The Penicillin-Binding Proteins: Structure and Role in Peptidoglycan Biosynthesis

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Abstract

Penicillin-binding proteins (PBPs) have been scrutinized for over 40 years. Recent structural information on PBPs together with the ongoing long-term biochemical experimental investigations, and results from more recent techniques such as protein localization by GFP-fusion immunofluorescence or double-hybrid assay, have brought our understanding of the last stages of the peptidoglycan biosynthesis to an outstanding level that allows a broad outlook on the properties of these enzymes. Details are emerging regarding the interaction between the peptidoglycan-synthesizing PBPs and the peptidoglycan, their mesh net-like product that surrounds and protects bacteria. This review focuses on the detailed structure of PBPs and their implication in peptidoglycan synthesis, maturation and recycling. An overview of the content in PBPs of some bacteria is provided with an emphasis on comparing the biochemical properties of homologous PBPs (orthologues) belonging to different bacteria.

1 # Introduction

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3 The bacterial peptidoglycan not only enables the bacteria to resist the intracellular pressure
4 of several atmospheres that exists in the cell but also provides the bacterium with a well defined
5 cell-shape that is reproduced from generation to generation. The peptidoglycan is made of glycan
6 chains of alternating N-acetylglucosamine and N-acetylmuramic acid cross-linked by short stem
7 peptides attached to the N-acetylmuramic acid (Ghuysen, 1968, Schleifer & Kandler, 1972). PBPs
8 catalyze the polymerization of the glycan strand (transglycosylation) and the cross-linking between
9 glycan chains (transpeptidation). Some PBPs can hydrolyze the last D-alanine of the stem peptide
10 (DD-carboxypeptidation) or hydrolyze the peptide bond connecting two glycan strands
11 (endopeptidation). Endopeptidation and transpeptidation are reverse activities. Because of the
12 structural resemblance between their natural substrate, the D-Ala-D-Ala end of the stem peptides,
13 and penicillin, the late stage peptidoglycan synthesizing enzymes are sensitive to penicillin with
14 which they form a long-lived acyl-enzyme that impairs their peptidoglycan cross-linking capability.

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17 # Classification and overview of the content in PBPs of selected bacteria

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19 Bacteria possess a variable number of PBPs (figure 1). Since the sequencing of many
20 bacterial genomes has been achieved, the number of PBPs of each bacterium is well determined.
21 PBPs have been divided into two main categories, the high molecular mass (HMM) PBPs and the
22 low molecular mass (LMM) PBPs. HMM PBPs are multimodular penicillin-binding proteins
23 responsible for peptidoglycan polymerization and insertion into preexisting cell wall (Goffin &
24 Ghuysen, 1998). Their topology consists of a cytoplasmic tail, a transmembrane anchor, and
25 essentially two domains joined by a β-rich linker located in the outer surface of the cytoplasmic
26 membrane where peptidoglycan synthesis takes place (Goffin & Ghuysen, 1998, Macheboeuf, *et*

al., 2006, Lovering*, et al.*, 2007). Depending on the structure and the catalytic activity of their N-terminal domain, they belong either to class A or class B PBPs. The C-terminal penicillin-binding domain of both classes has a transpeptidase (TP) activity catalyzing peptide cross-linking between two adjacent glycan chains. In class A, the N-terminal domain is responsible for their glycosyltransferase activity, catalyzing the elongation of uncross-linked glycan chains. In class B, the N-terminal domain is believed to play a role in cell morphogenesis by interacting with other proteins involved the cell cycle (Holtje, 1998). Monofunctional enzymes (MGTs) similar to the glycosyltransferase (GT) domain of class A PBPs (A-PBPs) also exist in some bacteria but their exact role is still unknown (Spratt*, et al.*, 1996).

LMM PBPs are sometimes referred to as LMM PBPs of class A, B and C. As a whole LMM PBPs are frequently described with the general term of class C PBPs, sometimes with C1, C2 and C3 as subdivisions. We will use four subcategories and will refer to their main PBP representative in *Escherichia coli*, i.e. type-4 for PBPs similar to *E. coli* PBP4, type-5 for enzymes similar to *E. coli* PBP5, type-7 for PBPs similar to *E. coli* PBP7 and type-AmpH for PBPs similar to *E. coli* AmpH. The numbering of PBPs is historically based on SDS-PAGE migration and this may lead to some confusion. For example *Staphylococcus aureus* PBP2 is a class A PBP similar to *E. coli* PBP1a, *S. aureus* PBP3 is similar to *E. coli* PBP2 and *S. aureus* PBP1 is similar to *E. coli* PBP3. The complete set of PBPs of 10 important and widely studied bacteria, with their numbering and grouping in subclasses on the base of their sequence is provided in figure 1. The classification used herein is based on amino acid sequence alignment aided by the knowledge of structural features.

The twelve PBPs of *E. coli* have been the subject of numerous investigations. *E. coli* possesses three class A PBPs. PBP1a and PBP1b are the major transpeptidases-transglycosylases. Deletion of one of them is not lethal for the bacteria (Suzuki*, et al.*, 1978, Denome*, et al.*, 1999, Meberg*, et al.*, 2001). The role of PBP1c is not well understood. PBP1c is unaffected by most β-lactams and overexpression of PBP1c does not suppress the autolysis phenotype of a mutant lacking both PBP1a and PBP1b(Schiffer & Holtje, 1999). The two class B PBPs of *E. coli* are

monofunctional transpeptidases. PBP2 is involved in the elongasome, a putative complex specific to cell elongation while PBP3 is a major protein of the divisome, the cell division complex, (these complexes are dealt with in the chapter "Morphogenesis of the rod shaped sacculus" by den Blaauwen *et al.*).

The seven LMM PBPs of *E. coli* are involved in cell separation, peptidoglycan maturation or recycling. PBP4 and PBP7 are two endopeptidases that cleave cross-bridges between two glycan strands. They probably should be considered as hydrolase members of the autolysins pool of *E. coli* (Vollmer & Holtje, 2004). PBP5 is the major carboxypeptidase, i.e. the most abundant, and it only cleaves the terminal D-Ala-D-Ala bond, making the stem peptide unavailable for transpeptidation. PBP6 and PBP6b both have sequences homologous to PBP5 and their activity is, like PBP5, that of a strict carboxypeptidase. PBP4b and AmpH both have a sequence close to the paradigmatic *Streptomyces* R61 DD-peptidase. The role of PBP4b is undetermined whereas AmpH is associated with peptidoglycan recycling.

The Gram-negative *Neisseria gonorrhoeae* has only four PBPs. PBP1 is analogous to *E. coli* PBP1a (Ropp*, et al.*, 2002) and PBP2 is homologous to *E. coli* PBP3 (Spratt & Cromie, 1988, Dowson*, et al.*, 1989, Zhang & Spratt, 1989). *Neisseria* incorporates new peptidoglycan through its divisome complex. The absence of elongasome is coherent with the coccoïd shape of *Neisseria*. *N. gonorrhoeae* PBP3 and PBP4 have a sequence similar to *E. coli* PBP4 and PBP7, respectively (Stefanova*, et al.*, 2003, Stefanova*, et al.*, 2004).

Bacillus subtilis is the model organism for sporulating Gram-positive bacteria. Most of its sixteen PBPs have been extensively studied for their role in vegetative peptidoglycan synthesis and in sporulation. *B. subtilis* has four class A PBPs. PBP1 is part of the cell division machinery and is required for the efficient formation of the asymmetric sporulation septum (Scheffers & Errington, 2004). Among the six class B PBPs, PBP2b is the cell division specific class B transpeptidase (Daniel*, et al.*, 2000). PBP4a is the equivalent of *E. coli* PBP4. PBP5 is the major carboxypeptidase. Two other carboxypeptidases similar to PBP5 are present in *B. subtilis*, PBP5* and dacF. PBP5* is

1 required for proper spore cortex synthesis (Popham*, et al.*, 1995) and dacF regulates the degree of
2 cross-linking of spore peptidoglycan (Popham*, et al.*, 1999). PBP4* and PBPX, two class C PBPs
3 of type-AmpH, are supposed to be involved somehow in sporulation (Scheffers, 2005). Of note is
4 the absence of type-7 PBP in *B. subtilis*.
5 *Staphylococcus aureus* is a Gram-positive coccus. It incorporates peptidoglycan at the
6 division site and has no elongasome. Consistantly its unique class A PBP localizes to the septum.
7 Strains of *S. aureus* susceptible to β-lactam antibiotics have two class B PBPs (Pinho*, et al.*, 2000,
8 Pereira*, et al.*, 2007) but resistant strains have acquired an additional PBP, PBP2a, with a low
9 sensitivity to β-lactams. *S. aureus* has only one LMM PBP, which is of type-5 but unlike *E. coli*
10 PBP5, *S. aureus* PBP4 has a transpeptidase activity necessary to achieve the high degree of cross-
11 linkage observed in the peptidoglycan of staphylococci (Wyke*, et al.*, 1981).
12 *Listeria monocytogenes* has six PBPs (Guinane*, et al.*, 2006), including two class A PBPs (PBP1
13 and PBP4), three class B PBPs (PBP2, PBP3 and *lmo0441*), and one class C PBP of type-5 (PBP5)
14 (Vicente*, et al.*, 1990, Korsak*, et al.*, 2005, Zawadzka-Skomial*, et al.*, 2006). Their respective role
15 is not well known. Increased expression of PBP4 together with a histidine kinase was associated
16 with resistance to nisin (Gravesen*, et al.*, 2001, Gravesen*, et al.*, 2004), an antibacterial peptide
17 which exerts its action by forming pores in the cytoplasmic membrane through interaction with the
18 PG precursor lipid II (Brotz*, et al.*, 1998).
19 *Enterococcus faecalis* has three class A and three class B PBPs (Arbeloa*, et al.*, 2004).
20 PBP4 is responsible for the resistance of *E. faecalis* to β-lactam antibiotics (Duez*, et al.*, 2001). As
21 in most Gram-positive cocci, enterococci have one type-5 LMM PBP (el Kharroubi*, et al.*, 1989).
22 *E. faecalis* also possesses a type-AmpH PBP, similar to the PBPX of *B. subtilis.*
23 *Streptococcus pneumoniae* has three class A PBPs (Hoskins*, et al.*, 1999), two class B PBPs
24 and a type-5 class C PBP (Morlot*, et al.*, 2005). Unlike *S. aureus*, *E. faecalis* or *L. monocytogenes*,
25 *S. pneumoniae* doesn't have a penicillin-resistant PBP resembling *S. aureus* PBP2a and the

1 resistance to β-lactams in *S. pneumoniae* arises from the alteration of the sequence and structure of

2 its PBPs (Hakenbeck, 2000, Hotomi*, et al.*, 2006).

3 Probably because of their complex life cycle and their ability to produce β-lactam molecules

4 (e.g. *S. clavuligerus* produces cephamycin and clavulanic acid (Ward & Hodgson, 1993)) the

5 Actinomycetales of the *Streptomyces* genera have a great number of PBPs that can be expressed at

6 the different stages of the bacterial development or when the bacterium effectively produces β-

7 lactam metabolites that can interfere with peptidoglycan biosynthesis. *S. coelicolor* has 21 PBPs:

8 three class A, nine class B, and nine class C PBPs. The time schedule of their expression is

9 unknown.

10 *Mycobacterium tuberculosis* produces 2 class A PBPs, two class B PBPs and a lipoprotein

11 sharing some motifs with the class B PBPs. It has also one type-4 PBP, one type-5 PBP and one

12 type-7 PBP. Three type-AmpH putative PBPs complete the set of PBPs of *M. tuberculosis*.

13 The cyanobacterium *Anabaena sp*. PCC7120 has twelve PBPs: six class A PBPs, two class

14 B PBPs, two type-4 PBPs and two type-AmpH PBPs (Lazaro, et al., 2001, Leganes, et al., 2005).

15 Strikingly, *A. sp*. is devoid of type-5 (and type-7) PBP a property shared by all cyanobacteria

16 analysed by Leganes *et al*.

17

The penicillin binding domain and the DD-peptidase activity

PBPs share a common DD-peptidase activity, whether a DD-transpeptidase, a DD-carboxypeptidase or a DD-endopeptidase activity (figure 2). The carboxypeptidation and transpeptidation reactions catalyzed by PBPs follow a three-step mechanism: the rapid reversible formation of a non covalent Henri-Michaelis complex between the enzyme and a peptidoglycan stem peptide, called the donor strand, is followed by the attack of the active serine on the carbonyl carbon atom of the C-terminal D-Ala-D-Ala peptide bond, leading to the formation of an acyl-enzyme intermediate and the concomitant release of the C-terminal D-Ala (acylation). The final step (deacylation) consists in either hydrolysis, with release of the shortened peptidoglycan strand (carboxypeptidation), or cross-link formation with a second peptidoglycan stem peptide called the acceptor strand (transpeptidation). The DD-endopeptidase activity of PBPs consists in the hydrolysis of the cross-bridge resulting from the DD-transpeptidase activity. The endopeptidation is in fact a carboxypeptidation as it removes the last peptide of the stem pentapeptide. Carboxypeptidation is the term used for the removal by a PBP of the last D-alanine of the stem peptide and endopeptidation for the hydrolysis of the cross-bridge between two peptidoglycan strands.

The DD-peptidase activity of PBPs is materialized by a common penicillin-binding (PB) domain, which binds β-lactam antibiotics (figure 2). The latter are aimed to inhibit the enzymatic DD-peptidase activity of the PB domain playing on the structural similarity between penicillin and the D-Ala-D-Ala dipeptide that ends the natural substrate of PBPs, the disaccharide pentapeptide. The PB domain of PBPs is made of two subdomains, a β-sheet covered by three α helices and an all helical domain. The active site lies at the interface of the two subdomains (figure 3A). There may be some flexibility between these two subdomains, and this can influence the binding capability of some PBPs towards various ligands (Lim & Strynadka, 2002). The active site encompasses nine residues broadly conserved in PBPs. The active serine is positioned at the beginning of helix α2 and

1 is followed by a lysine to form a S*xxK motif. A second motif, SxN, is situated in a loop between
2 helices α4 and α5. Four conserved residues form the third motif KTG(T/S). A ninth residue, a
3 glycine situated in the rear of the active site, is also strictly conserved (figure 3A).

