

Secretion by overexpression and purification of the water-soluble *Streptomyces* K15 DD-transpeptidase/penicillin-binding protein

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Though synthesized with a cleavable signal peptide and devoid of membrane anchors, the 262-amino-acid-residue *Streptomyces* K15 DD-transpeptidase/penicillin-binding protein is membrane-bound. Overexpression in *Streptomyces lividans* resulted in the export of an appreciable amount of the synthesized protein (4 mg/litre of culture supernatant). The water-soluble enzyme was purified close to protein homogeneity with a yield of 75 %. It requires the presence of 0.5 M-NaCl to remain soluble. It is indistinguishable from the detergent-extract wild-type enzyme with respect to molecular mass, thermostability, transpeptidase activity and penicillin-binding capacity.

INTRODUCTION

The monofunctional 262-amino-acid-residue *Streptomyces* K15 DD-peptidase/penicillin-binding protein (PBP) is of known primary structure and is synthesized with a cleavable 29-amino-acid-residue signal peptide [1]. The enzyme has been isolated and purified from the mycelium in the presence of cetyltrimethylammonium bromide (CTAB) [3]. It functions *in vitro* almost as a strict DD-transpeptidase, using diacetyl(Ac₂)-L-Lys-D-Ala-D-Ala as carbonyl donor and a properly structured amino compound, such as Gly-Gly, as acceptor [2]. The reaction products are D-alanine and Ac₂-L-Lys-D-Ala-Gly-Gly. This property is the consequence of the high efficacy with which Gly-Gly performs attack of the serine ester-linked Ac₂-L-Lys-D-alanyl-enzyme, when compared with that of water and D-alanine (i.e. the leaving group of the enzyme-acylation step). Hence the *Streptomyces* K15 enzyme mimics well the transpeptidation reaction through which PBPs catalyse peptide cross-linking during bacterial wall peptidoglycan synthesis.

The *Streptomyces* K15 enzyme is also peculiar in that it behaves as a membrane-bound protein [3], though it lacks membrane anchors. Predictional studies [1] suggest that, by reference to the β -lactamases of class A, the *Streptomyces* K15 enzyme has a five-stranded β -sheet partially exposed on one face, thus providing a zone of hydrophobicity potential that may be the site of interaction of the protein with the membrane.

As described below, amplified expression of the encoding gene in *Streptomyces lividans* resulted in the export of part of the synthesized DD-transpeptidase in the culture medium from which the enzyme was isolated in the absence of detergent.

MATERIALS AND METHODS

Streptomyces strains, media maintenance and growth conditions

The following strains were used: *Streptomyces* K15, a local strain; *S. griseofuscus*, A.T.C.C. 29916; *S. vinaceus* N.C.I.B. 8852; *S. ambofaciens* A.T.C.C. 15154; *S. venezuelae* D.S.M. 40755; *S. pristinae spiralis* A.T.C.C. 25486; *S. coelicolor* A3(2)

[4]; *S. fradiae* A.T.C.C. 10745; *S. fradiae* PM76 [5] (a gift from Dr. R. H. Baltz, Lilly Research Laboratories, Indianapolis, IN, U.S.A.); *S. lividans* TK24 [6] and *S. albus* G R2 [7] (gifts from the John Innes Institute, Norwich, U.K.).

The following solid media were used: the AG agar (containing, per litre: asparagine, 1 g; glycerol, 10 g; K₂HPO₄, 1 g; FeSO₄·7H₂O, 0.1 g; MnCl₂·4H₂O, 0.1 g; ZnSO₄·7H₂O, 0.1 g), S agar [8], R2YE agar [9] and modified R2YE agar (which is the R2YE medium, except that it contains, per litre: sucrose, 206 g; MgCl₂·6H₂O, 2.03 g; valine and isoleucine, 300 mg each).

The aforementioned *Streptomyces* strains were maintained on AG agar except *S. venezuelae*, which was maintained on S agar, and *S. lividans* TK24, which was maintained on R2YE agar. Unless otherwise stated, liquid cultures were carried out in TSB Oxoid-trypton soya broth at 28 °C and with vigorous orbital shaking in Erlenmeyer flasks.

Plasmids

Plasmid pIJ702 [9] was from the John Innes Institute, Norwich, U.K., and plasmid pDML223, which contains the *Streptomyces* K15 DD-peptidase-encoding gene, was that described in [1].

