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## Divergence in Non-Cognate Amino Acid Recognition Between Class I and Class II Lysyl-tRNA Synthetases

### Comments

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## Divergence in Noncognate Amino Acid Recognition between Class I and Class II Lysyl-tRNA Synthetases\*

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**Lysine insertion during coded protein synthesis requires lysyl-tRNA<sup>Lys</sup>, which is synthesized by lysyl-tRNA synthetase (LysRS). Two unrelated forms of LysRS are known: LysRS2, which is found in eukaryotes, most bacteria, and a few archaea, and LysRS1, which is found in most archaea and a few bacteria. To compare amino acid recognition between the two forms of LysRS, the effects of L-lysine analogues on aminoacylation were investigated. Both enzymes showed stereospecificity toward the L-enantiomer of lysine and discriminated against noncognate amino acids with different R-groups (arginine, ornithine). Lysine analogues containing substitutions at other positions were generally most effective as inhibitors of LysRS2. For example, the  $K_i$  values for aminoacylation of S-(2-aminoethyl)-L-cysteine and L-lysine were over 180-fold lower with LysRS2 than with LysRS1. Of the other analogues tested, only  $\gamma$ -aminobutyric acid showed a significantly higher  $K_i$  for LysRS2 than LysRS1. These data indicate that the lysine-binding site is more open in LysRS2 than in LysRS1, in agreement with previous structural studies. The physiological significance of divergent amino acid recognition was reflected by the *in vivo* resistance to growth inhibition imparted by LysRS1 against S-(2-aminoethyl)-L-cysteine and LysRS2 against  $\gamma$ -aminobutyric acid. These differences in resistance to naturally occurring noncognate amino acids suggest the distribution of LysRS1 and LysRS2 contributes to quality control during protein synthesis. In addition, the specific inhibition of LysRS1 indicates it is a potential drug target.**

The fidelity of coded protein synthesis is dependent on the accuracy of two processes: the matching of codons in mRNA with their corresponding anticodons in tRNA and the aminoacylation of these tRNAs with amino acids defined by the anticodon. Aminoacyl-tRNA synthesis is a highly specific reaction catalyzed by the aminoacyl-tRNA synthetase (aaRS)<sup>1</sup> protein family, each member of which is specific for a particular amino acid and tRNA (1, 2). Despite the remarkable precision aaRSs display in the recognition and selection of the correct amino acid and tRNA, proofreading and editing mechanisms are both required to maintain accuracy at a level consistent

with faithful translation of the genetic code (3, 4). Elongation factor Tu, which takes aminoacyl-tRNAs to the ribosomal decoding site, provides an additional level of quality control by screening for incorrectly aminoacylated tRNAs (5).

The 20 aaRS proteins, as for example found in *Escherichia coli* (6), are divided into two mutually exclusive structural groups of 10 members each termed class I and class II (7, 8). Structural studies have shown that in class I synthetases, the active site contains a Rossmann dinucleotide-binding domain, whereas this fold is absent from the active site of class II enzymes, which instead contain a novel anti-parallel  $\beta$ -fold. One result of this difference in active site structure is that class I enzymes bind ATP in an extended conformation and class II in a bent conformation (7). The other major difference between the two aaRS classes is in their binding of tRNA and the site at which the amino acid is subsequently attached to it. With a few exceptions, class I enzymes approach the acceptor stem of tRNA from the minor groove side and acylate the 2'-hydroxyl of the terminal adenosine, whereas class II synthetases approach the major groove side and acylate the 3'-hydroxyl (9).

The assignment of an aaRS specific for a particular amino acid to one or the other structural class is almost completely conserved in all species, reflecting the antiquity of this dichotomy (10). The only widespread exception observed to date is LysRS, which is found in both class I and class II (11). Comparative genomic analysis has established that class I LysRSs are present in most archaea, a few bacteria, but no eukaryotes (12). As a result of microbial genome sequencing, over 40 class I LysRSs have now been identified. Despite their comparative rarity, class I LysRSs conform to the canonical archaeal/bacterial division of the universal phylogenetic tree (13, 14). Strikingly, the class I (LysRS1) and class II (LysRS2) proteins are almost never found together, with organisms generally containing one or the other but not both. The only well documented example of the co-existence of LysRS1 and LysRS2 is in the *Methanosarcinaceae*, where they function together to aminoacylate the specialized tRNA<sup>Pyl</sup> suppressor species (15).

Functional (16) and structural (17) characterizations have shown that LysRS1 and LysRS2 are functionally equivalent but structurally unrelated. Consequently, despite their lack of sequence similarity, the class I and II LysRSs are able to recognize the same amino acid and tRNA substrates both *in vitro* and *in vivo*, providing an example of functional convergence by divergent enzymes (12). The two classes of LysRS proteins approach their RNA substrates from opposite sides but recognize the same regions of tRNA<sup>Lys</sup>, namely the anticodon, acceptor stem, and discriminator base (16). Within these common recognition sites in tRNA<sup>Lys</sup>, the relative importance of particular nucleotides varies for the two classes of LysRS (12). These results show how the unrelated forms of LysRS perform the same cellular function, in this case tRNA<sup>Lys</sup> recognition, using different molecular mechanisms.

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<sup>1</sup> The abbreviations used are: aaRS, aminoacyl-tRNA synthetase; LysRS, lysyl-tRNA synthetase; AEC, S-(2-aminoethyl)-L-cysteine.

Variation between LysRS1 and LysRS2 has also been observed for lysine activation. LysRS2 initiates lysyl-tRNA synthesis using only lysine and ATP to generate an enzyme bound aminoacyl-adenylate, as do all class II and the majority of class I aaRSs, whereas the class I LysRS requires tRNA<sup>Lys</sup> binding prior to aminoacyl-adenylate synthesis, a feature shared by only a small sub-group of class I aaRSs (16, 18–20). Crystal structures of LysRS1 and LysRS2 complexed with L-lysine reveal that, whereas their active site architectures are fundamentally different, the strategies for recognition of the R-group of L-lysine (but not the remainder of the molecule) are quite similar (17). To compare the amino acid recognition strategies of LysRS1 and LysRS2 in more detail, we have now studied the effects of L-lysine analogues on the aminoacylation reaction *in vitro* and *in vivo*. Significant differences in substrate recognition were found, providing both a rationale for the existence of two forms of LysRS and also suggesting a means of developing LysRS1 as a species-specific target for novel anti-infective agents.

