The Nondiscriminating Aspartyl-tRNA Synthetase from *Helicobacter pylori*: Anticodon-Binding Domain Mutations That Impact tRNA Specificity and Heterologous Toxicity†

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ABSTRACT: Divergent tRNA substrate recognition patterns distinguish the two distinct forms of aspartyl-tRNA synthetase (AspRS) that exist in different bacteria. In some cases, a canonical, discriminating AspRS (D-AspRS) specifically generates Asp-tRNA^{Asp} and usually coexists with asparaginyl-tRNA synthetase (AsnRS). In other bacteria, particularly those that lack AsnRS, AspRS is nondiscriminating (ND-AspRS) and generates both Asp-tRNA^{Asp} and the noncanonical, misacylated Asp-tRNA^{Asn}; this misacylated tRNA is subsequently repaired by the glutamine-dependent Asp-tRNA^{Asn}/Glu-tRNA^{Gln} amidotransferase (Asp/Glu-Adt). The molecular features that distinguish the closely related bacterial D-AspRS and ND-AspRS are not well-understood. Here, we report the first characterization of the ND-AspRS from the human pathogen *Helicobacter pylori* (*H. pylori or Hp*). This enzyme is toxic when heterologously overexpressed in *Escherichia coli*. This toxicity is rescued upon coexpression of the *Hp* Asp/Glu-Adt, indicating that *Hp* Asp/Glu-Adt can utilize *E. coli* Asp-tRNA^{Asn} as a substrate. Finally, mutations in the anticodon-binding domain of *Hp* ND-AspRS reduce this enzyme's ability to misacylate tRNA^{Asn}, in a manner that correlates with the toxicity of the enzyme in *E. coli*.

The aminoacyl-tRNA synthetases (aaRSs)[1] are critical in ribosomal protein translation because each canonical aaRS accurately pairs its cognate amino acid to the correct tRNA isoacceptor(s) (*1*−*3*). Recently, it has become clear that this tenet of one aaRS per amino acid/tRNA isoacceptor pair is hardly set in stone: aaRSs have now been discovered in different organisms that (1) aminoacylate two different sets of tRNA isoacceptors (*4*−*14*), (2) only aminoacylate noncognate tRNAs (*15*−*17*), and (3) naturally aminoacylate tRNAs with essential noncoded amino acids (*18*−*24*). These variations highlight the importance of characterizing unexpected genetic variations in aaRSs and related genes.

Aspartyl-tRNA synthetase (AspRS) is an example of an aaRS that can aminoacylate two different tRNA isoacceptors: tRNA^{Asp} and tRNA^{Asn} (*3*, *5*−*7*, *12*, *14*). In eukaryotes, some bacteria, and some archaea, AspRS is a canonical, discriminating enzyme (D-AspRS) that only generates Asp-tRNA^{Asp} (*14*, *25*). In other bacteria, archaea, and some organelles, however, AspRS is a noncanonical, nondiscriminating enzyme (ND-AspRS) that generates both Asp-tRNA^{Asp} and the misacylated Asp-tRNA^{Asn} (*3*, *5*−*7*, *12*, *14*). This Asp-tRNA^{Asn} is converted to Asn-tRNA^{Asn} by a glutamine-dependent Asp-tRNA^{Asn}/Glu-tRNA^{Gln} amidotransferase (Asp/Glu-Adt) (*9*, *12*, *26*−*28*). ND-AspRS is most commonly found in organisms lacking asparaginyl-tRNA synthetase (AsnRS), and in these cases, the combination of ND-AspRS and Asp/Glu-Adt provides the sole route for Asn-tRNA^{Asn} biosynthesis and, in some cases, for asparagine biosynthesis as well (*7*, *29*). Rarely, a given bacterium will encode for both a D-AspRS and an ND-AspRS (e.g., *Thermus thermophilus (8, 30)* and *Deinococcus radiodurans (7)*); however, in these cases, the ND-AspRS tends to be archaeal rather than bacterial in origin (*8*).

Crystal structures of the two AspRSs from *T. thermophilus* (a bacterial D-AspRS and an archaeal-type ND-AspRS) have been reported (*10*, *31*, *32*). These structures, in conjunction with biochemical analyses, are beginning to paint a picture of how an archaeal-type ND-AspRS can recognize both tRNA^{Asp} and tRNA^{Asn} (*6*, *8*, *30*, *33*). However, little is known about tRNA recognition by bacterial ND-AspRS, as no crystal structure has been reported. To date, only two mutations, H31L and G83K, both in the anticodon-binding domain of the ND-AspRS from *Pseudomonas aeruginosa*, have been analyzed (*34*). Interestingly, each of these single mutations introduced greater specificity for tRNA^{Asp} (over tRNA^{Asn}) into this ND-AspRS only when tested against total tRNA from *Escherichia coli*; assays with *P. aeruginosa* tRNA showed little perturbation in tRNA specificity.

Like *P. aeruginosa*, the *Helicobacter pylori* (*H. pylori* or *Hp*) genome does not encode an AsnRS (*35*); thus, it can be inferred that the *H. pylori* AspRS is nondiscriminating. *H. pylori* is also one of several bacteria that utilize two divergent

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[1] Abbreviations: AaRS, aminoacyl-tRNA synthetase; AspRS, aspartyl-tRNA synthetase; D-AspRS, discriminating AspRS.; D-GluRS, discriminating GluRS; Asp/Glu-Adt, glutamine-dependent Asp-tRNA^{Asn}/Glu-tRNA^{Gln} amidotransferase; GluRS, glutamyl-tRNA synthetase; ND-AspRS, nondiscriminating AspRS; Q, queosine.

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glutamyl-tRNA synthetases (GluRS1 and GluRS2) (*15*−*17*); *Hp* GluRS2 is uniquely specific for $tRNA^{Gln}$ and generates Glu-$tRNA^{Gln}$, which is subsequently converted to Gln-$tRNA^{Gln}$ by the same Asp/Glu-Adt mentioned above. Because of the unexpected tRNA specificity of *Hp* GluRS2, we sought to verify that the *Hp* AspRS is, as predicted, a canonical ND-AspRS. Here, we report the first characterization of this enzyme. *Hp* AspRS is nondiscriminating; however, it preferentially aminoacylates $tRNA^{Asp}$ (its cognate substrate) over $tRNA^{Asn}$ (its noncognate substrate). Consistent with its ability to misacylate $tRNA^{Asn}$, heterologous overexpression of this ND-AspRS is toxic to *E. coli*. Two mutations have been identified which increase the $tRNA^{Asp}$/$tRNA^{Asn}$ specificity ratio of this enzyme, making it more D-AspRS-like. The most effective of these mutations reduces the toxicity of this ND-AspRS in *E. coli*.

