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Mapping the Stability Determinants of Bacterial Tyrosyl Transfer RNA Synthetases by an Experimental Evolutionary Approach

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The tyrosyl-tRNA synthetases from *Bacillus stearothermophilus* (Bst-TyrTS) and *Escherichia coli* (Eco-TyrTS) are 56% identical in amino acid sequence. To map and characterize the set of interactions that makes Bst-TyrTS more stable than Eco-TyrTS, a family of nine hybrid proteins was constructed between the two enzymes. The N-terminal part of each hybrid came from Eco-TyrTS and the C-terminal part from Bst-TyrTS. The stability and activity of these hybrids were estimated by experiments of thermal inactivation and tRNA charging. For all the hybrids, the temperature of half-inactivation in 30 minutes was above 44 °C and the rate of charging was at least 40% that of Bst-TyrTS. In general, the temperature of half-inactivation increased and the rate of charging decreased monotonically when the number of residues coming from the more stable and less active Bst-TyrTS increased. As a result, the rate of charging decreased when the temperature of half-inactivation increased. These results show that the sequences and structures of the two enzymes can replace each other locally and still give a stable and active TyrTS, and that the greater stability of Bst-TyrTS is due to cumulative changes of residues scattered along the sequence. They suggest that Bst-TyrTS is more rigid than Eco-TyrTS at low temperature. The existence of a few exceptional hybrids, having stabilities or activities lower than those of the neighbouring hybrids, shows that compensatory changes of residues have occurred between the two sequences during evolution. These exceptions could be explained by the systematic identification of the couples of residues that are in contact in the Bst-TyrTS structure and became heterologous in some hybrids.

Keywords: aminoacyl transfer-RNA synthetase; hybrid protein; thermostability; evolution; compensatory mutations

1. Introduction

The comparison of proteins performing similar functions in different organisms, at the levels of sequence and three-dimensional structure, has led to important concepts about the mechanisms of protein folding and evolution. The amino acid sequence of a protein contains the information that determines its structure. The finding that this information is degenerate, in the sense that proteins having essentially the same structure and activity can have very different sequences, has raised two main questions: (1) By which mechanisms does structure adapt to sequence changes (Chothia & Lesk, 1987; Bordo & Argos, 1990; Perry *et al.*, 1990; Bowie *et al.*, 1990)? (2) What are the sequence characteristics that determine the three-dimensional structure (Perutz *et al.*, 1965; Bashford *et al.*, 1987; Overington *et al.*, 1990)? On the answers to these questions depend our abilities to anticipate the effects of mutations, to design new proteins and to predict the structure and function of a protein from its amino acid sequence.

The comparison of evolutionary related proteins has also been used to understand the structural bases of their differences in stability (Perutz & Raidt, 1975). This approach has met with little success (Jaenicke, 1991) because the stability of proteins is marginal and due to a delicate balance

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between numerous stabilizing and destabilizing interactions. Moreover, these interactions act co-operatively. How the stability of proteins evolves therefore remains partially unknown.

Parallel with the above comparisons, studies of evolutionary rates and of protein polymorphisms have led to the hypothesis that many sequence changes are selectively neutral and were fixed by a random genetic drift (Kimura & Ohta, 1971). Several models, "context effect" (Hardies & Garvin, 1991), "directed pathway" (Lim & Sauer, 1989; Bordo & Argos, 1990), describe how the interplay between the limited plasticity of structure and the necessity of maintaining function constrain the sequence changes during the evolution of a protein. All the above ideas have been tested in great detail by creating single or multiple mutations in proteins, using the techniques of site-directed mutagenesis and protein engineering (Matthews, 1987; Malcolm *et al.*, 1990; Vernet *et al.*, 1992; Eigenbrot & Kossiakov, 1992; Fersht & Winter, 1992, and references therein).

To understand how the stability of a protein evolves, we want to map and characterize the set of interactions that makes an enzyme from a thermophilic organism more stable than its homologue from a mesophilic organism. In this work, as a first step towards these goals, we constructed a family of hybrid proteins between the tyrosyl-tRNA synthetases from *Escherichia coli* (Eco-TyrTS) and *Bacillus stearothermophilus* (Bst-TyrTS). The N-terminal part of each hybrid came from Eco-TyrTS and its C-terminal part from Bst-TyrTS. This family can be considered as a set of artificial evolutionary intermediates. We measured the stability and activity of these hybrids and analysed their variations when the position of the fusion point moved along the protein sequence. We then interpreted the data in light of the crystal structure of Bst-TyrTS.

TyrTS catalyses the aminoacylation of $tRNA^{Tyr}$ with tyrosine. The *tyrS* genes from *E. coli* (*Eco-tyrS*) and *B. stearothermophilus* (*Bst-tyrS*) are 58% identical in nucleotide sequence and the two corresponding proteins are 56% identical in amino acid sequence (Barker *et al.*, 1982; Winter *et al.*, 1983). Eco-TyrTS is a thermolabile protein whereas Bst-TyrTS is thermostable (Barker, 1982; Jones *et al.*, 1986). The crystal structure of Bst-TyrTS has been solved at 2·3 Å resolution. It is a dimer whose subunits are symmetrical through a 2-fold rotational axis. Each monomer has three domains, an α/β-domain (residues 1 to 220) containing a six-stranded β-sheet, an α-helical domain (248 to 318) containing five α-helices and a C-terminal domain (319 to 419) for which it was not possible to trace the polypeptide chain (Fig. 1; Brick & Blow, 1987; Brick *et al.*, 1989). The crystal structure of Eco-TyrTS has not been characterized but, given its

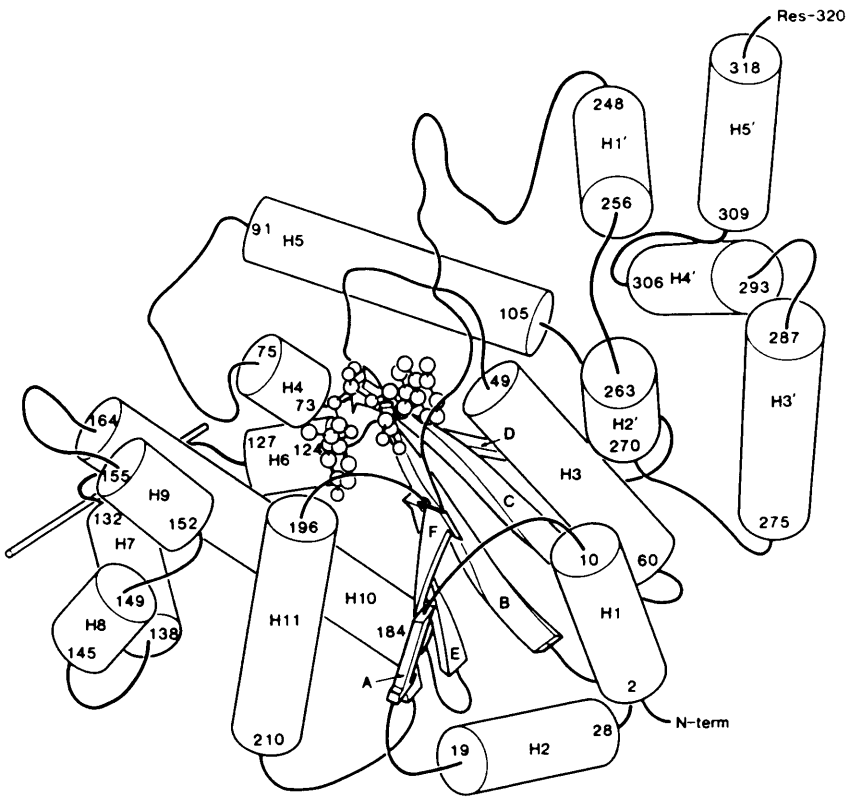

Figure 1. Schematic diagram of the ordered domains of the TyrTS structure showing the tyrosyl-adenylate binding site and the arrangement of the α-helices and β-sheets within one monomer. The position of the crystallographic 2-fold axis is indicated by the diagonal rod at the left. Reproduced from Brick *et al.* (1989) with permission.

strong identity of sequence with Bst-TyrTS, the structures of the two proteins should be rather similar (Chothia & Lesk, 1987).