#### EXPERIMENTAL PROCEDURES

**Bacterial Strains and Plasmids**—Plasmid pKS-*lysS* (21) was used as the template for amplification of the *E. coli lysS* gene, with primers designed to generate a product flanked by NdeI and SapI sites. PCR was carried out using Platinum Pfx DNA polymerase (Stratagene) and the resulting product cloned into the TOPO-TA blunt end (Invitrogen). The gene was sequenced with two times coverage. Subsequently, the gene was excised and inserted into the pTYB1 vector to allow production of a LysRS2-intein fusion protein (IMPACT System, New England Biolabs). *Bacillus subtilis* strain 168 (encoding *B. subtilis* LysRS2) and its derivative 157.1 (encoding *Borrelia burgdorferi* LysRS1 but not *B. subtilis* LysRS2) have been described previously (22). The cells were routinely grown aerobically in LB medium or in Spizizen's minimal medium at 37 °C (23).

**Substrates**—All lysine analogues and other amino acids were from Sigma except for L-lysine methyl ester and L-lysine ethyl ester (ICN Biomedical), DL-5-hydroxylysine (Fluka, Buchs, Switzerland) and 5'-O-[N-(L-lysyl)-sulfamoyl] adenosine (RNA-TEC, Leuven, Belgium). All substrates tested as inhibitors of aminoacylation, with the exception of D-lysine, were shown by mass spectrometry (Campus Chemical Instrument Center, Ohio State University, Columbus, OH) to lack contamination with lysine. L-[U-<sup>14</sup>C]lysine and L-[U-<sup>3</sup>H]lysine were from PerkinElmer Life Sciences. Transcripts corresponding to *B. burgdorferi* tRNA<sup>Lys1</sup> were prepared as described previously (16).

**Lysyl-tRNA Synthetase Purification**—The *E. coli lysS* encoded LysRS2 cloned into the pTYB1 vector was expressed in *E. coli* BL21(DE3) cells. Transformants were grown at room temperature in LB supplemented with ampicillin (100 µg/ml) to cell density  $A_{600} = 0.6$ . Expression of *lysS* was induced by isopropyl-1-thio-β-D-galactopyranoside (1 mM) for 16 h at room temperature. Subsequent steps were performed at 4 °C. The cells were harvested by centrifugation and washed in column buffer (20 mM Tris-HCl, pH 7.4, 500 mM NaCl, 1 mM MgCl<sub>2</sub>, 10% glycerol). The cells were resuspended in column buffer supplemented with protease inhibitor (Hoffmann-La Roche), passed through a French pressure cell, and then centrifuged at 20,000 × *g* for 30 min. The resulting supernatant was loaded onto a chitin affinity bead column (New England Biolabs) according to the manufacturer's instructions. Protein was eluted from the chitin affinity column in a buffer of 50 mM Tris-HCl, pH 8.0, 1 mM MgCl<sub>2</sub>, 50 mM NaCl, 10% glycerol, and 10 mM β-mercaptoethanol. The fractions containing LysRS2 (judged to be >99% pure by Coomassie Blue staining after SDS-PAGE) were pooled, concentrated by ultrafiltration using Amicon Ultra-15 (Millipore), dialyzed against storage buffer (50 mM Tris-HCl, pH 8.0, 1 mM MgCl<sub>2</sub>, 10% glycerol, 10 mM β-mercaptoethanol), and stored at -80 °C. The concentration of LysRS2 was determined by active site titration as described previously (24), except that [<sup>14</sup>C]tyrosine was replaced with [<sup>14</sup>C]lysine, and reactions were performed for 10 min. Calculations were based upon half of the sites' reactivity for *E. coli* LysRS (25). *B. burgdorferi* LysRS1 was prepared as described previously (16).

**Aminoacylation Assays**—Aminoacylation was performed at 37 °C in 100 mM Hepes, pH 7.2, 25 mM KCl, 10 mM MgCl<sub>2</sub>, 4 mM dithiothreitol, 5 mM ATP, 5 µM tRNA tRNA<sup>Lys</sup>, 100 nM (LysRS1) or 10 nM (LysRS2) enzyme and [<sup>14</sup>C]L-Lys at concentrations varying between 0.2 and 5 times the  $K_m$ , 10 (LysRS2)- or 20-µl (LysRS1) aliquots were taken every 15–30 s and spotted onto Whatman No. 3MM filter disks presoaked in

5% trichloroacetic acid (w/v) containing 0.5% (w/v) <sup>12</sup>C-L-Lys. Sample disks were washed three times for 10 min at room temperature in 5% trichloroacetic acid (w/v) containing 0.5% (w/v) <sup>12</sup>C-L-Lys, dried at 80 °C, and the level of [<sup>14</sup>C]L-Lys-tRNA quantified by the addition of liquid scintillant (Ultima Gold, Packard Corp.) and scintillation counting. Analogues were added as indicated during determination of  $K_i$  values and [<sup>14</sup>C]L-Lys adjusted so that concentrations varied between 0.2 and 5 times the apparent  $K_m$ . The direct attachment of analogues to *in vitro* transcribed tRNA<sup>Lys1</sup> was monitored as described earlier (26). Transcript tRNA<sup>Lys</sup> from *B. burgdorferi* and pure tRNA<sup>Lys</sup> from *E. coli* were radiolabeled by an [α-<sup>32</sup>P]ATP-PP<sub>i</sub> exchange reaction catalyzed by the *E. coli* tRNA-terminal nucleotidyltransferase (27). The aminoacylation reaction was performed in the presence of 0.1 µM *B. burgdorferi* LysRS1 or 10 nM *E. coli* LysRS2, 10 mM of lysine or analogues, 2 µM radiolabeled transcript from *B. burgdorferi*, or a trace of pure 3'-radiolabeled tRNA<sup>Lys</sup> from *E. coli* with 1.3 mg/ml of total tRNA. Following 30 min of incubation at 37 °C, an aliquot was removed and treated with RNase P1. The liberated [α-<sup>32</sup>P]AMP and aminoacyl-[α-<sup>32</sup>P]AMP were separated by TLC and visualized as described previously (26). The levels of tRNA charging are uniformly low (~10%) as a result of significant loss of activity during the labeling and purification of both *E. coli* and *B. burgdorferi* tRNA<sup>Lys</sup> (both tRNAs are 80–90% active initially). This problem is, to date, unique to tRNA<sup>Lys</sup> as both tRNA<sup>Phe</sup> 2 and tRNA<sup>Ala</sup> (26) retain 40–50% activity during the same procedure.