EXPERIMENTAL PROCEDURES

Materials. Unless otherwise noted, all reagents were purchased from Sigma or Fisher Scientific. Aspartic acid [2,3-^{3}H] was purchased from Amersham Biosciences. Oligonucleotides were purchased from Invitrogen. Plasmids were purified using plasmid isolation kits from Promega. DNA sequencing was performed at the Johns Hopkins University School of Medicine Synthesis & Sequencing Facility. The entire gene insert was completely sequenced for each plasmid construct.

Cloning, in Vivo Transcription, and Purification of Hp $tRNA^{Asp}$ and $tRNA^{Asn}$. Inserts encoding *Hp* $tRNA^{Asp}$ and $tRNA^{Asn}$ were generated by PCR from self-annealing primer pairs Pt#37F/Pt#37R and Pt#38F/Pt#38R, respectively, and short extension times (see Supporting Information for all oligonucleotide sequences). These primers appended an *EcoRI* and a *BamHI* site, respectively, onto the 5′- and 3′-ends of each gene. Each PCR product was first inserted into pCR 2.1 TOPO (Invitrogen) and then ligated into the *EcoRI* and *BamHI* sites of pSS007 (*16*). The final plasmids were designated pPTC010 ($tRNA^{Asp}$) and pPTC011 ($tRNA^{Asn}$).

Hp $tRNA^{Asp}$ and $tRNA^{Asn}$ were each overtranscribed in *E. coli* (strain MV1184) that had been transformed with either pPTC010 ($tRNA^{Asp}$) or pPTC011 ($tRNA^{Asn}$), as previously described (*16*). Total tRNA was isolated by Nucleobond purification (Clontech), as per the manufacturer's instructions. This in vivo overtranscription approach yields a mixture of *E. coli* tRNAs that is enriched with the *Hp* tRNA of interest. Because many of the post-transcriptional tRNA modification machinery is conserved in different bacteria (*36*), these tRNAs are presumed to be post-transcriptionally modified in a manner similar to wild-type *Hp* tRNAs.

Hp ND-AspRS (see below for cloning and purification) was used in plateau aminoacylation assays to quantify the amount of $tRNA^{Asp}$ and $tRNA^{Asn}$ in each overtranscribed mixture of tRNAs. Each tRNA mixture was denatured at 75 °C for 5 min and then refolded via the addition of 8 mM $MgCl_2$ at 65 °C and slow cooling to room temperature. Each tRNA solution was incubated at 37 °C in 100 mM Hepes buffer (pH 7.5), with 2 mM ATP, 4 mM $MgCl_2$, 10 μM aspartic acid, 50 μCi/mL [2,3-^{3}H] aspartic acid, and 0.2 μM *Hp* AspRS. Reaction aliquots (10 μL) were removed at specific time points and quenched onto filter paper saturated with trichloroacetic acid (TCA), washed three times with a chilled 5% TCA solution, and counted via scintillation to determine the amount of ^{3}H-Asp-$tRNA^{Asp/Asn}$ that had been generated. Quantification was performed in triplicate using conditions previously reported (*16*). Total stock concentrations of $tRNA^{Asp}$ (typically ∼280 μM, 1400 pmol/OD) and $tRNA^{Asn}$ (typically ∼270 μM, 1500 pmol/OD) were back-calculated from these values.

Cloning of Hp AspRS into pQE-80L. The *Hp aspS* gene was PCR-amplified from *Hp* genomic DNA (strain 26695, ATCC) using primers Pt#2 and Pt#3 (see Supporting Information). These primers introduced flanking *BamHI* and *PstI* sites onto the 5′- and 3′-ends of the *aspS* gene, respectively. The gene was inserted into the pCR 2.1 TOPO vector (Invitrogen) and then subcloned into the *BamHI* and *PstI* sites of pQE-80L (Qiagen) in order to append an N-terminal, six-histidine tag onto AspRS (pPTC001) upon overexpression.

Cloning of Hp AspRS into pCDF-1b Vector. The *Hp aspS* gene was PCR-amplified from pPTC001 using primers Pt#45 and Pt#3 (see Supporting Information). These primers introduced flanking *KpnI* and *PstI* sites onto the 5′- and 3′-ends of the *aspS* gene, respectively. The gene was inserted into the pCR 2.1 TOPO vector (Invitrogen) and then subcloned into the *KpnI* and *PstI* sites of pCDF-1b (Novagen) to yield pPTC022.

Construction of L81N, L86M, and L81N/L86M Single and Double Mutants. The two single mutations (L81N and L86M) were introduced into pPTC001 via QuikChange Site-Directed Mutagenesis (Stratagene), according to the manufacturer's instructions. Plasmid pPTC024, encoding for L81N AspRS, was generated using primers Pt#54 and Pt#55. Plasmid pPTC026, encoding L86M AspRS, was generated using primers Pt#62 and Pt#63. (See Supporting Information for primer sequences.) Plasmid pPTC026 was used as the template for construction of the double mutant, using primers Pt#65 and Pt#66, to generate pPTC029. In each case, correct insertion of the desired mutation was confirmed by DNA sequencing of the entire AspRS open reading frame.

Expression and Purification of Hp AspRS. For overexpression of *Hp* AspRS, *E. coli* (DH5α) calcium-competent cells were transformed with pPTC001 and selected on Luria-Broth (LB) agar plates containing 50 μg/mL ampicillin. *Hp* AspRS was overexpressed (for 30 min or less to avoid toxicity effects) and purified by Ni−NTA affinity chromatography as described for *Hp* GluRS2 (*16*). Sodium dodecyl sulfate−polyacrylamide gel electropheresis (SDS−PAGE) confirmed that the protein was purified to homogeneity (>95% pure, data not shown). The final purified protein was stable for months when stored at −20 °C in 50% glycerol, 33 mM phosphate buffer, pH 7.4, 3 mM Tris-Cl, 1.5 mM β-mercaptoethanol (BME), and 0.5 mM phenylmethanesulfonyl fluoride (PMSF). Final protein concentrations were determined by the Bradford Protein Assay (Bio-Rad) in triplicate.

Growth Curves. Toxicity and viability profiles of different *E. coli* cultures were determined according to the following general protocol. A single colony was grown overnight in 5 mL of LB supplemented with the appropriate antibiotic(s). This culture was used to inoculate 200 mL of the same medium to an initial OD_{600} of 0.05, and the culture was incubated at 37°C with agitation. Cell growth was assessed every 30 or 60 min. When cell density reached an OD_{600} of

approximately 0.2, the culture was induced by addition of IPTG to a final concentration of 0.3−1 mM. Growth was monitored for an additional 8 h. Reported data represents the average of experiments conducted at least in triplicate.