4 Structural information regarding the interaction of PBPs with their substrate mainly comes
5 from structures of LMM PBPs in complex with substrates that mimic the natural substrate.
6 McDonough *et al.* obtained two high resolution structures with the DD-peptidase of *Streptomyces*
7 R61, one showing the non-covalent binding of the two products of the carboxypeptidase reaction
8 and the other showing the enzyme-substrate Henri-Michaelis complex resulting from the utilization
9 of an inactivated enzyme (McDonough*, et al.*, 2002). Nicola *et al.* reported a 1.6 Å resolution
10 structure of *E. coli* PBP5 in complex with a substrate-like peptide boronic acid. The boronyl group
11 mimics the transition state of a PBP-catalyzed deacylation reaction (Nicola*, et al.*, 2005). We
12 recently reported on the structure of *B. subtilis* PBP4a with α-aminopimelyl-ε-D-alanyl-D-alanine,
13 a peptide that mimics the peptidoglycan ending tripeptide of *Bacillus* (Sauvage*, et al.*, 2007). This
14 structure shows the acyl-enzyme α-aminopimelyl-ε-D-alanyl-PBP4a and an unbound D-alanine, the
15 result of the PBP-catalyzed acylation reaction. From these structures, it emerges that the
16 penultimate D-alanine of the donor stem peptide fits tightly in the active site with its amide group
17 wedged between the side chain of the asparagine of the second motif and the backbone of the β3
18 strand that lines the active site (figure 3B). The carbonyl oxygen of the penultimate D-alanine lies
19 in the oxyanion hole and its methyl group is inserted into a hydrophobic pocket, underlining the
20 importance of the conserved glycine at the rear of the active site. The carboxylate of the leaving D-
21 alanine is oriented towards the two hydroxyl groups of the third motif. In brief, from the nine
22 conserved residues, the asparagine of the second motif and both hydroxyl group of the third motif
23 are important for the correct positioning of the substrate. The glycine of the third motif is needed to
24 avoid sterical hindrance as a bulkier residue would block the entry of the active site to an incoming
25 substrate. The glycine in the rear of the active site is important for the binding specifity of PBPs

towards the penultimate D-alanine of the peptidoglycan stem peptide (Adediran, *et al.*, 2005). Finally, both serines and both lysines are important for the catalytic mechanism.

Transpeptidase mechanism

The acylation of PBPs by the penultimate D-alanine of the donor strand, with the concomitant release of the last D-alanine, requires the abstraction of a proton from the active serine. The lysine of the first motif, assumed deprotonated, can perform the withdrawal of the proton (Gordon, *et al.*, 2000, Lim & Strynadka, 2002, Nicola, *et al.*, 2005, Macheboeuf, *et al.*, 2006, Sauvage, *et al.*, 2007). The proton can subsequently be back-donated to the leaving D-alanine amine group *via* the serine of the second motif. Alternatively, the direct transfer of a proton from the active serine to the serine of the second motif concomitant to the transfer of a proton from the latter to the amine group of the leaving D-alanine in a one step process may be considered (Dive & Dehareng, 1999).

In the deacylation step, a proton of the acceptor (an amino group in transpeptidation, water in carboxypeptidation) has to be abstracted to allow the activated group to attack the carbonyl carbon atom of the ester bond of the acyl-enzyme (Ghuysen, 1991, Goffin & Ghuysen, 1998). The proton can subsequently be back-donated to the active serine. Withdrawal of a proton from the acceptor group can be performed by the deprotonated serine of the second motif with the assistance of the lysine of the third motif (Nicola, *et al.*, 2005).

The active site of PBPs can be seen as a double lysine-serine system, one for acylation and one for deacylation, although both systems are not independent. Simple catalytic hydroxyl/amine dyads (Lys-Ser) are frequently encountered in proteins (Paetzel & Dalbey, 1997, Lee, *et al.*, 2007). Withdrawal of a proton from the serine by the deprotonated lysine might be a too simplistic view. The proton must lie somewhere between the hydroxyl oxygen atom of the serine and the nitrogen atom of the lysine, and the resulting orientation of the lone pairs of the oxygen should be crucial for

1 the catalytic mechanism. Quantum level calculation on lysine-serine dyads is worth of
2 consideration.
3
4 *Interaction with β-lactams*

5 PBPs are to a variable degree sensitive to β-lactam antibiotics, with which they form a stable
6 acyl-enzyme. The acylation rates of benzylpenicillin with PBPs range from about 20 $M^{-1}s^{-1}$ for the
7 penicillin-resistant class B PBPs (subclass B1) (Zorzi*, et al.*, 1996, Lu*, et al.*, 1999, Hujer*, et al.*,
8 2005) to about 300.000 $M^{-1}s^{-1}$ in the case of type-4 PBPs (Granier*, et al.*, 1992, Stefanova*, et al.*,
9 2003).

10 Crystallographers have been successful in trapping acyl-enzymes of PBPs and β-lactam antibiotics.
11 Most of the structures of PBPs have been solved in the apo form and in complex with β-lactams
12 (Kuzin*, et al.*, 1995, Gordon*, et al.*, 2000, Lim & Strynadka, 2002, Sauvage*, et al.*, 2002,
13 Macheboeuf*, et al.*, 2005, Nicola*, et al.*, 2005, Sauvage*, et al.*, 2005, Silvaggi*, et al.*, 2005, Kishida*,*
14 *et al.*, 2006). X-ray structures show that many antibiotics covalently linked to the active site serine
15 adopt a common standard positioning that shares some characteristics with the acyl-enzyme PBP4a-
16 α-aminopimelyl-ε-D-alanyl: (i) the amide group of the side chain is inserted between the asparagine
17 of the second motif and the backbone of β3 strand, (ii) the carboxylate associated to the thiazolidine
18 or to the dihydrothiazine ring of the antibiotic is hydrogen bonded to one or both hydroxyl groups
19 of the KTGT motif, (iii) the carbonyl oxygen lies in the oxyanion hole. The acyl-enzyme PBP2a-
20 benzylpenicillin (Lim & Strynadka, 2002) is a typical example of this "canonical" conformation
21 (figure 3C). The catalytic mechanism can follow the scheme described for PBPs with the D-Ala-D-
22 Ala end of the stem pentapeptide although a different mechanism can be at work.

Class A PBPs and MGTs

<u>Subclasses</u>

From sequence alignment, class A PBPs can be grouped in at least 7 subclasses (figure 1). As defined by Goffin & Ghuysen (Goffin & Ghuysen, 1998), subclasses A1 and A2 group Gram-negative PBPs, whereas subclasses A3, A4, and A5 form three clusters of Gram-positive PBPs. Subclass A6 contains *E. coli* PBP1c and *Anabaena sp*. PBP2. The three *S. coelicolor* class A PBPs are grouped with the penicillin-resistant *M. tuberculosis* PBP1a in subclass A7.

The presence of a class A PBP is necessary for cell growth in most but not all bacteria. Loss of *E. coli* PBP1a or PBP1b is tolerated but loss of both proteins is lethal even in the presence of PBP1c. Deletion of PBP1, the only class A PBP in *N. gonorrhoeae*, results in the loss of viability (Ropp*, et al.*, 2002). In *S. pneumoniae*, the three class A PBPs are dispensable individually but PBP1a or PBP2a is required for growth *in vitro* (Hoskins*, et al.*, 1999).

The absence of all its class A PBPs is not lethal to *B. subtilis*. A strain lacking all four class A PBPs is still viable and produces a peptidoglycan with only small structural differences from that of the wild type. The growth rate of the quadruple mutant is much lower than that of a strain lacking only three of the class A PBPs and wall abnormalities are more frequent (McPherson & Popham, 2003). PBP1 is mainly responsible for the morphological abnormalities (Popham & Setlow, 1995). A situation similar to the one observed in *B. subtilis* prevails in *E. faecalis*. Deletion of the three class A PBPs is not lethal although this leads to increase in generation time and decrease in peptidoglycan cross-linking. As there is no monofunctional glycosyltransferase in *E. faecalis*, the glycan chain polymerization in the triple mutant must be performed by a novel type of glycosyltransferase, that is not inhibited by moenomycin (Arbeloa*, et al.*, 2004).

In Gram-positive bacteria, the PBPs of subclass A3 are the major class A PBPs and are recruited to the septum site (Scheffers & Errington, 2004, Leski & Tomasz, 2005). Recruitment of

S. aureus PBP2 to the division site depends on its transpeptidation substrate (Pinho & Errington, 2005).

E. coli PBP1c is a penicillin-insensitive class A PBP. It binds only specific β-lactams and its transpeptidase activity could not be measured, which suggests that it may function *in vivo* as GT only. Interestingly, the *pbpB* gene coding for *Anabaena sp*. PBP2, a class A PBP with a sequence similar to *E. coli* PBP1c, is required for anaerobic nitrogen fixation in the cyanobacterium *Anabaena sp* strain PCC7120 (Lazaro*, et al.*, 2001).. A PBP similar to PBP1c exists only in heterocyst-forming filamentous cyanobacteria (Leganes*, et al.*, 2005). *Anabaena sp* PCC7120 has three class A PBPs similar to the three class A PBPs from *E. coli* and three additional class A PBPs similar to *E. coli* PBP1b (Lazaro*, et al.*, 2001).

Structural basis

In their attempt to determine the structure of a class A PBP, the group of Dessen in Grenoble crystallized both PBP1a and PBP1b of *S. pneumoniae* (Macheboeuf*, et al.*, 2005, Contreras-Martel*, et al.*, 2006). In the same time, the group of Strynadka also solved the structure of PBP1b of *S. pneumoniae* (Lovering*, et al.*, 2006). The structure of the transpeptidase domain associated with a linker domain could be determined but the structure of the transglycosylase domain remained undecephired. The determination of an X-ray structure of a class A PBP (*S. aureus* PBP2) with its transglycosylase domain was achieved recently by the group of Strynadka (Lovering*, et al.*, 2007). The general fold of class A PBPs is made of a N-terminal domain coupled to the C-terminal penicillin-binding domain. The interdomain linker is composed of a small β-sheet, and one α helix (figure 4A). A small number of class A PBPs, e.g. the (penicillin-resistant) PBP1 of *M. tuberculosis*, contain an additional domain made of one or two repeating units known as PASTA domains (Penicillin-binding protein And Serine/Threonine kinase Associated, because this domain is also found in the C-termini of serine/threonine kinases (Yeats*, et al.*, 2002)). The PASTA domain is a small globular domain consisting of three β strands and one α helix. An Fn3 (fibronectin type

III) domain can also be found at the C-terminus of some class A PBPs whereas a FHA (forkhead-associated) domain, consisting in a typical 11-stranded beta sandwich fold, may be found at the N-terminus. This observation suggests a more complex organization and other functions acquired by these PBPs. The significance and role of these domains are still unknown, but most often Fn3 domains are involved in some manner in cell surface binding and FHA domains are protein-protein interaction domains.

Transpeptidase domain

The global fold of the transpeptidase (TP) domain, as observed in PBP1a and PBP1b of *S. pneumoniae* and PBP2 of *S. aureus,* is identical to the general structure of the penicillin binding domaine described previously. However, both structures described for *S. pneumoniae* PBP1b show important conformational differences in the active site conformation of the apoenzyme. Macheboeuf *et al*. observed a "closed" active site in the absence of substrate or antibiotic (Macheboeuf*, et al.*, 2005). Residues following the KTGT motif on strand β3 move away from strand β4 and an asparagine situated in the loop connecting these two strands makes hydrogen bond contacts with the backbone of a loop situated on the other side of the active site, thus blocking the entry of the active site. When crystals of PBP1b are grown in the presence of substrate, then washed and back-soaked in the presence of nitrocefin or cefotaxime, the active site retains a "canonical" configuration and the antibiotic forms with the PBP1b a classical acyl-enzyme. Analysis of the structure solved by Strynadka *et al.* reveals an ''open'' active site which resembles the acyl-enzyme active-site topology and is more similar to the one observed in the penicillin binding domain of other PBPs (Lovering*, et al.*, 2006). It has been suggested that both observed apoenzyme structures may illustrate a conformational sampling of the ''open'' and ''closed'' forms that may play a regulatory role in PBP1b catalytic activity.

Transglycosylase domain

1 Despite the difficulties of finding well behaved glycosyltransferases (GT), the efforts
2 deployed by several laboratories led recently to the determination of two X-ray structures. The
3 bifunctional *S. aureus* PBP2 (lacking the cytoplasmic and transmembrane anchor) was solved by
4 Strynadka's group (Lovering*, et al.*, 2007) in apo form and in complex with moenomycin (figure
5 4B), and the isolated GT domain of *Aquifex aeolicus* PBP1a was solved by Walker's group (Yuan*,*
6 *et al.*, 2007). The two structures revealed that the GT domain contains almost only α-helices and its
7 fold resembles that of λ-lysozyme but differs from all known GT structures. Like lysozyme the GT
8 domain consists of a large and a small lobes separated by an extended cleft which contains the
9 active site. The two structures differ in the small lobe, which of the GT is mainly α-helical and
10 presents a hydrophobic region which probably mediates the interaction of the GT domain with the
11 membrane while that of λ-lysozyme is hydrophilic and contains a β-sheet. Sequence alignments
12 have revealed that all GT domains shared five conserved motifs. The first three motifs are found in
13 the cleft. Motif 1 (EDxxFxxHxG) and motif 3 (RKxxE) contain the first and second putative
14 catalytic glutamic acids respectively (figure4B). The essential glutamic acid in motif 1 of the GT
15 and the catalytic Glu19 of λ-lysozyme are located at the same position. Motif 2 which divides the
16 cleft in two pockets was proposed to be involved in substrate recognition. Motif 4 forms the back
17 wall of the cleft and motif 5 is located in the large lobe farther from the active site. They were both
18 suggested to play mainly structural role (Lovering*, et al.*, 2007). Comparison of the ligand-free and
19 moenomycin bound PBP2 structures shows that moenomycin induces conformational change in the
20 small subdomain and that some flexibility occurs around the linker region connecting the GT and
21 the TP domains.
22 Monofunctional enzymes (MGTs) similar to the GT domain of class A PBPs also exist in
23 some bacteria but their exact role is still unknown (Spratt*, et al.*, 1996). Both the GT domain of A-
24 PBPs and MGTs belong to GT51 family which is characterized by the five conserved motifs and
25 uses lipid II (undecaprenyl-P-P-MurNAc-(pentapeptide)-GlcNAc) as substrate. The MGTs
26 represent about 20 % of the sequences in the GT51 database (CAZy.org) showing that the majority

of the GT are bifunctional PBPs. This proportion reflects the distribution and the number of class A PBPs and MGT among bacteria.

