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Protein-Protein Interaction Between *Bacillus stearothermophilus* Tyrosyl-tRNA Synthetase Subdomains Revealed by a Bacterial Two-Hybrid System

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Abstract

We have recently developed a bacterial two-hybrid system (BACTH), based on functional complementation between two fragments of the catalytic domain of *Bordetella pertussis* adenylate cyclase (AC), that allows an easy *in vivo* screening and selection of functional interactions between two proteins in *Escherichia coli.* In this work, we have further explored the potentialities of the BACTH system to study protein-protein interactions, using as a model, the interactions between various subdomains of the dimeric tyrosyl-tRNA synthetase (TyrRS) of *Bacillus stearothermophilus.* Using the BACTH system we confirmed the known interactions of the α/β domains and those between the α/β domain and the α domain that could be anticipated from the three-dimensional structure of TyrRS. Interestingly, the BACTH system revealed the unexpected interaction between the TyrRS α domains which is presumably mediated by a pseudo-leucine zipper motif. This study illustrates the interest of the bacterial two-hybrid system to delineate interacting domains of proteins and shows that it can reveal interactions that occur *in vivo* and that were not anticipated from the three-dimensional structure of the protein of interest.

Introduction

Specific protein-protein interactions are essential in most biological processes. Therefore, the identification of these interactions is important and helpful in understanding of biological phenomena. Presently, the yeast two-hybrid system represents the most powerful genetic approach to analyze interactions between macromolecules and to screen for polypeptides that bind to a given "bait" (Fields and Song, 1989; Fields and Sternglanz, 1994; Vidal and Legrain, 1999; Uetz *et al*., 2000). In the last years, several one and two-hybrid systems, which allow screening and/or selection of functional interactions between two proteins *in vivo,* have been described in *Escherichia coli* (Hu *et al*., 1990; Dmitrova *et al*., 1998; Dove and Hochschild, 1998; Jappelli and Brenner, 1998; Karimova *et al*., 1998; Kornacker *et al*., 1998; Pelletier *et al*., 1998; Hu *et al*., 2000). We designed a particular bacterial two-hybrid system based on a reconstituted cAMP signal transduction pathway in *E. coli*, that relies on functional complementation between two fragments (T25 and T18) of the catalytic domain (AC) of *Bordetella pertussis* adenylate cyclase (Karimova *et al*., 1998). When these two fragments are co-expressed as separated entities, in an *E. coli* strain lacking its endogenous adenylate cyclase (*cya*), they are unable to reassociate spontaneously and they cannot reconstitute a functional enzyme. However, when the T25 and T18 fragments are fused to peptides or proteins that can interact, heterodimerization of these chimeric polypeptides results in a functional complementation and, consequently, in cAMP synthesis. This can be easily monitored in *E. coli cya*, because cAMP is a pleiotropic regulator of gene transcription in this organism. Cyclic AMP binds to the transcriptional regulator CAP (catabolite activator protein), and the cAMP/CAP complex can trigger the transcriptional activation of a number of catabolic operons such as *lac* or *mal*. Therefore, Cya^+ bacteria, unlike Cya^- ones, are able to utilize lactose or maltose as unique carbon sources and can be easily distinguished from *cya* cells either on indicator media or selective media.

We have already demonstrated the efficiency of the BACTH (bacterial adenylate cyclase two-hybrid) system in detecting protein-protein interactions, such as homodimerization of GCN4 leucine zipper, homodimerization of tyrosyl-tRNA synthetase of *Bacillus stearothermophilus* and association between yeast-splicing factors, Prp21 and Prp11 (Karimova *et al*., 1998).

In the present study we describe further developments of the BACTH system, and its application to analyze interactions between various subdomains of the dimeric tyrosyl-tRNA synthetase (TyrRS) of *B. stearothermophilus*. This enzyme was chosen as a model, because its three-dimensional structure is known and numerous studies have identified the role of critical residues in the dimerization process (Ward *et al*., 1986; Brick and Blow, 1987; Ward *et al*., 1987; Brick *et al*., 1989; Park *et al.,* 1999).

Results

The tyrosyl-tRNA synthetase (TyrRS) catalyzes the activation of tyrosine and its coupling to the cognate tRNA (Bedouelle *et al.,* 1993). The structure of TyrRS of *B. stearothermophilus* has been determined at high resolution (Brick and Blow, 1987; Brick *et al*., 1989). This enzyme is made of two domains: an N-terminal catalytic domain (residues 1-320), which is dimeric and has the characteristic

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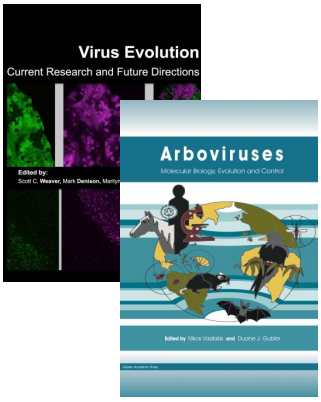