To determine whether overexpression of *Hp* AspRS is toxic to *E. coli*, growth curves were determined as described above for *E. coli* (DH5α) transformed with pPTC001. All cultures were supplemented with 50 μg/mL ampicillin. This same protocol was used to evaluate growth of each of the mutant ND-AspRS variants. These experiments were repeated 6−9 times in order to be certain that the trends shown in Figure 5 are statistically significant.

To evaluate culture sustainability upon coexpression of *Hp* AspRS and *Hp* Asp/Glu-Adt, growth curves were determined for *E. coli* (BL21(DE3)) that had been co-transformed with pPTC022 and pSS015 (*Hp gatCAB* in pET-28a, Skouloubris and Hendrickson, unpublished results) in media supplemented with 50 μg/mL ampicillin and 50 μg/mL kanamycin. The viability of this strain was compared to BL21(DE3) containing pPTC022 alone.

Aminoacylation Assays and Kinetic Constant Determination. Aminoacylation assays were performed in 100 mM Hepes (pH 7.5), 2 mM ATP, 4 mM $MgCl_2$, 10 μM aspartic acid, and 100 μCi/mL L-[2,3-^{3}H] aspartic acid. AspRS was added to a final concentration of 0.2 μM. Each tRNA was pre-folded with $MgCl_2$ as described above. Each tRNA was assayed over a range of concentrations (0.25−24 μM). These experiments were always run in parallel (e.g., a single enzyme batch was assayed over a range of tRNA concentrations with both tRNAs at the same time) to minimize errors and ensure that the V_{max} values shown in Figure 4 would be internally comparable. Michaelis−Menten kinetic parameters were determined by least-squares analyses using KaleidaGraph Version 3.6.2 (Synergy Software). Error bars represent the error range of experiments conducted at least in triplicate.

Acid Gel Electrophoresis and Northern Blot Analyses. Total *E. coli* tRNA was pre-folded and aminoacylated for 90 min as described above. Each reaction was quenched by addition of phenol (saturated with citric acid, pH 4.5). The aqueous layer was isolated and extracted with phenol two more times. The mixture of tRNAs and aminoacyl-tRNAs was then precipitated with ethanol. The resultant pellet was dissolved in 10 mM NaOAc (pH 4.5).

Each tRNA sample (320 pmol) was analyzed by acid gel electrophoresis as previously described (*17*, *37*). Gels were typically run at 500 V for 18 h. The separated tRNAs were then blotted onto nitrocellulose membrane and hybridized with a ^{32}P-radiolabeled DNA probe specific for *E. coli* $tRNA^{Asp}$ (Pt#01P) or *Hp* $tRNA^{Asn}$ (Pt#02P). (See Supporting Information for probe sequences.)

RESULTS

Heterologous Expression of Hp AspRS in E. coli Is Toxic but Can Be Partially Rescued by Coexpression with Hp Asp/Glu-Adt. It is well-established that ND-GluRS (*38*, *39*) and GluRS2 (*16*) variants are toxic when heterologously overexpressed in *E. coli* due to misacylation of a single $tRNA^{Gln}$ isoacceptor. This toxicity is likely due to a buildup of Glu-$tRNA^{Gln1}$ causing a dearth of Gln-$tRNA^{Gln}$ as a substrate for protein biosynthesis or catastrophic errors in protein biosynthesis (*16*, *39*−*41*). (*E. coli* lacks the three genes encoding for the bifunctional, bacterial Asp/Glu-Adt (*gatCAB*) and subsequently cannot repair misacylated Glu-$tRNA^{Gln}$ (*42*).) Toxicity could also arise from misincorporation of glutamate into proteins at glutamine codons; however, neither the chloroplast (*43*) nor the *T. thermophilus* (*44*) EF-Tu binds Glu-$tRNA^{Gln}$.

A similar toxicity profile should correlate to ND-AspRS overexpression, if a given ND-AspRS is capable of misacylating *E. coli* $tRNA^{Asn}$ in vivo. In fact, in a recent report, *E. coli* transformants containing a plasmid for expression of the ND-AspRS from *P. aeruginosa* were not isolable, even in the absence of IPTG (*34*). Only once the enzyme was mutated to enhance specific $tRNA^{Asp}$ aminoacylation were these constructs stable in vivo.

To determine whether *H. pylori* AspRS expression is toxic to *E. coli*, the *Hp aspS* gene was PCR-amplified (strain 26695, Hp0617, TIGR annotation (*35*)) and cloned into pQE-80L (Qiagen). This plasmid was chosen because it contains a copy of the *rep4* repressor gene; thus, protein expression is tightly inhibited until IPTG induction. As shown in Figure 1A, IPTG induction of *Hp* AspRS overexpression is toxic, similar to previously reported phenotypes for ND-GluRS (*38*, *39*, *41*) and GluRS2 (*16*).

To verify that the observed AspRS toxicity was due to the misacylation of *E. coli* $tRNA^{Asn}$, *Hp* AspRS was purified by Ni−NTA affinity chromatography and tested in aminoacylation assays with total *E. coli* tRNA as substrates. [As previously described for the purification of the similarly toxic *Hp* GluRS2 (*16*), heterologous overexpression of *Hp* AspRS was limited to 30 min or less to minimize any deleterious consequences that could arise from its toxicity in *E. coli*.] *Hp* AspRS was readily purified by Ni−NTA affinity chromatography to homogeneity (data not shown).

The purified *Hp* AspRS was incubated with total *E. coli* tRNA in the presence of aspartic acid in order to verify whether this enzyme can heterologously aminoacylate *E. coli* $tRNA^{Asp}$ and $tRNA^{Asn}$. After a 90 min incubation and reaction workup (see Experimental Procedures), the aminoacylated and deacylated tRNAs were separated by acid gel electrophoresis (*17*, *37*). In these gels, aminoacylated tRNAs migrate more slowly than their deacylated counterparts because of the addition of the positively charged α-amino group of the appended amino acid; thus, an upward shift in a tRNA band is indicative of aminoacylation. Furthermore, Northern blot analyses using oligonucleotide probes that are specific for different tRNAs can be used to identify which tRNA(s) was aminoacylated. Figure 1B shows the results of these experiments when probes specific for $tRNA^{Asp}$ (top panel) and $tRNA^{Asn}$ (bottom panel) were used. In each gel, lane 1 shows a deacylated tRNA control and lane 2 shows the tRNA upon incubation with *Hp* AspRS and aspartic acid. As expected, *Hp* AspRS readily aminoacylates *E. coli* $tRNA^{Asp}$ (Figure 1B, lane 2, top panel), as indicated by the upward shift observed in lane 2. Consistent with the toxicity of this AspRS when expressed in *E. coli*, *Hp* AspRS also aminoacylates *E. coli* $tRNA^{Asn}$ to generate the misacylated Asp-$tRNA^{Asn}$ (Figure 1B, lane 2, bottom panel). Note that $tRNA^{Asn}$ migrates as two bands in this gel—this pattern has been seen with other post-transcriptionally modified tRNAs (*16*) and is presumably due to hypomodification at one or more tRNA bases. Both bands shift upward in lane 2, indicating that both variants are misacylated by *Hp* AspRS.

