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Identification of a Penicillin-Binding Protein 3 Homolog, PBP3x, in *Pseudomonas aeruginosa*: Gene Cloning and Growth Phase-Dependent Expression

XIAOWEN LIAO AND ROBERT E. W. HANCOCK*

Department of Microbiology and Immunology, University of British Columbia, Vancouver, British Columbia, Canada V6T 1Z3

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A homolog of *Pseudomonas aeruginosa* penicillin-binding protein 3 (PBP3), named PBP3x in this study, was identified by using degenerate primers based on conserved amino acid motifs in the high-molecular-weight PBPs. Analysis of the translated sequence of the *pbpC* gene encoding this PBP3x revealed that 41 and 48% of its amino acids were identical to those of *Escherichia coli* and *P. aeruginosa* PBP3s, respectively. The downstream sequence of *pbpC* encoded convergently transcribed homologs of the *E. coli soxR* gene and the *Mycobacterium bovis adh* gene. The *pbpC* gene product was expressed from the T7 promoter in *E. coli* and was exported to the cytoplasmic membrane of *E. coli* cells and could bind [^{3}H]penicillin. By using a broad-host-range vector, pUCP27, the *pbpC* gene was expressed in *P. aeruginosa* PAO4089. [^{3}H]penicillin-binding competition assays indicated that the *pbpC* gene product had lower affinities for several PBP3-targeted β-lactam antibiotics than *P. aeruginosa* PBP3 did, and overexpression of the *pbpC* gene product had no effect on the susceptibility to the PBP3-targeted antibiotics tested. By gene replacement, a PBP3x-defective interposon mutant (strain HC132) was obtained and confirmed by Southern blot analysis. Inactivation of PBP3x caused no changes in the cell morphology or growth rate of exponentially growing cells, suggesting that *pbpC* was not required for cell viability under normal laboratory growth conditions. However, the upstream sequence of *pbpC* contained a potential σ^s recognition site, and *pbpC* gene expression appeared to be growth rate regulated. [^{3}H]penicillin-binding assays indicated that PBP3 was mainly produced during exponential growth whereas PBP3x was produced in the stationary phase of growth.

β-Lactam antibiotics exert their functions by acting as analogs of the D-alanyl-D-alanine moiety of the lipid-linked disaccharide-pentapeptide, the substrate for a group of enzymes catalyzing cross-linking of the peptide side chains of nascent cell wall peptidoglycan (4). These enzymes bind covalently to their substrates and bind covalently and irreversibly to β-lactams, which enabled the development of a convenient autoradiographic method for the detection of penicillin-sensitive enzymes as penicillin-binding proteins (PBPs) (27). The most extensively studied targets of β-lactam antibiotics in gram-negative bacteria are the PBPs of *Escherichia coli*. In *E. coli* cells, there are at least eight PBPs that can be detected by radiolabeled penicillin G (27). Based on their structural features and enzyme activities, PBPs of *E. coli* are classified as high-molecular-weight PBPs, which include PBP1a, PBP1b, PBP2, and PBP3, and low-molecular-weight PBPs, which include PBP4, PBP5, PBP6, and PBP7. The high-molecular-weight PBPs of *E. coli* are generally regarded as bifunctional enzymes, having transglycosylase and transpeptidase activities which are essential for the completion of peptidoglycan biosynthesis, whereas the low-molecular-weight PBPs of *E. coli* act mainly as DD-carboxypeptidases and have been suggested to be nonessential for cell survival (28). The high-molecular-weight PBPs are essential for cell growth and are thought to be the lethal targets of the β-lactam antibiotics (28). PBP1a and PBP1b appear to be redundant or at least capable of fulfilling compensatory roles in cell elongation, PBP2 is essential for lateral cell wall elongation and maintenance of the rod shape, and PBP3 is involved in the formation of the septum during cell division. The PBP3-encoding gene, *ftsI* or *pbpB*, is found proximal to a cluster of genes required for the synthesis of the peptidoglycan precursors (e.g., *murE*, *murF*, *murG*, *murG*, *murC*, and *ddl*) or for cell division and septum formation (e.g., *ftsW*, *ftsQ*, *ftsA*, and *ftsZ*) (3).

PBPs of *Pseudomonas aeruginosa* show an electrophoretic pattern similar to that of *E. coli* but are not well studied. Comparative studies of the binding affinities for various β-lactam antibiotics indicated that *P. aeruginosa* PBP1a, -1b, -2, -3, -4, and -5 corresponded to *E. coli* PBP1b, -1a, -2, -3, -4, and -5, respectively, and that *P. aeruginosa* PBP6 and PBP7 were not readily detected (21). Binding between the *P. aeruginosa* PBPs and β-lactam antibiotics generally results in morphological changes similar to those observed in *E. coli* (6). Previously we cloned and characterized the *P. aeruginosa* PBP3-encoding gene *pbpB*, which encoded a protein sequence with 58% conserved amino acids compared to that of *E. coli* PBP3 (16). The *pbpB* gene was found to be located upstream of a gene encoding a homolog of the *E. coli murE* gene product, and these two genes mapped to the same region as did certain other cell division genes including *ftsA*, *ftsZ*, and *envA* (17). Taken together, these data suggested that *pbpB* is essential for cell division. During the course of generating a probe for cloning the *pbpB* gene by degenerate primer PCR, a second PCR product was found to translate into an amino acid sequence with significant homology to that of *E. coli* PBP3. This PCR product was mapped 2 megabase pairs from the *pbpB* locus on the *P. aeruginosa* chromosome (17). These results suggested that *P. aeruginosa* contains two copies of genes encoding proteins having functions similar to that of *E. coli* PBP3. To test

* Corresponding author. Mailing address: Department of Microbiology and Immunology, 300-6174 University Blvd., University of British Columbia, Vancouver, B.C., Canada V6T 1Z3. Phone: (604) 822-2682. Fax: (604) 822-6041. E-mail: bob@cbdn.ca.

