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Spiroplasma citri UGG and UGA Tryptophan Codons: Sequence of the Two Tryptophanyl-tRNAs and Organization of the Corresponding Genes

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From the total tRNAs of *Spiroplasma citri*, we isolated and purified two $tRNA^{Trp}$ species by using chromatography on an RPC-5 column followed by denaturing polyacrylamide gel electrophoresis. The sequence of the two tRNAs, as well as the sequences of the corresponding genes, were determined. One of the two $tRNA^{Trp}$ species has a CCA anticodon and is able to pair with the universal UGG tryptophan codon, while the second has a U*CA (U* is a modified uridine) anticodon and is able to pair with UGA but also with UGG in accordance with the "U:N wobble" rule. Thus, in *S. citri*, UGA is not a stop codon but codes for tryptophan. The two $tRNA^{Trp}$ genes, together with a third tRNA gene, $tRNA^{Ser}$(CGA), belong to a single transcription unit. The nucleotide sequences of the two $tRNA^{Trp}$ species show 82.9% similarity. The two spiroplasmal $tRNA^{Trp}$ species can be aminoacylated by using an aminoacyl-tRNA synthetase fraction from *S. citri*. In contrast, the enzyme fraction from *Escherichia coli* aminoacylates $tRNA^{Trp}$(CCA) but not $tRNA^{Trp}$(U*CA).

Members of the class *Mollicutes* are wall-less eubacteria. They have the smallest genomes of self-replicating, free-living organisms. Phylogenetic studies based on 16S rRNA sequence comparisons have shown that the class *Mollicutes* arose by regressive evolution, i.e., genome reduction, from common ancestors of gram-positive eubacteria with low guanine-plus-cytosine content, such as *Clostridium ramosum* and *C. innocuum* (39, 40). The class *Mollicutes* is divided into six genera: *Acholeplasma*, *Anaeroplasma*, *Asteroplasma*, *Mycoplasma*, *Spiroplasma*, and *Ureaplasma*. Members of the genus *Spiroplasma* are motile and have helical morphology (6).

In spiroplasmas, UGA is not used as a stop codon but codes for tryptophan (27, 33). This peculiar codon usage was discovered while studying the capsid protein gene of *Spiroplasma melliferum* virus SPV4. This protein has a molecular mass of 63 kDa. No open reading frame large enough to code for a 63-kDa protein could be identified on the sequenced viral genome when UAG, UAA, and UGA were all considered as stop (nonsense) codons. Only when UGA was taken as a sense codon could an open reading frame with the right size be identified (28). This open reading frame was shown to be the capsid protein gene by comparison of its deduced amino acid sequence with the N-terminal sequence of the purified capsid protein. The capsid protein gene contains nine UGA codons. It could be cloned in a plasmid vector and amplified in *Escherichia coli*, but no capsid protein was found in the bacterial clones (25), whatever the cloning strategy. In *E. coli*, UGA is recognized as a stop codon, and translation of the viral protein is expected to stop at the first UGA codon encountered by the ribosome. In contrast, the gene for spiralin, the major membrane protein of *S. citri*, was efficiently translated when introduced and amplified in *E. coli* (19). No UGA codon occurs in the spiralin gene (7), and hence, translation of this gene is not affected by premature termination at a UGA codon. The interpretation of these data benefited from the discovery of Yamao et al. (42) that in *Mycoplasma capricolum* UGA is read as tryptophan. This suggested that in spiroplasmas UGA also codes for tryptophan. In agreement with this hypothesis, the absence of UGA (and UGG) codons in the spiralin gene explained the absence of tryptophan in the protein. *M. capricolum* has two $tRNA^{Trp}$ species (41, 42). One $tRNA^{Trp}$ has a CCA anticodon and is able to decode the normal UGG tryptophan codon in accordance with the universal genetic code; the other $tRNA^{Trp}$ has a U*CA (U* is a modified uridine) anticodon and is able to recognize both UGA and UGG in accordance with the "U:N wobble" mechanism (9). *M. gallisepticum* also has the two $tRNA^{Trp}$ species. However, other mycoplasmas, such as *M. pneumoniae* and *M. genitalium*, possess only $tRNA^{Trp}$(UCA) (12).

In contrast, in *Acholeplasma laidlawii*, UGA functions as a stop codon and no $tRNA^{Trp}$(UCA) has been found (8, 35). The situation in ureaplasmas is not known.

Sequence determination of several spiroplasmal genes has shown that both UGG and UGA are used as tryptophan codons (5, 7, 28, 38). In the present study, we analyzed the $tRNA^{Trp}$ situation in *S. citri*. Two different $tRNA^{Trp}$ species were found. The sequences of these two $tRNA^{Trp}$ species and the sequences of the corresponding genes were determined. The comparative organization of $tRNA^{Trp}$ genes in *S. citri* and other members of the class *Mollicutes* is discussed.

MATERIALS AND METHODS

Bacterial and spiroplasmal strains. *E. coli* DH5α(F') was used as the host for propagation of plasmid pUC18 and bacteriophage M13.

S. citri Morocco strain R8A2 HP (high passage) (ATCC 27556) was used to prepare total tRNAs, genomic DNA, and the enzymatic fraction containing the aminoacyl-tRNA synthetase activities (fraction S100).

