Comparative analysis of *Pseudomonas aeruginosa* penicillin-binding protein 7 in the context of its membership in the family of low-molecular-mass PBPs

Jian Song,† Gary Xie, Pamela K. Elf, Kevin D. Young and Roy A. Jensen

Author for correspondence: Roy A. Jensen Tel: +1 352 392 9677. Fax: +1 352 392 5922. e-mail: rjensen@micro.ifas.ufl.edu

The *Pseudomonas aeruginosa* *pbpG* gene encoding penicillin-binding protein 7, a homologue of the *Escherichia coli* gene encoding a DD-endopeptidase, was cloned and sequenced. *pbpG* was located immediately downstream of the phenylalanine hydroxylase (*phh*) operon. DNA sequencing revealed an open reading frame of 936 bp (starting with a GTG codon) which encodes a protein of 34115 Da. N-terminal amino acid sequencing confirmed the presence of a cleavable N-terminal signal peptide of 23 amino acids. Verification that the protein is a penicillin-binding protein was directly demonstrated by labelling with 125I-labelled penicillin X. Inactivation of *P. aeruginosa* *pbpG* by interposon mutagenesis resulted in no obvious phenotypic changes, but when *P. aeruginosa* PbpG was overexpressed in *E. coli* using a T7 expression system, cell lysis resulted. *P. aeruginosa* PbpG resembled *E. coli* PbpG in being associated with the membrane fraction. Two additional members of the PbpG subfamily were identified in the database. *P. aeruginosa* PbpG shows 63% identity with *E. coli* penicillin-binding protein 7 (PbpG) and 60% identity with *Vibrio cholerae* PbpG, but only 23% identity with *Haemophilus influenzae* PbpG. The PbpG subfamily and three other subfamilies constituting the low-molecular-mass PBP protein family were analysed by multiple alignment of 26 sequences. PbpG exhibited the consensus motifs of other penicillin-binding proteins. Ten anchor residues were identified that are conserved at the family level within the superfAMILY of serine-active-site penicillin-interacting proteins.

**Keywords:** penicillin-binding proteins, PBP protein family, *Pseudomonas aeruginosa*

**INTRODUCTION**

The murein (peptidoglycan) sacculus is a shape-determining structure in bacterial cell walls that consists of a complex polymer made up of polysaccharides and peptides (Van Heijenoort, 1996). The murein sacculus is located in the periplasmic space and is essential for maintenance of the integrity of the cytoplasmic membrane. It is intimately related to cell growth and division (Waxman & Strominger, 1983). In endospore-forming bacilli the sacculus also plays a crucial role in the morphogenetic processes of endospore formation and germination (e.g. Murray et al., 1997 and references therein). Elongation of the sacculus during growth is a dynamic process requiring an appropriate balance of formation and hydrolysis of cross-links. High-molecular-mass penicillin-binding proteins (PBPs) possess a transglycosylase domain, which participates in new cross-linking, and a transpeptidase domain. Low-molecular-mass PBPs are DD-peptidases of two types. DD-Carboxypeptidases cleave between two d-alanines, thus facilitating the interpeptide cross-linking between an activated terminal d-alanyl moiety of one subunit and meso-diaminopimelate of another subunit. DD-
Endopeptidases hydrolyse the latter interpeptide cross-links.

The transpeptidase domain of high-molecular-mass PBPs and the dipeptidase enzymes (low-molecular-mass PBPs) possess a common active-site serine residue associated with a configuration of conserved motifs found in \( \beta \)-lactamase (Ghuysen, 1991). PBPs covalently bind penicillin or penicillin derivatives, a property which has facilitated their detection with labelled penicillin following SDS-PAGE. Escherichia coli produces at least 11 (1a, 1b, 1c, 2, 3, 4, 5, 6, 6b, 7 and 8) PBPs (Baquero et al., 1996). Among the low-molecular-mass PBPs, three (PBP5, PBP6, and PBP6b) are DD-carboxypeptidases, whereas PBP4 and PBP7 are DD-endopeptidases.

