Characterization of the *Pseudomonas aeruginosa* penicillin-binding proteins 3 and 3x: gene cloning, expression and role in susceptibility to β-lactam antibiotics

by

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ABSTRACT

Two degenerate oligonucleotides primers were synthesized based on the amino acid sequences found in the conserved SXXK and KTG motifs of *Escherichia coli* high-molecular-weight PBPs and *Nesseria gonorrhoeae* PBP2. The primers were subsequently used in a PCR amplification experiment using *Pseudomonas aeruginosa* PAO1 chromosomal DNA as the template. Five of the resulting PCR products were cloned and sequenced: two products that translated to sequences with strong homology to *E. coli* PBP3 and *N. gonorrhoeae* PBP2 were subsequently used as probes to clone the complete *pbpB* and *pbpC* genes; the other three PCR products were identified as the homologues of the *E. coli sucC/sucD*, *yhhF* and *cypH* gene products.

The derived amino acid sequence of *pbpB* gene had 45.1% identity to that of *E. coli* PBP3. The downstream sequence of *pbpB* encoded an amino acid sequence homologous to the *E. coli murE* gene product. These two genes mapped to the same region of the chromosome as did other cell division genes including *ftsA*, *ftsZ* and *envA*. Analyses of the translated sequence of the *pbpC* gene revealed that it had 40.7% identity to that of *E. coli* PBP3. The downstream sequence of *pbpC* encoded convergently transcribed homologues of the *E. coli soxR* and *Mycobacterium bovis adh* gene products. Its upstream sequence, about 370 bp in length, did not resemble any sequences in the GenBank database. The *pbpC* gene mapped 2 megabase pairs from the *pbpB* gene on the *P. aeruginosa* chromosome and apparently was not associated with genes involved in cell division. The upstream sequence of *pbpC* contained a potential σ^s recognition site, suggesting that the expression of this gene may be growth or stress regulated.

The *pbpB* and *pbpC* genes were expressed in *E. coli* by the T7 RNA polymerase and promoter system. The produced proteins were exported to the cytoplasmic membrane of *E. coli* cells and bound ^{3}H-penicillin. They had an apparent

molecular mass of 60 and 58 kDa respectively, whereas the calculated molecular mass were 63.69 and 61.128 kDa. The N-terminal amino acid sequences of the proteins produced in *E. coli* were identical to those deduced from the nucleotide sequences of the *pbpB* and *pbpC* genes, suggesting that there was no N-terminal processing.

The *pbpB* and *pbpC* genes were expressed in *P. aeruginosa* PAO4089 using a broad-host-range vector pUCP27. Results from the minimal inhibitory concentration testing and ^{3}H-penicillin binding competition assays indicated that overproduction of *pbpB* gene product led to increased resistance to the PBP3-targeted antibiotics aztreonam, cefepime, cefsulodin and ceftazidime whereas the presence of the *pbpC* gene product in PAO4089 did not have any effect on susceptibility to the tested PBP3-targeted antibiotics. *E. coli pbpB* gene was expressed in PAO4089 using pUCP27. Overproduction of the *E. coli pbpB* product in PAO4089 resulted in increased resistance to aztreonam, cefepime and ceftazidime.

The attempt to construct a PBP3-defective mutant using a gene replacement technique was not sucessful. This result could be due to the location of the *pbpB* gene at the proximal end of an operon containing a cluster of cell division genes, where the placement of a polar mutation would be lethal to the cells. Using the same approach, a PBP3x-defective mutant (strain HC132) was obtained and confirmed by Southern blot analysis. The PBP profiles of wild type strain H103 and mutant strain HC132 were similar, suggesting that the *pbpC* gene was not visibly expressed under the physiological conditions tested. Furthermore, inactivation of PBP3x did not cause any changes in cell morphology or growth rate, suggesting that *pbpC* was not required for cell viability under normal laboratory growth conditions.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

Ap	ampicillin
AZT	aztreonam
bp	base pair
CEPH	cephaloridine
CFPM	cefepime
CFS	cefsulodin
CFU	colony forming unit
Cm	chloramphenicol
CTZ	ceftazidime
dH_2O	distilled water
DIG	digoxigenin
hr	hour
HPLC	high performance liquid chromatography
I_{50}	concentration inhibiting ^{3}H-penicillin binding by 50%
IMIP	imipenem
IPTG	isopropyl-β-D-thiogalactoside
kb	kilobase
Km	kanamycin
kDa	kilodaltons
LB	Luria-Bertani

mb	megabase
MH	Mueller-Hinton
MIC	minimal inhibitory concentration
min	minute
OD_{600}	optical density at 600 nm
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBP	penicillin binding protein
PCR	polymerase chain reaction
PVDF	polyvinylidene difluoride
RBS	ribosome binding site
SDS	sodium dodecyl sulfate
sec	second
Tc	tetracycline

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INTRODUCTION

1. *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a motile, rod-shaped, Gram negative bacterium. It is commonly found in the environment, especially in soil, water and sediments. The broad environmental distribution of this organism is afforded by its minimal nutritional requirements (Palleroni, 1981; Durack, 1989).

P. aeruginosa has been recognized as an opportunistic pathogen that causes a variety of infections, usually in immunocompromised hosts such as severe burn patients, children with cystic fibrosis and patients treated with immunosuppresive drugs. Consequently, *P. aeruginosa* is almost exclusively a nosocomial pathogen and was found in nosocomial lung infections to cause the highest mortality rate of any bacterium (Bodey *et al.*, 1983, Cross *et al.*, 1983). It is intrinsically resistant to many commonly used antibiotics. Among the few effective agents are some newer β-lactams, aminoglycosides and quinolones (Durack, 1989; Korvick & Yu, 1991). Combination therapy with two or three antibiotics is frequently chosen for serious infections. Although new and supposedly more effective anti-pseudomonal antibiotics have been developed, *P. aeruginosa* quickly acquired resistance to these agents. Production of antibiotic-inactivating enzymes such as β-lactamase, changes in the permeability of the cell envelope and reduction in the affinity of target proteins

such as penicillin-binding proteins (PBPs) are believed to be the major mechanisms responsible for the high antibiotic resistance among most *P. aeruginosa* isolates (see section 3).

2. Penicillin-binding proteins (PBPs)

2.1 General properties of PBPs

Early studies on the mechanism of action of β-lactam antibiotics revealed that this group of compounds exerted their functions by acting as analogues of the acyl-D-alanyl-D-alanine (Fig. 1) moiety of the lipid-linked disaccharide-pentapeptide, the substrate of the enzymes catalyzing the crosslinking of the peptide side chains of nascent peptidoglycan (Tipper & Strominger, 1965). Later Blumberg & Strominger (1974) demonstrated that penicillin bound covalently to the enzymes that it inhibited. This discovery led to the development of a convenient autoradiographic method for the detection of penicillin-sensitive enzymes as penicillin-binding proteins (PBPs) (Spratt, 1977a). With this method, PBPs are readily detected and their relative amounts quantitated by incubation of bacterial membranes with radio-labeled penicillin G, followed by sodium dodecylsulfate-polyacrylamide gel electrophoresis and fluorography. The PBP assay proved to be very useful as it allowed the study of all of the penicillin-sensitive enzymes of bacterial cells and is still widely used by many researchers for PBP studies. It can also be used to assess the relative affinity of a PBP for a nonradiolabeled β-lactam antibiotic by determining the concentration of

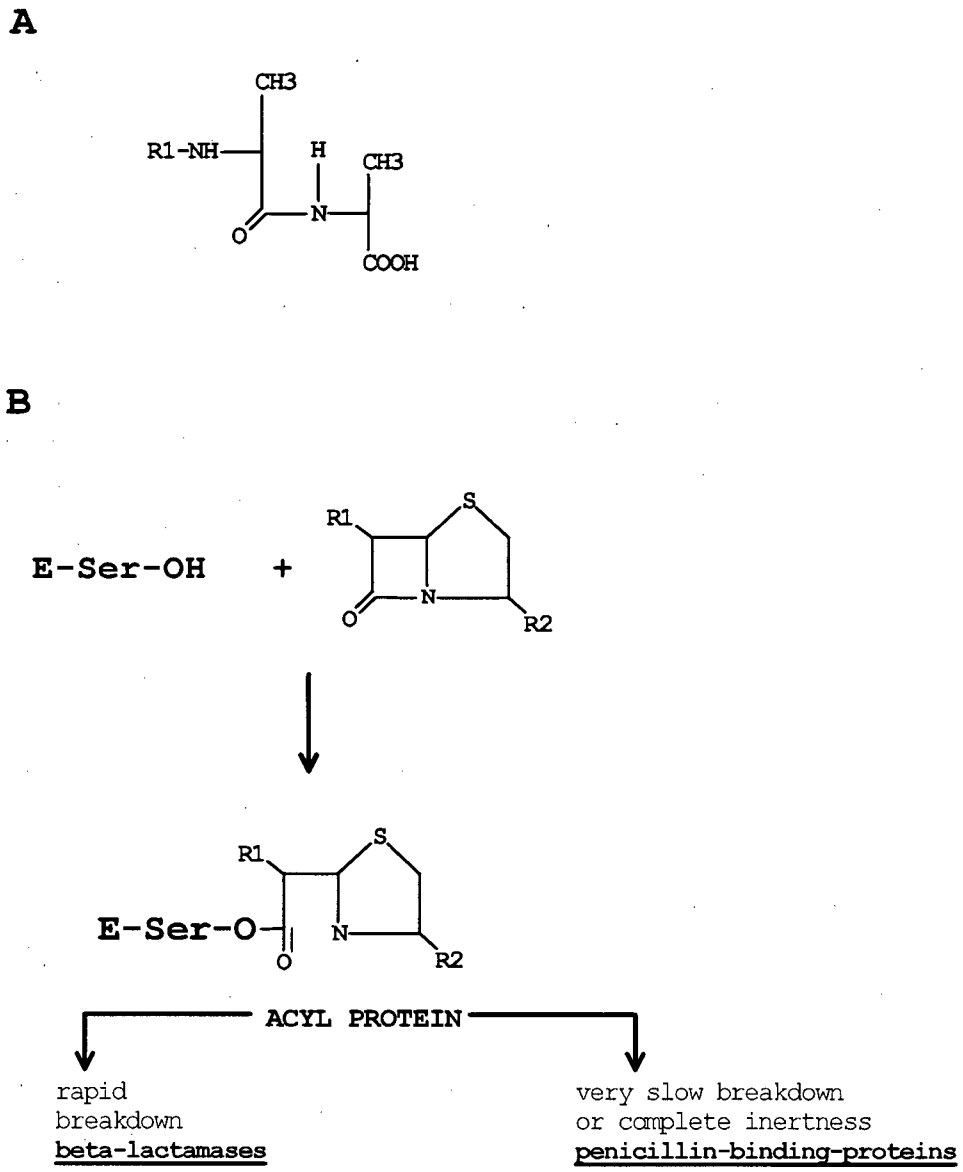

Figure 1. (A) Structure of acyl-D-alanyl-D-alanine illustrating its similarity to β-lactam molecule as shown in (B). (**B**) Biochemical reaction of β-lactam and the penicillin-interactive, active-site serine proteins (E-ser-OH) (Ghuysen, 1991).

the β-lactam antibiotic required to reduce the binding of radiolabeled penicillin G to the PBP by 50%, after preincubation with the unlabeled β-lactam antibiotic.

All bacteria possess multiple PBPs. The affinities of each PBP for penicillin and other β-lactam antibiotics vary widely, which is presumably due to small differences in the structure of the β-lactam compounds and to differences in the sequence (and thus presumably the configuration) of the amino acid residues around the active-site serine (see below) of the PBP. Each bacterial species has its own assortment of PBPs. In a given organism, PBPs are numbered in order of decreasing apparent molecular weight. Usually there is no equivalent relationship between identically numbered PBPs of two distantly related organisms, although more closely related bacteria show similar PBP patterns.

The most extensive studies on the targets of β-lactam antibiotics have been focused on the PBPs of *E. coli*. In *E. coli* cells, there are at least eight PBPs that can be detected by radiolabeled-penicillin G (Spratt, 1977a, Henderson *et al.* 1994), namely PBP1a, 1b, PBP2, PBP3, PBP4, PBP5, PBP6 and PBP7. Each of the genes coding for these proteins has been cloned and mapped on the *E. coli* chromosome (Table 1). The PBPs of *E. coli* are localized in the cytoplasmic membrane (Spratt, 1977a) and each of these proteins has unique structural features that result in distinct penicillin-sensitive enzymatic functions which enable the protein to function in cell growth and morphogenesis (see below). Based on their structural features and

Table 1. Properties of the penicillin-binding proteins of *Escherichia coli*

PBP	Relative molecular mass	Gene / location on chromosome	Mutant phenotype	Physiological function	Enzymatic activities
1a	93 500	*ponA* / 73.5 min	Cell lysis[a]	Cell elongation	Peptidoglycan transglycosylase & transpeptidase
1b	94 100[b]	*ponB* / 3.3 min	Cell lysis[a]	Cell elongation	Peptidoglycan transglycosylase & transpeptidase
2	70 867	*pbpA* / 14 min	Round cell[c]	Cell shape maintenance	Peptidoglycan transglycosylase & transpeptidase
3	63 850	*pbpB* / 2 min	Filamentous cell[c]	Cell division in association with the FtsW protein	Peptidoglycan transglycosylase & transpeptidase[d]
4	49 568	*dacB* / 70 min	None[e]	Cell wall lysis and cell wall maturation	D-ala-carboxy-peptidase & DD-endopeptidase
5	44 330	*dacA* / 14 min	None[e]	Cell wall maturation	D-ala-carboxy-peptidase
6	44 000	*dacC* / 19 min	None[e]	Cell wall maturation	D-ala-carboxy-peptidase
7	30 910	*pbpG* / 48 min	None[e]	Prevents autolysis in nongrowing cells	nt

a. Lethal when both *ponA* and *ponB* are mutated.
b. Four components of PBP1b were produced from the *ponB* gene, α (94 100 Da), β, γ and δ.
c. Only temperature sensitive mutants could be obtained; deletion mutation is lethal.
d. Measured in crude membrane preparations containing disproportionately large amounts of PBP3 and FtsW.
e. Single mutations are non-lethal. Double mutations of PBP4 and PBP5 or PBP5 and PBP6 are also non-lethal.

nt. not tested

enzyme activities, PBPs of *E. coli* are classified as the high-molecular-weight PBPs which include PBP1a, PBP1b, PBP2 and PBP3, and the low-molecular-weight PBPs which include PBP4, PBP5, PBP6 and PBP7.

2.2 PBPs: enzymes involved in the final steps of peptidoglycan biosynthesis

Nearly all bacteria contain a peptidoglycan layer that surrounds the cytoplasmic membrane. This peptidoglycan layer is a network structure composed of similar building blocks, but with different crosslinking moieties among the bacteria. Alternating residues of *N*-acetylglucosamine and *N*-acetylmuramic acid are linked through the D-lactate of *N*-acetylmuramic acid with a tetrapeptide of the structure L-Ala-D-Glu-L-Xaa-D-Ala, for which Xaa is often a diamino acid. Crosslinking varies extensively among bacteria.

PBPs are the enzymes responsible for the final steps of peptidoglycan biosynthesis. These final steps, with *E. coli* as an example, consist of the polymerization of the repeating disaccharide unit (transglycosylation) and the formation of the peptide crosslinking the amino terminal of *meso*-diaminopimelic acid and the carboxyl terminal of the penultimate D-alanine residue of the pentapeptide side chains accompanied by the removal of the terminal D-alanine residue (transpeptidation). The extent of the peptide crosslinking is controlled by a third reaction, DD-carboxypeptidation, which involves the transfer of the carbonyl group of the penultimate D-alanine residue of the pentapeptide to water, rather than to

an amino group (Fig. 2). β-lactam antibiotics, due to their structural similarity to the D-alanyl-D-alanine carboxyl terminal residues of the pentapeptide, inhibit the transpeptidation and DD-carboxypeptidation. The transglycosylation reaction is not penicillin-sensitive.

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. The dual enzymatic activities of the high-molecular-weight PBPs of *E. coli* were demonstrated by *in vitro* catalysis reactions on lipid-linked disaccharide-pentapeptide precursors, using purified PBP preparations [PBP1a (Tomioka *et al.*, 1982), 1b or PBP3 (Ishino *et al.*, 1981)] or membrane preparations lacking PBP1b and containing disproportionately large amounts of PBP2 or PBP3 (Ishino *et al.*, 1986).

The low-molecular-weight PBPs of *E. coli* act mainly as DD-carboxypeptidases. PBP4 has a highly penicillin-sensitive DD-carboxypeptidase activity (Matsuhashi *et al.*, 1977, Iwaya *et al.*, 1977) and a DD-endopeptidase activity which might account, in part, for the turnover of peptide cross-bridges in peptidoglycan (Korat *et al.*, 1991; Matsuhashi, 1994). PBP5 and PBP6 catalyze a DD-carboxypeptidation reaction that is moderately sensitive to penicillin (Amanuma *et al.*, 1980; Matsuhashi *et al.*, 1979). There are no reports on PBP7 enzymatic activity in peptidoglycan biosynthesis. The major functions of low-molecular weight PBPs possibly involve the maturation of peptidoglycans (Izaki *et al.*, 1968) and

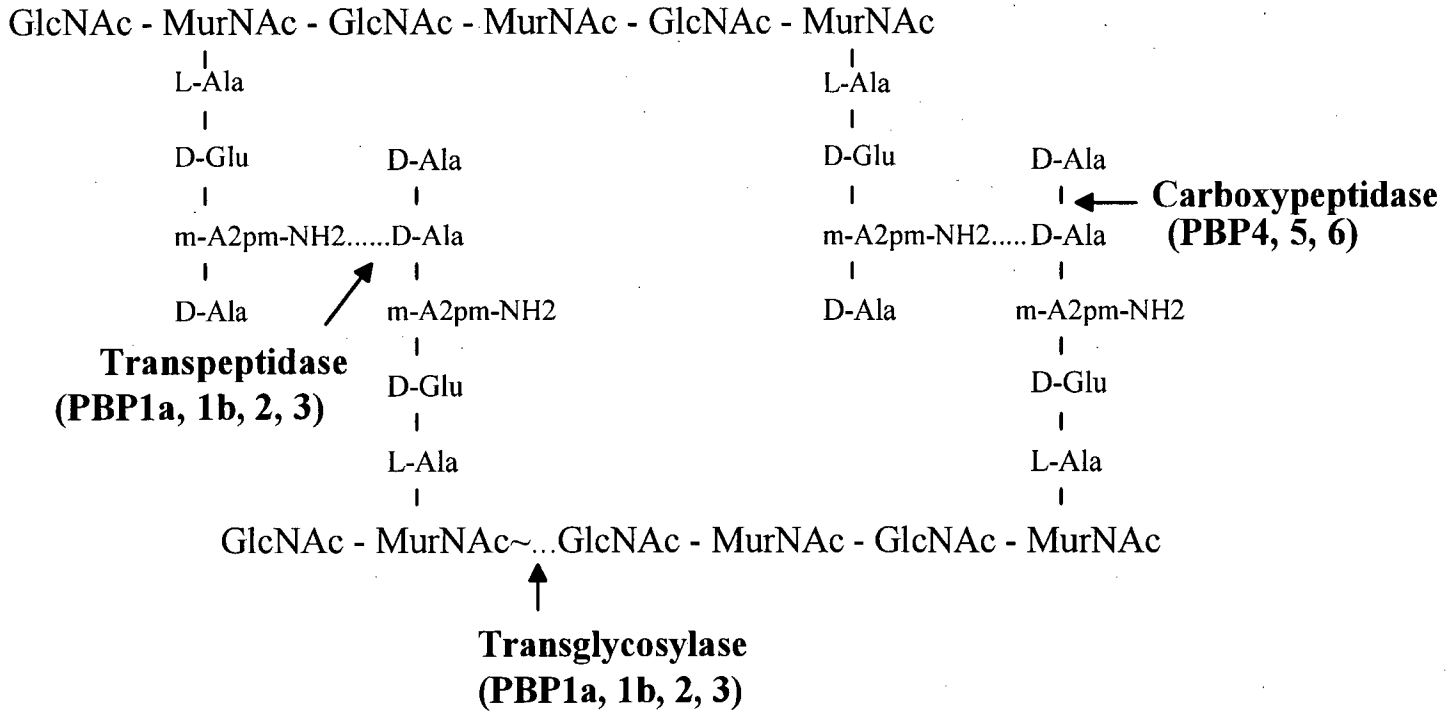

Figure 2. Schematic representation of *E. coli* peptidoglycan structure

regulation of the availability of peptidoglycan precursors (Begg *et al.*, 1990).

2.3 Structures of PBPs

2.3.1 Conserved motifs

PBPs are penicillin-interactive, active-site serine enzymes (or proteases) (Ghuysen, 1991). The acyl enzyme mechanism was proposed following the studies on the mechanism of β-lactam antibiotic action (Tipper & Strominger, 1965). Involvement of an essential serine at the active site was biochemically demonstrated for the reactions with low-molecular-weight PBP from *Streptomyces sp.* R61 (Frere *et al.*, 1976) and several class A and class C β-lactamases (Knott-Hunziker *et al.*, 1979; Cohen *et al.*, 1980; Fisher *et al.*, 1981). Site-directed mutagenesis experiments performed on the PBPs of *E. coli* and β-lactamases have confirmed the active-site serine mechanism (Keck *et al.*, 1985; Nicholas *et al.*, 1988). Central to this mechanism is the transfer of the electrophilic group R-C(=O)- of the scissile (peptide or amide) bond to the hydroxyl group of the active-site serine reside (Fig. 1B). The ester-linked acyl-enzymes formed by reaction between the β-lactam antibiotics and the β-lactamases are usually very short-lived. In contrast, those formed by reaction with the PBPs are usually very long-lived (Ghuysen, 1991). Consequently, the β-lactam antibiotics are substrates of the β-lactamases (thus being hydrolyzed) and covalent inactivators of transpeptidases and DD-carboxypeptidases, which can thus be detected

as penicillin-binding proteins.

The penicillin-interactive, active-site serine protein family consists of three members, class A and class C β-lactamases, low-molecular-weight PBPs, and high-molecular-weight PBPs. The primary sequences of the proteins in this family are highly divergent (Spratt *et al.*, 1988). However, they share certain common features in their primary and secondary structures. Common among this protein family are three conserved amino acid motifs: the tetrad active-site serine-x-x-lysine (SXXK), where x is a variable amino acid, the triad serine or tyrosine-x-asparagine [S(Y)XN] and the triad lysine or histine-threonine or serine-glycine [K(H)T(S)G]. When the protein folds, these motifs are brought close to each other, generating an active-site at the junction between an all-α domain, and an α-β domain whose five-stranded β-sheet is protected by additional α-helices on both faces. In this structure, the serine of the SXXK motif is in a central position in the cavity whereas the SXN and the KTG motifs are on either side (Ghuysen, 1991). When penicillin enters this cavity, the active-site serine covalently binds to the carbonyl moiety of the compound whereas the lysine or histidine of the K(H)T(S)G motif is in a position to promote the initial binding by providing a positive charge that can interact with the carboxylate moiety of the substrate (Malhotra *et al.*, 1992). Other amino acid residues surround the cavity also play certain roles in facilitating the binding to the substrate by hydrogen bonding or electrostatic interactions.

2.3.2 Domain structures

In the low-molecular weight PBPs and β-lactamases, the active-site serines reside close to the amino terminus of the proteins (Frere *et al.*, 1985). In the high-molecular weight 'bifunctional' PBPs, the acylated serine residues are located towards the middle of the sequences (Keck *et al.*, 1985) and the penicillin-sensitive transpeptidase domains (or the penicillin-binding domains) are located towards the carboxy-terminus of the proteins (Hedge & Spratt, 1984). The penicillin-binding domain is assumed to start 60 residues upstream of the essential SXXK motif and to terminate 60 residues downstream from the KTG motif (Ghuysen, 1991). In most high molecular weight PBPs, the penicillin-binding domain has a "tail" in the form of an approximately 100 amino acid carboxyl terminal extension (Ghuysen, 1994). The amino-terminal region, which has no counterpart in the low-molecular-weight PBPs and β-lactamases, is believed to contain the transglycosylase domain, although the evidence for this assignment is weak in most cases (Nakagawa *et al.*, 1984).

The high molecular weight PBPs are not synthesized as preproteins but have a highly hydrophobic region near the amino terminus that acts as a noncleaved signal-like sequence. Thus, this sequence could act not only to transport the protein to the periplasm but also to anchor the protein to the cytoplasmic membrane. This is consistent with fusion studies of PBP1b and PBP3 (with β-lactamase as a reporter),

which each has only a single transmembrane segment located near the amino-terminal end, with the remainder of the polypeptide located in the periplasm (Edelman *et al.*, 1987; Bowler & Spratt 1989). Of note is the sequence that precedes the hydrophobic segment. PBP1b contains a highly charged segment that is 63 amino acids in length, while PBP2 and PBP3 contain segments of 20 and 23 residues, respectively. PBP1a, an exception, contains a sequence that is only five residues in length (Ghuysen, 1994). Although PBP3 of *E. coli* contains the consensus sequence for modification and processing of lipoproteins ($L_{27}CGC_{30}$), only a small fraction appears to be modified along this pathway (Hayashi *et al.*, 1988). Instead PBP3 undergoes maturation by elimination of a 10-amino acid stretch from its carboxy-terminus (Nagasawa *et al.*, 1989). This reaction is catalyzed by the product of the *prc* gene (Hara *et al.*, 1991), a C-terminal-specific protease localized in the periplasm (Keiler *et al.*, 1995). The function of this posttranslational modification remains to be elucidated.

The low-molecular-weight PBPs contain a carboxy-terminal extension which is about 50 to 100 amino acids in length and starts approximately 60 residues down stream from the KT(S)G motif (Ghuysen, 1991). The end of the carboxy-terminal extension contains a signal-like peptide segment that serves as a membrane anchor. As a consequence, the bulk of the protein is on the outer surface of the cytoplasmic membrane (Ghuysen, 1991; van der Linden *et al.*, 1993).

2.4 Role of PBPs in *E. coli* growth and cell morphology

The role of PBPs in *E. coli* cell growth and morphogenesis has been elucidated by two approaches: i) morphological analyses of mutants with altered PBP patterns, and ii) correlation between the binding affinities of β-lactam antibiotics to particular PBPs and the morphological effects of the β-lactam antibiotics on *E. coli* cells (β-lactam antibiotics cause at least three morphological effects: rapid cell lysis, formation of spherical cells, filamentation).

The four high-molecular-weight PBPs are essential for cell growth and are thought to be the lethal targets of the β-lactam antibiotics (Table 1). Rapid lysis of *E. coli* occurs when both PBP1a and 1b are bound. However, deletion of either the PBP1a- or the PBP1b- encoding gene is tolerated, suggesting that PBP1a and 1b are redundant or at least capable of fulfilling compensatory roles in cell elongation (Spratt, 1975, 1977b; Tamaki *et al.*, 1977; Suzuke *et al.*, 1978).

Inactivation of PBP2, either with mecillinam (a PBP2-targeted β-lactam antibiotic) or by growing a mutant that produces a thermolabile form of PBP2 at the restrictive temperature, results in the growth of *E. coli* cells as spherical shapes (Spratt, 1975, 1978). Thus, PBP2 is essential for lateral cell wall elongation and maintenance of the rod shape.

E. coli PBP3 is involved in the formation of the septum during cell division. Mutations affecting PBP3 (temperature sensitive mutants) or the selective binding of

the protein by β-lactam antibiotics (*e.g.*, treatment with cephalexin, a PBP3-targeted antibiotic) result in the inhibition of cell division and the growth of *E. coli* as filamentous cells, and eventual cell death (Spratt, 1975, 1977b). 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, murC, ddl*) or for cell division and septum formation (*e.g., ftsW, ftsQ, ftsA and ftsZ*) (Ayala *et al.*, 1994). The *pbpB* gene is essential for *E. coli* cell growth. Only conditional mutants have been isolated (Hara *et al.*, 1992).

The low-molecular-weight PBPs 4, 5 and 6 of *E. coli* were suggested to be nonessential for cell survival and were therefore not considered to be of major importance in the killing mechanism of β-lactam antibiotics. This hypothesis is supported by the observation that mutation of *E. coli* PBP4 or double deletions of PBP5 and PBP6, do not produce any significant morphological abnormalities (Matsuhashi *et al.*,1977; Broome-Smith & Spratt, 1985). Recently, it was observed that an insertional mutation of the *E. coli pbpG* gene, which encodes PBP7, did not produce any obvious growth defects (Henderson *et al.*, 1995). Overall, low-molecular-weight PBPs are dispensable for *E. coli* cell viability under laboratory conditions.

2.5 PBPs in *P. aeruginosa*

PBPs of *P. aeruginosa* show an electrophoretic pattern similar to that of *E.*

coli, but are not well studied and their genes have largely not been isolated. Binding between the *P. aeruginosa* PBPs and β-lactam antibiotics generally results in morphological changes similar to those observed in *E. coli* (Curtis *et al.*, 1979a). Comparative studies of the binding affinities for various β-lactam antibiotics indicated that *P. aeruginosa* PBP1a, 1b, 2, 3, 4 and 5, respectively, corresponded to *E. coli* PBP1b, 1a, 2, 3, 4 and 5, and that *P. aeruginosa* PBP6 and PBP7 were not always detected (Noguchi *et al.*, 1979). The enzyme activity study by Noguchi *et al* is the only report on *P. aeruginosa* PBP function in peptidoglycan biosynthesis (Noguchi *et al.*, 1985). It indicated that PBP5 of *P. aeruginosa* had a moderately penicillin-sensitive D-alanine carboxypeptidase activity and that a defect in this enzyme activity was not lethal, as was found for the equivalent *E. coli* enzyme. Overall, very little is known about *P. aeruginosa* PBPs.

3. β-lactam susceptibility and resistance in Gram-negative bacteria

3.1 Antimicrobial activities of β-lactam antibiotics

Since penicillin G was first discovered 50 years ago, enormous numbers of mostly semi-synthetic β-lactam compounds have been produced. These compounds contain an essential four-membered lactam ring (see Figure 1, B) that can exist as an isolated ring, as depicted by the monobactams (*e.g.*, aztreonam), or may be fused to a five-membered ring (*e.g.*, penicillin, imipenem) or a six-membered ring (*e.g.*,

cephaloridine, ceftazidime, cefepime) to form bicyclic ring structures. β-lactam antibiotics are effective antimicrobial agents of low toxicity to eukaryotes. These agents often represent the first line of the therapy for the treatment of bacterial infections and have been used successfully for more than 50 years. The activity of a particular β-lactam is influenced by the type of substitutions attached to the basic nucleus.

