A tRNA-independent Mechanism for Transamidosome Assembly Promotes Aminoacyl-tRNA Transamidation

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Background: Some microorganisms use indirect tRNA aminoacylation to produce Asn-tRNAAsn; the necessary components are assembled into a tRNAAsn-dependent transamidosome complex.

Results: A new protein, Hp0100, facilitates formation of an alternative, tRNA-independent transamidosome and increases the efficiency of Asp-tRNAAsn transamidation.

Conclusion: Hp0100 is a component of a stable efficient Helicobacter pylori transamidosome.

Significance: The Hp0100-containing transamidosome allows for optimal indirect biosynthesis of Asn-tRNAAsn.

Many bacteria lack genes encoding asparaginyl- and/or glutaminyl-tRNA synthetases, and consequently rely on an indirect pathway for the synthesis of both Asn-tRNAAsn and Gln-tRNAGln. In some bacteria such as Thermus thermophilus, efficient delivery of misacylated tRNA to the downstream amidotransferase (AdT) is ensured by formation of a stable, tRNA-dependent macromolecular complex called the Asn-transamidosome. This complex enables direct delivery of Asp-tRNAAsn from the non-discriminating aspartyl-tRNA synthetase (ND-AspRS) to AdT, where it is converted into Asn-tRNAAsn. Previous characterization of the analogous Helicobacter pylori Asn-transamidosome revealed that it is dynamic and cannot be stably isolated, suggesting the possibility of an alternative mechanism to facilitate assembly of a stable complex. We have identified a novel protein partner called Hp0100 as a component of a stable, tRNA-independent H. pylori Asn-transamidosome; this complex contains a non-discriminating aspartyl-tRNA synthetase, AdT, and Hp0100 but does not require tRNAAsn for assembly. Hp0100 also enhances the capacity of AdT to convert Asp-tRNAAsn into Asn-tRNAAsn by 35-fold. Our results demonstrate that bacteria have adopted multiple divergent methods for transamidosome assembly and function.

Protein synthesis proceeds with high fidelity to ensure optimal survival. This process requires a full set of aminoacyl-tRNAs that are accurately aminoacylated so that the identity of the tRNA anticodon corresponds to the amino acid attached to its acceptor stem. Many microorganisms are missing one or more aminoacyl-tRNA synthetases, the enzymes that typically aminoacylate each tRNA. The obligate human pathogen Helicobacter pylori does not have either glutaminyl- or asparaginyl-tRNA synthetase (1, 2). To compensate for the missing aminoacyl-tRNA synthetases, H. pylori utilizes an indirect pathway to produce Gln-tRNAGln and Asn-tRNAAsn (3–5). The first step in the synthesis of Asn-tRNAAsn relies on a bacterial-type, non-discriminating aspartyl-tRNA synthetase (ND-AspRS) (2), which aminoacylates tRNAAsp to generate Asp-tRNAAsp and misacylates tRNAAsn to produce Asp-tRNAAsn (6). Next, Asp-tRNAAsn is converted to Asn-tRNAAsn by a heterotrimeric, glutamine-dependent amidotransferase called AdT (or GatCAB). A similar pathway exists in H. pylori for Gln-tRNAGln synthesis, where misacylated Glu-tRNAGln is produced by a tRNAGln-specific glutamyl-tRNA synthetase (GluRS2) (1, 7). The same AdT converts Gln-tRNAGln into Gln-tRNAGln.

H. pylori and other organisms that utilize indirect tRNA aminoacylation must rely on mechanisms to prevent their misacylated tRNA intermediates from entering the ribosome, where they would cause errors in translation. Elongation factor Tu (EF-Tu) provides one such mechanism (8–10). In Thermus thermophilus, a thermophilic bacterium, formation of a stable complex called the Asn-transamidosome also promotes accuracy. This macromolecular complex requires tRNAAsn for assembly with AdT and AspRS2 (an archaeal-type ND-AspRS) (11, 12). The Asn-transamidosome ensures the stability of the aminoacyl ester bond of Asp-tRNAAsn, promotes its efficient conversion to Asn-tRNAAsn, and isolates it from EF-Tu and the ribosome until it is converted to Asn-tRNAAsn.

