The wobble hypothesis revisited: Uridine-5-oxyacetic acid is critical for reading of G-ending codons

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ABSTRACT

According to Crick's wobble hypothesis, tRNAs with uridine at the wobble position (position 34) recognize A- and G-, but not U- or C-ending codons. However, U in the wobble position is almost always modified, and *Salmonella enterica* tRNAs containing the modified nucleoside uridine-5-oxyacetic acid (cmo⁵U34) at this position are predicted to recognize U- (but not C-) ending codons, in addition to A- and G-ending codons. We have constructed a set of *S. enterica* mutants with only the cmo⁵U-containing tRNA left to read all four codons in the proline, alanine, valine, and threonine family codon boxes. From the phenotypes of these mutants, we deduce that the proline, alanine, and valine tRNAs containing cmo⁵U read all four codons including the C-ending codons, while the corresponding threonine tRNA does not. A *cmoB* mutation, leading to cmo⁵U deficiency in tRNA, was introduced. Monitoring A-site selection rates in vivo revealed that the presence of cmo⁵U34 stimulated the reading of CCU and CCC (Pro), GCU (Ala), and GUC (Val) codons. Unexpectedly, cmo⁵U is critical for efficient decoding of G-ending Pro, Ala, and Val codons. Apparently, whereas G34 pairs with U in mRNA, the reverse pairing (U34-G) requires a modification of U34.

Keywords: wobble hypothesis; translation; tRNA; modified nucleoside; decoding

INTRODUCTION

The genetic message is read by tRNAs that decode one triplet at a time. Of the 64 codons, 61 are sense codons and represent an amino acid in the final protein. Triplets with the same first two nucleosides constitute a codon box, and if all four codons represent one amino acid, such a box is called a family codon box. In all organisms there are eight family codon boxes (Fig. 1, shaded), and in *Salmonella enterica* serovar Typhimurium, six of them are decoded by tRNAs of which one has uridine-5-oxyacetic acid (cmo⁵U34) or its methylester (mcmo⁵U34) in position 34 (the wobble position) (Fig. 2). These six family codon boxes are specific for leucine, valine, serine, proline, threonine, and alanine (Fig. 1, light shade). To read the four codons in such a family codon box, there are, besides the cmo⁵U34-containing tRNA, one (valine and alanine) or two (leucine, serine,

threonine, and proline) additional isoacceptor tRNAs. One of these isoacceptors has G as the wobble nucleoside, and in four boxes (leucine, proline, threonine, and serine) the third isoacceptor has C as the wobble nucleoside (Fig. 1). According to the wobble hypothesis (Crick 1966), G34 base-pairs with C and U as the third nucleoside of the codon [denoted C(III) and U(III)], whereas C34 only base-pairs with G(III). Uridine as the wobble nucleoside cannot interact with a pyrimidine in the mRNA, since two pyrimidines are too "short" to form a base pair. Therefore, it was thought that the G34-containing tRNAs are essential for decoding the U- and C-ending codons. However, U as the wobble nucleoside is almost always modified, and the cmo⁵-modification and the related modification 5-methoxy-uridine, mo⁵U, present in tRNA of *Bacillus subtilis*, is predicted to extend the wobble capacity to read not only A(III) and G(III), as predicted by the wobble hypothesis, but also U(III), but not C(III) (Yokoyama et al. 1985). Thus, the G34-containing tRNAs seems to be required to decode the C-ending codons in these family codon boxes. Most in vitro experiments with *Escherichia coli* tRNAs or anticodon stem-loops (ASLs) support the theoretical considerations that a U reads A(III) and G(III) and that cmo⁵U enhances the wobble to include U(III), but not C(III) (Oda

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		Second position of codon				Third position of codon (3' end)
		U	C	A	G	
First position of codon (5' end)	U	Phe Leu	Ser Cmo5U	Tyr Stop	Cys Trp	U C A G
	C	Leu Cmo5U	Pro Cmo5U	His Gln	Arg Cmo5U	U C A G
	A	Ile Met	Thr Cmo5U	Asn Lys	Ser Arg	U C A G
	G	Val Cmo5U	Ala Cmo5U	Asp Glu	Gly Cmo5U	U C A G

FIGURE 1. The genetic code. The eight codon boxes with shaded background are the family codon boxes, containing four codons encoding one amino acid (fourfold degenerate). The six lighter-shaded boxes contain tRNAs having cmo^5U as wobble nucleoside. The boxes with white background are the mixed codon boxes. 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. Filled circles indicate codon reading as predicted by the wobble hypothesis (Crick 1966) or the revised wobble rules (Yokoyama et al. 1985). Open circles indicate that those tRNAs are able to read also the C-ending codons (results presented in this study and in Näsvall et al. 2004). Next to the symbol for each tRNA is indicated which wobble nucleoside it contains. The letters within parentheses below the wobble nucleoside in the family boxes for proline, threonine, alanine, and valine indicate the last letter in the name of the genes encoding the corresponding tRNAs (e.g., $\text{tRNA}_{\text{cmo}^5\text{UAC}}^{\text{Val}}$ is encoded by the four genes *valT*, *valU*, *valX*, and *valY*, and $\text{tRNA}_{\text{GGG}}^{\text{Pro}}$ is encoded by the gene *proL*).

et al. 1969; Ishikura et al. 1971; Mitra et al. 1979; Samuelsson et al. 1980; Takai et al. 1999; Phelps et al. 2004; Sørensen et al. 2005).

In contrast to the above-mentioned results obtained in vitro, there is evidence from in vivo experiments that $\text{cmo}^5\text{U34}$ -containing tRNAs base-pair also with C(III). A strain that lacks the G34-containing $\text{tRNA}_{\text{GGG}}^{\text{Pro}}$ (the subscript indicates the sequence of the anticodon in the 5' → 3' direction) and the C34-containing $\text{tRNA}_{\text{CGG}}^{\text{Pro}}$ and thus only has the $\text{cmo}^5\text{U34}$ -containing $\text{tRNA}_{\text{cmo}^5\text{UGG}}^{\text{Pro}}$ is viable, demonstrating that $\text{tRNA}_{\text{cmo}^5\text{UGG}}^{\text{Pro}}$ with $\text{cmo}^5\text{U34}$ as the wobble nucleoside can read all four proline codons (Näsvall et al. 2004). Based on a synergistic growth defect in mutants that lack $\text{tRNA}_{\text{GGG}}^{\text{Pro}}$ and are hypo-modified in the wobble position of $\text{tRNA}_{\text{cmo}^5\text{UGG}}^{\text{Pro}}$, the presence of $\text{cmo}^5\text{U34}$ was suggested to promote an efficient reading of C- and U-ending proline codons (Näsvall et al. 2004). Similarly, a strain having only $\text{tRNA}_{\text{cmo}^5\text{UGC}}^{\text{Ala}}$ with $\text{cmo}^5\text{U34}$ as the wobble nucleoside is also viable (Gabriel et al. 1996). It was recently shown that the binding of $\text{tRNA}_{\text{cmo}^5\text{UGC}}^{\text{Ala}}$ to GCC codons is only slightly weaker than binding to GCA, and that the kinetics of A-site binding at GCC is within the

range for cognate interactions (Kothe and Rodnina 2007). Thus, at least $\text{tRNA}_{\text{cmo}^5\text{UGG}}^{\text{Pro}}$ and $\text{tRNA}_{\text{cmo}^5\text{UGC}}^{\text{Ala}}$ are able to read codons ending with C(III), contrary to the theory and to most results obtained in vitro. However, the impact of $\text{cmo}^5\text{U34}$ on decoding by $\text{tRNA}_{\text{cmo}^5\text{UGC}}^{\text{Ala}}$ was not addressed by Gabriel et al. (1996) or Kothe and Rodnina (2007), since their analysis was performed with fully modified $\text{tRNA}_{\text{cmo}^5\text{UGC}}^{\text{Ala}}$ (Gabriel et al. 1996; Kothe and Rodnina 2007). Here, we extend these studies to elucidate whether the $\text{cmo}^5\text{U34}$ -containing tRNAs specific for valine and threonine are also able to read the four codons in the corresponding family boxes and if the presence of $\text{cmo}^5\text{U34}$ is required for such a reading in the family codon boxes specific for valine, alanine, and threonine.