PBPs from different bacterial species often have different binding affinities for β-lactam antibiotics. The inactivation of PBP1a and 1b, or PBP2, or PBP3, is lethal for *E. coli*. The β-lactam antibiotics can therefore kill by three completely different routes (rapid cell lysis, production of spherical cells and eventual cell death, or inhibition of cell division and eventual cell death). Some effective derivatives are known that kill exclusively by rapid lysis (*e.g.*, cefsulodin), by production of spherical cells and eventual cell death (mecillinam), or by filamentation and eventual cell death (cephalexin) (Curtis *et al.,* 1979a; Spratt, 1983). More typically, β-lactam antibiotics kill bacteria by two or all three of these mechanisms. Monobactams and many cephalosporins, which have structures containing the essential four-membered lactam ring and the adjacent six-membered ring, have very high binding affinity to *E. coli* PBP3 (Curtis *et al.*, 1979b; Georgopapadakou *et al.*, 1983; Hayes *et al.*, 1983), with filamentation being the initial response of the cell on exposure to the antibiotics. However, they also bind weakly but significantly to the bifunctional PBP1a and 1b,

eventually causing lysis of the organism. Carbapenems, a group of β-lactams containing the essential four-membered lactam ring and an adjacent five-membered ring, have very high binding affinity to PBP2 in *E. coli* and other enteric bacteria. Although this interaction initially results in the formation of spherical cells, lysis subsequently occurs resulting from additional binding to PBP1a or 1b, or both (Spratt *et al.,* 1977a). In *P. aeruginosa*, PBP3 is a major target of most of the third and fourth generation cephalosporins (Hayes *et al.*, 1983; Maejima *et al.,* 1991; Watanabe *et al.,* 1992).

Gram-negative bacteria are surrounded by a complex envelope (Fig. 3). In addition to the cytoplasmic membrane, these organisms usually have a thin peptidoglycan layer covered by an outer membrane. Between the peptidoglycan layer and the cytoplasmic membrane lies the periplasmic space, the location of most of the β-lactamases found in Gram-negative bacteria. The target proteins of β-lactam antibiotics, PBPs, are located on the outer surface of the cytoplasmic membrane and the polypeptides extend into the periplasm. Therefore β-lactam antibiotics need to traverse the outer membrane to gain their access to their target proteins. Thus, the antimicrobial activity of β-lactam antibiotics depends on a combination of factors including not only their affinity with the target PBPs, but also their rate of passage through the outer membrane barrier and their resistance to hydrolysis by β-lactamases.

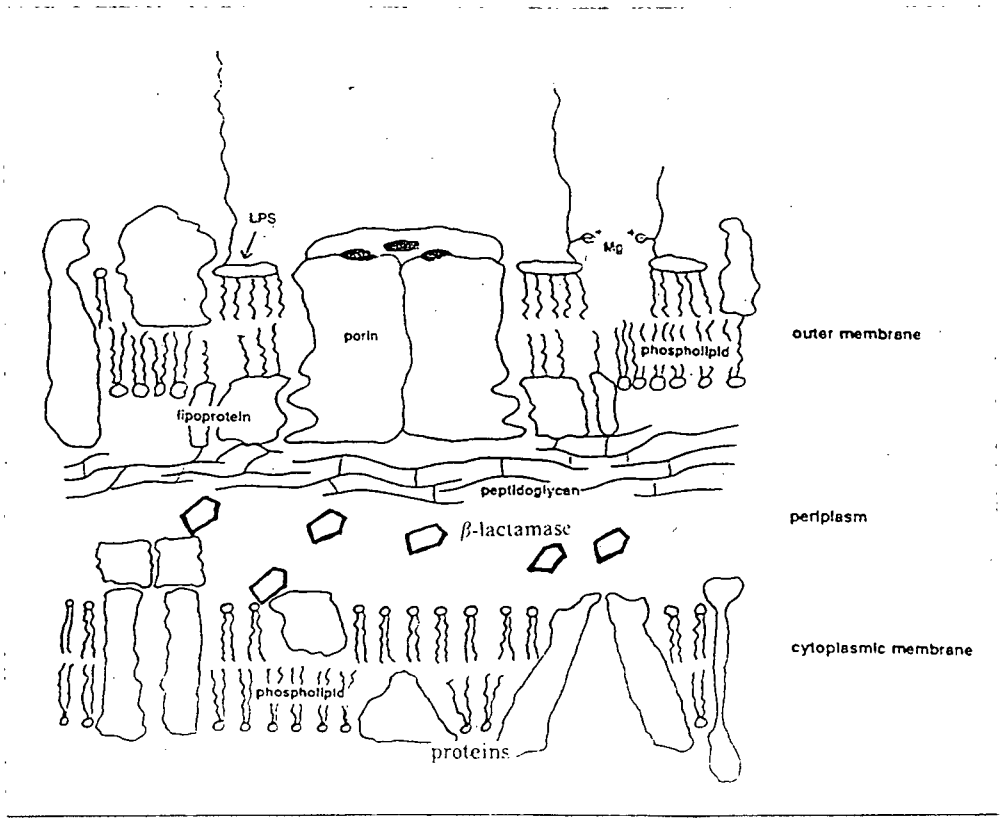

Figure 3. Schematic representation of a cross section of the cell envelope of a Gram-negative bacterium

The outer membrane is an asymmetric bilayer consisting of lipopolysaccharide (LPS) (in the outer leaf) and phospholipid (in the inner leaf) bilayers studded with proteins (Fig. 3). The outer membrane is usually described as a semi-permeablebarrier, in which hydrophilic molecules of sizes below a given exclusion limit can pass through protein channels called porins. Porins are water-filled, non-specific, transmembrane diffusion channels (diameter 0.6 - 2.3 nm) (Nikaido & Vaara 1985). The permeability of the porin channels for a β-lactam antibiotic is a function of the size, charge, and hydrophobic nature of the β-lactam molecule. β-lactam antibiotics differ considerably in charge, a factor that affects their rate of passage through porin channels. In *E. coli* and related *Enterobacteriaceae*, the porin channels favour hydrophilic, cationic molecules (Benz, Schmid & Hancock, 1985; Nikaido, 1989). The total area of channels per outer membrane influences antibiotic uptake. This explains the greater intrinsic resistance to antibiotics of *P. aeruginosa* compared to *E. coli*, since in *P. aeruginosa* only a small fraction of the porin molecules form open channels (Nicas & Hancock, 1983).

3.2 β-lactam resistance in Gram-negative bacteria

Resistance of Gram-negative bacteria to β-lactam antibiotics can result from a decrease in diffusion across the outer membrane, from the destruction of the antibiotics by β-lactamases, or from a reduction in the affinity of the target penicillin-

binding proteins (Hancock *et al.*, 1988; Malouin *et al.*, 1986).

Alteration in outer membrane permeability resulting from porin loss or modification have been described in Gram-negative bacteria including *P. aeruginosa* (Angus *et al.*, 1982; Godfrey *et al.*, 1987). However, most strains with altered permeability have been generated in the laboratory. These mutants confer only moderate increases in β-lactam resistance (Harder *et al.*, 1981; Jaffe *et al.*, 1983). There are fewer reports of the isolation of similar mutants from clinical samples (Nikaido, 1989b). It is possible that there is a limit to the extent that the permeability of the outer membrane can be reduced without seriously impairing the growth of the bacteria by limiting the uptake of nutrients (especially for *P. aeruginosa* given its already low level permeability compared to *E. coli*). Reports on the mechanisms responsible for β-lactam antibiotic resistance in clinical isolates often involve the production of β-lactamases (Sanders *et al.*, 1992), which wholly or partly account for the observed β-lactam resistance (Livermore, 1993). High levels of resistance usually result from the combined effect of decreased permeability and the presence of a β-lactamase (Angus *et al.*, 1982; Hancock *et al.*, 1988) or the alteration of PBPs (Mirelman *et al.*, 1981). The latter is found particularly in non-β-lactamase-producing bacteria (Malouin *et al.*, 1986).

Clinical resistance to β-lactam antibiotics in Gram-negative bacteria is not commonly associated with altered PBPs (Georgopapadakou, 1993). This is probably

due to the effectiveness of β-lactamase, coupled with reduced outer membrane permeability, in producing resistance. There are no reports of PBP-mediated clinical resistance in *E. coli*. However, PBP-mediated resistance to β-lactam antibiotics is well documented in *Haemophilus influenzae* (Malouin *et al.* 1986; Spratt, 1994) and *Neisseria gonorrhoeae* (Spratt, 1994). Non-β-lactamase-mediated resistance to β-lactams in clinical isolates and laboratory mutants of *P. aeruginosa* has been reported and is associated with a reduction in PBP3 binding affinity (Godfrey *et al.,* 1981; Gotoh *et al.*, 1990).

4. Aims of this study

The initial aims of this study were to clone and characterize the *P. aeruginosa* gene encoding PBP3 and investigate the role of this protein in susceptibility to β-lactam antibiotics. PBP3 was chosen for study since it is the primary target of newer generation β-lactams (Maejima *et al.*, 1991, Watanabe *et al.*, 1992). Furthermore, it has been reported that non-β-lactamase-mediated resistance to β-lactams in clinical isolates and laboratory mutants of *P. aeruginosa* was associated with a reduction in PBP3 binding affinity (Godfrey *et al.,* 1981; Gotoh *et al.*, 1990). Therefore, it seemed likely that *P. aeruginosa* PBP3 plays an important role in susceptibility to β-lactam antibiotics.

Preliminary results from the experiments of cloning the PBP3-encoding gene

indicated that *P. aeruginosa* might contain two copies of a gene encoding proteins with similar functions to *E. coli* PBP3. To test this hypothesis, further research was proposed to clone and characterize both the *P. aeruginosa* genes encoding *E. coli* PBP3-like proteins. The final goal of this project also included the investigation of roles of these two proteins in cellular functions and susceptibility to β-lactam antibiotics.

MATERIALS AND METHODS

I. Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in this study are described in Table 2 and Table 3. Bacterial strains were maintained as frozen stocks in LB broth plus 7% dimethyl sulphoxide at -70°C. *E. coli* strains were grown in LB broth (Difco, Detroit, MI). *P. aeruginosa* strains were grown in MH broth (Becton Dickinson, Cockeysbille, MD). Solid media were made by the addition of 2% Bacto-agar (Difco, Detroit, MI). VBMM (3 g/L sodium citrate, 2 g/L citric acid, 10 g/L K_2HPO_4, 3.5 g/L $NaNH_4HPO_4{\cdot}4H_2O$, pH 7.0; 0.8 M $MgSO_4$, 0.08 M $CaCl_2$) (Vogel & Bonner, 1956) was used to select growth of *P. aeruginosa,* since *E. coli* can not 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.

II. DNA manipulation

DNA manipulations were performed essentially as described by Maniatis *et al.* (1982). Chromosomal DNA was prepared by the method of Ausubel *et al.* (1987). DNA probes for Southern hybridization experiments were labeled with

Table 2. Bacterial strains

Strain	Relevant characteristics	References or sources
E. coli DH5α	*supE44 lacU169* (*80lacZ M15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	BRL
E. coli JM110	*dam dcm supE44 thi leu rpsL lacY galK galT ara tonA thr tsx* (*lac-proAB*) *F'*[*traD36 proAB*$^+$ *lacI*^{q} *lacZ M15*]	BRL
E. coli K38	HfrC (λ)	Russel & Model (1984)
E. coli BL21(DE3)	*hsdS gal* (λcIts857 *ind*1 *Sam*7 *nin*5 *lac uv*5-T7 gene 1)	Studier & Moffat (1986)
E. coli S17-1	*thi pro hsdR recA*; mobilizer strain	Simon *et al.* (1986)
P. aeruginosa H103	PAO1, prototroph	Hancock & Carey (1979)
P. aeruginosa PAO4089	*met*-9020 *pro*-9024 *bla*J9111 *bla*P9202	Gotoh *et al.* (1990)
P. aeruginosa HC132	*pbpC* mutant of PAO1, Km^r	This study

Table 3. Plasmids

Plasmid	Relevant characteristics	References or sources
pTZ18U	General cloning vector, Ap^r	USB
pTZ19U	General cloning vector, Ap^r	USB
pUCP27	Broad host-range vector, Tc^r	Schweizer (1994)
pT7-7	Cloning vector containing the T7 RNA polymerase promoter and RBS sequence, Ap^r	Studier Moffat (1986)
pGP1-2	Contains heat inducible gene for T7 RNA polymerase, Cm^r	Studier & Moffat, (1986)
pBBR1MCS	Broad-host-range vector containing the β-galactosidase gene and the T3 and T7 promoters, Cm^r	Kovach *et al.* (1994)
pUC4KPA	Gene cartridge vector containing a 1.3 kb fragment which encodes the enzyme conferring kanamycin resistance, Km^r	Pharmacia
pNOT19	pUC19 with 10 bp *Nde*I-*Not*I adapter in *Nde*I site, Ap^r	Schweizer (1992)
pMOB3	Derivative of pHSS21 containing *oriT* and *sacB*, Km^r, Cm^r	Schweizer (1992)
pPH125	Analogue of pBR322 and pAT153 in which Km^r replaces Ap^r, Km^r, Tc^r	Spratt *et al.* (1986)

Table 2-continued

pPH115	*E. coli pbpB* gene on a 2.6 kb *Bam*HI-*Eco*RI fragment cloned at *Hind*III site within the Tc^r gene of pPH125, Km^r	Spratt *et al.* (1986)
pXL2	A 580 bp PCR product corresponding to portion of *pbpB* gene cloned in pTZ18U, Ap^r	This study
pXL3	A 580 bp PCR product corresponding to portion of *pbpC* gene cloned in pTZ18U, Ap^r	``
pXL5	A 380 bp PCR product corresponding to portion of *yhhF* gene cloned in pTZ18U, Ap^r	``
pXL8	A 510 bp PCR product corresponding to portion of *sucC/sucD* genes cloned in pTZ18U, Ap^r	``
pXL12	A 460 bp PCR product corresponding to portion of *cypH* gene cloned in pTZ18U, Ap^r	``
pSPH1	A 1.4 Kb *Sph*I fragment of PAO1 DNA cloned in pTZ19U, Ap^r	``
pPST18	A 1.8 Kb *Pst*I fragment of PAO1 DNA cloned in pTZ19U, Ap^r	``
pXSm16	A 4.4 Kb *Sma*I-*Xho*I fragment of PAO1 DNA cloned in pTZ19U, Ap^r	``
pXLSH36	A 5.4 Kb *Sph*I-*Xho*I fragment of PAO1 DNA containing the *pbpB* and *murE* cloned in pTZ19U, Ap^r	``
pXLBI3	A 5.4 Kb *Sph*I-*Xho*I fragment of PAO1 DNA containing the *pbpB* and *murE* cloned in pTZ18U, Ap^r	``

Table 2-continued

pXL706	*P. aeruginosa pbpB* cloned between *Nde*I and *Bam*HI sites of pT7-7, Ap^r	``
pXL608	A 1.7 kb *Xba*I-*Bam*HI fragment from pXL706 containing the RBS sequence and *pbpB* cloned in pBBR1MCS, Cm^r	``
pXL506	A 1.7 kb *Xba*I-*Bam*HI fragment from pXL706 containing the RBS sequence and *pbpB* cloned in pUCP27, Tc^r	``
pXL546	A 300 bp of *Sph*I-*Bam*HI fragment from pXL706 containing 3′end of the *pbpB* gene cloned in pUCP27, Tc^r	``
pXL706::Km	A 1.3 kb Km^r cartridge cloned at *Sma*I site of the *pbpB* in pXL706, Ap^r	``
pBPB::Km	A 5.8 kb *Not*I fragment containing the *OriT*, *sacB* and Cm^r sequences cloned in pBPB1::Km, Ap^r, Km^r, Cm^r	``
pXL-Xh401	A 2.5 kb *Xho*I fragment of PAO1 DNA cloned in pTZ19U, Ap^r	``
pXL-PS406	A 4.0 kb *Pst*I-*Sal*I fragment of PAO1 DNA cloned in pTZ19U, Ap^r	``
pXL-Ec405	A 2.0 kb *Eco*RI fragment of PAO1 DNA cloned in pTZ19U, Ap^r	``
pXL-KE24	A 3.7 kb *Kpn*I-*Eco*RI fragment of PAO1 DNA containing the *pbpC* cloned in pTZ19U, Ap^r	``
pXL-Hd2	A 2.7 kb *Hind*III-*Eco*RI fragment of PAO1 DNA containing the *pbpC* cloned in pTZ19U, Ap^r	``

Table 2. continued

pXL-Xb5	A 2.3 *Xba*I-*Eco*RI fragment of PAO1 DNA containing the *pbpC* cloned in pTZ19U, Ap^r	``
pXL732	*P. aeruginosa pbpC* cloned between *Nde*I and *Bam*HI sites of pT7-7, Ap^r	``
pXL629	A 1.7 kb *Xba*I-*Bam*HI fragment from pXL732 containing the RBS sequence and *pbpC* cloned in pBBR1MCS, Cm^r	``
pXL519	A 1.7 kb *Xba*I-*Bam*HI fragment from pXL732 containing the RBS sequence and *pbpC* cloned in pUCP27, Tc^r	``
pXL732::Km	A 1.3 kb Km^r cartridge cloned at *Sma*I site of the *pbpC* gene in pXL732, Ap^r	``
pBPC::Km	A 5.8 kb *Not*I fragment containing the *OriT*, *sacB* and Cm^r sequences cloned in pBPC1::Km, Ap^r, Km^r, Cm^r	``
pXLK20	A 2.6 kb *Eco*RI-*Bam*HI fragment from pPH115 containing *E. coli pbpB* cloned in pUCP27 behind the *lac* promoter, Tc^r	``

^{32}P-dATP (Amersham, Canada) or digoxigenin-dUTP (Boehringer Mannheim) by the random primer labeling method. Restriction enzymes and DNA modification enzymes were purchased from either BRL, Pharmacia Canada, Boehringer-Mannheim Canada or New England Biolabs Canada, and used according to the manufacturer's directions.

Oligodeoxyribonucleotides were synthesized with an ABI model 392 DNA/RNA synthesizer (Applied Biosystems Inc., Foster City, CA). Synthesized oligonucleotides were purified by the method of Sawadogo and Dyke (1990).

III. DNA sequencing

Plasmid DNA for sequencing was prepared by the Qiawell-8 plasmid purification system (Qiagen Inc., Chatsworth, CA) according to the manufacturer's directions. DNA concentrations were determined with a mini-fluorometer (model TKO 100 , Hoefer Scientific Instruments, San Francisco, CA). DNA sequencing was done with an ABI Model 373 automated DNA sequencer and dye terminator chemistry following the protocols from ABI using the universal forward and reverse primers. Both DNA strands were sequenced. Nested deletions were created with the Erase-a-base kit (Promega, Madison, WI). Oligonucleotide primers were constructed to fill in gaps.

IV. DNA and amino acid sequence analyses

DNA and amino acid sequences were analyzed with the PC Gene, ESEE and DNAMAN computer programs. Sequences were compared to the GenBank database with the BLASTN, BLASTP and BLASTX programs (Altschul *et al.* 1990).

V. Transformation

E. coli was transformed by the $CaCl_2$ method (Maniatis *et al.* 1982). *P. aeruginosa* competent cells were prepared by the procedure of Schweizer (1991). Briefly, cells from exponential phase cultures were washed with ice cold 100 mM $MgCl_2$, resuspended in 100 mM $CaCl_2$ and incubated on ice for 30 min. Subsequently, the cells were pelleted by centrifugation and resuspended in ice cold 100 mM $CaCl_2$ plus 10% glycerol, aliquoted and stored at -70°C until needed. For transformation, competent cells were thawed on ice and mixed with plasmid DNA. The cells were then incubated on ice for 30 to 60 min, heat shocked for 3 min at 37°C followed by incubation on ice for 5 min; 1 ml of LB broth was added and the cells incubated at 37°C with agitation for 1.5 to 2.5 hrs to allow for the expression of the antibiotic resistance gene. The cells were plated on selective media and incubated at 37°C for 24 to 48 hrs.

VI. Construction of a PBP3 or PBP3x-defective mutant

An improved gene replacement technique described by Schweizer (1992) was

used to construct a PBP3-defective or PBP3x-defective mutant. Plasmids pNOT19, pMOB3 and pUC4KAPA were used. pNOT19 is derived from pUC19 with the unique *Nde*I site changed to a *Not*I site. pMOB3 contains the MOB3 cassette as a 5.8 kb *Not*I fragment which is composed of *oriT*, the *Bacillus subtilis sacB* gene as a counter-selectable marker, and a chloramphenicol resistance gene allowing positive selection of both the *oriT* and the *sacB*. pUC4KAPA contained a 1.3 kb fragment which was derived from Tn901, which encoded the enzyme conferring kanamycin resistance, flanked by identical restriction enzyme recognition sites.

6.1 Construction of plasmids pBPB::Km and pBPC::Km

The *pbpB* and *pbpC* genes on the plasmid pXL706 (see section VII) and pXL732 (see section VII) were mutated by the insertion of a 1.3 kb blunt ended *Hinc*II fragment of Km^r cartridge (isolated from pUC4KPA) at the unique *Sma*I site in the *pbpB* or *pbpC* gene. This procedure permitted the generation of plasmid pXL706::Km which had 0.96 kb and 0.82 kb of chromosomal DNA sequence on either side of the Km^r cartridge, and the plasmid pXL732::Km which had 1.1 kb and 0.5 kb of chromosomal DNA sequence on either side of the Km^r cartridge. The 3.0 kb fragment containing the *pbpB*::Km^r or *pbpC*::Km^r was isolated from pXL706::Km or pXL732::Km and cloned into pNOT19, respectively. Subsequently, the MOB3 cassette was isolated as a 5.8 kb *Not*I DNA fragment from pMOB3 and cloned into the unique *Not*I site on pNOT19 with *pbpB*::Km^r or *pbpC*::Km^r to

generate plasmids pBPB::Km (Fig. 20, see Results Chapter Two) and pBPC::Km (Fig. 30, see Results Chapter Three) respectively, which were then separately transformed into an *E. coli* mobilizing strain S17-1.

6.2 Conjugation

Transfer of plasmids from the *E. coli* mobilizing strain S17-1 to *P. aeruginosa* PAO1 strain H103 was achieved by biparental mating as follows. The donor (*E. coli* S17-1) was grown overnight in LB broth at 30°C. The recipient (*P. aeruginosa* H103) was grown overnight in LB broth at 42°C. Samples of the donor and recipient cultures (100 ul of each) were mixed, diluted into 2 ml LB broth and incubated at room temperature for 30 min. The cell mixture was then filtered onto a membrane (0.45 um; Nalgene, Rochester, NY). The filter was placed cell-side up on a LB agar plate and incubated at 30°C overnight. The cells were then washed off the membrane into 1 ml of sterile saline, diluted and spread onto VBMM agar plates containing carbenicillin, chloramphenicol and kanamycin to allow for the selection of plasmid integration. The transconjugates were then plated onto MH agar containing kanamycin and 5% sucrose to select for the cointegrates and deletion of plasmid sequences.

VII. Production of *pbpB* and *pbpC* DNA probes by degenerate PCR

Degenerate PCR primers for the amplification of portions of the *pbpB* and

pbpC genes were designed based on amino acid sequences surrounding the conserved SXXK and KTG motifs found in amino acid alignments of *E. coli* PBP1A, 1B, 2, 3 and *N. gonorrhoeae* PBP2 proteins, after adjusting the sequences according to the codon usage of *P. aeruginosa* (West & Iglewski, 1988). The sequence of the degenerate upstream primer corresponding to the SXXK motif was 5'-TTTGAATTCGG(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 corresponding to the KTG motif was 5'-AAAGAATTCG(CT)T(C)T(G)C(G)GT(C)C(G)GTGCCG (C)G(C)T(A)CTT-3' which corresponded to the amino acid sequence KT(S)GTT(A)K(QRN); the letters in parentheses represent alternative nucleotides or amino acids at given positions in the sequence corresponding to the preceding nucleotide or amino acid. An *EcoR*I restriction site sequence (underlined) was included at the 5' end of both primers to facilitate subsequent cloning of PCR amplification products.

PCR amplification 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 sec, 37°C for 30 sec and 72°C for 90 sec. The primer annealing temperature was raised from 37°C to 55°C for the remaining 25 cycles.

VIII. Protein expression using the T7 RNA polymerase/promoter system

8.1 Construction of recombinant clones

The *P. aeruginosa pbpB* and *pbpC* genes cloned in pT7-7 were amplified by PCR. The upstream primer contained an *Nde*I recognition sequence and the sequences coding for the N-terminus of PBP3 or PBP3x. Their sequences were respectively, 5'-TAAACATATGAAACTGAATTATTTCCAGGGCGCCCT-3' and 5'-AAACATATGAGCAGTCAACGCCGAAACTACCGCTTCA-3'. The downstream primer contained the sequences coding for the C-terminus of PBP3 or PBP3x followed by a stop codon and the sequence for a *Bam*HI recognition site. Their sequences were 5'-AAAGGATCCTCAGCCACGCCCTCCTTTTGCGG GCGCA-3' and 5'-AAAGGATCCTCAGCCGTGGTGCTGGCGGTCGGCGA-3' respectively. The PCR was performed under conditions involving 25 temperature cycles of 96°C for 60 sec, 63°C for 60 sec and 72°C for 90 sec, followed by a 10 min primer extension at 72°C. $Vent_R$ DNA polymerase was used for all PCR amplifications according to the manufacturer's recommendations. The resulting PCR product of 1.7 kb in length, corresponding to the size of the *pbpB* or *pbpC* gene, was digested with *Nde*I and *BamH*I and cloned into the *Nde*I/*BamH*I digested pT7-7. The resulting plasmids pXL706 (Fig. 10B, see Results Chapter Two) and pXL732 (Fig. 21B, see Results Chapter Three) were transformed into *E. coli* K38/pGP1-2.

The following procedure was used for the construction of the recombinant clone containing the *pbpB* or *pbpC* gene in the broad-host range vector pBBR1MCS.

A 1.7 kb *Xba*I-*Bam*HI fragment isolated from pXL706 or pXL732, which contained an RBS sequence from pT7-7 and the *pbpB* or *pbpC* gene, was cloned behind the T7 promoter on the vector pBBR1MCS to generate plasmids pXL608 (Fig. 13, see Results Chapter Two) or pXL629 (Fig. 24, see Results Chapter Three), which were then separately transformed into *E. coli* BL21(DE3).

8.2 Analyses of protein expression

LB broth containing ampicillin and kanamycin was inoculated at a 1:40 ratio with overnight cultures of *E. coli* K38/pGP1-2 (pXL706), K38/pGP1-2 (pXL732) or K38/pGP1-2 (pT7-7) grown at 30°C in LB broth. The freshly inoculated cultures were grown at 30°C until they reached an OD_{600} of 0.45. The cultures were then incubated at 42°C for 30 min to induce expression of T7 RNA polymerase from pGP1-2 and the resulting expression of *pbpB* or *pbpC* under the control of the T7 promoter. Cell cultures were grown for an additional 90 min at 37°C before harvesting.

Examination of protein expression for the genes cloned in pBBR1MCS was done as follows. LB broth plus chloramphenicol was inoculated at a ratio of 1:50 with overnight cultures of BL21(DE3)/pXL608, BL21(DE3)/pXL629 or BL21(DE3)/pBBR1MCS. The freshly inoculated cultures were grown at 37°C to an OD_{600} of 0.6. IPTG was then added into the cultures to a final concentration of 0.5 mM to induce expression of T7 RNA polymerase and *pbpB* or *pbpC* under the

control of the T7 promoter. Cultures were further incubated at 37°C for 1 to 4 hrs before harvesting.

IX. SDS-PAGE

Protein profiles were analyzed by SDS-PAGE as described previously (Hancock & Carey, 1979). Proteins were solubilized in Laemmli solubilization buffer for 5 min at 100°C before being separated by SDS-PAGE. Proteins were visualized by staining with Coomassie Brilliant Blue R250 (Bio-Rad, Richmond, CA).

X. Whole cell lysates

Whole cell lysates were prepared by an SDS boiling method (Nicas & Hancock, 1980). Briefly, samples from *E. coli* cultures were taken at different time points of growth. Cells were collected by centrifugation and resuspended in solubilization sample buffer and the proteins were analysed by SDS-PAGE.

XI. N-terminal amino acid sequence determination

Partially purified protein preparations were separated by SDS-PAGE and electroblotted onto a polyvinylidene difluoride (PVDF) membrane (Millipore, San Francisco, CA) (Matsudaria, 1990). Briefly, protein samples were solubilized in Laemmli solubilization buffer that was prepared without bromphenol blue.

Following SDS-PAGE, the proteins were electroblotted onto PVDF membrane using 10% methanol and 0.2% 3'-[cyclohexylamino]-1-propanesulfonic acid (CAPS) (Sigma), pH 11.0, as the transfer buffer in an electroblotting apparatus (Bio-Rad); electroblotting was performed at 100V for 60 min. The PVDF membrane was then washed twice with fresh transfer buffer and stained with Ponceau-S Red solution (0.5% Ponceau-S and 1% acetic acid) at room temperature for 20 min to visualize the electroblotted proteins. The membrane was then rinsed with dH_2O until the protein bands became visible above the background. The membrane was air dried and the appropriate protein bands excised from the membrane and sent to S. Kielland, Department of Biochemistry and Microbiology, University of Victoria, for N-terminal sequence determination using a gas phase sequencer (ABI, 470A).

XII. Membrane protein preparation

E. coli and *P. aeruginosa* cells were harvested in the mid-log phase of growth and washed in 10 mM Tris-HCl buffer, pH 8.0. The washed cells were lysed by passage through a French Press (14000 pounds / square inch) and the particulate fraction was collected by ultracentrifugation at 50,000 rpm (Beckman 70.1 Ti) at 4°C for 1 h. The membrane-containing pellet was resuspended in 10 mM Tris-HCl buffer, pH 8.0 and sonicated twice on ice at maximum power for 15 sec (Fisher Sonic Dismembrator 300 fitted with a microtip 3.5 mm in diameter). The sonicate was centrifuged at 50,000 rpm (Beckman 70.1 Ti) at 4°C for 1 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.

XIII. Protein assay

Protein concentrations were determined by a modified Lowry assay (Sandermann & Strominger, 1972) which contained 1% SDS. This method allowed the analysis of protein samples which contained detergents. Bovine serum albumin was used as a standard.

IVX. Antibiotic susceptibility testing

β-lactam antibiotics used in this study included cefsulodin (Ciba Ceigy, Basel, Switzerland); cefepime (Bristol-Myers-Squibb Inc., Wallingford, CT); ceftazidime (Glaxo Canada Inc.); aztreonam (E. R. Squibb & Sons, Inc.); imipenem (Merck Sharp & Dohme, division of Merck & Co. Inc.) and cephaloridine (Sigma).

Minimal inhibitory concentrations (MICs) were determined by the agar dilution method on MH agar plates using two-fold serial dilutions of the antibiotics. Overnight cultures of the bacteria to be tested were diluted in LB broth to approximately 10^5 colony forming unit per ml. Five ul of this diluted culture was inoculated onto the surface of the MH agar plates containing antibiotic, or in control experiments, without any antibiotics. The recorded MIC corresponded to the lowest

concentration of antibiotic causing greater than 90% inhibition of bacterial growth after 18 h incubation at 37°C. Final MIC values were the average of at least three determinations.

