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Cloning and Characterization of the *Pseudomonas aeruginosa pbpB* Gene Encoding Penicillin-Binding Protein 3

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Clones containing the *pbpB* gene which encodes penicillin-binding protein (PBP) 3 of *Pseudomonas aeruginosa* were detected by hybridization by PCR amplification with primers based on the conserved sequences of high-molecular-weight PBPs. The translated amino acid sequence demonstrated 45% identity and had a total of 66% conserved amino acids relative to the *Escherichia coli* PBP3. The *pbpB* gene was located upstream of a gene homologous to the *E. coli murE* gene, which encodes uridine diphosphate-*N*-acetyl muramic acid-tripeptide synthetase. The overexpressed *pbpB* gene product reacted with ^{3}H-penicillin G and had an apparent molecular weight of 60,000.

Pseudomonas aeruginosa is an opportunistic pathogen that is intrinsically resistant to a wide range of antibiotics. Infections by this bacterium are often treated with β-lactam antibiotics. β-Lactams exert their effects by acting as the substrate analogs of the peptidoglycan biosynthetic enzymes transpeptidase and D-alanine carboxypeptidase (21), which are located within the cytoplasmic membrane and are commonly named penicillin-binding proteins (PBPs) because of their abilities to covalently bind radiolabelled penicillin (16). It has been found that there are six to seven PBPs in *P. aeruginosa* (14). However, PBPs of *P. aeruginosa* are not as well studied as those in *Escherichia coli* (17, 18) and their genes have not been characterized. Maejima et al. (10) and Watanabe et al. (23) reported that *P. aeruginosa* PBP3 was the primary target for the expanded-spectrum and "fourth generation" cephalosporins. It was observed by Godfrey et al. (5) that some β-lactam-resistant clinical isolates of *P. aeruginosa* from cystic fibrosis patients had apparently lost PBP3 and/or PBP6. Therefore, it seems highly likely that *P. aeruginosa* PBP3 plays an important role in susceptibility to β-lactam antibiotics. To further characterize this protein, in this study we have cloned and sequenced the *pbpB* gene encoding PBP3.

P. aeruginosa PAO1 strain H103 (6) was used for cloning. All DNA techniques were performed essentially as described previously (2, 11), except for isolation of chromosomal DNA, for which hexadecyltrimethyl ammonium bromide was used (1). DNA fragments were isolated by using the Geneclean kit (BIO 101 Inc., La Jolla, Calif.). DNA probes were labelled with digoxigenin-dUTP (Boehringer Mannheim) by random primer labelling. Southern hybridization and colony hybridization were performed by using the Genius system (Boehringer Mannheim). LB broth (0.8% Bacto-tryptone, 0.5% yeast extract) with 0.5 or 0.05% NaCl was used for *E. coli* and *P. aeruginosa* growth, respectively; 2% Bacto-agar was added when solid medium was required. Antibiotics used in selection media included ampicillin at 75 μg/ml, chloramphenicol at 30 μg/ml, and kanamycin at 50 μg/ml.

