Conserved amino acids near the carboxy terminus of bacterial tyrosyl-tRNA synthetase are involved in tRNA and Tyr-AMP binding

J.C. Salazar[a], R. Zuñiga[a], C. Lefimil[a], D. Söll[b], O. Orellana[a,*]

[a] *Programa de Biología Celular y Molecular, Instituto de Ciencias Biomédicas, Facultad de Medicina, Universidad de Chile, Casilla 70086 Santiago 7, Chile*
[b] *Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, CT 06520-8114, USA*

Received 28 January 2001; accepted 2 February 2001

First published online 15 February 2001

Edited by Lev Kisselev

Abstract Bacterial tyrosyl-tRNA synthetases occur in two large subfamilies, TyrRS and TyrRZ, that possess about 25% amino acid identity. Their amino-terminal region, the active site domain, is more conserved (>36% identity). The carboxy-terminal segment of these enzymes includes the tRNA binding domain and contains only few conserved residues. Replacement of three of these residues in *Acidithiobacillus ferrooxidans* TyrRZ revealed that S356 and K395 play roles in tRNA binding, while H306, a residue at the junction of the catalytic and tRNA binding domains, stabilizes the Tyr-AMP:TyrRZ complex. The replacement data suggest that conserved amino acids in *A. ferrooxidans* TyrRZ and *Bacillus stearothermophilus* TyrRS play equivalent roles in enzyme function.

Key words: Overexpression; Fusion protein; Mutagenesis; tRNA binding domain

1. Introduction

Tyrosyl-tRNA synthetase (TyrRS), a class I aminoacyl-tRNA synthetase [1] forming tyrosyl-tRNA for protein synthesis, has been well characterized [2–4]. The structure of the class defining the amino-terminal catalytic core of *Bacillus stearothermophilus* TyrRS complexed with tyrosyl-adenylate has been determined [4,5]. Crystallographic and hydrodynamic data led to the proposal that the enzyme is a homodimer. Structural and kinetic data provided a detailed description of the enzyme's interactions with ATP, tyrosine and analogs of intermediates in the activation reaction [4]. The more conserved N-terminal domain with the HIGH region includes the catalytic core [5]; this is followed by the more idiosyncratic C-terminal tRNA binding domain. Based on spectroscopic analysis the carboxy terminus of *B. stearothermophilus* TyrRS is structured in solution. The lack of structural information on this region of the protein by X-ray crystallography is probably due to flexibility at the junction of the catalytic and the tRNA binding domain [6,7]. No structural data are yet known for the complex of the enzyme with tRNA. However, mutational analysis has led to a model of the interaction of one molecule of tRNA with the dimer [8].

Previously we reported the sequence of *Acidithiobacillus ferrooxidans* tyrosyl-tRNA synthetase (TyrRZ) predicted from the sequence of the *tyrZ* gene [9]. This protein, which is active when produced in vivo in *Escherichia coli*, shows 44% identity at the amino acid level with the *tyrZ* gene product from *Bacillus subtilis* [10]. Recent advances in genome sequencing confirmed that the two subfamilies of tyrosyl-tRNA synthetases (TyrRS and TyrRZ) are widely represented in the bacterial domain (see below [11,12]). Members of the TyrRZ subfamily share no more that 25% identity with TyrRS. Multiple sequence alignments of members of the two subfamilies revealed that certain amino acids known to be involved in either the binding of substrates or the catalysis are conserved [9]. The majority of these residues are clustered at the N-terminal domain. However, a few amino acids in the putative tRNA binding domain are also conserved. In this work we attempted to define the role of some of those conserved amino acids in TyrZ.

2. Materials and methods

2.1. DNA manipulations

All DNA manipulations were carried out as described [13].

