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Dimerization of the penicillin-binding proteins in Escherichia coli

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Title (English) **Dimerization of the penicillin-binding proteins in *Escherichia coli***	
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Abstract The penicillin-binding proteins (PBP's) play a crucial role in the bacterial cell cycle by synthesizing the peptidoglycan. They are popular drug targets and have been studied for decades as they are the target for the first antibiotic discovered – penicillin. However, due to an increasing resistance to antibiotics, new means of disrupting their function are needed. A complete understanding of the multi-protein complexes that make up the peptidoglycan synthesizing machinery is therefore of interest. A fundamental knowledge for elucidating the multi-protein complexes are the biological conformation of the proteins. In this work we provide evidence that most PBP's form dimers and that for PBP5 dimerization occurs in the membrane anchor.	
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Dimerization of the penicillin-binding proteins in *Escherichia coli*

Mårten Hellberg

Sammanfattning

Det första antibiotikumet som upptäcktes var penicillinet. Det verkar genom att slå ut funktionen hos de penicillinbindande proteinerna vilka bygger upp bakteriernas cellvägg. Om cellväggen inte kontinuerligt förnyas leder det till att bakterierna slutar att växa och dör.

Eftersom mänskliga celler saknar cellvägg är penicillinbindande proteiner bra mål för läkemedel. Emellertid har ökad resistans mot penicillin gett upphov till behov av att finna nya antibiotikum. En förståelse för hur proteinerna fungerar och interagerar med varandra är därför av betydelse.

Även fast penicillinbindande proteiner har studerats under decennier finns inte en förståelse för hur de sammanfogas i makromolekylära komplex. För en full förståelse av proteinkomplexen är kunskap om proteinernas biologiska konformation viktig. Vi har i denna studie visat att de flesta av de penicillinbindande proteinerna består av homodimerer (proteinerna binder till sig själva i par).

Dimerisering har visats viktig för flera proteiners funktion. För att kunna studera dimeriseringens funktionella betydelse behöver man veta vilka aminosyror som binder till varandra mellan proteinerna. Vi undersökte detta i penicillinbindande proteinet 5. Resultaten visade att proteinets membranankare är involverad i dimeriseringen. Via denna kunskap är vårt mål att hitta liknande motiv i alla penicillinbindande proteiner, vilket kan leda till tänkbara mål för nya antibiotika.

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Introduction

Antibiotic resistance is a growing problem in a big part of the world. As more and more bacterial strains gain resistance to commonly used antibiotics such as penicillin, the need for new innovative antibiotics has increased (Arbeloa *et al.*, 2004). The main problem that exists is to obtain specificity for bacterial cells within the contact of the human body. Essential proteins that are unique to bacterial cells are therefore of special interest as drug targets. One class of proteins that has these distinct characteristics are the penicillin binding proteins (PBP's): the target for the oldest used antibiotic, penicillin (Georgopapadakou *et al.*, 1980, Macheboeuf *et al.*, 2006).

The PBP's are a family of enzymes involved in synthesis of the bacterial cell wall (Cabeen *et al.*, 1999, Dmitriev *et al.*, 2005, Höltje *et al.*, 1998, Matsuhashi *et al.*, 1990). The cell wall of gram negative bacteria is composed of three distinct layers, the inner membrane, the periplasm, and the outer membrane. The outer and inner membranes are composed of a lipid bilayer containing a large number of proteins with a wide diversity of functions, including transport, cell division, and biogenesis (Dmitriev *et al.*, 2005, Höltje *et al.*, 1998, Natividad *et al.*, 2005, Scheffers *et al.*, 2005) . The periplasm is composed of peptidoglycan that is made from stiff glycan chains which are crosslinked by flexible peptide bridges (Höltje *et al.*, 2001, van Heijenhoort *et al.*, 2001). Each subunit within the peptidoglycan layer is composed of two amino sugars, N-acetylglucosamine (GlcNAc), and N-actylmuramic acid (MurNAc), which are connected to each other through a transglycosylation reaction. A pentapeptide is connected to each MurNAc perpendicularly, which can be crosslinked to an adjacent pentapetide by a transpeptidation reaction. In this way, a mesh of crosslinked glycan strands are formed which gives the peptidoglycan layer a rigidity and stability that is essential for withstanding osmotic stress (figure 1).

The synthesis of peptidoglycan is initiated in the cytoplasm with the synthesis of the amino sugars by the so called Mur-enzymes (Höltje *et al.*, 1998, Scheffers *et al.*, 2005). These amino sugars are transported to the periplasm by an unknown mechanism, but a flippase activity by the proteins FtsW and RodA has been proposed (Matsuhashi *et al.*, 1994). Within the periplasm the PBP's finalize the synthesis of the peptidoglycan by connecting them to each other in a number of enzymatic reactions.

The PBP's can be divided into 2 broad categories: the high molecular weight (HMW) PBP's, and the low molecular weight (LMW) PBP's (table 1) (Georgopapadakou *et al.*, 1980). The HMW PBP's can be further subdivided into class A PBP's and class B PBP's

based on their enzymatic activity. The HMW class A PBP's include PBP 1a, PBP 1b and PBP 1c, all which have both transglycosylation and transpeptidation activity (Bertsche *et al.*, 2005, Born *et al.*, 2006) . They are essential for cell survival and have been a popular target for antibiotics. The HMW class B PBP's include PBP 2 and PBP 3, which both act as transpeptidases. PBP 2 has been shown to be essential for transpeptidation during cell elongation, and PBP 3 during cell division in *Escherichia coli* (Begg *et al.*, 1990, Höltje *et al.*, 2001, Signoretto *et al.*, 1998, Spratt *et al.*, 1975). PBP 3 is also positioned in the so called Z-ring, at the site of septation during cell division, and been shown to interact with other cell division proteins (Bertsche *et al.*, 2006, Errington *et al.*, 2003, Karimova *et al.*, 2005).

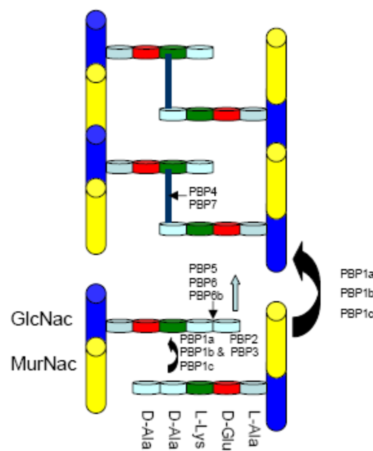

Figure 1. Peptidoglycan synthesis. In the periplasm, the precursors made of aminosugars are connected to each other. Long strands of aminosugars are synthesized through transglycosylation by the enzymes PBP1a, 1b, and 1c, and a meshwork of peptidoglycan is created through transpeptidation between L-Lys, and the fourth D-Ala, by PBP2, and PBP3. Small changes are later made to the peptidoglycan by the carboxypeptidases PBP5, PBP6, and 6b, which cleave the terminal D-Ala:D-Ala bond, or by the endopeptidases PBP4, and PBP7 which cleave the transpeptide bond.

The LMW PBP's have a different enzymatic activity as they mainly perform small changes to the pentapeptides. The carboxypeptidases (PBP5, PBP6 and PBP6B) remove the terminal amino acids of the pentapeptides (Amanuma *et al.*, 1980, Broome-Smith *et al.*, 1982, Korsak *et al.*, 2005, Matsuhashi *et al.*, 1979, Nishimura *et al.*, 1980, Spratt *et al.*, 1976, Tamura *et al.*, 1976) , and the endopeptidases (PBP 4 and PBP 7) break up the transpeptide bond between adjacent pentapeptides (Henderson *et al.*, 1995). None of the LMW PBP's have been shown to be essential for cell survival, however several studies have shown that they have roles in defining the morphology of the cell. Especially important is PBP 5, which cleaves the terminal D-ala:D-ala bond on the pentapeptide. Mutational studies have shown that deletions of PBP 5 together with other LMW PBP's give rise to kinks and bends in the cell envelope, as well as branching of the cells (Denome *et al.*, 1999, Nelson *et al.*, 2000, Nelson *et al.*, 2001, Popham *et al.*, 2003). The sites of the kinks and bends are in some way

connected to so-called inert peptidoglycan, where no natural turn-over of the peptidoglycan occurs (de Pedro *et al.*, 1997, de Pedro *et al.*, 2003, de Pedro *et al.*, 2003, Korsak *et al.*, 2005, Nilsen *et al.*, 2004). However, what role PBP5 and its enzymatic activity have in the synthesis of inert peptidoglycan is not understood.

