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Three Modifications in the D and T Arms of tRNA Influence Translation in *Escherichia coli* and Expression of Virulence Genes in *Shigella flexneri*

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The modified nucleosides 2′-*O*-methylguanosine, present at position 18 (Gm18), 5-methyluridine, present at position 54 (m^5U54), and pseudouridine, present at position 55 (Ψ55), are located in the D and T arms of tRNAs and are close in space in the three-dimensional (3D) structure of this molecule in the bacterium *Escherichia coli*. The formation of these modified nucleosides is catalyzed by the products of genes *trmH* (Gm18), *trmA* (m^5U54), and *truB* (Ψ55). The combination of *trmH*, *trmA*, and *truB* mutations resulting in lack of these three modifications reduced the growth rate, especially at high temperature. Moreover, the lack of three modified nucleotides in tRNA induced defects in the translation of certain codons, sensitivity to amino acid analog 3,4-dehydro-DL-proline, and an altered oxidation of some carbon compounds. The results are consistent with the suggestion that these modified nucleosides, two of which directly interact in the 3D structure of tRNA by forming a hydrogen bond between Ψ55 and Gm18, stabilize the structure of the tRNA. Moreover, lack of Ψ55 in tRNA of human pathogen *Shigella flexneri* leads to a reduced expression of several virulence-associated genes.

In cells of all organisms there are several species of stable RNA, of which the most prominent are rRNA and tRNA. Most of the stable RNAs contain modified nucleosides, which are derivatives of the four ordinary nucleosides. In a global survey, 81 of 95 modified nucleosides were found in tRNA (49). Pseudouridine (Ψ) and 2′-O-methylated nucleosides are the most abundant modified residues in RNAs (33, 41). In the tRNA of the bacterium *Escherichia coli*, formation of Ψ occurs at positions 32 (48, 60), 38 to 40 (26), and 55 (39). The Ψ55 is present in the TΨC loop, which is present in all tRNA species of this bacterium. Deletion of the *truB* gene, which is responsible for the modification of Ψ55, has no major effect on growth rate, but a strain lacking the *truB* gene was outcompeted by wild-type cells in a growth competition experiment (23). Another modified nucleoside which is also present in the TΨC loop of all tRNA species in *E. coli* and whose function remains obscure is 5-methyluridine (ribothymidine, m^5U[T]54). The *trmA*::*cat* insertion relatively early in the *trmA* gene (codon 161 out of 366), which is responsible for the formation of m^5U54, is lethal for *E. coli* (44). However, several *trmA* point mutants lacking the methyl group in tRNA and producing full-length TrmA proteins (44, 57) have no apparent growth defects. The only observed disadvantage for cells lacking m^5U54 is that they are outcompeted by wild-type cells in a mixed-population experiment (4).

2′-*O*-Methylguanosine (Gm18) is present in the conserved D loop in 13 out of the 46 *E. coli* tRNA species sequenced (54). Gm18 methylating activity has been established (19), and the *trmH* gene, the product of which is responsible for its synthesis, has been identified (45). Absence of Gm18 in tRNA of *E. coli* has no effect on the activity of the *supF* amber suppressor tRNA or on the growth rate of cells (45).

Nucleosides Gm18 and Ψ55 interact in the tRNA tertiary structure by hydrogen bonding, thereby stabilizing the L shape of tRNA (30, 46) (Fig. 1). Generally, 2′-O methylation of the ribose stabilizes the nucleoside in the $C_{3'}$-endo form, thereby causing conformational rigidity (27, 28). The 2′-O methylation of Gm18 may contribute to the rigidity of G18, stabilizing Gm18-Ψ55 base pairing. Both 5-methylation of U54 and the isomerization of U55 into Ψ55 stabilize the tRNA, probably by enhancing base stacking (12, 13, 59). Also, a water-mediated bridge between Ψ55 and the phosphate backbone provides an additional stabilizing effect (1). Therefore, we expected that a combination of mutations that lead to the absence of Gm18, m^5U54, and Ψ55 in tRNA of *E. coli* would destabilize the tRNA, resulting in a possible effect on the efficiency of translation, the growth rate, and/or the metabolism. The present work addresses this question, and indeed this expectation was verified. Moreover, we also show that expression of virulence genes in *Shigella flexneri* was affected by lack of Ψ55 and that the *Pseudomonas aeruginosa orp* mutant, deficient in the expression of virulence genes, lacks Ψ55-modifying activity.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used are listed in Tables 1 and 2.

Growth conditions and genetic procedures. Cultures were grown in either Luria-Bertani (LB) medium (2), rich-MOPS (37a), or MOPS-glucose medium (37). For growth of *S. flexneri*, MOPS-glucose medium was supplemented with 0.005% nicotinic acid (NA). As solid medium, TYS (10 g of Trypticase peptone, 5 g of yeast extract, 5 g of NaCl, and 15 g of agar per liter) or BHI (37 g of brain heart infusion [Difco Laboratories, Detroit, Mich.] and 15 g of agar per liter) was used. When needed, carbenicillin (50 μg/ml), chloramphenicol (15 μg/ml), or tetracycline (15 μg/ml) was added to the growth media. Phage P1 transductions and F′ conjugations in *E. coli* were performed as described previously (35). *S.*

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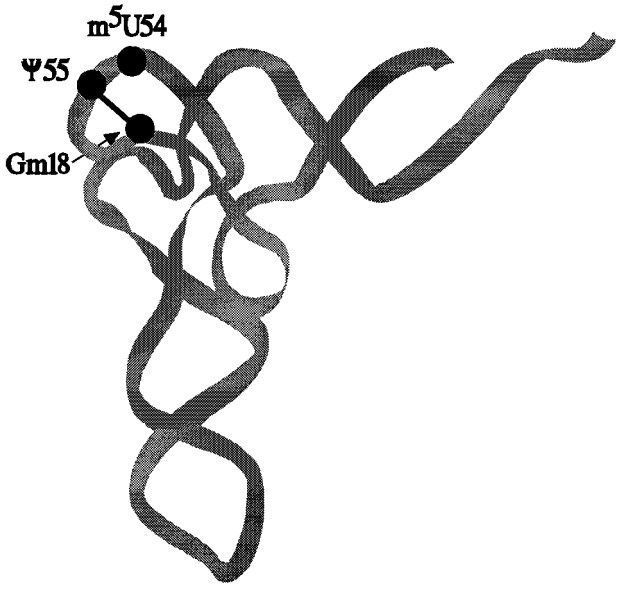

FIG. 1. Schematic model of three-dimensional structure of the yeast $tRNA^{Phe}$ molecule, adapted from reference 29. The locations of the modified nucleosides in positions 18, 54, and 55 are demonstrated. Black line, hydrogen bonding between nucleosides Gm18 and Ψ55.

flexneri strains were constructed by P1-mediated transduction with *E. coli* strains as donors.