2.2. Cloning of tyrZ

The *A. ferrooxidans* gene was obtained from the plasmid pTR1 [14] and cloned in pGEX-2T (Pharmacia) generating recombinant plasmid pGT9. The cloning procedure was essentially as is described [11] except that pGEX-2T was the vector and the primers for polymerase chain reaction (PCR) amplification were (i) 5′-GAGAATTCCTATGAAGCATCAGG-3′ (underlined nucleotides, *Eco*RI recognition site) and (ii) 5′-GCGGCAATCTGGATCAGCTCC-3′. To obtain a His-tagged TyrRZ, PCR amplified *tyrZ* derived from plasmid pGT9 was cloned in the *Eco*RI site of a modified version of plasmid pET15b (Novagen) obtaining plasmid pETYRZ. All cloning procedures were confirmed by DNA sequencing.

2.3. Mutagenesis of tyrZ

PCR was used for the generation of mutations in *tyrZ* according to the method described by Higuchi et al. [15]. Mutations were confirmed by DNA sequencing. Mutated *tyrZ* was cloned back into pGT9.

2.4. Complementation of the E. coli HB2109 tyrS^{ts} strain

Complementation of the ts mutation in *E. coli* HB2109 *tyrS*^{ts} with pGT9 and mutated derivatives was carried out as described [9].

2.5. Overexpression and purification of TyrRZ

E. coli JM105, transformed by electroporation with pGT9 or mutagenized *tyrZ* were cultured at 25°C in Luria broth (LB) with 100 μg/ml ampicillin and subjected to induction of expression and purification of the protein product essentially as described [16] except that the lysis buffer contained 50 mM Tris pH 8.0, 2 mM EDTA, 0.1% v/v

*Corresponding author. Fax: (562)-737-6320.
E-mail: oorellan@bitmed.med.uchile.cl

PII: S0014-5793(01)02214-1

Triton X-100, 20 mM 2-mercaptoethanol, 100 μg/ml lysozyme, 1 mM phenylmethylsulfonyl fluoride and the fusion protein was eluted with 20 mM glutathione and stored at −20°C in 45% glycerol in the elution buffer. The fusion protein was, in some cases, subjected to digestion with thrombin in the elution buffer plus 2.5 mM $CaCl_2$ for 1 h at 25°C or 4°C overnight. To express the His-tagged TyrRZ, pETYRZ was electroporated in *E. coli* BL21(DE3). Cells were cultured at 37°C and TyrRZ was induced as described above. Under these conditions the overexpressed protein was in inclusion bodies that were solubilized by treatment with guanidine hydrochloride [17]. The soluble protein was concentrated by ultrafiltration on Centricon filters.

2.6. In vitro enzymatic activity of TyrRZ

All enzymatic analyses were performed with affinity chromatography-purified GST–TyrRZ fusion product or derivatives. (A) Aminoacylation of tRNA was carried out in a reaction buffer containing 50 mM Tris–HCl pH 8.0, 10 mM $MgCl_2$, 5 mM ATP, 5 mM dithiothreitol, 100 μM [^{3}H]Tyr (specific activity: 100 mCi/mmol) and 8.0 mg/ml of total *E. coli* tRNA or variable concentrations of tyrosine, ATP or *E. coli* $tRNA^{Tyr}$ (Sigma), essentially as is described [18]. (B) The amino acid activation assay was carried out by the [^{32}P]PPi (NEN) (1–2 cpm/pmol) exchange assay as described [19]. Kinetic analysis was carried out with variable concentrations of ATP or tyrosine. The radioactivity was counted in a scintillation counter. (C) The active site titration assay was carried out essentially as described [20]. The enzyme (TyrRZ or mutant) was incubated in the proper buffer containing 2 mM ATP, 4 U/ml of inorganic pyrophosphatase and 5 μM [^{14}C]Tyr (497.7 mCi/mmol). The radioactive complex retained on the nitrocellulose filter (Schleicher and Schuell BA 85) was counted as previously described. (D) The stability of the Tyr-AMP:TyrRZ complex was determined as follows. Preparation of the Tyr-AMP bound to the enzyme was carried out as in the active site titration reaction but at 4°C. Known amounts of the ternary complex were retained on nitrocellulose filters and incubated at room temperature (21 ± 1°C) in 700 μl of a reaction buffer containing 10 mM Bis–Tris propane pH 6.3, 150 mM NaCl and 10 mM $MgCl_2$. At different times, a 100 μl aliquot was spotted on filter paper (3MM Whatman) and dried to measure the total radioactivity released or on a new nitrocellulose filter to measure the released ternary complex (this filter was washed as in the active site titration analysis). The radioactivity bound to filters was counted in a scintillation counter. The chemical identity of the free radioactive compound was tested by thin layer chromatography (TLC) on silica gel (Merck) in a solvent containing *n*-propanol:water (70:30). [^{14}C]Tyr was used as marker. The radioactivity on the plate was processed in a phosphorimager (Bio-Rad).

