REVIEW ARTICLE

Penicillin-binding proteins and β-lactam resistance

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Abstract

A number of ways and means have evolved to provide resistance to eubacteria challenged by β-lactams. This review is focused on pathogens that resist by expressing low-affinity targets for these antibiotics, the penicillin-binding proteins (PBPs). Even within this narrow focus, a great variety of strategies have been uncovered such as the acquisition of an additional low-affinity PBP, the overexpression of an endogenous low-affinity PBP, the alteration of endogenous PBPs by point mutations or homologous recombination or a combination of the above.

What are PBPs?

Since their discovery as targets of the β-lactams, the penicillin-binding proteins (PBPs) have been the subject of intense research, particularly regarding their role in the resistance to β-lactams of some important pathogens such as *Staphylococcus aureus*, Enterococci and *Streptococcus pneumoniae*.

The penicillin-binding domains of PBPs are transpeptidases or carboxypeptidases involved in peptidoglycan metabolism (Goffin & Ghuysen, 2002; Sauvage *et al.*, 2008). These domains harbor three specific motifs: SXXK, (S/Y)XN and (K/H)(S/T)G, which define the active-site serine penicillin-recognizing enzymes family (for ASPRE) that also includes the class A and C β-lactamases. The serine of the SXXK motif is central to the catalytic mechanism. The serine attacks the carbonyl of the penultimate D-Ala amino acid of the stem peptide, which releases the last D-Ala amino acid from the 'donor' peptide and forms a covalent acyl–enzyme complex. In transpeptidases, the carbonyl of the D-Ala amino acid, now forming an ester linkage with the active site serine, then undergoes an attack from a primary amine linked in various ways to the third residue of a second 'acceptor' stem peptide. A peptide bridge is then created between two stem peptides, forming a link between glycan strands (Fig. 1a). In DD-carboxypeptidases, the acyl-enzyme intermediate is hydrolyzed; this process eliminates 'donor' stem peptides from the peptidoglycan.

β-Lactams mimic the D-Ala-D-Ala dipeptide in an elongated conformation, particularly regarding the distribution of three electrostatic-negative wells (Fig. 1b–d), and act as suicide inhibitors. The active site serine attacks the carbonyl of the β-lactam ring, resulting in the opening of the ring and formation of a covalent acyl-enzyme complex. This complex is hydrolyzed very slowly, thus effectively preventing further reactions.

The following kinetic model describes the reaction of PBPs with β-lactams. A noncovalent complex EI is formed between the enzyme E and the inhibitor I, with the dissociation constant K_d, from which acylation proceeds to form the covalent complex EI* with the rate k_2. EI* is hydrolyzed at the rate k_3 to regenerate E and an inactivated product P (Fig. 2). The rate described by k_3 is negligible on the time scale of a bacterial generation. The following nomenclature

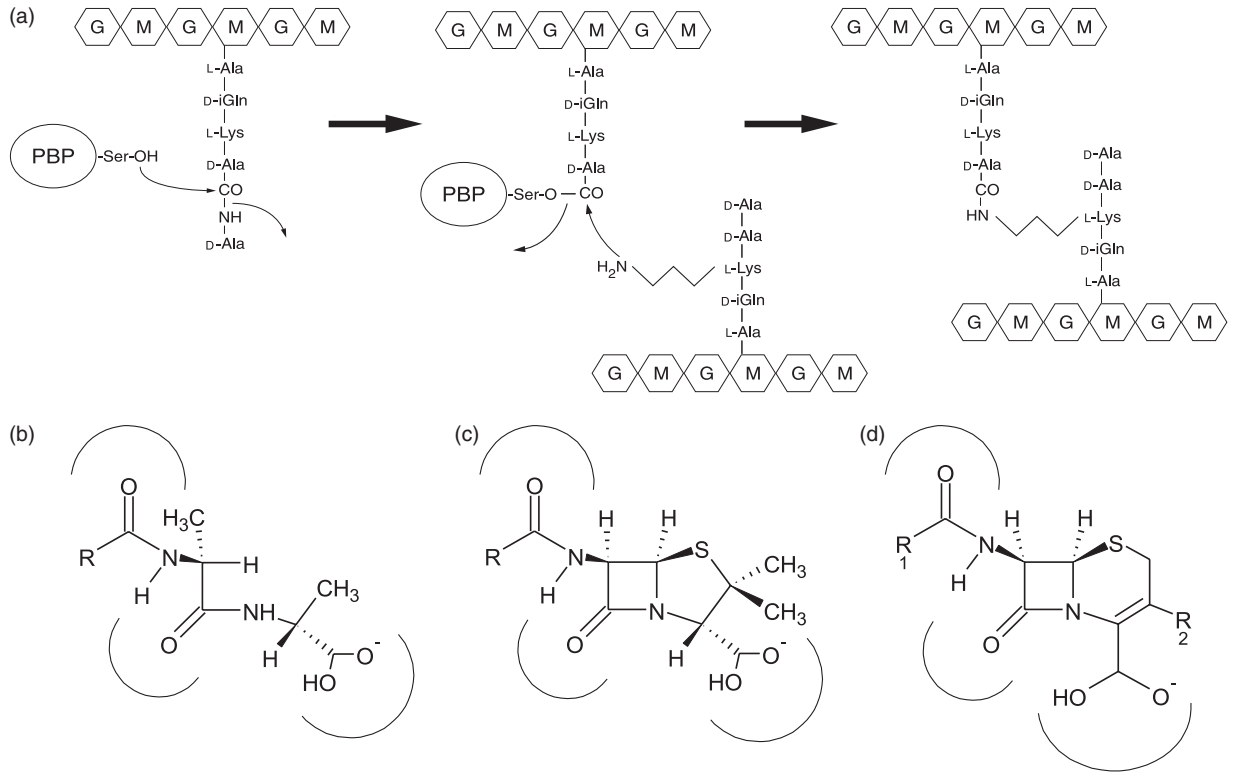

Fig. 1. (a) Catalysis of transpeptidation. Fragments of glycan strands are represented by chains of hexagones standing for the hexoses *N*-acetyl glucosamine (G) and *N*-acetyl muramic acid (M). The 'donor' pentapeptide is depicted on the upper glycan strand, whereas the 'acceptor' is attached here on the lower strand. The peptides shown are those from *Streptococcus pneumoniae*. The second and third amino acids may differ in various species. Note that in many instances, including in *Streptococcus pneumoniae*, various intervening amino acids are attached to the third residue of the acceptor peptide, and provide the free amine that attacks the acyl-enzyme intermediate. Such stem peptides are called 'branched'. Structural similarity between β-lactams and the natural substrate of the PBPs. (b) *N*-Acyl-D-Alanyl-D-Alanine peptide. (c) Penicillin backbone. (d) Cephalosporin backbone. The regions of negative electrostatic potential are indicated by arcs.

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EI \xrightarrow{k_2} EI^* \xrightarrow{k_3} E + P$$

$$K_d = k_{-1}/k_1$$

Fig. 2. Kinetic scheme of the reaction between a PBP (E) and a β-lactam (I). EI represents a preacylation noncovalent complex. EI* represents the covalent acyl-enzyme complex. P is the open inactivated product.

will be used throughout this review. The constants k_2 and k_3 describe the acylation and deacylation rates, respectively. The second-order rate constant k_2/K_d is the efficiency of acylation, which allows calculation of the overall acylation rate at a given antibiotic concentration. The inhibitory potency of a β-lactam for a PBP is given by the c_{50}, which is the antibiotic concentration resulting in the inhibition of half the PBP molecules at a steady state (when the acylation and deacylation reactions proceed at the same rate). The value of c_{50} is equal to the ratio $k_3/(k_2/K_d)$. Here, as in the literature in general, PBPs are said to be or to have high or low affinity for β-lactams. This affinity implicitly refers to the c_{50}, and should not be confused with the strength of a noncovalent interaction.

Despite detailed kinetic studies and several crystal structures of PBPs, the reaction mechanism is still a matter of debate, and likely differs between various ASPRE enzymes, and even for a single protein and different β-lactams (Oliva *et al.*, 2003).

PBP-based β-lactam resistance

Inhibition of PBPs produces an imbalance in cell wall metabolism, resulting in growth inhibition or lysis. The link between PBP inhibition and the biological outcome remains poorly understood [e.g. *Escherichia coli* (de Pedro *et al.*, 2002), *Staphylococcus aureus* (Giesbrecht *et al.*, 1998), *Enterococcus hirae* (Pucci *et al.*, 1986), *Streptococcus pneumoniae* (Mascher *et al.*, 2006)]. Despite the ignorance of the detailed consequences of β-lactam treatment, various means of resistance have been uncovered and investigated, such as a

decreased permeability of the outer membrane, export of the antibiotics (these mechanisms are restricted to gram-negative bacteria), degradation of the antibiotic by β-lactamases or utilization of PBPs with low affinity for the β-lactams. The following sections will be concerned with PBPs of organisms that exploit this latter strategy. Note that the emphasis of this review is mostly biochemical, and that the term resistance is often used to qualify sometimes modest decreases of susceptibility; the definition of resistance in this review is thus rather biological, meaning a decreased susceptibility resulting from selection, rather than medical according to susceptibility thresholds set by various national agencies.

Staphylococcus aureus

After the spread of *Staphylococcus aureus* strains resistant to penicillin through the acquisition of a β-lactamase, methicillin was introduced, which was not degraded by β-lactamases. A methicillin-resistant strain was isolated soon afterwards (Jevons *et al.*, 1963). The so-called methicillin–resistant *Staphylococcus aureus* (MRSA) strains are particularly dangerous as they exhibit blanket resistance to virtually all β-lactams, often associated with resistance to other classes of antibiotics.

The β-lactam resistance of MRSA strains results from the expression, in addition to the four native PBPs, of a fifth low-affinity PBP termed PBP2a or PBP2' (Hartman & Tomasz, 1984; Utsui & Yokota, 1985). Note that the expression of PBP2a (about 800 molecules cell^{-1}) does not alter the amount of the other PBPs (comprised individually between 150 and 450 molecules cell^{-1}) (Pucci & Dougherty, 2002). PBP2a is the product of the *mecA* gene whose transcription is controlled by the *mecI* and *mecR1* regulatory elements. MecI represses *mecA* transcription (Garcia-Castellanos *et al.*, 2004). By analogy with the homologous BlaI and BlaR1 system that controls the expression of the β-lactamase BlaZ, the Mec system is thought to function as follows (Zhang *et al.*, 2001): MecR1 is a signal-transduction protein with an extracellular penicillin-binding domain that senses the presence of β-lactams and activates its cytoplasmic domain. The intracellular domain of MecR1 is a protease that undergoes activation, which results directly or indirectly in the cleavage of the MecI repressor. The *mecA* gene and its regulatory system are found on a mobile genetic element called the staphylococcal cassette chromosome *mec* that integrates in the chromosome (Ito *et al.*, 2001). A detailed presentation of these genetic elements can be found elsewhere (Hiramatsu *et al.*, 2001; Ender *et al.*, 2004).

