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A novel peptidyl-prolyl *cis/trans* isomerase from *Escherichia coli*

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Abstract

A novel peptidyl-prolyl *cis/trans* isomerase was isolated from *Escherichia coli* cell extract and characterized partially. Determination of the molecular mass by electrospray mass spectrometry indicated a protein of 10102 ± 2 Da, smaller than cyclophilins or FK506 binding proteins currently known. The specificity constant k_{cat}/K_m determined with Succinyl-Ala-Xaa-Pro-Phe-4-nitroanilide (Xaa = Leu) had a value comparable to those from cyclophilins from the same organism. However, the pattern of subsite specificity (Xaa = Gly, Ala, Val, Ile, Leu, Phe, Trp, His, Lys and Glu) was reminiscent of FK506 binding peptidyl-prolyl *cis/trans* isomerases. The enzyme activity was not inhibited by cyclosporin A or FK506 at inhibitor concentrations of < 5 μM, concentrations that affect most bacterial peptidyl-prolyl *cis/trans* isomerases. Computer-assisted analysis of 21 amino acid residues of the N-terminus determined by Edman degradation revealed no homology to known peptidyl-prolyl *cis/trans* isomerases.

Key words: Escherichia coli; Peptidyl-prolyl *cis/trans* isomerase; FK506; Cyclosporin A; Proline

1. Introduction

Peptidyl-prolyl *cis/trans* isomerases (PPIases; EC 5.2.1.8) catalyse the *cis/trans* isomerization of the peptidyl-prolyl peptide bond in oligopeptides and are thought to be able to accelerate slow steps in protein refolding that are limited in rate by *cis/trans* isomerizations of peptidyl-propyl peptide bonds [1–3]. PPIases occur in mammals as well as in plants, fungi and bacteria. Perhaps they control the binary *cis/trans* switch of prolyl peptide bonds at various conformational states of the polypeptide chains within the cell [4]. Currently they are divided into two families, cyclophilins (Cyp) and FK506 binding proteins (FKBP), which have little if any amino acid sequence similarity to each other [5]. However, within each family a core containing highly conserved amino acids exists which has been transmitted from prokaryotes to eukaryotes during evolution [6]. Typically, the core includes about 80 and 100 residues for FKBPs and cyclophilins, respectively. Cyclophilins from all sources bind tightly ($K_i \ll 0.1$ mM) to the immunosuppressive undecapeptide cyclosporin A with a time-dependent loss of enzymatic activity. A related reversible reaction occurs between FKBPs and the immunosuppressive macrolides FK506 and rapamycin ($K_i < 1$ μM). The inhibition of PPIase activity, however, is not responsible for the suppression of the clonal expansion of T-cells induced by these effectors [7,8]. Instead, PPIase activity is important for protein folding and trafficking [9–11].

As much as 9 different PPIases were detected and characterized in human cells until now. This contrasts with only two cyclophilins expressed in *E. coli*, one of which is found in the cytosol and one in the periplasmic space [12–14]. FKBP-like proteins have not yet been detected in this organism although an open reading frame (ORF) in the LspA-DapB intergenic region exists which may code for a 16.1 kDa protein [15], showing 33% identity to the cytosolic 12 kDa human FKBP (human FKBP12cy) [6]. Furthermore, an *E. coli* sequence of 200 amino acids in length was reported by Wuelfing and Plueckthun (EMBL:S33444) that shows a N-terminal region with clusters of residues found to be fully conserved throughout the FKBP family. C-terminally a histidine-rich region, proposed to be a metal ion binding motif is linked to the FKBP domain. However, there is no information about an enzymatic activity of this protein.

In the pathogenic Gram-negative bacterium *Legionella pneumophila*, the causative agent of Legionnaires disease, the FKBP-homologous Mip protein (25 kDa) has been characterized as a FK506-inhibitable PPI-ase [16]. From the same organism a cyclophilin of 18 kDa has been isolated, sequenced and tested for catalytic function in prolyl *cis/trans* isomerization [17] indicating that bacteria may simultaneously express both types of PPIase activity. Cyclophilins also occur in *Bacillus subtilis* [13] and *Streptomyces chrysomallus* [14], while FKBPs have been found in *Streptomyces chrysomallus* [20], *Neisseria meningitidis* [21] and *Chlamydia trachoma-*

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tis [22]. In addition some ORFs were identified coding for proteins with some similarity to the core region of cyclophilins or FKBPs in *Salmonella typhimurium* [23], *Synechococcus sp.* [24], and *Pseudomonas aeruginosa*[25].

