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Identification and Overexpression in *Escherichia coli* of a *Mycobacterium leprae* Gene, *pon*1, Encoding a High-Molecular-Mass Class A Penicillin-Binding Protein, PBP1

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Cosmid B577, a member of the collection of ordered clones corresponding to the genome of *Mycobacterium leprae*, contains a gene, provisionally called *pon*1, that encodes an 821-amino-acid-residue high-molecular-mass class A penicillin-binding protein, provisionally called PBP1. With similar amino acid sequences and modular designs, *M. leprae* PBP1 is related to *Escherichia coli* PBP1a and PBP1b, bienzymatic proteins with transglycosylase and transpeptidase activities. When produced in *E. coli*, His tag-labelled derivatives of *M. leprae* PBP1 adopt the correct membrane topology, with the bulk of the polypeptide chain on the surface of the plasma membrane. They defy attempts at solubilization with all the detergents tested except cetyltrimethylammonium bromide. The solubilized PBP1 derivatives can be purified by affinity chromatography on Ni^{2+}-nitrilotriacetic acid agarose. They have low affinities for the usual penicillins and cephalosporins.

Synthesis of the bacterial wall peptidoglycan involves a set of membrane-bound monofunctional (low-molecular-mass) and multimodular (high-molecular-mass) penicillin-binding proteins (PBPs) and an assortment of cell cycle proteins that do not bind penicillin (7). The PBP patterns of *Mycobacterium smegmatis* (1) and *Mycobacterium fortuitum* (5) have been described elsewhere, and a low-molecular-mass PBP of *M. smegmatis* has been studied.

Mycobacterium leprae, the causative agent of leprosy, is an obligatory intracellular gram-positive bacillus. It is not cultivable, and it has an extremely long generation time of 2 weeks or more in experimentally infected animals. An *M. leprae* gene project has been launched, the aim of which is to obtain an ordered cosmid library that covers the entire chromosome and, ultimately, to obtain the complete nucleotide sequence (3, 6, 10, 16).

As shown below, cosmid B577 (3), after correction for a frameshift, contains a gene, provisionally called *pon*1, that encodes a class A multimodular PBP. *M. leprae* PBP1 has been produced in *Escherichia coli*, and its properties were studied.

MATERIALS AND METHODS

Strains, oligonucleotides, and plasmids. The transformations were carried out in *E. coli* DH5α. The expression of the modified *pon*1 genes was carried out in *E. coli* BL21 (DE3). Oligonucleotides A, B, and C (Fig. 1) were from Pharmacia, Biotech Benelux, Roosendaal, The Netherlands.

The plasmids (Fig. 2) were constructed as follows.

(i) pDML901. Cosmid B577 was amplified in *E. coli* DH5α and digested with *Bam*HI and *Eco*RI, and the excised 6,078-bp fragment was cloned into the high-copy-number pUC18. pDML901 served as the source of the *M. leprae* PBP1-encoding *pon*1.

(ii) pDML904. pET-28a(+) (Novagen, Madison, Wis.) was digested with *Nde*I and *Not*I, and the excised 72-bp fragment was replaced by oligonucleotide A.

(iii) pDML905. pDML901 was digested with *Bam*HI and *Sca*I, and the excised 3,232-bp fragment (purified with the Gibco BRL Life Technologies Glassmax kit) was digested partially with *Hin*dIII. The 2,698-bp *Hin*dIII-*Sca*I fragment (encoding the truncated PBP1 devoid of the 14 amino-terminal amino acid residues) was inserted between *Hin*dIII and *Sca*I into pDML904.

(iv) pDML906. The *Dra*III-*Alw*NI segment of pET-22b(+) (Novagen) containing the ampicillin resistance determinant was replaced by the *Dra*III-*Alw*NI segment of pET-28a(+) containing the kanamycin resistance determinant, yielding pET22b/kan. The 67-bp *Msc*I-*Xho*I segment of pET22b/kan was replaced by oligonucleotide B.

(v) pDML907. pDML905 was digested with *Eco*RV (with elimination of the segment encoding the 39 amino-terminal amino acid residues of PBP1) and *Rsr*II (with elimination of the sequence encoding the 4 carboxy-terminal amino acid residues of PBP1). The excised 2,338-bp *Eco*RV-*Rsr*II fragment was inserted between *Sca*I and *Rsr*II into pDML906.

