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Occurrence of unmodified adenine and uracil at the first position of anticodon in threonine tRNAs in *Mycoplasma capricolum*

(codon recognition/unmodified anticodon/biased mutation pressure)

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ABSTRACT **Codon usage pattern in the threonine four-codon (ACN) box in *Mycoplasma capricolum* is strongly biased towards adenine and uracil for the third base of codons. Codons ending in uracil or adenine, especially ACU, predominate over ACC and ACG. This bacterium contains two isoacceptor threonine tRNAs having anticodon sequences AGU and UGU, both with unmodified first nucleotides. It would thus appear that ACN codons are translated in an unusual way; tRNA^{Thr}(AGU) would translate the most abundantly used codon ACU exclusively, because adenine at the first anticodon position can, according to the wobble rule, pair only with uracil of the third codon position. The tRNA^{Thr}(UGU) would mainly be responsible for translation of three other codons, ACA, ACG, and ACC. Anticodon UGU would also be used for reading codon ACU as a redundancy of tRNA^{Thr}(AGU), as deduced from the mitochondrial code where unmodified uracil at the first anticodon position can pair with adenine, cytosine, guanine, and uracil by four-way wobble. The tRNA^{Thr}(AGU) has much higher sequence homology to tRNA^{Thr}(UGU) from *M. capricolum* (88%), *Bacillus subtilis* (77%) and *Escherichia coli* (86%) than to tRNA^{Thr}(GGU) from *B. subtilis* (66%) and *E. coli* (63%), suggesting that tRNA^{Thr}(AGU) has been derived from tRNA^{Thr}(UGU), but not from tRNA^{Thr}(GGU).**

In the eubacterial genetic code, the first bases of tRNA anticodons for four-codon boxes are generally guanine and uracil (uracil almost always modified), with, additionally, frequent occurrence of cytosine (see ref. 3). Anticodon GNN translates codons NNU and NNC, and anticodon hoUNN [hoU = a 5-hydroxyuridine derivative (see ref. 4)] translates codons NNA, NNG, and possibly NNU by wobbling (4). Anticodon CNN translates only NNG codons. Presumably, predominance of codons NNG in the G+C-rich bacterial lines leads to a need for anticodon CNN (3). The only exception is the arginine four-codon box (CGN), where anticodon ICG (I for the hypoxanthine of inosine) translates codons CGU, CGC, and CGA; and anticodon CCG translates codon CGG.

During analyses of the tRNA gene sequence in *Mycoplasma capricolum*, we have found a gene cluster containing five tRNA genes, one of which is a gene for putative threonine tRNA with anticodon sequence AGU. Anticodon AGU, unmodified, can, according to the wobble rule (1), translate only codon ACU, but not ACC. Because threonine codon ACC rarely appears in reading frames in *M. capricolum*, the most plausible explanation for the above finding would be that the first nucleoside of this tRNA anticodon is replaced by inosine (5) after transcription, so that anticodon IGU can translate codon ACC, as well as ACU and ACA, by three-way wobble, as in the case of ICG in the arginine four-codon box. Anticodon IGU appears in yeast (6). Another possibility would be that tRNA^{Thr}(GGU) exists in addition to tRNA^{Thr}(AGU), so that codons ACU and ACC can be translated, although coexistence of anticodons GNN and ANN (or INN) has never been reported.

We have thus analyzed the product of the gene for tRNA^{Thr}(AGU), as well as the other threonine isoacceptor tRNA(s). Unexpectedly, the results have shown that the two possibilities mentioned above are both incorrect; there exist two isoacceptor tRNAs, one having anticodon sequence AGU and the other having UGU and both having unmodified first nucleotides. Thus far, unmodified adenine at the first position of the anticodon has only been found for tRNA^{Arg}(ACG) in yeast mitochondria (7, 8).

MATERIAL AND METHODS

DNA Cloning and Sequencing. The gene library of *Mycoplasma capricolum* [American Type Culture Collection 27343 (Kid.)] was constructed with *Hin*dIII-digested genomic DNA ligated to plasmid pBR322. The recombinant plasmid pMCH502 containing a 4.0-kilobase-pair (kbp) DNA insert was isolated as one of the clones that hybridized with unfractionated *M. capricolum* tRNA. DNA sequencing was done by the dideoxynucleotide chain-termination method (9).

