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

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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 ^{125}I-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-

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Abbreviation: PBP, penicillin-binding protein.

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Endopeptidases hydrolyse the latter interpeptide cross-links.

The transpeptidase domain of high-molecular-mass PBPs and the DD-peptidase enzymes (low-molecular-mass PBPs) possess a common active-site serine residue associated with a configuration of conserved motifs found in β-lactamase (Ghuysen, 1991; Ghuysen & Dive, 1994). 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 OmpT (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*, *pbpG*, was recently cloned (Henderson *et al.*, 1995). PBP7 has been reported to be a target for β-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 ceftazidime has been correlated with increased expression of PBP8 (Malouin *et al.*, 1991).

PBPs of *Pseudomonas aeruginosa* have received relatively little attention, even though β-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. aeruginosa* were 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. aeruginosa* has been 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. 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 *pbpG* in the *P. 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. aeruginosa*. Pseudomonas isolation agar (Difco) was used for isolating *P. aeruginosa pbpG* knockout mutants. Additions of ampicillin (100 μg ml^{-1}), chloramphenicol (40 μg ml^{-1}), tetracycline (25 μg ml^{-1}) and mercuric chloride (15 μg 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 (Bio101). 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 *pbpG* was placed into a translational fusion vector (pET11a). The coding region was amplified using PCR with the upper 30mer primer 5′-TTCCAT**ATG**AGAAACC-GTCTCCTGTCACTG-3′ (a built-in *Nde*I site is underlined and the translational start codon is in bold) and the lower 18mer primer (RJ 105) 5′-CTTCGCGACGGATCAGCG-3′ (complementary to nucleotides 1504–1521). The PCR fragment was first cloned into the *Sma*I site of pUC18 and was subsequently excised with *Nde*I and *Bam*HI. The latter fragment carrying the *pbpG* gene was then ligated with pET11a digested with *Nde*I and *Bam*HI, to create the PbpG overexpression plasmid, pJS87. To avoid the toxicity resulting from PbpG overexpression, the *Bgl*II–*Bam*HI fragment from pJS87 carrying the *pbpG* gene was cloned into the low-copy-number plasmid pACYC184, to create pJS89S.

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

Procedure for 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 μg 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 (wet 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 × 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 pelleted 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

Strain or plasmid	Relevant genotype or description	Source or reference
E. coli		
BL21(DE3)	F^- *ompT hsdS*$_B$ (r_B^- m_B^-) *gal dcm*; with DE3, a λ prophage carrying the T_7 RNA polymerase gene	Novagen
CS109	W1485, λ^- F^- *thi glnV(supE) rph rpoS*	Henderson *et al.* (1997)
CS9-19	*pbpG*::*res* in CS109 background	S. A. Denome and others, unpublished
DH5α	F^- *e14*$^-$ (*mcrA*) *hsdR514* (r_k^- m_k^+) *recA1 endA1 gyrA96 thi-1 relA1 supE44*	Gibco-BRL
S17-1	[RP4-2(Tc::Mu) (Km::Tn7) Tra (IncP)] *pro hsdR recA* Tp^r Sm^r	Simon *et al.* (1983)
P. aeruginosa		
PAO-1	Prototroph	D. H. Calhoun, City College, New York
JS105	PAO-1, Δ*pbpG*, Hg^r	This study
Plasmids		
pACYC184	Cm^r Tc^r; low-copy-number cloning vector	Chang & Cohen (1978)
pET11a	T7*lac* promoter, *lacI*$^+$ Amp^r	Novagen
pJS6	*Sph*I–*Hin*dIII fragment carrying *pbpG* cloned into pUC18	This study
pJS87	PbpG overexpression plasmid, PbpG-coding region fused with T7 translational initiation signal at *Nde*I site of pET11a	This study
pJS89	*Xba*I–*Bam*HI fragment carrying *pbpG* from pJS87 cloned into pUC19	This study
pJS89S	*Bgl*II–*Bam*HI fragment carrying *pbpG* from pJS87 cloned into pACYC184	This study
pJZ9	*phhRABC*, Amp^r	Zhao *et al.* (1994)
pUC18	Amp^r; high-copy-number cloning vector	Yanisch-Perron *et al.* (1985)
pUC19	Amp^r; high-copy-number cloning vector	Yanisch-Perron *et al.* (1985)
pUFR004	ColE1, Cm^r Mob^+ *mobP lacZa*$^+$	Defeyter *et al.* (1990)