(vi) pDML908. pDML907 was digested with *Asc*I and *Rsr*II, and the excised 314-bp segment was replaced by oligonucleotide C.

Preparation and protease treatment of spheroplasts of *E. coli* transformants. *E. coli* cells were pelleted and resuspended in 15 mM Tris-HCl (pH 8.0) containing 12.5% (wt/vol) sucrose at a concentration of 2×10^{10} cells per ml. The cell suspensions were then treated with lysozyme (2 mg/ml)-EDTA (5 mM) in the same buffer for 25 min at 30°C. Under these conditions, all the cells were converted to spheroplasts.

Spheroplasts were incubated at 30°C for 25 min with proteinase K at final concentrations ranging from 0.016 to 2 mg/ml. Digestion was stopped by adding 100 mM phenylmethylsulfonyl fluoride. Lysis was not observed during this treatment. Samples were then denatured and fractionated on 10% sodium dodecyl sulfate (SDS) gels. The proteins were electrophoretically transferred to a nitrocellulose membrane.

Preparation of plasma membranes of *E. coli* transformants. *E. coli* cells collected from 250-ml isopropyl-β-D-thiogalactopyranoside (IPTG)-induced cultures (optical density at 600 nm of about 1.0) were resuspended in 10 ml of 10 mM Tris-HCl (pH 7.4)–1 mM $MgCl_2$. The suspensions were supplemented with 1 μg of DNase per ml and sonicated at 200 W for 2 min. After centrifugation at $10,000 \times g$ for 15 min to remove the unbroken cells and debris, the membranes were isolated by centrifugation at $100,000 \times g$ for 30 min. They were washed twice and stored at −20°C in the above buffer at a concentration of 20 mg of total proteins per ml.

Purification of membrane-bound His tag-labelled PBP1 derivatives. Membranes (20 mg of total proteins) were suspended in 20 ml of 30 mM potassium phosphate (pH 7.6)–0.5 M NaCl–2% cetyltrimethylammonium bromide (CTAB) and maintained for 30 min at 37°C with occasional shaking. After centrifugation at $100,000 \times g$ for 30 min, the supernatant was loaded on a 2-ml Ni^{2+}-nitrilotriacetic acid (NTA) agarose column (Affiland, Liège, Belgium) equilibrated with the above phosphate-CTAB-NaCl buffer. The column was washed with 50 mM imidazole, pH 7.4. Elution was carried out with 100 mM imidazole buffer (pH 7.4) containing 2% CTAB.

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A

NdeI                                                HindIII     ScaI    NotI
T ATG TCA GAG CGC CTC CCA GCC GTC CTC GCA GTT CTC AAG CTT CTG CAG TAC TGC
   AC AGT CTC GCG GAG GGT CGG CAG GAG CGT CAA GAG TTC GAA GAC GTC ATG ACG CCG G

1M  S  E  R  L  P  A  V  L  A  V  L  K13

B                                     C

ScaI      RsrII        XhoI          AscI
CCGG AGT ACT CGA TCG GTC CGA GAC C   CG CGC CAG CGC CTG AAG GGG GCA
GGCC TCA TGA GCT AGC CAG GCT CTG G AGCT   G GTC GCG GAC TTC CCC CGT CAG

     819V  R  D821                   715R  Q  R  L  K  G  A721
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FIG. 1. Oligonucleotides used in the construction of plasmids. The peptides (A, B, and C) and amino acid numbering refer to the sequence shown in Fig. 3.

Anti-*M. leprae* PBP1 antibodies and Western blotting (immunoblotting). Purified PBP1 (100 μg) was injected subcutaneously with incomplete Freund's adjuvant into one male rabbit. Successive injections of 40 μg each with incomplete Freund's adjuvant were repeated at the end of the second, third, and fourth weeks; bleeding was done at the end of the fifth week. Western blotting using anti-PBP1 antibodies at a dilution of 1:5,000 was performed according to the Bio-Rad protocol, using horseradish peroxidase-linked anti-rabbit immunoglobulin G as the second antibody and 4-chloro-1-naphthol as the color development reagent.

