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Transfer RNA Identity Change in Anticodon Variants of *E. coli* $tRNA^{Phe}$ *in Vivo*

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The anticodon sequence is a major recognition element for most aminoacyl-tRNA synthetases. We investigated the *in vivo* effects of changing the anticodon on the aminoacylation specificity in the example of *E. coli* $tRNA^{Phe}$. Constructing different anticodon mutants of *E. coli* $tRNA^{Phe}$ by site-directed mutagenesis, we isolated 22 anticodon mutant $tRNA^{Phe}$; the anticodons corresponded to 16 amino acids and an opal stop codon. To examine whether the mutant tRNAs had changed their amino acid acceptor specificity *in vivo*, we tested the viability of *E. coli* strains containing these $tRNA^{Phe}$ genes in a medium which permitted tRNA induction. Fourteen mutant tRNA genes did not affect host viability. However, eight mutant tRNA genes were toxic to the host and prevented growth, presumably because the anticodon mutants led to translational errors. Many mutant tRNAs which did not affect host viability were not aminoacylated *in vivo*. Three mutant tRNAs containing anticodon sequences corresponding to lysine (UUU), methionine (CAU) and threonine (UGU) were charged with the amino acid corresponding to their anticodon, but not with phenylalanine. These three tRNAs and $tRNA^{Phe}$ are located in the same cluster in a sequence similarity dendrogram of total *E. coli* tRNAs. The results support the idea that such tRNAs arising from *in vivo* evolution are derived by anticodon change from the same ancestor tRNA.

Keywords: Anticodon; Evolution; Identity; tRNA.

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Introduction

The major role of tRNA is to specify the amino acid transferred during the translation of genetic information into protein. This process is crucial for precise execution of the genetic code. The fidelity of this step depends on specific interactions between aminoacyl-tRNA synthetases and their cognate tRNAs. In a majority of organisms, there is an aminoacyl-tRNA synthetase and one or more cognate tRNAs for each amino acid for protein synthesis. However, some organisms appear to be missing one or more of the aminoacyl-tRNA synthetases (Ibba *et al.*, 1997; Low and Berry, 1996). Because tRNAs are regarded as one of earliest evolved molecules, it is possible that the tRNA existed before the advent of cognate aminoacyl-tRNA synthetases (Nagel and Doolittle, 1995; Ribas *et al.*, 1998). The generation of tRNA specificities is also important for the formation of the genetic code (Osawa *et al.*, 1992).

Except for several well-conserved nucleotides, tRNA sequences vary widely from acceptor to acceptor and organism to organism (Sprinzl *et al.*, 1991). But most tRNAs share a common secondary and tertiary structure. An aminoacyl-tRNA synthetase recognizes a set of nucleotides, known as tRNA identity elements (McClain, 1995), on their cognate tRNAs. In the last decade, tRNA identity elements have been the subject of much *in vitro* and *in vivo* study (Giege *et al.*, 1998; McClain, 1995; Normanly and Abelson, 1989) they concluded that tRNA identity is governed by positive and negative elements that respectively modulate acceptance and rejection by cognate or non-cognate aminoacyl-tRNA synthetases. Many reports demonstrate that, among tRNA identity elements, the anticodon and discriminator base (N73) are the most important positive identity elements (McClain, 1995). However, because many *in vivo* experiments for tRNA identity are based on suppressor tRNA, the effects of all tRNA anticodons could not be tested *in vivo*. Some studies

Abbreviations: IPTG, isopropyl-β-D-thiogalactopyranoside.

