Context-dependent anticodon recognition by class I lysyl-tRNA synthetases

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Lysyl-tRNA synthesis is catalyzed by two unrelated families of aminoacyl-tRNA synthetases. In most bacteria and all eukarya, the known lysyl-tRNA synthetases (LysRSs) are subclass IIb-type aminoacyl-tRNA synthetases, whereas many archaea and a scattering of bacteria contain an unrelated class I-type LysRS. Examination of the recognition of partially modified $tRNA^{Lys}$ anticodon variants by a bacterial (from *Borrelia burgdorferi*) and an archaeal (from *Methanococcus maripaludis*) class I lysyl-tRNA synthetase revealed differences in the pattern of anticodon recognition between the two enzymes. U35 and U36 were both important for recognition by the *B. burgdorferi* enzyme, whereas only U36 played a role in recognition by *M. maripaludis* LysRS. Examination of the phylogenetic distribution of class I LysRSs suggested a correlation between recognition of U35 and U36 and the presence of asparaginyl-tRNA synthetase (AsnRS), which also recognizes U35 and U36 in the anticodon of $tRNA^{Asn}$. However, the class II LysRS of *Helicobacter pylori*, an organism that lacks AsnRS, also recognizes both U35 and U36, indicating that the presence of AsnRS has solely influenced the phylogenetic distribution of class I LysRSs. These data suggest that competition between unrelated aminoacyl-tRNA synthetases for overlapping anticodon sequences is a determinant of the phylogenetic distribution of extant synthetase families. Such patterns of competition also provide a basis for the two separate horizontal gene transfer events hypothesized in the evolution of the class I lysyl-tRNA synthetases.

The aminoacyl-tRNA synthetases (AARS) are an ancient family of enzymes whose function is to match amino acids with their corresponding tRNAs, leading to aminoacyl-tRNA synthesis (1). The main cellular function of aminoacyl-tRNAs is to serve as substrates for messenger RNA-templated ribosomal protein synthesis. In accord with the essential function of aminoacyl-tRNA synthetases, they are a well conserved family of enzymes found in all organisms. However, whereas protein synthesis universally requires tRNAs aminoacylated with all 20 canonical amino acids, these are not always synthesized by a full complement of 20 canonical AARSs (2). The most widespread exceptions are Asn-$tRNA^{Asn}$ and Gln-$tRNA^{Gln}$, which in most archaea, many bacteria, and some organelles are synthesized by tRNA-dependant transamidation pathways rather than by direct aminoacylation using asparaginyl-tRNA synthetase (AsnRS) or glutaminyl-tRNA synthetase (GlnRS), respectively (3–5). Similar indirect aminoacylation pathways also account for the synthesis of formylmethionyl-tRNA (6, 7) and selenocysteinyl-tRNA (8, 9). The exclusive use of these nonaminoacyl-tRNA synthetase pathways in a wide range of organisms was supported by the analysis of numerous complete genome sequences (10, 11). In addition to confirming the predicted absence of genes encoding AsnRS and GlnRS in a number of organisms, whole genome sequence analyses also failed to identify genes encoding other AARSs in several cases. Most notably, the genomes of two thermophilic archaea [*Methanococcus jannaschii* (12) and *Methanobacterium thermoautotrophicum* (13)] did not contain recognizable genes encoding canonical cysteinyl-(CysRS) or lysyl-tRNA synthetases (LysRS). Subsequent experimentation revealed that CysRS is indeed absent, with Cys-tRNA instead being synthesized by a dual specificity prolyl-tRNA synthetase (14). The lack of a recognizable LysRS was found to result from the existence of a novel class I-type enzyme in certain archaea which is unrelated to the class II-type enzyme previously characterized in bacteria and eukaryotes (15).