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2 The abbreviations used are: ND-AspRS, non-discriminating aspartyl-tRNA synthetase; AdT, amidotransferase; GluRS, glutamyl-tRNA synthetase; EF-Tu, elongation factor Tu; D-AspRS, discriminating AspRS; DLS, dynamic light scattering; SPR, surface plasmon resonance; MARS, multi-aminoacyl-tRNA synthetase.
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structure of the *T. thermophilus* Asn-transamidosome shows that it contains two tRNAs in scaffold positions and two positioned to act as substrates for both ND-AspRS and AdT (12). Thus, this transamidosome is a tRNA-containing ribonucleoprotein complex. Similarly, the thermophilic archaeon *Methanothermobacter thermautotrophicus* assembles a Gln-transamidosome from ND-GluRS and GatDE. (GatDE is a heterodimeric homolog of AdT that is specific for Gln-tRNA<sub>Gln</sub> biosynthesis (13)). Assembly of this Gln-transamidosome does not require tRNA<sub>Gln</sub>, but it is also less stable and more dynamic than the *T. thermophilus* Asn-transamidosome. Recently, pre-steady-state kinetic experiments and unsuccessful efforts to isolate the *M. thermoautotrophicus* Gln-transamidosome argue that Gln-transamidosome formation in vitro may depend on the presence of H. *pylori*—AdT and purified Gln-transamidosome formation and function. We recently examined the formation of Asn- and/or Gln-transamidosome formation and function. We recently examined the formation of Asn- and Gln-transamidosomes from *H. pylori* components (e.g. ND-AspRS, tRNA<sub>Asn</sub>, and AdT or GluRS2, tRNA<sub>Gln</sub>, and AdT, respectively). Stable assembly of a macromolecular transamidosome complex was not observed in either case. However, quantitative steady-state kinetic analyses argued for transient dynamic assembly of both transamidosome particles (15, 16). These analyses left open the intriguing possibility that an additional mechanism to prevent translational errors remains undiscovered.

Here, we examined the hypothesis that *H. pylori* uses other proteins or enzymes either to facilitate transamidosome assembly or to stabilize and deliver each aminoacyl-tRNA from the misacylating aminoacyl-tRNA synthetase to AdT. A yeast two-hybrid interaction profile of the *H. pylori* proteome (17) was used to identify a protein of unknown function called Hp0100. Hp0100 was selected for further studies based on its weak reported interactions with GatA (a subunit of AdT) and its strong interactions with ND-AspRS. Here, we demonstrate that Hp0100 is a component of a new, stable, tRNA-independent transamidosome enzyme complex consisting of ND-AspRS, AdT, and Hp0100. The addition of Hp0100 also significantly accelerates the AdT-catalyzed rate of Asp-tRNA<sub>Asn</sub> transamidation. Consequently, Hp0100 simplifies the process of indirect tRNA aminocacylation by bringing all enzymatic players into proximity in a distinct Asn-transamidosome.

**EXPERIMENTAL PROCEDURES**

**Overexpression and Purification of *H. pylori* ND-AspRS and AdT**—*H. pylori* ND-AspRS and AdT were overexpressed in *Escherichia coli* and purified as described previously (6, 18). To avoid possible contamination by *E. coli* tRNA, cell lysates were treated with USR<sub>R</sub> RNase A (6.25 μg/ml; Affymetrix Inc.) prior to affinity purification.

In Vivo Production and Purification of *H. pylori* tRNA<sub>Asp</sub> and tRNA<sub>Asn</sub>—*H. pylori* tRNA<sub>Asp</sub> and tRNA<sub>Asn</sub> were produced in vivo in *E. coli* MV1184. They were purified, and their concentrations were determined as described previously (6). Each tRNA was diluted to a stock solution of 150 μM prior to use.

**Cloning, Overexpression, and Purification of Hp0100**—The *hp0100* gene was amplified from *H. pylori* strain 26695 genomic DNA (American Type Culture Collection) and cloned into pQE-80L (Novagen) to encode the full-length protein with an N-terminal His<sub>6</sub> tag (pPTC034). *E. coli* DH5α calcium chloride competent cells were transformed with the pPTC034 plasmid. Cultures were grown, beginning with single colonies, at 37 °C in LB medium. Protein expression was induced with isopropyl β-D-thiogalactopyranoside (1 mM) when the absorbance at 600 nm reached 0.6 to 1.0. After 1 h, cells were harvested by centrifugation at 5000 rpm for 5 min. Hp0100 was first purified using nickel-nitrilotriacetic acid spin columns (Qiagen) following the manufacturer’s instructions. Purification of Hp0100 resulted in co-purification of a contaminant that was the same approximate size as *E. coli* discriminating AspRS (D-AspRS).

