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Structural alterations of the tRNA(m^{1}G37)methyltransferase from *Salmonella typhimurium* affect tRNA substrate specificity

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ABSTRACT

In *Salmonella typhimurium*, the tRNA(m^{1}G37)methyltransferase (the product of the *trmD* gene) catalyzes the formation of m^{1}G37, which is present adjacent and 3′ of the anticodon (position 37) in seven tRNA species, two of which are tRNA$^{Pro}_{CGG}$ and tRNA$^{Pro}_{GGG}$. These two tRNA species also exist as +1 frameshift suppressor *sufA6* and *sufB2*, respectively, both having an extra G in the anticodon loop next to and 3′ of m^{1}G37. The wild-type form of the tRNA(m^{1}G37)methyltransferase efficiently methylates these mutant tRNAs. We have characterized one class of mutant forms of the tRNA(m^{1}G37)methyltransferase that does not methylate the *sufA6* tRNA and thereby induce extensive frameshifting resulting in a nonviable cell. Accordingly, pseudorevertants of strains containing such a mutated *trmD* allele in conjunction with the *sufA6* allele had reduced frameshifting activity caused by either a 9-nt duplication in the *sufA6* tRNA or a deletion of its structural gene, or by an increased level of m^{1}G37 in the *sufA6* tRNA. However, the *sufB2* tRNA as well as the wild-type counterparts of these two tRNAs are efficiently methylated by this class of structural altered tRNA(m^{1}G37)methyltransferase. Two other mutations (*trmD3*, *trmD10*) were found to reduce the methylation of all potential tRNA substrates and therefore primarily affect the catalytic activity of the enzyme. We conclude that all mutations except two (*trmD3* and *trmD10*) do not primarily affect the catalytic activity, but rather the substrate specificity of the tRNA, because, unlike the wild-type form of the enzyme, they recognize and methylate the wild-type but not an altered form of a tRNA. Moreover, we show that the TrmD peptide is present in catalytic excess in the cell.

Keywords: 1-methylguanosine; modification; recognition

INTRODUCTION

Transfer RNAs from all organisms contain modified nucleosides (Björk, 1995; Sprinzl et al., 1998) that are derivatives of the four normal nucleosides, adenosine (A), guanosine (G), uridine (U) and cytosine (C). The nucleoside modifications occur after transcription, once the polynucleotide has been formed (Svensson et al., 1963). A few modified nucleosides, for example pseudouridine and 5-methyluridine, are present in all tRNA species in a given organism, whereas most of the modified nucleosides are present in a subset of tRNAs. The enzymes catalyzing the formation of this latter type of modified nucleoside must therefore recognize this subset of tRNAs and disregard all other tRNAs as substrates. As many as 15 modified nucleosides are present in corresponding positions in tRNAs from the three domains—Archaea, Bacteria, and Eucarya (Björk, 1986; Cermakian & Cedergren, 1998). This suggests a common evolutionary origin for these modified nucleosides (Björk, 1986; Cermakian & Cedergren, 1998) and indicates that the tRNA present in the progenitor may have contained these modified nucleosides. One of these conserved modified nucleosides is 1-methylguanosine (m^{1}G), present in position 37 (adjacent to and 3′ of the anticodon) in tRNAs from all organisms' reading codons of the type C(U,C)N and CGG, that is, tRNA-specific for leucine, proline, and arginine (Björk, 1995). The formation of m^{1}G37 is catalyzed by the tRNA(m^{1}G37)methyltransferase, which uses AdoMet as methyl donor. Evidently, this enzyme must both bind the AdoMet and exclusively recognize this subset of tRNAs. The enzymes in the various organisms of today may, therefore, besides having the conserved AdoMet binding motif, also contain conserved features reflecting common recognition determinants. Indeed, the tRNA(m^{1}G37)methyltransferase has stretches of amino acids with high similarity between organisms as evo-

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lutionary far apart as *Salmonella typhimurium* (official designation, *Salmonella enterica* serovar Typhimurium) and *Mycoplasma* (Fig. 1). The function of this enzyme and the way it recognizes the tRNA may therefore be similar among various bacterial species and perhaps even in other organisms.

There have been several reports that address the question of which sequences in the tRNA are determining the specificity and the efficiency of the tRNA modification reaction (see, e.g., Droogmans et al., 1986; Edqvist et al., 1994; Nakanishi et al., 1994; Björk, 1995; Curnow & Garcia, 1995; Grosjean et al., 1996; Gu et al., 1996, 1998; Motorin et al., 1997; Qian & Björk, 1997; Redlak et al., 1997). However, only one report has addressed the question of which features of a tRNA-modifying enzyme are involved in tRNA binding

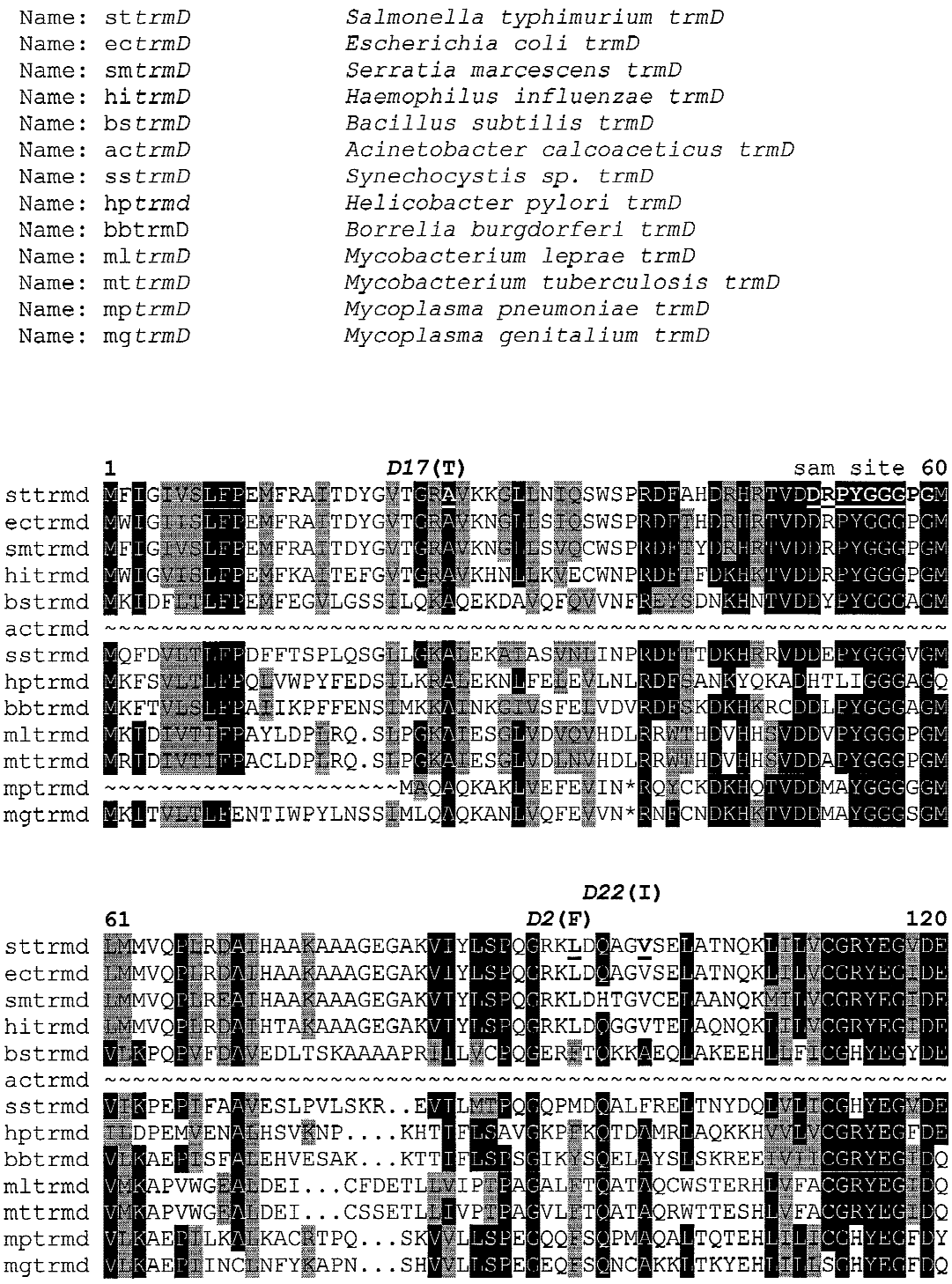


FIGURE 1. (*Legend on facing page.*)