Enzymes and chemicals. Voltalef 300 LDPL micro (Atochem)

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was used as RPC-5 support. Trioctylmethylammonium chloride (Serva, Heidelberg, Germany) was used for RPC-5 preparation.

Nuclease P1 from *Penicillium citrum*, T4 DNA ligase, isopropyl-β-D-thiogalactopyranoside, and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside were purchased from Boehringer (Mannheim, Germany). *N,N'*-Methylenebisacrylamide, acrylamide, and agarose were from Appligene (Strasbourg, France), and nylon N+ membrane, vector pUC18, T4 polynucleotide kinase, T4 RNA ligase, and radiolabelled compounds [^{32}P]pCp (110 TBq/mol) [γ^{32}P]ATP (110 TBq/mol), [α-^{35}S]dATP (22 TBq/mol), and [^{3}H]tryptophan (110 TBq/mol) were from Amersham Corp. (Arlington Heights, Ill.). Restriction endonucleases were obtained from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.).

M13 DNA sequencing was performed by using the Sequenase version 2.0 sequencing kit from United States Biochemical Corporation (Cleveland, Ohio). Polygram cel 300 DEAE/AR-2/15 for thin-layer chromatography was from Macherey-Nagel (Düren, Germany).

Isolation of total tRNA. *S. citri* cells were grown in 5 liters of BSR medium (37) at 32°C and harvested by centrifugation (20,000 × *g* for 30 min). The following procedure for total tRNA extraction was based on the method of Zubay (44). After direct phenol extraction, total tRNA was deacylated by incubation in 1 M Tris-HCl (pH 9.0) at 37°C for 1 h. The pH was adjusted to 7 by addition of 2 M HCl, and the tRNAs were precipitated by addition of a 0.01% solution of 20% (wt/vol) potassium acetate and 2.5 volumes of absolute ethanol. Nucleic acids were sedimented by centrifugation. The pellet was suspended in 1 ml of 10^{-4} M potassium acetate and dialyzed overnight against 2 liters of the same buffer.

Fractionation of total tRNA. Total tRNA of *S. citri* was fractionated on an RPC-5 column (13) by using an NaCl gradient from 0.4 to 0.8 M in 0.01 M sodium acetate buffer (pH 7.4) containing 0.01 M $MgCl_2$ at a final volume of 100 ml and a flow rate of 1 ml/min. Two peaks showing tryptophan-accepting activity were identified by aminoacylation in the presence of [^{3}H]tryptophan using aminoacyl-tRNA synthetase S100 fractions from *S. citri* or *E. coli* (see below). Fractions containing the $tRNA^{Trp}$ present in peak 1 ($tRNA^{Trp}1$) were pooled, concentrated, and further subjected to 10% polyacrylamide gel electrophoresis followed by denaturing 15% polyacrylamide gel electrophoresis as described by Green et al. (10). Purification of the second $tRNA^{Trp}$ ($tRNA^{Trp}2$), contained in fractions of peak 2, was achieved directly by denaturing 15% polyacrylamide gel electrophoresis. The presence of the two $tRNA^{Trp}$ species was monitored at each purification step by aminoacylation assays (see below).

Preparation of aminoacyl-tRNA synthetase fraction S100 from *S. citri* and *E. coli* and conditions of aminoacylation. The aminoacyl-tRNA synthetase fractions (S100) of *S. citri* and *E. coli* were prepared as described by Andachi et al. (1). For aminoacylation assays, 0.05- to 20-μg samples of tRNAs were incubated at 37°C for 20 min in 100 μl of a mixture containing 10 mM ATP, 20 mM $MgCl_2$, 50 mM Tris-HCl (pH 7.5), 0.4 mM glutathione, 0.1 mg of bovine serum albumin per ml, 2 μCi of [^{3}H]tryptophan, and 10 to 50 μl of enzymatic extract (depending of the enzymatic activity).

Sequencing of $tRNA^{Trp}$. The nucleotide sequences of the two *S. citri* $tRNA^{Trp}$ species were determined by using in vitro postlabelling methods (30) and approaches described previously (26). Most data were obtained by using the technique of Stanley and Vassilenko (34). Some RNA fragments were also sequenced by using mobility shift analysis (30). All suspected modified residues were identified by two-dimensional thin-layer chromatography in different solvent systems (22).

Oligonucleotide synthesis and labelling. Oligonucleotides were synthesized by J. C. Gandar, Institut de Biochimie Cellulaire et de Neurochimie, Bordeaux, France. Oligonucleotide TRP1 (5'GGGGTAGCAGGACTTGAAC3') was 5' end labelled by using [γ-^{32}P]ATP (110 TBq/mol) and T4 polynucleotide kinase as described by Maxam and Gilbert (16). The labelled oligonucleotide was purified by using a Nensorb 20 Nucleic Acid Purification Cartridge (Dupont Co., Boston, Mass.) as recommended by the manufacturer.

DNA isolation and Southern hybridization. Isolation of genomic DNA from *S. citri* was performed by the method of Marmur (15). Recombinant plasmid DNAs were analyzed by rapid alkaline extraction (2). Extraction of large amounts of plasmid DNA was performed by using the same method; purification was achieved by CsCl gradient as the standard procedure (14).

Southern blot (31) hybridization with an oligonucleotide as the probe was performed as described by Zeff and Geliebter (43).

