

# Single amino acid changes in AspRS reveal alternative routes for expanding its tRNA repertoire *in vivo*

Franck Martin, Sharief Barends and Gilbert Eriani\*

UPR 9002 SMBMR du CNRS, Institut de Biologie Moléculaire et Cellulaire, 15, rue René Descartes, 67084 Strasbourg, France

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## ABSTRACT

**Aminoacyl-tRNA synthetases (aaRSs) are enzymes that are highly specific for their tRNA substrates. Here, we describe the expansion of a class IIb aaRS-tRNA specificity by a genetic selection that involves the use of a modified tRNA displaying an amber anticodon and the *argE<sub>amber</sub>* and *lacZ<sub>amber</sub>* reporters. The study was performed on *Escherichia coli* aspartyl-tRNA synthetase (AspRS) and amber tRNA<sup>Asp</sup>. Nine AspRS mutants able to charge the amber tRNA<sup>Asp</sup> and to suppress the reporter genes were selected from a randomly mutated library. All the mutants exhibited a new amber tRNA<sup>Asp</sup> specificity in addition to the initial native tRNA<sup>Asp</sup>. Six mutations were found in the anticodon-binding site located in the N-terminal OB-fold. The strongest suppressor was a mutation of residue Glu-93 that contacts specifically the anticodon nucleotide 34 in the crystal structure. The other mutations in the OB-fold were found at close distance from the anticodon in the so-called loop L45 and strand S1. They concern residues that do not contact tRNA<sup>Asp</sup> in the native complex. In addition, this study shows that suppressors can carry mutations located far from the anticodon-binding site. One such mutation was found in the synthetase hinge-module where it increases the tRNA<sup>Asp</sup>-charging rate, and two other mutations were found in the prokaryotic-specific insertion domain and the catalytic core. These mutants seem to act by indirect effects on the tRNA acceptor stem binding and on the conformation of the active site of the enzyme. Altogether, these data suggest the existence of various ways for modifying the mechanism of tRNA discrimination.**

## INTRODUCTION

The aminoacyl-tRNA synthetases (aaRSs) form a family of enzymes that catalyze the covalent attachment of a given

amino acid onto its cognate tRNA during a two-step reaction that starts by the activation of the amino acid by ATP followed by its transfer onto the 3' terminal adenosine of the cognate tRNA (1). Aminoacyl-tRNA molecules exhibit, in addition to the attached amino acids, specific nucleotide triplets (anticodons) that are complementary to the codons on the mRNA they decode. Hence, they act as adapters that link the genetic information with the protein sequence. Therefore, accurate aminoacylation of tRNAs by aaRSs is crucial for translation fidelity since after aminoacylation, the delivery of amino acid to the nascent peptide chain depends essentially on base-pair interactions between the codon and anticodon triplet on the ribosome.

Genetic, biochemical and biophysical studies have elucidated the basis for the highly specific recognition of a tRNA by its aaRS (also referred to as 'tRNA identity'). It is generally accepted that accurate recognition results from direct interactions between groups called identity elements or determinants and from the presence of negative elements also called anti-determinants that prevent the binding of non-cognate molecules. The complete set of identity elements from *E. coli* has been elucidated and those from *Saccharomyces cerevisiae* and *Homo sapiens* are in progress [for a review see (2,3)]. In addition, a proper *in vivo* balance of the relative concentrations of tRNA and its cognate aaRS is also crucial for the maintenance of an accurate aminoacylation (4,5). For a long time it was accepted that 20 of these enzymes, one per amino acid, formed the essential set for protein biosynthesis. This distribution agreed with an early apparition and an evolutionary history associated with the co-development of the genetic code and biosynthetic pathways for amino acids. However, results from systematic genome sequencing programs led to revision of this concept for prokaryotes and archaea. Indeed, it appears that many of these organisms lack one or two aaRSs. Most bacteria, archaea and organelles lack glutamyl-tRNA synthetase (GlnRS) (6), and most archaea and some bacteria lack asparaginyl-tRNA synthetase (AsnRS) (7). In these organisms, non-discriminating glutamyl- and aspartyl-tRNA synthetases charge tRNA<sup>Glu,Gln</sup> and tRNA<sup>Asp,Asn</sup> with Glu and Asp, respectively. In a second step, the misacylated Glu-tRNA<sup>Gln</sup> and Asp-tRNA<sup>Asn</sup> are transamidated by a transamidase in order to give the correct

\*To whom correspondence should be addressed. Tel: +33 3 88 41 70 42; Fax: +33 3 8860 2218; Email: G.Eriani@ibmc.u-strasbg.fr

Present address:

Sharief Barends, Department of Biochemistry, Leiden Institute of Chemistry, Leiden University, P.O. Box 9502, 2300 RA Leiden, The Netherlands

products required for accurate protein synthesis (8,9). These spontaneous examples of misacylation contrast with the property of strict specificity claimed for these enzymes during several decades. For this reason, the elucidation of the molecular mechanisms used by these misacylating enzymes to recognize their non-cognate tRNA became recently a subject of interest (10,11). Mutagenesis experiments performed on *T. thermophilus* GluRS showed that the discriminating properties for the Glu and Gln anticodons is achieved by a single Arg residue (Arg-358) that recognizes C36 in tRNA<sup>Glu</sup> (10). On the other hand, phylogenetic analyses on discriminating and non-discriminating AspRS sequences identified two amino acid residues that are responsible for the discrimination of tRNA<sup>Asp</sup> from tRNA<sup>Asn</sup> (11). These two studies showed that only limited amino acid changes could induce the switch between the discriminating and non-discriminating enzymes. However, the fact that in both cases the tRNA specificity could only be expanded and not strictly changed suggests that the mechanism of evolution of tRNA recognition is a subtle process that does not depend on single amino acid changes but on several consecutive mutational steps. Recent studies performed on organisms that harbor duplicated GluRSs reported the presence of such a transient GluRS-like ancestor of GlnRS that is a specific Glu-tRNA<sup>Gln</sup>-forming enzyme unable to catalyze the Glu-tRNA<sup>Glu</sup> formation (12,13). This example confirms that sequential events probably played a preponderant role in the evolutionary process that led to the contemporary aaRSs. Interestingly, several attempts to reprogram the tRNA recognition properties of aaRS have been performed on MetRS (14–17) and class II LysRS (18). Elements that control the recognition of the tRNA were identified far from the catalytic core structure, usually in small idiosyncratic peptides. Likewise, the reprogramming of the tRNA recognition was almost achieved when working on the closely related anticodon-binding sites of MetRS and IleRS (15).

