The organization and evolution of transfer RNA genes in *Mycoplasma capricolum*

Akira Muto, Yoshiki Andachi, Harumi Yuzawa*, Fumiaki Yamao+ and Syozo Osawa
Department of Biology, School of Science, Nagoya University, Chikusa, Nagoya 464-01, Japan

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ABSTRACT

The genes for presumably all the tRNA species in *Mycoplasma capricolum*, a derivative of Gram-positive eubacteria, have been cloned and sequenced. There are 30 genes encoding 29 tRNA species. This number is the smallest in all the known genetic systems except for mitochondria. The sequences of 9 tRNA genes of them have been previously reported (1 – 3). Twenty-two genes are organized in 5 clusters consisting of nine, five, four and two genes (2 sets), respectively. The other eight genes exist as a single transcription unit. All the tRNAs are encoded each by a single gene, except for the occurrence of two $tRNA^{Lys}$(TTT) genes. The arrangement of tRNA genes in the 9-gene cluster, the 5-gene cluster, the 4-gene cluster and one of the 2-gene clusters reveals extensive similarity with a part of the 21-tRNA gene cluster and/or the 16-tRNA gene cluster in *Bacillus subtilis*, respectively. The results suggest that the present *M. capricolum* tRNA genes have evolved from large tRNA gene clusters in the ancestral Gram-positive bacterial genome common to *M. capricolum* and *B. subtilis*, by discarding genes for redundant as well as non-obligate tRNAs, so that all the codons may be translated by as small a number of tRNAs as possible.

INTRODUCTION

Mycoplasmas are wall-less eubacteria and parasitic in eukaryotic tissues and organs. They are phylogenetically related to Gram-positive bacteria such as *Bacillus* spp. and *Clostridium* spp. (4–7), however, with the genome size much smaller (about 800 Kbp). They are thus regarded as a degenerated form of Gram-positive bacteria. *M. capricolum* contains the genes for only about 400 proteins (8) and two sets of rRNAs (9), suggesting that many non-obligate genes have been discarded during evolution.

In the previous paper (10), we have reported sequences for the complete set of tRNA species from *M. capricolum*, that consists of only 29 tRNA species with 28 different anticodons, the smallest in number of all the known genetic systems except for mitochondria. Many non-obligate tRNAs do not exist, so that a set of synonymous codons may be translated by a single tRNA species in most of the cases. This suggests that many tRNA genes, like genes for proteins and rRNAs, have been discarded during evolution, so as to read all the codons by as small a number of tRNA anticodons as possible. In this study, we have cloned and sequenced all the genes for tRNAs in the *M. capricolum* genome including the nine tRNA genes previously reported (1–3). The results show that the genome contains 30 tRNA genes encoding 29 tRNA species. The organization of these tRNA genes is in many ways similar to that of *Bacillus subtilis* and suggests that the tRNA genes in *M. capricolum* have evolved by discarding redundant as well as non-obligate tRNA genes from large tRNA gene clusters like those conserved in the *B. subtilis* genome.

MATERIALS AND METHODS

Preparation of DNA and tRNA

Mycoplasma capricolum [American Type Culture Collection 27343 (Kid)] cells were grown at 37°C in a medium containing 2.2% (w/v) PPLO broth, 0.1% yeast extract, 0.2% glucose, 20 mM-Tris.HCl (pH 7.6), 0.01% thallous acetate, 400 U/ml-penicillin G and 1% calf serum (9). Cells were collected at late log phase by centrifugation and stored at −20°C. The genomic DNA was prepared as described previously (11).

Total tRNAs were prepared from the cells by the direct phenol extraction method (12), purified by 12% polyacrylamide gel-electrophoresis (10), and deacylated by incubation in 1 M-Tris.HCl (pH 9.0) at 37°C for 2 h.

For isolation of purified tRNA species, benzoylated DEAE-cellulose column chromatography was used as described (10).

Hybridization

The 3'-end-labelled tRNA was used as a probe for hybridization experiments. The total or purified tRNA (0.2–2.0 μg) was incubated with 10 μCi of [5'-^{32}P]pCp (300 Ci/mmol) and 1 unit of T4 RNA-ligase at 4°C for 15 h in a reaction mixture (10 μl) as described by England *et al.* (13). The labelled tRNAs were recovered from the mixture by ethanol precipitation together with 5 μg carrier *M. capricolum* ribosomal RNAs, washed twice with 70% ethanol and dissolved in 20 μl 5×SSC.

The DNA-blotted nitrocellulose filter was incubated in a solution containing 5×SSC, 50% formamide and ^{32}P-labelled tRNA at 45°C for 20 h. The filter was washed with 5×SSC and 50% formamide at 45°C twice and then with 0.5×SSC at 50°C three times, dried and exposed to an X-ray film.