Protoplasting and transformation conditions

S. lividans and *S. pristinae spiralis* were grown at 30 °C for 38 h (*S. lividans*) or 46 h (*S. pristinae spiralis*) in the YEME medium [10] supplemented with 0.5 % (*S. lividans*) or 0.8 % (*S. pristinae spiralis*) (w/v) glycine. *S. albus* GR2 was grown at 34 °C for 16 h in TSB medium (in Erlenmeyer flasks containing a stainless-steel spring). The protoplasts were prepared as described by Hopwood *et al.* [4], except that the *S. albus* GR2 protoplasts were regenerated on modified R2YE plates. From 30 ml cultures, 10¹⁰ (*S. lividans*), 8 × 10⁹ (*S. pristinae spiralis*) and 4 × 10⁸ (*S. albus* GR2) regeneratable protoplasts were obtained.

Protoplasts of *S. lividans* were transformed as described in [4]. Protoplasts of *S. pristinae spiralis* and *S. albus* GR2 were transformed according to the same procedure, except that the concentration of poly(ethylene glycol) was increased to 21 %

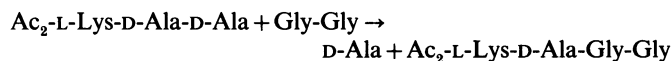
Abbreviations used: PBP, penicillin-binding protein; CTAB, cetyltrimethylammonium bromide; Ac₂, diacetyl; *m*-A₂pm, *m*-diaminopimelic acid.

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(w/v). The transformation rates with pIJ702 were 1×10^5 and 5×10^4 transformants/ μg of plasmid respectively.

Reference enzyme and enzyme unit

When using the donor (5 mM-Ac₂-L-Lys-D-Ala-D-Ala)-acceptor (1.5 mM-Gly-Gly) system, 1 enzyme unit corresponded to 1 nmol of tripeptide consumed/min according to the reaction:



The reaction was monitored by using radioactive/non-radioactive substrate pairs ($[^{14}\text{C}]\text{Ac}_2\text{-L-Lys-D-Ala-D-Ala} + \text{Gly-Gly}$ or $\text{Ac}_2\text{-L-Lys-D-Ala-D-Ala} + [^{14}\text{C}]\text{Gly-Gly}$) as described in [2]. Hydrolysis (reaction product: D-Ala + Ac₂-L-Lys-D-Ala) was negligible. The *Streptomyces* K15 DD-transpeptidase purified to protein homogeneity from the mycelium CTAB extract [3] had a specific activity of 360 units/mg. It served as a reference.

Screening of *Streptomyces* K15 DD-peptidase-producing clones: immunological test

Rabbit anti-(purified *Streptomyces* K15 enzyme) antiserum was prepared by Gamma S.A. (Parc Industriel de Recherches, Sart Tilman, Belgium) and used to screen *Streptomyces* clones. The protocol described below was based on a procedure developed to detect the intracellular β -galactosidase in *Escherichia coli* [11,12]. *Streptomyces* clones (25 clones/plate; each plate in triplicate) were grown for 48 h on AG plates. With each strain, one plate was kept untreated. Another plate was covered with a nitrocellulose (Millipore HATF) filter, itself overlaid by a 3MM Whatman paper soaked in 25 mM-Tris/HCl, pH 8. The third plate was covered with a nitrocellulose filter itself overlaid by a 3MM Whatman paper soaked in buffer containing 2% (w/v) SDS. The plates were incubated at 37 °C for 30 min, overlaid with a fresh 3MM Whatman paper soaked (depending on the case) in buffer or in SDS-containing buffer and reincubated at 37 °C for 30 min. This operation was repeated two more times. The four Whatman papers were then removed from the plates and the nitrocellulose filters submitted to the immunological test using the Bio-Rad Immuno Blot Alkaline Phosphatase Assay systems.

Protein estimation, identification of *Streptomyces*-K15-enzyme-immunologically-related proteins and amino acid sequencing

Proteins were measured by the Lowry method in the presence of SDS [13], by the Bradford [14] method and on the basis of A_{280}/A_{260} values [15]. Proteins were separated by SDS/PAGE (10% acrylamide) and electroblotted on nitrocellulose filters or on poly(vinylidene difluoride) Immobilon membrane filters by using a Bio-Rad Minitransblot cell. The nitrocellulose filters were submitted to the immunological test described above. The Immobilon filters served for automated amino acid micro-sequencing. Sequencing was performed on a 477-A pulsed liquid sequenator with on-line analysis of the amino acid phenylthiohydantoin derivatives using a 120-A analyser (Applied Biosystems, Foster City, CA, U.S.A.).