this hypothesis, this study describes the cloning and characterization of the *P. aeruginosa* PBP3 homolog PBP3x. The regulation of *pbpB* and *pbpC* gene expression in *P. aeruginosa* was investigated.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The major bacterial strains used in this study included *E. coli* DH5α (*supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1 lacU169*/ϕ80*lacZ-M15*) (Gibco BRL, Burlington, Ontario, Canada), K38 (HfrC/λ) (22), and BL21(DE3) (*hsdS gal*/λc*I*ts857 *ind1 sam7 nin5 lacUV5*-T7 gene 1) (30) and *P. aeruginosa* PAO1 protrophic strain H103 (16) and PAO4089 *met*-9020 *pro*-9024 *blaJ*9111 *blaP*9202 (12). Plasmids included the general cloning vectors pTZ19U (U.S. Biochemicals, Cleveland, Ohio) and pT7-7 (containing a T7 RNA polymerase promoter and ribosomal binding site [30]) and the broad-host-range cloning vectors pUCP27 (26) and pBBR1MCS (16), as well as pGP1-2 containing the heat-inducible gene for T7 RNA polymerase (30).

E. coli strains were grown in Luria-Bertani (LB) broth (Difco, Detroit, Mich.). *P. aeruginosa* strains were grown in Mueller-Hinton (MH) broth (Becton Dickinson, Cockeysville, Md.). Solid media were made by the addition of 2% Bacto Agar (Difco). VBMM (25) was used selectively for the growth of *P. aeruginosa*, since *E. coli* cannot use citrate as a carbon source. Antibiotics were used in selective media at the following concentrations: for *E. coli*, ampicillin at 75 μg/ml, chloramphenicol at 30 μg/ml, kanamycin at 50 μg/ml, and tetracycline at 12 μg/ml; for *P. aeruginosa*, carbenicillin at 500 μg/ml, kanamycin at 300 μg/ml, and tetracycline at 100 μg/ml.

DNA manipulation. DNA manipulations were performed essentially by the method of Maniatis et al. (18) modified slightly as described previously (16). Oligodeoxyribonucleotides were synthesized with a DNA-RNA synthesizer (model 392; Applied Biosystems Inc. [ABI], Foster City, Calif.). Plasmid DNA for sequencing was prepared by the Qiawell-8 plasmid purification system (Qiagen Inc., Chatsworth, Calif.). DNA sequencing was done by using an ABI model 373 automated DNA sequencer and dye terminator chemistry following the protocols from ABI with the universal forward and reverse primers. Both DNA strands were sequenced. Nested deletions were created with the Erase-a-base kit (Promega, Madison, Wis.). Oligonucleotide primers were constructed to fill in gaps. DNA and amino acid sequences were analyzed with the PC Gene, ESEE, and DNAMAN computer programs. Sequences were compared to sequences in the GenBank database with the BLASTN, BLASTP, and BLASTX programs (1).

Cloning of the *pbpC* gene. *P. aeruginosa* PAO1 chromosome DNA, which had been digested with various restriction enzymes individually or in combination, was resolved by agarose gel electrophoresis, transferred to a nylon membrane, and probed with a radioactively labeled 580-bp PCR product homologous to the PCR product (17) that was part of the *P. aeruginosa pbpC* gene (16). This procedure was used to create a restriction enzyme map (Fig. 1A). To clone the *pbpC* gene, an attempt was made to construct a library with the 6.0-kb *Sal*I fragments of PAO1 chromosomal DNA. However, this was not successful. The cloning strategy was therefore changed to isolate three restriction fragments containing three different portions of the PAO1 chromosomal DNA corresponding to the 2.5-kb *Xho*I, 4.0-kb *Pst*I-*Sal*I, and 2.0-kb *Eco*RI fragments, respectively. These fragments were separately ligated with the digested vector pTZ19U and transformed into *E. coli* DH5α. Three positive clones, pXL-Xh401, pXL-PS406, and pXL-Ec405 (Fig. 1B), were obtained after colony hybridization with the PCR probe. However, none of these clones contained the entire *pbpC* gene. Therefore, a 1.9-kb *Kpn*I-*Xho*I fragment from pXL-PS406 was ligated with a 1.8-kb *Xho*I-*Eco*RI fragment from pXL-E405, and the resultant fragment was cloned into the *Kpn*I and *Eco*RI sites on vector pTZ19U to create plasmid pXL-KE24 (Fig. 1B). A subclone pXL-Hd2 was subsequently created after deletion of the 1-kb *Kpn*I-*Hin*dIII fragment from pXL-KE24 (Fig. 1B).

Construction of the PBP3x-defective mutant. A gene replacement technique described by Schweizer (25) was used for construction of the PBP3x-defective mutant. To construct the insertion plasmid pBPC::Km, the *pbpC* gene on pXL732 (see below) was mutated by the insertion of a 1.3-kb blunt-ended *Hin*cII interposon encoding a Km^r gene (isolated from pUC4KPA [Pharmacia Biotech Inc., Quebec, Canada]) into the unique *Sma*I site in the *pbpC* gene to generate plasmid pXL732::Km. The 3.0-kb *Eco*RI-*Bam*HI fragment containing *pbpC*::Km^r was isolated from pXL732::Km and cloned into pNOT19. Subsequently, a 5.8-kb *Not*I DNA fragment from pMOB3 containing *oriT*, the *Bacillus subtilis sacB* gene as a counter-selectable marker, and a chloramphenicol resistance gene was cloned into the unique *Not*I site on pNOT19 with *pbpC*::Km^r to generate plasmid pBPC::Km, which was then transformed into *E. coli* mobilizing strain S17-1 (23). This plasmid was then mobilized into *P. aeruginosa* PAO1 strain H103, and recipients in which the plasmid *pbpC*::Km^r gene had replaced the chromosomal *pbpC* gene were selected by the method of Schweizer (25).