2. Materials and Methods

(a) *Parental strains and growth conditions*

The bacterial strains of *E. coli K12*, HB2109 (*tyrS*(Ts) *recA srl*::Tn*10 argG thi lac*), TG1 and TG2, the bacteriophages M13KO7 and M13(4am)-BY(M24.89), and phagemids pEMBL9$^+$, pEMBL8-EY, pEMBL9-BY(Ptac) and pEMBL9-BY(Ptac, Δ1) have been described (Bedouelle & Winter, 1986; Bedouelle *et al.*, 1990; Vidal-Cros & Bedouelle, 1992; Sambrook *et al.*, 1989). M13(4am)-BY(M24.89) carries the *Bst-tyrS* gene under control of a mutant promoter. The phagemids carry the replication origin of the filamentous phage fd. pEMBL8-EY carries the *Eco-tyrS* gene under control of its own promoter; pEMBL9-BY(Ptac) carries the *Bst-tyrS* gene under control of promoter *tac*, which is inducible with isopropyl-βD-thiogalactopyranoside (IPTG); pEMBL9-BY(Ptac, Δ1) is a derivative of pEMBL9-BY(Ptac) that carries a deletion of the 3′-terminal part of *Bst-tyrS*. The media were prepared and the bacteria grown as described (Bedouelle *et al.*, 1990). In particular, the strains that harboured a recombinant plasmid were grown at 30°C because some of these plasmids were toxic at 37°C.

(b) *Mutagenesis and sequencing*

The single-stranded DNA of the phages and phagemids was prepared as described (Sambrook *et al.*, 1989), taking TG2 as a cellular host and M13KO7 as a helper phage. We used this single-stranded DNA as template for mutagenesis and sequencing. We determine the nucleotide sequences of the mutant and hybrid *tyrS* genes by a variation of the dideoxy technique (Tabor & Richardson, 1987), using a universal primer that hybridizes within the *lacZ* gene and oligonucleotides that hybridized within the *Bst-tyrS* gene as primers.

We introduced a *Bsm*I restriction site into the *Bst-tyrS* gene by replacing the codon (CGC, Ala) at position 321 by (TGC, Cys), which is present in the *Eco-tyrS* gene at the homologous position 325. We made this replacement by site-directed mutagenesis of phage M13(4am)-BY(M24.89) with oligonucleotide 5′CTA AAG AGG CAT TCA GAA ATG3′ and an amber selection (Carter *et al.*, 1985). We named the mutant phage M13-BY(BsmI) and the corresponding gene, *Bst-tyrS(BsmI)*.

We corrected the mutation that changed codon 123 from GCG (Ala) to ACG in hybrid gene *Eco/Bst-tyrS3**, by site-directed mutagenesis (Kunkel *et al.*, 1987) of phagemid pEMBL-E/BY3* with an oligonucleotide, 5′GTT GTT CGC CGC GAT AG3′, that straddled the fusion point between the *Eco-tyrS* and *Bst-tyrS* genes in this hybrid. We checked the complete sequence of the corrected gene and of its promoter and named it *Eco/Bst-tyrS3*.

(c) *Phagemid constructions*

The construction of phagemid pEMBL-EY-BY(Ptac) by *in vitro* recombination and the obtaining of phagemids pEMBL-E/BY1 to 8 by *in vivo* recombination are described in Results. We created the *Eco/Bst-tyrS9* hybrid gene by recombining the *Bsm*I restriction sites of the *Eco-tyrS* and *Bst-tyrS(BsmI)* genes *in vitro*. We assembled phagemid pEMBL-E/BY9, which carried this hybrid gene, through the following steps. The *Pst*I-*Eco*RI restriction fragment of phage M13-BY(BsmI), that carried codons 173 to 419 of *Bst-tyrS(BsmI)*, was inserted between the corresponding sites of phagemid pEMBL9-BY(Ptac, Δ1). The resulting phagemid carried the mutant allele, *Bst-tyrS(BsmI)*, and we named it pEMBL9-BY(Ptac, BsmI). Phagemids pEMBL-BY(Ptac, BsmI) and pEMBL8-EY were digested with *Bsm*I and with *Sca*I, which cuts in the β-lactamase gene, then the fragments were mixed and ligated. pEMBL-E/BY9, which carried the 5′-terminal part of *Eco-tyrS* and the 3′-terminal part of *Bst-tyrS*, was identified by restriction analysis with the *Bam*HI and *Hin*dIII endonucleases.

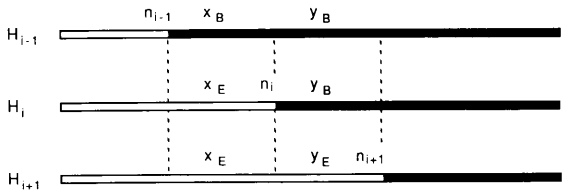

Figure 2. Couples of heterologous residues in the hybrids. Let H_i be a hybrid TyrTS, n_i be the position of its fusion point in the Bst-TyrTS sequence, x and y be 2 residue positions in this sequence ($x<y$), x_B be the side-chain at position x in Bst-TyrTS and x_E be the side-chain at the homologous position in Eco-TyrTS. For the couple of residues at positions x and y to become heterologous in one hybrid, it is necessary that $x_E \neq x_B$ and $y_E \neq y_B$. For a couple of heterologous residues at positions x and y to be specific H_i, it is necessary that x be in the sequence to interval $[n_{i-1}, n_i]$ and y be in $[n_i, n_{i+1}]$.

(d) *Preparation of cellular extracts*

The soluble extracts were prepared as described (Bedouelle *et al.*, 1990) except that, after washing, the cells were resuspended in 0·1 vol. standard buffer (44 mM Tris, 100 mM Tris·HCl, pH 7·78, 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 0·1 mM phenyl-methylsulphonide fluoride (PMSF)) when the preparation was for the aminoacylation of $tRNA^{Tyr}$ and in 1/40 vol. for the thermal-inactivation experiments, taking the culture volume as the reference. The soluble extracts were used immediately after their preparation. When the soluble extracts were prepared only for an analysis by gel electrophoresis, the bacterial cultures were centrifuged, resuspended in 0·1 vol. (50 mM Tris·HCl, pH 7·5, 10 mM 2-mercaptoethanol, 0·1 mM PMSF), frozen at −20°C, then processed as described (Bedouelle *et al.*, 1990). The gels were 8% (w/v) in polyacrylamide (acrylamide/bisacrylamide = 29 : 1) and 0·1% (w/v) in SDS; they were stained with Coomassie blue.

(e) *Active site titration*

We determined the concentration of TyrTS active sites in soluble extracts as described (Wilkinson *et al.*, 1983). The reactions were performed at 25°C in standard buffer. Sodium pyrophosphate was added to the reaction mixture to displace unlabelled Tyr-AMP from TyrTS and then hydrolysed with pyrophosphatase to initiate formation of ^{14}C-labelled Tyr-AMP. We introduced the following modifications. The reaction mixture (60 μl) contained 20 μM

[^{14}C]Tyr, 10 mM ATP-Mg^{2+} and 32 μl soluble extract. Portions (25 μl) were spotted onto nitrocellulose filters after 5 and 30 min then washed with 25 ml of ice cold, 5 times diluted standard buffer.