To study the function of $\text{cmo}^5\text{U34}$ in vivo, we need a way to manipulate the presence of $\text{cmo}^5\text{U34}$ in tRNA. We have recently identified two genes (*cmoA* and *B*) whose products are required for the synthesis of $\text{cmo}^5\text{U34}$ (Näsvall et al. 2004). Deletion of the *cmoB* gene results in a complete absence of $\text{cmo}^5\text{U34}$ in tRNA, and all of the cmo^5U found in the wild type is present as the biosynthetic intermediate 5-hydroxyuridine (ho^5U) (Fig. 2; Näsvall et al. 2004). Therefore, we have changed the allelic state of the *cmoB* gene in our attempt to demonstrate the coding capacities of cmo^5U versus ho^5U in tRNAs specific for proline, alanine, and valine. Surprisingly, considering the wobble hypothesis and other predictions (Crick 1966; Yokoyama et al. 1985), our results show that cmo^5U is required for efficient decoding of G-ending codons by $\text{tRNA}_{\text{cmo}^5\text{UGC}}^{\text{Ala}}$, $\text{tRNA}_{\text{cmo}^5\text{UAC}}^{\text{Val}}$, and $\text{tRNA}_{\text{cmo}^5\text{UGG}}^{\text{Pro}}$. Thus, whereas G as the wobble nucleoside can base-pair with U in mRNA, apparently the reverse pairing [U34-G(III)] requires a modification of uridine.

RESULTS

$\text{tRNA}_{\text{cmo}^5\text{UGU}}^{\text{Thr}}$ containing $\text{cmo}^5\text{U34}$ cannot read all four threonine codons

S. enterica has three threonine isoacceptors ($\text{tRNA}_{\text{cmo}^5\text{UGU}}^{\text{Thr}}$, $\text{tRNA}_{\text{GGU}}^{\text{Thr}}$, and $\text{tRNA}_{\text{CGU}}^{\text{Thr}}$) (see Fig. 1). The G34-containing $\text{tRNA}_{\text{GGU}}^{\text{Thr}}$ is encoded by the genes *thrT* and *thrV* and the C34-containing $\text{tRNA}_{\text{CGU}}^{\text{Thr}}$ is encoded by the gene *thrW* (Fig. 3A). Strains lacking the genes encoding $\text{tRNA}_{\text{GGU}}^{\text{Thr}}$ and $\text{tRNA}_{\text{CGU}}^{\text{Thr}}$ were constructed by inserting a kanamycin resistance cassette flanked by FLP recombinase target sequences (FRTs) (Datsenko and Wanner 2000) into the *thrT*, *thrV*, or *thrW* genes. The three resulting single mutants lacking either the C34-containing $\text{tRNA}_{\text{CGU}}^{\text{Thr}}$ or one of the two genes encoding the G34-containing $\text{tRNA}_{\text{GGU}}^{\text{Thr}}$ were all viable, with no apparent growth phenotype on solid rich medium at 37°C (data not shown).

To test if $\text{tRNA}_{\text{cmo}^5\text{UGU}}^{\text{Thr}}$ is able to read all four threonine codons, we attempted to generate a mutant having $\text{tRNA}_{\text{cmo}^5\text{UGU}}^{\text{Thr}}$ as the only remaining threonine isoacceptor

FIGURE 2. The proposed biosynthetic pathway for the synthesis of *cmo*⁵U and *mcmo*⁵U. (Gray arrows) Indicate the link between chorismic acid (or an unknown derivative of it) and different steps in the synthesis of *cmo*⁵U according to Näsval et al. (2004). (U) Uridine; (*ho*⁵U) 5-hydroxyuridine; (*mo*⁵U) 5-methoxyuridine; (*cmo*⁵U) uridine-5-oxyacetic acid; (*mcmo*⁵U) uridine-5-oxyacetic acid methyl ester. (Adapted from Näsval et al. 2004 and reprinted with permission from the RNA Society ©2004.)

by deleting the two genes encoding the G34-containing tRNA^{Thr}_{GGU} (*thrT* and *V*) and the C34-containing tRNA^{Thr}_{CGU} (*thrW*). Whereas construction of mutants with one remaining gene encoding the G34-containing tRNA^{Thr}_{GGU} was possible, combining mutations in both genes encoding this tRNA failed (Table 1). These results suggest that a double mutant (*thrT thrV*) having only the *cmo*⁵U34- and C34-containing threonine isoacceptors is not viable and consequently indicates that tRNA^{Thr}_{GGU} is essential. Still, a few transductants appeared in the attempts to make the *thrT thrV* double mutant. Since it is known that in a growing culture of *Salmonella*, different loci can be transiently duplicated, the rare Km^R transductants may have both the wild-type allele and the mutated allele of *thrT* or *thrV*. Indeed, all of the 37 tested transductants possessed both the wild-type and the *thrT*<>*kan* alleles. Moreover, purification of 10 different Km^R transductants on nonselective plates revealed segregation of Km^R and Km^S clones as expected if the original transductant contains a duplication of the wild-type and the *thrT*<>*kan* alleles. We conclude that the G34-containing tRNA^{Thr}_{GGU} is essential, most likely because the *cmo*⁵U34-containing tRNA^{Thr}_{cmo5UGU} cannot recognize C-ending threonine codons.

The *cmo*⁵U-containing tRNA^{Val}_{cmo5UAC} by itself can only support growth at an extremely reduced rate

The two genes *valV* and *valW*, encoding two slightly different tRNA^{Val}_{GAC}s, are present as a tandem repeat in a dicistronic operon with no other genes (Fig. 3B). A mutant (Δ *valVW*) lacking tRNA^{Val}_{GAC}, and thus having only the *cmo*⁵U-containing tRNA^{Val}_{cmo5UAC} to read the four valine codons (Fig. 1), was viable but showed a 70% reduction in growth rate (Table 2). These results indicate that, similarly to tRNA^{Pro}_{cmo5UGG} (Näsval et al. 2004), tRNA^{Val}_{cmo5UAC} is also

able to read all four valine codons, albeit with low efficiency.

As earlier reported, *cmoB* mutants have *ho*⁵U34 instead of *cmo*⁵U34 in their tRNA (Näsval et al. 2004). To test the impact of such hypo-modification on the decoding capacity of tRNA^{Val}_{cmo5UAC}, we disrupted the *cmoB* gene in a strain lacking the G34-containing tRNA^{Val}_{GAC}. This strain is viable but showed a decrease in growth rate compared to the parent strain (Table 2), and it also accumulated faster-growing suppressor mutants (data not shown). In addition to the slow-growth phenotype, cultures sometimes formed visible aggregates, which were caused by part of the population of cells forming long filaments (data not shown). Clearly, the presence of the *cmo*⁵-modification improves the decoding efficiency of tRNA^{Val}_{cmo5UAC}.

To test if increased levels of tRNA^{Val}_{cmo5UAC} would help mutants lacking tRNA^{Val}_{GAC} and *cmo*⁵U, we compared the growth of strains harboring either plasmid p815 (O'Connor 2002) (carrying the *E. coli valU* operon, containing three genes encoding tRNA^{Val}_{cmo5UAC} and one gene encoding tRNA^{Lys}_{mnm5s2UUU}) or plasmid pLG339 (vector control). Overexpression of tRNA^{Val}_{cmo5UAC} partially suppressed the growth phenotypes of the strains having only this tRNA (Fig. 4). Also, overexpression of the hypo-modified tRNA^{Val}_{ho5UAC} improved the growth of the Δ *valVW cmoB2* mutant, demonstrating that tRNA^{Val}_{cmo5UAC} at normal concentration is, indeed, dependent on the *cmo*⁵-modification.

