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Primary and predicted secondary structures of the *Actinomadura* R39 extracellular DD-peptidase, a penicillin-binding protein (PBP) related to the *Escherichia coli* PBP4

Benoît GRANIER,* Colette DUEZ,* Sophie LEPAGE,* Serge ENGLEBERT,* Jean DUSART,*‡
Otto DIDEBERG,* Jozef VAN BEEUMEN,† Jean-Marie FRÈRE* and Jean-Marie GHUYSEN*

*Centre d'Ingénierie des Protéines, Université de Liège, Institut de Chimie, B6, B-4000 Sart Tilman (Liège 1), and †Laboratorium voor Microbiologie en Microbiële Genetica, Rijksuniversiteit-Gent, K.L. Ledeganckstraat 35, B-9000 Gent, Belgium

As derived from gene cloning and sequencing, the 489-amino-acid DD-peptidase/penicillin-binding protein (PBP) produced by *Actinomadura* R39 has a primary structure very similar to that of the *Escherichia coli* PBP4 [Mottl, Terpstra & Keck (1991) FEMS Microbiol. Lett. **78**, 213–220]. Hydrophobic-cluster analysis of the two proteins shows that, providing that a large 174-amino-acid stretch is excluded from the analysis, the bulk of the two polypeptide chains possesses homologues of the active-site motifs and secondary structures found in the class A β-lactamase of *Streptomyces albus* G of known three-dimensional structure. The 174-amino-acid insert occurs at equivalent places in the two PBPs, between helices α2 and α3, away from the active site. Such an insert is unique among the penicilloyl serine transferases. It is proposed that the *Actinomadura* R39 PBP and *E. coli* PBP4 form a special class, class C, of low-M_r PBPs/DD-peptidases. A vector has been constructed and introduced by electrotransformation in the original *Actinomadura* R39 strain, allowing high-level expression and secretion of the DD-peptidase/PBP (250 mg·l^{-1}). The gene encoding the desired protein is processed differently in *Actinomadura* R39 and *Streptomyces lividans*. Incorrect processing in *Streptomyces lividans* leads to a secreted protein which is inert in terms of DD-peptidase activity and penicillin-binding capacity.

INTRODUCTION

The β-lactamases, the low-M_r penicillin-binding proteins (PBPs)/DD-peptidases and the penicillin-binding domains of the bifunctional high-M_r PBPs are believed to form a superfamily of evolutionarily related penicilloyl serine transferases [1]. However, only a few β-lactamases of class A, one β-lactamase of class C and the *Streptomyces* R61 low-M_r PBP/DD-peptidase of class B are of known three-dimensional structures [1]. Hence the case of a divergent evolution is mainly supported by predictional studies.

Hydrophobic-cluster analysis [2,3] is a powerful method for analysing proteins that are weakly related in the primary structure. By using this method, it has been shown that, among the low-M_r PBPs/DD-peptidases, the *Streptomyces* K15 PBP, the *Escherichia coli* PBP5 and the *Bacillus subtilis* PBP5 of class A have similarity in the polypeptide folding with the class A β-lactamases [4]. Recently, Mottl *et al.* [5] reported that the *E. coli* PBP4 has a type of primary structure which is unique among the penicillin-interactive proteins. Gene cloning and sequencing has shown that the secretory PBP produced by a very taxonomically distant species, *Actinomadura* R39, has a primary structure which is extremely similar to that of PBP4. It is proposed that these two proteins form a distinct class, class C, of low-M_r PBPs/DD-peptidases.

MATERIALS AND METHODS

Bacterial strains, plasmids and media

Actinomadura R39 (from this laboratory) was grown at 28 °C with vigorous orbital shaking in tryptone soya broth (Oxoid). Plasmids pBR322 and pBR325 were used for cloning experiments in *Escherichia coli* HB101. Growth was at 37 °C in Luria–Bertani medium. *Streptomyces lividans* TK24 and plasmids pIJ486 and pIJ702 were from the John Innes Institute, Norwich, U.K.; *S. lividans* was grown in MYEME medium [6] and recombinant *Actinomadura* R39 was grown in TAU medium [7]. The R2YE medium [8] was also used.

Recombinant DNA techniques, radioactive oligonucleotide probe and nucleotide sequence

The *Actinomadura* chromosomal DNA was prepared as described in [8], and the recombinant DNA techniques were performed essentially as described in [9].

The sequence of the 58-amino-acid *N*-terminal region of the *Actinomadura* R39 PBP was determined on 3.2 nmol of protein (purified as described in [10]), using a 477-A pulsed liquid sequenator with on-line analysis of the amino acid phenylthiohydantoin derivatives and a 120-A analyser (Applied Biosystems, Forster City, CA, U.S.A.). On the basis of these data and the known Actinomycetes codon usage, and by using the PCR procedure [11], a 109 bp DNA segment was prepared and, from this, the non-degenerated 27-mer probe 3′-CGG-CAC-AGC-CCC-CAC-CAG-CAG-CAC-CTG-5′ was synthesized. This probe was complementary to the nucleotide sequence encoding the nonapeptide A^{21}VSGVVVVD^{29} of the PBP. The probe was labelled with [γ-^{32}P]ATP and served to screen gene libraries by hybridization [12,13].

DNA segments cloned into M13 vectors were sequenced by the dideoxynucleotide-chain-termination method [14]. Zones of base compression due to high GC content were resolved using dITP instead of dGTP (Sequenase kit; USB, Cleveland, OH, U.S.A.). Codon usage was analysed with Staden's program [15] and Fickett's test [16] using the *Actinomadura* R39 β-lactamase [17] as reference.