Interestingly, the intact *mec* system does not confer resistance, as the expression of PBP2a is normally well repressed. Only a few β-lactams can alleviate this repression. Mutations, for example in *mecI* or in the operator region, lead to de-repression of *mecA*. Even so, strains with unrestricted expression of PBP2a exhibit resistance only in a small subpopulation, when maintained without β-lactam selection. A homogenous resistant population is selected only upon exposure to β-lactams. When the antibiotic pressure is removed, heterogeneity is restored, indicating that the functioning of PBP2a in cell wall synthesis bears a cost that is best avoided in the absence of β-lactams. The genetic determinants of homogenous resistance in wild strains remain mysterious.

The class B PBP2a lacks the glycosyltransferase activity that is also required for peptidoglycan synthesis. Although PBP2a supports all the transpeptidase activity when this activity is inhibited by β-lactams in the four native PBPs, the presence of the glycosyltransferase domain of the class A PBP2 is nevertheless required (Pinho *et al.*, 2001a, b). Most surprisingly, the presence of the native class B PBP1 is also required, even though the transpeptidation can be carried out by PBP2a (Pereira *et al.*, 2007).

Other genes are necessary for the expression of the resistance conferred by PBP2a. Over 30 of these auxiliary genes, *aux* or *fem* (for *f*actor *e*ssential for *m*ethicillin resistance) have been identified (De Lencastre *et al.*, 1999). Several *fem* genes are involved in cell wall metabolism; other genes participate into putative regulatory or sensory functions. The *femAB* operon products, for example, add the second to fifth glycine residues to the peptidoglycan precursor to form the pentaglycine branch that serves as the cross-bridge of staphylococcal peptidoglycan (Henze *et al.*, 1993; Stranden *et al.*, 1997). The trivial expectation that PBP2a requires 'acceptor' peptides with a pentaglycine branch turned out to be naïve, for PBP2a can confer resistance to Enterococci, which lack *femAB* and have alternative peptidoglycan cross-bridges (Arbeloa *et al.*, 2004a).

PBP2a belongs to a subgroup of class B PBPs characterized by an insertion of about 100 residues following the transmembrane anchor. This group includes chromosomally encoded PBP5 from Enterococci, which are low-affinity PBPs involved in some degree of β-lactam resistance. Other members of this subgroup are present in *Bacillus subtilis* and related species, in *Listeria monocytogenes* and *innocua* and in *Clostridium acetobutylicum*, although these do not appear to confer reduced susceptibility to β-lactams. The origin of PBP2a remains mysterious. Close homologues have been found in susceptible and resistant *Staphylococcus sciuri* strains, and in resistant *Staphylococcus vitulinus*, *capitis* and *kloosii* (Wu *et al.*, 1998; Couto *et al.*, 2003; Schnellmann *et al.*, 2006) (Fig. 3). The *mec* system may thus have spread from a hitherto unidentified species, not only to *Staphylococcus aureus* but also to at least 14 other pathogenic or commensal staphylococcal species (Pierre *et al.*, 1990b; Yasuda *et al.*, 2000; Busscher *et al.*, 2006; Schnellmann *et al.*, 2006).

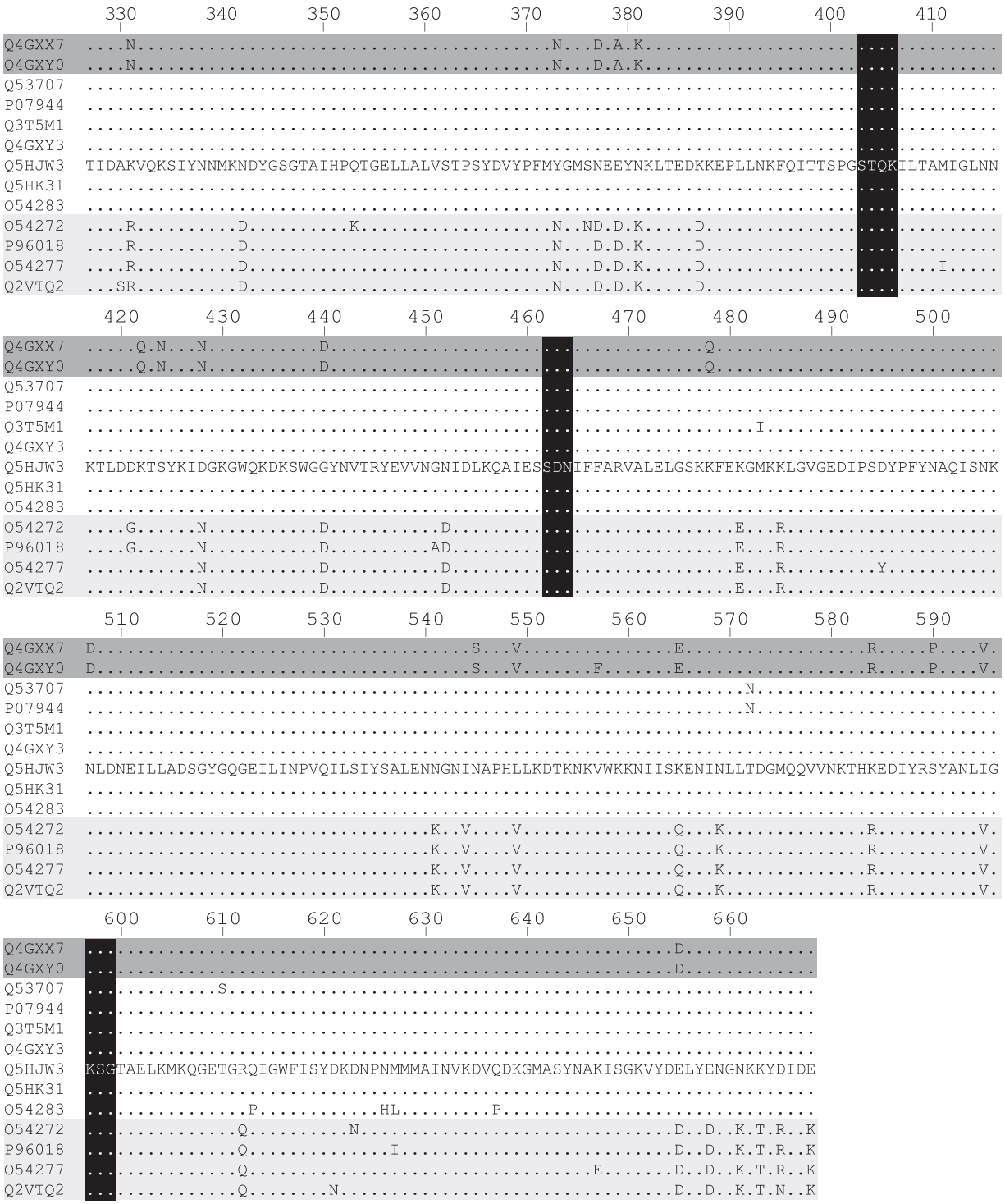

Fig. 3. Sequence alignment of staphylococcal PBP2a transpeptidase domains 327–668 (designated by their Uniprot accession numbers). Three types of sequences have been found. Sequences on a white background are from MRSA. The most commonly found sequence (Q5HJW3) in MRSA has also been isolated from *Staphylococcus kloosii, sciuri, vitulinus, haemolyticus, epidermidis* and *capitis*. The catalytic motifs are in black boxes. Sequences in dark gray were isolated from *Staphylococcus vitulinus*, *capitis* and *kloosii*. Sequences in light gray are from *Staphylococcus sciuri* strains.

PBP2a reacts with β-lactams extremely slowly. The acylation efficiency of PBP2a by penicillin G, characterized by the second-order rate constant $k_2/K_d = 15\,M^{-1}\,s^{-1}$, is one to three orders of magnitude slower than that of the four native PBPs from *Staphylococcus aureus* (Chambers *et al.*, 1994; Graves-Woodward & Pratt, 1998; Lu *et al.*, 1999). The acylation rate of PBP2a at therapeutic concentrations of β-lactams is thus negligible compared with the bacterial generation time ($t_{1/2}$ for acylation > 1 h with 10 μM of penicillin).

The low efficiency of acylation appears to result both from a poor 'true' affinity of PBP2a for the β-lactams, with dissociation constants (K_d) of the preacylation complex in the millimolar range, and extremely slow acylation rates (k_2) ranging from 0.2 to 0.001 s^{-1} (Lu *et al.*, 1999; Fuda *et al.*, 2004). The acylation rate k_2 of PBP2a by penicillin G, for example, is three orders of magnitude slower than that of the susceptible PBP2x from *Streptococcus pneumoniae* (Lu *et al.*, 1999, 2001).

The structure of a soluble form of PBP2a has been solved to a resolution of 1.8 Å (Lim & Strynadka, 2002). The active site of PBP2a appears to be somewhat closed without a bound antibiotic, with the active site S403 poorly positioned for a nucleophilic attack and a twisting of strand β3. The penicillin-binding domain of ASPRE proteins can be described as consisting of two subdomains, α-helical and α/β, surrounding the active site. The structures of PBP2a with bound antibiotics revealed a tilt of the whole helical subdomain with respect to the α/β-subdomain (O. Dideberg, pers. commun.). This rotation opens and substantially rearranges the active site. The Oγ of S403 is displaced by 1.8 Å (with nitrocefin), whereas strand β3 is straightened (Fig. 4). It has been argued that this rearrangement is costly and impedes acylation. Although possible, this and other explanations rely on the assumption that the conformation of the acyl-enzyme complexes are relevant to the transition state of the acylation reaction. In this regard, it must be remembered that there is a complete absence of correlation between the efficiency of acylation (k_2/K_d) and the strength of the noncovalent interaction between the covalently bound antibiotic and the PBP, as demonstrated with *Escherichia coli* PBP5 (Beadle *et al.*, 2001). Therefore, analysis of the complementarity of bound open antibiotics may bear little relevance to the understanding of the acylation process.

However, support for a substantial rearrangement of PBP2a upon acylation is provided by circular dichroism measurements that showed a diminution of the helix content with kinetics consistent with the acylation rates by several β-lactams (Fuda *et al.*, 2004, 2006). Binding of synthetic peptidoglycan fragments also caused rearrangements detected by circular dichroism (Fuda *et al.*, 2005). As binding of the peptidoglycan surrogate molecules led to an increase of the acylation rate by β-lactams, it has been proposed that the catalytic activity of PBP2a, and possibly of PBPs in general, might be stimulated allosterically by the peptidoglycan (Fuda *et al.*, 2004, 2006).

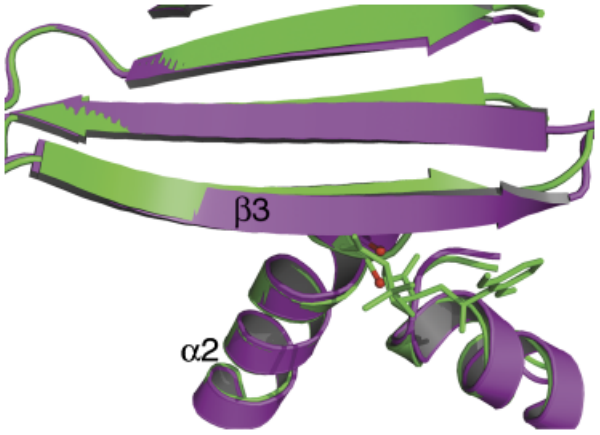

Fig. 4. Superposition of the active site of *Staphylococcus aureus* PBP2a without (magenta) and with (green) bound methicillin (shown green sticks). The Oγ of the catalytic S403 is shown as a red ball. Note the displacement of the active site serine and the conformational change of strand β3, which are further apart following acylation by methicillin. The figure was prepared using the PDB entries #1 MWU and #1 MVR (Lim & Strynadka, 2002).