Protein expression with the T7 RNA polymerase-promoter system. The *P. aeruginosa pbpC* gene cloned in pXL-Hd2 was amplified by PCR. The upstream primer 5′-AAACATATGAGCAGTCAACGCCGAAACTACCGCTTCA-3′ contained an *Nde*I recognition sequence and the sequence coding for the N terminus of PBP3x. The downstream primer 5′-AAAGGATCCTCAGCCGTGGTGCTGG CGGTCGGCGA-3′ contained the sequence coding for the C terminus of PBP3x followed by a stop codon and the sequence for a *Bam*HI recognition site. PCR was performed under the conditions described previously (16). The resulting 1.7-kb PCR product, which based on DNA sequencing, corresponded to the *pbpC* gene, was digested with *Nde*I and *Bam*HI and cloned into the *Nde*I-*Bam*HI-digested pT7-7, yielding plasmid pXL732, and transformed into *E. coli* K38/pGP1-2. Examination of protein production was done by the procedure by Ausubel et al. (2).

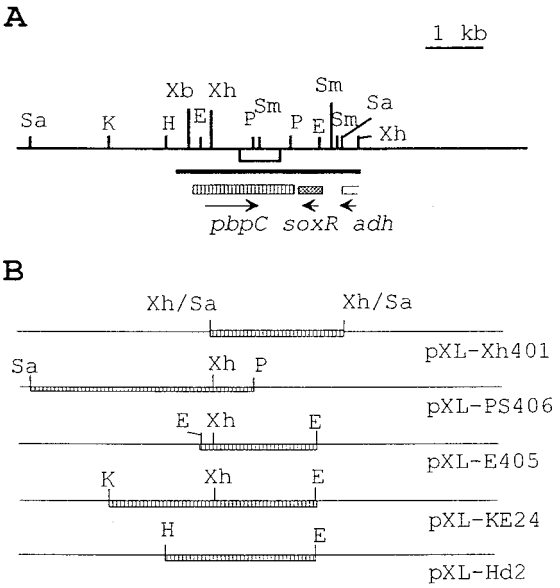

FIG. 1. (A) Restriction map of the *P. aeruginosa pbpC* region. The locations of the 580-bp PCR product (open box), the sequenced 3.2-kb *Hin*dIII-*Xho*I region (solid bar), the *pbpC*, *soxR*, and *adh* ORFs, and the direction of transcription (arrows) are indicated. (B) Restriction maps of *pbpC* subclones. The striped boxes represent the PAO1 chromosomal DNA cloned into vector pTZ19U. Abbreviations: E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; Sa, *Sal*I; Sm, *Sma*I; Xb, *Xba*I; Xh, *Xho*I; Xh/Sa: *Xho*I-*Sal*I.

To construct the plasmid containing the *pbpC* gene in the broad-host-range vector pBBR1MCS, a 1.7-kb *Xba*I-*Bam*HI fragment, which contained a ribosomal binding sequence (RBS) sequence from pT7-7 and the *pbpC* gene, was isolated from pXL732 and cloned behind the T7 promoter on vector pBBR1MCS to generate pXL629. This plasmid was transformed into *E. coli* BL21(DE3). Examination of protein expression from BL21(DE3)/pXL629 was done as follows. LB broth plus chloramphenicol was inoculated at a ratio of 1:50 with cultures of BL21(DE3)/pXL629 or BL21(DE3)/pBBR1MCS grown overnight. The freshly inoculated cultures were grown at 37°C to an optical density at 600 nm of 0.6. Isopropyl-β-D-thiogalactopyranoside (IPTG) was then added to a final concentration of 0.5 mM to induce expression of T7 RNA polymerase and consequently *pbpC* under the control of the T7 promoter. Cultures were further incubated at 37°C for 3 h before harvesting.

Whole-cell lysates. Whole-cell lysates were prepared by a sodium dodecyl sulfate (SDS) boiling method (20). Cells collected by centrifugation were resuspended in solubilization sample buffer and boiled, and the proteins were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE).

Membrane protein preparation. Cells were harvested at various points of growth and washed in 10 mM Tris-HCl buffer, pH 8.0. The washed cells were lysed by passage through a French press (14,000 lbs/in^2), and the membrane-containing pellet was collected by ultracentrifugation at 44,000 rpm (Beckman 70.1 Ti) at 4°C for 2 h. Samples were resuspended in 10 mM Tris-HCl buffer, pH 8.0, and sonicated twice on ice at maximum power for 15 s (Fisher Sonic Dismembrator 300 fitted with a 3.5-mm-diameter microtip) to remove β-lactamase associated with the membrane. The sonicate was centrifuged at 44,000 rpm (Beckman 70.1 Ti) at 4°C for 2 h, and the membrane-containing pellet was retained. The membrane-containing pellet was washed once in 10 mM Tris-HCl buffer, pH 8.0, then resuspended in the same buffer, and used immediately or stored at −70°C until needed.

Penicillin-binding protein assays. PBPs were assayed essentially by the method of Spratt (27) modified slightly as described previously (16). For the competition assay, membrane proteins (100 μg in each sample) were incubated with different concentrations of the tested β-lactam antibiotic or with distilled water (for control experiments) at 25°C for 10 min. Subsequently, 4 μCi of [^{3}H]penicillin G (3.7 μg/ml; 22 Ci/mol; Amersham) was added. After incubation at 25°C for 10 min, the reaction was stopped by the addition of excess (1,000x)