(f) *Aminoacylation of $tRNA^{Tyr}$*

We measured the initial rate of $tRNA^{Tyr}$ charging with [^{14}C]tyrosine as described (Vidal-Cros & Bedouelle, 1992) with the following modifications. The reactions were performed at 25°C in standard buffer. The final reaction mixture (125 μl) contained 20 μM [^{14}C]tyrosine, 10 mM ATP-Mg^{2+}, 5 mg/ml crude *E. coli* tRNA, 10 units/ml inorganic pyrophosphatase and 25 μl of a dilution of the soluble extract in standard buffer. We omitted bovine serum albumin in the buffers. The concentration of TyrTS in the reaction was between 0·18 and 1·7 nM. We determined the concentration of aminoacylable $tRNA^{Tyr}$ in the preparation of crude *E. coli* tRNA (356 pmol of tyrosine acceptance/mg) by using a large quantity of soluble extract from strain TG2(pEMBL8/EY), corresponding to 0·1 μM Eco-TyrTS in the reaction mixture.

(g) *Thermal inactivation*

The cellular extracts of strains producing wild-type or hybrid TyrTS were divided in identical portions and these portions were simultaneously incubated at varying temperatures during 30 min. The samples were cooled 10 min on ice and the precipitate of protein was eliminated by centrifugation. The active sites of TyrTS in the heated samples were then titrated. As a control, the same experiment was performed with purified Bst-TyrTS, prepared as described (Vidal-Cros & Bedouelle, 1992). The concentration of TyrTS active-sites in the unheated samples was around 1 μM.

(h) *Identification of heterologous contacts*

To provide a structural interpretation of the hybrid properties, we looked for the couples of residues that are in contact in the Bst-TyrTS structure and became heterologous in some hybrids (Fig. 2). Let x and y be 2 residue positions in the Bst-TyrTS sequence ($x<y$). We note x_B the side-chain at position x in Bst-TyrTS and x_E the side-chain at the homologous position in Eco-TyrTS. For the couple of residues at positions x and y to become heterologous in one of the hybrids, it is necessary that $x_E \neq x_B$ and $y_E \neq y_B$. Let H_i be a hybrid TyrTS and n_i be the position of its fusion point in the sequence ($i=1$ to 9). For a couple of heterologous residues at positions x and y to be specific to H_i, it is necessary that x belongs to the sequence interval $[n_{i-1}, n_i]$, between n_{i-1} and n_i, and that y belongs to $[n_i, n_{i+1}]$. Let $d(x, y)$ be the minimal distance between atoms of the side-chains of x_B and y_B (C^α excluded) in the structure of Bst-TyrTS. For each value of i, we looked for the heterologous contacts specifically created in H_i, i.e. couples (x, y) of positions such that x be in $[n_{i-1}, n_i]$ y in $[n_i, n_{i+1}]$, $x_B \neq x_E$, $y_B \neq y_E$ and $d(x, y) \leq 6$ Å. In the case of H_3, we did not find any couple (x, y) of positions satisfying the above conditions. We therefore broadened our search to couples of residues that are in contact in the structure through the intermediate of a third residue or a water molecule and became heterologous specifically in H_3, i.e. we looked for triplets (x, y, z), where z is either a residue position in the Bst-TyrTS sequence or a water molecule in its structure, such that x be in $[n_2, n_3]$, y in $[n_3, n_4]$, $x_B \neq x_E$, $y_B \neq y_E$, $d(x, z) \leq 6$ Å and $d(z, y) \leq 6$ Å.

3. Results

(a) *Construction of hybrid genes between* Eco-tyrS *and* Bst-tyrS

We constructed hybrid genes between *Eco-tyrS* and *Bst-tyrS* by homologous recombination *in vivo* as follows. In a first step, we constructed phagemid pEMBL-EY-BY(Ptac), which carried *Eco-tyrS* under control of its own promoter and *Bst-tyrS* under control of promoter *tac*, in tandem and in the same orientation (Fig. 3(c)). As the sequences of *Eco-tyrS* and *Bst-tyrS* are 58% identical, pEMBL-EY-BY(Ptac) could undergo intramolecular homologous recombinations (Fig. 3(d)) that precisely fuse the two genes and create hybrids, *Eco/Bst-tyrS*, in which the promoter and the 5′ part of the gene come from *E. coli* while the 3′ part of the gene comes from *B. stearothermophilus* (Fig. 3(e)). We used strain TG1, which is *recA*$^+$ and thus capable of homologous recombination, as a host to prepare the DNA of pEMBL-EY-BY(Ptac).

In a second step, we selected the recombinants by using the property of circular DNA to transform *E. coli* more efficiently than linear DNA. We digested the DNA of pEMBL-EY-BY(Ptac) with restriction endonucleases that cut this phagemid only once, between the *Eco-tyrS* and *Bst-tyrS* genes (*Hin*dIII, *Eco*RV and *Kpn*I, Fig. 3(c)). The phagemid molecules that had not undergone an homologous recombination, were linearized by these digestions whereas those molecules that had recombined, remained circular since their intergenic

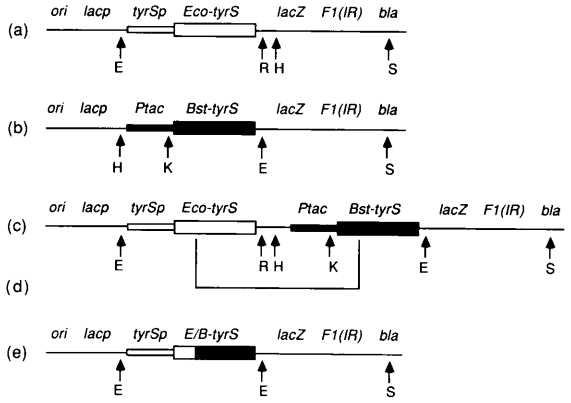

Figure 3. Structures of the recombinant phagemids. E, *Eco*RI; R, *Eco*RV; H, *Hin*dIII; S, *Sca*I; K, *Kpn*I. (a) pEMBL-EY: a derivative of pEMBL8$^+$ carrying the *Eco-tyrS* gene under control of its own promoter, *Eco-tyrSp*. (b) pEMBL9-BY(Ptac): a derivative of pEMBL9$^+$ carrying *Bst-tyrS* under control of the *tac* promoter. (c) Digestion of pEMBL-EY and pEMBL9--BY(Ptac) with *Hin*dIII and *Sca*I then ligation gave phagemid pEMBL-EY-BY(Ptac) carrying *Eco-tyrS* and *Bst-tyrS* in tandem and in the same orientation. (d) *In vivo* intramolecular recombinations between homologous sequences of pEMBL-EY-BY(Ptac) gave (e), i.e. phagemids pEMBL-E/BY carrying hybrid genes *Eco/Bst-tyrS* under control of promoter *Eco-tyrSp*. (e) also schematizes the structure of pEMBL-E/BY9, which was assembled by *in vitro* recombination.

region had been deleted during the recombination event. After performing the digestions, we introduced the whole of the molecules derived from pEMBL-EY-BY(Ptac) into strain TG2, which is *recA*, by transformation.

Whatever the point of homologous recombination between the two parental genes, the hybrid gene is located on an *Eco*RI restriction fragment of about 2000 bp (Fig. 3(e)). This property allowed us to identify the phagemids that had undergone the desired recombination. After transformation, we prepared the replicative form of the phagemid DNA from 154 colonies that were resistant to ampicillin and found 42 phagemids potentially carrying a hybrid gene, *Eco/Bst-tyrS*. We called these phagemids pEMBL-E/BY.

We created an additional hybrid gene by *in vitro* recombination. The *Eco-tyrS* gene carries a unique *Bsm*I site at the level of codons 324 to 326. We introduced a *Bsm*I site into the *Bst-tyrS* gene, at the level of the homologous codons, 320 to 322, by oligonucleotide site-directed mutagenesis. We then fused the 5′-terminal part of the wild-type *Eco-tyrS* gene to the 3′-terminal part of the mutant gene, *Bst-tyrS(BsmI)*, by digestion and ligation at the *Bsm*I sites. The recombinant gene, *Eco/Bst-tyrS9*, coded for a hybrid TyrTS comprising the N-terminal domain of Eco-TyrTS and the C-terminal domain of Bst-TyrTS.