In the past decade, several novel carbapenems and cephalosporins have been described that are good inhibitors of PBP2a, including some in clinical trials (e.g. Hebeisen *et al.*, 2001 and references therein; Fuda *et al.*, 2006; Page, 2006). Detailed kinetic studies have shown that the improved efficacy of three novel cephalosporins resulted from better preacylation binding, with K_d in the range of tens of micromolar, whereas the acylation rate k_2 was unchanged compared with other antibiotics (Fuda *et al.*, 2006).

Mildly resistant strains of *Staphylococcus aureus* have also been isolated that lack both *mecA* and β-lactamases. In one clinical strain, resistance to a small set of structurally related cephalosporin was correlated with poor binding to PBP2, while PBP3 was absent or did not react with penicillin (Georgopapadakou *et al.*, 1982). Alterations of penicillin binding by PBP1, PBP2 and an elevated amount of PBP4 were observed in some strains (Tomasz *et al.*, 1989; Henze & Berger-Bachi, 1996). The acylation rates of PBP1 and PBP2 were decreased, and the deacylation rates increased (Chambers *et al.*, 1994). The S569A and A576S substitutions were shown to cause a 10-fold decrease of the acylation efficiency of PBP2 (Hackbarth *et al.*, 1995). Another variant with the A450D and A462V substitutions surrounding the SXN motif and the Q629D mutation has a k_2/K_d reduced 20-fold (Hackbarth *et al.*, 1995). A laboratory mutant selected with ceftizoxime has the single substitution P458L close to the SXN catalytic motif (Leski & Tomasz, 2005).

Thus, *Staphylococcus aureus* has been found to resist β-lactams in three ways: using β-lactamases to degrade

the antibiotic, by lowering the affinity of its endogenous PBPs for β-lactams and most dangerously through the recruitment of an additional PBP that is unaffected by β-lactams.

Enterococci

Enterococci have an intrinsic low susceptibility or resistance to β-lactams. *Enterococcus faecalis* typically has minimum inhibitory concentrations (MICs) for penicillin of 2–8 mg L^{-1} (e.g. Perez *et al.*, 1987), and *Enterococcus faecium* of 16–32 mg L^{-1} (e.g. Moellering *et al.*, 1979). These important human pathogens have been the subject of intense molecular studies, together with *Enterococcus hirae*, which is more of a veterinary concern.

The intrinsic moderate resistance to β-lactams results from the presence of a particular high-molecular-weight (MW) class B PBP, which takes over the transpeptidase function of the other PBPs when these are inhibited by antibiotics (Fontana *et al.*, 1983, 1985). Three lines of evidence in early studies of an *Enterococcus hirae* strain and several derivatives (initially identified as *Streptococcus faecium* ATCC 9790) led to this conclusion. Firstly, one of the high-MW PBPs (PBP5) has a much lower affinity for penicillin, and spontaneous mutants with greater resistance have elevated amounts of this PBP (Fontana *et al.*, 1983). Secondly, a mutant hypersensitive to penicillin lacks PBP5 expression (Fontana *et al.*, 1985). Finally, saturation of PBP5 with β-lactams leads to bacterial death (Lleo *et al.*, 1987). Note that a peculiar strain of *Enterococcus hirae* were found to express a second low-affinity PBP of the same family, termed PBP3r, in addition to PBP5. In contrast to PBP5, PBP3r is plasmid-encoded (Raze *et al.* 1998).

Subsequent studies uncovered the same mechanism causing intrinsic β-lactam resistance in *Enterococcus faecium* (Williamson *et al.*, 1985; Sifaoui *et al.*, 2001) and *Enterococcus faecalis* (Duez *et al.*, 2001). Note that, confusingly, the low-affinity PBP of *Enterococcus faecalis* is sometimes termed PBP4. The elevated levels of resistance exhibited by clinical isolates of *Enterococcus faecium* was found to arise from increased expression of PBP5 and mutations of PBP5 that further decrease its affinity for β-lactams (Klare *et al.*, 1992; Fontana *et al.*, 1994). Strains with low susceptibility (MIC for ampicillin of 8 mg L^{-1}) appear to rely mainly on the overexpression of PBP5, the reasons for which are still unclear. Extremely resistant strains (MIC for ampicillin of up to 512 mg L^{-1}) appear to combine both overexpression and reduced affinity (Klare *et al.*, 1992; Fontana *et al.*, 1994) or use only the latter mechanism (Zorzi *et al.*, 1996; Rybkine *et al.*, 1998). Note that the exclusive use of the PBP5 transpeptidase, when the others are inhibited by β-lactams, does not modify the composition of the peptidoglycan cross-bridges (Sifaoui *et al.*, 2001).

Several point mutations in PBP5 were found to be correlated with a low affinity for β-lactams and high resistance of *Enterococcus faecium* (Fig. 5a, Table 1) (Ligozzi *et al.*, 1996; Zorzi *et al.*, 1996; Rybkine *et al.*, 1998). However, as isolates are not isogenic, assessment of the effect of various PBP5 sequences awaited their introduction into a single strain. When three PBP5 sequences from strains with MICs for ampicillin of 2, 24 and 512 mg L^{-1} were introduced in a strain with no PBP5 expression (MIC ampicillin of 0.03 mg L^{-1}), the resulting strains had MICs of 6, 12 and 20 mg L^{-1}, respectively (Sifaoui *et al.*, 2001). These results show that variants of PBP5 confer different MICs, but that this effect is strongly modulated by unknown factors (Sifaoui *et al.*, 2001; Jureen *et al.*, 2004). The particular mutation M485A was hypothesized to have a very important effect as it was found in two highly resistant strains and is located close to the second catalytic motif SXN482 (Zorzi *et al.*, 1996). When introduced individually, this mutation caused only a small increase of resistance, correlated with a modest decrease of the affinity for penicillin (Zorzi *et al.*, 1996; Sifaoui *et al.*, 2001; Rice *et al.*, 2004). Smaller effects resulted from three other mutations investigated individually: I499T, E629V and the introduction of an additional Ser466's. However, when combined with the other mutations, in particular M485A, the additional Ser466' caused a nearly threefold increase of the MIC for ampicillin (Rice *et al.*, 2004). This study also revealed that the various individual mutations tested had different effects on the MICs of different β-lactams.

Enterococcal PBP5 belongs to the same subgroup of class B PBPs as staphylococcal PBP2a (el Kharroubi *et al.*, 1991). The crystal structure of *Enterococcus faecium* PBP5 bound to penicillin was solved to a resolution of 2.4 Å (Sauvage *et al.*, 2002). The resistance of the originating strain (D63r, MIC for penicillin of 70 mg L^{-1}) appears to result from overproduction of the same PBP5 found in the parental strain (D63) (Zorzi *et al.*, 1996). The structure is therefore that of a 'wild-type' PBP5, without substitutions that further decrease the affinity for β-lactams. The efficiency of acylation by penicillin of D63r *Enterococcus faecium* PBP5 defined by the second-order rate constant $k_2/K_d = 20\ M^{-1}\ s^{-1}$ is similar to that of *Staphylococcus aureus* PBP2a, that is two to three orders of magnitude slower than that of a 'regular' high-affinity PBP (Zorzi *et al.*, 1996). Investigation of the apparent affinity and reaction rate of a variety of β-lactams with a soluble form of PBP5 has shown that the acylation is sensitive to the various substituents grafted onto the antibiotic, thus offering some scope for improving drugs against enterococci (Hujer *et al.*, 2005).

As no structure was obtained in the absence of antibiotic, no comment could be made regarding a possible rearrangement upon acylation, although some loop residues, which are conserved in this subgroup of PBPs (residues 461–465),

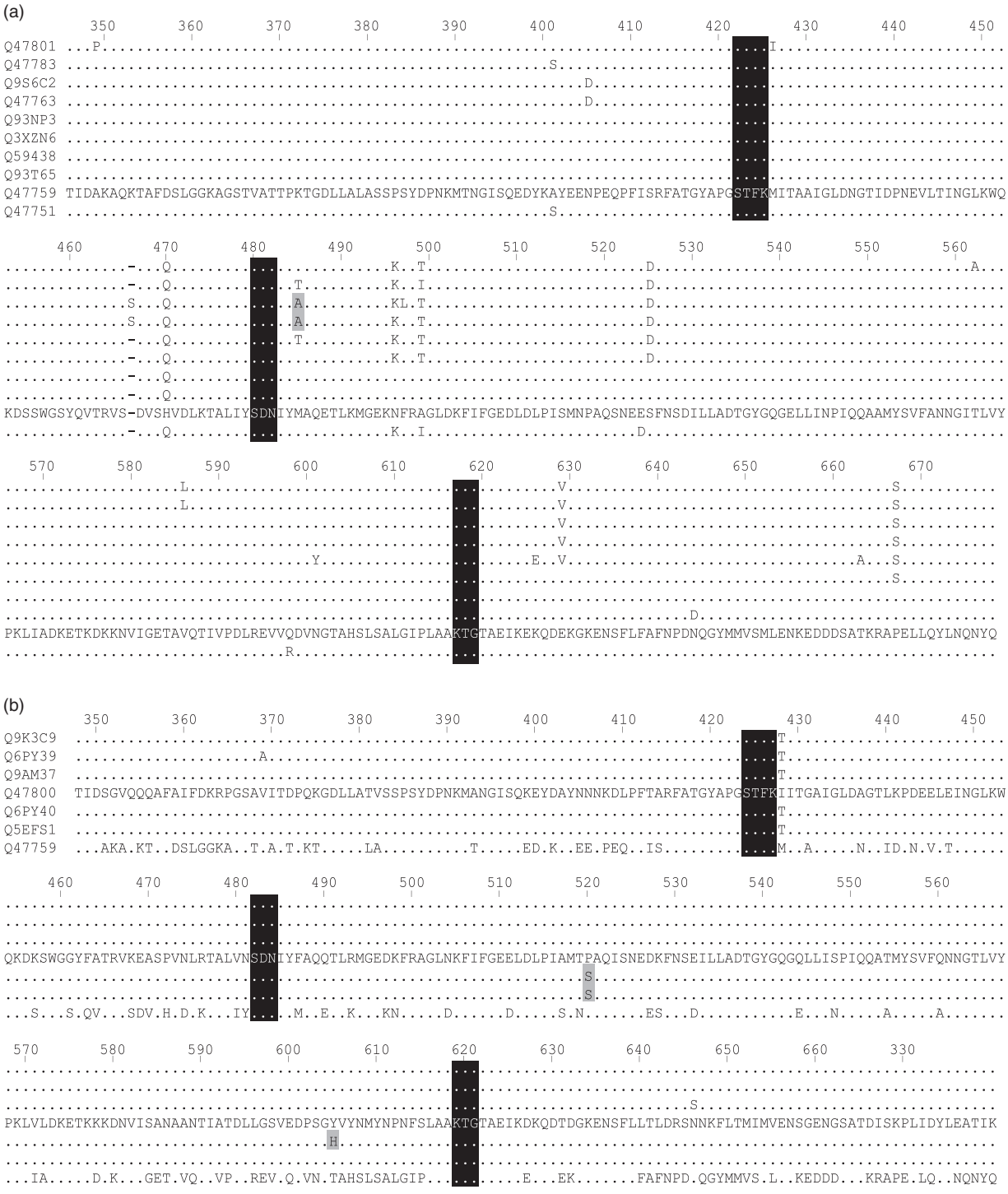

Fig. 5. Sequence variation of *Enterococcus faecium* PBP5 (a) and *Enterococcus faecalis* PBP4 (b) transpeptidase domain. An *Enterococcus faecium* PBP5 sequence (Uniprot number #47759) is also shown in (b) to illustrate the degree of divergence with *Enterococcus faecalis* PBP4. Catalytic motifs are blackened. The PBP5 M485A substitution that was shown to increase resistance is highlighted in gray. The PBP4 mutations in positions 520 and 605 associated with greater resistance are in gray.