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′-GTGAAGCCGGTCT-TGGTCAACTGGAT-3′ (complementary to nucleotides 1249–1274) were used. Interruption of the *pbpG* gene in a *P. aeruginosa* Hg^r 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 *phhABC* 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 *phh* operon. Indeed, the upstream region proved to house a positively acting regulatory gene, *phhR* (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 *phh* operon genes was indirect. Fig. 1 shows the subclone construct (pJS6) used for nucleotide sequencing.

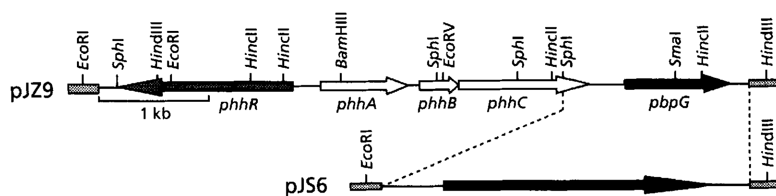

Fig. 1. Physical map of *P. aeruginosa pbpG* (solid arrow), and the upstream flanking region containing the *phhR* regulatory gene (shaded arrow) and the structural gene members (open arrows) of the *phh* operon. Positions of restriction sites in the cloned DNA and in the pUC polylinker region (shaded bars) are shown at the top. pJZ9 is the original clone isolated, and pJS6 is the subclone 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 *phh* 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 σ^{70}, σ^{54} or σ^{E} were all too marginal to merit speculation. The deduced mature PbpG protein is positively charged and exhibits a calculated isoelectric point of 10·37. It possesses only a single cysteine residue.

Downstream of *pbpG* is a convergently transcribed gene (*yaeJ*) of unknown function. However, it is a homologue (52% identity) of a 15·6 kDa protein in *E. coli* which is transcribed immediately ahead of *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 *pcaJ*, 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

PpbG was overexpressed in *E. coli* BL21(DE3)/pJS87 using the T7 expression system (Novagen). PpbG was readily visible as a prominent band of the expected size following SDS-PAGE of samples from whole-cell lysates

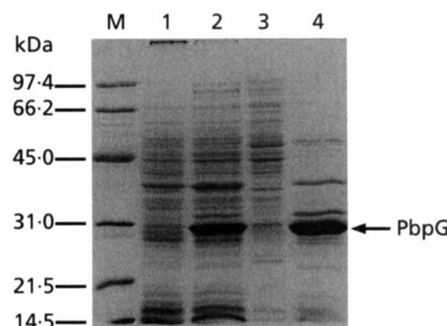

Fig. 2. Overexpression and subcellular location of *P. aeruginosa* PbpG in *E. coli* BL21(DE3) using the T7 expression system. Proteins in whole-cell lysates or subcellular fractions were separated on SDS-12% PAGE gels. Lane M, molecular-mass standards; Lanes: 1, whole-cell lysate BL21(DE3)/pJS87 in the absence of IPTG induction; 2, whole-cell lysate of BL21(DE3)/pJS87 following induction with 0·4 mM IPTG; 3, cytoplasmic fraction of BL21(DE3)/pJS87 prepared from an IPTG-induced culture; 4, membrane fraction of BL21(DE3)/pJS87 prepared from an IPTG-induced culture.

(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 (MRNRLLSLVTLFLSLSVATAV-SA) fulfils the three standard criteria for cleavable signal peptides (Nakai & Kanehisa, 1991).