Cloning and sequencing of the $tRNA^{Trp}$ genes. *S. citri* genomic DNA was digested to completion with endonuclease *Eco*RI. The resulting DNA fragments were ligated with dephosphorylated, *Eco*RI-digested pUC18 plasmid DNA. Bacterial transformants containing a recombinant plasmid carrying $tRNA^{Trp}$ genes were screened for by colony hybridization with the oligonucleotide TRP1 probe by standard procedures (14).

A partial restriction map of cloned inserts was constructed, and restriction fragments were subcloned into vector M13mp18. Procedures used for restriction enzyme digestions, electrophoresis, and cloning of DNA were those described by Maniatis et al. (14).

Preparation of single-stranded DNA templates and annealing and sequencing reactions were performed in accordance with the Sequenase version 2.0 kit handbook (United States Biochemical Corp.). Double-stranded plasmid DNAs were prepared as described by to Mierendorf et al. (18) and partially sequenced by using synthesized oligonucleotides as primers. Sequencing gel electrophoresis and computer programs used for analysis of nucleotide sequences have been described by Renaudin et al. (28).

Sequences which are not of *S. citri* origin were imported through the GenBank server (Los Alamos, N.Mex.) on the Internet network.

Nucleotide sequence accession number. The accession number of the nucleotide sequence reported here is M91385.

RESULTS

Purification and sequencing of *S. citri* $tRNA^{Trp}$ species. Fractionation of the total tRNA of *S. citri* was achieved by RPC-5 column chromatography. The tRNAs in each eluted fraction were tested for tryptophan-accepting activity with *S. citri* and *E. coli* aminoacyl-tRNA synthetase fractions (Fig. 1). When the spiroplasmal enzyme fraction was used, two peaks of tryptophan-accepting activity, numbered 1 and 2 on the basis of their elution order, were detected, while a single peak (corresponding to peak 2) was found when the *E. coli* enzymatic extract was used.

Each of these two peaks yielded several tRNA bands after fractionation on denaturing polyacrylamide gel. For each peak, one of the tRNA bands was found by aminoacylation

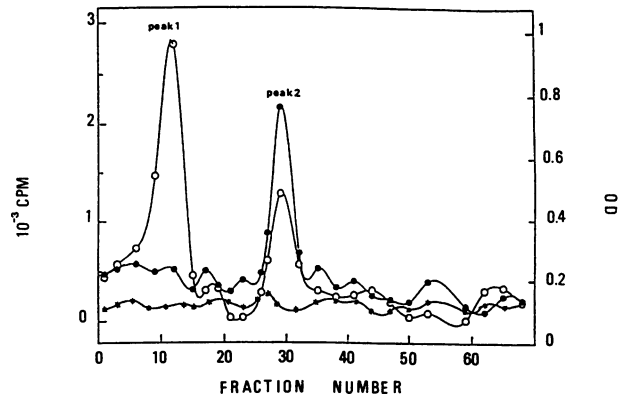

FIG. 1. RPC-5 fractionation of *S. citri* total tRNA. One milligram of total tRNA in 0.01 M sodium acetate buffer (pH 4.7), containing 0.01 M $MgCl_2$ and 0.3 M NaCl was loaded on the column (70 by 0.45 cm). Elution was performed in the same buffer with a linear gradient (2 × 50 ml) from 0.4 to 0.8 M NaCl. Fractions of 1 ml were collected. Symbols: *, optical density (OD) at 260 nm; ○, [^{3}H]tryptophan-accepting activity determined by using the *S. citri* enzyme fraction; ●, [^{3}H]tryptophan-accepting activity detected with the *E. coli* enzyme fraction.

to contain a $tRNA^{Trp}$. Two $tRNA^{Trp}$ species were thus detected.

The nucleotide sequences of these two *S. citri* $tRNA^{Trp}$ isoacceptors are shown in Fig. 2. Although the sequences of the two $tRNA^{Trp}$ species show high similarity (82.9%), one of the main differences between the two $tRNA^{Trp}$ species is at the anticodon level: $tRNA^{Trp}_1$ has an NCA anticodon, whereas $tRNA^{Trp}_2$ has a CCA anticodon. The NCA anticodon found in $tRNA^{Trp}_1$ contains a modified nucleotide in the wobble position (position 34) of the anticodon. The nucleotide 34-nucleotide 35 phosphodiester bond is resistant to water, partially resistant to nuclease P1, and digested by snake venom phosphodiesterase. These data suggest that nucleotide 34 contains an O-methylated ribose. In addition, the sequence of the $tRNA^{Trp}_1$ gene revealed that nucleotide 34 is a derivative of U (U*) (see below). $tRNA^{Trp}$(U*CA) (Fig. 2A) is 76 nucleotides long and contains one more nucleotide, a C at position 17, than $tRNA^{Trp}$(CCA) (Fig. 2B).