Table 1. Characteristics of *Enterococcus faecium* strains and their PBP5, for which sequences are publicly available

Strain	MIC ($mg\,L^{-1}$)	Uniprot #	Expression level	k_2/K_d ($M^{-1}\,s^{-1}$)*	MIC ($mg\,L^{-1}$) conferred to a recipient strain†
BM4107	2 (Amp)†	Q93T65	Unchanged†		6
D366	16 (Pen)*	Q47751	Unchanged*	17	
D63	5 (Pen)*	Q47759	Unchanged*	24	
D63r	70 (Pen)*		Highly increased*	20	
D344	24 (Amp)†	Q93NP3	Highly increased*	17	12
	64 (Pen)*				
EFM-1	90 (Pen)*	Q47783	Unchanged*	1.5	
9439	128 (Amp)‡	Q47801	Increased§		
C68	256 (Amp)‖	Q9S6C2			
H80721	512 (Amp)†	Q47763	Unchanged*	< 1.3	20
	512 (Pen)*				

*Zorzi *et al*. (1996).
†Sifaoui *et al*. (2001).
‡Ligozzi *et al*. (1996).
§Fontana *et al*. (1994).
‖Rice *et al*. (2001).

may have been pushed aside (Sauvage *et al*., 2002). Based on this structure, explanations have been advanced for the various mutations that increase the resistance (Sauvage *et al*., 2002).

PBP5, as a class B PBP, does not support the necessary glycosyltransferase activity for peptidoglycan synthesis. Deletion studies in *Enterococcus faecalis* have demonstrated that the glycosyltransferase activity must be provided by at least one of the two class A PBPs encoded by *ponA* or *pbpF* (Arbeloa *et al*., 2004b).

Note also that four isolates of *Enterococcus faecalis* were found to exhibit high resistance to ampicillin and imipenem without overexpression of PBP4. Instead, the resistance is due to two substitutions, P520S and Y605H, in PBP4 (Ono *et al*., 2005). β-Lactamase-producing strains of *Enterococcus faecalis* have also been isolated in nosocomial infections (Murray, 1992), demonstrating again that several mechanisms can be exploited by the same pathogen.

Streptococcus pneumoniae

The expression of β-lactamases or additional low-affinity PBPs has never been reported in pneumococcus. Instead, β-lactam-resistant strains of *Streptococcus pneumoniae* always harbor modified versions of their own class A and class B PBPs that are poorly acylated by β-lactams (Hakenbeck *et al*., 1980; Zighelboim & Tomasz, 1980).

Of the six PBPs from *Streptococcus pneumoniae*, PBP1a, PBP2b, PBP2x and sometimes PBP2a were found to be altered in resistant clinical isolates. These modified PBPs bound less radio-labeled antibiotic, whereas the affinity of PBP1b and PBP3 was unchanged (Laible *et al*., 1991). Sequencing revealed that mosaic genes encode PBP2b (Dowson *et al*., 1989a), PBP2x (Laible *et al*., 1991) and PBP1a (Martin *et al*., 1992) in most resistant clinical strains. Mosaicity is the product of recombination events between different alleles within a species or between orthologous genes of related species.

Mosaic sequences of *pbp* genes are very difficult to classify and organize. Comparison of nucleotide sequences originating from susceptible strains show that they exhibit the same level of polymorphism as other loci, with one or two amino acid substitutions over the protein length (Dowson *et al*., 1989a; Laible *et al*., 1991). In contrast, mosaic *pbp* genes show blocks of sequences that differ from nonmosaic alleles by about 14–23% [PBP2b (Dowson *et al*., 1989a; Smith & Klugman, 1995); PBP1a (Martin *et al*., 1992); PBP2x (Laible *et al*., 1991)]. The diverging blocks span various lengths of the transpeptidase domain or even most of the extracellular domain. The degree of difference suggested that the diverging sequence blocks originate from other streptococcal species, and that multiple sources had been tapped by pneumococcal strains to survive antibiotic selection (Dowson *et al*., 1989a; Laible *et al*., 1991; Sibold *et al*., 1994). Multiple recombinational events in the history of individual *pbp* alleles further complicates the analysis, although favored sites of recombination can be identified (Sibold *et al*., 1994).

The origin of the sequence blocks found in mosaic *pbp* genes remains largely unknown, with some exceptions for *pbp2x*. Fragments of *pbp2x* sequences of susceptible *Streptococcus mitis* and *Streptococcus oralis* could be identified in many *pbp2x* alleles from resistant pneumococci (Sibold *et al*., 1994; Chi *et al*., 2007), although the identity is not perfect. This finding supports the following scenario for the

emergence of pneumococcal resistance. Commensal streptococci, such as *Streptococcus oralis* and *Streptococcus mitis*, have acquired resistance through point mutations selected by exposure to β-lactam treatment for various diseases. Fragments of genes encoding low-affinity PBPs were subsequently exchanged between related streptococcal species, including *Streptococcus pneumoniae*, and selected by antibiotherapy (Dowson *et al*., 1990). The recognition of these gene transfers in streptococci has led to the concept of a global gene pool of altered *pbp* sequences for β-lactam resistance (Reichmann *et al*., 1997).

Comparison of the PBP2x sequence of a drug-susceptible strain of *Streptococcus mitis*, and sequence blocks, derived from this ancestral sequence, but found in resistant pneumococcal strains identified a few substitutions that are likely important for the resistance (Chi *et al*., 2007). The role of some of these substitutions in the resistance has been verified experimentally [e.g. in positions 338, 339, 364, 369, 371, 605, (Smith & Klugman, 2005; Carapito *et al*., 2006)] , thus supporting the proposed scenario for the emergence of resistance.

Because *Streptococcus pneumoniae* can easily exchange genetic material, closely related strains can differ in capsular biosynthetic genes (hence serotype) and *pbp* genes. Conversely, identical *pbp* alleles or capsular biosynthetic genes can be found in unrelated strains (Coffey *et al*., 1991; Zhou *et al*., 2000). Nevertheless, despite the complications that horizontal gene transfers bring to the definition of pneumococcal lineage, it appears that the worldwide spread of pneumococcal β-lactam resistance results from the dispersion of a limited number of successful clones (McGee *et al*., 2001).

Besides recombination, point mutations that occurred in pneumococcus have certainly contributed to the resistance phenomenon. A case in point is the T550A substitution in PBP2x that confers resistance to cephalosporins but susceptibility to penicillin. This substitution was found in the laboratory (Grebe & Hakenbeck, 1996; Krauss *et al*., 1996; Sifaoui *et al*., 1996), as well as in PBP2x from clinical isolates where it was caused by a mutation either within a mosaic (Coffey *et al*., 1995) or a 'virgin' *pbp2x* gene (Asahi *et al*., 1999).

Selection in the laboratory demonstrated that PBP2x and PBP2b are the primary resistance determinants for cefotaxime and piperacillin, respectively (Laible & Hakenbeck, 1987; Grebe & Hakenbeck, 1996), suggesting that PBP2x and PBP2b are the essential PBPs most reactive towards cefotaxime and piperacillin, respectively. Indeed, cefotaxime does not react with PBP2b (Hakenbeck *et al*., 1987). Surprisingly, the point mutations selected in the laboratory do not match the substitutions of clinical isolates, with the exception of the aforementioned T550A in PBP2x (Grebe & Hakenbeck, 1996; Krauss *et al*., 1996; Sifaoui *et al*., 1996) and T446A in PBP2b (Grebe & Hakenbeck, 1996). This discrepancy could reflect limited sampling, or the most useful substitutions may be different in the native PBPs from *Streptococcus pneumoniae*, as selected in the laboratory, or in the PBPs from the commensal species where they were originally selected in their host.

Transfer to a susceptible strain of *pbp2x* genes from clinically resistant isolates decreases the susceptibility to cephalosporins and most penicillins (Dowson *et al*., 1989b; Laible *et al*., 1991; Reichmann *et al*., 1996; Smith & Klugman, 1998; Asahi *et al*., 1999; du Plessis *et al*., 2002; Chesnel *et al*., 2003; Pernot *et al*., 2004). Introduction of mosaic *pbp2b* genes causes a modest reduction of the susceptibility to piperacillin (Pagliero *et al*., 2004). Lower susceptibility is achieved on transfer of both mosaic *pbp2x* and *pbp2b* genes (Dowson *et al*., 1989b; Reichmann *et al*., 1996; Smith & Klugman, 1998; du Plessis *et al*., 2002). Still higher levels of resistance result from the additional introduction of a mosaic *pbp1a* gene (Barcus *et al*., 1995; Reichmann *et al*., 1996; Smith & Klugman, 1998; du Plessis *et al*., 2002; Chesnel *et al*., 2003). A high level of resistance restricted to the cephalosporins is obtained following transformation of a susceptible strain with mosaic *pbp2x* and *pbp1a* (Munoz *et al*., 1992; Coffey *et al*., 1995; Reichmann *et al*., 1996; Smith & Klugman, 1998; Chesnel *et al*., 2003). These observations can be rationalized by a threshold effect (Fig. 6).

