The modified wobble nucleoside uridine-5-oxyacetic acid in $tRNA^{Pro}_{cmo^5UGG}$ promotes reading of all four proline codons in vivo

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ABSTRACT

In *Salmonella enterica* serovar Typhimurium five of the eight family codon boxes are decoded by a tRNA having the modified nucleoside uridine-5-oxyacetic acid (cmo^5U) as a wobble nucleoside present in position 34 of the tRNA. In the proline family codon box, one ($tRNA^{Pro}_{cmo5UGG}$) of the three tRNAs that reads the four proline codons has cmo^5U34. According to theoretical predictions and several results obtained in vitro, cmo^5U34 should base pair with A, G, and U in the third position of the codon but not with C. To analyze the function of cmo^5U34 in $tRNA^{Pro}_{cmo5UGG}$ in vivo, we first identified two genes (*cmoA* and *cmoB*) involved in the synthesis of cmo^5U34. The null mutation *cmoB2* results in tRNA having 5-hydroxyuridine (ho^5U34) instead of cmo^5U34, whereas the null mutation *cmoA1* results in the accumulation of 5-methoxyuridine (mo^5U34) and ho^5U34 in tRNA. The results suggest that the synthesis of cmo^5U34 occurs as follows: U34 $\xrightarrow{?}$ ho^5U $\xrightarrow{CmoB}$ mo^5U $\xrightarrow{CmoA?}$ cmo^5U. We introduced the *cmoA1* or the *cmoB2* null mutations into a strain that only had $tRNA^{Pro}_{cmo5UGG}$ and thus lacked the other two proline-specific tRNAs normally present in the cell. From analysis of growth rates of various strains and of the frequency of +1 frameshifting at a CCC-U site we conclude: (1) unexpectedly, $tRNA^{Pro}_{cmo5UGG}$ is able to read all four proline codons; (2) the presence of ho^5U34 instead of cmo^5U34 in this tRNA reduces the efficiency with which it reads all four codons; and (3) the fully modified nucleoside is especially important for reading proline codons ending with U or C.

Keywords: tRNA; modified nucleoside; wobble; family codon box; uridine-5-oxyacetic acid; synthesis

INTRODUCTION

When the ribosome translates a genetic message, correct aminoacyl-tRNAs are selected one at a time through recognition of the anticodon that fit the A-site triplet in mRNA. The genetic code is composed of 64 triplets, of which three are recognized as stop codons. This leaves 61 codons as sense codons, all of which represents an amino acid in the final protein (Fig. 1A). Triplets with the same first two letters represent a codon box, resulting in 16 codon boxes. All four codons in eight of the codon boxes code for a single amino acid; these boxes are denoted family codon boxes (fourfold degenerate codon box). The remaining eight codon boxes are denoted mixed codon boxes (twofold degenerate codon box). Because each codon does not have a complementary tRNA, this requires that one tRNA be able to read more than one codon triplet that differs only in the third letter of the triplet as explained by the wobble hypothesis (Crick 1966). According to this hypothesis, uridine present in the first position (denoted position 34) of the anticodon (U34), base pairs with adenosine (A) and guanosine (G) but not with uridine (U) or cytidine (C), because the latter base pairs would be too short. However, not long after the wobble hypothesis was proposed, Nishimura and colleagues discovered that some tRNAs with modified nucleosides in the wobble position have decoding capacities that are more extensive than the wobble hypothesis would allow for (for review, see Nishimura 1979). One of these modified nucleosides is uridine-5-oxyacetic acid (cmo^5U34, earlier called V nucleoside), which base pairs not only with A and G as unmodified U would do as predicted by the wobble hypothesis, but also with U. In *Escherichia coli* and in *Salmonella enterica* serovar Typhimurium, tRNAs with genetically encoded U34 reading codons in the family boxes specific for valine, alanine, threonine, proline, and serine have cmo^5U34 or, at least in the alanine- and serine-specific tRNAs (Pope et al. 1978), its methylester $mcmo^5U34$ as a wobble nucleoside. The extended wobble capacity is caused by the ability of the cmo^5-side chain to influence the puckering equilibrium of the ribose ring in

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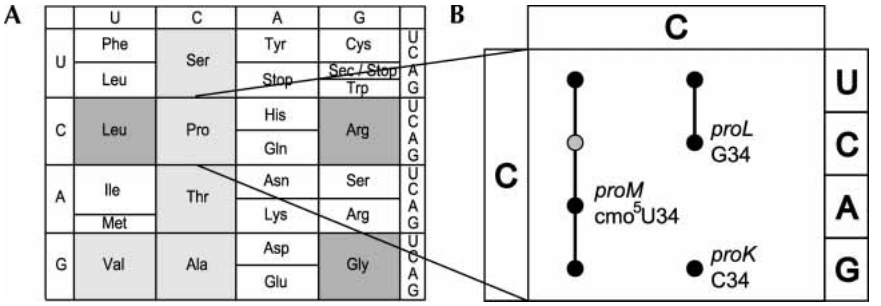

FIGURE 1. (*A*) The genetic code. The eight codon boxes with shaded background are the family codon boxes, containing four codons representing one amino acid (fourfold degenerate). The five family codon boxes in lighter shade contain tRNA having cmo⁵U as a wobble nucleoside. The codon boxes with white background are the mixed codon boxes. (*B*) The proline family codon box (CCN). *proK*, *L*, and *M* denote the genes encoding $tRNA^{Pro}_{CGG}$, $tRNA^{Pro}_{GGG}$, and $tRNA^{Pro}_{cmo5UGG}$, respectively, and the wobble nucleosides, which are present in position 34, are indicated. A circle corresponds to a codon read by a tRNA and a line connecting two or more circles indicates that the same tRNA is able to read those codons (e.g., the *proL* tRNA contains G34 and reads the CCU and CCC codons). The gray circle for $tRNA^{Pro}_{cmo5UGG}$ (codon CCC) indicates that this tRNA reads the CCC codon (results presented in this article), and the black circles show the codon reading abilities predicted by the wobble hypothesis and the revised wobble rules.

such a way that the majority of the cmo⁵U molecules are in the C2′-endo conformation, whereas the conformation of the unmodified U is equally distributed between the C2′- and C3′-endo conformations. cmo⁵U34 in the C2′-endo conformation base pairs not only with A and G, but also with U, thus explaining the codon binding capacities of a cmo⁵U34-containing tRNA (Yokoyama et al. 1985; Agris et al. 1992).

