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Recognition of $tRNA^{Gln}$ by *Helicobacter pylori* GluRS2—a $tRNA^{Gln}$-specific glutamyl-tRNA synthetase

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ABSTRACT

Accurate aminoacylation of tRNAs by the aminoacyl-tRNA synthetases (aaRSs) plays a critical role in protein translation. However, some of the aaRSs are missing in many microorganisms. *Helicobacter pylori* does not have a glutaminyl-tRNA synthetase (GlnRS) but has two divergent glutamyl-tRNA synthetases: GluRS1 and GluRS2. Like a canonical GluRS, GluRS1 aminoacylates $tRNA^{Glu1}$ and $tRNA^{Glu2}$. In contrast, GluRS2 only misacylates $tRNA^{Gln}$ to form Glu-$tRNA^{Gln}$. It is not clear how GluRS2 achieves specific recognition of $tRNA^{Gln}$ while rejecting the two *H. pylori* $tRNA^{Glu}$ isoacceptors. Here, we show that GluRS2 recognizes major identity elements clustered in the $tRNA^{Gln}$ acceptor stem. Mutations in the tRNA anticodon or at the discriminator base had little to no impact on enzyme specificity and activity.

INTRODUCTION

In protein translation, each aminoacyl-tRNA synthetase (aaRS) recognizes and connects its cognate tRNA to its cognate amino acid (aa), forming a specific aminoacyl-tRNA (or isoacceptor set). These aminoacyl-tRNAs are then brought into the ribosome by elongation factor (EF-Tu) where they are used in protein translation. Intuitively, a complete set of 20 aaRSs is required with one enzyme matching each of the cognate 20 amino acids to the appropriate tRNA(s) (1). However, many microorganisms lack a full set of aaRSs. For example, *Helicobacter pylori* does not have glutaminyl-tRNA synthetase (GlnRS) or asparaginyl-tRNA synthetase (AsnRS) (2). In fact, many bacteria and archaea are missing one or both of these enzymes (3).

In the absence of GlnRS or AsnRS, Gln-$tRNA^{Gln}$ and Asn-$tRNA^{Asn}$ are generated indirectly through similar two-step processes (4–11). Typically, non-discriminating glutamyl- and aspartyl-tRNA synthetases (ND-GluRS and ND-AspRS) misacylate $tRNA^{Gln}$ and $tRNA^{Asn}$, respectively, to generate Glu-$tRNA^{Gln}$ and Asp-$tRNA^{Asn}$ (Reactions 1 and 3, respectively, misacylated tRNAs are shown in bold) (1,12). These enzymes are non-discriminatory because they still recognize and aminoacylate their cognate tRNAs to generate Glu-$tRNA^{Glu}$ and Asp-$tRNA^{Asp}$ (Reactions 2 and 4, respectively). Next, a glutamine-dependent amidotransferase (AdT) identifies and repairs Glu-$tRNA^{Gln}$ and Asp-$tRNA^{Asn}$ to generate Gln-$tRNA^{Gln}$ and Asn-$tRNA^{Asn}$, respectively (AdT rxn not shown) (4,6,9).

(1) $ND\text{-}GluRS:\ Glu + ATP + tRNA^{Gln} \rightarrow \mathbf{Glu\text{-}tRNA^{Gln}} + AMP + PPi$
(2) $ND\text{-}GluRS:\ Glu + ATP + tRNA^{Glu} \rightarrow Glu\text{-}tRNA^{Glu} + AMP + PPi$
(3) $ND\text{-}AspRS:\ Asp + ATP + tRNA^{Asn} \rightarrow \mathbf{Asp\text{-}tRNA^{Asn}} + AMP + PPi$
(4) $ND\text{-}AspRS:\ Asp + ATP + tRNA^{Asp} \rightarrow Asp\text{-}tRNA^{Asp} + AMP + PPi$

Instead of a canonical ND-GluRS, *H. pylori* and a small subset of other bacteria utilize two paralogous GluRSs—GluRS1 and GluRS2. *Helicobacter pylori* GluRS1 is discriminatory and only generates Glu-$tRNA^{Glu1}$ and Glu-$tRNA^{Glu2}$ (*H. pylori* has two $tRNA^{Glu}$ isoacceptors; Figure 1). GluRS2 is consequently responsible for the biosynthesis of Glu-$tRNA^{Gln}$. Interestingly, although GluRS1 and GluRS2 are closely related, GluRS2 does not make Glu-$tRNA^{Glu}$ (13,14).

(5) $GluRS1:\quad Glu + ATP + tRNA^{Glu} \rightarrow Glu\text{-}tRNA^{Glu} + AMP + PPi$
(6) $GluRS2:\quad Glu + ATP + tRNA^{Gln} \rightarrow \mathbf{Glu\text{-}tRNA^{Gln}} + AMP + PPi$

The close evolutionary relationship between GluRS1 and GluRS2 and the unusual non-cognate $tRNA^{Gln}$ specificity of GluRS2 led to the proposal that GluRS2 could represent an abortive or ongoing attempt by bacteria to evolve a bacterial GlnRS (14). (All known