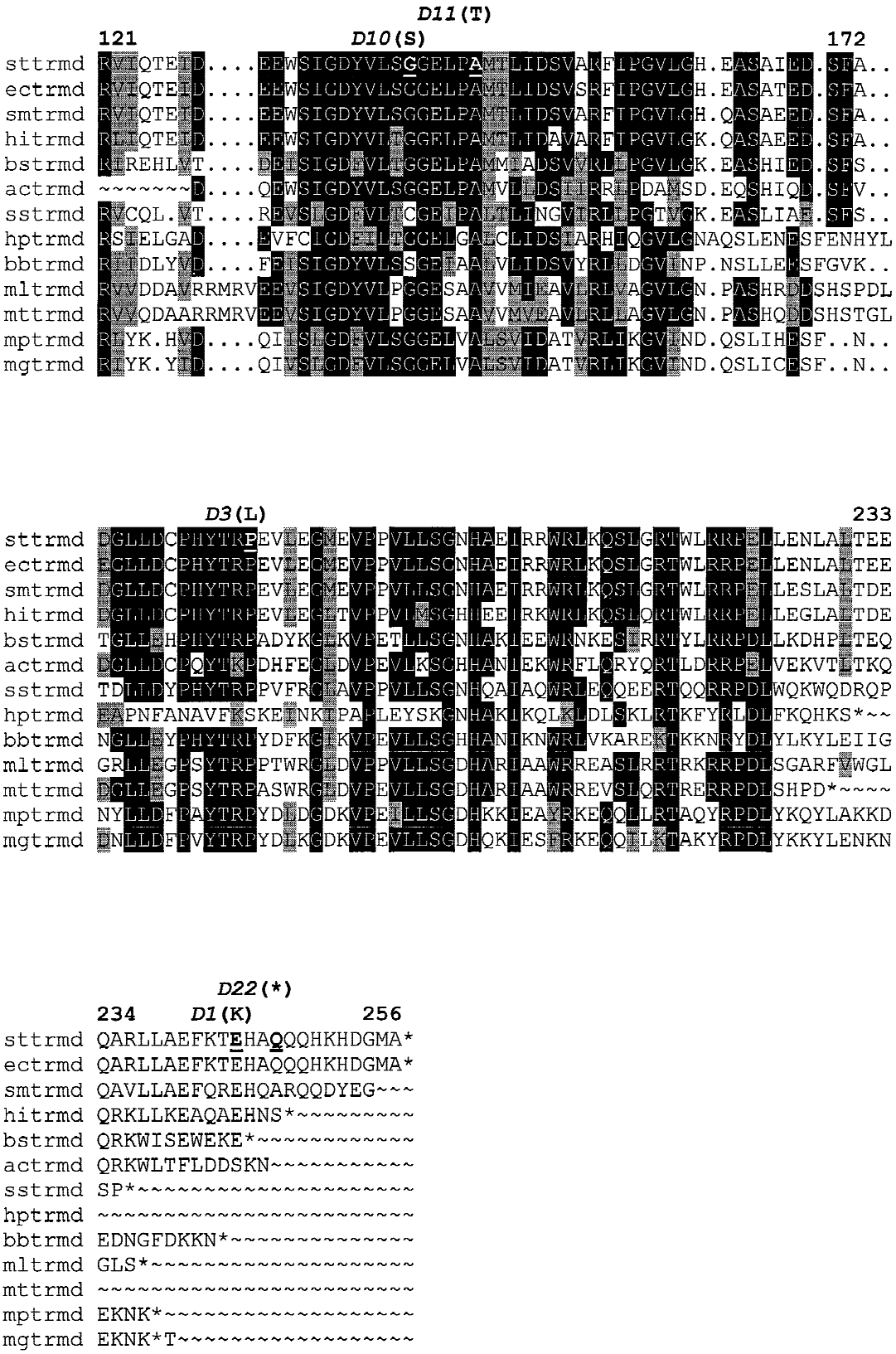


FIGURE 1. Deduced amino acid sequence of the *trmD* gene from *S. typhimurium* and alignment of all the known TrmD sequences obtained from the database using the box and shade program. Numbers above the alignment correlate to amino acid positions in *S. typhimurium* and amino acid substitutions identified in different mutants are underlined. Black shade indicates identical amino acids and gray shade indicate similar amino acids. The stop codons are marked with asterisks. The putative S-adenosyl methionine binding site is also underlined. The *Acinetobacter calcoaceticus trmD* gene is only partially sequenced. Translational starts of the *trmD* genes from the two *Mycoplasma* species are not identified.

(Romier et al., 1996). Although the three-dimensional structure of enzyme-tRNA complex is required to fully understand in detail how a tRNA-modifying enzyme recognizes the tRNA substrate, identification of those amino acids that are influencing tRNA substrate specificity is a first step in such an analysis. Such information will be valuable when the three-dimensional structure is known to identify which surface of the enzyme is engaged in the tRNA recognition. As a first step to identify such amino acids in a tRNA-modifying enzyme, we have characterized mutant forms of the tRNA(m^1G37)methyltransferase that do not methylate a structurally altered tRNA, but are still able to methylate its wild-type counterpart.

RESULTS

Isolation and genetic characterization of *trmD* mutants

sufA6 is a derivative of the major proline $tRNA^{Pro}_{CGG}$ (Riddle & Roth, 1970) and functions as a +1 frameshift suppressor at CCC-U site (CCC is in the zero frame, a +1 frameshift moves the ribosome to the CC-U codon). The wild-type (Kuchino et al., 1984) as well as the *sufA6* tRNA (see below) contain m^1G adjacent to and 3′ of the anticodon. The absence of m^1G37 reduces the rate with which the $tRNA^{Pro}$ isoacceptors are selected at the ribosomal A-site (Li et al., 1997). Therefore, lack of this modified nucleoside may reduce the activity of the frameshifting suppressor tRNA if it is poorly selected. Since m^1G37 also prevents frameshifting (Björk et al., 1989), the lack of it in the *sufA6* derivative of $tRNA^{Pro}_{CGG}$ may also increase the frameshifting activity. Thus, by monitoring the frameshifting activity we may isolate mutations in the *trmD* gene that either reduce or increase the frameshifting activity of the *sufA6* $tRNA^{Pro}_{CGG}$ caused by the lack of m^1G37. We also screened for mutants unable to grow at high temperature on rich medium. Such potential *trmD* mutations were combined with another frameshift suppressor mutation, *sufB2*, which is a G insertion in the anticodon loop of another proline tRNA species, the $tRNA^{Pro}_{GGG}$. The *sufB2* tRNA is also a substrate for the wild-type tRNA(m^1G37)methyltransferase. By comparing the phenotype of these double mutants (*trmD, sufA6* versus *trmD, sufB2*) and the single mutant (*trmD*), we tried to identify structural alterations in the TrmD peptide that mediated different ability to methylate these two mutant forms of $tRNA^{Pro}_{CGG}$ and $tRNA^{Pro}_{GGG}$ as well as their wild-type counterparts.

A stock of phage P22 grown on strain TT126 (*tyrA555::*Tn*10*) was treated with hydroxylamine (Hong & Ames, 1971) to mutagenize the *trmD* gene that is 52% linked to *tyrA* (Björk et al., 1989). Strain TR1457 (*sufA6*, *hisO1242*, *hisD3749*) was used as recipient. The *hisD3749* mutation is a C insertion resulting in the sequence CCC-UGA, which can be suppressed by the +1 frameshift suppressor *sufA6* (CCC is in the zero frame, a +1 frameshift moves the ribosome to the CC-U codon). Among the Tet^R transductants selected, we screened for His^- clones and also tested the growth ability at high temperature on rich medium. All transductants were His^+, indicating that no mutations were obtained that reduced the activity of the *sufA6* $tRNA^{Pro}_{CGG}$ as a +1 frameshift suppressor. However, several mutants were obtained that were unable to grow at high temperature on rich medium. Introduction of various plasmids harboring the $trmD^+$ gene from *Escherichia coli* or the vector control showed that all these mutations were in the *trmD* gene (data not shown; for the *trmD3* mutation and experimental design, see Björk et al., 1989). Based on various phenotypes, these mutations were divided into three classes (Table 1). Only the *trmD3* mutation caused a temperature-sensitive-growth phenotype in the absence of any other mutations (Class III). Furthermore, only this mutation mediated the ability to suppress +1 frameshift mutations at 37 °C (Björk et al., 1989). Mutations *trmD1*, *D2, D17* and *D22* were not temperature sensitive alone or in combination with the *sufB2* allele (Class I), whereas two mutations, *trmD10* and to some degree *trmD11*, induced temperature sensitivity when combined with the *sufB2* allele (Class II).

TABLE 1. Phenotypes induced by the three classes of *trmD* mutations.