In this study, we investigated whether we can reprogram the tRNA<sup>Asp</sup> recognition property of AspRS by mutating the whole gene and by selecting active mutants of AspRS with a new tRNA identity. The strategy used is based on the early concept that the anticodon of tRNA is a major recognition site for aaRSs to discriminate between various tRNAs and to confer the 'superspecificity' of aminoacyl-tRNA formation [for a review see (19)]. We modified the aspartic anticodon GUC that is crucial for tRNA<sup>Asp</sup> identity and replaced it by an amber anticodon CUA that inactivated aminoacylation of the tRNA<sup>Asp</sup><sub>CUA</sub>. Then, we selected reprogrammed AspRSs able to charge this tRNA by using an *in vivo* screen based on the suppression of *argE* and *lacZ* amber mutations. Most of the mutations were confined in the OB-fold domain that binds the tRNA anticodon. Interestingly, three additional mutations were located far from the anticodon-binding domain, in the catalytic core and hinge module that connects the OB-fold to the catalytic site. These data suggest that several efficient ways can be used to remodel the tRNA specificity of a pre-existing aaRS. Furthermore, it is not excluded that the change of specificity can be improved by the acquisition of appended domains. The multidomain organization and the structural diversity

actually seen with modern synthetases clearly support this hypothesis.

## MATERIALS AND METHODS

### Bacterial strains and vectors

TG1 [ $\Delta(lac-proAB)$ , *supE*, *thi*, *hsdD5*, *F'(traD36, proAB<sup>+</sup>, lacI<sup>q</sup>ZAM15)*] was used for plasmid-DNA transformation. JM103 [ $\Delta(lac-proAB)$ , *thi-1*, *strA*, *supE*, *endA*, *sbcB15*, *hsdR4*, *F'(traD36, proAB<sup>+</sup>, lacI<sup>q</sup>ZAM15)*] (20) was used to overexpress the mutated *aspS* genes. The mutator XL1-red strain [*F<sup>-</sup>*, *endA*, *gyrA96*, *thi-1*, *hsdR17*, *supE44*, *relA1*, *lac*, *mutD5*, *mutS*, *mutT*, *Tn10 (tet<sup>r</sup>)*] (21) was from Stratagene (La Jolla, CA). 121R [*ara*, *argE<sub>am</sub>53*,  $\Delta(lac-proB)$ , *nalA*, *rpoB*, *thi*, *recA56n*, *srl300::Tn10 F'(proA<sup>+</sup>B<sup>+</sup>, lacI-Z<sub>am</sub>181)*] (22) was used for the selection of mutated *aspS* genes able to charge amber tRNA<sup>Asp</sup> (tRNA<sup>Asp</sup><sub>CUA</sub>) that suppresses *argE<sub>am</sub>* and *lacI-Z<sub>am</sub>*, conferring the Arg<sup>+</sup> and Lac<sup>+</sup> phenotypes, respectively. CS143 (*F<sup>-</sup>*, *thi-1*, *hisG4*,  $\Delta(gpt-proA)62$ , *argE3*, *thr-1*, *leuB6*, *kdgK51*, *rfbD1*, *ara-14*, *lacY1*, *galK2*, *xyl-5*, *mtl-1*, *supE44*, *tsx-33*, *rpsL31*, *tls-1*, *eda-51*, *ter<sup>s</sup>*) (23) contains the *aspS* mutation conferring a thermosensitive phenotype on low salt medium at 42°C (LB complemented with 0.5 g/l NaCl) (24).

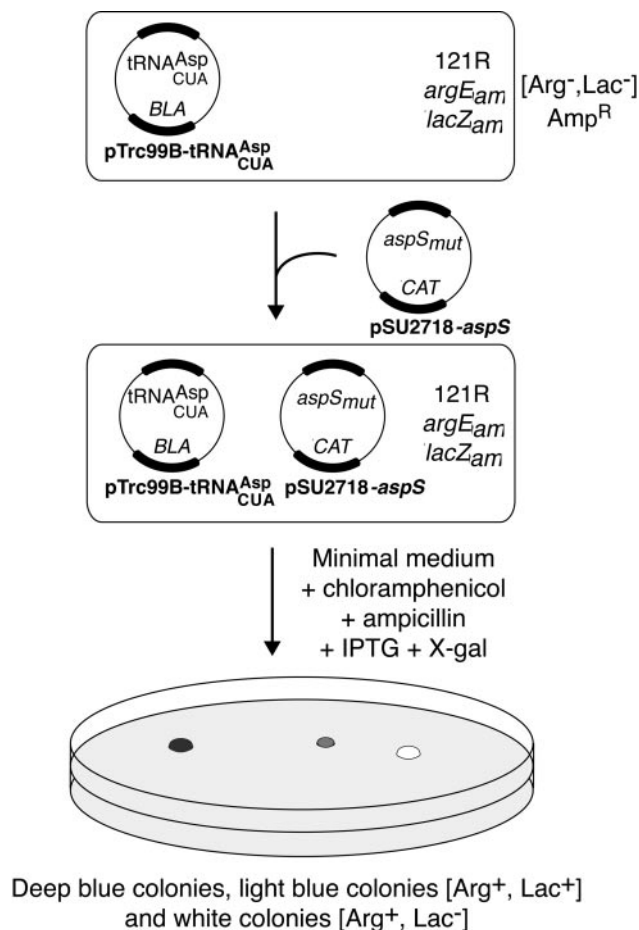
Plasmid pSU2718-*aspS* (25) contains a P15A replicon and the chloramphenicol resistance gene, which allows a moderate expression of the *aspS* gene. Plasmid pTrc99B-tRNA<sup>Asp</sup><sub>CUA</sub> (26) contains the strong hybrid promoter *trc* upstream of the tRNA<sup>Asp</sup><sub>CUA</sub> gene (27).