Although the amino acid sequences of PBPs are only moderately conserved, the sequences around the active-site motif S*XXK (where S* is the binding residue) and the KTG motif are highly homologous among the high-molecular-weight PBPs of *E. coli* and *Neisseria gonorrhoeae* PBP2. Primers for PCR were designed according to the sequences at and surrounding the conserved motifs S*XXK and KTG of *E. coli* PBP1A, -1B, -2, and -3 and *N. gonorrhoeae* PBP2 (18), adjusted to the codon usage of *P. aeruginosa* (24). The sequence of the degenerate upstream primer based on the S*XXK motif was 5′-TTTG AATTCGG(C)CA(T)C(G)C(G)G(AC)C(AT)G(C)G(A)C(T) G(C)AAGCC-3′, which corresponded to the amino acid sequence G(A)ST(ANL)V(IAM)KP. The sequence of the degenerate downstream primer based on the KTG motif was 5′-AAAGAATTCG(CT)T(C)T(G)C(G)GT(C)C(G)GTGCC G(C)G(C)T(A)CTT-3′, which corresponded to the amino acid sequence KT(S)GTT(A)N(QRK). Both of these primers were synthesized with nine extra nucleotides at the 5′ end containing an *Eco*RI recognition site, which ensured that the PCR products could be subsequently digested by *Eco*RI and then cloned into the vector pTZ18U. The PCR was performed in the presence of 5% formamide, 10% glycerol, and 15 mM Mg^{2+}, under conditions whereby the first 5 cycles involved temperature cycles of 94°C for 15 s, 37°C for 30 s, and 72°C for 90 s whereas for the remaining 25 cycles the primer-annealing temperature was raised from 37 to 55°C. A mixture of PCR products ranging from 200 to 750 bp was obtained. All of these products were gel purified, digested with *Eco*RI, and cloned into a vector, pTZ18U. One of these PCR products, 580 bp in length, was found to translate to a sequence with 71 and 72% conservation of amino acids compared with *E. coli* PBP3 and *N. gonorrheae* PBP2, respectively. None of the other PCR products demonstrated any homology to PBP1A, -1B, or -2 of *E. coli*. With the 580-bp PCR product as a probe, Southern blots of the *P. aeruginosa* PAO1 chromosome DNA, which had been digested with various restriction enzymes individually or in combination, permitted the creation of a restriction enzyme map (Fig. 1A). In our hands, the entire gene could not be cloned as a single fragment. Therefore, restriction fragments containing three different portions of the PAO1 chromosomal DNA corresponding to the 1.4-kb *Sph*I, 1.8-kb *Pst*I, and 4.4-kb *Sma*I-*Xho*I fragments were cloned into a vector, pTZ19U, respectively. The positive clones pSPH1, pPST18, and pXSm16 were detected by colony hybridization (Fig. 1B). None of these clones contained the entire gene. Therefore, a 1-kb *Sma*I fragment from plasmid pSPH1 was

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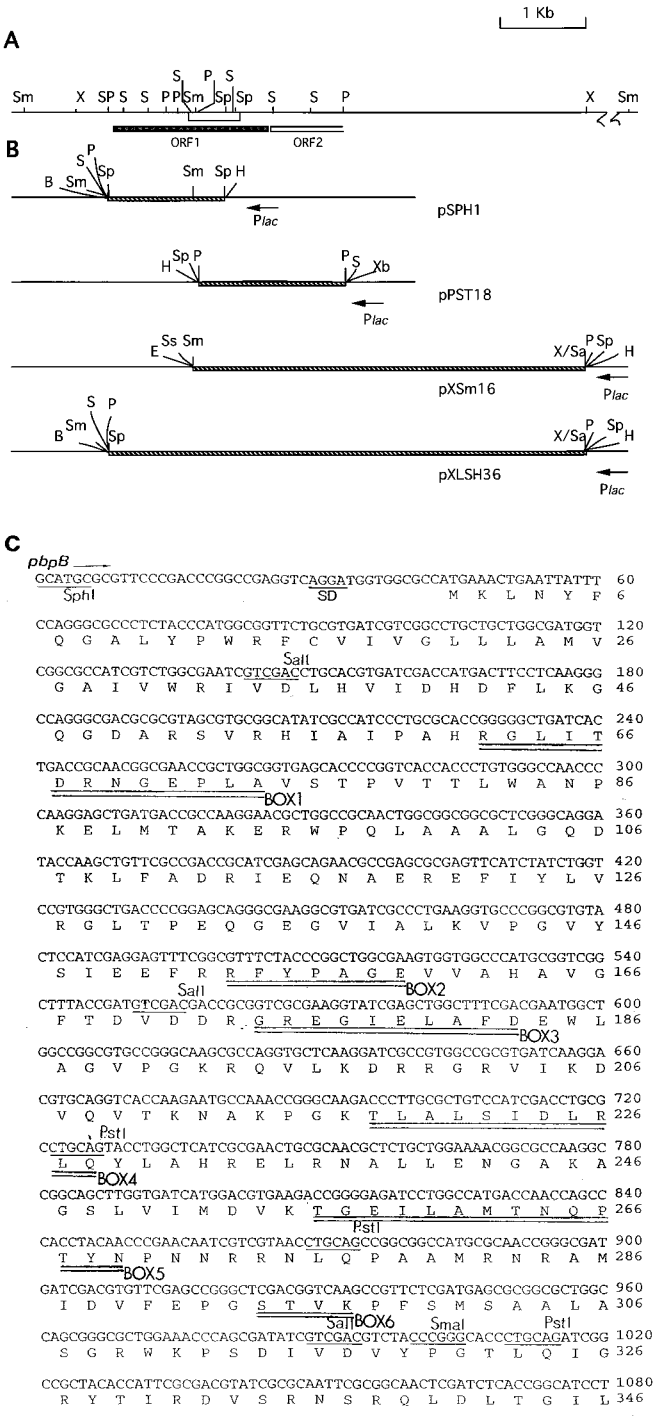