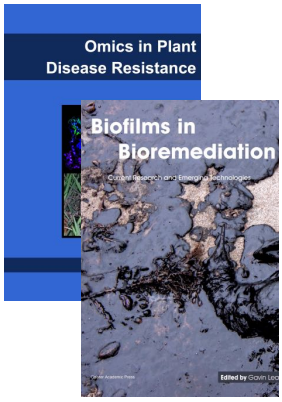

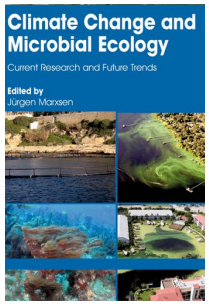

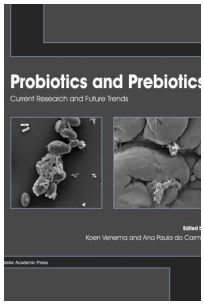

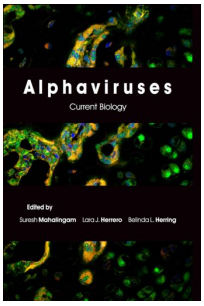

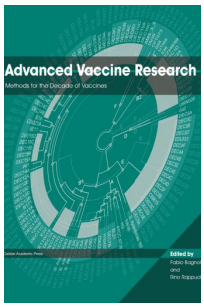

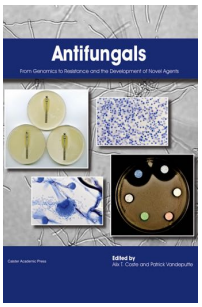

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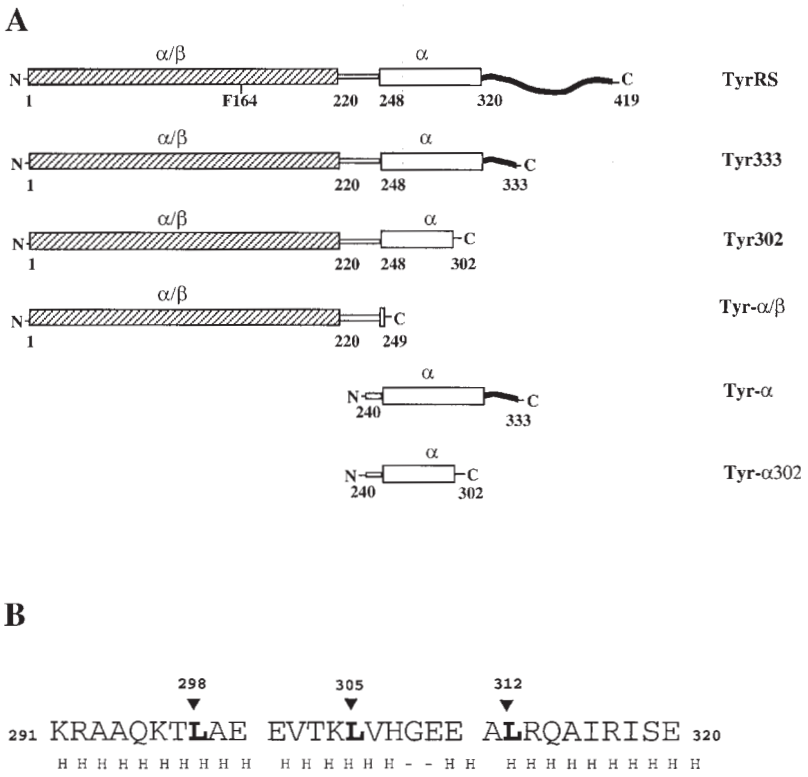

Figure 1. Schematic representation of the different sub-domains of TyrRS tested in complementation assays (A) and amino-acid sequence of TyrRS from residues 291 to 320 (B). The Leucine residues at position 298, 305, and 312 are indicated by triangles. The secondary structure of this segment is indicated on the bottom line (H marks α-helical residues).

fold of the class I aminoacyl-tRNA synthetases, and a C-terminal domain (residues 321-419), which binds the anticodon arm of tRNA Tyr and is disordered in the crystal structure (Brick and Blow, 1987; Brick *et al.*, 1989). Deletion of the C-terminal domain (residues 321-419) of TyrRS produces a truncated ΔTyrRS (residues 1-320) which forms a dimer that closely resembles the wild-type dimer (Bedouelle and Winter, 1986, Brick and Blow, 1987; Brick *et al.*, 1989). The truncated ΔTyrRS is itself made of two structural subdomains: the α/β domain (residues 1-220) containing a classical Rossman fold made of six parallel β–sheets flanked by α-helices, and the α-helical domain (residues 248-320) containing five helices. The dimer is formed by the association of a hydrophobic surface encompassing residues 128-167 within the α/β domain of each monomer (Brick and Blow, 1987).

The aim of the present study was to examine interactions between various subdomains of TyrRS using the BACTH system. For this purpose, we constructed a set of plasmids expressing various fragments of the TyrRS molecule (Figure 1) fused to either the T25 or the T18 fragment of the catalytic domain of *B. pertussis* adenylate cyclase (Figure 2). These plasmids were then tested in complementation assays in the *E. coli cya* strain DHP1.

Interaction of Tyr333 with the Isolated α/β and α Subdomains.

We first analyzed the interaction between the isolated α/β or α subdomains of TyrRS and Tyr333, a truncated TyrRS polypeptide spanning residues 1 to 333 (Figure 1A). For this purpose, the isolated α/β and α subdomains of TyrRS were fused to the C-terminus of the T25 fragment (encoded by plasmids pT25-α/β and pKT25-α, respectively) whereas the first 333 residues of TyrRS were fused to the N-terminus of the T18 fragment of AC (encoded by plasmid pT18-Tyr333). DHP1 was cotransformed with the corresponding plasmids, and bacteria were plated on MacConkey/maltose plates (supplemented with the relevant antibiotics). The efficiency of complementation was further assessed by measuring β-galactosidase activities of liquid cultures. As shown in Figure 3, control transformation of DHP1 with the pT18 and pT25 vectors yielded colorless colonies exhibiting background level of β-galactosidase activity (line 1). When, as a positive control, DHP1 was cotransformed with pT25-Tyr-333, a plasmid that expresses the first 333 residues of TyrRS fused to the C-terminus of the T25, and pT18-Tyr333, the cotransformants were red on MacConkey/maltose medium (indicating that they became Cya^+) and expressed high β-galactosidase activity (Figure 3, line 2). This indicates that heterodimerization of the chimeric proteins T25-Tyr333 and T18-Tyr333 resulted in efficient functional complementation between the adenylate cyclase fragments, in agreement with our previous results (Karimova *et al.*, 1998).

As shown in Figure 3, lines 3 and 4, both hybrid proteins T25-α/β and T25-α were able to complement T18-Tyr333 thus showing that both isolated α/β and α subdomains can interact *in vivo* with the Tyr333 polypeptide. The interaction between the isolated α/β domain and the Tyr333 moiety was indeed expected from the crystal structure of TyrRS which shows that the dimer is formed through the association of a hydrophobic surface of the α/β domain (Brick and Blow, 1987). In contrast, the interaction of the isolated α domain with Tyr333 was not