XV. Penicillin-binding protein assays

15.1 Direct assay

PBPs were assayed essentially by the method of Spratt (1977b). Membrane proteins were incubated with ^{3}H-penicillin G (3.7 μg/ml, 22 Ci/mol) at 25°C for 10 min. The reaction was stopped by the addition of an excess (1000x) of nonradioactive penicillin G. The samples were separated by SDS-PAGE (7.5%, 8.5% or 10% polyacrylamine) and the gel treated with 1 M sodium salicylate, pH 6.0, at room temperature for 30 min, dried at 80°C under vacuum for 2 hrs. The dried gel was then exposed to X-ray film (X-Omat K XK-1, Kodak) for 3 to 30 days at -70°C to visualize the radiolabelled proteins.

15.2 Competition assay

Membrane proteins were incubated with a β-lactam antibiotic or for control experiments with dH_2O, 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 an excess (1000x) of nonradioactive

penicillin G. The proteins were separated by SDS-7.5% PAGE. The resulting gel was treated with 1M 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 with a scanning densitometer (Studio Scan II, AGFA) in combination with a Macintosh computer using the public domain NIH Image program. The software was used to integrate scan peaks for quantitation of PBP binding to ^{3}H-penicillin. Binding inhibition was evaluated as concentration of the antibiotic inhibiting binding by 50% relative to binding in the absence of competing antibiotic.

XVI. Growth experiment

Overnight cultures of *P. aeruginosa* were inoculated into fresh MH broth at a ratio of 1:100. Cultures were then incubated at 37°C with shaking (180 rpm). The absorbance at 600 nm of the culture was determined at various times and a growth curve was plotted.

XVII. Cell shape examination

Cell shape was examined by phase contrast microscopy (BH2-PC, Olympus BHT, Tokyo, Japan). Cell photograph was taken with an Axiophot photomicroscope (Zeiss, Germany).

RESULTS

CHAPTER ONE Gene Cloning and Sequence Analyses of *P. aeruginosa pbpB* and *pbpC*

1. Introduction

Sequences surrounding the active-site motif SXXK and the KTG motif are highly conserved among *E. coli* high-molecular-weight PBPs and *Nesseria gonorrhoeae* PBP2 (Ghuysen, 1991). *N. gonorrhoeae* PBP2 is an *E. coli* PBP3-like protein responsible for cross-wall formation during cell division, and its overall amino acid sequence is 47.7% homologous to that of *E. coli* PBP3 (Spratt, 1988). I hypothesized that *P. aeruginosa* PBP3 also contained the conserved SXXK and KTG motifs. Based on this hypothesis, two degenerate oligonucleotides were synthesized according to the amino acid sequences found in the conserved motifs of *E. coli* high-molecular-weight PBPs and *N. gonorrhoeae* PBP2. The strategy for cloning the gene encoding *P. aeruginosa* PBP3 thus involved the use of PCR to amplify a portion of the gene which was then used as a probe to clone the complete gene.

2. Generation of DNA probes for cloning the *pbpB* and *pbpC* genes by degenerate PCR

A pool of degenerate primers was synthesized based on the sequences at and

surrounding the conserved functional motifs SXXK and KTG of *E. coli* PBP1A, 1B, 2, 3 and *N. gonorrhoeae* PBP2, after adjustment to the codon usage of *P. aeruginosa* (for sequences, see Materials and Methods section VII). These primers were used for PCR amplifications using *P. aeruginosa* PAO1 chromosomal DNA as the template. A mixture of PCR products ranging from 220 bp to 600 bp was amplified. Since the spacing between the SXXK and KTG motifs of *E. coli* PBP3 is 184 amino acid residues (Nakamura *et al.*, 1983), the fragment of about 600 bp in length was likely the product amplified from the DNA sequence coding for *P. aeruginosa* PBP3. Nevertheless, all of the PCR products were gel-purified, digested with *Eco*RI and cloned into a vector pTZ18U. The resultant clones were grouped by insert size and representative clones from each group were sequenced. The resulting DNA sequences were used for BLASTX homology searches of the GenBank database. The results of these searches are shown in Table 4.

One of the PCR products, a 580 bp DNA fragment cloned in pXL2, was found to translate to a protein sequence with 63.2% and 52.8% conservation of amino acids compared with *E. coli* PBP3 and *N. gonorrhoeae* PBP2, respectively. This PCR product was subsequently used as a probe to clone the complete *P. aeruginosa pbpB* gene.

A second PCR product, which was 580 bp in length and cloned in pXL3, was found to translate to a protein sequence with 62.5% and 56.6% conservation of

Table 4. *P. aeruginosa* PCR products with significant homology to GenBank sequences

Plasmid	Size of PCR product (bp)	Homologous genes	Function	Original organism	Homology %Identity	Homology %Conservation
pXL2	580	*pbpB*	PBP3	*E. coli*	39.4	63.2
		penA	PBP2	*N. gonorrhoeae*	35.4	52.8
pXL3	580	*pbpB*	PBP3	*E. coli*	37.5	62.5
		penA	PBP2	*N. gonorrhoeae*	38.1	56.6
pXL5	380	*yhhF*	hypothetical protein	*E. coli*	53.6	62.7
pXL8	510	*sucC*	β-subunit of succinyl coenzyme A synthetase	*E. coli*	87.3	91.4
		sucD	α-subunit of succinyl coenzyme A synthetase	*E. coli*	73.7	89.5
pXL12	460	*cypH*	periplasmic peptidyl-propyl-*cis-tran* isomerase	*E. coli*	63.4	76.0

amino acid compared with *E. coli* PBP3 and *N. gonorrhoeae* PBP2, respectively. The nucleotide sequences of these PCR products were 64.5% identical. The second PCR fragment was likely the product amplified from another region of the PAO1 chromosome. Thus it seemed likely that *P. aeruginosa* has two copies of a PBP3-like-encoding gene. The second PCR product was subsequently used as a probe to clone the complete gene named *pbpC*.

Three other PCR products were amplified by the same primers. One of the sequences cloned in pXL8 was 87.3% identical to amino acid residues 239-388 of the *E. coli* β-subunit of succinyl coenzyme A synthetase encoded by *sucC*. The last nucleotide of the TAA stop codon was the first nucleotide of the probable methionine start codon of a stretch encoding 19 amino acids, 14 of which were identical to the product of the *E. coli sucD* gene which encodes the α-subunit of succinyl coenzyme A synthetase and is linked to the *sucC* gene in *E. coli*. The %G+C of the third position of codons was 84%, which is typical of a high G+C organism like *P. aeruginosa* (West & Iglewski, 1988). A second sequence cloned in pXL5 was 53.6% identical to amino acids 1-104 of a hypothetical *E. coli* protein encoded by *yhhF* (formerly called *ftsS*) (Sofia *et al.*, 1994). A third sequence cloned in pXL12 was 63.4% identical to residues 24-176 of an *E. coli* periplasmic peptidyl-prolyl cis-trans isomerase ('rotamase') encoded by *rot* or *cypH*. These sequences also had a high G+C content (83 % and 90 % for *yhhF* and *cypH*, respectively) in the third codon position.

3. Cloning of the *pbpB* and *pbpC* genes

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 the radioactive labeled PCR products (probes for *pbpB* and *pbpC*), respectively. This procedure was used to create two restriction enzyme maps (Fig. 4A & 5A). The resulting restriction maps were used for the construction of various subgenomic libraries to facilitate the cloning of the *P. aeruginosa pbpB* and *pbpC* genes.

To clone the *pbpB* gene, an attempt was made to construct a library using the 6.0 kb *Xho*I fragments of PAO1 chromosomal DNA. However, this approach was not successful. The cloning strategy was then changed to isolate three 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 respectively. These fragments were then ligated with digested pTZ19U and transformed into *E. coli* DH5α. Three positive clones pSPH1, pPST18 and pXSm16 (Fig. 4B) were obtained after colony hybridization. However, none of these clones contained the entire *pbpB* gene. Therefore a 1 kb *Sma*I fragment from pSPH1 was cloned into the *Sma*I site of pXSm16 to generate pXLSH36 (Fig. 4B). This plasmid contained a 5.4 kb *Sph*I-*Xho*I fragment of the PAO1 chromosome DNA cloned in the vector pTZ19U. The 5.4 kb *Sph*I-*Xho*I fragment was isolated from pXLSH36 and cloned into a vector pTZ18U to generate pXLBI3. pXLBI3 contained the *P.*

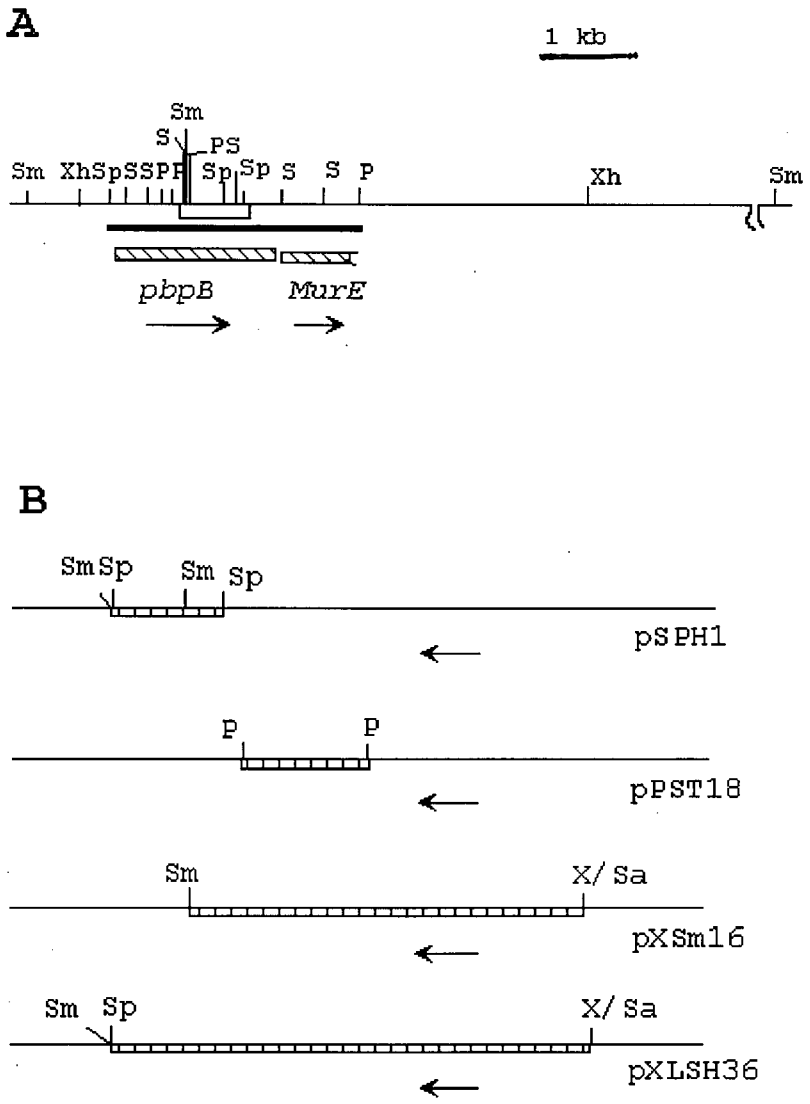

Figure 4. **(A)** Restriction map of the *P. aeruginosa pbpB* region. The open box indicates the location of the 580 bp PCR product. The solid bar indicates the sequenced 2.7 kb *Sph*I-*Pst*I region. The dashed boxes indicate the locations of the *pbpB* and *murE* ORFs. The arrows indicate the directions of transcriptions. **(B)** Restriction map of the various *pbpB* subclones. The dashed boxes represent the PAO1 chromosomal DNA cloned in the vector pTZ19U. Abbreviations: P, *Pst*I; S, *Sal*I; Sm, *Sma*I; Sp, *Sph*I; X, *Xho*I; X/Sa: *Xho*I-*Sal*I.

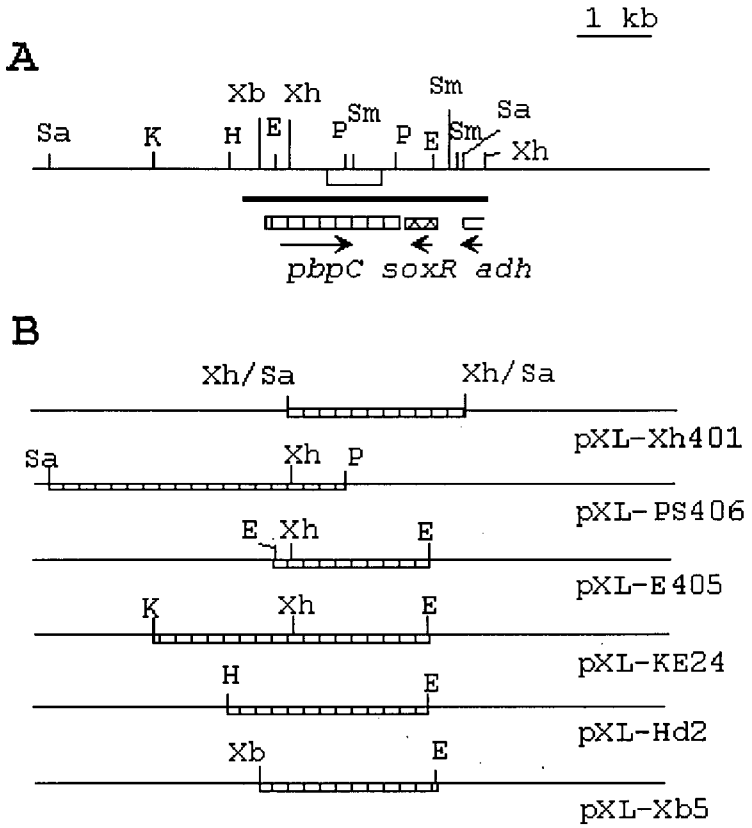

Figure 5. **(A)** Restriction map of the *P. aeruginosa pbpC* region. The open box indicates the location of the 580 bp PCR product. The solid bar indicates the sequenced 3.2 kb *Hind*III-*Xho*I region. The dashed boxes indicate the locations of the *pbpC*, *soxR* and *adh* ORFs respectively. The arrows indicate the directions of transcriptions. **(B)** Restriction map of the various *pbpC* subclones. The dashed boxes represent the PAO1 chromosomal DNA cloned into the 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; X/Sa: *Xho*I-*Sal*I.

aeruginosa pbpB gene under the control of the *lac* promoter (Liao & Hancock, 1995).

To clone the *pbpC* gene, an attempt was made to construct a library using 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 then ligated with the digested pTZ19U and transformed into *E. coli* DH5α. Three positive clones pXL-Xh401, pXL-PS406 and pXL-E405 (Fig. 5B) were obtained after colony hybridization. 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 the vector pTZ19U to create the plasmid pXL-KE24 (Fig. 5B). Two subclones pXL-Hd2 and pXL-Xb5 were subsequently created after the deletion of the 1 kb *Kpn*I-*Hind*III fragment and the 1.4 kb *Kpn*I-*Xba*I fragment from pXL-KE24, respectively (Fig. 5B).

4. DNA sequence analyses of the *pbpB* and *pbpC* loci

4.1 *pbpB*

Both strands of the 2.7 kb *Sph*I-*Pst*I region were sequenced after a series of overlapping nested deletion clones were created from pSPH1 and pPST18, respectively. Two putative open reading frames (ORFs) were found in this sequence

(Fig. 6). They were located at nucleotides 44 through 1783 and 1783 through 2757. The second ORF appeared incomplete at its 3' end. The first ORF (ORF1) encoded a 579 amino acid sequence with 45% identity and a total of 57.7% conserved amino acids compared to the *E. coli* PBP3 (Table 5). It was preceded by a putative RBS sequence AGGA at nucleotides 30-34, which matched reasonably to a consensus RBS sequence. The N-terminal amino acid sequence of the *pbpB* gene product was MKLNYF (see below) which showed that the potential initiation codon ATG at nucleotides 116-118 was not used. The third position of codons comprised 85.8% G+C, typical of *P. aeruginosa* genes. No potential transcription terminator sequences were identified downstream of the ORF1.

The second putative ORF (ORF2) was incomplete and encoded 325 amino acids. The first nucleotide of the putative ATG start codon for ORF2 is the last nucleotide of the TGA stop codon for the ORF1. The sequence of the ORF2 was 43% identical and 65% similar to amino acids 1-337 of the *E. coli murE* gene product, which encodes uridine diphosphate-N-acetyl muramic acid-tripeptide synthetase (Michand *et al.*, 1990). A putative RBS sequence AGGA was located at nucleotides 1768-1771. The third position of codons comprised 86.8% G+C.

4.2 *pbpC*

Both strands of the 3.2 kb *Hind*III-*Xho*I region were sequenced using the nested deletion clones of pXL-Xh401 and pXL-Hd2. Three putative open reading

Fig.6

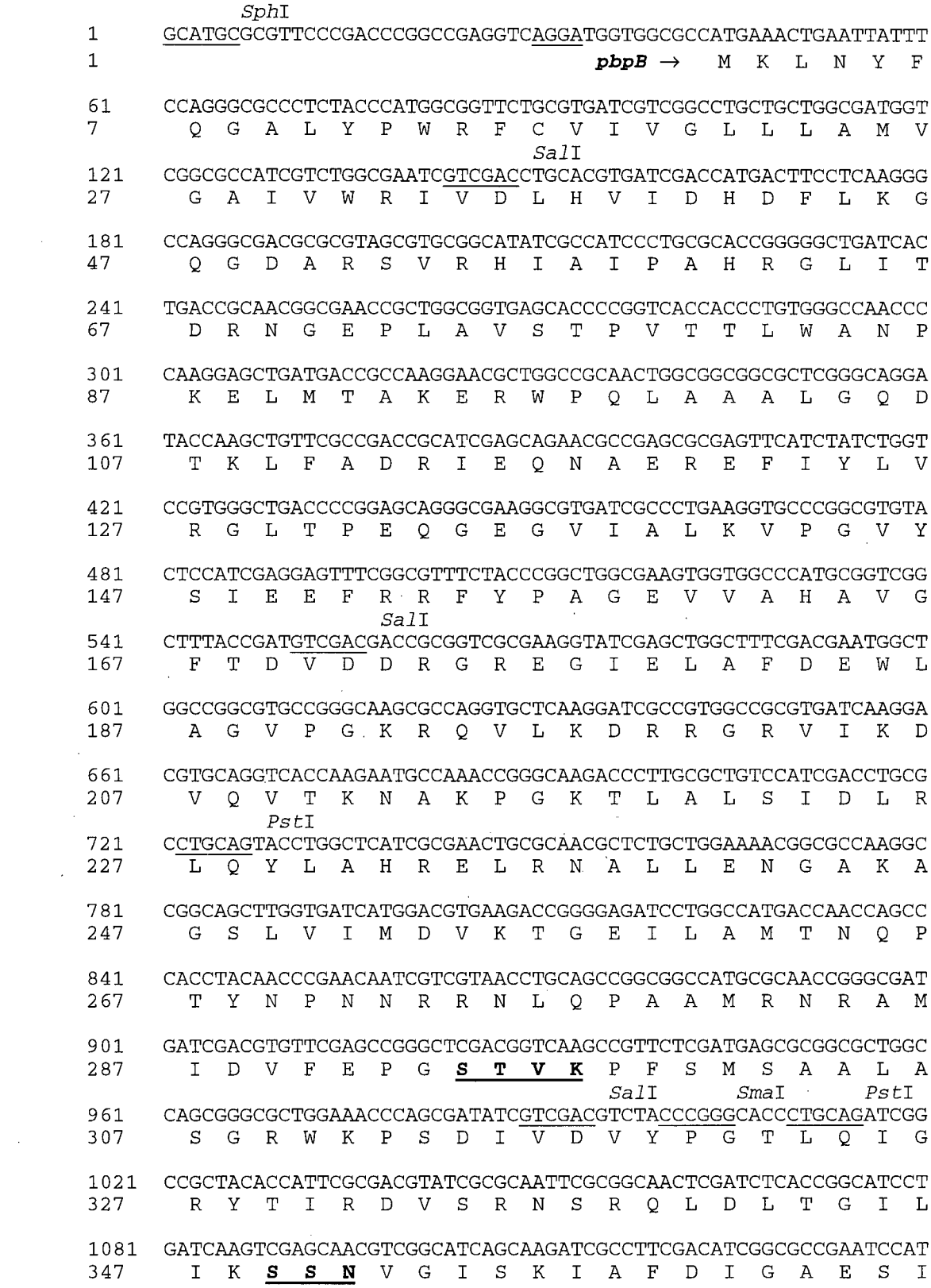
SphI
1 GCATGCGCGTTCCCGACCCGGCCGAGGTCAGGATGGTGGCGCCATGAAACTGAATTATTT
1 pbpB → M K L N Y F
61 CCAGGGCGCCCTCTACCCATGGCGGTTCTGCGTGATCGTCGGCCTGCTGCTGGCGATGGT
7 Q G A L Y P W R F C V I V G L L L A M V
SalI
121 CGGCGCCATCGTCTGGCGAATCGTCGACCTGCACGTGATCGACCATGACTTCCTCAAGGG
27 G A I V W R I V D L H V I D H D F L K G
181 CCAGGGCGACGCGCGTAGCGTGCGGCATATCGCCATCCCTGCGCACCGGGGGCTGATCAC
47 Q G D A R S V R H I A I P A H R G L I T
241 TGACCGCAACGGCGAACCGCTGGCGGTGAGCACCCCGGTCACCACCCTGTGGGCCAACCC
67 D R N G E P L A V S T P V T T L W A N P
301 CAAGGAGCTGATGACCGCCAAGGAACGCTGGCCGCAACTGGCGGCGGCGCTCGGGCAGGA
87 K E L M T A K E R W P Q L A A A L G Q D
361 TACCAAGCTGTTCGCCGACCGCATCGAGCAGAACGCCGAGCGCGAGTTCATCTATCTGGT
107 T K L F A D R I E Q N A E R E F I Y L V
421 CCGTGGGCTGACCCCGGAGCAGGGCGAAGGCGTGATCGCCCTGAAGGTGCCCGGCGTGTA
127 R G L T P E Q G E G V I A L K V P G V Y
481 CTCCATCGAGGAGTTTCGGCGTTTCTACCCGGCTGGCGAAGTGGTGGCCCATGCGGTCGG
147 S I E E F R R F Y P A G E V V A H A V G
SalI
541 CTTTACCGATGTCGACGACCGCGGTCGCGAAGGTATCGAGCTGGCTTTCGACGAATGGCT
167 F T D V D D R G R E G I E L A F D E W L
601 GGCCGGCGTGCCGGGCAAGCGCCAGGTGCTCAAGGATCGCCGTGGCCGCGTGATCAAGGA
187 A G V P G K R Q V L K D R R G R V I K D
661 CGTGCAGGTCACCAAGAATGCCAAACCGGGCAAGACCCTTGCGCTGTCCATCGACCTGCG
207 V Q V T K N A K P G K T L A L S I D L R
PstI
721 CCTGCAGTACCTGGCTCATCGCGAACTGCGCAACGCTCTGCTGGAAAACGGCGCCAAGGC
227 L Q Y L A H R E L R N A L L E N G A K A
781 CGGCAGCTTGGTGATCATGGACGTGAAGACCGGGGAGATCCTGGCCATGACCAACCAGCC
247 G S L V I M D V K T G E I L A M T N Q P
841 CACCTACAACCCGAACAATCGTCGTAACCTGCAGCCGGCGGCCATGCGCAACCGGGCGAT
267 T Y N P N N R R N L Q P A A M R N R A M
901 GATCGACGTGTTCGAGCCGGGCTCGACGGTCAAGCCGTTCTCGATGAGCGCGGCGCTGGC
287 I D V F E P G S T V K P F S M S A A L A
SalI SmaI PstI
961 CAGCGGGCGCTGGAAACCCAGCGATATCGTCGACGTCTACCCGGGCACCCTGCAGATCGG
307 S G R W K P S D I V D V Y P G T L Q I G
1021 CCGCTACACCATTCGCGACGTATCGCGCAATTCGCGGCAACTCGATCTCACCGGCATCCT
327 R Y T I R D V S R N S R Q L D L T G I L
1081 GATCAAGTCGAGCAACGTCGGCATCAGCAAGATCGCCTTCGACATCGGCGCCGAATCCAT
347 I K S S N V G I S K I A F D I G A E S I

Fig. 6

```
1141  CTACTCGGTCATGCAACAGGTCGGTCTCGGGCAGGACACGGGGTTGGGCTTCCCCGGCGA
367     Y  S  V  M  Q  Q  V  G  L  G  Q  D  T  G  L  G  F  P  G  E

1201  GCGCGTCGGCAACCTGCCCAACCACCGCAAGTGGCCGAAGGCGGAAACCGCGACCCTGGC
387     R  V  G  N  L  P  N  H  R  K  W  P  K  A  E  T  A  T  L  A
                                                    SphI
1261  CTACGGCTACGGTCTCTCGGTAACCGCGATCCAGTTGGCGCATGCCTATGCGGCCCTGGC
407     Y  G  Y  G  L  S  V  T  A  I  Q  L  A  H  A  Y  A  A  L  A

1321  CAACGACGGCAAGAGCGTGCCGCTGAGCATGACCCGAGTCGACCGCGTGCCGGATGGTGT
427     N  D  G  K  S  V  P  L  S  M  T  R  V  D  R  V  P  D  G  V
                                                    SphI
1381  GCAGGTGATCTCGCCTGAAGTGGCTTCCACCGTGCAGGGCATGCTGCAACAAGTGGTCGA
447     Q  V  I  S  P  E  V  A  S  T  V  Q  G  M  L  Q  Q  V  V  E

1441  GGCCCAGGGCGGGGTGTTCCGCGCCCAGGTGCCGGGTTACCACGCCGCCGGCAAGAGCGG
467     A  Q  G  G  V  F  R  A  Q  V  P  G  Y  H  A  A  G  K  S  G

1501  GACCGCGCGCAAGGTCTCGGTCGGCACCAAGGGCTACCGGGAAAACGCCTATCGCTCGCT
487     T  A  R  K  V  S  V  G  T  K  G  Y  R  E  N  A  Y  R  S  L

1561  GTTCGCCGGTTTCGCCCCGGCCACCGATCCGCGCATCGCGATGGTCGTGGTGATCGACGA
507     F  A  G  F  A  P  A  T  D  P  R  I  A  M  V  V  V  I  D  E

1621  GCCGAGCAAGGCGGGCTACTTCGGCGGCCTGGTGTCGGCGCCGGTGTTCAGTAAGGTCAT
527     P  S  K  A  G  Y  F  G  G  L  V  S  A  P  V  F  S  K  V  M

1681  GGCTGGCGCGCTGCGCCTGATGAACGTGCCGCCGGATAACCTGCCGACGGCCACCGAACA
547     A  G  A  L  R  L  M  N  V  P  P  D  N  L  P  T  A  T  E  Q

1741  GCAGCAGGTCAATGCTGCGCCCGCAAAAGGAGGGCGTGGCTGATGCCTATGAGCCTGAGC
567     Q  Q  V  N  A  A  P  A  K  G  G  R  G  *  M  P  M  S  L  S
                                                    murE →
1801  CAACTGTTTCCCCAGGCCGAGCGCGATCTGCTGATCCGCGAGCTGACCCTGGATAGCCAC
7       Q  L  F  P  Q  A  E  R  D  L  L  I  R  E  L  T  L  D  S  H
                                SalI
1861  GGCGTTCGTCCGGTCGACCTGTTCCTGACGGTTCCGGGCGGGCACCAGGATGGTCGTGCG
27      G  V  R  P  V  D  L  F  L  T  V  P  G  G  H  Q  D  G  R  A

1921  CACATCGCCGATGCCCTGACCAAGGGCGCGACTGCCGTGGCTTACGAGGCGGAAGGCGCC
47      H  I  A  D  A  L  T  K  G  A  T  A  V  A  Y  E  A  E  G  A

1981  GGAGAGTTGCCGCCCAGCGATGCGCCGCTGATCGCGGTGAAGGGGCTGGCCGCGCAACTG
67      G  E  L  P  P  S  D  A  P  L  I  A  V  K  G  L  A  A  Q  L

2041  TCGGCGGTCGCCGGGCGTTTCTACGGCGAGCCGAGCCGCGGGCTGGACCTGATCGGCGTC
87      S  A  V  A  G  R  F  Y  G  E  P  S  R  G  L  D  L  I  G  V

2101  ACCGGCACCAACGGCAAGACCAGCGTCAGCCAACTGGTGGCCCAGGCCCTGGATCTGCTC
107     T  G  T  N  G  K  T  S  V  S  Q  L  V  A  Q  A  L  D  L  L

2161  GGCGAGCGCTGCGGCATCGTCGGCACCCTCGGCACCGGTTTCTACGGCGCCCTGGAGAGC
127     G  E  R  C  G  I  V  G  T  L  G  T  G  F  Y  G  A  L  E  S

2221  GGCCGGCACACCACGCCGGACCCGCTCGCGGTGCAGGCCACGCTGGCCACGCTGAAGCAG
147     G  R  H  T  T  P  D  P  L  A  V  Q  A  T  L  A  T  L  K  Q

2281  GCCGGCGCCCGCGCGGTAGCGATGGAAGTGTCTTCCCACGGCCTCGACCAGGGCCGCGTG
167     A  G  A  R  A  V  A  M  E  V  S  S  H  G  L  D  Q  G  R  V
```

Fig. 6

```
2341  GCGGCGCTCGGCTTCGATATCGCGGTGTTCACCAATCTGTCCCGCGACCACCTCGACTAT
187    A  A  L  G  F  D  I  A  V  F  T  N  L  S  R  D  H  L  D  Y

2401  CACGGTTCGATGGAAGCCTATGCCGCCGCCAAGGCCAAGCTGTTCGCCTGGCCGGACCTG
207    H  G  S  M  E  A  Y  A  A  A  K  A  K  L  F  A  W  P  D  L

2461  CGCTGCCGGGTGATCAACCTGGACGACGATTTCGGCCGTCGACTGGCCGGCGAGGAGCAG
227    R  C  R  V  I  N  L  D  D  D  F  G  R  R  L  A  G  E  E  Q

2521  GACTCGGAGCTGATCACCTACAGCCTCACCGACAGCTCGGCGTTCCTCTATTGCCGCGAA
247    D  S  E  L  I  T  Y  S  L  T  D  S  S  A  F  L  Y  C  R  E

2581  GCGCGCTTCGGCGACGCCGGCATCGAGGCGGCGCTGGTCACTCCGCACGGCGAGGGCCTG
267    A  R  F  G  D  A  G  I  E  A  A  L  V  T  P  H  G  E  G  L

2641  CTGCGCAGCCCGTTGCTCGGCCGCTTCAACCTGAGCAACCTGCTGGCGGCGGTCGGTGCG
287    L  R  S  P  L  L  G  R  F  N  L  S  N  L  L  A  A  V  G  A
                                                                PstI
2701  TTGCTTGGCCTGGGTTATCCCCTGGGCGATATCCTCCGCACTTTGCCGCAGCTGCAG
307    L  L  G  L  G  Y  P  L  G  D  I  L  R  T  L  P  Q  L  Q
```

Figure 6. Nucleotide sequence of the *P. aeruginosa pbpB* region and the deduced amino acid sequences of two ORFs. The numbers on the left designate the nucleotide or amino acid at the left end of each row. The conserved motifs of PBP3 are bold and underlined. The putative RBS sequences are underlined. The arrows indicate direction of transcription. Sequences recognized by the restriction enzymes are underlined and indicated by the name beside.