FIG. 1. (A) Physical map of the *P. aeruginosa pbpB* gene region. The light grey box indicates the location of the 580-bp PCR product. The dark grey and open boxes indicate the locations of ORF1 and ORF2, respectively. (B) Restriction enzyme map of the various *pbpB* subclones. The hatched boxes represent the PAO1 chromosomal DNA cloned into the vector pTZ19U. The arrows indicate the orientation of the *lac* promoter on the vector. B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; S, *Sal*I; Sm, *Sma*I; Sp, *Sph*I; Xb, *Xba*I; X, *Xho*I; X/Sa, *Xho*I- *Sal*I. (C) Nucleotide sequence of the *pbpB* region from *P. aeruginosa* and deduced amino acid sequences of the two ORFs. The putative ribosome binding (Shine-Dalgarno [SD]) sites are underlined. The residues corresponding to the conserved boxes are indicated by double underlining. The domain A motifs of ATP-binding proteins are indicated by dashed underlining. Asterisk indicates stop codon.

cloned into the *Sma*I site of the plasmid pXSm16 to obtain pXLSH36 (Fig. 1B). This clone contained a 5.4-kb *Sph*I-*Xho*I fragment of the PAO1 chromosome DNA cloned into the vector pTZ19U. Both strands of a 2.7-kb *Sph*I-*Pst*I region were sequenced with an Applied Biosystems Inc. (Foster City, Calif.) model 373 automated fluorescence sequencer, after creation of nested deletions (8). Two translated open reading frames (ORFs) were found from this sequence (Fig. 1C). They were located at nucleotides 44 through 1783 and 1783 through 2757. The second ORF appeared incomplete at its 3′ end. The first ORF encoded a 579-amino-acid sequence with 45% identity and a total of 66% conserved amino acids compared with the *E. coli* PBP3. All nine conserved motifs of the high-molecular-weight PBPs (4, 15) were strongly conserved in this sequence. The *pbpB* gene was preceded by a Shine-Dalgarno sequence, AGGA, at positions 30 to 34, which matched reasonably the consensus ribosome binding site. Another initiation codon, ATG at positions 116 to 118, was believed unlikely to encode the first amino acid of the protein, since its upstream

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GGCCGGCACACCACGCCGGACCCGCTCGCGGTGCAGGCCACGCTGGCCACGCTGAAGCAG 2280
 G  R  H  T  T  P  D  P  L  A  V  Q  A  T  L  A  T  L  K  Q  166

GCCGGCGCCCGCGCGGTAGCGATGGAAGTGTCTTCCCACGGCCTCGACCAGGGCCGCGTG 2340
 A  G  A  R  A  V  A  M  E  V  S  S  H  G  L  D  Q  G  R  V  186

GCGGCGCTCGGCTTCGATATCGCGGTGTTCACCAATCTGTCCCGCGACCACCTCGACTAT 2400
 A  A  L  G  F  D  I  A  V  F  T  N  L  S  R  D  H  L  D  Y  206