The HMW PBP's are predominantly localised in the periplasm where their enzymatic domains (transglycosylation and transpeptidation sites) are situated (figure 2). All of them are attached to the inner membrane through a single transmembrane helix at their N-terminus, and PBP1b and PBP3 also have a small domain in the cytosol (Gittins *et al.*, 1994). Their molecular weights range from 93,4 kDa (PBP1a) to 61 kDa (PBP3) (table 1).

Table 1. The penicillin binding proteins. The high molecular weight class A (PBP 1a, 1b, and 1c), class B (PBP 2, and 3), and the low molecular weight protein's (PBP 4, 5, 6, 6b, and 7). Different enzymatic activities are listed below.

Protein	Gene	Gene lenght (base pairs)	Protein length (amino acids)	Molecular weight (kDa)	Enzymatic activity
PBP 1a	*mrcA*	2553	850	93,4	Transglycosylase/Transpeptidase
PBP 1b	*mrcB*	2553	844	94,1	Transglycosylase/Transpeptidase
PBP 1c	*pbpC*	2313	770	84,9	Transglycosylase
PBP 2	*mrdA*	1902	633	70,7	Transpeptidase (cell elongation)
PBP 3	*ftsI*	1767	588	63,7	Transpeptidase (cell division)
PBP 4	*dacB*	1434	477	51,6	Endopeptidase/Carboxypeptidase
PBP 5	*dacA*	1212	403	44,3	Carboxypeptidase
PBP 6	*dacC*	1212	400	43,4	Carboxypeptidase
PBP 6B	*dacD*	1167	388	43,2	Carboxypeptidase
PBP 7	*pbpG*	933	310	33,7	Endopeptidase

The LMW PBP's have a different structure and are smaller. Their molecular weights range from 51,6 kDa (PBP4) to 33,7 kDa (PBP7) (table 1). They are attached to the inner membrane by an amphipathic helix that acts like a membrane anchor (Brandenburg *et al.*, 2002, Gittins *et al.*, 1994, Harris *et al.*, 95, Harris *et al.*, 97, Harris *et al.*, 98, Harris *et al.*, 2002, Phoenix *et al.*, 1993, Pratt *et al.*, 1986, Siligardi *et al.*, 1997). Their catalytic activity is performed by the N-terminal domain (van der Linden *et al.*, 1993, Nelson *et al.*, 2002). This domain has been studied in some detail, especially in PBP5 where it has been crystallized (Nicholas *et al.*, 2003). The crystal structure revealed that the active site is situated in the N-terminal domain of the protein which reaches out to the peptidoglycan through a linker domain between the N-terminal domain and the membrane anchor. Mutational studies have also revealed the exact amino acids involved in the enzymatic activity, which comprises a SXN-motif which binds to the pentapeptide and cleaves the D-Ala:D-Ala bond (van der Linden *et al.*, 1994, Nicholas *et al.*, 2003). In the structure PBP5 crystallized as a monomer, however the membrane anchor had to be removed to make the protein soluble.

The membrane anchor of PBP5 has been shown to be necessary for the correct function of PBP5. By creating fusion proteins and point mutations, Young and co-workers have shown that single point mutations in the membrane anchor could create a non-functional PBP5 (Nelson *et al.*, 2002). Whether this was due to a loss of interaction with the membrane, or because it lost interaction to other proteins was not elucidated.

Possible interactions between the PBP's have been studied, but are not well characterized. It has been suggested that they exists in macromolecular complexes, possibly one implicated in cell division, and one in cell elongation (Höltje *et al.*, 1998). For instance, studies have shown that PBP 3 interacts with PBP 1b, as well as the cell division machinery (Bertsche *et al.*, 2005, Karimova *et al.*, 2005 Matsuhashi *et al.*, 1990, Spratt *et al.*, 1975). In addition, movement of PBP3 (encoded by *dacA* in *Streptomyces pneumoniae*) in the cell has been observed to be in syncrony with the cell cycle (Morlot *et al.*, 2004). However, the exact composition, the sizes and the functional relevancies of the complexes are unknown.

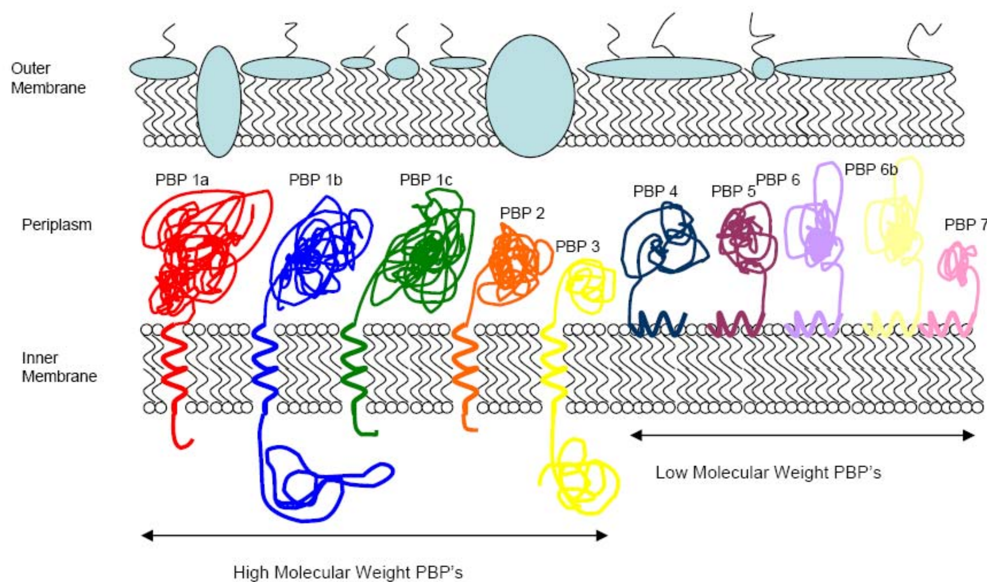

Figure 2. Structure and localisation of the penicillin binding proteins. The high molecular weight PBP's, PBP1a, PBP1b, PBP1c, PBP2, and PBP3 are all attached to the inner membrane through a trans-membrane helix with their enzymatic domain pointing into the periplasm. PBP1a, 1b, and 1c have two enzymatic domains, a transpeptidase domain and a transglycosylase domain. PBP 2 and PBP 3 have a single transpeptidase domain. The low molecular weight PBP's are attached to the inner membrane through an amphipathic helix. They have one enzymatic domain with endopeptidase activity (PBP4, and 7), or carboxypeptidase activity (PBP5, 6, and 6b).

Some HMW PBP's (e.g. PBP1a, PBP1b, and PBP3) have been shown to dimerize (Bertsche *et al.*, 2005, Chalut *et al.*, 1999, Charpentier *et al.*, 2002, Karimova *et al.*, 2005). In

addition to its structural relevance, the dimerization was also shown to have implications for function. By breaking the dimer in PBP1b almost half of its activity was lost, making this complex a possible new target for antibiotics (Bertsche *et al.*, 2005). The binding motif in PBP1b has been investigated, and proposed to be near the transglycosylation site. However, this field is poorly understood, and the oligomeric state of the PBP's has not been studied.

In this study we have made a full scale investigation of the oligomeric states for all PBP's, using a biochemical protein interaction assay (Stenberg *et al.*, submitted), and based on previous results indicating dimerization of PBP5 and PBP6 (Hellberg *et al.*, 2006). From our experiments we provide evidence that all HMW PBP's form dimers, as well as most of the LMW PBP's (PBP4, PBP5, and PBP6). Further, we have started to characterize the dimerization motif in PBP5 and show that the membrane anchor is necessary for dimerization, and that the dimerization can be disrupted by single point mutations.