(b) *Mapping of the fusion points*

To determine the precise nature of the recombination events, we first mapped the fusion sites of the hybrid genes in eight restriction fragments, using endonucleases that cut rarely in the *Bst-tyrS* gene (*Bal*I, *Aat*II, *Pst*I and *Bss*HII) or in the *Eco-tyrS* gene (*Bam*HI, *Bss*HII, *Bsm*I). For each hybrid gene, we then sequenced at least the physical interval where the fusion point was located, using oligonucleotides hybridizing within *Bst-tyrS* as primers. Distinct clones gave the same fusion point and fusion points that were different but close sometimes created identical hybrid proteins. Twenty one clones had recombination sites that were identical and brought the wild-type *Bst-tyrS* gene under control of a hybrid regulatory region, *Eco/Bst-tyrSp*, between those of *Eco-tyrS* and *Bst-tyrS*. One of the hybrid genes, corresponding to a recombination event at the level of codons 124 to 126 of *Eco-tyrS* and 122 to 124 of *Bst-tyrS*, carried an additional mutation, located two nucleotides upstream of the recombination site. This mutation changed codon 123 of *Eco-tyrS* from GCG (Ala) to ACG (Thr). We corrected this additional mutation by oligonucleotide site-directed mutagenesis to restore the wild-type codon, GCG. In the following, we shall only consider the corrected hybrid. The hybrid genes that we selected *in vivo* corresponded to 13 different events of recombination and to 8 distinct hybrid proteins (Table 1) that we called H1 to H8. We called H9, the product of the hybrid gene that we constructed *in vitro*.

Table 1
Positions of the fusion points

Class	No.	Codons	Amino acids
H1	1	35–36	33–45
H2	3	57–62	57–63
H3	1	122–124	122–126
H4	3	140–141	140–146
H5a	1	188–190	188–200
H5b	1	194–195	188–200
H5c	1	196–197	188–200
H5d	2	198–200	188–200
H6	2	232	224–235
H7	2	236–237	237
H8a	1	252–254	251–258
H8b	2	255–258	251–258
H9	1	322	322–325

Positions of the fusion points in the hybrid genes and proteins. The classes (1 to 9) correspond to different hybrid proteins between Eco-TyrTS and Bst-TyrTS, and the subclasses (a to d) to different events of homologous recombination between the *Eco-tyrS* and *Bst-tyrS* genes. The positions are given in the form of an interval of codons or amino acid residues that are identical in the 2 species. They are numbered according to the sequences of *Bst-tyrS* and Bst-TyrTS. No., number of occurrences.

(c) *Production of the hybrid synthetases*

We analysed the production of hybrid TyrTS from the recombinant phagemids by electrophoresis of the proteins through SDS/polyacrylamide gels and active site titration of the enzymes. These analyses were performed on soluble extracts of strain TG2, taken as a cellular host. When the wild-type *Bst-tyrS* gene was under control of the hybrid promoter, *Eco/Bst-tyrSp*, the production of TyrTS was undetectable in gels. All the other hybrid genes were under control of the wild-type promoter, *Eco-tyrSp*. The strains harbouring these hybrid genes synthesized polypeptides migrating as Eco-TyrTS and Bst-TyrTS. The migrations varied slightly from one hybrid to another. The levels of production of the hybrids were close to that of Eco-TyrTS, as judged from the gels (not shown).

The control strain, TG2(pEMBL9$^+$), produced 2000 molecules of TyrTS per cell, as determined by active site titration. The strain harbouring the *Bst-tyrS* gene under control of the hybrid promoter, *Eco/Bst-tyrSp*, synthesized 8600 molecules of TyrTS per cell. The production of hybrid TyrTS from phagemids pEMBL-E/BY comprised between 61% and 101% of the production of Eco-TyrTS from pEMBL8-EY (71,000 molecules/cell), except for the production of hybrid H1 which was 183% that of Eco-TyrTS (Fig. 4). These experiments of active site titration were a first indication that the hybrid TyrTSs were at least partially active.

(d) *Activity of the hybrids* in vivo *and* in vitro

Strain HB2109 does not grow at 42°C because it carries a thermosensitive mutation in its *tyrS* gene, which is essential. We introduced the vector pEMBL9$^+$, the parental phagemids pEMBL8-EY and pEMBL9-BY(Ptac), and the recombinant

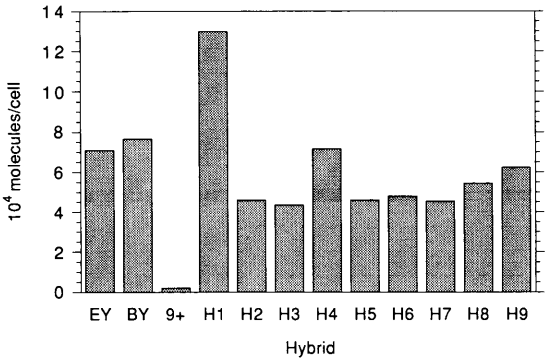

Figure 4. Production of active TyrTS by the parental and recombinant phagemids. 9^+, pEMBL9$^+$; EY, pEMBL-EY; BY, pEMBL-BY(Ptac); H1 to H9, phagemids pEMBL-E/BY1 to 9. The host strain was TG2. Ptac was induced with 1 mM IPTG. The concentration of TyrTS in the cellular extracts was determined by active site titration.

phagemids pEMBL-E/BY, individually into HB2109 by transformation. All the derivatives of HB2109 grew at 42°C, except HB2109(pEMBL9$^+$). These experiments of genetic complementation showed that all the hybrids were functional *in vivo* at 42°C, for the charging of $tRNA^{Tyr}$ by tyrosine and also for the discrimination in favour of $tRNA^{Tyr}$ and against the 19 other species of tRNAs.

To characterize the activity of the hybrids quantitatively, we performed kinetic assays *in vitro*. We prepared soluble cellular extracts of the recombinant strains, TG2(pEMBL-E/BY), and, for each preparation, we measured both the concentration of TyrTS active sites and the rate of tyrosylation of crude *E. coli* tRNA, so that the rates (V) are expressed in s^{-1} (Table 2). The rates of aminoacylation by the hybrids varied between 0·43 and 2·76 times that of Bst-TyrTS and between 0·15 and 0·96 times that of Eco-TyrTS. Thus, all the hybrids were highly active. Figure 6a shows the variation of the rate of tyrosylation by the hybrids as a function of the position of the fusion point in the Bst-TyrTS sequence.

(e) *Thermal inactivation of the hybrids*

We compared the stability of the hybrids in a thermal-inactivation assay. Samples of a soluble extract, prepared from producing cells, were heated during 30 minutes at different temperatures. After elimination of the protein precipitate by centrifugation, the concentration of active TyrTS in the supernatant was determined by active-site titration. Figure 5 gives the profiles of thermal inactivation as a function of temperature for the parental and hybrid TyrTSs. The profiles have sigmoid shapes and their relative positions can be conveniently compared through the temperatures of half-inactivation, that we call t_m. Note that t_m is an approximate measure of the stability of the hybrids and not a thermodynamic parameter. Table 2 gives

Table 2
Activity and stability of the hybrids

Hybrid	V (s^{-1})	t_m (°C)	Slope
Bst	1·61±0·13	67·19±0·04	1·10±0·05
H1	2·18±0·32	62·29±0·04	0·89±0·03
H2	0·95±0·10	56·13±0·11	0·77±0·06
H3	3·91±0·23	44·32±0·13	0·72±0·06
H4	3·04±0·15	55·21±0·12	0·87±0·09
H5	1·07±0·09	44·44±0·06	0·83±0·04
H6	4·31±0·23	50·29±0·02	1·55±0·04
H7	4·44±0·10	49·79±0·07	1·38±0·11
H8	4·02±0·15	47·98±0·12	0·60±0·04
H9	0·70±0·03	45·22±0·18	0·51±0·05
Eco	4·61±0·11	48·58±0·01	0·86±0·05

