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Role of Residue Glu152 in the Discrimination between Transfer RNAs by Tyrosyl-tRNA Synthetase from *Bacillus stearothermophilus*

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Residue Glu152 of tyrosyl-tRNA synthetase (TyrTS) from *Bacillus stearothermophilus* is close to phosphate groups 73 and 74 of $tRNA^{Tyr}$ in the structural model of their complex. TyrTS(E152A), a mutant synthetase carrying the change of Glu152 to Ala, was toxic when overproduced in *Escherichia coli*. The toxicity strongly increased with the growth temperature. It was measured by the ratios of the efficiencies with which the producing cells plated in induced or repressed conditions and at 30°C or 37°C. TyrTS(E152Q), TyrTS(E152D) and the wild-type synthetase were not toxic in conditions where TyrTS(E152A) was toxic. The toxicity of TyrTS(E152A) was abolished by additional mutations of the synthetase that prevent the binding of $tRNA^{Tyr}$ but not by a mutation that prevents the formation of Tyr-AMP. Because TyrTS(E152A) was active for the aminoacylation of $tRNA^{Tyr}$, its toxicity could only be due to faulty interactions with non-cognate tRNAs, either their non-productive binding or their mischarging with tyrosine. TyrTS(E152A) and TyrTS(E152Q) mischarged $tRNA^{Phe}$ and $tRNA^{Val}$ *in vitro* with tyrosine unlike TyrTS(E152D) or the wild-type enzyme. Thus, several features of the side-chain in position 152 of TyrTS, including its negative charge, are important for the rejection of non-cognate tRNAs. TyrTS(E152A), TyrTS(E152D) and TyrTS(E152Q) had similar steady-state kinetics parameters for the charging of $tRNA^{Tyr}$ with tyrosine *in vitro*, with k_{cat}/K_M ratios improved 2·5 times relative to the wild-type synthetase. We conclude that the side-chain of residue Glu152 weakens the binding of TyrTS to $tRNA^{Tyr}$ and prevents its interaction with non-cognate tRNAs.

Keywords: aminoacyl-tRNA synthetase; transfer RNA identity; $tRNA^{Tyr}$; protein–nucleic acid recognition; protein toxicity

1. Introduction

Although the genetic code was formally deciphered 30 years ago, the concrete mechanisms by which the living cell reads this code remain partially unknown. The aminoacyl-tRNA synthetases are the enzymes that translate the genetic code since they attach the amino acids to the corresponding tRNAs. During the past five years, many data have been gained on the sequence elements of tRNAs that determine their identity, i.e. the amino acid to which they are attached (for reviews, see Schimmel, 1989; Normanly & Abelson, 1989; Schulman & Abelson, 1988; Yarus, 1988). There is less information on the binding sites for tRNAs at the surface of aminoacyl-tRNA synthetases or on the chemical nature of the interactions that are responsible for the binding of tRNAs, the specific recognition of their identity elements and the rejection of non-cognate tRNAs (Bedouelle & Winter, 1986; Labouze & Bedouelle, 1989; Rould *et al.*, 1989, 1991; Ruff *et al.*, 1991; Perona *et al.*, 1991).

We wish to understand how the tyrosyl-tRNA synthetase (TyrTS§) from *Bacillus stearothermophilus* interacts with $tRNA^{Tyr}$ and how it discriminates

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§ Abbreviations used: TyrTS, tyrosyl-tRNA synthetase; Tyr-AMP, tyrosyl-adenylate; IPTG, isopropyl-β-D-thiogalactopyranoside; e.o.p., efficiency of plating.

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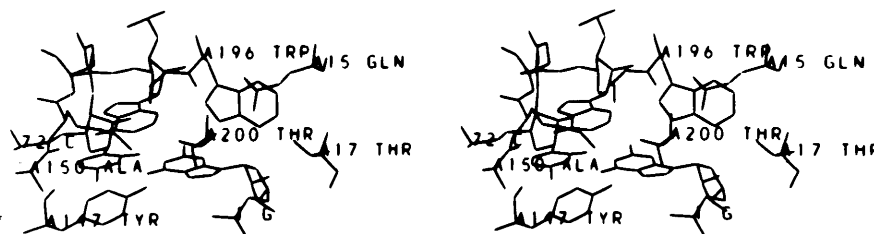


Figure 1. Environment of adenosine 73 in the structural model of the complex between TyrTS and tRNA^{Tyr}. The relevant fragments of TyrTS, guanine 1, cytosine 72 and adenosine 73 are represented. Only the N or 5'-terminal residue of each TyrTS or tRNA^{Tyr} fragment is labelled. The N-6–H-2 group of adenine 73 points toward the main-chain oxygen atom of Ala150, at hydrogen-bonding distance, and its C-2 and N-3 atoms are in contact with the C$^{\delta 1}$ of Trp196, at van der Waals' distances. Glu152 is close to phosphate groups 73 and 74. See Labouze & Bedouelle (1989) for a complete description of the model.

between tRNA^{Tyr} and the non-cognate tRNAs. TyrTS catalyses the aminoacylation of tRNA^{Tyr} in a two-step reaction. The tyrosine is first activated with ATP to form tyrosyl-adenylate (Tyr-AMP), then Tyr-AMP is attacked by tRNA^{Tyr} to form tyrosyl-tRNA^{Tyr} (Fersht, 1987). TyrTS is a homodimer. Each subunit has 419 residues, subdivided into two domains: an N-terminal domain (residues 1 to 319) of known structure, which is sufficient to catalyse the formation of Tyr-AMP, and a C-terminal domain (residues 320 to 419) of unknown structure, which is necessary for tRNA^{Tyr} binding (Brick *et al.*, 1989; Waye *et al.*, 1983).

Studies of mutant *Escherichia coli* tRNA^{Tyr}, obtained through *in vivo* selections or *in vitro* constructions, have shown that bases G34 and U35 in the anticodon and A73, the fourth base from the 3' end, in the acceptor arm are recognized by TyrTS. The orientation of the extra stem and loop also plays a role in this recognition (Celis & Piper, 1982; Hou & Schimmel, 1989; Himeno *et al.*, 1990). Previously, we introduced a large number of mutations at the surface of TyrTS, by site-directed mutagenesis, and analysed their properties. On the basis of these data, we constructed a structural model of the complex between TyrTS and tRNA^{Tyr} (Bedouelle & Winter, 1986; Labouze & Bedouelle, 1989). In the model, the acceptor arm of tRNA^{Tyr} interacts with the N-terminal domain of one TyrTS subunit and its anticodon arm interacts with the C-terminal domain of the other subunit. Adenine 73 lodges in a pocket, at the surface of TyrTS, surrounded by residues Trp196, Lys151 and Glu152. The carboxyl group of Glu152 is close to phosphate groups 73 and 74 (Fig. 1). The nature of the contacts between Trp196, Lys151, Glu152 and tRNA^{Tyr} was investigated by mutagenesis of the three aminoacyl residues (Labouze & Bedouelle, 1989).

In vitro, the study of the discrimination between tRNAs by an aminoacyl-tRNA synthetase is a difficult task because one needs both the cognate and the non-cognate tRNAs in pure form. In *E. coli*, there are about 60 different tRNA species (Fournier & Ozeki, 1985). *In vivo*, a mutation of synthetase that weakens its discrimination between tRNAs may be deleterious to the cell by interfering with the translation process and thus may be recognized by a global phenotype. In the present work, we used both *in vivo* and *in vitro* approaches to show that residue Glu152 of *B. stearothermophilus* TyrTS prevents its interaction with non-cognate tRNAs without contributing to the binding of the cognate tRNA^{Tyr}.