Materials and methods

Strains and growth media

Inner membrane vesicles were prepared from the *E. coli* strain BL21 (DE3) pLysS (F^{-} ompT $hsdS_B$ (r_B^{-} m_B^{-}) gal dcm (DE3) pLysS). All cloning steps were undertaken in the *E. coli* strain MC1061. For the protein interaction assay the *E. coli* strain BL21 (DE3) was used.

Bacteria were grown at 37 ˚C in Luria-Bertani (LB) broth, supplemented with kanamycin at 50 µg/ml if nothing else stated. Transformed colonies were screened on LA agar plates supplemented with kanamycin. Chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, U.S.A.).

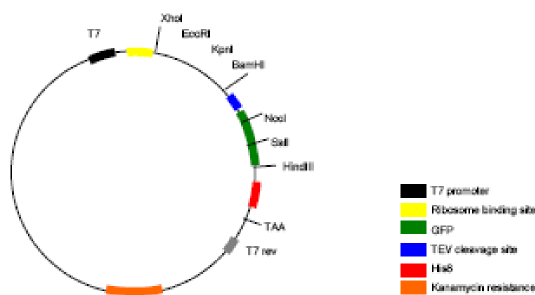

Figure 3. The GFPe-vector. The GFPe-vector is under control of a T7-promoter. A ribosome binding site is followed by a number of restriction sites giving several options for ligation of the gene of interest. GFP, and/or a His8-tag can be fused to the gene if wanted. The vector has a kanamycin resistance cassette for screening of positive clones.

Molecular cloning

Vectors

To construct recombinant plasmids for the protein interaction assay the GFPe-plasmid (Rapp *et al.*, 2004) (figure 3) was used.

PCR amplification

All ORFs were PCR amplified from *E. coli* genomic DNA using appropriate primers, purchased from Cybergene AB (Huddinge, Sweden) (table 2) with a Thermocycler from Biometra.

Table 2. Primers used for cloning of wild-type PBP's. The different genes were subcloned into the GFPe-vector . For each protein the restriction sites used are listed.

Gene name	Vector	Restriction sites	Forward primer	Reverse primer
mrcA	GFPj	5' EcoRI/3' BamHI	GCGCGCGAATTCGTGAAGTTCGTAAAGTATTTT	GCGCGCGGATCCTCAGAACAATTCCTGTGCCTC
mrcB	GFPe	5' XhoI/3' EcoRI	GCGCGCCTCGAGATGGCCGGGAATGACCGCGAG	CGCGCGGAATTCTTAATTACTACCAAACATATC
pbpC	GFPe	5' XhoI/3' EcoRI	GCGCGCCTCGAGATGCCTCGCTTGTTAACCAAA	GCGCGCGAATTCCTATTGCATGACAAATTTCAC
mrdA	GFPe	5' XhoI/3' EcoRI	GCGCGCCTCGAGATGAAACTACAGAACTCTTTT	GCGCGCGAATTCTTAATGGTCCTCCGCTGCGGC
ftsI	GFPe	5' XhoI/3' EcoRI	GCGCGCCTCGAGATGAAAGCAGCGGCGAAAACG	GCGCGCGAATTCTTACGATCTGCCACCTGTCCC
dacB	GFPe	5' XhoI/3' EcoRI	GCGCGCCTCGAGATGCGATTTTCCAGATTTATC	GCGCGCGAATTCCTAATTGTTCTGATAAATATC
dacA	GFPe	5' XhoI/3' BamHI	GCGCGCCTCGAGATGAATACCATTTTTTCCGC	GCGCGGGATCCTTAACCAAACCAGTGATGGAA
dacC	GFPe	5' XhoI/3' HindIII	GCGCGCCTCGAGGATGACGCAATACTCCTCTCTCCTTCG	GCGCGAAGCTTTTAAGAGAACCAGCTGCCG
dacD	GFPe	5' XhoI/3' EcoRI	GCGCGCCTCGAGTTGAAACGCCGTCTTATTATT	GCGCGCGAATTCTCAGGCCTTATGGTGGAAATA
pbpG	GFPe	5' XhoI/3' EcoRI	GCGCGCCTCGAGATGCCGAAATTTCGAGTTTCT	GCGCGCGAATTCTTAATCGTTCTGTGCCGTCTG

Amplified DNA fragments and corresponding vector were digested with 5' XhoI/3' BamHI for *dacA*, 5' XhoI/3' Hind III for *dacC*, 5' XhoI/3' EcoRI for *mrcB, mrdA, ftsI, dacB, dacC, dacD,* and *pbpG* (figure 4). These restriction sites were added to the PCR-primers for the construction of the different clones. All PCR-fragments were purified by using the Qia-quick PCR purification system, verified by agarose gel electrophoresis, and ligated into the GFPe-vector. Verification of clones was performed by digestion with appropriate restriction enzymes, followed by agarose gel electrophoresis. All clones were sequenced using the Big Dye PCR sequencing kit (Applied Biosystems), and analysed by BM labbet AB, (Furulund, Sweden).

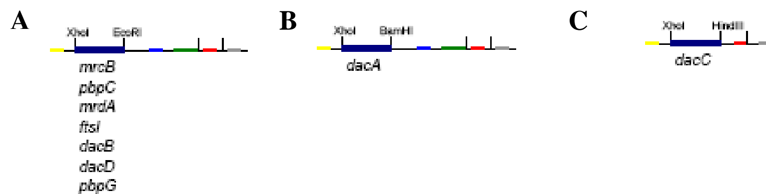

Figure 4. Genes subcloned into the GFPe-vector. The genes *mrcB, mrdA, ftsI, dacB, dacD,* and *pbpG* were subcloned in between the XhoI and EcoRI sites (A). *dacA* was subcloned in between the XhoI and BamHI sites (B), and *dacC* between the XhoI and HindIII sites (C).

Transformation

Transformations were performed by adding 5 µl of purified plasmid to 100 µl of competent cells. The samples were incubated on ice for 30 minutes followed by a heat-shock at 42 ˚C for 75 seconds, and incubation on ice for 2 minutes. 750 µl of LB was added to the samples, followed by incubation at 37 ˚C with shaking for 30 minutes. Cells were pelleted by

centrifugation for 5 minutes at 850 × *g*, and 500 µl of the supernatant was removed. The cells were resuspended in the remaining supernatant, and streaked out on a LA-plate supplemented with kanamycin.

Agarose gel electrophoresis

Agarose gel electrophoresis was performed according to standard protocols (Sambrook *et al.*, 1989). 10 µl of DNA was supplemented with loading buffer (43.5 % (v/v) glycerol, 0.1 % (w/v) bromophenol blue) was run at 100 V, 100 mA for an hour.

Site directed mutagenesis

Site-directed mutagenesis was performed using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, Sweden) using appropriate primers purchased from Cybergene AB (Huddinge, Sweden).

Table 3. Primers used for site directed mutagenesis. The site directed mutagenesis for *dacA* was performed using the primers listed below.

Mutant	Forward primer	Reverse primer
dacA(G384L)	GAAATCCCGGAACTTAACTTCTTCGGC	GCCGAAGAAGTTAAGTTCCGGGATTTC
dacA(G384W)	GAAATCCCGGAATGGAACTTCTTCGGC	GCCGAAGAAGCCCCATTCCGGGATTTC
dacA(G384LG388L))	CTTAACTTCTTCCTCAAAATCATTGAT	ATCAATGATTTTGAGGAAGAAGTTAAG
dacA(F387L)	GAAGGTAACTTCCTCGGCAAAATCATT	AATGATTTTGCCGAGGAAACCTTC
dacA(F387D)	GAAGGTAACTTCGACGGCAAAATCATT	AATGATTTTGCCGTCGAAGTTACCTTC
dacA(I394D)	ATCATTGATTACGATAAATTAATGTTC	GAACATTAATTTATCGTAATCAATGAT
dacA(H400A)	TTAATGTTCCATGCCTGGTTTGGTTAA	TTAACCAAACCAGGCATGGAACATTAA
dacA(Δ383-403)	CAAGAAATCCCGTAAGGTAACTTCTTC	GAAGAAGTTACCTTACGGGATTTCTTG

Pulse-labelling, BN-PAGE protein interaction assay

The protein interaction assay used (Stenberg *et al.*, submitted) is based on radioactive labelling of the proteins *in vivo*, followed by a BN/SDS-PAGE (Schägger *et al.*, 1991, Stenberg *et al.*, 2005).