The aminoacyl-tRNA synthetases can be divided into two classes (I and II) of ten members each based on the presence of mutually exclusive amino acid sequence motifs (16, 17). This division reflects structurally distinct topologies within the active site, class I AARSs containing a Rossmann fold and class II a unique antiparallel β fold. In addition, it has been observed that class I enzymes bind the acceptor helix of tRNA on the minor groove side, whereas class II enzymes bind the major groove side. An aminoacyl-tRNA synthetase of particular substrate specificity will always belong to the same class regardless of its biological origin, reflecting the ancient evolution of this enzyme family. The first known exception to this rule was found among the lysyl-tRNA synthetases with the discovery of a class I enzyme in certain archaea (15), all previously characterized members of this family belonging to class II. Subsequent work originating from analysis of whole genome sequences showed that the class I-type LysRS is found in the majority of archaea and a scattering of bacteria, to the exclusion of the more common class II-type protein (18, 19). To date, all known eukaryotic LysRSs (both cytoplasmic and organellar) are of the class II type. Despite their lack of sequence similarity, both class I and class II LysRSs are able to recognize the same amino acid and highly similar tRNA substrates, providing an example of functional convergence by divergent enzymes (19) and supporting the hypothesis that $tRNA^{Lys}$ itself predates at least one of the two extant forms of LysRS (19, 20).

Examination of the molecular phylogeny of the class I-type LysRSs indicates that the archaeal and bacterial examples are intermixed, suggesting that horizontal gene transfer has contributed to their current distribution (19), as is the case for the majority of the AARS families (21). The existence of two unrelated forms of LysRS, which recognize similar elements in $tRNA^{Lys}$, provides an ideal system with which to investigate this evolutionary dynamic in the AARSs. Previous studies identified differences in acceptor stem recognition between class I and class II LysRS as a possible partial determinant of the displacement of class II by class I LysRS in bacteria (19). More detailed comparisons of anticodon recognition between phylogenetically

Abbreviations: AARS, aminoacyl-tRNA synthetase; AsnRS, asparginyl-tRNA synthetase; LysRS, lysyl-tRNA synthetase; $tRNA^{Lys}$, transfer RNA specific for lysine; AspRS, aspartyl-tRNA synthetase.

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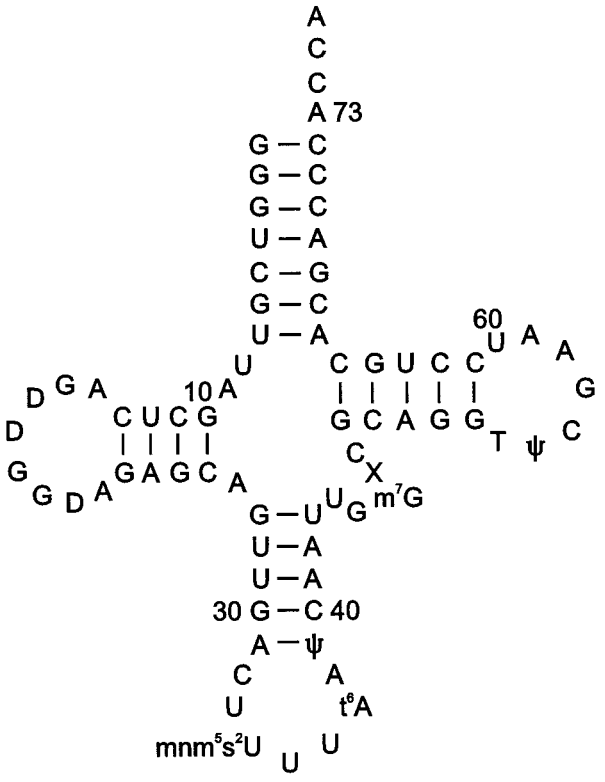

Fig. 1. Secondary structure of wild-type *E. coli* $tRNA^{Lys}$. D, dihydrouridine; mnm^5s^2U, 5-methylaminomethyl-2-thiouridine; t^6A, N^6-threonylcarbamoyladenosine; ψ, pseudouridine; m^7G, 7-methylguanosine; X, putative 3-(3-amino-3-carboxypropyl) uridine modification. The undermodified variants used in this study contain U34 in place of mnm^5s^2U34 and A37 in place of t^6A37.

distant class I LysRS and class II LysRS enzymes now provide evidence that competition between LysRS and other AARSs for overlapping anticodon sequences may also have contributed to the distribution of extant class I LysRSs.