Although PBP8 of E. coli is also a DD-endopeptidase it has recently been shown to result from processing of PBP7 by the outer-membrane protease OnmpT (Henderson et al., 1994). Evidence was obtained suggesting the processing to be an in vitro artifact caused by abnormal contact of these proteins following cell disruption. The gene encoding PBP7 in E. coli, \( \text{pbpG} \), was recently cloned (Henderson et al., 1995). PBP7 has been reported to be a target for \( \beta \)-lactam antibiotics that have the unusual property of lysing nongrowing E. coli cells (Tuomanen & Schwartz, 1987). An increased resistance of E. coli to cephaloridine and cefadiazime has been correlated with increased expression of PBP8 (Maloun et al., 1991).

PBPs of Pseudomonas aeruginosa have received relatively little attention, even though \( \beta \)-lactam antibiotics have been among the few that have proven effective in treating infections caused by this notorious opportunistic pathogen. At least six PBPs in \( P. \text{aeruginosa} \) have been described in an early study in which the electrophoretic pattern of PBPs roughly resembled that of E. coli (Noguchi et al., 1979). However, the low-molecular-mass counterparts of E. coli PBP6 and PBP7 were not definitively detected. The most comprehensive recent work in \( P. \text{aeruginosa} \) was with the high-molecular-mass PBP species, PBP3 (Liao & Hancock, 1995) and PBP3x (Liao & Hancock, 1997). These are both homologues of E. coli PBP3. Although they may be catalytically redundant, \( P. \text{aeruginosa} \) PBP3 and PBP3x probably have specialized roles, since they are differentially regulated (Liao & Hancock, 1997).

In this paper we present molecular-genetic data supporting the existence of \( \text{pbpG} \) in the \( P. \text{aeruginosa} \) genome, encoding a homologue of E. coli PBP7. A comparative analysis of four known PBP7 proteins in the context of their membership in the family of low-molecular-mass PBPs is presented.

**METHODS**

**Materials.** Bacterial strains and plasmids used in this study are described in Table 1. The LB formulation (Sambrook et al., 1989) was used as growth medium for both E. coli and \( P. \text{aeruginosa} \). Pseudomonas isolation agar (Difco) was used for isolating \( P. \text{aeruginosa} \) \( \text{pbpG} \) knockout mutants. Additions of ampicillin (100 \( \mu \)g ml\(^{-1} \)), chloramphenicol (40 \( \mu \)g ml\(^{-1} \)), tetra-cycline (25 \( \mu \)g ml\(^{-1} \)) and mercuric chloride (15 \( \mu \)g ml\(^{-1} \)) were made when required. Ampicillin was never used in growth media when penicillin-binding proteins were to be assayed. Agar was added at 20 g l\(^{-1} \) for preparation of solid medium.

Restriction enzymes, T4 DNA ligase, DNA modifying enzymes (New England Biolabs), and Taq DNA polymerase (Perkin-Elmer) were used as recommended by the suppliers. Other biochemicals were purchased from Sigma. Inorganic chemicals (analytical grade) were from Fisher Scientific.

**DNA manipulations and protein assays.** Procedures for general DNA manipulation, including plasmid purification, subcloning and restriction analysis were conducted by standard methods (Sambrook et al., 1989). DNA fragments were purified from agarose gels with a Geneclean kit (Biolol). Protein concentrations were estimated by the use of Bradford reagent (Bradford, 1976).

**Expression and labelling of PBPs.** For overexpression of PbpG in E. coli, we used the T7 expression system (Novagen). The coding region of \( \text{pbpG} \) was placed into a translational fusion vector (pET11a). The coding region was amplified using PCR with the upper 30mer primer 5'-TTCCATATGAAAGCGTCTCTCTGCTGACTG-3' (a built-in NdeI site is underlined) and the translational start codon is in bold) and the lower 18mer primer (RJ 105) 5'-CTTCGCGGACGATCGCAGG-3' (complementary to nucleotides 1504–1521). The PCR fragment was first cloned into the Smal site of pUC18 and was subsequently excised with Ndel and BamHI. The latter fragment carrying the \( \text{pbpG} \) gene was then ligated with Ndel and BamHI to create the PbpG overexpression plasmid, pJS87. To avoid the toxicity resulting from PbpG overexpression, the BglII–BamHI fragment from pJS87 carrying the \( \text{pbpG} \) gene was cloned into the low-copy-number plasmid pACYC184, to create pJS895.