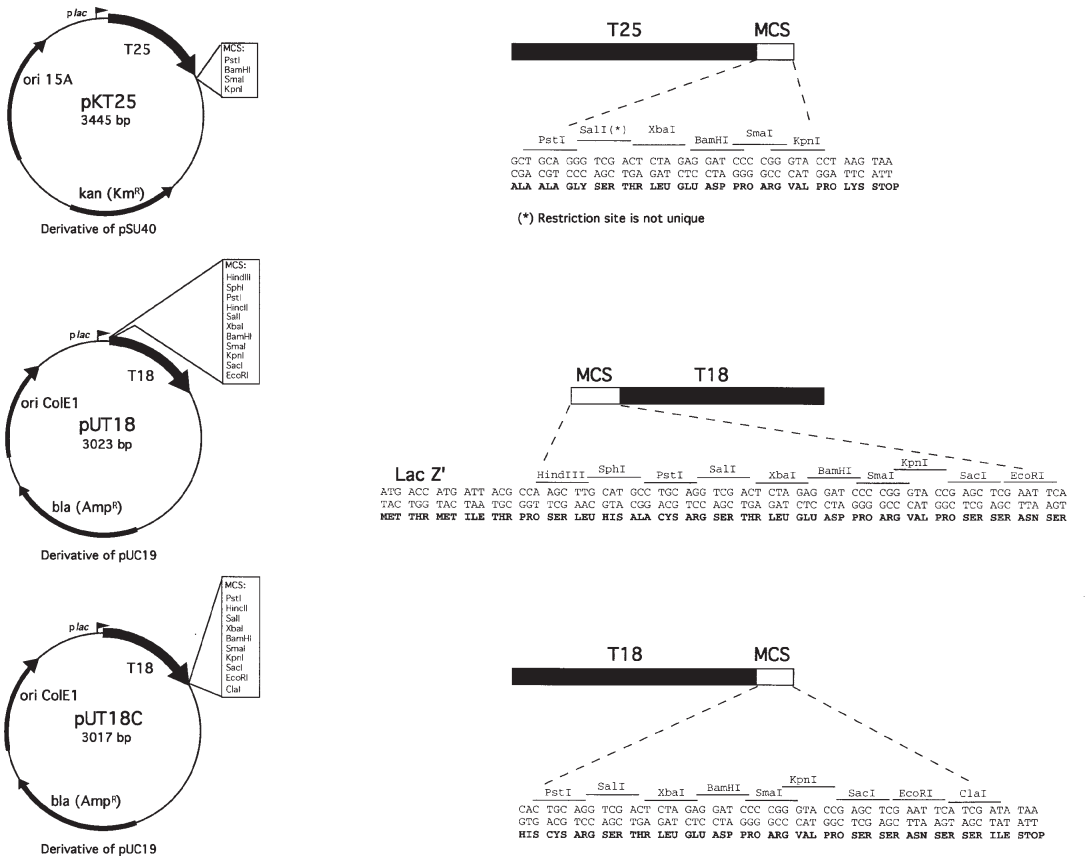

Figure 2. Schematic representation of novel vectors described in this work. The maps on the left depict the novel vectors and show the location of the relevant genetic elements. The sequences of multiple cloning sites (MCS) of the vectors and corresponding open reading frames (ORF) are present on the right.

anticipated from the 3D structure.

To test for possible *in vivo* interactions between the C-terminal domain of TyrRS and Tyr333, we fused residues 321-419 to the T25 fragment and cotransformed the resulting plasmid pT25-tyrC and pT18-Tyr333 in DHP1. This combination of plasmids did not yield any detectable complementation in DHP1, suggesting, in agreement with recent biochemical data (Guez *et al.*, 2000), that the N and C domains of TyrRS do not interact directly.

Interaction Between Isolated Subdomains of TyrRS. DHP1 cotransformed with plasmids pT18-α/β and pT25-α/β, that express the α/β domain fused to the T18 and T25 fragments, respectively, yielded red colonies on MacConkey/maltose indicator plates and expressed β–galactosidase activity (Figure 3, line 5). This indicates that the isolated α/β subdomain was indeed able to dimerize *in vivo* as was predicted from the TyrRS 3D structure (Brick and Blow, 1987).

We further showed that the α/β subdomain was able to associate with the α subdomain. Two reciprocal BACTH complementation assays were carried out. In the first one (Figure 3, line 6), the α/β domain was fused to the T25 fragment (T25-α/β) while the T18 fragment was fused to the α domain of TyrRS (T18-α). In the second one (Figure 3, line 7), the α/β domain was fused to the T18 fragment (T18-α/β) and the α domain to T25 (T25-α). Both pairs of hybrid proteins yielded functional complementation as indicated by the red phenotype of the colonies on the indicator medium and by the level of expression of β–galactosidase. It is noteworthy that the two reciprocal complementation tests gave slightly different β–galactosidase activities. This most likely reflects the difference in topological positioning of the TyrRS subdomains with respect to the adenylate cyclase fragments.

When plasmids pKT25-Tyr-α and pUT18-Tyr-α, which express the α domain fused to T25 and T18 respectively, were cotransformed in DHP1, the colonies appeared red on MacConkey/maltose plates and exhibited high β–galactosidase activity (Figure 3, line 8), comparable to that obtained in the complementation assay between the isolated α domain and full-length Tyr333 (Figure 3, line 4). This result suggests that the α domain of TyrRS is able to dimerize *in vivo*.

The Dimerization Motif of the α Domain of TyrRS: a Pseudo Leucine Zipper

In order to identify the amino acid sequences that could be involved in the dimerization of the α domain, we examined its primary sequence. We noticed the presence of three leucine residues at positions 298, 305 and 312

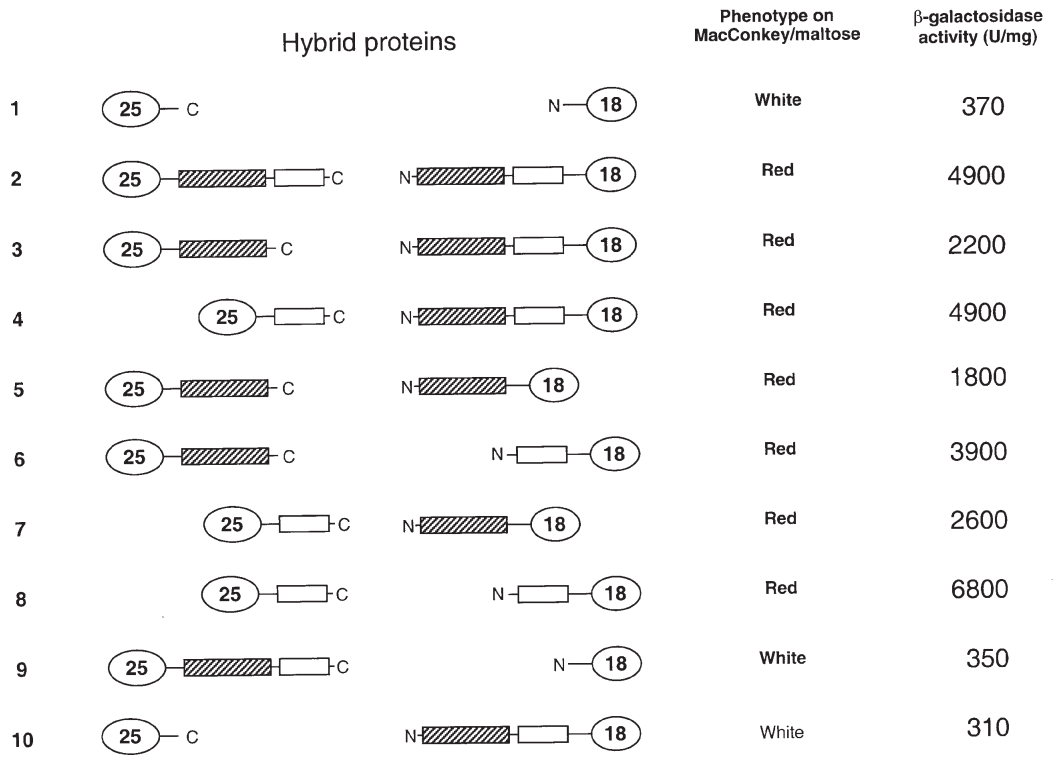

Figure 3. Functional complementation between hybrid proteins made of TyrRS sub-domains fused to AC fragments. The two AC fragments T25 and T18 are schematized by ovals and the different sub-domains of TyrRS by rectangles: hatched rectangle for TyrRS-α/β (residues 1-249) and open rectangle for TyrRS-α (residues 240-333). For all hybrids N-terminus is on the left and C-terminus on the right. The phenotypes of the DHP1 transformants expressing the indicated pair of hybrid proteins were scored on MacConkey/maltose plates and their β-galactosidase activities (expressed in units/mg dry weight bacteria) were measured as described in Experimental procedure. The plasmids expressing the indicated hybrid proteins were as follows: line 1) pT25 / pT18; line 2) pT25-Tyr333 / pT18-Tyr333; line 3) pT25-Tyrα/β / pT18-Tyr333; line 4) pKT25-Tyrα / pT18-Tyr333; line 5) pT25-Tyrα/β / pT18-Tyrα/β ; line 6) pT25-Tyrα/β / pUT18-Tyrα; line 7) pKT25-Tyrα / pT18-Tyrα/β ; line 8) pKT25-Tyrα / pUT18-Tyrα; line 9) pT25-Tyr333 / pT18; line 10) pT25 / pT18-Tyr333.