with initiator tRNA^{Met} anticodon mutants also showed identity changes corresponding to the anticodon sequence (Chattapadhyay *et al.*, 1990; Pallanck and Schulman, 1991). However, their results are based on a translational level of amino acid analysis rather than aminoacylation. Here we attempt to examine the aminoacylation consequences of anticodon changes in tRNA^{Phe}. tRNA genes are regarded as one of the earliest genes to arise (Eigen *et al.*, 1989). Many efforts to elucidate the evolution of tRNAs have already been reported (Dick and Schamel, 1995; DiGiulio, 1995; Eigen *et al.*, 1989; Rodin and Ohno, 1997; Saks *et al.*, 1998). From homology comparisons among primary sequences, tRNAs can be divided in several clusters (Saks *et al.*, 1998). Isoacceptors are usually contained in one cluster, but some clusters contain tRNAs of different amino acid specificity. We can assume that these tRNAs originated from the same ancestor tRNA. Based on the above, we have provided a possible mechanism for generating new tRNAs from existing tRNA genes. Using *E. coli* tRNA^{Phe} anticodon mutants, we deduce how these mutant tRNAs interact with cellular components *in vivo*.

Materials and Methods

Construction of the recombinant plasmids The sequence of the tRNA^{Phe} gene and its 3′ and 5′ flanking regions is shown in Fig. 1. This tRNA^{Phe} gene expression cassette segment was ligated to pUC118/119 plasmid. Before the introduction of this recombinant plasmid into an *E. coli* host, we confirmed the

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CGGTC  CCAAA  AGGGT  CAGTG  CTGCA  ACATT  TGCTG  CCGGT

                                    +1
CAGAA  TTCTA  ATACG  ACTCA  CTATA  GCCCG  GATAG  CTCAG
  EcoRI          T7 promoter          tRNAPhe

TCGGT  AGAGC  AGGGG  ATTGA  AAATC  CCCGT  GTCCT  TGGTT

                              +76
CGATT  CCGAG  TCCGG  GCACC  AGGTT  CATAT  AAACG  GACCC
                      BstNI

TGCAG  CCCAA  GCTGA  CGGAT  CCGGC  TGCTA  ACAAA  GCCCG
 PstI                 BamHI

AAAGG  AAGCT  GAGTT  GGCTG  CTGCC  ACCGC  TGAGC  AATAA

CTAGC  ATAAC  CCCTT  GGGGC  CTCTA  ACGG  GTCTT  GAGGG
                      Tϕ terminator

GTTTT  TTGCT  GAAAG  GAGGA  ACTAT  ATCCG  GATCT  GGCGT
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Fig. 1. The nucleotide sequence and restriction enzyme sites of the *E.coli* tRNA^{Phe} gene and its 5′ and 3′ flanking regions. The nucleotide numbered +1 is the transcriptional initiation site, and the nucleotide A, numbered +76, is the 3′ end of the tRNA^{Phe}. T7 promoter and Tϕ terminator sequences are indicated by bold letters.

production of chargeable tRNA^{Phe} by the *in vitro* transcription method (Kim and Lee, 1990). Because the transcription start +1G corresponds to the first base of the *E. coli* tRNA^{Phe}, the synthesized precursor requires no processing at the 5′-end. As the expression of mutant tRNAs can be very toxic to the cell, strict regulation of tRNA expression is necessary. In order to strictly regulate T7 RNA polymerase, the *lac* promoter region (*Pvu*II – *Eco*RV) was deleted from the plasmid. Plasmids were introduced into *E. coli* JM109 (DE3) that possessed a T7 RNA polymerase gene on the chromosome under the control of *lac*UV5 promoter. The tRNA^{Phe} was overexpressed by addition of isopropyl-β-D-thiogalactopyranoside (IPTG).

The isolated RNAs were separated on 6% polyacylamide gel containing 7.5 M urea, and stained with silver nitrate. The image of the visible bands was duplicated on EDF film (Promega), and the quantity of 76 nucleotide-sized tRNAs was determined by a densitometric method using 5S rRNA as an internal standard. The tRNA (76nt)/5S rRNA ratio is 0.27 from the *E. coli* host, 0.29 from the uninduced host containing recombinant plasmid, and 6.90 from the induced cell. Accordingly, tRNA^{Phe} is overproduced by 24-fold compared to the uninduced cell. The ratios of mutant tRNAs are mostly same as the wild-type (3.9–7.0), but both of the arginine anticodon mutants are significantly lower than the wild-type (0.5 and 2.3).