Transglycosylation

In class A PBPs, the N-terminal domain is responsible for their glycosyltransferase (GT) activity, catalyzing the elongation of uncross-linked glycan chains of the peptidoglycan. Their GT domain uses undecaprenyl-P-P-MurNAc-(pentapeptide)-GlcNAc lipid II as substrate**.** In recent years, a substantial progress has been made in the synthesis of lipid II (either radioactive or labeled with different fluorescent probes) using enzymatic route (van Heijenoort*, et al.*, 1992, Breukink*, et al.*, 2003), chemoenzymatic synthesis (Ye*, et al.*, 2001, Schouten*, et al.*, 2006) or complete chemical synthesis (Schwartz*, et al.*, 2001, Schwartz*, et al.*, 2002, VanNieuwenhze*, et al.*, 2002). The availability of sufficient amount of fluorescent substrate allowed detailed kinetic characterization and additives (DMSO, detergent, metal ions) requirement of *E.coli* PBP1b GT activity (Schwartz*, et al.*, 2002), the most biochemically characterized class A PBP.

Several difficulties related to the intrinsic properties of the GT were though encountered during the purification and studies of these proteins. Most GT have a tendency to aggregate even in the presence of detergents and proteolysis was also often observed. In most cases the treatment of entire class A PBPs with trypsin results in stable transpeptidase domain and the complete degradation of the GT domain (Di Guilmi*, et al.*, 1998, Di Guilmi*, et al.*, 2003). It was shown that the expression of a stable class A PBPs GT domain greatly depends on the choice of the C-terminal boundaries (Barrett*, et al.*, 2004). Finally, the numbers of GT that have been successfully purified and characterized (with kinetic parameters determined *in vitro* with lipid II) do not exceed a dozen candidates (Table 1).

Properties of class A PBPs and GT domain derivatives

1 *E. coli* PBP1a and 1b use monomeric -tri, -tetra and -pentapeptide as acceptor in the *in vitro*
2 assay transpeptidase reaction with lipid II as substrate (Bertsche*, et al.*, 2005, Born*, et al.*, 2006).
3 PBP1a catalyzes the polymerization of cross-linked peptidoglycan from lipid II with an efficiency
4 of 33,000 $M^{-1}s^{-1}$ (Mottl*, et al.*, 1995). The glycan strands have an average length of 20 disaccharide
5 units with 18-26% of the peptides involved in cross-linking (Born*, et al.*, 2006). In addition PBP1a
6 catalyzes the attachment of nascent peptidoglycan to mature cell wall by transpeptidation (Born*, et*
7 *al.*, 2006). The catalytic efficiency of the PBP1b GT is 320,000 $M^{-1}s^{-1}$ (Schwartz*, et al.*, 2002), and
8 the length of glycan strands obtained by polymerization is superior to 25 disaccharide units with
9 almost 50 % of the peptides engaged in cross-linking (Bertsche*, et al.*, 2005). The activity of the
10 two PBPs is inhibited by moenomycin (IC_{50} value for PBP1b are within 2-14 nM) (Terrak*, et al.*,
11 1999, Chen*, et al.*, 2003, Welzel, 2005). Different GT domain derivatives of PBP1b were produced.
12 They show similar moenomycin sensitivity and their catalytic activity is lower or comparable to
13 that of the full length protein. For example, the fragments M46-D478 and M46-Q423 retain 23%
14 and 13% of the entire PBP1b activity respectively (Terrak*, et al.*, 1999). The C-terminal boundary
15 of the GT domain was also identified between residues 385-478 (Barrett*, et al.*, 2004).
16 The full length PBP2 of *S. aureus* was purified and characterized with heptaprenyl-lipid II
17 (C_{35}) substrate. Its catalytic efficiency is 3,400 $M^{-1}s^{-1}$. The PBP4 of *L. monocytogenes* EGD was
18 overexpressed in *E. coli* and purified (Zawadzka-Skomial*, et al.*, 2006)**.** It catalyzes *in vitro*
19 peptidoglycan polymerization with an efficiency of 1,400 M^{-1} s^{-1} from lipid II substrate. Disruption
20 of the *lmo2229* gene encoding PBP4 increased the resistance of *L. monocytogenes* EGD to
21 moenomycin.
22 *S. pneumoniae* PBP1a, PBP1b and PBP2a were overexpressed without transmembrane
23 segment (Δtm) as GST fusions in *E. coli*, purified and characterized. GST-PBP2aΔtm fusion
24 required detergent for solubility (Di Guilmi*, et al.*, 1999) but not GST-PBP1bΔtm. A GT domain
25 derivative of PBP1b (82-300) was also produced in the absence of detergent. These three proteins
26 catalyze the polymerization of peptidoglycan from dansyl-lipid II substrate and the GT activity is

inhibited by moenomycin. Several observations suggest that moenomycin induces conformational modification of the GT domain (Lovering*, et al.*, 2007).

Since the expression of the full length PBP1a of *A. aeolicus* was poor (Yuan*, et al.*, 2007), three derivatives of its GT domain (29/51/67-243) were realized and expressed in *E. coli* (Yuan*, et al.*, 2007). The 67-243 derivative was active and catalyzes peptidoglycan polymerization from heptaprenyl lipid II with an efficiency of 10,000 $M^{-1}s^{-1}$. The glycan chains have a length of at least 40 disaccharides. The 51-243 derivative leads to the X-ray structure solution. The role of residues Glu83, Asp84, H90 and K153 of the PBP1a GT domain was analyzed by mutagenesis. The results confirm the role of Glu83 as the catalytic glutamic acid residue of the GT51 family (Terrak*, et al.*, 1999, Terrak & Nguyen-Disteche, 2006).

The GT domain of *Thermotoga maritima* PBP1a (Glu34–Thr244) was expressed and purified (Offant*, et al.*, 2006). The use of detergents and/or moenomycin helped in reducing the aggregation state and increasing the protein homogeneity. A glycosyltransferase activity was identified with fluorescent lipid II and was inhibited by moenomycin with an IC_{50} value of moenomycin was 4.8 μM. Mutagenesis experiments showed that Glu133 may be involved in catalysis.

<u>Properties of monofunctional glycosyltransferases</u>

A soluble and active form of *S. aureus* MGT was overexpressed in *E. coli* and purified (Wang*, et al.*, 2001, Liu & Wong, 2006, Terrak & Nguyen-Disteche, 2006). The enzyme catalyzes glycan chain polymerization from lipid II with an efficiency of 5,800 M^{-1} s^{-1}. The properties of *S. aureus* MGT were more similar to those of *E. coli* PBP1b and distinct from those of *S. aureus* PBP2 and *E. coli* MGT (Terrak & Nguyen-Disteche, 2006). A protein of ~34 kDa able to polymerize uncross-linked PG *in vitro* from lipid II substrate was isolated from *E. coli* (Hara & Suzuki, 1984). The membrane bound MGT was active but was insensitive to moenomycin (Di Berardino*, et al.*, 1996).

1

2 *Glycosyltransferase mechanism*

3 The crystal structures of PBP2 and its complex with moenomycin, brought direct evidence
4 that the lipid II is the acceptor and the growing chain is the donor (Lovering*, et al.*, 2007). From the
5 kinetic characterization of *E. coli* PBP1b (Schwartz*, et al.*, 2002) and its interaction with
6 moenomycin (Welzel, 2005), mutagenesis studies (Terrak*, et al.*, 1999) and the recently determined
7 crystal structure of *S. aureus* PBP2 (Lovering*, et al.*, 2007), it was concluded that the mechanism of
8 glycan chain elongation by the GT proceeds by successive attacks of the growing chain (donor) at
9 the reducing end by lipid II (acceptor) catalyzed by deprotonation of the 4-OH nucleophile of
10 GlcNAc by the active site base (Glu of motif 1), concomitant with stabilization of the leaving
11 diphospho-undecaprenyl group by the second glutamate (motif 3) probably via a divalent metal
12 (Schwartz*, et al.*, 2002). The exact role of metal ion in the activity of the GT needs to be
13 determined. It should be noted that apart from *S. aureus* PBP2 (Barrett*, et al.*, 2005), the activity of
14 most characterized GTs was dependent on metal ions (Schwartz*, et al.*, 2002, Terrak & Nguyen-
15 Disteche, 2006, Zawadzka-Skomial*, et al.*, 2006).

16

17 *Relation transglycosylation/transpeptidation in peptidoglycan synthesis*

18 For the bifunctional PBPs, the transglycosylation can proceed while the transpeptidase
19 domain is either inhibited by penicillin, mutation or completely deleted (Terrak*, et al.*, 1999, Born*,*
20 *et al.*, 2006). In contrast, inactivation of the glycosyltransferase domain by mutation (Terrak*, et al.*,
21 1999, Born*, et al.*, 2006) or by moenomycin completely blocks the peptidoglycan polymerization.
22 Study with *E. coli* PBP1a showed that, for the transpeptidation to occur, it must be coupled to the
23 transglycosylase activity on the same polypeptide with stem donor peptide from oligomerized
24 glycan strands (Born*, et al.*, 2006). Time course of PG synthesis *in vitro* by PBP1a and PBP1b
25 showed that peptide cross-linking catalyzed by PBP1b occurs simultaneously with GT of lipid II
26 (Bertsche*, et al.*, 2005). With PBP1a the transpeptidation is delayed until oligomerized glycan

1 strand are formed indicating that PBP1a transpeptidase prefers peptide of elongating glycan strands

2 (Born*, et al.*, 2006).

Class B PBPs

Structural basis

S. pneumoniae PBP2x was the first HMM PBP whose crystal structure was ever solved (Pares*, et al.*, 1996). More recently, the structures of *S. aureus* PBP2a (Lim & Strynadka, 2002) and *E. faecium* PBP5 (Sauvage*, et al.*, 2002) were solved at the same time. *S. aureus* PBP2a and *E. faecium* PBP5 are orthologous proteins responsible for the high level of resistance encountered in *S. aureus* and *E. faecium* strains. Sequence alignment allowed Goffin and Ghuysen (Goffin & Ghuysen, 1998) to define five subclasses. *S. aureus* PBP2a and *E. faecium* PBP5 are in the subclass B1 and all the PBPs in this subclass are supposed to show a low affinity for penicillin. The subclass B2 contains elongasome specific type-2 PBPs of Gram-negative bacteria (e.g. *E. coli* PBP2), whereas the subclass B3 contains divisome specific type-3 PBPs of Gram-negative bacteria (e.g. *E. coli* PBP3). *S. pneumoniae* PBP2x is part of subclass B4 together with other enzymes involved in division of Gram-positive bacteria, and subclass B5 contains enzymes from Gram-positive not directly involved in septation. Subclasses B-like I, II and III are defined by Goffin & Ghuysen (Goffin & Ghuysen, 2002) and contains PBPs from mycobacteria, streptomycetes, and related bacteria.

The overall fold of class B PBPs is made of a complex N-terminal domain coupled to the C-terminal transpeptidase domain. In the transpeptidase domain, the residue following motif 3 (KTG(T/S)) is, with very few exceptions, an alanine in class B PBPs (KTG(T/S)A) while it is a threonine or a serine in class A PBPs (KTG(T/S)(T/S)), and this might be related to their specific role as class A or class B PBPs. Gram-negative type-2 PBPs are also distinguished in their active site by the presence of an aspartic acid at the third position of motif 2 (SxD). The other subclasses have an asparagine as in the canonical active site of PBPs (SxN).

1 The N-terminal domain was hypothesized to interact with other proteins (Holtje, 1998) or to
2 serve as a pedestal for PBPs to reach their target (Macheboeuf*, et al.*, 2006). Small loops or
3 domains may interact with other proteins as shown by a complementation analysis of the PBP3
4 deficiency in *E. hirae* (Leimanis*, et al.*, 2006). The interdomain linker is made of four to six β-
5 strands. Five conserved motifs (I to V) in or close to the linker constitute the signature of class B
6 PBPs (Goffin & Ghuysen, 1998). The N-terminal domain should be seen as small subdomains or
7 loops that are connected to the interdomain linker (figure 5). The number of residues in each loop or
8 subdomain between the conserved motifs is characteristic of each subclass. In the absence of a type-
9 2 PBP structure, the features distinguishing *E. coli* PBP2 from *E. coli* PBP3 are unknown.
10 Interestingly, in Gram-negative bacteria, the length of the loop between motif II and motif III is
11 similar in all type-2 PBPs and in all type-3 PBPs but differs between type-2 and type-3. This holds
12 as well for the loop between motif IV and motif V, but the significance of such characteristics
13 awaits further structural information.
14 The length of the loop between motif III and motif IV (figure5) is similar in subclasses B1
15 and B2. As well, the loop between motifs IV and V and the loop between motif V and motif 1 of
16 the transpeptidase domain are of same length in subclasses B1 and B2 whereas they have different
17 length in the other subclasses. Sequence alignment suggests that enzymes of subclass B1 are the
18 equivalent in Gram-positive bacteria of *E. coli* PBP2 but experimental evidence with *B. subtilis*
19 PBP2a and PBPH suggests rather that the equivalent of Gram-negative type-2 PBPs are enzymes of
20 subclass B5 (Murray*, et al.*, 1998, Wei*, et al.*, 2003).
21
22 *Role of class B PBPs in peptidoglycan biosynthesis*
23 *B. subtilis* PBP2a is required for normal outgrowth of spores (Murray*, et al.*, 1998). PBP2a
24 and PBPH play redundant roles in determining the rod cell shape and the activity of one of these
25 proteins is required for viability (Wei*, et al.*, 2003). In the double mutant, septa are formed
26 extremely irregularly and cells have a greater diameter (Wei*, et al.*, 2003), a phenotype also

1 associated with the loss of PBP5 (Nelson & Young, 2000). PBPH is expressed most strongly in late
2 log phase and during the transition to stationary phase and may play a unique role during that part
3 of the life cycle.