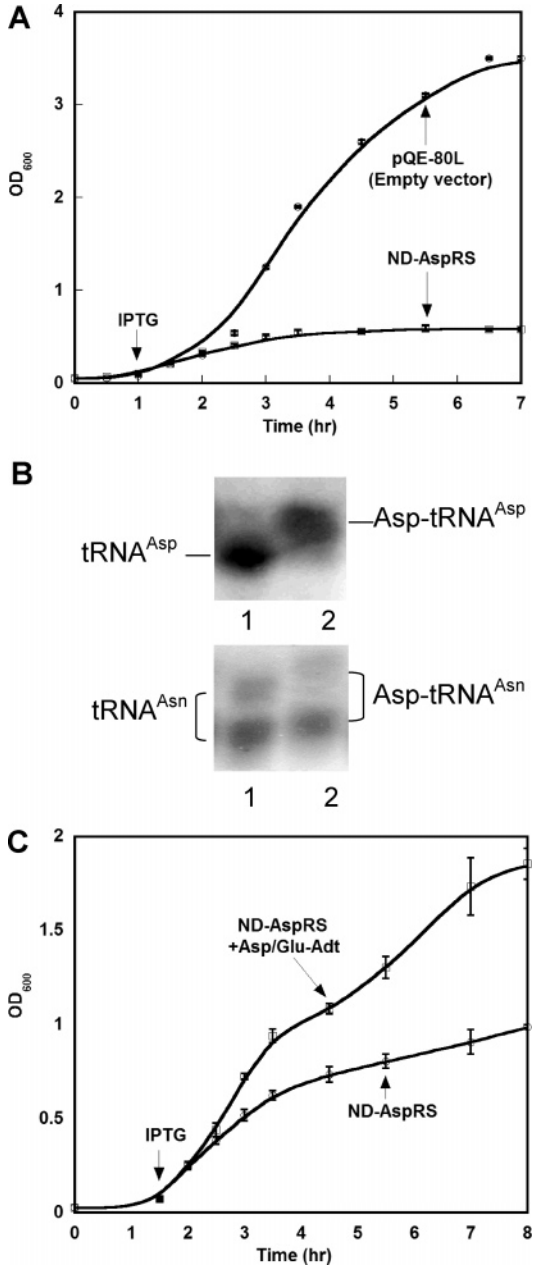

FIGURE 1: Heterologous expression of *H. pylori* ND-AspRS is toxic to *E. coli* and can be rescued by coexpression of the *H. pylori* Asp/Glu-Adt. **(**A) Overexpression of *Hp* ND-AspRS was monitored upon induction with IPTG. This overexpression was toxic to *E. coli*, when compared to a cell culture containing the empty plasmid, pQE-80L, grown under identical conditions. (B) ND-AspRS aminoacylates *E. coli* $tRNA^{Asp}$ (top gel) and misacylates *E. coli* $tRNA^{Asn}$ (bottom gel) (*17*, *37*). In each gel, lane 1 is a deacylated tRNA control and lane 2 represents the tRNA pool treated with *Hp* ND-AspRS. Primers specific for $tRNA^{Asp}$ (top) or $tRNA^{Asn}$ (bottom) were used for Northern blot analyses to specifically visualize each tRNA of interest. The upward shift of the two bands in lane 2 demonstrates ND-AspRS aminoacylates *E. coli* $tRNA^{Asn}$, consistent with the toxicity of this enzyme when overexpressed in *E. coli*. (C) The toxicity of ND-AspRS overexpression can be alleviated by co-overexpression of *H. pylori* Asp/Glu-Adt (GatCAB). See Experimental Procedures for details. All growth curves and acid gels were conducted in triplicate.

Thus, the nature of the low modification is not critical under these experimental conditions. These data confirm that *Hp* AspRS is toxic to *E. coli* due to misacylation of *E. coli* $tRNA^{Asn}$.

It has been hypothesized that the toxicity of the misacylating aminoacyl-tRNA synthetases is due to either accumulation of misacylated tRNAs in conjunction with the lack of a repair mechanism or errors in protein synthesis (*16*, *38*, *39*, *41*). To further probe this scenario, we decided to test whether the toxicity of *Hp* AspRS could be alleviated by coexpression with Asp/Glu-Adt, the amidotransferase that converts both Glu-$tRNA^{Gln}$ and Asp-$tRNA^{Asn}$ into Gln-$tRNA^{Gln}$ and Asn-$tRNA^{Asn}$, respectively. For this experiment to work, not only would the toxicity have to be due to Asp-$tRNA^{Asn}$ accumulation, but *Hp* Asp/Glu-Adt would have to be able to recognize *E. coli* Asp-$tRNA^{Asn}$ as a substrate. The three subunits of Asp/Glu-Adt (*gatCAB*) were combined into a single operon in pSS015, derived from the pET-28a vector (Novagen). To resolve plasmid compatibility issues, the *Hp aspS* gene was transferred from pPTC001 into pCDF-1b (generating pPTC022). Plasmid pCDF-1b is fully compatible with most pBR322-type plasmids, including pET-28a, because it has an origin of replication derived from CloDF13 and spectinomycin/streptomycin antibiotic resistance; this plasmid is IPTG-inducible. A single strain of *E. coli* BL21-(DE3) was co-transformed with pSS015 and pPTC022 and selected on both kanamycin and streptomycin. The viability of this strain, upon IPTG-induction of protein expression, was compared to a strain containing pPTC022 alone. As shown in Figure 1C, coexpression of Asp/Glu-Adt with *Hp* AspRS nearly doubled the achievable cell density. This result supports the hypothesis that *Hp* AspRS is toxic to *E. coli* because of sequestration of $tRNA^{Asn}$ as Asp-$tRNA^{Asn}$. It also demonstrates that *Hp* Asp/Glu-Adt can recognize *E. coli* Asp-$tRNA^{Asn}$ as a substrate in vivo.