2. Materials and Methods

(a) *Media, strains and bacterial techniques*

The genotypes of strains TG2, DH1 and HB101 have been described (Sambrook *et al.*, 1989). The derivatives of phage M13 were propagated in strain TG2. During the *in vitro* constructions, we used the female strain DH1(pTT15) to counterselect the parental phages derived from M13; the male strain HB2151 (su^-) to counterselect the parental phages that carried an amber mutation in gene *4* (Carter *et al.*, 1985); strain HB101 (*galK*) to counterselect plasmid pDR540, which codes for galactokinase. Plasmid pTT15 is compatible with pEMBL9 and codes for resistance to tetracycline and for the repressor, LacI, of promoter *tac* (Terwilliger *et al.*, 1988). Strain HB2202 is identical to TG2, except that it carries a $lacZ^+$ allele (Bedouelle *et al.*, 1990). Ampicillin was used at 100 μg/ml and tetracycline at 15 μg/ml. Isopropyl-β-D-thiogalactopyranoside (IPTG; at 1 mM) was added to the medium to induce the *tac* promoter when necessary. The plating experiments were performed from resuspended colonies, exactly as described (Bedouelle *et al.*, 1990).

(b) *Recombinant DNA and sequencing*

The phage and plasmid constructions were done by *in vitro* recombinations between restriction fragments of DNA, using techniques already described (Bedouelle & Duplay, 1988). The presence of point mutations in the recombinant plasmids and phages was checked by DNA sequencing with the phage T7 polymerase (Tabor & Richardson, 1987). We used synthetic oligonucleotides complementary to internal sites in the *tyrS* gene as primers for sequencing. The single-stranded DNA forms of the plasmids and phages were prepared as described (Sambrook *et al.*, 1989) and used as templates for DNA sequencing.

(c) *TyrTS purification*

The wild-type and mutant enzymes were produced in strain TG2 from recombinant M13 phages and purified by

modifications of existing procedures (Fersht *et al.*, 1988). A portion (5 ml) of an overnight culture of TG2 in 2 × TY broth was infected with an M13 derivative at high multiplicity of infection (> 10) and the mixture incubated for 20 min at 37 °C. The infected cells were added to 500 ml of 2 × TY broth, prewarmed at 37 °C, and grown for 6 h. The cells were harvested by centrifugation at 6000 revs/min at 4 °C for 15 min and resuspended in 0·1 vol. buffer A (50 mM-Tris·HCl (pH 7·5), 10 mM-2-mercaptoethanol, 0·1 mM-phenylmethylsulphonyl fluoride) containing 1 mM-EDTA. The cell suspension was kept at −70 °C. After thawing, the cells were disrupted by passage through a French press. The cell debris was removed by centrifugation at 18,000 revs/min at 4 °C for 20 min. The soluble extract was heated at 58 °C for 40 min to precipitate the endogeneous TyrTS and the thermolabile proteins of *E. coli*, and clarified by centrifugation as above. The clear lysate was applied to a 2-ml column containing Whatman DE52 resin and equilibrated with buffer A. The column was washed with 10 ml of buffer A, 10 ml of buffer A containing 50 mM-NaCl, then eluted with successive additions of 1 ml portions of buffer A containing 150 mM-NaCl. The fractions containing the enzyme were dialysed for 4 h at 4 °C against buffer A containing 0·1 mM-tetrasodium pyrophosphate to remove any enzyme-bound Tyr-AMP. After dialysis overnight against buffer A, the enzyme was further purified on a FPLC Mono Q column (Pharmacia) equilibrated with buffer A and eluted with a gradient of 0 mM to 300 mM-NaCl in buffer A. The enzyme eluted at about 200 mM-NaCl and was at least 90 % pure, as judged by SDS/polyacrylamide gel electrophoresis. It was dialysed against standard buffer (144 mM-Tris·HCl (pH 7·78), 10 mM-$MgCl_2$, 10 mM-2-mercaptoethanol, 0·1 mM-phenylmethylsulphonyl fluoride). Fractions were kept at 4 °C or rapidly frozen at −70 °C.

(d) *Kinetics experiments*

Active-site titration of pure TyrTS or of TyrTS in crude extracts was performed as described (Wilkinson *et al.*, 1983) except that [^{14}C]tyrosine was used at 20 μM and the nitrocellulose filters were washed with 10 ml of standard buffer diluted 1 : 4 (v/v). For the tRNA charging experiments, the purified enzyme was diluted in standard buffer containing 0·1 mg of bovine serum albumin/ml. The reaction mixtures contained 0·1 mg of bovine serum albumin/ml, 20 μM-[^{14}C]tyrosine, 10 mM-ATP.Mg^{2+}, 10 units of inorganic pyrophosphatase/ml, 0·4 nM-wild-type or mutant-type TyrTS and tRNA in standard buffer, in a total volume of 125 μl. Pure *E. coli* $tRNA^{Tyr}$ (1479 pmol of tyrosine acceptance/A_{260} unit) was used at concentrations between 0·25 and 8 μM, and crude *E. coli* tRNA (300 pmol tyrosine acceptance/mg) at 0·1 to 1·2 mg/ml. Every minute during the first 4 min, 25-μl portions were spotted onto Whatman 3MM paper discs and, after 10 s, the discs were immersed into 5 % (w/v) trichloroacetic acid and washed as described (Bollum, 1959). Portions (25 μl) of the reaction mixtures were spotted onto filter discs and the filters dried to obtain the total count number. The radioactivity retained by the filters was counted after addition of ASCII scintillation liquid (Amersham). The charging capacity of $tRNA^{Tyr}$, which depends on the enzyme concentration (Bonnet & Ebel, 1972), was determined with 80 nM-pure TyrTS. The kinetic data were analysed with the Enzfitter software (Leatherbarrow, 1990). The tRNA mischarging experiments were performed in the same conditions as the $tRNA^{Tyr}$ charging experiments, except that the wild-type or mutant-type TyrTS was present at 1·0 mM, $tRNA^{Phe}$ (1321 pmol/A_{260} unit) at 2·3 μM, $tRNA^{Val}$ (1067 pmol/A_{260} unit) at 1·7 μM and crude *E. coli* tRNA at 1 mg/ml. The tRNAs were purchased from Boehringer-Mannheim.

3. Results

(a) *Construction of point mutation E152A of TyrTS*

We initially constructed the mutation of residue Glu152 to Ala by oligonucleotide site-directed mutagenesis of phage M13(4am)-BY(M24.89), which carries the *tyrS* gene under control of a mutant promoter, *tyrSp(M24.89)*, and constitutively overexpresses TyrTS. After mutagenesis, we screened the phage clones by sequencing their DNA around the site of the mutation. Among ten clones carrying the nucleotide change corresponding to mutation E152A, only one directed the synthesis of a protein with an apparent molecular mass close to that of the wild-type TyrTS in SDS/polyacrylamide gels. We determined the entire sequence of the *tyrS* gene for this particular phage clone and found three different changes, corresponding to mutations E152A, T224A and a deletion, Δ2, of residues 377 to 401 of TyrTS. We named this clone M13-BY(3 × M).