**PBp labelling of whole-cell extracts and visualization by SDS-PAGE.** was performed as described previously (Henderson et al., 1994).

**Preparation of osmotic shock.** Osmotic shock was performed by a procedure similar to that described by Neu & Chou (1967). E. coli BL21(DE3)/pJS87 was grown in LB broth supplemented with 100 \( \mu \)g ampicillin ml\(^{-1} \) to an OD\(_{600}\) of about 1. Expression of PbpG was then induced by adding 0.4 mM IPTG. Cells were harvested after 2 h induction by centrifugation at 12000 \( g \) for 15 min at 4°C. The cell pellets were washed with 0.85% NaCl and resuspended in 20% sucrose:30 mM Tris/HCl (pH 7.3) at room temperature at a ratio of 80 ml sucrose:Tris to 1 g cells (weight). EDTA was added to a concentration of 1 mM. After 10 min of mixing at room temperature, the cells were pelleted by centrifugation at 4°C. The cell pellets were resuspended in 0.5 mM MgCl\(_2\) at 0°C and mixed on ice for 10 min. The cells were removed by centrifugation, and the supernatant was collected as the osmotic fraction. The shocked cells were sonicated and the cytoplasmic and membrane fractions were prepared as described below.

**Preparation of cytoplasmic and membrane fractions.** Cells grown in LB broth until the late-exponential stage were harvested by centrifugation at 12000 \( g \) for 15 min at 4°C. The cell pellets were resuspended in 30 mM Tris/HCl (pH 7.3), and sonicated for 2 x 30 s with a Labline sonicator. Cell debris and unbroken cells were removed by centrifugation at 3000 \( g \) for 10 min at 4°C. The supernatant was centrifuged at 150000 \( g \) for 65 min at 4°C. The pellets membranes were washed and resuspended in the same buffer. The supernatant is referred to as the cytoplasmic fraction.
**Table 1.** Strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant genotype or description</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BL21(DE3)</td>
<td>F^- ompT hsdS6 (rK^- mK^-) gal dcm; with DE3, a λ prophage carrying the T7 RNA polymerase gene</td>
<td>Novagen</td>
</tr>
<tr>
<td>CS109</td>
<td>W1485, l^- F^- thi glnV(supE) rph tpoS pbpG::res in CS109 background</td>
<td>Henderson et al. (1997)</td>
</tr>
<tr>
<td>CS9-19</td>
<td></td>
<td>S. A. Denome and others, unpublished</td>
</tr>
<tr>
<td>DHSa</td>
<td>F^- e14^- (mcrA) hsdRS14 (rK^- mK^-) recA1 endA1 gyrA96 thi-1 relA1 supE44</td>
<td>Gibco-BRL</td>
</tr>
<tr>
<td>S17-1</td>
<td>[RP4-2(Tc::Mu)] (Km::Tn7) Tra (IncP) pro hsdR recA Tp' Sm'</td>
<td>Simon et al. (1983)</td>
</tr>
<tr>
<td><strong>P. aeruginosa</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAO-1</td>
<td>Prototroph</td>
<td>D. H. Calhoun, City College, New York</td>
</tr>
<tr>
<td>JS105</td>
<td>PAO-1, ΔpbpG, Hg'</td>
<td>This study</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pACYC184</td>
<td>Cm' Tc'; low-copy-number cloning vector</td>
<td>Chang &amp; Cohen (1978)</td>
</tr>
<tr>
<td>pET11a</td>
<td>T7lac promoter, lacI' Amp'</td>
<td>This study</td>
</tr>
<tr>
<td>pJS6</td>
<td>Sphi-HindIII fragment carrying pbpG cloned into pUC18</td>
<td>This study</td>
</tr>
<tr>
<td>pJS87</td>
<td>PbpG overexpression plasmid, PbpG- coding region fused with T7 translational initiation signal at NdeI site</td>
<td>This study</td>
</tr>
<tr>
<td>pJS89</td>
<td>Xbal-BamHI fragment carrying pbpG from pJS87 cloned into pUC19</td>
<td>This study</td>
</tr>
<tr>
<td>pJS89S</td>
<td>BglII-BamHI fragment carrying pbpG from pJS87 cloned into pACYC184</td>
<td>This study</td>
</tr>
<tr>
<td>pJZ9</td>
<td>pbhRABC, Amp'</td>
<td>Zhao et al. (1994)</td>
</tr>
<tr>
<td>pUC18</td>
<td>Amp'; high-copy-number cloning vector</td>
<td>Yanisch-Perron et al. (1985)</td>
</tr>
<tr>
<td>pUC19</td>
<td>Amp', high-copy-number cloning vector</td>
<td>Yanisch-Perron et al. (1985)</td>
</tr>
<tr>
<td>pUFR004</td>
<td>ColE1, Cm' Mob' mobP lacZa'</td>
<td>Defeyter et al. (1990)</td>
</tr>
</tbody>
</table>