4 In Gram-positive cocci, the situation is different. There is no apparent phenotype associated
5 with the inactivation of *pbp3* (PBP3) in *S. aureus* regarding growth rate, cell wall muropeptide
6 profile, and impact on methicillin resistance in a MRSA strain. The only observable effect was a
7 decrease in autolysis rate (Pinho*, et al.*, 2000). The problem of the presence of two or three class B
8 PBPs in Gram-positive cocci and the role of class B PBPs not involved in bacterial division is
9 addressed by Zapun et al in the chapter "The different shapes of cocci".

10 Gram-positive division specific PBPs belong to subclass B4. They all possess a supplementary
11 domain made of two repeating units known as PASTA domains (cf class A PBPs). An interesting
12 example is the serine/threonine kinase PknB of *M. tuberculosis* that phosphorylates PBPA, a class
13 B PBP required for cell-division (Dasgupta*, et al.*, 2006). The PASTA domain is believed to bind
14 unlinked peptidoglycan because a cefuroxime was observed associated to the first PASTA domain
15 in the X-ray structure of *S. pneumoniae* PBP2x (Gordon*, et al.*, 2000) but further experimental
16 evidence is needed to prove this assertion.

17 *M. tuberculosis* has one type-3 PBP (*pbpB* or *mtu3)*, one in subclass B-like I (PBPA) and one in
18 subclass B-like II, which is annotated as Penicillin-binding lipoprotein. *S. coelicolor* has one PBP
19 ("PBP2", *sco2608*) classified in subclass B2, but with a weak similarity to other members and one
20 in subclass B3 ("PBP3", *sco2090*). *S. coelicolor* has also four B-like I and three B-like III PBPs.
21 *Anabaena sp.* PCC7120 has one type-2 PBP (PBP5; *alr5045*) and one type-3 PBP (PBP6;
22 *alr0718*). Their sequences are close to those of their respective *E. coli* orthologues. The roles of
23 these proteins are largely unknown.

24

25 <u>*Resistance to β-lactams*</u>

Class B PBPs play an important role in the resistance to β-lactams of many bacteria. One resistance mechanism found in some Gram-positive bacteria is the presence of an endogenous or acquired penicillin-resistant PBP that can take over the transpeptidase function of all other PBPs, like *S. aureus* PBP2a and *E. faecium* PBP5. Strikingly a similar PBP in *L. monocytogenes* (*lmo0441*) is responsible for the resistance to monobactams and some cephalosporins but not penicillin (Guinane*, et al.*, 2006).

Mutations or mosaic gene transfer is also frequently observed with naturally competent bacteria, mainly involving the division specific class B PBP (type-3 PBP). Alterations of the *N. gonorrhoeae* or *meningitidis* PBP2 (a type-3 PBP) is associated with reduced susceptibility to penicillin G (Dougherty*, et al.*, 1980, Barbour, 1981, Spratt, 1988, Antignac*, et al.*, 2001), a situation that also exists in *Pseudomonas aeruginosa*, *S. pneumoniae* and *Bacteroides fragilis* (Ayala*, et al.*, 2005). This situation also occurs in class A PBPs. For example, alterations in penicillin-binding protein 1A confer resistance to beta-lactam antibiotics in *Helicobacter pylori* (Kwon, 2003). (see chapter “Penicillin-Binding Proteins and -lactam resistance” by Zapun *et al.*)

Class C PBPs of type-4

<u>*Structural basis*</u>

There is generally one or zero type-4 class C PBP in bacteria. Of note is the absence of this type of protein in Gram-positive cocci like staphylococci, enterococci, streptococci or listeria. *S. coelicolor* and some cyanobacteria are the only presumably known bacteria possessing two type-4 PBPs.

The three X-ray structures of type-4 PBPs available to date show a great similarity in their overall folding (Sauvage*, et al.*, 2005, Kishida*, et al.*, 2006, Sauvage*, et al.*, 2007) (figure 6). The transpeptidase domain is associated with two other domains, which are not in N- or C-terminal positions but are inserted in the transpeptidase domain in the way of matryoschka dolls (Sauvage*, et al.*, 2005), i.e. the third domain is inserted in the second domain, which itself is inserted in the penicillin-binding domain between the conserved motifs 1 and 2 (of the PB domain). The role of these domains is unknown. Domain II has the topology of half a Rossmann fold, which is also the case of the N-terminal domain MinC and the 1A region of FtsA, two proteins interacting with FtsZ and involved in the regulation of the septum formation in cell division, but the resemblance of these proteins to the domain II of type-4 PBPs remains limited (Sauvage*, et al.*, 2005). Interestingly a positively charged surface appears in domain II of the DD-peptidase of *Actinomadura* R39 and *B. subtilis* PBP4a (figure 6), and it has been suggested that this positive surface could interact with the teichoic acid present in *Bacillus* and Actinomycetales (Sauvage*, et al.*, 2007).

Strikingly, the number of residues between motifs 1 and 2 is reduced by 50 for type-4 PBPs of some bacteria (*Streptomyce avermitidilis*, *S. coelicolor*, *Corynebacterium diphtheriae*, *M. tuberculosis*, *Anabaena sp* (PBP11 but not PBP10), …), which corresponds approximatively to the length of the sequence of domain III and our sequence alignment supports the idea that this domain is absent from those bacteria.

The surroundings of the active site are specific to type-4 PBPs. The residue following motif 3 is invariably a leucine or a methionine, a hydrophobic residue turned inside the cavities that pushes the C-terminus of β3 strand slightly in the forefront of the active site. Together with residues pertaining to domain II, the residues of the C-terminus of β3 strand form a pocket that can accommodate the terminal $H3N^{+}$-CH-COO^{-} group of the diaminopimelic acid, the antepenultimate amino-acid of the peptidoglycan stem peptide (Sauvage*, et al.*, 2007). The residues involved in the binding pocket are widely conserved with some variations (Sauvage*, et al.*, 2005).

Type-4 LMM PBPS have medium to high β-lactam binding activities (Duez*, et al.*, 2001, Stefanova*, et al.*, 2003). High acylation rates and very low deacylation rates make them good candidates to trap crystallographic acyl-enzyme with β-lactams or other interacting ligands (Jamin*, et al.*, 1991, Sauvage*, et al.*, 2005, Kishida*, et al.*, 2006, Sauvage*, et al.*, 2007) (unpublished results with *Actinomadura* R39).

Role of type-4 PBPs in peptidoglycan biosynthesis

Type-4 PBPs are not anchored into the cytoplasmic membrane via a transmembrane helix (Harris*, et al.*, 2002, Stefanova*, et al.*, 2003) (Gittins*, et al.*, 1994, Harris*, et al.*, 2002). Nearly all of *E. coli* PBP4 in overproducing cells is in a soluble form (Korat*, et al.*, 1991, Gittins*, et al.*, 1994). *N. gonorrhoeae* PBP3 has been identified to an outer membrane protein that interacts with peptidoglycan (Judd*, et al.*, 1991, Shafer & Judd, 1991). The DD-peptidase of *Actinomadura* R39 is found in the culture medium, and *B. subtilis* PBP4a is easily washed away from the cells with a buffer containing 1M KCl (C. Duez, personal communication). These results suggest that type-4 PBPs are very loosely associated with the cytoplasmic membrane. They could be associated directly with the peptidoglycan as suggested for PBP4a. Gram-positive type-4 PBPs could interact with anionic polymers that decorate the peptidoglycan whereas Gram-negative type-4 PBPs could be associated with the outer membrane or directly with the peptidoglycan.

1 Localization studies by green fluorescent protein fusions have shown that *B. subtilis* PBP4a
2 is recruited to the lateral wall and is absent from the septum (Scheffers*, et al.*, 2004). This result
3 must be paralleled with the presence of a type-4 PBP in *N. gonorrhoeae*, a coccoïd bacterium
4 without lateral wall.
5 An *in vivo* endopeptidase activity has been shown for *E. coli* PBP4 (Korat*, et al.*, 1991) and *N.*
6 *gonorrhoeae* PBP3 (Stefanova*, et al.*, 2003). Type-4 PBPs are dispensable under laboratory growth
7 conditions (Duez*, et al.*, 2001, Stefanova*, et al.*, 2003) but an endopeptidation function might be
8 necessary for correct peptidoglycan incorporation and cell growth.
9 The absence of a membrane anchoring helix and the *in vivo* endopeptidase activity are two
10 arguments in favour of type-4 PBPs being envisioned as members of the pool of autolysins, as
11 suggested by Höltje and colleagues for *E. coli*. Type-4 PBPs could be inserted into the
12 peptidoglycan or exposed on the outer face of peptidoglycan where it would exert its endopeptidase
13 activity. It could thereby be indirectly involved in cell morphology (Meberg*, et al.*, 2004), in
14 daughter cells separation (Priyadarshini*, et al.*, 2006), and could be implicated in biofilm formation
15 (Gallant*, et al.*, 2005).
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19
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Class C PBPs of type-5

We distinguish the groups of type-5 and type-7 class C PBPs on the basis of the presence in type-5 PBPs of a C-terminal domain that was shown in *E. coli* to be essential for the correct functioning of the PBP5 (Nelson & Young, 2001). The PBP5 C-terminal domain ends with an amphipathic helix that associates PBP5 to the cytoplasmic membrane. The truncated PBP5, lacking the C-terminal domain, is soluble (like PBP7) and its overproduction leads to cell lysis (Nelson & Young, 2001).

Structural basis

The crystal structures of *E. coli* PBP5 (Davies*, et al.*, 2001, Nicholas*, et al.*, 2003), *S. pneumoniae* PBP3 (Morlot*, et al.*, 2005) and *S. aureus* PBP4 have been determined (figure 7A). The overall fold of type-5 PBPs is biglobular. The penicillin binding domain is associated with a β-strand rich C-terminal domain that ends with an associated membrane amphipathic helix. The penicillin binding domain lacks the N-terminal helix and a small loop extends on the top of the active site.

Role of type-5 PBPs in peptidoglycan biosynthesis

With the exception of *S. aureus* PBP4, type-5 PBPs are strict DD-carboxypeptidases unable to catalyze a transpeptidation reaction (Matsuhashi*, et al.*, 1979). They play a major role in the control of cell diameter and correct septum formation. Cells of an *E. coli* mutant lacking only PBP5 had aberrant morphology (Meberg*, et al.*, 2004), exacerbated by the deletion of other DD-carboxypeptidases (Nelson & Young, 2000, Nelson & Young, 2001). In *L. monocytogenes*, cells lacking PBP5 displayed an irregular morphology and shape (Guinane*, et al.*, 2006), and a thicker cell wall (Korsak*, et al.*, 2005). Moreover the ratio of pentapeptides to tripeptides was increased in cells lacking PBP5 (Korsak*, et al.*, 2005). The ratio of pentapeptides to tripeptides was also largely

increased in a *dacA* (PBP5) mutant of *B.subtilis* (Atrih*, et al.*, 1999). In *S. pneumoniae*, PBP3 plays a major role in organizing the growth and division process (Morlot*, et al.*, 2004). PBP3 is present on the whole surface of the cell except at the septum, where the pentapeptides are left intact as substrates of the division specific PBPs.

In contrast to previous results, *S. aureus* PBP4 was shown to have a secondary transpeptidase activity (Wyke*, et al.*, 1981). Disruption of *pbpD*, the structural gene of *S. aureus* PBP4, caused a large decrease in the highly cross-linked muropeptide components of the cell wall (Leski & Tomasz, 2005). The striking difference between the activity, function and physiological role of *S. aureus* PBP4 compared to the concordant activity, function and physiological role of its structural equivalent in other bacteria remains to be explained. A possibility is that *S.aureus* PBP4 plays a similar role in restricting the availability of pentapeptides to its division specific PBPs, but instead of simply removing the ultimate D-alanine of the stem peptide, PBP4 utilizes the available D-Ala-D-Ala to further cross-link the glycan chains.

Bacteria most often have a major type-5 PBP which is the most abundant LMM PBP they produce. *E. coli* produces two other type-5 PBPs (figure 1). The expression level of *E. coli* PBP6 considerably rises in stationary-phase cells compared with exponentially growing cells(Buchanan & Sowell, 1982). The level of transcription of *dacA* (PBP5), *dacC* (PBP6) and *ampC* (AmpC) is controlled by the *bolA* gene (Santos*, et al.*, 2002). PBP6 is supposed to play a major role at the onset of stationary-phase (van der Linden*, et al.*, 1992). Interestingly, by constructing mosaic proteins in which a 20 amino-acids sequence were grafted from PBP5 to PBP6, and vice versa, Ghosh and Young showed that the shape maintaining characteristics of PBP5 relies on residues following the KTGT motif (Ghosh & Young, 2003).

B subtilis produces three type-5 PBPs (figure 1). PBP5 (*dacA*) is the major carboxypeptidase found in vegetative cells (Lawrence & Strominger, 1970). PBP5 has no role in spore peptidoglycan synthesis but PBP5* (*dacB*) and DacF (*dacF*) both function in regulating the degree of cross-linking of spore peptidoglycan (Popham*, et al.*, 1999). *dacB* is expressed only in the mother cell

1 compartment of the developing sporangium whereas *dacF* is expressed in the forespore

2 compartment and they can act differently on the nascent spore peptidoglycan (Popham*, et al.*,

3 1999).