### Construction of the mutated library and selection of *aspS* suppressor genes

The randomly mutated library of *aspS* was generated by amplifying pSU2718-*aspS* into the mutator strain XL1-red. The frequency of the mutation was controlled by direct sequencing of several *aspS* genes. An aliquot of the library was introduced into the strain (121R, pTrc99B-tRNA<sup>Asp</sup><sub>CUA</sub>) and plated on Luria-Bertani (LB) medium containing chloramphenicol (20 µg/ml) and ampicillin (100 µg/ml) (Figure 1). After 16-h of incubation at 37°C the colonies were replicated on M9 medium supplemented with chloramphenicol, ampicillin, isopropyl-β-D-thiogalactopyranoside (IPTG) (1 mM) and the chromogenic substrate X-Gal (20 µg/ml). After 24 up to 96 h of incubation at 37°C small white colonies (Arg<sup>+</sup>) and normal-sized blue colonies (Arg<sup>+</sup>, Lac<sup>+</sup>) appeared on the M9 medium. To verify that the phenotype was linked to the *aspS* allele, the plasmid DNA was extracted from the positive clones and used to transform again the indicator strain (121R, pTrc99B-tRNA<sup>Asp</sup><sub>CUA</sub>) under the same selective conditions. Only those that confirmed the phenotypes (Arg<sup>+</sup>) and (Arg<sup>+</sup>, Lac<sup>+</sup>) were purified and sequenced.

### Purification of tRNA<sup>Asp</sup><sub>CUA</sub> and of the mutated AspRSs

The bacterial cells, 121R, expressing tRNA<sup>Asp</sup>, and tRNA<sup>Asp</sup><sub>CUA</sub> from the *trc* promoter (pTrc99B) were induced with IPTG and purified as previously described (28). To overproduce the mutated AspRS enzymes, their genes were first excised from pSU2718 as BamHI-HindIII fragments and cloned into pBluescript KS vector, then expressed in strain JM103. Protein purification was essentially performed as described previously (29).



**Figure 1.** Schematic representation of the genetic screening procedure used for the selection of the lethal ArgRS genes. The 121R strain [Arg<sup>-</sup>, Lac<sup>-</sup>] expressing tRNA<sup>Asp</sup><sub>CUA</sub> from pTrc99B-tRNA<sup>Asp</sup><sub>CUA</sub> was transformed with the mutated *aspS* library (pSU2718-*aspS*). The resulting strain was grown on minimal medium supplemented with chloramphenicol, ampicillin, IPTG and X-gal. Blue colonies were [Arg<sup>+</sup>, Lac<sup>+</sup>], whereas white colonies were only [Arg<sup>+</sup>]. pSU2718 vectors were extracted and phenotypes were confirmed by a second transformation of strain 121R.

### Aminoacylation assays

Aminoacylation activities of the purified enzymes was assayed by adding the adequate amount of enzyme to the reaction mixture containing 100 mM Tris-HCl (pH 7.5), 30 mM KCl, 10 mM ATP, 22 mM MgCl<sub>2</sub>, 5 mM glutathione, 0.1 mg/ml bovine serum albumin, 0.1 mM [<sup>14</sup>C]Asp (25 000 c.p.m./nmol) (CEA, France), and 10 mg/ml unfractionated *E. coli* tRNA. At constant time intervals of incubation at 37°C, aliquots of the reaction mixture were assayed for [<sup>14</sup>C]Asp-tRNA<sup>Asp</sup> formation. The *K<sub>M</sub>* values for aspartic acid, ATP and tRNA<sup>Asp</sup> in the aminoacylation reaction were determined by varying the concentration of the assayed substrate over a 10-fold range. Amber tRNA<sup>Asp</sup><sub>CUA</sub> was an extremely poor substrate for wild-type AspRS. Increasing tRNA<sup>Asp</sup><sub>CUA</sub> concentrations up to 15 μM showed linear increases of the aspartate acceptance, indicating that this concentration was far below the *K<sub>M</sub>* for the tRNA. Under these conditions, accurate values for the kinetic parameters for the aminoacylation with aspartate could not be obtained. Consequently, the catalytic efficiencies, *k<sub>app</sub>*/*K<sub>M</sub>* were determined as described (30).

**Table 1.** Mutations selected into the *E. coli* AspRS, phenotypes and corresponding residues in the yeast AspRS

<i>E. coli</i> AspRS substitution	Color phenotype	Corresponding yeast AspRS residue
D29N	Deep-blue	Q120
L30F	Deep-blue	Q121
T89I	Light-blue	V184
G90S	Light-blue	Q185
G90V	Light-blue	Q185
E93K	Deep-blue	E188
E118K	Light-blue	L228
R383C	White	No equivalent <sup>a</sup>
A471T	White	S467

<sup>a</sup>R483C is localized in the prokaryotic-specific insertion domain.

The amino acid activation rate was measured by the ATP-PPi exchange rate. The reaction mixture contained 100 mM HEPES (sodium salt) (pH 7.2), 10 mM MgCl<sub>2</sub>, 2 mM [<sup>32</sup>P]PPi (1–2 c.p.m./pmol) (Amersham Biosciences), 2 mM ATP, 10 mM aspartic acid and 10 mM KF. The reaction was started by addition of the enzyme and incubated at 37°C. At regular time intervals, aliquots were removed and measured for synthesized [<sup>32</sup>P]ATP.