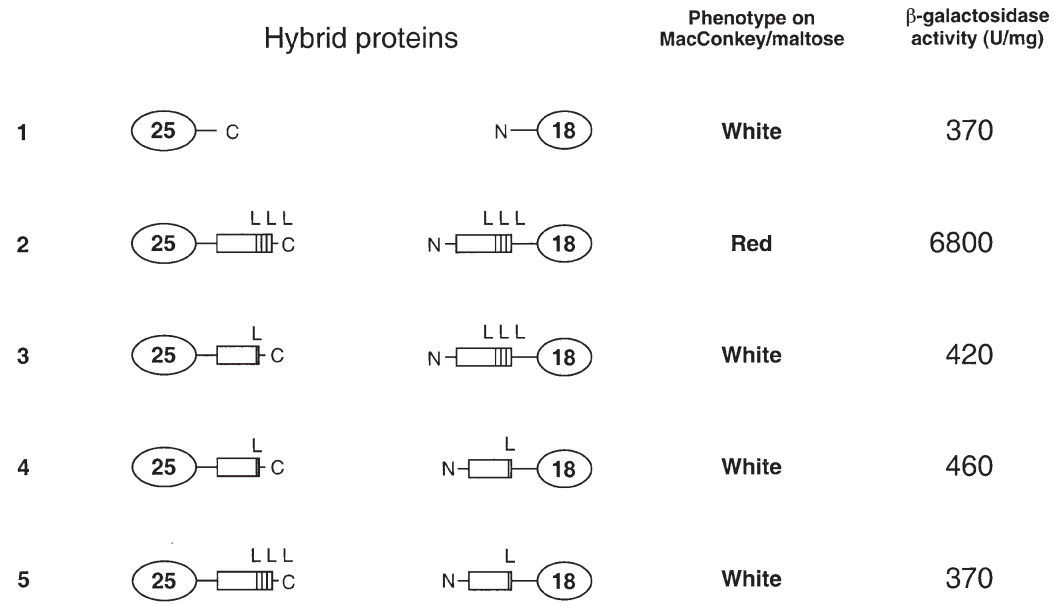

Figure 4. Functional complementation between hybrid proteins made of TyrRS-α sub-domains. The T25 and T18 fragments are schematized by ovals and the TyrRS-α sub-domain by open rectangle. The 3 Leucine residues at the C-terminus of TyrRS-α sub-domain (residues 240-333) are indicated by 3 L. The single L corresponds to Leucine residue 298 of the truncated TyrRS-α (residues 240-302). The phenotype of the DHP1 transformants and β-galactosidase activities (units/mg dry weight) were determined as indicated in Experimental procedure. The plasmids expressing the indicated hybrid proteins were as follows: line 1) pT25 / pT18; line 2) pKT25-Tyrα / pUT18-Tyrα; line 3) pKT25-Tyrα302 / pUT18-Tyrα; line 4) pKT25-Tyrα302 / pUT18C-Tyrα302; line 5) pT25-Tyrα / pUT18C-Tyrα302.

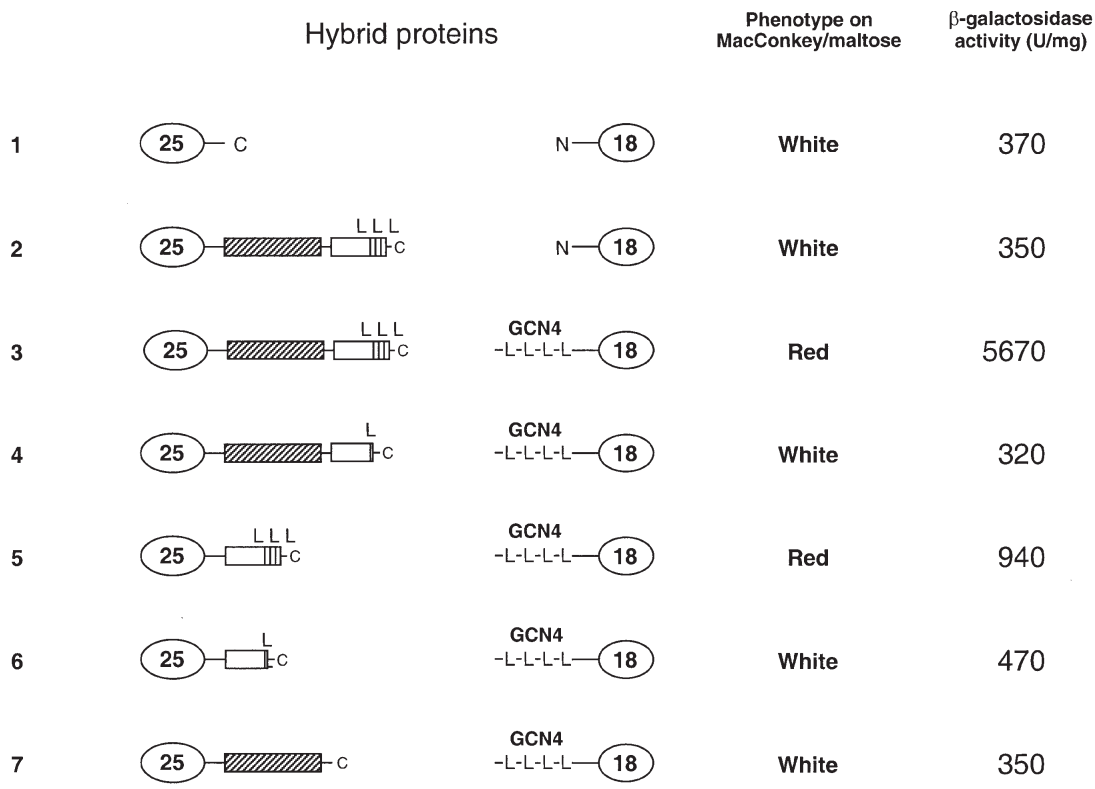

Figure 5. Interactions between GCN4 leucine zipper and TyrRS sub-domains. The AC fragments and the TyrRS sub-domains are schematized as in Figures 3 and 4. Four L represent the 4 Leucine residues from the GCN4 leucine zipper. The phenotype of the DHP1 transformants and β-galactosidase activities were determined as indicated in Experimental procedure. Line 1) pT25 / pUT18; line 2) pT25-Tyr333 / pUT18; line 3) pT25-Tyr333 / pUT18-zip; line 4) pT25-Tyr302 / pUT18-zip; line 5) pKT25-Tyrα / pUT18-zip; line 6) pKT25-Tyrα302 / pUT18-zip; line 7) pT25-Tyrα/β / pUT18-zip.