Class	*trmD*	*suf*	Growth on rich plate at 42.5 °C[a]	Suppression of *hisD3749* at 37 °C[b]
	0	+	++	−
		A6	++	++
		B2	++	++
I	*D1; D2; D17; D22*	+	++	−
		A6	−	++
		B2	++	++
II	*D10; D11*	+	++	−
		A6	−	++
		B2	+	++
III	*D3*	+	+	+
		A6	−	++
		B2	−	++

[a]Growth of indicated strains were monitored on agar plates following incubation at 42.5 °C for 24 h before the size of the colonies were scored. ++: colonies as large as the colonies formed by the wild-type cell; +: small colonies; and −: no colonies formed. All strains (GT5473, GT5475, GT5477, GT5479, GT2772, GT2774, GT2780, GT2784) contained the *tyrA555::Tn10*, *hisO1242*, and the *hisD3749* mutations and the indicated *trmD* allele.

[b]Frameshift suppressor activity was scored as the ability to suppress the frameshift mutations *hisD3749* or *hisD3018*. ++: growth was observed after 12 h incubation at 37 °C on minimal plate lacking His. +: growth was observed after 24 h incubation under the same condition; −: no growth was observed after 3 days.

Sequence alterations in the *trmD* gene induced by the various mutations

The *trmD* gene from 13 bacterial species, *E. coli*, *S. typhimurium*, *Serratia marcescens*, *Hemophilus influenzae*, *Bacillus subtilis*, *Synechocystis sp.*, *Borrelia burgdorferi*, *Mycobacterium leprae*, *Mycobacterium tuberculosis*, *Mycoplasma genitalium, Mycoplasma pneumoniae*, *Helocobacter pylori*, and *Acinetobacter calcoaceticus* (partly), have been sequenced and are aligned in Figure 1.

Chromosomal fragments carrying the *trmD* gene from the different *trmD* mutants were amplified by PCR and sequenced. Amino acid changes mediated by the mutations are presented in Figure 1. Three class I mutations (*trmD2, 17* and *22*) mediate changes within the first 100 amino acids in the N-terminus of the 255-amino-acid-long TrmD peptide: L94F (*trmD2*); A25T (*trmD1*); and V99I, Q246Stop(*trmD22*). However, the *trmD1* mutation, which phenotypically belonged to the class I mutations, was found to result in a change close to the C-terminus of the enzyme (E243K). This was close to one of the two mutations in the Class I mutant *trmD22*. The *trmD3* mutation (the only Class III obtained) caused an amino acid alteration in position 184 (P184L). The two class II mutations (*trmD10* and *D11*) whose phenotypes are in between the class I and class III mutations had the amino acid substitutions, G140S and A145T, respectively. These mutations were not far from the class III mutation *trmD3* in position 184. Interestingly, most of the mutations obtained induced changes in conserved positions (Fig. 1) but none changed the highly conserved putative AdoMet binding site (three out of seven positions are identical in all species). Any mutation in the AdoMet binding site would most likely affect the methylation reaction but not necessarily the tRNA substrate specificity. This suggests that the mutations characterized affected some important features in the interaction between the TrmD protein and the tRNA, the expression, or the stability of the enzyme.

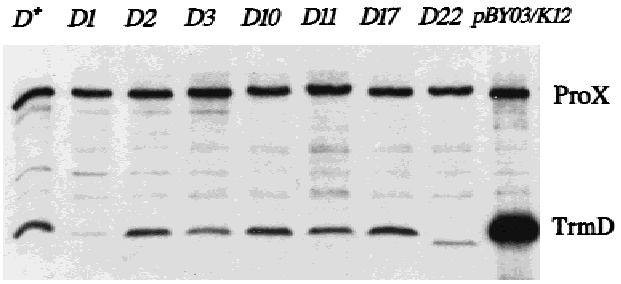
Effects of the *trmD* mutations on the level and the activity of the TrmD protein

Introduction of various mutations in the *trmD* gene may influence the synthesis, stability, or the catalytic activity of the TrmD protein. Changes in these parameters may result in m^1G-deficient tRNAs. Therefore, we measured the level of m^1G in total tRNA by high performance liquid chromatography (HPLC), and in specific tRNA species by thin-layer chromatography. Moreover, we monitored by Western blot analysis the steady-state level of the TrmD protein. As shown in Figure 2 and Table 2 all but one (*trmD10*) mutation reduced the level of the TrmD peptide to various degrees. The reduction was more pronounced at 42 °C compared to 37 °C. Among the mutations tested, *trmD1* and *trmD22* reduced the steady-state level of the respective protein by more than 50% at 42 °C. Both these mutations caused changes in the C-terminal part of the TrmD peptide (*trmD1*, E243K and *trmD22*, Q246stop; *trmD22* has an additional mutation, Q246Stop). The *trmD1* mutant has at 42 °C only 16% of the wild-type level of the peptide, but is still able to fully modify the wild-type tRNAs (Table 2). The TrmD3 and the TrmD10 peptides, however, have reduced modification capacity but relatively much higher level of the respective peptide as compared to TrmD1. This suggests that the *trmD3* and the *trmD10* mutations must affect primarily the catalytic activity of the enzyme, whereas the *trmD1* mutation does not. This is also true for all other mutations in Class I as well as the *trmD11* mutation of Class II (Table 1). These results suggest also that TrmD peptide is normally present in catalytic excess in the cell.

Growth ability of the *trmD*, *suf* mutants at high temperature reflects the level of m^1G37 in tRNA

In the presence of the *sufA6* allele, all *trmD* mutants were temperature sensitive for growth. However, they behaved differently in the presence of the *sufB2* allele (classification based on this phenotype, Table 1). To investigate whether the presence of different mutant tRNAs affects the modification level and therefore the cell growth, we prepared bulk and specific tRNAs from the double mutant strains. *trmD1* was chosen as a representative of the Class I mutants and *trmD3* of Class III.

Both the *sufA6* $tRNA^{Pro}_{CGG}$ (this work) and the *sufB2* $tRNA^{Pro}_{GGG}$ (Sroga et al., 1992) has a G insertion in the anticodon loop. These two tRNA species were fully methylated by the wild-type ($TrmD^+$) enzyme and the strains carrying the *sufA6* or the *sufB2* mutation grew normally at 42.5 °C (Table 3). We also know that m^1G is adjacent to and 3′ of the anticodon in these mutant tRNAs, as it is in their wild-type counterparts (Qian et al., 1998). Therefore a G insertion in the anticodon loop of these tRNA species does not affect the substrate specificity of the wild-type form of the enzyme. Also the TrmD1 peptide methylated efficiently the wild-type and the *sufB2* tRNAs at 42 °C (Table 2). Since the *trmD1, sufA6* strain did not grow at high temperature, a shift of temperature was performed after the culture had been pregrown at 30 °C. In such a shift experiment, a tenfold increase in mass occurred before the cells stopped growing, because the tRNA made at 30 °C is fully methylated and therefore allows the cell to grow, albeit at reduced rate. By this method we were able to prepare tRNA from the *trmD1, sufA6* strain at 42 °C and found that no m^1G was synthesized (Table 3). Since the TrmD3 peptide is functionally inactive, even in the presence of the wild-type substrate tRNAs at 37 °C



	Integrated intensity	*trmD*$^+$	*trmD1*	*trmD2*	*trmD3*	*trmD10*	*trmD11*	*trmD17*	*trmD22*
37°C	TrmD/ProX	0.64±0.03	0.20±0.07	0.62±0.11	0.49±0.07	0.78±0.08	0.43±0.05	0.69±0.10	0.34±0.05
	Mu/Wt	1	0.32±0.12	0.97±0.13	0.75±0.12	1.2±0.1	0.64±0.08	1.1±0.1	0.53±0.10
42°C	TrmD/ProX	0.91±0.09	0.15±0.07	0.66±0.17	0.50±0.10	0.91±0.05	0.56±0.13	0.68±0.08	0.30±0.10
	Mu/Wt	1	0.16±0.06	0.72±0.12	0.55±0.05	0.97±0.05	0.59±0.08	0.75±0.02	0.32±0.08

FIGURE 2. Level in vivo of the TrmD polypeptide in different *trmD* mutants (GT5473, GT5475, GT5477, GT5479, GT2772, GT2774, GT2780, GT2784). The various strains are only differing in the allelic state of the *trmD* gene. Cells were grown at 37 °C or 42 °C (gel not shown) and cell extracts containing 20 μg of protein prepared from each strain were electrophoresed on a SDS-15% polyacrylamide gel. TrmD protein was visualized by immunoblotting with polyclonal antiserum directed against TrmD and the streptavidin-HRP labeled secondary antibody specific for rabbit Ig. An unknown protein (ProX), which was present at the same level in all extracts, was used as an internal control and the intensity of each band was quantified with a Howtek scanmaster 3+. A wild-type *E. coli* strain carrying a plasmid overexpressing the *trmD* gene (pBY03/*K12*) was used as a marker to identify the TrmD polypeptide. One of the two mutations in the *trmD22* mutant introduced a stop codon at amino acid 246 and therefore gave rise to a 10-amino-acid-shorter product. The values presented in the table are the average of two or three independent measurements with experimental errors indicated.