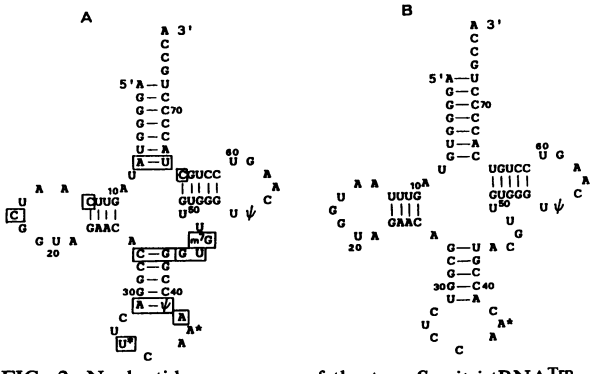

FIG. 2. Nucleotide sequences of the two *S. citri* $tRNA^{Trp}$ species. (A) *S. citri* $tRNA^{Trp}$(U*CA). (B) *S. citri* $tRNA^{Trp}$(CCA). Boxes in panel A indicate the nucleotides of $tRNA^{Trp}$(U*CA) which differ from those of $tRNA^{Trp}$(CCA). U* at position 34 is a hypermodified nucleotide which has been identified as a derivative of U by gene sequencing, and A* at position 37 is a hypermodified adenosine.

The two $tRNA^{Trp}$ species also differ in their modified nucleotide contents. While $tRNA^{Trp}$(CCA) contains only two modified nucleotides, a hypermodified adenosine (A*) at position 37 and a pseudouridine (ψ) at position 55, $tRNA^{Trp}$(U*CA) has five modified nucleotides, namely, two ψ's at positions 39 and 55, a modified U (U*) at position 34, A* at position 37, and a 7-methylguanosine (m^7G) at position 46. The hypermodified A (A*) found at position 37 in both $tRNA^{Trp}$ species shows similar chromatographic behavior and probably corresponds to the same posttranscriptional modification in the two $tRNA^{Trp}$ species. Furthermore, the chromatographic mobilities (in the two systems used) of this modified A are very close to the mobilities found for N^6-Δ^2-isopentenyladenosine or 2-methylthio-N^6-Δ^2-isopentyladenosine. This suggests that this modified A is a derivative of these two hypermodified adenosines.

Localization of the $tRNA^{Trp}$ genes on the spiroplasmal DNA. Each purified $tRNA^{Trp}$ species was labelled at its 5′ end with ^{32}P and used as a probe in Southern blot hybridization with *Eco*RI- or *Hin*dIII-restricted *S. citri* genomic DNA. A single *Eco*RI fragment of 3.8 kbp and a single *Hin*dIII fragment of 6.2 kbp hybridized with both $tRNA^{Trp}$ probes (data not shown), suggesting that the two $tRNA^{Trp}$ genes were located within the same *Eco*RI or *Hin*dIII restriction fragment. To determine the position of one $tRNA^{Trp}$ gene with regard to the other, it was necessary to clone and sequence them. For this purpose, a 19-mer oligodeoxynucleotide (TRP1) complementary to the 3′ end of $tRNA^{Trp}$(U*CA) and 89.5% homologous to the 3′ end of $tRNA^{Trp}$(CCA) was used as a probe to screen an *S. citri* genomic library. To check the specificity of this oligonucleotide, it was first used to probe Southern blots of restricted *S. citri* genomic DNA. This TRP1 oligonucleotide hybridized to only one fragment, regardless of the restriction enzyme used; the *Eco*RI and *Hin*dIII hybridizing fragments had sizes of 3.8 and 6.2 kbp, respectively, in agreement with the hybridization pattern obtained when pure $tRNA^{Trp}$(U*CA) was used as the probe. Thus, TRP1 was suitable for screening of a genomic DNA library of *S. citri*.

Screening of an *S. citri* genomic library and sequencing. Chromosomal *S. citri* DNA fragments that resulted from *Eco*RI digestion were inserted into the unique *Eco*RI site of vector pUC18. The ligation mixture was used to transform competent *E. coli* cells, and the resulting genomic library was screened with oligonucleotide TRP1 as the probe. One clone, that carried a recombinant plasmid (PT2) with a 3.8-kbp *Eco*RI DNA insert and hybridized strongly with the probe, was isolated. Southern blot experiments showed that total *S. citri* tRNA (Fig. 3A) and oligodeoxynucleotide TRP1 (Fig. 3B), used as probes, hybridized with only one *Eco*RI-*Eco*RV DNA fragment (2.1 kbp) of PT2 (Fig. 3, lanes 5). To determine the nucleotide sequence of this fragment, it was purified from agarose gel and *Rsa*I and *Alu*I enzymes were used to generate shorter fragments which were subcloned in vector M13mp18. In addition, direct sequencing on the double-stranded DNA plasmid was performed by using oligodeoxynucleotides as primers to overlap the junction.

Nucleotide sequence of the *S. citri* $tRNA^{Trp}$ genes. Nucleotide sequence analysis confirmed the presence of the two expected $tRNA^{Trp}$ genes on the 2.1-kbp *Eco*RI-*Eco*RV fragment. The sequences of these $tRNA^{Trp}$ genes and their flanking regions are shown in Fig. 4. The $tRNA^{Trp}$(U*CA) gene is separated from the $tRNA^{Trp}$(CCA) gene by an 83-bp spacer. The nucleotide sequences of these two $tRNA^{Trp}$ genes were found to be identical to the sequences obtained by direct RNA sequencing and have shown that the modified