## RESULTS

### Nine mutants of AspRS are able to suppress the Arg<sup>-</sup> phenotype

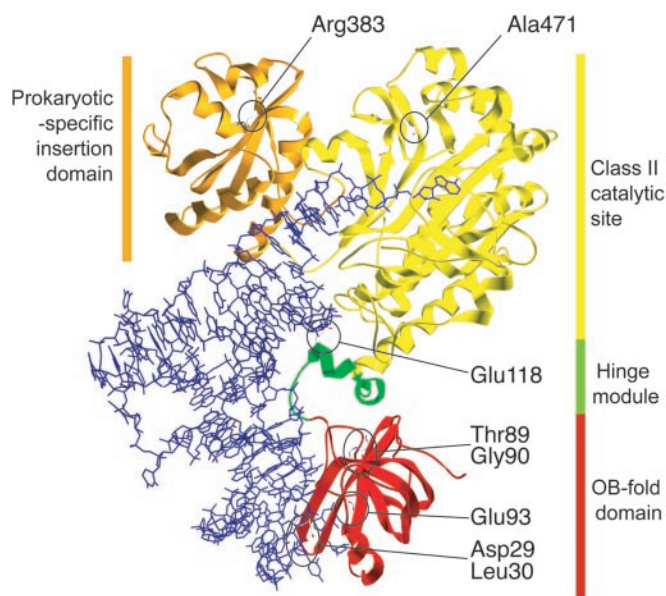
Strain 121R harboring pTrc99B-tRNA<sup>Asp</sup><sub>CUA</sub> was transformed with the pSU2718-*aspS* mutated library and 10<sup>5</sup> colonies were screened (Figure 1). On selective medium, a total of 20 blue (Lac<sup>+</sup> and Arg<sup>+</sup>) and white (Arg<sup>+</sup>) colonies appeared after 24 up to 96 h of growth. Only nine of them confirmed the suppression phenotype after recloning and selective growth. Three clones were deep blue, four were only light blue and two were white. The color difference was used as the first indicator for the suppression strength. Indeed, strain 121R contains two amber mutations that differ in their response to suppression. The mutation *argE<sub>am</sub>53*, which is more sensitive to suppression, is responsible for the growth on minimal medium with a white phenotype. On the other hand, the *lacI-Z<sub>am</sub>181* mutation requires a much higher level of suppression to confer the blue phenotype on X-gal-containing medium (22,27).

The entire *aspS* genes (1770 bp), as well as >100 nt on both 5' and 3' ends, were sequenced in order to control the promoter and terminator regions. Nine isolated substitutions were found (Table 1). Six were located in the OB-fold domain (D29N, L30F, T89I, G90S, G90V and E93K), one in the hinge region (E118K), one in the prokaryotic insertion domain (R383C) and the last in the catalytic site (A471T) (Figures 2 and 3).

### The mutated AspRSs are still able to charge the wild-type tRNA<sup>Asp</sup>

The nine pSU2718 plasmids carrying the mutated *aspS* genes were introduced into the strain CS143, which exhibits a thermo-sensitive phenotype on low salt medium due to a single mutation (P555S) in the chromosomal allele of *aspS* (24). Transformants were first plated on LB medium in the presence

of chloramphenicol and incubated at 30°C for 36 h. Then, the colonies were replicated on two series of low-salt LB-cam plates and one was incubated at 42°C and the other at 30°C (control). All nine transformants gave the same growth phenotype as the wild-type pSU2718-*aspS*, whereas the pSU2718-*aspS* (P555S) mutant failed to complement the mutated strain (24) (Figure 4). This result shows that the nine AspRSs are still able to aminoacylate the native tRNA<sup>Asp</sup> *in vivo* at a level

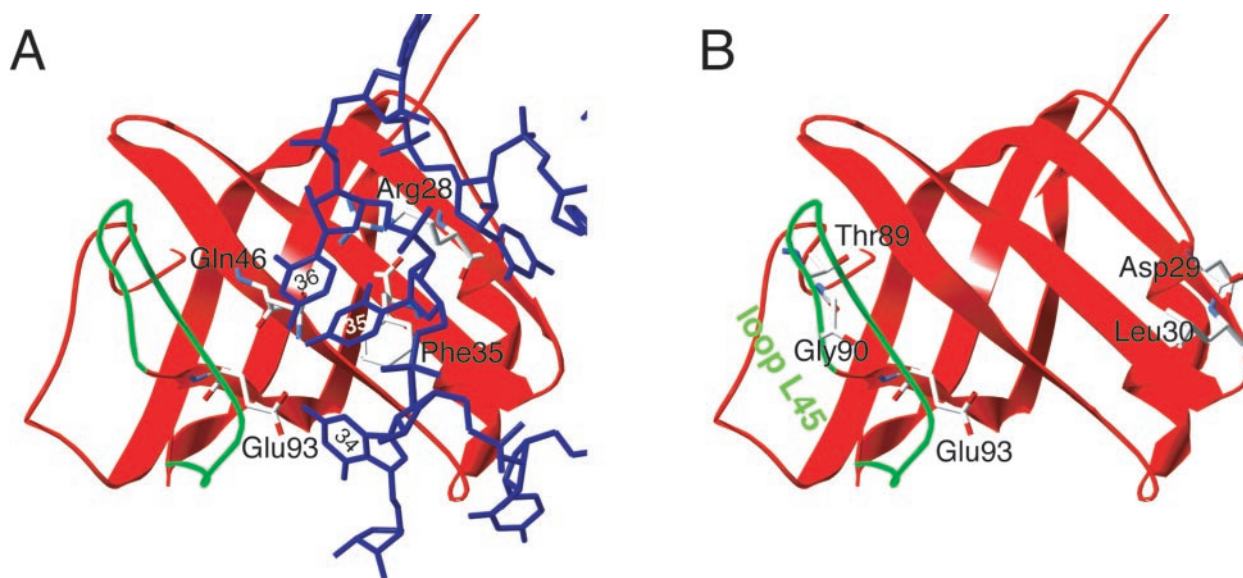


**Figure 2.** Overview of the *E. coli* AspRS:tRNA<sup>Asp</sup> complex [Protein Data Bank (PDB) ID code: 1COA] (31). Only one monomer is shown. The protein modules are colored in red (OB-fold domain), green (hinge module), yellow (class II catalytic site) and orange (prokaryotic-specific insertion module). The tRNA<sup>Asp</sup> molecule is colored in blue. The mutated protein residues are indicated.

sufficient to complement the defective strain CS143. However, we cannot exclude that complementation resulted from the reactivation of the thermosensitive AspRS subunits by heterodimer-assembly with the suppressor AspRS subunits.