**$K_i$  Determination**—To determine  $K_i$  values for lysine analogues, at least five different concentrations of analogues were first screened in the aminoacylation reaction under standard conditions with 10 or 4.5 µM of [<sup>14</sup>C]L-Lys for LysRS1 and LysRS2, respectively. Analogue concentrations were then established, at which the initial rate of aminoacylation was decreased by 20–50% when compared with the reaction lacking analogue, and these levels were used for  $K_i$  determinations. Concentrations of analogues used to determine the  $K_i$  for LysRS1 and LysRS2 were, respectively: 150 or 100 µM L-lysine hydroxamate; 650 or 25 µM S-(2-aminoethyl)-L-cysteine (AEC); 650 or 50 µM L-lysineamide; 400 or 250 µM L-lysine methyl ester; 250 or 100 µM L-lysine ethyl ester; 650 µM or 1 mM DL-5-hydroxylysine; 2.5 or 5 mM L-ornithine; 4 or 40 mM D-lysine; 200 or 500 µM L-cadaverine; 20 or 25 mM lysyl-sulfamoyl adenosine; 8 or 40 mM L-α-aminobutyric acid; 2 or 200 mM L-γ-aminobutyric acid; 2.5 or 30 mM L-arginine; 10 or 50 mM L-glutamic acid. In all cases, stock solutions of inhibitors were maintained at neutral pH. The  $K_i$  values presented represent the average of at least two independent experiments for LysRS1 and three independent experiments for LysRS2 where values deviated by no more than 10% between individual determinations.

***In Vivo* Growth Inhibition**—*B. subtilis* strains 168 and 157.1 were grown aerobically in LB medium until  $A_{600} = 1$ . 1 ml of this culture was then spun down and washed and resuspended in 1 ml of Spizizen's minimal medium. 250 µl of these cells were then inoculated in 25 ml of Spizizen's minimal medium supplemented with 2 mM L-lysine or 5 µM of AEC or 400 mM L-γ-aminobutyric acid at 37 °C.

#### RESULTS

**Inhibition of LysRS1- and LysRS2-catalyzed *In Vitro* Aminoacylation**—The aaRS-catalyzed aminoacylation of tRNA is a two-step reaction. In the first step, an amino acid is activated to form an enzyme-bound aminoacyl adenylate. The second step of the reaction involves binding of this complex by tRNA, whose 3'-end is then esterified with the aminoacyl moiety followed by release of the resulting aminoacyl-tRNA. Although LysRS1 and LysRS2 both utilize this overall reaction mechanism, they show a key difference at the first step; lysyl-adenylate synthesis by LysRS1 requires the presence of tRNA, whereas LysRS2 can perform the reaction in the absence of tRNA. Given this difference between LysRS1 and LysRS2, we chose to compare their ability to recognize lysine and lysyl-adenylate analogues by determining the kinetics of inhibition of steady-state aminoacylation. This approach, rather than measurement of the inhibition of amino acid activation, would then allow more direct comparisons to be made between the two systems.

All compounds tested (Fig. 1) were found to act as competitive inhibitors of both LysRS1 and LysRS2, as judged by the observation of significant changes in  $K_m$  but not  $k_{cat}$  when

<sup>2</sup> M. Ibba and H. Roy, unpublished results.