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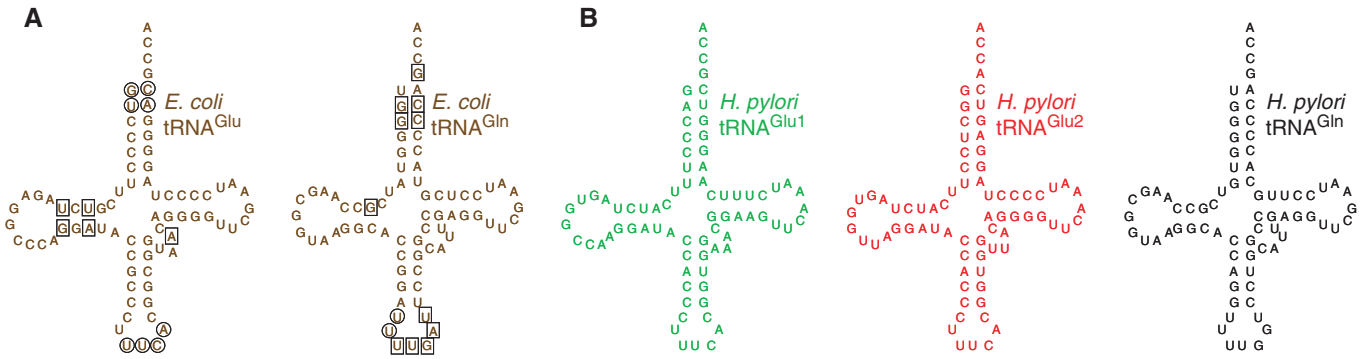

Figure 1. Sequences and secondary structures of tRNAs. (**A**) The known major identity elements in *E. coli* $tRNA^{Glu}$ and $tRNA^{Gln}$ are boxed (21–23); minor elements are circled. (**B**) *Helicobacter pylori* $tRNA^{Glu1}$ is shown in green, *H. pylori* $tRNA^{Glu2}$ is shown in red and *H. pylori* $tRNA^{Gln}$ is in black.

GlnRSs originated in eukarya (15–18) and the factors that have prevented the emergence and/or utilization of GlnRS in most bacteria are not well understood.) It has also been proposed that the divergence of GluRS1 and GluRS2 occurred to accommodate changes in the length of the $tRNA^{Glu}$ and $tRNA^{Gln}$ D-stems (4 versus 3 base pairs, respectively) (13).

We are interested in understanding how GluRS2 diverged from GluRS1 to gain unique specificity for $tRNA^{Gln}$, while rejecting the two $tRNA^{Glu}$ isoacceptors $tRNA^{Glu1}$ and $tRNA^{Glu2}$. We have previously shown that a single point mutation in the anticodon-binding domain of GluRS2 converts this enzyme into one that only aminoacylates $tRNA^{Glu1}$ instead of $tRNA^{Gln}$, demonstrating recognition of the tRNA anticodon by GluRS2. Unexpectedly, this G417T mutation did not induce aminoacylation activity towards $tRNA^{Glu2}$, despite the fact that this tRNA has the same UUC anticodon (19). In order to identify the mechanisms used by GluRS2 to select $tRNA^{Gln}$ and reject $tRNA^{Glu2}$, here we have introduced varying degrees of $tRNA^{Glu2}$ character into $tRNA^{Gln}$. Analysis of these tRNAs demonstrates that the anticodon loop and the discriminator base are not identity elements for GluRS2 aminoacylation of $tRNA^{Gln}$. Instead, the major identity elements are localized in the acceptor stem of $tRNA^{Gln}$. These results are put into an evolutionary context.

MATERIALS AND METHODS

Materials

Oligonucleotides were purchased from Invitrogen and used without further purification. The pCR 2.1 TOPO plasmid was also from Invitrogen. Pfu polymerase was purchased from Stratagene. Taq polymerase was from New England Biolabs. Radiolabeled glutamate (L-[3,4-^{3}H]-glutamic acid) was purchased from Perkin Elmer. All buffers were filtered through a 0.22 µm filter prior to use. When appropriate, solutions were autoclaved. Unless otherwise stated, reagents were used without further purification. All gene constructs were verified by DNA sequencing of the entire gene insert.

Cloning of Hp tRNA variants

For each tRNA chimera, two partially overlapping primers were designed to reconstitute the entire tRNA gene with appended BamHI and EcoRI restriction sites onto the 5′- and 3′-ends of the gene, respectively (see Supplementary Table S1). Each primer pair was used in a template-independent polymerase chain reaction (PCR) with Pfu polymerase. Each PCR product was inserted into the pCR2.1 TOPO vector after incubation with Taq polymerase. The correct insert was verified by DNA sequencing and then sub-cloned into the BamHI and EcoRI sites of the pES300 vector to enable isopropyl β-D-1-thiogalactopyranoside (IPTG)-induced *in vivo* overtranscription of the cloned tRNA gene (14).

Mutations were introduced by QuikChange© mutagenesis according to the directions provided by Stratagene. Primer sequences are provided in Supplementary Table S1.