Materials and Methods

General. Expand High Fidelity PCR system was from Roche, pBAD-TOPO cloning and expression kit was from Invitrogen, Ni-NTA matrix was from Qiagen (Chatsworth, CA), and L(+) arabinose was from Sigma. Mature *Escherichia coli* $tRNA^{Lys}$ was purchased from Sigma. *In vivo* overproduced *E. coli* $tRNA^{Lys}$ variants, in which the anticodon loop nucleotides 34 and 37 are unmodified (Fig. 1), were prepared as previously described (22). *B. burgdorferi* LysRS and *M. maripaludis* LysRS were overproduced and purified as previously described (19). Aminoacylation assays were performed as previously described (19) with the concentration of the substrate under investigation varied over the range 0.2–5 times K_M.

Cloning of the *H. pylori lysS* Gene. The *H. pylori* lysyl-tRNA synthetase gene was PCR-amplified by using 100 ng of a genomic library as a template and 100 pmol of the following sense (5′-TGAAAGGAGTTAATTAAATGTTTTCTAACCAATACATCCAACAACGC-3′) and antisense (5′-TTCTTCACCCTCAACATTAAAATCG-3′) primers, and subcloned into the pBAD-TOPO (Invitrogen) cloning and expression vector. Primers were designed to avoid fusion of the N-terminal leader but allowed C-terminal His_6-tagging of the *lysS* gene.

Overexpression of *H. pylori* LysRS. 750 ml of LB medium supplemented with 100 μg/ml of ampicillin were inoculated with 20 ml of an overnight culture of the overproducing strain, and cells were grown at 37°C. Overexpression of LysRS was induced by addition of 0.02% (wt/vol) of L(+) arabinose when $A_{600\ nm}$ reached 0.5. After 6 h of incubation the culture was harvested and yielded 2 g of cells.

Purification of *H. pylori* LysRS. All steps were conducted at 4°C and, unless indicated, all buffers contained 500 mM NaCl, 1 mM benzamidine, 10% (vol/vol) of glycerol, and 5 mM of 2-mercaptoethanol. The cells were suspended in 5 ml of 50 mM Tris·HCl (pH 8.0), containing 0.1% (vol/vol) Triton X-100, 10 μg/ml of lysozyme, and submitted to 10 cycles of 30 s sonication each at 60 V. The supernatant obtained after 2 h centrifugation at 45,000 rpm was loaded on a 2 ml Ni-NTA-agarose column equilibrated with 50 mM Tris·HCl (pH 8.0). After extensive washing of the column with 50 mM Tris·HCl (pH 8.0) containing 30 mM of imidazole, elution of the LysRS was performed by using 750 mM of imidazole in 50 mM Tris·HCl (pH 8.0). Presence of LysRS was ascertained by using SDS/PAGE, and the corresponding fractions pooled and dialyzed against 100 mM Hepes-Na (pH 7.2) containing 50% glycerol. LysRS (7.3 mg) was obtained with a purity of more than 95% according to SDS/PAGE analysis.

Genomic and Phylogenetic Analyses. All predicted amino acid sequences were obtained from GenBank (http://www.ncbi.nlm.nih.gov/GenBank) except as follows. The class I LysRS sequences from *Caulobacter crescentus* and *Treponema denticola* were obtained from preliminary sequence data from The Institute for Genomic Research (http://www.tigr.org), for *Streptomyces coelicolor* from the Sanger Centre (http://www.sanger.ac.uk/Projects/S_coelicolor), and for *Rhodobacter capsulatus* from the What Is There database (http://wit.mcs.anl.gov/WIT2). The class I LysRS sequence from *Methanosarcina mazei* was kindly provided by the Göttingen Genomics Laboratory (Institute of Microbiology and Genetics, University of Göttingen, Germany). Sequences were aligned by using the program CLUSTAL X (23). Phylogenies were generated by using the maximum likelihood method as implemented in the program TREE-PUZZLE 4.0.2 (24).