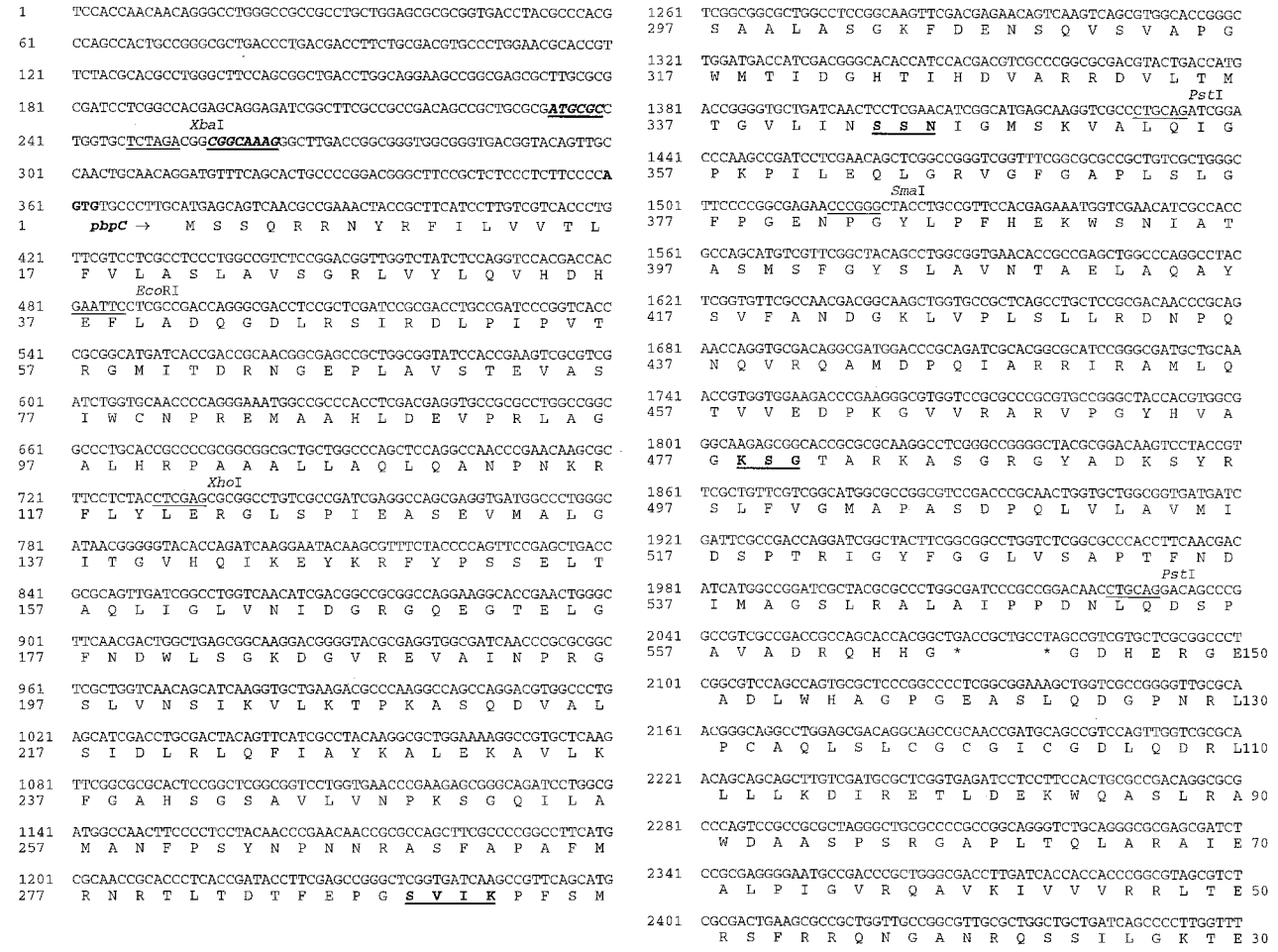

FIG. 2. Nucleotide sequence of the *P. aeruginosa pbpC* region and the deduced amino acid sequences of three ORFs. The numbers on the sides designate the nucleotide and amino acid at the end of each row. The conserved motifs of PBP3x are in bold type and underlined. The putative RBS sequence is in bold type. The putative promoter sequences recognized by σ^s are in bold italic type and underlined. The arrows indicate the direction of transcription. Sequences recognized by a restriction enzyme are underlined.

nonradioactive penicillin G. The proteins were separated by SDS–7.5% PAGE. The resulting gel was treated with 1 M sodium salicylate, dried, and exposed to X-ray film (X-Omat K XK-1; Kodak) for 3 to 6 days at −70°C. The intensities of the bands on the fluorograms were quantitated by using a scanning densitometer (Studio Scan II; AGFA) in combination with a Macintosh computer using the public domain National Institutes of Health Image program. The software was used to integrate scan peaks for quantitation of PBP binding to [^{3}H]penicillin. Binding inhibition was evaluated as the I_{50}, which was the concentration of the antibiotic inhibiting binding by 50% relative to binding in the absence of competing antibiotic.

N-terminal amino acid sequence determination. Partially purified protein preparations were separated by SDS-PAGE and electroblotted onto polyvinylidene difluoride membranes (Millipore, San Francisco, Calif.) by the procedure of Matsudaira (19). Protein bands were excised from the membrane after staining with Ponceau S red solution (0.5% Ponceau S red and 1% acetic acid), air dried, and sent to S. Kielland, Department of Biochemistry and Microbiology, University of Victoria, for N-terminal sequence determination with a gas-phase sequencer (ABI model 470A).

Protein assay. Protein concentrations were determined by a modified version (24) of the Lowry assay. Bovine serum albumin was used as a standard.

Antibiotic susceptibility testing. β-Lactam antibiotics used in this study included aztreonam, cefepime (Bristol-Myers-Squibb Inc., Wallingford, Conn.), cefsulodin (Ciba Ceigy, Basel, Switzerland), ceftazidime (Glaxo Canada Inc.), cephaloridine (Sigma), and imipenem (Merck, Rahway, NJ). MICs were determined by the agar dilution method on MH agar plates with twofold serial dilutions of the antibiotics. Overnight-grown cultures of the bacteria to be tested were diluted in MH broth to approximately 10^5 CFU per ml. Five-microliter samples of this diluted culture were inoculated onto the surfaces of MH agar plates containing antibiotic or control plates without any antibiotics. The recorded MIC corresponded to the lowest concentration of antibiotic causing greater than 90% inhibition of bacterial growth after 18 h of incubation at 37°C. Final MICs were the averages of at least three determinations.