3. Results

3.1. Functional equivalence of TyrRS and TyrRZ

Based on the divergence in primary structure between TyrRS and TyrRZ, an important consequence of the multiple alignment between TyrRS and TyrRZ is that it makes possible a rational identification of conserved residues that might be important for the enzyme function. To develop a system amenable to further biochemical and molecular genetic analysis of TyrRZ, we constructed plasmid pGT9 carrying the coding region of *tyrZ* as a *gst* (encoding glutathione-*S*-transferase) fusion expressed under the control of isopropyl β-D-thiogalactopyranoside. The in vivo functionality of the fusion protein was tested by complementing *E. coli* HB2109, harboring a temperature-sensitive *tyrS* gene, with pGT9. Growth at the non-permissive temperature (42°C) was observed in the transformant with pGT9 but not with pGEX-2T vector alone

ATP Binding site

```
Aferr     PLRIKL-GMDPTAPDLHLGHTVL   59
Aaeol     PLRVKA-GFDPTAPDLHLGHVVL   53
Hinfl     PLKVKL-GADPTAPDIHLGHTVV   54
Hpylo     RFIVKA-GFDPTAPDLHLGHTVL   59
BsubZ     PLKIKL-GLDPSAPDVHLGHTVV   69
Synec     PLRVKL-GIDPTGTDIHLGHSIP   61
Bstea     ERVTLYCGFDPTADSLHIGHLAT   51
Bcald     ERVTLYCGFDPTADSLHIGNLAA   51
BsubS     EKIRLYSGFDPTADSLHIGHLLP   52
Ecoli     GPIALYCGFDPTADSLHLGHLVP   53
                * ***     * **
```