Radioactive labelling

Plasmids were transformed into BL21(DE3). Colonies were grown in 1 ml LB broth supplemented with kanamycin at 50 µg/ml (except cells without plasmids), and incubated at 37 ˚C with shaking overnight. 50 µl of culture was back diluted into 1 ml of fresh LB broth and grown until an OD_{600} of 0.3 was reached. Cells were pelleted by centrifugation for 5 minutes at 850 × *g*, and resuspended in 1 ml minimal media (1×M9, amino acids minus Met at 1mg/ml, 0.2 % (w/v) glucose, 1 mM $MgSO_4$, 0.25 mM $CaCl_2$, and 10 mM Thiamine), and grown for 90 minutes.

Protein synthesis was induced by adding 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) followed by incubation for 10 minutes, at 37 ˚C with shaking. To inhibit transcription of the genomic DNA before the radioactive pulse labelling, 0.2 mg/ml rifampicin was added to the samples, followed by incubation for 10 minutes at 37 ˚C with shaking. Finally 15 µCi ^{35}S-Met was added to each sample, which was incubated at different time intervals depending on the level of expression desired (*dacC, pbpG* were incubated for 40 minutes, *mrcB, pbpC, mrdA, ftsI,* and *dacB* were incubated for 30 minutes, and *dacA,* and *dacD* were incubated for 20 minutes). Cells were pelleted by centrifugation for 5 minutes at 850 × *g*, and resuspended in 1 ml of fresh LB media supplemented with kanamycin at 50 µg/ml, and grown at 37 ˚C with shaking for 30 minutes.

The samples were divided into two tubes, one containing 100 µl and the other 900 µl of the culture. Cells were pelleted by centrifugation (2 minutes at 17949 × *g*), and the supernatant was removed. The pellet from the tube containing 100 µl was analysed by SDS-PAGE, and the pellet from the tube containing 900 µl was analysed by a BN- / SDS-PAGE.

SDS-PAGE

The pellet from the tube containing 100 µl of cell culture was resuspended in 20 µl of Laemmli-loading buffer (125 mM Tris-HCl, pH 6.8; 4 % (w/v) SDS; 3 % (v/v) glycerol; 10 % (v/v) β-mercapto-ethanol; 0.05 % (w/v) bromophenol blue), and SDS-PAGE was performed according to standard protocols, with a X Cell SureLock (Invitrogen, Novex Mini-Cell) using a 14 % separating gel, at 12 mA for 3 hours. The gel was fixed (30 % (v/v) methanol, 10 % (v/v) acetic acid) for 30 minutes, and dried on a vacuum Slab Gel Dryer, SGD 2000 (Savant) at 60 ˚C for 1 hour. The dried gels were pressed against a phosphor-image plate, using a Fuji-EC-A-Cassette (20 × 40 cm) and exposed overnight. The phosphor-image plates were analysed using a FLA-3000 (Fujifilm), and the software Image Reader v1.8, and Image Gauge v3.45 (Fujifilm).

BN-PAGE

The pellet from the tube containing 900 µl of cell culture was resuspended in 1 ml of H_20 supplemented with 0.4 mg / ml lysozyme, and the samples were incubated at 30 ˚C with shaking for 45 minutes. The crude membrane fraction was pelleted by centrifugation at 284 000 × *g*, 4 ˚C for 30 minutes, and the supernatant was removed. Crude membrane pellets were resuspended in 170 µl of ACA_{750}-buffer (750 mM amino-n-caproic acid, 50 mM Bis-Tris, 0.5 mM Na_2EDTA, pH 7.0), and membrane proteins solubilised with 0.5 % (w/v) n-

dodecyl-β-D-maltoside (DDM). Unsolubilised membranes were removed by centrifugation at 284 000 × *g*, 4 ˚C for 30 minutes and the supernatant was added to 30 µl of G250-buffer (5 % (w/v) Coomassie G250 in ACA_{750} buffer).

The samples were subjected to a BN-PAGE (Schägger *et al.*, 1991, Stenberg *et al.*, 2005) at 4 ˚C for 17 hours, using a 5-15 % gradient gel. The gel was fixed and dried and analysed as described above.

Solubilization test

Samples were taken from each step of the sample preparation for BN-PAGE, i.e. 100 µl of whole cells, taken after the radioactive pulse labelling, 10 µl of supernatant taken after the cells had been treated with lysozyme and centrifuged at 284 000 × *g*, 4 ˚C for 30 minutes, 10 µl of supernatant after the cell membrane had been dissolved with 0.5 % (w/v) DDM and centrifuged at 284 000 × *g*, 4 ˚C for 30 minutes, and finally a sample was taken from the remaining membrane pellet that was dissolved in 180 µl of Laemmli-buffer. Each sample was dissolved in 1 × Laemmli-buffer, and 20 µl was used for a SDS-PAGE, which was performed with a X Cell SureLock (Invitrogen, Novex Mini-Cell) using a 14 % separating gel, on 100 V, 12 mA for 3 hours. The gels were fixed for 30 minutes, and dried at 60 ˚C for 1 hour, using a Slab Gel Dryer, SGD 2000 (Savant). The dried gels were pressed against a phosphor-image plate, using a Fuji-EC-A-Cassette (20 × 40 cm) and exposed overnight. The phosphor-image plates were analysed by autoradiography, using FLA-3000 (Fujifilm), and the software Image Reader v1.8 and Image Gauge v3.45 (Fujifilm).

SDS-PAGE protein interaction assay

The SDS-PAGE protein interaction assay is based on previous studies of the penicillin binding proteins (Charpentier *et al.*, 2002) but with some modifications. The proteins were radio-labelled as described above. 100 µl from each sample was transferred to 2 separate tubes, and cells collected by centrifugation for 2 minutes at 17949 × *g*. The pellets were resuspended in 50 µl “non-denaturating” SDS-loading buffer (60 mM Tris-HCl pH 6.8, 1 % (w/v) SDS, 10% (v/v) glycerol, and 0.01 % (w/v) bromophenol blue). One of the samples was boiled for 5 minutes in 100 ˚C, and both samples were subjected to a SDS-PAGE as described earlier.

Homology analyses and helical wheel analysis

Homology analyses was performed with ClustalW v. 1.82 (PIR, Protein Information Resource: http://pir.georgetown.edu/pirwww/search/multialn.shtml (15 Jan. 2007)).

The helical wheel were created with the Interactive Java helical wheel program (http://kael.net/helical.htm (15 Jan. 2007)).

Results

To determine whether the penicillin binding proteins exist as monomers, dimers or in higher homo-oligomeric complexes we used a protein interaction assay recently developed in our lab (Stenberg *et al.*, 2006). Nine penicillin binding proteins were subcloned into the GFPe-vector according to materials and methods. *mrcA* (endoding PBP1a) was not cloned because it contained restriction sites that were not compatible with our vector.

Expression patterns and solubility of the PBPs

Constructs were transformed into the *E. coli* strain BL21 (DE3), and proteins radio-labelled with ^{35}S-Methionine. Protein expression was verified by analysis of whole cells by SDS-PAGE (Figure 5). All nine proteins could be detected (Figure 5, lanes 2-10 vs. lane 1), although the expression levels differed.