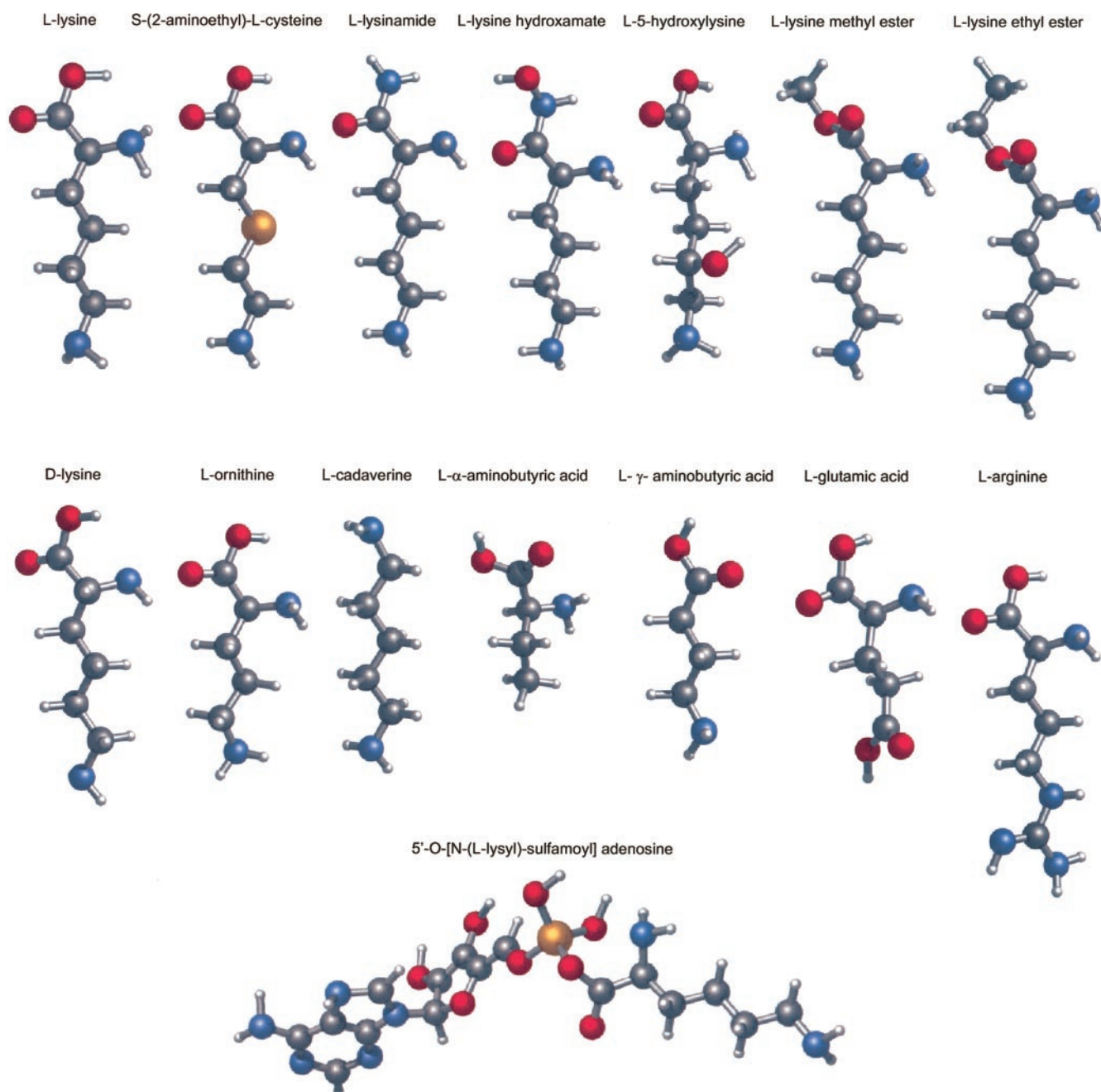


FIG. 1. **Structures of L-lysine and analogues.** Geometries of structures were optimized using ArgusLab 3.1 (Planaria Software). Carbon, nitrogen, oxygen, sulfur, and hydrogen atoms are represented in gray, blue, red, yellow, and white, respectively.

comparing steady-state aminoacylation kinetic parameters with and without the addition of analogues (Table I). The most potent inhibitor of both LysRS1 and LysRS2 was the lysyl-adenylate analogue lysylsulfamoyl-adenosine, which inhibited both enzymes equally well (Table I). Analogues of L-lysine, rather than the adenylyl derivative, were less potent inhibitors with  $K_i$  values ranging from low  $\mu\text{M}$  ( $3.9 \mu\text{M}$  for AEC with LysRS2) to low mM ( $12 \text{ mM}$  for D-lysine with LysRS2). The least effective inhibitors were the noncognate amino acids, whose  $K_i$  values varied from  $5 \text{ mM}$  (L-arginine with LysRS1) to  $470 \text{ mM}$  (L- $\gamma$ -aminobutyric acid with LysRS2).

For LysRS2, the  $K_m$  for lysine (28) and  $K_i$  values for competitors determined in this study generally correlated well with previously determined values where comparable data exists. For AEC (29), lysynamide (30), lysine methyl ester (30), lysine ethyl ester (30), ornithine (31), and cadaverine (28), previously determined  $K_i$  values for aminoacylation by LysRS2 are all

within 1–5-fold of the values reported here. The only exception is D-lysine, where the previously reported value of  $220 \mu\text{M}$  (30) is over 50-fold lower than the  $K_i$  determined here. For other compounds tested, either kinetic parameters were only determined previously in the amino acid activation reaction (5-hydroxylysine, lysine hydroxamate (32)) or no other data are, to the best of our knowledge, currently available. For LysRS1, although we recently reported (in Ref. 22 and accompanying inhibition plots) that AEC acts a competitive inhibitor with a nearly identical  $K_i$  to that described here, no other kinetic parameters for the inhibition of aminoacylation have been reported for comparison.

**Growth Inhibition by Lysine Analogues**—Comparison of the kinetics of inhibition of *in vitro* aminoacylation by LysRS1 and LysRS2 indicated that several compounds preferentially inhibit one form of LysRS rather than the other. Lysine analogues with the strongest preferences were lysynamide and

TABLE I  
Kinetic parameters for the inhibition of steady-state aminoacylation by *B. burgdorferi* LysRS1 and *E. coli* LysRS2 (*lysS* encoded)

Analogue	$K_i^a$		$K_i$ LysRS1/ $K_i$ LysRS2	$k_{cat}$ (R) <sup>b</sup>	
	LysRS1	LysRS2		LysRS1	LysRS2
	$\mu\text{M}$	$\mu\text{M}$			
L-Lysine hydroxamate	360 ± 70	86 ± 7	4	1.1 ± 0.06	0.7 ± 0.02
S-(2-Aminoethyl)-L-cysteine	1140 ± 230	3.9 ± 0.4	290	1 ± 0.09	0.8 ± 0.02
L-Lysinamide	2120 ± 450	17 ± 2	180	1 ± 0.07	1.1 ± 0.01
L-Lysine methyl ester	478 ± 100	74 ± 7	6	1 ± 0.07	0.8 ± 0.03
L-Lysine ethyl ester	303 ± 45	55 ± 6	6	1.1 ± 0.05	0.8 ± 0.02
DL-5-Hydroxylysine	1200 ± 140	500 ± 52	2	1.2 ± 0.03	0.8 ± 0.02
L-Ornithine	8800 ± 1300	6300 ± 600	1	1.1 ± 0.02	0.9 ± 0.02
D-Lysine	6900 ± 2500	12,000 ± 1400	1	1.1 ± 0.1	0.7 ± 0.04
L-Cadaverine	320 ± 45	260 ± 28	1	0.9 ± 0.03	1 ± 0.03
Lysyl-sulfamoyl adenosine	0.025 ± 0.004	0.028 ± 0.003	1	1 ± 0.05	0.9 ± 0.03
L- $\alpha$ -Amino butyric acid	21,200 ± 5300	14,200 ± 1700	1	1.1 ± 0.09	1.2 ± 0.02
L- $\gamma$ -Aminobutyric acid	8040 ± 2200	470,000 ± 51,000	0.02	1 ± 0.07	1 ± 0.02
L-Arginine	5060 ± 860	64,000 ± 5000	0.08	1.1 ± 0.04	0.9 ± 0.03
L-Glutamic acid	37,000 ± 7800	130,000 ± 13,000	0.3	0.9 ± 0.06	1.2 ± 0.03

Analogue	$K_m$		$k_{cat}$	
	LysRS1	LysRS2	LysRS1	LysRS2
	$\mu\text{M}$		$s^{-1}$	
L-Lysine	34 ± 3	2.6 ± 0.2	0.06 ± 0.002	1.84 ± 0.02

<sup>a</sup>  $K_i$  values were determined from the following formula  $K_{m(\text{app})} = K_{m(\text{real})} (1 + [\text{inhibitor}]/K_i)$ , using the  $K_m$  values shown and inhibitor concentrations indicated in the text.