We assumed that mutation E152A rendered TyrTS toxic to the host cell when overexpressed from a derivative of phage M13-BY(M24.89). We therefore introduced the corresponding nucleotide change into phage M13-BY(Ptac), which carries the *tyrS* gene under control of the repressible promoter *tac*. This construction was done by *in vitro* recombinations between restriction fragments, as described in Table 1 and Figure 2. We used strain TG2, which overproduces repressor LacI of *Ptac*, as a cellular host for the phage propagation. We found that the recombinant phage M13-BY(Ptac, E152A) gave identical plaques and titers when it infected TG2 either in the presence or in the absence of

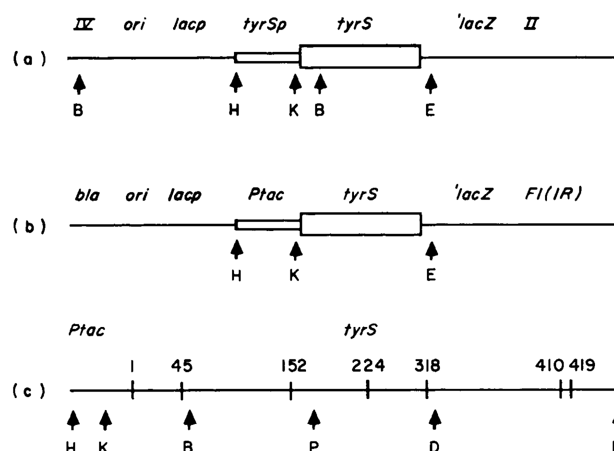


Figure 2. Structures of the parental plasmids and phages. (a) M13-BY(M24). (b) pEMBL9-BY(Ptac). (c) Restriction map of the *Ptac-tyrS* transcriptional unit; the codon positions in the *tyrS* gene are indicated above the line. B, *Bal*I; D, *Dra*II; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I.

Table 1
Phage and plasmid strains

Strain	Relevant characteristics	References
A. *M13 phages*		
M13-BY(M24)	*B. stearothermophilus tyrSp^{+} tyrS^{+}*†	10
M13-BY(M24, H45N)	Like M13-BY(M24) but codes for a mutant TyrTS (His45 → Asn)	2
M13-BY(M24.89)	Like M13-BY(M24) but *tyrSp* is deleted of residues −44 to −98; overproduces TyrTS	10
M13(4am)-BY(M24.89)	Like M13-BY(M24.89) but with an amber mutation in gene *4* of M13	1
M13-BY(Δ1)	Like M13-BY(M24.89) but codes for a TyrTS with a deletion, Δ1, of amino acid residues 318 to 417	9
M13(4am)-BY(Δ1)	Like M13-BY(Δ1) but with an amber mutation in gene *4*	1
M13-BY(E152D)	Like M13-BY(M24.89) but codes for a mutant TyrTS (Glu152 → Asp)	5
M13-BY(E152Q)	Like M13-BY(M24.89) but codes for a mutant TyrTS (Glu152 → Gln)	5
M13-BY(K410N)	Like M13-BY(M24.89) but codes for a mutant TyrTS (Lys410 → Asn)	5
M13-BY(K411N)	Like M13-BY(M24.89) but codes for a mutant TyrTS (Lys411 → Asn)	5
M13-BY(3 × M)	Like M13-BY(M24.89) but codes for a TyrTS with 3 changes, Glu152 → Ala, Thr224 → Ala and a deletion, Δ2, of amino acid residues 377 to 401	5
M13-BY(Ptac)	Like M13-BY(M24) but the nucleotides upstream from −78 replaced by *Ptac*; from pHB17 and M13-BY(M24) by *Hin*dIII and *Kpn*I	This work
M13(4am)-BY(Ptac, Δ1)	*4am Ptac tyrS(Δ1)*; from M13-BY(Ptac) and M13(4am)-BY(Δ1) by *Hin*dIII and *Pst*I	This work
M13-BY(Ptac, 3 × M)	*Ptac tyrS(E152A, T224A, Δ2)*; from M13(4am)-BY(Ptac, Δ1) and M13-BY(3 × M) by *Bal*I	This work
M13-BY(Ptac, E152A)	*Ptac tyrS(E152A)*; from M13-BY(Ptac, 3 × M) and M13(4am)-BY(M24.89) by *Pst*I and *Eco*RI	This work
M13-BY(Ptac, E152A, Δ1)	*Ptac tyrS(E152A, Δ1)*; from M13-BY(Ptac, 3 × M) and M13(4am)-BY(Δ1) by *Pst*I and *Eco*RI	This work
M13-BY(Ptac, E152A, T224A)	*Ptac tyrS(E152A, T224A)*; from M13-BY(Ptac, 3 × M) and M13(4am)-BY(M24.89) by *Dra*II and *Eco*RI	This work
B. *Plasmids*		
pEMBL9^{+}	*bla^{+}*, carries the intergenic region of phage f1	3
pTZ19R	*bla^{+}*, carries the multiple cloning region of phage M13mp19	6
pUC-4K	*bla^{+}*, carries a kanamycin-resistance cartridge	8
pDR540	Carries promoter *Ptac* between *Hin*dIII and *Bam*HI sites	7
pEMBL9^{+}K	Like pEMBL9^{+} but with the kanamycin-resistance cartridge of pUC-4K inserted into the *Pst*I site	4
pTZ19RK	Like pTZ19R but with the kanamycin-resistance cartridge of pUC-4K inserted into the *Pst*I site	4
pHB17	Carries promoter *Ptac* between *Hin*dIII and *Kpn*I sites; from pTZ19RK and pDR540 by *Hin*dIII and *Bam*HI	This work
pEMBL9-BY(Ptac)	*Ptac tyrS^{+}*; from M13-BY(Ptac) and pEMBL9^{+}K by *Hin*dIII and *Eco*RI	This work
pEMBL9-BY(Ptac, Δ1)	*Ptac tyrS(Δ1)*; from M13-BY(Δ1) and pEMBL9-BY(Ptac) by *Dra*II and *Eco*RI	This work
pEMBL9-BY(E152A)	*Ptac tyrS(E152A)*; from M13-BY(Ptac, E152A) and pEMBL9^{+}K by *Hin*dIII and *Eco*RI	This work
pEMBL9-BY(E152A, Δ1)	*Ptac tyrS(E152A, Δ1)*; from M13-BY(Ptac, E152A, Δ1) and pEMBL9^{+}K by *Hin*dIII and *Eco*RI	This work

The mutations are designated by the wild-type amino acid side-chain, the residue number and the mutant side-chain in the 1-letter amino acid code. References: 1, Carter *et al.*, 1985; 2, Carter *et al.*, 1986; 3, Dente & Cortese, 1987; 4, Hermann & Bedouelle, 1990; 5, Labouze & Bedouelle, 1989; 6, Meade *et al.*, 1986; 7, Russell & Bennett, 1982; 8, Vieira & Messing, 1982; 9, Waye *et al.*, 1983; 10, Waye & Winter, 1986.

† The *B. stearothermophilus* fragment extends from −325 to +1368, taking the 1st nucleotide residue of the initiation codon for the *tyrS* gene as +1. TyrTS has 419 amino acid residues (Winter *et al.*, 1983).

1 mM-IPTG, an inducer of *Ptac*. This result showed that the mutant enzyme TyrTS(E152A) was not toxic for the cell when expressed from phage M13-BY(Ptac, E152A). We used this property to produce, purify and study the mutant synthetase TyrTS(E152A) *in vitro*.