Site specific mutagenesis and selection of anticodon variants The plasmids were named by their anticodon sequence followed by the base in position 32 (e.g. 32T/GAA is the wild-type tRNA^{Phe} with the anticodon GAA and a U in position 32; see Fig. 1). The mutants were constructed by slightly modifying the standard methods of site-directed mutagenesis (Kunkel, 1985). Figure 2 shows the strategy for generating anticodon mutants. In order to simplify the enrichment of mutant tRNAs, a single-stranded DNA template was prepared from a recombinant plasmid carrying a mutant *E. coli* tRNA^{Phe} gene containing a unique *Sma*I restriction enzyme site in the tRNA's variable loop region (T45G). The mutagenic oligonucleotide was designed for reversion of the mutant gene (G45T) as well as generation of randomized anticodon sequences (34N, 35N, 36N). The nucleotide in position 32 was also changed to C, which is generally found in *E. coli* tRNA. The nucleotide sequence of the random mutagenic oligonucleotide is shown in Fig. 2. The heteroduplex DNA resulting after mutagenesis and T4 DNA polymerase treatment was transformed into the *E. coli* WK6*mut*S host strain. After inoculation into 2X YT medium containing ampicillin (50 μg/ml) and growth of transformants overnight, plasmid DNA was isolated from the cells. This plasmid DNA was digested with *Sma*I (to remove the anticodon-unchanged tRNA^{Phe} gene), the undigested plasmid was isolated from the agarose gel, and then transformed into *E. coli* JM109(DE3). To select anticodon mutant clones, two different solid media were used. The first one, the tRNA repression medium, contained 0.2% glucose for repressing T7 RNA polymerase, as the *lac*UV5 promoter can be repressed completely by adding glucose. The second, the tRNA induction medium contained 0.5 mM IPTG for T7 RNA polymerase induction. The total mixture of transformants possessing the randomized anticodon were spread on the tRNA repression plate and incubated overnight at 37°C. To choose growth positive and negative clones, each colony of the transformant was streaked out on both media. Some

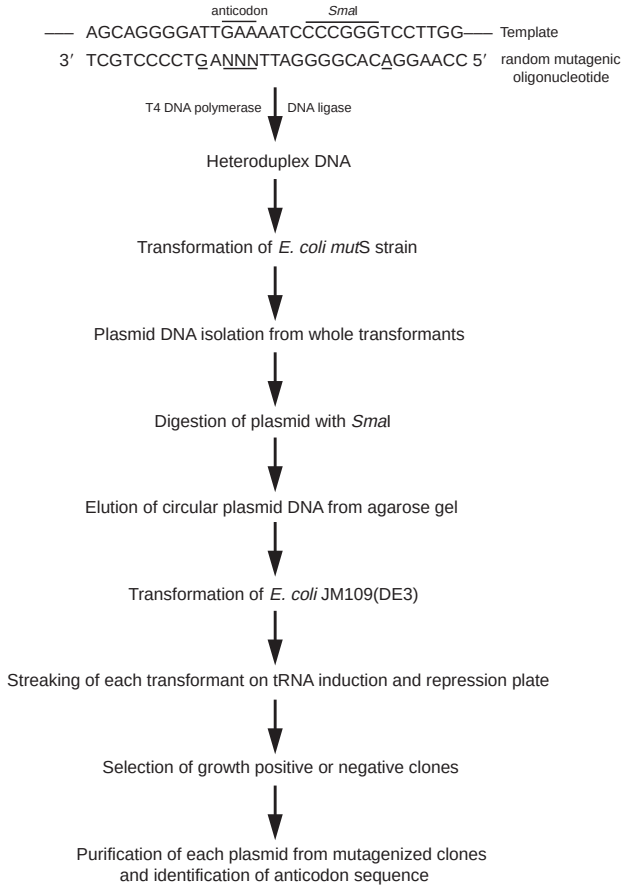

Fig. 2. Strategy for the generation of anticodon mutagenized clones by random mutagenesis. Mutagenic oligonucleotide has equal proportion of the four bases (A, G, C and T) at the corresponding anticodon sequence. LB solid medium containing ampicillin (50 μg/ml) and 0.2% (w/v) glucose was used as a tRNA repression plate, and the tRNA induction plate contained IPTG (0.5 mM) instead of glucose. The altered bases in the mutagenic oligonucleotide are underlined.