<sup>b</sup>  $k_{cat}$  determined in the presence of inhibitor relative to  $k_{cat}$  in the absence of inhibitor.

AEC, which showed 180- and 290-fold, respectively, lower  $K_i$  values for LysRS2 than LysRS1, and  $\gamma$ -aminobutyric acid, which had a 60-fold lower  $K_i$  for LysRS1 than LysRS2 (Table I). To investigate whether these *in vitro* differences could be correlated with specific *in vivo* growth phenotypes, two related strains of *B. subtilis* were employed. 168 is a wild-type strain that employs LysRS2 for lysyl-tRNA synthesis, and 157.1 is a derivative of 168, where the endogenous LysRS2-encoding gene has been replaced by a gene encoding *B. burgdorferi* LysRS1 (22). The growth of these strains in minimal medium was monitored with and without the addition of varying concentrations of AEC, lysinamide, and  $\gamma$ -aminobutyric acid. Addition of lysinamide at concentrations up to 46 mM had no detectable effect on the growth rates of either strain 168 or 157.1 (data not shown). Growth in the presence of 5  $\mu\text{M}$  AEC completely prevented growth of 168 but only resulted in an ~50% reduction in the growth rate of 157.1 (Fig. 2A). Conversely, the addition of 400 mM  $\gamma$ -aminobutyric acid completely inhibited the growth of 157.1 but only lowered the growth rate of 168 by about 50% (Fig. 2B). These results are consistent with data from *in vitro* steady-state kinetics, confirming the selectivity of AEC and  $\gamma$ -aminobutyric acid as preferential inhibitors of LysRS2 and LysRS1, respectively.

**Aminoacylation with Noncognate Amino Acids Using LysRS1 and LysRS2**—Previous studies of *E. coli* LysRS2 (*lysS*-encoded) showed that the enzyme is able to activate, aminoacylate and edit a number of noncognate amino acids (33), and we have shown previously (22) that AEC is a substrate for aminoacylation by LysRS1. We investigated the ability of LysRS1 and LysRS2 to aminoacylate tRNA<sup>Lys</sup> with a number of naturally occurring noncognate amino acids shown above to be competitive inhibitors of the enzyme. Of the analogues tested, L-lysine methyl ester, L-lysine ethyl ester, AEC, and, to a lesser extent, L-ornithine were found to be substrates for aminoacylation of tRNA<sup>Lys</sup> by LysRS1 (Fig. 3B). LysRS2 displayed a significantly broader substrate spectrum, catalyzing aminoacylation with D-lysine, L-lysine methyl ester, L-lysine ethyl ester, L-ornithine, AEC, L-lysine hydroxamate, L- $\alpha$ -aminobutyric acid, and, to a lesser extent, lysinamide, arginine, and glutamate (Fig. 3A).

**Active Site Homology Plots**—The ability of certain compounds to selectively inhibit *B. burgdorferi* LysRS1 or *E. coli* LysRS2 *in vitro* and *B. burgdorferi* LysRS1 or *B. subtilis* LysRS2 *in vivo* suggests differences between the active site architectures of the two enzymes. To estimate the degree to which this divergent substrate discrimination might be conserved, sequence alignments were constructed from 44 LysRS1 and 137 representative LysRS2 predicted protein sequences using ClustalX (34). Conservation of amino acids (identity) was then scored for each position in the two LysRS alignments. These data were mapped onto the three-dimensional structures of *E. coli* LysRS2 (*lysS*) (35) and *P. horikoshii* LysRS1 (17) (Fig. 4, A and B). Examination of the three-dimensional identity plots for both LysRS1 and LysRS2 showed a strikingly high degree of conservation throughout the lysine-binding sites of both proteins (Fig. 4, A and B). This conservation of residues was seen in regions binding both the R-groups and the remainder of the lysine molecules, suggesting that the patterns of noncognate amino acid discrimination observed above might be conserved in the LysRS1 and LysRS2 protein families.

## DISCUSSION

**Comparison of Amino Acid Discrimination by LysRS1 and LysRS2**—The inhibition of aminoacylation by lysine analogues suggests several key similarities and differences between the two forms of LysRS. Both LysRSs showed a comparably strong enantiomeric selectivity for L-lysine over D-lysine, consistent with the general observation that L-amino acids are strongly favored throughout protein synthesis (Ref. 36 and references therein). Although LysRS2 was able to more easily aminoacylate tRNA<sup>Lys</sup> with D-lysine (Fig. 3A), the level of D-lysine required was significantly higher than would be expected *in vivo* given estimates of microbial total lysine pools under normal growth conditions (37). Similarly, the levels of arginine and ornithine required for the inhibition of aminoacylation by both LysRS1 and LysRS2 are significantly higher than have been observed *in vivo* (38), indicating an adequate level of discrimination by both enzymes. Estimates of cellular concentrations of cadaverine are comparable with the  $K_i$  values determined here, indicating specific protection exists against cadaverine