Overtranscription and purification of *Hp* tRNAs and tRNA chimeras

Each tRNA and chimera was overexpressed in the *Escherichia coli* strain MV1184 at 37°C in Luria Broth (500 ml LB) supplemented with ampicillin (100 µg/ml) and glucose (0.5% w/v). When the A600 nm was between 0.4 and 0.6, IPTG (to a final concentration of 1 mM) was added to induce production of the tRNA. Cells were pelleted by centrifugation 4–5 h after induction and stored at −80°C for future use. Each overproduced tRNA was purified by Nucleobond affinity chromatography (Clontech) as previously described (14). This procedure generates a mixture of *E. coli* tRNAs that is enriched with the encoded *H. pylori* tRNA of interest. *In vivo* tRNA production was conducted (instead of *in vitro* transcription) to allow for the introduction of a 2-thiouridine at position 34 of all three wild-type tRNAs and all mutations. This modification is essential for aminoacylation by GluRS (20). Thus, any tRNAs that lack this modification will have negligible activity in the experiments described below.

The concentration of each tRNA was determined using GluRS1 or GluRS2, depending on the substrate specificity

of the tRNA, in standard aminoacylation assays (see below). Expression of tRNA variants that were not robust substrates for either GluRS1 or GluRS2 was verified by urea gel and northern blot (see next section). Final tRNA concentrations were highly variable and ranged from being too low to accurately quantify (<100 pmol/A260 nm) to ~700 pmol/A260 nm (see Supplementary Table S3).

Northern blots of acceptor mutations and D stem/loop tRNAs

Northern blots were performed for tRNAs that could not be robustly quantified by aminoacylation. Total tRNA concentration was measured by A260 nm value; either 0.5 or 0.05 A260 nm aliquots were used for analysis of each tRNA. Each tRNA was diluted to 40 µl in 100 mM NaOAc, pH 5.0, 8 M urea, 0.05% (w/v) bromophenol blue and 0.05% (w/v) xylene cyanol; the sample was boiled for 5 min. Each sample (20 µl) was immediately loaded onto a 12% urea gel. Electrophoresis was performed for 75 min at 150 V. The tRNAs were transferred from the gel to an Immobilon-NY+ membrane (Millipore) using a Semi-Dry Blotting Unit (Fisher Biotech); transfer was conducted for 90 min at 320 mA. The membrane was baked for 90 min at 75°C and then subjected to overnight hybridization with a ^{32}P-labeled oligonucleotide (TLH-14C: 5′ C TCGGAATGCCAGGACCAA 3′) selected to be specific for all mutant tRNAs (15). The membrane was washed four times with 20 ml of the following buffer: 450 mM NaCl, 90 mM Tris–HCl, pH 8.0, 6 mM Na_2EDTA, 0.1% SDS w/v, before exposure to a storage phosphor screen (Molecular Dynamics). Bound radioactivity was visualized using a Typhoon 9210 (Amersham Biosciences).

Aminoacylation assays with GluRS1 and GluRS2

Helicobacter pylori GluRS1 and GluRS2 were purified to homogeneity as previously described (14). Aminoacylation reactions were conducted in 40 mM HEPES-OH, pH 7.5, 4 mM ATP, 8 mM $MgCl_2$, 200 µM unlabelled Glu and 50 µCi ^{3}H-Glu at 37°C. For assays aimed at measuring the expression level of different tRNAs, the experiments were performed for 90 min with 1 µM GluRS1 or GluRS2. For initial rate assays, 0.1 µM GluRS1 or GluRS2 was used with 10 µM enriched tRNA (concentration was estimated from A260 nm readings) and time points were taken at shorter intervals. The unit definition of GluRS1 is defined as the amount of enzyme that aminoacylates 0.1 pmol $tRNA^{Glu1}$ per second; a unit of GluRS2 aminoacylates $tRNA^{Gln}$ at a rate of 0.1 pmol per second (14). All assays were conducted in triplicate and the reported error measurements reflect standard deviation.

RESULTS

The tRNA acceptor stem is important for the $tRNA^{Gln}$ specificity of GluRS2

There are two $tRNA^{Glu}$ isoacceptors in *H. pylori*—$tRNA^{Glu1}$ and $tRNA^{Glu2}$—and one $tRNA^{Gln}$ (2)

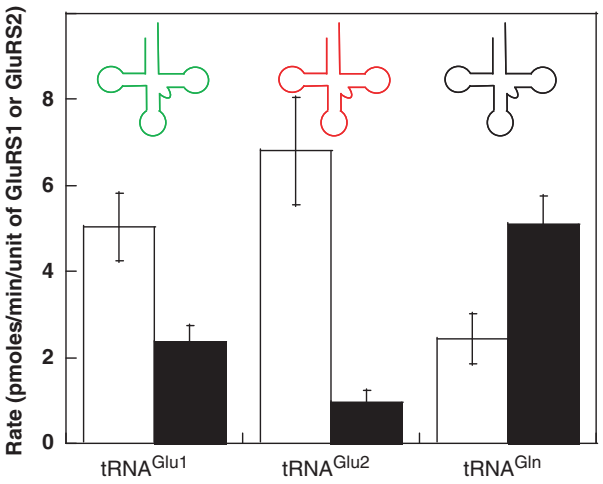

Figure 2. Initial rates of aminoacylation for *H. pylori* $tRNA^{Glu1}$ (green), $tRNA^{Glu2}$ (red) and $tRNA^{Gln}$ (black) using *H. pylori* GluRS1 (white bars) and GluRS2 (black bars). GluRS2 preferentially aminoacylates $tRNA^{Gln}$, whereas GluRS1 aminoacylates $tRNA^{Glu1}$ and $tRNA^{Glu2}$. The aminoacylation rate is determined by measuring the rate of formation of glutamyl-tRNAs in pmoles per unit of GluRS1 or GluRS2. Error bars represent standard deviation from triplicate assays.