transformants grew on the tRNA induction plate, while others did not. Plasmids were isolated from selected clones, and the anticodon sequence of each transformant was determined by DNA sequencing.

tRNA induction and isolation of aminoacyl-tRNA The isolation of tRNA was done as described by Varshney *et al.* (1991). LB medium (10 ml) containing 50 μg/ml ampicillin and 0.5 mM IPTG was inoculated with 0.5 ml of a fresh overnight culture of *E. coli* JM109(DE3) transformants, grown for 4–5 h on a rotary shaker at 37°C, and chilled in wet ice. All subsequent steps were carried out in the cold. The cells were pelleted at 4°C, resuspended in 0.3 ml of 0.3 M sodium acetate (pH 4.5) and 10 mM Na_2EDTA, transferred to 1.5 ml Eppendorf tubes, and subjected to two extractions with equal volumes of phenol equilibrated with the same buffer. The aqueous layer was transferred to new tubes, mixed with 2.5 volumes of ethanol, and left on ice for 30 min. Total nucleic acids were recovered by centrifugation for 15 min. The pellet was dissolved in 60 ml of 0.3 M sodium acetate (pH 4.5). Nucleic acids were reprecipitated with 2.5 volumes of ethanol, left on ice for 60 min, and recovered by centrifugation for 15 min. The pellet was dissolved in 10 mM sodium acetate (pH 4.5) and 1mM Na_2EDTA.

Analysis of charged amino acid To deacylate isolated tRNAs, 25 μg of tRNA (~1 nmole) was dissolved in 50 mM ammonium bicarbonate buffer (pH 9.0) and incubated at 37°C for 1 h. The deacylated tRNAs were removed by ultrafiltration through a membrane filter (MW 3000 cut-off, Microcon-3TM from Amicon company). Deacylated amino acid mixtures were dried completely in a centrifugal concentrator *in vacuo*. Following this, the amino acid mixture was derivatized with phenylisothiocyanate (Bidlingmeyer *et al.*, 1984), and analyzed by HPLC using the reversed-phase chromatography protocol provided by Waters.

Results and Discussion

Selection of mutant clones The two bases preceding the anticodon in tRNAs are almost always C and U (positions 32 and 33). However, in *E. coli* $tRNA^{Phe}$, position 32 is a pseudouridine, a U derivative. In *E. coli* $tRNA^{Phe}$, the pseudouridine at position 32 has been known to affect translocation (Lee *et al.*, 1991). In order to create a similar environment in the anticodon loop sequence of the mutant tRNAs, we changed T32 to C. Lee *et al.* (1992) also show that the identity of the $tRNA^{Phe}$ transcript could be changed to methionine by alteration of the anticodon as well as by transforming U32 to C.

As the three nucleotides of anticodon were fully randomized, all 64 anticodon variants could be generated. However, we obtained only 22 different anticodon variants from our randomized anticodon sequences. These anticodons corresponded to 16 amino acids and an opal nonsense codon (Table1). Because we selected the clones that were obviously growth-positive or growth-negative on tRNA induction media, some variants that show unstable growth may have been excluded at this step.

The isolated RNAs were separated on 6% polyacylamide gel containing 7.5 M urea, and stained with silver nitrate. The image of the visible bands was duplicated on EDF film (Promega), and the quantity of 76 nucleotide-sized tRNAs were determined by the densitometric method using 5S rRNA as an internal standard. The tRNA (76nt)/5S rRNA ratio is 0.27 from the *E. coli* host, 0.29 from the uninduced host containing recombinant plasmid, and 6.90 from the induced cell (data not shown). Accordingly, $tRNA^{Phe}$ is overproduced by 24-fold compared to the uninduced cell. The ratios of mutant tRNAs are mostly the same as the wild-type (3.9–7.0), but both the arginine anticodon mutants are somewhat lower than the wild-type (0.5 and 2.3) (data not shown).