Class C PBPs of type-7

As we mention for type-5 PBPs, *E. coli* PBP7 and *E. coli* PBP5 have homologous sequences. The structure of PBP7 differs from that of PBP5 by the absence of the C-terminal domain and the amphiphatic helix that anchors PBP5 to the cytoplasmic membrane.

Structural basis

The crystal structures of *Mycobacterium tuberculosis* PBP7 (Krieger, unpublished) and the DD-peptidase of *Streptomyces* K15 (Fonzé*, et al.*, 1999) (K15) have been determined. The sequence of K15 is slightly different from the sequences of the other type-7 PBPs (Palomeque-Messia*, et al.*, 1991) and close orthologues have been found only in *S. coelicolor* and *S. avermitidilis*. The global fold of *M. tuberculosis* PBP7 and K15 proteins is similar to the penicillin-binding domain of *E. coli* PBP5. As type-5 PBPs, type-7 PBPs lack the N-terminal helix and exhibit on the top of the active site a β-hairpin protuberance, which in K15 is relatively long and was hypothesized to anchor the protein to the plasma membrane (Fonzé*, et al.*, 1999) (figure 7B).

Role of type-7 PBPs in peptidoglycan biosynthesis

E. coli PBP7 was shown to be loosely associated with the membrane and totally released in the supernatant in the presence of 1M NaCl (Romeis & Holtje, 1994). PBP7 is an endopeptidase that hydrolyses the D-alanyl-ε-*meso*-2,6-diaminopimelyl cross-bridge bond in high-molecular mass sacculi (not with soluble low molecular mass muropeptides) but had no carboxypeptidase activity. Interestingly, a mutant of *E. coli* lacking PBP7 showed no morphological change but a double mutant ΔPBP5-ΔPBP7 was more abnormal than the single mutant lacking only PBP5 (Meberg*, et al.*, 2004). *E. coli* PBP8, a proteolytic degradation product of PBP7, was also shown to stabilize the soluble lytic transglycosylase Slt70, and indirectly interact with PBP3 (Romeis & Holtje, 1994). As we suggsested for type-4 PBPs, *E. coli* PBP7 should probably be looked at as an autolysin but its

precise role remains unclear and the difference between the role of *E. coli* PBP4 and the role of *E. coli* PBP7 is undetermined. Both are endopeptidases implicated in cell morphology (Meberg*, et al.*, 2004) and in daughter cells separation.

N. gonorrhoeae PBP4 is close to *E. coli* PBP7. It lacks the characteristic C-terminal domain of type-5 PBPs. PBP4 has a carboxypeptidase activity on L-Lys-D-Ala-D-Ala based substrates, preferentially with Nε substituted substrates. It showed no transpeptidase activity in a transpeptidation reaction with Ac2-L-Lys-D-Ala-D-Ala as donor and glycine as acceptor. Endopeptidation was not tested. One of the two LMM PBPs (PBP3 or PBP4) can be deleted without severely affecting the cells but the removal of both of them leads to a modest decrease of cell growth and a change in morphology, suggesting that *N. gonorrhoeae* PBP3 and PBP4 have a redundant function in normal cell wall biosynthesis (Stefanova*, et al.*, 2004).

Type-7 PBPs are absent from the Gram-positive bacteria that we have analyzed, with the exception of the $vanY_D$ DD-carboxypeptidase (Arthur*, et al.*, 1994, Casadewall*, et al.*, 2001, Reynolds*, et al.*, 2001). The $vanY_D$ DD-carboxypeptidase is included in the *vanD* gene cluster responsible for the resistance to vancomycin of enterococci (Courvalin, 2006). $VanY_D$ lacks the C-terminal domain of *E. coli* PBP5 and hence cannot be associated with the membrane *via* the C-terminal amphiphatic helix characteristic of type-5 PBPs. The hydrophobicity profile of $VanY_D$ rather suggests that there is a single transmembrane segment close to the N-terminus (Reynolds*, et al.*, 2001).

The *in vivo* endopeptidation activity and the carboxypeptidation/transpeptidation activities might result from the location of the protein active site at the outer face (endopeptidase) or inner face (carboxypeptidase/transpeptidase) of the peptidoglycan.

Although lacking transmembrane segments, *S.* K15 enzyme is associated with the cytoplasmic membrane. Its overexpression results in the secretion of ~30% of the synthesized enzyme in the culture medium. K15 can be considered as a strict transpeptidase, for which the rate of transpeptidation depends on the nature of both the scissile bond (peptide, thiolester, or ester) of the carbonyl donor and the acceptor. When assayed on Ac2-L-Lys-D-Ala-D-Ala, the enzyme

behaves as a poor hydrolase. It utilizes the small amount of the released D-Ala as an acceptor to catalyze a silent exchange between the tripeptide C-terminal D-Ala and the free amino acid. In the presence of Gly-Gly or Gly-L-Ala, which mimic the structure of the acceptor in the nascent peptidoglycan, virtually no hydrolysis product is formed, and the enzyme behaves as a strict and efficient transpeptidase (Nguyen-Disteche*, et al.*, 1982, Fonzé*, et al.*, 1999).

Class C PBPs of type-AmpH

<u>*Structural basis*</u>

The structure of type-AmpH PBPs is mainly based on the structure of the DD-peptidase of *Streptomyces* R61 (R61) (reviewed in (Frère, 2004)). R61 folding is similar to the PB domain described for the other classes of PBPs although differences occur in loops or even secondary structures (figure 8A). The active site lies at the interface of the two subdomains and the first motif SxxK is similar to other PBPs. The conserved serine of the second motif (SxN) is, in type-AmpH PBPs, replaced by a tyrosine and this is the main feature that distinguishes the enzymes of this family. Nevertheless, when superposing the active site of R61 to that of other PBPs, the tyrosine hydroxyl group of R61 is roughly at the same position as the hydroxyl group of the serine of the second motif of the other PBPs. The third residue of the second motif is generally an asparagine but is an aspartic acid in *E. coli* PBP4b. The third motif (KTG(T/S)) is not well conserved in this family. A histidine is frequently found at the first position but a lysine can also occur, as in *E. coli* AmpH. The second position also suffers variations. An arginine and a serine residue are respectively found in the two type-AmpH PBPs (PBP9 and PBP12) of the cyanobacterium *Anabaena sp*. PCC7120, whereas the type-AmpH PBP of *E. faecalis* have a HGG motif.

The exact enzymatic function of these enzymes is generally not determined except in a few cases and we cannot be sure that these proteins have a role in peptidoglycan biosynthesis or that they have another function, for example a β-lactamase function or a D-aminopeptidase function. A D-amino acid amidase from *Ochrobactrum anthropi* was shown to have a fold similar to that of R61 (Bompard-Gilles*, et al.*, 2000, Okazaki*, et al.*, 2007). Because a loop impairs its active site entrance, the D-aminopeptidase lacks transpeptidase and carboxypeptidase activities (figure 8B).

<u>*Class C β-lactamases*</u>

Class C β-lactamases also have a structure close to R61, and although β-lactamases are not included in our analysis of penicillin-binding proteins, we will briefly discuss them because it has been suggested that the AmpC β-1actamase may have an additional cellular function as a peptidoglycan hydrolase (Bishop & Weiner, 1993, Henderson*, et al.*, 1997). From the high structural similarity, first observed between R61 and the class C β-lactamase of *Enterobacter cloacae* P99, has emerged the hypothesis of an evolution from a common ancestral enzyme. The evolution scenario includes structural changes leading in β-lactamases to rejection of the D-methyl substituent of the penultimate D-alanine residue of the DD-peptidase substrate. However molecular modelling studies have shown that the D-alanyl methyl group fits tightly into the space originally occupied by the motif 2 tyrosine side chain and thus allows the bound substrate to adopt a conformation similar to that observed in the R61 active site, which has a hydrophobic pocket for this substituent. Accommodation of the penultimate D-alanyl methyl group seems therefore to be necessary, but not sufficient for an efficient DD-peptidase activity (Adediran*, et al.*, 2005).

In *E. coli*, *ampC* deletion mutant exhibited a decline in growth rate at mid-exponential phase which could be delayed by expression of AmpC at early-exponential phase. Maintenance and expression of the *ampC* gene represents an unbearable cost for *Salmonella enterica* in terms of reduction of the growth rate and invasiveness (Morosini*, et al.*, 2000). The deleterious AmpC burden could be eliminated by decreasing the production of AmpC when both regulatory *ampR* and *ampC* genes were present in *S. enterica.* Even if AmpC synthesis did not produce major variations in the peptidoglycan composition of *S. enterica*, data suggested that overexpression may interfere with normal PG synthesis or turnover.

Role of type-AmpH PBPs in peptidoglycan biosynthesis

Although closely related to AmpC and other class C β-lactamases, and despite the fact that they strongly bind penicillin, AmpH and Pbp4b show no β-lactamase activity (Henderson*, et al.*, 1997, Vega & Ayala, 2006). Mutation of the *ampC* and/or *ampH* genes in *E. coli* lacking PBPs 1a

and 5 produced morphologically aberrant cells, particularly in cell filaments induced by aztreonam. Thus, these traits suggest that AmpC and AmpH play roles in the normal course of peptidoglycan synthesis, remodeling, or recycling. Pbp4b has been shown to have a weak DD-carboxypeptide activity in vitro (Vega & Ayala, 2006), but extensive assays to identify any enzymatic activity with natural isolated muropeptides have been unsuccessful (unpublished results, J. Ayala).

On the opposite, *in B. subtilis*, a weak β-lactamase activity is associated with PBP4* (Popham & Setlow, 1993). Dynamic localization of PBP4* and PBPX, in fusion with the green fluorescent protein (GFP), during spore development, has shown that PBP4* is implicated as having a function in sporulation. Moreover it was seen that PBPX changes its localization and accumulates specifically at the prespore. It starts by localizing to the division septum at midcell, and then appears to spiral out in a pattern similar to FtsZ. GFP-PBPX is seen to appear at both poles, as observed for cell-division proteins like FtsZ and the sporulation-specific SpoIIE. Despite these observations, PBPX does not seem to be an essential component of the cell-division machinery of *B. subtilis*. Indeed, the inactivation of PBPX does not affect cell growth, or cell shape or sporulation efficiency. A possible role for the PBPX could be the quick removal of PG connecting two cells after vegetative division, or thinning of the sporulation septum prior to engulfment (Scheffers, 2005).

1 **Peptidoglycan manufacture**

2

3 Resting on the information reviewed in the previous chapters, and using a peptidoglycan model
4 based on glycan chains running parallel to the cytoplasmic membrane, we propose a simplified
5 version of the three-to-one model of peptidoglycan biosynthesis described by J. Höltje (Holtje,
6 1998). The purpose of this model is to specify for each type of PBPs a role in peptidoglycan
7 biosynthesis and in the incorporation of new glycan chains in the preexisting peptidoglycan (figure
8 9A) (see chapter "Peptidoglycan Structure and Architecture" by W. Vollmer *et al*. for a review of
9 peptidoglycan models)
10 Lipid II is synthesized in the cytoplasm by the *mur* family enzymes (see chapter "Cytoplasmic steps
11 of peptidoglycan biosynthesis" by H. Barreteau *et al*.) and then transferred to the class A PBP
12 glycosyltransferase active site where it is transglycosylated onto a donor strand that may run along
13 the surface of the whole class A PBP (figure 9B). The donor strand can adopt a three- or fourfold
14 rotation axis (Holtje, 1998, Meroueh*, et al.*, 2006) while running along the class A PBP surface.
15 The stem peptide of a disaccharide unit pentapeptide of the elongating strand (about nine
16 disaccharide units from the glycosyltransferase active site) can insert into the groove of the
17 penicillin-binding domain active site where it may undergo a transpeptidase attack from a
18 preexisting peptidoglycan transpeptidase-donor strand (figure 9B). The orientation of the active site
19 of the transpeptidase domain suggests that class A PBPs can hook the elongating glycan strand
20 beneath the old peptidoglycan.
21 Although class A PBPs have a transpeptidase domain, the transpeptidase domain of a class B PBP
22 is needed. The precise role of each transpeptidase domain in peptidoglycan biosynthesis is not
23 known and we hypothesize that class B PBPs undertake to cross-link the glycan chain hooked by
24 class A PBPs beneath the old peptidoglycan to a second strand of the peptidoglycan (figure 9C).
25 After hydrolysis by an endopeptidase of the bond that cross-links the two old glycan chains, the
26 new strand reaches the surface as a result of the internal pressure. The endopeptidase activity of

1 type-4 or type-7 PBPs can serve to cleave the cross-links between the glycan chains (figure 10).
2 These enzymes don't need to be directly associated with the elongasome complex but they could be
3 associated with other amidases.
4 Type-5 PBPs removes the ultimate D-Ala of the pentapeptide. They seem not to be directly
5 associated with the proteins of the divisome or the elongasome. They are dispersed on the whole
6 surface and prevent the septum formation at inappropriate places by removing the pentapeptide
7 substrates. The C-terminal domain can act as a pedestal that positions the penicillin-binding domain
8 close to the D-Ala stem peptide end of the peptidoglycan (figure 11).

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LEGENDS

Figure 1: PBPs classification.

Complete set of PBPs from 10 bacteria. Class A and class B subdivisions are adapted from Goffin & Ghuysen. Subdivisions of class C are explained in the text. The name of the PBP is given with its encoding gene (for most PBPs). Abbreviations for PBPs from other bacteria: PBP5fm: *Enterococcus faecium* PBP5; R39: DD-peptidase from *Actinomadura* R39; K15: DD-peptidase from *Streptomyces* K15; R61: DD-peptidase from *Streptomyces* R61.

PBPs for which the X-ray structure has been determined are highlighted in orange.

PDB codes: *E. coli* PBP4 (2EX2); *E .coli* PBP5 (1NZ0); *E. faecium* PBP5 (not deposited); *B. subtilis* PBP4a (1W5D); *S. aureus* PBP2 (2OLU); *S. aureus* PBP2a (1MWS); *S. aureus* PBP4 (1TVF); *S. pneumoniae* PBP1a (TP domain 2C6W); *S. pneumoniae* PBP1b (TP domain 2BG1); *S. pneumoniae* PBP2x (1QME); *S. pneumoniae* PBP3 (1XP4); *A.* R39 (1W79); *S.* K15 (1SKF); *S.* R61 (3PTE); *M. tuberculosis* PBP7 (2BCF).