Table 5. Percent identities and total conservation of the amino acid sequences of PBP3 and PBP3x to those of other PBPs*

Protein	Accession number	Number of amino acids	% PBP3		% PBP3x	
			identity	conservation	identity	conservation
PBP3	X84053	579	100	100	48.3	63.2
PBP3x	X95517	565	48.3	63.2	100	100
EcoliPBP3	K00137	588	45.1	57.7	40.7	52.9
NgorPBP2	X07469	583	37.7	52.6	35.8	49.6
HinfPBP3	L45768	610	32.6	53.7	31.9	45.4
BsubSPOVD	Z25865	645	25.7	39.0	26.7	38.9
HinfPBP2	L44676	651	23.5	36.3	21.9	35.4
EcoliPBP2	X04516	633	22.8	36.4	20.4	33.9
BsubPBP2B	L09703	716	21.8	34.4	23.0	35.9

* The protein sequences were analyzed by the PC gene program using the genetic-code matrix with an open gap cost of 6 and an unit gap cost of 20. Similar amino acids include: AST; DE; NQ; RK; ILMV and FYW. EcoliPBP3, *E. coli* PBP3; NgorPBP2, *N. gonorrhoeae* PBP2; HinfPBP3, *H. influenzae* PBP3; BsubSPOVD, *B. subtilis* SPOVD protein; HinfPBP2, *H. influenzae* PBP2; EcoliPBP2, *E. coli* PBP2; BsubPBP2B, *B. subtilis* PBP2B.

frames were found in this sequence (Fig. 7). They were located at nucleotides 373 through 2070 (ORF1), 2078 through 2548 (ORF2) and 3028 through 3274 (ORF3) respectively. The third ORF was incomplete at its 5' end. The first ORF would be transcribed in the opposite orientation to the second and third ORFs.

ORF1 encoded a 565 amino acid sequence with 40.7% identity and a total of 52.9% conserved amino acids compared to *E. coli* PBP3 (Table 5). A putative RBS sequence and two promoter-like sequences potentially recognized by σ^s and σ^{70}, respectively, were identified in the upstream region (Fig. 7). The third position of codons comprised 93% G+C. This amino acid sequence showed 48.3% identity and 63.2% conserved amino acids relative to that of the *pbpB* gene product (Table 5).

ORF2 encoded a protein sequence of 156 amino acids which had 62% identity and a total of 78% conserved amino acids relative to *E. coli soxR* gene product. *E. coli* SoxR protein is activated by superoxide-generating agents or nitric oxide and is a transcriptional activator of the *soxS* gene. The *soxS* gene product activates approximately 10 other promoters (Hidalgo *et al.*, 1994; Li *et al.*, 1994). In *E. coli soxR* and *soxS* are adjacent to one another (Wu *et al.*, 1991). However, between the *P. aeruginosa soxR* (ORF2) and the 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% identity and 76.8% conserved amino acids compared to amino acids

Fig. 7

```
1      TCCACCAACAACAGGGCCTGGGCCGCCGCCTGCTGGAGCGCGCGGTGACCTACGCCCACG

61     CCAGCCACTGCCGGGCGCTGACCCTGACGACCTTCTGCGACGTGCCCTGGAACGCACCGT

121    TCTACGCACGCCTGGGCTTCCAGCGGCTGACCTGGCAGGAAGCCGGCGAGCGCTTGCGCG

181    CGATCCTCGGCCACGAGCAGGAGATCGGCTTCGCCGCCGACAGCCGCTGCGCGATGCGCC
                      XbaI
241    TGGTGCTCTAGACGGCGGCAAAGGGCTTGACCGGCGGGTGGCGGGTGACGGTACAGTTGC

301    CAACTGCAACAGGATGTTTCAGCACTGCCCCGGACGGGCTTCCGCTCTCCCTCTTCCCCA

361    GTGTGCCCTTGCATGAGCAGTCAACGCCGAAACTACCGCTTCATCCTTGTCGTCACCCTG
1        pbpC →    M  S  S  Q  R  R  N  Y  R  F  I  L  V  V  T  L

421    TTCGTCCTCGCCTCCCTGGCCGTCTCCGGACGGTTGGTCTATCTCCAGGTCCACGACCAC
17      F  V  L  A  S  L  A  V  S  G  R  L  V  Y  L  Q  V  H  D  H
             EcoRI
481    GAATTCCTCGCCGACCAGGGCGACCTCCGCTCGATCCGCGACCTGCCGATCCCGGTCACC
37      E  F  L  A  D  Q  G  D  L  R  S  I  R  D  L  P  I  P  V  T

541    CGCGGCATGATCACCGACCGCAACGGCGAGCCGCTGGCGGTATCCACCGAAGTCGCGTCG
57      R  G  M  I  T  D  R  N  G  E  P  L  A  V  S  T  E  V  A  S

601    ATCTGGTGCAACCCCAGGGAAATGGCCGCCCACCTCGACGAGGTGCCGCGCCTGGCCGGC
77      I  W  C  N  P  R  E  M  A  A  H  L  D  E  V  P  R  L  A  G

661    GCCCTGCACCGCCCCGCGGCGGCGCTGCTGGCCCAGCTCCAGGCCAACCCGAACAAGCGC
97      A  L  H  R  P  A  A  A  L  L  A  Q  L  Q  A  N  P  N  K  R
                      XhoI
721    TTCCTCTACCTCGAGCGCGGCCTGTCGCCGATCGAGGCCAGCGAGGTGATGGCCCTGGGC
117     F  L  Y  L  E  R  G  L  S  P  I  E  A  S  E  V  M  A  L  G

781    ATAACGGGGGTACACCAGATCAAGGAATACAAGCGTTTCTACCCCAGTTCCGAGCTGACC
137     I  T  G  V  H  Q  I  K  E  Y  K  R  F  Y  P  S  S  E  L  T

841    GCGCAGTTGATCGGCCTGGTCAACATCGACGGCCGCGGCCAGGAAGGCACCGAACTGGGC
157     A  Q  L  I  G  L  V  N  I  D  G  R  G  Q  E  G  T  E  L  G

901    TTCAACGACTGGCTGAGCGGCAAGGACGGGGTACGCGAGGTGGCGATCAACCCGCGCGGC
177     F  N  D  W  L  S  G  K  D  G  V  R  E  V  A  I  N  P  R  G

961    TCGCTGGTCAACAGCATCAAGGTGCTGAAGACGCCCAAGGCCAGCCAGGACGTGGCCCTG
197     S  L  V  N  S  I  K  V  L  K  T  P  K  A  S  Q  D  V  A  L

1021   AGCATCGACCTGCGACTACAGTTCATCGCCTACAAGGCGCTGGAAAAGGCCGTGCTCAAG
217     S  I  D  L  R  L  Q  F  I  A  Y  K  A  L  E  K  A  V  L  K

1081   TTCGGCGCGCACTCCGGCTCGGCGGTCCTGGTGAACCCGAAGAGCGGGCAGATCCTGGCG
237     F  G  A  H  S  G  S  A  V  L  V  N  P  K  S  G  Q  I  L  A

1141   ATGGCCAACTTCCCCTCCTACAACCCGAACAACCGCGCCAGCTTCGCCCCGGCCTTCATG
257     M  A  N  F  P  S  Y  N  P  N  N  R  A  S  F  A  P  A  F  M

1201   CGCAACCGCACCCTCACCGATACCTTCGAGCCGGGCTCGGTGATCAAGCCGTTCAGCATG
277     R  N  R  T  L  T  D  T  F  E  P  G  S  V  I  K  P  F  S  M
```

Fig. 7

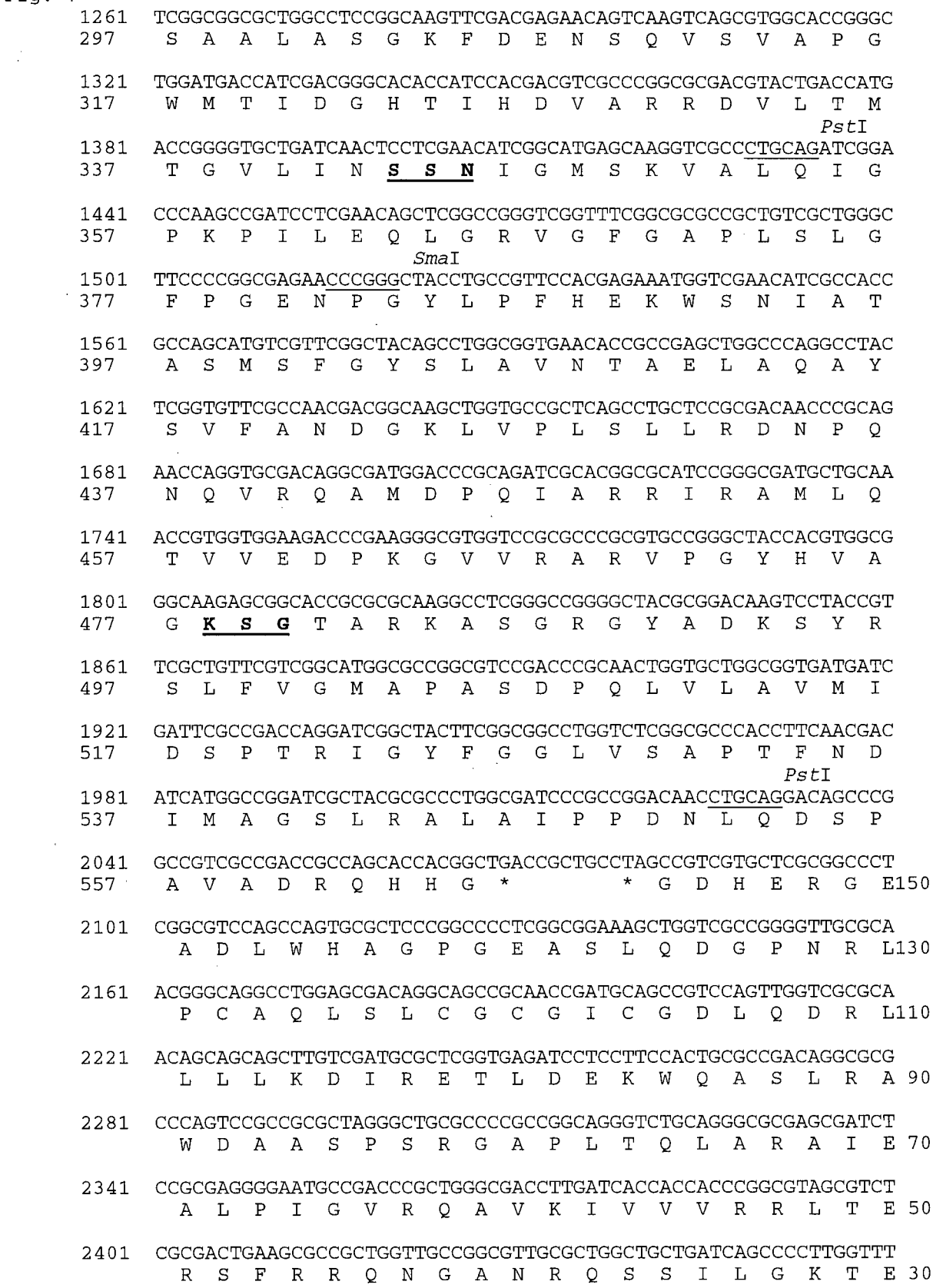
1261 TCGGCGGCGCTGGCCTCCGGCAAGTTCGACGAGAACAGTCAAGTCAGCGTGGCACCGGGC
297 S A A L A S G K F D E N S Q V S V A P G
1321 TGGATGACCATCGACGGGCACACCATCCACGACGTCGCCCGGCGCGACGTACTGACCATG
317 W M T I D G H T I H D V A R R D V L T M
PstI
1381 ACCGGGGTGCTGATCAACTCCTCGAACATCGGCATGAGCAAGGTCGCCCTGCAGATCGGA
337 T G V L I N S S N I G M S K V A L Q I G
1441 CCCAAGCCGATCCTCGAACAGCTCGGCCGGGTCGGTTTCGGCGCGCCGCTGTCGCTGGGC
357 P K P I L E Q L G R V G F G A P L S L G
SmaI
1501 TTCCCCGGCGAGAACCCGGGCTACCTGCCGTTCCACGAGAAATGGTCGAACATCGCCACC
377 F P G E N P G Y L P F H E K W S N I A T
1561 GCCAGCATGTCGTTCGGCTACAGCCTGGCGGTGAACACCGCCGAGCTGGCCCAGGCCTAC
397 A S M S F G Y S L A V N T A E L A Q A Y
1621 TCGGTGTTCGCCAACGACGGCAAGCTGGTGCCGCTCAGCCTGCTCCGCGACAACCCGCAG
417 S V F A N D G K L V P L S L L R D N P Q
1681 AACCAGGTGCGACAGGCGATGGACCCGCAGATCGCACGGCGCATCCGGGCGATGCTGCAA
437 N Q V R Q A M D P Q I A R R I R A M L Q
1741 ACCGTGGTGGAAGACCCGAAGGGCGTGGTCCGCGCCCGCGTGCCGGGCTACCACGTGGCG
457 T V V E D P K G V V R A R V P G Y H V A
1801 GGCAAGAGCGGCACCGCGCGCAAGGCCTCGGGCCGGGGCTACGCGGACAAGTCCTACCGT
477 G K S G T A R K A S G R G Y A D K S Y R
1861 TCGCTGTTCGTCGGCATGGCGCCGGCGTCCGACCCGCAACTGGTGCTGGCGGTGATGATC
497 S L F V G M A P A S D P Q L V L A V M I
1921 GATTCGCCGACCAGGATCGGCTACTTCGGCGGCCTGGTCTCGGCGCCCACCTTCAACGAC
517 D S P T R I G Y F G G L V S A P T F N D
PstI
1981 ATCATGGCCGGATCGCTACGCGCCCTGGCGATCCCGCCGGACAACCTGCAGGACAGCCCG
537 I M A G S L R A L A I P P D N L Q D S P
2041 GCCGTCGCCGACCGCCAGCACCACGGCTGACCGCTGCCTAGCCGTCGTGCTCGCGGCCCT
557 A V A D R Q H H G * * G D H E R G E150
2101 CGGCGTCCAGCCAGTGCGCTCCCGGCCCCTCGGCGGAAAGCTGGTCGCCGGGGTTGCGCA
A D L W H A G P G E A S L Q D G P N R L130
2161 ACGGGCAGGCCTGGAGCGACAGGCAGCCGCAACCGATGCAGCCGTCCAGTTGGTCGCGCA
P C A Q L S L C G C G I C G D L Q D R L110
2221 ACAGCAGCAGCTTGTCGATGCGCTCGGTGAGATCCTCCTTCCACTGCGCCGACAGGCGCG
L L L K D I R E T L D E K W Q A S L R A 90
2281 CCCAGTCCGCCGCGCTAGGGCTGCGCCCCGCCGGCAGGGTCTGCAGGGCGCGAGCGATCT
W D A A S P S R G A P L T Q L A R A I E 70
2341 CCGCGAGGGGAATGCCGACCCGCTGGGCGACCTTGATCACCACCACCCGGCGTAGCGTCT
A L P I G V R Q A V K I V V V R R L T E 50
2401 CGCGACTGAAGCGCCGCTGGTTGCCGGCGTTGCGCTGGCTGCTGATCAGCCCCTTGGTTT
R S F R R Q N G A N R Q S S I L G K T E 30

Fig. 7

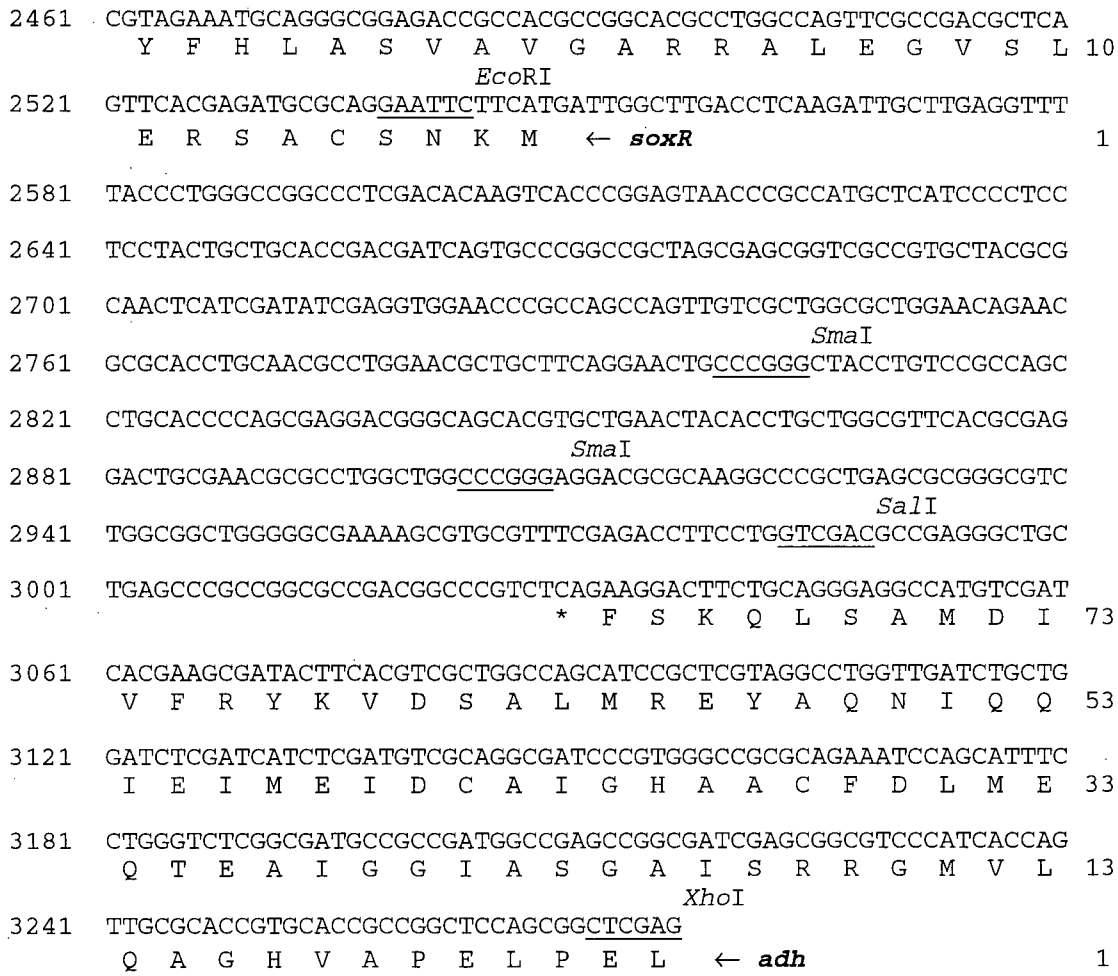

Figure 7. Nucleotide sequence of the *P. aeruginosa pbpC* region and the deduced amino acid sequences of three ORFs. The numbers on both the left and right designate the nucleotide or amino acid at the end of each row. The conserved motifs of PBP3x are bold and underlined. The putative RBS sequence is bold. The putative promoter sequences recognized by σ^S are bolded italic and underlined. The putative promoter sequences recognized by σ^{70} are italic and underlined. The arrows indicate the directions of transcriptions. The conserved motifs of PBP3x are bold and underlined. Sequences recognized by the restriction enzymes are underlined and indicated by the name beside.

219-346 of the *Mycobacterium bovis adh* gene product, an NADP-dependent alcohol dehydrogenase (Stelandre *et al.*, 1992).

5. Features of the *pbpB* and *pbpC* gene products and comparison with other PBPs

All of the high-molecular-weight PBPs studied to date are known to be cytoplasmic membrane proteins with their hydrophobic amino terminus anchored in the cytoplasmic membrane. Hydropathy analyses of the deduced amino acid sequences of both the *P. aeruginosa* PBP3 and PBP3x showed similar profiles to that of *E. coli* PBP3 (data not shown). Analysis of membrane spanning segments by the PC Gene computer program predicted that there was one transmembrane segment for each of the *pbpB* and *pbpC* gene product. The transmembrane domains were localized to residues 15 to 31 and 10 to 26 as the inner boundaries and residues 8 to 39 and 7 to 31 as the outer boundaries for the *pbpB* and *pbpC* gene products, respectively. These results suggested that *P. aeruginosa* PBP3 and PBP3x were cytoplasmic membrane-associated proteins.

Nine conserved motifs or boxes within the amino acid sequences of the high molecular weight PBPs have been identified as being unique to the class B high-molecular-weight PBPs by Piras *et al.* (1993) and Ghuysen (1994). As shown in Fig. 8, *P. aeruginos*a PBP3, PBP3x and *E. coli* PBP3 were conserved in all of the nine boxes. The motifs SXXK, SXN and KTG were located in the C-terminal portion and

```
PBP3      MKLNYFQGALYP.........WRFCVIVGLLLAMVGAIVWRIVDLHVIDHDFLKGQGDAR   51
PBP3x     -ssqrrnyrfil.........vvtlfvlas-avsgrlvylqvh-heflad.....---l-   46
ECPBP3    --aaaktqkpkrqeehanfis---allc-ci-lalafllg-vaw-q--sp-m-vke--m-   60

PBP3      SVRHIAIPAHRGLITDRNGEPLAVSTPVTTLWANPKELMTAKERWPQLAAALGQDTKLFA  111
PBP3x     -i-dlp--vt--m-------------e-asi-c--r-maahldev-r--g--hrpaaall  106
ECPBP3    -l-vqqvsts--m----s-r-----v--kai--d---vhd-ggisvgdrwkalanalnip  120
                     Box 1
PBP3      DRIEQNAERE.....FIYLVRGLTPEQGEGVIALKVPGVYSIEEFRRFYPAGEVVAHAVG  166
PBP3x     aqlqa-pnkr.....-l--e---s-iease-m--git--hq-k-yk----ss-lt-qli-  161
ECPBP3    ldqlsarinanpkgr----a-qvn-dmadyikk--l--ihlr--s--y--s---t--li-  180
                                                          Box 2
PBP3      FTDVDDRGREGIELAFDEWLAGVPGKRQVLKDRRGRVIKDVQVTKNAKPGKTLALSIDLR  226
PBP3x     lvni-g--q--t--g-nd--s-kd-v-e-ainp--slvnsik-l-tp-asqdv-------  221
ECPBP3    --n--sq-i--v-ks--k--t-q--e-i-r---y----e-iss-dsqaah.n------e-  239
                    Box 3                                      Box 4
PBP3      LQYLAHRELRNALLENGAKAGSLVIMDVKTGEILAMTNQPTYNPNNRRNLQPAAMRNRAM  286
PBP3x     --fi-yka-ek-v-kf--hs--a-lvnp-s-q----a-f-s------asfa--f----tl  281
ECPBP3    --a-vy---n--vaf-k-es--a-lv--n---v---a-s-s-----lsgtpke-----ti  299
                                        Box 5
PBP3      IDVFEPGSTVKPFSMSAALASGRWKPSDIVDVYPGTLQIGRYTIRDVSRNSRQLDLTGIL  346
PBP3x     t-t-----vi-----------kfdensq-s-a--wmt-dgh--h--a-rdvltmtgvli  341
ECPBP3    t----------mvvmt--qr-vvrensvlnti-yringheikdvaryselt...---v-  356
                Box 6
PBP3      IKSSNVGISKIAFDIGAESIYSVMQQVGLGQDTGLGFPGERVGNLPNHRKWPKAETATLA  406
PBP3x     n.---i-m--v-lq--pkp-leqlgr--f-apls------np-y--f-e--snia--sms  400
ECPBP3    q-----v--l-lampssalvdtysrf---ka-n--lv---s-ly-qkqr-sdi-r--fs  416
            Box 7
PBP3      YGYGLSVTAIQLAHAYAALANDGKSVPLSMTRVDRVPDGVQVISPEVASTVQGMLQQVVE  466
PBP3x     f--s-a-ntae--q--svf-----l----ll-dnpqnqvr-amd-qi-rrira---t---  460
ECPBP3    f----m--pl---rv--tigsy-iyr---i-k--ppvp-er-fpesivr--vh-mes-al  476

PBP3      AQGGVFRAQVPGYHAAGKSGTARKVSVGTKGYRENAYRSLFAGFAPATDPRIAMVVVIDE  526
PBP3x     dpk--v--r-----v---------a-grgyadks..-----v-m--s--qlvla-m--s  518
ECPBP3    pg--gvk-aik--ri-i-t---k--gpdgryinkyiaytagv..---sq--f-l----nd  534
                       Box 8                          Box 9
PBP3      PSKAGYFGGLVSAPVFSKVMAGALRLMNVPPDNLPTATEQQQVNAAPAKGGRG          579
PBP3x     -tri----------t-ndi---s--alai-----qdspavadrqhhg                565
ECPBP3    -qagk-y--a------gai-g-v--t--ie--a-t-gdknef-inqgegt-grs         588
```

Figure 8. Amino acid sequence alignments of *P. aeruginosa* PBP3, PBP3x and *E. coli* PBP3. The protein sequences were aligned by the DNAMAN software program. Gaps introduced into the sequences are indicated by periods. Positions at which identical residues are found are indicated by - . The residues corresponding to the conserved boxes are bold. The numbers on the right correspond to the last residue in that row for each protein. The sequences are as follows: PBP3, *P. aeruginosa* PBP3; PBP3x, *P. aeruginosa* PBP3x; ECPBP3, *E. coli* PBP3.

belonged to boxes 6, 7 and 8 respectively. The active-site serine residue that binds to penicillin and is typically a part of the SXXK motif (box 6), was presumably located at residues 294 and 289 of *pbpB* and *pbpC* gene products. The SXN motif (boxes 7) was located at residues 349 to 351 and 343 to 345, while the KTG motif (box 8) was found at residues 484 to 486 and 478 to 480 of *pbpB* and *pbpC* gene product, respectively. As observed for other class B high molecular weight PBPs, the spacings between these boxes in the protein sequences were highly conserved.

Further analyses of the amino acid sequences of PBP3 and PBP3x with those of other PBPs indicated that both of these two proteins were more related to *E. coli* PBP3, *N. gonorrhoeae* PBP2 and *H. influenzae* PBP3, a group of PBPs functioning in cell-wall septum formation of Gram negative bacteria. The sequences of these two proteins were found to be similar, but to a lesser degree, to the *E. coli* PBP3-like proteins in *B. subtilis*, PBP2B and spoVD protein, and to the *E. coli* and *H. influenzae* PBP2 (Table 5).

6. Chromosomal locations of the genes cloned in this study

All plasmids described in Table 4 were examined by Dr. R. Levesque, Laval University and Dr. J. Lam, University of Guelph, respectively, to assist in the *P. aeruginosa* genome sequencing project. As the result of these collaborations, the physical locations of the genes cloned in this study were mapped by hybridization to the PAO1 chromosome (Fig. 9) (Liao *et al.*, 1996). Of note, *pbpB* and *pbpC* mapped

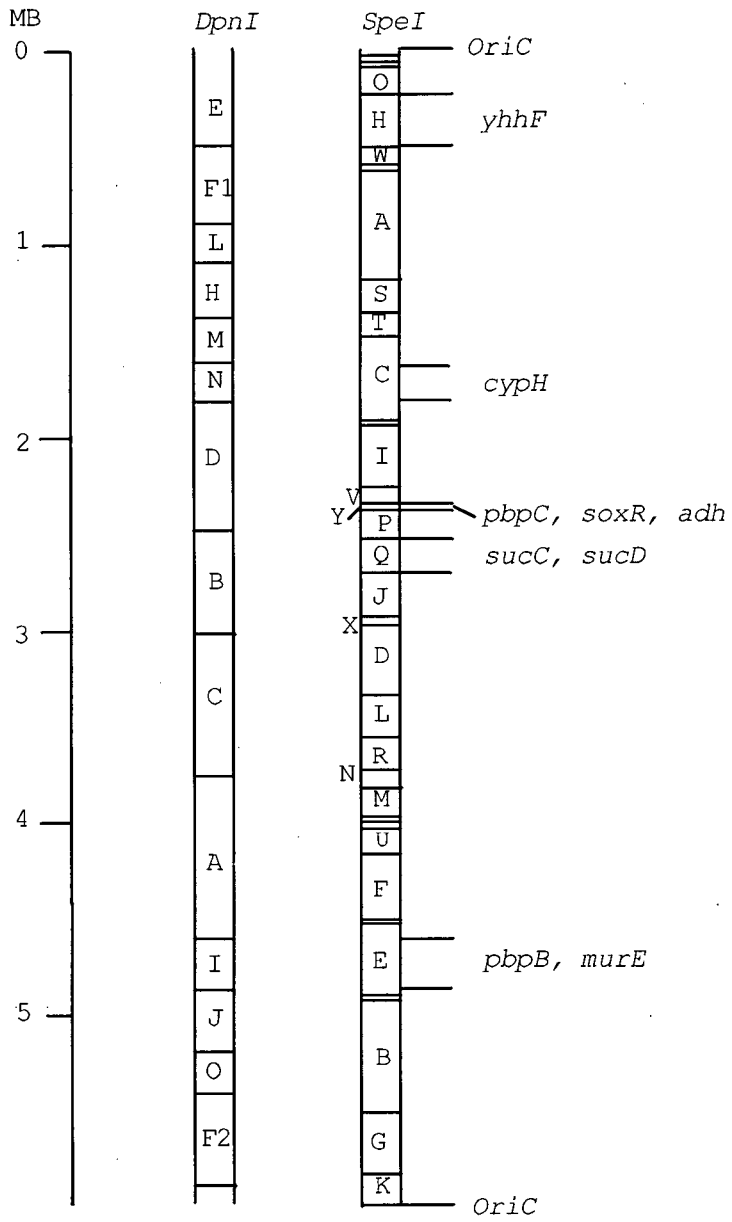

Figure 9. Physical map of the 5.94 Mb genome of PAO1. The locations of the genes are indicated by the horizontal lines. *Dpn*I and *Spe*I fragments are identified in capital letters. The smaller *Spe*I fragments are not indicated.

more than 2 Mb apart on the chromosome. The *pbpB* gene mapped to exactly the same genomic fragments as the *ftsA* and *envZ* genes, suggesting that *pbpB* and *murE* may be part of a large cluster of genes involved in cell division as observed in *E. coli*.