**Inactivation of pbpG in P. aeruginosa.** pbpG was inactivated by marker interruption (Kamoun et al., 1992) as described by Song & Jensen (1996). To generate the truncated ‘pbpG’ fragment (614 bp), the upper 26mer primer 5'-GGG-CTGCCGCTGCAACAGGAGCTGGC-3' (nucleotides 661-686), and the lower 26mer primer 5'-GTGAAGCCGGTCTTGGTCAACTGGAT-3' (complementary to nucleotides 1249-1274) were used. Interruption of the pbpG gene in a P. aeruginosa Hg' isolate was confirmed by Southern hybridization.

**Nucleotide sequencing and data analysis.** Plasmid pJS6 was sequenced by the DNA Sequencing Core Lab of the Interdisciplinary Center for Biotechnology Research (ICBR) of the University of Florida. Primers were made by the DNA Synthesis Core Lab of the ICBR. The nucleotide sequence and the deduced amino acid sequence were analysed by using the Genetics Computer Group (University of Wisconsin) software packages. Amino acid sequences were analysed for N-terminal signal sequences and transmembrane domains using PSORT (http://psort.nibb.ac.jp) (Nakai & Kanehisa, 1991).

**N-terminal amino acid sequencing.** Samples for N-terminal amino acid sequencing were processed with an Applied Biosystems model 407A protein sequencer with an on-line 120A phenylthiohydantoin analyser in the Protein Core Facility of the ICBR at the University of Florida.