Isolation of tRNA by DNA Column. Total tRNAs were prepared from *M. capricolum* by direct phenol extraction. A column of Sephacryl S-500 conjugated with 1.3-kbp *Acc* II fragment of the 4.0-kbp DNA (see above) was prepared according to the method of Bünemann and Westhoff (10). The 1.3-kbp fragment contained genes for tRNA^{Thr}(AGU) and a part of the 5′ end of tRNA^{Tyr}(GUA). Hybridization to and elution from the DNA column of the tRNAs were done as described (11). The eluted tRNAs were further purified by 12% polyacrylamide gel electrophoresis (11).

Isolation of tRNA by Benzoylated DEAE-Cellulose Column. Deacylated total tRNAs were incubated with threonine in the presence of the *M. capricolum* S100 fraction. The threonine-charged tRNAs were separated from other tRNAs by chromatography on benzoylated DEAE-cellulose column after 2-naphthoxyacetylation of the tRNAs^{Thr} according to the method of Gillam *et al.* (12). The threonine tRNA fraction was then subjected to 12% gel electrophoresis to separate the tRNA species; the tRNA bands were eluted from the gel and used for RNA sequencing.

RNA Sequencing. The nucleotide sequence was determined by the chemical methods of Peattie (13) and the enzyme method of Donis-Keller (14) using 3′ or 5′ ^{32}P-labeled RNA. The sequence was also determined by the postlabeling method of Kuchino *et al.* (15). Modified nucleotides were identified in the two-dimensional TLC using two different solvent systems: System I, isobutyric acid/0.5 M ammonia (5:3) in the first dimension and isopropyl alcohol/HCl/water (70:15:15) in the second dimension (16); System II, isobutyric

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acid/ammonia/water (60:1:33) in the first dimension and 100 ml of 0.1 M sodium sulfate (pH 6.8), 60 g of ammonium sulfate, and 2 ml of *n*-propylalcohol in the second dimension (17).

Chemicals. Enzymes were purchased from Takara-Shuzo (Kyoto), [5′-^{32}P]pCp and [γ-^{32}P]ATP were from Amersham (Japan), Sephacryl S-500 was from Pharmacia Fine Chemicals, benzoylated DEAE-cellulose was from Serva (Heidelberg), and cellulose thin-layer plates were from Funakoshi Pharmacological (Tokyo).

RESULTS

We isolated the recombinant plasmid pMCH502 containing a 4-kbp *Hin*dIII fragment of *M. capricolum* DNA as one of the clones that hybridizes with unfractionated tRNA. The fragment contained a cluster of five tRNA genes. The order was 5′–tRNA^{Thr}(AGU)–tRNA^{Tyr}(GUA)–tRNA^{Gln}(UUG)–tRNA^{Lys}(UUU)–tRNA^{Leu}(UAA)–3′ (Fig. 1). The tRNA gene cluster was preceded by a putative promoter structure and followed by a probable termination signal. The tRNA genes were divided by short (4- to 8-base-pairs) spacers. Thus these genes seem to consist of a single transcriptional unit. Of special interest is that the anticodon sequence of tRNA^{Thr} gene was AGU, because in the eubacterial code, the first base of anticodons is always guanine, uracil, or cytosine, with an exception of anticodon ICG for arginine (see above).

To determine whether the tRNA^{Thr}(AGU) gene described above is transcribed and the product tRNA has the anticodon sequence AGU or IAU, we purified the tRNAs that hybridized with a DNA fragment (a derivative of pMCH502) containing a complete sequence of tRNA^{Thr}(AGU) and the 5′-half of tRNA^{Thr}(GUA) genes. The purification procedure consists of hybridization of total tRNAs with the DNA fragment, followed by identification of the hybridized tRNAs by sequencing. Two tRNAs were detected; one of them agreed with the DNA sequence for the tRNA^{Thr}, and the other agreed with the DNA sequence for the tRNA^{Tyr}, as expected. The anticodon sequence of tRNA^{Thr} was AGU in which adenine was unmodified as judged by TLC (see below).

In the eubacterial code, threonine codons ACU and ACC are read by anticodon GGU; and codons ACA, ACG, and possibly ACU are read by anticodon hoUCU. Anticodon AGU would be able to recognize only ACU, but not ACC. Table 1 shows that threonine codon usage pattern in *M. capricolum* is strongly A+T(or U)-biased, as expected from a high A+T content of the genome (18). Codons ending in uracil or adenine, especially ACU, predominated, and yet codon ACC was used in a low frequency. Codon ACG has not been found thus far.

