringent response) necessary for the uncharged tRNA to induce, rather than cause, a direct interference.

The balance between tRNA and aminoacyl-tRNA synthetase levels is likely to be under tight control (25). Overproduction of tRNAs or tRNA synthetases has been shown to cause misacylation in vivo (37, 40) and can result in toxic effects. In this study, expression of tRNA variants was carried out under P_{spac} control to reduce the possibility of such effects. This control was very efficient, as monitored by suppression of an amber mutation by IPTG-induced expression of the amber suppressor variant of $tRNA^{Tyr}$ (19). The proposed mechanism for regulation of tRNA synthetase genes in gram-positive bacteria in direct response to the levels of charged and uncharged cognate tRNA would provide the cell with a means to couple synthetase and tRNA levels to permit optimal translation and prevent misacylation.

In *E. coli*, expression of a number of aminoacyl-tRNA synthetase genes has been shown to increase as a function of growth rate (15). This has not been explored in detail in *B. subtilis*. We observed that expression of certain *tyrS-lacZ* fusions was increased during growth in LB medium (doubling time, 30 min) compared with growth in minimal medium with all required amino acids (120 min), suggesting a possible effect of growth rate (Table 1). This was not observed for the most severely affected mutants (TyrKp5 and TyrKpΔU). The basis for this differential effect is not known, and future studies will be necessary to determine if the growth rate effect is mediated at the level of antitermination or at some other level.

This study was designed to determine if there are positions of interaction between the tRNA and the leader in addition to the specifier sequence-anticodon interaction. A second site, at the antiterminator and the acceptor end of the tRNA, was demonstrated. It may be that the specifier and antiterminator sequences represent the dominant specificity determinants, while other leader region features may assist in forming an appropriate three-dimensional pocket for recognition of the cognate tRNA and discrimination against noncognate tRNA species. Elucidation of these additional features may provide new insight into RNA-RNA interactions. The possibility that protein factors, either general or specific to individual genes, act in concert with uncharged tRNA to mediate antitermination remains to be explored.

ACKNOWLEDGMENTS

We thank S. A. Zahler and S. D. Ehrlich for communication of unpublished results and Wayne L. Nicholson for providing plasmid pDG148.

This work was supported by Public Health Service grants GM40650 and GM47823 from the National Institutes of Health.

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