7. Summary

Two degenerate oligonucleotides were synthesized according to the amino acid sequences found in the conserved motifs of *E. coli* high-molecular-weight PBPs and *N. gonorrhoeae* PBP2 and subsequently used in a degenerate PCR amplification experiment using *P. aeruginosa* PAO1 chromosomal DNA as the template. Five of the PCR products were cloned and sequenced: two were found to translate to sequences with strong homology to *E. coli* PBP3 and *N. gonorrhoeae* PBP2 and were subsequently used as probes to clone the complete *pbpB* and *pbpC* genes; the other three PCR products were identified as the homologues of the *E. coli* gene products *sucC*/*sucD*, *yhhF* and *cypH* respectively. The physical locations of these genes on the genome of *P. aeruginosa* PAO1 were established in collaboration with others.

Probing of chromosomal digests separately with the two PCR products allowed the *pbpB* and *pbpC* genes to be cloned in *E. coli*. DNA sequence analysis of the *pbpB* region confirmed the cloning and led to the identification of an ORF in the downstream sequence, which encoded an amino acid sequence homologous to the *E. coli murE* gene product. DNA sequence analysis of the *pbpC* region revealed that *P. aeruginosa* contains a second copy of the gene coding for PBP3. This was confirmed

by the physical locations of the *pbpB* and *pbpC* genes on the PAO1 genome. Two putative ORFs were identified downstream of *pbpC*. These ORFs were found to be homologues of the *E. coli soxR* and *M. bovis adh* gene products, respectively.

Analyses of the deduced amino acid sequences of the *pbpB* and *pbpC* gene products suggested they were both integral cytoplasmic membrane proteins containing the nine conserved amino acid sequence motifs which have been identified as common to the class B, high-molecular-weight PBPs.

CHAPTER TWO Expression of the *P. aeruginosa pbpB* Gene Product and Mutational Analysis of the *pbpB* Gene

1. Introduction

Evidence of similar amino acid sequences and modular design between the *P. aeruginosa* and *E. coli pbpB* gene products strongly supported the hypothesis that the cloned *P. aeruginosa pbpB* gene encoded an *E. coli* PBP3-like protein. *P. aeruginosa* PBP3 is an important killing target for the newer generation β-lactam antibiotics (Watanabe *et al.*, 1988; Maejima *et al.,* 1991). It was observed by Godfrey *et al.* (1981) that PBP3 and /or PBP6 of some β-lactam-resistant clinical isolates of *P. aeruginosa* from cystic fibrosis patients had apparently lost their binding abilities to radiolabeled penicillin. Moreover, a laboratory mutant strain that is resistant to cefsulodin has been reported and its phenotype is associated with reduced PBP3 binding affinity (Gotoh *et al.*, 1990). Therefore, it seems likely that *P. aeruginosa* PBP3 plays an important role in susceptibility to β-lactam antibiotics. This chapter describes the characterization of this protein and the effect of its overproduction on the susceptibility of *P. aeruginosa* to β-lactam antibiotics.

2. Expression of the *P. aeruginosa pbpB* gene product in *E. coli*

2.1. Preliminary work

E. coli DH5α was transformed with pXLBI3 which contained the *P. aeruginosa pbpB* gene as a 5.4 kb *Sph*I-*Xho*I fragment cloned in the same orientation as the *lac* promoter on the vector pTZ18U (see Chapter One, section 3). Following induction with IPTG, membrane protein samples were collected and used in ^{3}H-penicillin binding assays. However, no novel PBP protein corresponding to the molecular mass of *P. aeruginosa* PBP3 was detected (data not shown). This might have been due to weak expression of the *pbpB* gene product or instability of the mRNA or the translational product. Accordingly, a more efficient expression system based on the T7 RNA polymerase and the corresponding promoter (Tabor & Richardson, 1985) was used for the expression of the *P. aeruginosa pbpB* gene product in *E. coli*.

2.2 pT7-7 as the expression vector

The expression vector pT7-7 contains the T7 RNA polymerase promoter and a RBS sequence followed by multiple cloning sites. The multiple cloning site contains an *Nde*I restriction site (CATATG, Fig. 10A). As part of the *Nde*I recognition sequence, ATG can be used as the translation start codon for appending in frame protein-encoding sequences. To permit cloning into this site, an *Nde*I site was engineered upstream of the *pbpB* gene. The *pbpB* gene was amplified by PCR using an upstream primer containing an *Nde*I recognition sequence in front of the sequence

A

GAAATTAATACGACTCACTATAGGGAGACCACAACGGTTTCCCTCTAGAAATAATTTTGTTTAACT
T7 promoter *Xba*I

TTAAGAAGGAGATATACATATGGCTAGAATTCGCGCGCCCGGGGATCCTCTAGAGTCGACC
RBS *Nde*I *Eco*RI *Sma*I *Bam*HI *Xba*I

B

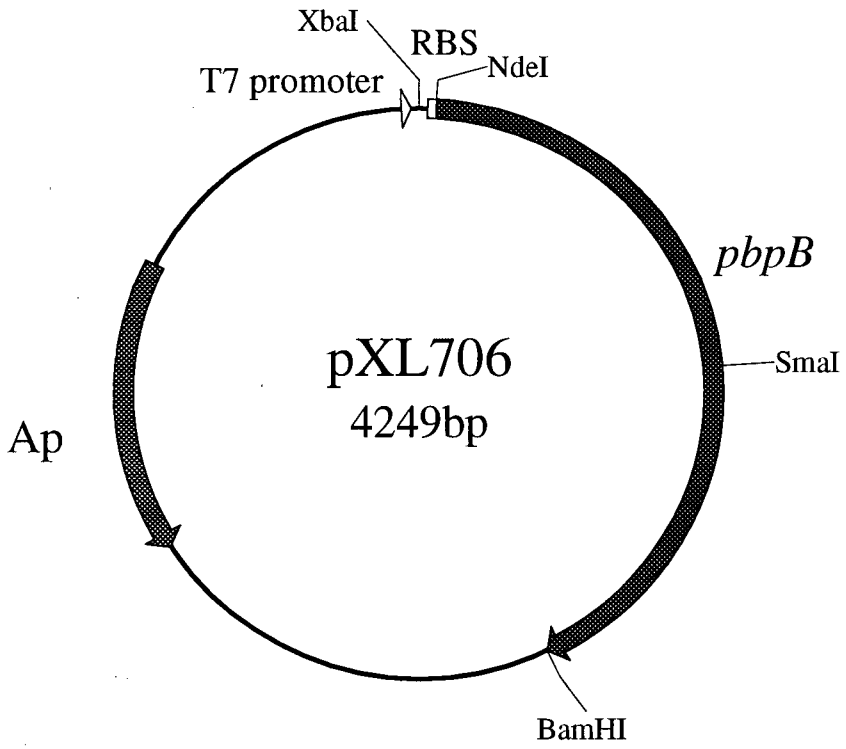

Figure 10. (**A**) Nucleotide sequence of the region containing the T7 RNA polymerase promoter, RBS and multiple cloning site on the vector pT7-7. (**B**) Diagram of pXL706 used for the expression of the *P. aeruginosa pbpB* gene product. The regions shown include the ampicillin resistance gene (Ap), promoter and RBS of T7 RNA polymerase, and the *P. aeruginosa pbpB* gene (*pbpB*). The plasmid is not drawn to scale. Only selected restriction enzyme sites are shown.

coding for the N-terminus of PBP3, and a downstream primer encoding the C-terminus of PBP3 followed by a stop codon and the sequence for a *Bam*HI recognition site (for the sequences utilized, see Materials and Methods section 8.1). The PCR product corresponding to the size of the *pbpB* gene was purified and cloned into the vector pT7-7 between the *Nde*I and *Bam*HI sites to generate the plasmid pXL706 (Fig. 10B).

The host strain for the expression of the *pbpB* gene product was *E. coli* K38 containing pGP1-2. pGP1-2 contains the gene for T7 RNA polymerase and the expression of this gene is temperature regulated. A novel protein was observed from the SDS-PAGE of whole cell lysate sample of *E. coli* K38/pGP1-2 (pXL706) after the induction of the T7 RNA polymerase expression at 42°C, indicating that the PBP3 was expressed in *E. coli* (Fig. 11A). ^{3}H-penicillin assay showed that the *pbpB* gene product bound penicillin (Fig. 12). However, the penicillin binding ability of the protein detected by the ^{3}H-penicillin assay appeared to be lower than expected, given its abundance in the membrane protein sample (Fig. 11B). This could possibly be due to incomplete removal of the β-lactamase produced by pT7-7, which might also explain the weak reaction between the PBP5/6 (42 kDa) and the ^{3}H-penicillin in the similar types of samples as demonstrated previously by Parr *et al.* (1988).

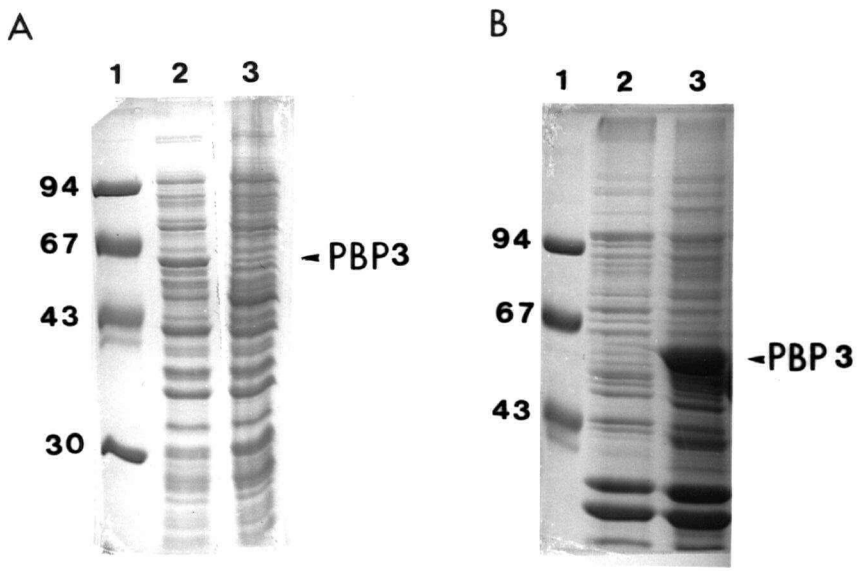

Figure 11. (**A**) SDS-10% PAGE of whole cell lysates. Lane 1, Standard molecular mass markers. Lane 2, K38/pGP1-2(pXL706), containing the cloned *P. aeruginosa pbpB* gene. Lane 3, K38/pGP1-2(pT7-7), vector control for the lane 2. (**B**) SDS-8.5% PAGE of cell membrane proteins. Lane 1, Standard molecular mass markers. Lane 2, K38/pGP1-2(pT7-7), vector control for the lane 3. Lane 3, K38/pGP1-2(pXL706), containing the cloned *P. aeruginosa pbpB* gene. Numbers indicate molecular mass in kDa. PBP3 in lane 2 (A) and lane 3 (B) are indicated by the arrows.

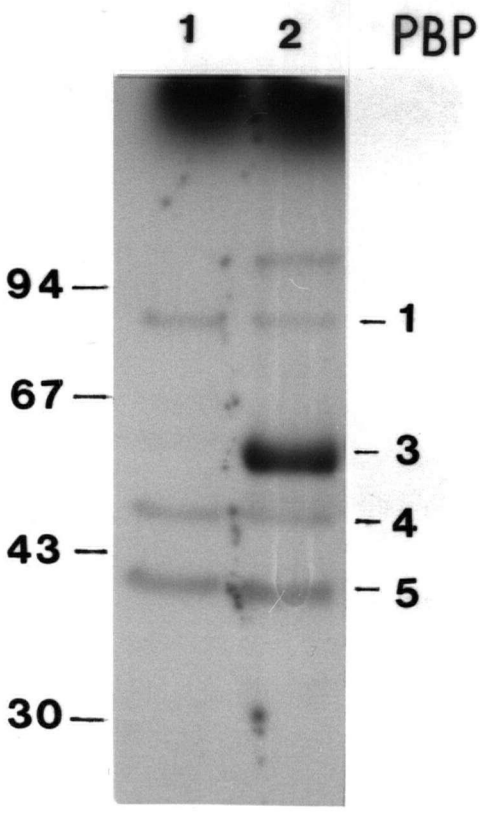

Figure 12. Autoradiogram of cell membrane proteins after incubation with ^{3}H-penicillin and separation by SDS-8.5% PAGE. Lane 1, K38/pGP1-2(pT7-7), vector control for the lane 2. Lane 2, K38/pGP1-2(pXL706), containing the cloned *P. aeruginosa pbpB* gene. Numbers on the left indicate molecular mass in kDa. PBPs are indicated on the right.

2.3 pBBR1MCS as the expression vector

To overcome the problem of the presence of the β-lactamase, a broad-host-range vector pBBR1MCS, which carries the T7 promoter (however, without the RBS sequence) and a chloramphenicol resistance gene, was used in combination with an *E. coli* strain BL21(DE3) in which the T7 RNA polymerase gene is present in the chromosome with its expression under the control of the *lac* promoter. The recombinant clone pXL608 (Fig. 13) was generated by cloning the 1.7 kb *Xba*I-*Bam*HI fragment isolated from pXL706 into the *Xba*I/*Bam*HI digested pBBR1MCS. This strategy moved the RBS from the pT7-7 along with the *pbpB* gene. The resultant plasmid pXL608 contained the *pbpB* gene under the control of the T7 promoter and in the opposite orientation to the *lac* promoter.

Upon induction with IPTG (0.5 mM), a novel protein was observed in the SDS-PAGE of the whole cell lysate sample of *E. coli* BL21(DE3)/pXL608, indicating that PBP3 was efficiently expressed (Fig. 14). The protein cofractionated with cytoplasmic membrane proteins (Fig. 15), indicating that it was incorporated efficiently into the membrane in the *E. coli* cells. An ^{3}H-penicillin assay showed that the *pbpB* gene product bound penicillin (Fig. 16). The protein migrated to a location similar to that of *E. coli* PBP3, which has an apparent molecular mass of 60 kDa.

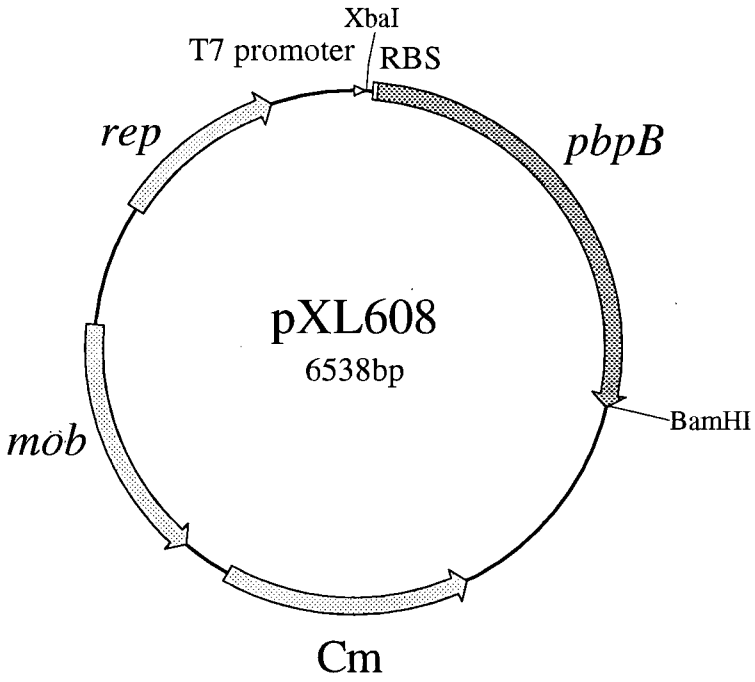

Figure 13. Diagram of pXL608 used for the expression of the *P. aeruginosa pbpB* gene product. The regions shown include the T7 RNA polymerase promoter, RBS, *pbpB* gene (*pbpB*), chloramphenicol resistance gene (Cm), gene required for plasmid mobilization (*mob*) and the gene required for plasmid replication (*rep*). The plasmid is not drawn to scale. Only selected restriction enzyme sites are shown.

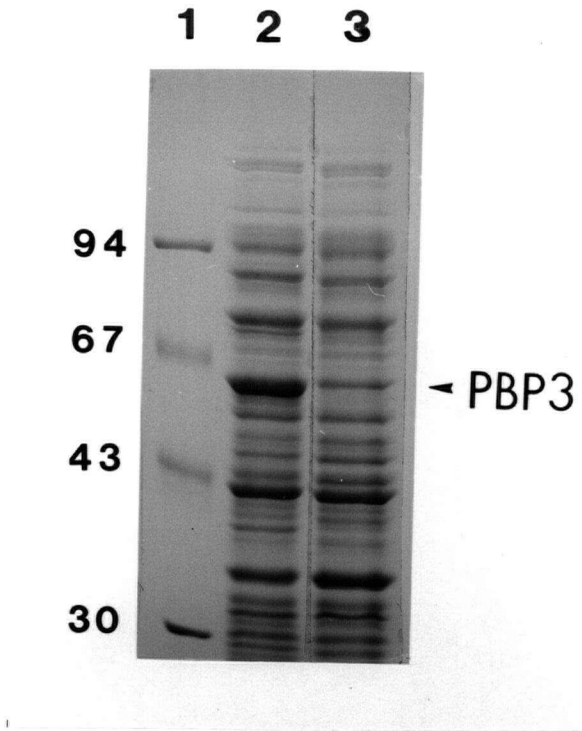

Figure 14. SDS-8.5% PAGE of whole cell lysates. Lane 1, Standard molecular mass markers. Lanes 2, BL21(DE3)/pXL608, containing the cloned *P. aeruginosa pbpB* gene. Lanes 3, BL21(DE3)/pBBR1MCS, the vector control for the lane 2. Numbers on the left indicate molecular mass in kDa. PBP3 present in the lane 2 is indicated by the arrow.

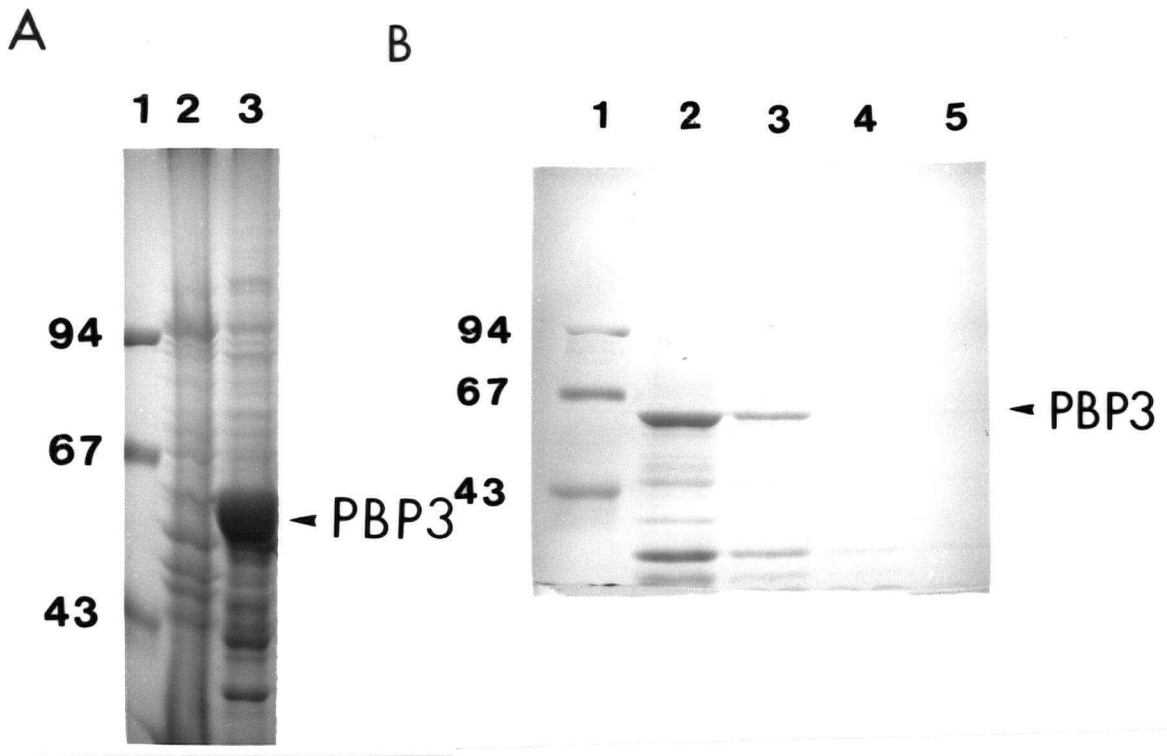

Figure 15. **(A)** SDS-8.5% PAGE of cell membrane proteins. Lane 1, Standard molecular mass markers. Lane 2, BL21(DE3)/pBBR1MCS, vector control for the lane 3. Lane 3, BL21(DE3)/pXL608, containing the cloned *P. aeruginosa pbpB* gene. **(B)** SDS-8.5% PAGE of cell membrane proteins. Lane 1, Standard molecular weight markers. Lanes 2, 3, 4 and 5, BL21(DE3)/pXL608 in 5 fold series dilutions (lane 2 contains 1/5 of the proteins in lane 3[A]). Numbers on the left indicate molecular mass in kDa. PBP3 is indicated by the arrow.

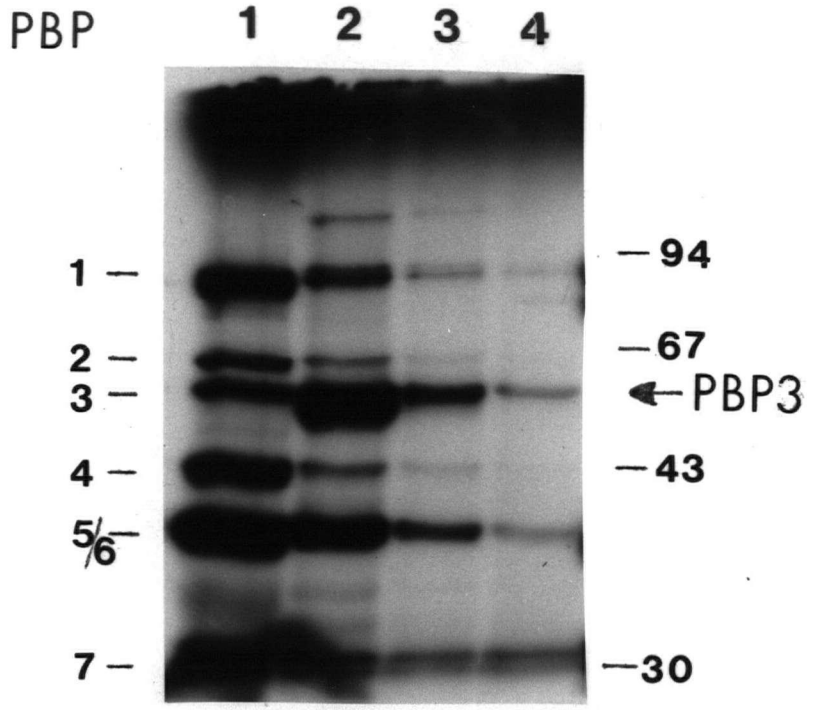

Figure 16. Autoradiogram of cell membrane proteins after incubation with ^{3}H-penicillin and separation by SDS-8.5% PAGE. Lane 1, BL21(DE3)/pBBR1MCS, containing same amount of membrane protein as in lane 2 Figure 15 (A). Lanes 2, 3 and 4, BL21(DE3)/pXL608, containing same amount of membrane proteins as lanes 2, 3 and 4 in Figure 15 (B) respectively. Numbers on the right indicate molecular mass in kDa. Numbers on the left indicate the locations of PBPs. *P. aeruginosa* PBP3 is indicated by the arrow.

2.4 Processing of the *P. aeruginosa pbpB* gene product in *E. coli*

The derived amino acid sequence of the *pbpB* gene product containing 579 amino acids had a calculated molecular mass of 62.856 kDa. However, the protein produced in *E. coli* migrated with apparent molecular mass of 60 kDa. The PBP3 protein was thus subjected to N-terminal amino acid sequencing after being transferred to a PVDF membrane following SDS-PAGE separation of the membrane protein sample prepared from *E. coli* K38/pGP1-2 (pXL706) (see figure 11B). The first six N-terminal amino acids were MKLNYF for PBP3, which was identical to that of the translated sequences. Therefore, the sequences at the N-terminus of PBP3 were apparently not removed, and did not appear to be characteristic of typical signal peptides. Nor did it contain a putative lipoprotein signal processing sequence as does the *E. coli* PBP3 (Hayashi *et al.*, 1988). It is possible that the disparity in molecular weights may reflect post-translational C-terminal processing, as is known to occur with the *E. coli* PBP3 (Nagasawa *et al.*, 1989; Hara *et al.*, 1991).

3. Overproduction of the *P. aeruginosa* and *E. coli pbpB* gene products in *P. aeruginosa*

P. aeruginosa PAO4089, a strain deficient in production of chromosomal β-lactamase, was chosen to be the host for expression of the *pbpB* gene product. This strain would reduce the problem of β-lactamase interference in the ^{3}H-penicillin assay.

The broad-host-range vector pUCP27, which is a derivative of the vector pUC19 and contains a tetracycline resistance gene and a stabilizing fragment for maintenance in *P. aeruginosa* was used as the expression vector. The 1.7 kb *Xba*I-*Bam*HI DNA fragment isolated from pXL706, which contained the RBS sequence from the pT7-7 and the *pbpB* gene, was cloned into pUCP27 behind the *lac* promoter to generate pXL506. This plasmid was transformed into PAO4089.

To address whether *E. coli* PBP3 functions in *P. aeruginosa*, an attempt was made to express the *E. coli pbpB* gene product in PAO4089. The 2.6 kb *Bam*HI-*Eco*RI fragment isolated from the plasmid pPH115 (obtained from Dr. B Spratt), which contained the *E. coli pbpB* gene and the upstream putative promoter sequence, was cloned into the vector pUCP27 behind the *lac* promoter to create pXLK20. This plasmid was then transformed into PAO4089.

Both the *P. aeruginosa* and *E. coli pbpB* gene products were expressed in PAO4089(pXL506) and PAO4089(pXLK20) with apparent molecular masses of 60 kDa, and could be detected by a ^{3}H-penicillin binding assay of membrane protein preparations (Fig. 17). The proteins were not visible by Coomassie Blue staining following SDS-PAGE of the membrane protein preparations. Using a scanning densitometer, the amounts of *P. aeruginosa* and *E. coli* PBP3 produced from the recombinant clones were estimated to be 7-fold that of the native PBP3 present in PAO4089(pXL546). pXL546, which contained a DNA fragment of 300 bp in length (corresponding to the 3′ end of the *P. aeruginosa pbpB* gene) cloned in the

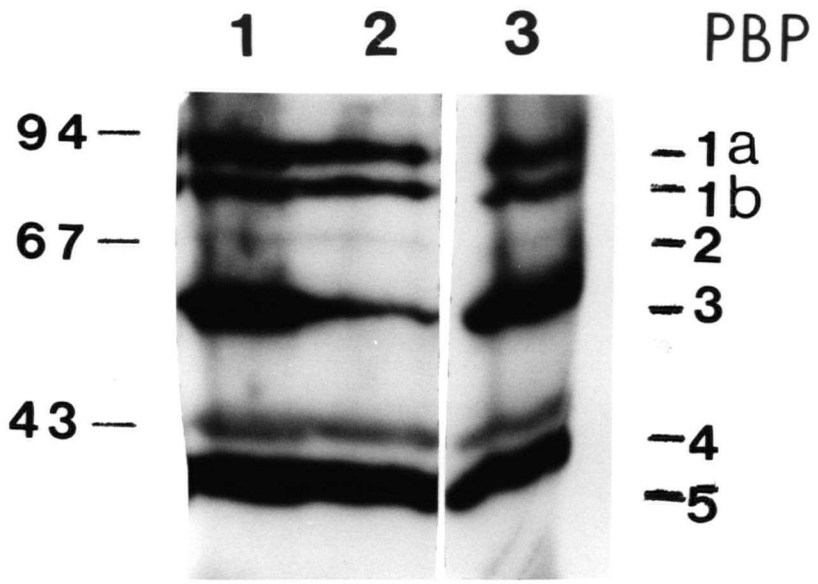

Figure 17. Autoradiogram of cell membrane proteins after incubation with ^{3}H-penicillin and separation by SDS-8.5% PAGE. Lane 1, PAO4089(pXL506), containing the cloned *P. aeruginosa pbpB* gene. Lane 2, PAO4089(pXL546), containing the 300 bp at 3′end of the *P. aeruginosa pbpB* gene and used as the control for the lane 1 and lane 3. Lane 3, PAO4089(pXLK20), containing the cloned *E. coli pbpB* gene. Numbers on the left indicate molecular mass in kDa. PBPs are indicated on the right.

pUCP27, failed to express any novel product.

4. Effect of overproduction of the *pbpB* gene products on the susceptibility of *P. aeruginosa* to β-lactam antibiotics

To investigate the effect of overproduction of the *P. aeruginosa* and *E. coli* PBP3 on antibiotic susceptibility, several β-lactam antibiotics, including the PBP3-targeted compounds cefsulodin (PBP1-targeted in *E. coli*), ceftazidime, cefepime and aztreonam, PBP2-targeted imipenem and PBP1-targeted cephaloridine, were tested for the MICs. Table 6 summarizes the MIC results for these antibiotics against *P. aeruginosa* PAO4089 producing *P. aeruginosa* or *E. coli* PBP3. Overproduction of PBP3 from the cloned *P. aeruginosa pbpB* gene led to 2-8 fold increased MICs of PBP3-targeted antibiotics. MICs of the PBP2-targeted and PBP1-targeted antibiotics were not influenced by the presence of the extra copy of this PBP3 protein. A similar result was observed in PAO4089 which overproduced the *E. coli pbpB* gene product, except that cefsulodin susceptibility was not affected, a result consistent with the fact that cefsulodin has a different target in *E. coli* as compared to *P. aeruginosa*.