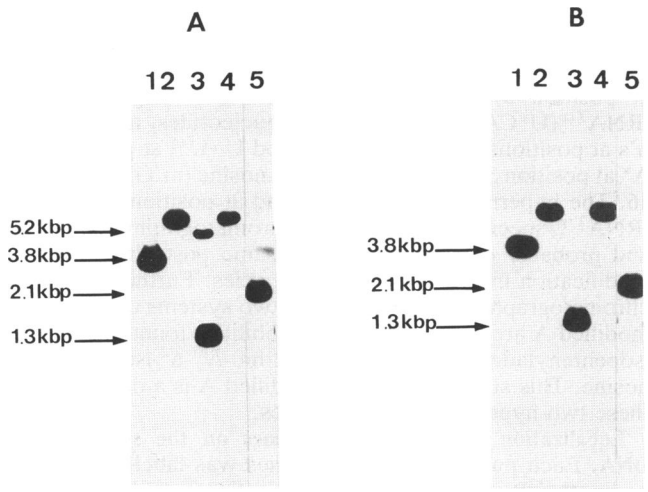

FIG. 3. Southern blot hybridization of recombinant plasmid PT2 DNA with total spiroplasmal tRNA (A) and TRP1 (B) used as probes. Lanes: 1, plasmid PT2 DNA restricted by *Eco*RI; 2, *Eco*RV 3, *Acc*I; 4, *Hin*dIII; 5, *Eco*RI and *Eco*RV.

nucleotide at position 34 of $tRNA^{Trp}$(U*CA) is a uridylic acid residue.

Another tRNA gene was found 10 bp downstream of the $tRNA^{Trp}$(CCA) gene. This tRNA has a CGA anticodon and is a serine tRNA, in accordance with the universal genetic code. A potential promoterlike region, with a 5′TAAAAT3′ −10 region and a 5′TTGCTA3′ −35 region, is present upstream of the $tRNA^{Trp}$(U*CA) gene. The sequences of these −10 and −35 regions are similar to the −10 and −35 consensus sequences, TATPuAT and TTGACA, respectively, of bacterial promoters recognized by RNA polymerase functioning with the general sigma factor (29).

An inverted repeat sequence occurs 18 bp downstream of the $tRNA^{Ser}$ gene and is a potential transcription termination site (11). These data suggest that the three tRNA genes belong to a single transcription unit. Interestingly, $tRNA^{Ser}$ (CGA) does not exist in *M. capricolum* (1, 20). To verify that this tRNA is expressed in *S. citri*, we used the total *S. citri* tRNA as a probe in Southern blot experiments (Fig. 3A) with *Acc*I-restricted plasmid DNA (lanes 3). According to the sequencing results, the *Acc*I enzyme should cleave the spiroplasmal DNA insert at the 5′ end of the $tRNA^{Ser}$ gene (Fig. 4). Total tRNA hybridized with two *Acc*I-*Acc*I DNA fragments (Fig. 3A, lane 3), while oligodeoxynucleotide TRP1 hybridized with a single fragment (Fig. 3B, lane 3), showing that $tRNA^{Ser}$ is expressed.

Sequence comparison with other $tRNA^{Trp}$ gene sequences. Alignment of the 994-bp DNA fragment of *M. capricolum* (42) which carries the two $tRNA^{Trp}$ genes and the corresponding *S. citri* fragment is shown in Fig. 4. Comparison of the regions flanking *M. capricolum* $tRNA^{Trp}$ genes and those of *S. citri* showed 44.4% similarity. Higher similarity values were obtained, as expected, between the $tRNA^{Trp}$ genes themselves.

Comparison of the $tRNA^{Trp}$ gene sequences from members of the class *Mollicutes* (12, 42) and bacteria (32) delineating various domains in their secondary structure (Fig. 5), was also performed. With this representation, conserved and variable regions can be distinguished. Figure 5 shows that the length of the D loop can vary by 1 residue. The TF loop is highly conserved, except in both spiroplasmal $tRNA^{Trp}$(CCA) and $tRNA^{Trp}$(U*CA), where the conserved G residue is replaced by an A. Similarly, in the anticodon stem of *S. citri* $tRNA^{Trp}$(U*CA), two bases involved in base pairing are different from those found in the other nine tRNAs. The extra arm is relatively less conserved in tRNA(CCA) than in tRNA(UCA). In general, the sequences of $tRNA^{Trp}$(CCA) are more variable than those of $tRNA^{Trp}$(UCA).

DISCUSSION

We isolated and sequenced two *S. citri* $tRNA^{Trp}$ species. One has a CCA anticodon and is able to read the universal UGG tryptophan codon. The second has a U*CA anticodon that is able to pair with UGA but also with UGG in accordance with the U:N wobble rule (9). Furthermore, $tRNA^{Trp}$(U*CA) was shown to be correctly aminoacylated in vitro by using an *S. citri* crude extract as a source of tryptophanyl-tRNA synthetase. These results show that in *S. citri*, UGA is not a stop codon but in fact codes for tryptophan.