(b) *Toxicity of plasmid pEMBL9-BY(E152A)*

The toxicity of a protein can depend on its level of cellular production and on the temperature of growth. Production, in turn, depends on the strength of the promoter and the copy number of the coding gene (Bedouelle & Duplay, 1988; Bedouelle *et al.*, 1990). To provide evidence for a potential toxicity of the mutant synthetase TyrTS(E152A), we inserted the wild-type *Ptac tyrS^{+}* and the mutant *Ptac tyrS(E152A)* transcriptional units into plasmid pEMBL9^{+}, which has a very high copy number (Dente & Cortese, 1987). We transformed strain HB2202 with the recombinant plasmids and measured the plating efficiencies of its derivatives under four experimental conditions: in the presence or in the absence of the inducer, IPTG, and at a temperature of 30°C or 37°C. Table 2 gives the ratios of these efficiencies of plating (e.o.p.). The

Table 2
Toxicity and cellular concentration of TyrTS

Mutation	Ratio of plating efficiencies at: 30° − IPTG / 30° + IPTG	37° − IPTG / 37° + IPTG	30° − IPTG / 37° − IPTG	30° + IPTG / 37° + IPTG	% of soluble proteins (30 °C)†
E152	0·89(±0·11)	0·97(±0·13)	0·97(±0·10)	1·04(±0·16	5·4
E152A	5·58(±0·17)	1·15(±0·28) × 10^4	2·67(±1·13)	6·41(±4·02) × 10^3	1·2
E152A, Δ1	1·15(±0·20)	0·92(±0·09)	0·90(±0·13)	0·72(±0·10)	6·2
E152D	1·17(±0·12)	1·05(±0·22)	1·05(±0·06)	0·91(±0·09)	3·9
E152Q	1·66(±0·37)	2·50(±0·26)	1·38(±0·23)	2·22(±0·40)	4·1
H45N	1·18(±0·12)	3·95(±0·87)	1·12(±0·10)	3·18(±0·59)	10·0
H45N, E152A	2·07(±0·42) × 10^4	3·08(±1·65) × 10^5	1·11(±0·02)	26·9(±14·3)	3·6
T224A	1·04(±0·16)	1·06(±0·08)	0·81(±0·12)	0·83(±0·02)	4·4
E152A, T224A	171(±82)	2·46(±0·83) × 10^5	0·86(±0·08)	5·34(±4·73) × 10^3	3·4
E152A, K410N	0·91(±0·09)	0·78(±0·04)	1·21(±0·17)	1·06(±0·16)	6·7
E152A, K411N	0·89(±0·08)	0·50(±0·04)	1·21(±0·17)	0·69(±0·03)	6·0

The *tyrS* mutations that correspond to the amino acid changes listed in column 1 were introduced into plasmid pEMBL9-BY(Ptac) by recombination between M13 and pEMBL9 derivatives (Table 1), as described in Fig. 2 (see also, Hermann & Bedouelle, 1990; Bedouelle *et al.*, 1990).

Comparison of plating efficiencies: an isolated colony of strain HB2202, grown at 30 °C and containing plasmid pEMBL9-BY(Ptac) or a mutant derivative, was suspended in buffer and dilutions of the suspension were spread on LB plates containing ampicillin. The colonies were counted after 42 h of growth, except those of HB2202(pEMBL9-BY(H45N)), which grew slowly at 37 °C in the presence of IPTG and were counted after 72 h. Columns 2 to 5 give the ratios of the colony counts obtained in the following conditions: 30° − IPTG, 30 °C without IPTG; 30° + IPTG, 30 °C with IPTG; 37° − IPTG, 37 °C without IPTG; 37° + IPTG, 37 °C with IPTG. The average values and the standard errors from 3 independent experiments are shown. On average, a resuspended colony gave progeny to 8·5 × 10^6 colonies at 30 °C without IPTG, so that we could detect 1 survivor in 1·7 × 10^6 cells in our experimental conditions.

Cellular concentration of *TyrTS*: the HB2202 derivatives were grown in LB broth containing ampicillin and IPTG at 30 °C for 4 generations, until an A_{600} of 1·0 cm^{-1} to 1·2 cm^{-1} was reached. The cultures of HB2202(pEMBL9-BY(H45N, E152A)) and HB2202(pEMBL9-BY(E152A, T224A)) grew for only 1·5 to 2·5 generations and then remained stationary at A_{600} = 0·2 to 0·3 cm^{-1}. The concentrations of TyrTS were normalized with respect to the total concentrations of protein in the soluble extracts and are expressed as percentages. The molecular masses of TyrTS and TyrTS(Δ1) were taken as 94,632 Da and 72,648 Da, respectively (Winter *et al.*, 1983; Waye *et al.*, 1983). Average values of 2 independent experiments are shown.

† The concentration of the endogeneous *E. coli* TyrTS is 0·15% of the soluble proteins (Bedouelle *et al.*, 1990).

recombinant strain HB2202(pEMBL9-BY(Ptac)), producing the wild-type TyrTS, had the same e.o.p. under the four conditions. In contrast, the e.o.p. of HB2202(pEMBL9-BY(E152A)), producing the mutant TyrTS, decreased 11,000-fold at 37 °C and sixfold at 30 °C when promoter *tac* was induced. Its e.o.p. decreased about 6400-fold in the presence of IPTG, and twofold in its absence when the temperature went from 30 °C to 37 °C. These results showed a high level of toxicity of TyrTS(E152A) for the cell, and a strong effect of temperature on this toxicity.

(c) *Mutations that prevent the binding of* $tRNA^{Tyr}$

Deletion Δ2 removed 24 residues of the C-terminal domain of TyrTS. Several mutations in the C-terminal domain, e.g. deletion Δ1 of residues 318 to 417 or the point mutations K410N and K411N, strongly diminish the binding of $tRNA^{Tyr}$ without affecting the formation of Tyr-AMP (Waye *et al.*, 1983; Bedouelle & Winter, 1986). We suspected that deletion Δ2 had the same effect on the function of TyrTS as K410N, K411N or Δ1, and that it had been selected during the construction of mutation E152A for this reason. To investigate the effect of well-characterized mutations of this class on the toxicity of E152A, we constructed derivatives of plasmid pEMBL9-BY(Ptac) encoding doubly mutant TyrTS that carried mutation K410N, K411N or Δ1 in addition to E152A. In the three cases, the e.o.p. of the cellular host was identical under the four experimental conditions tested, i.e. in the presence or in the absence of IPTG and at 30 °C or 37 °C (Table 2). Thus, the toxicity of mutation E152A was abolished by mutations of TyrTS that prevent the binding of $tRNA^{Tyr}$.

(d) *A mutation that prevents the formation of Tyr-AMP*

The experiments described in section (c), above, suggested to us that a faulty interaction with tRNAs and not the formation of Tyr-AMP was the cause for the toxicity of TyrTS(E152A). This interaction could be a non-productive binding of tRNAs to the mutant synthetase and their depletion from the cell or their mischarging with tyrosine. In an attempt to distinguish between these two possibilities, we used mutation H45N of TyrTS. The side-chain of residue His45 is believed to bind to the γ-phosphoryl group of ATP in the transition state for the formation of Tyr-AMP. Mutation H45N decreases 10,000-fold the rate of formation of Tyr-AMP by TyrTS and, as a consequence, abolishes the aminoacylation of $tRNA^{Tyr}$ (Carter *et al.*, 1986; Ward & Fersht, 1988). We constructed derivatives of plasmid pEMBL9-BY(Ptac) encoding mutant TyrTS that carried mutation H45N either

alone or combined with mutation E152A. The production of the singly mutant synthetase TyrTS(H45N) had no effect on the growth of the cellular host at 30 °C and only a very modest effect at 37 °C; it approximately doubled the time necessary for this host to form colonies and decreased its e.o.p. fourfold. In contrast, the production of the doubly mutant synthetase TyrTS(H45N, E152A) strongly decreased the e.o.p. of the host, 20,000-fold at 30 °C and 300,000-fold at 37 °C; increasing the temperature of growth from 30 °C to 37 °C decreased this e.o.p. about 27-fold (Table 2). Comparison of the results obtained for TyrTS(E152A) and TyrTS(H45N, E152A) shows that mutation H45N increased the toxicity of E152A 3700 times at 30 °C and 27 times at 37 °C. Thus, mutation E152A was more toxic when it was introduced into a TyrTS that does not form Tyr-AMP and, as a consequence, does not charge $tRNA^{Tyr}$ with tyrosine, than when it was introduced into an otherwise wild-type enzyme. Remarkably, temperature had much less effect on the toxicity of TyrTS(H45N, E152A), with a factor of 27, than on that of TyrTS(E152A), with a factor of about 6400.