Penicillin binding. Isolated membranes and purified PBP1 derivatives were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE), Coomassie blue staining, and fluorography of the gels. In this latter case, the protein preparations were previously labelled with 1 mM [^{3}H]benzylpenicillin (5 Ci/mmol; The Radiochemical Center, Amersham, Buckinghamshire, United Kingdom) for 30 min at 37°C. The values of the second-order rate constant of enzyme penicilloylation and the first-order rate constant of acyl enzyme breakdown were estimated by previously described methods (8, 9).

Similarity searches and amino acid sequencing. The algorithms (Pile Up, Distances, and Plot similarity) were from the Genetics Computer Group package (2). Automated microsequencing was performed on a 477A pulsed liquid sequenator with a one-line 120A PTH-analyzer (Applied Biosystems, Foster City, Calif.).

RESULTS

Amino acid sequence and molecular organization of *M. leprae* PBP1. The sequence of cosmid B577 (4a) was corrected for a frameshift at nucleotide 3437 by dideoxy sequencing reactions using the 21-mer T365-T385 oligonucleotide, an automated laser fluorescent DNA sequencer, and internal labelling with fluoro-dATP. The corrected nucleotide sequence contained an open reading frame, called *pon*1, that encoded the 821-amino-acid-residue multimodular class A PBP1 (Fig. 3).

PBP1 consisted of an M1-I39 pseudo-signal peptide (the membrane anchor), fused to an S40-A336 non-penicillin-binding (n-PB) module; the n-PB module was fused to an A337-A674 penicillin-binding (PB) module, and the PB module was fused to an M675-D821 carboxy-terminal extension. The n-PB module contained motifs 1 to 4 common to the multimodular class A PBPs, and the PB module contained motifs 5 to 7 characteristic of the penicilloyl serine transferase superfamily. S-398 of motif 5 was assumed to be the active-site serine. Motif 6 was ambiguous, being either S454MN or S464PN. By analogy with the monofunctional penicilloyl serine transferases, the PB module was assumed to start about 60 residues upstream from S-398 of motif 5 and to terminate about 60 residues downstream from G-615 of motif 7.

E. coli PBP1a and PBP1b contain 850 and 844 amino acid residues, respectively. They are regarded as prototypes of the multimodular class A PBPs because they are the only PBPs which have been identified as bienzymatic polypeptides performing, in vitro, transglycosylase (the n-PB module) and transpeptidase (the PB module) activities on isolated disaccharide-peptide lipid II precursors. In contrast to *M. leprae* PBP1, *E. coli* PBP1a contains large inserts occurring at the junctions between the n-PB and PB modules and between motifs 6 and 7 of the PB module. Similarly, *E. coli* PBP1b contains large inserts immediately upstream and downstream from the membrane anchor (7).

For comparison purposes, the inserts and carboxy-terminal extensions were eliminated from the amino acid sequences. As a result, the n-PB modules of *M. leprae* PBP1, *E. coli* PBP1a, and *E. coli* PBP1b contained 296 (S-40 to A-336), 277 (V-42 to P-319), and 273 (M-186 to P-459) amino acid residues, respectively, and the corresponding PB modules contained 322 (A-337 to M-659), 309 (Q-412 to P-775 minus the insert from D-263 to T-677), and 299 (L-461 to P-760) amino acid residues, respectively. Multisequence amino acid alignment of the n-PB modules revealed identities of 30% for the PBP1-PBP1a pair and 27% for the PBP1-PBP1b pair in comparison with 37% for the PBP1a-PBP1b pair. Alignment of the PB modules revealed 19 to 20% identity for the PBP1-PBP1a and PBP1-PBP1b pairs in comparison with 29% identity for the PBP1a-PBP1b pair. In addition, the triad S464PN, not the triad S454MN, of motif 6 of PBP1 aligned with the triad S524KN of PBP1a and the triad S571MN of PBP1b.