Immunological screening

Rabbit anti-(*Actinomadura* R39 DD-peptidase/PBP) antiserum was prepared by Gamma S.A. (Tavier, Belgium) and was

Abbreviations used: PBP, penicillin-binding protein; ORF, open reading frame.

‡ To whom correspondence should be addressed.

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases.

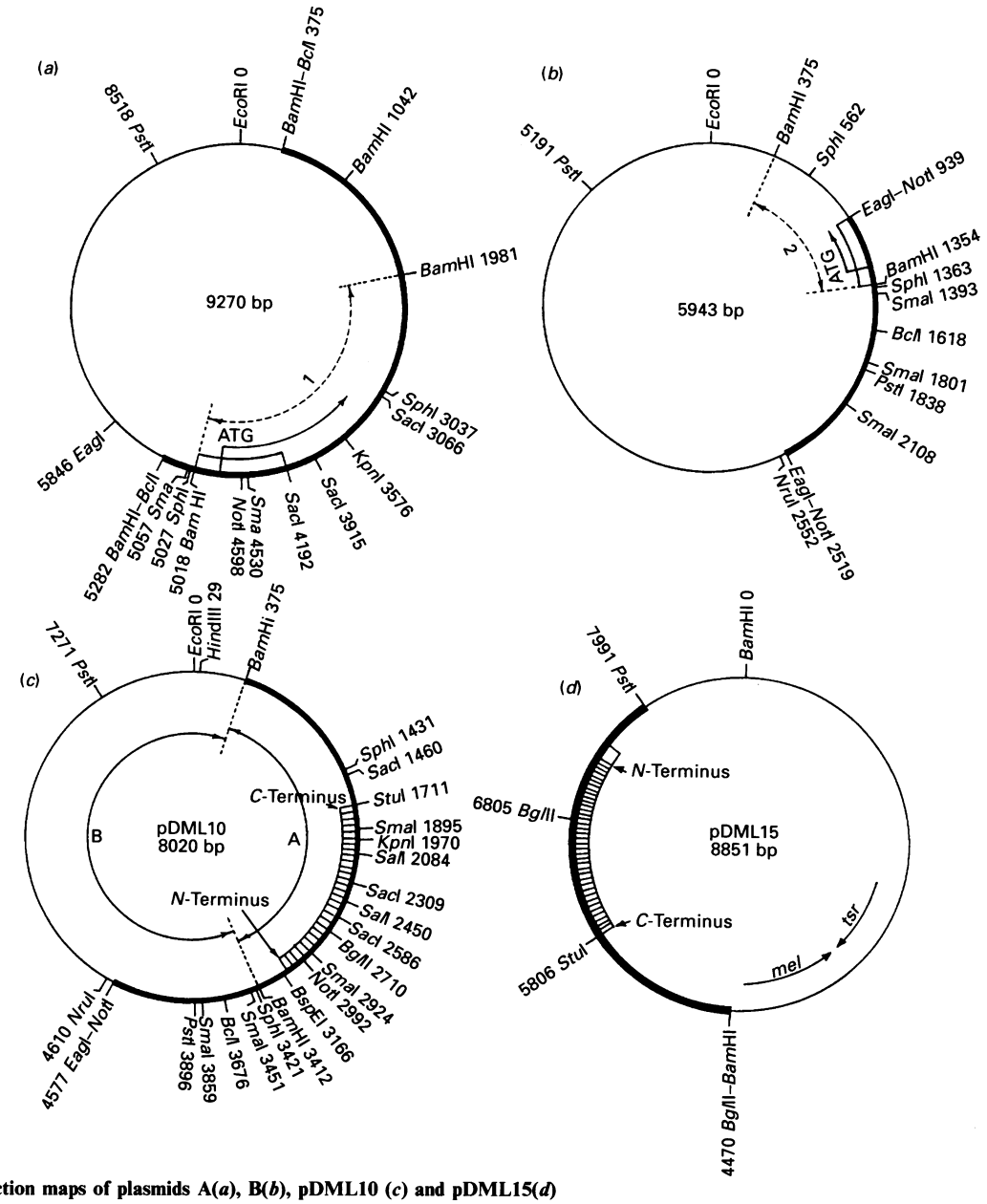

Fig. 1. Restriction maps of plasmids A(*a*), B(*b*), pDML10 (*c*) and pDML15(*d*)

Inserts are shown in heavy lines. The open boxes in plasmids A and B indicate the hybridizing regions. The position of the initiation codon ATG and the orientation of the ORF in plasmids A and B are indicated. The hatched line in pDML10 and pDML15 specifies the *Actinomadura* R39 DD-peptidase/PBP-encoding gene. Abbreviations: *mel*, tyrosinase; *tsr*, thiostrepton resistance.

used to screen PBP-secretory clones. Bio-Rad Immuno Blot Alkaline Phosphatase Assay Systems were employed.

DD-Peptidase activity

Measurement of the DD-carboxypeptidase activity was performed on Acetyl$_2$-L-Lys-D-Ala-D-Ala (release of the *C*-terminal D-Ala) as described in [18].

