Coevolution of an aminoacyl-tRNA synthetase with its tRNA substrates

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Glutamyl-tRNA synthetases (GluRSs) occur in two types, the discriminating and the nondiscriminating enzymes. They differ in their choice of substrates and use either $tRNA^{Glu}$ or both $tRNA^{Glu}$ and $tRNA^{Gln}$. Although most organisms encode only one GluRS, a number of bacteria encode two different GluRS proteins; yet, the tRNA specificity of these enzymes and the reason for such gene duplications are unknown. A database search revealed duplicated GluRS genes in >20 bacterial species, suggesting that this phenomenon is not unusual in the bacterial domain. To determine the tRNA preferences of GluRS, we chose the duplicated enzyme sets from *Helicobacter pylori* and *Acidithiobacillus ferrooxidans*. *H. pylori* contains one $tRNA^{Glu}$ and one $tRNA^{Gln}$ species, whereas *A. ferrooxidans* possesses two of each. We show that the duplicated GluRS proteins are enzyme pairs with complementary tRNA specificities. The *H. pylori* GluRS1 acylated only $tRNA^{Glu}$, whereas GluRS2 was specific solely for $tRNA^{Gln}$. The *A. ferrooxidans* GluRS2 preferentially charged $tRNA^{Gln}_{UUG}$. Conversely, *A. ferrooxidans* GluRS1 glutamylated both $tRNA^{Glu}$ isoacceptors and the $tRNA^{Gln}_{CUG}$ species. These three tRNA species have two structural elements in common, the augmented D-helix and a deletion of nucleotide 47. It appears that the discriminating or nondiscriminating natures of different GluRS enzymes have been derived by the coevolution of protein and tRNA structure. The coexistence of the two GluRS enzymes in one organism may lay the groundwork for the acquisition of the canonical glutaminyl-tRNA synthetase by lateral gene transfer from eukaryotes.

Faithful protein biosynthesis relies on the correct attachment of amino acids to their corresponding (cognate) tRNAs catalyzed by the aminoacyl-tRNA synthetases (AARSs) (1). Given the presence of 20 canonical amino acids, one would expect each organism to contain at least 20 AARSs to ensure high accuracy during aminoacylation of the complete set of tRNAs. However, this is true only for eukarya and some bacteria. Most of the other bacterial organisms (2, 3), all known archaea (4, 5), and eukaryotic organelles (6) lack the AARS specific for glutamine. In addition, a canonical asparaginyl-tRNA synthetase is missing from many archaeal and bacterial genomes (7), whereas a canonical cysteinyl-tRNA synthetase is not distinguishable in some methanogenic archaea (8).

In addition to direct acylation of the cognate tRNA by AARSs, an indirect pathway was found to be responsible for the formation of both Gln-$tRNA^{Gln}$ and Asn-$tRNA^{Asn}$. In these cases, formation of the mischarged product (Glu-$tRNA^{Gln}$ and Asp-$tRNA^{Asn}$, respectively) is a required intermediate in the synthesis of the cognate amino acid–tRNA pairs. To accomplish this, the indirect pathway to Gln-$tRNA^{Gln}$ formation uses a nondiscriminating (ND) form of glutamyl-tRNA synthetase (GluRS) that is able to efficiently glutamylate both $tRNA^{Glu}$ and $tRNA^{Gln}$ (9). In the next step, a tRNA-dependent amidotransferase, Glu-$tRNA^{Gln}$ amidotransferase, amidates Glu-$tRNA^{Gln}$ to the correctly charged Gln-$tRNA^{Gln}$, which is then used as a substrate for protein synthesis (reviewed in ref. 10).

In bacteria, the ND *Bacillus subtilis* GluRS has been extensively studied (9). Due to the lack of a canonical glutaminyl-tRNA synthetase (GlnRS) in this organism, the ND-GluRS is an essential enzyme in Gln-tRNA formation as it generates Glu-$tRNA^{Gln}$. This product is then converted to Gln-$tRNA^{Gln}$ by Glu-$tRNA^{Gln}$ amidotransferase (10–12). However, the mechanism of how the ND-GluRS recognizes two different tRNA substrates is not known, and the tRNA identity set for such an enzyme has never been determined. In contrast, organisms with a canonical GlnRS enzyme possess a discriminating GluRS (D-GluRS), which recognizes only $tRNA^{Glu}$. The tRNA identity elements of a D-GluRS enzyme were reported for *E. coli* (13). Major identity determinants for this enzyme are located in the augmented D-helix (indicated in Fig. 1), which is formed by nucleotides in the D-stem and D-loop, as well as the bases connecting the acceptor helix and D-stem, and some bases in the extra arm (13). *In vitro* mutagenesis of *Thermus thermophilus* D-GluRS expanded its tRNA specificity to the recognition of $tRNA^{Glu}$ with a glutamine-specific anticodon (14). However, this work did not afford real insight into a ND-GluRS, because the tRNA specificity was not changed.