To avoid *E. coli* D-AspRS contamination, Hp0100 was purified by ion-exchange chromatography instead of nickel affinity. Harvested cells were homogenized in 3 ml of lysis buffer (20 mM Na<sub>2</sub>PO<sub>4</sub>, 5 mM β-mercaptoethanol, 6.25 μg/ml RNase A, 15 μl/ml saturated PMSF, and 1 mg/ml lysozyme at pH 7.4) and incubated on ice for 30 min. The lysate was then sonicated six times at 38% for 10 s on a Branson Digital Sonifier® with 2-min recovery intervals on ice between each pulse. Cell debris was removed by centrifugation at 14,000 rpm for 1 h at 4 °C. The filtered lysate (1 ml) was loaded onto a UNO S ion-exchange column (12-ml column volume; Bio-Rad). Hp0100 eluted at ~400 mM NaCl. After ion-exchange purification, Hp0100 did not contain detectable levels of contaminating *E. coli* D-AspRS (supplemental Fig. S1A) or tRNA (supplemental Fig. S2). The concentration of Hp0100 was determined by UV-visible spectroscopy (280 nm) using an extinction coefficient of ε = 30620 M<sup>−1</sup> cm<sup>−1</sup> as determined by the ExPASy Proteomics server (19).

**ND-AspRS Aminoacylation Assays**—Aminoacylation assays were performed with tRNA<sub>Asp</sub> and tRNA<sub>Asn</sub> as described previously (6). For titration experiments, each tRNA concentration was 10 μM, and ND-AspRS was used at 0.2 μM. The concentration of Hp0100 was varied from 0.2 to 4 μM as indicated.

Preparation of <sup>32</sup>P-Labeled Asp-tRNA<sub>Asn</sub>—The CCA-adding enzyme was used to incorporate [α-<sup>32</sup>P]AMP into the 3’-end of *H. pylori* tRNA<sub>Asp</sub> and tRNA<sub>Asn</sub> by replacing the unlabeled 3’-AMP with [α-<sup>32</sup>P]AMP (from [α-<sup>32</sup>P]ATP; American Radiolabeled Chemicals) as described previously (4, 20, 21). The plasmid expressing the CCA-adding enzyme was generously provided by Dr. Rebecca Alexander (Wake Forest University).

**P1 Nuclease AdT Transamidation Assay**—Transamidation activity was measured essentially as described previously (4, 20, 21). The assay buffer contained 5 mM <sup>32</sup>P-labeled Asp-tRNA<sub>Asn</sub> (specific activity of ~4.5 Ci/mmol), 50 mM HEPES-OH (pH 7.5), 4 mM ATP, 8 mM MgCl<sub>2</sub>, 25 mM KCl, and 1 mM Gln with 10 mM AdT. Where indicated, Hp0100 was preincubated with AdT for 10 min at 4 °C at final concentrations that ranged from 25 to 500 nM. Aliquots (5 μl) were quenched at each time point, digested with P1 nuclease, and processed as described previ-
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ously (4, 20, 21). Reaction progress was monitored by developing each time point on a 20 × 20 cm PEI-cellulose TLC plate (EMD Millipore) in a freshly prepared 100-mL solution of 10 mM ammonium chloride and 5% glacial acetic acid. For better resolution, the TLC plates were pretreated with water for 15 min and then dried prior to use. Plates were phosphorimaged after ~16 h of screen exposure. Asn-tRNA_Asn production was normalized against Asp-tRNA_Asn to eliminate errors arising from differences in sample spotting. For this reason, Asn-tRNA_Asn production is reported as a percentage relative to Asp-tRNA_Asn.

**Electrophoretic Mobility Shift Assays**—Macromolecular complex formation between AdT, ND-AspRS, and Hp0100 was examined by electrophoretic mobility shift assay. AdT (0.5 μM), ND-AspRS (0.5 μM), Hp0100 (2.5 μM), and tRNA_Asn (5 μM) were preincubated for 30 min at 4°C in the indicated combinations. Complexes were examined on an 8% native polyacrylamide gel (330 × 430 × 1.5 mm^2) in Tris/glycine buffer (25 mM Tris and 200 mM glycine at pH 8.3). After electrophoresis at 20 V for 16 h at 4°C, bands were visualized by Western blotting using anti-His_6 antibody (AnaspSpec) according to the manufacturer’s instructions. Hp0100 and ND-AspRS both contain single N-terminal His_6 tags. However, His_6-Hp0100 was not detectable with anti-His_6 antibody under native gel conditions (supplemental Fig. S3). His_6-Hp0100 was readily visualized by Western blotting under denaturing conditions (data not shown). For AdT, both the GatC and GatB subunits contain His_6 tags.

