Chapter 13
Penicillin-Binding Proteins and β-Lactam Resistance

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1 What Are PBPs?

Penicillin-binding proteins (PBPs) are the targets of β-lactam antibiotics. These enzymes catalyze the last stages in the polymerization of peptidoglycan, the major constituent of the cell wall. The peptidoglycan, or murein, is a giant molecule, which forms a molecular mesh around the plasma membrane. Chains of tandemly repeated disaccharides form the glycan strands that are linked to each other by short peptide bridges. The discoveries of the PBPs and their cross-bridging mechanism were intimately intertwined. On the basis of studies of the effect of penicillin on peptidoglycan synthesis, it was concluded that cross-linking of the glycan chains resulted from a transpeptidation reaction, which is inhibited by β-lactams (1, 2). The first PBPs were isolated a few years later by covalent affinity chromatography on penicillin-substituted resin (3). Some of these PBPs were DD-carboxypeptidases or endopeptidases rather than transpeptidases. In the intervening three decades, intense research has been carried out on PBPs, particularly on their role in the resistance to β-lactams of some important pathogens such as *Staphylococcus aureus*, *Enterococci* and *Streptococcus pneumoniae*.

PBPs are characterized by the presence of a penicillin-binding domain, which harbors three specific motifs: SXXK, (S/Y)XN and (K/H)(S/T)G. This signature is common to the ASPRE protein family (for active-site serine penicillin-recognizing enzymes), which includes the class A and C β-lactamases. The topology of these β-lactamases is shared with the penicillin-binding domain of the PBPs (4, 5). The penicillin-binding domain is characterized by an active-site cleft between an α-helical sub-domain and an α/β-sub-domain, which consists of a 5-stranded β-sheet covered by a C-terminal α-helix. Following the topological nomenclature for β-lactamases (4, 6), the first motif SXXK is on the N-terminus of helix α2 of the helical sub-domain, on the bottom of the active-site groove, in the standard representation. The third KTG motif on strand β3 of the α/β sub-domain is located on the right side of the active site. Note that this strand is termed β3 as a result of the connectivity of the polypeptide chain, although it forms the margin of the 5-stranded β-sheet. The second SXN motif is on the left side of the active site, on a loop between helix 4 and 5 of the helical sub-domain (Fig. 1).

The serine of the SXXK motif is central to the catalytic mechanism, which is thought to occur in the following manner (Fig. 2). The Oγ of the serine carries out a nucleophilic attack on the carbonyl of the penultimate D-Ala amino acid of the stem peptide, which results in the removal of the last D-Ala amino acid and the formation of a covalent acyl–enzyme complex between the "donor" stem peptide and the protein. The carbonyl of the D-Ala amino acid, now forming an ester linkage with the active-site serine, then undergoes a nucleophilic attack from a primary amine linked in various ways to the third residue of a second "acceptor" stem peptide. This second reaction forms a peptide bond between the two stem peptides and regenerates a free active-site serine. What was just described is the catalysis of transpeptidation (Fig. 2a). In the case of DD-carboxypeptidases, the acyl–enzyme intermediate is hydrolyzed (Fig. 2b).

β-lactams resemble the D-Ala-D-Ala dipeptide in an elongated conformation (Fig. 3). More than the similarity of linked atoms, it is the distribution of three electrostatic negative wells that accounts for the resemblance. With PBPs, β-lactams 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 the active-site serine from engaging in further productive reactions. β-lactamases differ in that they react with β-lactams rather than with D-Ala-D-Ala dipeptides, and that hydrolysis of the acyl–enzyme complex is extremely fast, thus releasing an active enzyme and an inactive compound.

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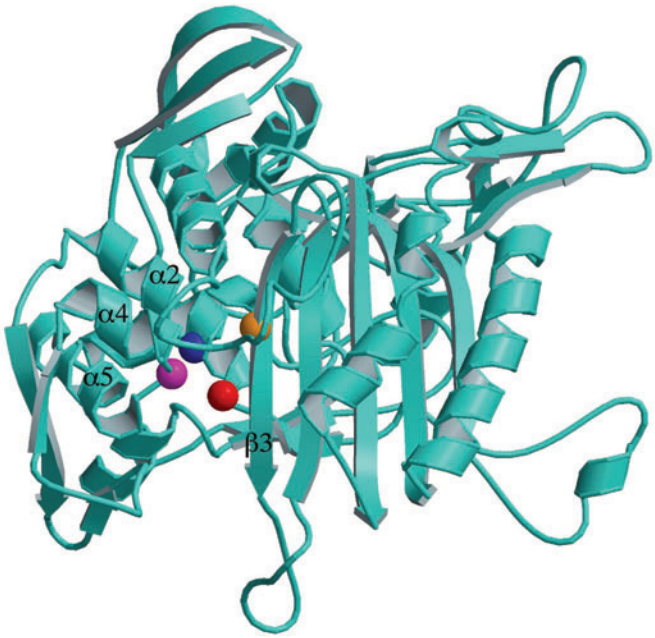

Fig. 1 Topology of the penicillin-binding domain. The example presented is the transpeptidase domain of *S. pneumoniae* PBP2x. The positions of the serine and lysine of the first SXXK motif are shown by red and blue spheres, respectively. The serine of the second SXN motif is indicated by a purple sphere. The lysine of the third KTG motif is shown in yellow. The elements of secondary structure, which bear the catalytic motifs, are indicated with the standard nomenclature (*See Color Plates*)

The reaction of PBPs and serine β-lactamases with β-lactams can be described kinetically as follows (Fig. 4). 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 finally hydrolyzed with the rate k_3 to regenerate the enzyme E and an inactivated product P. The rate described by k_3 is extremely rapid with β-lactamases, whereas it is negligible for PBPs on the time scale of a bacterial generation. The following nomenclature will be used throughout this review. The rate constants k_2 and k_3 describe the acylation and deacylation reactions respectively. The second order rate constant k_2/K_d will be referred to as the efficiency of acylation, which allows calculation of the overall acylation rate at a given concentration of antibiotic. Note that the inhibitory potency of a particular β-lactam for a PBP is given by c_{50}, which is the antibiotic concentration resulting in the inhibition of half the PBP molecules at steady state (i.e., when the acylation and deacylation reactions proceed at the same rate). The value of c_{50} is equal to the ratio $k_3/(k_2/K_d)$. In this review as in the literature in general, PBPs are referred to as being (or having) high or low "affinity" for β-lactams. This "affinity" implicitly refers to c_{50}, and should not be confused with the strength of a noncovalent interaction, which can be described by an association–dissociation equilibrium with a K_d constant, such as the formation of the preacylation complex.

Despite the availability of several crystal structures of PBPs and β-lactamases, and detailed kinetic studies, the enzymatic mechanism is still a matter of debate. Several mechanisms have been proposed that involve various residues of the conserved catalytic motifs and the carboxylate of the antibiotic. It is likely that the precise mechanisms differ between various ASPRE enzymes, and even for a single protein between different β-lactams (7).

2 Classification of PBPs

PBPs are commonly classified into three groups according to their molecular weight and domain structure: high molecular weight PBPs, which fall into two broad families called class A and B, and low molecular weight PBPs. Note that the nomenclature of the PBPs is particularly confusing as it is historically based on the observed electrophoretic pattern exhibited by proteins labeled with radioactive penicillin. Thus there is no necessarily functional or genetic relationship between homonymic PBPs of various organisms.

Class A PBPs comprise a single transmembrane segment, sometimes preceded by a short N-terminal cytoplasmic region, and two extracellular domains. The first extracellular domain carries the glycosyltransferase activity that is responsible for the polymerization of the glycan strands. The glycosyltransferase activity has been demonstrated for various purified recombinant class A PBPs including *Escherichia coli* PBP1b and PBP1a (8–12) and *S. pneumoniae* PBP1b and PBP 2a (13). The glycosyl transferase activity is inhibited by the glycopeptide antibiotic moenomycin (14), which is not used in therapy due to poor pharmacokinetic properties. As the focus of this review is on β-lactam resistance, the glycosyltransferase domain of the class A PBPs will not be discussed further. The C-terminal region of class A PBPs constitutes the penicillin-binding domain that catalyzes transpeptidation, thus bridging adjacent glycan strands. Demonstration of the transpeptidase activity in vitro with a purified recombinant protein has been achieved only recently for *E. coli* PBP1b and PBP1a (8, 11, 12).

Class B PBPs consist of a transmembrane anchor, a domain of unknown function, and a transpeptidase penicillin-binding domain. Although the transpeptidase activity of class B PBPs has never been demonstrated with recombinant proteins, studies of the peptidoglycan composition of *E. coli* cells following treatment with aztreonam, a β-lactam specific to PBP3, indicated that this class B PBP is indeed a transpeptidase (15). The transmembrane segment and non-penicillin-binding domain are certainly involved in proper cellular targeting through probable interactions with other proteins, as demonstrated in the case of *E. coli* PBP3 (16).