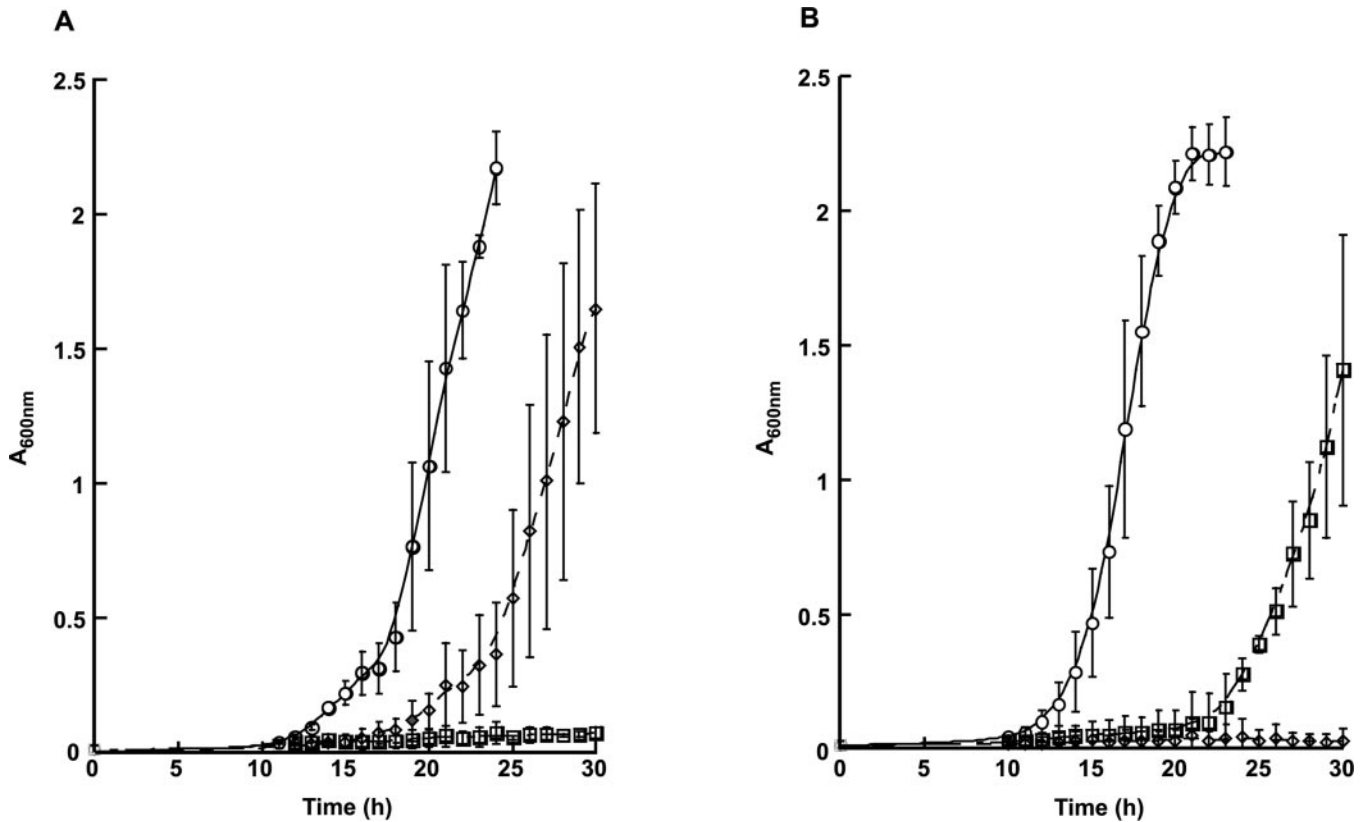
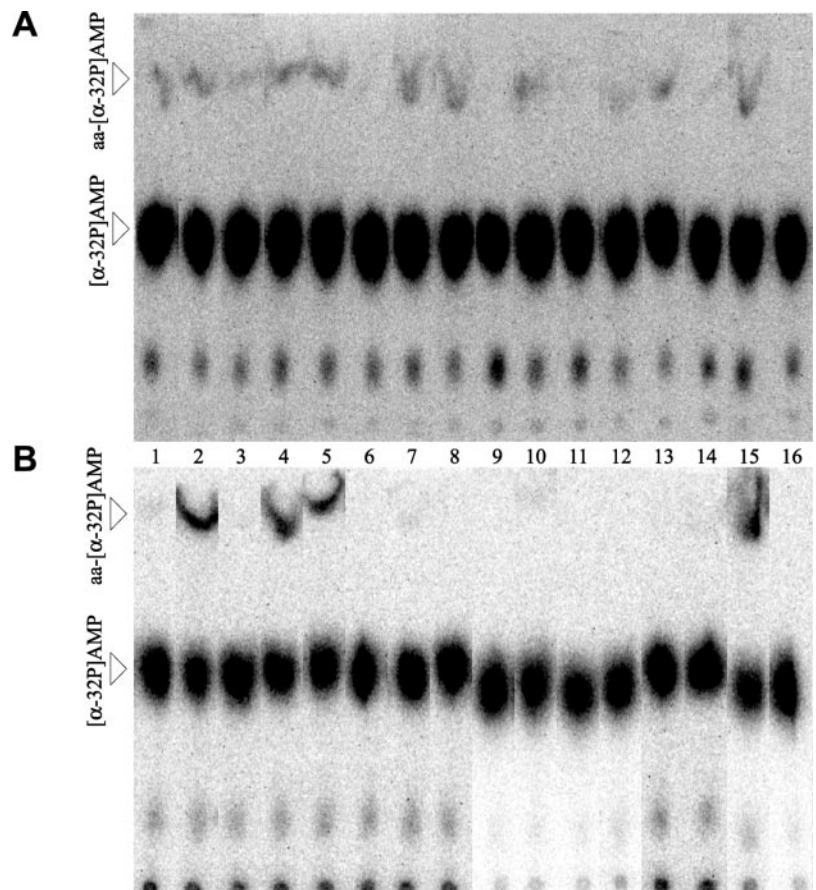
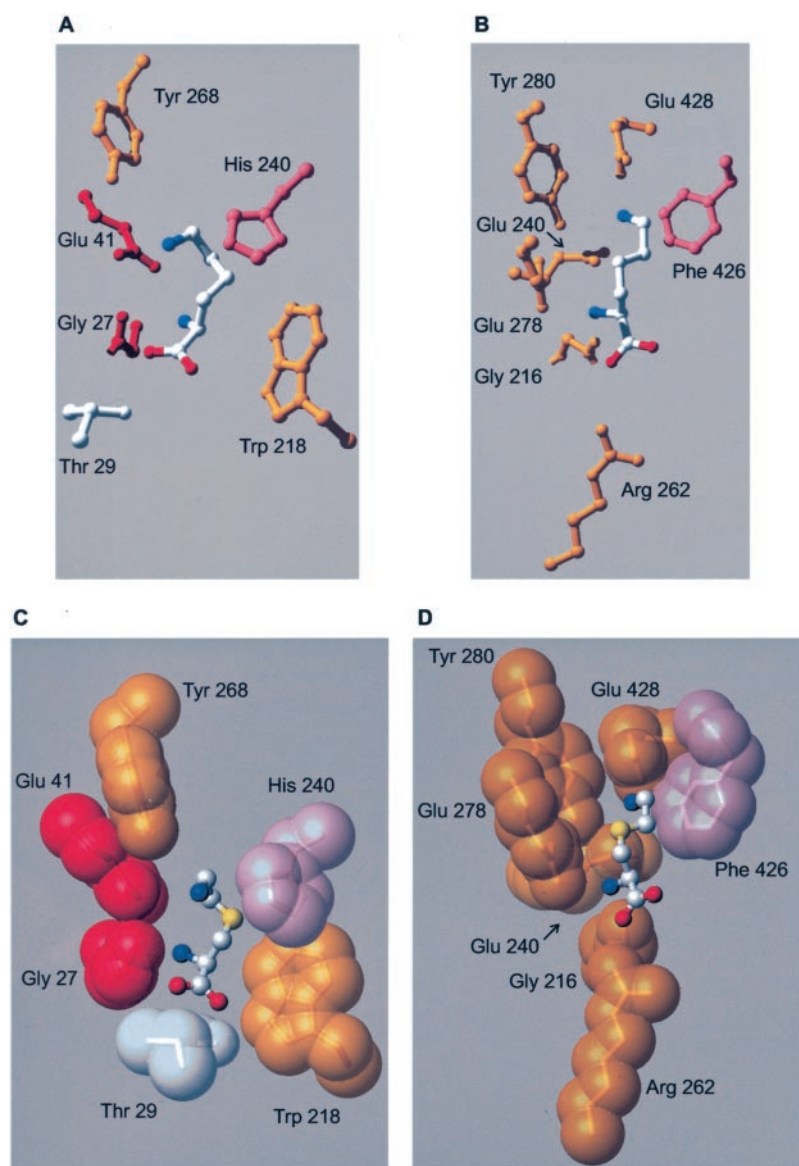