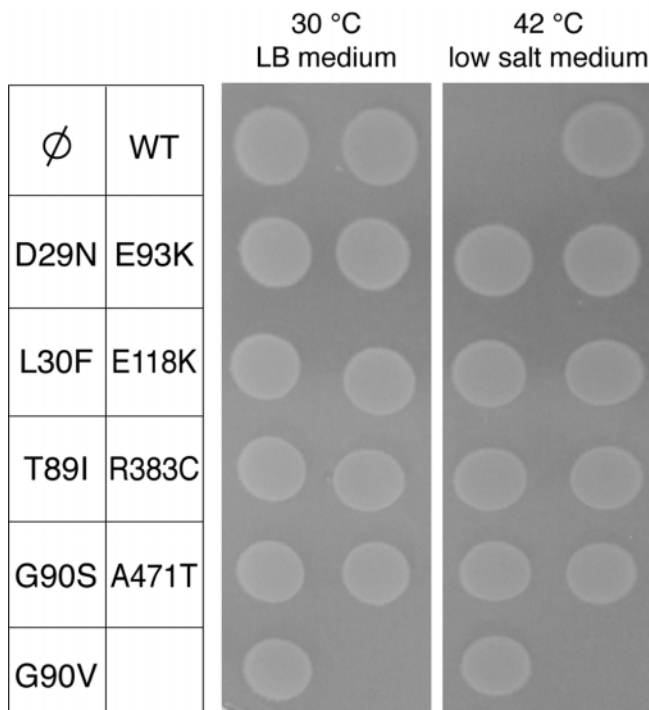
### Aminoacylation properties of the mutated AspRSs

Four mutated AspRSs and the wild-type enzyme were purified and analyzed for their enzymatic properties. Mutant E93K was chosen because the wild-type residue interacts with Q34 of tRNA<sup>Asp</sup><sub>QUC</sub> (31) and the selected mutation Lys-93 now permits the recognition of tRNA<sup>Asp</sup><sub>CUA</sub>. Moreover, the yeast counterpart of Glu-93 is Glu-188, which has been well studied (28,32). Leu-30 is the spatial equivalent of yeast Gln-121, which interacts with nucleotides 35 and 38 (32,33). E118K is located in the hinge region and could act on the tRNA-anticodon binding or on the tRNA aminoacylation by a distal effect. The last mutant is A471T that is located in the catalytic core far away from the tRNA-anticodon binding site. The yeast spatial equivalent, Ser-467, was selected as a lethal mutation in a previous study (34). Enzymes were purified to homogeneity and assayed for aminoacylation with purified tRNA<sup>Asp</sup><sub>QUC</sub> and tRNA<sup>Asp</sup><sub>CUA</sub> (Table 2). It appears that all the mutated AspRSs are now more efficient for the aminoacylation of the amber tRNA<sup>Asp</sup><sub>CUA</sub>. Compared to the basal level of aminoacylation of the wild-type AspRS, it appears that a modest 2.5-fold increase in the acylation rate of the amber tRNA is sufficient to suppress *argE* mutation *in vivo*, as shown for mutant A471T, which has a white phenotype. Above a 3-fold increase in the acylation rate of the amber tRNA, mutants exhibited a blue phenotype resulting from the suppression of the *lacZ* amber mutation. Analysis of charging properties of the native tRNA<sup>Asp</sup><sub>QUC</sub> shows various effects on the enzyme properties. Mutant L30F shows basically no effect. Mutant A471T displays significant decreases in the ATP-PP<sub>i</sub> exchange activity with a concomitant reduction of ATP binding strength (Table 3). Mutant E93K exhibits a perfect uncoupling of the



**Figure 3.** View of the anticodon-binding site of AspRS (red color) complexed with tRNA<sup>Asp</sup> (blue color). (A) Nucleotides of the tRNA<sup>Asp</sup> anticodon loop are shown. U35 interacts with the class IIb triad Arg-28-Phe-35-Gln-46, and residue Glu-93 binds Q34. (B) The five residues that were selected as suppressors of amber tRNA<sup>Asp</sup> are numbered. Residues Asp-29 and Leu-30 are located in strand S1. Residues Thr-89 and Gly-90 are located in loop L45 (green color) and residue Glu-93 in strand S5. The OB-fold strands have been numbered.

aminoacylation and activation reaction. This enzyme is able to form the aspartyl-adenylate but shows a very low aminoacylation capacity that was nevertheless enough to complement the thermosensitive *aspS* strain (see above). On the other hand, E118K displays a 6.5-fold increase in aminoacylation rate, and a 3-fold decrease in the amino acid activation reaction. Altogether, these data show that there are different ways to transform the AspRS molecule in a new enzyme with an additional tRNA specificity. The common requirement is to increase the aminoacylation rate of the amber tRNA, but this can be obtained with or without any effect on the aminoacylation properties of the native tRNA.



**Figure 4.** Complementation assay of the thermosensitive CS143 strain carrying the *aspS tls-1* mutation (23). pSU2718 vectors, carrying the different mutations and the wild-type *aspS* were introduced into CS143 strain. Cam<sup>R</sup> colonies were subjected to drop test on LB or low-salt LB medium at 30 and 42°C, respectively. The  $\emptyset$  label is for the pSU2718-*aspS-tls-1* vector expressing the thermosensitive AspRS (24), which does not rescue CS143 strain at 42°C.

## DISCUSSION

### Efficient suppression results from an increase of a basal suppressing property

The present work describes the search for *E. coli* AspRS mutants able to recognize the amber tRNA<sup>Asp</sup><sub>CUA</sub>. The substitution of the aspartic GUC anticodon by the amber CUA anticodon leads to a drastic reduction in aminoacylation rate (30 000-fold) and aminoacylation efficiency ( $\sim 10^6$ -fold) (Table 2). *In vivo*, this residual charging activity is too low to confer growth to the 121R strain expressing amber tRNA<sup>Asp</sup><sub>CUA</sub> and the wild-type AspRS from the chromosome. Strikingly, even when the AspRS gene number was increased by means of a plasmid with a p15A replicon (about 15 copies per cell), the strain 121R was still unable to grow. However, when we further increased the overexpression of AspRS, by using the high copy plasmid pBluescript, we observed a suppression of the *argE* and *lacZ* amber codons of strain 121R (data not shown). This was not unexpected considering the high copy number of the pBluescript vectors (at least 100 copies), which significantly increased the basal level of amber tRNA<sup>Asp</sup><sub>CUA</sub> charging by AspRS. Consequently, to avoid the spontaneous suppression, a pSU2718-*aspS* library based on the p15A replicon was constructed and screened for active suppressors in AspRS. Under these conditions, assuming that about 15 copies of this plasmid are present per cell (35), and that no regulation of AspRS expression takes place in *E. coli*, we expect an overexpression of the AspRS mutants of 15-fold compared to the basal genomic level. Thus, to determine the *in vivo* charging activity of the mutants, the modest increases of the tRNA<sup>Asp</sup><sub>CUA</sub> charging activities measured *in vitro* with the AspRS suppressors (2.5- to 6-fold) should be multiplied by a factor corresponding to the plasmid number. Nevertheless, these theoretical charging activities present in the mutated strains remain moderate compared to the wild-type activity of tRNA<sup>Asp</sup> charging. To isolate more efficient synthetase mutants several problems should be overcome. One is inherent to the *in vivo* procedure and concerns the toxicity that a very efficient suppressor could have for the cell. The synthesis of aberrant proteins could reach levels incompatible with cell survival and it is predictable that these highly efficient suppressors could not be selected due to the lethality. A second problem is that the improvement of the suppression efficiency may require more than one mutation. As we have used mutagenic conditions to only mutate one residue, we missed these combinations of mutants in our library.