Activity and stability of the hybrids. Column 1: Bst, Bst-TyrTS; Eco, Eco-TyrTS; H1-H9, hybrid TyrTS. Column 2: Rates of tyrosylation (V) of crude *E. coli* tRNA (5 mg/ml, 356 pmol of tyrosine incorporation/mg) by soluble extracts of strain TG2 derivatives producing the hybrids listed in column 1. The concentration of TyrTS in the extracts was determined by active site titration. If v was the tyrosylation rate by the extract of strain TG2(pEMBL-E/BY), e, the concentration of TyrTS active sites in this extract, v_0 and e_0 the same parameters for a similar extract prepared from the control strain TG2(pEMBL9$^+$), then V was calculated using the relation $V=(v-v_0)/(e-e_0)$ to correct the rate for the contribution of the *tyrS* gene on the bacterial chromosome to the production of TyrTS activity. The average V value and the standard error of 3 different measures are shown. Column 3: temperatures of half-inactivation in 30 min (t_m) of the hybrids listed in column 1, calculated from the curves of Fig. 5. Column 4: slopes of the inactivation curves of Fig. 5, at t_m. The slopes and t_m are given with their standard errors in the curve fit. Purified Bst-TyrTS (1·2 μM) gave a t_m equal to 69·88(±0·03)°C and a slope at t_m equal to 2·90(±0·20).

the values of t_m deduced from the profiles of Figure 5, and Figure 6b shows the variation of the t_m of the hybrids as a function of the position of their fusion point in the Bst-TyrTS sequence. We performed three control experiments.

(1) The profile of thermal inactivation and the value of t_m for Bst-TyrTS did not vary when the

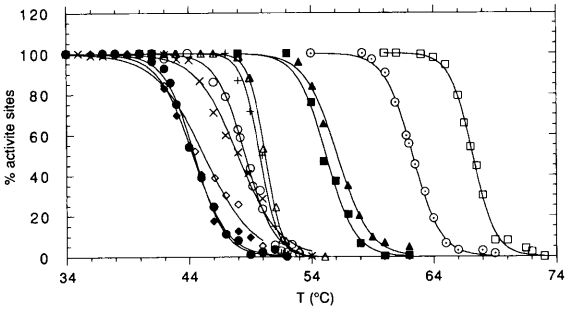

Figure 5. Thermal inactivation of the hybrids. (□), Bst-TyrTS; (⊙), hybrid H1; (▲), H2; (◆), H3; (■), H4; (●), H5; (△), H6; (+), H7; (×), H8; (◇), H9; (○), Eco-TyrTS. The parental and hybrid TyrTSs were produced in strain TG2 from recombinant phagemids. Portions of a cellular extract were heated at different temperatures during 30 min, the protein precipitates were eliminated and the concentrations of soluble TyrTS determined by active site titration, as described in Materials and Methods. 100% active sites corresponds to the concentration of TyrTS in the extract before heating (around 1 μM).

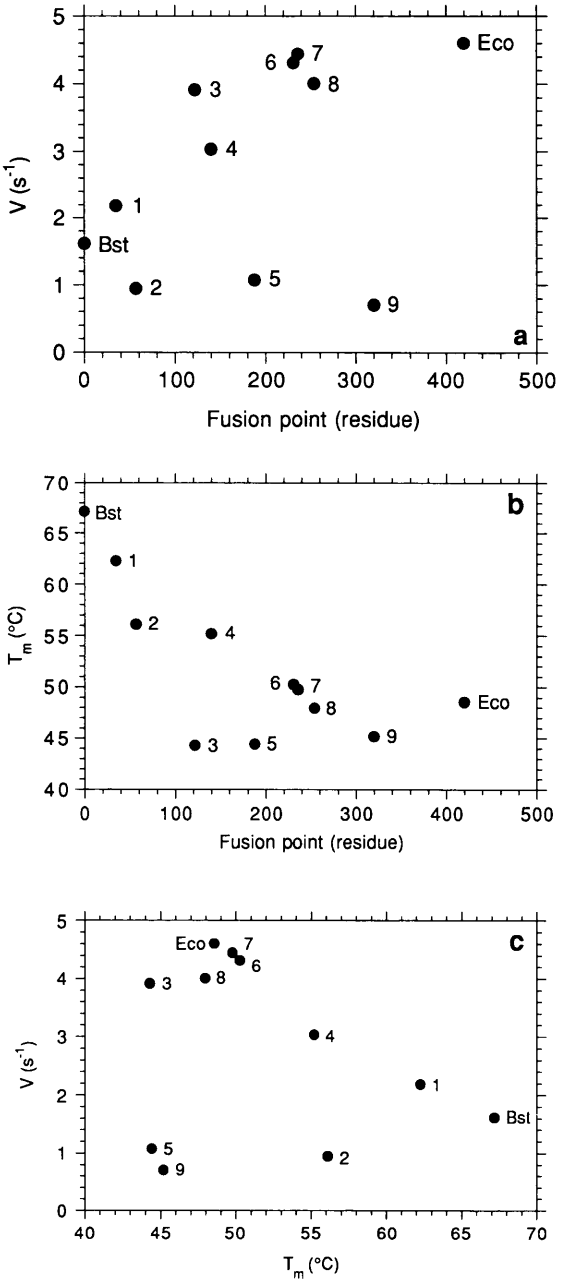

Figure 6. Comparison of the hybrids properties. V, rate of tRNA charging; t_m, temperature of half-inactivation. (a) V and (b) t_m as functions of the position of the fusion point in the Bst-TyrTS sequence. (c) V as a function of t_m. Eco, Eco-TyrTS; Bst, Bst-TyrTS; 1 to 9, hybrids H1 to H9. Data from Table 2.

soluble extract used in the assay was diluted (in a ratio of 1 to 8). In fact, because the production of the various TyrTSs by *E. coli* was nearly constant (Fig. 4), all the inactivation experiments were done with concentrations of the wild-type and hybrid TyrTSs that were similar and around 1 μM.

(2) A dialysis of the extract against pyrophosphate, to displace any enzyme bound Tyr-AMP, had no effect on the inactivation profile and t_m of Bst-TyrTS.

(3) Purified and unpurified Bst-TyrTS (1 μM) had t_m values equal to 69·9 °C and 67·2 °C respectively. The slopes of the inactivation profiles at t_m were 2·9 and 1·1 respectively (Table 2). Thus, the purified Bst-TyrTS was slightly more stable than the unpurified Bst-TyrTS and its inactivation occurred in a narrower interval of temperature. These small differences in t_m in no way affect the conclusions that we drew from our measurements on the unpurified hybrids.

4. Discussion

(a) *Location of the fusion points*

The characterization of the hybrid *tyrS* genes by DNA-sequencing showed that the fusion points belonged to sequences that could pair along at least eight consecutive residues during the strand exchange that led to recombination, when the G·T pairs are taken into account. There are 36 recombination sites between the two *tyrS* genes that satisfy this criterion; we only obtained 12 of them in our *in vivo* experiments, some repeatedly. The 36 potential recombination sites would generate 43 different hybrid proteins and we only obtained eight of them. The sequence identities between the *tyrS* genes are more numerous upstream from codon 259 (62%) than downstream (50%), which could explain why we did not find recombination events in the 3′-terminal parts of the genes. The expression of hybrid H9, that we constructed *in vitro*, was slightly toxic to the host cell. The construction of additional hybrids, by *in vitro* recombinations, could help us to determine whether we did not obtain some hybrids because of their potential toxicity for the host.