(Figure 1; the sequences of *E. coli* $tRNA^{Glu}$ and $tRNA^{Gln}$ are given for comparison, with known identity elements marked by circles and squares) (21–23). While $tRNA^{Glu1}$ and $tRNA^{Glu2}$ share 78% sequence identity, *H. pylori* GluRS2 apparently uses different mechanisms to reject these two tRNAs. It has been shown that a single mutation in the GluRS2 anticodon-binding domain can switch this enzyme's tRNA substrate specificity from $tRNA^{Gln}$ to $tRNA^{Glu1}$. However, this mutated GluRS2 failed to aminoacylate $tRNA^{Glu2}$ (19). The main aim of the present work is to investigate the important elements that distinguish $tRNA^{Glu2}$ from $tRNA^{Gln}$, with respect to *H. pylori* GluRS1 and GluRS2.

A series of four tRNA chimeras were designed according to different domains of the tRNA. These $tRNA^{Gln/Glu2}$ chimeras each contain ~75% $tRNA^{Gln}$ and ~25% $tRNA^{Glu2}$ character (Figures 2 and 3; Supplementary Table S2). Chimera 2 was further modified to contain the $tRNA^{Glu2}$ variable loop in order to maintain stable tertiary structure (21). All chimeras, including chimera 3, retain the $tRNA^{Gln}$ anticodon. Each tRNA was overtranscribed *in vivo* and purified by ion exchange chromatography, as previously described (14). Levels of overexpression were quantified by aminoacylation assays using excess GluRS1 and GluRS2, and results from the assay that produced the highest aminoacylation levels were used. (Note: The calculated expression levels for each tRNA are included in Supplementary Table S3.)

Each chimeric tRNA was assayed with GluRS1 and with GluRS2; for comparison, identical assays were conducted with the three wild-type *H. pylori* tRNAs. The results of these initial rate assays are shown in Figure 2 (for wild-type tRNAs) and in Figure 3 (for the chimeric

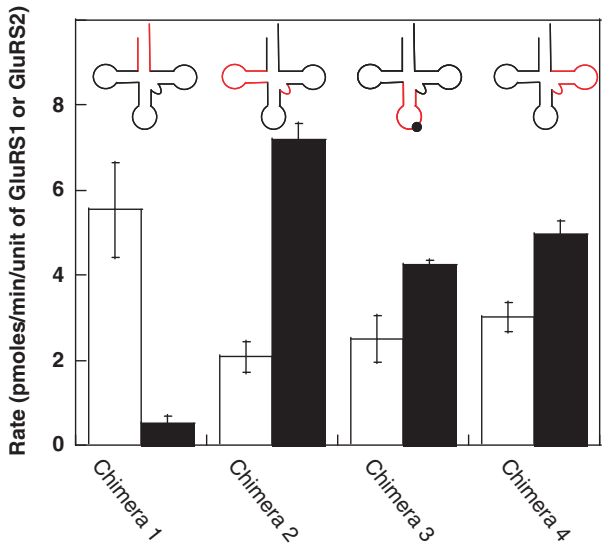

Figure 3. Initial rates of aminoacylation for different tRNA chimeras using *H. pylori* GluRS1 (white bars) and GluRS2 (black bars). Each tRNA chimera represents the insertion of different regions of *H. pylori* $tRNA^{Glu2}$ (red) into *H. pylori* $tRNA^{Gln}$ (black). Chimera 1 is a better substrate for GluRS1 than GluRS2, while the remaining chimeras retain $tRNA^{Gln}$-like specificity and are substrates for GluRS2. The $tRNA^{Gln}$ anticodon was retained in chimera 3. Error bars represent standard deviation from triplicate assays.

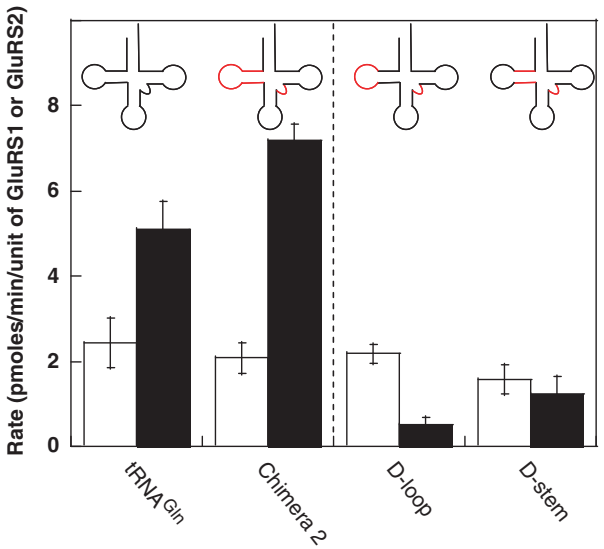

Figure 4. Investigation of the role of the D-stem and D-loop. Transfer RNAs were constructed to contain only the $tRNA^{Glu2}$ D-stem (red) or D-Loop (red) in a $tRNA^{Gln}$ (black) background. Neither mutant tRNA was a robust substrate for either GluRS1 or GluRS2. Data to the left of the dashed line are reproduced from Figures 1 and 2 for comparison. The white and black bars indicate data obtained from GluRS1 and GluRS2 assays, respectively. Error bars represent standard deviation from triplicate assays.