Carboxy terminal region

```
Aferr     273 AVEQTRLQKEAASGARNPRDIKLDLAGELVRRFHGTARAQEAHIAFLARFQ-----------RHETPEDLPLQAIKLSE---APRLSQLLVQVHLAASTS   358
Aaeol     227 KEEIEKMRREM-----HPMEAKKLLAFTIVKRFHSEEEARKAKEWWEKTFS-----------QREFPEDAPLVKLN-EK---KLRAVDFLVKIGAVKSKN   346
Hinfl     268 LNEIAQLKSEV-ENGKNPRDVKILLAKELIARFHNEEAANAAEQEFINRFQ-----------KGAMPDEMPEFTFS-GE----MGLATLLKEAGLVPSTS   350
Hpylo     274 LEEIEDLKHGILNQTLHPKAVKEDLASEIVARYYDNDQAIKAKEQFSKVFS-----------ANLLPEILSESDFD-EG----VGILDVLKQIGFCPSTS   357
BsubZ     283 LEEKKQLVKDLETGAVHPRDAKMLLARTIVRMYHGEKAAEAAEHSFKTVFQ-----------ENSLPEDIPAVNWKGEK---TIAMIDLLVKLKLLSSKS   368
Synec     278 LAELPE----------NPRECQKLLAKEVTAQFHGVAGAIAAQKTAEDIVT-----------QGKAGNTDSVPEFSLAEITFPVKLAYLLSASGLCPSSS   356
Bstea     275 KEEIEALEQELREAP-EKRAAQKTLAEEVTKLVHGEEALRQAIRISEALFSGDIANLTAAEIEQGFKD-VPSF-VH-EGGDVP--LVELLVSAGISPSKR   368
Bcald     275 KEEIEALEQELREAP-EKRAAQKALAEEVTKLVHGEEALRQAIRISEALFSGDIANLTAAEIEQGFKD-VPSF-VH-EGGDVP--LVELLVSAGISPSKR   368
BsubS     277 KEEIEAYAEK-TEAP-EKREAQKRLAEEVTSLVHGREALEQAINISQALFSGNIKELSAQDVKVGFKD-VPSMEVD-STQELS--LVDVLVQSKLSPSKR   370
Ecoli     278 IEEINALEEEDKNSG-KAPRAQYVLAEQVTRLVHGEEGLQAAKRITECLFSGSLSALSEADFEQLAQDGVPMVEME-KGAD----LMQALVDSELQPSRG   372
               *                    **      *                                                  *       *

Aferr     359 EAMRKMKEGAVRVDGERVVDPATILALDAVYL-----LQFGKRHFARVALQKGE 407
Aaeol     347 EARRVIQGGGLKINGEKVTDPNTEIEINGELK-----VKVGKKKFYRVVSG    392
Hinfl     351 EAIRSAQQGGVKINGEKVDNVKDN-APKGTNV-----YQVGKRKFARVRL     401
Hpylo     358 QARRDIQGGGVKINQEVIKNESYRF-VKGNYV-----IQLGKKRFMKLNIN    402
BsubZ     369 EARRMIQNGGVRIDGEKVTDVHAKAEIRENMI-----IQVGKRKFLKLQ      412
Synec     357 EGRRQIKGGAVRLDGDRLEDVNQEYADPKMLINK--VLQMGKKKFIRLIS     404
Bstea     367 QAREDIQNGAIYVNGERLQDVGAILTAEHRLEGRFTVIRRGKKKYYLIRYA    419
Bcald     367 QAREDIQNGAIYVNGERLQDVGAILTAEHRLEGRFTVIRRGKKKYYLIRYA    419
BsubS     371 QAREDIQNGAVYINGERQTEINYTLSGEDRIENQFTVLRRGKKKYFLVTYK    421
Ecoli     373 QARKTIASNAITINGEKQSDPEYFFKEEDRLFGRFTLLRRGKKNYCLICWK    423
               *          **                    ***
```

Fig. 1. Amino acid sequence alignment of two regions of bacterial tyrosyl-tRNA synthetases. The alignment was carried out with the Clustal W program [24]. An asterisk (*) represents identical or nearly identical amino acids in all sequences. The HIGH motif is underlined. Positions of amino acid mutations in *A. ferrooxidans* TyrRZ are shown in black background. Aferr, *A. ferrooxidans*; Aaeol, *A. aeolicus*; Hinfl, *Haemophilus influenzae*; Hpylo, *Helicobacter pylori*; Bsub (S or Z), *B. subtilis*; Synec, *Synechocystis* sp.; Bstea, *B. stearothermophilus*; Bcald, *Bacillus caldotenax*; Ecoli, *E. coli*.

Table 1
Complementation of thermosensitive mutation in *E. coli tyrS* by *tyrZ* derivatives

tyrZ derivative	Growth of *E. coli* HB2109 (42°C)
pGT9	++
H53A	−
H306A	−
H306D	−
S356A	−
K395N	+/−
pGEX-2T	−

Growth of *E. coli* HB2109 transformed with plasmids carrying wild-type or mutant *tyrZ* at 42°C. pGT9, wild-type *tyrZ*; H53A, H306A,D, S356A and K395N are the *tyrZ* mutants; pGEX-2T, vector alone. ++: full growth was observed after 16 h of incubation. +/−: few and small colonies were observed at 16 h of incubation (full growth observed after 48 h). −: no colonies were observed at 16 h of incubation.

(Table 1), demonstrating the in vivo activity of the fusion protein in *E. coli*.