The present paper describes the isolation and preliminary characterisation of a new PPIase with an exceptionally low molecular mass from *E. coli*.

2. Experimental

2.1. Materials

4-(2-hydroxyethyl)-1-Piperazineethanesulphonic acid (HEPES) and *N*-tris(hydroxymethyl)-methylglycine (Tricine) were obtained from Serva, Heidelberg; 1.4-Dithiothreitol (DTT) was from Roth, Karlsruhe; chymotrypsin, Fractogel EMD DEAE-650(M), Fractogel EMD SO_3-650(M) Fractogel TSK AF-Blue and Benzon Nuclease solution (Benzonase, 25 U/μl) were purchased from Merck, Darmstadt; the HPLC column (300-C_4-5 μm, 50 × 3 mm) was from Macherey-Nagel, Düren; the HiLoad 16/60 Superdex 75 column was obtained from Pharmacia LKB, Uppsala and all substrates for the PPIase activity assay were purchased from Bachem, Heidelberg.

2.2. Purification

E. coli K12 cells were grown overnight at 37°C in LB-medium, containing sodium chloride (5 g/l), yeast extract (10 g/l) and tryptone (5 g/l). The cells were harvested by centrifugation at 4°C for 10 min at 6,000 × *g* in a Centricon T-324 centrifuge. The pellet was resuspended in 20 mM Tricine buffer, pH 8.5 (buffer A) and the cells were disrupted in a SLM Aminco FRENCH pressure cell with 20,000 psi. The cell lysate was stirred for 15 min at 25°C with 0.1% (vol/vol) Benzonase. The cell debris were removed by centrifugation at 4°C for 30 min at 20,000 g in a Beckman L8 60M ultracentrifuge. An equal volume of buffer A was added to the supernatant, the pH was adjusted to pH 8.5 and the protein solution was passed through a Fractogel EMD DEAE-650(M) column (2.5 × 20 cm). The flow-through fractions were collected and applied to a Fractogel TSK AF-Blue column (1 × 5 cm), equilibrated with buffer A. Using a linear gradient of 0–2 M KCl in buffer A, PPIase-active fractions were eluted at KCl concentrations of 100 to 150 mM. The active fractions were concentrated with a Filtron OMEGACELL, 5,000 Da and applied to a HiLoad 16/60 Superdex 75 gel filtration column equilibrated with 10 mM HEPES buffer, pH 7.8, containing 150 mM KCl and 0.5 mM DTT (buffer B). PPIase-active fractions were pooled and dialysed against 10 mM HEPES buffer, pH 7.8 (buffer C). Final purification to homogeneity was obtained by cation-exchange chromatography on a Fractogel EMD SO_3-650(M) column (1 × 2 cm) equilibrated with buffer C. PPIase-active fractions were eluted using a linear gradient of 0–2 M NaCl in buffer C at approximately 200 mM NaCl.

2.3. PPIase assay, substrate specificity and inhibition studies

PPIase activity was determined using a protease-coupled assay with chymotrypsin and the substrate Succinyl-Ala-Phe-Pro-Phe-4-nitroanilide [16] in 0.035 M HEPES buffer, pH 7.8, at 10°C. Stock solutions of substrates were made in dimethylsulfoxide. The reaction was started by the addition of 2 μl substrate solution to the reaction mixture. For inhibition studies the PPIase assay was carried out with addition of FK506 or cyclosporin A from a stock solution in 50% (vol/vol) aqueous ethanol. A Hewlett Packard 8452 diode array UV/VIS spectrophotometer was used for monitoring the time course of the reaction. The obtained first-order kinetics can be described by the rate equation $v = k_{obs} \cdot [cis]$; $k_{obs} = k_o + k_{enz} \cdot ([cis]$ is the time-dependent concentration of the *cis* conformer, k_o is the uncatalysed, k_{enz} is the PPIase-catalysed *cis* to *trans* interconversion of the substrate). The specificity constant k_{cat}/K_m was obtained from the equation $k_{cat}/K_m = (k_{obs} - k_o)/[PPIase]_{total}$ assuming that the entire amount of the pure enzyme protein represents catalytically active molecules. For determination of the volume-dependent activity of crude enzyme fractions in the assay we used arbitrary units, $AU = (k_{obs}/k_o) - 1$. Protein concentration was determined with the Bradford assay [26] using a bovine serum albumin standard.