Results

Anticodon Recognition by Class I Lysyl-tRNA Synthetases. Comparison of tRNA recognition by class I and class II LysRSs was previously investigated by using mutant transcripts of the *E. coli* $tRNA^{Lys}$ gene synthesized *in vitro*, which serves as a substrate both for the class I enzyme of *B. burgdorferi* and the class II enzyme of *E. coli* (19). While these studies revealed the role of the anticodon and discriminator base (N73) in tRNA recognition by class I LysRS, the *in vitro* transcripts were relatively poor substrates preventing precise assignment of function to individual nucleotides. Here we investigated anticodon recognition by using undermodified $tRNA^{Lys}$ variants generated *in vivo*, which have previously been shown to be more efficient substrates for the *E. coli* class II LysRS than their *in vitro* transcribed counterparts (22). Steady-state aminoacylation kinetic data showed undermodified *in vivo* synthesized tRNA to be approximately 3-fold less efficient than fully modified $tRNA^{Lys}$, but 10-fold more efficient (in terms of k_{cat}/K_M) compared with the equivalent *in vitro* transcript as a substrate for *B. burgdorferi* LysRS (Table 1). Analysis of undermodified tRNAs containing mutations in the anticodon clearly indicated that U35 and U36, but not U34, of $tRNA^{Lys}$ are major recognition elements for *B. burgdorferi* LysRS (Table 1).

The ability of the class I LysRS of *M. maripaludis* to recognize undermodified *E. coli* $tRNA^{Lys}$ variants was also investigated. In all cases, insufficient charging was observed to allow accurate determination of steady-state kinetic parameters (data not

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Table 1. Recognition of *E. coli* tRNA^{Lys} variants by *B. burgdorferi* LysRS

Anticodon	K_M tRNA (μM)	k_{cat} (min^{-1})	k_{cat}/K_M (R)*
UUU	0.4 ± 0.1	0.57 ± 0.06	1
CUU	0.56 ± 0.09	0.84 ± 0.04	0.9
GUU	0.33 ± 0.03	0.57 ± 0.02	0.8
UCU	NA	NA	—
UGU	1.8 ± 0.3	0.024 ± 0.001	107
UUC	2.8 ± 0.9	0.011 ± 0.002	365
mnm^{5}s^{2}UUU†	1.5 ± 0.2	7.2 ± 0.3	0.3

NA, no detectable activity.

*Relative value expressed as fold decrease in k_{cat}/K_M compared to aminoacylation of *in vivo* produced *E. coli* tRNA^{Lys} containing an unmodified anticodon.

†Fully modified, including t^6 modification of A37, which is absent from the other variants investigated.

shown). tRNA recognition was therefore investigated by performing extended time courses of aminoacyl-tRNA synthesis at elevated enzyme and tRNA concentrations (Fig. 2). The results of these experiments indicate that mutation of U34 (Fig. 2*A*) and U35 (Fig. 2*B*) do not affect aminoacylation by *M. maripaludis* LysRS, whereas mutation of U36 is detrimental to recognition of tRNA^{Lys} (Fig. 2*C*). The observed differences in recognition of the U35 variants of tRNA^{Lys} between *B. burgdorferi* and *M. maripaludis* LysRS prompted the reexamination described below of the phylogeny of the known class I enzymes.