Present addresses: *Institute of Virus Research, Kyoto University, Kyoto 606 and +National Institute of Genetics, Mishima 411, Japan

Cloning and sequencing

A DNA library was made by ligating 15−20 Kbp (Kilo-base pairs) *Sau*3AI partial digestion fragments of *M. capricolum* genomic DNA into the *Bam*HI-site of EMBL4 phage DNA (14). Among 350 recombinant phages, 30 phages that hybridized with total tRNAs were selected by plaque hybridization. The DNA was prepared from each recombinant phage, digested with *Hin*dIII and separated by agarose gel electrophoresis. The DNA in the gel was blotted to nitrocellulose filter, and hybridized with ^{32}P-labelled total tRNAs to identify the DNA fragments containing tRNA genes. The DNA fragment was isolated and subcloned into the *Hin*dIII site of the plasmid vector PUC118 or PUC119 DNA (15) for sequencing analysis.

Some tRNA genes were isolated using purified tRNA as a hybridization probe. The total DNA of *M. capricolum* was digested with *Hin*dIII, separated by agarose gel electrophoresis and blotted to nitrocellulose filter. The filter was used for hybridization assay with purified tRNA to know the size of the DNA fragment containing the gene. The DNA fragment having an appropriate size was prepared from agarose gel, ligated to PUC118 DNA and transformed into *Escherichia coli* JM109 cells. Colony hybridization was performed to select the cell which carries the recombinant plasmid including the tRNA gene. The DNA fragment in the isolated recombinant plasmid was deleted stepwise from one end by ExoIII and ExoVII nuclease (16), and the deleted plasmids were used directly for plasmid sequencing analysis (17,18).

For sequencing the tRNA^{Ile}(GAT) gene and its flanking regions, the inverse polymerase chain reaction (PCR) (19) was carried out: the total *M. capricolum* DNA was digested with *Hin*dIII and circularized by self-ligating the digestion product at the concentration of 1 μg DNA/ml with T4 DNA-ligase (20). Two intragenic sequences of tRNA^{Ile}(GAT) (5′-CCAGCTGAG-CTATATTCC-3′ and 5′-GATAACGGAGAGGTCGTT-3′) (4) were synthesized by Pharmacia-LKB DNA synthesizer (Gene Assembler Plus) and used as primers. PCR reaction (40 cycles) was carried out by DNA Thermal Cycler (Perkin Elmer Cetus) under the standard conditions using about 10 ng DNA as a template and *Taq* DNA-polymerase. The reaction products were separated by 1% agarose gel electrophoresis. The DNA band (0.9 Kbp) was eluted from the gel, phosphorylated at the 5′-end, cloned in the *Sma*I-site of PUC119 and sequenced. To confirm the intragenic sequence of the tRNA^{Ile}(GAT) gene, the second PCR reaction was carried out using two synthetic DNA primers (5′-GCTATAATTGTTAGAAGA-3′ and 5′-GGCTTGTGT-GAAAATC-3′), which are complementary to the sequences of about 180 bp upstream region from the 5′-end of the gene (sense strand) and of about 130 bp downstream region from the 3′-end of the gene (antisense strand)(see Fig. 2). The sequence of the amplified DNA fragment (373 bp) was determined by the direct sequencing method (21).

The other DNA handling techniques were performed by the established methods (22).

Materials

All restriction endonucleases, T4 RNA-ligase, *Taq*-polymerase, ExoIII and ExoVII nucleases were purchased from Takara-Shuzo Co. Ltd. (Kyoto, Japan); Radioactive compounds (^{32}P and ^{35}S) were from Amersham Japan (Tokyo, Japan). For sequencing DNA, 'Sequenase' kit of Toyobo Co., Ltd. (Osaka, Japan) was used.