The occurrence of a $tRNA^{Trp}$ that reads UGA should be considered from the point of view of evolution. Acholeplasmas (an anaerobic members of the class *Mollicutes*) are seen phylogenetically as the first members of the class *Mollicutes* to have appeared by regressive evolution from their bacterial (clostridial) ancestors. The spiroplasmas have evolved from an early splitting of the acholeplasma branch, and the mycoplasmas and ureaplasmas are thought to have spiroplasmal precursors (36). *A. laidlawii*, like its eubacterial ancestors, uses UGG as its sole tryptophan codon; to do that, it has a $tRNA^{Trp}$ with a CCA anticodon (8, 35). *S. citri* and *M. capricolum* belong to the same phylogenetic group (spiroplasma group) (36). They use both UGG and UGA as tryptophan codons (7, 28, 38) and have two $tRNA^{Trp}$ species. *M. gallisepticum*, *M. pneumoniae*, *M. genitalium*, and *Ureaplasma urealyticum* are members of the pneumoniae phylogenetic group (36). *M. gallisepticum* resembles *S. citri* and *M. capricolum* in that it has the two $tRNA^{Trp}$ species (12). However, *M. pneumoniae* and *M. genitalium* have lost the usual tryptophan tRNA, $tRNA^{Trp}$(CCA), and have only one left: $tRNA^{Trp}$(UCA) (12). *U. urealyticum* reads UGA as tryptophan (4) and very probably has $tRNA^{Trp}$(UCA), but it remains to be seen whether it still possesses the ancestral tryptophan tRNA, $tRNA^{Trp}$(CCA). Anaeroplasmas and asteroleplasmas have not been examined for the use of UGA as a tryptophan codon, but these anaerobic members of the class *Mollicutes* are members of the same phylogenetic group as the acholeplasmas (anaeroplasma group). It is therefore predictable that they have not evolved UGA as a tryptophan codon and possess only one tryptophan tRNA: $tRNA^{Trp}$(CCA), which reads UGG.

A very interesting case is that of *Acholeplasma florum*. This member of the class *Mollicutes*, as well as several others, of plant or insect origin, does not need sterol for growth and was therefore classified as an acholeplasma, even though it does require 0.04% Tween in its medium. Phylogenetic analysis (36) has shown that this organism is a member of the spiroplasma phylogenetic group! It has been found that this so-called acholeplasma, unlike *A. laidlawii*, uses UGA as a tryptophan codon (21). Therefore, determination of UGA as a tryptophan codon confirms its phylogenic position.

A scenario for the reassignment of UGA from a stop codon to a tryptophan codon in the *M. capricolum* lineage has been proposed by Osawa et al. (24) and might be valid in