To test the role of conserved amino acids (Fig. 1) in the functional equivalence of TyrRS and TyrRZ, we carried out site-directed mutagenesis of *tyrZ*, based on the known function of these residues in *B. stearothermophilus* TyrRS [2,3,21]. The ability of the mutated *tyrZ* genes to rescue the $tyrS^{ts}$ mutation of *E. coli* strain HB2109 was then tested (Table 1). A mutant in H53 (H53A), which is part of the Class I HIGH signature sequence, was unable to complement. This replacement also abolishes the activation of tyrosine by TyrRZ in vitro (data not shown). K395 aligns with K410 in *B. stearothermophilus* TyrRS and is known to participate in tRNA binding [21]; the K395N *tyrZ* gene complements very poorly. Kinetic analysis revealed that the K395N mutation raises the K_M for *E. coli* tRNA about 17-fold, without affecting the K_M for ATP or tyrosine (Table 2). Three other mutants of conserved residues in the carboxy-terminal region of *tyrZ* did not complement (see below for the biochemical analysis). These data suggest that certain structural features in *A. ferrooxidans* TyrZ are functionally equivalent to those in TyrRS.

3.2. *A. ferrooxidans TyrRZ is a homodimer*

Affinity chromatography on glutathione–agarose of an *E. coli* S-100 extract containing heterologously expressed GST–TyrRZ yielded a nearly 90% pure protein. In agreement with the prediction from the deduced amino acid sequence a 71 kDa protein was observed. After digestion of the purified fusion protein with thrombin, TyrRZ (MW = 46 kDa) and GST (MW = 27 kDa) were obtained. The kinetic parameters in the aminoacylation reaction of thrombin-digested and GST-fused TyrRZ are indistinguishable (data not shown). The quaternary structure of His-tagged TyrRZ was tested by gel filtration on a Superose 12 column. A molecular mass of 105 kDa was obtained. As the molecular mass of this protein was 48 kDa as judged by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (data not shown), we conclude that native His-tagged TyrRZ is a dimer. Although some residues (e.g. P164) involved in dimerization of TyrRS [22] are not conserved in *A. ferrooxidans* TyrRZ, this enzyme is also a dimer.

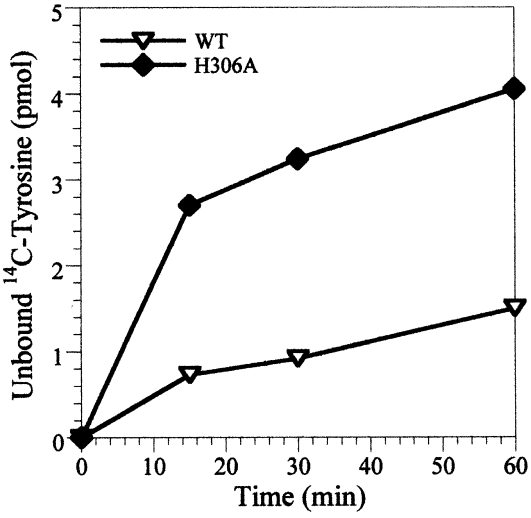

Fig. 2. Stability of the Tyr-AMP intermediate. Release of radioactivity from Tyr-AMP after binding of the Tyr-AMP:TyrRZ complex to nitrocellulose filters. ▽, wild-type TyrRZ; ◆, H306A mutant TyrRZ.

3.3. *Role of conserved amino acids in the carboxy-terminal region of TyrRZ*

A sequence alignment of the carboxy-terminal regions of 10 TyrRZ and TyrRS sequences (Fig. 1) highlighted H306 and S356 as conserved residues. In order to examine the potential function of these amino acids the following replacements were made: H306A or D and S356A. As shown above, none of these mutant *tyrZ* genes was able to complement the $tyrS^{ts}$ mutation in *E. coli* strain HB2109 (Table 1) suggesting that preservation of these amino acids is crucial for TyrRZ func-