According to the revised wobble rules, cmo⁵U34, which is present only in tRNAs reading family codon boxes, should read A, G, and U, but not C. Triplet-dependent binding and in vitro protein synthesis have shown that this is indeed the case (Oda et al. 1969; Ishikura et al. 1971; Mitra et al. 1979; Samuelsson et al. 1980). The presence of cmo⁵U34 in the anticodon stem and loop construct (ASL) of $tRNA^{Val}_{cmo5UAC}$ enables this ASL to bind to A- and P-sites programmed with the A-, G-, and U- ending Val codons and cmo⁵U34 is required for translocation from the A- to the P-site at these codons (Phelps et al. 2004; for review, see Agris 2004). Whereas a completely unmodified $tRNA^{Ser}_{UGA}$ reads UCA well and UCU poorly, introduction of 5-methoxy-uridine (mo⁵U34) in the wobble position of an otherwise unmodified $tRNA^{Ser}_{UGA}$ enhances reading of UCU and UCG at the expense of less efficient reading of UCA (Takai et al. 1999). However, no ability to base pair with C(III) was observed (N34 denotes the nucleoside in the first position of the anticodon [wobble position] and N(III) denotes the third nucleoside in the codon). Thus, in vitro experiments support the theoretical considerations that xmo⁵U34 derivatives extend the binding capacities of U from only base pairing with A(III) and G(III) to base pairing with U(III) as well. Accordingly, one would expect that a bacterium having only a cmo⁵U34-containing tRNA to read the codons in a family box would not be viable because such a tRNA would not be able to read all four codons in such a box. However, in vivo there is evidence that some cmo⁵U34-containing tRNAs may base pair with C(III). First, a strain containing only the $tRNA^{Ala}_{cmo5UGC}$ is viable, demonstrating that $tRNA^{Ala}_{cmo5UGC}$ can read all four Ala codons (Gabriel et al. 1996). Second, according to the model that explains how an aberrant tRNA may induce frameshifting (Qian et al. 1998), a near cognate tRNA may outcompete the cognate tRNA, provided that the latter is defective in some manner. Following a 3-nt translocation, this near cognate tRNA resides in the P-site. If for some reason the ribosome pauses at this site (e.g., caused by low concentration of the next aminoacyl-tRNA, a rare codon, or a stop codon), the near cognate tRNA in the P-site may shift into the +1 frame. In the proline family codon box, three *Salmonella* tRNAs, encoded by the *proK*, *proL*, and *proM* genes, read the proline codons (Fig. 1B). The CCC codon is, according to the above theory, only read by the *proL* $tRNA^{Pro}_{GGG}$, because the *proM* $tRNA^{Pro}_{cmo5UGG}$ should not read the CCC codon. However, a frameshifting event at a CCC codon is dependent on $tRNA^{Pro}_{cmo5UGG}$, suggesting that this tRNA may read the CCC codon (Qian et al. 1998). It is not clear from this observation how efficient the cmo⁵U34-containing proline-specific tRNA reads the CCC codon. Third, a strain lacking the *proL* $tRNA^{Pro}_{GGG}$, which reads CCC and CCU codons, is viable, supporting the notion that the *proM* $tRNA^{Pro}_{cmo5UGG}$ is able to read the CCC codon (Chen et al. 2002), unless the *proK* $tRNA^{Pro}_{CGG}$ contributes to the CCC reading (Qian et al. 1998). Thus in vivo, at least $tRNA^{Ala}_{cmo5UGC}$ and perhaps $tRNA^{Pro}_{cmo5UGG}$ are able to read C-ending codons contrary to the theory and to several results obtained in vitro. Because the impact of some modified nucleosides is tRNA species dependent (Li et al. 1997), results from an analysis of decoding capacities for one tRNA, like $tRNA^{Ala}_{cmo5UGC}$, cannot be extrapolated to another tRNA, for example, $tRNA^{Pro}_{cmo5UGG}$. Moreover, the role of cmo⁵U34 was not addressed in these studies, as well-defined mutants defective in the synthesis of this modified nucleoside were not available.

Mutants defective in the synthesis of the aromatic amino acids are also deficient in cmo⁵U and mcmo⁵U in their tRNA (Björk 1980). The presence of shikimic acid in the growth medium restores the synthesis of these modified nucleosides in an *aroD* mutant but not in an *aroC* mutant (distal to shikimic acid but prior to chorismic acid; Fig. 2), demonstrating that there is a metabolic link between the synthesis of chorismic acid and the synthesis of cmo⁵U and mcmo⁵U (Björk 1980). One carbon atom in the $–O–CH_2–COO^-$ side chain of cmo⁵U originates from the methyl

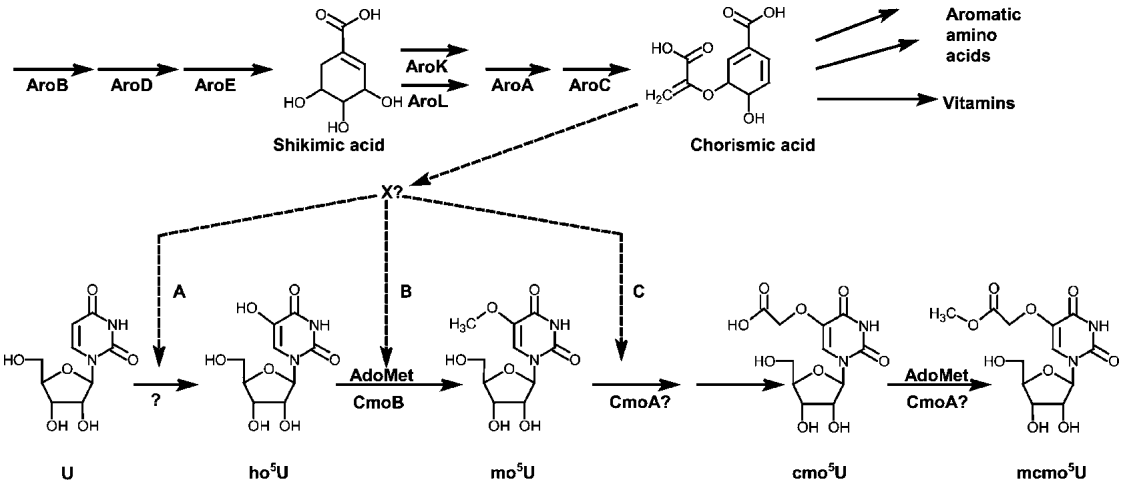

FIGURE 2. The proposed biosynthetic pathway for the synthesis of cmo^{5}U and mcmo^{5}U. The dashed reaction arrows indicate the link between the synthesis of cmo^{5}U and chorismic acid. The arrow denoted A is according to Hagervall et al. (1990) and the arrows denoted B and C are the suggested link between chorismic acid and the synthesis of cmo^{5}U34 according to present results. (X?) A possible unknown derivative of chorismic acid. (U) Uridine, (ho^{5}U) 5-hydroxyuridine, (mo^{5}U) 5-methoxyuridine (cmo^{5}U) uridine-5-oxyacetic acid, (mcmo^{5}U) uridine-5-oxyacetic acid methyl ester.

group of L-methionine whereas the other originates neither from carbonate nor from the C1 pool (Hagervall et al. 1990). Because no extra compound, except an occasional presence of ho^{5}U (at 0%–15% of the level of cmo^{5}U), was detected in tRNA from the Aro$^-$ mutants, it was suggested that chorismic acid or a metabolic derivative of it (X in Fig. 2) is required for the first step in the synthesis of cmo^{5}U (Hagervall et al. 1990). As the occasional presence of ho^{5}U might be unevenly distributed among the various tRNA species, it was not possible to state conclusively that a specific tRNA has an unmodified U34 or not. Clearly, the nonexistence of well-defined mutants defective in the biosynthesis of cmo^{5}U34 has hampered the in vivo analysis of the function of this modified wobble nucleoside. Moreover, to unravel the molecular mechanism of the metabolic link between the synthesis of aromatic amino acids and tRNA modification, it is necessary to identify the enzymes required for the synthesis of cmo^{5}U. Here, we report the identification of two genes encoding proteins required for the synthesis of cmo^{5}U. As one of the mutants (*cmoA*) accumulates mo^{5}U in tRNA whereas the other mutant (*cmoB*) accumulates ho^{5}U, the biosynthetic pathway can be schematized as shown in Figure 2. Using these well-defined mutants, we also show that the presence of cmo^{5}U34 in tRNA$^{Pro}_{cmo5UGG}$ is required for efficient reading of the U- and C-ending proline codons.