(b) *A hybrid promoter:* Eco/Bst-tyrSp

We found that 21 among the 42 hybrid genes that we obtained *in vivo*, corresponded to the same recombination event, between the promoter regions of the *Eco-tyrS* and *Bst-tyrS* genes. The recombination event occurred within the *Kpn*I restriction site of *Bst-tyrSp*, (−78)GGTAC/C(−73), and within a sequence of *Eco-tyrSp*, (−99)GGTACT(−94), nearly identical to a *Kpn*I site (taking the first nucleotide of the initiation codon of *tyrS* as residue +1). The recombination could have occurred *in vivo* between the two stretches of homologous sequences; alternatively, the *E. coli* sequence, GGTACT, could have been cut with a low efficiency by the *Kpn*I endonuclease during the *in vitro* selection for recombination, generating a *Kpn*I cohesive end which could have paired with the complementary sequence in *Bst-tyrSp*.

The recombination event deleted the major site of transcription initiation at the *Eco-tyrSp* promoter, located around A(−47). The residual transcription of the *Bst-tyrS* gene from the recombinant plasmid could come from a minor site of transcription initiation located about 51 nucleotides upstream from the major site (see Figure 5 of Lam & Winkler, 1992).

The sequence (−132)CTGAAA-17 bp-TTTAAT-4 bp-GGT(−97), which occurs immediately upstream from the recombination site and is close to the consensus sequence of the *E. coli* promoters, could be the entry site of RNA-polymerase at this minor promoter.

(c) *Activity and stability of the hybrids: general case*

The recombinant strains producing the hybrid TyrTSs, TG2(pEMBL-E/BY), were perfectly isogenic except for the position of the fusion point between the *Eco-tyrS* and *Bst-tyrS* genes on the plasmid. Therefore, any variation of the properties of the hybrids in our experiments resulted necessarily from the variable position of this fusion point, either directly or indirectly.

We found that the hybrids were produced in large quantities in *E. coli* and that the synthesized molecules were at least partially active since they could form Tyr-AMP (Fig. 4). The temperature of half-inactivation (t_m) of the hybrids was higher than 44 °C in all the cases; it was higher than that of Eco-TyrTS in most cases, except H3, H5 and H9 which will be discussed later (Table 2). These results show that the global folding of TyrTS was preserved in the hybrids and gave fairly stable structures.

Most of the TyrTS residues that are involved in the binding of the substrates and in catalysis have identical side-chains in *E. coli* and *B. stearothermophilus* (Bedouelle, 1990). These residues are distant in the sequence and in the structure: for example, $tRNA^{Tyr}$ interacts with both N-terminal and C-terminal domains of TyrTS (Bedouelle & Winter, 1986). Therefore, the residues of the active sites were carried by segments of polypeptide chains coming from both *E. coli* and *B. stearothermophilus* in the hybrids. We found that all the hybrids were active *in vivo* in a genetic complementation assay, and *in vitro* in a kinetic assay for the aminoacylation of *E. coli* $tRNA^{Tyr}$. For every hybrid the rate of charging was at least 40% that of Bst-TyrTS. This rate was between those of the parental TyrTSs for most hybrids, except H2, H5 and H9 which will be discussed later (Table 2). These results show that the geometry of the TyrTS active sites was precisely conserved in the hybrids.

Let H_i and H_j be any two hybrids, and n_i and n_j be the positions of their fusion points in the Bst-TyrTS sequence. Our results show that the sequence interval $[n_i, n_j]$, between n_i and n_j, could come from either *E. coli* or *B. stearothermophilus* and still give a stable and active TyrTS. We conclude that, as a first approximation, the two sequences can replace each other locally and the two structures are locally compatible.

In general, the t_m values of the hybrids increased with the number of residues coming from *B. stearothermophilus* (Fig. 6b). This shows that the greater thermostability of Bst-TyrTS is obtained by the cumulative effect of several residue changes scattered along the sequence. This conclusion is compatible with mutagenesis studies on other proteins showing that amino acid substitutions can have additive effects on stability (Jaenicke, 1991).

In general, the rates of $tRNA^{Tyr}$ charging by the hybrids increased with the number of residues coming from Eco-TyrTS (Fig. 6a). If the progressive increase in the stability of TyrTS with the number of residues coming from *B. stearothermophilus* could be explained by the cumulative effects of residue changes, the same kind of argument can hardly explain the progressive increase in activity with the number of residues coming from *E. coli* since the residues of the active sites are conserved between the two enzymes. We propose the following explanation. The active sites of enzymes are generally flexible (Tsou, 1986). This is especially true for TyrTS which functions by an induced fit mechanism (Fersht *et al.*, 1988). The proteins from mesophiles and from thermophiles have flexibilities and dynamics that are comparable at their optimal temperatures of activity, so that the proteins from thermophiles are more rigid at low temperature (Vihinen, 1987; Jaenicke, 1991). At low temperature, the lower activity of Bst-TyrTS could be due to a greater rigidity and the increase in the activity of the hybrids with the number of residues coming from *E. coli* could be due to a progressive decrease in the rigidity of the molecule. The rough correlation that exists (except for H2, H5 and H9) between the values of t_m and the rates of aminoacylation supports this explanation (Fig. 6c).

(d) *Compensatory mutations*

Four among the nine hybrids that we studied had an abnormally low activity (H2, H5 and H9) or stability (H3, H5 and H9) when compared to the hybrids whose fusion points were neighbouring (Fig. 6a and b). Let H_i ($i=1$ to 9) be any single hybrid. The finding that the activity or the stability of H_i was abnormally lower than those of H_{i-1} and H_{i+1} implies the existence of defective interactions between residues of H_i, that existed neither in H_{i-1} nor in H_{i+1}. Otherwise stated, there exist compensatory changes of residues between Eco-TyrTS and Bst-TyrTS, some partners of which are in the sequence interval $[n_{i-1}, n_i]$ defined by the fusion points of H_{i-1} and H_i, and some other partners in $[n_i, n_{i+1}]$ (Fig. 2).

We tried to identify these compensatory changes by looking for couples of residues whose side-chains form contacts within 6 Å in the crystal structure of Bst-TyrTS and differed in hybrids H_{i-1}, H_i and H_{i+1} (Materials and Methods). We found such couples of residues for $i=2$, 4 and 5 but not for the other values of i. For $i=3$, we then looked for couples of residues that form contacts through a third residue or a water molecule (Materials and Methods). In the following, we describe the potential compensatory changes thus identified (Fig. 7), the tertiary or quaternary interactions to which they correspond

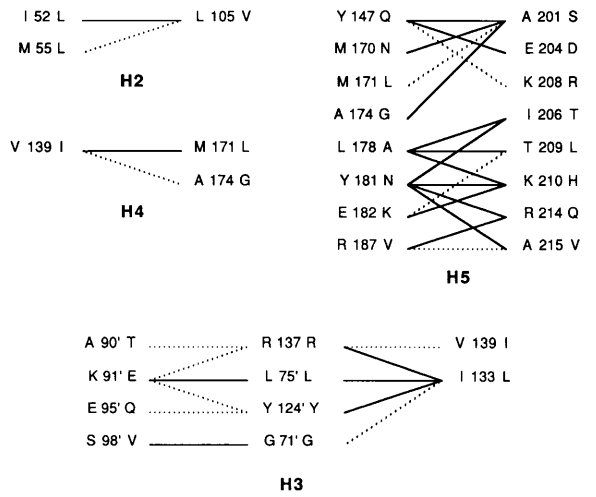

Figure 7. Potential compensatory changes between Eco-TyrTS and Bst-TyrTS. For each residue, the nature of its side-chain in Bst-TyrTS, its position in the sequence of Bst-TyrTS and the side-chain at the homologous position of Eco-TyrTS are indicated from left to right. A prime after the position indicates that the residue belongs to another subunit of the TyrTS dimer. The plain (dotted) lines linking residues indicate that the minimal distance between atoms of their side-chains (C^{α} excluded) are below 4·5 Å (6·0 Å). The non-conserved tertiary or quaternary interactions in TyrTS could explain the abnormal properties of hybrids H2 to H5, indicated below the different parts of the Figure. In the case of H5, the tertiary interactions divide into 2 independent groups. In the case of H3, the quaternary interactions occur through a third layer of residues that are identical in Eco-TyrTS and Bst-TyrTS.