Table 2
Kinetic parameters of wild-type and mutant tyrosyl-tRNA synthetases

Enzyme	K_m ATP (mM)	K_m tyr (μM)	K_m $tRNA^{tyr}$ (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (ATP)	k_{cat}/K_m (tyr)
Aminoacylation:						
TyrRZ (*A. ferrooxidans*)	0.13	26.8	2.54	7.7		
H306A	–	–	3.96	7.2		
S356A	–	–	19.9	–		
K395N	0.13	–	36.7	–		
TyrRS (*B. stearothermophilus*)[a]	2.2	2.0	1.4	1.2		
ATP–PPi exchange:						
TyrRZ (*A. ferrooxidans*)	0.07	21		5.29	75.6	0.25
H306A	0.035	24		1.63	46.6	0.068
S356A	0.094	21.6		4.28	45.53	0.198
K395N	–	12		–	–	–
TyrRS (*B. stearothermophilus*)	0.9	2.4		7.6	8.4	32

H306D, S356A, K395N = TyrRZ with the corresponding mutations.
[a]Obtained from [19,21].

tion. In vitro aminoacylation showed that the S356A protein has a seven-fold increase in the K_M for tRNA (Table 2); no effect in other kinetic parameters was observed. This strongly suggests the involvement of S356 in tRNA binding.

Sequence comparison of TyrRS and TyrRZ revealed that H306 in *A. ferrooxidans* TyrRZ is located at the junction of the catalytic and tRNA binding domains. Kinetic analysis of H306A TyrRZ showed that the K_M for neither tyrosine nor ATP was significantly altered by the mutation. A three-fold decrease in the k_{cat} for amino acid activation was observed (Table 2). The mutation does not appear to affect enzyme stability or mobility on native PAGE (data not shown) suggesting that the mutant protein does not have a significantly altered tertiary structure. An explanation of the decreased activity of the H306A TyrRZ mutant might be that the stability of the Tyr-AMP:TyrRZ complex is altered. To test this, [^{14}C]Tyr-AMP bound to the enzyme was immobilized to nitrocellulose filters and the release of radioactivity (from the filter to the incubation solution) was determined. A 3–4-fold increase in the release of radioactivity of the mutant TyrRZ complex was observed when compared to wild-type (Fig. 2). The released compound was tyrosine (identified by TLC). These data suggest that the conserved H at the junction of catalytic and tRNA binding domains is involved in the stability of the Tyr-AMP:TyrRZ ternary complex.

4. Discussion

Sequence alignments of the two tyrosyl-tRNA synthetase subfamilies TyrRS and TyrRZ highlighted some conserved residues. Replacement in TyrRZ of some amino acids conserved between TyrRZ and TyrRS, whose function was known from studies of *B. stearothermophilus* TyrRS, led to changes in TyrRZ activity consistent with the predictions. Thus, in spite of the divergence in their primary structures, conserved amino acids apparently perform the same function in both TyrRZ and TyrRS. These findings extend our earlier observation that *A. ferrooxidans* TyrRZ can functionally replace *E. coli* TyrRS in vivo [9].

Early duplication of the *tyrS* gene and faster evolution of one copy due to different functional constrains has been invoked as the reason for the presence of both *tyrS* and *tyrZ* genes in *B. subtilis* [10]. The availability of a significant number of bacterial genome sequences showed that at least 24 species of bacteria possess *tyrZ* genes. There are nine bacterial lineages in which *tyrZ* is found. They include the Aquificales, green sulfur bacteria, Cyanobacteria, low G+C Gram-positive bacteria, β-Proteobacteria, γ-Proteobacteria, ε-Proteobacteria, Thermotogales and the Thermus/Deinococcus family. Deep-rooted organisms like *Thermotoga maritima* and *Aquifex aeolicus* contain *tyrZ* as the only gene encoding tyrosyl-tRNA synthetase. Others (e.g. *Clostridium acetobutylicum*, *Pseudomonas aeruginosa*, *Vibrio cholerae* and *B. subtilis* contain one copy of each *tyrS* and *tyrZ*). Thus the origin of the two different tyrosyl-tRNA synthetases in the bacterial domain is still an open question. In light of the currently prevailing view that contemporary aminoacyl-tRNA synthetases are composed of functional domains that have been acquired during evolution to enhance substrate specificity [23], a more detailed analysis of the tRNA^{Tyr}:TyrRZ complex will be needed to shed more light on the possible evolutionary divergence of the protein–RNA interaction in the TyrRS and TyrRZ subfamilies.