Two types of mutant tRNAs based on transformant growth It is plausible that some of the mutant tRNAs

Table 1. Characterization of the acylated amino acids from anticodon mutant tRNAs. The charged amino acid that was released by alkaline hydrolysis from mutant tRNA was identified by HPLC.

Variants	Growth on tRNA induction plate	Charged amino acid
32T/GAA(Phe)	+	Phe
32C/GAA(Phe)	+	Phe
32C/AAA(Phe)	+	Phe
32C/CAT(Met)	+	Met
32C/TGT(Thr)	+	Thr
32C/TTT(Lys)	+	Lys
32C/CCA(Trp)	+	none
32C/CTC(Glu)	+	none
32C/TCC(Gly)	+	none
32C/TCG(Arg)	+	none
32C/TCA(opal)	+	none
32C/TTG(Gln)	+	none
32C/ACG(Arg)	+	none
32C/GTC(Asp)	+	none
32T/GTG(His)	+	none
32T/GCA(Cys)	−	ND
32C/GCA(Cys)	−	ND
32C/GCC(Gly)	−	ND
32C/GAG(Leu)	−	ND
32C/GGA(Ser)	−	ND
32C/GAC(Val)	−	ND
32C/CGA(Ser)	−	ND
32C/CGC(Ala)	−	ND

ND; not determined.

may be charged with more than one amino acid or the mutation may make them less fit or detrimental to protein synthesis. Therefore it was expected that not all the mutant tRNA genes would give rise to normal host cell growth. For this reason, the isolated plasmids containing anticodon mutants of $tRNA^{Phe}$ were re-transformed into *E. coli* strain JM109 (DE3) to probe the effects the mutant tRNA on the host. LB broth containing 0.5 mM IPTG agar plate was used as a tRNA induction medium, and 0.2% (w/v) glucose instead of IPTG was used as a repression medium, which permitted all transformants to grow. Every transformant containing anticodon mutant tRNA was streaked on both agar plates.

The studies showed that the tRNA mutants fell into two groups, growth-positive and growth-negative. The first group could comprise mutant tRNAs which are charged with an amino acid or are uncharged (and thus "neutral" to cell growth). The growth-negatives are very likely tRNAs that are charged but whose amino acid no longer matches the anticodon. This would lead to misincorporation of amino acids in protein synthesis. Sequence analysis (see Table 1) showed that most of the growth-negative clones were altered in positions 35 or 36; positions known to be less important for phenylalanine identity than G34 (Chattapadhyay *et al.*, 1990; Pallanck and Schulman, 1991; Peterson and Uhlenbeck, 1992). Because these anticodons are similar to phenylalanine anticodon (GAA), these mutant tRNAs may be still charged with phenylalanine. Accordingly, those mutant tRNAs charged with amino acids do not match the anticodons. The other two anticodon sequences of growth negative clones (CGA and CGC) may be charged with threonine, because G35 nucleotide is known as a critical positive identity element for $tRNA^{Thr}$ (Hasegawa *et al.*, 1992; Saks *et al.*, 1998). As a result, these mutant tRNA cause translational errors during protein synthesis.

Analysis of the charged amino acids Cells of the growth-positive strains were prepared for extraction of aminoacylated tRNA. Analysis of the amino acids bound to the tRNA would reveal the nature of the amino acid charged to the mutant tRNA. The *in vivo* aminoacyl-tRNA was isolated in acid buffer and then discharged in slightly alkaline medium (See Materials and Methods). The released amino acids were identified by standard amino acid analysis (see Fig. 3 for the example of phenylalanine). Wild-type $tRNA^{Phe}$, which is over expressed in *E. coli* (about 25-fold, data not shown), is charged only with phenylalanine.