Table 1. Threonine codon usage in *Mycoplasma capricolum*

Codons	ACU	ACC	ACA	ACG
Occurrence*	131	4	85	0
Percent	(59.5)	(1.8)	(38.6)	(0)

*Number of threonine codons in 20 ribosomal protein genes, *secY* and *adk* of *M. capricolum* (from ref. 27).

We then isolated the isoacceptor threonine tRNAs, and their sequences were determined. The deacylated total tRNAs were incubated for a short period with threonine in the presence of the *M. capricolum* S100 fraction to reacylate threonine tRNAs. The charged tRNAs were 2-naphthoxyacetylated and separated from other nonacylated tRNAs on a benzoylated DEAE-cellulose column, followed by further purification with polyacrylamide gel electrophoresis. Fig. 2 shows an electrophoretic separation of the tRNAs. Three RNAs (a, b, and c in Fig. 2), differing slightly in length, predominated. A longer RNA (d) was also found as a minor component. All the four RNA species were eluted and sequenced. The band d was tRNA^{Leu}(UAA). The tRNAs of band a and b were tRNA^{Thr}(AGU) and tRNA^{Thr}(UGU), respectively. The tRNA of band c had anticodon sequence NAU (N, unidentified modified base) and is probably tRNA^{Ile}. The first modified base (*C) of the anticodon for the *E. coli* tRNA^{Ile}(*CAU) contains a nonaromatic amino acid (Yokoyama *et al.*, personal communication). If the same is applied to the *M. capricolum* tRNA^{Ile}(NAU), then the amino acid residue in this tRNA would be 2-naphthoxyacetylated and be recovered together with tRNA^{Thr}. TLC identification, using two different solvent systems, of the first (5′) nucleotide of anticodons clearly revealed that adenine in tRNA^{Thr}(AGU) and uracil in tRNA^{Thr}(UGU) were both unmodified; radioactive spots of these nucleotides perfectly agreed with the UV spots of authentic adenylic acid and uridylic acid, respectively (Fig. 3). Fig. 4 shows the total sequences of the two threonine tRNAs including modified nucleotides.

Because the bulk, if not all, of the threonine-charged tRNAs should be recovered from the benzoylated DEAE-cellulose chromatography, we conclude that *M. capricolum* contains two isoacceptor tRNAs with anticodon sequences of

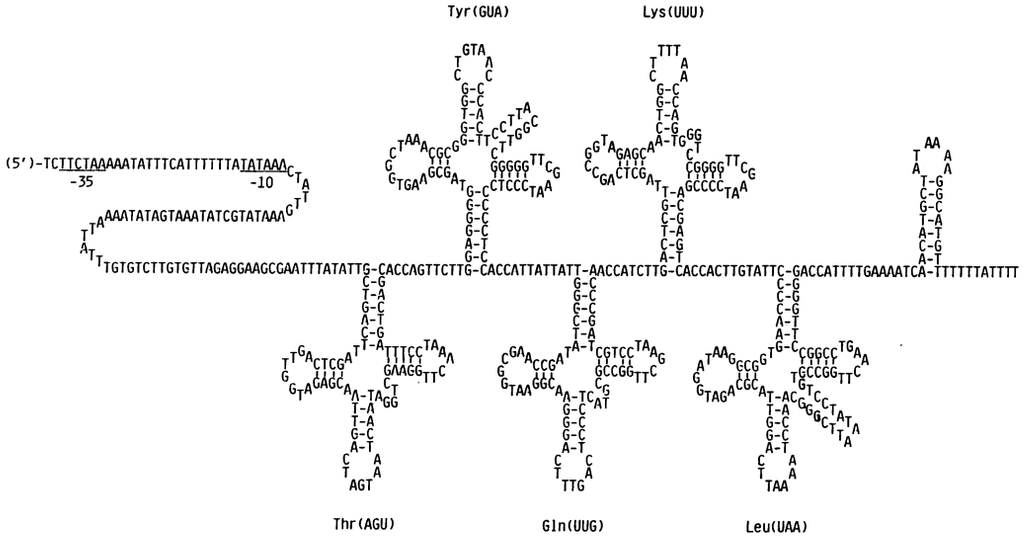

FIG. 1. DNA sequence of a *M. capricolum* tRNA gene cluster. Noncoding (RNA-like) strand of a part of pMCB502 DNA including tRNA genes is shown.