that belong to two successive α helices separated by a kink at position Gly 308 (Figure 1B). The disposition of these leucine residues spaced every seven residues on helical segments is reminiscent of the classical leucine zipper motif that can dimerize in a parallel two stranded coiled-coil. Although leucine zippers are made of at least 4 heptad repeats, we hypothesized that this polypeptide segment might form a pseudo leucine zipper motif that could be responsible for the dimerization of the α domain of TyrRS.

Indeed, as shown in Figure 4 (lines 3, 4 and 5), when this motif was deleted from the C-terminus of the α domain, the truncated α domain (residues 240-302) no longer interacted with the full-length α domain (residues 240-333). This result strongly suggests that the pseudo leucine zipper motif is required for the dimerization of the isolated α domain of TyrRS.

We then examined whether this pseudo leucine zipper sequence could interact with the typical leucine zipper derived from the yeast transcriptional regulator GCN4 (O'Shea *et al.*, 1989). For this purpose we constructed the plasmid pUT18-zip, encoding the GCN4 leucine zipper polypeptide fused to the N-terminus of T18, and tested it in complementation assays with pT25-Tyr333 and pKT25-Tyr-α. The results presented in Figure 5, show that the full-length Tyr333 molecule containing the α/β and α domains as well as the intact isolated α domain could interact with the GCN4 leucine zipper, albeit with quite different efficiencies (lines 3 and 5). Deletion of the main part of the pseudo leucine zipper from either Tyr333 (resulting in Tyr302 encompassing residues 1 to 302) or from the α domain (i.e. residues 240-302) totally abrogated their interaction with GCN4 leucine zipper as revealed by lack of complementation (Figure 5, lines 4 and 6). Moreover, as expected, no interaction could be detected by the BACTH between GCN4 leucine zipper and the α/β domain (Figure 5, line 7). Taken together, these results strongly suggest that dimerization of the TyrRS α domain is mediated by its C-terminal region *via* its pseudo leucine zipper motif. The structural and/or functional role of this motif in the full-length TyrRS awaits further investigation.

Complementation Between Dimerization-Defective TyrRS Mutants

We next examined whether point mutations which are known to prevent association of TyrRS monomers, would also abolish functional complementation in the BACTH system. TyrRS forms dimers through interaction between the hydrophobic regions of the two α/β domains (Figure 1A) and the two symmetrical Phe164 residues at the interface between the two monomers make the greatest contribution to this interaction (Brick and Blow, 1987; Brick *et al.*, 1989). Previous *in vitro* studies showed that introduction of charged residues into the hydrophobic

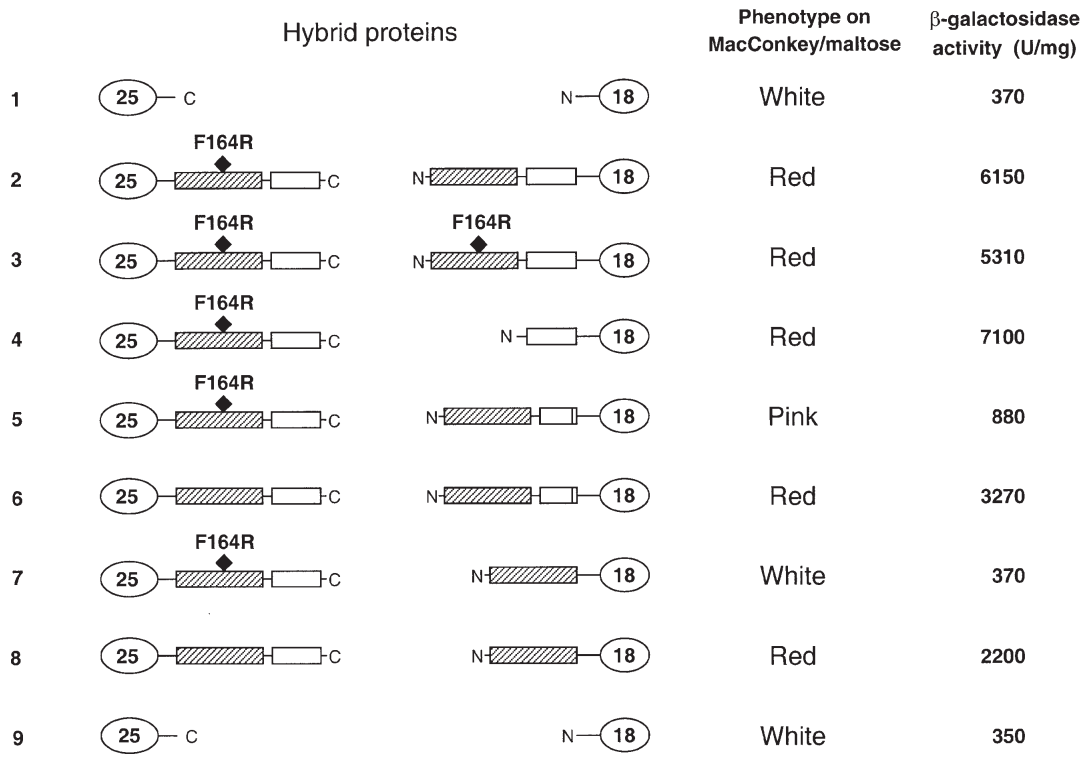

Figure 6. Functional complementation between dimerization-defective TyrRS mutants. The AC fragments and the various TyrRS sub-domains are schematized as in Figures 3 and 4. The black diamond represents the single modification of Phe164 to Arg in Tyr333F164R. The phenotype of the DHP1 transformants and β-galactosidase activities were determined as indicated in Experimental procedure. The plasmids expressing the indicated hybrid proteins were as follows: line 1) pKT25 / pT18; line 2) pT25-Tyr333F164R / pT18-Tyr333; line 3) pT25-Tyr333F164R / pT18-Tyr333F164R; line 4) pT25-Tyr333F164R / pUT18-Tyrα; line 5) pT25-Tyr333F164R / pT18-Tyr302; line 6) pT25-Tyr333 / pT18-Tyr302; line 7) pT25-Tyr333F164R / pT18-Tyrα/β ; line 8) pT25-Tyr333 / pT18-Tyrα/β ; line 9) pT25 / pT18.

subunit interface of dimeric TyrRS induces dissociation of the homodimeric enzyme into inactive monomers (Ward *et al.*, 1986; Ward *et al.*, 1987). To assess whether such a modification would prevent the *in vivo* dimerization of the α/β domain, the Phe164 codon of TyrRS was changed to an Arg codon by site-directed mutagenesis (Ward *et al.*, 1987) in plasmids pT25-Tyr333 and pT18-Tyr333. The resulting plasmids encoding the modified Tyr333 (labeled Tyr333F164R) fused to either T25 or T18, were then tested in complementation assays.