Table 3
Kinetic parameters for $tRNA^{Tyr}$ charging by wild-type and mutant-type TyrTS

Enzyme	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M (10^{-6} M^{-1} s^{-1})	ΔG_T (kcal mol^{-1})
E152	1·38 ±0·16	3·64 ±0·26	2·65 ±0·15	0·00
E152A	0·53 ±0·03	3·58 ±0·06	6·74 ±0·34	−0·55 ±0·03
E152D	0·62 ±0·05	4·62 ±0·26	7·40 ±0·48	−0·60 ±0·04
E152Q	0·52 ±0·04	4·64 ±0·01	8·91 ±0·60	−0·71 ±0·04

$\Delta G_T = -RT \ln (k_{cat}/K_M)_{mut}/(k_{cat}/K_M)_{wt}$, where k_{cat} and K_M are the kinetic parameters for the aminoacylation of pure *E. coli* $tRNA^{Tyr}$ with [^{14}C]tyrosine, mut indicates mutant and wt indicates wild-type (Wilkinson *et al.*, 1983). Each number is the average of the values obtained in 3 independent assays and is given with their standard deviation. We found that the addition of bovine serum albumin in the dilution buffer of the enzyme and in the reaction mixture was necessary to obtain reproducible results (see Materials and Methods). The k_{cat} values were about 3 times higher when the buffers contained bovine serum albumin.

(e) *Mutations that change the side-chain of residue 152*

We reported above that, after mutagenesis of phage M13(4am)-BY(M24.89) with synthetic oligonucleotides, we screened the mutant phages by DNA sequencing and that, among ten phage clones carrying the nucleotide change of mutation E152A, only one directed the synthesis of a protein having an apparent molecular mass close to that of the wild-type TyrTS in SDS/polyacrylamide gels. The ratios were 2/3 and 3/3 for mutations E152D and E152Q, respectively (Labouze & Bedouelle, 1989). These figures suggested to us that the mutant synthetases TyrTS(E152D) and TyrTS(E152Q) were not toxic to the cell in conditions where TyrTS(E152A) was toxic. To test this hypothesis, we constructed plasmids pEMBL9-BY(E152D) and pEMBL9-BY(E152Q). The cellular host had the same e.o.p. under the four conditions tested when containing these two plasmids (Table 2). These results showed that the side-chains of Glu, Gln and Asp were tolerated in residue position 152 of TyrTS in conditions where Ala was toxic.

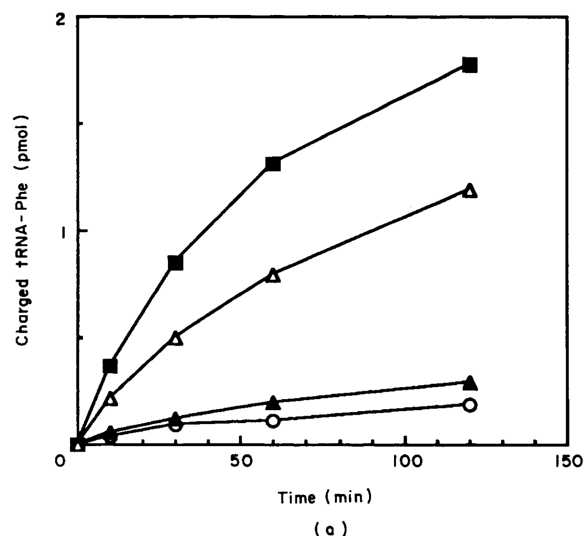
(f) *Role of mutation T224A*

To determine whether mutation T224A had an effect on the toxicity of E152A, we constructed derivatives of plasmid pEMBL9-BY(Ptac) encoding mutant TyrTS that carried mutation T224A either alone or combined with mutation E152A. The e.o.p. of the cellular host showed that mutation T224A was not toxic in itself and that it enhanced the toxicity of mutation E152A 20 to 30 times at both 30 °C and 37 °C (Table 2). This enhancement of toxicity indicates that mutation T224A was not selected but arose by chance. Unwanted additional mutations are frequent during oligonucleotide site-directed mutagenesis (Craik, 1985). We have no simple explanation for the effect of T224A on toxicity when combined with mutation E152A but it is interesting to note that residue Thr224 belongs to a loop of TyrTS that is involved in and moves during the formation of Tyr-AMP (Fersht *et al.*, 1988).

(g) *Charging and mischarging with tyrosine*

The three mutant TyrTS, carrying mutations E152A, -D or -Q, had similar steady-state kinetics parameters for the aminoacylation of pure *E. coli* $tRNA^{Tyr}$ with tyrosine *in vitro* (Table 3). Indeed, their K_M values varied between 0·35 and 0·45 times, and their k_{cat} values between 0·99 and 1·27 times those of the wild-type TyrTS. The ratio of the k_{cat}/K_M values for TyrTS(E152A) and for the wild-type enzyme was equal to 2·5 when pure *E. coli* $tRNA^{Tyr}$ was the substrate for aminoacylation with tyrosine (Table 3) and was equal to 3·4 when it was crude *E. coli* tRNA.

We tested the misaminoacylation of *E. coli* $tRNA^{Phe}$, $tRNA^{Val}$ and crude tRNA with tyrosine *in vitro* by the wild-type TyrTS and those mutated at residue 152. TyrTS(E152D) did not charge $tRNA^{Phe}$ and $tRNA^{Val}$ more than the wild-type TyrTS, whereas TyrTS(E152A) and TyrTS(E152Q) did mischarge them significantly more, although to low levels (about 0·5%; see Fig. 3). For crude *E. coli* tRNA, the levels of charging were about 40% higher with TyrTS(E152A) and TyrTS(E152Q) than with TyrTS(E152D) or the wild-type enzyme. For $tRNA^{Phe}$, the levels of mischarging after 120 minutes of reaction were about twice as high at 37 °C than at 25 °C. Therefore, we did not see the strong temperature effect on *in vitro* mischarging that we saw on *in vivo* toxicity.



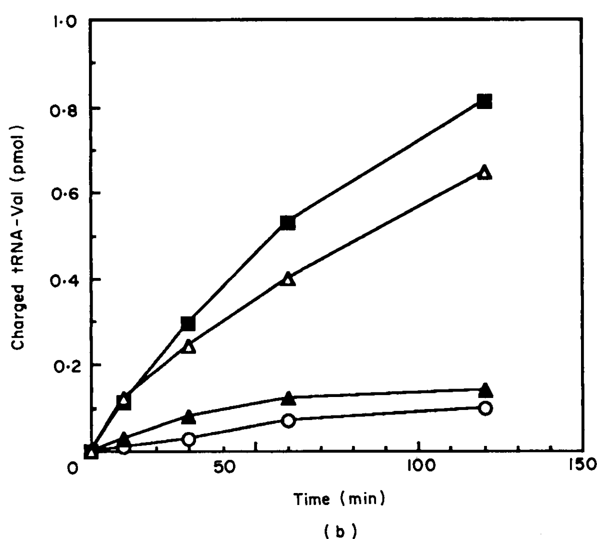


Figure 3. Mischarging of *E. coli* (a) $tRNA^{Phe}$ and (b) $tRNA^{Val}$ with tyrosine. The wild-type (○) or mutant-type TyrTS (E152A (■), E152D (▲) and E152Q (△)) were at 1·0 μM, pure *E. coli* $tRNA^{Phe}$ and $tRNA^{Val}$ at 2·3 and 1·7 μM, respectively. The reactions were performed at 25 °C. Similar results were obtained with $tRNA^{Phe}$ at 37 °C.