**Table 2.** Aminoacylation properties of the wild-type and mutated *E. coli* AspRSs

	tRNA <sup>Asp</sup> <sub>QUC</sub> $k_{cat}$ (s <sup>-1</sup> )	Aminoacylation		Relative	tRNA <sup>Asp</sup> <sub>CUA</sub> $k_{app}^a$ ( $\times 10^{-4}$ ) (s <sup>-1</sup> )	Aminoacylation	Relative	L <sup>c</sup> ( $\times$ fold)
		$K_M$ ( $\mu$ M)	$k_{cat}/K_M$ (s <sup>-1</sup> · $\mu$ M <sup>-1</sup> )	( $k_{cat}/K_M$ )		$k_{app}/K_M^b$ ( $\times 10^{-6}$ ) (s <sup>-1</sup> · $\mu$ M <sup>-1</sup> )	( $k_{app}/K_M$ ) <sup>b</sup>	
Wild type	12 $\pm$ 1	0.5	24	1	4 $\pm$ 0.5	7	1	3.4 $\times 10^6$
L30F	12 $\pm$ 0.5	1.0	12	0.5	12 $\pm$ 1	16	2.3	0.75 $\times 10^6$
E93K	0.05 $\pm$ 0.01	nd	nd	nd	25 $\pm$ 2	40	5.7	nd
E118K	79 $\pm$ 4	0.55	144	6	20 $\pm$ 2	26	3.7	5.5 $\times 10^6$
A471T	8 $\pm$ 1	0.65	12	0.5	10 $\pm$ 1	14	2.0	0.85 $\times 10^6$

nd, not determined.

<sup>a</sup>Aminoacylation with 10  $\mu$ M of tRNA<sup>Asp</sup><sub>CUA</sub>.

<sup>b</sup>According to (30).

<sup>c</sup>L values are losses of catalytic efficiencies of amber tRNA<sup>Asp</sup><sub>CUA</sub> compared to native tRNA<sup>Asp</sup><sub>QUC</sub> [ $L = (k_{cat}/K_M)_{native}/(k_{app}/K_M)_{amber}$ ].

**Table 3.** Michaelis constants of the wild-type and mutated AspRSs for aspartate and ATP

	Aspartate <sup>a</sup> $K_M^a$ (mM)	$k_{cat}^a$ (s <sup>-1</sup> )	ATP <sup>b</sup> $K_M$ ATP <sup>b</sup> ( $\mu$ M)	$k_{cat}^b$ (s <sup>-1</sup> )
Wild type	1.3 $\pm$ 0.15	30 $\pm$ 3	91 $\pm$ 7	12 $\pm$ 1
L30F	1.4 $\pm$ 0.2	30 $\pm$ 4	92 $\pm$ 8	12 $\pm$ 0.5
E93K	1.4 $\pm$ 0.15	32 $\pm$ 5	50 $\pm$ 5	0.05 $\pm$ 0.01
E118K	1.2 $\pm$ 0.2	11 $\pm$ 2	100 $\pm$ 5	79 $\pm$ 4
A471T	1.3 $\pm$ 0.2	3 $\pm$ 0.5	1110 $\pm$ 15	8 $\pm$ 1

<sup>a</sup>Measured in the ATP—PPi exchange reaction.<sup>b</sup>Measured in the aminoacylation reaction of tRNA<sup>Asp</sup><sub>CUA</sub>.

To solve this problem, a second round of mutation and selection should be performed on these mutated synthetases.

### Most of the selected mutations are located in the anticodon-binding domain of AspRS

AspRS belongs, with asparaginyl-, and lysyl-tRNA synthetases (AsnRS and LysRS, respectively) to class IIb of aaRSs. In this subclass of enzymes, the discriminator base and the anticodon are the major identity elements (3). In addition, AspRS also recognizes base pairs G10:U25, G2:C71 and base C38 (36–39). One key element of the tRNA–anticodon recognition of these enzymes is the appended N-terminal domain, which has an OB-folded conformation (Figure 3). Such a fold is characterized by a five-stranded Greek-key  $\beta$ -barrel capped by an  $\alpha$ -helix between the third and the fourth strand. This fold has been found in various nucleic acid-binding proteins, in oligosaccharide-binding proteins, in inhibitors of metalloproteinases and in histidine kinases (40,41). In class IIb aaRSs, the presence of this fold is related to the recognition of the central base of the anticodon, which is always a U in the aspartate, asparagine and lysine systems. In various three-dimensional (3D) structures of class IIb aaRSs:tRNA complexes, the binding mode of the anticodon stem and loop of the tRNA to the OB-fold is nearly the same (31,33,42–44). The OB-fold provides a rigid scaffold on which some almost strictly conserved residues are invariably found in the synthetases of this group (AspRS, AsnRS and LysRS). The conserved interactions that take place with the tRNA XUX–anticodons involve residue Arg-28 (interacts with O2 of U35), Phe-35 (stacks with U35) and Gln-46 (interacts with the N3 of U35) in *E. coli* AspRS (Figure 3A) (31).

To restrict the tRNA specificity of the OB-fold to the GUX–anticodons of tRNA<sup>Asp</sup> and tRNA<sup>Asn</sup>, AspRS and AsnRS use in addition an acidic residue located at the first position of the fifth strand (Glu-93 and Glu-91 in the *E. coli* enzymes, respectively). In AspRS, Glu-93 is hydrogen-bonded with the N1 and N2 atoms of base 34, which can either be a queueine (Q) or a guanine in prokaryotic or eukaryotic tRNA<sup>Asp</sup>, respectively.