Gel fractionation, membrane preparation, mycelium CTAB extraction, PBPs analysis, enzyme thermostability and sensitivity to benzylpenicillin

The procedures were those described in [2,3,16].

Substrates, antibiotics and enzymes

Ac₂-L-Lys-D-Ala-D-Ala was a gift from Reanal (Budapest, Hungary), $[^{14}\text{C}]\text{Ac}_2\text{-L-Lys-D-Ala-D-Ala}$ was prepared as described in [2], Gly-Gly was from Sigma (St. Louis, MO, U.S.A.), $[^{14}\text{C}]\text{Gly-Gly}$ ($1.9 \text{ mCi} \cdot \text{mmol}^{-1}$) was from Amersham

International, Amersham, Bucks., U.K., $[^{35}\text{S}]\text{benzylpenicillin}$ ($500 \text{ mCi} \cdot \text{mmol}^{-1}$) was from New England Nuclear (du Pont de Nemours, Brussels, Belgium), thiostrepton was a gift from Dr. S. L. Lucania (Squibb and Sons, New Brunswick, NJ, U.S.A.); enzymes for recombinant DNA techniques were from Boehringer (Mannheim, Germany), Bethesda Research Laboratories (Gaithersburg, MD, U.S.A.) and Amersham International.

Production and purification of the exocellular *Streptomyces* K15 DD-transpeptidase

Streptomyces lividans transformed with plasmid pDML225 (see the Results section) was grown for 48 h at 28 °C with orbital shaking (240 rev./min) in 500 ml flasks containing 100 ml of TSB medium supplemented with 2 g of $(\text{NH}_4)_2\text{SO}_4$ and 50 mg of thiostrepton/litre. After two successive subcultures, 300 ml of the second subculture were used to inoculate 10 litres of the same medium contained in a Biolafitte 15-litre fermentor. The fermentation conditions were 28 °C for 48 h, with an agitator speed of 250 rev./min and an airflow of 5 litres $\cdot \text{min}^{-1}$. After centrifugation (using an Alpha-Laval LAP 202 \times dish-stack centrifuge), the supernatant was clarified by filtration through a 0.2 μm -pore-size Millipore poly(vinylidene difluoride) membrane and 10-fold-concentrated by using a Flowgen Ultrasette Tangential Flow ultrafiltration cell (10 kDa-cut-off membrane).

A 1 vol. portion of the resulting solution (1 litre) was supplemented with 1 vol. of cold (-20°C) acetone. After maintaining the mixture at -20°C for 30 min, the pellet, collected by centrifugation at 7500 g for 30 min, was dissolved in 0.1 vol. (100 ml) of 25 mM-Tris/HCl (pH 8)/0.2 mM-dithiothreitol/0.5 M-NaCl. The enzyme preparation thus obtained (13 500 enzyme units; 205 mg total protein) was dialysed against the Tris/HCl/dithiothreitol buffer without NaCl, the pellet was dissolved in the same buffer with 0.5 M-NaCl and the solution was incubated with 1 mg of RNAase for 45 min at 37 °C and then dialysed against buffer with NaCl.

RESULTS

Host strains

Attempts to overexpress the *Streptomyces* K15 DD-peptidase/PBP in *Streptomyces* strains were made using, as vector, the *Streptomyces* high-copy-number plasmid pIJ702, in which the relevant encoding gene was inserted (see below). *Streptomyces* K15, which possesses an endogenous plasmid, could not be transformed with pIJ702 under the conditions tested. Hence other prospective hosts were sought. Among the *Streptomyces* strains listed in the Materials and methods section, three did not produce detectable amounts of a DD-peptidase/PBP identical with the genuine *Streptomyces* K15 enzyme and gave high transformation rates with pIJ702.

Colonies of *S. albus* GR2 and *S. pristinae spiralis* did not react with the anti-(*Streptomyces* K15 enzyme) antiserum using the described protocol with or without SDS treatment (Figs. 1a and 1b). The isolated membranes, mycelium CTAB extracts and culture supernatants did not exhibit DD-transpeptidase activity, had no 27000- M_r PBP and lacked proteins immunologically related to the *Streptomyces* K15 enzyme (results not shown).