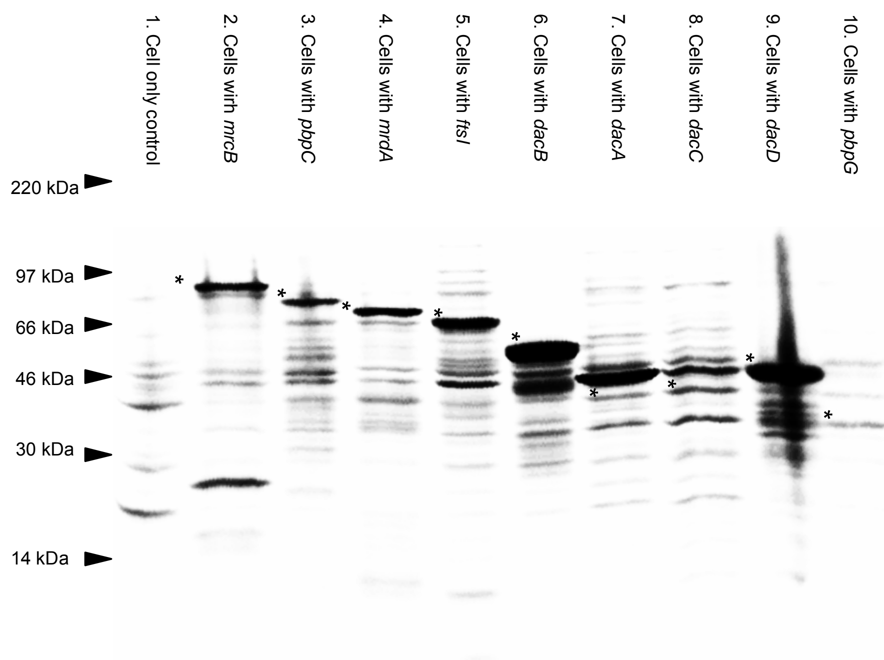

Figure 5. Radio-labelling of the penicillin-binding proteins. Plasmids containing each of the penicillin-binding proteins were subcloned into the wild type *E. coli* strain BL21 (DE3). The proteins were pulse-labelled with ^{35}S-Met and the expression verified by SDS-PAGE of whole cells. As can be seen in this figure PBP1b (lane 2), PBP1c (lane 3), PBP2 (lane 4), and PBP3 (lane 5) all expressed well. PBP4 (lane 6), PBP5 (lane 7), and PBP6b (lane 9) had a slighter higher expression level, and PBP6 (lane 8) and PBP7 (lane 10) expressed poorly. Stars indicate radiolabelled proteins from the plasmid.

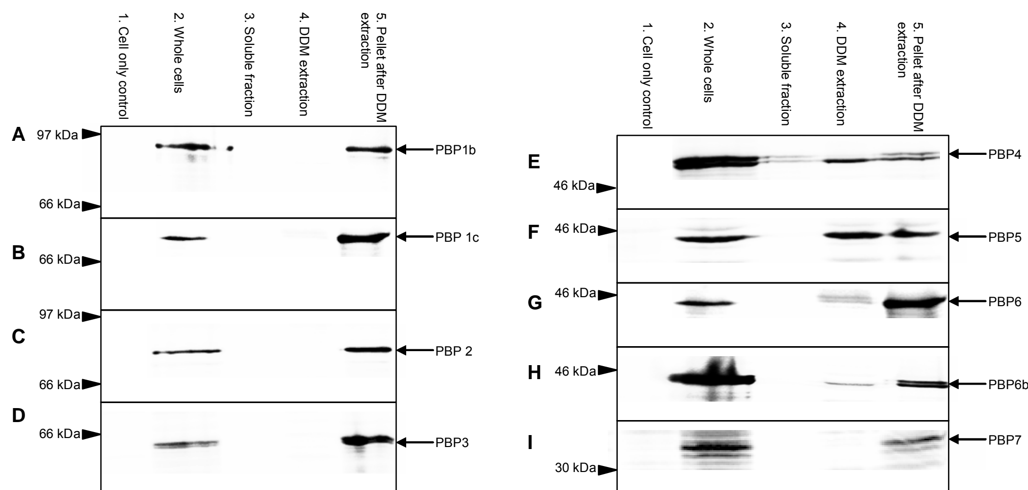

Figure 6. Solubility in DDM for the penicillin-binding proteins. In each figure lane nr 1 is a control, i.e. cells with no construct, lane nr 2 proteins from whole cells dissolved in Laemmli-buffer, lane nr 3 proteins from lysed cells (water-soluble fraction), lane nr 4 from membranes dissolved in 0,5 % DDM, and lane nr 5 the remaining crude membrane pellet dissolved in Laemmli-buffer.

To determine if the PBP's were compatible with our protein interaction assay, we tested their solubility in the mild detergent, DDM. PBP4, 5, and 6 could all be solubilised in 0,5 % (w/v) DDM (figure 6E, F, and G, lane 4), but PBP6b and 7 solubilised poorly (fig. 6H and I, lane 4). PBP4 was already in the soluble fraction after the cells had been lysed, indicating that is not attached to the inner membrane with the same strength as the other LMW PBP's. None of the HMW PBP's solubilised in DDM at any concentration (figure 6A, B, C, and D, lane 4).

PBP4, PBP5, and PBP6 form dimers

Whole cells containing radio-labelled PBP4, 5, and 6 were dissolved with DDM, and protein complexes separated by BN-PAGE. We detected bands corresponding to the pulsed proteins, which indicated that they formed homo-dimers (i.e. by the apparent sizes of the bands) (figure 7 A-C, lane 2).

As a further control we denaturated the pulse-labelled proteins by adding 2 % SDS to half of the sample before performing the BN-PAGE (figure 7 A-C, lane 3). In this way we could compare the monomeric states of the proteins with their oligomeric states. As can be seen from these experiments, we found that PBP4, PBP5, and PBP6 all formed dimers. For PBP7 we could not detect any higher oligomers than its monomeric state (data not shown). We cannot say whether this is due to the detergent used, its poor expression level, or because it does not form any higher oligomers.