**Dynamic Light Scattering**—Dynamic light scattering (DLS) analyses of ND-AspRS, AdT, and Hp0100, and various binary and ternary complexes were performed at 25°C in a Zetasizer Nano S instrument (Malvern Instruments Ltd., Worcestershire, United Kingdom). Protein samples were diluted in buffer containing 50 mM NaH_2PO_4 and 300 mM NaCl (refractive index = 1.34) to prepare the required concentrations; 40-μL samples were used for analyses. For particles that are smaller than the wavelength of incident light (λ), the intensity of light scattered (I) is related to the diameter of the particle (d) by the Rayleigh equation (I∝d^4/λ^4). We used the Mie theory within the Nano S software to convert the intensity (I) versus size (d in nanometers) distribution data to volume versus size (d) distribution (supplemental Fig. S4). Mie theory provides a more realistic view of the importance of the observed peak/s.

**Surface Plasmon Resonance**—Surface plasmon resonance (SPR) was used to characterize the binary interactions between Hp0100 and each of its possible binding partners (ND-AspRS, AdT, and tRNA_Asn). Hp0100 was immobilized on a CM5 sensor chip, and increasing concentrations of analyte (either protein or tRNA) were delivered to the chip. Dissociation constants (K_d) for each pair of interactions were determined either by fitting the SPR data directly to a Langmuir model using BIACore evaluation version 4.0.1 or by plotting the equilibrium response units versus analyte concentration and fitting the resulting curve to the following equation: H-A = [A_0]R_max/(K_d + [A_0]) (using KaleidaGraph version 4.0), where H represents the complex between Hp0100 (H) and the analyte (A) and is given in SPR equilibrium response units, [A_0] is the initial concentration of the analyte in solution, and R_max represents the theoretical response units that would be observed upon 100% complex formation.

**RESULTS**

**Published Yeast Two-hybrid Results Suggest Biologically Significant Interactions between AdT, ND-AspRS, and Hp0100, a Protein of Unknown Function**—In contrast to the stable, tRNA-dependent Asn-transamidosome from *T. thermophilus*, an analogous *H. pylori* Asn-transamidosome could not be isolated (15). We investigated the possibility that a protein of unknown function is needed to promote assembly of a more stable complex. By mining published results of a protein-protein interaction map of the *H. pylori* proteome (17), we identified Hp0100 as a possible candidate because it showed yeast two-hybrid interactions with both AdT and ND-AspRS, the two enzymes in the *T. thermophilus* Asn-transamidosome. Hp0100 is a 368-amino acid protein that is conserved throughout the *e*-proteobacteria; many bacterial genomes outside of this clade contain genes encoding the N-terminal half of Hp0100. The primary sequence of Hp0100 is not related to any protein of known function. However, sequence analyses suggest that its N terminus contains an ATP-binding motif that belongs to the adenine nucleotide α-hydrase superfamily (AANH-like superfamily) (22).

*E. coli* D-AspRS Is Purified with His_6-Hp0100—Building on the observation that Hp0100 interacts with both ND-AspRS and AdT by yeast two-hybrid screening, we hypothesized that this protein is a component of a novel bacterial-type transamidosome. We cloned the *hp0100* gene and expressed Hp0100 in *E. coli* with an N-terminal His_6 tag. Nickel affinity chromatography led to the purification of Hp0100 with a single co-purifying contaminant that was approximately the same size as *E. coli* discriminating D-AspRS (supplemental Fig. S1A). Aminoacylation assays with tRNA_Asp confirmed that the mixture of Hp0100 and this protein contaminant produced Asp-tRNA_Asp, supporting our presumption that this contaminant is *E. coli* D-AspRS (supplemental Fig. S1B). These results offered the first in vivo and in vitro evidence for a relevant noncovalent interaction between Hp0100 and AspRS. *E. coli* does not have a full-length ortholog of Hp0100. Hp0100 was subsequently purified by ion-exchange chromatography to >95% purity with complete removal of the contaminating D-AspRS (supplemental Fig. S1A) (4, 20, 21).

Hp0100, AdT, and ND-AspRS Assemble into Binary and Ternary Complexes in the Absence of tRNA_Asn—*H. pylori* ND-AspRS, tRNA_Asn, and AdT do not assemble into a stable *T. thermophilus*-like Asn-transamidosome in gel-shift experiments (Fig. 1C, lane 5) (15). We recently showed that the *H. pylori* Asn-transamidosome is dynamic and unstable (15). We proposed a kinetic mechanism for translational fidelity wherein Asp-tRNA_Asn would not dissociate from ND-AspRS prior to uptake by AdT (15). This mechanism was consistent with the data available at that time, but it was dissatisfyingly complex.