Analysis of modified nucleotides in tRNA by TLC. Bacteria were grown in 10 ml of LB medium at 37°C, harvested at a cell density of about 4×10^8 cells/ml by centrifugation, washed once with buffer B (25 mM Tris-HCl [pH 7.4], 10 mM $Mg[CH_3COO]_2$, 0.1 mM dithiothreitol, 1 mM EDTA, 10% [by volume] ethylene glycol) (40), and resuspended in 0.5 ml of buffer B. Cells were disrupted by sonication three times for 5 s at 20% power on a VCX400 sonicator (Sonics and Materials Inc., Danbury, Conn.). Cell debris was removed by centrifugation with an Eppendorf centrifuge (5415D) for 15 min at 4°C, and the supernatant was transferred to new tube. The obtained supernatant was used as enzyme extract. To assay enzymatic activity of the TruB protein and/or TrmA protein, [α-^{32}P]UTP-labeled transcripts of $tRNA^{Val}$ (about 10^4 cpm) were incubated with a mixture containing 80 μl of enzyme extract, 50 μM *S*-adenosylmethionine, 25 mM Tris-HCl, pH 8.0, 2.5 mM $MgCl_2$, and 0.1 mM dithiothreitol in a final volume of 100 μl for 30 min at 37°C. The reaction was stopped by adding equal volumes of Tris-buffered phenol, pH 7.5, and chloroform-isoamyl alcohol (24:1) and vortexing. The tRNA in the aqueous phase was precipitated by adding 20 μg of yeast RNA as a carrier and 2 volumes of ethanol. The precipitate was washed with 70% ethanol, dried, and digested to nucleotides by P1 nuclease (21). The distribution of modified uridine derivatives was determined by two-dimensional thin-layer chromatography (TLC) as described previously (38). The radioactive compounds were detected with a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.).

Analysis of modified nucleotides in tRNA by HPLC and determination of growth rates. High-pressure liquid chromatography (HPLC) analysis was performed as described previously (20, 21). Growth rates at 37°C in rich-MOPS and MOPS-glucose were determined as described previously (4).

Determination of frameshifting levels. The level of frameshifting was determined by using a system which contains the *lacZ* gene placed downstream of a short frameshifting window in such a way that the β-galactosidase activity is a direct measurement of the frequency with which the ribosomes shift reading frame (58).

Determination of suppression by *tyrT* (*supF*) tRNA. The efficiency of *supF* amber (UAG) suppressor tRNA, which is a mutated $tRNA^{Tyr}$, was measured by introducing an F′ plasmid with a *lacI-lacZ* fusion with or without nonsense mutations in the *lacI* part into a *tyrT* (*supF*) mutant *E.* coli (36). Strains were grown in LB medium, and the β-galactosidase activity was determined as described previously (35).

Determination of sensitivity to amino acid analogs. Strains were grown at 37°C overnight in LB medium. A sample of each culture (0.1 ml) was mixed with 2 ml of 0.5% agar in 0.9% NaCl, and the mixture was poured onto plates containing medium E plus 0.2% glucose. Paper disks (6 mm in diameter) were placed on the surfaces of the plates, and 50 μg of various amino acid analogs was applied to each disk. The plates were incubated at 37°C for 24 h before being scored. Analogs to which strains responded equally were DL-aspartic-β-hydroxamate, 1,2,4-triazole, azaserine, L-glutamic acid-γ-hydrazide, L-methionine-DL-sulfoximine, 1,2,4-DL-triazole-3-alanine, 3-amino-1,2,4-triazole, β-chloro-L-alanine, 4-aza-DL-leucine, 5,5,5-trifluoro-DL-leucine, *S*-2-aminoethyl-L-cysteine, L-methionine, DL-methionine-hydroxamate, α-methyl-DL-methionine, L-norleucine, *m*-fluoro-DL-phenylalanine, *p*-fluoro-DL-phenylalanine, β-(2-thienyl)-DL-alanine, β-3-thienyl-DL-alanine, L-2-acetidine-carboxylic acid, thioproline, DL-serine-hydroxamate, DL-β-hydroxynorvaline, DL-7-azatryptophane, DL-5-fluoro-tryptophane, 5-methyl-DL-tryptophane, 3-amino-L-tyrosine, *m*-fluoro-DL-tyrosine, 3-nitro-L-tyrosine, azatyrosine, and fluoracetate.

Measurement of carbon source oxidation. Cells were grown at 37°C in either LB or MOPS-glucose medium to a density of about 5×10^8 to 1×10^9 cells/ml. Cells were harvested on ice, pelleted, and diluted to about 5×10^7 cells/ml in 0.9% NaCl. Samples (150 μl) were transferred to each well of an ES Microplate purchased from Biolog Inc. (Hayward, Calif.). The Biolog Microplate tests the ability of bacteria to oxidize 95 different compounds. Oxidation is measured as transfer of electrons from NADH to a tetrazolium dye, which results in formation of a purple color. After inoculation, plates were incubated at 37°C for 15 to 18 h, and optical density at 620 nm (OD_{620}) was measured by a Titertrek Multiscan MCC/340 reader.

Virulence phenotype assays. *S. flexneri* strains were grown at 37°C. The contact hemolytic assay (52) was used to measure the production of invasins. Expression of the *mxiC* gene was measured by using a *vir-83*::MudI1734 (*mxiC-lacZ*) operon fusion (34).

Immunoblotting. Bacterial samples were prepared as described previously (16). Bacterial proteins were separated by sodium dodecyl sulfate–12% polyacrylamide gel electrophoresis. Separated proteins were blotted onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, Calif.). Antisera specific to VirF (15) and IpaBCD (43) have been described previously. The ECF Western blotting kit (Amersham Life Science Ltd., Little Chalfont, Buckinghamshire, England) was used for detection of primary antibodies. The PVDF membrane was scanned with a Storm 860 optical scanner and quantified with ImageQuant software (Molecular Dynamics).

Statistical analysis. A *t* test of the means with two tails was used to evaluate whether different parameters were statistically different for the wild type and the respective mutant ($P < 0.05$; see Tables 3 to 5 and Fig. 2, 3, 4A, and 5), except when measuring carbon source oxidation (see Table 6), where parameters to determine differences in values were set arbitrarily.

RESULTS

Construction of strains containing the *trmH*::Km^r, *trmA5*, *truB2422*::mini-Tn*10*Cm mutations and their combinations. To determine the influence of Gm18, m^5U54, and Ψ55 on the physiology of *E. coli*, we transferred one or more of the *trmH*::Km^r, *trmA5*, and *truB2422*::mini-Tn*10*Cm mutations into different genetic backgrounds by P1 transduction. Since the *trmA* gene is essential (44), we used the *trmA5* allele, which abolishes the synthesis of m^5U54 (3). Therefore, to address the function of the methyl group of m^5U, the *trmA5* allele is suitable. We showed by TLC and HPLC analyses that the levels of Gm18, m^5U54, and Ψ55 were not detectable in mutants containing the respective mutations (data not shown).