Acknowledgements: We thank M. Ibba and J. Tapia for advice on some experiments. This work was supported by grants from Fondecyt Chile (1950702, 1981083) and ICGEB (CRP/CHI94-05(a2) to O.O., from Fondecyt Chile (2990034) to J.C.S. and from NIH (TW00481 and GM22854) to D.S.

References

[1] Ibba, M. and Söll, D. (2000) Annu. Rev. Biochem. 69, 617–650.
[2] Bedouelle, H. (1990) Biochimie 72, 589–598.
[3] Bedouelle, H., Guez-Ivanier, V. and Naggeotte, R. (1993) Biochimie 75, 1099–1108.
[4] Brick, P., Bhat, N. and Blow, D.M. (1989) J. Mol. Biol. 208, 83–98.
[5] Waye, M.M., Winter, G., Wilkinson, A.J. and Fersht, A.R. (1983) EMBO J. 2, 1827–1829.
[6] Pintar, A., Guez, V., Castagné, C., Bedouelle, H. and Delepierre, M. (1999) FEBS Lett. 446, 81–85.
[7] Guez, V., Nair, Sh., Chaffotte, A. and Bedouelle, H. (2000) Biochemistry 39, 1739–1747.
[8] Carter, P., Bedouelle, H. and Winter, G. (1986) Proc. Natl. Acad. Sci. USA 83, 1189–1192.
[9] Salazar, O., Sagredo, B., Jedlicki, E., Söll, D., Weygand-Duracevic, I. and Orellana, O. (1994) J. Bacteriol. 176, 4409–4415.
[10] Glaser, P., Kunst, F., Debarbouille, M., Vertes, A., Danchin, A. and Dedonder, R. (1991) DNA Seq. 1, 251–261.
[11] Wolf, Y.I., Aravind, L., Grishin, N.V. and Koonin, E.V. (1999) Genome Res. 9, 689–710.
[12] Woese, C.R., Olsen, G.J., Ibba, M. and Söll, D. (2000) Microb. Mol. Biol. Rev. 64, 206–236.
[13] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: a Laboratory Manual, 2nd edn. Cold Spring Harbor, New York.
[14] Salazar, O., Takamiya, M. and Orellana, O. (1989) FEBS Lett. 242, 439–443.
[15] Higuchi, R., Krummel, B. and Saiki, R.K. (1988) Nucleic Acids Res. 16, 7351–7367.
[16] Smith, D.B. and Johnson, K.S. (1988) Gene 67, 31–40.
[17] Maldonado, E., Drapkin, R. and Reinberg, D. (1996) Methods Enzymol. 274, 72–100.
[18] Vidal-Cross, A. and Bedouelle, H. (1992) J. Mol. Biol. 223, 801–810.
[19] Bunjun, S., Stathopoulos, C., Graham, D., Min, B., Kitabatake, M., Wang, A.L., Wang, Ch.C., Vivares, C.P., Weiss, L.M. and Söll, D. (2000) Proc. Natl. Acad. Sci. USA 97, 12997–13002.
[20] Wilkinson, A.J., Fersht, A.R., Blow, D.M. and Winter, G. (1983) Biochemistry 22, 3581–3586.
[21] Bedouelle, H. and Winter, G. (1986) Nature 320, 371–373.
[22] Jones, D.H., McMillan, A.J., Fersht, A.R. and Winter, G. (1985) Biochemistry 24, 5852–5857.
[23] Ibba, M., Curnow, A.W. and Söll, D. (1997) Trends Biochem. Sci. 22, 39–42.
[24] Higgins, D.G. and Sharp, P.M. (1988) Comput. Appl. Biosci. 5, 151–153.