Electrotransformation

Cells of *Actinomadura* R39 [a 48 h culture made in 25 ml of tryptone soya broth containing 0.5% (w/v) glycine] were dispersed by gentle sonication on ice (5 bursts of 30 s each; 20 kHz), washed by centrifugation (4 °C; 4000 *g*; 15 min) first with cold water (three times) and then with a 10% (v/v) glycerol/water solution (once) and resuspended in 1 ml of glycerol/water. The cell suspension (100 μl) and the plasmid (50 ng; see the Results section) were mixed in a 1.5 ml polypropylene tube. The mixture was successively transferred to a 0.2 cm electroporation cuvette, pulsed once (Celljet, Eurogentec S.A., Liège, Belgium; 40 μF; 2.5 kV; 192 Ω), supplemented with 1 ml of SOC medium [19] and homogenized with a Pasteur pipette (all these operations were carried out at 4 °C). The suspension was shaken at 250 rev./min for 1 h at 37 °C in a 17 mm × 100 mm polypropylene tube. The cells were plated on TAU agar medium and, after 10 h at 28 °C, overlaid with 3 ml of soft agar containing 50 μg of thiostrepton/ml.

Hydrophobic-cluster analysis

This procedure [2,3] rests upon a representation of the amino acid sequences on an α-helical two-dimensional pattern in which

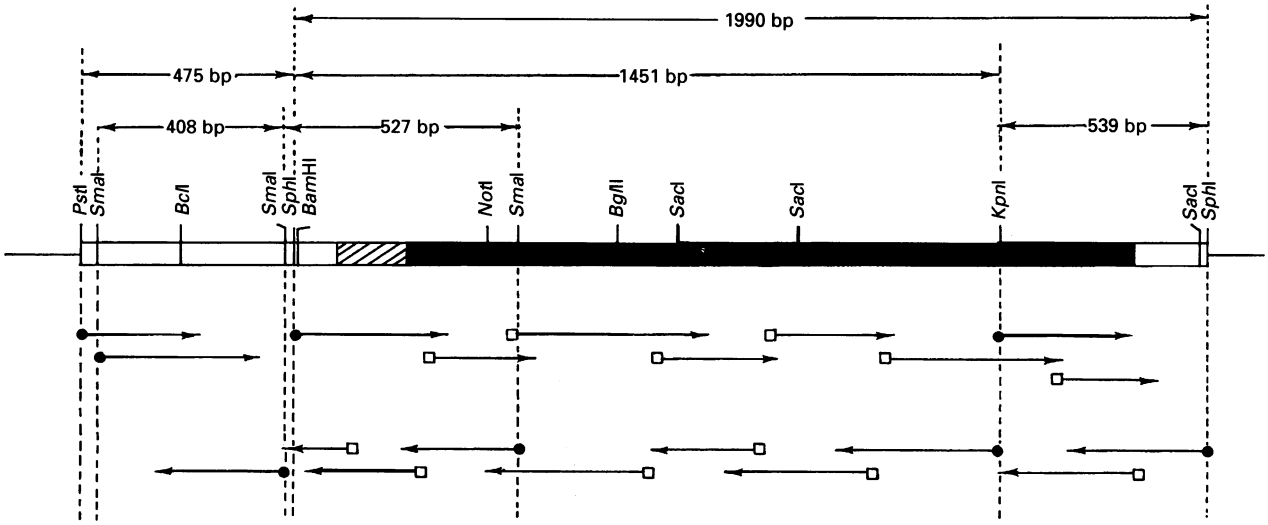

Fig. 2. Strategy of nucleotide sequencing

The phage vectors M13mp18 tg 130 and tg 131 were used to clone the 1990 bp *Sph*I, the 1926-bp *Pst*I–*Kpn*I and the 539 bp *Sph*I–*Kpn*I subfragments. The phage vector M13mp10 (cut with *Sam*I and dephosphorylated) was used to clone the 408 bp and 527 bp *Sma*I subfragments. Nucleotide sequences initiated with the M13 universal primer are marked by '●'. Those initiated with the DNA probe are marked '□'. The arrows indicate the orientation and length of the sequenced segments.

the hydrophobic residues tend to form clusters that usually correspond to the secondary structure elements. Clusters of similar shapes, sizes and relative positions express similarity in the polypeptide folding of the proteins. When compared with methods based only on single-amino-acid property/identity (Goad & Kanehisa [20]; BESTFIT [21]), the hydrophobic-cluster analysis allows distant information to become more visible and allows deletions or insertions to be introduced more easily between the secondary structures.

RESULTS

Gene cloning

The genomic DNA of *Actinomadura* R39 was cleaved with *Bam*H1, *Bcl*I, *Bgl*II, *Not*I, *Sal*I, *Sph*I and *Nco*I, and the DNA fragments were cloned in pBR322 or pBR325 (for the *Nco*I library). Among the 4500 ampicillin-resistant *E. coli* transformants obtained, one clone A from the *Bcl*I library and one clone B from the *Not*I library gave a strong signal with the radioactive probe after washing the filters at 70 °C ($T_m - 6$ °C). The restriction maps are shown in Figs. 1(*a*) and 1(*b*). The DNA segments responsible for the hybridization signal occurred at the extremity of each of the inserts (open boxes in the Figures). The 500 bp *Sma*I subfragment from plasmid A and the 800 bp *Sph*I subfragment from plasmid B were prepared, cloned into M13 and sequenced by using the M13 universal primer. Both inserts encoded the *N*-terminal region of the *Actinomadura* R39 PBP.