2.4. Determination of the N-terminal amino acid sequence and data analysis

Protein from 400 μl of the solution eluted from the Fractogel EMD SO_3-650(M) column was blotted on ProSpin-PVDF membrane (Applied Biosystems Inc.) with centrifugation at 5,000 × *g*. After rinsing the membrane with methanol/water 1:5 (vol/vol) the protein was sequenced on an Applied Biosystems gas-phase sequencer modul 470A.

In order to achieve optimal alignment of the resulting N-terminal part of the amino acid sequence, the program BESTFIT (University of Wisconsin Genetics Computer Group package) for pairwise homology comparison was used. Cyclophilin and FKBP sequences were taken from Protein Identification Resource release 37.x and Swissprot release 25.0. Data base searches were performed using the program FASTA.

2.5. Determination of the molecular mass

A 15 μg protein sample eluted from the Fractogel EMD SO_3 column was concentrated, desalted and transferred into a volatile solvent by means of narrowbore reversed-phase HPLC. Portions of 1 ml were loaded on a 50 × 3 mm C_4 column equilibrated with 0.1% aqueous trifluoroacetic acid. The protein was eluted with a gradient of 0 to 60% acetonitrile in 0.1% trifluoroacetic acid within 12 min at a flow rate of 1 ml/min. The eluted enzyme was still active.

Molecular mass was determined by electrospray MS on a VG BIO-Q (Fisons Instruments), consisting of an electrospray ion source followed by a triple quadrupole mass analyser with a mass range of 4000. An aliquot of 5 μl of the HPLC-purified protein solution was injected directly into the electrospray device by way of a loop injector (Rheodyne 5717) at a solvent (acetonitrile/water 1:1 (vol/vol), 1% formic acid) flow rate of 4 μl/min (pump HP 1050). Scanning was performed from *m/z* 500 to *m/z* 1300 in 10 s and this mass scale was calibrated using horse heart myoglobin.

3. Results and discussion

A typical preparation was started from a 10 l overnight culture of *E. coli* cells. After cell disruption and centrifugation 2.7 g of protein were obtained. A cyclosporin A-insensitive PPIase activity could be demonstrated only after passage of the protein through Fractogel EMD DEAE-650(M) and the Fractogel TSK AF-Blue column. During earlier stages in purification the

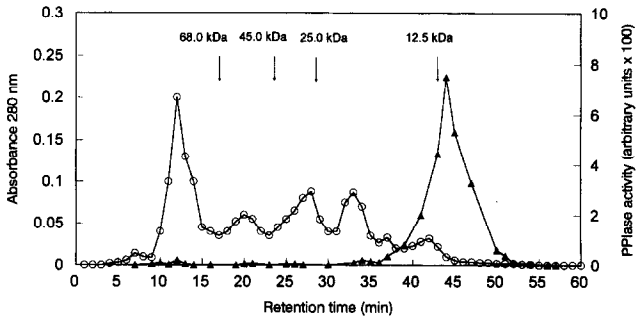

Fig. 1. Elution profile of HiLoad 16/60 Superdex 75 gel filtration of *E. coli* proteins desorbed from a Fractogel TSK AF-Blue column. The active fractions received from the Fractogel TSK AF-Blue column were concentrated. Aliquots of 1 ml were loaded onto a gel filtration column, equilibrated with 10 mM HEPES buffer, pH 7.8, containing 150 mM KCl and 0.5 mM DTT. The separation was done at a flow rate of 1 ml/min. The column was calibrated with bovine serum albumin (68000 Da), ovalbumin (45000 Da), chymotrypsinogen A (25000 Da) and cytochrome *c* (12500 Da). PPIase activity (▲); Absorbance at 280 nm (○).

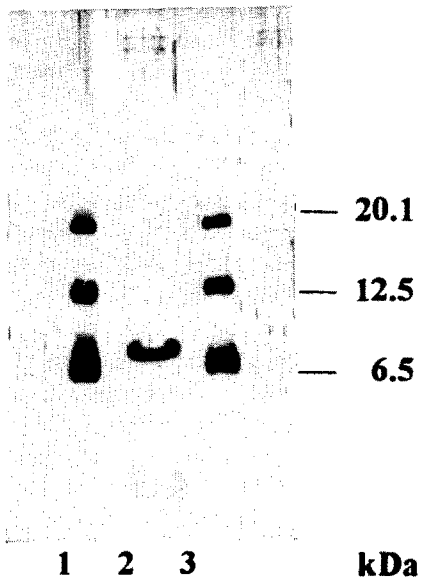

Fig. 2. SDS-PAGE of purified *E. coli* 10.1 kDa PPIase. Electropherogram of a silver stained SDS-polyacrylamide gel (17.5% acrylamide). Lanes 1 and 3 (molecular mass markers): soybean trypsin inhibitor (21000 Da), cytochrome *c* (12500 Da) and aprotinin (6500 Da); lane 2, 0.07 μg *E. coli* 10.1 kDa PPIase.