The growth rate is decreased in an *E. coli* mutant lacking both Gm18 and Ψ55. Strains MW100 (wild type), GOB113 (*truB2422*::mini-Tn*10*Cm), GRB1882 (*trmA5*), GRB1756 (*trmH*::Km^r), GRB1737 (*truB2422*::mini-Tn*10*Cm *trmH*::Km^r), GRB1887 (*truB2422*::mini-Tn*10*Cm *trmA5*), GRB1814 (*trmH*::Km^r *trmA5*), and GRB1777 (*truB2422*::mini-Tn*10*Cm *trmH*::Km^r *trmA5*) were grown under steady-state conditions in rich-MOPS or MOPS-glucose medium at 30, 37, or 42°C (rich-MOPS) or at 40°C (MOPS-glucose). The *trmA* single mutation did not reduce the growth rate at any of the conditions tested,

TABLE 1. Bacterial strains used

Strain	Relevant characteristic(s)	Source or reference
E. coli		
MW100	Hfr P4X	M. Wikström
GOB113	Hfr P4X *sdr*$^+$ *truB2422*::mini-Tn*10*Cm; donor for P1-mediated transduction	7
G11-5-18	*ilvA trmA5;* donor for P1-mediated transduction	L. Isaksson
GRB1882	Hfr P4X *trmA5*	This work
CF4666	Δ*trmH*::*kan* (Δ*spoU3*::*kan*); donor for P1-mediated transduction	M. Cashel
CAG 12185	*argE86*::Tn*10;* donor for P1-mediated transduction.	C. Gross
GRB1756	Hfr P4X Δ*trmH*::*kan*	This work
GRB1737	Hfr P4X *truB2422*::mini-Tn*10*Cm Δ*trmH*::*kan*	This work
GRB1887	Hfr P4X *truB2422*::mini-Tn*10*Cm *trmA5*	This work
GRB1814	Hfr P4X Δ*trmH*::*kan trmA5*	This work
GRB1777	Hfr P4X *truB2422*::mini-Tn*10*Cm *trmH*::Km *trmA5*	This work
GOB083	Hfr P4X *sdr-43 truB2422*::mini-Tn*10*Cm	7
GT527	MC4100 with $\Phi(trhA_1\text{-}lacZ)_{hyb2\text{-}1}$	51
GRB1536	MC4100 with $\Phi(trhA_1\text{-}lacZ)_{hyb2\text{-}1}$ *truB2422*::mini-Tn*10*Cm	This work
GBEC384 (-CSH41)	Δ(*lac-pro*) *galE*	Cold Spring Harbor Laboratory
GRB1490	Δ(*lac-pro*) *galE truB2422*::mini-Tn*10*Cm	This work
GRB1886	Δ(*lac-pro*) *galE trmA5*	This work
GRB1739	Δ(*lac-pro*) *galE* Δ*trmH*::*kan*	This work
GRB1741	Δ(*lac-pro*) *galE truB2422*::mini-Tn*10*Cm Δ*trmH*::*kan*	This work
GRB 1890	Δ(*lac-pro*) *galE truB2422*::mini-Tn*10*Cm *trmA5*	This work
GRB1817	Δ(*lac-pro*) *galE* Δ*trmH*::*kan trmA5*	This work
GRB1780	Δ(*lac-pro*) *galE truB2422*::mini-Tn*10*Cm Δ*trmH*::*kan trmA5*	This work
GRB1655	Δ(*lac-pro*) *galE sdr-43 truB2422*::mini-Tn*10*Cm	This work
UB585	*argE*(UAG) *ara* Δ(*lac-pro*) *gyrA rpoB tni tyrT* (*supF*) *metB*	L. Isaksson
GRB1734	*argE*(UAG) *ara* Δ(*lac-pro*) *gyrA rpoB tni tyrT* (*supF*) *metB truB2422*::mini-Tn*10*Cm	This work
GRB1743	*argE*(UAG) *ara* Δ(*lac-pro*) *gyrA rpoB tni tyrT* (*supF*) *metB trmA5*	This work
GRB1893	*argE*(UAG) *ara* Δ(*lac-pro*) *gyrA rpoB tni tyrT* (*supF*) *metB* Δ*trmH*::*kan*	This work
GRB1894	*argE*(UAG) *ara* Δ(*lac-pro*) *gyrA rpoB tni tyrT* (*supF*) *metB truB2422*::mini-Tn*10*Cm *trmA5*	This work
GRB1754	*argE*(UAG) *ara* Δ(*lac-pro*) *gyrA rpoB tni tyrT* (*supF*) *metB truB2422*::mini-Tn*10*Cm Δ*trmH*::*kan*	This work
GBR1819	*argE*(UAG) *ara* Δ(*lac-pro*) *gyrA rpoB tni tyrT* (*supF*) *metB* Δ*trmH*::*kan trmA5*	This work
GBR1783	*argE*(UAG) *ara* Δ(*lac-pro*) *gyrA rpoB tni tyrT* (*supF*) *metB truB2422*::mini-Tn*10*Cm Δ*trmH*::*kan trmA5*	This work
S. flexneri		
2457T	2a, *truB*$^+$	17
GBOB8	2a, *truB2422*::mini-Tn*10*Cm	This work
BS184	2a, *vir-83*::MudI1734 (*mxi-lacZ*)	34
GBOB4	2a, *vir-83*::MudI1734 (*mxi-lacZ*) *truB2422*::mini-Tn*10*Cm	This work
YSH6200	Avirulent 2a strain	53
P. aeruginosa		
PAO1	Prototroph, *chl-3*	50
Tn5T1	*orp* (*truB*)::Tn5Tc	50

consistent with earlier results (4), whereas the *truB* and *trmH* mutations reduced the growth rate in rich-MOPS (Table 3). In glucose minimal medium, only the *trmH* mutation reduced the growth rate and then only at 40°C. The *trmH truB* and the *trmH trmA* double mutants showed a reduced growth rate in the rich medium that was similar to that of the *trmH* single mutant but not to that of the *trmH truB* double mutant at 37°C (Table 3). In MOPS-glucose medium the *truB* mutation augmented at all temperatures the reduction of growth rate observed for the single *trmH* mutant (Table 3; 30, 37, and 40°C). In this medium the *trmA* mutation also augmented the *trmH* effect, but only at 30°C. The growth rate of the *trmH truB trmA* triple mutant was in general similar to that of the *trmH truB* double mutant in both rich and minimal media. We conclude that the parallel lack of Gm18 and Ψ55 in the tRNA reduced the growth rate, consistent with the suggestion that the presence of these modifications stabilizes the tRNA structure. However, lack of m^5U54 had no, or only a minor, influence on the growth rates of strains lacking the other two modifications.