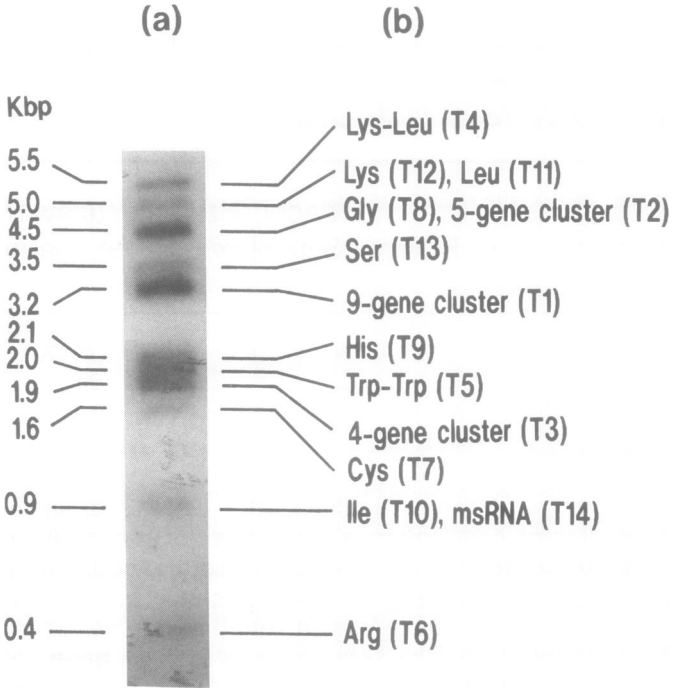


Fig. 1. Distribution of tRNA genes in the *Mycoplasma capricolum* genome. (a) Southern hybridization of *Hin*dIII-digests of total DNA and total tRNA labelled with [^{32}P]pCp. (b) Correspondence of the hybridized bands and the cloned tRNA genes or clusters. For organization of each clone, see Fig. 3.

RESULTS

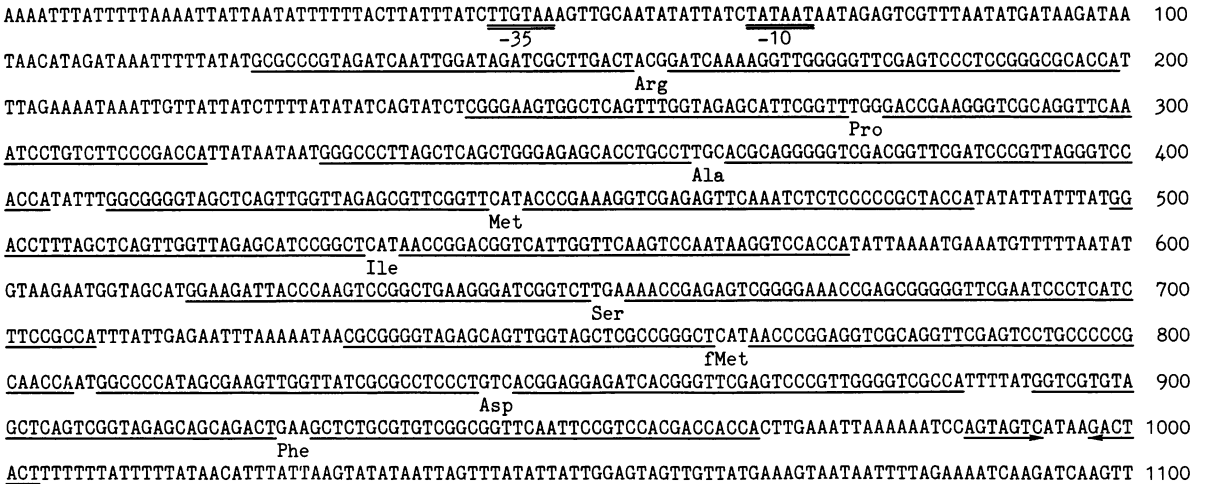
Cloning and Sequencing of tRNA genes

Southern hybridization of the *Hin*dIII-digested total DNA and ^{32}P-labelled total tRNAs of *M. capricolum* revealed at least 11 distinct bands with the size ranging from 0.4 to 5.5 Kbp (Fig. 1(a)). The tRNA genes corresponding to all of these bands were identified by cloning and sequencing the DNAs (Fig. 1(b)). They were distributed in 13 different *Hin*dIII DNA fragments, T1 to T13. The 5.0 Kbp and 4.0 Kbp bands included two different clones, T11 and T12, and T2 and T8, respectively. Most of the clones were selected from the genomic DNA library of *M. capricolum* made with lambda phage (EMBL4) DNA as a vector (14), and subcloned into plasmid vector PUC118 or PUC119. The tRNA^{Gly}(TCC) and tRNA^{Leu}(CAA) genes were identified by genomic Southern hybridization using the purified respective tRNAs.

The tRNA^{Ile}(GAT) gene present in 0.9 Kbp *Hin*dIII-fragment (T10) could not be cloned in the plasmid vector. For sequencing the gene and its flanking regions, inverse polymerase chain reaction (PCR) was carried out using the synthesized DNA primers having the sequences of two different parts of the known tRNA^{Ile}(GAT) sequence (10)(see Fig. 2). The sequences of the tRNA^{Trp}(TCA)-tRNA^{Trp}(CCA) gene cluster in 2.0 Kbp *Hin*dIII-fragment (T5), the tRNA^{Lys}(TTT)-tRNA^{Leu}(TAG) gene cluster in 5.5 Kbp *Hin*dIII-fragment (T4) and the 5-tRNA gene cluster in 4.5 Kbp *Hin*dIII-fragment (T2) were reported previously (1−3).