These laboratory findings are mirrored in clinical strains (Sanbongi *et al*., 2004; Ubukata *et al*., 2004). Most resistant clinical isolates harbor three mosaic *pbp* genes encoding PBP1a, PBP2b and PBP2x (e.g. Ferroni & Berche, 2001; Overweg *et al*., 2001; du Plessis *et al*., 2002; Nagai *et al*., 2002; Nichol *et al*., 2002). However, some weakly resistant strains have mosaic alleles of *pbp2x* and *pbp2b* only (e.g. Ferroni & Berche, 2001; Nichol *et al*., 2002), and at least one strain has only *pbp2x* modified (Nichol *et al*., 2002). Some isolates with cephalosporin resistance, yet susceptible to penicillin, were found to have mosaic *pbp2x* and *pbp1a*

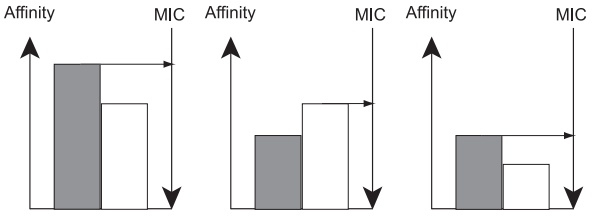

Fig. 6. Schematic representation of the threshold effect that may account for the relationship between the identity of the altered PBPs and the level of pneumococcal resistance. The PBP with the highest affinity for the β-lactam considered sets the susceptibility threshold of the recipient strain (horizontal arrow). The sequence of introduction of altered PBPs, which produces an incremental increase of resistance, depends on the relative affinities of the PBPs of the susceptible strain for a particular β-lactam. The depicted example could apply to PBP2x (gray) and PBP1a (white) with cefotaxime.

while retaining a 'virgin' *pbp2b* (McDougal *et al.*, 1995; Smith & Klugman, 2001).

By measuring the decrease in intrinsic fluorescence of a recombinant soluble form of PBP2x upon antibiotic binding, the overall acylation efficiency k_2/K_d was found to be between 60 000 and 110 000 $M^{-1}\,s^{-1}$ for penicillin (Jamin *et al.*, 1993; Mouz *et al.*, 1998; Di Guilmi *et al.*, 2000; Chesnel *et al.*, 2002). The deacylation rate k_3 measured in different ways is between 0.8 and 5 s^{-1} (Jamin *et al.*, 1993; Di Guilmi *et al.*, 2000; Lu *et al.*, 2001; Chesnel *et al.*, 2002). The very fast acylation and slow deacylation reactions result in a c_{50} that lies in the micromolar range, which is consistent with MIC of susceptible strains (Lu *et al.*, 2001; Chesnel *et al.*, 2003). Attempts have been made to delineate the dissociation constant K_d and the rate of acylation k_2 with penicillin. One study found a K_d of 0.9 mM and a k_2 of 180 s^{-1} (Lu *et al.*, 2001), whereas a second study reported a K_d of 20 mM and a k_2 of 1600 s^{-1} (Thomas *et al.*, 2001). Penicillin thus has a very poor 'true' affinity for PBP2x, a finding that presumably applies to β-lactams and PBPs in general. The efficacy of β-lactams does not result from a particularly good fit of the antibiotic to its target (K_d), but rather from the extremely high rate of acylation (k_2).

The crystal structure of PBP2x from the susceptible strain R6 was solved to a resolution of 2.4 Å (Pares *et al.*, 1996; Gordon *et al.*, 2000). The extracellular part of PBP2x consists of a transpeptidase domain, flanked by an elongated N-terminal domain and a small globular C-terminal domain. Regarding the precise mechanism of acylation by antibiotics, the crystal structure and theoretical studies have left the question open (e.g. Oliva *et al.*, 2003).

Identifying amino acid substitutions that are relevant to the reduction of affinity of a particular PBP is a difficult task. Owing to the process of recombination, neutral substitutions have likely been imported together with the ones that provide antibiotic resistance ('hitchhiking'). Indeed, even genes neighboring *pbp2b* or *pbp1a* have been incidentally modified through recombination of large DNA fragments (Coffey *et al.*, 1999; Enright & Spratt, 1999). Nevertheless, a number of likely important substitutions were proposed based on their absence in susceptible strains, presence in many resistant strains and proximity to the catalytic motifs: T338A, T338G, T338P and M339F found within the SXXK motif (Coffey *et al.*, 1995; Reichmann *et al.*, 1996; Mouz *et al.*, 1998; Asahi *et al.*, 1999), H394Y and M400T that surround the SXN motif (Laible & Hakenbeck, 1987; Coffey *et al.*, 1995; Nagai *et al.*, 2002; Nichol *et al.*, 2002) and L546V, T550A and Q552E, which are close to the KTG motif (Coffey *et al.*, 1995; Asahi *et al.*, 1999; Mouz *et al.*, 1999; Ferroni & Berche, 2001). These susbstitutions do not appear randomly, and some sequence families can be identified.

Examination of about one hundred public sequences of the transpeptidase domain of PBP2x reveals three broad families. An alignment is provided in Fig. 7 to convey graphically a sense of the diversity of sequences, and of the difficulty of classifying those sequences resulting from the mosaicity. One family contains nonmosaic sequences. The mosaicity complicates the two other families and the grouping would differ for various sequence blocks. Nevertheless, the pattern emerging from a minimal overlap of substitutions in the transpeptidase domain suggests that two mechanisms have been selected to reduce the affinity of PBP2x for β-lactams (Pernot *et al.*, 2004).

One family of sequences is characterized by the T338A substitution. A subset of these sequences also have the adjacent M339F substitution. These latter sequences are from strains with particularly high levels of resistance (Coffey *et al.*, 1995; Asahi *et al.*, 1999; Ferroni & Berche, 2001; Nagai *et al.*, 2002; Chesnel *et al.*, 2003; Sanbongi *et al.*, 2004). PBP2x with the T338A/M339F substitutions have an efficiency of acylation by penicillin reduced more than 1000-fold (Lu *et al.*, 2001; Chesnel *et al.*, 2003), mostly due to slower acylation (k_2 decreased 300-fold), although weaker preacylation binding (K_d fourfold higher) also contributes to the extremely poor affinity (Lu *et al.*, 2001; Chesnel *et al.*, 2003). These PBP2x variants also have significantly faster deacylation kinetics (k_3 increased 40–70-fold), an effect mostly due to the M339F substitution (Di Guilmi *et al.*, 2000; Chesnel *et al.*, 2003). The slow acylation and fast deacylation combine to elevate the c_{50} (concentration of antibiotic resulting in the steady-state acylation of half the enzyme) by four to five orders of magnitude (Lu *et al.*, 2001; Chesnel *et al.*, 2003).

The individual reversion of the 41 substitutions of a transpeptidase domain of PBP2x from a particularly resistant strain uncovered the role of four mutations, I371T, R384G, M400T and N605T, in addition to those in positions 338 and 339 (Carapito *et al.*, 2006) (Fig. 8). Reversion of the six substitutions nearly restored a normal and rapid acylation rate. Introduction of five of the substitutions into PBP2x from a susceptible strain diminished the reactivity with β-lactams, almost to the level of the original PBP2x from the resistant strain. These effects measured *in vitro* were mirrored by the expected phenotypic consequences *in vivo* (Carapito *et al.*, 2006). With a different PBP2x, a similar experiment where the effect of individual reversions was directly monitored *in vivo* also identified positions 371 and 384 as important (Smith & Klugman, 2005). A surprising discrepency is the identification of Y595F as important, but not of N605T, into the *in vivo* study (Smith & Klugman, 2005), whereas the opposite was found in the *in vitro* screening (Carapito *et al.*, 2006).

The side chain of T338 is pointing away from the active site cavity and is hydrogen bonded to a buried water molecule. It has been proposed that suppression of the hydrogen bonding by replacement of T338 can lead to

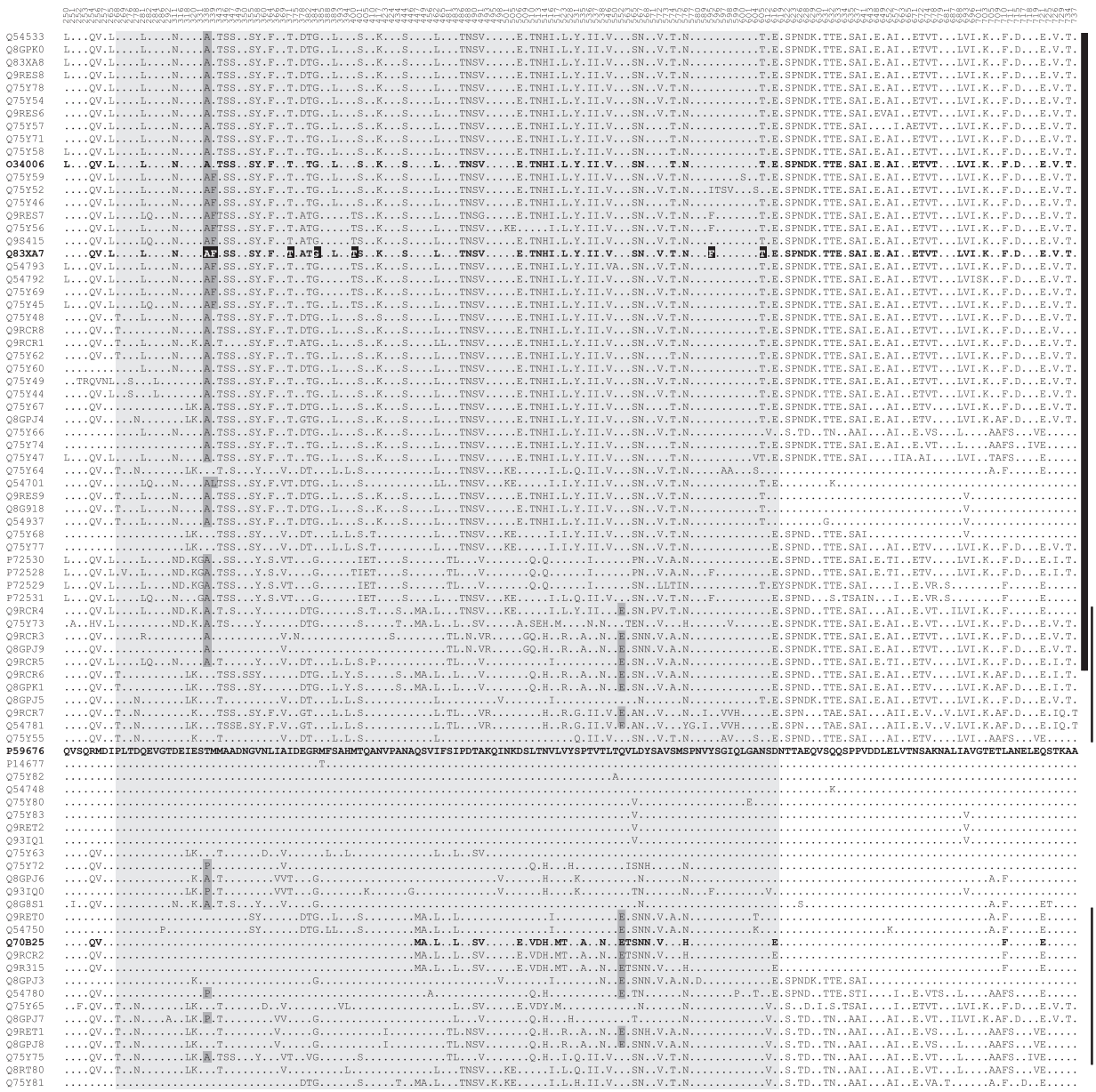

Fig. 7. Alignment of PBP2x publicly available sequences (aligned and clustered with CLUSTALW). Only positions where at least one sequence differs from the R6 reference (Uniprot accession number #P59676) are shown. The tranpeptidase domain is in light gray. Substitutions at positions 338, 339 and 552 are highlighted in dark grays. Although the mosaicity confounds efforts to classify these sequences unambiguously, this representation allows to visualize that sequences characterized by a mutation in position 338 (denoted by the thick black line on the right of the alignment) differ substantially from sequences with the Q552E substitution (denoted by the thin black on the right of the alignment), although a few sequences harbor both mutations. The absence of a line on the right of the alignement denotes nonmosaic sequences or sequences with few substitutions. The crystal structures of the high-affinity PBP2x from strains R6 (# P59676), as well as the two low-affinity proteins from strains Sp328 (# O34006) and 5259 (# Q70B25) have been solved, revealing two modes of reducing the affinity for β-lactams. The corresponding sequences are in bold characters. Also in bold is PBP2x from strain 5204 (# Q83KA7) with substitutions experimentally identified as contributing to the resistance highlighted in black.

destabilization of the active site due to the loss of the water molecule (Mouz *et al.*, 1998). Resolution of the structure of PBP2x from the resistant strain Sp328, which belongs to the family defined by the T338A substitution, has confirmed the absence of the buried water molecule (Dessen *et al.*, 2001).