tRNAs). (Note: the primary sequences of these chimeras and all other tRNA constructs are included in the Supplementary Table S2). Only Chimera 1, wherein the acceptor stem of $tRNA^{Glu2}$ was transplanted into $tRNA^{Gln}$, has $tRNA^{Glu2}$-like activity. Unlike the parent $tRNA^{Gln}$, Chimera 1 is a strong substrate for GluRS1 but not for GluRS2. In contrast, Chimeras 2, 3 and 4 all retain $tRNA^{Gln}$-like activity as they are predominantly aminoacylated by GluRS2 but not by GluRS1. These results argue that the key identity elements for GluRS2 recognition of $tRNA^{Gln}$ are localized only within the acceptor stem of $tRNA^{Gln}$. In fact, the aminoacylation profiles of Chimeras 3 and 4 are virtually indistinguishable from that of $tRNA^{Gln}$. (Note: For $tRNA^{Gln}$, the low observed rate of GluRS1-catalyzed aminoacylation is the result of aminoacylation of contaminating *E. coli* tRNAs (14); thus, it is likely that the GluRS1 data for chimeras 2–4 is misleadingly high. Given the negligible impact that these chimeras had on GluRS2 activity, the role of contaminating tRNAs was not investigated further). Interestingly, Chimera 2 is actually a better substrate for GluRS2 than is $tRNA^{Gln}$. This result is seemingly in contradiction with the proposed role of the D-stem length in the emergence of GluRS2 (13); see below for further discussion and analysis.

Based on these results and the known identity elements for other $tRNA^{Gln}$ aminoacylation systems (21–23), we chose to further dissect the acceptor stem, D-stem/loop, and anticodon stem/loop to more precisely define the role(s) of these regions and to confirm the unexpected results that neither the D-stem/loop nor the anticodon stem/loop are strong sources of identity for GluRS2 recognition of $tRNA^{Gln}$.

The role of the D-stem/loop

Transfer RNA^{Glu} isoacceptors typically have an augmented D-stem, containing four base pairs instead of the three base pairs seen in $tRNA^{Gln}$. In *E. coli* $tRNA^{Glu}$, this larger D-stem contains major identity elements that are recognized by the discriminating *E. coli* GluRS (Figure 1) (21). Moreover, it has also been proposed that the size of the $tRNA^{Glu}$ D-stem is an important feature for the divergence in tRNA specificity between GluRS1 and GluRS2; this hypothesis was partially based on the observation that *Acidithiobacilus ferrooxidans* GluRS1 aminoacylates one of its $tRNA^{Gln}$ isoacceptors and this $tRNA^{Gln}$ has a four base pair D-stem (13). Because of the apparent contradiction between these previous observations and our data showing that Chimera 2, which contains an engineered four base pair D-stem, is still $tRNA^{Gln}$-like in activity, we evaluated two additional D-stem/loop constructs. In the first construct, the D-loop of $tRNA^{Glu2}$ was transplanted into $tRNA^{Gln}$; in the second, the $tRNA^{Glu2}$ D-stem was introduced into $tRNA^{Gln}$. Neither of these tRNAs were robust substrates for either GluRS1 or GluRS2, a result that is strikingly different from both $tRNA^{Gln}$ and Chimera 2 (Figure 4A). Interestingly, when the concentration of GluRS1 or GluRS2 and the length of the assay is increased (the conditions we use to quantify tRNA expression levels), both of these tRNAs can be aminoacylated by either GluRS1 or GluRS2 (see Supplementary Table S3). Consequently, neither the D-stem nor the D-loop contain major identity elements for GluRS1 or GluRS2. However, because both the D-stem and the D-loop tRNAs have diminished activity

towards GluRS2, the possibility that this region contains minor identity elements cannot be ruled out.

Mutagenesis of the anticodon loop

Next, we focused our attention on the anticodon stem/loop of $tRNA^{Gln}$. In *Thermus thermophilus* D-GluRS, a single mutation in the anticodon-binding domain switched this enzyme to an ND-GluRS with dual specificity for $tRNA^{Glu}$ and $tRNA^{Gln}$ (24). And in *H. pylori*, a G417T mutation in the GluRS2 anticodon-binding domain was sufficient to introduce $tRNA^{Glu1}$, but not $tRNA^{Glu2}$, aminoacylation activity into GluRS2 (19). It has also been shown that both the anticodon of *E. coli* $tRNA^{Glu}$ (UUC) and *E. coli* $tRNA^{Gln}$ (UUG) are important for *E. coli* GluRS and *E. coli* GlnRS recognition, respectively (21–23). Also, post-transcriptional modification of position 34 in the anticodon, to generate 5-methylaminomethyl 2-thiouridine (mnm^5s^2U34) enhances both aminoacylation activity and specificity (20). This modification is expected to be present in all three *H. pylori* tRNA isoacceptors (2) and in our different tRNAs (14). Because U34 is present in all three tRNAs, it was not evaluated. The *H. pylori* $tRNA^{Glu1}$, $tRNA^{Glu2}$, and $tRNA^{Gln}$ anticodon loops vary at four positions (Figure 1). Among these four nucleotides, N36, N37, and N38 have been shown to be important identity elements for both *E. coli* GluRS and GlnRS (21–23). We individually evaluated each of these positions by mutating the nucleotide in $tRNA^{Gln}$ into that of $tRNA^{Glu2}$ (Figure 5). Consistent with the wild-type behavior of Chimera 3, mutagenesis at each of these positions had no effect on the substrate behavior of $tRNA^{Gln}$. These results show that the anticodon loop is not important for GluRS2 recognition, in contrast to patterns seen with other GluRSs and with many other tRNA/aaRS pairs (12,19,24,26,27).