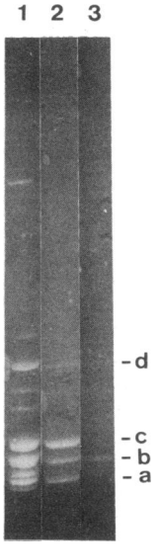

FIG. 2. Polyacrylamide gel electrophoresis of tRNAs. Deacylated total tRNAs of *M. capricolum* were incubated for 10 min at 37°C with 2 mM threonine in the presence of the S100 fraction to acylate threonine tRNAs. The charged tRNAs were 2-naphthoxyacetylated and separated from noncharged tRNAs by a benzoylated DEAE-cellulose column. (Lane 1) Total tRNAs; (lane 2) "charged" tRNAs isolated by benzoylated DEAE-cellulose column chromatography; (lane 3) $tRNA^{Thr}$(AGU) isolated by using a column of Sephacryl S-500 conjugated with the DNA fragment containing the gene for $tRNA^{Thr}$(AGU). a, $tRNA^{Thr}$(AGU); b, $tRNA^{Thr}$(UGU); c, (NAU; N, unidentified modified base) probably $tRNA^{Ile}$; and d, $tRNA^{Leu}$ (UAA).

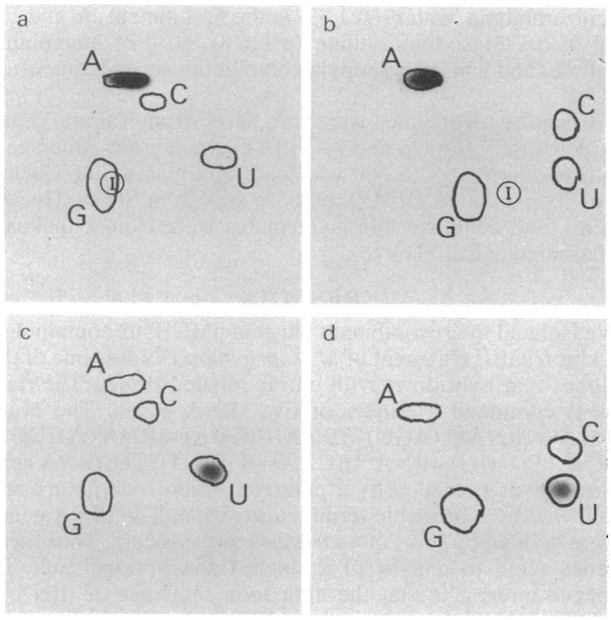

FIG. 3. TLC identification of the first (5′) nucleotide of anticodons of $tRNA^{Thr}$(AGU) and $tRNA^{Thr}$(UGU). $tRNA^{Thr}$(AGU) and $tRNA^{Thr}$(UGU) were isolated by DNA column and benzoylated DEAE-cellulose column, respectively. The tRNA was partially digested with formamide, and the 5′ ends of the digests were labeled with [γ-^{32}P]ATP and polynucleotide kinase. The labeled products were separated by 20% polyacrylamide gel electrophoresis according to their chain lengths and autoradiographed. The 5′ end-labeled oligonucleotide in each radioactive band was eluted from the gel and digested with ribonuclease P1 to produce N-[5′-^{32}P]monophosphate. The product was mixed with nonlabeled specific N,N,N,N-5′-monophosphates and displayed by two-dimensional TLC (15). The anticodon sequence could be identified from the total tRNA sequence determined by the above method. To see the modification of the first nucleotide of anticodon, the corresponding nucleotide was further identified by TLC. The labeled first nucleotide of the anticodon from $tRNA^{Thr}$(AGU) (*a* and *b*) or from $tRNA^{Thr}$(UGU) (*c* and *d*) was displayed by TLC using solvent system I (*a* and *c*) and system II (*b* and *d*), respectively, and exposed on an x-ray film. Positions of authentic unmodified nucleotides were determined by UV irradiation. I (inosinic acid) should be positioned as indicated in the figures. Positions of various modified uridine nucleotides do not agree with that of the unmodified one (see refs. 16 and 17).

AGU and UGU. The first anticodon nucleotide is not modified in either of these tRNAs.