minor enzymatic activity signaling the new PPIase was obscured by enzyme activity resulting from the bulk of cyclophilins occurring in *E. coli* cells. This also prevented the calculation of the yield of the purification procedure. Gel filtration of the PPIase resulted in an active protein peak with a retention time corresponding to a molecular mass of about 10 kDa (Fig. 1). In the next purification step nearly pure protein was obtained using a cation-exchange column. The protein was analysed by SDS-PAGE (17.5% acrylamide). The silver-stained gel showed a single band with an apparent molecular mass of approximately 8 kDa (Fig. 2). The absence of other bands on a heavily overloaded polyacrylamide gel confirmed that the PPIase activity in the enzyme assays originated from the 8 kDa protein. The final yield was 18 μg pure enzyme.

The accurate molecular mass of the novel PPIase was determined by electrospray ionisation mass spectrometry after a final purification step on reversed-phase HPLC. The measurement indicated a protein with a molecular mass of 10101 ± 2 Da (Fig. 3). This value does not correspond to any of the known *E. coli* PPIases. All of them, the cytoplasmic (18.2 kDa) and the mature periplasmic cyclophilin (18.1 kDa) as well as the FKBP-like histidine-rich protein (21.3 kDa) and the ORF encoding for a PPIase-related protein (16.1 kDa) considerably exceed this value. In comparison to human FKBP12 (11824 Da; 107 amino acid residues) the smallest PPIase known to date, the 10.1 kDa *E. coli* PPIase may be considerably shorter than 100 amino acids.

Until now it is not known if the 10.1 kDa enzyme represents a proteolytic fragment of a more extended polypeptide chain.

The first 21 amino acid residues found by Edman degradation of the new PPIase represent 22% of the molecular mass of the entire enzyme (Fig. 4). Screening of the Pir, Mipsown and Swissprot data collections with the FASTA program did not reveal any convincing similarity to this novel sequence. Fig. 4 shows that only weak similarities can be detected by pairwise comparisons to known PPIase sequences with the BESTFIT program. Except for a single residue (Leu-96) in FKBP12cy the amino acids possessing a certain degree of similarity in the matched sequences do not include those conserved amino acids thought to be responsible for enzymatic activity. Thus, the new PPIase is not a truncated form of cyclophilin or FKBP. FKBPs as well as cyclophilins purified from mammalian species can be inhibited by the respective immunosuppressive drug in the nanomolar range [27], whereas the *E. coli* cyclophilins are 1000-fold less sensitive to cyclosporin A. This is caused by the