Alternatively, another unknown protein factor such as Hp0100 could induce stable transamidosome assembly, offering additional protection above that provided by a kinetic mechanism. To accurately assess the role of Hp0100 in the absence of tRNA, all enzymes used in experiments herein were
pretreated with RNA. RNA gel evaluation in comparison with tRNA standards confirmed that each enzyme contained <0.1% tRNA contamination (supplemental Fig. S2). Where indicated, tRNAAsn was added. To determine whether or not Hp0100 participates in stable Asn-transamidosome formation, we first sought to recapitulate the observed yeast two-hybrid interactions between Hp0100 and AdT and between Hp0100 and ND-AspRS. Native gel electrophoresis showed upward shifts when Hp0100 was combined with ND-AspRS (Fig. 1A) and with AdT (Fig. 1B), demonstrating that both of these indirect tRNA aminoacylation components separately interact with Hp0100, as was suggested by yeast two-hybrid screening. The extent of complex formation appears to be nearly complete in Hp0100, as was suggested by yeast two-hybrid screening. The recttRNAaminoacylationcomponentsseparatelyinteractwith

Hp0100 alone has a propensity to aggregate (observed upon the addition of equimolar Hp0100, indicating binary complex formation. AdT alone had an observed hydrodynamic diameter of 10.6 ± 0.2 nm, which increased negligibly to 11.0 ± 0.3 nm upon the addition of Hp0100. It is unclear why this shift in particle size was statistically insignificant. The particle diameters determined by DLS represent the diameters of a perfect hard sphere. It is possible that the Hp0100-AdT complex is more tubular and, consequently, less amenable to characterization by DLS or that assembly of this complex could result in compaction such that the observed diameter is similar to AdT alone. (The solution dimer between Hp0100 and AdT was further confirmed by SPR (Fig. 3B; see below).) Finally, the ternary complex of Hp0100, ND-AspRS, and AdT showed a diameter of 13.2 ± 0.2 nm, a significant increase from the diameters of either dimeric complex. These results were insufficient to

FIGURE 1. ND-AspRS and AdT assemble into an Hp0100-dependent transamidosome that does not require tRNAAsn. The formation of different complexes was evaluated by native gel electrophoresis and anti-His6 Western blotting. ND-AspRS, GatC, GatB, and Hp0100 were all modified with His6 tags. Upward shifting bands indicate the assembly of macromolecular complexes. As indicated, ND-AspRS (0.5 μM), AdT (0.5 μM), tRNAAsn (5 μM), and Hp0100 (2.5 μM) were combined prior to sample loading (40-μl samples). Asterisks highlight the band shifts that demonstrate formation of relevant binary and ternary complexes. A, Hp0100 forms a complex with ND-AspRS in the presence (lane 2) and absence (lane 3) of tRNAAsn. B, Hp0100 assembles into a complex with AdT in the absence (lane 3) and presence (lane 4) of tRNAAsn. C, Hp0100 forms a complex with AdT and ND-AspRS that is independent of tRNAAsn (lane 4).

FIGURE 2. Hp0100, AdT, and ND-AspRS assemble into an Asn-transamidosome in solution. DLS was used to quantify changes in particle size with different binary and ternary combinations of Hp0100, AdT, and ND-AspRS as indicated. Results are reported only from spectra that showed a single predominant particle. Error bars represent standard (Std.) deviation from triplicate measurements. Molecular weights (Mol. Wt.) are given for each monomer enzyme. The molecular weight for AdT is based on one copy of the heterotrimeric GatCAB enzyme. The molecular weights calculated for each complex are also based on 1:1 stoichiometries. Exact stoichiometries are given when evident. ND, not determinable from these DLS results. All spectra are provided in supplemental Fig. S4.
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Determine complex stoichiometry, but they do confirm that Hp0100 promotes the formation of a stable transamidosome-like complex in solution.

Hp0100 Has Low Micromolar Affinities for Both ND-AspRS and AdT—SPR was used to quantitatively assess binary interactions between Hp0100 and ND-AspRS, between Hp0100 and AdT, and between Hp0100 and tRNA^Asn (Fig. 3). Both AdT and ND-AspRS separately interacted with Hp0100 with low micromolar \(K_a\) values (1.6 ± 0.3 and 1.3 ± 0.2 \(\mu\)M, respectively) (Fig. 3). These dissociation constants are indistinguishable within error, with consistent assembly of a ternary complex.