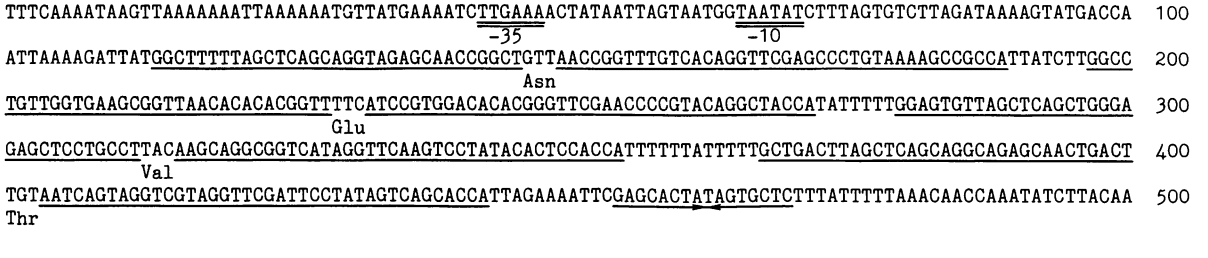
Altogether, the sequences of the 30 tRNA genes in the 13 different clones were determined. The tRNA^{Lys}(UUU) was only tRNA species encoded by two genes with identical sequence.

(T1) 9-tRNA gene cluster

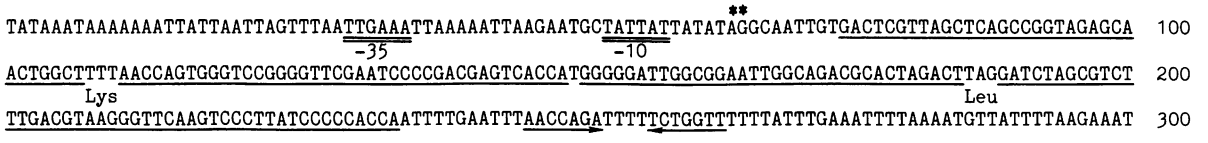


(T2) 5-tRNA gene cluster

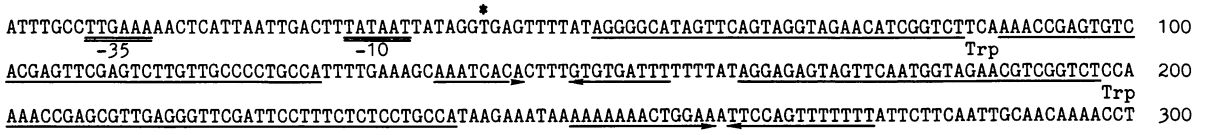
(T3) 4-tRNA gene cluster



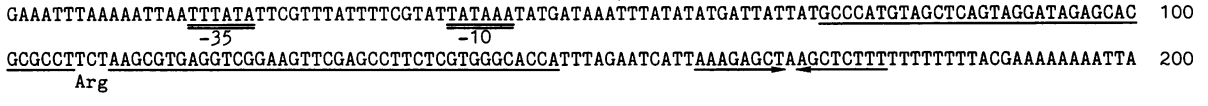
(T4) $tRNA^{Lys}(TTT)$-$tRNA^{Leu}(TAG)$