Colonies of *Streptomyces lividans* TK24 gave a weak response in the immunological test (Figs 1a and 1b), and the isolated membranes had a specific DD-transpeptidase activity similar to those of *Streptomyces* K15 (0.05 unit/mg of protein). But the *Streptomyces lividans* TK24 DD-transpeptidase was CTAB-sensitive and, thereby, the CTAB-treated membranes and the mycelium CTAB extract had no detectable transpeptidase activity and no 27000- M_r PBP (Fig. 2; lanes 1).

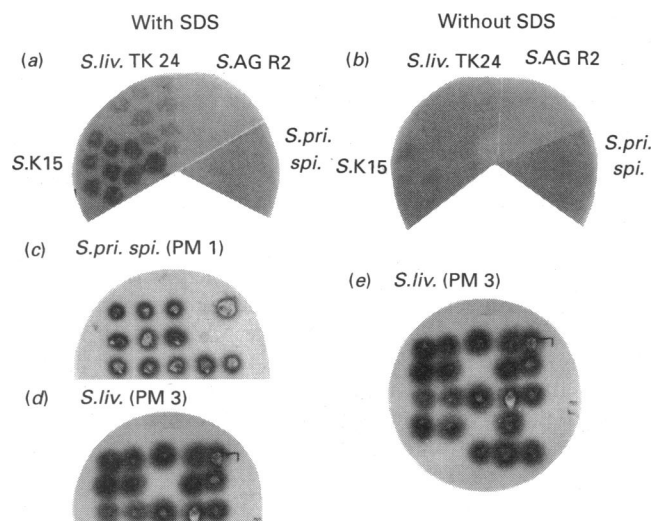


Fig. 1. Screening of *Streptomyces* colonies producing the *Streptomyces* K15 DD-peptidase or *Streptomyces* K15 DD-peptidase-related proteins: immunodetection with (a, c and d) and without (b, e) prior SDS treatment

Abbreviations: S.K15, *Streptomyces* K15; S.liv., *S. lividans*; S.AG R2, *S. albus* G R2; S.pri.spi., *S. pristinae spiralis*. *S. pristinae spiralis* PM1 and *S. lividans* PM3 (harbouring pDML225) were among the clones that gave a positive reaction.

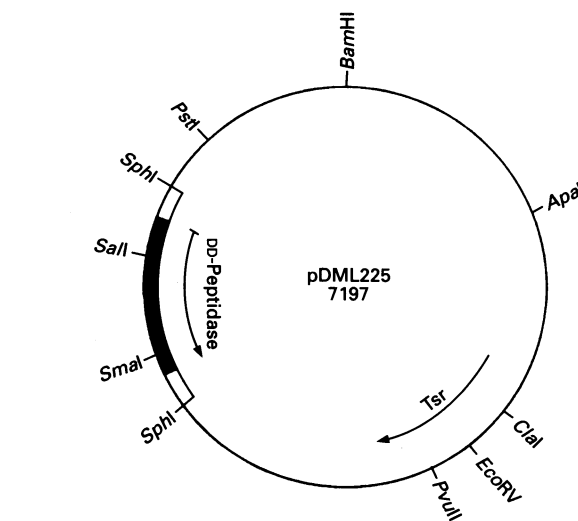


Fig. 3. Restriction map of pDML225 carrying the K15 DD-peptidase encoding gene

Abbreviation: Tsr, thiostrepton-resistance-encoding gene.

the DD-peptidase/PBP. The *SphI* DNA insert was excised from pDML223 and ligated to pIJ702 (previously cleaved with *SphI* and treated with bacterial alkaline phosphatase), and the ligation mixture was used to transform protoplasts of *S. pristinae spiralis*.

Among the 450 clones selected on R2YE agar containing 25 µg of thiostrepton/ml (transformation rate $1.8 \times 10^4/\mu\text{g}$ of DNA), 48 were tested and 37 gave rise to colonies which, after SDS treatment, reacted with the anti-(*Streptomyces* K15 enzyme) antiserum (Fig. 1c). One of them, strain PM1, was grown for 48 h in TSB medium containing 25 µg of thiostrepton/ml. The isolated plasmid was designated pDML225, and its restriction map is shown in Fig. 3. The membrane fraction and mycelium CTAB extract of strain PM1 had specific DD-transpeptidase activities of 0.08 and 5.5 units/mg of protein respectively. Though with reference to *Streptomyces* K15 there was a 6-fold enzyme enrichment in the CTAB extract, strain PM1 secreted only trace amounts of the 27000- M_r DD-transpeptidase/PBP. Moreover, the enzyme production decreased after several subcultures.