The M339F mutation alone, introduced in the reference R6 PBP2x, reduces the efficiency of acylation by penicillin by sixfold and reduces the susceptibility (Chesnel *et al.*, 2003). A combination of the M339F and T338A mutations produces a greater effect. The structure of the latter double

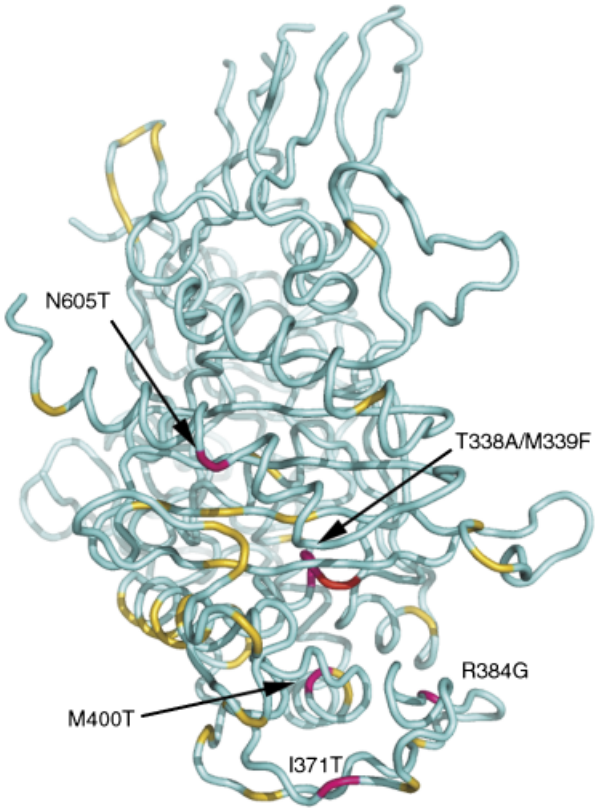

Fig. 8. Overall structure of the transpeptidase domain of PBP2x from strain R6, showing the distribution of the 41 positions substituted in the β-lactam-resistant strain 5204. The position of the active site S337 is in red. Positions found to affect the efficiency of acylation experimentally are in magenta.

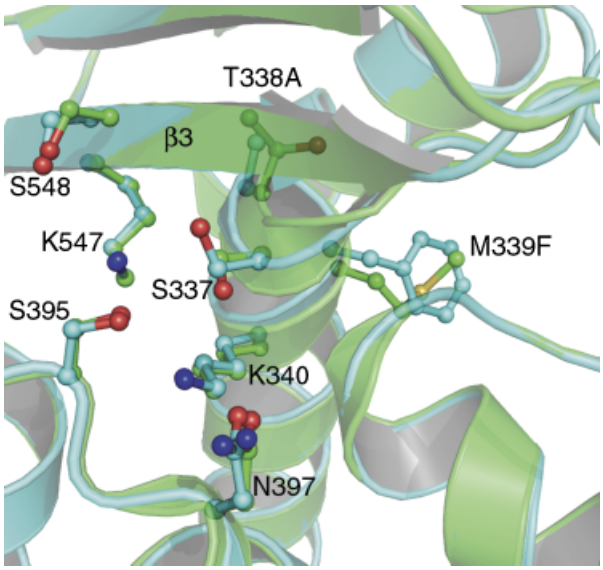

Fig. 9. Superposition of the active site of wild-type R6 PBP2x (green) and of the double mutant T338A/M339F (cyan). Residues from the catalytic motifs are shown along the mutated amino acids. Note that the hydroxyl of the catalytic S337 is pointing towards strand β3 in the mutant, whereas it is pointing to the active site cavity in the wild-type enzyme.

mutant has been solved to a resolution of 2.4 Å. The salient feature of the mutated active site is the reorientation of the hydroxyl of the catalytic S337 that is now pointing away from the active site center and is hydrogen-bonded to the main chain nitrogen of T550 instead of to K340 (Chesnel *et al.*, 2003) (Fig. 9).

Mutations I371T and R384G impart a great flexibility to the loop spanning residues 365–394, as noted from the structure of Sp328. This instability extends in part to the SXN motif in positions 395–397, with S395 being somewhat displaced. The flexibility of this region generates a more accessible 'open' active site that may better accommodate alternative physiological substrates with branched stem peptides (Dessen *et al.*, 2001). Mutations M400T and N605T certainly have indirect effects as they are quite far from the active site.

A second family of PBP2x molecules from resistant strains can be defined by the presence of the Q552E substitution. Introduction of this single substitution into PBP2x reduces about fourfold the efficiency of acylation and modestly reduces the susceptibility of the recipient R6 strain (Mouz *et al.*, 1999; Pernot *et al.*, 2004). The structure of a PBP2x from a clinical strain that possesses the Q552E substitution has been solved to a resolution of 3 Å. This PBP2x has an efficiency of acylation reduced more than 15-fold (Pernot *et al.*, 2004). The only significant difference found in comparison with the structure of R6 PBP2x is the displacement of strand β3, which carries the KTG motif (Pernot *et al.*, 2004). This displacement of 0.5 Å narrows the active site, and is reminiscent of the closed conformation of PBP2a from *Staphylococcus aureus* (Lim & Strynadka, 2002). In addition to this conformational effect, the introduction of a negative charge into position 552 greatly affects the entry of the active site and does not favor binding of β-lactams, which are negatively charged (Pernot *et al.*, 2004).

Consequently, it appears that two distinct mechanisms have been selected that reduce the reactivity of PBP2x towards β-lactams. One mechanism primarily affects the chemistry of the active site S337, whereas the second mechanism hinders acylation by requiring an opening of the active site. These two mechanisms may be a reflection of two major sources of exogenous genetic material that have been incorporated into strains of *Streptococcus pneumoniae*. Note that a few sequences of PBP2x have both T338A and Q552E substitutions and may thus combine the effect of both mechanisms.

Another significant substitution is T550A, which confers resistance to cephalosporins only, both in laboratory and clinical strains (Coffey *et al.*, 1995; Grebe & Hakenbeck,

1996; Sifaoui *et al.*, 1996; Asahi *et al.*, 1999). When the T550A point mutation occurs within a mosaic PBP2x, which contains the T338A/M339F double mutation, it further increases the resistance to cephalosporins, while it almost abolishes resistance to penicillin (Coffey *et al.*, 1995). This effect is mirrored in the acylation efficiency of a T550A point mutant of R6 PBP2x, which is decreased 20-fold towards cefotaxime and unaffected towards penicillin (Mouz *et al.*, 1999). This effect has been rationalized by the abolition of the hydrogen-bond between T550 and the carboxylate that is attached to the six-member ring of second- and third-generation cephalosporins (Gordon *et al.*, 2000).

Note that ceftobiprole, a novel cephalosporine active against MRSA, shows great promise for the treatment of infections caused by *Streptococcus pneumoniae* with resistance to most β-lactams. Ceftobiprole MICs are not $> 1\,mg\,L^{-1}$, even with strains that have altered PBP2x, PBP2b and PBP1a, including PBP2x with the major substitutions T338A, M339F, I371T, R384G and M400T (Davies *et al.*, 2006). Whether ceftobiprole reacts with PBP2x molecules that have the cephalosporine-specific mutation T550A remains to be established.

PBP2b, the other class B PBP from *Streptococcus pneumoniae*, has not been subjected to such thorough investigations, presumably because of the absence of a high-resolution structure. Over 90 sequences are available and two substitutions, T446A or T446S and E476G, are always found in PBP2b from clinically resistant strains. The probable importance of these two substitutions was pointed out in numerous studies (Dowson *et al.*, 1989b; Smith & Klugman, 1995; Ferroni & Berche, 2001; Sanbongi *et al.*, 2004). The T446A mutation, which is immediately adjacent to the SXN motif, is also selected by piperacillin in the laboratory (Grebe & Hakenbeck, 1996). T446A is the only substitution that has been characterized biochemically (Pagliero *et al.*, 2004) and it reduces the affinity for penicillin by 60%. The affinity of various PBP2b molecules from clinical isolates with six to 43 mutations in addition to T446A is reduced by 90–99%.

In addition to the mutations in positions 446 and 476, some PBP2b sequences are distinguished by other salient features such as the substitution of six to seven adjacent residues at position 426/427–432 (Dowson *et al.*, 1989a; Smith & Klugman, 1995). Three related PBP2b sequences from Japanese isolates are noteworthy by the insertion of three residues (SWY) after position 422 (Yamane *et al.*, 1996). This is one of two occurrences of a change in the number of residues in a mosaic PBP. The other case was found in PBP1a. In all other cases, the total length of the proteins and the position of the catalytic motifs are fully conserved, despite extensive sequence remodeling. Seven related sequences from Korean clinical strains show a substitution within the third catalytic motif KTG, which is changed to KSG (Song *et al.*, 2000). In contrast to PBP2x and PBP1a, where mutations within the first catalytic motif are commonplace, a single case was reported of a V388A substitution within the SVVK motif (Kell *et al.*, 1993). The recent emergence of strains that show a particularly high resistance to amoxicillin, relative to other β-lactams, appears to result from a set of 10 susbstitutions in the region 591–640 surrounding the third catalytic motif KTG (du Plessis *et al.*, 2002; Kosowska *et al.*, 2004). The relative importance of these and other mutations for the resistance awaits investigation, and mechanistic insight will require the resolution of the crystal structure of PBP2b from susceptible and resistant strains.