(Table 2), no m^1G should be expected in the *trmD3, sufA6* or the *trmD3, sufB2* double mutants at high temperature (these two strains do not grow at all at 42 °C, as at 30 °C they already are deficient in m^1G37). Thus, all these strains that cannot grow at high temperature, the *trmD1, sufA6* and the *trmD3, sufA6* or *trmD3, sufB2*, were also deficient of m^1G. By Western blot analysis, we found that the level of the TrmD1 protein was reduced upon introduction of the *sufA6* allele, but not upon introduction of the *sufB2* allele (Table 3). Therefore, the lack of m^1G in the *trmD1, sufA6* mutant is correlated to the reduced level of the TrmD1 polypeptide in this particular strain. In contrast, the level of the TrmD3 polypeptide was reduced upon introduction of either the *sufA6* or the *sufB2* allele (Table 3).

Extensive frameshifting by the *sufA6* tRNA causes reduction of the TrmD1 and the TrmD3 peptides

To further analyze how the different mutant TrmD peptides and the substrate tRNAs in combination induced a temperature-sensitive phenotype, we isolated spontaneous temperature resistant revertants of the double mutants *trmD1*, *sufA6* and *trmD3*, *sufA6*. These revertants were isolated at a frequency of 10^{-6}–10^{-7} and genetic and biochemical characterizations are summarized in Table 3.

Two classes of revertants derived from the *trmD1*, *sufA6* double mutant were characterized (at least two independent isolates from each class were analyzed and were caused by the same mutational event). Both classes had lost their frameshift suppressor activity and the mutations were linked to the *sufA6* allele. The *sufA6* mutation, similar to the *sufB2* mutation established by Sroga et al. (1992), was shown to have an extra G inserted in the anticodon loop (Fig. 3). One class of the revertants, *trmD1, sufA6+9*, had a duplication of a 9-nt-long DNA sequence corresponding to A39 to G47 in the *sufA6* $tRNA^{Pro}_{CGG}$ sequence. The resulting $tRNA^{Pro}_{CGG}$ can be drawn in the cloverleaf form as having an extended variable loop (14 nt instead of 5; Fig. 3) besides retaining the extra G in the anticodon loop. The second class of the revertants, the *trmD1,* Δ*sufA6* revertant,

TABLE 2. Growth ability and m^1G level in tRNAs of various *trmD* or *suf* mutants.

Relevant genetic markers	Growth at 42.5 °C[a]	Suppression of *hisD3749* at 37 °C[b]	m^1G level in bulk tRNA 37 °C[c]	Level of TrmD 37 °C[d]		Level of m^1G (37 °C)[e]	Mutation identified
				$tRNA^{Pro}_{CGG}$	$tRNA^{Pro}_{GCGG}$		
wt	++	−	0.13 ± 0.02 0.15 ± 0.02 (42 °C)	0.93	1.1	1	
sufA6	++	++	0.12 ± 0.00 0.14 ± 0.01 (42 °C)	1.2 ± 0.3	ND[g]	0.89 ± 0.09	+G in the anticodon of $tRNA^{Pro}_{CGG}$
sufB2	++	++	0.12 ± 0.01 0.14 (42 °C)	ND	1.0 ± 0.2	1.0 ± 0.1	+G in the anticodon of $tRNA^{Pro}_{GGG}$
trmD1 (Class I[g])	++	−	0.14 0.13 (42 °C)	1.3 ± 0.1	1.0 ± 0.1	0.32 ± 0.12	E243K substitution in *trmD*
trmD2 (Class I[g])	++	−	0.14 0.14 (42 °C)	ND[f]	ND[f]	0.97	L94F substitution in *trmD*
trmD10 (Class II)	++	−	0.075 ± 0 0.087	ND[f]	ND[f]	1.2	G140S substitution in *trmD*
trmD11 (Class II)	++	−	0.12 ± 0.03 0.13	ND[f]	ND[f]	0.64	A145T substitution in *trmD*
trmD3 (Class III)	+	+	0.018 ± 0.003 <0.01 (42 °C)	undetectable	undetectable	0.75 ± 0.12	P184L substitution in *trmD*

[a]Growth of strains were monitored on agar plates following incubation at 42.5 °C for 24 h before the sizes of the colonies were scored. ++: colonies as large as those of the wild-type cells; +: medium; and −: no colonies formed. All strains (GT5473, GT476, GT477, GT5475, GT5479, GT686, GT2732, GT3162, GT2756, GT3645, GT3647, GT3649, GT3651) used contain the *hisO1242* and *hisD3749* or *hisD3018* or *hisC3737* alleles and the indicated alleles of the *trmD* gene, the *sufA6* or the *sufB2* alleles.

[b]++: growth was observed after 12 h incubation at 37 °C on glucose minimal plate lacking His; +: growth after 24 h; (+): weak growth after 48 h incubation; −: no growth was observed after 3 days under the same condition.

[c,d]The level of m^1G in bulk tRNAs are presented as the amount of m^1G relative to the amount of Ψ. Level of m^1G in *sufA6* tRNA or its derivatives are presented as mole of m^1G per mole of tRNA. Experimental variations are shown when more than one (2–3) measurements were performed.

[e]Levels of TrmD polypeptide of cells grown at 37 °C were measured as described in Figure 2.

[f]ND: not determined.

[g]The two other Class I mutants, *trmD17* and *trmD22*, had 0.12 ± 0.01 and 0.13 ± 0.01 m^1G per Ψ in tRNA from cells grown at 37 °C.

had an 18-nt-long deletion within the structural gene (*proK*) for $tRNA^{Pro}_{CGG}$. This corresponded to nt A59 to A76 in the tRNA. A Northern blot experiment using a DNA probe specific for the anticodon region of $tRNA^{Pro}_{CGG}$ confirmed that this tRNA species was not present in the cell (data not shown). Thus, although $tRNA^{Pro}_{CGG}$ is a major tRNA species, it is not essential for growth. The $tRNA^{Pro}_{CGG}$ from the *trmD1, sufA6+9* revertant was overexpressed (more than fivefold) and partially (8.5%) aminoacylated as measured by Northern blot using an acidic gel (data not shown, for experimental design see Materials and Methods). Since the revertant *trmD1, sufA6+9* was also unable to suppress the *hisD3749* mutation, the extended variable loop may counteract the frameshifting ability induced by the G insertion in the anticodon. Alternatively, because $tRNA^{Pro}_{CGG}$ is nonessential, this mutant form of the $tRNA^{Pro}_{CGG}$ with its 9-nt duplication might be unable to function in the decoding step of translation although it is able to partially accept amino acid in vivo. The *sufA6+9* mutation mediated an 8.5-fold increase and the deletion of *sufA6* mediated a fourfold increase in the level of the TrmD1 polypeptide as compared to that in the *trmD1, sufA6* strain (Table 3). Thus, a correlation exists between low frameshifting activity and a high level of the TrmD peptide. Moreover, these findings suggest that the cause of lethality induced by the *trmD1* mutation in the *trmD1, sufA6* mutant is a too extensive frameshifting by the unmethylated *sufA6* tRNA.