Streptomyces lividans TK24 was transformed with pDML225. Among the 28600 clones selected on thiostrepton-containing R2YE agar plates (transformation rate 6.5×10^5 transformants/µg of plasmid), 25 were tested and 20 gave a strong positive response in the immunological test (Figs. 1d and 1e). At variance with *Streptomyces* K15, colonies of pDML225-transformed *S. lividans* TK24 were surrounded, without prior SDS treatment, by a large zone that reacted with the antibodies, suggesting that an appreciable proportion of the enzyme had diffused into the agar medium. One of these clones, strain PM3, was grown for 48 h in TSB medium containing 50 mg of thiostrepton and 2 g of $(\text{NH}_4)_2\text{SO}_4/\text{litre}$. It possessed pDML225. The membrane fraction and the mycelium CTAB extract had specific enzyme activities of 0.3 and 8 units/mg of protein respectively, and, in addition, the culture supernatant contained 7 units/mg of protein. The three fractions possessed the expected 27000- M_r PBP, and this protein reacted with the anti-(*Streptomyces* K15 enzyme) antiserum (Fig. 2, lanes 2). Other immunologically related proteins were also secreted, but they lacked penicillin-binding capacity.

S. lividans PM3 grown under the aforementioned conditions produced, per litre of culture, about 4700 enzyme units (i.e. 13 mg of enzyme), of which 70% was bound to the mycelium

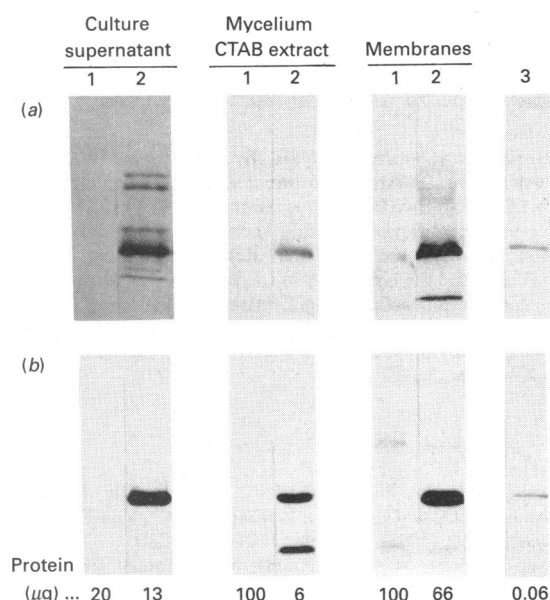


Fig. 2. SDS/PAGE of culture supernatants, mycelium CTAB extracts and plasma membranes of *S. lividans* TK24 (lanes 1) and *S. lividans* PM3 (lanes 2): immunodetection (a) and fluorography (b)

In (b) the fractions were treated with 0.1 mM-[^{14}C]benzylpenicillin for 10 min at 37 °C before SDS/PAGE. Lane 3, genuine *Streptomyces* K15 DD-peptidase used as control. The amounts of protein are indicated; they were estimated using the procedures described in [14] for the culture supernatants, in [15] for the mycelium CTAB extracts and in [13] for the membranes.

Expression of the *Streptomyces* K15 DD-transpeptidase/PBP in *S. pristinae spiralis* and *S. lividans* TK24

The 5720 bp pDML223 plasmid described previously [1] is pBR322 with a 1357 bp *SphI* insert originating from the genomic DNA of *Streptomyces* K15 and carrying the gene that codes for

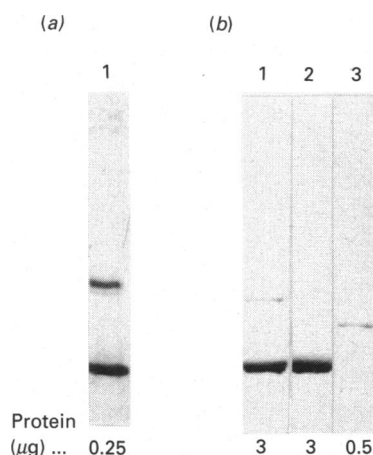


Fig. 4. SDS/PAGE of the overexpressed and purified water-soluble *Streptomyces* K15 DD-peptidase: immunodetection (a) and Coomassie Blue staining (b)

The enzyme was not treated (lanes 1) or was treated (lane 2) with guanidinium chloride before SDS/PAGE. In lane 3 in (b) the 349-amino-acid-residue *Streptomyces* R61 PBP was used as a control [17]. The amounts of protein, estimated as described in [14], are indicated.

and 30 % (i.e. 1350 enzyme units or 4 mg of enzyme) was secreted into the medium.