In addition to the lack of some synthetases (see above), whole genome analysis also revealed several organisms containing more than one AARS for certain amino acids. In many cases, the function of such a redundancy is unknown; however, in some organisms, the roles of the duplicated AARSs have been explained (15). A second aspartyl-tRNA synthetase in *Deinococcus radiodurans*, for instance, is shown to be involved in tRNA-dependent asparagine biosynthesis (7). In *E. coli*, two genes for lysyl-tRNA synthetase (LysRS) exist; one LysRS gene (*lysS*) is constitutively expressed, and the other one is heat-inducible (*lysU*) (16, 17). Similarly, two paralogous *gltX* genes (encoding GluRS) exist in some proteobacteria and a scattering of other bacterial groups (18); however, the function of this duplication is not clear. There are indications that the two GluRSs in *Helicobacter pylori* preferentially glutamylate either $tRNA^{Glu}$ or $tRNA^{Gln}$.** To investigate further the duplicated *gltX* genes, we chose the *Acidithiobacillus ferrooxidans* and *H. pylori* systems and examined the substrate preferences of the two GluRS enzymes that coexist in these organisms.

Materials and Methods

Oligonucleotides, DNA Sequencing, and Radiochemicals. Oligonucleotides were synthesized, and DNAs were sequenced by the Keck Foundation Biotechnology Resource Laboratory at Yale University. Uniformly labeled [^{14}C]Glu (254 mCi/mmol) and

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Abbreviations: AARS, aminoacyl-tRNA synthetase; ND, nondiscriminating; GluRS, glutamyl-tRNA synthetase; GlnRS, glutaminyl-tRNA synthetase.

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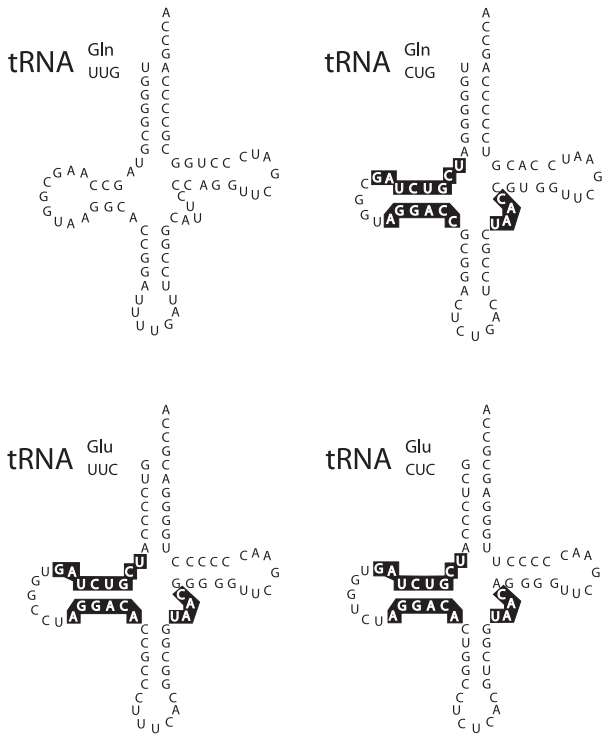

Fig. 1. Cloverleaf representation of *A. ferrooxidans* $tRNA^{Glu}$ and $tRNA^{Gln}$ species. The bases that form part of the augmented D-helix (13), in both isoacceptors of $tRNA^{Glu}$ and in $tRNA^{Gln}_{CUG}$, are shown in white letters and black shadow.

[^{14}C]Gln (244 mCi/mmol) and [γ-^{32}P]ATP (6,000 Ci/mmol) were from Amersham Pharmacia Biosciences.

Cloning of *gltX* Genes from *H. pylori* and *A. ferrooxidans*. The *H. pylori* genes (HP0476, *gltX1*; HP0643, gltX2) were obtained by PCR from the genomic DNA (strain 26695) by using the Expand High Fidelity PCR System (Roche Molecular Biochemicals). Oligonucleotide primers introduced an *Nde*I site at the 5′ end and a *Sap*I site at the 3′ end of each gene. The PCR products, digested with *Nde*I and *Sap*I, were cloned into the pTYB1 vector (New England BioLabs) digested with the same enzymes, to form the plasmids pHPgltX1 and -X2. The identity of the clones was determined by DNA sequencing.

Analysis (http://tigrblast.tigr.org/ufmg/index.cgi?database = a_ferrooxidans seq) of the incomplete *A. ferrooxidans* genome sequence revealed two *gltX* genes. They were cloned into the pCR2.1-TOPO vector (Invitrogen) after PCR amplification of genomic *A. ferrooxidans* DNA (ATCC 23270) by using oligonucleotides that introduced an *Eco*RI site on both ends of each gene. After sequence confirmation, the genes were cloned into the *Eco*RI site of pGEX-2T (Amersham Biosciences) to yield the plasmids pAFgltX1 and -X2.

Expression and Purification of GluRSs. The plasmids pHPgltX1 and -X2 were transformed into *Escherichia coli* BL21(DE3) (Invitrogen). The expression of both genes and the purification and cleavage of the intein fusion proteins were done as described (New England BioLabs). For the overexpression of *A. ferrooxidans* genes and purification of the GST-fusion protein, we followed the previously described protocols (19).