As shown in Figure 6, when pT25-Tyr333F164R was cotransformed with either pT18-Tyr333 or pT18-Tyr333F164R, the colonies were red on MacConkey/maltose plates and expressed high β–galactosidase levels (Figure 6, lines 2 and 3), indicating that *in vivo*, the chimeric T25-Tyr333 F164R protein can interact with both wild type T18-Tyr333 and mutated T18-Tyr333F164R molecule. However, this interaction is mediated essentially by the α domain of the chimeric TyrRS molecules as indicated by (i) the efficient complementation between T25-Tyr333F164R and T18-α (Figure 6, line 4) and (ii) the weak complementation between T25-Tyr333F164R and pT18-Tyr302, that lacks the C-terminal pseudo leucine zipper (Figure 6, line 5), as compared to the high level complementation between T25-Tyr333 and pT18-Tyr302 (Figure 6, line 6). Furthermore, T25-Tyr333F164R failed to complement T18-α/β (Figure 6, line 7) indicating that the Phe164Arg modification in the α/β domain of Tyr333 abolishes its heterodimerization with the isolated α/β domain. Taken together, these results show that a point mutation that converts Phe164 to Arg and eliminates *in vitro* interaction between two TyrRS α/β domains (Ward *et al.*, 1987), also abolishes *in vivo* functional complementation in the bacterial two-hybrid assay. This demonstrates that single amino acid residues critical for specific protein-protein interactions can be detected by the BACTH system.

Functional Complementations Mediated by C-Terminal Fusions to T18

To further expand the potentialities and the flexibility of the BACTH system we constructed a new plasmid, pUT18C, that allows to fuse polypeptides to the C-terminus of T18 instead of the N-terminus, as it is the case with pT18 and pUT18 vectors (Figure 1). Various polypeptides, including the isolated α/β or α domains of TyrRS and the GCN4 Leucine zipper were then fused to the C-terminus of T18 and the resulting plasmids, pUT18C-Tyr-α/β, pUT18C-Tyr-α and pUT18C-zip, were tested for complementation with different interacting proteins fused to T25. The complementation efficiencies of these chimeric proteins, assessed by β–galactosidase expression, were compared to complementation occurring between the same T25 hybrids and the N-terminal T18 fusions. As shown in Figure 7, lines 2 to 5, for some interactions, like the Tyr333 / GCN4 leucine zipper association or the dimerization of the α domain of TyrRS, the complementation efficiencies were similar with both the N- and C-terminal fusions to T18. For

	Plasmids		Hybrid proteins	β-galactosidase activity (U/mg)
1	pKT25	pT18	25—C N—18	370
2	pKT25-Tyr333	pUT18-zip	25—C GCN4 -L-L-L-L—18	5660
3	pKT25-Tyr333	pUTC18-zip	25—C 18—L-L-L-L-	6330
4	pKT25-Tyr-α	pUT18-Tyr-α	25—C N—18	6800
5	pKT25-Tyr-α	pUT18C-Tyr-α	25—C 18—C	5830
6	pKT25-Tyr-α	pUT18-Tyr-α/β	25—C N—18	2600
7	pKT25-Tyr-α	pUT18C-Tyr-α/β	25—C 18—C	1460
8	pKT25-Tyr-α/β	pUT18-Tyr-α/β	25—C N—18	1800
9	pKT25-Tyr-α/β	pUT18C-Tyr-α/β	25—C 18—C	320
10	pKT25-Tyr-α	pUT18-zip	25—C -L-L-L-L—18	840
11	pKT25-Tyr-α	pUTC18-zip	25—C 18—L-L-L-L-	2310

Figure 7. Functional complementations mediated by C-terminal fusions to T18. The AC fragments, the various TyrRS sub-domains, and the GCN4 leucine zipper are schematized as previously. The β-galactosidase activities of the DHP1 transformants were determined as indicated in Experimental procedure.

other interacting couples, the efficiency of complementation appeared more dependent upon the site of fusion: for the Tyr-α/Tyr-α/β interaction, fusion of Tyr-α/β to the N-terminus of T18 resulted in twice as efficient complementation than fusion to the C-terminus (Figure 7, lines 6 and 7). The difference was even more dramatic for the dimerization of the isolated Tyr-α/β domain: no complementation could be detected when the α/β domain was fused to the C-terminus of T18, whereas significant β–galactosidase activity was measured with the N-terminal fusion (Figure 7, lines 8 and 9). Conversely, the Tyr-α/ GCN4 leucine zipper interaction, was barely detectable when the leucine zipper was fused to the N-terminus of T18 while it was quite efficient with the C-terminal fusion (Figure 7, lines 10 and 11). In summary, these results indicate that heterologous polypeptides can be fused to either ends of the T18 fragment, although in each particular case complementation might be affected by the topological orientation of the T18 fragment with respect to the fused polypeptide. These results also emphasize that in the BACTH system, as in other two-hybrid techniques, the lack of *in vivo* complementation does not necessarily imply absence of interaction between the hybrid proteins.

Discussion

In this work we have further explored the potentialities of the BACTH system to study protein-protein interactions. As a model, we analyzed the interactions between various subdomains of the dimeric tyrosyl-tRNA synthetase (TyrRS) of *B. stearothermophilus*. Different types of interactions between TyrRS monomers as well as between TyrRS subdomains were revealed with this genetic assay :

(i) Dimerization through the α/β domains was already known and characterized both at the biochemical and structural level (Ward *et al.*, 1986; Brick and Blow 1987; Ward *et al.*, 1987; Brick *et al.*, 1989). The 3D-structure revealed that TyrRS dimer is formed by the association of a hydrophobic surface encompassing residues 128-167 within the α/β domain of each subunit. Importantly, we have shown that the modification of Phe164 to an Arg residue, which was known to prevent association of TyrRS monomers, also abolishes *in vivo* functional complementation between mutated TyrRS and the isolated α/β domain. These results suggest that this two-hybrid system could be a method of choice to identify important residues or group of residues that are involved in the association of two polypeptides.

(ii) Interaction between the α/β domain and the α domain could be anticipated from the crystal structure of TyrRS as these two sub-domains exhibit an extensive contact area (Brick and Blow, 1987); however a direct biochemical interaction between these two isolated sub-domains has not yet been reported. Our present results clearly indicate that these isolated sub-domains fused to AC fragments can associate *in vivo*. Indeed, functional complementation was observed with three independent combinations of hybrid proteins made of α/β and α domains fused to the T25 and T18 fragments in different topological orientations (Figure 3, lines 5 and 6, Figure 7, lines 8 and 9).