Low molecular weight PBPs constitute the third group. These consist mainly of a penicillin-binding domain with a

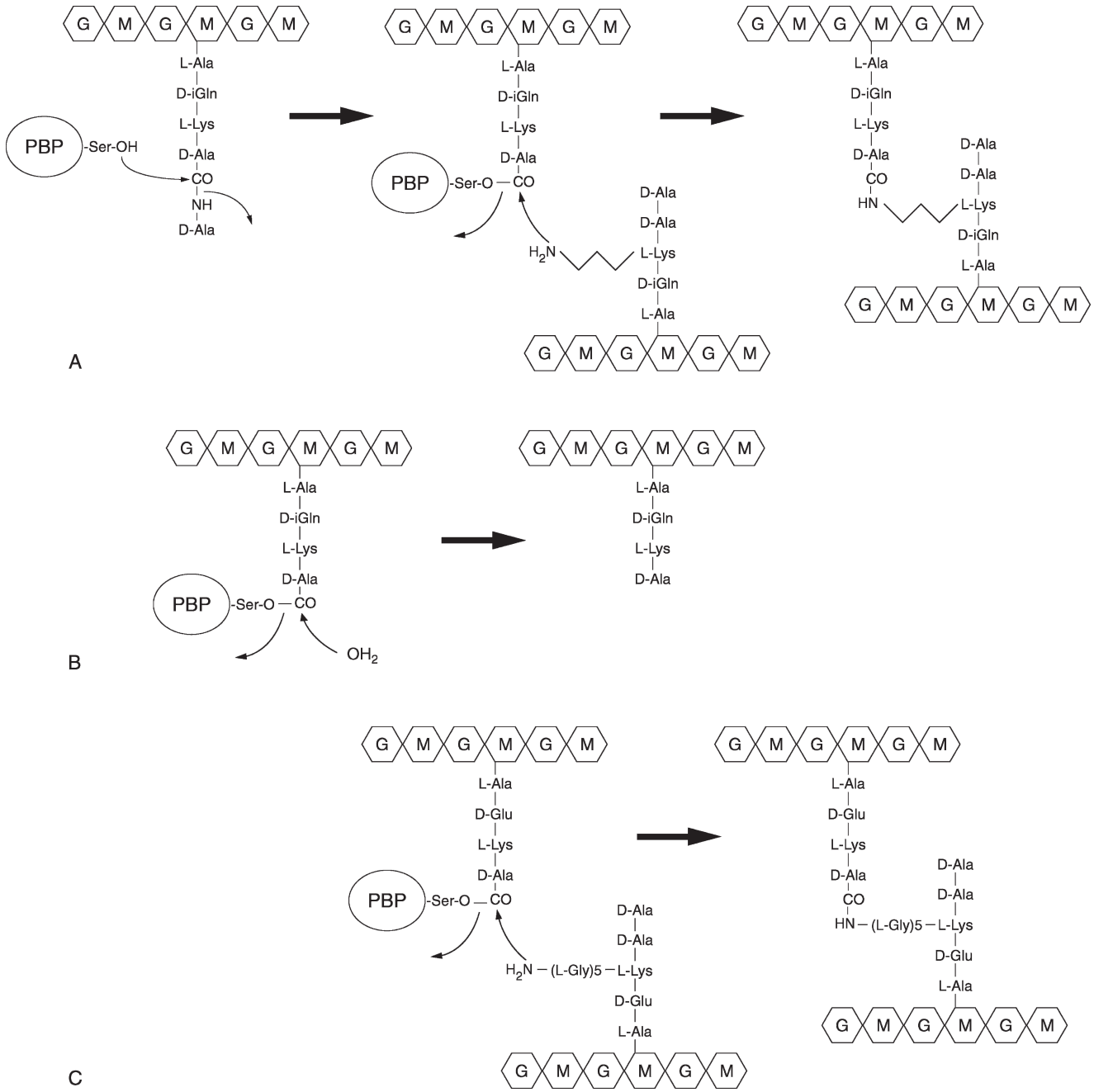

Fig. 2 (**a**) Catalysis of transpeptidation. Fragments of glycan strands are represented by chains of hexagons standing for the hexoses *N*-acetyl glucosamine (G) and *N*-acetyl muramic acid (M). The "donor" pentapeptide is depicted on the upper glycan strand, whereas the "acceptor" is attached here on the lower strand. The peptides shown are those from *Streptococcus pneumoniae*. The second and third amino acids may differ in various species. (**b**) Reaction catalyzed by DD-carboxypeptidase PBPs. With such proteins, the acyl–enzyme inter-mediate is hydrolyzed. (**c**) Transpeptidation reaction scheme in *Staphylococcus aureus*. Note that in many instances, including in *S. 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"

small additional C-terminal domain, which is anchored to the plasma membrane either through a transmembrane segment or an amphipathic helix presumably lying on the lipid bilayer (17). Low molecular PBPs have either demonstrated DD-carboxypeptidase or DD-endopeptidase activities (18–21).

3 Physiological Function of PBPs

The cellular function of some PBPs has been inferred from various lines of evidence, but our knowledge remains sketchy. One type of data is the phenotype of mutant strains, or of cells treated with β-lactams that are specific to particular

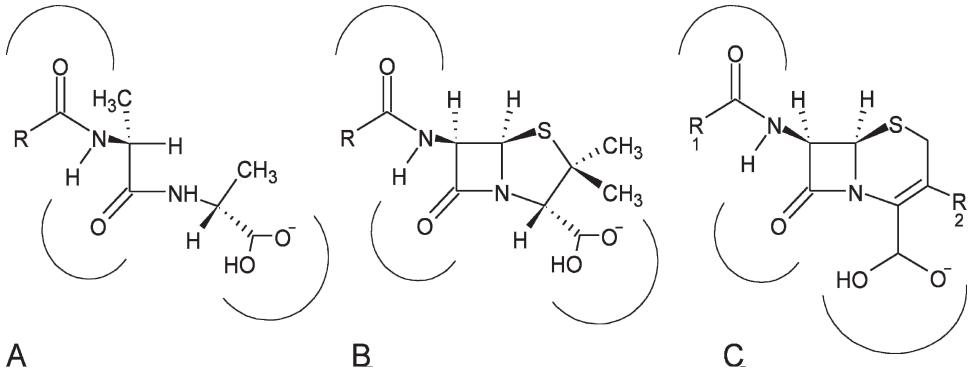

Fig. 3 Structural similarity between β-lactams and the natural substrate of the PBPs. (**a**) *N*-acyl-D-alanyl-D-alanine peptide. (**b**) Penicillin backbone. (**c**) Cephalosporin backbone. The regions of negative electrostatic potential are indicated by arcs

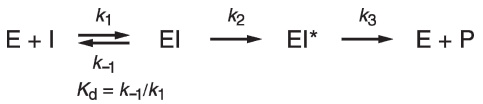

Fig. 4 Kinetic scheme of the reaction between a PBP (E) and a β-lactam (I). EI is a preacylation non-covalent complex. EI* is the covalent acyl–enzyme complex. P is the open inactivated product

PBPs. The second type of result is the cellular localization of various PBPs, determined by immunofluorescence or fusion with the green fluorescent protein. Thus, various class A and B PBPs are involved in peptidoglycan synthesis during cell enlargement, cell division, or sporulation. In *E. coli*, for example, the class B PBP3 plays a role in division (22), whereas the class B PBP2 is involved in cell elongation and the onset of the division (23–25). Nothing is known of the specific role of the *E. coli* class A PBPs, as they show some degree of functional redundancy. In *B. subtilis*, the class A PBP1 and PBP4a appear to participate in cell division and elongation, respectively (26, 27), while the class B PBP2b is specific to the division (28) and the class B PBP5, PBP3, and PBP2a take part in the enlargement process (27, 29). In *S. pneumoniae*, and similarly shaped streptococci, enterococci, and lactococci, there certainly are two mechanisms of peptidoglycan synthesis (30, 31), with class B PBP2x and PBP2b participating in septal and peripheral cell wall synthesis, respectively. However, the peripheral localization reported is incorrect (32), and all the high molecular weight PBPs are present at mid cell. The function of the class A PBPs remains therefore unknown in *S. pneumoniae*. *S. aureus* is a spherically shaped coccus, whose division appears to produce entirely the new hemisphere of the daughter cell, in a process that involves its single class A PBP2 (33). The relative role of the two class B PBP1 and PBP3 in *S. aureus* is not clear.

In summary, peptidoglycan synthesis occurs in different phases, sometimes at different locations, depending on the morphology of the organism considered, with different participating PBPs. The class B PBP strictly involved in cell division can be generally identified by sequence comparisons with well-characterized examples and by the localization of its gene in a cluster coding for division proteins (34). The specific cellular function of the other class B and class A PBPs is more difficult to determine without dedicated genetic and localization studies.

4 PBP-Based β-Lactam Resistance

Inhibition of PBPs produces an imbalance in cell wall metabolism resulting in lysis or growth inhibition. The link between PBP inhibition and the biological outcome, lysis or growth arrest, remains poorly understood (e.g., *Escherichia coli* (35), *Staphylococcus aureus* (36), *Enterococcus hirae* (37)). Despite our ignorance of the detailed physiological consequences of β-lactam treatment, various means of resistance have been uncovered and investigated. Resistance to β-lactams arose from decreased permeability of the outer membrane, export of the antibiotics by efflux pumps (these two 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 devoted to the PBPs of organisms that exploit this latter strategy.

4.1 Staphylococcus aureus

After the spread of *S. aureus* strains that were resistant to penicillin through the acquisition of a β-lactamase, the semisynthetic β-lactam methicillin was introduced, which was not degraded by β-lactamases. A methicillin-resistant clinical strain was isolated soon afterwards (38). The so-called MRSA (*m*ethicillin *r*esistant *S. aureus*) strains are particularly dangerous in that they exhibit blanket resistance to virtually all β-lactams, often associated with resistance to other classes of antibiotics. MRSA strains were initially found in hospitals causing difficulty in treating nosocomial infections which are increasingly found in the community. The true

origin of community-acquired MRSA strains is debated (39). Vancomycin, a glycopeptide antibiotic, has long been used as a last resort weapon to fight MRSA strains. However, strains exhibiting both high methicillin and vancomycin resistance have appeared recently (40).