Two classes of revertants were characterized from the *trmD3, sufA6* double mutant (also in this case at least two independent isolates of each class were analyzed and were caused by the same mutational events). The first Class *trmD3, sufA6-A* revertants appeared as large colonies as the wild-type cells at 42.5 °C and the frameshift suppressor activity of the revertants was reduced but not completely abolished as scored by the His-phenotype (Table 3). This revertant had a deletion of an A in the anticodon loop of the *sufA6* tRNA (Fig. 3). This created a tRNA that has the same sequence as the wild-type $tRNA^{Pro}_{CGG}$ except for a base substitution in the anticodon loop (A38 to G38). The level of m^1G in this tRNA species was increased from 0 to 0.37 mole per mole tRNA. In bulk tRNA, an increase from 0.014 to 0.042 mole m^1G per Ψ was observed, which can be attributed to the increased

TABLE 3. Growth ability and m^1G37 level in tRNA of various *trmD, suf* double mutants and their temperature resistant revertants.

trmD allele	*suf* allele	Growth at 42.5 °C[a]	Suppression of *hisD3749* at 37 °C[b]	m^1G level in bulk tRNA 37C[d]	Level of m^1G (37 °C)[e] $tRNA^{Pro}_{CGG}$	Level of m^1G (37 °C)[e] $tRNA^{Pro}_{GGG}$	Level of TrmD 37 °C[f]	Mutation identified
trmD1	*sufB2*	++	++	0.12 ± 0.03 0.13 ± 0.01 (42 °C)	1.2	0.97 ± 0.14	0.36 ± 0.03	
	sufA6	−	++[c]	0.14 ± 0.02 <0.01(42 °C)	0.80 ± 0.2	0.73 ± 0.02	0.03 ± 0.01	
	sufA6+9 (Rev.)	++	−	0.11 ± 0.02	0.24 ± 0.05	ND[g]	0.25 ± 0.06	+9 nt in *sufA6* tRNA
	Δ*sufA6* (Rev.)	++	−	0.082 ± 0.014[h]	ND[g]	ND[g]	0.12 ± 0.04	Deletion of *sufA6* tRNA
trmD3	*sufB2*	−	++	ND[i]	ND[g]	ND[g]	0.03 ± 0.02	
	sufA6	−	++	0.014	undetectable	ND[g]	0.08 ± 0	
	sufA6-A (Rev.)	++	(+)	0.042 ± 0.003	0.37	ND[g]	0.43 ± 0.05	GGGG instead of GGGGA in the anticodon of *sufA6* tRNA
trmD3, *trmD23* (Rev)	*sufA6*	+	+	0.08 ± 0.02	0.44	ND[g]	1.8 ± 0.2	TGGGGA → TGGGGG in *trmD* SD-sequence

[a]Growth of strains was monitored on agar plates following incubation at 42.5 °C for 24 h before the sizes of the colonies were scored. ++: colonies as large as those of the wild-type cells; +: medium; and −: no colonies formed. All strains (GT5473, GT476, GT477, GT5475, GT5479, GT686, GT2732, GT3162, GT2756, GT3645, GT3647, GT3649, GT3651) used contain the *hisO1242* and *hisD3749* or *hisD3018* or *hisC3737* alleles and the indicated alleles of the *trmD* gene, the *sufA6*, or the *sufB2* alleles.

[b]++: growth was observed after 12 h incubation at 37 °C on glucose minimal plate lacking His; +: growth after 24 h; (+): weak growth after 48 h incubation; −: no growth was observed after 3 days under the same condition.

[c]Mucoid.

[d,e]The level of m^1G in bulk tRNAs is presented as the amount of m^1G relative to the amount of Ψ. Level of m^1G in *sufA6* tRNA or its derivatives is presented as mole of m^1G per mole of tRNA. Experimental variations are shown when more than one (2–3) measurements were performed.

[f]Levels of TrmD polypeptide of cells grown at 37 °C were measured as described in Figure 2.

[g]ND: not determined.

[h]$tRNA^{Pro}_{CGG}$ contributes to 29% of the total m^1G content (calculated according to Riddle & Roth, 1972; Ikemura, 1981). The observed reduction in m^1G in *trmD1,* Δ*sufA6 double mutant can be attributed entirely to the absence of* $tRNA^{Pro}_{CGG}$ and therefore the other tRNA species contain the normal level of m^1G37.

[i]The strain containing both *trmD3* and *sufB2* mutations was not possible to grow in liquid medium because the mutations lysed and quickly reverted before reaching a cell density suitable for preparing tRNA.

level of m^1G in *sufA6-A* tRNA, as it is a major tRNA (Table 3). Thus, the *sufA6-A* tRNA with its normal-sized anticodon loop is a better substrate for the TrmD3 polypeptide as compared to the *sufA6* tRNA with its oversized anticodon loop.

The other class of revertants, *trmD3, sufA6, trmD23*, was still able to frameshift, although less efficiently compared to its parent, the double mutant *trmD3*, *sufA6*. The *trmD23* mutation was found to be linked to the *trmD* gene and sequence analysis showed that the mutation was an A-to-G change at −8 in the putative translation initiation region (TIR)(TGGGGG instead of TGG GGA). Compared to the original double mutant *trmD3*, *sufA6*, which has no m^1G37 in $tRNA^{Pro}_{CGG}$ and only about 10% of the wild-type level of m^1G37 in bulk tRNA, 44% of the *sufA6* tRNA had regained m^1G and 80% of the bulk tRNA was modified in the revertant strain (Table 3). The changed Shine-Dalgarno sequence increased the synthesis of the TrmD3 polypeptide, resulting in an increased synthesis of m^1G37. A Western blot experiment using antibody against the TrmD polypeptide showed that the amount of the TrmD3 polypeptide was indeed increased 23-fold compared to that in the *trmD3, sufA6* mutant (Table 3). Similarly, but to a lesser extent (fivefold), the TrmD polypeptide was also increased in the *trmD3, sufA6-A* revertant. This is consistent with a correlation between a low frameshifting activity and an increased level of the TrmD polypeptide.

In summary, the analysis of the revertants obtained from class I (*trmD1*, *sufA6*) and from class III (*trmD3*, *sufA6*) mutants showed that in both cases the revertants had reduced frameshifting activities. This was correlated with an increased ability to methylate the tRNA and an increased synthesis of the mutated TrmD peptide. The cause of lethality in the double mutants seems to be an extensive frameshifting activity mediated by the unmethylated *sufA6* tRNA.

DISCUSSION

In this work, we have described the isolation and characterization of various mutant forms of the tRNA(m^1G37)methyltransferase of *S. typhimurium* encoded by the *trmD* gene. The enzyme is highly con-