The Hp AspRS Is a Nondiscriminating Enzyme. The in vivo results described above offer the first evidence that *Hp* AspRS is a nondiscriminating enzyme that aminoacylates both $tRNA^{Asp}$ and $tRNA^{Asn}$. To confirm this relaxed tRNA specificity using *H. pylori* tRNAs, traditional aminoacylation assays were set up using *Hp* $tRNA^{Asp}$ and $tRNA^{Asn}$ that had been overtranscribed in vivo and purified from *E. coli*. The anticodons of both $tRNA^{Asp}$ and $tRNA^{Asn}$ contain a post-transcriptionally modified queosine (Q) at position 34, and this modified base is an identity element for recognition by *E. coli* AspRS and AsnRS (*36*, *45*, *46*). The Q biosynthetic machinery is conserved throughout life (*36*); thus, the decision to use in vivo-transcribed tRNAs was predicated on the hypothesis that the *E. coli* machinery would introduce this essential modification into the two different *Hp* tRNAs. This transcription method produces a mixture of tRNAs, highly enriched with the *Hp* tRNA of interest, but contaminated with total *E. coli* tRNA.

The results of triplicate aminoacylation assays conducted at a single concentration of $tRNA^{Asp}$ and $tRNA^{Asn}$ (both at 2 μM) are shown in Figure 2. As expected, both tRNAs were substrates for *Hp* AspRS, confirming that this enzyme is indeed an ND-AspRS. *Hp* ND-AspRS is more efficient at aminoacylating its cognate substrate $tRNA^{Asp}$ than $tRNA^{Asn}$. A comparison of the Michaelis−Menten kinetics for each tRNA (Figure 4A, see below) confirmed that *Hp* ND-AspRS is 1.7 times more efficient at aminoacylating $tRNA^{Asp}$ over $tRNA^{Asn}$, under the conditions used in this study. Interestingly, this specificity ratio is nearly identical to the ratio reported for the *P. aeruginosa* ND-AspRS when evaluated with *E. coli* tRNAs (*34*), suggesting that it may be a general

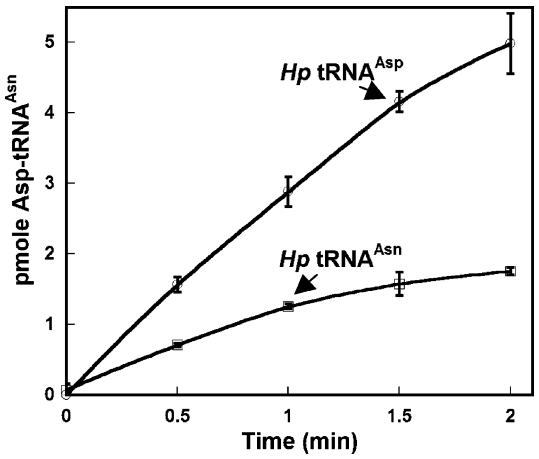

FIGURE 2: *H. pylori* preferentially aminoacylates tRNA^{Asp} over tRNA^{Asn}. When aminoacylation assays are run under identical conditions (2 μM tRNA^{Asp} or 2 μM tRNA^{Asn}, 200 nM ND-AspRS), tRNA^{Asp} (open circles) is aminoacylated faster than tRNA^{Asn} (closed circles). Error bars represent the standard deviation arising from experiments run in triplicate.

trend for bacterial ND-AspRSs to have retained slight specificity for their cognate tRNA^{Asp} isoacceptors.

Mutations in the Anticodon-Binding Domain of Hp ND-AspRS Enhance tRNA^{Asp} Specificity. Bacterial ND-AspRS and D-AspRS share a high level of sequence identity and similarity. Consequently, the molecular features that differentiate these two types of enzymes are not well-understood. Key identity determinants for D-AspRS aminoacylation of tRNA^{Asp} include the three anticodon nucleotides (QUC, base 34 is post-transcriptionally modified to Q) and the discriminator base (G73) (*36*, *45*, *46*). These bases are conserved in tRNA^{Asn}, including Q34, but excluding U36, the anticodon base that defines tRNA^{Asn}. It seems likely that the C/U swap at position 36 would be a critical site for discrimination in D-AspRS but would have to be a site of relaxed recognition for ND-AspRS.

Several crystal structures of AspRS have been reported, including the archaeal-type ND-AspRS from *T. thermophilus* (*10*) and the D-AspRS from *E. coli*, complexed with *E. coli* tRNA^{Asp} (*10*, *46*). In the cocrystal structure, the three bases of the tRNA anticodon are nestled within a cleft of the N-terminal anticodon-binding domain of D-AspRS (Figure 3B) (*46*). We constructed an alignment of this domain using several bacterial D-AspRS and ND-AspRS sequences, a portion of which is shown in Figure 3A. This alignment shows the high level of conservation found in the anticodon-binding domains of both D- and ND-AspRS, with slightly different patterns of conservation between the two types of enzymes. (Unfortunately, the *T. thermophilus* ND-AspRS more closely resembles a mix of an archaeal and eukaryal AspRS, so its sequence homology to the *Hp* ND-AspRS is low and it was omitted from the alignment (*10*).)

Using this alignment and the *E. coli* D-AspRS:tRNA^{Asp} cocrystal, we focused our mutagenesis efforts on two amino acids in *Hp* ND-AspRS: Leu81 and Leu86. These two residues correspond to Asn82 and Met87 in the *E. coli* enzyme (Figure 3C, magenta) and are proximal to the tRNA anticodon bases. Met87 sits on the far edge of the C36 binding pocket and is typically either a Met or a Leu in both D- and ND-AspRS sequences. Asn82 forms a backbone hydrogen bond to N4 in C36, and the γ-carbonyl hydrogen-bonds to the 2′-OH of Q34. This residue is highly conserved as either an Asn or Gln in D-AspRS sequences but is more commonly a small amino acid in ND-AspRS sequences.

We constructed three mutant variants of *Hp* ND-AspRS: L81N, L86M, and the double mutant, L81N/L86M. Each of these enzymes was overexpressed and purified to homogeneity (>95% pure by SDS−PAGE), according to the protocols optimized for wild-type *Hp* ND-AspRS. Kinetic constants were determined separately for tRNA^{Asp} and tRNA^{Asn} with wild-type ND-AspRS and each mutant enzyme; these data are summarized in Table 1 and shown in Figure 4.

Each of the two single mutations enhanced enzyme specificity for tRNA^{Asp} over tRNA^{Asn} (Figure 4A−C). In both cases, this increased specificity for tRNA^{Asp} predominantly results from increases in the K_M for tRNA^{Asn}, with each mutation leaving the K_M for tRNA^{Asp} unperturbed (within error). This effect is most dramatic for the L86M mutant enzyme where its tRNA^{Asp} specificity is doubled as compared to wild-type (from 1.7 to 3.4, Table 1). Without a crystal structure of a bacterial ND-AspRS (ideally cocrystallized with tRNA^{Asn}), it is difficult to understand the impact of this L86M mutation at a molecular level, particularly because this methionine is found in the sequences of many ND-AspRS and is not rigorously conserved in D-AspRS sequences. Further analyses to identify the rate-limiting step in *Hp* ND-AspRS catalysis or to determine the dissociation constant for each tRNA, using both the wild-type enzyme and this mutant variant, may further clarify the role of this residue in tRNA specificity.