The wide spectrum β-lactam resistance of MRSA strains results from the expression, in addition to the four native PBPs, of a fifth PBP termed PBP2a or PBP2' with low affinity for the antibiotics (41, 42). PBP2a is the product of the *mecA* gene whose transcription is controlled by the *mecI* and *mecR1* regulatory elements. MecI is a DNA-binding protein that represses *mecA* transcription (43). By analogy with the homologous BlaI and BlaR1 systems that control the expression of the β-lactamase BlaZ, the Mec system is thought to function in the following manner (44). MecR1 is a signal-transduction protein with an extracellular penicillin-binding domain that senses the presence of β-lactams in the medium, and activates its cytoplasmic domain. The intracellular domain of MecR1 is a protease that undergoes activation through autocatalytic cleavage, which results directly or indirectly in the cleavage of the MecI repressor. The *mecA* gene and its regulatory system are found on a large mobile genetic element called the staphylococcal cassette chromosome *mec* that integrates at a unique site in the chromosome (45). Several variants of the cassette have been found that include in addition to the *mec* genes, several genes encoding resistance to other types of antibacterial agents. A thorough presentation of the current understanding of these genetic elements and their history can be found elsewhere (46, 47).

Interestingly, the intact *mec* system does not confer resistance, as the expression of PBP2a is normally well repressed. Only few β-lactams, not including methicillin, can alleviate this repression. Mutations, for example in *mecI* or in the *mecA* operator region, lead to derepression of *mecA*. Even so, strains with unrestricted expression of PBP2a exhibit methicillin resistance only in a small sub-population (at a frequency of 10^{-4}–10^{-6}), when maintained without β-lactam selective pressure. Following exposure to β-lactams, a homogenous resistant population is selected. When the antibiotic selective pressure is removed, heterogeneity is rapidly restored, with only a small sub-population retaining resistance. These observations indicate that the functioning of PBP2a in cell-wall synthesis bears a cost that is best avoided in the absence of β-lactams. The nature of the genetic determinants of homogenous methicillin resistance in wild strains remains mysterious.

PBP2a is of class B and therefore 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 high molecular weight PBPs, the presence of the class A PBP2 with an active glycosyltransferase domain is nevertheless required (48, 49).

Other genes have been found to be necessary for the full expression of the resistance offered by PBP2a. Over 30 of these auxiliary genes, often termed *fem* (*f*actor *e*ssential for *m*ethicillin resistance, or *aux* for auxiliary) have been identified (50). Several *fem* genes are involved in cell-wall metabolism, other genes take part in regulatory or putative sensory functions. How they cooperate to allow the *mecA*-based resistance is a complex and unresolved issue. The *fem AB* operon, for example, adds the second to fifth glycine residues to the peptidoglycan precursor to form the pentaglycine branch that serves afterwards as the cross-bridge of staphylococcal peptidoglycan (51, 52) (Fig. 2c). A trivial conclusion is that PBP2a has a specific requirement for "acceptor" peptides with a pentaglycine branch. This expectation may be naïve, for PBP2a can confer resistance to *Enterococcus faecalis* and *faecium*, which lack *fem AB* and have the alternative peptidoglycan cross-bridges $(Ala)_2$ and D-Asx, respectively (53).

PBP2a belongs to a sub-group of class B PBPs characterized by the presence of an insertion of about 100 residues following the transmembrane anchor (Fig. 5). This group also includes chromosomally encoded PBP5 from *Enterococcus faecium*, *hirae,* and *faecalis*, and plasmid-encoded PBP3 conferring *Enterococcus hirae*, which are all low-affinity PBPs conferring β-lactam resistance (see below). There are other members of this sub-group of PBPs in *Bacillus subtillis* and related species, in *Listeria monocytogenes* and *L. innocua* and in *Clostridium acetobutylicum*, although these do not appear to confer reduced susceptibility to β-lactams. The origin of PBP2a remains mysterious. A close *mecA* homologue has been found both in susceptible and resistant *Staphylococcus sciuri* strains (54, 55). The *mec* system may thus have spread from a hitherto unidentified staphylococcal species, not only to *S. aureus*, but also to *S. epidermitis*, *S. haemolyticus*, *S. hominis,* and *S. simulans* (56).

The reaction of PBP2a with β-lactams is extremely slow. The acylation efficiency of PBP2a by penicillin G, characterized by the second order rate constant k_2/K_d of approximately $15\,M^{-1}s^{-1}$, is roughly 500-, 800-, 900- and 20-fold smaller than that of the native PBP1, PBP2, PBP3, and PBP4 from *S. aureus*, respectively (57–59). When compared to PBP2x from the susceptible *S. pneumoniae* strain R6, PBP2a is acylated three to four orders of magnitude more slowly (57, 60, 61). With such a poor acylation efficiency (57, 58), the acylation rate of PBP2a at therapeutic concentrations of β-lactams is negligible compared to the bacterial generation time ($t_{1/2}$ for acylation greater than 1 h with 10 μM of penicillin).

The low efficiency of acylation results 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 $0.001\,s^{-1}$ (57, 62). Although published values differ for various β-lactams and means of measurement, the acylation rate k_2 of PBP2a by penicillin G, for example, is three orders

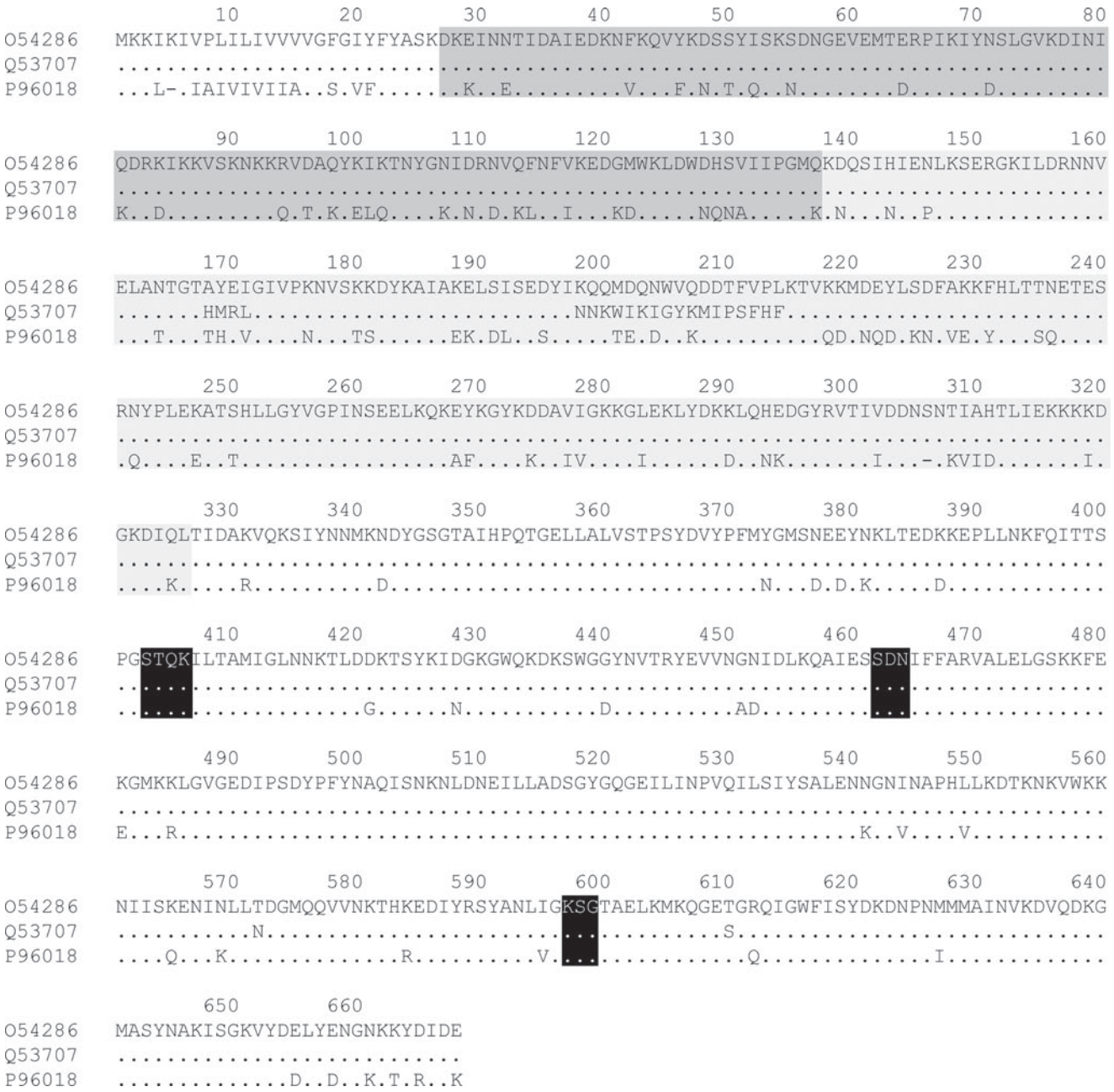

Fig. 5 Sequence alignment of staphylococcal PBP2a (designated by their Uniprot accession numbers). Three out of 16 available sequences are shown. There are three additional sequences that closely resemble # O54286, differing at one or six positions. One additional sequence is very similar to # Q53707, with one substitution and the insertion of two residues. Finally two other sequences resemble # P96018, differing each at 15 positions. The # P96018 sequence and close variants are from *S. sciuri*. The N-terminal domain is shaded. *Dark shading* indicates the extension specific to the sub-group of class B PBPs that includes PBP2a. The catalytic motifs are in *black boxes*

of magnitude slower than that of the susceptible PBP2x from *S. pneumoniae* (57, 60).