Growth experiment and cell shape examination. Cultures of *P. aeruginosa* grown overnight were inoculated into fresh MH broth at a ratio of 1:100. Cultures were then incubated at 37°C with shaking (180 rpm). The absorbency at 600 nm of the culture was determined at various times, and a growth curve was plotted. Cell shape was examined by phase-contrast microscopy (BH2-PC microscope; Olympus BHT, Tokyo, Japan).

Nucleotide sequence accession number. The *pbpC*, *soxR*, and *adh* sequence shown in Fig. 2 has been submitted to the EMBL data library and assigned accession number X95517.

RESULTS

Gene cloning and sequence analysis of *pbpC*. The *pbpC* gene, encoding the PBP3 homolog PBP3x, and surrounding regions were cloned as described in Materials and Methods. Both strands of the 3.2-kb *Hin*dIII-*Xho*I region (Fig. 1A) were sequenced by using nested deletion clones of pXL-Xh401 and pXL-Hd2 (Fig. 1B). Three putative open reading frames (ORFs) were found in this sequence (Fig. 2). They were located at nucleotides 373 through 2070 (ORF1), 2078 through 2548 (ORF2), and 3028 through 3274 (ORF3). ORF1 would be transcribed in the orientation that of ORF2 and ORF3. ORF1 encoded a 565-amino-acid sequence with approximately

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2461  CGTAGAAATGCAGGGCGGAGACCGCCACGCCGGCACGCCTGGCCAGTTCGCCGACGCTCA
        Y  F  H  L  A  S  V  A  V  G  A  R  R  A  L  E  G  V  S  L 10
                          EcoRI
2521  GTTCACGAGATGCGCAGGAATTCTTCATGATTGGCTTGACCTCAAGATTGCTTGAGGTTT
        E  R  S  A  C  S  N  K  M   ← soxR                        1

2581  TACCCTGGGCCGGCCCTCGACACAAGTCACCCGGAGTAACCCGCCATGCTCATCCCCTCC

2641  TCCTACTGCTGCACCGACGATCAGTGCCCGGCCGCTAGCGAGCGGTCGCCGTGCTACGCG

2701  CAACTCATCGATATCGAGGTGGAACCCGCCAGCCAGTTGTCGCTGGCGCTGGAACAGAAC
                                                  SmaI
2761  GCGCACCTGCAACGCCTGGAACGCTGCTTCAGGAACTGCCCGGGCTACCTGTCCGCCAGC

2821  CTGCACCCCAGCGAGGACGGGCAGCACGTGCTGAACTACACCTGCTGGCGTTCACGCGAG
                          SmaI
2881  GACTGCGAACGCGCCTGGCTGGCCCGGGAGGACGCGCAAGGCCCGCTGAGCGCGGGCGTC
                                                  SalI
2941  TGGCGGCTGGGGGCGAAAAGCGTGCGTTTCGAGACCTTCCTGGTCGACGCCGAGGGCTGC

3001  TGAGCCCGCCGGCGCCGACGGCCCGTCTCAGAAGGACTTCTGCAGGGAGGCCATGTCGAT
                                * F  S  K  Q  L  S  A  M  D  I  73

3061  CACGAAGCGATACTTCACGTCGCTGGCCAGCATCCGCTCGTAGGCCTGGTTGATCTGCTG
        V  F  R  Y  K  V  D  S  A  L  M  R  E  Y  A  Q  N  I  Q  Q  53

3121  GATCTCGATCATCTCGATGTCGCAGGCGATCCCGTGGGCCGCGCAGAAATCCAGCATTTC
        I  E  I  M  E  I  D  C  A  I  G  H  A  A  C  F  D  L  M  E  33

3181  CTGGGTCTCGGCGATGCCGCCGATGGCCGAGCCGGCGATCGAGCGGCGTCCCATCACCAG
        Q  T  E  A  I  G  G  I  A  S  G  A  I  S  R  R  G  M  V  L  13
                                      XhoI
3241  TTGCGCACCGTGCACCGCCGGCTCCAGCGGCTCGAG
        Q  A  G  H  V  A  P  E  L  P  E  L   ← adh                1
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FIG. 2—*Continued.*

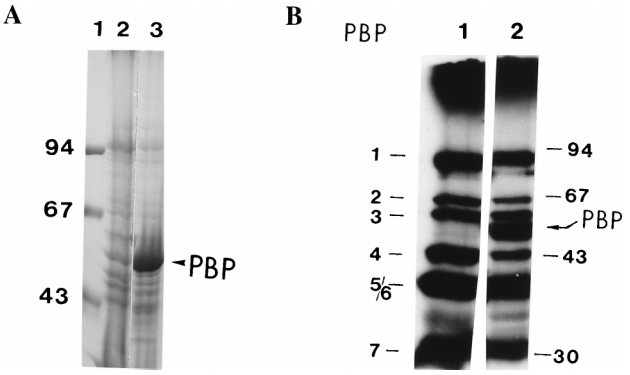

FIG. 3. (A) SDS–8.5% PAGE of cell membrane proteins. Lane 1, standard molecular mass markers; lane 2, BL21(DE3)/pBBR1MCS, vector control for lane 3; lane 3, BL21(DE3)/pXL629, containing the cloned *P. aeruginosa pbpC* gene. Numbers to the left of the gel are molecular masses (in kilodaltons) of the proteins. (B) Autoradiogram of cell membrane proteins after incubation with [^{3}H]penicillin and separation by SDS–8.5% PAGE. Lane 1, BL21(DE3)/pBBR1MCS, containing the same amount of membrane protein as in lane 2 of panel A; lanes 2, BL21(DE3)/pXL608, containing one-fifth the amount of membrane proteins as in lane 3 of panel A. Numbers to the right of the gel are molecular masses (in kilodaltons) of the proteins. Numbers to the left of the gel indicate the positions of PBPs. PBP3x is indicated by the arrow labeled PBP.