CACGGTTCGATGGAAGCCTATGCCGCCGCCAAGGCCAAGCTGTTCGCCTGGCCGGACCTG 2460
 H  G  S  M  E  A  Y  A  A  A  K  A  K  L  F  A  W  P  D  L  226

                                SalI
CGCTGCCGGGTGATCAACCTGGACGACGATTTCGGCCGTCGACTGGCCGGCGAGGAGCAG 2520
 R  C  R  V  I  N  L  D  D  D  F  G  R  R  L  A  G  E  E  Q  246

GACTCGGAGCTGATCACCTACAGCCTCACCGACAGCTCGGCGTTCCTCTATTGCCGCGAA 2580
 D  S  E  L  I  T  Y  S  L  T  D  S  S  A  F  L  Y  C  R  E  266

GCGCGCTTCGGCGACGCCGGCATCGAGGCGGCGCTGGTCACTCCGCACGGCGAGGGCCTG 2640
 A  R  F  G  D  A  G  I  E  A  A  L  V  T  P  H  G  E  G  L  286

CTGCGCAGCCCGTTGCTCGGCCGCTTCAACCTGAGCAACCTGCTGGCGGCGGTCGGTGCG 2700
 L  R  S  P  L  L  G  R  F  N  L  S  N  L  L  A  A  V  G  A  306

                                                PstI
TTGCTTGGCCTGGGTTATCCCCTGGGCGATATCCTCCGCACTTTGCCGCAGCTGCAG     2757
 L  L  G  L  G  Y  P  L  G  D  I  L  R  T  L  P  Q  L  Q     325
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sequence had no suitable ribosome binding site. This was further confirmed by the N-terminus amino acid sequence analysis (see below). The third position of codons constituted 85.8% of G+C content, typical of a high-G+C organism like *P. aeruginosa* (24). No promoter-like sequences were identified between the *Sph*I site and Shine-Dalgarno sequence.

The second ORF was incomplete and encoded 325 amino acids. This ORF started 2 nucleotides downstream from the end of the *pbpB* gene (Fig. 1C). Its sequence showed 43% identity and a total of 65% conserved amino acids compared with amino acids 1 to 337 of the *E. coli murE* gene product, uridine diphosphate-*N*-acetyl muramic acid-tripeptide synthetase. A putative Shine-Dalgarno sequence, AGGA, was located at positions 1768 to 1771. The third position of codons constituted 86.8% of G+C content. The putative ATP-binding sequences of the *murE* gene product were conserved. The *pbpB* and *murE* genes are also clustered in *E. coli* (12). The two genes were spaced 2 nucleotides apart in *P. aeruginosa*, whereas in *E. coli* the coding region for *murE* overlapped the end of the *pbpB* coding region by 11 bp. Interestingly, these genes mapped physically to the same region of the chromosome, *Spe*I fragment D2, *Dpn*I fragment I, as did the *ftsA-ftsZ-envA* genes (9a), suggesting that *P. aeruginosa*, like *E. coli*, may have a major cell division gene cluster.

Having observed that a novel PBP3 protein was not detected by the ^{3}H-penicillin G assay in *E. coli* DH5α containing plasmid pXLSH36, which had the *pbpB* gene cloned in the opposite orientation relative to the *lac* promoter in the vector pTZ19U, we then cloned the same 5.4-kb *Sph*I-*Xho*I fragment into the vector pTZ18U to create plasmid pXLBI3, which had the *pbpB* gene in the same orientation relative to the *lac* promoter. However, no novel protein was detected by ^{3}H-penicillin, probably because of a weak expression of the protein. Therefore, an efficient expression system (20) utilizing plasmid pT7-7 in combination with pGP1-2 was used. For the purpose of conveniently cloning the *P. aeruginosa pbpB* gene into the vector pT7-7 between the *Nde*I and *Bam*HI sites, PCR was utilized to amplify the *pbpB* gene. Primers for the PCR were 5′-TAAACATATGAAACTGAATTATTTCCAGGGCGC CCT-3′, which contained an *Nde*I recognition sequence and the sequence coding for the N terminus of PBP3, and 5′-AAAGGATCCTCAGCCACGCCCTCCTTTTGCGGGC GCA-3′, which contained the sequence coding for the C terminus of PBP3 followed by a stop codon and the sequence for a *Bam*HI recognition site. The PCR utilized Vent$_R$ DNA polymerase (New England Biolabs), which contains a 3′→5′ proofreading exonuclease activity resulting in