**RESULTS**

**Cloning and sequencing of pbpG**

The original clone isolated by Zhao et al. (1994) is shown in Fig. 1. Both upstream and downstream flanking regions of the pbhABC operon were originally regarded as candidate locations for regulatory genes because presence or absence of these flanking regions in subclone derivatives influenced the expression levels of genes in the pbh operon. Indeed, the upstream region proved to house a positively acting regulatory gene, pbhR (Song & Jensen, 1996). Elevated expression of pbpG in the downstream region was found to be deleterious to growth, causing cells to be prone to lysis. Hence, the negative effect of pbpG on expression of pbh operon genes was indirect. Fig. 1 shows the subclone construct (pJS6) used for nucleotide sequencing.
pbpG utilizes a GTG start codon. The possible use of ATG start codons further upstream was discounted because of the presence of tandem stop codons (TAG TGA). An AGTGAG hexamer provides a putative ribosome-binding site with suitable spacing (8 nucleotides). The 134 nucleotide region between the terminator following the pbpG operon and the ribosome-binding site ahead of pbpG was examined in an attempt to identify a likely promoter, but possible promoters corresponding to consensus sequences for σA, σE or σK were all too marginal to merit speculation. The deduced mature PbpG protein is positively charged and exhibits a to consensus sequences for Pb pG was overexpressed in a likely promoter, but possible promoters corresponding following SDS-PAGE of samples from whole-cell lysates transcribed immediately ahead of Pb pG was located in the membrane fraction (lane 4), and nplE (cutF), both apparently belonging to a common operon (Gupta et al., 1995). Another homologue (68% identity) is a 15.2 kDa protein from Pseudomonas putida, which is transcribed convergently with pceA, and encodes a subunit of β-ketoadipate:succinyl-Coenzyme A transferase (Parales & Harwood, 1992). The truncated C-terminal portion of P. aeruginosa YaeJ has a high content of basic amino acids and only few acidic amino acids (8K, 8R, 2D, 0E). Of the 16 basic residues in P. aeruginosa YaeJ, 9 are identical conserved residues in all three YaeJ homologues. The extreme positive charge of these proteins is consistent with DNA- or RNA-binding properties and a regulatory function. Since yaeJ is transcribed convergently with pbpG in P. aeruginosa, the gene organization in this region differs from that of the closely related P. putida. Only 22 bp separate the stop codons of the convergently transcribed pbpG and yaeJ genes. The mechanism used for transcript termination in this region would be of interest. Several potential stem–loop structures span this intergenic region. YaeJ possesses two regions that have been reported to show significant homology to peptide chain release factors in prokaryotes (Gupta et al., 1995).

**Subcellular location of PbpG**

PbpG was overexpressed in E. coli BL21(DE3)/pJS87 using the T7 expression system (Novagen). PbpG was readily visible as a prominent band of the expected size following SDS-PAGE of samples from whole-cell lysates (Fig. 2, lane 2). Osmotic shock experiments were done using cyclohexadienyl dehydratase as a positive control for periplasm localization (Zhao et al., 1993). It was qualitatively apparent from the SDS-PAGE analysis that little or no PbpG was present in the periplasmic fraction.

An IPTG-induced culture strongly expressing PbpG in whole-cell lysates (Fig. 2, lane 2) was fractionated into a cytoplasmic fraction and a membrane fraction (as described in Methods). Fig. 2 shows that most or all PbpG was located in the membrane fraction (lane 4), and not in the cytoplasmic fraction (lane 3).

PbpG protein overproduced in E. coli BL21(DE3)/pJS87 was first isolated in the membrane fraction and then separated from other membrane proteins by SDS-PAGE. The gel was then blotted onto a PVDF membrane (Bio-Rad) and stained with Coomassie brilliant blue R-250 (Sigma). The band corresponding to PbpG was excised from the membrane and used for N-terminal amino acid sequencing. The N-terminal amino acid sequence obtained (SPPPKA) confirmed the existence of a signal peptide. The 23-residue signal peptide deduced from the nucleotide sequence (MRNRLSSLVTLFLSLSVVATVSA) fulfills the three standard criteria for cleavable signal peptides (Nakai & Kanehisa, 1991).
Terminal amino acids by the OmpT protease at the RR carries an interposon mutation within Pbp7/8 in a pbpG mutant. Overexpression were labelled with 35S-penicillin X as described in Methods. Fig. 3. Identification of PBP7 in E. coli and P. aeruginosa. PBP7 were labelled with 35S-penicillin X as described in Methods. Lane 1, wild-type E. coli CS109; lane 2, E. coli CS109/pJS895 expressing cloned P. aeruginosa Pbp7; lane 3, E. coli CS9-19 (mutant lacking Pbp7/8); lane 4, E. coli CS9-19/pJS895; lane 5, P. aeruginosa PAO-1 (wild-type); lane 6, P. aeruginosa JS105 (mutant lacking Pbp7/8). E. coli PBP7 are identified on the left margin. AmpH marks the position of a newly described β-lactamase (Henderson et al., 1997).