An additional major structural element that achieves tRNA recognition is the loop that joins the fourth and fifth strand of the  $\beta$ -barrel [L45; (40)]. In *E. coli* AspRS, this loop contains residues that contact the three anticodon bases (residues 77–92) (31). Idiosyncratic residues that specify aspartic, asparagine and lysine identities have been found at this level, and specific motifs have been derived from synthetase

sequences (18). It was also shown that transplanting the AspRS loop into the LysRS sequence generates a LysRS inactive in tRNA<sup>Lys</sup> charging, which has acquired a new tRNA<sup>Asp</sup> charging activity with lysine (18).

We have selected seven mutants in the OB-fold that have acquired new capacity of charging the amber suppressor of tRNA<sup>Asp</sup>. The most remarkable one is probably the mutation E93K that has lost most of its capacity to interact with Q34. The resulting aminoacylation rate of tRNA<sup>Asp</sup> is very low, whereas the one of tRNA<sup>Asp</sup><sub>CUA</sub> is the highest measured. This effect suggests that the original Glu-93–Q34 interaction has been substituted by a Lys-93–C34 interaction. This also confirms the crucial role of amino acid residue 93 for the recognition of nucleotide 34, which can either be a Q34 when a Glu residue is found or a C34 when a Lys residue is found in AspRS. Strikingly, this effect is transposable to the *S. cerevisiae* system since an orthogonal suppressor pair was derived from *E. coli* based on the yeast tRNA<sup>Asp</sup><sub>CUA</sub> and the E93K corresponding AspRS mutation (E188K) (45). Mutant E188K was also found in yeast genetic selection for lethal mutations of the yeast AspRS. The aminoacylation rate of this mutant was three-orders of magnitude lower accompanied with a 75-fold increased  $K_d$  for tRNA<sup>Asp</sup> (34). In another study, the deficit of tRNA binding energy in the ground state ( $\Delta\Delta G_b$ ) for the E188A mutation was found to be 1.56 kcal/mol, a value that could account for the loss of the two hydrogen bonds with G34 (32). Thus, Glu-93 in *E. coli* or Glu-188 in *S. cerevisiae*, are necessary elements of the tRNA<sup>Asp</sup> recognition. They show the same drastic defects on tRNA<sup>Asp</sup> charging when mutated, and in addition, they have a latent capacity for a moderate but new specificity for C34.

The relatively modest tRNA<sup>Asp</sup><sub>CUA</sub> charging efficiency observed for the E93K mutation when compared to the wild-type charging activity suggests that other determinants of the tRNA recognition are present in AspRS that can limit the specificity change. Loop L45 that connects the fourth to the fifth strand, contacts the three bases of tRNA<sup>Asp</sup> anticodon (Figure 3B) (18,31). In this loop, we isolated the T89I, G90S and G90V mutations that induce a weak phenotype of suppression (light-blue colonies). These residues do not directly contact the tRNA but are suspected to play indirect roles in the suppression phenomenon. Thr-89 is located at >7 Å away from the tRNA, and its side chain is oriented toward strand S4. Thus, the T89I mutation might destabilize this environment and induce some structural modifications responsible for the new recognition property. The adjacent residue (Gly-90) exhibited the same phenotype when mutated in Ser or Val. In this case, the addition of new polar or hydrophobic side chains might create extra contacts that may modify the angle formed by the loop at this level. Thus, mutations of residues 89 and 90 might induce a different positioning of the loop L45 that contacts the three bases of the anticodon. This would allow the recognition of the modified CUA anticodon instead of the GUC anticodon.

The two D29N and L30F mutations affect the proximal environment of residue Arg-28, one of the key class IIb-specific residues responsible of U35 and C36 binding (see above). The amido group of the Asn-29 mutant is susceptible to prevent the formation of the hydrogen bond formed with the main chain of Gly-31. This would alter the conformation of the end of strand S1 and modify the structure of the turn and



the strand S2 on which the crucial Phe-35 is found. As the D29N mutation, the L30F mutation is a conservative change that might only slightly affect the hydrophobic core made of Leu-30, Leu-33 and Phe-35 (a class IIb-specific residue). No effect is observed on the charging of native tRNA<sup>Asp</sup>, suggesting that a simple relaxation of the tRNA specificity has been generated upon mutation.

#### **A mutation isolated in the hinge domain increases the tRNA charging rate**

In the hinge domain of *E. coli* AspRS, four interactions between Asn-113, Asn-116, Glu-119 and Thr-117 and the ribose groups of nucleotides 25, 11, 68 and the phosphate group of nucleotide 12 respectively have been described (31). Close to these positions, the E118K mutant is located, which we have selected in this study. This residue does not directly interact with the tRNA molecule, moreover, its side chain points in the opposite direction, towards the protein. Thus, Glu-118 appears to be involved in the stabilization of the hinge-domain structure and how the Lys mutation triggers the suppression phenotype is probably related to an indirect effect. The E118K mutation induces a concomitant increase in the charging rate of tRNA<sup>Asp</sup><sub>QUA</sub> and tRNA<sup>Asp</sup><sub>CUA</sub> by nearly the same factor of 7- and 5-fold, respectively. This suggests that the E118K mutation does not increase the selectivity for tRNA<sup>Asp</sup><sub>CUA</sub>, but increases unspecifically the catalytic process of aminoacylation by a distal effect. This hypothesis finds support in previous results obtained on yeast AspRS showing that the interactions occurring with the hinge domain are crucial for the correct positioning of A76 in the active site and for the transition-state stabilization of the aminoacylation reaction (32). Thus, the E118K mutation probably triggers a conformational change in the 3D structure of the hinge domain that might favor the fit of the acceptor stem into the active site. In a previous study, we showed that the anticodon nucleotides and the discriminator base do not bind the enzyme independently but rather connectively in favoring catalysis (32). Therefore, the hinge module was supposed to play an active role in transmitting the anticodon recognition signal to the catalytic site since it connects the tRNA-anticodon binding domain to the acceptor stem. Here we propose that the signal transmission might be modified by the E118K mutation in a way that it renders the enzyme less specific for a tRNA exhibiting anticodon nucleotide changes. This would explain the 5-fold increase of tRNA<sup>Asp</sup><sub>CUA</sub> charging rate compared to the native rate.

