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Dual Multimodular Class A Penicillin-Binding Proteins in *Mycobacterium leprae*

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The *ponA* gene of cosmid L222 of the *Mycobacterium leprae* genome library encodes a multimodular class A penicillin-binding protein (PBP), PBP1*. The PBP, labelled with a polyhistidine sequence, has been produced in *Escherichia coli*, extracted from the membranes with 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) and purified by Ni^{2+}-nitrilotriacetic acid-agarose chromatography. In contrast to the *pon1*-encoded class A PBP1, PBP1* undergoes denaturation at temperatures higher than 25°C, it catalyzes acyl transfer reactions on properly structured thiolesters, and it binds penicillin with high affinity.

A set of membrane-bound proteins, known as penicillin-binding proteins (PBPs), are involved in the final assembly of the bacterial cell wall peptidoglycan. *Escherichia coli* possesses four multimodular PBPs. PBP1a and PBP1b of class A are bienzymatic polypeptides performing, in vitro, transglycosylase (the non-penicillin-binding [n-PB] module) and transpeptidase (the penicillin-binding [PB] module) activities on the disaccharide peptide lipid II precursor. PBP2 and PBP3 of class B are essential members of the morphogenetic networks involved in wall expansion and septum formation, respectively (8).

Until recently, the peptidoglycan-synthesizing machinery of *Mycobacterium leprae* was inaccessible to direct biochemical investigation. As an ordered cosmid library covering the mycobacterial 2.8-Mb chromosome has been established (4), important genes are being identified, allowing the encoded proteins to be produced in heterologous systems and characterized. *pon1* of cosmid B577 encodes a class A PBP1. Expression of *pon1* in *E. coli* results in the production of *M. leprae* PBP1 bound to the plasma membrane of the host. PBP1 has the expected membrane topology, it is thermostable, and it binds penicillin with a low affinity (1).

As shown below, *M. leprae* contains another class A PBP, PBP1*, the biochemical and enzymatic properties of which differ markedly from those of PBP1.

Modular design of *M. leprae* PBP1*. *ponA* of cosmid L222 (nucleotides 7365 to 5305) encodes the 686-amino-acid residue PBP1* (7). This PBP bears the signature amino acid sequence of the multimodular class A PBPs. The nine motifs shown in Fig. 1 are the consensus derived from the alignment of 11 class A PBPs. PBP1* consists of a V1-to-L225 n-PB module fused to an R302-to-P686 PB module via a G226-to-Q301 connecting module. The n-PB module has a pseudo-signal peptide (the membrane anchor) at the amino end of the polypeptide chain, and the PB module possesses, downstream from motif 9, a 130-amino-acid residue carboxy-terminal extension. In comparison with PBP1*, *M. leprae* PBP1 has a much longer tail, *E. coli* PBP1a has an ~100-amino-acid residue insert between motifs 6 and 7, and *E. coli* PBP1b has an ~150-amino-acid residue insert upstream from motif 1.

In spite of the highly conserved molecular organization adopted by the *M. leprae* and *E. coli* class A PBPs, similarity in the amino acid sequences (after elimination of the inserts and extensions) is low or even statistically insignificant. At the level of the n-PB modules, the members of the pairs *M. leprae* PBP1*-*M. leprae* PBP1, *M. leprae* PBP1*-*E. coli* PBP1a, and *M. leprae* PBP1*-*E. coli* PBP1b have 25 to 30% identities. At the level of the PB modules, the members of the pair *M. leprae* PBP1*-*M. leprae* PBP1 have 25% identity, and the members of the pairs *M. leprae* PBP1*-*E. coli* PBP1a and *M. leprae* PBP1*-*E. coli* PBP1b have only 17% identity. For comparative purposes, the n-PB modules of *E. coli* PBP1a and *E. coli* PBP1b have 31% identity, and the corresponding PB modules have 28% identity.

Production of *M. leprae* (His tag)(R2-P686) PBP1* in *E. coli*. *M. leprae* PBP1* with its membrane anchor (lacking residue V1) was produced in *E. coli* and labelled with a polyhistidine sequence fused to residue R2. Plasmid pDML909, which encodes PBP1* with a polyhistidine tag fused to R2 residue P686 [(His tag)(R2-P686) PBP1*], was constructed (Fig. 2) as follows. (Step 1) The sense and antisense primers were used to synthesize by PCR a 167-bp DNA segment encoding the R2-to-L49 sequence of PBP1*, flanked by an *Eco*RI site fused to an *Nde*I site at the 5′ end and by an *Xcm*I site fused to an *Apa*I site at the 3′ end. (Step 2) The *ponA*-containing *Xba*I-*Eco*RV 3,800-bp segment of cosmid L222 was inserted into pUCBM20, the resulting plasmid was digested with *Eco*RI and *Xcm*I (partially), and the excised 1,159-bp fragment was replaced by the *Eco*RI-*Xcm*I PCR product. (Step 3) The latter plasmid was digested with *Nde*I and *Hin*dIII, and the excised 2,832-bp fragment carrying the R2-P686 PBP1*-encoding sequence was cloned into pET28a(+) between *Nde*I and *Hin*dIII, yielding pDML909.