Figure 2: Overview of PBPs structures.

Examples of each class of PBPs. High molecular mass PBPs are on the left and low molecular mass PBPs are on the right. PB: penicillin binding domain, with either transpeptidase or carboxypeptidase or endopeptidase activity. GT: transglycosylase domain.

Figure 3: Penicillin binding domain active site

A: Penicillin binding domain and conserved motifs of the active site. The first motif SxxK is coloured yellow with black letters. The second motif SxN is coloured green with green letters. The third motif is coloured cyan with white letters. The glycine in the rear of the active site is coloured red.

B: Penicillin binding domain active site (*B. subtilis* PBP4a) acylated by a peptidoglycan mimetic peptide. The dipeptide D-α-aminopimelyl-ε-D-alanine (magenta) is covalently linked to the active serine S*. OH indicates the oxyanion hole defined by the two backbone amine groups shown in dark blue. The released D-alanine is shown in orange.

C: Penicillin binding domain active site (*S. aureus* PBP2a) acylated by benzylpenicillin. Benzylpenicillin and the active serine are shown in pink. The amide bond of benzylpenicillin side chain is wedged between strand β3 and the asparagine of the second motif (green). The carboxylate is hydrogen bonded to both hydroxyl group of the third motif (cyan).

Figure 4: Structure of class A PBPs (*S. aureus* PBP2a)

A: Overall view of the structure of *S. aureus* PBP2. The catalytic serine of the penicillin binding/transpeptidase domain is shown as a red sphere and the catalytic glutamic acid (E114 of motif 1) of the transglycosylase domain is shown as a green sphere.

B: Transglycosylase active site with binding of the moenomycin molecule.The moenomycin is shown in orange and the two putative catalytic residues E114 (green) and E171 (cyan) are shown in stick form.

Figure 5: Structure of class B PBPs (*E. faecium* PBP5)

Overall view of the structure of *E. faecium* PBP5. The catalytic serine of the penicillin binding/transpeptidase domain is shown as a red sphere. The subdomai in ellow is delimited by the N-terminal helix and motif I. The red, green and blue sudomains are respectively delimited by

1 motifs I and II, motifs II and III, and motifs III and IV. The subdomain in orange is inserted in the
2 penicillin binding domain between motif V and the active serine (motif 1 of the PB domain)
3
4
5 **Figure 6: Structure of type-4 PBPs (*B. subtilis* PBP4a)**
6 Overall view of the structure of *B. subtilis* PBP4a. The catalytic serine of the penicillin
7 binding/endopeptidase domain is shown as a red sphere. Seven lysine residues (Lys83, Lys85,
8 Lys86, Lys114, Lys119, Lys122 and Lys265) forming a positively charged surface in domain II are
9 represented as blue sticks.
10
11 **Figure 7: Structures of type-5 PBPs (*E. coli* PBP5) and type-7 PBPs (*S*. K15)**
12 Overall view of the structures of *E. coli* PBP5 (A) and *S*. K15 (B). The catalytic serine of the
13 penicillin binding/DD-peptidase domain is shown as a red sphere. On the top of both active sites
14 the β-hairpin protuberance is coloured in green.
15
16 **Figure 8: Structures of type-AmpH PBPs (*S*. R61) and the D-aminopeptidase (*O. anthropi)***
17 Overall view of the structures of *S.* R61 (A) and the D-aminopeptidase from *O. anthropi* (B). The
18 catalytic serine of the penicillin binding/peptidase domain is shown as a red sphere. The two
19 additional β-barrels domains of the D-aminopeptidase are oriented in the front of the penicillin-
20 binding domain, and the loop impairing the active site entrance is highlighted in green.
21
22 **Figure 9: Role of PBPs in biosynthesis**
23 **A:** Overview of glycan chain synthesis and its incorporation in old peptidoglycan. The pre-existing
24 peptidoglycan is shown in blue. The glycan chain newly synthesized is shown in green. The glycan
25 chain is elongated by incorporation of lipid II in the glycosytransferase domain of the class A PBP
26 (yellow). Transpeptidation between the glycan chain and the old peptidoglycan occurs both in the

1 transpeptidase domain of the class A PBP and the transpeptidase domain of the class B PBP
2 (magenta). The new chain is incorporated after endopeptidation of the pre-existing peptidoglycan
3 by the type-4 PBP (orange). CM: cytoplasmic membrane (Heller, *et al.*, 1993). OM: Outer
4 membrane. PG: peptidoglycan.
5
6 B: The elongating glycan chain running along the surface of a classA PBP and its attachment to the
7 preexisting peptidoglycan.
8
9 C: Transpeptidation by a class B PBP of a glycan chain. The new glycan chain (green) previously
10 hooked to the old peptidoglycan by a class A PBP is transpeptidated by a class B PBP.
11
12 **Figure 10: Endopeptidation by type-4 PBP**
13 View of the peptidoglycan from the cytoplasmic membrane side. The type-4 PBP (orange) can
14 hydrolyze the cross-link between two glycan chains. The active site is shown with a white arrow.
15
16 **Figure 11 DD-carboxypeptidation by a type-5 PBP.**
17 Type-5 PBP can remove the D-alanine from the pentapeptide. Type-5 PBP is coloured pink. CM:
18 cytoplasmic membrane. OM: Outer membrane. PG: peptidoglycan.
19

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51

Table 1

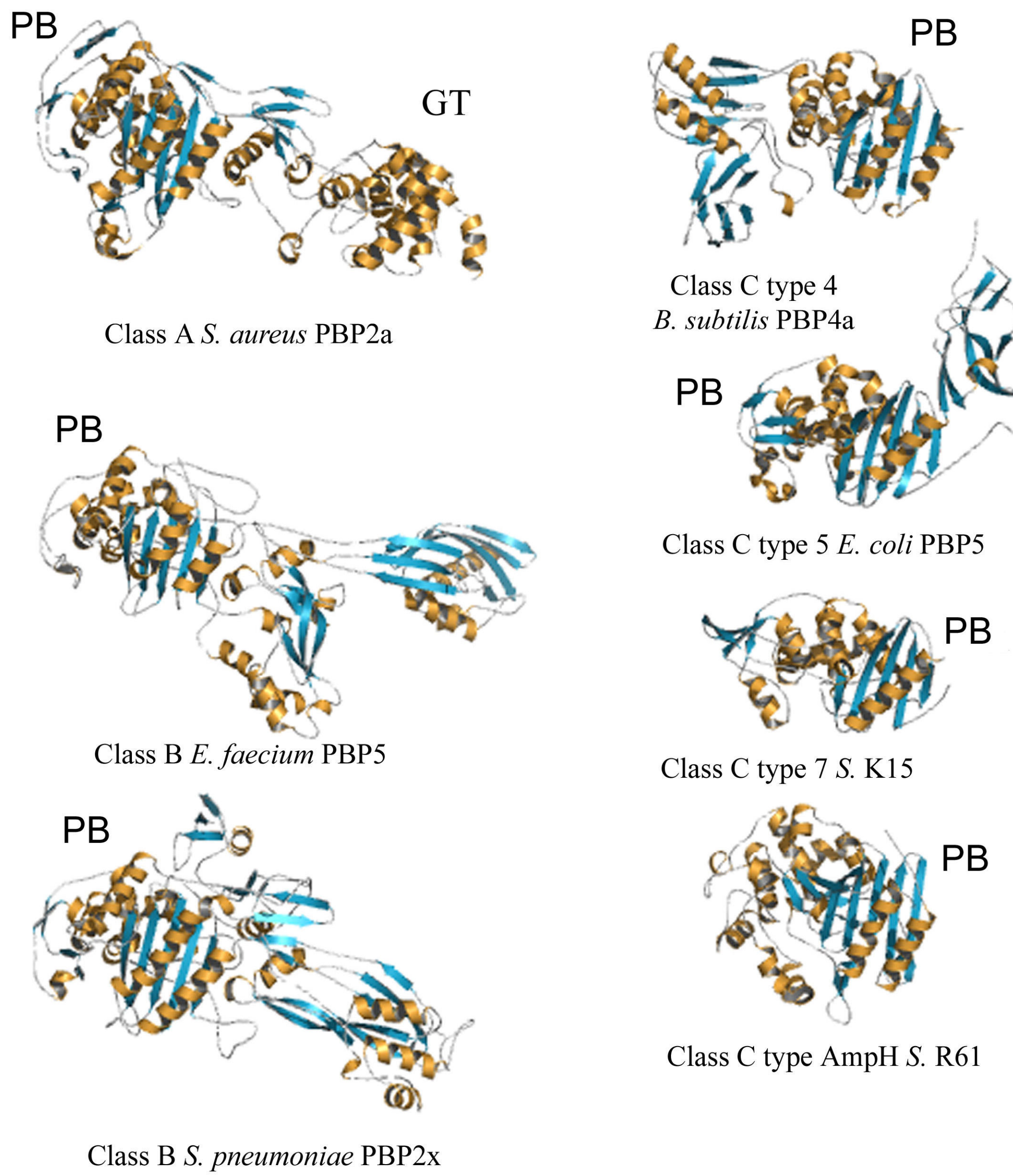
	k_{cat} (s^{-1})	K_m (µM)	k_{cat}/K_m ($M^{-1}s^{-1}$)	references
A-PBPs				
PBP1b_*Eco*	0.64 70 10^{-3}	2 1.8	3.2 10^5 3.9 10^4	(Schwartz, *et al.*, 2002) (Terrak, *et al.*, 1999)
PBP1a_*Eco*	0.03	1	3.3 10^4	(Mottl, *et al.*, 1995)
PBP2_*Sau*	1.5 10^{-2}	4	3.4 10^3	(Barrett, *et al.*, 2005)
PBP4_*Lmo*	-	> 5	1.4 10^3	(Zawadzka-Skomial, *et al.*, 2006)
PBP2a_*Spn*	4.3 10^{-8}	40.6	1.0 10^{-3}	(Di Guilmi, *et al.*, 2003)
A-GTs				
PBP1b_*Eco* M1-S409 M1-L433	 0.83 10^{-2} 2 10^{-2}	 0.87 2.6	 9700 7700	(Barrett, *et al.*, 2004)
PBP1_*Aae*	0.058	5.8	1.0 10^4	(Yuan, *et al.*, 2007)
PBP1b_*Spn*	0.76	62	1.2 10^4	(Liu & Wong, 2006)
MGTs				
MGT_*Sau*	13 10^{-3}	2.2	5.8 10^3	(Terrak & Nguyen-Disteche, 2006)

Eco = *E. coli*; Sau = *S. aureus*; Lmo = *L. monocytogenes*; Spn = *S. pneumoniae*; Aae = *A. aeolicus*.

Figure 1

	Class A									Class B										Class C									
																				Type-4		Type-5			Type-7		Type-AmpH		
	A1	A2	A3	A4	A5	A6		A7		B1	B2	B3	B4	B5	B5	B6	B-like-I	B-like-II	B-like-III										
Gram -																													
Escherichia coli K12	PBP1a *ponA*	PBP1b *ponB*				PBP1c *pbpC*			MGT *mgt*		PBP2 *pbpA*	PBP3 *ftsI*								**PBP4** *dacB*		**PBP5** *dacA*	PBP6 *dacC*	PBP6b *dacD*	PBP7 *pbpG*			PBP4b *yefw*	AmpH *ampH*
Neisseria gonorrhoeae FA 1090	PBP1 *ponA*											PBP2 *ftsI*								PBP3 *pbp3*					PBP4 *pbp4*				
Gram +										**PBP5fm**																			
Bacillus subtilis 168			PBP1 *ponA*	PBP2c *pbpF*	PBP4 *pbpD*		PBP2d *pbpG*			PBP3 *pbpC*		SpoVD *spoVD*	PBP2b *pbpB*	PBP2a *pbpA*	PbpH *pbpH*	PBP4b *yrrR*				**PBP4a** *dacC*		DacF *dacF*	PBP5 *dacA*	PBP5* *dacB*				PBP4* *pbpE*	PbpX *pbpX*
Staphylococcus aureus MRSA252			PBP2 *pbp2*						MGT *mgt*	**PBP2a** *mecA*			PBP1 *pbpA*	PBP3 *pbp3*								**PBP4** *pbp4*							
Listeria monocytogenes 4b F236			PBP1 *lmo1892*	PBP4 *lmo2229*						PBP *lmo0441*			PBP2 *lmo2039*	PBP3 *lmo1438*								PBP5 *lmo2754*							PBP *lmo0540*
Enterococcus faecalis V583			PBP1a *EF_1148*	PBP2a *EF_0680*	PBP1b *EF_1740*					PBP4 *EF_2476*			PBP2 *EF_0991*	PBP2b *EF_2857*								DacF *EF_3129*							PBP *EF_0746*
Streptococcus pneumoniae R6			**PBP1a** *pbpA*	PBP2a *pbp2&*	**PBP1b** *pbp1b*								**PBP2x** *pvpX*	PBP2b *pbp2b*								**PBP3** *pbp3*							
Actinomycetes																				**R39**						**K15**	**R61**		
Streptomyces coelicolor A3(2)								3 PBP-A *sco3901* *sco2897* *sco5039*			PBP2 *sco2608*	PBP3 *sco2090*					4 PBP-B *sco3771* *sco3156* *sco4013* *sco3847*		3 PBP-B *sco3157* *sco3771* *sco3156*	PBP4 *sco3408*	PBP4 *sco6131*	PBP *sco4439*			PBP7 *sco3811*	PBP *sco4847* *sco7050*	PBP *sco0830*	PBP *sco7561*	PBP *sco2283*
Mycobacterium tuberculosis H37Rv				PBP1 *ponA1*				PBP1A (r) *ponA2*		PBPA *pbpA*		PBP2 *pbpB*						PBP-lipo *Rv2864c*		PBP4 *Rv3627*		PBP5 *dacB1*			**PBP7** *dacB2*		PBP *Rv0907*	PBP	PBP *Rv1367c*
Cyanobacteria																													
Anabaena species PCC7120	PBP1 *all2952*	3-4-5-6 *alr4579* *alr5324* *alr5326* *all2981*				PBP2 *alr5101*					PBP7 *alr5045*	PBP8 *alr0718*								PBP10 *alr1666*	PBP11 *alr0054*							PBP9 *alr0153*	PBP12 *all2656*