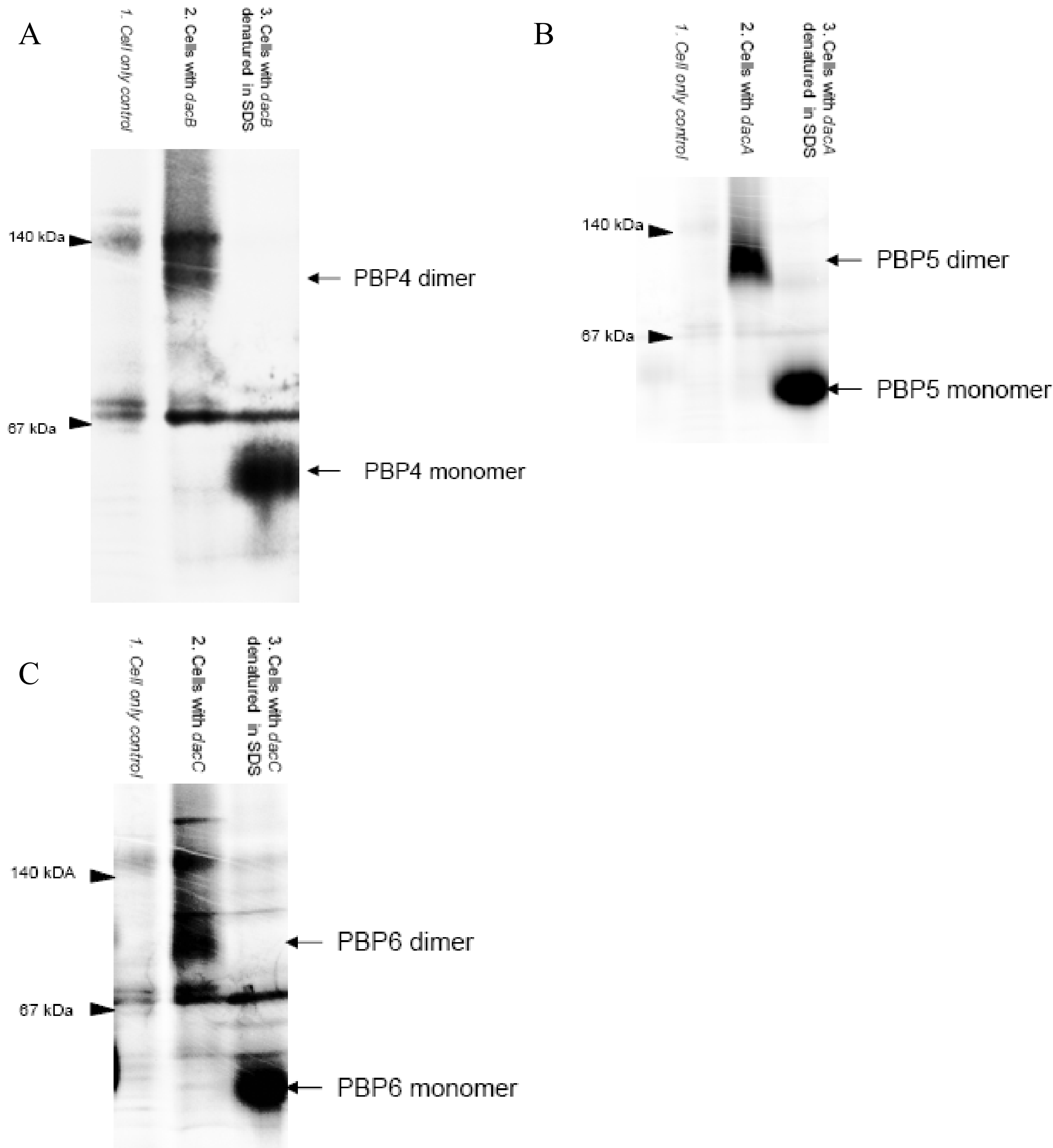

Figure 7. Oligomerization of the LMW PBP's. The pulse-labelled proteins were solubilised in 0,5 % DDM and analysed by BN-PAGE to detect their oligomeric states (i.e. by looking at the apparent size of the specific bands (lane 2 in each figure)). As a control we used cells without any constructs (lane 1) and proteins where we denatured the proteins by adding 2 % SDS to the sample before the BN-PAGE (lane 3). From these experiments we concluded that PBP4, 5, and 6 dimerize but PBP7 does not (data not shown).

All HMW PBP's form dimers

As we could not solubilise the HMW PBP's in DDM we performed a protein interaction assay previously used for characterising the dimeric forms of PBP1a, and PBP1b (Charpentier *et al.*, 2002). This interaction assay is based on SDS-PAGE but uses a non-denaturing loading-buffer for the proteins (see materials and methods). We performed this assay for all PBP's (figure 8) and found specific bands corresponding to higher oligomers for PBP1b, 1c, 2, and 3 as well as their monomeric state. However for the LMW PBP's where we previously had characterized dimeric states in the mild detergent DDM, we could only detect their monomeric states (data not shown).

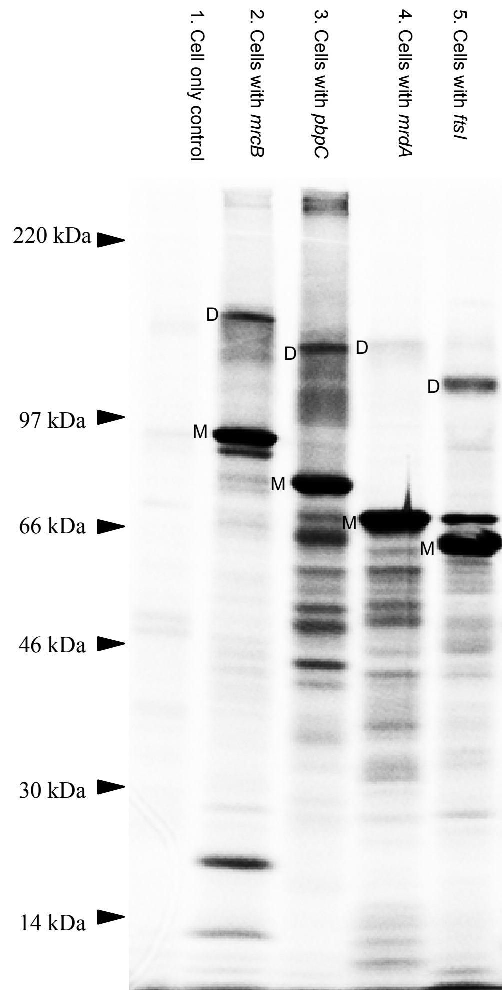

Figure 8. Dimerization of PBP1b, 1c, 2 and 3. By performing a non-denaturating SDS-PAGE of radio labelled proteins in whole cells, we could detect the oligomeric states of PBP1b, 1c, 2 and 3. As can be seen all HMW PBP's form dimers in addition to monomers. M indicates monomers, D indicates dimers.

By looking at the apparent size of the specific bands we could conclude that all HMW PBP's form dimers. To verify that the specific bands corresponded to a dimeric state we performed an additional SDS-PAGE (Charpentier *et al.*, 2002) with some modifications. We prepared the proteins as previously, but divided the sample in two, of which one was boiled at 100 ˚C for 5 minutes which previously had shown to break up the dimers of PBP1a, and PBP1b (Charpentier *et al.*, 2002). The SDS-PAGE revealed that the band corresponding to the dimeric state disappeared upon boiling but not the band corresponding to the monomeric state (figure 9). From these experiments we conclude that PBP1b, 1c, 2, and 3 form dimers.

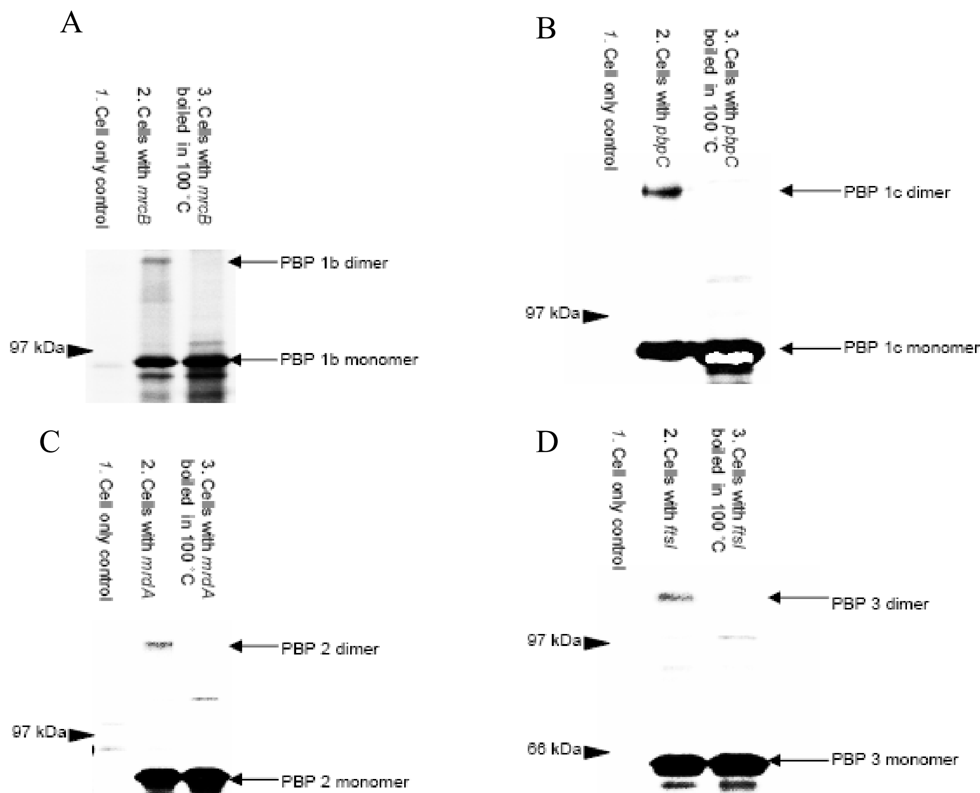

Figure 9. HMW PBP dimers disappear upon boiling. To verify that the specific bands from the non-denaturating SDS-PAGE corresponded to dimers we performed a similar SDS-PAGE, but boiled half of the samples for 5 minutes to disrupt the dimer (lane three in all figures). As can be seen, boiling of the samples breaks up the dimer. Lane one is cells without any constructs, lane two unboiled samples and lane three boiled samples.