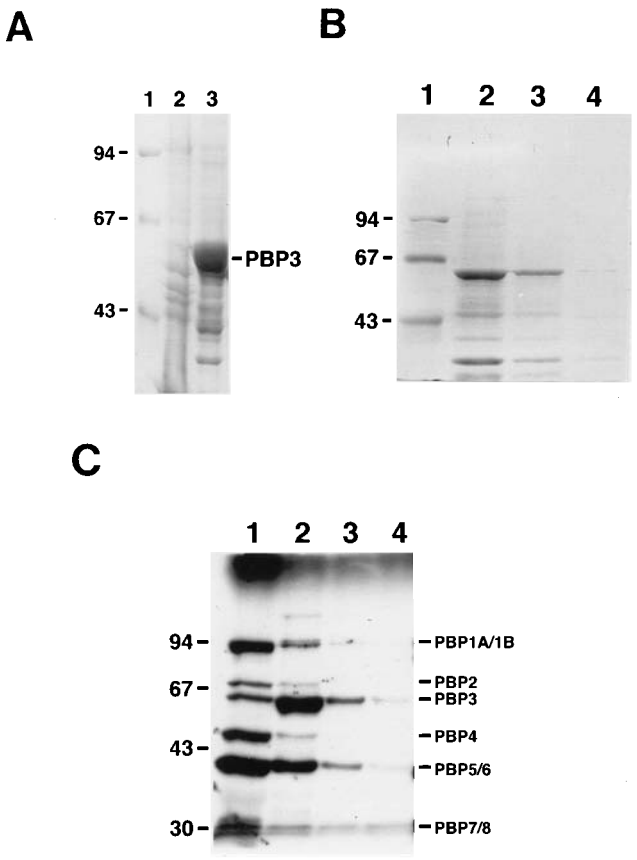

FIG. 2. (A and B) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of cell membrane proteins. *E. coli* BL21(DE3) cells grown in LB broth, supplemented with chloramphenicol for plasmid-containing bacteria, were harvested after induction with 1 mM IPTG for 3 h and washed in 10 mM $NaKHPO_4$ buffer, pH 7.0. After French Press (14,000 lb/in^2) treatment of cells and ultracentrifugation at 55,000 rpm (Beckman 70.1 Ti rotor) at 4°C for 1 h, membrane proteins were resuspended in the $NaKHPO_4$ buffer (10 mM, pH 7.0). (A) Lane 1, standard molecular mass markers; lane 2, *E. coli* BL21(DE3)/pBBR1MCS, the vector control for lane 3, lane 3, *E. coli* BL21(DE3)/pXL608, containing the cloned *P. aeruginosa pbpB* gene. (B) Lane 1, standard molecular mass markers; lane 2, *E. coli* BL21(DE3)/pXL608, 1/10 the amount of membrane proteins loaded in lane 3 of panel A; lane 3, *E. coli* BL21(DE3)/pXL608, one-fifth the amount of membrane proteins loaded in lane 2; lane 4, *E. coli* BL21(DE3)/pXL608, one-fifth the amount of membrane proteins loaded in lane 3. Numbers indicate molecular mass in kilodaltons. (C) Autoradiogram of cell membrane proteins after incubation with ^{3}H-penicillin G and separation by SDS-PAGE. PBPs were assayed by the method of Spratt (16). Membrane proteins were incubated with ^{3}H-penicillin G (3.7 μg/ml, 22 Ci/mol) at 23°C for 10 min. The reaction was stopped by the addition of an excess (1,000-fold) of nonradioactive penicillin G. The samples were separated by SDS-PAGE (8.5% acrylamide gel), and the resultant gel was autoradiographed on Kodak X-ray film for 33 days at −70°C, after being pretreated with 1 M sodium salicylate, pH 6.0, at 23°C for 30 min and dried at 80°C under a vacuum for 2 h (3). Lane 1, *E. coli* BL21(DE3)/pBBR1MCS, the same amount of membrane proteins as loaded in lane 2 of panel A and used as the vector control for lanes 2, 3, and 4; lane 2, *E. coli* BL21(DE3)/pXL608, the same amount of membrane proteins as loaded in lane 2 of panel B; lane 3, *E. coli* BL21(DE3)/pXL608, the same amount of membrane proteins as loaded in lane 3 of panel B; lane 4, *E. coli* BL21(DE3)/pXL608, the same amount of membrane proteins as loaded in lane 4 of panel B. Numbers indicate molecular mass in kilodaltons.