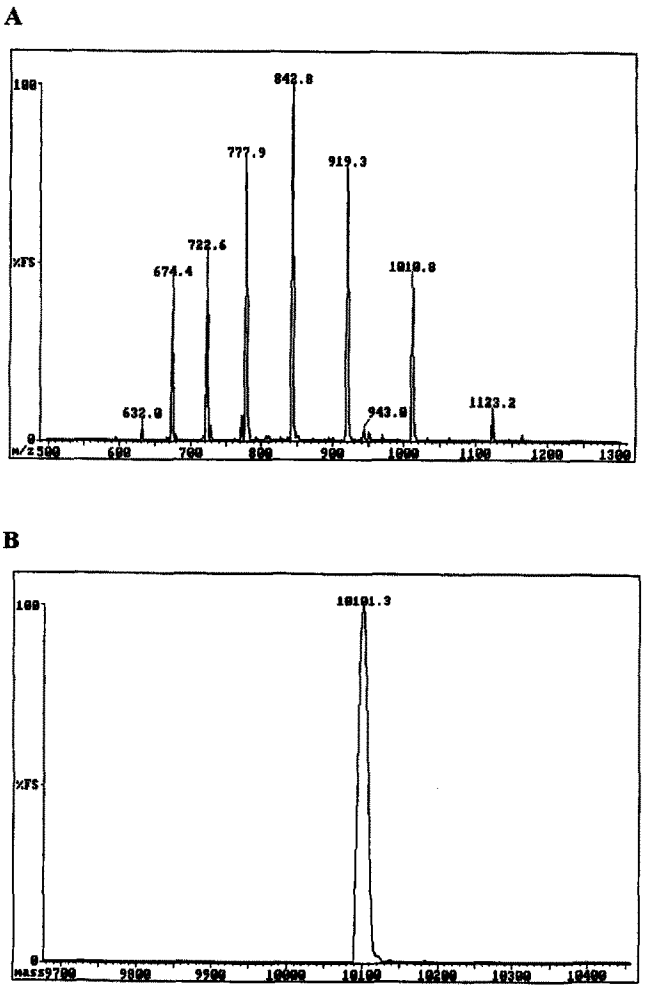

Fig. 3. Electrospray ionization mass spectrum of *E. coli* 10.1 kDa PPIase. The spectrum (B) was received from deconvolution of eight peaks from the mass/charge spectrum (A).

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                1 AKTAAALHILVKEEKLALDLL 21
E.coli            ||..: : : ...:.:.|:|
Cyp21peri      25 AKGDPHVLLTTSAGNIELELD 45

                1 AKTAAALHILVKEEKLALDLL 21
E.coli            |..  .::|  |..|.: :: :
Cyp18cy       140 ATGRSGMHQDVPKEDVIIESV 160

                1 AKTAAALHILVKE...EKLALDLL 21
E.coli            |.:.:.:. :::|    :.:.:|:
16.1kDa (ORF) 103 AMDGSEMPGVIREINGDSITVDFN 126

                1 AKTAAALHILVKEEKLALDLL 21
Human             ..:    |:.....|.:|:
FKBP12cy       84 YGATGHPGIIPPHATLVFDVE 102
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Fig. 4. The NH_2-terminal 21 amino acids of the *E. coli* 10.1 kDa PPIase aligned with the most similar stretches from *E. coli* cyclophilins, an ORF with FKBP homology from the same organism and human FKBP12cy. Identity and similarity were calculated by pairwise comparison using the program BESTFIT. The percent similarity (*E. coli* Cyp21peri, 50.0; *E. coli* Cyp18cy, 47.6; *E. coli* 16.1ORF, 60.0; human FKBP12cy, 38.8) and the percent identy (*E. coli* Cyp21peri, 20.0; *E. coli* Cyp18cy, 19.0; *E. coli* 16.1ORF, 15.0; human FKBP12cy, 16.7) were calculated. Strongly conserved amino acids in the cyclophilins and FKBPs are shown in bold letters.

absence of the tryptophan residue in position 121 which has been shown to be important for the binding of this cyclopeptide [28]. When exposed to concentrations of either 5 μM FK506 or 5 μM cyclosporin A, there is no indication for inhibition of the enzymatic activity of the *E. coli* 10.1 kDa PPIase.

Despite its insensitivity to micromolar concentrations of FK506, the *E.coli* 10.1 kDa PPIase shows the typical pattern of substrate specificity of FKBPs as indicated in Table 1. The *E. coli* 10.1 kDa PPIase prefers amino acid residues with hydrophobic side chains like leucine and phenylalanine in the P_1-position of the peptide substrates [29]. The unusually high PPIase activity for the substrate with an alanyl residue in this position allowed to differentiate the enzyme from FKBPs.

Table 1
Substrate specificity of the *E. coli* 10.1 kDa PPIase compared with Cyp21peri (*E. coli*) [14] and human recombinant FKBP12cy

Xaa	*E. coli* 21 peri	*E. coli* 10.1 kDa PPIase	rhFKBP12cy
Gly	93	0	0
Ala	345	60	12
Val	nt	32	48
Ile	nt	40	88
Leu	120	150	145
Phe	100	100	100
Trp	nt	44	19
His	55	34	4
Lys	17	30	4
Glu	86	15	0

The relative specificity constants k_{cat}/K_m for the substrates of the type Succinyl-Ala-Xaa-Pro-Phe-4-nitroanilide are shown. Succinyl-Ala-Phe-Pro-Phe-4-nitroanilide was set to 100%. Measurements were carried out in 35 mM HEPES buffer, pH 7.8, at 10°C.

The *E. coli* 10.1 kDa PPIase showed a specificity constant k_{cat}/K_m of $1.69 \cdot 10^7$ $M^{-1} \cdot s^{-1}$ for the substrate succinyl-Ala-Leu-Pro-Phe-4-nitroanilide. This value considerably exceeds enzymatic activities published for FKBPs and corresponds to the highest catalytic constants measured for the *E. coli* cyclophilins [17].