Ψ55, but not m^5U54, improves the reading of the CGA codon. To measure the efficiency of translation of arginine CGA and CGU codons, we introduced plasmids containing nine CGA or CGU codons in a row (9) into the wild-type strain, the *trmA* and *truB* single mutants, and the *trmA truB* double mutant. Since Gm18 is not present in tRNAs reading these codons, the plasmids were not introduced into the *trmH* mutant. The nine CGA or CGU codons are translationally coupled to the *lacZ* gene such that the ribosomes translate the CGA- or CGU-containing cistrons, terminate, and reinitiate at *lacZ*. Therefore, β-galactosidase activity is a measure of the

TABLE 2. Plasmids used

Plasmid	Relevant characteristic(s)	Source or reference
pCGA9	Contains nine consecutive CGA codons translationally coupled to *lacZ*	11
pCGU9	Contains nine consecutive CGU codons translationally coupled to *lacZ*	11
pCPF4	Contains UUU-UAU frameshifting window fused to *lacZ*	18
pCPF7	Contains UUU-AAU frameshifting window fused to *lacZ*	18
pCPF8	Contains UUU-CAU frameshifting window fused to *lacZ*	18
pTHF32	Contains CCC-AAA frameshifting window fused to *lacZ*	58
pTHF33	Contains CCC-AAG frameshifting window fused to *lacZ*	58
pJC27tet CUU-UAU	Contains CUU-UAU frameshifting window fused to *lacZ*	58
pJC27tet CUU-UAC	Contains CUU-UAC frameshifting window fused to *lacZ*	58
pJC27tet UAU-UAG	Contains UAU-UAG frameshifting window fused to *lacZ*	58
pJC27tet UAC-UAG	Contains UAC-UAG frameshifting window fused to *lacZ*	58
F′ am117	F′ Δ-14 *lacI* am117 *proA*$^+$B$^+$	36
F′ am121	F′ Δ-14 *lacI* am121 *proA*$^+$B$^+$	36
pTrc99A	Plasmid vector	Pharmacia
pTrc99A-truB	Contains the *truB*$^+$ gene	23
pTrc99A-truBD48C	Contains the *truB*D48C gene	23

rate with which the ribosome translates these nine codons in a row. The more efficiently the codons upstream of the initiation site of the *lacZ* mRNA are translated, the more ribosomes will initiate *lacZ* mRNA and thus the more β-galactosidase that is synthesized. The *truB* mutation reduced the translation of the $(CGA)_9$ codons 3.4-fold, whereas the *trmA* mutation had no effect on the translation of these codons (Fig. 2A). The effect mediated by the *truB* mutation was not influenced by the *trmA* mutation. However, there was no influence on the translation of the $(CGU)_9$ codons by any of the mutations (Fig. 2B). Strain GRB1655 [DUP(*truB*$^+$)(*truB*)] contains both the *truB*$^+$ allele and the *truB* mutant allele in a chromosomal duplication (6). Accordingly, this strain contains Ψ55 in its tRNA, as shown by TLC analysis (data not shown). The level of β-galactosidase in this strain was the same as that observed in wild-type cells (Fig. 2A, *truB*$^+$ *truB*). We conclude that presence of Ψ55 in tRNA (or the TruB protein) improves the translation of CGA codons but not that of CGU codons, whereas m^{5}U54 does not influence the reading of these codons.

Ψ55 does not affect expression of the *thr* operon. The expression of the threonine (*thrABC*) operon of *E. coli* is regulated by an attenuator located upstream of the first gene, *thrA* (51). The rate with which the ribosomes traverse through four Ile and eight Thr control codons in the leader mRNA regulates the expression of the *thrABC* operon (32). The *truB* mutation was introduced into a strain containing a *thrA-lacZ* transcription fusion in the *thrA* gene. Thus, the activity of β-galactosidase reflects the level of transcription of the *thr* operon (51). The fact that the levels of expression of β-galactosidase in strains GRB1212 (wild type) and GRB1536 (*truB*) grown in either rich-MOPS or MOPS-glucose minimal medium were the same suggests that Ψ55 does not influence translation of the Ile and/or Thr codons (data not shown).

A-site selection of Gm18-deficient $tRNA^{Tyr}_{QUA}$ is decreased. The rate of selection of aminoacyl-tRNA in the A site of the ribosome during translation influences frameshifting by the P-site tRNA (11). To study the importance of Ψ55, m^{5}U54, and Gm18 on A-site selection, we used several assay systems containing a short frameshifting window: the nonprogrammed +1 frameshifting in the *argI* gene at a UUU-U/CAU site (18), the $tRNA^{Pro}$-induced +1 frameshifting system at CCC-NNN sites (24), and the *prfB* gene-based +1 frameshifting at CUU-NNN sites (11). In these assay systems, the first three letters denote a codon in the P site and next three letters denote a codon in the A site. The *lacZ* gene is placed downstream of the frameshifting window such that the β-galactosidase activity is a direct measure of the frequency with which the ribosome shifts frame within this window.

TABLE 3. Growth rate determination for the *truB, trmA,* and *trmH* mutants, lacking the Ψ55, m^{5}U54, and Gm18 tRNA modifications

Strain	Genotype	Growth rate (% difference from wt)[a] in indicated medium at:					
		30°C		37°C		42°C, rich-MOPS	40°C[b], MOPS-glucose
		Rich-MOPS	MOPS-glucose	Rich-MOPS	MOPS-glucose		
MW100	wt	0 (1.04 ± 0.01)	0 (0.62 ± 0.05)	0 (1.44 ± 0.06)	0 (0.81 ± 0.03)	0 (1.35 ± 0.04)	0 (0.70 ± 0.02)
GOB113	*truB*	−6	0	−7	0	−17	0
GRB1882	*trmA*	0	0	0	0	0	0
GRB1756	*trmH*	−29	0	−33	0	−27	−11
GRB1737	*trmH truB*	−27	−15	−9	−33	−30	−46
GRB1814	*trmH trmA*	−27	−16	−35	0	−33	−9
GRB1887	*truB trmA*	0	0	−18	0	−7	0
GRB1777	*trmH truB trmA*	−29	−19	−17	−33	−37	−44

[a] The results presented are averages from three independent experiments. Specific growth constant values (in parentheses; averages ± standard deviations) are presented only for wild-type (wt) bacteria.

[b] In MOPS-glucose medium, the growth rate of the wild-type strain was reduced more at 40°C than it was in rich-MOPS at 42°C (cf. rich-MOPS at 37 and 42°C with MOPS-glucose at 37 and 40°C); this is why 40°C was chosen instead of 42°C for MOPS-glucose medium.