DISCUSSION

We have shown that in *M. capricolum* there exist at least two threonine isoacceptor tRNAs having anticodon sequences of AGU and UGU, the first bases adenine and uracil being both unmodified. No tRNA having anticodon GGU or CGU has been found, although a possibility of the presence of these two tRNA species as minor components cannot be excluded. Assuming that $tRNA^{Thr}$(AGU) and $tRNA^{Thr}$(UGU) are the only threonine tRNAs in this organism, codon ACU, which is the most abundantly used threonine codon (18) (see Table 1), would appear to be translated by anticodon AGU. Anticodon UGU would mainly be responsible for three other threonine codons, ACA, ACG, and ACC. Because unmodified uracil at the first anticodon position has been reported to be able to pair with adenine, guanine, cytosine, and uracil by four-way wobble in the mitochondrial code (2), $tRNA^{Thr}$-(UGU) could also be used for reading codon ACU as a redundancy of $tRNA^{Thr}$(AGU).

As postulated previously (3), the early code would have used GNN anticodon for translation of codons NNY (where Y = uracil or cytosine) and anticodon hoUNN for codons NNR (where R = adenine or guanine) in all the four-codon boxes. This rule can be applied to most of the eubacterial code, except for the arginine four-codon box. The evolution of AGU and UGU anticodons in the *Mycoplasma* line can be deduced as follows.

The simplest explanation for the appearance of anticodon AGU would be that GGU mutated to AGU by A+T-biased mutation pressure as assumed from a very high A+T-content (75%) of the *M. capricolum* genomic DNA (19). However, this seems not to be the case. The $tRNA^{Thr}$(AGU) would most probably have been derived from $tRNA^{Thr}$(UGU), but not from $tRNA^{Thr}$(GGU), as assumed from the following facts. The *M. capricolum* tRNA gene arrangement, $tRNA^{Thr}$(AGU)–$tRNA^{Tyr}$(GUA)–$tRNA^{Gln}$(UUG)–$tRNA^{Lys}$-(UUU)–$tRNA^{Leu}$(UAA) studied here (Fig. 1), is similar to a part of the *B. subtilis* "16 tRNA gene cluster," the arrangement of which is *$tRNA^{Thr}$(UGU)–$tRNA^{Tyr}$(GUA)*–$tRNA^{Trp}$-(CCA)–$tRNA^{His}$(GUG)–*$tRNA^{Gln}$(UUG)*–$tRNA^{Gly}$(GC-C)–$tRNA^{Cys}$(GCA)–*$tRNA^{Leu}$(UAA)* (20). The *B. subtilis* genes shown in italic letters are those also found in *M. capricolum*. The sequence similarity between presumed homologous tRNA genes of *M. capricolum* with those of *B. subtilis* is high—i.e., 79% between $tRNA^{Thr}$(AGU) (*M, M. capricolum*) and $tRNA^{Thr}$(UGU) (*B, B. subtilis*), 78% between $tRNA^{Tyr}$(GUA) (*M*) and $tRNA^{Tyr}$(GUA) (*B*), 90% between $tRNA^{Gln}$(UUG) (*M*) and $tRNA^{Gln}$(UUG) (*B*), and 69% between $tRNA^{Leu}$(UAA) (*M*) and $tRNA^{Leu}$(UAA) (*B*). On the other hand, *B. subtilis* $tRNA^{Thr}$(GGU), the gene of which is a member of the "6 tRNA gene cluster" (21), has only 64% similarity to the *M. capricolum* $tRNA^{Thr}$(AGU). These observations suggest that the *M. capricolum* $tRNA^{Thr}$(AGU) gene has resulted from a mutation of the $tRNA^{Thr}$(UGU) gene that is homologous with the *B. subtilis* $tRNA^{Thr}$(UGU) gene mentioned above. *B. subtilis* has one more tRNA gene cluster—i.e., the "21 tRNA gene cluster,"