Hp0100 Does Not Affect ND-AspRS Activity—Next, we evaluated the impact of Hp0100 on ND-AspRS activity in the absence and presence of AdT. ND-AspRS aminoacylation assays were performed in the presence of increasing concentrations of Hp0100 (0–4 \(\mu\)M) without AdT; all substrates were at saturating concentrations, so the observed rates approximate \(k_{cat}\). Initial rates of aminoacylation were examined using both tRNA^Asp and tRNA^Asn to determine whether any impact could be observed and, if so, at what Hp0100 concentration this effect would reach saturation. Our results show that the addition of Hp0100 alone did not affect the ND-AspRS-catalyzed initial rates of aminoacylation of either tRNA even at concentrations of Hp0100 above the \(K_a\) (supplemental Fig. S5).

To evaluate the impact of the ternary complex on ND-AspRS activity, Hp0100 and AdT were added to ND-AspRS at saturating concentrations (2 and 10 \(\mu\)M, respectively), and ND-AspRS activity was measured (supplemental Fig. S6). Under these conditions, the addition of AdT and Hp0100 did not increase the rate of tRNA^Asn aminoacylation. In fact, under these conditions, the addition of AdT alone or with Hp0100 caused slight drops in this rate, presumably because the excess AdT competed for tRNA^Asn.

Hp0100 Increases the Rate of AdT-catalyzed Transamidation of Asp-tRNA^Asn—We also quantitatively examined the impact of Hp0100 on the initial rate of transamidation of Asp-tRNA^Asn by AdT (Fig. 4). Results from representative transamidation assays (Fig. 4, A and B) show that Hp0100 accelerated the rate of conversion of Asp-tRNA^Asn to Asn-tRNA^Asn. This rate enhancement reached saturation at ~400 nM Hp0100 (Fig. 4C), with an ~35-fold maximal increase in the rate of AdT catalysis. Because Hp0100 and AdT formed a stable complex at low micromolar concentrations (see Fig. 4C), one can hypothesize that these titration data reflect the uptake of Asp-tRNA^Asn into the Hp0100-AdT complex. Thus, these data allowed us to approximate a \(K_d\) of ~100 nM for Asp-tRNA^Asn and the Hp0100-AdT complex. This value likely represents an upper limit because competition for Asp-tRNA^Asn from free Hp0100 cannot be excluded.

DISCUSSION

The thermophilic bacterium *T. thermophilus* utilizes a tRNA^Asn-dependent Asn-transamidosome to sequester Asp-tRNA^Asn and deliver it directly to AdT, where it is converted to Asn-tRNA^Asn. This transamidosome assembles from an archaeal-type ND-AspRS, tRNA^Asn, and AdT; two of the four tRNAs bound to this complex are structural, making this complex a tRNA-containing ribonucleoprotein complex (12). Here, we have demonstrated that the *H. pylori* Asn-transamidosome is not a tRNA-containing ribonucleoprotein complex because the catalytically competent complex assembles in the absence of tRNA^Asn, relegating this tRNA to the role of substrate only.

In looking at the *H. pylori* Asn-transamidosome, it is important to consider the differences between the archaeal- and bacterial-type ND-AspRS orthologs. The *T. thermophilus* Asn-transamidosome contains an archaeal-type ND-AspRS; *H. pylori* has a bacterial-type ND-AspRS with an extra insertion domain positioned where it could interfere with Asn-transamidosome assembly, at least based on the structure of the *T. thermophilus* transamidosome (12, 15). Consistently, our previous characterization of the putative *H. pylori* Asn-transamidosome (prior to our discovery of Hp0100) revealed a dynamic complex that could not be stably isolated (15). It is intriguing to consider that this unstable association between ND-AspRS and AdT may be a result of the bacterial ND-AspRS insertion domain causing steric interference.
Because *H. pylori* ND-AspRS, AdT, and tRNA<sub>Asn</sub> did not assemble into a stable Asn-transamidosome, we originally proposed a kinetic model for sequestration of Asp-tRNA<sub>Asn</sub> by ND-AspRS until delivery to AdT via transient association (15). This model fit the data at hand and likely contributes to translational accuracy to some extent. However, our discovery of Hp0100 enabled revision and simplification of this model (Fig. 5). By adding Hp0100 to a tRNA-independent Asn-transamidosome, it is clear that this complex exists in two forms. The first, which we call the “apo-transamidosome,” contains ND-AspRS, Hp0100, and AdT. The second, “holo-transamidosome,” arises upon tRNA<sub>Asn</sub> binding; AdT activity is enhanced within this complex. The formation of these complexes minimizes the need for a kinetic Asp-tRNA<sub>Asn</sub> retention mechanism. Apo-transamidosome formation does not require tRNA<sub>Asn</sub>, further distinguishing this complex from the *T. thermophilus* Asn-transamidosome.