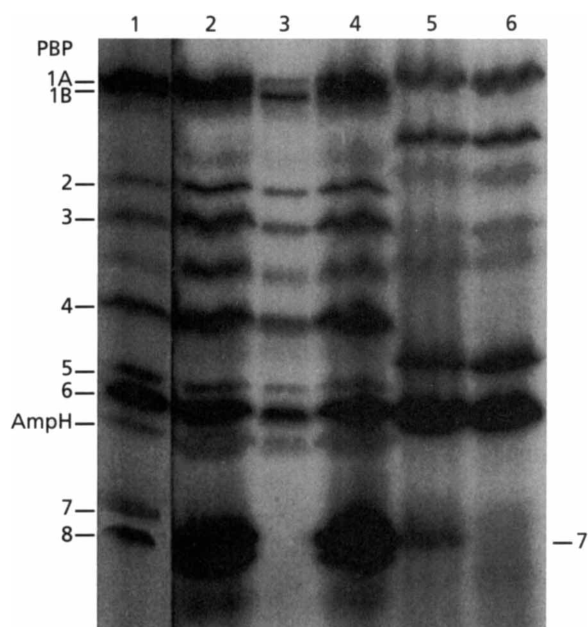

Fig. 3. Identification of PBP7 in *E. coli* and *P. aeruginosa*. PBPs were labelled with ^{125}I-penicillin X as described in Methods. Lane 1, wild-type *E. coli* CS109; lane 2, *E. coli* CS109/pJS89S expressing cloned *P. aeruginosa* Pbp7; lane 3, *E. coli* CS9-19 (mutant lacking Pbp7/8); lane 4, *E. coli* CS9-19/pJS89S; lane 5, *P. aeruginosa* PAO-1 (wild-type); lane 6, *P. aeruginosa* JS105 (mutant lacking Pbp7/8). *E. coli* PBPs 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 PpbG 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, over-expression 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 163–166 of *P. aeruginosa* PbpG). The other motif is SxNxxA (corresponding to residues 121–126 of *P. aeruginosa* PbpG).

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 PBP paralogues in such 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