In order to further study the efficiency of tRNA^{Val}_{cmo5UAC} in reading the four valine codons and the effect of having *ho*⁵U in place of *cmo*⁵U, we used the system described by Curran and Yarus (1989) to measure the in vivo A-site selection rates (Fig. 5). The mutant lacking both tRNA^{Val}_{GAC} and *cmo*⁵U (Δ *valVW cmoB2*<>*frt*) was not included because of difficulties in keeping the culture suppressor free, but also because of the filamentous growth phenotype,

FIGURE 3. Locations of tRNA genes in the *S. enterica* genome. (A) Threonine tRNAs. (Upper line) The *thrW* gene, encoding tRNA^{Thr}_{CGU}. (Middle line) The *rrnD* rRNA operon containing one of the two genes encoding tRNA^{Thr}_{GGU}. (Lower line) The *tufB* operon, containing the gene encoding tRNA^{Thr}_{cmo5UGC} as well as the second gene encoding tRNA^{Thr}_{GGU}. (B) Valine tRNAs. (Upper and middle lines) The two tRNA operons containing the four genes encoding tRNA^{Val}_{cmo5UAC}. (Lower line) The dicistronic *valV*, *valW* operon containing the two genes encoding tRNA^{Val}_{GAC}. (C) Alanine tRNAs. (Upper line) The three rRNA operons *rrnH*, *rrnA*, and *rrnB*, containing the genes encoding tRNA^{Ala}_{cmo5UGC}, have the same basic organization except an additional tRNA gene (*aspU*) at the end of *rrnH*. (Lower line) The *alaW*, *alaX* tRNA operon encoding tRNA^{Ala}_{GGC}. (D) Proline isoacceptors. (Upper line) The monocistronic *proL* gene, encoding tRNA^{Pro}_{GGG}. (Middle line) The *proK* gene, encoding tRNA^{Pro}_{CGG}. (Lower line) The operon containing the gene encoding tRNA^{Pro}_{cmo5UGG} as well as three other tRNA genes. (Black arrows) tRNA genes encoding threonine, valine, alanine, or proline tRNAs; (dark gray arrows) other tRNA genes; (light gray arrows) other genes. The anticodons of the relevant tRNAs are written below the genes. The drawings are not to scale. The asterisk (*) after *gltT* (which encodes tRNA^{Glu}_{mnm5s2UUC}) in A, middle line, indicates that the gene (STM3397) is not named in *Salmonella*. *gltT* is the name of an identical gene in the *E. coli* *rrnB* operon, which in *Salmonella* instead contains the genes *ileU* and *alaU* (asterisks in C).

which would produce unreliable OD values. As expected, the rate of A-site selection is severely reduced at all four codons in the strain lacking the G34-containing tRNA^{Val}_{GAC} (Fig. 5). Most severely affected was the rate at the GUC codon. This is not surprising considering the fact that this

strain lacks the tRNA (tRNA^{Val}_{GAC}) that is the major tRNA recognizing GUC codons. The data show the relative efficiency of fully modified tRNA^{Val}_{cmo5UAC} at recognizing the different codons; it recognized GUA, GUU, and GUG with equal efficiency, while, as expected, it was quite poor

TABLE 1. The cmo⁵U-containing tRNA^{Thr}_{cmo5UGU} is unable to decode all four threonine codons

Donor genotype → Recipient genotype ↓	<i>thrT</i> <> <i>kan</i> ^a (G34)	<i>thrV</i> <> <i>kan</i> ^a (G34)	<i>thrW</i> <> <i>kan</i> ^a (C34)
LT2 ^a	1.0 (2388) ^c	1.0 (~4700) ^c	1.0 (1954) ^c
<i>thrT</i> <> <i>frt</i> (G34) ^a	0.95 (2276) ^c	6.4 × 10⁻⁴ (3) ^c	0.99 (1934) ^c
<i>thrV</i> <> <i>frt</i> (G34) ^a	8.4 × 10⁻³ (20) ^{b,c}	1.2 (~5500) ^c	0.91 (1786) ^c
<i>thrW</i> <> <i>frt</i> (C34) ^a	0.90 (2157) ^c	1.1 (5224) ^c	0.92 (1807) ^c

^aP22 transductions designed to construct mutants lacking both genes encoding the G34-containing tRNA^{Thr}_{GGU} (*thrV* and *thrT*). Mutants carrying alleles disrupted with Km^R cassettes (indicated by <>*kan*) were used as donors, and mutants with disrupted alleles without resistance cassettes (indicated by <>*frt*) were used as recipients. Strain LT2 (wild-type) and strains lacking the C34-containing tRNA^{Thr}_{CGU} (encoded by *thrW*) were used as controls. For each individual transduction, the same volume of phage lysate was used.

^bSeven colonies were visible after 24 h of incubation at 37°C. Thirteen more colonies appeared during the following 48 h.

^cShown are frequencies of transduction relative to the frequency obtained when LT2 was used as recipient. The numbers within parentheses are the actual numbers of Km^R transductants. Numbers in boldface show the combinations that gave a significantly lower frequency of transduction.

TABLE 2. tRNA^{Val}_{cmo⁵UAC} by itself supports growth at an extremely reduced rate

Genotype	Anticodons present	Specific growth rate k (h ⁻¹)	Relative change $(k - k_{LT2})/k_{LT2}$
Wt (LT2)	GAC cmo ⁵ UAC	1.79 ± 0.021 ^a	0.00
<i>cmoB2</i>	GAC ho ⁵ UGU	1.82 ± 0.041 ^a	0.02
$\Delta val/VW$	cmo ⁵ UGU	0.53 ± 0.028 ^{b,c}	-0.70
$\Delta val/VW$ <i>cmoB2</i>	ho ⁵ UGU	0.34 ± 0.006 ^{b,d}	-0.81

^aThe values are averages from two or three dilutions from two separate cultures (LT2) or only from two dilutions from one culture (*cmoB2*).

^bDuring growth, samples were taken to check for the presence of faster-growing suppressor mutants. The cultures used for these calculations all contained <5% suppressors at the end of the experiment.

^cThe values are averages calculated from five separate cultures. We do not consider these cultures to have been in balanced growth (See Materials and Methods).

^dOwing to difficulties keeping pure cultures of this strain, this value is calculated from only two different cultures grown on separate days. We do not consider these cultures to have been in balanced growth (See Materials and Methods).

at recognizing the GUC codon. Whereas the *cmoB2* mutation did not influence the rate of valyl-tRNA^{Val} selection to the GUU- and GUA-programmed ribosomal A-site (Fig. 5), significant decreases in the rates at GUC and, unexpectedly, also at GUG codons were observed.

tRNA^{Ala}_{cmo⁵UGC} requires cmo⁵U34 for efficient wobble reading of GCC

The two identical genes *alaX* and *alaW* (sometimes referred to as *alaW_α* and *alaW_β*) (Fig. 3C) encoding tRNA^{Ala}_{GGC}, are arranged as a tandem repeat in a single operon containing no other genes. A strain lacking tRNA^{Ala}_{GGC} ($\Delta alaXW$) is viable, as is also the case in *E. coli* (Gabriel et al. 1996), but has a clear reduction in growth rate compared to the wild-type strain (LT2) (Table 3), a phenotype that is further enhanced at higher temperature, seen as a decreased colony size on plates at 44°C (data not shown). These results indicate that, similarly to tRNA^{Pro}_{cmo⁵UGG} (Näsvall et al. 2004), tRNA^{Ala}_{cmo⁵UGC} is able to read all four alanine codons, although not efficiently enough to support a maximum growth rate.

When *cmoB*<>*kan* was transduced into a strain ($\Delta alaXW$) lacking tRNA^{Ala}_{GGC}, tiny colonies (barely visible without magnification) started to appear after 2 d of incubation at 37°C. A few larger colonies (~0.2% of totally 409 transductants in one transduction) were also apparent, indicating the presence of suppressor mutants in some colonies. The “tiny” colonies were purified on selective medium and found to be viable but to accumulate suppressor mutations that partially restored growth (Fig. 6). When we transduced a wild-type strain (LT2) with the same amount of the same phage lysate, we received about

the same number of transductants as when strain GT7365 ($\Delta alaXW$) was used as recipient, but the obtained transductants showed a normal growth phenotype. These results show that a mutant lacking tRNA^{Ala}_{GGC} is viable even when it is hypo-modified at the wobble position in the only remaining alanine tRNA, but it has an extremely reduced growth rate, and mutations that partially restore growth are relatively frequent. If the growth phenotype would be caused by poor reading of one or more of the alanine codons, expression of more of the hypo-modified tRNA^{Ala}_{cmo⁵UGC} would allow the mutant to grow faster. To test this, we introduced the *cmoB2*<>*kan* allele into strains harboring plasmid p70 (Vila-Sanjurjo et al. 1999), carrying *E. coli* genes encoding tRNA^{Ala}_{cmo⁵UGC} and four other tRNAs (tRNA^{Asp}_{QUC}, tRNA^{Trp}_{CCA}, tRNA^{Ile}_{GAU}, and tRNA^{Thr}_{GGU}) expressed from the *tac* promoter. The growth phenotypes (seen as relative colony sizes on plates) of these strains were compared to the corresponding plasmid-free strains (Fig. 6). The relatively mild growth defect of the $\Delta alaXW$ strain, which has only the cmo⁵U34-containing alanine tRNA, seems to be fully suppressed, and the $\Delta alaXW$ *cmoB2*<>*kan* mutant is partially suppressed by overexpression of tRNA^{Ala}_{cmo⁵UGC}. Thus tRNA^{Ala}_{cmo⁵UGC}, when expressed at normal levels, is very dependent on the presence of the modification for its ability to read some of the four codons, but less so if it is overexpressed. We also measured the A-site selection rate at the four alanine codons. The $\Delta alaXW$ *cmoB2* mutant was considered too slow growing and unstable to be included in such an analysis. The *cmoB2* mutant shows a large reduction in the rate of reading the