Molecular Phylogeny of Class I Lysyl-tRNA Synthetases. To date, the sequences of class I LysRS encoding genes have been reported from 19 organisms. The molecular phylogeny of the predicted amino acid sequences of class I LysRS proteins shows three main groupings, with the archaeal and bacterial examples intermixed in two of these (Fig. 3). The class I LysRS proteins of *B. burgdorferi* and *M. maripaludis* are found in two distinct groupings, raising the possibility that their differences in anticodon recognition may correlate with other genotypic features of the organisms represented in Fig. 3. The phylogenetic pattern in Fig. 3 does not show a strong resemblance to any of the other known AARS phylogenies (21), but does correlate with the known occurrence of genes encoding the AsnRS, which have previously been shown to be absent from a significant number of completed genome sequences (5). A search of genomic sequences for genes encoding AsnRS (*asnS*) revealed a strong correlation between the presence/absence of AsnRS and the placement of class I LysRSs in the corresponding phylogenetic tree (Fig. 3). Of the three major class I LysRS groupings, the organisms composing two (with a single exception) do not contain AsnRS with only the third (which includes *B. burgdorferi*) containing AsnRS.

Anticodon Recognition by *H. pylori* Lysyl-tRNA Synthetase. AsnRS is absent from a significant number of organisms whose genomes encode a class I LysRS, and is also missing from several genomes which instead encode class II LysRS proteins. This raises the question as to whether the presence of AsnRS correlates with the pattern of tRNA^{Lys} anticodon recognition by class II LysRS proteins, as described above for their class I counterparts. This was investigated by using the class II LysRS from *Helicobacter pylori*, the genome of which does not encode an AsnRS protein (25). The *H. pylori lysS* gene was directly cloned from genomic DNA and heterologously expressed in *E. coli* with a 3′ His$_6$ epitope tag. Following induction of expression, His$_6$-tagged *H. pylori* LysRS was overproduced and subsequently purified by affinity chromatography using Ni-NTA agarose. The ability of *H. pylori* LysRS to recognize undermodified *E. coli* tRNA^{Lys} variants was then investigated *in vitro*. Steady-state kinetic analyses showed that *H. pylori* LysRS could recognize the unmutated variant of tRNA^{Lys} ($U_{34}U_{35}U_{36}$) almost as well as could *E. coli* LysRS [for *H. pylori*, $k_{cat}/K_M = 0.92\ s^{-1}\ \mu M^{-1}$; for *E. coli*, $k_{cat}/K_M = 1\ s^{-1}\ \mu M^{-1}$ (22)]. tRNA recognition by the *H. pylori* enzyme was less sensitive to mutation of the first and third anticodon nucleotides, U34 and U36, than *E. coli* LysRS (Table 2). Mutation of the second anticodon nucleotide, U35, severely impaired recognition by *H. pylori* LysRS, implicating this position as a major identity element.

Discussion

Divergence in tRNA^{Lys} Recognition by Class I and II Lysyl-tRNA Synthetases. Previous studies have shown that class I- (19) and class II-type LysRS proteins (22, 26–28) recognize common nucleotides in their tRNA substrates, namely the anticodon and the discriminator base (N73). However, the limited utility of *in vitro* transcribed tRNAs as substrates of the class I LysRS enzymes meant that detailed comparisons could not be made between the two forms of LysRS. This problem has now been addressed through the use of undermodified *in vivo* transcribed variants of *E. coli* tRNA^{Lys} which serve as substrates for a number of class I and class II LysRS proteins. Although the use of these tRNAs confirmed that class I LysRSs recognize U35 and U36, recognition of these positions is significantly weaker than previously observed with class II LysRSs indicating that these interactions