(T5) $tRNA^{Trp}(TCA)$-$tRNA^{Trp}(CCA)$



(T6) $tRNA^{Arg}(TCT)$



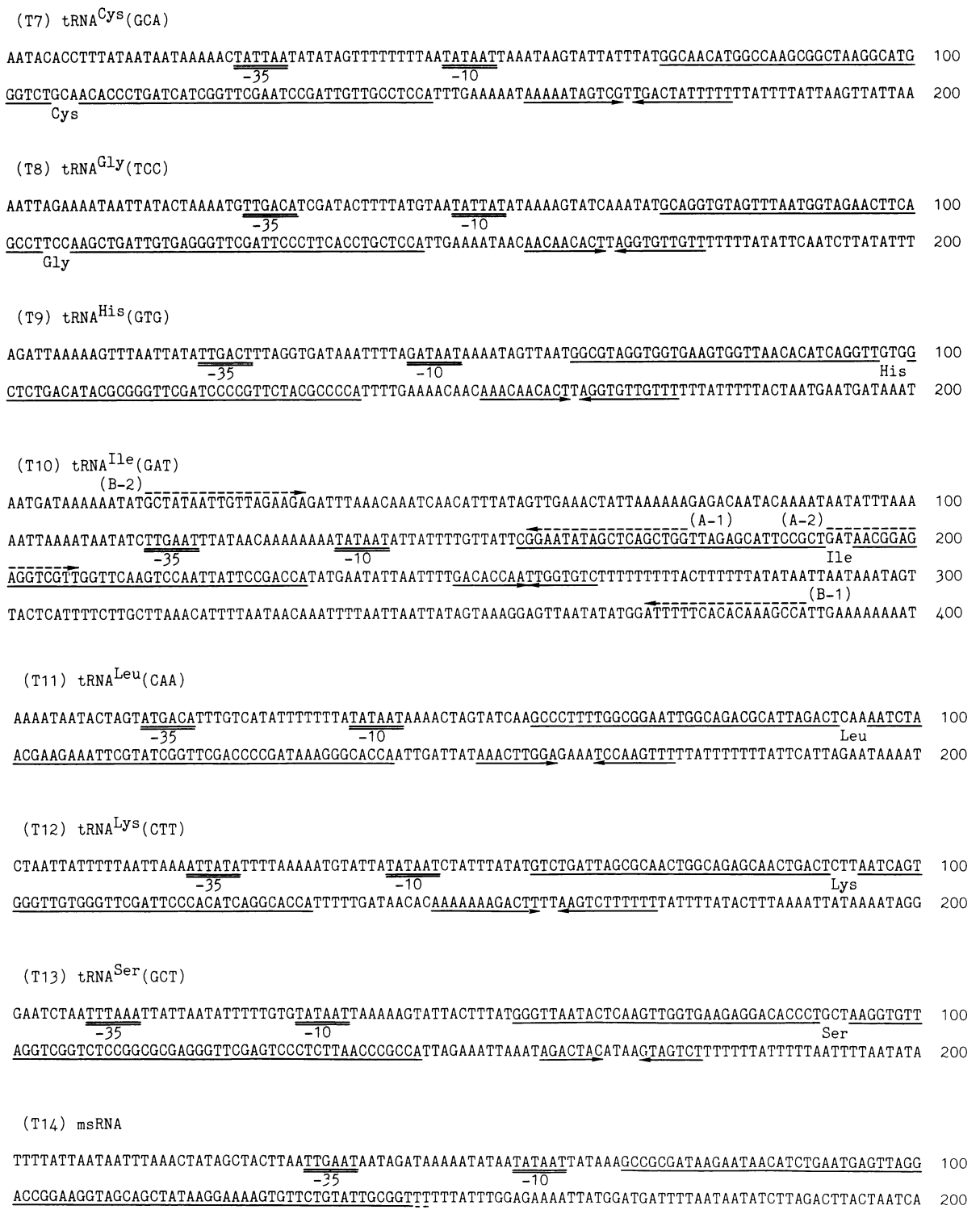


Fig. 2. Nucleotide sequences of tRNA genes and their flanking regions. Only RNA-like sequences are shown. tRNA sequences are underlined. Double underlines show putative promoter, −10 and −35 sequences, and arrows show terminator-like dyad-symmetrical sequences. The transcription initiation sites for the two 2-gene clusters (T4 and T5), determined by Gafny *et al.* (3) and Yamao *et al.* (27), respectively, are shown by asterisks. For sequencing tRNA^{Ile}(GAT) gene (T10), PCR method was used: The first PCR (inverse PCR) reaction was carried out using circularized *Hin*dIII-DNA and synthetic DNA primers A1 and A2 (dotted arrows above the sequence), having intragenic sequences of the tRNA gene; the second reaction was performed using linear DNA and primers B1 and B2, having 5′- and 3′-flanking sequences. The sequence of a new small RNA gene (see *Appendix 2*) is also shown (msRNA:T14). The 3′-end of the RNA is redundant as shown by dotted underline. The nucleotide sequence data will appear in the DDBJ, EMBL and GenBank Nucleotide Sequence Databases under the accession numbers from D00547 (T1) to D00560 (T14).

Thus, the 30 genes encode 29 different tRNA species, corresponding to those reported in the previous paper (10). The sequences (RNA-like strand) of the 30 genes and their flanking regions are shown in Fig. 2.