The expression of the modified *ponA* in *E. coli* BL21(DE3)/pDML909 was IPTG (isopropyl-β-D-thiogalactopyranoside) inducible and under the control of the T7 promoter and *lac* operator. Transformants were grown at 37°C in Luria-Bertani medium containing 50 μg of kanamycin per ml. When an optical density at 600 nm of 0.6 was reached, various concentrations of IPTG, from 1 μM to 2 mM, were added and the

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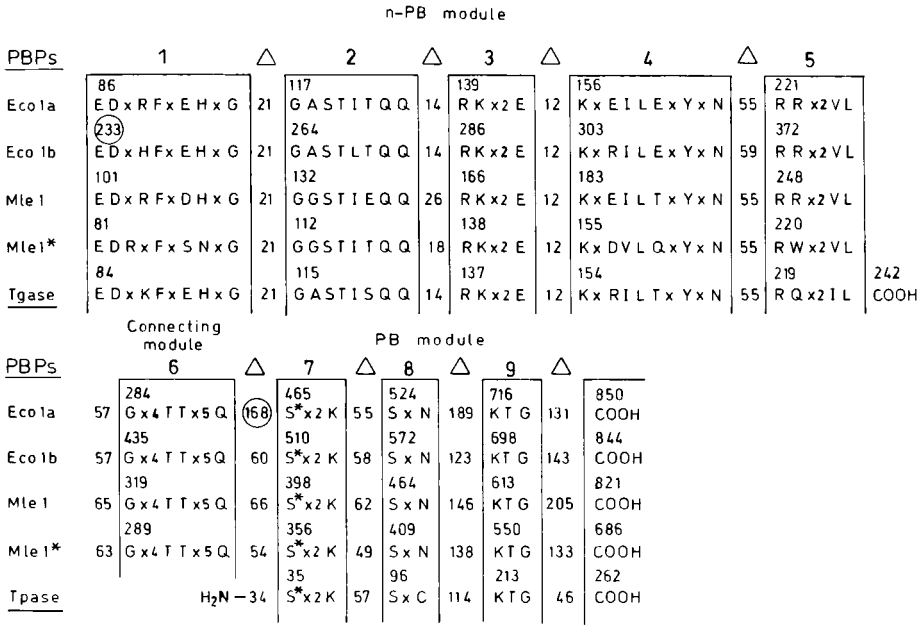

FIG. 1. Modular design of the multimodular class A PBP1a and PBP1b of *E. coli* and PBP1 and PBP1* of *M. leprae* and occurrence of the conserved motifs along the amino acid sequences. Intervals (△) between conserved motifs are expressed in numbers of amino acid residues. Circled numbers indicate the presence of inserts. *E. coli* possesses a 34,000-M_r monofunctional transglycosylase (10), and the genes whose products have the signature amino acid sequence of the class A PBPs are present in several bacterial species (15). The transglycosylase (Tgase) motifs shown are those derived from the *E. coli* gene sequence. The transpeptidase (Tpase) motifs shown are those of the *Streptomyces* strain K15 PBP (13), which has been biochemically characterized as a DD-transpeptidase (9, 12). The sequences of *E. coli* PBP1a and PBP1b (Eco1a and Eco1b, respectively) are from reference 2, and the sequences of *M. leprae* PBP1 and PBP1* (Mle1 and Mt1*, respectively) are from references 1 and 7.

cultures were allowed to grow at various temperatures, from 20 to 37°C, for 1 to 20 h. The plasma membranes of the *E. coli* transformants were prepared by transforming the cells into spheroplasts at 4°C in the presence of lysozyme and EDTA as described previously (5). The membranes suspended in 40 mM sodium phosphate, pH 7.0, were labelled with 10^{-4} M [^{3}H]benzylpenicillin (5 μCi/mmol) for 15 min at 37°C and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining and fluorography of the gels.