The results immediately showed that there are two groups of tRNAs among the growth-positive clones. A significant number of the tRNAs were not charged with any amino acid to a detectable level (Table 1). Thus, these tRNAs may have lost the identity elements for proper charging with phenylalanine while not acquiring a new specificity. Almost all growth positive clones have 34C or T, which can play a role as a negative element for phenylalanyl-tRNA synthetase (PheRS), and these mutants may not be charged with any amino acid. Although 32C/GTC (Asp) and 32T/GTG (His) mutant possess 34G, the next nucleotide 35U acts sufficiently as a negative element for PheRS. Accordingly, not only 34G, but also 35Pu, are important recognition elements of $tRNA^{Phe}$.

The most exciting mutant tRNAs were four mutant species. Interestingly, among the growth positive clones, 32C/TTT (Lys), 32C/TGT (Thr), and 32C/CAT (Met) mutant clones swapped tRNA identity with those of their anticodon sequence. In other words, the $tRNA^{Phe}$ identity changed from phenylalanine to lysine, or threonine, or methionine by alteration of the anticodon respectively. This result supports the idea that these four kinds of tRNA not only use the anticodon as a major identity element (Normanly and Abelson, 1989), but also share a common backbone structure used for cognate aminoacyl-tRNA synthetase recognition. Actually, these four tRNAs have 73A nucleotide as a discriminator (Sprinzl *et al.*, 1991). As another example of this, Schulman and Pelka (1990) also