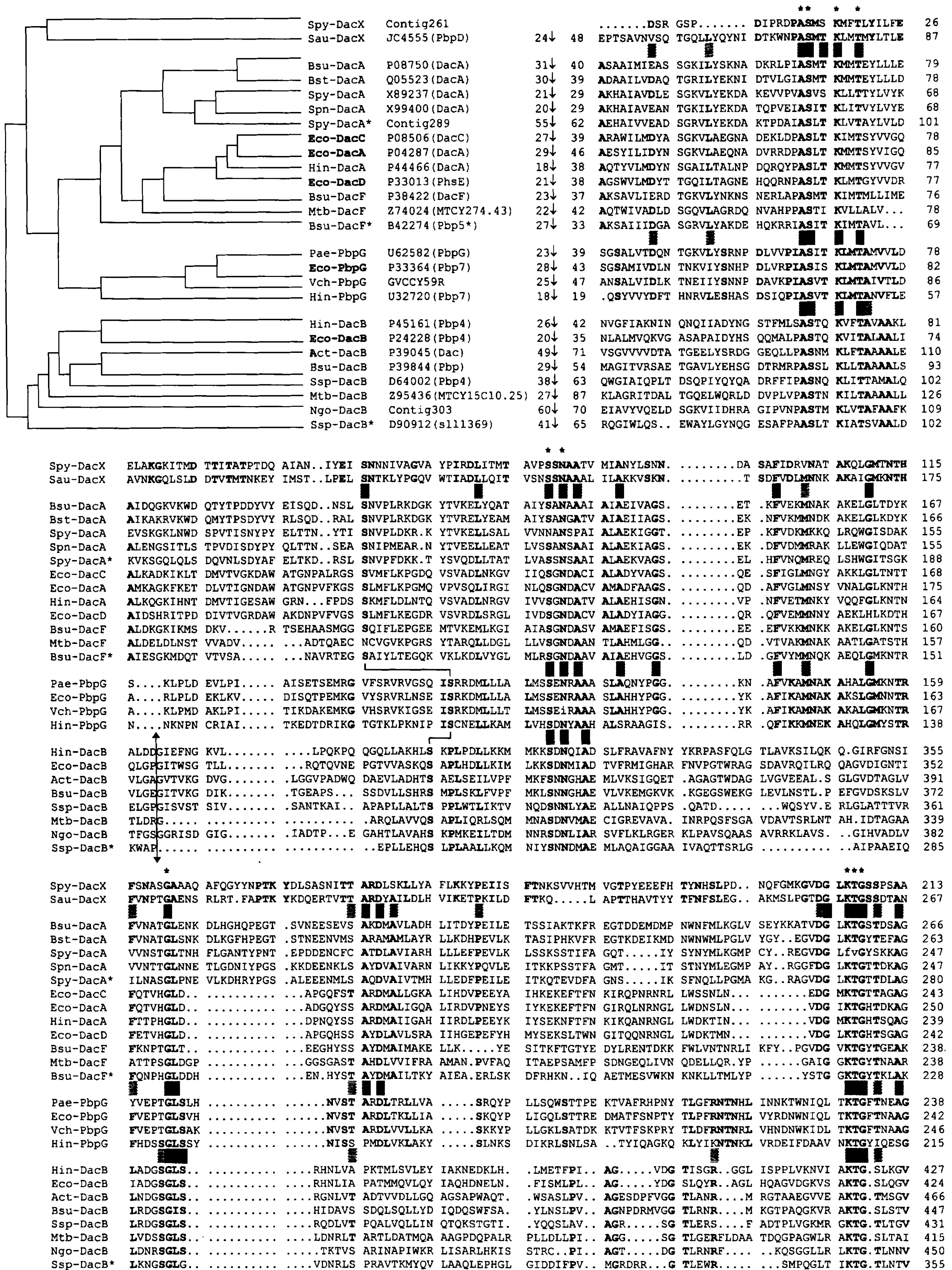

Fig. 4. For legend see facing page.

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Spy-DacX   FNAMITAKRG .KTRLITIVM GVGDWSDQNG EFYRHPFVNA LTEKGF...K  DSKTLSKKAR QKLEK..... ..LVPQTKKE TSSKQQHFKA TKKQSY  298/363
Sau-DacX   YNHTITTKRG .KFRINQVIM GAGDYKNLGG EKQRNMMGNA LMERSFDQYK  YVKILSKGEQ RINGKKYYVE NDLYDVLPSD FSKKDYKLVV EDGKVH  362/431