(Fig. 8) and how they could account for the abnormal properties of hybrids H2, H3 and H5.

(i) *H2*

We found two couples of residues differing in hybrids H1, H2 and H3, and forming contacts within 6·0 Å in the crystal structure of Bst-TyrTS (Fig. 7). These contacts occur between residues Ile52 and Met55 of helix 3 and Leu105 of helix 5 (Fig. 1). The couple corresponding to the closest contact was equal to (I52, L105) in Bst-TyrTS and H1, (L52, L105) in H2, and (L52, V105) in H3 and Eco-TyrTS. The change of Ile52 into Leu could lead to a steric clash with Leu105 and perturb the tertiary interactions between helices 3 and 5 (Fig. 8). As helix 3, the adjacent strand B and the loop between them carry several residues involved in the formation of Tyr-AMP (Fersht, 1987; Brick *et al.*, 1989), the occurrence of (L52, L105) in H2 could modify the positions of active site residues and be responsible for the slow rate of aminoacylation by this hybrid. (I52L, L105V) could thus be a couple of compensatory mutations. These hypotheses are compatible with the results of a crystallographic study on mutation C35G of Bst-TyrTS. This study has shown the existence of a structural coupling between Ile52 and Cys35, a residue involved in the binding of the reaction intermediate, Tyr-AMP (Fothergill & Fersht, 1991).

(ii) *H3*

We found several couples of residues differing in hybrids H2, H3 and H4 and forming indirect contacts in the structure of Bst-TyrTS, through a third residue (Fig. 7). The triplet corresponding to the closest contacts was equal to (K91′, L75′, I133) in Bst-TyrTS and H2, to (E91′, L75′, I133) in H3 and to (E91′, L75′, L133) in H4 and Eco-TyrTS, where the primes designate residues of a different subunit in the TyrTS dimer. Mutations K91E and S98V of helix 5 are non-conservative. They could perturb the subunit interface in H3 and be responsible for the low stability of this hybrid. They could be compensated by mutations I133L and V139I of helix 7 in hybrid H4 and Eco-TyrTS (Fig. 8).

(iii) *H4*

We found two couples of residues differing in hybrids H3, H4 and H5 and forming contacts within 6·0 Å in the structure of Bst-TyrTS (Fig. 7). The contacts occur between Val139 at the C-terminal end of helix 7, and residues Met171 and Ala174 of helix 10 (Fig. 1). The change of Val139 into Ile could lead to a steric clash with Met171 and Ala174 and perturb the position of helix 10 (Fig. 8). As helix 10 carries residues (169, 173 and 176) of the binding site for tyrosine (Brick & Blow, 1987), a modification of its position could explain the lower rate of aminoacylation by hybrid H4 compared to H3 and H6 (Fig. 6a). Mutation V139I could be compensated by mutations M171L and A174G in the *E. coli* enzyme.

(iv) *H5*

We found many couples of residues differing in hybrids H4, H5 and H6 and forming contacts within 6·0 Å (Fig. 7). These contacts correspond to the docking of helix 11 and the ensuing loop onto helix 8, helix 10 and the N-terminal end of strand E (Fig. 1). As mentioned above, helix 10 carries residues of the binding site for tyrosine. Helix 11 carries residues (192, 194, 195) of the Tyr-AMP binding site at its N-terminal end (Brick *et al.*, 1989) and residues (196, 207, 208) of the $tRNA^{Tyr}$ binding site at both ends (Bedouelle & Winter, 1986; Labouze & Bedouelle, 1989). The large number of tertiary interactions that were modified by the formation of hybrid H5 (Fig. 8) and the presence of many active site residues in the corresponding region of TyrTS are compatible with the loss of stability and activity associated with H5, in comparison with H4 and H6.

(v) *H9*

Hybrid H9 was active for the aminoacylation of $tRNA^{Tyr}$. This result shows that it is possible to precisely replace the C-terminal domain of Eco-TyrTS by the disordered C-terminal domain of Bst-TyrTS and keep a functional enzyme. The much lower activity of H9 compared to H8 and

Eco-TyrTS shows that unfavourable interactions existed between the α-helical domain from *E. coli* and the C-terminal domain from *B. stearothermophilus* in this hybrid. The slightly lower stability of H9 compared to H8 and Eco-TyrTS suggests that some of these interactions were direct rather than mediated by the bound tRNA.

The properties of hybrids H2, H3, H5 and H9 have revealed the existence of compensatory changes between the sequences of Eco-TyrTS and Bst-TyrTS. We roughly mapped these changes using our genetic data and made precise assumptions on the residues involved using the crystal structure of Bst-TyrTS. The construction of single and double changes in Bst-TyrTS by oligonucleotide site-directed mutagenesis will allow us to demonstrate the role of these residues in stability and folding, and to characterize the nature of their interactions. Our analysis suggests a rational