The structure of a soluble form of PBP2a without its transmembrane anchor has been solved to a resolution of 1.8 Å (63). The N-terminal non-penicillin-binding domain (residues 27–328) is bilobal, with the first lobe (27–138) formed by the sub-group specific extension. The transpeptidase domain shares its overall fold with other PBPs. The N-terminal domain confers a rather elongated shape to the whole molecule with the active site reaching approximately 100 Å from the membrane anchor.

In the absence of bound antibiotic, the active site of PBP2a appears to be rather closed with the active site S403 poorly positioned for a nucleophilic attack and a twisting of strand β3 that is required to accommodate the N-terminus of helix α2 and the active site S403. The structures of PBP2a with covalently bound nitrocefin, methicillin, and penicillin G revealed a tilt of the whole helical subdomain with respect to the

α/β-subdomain (2.3° with nitrocefin, O. Dideberg, personal communication). This rotation opens the active site and is accompanied by a substantial local rearrangement of the active site (Fig. 6). The Oγ of S403 is displaced by 1.8 Å (with nitrocefin), whereas the strand β3 is straightened. It has been argued that this conformational rearrangement is costly and impedes acylation. The 20-fold slower acylation by methicillin (k_2 = 0.008 s^{-1}) compared to penicillin G (k_2 = 0.2 s^{-1}), has likewise been rationalized on the basis that bound methicillin is translated along the active-site cleft relative to bound penicillin G. This relative displacement increases the distance between the putative proton donor (S462) of the second catalytic motif and the nitrogen group of the opening β-lactam ring. Although possible, these explanations rely on the assumption that the conformations of the acyl–enzyme intermediates are relevant to the transition states of the acylation reaction. However, 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 *E. coli* PBP5 (64). Therefore, analysis of the complementarity of bound open antibiotics may bear little relevance to the understanding of the acylation process.

Mildly β-lactam resistant strains of *S. aureus* that lack both *mecA* and β-lactamases have also been isolated. There are good indications that the resistance of these strains is due to modified native PBPs. Alterations of penicillin binding by PBP1, PBP2 and elevated amount of PBP4 were observed in such strains (65, 66). The acylation rates of PBP1 and PBP2 were decreased, and the deacylation rates increased (59). The kinetic modifications result from point mutations, as demonstrated for PBP2 (67). A tenfold decrease of the acylation efficiency results from the double substitution S569A and A576S. Another variant with the A450D and A462V substitution surrounding the SXN motif and the Q629D mutation has a k_2/K_d lowered 20-fold (67). A laboratory mutant selected with ceftizoxime has the single substitution P458L close to the SXN catalytic motif (68).

Thus, *Staphylococcus aureus* has been found to resist β-lactams in three ways, by 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.

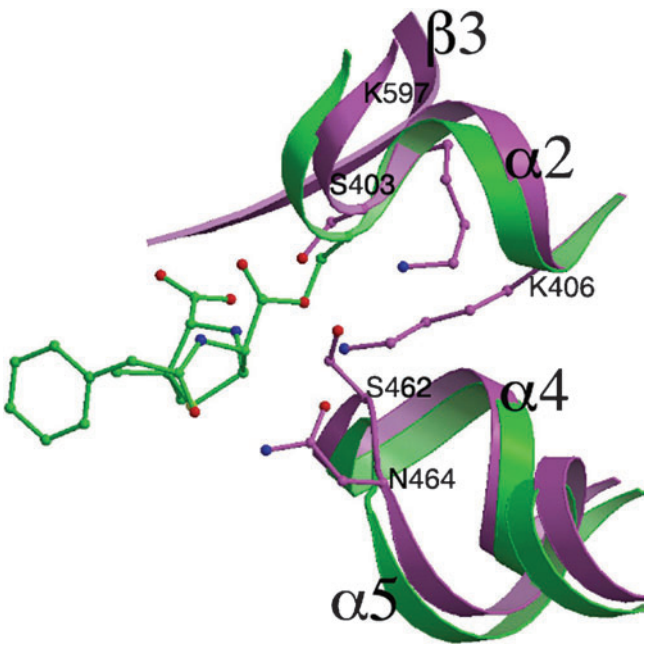

Fig. 6 Superposition of the active site of *S. aureus* PBP2a without (purple) and with (green) bound penicillin (shown in balls and sticks). The first motif on helix α2 and the second motif between α4 and α5 are moved away from strand β3, which bears the third catalytic motif (*See Color Plates*)

4.2 Enterococci

The intrinsic resistance to β-lactams is a characteristic of enterococci. Isolates of *Enterococcus faecalis* typically exhibit MICs for penicillin of 2–8 mg/L (e.g., (69)), and *E. faecium* of 16–32 mg/L (e.g., (70)). These two species, which cause important human health problems, particularly nosocomial infections, have been the subject of intense molecular studies over the past two decades, together with *E. hirae*, which is more of a concern in veterinary medicine.

Enterococci morphologically resemble streptococci, which may be related to the fact that they share the same set of three class A and two class B high molecular weight PBPs (71). However, the intrinsic moderate resistance to β-lactams results from the presence of an additional sixth high molecular weight PBP, which takes over the transpeptidase function of the other PBPs when these are inhibited by the antibiotics (72, 73). This was concluded from three lines of evidence in early studies of an *E. hirae* strain and several derivatives (initially identified as *Streptococcus faecium* ATCC 9790). Firstly, it was found that one of the high molecular weight PBPs (PBP5) had a much lower affinity for penicillin, and spontaneous mutants with greater resistance had elevated amounts of this PBP (73). Secondly, a mutant hypersensitive to penicillin was found to lack PBP5 expression (72). Finally, saturation of PBP5 with β-lactams led to bacterial death (74).

Subsequent and parallel studies uncovered the same mechanism underlying intrinsic β-lactam resistance in *E. faecium* (75, 76) and *E. faecalis* (77). The wide range of elevated levels of resistance exhibited by clinical isolates of *E. faecium* was found to arise from two mechanisms: increased expression of PBP5 and mutations of PBP5 that further decrease its affinity for β-lactams (78, 79). Strains with intermediate level of resistance (MIC for ampicillin of 8 mg/mL) appear to rely mainly on the first mechanism, while extremely resistant strains (MIC for ampicillin of up to 512 mg/L) appear to combine both overexpression and reduced affinity (78, 79) or use

only the latter mechanism (80, 81). 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 (75). A peculiar strain of *E. hirae* (S185) was found to express, in addition to its chromosomally encoded PBP5, a second PBP with low β-lactam affinity. This related but plasmid-encoded PBP is termed PBP3r (82, 83).

When the genes encoding PBP5 from various *E. faecium* clinical isolates were sequenced (Fig. 7, Table 1), several point mutations were found to be correlated with a low affinity for β-lactams and high resistance (80, 81, 84). However, as clinical isolates are not isogenic, assessment of the effect of various PBP5 sequences awaited their introduction in a single strain. When three PBP5 sequences originating from strains with MICs for ampicillin of 2, 24, and 20 mg/L were introduced in a strain with no PBP5 expression (MIC ampicillin of 0.03 mg/L), the resulting strains had MICs of 6, 12, and 512 mg/L, respectively (75). These results demonstrate that variants of PBP5 indeed confer different MICs, but that this effect is strongly modulated by other unknown factors (75, 85). 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 (80). When introduced individually, this mutation caused only a modest increase of resistance, when compared to the resistance of the clinical strains that harbor this substitution (75, 80). However, in an isogenic background, the M485A substitution accounted for most of the difference