48 and 41% of its amino acids identical to those of *P. aeruginosa* and *E. coli* PBP3s and was designated the *pbpC* gene encoding PBP3x (Table 1). Ninety-three percent of the nucleotides in the third position of codons were either G or C, typical of high-G+C organism like *P. aeruginosa* (32). ORF2 encoded a protein sequence of 156 amino acids which had 62% identical and 78% conserved amino acids relative to the *E. coli soxR* gene product. In *E. coli*, *soxR* and *soxS* are adjacent to one another (33). However, between the *P. aeruginosa soxR* (ORF2) and ORF3, no *soxS*-like gene product was identified, and this sequence showed no significant homology to any sequences in the GenBank database. ORF3 was incomplete at its 5′ end and encoded 82 amino acids. This sequence showed 57.3% identical and 76.8% conserved amino acids compared to amino acids 219 to 346 of the *Mycobacterium bovis adh* gene product, an NADP-dependent alcohol dehydrogenase (29).

Analysis of PBP3x for membrane-spanning segments using the PC Gene computer program predicted that there was one transmembrane segment for the *pbpC* gene product which localized to residues 10 to 26 as the inner boundaries and residues 7 to 31 as the outer boundaries. The nine conserved amino acid sequence motifs or boxes that have been identified as being unique to the class B high-molecular-weight PBPs by Ghuysen (11) were highly conserved in *P. aeruginosa* PBP3, PBP3x, and *E. coli* PBP3, as were the spacings between these boxes. Comparison of the amino acid sequence of PBP3x with those of other PBPs (Table 1) indicated that the PBP3x protein was more related to *P. aeruginosa*, *E. coli*, and *Haemophilus influenzae* PBP3s and *Neisseria gonorrhoeae* PBP2, a group of PBPs that have been suggested to function in cell-wall septum formation of gram-negative bacteria. The sequence of the protein was found to be similar but to a lesser degree to the *E. coli* PBP3-like proteins in *B. subtilis* (PBP2B and SPO*VD*) and to the *E. coli* and *H. influenzae* PBP2s (Table 1).

TABLE 1. Percent identity and total conservation of the amino acid sequences of PBP3 and PBP3x to those of other PBPs[a]

Protein[b]	No. of amino acids	PBP3x		PBP3	
		% Identity	% Conservation	% Identity	% Conservation
PBP3	579	48.3	63.2	100	100
PBP3x	565	100	100	48.3	63.2
EcoliPBP3	588	40.7	52.9	45.1	57.7
NgorPBP2	583	35.8	49.6	37.7	52.6
HinfPBP3	610	31.9	45.4	32.6	53.7
BsubSPOVD	645	26.7	38.9	25.7	39.0
HinfPBP2	651	21.9	35.4	23.5	36.3
EcoliPBP2	633	20.4	33.9	22.8	36.4
BsubPBP2B	716	23.0	35.9	21.8	34.4

[a] The protein sequences were analyzed by the PC Gene program (Intelli Genetics, Inc.) using the genetic code matrix with an open gap cost of 6 and an unit gap cost of 20. Similar or conserved amino acids are as follows: A, S, and T; D and E; N and Q; R and K; I, L, M, and V; and F, Y, and W.

[b] Abbreviations: EcoliPBP3, *E. coli* PBP3; NgorPBP2, *N. gonorrhoeae* PBP2; HinfPBP3, *H. influenzae* PBP3; BsubSPOVD, *B. subtilis* SPOVD protein; Hinf PBP2, *H. influenzae* PBP2; EcoliPBP2, *E. coli* PBP2; BsubPBP2B, *B. subtilis* PBP2B.

Expression of *pbpC* gene in *E. coli*. An efficient expression system using the RBS from pT7-7 (30) and the T7 RNA polymerase and its promoter in pBBR1MCS (16) was used for *pbpC* expression. The recombinant plasmid pXL629 containing the *pbpC* gene under control of the T7 promoter was transformed into *E. coli* BL21(DE3) in which the T7 RNA polymerase, under the control of the *lac* promoter, is integrated into the chromosome (30). Upon induction with IPTG (0.5 mM), a novel protein was observed by SDS-PAGE of the whole-cell-lysate samples of *E. coli* BL21(DE3)/pXL629, indicating that *pbpC* was efficiently expressed. The protein cofractionated with the cell membrane proteins (Fig. 3A), indicating that it was efficiently incorporated into the membranes of the *E. coli* cells. The [^{3}H]penicillin assay confirmed that the *pbpC* gene product bound penicillin (Fig. 3B). The PBP3x protein migrated faster than *E. coli* PBP3, with an apparent molecular mass of 55 kDa.

The amino acid sequence derived from the *pbpC* gene containing 565 amino acids had a calculated molecular mass of 61,128 kDa. However, the protein produced in *E. coli* migrated with an apparent molecular mass of 55 kDa. PBP3x did not appear to contain an N terminus characteristic of a typical signal peptide, nor did it contain a putative lipoprotein signal

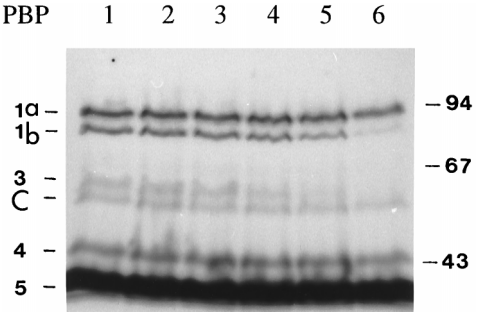

FIG. 4. Autoradiogram of cell membrane proteins illustrating competition of cefsulodin with [^{3}H]penicillin for the PBPs of *P. aeruginosa* PAO4089 expressing the *pbpC* gene product. The cefsulodin concentration increased from 0.0375 to 9.6 μg/ml in fourfold increases per lane from lanes 2 to 6; lane 1 is the control and contains no cefsulodin. Membrane proteins were prepared from cells grown in MH broth containing tetracycline and harvested at exponential phase. Numbers to the right of the gel are molecular masses (in kilodaltons). The positions of PBPs are indicated to the left of the gel, with the *pbpC* gene product indicated by the C. PBP2 was not observed in this experiment.

processing sequence as proposed for *E. coli* PBP3 (14). To confirm the lack of the N-terminal processing, the PBP3x protein was subjected to N-terminal amino acid sequencing after transfer to a polyvinylidene difluoride membrane following SDS-PAGE separation of the membrane protein sample prepared from *E. coli* K38/pGP1-2 (pXL732). The six N-terminal amino acids analyzed were SSQRRN, which was identical to that of the derived sequence, except that the first residue methionine was cleaved from the gene product of *pbpC*. Similar to *P. aeruginosa* PBP3 (16), the sequence at the N terminus of PBP3x produced in *E. coli* do not appear to have been removed. Therefore, the disparity in molecular mass may reflect posttranslational C-terminal processing, as observed for *E. coli* PBP3 (13).