The finding that the overexpression of the *P. aeruginosa* or *E. coli pbpB* gene product led to increased MICs, was further verified by an indirect ^{3}H-penicillin binding assay, or competition assay. ^{3}H-penicillin was used in a competition assay because it is the only radioactive-labeled β-lactam commercially available. The

Table 6. MICs of β-lactam antibiotics against PAO4089 expressing the *P. aeruginosa* and *E. coli* *pbpB* gene products

Strain	PBP gene expressed	MIC (ug/ml)					
		CTZ	CFS	CFPM	AZT	IMIP	CEPH
PAO4089	/	1.25	0.78	0.5	4	0.25	16
PAO4089(pXL546)	Δ*pbpB* ^{p}	1.25	0.78	0.5	4	0.25	16
PAO4089(pXL506)	*pbpB* ^{p}	**10**	**6.25**	**2**	**8**	0.25	16
PAO4089(pXLK20)	*pbpB* ^{e}	**5**	0.78	**2**	**8**	0.25	16

p, *P. aeruginosa* gene. e, *E. coli* gene.

parameter for evaluating the affinity of a PBP and a selected β-lactam antibiotic was the I_{50}, which is defined as the concentration of the β-lactam antibiotic that reduces the ^{3}H-penicillin binding by 50%. Therefore, the lower the measured I_{50}, the higher the affinity. The results of the competition binding assays indicated that the PBP3-targeted β-lactams, ceftazidime (Fig. 18), cefepime and aztreonam reacted preferentially with the *P. aeruginosa* and *E. coli* PBP3s (Table 7). In contrast, cefsulodin bound preferentially to *P. aeruginosa* PBP3 but did not influence penicillin binding to *E. coli* PBP3, a result consistent with its known binding properties. The data presented in Table 7 were consistent with literature data and demonstrated an overall pattern that was consistent with the MIC testing. Thus, the competition study confirmed that increases of the MICs of the PBP3-targeted antibiotics were due to the overproduction of the *P. aeruginosa* and *E. coli pbpB* gene products.

5. Mutational analysis of the *pbpB* gene

5.1 Rationale for carrying out the mutational analysis

PBP3 and PBP3x of *P. aeruginosa*, having similar amino acid sequences and modular designs, were related to *E. coli* PBP3 (Chapter One). Genes encoding the PBP3 and PBP3x were cloned independently and they were mapped to different loci on the *P. aeruginosa* chromosome (Chapter One). Taken together, these data suggest that *P. aeruginosa* contains two copies of a gene encoding proteins having similar

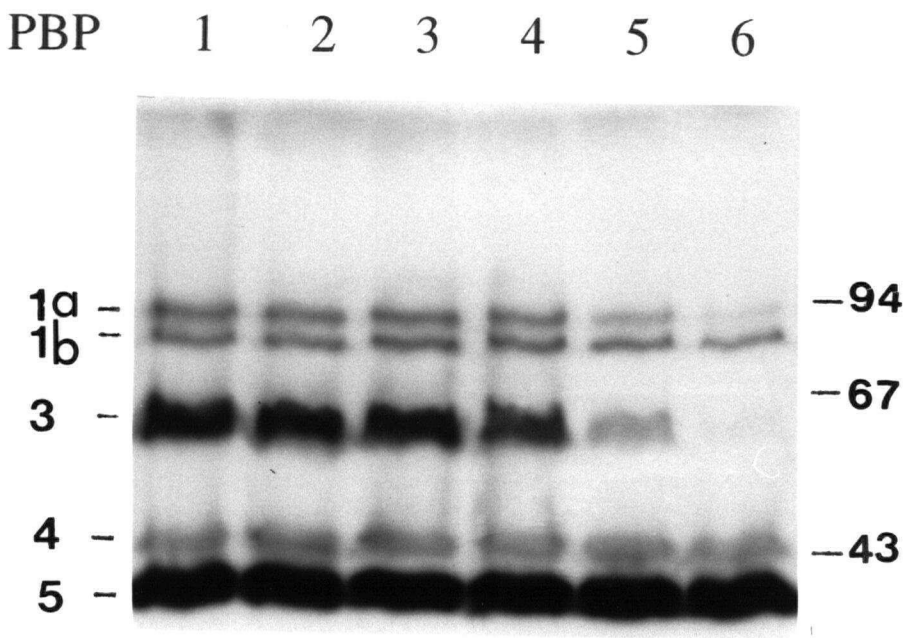

Figure 18. Autoradiogram of cell membrane proteins illustrating competition of ceftazidime with ^{3}H-penicillin for the PBPs of PAO4089 expressing the *P. aeruginosa pbpB* gene product. The ceftazidime concentration increases from 0.0125 to 3.2 ug/ml (4-fold increase/lane from lane 2 to 6) and lane 1 is the control containing no ceftazidime. Numbers on the right indicate molecular mass in kDa. PBPs are indicated on the left.

Table 7. I_{50} of β-lactam antibiotics for PBPs from PAO4089 expressing the *P. aeruginosa* and *E. coli pbpB* gene products

Strain	PBPs	I_{50} (ug/ml)				
		CTZ	CFS	CFPM	AZT	CEPH
PAO4089	1A	0.8	>9.6	>0.0625	>1.25	0.5
(pXL506)	1B	>3.2	1.6	>0.0625	>1.25	0.2
	3	0.4	0.6	0.0125	0.05	2.5
	4	>3.2	>9.6	>0.0625	>1.25	0.05
	5/6	>3.2*	>9.6*	>0.0625*	>1.25*	>5.0*
PAO4089	1A	0.8	25	0.15	>1.25	0.5
(pXLK20)	1B	>1.5	2.5	0.4	>1.25	0.2
	3	0.3	>25	0.15	0.25	8.0
	4	>1.5	>25	>0.75	>1.25	0.05
	5/6	>1.5*	>25*	>0.75*	>1.25*	>25*

pXL506. *P. aeruginosa pbpB* gene cloned in the vector pUCP27; pXLK20, *E. coli pbpB* gene cloned in the vector pUCP27.
* indicating the highest concentration used in the competition assay.
PBP2 was not observed in these experiments.

functions to *E. coli* PBP3. Subsequently, it was found that PBP3 and PBP3x had different affinities for various PBP3-targeted β-lactam antibiotics (see Chapter Two, section 3 and next Chapter, section 3). This result suggested that these two proteins might have different roles in cell growth. To test if PBP3 and PBP3x were redundant in *P. aeruginosa*, whether they both functioned in concert, or if one of them was silent, an attempt was made to construct PBP3-defective or PBP3x-defective mutants (see below).

5.2 Mutagenesis of the *pbpB* gene

The strategy for constructing a PBP3-defective mutant, as illustrated in Fig. 19, involved the use of a gene replacement procedure (Schweizer, 1992) which involved a ColE1-type plasmid pNOT19, which contains a unique *Not*I site, a MOB3 cassette as a *Not*I fragment containing *oriT*, and the *B. subtilus sacB* gene as a counter-selectable marker for promotion of gene replacement. The insertion plasmid pBPB::Km (Fig. 20) was generated by cloning the mutated chromosomal sequences of *pbpB* (disrupted by a kanamycin resistance cartridge) and the MOB3 cassette into pNOT19 (Fig. 19, step 1). The mobilization of the insertion plasmid into *P. aeruginosa* and subsequent growth of the transconjugants on VBMM medium (selective for *P. aeruginosa* growth) containing kanamycin and carbenicillin should select for plasmid integration into the chromosome, *i.e.* a tandem duplication of the wild-type and mutant alleles of the cloned gene with the plasmid sequence in between the two copies (Fig. 19, step 2).

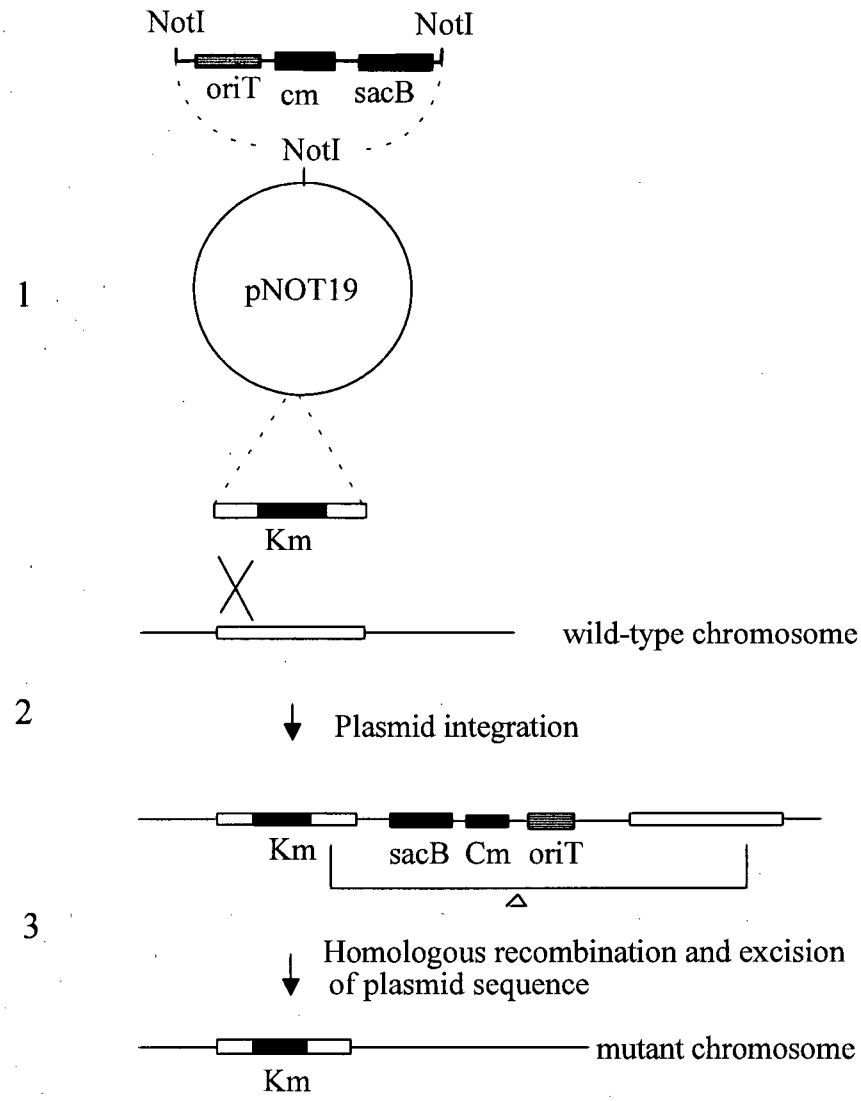

Figure 19. Schematic summary of gene replacement procedure for constructing a PBP3-defective or PBP3x-defective mutant. 1, Cloning of the mutated *pbpB* or *pbpC* gene and the MOB3 cassette into pNOT19 to generate an insertion plasmid. 2, Mobilization of the insertion plasmid into *P. aeruginosa* followed by growth on a selective medium for obtaining cointegrates. 3, Growth on sucrose containing medium to select homologous recombination and excision of plasmid sequences.

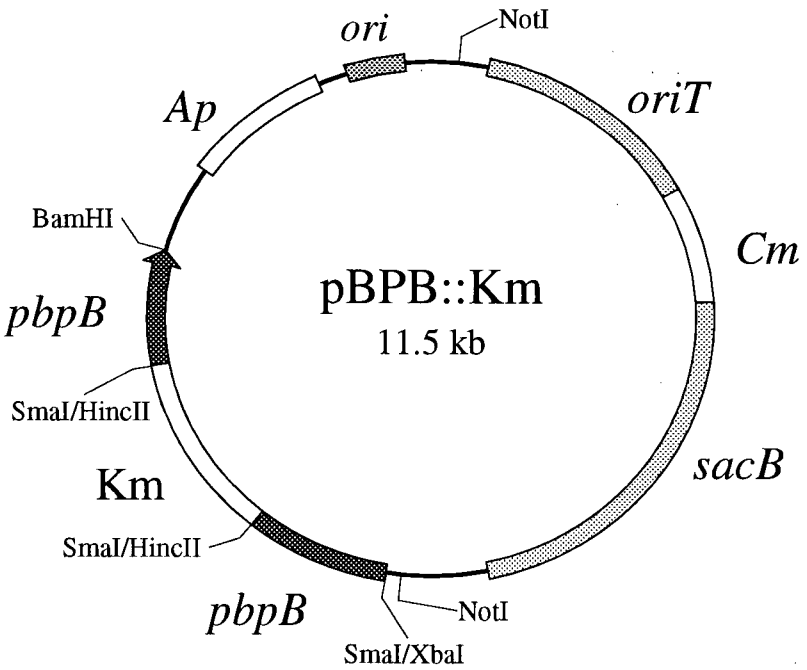

Figure 20. Diagram of pBPB::Km used for allele replacement mutagenesis. The lightly shaded arrow between the *Xba*I and *Bam*HI restriction site represents the *P. aeruginosa pbpB* gene and the open bar in the middle represents the 1.3 kb kanamycin resistance cartridge that was used to interrupt the *pbpB* gene. The fragment between two *Not*I site is the 5.8 kb MOB3 cassette including origin of transfer (*oriT*), chloramphenicol resistance gene (Cm) and the *sacB* loci from *B. subtilus*. *Ori* indicated the colE1 origin of replication. The fragments are not drawn to scale. Only selected restriction sites are shown.

A subsequent shift to a sucrose-containing medium should promote the deletion of the plasmid sequence by homologous recombination, since the expression of the *sacB* gene is lethal to *P. aeruginosa* in the presence of sucrose at 37°C (Fig. 19, step 3).

In an attempt to generate integration of the plasmid pBPB::Km into the chromosome of PAO1 strain H103, it was found that in repeated attempts no transconjugant could grow on the VBMM medium containing kanamycin and carbenicillin. The lack of cointegrates did not prove the *pbpB* gene was essential, since at this stage one normal and one defective copy of the gene would be present. Instead this could possibly be due to the location of the *pbpB* gene within a cluster of cell division genes, likely causing a deleterious polar effect on the expression of the downstream genes.

6. Summary

The *P. aeruginosa pbpB* gene product was expressed in *E. coli* using the T7 RNA polymerase and promoter system. The expressed protein was exported to the cytoplasmic membrane of *E. coli* cells and bound ^{3}H-penicillin. It had an apparent molecular mass of 60 kDa, whereas the calculated molecular mass was 62.856 kDa. The N-terminal amino acid sequence of the expressed protein was identical to that of PBP3 deduced from the nucleotide sequence of the *pbpB* gene, suggesting that there was no N-terminal processing.

The *P. aeruginosa* and *E. coli pbpB* gene products were expressed in *P. aeruginosa* PAO4089 using a broad-host-range vector pUCP27. Results from MIC testing and ^{3}H-penicillin binding competition assays indicated that overproduction of both the *P. aeruginosa* and *E. coli pbpB* gene products in *P. aeruginosa* led to increased resistance to the PBP3-targeted compounds aztreonam, cefepime, cefsulodin (except for *E. coli pbpB* gene product) and ceftazidime.

An attempt was made to construct a PBP3-defective mutant using a gene replacement technique. However, no PBP3-defective mutant was obtained. This could be due to the location of the *pbpB* gene at the proximal end of a cluster of cell division genes, where the placement of a polar mutation would be lethal to the cells.

CHAPTER THREE Expression of the *P. aeruginosa pbpC* Gene Product and Mutational Analysis of the *pbpC* Gene

1. Introduction

In Chapter two, it was reported that the *P. aeruginosa pbpB* gene was efficiently expressed in *E. coli* by the T7 RNA polymerase/promoter system and the expressed *pbpB* gene product was subsequently characterized by a ^{3}H-penicillin binding assay. It was found that the *P. aeruginosa pbpB* gene product produced in *E. coli* was not processed at its N-terminus, however, a disparity between the calculated and apparent molecular mass was observed. Overproduction of both the *P. aeruginosa* and *E. coli pbpB* gene products in *P. aeruginosa* results in increased resistance to selected PBP3-targeted β-lactam antibiotics. The *P. aeruginosa pbpC* gene product has similar amino acid sequence and modular design to those of both the *P. aeruginosa* and *E. coli pbpB* gene products (Chapter One). This chapter describes further characterization of the *P. aeruginosa pbpC* gene product by the approaches described in Chapter two. The influence of PBP3x overproduction on the susceptibility to β-lactam antibiotics was also investigated. To further test whether *P. aeruginosa* contains two genes encoding proteins having similar function to *E. coli* PBP3, a PBP3x-deficient mutant was characterized and is described in the last section of this chapter.

2. Expression of the *P. aeruginosa pbpC* gene product in *E. coli*

2.1. Preliminary work

The membrane protein samples from *E. coli* DH5α (pXLHd2) were examined for the expression of the PBP3x protein. pXLHd2 (see figure 5B) had the *pbpC* gene and the upstream putative RBS and promoter sequences cloned in the opposite orientation to the *lac* promoter in the vector pTZ18U. However, no novel PBP protein corresponding to the molecular mass of *P. aeruginosa* PBP3x was detected by the ^{3}H-penicillin assay (data not shown). Therefore, an expression system using the T7 RNA polymerase and promoter was applied to the expression of the *P. aeruginosa pbpC* in *E. coli.*

2.2 pT7-7 as the expression vector

As described for the expression of *pbpB* gene, an *Nde*I site was engineered upstream of the *pbpC* gene by using an upstream PCR primer containing an *Nde*I recognition sequence in front of the sequence coding for the N-terminus of PBP3x. The ATG in the *Nde*I recognition sequence was the translational start codon for the *pbpC* gene, and a downstream primer containing the sequence coding for the C-terminus of PBP3x followed by a stop codon and the sequence for a *Bam*HI recognition site (for sequences, see Materials and Methods section 8.1). The amplified *pbpC* gene was purified and cloned into the vector pT7-7 between the *Nde*I and *Bam*HI sites, to generate pXL732 (Fig. 21).

A

GAAATTAATACGACTCACTATAGGGAGACCACAACGGTTTCCCTCTAGAAATAATTTTGTTTAACT
T7 promoter *Xba*I

TTAAGAAGGAGATATACATATGGCTAGAATTCGCGCGCCCGGGGATCCTCTAGAGTCGACC
RBS *Nde*I *Eco*RI *Sma*I *Bam*HI *Xba*I

B

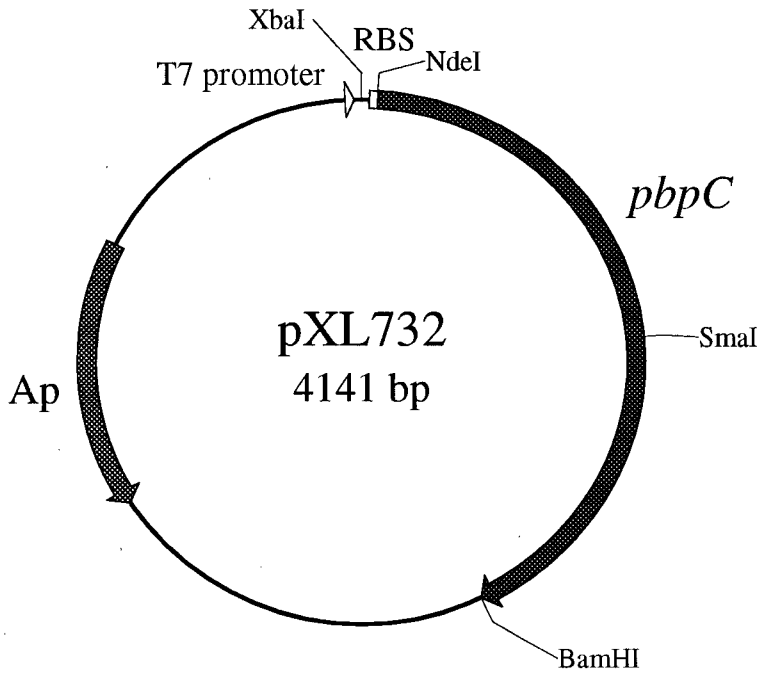

Figure 21. **(A)** Nucleotide sequence of the region containing the T7 RNA polymerase promoter, RBS and multiple cloning site on the vector pT7-7. **(B)** Diagram of pXL732 used for the expression of the *P. aeruginosa pbpC* gene product. The regions shown include the ampicillin resistance gene (Ap), T7 RNA polymerase promoter, RBS and the *P. aeruginosa pbpC* gene. The plasmid is not drawn to scale. Only selected restriction enzyme sites are shown.

The host strain for the expression of the *pbpC* gene was *E. coli* K38 containing pGP1-2. A novel protein was observed from the SDS-PAGE of whole cell lysate samples of *E. coli* K38/pGP1-2 (pXL732) after the induction of the T7 RNA polymerase expression at 42°C, indicating that the PBP3x was produced in *E. coli* (Fig. 22A). An ^{3}H-penicillin assay showed that the *pbpC* gene product bound penicillin (Fig. 23). As observed in the expression of the *pbpB* gene product using pT7-7 (Chapter Two), the penicillin binding ability of the PBP3x protein detected by ^{3}H-penicillin assay appeared to be lower than expected given its abundance in the membrane protein sample (Fig. 22B). This could possibly be due to incomplete removal of the β-lactamase produced by pT7-7.

2.3 pBBR1MCS as the expression vector

To avoid the problem of the presence of the β-lactamase, pBBR1MCS was used as an alternative expression vector. The recombinant clone pXL629 (Fig. 24) was generated after ligation of pBBR1MCS with the 1.7 kb *Xba*I-*Bam*HI fragment isolated from pXL732, which contained the *pbpC* gene and the RBS sequence from the vector pT7-7. The resultant plasmid pXL629 contained the *pbpC* gene under control of the T7 promoter and in the opposite orientation to the *lac* promoter.

After induction with IPTG (0.5 mM), a novel protein was observed

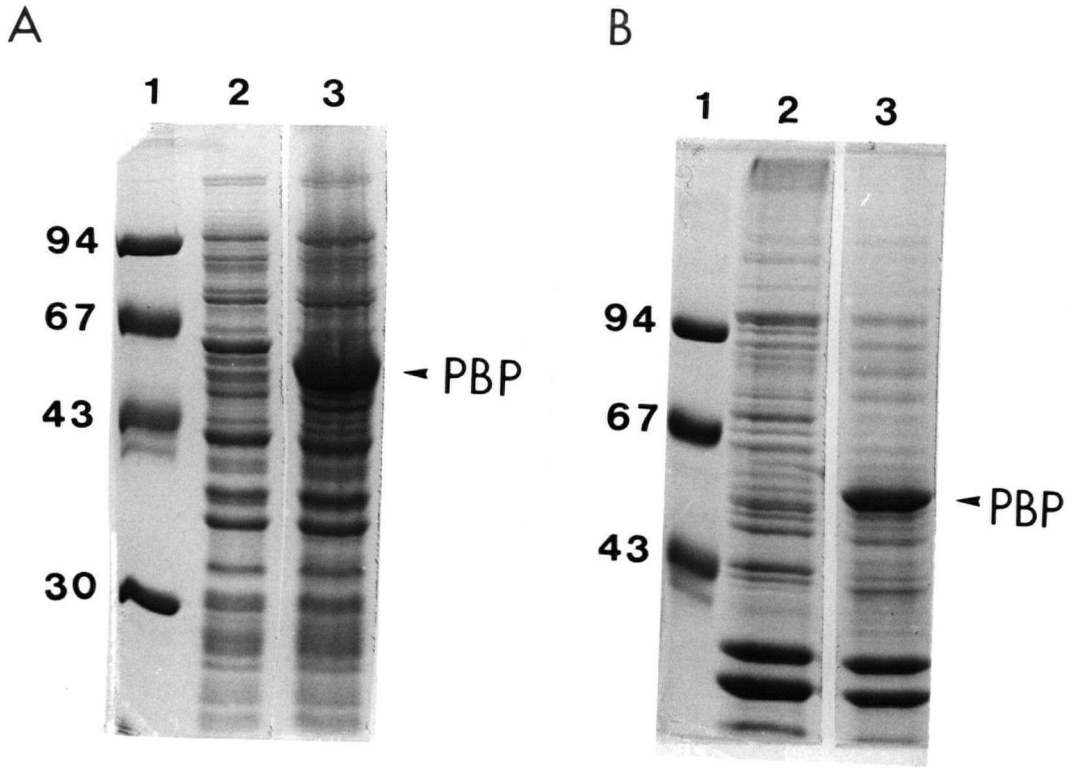

Figure 22. **(A)** SDS-10% PAGE of whole cell lysates. Lane 1, Standard molecular mass markers. Lane 2, K38/pGP1-2(pXL506), containing the cloned *P. aeruginosa pbpB* gene. lane 3, K38/pGP1-2(pXL732), containing the cloned *P. aeruginosa pbpC* gene. **(B)** SDS-8.5%PAGE of cell membrane proteins. Lane 1, Standard molecular mass markers. Lane 2, K38/pGP1-2(pT7-7), vector control for the lane 3. Lane 3, K38/pGP1-2(pXL732), containing the cloned *P. aeruginosa pbpC* gene. Numbers on the left indicate molecular mass in kDa. PBP3x is indicated by the arrow.

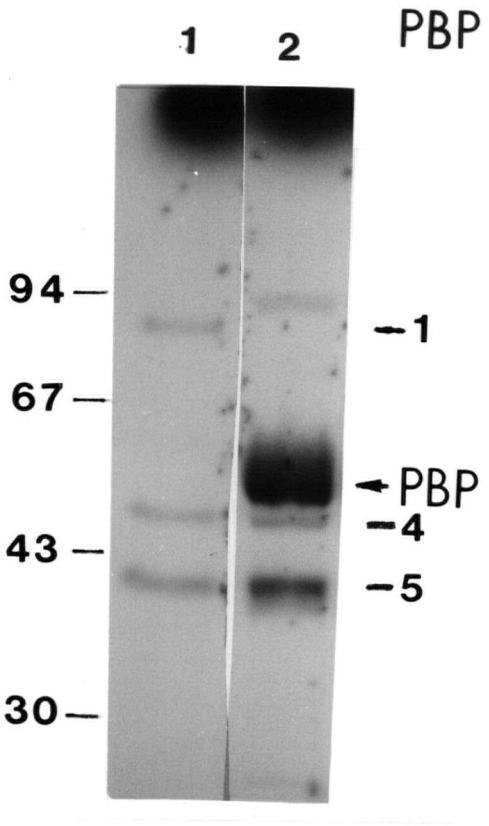

Figure 23. Autoradiogram of cell membrane proteins after incubation with ^{3}H-penicillin and separation by SDS-8.5% PAGE. Lane 1, K38/pGP1-2(pT7-7), vector control for the lane 2. Lane 2, K38/pGP1-2(pXL732), containing the cloned *P. aeruginosa pbpC* gene. Numbers on the left indicate molecular mass in kDa. PBPs are indicated on the right. PBP3x is indicated by the arrow.

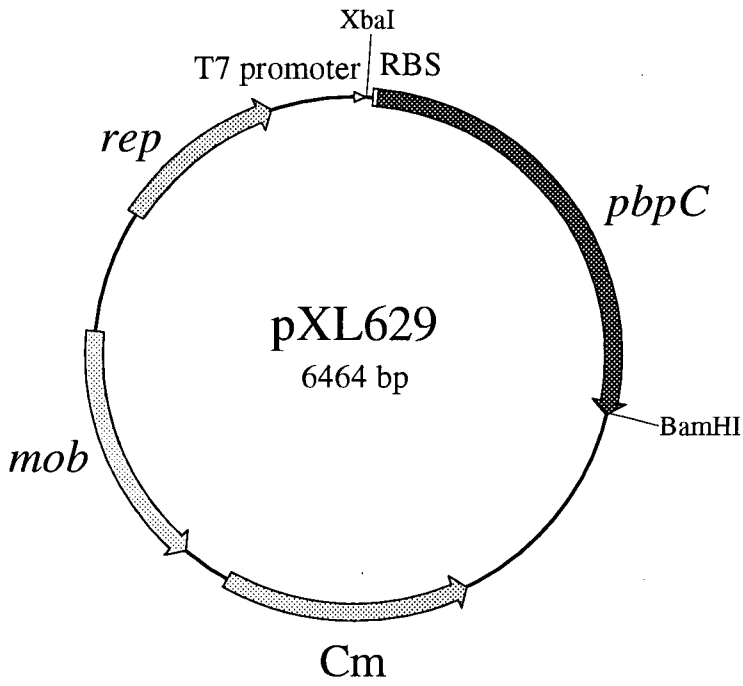

Figure 24. Diagram of pXL629 used for the expression of the *P. aeruginosa pbpC* gene product. The regions shown include the T7 RNA polymerase promoter, RBS sequence, *P. aeruginosa pbpC* gene, chloramphenicol resistance gene (Cm), gene required for plasmid mobilization (*mob*) and the gene required for plasmid replication (*rep*). The plasmid is not drawn to scale. Only selected restriction enzyme sites are shown.

in the SDS-PAGE of the whole cell lysate sample of *E. coli* BL21(DE3)/pXL629, indicating that *pbpC* was efficiently expressed (Fig. 25). As with PBP3, protein PBP3x was produced efficiently after induction with IPTG for 1 hr, and levels of protein production increased with longer time (up to 4 hr examined) of IPTG induction. The protein cofractionated with the cytoplasmic membrane proteins (Fig. 26), indicating that it was incorporated efficiently into the membrane of the *E. coli* cells. The ^{3}H-penicillin assay confirmed that the *pbpC* gene product bound penicillin (Fig. 27). The PBP3x protein migrated to a gel location slightly lower than that of *E. coli* PBP3, with apparent molecular mass of 58 kDa.

2.4 Processing of the *P. aeruginosa pbpC* gene product in *E. coli*

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 apparent molecular mass of 58 kDa. PBP3x did not appear to contain an N-terminus characteristic of a typical signal peptide, nor did it contain a putative lipoprotein signal processing sequence as proposed for *E. coli* PBP3. To confirm the lack of the N-terminal processing, the PBP3x protein was subjected to N-terminal amino acid sequencing after transfer to a PVDF membrane following SDS-PAGE separation of the membrane protein sample prepared from *E. coli* K38/pGP1-2 (pXL732) (see figure 22B). The six N-terminal amino acids analyzed were SSQRRN, which was identical to that of the derived sequence, except that the first residue

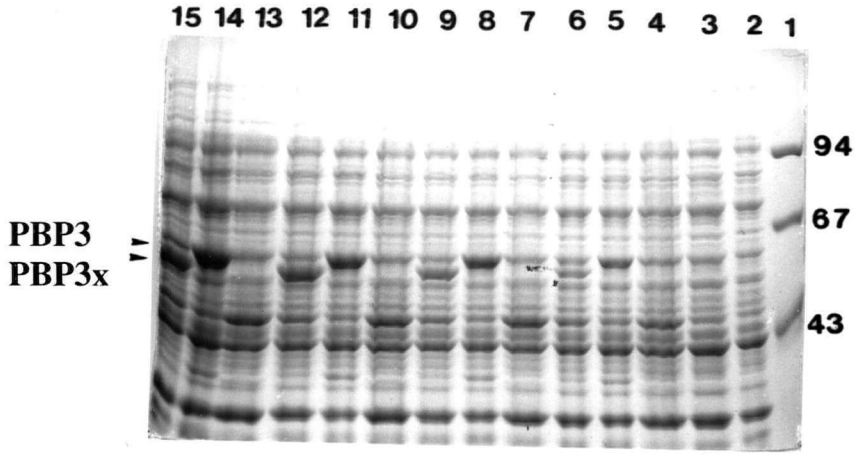

Figure 25. SDS-8.5% PAGE of whole cell lysates. Lane 1, Standard molecular mass markers. Lanes 2, 5, 8, 11 and 14, BL21(DE3)/pXL608, containing the cloned *P. aeruginosa pbpB* gene. Lanes 3, 6, 9, 12 and 15, BL21(DE3)/pXL629, containing the cloned *P. aeruginosa pbpC* gene. Lanes 4, 7, 10 and 13, BL21(DE3)/pBBR1MCS, the vector control for the clones pXL608 and pXL629. Lanes 2 and 3, IPTG induction for 0 hr. Lanes 4, 5 and 6, IPTG induction for 1 hr. Lanes 7, 8 and 9, IPTG induction for 2 hr. Lanes 10, 11 and 12, IPTG induction for 3 hr. Lanes 13, 14 and 15, IPTG induction for 4 hr. Numbers on the right indicate molecular mass in kDa. PBP3 and PBP3x are indicated by the arrows.