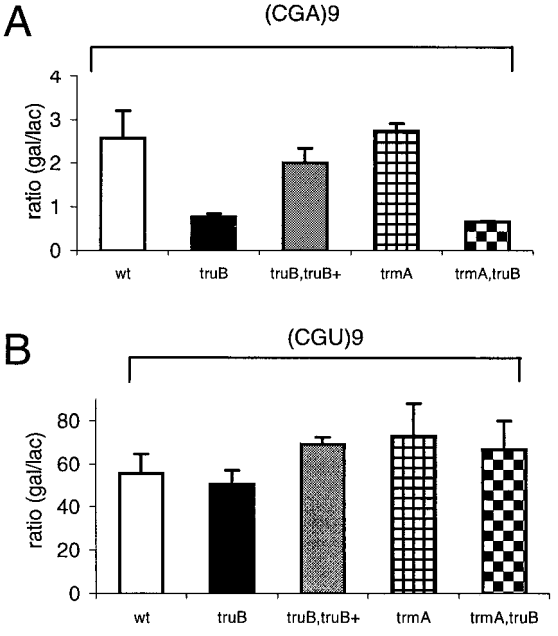

FIG. 2. Effect of the *truB* and *trmA* mutations on translation of the nine consecutive CGA (A) and CGU (B) codons. The results presented are averages from three independent experiments and are expressed as ratios of the β-galactosidase (gal) to β-lactamase (lac) enzyme activities. Variations are standard deviations. wt, wild type.

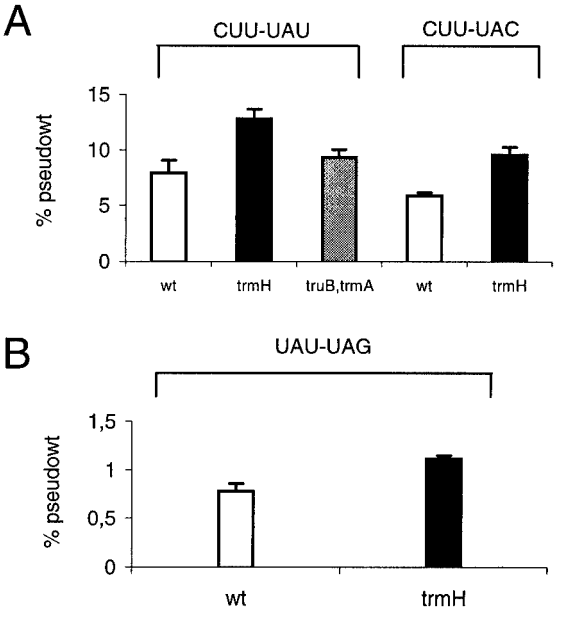

FIG. 3. (A) $tRNA^{Tyr}_{QUA}$ selection in the wild-type (wt) and *trmH* and *truB trmA* mutants, lacking Gm18 (*trmH*) or Ψ55 and m^5U54 (*truB trmA*) tRNA modifications. The results are expressed as the activities of the β-galactosidase produced from the test plasmids pJC27tet CUU-UAU and -UAC compared to that from pseudo-wild-type plasmid pJC27tet. (B) Influence on P-site slippage by Gm18-deficient $tRNA^{Tyr}_{QUA}$. The results are expressed as the activities of the β-galactosidase produced from the test plasmid pJC27tet CUU-UAU compared to that from pseudo-wild-type plasmid pJC27tet. The results (A and B) are averages from three independent experiments, and variations are standard deviations.

Plasmids pCPF4 (UUU-UAU), pCFP7 (UUU-AAU), pCFP8 (UUU-CAU), pTHF32 (CCC-AAA), and pTHF33 (CCC-AAG) were introduced into strains GBEC384 (wild type) and GRB1490 (*truB*). There was no difference in the level of frameshifting between the wild type and the *truB* mutant at any of these sites tested (data not shown). The $tRNA^{Tyr}_{QUA}$, which reads codon UAU/C, contains the Ψ55, m^5U54, and Gm18 modifications. We introduced plasmids pJC27tet CUU-UAU and CUU-UAC into strains lacking one, two, or all three of the modifications. Frameshifting was increased by 60% at the CUU-UAU and -UAC sites in the single *trmH* mutant. In the *truB trmA* double mutant frameshifting was increased by 20% at the CUU-UAU site (Fig. 3A), whereas no difference at the CUU-UAC site was observed (data not shown). No difference in the level of frameshifting between the wild-type strain and the other mutants tested (*trmA*, *truB*, *trmA trmH*, *truB trmH*, and *trmA truB trmH* mutants) was observed at either of these two sites (data not shown).

P-site slippage of Gm18-deficient $tRNA^{Tyr}_{QUA}$ is increased. Recently, we have shown that undermodification of tRNA leads to an increased slippage in the P site of the ribosome, resulting in an elevated level of frameshifting (58). To measure the P-site effect, a stop codon was placed just downstream of the P-site codon (8). Since the release factor acts at the A site (55) and since the *lacZ* gene is placed in the +1 frame downstream of this site, β-galactosidase activity is a measure of a P-site event. We introduced plasmids pJC27tet UAU-UAG and UAC-UAG into strains lacking either Gm18, m^5U54, or Ψ55, any two of them, or all three. A lack of Gm18 increased the level of frameshifting by 40% at the UAU-UAG site (Fig. 3B), whereas no difference in frameshifting level compared to the wild-type strain was observed for any of the other mutant strains (*trmA*, *truB*, *truB trmA*, *trmA trmH*, *truB trmH*, *trmA truB trmH* mutants; data not shown).

The Gm18-deficient *tyrT* (*supF*) tRNA reads inefficiently stop codon UAG. The amber (UAG) *supF* suppressor $tRNA^{Tyr}_{CUA}$, which is a mutated $tRNA^{Tyr}_{QUA}$, contains Ψ55, m^5U54, and Gm18 modifications. We measured the suppressor activity of this tRNA in various mutants lacking one to three of these modifications. For this purpose, we introduced two different F′ plasmids harboring an in-frame *lacI′-′lacZ* fusion with or without amber nonsense codons in the *lacI* part (36) into strains lacking one to three of these modifications. The suppression efficiency was measured at three different temperatures, since we suspected that the tRNA modifications tested could have different effects at different temperatures. Lack of Gm18 resulted in a reduced efficiency of the *supF* $tRNA^{Tyr}_{CUA}$ in reading the *lacI* am117 codon at 37°C and the *lacI* am121 codon at 30 and 37°C but not at 42°C (Table 4). Also, decreased suppression was observed in the mutant lacking both Gm18 and m^5U54. These results suggest that the major contributor to the reduced efficiency was the lack of Gm18. Increased suppression was observed when Gm18 and Ψ55 (*lacI* am121 at 42°C) or Ψ55 and m^5U54 (*lacI* am121 at 37°C) were lacking. Thus, of these three modifications, only the Gm18 modification alone influenced the efficiency of the *supF* $tRNA^{Tyr}_{CUA}$, whereas a lack of two modifications both increased and decreased efficiency.