Figure 2



Class A *S. aureus* PBP2a

Class B *E. faecium* PBP5

Class B *S. pneumoniae* PBP2x

Class C type 4
B. subtilis PBP4a

Class C type 5 *E. coli* PBP5

Class C type 7 *S.* K15

Class C type AmpH *S.* R61

Figure 3A

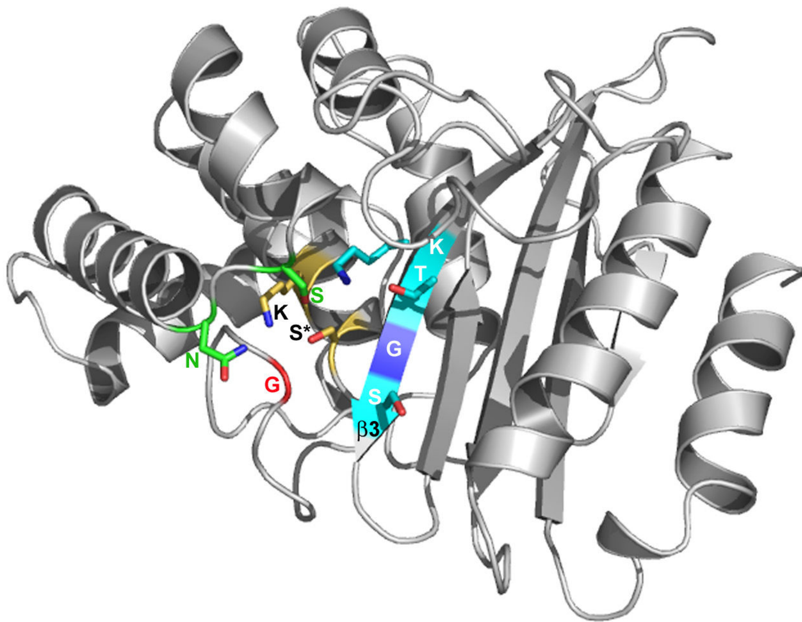


Figure 3B

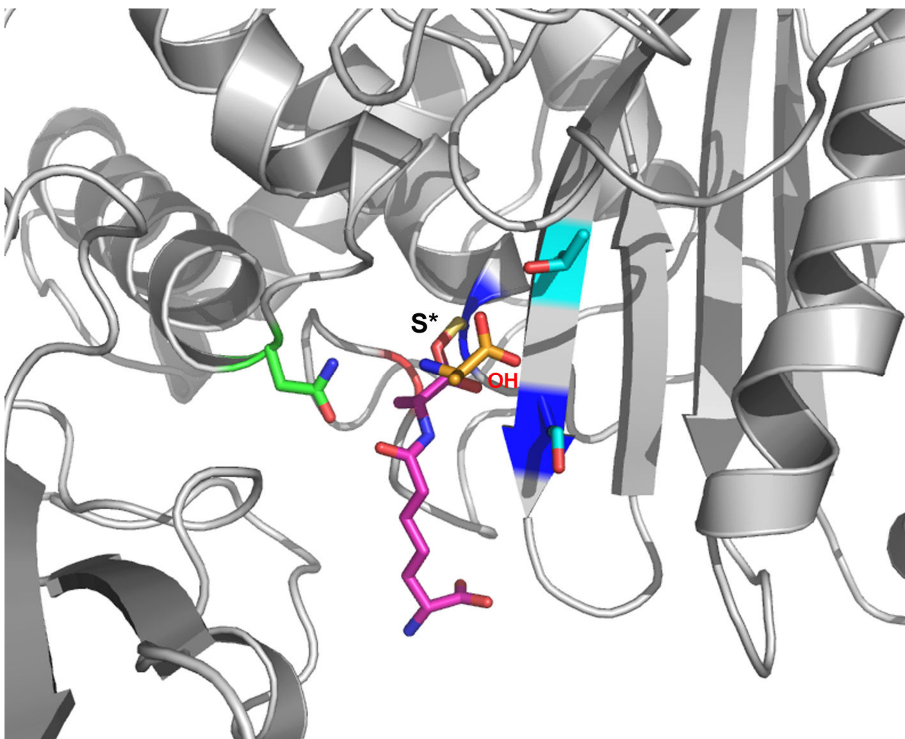


Figure 3C

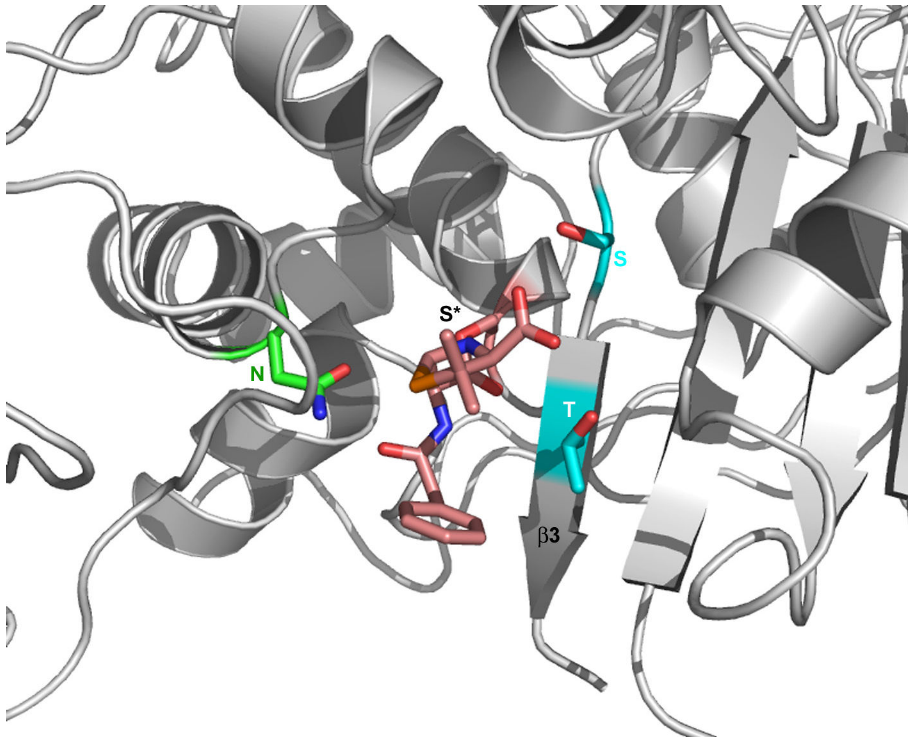


Figure 4A

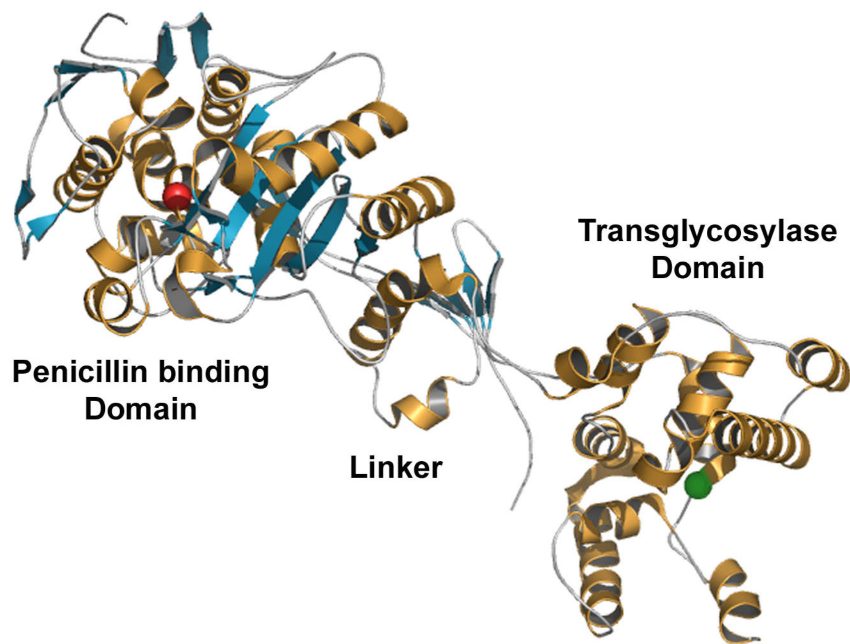


Figure 4B