RESULTS

Isolation of a mutant lacking cmo^{5}U34

At the +1 frameshift site -NNN-CCC-UGA- (the triplets are in zero frame and one of the Cs in the sequence CCC is the inserted nucleoside resulting in the +1 frameshift mutation) the near cognate tRNA$^{Pro}_{cmo5UGG}$, encoded by the *proM* gene, may read the codon CCC provided either that the cognate CCC-reading tRNA$^{Pro}_{GGG}$ is defective (Qian et al. 1998) or absent (this article). Following a normal 3-nt translocation, the P-site-located near cognate tRNA$^{Pro}_{cmo5UGG}$ slips forward 1 nt provided that the ribosome is stalled, which may happen if the codon in the A-site is a stop codon (Qian et al. 1998). If such a frameshift mutation is in the *hisD* gene, and the strain is also deleted for the *proL* gene, the strain will be phenotypically His$^+$, because the +1 frameshift mutation in the *hisD* gene will be suppressed according to the model presented above. However, the presence of the cmo^{5}U34 wobble nucleoside is a prerequisite for the His$^+$ phenotype (Qian et al. 1998). Therefore, we predict that a mutation in an enzyme required for the synthesis of cmo^{5}U34 would induce a His$^-$ phenotype of the His$^+$ strain having the mutations Δ*proL* and a frameshift mutation in the *his*-operon.

We have devised a screening method based on these observations. The parent strain GT6606 (pTHF14/Δ*proL*, *hisO1242*, *hisD3749*) contains the *hisD3749* allele, which is a +1 frameshift mutation resulting in the sequence -CCC-UGA in the *hisD* gene. This strain lacks tRNA$^{Pro}_{GGG}$, caused by the Δ*proL* deletion, which induces suppression of the *hisD3749* mutation, and therefore the strain GT6606 is His$^+$. Mutations were induced in strain GT6604, which is a pool of *EZ::TN* insertions, by overexpression of DinB, which induces single-base deletions and base substitutions at a ratio of 2:1 (Wagner and Nohmi 2000). Phage P22 was grown on such mutagenized cells and the resulting phage stock was used to infect the parent strain GT6606 (pTHF14/Δ*proL*, *hisO1242*, *hisD3749*). Clones resistant to kanamycin (Km^R) were selected on medium E plates containing 0.2% glucose and "low histidine" (0.1 μM histidine). Using this concentration of histidine, 3 to 4 days of incubation at 37°C

results in a clear difference in colony size between the parent strain GT6606, which is His^+, and strain GT6607, which is His^- due to lack of cmo^5U34 in $tRNA^{Pro}_{cmo5UGG}$ caused by the *aroD*::Tn*10* mutation (Björk 1980). The smaller colony size of the latter strain is a result of less frameshift suppression caused by the cmo^5U34 deficiency of the $tRNA^{Pro}_{cmo5UGG}$. Among approximately 45,000 Km^R colonies, 534 tiny colonies were found that potentially were defective in suppression of the *hisD3749* mutation. Histidinol is the substrate for the HisD enzyme (for review, see Winkler 1996). A mutant of interest should grow poorly on a histidinol-containing plate but normally on a histidine plate. Such a screen should exclude all mutations causing slow growth for reasons other than poor frameshift suppression of the *hisD3749* mutation. Ninety-five of the Km^R transductants that grew poorly or not at all on histidinol but grew normally or nearly normally on histidine containing plates were saved for further testing.

The parent strain GT6606 (pTHF14/Δ*proL*, *hisO1242*, *hisD3749*) also contains the plasmid pTHF14, which harbors a *lacZ* gene with a similar frameshift mutation as *hisD3749* in the beginning of the *lacZ* gene (Hagervall et al. 1993). These cells are Lac^+ because of suppression of this frameshift mutation in the *lacZ* gene in a way similar to the suppression of the *hisD3749* mutation. To identify true frameshift antisuppressors from other mutations of no interest, we measured the level of suppression of the frameshift mutation in the *lacZ* gene. Of the 95 His^- mutants isolated above, one mutant, strain GT6631, has a significant decrease in β-galactosidase activity (Fig. 3). Apparently a low but significant suppression of the frameshift mutation in the *lacZ* gene still occurs in strain GT6631. Analysis of modified nucleosides in total tRNA from this mutant showed a complete loss of cmo^5U34 (data not shown).

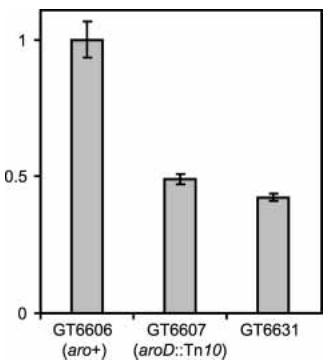

FIGURE 3. β-Galactosidase assay showing suppression of the +1 frameshift mutation in *lacZ* of pTHF14. GT6631 is a mutant of the parental strain GT6606 that grew poorly on plates containing histidinol, but grew normally on plates containing histidine. All values are averages of three independent cultures and relative to the values in strain GT6606 (set to 1.0, which equals 1345 ± 88 miller units). A Student's *t*-test (two-tailed; two sample equal variance) shows that both GT6607 and GT6631 are significantly lower than GT6606 ($p < 0.001$).

The EZ::TN transposon is inserted in yecO, a gene of unknown function

The transposon *EZ::TN* used to make the pool of randomly inserted transposons contains the plasmid origin R6Kγori. Upon digestion of DNA containing this transposon, plasmids can therefore be generated that can only replicate in an *E. coli* strain containing the λ pir protein, which is required for R6Kγori to be functional. To clone the *EZ::TN* insertion point, chromosomal DNA prepared from strain GT6631 was digested, ligated, and transformed into *E. coli* strain GRB1369 (λ *pir*$^+$). Plasmids were isolated from three different transformants and the DNA next to the transposon was sequenced. All three plasmids had the transposon inserted in the *proQ* gene. Various P22 crosses to different strains revealed a genetic inconsistency and it was shown that the original mutant GT6631 unexpectedly contained two transposons, one inserted in *proQ* and the other in the *yecO* gene (data not shown). A strain having only a transposon in *proQ* is still His^+, whereas a strain [GT6811 (*yecO*::EZ)] with only a transposon inserted in the *yecO* gene is His^-. When a phage lysate was grown on the latter strain and used as donor in a cross with strain GT6787 (Δ*proL*, *hisO1242*, *hisD3749*) as recipient, 100% (300 tested) of the Km^R transductants were also His^-, implying that the His^- phenotype is due to either the insertion in *yecO* itself or a mutation in a gene very close to it.

The *yecO* gene is the first gene in a potential two-cistron operon, predicted in RegulonDB (Salgado et al. 2004), containing another gene, *yecP*, downstream from *yecO*. We have denoted these genes *cmoA* (*yecO*) and *cmoB* (*yecP*), respectively (Fig. 4). Both CmoA and CmoB are predicted S-adenosyl-L-methionine (SAM)-dependent methyltransferases, making them likely candidates for the two methyl transfer reactions potentially involved in the synthesis of cmo^5U34 and $mcmo^5U34$ (see Fig. 2 and Discussion).