FIGURE 4. Overexpression of tRNA^{Val}_{cmo⁵UAC} restores growth of a $\Delta valVW$ mutant. (A) Growth after 25 h of incubation at 37°C. (Sectors 1–4) Strains carrying pLG339 (vector control); (sectors 5–8) strains carrying p815 (*valU valX valY lysV*). The chromosomal genotypes are (1,8) LT2 (wt); (2,7) *cmoB2*<>*cat*; (3,6) $\Delta valVW$; (4,5) $\Delta valVW$ *cmoB2*<>*cat*. (B) Sectors 3 and 4 of the same plate as in A, but after 44 h at 37°C. No suppressor mutants were apparent in this particular experiment. The relative colony sizes after 15 h of growth were (LT2/pLG339 and *cmoB2*/pLG339) 1.0 ± 0.03; (LT2/p815) 1.0 ± 0.07; (*cmoB2*/p815) 1.0 ± 0.03; ($\Delta valVW$ /p815) 0.68 ± 0.04. Colonies of $\Delta valVW$ *cmoB2*/p815 were visible but still too small to measure, and no colonies were visible of $\Delta valVW$ /pLG339 or $\Delta valVW$ *cmoB2*/pLG339. After 25 h, colonies of $\Delta valVW$ *cmoB2*/p815 were ~30% smaller than $\Delta valVW$ /p815, and after 44 h, (B) colonies of $\Delta valVW$ *cmoB2*/pLG339 were approximately half the size compared to $\Delta valVW$ /pLG339.

FIGURE 5. A-site selection rates at valine (GUN) codons. (*) Values in the *cmoB2* mutant are significantly different from the control (LT2), as determined by a student's *t*-test (two sample, equal variance, $p < 0.05$). All values for the $\Delta valVW$ mutant are significantly different from LT2 ($p < 0.005$). The values are averages of four experiments, with at least two independent cultures of each strain.

GCG codon (Fig. 7). The $\Delta alaXW$ mutant has a reduction in the rate of alanyl-tRNA entry on all four codons, and the most severe reduction is on the GCC codon. Taken together, these results show that fully modified tRNA^{Ala}_{cmo⁵UGC} reads GCA, GCG, and GCU efficiently and GCC poorly and that cmo⁵U improves reading of the G-ending codon.

cmo⁵U34 in tRNA^{Pro}_{cmo⁵UGG} mainly enhances wobble reading of G

A mutant having only the cmo⁵U-containing tRNA^{Pro}_{cmo⁵UGG} is viable without any apparent phenotype, demonstrating that this tRNA reads efficiently all four proline codons. If this tRNA in such a mutant contains ho⁵U instead of cmo⁵U34, a clear reduction in growth rate caused by the hypo-modification is observed (Näsvall et al. 2004). Furthermore, a mutant lacking the G34-containing tRNA^{Pro}_{GCG} and thus having the cmo⁵U34- and C34-containing tRNAs has a significant growth rate reduction in conjunction with hypo-modification of the wobble nucleoside in tRNA^{Pro}_{cmo⁵UGG} (Näsvall et al. 2004). Based on these data and the theoretical prediction that cmo⁵U34 reads U-ending codons (but not C-ending codons) (Yokoyama et al. 1985), we suggested that the reason for the observed phenotypes of the various mutants being deficient in tRNA^{Pro} and cmo⁵U was the slower reading of mainly the U- and C- ending proline codons (Näsvall et al. 2004). To verify this suggestion, we measured the A-site selection rates on each of the four proline codons. Lack of tRNA^{Pro}_{GCG} and tRNA^{Pro}_{GCG} leads to a large reduction in the rate of reading all four proline codons (Fig. 8, cf. LT2 and $\Delta proKL$). This is not surprising, since the two missing tRNAs together make up about two-thirds of the total proline tRNA pool (Dong et al. 1996)

and should normally read most of the CCC, CCU, and CCG codons. Similarly to the alanine tRNA (Fig. 7), the largest effect of hypo-modification of tRNA^{Pro}_{cmo⁵UGG} (Fig. 8, cf. LT2 and *cmoB2* and $\Delta proKL$ and *cmoB2* $\Delta proKL$) seems to be a reduced rate of reading CCG. We also measured the effect of not having cmo⁵U on the A-site selection rates in mutants only lacking the C34-containing tRNA^{Pro}_{GCG} (*proK* <> *frt*). In two separate experiments, the rate of reading the CCG codon in the *cmoB2* mutant was reduced by 42% and 39%, respectively (data not shown), further strengthening our observations that cmo⁵U34 is important for recognizing the G-ending proline codon.

DISCUSSION

In this study, we show that the function of the modified nucleoside cmo⁵U34 is different from what has previously been hypothesized and that the impact on hypo-modification of the wobble position is different in different cmo⁵U34-containing tRNAs. According to a theoretical model, cmo⁵U is predicted to allow reading of U-ending codons (Yokoyama et al. 1985). This model was based on how the modification (cmo⁵ or mo⁵), by interacting with the 5'-phosphate, affects the equilibrium between two different conformations (C2'-endo and C3'-endo) of the ribose moiety of synthetic nucleotides in solution. 5-Hydroxy uridine would not be able to make this interaction and would thus have decoding properties similar to uridine, which should only read A- and G-ending codons according to the wobble hypothesis (Crick 1966). The model did not explain why some cmo⁵U-containing tRNAs can read C-ending codons, and, in fact, it was predicted that a cmo⁵U-C pair would be impossible due to steric repulsion between ribose 34 and ribose 35 (Yokoyama et al. 1985). Moreover, this model would predict that a tRNA having ho⁵U in place of cmo⁵U would have a dramatically reduced rate of reading U-ending (and probably also C-ending in the cases where it does happen) codons, while the rates of reading the A- and G-ending codons should not be

TABLE 3. tRNA^{Ala}_{cmo⁵UGC} alone can support growth only at a reduced rate

Genotype	Anticodons present	Specific growth rate k (h ⁻¹)	Relative change $(k - k_{LT2})/k_{LT2}$
Wt (LT2)	GGC cmo ⁵ UGC	$1.73^a \pm 0.011$	0.00
$\Delta alaXW$	cmo ⁵ UGC	$1.13^a \pm 0.016$	-0.35
$\Delta alaXW$ <i>cmoB2</i>	ho ⁵ UGC	NA ^b	NA ^b

^aThe values are averages from two consecutive dilutions (see Materials and Methods) of three different cultures of each strain, all grown on the same day.

^b(NA) Not applicable. This mutant was not included because it accumulates suppressor mutants too quickly to be able to keep as a pure culture.