Preparation of Unfractionated tRNA. The *A. ferrooxidans* $tRNA^{Gln}$ genes (sequence from the TIGR database), were constructed by synthesis of the corresponding oligonucleotides and subsequent ligation into the pKK223-3 vector (Amersham Biosciences) and digested with *Eco*RI and *Hin*dIII enzymes, generating the plasmids pJSQUUG and pJSQCUG. These were transformed into *E. coli* DH5α. Purification of unfractionated tRNA containing the specified tRNA gene transcript was carried out as described (20). Comparison of aminoacylation reactions by using *E. coli* GlnRS and unfractionated tRNA obtained from the cells, transformed with pJSQUUG, pJSQCUG, or empty vector, showed that the *A. ferrooxidans* $tRNA^{Gln}_{UUG}$ and $tRNA^{Gln}_{CUG}$ isoacceptors comprised between 10% and 20% of the total tRNA.

To obtain mature unfractionated tRNA from both organisms, *H. pylori* cells (strain 43526) were grown (21), and the unfractionated tRNA was prepared as described (20). *A. ferrooxidans* (strain 19859) was grown in a bioreactor in 9-K medium [0.4 g/liter $(NH_4)_2SO_4$, 0.4 g/l $MgSO_4(7H_2O)$, 0.056 g/l $K_2HPO_4(3H_2O)$/5 g/l $FeSO_4(7H_2O)$ adjusted to pH 1.6 with concentrated H_2SO_4, and sterilized by filtration. The ferrous ion was regenerated by 0.5 A of current]. Total tRNA extracted from 0.5 g of *A. ferrooxidans* cells was purified by the Qiagen RNA/DNA kit as described by the manufacturer (Qiagen, Valencia, CA).

Purification of $tRNA^{Gln}$ and $tRNA^{Glu}$ by Affinity Chromatography. The *A. ferrooxidans* $tRNA^{Gln}_{UUG}$ and $tRNA^{Gln}_{CUG}$ (see above) and *E. coli* $tRNA^{Gln}$ and $tRNA^{Glu}$ were purified from unfractionated tRNA by affinity chromatography on immobilized *T. thermophilus* EF-Tu (22) with the following specifications. Unfractionated *E. coli* tRNA was aminoacylated with *E. coli* GlnRS or GluRS. The Gln-$tRNA^{Gln}$ or Glu-$tRNA^{Glu}$ was separated from the uncharged tRNA by the formation of a ternary complex with the EF-Tu-GTP immobilized on a Ni-NTA-agarose column. After the elution of Gln-$tRNA^{Gln}$ or Glu-$tRNA^{Glu}$, the tRNA was deacylated for 15 min at 65°C in 0.1 mM borate-KOH, pH 9.0. The pure $tRNA^{Gln}$ was not contaminated with $tRNA^{Glu}$ and vice versa, as judged by aminoacylation with pure *E. coli* GluRS or GlnRS, respectively.

Aminoacylation Assays with GluRS1 and -RS2. *In vitro* acylations with *H. pylori* and *A. ferrooxidans* GluRS enzymes was carried out at 37°C in an 80-μl reaction mixture containing 100 mM Hepes-KOH, pH 7.2; 30 mM KCl; 12 mM $MgCl_2$; 5 mM ATP; 2 mM DTT; 25 μM [^{14}C]Glu plus 75 μM unlabeled glutamate and 40–60 μg of unfractionated tRNA or 0.7–1.3 μg of pure tRNA. The reactions were started by adding the enzyme (100–150 nM). Aliquots (15 μl) were taken at different times, spotted on 3MM filter paper discs, and washed twice with 5% trichloroacetic acid. For acidic gel separations (see below), unfractionated *H. pylori* or *A. ferrooxidans* tRNA was charged in the presence of 0.1 mM unlabeled glutamate.

Acid Urea Gel Electrophoresis of tRNA and Aminoacyl-tRNA. This method (23) allows the separation of charged from uncharged tRNA due to a difference in electrophoretic mobility between the two species. Hybridization of a sequence-specific probe permits the determination of the identity of the tRNA on the gel. Unfractionated tRNA (80 μg) from *H. pylori* or *A. ferrooxidans* was glutamylated for 15 min at 37°C with the homologous synthetases (see above), and half of the reaction was deacylated for 15 min at 65°C with 0.1 mM borate-KOH, pH 9.0. After extraction with phenol (saturated with 0.3 M sodium acetate, pH 5.0/10 mM EDTA), the charged and uncharged tRNAs were recovered by ethanol precipitation. They were dissolved (at a final concentration of 5 μg/μl) in sample buffer (0.1 M sodium acetate, pH 5.0/8 M urea/0.05% bromophenol blue/0.05% xylene cyanol) The RNA samples (10 μg) were loaded on a 9.5% polyacrylamide gel (50 × 20 cm, 0.4 mm thick) containing 7 M urea and 0.1 M sodium acetate, pH 5.0 and run at 4°C, 600 V in 0.1 M sodium acetate, pH 5.0, for 40 h.

Detection of the tRNAs was performed by Northern blotting.