Overproduction of the *pbpC* gene product in *P. aeruginosa*. *P. aeruginosa* PAO4089, deficient in producing chromosomal β-lactamase (12) was used as the host for gene expression experiments, since the wild-type strain of *P. aeruginosa* contains an inducible chromosomal β-lactamase which has been found to interfere with penicillin-binding assays (16). A 1.7-kb *Xba*I-*Bam*HI DNA fragment, containing the T7 RBS sequence from the pT7-7 plasmid and the *pbpC* gene, was isolated from pXL732 and cloned into the broad-host-range vector pUCP27 behind the *lac* promoter to generate pXL519. This plasmid was transformed into strain PAO4089. By [^{3}H]penicillin assay, a novel protein with a molecular mass of 55 kDa was detected from the cell envelope protein sample of PAO4089(pXL519) (Fig. 4, lane 1). By using a scanning densitometer, the amount of the recombinant *P. aeruginosa* PBP3x protein was estimated to be approximately the same as the amount of native PBP3 present in PAO4089(pXL546). Plasmid pXL546, which contained a DNA fragment 300 bp long (corresponding to the 3′ end of the *P. aeruginosa pbpB* gene) (16)) cloned in pUCP27, failed to direct the expression of novel product (data not shown) (16).

Effect of overproduction of PBP3x on susceptibility to β-lactam antibiotics. Previously we observed that overproduction of PBP3 in *P. aeruginosa* PAO4089 resulted in twofold-increased resistance to aztreonam, fourfold-increased resistance to cefepime, and eightfold-increased resistance to ceftazidime and cefsulodin (PBP3-targeted β-lactam antibiotics) but did not affect the susceptibility to the PBP1-targeted antibiotic cephaloridine and PBP2-targeted imipenem (16a). However, the production of PBP3x in *P. aeruginosa* had no effect on the susceptibility of PAO4089 to the β-lactam antibiotics mentioned above. To further examine this issue, we carried out a [^{3}H]penicillin-binding competition assay (e.g., Fig. 4). The results of competition binding assays indicated that the PBP3-targeted β-lactams, ceftazidime, cefsulodin, cefepime, and aztreonam, reacted primarily with the *P. aeruginosa* PBP3 and to a 3- to >16-fold-lesser extent with *P. aeruginosa* PBP3x (I_{50}s of = 0.2 versus 0.6 μg/ml for ceftazidime, 0.6 versus >9.6 μg/ml for cefsulodin, 0.0125 versus 0.06 μg/ml for cefepime, and 0.05 versus 0.32 μg/ml for aztreonam, for PBP3 versus PBP3x, respectively). This suggested that the *pbpC* gene product had lower affinity for the PBP3-targeted β-lactams tested than did the PBP3 protein and thus would not be expected to affect antibiotic susceptibility when PBP3 was present in the cells.

Mutational analysis of the *pbpC* gene. *P. aeruginosa* PBP3 and PBP3x with similar amino acid sequences and modular designs were both related to *E. coli* PBP3, but their genes mapped to different loci on the *P. aeruginosa* chromosome (17). The *pbpB* gene, located upstream of a gene encoding a homolog of the *E. coli murE* gene product (16), mapped within the major cell division gene cluster, whereas *pbpC*, which mapped 2 megabase pairs from *pbpB*, appeared not to associate with any gene involved in cell division. To test whether PBP3x was redundant in *P. aeruginosa* or whether it functioned in concert with PBP3, we constructed a PBP3x-defective interposon mutant by gene replacement. One such mutant, HC132, was isolated, digested with *Eco*RI and *Xho*I, and analyzed by Southern blot hybridization, confirming that it contained a 1.3-kb Km^r interposon inserted in the *pbpC* gene. HC132 grew at the same rate as its parent H103 in MH broth. Exponential- and stationary-phase cells of H103 and HC132 grown in MH broth were used to observe cell morphology with a light microscope. The mutation of the *pbpC* gene in HC132 did not cause any major change of cell shape. These results suggested that the *pbpC* gene product was not essential for normal cell morphology or viability under the conditions tested.

PBP profiles of the PBP3x-defective mutant. To further characterize the PBP3x-defective mutant, a [^{3}H]penicillin-binding assay was carried out using the membrane proteins of wild-type strain H103 and the mutant strain HC132. The membrane proteins were prepared from cells isolated from the exponential phase of growth. The results of this assay showed no difference between the PBP profiles of the wild-type cells and the mutant cells. Consistent with this, a potential σ^{70} binding promoter which had 19 nucleotides between the −10 and −35 sites (Fig. 2, nucleotides 267 to 272 and 292 to 297, respectively) was decidedly suboptimal. As shown in Fig. 2, the upstream sequence of *pbpC* contained a putative consensus sequence recognized by σ^s (31). Thus, we considered the possibility that the expression of the *pbpC* gene was regulated by growth stage. The results of PBP assays using membrane proteins of H103 and HC132, which were prepared from cells isolated at mid-exponential, late exponential, stationary, and late stationary phases of growth, suggested that *pbpC* gene expression was inversely dependent on growth rate (Fig. 5). In the exponential phase, PBP3 was efficiently produced, whereas no PBP3x was detected by [^{3}H]penicillin. When cells reached stationary phase, the PBP3 production level was dramatically decreased, whereas PBP3x appeared to be upregulated. The pattern of production of PBP2 seemed to be similar to that of PBP3x, suggesting that the expression of this PBP is also inversely growth rate regulated. The production of the other high-molecular-weight PBPs, PBP1a and PBP1b, seemed to steadily decrease as cells entered stationary phase, with PBP1a being more profoundly affected.