CmoB and CmoA mediate the conversion of hydroxyuridine (ho^5U) to 5-methoxyuridine (mo^5U) and of mo^5U to cmo^5U, respectively

Strains carrying a replacement of *cmoA*, *cmoB*, or both genes by a Km^R cassette (Fig. 4) were generated according to the method devised by Datsenko and Wanner (2000). HPLC analysis of modified nucleosides in tRNA from these mutants, still containing the Km^R cassettes, revealed that they all lack cmo^5U. The *cmoB2<>kan* mutant contained instead of cmo^5U an extra compound identified as ho^5U (data not shown) and the same results were obtained when the Km^R cassette had been removed (Fig. 5C). These results suggest that the CmoB peptide is involved in the conversion of ho^5U to mo^5U. The *cmoA1<>kan* mutant contained two additional compounds identified as ho^5U and mo^5U. After FLP-mediated removal of the resistance markers the results were the same (Fig. 5B). Thus, the *cmoA* deletion mutant contained both ho^5U and mo^5U in tRNA prepared from

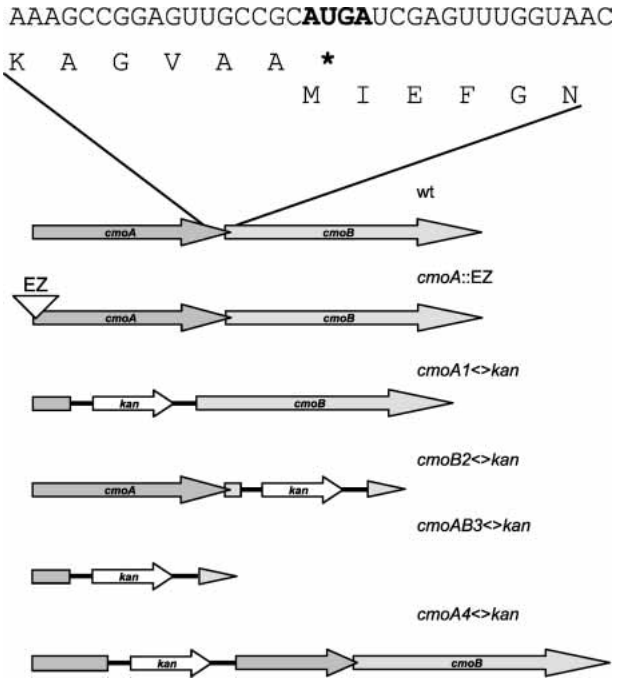

FIGURE 4. The gene organization of the *cmoAB* operon. (*Top*) The translational overlap between *cmoA* and *cmoB* is indicated with the RNA sequence and the corresponding peptide sequences. The start (AUG) codon for *cmoB* and the stop (UGA) codon for *cmoA* are indicated in bold. (*Bottom*) Schematic representation of the different mutants constructed in this study. The transposon *EZ*::*TN*⟨*R6K*γ*ori/KAN-2*⟩ is abbreviated EZ, the white triangle indicates the insertion point within the first few codons of the *cmoA* gene; *kan* is the kanamycin resistance cassette from plasmid pKD4.

exponentially growing cells. However, preparing tRNA from cells in stationary phase resulted in the accumulation of mo^5U and almost no ho^5U (Table 1, "24 h"). These results suggest that the insertions (the Km^R cassette or the "scar" sequence, see Materials and Methods) in the *cmoA* gene, which is located upstream of the *cmoB* gene, is polar on the expression of the latter gene, consistent with the suggestion that the CmoB peptide is involved in the conversion of ho^5U to mo^5U. To further test this suggestion, we replaced the DNA sequence corresponding to amino acids 60–68 in CmoA, which contains the AdoMet binding site, with the scar sequence. This resulted in an even lower level of mo^5U than the level observed in the former deletion (*cmoA1*; Table 1, compare strains GT6945 (*cmoA4<>frt*) and GT6854 (*cmoA1<>frt*)). Apparently, this *cmoA* construct was even more polar on the expression of *cmoB* than the first construct, consistent with the suggestion that insertions in the *cmoA* gene may be polar to different degree on the expression of the downstream gene *cmoB*. Thus, whereas the *cmoA* mutations did not induce one specific block in the synthesis of cmo^5U, the *cmoB* mutations did, making the latter mutation an excellent tool to study the influence of hypomodification of cmo^5U on translation efficiency.

We have shown earlier the occasional occurrence (0%–15% of the level of cmo^5U) of ho^5U in tRNA prepared from an *aroD* mutant (Hagervall et al. 1990). Analysis of tRNA from strain GT6909 (*aroD553*::Tn*10*) confirmed the absence of cmo^5U and a low level of ho^5U but we also observed a low level of mo^5U (Fig. 5D). Analysis of tRNA from strain GT6909 (*aroD553*::Tn*10*) grown into the stationary phase revealed an increased level of mo^5U at the expense of ho^5U and still no detectable level of cmo^5U (Table 1, *aroD*, "24 h"). Apparently, the *aroD* mutation does not induce a specific block in the synthesis of cmo^5U, and chorismic acid or a derivative of it participates in all steps known leading to cmo^5U, although the degree of requirement seems to be quite different in different biosynthetic steps. Because of this heterogeneous accumulation of various intermediates in the synthesis of cmo^5U in an *aroD* mutant, an *aroD* mutation is less suited as a tool to study the functional aspect of cmo^5U.

ho^5U and mo^5U in proM tRNA influences +1 frameshift suppression differently

To investigate the effect on +1 frameshifting by the partially modified $tRNA^{Pro}_{ho5UGG}$ and $tRNA^{Pro}_{mo5UGG}$ we constructed a series of strains (GT6844–GT6851) having Δ*proL*, *hisO1242*, *hisD2504*::mu*dK* with or without the *hisD3749* +1 frameshift mutation, and the three deletions *cmoA1<>frt*, *cmoB2<>frt*, and *cmoAB3<>frt*. The mu*dK* is inserted after the 71st codon of the *hisD* gene, so that an

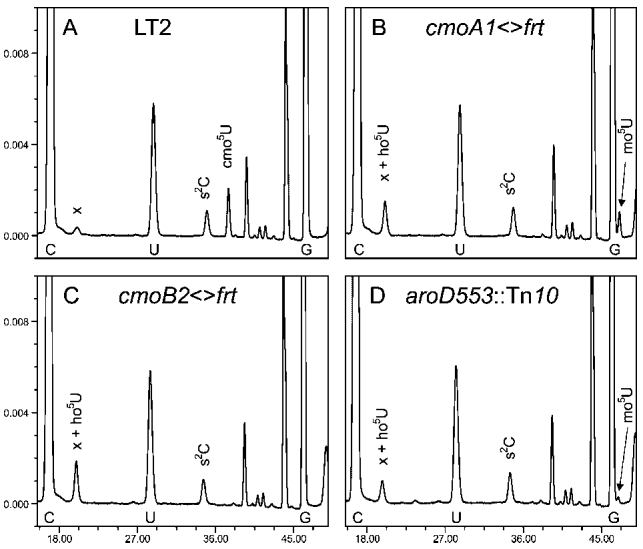

FIGURE 5. HPLC chromatograms monitored at 290 nm. The positions of cmo^5U, ho^5U, and mo^5U are indicated *above* the corresponding peaks. The three major nucleosides C, U, and G are indicated *below* the corresponding peaks. "x" indicates an unidentified compound with a spectrum that is not similar to ho^5U, comigrating with ho^5U in all analyses. About 25 μg of total tRNA was digested to nucleosides and analyzed. The degradation procedure used, the treatment by alkaline phosphatase at pH 8.3, hydrolyzes most of $mcmo^5U$ to cmo^5U.