Table 1. Organisms with two *gltX* genes

Organism	Bacterial class	$tRNA^{Glu}$ species	$tRNA^{Gln}$ species	Gat CAB	*gluX*	GlnRS
Anaplasma marginale	α-Proteobacteria	1	1	+	−	−
Anaplasma phagocytophilum	α-Proteobacteria	1	1	+	−	−
Brucella melitensis	α-Proteobacteria	2	2	+	+	−
Brucella suis	α-Proteobacteria	2	2	+	+	−
Ehrlichia chaffeensis	α-Proteobacteria	1	1	+	−	−
Magnetospirillum magnetotacticum	α-Proteobacteria	2	2	+	+	−
Mesorhizobium loti	α-Proteobacteria	2	2	+	+	−
Neorickettsia sennetsu	α-Proteobacteria	1	1	+	−	−
N. aromaticivorans	α-Proteobacteria	2	2	+	+	−
Rhodobacter sphaeroides	α-Proteobacteria	1	1	+	+	−
Rhodospirillum rubrum	α-Proteobacteria	2	2	+	+	−
Rickettsia conorii	α-Proteobacteria	1	1	+	−	−
Rickettsia prowazekii	α-Proteobacteria	1	1	+	−	−
Silicibacter pomeroyi	α-Proteobacteria	1	1	+	+	−
Wolbachia sp.	α-Proteobacteria	1	1	+	−	−
A. ferrooxidans	γ-Proteobacteria	2	2	+	+	−
Coxiella burnetii	γ-Proteobacteria	1	1	+	−	−
Methylococcus capsulatus	γ-Proteobacteria	2	2	+	+	−
C. jejuni	ε-Proteobacteria	1	1	+	−	−
H. pylori sp.	ε-Proteobacteria	1	1	+	−	−
Helicobacter hepaticus	ε-Proteobacteria	1	1	+	−	−
D. hafniense	Clostridia	2	1	+	−	+
Thermoanaerobacter tengcongensis	Clostridia	2	2	+	−	−
T. maritima	Thermotogae	2	2	+	−	−

gluX, a truncated *gltX* gene (see text); GatCAB, Glu-$tRNA^{Gln}$ amidotransferase; + or −, presence or absence, respectively.

For this purpose, the portion of the gel containing the tRNAs was electroblotted onto a Hybond-*N* + membrane (Amersham Bioscience) by using a Hoefer Electroblot apparatus (Amersham Bioscience) at 10 V for 10 min and then at 30 V for 90 min with 10 mM Tris acetate, pH 7.8/5 mM sodium acetate/0.5 mM Na-EDTA as transfer buffer. The membranes were then baked at 72°C for 2 h. The tRNAs were detected by hybridization with a 5′-^{32}P-labeled oligodeoxyribonucleotide probe. For *H. pylori* $tRNA^{Gln}$, the probe complementary to nucleotides 1–21. *H. pylori* has two slightly different $tRNA^{Glu}_{UUC}$ genes; however, by Northern blot, we detected the product of one gene as the main $tRNA^{Glu}$ (data not shown); the probe used to detect that tRNA was complementary to nucleotides 1–21 (5′-TAACCACTA-GATGAAGGAGCC-3′).

The *A. ferrooxidans* genome sequence reveals two $tRNA^{Gln}$ isoacceptors, $tRNA^{Gln}_{UUG}$ and $tRNA^{Gln}_{CUG}$ (see Fig. 1). The probes were complementary to nucleotides 13–37 and 53–75 for $tRNA^{Gln}_{UUG}$ and to nucleotides 14–37 for $tRNA^{Gln}_{CUG}$. In the case of $tRNA^{Glu}$, we also found two isoacceptors that were detected by using the probes complementary to nucleotides 15–39 for each tRNA.

Results

Selection of the Experimental Systems. The presence of two paralogous *gltX* genes has been previously noticed in a few bacterial genomes (18). To survey the presence of duplicated GluRS genes or pieces thereof, we performed TBLASTN searches (24) of all currently available bacterial genomes. As a result, *gltX* duplications were found in the genomes of >20 organisms (Table 1). With the exception of two members of the Clostridiales and the deep-rooted *Thermotoga maritima,* all organisms with duplicated *gltX* genes are proteobacteria.

Apart from duplications of the entire *gltX* gene, many genomes were found to contain *gluX (yadB* in *E. coli*), a truncated form of *gltX* encoding a GluRS protein that lacks the enzyme's entire C-terminal anticodon-binding domain (≈35% of the total protein) and has no ascribed function; it was suggested to be a pseudogene (25) and shown to be not essential in *E. coli* (ref. 26, J.C.S., unpublished results). The relatively wide distribution (from *E. coli* to *D. radiodurans* and *Corynebacterium*) of the genes encoding these precisely truncated GluRS fragments suggests that these proteins may have a yet unknown function. Nonetheless, GluX was not analyzed further.

Class I lysyl-tRNA synthetase (LysRS) is structurally very related (27) and displays sequence homology to GluRS near its C terminus (COG 0008). Its rare occurrence in bacteria is confined largely to α-proteobacteria. It is interesting to note that the entire set of α-proteobacteria with two *gltX* genes (Table 1) is identical to that containing a class I LysRS. Whether this is a functionally significant correlation remains to be seen.