The membrane anchor of PBP5 is necessary for dimerization

PBP5 is the most well studied of the LMW PBP's, and several studies have shown the importance of its membrane anchor. One study performed by Young and co-workers showed

that single point mutations in the membrane anchor could disrupt the functionality of the whole protein (Nelson *et al.*, 2002). In addition, a soluble form of PBP5, lacking the membrane anchor has been crystallized and the structure showed that it crystallized as a monomer (Nicholas *et al.*, 2003). From these observations, we hypothesized that the membrane anchor could be the site for dimerization.

We created 8 mutations to the C-terminal membrane anchor of PBP5: *(G384L), (G384W), (G384LG388L), (F387L), (F387D), (I394D), (H400A),* and *dacA(Δ383-403)* by site directed mutagenesis

The point mutations were the same as Young and colleagues previously had shown caused a dysfunctional PBP5 (Nelson *et al.*, 2002). In addition we made point mutations within a conserved GxxxG motif found in the membrane anchor. The GxxxG motif is a well characterized binding motif for both dimerization and helix-helix interactions (Curran *et al.*, 2003, Kleiger *et al.*, 2002, Lemmon *et al.*, 1992, MacKenzie *et al.*, 1998, Senes *et al.*, 2004, Walters *et al.*, 2006).

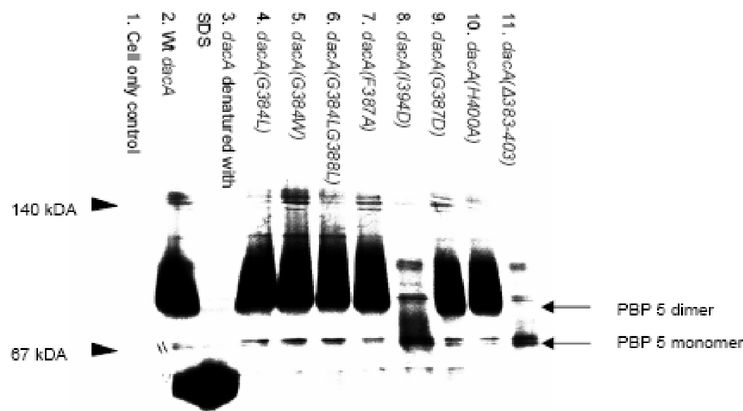

Figure 10. The membrane anchor of PBP5 is necessary for dimerization. By site-directed mutagenesis we created 7 different point mutations in the membrane anchor of PBP5, as well as one mutant where the whole membrane anchor was removed. By performing a BN-PAGE protein interaction assay, we could detect that the mutant lacking the membrane anchor did not dimerise (lane 11) as well as the *(I394D)* mutant (lane 8). Lane one corresponds to cells with no construct, lane two to wt PBP5 , lane three to PBP5 with 2 % SDS, lane four to PBP5(G384L), lane five to PBP5(G384W), lane six to PBP5(G384L/G388L), lane seven to PBP5(G387L), lane eight to PBP5(G387D), lane nine to PBP5(I294D), lane ten to PBP5(H400A), and lane eleven to PBP5(Δ383-403).

We performed the BN-PAGE protein interaction assay for all mutants of PBP5. It was evident that the membrane anchor indeed is necessary for dimerization (figure 10, lane 11) but the GxxxG motif is not involved in the dimerization (figure 10, lane 4-6). However, one of the point mutations (*I394D)* made previously by Young and co-workers, which had shown to affect the activity of PBP5, also disrupted the dimer.

To verify that all mutants were attached to the membrane, we performed a similar solubilisation test as described previously (fig. 11). All PBP5 mutants could be detected in the membrane fraction (fig. 11, lanes 4 and 5). In addition we found that *dacA(Δ383-403)* and *dacA(I394D)* also could be detected in the water-soluble fraction (fig. 11, lane 3). None of the other mutants could be detected in this fraction. This indicates that *dacA(Δ383-403)* and *dacA(I394D)* do not bind as effectively to the membrane as the other mutants.

From these experiments we conclude that the membrane anchor as well as localisation of PBP5 to the membrane is necessary for dimerization. However, we have not been able to fully characterize the interaction motif and further studies are needed.

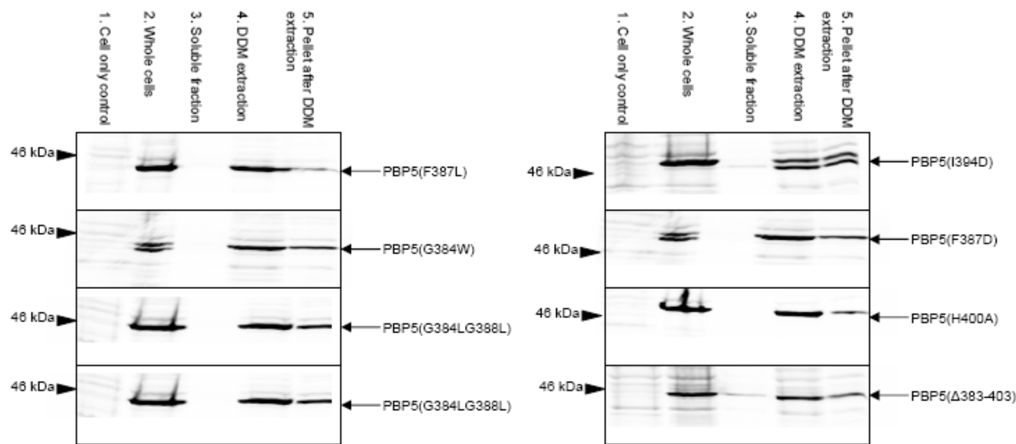

Figure 11. Solubility test for *dacA* mutants. We performed a solubility test as previously described for the PBP mutants. As can be seen in this figure all *dacA* mutants solved well in 0,5 % DDM. The *(I394D)* and *(Δ383-403)* mutants was also detected in the water-soluble fraction, indicating that they had a weaker attachment to the membrane than the other PBP5 mutants.

Discussion

The penicillin binding proteins have been studied for decades, as well as the effect that penicillin and its derivatives have on them (Georgopapadakou *et al.*, 1980, Macheboeuf *et al.*, 2006). As they are highly specific for bacteria and crucial for bacterial survival they are considered as perfect drug targets. However, due to an increasing resistance among virulent bacteria to penicillin, finding new ways to disrupt their function is important.

An emerging field within drug development are the so-called peptide inhibitors (Arkin *et al.*, 2004, Killian *et al.*, 2006). They work by binding to surfaces and sequence motifs in proteins that are important for interactions and thereby inhibiting interactions either in multi protein complexes or in dynamic interactions crucial for cell signalling (Blundell *et al.*, 2006, Curran *et al.*, 2003).

To effectively design peptide inhibitors for the PBP's, we need a fundamental understanding of how they interact with each other (i.e. their binding motifs), and if they exist in stable complexes or interact briefly upon stimulation (Blundell *et al.*, 2006). However, even though extensively studied, a full scale investigation of their internal interactions has never been done. In this study we add a few more pieces to the puzzle by showing that most of the PBP's form dimers. Our results also confirm the previous results showing that PBP1b, and PBP3 dimerize (Bertsche *et al.*, 2005, Chalut *et al.*, 1999, Charpentier *et al.*, 2002, Karimova *et al.*, 2005). They also confirm our previous study which indicated that PBP5 and PBP6 dimerized (Hellberg *et al.*, 2006).

The functional relevance of dimerization has been shown previously for PBP1b (Bertsche *et al.*, 2005). By disrupting the dimer interface the protein lost up to 50 % of its function. In addition, dimerization in several proteins from other families including the GPCR's and GlycophorinA, have been shown to have functional relevance (Bai *et al.*, 2004, Breitwieser *et al.*, 2004, Terillon *et al.*, 2004, Lutkenhaus *et al.*, 2003, MacKenzie *et al.*, 1997).