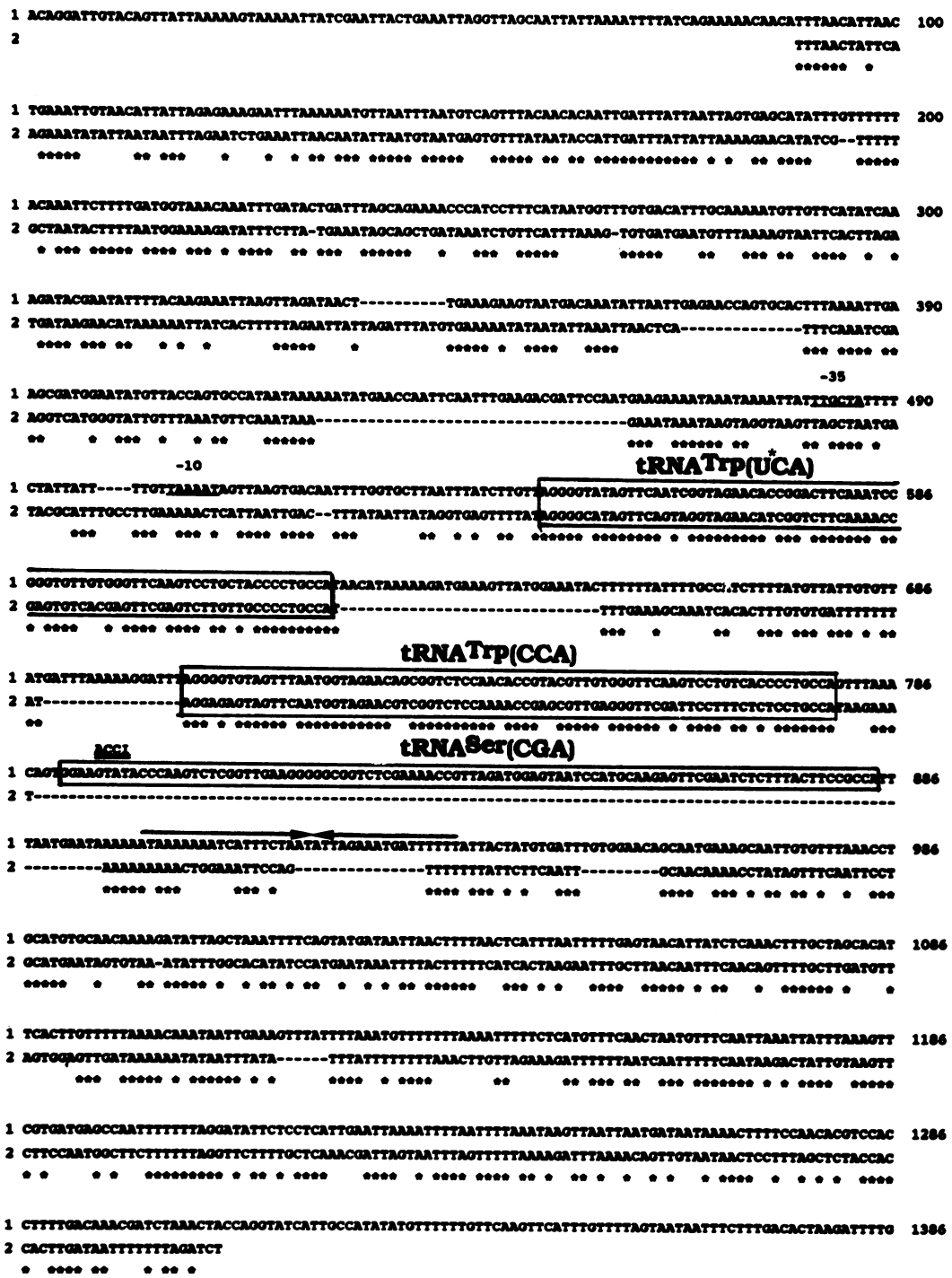

FIG. 4. Nucleotide sequences of the regions containing the tRNA^{Trp} genes from *S. citri* (line 1) and *M. capricolum* (line 2). These sequences are aligned on the tRNA^{Trp} genes. The tRNA^{Trp}(U*CA), tRNA^{Trp}(CCA), and tRNA^{Ser}(CGA) genes are boxed. Sequence identities are shown by stars, and gaps are shown by dashes. The putative promoter sequences are underlined, and the potential termination signal is indicated by arrows.

the case of the spiroplasma lineage. Under high AT pressure, the stop codon UGA undergoes a neutral mutation to UAA. The tRNA^{Trp}(CCA) gene becomes duplicated, and one of the copies is converted to tRNA^{Trp}(U*CA). UGG mutates into UGA and can be read as tryptophan because of the new tRNA^{Trp}(U*CA). In agreement with this hypothesis, Yamao et al. (42) have shown that the two tRNA^{Trp} genes are arranged in a single transcription unit, and we found a similar situation in the two *S. citri* tRNA^{Trp} genes in this study (Fig. 6). However, in *M. capricolum*, the spacer region between the two tRNA^{Trp} genes contains an attenuator which represses transcription of tRNA^{Trp}(CCA) (41), whereas no

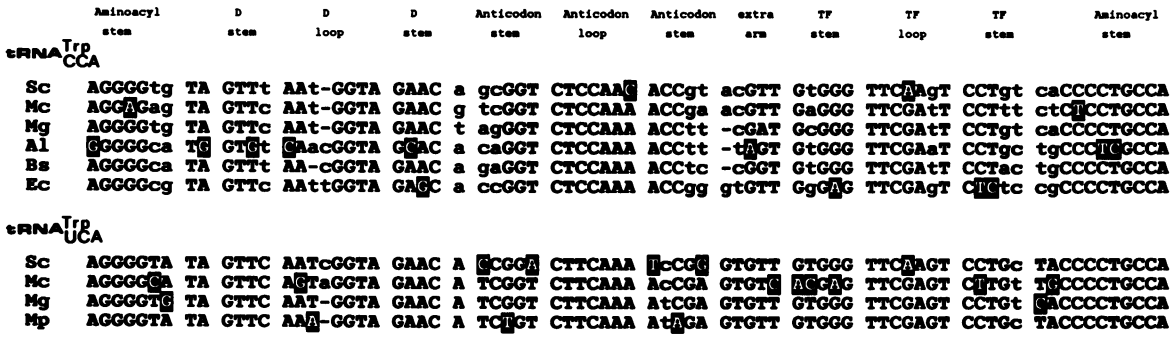

FIG. 5. Alignment of tRNA^{Trp} genes delineating various domains in their secondary structure. Boxed nucleotides in black are exceptions at the positions where they occur; uppercase letters indicate conserved nucleotides; lowercase letters indicate variable nucleotides. Dashes indicate gaps in the alignment. Al, *A. laidlawii*; Bs, *Bacillus subtilis*; Ec, *E. coli*; Mc, *M. capricolum*; Mg, *M. gallisepticum*; Mp, *M. pneumoniae*; Sc, *S. citri*.

attenuator was found between the two spiroplasmal tRNA^{Trp} genes. The organization of the tRNA^{Trp} genes in three other mycoplasma species was also determined (12): *M. pneumoniae* and *M. genitalium* possess only the tRNA^{Trp}(UCA) gene, while *M. gallisepticum* has the two tRNA^{Trp} genes but they are located in two distinct transcription units. This suggests that, during evolution, members of the class *Mollicutes* tend to discard tRNA^{Trp}(CCA).

In *S. citri*, tRNA^{Ser}(CGA), able to read the UCG codon, is encoded by a gene located downstream of the two tRNA^{Trp} genes. In *M. capricolum*, the complete set of tRNA genes has been determined (20) and no tRNA^{Ser}(CGA) gene was found. In this member of the class *Mollicutes*, the four serine UCN codons are read by tRNA^{Ser}(UGA), in accordance with the U:N wobble rule. Our results suggest that in *S. citri* an additional tRNA^{Ser} should exist to decode the UCU, UCC, and UCA codons. *S. citri* and *M. capricolum* share a common ancestor. During evolution, *S. citri* has kept tRNA^{Ser}(CGA) while *M. capricolum* has discarded it because of nonobligatory use.