Phylogenetic analysis of GluRS sequences in a general tree showed a segregation of the duplicated GluRS proteins into two groups (Fig. 2). The first enzyme, GluRS1, branched with discriminating GluRS of *E. coli* and other proteobacteria. The second enzyme, GluRS2, appeared quite diverged but was in some cases closer to the ND-GluRSs, e.g., *B. subtilis* GluRS. Therefore, we assumed that GluRS1 is a canonical discriminating synthetase, whereas GluRS2 is the ND enzyme capable of forming Glu-$tRNA^{Gln}$, the tRNA substrate in the transamidation pathway of Gln-tRNA formation (reviewed in ref. 10). The key enzyme in this pathway, the Glu-$tRNA^{Gln}$ amidotransferase (encoded by *gatCAB*) (10–12), is present in all of the organisms with two *gltX* genes (Table 1). A relationship, mediated by a common partner, has been established between *H. pylori gltX2* and *gatA* in a genomic protein–protein interaction map (29).

To complement the enzyme analysis, we proceeded to examine the $tRNA^{Glu}$ and $tRNA^{Gln}$ species in these organisms. Our survey showed that some of the organisms possess only one isoacceptor of each $tRNA^{Glu}$ and $tRNA^{Gln}$ (e.g., *H. pylori* and *Campylobacter jejuni*), whereas others have two (e.g., *A. ferrooxidans* and *Novosphingobium aromaticivorans*). Closer examination brought better insight: in most cases where a genome contains two $tRNA^{Gln}$ isoacceptors, they are dissimilar and

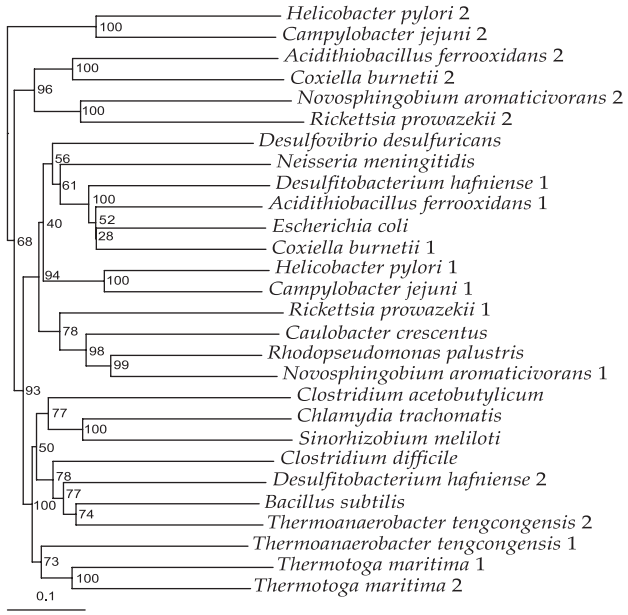

Fig. 2. Phylogeny of bacterial-type GluRS proteins. Amino acid sequences were aligned by using the CLUSTALW program (Version 1.82) (28). Initial analyses were performed with GluRS proteins from numerous bacterial organisms, and later the same was confirmed with a set of representative GluRSs. Phylogeny was inferred by using the neighbor-joining method to create and evaluate 1,000 resampled alignments. Bootstrap percentages are given for each branch. (Bar = 10 aa replacements per 100 positions.)

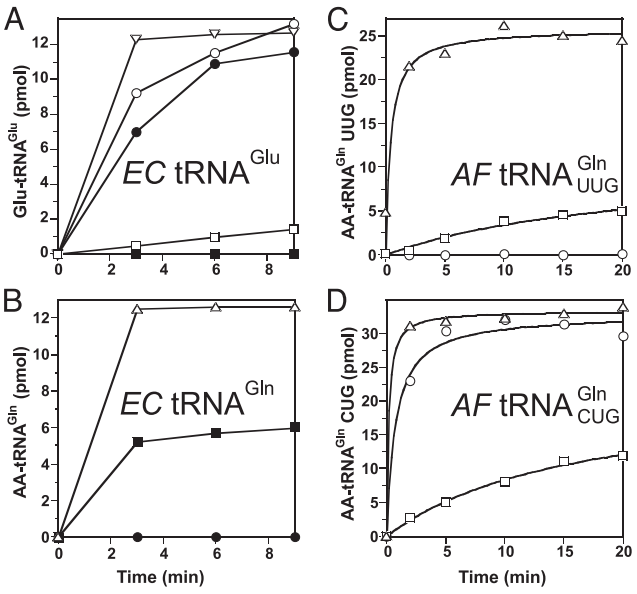

Fig. 3. Aminoacylation of $tRNA^{Glu}$ and $tRNA^{Gln}$ from *E. coli* and *A. ferrooxidans*. The reaction conditions were as indicated in *Materials and Methods* by using the pure tRNAs. (*A*) *E. coli* $tRNA^{Glu}$. (*B*) *E. coli* $tRNA^{Gln}$. (*C*) *A. ferrooxidans* $tRNA^{Gln}_{UUG}$. (*D*) *A. ferrooxidans* $tRNA^{Gln}_{CUG}$. The enzymes used were *E. coli* GluRS (▽) and GlnRS (Δ), *H. pylori* GluRS1 (●) and GluRS2 (■), and *A. ferrooxidans* GluRS1 (*o*) and GluRS2 (□). Glutamic acid was used with the GluRS, and glutamine was used with GlnRS.