high fidelity of base incorporation. The 1,750-bp PCR product, corresponding to the size of the *pbpB* gene, was digested with *Nde*I and *Bam*HI and cloned into pT7-7 to make plasmid pXL706. This plasmid carried the *pbpB* gene behind the T7 promoter with the start codon of the *pbpB* gene 8 bp downstream from the ribosomal binding site on the vector. The protein was efficiently expressed in *E. coli* K38/pGP1-2 after T7 RNA polymerase was induced at 42°C. The amount of protein detected by ^{3}H-penicillin appeared to be somewhat less than expected given its abundance in the membrane protein sample (data not shown). This could possibly be due to incomplete removal of the β-lactamase produced by pT7-7. Therefore, an alternative approach utilizing a broad-host-range vector, pBBR1MCS, which carries a chloramphenicol resistance gene and T7 promoter (9), was used to express the *pbpB* gene product. A 1.75-kb *Xba*I-*Bam*HI fragment isolated from plasmid pXL706, which contained the ribosomal-binding-site sequence and the *pbpB* gene, was cloned in the vector pBBR1MCS behind the T7 promoter. After induction with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), the protein was expressed in *E. coli* BL21(DE3), which carries the T7 RNA polymerase gene under the control of the *lacUV5* promoter (19). The expressed protein had an apparent molecular weight of 60,000 (Fig. 2A and B). This *pbpB* gene product cofractionated with cytoplasmic membrane proteins. The ^{3}H-penicillin G assay showed that the *P. aeruginosa pbpB* gene product covalently bound penicillin (Fig. 2C).

The translated PBP3 protein containing 579 amino acids would have a calculated molecular mass of 63.69 kDa. However, the protein expressed from *E. coli* BL21(DE3)/pXL608 migrated with an apparent molecular mass of 60 kDa. N-terminal amino acid sequence analysis of this protein showed that it was identical to the translated sequence for the first 6 amino acids. This may reflect posttranslational C-terminal processing, as shown to occur with *E. coli* PBP3 before it is localized to the cytoplasmic membrane (13). The sequence at the N terminus was apparently not removed and did not appear to be characteristic of a typical signal peptide (22), nor did it contain a putative lipoprotein signal processing sequence as proposed for *E. coli* PBP3 (7).

All of the high-molecular-weight PBPs studied to date are known to be inner membrane proteins with their hydrophobic amino terminus anchored in the cytoplasmic membrane. Hydropathy analysis of the deduced amino acid sequence of *P. aeruginosa* PBP3 showed a profile similar to that of *E. coli* PBP3 (data not shown). Prediction of membrane-spanning segments by using the computer program PC gene indicated that there was only one transmembrane segment, stretching from residues 15 to 31 as the inner boundaries and residues 8 to 39 as the outer boundaries. The protein was thus predicted to be an integral inner membrane protein.

Nucleotide sequence accession number. The *pbpB* and *murE* sequence shown in Fig. 1C has been submitted to the EMBL data library and assigned the accession number X84053.

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