PBP1a may be considered as the most important and troublesome PBP clinically. Indeed, the resistance potentially provided by mosaic PBP2x and PBP2b is capped by the presence of a 'virgin' PBP1a, which still warrants some efficacy to β-lactam therapy. A high level of resistance depends on a modified PBP1a. About 50 PBP1a sequences are publicly available. The T371A substitution within the first catalytic motif, analogous to the T338A mutation in PBP2x, is commonly found in PBP1a sequences from resistant strains (Asahi & Ubukata, 1998; Smith & Klugman, 1998; Ferroni & Berche, 2001; Nagai *et al.*, 2002; Nichol *et al.*, 2002) (Fig. 10). Reversion of this substitution reduced

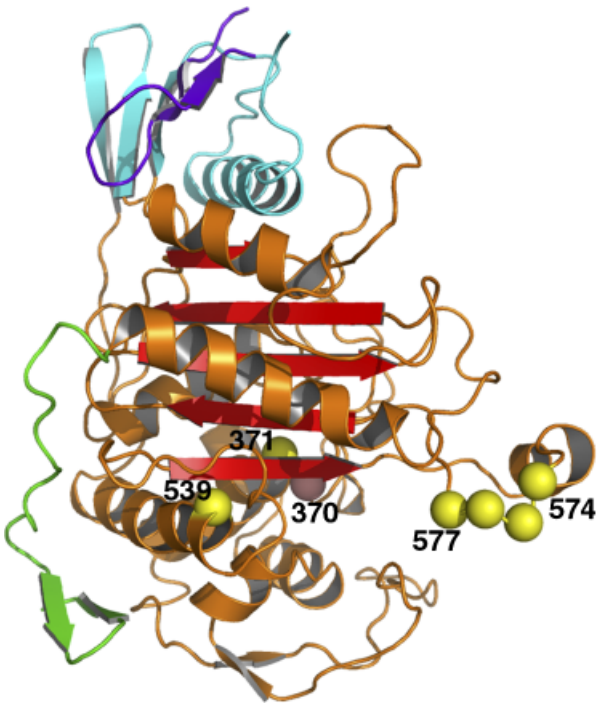

Fig. 10. Overall structure of the transpeptidase domain of PBP1a. The position of the active site S370 is shown with a red sphere. Yellow spheres mark the positions of substitutions that are important for resistance, T371A and TSQF(574-577)NTGY.

but did not abrogate the resistance that PBP1a confers in addition to PBP2x and PBP2b (Smith & Klugman, 1998). Some mosaic sequences lack the T371A mutation, including PBP1a from a highly resistant Hungarian isolate (MIC for penicillin of 16 mg L^{-1}) (Smith & Klugman, 2003). Another remarkable feature is the mutation of a stretch of four residues (TSQF to NTGY) at positions 574–577, which is observed in all the mosaic sequences (Fig. 10). Reversion of this set of substitutions decreased the additional resistance conferred by PBP1a (Smith & Klugman, 2003). A similar effect of the reversion was found for the L539W substitution, although the sequence in which the experiment was performed is the only one that presents this particular mutation (Smith & Klugman, 2003). Much remains to be learned about the detailed mechanism by which the reactivity of PBP1a is reduced.

Although PBP2x, PBP2b and PBP1a are the major PBPs responsible for the resistance of *Streptococcus pneumoniae*, a number of studies have hinted at the possible involvement of various other PBPs. Transfer of a high level of resistance from a strain of *Streptococcus mitis* to a laboratory strain of *Streptococcus pneumoniae* was shown to require transfer of the genes encoding the five high-MW PBPs (Hakenbeck *et al.*, 1998). A point mutation in the low-MW PBP3 was found to contribute to the resistance of a strain selected on cefotaxime in the laboratory (Krauss *et al.*, 1996). In contrast to these laboratory experiments, examination of the PBPs from clinical isolates failed to reveal significant modification of PBP1b or PBP3 (Du Plessis *et al.*, 2000; Sanbongi *et al.*, 2004). Early studies, which examined various strains through the labeling of PBPs with radioactive penicillin, found several instances where binding to PBP2a was diminished in resistant strains (Laible *et al.*, 1991; Reichmann *et al.*, 1997). Also, transfer in the laboratory of resistance from a *Streptococcus mitis* strain to *Streptococcus pneumoniae* involved modification of PBP2x, PBP2b, PBP1a and PBP2a, but not of PBP1b and PBP3 (Reichmann *et al.*, 1996). Various combinations of point mutations, including silent ones, were observed in some PBP2a sequences, suggesting events of intraspecies recombination (Chesnel *et al.*, 2005). The role of PBP2a in β-lactam resistance is now firmly established in at least one instance (Smith *et al.*, 2005). A strain isolated from an AIDS patient was found to harbor a mosaic PBP2a in addition to mosaic PBP2x, PBP2b and PBP1a. Transformation experiments demonstrated that this PBP2a variant is indeed responsible for an elevated resistance to various β-lactams. The sequence shows 25 substitutions including 12 within the transpeptidase domain. The absence of a crystal structure precludes a detailed analysis, but it is noteworthy that the threonine following the catalytic serine is replaced by an alanine, like in numerous variants of PBP2x and PBP1a.

Both class B PBPs, PBP2x and PBP2b, are essential in *Streptococcus pneumoniae*, which is consistent with the selection of variants of these proteins by β-lactams (Kell *et al.*, 1993). PBP1b and PBP3 are not essential (Schuster *et al.*, 1990; Hoskins *et al.*, 1999), which is again consistent with the fact that these proteins are not involved in the resistance process. PBP1a and PBP2a are not essential individually, but one of them must be present and functional (Hoskins *et al.*, 1999; Paik *et al.*, 1999). The fact that PBP1a, rather than PBP2a, is the main target of antibiotic selective pressure may be due to PBP2a having a low intrinsic affinity for β-lactams (Zhao *et al.*, 2000).

A puzzling discovery was made, which is directly related to PBP-based β-lactam resistance. Clinically resistant isolates have an abnormal peptidoglycan structure with an elevated proportion of cross-bridges that involve branched stem-peptides (Garcia-Bustos & Tomasz, 1990). Instead of having the L-Lys of the 'acceptor' peptide cross-linked directly to the D-Ala of the 'donor' peptide, there are intervening L-Ala-L-Ala or L-Ala-L-Ser dipeptides. The genetic determinants of this cell wall abnormality could nevertheless be separated from the resistance determinants (the mosaic *pbp* genes) (Severin *et al.*, 1996). The genes responsible for the synthesis of branched precursors were found to constitute the *murMN* operon (Filipe & Tomasz, 2000), also known as the *fibAB* operon (Weber *et al.*, 2000). Mosaic *murM* genes often increase the resistance level conferred by a set of mosaic *pbp* genes (Filipe & Tomasz, 2000; Smith & Klugman, 2001). A naïve explanation is that mosaic PBPs prefer branched substrates. However, deletion of *murM* abolishes the resistance but does not impact the growth rate in the absence of antibiotic challenge (Filipe & Tomasz, 2000), demonstrating that mosaic PBPs can efficiently use linear precursors. The situation is reminiscent of the role of the *femAB* operon in *Staphylococcus aureus*, which is required for expression of *mecA*-based resistance, while the *mecA*-encoded PBP2a can nevertheless function with alternative substrates produced in the absence of *femAB* (Stranden *et al.*, 1997; Arbeloa *et al.*, 2004a). It has been proposed that branched stem-peptides may be superior competitors against β-lactams for the active site of some PBPs of resistant strains, or that they may be involved in some signaling function of cell wall metabolism, or that they play a particular role in the integrity of the peptidoglycan, a role that becomes critical when some PBPs are inhibited by antibiotics (Filipe & Tomasz, 2000).

Besides MurM, other unknown factors modulate β-lactam resistance. Indeed, five clinical isolates with significantly different levels of resistance were found to have the same MurM allele and strictly identical sequence of their penicillin-binding domains, for the six PBPs (Chesnel *et al.*, 2005). For example, a functional two-component signal-transducing system CiaRH is necessary to tolerate

low-affinity PBP2x variants, even in the absence of antibiotic challenge (Mascher *et al*., 2006).

Although much is known about the biochemistry of the PBPs, the MurM and CiaRH complications highlight the limited understanding of the physiological function of the PBPs in cell wall metabolism, both in the absence and in the presence of antibiotics.

Neisseria

Neisseria meningitidis and *Neisseria gonorrhoeae* have acquired reduced susceptibility to penicillin via two routes: β-lactamase production (Bergström *et al*., 1978) and modified PBPs. The modification of at least one chromosomally encoded PBP will be discussed below.

Neisseria contain only three PBPs called PBP1, PBP2 and PBP3, respectively of class A, of class B and a low-MW carboxypeptidase. Gonococcal strains with reduced susceptibility to β-lactams without β-lactamase were found to exhibit reduced labeling of PBP2 and PBP1 by radiolabeled penicillin (Dougherty *et al*., 1980). The full level of resistance of a donating clinical strain could be conferred to a susceptible recipient strain upon transformation, resulting in diminished binding of radiolabeled penicillin to PBP1 and PBP2 (Dougherty, 1986). Reduced labeling of PBP2 was observed in meningococci (Mendelman *et al*., 1988). PBP2 is encoded by the *penA* gene, which is mosaic in resistant strains of *N. gonorrhoeae* (Spratt, 1988) and *N. meningitidis* (Spratt *et al*., 1989). The mechanism of the acquisition of nonplasmidic resistance in *Neisseria* is therefore similar to that of *Streptococcus pneumoniae*.

The origin of the sequence fragments that are found in mosaic *penA* genes of gonococci and meningococci has been investigated in some depth. Several commensal species, such as *Neisseria flavescens*, *Neisseria cinerea* or *Neisseria perflava*, appear to have each contributed sequence blocks (Spratt *et al*., 1989, 1992; Bowler *et al*., 1994; Ameyama *et al*., 2002). *Neisseria flavescens* isolates from the preantibiotic era are relatively resistant to penicillin and have a PBP2 with an intrinsic low affinity for penicillin (Bowler *et al*., 1994). Transfer in the laboratory of the *penA* gene from such *N. flavescens* isolates could indeed reduce the susceptibility of *N. meningitidis* (Bowler *et al*., 1994). In contrast, *N. cinerea* is not naturally resistant, and accordingly, no resistance was conferred upon transfer of the *penA* gene from this species (Bowler *et al*., 1994). Instead, PBP2 sequences of *N. cinerea* found in resistant meningococci have an additional aspartic acid following D345, which is not present in susceptible *N. cinerea* strains (Bowler *et al*., 1994). This insertion was also found in mosaic PBP2 sequences from most resistant gonococcal strains. It is sufficient to decrease the reactivity of PBP2 for β-lactams and to confer some resistance to *N. gonorrhoeae* (Brannigan *et al*., 1990). A clinically resistant strain was later discovered that only has this additional aspartic acid (Ito *et al*., 2005). The sequence identity between *Neisseria* PBP2 and PBPs of known structures is too low to obtain a reliable alignment. Nevertheless, by simply aligning the SXXK and SXN motifs, it appears that the insertion following position 345 is in a region close in space from the SXN motif. This loop was found to be destabilized in a low-affinity PBP2x of *Streptococcus pneumoniae* (Dessen *et al*., 2001).

Thus, *penA* alleles conferring penicillin resistance have arisen both from the recruitment of sequence blocks from naturally resistant species, such as *N. flavescens*, and new mutations such as a codon insertion. As commensal *Neisseria* species readily exchange genetic material, the *penA* alleles conferring resistance may be considered as forming a common gene pool shared by several species (Saez-Nieto *et al*., 1990; Lujan *et al*., 1991).