Expression of *pon*1 in *E. coli* and properties of PBP1. As *M.*

pDML901 (8743 bp) — BamHI, HindIII, EcoRV, HindIII, RsrII, ScaI, EcoRI, BamHI; pon 1 (2698bp); amp

pDML904 (5352 bp) — AlwNI; kan; lacI; NdeI HindIII ScaI NotI; Oligo A

pDML905 (8042 bp) — HindIII; NdeI; EcoRV; RsrII; ScaI; AlwNI; kan; lacI; pon 1

pDML906 (5323 bp) — AlwNI; kan; lacI; ScaI RsrII XhoI; Oligo B

pDML907 (7920 bp) — ScaI/EcoRV; RsrII XhoI; AlwNI; kan; lacI; pon 1*

*: . . . ATG GCC GGA GTA TCC// GTC CGA GAC CTC GAG CAC . . .
. . Ala Met Ala Gly Val Ser// Val Arg Asp Leu Glu His . . .
PelB signal peptide; 40; PBP1; 821; His-Tag

T7 promoter lac operator His Tag
T7 promoter lac operator pelB signal peptide

FIG. 2. Plasmids used for the production of *M. leprae* PBP1 in *E. coli*. Oligonucleotides (Oligo) A and B are shown in Fig. 1. Oligonucleotide C was used to construct pDML908 (not shown; see text).

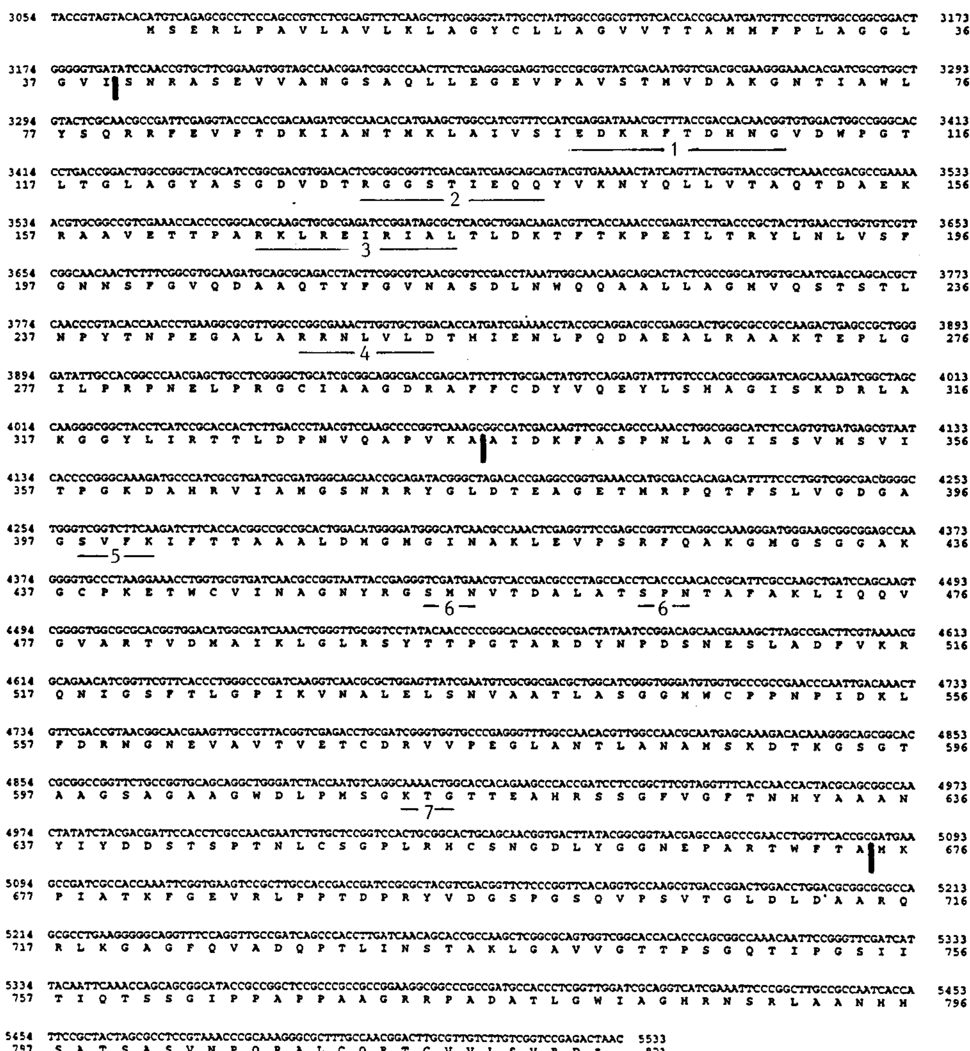

FIG. 3. Nucleotide sequence of the *M. leprae pon*1 gene and amino acid sequence of PBP1. Vertical lines define the membrane anchor, the n-PB module, and the PB module and carboxy-terminal extension of PBP1, respectively. Conserved motifs 1 to 4 (n-PB module) and 5 to 7 (PB module, with some ambiguity for motif 6) are underlined.