In order to allow the orientation of the gene, the 1.45 kb *Sph*I–*Kpn*I subfragment from plasmid A and the 1 kb *Sph*I–*Bam*HI subfragment from plasmid B were prepared and cloned into M13. Nucleotide sequencing, initiated with the M13 universal primer from the *Sph*I site, revealed that plasmid A contained the complete PBP-encoding gene (with 4500 bp downstream of the initiation codon ATG), but probably not the complete promoter (with only 370 bp upstream of ATG). Plasmid B contained only part of the PBP-encoding gene (with 300 bp downstream of ATG), but a large 1.25 kb segment upstream of ATG. Consequently, the *Bam*HI 375–*Bam*HI 1354 DNA segment was excised from plasmid B and replaced by the *Bam*HI 1981–*Bam*H1 5018 DNA segment of plasmid A, yielding pDML10 (Fig. 1*c*), where the structural gene is very probably preceded by its own promoter.

Gene sequencing and primary structure

Establishment of the nucleotide sequence of the *Actinomadura* R39 PBP-encoding gene (using the strategy shown in Fig. 2) revealed a 1614-nucleotide open reading frame (ORF) (Fig. 3). The ORF started with an ATG codon, presented the biased pattern of codon usage typical of Actinomycetes genes and terminated with an Amber codon TGA. This ORF translated into a 538-amino-acid protein precursor whose 49-amino-acid *N*-terminal region had the features of a long signal peptide. It contained one lysine residue at position −48, six arginine residues at positions −40, −38, −37, −33, −29 and −28, and a long hydrophobic stretch from Ala-27 to Ala-6. The amino acid sequence of the protein from Arg-1 to Ser-38 was that of the *N*-terminal region of the mature *Actinomadura* R39 DD-peptidase/PBP.

Expression of the cloned gene in *S. lividans* and *Actinomadura* R39

The *Bam*HI 375–*Pst*I 3896 DNA segment, containing the complete structural gene and the 590 bp upstream region was excised from pDML10 and ligated to the *Streptomyces* high-copy-number plasmid pIJ702 (previously cleaved with *Bgl*II and *Pst*I and treated with bacterial alkaline phosphatase). The resulting plasmid pDML15 (Fig. 1*d*) was used to transform *S. lividans* TK24 protoplasts. Among the transformants (selected on R2YE agar plates containing 6 μg of thiostrepton/ml), *S. lividans* BG2 was the best producer of the expected protein, as evidenced by the immunological test. However, when *S. lividans* BG2 was cultivated in MYEME liquid medium, the secreted protein, though reacting with the anti-(*Actinomadura* R39 DD-peptidase/PBP) antiserum, lacked both DD-peptidase activity and penicillin-binding capacity. Moreover, it migrated on SDS/PAGE with an apparent molecular mass about 3 kDa larger than that of the original DD-peptidase/PBP.

In spite of these abnormalities, pDML15 was re-isolated from *S. lividans* BG2 and introduced in the *Actinomadura* R39 strain