Bsu-DacA   SCFTGTAERN .GMRVITVVL NAKG....NL HTGRFDETKK MFDYAFDNFS  MKEIYAEGDQ VKGHKTISVD KGKEKEVGIV TNKAFSLPVK NGEEKN  357/443
Bst-DacA   NCFTGTAKRN .GVRLISVVM NAKDASGKTT KEARFKETEK LFNYGFNQYS  LETLYPKGYQ LKGKETLPVV KGKEKEVRVA TGKNLDLLVK NGEEKQ  358/452
Spy-DacA   ASFVATSVEN .QMRVITVVL NADQ..SHED DLAIFKTTNQ LLQYLLINFQ  KVQLIENNKP V...KTLYVL DSPEKNCQTC SPK....... ......  324/324
Spn-DacA   ESFVGTTVEK .GMRVITVVL NADH..QDNN PYARFTATSS LMDYISSTFT  LRKIVQQGDA YQD.SKAPVQ DGKEDTVIAV APEDIYLIER VGNQSS  339/413
Spy-DacA*  HCLVVTSIEN .GMRLITVIL NADG..SDKN QNTRFEQANR LLDYVARTYC  RRKILKKGSL VSE.RSLPIQ DGQVKELPIS VAEDVTIILQ QGEQVP  372/442
Eco-DacC   YNLVASATQG .DMRLISVVL GAK......T DRIRFNESEK LLTWGFRFFE  TVTPIKPDAT F...VTQRVW FGDKSEVNLG AGEAGSVTIP RGQLKN  329/400
Eco-DacA   YNLVASATEG .QMRLISAVM GGR......T FKGREAESKK LLTWGFRFFE  TVNPLKVGKE F...ASEPVW FGDSDRASLG VDKDVYLTIP RGRMKD  336/403
Hin-DacA   YNLVASATTS NNMRLISVVM GVP......T YKGREVESKK LLQWGFANFE  TFKTLEAGKE I...SEQRVY YGDKNSVKLG ALMDHFITIP KGKQSE  326/393
Eco-DacD   FNLIASAVDG .QRRLIAVVM GAD......S AKGREEEARK LLRWGQQNFT  TVQILHRGKK V...GTERIW YGDKENIDLG TEQEFWMVLP KAEIPH  328/390
Bsu-DacF   YCLTASAKKG .NMRAIAVVF GAS......T PKERNAQVTK MLDFAFSQYE  THPLYKRNQT V...AKVKVK KGKQKFIELT TSEPISILTK KGEDMN  324/389
Mtb-DacF   KTFVGAAARG .GRRLVIAMM YGLVKEGGPT Y...WDQAAT LFDWGFA...  ...LNPQA.. .....SVGSL .......... .......... ......  291/291
Bsu-DacF*  RTLVSTASKD .GIDLIA... ...VTINDPN D...WDDHMK MFNYVFEHYQ  TYLIAKKG.. .....DIPKL KGTFYESKAF IKRDITYLLT EEEKEN  307/382

Pae-PbpG   HCLVM..RTT INRSPVNLVV .....LDAFG KYTHFADATR LRRW....LE  TGQVTAIPAA AKAYRLQRDD RERGLGQPVA QAVR...... ......  311/311
Eco-PbpG   HCLVM..RTV INNKPVALVV .....MDAFG KYTHFADASR LRTW....IE  TGKVMPVPAA ALSYKKQKAA QMAAAGQTAQ ND........ ......  313/313
Vch-PbpG   HCLVM..RTQ MGRRQVAFVV .....LDTKG KLSPVGDANR LRTW....IE  TGKVTPLSSE AKVI...... .......... .......... ......  299/299
Hin-PbpG   YNLVFINKHR CKNATIGVIS .....LN... .....NTSSA YRSS....FT  KSKLEKFGCT ALNGRTIRDV AGEAQYEDGY DEVGFNTLIQ KLSK..  292/292