leprae is not cultivable, PBP1 is inaccessible to direct investigation. PBP1, with its genuine membrane anchor, was produced in *E. coli*, the peptidoglycan of which is of the same *meso*-diaminopimelic acid type as that of *Mycobacterium* sp. To facilitate the purification, PBP1 was labelled with a polyhistidine [His tag] sequence fused to the amino end of the protein (plasmid pDML905 encoding [His tag]PBP1).

Expression of the modified *pon*1 in *E. coli* BL21 (DE3)/pDML905 was IPTG inducible and under the control of the T7 promoter and *lac* operator. Transformants were grown at 37°C in Luria broth containing 50 μg of kanamycin per ml. When an optical density at 600 nm of 0.5 to 0.6 was reached, 1 mM IPTG was added and the culture was allowed to grow for 1 more h. Cell lysis did not occur. The overproduced membrane-bound [His tag]PBP1 represented about 20% of the total membrane proteins.

The membrane-bound [His tag]PBP1 had a low affinity for β-lactam antibiotics. The antibiotic concentrations required to achieve half-saturation at 37°C were 5×10^{-5} M for ceftriaxone, 5×10^{-4} M for ampicillin, amoxycillin, and cefoxitin, and $>10^{-3}$ M for ticarcillin, temocillin, and cephaloridine. The value of the second-order rate constant of acylation by benzylpenicillin was 5 to 10 $M^{-1}\ s^{-1}$ in comparison with values of 800 $M^{-1}\ s^{-1}$ for *E. coli* PBP1a and 150 $M^{-1}\ s^{-1}$ for PBP1b. The penicilloyl-PBP1 intermediate decayed spontaneously with a first-order rate constant of about $1.7 \times 10^{-4}\ s^{-1}$ and therefore had a relatively short half-life of about 90 min. The penicillin-binding activity of PBP1 had a high thermostability comparable to that of the *E. coli* PBP1b, with no loss after 10 min at 60°C.

To establish that the bulk of the polypeptide chain of the membrane-bound [His tag]PBP1 was exposed in the periplasm, *E. coli* transformants grown and induced at 37°C were converted to spheroplasts and the spheroplasts were treated with increasing concentrations of proteinase K for 25 min at 30°C. The amount of PBP1 left intact in the protease-treated spheroplasts was estimated by SDS-PAGE and Western blotting using anti-PBP1 antibodies (Fig. 4). The result was that at a certain concentration of proteinase K, PBP1 could be totally degraded in intact spheroplasts, showing that the overproduced PBP1 adopted the expected membrane topology.

To check the effects of various levels of *pon*1 expression on

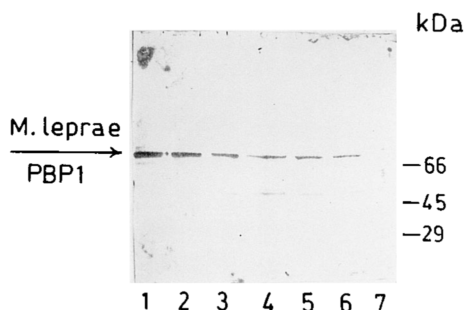

FIG. 4. Protease digestion of PBP1 in spheroplasts. Spheroplasts of *E. coli* BL21 (DE3) harboring pDML905 were incubated with proteinase K as described in Materials and Methods at concentrations of 0, 16, 40, 80, 200, 400, and 2,000 μg/ml. Samples were fractionated by SDS-PAGE, transferred to nitrocellulose membranes, and probed with anti-PBP1 antibodies. The arrow indicates the position of PBP1. The relative mobilities of molecular mass markers are indicated to the right of the gel.

the folding of PBP1, *E. coli* transformants were grown under the same conditions as described above, except that the induction with IPTG was carried out at 20°C, causing a 50% decreased production of PBP1 in the membrane from that obtained at 37°C. The result was that the membrane topology and the low penicillin affinity of PBP1 were independent of the level of gene expression.