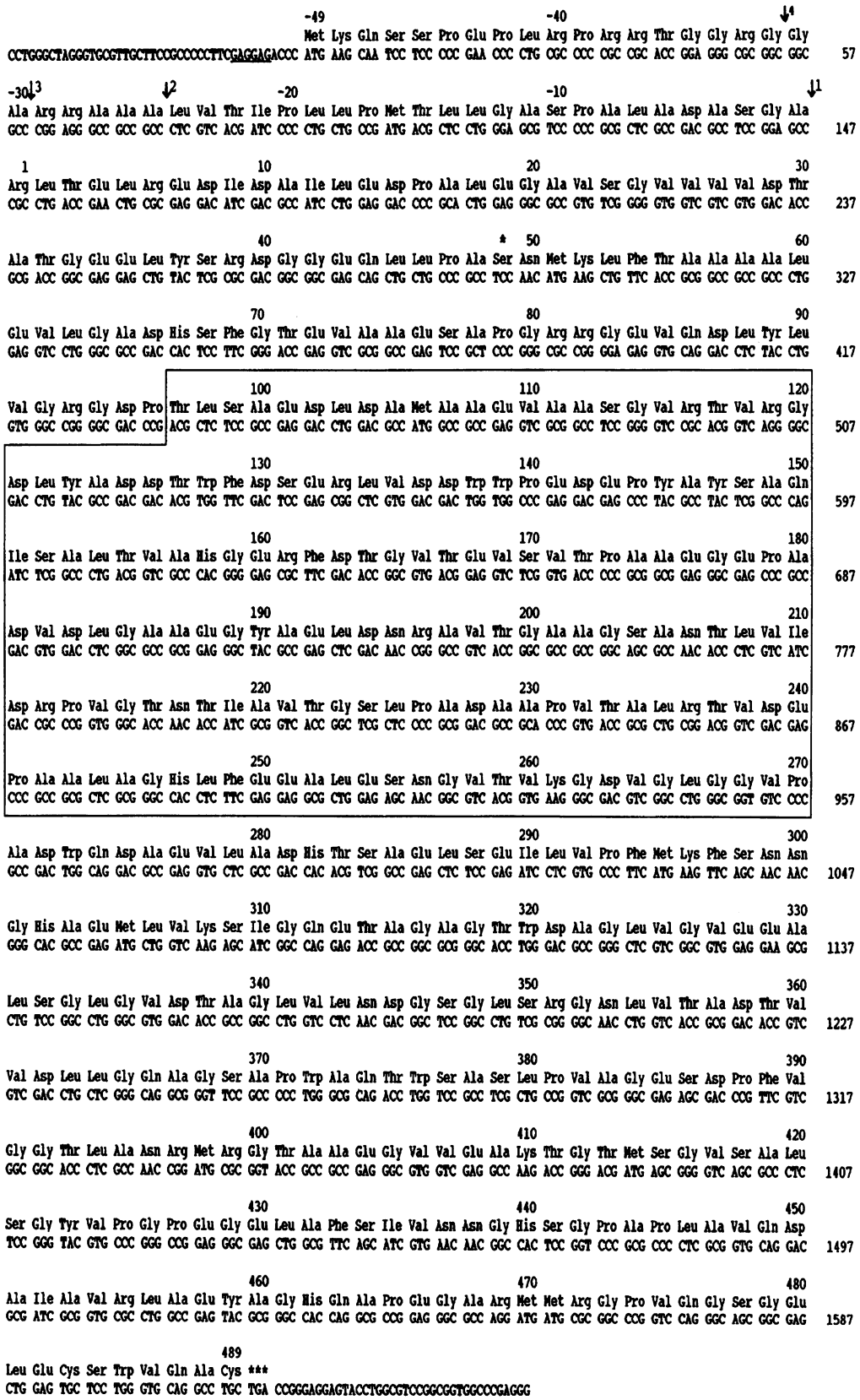

Fig. 3. Nucleotide sequence of the gene encoding the *Actinomadura* R39 PBP precursor and deduced amino acid sequence

The site of cleavage by the leader peptidase is indicated by the vertical arrow 1. Other potential cleavage sites 2, 3 and 4 are also indicated (see the text). Ser*, active-site serine. The T^{97}–P^{270} insert (see the text) is boxed. The putative ribosome-binding site GAGGAG is underlined.

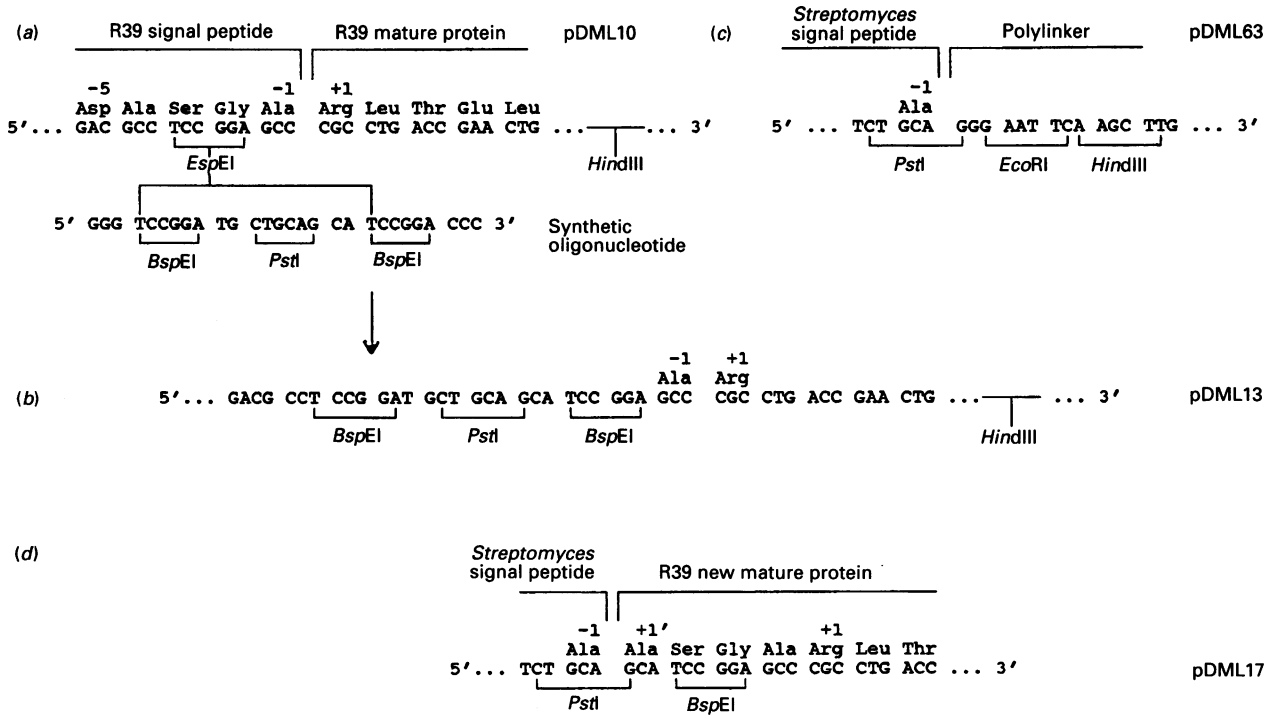

Fig. 4. Introduction of the DNA sequence encoding the mature *Actinomadura* R39 DD-peptidase/PBP in the *Streptomyces* secretion vector pDML 63: construction of pDML17

(*a*) A perfect palindromic oligonucleotide was synthesized and allowed to self-hybridize, giving a DNA segment with a *Pst*I site flanked on each side with a *Bsp*EI site. The hybrid was digested with *Bsp*EI and inserted in the unique *Bsp*EI 3166 site of pDML10 (see Fig. 1*c*), yielding pDML13 (*b*), in which the natural signal peptidase cleavage site (Ala^{-1}–Ala^{+1}) of the *Actinomadura* R39 DD-peptidase/PBP is thus preceded by a 17-nucleotide sequence containing a *Pst*I and a *Bsp*EI site separated by a dinucleotide CA. Digestion of pDML13 with *Pst*I and *Hin*dIII liberated a 3100-bp segment that contained the region encoding the mature part of the PBP-encoding gene downstream of a 12-nucleotide sequence coding for Ala, Ser, Gly and Ala. This 3100-bp segment was introduced in the polylinker of pDML63 (*c*), a *Streptomyces* high-copy-number secretion vector (see the text), giving rise to pDML17 (*d*) in which the *Streptomyces* signal sequence is in phase with the gene encoding the *Actinomadura* R39 mature protein (with a four-amino-acid *N*-terminal extension).

by electrotransformation, yielding *Actinomadura* BG3 (note that transformation of the *Actinomadura* strain by the usual PEG-assisted procedure failed and that electrotransformation gave only one transformant in several assays). Under optimal growth conditions in TAU medium, *Actinomadura* BG3 produced 250 mg of active DD-peptidase/PBP/litre of culture, instead of 15 mg/litre for the original strain (grown under identical conditions). The secreted protein had the 'correct' molecular mass.