4. Discussion

We used three different tests to analyse the effects of mutations of residue Glu152 on the interaction between TyrTS and the *E. coli* tRNAs: (1) the toxicity of TyrTS when overproduced *in vivo*; (2) the charging of $tRNA^{Tyr}$; and (3) the mischargings of $tRNA^{Phe}$ and $tRNA^{Val}$ with tyrosine *in vitro*. The results are summarized in Table 4. In our structural model of the complex between TyrTS and $tRNA^{Tyr}$ (Fig. 1), the side-chain of Glu152 is close to phosphate groups 73 and 74. We found that the mutations of Glu152 affected the interactions between TyrTS and tRNAs, in particular $tRNA^{Tyr}$. Therefore, our new data confirmed this aspect of the model. The four side-chains that we studied in position 152 of TyrTS, Glu, Asp, Gln and Ala, could be distinguished on the basis of the three tests that we used. The interaction between TyrTS and tRNAs therefore appears sensitive to the nature of the side-chain at position 152.

Table 4
Properties of TyrTS modified at residue Glu152

Side-chain	Glu	Asp	Gln	Ala
In vivo toxicity†	−	−	−	+
In vitro mischarging‡	−	−	+	+
Decreased K_M§	−	+	+	+

A minus (−) indicates wild-type properties, a plus (+) indicates altered properties.
† See Table 2.
‡ See Fig. 3.
§ See Table 3.

(a) *Toxicity as a probe of the interaction between TyrTS and tRNAs* in vivo

We showed that mutation E152A rendered TyrTS from *B. stearothermophilus* toxic to *E. coli*. The toxicity was abolished by additional mutations of TyrTS that strongly diminish the binding of $tRNA^{Tyr}$. Thus, it was due to the interaction of TyrTS with $tRNA^{Tyr}$ or other tRNA-like molecules. It is unlikely that TyrTS(E152A) was toxic because it bound other macromolecules than tRNAs; indeed mutations of residues that belong to distant parts of the $tRNA^{Tyr}$-binding site, E152A, K410N and K411N (Bedouelle & Winter, 1986), affected toxicity.

The toxicity of TyrTS(E152A) was not due to a defective interaction with $tRNA^{Tyr}$. Indeed, TyrTS(E152A) charged $tRNA^{Tyr}$ *in vitro* with a kinetic parameter, k_{cat}/K_M, increased 2·5-fold relative to the wild-type enzyme. Moreover, three mutant TyrTS, carrying mutations E152A, -D or -Q, had similar steady-state kinetics parameters for the aminoacylation of $tRNA^{Tyr}$, whereas only TyrTS(E152A) was toxic. We compared the kinetics of aminoacylation with tyrosine for pure $tRNA^{Tyr}$ and crude tRNA. The ratio of the k_{cat}/K_M values for the mutant-type TyrTS(E152A) and the wild-type TyrTS was 2·5 for pure $tRNA^{Tyr}$ and 3·4 for crude tRNA. These values are close and indicate that the toxicity of TyrTS(E152A) was not due to inhibition of the charging of $tRNA^{Tyr}$ by non-cognate tRNAs. As $tRNA^{Tyr}$ was not involved, we conclude that mutation E152A was toxic because it altered the discrimination of TyrTS against non-cognate tRNAs.

TyrTS(E152A) was not toxic to the cell due to a defective folding for the following reasons. Residue Glu152 and its side-chain are located on the surface of TyrTS (Fig. 1; Brick *et al.*, 1989). TyrTS(E152A) was totally active for the charging of $tRNA^{Tyr}$ and thermostable. Doubly mutant TyrTS, carrying mutation E152A in the N-terminal domain and either K410N or K411N in the C-terminal domain,

were not toxic. Our measures of the cellular concentrations of the wild-type and mutant-type TyrTS in cells grown at 30 °C also indicate that the toxicity of TyrTS(E152A) was not due to its overproduction relative to TyrTS of other types (Table 2).

(b) *Potential mechanisms of toxicity*

A priori, the faulty interaction of TyrTS(E152A) with non-cognate tRNAs could have two causes, either their mischarging with tyrosine or their non-productive binding. Mischarging would result in the incorporation of tyrosine, in place of other amino acids, into proteins and in their destabilization. Non-productive binding would drain the cellular pools of some tRNAs species and, in this way, would stop polypeptide elongation. It could result in the misincorporation of amino acids into proteins, indirectly, because the respective concentrations of tRNAs, aminoacyl-tRNA synthetases and translation apparatus are all involved in the fidelity of translation (Yarus, 1972; Swanson *et al.*, 1988). TyrTS(E152A) was weakly toxic at 30 °C, whereas TyrTS(H45N, E152A) was strongly toxic at this temperature. The effect of temperature on toxicity was large (about 6400-fold) for TyrTS(E152A) and moderate (27-fold) for TyrTS(H45N, E152A). These results indicate that the mechanisms of toxicity were different for the two mutant synthetases (Table 2).

The strong effect of temperature on the toxicity of TyrTS(E152A) suggests that this toxicity was due to the misincorporation of amino acids and the destabilization of some vital proteins. The destabilization of proteins, such as HtrB, that are themselves essential for *E. coli* growth above 33 °C (Karow *et al.*, 1991), could amplify the effect of temperature on toxicity and explain its magnitude for TyrTS(E152A).

Because it carried mutation H45N, TyrTS(H45N, E152A) was unable to form Tyr-AMP and aminoacylate tRNAs with tyrosine. Therefore, its own toxicity could only be due to their non-productive binding. This binding could sequester some tRNA species, prevent the incorporation of the corresponding amino acids into proteins and arrest cell division in this way. Such a mechanism can explain why TyrTS(H45N, E152A) was toxic at both 30 °C and 37 °C. The moderate increase (27-fold) in the toxicity of TyrTS(H45N, E152A) with temperature could be due to an indirect effect on the fidelity of translation, induced by the sequestration of tRNAs as explained above. Did the non-productive binding of $tRNA^{Tyr}$, in addition to non-cognate tRNAs, contribute to the toxicity of TyrTS(H45N, E152A)? TyrTS(H45N), which is unable to charge tRNAs with tyrosine, had only a weak (4-fold) toxicity, although it significantly reduced the growth-rate of the bacterial host. Thus, TyrTS(H45N) did not bind $tRNA^{Tyr}$ strongly enough to alter the e.o.p. of the host. Mutation E152A might increase the affinity of TyrTS for $tRNA^{Tyr}$, as indicated by the 2·5-fold decrease in the K_M for charging (Table 3), sufficiently to make TyrTS(H45N, E152A) able to sequester $tRNA^{Tyr}$ when overproduced.

(c) *Role of the side-chain at position 152*

The interaction between TyrTS and $tRNA^{Tyr}$ was strengthened by the mutations of residue 152 by more than 0·5 kcal/mol (1 kcal = 4·184 J: Table 3). This result is compatible with an electrostatic repulsion between the wild-type side-chain, Glu152, and phosphate groups 73 and 74, which are located close by in our structural model of the complex between TyrTS and $tRNA^{Tyr}$ (Fig. 1; Labouze & Bedouelle, 1989). Mutations E152A and E152Q could decrease this repulsion by removing the negative charge of the side-chain, and E152D by shortening it and setting the negative charges wider apart.

E. coli $tRNA^{Phe}$, $tRNA^{Val}$ and crude tRNA were charged more efficiently with tyrosine *in vitro* when the negative charge was removed from position 152, as with mutations E152A and E152Q, than when the charge was maintained, as with E152D or the wild-type Glu152. These results show that the negative charge in position 152 was involved in the rejection of $tRNA^{Phe}$, $tRNA^{Val}$ and possibly other non-cognate tRNAs. Compatible with the functional role of this negative charge, the pair Lys151-Glu152 is conserved in the TyrTS from *E. coli*, *B. stearothermophilus* and *Bacillus caldotenax*, and semi-conserved, as Lys-Asp or Arg-Asp, in the TyrTS from *Bacillus subtilis* and from the mitochondria of *Neurospora crassa* and yeast (Barker *et al.*, 1982; Winter *et al.*, 1983; Jones *et al.*, 1986; Glaser *et al.*, 1991; T. Henkin *et al.*, personal communication; Akins & Lambowitz, 1987; J. Hill & A. Tzagoloff, personal communication).