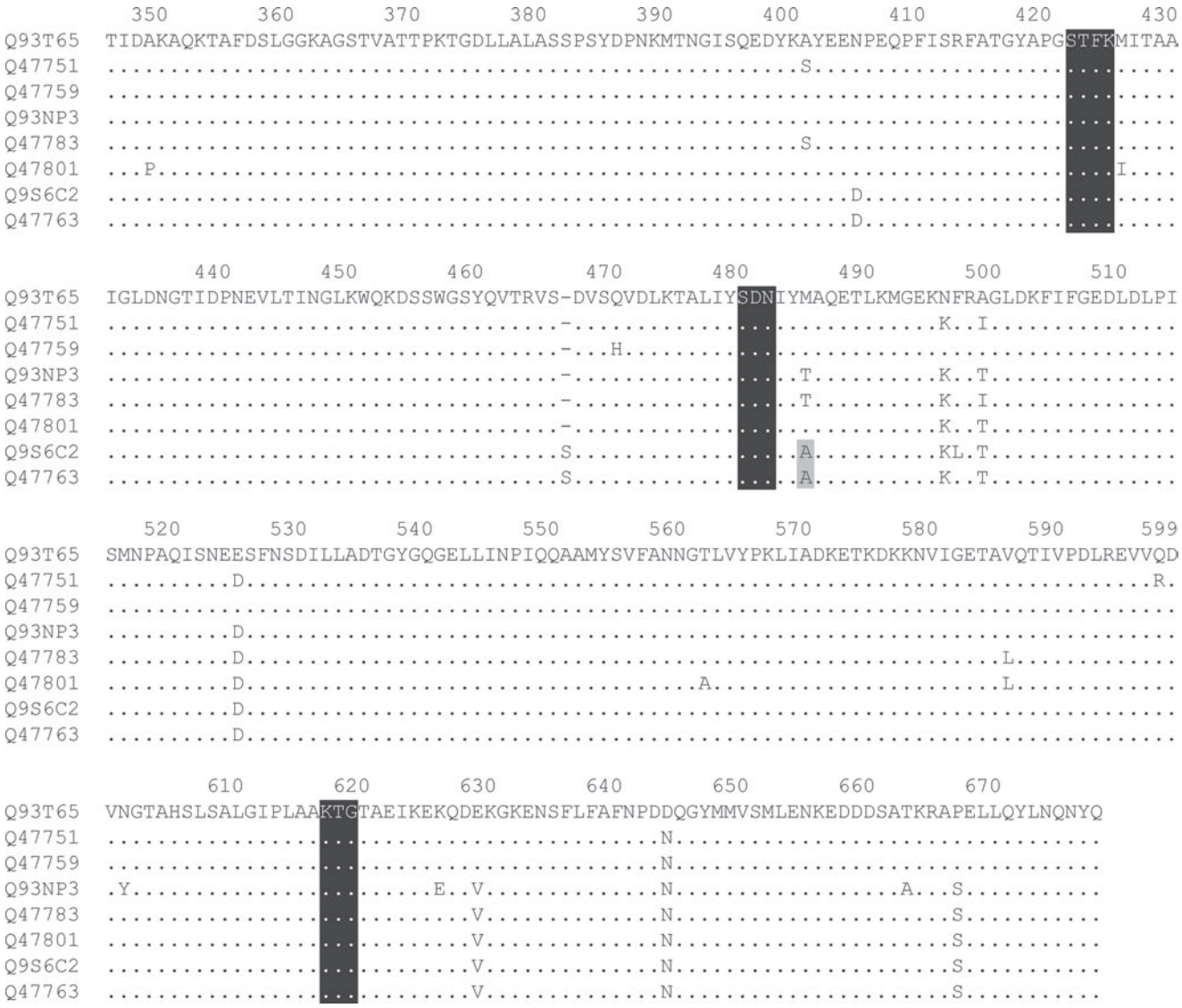

Fig. 7 Alignment of publicly available sequences of *E. faecium* PBP5 transpeptidase domain. Sequences are ordered according to the MIC of their originating strain (see Table 1). Catalytic motifs are blackened. The M485S substitution that was investigated and shown to increase resistance is *highlighted in gray*

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

Uniprot #	Strain	MIC (mg/L)	MIC (mg/L) in isogenic strains[b]	Expression level	k_2/K_d ($s^{-1}M^{-1}$)[a]
Q93T65	BM4107	2 (Amp)[b]	6	→[b]	
Q47751	D366	16 (Pen)[a]		↗[a]	17
Q47759	D63	5 (Pen)[a]		→[a]	24
	D63r	70 (Pen)[a]		↗↗[a]	20
Q93NP3	D344	24 (Amp)[b]	12	↗↗[a]	17
		64 (Pen)[a]			
Q47783	EFM-1	90 (Pen)[a]		→[a]	1.5
Q47801	9439	128 (Amp)[d]		↗[c]	
Q9S6C2	C68	256 (Amp)[e]			
Q47763	H80721	512 (Amp)[b]	20	→[a]	<1.3
		512 (Pen)[a]			

[a](80); [b](75); [c](78); [d](84); [e](89)

of resistance conferred by two PBP5 variants that otherwise differed at seven positions in the transpeptidase domain (75).

Enterococcal PBP5 belongs to the same sub-group of class B PBPs as the acquired *S. aureus* PBP2a, with an insertion of about 120 residues following the transmembrane helix. The crystal structure of *E. faecium* PBP5 bound to penicillin was solved to a resolution of 2.4 Å (86). The originating strain (D63r) had an MIC for penicillin of 70 mg/L that appears to result solely from overproduction of the same PBP5 found in the parental strain (D63), which has the basal MIC of 5 mg/L (80). Therefore, the structure is that of a "wild-type" PBP5, without substitutions that further decrease the affinity for β-lactams. The efficiency of acylation of D63r *E. faecium* PBP5 defined by the second order rate constant $k_2/K_d = 20\,M^{-1}s^{-1}$ is similar to that of *S. aureus* PBP2a, that is 2–3 orders of magnitude slower than that of a "regular" high-affinity PBP (80).

As no structure was obtained in the absence of antibiotic, no comment could be made regarding a possible rearrangement upon acylation, although the authors speculate that some loop residues, which are conserved in this sub-group of PBPs (residues 461–465), may have been pushed aside to allow antibiotic binding (86). Another proposal is that S480 of the second catalytic motif may not be appropriately positioned to act as the proton donor for the nitrogen of the opening β-lactam ring (86), much as proposed in the case of *S. aureus* PBP2a and methicillin (63). The important role of the substitution of M485 by Ala or Thr in the expression of high resistance (75, 80, 81) was rationalized as follows. The side chain of M485 lies behind K425 of the first catalytic site, which may be involved in the proton abstraction of the catalytic S422. Smaller residues in position 485 may result in greater conformational freedom of K425 and thus hinder acylation. The same argument might apply to the M426I substitution found in a highly resistant strain (84). The addition of a second serine after S466 that is found in a PBP5 with an extremely low efficiency of acylation (80) was tentatively explained by a reinforcement of the steric hindrance due to the rigid loop 451–466 (86).

PBP5, as a class B PBP, does not support the necessary glycosyltransferase activity for peptidoglycan synthesis, although it can take over all the required transpeptidase activity. Deletion studies in *E. 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* (71). The third class A PBP encoded by *pbpZ* is not required.

Although the high resistance of many enterococcal clinical strains results from their greater amount of PBP5, the reasons underlying this overexpression are still unclear. An open-reading frame upstream of the gene encoding PBP5 is truncated in an *E. hirae* strain overproducing PBP5. This finding suggested that this gene might be a PBP5 synthesis repressor (*psr*) (87). However, subsequent tests of this hypothesis in *E. hirae* using isogenic strains have ruled out a role of *psr* in the regulation of PBP5 expression (88). Similarly, no role for *psr* was found in PBP5 expression in *E. faecium* (89) or *E. faecalis* (77).

Four isolates of *E. faecalis* were found to exhibit high resistance to ampicillin and imipenem without overexpression of PBP5. Instead, the resistance is due to two substitutions, P520S and Y605H, in PBP4 (the orthologue of streptococcal PBP2x) (90).

In addition to the modes of resistance presented above, the plasmid-borne expression of β-lactamases has been documented in some clinical strains of *E. faecalis*, and less frequently in *E. faecium* (91). Although not found in clinical isolates (yet?), an intriguing mechanism of β-lactam resistance was selected in the laboratory strains of *E. faecium* (92–94). These mutants appear to by-pass altogether the need for PBPs. A β-lactam insensitive L,D-transpeptidase activity appears to be responsible for cross-linking of the peptidoglycan, generating L-Lys-D-Asx-L-Lys instead of D-Ala-D-Asx-L-Lys bridges. However, increased resistance does not result from higher L,D-transpeptidase activity, but from a greater amount of precursor that lacks the terminal D-Ala. This elevated amount of truncated precursor is due to the cytoplasmic overexpression of a β-lactam insensitive DD-carboxypeptidase (93). This precursor cannot be a

"donor" substrate for the PBPs but is adequate for the L,D-transpeptidase activity. If ever found in clinical isolates, this mechanism would spell the end of β-lactam-based therapy for enterococci, as it completely obviates the transpeptidase function of the PBPs.

4.3 *Streptococcus pneumoniae*

Expression of a β-lactamase or an additional low-affinity PBP has never been reported in pneumococcus. Instead, β-lactam-resistant strains of *S. pneumoniae* always harbor modified versions of their own PBPs that are inefficiently acylated by β-lactams (95, 96).

Once electrophoretic techniques were good enough to resolve the six PBPs from *S. pneumoniae*, it became apparent that PBP1a, PBP2b, PBP2x, and sometimes PBP2a were altered in resistant clinical isolates. These modified PBPs bound less radio-labeled antibiotic, whereas the affinity of PBP1b and PBP3 was unchanged (97). Sequencing revealed that mosaic genes encode PBP2b (98), PBP2x (97), and PBP1a (99) in resistant clinical strains. Mosaicity is the product of recombination events between different alleles within a species or between homologous genes of related species. *S. pneumoniae* as a naturally competent organism is particularly apt to this type of genomic plasticity (100).

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 less than 1% of difference leading to one or two amino acid substitutions over the protein length (97, 98). In contrast, mosaic *pbp* genes show blocks of sequences that differ from non-mosaic alleles by about 14–23% (PBP2b (98, 101); PBP1a (99); PBP2x (97)). The diverging blocks span various lengths of the regional coding for the transpeptidase domain or even most of the extracellular domain. The degree of difference compared to the normal level of intraspecies polymorphism suggested that the diverging sequence blocks originate from other streptococcal species (97, 98). Parallel examination of various mosaic *pbp* genes showed that multiple sources of homologous DNA had been tapped by pneumococcal strains to survive antibiotic selection (97, 98, 102). Evidence of multiple recombinational events in the history of individual *pbp* alleles further complicates the analysis, although favored sites of recombination can be identified (102).