A likely hypothesis derived from the above studies was that, in *S. lividans*, incorrect processing of the DD-peptidase/PBP precursor occurred, producing an inactive protein with an extended *N*-terminal region. Examination of the ORF shows that the signal peptide possesses, upstream of the site cleaved in *Actinomadura* R39 (marked 1 in Fig. 3), other potential cleavage sites (marked 2, 3 and 4) [22]. In particular, site 2 is identical with the *S. lividans* β-galactosidase signal-peptide-cleavage site [23]. Cleavage of the DD-peptidase precursor at this site would generate a protein with a molecular mass of 52339 (instead of 50053 for the protein processed at site 1).

In order to test the hypothesis, the mature-protein-encoding DNA was introduced into the *Streptomyces* high-copy-number secretion vector pDML63 (a derivative of pIJ702; A. Brans, M. V. Lenzini, C. Fraipont-Piron & J. Dusart, unpublished work). This vector possesses *Streptomyces* transcription, translation and secretion signals, followed by a polylinker (*Pst*I, *Eco*RI, *Hin*dIII, *Sma*I, *Xba*I) whose *Pst*I site is in phase with the translation signal. Therefore a *Pst*I site was introduced in the unique *Bsp*EI site of the cloned gene, close to the junction between cleavage site 1 and the mature-protein-encoding DNA (Fig. 1*c*), the strategy shown in Fig. 4 being followed. The final construction was called pDML17. *S. lividans* transformed with pDML17 and grown in MYEME medium secreted at least 18 mg of the active DD-peptidase/PBP per litre.

Hydrophobic-cluster analysis: similarity to the *E. coli* PBP4 and structural relatedness with *Streptomyces albus* G β-lactamase of class A

Both the Goad & Kanehisa algorithm [20] and BESTFIT program revealed high similarity, in the primary structure, between the *Actinomadura* R39 PBP and the *E. coli* PBP4 (results not shown). By using the same procedure, no, or only marginal, similarity was observed with the other groups and classes of penicilloyl serine transferases. Hydrophobic-cluster analysis (Fig. 5) confirmed that the *Actinomadura* R39 PBP and the *E. coli* PBP4 were indeed remarkably similar. Providing that a large deletion was made in the PBPs, the analysis also revealed similarity, in the polypeptide folding, between the two PBPs and the class A *Streptomyces albus* G β-lactamase of known three-dimensional structure [24,25]. Fig. 6 shows the amino acid alignments as derived from this analysis.

DISCUSSION

As a first attempt to obtain the desired *Actinomadura* R39 gene, a *S. lividans* TK24-pIJ702 cloning system and an immunological screening test were used (B. Granier, unpublished work). Clones were isolated which produced a protein that effectively reacted with the anti-(*Actinomadura* R39 DD-peptidase/PBP)