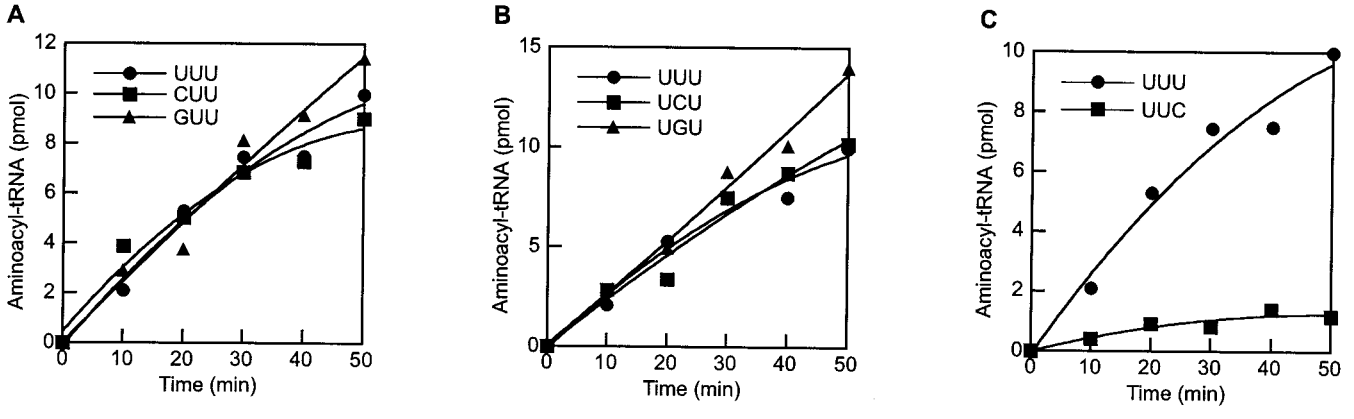

Fig. 2. Aminoacylation of *E. coli* tRNA^{Lys} variants by *M. maripaludis* His$_6$-LysRS. Aminoacylation reactions were performed as described (20 μl samples) in the presence of 200 nM enzyme, 8 μM tRNA, and 500 μM [^{3}H] lysine. Comparison of charging of wild-type tRNA (UUU anticodon) to variants of: (*A*) U34; (*B*) U35; (*C*) U36.