[His tag]PBP1 could not be extracted from the membranes by treatment with 2% Triton X-100, 1% Genapol X-100, 1% Nonidet P-40, 1% octylglucoside, 1% deoxycholate, or 1% Sarkosyl. It was solubilized by 2% CTAB–0.5 M NaCl in 10 mM Tris-HCl, pH 7.4. The CTAB-solubilized PBP1 had the same penicillin affinity as the membrane-bound PBP1. It was adsorbed on a Ni^{2+}-NTA agarose column (Fig. 5). After elution with 100 mM imidazole in Tris-HCl (pH 7.4)–2% CTAB and acetone precipitation (7 volumes), SDS-PAGE revealed the presence of a single protein with the expected molecular mass. The purified protein bound penicillin with the same low affinity as the membrane-bound [His tag]PBP1. Upon storage, degradation products were observed.

Attempts at obtaining water-soluble, truncated forms of PBP1 in the periplasm of *E. coli* were made. The pseudo-signal peptide M1-I39 was replaced by the PelB leader peptide, and the His tag label was fused to the carboxy end of the protein (plasmid pDML907 encoding PelB[ΔM1-I39]PBP1[His tag]). In addition, the pseudo-signal peptide was replaced by the PelB leader peptide, the last 100 amino acid residues of the carboxy-terminal extension were eliminated, and the His tag label was fused at the carboxy end of the truncated protein (plasmid pDML908 encoding PelB[ΔM1-I39]PBP1[ΔG722-D821][His tag]). Upon induction at 37°C, the *E. coli* transformants produced large amounts of PBPs, but contrary to the expectations, the truncated forms of PBP1 each remained bound or associated with the plasma membrane. Amino acid sequencing of the doubly truncated [ΔM1-I39]PBP1[ΔG722-D821][His tag], purified by chromatography on Ni^{2+}-NTA agarose, showed that the PelB leader sequence was not processed.

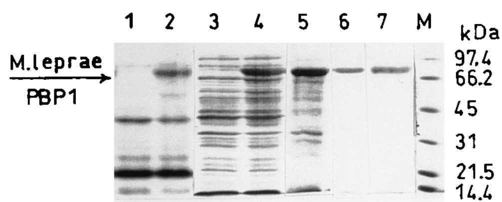

FIG. 5. Overproduction of the *M. leprae* membrane-bound [His tag]PBP1 in *E. coli* BL21 (DE3)/pDML905 and purification of the CTAB-solubilized PBP1. The membranes and proteins were analyzed by SDS-PAGE. Lanes 1 to 4 contain membranes (2 μg of proteins) isolated from noninduced (lanes 1 and 3) and IPTG-induced (lanes 2 and 4) cells. Lanes 1 and 2 show PBP patterns after fluorography. The membranes were labelled with 1 mM [^{3}H]benzylpenicillin for 30 min at 37°C before electrophoresis; the gels were exposed for 2 days. Lanes 3 and 4 show protein patterns after Coomassie blue staining. Lane 5 contains the soluble fraction obtained after 2% CTAB–0.5 M NaCl treatment of membranes isolated from IPTG-induced cells and after Coomassie blue staining. Lanes 6 and 7 contain Ni^{2+}-NTA agarose-purified PBP1 after Coomassie blue staining (lane 6) and fluorography (lane 7). Lane M contains molecular mass markers.

DISCUSSION

It is known that proteins having 25% or more of their sequences in common adopt the same folded structures. At the same time, an increasing number of proteins that have similar folds but statistically insignificant sequence similarity are being revealed. In consequence, a classification which extends the sequence-based superfamilies to include proteins with similar three-dimensional structures but no sequence similarity has been proposed (13).

On the basis of the percentages of identities that they contain, one can be confident that the n-PB module of *M. leprae* PBP1 and the n-PB (transglycosylase) module of *E. coli* PBP1a and PBP1b adopt a similar fold. The percentages of identities between the PB modules are below the cutoff borderline of statistically significant similarity, but the motifs characteristic of the penicilloyl serine transferases are conserved along the sequences. In all likelihood, the PB module of the *M. leprae* PBP1 also has a folded structure similar to that of the PB (transpeptidase) module of *E. coli* PBP1a and PBP1b.