Other tRNA aminoacylation systems offer precedence for the use of a bridging protein in complex assembly. For example, in yeast, Arc1p enables the assembly of a complex between Arc1p, GluRS, and methionyl-tRNA synthetase (23, 24), and in *Metazoa*, at least nine aminoacyl-tRNA synthetases are assembled into a multiaminoacyl-tRNA synthetase (MARS) complex with three other proteins that are essential for MARS assembly (25, 26). To date, we have no evidence for formation of an Hp0100-driven MARS-like complex. GluRS2 is the most likely candidate for assembly into this kind of macromolecular complex because it produces Glu-tRNA<sub>Gln</sub>, which is subsequently converted to Gln-tRNA<sub>Gln</sub> by AdT with a 35-fold acceleration in k<sub>cat</sub>. Step 5, Asn-tRNA<sub>Asn</sub> is released from the complex, and the apo-transamidosome is regenerated.

Our discovery of the Hp0100-dependent Asn-transamidosome does not explain how *H. pylori* manages simultaneous faithful transamidation of both Asp-tRNA<sub>Asn</sub> and Glu-tRNA<sub>Gln</sub>. The solution to this challenge is likely to be one of two possibilities. Either AdT is expressed at sufficiently high levels to enable Asn-transamidosome formation with excess unbound AdT remaining available for Glu-tRNA<sub>Gln</sub> transamidation, or other as yet unidentified protein partners bring GluRS2 into this Asn-transamidosome, making it a MARS-like complex that contains ND-AspRS, GluRS2, and AdT. Echoes of this putative MARS-like complex can be found within the published yeast two-hy-

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3 G. N. Silva, S. Fatma, and T. L. Hendrickson, unpublished data.
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brid protein-protein interact map for H. pylori (15), but experimental evidence to distinguish between these two possibilities has remained elusive.

Because full-length Hp0100 is unique to the ε-proteobacteria, its role in transamidosome function and assembly is also likely to be limited to this bacterial clade. This scenario presents two intriguing possibilities for future consideration. First, one can imagine targeting assembly of the apo-transamidosome for the development of clade-specific antibiotics. Second, given that the ε-proteobacteria and T. thermophilus use different mechanisms for Asn-transamidosome assembly and function, it seems likely that other mechanisms for this critical process remain undiscovered. This mechanistic divergence suggests the exciting possibility that different branches of the tree of life elected different unique strategies for optimal indirect tRNA aminocoylation, sequestration of misacylated tRNAs, and translational accuracy.

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SUPPLEMENTARY MATERIAL

A tRNA-Independent Mechanism for Transamidosome Assembly that Promotes Aminoacyl-tRNA Transamidation