TABLE 1. Quantification of cmo⁵U, ho⁵U, and mo⁵U compared to Ψ in tRNA from different strains

Strain	Relevant genotype	Growth conditions	ho⁵U/Ψ[a]	mo⁵U/Ψ	cmo⁵U/Ψ
LT2	Wild type	100 klett	0	N/D[b]	0.082
LT2	Wild type	24 h	0	N/D	0.079
GT6854	*cmoA1<>frt*	100 klett	0.041	0.036	N/D
		24 h	0.0017	0.087	N/D
GT6945	*cmoA4<>frt* (ΔSAM)	100 klett	0.084	N/D	N/D
GT6856	*cmoB2<>frt*	100 klett	0.072	N/D	N/D
GT6858	*cmoAB3<>frt*	100 klett	0.072	N/D	N/D
GT6909	*aroD553*::Tn*10*	100 klett	0.014	0.0081	N/D
		24 h	0.011	0.023	N/D

The area of each compound monitored at 290 nm was divided with that of pseudouridine (Ψ) at 254 nm. Cells were either harvested at 100 klett units (about 4×10^8 cells/mL) or after incubation of the culture for 24 h.
[a]An unknown compound, comigrating with ho⁵U, was present in all samples, so all values for ho⁵U are corrected by subtracting the value found in tRNA from strain LT2 at the same conditions.
[b](N/D) Not detected.

in-frame fusion between *hisD* and *lacZ* is produced downstream from the mutation in *hisD3749* (the extra C in *hisD3749* is inserted in codon 14). The strains having the *hisD3749* mutation require a +1 frameshift to produce β-galactosidase, whereas strains without the *hisD3749* mutation were used as in-frame controls. Therefore, the β-galactosidase activity is a measurement of the level of the HisD–LacZ fusion protein and thereby a direct measurement of the frequency of frameshifts that correct the *hisD3749* frameshift mutation. In the wild-type strain, GT6850, frameshifting is about twice that observed in the *cmoB2<>frt* and *cmoAB3<>frt* mutants, which both have ho⁵U34 instead of cmo⁵U (Fig. 6). However, in the *cmoA1<>frt* mutant, which in addition to ho⁵U also has mo⁵U (Table 1), the level of frameshifting was reduced by 30% compared to that observed in the wild type. Apparently, the presence of mo⁵U is less detrimental to the ability of *proM* tRNA to induce frameshifting compared to the presence of ho⁵U. We conclude that the presence of ho⁵U instead of cmo⁵U34 in *proM* tRNA induces frameshifting less efficiently and thus decodes CCC less well compared to the *proM* $tRNA^{Pro}_{cmo5UGG}$ containing cmo⁵U.

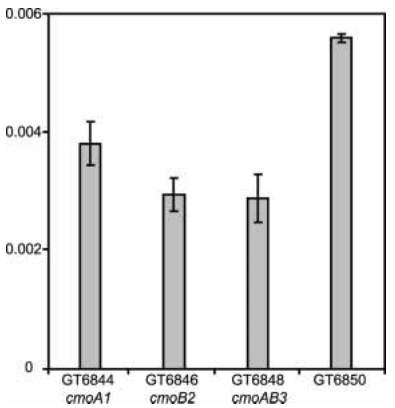

FIGURE 6. Relative frameshifting frequencies at the CCC-UGA frameshifting site in *hisD3749*. All strains are Δ*proL*, *hisO1242*, *hisD3749*, *hisD2504*::Mu*dK*, and the values are relative to the corresponding in-frame construct (same genotype except without *hisD3749*) and are averages of three cultures. The cells were grown in LB + 100 mg/L kanamycin at 37°C. A Student's *t*-test (two-tailed; two sample equal variance) shows that *cmoA1<>frt*, *cmoB2<>frt*, and *cmoAB3<>frt* significantly differ from GT6850 (wild type; $p \leq 0.001$). *cmoA1<>frt* is also different from *cmoB2<>frt* and *cmoAB3<>frt* ($p < 0.05$).

Presence of cmo⁵U34 in proM $tRNA^{Pro}_{cmo5UGG}$ is required for efficient reading of all four proline codons, including the CCU and CCC codons

Introduction of the *cmoA1<>frt* or the *cmoB2<>frt* mutations into the wild-type strain did not influence the growth rate in any of the three growth media tested (Table 2, part A). Because the cmo⁵U-containing *proM* $tRNA^{Pro}_{cmo5UGG}$, which should read CCA, CCG, and CCU but not CCC, is only one of three proline specific tRNAs, such an experiment may be too insensitive to reveal a small decrease in the efficiency of this tRNA in the presence of the other two proline-specific tRNAs. Therefore, we deleted the genes (*proK* and *proL*) for $tRNA^{Pro}_{CGG}$ reading CCG and $tRNA^{Pro}_{GGG}$ reading CCU/C, leaving the *proM* $tRNA^{Pro}_{cmo5UGG}$ as the only tRNA reading the proline codons (Fig. 1B). Surprisingly, such a strain is viable, showing that the *proM* $tRNA^{Pro}_{cmo5UGG}$ reads all four proline codons. In fact, such a strain grew as well as the wild-type strain in Rich-MOPS medium and showed only about 10% reduction in growth rate in MOPS-glucose and MOPS-acetate media, caused by the lack of the *proL* and *proK* tRNAs (Table 2, compare strain LT2 with strain GT6902 (*proK<>frt*, Δ*proL*)). Such a strain, which only has one tRNA (*proM* $tRNA^{Pro}_{cmo5UGG}$) reading the four proline codons, is ideal to study the effect of hypomodification of $tRNA^{Pro}_{cmo5UGG}$. We therefore introduced into strain GT6902 (*proK<>frt*, Δ*proL*), the *cmoA1<>frt*, *cmoB2<>frt*, or the *cmoAB3<>frt* alleles. In the

TABLE 2. cmo^{5}U34 in *proM* tRNA is required for efficient reading of proline codons

	Strain	Genotype	Rich MOPS		MOPS glucose		MOPS acetate	
			k[a]	$(k_m - k_c)/k_c$[b]	k	$(k_m - k_c)/k_c$	k	$(k_m - k_c)/k_c$
A	LT2	Wild type **(control)**	1.36	0.00	0.95	0.00	0.33	0.00
	GT6854	*cmoA1<>frt*	1.36	0.00	0.93	–0.02	0.34	0.02
	GT6856	*cmoB2<>frt*	1.36	0.01	0.95	–0.01	0.34	0.02
	GT6858	*cmoAB3<>frt*	1.36	0.00	0.93	–0.02	0.34	0.02
B	GT6902	Δ*proL, proK<>frt* **(control)**	1.35	0.00 (–0.01)	0.86	0.00 (**–0.09**)	0.29	0.00 (**–0.12**)
	GT6913	*cmoA1<>frt,* Δ*proL, proK<>frt*	1.09	**–0.20**	0.68	**–0.21**	0.24	**–0.16**
	GT6914	*cmoB2<>frt,* Δ*proL, proK<>frt*	1.01	**–0.25**	0.63	**–0.27**	0.22	**–0.22**
	GT6915	*cmoAB3<>frt,* Δ*proL, proK<>frt*	1.01	**–0.25**	0.63	**–0.26**	0.22	**–0.25**
C	GT6877	*proK<>frt* (**control**)	N.D.[c]	N.D.	0.96	0.00 (0.01)	N.D.	N.D.
	GT6899	*cmoAB3<>frt, proK<>frt*	N.D.	N.D.	0.93	–0.03	N.D.	N.D.
D	GT6879	Δ*proL* (**control**)	N.D.	N.D.	0.94	0.00 (–0.01)	N.D.	N.D.
	GT6912	*cmoAB3<>frt,* Δ*proL*	N.D.	N.D.	0.81	**–0.14**	N.D.	N.D.