structurally distinguished by the presence or absence of an augmented D-helix (13). The $tRNA^{Glu}$ and $tRNA^{Gln}$ species of *A. ferrooxidans* are depicted in Fig. 1; the $tRNA^{Gln}_{CUG}$ has an augmented D-helix, whereas the $tRNA^{Gln}_{UUG}$ lacks this element; in addition it has a different acceptor stem sequence. Thus, the *A. ferrooxidans* $tRNA^{Gln}_{CUG}$ may be a substrate for the *E. coli*-type GluRS (GluRS1). Together, these findings suggest that the two GluRS proteins may bind structurally different tRNA substrates. Therefore, we chose *A. ferrooxidans* and *H. pylori*, organisms with duplicated *gltX* genes, to examine the possible reason for the presence of two GluRS proteins by determining the tRNA substrate preferences of these enzymes.

The Two *H. pylori* GluRSs Possess Complementary tRNA Specificities. The *gltX1* and *gltX2* genes from *H. pylori* and *A. ferrooxidans* were cloned into expression vectors to encode intein or GST fusion proteins, respectively. After expression in *E. coli* and affinity chromatography, the native *H. pylori* enzymes were generated by intein cleavage, whereas the *A. ferrooxidans* GluRS enzymes were used as GST fusion proteins. Preliminary experiments showed that GluRS1 and -RS2 from both organisms efficiently charge total *E. coli* tRNA with glutamate but, as expected, not glutamine (data not shown). We then proceeded to determine the enzymes' tRNA specificities.

Because *H. pylori* possess only one $tRNA^{Glu}$ and one $tRNA^{Gln}$ species, we analyzed this system first. Because *H. pylori* tRNAs are very similar in sequence to *E. coli* tRNAs (>80% sequence identity for both species), we proceeded to purify $tRNA^{Glu}$ and $tRNA^{Gln}$ from unfractionated *E. coli* tRNA by EF-Tu affinity chromatography (22). The resulting pure *E. coli* tRNA species were then acylated with glutamate by the *H. pylori* GluRS1 and -RS2 enzymes. GluRS1 charged $tRNA^{Glu}$ well, whereas GluRS2 did not recognize this tRNA (Fig. 3*A*). In contrast, the result with $tRNA^{Gln}$ was completely reversed, because this tRNA was charged exclusively by GluRS2 (Fig. 3*B*). To ascertain the validity of these results obtained in charging the heterologous *E. coli* tRNA, we also analyzed glutamylated mature *H. pylori* tRNA samples by Northern blot of acid/urea gels (23). Glu-tRNAs generated by *H. pylori* GluRS1 and -RS2 and the control Gln-tRNA formed by *E. coli* GlnRS were used. After running the gel, Northern blot analysis was performed by using a specific probe for each tRNA of interest. Glu-$tRNA^{Gln}$ was formed only in the presence of GluRS2 (Fig. 4*A*, lane 3), whereas Glu-$tRNA^{Glu}$ was formed only in the presence of the GluRS1 (Fig. 4*A*, lane 6). These results confirmed that GluRS1 and -RS2 recognize the homologous $tRNA^{Glu}$ and $tRNA^{Gln}$, respectively (Fig. 4*A*, compare lanes 2 and 3 to lanes 6 and 8). As expected, Gln-$tRNA^{Gln}$, made by *E. coli* GlnRS, moved more slowly than Glu-$tRNA^{Gln}$ (Fig. 4*A*, compare lanes 1 and 3), which suggests that this method could also be used to provide a clue to the amino acid identity of the reaction products.

tRNA Specificity of GluRS Is Linked to Idiosyncratic tRNA Features. The *A. ferrooxidans* GluRS system is expected to be more complex than that of *H. pylori*, because there are two $tRNA^{Glu}$ and two $tRNA^{Gln}$ isoacceptors (Fig. 1). Although these four tRNAs have different anticodons, both $tRNA^{Glu}$ isoacceptors are uniform in their secondary structure and similar to *E. coli* and *H. pylori* $tRNA^{Glu}$, one of the $tRNA^{Gln}$ isoacceptors shows a striking divergence in its structure and is in some aspects more similar to $tRNA^{Glu}$ than $tRNA^{Gln}$ (Fig. 1). We expressed the *A. ferrooxidans* tRNA genes in *E. coli* and purified them by EF-Tu chromatography (22). The aminoacylation results show clearly that $tRNA^{Gln}_{UUG}$ was not charged by *A. ferrooxidans* GluRS1 but moderately by GluRS2 and well by *E. coli* GlnRS (Fig. 3*C*).†† However, $tRNA^{Gln}_{CUG}$ was charged well by GluRS1 and *E. coli* GlnRS but less well by GluRS2 (Fig. 3*D*). As expected, only *A. ferrooxidans* GluRS1 could charge *E. coli* $tRNA^{Glu}$ (Fig. 3*A*).