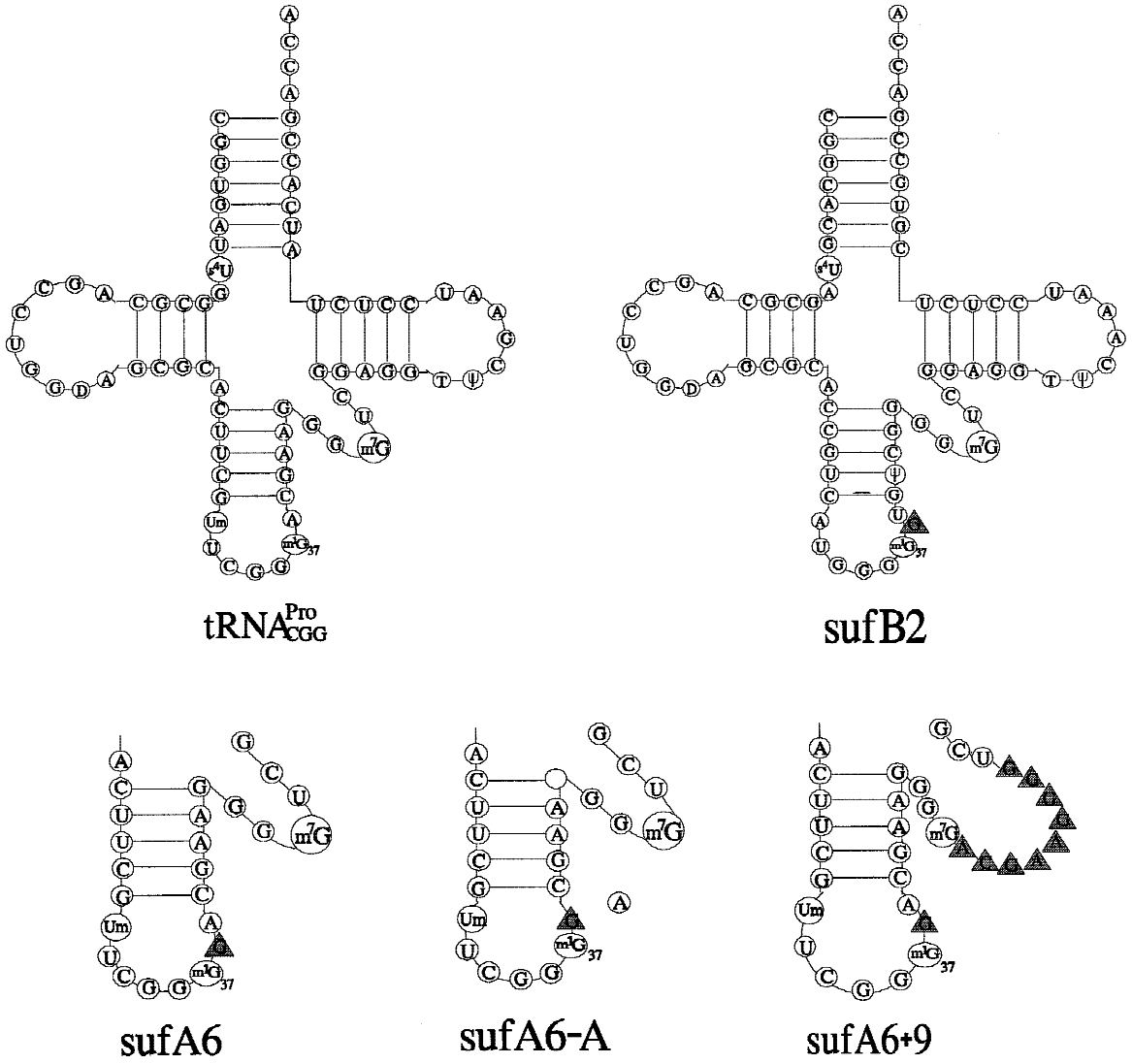


FIGURE 3. Cloverleaf structure of tRNA$^{Pro}_{CGG}$ (Kuchino et al., 1984) and of *sufB2* tRNA (Qian et al., 1998). Only the anticodon region and the variable loop are shown for the mutant derivatives of the *sufA6* tRNA. ▲ in the *sufA6* and in the *sufB2* tRNAs indicates the position of the inserted G adjacent and 3′ of the m^{1}G37 (Qian et al., 1998). The revertant *sufA6-A* has deleted an A (symbolically drawn outside the tRNA) compared to the *sufA6* tRNA resulting in tRNA with a normal sized anticodon loop and an A38 to G38 base substitution compared to the wild-type *proK* tRNA. The *sufA6+9* tRNA has an internal nine base duplication (bold triangles) creating in the cloverleaf model an extended variable loop.

served among closely related organisms such as *E. coli*, *S. marcescens*, and *H. influenzae* (92%, 84%, and 78% identities, respectively, to *S. typhimurium*) but also conserved in many regions among more distantly related species such as *Mycoplasma* (Fig. 1). These conserved regions must be functionally important, as the same subset of tRNAs are modified at the same position in all these organisms and most *trmD* mutations obtained in this work were changed in these highly conserved positions.

The *sufA6* tRNA, which has an extra G 3′ of m^{1}G37 as compared to its wild-type form tRNA$^{Pro}_{CGG}$ (Fig. 3), induces +1 frameshifting at CCC-N sites (Riddle & Roth, 1970) (CCC is in the zero frame and a +1 frameshift moves the ribosome to the CC-N codon). Also lack of m^{1}G37, induced by the *trmD3* mutation, mediates +1 frameshifting at similar sites (Björk et al., 1989). Combining the *sufA6* mutation with the *trmD3* mutation therefore results in a hypomethylated derivative of the *sufA6* tRNA at 42 °C and such a double mutant is not viable at this temperature (Table 3). The fact that whereas a small amount of m^{1}G is present at 37 °C in the *trmD3* mutant, no m^{1}G was detected at 42 °C (Table 2) raises the question of why the observed lethality at 42 °C of these double mutants is correlated to lack of m^{1}G at this temperature. We suggest that the reason for lethality of these double mutants is extensive frameshifting; that is, the undermethylated derivative of these frameshifting tRNA are inducing more +1 frameshifting than the methylated derivative of them. Indeed, using a

plasmid in which the *lacZ* gene expression is dependent on a +1 frameshifting event at a CCC-U site, a higher β-galactosidase activity was observed in these double mutants as compared to the *sufA6* or *sufB2* single mutants (Q. Qian & G.R. Björk, unpubl.). The TrmD3 peptide is also strongly reduced in these double mutants, suggesting that its synthesis is affected by extensive frameshifting. Indeed, a potential frameshifting site (CCC-U) is present in the structural gene of *trmD* at codon 66. A frameshifting event at this site will result in premature termination after translation has proceeded for an additional 17 amino acids in the +1 frame. The truncated polypeptide is most likely not functional and will be quickly degraded. Thus, the results obtained with the double mutants *trmD3*, *sufA6* and *trmD3*, *sufB2* suggest that lack of m^1G37 in these tRNAs causes extensive frameshifting that also affects the level of the TrmD3 peptide, perhaps by a +1 frameshifting event early in the *trmD* mRNA.

In contrast to the *trmD3* mutation, the Class I mutation *trmD1* has normal capacity to methylate wild-type tRNA and causes temperature sensitivity only in the presence of the *sufA6* allele (Table 4). A reduced capacity to methylate tRNA and a reduction of the level of the TrmD1 peptide was observed in the *trmD1, sufA6* double mutant (Table 3). This phenotype is reminiscent of that shown by the double mutant *trmD3*, *sufA6*, which suggests that the TrmD1 peptide, like the TrmD3 peptide, does not methylate the *sufA6* tRNA. Lack of m^1G37 in the *sufA6* tRNA results in an extensive +1 frameshifting and consequently a reduced level of the TrmD1 peptide. Indeed, using the same plasmid as above in which the *lacZ* gene expression is dependent on the +1 frameshifting event at a CCC-U site, a tenfold higher β-galactosidase activity was observed in the *trmD1, sufA6* mutant compared to that in the *sufA6* mutant (Q. Qian, J.-N. Li, & G.R. Björk, unpubl. result). These results suggest that the TrmD1 polypeptide recognizes inefficiently the *sufA6* tRNA with its extra G in the anticodon loop, although it fully methylates its wild-type (*proK*$^+$) counterpart and the *sufB2* tRNA as well as its wild-type (*proL*$^+$) counterpart. Note that the wild-type form of the enzyme does not discriminate between these two forms of the same tRNA, as it fully methylates these tRNAs (Table 2). Thus, we suggest that the E243K alteration in the TrmD1 polypeptide influences primarily the substrate specificity, which results in a discrimination between the *proK*$^+$ and *sufA6* tRNA substrates only differing in the insertion of a G in the anticodon loop of the *sufA6* tRNA.

Earlier we suggested that the reason for the temperature sensitivity of the *trmD1*, *sufA6* double mutant is extensive frameshifting caused by the inability of the TrmD1 peptide to methylate *sufA6* tRNA. A further

TABLE 4. *S. typhimurium* strains used in this study.

Name	Genetic characteristics	Source
LT2	wt	John Roth
GT476(TR1457)	*hisO1242*, *hisD3749*, *sufA6*	John Roth
GT477(TR936)	*hisO1242*, *hisD3018*, *sufB2*	John Roth
GT686	*hisO1242*, *hisC3737, tyrA555*::Tn*10*, *trmD1*, *sufA6*	This work
GT688	*hisO1242*, *hisC3737, tyrA555*::Tn*10*, *trmD2*, *sufA6*	This work
GT907	*metA22*, *metE551*, *ilv-452*, *trpB2*, *xyl-404*, *rpsL120*, *flaA66*, *hsdL66*, *hsdA29*, *galE503*	
GT2677	*hisO1242*, *hisD3749*, *tyrA555*::Tn*10*, *trmD10*, *sufA6*	This work
GT2678	*hisO1242*, *hisD3749*, *tyrA555*::Tn*10*, *trmD11*, *sufA6*	This work
GT2685	*hisO1242*, *hisD3749*, *tyrA555*::Tn*10*, *trmD17*, *sufA6*	This work
GT2690	*hisO1242*, *hisD3749*, *tyrA555*::Tn*10*, *trmD22*, *sufA6*	This work
GT2732	*hisO1242*, *hisD3749*, *tyrA555*::Tn*10*, *trmD3*, *sufA6*	This work
GT2750	*hisO1242*, *hisD3018*, *tyrA555*::Tn*10*, *trmD10*, *sufB2*	This work
GT2752	*hisO1242*, *hisD3018*, *tyrA555*::Tn*10*, *trmD11*, *sufB2*	This work
GT2756	*hisO1242*, *hisD3018*, *tyrA555*::Tn*10*, *trmD3*, *sufB2*	This work
GT2772	*hisO1242*, *hisD3749*, *tyrA555*::Tn*10*, *trmD10*	This work
GT2774	*hisO1242*, *hisD3749*, *tyrA555*::Tn*10*, *trmD11*	This work
GT2780	*hisO1242*, *hisD3749*, *tyrA555*::Tn*10*, *trmD17*	This work
GT2784	*hisO1242*, *hisD3749*, *tyrA555*::Tn*10*, *trmD22*	This work
GT3162	*hisO1242*, *hisD3018*, *tyrA555*::Tn*10*, *trmD1*, *sufB2*	This work
GT3164	*hisO1242*, *hisD3018*, *tyrA555*::Tn*10*, *trmD2*, *sufB2*	This work
GT3645	*hisO1242*, *hisC3737*, *tyrA555*::Tn*10*, *trmD1*, *sufA6+9*	This work
GT3647	*hisO1242*, *hisC3737*, *tyrA555*::Tn*10*, *trmD1*, Δ*sufA6*	This work
GT3649	*hisO1242*, *hisD3749*, *tyrA555*::Tn*10*, *trmD3*, *sufA6-A*	This work
GT3651	*hisO1242*, *hisD3749*, *tyrA555*::Tn*10*, *trmD3*, *trmD23*	This work
GT5473	*hisO1242*, *hisD3749*, *tyrA555*::Tn*10*, *trmD*$^+$	This work
GT5475	*hisO1242*, *hisD3749*, *tyrA555*::Tn*10*, *trmD1*	This work
GT5477	*hisO1242*, *hisD3749*, *tyrA555*::Tn*10*, *trmD2*	This work
GT5479	*hisO1242*, *hisD3749*, *tyrA555*::Tn*10*, *trmD3*	This work