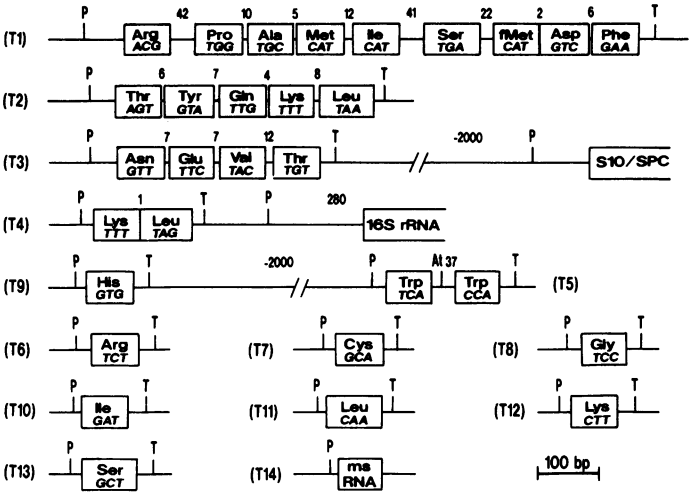


Fig. 3. Organization of tRNA genes. The gene arrangements in the 14 clones including tRNA genes (T1-T13) and a new small RNA species (T14: see *Appendix 2*) are schematically shown. The tRNA genes are indicated by box with the specifying amino acid and the anticodon. Abbreviations: P, promoter; T, terminator; At, attenuator; S10/SPC, ribosomal protein operon (14). Numbers above the lines indicate the number of base pairs in the spacers. T3 cluster locates about 2 Kbp upstream of S10/Spc operon; T4 locates about 280 bp upstream of the 16S rRNA gene in *rrnA* (7); T7 ($tRNA^{His}$) is about 2 Kbp upstream of T5. Attenuator sequence exists in the spacer between the two $tRNA^{Trp}$ genes (T5) (27).

Organization of tRNA genes

Fig. 3 shows the organization of the 30 tRNA genes, of which 22 genes are organized in five tRNA gene clusters, the 9-gene cluster (T1), the 5-gene cluster (T2), the 4-gene cluster (T3) and two sets of 2-gene cluster (T4 and T5). The other eight tRNA genes are present each as a putative single transcription unit (T6-T13). The 3′-terminal CCA end is all encoded in DNA.

Two tRNA gene clusters with the gene orders identical to the 9-gene (T1) and the 4-gene (T3) clusters, respectively, have been isolated from *M. mycoides*, a closely related species of *M. capricolum* (23,24). Rogers *et al.* (25) have reported a gene cluster of *Spiroplasma meliferm* containing 10 tRNA genes, in which the order of 9 tRNA genes is idential to that in the 9-gene cluster of *M. capricolum* and *M. mycoides*. A 2-gene cluster homologous to the $tRNA^{Lys}$(TTT)-$tRNA^{Leu}$(TAG) culster (T5) has been found in *Mycoplasma* PG50 (26). These similarities in the organizations as well as in the primary sequences suggest that tRNA genes are well conserved among mycoplasma species.

The transcription initiation regions for two *M. capricolum* tRNA gene clusters, the $tRNA^{Trp}$(TCA)-$tRNA^{Trp}$(CCA) (T5) and the $tRNA^{Lys}$(TTT)-$tRNA^{Leu}$(TAG) gene (T4) clusters, have promoter-like sequences, resembling *Escherichia coli* −10 and −35 consensus sequences, at 5′-upstreams of the initiation sites as reported by Yamao *et al.* (27) and Gafny *et al.* (3), respectively. There exists a similar promoter-like sequences in all the other three gene clusters and eight single tRNA genes (see Fig. 2). The space between the −10 and −35 boxes is 15 to 17 bp. At 3′-downstream region, there is a *rho*-independent terminator-like structure, consisting of a dyad-symmetrical sequence and T-cluster, in all the clusters or the genes (Fig. 2). These suggest that *M. capricolum* uses the promoter and terminator signals resembling those of *E. coli*, and that each cluster or gene constructs a transcription unit (operon).