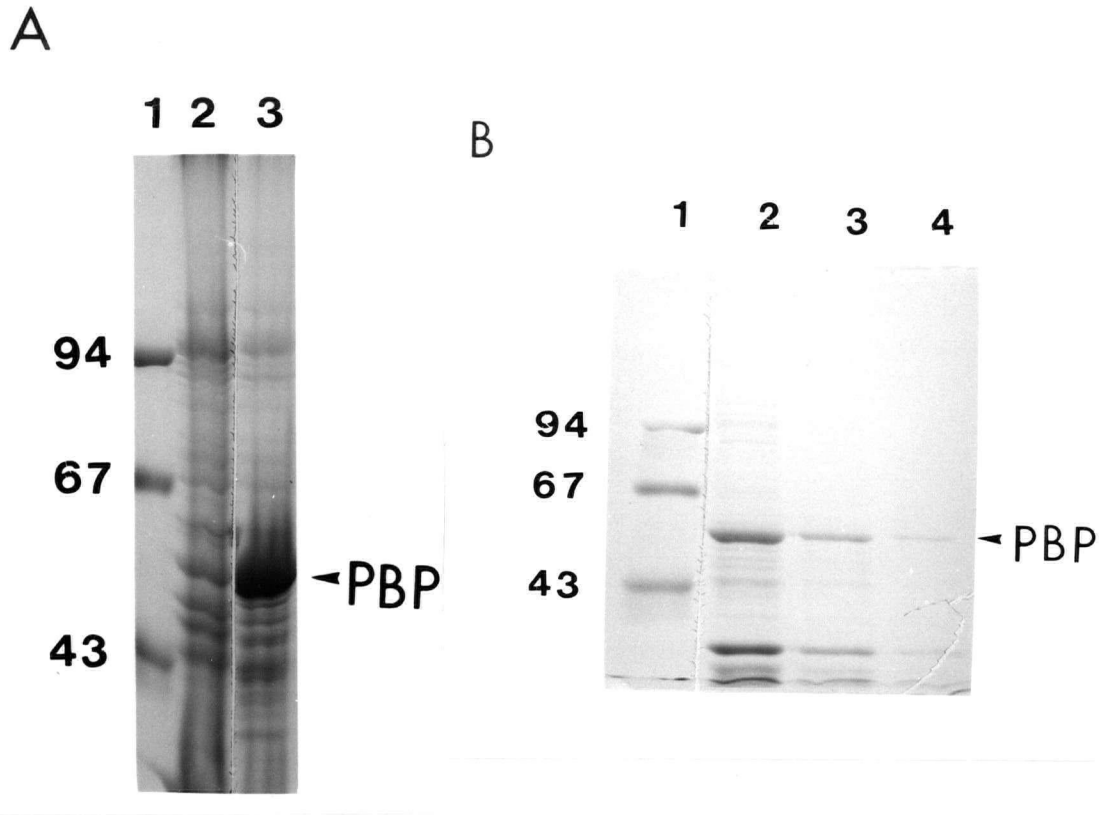

Figure 26. **(A)** SDS-8.5% PAGE of cell membrane proteins. Lane 1, Standard molecular mass markers. Lane 2, BL21(DE3)/pBBR1MCS, vector control for the lane 3. Lane 3, BL21(DE3)/pXL629, containing the cloned *P. aeruginosa pbpC* gene. **(B)** SDS-8.5% PAGE of cell membrane proteins. Lane 1, Standard molecular mass markers. Lanes 2, 3, and 4, BL21(DE3)/pXL629 in 5 fold series dilutions (lane 2 contains 1/5 of the proteins in lane 3 [A]). Numbers on the left indicate molecular mass in kDa. PBP3x is indicated by the arrow.

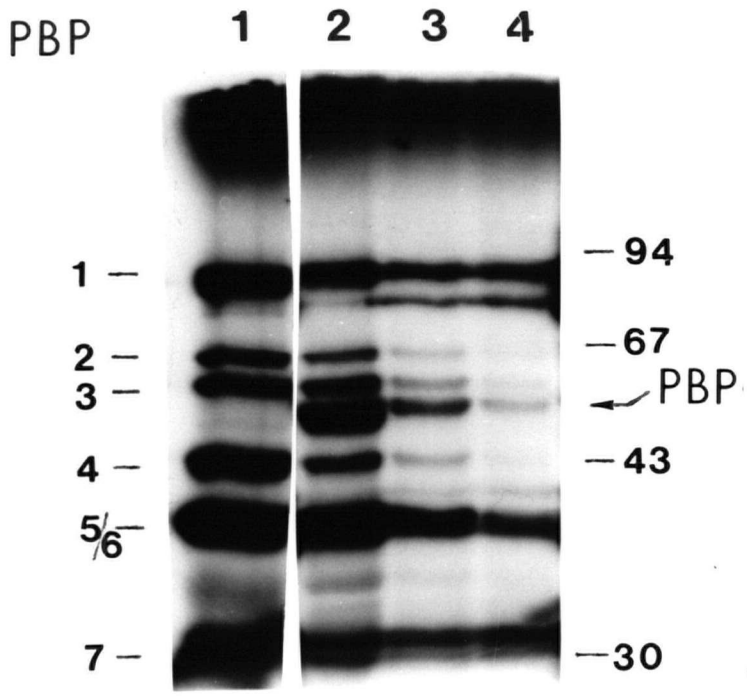

Figure 27. Autoradiogram of cell membrane proteins after incubation with ^{3}H-penicillin and separation by SDS-8.5%PAGE. Lane 1, BL21(DE3)/pBBR1MCS, containing same amount of membrane protein as in lane 2 Figure 15 (A). Lanes 2, 3 and 4, BL21(DE3)/pXL629, containing same amount of membrane proteins as lanes 2, 3 and 4 in Figure 26 (B) respectively. Numbers on the right indicate molecular mass in kDa. Numbers on the left indicate the locations of PBPs. PBP3x is indicated by the arrow.

methionine was cleaved from the gene product of *pbpC*. Similar to *P. aeruginosa* PBP3, the sequences at the N-terminus of PBP3x were not apparently removed. It is possible therefore that the disparity in molecular mass may reflect post translational C-terminal processing.

3. Overproduction of the *pbpC* gene product in *P. aeruginosa*

P. aeruginosa PAO4089 was chosen as the host for expression of the *pbpC* gene product. The broad-host-range vector pUCP27 was used as the expression vector. The 1.7 kb *Xba*I-*Bam*HI DNA fragments isolated from pXL732, which contained the RBS sequence from the pT7-7 and the *pbpC* gene, was cloned into pUCP27 behind the *lac* promoter to generate pXL519. This plasmid was transformed into PAO4089.

A novel protein with a molecular mass of 58 kDa was detected by ^{3}H-penicillin assay of the membrane protein sample of PAO4089(pXL519) (Fig. 28). The protein was not visible from Coomassie blue staining on SDS-PAGE of the membrane protein preparations. The amount of the *P. aeruginosa* PBP3x produced from the recombinant clone was estimated to be about the same as the native PBP3 present in PAO4089(pXL546).

4. Effect of overproduction of the *pbpC* gene product on the susceptibility of *P. aeruginosa* to β-lactam antibiotics

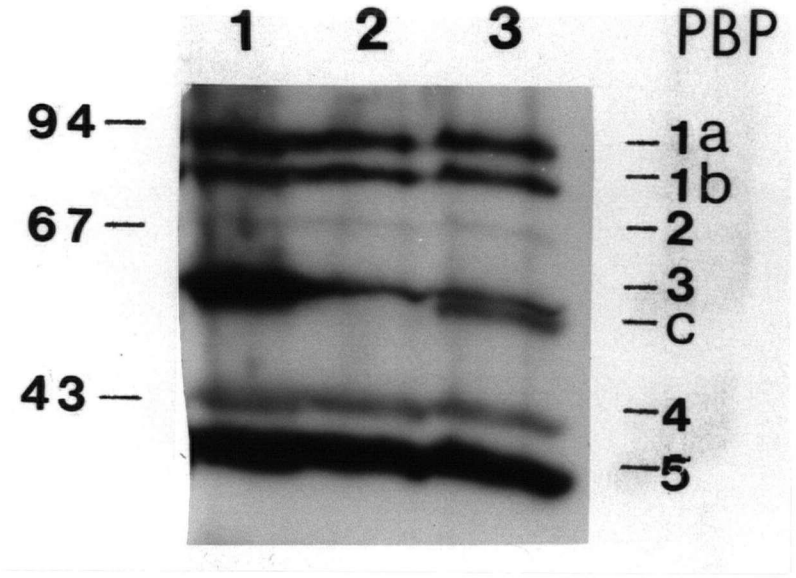

Figure 28. Autoradiogram of cell membrane proteins after incubation with ^{3}H-penicillin and separation by SDS-8.5%PAGE. Lane 1, PAO4089(pXL506), containing the cloned *P. aeruginosa pbpB* gene. Lane 2, PAO4089(pXL546), containing the 300 bp at 3′end of the *P. aeruginosa pbpB* gene and used as the control for the lane 1 and lane 3. Lane 3, PAO4089(pXL519), containing the cloned *P. aeruginosa pbpC* gene. Numbers on the left indicate molecular mass in kDa. PBPs are indicated on the right (PBP3x is indicated by C).

β-lactam antibiotics, including the PBP3-targeted compounds cefsulodin (PBP1-targeted in *E. coli*), ceftazidime, cefepime and aztreonam, PBP2-targeted imipenem and PBP1-targeted cephaloridine, were used to determine the MICs of *P. aeruginosa* PAO4089 expressing the *pbpC* gene product. It appeared that the presence of an extra *P. aeruginosa pbpC* gene had no effect on the susceptibility of PAO4089 to the β-lactam antibiotics tested in this study (Table 8). The results of competition binding assays indicated that the PBP3-targeted β-lactams, ceftazidime (Fig. 29), cefsulodin, cefepime and aztreonam, primarily reacted with the *P. aeruginosa* PBP3 (Table 9), and to a much lesser (*i.e.*, 3 to >16 fold) extent with the *P. aeruginosa* PBP3x. This suggested that the *pbpC* gene product had lower affinity to the PBP3-targeted β-lactams than did the PBP3 protein, and thus would not be expected to affect antibiotic susceptibility when PBP3 was present in the cells (for the antibiotics that primary bind to PBP3).

5. Mutational analysis of the *pbpC* gene

5.1 Construction of a PBP3x-defective mutant

The strategy for constructing a PBP3x-defective mutant is illustrated in Fig. 19. The insertion plasmid pBPC::Km (Fig. 30) was generated by cloning the mutated chromosomal sequences of *pbpC* (disrupted by a kanamycin resistance cartridge) and the MOB3 cassette into pNOT19 (Fig. 19, step 1). This plasmid was introduced into

Table 8. MICs of β-lactam antibiotics against PAO4089 expressing the *P. aeruginosa* *pbpC* gene product

Strain	PBP gene expressed	MIC (ug/ml)					
		CTZ	CFS	CFPM	AZT	IMIP	CEPH
PAO4089	/	1.25	0.78	0.5	4	0.25	16
PAO4089(pXL546)	Δ*pbpB* ^{p}	1.25	0.78	0.5	4	0.25	16
PAO4089(pXL519)	*pbpC* ^{p}	1.25	0.78	0.5	4	0.25	16

p, *P. aeruginosa* gene.

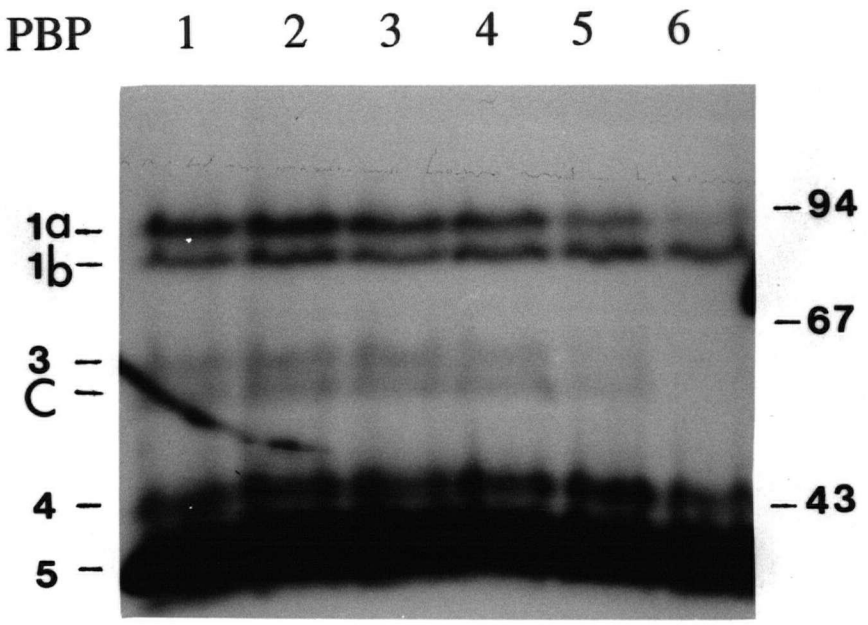

Figure 29. Autoradiogram of cell membrane proteins illustrating competition of ceftazidime with ^{3}H-penicillin for the PBPs of PAO4089 expressing the *P. aeruginosa pbpC* gene product. The ceftazidime concentration increases from 0.0125 to 3.2 ug/ml (4-fold increase/lane from lane 2 to 6) and lane 1 is the control containing no ceftazidime. Numbers on the right indicate molecular mass in kDa. PBPs are indicated on the left (PBP3x is indicated by C).

Table 9. I_{50} of β-lactam antibiotics for PBPs from PAO4089 expressing the *P. aeruginosa pbpC* gene product

Strain	PBPs	I_{50} (ug/ml)				
		CTZ	CFS	CFPM	AZT	CEPH
PAO4089	1A	0.8	>9.6	0.14	>1.25	0.5
(pXL519)	1B	>3.2	2.8	>0.3125	>1.25	0.2
	3	0.2	0.6	0.0125	0.05	2.5
	C	0.6	>9.6	0.06	0.32	2.5
	4	>3.2	>9.6	>0.3125	>1.25	0.05
	5/6	>3.2*	>9.6*	>0.3125*	>1.25*	>5.0*

pXL519, *P. aeruginosa pbpC* gene cloned in the vector pUCP27.
*, indicating the highest concentration used in the competition assay.
PBP2 was not observed in these experiments.

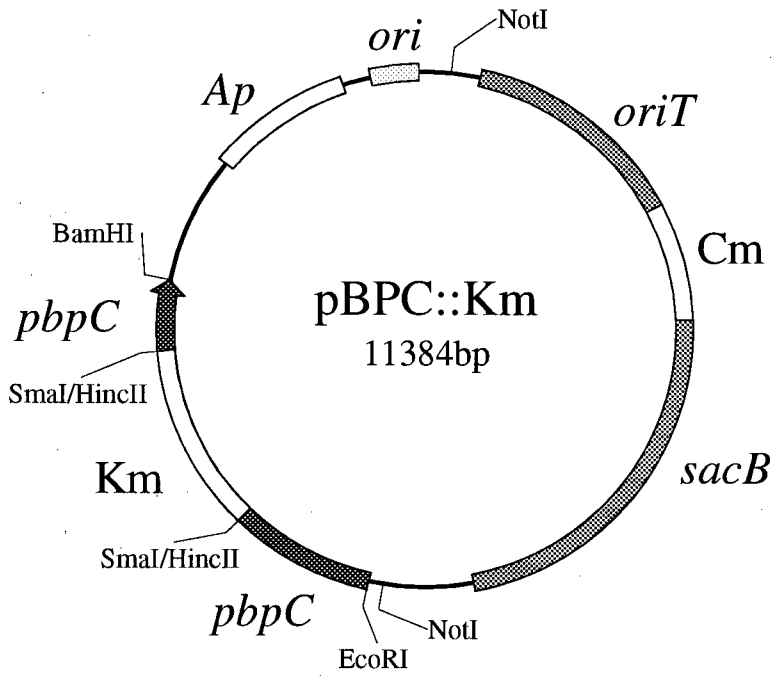

Figure 30. Diagram of pBPC::Km used for allele replacement mutagenesis. The lightly shaded arrow between the *Eco*RI and *Bam*HI restriction site represents the *P. aeruginosa pbpC* gene and the open bar in the middle represents the 1.3 kb kanamycin resistance cartridge that was used to interrupt the *pbpC* gene. The fragment between two *Not*I site is the 5.8 kb MOB3 cassette which includes origin of transfer (*oriT*), chloramphenicol resistance gene (Cm) and the *sacB* loci from *B. subtilus*. Ori indicates the colE1 origin of replication. The fragments are not drawn to scale. Only selected restriction sites are shown.

P. aeruginosa PAO1 strain H103 and plasmid cointegrates were selected on VBMM containing kanamycin and carbenicillin. An estimated 160 colonies were then patched onto LB agar containing kanamycin and carbenicillin to confirm the cointegration; a single colony was found to grow on this plate. Transfer to MH agar containing kanamycin and 5% sucrose resulted in growth, indicating that the plasmid sequences had possibly been eliminated from the chromosome. The chromosomal DNA of this strain, named HC132, was subsequently isolated, digested with *EcoR*I and *Xho*I, and analyzed by Southern blot hybridization. A probe derived from the 0.78 kb *Xho*I-*Sma*I fragment within the 5′ region of the *pbpC* gene hybridized to a 3.3 kb *EcoR*I fragment and a 1.9 kb *Xho*I fragment (Fig. 31). These results confirmed that the 1.3 kb kanamycin resistance cartridge had been inserted into the *pbpC* gene on the chromosome.

5.2 PBP profile of the PBP3x-defective mutant

^{3}H-penicillin binding assays of the membrane proteins of PAO1 strain H103 and the mutant strain HC132 showed the same PBP profile (Fig. 32). These data indicate that the *pbpC* gene product was not expressed under the conditions tested.

5.3 Cell morphology and growth of PBP3x-defective mutant

Stationary phase cells of H103 and HC132 grown in MH broth were harvested

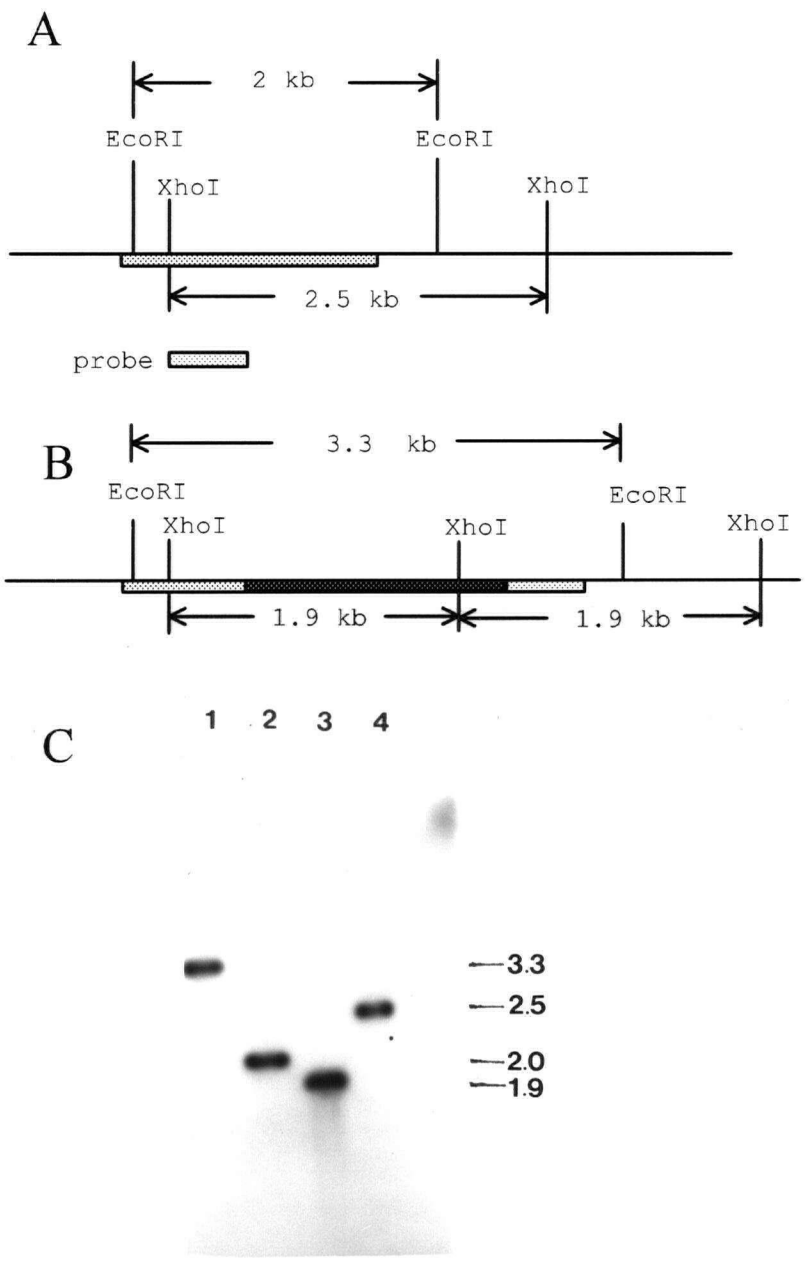

Figure 31. Southern hybridization demonstrating the interruption of the *pbpC* gene (dotted box) with a kanamycin resistance cartridge (dark-dotted box). The physical maps of the wild-type **(A)** and the mutant **(B)** *pbpC* locus are shown. The chromosomal DNAs were digested **(C)** with *Eco*RI and *Xho*I respectively and were hybridized to the DIG-labelled 0.78 kb *Xho*I-*Sma*I fragment shown in panel A. Lane 1, HC132 DNA digested with *Eco*RI. Lane 2, H103 DNA digested with *Eco*RI. Lane 3, HC132 DNA digested with *Xho*I. Lane 4, H103 DNA digested with *Xho*I. The molecular sizes of the fragments in kilobases are indicated on the right.

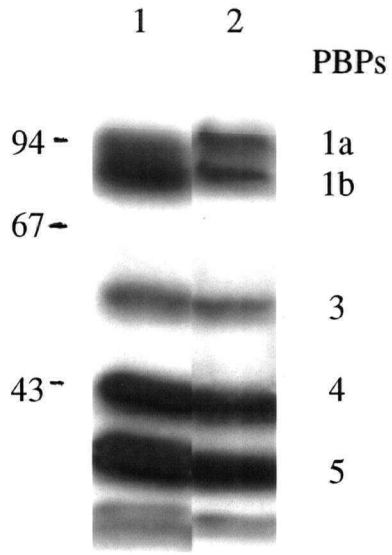

Figure 32. Autoradiogram of cell membrane proteins after incubation with ^{3}H-penicillin and separation by SDS-7.5%PAGE. Lane 1, HC132, PBP3x-defective mutant. Lane 2, H103, wild type. Numbers on the left indicate the molecular mass in kDa. PBPs are indicated on the right. PBP2 was not observed in this experiment.

and observed under a light microscope. As shown in Fig. 33 A & B, mutation of the *pbpC* gene did not cause any major change of cell shape. Growth curve experiments using MH broth showed no difference between H103 and HC132 (Fig. 34). These results suggested that the *pbpC* gene product was not essential for normal cell morphology or viability under the conditions tested. Results of MIC testing indicated that *pbpC* deficient did not change the susceptibility of *P. aeruginosa* to the tested β-lactam antibiotics mentioned in the previous section.

6. Summary

The *P. aeruginosa pbpC* gene was expressed in *E. coli* using the T7 RNA polymerase and promoter system. The protein was exported to the cytoplasmic membrane of *E. coli* cells and reacted with ^{3}H-penicillin. The protein had the apparent molecular mass of 58 kDa, whereas the calculated molecular mass was 61.128 kDa. The N-terminal amino acid sequence of the expressed protein was identical to that of PBP3x except that the N-terminal methionine was removed.

The *P. aeruginosa pbpC* gene was expressed in *P. aeruginosa* PAO4089 using a broad-host-range vector pUCP27. Data from MIC testing and ^{3}H-penicillin binding competition assays indicated that the presence of the *pbpC* gene product in the PAO4089 did not have any effect on susceptibility to PBP3- targeted antibiotics, in contrast to the properties of PAO4089 overproducing the *P. aeruginosa* and *E. coli* *pbpB* gene products.

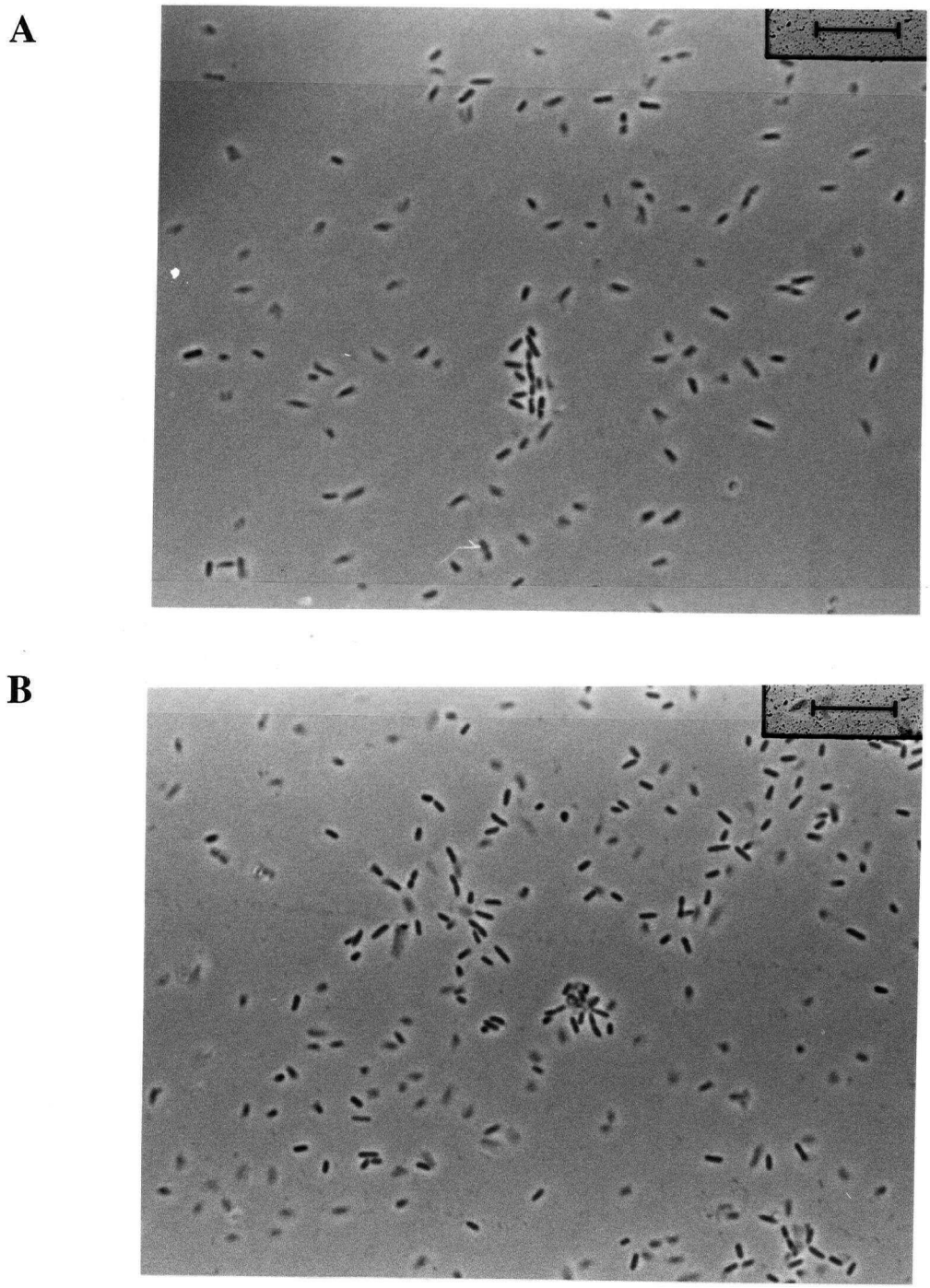

Figure 33. Cell morphology of *P. aeruginosa*. **(A)** H103, wild type strain. **(B)** HC132, PBP3x-defective mutant strain. The bar markers represent 10 μm.

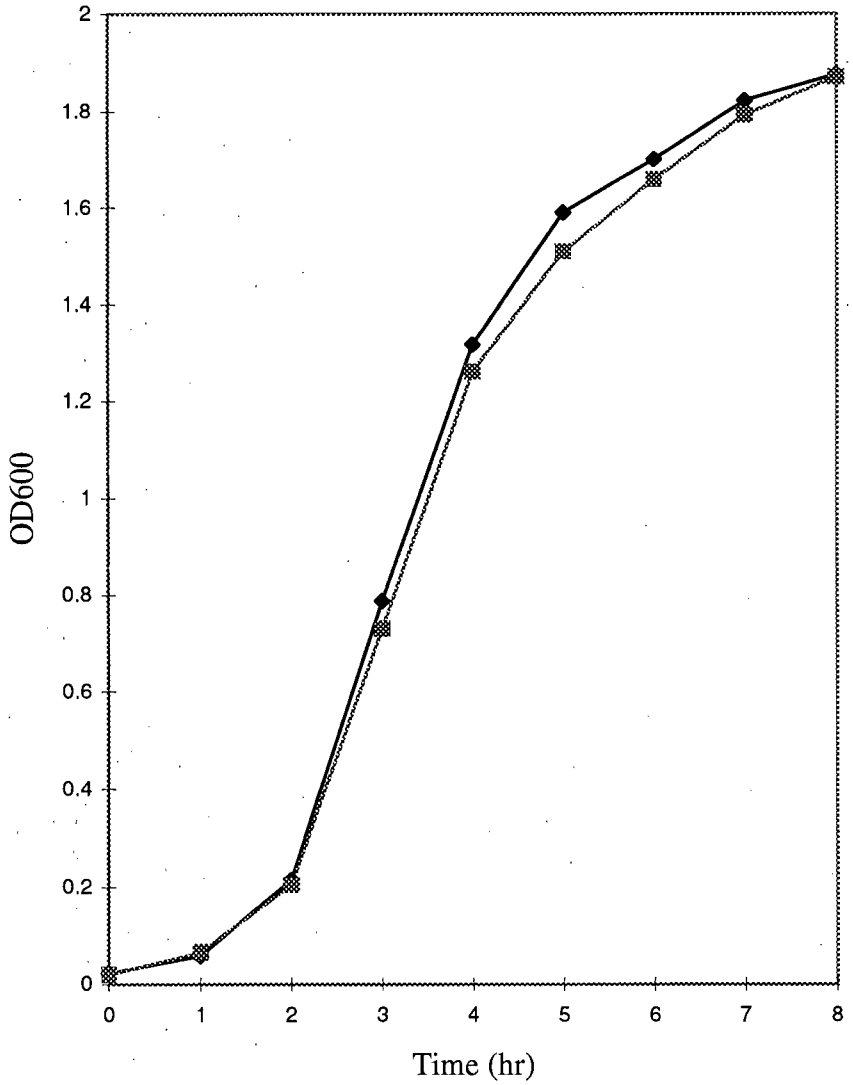

Figure 34. Growth of *P. aeruginosa* PBP3x-defective mutant. Strains were grown at 37°C in MH broth and the optical density was monitored. Solid line, H103, wild type strain. Dashed line, HC132, PBP3x-defective mutant strain.