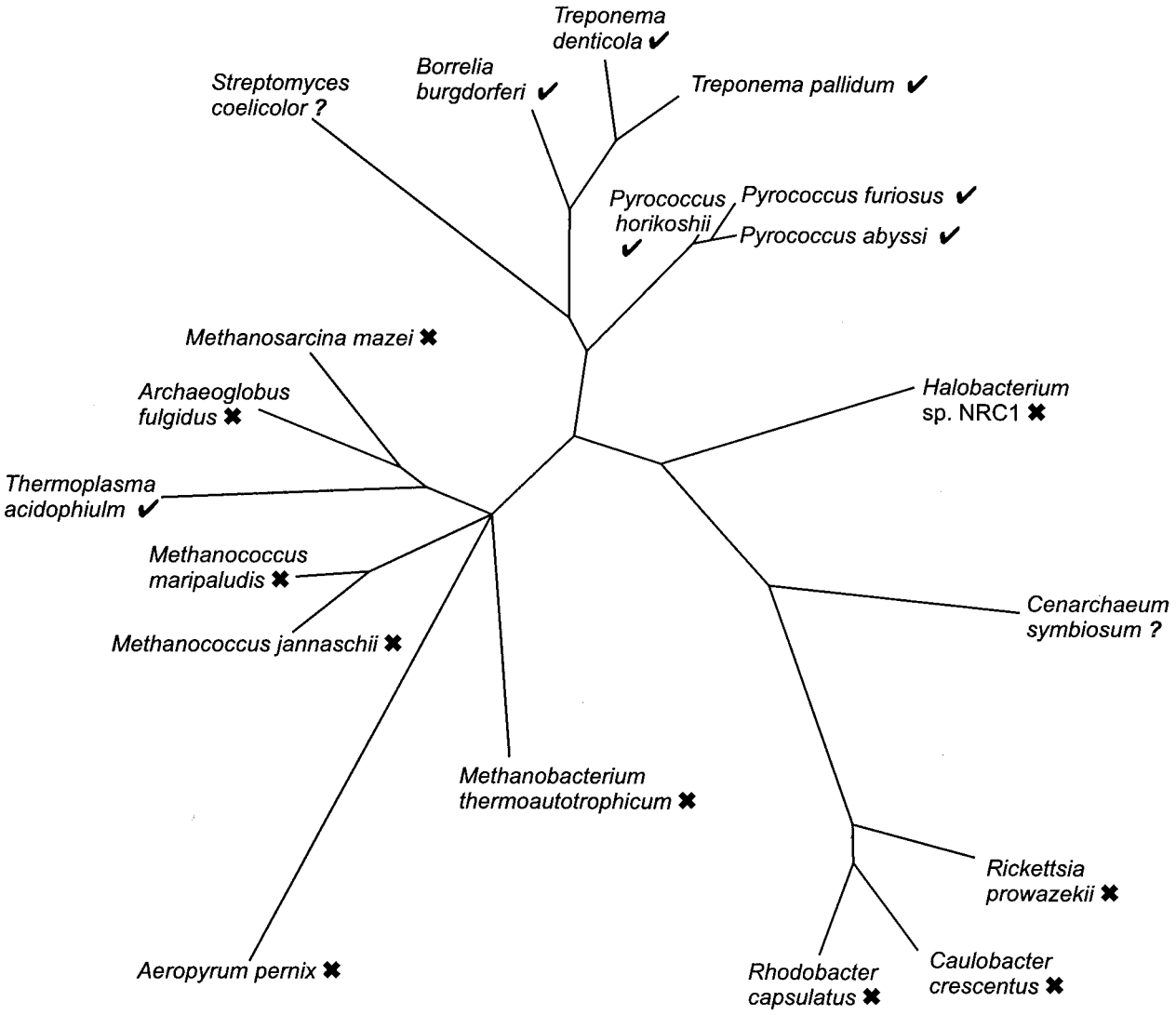

Fig. 3. Unrooted phylogeny of class I LysRS proteins. The phylogeny was constructed by using the maximum likelihood method (5,000 puzzling steps) implemented in the program TREE-PUZZLE 4.0.2 under the JTT model. ✖, genome does not encode AsnRS; ✓, genome encodes AsnRS; ?, genome sequence incomplete, AsnRS encoding gene not yet detected.

alone are probably not sufficient to determine tRNA identity. These differences are most pronounced for the class I LysRS of *M. maripaludis*, which does not recognize U35, the major identity element for class II LysRS, under the conditions used in this study. This suggests that significant interactions may also exist between class I LysRSs and other regions of $tRNA^{Lys}$ such as the acceptor stem and/or the D-stem and loop, a conclusion supported by preliminary footprinting (A. Ambrogelly and D.S., unpublished results) and alignment-based homology analyses (N. Jensen and M.I., unpublished results). Taken together, these results indicate that whereas class I and II LysRSs clearly recognize a number of the same nucleotides in $tRNA^{Lys}$, the overall contribution of these interactions to tRNA identity may be significantly different for the two protein families.

Asparaginyl-tRNA Synthetase Provides a Functional Context for the Class I Lysyl-tRNA Synthetases. The pattern of anticodon recognition by LysRSs is dictated by their need to effectively compete for closely related tRNAs with AsnRS and aspartyl-tRNA synthetase (AspRS). $tRNA^{Lys}$, $tRNA^{Asn}$, and $tRNA^{Asp}$ form a unique subgroup of tRNAs which share the degenerate anticodon sequence G/U34–U35–U/C36. Previous studies have shown that selection of $tRNA^{Lys}$, $tRNA^{Asn}$, and $tRNA^{Asp}$ by their cognate AARS is achieved through extensive recognition of all three anticodon nucleotides: G34, U35, and C36 are all recognized by AspRS (29), G34, U35, and U36 by AsnRS (30); and (U/C)34, U35, and U36 by LysRS (22, 26, 28). However, AsnRS is absent from a number of organisms (5) in which case a nondiscriminating AspRS instead aminoacylates both $tRNA^{Asn}$ and $tRNA^{Asp}$ with aspartate. The broader substrate specificity of such nondiscriminating AspRS proteins is achieved through structural changes in the enzyme that restrict recognition to the two anticodon nucleotides shared by $tRNA^{Asn}$ and $tRNA^{Asp}$, G34 and U35, with the third anticodon nucleotide no longer recognized. Thus, in the absence of AsnRS, the discrimination of $tRNA^{Lys}$ from $tRNA^{Asn}$ and $tRNA^{Asp}$ is no longer dependent on the simultaneous recognition of all three nucleotides in the anticodon. Under such circumstances, where position 36 is no longer recognized by either AsnRS or AspRS, recognition of U36 alone in the anticodon of $tRNA^{Lys}$ could be sufficient to allow discrimination against the recognition of $tRNA^{Asn}$ and $tRNA^{Asp}$ by LysRS. The data presented here indicate that this is