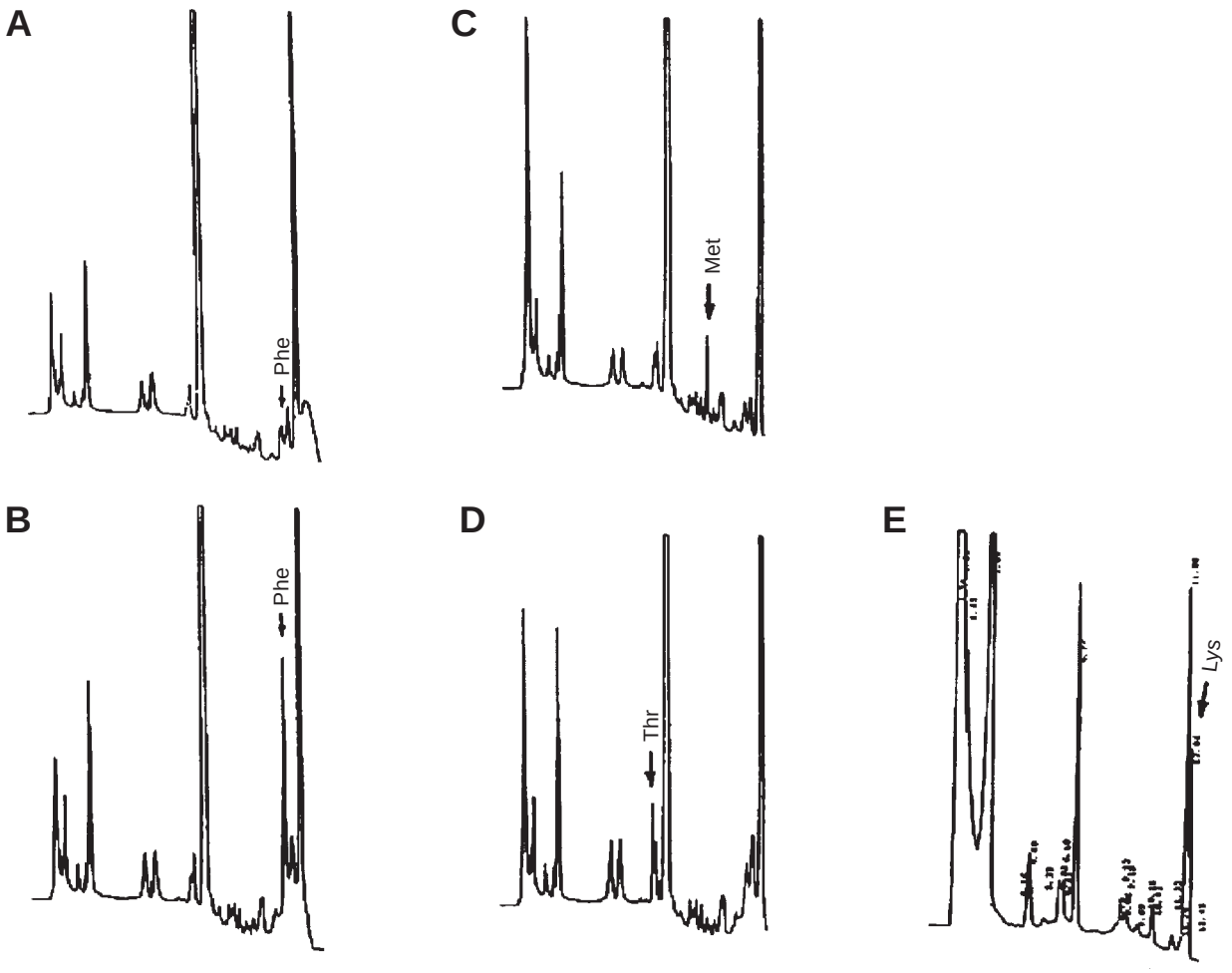

Fig. 3. Amino acid analysis from the deacylated tRNA by HPLC. The analysis of deacylated amino acids from the uninduced host tRNA (**A**), and that of the induced host by the addition of IPTG (**B**). The HPLC profiles of decaylated amino acid from induced mutant tRNA having methionine anticodon (**C**), threonine anticidon (**D**), and lysine anticodon (**E**) are also shown. The total isolated tRNA was deacylated by treatment with 50 mM ammonium bicarbonate buffer (pH 9) at 37°C for 60 min, and tRNA was removed by ultrafiltration. The amino acids in deacylated mixture were derivatized with PITC and separated by reverse phase column.

showed that a mutant $tRNA^{Met}$ possessing threonine anticodon could be charged with threonine. The codons for phenylalanine are UUU and UUC, but natural $tRNA^{Phe}$ has only GAA anticodon. The 32C/AAA clone shows good phenylalanine charging. There is a report that $tRNA^{Phe}$(AAA) can be charged with phenylalanine as well as wild-type $tRNA^{Phe}$(GAA) in the presence of 15mM magnesium ion (Kholod *et al*., 1997). However, other reports state that $tRNA^{Phe}$(AAA) did not influence the expression of the pheA gene, of which translation is controlled by phenylalanylated $tRNA^{Phe}$-mediated attenuation (Gavini and Pulakat, 1992). Even though $tRNA^{Phe}$(AAA) can be charged with phenylalanine, we could not conclude whether this phenylalanylated $tRNA^{Phe}$(AAA) has biological functions in translation.

In the case of growth-negative tRNA mutants, we could not isolate enough tRNA from the induced cell. Therefore, we could not determine what amino acids were acylated to these mutant tRNAs.

Evolutionary aspects The result of our *in vivo* analysis is an example of the changes that can be made in a tRNA scaffold which give rise to a viable tRNA with a different identity. As expected, it is apparent that duplication of tRNA genes and anticodon changes will lead to the evolution of a new tRNA. This encouraged us to think about the generation of new tRNAs by anticodon change and we constructed a tree of *E. coli* tRNA sequence similarity. We took all *E. coli* tRNA sequences, and trimmed them to the same length. So, the bases in variable loops were removed from the tRNA sequences. The dendrogram was generated from the sequence aligned with the Pileup algorithm (Genetic Computer Group package).

In the case of 32C/TTT (Lys), 32C/TGT (Thr), and 32C/

CAT (Met), the anticodon change was sufficient to swap tRNA identity *in vivo.* Accordingly, we assume that these four tRNAs evolved from the same ancestor tRNA. Moreover $tRNA^{Met}$, $tRNA^{Lys}$, $tRNA^{Thr}$ and $tRNA^{Phe}$ are located in the same cluster in a sequence similarity dendrogram of *E. coli* tRNAs (Fig. 4). This suggests that the anticodon is the first target to change in the recruitment of new tRNAs on the evolutionary pathway. However, their aminoacyl-tRNA synthetases have no significant relationship. PheRS, ThrRS and LysRS belong to a class II enzyme, but MetRS is class I, and ThrRS, LysRS and MetRS have $\alpha 2$ multimeric state, but PheRS is $\alpha 2\beta 2$ (Meinnel *et al.*, 1995). This fact implies that evolution mechanisms between tRNA and their aminoacyl-tRNA synthetases are not related.