The origin of the sequence blocks found in mosaic *pbp* genes remains largely mysterious with the possible following exceptions for *pbp2x*. Fragments of the *pbp2x* sequences of two penicillin-susceptible strains of the commensal *Streptococcus mitis* and *Streptococcus oralis* could be identified in many alleles encoding PBP2x from resistant pneumococci (102). Although large fragments of these *S. oralis* and *S. mitis pbp2x* sequences can be recognized in resistant strains of *S. pneumoniae*, the identity in these blocks is not perfect. Differences are found in some codons that are important for the resistance including positions 338 and 339 of the first catalytic motif. This observation supports the following scenario for the emergence of pneumococcal resistance. Commensal streptococci sharing the same niche, such as *S. oralis* and *S. mitis*, have acquired resistance through point mutations selected by repeated exposure to β-lactam treatment for various ailments. Fragments of genes encoding PBPs with reduced affinity were subsequently exchanged between closely related streptococcal species, including *S. pneumoniae*, and selected by antibiotherapy (103). The recognition of these multiple horizontal gene transfers in commensal *Streptococci* and *Pneumoccus* has led to the concept of global gene pool of altered *pbp* sequences for β-lactam resistance (104). Since *S. 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 (105, 106). Nevertheless, despite the complications that horizontal gene transfers bring to the definition of pneumococcal lineage, it appears from numerous studies that the worldwide spread of pneumococcal β-lactam resistance results from the dispersion of a limited number of successful clones (107).

Besides mosaicity resulting from inter- and intra-species homologous recombination, point mutations in *pbp* genes 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 upon selection with cefpodoxime or cefotaxime (108–110), as well as in PBP2x from clinical isolates where it was caused by a mutation either within a mosaic (111) or a "virgin" *pbp2x* gene (112).

Selection in the laboratory has demonstrated that PBP2x and PBP2b are the primary resistance determinants for cefotaxime (a cephalosporin) and piperacillin (a penicillin), respectively (110, 113). This can be interpreted as PBP2x and PBP2b being the essential PBPs most reactive towards cefotaxime and piperacillin, respectively. Indeed, cefotaxime does not react with PBP2b (114). Surprisingly, the amino acid substitutions selected in the laboratory do not match those found in clinical isolates, with the exception of the aforementioned T550A in PBP2x (108–110) and T446A in PBP2b (110). This discrepancy may simply reflect the limited sampling. Alternatively, the most useful substitutions may be different in the molecular context of the native PBPs from *S. pneumoniae*, as selected in the laboratory, or in the PBPs from the commensal streptococcal species where they were probably originally selected by their host.

Like the laboratory point mutants, transfer of *pbp2x* genes from clinical resistant isolates to a susceptible strain can confer a moderate level of resistance to both cephalosporins and most penicillins (97, 112, 115–120). Introduction of mosaic *pbp2b* genes can be selected by a modest reduction of the susceptibility to piperacillin (121). Increased resistance to penicillins is achieved upon transfer of both mosaic *pbp2x* and *pbp2b* genes (115–118). Higher level of resistance to cephalosporins and penicillins results from the additional introduction of a mosaic *pbp1a* gene (116–119, 122). A high level of resistance restricted to the cephalosporins is obtained following transformation of a susceptible strain with mosaic *pbp2x* and *pbp1a* (111, 116, 117, 119, 123). The above observation can be rationalized by invoking a threshold effect, as depicted in Fig. 8.

These experimental findings are mirrored in clinical strains (124, 125). Most resistant clinical isolates harbor three mosaic *pbp* genes encoding PBP1a, PBP2b, and PBP2x (e.g., (118, 126–129)). However, some weakly resistant strains have mosaic alleles only of *pbp2x* and *pbp2b* (e.g., (127, 128)). At least one example was found of a clinical strain with barely reduced susceptibility to penicillin that has only *pbp2x* modified (128). Some isolates with cephalosporin resistance, yet susceptible to penicillin, were found to have mosaic *pbp2x* and *pbp1a* while retaining a "virgin" *pbp2b* (130, 131).

The identification of amino acid substitutions that are relevant to the reduction of affinity of a particular PBP is a difficult task. Due to the process of recombination, superfluous substitutions have probably been imported together with the ones that provide antibiotic resistance (the "hitchhiking" effect). Indeed, even genes neighboring *pbp2b* or *pbp1a* have been incidentally modified through recombination of large DNA fragments (132, 133). Nevertheless, a number of probable important substitutions were proposed based on their absence in susceptible strains, presence in many resistant strains, and proximity to the catalytic motifs. The role of some of these substitutions was probed by detailed genetic, enzymatic, and structural studies.

PBP2x has been the subject of the most detailed investigations. The transpeptidation reaction with substrates mimicking the physiological reaction (such D-Ala-D-Ala-L-Lys containing peptides) has never been achieved in vitro with PBP2x or other pneumococcal PBPs (134). In contrast, PBPs were shown to catalyze the hydrolysis of thiol–ester substrate analogues. With such a substrate called S2d, a benzyl-D-alanyl–enzyme intermediate is formed transiently and hydrolyzed (135). With PBP2x, some D-amino acids could provide their free primary amine to attack such acyl–enzyme intermediates, thus completing a transpeptidation reaction (134). However, the significance of these in vitro reactions is unclear as L-amino acids were ineffective, although the physiological primary amine is provided by the side-chain of an L-lysine (134).

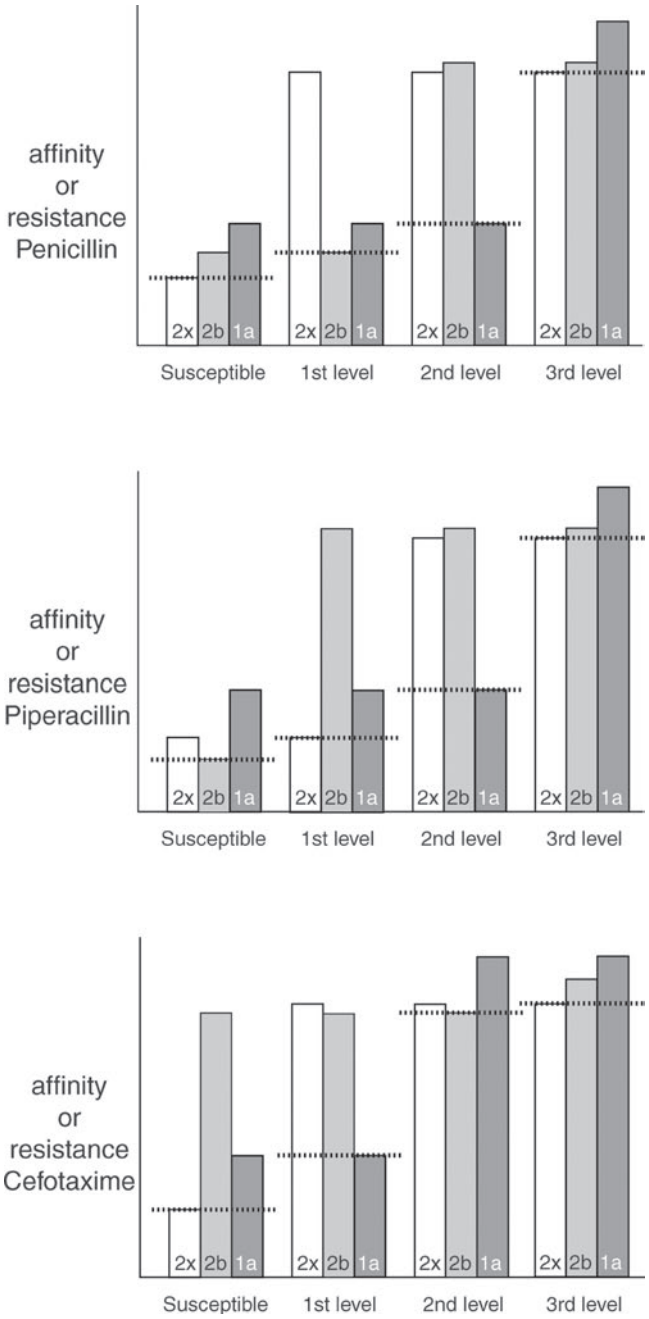

Fig. 8 Schematic representation of the threshold effects that may account for the relationship between which PBPs are modified and the level of pneumococcal resistance. Of PBP2x, PBP2b, and PBP1a, the one with the highest affinity for the β-lactam considered sets the susceptibility threshold of the recipient strain (*dashed line*). 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