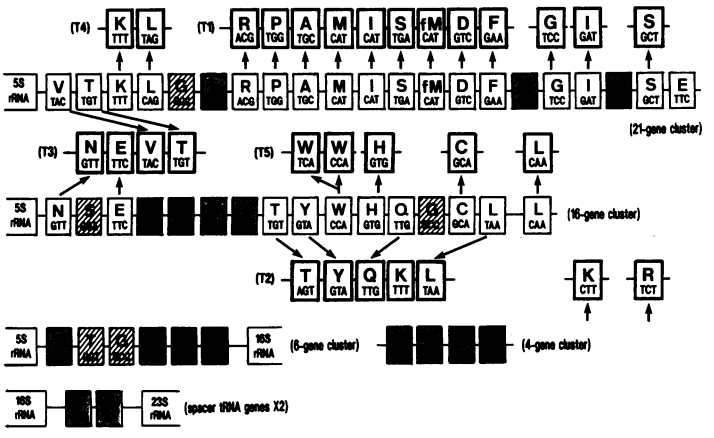


Fig. 4. Comparisons of the tRNA gene organizations between *M. capricolum* and *B. subtilis*. The tRNA genes of *M. capricolum* and *B. subtilis* are boxed by bold- and thin-line, respectively. The species of tRNA genes are shown by one-letter code of the specifying amino acid and the anticodon. Presumed correspondence of tRNA genes between the two species is indicated by arrows. The tRNA genes that possibly have been discarded in evolution to *M. capricolum* are shadowed by stripes (non-oblique tRNA genes) or by dots (redundant tRNA genes) (see Text).

The sequence comparisons of the tRNA clones with the clones containing genes for rRNAs and ribosomal proteins have indicated that the 4-tRNA gene cluster (T3) locates about 2 Kbp upstream of the S10/Spc-ribosomal protein gene cluster (14), and the $tRNA^{Lys}$(TTT)-$tRNA^{Leu}$(TAG) cluster (T5) is present at 280 bp upstream of the 5′-end of the 16S rRNA gene in *rrnA* operon as reported by Gafny *et al.*(3). The $tRNA^{His}$(GTG) gene (T9) and the $tRNA^{Trp}$(TCA)-$tRNA^{Trp}$(CCA) gene cluster (T5) are also linked on the chromosome separated by a space of about 2 Kbp (Fig. 3).

DISCUSSION

The codon usage and tRNA anticodon composition of *M. capricolum* are unique among eubacteria (10,28). The codon usage is strongly biased to A- and T(U)-richness, and more than 90% of the codon third positions are occupied by A or U. The amino acid assignment for one, and probably two, codon deviates from the universal genetic code: codon UGA from stop to Trp, and probably codon CGG from Arg to an unassigned, in accordance with the existence of anticodon UCA (Trp) and the absence of anticodon CCG (Arg), respectively. As discussed previously (10), the codon-anticodon recognition patterns of *M. capricolum* are unique in many ways as compared with those of other bacteria such as *E. coli* and *B. subtilis*. For example, most of four synonymous codons in family-boxes are read by a single anticodon UNN, U unmodified, with deletion of anticodon GNN and CNN (10,24,29), and many non-obligate CNN anticodons in two-codon sets are absent. As a result, most of the synonymous set of codons are translated each by a single

tRNA species. The absence of these non-obligate tRNA genes as well as the gene for $tRNA^{Arg}$(CCG) is evident by the present analyses.

M. capricolum genome contains 30 tRNA genes encoding 29 tRNA species, which are much smaller in number as compared with the genome of *E. coli* that carries 78 genes for 45 tRNA species (or 41 species of anticodon) (30), or with *B. subtilis* having at least 51 genes for 31 different tRNA species (31). Thus, in *E. coli* and *B. subtilis*, the genes for tRNA species occur in multiple, while all the tRNA species of *M. capricolum*, except for $tRNA^{Lys}$(UUU), are the single gene product, suggesting strongly that most of the redundant tRNA genes, together with the non-obligate tRNA genes, have been discarded during evolution. The presence of two identical $tRNA^{Lys}$(TTT) genes would accommodate of an extremely high usage of lysine codons (mostly AAA) in the *M. capricolum* protein genes (about 12% of the total codons analyzed: see ref. 10). In fact, $tRNA^{Lys}$(UUU) occurs in a very high amount in the cell (32).

Comparative studies of the tRNA gene organization between *M. capricolum* and its phylogenetically related species may be useful to deduce the evolutionary process of an economization of tRNA genes discussed above. For this purpose, *B. subtilis* may be used, because *M. capricolum* and *B. subtilis* share a common ancestor in the low GC Gram-positive bacterial lineage (4–7), and yet the *B. subtilis* genome is of the 'standard' size. Furthermore, the organization of tRNA genes in *B. subtilis* has been studied extensively (31).