The peptidoglycan composition is considerably altered in a gonococcal strain with altered PBP1 and PBP2, even in the absence of antibiotics (Dougherty, 1985). The cell wall of a meningococcal strain with altered *penA* alleles has a greater amount of unprocessed pentapeptides, suggesting that the transpeptidase and/or carboxypeptidase activity of low-affinity PBP2 is modified (Garcia-Bustos & Dougherty, 1987; Antignac *et al*., 2003).

Early studies showed that PBP1, the class A PBP, also had decreased reactivity for penicillin in gonococci (Dougherty *et al*., 1980), but subsequent studies failed to uncover mosaicity in the *ponA* gene encoding PBP1. More recently, an allele of *ponA* encoding PBP1 with the single substitution L421P was found to contribute to the high resistance of some *N. gonorrhoeae* strains (Ropp *et al*., 2002). The L421P substitution was shown *in vitro* to diminish about fourfold the acylation efficiency of PBP1 by various β-lactams (Ropp *et al*., 2002).

Note that three non-*pbp* loci have been found to contribute to β-lactam resistance in *Neisseria* species. The *mtr* locus encodes an efflux pump (Hagman *et al*., 1995), while *penB* codes for a porin (Gill *et al*., 1998). The nature of the third locus *penC*, which is required to allow phenotypic expression of the *ponA* mutation, remains undetermined (Ropp *et al*., 2002).

Other pathogens

Modified PBPs to resist β-lactams have been found in a few other pathogens, including species where the most frequent mode of resistance is the production of a β-lactamase.

Resistant clinical isolates of *Haemophilus influenzae* usually evade the action of β-lactams by producing one of two β-lactamases (Tristram *et al*., 2007). However, the number of β-lactamase-negative ampicillin-resistant (BLNAR) strains is increasing, particularly in Japan (Hasegawa *et al*.,

2004). First documented in 1980 (Markowitz, 1980), BLNAR strains express low-afinity PBP3, the division-specific class B PBP (Mendelman *et al.*, 1984; Parr & Bryan, 1984; Malouin *et al.*, 1987; Clairoux *et al.*, 1992).

Sequencing revealed in excess of 20 mutation patterns for the transpeptidase domain of PBP3, with a number of mutations ranging from one to nine, affecting 21 different positions (Ubukata *et al.*, 2001; Dabernat *et al.*, 2002; Kaczmarek *et al.*, 2004). These sequences are not mosaic but show an accumulation of point mutations. Some sequences are characterized by the presence of an R517H substitution, while others have the N526K mutation. Both substitutions are relatively close to the third KTG514 catalytic motif. Position 517 with respect to the KTG motif is analogous to the position 552 in PBP2x from *Streptococcus pneumoniae*, which is also mutated in some strains (Pernot *et al.*, 2004). Sequences that contain the N526K substitution may also possess the three additional mutations M377I, S385T and L389F surrounding the second SSN381 catalytic motif. These mutations are associated with a high resistance to cefotaxime in Japan (Dabernat *et al.*, 2002). S385T and L389F increase the resistance conferred by N526K (Osaki *et al.*, 2005). Modeling of the structure of *H. influenzae* PBP3 on that of *Streptococcus pneumoniae* PBP2x showed that residues 517, 526, 377, 385 and 389 are probably lining the active site cavity (Ubukata *et al.*, 2001).

The affinity for penicillin of a few *H. influenzae* PBP3 variants has been measured *in vitro* (Kaczmarek *et al.*, 2004). Variants with the N526K mutation have a lower affinity than without, in agreement with the resistance level of the originating strains. Surprisingly, a PBP3 with only the R517H substitution had the same high affinity as a wild-type PBP3. Therefore, this substitution cannot confer resistance in isolation.

BLNAR strains with a high level of resistance combine mechanisms that involve alteration of PBP3 and an efflux pump (Kaczmarek *et al.*, 2004). A low affinity PBP3 can also be found in strains expressing a β-lactamase, and both mechanisms can cooperate to increase the resistance or to resist to combinations of β-lactams and β-lactamase inhibitors such as the widely used amoxicillin/clavulanate formulations (Dabernat *et al.*, 2002; Matic *et al.*, 2003).

The genome of *Helicobacter pylori* encodes three PBPs. These are the homologues of the class B PBP2 and PBP3, and of the class A PBP1a from *Escherichia coli*. A few stable amoxicillin-resistant strains have been reported (van Zwet *et al.*, 1998; Han *et al.*, 1999; Gerrits *et al.*, 2002; Okamoto *et al.*, 2002). In one case, the resistance was shown to result entirely from the single point mutation S414R in PBP1a (Gerrits *et al.*, 2002). Two other resistant strains were found to have the three substitutions T556S, N562Y and T593A as well as the insertion of a Glu after residue 464 (Okamoto *et al.*, 2002). One strain had ten substitutions, all of them in the second half of the transpeptidase domain, including the T556S and N562Y mutations (Kwon *et al.*, 2003). T556S is within the third catalytic motif KTG. *In vitro* selection on amoxicillin also yielded strains with modified PBP1a (DeLoney & Schiller, 2000). The PBP1a of one such strain had four substitutions, including the S414R mutation (Paul *et al.*, 2001).

To the authors' knowledge, no clinical isolates of *Escherichia coli* are known to resist through the expression of modified PBPs. However, as a laboratory workhorse, *Escherichia coli* was used to demonstrate that β-lactam pressure can select for altered PBPs (Spratt, 1978). Several point mutations in PBP3, the class B PBP dedicated to division, were found to reduce susceptibility. Interestingly, the substitution T308A, next to the active site S307, is analogous to the PBP2x T338A and PBP1a T371A that contribute to resistance to *Streptococcus pneumoniae* (Hedge & Spratt, 1985a, b). Another mutation was found in the second catalytic motif, changing SSN361 into SSS361 (Hedge & Spratt, 1985a).

A few reports must be added to complete this overview of pathogens with modified PBPs. PBP alteration has also been found in imipenem-resistant clinical isolates of *Proteus mirabilis* (Neuwirth *et al.*, 1995) and *Pseudomonas aeruginosa* (Bellido *et al.*, 1990). A cefsulodin-resistant clinical isolate of *P. aeruginosa* also had one PBP with reduced affinity, although not the same as the imipenem-resistant isolate (Gotoh *et al.*, 1990). Overexpression of PBP3, in addition to decreased outer-membrane permeability, was found in a highly resistant strain of *Salmonella muenchen* (Bellido *et al.*, 1989). The various levels of resistance of several strains of *Acinetobacter calcoaceticus* could be correlated with the production of PBPs with altered expression or affinity for β-lactams (Obara & Nakae, 1991). Also, a nosocomial imipenem-resistant strain of *Acinetobacter baumanii* was found to have PBPs with low-affinity for β-lactamase inhibitors, in particular clavulanate, which are normally used against this bacteria (Urban *et al.*, 1995).

In the laboratory, imipenem could select a resistant clone of *Acinetobacter baumanii* with an altered PBP (Gehrlein *et al.*, 1991). Alterations of PBP3 or PBP2 were selected in laboratory mutants of *Listeria monocytogenes* (Gutkind *et al.*, 1990; Pierre *et al.*, 1990a). Altered PBPs were also found in laboratory-resistant mutants of the *Bacteroides fragilis* group (Wexler & Halebian, 1990) and of *Rhodococcus equi* (Nordmann *et al.*, 1993).

Pathogens have been submitted to severe antibiotic pressure over the past five decades, leading to the emergence of resistant strains. In a natural setting as well, β-lactam-producing bacteria need to be protected against the drugs of their own making. Two examples have been documented, which involve low-affinity PBPs. The expression of a particular PBP is responsible in part for the resistance of

Table 2. High molecular weight PBPs of organisms that resist β-lactams by expressing low affinity PBPs

	Class A (bifunctional)			Class B (monofunctional)		
Staphylococcus aureus	PBP2			PBP1	PBP3	**PBP2a** ↗ *mecA*
Enterococci	PBP1a *ponA*	PBP1b *pbpZ*	PBP2a *pbpF*	PBPC(B) *pbpB*	PBP2b *pbpA*	PBP5 ↗
Streptococcus pneumoniae	*PBP1a*	PBP1b	PBP2a	*PBP2x*	*PBP2b*	
Neisseria	PBP1 *ponA*			*PBP2* *penA*		
Haemophilus influenzae	PBP1a	PBP1b		PBP3 *ftsI*	PBP2	
Helicobacter pylori	PBP1a			PBP3	PBP2	

Low-affinity PBPs are underlined. Discontinuous underline indicate intrinsic low affinity. Italic characters indicate mosaicity. Nonitalic characters indicate point mutations. An arrow indicate overexpression. Bold characters indicate acquisition of exogenous origin. Alternative gene names are given below their respective product.

β-lactam-producing *Streptomyces clavuligerus* (Paradkar *et al.*, 1996). None of the eight PBPs of cephamycin C-producing *Nocardia lactamdurans* bind the β-lactam secreted by this bacteria, although it also expresses a β-lactamase (Coque *et al.*, 1993).

Concluding remarks: are the PBPs sustainable targets?

β-Lactam treatments for staphylococcal, enterococcal and pneumococcal infections are increasingly inneffective. Table 2 summarizes the various PBP-based mechanisms of resistance that have been uncovered. The isolation of strains with modified PBPs from species producing β-lactamases is worrisome. The efficacy of β-lactams may thus be compromised even with the advent of efficient β-lactamase inhibitors. One should therefore ask whether PBPs are still valid targets for future antimicrobial therapies.

Five decades of β-lactam therapy have largely validated the targeting of PBPs. In which direction should the research effort be headed? Detailed kinetic studies of the reaction between PBPs and β-lactams have shown that these antibiotics are a poor fit to the enzyme active site, which guarantees the broad specificity of these drugs. However, clinically useful improvements of the affinity of the preacylation complex can be obtained, as demonstrated with novel cephalosporines active against MRSA and a host of other resistant pathogens (Fuda *et al.*, 2006; Page, 2006).

Restoring a rapid acylation rate in low-affinity PBPs might be more difficult. With this aim, instead of studying the reaction between PBPs and β-lactams, research should be directed towards the physiological reaction of transpeptidation. Indeed, the following paradox needs to be resolved. β-Lactams are mimics of the natural substrate of PBPs, so that explanations of the low reactivity of altered PBPs for the drugs should apply equally well to the reaction with the natural substrates. However, this is not the case, as resistant pneumococci are fit, indicating that the physiological reactions catalyzed by altered low-affinity PBPs are not severely affected. Understanding how the natural PBP substrates, or partner proteins, maintain the reactivity of the catalytic serine even in PBPs from resistant bacteria should aid in the design of novel compounds. Such new drugs could react with all PBPs, regardless of their reactivity with β-lactams (Zervosen *et al.*, 2004). Alternatively, new molecules might serve as adjuvants to restore or maintain the reactivity of all PBPs towards traditional β-lactams (Fuda *et al.*, 2005).

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