The similarities among all of the tRNAs^{Trp}(UCA) nucleotide sequences determined (from 80.3 to 92%) are higher than those observed between tRNA^{Trp}(CCA) sequences from members of the class *Mollicutes* (68.4 to 85.6%) or those observed between tRNAs^{Trp}(CCA) and tRNAs^{Trp}(UCA) (67.1 to 88%). The lowest similarity values were obtained when *A. laidlawii* tRNA^{Trp}(CCA) was compared to tRNA^{Trp} of other members of the class *Mollicutes*. This may be correlated to the early emergence of the acholeplasma branch in the phylogenetic tree of the class *Mollicutes*, as mentioned before. The two spiroplasmal tryptophan tRNAs show 82.9% sequence identity and possess, especially in the case of tRNA(CCA), a low number of modified nucleotides in comparison with other bacterial tRNAs (32) and tRNAs from members of the class *Mollicutes* (1). In particular, the D loop, which usually contains a dihydrouridine residue in most tRNAs, has no such modified nucleotide in both spiroplasmal tryptophan tRNAs. This feature was also observed in spiroplasmal tRNA^{Ile}(GAU) (7a). Furthermore, mycoplasmal tRNAs, spiroplasmal tRNA^{Trp}, and tRNA^{Ile}(GAU) share a common property: uridine in position 54 is unmodified, as opposed to the situation that exists in most eucaryotic and eubacterial tRNAs (3, 32). However, as in eucaryotic, archaebacterial, and eubacterial tRNAs that recognize UNN codons, spiroplasmal tRNA^{Trp} contains a hypermodified adenosine in position 37. Pseudouridine in position 38, 39, or 40 of the anticodon stem has been shown to increase the efficiency of the tRNA decoding activity (3) and might have a similar role in *S. citri* tRNA^{Trp}(U*CA), where pseudouridine is found in position 39.

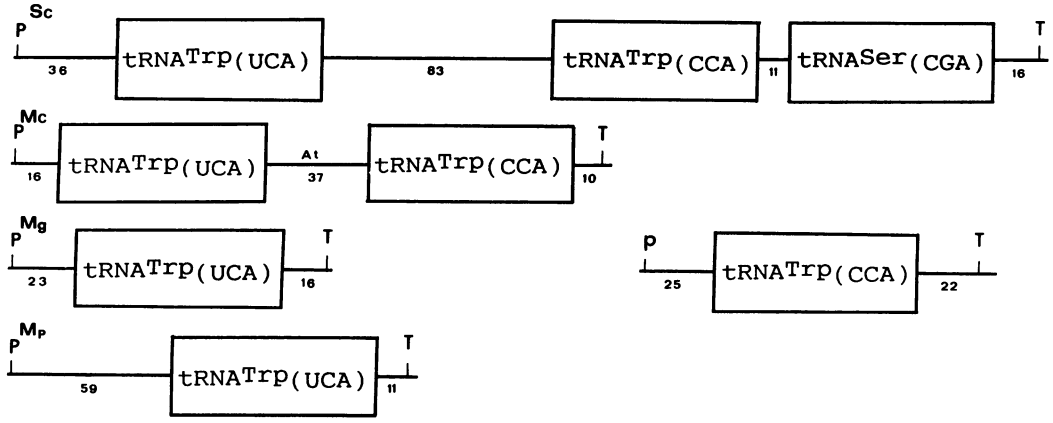

FIG. 6. Organization of tRNA^{Trp} genes in members of the class *Mollicutes*. Mc, *M. capricolum*; Mg, *M. gallisepticum*; Mp, *M. pneumoniae*; Sc, *S. citri*; P, promoter; T, terminator; At, attenuator. Numbers under the lines indicate the numbers of base pairs in the spacers.

Interestingly, aminoacylation of the two spiroplasmal $tRNA^{Trp}$ species with tryptophan by tryptophanyl-tRNA synthetase is slightly different. *S. citri* tryptophanyl-tRNA synthetase seems to aminoacylate $tRNA^{Trp}$(CCA) less efficiently than $tRNA^{Trp}$(U*CA) (Fig. 1). In *M. capricolum*, Yamao et al. (41) have observed that in vivo $tRNA^{Trp}$(CCA) was not only present in lower amount than $tRNA^{Trp}$(U*CA) but was also less efficiently charged. In our case, the difference of aminoacylation cannot be explained solely by a difference in the relative amounts of the two $tRNA^{Trp}$ species since fixation of tryptophan to $tRNA^{Trp}$(CCA) was higher with the *E. coli* enzyme than with the spiroplasmal enzyme. Because $tRNA^{Trp}$(U*CA) is not charged in the presence of the *E. coli* enzyme, while $tRNA^{Trp}$(CCA) is, comparison between *S. citri* and *E. coli* $tRNA^{Trp}$ sequences could help to determine the nucleotides involved in the recognition of $tRNA^{Trp}$ by tryptophanyl-tRNA synthetase. Six nucleotides in $tRNA^{Trp}$(U*CA) are not shared by *S. citri* and *E. coli* $tRNA^{Trp}$(CCA): A in position 7, C in position 17, A in position 31, U* in position 34, ψ in position 39, and U in position 66 (Fig. 2). A in position 7 is hydrogen bonded to the U in position 66, and A in position 31 is hydrogen bonded to ψ in position 39. Among these six nucleotides, the modified U in the wobble position of the anticodon (U*) might be a major determinant in its nonrecognition by *E. coli* tryptophanyl-tRNA synthetase. This is in line with the hypothesis that in a number of tRNAs the anticodon could be involved in the recognition process (17, 23).

Further experiments, including studies on *S. citri* tryptophanyl-tRNA synthetase and in vitro mutagenesis of $tRNAs^{Trp}$ may help identify the determinants of the recognition process.

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