(iii) Interaction between the α domains, revealed by the BACTH was not predictable from the crystal structure of TyrRS. Complementation studies between various truncated fragments of TyrRS indicated that this dimerization is mediated by the C-terminal region of the α domain, which harbors a pseudo-leucine zipper motif made of three leucine residues (298, 305 and 312). This region was also shown to mediate *in vivo* interaction with a typical leucine zipper. A direct physical interaction between T25-Tyr-α and T18-Tyr-α or T25-Tyr333 and T18-zip hybrids remains to be established biochemically, although it is possible that such interaction might not be detectable by *in vitro* binding assays (e.g. if affinity is low). At present, it is not known whether *in vivo* this motif might participate in interactions of the native enzyme with putative ligands which could modulate enzymatic activity. For example, binding of cellular polypeptides or proteins to the pseudo-leucine zipper motif in *B. stearothermophillus* TyrRS might control the accessibility of the tRNA and therefore the efficacy of aminoacylation of Tyr-tRNA.

It should be stressed that we have never observed functional complementation when a hybrid protein made of one of the AC fragments fused to a particular TyrRS sub-domain was co-expressed with the complementary AC fragment not fused to a cognate interacting domain. This clearly indicates that functional complementation directly reflects the interaction between the hybrid proteins through the polypeptide moieties fused to the T25/T18 fragments. In contrast, the absence of complementation between two hybrid proteins does not necessarily imply a lack of interaction between the corresponding domains. We provided here evidence for large differences in efficiency of complementation between different hybrids that are made up of the same interacting domains but fused to either T25 or T18 or to either ends of the T18 fragments. Such results are not unexpected for at least two reasons:

1- As functional complementation requires the spatial proximity between the T25 and T18 fragments, the association of two hybrid proteins might impose steric constraints that could decrease or even abolish the adenylate cyclase enzymatic activity of the reconstituted complex. This should be the case if the hybrid proteins interact in such a way that the fused T25 and T18 fragments are hold too far in space to properly associate in an active complex. An example is given here by the dimerization of the isolated Tyr-α/β domain. The T25-Tyr-α/β could not complement the T18-Tyr-α/β when the α/β domain was fused to the C-terminus of T18, whereas a significant complementation was observed when the same α/β domain was fused to the N-terminus of T18. From the crystal structure of the TyrRS dimer, the amino acid residues of Tyr-α/β domain that were directly connected to the T25 and T18 fragments (i.e. Met1 and/or Pro248) are about 80 Angströms distant in both cases (Brick and Blow, 1987). However, in the case of the functional hybrid (i.e. with Tyr-α/β fused to the N-terminus of T18), the two AC fragments are localized on the same face of the Tyr-α/β dimer whereas in the case of the non-functional hybrid (i.e. with Tyr-α/β fused to the C-terminus of T18), they are located on the opposite faces of the rod-like dimer. It is likely that in this latter case, the Tyr-α/β dimer itself imposes sterical constraints that prevents the association between the T25 and T18 fragments. It will be of interest to examine whether such steric hindrance could be overcome by using longer linkers to connect the interacting polypeptides to the AC fragments; they could offer greater flexibility and better possibility for association.

2- Formation of a functional heterodimer between hybrid proteins is a complex process that will depend on the kinetics of synthesis and folding of the individual chimeric proteins and on the kinetics of association of the refolded hybrids. In addition, the molecules could be subject to proteolytic degradation at each of the steps during this association process. Therefore, it is obvious that any change in the kinetics of any given step could dramatically affect the overall reconstitution of a functional complex. These factors are, unfortunately, difficult to appreciate quantitatively. Furthermore, because these processes occur *in vivo*, it is likely that specific cellular factors like chaperon proteins and/or proteases directly influence the fate of the individual hybrid proteins.

The direct consequence of these biological and biophysical constraints is that two chimeric proteins that can physically interact will not necessarily complement *in vivo*. In other words, the absence of complementation will not necessarily mean the absence of interactions. In reality, this rule applies similarly to all two-hybrid methodologies, including the yeast two-hybrid system (Bartel and Fields, 1995; Phizicky and Fields, 1995; Hu *et al*., 2000). Future work with this bacterial system will give more insight into its flexibility and tolerance. It is particularly interesting that the new vectors described here can allow fusions at either ends of the T18 fragment, a possibility that clearly expands the applicability of this system.

In conclusion, this work shows that the bacterial two-hybrid system based on complementation of adenylate cyclase fragments can be used as a general methodology to investigate protein-protein interactions *in vivo*. We are currently using this system to dissect interactions of different couples of interacting polypeptides and to search on a genome wide scale for interacting partners for given "bait" proteins.

Experimental Procedure

Bacterial Strains and Growth Media

The *E. coli* strain XL1-Blue (Stratagen) was used in all of the cloning steps. The *E. coli* adenylate cyclase deficient (*cya*) strain DHP1 (Karimova *et al*., 1998), a derivative of DH1 (F^-, *glnV44(AS), recA1, endA1, gyrA96 (Nal^r), thi1, hsdR17, spoT1*, *rfbD1*) was used in all complementation assays. Cells were grown in Luria-Bertani (LB) broth or on LB agar (Miller, 1992) supplemented, when appropriate, with ampicillin 100 μg/ml, chloramphenicol 30 μg/ml or kanamycin 50 μg/ml. Screening for the ability to utilize sugar was performed on MacConkey agar plates containing 1% maltose (Miller, 1992) plus 0.5mM isopropyl-β-D-galactopyranoside (IPTG) and, when appropriate, antibiotics. All procedures were performed at 30°C.

Plasmids

Bacterial Two-Hybrid System Vectors

The initial pair of compatible vectors, pT25 and pT18, derivatives of pACYC184 and pBluescript II KS (Stratagene), respectively, was already described (Karimova *et al*., 1998). To facilitate constructions and screening of libraries, using the BACTH system, we created three new vectors.

Plasmid pKT25 (3445-bp), encoding the T25 fragment (residues 1-224 of CyaA) and expressing a kanamycin resistance selectable marker, is a derivative of the low copy vector pSU40 (Bartolome *et al*., 1991). It was constructed as follows: a 1044-bp *Hind*III-*EcoR*I fragment of pT25 was first subcloned into pSU40 linearized with *Hind*III and *EcoR*I, resulting in pKT25L. pKT25 was created from pKT25L by deleting a 236-bp *Nhe*I-*Hind*III fragment. pKT25 was designed to create chimeric proteins in which a heterologous

polypeptide can be fused to the C-terminal end of T25 (Figure 2).