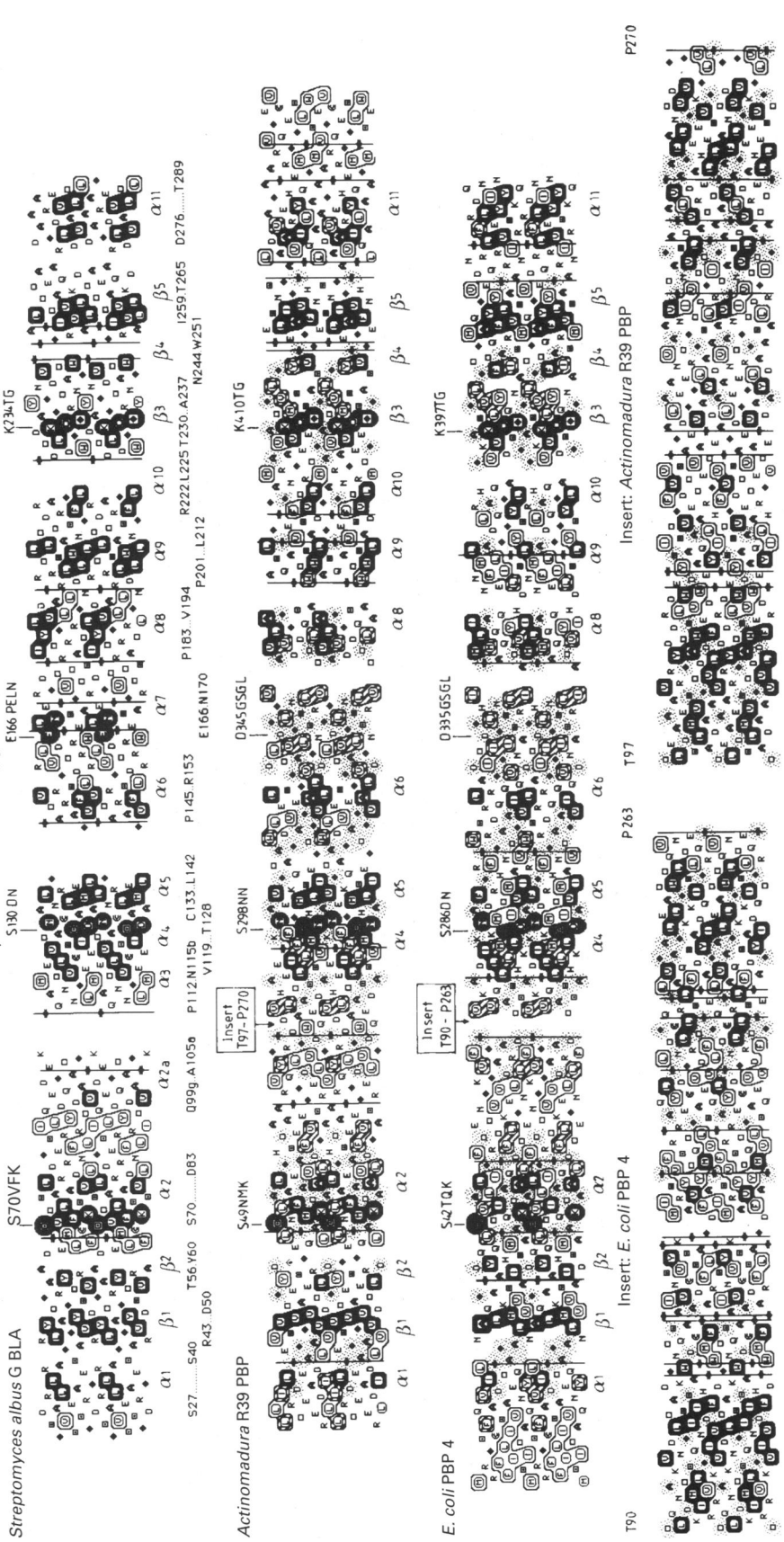

Fig. 5. Hydrophobic-cluster analysis of the amino acid sequences of the *Actinomadura* R39 PBP, the *E. coli* PBP4 and the *Streptomyces albus* G β-lactamase (BLA) of class A used as reference

The one-letter code is used except for Pro (*), Gly (◆), Cys (Ⓒ), Ser (□) and Thr (□). Hydrophobic residues are encircled and hydrophobic clusters are also delineated. The hydrophobic residues and clusters that belong to the secondary structures of the *Streptomyces albus* G β-lactamase (as indicated) are written in full above the sequence. Those that occur at equivalent places along the amino acid sequences of the two PBPs are also in bold. The hydrophobic residues and clusters indicated by black transverse bars and identical hydrophilic residues marked by scattered points are common only to the two PBPs. The ABL amino acid numbering is used. The four motifs that form the active site of the β-lactamase and the equivalent amino acid groupings in the two PBPs are written in full. The alignment between the three proteins requires very few deletions, providing that stretches T^{97}–P^{270} in the *Actinomadura* R39 PBP and T^{90}–P^{263} in the *E. coli* PBP are excluded from the analysis. These inserts are shown in the lower part of the Figure. The hydrophobic residues and clusters shown in bold and the identical hydrophilic residues and clusters marked by scattered points occur at identical places along the amino acid sequences of the inserts.

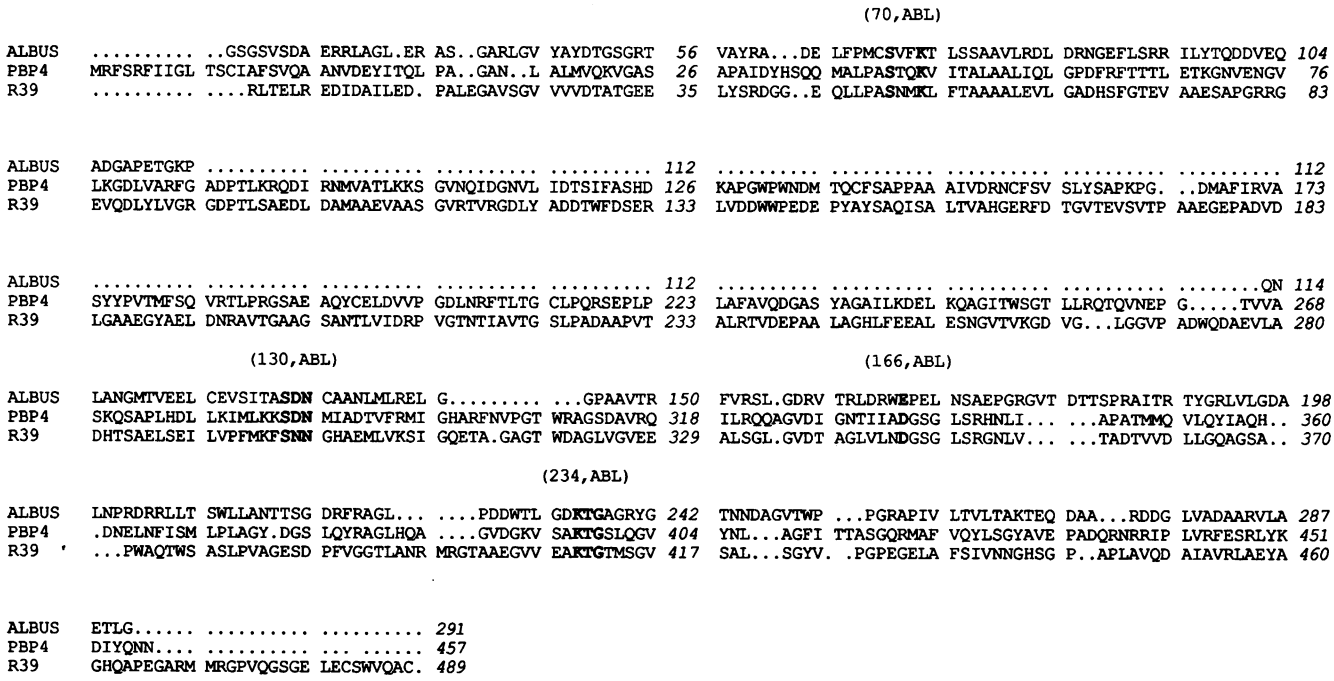

Fig. 6. Amino acid sequence alignment of the *Actinomadura* R39 PBP, the *E. coli* PBP4 and the *Streptomyces albus* G β-lactamase of class A