Numbers in bold indicate those that we consider significantly different from the relevant control. Numbers within parentheses are the differences in growth rates of the control strains compared to LT2.
[a]Growth rate (h^{-1}), $k = \ln 2/g$, where g (h) is the generation time.
[b](k_m) Growth rate of mutant, (k_c) growth rate of control strain (A: LT2, B: GT6902, C: GT6877, D: GT6879).
[c](N/D) Not determined.

three media tested, there was a 16%–27% reduction in growth rate for all mutants compared to the control strain GT6902 (*proK<>frt*, Δ*proL*) (Table 2, part B). Thus, the presence of ho^{5}U or mo^{5}U instead of cmo^{5}U in *proM* tRNA reduced the efficiency of this tRNA to read the four proline codons. According to the theory (Crick 1966; Nishimura 1979) and to in vitro results (Oda et al. 1969; Ishikura et al. 1971; Mitra et al. 1979; Samuelsson et al. 1980; Takai et al. 1999; Agris 2004; Phelps et al. 2004), $tRNA^{Pro}_{cmo5UGG}$ should not read the CCC codon, which is normally decoded by the *proL* $tRNA^{Pro}_{GGG}$. Therefore, we expected that the reduction caused by the hypomodification was mainly due to an inefficient reading of the CCC codon. If so, by combining the *cmoAB3<>frt* mutation, which results in the presence of ho^{5}U instead of cmo^{5}U in the *proM* tRNA, with either *proK<>frt* or Δ*proL* mutations, a differential effect would be observed, that is, a larger decrease in growth rate would be observed when the *proL* $tRNA^{Pro}_{GGG}$ was lacking than when *proK* $tRNA^{Pro}_{CGG}$ was absent in conjunction with the *cmoAB3<>frt* mutation (Fig. 1B). Indeed, this was the case, because strain GT6912 (*cmoAB3<>frt*, Δ*proL*) grew 14% slower than the corresponding control strain whereas strain GT6899 (*cmoAB3<>frt*, *proK<>frt*) grew as its control strain (Table 2, parts C and D). Therefore, ho^{5}U can only partially substitute for the cmo^{5}U in base pairing with U and C. We conclude that the *proM* $tRNA^{Pro}_{cmo5UGG}$ is able to read all four proline codons, including the CCC codon, but requires the presence of cmo^{5}U for an efficient reading.

DISCUSSION

We know from earlier work that chorismic acid is required at an early step in the synthesis of cmo^{5}U (Björk 1980). However, how and at what step chorismic acid is required in the synthesis of cmo^{5}U is not known. One of the two carbon atoms of the side chain –O–CH_2–COOH originates from the methyl group of L-methionine, suggesting that at least one step in the synthesis of cmo^{5}U is an AdoMet-dependent transmethylation reaction. We describe here the identification of two genes (*cmoA* and *B*) involved in the synthesis of cmo^{5}U. Both proteins contain an AdoMet-binding motif and accordingly two biosynthetic steps in the synthesis of cmo^{5}U should be AdoMet transmethylation reactions. One such step is the conversion of ho^{5}U to mo^{5}U in which CmoB is involved. Even though CmoA, which apparently is involved in the conversion of mo^{5}U to cmo^{5}U, has an AdoMet-binding motif, it is not obvious how a methylation would be involved in this step, because the following carbon atom of the side chain –O–CH_2–COOH apparently does not originate from AdoMet (Hagervall et al. 1990). However, the CmoA peptide may be in a complex that, in addition to the conversion of mo^{5}U to cmo^{5}U, also participates in the methylation of cmo^{5}U to mcmo^{5}U. If so, the CmoA peptide is the AdoMet-binding peptide in the tRNA(mcmo^{5}U34)methyltransferase partially purified by Pope and Reeves (1978) that catalyzes the formation of mcmo^{5}U in at least the tRNAs specific for alanine and serine (Pope et al. 1978). Clearly, purification of the CmoA and CmoB peptides and analysis of the in vitro reactions must be performed before the reaction in which these peptides participate can be unraveled.

Present results confirm our earlier observation that the presence of chorismic acid or a derivative of it (X in Fig. 2) is critical in the formation of cmo^{5}U (Table 1; Björk 1980; Hagervall et al. 1990). As demonstrated earlier (Hagervall et al. 1990), we also observed accumulation of a small amount

of ho^{5}U, but also of mo^{5}U, in tRNA from cells lacking chorismic acid as in an *aroD* mutant (Table 1). Upon prolonged incubation of the culture, some of the ho^{5}U was converted to mo^{5}U (Table 1, "24 h"). Still, no cmo^{5}U was observed, suggesting that chorismic acid or a derivative thereof (X in Fig. 2) is required for the conversion of mo^{5}U to cmo^{5}U. If so, the chorismic acid requirement would be at the same step(s) in which the CmoA peptide participates. We noticed that upon prolonged incubation of cells in stationary phase in the absence of chorismic acid the amount of mo^{5}U increased at the expense of ho^{5}U, suggesting that this conversion can proceed without chorismic acid although at a low efficiency. Still, the level of mo^{5}U was much less than that observed in the *cmoA1* mutant under similar conditions (Table 1). We can not rule out the possibility that chorismic acid is also required earlier than at the mo^{5}U to cmo^{5}U conversion step, because ho^{5}U also accumulates under these conditions. The level of ho^{5}U accumulated in the absence of chorismic acid never reached the level observed in the *cmoB* mutant (Table 1), suggesting that this metabolite also stimulates the U to ho^{5}U conversion. Therefore, chorismic acid may play an integral role in the conversion of U to ho^{5}U, of ho^{5}U to mo^{5}U, and of mo^{5}U to cmo^{5}U. The requirement in the mo^{5}U to cmo^{5}U conversion seems to be stricter than in the earlier steps, as no cmo^{5}U was formed in the absence of chorismic acid.

We have earlier shown that m^{1}G37, which is present 3′ and next to the anticodon in all three proline tRNAs, improves reading frame maintenance (Björk et al. 1989). Deficiency of the wobble nucleoside cmo^{5}U34 may influence the synthesis of m^{1}G37. However, a base substitution in the wobble position does not influence the synthesis of m^{1}G37 (Qian and Björk 1997). Even if a deficiency of cmo^{5}U34 would cause a reduced level of m^{1}G37, such a deficiency would increase frameshifting rather than the decrease we observed (Fig. 6). It is also unlikely that cmo^{5}U34 deficiency would cause less charging of *proM* tRNA$^{Pro}_{cmo5UGG}$, because alterations in the anticodon loop and stem do not influence the prolinylation of tRNA (Qian and Björk 1997). Accordingly, the anticodon is not an important recognition determinant for prolyl-tRNA synthetase (McClain et al. 1994). Therefore, the observed effect on frameshifting and growth rates is caused by anticodon–codon aberrations and not from a reduced level of prolyl-tRNA.

We demonstrate here the viability of a cell having only the *proM* tRNA$^{Pro}_{cmo5UGG}$, thus showing that this tRNA can read all four proline codons including the C-ending codon. This was unexpected considering the stereochemical theory (Yokoyama et al. 1985) and several in vitro results (Oda et al. 1969; Ishikura et al. 1971; Mitra et al. 1979; Samuelsson et al. 1980; Takai et al. 1999; Agris 2004; Phelps et al. 2004), which suggest that the cmo^{5}U modification extends the wobble capacity of U to pair with A(III), G(III), and U(III) but not with C(III). However, this observation is similar to the report by Gabriel et al. (1996), who have shown that a strain having only the cmo^{5}U34-containing tRNA$^{Ala}_{cmo5UGC}$ is viable, suggesting that the tRNA$^{Ala}_{cmo5UGC}$ reads all four alanine codons in vivo. Moreover, Lim and Curran (2001) suggested, from theoretical considerations, that xo^{5}U derivatives may misread C(III). We also have to remember that the in vitro results were obtained using a tRNA devoid of all other modified nucleosides except for mo^{5}U (Takai et al. 1999) or an anticodon loop and stem derivative (Phelps et al. 2004), whereas our in vivo results were obtained with a tRNA lacking only the cmo^{5}U34. These differences in tRNA may partly explain the difference obtained between in vitro and in vivo. Although the mutant lacking the *proL* and *proK* tRNAs grew as wild type in one medium, it showed a clear growth disadvantage in the other two media tested (Table 2). Thus, under some physiological conditions cells containing *proL* and *proK* tRNAs have a selective advantage.