Mutagenesis of the $tRNA^{Gln}$ acceptor stem and discriminator base

Finally, we turned our attention to the acceptor stem of $tRNA^{Gln}$, the region that holds the most promise based on the original survey of tRNA chimeras. We first examined the discriminator base (N73), another position that is often used by aaRSs to achieve tRNA specificity (27,28). The G73 discriminator base of *E. coli* $tRNA^{Gln}$ is a major identity element for *E. coli* GlnRS (22,23). Interestingly, *H. pylori* $tRNA^{Glu1}$ and $tRNA^{Gln}$ have the same G73 discriminator base, despite being recognized by different GluRSs (GluRS1 and GluRS2, respectively); $tRNA^{Glu2}$ has an A73 in this position. A G73A-mutant $tRNA^{Gln}$ was evaluated to test the impact of the discriminator base on GluRS1 and GluRS2 aminoacylation. Unexpectedly, given the common importance of this position, the identity of the discriminator base is not important for GluRS2 recognition of $tRNA^{Gln}$ (Figure 6A).

Next, we individually evaluated each base pair in the acceptor stem of $tRNA^{Gln}$, in order to better understand how GluRS1 recognizes Chimera 1. In other GluRS and GlnRS systems, residues within the acceptor stem are known identity elements (The first two base pairs of

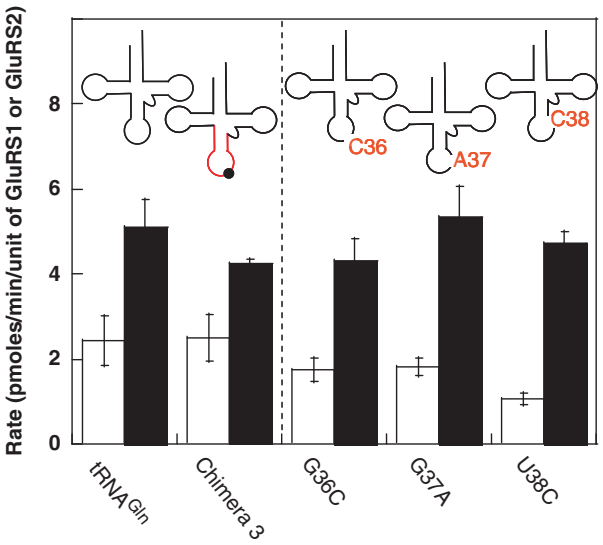

Figure 5. Impact of specific nucleotides in the anticodon loop on the initial aminoacylation rates of $tRNA^{Gln}$. The third nucleotide of the $tRNA^{Gln}$ anticodon G36, G37 and U38, were individually mutated to the corresponding nucleotides in $tRNA^{Glu2}$ (G36C, G37A, U38C) and assayed with both GluRS1 (white bars) and GluRS2 (black bars). These mutations did not impact the substrate specificity of $tRNA^{Gln}$. Data to the left of the dashed line are reproduced from Figures 1 and 2 for comparison. Error bars represent standard deviation from triplicate assays.

E. coli $tRNA^{Glu}$ with GluRS, and the second and third base pairs of *E. coli* $tRNA^{Gln}$ with GlnRS) (21–23). To more precisely characterize the impact of the tRNA acceptor stem on *H. pylori* GluRS1 and GluRS2 aminoacylation activity, each acceptor stem base pair in $tRNA^{Gln}$ was separately mutated to that found in $tRNA^{Glu2}$; as above, each tRNA was assayed with both GluRS1 and GluRS2 (Figure 6B). Consistent with the analysis of Chimera 1, all but two of these mutations are poor substrates for GluRS2. The exceptions are the third and sixth base pairs (G3:C70 and U6:A67 in $tRNA^{Gln}$). Inversion of the G3:C70 base pair to C3:G70 had no effect on this tRNA's specificity, whereas the C6:G67 mutation caused a slight decrease in the rate of aminoacylation by GluRS2 but did not induce GluRS1 recognition. In contrast, none of the remaining mutations were robust substrates for either GluRS1 or GluRS2.