Hin-DacB   YNLAGFMTNA RGEKVAFVQF INGYSTGDLE SKTKRAPLVQ FERNLYNELY  KY........ .......... .......... .......... ......  479/479
Eco-DacB   YNQAGFITTA SGQRMAFVQY LSGYAVEPAD QRNRRIPLVR FESRLYKDIY  QNN....... .......... .......... .......... ......  477/477
Act-DacB   SALSGYVPGP EG.ELAFSIV NNGH.SGPAP L.AVQDAIAV RLAEYAGHQA  PEGARMMRGP VQGSGELECS WVQAC..... .......... ......  538/538
Bsu-DacB   SSLSGYAETK SGKKLVFSIL LNGLIDEEDG K.DIEDQIAV ILANQ.....  .......... .......... .......... .......... ......  491/491
Ssp-DacB   VSLTGYVENQ QWGTVAFSFM VNNSDLGASV LREAMKQMVL WTAQVEKCQP  SDQGR..... .......... .......... .......... ......  486/486
Mtb-DacB   NSLVGVLTDR SGRVLTFAFI SNEAGPNGRN AMDALATKLW FCGCTT....  .......... .......... .......... .......... ......  461/461
Ngo-DacB   RALAGYWLGD KPMAVV.VII NSGRAVSLLP DLDNFVAKNI ISGGDGWLDA  KLMCKERRA. .......... .......... .......... ......  508/508
Ssp-DacB*  SALAGTIPTQ ERGTVWFAII NNGPNFDRLR VEQDRLLQQI AEHWQVLPEN  LNAGPMDKVL LGDPARNLTP PPSES..... .......... ......  430/430
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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 (↓) and beginning residue number shown in the multiple alignment. At the far lower right is given the number of the final residue presented followed (slash) by the number of the final sequence residue. Residues that are highly conserved within any one of the four clusters compared are printed in bold type. Solid bars join residues that are highly conserved between a given pair of groups. 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 and KTG motifs of PBPs from Vch-PbpG and Spy-DacA, respectively, are probably errors which can be expected because of the preliminary status of these genome sequencing projects.) A serine residue just prior to the SxN motif (corresponding to *P. aeruginosa* PbpG S_{111}) is conserved in each of the subgroups, but imperfectly aligned. The possibility that these serine residues are functionally equivalent is indicated by the double-bent lines between subgroups. The vertically divergent arrow shown within the DacB cluster indicates the region which was manually deleted to optimize the alignment as carried out by Mottl *et al.* (1991). The probable target sites for artifactual cleavage of *E. coli* and *P. aeruginosa* PBP7 (PpbG) proteins by OmpT to produce PBP8 species are double-underlined. Organism abbreviations: Spy, *Streptococcus pyogenes*; Sau, *Staphylococcus aureus*; Bsu, *Bacillus subtilis*; Bst, *B. stearothermophilus*; Spn, *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 a single paralogue represents the DacA-F group. The much more distant cyanobacterium *Synechocystis* sp. expresses only two paralogues of the low-molecular-mass PBPs, these being divergent members of the DacB cluster.

The extent to which the major groupings shown in Fig. 4 correspond to functionally specialized PBPs is currently incompletely known. The data available indicate that cluster DacA-F contains DD-carboxypeptidases, whereas cluster PbpG contains DD-endopeptidases. Within the DacB grouping, *E. coli* DacB is a DD-endopeptidase which has weak DD-carboxypeptidase activity. In view of the wide divergence within this group, different functional specialization within this group would not be surprising. For example, *Synechocystis* sp. DacB and DacB* are logical candidates for functional divergence, as *Synechocystis* sp. DacB* has diverged more from its DacB paralogue than have the functionally different *E. coli* DacA and PbpG paralogues.

Thus far, PbpG homologues in different organisms (orthologues) are restricted to a relatively closely related group of organisms: *E. coli*, *Vibrio cholerae*, *P. aeruginosa* and *H. influenzae*. We could not identify PbpG orthologues in the nearly completed sequences of *Neisseria gonorrhoeae*, *Staphylococcus aureus* or *Streptococcus pyogenes*. Perhaps the DacX group represented by the latter two organisms (Fig. 4) corresponds to another functionally specialized DD-endopeptidase cluster. DD-Endopeptidase function is also known to exist in *E. coli* for MepA, a non-homologue of PBPs. 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 β-lactamases share three major motifs: SxxK, S/YxN and K/HT/SG. At the family level within the superfamily, one can expect expanded motif signatures built around the invariant anchor residues. In the low-molecular-mass PBP family the first motif is ^{69}ASxxKxxT^{77} (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 ASxxKxxTxxAA.

The second motif is ^{127}SxN(x)$_{35}$G^{165}. If the DacB group is excluded, the remaining three clusters share the signature motif ^{127}SxNxA(x)$_4$A(x)$_9$F(x)$_3$M(x)$_7$G(x)$_{10}$ G^{165}. Just prior to the SxN motif is a conserved serine residue (^{115}S 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 ^{234}KTGS/T^{237}. A fourth motif is a peptide segment having one or two dicarboxylic acids between the SxN and KTG motifs. Henderson *et al.* (1995) speculated that residue ^{180}D might serve this function in *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 ^{177}S/TAxD^{180}.

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