The 30 tRNA genes of *M. capricolum* are distributed in 13 transcription units, of which 22 genes are in the clusters (Fig. 3). The organization in the *M. capricolum* tRNA gene clusters reveals extensive similarity with that in *B. subtilis*, where majority tRNA genes are organized in the four clusters, including 21 tRNA genes (21-gene cluster), 16 genes (16-gene cluster), 6 genes (6-gene cluster) and 4 genes (4-gene cluster), respectively, and some in the spacers of two rRNA operons (33–38). In Fig. 4 are compared the tRNA gene organizations between the two organisms. Strikingly, the order of tRNA genes, from $tRNA^{Arg}$(ACG) to $tRNA^{Phe}$(GAA), in the *M. capricolum* 9-gene cluster (T1) is identical to that of a portion of the 21-gene cluster of *B. subtilis*, and all these tRNAs have obligate anticodons for *M. capricolum* (see also refs. 23,25). The gene arrangement in the *M. capricolum* 5-gene cluster (T2), the 4-gene cluster (T3) and one (T4) of the 2-gene clusters is also similar to a part of the 21- and/or 16-gene clusters of *B. subtilis*. Most of the other *M. capricolum* tRNA genes, that exist in a single operon, seem to have resulted by translocation either from the 21- or from the 16-gene cluster, since the homologous tRNA genes exist in either one or both of the *B. subtilis* clusters. Altogether, 25 out of the total 30 *M. capricolum* tRNA genes may be located as the homologues in the *B. subtilis* 21- and/or 16-gene clusters.

The $tRNA^{Trp}$(TCA) gene does not exist in *B. subtilis*, because UGA is not used as a tryptophan codon. The $tRNA^{Trp}$(TCA) gene seems to have been produced by gene duplication of the $tRNA^{Trp}$(CCA) gene after translocation from the 16-gene cluster, followed by a mutation from CCA anticodon to TCA in one of the duplicates (1,27). The $tRNA^{Thr}$(AGT) gene in the 5-gene cluster (T2) may have resulted from a mutation of $tRNA^{Thr}$(TGT) in the 16-gene cluster, and not from $tRNA^{Thr}$(GGT) found in the *B. subtilis* 6-gene cluster, as discussed previously (2). For the $tRNA^{Lys}$(CTT), $tRNA^{Arg}$(TCT) and $tRNA^{Leu}$(TAG) genes, the corresponding genes have not been reported in *B. subtilis*.

These similarities of the tRNA gene organizations between the two species suggest that most of the *M. capricolum* tRNA genes share the same phylogenetic origin with the *B. subtilis* 21- and 16-gene clusters. Presumably, in the genome of ancestral Gram-positive bacteria common to the two species, tRNA genes were organized in a few large clusters like in the present *B. subtilis* genome. In the mycoplasma lineage, many redundant and non-obligate tRNA genes might have been discarded due to the evolutionary constraints reducing the genome size (27). These are $tRNA^{Gly}$(GCC)*, $tRNA^{Leu}$(TAA)$^+$, $tRNA^{His}$(GTG)$^+$, $tRNA^{Asn}$(GTT)$^+$ and $tRNA^{Glu}$(TTC)$^+$ in the 21-gene cluster, and $tRNA^{Ser}$(GGA)*, $tRNA^{Val}$(TAC)$^+$, $tRNA^{Met}$(CAT)$^+$, $tRNA^{Asp}$(GTC)$^+$, $tRNA^{Phe}$(GAA)$^+$ and $tRNA^{Gly}$(GCC)* in the 16-gene cluster (*:non-obligate anticodon in *M. capricolum* family box; +:redundant anticodon, see Fig. 4). Also, all the tRNA genes homologous with those present in the 6-gene, 4-gene clusters and in the 2-gene sets in rRNA operons in *B. subtilis* (if they existed in the ancestor) have been deleted in the *M. capricolum* evolution.

The mycoplasma genome is the smallest of all the known free-living organisms. In spite of the small number of the genes encoded, the basic organizations and structures of genes essential for growth, such as for ribosomal RNAs and ribosomal proteins are well conserved (11,14,28,39). The present study indicates that the *M. capricolum* genome encodes the smallest number of tRNA genes among eubacteria, and yet the encoded tRNAs fulfill the requirement for translation of all the codons used in this bacterium.

ACKNOWLEDGEMENTS

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APPENDIX 1

Discrepancies between RNA and DNA sequences

Comparisons of the sequences of the total tRNA species of *M. capricolum* (10) and those of tRNA genes (this study) revealed discrepancies in the sequences of $tRNA^{Ile}$(GAU) (position 27) and $tRNA^{Leu}$(CAA) (positions 4, 53 and 54). In all cases, U in the RNA sequence is C in the DNA sequence. These are probably due to technical difficulties in the RNA sequencing.

APPENDIX 2

Sequence of a new small RNA

During our study of sequencing the tRNAs from *M. capricolum* (10), a new small RNA (non-tRNA) species having about 77 base long has been detected (unpublished result). One of the clones carrying 0.9 Kbp *Hin*dIII-fragment, which hybridized with the total tRNA fraction, contained the same sequence as the small RNA species (msRNA : T14 in Fig. 2). No sequence homology was found with the known structural RNAs.