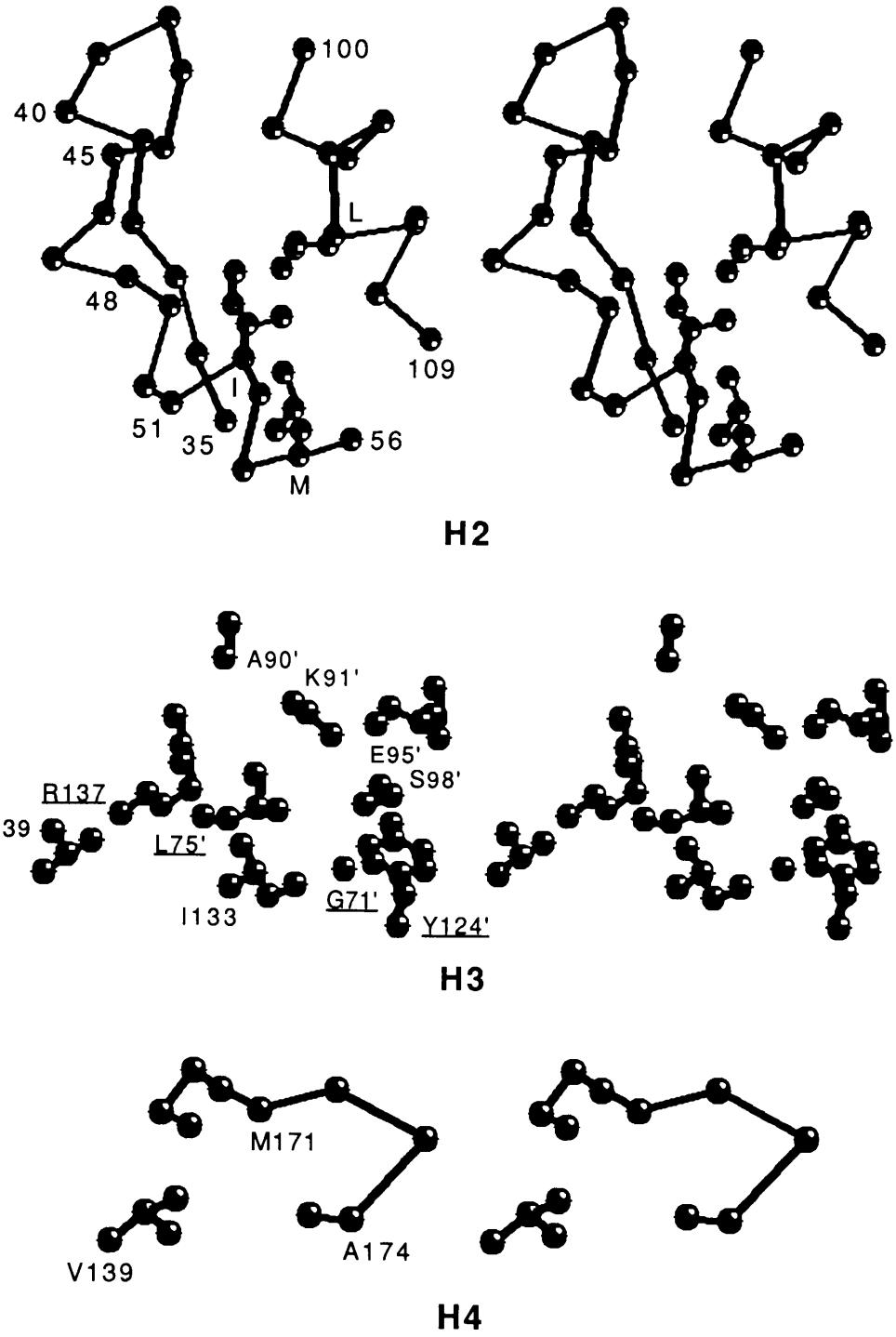

Fig. 8.

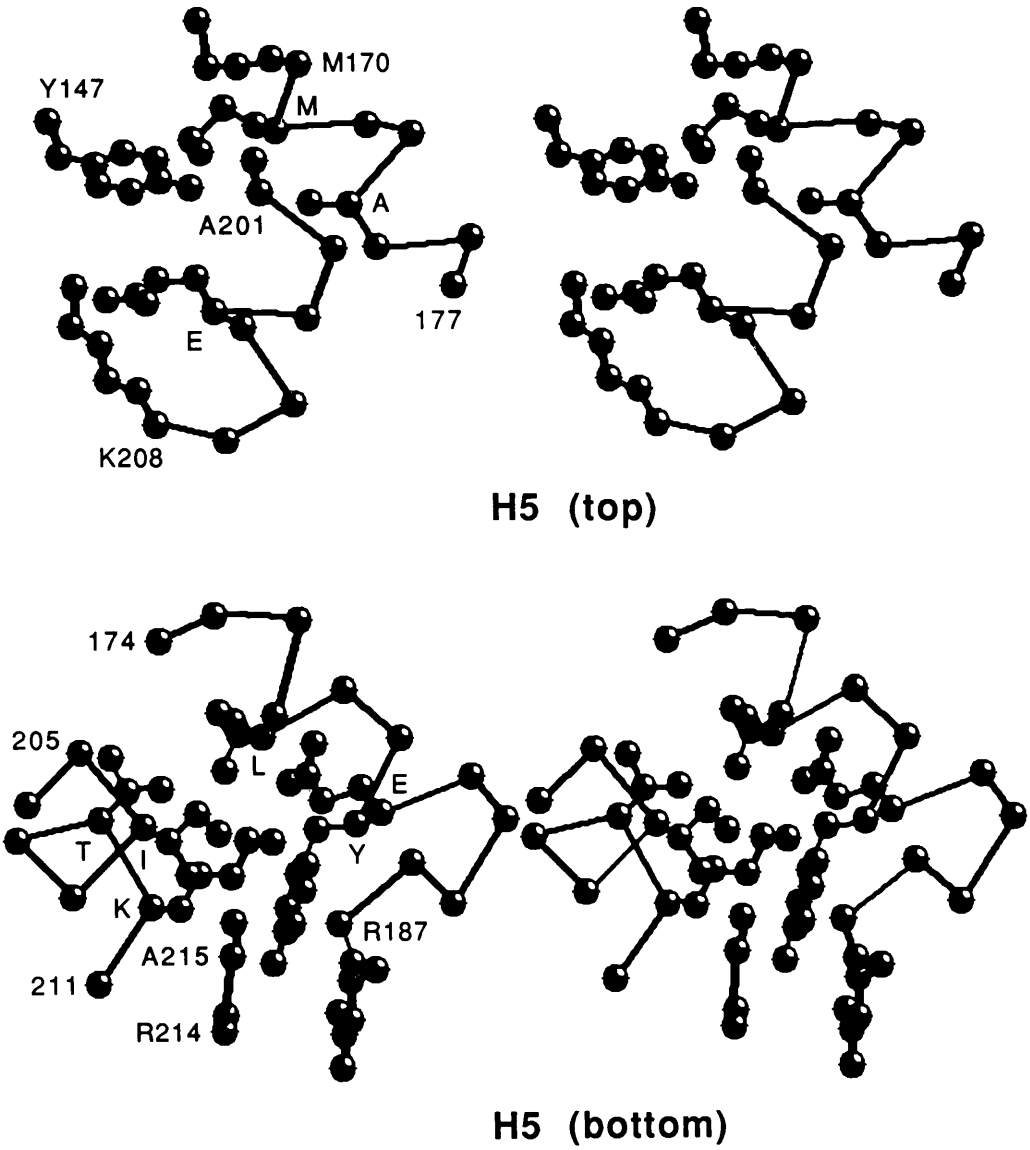

Figure 8. Tertiary and quaternary interactions in Bst-TyrTS. This stereo figure shows the positions, in the structure of Bst-TyrTS, of the main interactions listed in Fig. 7. Only the side-chains involved and, in some cases, the adjacent chain of C^{α} atoms are represented. The nature of the side-chain and/or the position of the residue in the Bst-TyrTS sequence are written close to the C^{α}. In the case of hybrid H3, the identification labels of the residues that belong to an intermediate layer, are underlined. The tertiary interactions that were modified in hybrid H5, are represented in 2 distinct parts for clarity. The side-chains of Lys91 and Arg214 are not visible in their entirety in the crystal structure of Bst-TyrTS.

approach to the construction of stable and active hybrids between homologous proteins for applications. This approach avoids the modification of tertiary or quaternary interactions in the three-dimensional structure upon formation of the hybrid.

(e) *Evolutionary consequences*

At a coarse level, the finding that all the hybrids were stable and active, shows that the residues that are essential for the folding, stability and activity of TyrTS, have been conserved during evolution. The finding that the properties of the hybrids, V and t_m, varied monotonically with the position of the fusion point (with few exceptions), suggests that most residue changes between Eco-TyrTS and Bst-TyrTS have cumulative effects at the resolution of our mapping.

At a finer level, we showed the existence of compensatory changes. The analysis of the crystal structure of Bst-TyrTS suggested that these compensatory changes involve residues that are in contact directly or by the intermediate of common residues. The existence of compensatory mutations raises the question of their mechanism of appearance. Indeed, the evolutionary changes occur through single point mutations, for probability reasons. How then is an unfavourable mutation maintained before a second compensatory mutation occurs? Two related theories offer solu-

tions to this paradox. Both are based on the observation that a substitution of residue is acceptable in a protein only if the neighbouring residues are compatible with it or can adapt to it. Otherwise stated, the structural environment of each residue restrains its changes of side-chain. In the theory of the "context effect", this structural environment could modify through the accumulation of neutral mutations, caused by a random genetic drift, until it becomes compatible with a replacement (Hardies & Garvin, 1991). After a change of side-chain, the environment of the concerned residue could similarly modify until it locks the replacement and makes its reversion unacceptable. In the theory of the "evolutionary pathway", constrained but additive, successive alterations of a residue and of its environment could progressively lead to a large change of its side-chain, along a non-lethal evolutionary pathway (Bordo & Argos, 1990; Bowie *et al.*, 1990).

Our results suggest the superposition of two levels in the evolution of TyrTS. At a first level, the folding and structure of TyrTS are robust and insensitive to a large number of residue changes, the effects of which seem neutral. At a second level, the fine tuning of this robust structure to particular conditions of activity or stability occurs through both independent and compensatory changes.

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