Direct confirmation that P. aeruginosa PbpG is a penicillin-binding protein on the classical criterion is shown in Fig. 3. Lane 3 shows the absence of E. coli Pbp7/8 in a pbpG mutant. Overexpression of P. aeruginosa pbpG in this mutant background is apparent from the appearance of high protein levels in the general electrophoretic positions of E. coli Ppb7/8. Bands corresponding to at least eight PBP species are evident in lysates prepared from wild-type P. aeruginosa PAO-1 (lane 5). The identity of the bottom band as Ppb7 was confirmed by results obtained with strain JS105, which carries an interposon mutation within pbpG (lane 6).

The P. aeruginosa PbpG band in lane 5 of Fig. 3 appears to have a lower molecular mass than the E. coli Pbp8 species. This would be consistent with cleavage of 37 C-terminal amino acids by the OmpT protease at the RR residues marked in Fig. 4. On the other hand, overexpression in the BL21(DE3) system (which lacks a functional OmpT) yielded a protein of the expected size (Fig. 2).

Physiological effects of perturbation of PbpG levels

As noted earlier, the original clone pJZ9 (Fig. 1) resulted in a tendency for the host cells to lyse. When PbpG was overproduced in E. coli BL21(DE3)/pJS87, obvious cell lysis began 2 h after IPTG induction, with progressively more marked lysis thereafter. On the other hand, when pbpG was inactivated in a P. aeruginosa knockout mutant, no phenotypic changes were observed in growth rate, survival or colony morphology on minimal salts-glucose medium or an enriched medium (LB).

DISCUSSION

The PbpG subfamily

P. aeruginosa PbpG appears to resemble E. coli PbpG, its nearest known homologue. We have located two other pbpG sequences in the database. Of these, Vibrio cholerae pbpG is quite similar to the foregoing two, whereas H. influenzae pbpG is the most divergent of the four. E. coli PbpG is membrane associated, but has been shown not to be an integral membrane protein (Romeis & Höltje, 1994). It can be dissociated from membranes in high salt and released by osmotic shock. P. aeruginosa PbpG is also membrane associated. Although psort analysis indicates a potential membrane-spanning region between residues 60 and 77, this is highly unlikely, since this region encompasses an established active-site motif (SxxK) for the PBP family. E. coli PbpG possesses a KK target for OmpT (residues 296–297) which generates Pbp8 as a processing artifact (Henderson et al., 1994). P. aeruginosa PbpG possesses an RR target for OmpT (residues 273–274) which also appears to generate a carboxy-cleaved gene product. As is the case in E. coli (Henderson et al., 1995) inactivation of pbpG produces no obvious phenotype under ordinary conditions of laboratory culture. However, overexpression of P. aeruginosa PbpG in E. coli was not tolerated, and dramatic cell lysis occurred.

As illustrated by the dendrogram in Fig. 4, the PbpG subfamily is closer to the DacA-F subfamily. However, the catalytic specificity of the PbpG subfamily is more similar to that of the DacB subfamily. Therefore, we compared the sequences of the latter two subfamilies for regions that might be conserved. Only two regions were found. One region provides the motif S/TGLS (corresponding to residues 60–67) of PbpG, its nearest known homologue. We have located two other pbpG sequences in the database. Of these, Vibrio cholerae pbpG is quite similar to the foregoing two, whereas H. influenzae pbpG is the most divergent of the four. E. coli PbpG is membrane associated, but has been shown not to be an integral membrane protein (Romeis & Höltje, 1994). It can be dissociated from membranes in high salt and released by osmotic shock. P. aeruginosa PbpG is also membrane associated. Although psort analysis indicates a potential membrane-spanning region between residues 60 and 77, this is highly unlikely, since this region encompasses an established active-site motif (SxxK) for the PBP family. E. coli PbpG possesses a KK target for OmpT (residues 296–297) which generates Pbp8 as a processing artifact (Henderson et al., 1994). P. aeruginosa PbpG possesses an RR target for OmpT (residues 273–274) which also appears to generate a carboxy-cleaved gene product. As is the case in E. coli (Henderson et al., 1995) inactivation of pbpG produces no obvious phenotype under ordinary conditions of laboratory culture. However, overexpression of P. aeruginosa PbpG in E. coli was not tolerated, and dramatic cell lysis occurred.