The proposed alignment derives from that of Fig. 5. ALBUS, *Streptomyces albus* G BLA; PBP4, *E. coli* PBP4; R39, *Actinomadura* R39 PBP. The motifs that form the β-lactamase active site are indicated (ABL numbering).

antiserum. This protein had an apparent molecular mass larger than that of the original DD-peptidase/PBP and it was inert in terms of DD-peptidase and penicillin-binding activity, suggesting that the use of a polyclonal antibody might have been misleading. Subsequently, and as described above, the gene was cloned using *E. coli* as host and a nucleotide probe as screening tool, but introduction of the gene in *S. lividans*, *via* pDML15, again resulted in the synthesis (in MYEME medium) of a protein which was devoid of enzymic activity and was also about 3 kDa larger than the original DD-peptidase/PBP. However, and at variance with this observation, *S. lividans* TK24 transformed with a secretion vector in which the signal sequence of the *Actinomadura* gene was replaced by a *Streptomyces* signal sequence, produced and secreted an active DD-peptidase/PBP of normal size. These studies thus support the view that the protein precursor is processed differently in *Actinomadura* and in *Streptomyces* and that the *N*-terminal extension resulting from the incorrect processing in *S. lividans* is sufficient to prevent correct folding of the protein.

Hydrophobic-cluster analysis of the *Actinomadura* R39 PBP and the *E. coli* PBP4 and pairwise comparison of the PBPs with the class A *Streptomyces albus* G β-lactamase (of known three-dimensional structure) leads to the following conclusions. (1) The two PBPs have, along the amino acid sequences, the same typical pattern of distribution of hydrophobic clusters and of hydrophilic residues between the conserved hydrophobic clusters. (2) The peptide stretches T^{97}–P^{270} in the *Actinomadura* PBP and T^{90}–P^{263} in the *E. coli* PBP have no equivalents in the β-lactamase. (3) When these adducts are eliminated from the amino acid sequences, the bulk of the polypeptide chain of the two PBPs exhibits similarity with that of the β-lactamase. (4) Secondary structures equivalent to the β-lactamase strands β1, β2, β3, β4 and β5 and helices α1, α2, α4, α5, α6, α8, α9, α10 and α11 are easily identified in the PBPs. (5) Identification in the PBPs of the S*XXK motif (where S* is the essential serine) on α2, the SXN motif between α4 and α5 and the KTG motif on β3 is not a matter of controversy. (6) D^{345} in the *Actinomadura* PBP and D^{335} in the *E. coli* PBP occur at a position comparable with that of E^{166} of the EPELN motif, between α6 and α8, in the β-lactamase. E^{166} functions as proton abstractor in the attack of the carbonyl carbon of the scissile amide bond of penicillin by the γ-OH of the β-lactamase active-site serine residue [25]. The homologues D^{345} and D^{335} might play a similar role in the PBPs. Note, however, that another aspartic acid, D^{337} or D^{327}, occurs immediately upstream from D^{345} and D^{335}. Site-directed-mutagenesis experiments will give answers to the question regarding the essentiality of these acidic side chains in the PBPs. (7) The inserts T^{97}–P^{270} in the *Actinomadura* R39 PBP and P^{90}–P^{263} in the *E. coli* PBP4 (whose position is slightly different from that proposed by Mottl *et al.* [5]) occur between α2 and α4, i.e. on the surface of the proteins. The structure and possible function of the inserts are unknown.

The *Actinomadura* R39 PBP is a secretory protein. The majority of the *E. coli* PBP4 is exported in the periplasm, at least in the overproducing strains [26]. By analogy to the β-lactamases, the polypeptide chain of these two water-soluble PBPs terminates immediately or almost immediately after α11. In contrast, the membrane-bound *E. coli* PBP5 and *B. subtilis* PBP5 are anchored in the membrane by a short peptide stretch located at the end of a long (> 100 amino acids) additional *C*-terminal extension [27].

The *Actinomadura* R39 PBP and the *E. coli* PBP4 are functionally homologous with respect to the reactions that they catalyse on D-alanyl-D-amino acid-terminated peptides [26–28]. The *Actinomadura* PBP, however, performs transpeptidation reactions *in vitro* when incubated in the presence of structured amino acids and peptides [28] and it is extremely susceptible to inactivation by benzylpenicillin (second-order rate constant of acylation of the essential serine: 300,000 $M^{-1} \cdot s^{-1}$ at 37 °C, as compared with about 7000 $M^{-1} \cdot s^{-1}$ for the *E. coli* PBP4) [29].

A last comment must be made. The immediate environment of the active-site serine in the *Actinomadura* R39 PBP is LPASNMK, not LPASNGV, as proposed previously [30]. It is likely that the

last two residues were impurities and the isolated peptide was in fact the pentapeptide LPASN.

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