In the less dramatic case of the L81N mutant enzyme, the impact of the higher K_M on the k_{cat}/K_M for tRNA^{Asn} is diminished by the fact that this mutation nearly doubles the k_{cat} for tRNA^{Asn} as compared to the wild-type enzyme (from 0.014 to 0.026 s^{-1}, an 86% increase in k_{cat}). Thus, the k_{cat} for tRNA^{Asn} is virtually identical to that of tRNA^{Asp} in the presence of the L81N mutation. (In contrast, wild-type AspRS has a k_{cat} for tRNA^{Asp} that is 60% higher than that of tRNA^{Asn}.) The overall increase in specificity for tRNA^{Asp}, as judged by the ratio of k_{cat}/K_M for the two tRNAs with this L81N mutation, is only about 40% higher than that for the wild-type ND-AspRS. In the cocrystal structure of *E. coli* D-AspRS and tRNA^{Asp}, Asn82 (the residue corresponding to Leu81 in *Hp* ND-AspRS) forms two hydrogen bonds with the tRNA: (1) The γ-carbonyl hydrogen bonds to the 2′OH of Q34, the hypermodified base conserved in the tRNA^{Asp} and tRNA^{Asn} anticodons and (2) the backbone (α) carbonyl H-bonds to the N4 nitrogen of C36. It can be hypothesized that the side chain H-bond helps to align the backbone carbonyl of this residue for ideal recognition of C36 in tRNA^{Asp} over U36 in tRNA^{Asn}; however, the verification of these fine details in molecular recognition will only be confirmed upon the determination of a cocrystal structure of a bacterial ND-AspRS with tRNA^{Asp} or tRNA^{Asn}. What is clear from this mutagenesis analysis is that neither L81 nor L86 is a strong participant in recognition of either tRNA. Instead, these two residues contribute more subtly to the overall tRNA specificity of this ND-AspRS.

Interestingly, when the two mutations were combined, they were not synergistic in enhancing tRNA^{Asp} specificity. Instead, the L81N/L86M AspRS showed a significant deviation form Michaelis−Menten kinetics, arising from substrate inhibition at high concentrations of either tRNA

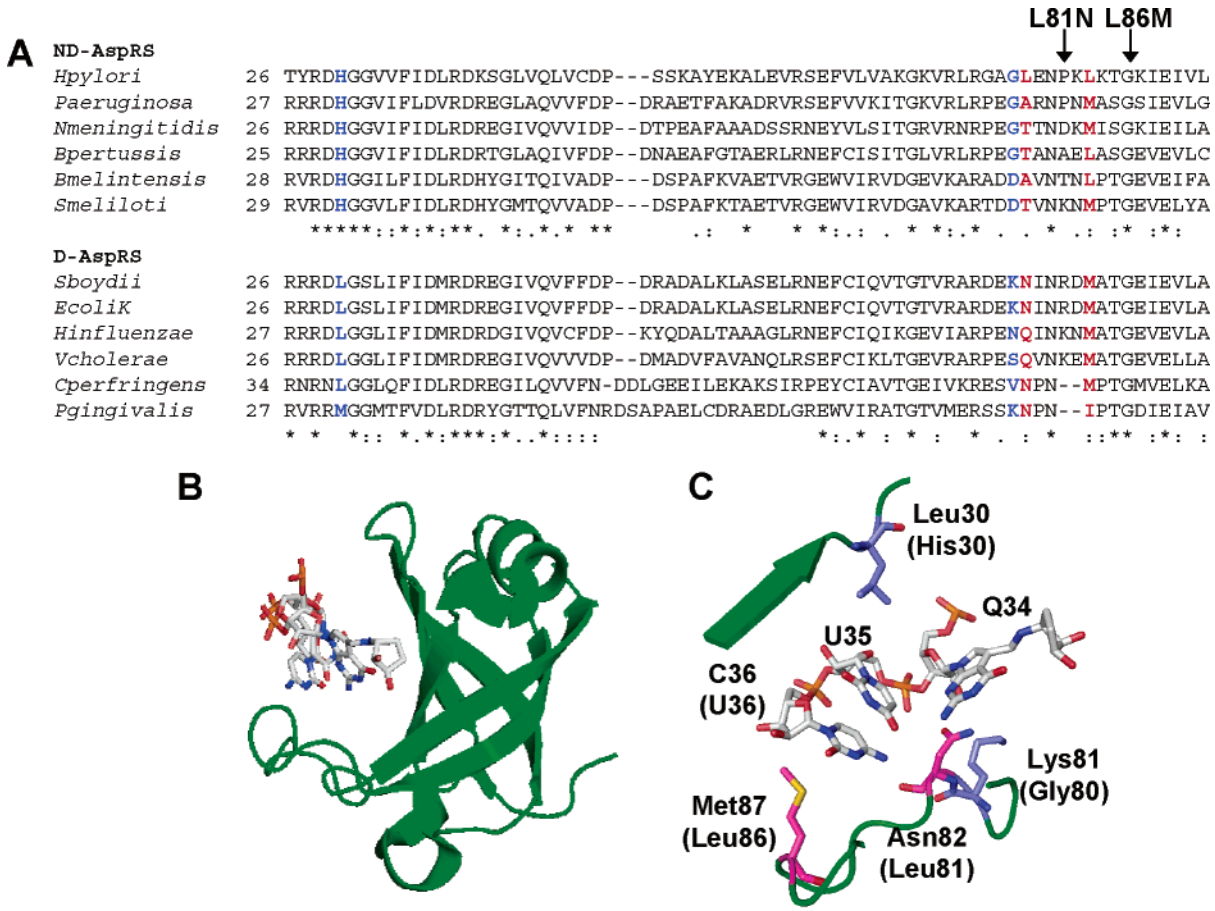

FIGURE 3: ND-AspRS and D-AspRS have similar but different anticodon-binding domains. (A) The anticodon-binding domains of select bacterial aspartyl-tRNA synthetase sequences were aligned (typically residues 1−100, only regions relevant to the present work are shown). Each AspRS sequence was assigned as a D-AspRS or an ND-AspRS based on genome analyses for the presence of AsnRS and Asp/Glu-Adt genes (*gatCAB*). Ambiguous AspRS sequences (e.g., enzymes from an organism that encodes both AsnRS and Glu-Adt) were omitted. The two residues highlighted in red were mutated (L81N and L86M) as part of the present study; those in blue were analyzed by Roy and colleagues (*34*). (B) The anticodon-binding domain of *E. coli* D-AspRS complexed with fully modified *E. coli* $tRNA^{Asp}$, showing the cleft that accommodates the anticodon trinucleotides (*46*). (C) A closeup of this anticodon-binding domain. Residues shown in magenta were mutated as part of this study; those shown in blue were analyzed in a study by Roy and colleagues (*34*). See Results for details.