The result of these analyses was that the produced protein lacked PB activity when *ponA* was expressed at 37°C. The optimal conditions for the production of an active (His tag)(R2-P686) PBP1* were to grow the *E. coli* transformant at 20°C for 7 h after induction with 100 μM IPTG. Under these conditions, the only PBPs detected in membrane samples of

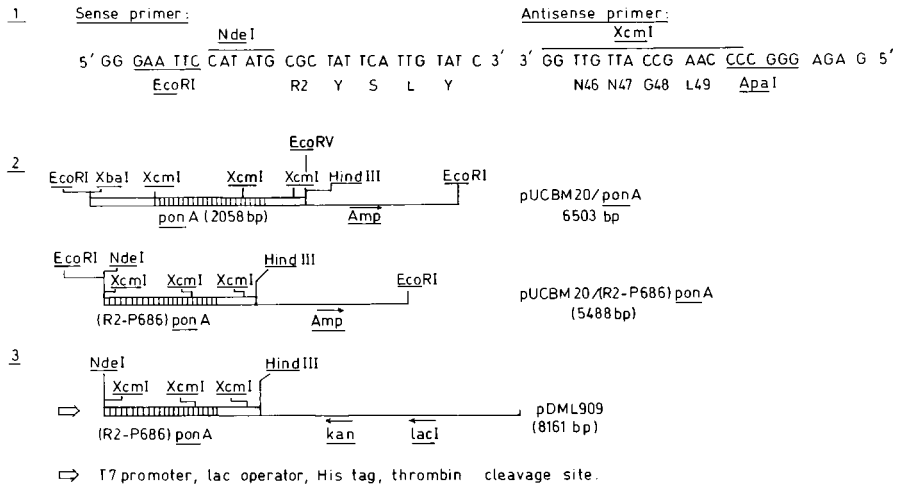

FIG. 2. Construction of pDML909, which encodes *M. leprae* (His tag)(R2-P686) PBP1*. PCR using the sense and antisense primers was carried out in 100 μl of 10 mM Tris-HCl, pH 9.0, containing 50 mM KCl, 0.1% Triton, 10% dimethyl sulfoxide, 1.5 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphate, and 2 U of *Taq* polymerase (Promega Corporation Benelux, Leiden, The Netherlands). The mixture was incubated successively at 94°C for 1 min, 35°C for 30 s, and 72°C for 30 s. The cycle was repeated six times. The *Eco*RI-*Apa*I PCR product was cloned into pUCBM20 and sequenced.

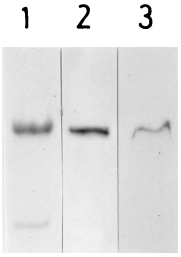

FIG. 3. SDS-PAGE (10% polyacrylamide) of the membranes of IPTG-induced *E. coli* BL21(DE3)/pDML909 (lane 1) and the Ni^{2+}-nitrilotriacetic acid-agarose-purified (His tag)(R2-P686) PBP1* (lanes 2 and 3). Samples were labelled with [^{3}H]benzylpenicillin before analysis. For lanes 1 and 2, fluorography was performed; for lane 3, Coomassie blue staining was performed. Amounts of proteins were as follows: 80 μg (lane 1), 150 ng (lane 2), and 2.5 μg (lane 3). The radioactive, ~40-kDa band in lane 1 is due to *E. coli* PBPs 5 and 6.

the *E. coli* transformant (containing 80 μg of total proteins) were *M. leprae* (His tag)(R2-P686) PBP1* and low levels of *E. coli* PBPs 5 and 6 (lane 1 in Fig. 3).

Properties of membrane-bound *M. leprae* (His tag)(R2-P686) PBP1*. A study of the membranes of the *E. coli* transformant led to the conclusion that *M. leprae* (His tag)(R2-P686) PBP1* differs from *M. leprae* (His tag) PBP1, *E. coli* PBP1a, and *E. coli* PBP1b in several respects.

PBP1* is thermolabile, having half-lives in 10 mM HEPES, pH 7.0, of 60 min at 25°C and less than 5 min at 37°C. *M. leprae* PBP1, *E. coli* PBP1a, and *E. coli* PBP1b have half-lives of 10 min at 60, 45, and 60°C, respectively.

PBP1* is very sensitive to inactivation by β-lactam antibiotics. The values of the rate of enzyme acylation and enzyme deacylation were determined as described previously (6). At 25°C and in 10 mM HEPES, pH 7.0, containing 0.5 M NaCl (half-life, 120 min), the values of the second-order rate constant of acylation by benzylpenicillin, ampicillin, cefotaxime, and cefuroxime are >50,000, 8,800, 8,700, and 1,200 M^{-1} s^{-1}, respectively, and the corresponding acyl-PBP1*s decay spontaneously with first-order rate constant values of 1.7×10^{-4} s^{-1} for cefotaxime and $<1 \times 10^{-4}$ s^{-1} for benzylpenicillin, ampicillin, and cefuroxime. For comparative purposes, the values of the rate of acylation of *M. leprae* PBP1, *E. coli* PBP1a, and *E. coli* PBP1b by benzylpenicillin are ~5 to 10, 800, and 150 M^{-1} s^{-1}, respectively.