For studying the functional relevance of dimerization, a knowledge about the binding motif is necessary. As a start for a full scale characterization of the dimerization motifs we chose to use PBP5 as our model protein. PBP5 is the most well studied LMW PBP (Amanuma *et al.*, 1980, Begg *et al.*, 1995, Brandenburg *et al.*, 2002, Ghosh *et al.*, 2003, Gittins *et al.*, 1994, Harris *et al.*, 1995, Harris *et al.*, Harris *et al.*, 1997, Harris *et al.*, 1998, Harris *et al.*, 2002, Korsak *et al.*, 2005, Matsuhashi *et al.*, 1979, Morlot *et al.*, 2004, Nelson *et al.*, 2000, Nelson *et al.*, 2001, Nelson *et al.*, 2002, Nicholas *et al.*, 2003, Nishimura *et al.*, 1980, Phoenix *et al.*, 1993, Pratt *et al.*, 1986, Siligardi *et al.*, 1997, Spratt *et al.*, 1976, Tamura

et al., 1976, van der Linden *et al.*, 1993, van der Linden *et al.*, 1994, Varma *et al.*, 2004) with a well characterized structure. We hypothesized that the motif would be in the membrane anchor as the crystal structure of PBP5, lacking the membrane anchor, did not crystallize as a dimer (Nicholas *et al.*, 2003).

The 21 amino acid residue membrane anchor has been studied extensively and is thought to attach to the membrane at an oblique angle (Brandenburg *et al.*, 2002, Gittins *et al.*, 1994, Harris *et al.*, 1995, Harris *et al.*, 1997, Harris *et al.*, 1998, Harris *et al.*, 2002, Phoenix *et al.*, 1993, Pratt *et al.*, 1986, Siligardi *et al.*, 1997, van der Linden *et al.*, 1993). In addition the membrane anchor has been shown to be relevant for correct function of PBP5 (Nelson *et al.*, 2001). Even single point mutations within the anchor have been shown to disrupt the function, suggesting that PBP5 interacts with other proteins (Nelson *et al.*, 2002).

Interestingly, the membrane anchor of PBP5 contains a GxxxG motif: a common motif for dimerization and helix-helix interactions (Curran *et al.*, 2003, Kleiger *et al.*, 2002, MacKenzie *et al.*, 1998, Senes *et al.*, 2004, Walters *et al.*, 2006). The GxxxG motif is also well conserved in the membrane anchor of PBP5 in gram negative bacteria (table 4). However, our study showed that the GxxxG motif is not relevant for dimerization. We could however show that the membrane anchor is necessary for dimerization. In addition we found that one of the point mutations (*dacA(I394D)*) previously shown to be important for function (Nelson *et al.*, 2002), disrupted the dimer, suggesting a functional relevance of dimerization.

As the membrane anchor is an amphipathic helix (Siligardi *et al.*, 1997) we created a hydrophobic wheel to study the individual residues (fig. 12). It showed that I394 is situated on the hydrophobic side. Interestingly, all residues shown to have implications for PBP5 function (Nelson *et al.*, 2002) are situated on the hydrophobic side except H400, which sits on the border between the hydrophobic and hydrophilic side of the helix.

Table 4. Multiple sequence alignment with PIR Multiple alignment (ClustalW v. 1.82), of the putative C-terminal membrane anchor of PBP5 from different gram negative bacteria. In *E. coli* the sequence begins with the residue E383. As can be seen the whole membrane anchor is a well conserved sequence.

Escherichia coli	EGNFFGKIIDYIKLMFHHWFG
Salmonella typhimurium	EGNFFGKIIDYIKLMFHHWFG
Shigella boydi	EGNFFGKIIDYIKLMFHHWFG
Photorhabdus luminescens	EGSIFGRFIDYIKLLFHHWFG
Yersinia pestis	EGGFFSRMVDYIKLMFHRWFG
Shewanella oneidensi	EGSWFSKLVDYFKQLFSGWFS
Pseudomonas putida	EGGFFRRMWDSIRLFFYGLFN
Haemophilus influenzae	EAGIFGKLWDWLVLTVKGLFS

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As only the *(Δ383-403)* and *(I394D)* mutants had implications for dimerization a question that remains unanswered is what function the other *dacA* mutants, showing phenotypes (Nelson *et al.*, 2002), play for creating a dysfunctional PBP5. Is there another interaction between the membrane anchor of PBP5 and an unknown protein as previously suggested (Nelson *et al.*, 2002, Varma *et al.*, 2004). Several studies have shown that PBP5 crosstalk with the cell division machinery. One study showed that deletion of PBP5 could reverse a thermo sensitive *ftsK* mutant (Begg *et al.,* 1995), and another study showed that over expression of *dacA* could reverse a thermo sensitive *ftsI23* mutant (Begg *et al.,* 1990). In addition, deletion of *dacA* also gives a more pronounced phenotype in a thermo sensitive *ftsZ84* mutant (Varma *et al.,* 2004). These observations points towards the existence of an unknown interaction between PBP5 and a protein involved in cell division, which could be mediated through the membrane anchor.

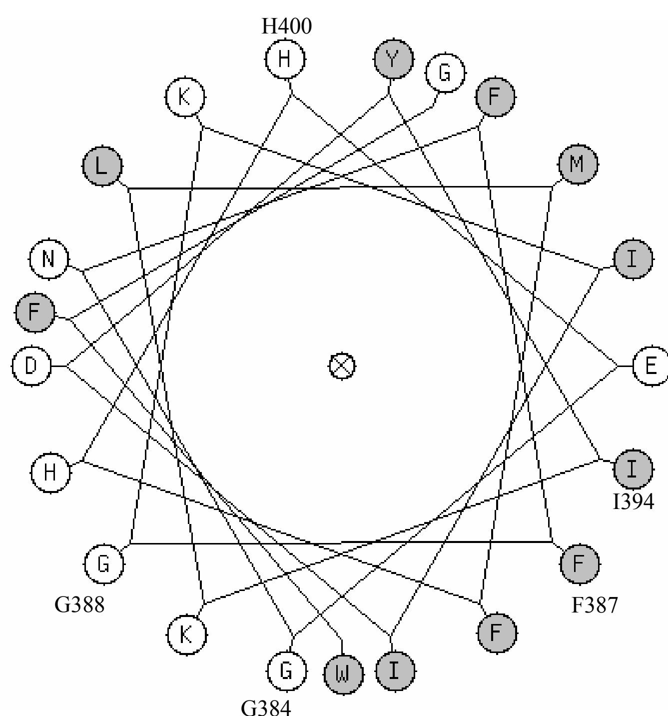

Figure 12. Helical wheel of the putative 21 amino acid membrane anchor of PBP5. We created a helical wheel with the Interactive Java helical wheel program (http://kael.net/helical.htm) of the terminal 21 amino acids (residue 383-403) of PBP5. The hydrophobic residues are shaded and as can be seen it is an amphipathic helix with a clear distinction between the hydrophobic and hydrophilic side. The residues that were mutated in our study are highlighted with their numbers. Interestingly, the residues that have implications for function (F387, I394, and H400) are situated on the border to, or in the hydrophobic side of the helix.

Interestingly, the membrane anchor of the LMW PBP's have shown to have different functions. In a previous study creating fusion proteins (Nelson 2002), exchanging the membrane anchor of PBP5 with the membrane anchor of PBP6 kept the function of PBP5 intact. However, if the membrane anchor of PBP4 or PBP6b was fused with PBP5, its function was lost. In addition, if another well characterized membrane anchor from a different

protein family was fused with PBP5 it also lost its function. This indicates that the LMW PBP's bind to the membrane in different ways or dimerize differently. Another possibility could be that the homologous membrane anchors of PBP5 and PBP6 interact with other proteins than PBP6b and PBP4, through their membrane anchor.

Apart from the common theme of dimerization among the PBP's our study also increases the complexity of the network that make up the peptidoglycan synthesising machinery. In addition to the already known interactions between PBP1b and PBP3 (Bertsche *et al.*, 2006) and PBP3 and the cell division proteins (Karimova *et al.*, 2005), future studies of the macro-molecular machines synthesizing the peptidoglycan, now need to take into account the interactions between PBP dimers.

As the dimerization is a very well conserved structural motif among the PBP's with a documented functional relevance in PBP1b (Bertsche *et al.*, 2005), studying the binding motifs as well as the functional relevance of dimerization for the other PBP's will be an interesting next step. These conserved structural features might be an interesting drug target for peptide inhibitors in the future.

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