Table 1: Aminoacylation Kinetics for Wild-Type and Mutant *Hp* ND-AspRS Variants[a]

enzyme	K_M $tRNA^{Asp}$ (μM)	k_{cat} $tRNA^{Asp}$ (s^{-1})	k_{cat}/K_M $tRNA^{Asp}$ (M^{-1} s^{-1})	K_M $tRNA^{Asn}$ (μM)	k_{cat} $tRNA^{Asn}$ (s^{-1})	k_{cat}/K_M $tRNA^{Asn}$ (M^{-1} s^{-1})	specificity ratio[b]
wild-type	0.77 ± 0.14	0.022 ± 0.001	2.9×10^4	0.83 ± 0.44	0.014 ± 0.001	1.7×10^4	1.7
L81N	0.85 ± 0.22	0.028 ± 0.001	3.3×10^4	1.87 ± 0.24	0.026 ± 0.001	1.4×10^4	2.4
L86M	0.87 ± 0.05	0.021 ± 0.001	2.4×10^4	2.23 ± 0.34	0.015 ± 0.001	0.7×10^4	3.4
L81N/L86M[c]	[0.6]	[0.021]	[3.5×10^4]	[1.3]	[0.018]	[1.4×10^4]	[2.5]

[a] Full kinetic analyses were conducted with wild-type ND-AspRS and its mutants. See Experimental Procedures and Figure 4 for details. All experiments were run at least in triplicate. [b] The specificity ratio is the k_{cat}/K_M for $tRNA^{Asp}$ divided by the k_{cat}/K_M for $tRNA^{Asn}$. [c] The L81N/L86M mutation did not follow Michaelis−Menten kinetics. The kinetic values presented here were extracted from tRNA concentrations ≤16 μM (dashed line, Figure 4D). They are shown in brackets to highlight the fact that they only represent an estimation based on low concentrations of tRNA; at tRNA concentrations ≥16 μM, significant inhibition was observed (solid lines, Figure 4D). See text and Figure 4 for further details.

([tRNA] ≥ 16 μM, Figure 4D, solid lines). We extracted crude values for K_M and k_{cat} by fitting the data for concentrations of tRNA between 0 and 4 μM (shown in Table 1 in brackets and derived from the curve fit analyses in Figure 4D shown as dashed lines). Using these values, the L81N/L86M AspRS still favored $tRNA^{Asp}$ over $tRNA^{Asn}$ with a specificity ratio of about 2.5. It is worth noting that this same substrate inhibition may be weakly occurring in wild-type ND-AspRS and the L81N mutant enzyme at higher tRNA concentrations (≥24 μM, Figure 4A,B); in the case of the double mutant, however, the effect was more dramatic, being observed at tRNA concentrations of 16 μM or less.

Increasing $tRNA^{Asp}$ Specificity in an ND-AspRS Diminishes in Vivo Toxicity. As discussed above, overexpression of wild-type *Hp* ND-AspRS in *E. coli* results in a toxic phenotype (see Figure 1A), presumably due to the lack of correctly acylated Asn-$tRNA^{Asn}$ for use in protein biosynthesis (*16*, *39*, *41*). We decided to evaluate whether the perturbations in tRNA specificity of our mutations would have any impact on the toxicity of *Hp* ND-AspRS in *E. coli*. In other words, we asked whether a 3-fold improvement in $tRNA^{Asp}$ aminoacylation over $tRNA^{Asn}$ aminoacylation, as we see with the L86M AspRS, is sufficient to induce a less toxic phenotype.

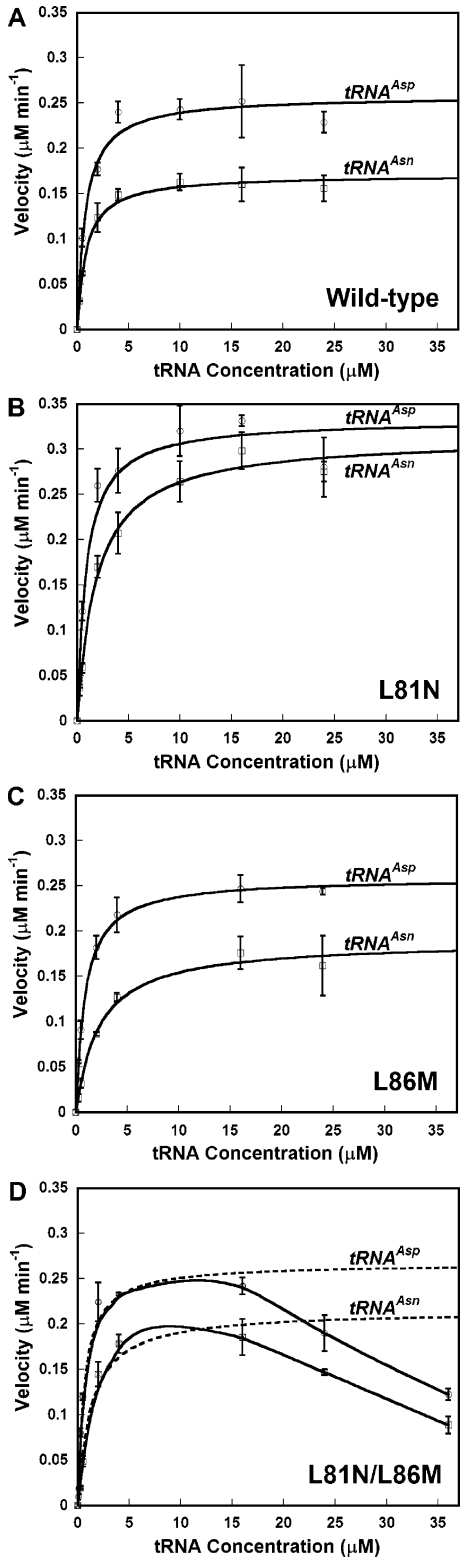

FIGURE 4: Michaelis-Menten kinetics for *Hp* wild-type AspRS and mutants. Plots for rate versus tRNA concentration are shown for each enzyme with $tRNA^{Asp}$ (open circles) and $tRNA^{Asn}$ (open squares). (A) Wild-type AspRS; (B) L81N; (C) L86M; and (D) L81N/L86M. For wild-type and each single mutant, the data were directly fit to the Michaelis−Menten equation using KaleidaGraph (Synergy Software); these fits are shown as solid lines. For the L81N/L86M double mutant (panel D), kinetic parameters were calculated using only the initial velocity values for tRNA concentrations ≤ 16 μM (broken line). At higher tRNA concentrations, significant substrate inhibition was observed (solid line); see Results for detail. Error bars represent the standard deviation of experiments run in triplicate.