E. coli PBP1a and PBP1b possess inserts that are not present in *M. leprae* PBP1, the three PBPs each have different carboxy-terminal extensions, the inserts and extensions are large enough to form additional modules having their own folds, and there is no cross-reaction between *E. coli* PBP1a or PBP1b and anti-*M. leprae* PBP1 antibodies or between *M. leprae* PBP1 and anti-*E. coli* PBP1b antibodies. Possibly the inserts and extensions are exposed at the surface of the proteins. They may affect, one way or another, the transglycosylase and transpeptidase activities of the PBPs. As a corollary, the functioning of PBP1 in cell wall peptidoglycan synthesis in *M. leprae* may be different from that of PBP1a and PBP1b in *E. coli.*

It is not possible to measure the penicillin affinity of the wild-type PBP1 in *M. leprae*. Consequently, PBP1 has been (over)produced in *E. coli*, with the bulk of the polypeptide chain exposed on the outer face of the membrane, i.e., with the correct membrane topology. However, solubilization of the membrane-bound PBP1 requires CTAB, a denaturing agent of many PBPs. It raises the possibility that the overexpression of *pon*1 may result in the formation of some inclusion bodies. As discussed below, the bulk of observations and experimental data support the view that the low penicillin affinity of PBP1 is not due to the misfolding of the polypeptide chain but, rather, is a property of the wild-type PBP1.

During membrane preparation of the *E. coli* transformants, the cell lysates are first centrifuged at 10,000 × *g*, and therefore, the membrane preparations (used to measure the penicillin affinity of PBP1) must be devoid of any inclusion bodies. The penicillin affinity of PBP1 is independent of the level of gene expression; it is exactly the same when expression is carried out at 37 and 20°C. The low penicillin affinity of PBP1 is comparable to that of PBP5 in *Enterococcus hirae* and PBP2′ in *Staphylococcus aureus* (4). Overexpression of the gene en-

coding the enterococcal low-affinity PBP5 in laboratory mutants and in *E. coli* (using expression vectors identical to those used for *pon*1) does not alter the penicillin-binding fold topology of the overproduced PBP; its penicillin affinity is the same as that of the wild-type PBP (13a).

The mode of binding of the PBPs to the membrane bilayer and the effects of the detergents are species specific. CTAB solubilizes the low-molecular-mass PBP (with DD-transpeptidase activity) of *Streptomyces* sp. strain K15 with preservation of the penicillin-binding fold topology; the value of the first-order rate constant of acylation by penicillin is exactly the same ($150\ M^{-1}\ s^{-1}$) for the wild-type membrane-bound enzyme and the CTAB-extracted enzyme (11). X-ray crystallography studies suggest that a secondary structure at the surface of the protein might function as a membrane association site (1a). *E. coli* PBP1b also possesses at least one membrane association site in addition to the amino-terminal transmembrane anchor (12). PBP1b is stable and active in Sarkosyl, a denaturing detergent for the other PBPs.

Classical β-lactam antibiotics are not effective agents for the treatment of leprosy. Yet, in the mouse footpad model, cefoxitin has low 90% inhibitory dose values (15), and ampicillin in combination with a β-lactamase inhibitor prevents growth of *M. leprae* strains, including strains resistant to dapsone or rifampin (14). The fact that PBP1 has a low penicillin affinity does not necessarily contradict these observations, since *M. leprae* may possess other essential PBPs that may be susceptible to penicillin action. A gene that is provisionally called *pon*2 and encodes a second high-molecular-mass class A PBP is present in cosmid L222 of the *M. leprae* library (unpublished data). This situation is reminiscent of *ponA* (encoding PBP1a) and *ponB* (encoding PBP1b) in *E. coli*. Deletion of *ponA* and *ponB* is fatal, but deletion of either *ponA* or *ponB* is tolerated, suggesting that one PBP can compensate for the other (17). Counterparts of the multimodular class B PBP2 and PBP3 of *E. coli* may also be present in *M. leprae*. These PBPs are key components of morphogenetic networks involved in cell septation and cell shape maintenance (7). In *E. coli*, PBPs are major killing targets of β-lactam antibiotics.

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