As illustrated by the dendrogram in Fig. 4, the PbpG subfamily is closer to the DacA-F subfamily. However, the catalytic specificity of the PbpG subfamily is more similar to that of the DacB subfamily. Therefore, we compared the sequences of the latter two subfamilies for regions that might be conserved. Only two regions were found. One region provides the motif S/TGLS (corresponding to residues 60–67) of PbpG, its nearest known homologue. We have located two other pbpG sequences in the database. Of these, Vibrio cholerae pbpG is quite similar to the foregoing two, whereas H. influenzae pbpG is the most divergent of the four. E. coli PbpG is membrane associated, but has been shown not to be an integral membrane protein (Romeis & Höltje, 1994). It can be dissociated from membranes in high salt and released by osmotic shock. P. aeruginosa PbpG is also membrane associated. Although psort analysis indicates a potential membrane-spanning region between residues 60 and 77, this is highly unlikely, since this region encompasses an established active-site motif (SxxK) for the PBP family. E. coli PbpG possesses a KK target for OmpT (residues 296–297) which generates Pbp8 as a processing artifact (Henderson et al., 1994). P. aeruginosa PbpG possesses an RR target for OmpT (residues 273–274) which also appears to generate a carboxy-cleaved gene product. As is the case in E. coli (Henderson et al., 1995) inactivation of pbpG produces no obvious phenotype under ordinary conditions of laboratory culture. However, overexpression of P. aeruginosa PbpG in E. coli was not tolerated, and dramatic cell lysis occurred.

The low-molecular-mass PBP family

Multiple homologues of the low-molecular-mass PBPs are often present in a single organism. Such protein families are called paralogues and presumably arose from a series of gene duplications. Sequencing of the entire genome of a growing number of prokaryotes is complete or nearly complete, thus allowing recognition of the total complement of PBPs in prokaryotic organisms. Escherichia coli and Bacillus subtilis represent widely divergent phylogenetic groupings in which PBPs have been intensively studied. Each exhibits three paralogues in what we will term the DacA-F cluster, which are often present in a single organism. Such protein families are called paralogues and presumably arose from a series of gene duplications. Sequencing of the entire genome of a growing number of prokaryotes is complete or nearly complete, thus allowing recognition of the total complement of PBPs in prokaryotic organisms. Escherichia coli and Bacillus subtilis represent widely divergent phylogenetic groupings in which PBPs have been intensively studied. Each exhibits three paralogues in what we will term the DacA-F cluster, although the B. subtilis paralogues are much more divergent. Each organism also exhibits a paralogue
Fig. 4. Multiple alignment of low-molecular-mass PBPs. The alignment was generated using the PILEUP program of the GCG package (Genetics Computer Group, 1995). The dendrogram generated is shown at the upper left. The five E. coli paralogues are shown in bold type. Immediately to the right of the dendrogram are our individual PBP designations, accession or contig numbers (with original designations in parentheses), last residue number of signal peptide prior to cleavage (I) and beginning residue number shown in the multiple alignment. At the far right lower right is given the number of the final residue present followed (slash) by the number of the final sequence residue. Residues that are highly conserved within any of the four clusters compared are printed in bold type. Solid bars join residues that are highly conserved between a given pair of clusters. Grey bars indicate less highly conserved residue groupings. Asterisks mark anchor residues conserved throughout the entire family of low-molecular-mass PBPs. (Lower-case letters within the SxN motif are functionally equivalent is indicated by the double-bent lines between subgroups. The vertically aligned as carried out by Mottl et al. [1991]. The probable target sites for artifactual cleavage of \( PBP_{7} \) from \( P. aeruginosa \), QNN, and the DD-endopeptidase activity appears to be due to redundant catalytic activities of DacB and PbpG. Perhaps the DacX group constitutes up to 30% of the total penicillin-binding proteins. In E. coli, B. subtilis, and \( S. aureus \), the DacX paralogue is more related to DacB than to PbpG. The DacX paralogue appears to be restricted to a relatively closely related group of organisms: E. coli, Vibrio cholerae, P. aeruginosa and H. influenzae. We could not identify PbpG homologues in the nearly completed sequences of Neisseria gonorrhoeae, Staphylococcus aureus or Streptococcus pneumoniae; Eco, Escherichia coli; Hin, Haemophilus influenzae; Mtb, Mycobacterium tuberculosis; Pae, Pseudomonas aeruginosa; Vch, Vibrio cholerae; Act, Actinomadura sp.; Ssp, Synechocystis sp.; Ngo, Neisseria gonorrhoeae.