Mutation E152Q of TyrTS was not toxic for *E. coli*. Thus, the toxicity of E152A was not due to the removal of the negative charge at position 152. The construction of additional mutations will be necessary to determine whether the toxicity of Ala in position 152 was due to the small dimensions of its side-chain, as compared to those of Glu, Gln or Asp, or to the absence of a hydrogen-bond acceptor. The close contact between residue 152 and phosphate groups 73 and 74 in our structural model of the complex between TyrTS and $tRNA^{Tyr}$ (Fig. 1; Labouze & Bedouelle, 1989), suggests that Glu152 could prevent the binding of non-cognate tRNAs by steric hindrance. Thus, several distinct features of the side-chain of residue Glu152, including its negative charge, are important for the discrimination by TyrTS between the tRNAs.

Both TyrTS(E152A) and TyrTS(E152Q) mischarged $tRNA^{Phe}$ and $tRNA^{Val}$ with tyrosine *in vitro* but only TyrTS(E152A) was toxic *in vivo*. These results indicate that the toxicity of TyrTS(E152A) was not due to the mischarging of $tRNA^{Phe}$ or $tRNA^{Val}$ with tyrosine *in vivo*. The non-cognate tRNAs that caused this toxicity remain to be identified.

The kinetic parameter K_M for the aminoacylation of $tRNA^{Tyr}$ was decreased 2·5-fold when going from

the wild-type TyrTS to the mutant TyrTS(E152A), TyrTS(E152D) and TyrTS(E152Q), whereas k_{cat} remained identical. Thus, the wild-type TyrTS was improved for the charging of $tRNA^{Tyr}$ by mutation. For TyrTS(E152A) and TyrTS(E152Q), the increase in the rate of aminoacylation was obtained at the expense of discrimination between tRNAs. We conclude that the synthetase residues that are involved in the binding of the cognate tRNA and in the avoidance of non-cognates can be distinct.

(d) *Relevance to the* in vivo *situation*

Our experiments were done with an heterologous experimental system. We studied the charging of *E. coli* $tRNA^{Tyr}$ and the discrimination between *E. coli* tRNAs by TyrTS from *B. stearothermophilus*. Strictly, our results showed that residue Glu152 of the TyrTS from *B. stearothermophilus* is involved in the discrimination between the *E. coli* tRNAs. The following reasons suggest that the conclusions still hold for the *E. coli* TyrTS. The TyrTS from *E. coli* and *B. stearothermophilus* have 58% identical and 70% conserved residues, which implies that their three-dimensional structures are very similar (Winter *et al.*, 1983; Chothia & Lesk, 1986). The pair Lys151-Glu152 is identical in the two synthetases. The *tyrS* gene from *B. stearothermophilus* can complement a thermosensitive mutation in the *tyrS* gene of *E. coli* for growth at the non-permissive temperature 42°C (Barker, 1982); this implies that the TyrTS from *B. stearothermophilus* recognizes and charges efficiently *E. coli* $tRNA^{Tyr}$ and that it can correctly discriminate between the *E. coli* tRNAs (Bedouelle, 1990). Thus, the TyrTS from *E. coli* and *B. stearothermophilus* have the same substrates and the same specificity, and the mechanisms of interaction with $tRNA^{Tyr}$ and of avoidance of the non-cognate tRNAs have been conserved between the two bacteria during evolution.

We showed that two mutant synthetases, TyrTS(E152A) and TyrTS(E152Q), significantly mischarged $tRNA^{Phe}$ and $tRNA^{Val}$ *in vitro* when the concentrations of tRNAs and synthetases were around 1 μM, i.e. those existing *in vivo* (Jakubowski & Goldman, 1984), and when the buffer conditions were standard. We chose these two *E. coli* tRNAs because they were commercially available. Mischarging by the mutant synthetases might be more important for other *E. coli* tRNAs that we could not test. We showed that the overall viability of *E. coli* cells was decreased 11,000 times when TyrTS(E152A) was overproduced about eight times (Table 2). The sequestration or mischarging of non-cognate tRNAs might already modify the bacterial growth when the synthetase is produced in normal quantities. Therefore residue E152 could have a role *in vivo*.

The strong toxicity of TyrTS(E152A) will enable us to select viable revertants of the mutant strain. The intragenic revertants that keep its activity to TyrTS will allow us to characterize some of the side-chains in position 152 that still permit a good discrimination between the tRNAs. The extragenic revertant mutations might enable us to identify the molecules that are involved in the toxicity, for example the non-cognate tRNAs that are wrongly recognized by TyrTS(E152A).

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References

Akins, R. A. & Lambowitz, A. M. (1987). A protein required for splicing group I introns in *Neurospora* mitochondria is mitochondrial tyrosyl-tRNA synthetase or a derivative thereof. *Cell,* **50**, 331–345.

Barker, D. G. (1982). Cloning and amplified expression of the tyrosyl-tRNA synthetase genes of *Bacillus stearothermophilus* and *Escherichia coli*. *Eur. J. Biochem.* **125**, 357–360.

Barker, D. G., Bruton, C. J. & Winter, G. (1982). The tyrosyl-tRNA synthetase from *Escherichia coli*. Complete nucleotide sequence of the structural gene. *FEBS Letters,* **150**, 419–423.

Bedouelle, H. (1990). Recognition of $tRNA^{Tyr}$ by tyrosyl-tRNA synthetase. *Biochimie,* **72**, 589–598.

Bedouelle, H. & Duplay, P. (1988). Production in *Escherichia coli* and one-step purification of bifunctional hybrid proteins which bind maltose. Export of the Klenow polymerase into the periplasmic space. *Eur. J. Biochem.* **171**, 541–549.

Bedouelle, H. & Winter, G. (1986). A model of synthetase/transfer RNA interaction as deduced by protein engineering. *Nature (London)*, **320**, 371–373.

Bedouelle, H., Guez, V., Vidal-Cros, A. & Hermann, M. (1990). Overproduction of tyrosyl-tRNA synthetase is toxic to *Escherichia coli*: a genetic analysis. *J. Bacteriol.* **172**, 3940–3945.

Bollum, F. J. (1959). Thermal conversion of nonpriming deoxyribonucleic acid to primer. *J. Biol. Chem.* **234**, 2733–2734.

Bonnet, J. & Ebel, J. P. (1972). Interpretation of incomplete reactions in tRNA aminoacylation. *Eur. J. Biochem.* **31**, 335–344.

Brick, P., Bhat, T. N. & Blow, D. M. (1989). Structure of tyrosyl-tRNA synthetase refined at 2·3 Å resolution. Interaction of the enzyme with the tyrosyl adenylate intermediate. *J. Mol. Biol.* **208**, 83–98.

Carter, P., Bedouelle, H. & Winter, G. (1985). Improved oligonucleotide site-directed mutagenesis using M13 vectors. *Nucl. Acids Res.* **13**, 4431–4443.

Carter, P., Bedouelle, H. & Winter, G. (1986). Construction of heterodimer tyrosyl-tRNA synthetase shows $tRNA^{Tyr}$ interacts with both subunits. *Proc. Nat. Acad. Sci., U.S.A.* **83**, 1189–1192.

Celis, J. E. & Piper, P. W. (1982). Compilation of mutant suppressor tRNA sequences. *Nucl. Acids Res.* **10**, r83–r91.