FIG. 2. *In vivo* growth inhibition of *B. subtilis* strains 168 (A) and 157.1 (B). Spizizen's minimal medium supplemented with 2 mM L-lysine ( $\circ$ ), 5  $\mu$ M AEC ( $\square$ ), or 400 mM L- $\gamma$ -aminobutyric acid ( $\diamond$ ) were inoculated with *B. subtilis* strain 168 or 157.1, and growth was monitored by absorbance at 600 nm. Each curve represents the average of at least three independent experiments with standard deviation indicated to each time point.

FIG. 3. TLC analysis of tRNA<sup>Lys</sup> aminoacylation with lysine analogues by (A) *E. coli* LysRS2 or (B) *B. burgdorferi* LysRS1. Lane 1, L-lysine hydroxamate; lane 2, *S*-(2-aminoethyl)-L-cysteine; lane 3, L-lysynamide; lane 4, L-lysine methyl ester; lane 5, L-lysine ethyl ester; lane 6, DL-5-hydroxylysine; lane 7, L-ornithine; lane 8, D-lysine; lane 9, L-cadaverine; lane 10, L- $\alpha$ -aminobutyric acid; lane 11, L- $\gamma$ -aminobutyric acid; lane 12, L-arginine; lane 13, L-glutamic acid; lane 14, L-glutamine; lane 15, L-lysine; lane 16, without amino acid. The samples were spotted on 10-cm polyethylenimine-cellulose plates (Sigma) prewashed and separated by TLC in glacial acetic acid/1 M NH<sub>4</sub>Cl/H<sub>2</sub>O (5:10:85). The significant proportion of uncharged tRNA reflects the low aminoacylation acceptance activity of tRNA<sup>Lys</sup> after labeling and purification (see "Experimental Procedures").



**FIG. 4. L-Lysine and S-(2-aminoethyl)-L-cysteine recognition by LysRS1 and LysRS2.** A, L-lysine in the active site of *P. horikoshii* LysRS1. B, L-lysine in the active site of *E. coli* LysRS2. C, model for the binding of S-(2-aminoethyl)-L-cysteine to the active site of *P. horikoshii* LysRS1. D, model for the binding of S-(2-aminoethyl)-L-cysteine to the active site of *E. coli* LysRS2. For C and D, the lysine ligand was modified to present an S instead of the  $\gamma$ C, and the energy of the resulting structures was minimized using Swiss-Pdb Viewer version 3.7. The resulting models were visualized in stick and van der Waals surface for active site residues and ball and stick for S-(2-aminoethyl)-L-cysteine in MOLMOL version 2k.2 (44). The residues are colored according to their conservation in corresponding sequence alignments: gold, 100% identity; red, 81–99%; pink, 61–80%; white, 41–60%. For the substrate lysine, the backbone is shown in white, and oxygen, nitrogen, and sulfur are colored red, blue, and yellow, respectively.



inhibition at normal lysine levels as proposed previously (28). Although the  $K_i$  values are significantly higher for L-glutamic acid than most of the other compounds tested, they are in fact not far removed from microbial glutamate concentrations, which may typically reach up to 80 mM or higher under certain growth conditions (e.g. 39). Taken together, our data confirmed that LysRS1 and LysRS2 are equally adept at discriminating against both the more common lysine analogues and the non-cognate canonical amino acids. The ability to discriminate lysine from several of the analogues tested here was also recently described for the L box of *B. subtilis*, a lysine-responsive leader RNA that directly binds lysine, indicating that RNA and protein based systems offer equally effective mechanisms for specific recognition of lysine (40, 41).