FIGURE 6. Overexpression of hypo-modified tRNA^{Ala}_{cmo5UGG} from plasmid p70 can partially restore growth of a Δ alaXW *cmoB2* mutant. (A) Growth of (sector 1) LT2/p70 (wild-type); (sector 2) *cmoB2*<>*kan*/p70; (sector 3) Δ alaXW/p70; and (sector 4) Δ alaXW *cmoB2*<>*kan*/p70 after 27 h at 37°C on an LA + tetracycline plate. The relative colony diameters (after 16 h) were (LT2/p70) 1.0 ± 0.05 ; (*cmoB2*/p70) 0.96 ± 0.04 ; and (Δ alaXW/p70) 0.92 ± 0.07 . The colonies of Δ alaXW *cmoB2*/p70 were visible but too small to measure. (B) Same as in A, but the strains do not contain any plasmid, and the plate is LA without any antibiotic. The relative colony diameters (after 16 h) were (LT2) 1.0 ± 0.07 ; (*cmoB2*) 1.0 ± 0.01 ; and (Δ alaXW) 0.71 ± 0.04 . At the time of the size measurements, no single colonies of the Δ alaXW *cmoB2* mutant had appeared, but after 27 h, very tiny colonies (<0.1 mm in diameter) as well as some faster-growing colonies (still too small to be clearly visible in the picture) could be seen. (C) Sector 4 of the plate in B, but after 75 h. The absolute majority of the colonies of the Δ alaXW *cmoB2* mutant were still <0.4 mm in diameter, while a few larger colonies ranging in sizes between ~0.5 and 2 mm were visible.

too much affected. However, our results do not support such a hypothesis, since the largest effect of hypo-modification of three tested tRNAs is on the rate of reading G-ending codons, while the effects (if any) on C- and U-ending codons are minor. This leads us to question the validity of the above model and suggests an alternative molecular mechanism for the decoding by *cmo⁵U*.

Interestingly, the effects on the growth rates or viability when removing all other tRNA isoacceptors for the different amino acids are quite different. One extreme is the *cmo⁵U*-containing threonine tRNA, which cannot at all support growth of a mutant lacking the G34-containing threonine isoacceptor (Table 1). This is similar to previously reported data for codon recognition by *cmo⁵U*-containing (or *mo⁵U*-containing) leucine (Nishiyama and Tokuda 2005; Sørensen et al. 2005) and serine (Takai et al. 1999) tRNAs. The *cmo⁵U*-containing valine and alanine tRNAs can support growth of mutants lacking the corresponding G34-containing isoacceptors (Tables 2, 3), although the growth rates of such mutants are reduced compared to a wild-type strain. At the other extreme is the *cmo⁵U34*-containing proline isoacceptor, which supports growth at a rate indistinguishable from a wild-type strain even when both the G34- and C34-containing proline isoacceptors are missing (Näsvall et al. 2004). These dramatic differences between the different tRNAs could be the result of tRNAs having different relative efficiencies of recognizing one or more of their specific codons.

Alternatively, the expression of the *cmo⁵U34*-containing tRNA^{Pro}_{cmo5UGG} could be high enough relative to the codon usage to allow efficient decoding even in the absence of the other proline tRNAs. However, among the *cmo⁵U*-containing alanine, valine, and proline tRNAs, the proline tRNA is the least abundant relative to the codon usage (Table 4). Thus, codon usage and tRNA levels alone cannot explain the differences in phenotypes we observe.

Comparing the relative rates of A-site selection for the different *cmo⁵U34*-containing proline, valine, and alanine tRNAs when they are the only isoacceptors present (Fig. 5, Δ valVW; Fig. 7, Δ alaXW; Fig. 8, Δ proKL) the A-, G-, and U-ending codons were recognized at similar rates, while the rates of recognizing the C-ending alanine and valine codons are much lower (about fourfold lower than the other codons). In all cases, the rates at the A-ending codons were lower in the strains lacking the other isoacceptors than in the wild type. This was expected, as the remaining tRNAs have to read more of the codons that are normally also read by the other isoacceptors, leading to fewer tRNAs available to read the A-ending codon. In vitro a slightly lower rate of recognition was also observed toward the GCC (Ala) codon compared to the GCA (Ala) codon (Kothe and Rodnina 2007). However, tRNA^{Pro}_{cmo5UGG} recognized the C-ending codon almost as efficiently as the other proline codons (Fig. 8), which could partly explain why the mutant (Δ proL *proK*<>*frt*) lacking the G34- and C34-containing proline isoacceptors has no apparent growth phenotype. One feature differentiating the anticodon loop in the *cmo⁵U*-containing proline tRNA from the anticodon loops in the other tested tRNAs is the presence of four consecutive purines (G35-G36-m¹G37-A38). As purine-purine stacking is the most stable stacking interaction (Saenger 1984), this may lead to an exceptionally stable anticodon loop through

FIGURE 7. A-site selection rates at alanine (GCN) codons. The asterisks (*) indicate values from the *cmoB2* mutant that are different from the control (LT2), as determined by a student's *t*-test [two sample, equal variance; (*) $p < 0.05$, (***) $p < 0.0005$]. All values from the Δ alaXW mutant are significantly different from LT2 ($p < 0.0005$). The values are averages from four experiments.

FIGURE 8. A-site selection rates at proline (CCN) codons. The asterisks (*) indicate values from the *cmoB2* mutant that are different from the control (LT2), as determined by a student's *t*-test [two sample, equal variance; (*) $p < 0.05$]. All values for the $\Delta proL proK<>frt$ and $\Delta proL proK<>frt cmoB2<>frt$ mutants are significantly different from LT2 ($p < 0.01$). The values are averages from at least three experiments. For simplicity, $\Delta proL proK<>frt$ is written $\Delta proKL$.

extensive stacking of these bases, perhaps contributing to the efficiency of decoding the CCC codon by tRNA^{Pro}_{cmo5UGG}.

Combining the lack of the G34-containing (and C34-containing) isoacceptors with hypo-modification (ho⁵U34 instead of cmo⁵U34) of the wobble nucleoside in the remaining isoacceptors also has different effects on the growth rates of the different mutants. All three mutants with only the cmo⁵U-containing isoacceptors left in the respective family codon boxes ($\Delta proL proK<>frt$, $\Delta alaXW$, and $\Delta valVW$) have significantly reduced growth rates when combined with a *cmoB* mutation (Tables 1, 3; Näsvall et al. 2004), but the severity of the synergistic phenotypes is very different. The $\Delta alaXW cmoB2$ mutant is virtually impossible to keep as a pure culture without accumulation of faster-growing suppressors (Fig. 6). The nature of the suppressors that accumulated in the $\Delta alaXW cmoB2$ and $\Delta valVW$ mutants was not examined in detail, but at least some of them segregated into small and large colonies, indicating that they may be amplifications of the tRNA genes (data not shown). As both mutants were suppressed by expressing more of the remaining tRNA (Figs. 4 and 6), it is likely that amplification of the genes encoding these tRNAs would account for some of the suppressors. The effects of hypo-modification on the $\Delta proL proK<>frt$ and the $\Delta valVW$ mutants seem to be similar (although the $\Delta valVW$ mutant is very slow growing even in the presence of cmo⁵U34) (Fig. 4). Comparing the effects of having ho⁵U in place of cmo⁵U on the A-site selection rate for the different tRNAs (Figs. 5, 7, 8, cf. LT2 and *cmoB2*; Fig. 8, cf. $\Delta proKL$ and $\Delta proKL cmoB2$), the only codons where we observed significant differences in all three family boxes are at the G-ending codons. The largest effect is at the

G-ending alanine codon. Taken together, the data from the A-site selection assays indicate that the extreme phenotype of the *cmoB2 ΔalaXW* mutant probably is a combination of very poor recognition of the GCC codon due to the lack of the G34-containing alanine isoacceptor and the reduced efficiency of recognizing the GCG codon caused by the modification deficiency. In addition to the decreases in the selection rates at the G-ending codons, we also observed decreased rates on the C-ending valine and proline codons and the U-ending alanine and proline codons. The effect of cmo⁵U deficiency on the ability of the $\Delta proL proK<>frt$ and $\Delta valVW$ mutants to recognize their corresponding G-ending codons is not as large as for the $\Delta alaXW$ mutant, which could be an explanation of why the different mutants have such different growth phenotypes. There

may be several reasons why cmo⁵U apparently is more important for the function of tRNA^{Ala}_{cmo5UGC} than it seems to be for the functions of tRNA^{Pro}_{cmo5UGG} and tRNA^{Val}_{cmo5UAC}. One might be that other features present in tRNA^{Pro}_{cmo5UGG} and tRNA^{Val}_{cmo5UAC} but absent in tRNA^{Ala}_{cmo5UGC} contribute to their efficient decoding. One candidate for such a feature would be the modification of position 37 (immediately 3' of the anticodon). tRNA^{Pro} has m¹G37 (Kuchino et al. 1984), and tRNA^{Val} has m⁶A37 (Kimura et al. 1971), while tRNA^{Ala} has an unmodified adenosine at position 37 (Lund and Dahlberg 1977). m¹G37 has previously been

TABLE 4. Codon usage and tRNA availability

Codon N(III)	Val	Ala	Pro
U	15.5 ^a	12.7 ^a	7.2 ^a
C	18.2 ^a	29.1 ^a	6.9 ^a
A	11.4 ^a	12.9 ^a	5.8 ^a
G	25.1 ^a	42.8 ^a	24.7 ^a
Total	70.3 ^a	97.6 ^a	44.5 ^a
tRNA N34			
G	4.0–7.2 μM ^b	2.0–3.6 μM ^b	2.3–4.0 μM ^b
cmo ⁵ U	11–20 μM ^b	10–21 μM ^b	1.8–2.6 μM ^b
C	–	–	2.4–3.5 μM ^b

^aCodon usage is expressed as the number of each codon found per 1000 codons in coding sequences. Data from the codon usage database (Nakamura et al. 2000) were re-calculated after removing data from other sources than the published genomic sequence of *Salmonella typhimurium* strain LT2.