Chothia, C. & Lesk, A. M. (1986). The relation between the divergence of sequence and structure in proteins. *EMBO J.* **5**, 823–826.

Craik, C. S. (1985). Use of oligonucleotides for site-specific mutagenesis. *Biotechniques,* **3**, 12–17.

Dente, L. & Cortese, R. (1987). pEMBL: a new family of

single-stranded plasmids for sequencing DNA. *Methods Enzymol.* **155**, 111–119.
Fersht, A. R. (1987). Dissection of the structure and activity of the tyrosyl-tRNA synthetase by site-directed mutagenesis. *Biochemistry,* **26**, 8031–8037.
Fersht, A. R., Knill-Jones, J. W., Bedouelle, H. & Winter, G. (1988). Reconstruction by site-directed mutagenesis of the transition state for the activation of tyrosine by the tyrosyl-tRNA synthetase: a mobile loop envelopes the transition state in an induced-fit mechanism. *Biochemistry,* **27**, 1581–1587.
Fournier, M. J. & Ozeki, H. (1985). Structure and organization of the transfer ribonucleic acid genes of *Escherichia coli* K12. *Microbiol. Rev.* **49**, 379–397.
Glaser, P., Kunst, F., Débarbouillé, M., Vertes, A., Danchin, A. & Dedonder, R. (1990). A gene encoding a tyrosine tRNA synthetase is located near *SacS* in *Bacillus subtilis. DNA Seq.* **1**, 251–261.
Hermann, M. & Bedouelle, H. (1990). A method for monitoring double restriction cuts within a polylinker. *Res. Microbiol.* **141**, 187–189.
Himeno, H., Hasegawa, T., Ueda, T., Watanabe, K. & Shimizu, M. (1990). Conversion of aminoacylation specificity from $tRNA^{Tyr}$ to $tRNA^{Ser}$ *in vitro. Nucl. Acids Res.* **18**, 6815–6819.
Hou, Y. M. & Schimmel, P. (1989). Modeling with in vitro kinetic parameters for the elaboration of transfer RNA identity in vivo. *Biochemistry,* **28**, 4942–4947.
Jakubowski, H. & Goldman, E. (1984). Quantities of individual aminoacyl-tRNA families and their turnover in *Escherichia coli. J. Bacteriol.* **158**, 769–776.
Jones, M. D., Lowe, D. M., Borgford, T. & Fersht, A R. (1986). Natural variation of tyrosyl-tRNA synthetase and comparison with engineered mutants. *Biochemistry,* **25**, 1887–1891.
Karow, M., Fayet, O., Cegielska, A., Ziegelhoffer, T. & Georgopoulos, C. (1991). Isolation and characterization of the *Escherichia coli htrB* gene whose product is essential for bacterial viability above 33 °C in rich media. *J. Bacteriol.* **173**, 741–750.
Labouze, E. & Bedouelle, H. (1989). Structural and kinetic bases for the recognition of $tRNA^{Tyr}$ by tyrosyl-tRNA synthetase. *J. Mol. Biol.* **205**, 729–735.
Leatherbarrow, R. J. (1990). Using linear and non-linear regression to fit biochemical data. *Trends Biochem. Sci.* **15**, 455–458.
Mead, D. A., Szczesna-Skorupa, E. & Kemper, B. (1986). Single-stranded DNA "blue" T7 promoter plasmids: a versatile tandem promoter system for cloning and protein engineering. *Protein Eng.* **1**, 67–74.
Normanly, J. & Abelson, J. (1989). tRNA identity. *Annu. Rev. Biochem.* **58**, 1029–1049.
Perona, J. J., Rould, M. A., Steitz, T. A., Risler, J. P., Zelwer, C. & Brunie, S. (1991). Structural similarities in glutaminyl- and methionyl-tRNA synthetases suggest a common overall orientation of tRNA binding. *Proc. Nat. Acad. Sci., U.S.A.* **88**, 2903–2907.
Rould, M. A., Perona, J. J., Söll, D. & Steitz, T. A. (1989). Structure of *E. coli* glutaminyl-tRNA synthetase complexed with $tRNA^{Gln}$ and ATP at 2·8 Å resolution. *Science,* **246**, 1135–1142.
Rould, M. A., Perona, J. J. & Steitz, T. A. (1991). Structural basis of anticodon loop recognition by glutaminyl-tRNA synthetase. *Nature (London),* **352**, 213–218.
Ruff, M., Krishnaswamy, S., Boeglin, M., Poterszman, A., Mitschler, A., Podjarny, A., Rees, B., Thierry, J. C. & Moras, D. (1991). Class II aminoacyl transfer RNA synthetases: crystal structure of yeast aspartyl-tRNA synthetase complexed with $tRNA^{Asp}$. *Science,* **252**, 1682–1689.
Russell, D. R. & Bennett, G. N. (1982). Construction and analysis of in vivo activity of *E. coli* promoter hybrids and promoter mutants that alter the −35 to −10 spacing. *Gene,* **20**, 231–243.
Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual,* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Schimmel, P. (1989). Parameters for the molecular recognition of transfer RNAs. *Biochemistry,* **28**, 2747–2759.
Schulman, L. H. & Abelson, J. (1988). Recent excitement in understanding transfer RNA identity. *Science,* **240**, 1591–1592.
Swanson, R., Hoben, P., Sumner-Smith, M., Uemura, H., Watson, L. & Söll, D. (1988). Accuracy of in vivo aminoacylation requires proper balance of tRNA and aminoacyl-tRNA synthetase. *Science,* **242**, 1548–1551.
Tabor, S. & Richardson, C. C. (1987). DNA sequence analysis with a modified bacteriophage T7 DNA polymerase. *Proc. Nat. Acad. Sci., U.S.A.* **84**, 4767–4771.
Terwilliger, T. C., Fulford, W. D. & Zabin, H. B. (1988). A genetic selection for temperature-sensitive variants of the gene *V* protein of bacteriophage f1. *Nucl. Acids Res.* **16**, 9027–9039.
Vieira, J. & Messing, J. (1982). The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. *Gene,* **19**, 259–268.
Ward, W. H. J. & Fersht, A. R. (1988). Asymmetry of tyrosyl-tRNA synthetase in solution. *Biochemistry,* **27**, 1041–1049.
Waye, M. M. Y. & Winter, G. (1986). A transcription terminator in the 5′ non-coding region of the tyrosyl tRNA synthetase gene from *Bacillus stearothermophilus. Eur. J. Biochem.* **158**, 505–510.
Waye, M. M. Y., Winter, G., Wilkinson, A. J. & Fersht, A. R. (1983). Deletion mutagenesis using an "M13 splint": the N-terminal structural domain of tyrosyl-tRNA synthetase (*B. stearothermophilus*) catalyses the formation of tyrosyl adenylate. *EMBO J.* **2**, 1827–1829.
Wilkinson, A. J., Fersht, A. R., Blow, D. M. & Winter, G. (1983). Site directed mutagenesis as a probe of enzyme structure and catalysis: tyrosyl-tRNA synthetase cysteine-35 to glycine-35 mutation. *Biochemistry,* **22**, 3581–3586.
Winter, G., Koch, G. L. E., Hartley, B. S. & Barker, D. G. (1983). The amino acid sequence of the tyrosyl-tRNA synthetase from *Bacillus stearothermophilus. Eur. J. Biochem.* **132**, 383–387.
Yarus, M. (1972). Intrinsic precision of aminoacyl-tRNA synthesis enhanced through parallel systems of ligands. *Nature New Biol.* **239**, 106–108.
Yarus, M. (1988). tRNA identity: a hair of the dogma that bit us. *Cell,* **55**, 739–741.

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