Hypomodification in conjunction with lack of *proK* tRNA$^{Pro}_{CGG}$did not influence the growth rate, whereas hypomodification in conjunction with lack of *proL* tRNA$^{Pro}_{GGG}$ did (Table 2). We therefore attribute the decrease in growth rate caused by the hypomodification in the *proL, proK* double mutant to the inefficient reading of the CCC and CCU codons, because these codons are normally read by the *proL* tRNA$^{Pro}_{GGG}$ (Fig. 1B). These results suggest that the cmo^{5}U modification is able to interact with C(III) and U(III). Therefore, the codon reading of the *proM* tRNA$^{Pro}_{cmo5UGG}$ is not the outcome of a "two-out-of-three" reading, which implies that only the two first nucleosides of the anticodon base pair with C(I) and C(II) and the third nucleoside does not interact with the third nucleoside in the codon (Lagerkvist 1978). We observed a larger decrease in growth rate in the Δ*proL*, *proK*<>*frt* double mutant than that observed in the Δ*proL* single mutant. This difference may be attributed to less hypomodified *proM* tRNA$^{Pro}_{ho5UGG}$ available to read the CCC and CCU codons, when it also has to read CCG codons, in the double mutant compared to that available in the single mutant.

The stability of the C3′- and C2′-endo conformations of pU is almost the same, whereas the C2′-endo form of pcmo^{5}U and pmo^{5}U is more stable than the C3′-endo conformation (enthalpy differences between C2′- and C3′-endo are 0.1 (pU), −0.72 (pmo^{5}U), and −0.67 (pcmo^{5}U) kcal/mole (Yokoyama et al. 1985). Interestingly, pho^{5}U favors the C2′-endo conformation (−0.28 kcal/mole difference) less than either pmo^{5}U or pcmo^{5}U but more than pU. Thus, from these considerations we would expect the largest difference in growth rate between the fully modified tRNA containing cmo^{5}U34 and tRNA containing ho^{5}U34. This was also the case when we analyzed the antisuppressor activity (Fig. 6, cf. wild type and *cmoB* mutant) and the reduction in growth rate caused by the *cmoB2*<>*frt* deletion, which results in only ho^{5}U being present in its tRNA (Table 2). The stability of the C2′-endo conformation for pmo^{5}U and pcmo^{5}U are almost the same, but we still observed a significant difference between *cmoA1*<>*frt*, which contains

mo^{5}U in its tRNA, and the wild type, both in the antisuppressor assay (Fig. 6) and in growth rate determinations (Table 2). However, the *cmoA1<>frt* mutant contains a mixture of ho^{5}U and mo^{5}U, but we do not know how these two intermediates are distributed in the *proM* tRNA pool. Therefore, the phenotypic differences we observed between the wild type and the *cmoA1<>frt* mutant might be attributed to the presence of ho^{5}U in a fraction of the *proM* tRNA. We conclude that although our results are not as expected from the stereochemical theory (the fact that the wild-type *proM* $tRNA^{Pro}_{cmo5UGG}$ in fact reads the CCC codon), they are consistent with the observed effect caused by the presence of ho^{5}U instead of cmo^{5}U in the *proM* tRNA.

The UGA suppressor *supK*, was originally thought to be the structural gene for the tRNA(mcmo^{5}U)methyltransferase (Atkins and Ryce 1974; Reeves and Roth 1975), because mutations in the *supK* gene reduce the activity of this enzyme in extracts (Reeves and Roth 1975). However, we know now that *supK* is allelic to *prfB*, which encodes release factor 2 (RF2), which recognizes the UGA and UAA stop codons (Kawakami et al. 1988). The translational stop codon for *cmoA*, which is UGA, overlaps the start codon AUG for *cmoB* (Fig. 4). Thus, a defective RF2 may allow read-through at the *cmoA* stop codon, resulting in less CmoB and a 45-amino-acid elongated CmoA, which may be inactive. Thus, a defective RF2 may result in less CmoA activity, consistent with the suggestion that the CmoA peptide is part of the tRNA(mcmo^{5}U)methyltransferase. Further work on the function of CmoA may solve the conundrum of how a defective RF2 can reduce the activity of the tRNA(mcmo^{5}U)methyltransferase in extracts.

MATERIALS AND METHODS

Bacteria and growth conditions

All strains are derivatives of *Salmonella enterica* serovar Typhimurium strain LT2 (Table 3). As rich liquid medium, either LB (Bertani 1951) or NB (0.8% Difco nutrient broth; Difco Laboratories) supplemented with the aromatic amino acids, aromatic vitamins, and adenine at concentrations as described previously (Davis et al. 1980) were used. MOPS (morpholinepropanesulfonic acid) medium supplemented with 0.4% (w/v) glucose or 0.4% (w/v) sodium acetate (Neidhardt et al. 1974) and rich MOPS (Neidhardt et al. 1977) were used as defined liquid media for growth rate determinations. As solid rich medium, TYS agar (10 g of Trypticase peptone, 5 g of yeast extract, 5 g of NaCl, and 15 g of agar/L) was used. Medium E (Vogel and Bonner 1956) containing 15 g agar/L and 0.2% glucose was used as solid minimal medium. All growth experiments were done at 37°C. To determine the growth rates, an overnight culture of the different strains were diluted to 0.05–0.1 OD_{420} units in prewarmed medium, pregrown to at least a fivefold increase in optical densities, never exceeding an $OD_{420} = 0.8$ during this pregrowth period. The cell suspension was then diluted to an OD_{420} of 0.05, and growth was monitored with a Shimadzu UV-1601 spectrophotometer at 420 nm during at least a 10-fold increase in cell density. Growth rate is expressed as k where $k = \ln 2$/mass doubling time in hours. Concentrations of the antibiotics used were, unless otherwise stated, Carbenicillin (Cb): 50 mg/L; Kanamycin (Km): 100 mg/L; Chloramphenicol (Cm): 12.5 mg/L.

Genetic procedures

Transduction with phage P22 HT105/1 (*int-201*; Schmieger 1972) was performed as described previously (Davis et al. 1980). Mutagenesis was performed as follows: A pool of transposon insertions was generated in strain GT6589 (pSMP24/Δ*proL*, *hisO1242*, *hisD3749*, *zef-201*::Tn*10d*Cm) using the *EZ::TN ⟨R6Kγori/KAN-2⟩Tnp Transposome Kit* (Epicentre), producing a pool of approximately 40,000 Km^R transposants. Colonies were pooled and pregrown in LB + Cb for 30 min at 37°C. To introduce point mutations, the pool was further mutagenized by inducing expression of DinB (DNA-polymerase IV, an error prone polymerase; Wagner et al. 1999; Wagner and Nohmi 2000) from the plasmid pSMP24, by diluting the culture into 10 tubes containing 1 ml LB + Cb + 0.04% L-arabinose. After overnight growth, phage P22 was added to make phage lysates on each of these 10 1-ml cultures. By transduction using these doubly mutagenized phage lysates and screening for a phenotype among the Km^R transductants, one may find mutants whose phenotype is either due to insertion of the transposon in a target gene or a point mutation within cotransduction distance from the transposon.

DNA sequencing was performed on chromosomal DNA, plasmid DNA, or PCR products as described in the manual for the Applied Biosystems ABI Prism cycle-sequencing BigDye Ready Reaction kit. Gene replacements were generated as described earlier by transforming with PCR products containing resistance markers flanked by the DNA sequence surrounding the sequence to be deleted (Datsenko and Wanner 2000). The resistance cassettes were later eliminated using FLP recombinase expressed from plasmid pCP20. This method leaves a "scar" sequence (denoted "*frt*") of 84 nt, containing stop codons in all reading frames and a Shine–Dalgarno site and a start codon at one end of the scar sequence. The deletion of *cmoA* (*cmoA1*) replaces nt 71–743 (of a total of 744 nt) of the *cmoA* gene with the scar sequence, so that the start codon of *cmoB* is replaced by the start codon of the scar sequence. This was done to try to avoid polar effects on the expression of *cmoB*. In *cmoA4* (ΔSAM) nt 177–204 (corresponding to the sequence encoding the SAM-binding site (VY**DLGCSLG**) were replaced. The deletion of *cmoB* (*cmoB2*) replaces nt 58–907 (of a total of 965 nt). The *cmoAB3* deletion replaces the region from nt 71 of *cmoA* to nt 907 of *cmoB*. The deletion of *proK* replaces the sequence corresponding to 3 nt before the 5′ end of the mature tRNA to 10 nt after the CCA end. Primers for amplifying the Km^R cassette from pKD4 were designed with 35–55 nt 5′-tails homologous to the sequences next to the sequence to be replaced. For PCR verification of the insertions, primers binding 100–350 bp away from the ends of the sequence to be replaced were used in combination with primers binding inside the Km^R cassette. The deletion of *proL* (Chen et al. 2002) was generated using the suicide plasmid pMAK705 (Hamilton et al. 1989), containing a temperature-sensitive replicon, carrying a 1.5-kb fragment containing a deletion from 26 bp before *proL* to 5 bp after *proL*.