In order to interpret these results, it was necessary to verify that these mutant tRNAs were overtranscribed and present in our assay mixtures. To this end, a sample of each tRNA was analyzed by northern blot (Figure 6C). Mutation at the first base pair (converting the U1:A72 in $tRNA^{Gln}$ into the G1:C72 of $tRNA^{Glu2}$), generated a tRNA that was overtranscribed *in vivo* at levels greater than that of wild-type $tRNA^{Gln}$. This high level of tRNA production and the lack of activity with GluRS2 clearly demonstrate that the G1:C72 base pair of $tRNA^{Glu2}$ is a major antideterminant for GluRS2. The fifth base-pair mutation (G5:C68 inverted to C5:G68) was overtranscribed at moderate levels, confirming that this position is also important as an antideterminant in $tRNA^{Glu2}$, preventing GluRS2 recognition. The second,

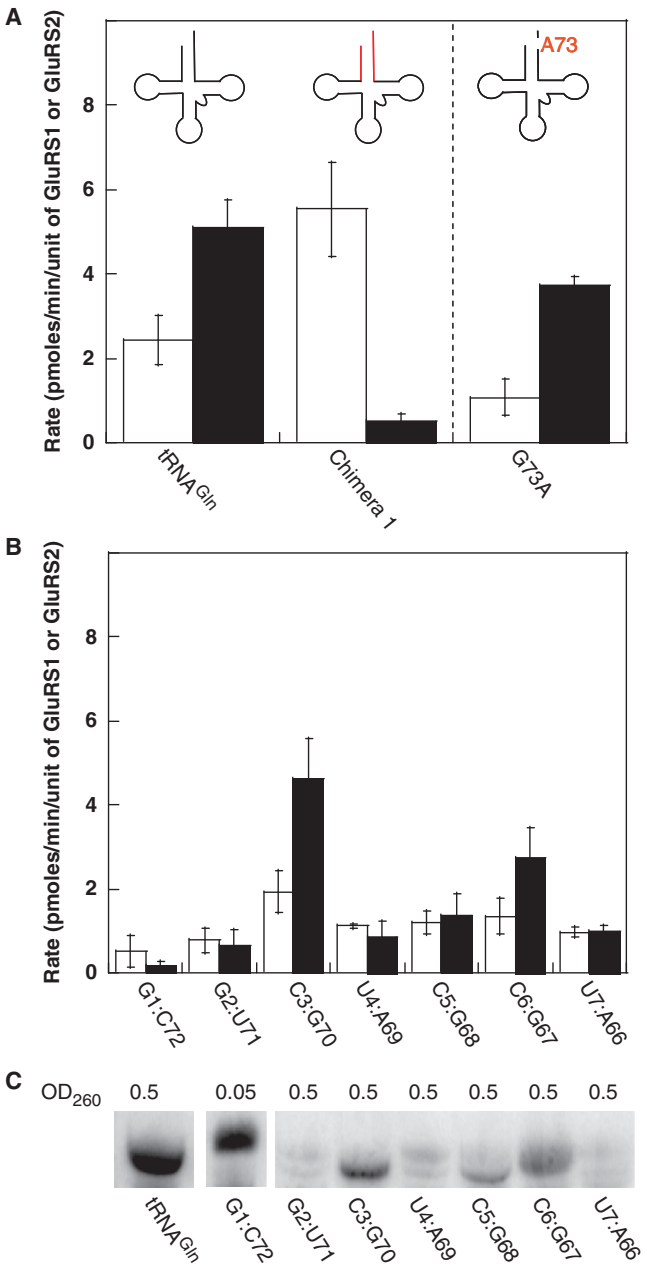

Figure 6. Influence of the discriminator base and acceptor stem on the initial aminoacylation rate of $tRNA^{Gln}$. (**A**) The discriminator base of *H. pylori* $tRNA^{Gln}$ was mutated to that of $tRNA^{Glu2}$ (A G73A mutation). This change did not affect substrate specificity of this tRNA. Initial rates of aminoacylation with GluRS1 (white bars) versus GluRS2 (black bars). Error bars represent standard deviation. (**B**) Evaluation of single base pair mutations in the acceptor stem of *H. pylori* $tRNA^{Gln}$. Initial rates of aminoacylation with GluRS1 (white bars) versus GluRS2 (black bars). Each mutation is labeled according to the base pair inserted into $tRNA^{Gln}$ (from $tRNA^{Glu2}$). Error bars represent standard deviation. (**C**) Evaluation of the expression efficiency of each acceptor stem mutant tRNA by Northern blot. Transfer RNA^{Gln} and the G1:C72 mutant tRNA were strongly expressed. The remaining mutant tRNAs were expressed at lower and varying levels. The white bars separating the first three lanes are the result of digital removal of empty lanes in the gel. The image shown was adjusted with uniform contrast using ImageQuant TL (Amersham Biosciences, v. 2005). Another image of the blot, showing the last 6 lanes with darker contrast, is provided in the Supplementary Figure S1.

fourth and seventh base pairs were not robustly overexpressed (Figure 6C and Supplementary Figure S1). Each of these tRNAs was assayed at the maximum possible concentration. Consequently, the poor aminoacylation activities with each of these mutations likely indicate that each of these positions is an important antideterminant that prevents $tRNA^{Glu2}$ from being aminoacylated by GluRS2, however the possibility that tRNA expression levels were simply too low to observe aminoacylation activity cannot be ruled out.

Interestingly, while many of the acceptor stem positions in $tRNA^{Glu2}$ are clearly important for rejection by GluRS2, no single base-pair mutation led to recognition by GluRS1. This observation is in sharp contrast to Chimera 1, which is a robust substrate for GluRS1. Clearly, some or all of these positions are $tRNA^{Glu2}$ determinants for GluRS1, but they are only strong enough to induce recognition when combined. Perhaps, $tRNA^{Gln}$ also contains an antideterminant for GluRS1 distal to the acceptor stem.