Purification and properties of the *Streptomyces* K15 DD-transpeptidase/PBP secreted by *Streptomyces lividans* PM3

The overproduced soluble DD-transpeptidase was purified as described in the Materials and methods section by a two-step procedure. Acetone precipitation gave a yield of 100 % enzyme recovery and a 10-fold increased enzyme specific activity (66 units/mg of protein). Dialysis against the Tris/HCl/dithiothreitol buffer without NaCl gave a yield of 75 % enzyme recovery. The estimated purity of the preparation was over 90 %. The specific enzyme activity, 340 units/mg of protein, was comparable with that of the enzyme purified from the mycelium of *Streptomyces* K15 [3]. Filtration on Sephadex G-75 in buffer with NaCl yielded one protein fraction with a K_D value corresponding to an M_r of 27000.

SDS/PAGE of the purified enzyme revealed the presence of a small amount of a 52 kDa PBP that reacted with the anti-*Streptomyces* K15 enzyme) antiserum (Fig. 4). The 27 kDa and 52 kDa proteins had the same VTKPT *N*-terminal end (as shown by transfer from the gel to an Immobilon membrane and amino acid sequencing). Moreover, the 52 kDa protein band completely disappeared when, before SDS/PAGE, the enzyme was treated with 7 M-guanidinium chloride in 0.1 M-Tris/HCl, pH 8.5, at 100 °C for 3 min and dialysed against 8 M-urea and then 0.1 % SDS. Hence, in all likelihood, the 52 kDa protein detected by SDS/PAGE under standard conditions was due to incomplete denaturation of the enzyme.

The purified water-soluble enzyme, whose properties were not affected by addition of the detergent, and the *Streptomyces* K15 enzyme, which had been purified from the mycelium after CTAB extraction, were indistinguishable with respect to thermostability, transpeptidase activity and penicillin-binding capacity [2,3,16]. Both preparations retained full activity after 10 min at 50 °C and 70 % after 10 min at 60 °C. They bound benzylpenicillin in a 1:1 molar ratio, and were inactivated by 50 % at the same ($\approx 5 \times 10^{-6}$ M) penicillin concentration. They utilized *m*-diaminopimelic acid (*m*-A₂pm) as acceptor of the transfer reaction with somewhat less efficacy than Gly-Gly. By using the system 5 mM-

[¹⁴C]Ac₂-L-Lys-D-Ala-D-Ala/1.5 mM-*m*-A₂pm donor-acceptor system, 90 % of the consumed tripeptide was channelled to transpeptidation (reaction product: [¹⁴C]Ac₂-L-Lys-D-Ala-*m*-A₂pm) and 10 % to hydrolysis (reaction product [¹⁴C]Ac₂-L-Lys-D-Ala).

DISCUSSION

Membrane-bound proteins are known which do not possess transmembrane sequences or membrane anchors [18]. They can exist in aqueous solutions as well as in membranes. In all likelihood this situation applies to the *Streptomyces* K15 DD-peptidase/PBP (see the Introduction). Overexpression probably causes saturation of the membrane and, thereby, results in the export of the enzyme synthesized in excess. Similar observations have been made with the membrane-bound DD-peptidase/PBP4 of *Escherichia coli*. When overexpressed, 80 % of the synthesized protein is secreted in the periplasm [19].

Overexpression of the *Streptomyces* K15 DD-peptidase/PBP in *S. lividans* implies the involvement of an effective promoter. This promoter may be part of the 1357 bp fragment which has been excised from the genomic DNA of *Streptomyces* K15 or it may be the *mel* promoter which, in the recombinant plasmid pDML225 (derived from pIJ702), is located upstream from the site at which the 1357 bp DNA fragment has been inserted. Whatever the case, 30 % of the overexpressed protein, i.e. 4 mg/litre of culture, is produced extracellularly. The soluble enzyme can be purified close to protein homogeneity by a two-step procedure and with a yield of 75 %. It requires 0.5 M-NaCl to remain soluble. It is indistinguishable from the membrane-bound enzyme with respect to molecular mass, thermostability, affinity to benzylpenicillin and efficacy to perform transpeptidation reactions.

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