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Table 2. Recognition of *E. coli* tRNA^{Lys} variants by *H. pylori* LysRS

Anticodon	K_M tRNA (μM)	k_{cat} (min^{-1})	k_{cat}/K_M (R)*
UUU	0.94 ± 0.1	51 ± 2	1
CUU	0.81 ± 0.3	32 ± 3	1.4
GUU	0.66 ± 0.1	1.6 ± 0.1	22
UCU	—	—	9,100†
UGU	NA	NA	—
UUC	0.82 ± 0.2	6.3 ± 0.7	7
mnm^{5}s^{2}UUU‡	1.6 ± 0.2	117 ± 8	0.7

NA, no detectable activity.

*Relative value expressed as fold decrease in k_{cat}/K_M compared to aminoacylation of *in vivo* produced *E. coli* tRNA^{Lys} containing an unmodified anticodon.

†Due to the high K_M compared to practical tRNA concentrations ([S]$\ll K_M$), k_{cat}/K_M was directly estimated from the equation $v = k_{cat}/K_M$([E][S]).

‡Fully modified, including t^6 modification of A37, which is absent from the other variants investigated.

indeed the case for certain class I-type LysRS enzymes, because the recognition of U35 and U36 or of U36 alone corresponds to the presence or absence, respectively, of AsnRS in particular organisms (Table 1 and Fig. 2).

Further evidence that the presence of AsnRS dictates recognition of the anticodon nucleotides in tRNA^{Lys} comes from the strong correlation between the phylogeny of class I LysRS sequences and AsnRS distributions (Fig. 3). To date, the only exception to this phylogenetic correlation comes from *Thermoplasma acidophilum*, which contains a gene encoding an AsnRS most similar to that found in *Pyrococcus* species (49% amino acid identity to *Pyrococcus abyssi*, 48% to *Pyrococcus horikoshii*). Given that widespread horizontal gene transfer is observed throughout the genome of *T. acidophilum* (31), the phylogenetic discrepancy may reflect the recent acquisition of either the class I LysRS or the AsnRS by this organism. In contrast, the observation that the LysRS of *H. pylori*, which lacks AsnRS, recognizes U35 in tRNA^{Lys} may relate to the above prediction that class II LysRSs are more dependent on anticodon binding than their class I counterparts. Thus, whereas the absence of AsnRS may result in some relaxation in substrate specificity (for example the less stringent recognition of U34 and U36 compared with the *E. coli* LysRS) strong interactions between the anticodon of tRNA^{Lys} and the *H. pylori* LysRS may have been retained to ensure a stable protein–RNA complex during the aminoacylation reaction.

Asparaginyl-tRNA Synthetase as a Determinant of Class I Lysyl-tRNA Synthetase Distribution. The molecular phylogeny of the class I LysRS proteins strongly suggests that the bacterial examples, which are not deeply rooted, arose through horizontal gene transfer from archaea. The division of the bacterial class I LysRS proteins into two distantly related groups indicates that they arose via separate transfer events from archaea. This scenario is now supported by the observation that one of these groups belongs to a branch containing AsnRS whereas the other does not. Thus, whereas a class I LysRS from an archaeal "donor" lacking AsnRS could be transferred into a bacterial "acceptor" also lacking AsnRS, it appears such LysRSs were not transferred to bacteria containing AsnRS. Similarly, it appears that LysRSs from archaea containing AsnRS were only transferred to bacteria containing AsnRS. Whether such a scheme also correlates with patterns of anticodon recognition now requires a more exhaustive sampling of tRNA recognition among the class I LysRS proteins.

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