TABLE 4. Suppression ability of *tyrT* (*supF*) tRNA in the *truB, trmA,* and *trmH* mutants, lacking the Ψ55, m^5U54, and Gm18 tRNA modifications

Strain	Genotype	Relative activity (%)[a] with mutation in indicated *lacI* codon at:					
		30°C		37°C		42°C	
		am117	am121	am117	am121	am117	am121
UB585	wt	14.0 ± 0.6	45.0 ± 3.5	11.7 ± 1.5	62.4 ± 2.5	2.7 ± 0.5	19.4 ± 1.1
GRB1734	*truB*	*	*	*	*	*	*
GRB1743	*trmA*	*	*	*	*	*	*
GRB1893	*trmH*	*	35.1 ± 0.6	8.2 ± 1.4	50.1 ± 2.7	*	*
GRB1754	*trmH truB*	*	*	*	*	*	34.0 ± 0.7
GRB1894	*truB trmA*	*	*	*	84.3 ± 7.5	*	*
GRB1819	*trmH trmA*	11.8 ± 0.6	*	8.2 ± 0.2	51.2 ± 5.3	*	*
GRB1783	*trmH truB trmA*	*	*	*	*	*	*

[a] The results are averages from three independent experiments ± standard deviations. The results are expressed as the activity of β-galactosidase expressed from the F′*lacZ* amber mutants relative to that of β-galactosidase expressed from the F′*lacZ*$^+$ plasmid. *, no difference in the efficiency of suppression between the wild type (wt) strain and the respective mutants.

Surprisingly, lack of all three modifications did not influence the efficiency of suppression at either *lacI* am117 or *lacI* am121 codons at any temperature. Although the reason for the lack of effect in the triple mutant is not fully understood, the fact that we observed both an increase and a decrease in the efficiency of suppression caused by modification deficiency suggests that at certain conditions the effect of one modification can counteract the effect of another modification.

Lack of both Ψ55 and Gm18 causes an increased sensitivity to an amino acid analog, whereas lack of Ψ55 is the major cause for increased oxidation of some carbon sources. It is known that undermodified tRNA influences the regulation of the synthesis of several amino acids (5, 22, 56, 61). We tested 31 different amino acid analogs for the growth response of the *trmH trmA5 truB* triple mutant (strain GRB1777). One of these analogs, 3,4-dehydro-DL-proline, to which the triple mutant was sensitive, was then tested on strains having one or two mutations. The inhibition zones for the triple mutant and the *trmH truB* double mutant were increased by 25% compared to that of the wild-type strain (Table 5), suggesting that the lack of both Gm18 and Φ55 in the tRNA influences the metabolism of the 3,4-dehydro-DL-proline in the cell.

Levels of oxidation of various carbon compounds by the wild-type and mutants lacking Ψ55, m^5U54, and/or Gm18 were compared. When cells were grown in the rich LB medium, all mutants, except the *trmH* and the *trmA trmA* mutants, oxidized DL-malic acid more efficiently than the wild-type cells (Table 6). The triple mutant and the *truB trmH* and *truB trmA* double mutants oxidized the three variants of malic acids and monomethylsuccinate more efficiently than the wild type. In general mutants pregrown in MOPS-glucose medium oxidized these compounds similarly to the wild-type cell. Apparently, the lack of Ψ55 is the major cause of the observed increased oxidation ability when cells were pregrown in LB medium.

TABLE 5. Sensitivity of the *truB, trmA,* and *trmH* mutants, lacking the Ψ55, m^5U54, and Gm18 modifications, to 3,4-dehydro-DL-proline[a]

Strain	Genotype	Inhibition zone diam (mm)
MW100	wt	30.5 ± 0.7
GOB113	*truB*	33.5 ± 0.7
GRB1882	*trmA*	32.0 ± 1.4
GRB1756	*trmH*	31.5 ± 4.9
GRB1737	*trmH truB*	**39.0 ± 1.4**
GRB1887	*truB trmA*	34.0 ± 4.2
GRB1814	*trmH trmA*	32.5 ± 3.5
GRB1777	*trmH truB trmA*	**37.0 ± 0.0**

[a] Fifty micrograms of 3,4-dehydro-DL-proline was placed on a 6-mm-diameter paper disk in the center of each agar plate. Diameters of zones without growth of bacteria were scored after 24 h of growth at 37°C. Average values of two experiments ± standard deviations are presented. Cases of significant difference between the wild type (wt) and mutant are in boldface.

A *truB* mutation reduces the expression of some virulence-associated genes of *S. flexneri*. The virulence of *Shigella* depends on the activity of the VirF protein, which belongs to the AraC family of transcription factors. The VirF protein activates directly the synthesis of two other transcription factors, VirG and VirB. In turn, VirB activates the *ipa* operon, which encodes invasines IpaB, -C, and -D, and the *mxi* operon. The hemolytic activity of the *truB* mutant was reduced about 30% compared with that of *S. flexneri* wild-type strain 2457T, although the level of VirF was the same (Fig. 4A and B). Of the three proteins, IpaB, -C, and -D, encoded in the *ipa* operon, only the level of IpaB was reduced (Fig. 4C). Since the vector influences hemolytic activity in the wild-type cells, we were unable to do a proper complementation experiment. To monitor a possible translational effect upstream of the regulation of the *mxi* operon, we used an *mxiC-lacZ* transcriptional fusion. Indeed, the *truB* mutation reduced the expression of the *mxi* operon by 25% in MOPS-glucose medium (Fig. 5), but not when cells were grown in rich-MOPS (data not shown). The decreased expression of the *mxi* operon in the *truB* mutant was restored by a plasmid containing the coding sequence for wild-type TruB but not by a plasmid containing the coding sequence for the TruBD48C mutant protein, which is able to bind to the tRNA but not to modify it (47). These results indicate that it was the lack of Ψ55 in tRNA rather than the absence of the TruB protein which was responsible for the reduced transcription of the *mxi* operon. In summary, our results suggest that Ψ55 in tRNA improves the translation of the IpaB protein and a protein(s) required for efficient expression of the *mxi* operon.

The *orp* gene in *P. aeruginosa* is homologous to the *truB* gene of *E. coli*. An *orp*::Tn*5*Tc mutant version of opportunistic human pathogen *P. aeruginosa* is impaired in growth on BHI plates at 43°C and has reduced amounts of virulence factor

TABLE 6. Carbon source utilization in the *truB, trmA,* and *trmH* mutants, lacking the Ψ55, m^{5}U54, and Gm18 modifications[a]

Strain	Genotype	OD_{620} for oxidation in indicated medium of:							
		DL-Malic acid		D-Malic acid		L-Malic acid		Monomethyl succinate	
		LB	MOPS-Glu	LB	MOPS-Glu	LB	MOPS-Glu	LB	MOPS-Glu
MW100	wt	0.02	0.01	0.00	*	0.02	0.02	0.01	*
GOB113	*truB*	0.16	*	*	*	*	*	*	*
GRB1882	*trmA*	0.12	*	*	*	*	*	*	*
GRB1756	*trmH*	*	*	*	*	*	*	*	*
GRB1737	*trmH truB*	0.15	0.06	0.11	*	0.09	*	0.14	*
GRB1887	*truB trmA*	0.27	0.16	0.31	*	0.15	*	0.23	*
GRB1814	*trmH trmA*	*	*	*	*	*	*	0.10	*
GRB1777	*trmH truB trmA*	0.17	*	0.15	*	0.11	0.12	0.17	*