††*A. ferrooxidans* GluRS2 does not reach the same plateau as *E. coli* GlnRS (Fig. 3*C*). This may be due to the lack of a nucleotide modification in the heterologously produced $tRNA^{Gln}_{UUG}$ or to the presence of a supernumerary one introduced by *E. coli*. This is plausible, because mature *A. ferrooxidans* $tRNA^{Gln}_{UUG}$ is fully charged by GluRS2 (Fig. 4*B*, lane 5).

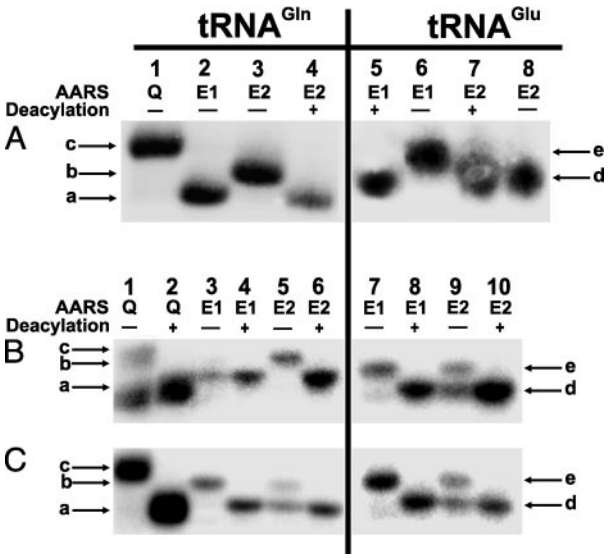

Fig. 4. Northern blot analysis of *H. pylori* and *A. ferrooxidans* tRNA after *in vitro* acylation by the different GluRS enzymes. The blots were probed with ^{32}P-labeled oligonucleotide complementary to the anticodon stem or plus acceptor stem (see *Materials and Methods*). Blots correspond to the detection of $tRNA^{Gln}$ (*Left*) and to $tRNA^{Glu}$ (*Right*). (*A*) Unfractionated tRNA from *H. pylori*. (*B*) Unfractionated tRNA from *A. ferrooxidans* probed for $tRNA^{Gln}_{UUG}$ or $tRNA^{Glu}_{UUC}$. (*C*) Unfractionated tRNA from *A. ferrooxidans* probed for $tRNA^{Gln}_{CUG}$ or $tRNA^{Glu}_{CUC}$. Lane 1, charging of unfractionated tRNA with glutamine by *E. coli* GlnRS. The enzymes used are: E1, GluRS1; E2, GluRS2; Q, *E. coli* GlnRS. The arrows indicate positions of AA-tRNA: a, uncharged $tRNA^{Gln}$; b, Glu-$tRNA^{Gln}$; c, Gln-$tRNA^{Gln}$; d, uncharged $tRNA^{Glu}$; and e, Glu-$tRNA^{Glu}$.

These tRNA specificities were confirmed by glutamylation of mature unfractionated *A. ferrooxidans* tRNA followed by acidic gel analysis, as described above for *H. pylori*. The *A. ferrooxidans* GluRS1 substrates are both $tRNA^{Glu}$ isoacceptors (Fig. 4 *B* and *C*, lane 7) and $tRNA^{Gln}_{CUG}$ (Fig. 4*C*, lane 3), but not $tRNA^{Gln}_{UUG}$ (Fig. 4*B*, lane 3). On the other hand, the *A. ferrooxidans* GluRS2 recognized primarily $tRNA^{Gln}_{UUG}$ (Fig. 4*B*, lane 5) but also exhibited some charging of the other $tRNA^{Gln}$ isoacceptor and $tRNA^{Glu}$ (Figs. 4*B*, lane 9, and *C,* lanes 5 and 9), thus retaining some characteristics of the ND-GluRS enzymes. That GluRS1 charged exclusively tRNAs with augmented D-helix structure strongly suggests this is the major identity element for this group of enzymes.

Altogether, these results imply the existence of a definite relationship between GluRS types and the specific structure of their tRNA substrates.

Discussion

RNA Sequence Suggests Structural Differences in Bacterial $tRNA^{Gln}$ Species. Inspection of the $tRNA^{Glu}$ and $tRNA^{Gln}$ sequences in the Genomic tRNA Database (http://rna.wustl.edu/tRNAdb) revealed that most archaeal and eukaryal $tRNA^{Glu}$ and $tRNA^{Gln}$ species (with the exception of *Saccharomyces cerevisiae*) possess sequences containing the augmented D-helix element. Thus, this RNA structural motif may be a relatively old element contributing to the function of these tRNAs. However, restricted to bacteria, structurally dissimilar (with respect to the augmented D-helix) $tRNA^{Glu}$ and $tRNA^{Gln}$ species, are frequently observed. In addition, $tRNA^{Gln}$ isoacceptors exist that differ in the presence or lack of augmented D-helix (e.g., *H. pylori*, one species with a nonaugmented D-helix; *A. ferrooxidans*, two $tRNA^{Gln}$ species, one with and one without augmented D-helix; *Caulobacter crescentus*, one species with an augmented D-helix). Thus, it is reasonable to assume the existence of different GluRS types as being related to this unusual tRNA diversity.