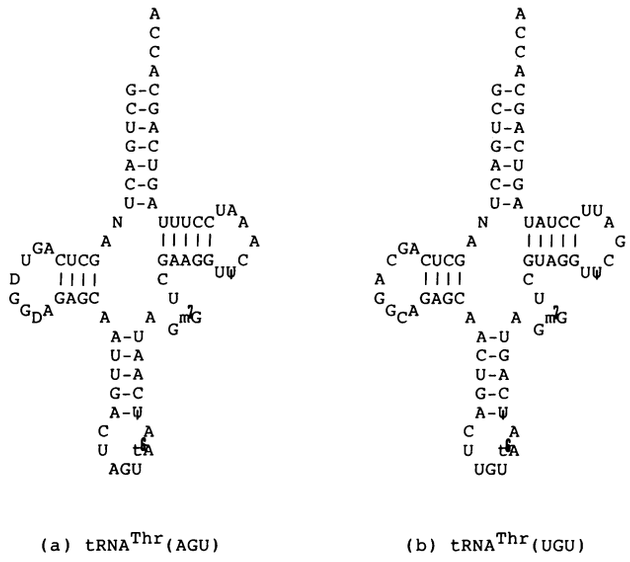

FIG. 4. Structures of the two threonine tRNAs of *M. capricolum*. The two tRNAs were purified through gel electrophoresis (see Fig. 2), and sequence was determined by chemical (13) and enzyme method (14) and also by a postlabeling method (15). Modified nucleotides were identified by TLC (16, 17). N represents unidentified modified uracil. (*a*) tRNA^{Thr}(AGU) and (*b*) tRNA^{Thr}(UGU).

containing the second tRNA^{Thr}(UGU) gene; its gene arrangement is unrelated to the "16 tRNA gene cluster," and yet the sequence of the second tRNA^{Thr}(UGU) is identical with that (first) in the "16 tRNA gene cluster" (20, 22). The sequence similarities of the first and the second *B. subtilis* tRNA^{Thr}(UGU) to *M. capricolum* tRNA^{Thr}(UGU) are high (77%), thus suggesting that the second *B. subtilis* tRNA^{Thr}(UGU) gene in the "21 tRNA gene cluster" and *M. capricolum* tRNA^{Thr}(UGU) gene are homologous counterparts. *M. capricolum* tRNA^{Thr}(AGU) also reveals a higher similarity to *E. coli* tRNA^{Thr}(UGU) (86%) than to tRNA^{Thr}(GGU) (63%).

From these observations, two possibilities would exist for the evolution of threonine tRNAs in the *Mycoplasma* line. (*i*) First, anticodon UGU became unmodified as a result of partial deprivation of the uracil-modification enzyme system, so that UGU could translate all the threonine codons by four-way wobble as deduced from the mitochondrial code, and GGU disappeared. This process would be simultaneous with a tendency to genomic economization in the *Mycoplasma* line (23). An increasingly A+T-biased mutation pressure led to an extreme predominance of ACU (and ACA) codons over ACC and ACG in the genes. Under these circumstances, one of the genes for tRNA^{Thr}(UGU) mutated to tRNA^{Thr}(AGU). (*ii*) UGU became unmodified, followed by mutation of one of the genes for UGU to AGU. This would be a transient stage where UGU, AGU, and GGU all existed. GGU then disappeared, for the low demand for translation of codon ACC would be supplied by anticodon UGU, and anticodon GGU was not needed. In any case, the appearance of tRNA^{Thr}(AGU) is adaptive to fulfill a heavy demand for translation of codon ACU. This situation may be somewhat analogous to an increase in G+C-rich bacterial lines in the content of anticodons CNN, which would have been called for by a predominance of NNG codons in the protein genes (see *Introduction*).

One significance of the presence of anticodon AGU would be that, as mentioned above, it would translate codon ACU more efficiently than anticodon UGU. Moreover, the codon–anticodon pairing 5′ACN3′·3′UGU5′ involves only one G·C pair, which might cause misreading. Thus the presence of anticodon AGU would be advantageous in view of both efficiency and correct reading of the most frequently used ACU threonine codon in this organism. In this connection, it is of interest to note that *Mycoplasma mycoides* seems to contain only one species of glycine tRNA having anticodon sequence UCC (24, 25). Because codon–anticodon pairing 5′GGN3′·3′CCU5′ in glycine four-codon box involves two G·C pairs, all four codons can be translated by four-way wobble more correctly, as compared with the case of the threonine box. The four-codon boxes where two G·C pairs are involved in codon recognition by the second and the third positions of the anticodon are for proline, alanine, and arginine in addition to glycine, whereas those containing only one G·C pair are for leucine, valine, and serine in addition to threonine. It is thus possible that codons in the former boxes containing one G·C pair could be read by anticodons ANN and UNN (N = cytosine or guanine, adenine or uracil), whereas in the latter containing two G·C pairs, four codons could be read only by anticodon UNN (N = guanine or cytosine), with an exception of arginine four-codon box. As already pointed out, all the eubacterial species so far studied use anticodon ICG for translation of arginine codons CGU, CGC, and CGA and anticodon CCG for codon CGG. In *M. mycoides*, a gene for tRNA with anticodon ACG has been reported (26), although whether adenosine is replaced by inosine after transcription and whether anticodon CCG exists have yet to be studied.

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