[a] The carbon sources shown are those that are oxidized inefficiently by the wild-type (wt) strain ($OD_{620} < 0.03$) and that are oxidized more efficiently ($OD_{620} > 0.05$) by the mutant in respective experiment. The results are averages of two separate experiments. The actual OD_{620} values are shown when a difference between the wild-type strain and the respective mutant was observed. *, no efficient oxidation in wild type and mutants. Glu, glucose.

phospholipase C compared to wild-type strain PAO1 (50). The predicted *orp* gene product possesses sequence similarity to the *truB* gene product of *E. coli*. Therefore, we tested whether the enzyme extracts from *P. aeruginosa* strains PAO1 (*orp*$^+$) and Tn*5*T1 (*orp*::Tn*5*Tc) have a Ψ55-modifying activity. The extract from the *orp*$^+$ strain was able to catalyze the synthesis of Ψ55 in vitro, whereas the extract from the *orp*::Tn*5*Tc mutant was not (Fig. 6). These results show that the *orp* gene is most likely the homologue of the *truB* gene of *E. coli*, and therefore the *orp*::Tn*5*Tc mutant should lack Ψ55 in its tRNA. Therefore, we suggest that the *orp* gene in *P. aeruginosa* be renamed *truB*.

DISCUSSION

In this work we show that a parallel lack of Gm18, m^{5}U54, and Ψ55 in tRNA of *E. coli* affects growth rate, translation of certain codons, sensitivity to amino acid analogs, and oxidation of some carbon compounds. Since nucleosides Gm18, m^{5}U54, and Ψ55 are located close to each other in the three-dimensional structure of tRNA and thereby stabilize it (12, 30, 46, 59), lack of these modifications could destabilize the tRNA. If so, one might see an effect on the physiology of *E. coli*. Indeed, a strain lacking all three of these modifications showed reduced growth rate in both rich-MOPS and MOPS-glucose minimal medium at all temperatures (Table 3). The major contributor to the growth rate reduction in rich-MOPS was the lack of Gm18. This was surprising since it was previously demonstrated that lack of Gm18, as in the *trmH* mutant, has no effect on the growth rate in the rich LB medium (45). We also did not find any growth rate difference between our wild-type strain MW100 and its *trmH* derivative when growth in LB medium was monitored (data not shown). Thus, the observed growth rate reduction caused by lack of Gm18 is medium dependent and not the result of differences in strain backgrounds among the different experiments. No growth rate difference between wild-type strain MW100 and its *trmH* derivative was observed for MOPS-glucose minimal medium, except at 40°C, confirming that the observed growth rate difference is medium dependent. Previously, it was reported that *truB*$^+$ and *truB* mutants grow with equal rates in LB and M-9 glucose minimal media (23), whereas we observed a minor growth rate reduction in rich-MOPS caused by the *truB* mutation (Table 3). Possibly, differences between the media used or differences in strain backgrounds might explain the different results. In some cases, when the lack of one of the Gm18, m^{5}U54, and Ψ55 modifications did not have any effect on the growth rate, a lack of two of the modifications resulted in a reduced growth rate, suggesting a cooperative effect by these modifications. These results suggest that small conformational changes in the tRNA may lead to changed interaction with rRNA (62). Absence of Gm18, m^{5}U54, and Ψ55 had no or only small effects on A-site-mediated frameshifting at codons UAU and UAC (Tyr), AUA (Asn), CAU (His), and Lys (AAA and AAG); peptidyl-tRNA slippage at codons UAU and UAC; and reading of the UAG stop codon by the *tyrT* tRNA. The observed effects can be explained by the effect of tRNA modification(s) on the stability of tRNA. We find such an explanation to our results unlikely, since single mutations in *Saccharomyces cerevisiae* resulting in Gm18, m^{5}U54, or Ψ55 deficiency have no major effect on the stability of $tRNA^{Ser}_{CGA}$ (25). An alternative explanation may be that the lack of modification(s) cause a small conformational change of the tRNA, which in turn could affect its interaction with rRNA and could result in decreased efficiency of translation (62). This can also explain both the increased and decreased reading of the UAG stop codon by the *tyrT* tRNA lacking Gm18, m^{5}U54, and/or Ψ55 (Table 4). Since the observed effects were small, we placed several copies of the same codon in a row to enhance the effect on translation by lack of these modifications. Indeed, the absence of Ψ55, but not of m^{5}U54, caused decreased efficiency in the translation of nine consecutive arginine CGA codons but not of nine consecutive arginine CGU codons (Fig. 2). Both CGA and CGU are read by the same $tRNA^{Arg}_{ICG}$, but CGA is decoded inefficiently due to the poor I34:A(III) [I34, I in position 34 (wobble position, first position of the anticodon) of the tRNA; A(III), third nucleoside of the codon] base pairing, whereas CGU uses the more efficient I34:U(III) base pairing (9). Apparently, the presence of the Ψ55 in $tRNA^{Arg}_{ICG}$ is more important for the poorly decoded CGA than for the more efficiently decoded CGU. It is unlikely that the observed decrease of the β-galactosidase activity is due to frameshifting instead of a decreased efficiency of translation of the nine-codon cistron. Previously, it was demonstrated that the CGA codon is not prone to frameshift or to premature termination by RF2 (9, 10). In addition, we have not observed any effect of Ψ55 on both +1 (see above) and −1 (57) frameshifting. Moreover, frameshifting in either