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FIGURE S1. Purification of His$_6$-Hp0100 from *E. coli* resulted in the co-purification of *E. coli* D-AspRS. A. SDS-PAGE gel showing D-AspRS (~66 kD) (lane 2, top band), which was co-purified with Hp0100 by Ni-affinity purification. The darker band is Hp0100 (~43 kD). Hp0100 was purified to homogeneity by ion-exchange chromatography (lane 3) as described in the accompanying manuscript; this alternate method eliminates co-purification of *E. coli* D-AspRS. Numerical values on the left indicate molecular weight markers in kD. B. Aminoacylation assays demonstrated that the 66 kD co-purified impurity aminoacylates tRNA$^{Asp}$, confirming its identity as *E. coli* D-AspRS (H. pylori ND-AspRS and tRNA$^{Asp}$ (●, 10 nM); Hp0100, co-purified *E. coli* D-AspRS, and tRNA$^{Asp}$ (▲); Hp0100 and co-purified *E. coli* D-AspRS (●, no tRNA$^{Asp}$ control)).
FIGURE S2. Assessment of tRNA contamination in protein preparations. An 8% RNA gel (8% Acrylamide:Bis 29:1, 7 M Urea) was run in Tris-borate buffer (90 mM Tris base, 90 mM boric acid, 12 mM EDTA, pH 8.3) at 25 °C to verify that the different protein samples are not significantly contaminated with *E. coli* tRNAs after RNase A treatment and purification. Lanes 1-3 were loaded with 5000 pmols of AdT, Hp0100 and ND-AspRS, respectively. Lanes 5-9 were loaded with increasing amounts of *H. pylori* tRNA^Asn^ (5, 10, 25, 50, 100 pmols respectively). These results provide an upper limit for tRNA^Asn^ contamination of 0.1% for any given protein sample.
FIGURE S3. Complete native gels illustrating ND-AspRS and AdT assemble into an Hp0100-dependent transamidosome that does not require tRNA^{Asn}. The formation of different complexes was evaluated by native gel electrophoresis and anti-His_{6} Western blots (ND-AspRS, GatC, GatB and Hp0100 are all modified with His_{6} tags). Excess His_{6}-Hp0100 did not respond to anti-His_{6} antibodies under native conditions (for example, see lanes 2 and 3, gel 1, wherein unbound His_{6}-Hp0100 was not detected.) As noted for each individual lane, samples contained ND-AspRS (0.5 μM), AdT (0.5 μM), tRNA^{Asn} (5 μM) and Hp0100 (2.5 μM); final volumes were adjusted to 40 μL. Under these conditions, ND-AspRS and AdT are predominantly incorporated into complexes with Hp0100. A. Hp0100 forms a complex with ND-AspRS in the absence (lane 2) and in the presence (lane 3) of tRNA^{Asn}. B. Hp0100 assembles into a complex with AdT in the absence (lane 3) and presence (lane 4) of tRNA^{Asn}. C. Hp0100 forms a complex with AdT and ND-AspRS that is independent of tRNA^{Asn} (lane 4).

The contaminating band marked with an asterisk in panels B and C is due to excess GatB. Although this GatB band is diffuse, it does appear to shift upward in the presence of Hp0100 and ND-AspRS, suggesting complex formation. The possibility that GatB is the site of Hp0100 interaction with AdT is currently under investigation.
Figure S4. DLS analysis of ND-AspRS, AdT, Hp0100 and various binary and ternary complexes. DLS traces showing particle size (in diameter nm) of individual, binary and ternary complexes plotted against the % volume. Black, Blue and red color traces in each plot represent replicate DLS analyses of each individual protein or complex. For binary and ternary complexes, equimolecular mixtures of each protein were incubated in ice for 15 min prior to analysis. A. AdT (20 μM). B. AdT+Hp0100 (20 μM each). C. ND-AspRS (60 μM). D. ND-AspRS+Hp0100 (30 μM each). E. Transamidosome (AdT+ND-AspRS+Hp0100) (20 μM each). F. AdT+ND-AspRS (20 μM each). G. Hp0100 (30 μM). Our results highlights that ND-AspRS and AdT do not assemble in to a stable complex (F). Hp0100 alone has a propensity to aggregate and we were unable to obtain reproducible results.
FIGURE S5. Surface plasmon resonance (SPR) was used to quantitatively assess the binary interactions between Hp0100 and tRNA\textsuperscript{Asn}. A. Hp0100 was immobilized on a CM5 sensor chip and increasing concentrations of tRNA (0-10 μM) were delivered to the chip. Interactions were observed between Hp0100 and tRNA\textsuperscript{Asn} but they did not approach saturation at concentrations up to 10 μM. B. The equilibrium response units (RU) were plotted against analyte concentration. Our results demonstrate that the dissociation constant (\(K_d\)) between Hp0100 and tRNA\textsuperscript{Asn} > 10 μM.
FIGURE S6. Titration curves evaluating the effects of increasing concentrations of Hp0100 on ND-AspRS relative to ND-AspRS aminoacylation rate. ND-AspRS-catalyzed aminoacylation of tRNA^{Asp} and tRNA^{Asn} was measured in the presence of increasing concentrations of Hp0100. Initial rates were normalized relative to the initial rate of ND-AspRS (no Hp0100). [ND-AspRS] = 0.2 μM; [tRNA^{Asp/Asn}] = 10 μM; [Hp0100] = 0 – 4.0 μM. Note: By comparing this figure to Figure 3B, it is clear that Hp0100 improves AdT catalysis and not ND-AspRS activity.
FIGURE S7. Impact of ternary complex on ND-AspRS activity. Aminoacylation assays were performed for tRNA^{Asn} (10 μM) to evaluate the impact of the ternary complex (AdT, Hp0100 and ND-AspRS) on the initial aminoacylation rate of ND-AspRS. Hp0100 and AdT were added to ND-AspRS (0.2 μM) at saturating concentrations (2 and 10 μM, respectively). Error bars represent the standard error of the mean from triplicate measurements.
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