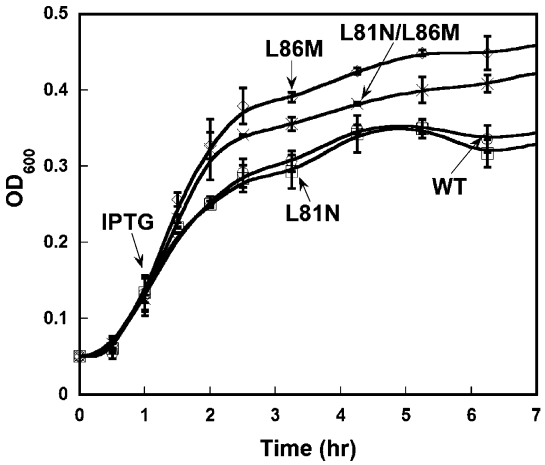

FIGURE 5: A single point mutation that optimizes $tRNA^{Asp}$ specificity is less toxic when expressed in *E. coli*. Wild-type and mutant AspRS were each evaluated for toxicity arising from heterologous expression in *E. coli*, as in Figure 1. Error analysis represents the average of three or more repetitions of each experiment.

We compared growth curves for cultures expressing wild-type *Hp* ND-AspRS and each of our mutant enzymes (Figure 5). Although still toxic, cultures expressing the L86M single mutant or the L81N/L86M double mutant grew to a greater extent than the cultures expressing either the L81N mutant enzyme or wild-type ND-AspRS. These toxicity curves are very consistent with the specificity ratios reported in Table 1, even including the double mutant. (Note: these experiments were performed 6−9 times to verify that the subtle trends shown were significant and reproducible.) These results demonstrate a direct correlation between tRNA substrate specificity and maximal growth density.

DISCUSSION

Here, we have reported the first characterization of the bacterial-type ND-AspRS from *H. pylori*. This enzyme is a canonical, nondiscriminating AspRS that is slightly more efficient at aminoacylating $tRNA^{Asp}$ over $tRNA^{Asn}$. To begin to understand how this relaxed tRNA specificity is achieved, we compared the anticodon-binding domains of several bacterial ND-AspRS and D-AspRS sequences to the crystal structure of the D-AspRS from *E. coli*. On the basis of these analyses, three mutant enzymes were evaluated (L81N, L86M, and L81N/L86M), all of which enhanced the specificity for $tRNA^{Asp}$ aminoacylation over $tRNA^{Asn}$ to varying, subtle degrees.

Figure 3B,C shows the anticodon-binding cleft of *E. coli* D-AspRS. The residues analyzed in the present work are highlighted in magenta, and the side chains mutated by Roy and co-workers in the *P. aeruginosa* ND-AspRS are highlighted in blue (*34*). Each of the mutations introduced at these different conserved and partially conserved positions led to only small perturbations in tRNA specificity. As reported here, introduction of an L86M mutation into *Hp* ND-AspRS doubled the $tRNA^{Asp}/tRNA^{Asn}$ specificity ratio from 1.7 to 3.4. In the *P. aeruginosa* study (*34*), the most dramatic mutation was G83K, which led to a specificity ratio of 6.2 when analyzed with heterologous tRNAs from *E. coli* (a 4-fold increase from the ratio of 1.5 determined with the wild-type enzyme). Interestingly, this G83K mutation had

no effect on the specificity of aminoacylation of *P. aeruginosa* tRNA, the enzyme's normal tRNA pool. This unusual observation supports the likely hypothesis that discriminating aminoacyl-tRNA synthetases evolved in conjunction with the evolution of their specific tRNA isoacceptors (*34*).

We were initially surprised that single mutations in *Hp* ND-AspRS were insufficient to dramatically perturb its tRNA specificity, particularly because a single mutation was sufficient to completely convert the archaeal-type D-AspRS from *Thermococcus kodakaraensis* into an ND-AspRS (*47*). One possible explanation is that the few mutations analyzed to date on bacterial ND-AspRSs are simply not the right ones for the desired effect, a theory that can only be verified by further analysis and experimentation. Another explanation might be that bacterial ND-AspRSs were (and/or are) under greater selective pressure to retain their nondiscriminating activity. Phylogenetic and experimental analyses have suggested that the divergence of AspRS into discriminating and nondiscriminating forms occurred twice, separately in bacteria and in archaea/eukarya (*9*, *25*, *33*, *48*−*50*), an example of convergent evolution that is consistent with the concept of different evolutionary pressures. Thus, information gained by mutagenesis experiments on archaeal AspRSs may not always translate into the bacterial system. Furthermore, some bacteria rely on ND-AspRS and Asp/Glu-Adt not only for Asn-tRNA^{Asn} biosynthesis but as the sole route for asparagine biosynthesis as well (*3*, *29*, *51*). Perhaps this amino acid anabolism requirement was sufficient to more tightly lock ND-AspRS activity into place in bacteria, leading to modern ND-AspRS orthologs that use multiple methods to ensure tRNA^{Asn} recognition and are consequently resistant to perturbations in their tRNA recognition patterns.

In the present work, we have also shown a correlation with the specificity ratio of Asp-tRNA^{Asp} over Asp-tRNA^{Asn} biosynthesis to in vivo toxicity of our heterologously overexpressed *Hp* ND-AspRS variants. At least in some organisms, EF-Tu plays a direct role in preventing Asp-tRNA^{Asn} and Glu-tRNA^{Gln} from entering the ribosome and causing errors in protein biosynthesis (*3*, *29*, *52*), arguing that this toxicity arises from sequestration of tRNA^{Asn} as the abortive Asp-tRNA^{Asn}. However, successful selection experiments in *E. coli* have relied on Asp-tRNA^{Asn}-mediated incorporation of Asp into proteins at Asn codons, arguing that *E. coli* EF-Tu binds this misacylated tRNA at least to an extent to be functionally significant (*11*, *33*). Thus, it remains to be seen whether the toxicity of *Hp* ND-AspRS is due to a dearth of Asn-tRNA^{Asn} or to the induction of catastrophic errors arising from low levels of Asp for Asn mutations at a proteome-wide level.

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SUPPORTING INFORMATION AVAILABLE

Table of primers used in cloning of *Hp* AspRS, tRNA^{Asp}, and tRNA^{Asn} and Northern hybridization experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

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