TABLE 3. *Salmonella enterica* serovar Typhimurium and *Escherichia coli* strains and plasmids

Strain	Relevant genotype	Source
Salmonella enterica serovar Typhimurium		
GT6589	pSMP24/Δ*proL, hisO1242, hisD3749, zef-201*::Tn*10d*Cm	This study
GT6604	Pool of EZ[a]-insertions in GT6589	This study
GT6606	Δ*proL, hisP1242, hisD3749, zef-201*::Tn*10d*Cm/pTHF14	This study
GT6607	*aroD553*::Tn*10*, Δ*proL, hisO1242, hisD3749, zef-201*::Tn*10d*Cm/pTHF14	This study
GT6631	*proQ*::EZ[a], *cmoA*::EZ[a], Δ*proL, hisO1242, hisD3749, zef-201*::Tn*10d*Cm/pTHF14	This study
GT6787	Δ*proL, hisO1242, hisD3749*	This study
GT6811	*cmoA*::EZ[a], Δ*proL, hisO1242, hisD3749*	This study
GT6831	*cmoA1<>kan*	This study
GT6833	*cmoB2<>kan*	This study
GT6835	*cmoAB3<>kan*	This study
GT6844	*cmoA1<>frt*, Δ*proL, hisO1242, hisD3749, hisD2504*::Mu*dK*	This study
GT6845	*cmoA1<>frt*, Δ*proL, hisO1242, hisD2504*::Mu*dK*	This study
GT6846	*cmoB2<>frt*, Δ*proL, hisO1242, hisD3749, hisD2504*::Mu*dK*	This study
GT6847	*cmoB2<>frt*, Δ*proL, hisO1242, hisD2504*::Mu*dK*	This study
GT6848	*cmoAB3<>frt*, Δ*proL, hisO1242, hisD3749, hisD2504*::Mu*dK*	This study
GT6849	*cmoAB3<>frt*, Δ*proL, hisO1242, hisD2504*::Mu*dK*	This study
GT6850	Δ*proL, hisO1242, hisD3749, hisD2504*::Mu*dK*	This study
GT6851	Δ*proL, hisO1242, hisD2504*::Mu*dK*	This study
GT6854	*cmoA1<>frt*	This study
GT6856	*cmoB2<>frt*	This study
GT6858	*cmoAB3<>frt*	This study
GT6877	*proK<>frt*	This study
GT6879	Δ*proL*	This study
GT6899	*cmoAB3<>frt, proK<>frt*	This study
GT6902	Δ*proL, proK<>frt*	This study
GT6909	*aroD553*::Tn*10*	This study
GT6912	*cmoAB3<>frt*, Δ*proL*	This study
GT6913	*cmoA1<>frt*, Δ*proL, proK<>frt*	This study
GT6914	*cmoB2<>frt*, Δ*proL, proK<>frt*	This study
GT6915	*cmoAB3<>frt*, Δ*proL, proK<>frt*	This study
GT6944	*cmoA4<>kan* (ΔSAM)	This study
GT6945	*cmoA4<>frt* (ΔSAM)	This study
Escherichia coli		
GRB1369	λ *pir*	Miller and Mekalanos 1988
Plasmids		
pCP20	FLP recombinase helper plasmid	Datsenko and Wanner 2000
pKD4	Template plasmid for amplification of kan^R-cassette	Datsenko and Wanner 2000
pKD46	λ-Red recombinase helper plasmid	Datsenko and Wanner 2000
pSMP24	*dinB* (Amp^R)	D. Andersson, pers. comm.
pTHF14	+1 frameshift site (suppressible at CCC-UGA) in *lacZ*	Hagervall et al. 1993

[a]EZ is short for the transposon *EZ::TN*™ ⟨*R6Kγori/KAN-2*⟩

Nomenclature of mutants: an allele number followed by <> and "*kan*" indicates that the gene is replaced by the Km^R cassette (e.g., *cmoB2<>kan*). After FLP-mediated removal of the cassette, the mutation is referred to with the same allele number, but with "*frt*" as description of the replacing scar sequence (e.g., *cmoB2<>frt*).

Analysis of modified nucleosides in tRNA

Bacterial strains were grown overnight in medium LB, diluted 100 times in 100 mL of the same medium and grown to 100 klett units (approximately 4×10^8 cells/mL) or in 50 mL and grown into the stationary phase for 24 h. Cells were harvested by centrifugation, and the pellets were resuspended in 5 mL TE buffer (10 mM Tris, 1 mM Na_2EDTA at pH 8.0). Three milliliters TRIzol (Invitrogen Life Technologies) reagent were added and the suspension was incubated on ice during 1.5–2 h. Cell debris was pelleted by centrifugation and the aqueous phase was extracted with chloroform until no white interphase was formed. RNA was precipitated by 2.5 volumes of cold 99.5% ethanol containing 1% (w/v) potassium acetate. After washing the pellet twice with 70% ethanol, the pellet was dissolved in 2 mL buffer R200 (10 mM Tris-H_3PO_4 at pH 6.3, 15% ethanol, 200 mM KCl) and applied to a Nucleobond AX500 column (Macherey-Nagel Gmbh & Co.), preequilibrated with the same buffer. The column was washed once with 6 mL R200 and once with 2.5 mL R650 (same composition as R200, except with 650 mM KCl). Finally, tRNA was eluted with 7 mL R650. tRNA was precipitated by 0.7 volumes isopropanol, washed once with 70% ethanol, and dissolved in water. tRNA was digested to nucleosides by nuclease P1 followed by treatment with bacterial alkaline

phosphatase at pH 8.3 (Gehrke et al. 1982). The mcmo^{5}U is alkili labile, why most mcmo^{5}U is converted to cmo^{5}U under the conditions used.

The hydrolysate was analyzed by HPLC as described earlier (Gehrke and Kuo 1990) except that a Develosil 5μ RP-AQUEOUS C-30 column (Phenomenex) was used and the gradient was slightly modified to obtain optimal separations of cmo^{5}U, ho^{5}U, and mo^{5}U. The first buffer (A) had 0.5% instead of 2.5% methanol, and the time for the isocratic part of the gradient was extended from 0–12 min to 0–22 min. To identify mo^{5}U and ho^{5}U, synthetic markers were added to the digested tRNA from *cmoA1<>frt* and *cmoB2<>frt* mutants, respectively, and when these spiked samples were analyzed, areas of the compounds identified as mo^{5}U and ho^{5}U increased compared to the nonspiked digested tRNA (data not shown).

β-Galactosidase assays

β-Galactosidase assays were performed as described earlier (Hagervall et al. 1993). During the screening procedure cultures for β-galactosidase assays were grown in 2.2-ml 96-well polypropylene blocks (Marsh Biomedical Products) and assays were performed essentially as described by Griffith and Wolf (2002).

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