member of the DacB cluster. Unlike \( E. coli \), \( B. subtilis \) apparently lacks a PbpG homologue. The completely sequenced genome of \( H. influenzae \) reveals a similar partitioning of paralogues into the three groups as found in \( E. coli \), but only one single parologue represents the DacA-F group. The much more distant cyanobacterium Synechocystis sp. expresses only two paralogues of the DacB grouping. In E. coli, B. subtilis, \( S. aureus \) and \( H. influenzae \), the DacX paralogue is more related to DacB than to PbpG. Perhaps the DacX group constitutes up to 30% of the total penicillin-binding proteins. In E. coli, the DD-endopeptidase activity appears to be due to redundant catalytic activities of DacB and PbpG, while DD-carboxypeptidase activity appears to be due to the redundant catalytic activities of \( E. coli \) DacA, DacC and DacD. In E. coli, PbpG has been shown to constitute up to 30% of the total penicillin-binding proteins (Dougherty et al., 1996).
Signature amino acid motifs

Members of the superfamily of serine-active-site penicillin-interacting proteins which includes the PBPs and \( \beta \)-lactamases share three major motifs: S\textsubscript{x}xK, S\textsubscript{x}xY\textsubscript{x}xN and K/H/T/SG. At the family level within the superfamly, one can expect expanded motif signatures built around the invariant anchor residues. In the low-molecular-mass PBP family the first motif is 69AS\textsubscript{xx}K\textsubscript{xx}T\textsubscript{xx}AA (superfamily anchor residues are in bold; residue numbers are according to the E. coli PbpG sequence), as seen in Fig. 4. At lower hierarchical clustering levels, an expanded signature may typify a given cluster. For example, the signature for the DacB cluster is AS\textsubscript{xx}K\textsubscript{xx}T\textsubscript{xx}AA.

The second motif is 127S\textsubscript{x}N\textsubscript{x}A(x)\textsubscript{4}A(x)\textsubscript{4}F(x)\textsubscript{4}M(x)\textsubscript{10}G\textsubscript{186}. Just prior to the S\textsubscript{x}N motif is a conserved serine residue (1165 in E. coli PbpG) which although imperfectly aligned between groups may be functionally equivalent.

The third major signature motif for low-molecular-mass PBPs is 234KTGS/T\textsubscript{237}. A fourth motif is a peptide 177S/TA\textsubscript{D}180. The motif is speculated that residue lsoD might serve this function in the S\textsubscript{x}N and KTG motifs. Henderson \textit{et al.} (1995) speculated that residue 149D might serve this function in \textit{E. coli} PbpG. Fig. 4 shows this and surrounding residues to be indeed highly conserved in all clusters except the DacB cluster. The motif is 175S/T\textsubscript{Ax}D\textsubscript{180}.

ACKNOWLEDGEMENTS

The authors thank the Institute for Genomic Research, the Gonococcal Genome Sequencing Project (University of Oklahoma), and the \textit{Streptococcus} Sequencing Project (University of Oklahoma) for availability of sequence data prior to publication. They appreciate the advice and help of Genshi Zhao. This publication is Florida Agriculture Experiment Station Journal Series no. R-05939.

REFERENCES


Received 10 October 1997; accepted 27 November 1997.