DISCUSSION

GluRS2 uses specialized mechanisms to recognize $tRNA^{Gln}$

These studies demonstrate that GluRS2 achieves its unique $tRNA^{Gln}$ specificity, rejecting $tRNA^{Glu2}$, solely by distinguishing between differences in the acceptor stems of these two tRNAs. It is surprising that neither the anticodon nor the discriminator base is important, as these positions are critical for $tRNA^{Glu}$ and $tRNA^{Gln}$ aminoacylation in other systems (21–23,27). In *E. coli* $tRNA^{Glu}$, identity elements are spread throughout the tRNA scaffold, with major determinants located in the augmented D-stem (Figure 1) (21). The identity elements of *E. coli* $tRNA^{Gln}$ are mainly located in the two distal ends of the tRNA, at the discriminator base, the second and third base pairs in the acceptor stem, and the anticodon loop (Figure 1) (22,23). Furthermore, in *T. thermophilus* D-GluRS the size and identity of the amino acid that interacts with the third nucleotide of the anticodon plays an important role in discrimination between $tRNA^{Glu}$ and $tRNA^{Gln}$ (24); this region is important for GluRS2 rejection of $tRNA^{Glu1}$ as well (19). In contrast, here we show that GluRS2 rejects $tRNA^{Glu2}$ by predominantly looking at only one region of the tRNA—the acceptor stem.

Although this work focused on the rejection of $tRNA^{Glu2}$, the results also provide some insight into how GluRS2 rejects $tRNA^{Glu1}$. As we have previously reported, a G417T mutant GluRS2 aminoacylates $tRNA^{Glu1}$ but not $tRNA^{Gln}$ or $tRNA^{Glu2}$ (19). In light of the present work, this result is surprising because $tRNA^{Glu1}$ contains the same G1:C72 base pair as $tRNA^{Glu2}$, a strong antideterminant for GluRS2 (Figure 6A and B). Thus, it appears that the G417T mutation unmasks a role for the $tRNA^{Glu1}$ anticodon that is sufficient to overcome the potency of the G1:C72 acceptor stem antideterminant. The combination of these results suggest that $tRNA^{Glu1}$, unlike $tRNA^{Glu2}$, contains

distal antideterminants for GluRS2 that are located in *both* the acceptor stem and the anticodon. This observation is unexpected since $tRNA^{Glu1}$ and $tRNA^{Glu2}$ contain the same UUC anticodon, and it suggests subtle differences in the shapes of the two tRNAs. Mutagenesis experiments within the $tRNA^{Glu1}$ framework are needed in order to truly understand the differences in how these tRNAs are rejected by GluRS2.

Our results indicate that the first acceptor stem base pair (U1:A72) is critically important for GluRS2s tRNA specificity—namely, the accurate recognition of $tRNA^{Gln}$ and the rejection of $tRNA^{Glu1}$ and $tRNA^{Glu2}$, which both contain a G1:C72 base pair. The importance of this position is conserved throughout indirect aminoacylation. Like GluRS2, AdT, the amidotranferase that converts Glu-$tRNA^{Gln}$ into Gln-$tRNA^{Gln}$, relies on the U1:A72 base pair for recognition of Glu-$tRNA^{Gln}$ (29,30); the archaeal type AdT (*Methanothermobacter thermautotrophicus* GatDE) also relies on this position for recognition of $tRNA^{Gln}$ (in this case, it is an A1:U72 base pair which is recognized by GatDE) (25). The archaeal GatCAB does not use the first base pair as a strong identity element (31).

Evolutionary implications

Substantial evidence has accumulated to suggest that progenitor tRNAs were smaller than their modern counterparts and consisted of either a single acceptor-stem microhelix or a minihelix comprised of the acceptor stem and TΨC-stem/loop (32–34). These smaller RNAs were putatively aminoacylated by ancestral aaRSs, comprised solely of catalytic domains (34,35). Acquisition of divergent anticodon-binding domains was a likely key step in the separation of GluRS and GlnRS (34,36). GluRS2 deviates from this picture, however, because it contains a GluRS-like anticodon-binding domain (13,14). Instead, this enzyme has capitalized on primordial mechanisms of tRNA recognition, in effect rendering the anticodon-binding domain useless, at least with respect to distinguishing between $tRNA^{Gln}$ and $tRNA^{Glu2}$.

A very recent report also demonstrated that the truncated catalytic domain of *E. coli* D-GluRS is capable of discriminating against $tRNA^{Gln}$, in favor of $tRNA^{Glu}$, even in the presence of a GlnRS anticodon-binding domain (37). While this D-GluRS truncation was large enough to include recognition of known D-stem/loop identity determinants, this report further supports the evolutionary hypothesis that ancestral identity elements were recognized solely by the catalytic domain of GluRSs, as we see predominate here for GluRS2.

The data presented herein also show that the strongest determinants for *H. pylori* GluRS1 are localized within the acceptor stem of $tRNA^{Glu}$. Chimera 1 was the only chimera to show robust aminoacylation activity with GluRS1. However, individual base pair mutations in the acceptor stem of $tRNA^{Gln}$ were insufficient to induce GluRS1 recognition. Thus, $tRNA^{Glu2}$ does not contain a single potent determinant for GluRS1, rather aminoacylation activity is induced by recognition of a set of identity elements apparently distributed throughout the acceptor stem. Thus, like GluRS2, GluRS1 uses ancestral mechanisms to recognize its tRNA substrates and to reject $tRNA^{Gln}$.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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