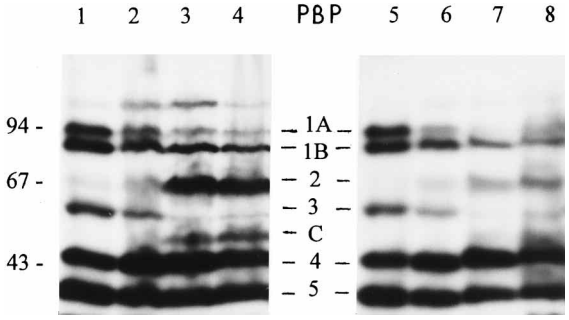

FIG. 5. Autoradiogram of cell membrane proteins after incubation with [^{3}H]penicillin and separation by SDS–7.5% PAGE. Lanes 1 to 4, wild-type strain H103; lanes 5 to 8, PBP3x-defective mutant strain HC132. Membrane proteins were prepared from cells growing in MH broth for 5 h (lanes 1 and 5), 9 h (lanes 2 and 6), 24 h (lanes 3 and 7), and 48 h (lanes 4 and 8). The numbers to the left of both gels are molecular masses (in kilodaltons). The positions of PBPs are indicated by the PBP numbers between the two gels, with PBP3x indicated by the C.

DISCUSSION

The data presented here are consistent with the suggestion that both *P. aeruginosa* PBP3 and PBP3x have structures and enzymatic functions similar to those of *E. coli* PBP3. Thus, the *pbpC* gene might have arisen as a gene duplication of the *pbpB* gene in *P. aeruginosa*. Interestingly, *B. subtilus* also has a second copy of its *pbpB* gene, known as *spoVD*, which is located between the *pbpB* and *murE* genes in *B. subtilis*. *spoVD* is expressed only during sporulation in *B. subtilis*, whereas the *pbpB* gene product is expressed during both vegetative growth and sporulation (7, 34). Thus, the two PBP3-like genes in *B. subtilis* have unique functions in cell growth, and their expression is differentially regulated. In *P. aeruginosa*, the *pbpB* gene is located in a cluster of genes involved in cell division and cell wall synthesis, whereas the *pbpC* gene is located 2 megabase pairs away (17) and is apparently not associated with genes involved in cell division. Data presented in this report suggested that the two genes encoding PBP3-like proteins in *P. aeruginosa* were also expressed differentially according to the growth phase. It appeared that *P. aeruginosa* PBP3 was downregulated when cells reached stationary phase. While this conventional PBP3 was downregulated, the production of the alternative PBP3, i.e., PBP3x, seemed to be upregulated. Similarly, it has been reported that σ^s downregulates the expression of *E. coli* PBP3 when cells enter stationary phase and cell division ceases (8). However, a second PBP3-like protein has not been identified in *E. coli* and is not evident in the *H. influenzae* genome sequence (9). In *E. coli*, PBP1a to PBP5 either remained unchanged during stationary phase or decreased over time, whereas the level of PBP6 was found to be 2- to 10-fold higher in stationary-phase cells than in exponentially growing cells (5). Our data showed that the production of *P. aeruginosa* PBP2 and PBP3x was upregulated in stationary phase. This implied that these proteins were especially required by the *P. aeruginosa* cells at this stage of growth. It has been shown that the cross-linkage index for cells increases after an extended stationary phase in *E. coli* cells (8). Thus, it might be that PBP2 (for the main body of the cells) and PBP3x (for the poles of the cells) are particularly required for peptidoglycan cross-linking or turnover during this stage when PBP3, PBP1a, and PBP1b are downregulated.

Several reports have described PBPs with a significant degree of functional overlap within a given species. This sometimes results in a failure to produce a phenotype different from that of the wild type upon mutation of a single PBP-encoding gene. This has been the case in studies of *E. coli* (28) and *B. subtilis* (22) PBP-encoding genes. In the former case, it has been shown that *E. coli* PBP1a and PBP1b appear to be redundant, since deletion of either the PBP1a- or PBP1b-encoding gene is tolerated. However, rapid lysis of *E. coli* occurs when both PBP1a and PBP1b are inhibited. In this study, we found that no differences from the wild-type phenotype were obvious in PBP3x-defective mutants. Therefore, the function of PBP3x in *P. aeruginosa* remains unclear. An attempt was made to construct a PBP3-defective mutant by using a gene replacement technique. However, no PBP3-defective mutant was obtained (15a). This could be due to the location of the *pbpB* gene at the proximal end of an operon for a cluster of cell division genes, where the placement of a polar mutation would be lethal to the cells. A conditional *pbpB* mutant or double mutation of *pbpB* and *pbpC* should provide information for testing whether PBP3 and PBP3x are indeed functionally redundant.

This work also revealed that production of the *pbpC* gene product in *P. aeruginosa* did not alter the susceptibility of the overexpressing strain to the PBP3-targeted β-lactam antibiotics tested. This probably resulted from the lower affinities of PBP3x (compared to PBP3) for the PBP3-targeted antibiotics, which could result from the nature of the various amino acid residues making up and surrounding the active binding site serine, since these are known to play roles in facilitating binding to the substrates (10, 15, 28). For example, it has been shown that replacement of threonine with proline in the second position of the SXXK motif of *E. coli* resulted in reduced affinity for cephalexin, a PBP3-targeted antibiotic (15). The amino acid sequences of the SXXK, SXN, and KTG motifs of *P. aeruginosa* PBP3 are STVK, SSN, and KSG, whereas for PBP3x they are SVIK, SSN, and KSG, respectively. Therefore, the different affinities of *P. aeruginosa* PBP3 and PBP3x for various substrates might be due to differences in the amino acids within the functional SXXK motif or in other amino acids that surround the active binding pocket.

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