support for this interpretation is our analysis of the temperature-resistant revertants isolated from the *trmD1, sufA6* and the *trmD3, sufA6* double mutants. Although these pseudorevertants are distinct, the underlying mechanism by which they were isolated may be the same—they all in different ways reduce or abolish the frameshifting activity of the m^1G37 deficient *sufA6* tRNA. The TrmD3 peptide has a reduced catalytic activity (Table 2) and therefore the revertants isolated from the *trmD3, sufA6* double mutant resulted in an increased modification capacity either by changing the tRNA to a better substrate (*sufA6-A*) for the TrmD3 peptide or by increasing the synthesis of the TrmD3 peptide (the *trmD23* mutation alters the sequence of the putative Shin-Dalgarno sequence of the *trmD* mRNA; Table 3). On the other hand, the TrmD1 peptide fails to recognize the *sufA6* tRNA but modifies normally other tRNAs; that is, its catalytic activity is normal if tRNAs other than *sufA6* tRNA are the substrates (Tables 2 and 3). Therefore, the isolated revertants had either lost the *sufA6* gene (Δ*sufA6*) or the tRNA was changed to a nonframeshifting derivative (*sufA6+9*). When the *sufA6+9* mutation (isolated as a revertant of *trmD1, sufA6*) was introduced into the various *trmD, sufA6* double mutants, the growth deficiency was only partially suppressed in the *trmD3, sufA6* mutant, but fully suppressed in the double mutants containing any of the Class I mutations (data not shown). This suggests that all Class I mutations induce the same type of physiological defect(s) as the *trmD1* mutation and, as stated above, they may all influence the tRNA substrate specificity.

These results are consistent with our suggestion, as discussed above, that the *trmD1* mutation and also the other Class I mutations influence primarily the tRNA substrate specificity, whereas the *trmD3* mutation has more influence on the catalytic activity in general. The allele specificity demonstrates that the TrmD1 polypeptide must interact in a distinct way with the *sufA6* tRNA compared to the TrmD3 peptide, and that this TrmD1-*sufA6* tRNA interaction can be counteracted by changes in the *sufA6* tRNA that is not counteracting the interaction(s) between the TrmD3 and *sufA6* tRNA. Clearly, the aberrant interaction between TrmD1-*sufA6* tRNA and the TrmD3-*sufA6* tRNA is different as the respective pseudorevertants isolated showed allele specificity.

Both *sufA6* and *sufB2* mutations induce strong frameshifting as monitored by their ability to suppress the *hisD3749* mutation, which contains the CCC-U potential frameshifting site. Still, these two frameshift suppressors did not significantly influence the level of the TrmD peptide (Table 3), although the same putative frameshifting site (CCC-U) is present in the *trmD* mRNA. However, even a very low *his*-expression (less than 1% of the wild-type *his*-expression; Riddle & Roth, 1970) results in His^+ phenotype. Indeed, the *his*-system is much more sensitive at monitoring frameshifting than many other systems are, for example, using a plasmid in which the *lacZ* gene expression is dependent on a +1 frameshifting event at the same frameshifting site (Qian et al., 1998). Thus, although the *sufA6* and *sufB2* frameshift suppressor tRNAs are reasonably strong as monitored by the His-phenotype, they may not induce frequent frameshift events at the many putative CCC-N sites that are present in the genome. Indeed, the growth rates in rich medium of these two mutants are similar to that of the wild-type strain (Table 2; see also Riddle & Roth, 1970), suggesting that significant frameshifting at many CCC-N sites may not occur and one such site seems to be the one present in the *trmD* gene. Strong frameshifting at this site apparently requires that the m^1G37 is absent, which creates an anticodon of 4 (*sufA6*) or 5(*sufB2*) Gs in a row (Fig. 3) as compared to only 2 (*sufA6*) or 3 (*sufB2*) Gs in a row in $trmD^+$ background (Fig. 3; presence of m^1G37 prevents Watson–Crick pairing with C; Newmark & Cantor, 1968). According to the new model of +1 frameshifting (Qian et al., 1998), the creation of a row of G in the anticodon may facilitate +1 slippage in the P-site and thereby induce a frameshifting event. Therefore, lack of m^1G37 in these frameshift suppressor tRNAs makes them able to induce a frameshifting event at most of the CCC-N sites present in the genome, resulting in a nonviable cell.

All Class I mutations [*trmD1* (E243K), *trmD2* (L94F), *trmD17* (A25T), and *trmD22* (V99I, Q246stop)] mediate alterations either in the N- or C-terminal part of the peptide. These mutations do not affect the modification of the wild-type tRNA even though the levels of the TrmD proteins were reduced. One mutant of this class, the *trmD1*, had the lowest amount of the TrmD polypeptide among all mutants isolated (32% of the wild-type level at 37 °C and 16% of the wild-type level at 42 °C); still the tRNAs were fully methylated even at 42 °C (Fig. 2, Table 2). This reduction of the TrmD1 level is not correlated to any observed increase in frameshifting as monitored by the suppression of the *hisD3749* mutation. Therefore, the TrmD1 peptide seems to be intrinsically more unstable than the wild-type $TrmD^+$ peptide. Moreover, in the double mutant *trmD1*, *sufA6* grown at 37 °C, surprisingly, only 3% of the wild-type level of the TrmD1 polypeptide was observed and still the level of m^1G37 in tRNA was the same or nearly the same as in the wild type (Table 3). Thus, the level of the TrmD polypeptide seems to be in surplus in relation to the need to methylate tRNA. Also the MiaA polypeptide, which catalyzes the first step in the formation of ms^2i^6A in tRNA reading codons starting with U, is present in catalytic excess (Leung et al., 1997). Whether this will be true for other tRNA modifying enzymes awaits further analysis. Still the tRNA modifying enzymes are present in low amount in the cell compared to other proteins involved in translation.

MATERIALS AND METHODS

Abbreviations

The enzyme (E.C. 2.1.1.31) that catalyzes the formation of 1-methylguanosine (m^1G) adjacent and 3′ of the anticodon (position 37) is denoted tRNA(m^1G37)methyltransferase or $TrmD^+$. The various mutant forms of it are denoted TrmD and the allele number of the mutation (e.g., TrmD3). The various mutated tRNAs are denoted by their allele designation in front of tRNA (e.g., *sufA6* tRNA is the $tRNA^{Pro}_{CGG}$ derivative that has an extra G next to and 3′ of the m^1G37.

Bacteria and growth conditions

The bacterial strains used were all derivatives *of S. typhimurium* LT2 (Table 4). Cells were grown in either MOPS minimal medium supplemented with 0.4% glucose (Neidhardt et al., 1974) or Rich-MOPS medium (Neidhardt et al., 1977) when defined media were used. As the complex medium (NAA), Difco nutrient broth (0.8%; Difco Laboratories, Detroit, Michigan), supplemented with 0.5% NaCl, adenine, tryptophan, tyrosine, phenylalanine, and the three aromatic vitamins p-hydroxybenzoate, 2,3-dihydroxybenzoate, and p-aminobenzoate, was used. All supplements were provided at concentrations recommended by Davis et al. (1980).

Genetic procedures

The *E. coli* plasmids used in this work were transferred by transformation first into the *Salmonella* strain GT907, which contains the *galE503*, *hsdL66*, and *hsdA29* mutations. The *galE503* mutation makes the *S. typhimurium* strain competent for transformation (MacLachlan & Sanderson, 1985), the *hsd*-mutations abolish the restriction of *E. coli* DNA, but the strain has an intact DNA modification system. Plasmids were moved from strain GT907 to various *Salmonella* strains by transduction with phage P22 HT105(*int-201*) (Schmieger, 1972; Davis et al., 1980).