A PBP3x-defective mutant (strain HC132) was obtained by a gene replacement procedure and confirmed by Southern blot analysis. The PBP profiles of wild type strain H103 and mutant strain HC132 were similar, suggesting that the *pbpC* gene was not visibly expressed under the physiological conditions tested. Furthermore, inactivation of *pbpC* did not cause any changes in cell morphology or growth rate, indicating that *pbpC* was not required for cell viability under normal laboratory growth conditions.

DISCUSSION

1. General

P. aeruginosa is a clinically important bacterium that is intrinsically resistant to a large number of β-lactam antibiotics. In addition, mutation resulting in high levels of resistance to β-lactam antibiotics is common with this species. Several hypotheses have been proposed to explain this property including low membrane permeability, the presence of β-lactamase activity and alterations in the affinity of drug targets (*i.e.* PBPs). The analysis of the resistance of *P. aeruginosa* to β-lactam antibiotics requires information on how specific *P. aeruginosa* PBPs interact with β-lactam antibiotics. Knowledge on how the *P. aeruginosa* PBPs interact with β-lactam antibiotics will facilitate the design of more efficient drugs and regimes for the treatment of *P. aeruginosa* infections.

Prior to the work presented here, PBPs in *P. aeruginosa* had not been well studied and none of the *P. aeruginosa* PBP genes had been cloned or sequenced, except for a partial sequence of PBP1a-encoding gene which was published three years ago (Martin *et al.*, 1993). Of particular interest was whether *P. aeruginosa* PBPs possess similar structures and functions to those in other bacterial species such as *E. coli*, and whether *E. coli* PBPs and *P. aeruginosa* PBPs could be functionally exchanged.

In previous reports it had been suggested that *P. aeruginosa* PBP3 was the primary target for the expanded-spectrum and fourth generation cephalosporins (Watanabe *et al.*, 1988; Maejima *et al.*, 1991). In addition, *P. aeruginosa* β-lactam-resistant clinical isolates from cystic fibrosis patients had apparently lost PBP3 and /or PBP6 (Godfrey *et al.*,1981). The work presented here was designed to address the hypothesis that *P. aeruginosa* PBP3 plays an important role in susceptibility to β-lactam antibiotics. Here I describe the cloning and characterization of the *P. aeruginosa pbpB* and *pbpC* genes. This work demonstrated that overproduction of the *P. aeruginosa* or *E. coli pbpB* gene product in *P. aeruginosa* could confer resistance to the PBP3-targeted β-lactam antibiotics aztreonam, cefepime, cefsulodin and ceftazidime. This study also revealed that the presence of the *P. aeruginosa pbpC* gene did not affect the susceptibility of *P. aeruginosa* to a number of PBP3-targeted antibiotics tested.

2. Cloning of the *P. aeruginosa pbpB* and *pbpC* genes

The strategy for cloning the *P. aeruginosa* PBP3 gene was based on constructing a pair of degenerate PCR primers according to the amino acid sequences found in the conserved SXXK and KTG motifs of the *E. coli* high-molecular-weight PBPs and *N. gonorrhoeae* PBP2. This pair of degenerate PCR primers were employed in PCR reactions using *P. aeruginosa* chromosomal DNA as template;

several PCR products were obtained and subsequently cloned. DNA sequencing and DNA sequence analyses of the cloned PCR products led to the identification of two independent and different clones that had significant similarity to *E. coli* PBP3 suggesting the presence of two *E. coli* PBP3-like genes in *P. aeruginosa*. Subsequently, the cloned PCR products were used as probes to clone both genes which were named *pbpB* and *pbpC*, respectively.

The genes encoding *P. aeruginosa* PBP3 and PBP3x mapped to two different loci on the *P. aeruginosa* chromosome. The *pbpB* gene mapped to a region of the chromosome located upstream of an *E. coli murE* homologue and was linked to a cluster of other essential genes involved in cell division. This finding suggests that the *P. aeruginosa pbpB* gene product, like *E. coli* PBP3, may be essential for cell division. The *pbpC* gene mapped approximately 2 megabase pairs from the *pbpB* gene and the gene appeared to be dispensable under normal laboratory growth conditions.

3. Structures of PBP3 and PBP3x

All of the high-molecular-weight PBPs examined to date are cytoplasmic membrane proteins with their hydrophobic amino-terminus traversing the cytoplasmic membrane and the reminder of the protein extending into the periplasm (Ghuysen, 1991). This type of membrane topology is not unexpected since the transglycosylation, transpeptidation and carboxypeptidation reactions, that are

catalyzed by PBPs, are known to occur either on the outer surface of the cytoplasmic membrane or within the periplasmic space (Bowler & Spratt, 1989; van Heijenoort, 1994). The membrane topology of *E. coli* PBP3 has been deduced from its primary amino acid sequence in combination with protein fusion studies (using β-lactamase as a reporter) and proteolytic digestion experiments. The results of these experiments have consistently shown that *E. coli* PBP3 is embedded in the cytoplasmic membrane at its amino terminus only and that the bulk of the protein is localized to the periplasmic space (Bowler & Spratt, 1989). The amino acid sequence of the *P. aeruginosa* PBP3 and PBP3x proteins showed that they were 45.1% and 40.7% identical to the *E. coli* PBP3 protein and that alignment with *E. coli* PBP3 was good. In addition, the hydropathy profiles of the *P. aeruginosa* PBP3 and PBP3x proteins were similar to that of *E. coli* PBP3 suggesting that there were only single transmembrane segments near their amino termini. This is consistent with the observation that the recombinant *P. aeruginosa pbpB* and *pbpC* gene products cofractionated with the membrane protein fractions of *E. coli* and *P. aeruginosa* (Figures 15, 17 and Figures 26, 28). Collectively, these data suggest that both the *P. aeruginosa* PBP3 and PBP3x proteins have similar tertiary structure and membrane topology as that of *E. coli* PBP3 protein.

The N-terminus of *E. coli* PBP3 has a sequence similar to typical *E. coli* lipoprotein signal sequences (Nakamura *et al.*, 1983). Modification and processing of lipoprotein signal sequences in *E. coli* occur at a conserved cysteine residue that is

modified by acetylation and becomes the new N-terminus. However, it is debatable whether the *E. coli* PBP3 is processed in this manner since only 15% of *E. coli* PBP3 was apparently lipid-modified when it was overexpressed (Hayashi *et al.*, 1988). *E. coli* PBP3 is processed *in vivo* by C-terminal proteolytic processing that results in the removal of the C-terminal 11 amino acids of the protein (Hara *et al.*, 1991). This C-terminal processing is mediated by a tail-specific periplasmic protease that cleaves the Val_{577}-Ile_{578} bond of *E. coli* PBP3.

The N-termini of *P. aeruginosa* PBP3 and PBP3x proteins had no sequence similarity to *E. coli* lipoprotein signal sequences or other known signal sequences. This observation makes it unlikely that *P. aeruginosa* PBP3 and PBP3x were processed by a similar mechanism to other lipoproteins. N-terminal amino acid sequencing of *P. aeruginosa* PBP3 and PBP3x that were expressed in *E. coli* showed that neither of these proteins were post-translationally processed at their N-terminus (Chapter Two; Chapter Three). It is possible that the amino termini of the *P. aeruginosa* PBP3 and PBP3x, similar to *E. coli* PBP3, function as noncleaved signal-like sequences to mediate translocation of the bulk of the protein into the periplasm and anchor the protein in the cytoplasmic membrane. However, there is a discrepancy between the apparent molecular weights of *P. aeruginosa* PBP3 and PBP3x proteins produced in *E. coli* (as determined by SDS-PAGE and calculated molecular mass). In addition, when both the *P. aeruginosa and E. coli pbpB* genes were expressed in *P. aeruginosa*, a similar discrepancy in observed and calculated molecular masses was

observed (Chapter Two, section 3). The same was observed when the *P. aeruginosa pbpC* gene was expressed in *P. aeruginosa* (Chapter Three, section 3). It is possible that these discrepancies resulted from C-terminal processing of the *P. aeruginosa* PBP3 and PBP3x. However, the substrate for the *E. coli* tail-specific protease (Val-Ile pair) that is responsible for the processing of *E. coli* PBP3 is not present within the C-termini of *P. aeruginosa* PBP3 or PBP3x. Studies on the substrate specificity of the C-terminal protease showed that it processes peptides with P1 residues that are small and uncharged, *e.g.,* alanine, serine or valine (Keiler *et al.*, 1995). Examination of the sequences of PBP3 and PBP3x of *P. aeruginosa* indicated that there would be several potential cleavage sites in each of the proteins. Whether these two proteins indeed undergo post-translational C-terminal processing has yet to be determined.

4. PBP3 maps to a cluster of conserved and essential cell division genes

PBP3 of *E. coli* functions in the formation of the septum during cell growth (Spratt, 1975, 1977a). The similarity of *P. aeruginosa* and *E. coli* PBP3 proteins strongly suggests that PBP3 of *P. aeruginosa* serves a comparable role in *P. aeruginosa* cell division. Supporting evidence for this hypothesis comes from the discovery that the *P. aeruginosa pbpB* gene is located upstream of an *E. coli murE* homologue (UDP-MurNAc-tripeptide synthetase) and that these two genes mapped to the same region of the chromosome as did other cell division genes including the *ftsA*, *ftsZ* and *envA* genes (Chapter One). Many genes involved in cell shape maintenance

and cell division are clustered in *E. coli* (van Heijenoort, 1994). The largest cluster at 2 min is referred to as the dcw (division and cell wall) cluster. The dcw cluster contains genes encoding proteins responsible for the cytoplasmic and periplasmic stages of peptidoglycan biosynthesis including *pbpB*, *murE*, *murF*, *mraY*, *murD*, *ftsW*, *murG*, *murC* and *ddl* (van Heijenoort, 1994; Vincente & Errington, 1996). Downstream of the *ddl* gene, are four additional cell division genes, *ftsQ*, *ftsA*, *ftsZ* and *envA*, followed by the *secA* gene which is involved in protein export. A similar cluster of cell division genes is found in *Bacillus subtilis* (Buchanan *et al.*, 1994). The fact that the *pbpB*, *murE* and *ftsA*, *ftsZ*, *envA* genes of *P. aeruginosa* all mapped to the same region is consistent with the possibility that *P. aeruginosa*, like *E. coli* and *B. subtilis*, may also have a conserved major cell division and cell wall synthesis gene cluster.

DNA sequence analysis of the *P. aeruginosa pbpB* and *murE* genes suggests that they are cotranscribed from a promoter upstream of *pbpB* which is similar to the situation found in *E. coli* (Ayala *et al.*, 1994); *pbpB* and *murE* are adjacent to each other in both *E. coli* and *P. aeruginosa*. Their spacing is only two nucleotides apart in *P. aeruginosa*, whereas in *E. coli* the coding region for *murE* overlaps the end of the *pbpB* coding region by 11 base pairs (Michaud *et al.*, 1990); there were no obvious signals for transcriptional termination downstream of the *P. aeruginosa pbpB* gene. This finding may explain the failure to obtain cointegrates during gene replacement mutagenesis, since a polar mutation in the *pbpB* gene that effected the expression of

essential downstream cell division genes (*i.e. murE*) would be lethal. It should be noted that one cannot rule out the possibility that *pbpB* is itself an essential gene. Isolation of *pbpB* conditional lethal or nonsense mutants would answer this question.

5. PBP3x: a second *E. coli* PBP3-like gene product

In this study two *E. coli* PBP3-like *P. aeruginosa* genes were identified among the products from PCR studies using degenerate primers, suggesting that *P. aeruginosa* may contain a second copy of an *E. coli* PBP3-like gene. The first product *pbpB* was described in the preceding section. To determine whether the *P. aeruginosa pbpC* gene product was functionally equivalent to *E. coli* PBP3, the gene was cloned and characterized. Analyses of the translated sequence of the *P. aeruginosa pbpC* gene revealed that, like the *pbpB* gene product, it was very similar to *E. coli* PBP3 in its amino acid sequence and the spacing between the nine conserved motifs, the signature of class B high-molecular-weight PBPs (Piras *et al.*, 1993; Ghuysen, 1994). This suggested that the *pbpC* might have been a duplicated *pbpB* gene. Interestingly, *B. subtilis* also has a second copy of its *pbpB* gene, known as *spoVD*. *SpoVD* is only expressed during sporulation in *B. subtilis*, whereas *pbpB* is expressed during both vegetative growth and sporulation (Yanouri *et al.*, 1993; Daniel *et al.*, 1994). Thus, the two PBP3-like genes in *B. subtilis* have unique functions in cell growth and their expression is differentially regulated. There are other examples of gene duplications for a variety of other genes including other PBPs. For example, *E. coli* contains two

copies of the gene encoding PBP1. *E. coli* PBP1a and PBP1b appear to be redundant and capable of fulfilling compensatory roles in cell elongation (Tamake *et al.*, 1977; Suzuki *et al.*, 1978). Whether the *P. aeruginosa pbpC* gene encodes a differentially expressed and functional equivalent of the *P. aeruginosa pbpB* homologue is difficult to determine without further studies. However, there is a potential σ^S recognition sequence upstream of the *P. aeruginosa pbpC* gene and in addition, mutation of the *P. aeruginosa pbpC* had no discernible effect on the growth rate or cell morphology of the bacterium under the laboratory conditions tested. The finding of a potential σ^S recognition site, upstream of the *pbpC* gene suggests the possibility that the expression of *pbpC* may be growth rate or stress regulated (see below).

Gene duplication has previously been demonstrated in *P. aeruginosa.* For example *OprO* and *OprP*, encoding outer membrane proteins which are adjacent to one another (Siehnel *et al.*, 1988; Siehnel *et al.*, 1992). OprO and OprP have 74% amino acids identity. These two proteins have certain functional similarities in anion specific binding, and yet also have distinct functions since OprO has a distinctly higher affinity for pyrophosphate than orthophosphate whereas for OprP the affinities are reversed (Hancock *et al.*, 1992).

P. aeruginosa PBP3 and PBP3x are similar to one another with 48.3% amino acid identity. Their C-terminal regions, or the penicillin-binding domains appear to be more conserved with 51.8% amino acids identity. However, this does not prove that these two proteins have closely related transpeptidase and yet distinct transglycosylase

functions, especially in the absence of direct enzymatic assays for these activities. Indeed, such assays would not even be definitive since all the high-molecular-weight PBPs have similar enzymatic functions. A more useful approach involves mutational studies, which proved to be of limited value in these studies. The results presented in this study suggest that *P. aeruginosa* PBP3 and PBP3x may have different penicillin-binding domain structures (thus resulting in distinct transpeptidase activities) since binding studies indicated that they had different affinities for various β-lactam antibiotics. These two proteins may have similar biochemical functions (transpeptidase and transglycosylase), but play different physiological roles during different stages of cell growth (see below).

Based on analyses of the nucleotide sequences flanking the *pbpC* gene, it appears to be located at the end of an operon or exists as a single gene. Its upstream sequence, about 370 bp in length, did not resemble any sequences in the GenBank database. The sequence downstream of the *pbpC* gene encoded convergently transcribed homologues of the *E. coli soxR* and *M. bovis adh* gene products. Thus, the second copy of the PBP3 gene in *P. aeruginosa* was apparently not associated with genes involved in cell division.

In an attempt to determine the function of the *pbpC* gene product, a PBP3x-defective mutant was constructed by the insertion of a Km^r cartridge. In this mutant strain, no phenotypic difference from the wild type strain could be detected. Failure to detect the loss of the *pbpC* gene product in the insertion mutant led to the tentative

conclusion that the *pbpC* gene product was not expressed under the conditions tested. The inability to prove the disappearance of the *pbpC* gene product in the insertion mutant by ^{3}H-penicillin binding studies could have been the result of a number of factors. For example it may be that this protein was not expressed under the physiological conditions tested and thus did not play any significant role in cell growth under the conditions tested. Alternatively, it may be that this protein comigrated with another PBP during SDS-PAGE, thus masking the disappearance of PBP3x in the insertion mutant, especially if the cell compensates for the loss of one PBP by elevating the production of another. It may also be that the *pbpC* gene product was labeled poorly by ^{3}H-penicillin, given the observation that it had a lower affinity for β-lactam antibiotics compared to PBP3 (Chapter Three, section 4) and that the overproduced PBP3x in *P. aeruginosa* PAO4089 (labeled by ^{3}H-penicillin) appeared to be only at the same level as native PBP3 (Chapter Three, section 3). It could also be that PBP3x was present in extremely low amounts because of low expression level or instability, especially when one considers that *E. coli* has been shown to have only 50 molecules / cell of PBP3 and 20 molecules / cell of PBP2 (Spratt, 1977a).

It has been reported that σ^{s} downregulates the expression of *E. coli* PBP3 when cells enter stationary phase and cell division ceases (Dougherty & Pucci, 1994). It may thus be significant that the upstream sequence of the *pbpC* gene contains a

consensus sequence recognized by σ^s (Chapter One, section 4.2). Therefore it is possible that the expression of the *pbpC* gene is regulated by growth rate or nutrient conditions. When cells reach stationary phase, σ^s might downregulate the production of the conventional PBP3 and produce the alternative PBP3, *i.e.*, PBP3x. Future studies on the expression of the *P. aeruginosa pbpC* gene under a variety of growth conditions and at various growth stages should provide insight into the possible functions of this gene.

6. Production of recombinant PBP3 and PBP3x

In preliminary studies, pXLBI3 and pXLHd2 were used for the expression of *pbpB* and *pbpC* respectively but the corresponding proteins could not be detected by ^{3}H-penicillin binding assay. pXLBI3 contained the *pbpB* gene and its putative upstream RBS cloned in the same orientation as the *lac* promoter on the high copy number vector pTZ18U. Failure to detect the *pbpB* gene product could possibly be explained by the low efficacy of the putative RBS of the *pbpB* gene. This is strongly similar to the RBS of the weakly expressed *E. coli pbpB* gene. A *lacZ* transcriptional fusion study has confirmed that the efficacy of the RBS of *E. coli pbpB* gene is very low (Ayala *et al.*, 1994). This could be one of the mechanisms by which *E. coli* and *P. aeruginosa* control PBP3 production at low levels. pXLHd2 had the *pbpC* gene, the upstream putative RBS, and the promoter sequence cloned in the opposite orientation to the *lac* promoter in the vector pTZ18U. Lack of expression in this

construction could be due to poor functioning of the RBS and promoter sequences in *E. coli*; the putative promoter sequence of the *pbpC* gene might be recognized by an alternative sigma factor σ^S (see above) and /or the RBS and promoter sequences not functioning under normal laboratory conditions.

In this study, recombinant *P. aeruginosa* PBP3 and PBP3x were efficiently produced using a T7 RNA polymerase system involving the promoter and RBS recognized by the T7 RNA polymerase. The optimization of translation was also attributed to the cloning of the *pbpB* or *pbpC* gene behind the RBS at the *Nde*I site on the vector pT7-7 such that the translational start signal for the T7 gene 10 was replaced by that for the *pbpB* or *pbpC*. Subsequently, the DNA fragment containing the T7 RBS and the *pbpB* or *pbpC* gene was cloned into a broad-host range vector pBBR1MCS which contains the T7 promoter. This cloning strategy led to the productions of the *pbpB* and *pbpC* gene products.

PBP3 produced in *P. aeruginosa* PAO4089(pXL506), as detected by ^{3}H-penicillin binding, was estimated to be at a 7-fold higher level than the native PBP3, whereas production of PBP3x in PAO4089(pXL519) was estimated to be nearly the same level as native PBP3. This may suggest that expression of the *pbpC* gene was under tight control. However, low production of the recombinant PBP3x as detected by ^{3}H-penicillin could also have been due to limitations of the detection method (given that PBP3x has a low binding affinity to β-lactam antibiotics) rather than that truly reflecting its low expression level.

7. Correlation of β-lactam antibiotic susceptibility and the overproduction of PBP3 and PBP3x

The MIC of an antibiotic for a given bacterium is the lowest concentration of the antibiotic present in the growth medium that results in the inhibition of bacterial growth. In the case of the β-lactam antibiotics and Gram-negative bacteria, the susceptibility to β-lactams, as measured by the MIC, is dependent upon at least three parameters, outer membrane permeability, hydrolysis rate by β-lactamase, and interaction with the target proteins (*i.e.*, PBPs). The alteration of either of the first two parameters can result in resistance to β-lactam antibiotics (Hancock *et al.*, 1988; Spratt, 1989; Livermore, 1993), which is experimentally observed by an increase in the MIC of the tested antibiotic against that bacterial strain. However, the interaction with the target PBPs has been expressed as a factor S_i (the periplasmic concentration of β-lactam antibiotics at the MIC) (Nikaido & Normark, 1987; Bellido *et al.*, 1991a). The relationship between this factor and the kinetic constants of β-lactam and PBP interactions, as well as their influence on MIC is unknown (see below).

The major impediments to β-lactam therapy have been the high level of resistance of some organisms, including *P. aeruginosa,* due to the intrinsic barrier properties of their outer membranes and the acquisition of high levels of β-lactamase due to either the presence of a plasmid or the derepression of a formerly inducible,

chromosomally encoded β-lactamase (Hancock *et al.*, 1988; Philippon *et al.*, 1989). The simple and convenient assay methods for β-lactamase activity have resulted in the generation of a substantial body of literature on β-lactamase-mediated resistance (Livermore, 1993; Moellering, 1993; Bush *et al.*, 1995).

Mutational alternations in the β-lactam targets, PBPs, have also been observed. Bacterial strains with altered PBPs have been generated in the laboratory and also found in clinical isolates (Malouin & Bryan, 1986; Georgopapadakou, 1993). It has been demonstrated that alteration of PBPs can result from either reduced affinities of PBPs for β-lactam antibiotics (Godfrey *et al.*, 1981; Hedge & Spratt, 1985; Gotoh *et al.*, 1990) or from acquisition of a resistant PBP by lateral gene transfer and homologous recombination (Spratt *et al.*, 1989; Spratt, 1994). However, reports on bacterial resistance mediated by PBP alteration seem to occur with somewhat less frequency as compared with ones on the resistance mediated by β-lactamase production. The rarity of finding this type of resistance mechanism in clinical isolates could also be due to the lack of sufficient biochemical investigation. In addition, the experimental demonstration of PBP binding affinity for most bacteria is difficult due to a lack of biochemical and genetic information about the PBPs of the bacterium in question and the lack of a convenient and simple assay method. Currently, radiolabeled penicillin G is the only commercially available probe for assaying alterations in the properties of PBPs.

As mentioned above, the efficacy of β-lactam antibiotics against Gram-negative bacteria, as measured by their MICs, depends on their rate of penetration across the outer membrane, their degree of resistance to β-lactamase inactivation, and their ability to inhibit the target proteins, the PBPs. Zimmermann and Rosselet (1977) first described outer membrane permeability to β-lactams in terms of Fick's first law of diffusion, *i.e.*, $V = P\ A\ (S_o - S_p)$, where V is the rate of diffusion across the outer membrane, S_o is the external and S_p the periplasmic concentration of the β-lactam, A is the area of the membrane per unit weight of cells (calculated by electron microscopy) and P the permeability coefficient. As noted by Zimmermann & Rosselet (1977), the outer membrane permeability is rate-limiting for hydrolysis of β-lactams by periplasmic β-lactamase, which follows the Michaelis-Menten equation $V' = V_{max}\ S_p / (S_p + K_m)$. At the steady state, the rate of hydrolysis of β-lactam is limited and thus balanced by the rate of permeation, *i.e.*, $V = V'$. This gives $V = P\ A\ (S_o - S_p) = V_{max}\ S_p / (S_p + K_m)$. The equation was modified by Nikaido and Normark (1987) to permit the prediction of the factors involved in β-lactam efficacy, *i.e.*, $S_o = S_p\ [1 + V_{max} / (K_m + S_p)\ P\ A]$. When S_o is equivalent to MIC, S_p corresponds to S_i which is the actual concentration of β-lactams in the periplasm at an external concentration equal to the MIC, assumed to be the target PBP inhibitory concentration. To predict the S_i, Nikaido and Normark (1987) used literature estimates of the I_{50} to one of the essential PBPs, *i.e.*, PBP1b, PBP2 or PBP3 to obtain a global estimate. However, this

might not be accurate. According to the rearrangement of the equation, S_p is related to the kinetic constants of the β-lactamase (V_{max}, K_m), the surface area of the bacterial cell (A) and the permeability coefficient of the outer membrane (P). The determination of the permeability coefficient of the outer membrane (P) can be influenced by the experimental approaches and has been a controversial issue. Bellido *et al.* (1991a) presented a direct method for assessing outer membrane permeability by using intact bacterial cells and high-performance-liquid-chromatography (HPLC), which avoids certain possible artifacts in those methods utilizing extrapolations from model membrane systems (Zimmermann & Rosselet, 1977; Nikaido *et al.*, 1983). With the HPLC method, the rate (V) of antibiotic uptake by intact cells (*i.e.*, disappearance from the medium) can be measured directly, and since $S_p = VK_m/(V_{max} - V)$, the coefficient P can then be assessed accurately according to $V = P A (S_o - S_p)$, where S_o is known as the β-lactam concentration initially used for measuring V. Using *Enterobacter cloacae* strain R1, Bellido *et al* (1991b) tested the use of the formula for predicting MICs, *i.e.* $MIC = S_i [1 + V_{max} / (K_m + S_p) P A]$, after calculation of P, V_{max} and K_m and estimation of S_i. Using variants of strain R1 with different levels of β-lactamase or different outer membrane permeability, the general reliability of this predictive equation was tested. In all cases calculated MICs were the same as measured MICs. However the influence of changes in the target PBPs on MIC was not tested.

In this study, the influence of overproduction of *P. aeruginosa* PBP3 on the susceptibility of *P. aeruginosa* to β-lactam antibiotics was investigated. It was found that increased levels of the *P. aeruginosa pbpB* gene product in *P. aeruginosa* resulted in increased levels of resistance to certain PBP3-targeted antibiotics. It was observed that a 7-fold increase in the amount of PBP3 in *P. aeruginosa* resulted in a 2-fold increase in the level of resistance to aztreonam, a 4-fold increase in resistance to cefepime, and 8-fold increases in resistance to ceftazidime and cefsulodin. The increased MICs in the strains that overproduced PBP3 suggests that the PBP is biologically functional. *P. aeruginosa* PAO4089, used in this study, is a β-lactamase non-inducible mutant; use of this strain eliminated complications due to the induction of chromosomal β-lactamase expression. The reaction of a β-lactam antibiotic and a PBP involves the initial formation of a reversibly bound enzyme-inhibitor complex followed by covalent modification (acylation) and hence irreversible inhibition. The reaction scheme is analogous to that of the Michaelis-Menten mechanism, $E + I = E{\cdot}I \rightarrow E\text{-}I^*$ and should show saturation kinetics with increasing inhibitor concentrations. Increased expression of the PBPs in the PAO4089 cells would therefore require a corresponding increase in the amount of periplasmic β-lactam antibiotic to effect the inhibition of peptidoglycan biosynthesis to a level sufficient to result in growth inhibition. Thus, the overexpression of PBP3 should result in increased S_i to PBP3 targeted antibiotic, which consequently resulted in increased concentration of the

antibiotic in the medium that is required to inhibit cell growth. This corresponded to the observed increase in the MIC. The differential increases in MIC among the four different PBP3-targeted antibiotics may have been due to their different binding affinities for PBP3, as indicated by their different I_{50} values (Table 7). Ceftazidime and cefsulodin had relatively lower affinities (*i.e.*, higher I_{50}) for PBP3 as compared to cefepime and aztreonam, and higher concentrations of these two antibiotics were thus required to inhibit the overproduced PBP3 (*i.e.*, higher MICs). Alternatively, it must be noted that MIC is not a simple function of S_i (see above) and that other parameters in the periplasm would influence MIC. Furthermore, binding to PBP1a, 1b or PBP2 might have played some role. In any event, the results presented in this study demonstrate that the interaction of β-lactam antibiotics and their targeted PBPs are influenced by the level of PBP production and support the notion that regulation of PBP expression is a possible mechanism for resistance to β-lactam antibiotics in *P. aeruginosa*. To my knowledge this is the first demonstration of PBP-overexpression-mediated resistance to β-lactam antibiotics.

In addition to the overproduction of *P. aeruginosa* PBP3, it was found that overproduction of the *E. coli pbpB* gene product in *P. aeruginosa* PAO4089 resulted in 2-fold increase in the level of resistance to aztreonam and 4-fold increases in resistance to ceftazidime and cefepime, which suggests that the *E. coli* PBP3 retained its biological function in *P. aeruginosa*. In addition, these results support the

assertion discussed in the preceding section that the *P. aeruginosa* PBP3 was a functional homologue of the *E. coli* PBP3.

Increased resistance to β-lactam antibiotics due to elevated expression of penicillin-sensitive PBPs has not been found in clinical isolates of Gram-negative bacteria and to my knowledge very little, if any, effort has been put forth to search for such mutants. The failure to identify this type of resistance mechanism in clinical isolates could have been due to the lack of an appropriate assay. Increased expression of PBPs alone probably would not contribute much to the overall resistance of a bacterium to β-lactam antibiotics. However, increased PBP expression coupled with reduced outer membrane permeability is potentially an effective mechanism for producing resistance.

This work also revealed that overproduction of the *P. aeruginosa pbpC* gene product did not alter the susceptibility of the overexpressing strain to the β-lactam antibiotics tested. This probably resulted from the lower affinity of PBP3x for the PBP3-targeted antibiotics as compared to PBP3. This could result from the nature of the various amino acid residues making up and surrounding the active binding-site serine, which are known to play roles in facilitating the binding to the substrates (Ghuysen, 1991; Malhotra *et al.*, 1992; Petrosino *et al.*, 1996). 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 (Hedge & Spratt, 1985). 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. The different affinities of *P. aeruginosa* PBP3 and PBP3x for various substrates might therefore have been due to differences in amino acids within the functional SXXK motif. Whether this is actually the case will require further study.

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