Plasmid pUT18 (3023-bp), compatible with pT25 and pKT25, encodes the T18 fragment (residues 225-399 of CyaA). It is a derivative of the high copy number vector pUC19 (Sambrook *et al*., 1989). In a first step, we constructed plasmid pUC19L by inserting a 24-bp double-stranded oligonucleotide 5'-AATTCATCGATATAACTAAGTAA-3' encoding a *Cla*I site and stop-codon sequences (underlined) between the *Eco*RI and *Nde*I sites of pUC19. Then, a 534-bp fragment harboring the T18 open reading frame was amplified by PCR (using appropriate primers and pT18 as target DNA) and cloned into pUC19L digested with *Eco*RI and *Cla*I. In the resulting plasmid, pUT18, the T18 open reading frame was fused in frame downstream from the multicloning site of pUC19. Plasmid pUT18 was designed to create chimeric proteins in which a heterologous polypeptide can be fused to the N-terminal end of T18 (Figure 2).

Plasmid pUT18C (3017-bp), a derivative of pUC19, was constructed by cloning the same 534-bp PCR-generated fragment, harboring the T18 open reading frame described above, into pUC19L linearized with *Hind*III and *Pst*I (the appropriate restriction sites were included into the PCR primers). In the resulting plasmid, pUT18C, the T18 open reading frame is fused in frame upstream from of the multicloning site of pUC19L. Plasmid pUT18C was designed to create chimeric proteins in which a heterologous polypeptide can be fused to the C-terminal end of T18 (Figure 2).

pTyr Plasmids

To construct the plasmids pT25-Tyr333 and pT18-Tyr333 which express, respectively, the T25 and T18 fragment fused in frame with the first 333 amino acids of TyrRS we amplified the corresponding part of the *tyrRS* gene from plasmid M13(4am)-BY(M24-89) (Vidal-Cros and Bedouelle, 1992) by PCR using primers tyrS1 (5'-AGAGGTACCGGACATGGATTTGCT-3') and tyrS2 (5'-GCCGGTACCGCCGCTGTCAAATTGGC-3'). The PCR-generated product was then cleaved with *Kpn*I and cloned into the *Kpn*I site of pT25 and pT18 vectors. Restriction sites to obtain the fragment were included into the PCR primers.

To construct pT25-Tyr-α/β, expressing the T25 fragment fused in frame with the α/β domain of TyrRS, pT25-Tyr333 was cut with *BsiW*I, blunt-ended with Klenow fragment in the presence of dNTP and religated. To create pT18-Tyr-α/β, expressing T18 fused in frame with the α/β domain of TyrRS, pT18-Tyr333 was digested with *BsiW*I and *Xho*I, treated with Klenow fragment in the presence of dNTP and religated.

To construct pKT25-Tyr-α, pUT18C-Tyr-α and pUT18-Tyr-α which encode, respectively, the T25 and T18 fragments fused in frame with the α domain of TyrRS, the corresponding DNA fragment of the *tyrRS* gene was amplified by PCR using pT18-Tyr333 as a template and primers tyrS240 (5'-CGCGGATCCCTGGCTCGACAAAGAG-3') and tyrS3 (Karimova *et al*., 1998). It was cloned into pKT25, pUT18C and pUT18 vectors linearized with *BamH*I-*Kpn*I digestion (the corresponding restriction sites were included into the PCR primers). Plasmid pKT25-Tyr-α302 expressing the T25 fragment fused to the truncated α domain of TyrRS (Tyr- α302) was constructed by PCR amplification (with appropriate primers) of the corresponding DNA fragment of TyrRS and subcloning into pKT25 linearized with *BamH*I-*Kpn*I digestion.

pT18-Tyr333F164R plasmid encoding a modified Tyr333F164R fused in frame with the T18 fragment was constructed by a method of an accurate polymerase chain reaction with Vent DNA polymerase using pT18-Tyr333 as a target (Ansaldi *et al*., 1996). Two primers 5'-CGGGCATTTCACGTACCGAGTTCAGCTATATGATG-3' and 5'-TGAACTCGGTACGTGAAATGCCCGTCTCGATGC-3' (mutated bases are underlined) were designed to construct the mutated allele Tyr333(F164R). Primers overlap for 24 nucleotides. PCR protocol involved a denaturation at 98°C (5 min) followed by 10 cycles of 1 min at 98°C, 1 min at 58°C and 5 min at 72°C. The final cycle was 1 min at 58°C, 10 min at 72°C. After *Dpn*I treatment the PCR mixture was transformed into XL1-Blue cells. The mutation was confirmed by DNA sequencing.

pT25-Tyr333F164R plasmid expressing Tyr333F164 fused to T25 was constructed by cloning a 1008-bp *KpnI* fragment of pT18-Tyr333F164R into pT25 linearized with *KpnI*.

Plasmids pT25-Tyr302 and pT18-Tyr302 were already described as pT25-Tyr and pT18-Tyr, respectively (Karimova *et al*., 1998).

pZip Plasmids

Plasmid pUT18-zip (3119-bp), a derivative of pUT18, was constructed by inserting a 114-bp DNA fragment (PCR-amplified using appropriate primers) encoding the leucine zipper region from the yeast transcriptional factor GCN4 (Blondel and Bedouelle, 1991) into pUT18 linearized with *KpnI* and *EcoRI*. The corresponding restriction sites were included into the PCR primers.

Plasmid pUT18C-zip (3119-bp), a derivative of pUT18C, was constructed by inserting the same DNA fragment encoding the GCN4 leucine zipper described above, into pUT18C digested with *Kpn*I and *Eco*RI. It encodes the leucine zipper of GCN4 fused in frame to the C-terminal part of T18.

All DNA manipulations, polymerase chain reaction (PCR) and $CaCl_2$ transformation procedures were performed according to standard protocols (Sambrook, *et al*., 1989).

β-Galactosidase Assay

Overnight cultures, grown in the presence of 0.5mM IPTG and appropriate antibiotics, were used. To permeabilize cells, before the assay 1 drop of toluene and 1 drop of a 0.1% SDS solution were added to 2-3 ml of bacterial suspension. The tubes were vortexed briefly and incubated in a shaker at 37°C for 30 minutes to evaporate toluene. The assay medium (PM2 buffer) contained 70 mM $Na_2HPO_4.12H_2O$, 30 mM $NaHPO_4$. H_2O, 1 mM $MgSO_4$ and 0.2 mM $MnSO_4$. For the enzymatic reaction, to aliquots of the permeabilized cells (0.2 ml) PM2 buffer containing 100 mM β-mercaptoethanol was added to a final volume of 2 ml and the tubes were placed in a 28°C water bath for 5 min. The reaction was started by adding 0.5 ml of ONPG in PM2 buffer without β-mercaptoethanol. After sufficient yellow color has developed, the reaction was stopped by adding 1 ml of a 1M Na_2CO_3 solution.

One unit of β-galactosidase activity corresponds to 1 nmol of ONPG hydrolyzed per min at 28°C (Pardee *et al*., 1959). The specific activity of β-galactosidase is defined as units/mg dry weight bacteria (Karimova *et al*., 1998).

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