#### **Mutations in the active site and prokaryotic-specific insertion domain confer weak amber tRNA acylating properties**

In the catalytic site of AspRS, tRNA<sup>Asp</sup> is deeply buried in a cleft closed by several mobile loops of the catalytic domain and by the prokaryotic-specific insertion domain. The loops that contact the tRNA acceptor arm are the motif 2-loop, the histidine loop (a eubacterial-specific loop) and the flipping loop. In addition, the prokaryotic-specific insertion domain contacts the tRNA by water-mediated interactions (31). Mutations R383C and A471T are located in the prokaryotic-specific insertion domain and in the active site domain, respectively. Both residues do not interact with the tRNA molecule or any other substrate of the aminoacylation reaction. The side chain

of residue Arg-383 is located in helix H9 (31) and it points toward the center of prokaryotic-specific insertion domain where it stabilizes the fold by interacting with Asp-294 and Asp-297. Presumably, the R383C substitution changes the 3D structure of the domain, and may modify the interactions with the tRNA. This would allow the recognition of the amber tRNA, however, at a low level since this mutant had only a white phenotype of X-gal plates (see Results). Thus, like the E118K-hinge mutant, the R383C mutation selected in the prokaryotic-specific insertion domain seems to indirectly increase the basal suppression activity. Here also, several interactions occur between the prokaryotic-specific insertion domain and the tRNA, but how the domain structure and the interactions are altered by the mutation is unexplained by the actual data available.

Residue Ala-471 is located in one of the six strands of the class II antiparallel  $\beta$ -sheet forming the catalytic site. The importance of this residue was previously revealed by the lethal mutant selected at this position in yeast AspRS (34). Here, the selected A471T mutation decreases significantly the ATP-PPi exchange reaction (10-fold) as well as the ATP affinity (10-fold). On the other hand, the mutation keeps the charging reaction essentially unchanged for native tRNA<sup>Asp</sup><sub>QUA</sub> and 2.5-fold increased for tRNA<sup>Asp</sup><sub>CUA</sub>. Ala-471 is located in a strand that belongs to the magnesium-binding site, the co-factor of the amino acid activation reaction. Thus, one might propose that the A471T mutation induces a structural change that decreases the activation catalysis by an effect on the Mg<sup>2+</sup> binding. Mg<sup>2+</sup> ions are known to stabilize the ATP binding in its bent conformation and are supposed to play a role in the stabilization of the pentavalent transition state by interacting with the highly charged  $\alpha$ -phosphate of ATP. Thus, a slight change in the Mg<sup>2+</sup> positioning might explain the observed decrease of adenylate rate formation. Remarkably, this enzyme alteration is followed by a positive effect on the tRNA<sup>Asp</sup><sub>CUA</sub> charging, in a way reminiscent of the yeast AspRS mutant in which the charging activity for the native tRNA<sup>Asp</sup> was improved while the activation reaction was reduced up to 9-fold (46).

#### **Expanding tRNA recognition is a crucial step for new aminoacyl-tRNA synthetase emergence**

The specific acylation of tRNAs with their respective amino acids by aaRSs is critical for protein synthesis. These enzymes display an extremely high degree of specificity as shown by the low frequency of errors found in protein sequences (47). The term 'superspecificity' has been applied to these enzymes when compared to other enzymes of the metabolism-like proteases (48). This high degree of specificity is achieved by the combination of the kinetic and binding specificity (49). In other words, the substrate discrimination is achieved by the conformational adaptation of the enzyme and substrate during both binding and catalytic steps.

The existence of non-discriminating AspRS and GluRS in the bacterial and archaeal kingdoms (and organelles) does not really rule out the fundamental principles of tRNA recognition required for viability. These enzymes misacylate tRNAs, but the potential toxic charged tRNAs do not enter the translation cycle due to the absence of interaction with EF-Tu (50,51). The non-discriminating AspRS and GluRS exhibit expanded tRNA repertoires that allow the recognition of several tRNAs,

which is a situation quite common for aaRSs. Some aaRSs even recognize four to six tRNA isoacceptors. One remarkable point concerns the process used by the organisms that contain non-discriminating enzymes to evolve toward discriminating aaRSs. It is widely accepted that GlnRS probably evolved from a eukaryotic ancestor through duplication of a *glx* gene and later spread by horizontal gene transfer to the bacterial kingdom (52). The initial gene duplication was probably followed by gene mutation that led to two enzymes with distinct tRNA and amino acid specificity. Because the addition of glutamine to the genetic code is a recent event, similar *glx* gene-duplication events may have occurred in other organisms to give rise to different forms of GlnRS. Some of these processes are possibly still going on, as suggested by the recent discovery of a duplicated GluRS which is in fact a transient GluRS-like ancestor of GlnRS able to catalyze the Glu-tRNA<sup>Gln</sup> formation but not the Glu-tRNA<sup>Glu</sup> formation (12,13). This extant enzyme, whose activity is intermediate between the ancestral and the modern, might become converted to a specific GlnRS after transformation of its amino acid-binding site, or more easily, it might also be lost after acquisition of a GlnRS gene by horizontal gene transfer (12,13).

The present study mimics one of the evolutionary processes that led to the construction of aaRSs with new tRNA specificities. This study shows that the evolution of the tRNA recognition properties of an aaRS can be mimicked with a genetic screen based on a strong phenotypic selection and a randomly mutated library of aaRS genes. A new amber tRNA<sup>ASP</sup> specificity was created by individual mutations that were scattered along the different domains of the synthetase. The effect of the single mutations remains moderate compared to the wild-type activity but their number and diversity suggest that combining positive effects by multiple rounds of mutagenesis might significantly increase the newly acquired activity. The selected enzymes exhibit a dual specificity for the original Asp anticodon and for the new amber anticodon. However, the consequent loss of charging of the original tRNA<sup>ASP</sup> was observed for mutant E93K underlining the inherent capacity of these ambiguous enzymes to gain high specificity after only limited mutational steps. In addition, we cannot exclude the possibility that the change of specificity can be improved by the acquisition of appended peptides. Successful fusions of synthetase-core domains with relatively simple peptides were obtained to build the catalytic site for tRNA charging (53) or to achieve tRNA-specific aminoacylation (54). Thus, during evolution multiple residue changes, together with insertion of peptides or larger domains, could have played a major role in constructing the new aaRSs (55). The modular organization and the structural diversity found in modern synthetases clearly support this model (56).

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