Among the other amino acids tested, all but one showed higher  $K_i$  values for LysRS1 than for LysRS2, in agreement with the more compact binding pocket for the lysine backbone predicted from the structure of the class I enzyme (Fig. 4C). L-Lysine hydroxamate, L-lysine methyl ester, L-lysine ethyl ester, and DL-5-hydroxylysine all show a marginal preference for inhibition of LysRS2 over LysRS1, with the  $K_i$  values 2–6-fold higher for the class I enzyme, whereas L- $\alpha$ -aminobutyric acid inhibits both enzymes to a similar degree. In contrast, AEC,

L-lysynamide, and L- $\gamma$ -aminobutyric acid were all found to be highly specific for a particular form of LysRS. AEC and lysinamide both show preferential inhibition of LysRS2 over LysRS1, with the  $K_i$  values being 290- and 180-fold lower for the class II enzyme, respectively. The differences in AEC and lysinamide recognition reflect the more closed structure of LysRS1 around the amino acid backbone, where two conserved aromatic residues make hydrophobic interactions with the side chain as opposed to a single residue in LysRS2 (Fig. 4, B and C). The role of these residues is illustrated from modeling the binding of AEC at both active sites. In LysRS1, which binds AEC relatively poorly, there is some steric exclusion of the sulfur atom by His-240 (Fig. 4C). In contrast, the orientation of bound AEC and the absence of a second “packing” residue in LysRS2 allow inhibitor binding without a potential steric clash (Fig. 4D), in agreement with the relatively strong binding of AEC. The importance of Trp-218 and His-240 in LysRS1 may be even more pronounced than is initially apparent from the existing tRNA-free structure. In a docking model of *Pyrococcus horikoshii* LysRS1 and tRNA (17), Trp-218 and His-240 (Trp-220 and His-242 in *B. burgdorferi*) make stacking interactions with the terminal adenosine of tRNA suggesting that they may be more closely packed in the active site during aminoacyla-



tion. Such tRNA-mediated rearrangements of active site residues have been observed previously in other class I aaRSs that, like LysRS1, require tRNA for amino acid activation (18–20, 42).

Of all the compounds compared as inhibitors of LysRS1 and LysRS2, only L- $\gamma$ -aminobutyric acid was a significantly better inhibitor of the class I enzyme. Examination of LysRS1 and LysRS2 active sites offers no obvious structural basis for this difference, although the relatively high  $K_i$  values compared with most of the other analogues may be indicative of poor binding in both cases. Although the kinetics of inhibition by L- $\gamma$ -aminobutyric acid suggest that neither form of LysRS binds this analogue well, *in vivo* data (discussed below) indicate that the difference in discrimination may be functionally significant.

*LysRS1 Displays a Narrower Substrate Spectrum than LysRS2*—The high degree of conservation of both LysRS1 and LysRS2 active site residues (Fig. 4, A and B) suggests that their marked differences in sensitivity to numerous inhibitors may be of functional significance. This was strongly supported by aminoacylation data, which showed that a far wider range of analogues could be stably attached to tRNA<sup>Lys</sup> by LysRS2 than by LysRS1. This difference could reflect the existence of a more proficient proofreading activity in LysRS1 or a more promiscuous active site in LysRS2. The possibility that proofreading prevents accumulation of mischarged tRNAs was not supported by our initial studies with LysRS1<sup>2</sup> and would not be expected given that the closely related class 1b aaRSs glutamyl- and glutamyl-tRNA synthetases have not been shown to catalyze such activities (reviewed in Ref. 4). Thus, the difference in substrate profiles between LysRS1 and LysRS2 can be attributed to a higher degree of substrate discrimination in the class I enzyme. This is in agreement with our recent study employing AEC, which suggested that inefficient analogue recognition by LysRS1 could prevent miscoding of lysine codons during protein synthesis (22). The data presented here supports this finding and suggests that this function in translation might also extend to other analogues, given the generally narrower substrate specificity of LysRS1. One important exception is L- $\gamma$ -aminobutyric acid, whose ability to preferentially inhibit LysRS1 indicates LysRS2 can also function in translational quality control by excluding particular noncognate amino acids. This was confirmed by the observation that production of LysRS2 allows growth of bacterial cells at L- $\gamma$ -aminobutyric acid concentrations inhibitory to cells producing solely LysRS1. It is worth noting, however, that any practical application of this difference in analogue recognition is dependent on the discovery of specific inhibitors of LysRS1 with  $K_i$  values several orders of magnitude lower than L- $\gamma$ -aminobutyric acid.

*Functional Consequences of Divergent Recognition of Noncognate Amino Acids*—The finding that LysRS1 and LysRS2 have substantially different noncognate amino acid substrate profiles has functional, evolutionary, and practical implications. Earlier work indicated that LysRS1 could prevent infiltration of the genetic code by AEC, but that now appears to simply be an example of a more general phenomenon whereby both forms of LysRS can provide translational quality control under appropriate conditions. The exact nature of the physiological conditions when such quality control might be critical awaits determination of lysine analogue pools in archaeal and bacterial metabolomes. LysRS-mediated quality control relies on the presence of one LysRS or the other but not both together, in agreement with the phylogenetic distributions observed for the majority of LysRS1 and LysRS2 sequences for which the corresponding complete genome sequences are known. Of the over 240 complete genome sequences publicly available, only

five encode both LysRS1 and LysRS2. Four examples are from the *Methanosarcinaceae*, where LysRS1 and LysRS2 apparently function together in suppressor tRNA charging (15), and the other is *Bacillus cereus*, where it is unclear if both LysRSs are produced.<sup>3</sup> In addition to providing a rationale for the existence and distribution of the two LysRSs, the divergence in substrate recognition confirms earlier proposals that LysRS1 may be a suitable target for the development of novel antimicrobials (43). LysRS1 is found alone in a number of bacterial pathogens (e.g. *B. burgdorferi*, various *Brucella* and *Rickettsia* species, *Treponema pallidum*, and *Tropheryma whippelii*), and our findings indicate that it may be practical to target Lys-tRNA<sup>Lys</sup> synthesis in these organisms without disrupting the LysRS2-mediated pathway of the human host.

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**Divergence in Noncognate Amino Acid Recognition between Class I and Class II  
Lysyl-tRNA Synthetases**

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