PBP1* can be solubilized by incubating *E. coli* membrane suspensions containing 1.25 mg of total proteins per ml in 50 mM sodium phosphate, pH 7.0, supplemented with 1 M NaCl and 1% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) for 40 min at 4°C. The yield, 50%, is independent of the temperature, from 4 to 25°C, and the incubation time, up to 1 h. In contrast, *M. leprae* PBP1 defies attempts at solubilization with all the detergents tested except cetyltrimethylammonium bromide (1), and *E. coli* PBP1b is stable and active in Sarkosyl, a denaturing detergent of *E. coli* PBP1a.

Properties of solubilized *M. leprae* (His tag)(R2-P686) PBP1*. The CHAPS-NaCl-solubilized PBP1* was adsorbed on a Ni^{2+}-nitrilotriacetic acid-agarose column, and the column was washed stepwise with 50 to 250 mM imidazole in 50 mM sodium phosphate (pH 7.4)–1% CHAPS–0.5 M NaCl. PBP1*, eluted at 250 mM imidazole, was dialyzed against the phosphate-CHAPS-NaCl buffer, concentrated on polyethylene glycol 4000 and dialyzed against the same phosphate-CHAPS-NaCl solution. All the steps were carried out at 4°C. SDS-PAGE revealed the presence of a single protein which bound penicillin and had the correct molecular mass (lanes 2 and 3 of Fig. 3).

The purified soluble PBP1* has a half-life of 10 min at 25°C in 50 mM sodium phosphate, pH 7.4, containing 0.5 M NaCl, 5% glycerol, 5% ethylene glycol, and 1% CHAPS. It is acylated by benzylpenicillin and cefuroxime with the same second-order rate constant values as the membrane-bound form. It catalyzes acyl transfer reactions on C_6H_5-CONH-CHR_2-COS-CHR_3-COOH thiolesters (the asymmetric carbon atoms of which have the D configuration). Hydrolysis with release of the HS-CHR_3-COOH leaving group proceeds until completion, with catalytic rate constant/K_m ratios of 4,500 M^{-1} s^{-1} when R_2 is CH_3 and R_3 is H and 3,300 M^{-1} s^{-1} when R_2 is H and R_3 is CH_3. These values were determined from initial rate measurements as described previously (11). PBP1* lacks detectable hydrolytic activity when R_2 and R_3 are both H. In contrast to PBP1*, *M. leprae* PBP1 is inert on the three thiolesters tested. One may note that the catalytic rate constant/K_m ratio is equivalent to the second-order rate constant of acylation of the protein by the thiolester.

Concluding remarks. The nine motifs characteristic of the class A PBPs are present in *M. leprae* PBP1, *M. leprae* PBP1*, *E. coli* PBP1a, and *E. coli* PBP1b protein sequences in the same order and with the same spacing. In spite of this close similarity in modular design and molecular organization, *M. leprae* PBP1 and *M. leprae* PBP1* differ markedly from each other and from *E. coli* PBP1a and *E. coli* PBP1b with respect to penicillin sensitivity, thermostability, and reactions to detergents. These differences can be related to the low levels of similarity in the corresponding amino acid sequences. The question of whether the mycobacterial PBPs are bienzymatic (transglycosylase-transpeptidase) polypeptides is left open. To resolve the issue, the peptidoglycan-synthesizing activity of the PBPs should be probed on the mycobacterial lipid II intermediate (or analog).

The lack of efficacy of classical β-lactam antibiotics against *mycobacteria* may be due to a combination of β-lactamase production and poor access to PBPs (3) rather than the inertness of the drugs towards the peptidoglycan cross-linking machinery. *M. leprae* PBP1* is a high-affinity PBP and is unstable at temperatures above 25°C, and the latter property may be related to the fact that *M. leprae* characteristically multiplies in the cooler tissues in humans (its optimum in vivo temperature has been shown to be 27 to 30°C in mice) (14). The question of whether the class A PBP1 and PBP1* are functionally redundant is also left open. *M. leprae* counterparts of the *E. coli* life cycle PBP2 and PBP3 are other potential targets that deserve to be investigated.

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