In conclusion, the generation of a new tRNA from existing tRNA requires duplication of an existing tRNA gene. Anticodon changes must take place before other changes in tRNA sequence, because a change in the anticodon is necessary to assign a new codon to a new amino acid. If an anticodon mutant can be recognized by an existing aminoacyl-tRNA synthetase, this tRNA can develop into an isoaccepting tRNA with a different anticodon sequence to the existing isoacceptor tRNAs. We can see examples of this in $tRNA^{Val}$(GAC), $tRNA^{Gly}$(GCC) and $tRNA^{Thr}$(GGU) on the tRNA sequence similarity dendrogram (Fig. 4). But if anticodon mutants are no longer recognized by any other aminoacyl-tRNA synthetase, they may remain as non-functional tRNA. Following the generation of new aminoacyl-tRNA synthetase, one of these functionless tRNA could be taken by the new synthetase for its cognate tRNA. It follows that sets of tRNA:aminoacyl-tRNA synthetase pairs generate and determine the codons for new amino acids. Because all organisms share universal codon, these codon-determining events occurred very early in evolution, before the division of the three kingdoms (Ribas *et al.*, 1998). Saks *et al.* (1998) also showed high homology between *E. coli* and *Haemophilus influenzae* class I tRNAs, and tested the possibility of a recruitment hypothesis of tRNA by a point mutation of anticodon. Our results also provide a plausible explanation for the evolution of tRNAs in $tRNA^{Phe}$ cluster by accumulate mutations include anticodon.

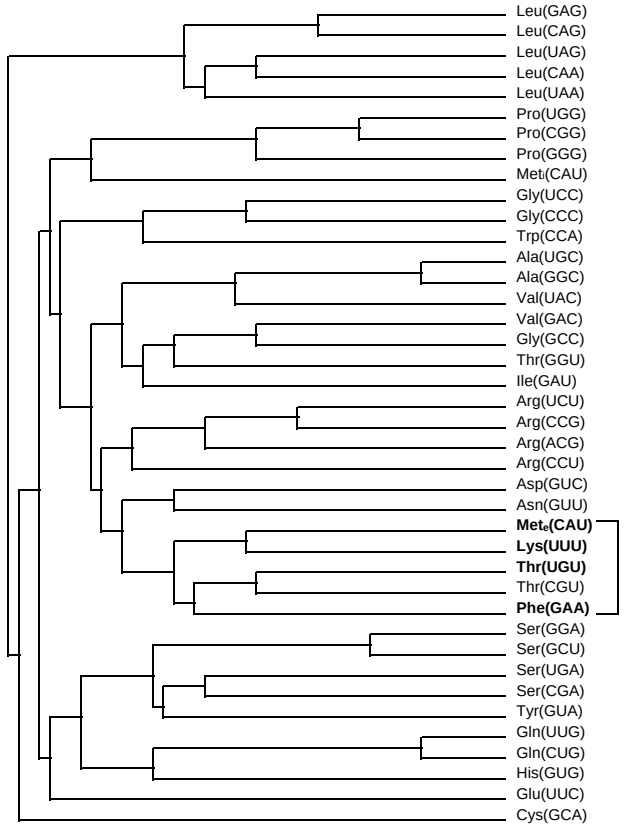

Fig. 4. *E. coli* tRNA sequence homology dendrogram. Genes are named using three-letter amino acid abbreviations and anticodon sequences. The dendrogram was generated from sequence aligned with the Pileup algorithm (Genetic computer group package). The several bases in variable loop were removed from certain tRNA sequences to create equal lengths of nucleic acid molecules.

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