Bacterial *gltX* Gene Duplications Occurred Several Times. Phylogenetic analyses suggest a common origin of the GluRS and GlnRS family of AARSs (25, 30, 31). It is widely accepted that GlnRS probably evolved within the eukaryal ancestor through duplication of a *gltX* gene and later spread by horizontal gene transfer to the bacterial domain (25). Thus, it is plausible that a similar *gltX* gene duplication event may have occurred in some bacterial species and gave rise to a new subgroup of genes encoding enzymes with the tRNA specificity of GlnRS. Phylogenetic analysis shows that the $tRNA^{Gln}$-specific GluRS (GluRS2) described here arose from the ancestral gene duplication within bacterial GluRSs (ref. 30; this work), possibly in α-proteobacteria. A standard ND-GluRS may have preceded the GluRS2-type enzyme, because *A. ferrooxidans* GluRS2 retains traces of $tRNA^{Glu}$-binding capability. Such a gene duplication must have taken place several times in bacterial evolution, because GluRS2 enzymes in proteobacteria, in *Thermoanaerobacter tengcongensis* and in *Thermotoga maritima*, for instance, are clearly not of the same origin. This gene duplication in *T. maritima* is a much more recent event, and its two GluRSs group together in the phylogenetic tree (Fig. 2).

GluRS Duplication, a Strategy to Enhance Fidelity of Aminoacylation? The consistent occurrence of structurally different $tRNA^{Glu}$ and $tRNA^{Gln}$ sequences in organisms with a *gltX1* and a *gltX2* gene suggests that the separation of tRNA specificities in the corresponding GluRS enzymes may have provided higher fidelity of aminoacylation. It is reasonable to assume that use of specialized enzymes like GluRS1, GluRS2, or GlnRS would be favorable in terms of aminoacylation accuracy, compared with the broader specificity of GluRS-ND enzymes.

GluRS2, a New Type of AARSs. The essential ND-GluRS (e.g., *B. subtilis* GluRS) found in many organisms is a synthetase specific for one amino acid (Glu) and two acceptor RNA families ($tRNA^{Gln}$ and $tRNA^{Glu}$). This simple view of a single type of ND-GluRS among bacteria is not universally correct. Our results uncovered at least two additional types of GluRS. The first is still a ND-GluRS that, in addition to $tRNA^{Glu}$, recognizes only the subset of the $tRNA^{Gln}$ isoacceptor having an augmented D-helix (e.g., *A. ferrooxidans* GluRS1). The second enzyme is unique among AARSs; it is a new "mischarging" synthetase with an amino acid specificity (Glu) different from its single tRNA specificity ($tRNA^{Gln}$). This GluRS (e.g., *H. pylori* GluRS2) is an enzyme halfway between the discriminating GluRS and GlnRS enzymes, each of which recognizes only (and correctly) their cognate amino acid/tRNA pair.

GluRS2 and GlnRS Evolved Independently. It is tempting to speculate that GluRS2 might be on its way to developing into a true bacterial-origin GlnRS that has not yet evolved a glutamine-binding capacity. However, all known bacterial GlnRS proteins show the highest similarity to eukaryal GlnRSs (25) and are apparently not descendants of the $tRNA^{Gln}$-specific GluRS2 proteins discussed here. Furthermore, examination of the still incomplete *Desulfitobacterium hafniense* genome revealed (Table 1) the coexistence of ORFs for GluRS1 and -RS2 proteins with the predicted complementary tRNA specificities and for a canonical GlnRS. This implies that the GluRS2 and GlnRS enzymes evolved independently. Possibly the coexistence of GluRS1 and -RS2 may have prepared the organism for the acquisition of a canonical GlnRS by horizontal gene transfer from eukarya and subsequent loss of the nonorthologous *gltX2* gene. This might have been an easier scenario than evolving a new amino acid specificity (Glu to Gln) in *gltX2*. These assump-

tions could explain the relatively narrow distribution of organisms with duplicated *gltX* genes and the very rare example (*D. hafniense*) of an organism with two *gltx* genes and a canonical GlnRS.

Diversity of Bacterial GluRS Enzymes. Based on currently available information, there appear to be three groups of GluRS covering most of the examples investigated. Many bacteria [e.g., *Sinorhizobium meliloti* (3) or *Chlamydia trachomatis* (20)] have a single ND-GluRS and $tRNA^{Gln}$ isoacceptor without the augmented D-helix. The GluRS2-type enzyme is adapted to recognize only $tRNA^{Gln}$ lacking the augmented D-helix, a tRNA species also recognized by the canonical GlnRS. Last, there is GluRS1, an enzyme on its way to becoming a discriminating GluRS and preferentially recognizing tRNA species with the augmented D-helix. Therefore, the discriminating or ND natures of GluRS may have formed by coevolution of protein and tRNA structure.

Considering all of the data, it is clear that the GluRS/GlnRS family of enzymes shows exquisite diversity of gene arrangement and pathways, which might be linked to a relatively young evolution of canonical GlnRS activity.

Note Added in Proof. During the course of publication, a similar study with comparable results was published (32).

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