In contrast to transpeptidation, the reaction of PBPs with β-lactams occurs readily in vitro. By measuring the decrease in intrinsic fluorescence of a recombinant soluble form of PBP2x upon antibiotic binding, the overall acylation efficiency defined by the second order rate constant k_2/K_d was

determined to be between 60,000 and 110,000 $M^{-1}s^{-1}$ for penicillin and about twice as fast for cefotaxime (61, 134, 136, 137). The deacylation rate k_3 measured in different ways (recovery of enzymatic activity, loss of bound radiolabeled penicillin, mass spectrometry) is between 0.8 and 5 s^{-1} for penicillin and somewhat slower for cefotaxime (60, 134, 136, 137). The very fast acylation and slow deacylation reactions result in a concentration of antibiotic at which half the enzyme is acylated at the steady state (c_{50}) that lies in the micromolar range. This value of c_{50} is consistent with MIC of susceptible strains (60, 119). Attempts have been made to delineate the dissociation constant of the noncovalent preacylation complex 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} (60), whereas a second study reported a K_d of 20 mM and a k_2 of 1,600 s^{-1} (138). The published data lend more credence to the latter higher numbers. Thus penicillin has a very poor "true" affinity for PBP2x, and this finding presumably applies to β-lactams and PBPs in general. The efficacy of β-lactams against susceptible bacteria 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, truncated of its cytoplasmic and transmembrane regions, was solved to a resolution of 2.4 Å (5, 139). The extracellular part of PBP2x consists of a transpeptidase domain within the common fold of the ASPRE proteins (residues 266–616), flanked by an elongated N-terminal domain (residues 49–265) and a small globular C-terminal domain (residues 617–750). The N-terminal domain is shaped like a pair of sugar tongs with a hole of about 10 Å in diameter (5). The function of this domain remains unknown although it was proposed to interact with other protein partners. Alternatively, this domain may recognize some chemical motif of the peptidoglycan. When all the amino acid substitutions found in different mosaic sequences of PBP2x are mapped onto the crystal structure (i.e., 30 of the 217 positions of the N-terminal domain), they are all distributed onto the outer surface of the domain and none is found within the hole. The conservation of the residues forming the inner surface of the hole supports the idea that the sugar tong serves to grasp an unknown partner (140). The function of the C-terminal domain is completely unknown, although it is found only in the class B PBPs involved in the division of some Gram-positive bacteria.

The main feature of the transpeptidase domain, with respect to other known structures of the ASPRE family, is the presence of a very long groove, at the center of which is found the active site. Modeling showed that this cleft can accommodate two molecules (NAG-NAM)-L-Ala-D-Glu-L-Lys-D-Ala, one of which is covalently bound to the active-site serine, and the other providing the Nζ of its L-Lys ready to complete the transpeptidation. Both disaccharide moieties can sit in the larger valleys at both ends of the groove (5).

Regarding the precise mechanism of acylation by antibiotics, the crystal structure of PBP2x and a number of theoretical studies have left the question open (e.g., (7)). The conservation of the hydrogen-bonding pattern involving residues of the three catalytic motifs SXXK, SXN, and KTG in PBP2x and the TEM-1 β-lactamase, suggests that the acylation mechanisms are similar (7, 139). The pH dependence of the acylation rate is consistent with a model where a residue with a pK_a of 4.9 functions as a base to help deprotonate the active-site serine, a group with a pK_a of 7.6 triggers upon deprotonation a rearrangement to a less reactive conformation, and a residue with a pK_a of 9.9 is hydrogen bonded in its protonated form to the free carboxylate of the substrate (138). The base was proposed to be K340 of the first motif with the unusual pKa of 4.9. T550, which binds the carboxylate of the antibiotic (139), would have the pKa of 9.9. Investigation of solvent isotope effects on the rate of acylation suggested a complex process partially rate-limited by the chemistry (the proton exchanges) and by solvation and/or conformational rearrangement (138).

Based upon sequence comparisons and the proximity to the catalytic motifs, the substitutions most likely to impart some resistance include T338A, T338G, T338P, and M339F found within the SXXK motif (61, 111, 112, 116), H394Y and M400T that surround the SXN motif (111, 113, 126, 128), and L546V, T550A, and Q552E, which are close to the KTG motif (111, 112, 127, 141). The effect of some of these substitutions has been characterized in detail as discussed below. These mutations do not appear randomly in sequences, but some families can be recognized.

Examination of approximately 100 publicly available sequences of the transpeptidase domain of PBP2x reveals three broad families (Fig. 9). One family contains non-mosaic sequences that are very similar to the PBP2x from the reference susceptible strain R6. The mosaicity complicates the picture of the two other families and the grouping would differ for various sequence blocks. Nevertheless, the emerging pattern suggests that two main mechanisms have been selected that reduce the affinity of PBP2x for the antibiotics (120). Figure 10 shows the distribution of the substitutions in the structure of the transpeptidase domain of PBP2x from two resistant isolates, representing two modes of reducing the affinity for β-lactams.

One family of sequences is characterized by the T338A substitution. About 30 other substitutions in the transpeptidase domain accompany this defining mutation, although no mutation is consistently found together with T338A, and never found in the absence of the T338A mutation. 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

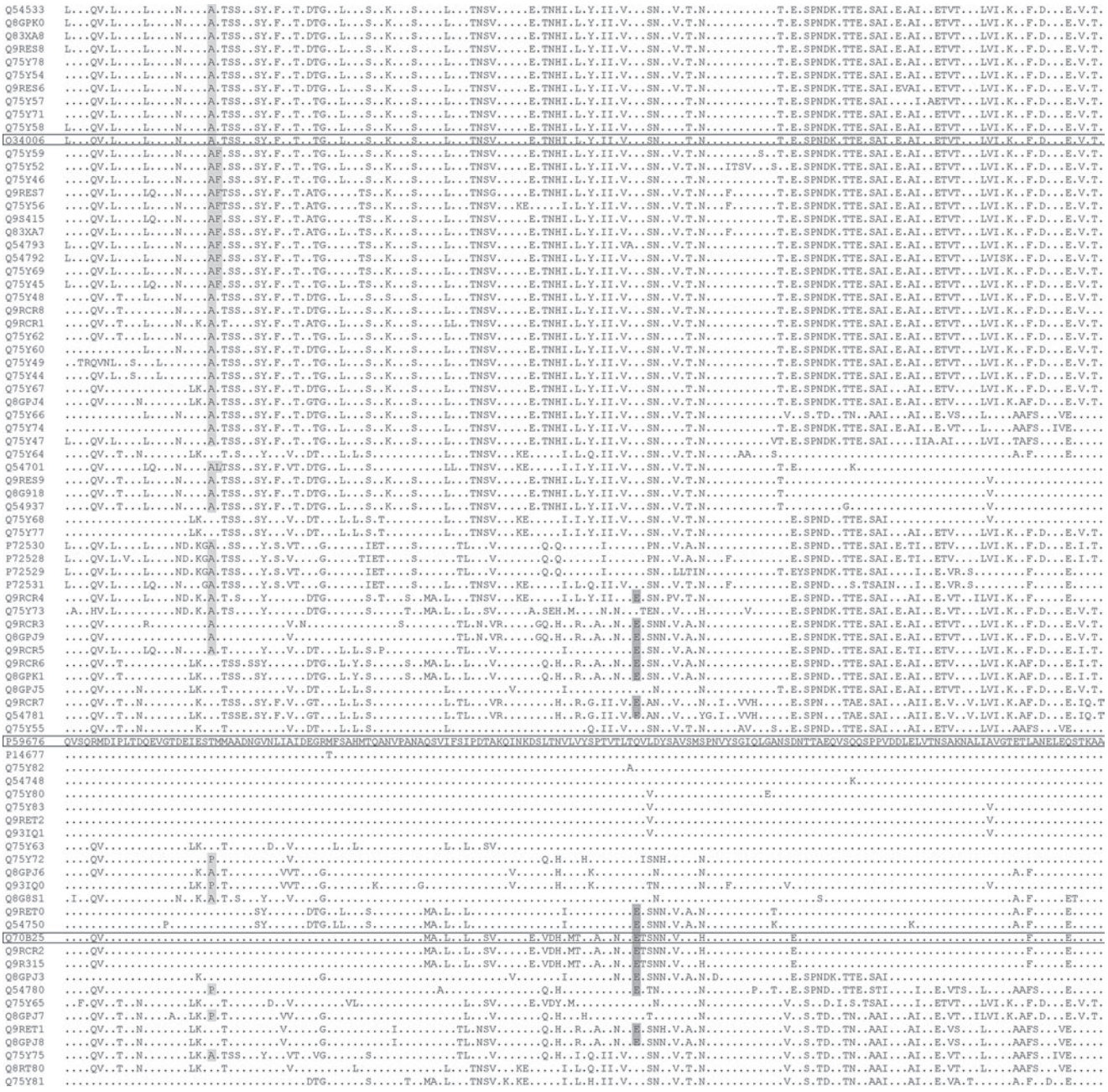

Fig. 9 Alignment of PBP2x publicly available sequences (aligned and clustered with CLUSTALW). Only positions where at least one sequence differs from the R6 reference (Uniprot accession number # P59676) are shown. Substitutions at position 338, 339, and 552 are highlighted. Although the mosaicity confounds effort to classify unambiguously these sequences, this representation allows to visualize that sequences characterized by a mutation in position 338 differ substantially from sequences with the Q552E substitution, although a few sequences harbor both mutations. The crystal structure of the high affinity PBP2x from strains R6 (# P59676), as well as the two low affinity protein from strains Sp328 (# O34006) and 5259 (# Q70B25) have been solved, revealing two modes of reducing the affinity for β-lactams

replacement of T338 can lead to destabilization of the active site due to the loss of the water molecule (61). Introduction of the sole T338A mutation in PBP2x from the susceptible strain R6 reduces its efficiency of acylation by penicillin by a factor of two (61), which is not enough to be selected following transformation into a susceptible strain (119). Reversion of the substitution in the related PBP2x from resistant strains Sp328 and 4,790 increases the acylation efficiency sixfold (61, 119).