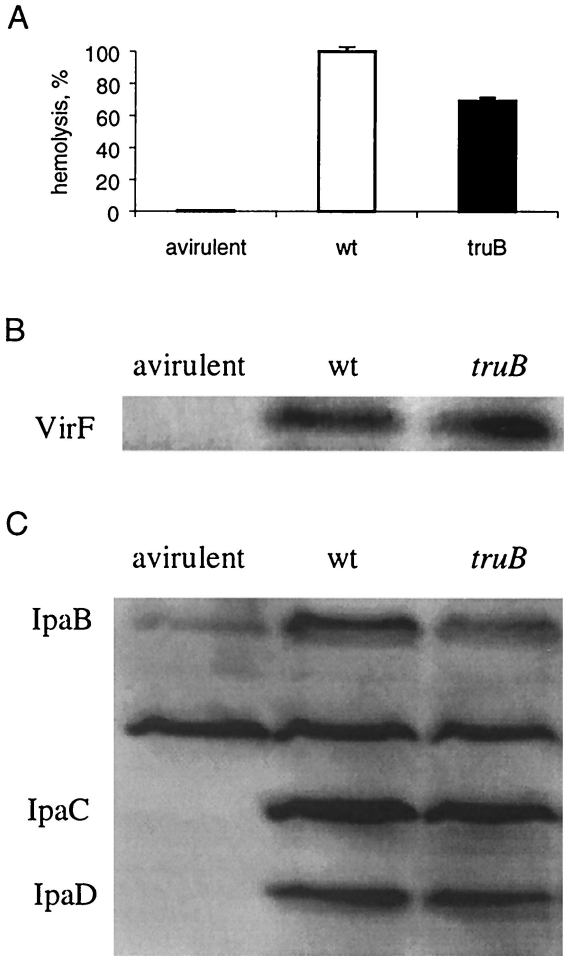

FIG. 4. Effect of the *truB* mutation on the expression of virulence-associated genes of *S. flexneri*. (A) Hemolytic activity of strains 2457T (wild type [wt]) and GBOB8 (*truB*) grown in MOPS-glucose–NA medium at 37°C. The contact hemolytic activity of the 2457T strain was arbitrarily defined as 100%. Each bar shows the mean and standard deviation of four measurements. Avirulent strain YSH6200 was used as a negative control. (B) Western blot of the VirF protein. An identical amount of total protein from each strain grown in MOPS-glucose–NA medium at 37°C was electrophoresed in sodium dodecyl sulfate–12% polyacrylamide gel and transferred to a PVDF membrane before being immunostained with antibodies specific to VirF. The quantification of the VirF-specific bands was performed by fluorescence scanning analysis (Storm), and values for different strains were compared. Lanes: left, YSH6200 (avirulent); middle, 2457T (wild type); right, GBOB8 (*truB*). (C) Western blot of the Ipa proteins. The procedure was the same as that for panel B except that antibodies specific to Ipa proteins were used. Lanes: left, YSH6200 (avirulent); middle, 2457T (wild type); right, GBOB8 (*truB*). The value for the avirulent strain, YSH6200, was subtracted when calculating the amounts of IpaB in the wild-type and *truB* strains.

+1 or −1 directions would create two mismatches in the new frame, including a purine-purine clash in the first position of the codon (CGA to GAC [+1] and CGA to ACG [−1]).

The requirement for Ψ55 for the efficient translation of the poorly decoded CGA codon and for *plcH* mRNA, encoding phospholipase C in *P. aeruginosa* (50), suggests that Ψ55 may also improve translation of the *virF* mRNA in *S. flexneri*. Surprisingly, whereas the expression of the *ipaB* and *mxiC* genes

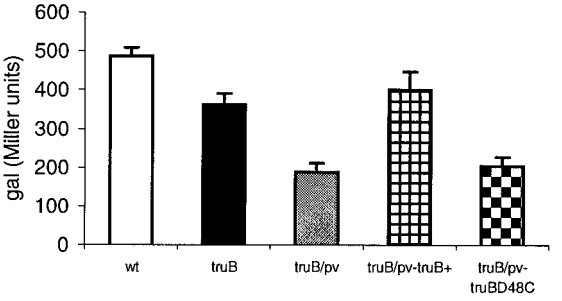

FIG. 5. Expression from the *mxiC-lacZ* transcriptional fusion in *S. flexneri* strains BS184 (wild type [wt]), GBOB4 (*truB*), and GBOB4 (*truB*) with plasmid Trc99A (pv) derivatives expressing the $TruB^{+}$ or TruBD48C protein. The results presented are averages from three independent experiments, and variations are standard deviations. Results are expressed as β-galactosidase (gal) activity.

was reduced in the *truB* mutant, (Fig. 4C and 5), the amount of the VirF protein was the same as that in the wild type (Fig. 4B), which is in contrast to the effect caused by lack of Q34 or ms^2i^6A37 (14, 16). Thus, whereas Q34 and ms^2i^6A37 exert

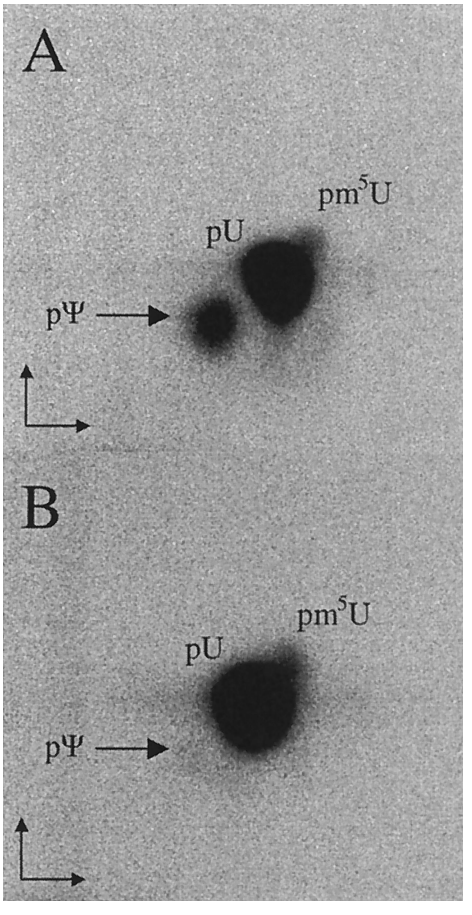

FIG. 6. Formation of Ψ55 in *P. aeruginosa* wild-type (wt) strain PAO1 and an *orp* mutant. In vitro-synthesized $tRNA^{Val}$, labeled by incorporation of [α-^{32}P]UTP, was incubated with the S16 enzyme extract prepared from strain PAO1 (A) and the *orp* mutant (B). After incubation, labeled tRNA was completely digested by nuclease P1 and two-dimensional TLC was performed. Arrows, positions of pΨ55.

their effects on virulence by reducing the translation of the *virF* mRNA, the lack of Ψ55 did not affect the synthesis of the main regulator VirF but affected the expression of VirF-regulated virulence genes *virB* and *ipaB* and/or a protein(s) required for efficient transcription of the *mxi* operon. Lack of Q34 in the *tgt* mutant of *S. flexneri* caused a delayed response in an animal infection model (Serény test) (42). Since the effect of Ψ55 was similar to that of Q34 in a contact hemolytic assay, the *truB* mutant may also influence infection of the host by *S. flexneri*.

It has been shown that various modified nucleosides have different impacts on the activities of various tRNA species; e.g., the lack of m^1G37 in $tRNA^{Leu}_{GAG}$ did not influence A-site selection but the lack of the same modification in $tRNA^{Pro}_{GGG}$ severely reduced the same reaction (31). Similar observations were also noted for Ψ in the anticodon stem (31). Here we show that Ψ55 improved the activity of $tRNA^{Arg}_{ICG}$ (Fig. 2) but not that of $tRNA^{Tyr}_{QUA}$ (Fig. 3, Table 4). Clearly, to clarify the function of some of the modified nucleosides, one will have to monitor the activity of specific tRNA species. Nonetheless, the fact that a reduced growth rate was observed for the triple mutant as well as for some of the double and single mutants demonstrates an important function of these modified nucleosides in the ability of the bacterium to grow and compete efficiently in the environment.

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