^bThe tRNA concentrations are the lowest and highest values reported by Dong et al. (1996) from an *E. coli* K12 strain grown at different growth rates.

demonstrated to be important for the A-site selection rate at all four proline codons (Li et al. 1997). As the effects on growth rates of some of the mutants appear to be larger than one would expect from the changes in the A-site selection rates, it is possible that part of the growth phenotypes are due to defects in a stage of the translation elongation cycle other than the A-site selection. We have previously shown that having ho⁵U instead of cmo⁵U in tRNA^{Pro}_{cmo⁵UGG} actually leads to decreased +1 frameshifting at CCC codons (Qian et al. 1998; Näsvall et al. 2004), which is why we do not think frameshifting in such a mutant reaches high enough levels to cause the observed growth-rate reduction.

It should be noted that there are organisms that have only a U34-containing tRNAs to read all four codons in some or all of the family codon boxes encoding Ser, Pro, Thr, Ala, Val, and Leu. *Bacillus subtilis* have only one proline tRNA (containing mo⁵U34) (Yamada et al. 2005), while it has two (mo⁵U34 and G34) or three (mo⁵U34, G34, and C34) tRNAs in the other five boxes (Sprinzl and Vassilenko 2005). Certain intracellular parasites, like the Mollicutes (to which the *Mycoplasmas* belong), have very small, A/T-rich genomes with a reduced number of tRNA genes compared to free-living bacteria. Some of these use only U34-containing tRNAs in all these boxes, while the others have additional G34-, C34-, or A34-containing tRNAs in some of the serine, threonine, alanine, valine, or leucine boxes (for review, see De Crécy-Lagard et al. 2007). The codon usages of these organisms are very strongly biased toward A- and U-ending codons. The modification status of tRNAs from most of these organisms are unknown, but at least *Mycoplasma capricolum* and *Mycoplasma mycoides* have unmodified uridines at the wobble position. This may indicate that during their reductive evolution, the Mollicutes (distantly related to *Bacillus*) have lost the genes required to synthesize mo⁵U, and have unmodified U34. As these bacteria use U34-containing tRNAs to read primarily A- and U-ending codons, while they use C- and G-ending codons very rarely, this suggests that U34-containing tRNAs in the family codon boxes are unexpectedly efficient at reading U-ending codons, while this requirement may not be relevant for the rarely used G- and C-ending codons. This may be an exception enabled by special features of the ribosomes and/or tRNAs from these highly specialized organisms, but it may also be a general rule for U34-containing tRNAs in other organisms as well. In fact, completely unmodified *E. coli* tRNA^{Ser}_{UGA} (which normally has cmo⁵U34) is capable of reading UCA and UCU in an in vitro translation system, but UCG is very poorly translated unless the wobble nucleoside is modified to mo⁵U (Takai et al. 1999). With this in mind, perhaps the unknown gene(s) encoding the enzyme(s) responsible for making ho⁵U34 from U34 in tRNA is essential for *Salmonella* and *E. coli* even in strains with the full complement of tRNAs, as the U34-containing alanine and valine tRNAs may require at least ho⁵U in

order to recognize their G-ending codons efficiently enough to support growth.

Relevant to the coding capacities in Mollicutes is the “two out of three” decoding model, which states that the third position of the codon–anticodon interaction can be disregarded, as long as the interactions in the first two positions are strong (Lagerkvist 1978). This would apply to codons where the first two positions form G-C pairs (Pro, Ala, Arg, and Gly) but not to codons forming only A-U pairs (such as the Phe/Leu, Ile/Met, Tyr/Stop, and Asn/Lys mixed codon boxes). The two out of three model is supported by in vitro translation experiments in which the *E. coli* cmo⁵U-containing alanine and valine tRNAs could incorporate the respective cognate amino acids at all four of their codons even in the presence of a competing cognate tRNA (Mittra et al. 1979; Samuelsson et al. 1980). The relative efficiency of the cmo⁵U-containing tRNA in reading the four alanine and valine codons in the presence of the cognate tRNA is similar to our results obtained using the A-site selection assay in cells with only the fully modified cmo⁵U-containing tRNA present to read all four codons (Figs. 5, 7). Lagerkvist assumed that the efficient reading of U-ending codons was caused by the presence of cmo⁵U, while the less efficient reading of C-ending codons was regarded as two-out-of-three reading that did not involve an interaction at the third position. However, in vivo (Figs. 5, 7, 8) the hypo-modified derivatives were less efficient to read the G-ending and sometimes the U- or C-ending codons, although the extent of the reductions at the C- and U-ending codons may be masked by the presence of the competing G34-containing tRNAs. Thus, the modification of the wobble base may contribute to the decoding efficiency even at C-ending codons and cannot be disregarded as suggested by the two out of three model. However, as we have no mutant that has unmodified U34, we cannot conclusively say if alanine, valine, or proline tRNA in such a mutant would still read U- or C-ending codons. Moreover, the fact that the Leu and Thr family boxes cannot be decoded by a single tRNA (Table 1; Nishiyama and Tokuda 2005; Sørensen et al. 2005) is not consistent with the two out of three decoding model. Why the cmo⁵U-containing Ala and Pro tRNAs can read C-ending codons but the Leu, Thr, and Ser tRNAs cannot may be related to the stability of the first two base pairs in the codon–anticodon complex (Ala and Pro make two G-C pairs, whereas Leu, Thr, and Ser make only one G-C pair). Still, it is unclear why tRNA^{Val}_{cmo⁵UAC} can read all four codons (although not efficiently enough to support normal growth) when it also makes only one G-C pair (Fig. 5; Table 2).

In his wobble hypothesis, Crick predicted that U34 in tRNA would be able to form a wobble pair with G(III) in the mRNA (Crick 1966), with two hydrogen bonds between U34 and G(III). In order to allow formation of such a base pair, either U34 has to be displaced toward the major

groove or G(III) has to be displaced toward the minor groove of the codon–anticodon mini-helix (a move of ~ 2.5 Å for one of the glycosidic bonds compared to a Watson–Crick base pair) (Fig. 9B; Crick 1966). It is unlikely that G(III) would be allowed to move, since its movement is restricted by its interactions with residues in the ribosomal 30S subunit (Ogle et al. 2001; Murphy et al. 2004). If U34 would move the entire distance required to form a U–G wobble pair, it would be unable to make a stacking interaction with the base in position 35 of the tRNA (Murphy et al. 2004), which could perhaps lead to poor recognition of G-ending codons by tRNAs with unmodified U34. Weixlbaumer et al. (2007) have recently solved the crystal structures of *Thermus thermophilus* 70S ribosomes containing an ASL^{Val}_{cmo⁵UAC} (with the modifications cmo⁵U34 and m⁶A37) bound to the four Val codons in the A-site. One interesting feature of the cmo⁵U–G base pair is that it has standard Watson–Crick geometry rather than the expected wobble geometry (Fig. 9). This means that the 4-carbonyl group of cmo⁵U34 has to be in the rare enol form rather than the normal keto form and that the base pair makes three hydrogen bonds (Fig. 9B). Hillen et al. (1978) showed that mo⁵U has a shifted keto–enol equilibrium compared to uridine and ho⁵U. Our data showing that tRNAs with cmo⁵U34 are significantly more efficient than tRNAs with ho⁵U at reading G-ending codons are consistent with the observed crystal structure and predict that cmo⁵U34 is, indeed, in the enol form. In all four of these structures, the