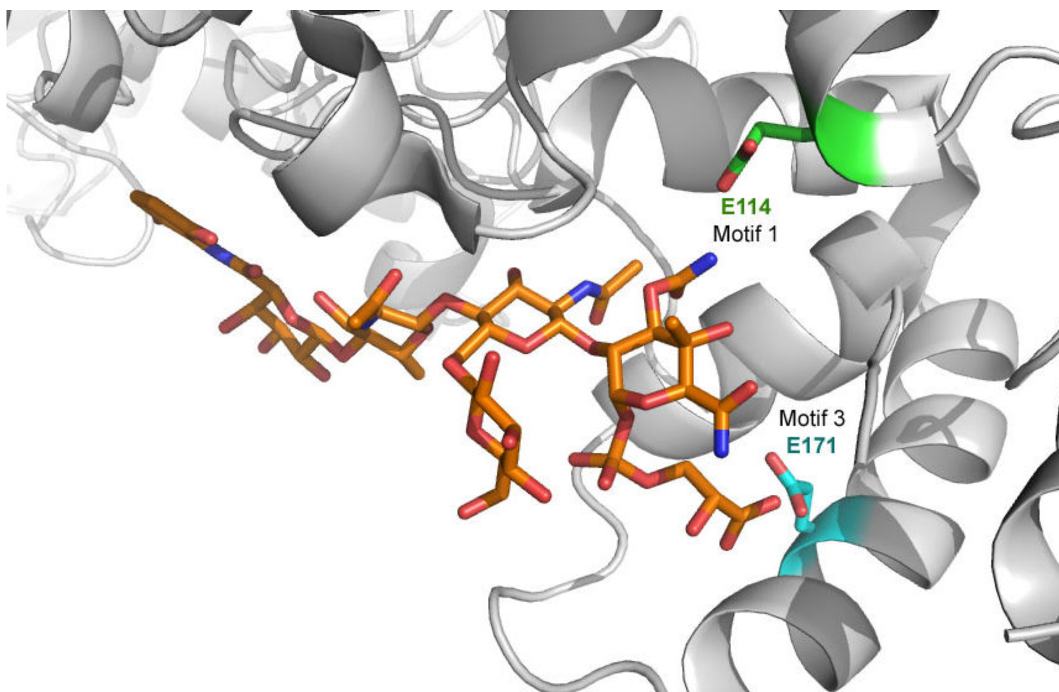


Figure 5

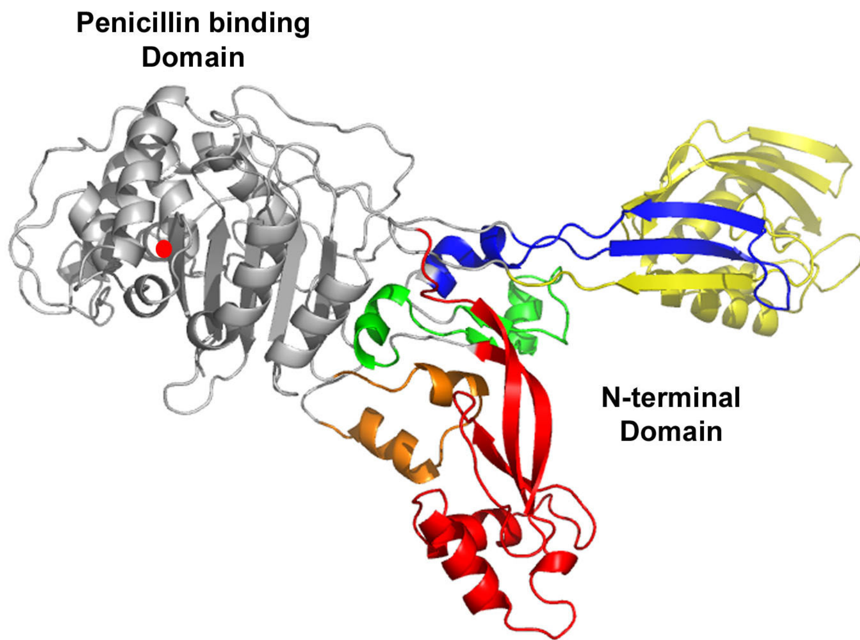


Figure 6

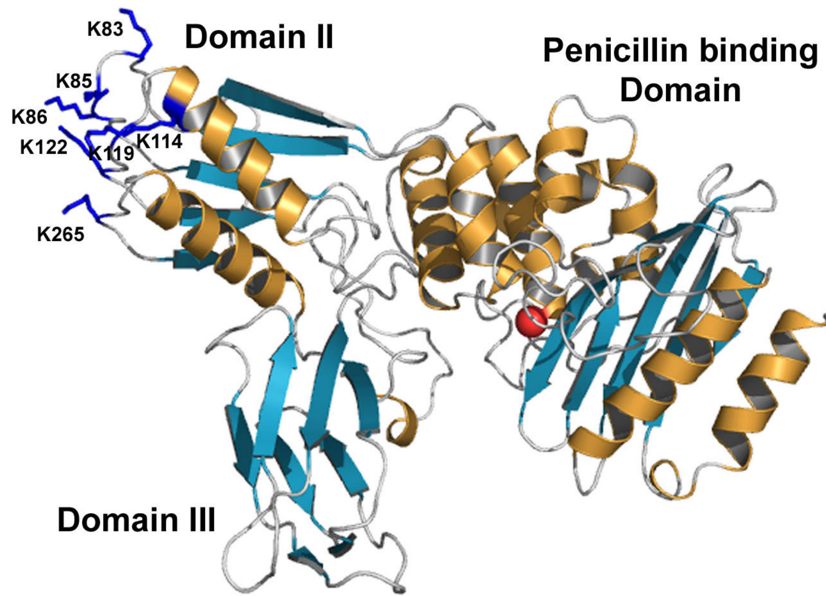


Figure 7

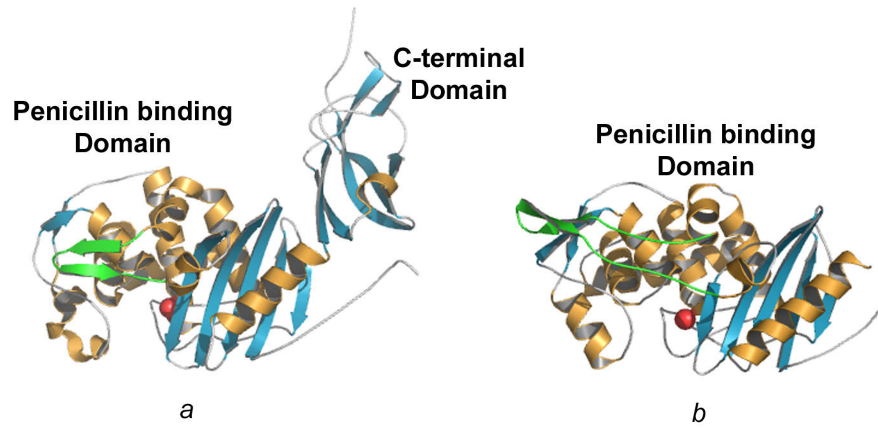


Figure 8

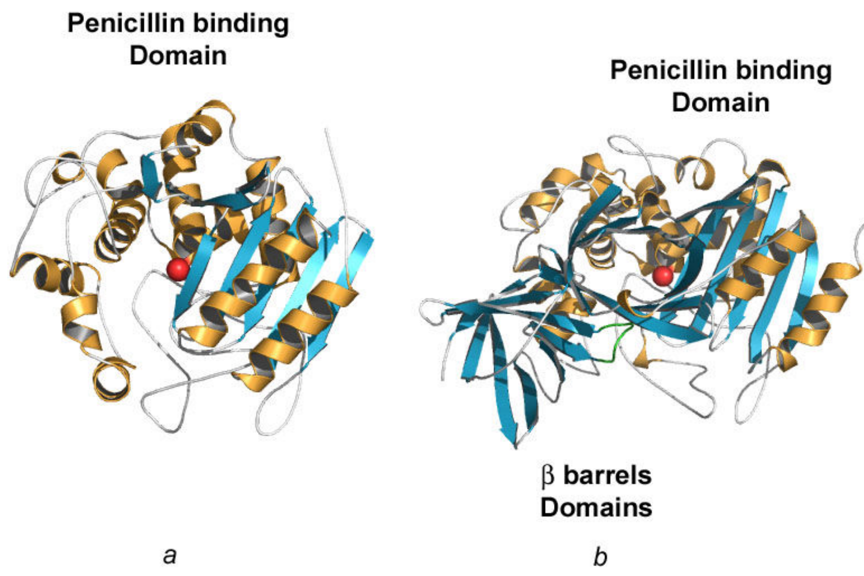


Figure 9A

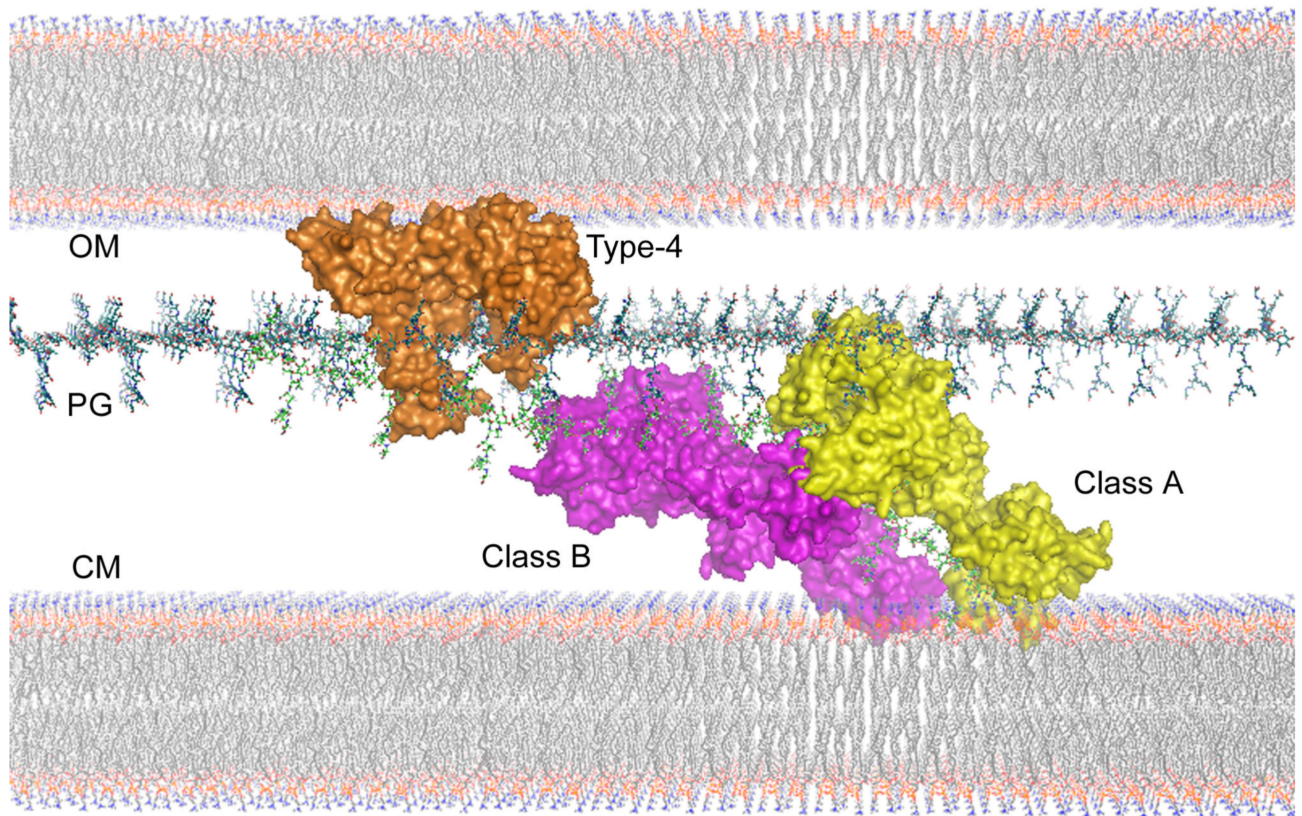


Figure 9B

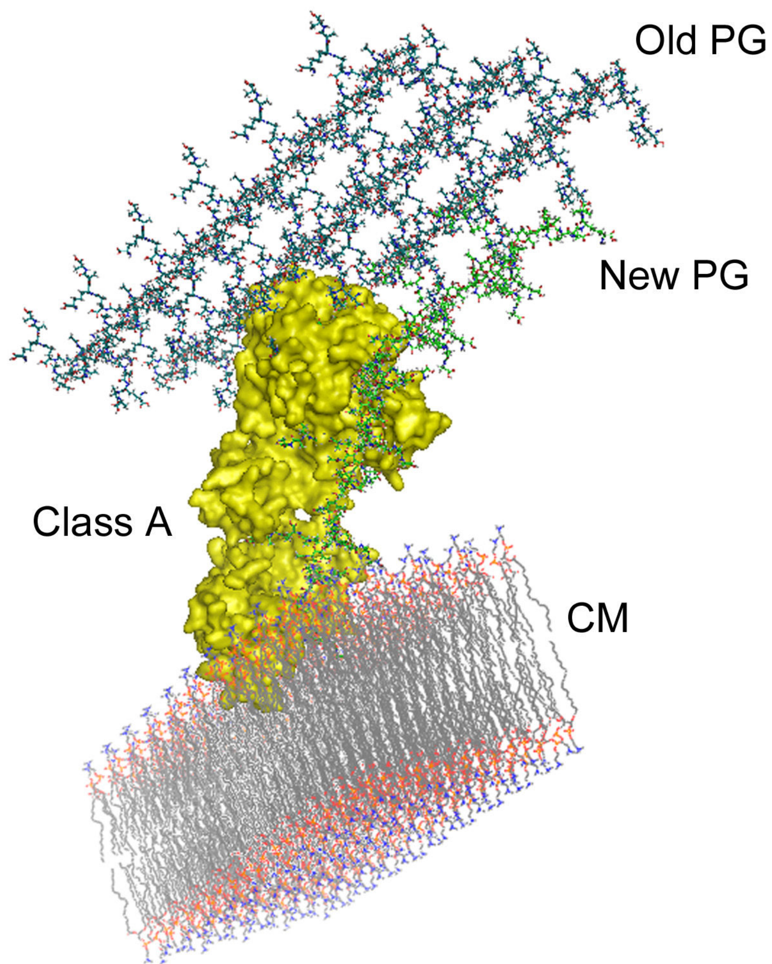


Figure 9C

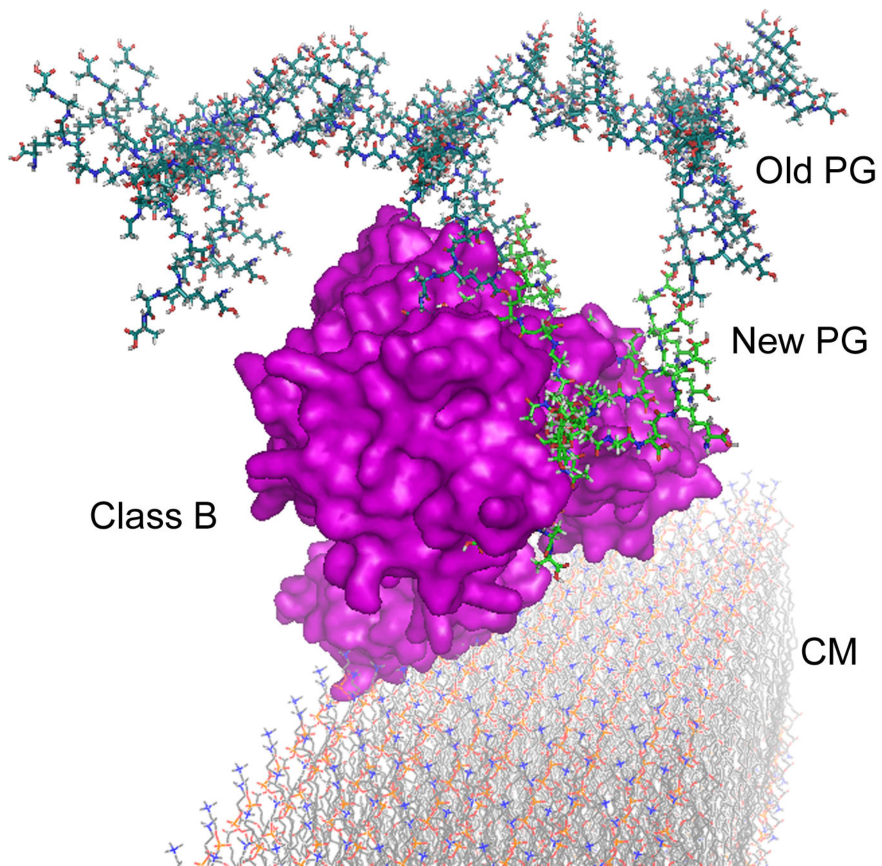


Figure 10

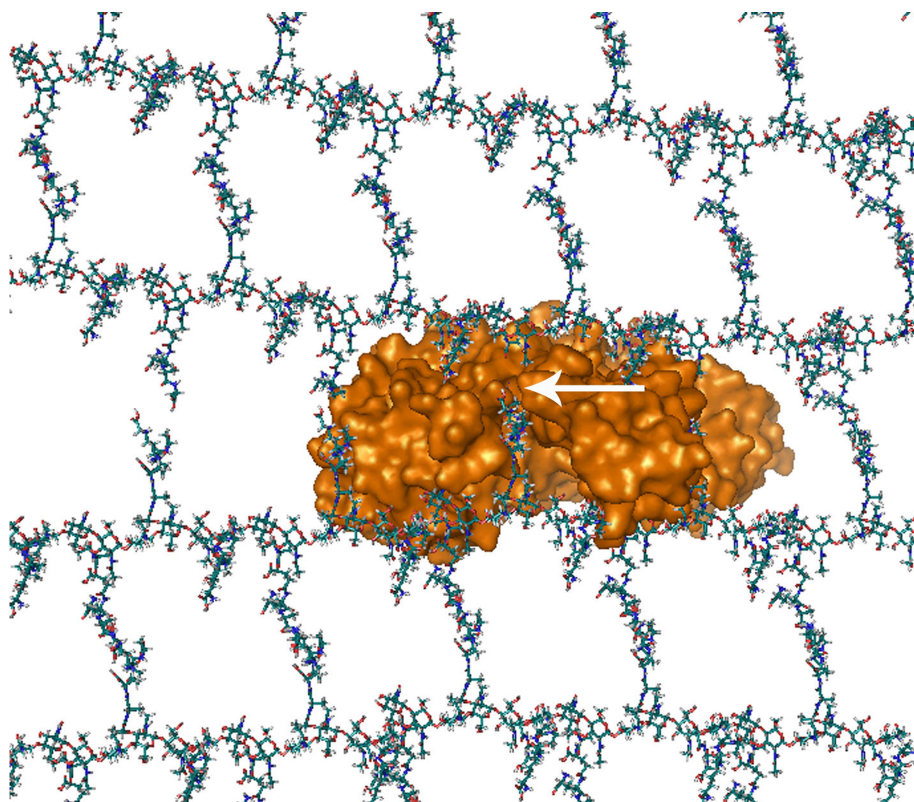


Figure 11