In conclusion, the finding of a previously undetected 10.1 kDa PPIase supplements the equipment of *E. coli* with PPIases. Three proteins have now been established for this organism differing in subsite specificity, inhibitor sensitivity, molecular mass and amino acid sequence. The unusually small enzyme characterized in the present report indicates the existence of a third family of PPIases in *E. coli*, unrelated to the previously known enzymes.

References

[1] Lang, K., Schmidt, F.X. and Fischer, G. (1987) Nature 329, 268–270.
[2] Lin, L.-N., Hasumi, H. and Brandts, J.F. (1988) Biochim. Biophys. Acta 956, 256–266.
[3] Schmid, F.X., Mayr, L.M., Mücke, M. and Schönbrunner, E.R. (1993) Adv. Prot. Chem. 44, 25–66.
[4] Fischer, G. (1994) Ang. Chem., in press.
[5] Wiederrecht, G., Brizuela, L., Elliston, K., Sigal, N.H. and Siekierka, J.J. (1991) Proc. Natl. Acad. Sci. USA 88, 1029–1033.
[6] Trandinh, C.C., Pao, G.M. and Saier, M.H. (1992) FASEB J. 6, 3410–3420.
[7] Schreiber, S.L. (1991) Science 251, 283–287.
[8] Flanagan, W.M., Corthesy, B., Bram, R.J. and Crabtree, G.R. (1991) Nature 352, 803–807.
[9] Steinmann, B., Bruckner, P. and Superti Furga, A. (1991) J. Biol. Chem. 266, 1299–1303.
[10] Stamnes, M.A., Shieh, B.H., Chuman, L., Harris, G.L. and Zuker, C.S. (1991) Cell 65, 219–227.
[11] Ondek, B., Hardy, R.W., Baker, E.K., Stamnes, M.A., Shieh, B.H. and Zuker, C.S. (1992) J. Biol. Chem. 267, 16460–16466.
[12] Liu, J. and Walsh, C.T. (1990) Proc. Natl. Acad. Sci. USA 87, 4028–4032.
[13] Hayano, T., Takahashi, N., Kato, S., Maki, N. and Suzuki, M. (1991) Biochemistry 30, 3041–3048.
[14] Compton, L.A., Davis, J.M., MacDonald, J.R. and Bächinger, H.P. (1992) Eur. J. Biochem. 206, 927–934.
[15] Bouvier, J. and Stragier, P. (1990) Nucleic Acid. Res. 19, 180.
[16] Fischer, G., Bang, H., Ludwig, B., Mann, K. and Hacker, J. (1992) Mol. Microbiol. 6, 927–934.
[17] Schmidt, B., unpublished results.
[18] Herrler, M., Bang, H., Brune, K., Fischer, G. and Marahiel, M.A. (1992) FEBS Lett. 309, 231–234.
[19] Pahl, A., Ühlein, M., Bang, H., Schlumbohm, W. and Keller, U. (1992) Mol. Microbiol. 6, 3551–3558.
[20] Pahl, A. and Keller, U. (1992) J. Bacteriol. 174, 5888–5894.
[21] Sampson, B.A. and Gotschlich, E.C. (1992) Proc. Natl. Acad. Sci. USA 89, 1164–1168.

[22] Lundemose, A.G., Kay, J.E. and Pearce, J.H. (1993) Mol. Microbiol. 7, 777–783.
[23] Tran, P.V., Bannor, T.A., Dotor, S.Z. and Nichols, B.P. (1990) J. Bacteriol. 172, 397–410.
[24] Hassidim, M., Schwarz, R., Liebman-Hurwitz, J., Marco, E., Ronen-Tarazi, M. and Kaplan, A. (1992) Plant. Physiol. 100, 1882–1886.
[25] Kato, J., Chu, L., Kitano, K., Devolt, J.D., Kimbara, K., Chakrabarty, A.M. and Misra, T.M. (1989) Gene 84, 31–34.
[26] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[27] Harrison, R.K. and Stein, R.L. (1990) Biochemistry 29, 3813–3816.
[28] Liu, J., Chen, C.-M. and Walsh, C.T. (1991) Biochemistry 30, 2306–2310.
[29] Schechter, I. and Berger, A. (1967) Biochem. Biophys. Res. Commun. 27, 157–162.