ether oxygen (O5) of the modification forms a hydrogen bond to the 2′-OH of U33 (Fig. 9A). This leads to a more constrained anticodon than if the wobble uridine would have been unmodified (Weixlbaumer et al. 2007), with the wobble nucleoside “locked” in position close to where it would be in a Watson–Crick base pair. The cmo⁵U–U and cmo⁵U–C pairs in the structures only form one hydrogen bond between the codon and anticodon bases (Weixlbaumer et al. 2007). Part of the stabilization needed to allow these pairs may come from the observed hydrogen bond between the O5 of cmo⁵U34 and the 2′-OH of U33. The cmo⁵U–U pair may be further stabilized by interactions with 16S rRNA, whereas the cmo⁵U–C pair may be slightly destabilized by poor stacking between C(III) and its 5′-base (Weixlbaumer et al. 2007). These differences between the cmo⁵U–U and cmo⁵U–C pairs may explain why the C-ending codons are poorly recognized by the cmo⁵U-containing alanine and valine tRNAs, while the U-ending codons are recognized efficiently (Figs. 5, 7). Weixlbaumer et al. also point out that the carboxyl group of cmo⁵U34 is close enough to the oxygen of the 4-carbonyl group of U(III) to be able to form a hydrogen bond if the carboxyl group is protonated (Weixlbaumer et al. 2007). This would provide even further stability to the cmo⁵U–U pair but not to the cmo⁵U–C pair. In such a case, the hypo-modified ho⁵U34-containing tRNAs in a *cmoB* mutant (lacking the carboxyl group) would be affected in their reading of U-ending codons. Since we see no large effect on the rates of reading U-ending codons, our data do not support this suggestion. Another important observation in these structures is that in all four pairs, the ribose of cmo⁵U34 adopts the 3′-endo conformation, and the modification does not interact with its 5′-phosphate as predicted by Yokoyama et al. (1985).

Based on the structures discussed above (Fig. 9; Weixlbaumer et al. 2007) and our in vivo data, we suggest that the function of cmo⁵U34 (and mo⁵U34) may be dual: Firstly, the ether oxygen (or the hydroxyl of ho⁵U) stabilizes the anticodon loop in a conformation where the wobble uridine is locked in position to form a base pair with Watson–Crick geometry. This leads to sufficient stabilization to compensate for the poor interactions between cmo⁵U34 and U(III) or C(III). Secondly, the rest of the modification (–CH₃ in mo⁵U or –CH₃COOH in cmo⁵U) stabilizes the enol form of the wobble nucleoside, promoting a nonstandard U34–G(III) base pair.

FIGURE 9. (A) The cmo⁵U34–G(III) base pair seen in the crystal structure solved by Weixlbaumer et al. (2007) (Protein Databank accession code 2UU9). The hydrogen bond between the 2′-OH of U33 and the O5 ether oxygen of cmo⁵U34 is indicated. (B) Keto–enol tautomerization of cmo⁵U. (Left) The keto tautomer of cmo⁵U (black), forming a wobble pair with G (gray) as predicted by Crick. (Right) The enol tautomer of cmo⁵U (black), engaged in a base pair with G (gray). The geometry of the cmo⁵U–G pair is the same as for a Watson–Crick (A–U or G–C) base pair.

MATERIALS AND METHODS

Bacteria and growth conditions

The strains and plasmids used are listed in Table 5. All *Salmonella* strains are derivatives of *Salmonella enterica* serovar Typhimurium strain LT2. As solid rich medium, TYS (10 g of Trypticase peptone, 5 g of yeast extract, 5 g of NaCl, and 15 g of agar per

TABLE 5. *Salmonella enterica* serovar Typhimurium and *Escherichia coli* strains and plasmids

Strain	Relevant genotype	Source/Reference
<i>Salmonella enterica</i> serovar Typhimurium		
GT3258*	LT2	
GT1796	(TT10186) <i>trp2482::MudA</i>	
GT6315	pKD46/LT2	
GT6648	<i>thrT</i> ↔ <i>kan</i>	This study
GT6647	<i>thrV</i> ↔ <i>kan</i>	This study
GT6683	<i>thrW</i> ↔ <i>kan</i>	This study
GT6940	<i>thrV</i> ↔ <i>frt</i>	This study
GT6925	<i>thrT</i> ↔ <i>frt</i>	This study
GT6926	<i>thrW</i> ↔ <i>frt</i>	This study
GT6300*	Δ <i>valVW</i>	This study
GT6971	Δ <i>valVW cmoB2</i> ↔ <i>kan</i>	This study
GT6972	Δ <i>valVW cmoB2</i> ↔ <i>frt</i>	This study
GT7365*	Δ <i>alaXW</i>	This study
GT6856*	<i>cmoB2</i> ↔ <i>frt</i>	Näsvall et al. (2004)
GT6902*	Δ <i>proL proK</i> ↔ <i>frt</i>	Näsvall et al. (2004)
GT6914*	Δ <i>proL proK</i> ↔ <i>frt cmoB2</i> ↔ <i>frt</i>	Näsvall et al. (2004)
GT6877*	<i>proK</i> ↔ <i>frt</i>	äsvall et al. (2004)
GT6898*	<i>cmoB2</i> ↔ <i>frt proK</i> ↔ <i>frt</i>	This study
<i>Escherichia coli</i>		
GRB1371	(S17-1) <i>thi pro hsdB hsdM⁺ recA</i> RP4-2-Tc::Mu-Km::Tn7 λ <i>pir</i>	Simon et al. (1983)
GRB1748	pUST300(pDM4+Δ <i>alaXW</i>)/GRB1371	This study
Plasmids		
pDM4	R6Kγ replication origin, <i>sacB</i>	Milton et al. (1996)
pMAK705	Ts replication origin	Hamilton et al. (1989)
pKD46	λ <i>red</i> recombinase under <i>araBAD</i> promoter	Datsenko and Wanner (2000)
pKD4	Template plasmid for kanamycin resistance cassette	Datsenko and Wanner (2000)
pKD3	Template plasmid for chloramphenicol resistance cassette	Datsenko and Wanner (2000)
pCP20	FLP recombinase helper plasmid	Datsenko and Wanner (2000)
pUST301	(pMAK705+Δ <i>valVW</i>)	This study
pUST300	(pDM4+Δ <i>alaXW</i>)	This study
pJC27	Pseudo-wild-type for A-site selection rate assays, Cm ^R	Curran and Yarus (1989)
pJC-CCU	Test construct for A-site selection rate assays, Cm ^R	Curran and Yarus (1989)
pJC-CCC	Test construct for A-site selection rate assays, Cm ^R	Curran and Yarus (1989)
pJC-CCA	Test construct for A-site selection rate assays, Cm ^R	Curran and Yarus (1989)
pJC-CCG	Test construct for A-site selection rate assays, Cm ^R	Curran and Yarus (1989)
pJC-GUU	Test construct for A-site selection rate assays, Cm ^R	This study
pJC-GUC	Test construct for A-site selection rate assays, Cm ^R	This study
pJC-GUA	Test construct for A-site selection rate assays, Cm ^R	This study
pJC-GUG	Test construct for A-site selection rate assays, Cm ^R	This study
pJC-GCU	Test construct for A-site selection rate assays, Cm ^R	This study
pJC-GCC	Test construct for A-site selection rate assays, Cm ^R	This study
pJC-GCA	Test construct for A-site selection rate assays, Cm ^R	This study
pJC-GCG	Test construct for A-site selection rate assays, Cm ^R	This study
p815	<i>E. coli valU</i> operon in pLG339, Km ^R	O'Connor (2002)
pLG339	Cloning vector, Km ^R , Tet ^R	Stoker et al. (1982)
p70	<i>E. coli aspT trpT ileU alaU thrV</i> , Tet ^R	Vila-Sanjurjo et al. (1999)

Strain numbers followed by an asterisk (*) indicate the strains used in the A-site selection assays.

liter) was used. As solid minimal medium, medium E (Vogel and Bonner 1956) containing 15 g of agar per liter and 0.2% glucose was used. As rich liquid medium, either LB or NAA (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) was used. For growth rate determination and assay of β-galactosidase activities, Rich MOPS (Neidhardt et al. 1977) was used. All growth was done at 37°C. Antibiotics were used at the following concentrations:

Carbenicillin (Cb): 50 mg/L; Kanamycin (Km): 100 mg/L; Chloramphenicol (Cm): 12.5 mg/L; Tetracycline (Tet): 15 mg/L.