Analysis of m^1G content in bulk tRNAs

Strains were grown in MOPS-Glucose medium at 37 °C from about 5 Klett units (approximately 2×10^7 cells/mL) to 100 Klett units (approximately 4×10^8 cells/mL). tRNA was prepared as described by Buck et al. (1983) and digested with nuclease P1 followed by alkaline phosphatase treatment (Gehrke et al., 1982). The hydrolysates were analyzed by HPLC according to the method of Gehrke & Kuo (1990). To determine the level of m^1G37 in tRNA from cells grown at 42 °C, we pregrew the strains at 30 °C to early log-phase and then diluted the cell suspensions to 8 Klett units in medium prewarmed to 42 °C. Cells were then grown to about 4×10^8 cells (100 Klett units) and tRNA was prepared and analyzed as described above. In such an analysis, no more than 10% of the cells would originate from culture grown at 30 °C. Although the double mutant *trmD1, sufA6* does not grow at 42 °C, still there is a tenfold increase in mass before this double mutant stops growing. Moreover, we have also specifically labeled the methylated nucleosides synthesized at 42 °C by supplementing the growth medium with ^{14}C-*methyl*-L-methionine. Transfer RNA was prepared and digested to nucleosides and the distribution of methylated nucleosides was analyzed by HPLC (Gehrke & Kuo, 1990). The radioactivity in the eluate was monitored by a flow scintillation analyzer (Radiomatic FLO-ONE beta; Packard Instrument Co., Meriden, Connecticut).

DNA sequencing with PCR amplified template

Two primers complementary to sequences upstream (−92 to −67) and downstream (+177 to +198) of the *E. coli proK* gene (structural gene for $tRNA^{Pro}_{CGG}$; Kuchino et al., 1985) were used to amplify by PCR the *Salmonella* counterpart. Primer 1: 5′-AGCGGCCTGCTGACTTTCTCGCCGAT-3′; primer 2: 5′-BIOTIN-GCCGTTGCTGGCGCAACGTTC-3′. The biotinylated primer binds to the antisense strand and is therefore complementary to the tRNA. After a standard PCR reaction, the amplified product was incubated at room temperature for 12 h with 1 mg Dynabeads M-280 pre-washed with TE buffer (10 mM Tris-HCl (pH 8.0) containing 1 M NaCl as recommended by the Dynal Company, Norway). The Dynabeads were then washed three times with deionized water and incubated in 20 μL of 0.15 M sodium hydroxide at room temperature for 1 h to separate the strands. The Dynabeads, now containing the sense strand of the tDNA, were used either as template for DNA sequencing or as probe to isolate $tRNA^{Pro}_{CGG}$. When *proL* (structural gene for $tRNA^{Pro}_{CGG}$) was amplified, primer 1 (5′-GGGAATTCTGCTGCTGCAAGTGC-3′) and primer 2 (5′-BIOTIN-GGGTCCCCCGTTGCTTTCCCG-3′) were used. These two primers were designed according to sequence data obtained by Sroga et al. (1992). The chromosomal fragment carrying the *trmD* gene was amplified with primers 5′-BIOTIN-GCTCTTGTTCAAGTTGCT-3′ and 5′-GGATCCTGGTTTTTAAACCACCG-3′ according to the earlier established sequence for trmD of *S. typhimurium* (Ref. EMBL data, accession number x74933) and the sequence of the $trmD^+$ allele obtained by us was the same as obtained earlier.

Purification of $tRNA^{Pro}_{CGG}$ and $tRNA^{Pro}_{GGG}$

Cells were pregrown at 37 °C in low phosphate (0.5 mM) MOPS-glucose medium from about 5 Klett units (approximately 2×10^7 cells/mL) for 2–3 generations. At this time, 5 mL of each culture were transferred to a tube containing 0.5 mCi ^{32}P-orthophosphate (10 mCi/mL, Amersham, England) according to the method described by Yarus et al. (1986). The growth of the control culture in medium containing no radioactive phosphate was monitored and the labeled culture was harvested when the control culture had reached 100 Klett units. Cells were pelleted and resuspended in 1 mL of buffer A (10 mM MgAc2, 50 mM NaAc, and 150 mM NaCl, pH 4.5) and an equal amount of phenol saturated with H_2O was added. The mixture was shaken at room temperature for 20 min. The supernatant containing the total RNA was extracted once more with chloroform to remove the phenol and was then lyophilized. tRNA and rRNA were separated on a 12% native polyacrylamide gel. After a short exposure (a few minutes) to an X-ray film, the tRNA band was cut out. The gel slices were mixed with 150 μL hybridization buffer (6 × SSC, 2 × Denhart, 50% formamide, and 0.1% SDS) and shaken

overnight at 37 °C to extract the tRNA. Such bulk tRNA preparations were then mixed with Dynabeads containing the respective sense strand of the tDNA and denatured at 95 °C for 5 min. After hybridization at 42 °C overnight, the beads were washed at 42 °C with 2 × SSC, 0.1% SDS for 15 min, 1 × SSC, 0.1% SDS for 30 min, and finally stringently washed with 0.1 × SSC, 0.1% SDS for 15 min. Each hybridized tRNA sample was dissociated from the DNA with 15 μL 0.15M NaOH at 65 °C for 5 min and purified once more by 12% PAGE. The last step was omitted in the latter part of the study, because we found that only one tRNA species was present in the eluted sample. Moreover, in the later stage of the investigation, the hybridized tRNA was dissociated from the DNA by incubating the sample in 200 μL of Milli-Q H_2O at 95 °C for 5 min instead of treatment with NaOH.

Measurement of the m^1G content in $tRNA^{Pro}_{CGG}$ and $tRNA^{Pro}_{GGG}$ by two-dimensional thin-layer chromatography

Purified tRNA (10,000–20,000 cpm) was digested to nucleotides with nuclease P1 (Gehrke et al., 1982). The hydrolyzed tRNAs were applied on cellulose-coated plastic thin-layer chromatography plates. The chromatograms were developed in the first dimension using the solvent isobutyric acid–25% NH_3-H_2O (50/1.1/28.9 by volume) and in the second dimension using solvent HCl-isopropanol-H_2O (15/70/10 by volume; Nishimura, 1979). The radioactivity of each of the nucleotides was measured using a PhosphorImager from Molecular Dynamics (England). The average radioactivity from each mole of nucleotide was calculated as the total amount of the radioactivity from the various spots divided by the number of nucleotides in the tRNA. The molar yield of m^1G was thereafter calculated as radioactivity of the spot correlated to m^1G divided by the average value.

Detection of $tRNA^{Pro}_{CGG}$ and $tRNA^{Pro}_{GGG}$ by Northern blot analysis

Total tRNA was prepared and fractionated at acidic pH in the cold according to Varshney et al. (1991) to preserve the aminoacylated tRNA species. Transfer RNA samples were deacylated by incubation for 20 min at 37 °C in 0.5 M Tris-HCl, pH 9.0. The Northern blot and hybridization experiments were carried out as described (von Pawel-Rammingen et al., 1992). ^{32}P end-labeled oligonucleotide 5′-CCCCCGACACCCC ATGACGGTGC-3′ (complementary to nucleosides 24–46 in $tRNA^{Pro}_{GGG}$) and 5′-CCCCTTCGTCCCGAACGAAGTGC-3′ (complementary to nucleosides 24–46 in $tRNA^{Pro}_{CGG}$) were used to detect these two tRNA species. The amount of each tRNA species synthesized was expressed as the total cpm of the bound probe per μg of total tRNA applied on the gel.

Western blot analysis

The level of the TrmD peptide in different mutants was measured by Western blot analysis. The rabbit polyclonal antibody used was raised against highly purified tRNA (m^1G37)methyltransferase from *E. coli* and kindly provided by Dr. Michael Holmes, Richmond, Virginia, USA. Cells were grown in MOPS-Glucose medium at 37 °C or NAA medium at 42 °C from 5 klett units to 100 klett units. Cells were concentrated 50-fold in buffer A (6 mM K_2PO_4, pH 7.3, 6 mM β-mercaptoethanol, 1 mM EDTA, 10% ethylene glycol) and disrupted by sonication. No significant difference in the level of the TrmD peptide in the various mutants was observed whether or not protease inhibitors (chymostatin, leupeptin, pepstatin A, PMSF, and benzamidin) were included in the sample buffer. Thus, we believe that the level of the various TrmD polypeptides reflects the in vivo level and is not caused by protein degradation during the extraction procedure. Samples containing 20 μg of total cell protein were separated on 15% SDS PAGE and electrophoretically transferred to a nitrocellulose membrane (Hybond-C, Amersham, England). The streptavidin-HRP labeled secondary antibody specific for rabbit Ig was provided by an ECL™ kit (Amersham, England).

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