Genetic procedures

To transfer chromosomal markers or plasmids between *Salmonella* strains, transductions were performed as described previously (Davis et al. 1980) with a derivative of phage P22 containing the mutations HT105/I (Schmieger 1972) and *int-201* (Scott et al.

1975). Green indicator plates (Chan et al. 1972) were used for testing that the clones were phage free and phage sensitive.

Molecular cloning procedures

PCR fragments were purified from agarose gels using Wizard DNA clean-up resin (Promega) or directly using PCR kleen-spin (Bio-Rad). DNA sequencing was done using the DYEnamic ET terminator cycle sequencing kit (Amersham Pharmacia Biotech Inc.) or the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems).

A 1.8-kb crossover PCR product containing the deletion of the *alaXW* operon (from 101 nt upstream to 55 nt downstream of *alaXW*) was generated as described by Link et al. (1997) and cloned into the suicide vector pDM4 (Milton et al. 1996). The plasmid (pUST300) was transformed into *E. coli* strain GRB1371, and the resulting strain (GRB1748) was used as donor in conjugation with the *Salmonella* strain GT1796 as recipient. Cointegrates were segregated by nonselective growth in LB supplemented with 5% sucrose. Sucrose-resistant segregants were screened by PCR to find clones carrying the *alaXW* deletion. To generate the *valVW* deletion, a 1.2-kb crossover fragment containing a deletion of the *valV* and *valW* genes (from 6 nt upstream of *valV* to 28 nt downstream of *valW*) was cloned into the temperature-sensitive suicide vector pMAK705 (Hamilton et al. 1989), generating pUST301. Cointegrates were obtained by growing at 44°C in the presence of chloramphenicol and segregated by growing nonselectively at 30°C. Chloramphenicol-sensitive segregants were screened by PCR to find clones carrying the *valVW* deletion on the chromosome.

We used the method described by Datsenko and Wanner (2000) to replace *thrT*, *thrV*, or *thrW* in strain GT6315 (an LT2 derivative with the λ -red recombinase plasmid pKD46) with the kanamycin resistance cassette amplified from plasmid pKD4 (Datsenko and Wanner 2000). The $\langle \rangle$ *kan* alleles were transferred by P22 transductions into strain LT2 before the FLP-recombinase helper plasmid pCP20 (Datsenko and Wanner 2000) was introduced to convert the $\langle \rangle$ *kan* alleles into $\langle \rangle$ *frt* alleles. To combine the different mutations, we used P22 lysates grown on strains containing the different *thr* $\langle \rangle$ *kan* alleles as donors and cultures of strains containing the different *thr* $\langle \rangle$ *frt* alleles as recipients in transductions, selecting Km^R transductants (Table 1). To examine the presence of duplications in the rare transductants that appeared when trying to construct a *thrT*, *thrV* double mutant, 37 transductants (from transductions using *thrT* $\langle \rangle$ *kan* as donor and *thrV* $\langle \rangle$ *frt* as recipient), including clones that appeared after up to 72 h of incubation, were checked by PCR reactions designed to distinguish between the wild-type and *thrT* $\langle \rangle$ *kan* alleles.

The *cmoB2* $\langle \rangle$ *cat* allele was constructed to be identical to the *cmoB2* $\langle \rangle$ *kan* allele (Näsvall et al. 2004), except the chloramphenicol resistance cassette from plasmid pKD3 was used instead of the kanamycin resistance cassette from pKD4.

Nomenclature of mutants

An allele number followed by $\langle \rangle$ and “*kan*” or “*cat*” indicates that the gene is replaced by the kanamycin or chloramphenicol resistance cassettes from plasmid pKD4 or pKD3, respectively (e.g., *cmoB2* $\langle \rangle$ *kan* or *cmoB2* $\langle \rangle$ *cat*). After FLP-recombinase-mediated removal of the cassette, the mutation is referred to with

the same allele number, but with “*frt*” as description of the resulting scar sequence (e.g., *cmoB2* $\langle \rangle$ *frt*). The same allele number is also used for replacements where removal of two different FRT (Flp Recombinase Target sequence) flanked antibiotic resistance cassettes would produce an identical end result; hence *cmoB2* $\langle \rangle$ *kan*, *cmoB2* $\langle \rangle$ *cat*, and *cmoB2* $\langle \rangle$ *frt* have the same allele number. A “ Δ ” before a gene name refers to a precise deletion rather than a replacement (e.g., “ Δ *proL*”).

Nomenclature of tRNAs

In most cases, tRNAs are referred to with their cognate amino acid in three-letter code in superscript and with their anticodon sequence (5' $\rightarrow$ 3') in subscript, i.e., tRNA^{Thr}_{cmo5UGC} refers to a threonine tRNA with the anticodon cmo⁵U34-G35-U36. In some cases, the names of tRNA genes are followed by the wobble nucleoside of the corresponding tRNA within parentheses.

Determination of growth rates

Overnight cultures of the different strains were diluted to ~ 0.05 – 0.1 OD₄₂₀ units in pre-warmed medium and were pre-grown to OD₄₂₀ ≈ 1.0 . The cultures were then diluted to OD₄₂₀ ≈ 0.02 – 0.06 , and growth was monitored with a Shimadzu UV-1601 spectrophotometer at 420 nm. When the OD of the cultures reached OD₄₂₀ ≈ 1.0 , they were again diluted into fresh pre-warmed medium. The cultures were judged to be in balanced growth when the growth rate determined after a dilution did not change more than 5% from what it was before dilution. We did not wait for the slow-growing Δ *valVW* (GT6300) or Δ *valVW*, *cmoB2* (GT6972) mutants (Table 2) to reach balanced growth; instead, they were treated as follows: Overnight cultures of GT6300 or still growing cultures (with an OD₄₂₀ below 1.0) of GT6972 were diluted once to OD₄₂₀ ≈ 0.05 , and growth was monitored until the OD again reached 1.0. Cultures of GT6300 were diluted once more, and monitoring continued until OD₄₂₀ ≈ 1.0 . Samples were withdrawn from the cultures and plated to estimate the proportion of faster-growing suppressor mutants. The data in Table 2 were calculated from cultures that contained $< 5\%$ suppressors at the end of the experiments. The specific growth rate is expressed as k (h^{−1}), where $k = \ln 2/g$ (where g is the generation time in hours). Strain LT2 (wild-type) was always grown in parallel with the mutants to get a reference value for each experiment. Because of this, the values for LT2 in Tables 2 and 3 differ slightly from each other. To estimate the colony diameters reported in the legends for Figure 4 and Figure 6, the plates were photographed with a ChemiDoc XRS (Bio-Rad). Quantity One software (Bio-Rad) was used to measure the approximate width and height of square boxes manually fitted to surround each colony. The values are averages of five representative colonies (except the *cmoB2* $\langle \rangle$ *kan* mutant in Fig. 6B, sector 2, where only two colonies were sufficiently separated from other colonies). The average diameter of strain LT2 (containing the relevant plasmids) growing on the same plate was set to 1.0.

Determination of A-site selection rates

Strains were grown overnight at 37°C in Rich MOPS medium supplemented with 12.5 μ g/mL chloramphenicol, subcultured and grown to mid-log phase (OD₆₀₀ ≈ 0.5 using a Shimadzu UV-1201 spectrophotometer, corresponding to $\sim 2 \times 10^8$ CFU/

mL). β -Galactosidase activity was measured as described by Miller (1972) (using the alternative method with chloroform and SDS instead of toluene to open the cells). ONPG was from Sigma. For each strain to be tested, at least two independent cultures were grown, and each strain was tested in at least three separate experiments. The plasmids for testing the alanine and valine codons were constructed by mutagenizing previously constructed plasmids (Curran and Yarus 1989) according to the protocol for the QuikChange Site-Directed Mutagenesis kit (Stratagene). The numbers presented in Figures 5, 7, and 8 are the A-site selection rates expressed as

$$\frac{R_t}{R_s} = \frac{1}{F} - 1,$$

where F is the frequency of frameshifting, determined by dividing the β -galactosidase activity of the test codon constructs with that of the pseudo-wild-type plasmid pJC27.

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