A subset of sequences that contain the T338A mutation also have the adjacent M339F substitution. These sequences are from strains with particularly high levels of resistance

Fig. 10 Distribution of the amino acid substitutions (red) in the PBP2x transpeptidase domain from *S. pneumoniae* strains Sp328 (sequence # O34006) and 5259 (# Q70B25), with respect to PBP2x from strain R6 (*See Color Plates*)

(111, 112, 119, 125–127). PBP2x molecules from such isolates have an efficiency of acylation by penicillin reduced more than 1,000-fold (60, 119). Most of this reduction is due to a slower rate of acylation (k_2 decreased 300-fold), although a weaker preacylation binding (K_d fourfold higher) also contributes to the overall extremely poor affinity of the PBP2x with the double T338A/M339F (60, 119). In addition, these PBP2x variants have significantly faster deacylation kinetics (k_3 increased 40- to 70-fold), an effect mostly due to the M339F substitution (119, 137). 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 (60, 119).

The M339F mutation alone, introduced in the reference R6 PBP2x, reduces the efficiency of acylation by penicillin by sixfold and is sufficient to confer a measurable level of resistance (119). Combination of the M339F and T338A mutations produces a greater effect. The structure of the latter double 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 (119) (Fig. 11). The active-site serine 337 may exist in an equilibrium between two rotamers, only one of which can be acylated. Mutations such as M339F, by subtly altering the active site, may shift the equilibrium towards the unproductive rotamer. Note that this effect could be restricted to the reaction with β-lactams if binding of the physiological substrates favors a conformation that offsets the effect of the mutations.

The detailed studies of a few mutations fell short of explaining the reduction of affinity measured for PBP2x from clinical resistant isolates. The individual reversions of the 41 mutations of the PBP2x transpeptidase domain from a highly resistant strain, revealed by in vitro kinetic and in vivo phenotypic characterization the importance of four substitutions, in positions 371, 384, 400 and 605, in addition to those in position 338 and 339 (142). The combined reversion of

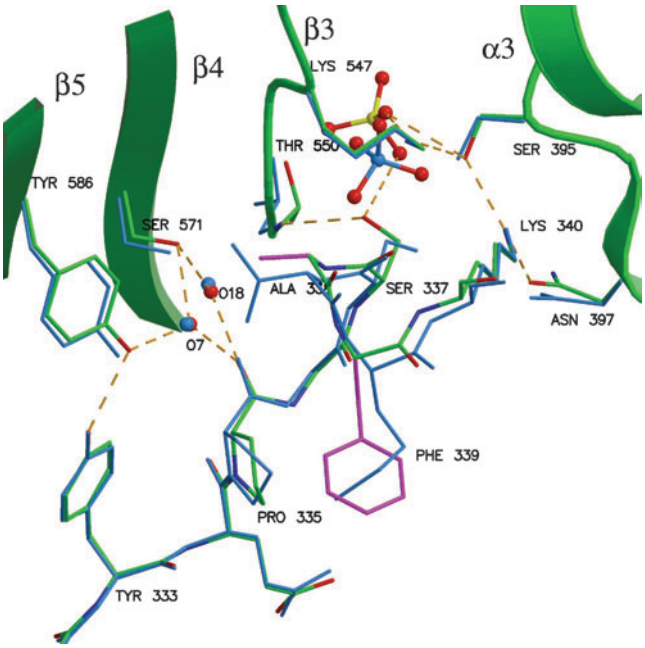

Fig. 11 Superposition of the active site of wild type R6 PBP2x (blue) and of the double mutant T338A/M339F (green carbon atoms and side chains of the mutated residues in purple). Note that the hydroxyl of the catalytic S337 is pointing in opposite directions (*See Color Plates*)

the six substitutions nearly restored the normal rapid rate of acylation by β-lactams. Conversely, introduction of five combined mutations diminished the reactivity towards β-lactams almost to the level of the original PBP2x with 41 substitutions. A conceptually similar study in vivo with a different PBP2x, also identified the I371T and R384G substitutions as central for the reduced acylation rate (143).

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 (140). The most striking feature of Sp328 PBP2x is the great flexibility of the loop-spanning residues 365–394. This instability extends in part to the SXN motif in positions 395 to 397, with S395 being somewhat displaced. Thus, the 60-fold reduction of the acylation efficiency by cefotaxime, for example, is due to a distortion of the active site (61, 119). The 365–394 segment forms one side of the groove leading to the active site. The flexibility of this region generates a more accessible "open" active site that may better accommodate alternative physiological substrates with branched stem peptides (140). The destabilization of the 365–394 region was shown to result from the I371T and R384G mutations (142).

A second family of PBP2x molecules from resistant strains can be defined by the presence of the Q552E substitution. Introduction of this single substitution in PBP2x reduces about fourfold the efficiency of acylation and confers a modest level of resistance to the recipient R6 strain

(120, 141). The structure of a PBP2x from a clinical strain that possess the Q552E substitution has been solved to a resolution of 3 Å. This PBP2x has an efficiency of acylation reduced more than 15-fold (120). The only significant difference found in comparison to the structure of R6 PBP2x is the displacement of strand β3, which carries the KTG motif (120) (Fig. 12). This displacement of 0.5 Å narrows the active site, and is reminiscent of the closed conformation of PBP2a from *S. aureus*, which is thought to cause the low efficiency of acylation of this enzyme by coupling the reaction to a major structural rearrangement (63). In addition to this conformational effect, the introduction of a negative charge in position 552 greatly affects the entry of the active site and does not favor binding of β-lactams, which are negatively charged (120).

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 in strains of *S. 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 (108, 110–112). 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 (111). 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 (141). 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 (139).

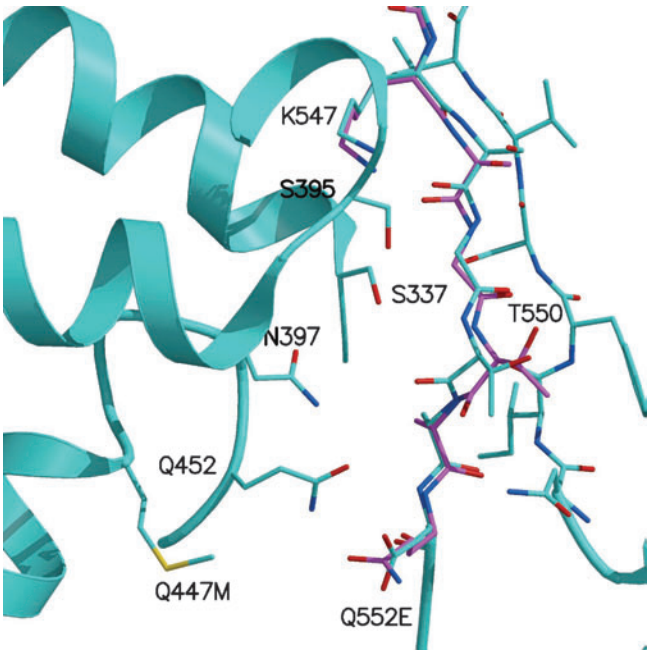

Fig. 12 Structure of the PBP2x active site from strain 5259 (cyan). The position of strand β3 from R6 PBP2x is shown in purple. Note the slight closure of the active site from 5259 PBP2x (*See Color Plates*)

PBP2b, the other class B PBP from *S. pneumoniae*, has not been subjected to such thorough investigations, presumably because of the absence of high-resolution structure. Over 90 sequences are available and two substitutions, T446A or T446S and E476G are always found in PBP2b from clinical resistant strains. The probable importance of these two substitutions was pointed out in numerous studies (101, 115, 125, 127). The T446A mutation, which is immediately adjacent to the SXN motif, is also selected by piperacillin in the laboratory (110). T446A is the only substitution that has been characterized biochemically (121) and it reduces the affinity for penicillin by 60%. The affinity of various PBP2b molecules from clinical isolates with 6 to 43 mutations in addition to T446A is reduced by 90 to 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 (98, 101). Three related PBP2b sequences from Japanese isolates are noteworthy by the insertion of three residues (SWY) after position 422 (144). 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 (see below). 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 (145). 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 (146). 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 ten substitutions in the region 591–640 surrounding the third catalytic motif KTG (118, 147). 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 clinically as the most important and troublesome PBP. 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. High level of resistance depends on a

modified PBP1a. Despite its clinical importance, PBP1a is the least studied of the three PBPs clearly involved in resistance. Biochemical studies have been limited, although the crystal structure of the transpeptidase domain is now available at the resolution of 2.6 Å (148). The acylation efficiency of PBP1a from the susceptible strain R6 was measured to be about 70,000 $M^{-1}s^{-1}$ for penicillin and the deacylation rate constant to be about $10^{-5}\,s^{-1}$ (149). These values are of the same magnitude as those reported for PBP2x. No biochemical data have been published for a mosaic PBP1a. About 50 PBP1a sequences are publicly available. The T471A substitution within the first catalytic motif, analogous to the T338A mutation in PBP2x, is commonly found in PBP1a sequences from resistant strains (117, 126–128, 150). Reversion of this substitution reduced but did not abrogate the resistance that PBP1a confers in addition to PBP2x and PBP2b (117). Some mosaic sequences lack the T471A mutation, including PBP1a from a highly resistant Hungarian isolate (MIC for penicillin of 16 mg/L) (151). Another remarkable feature is the mutation of a stretch of four residues (TSQF to NTGY) at position 574–577, which is observed in all the mosaic sequences. Amino acids at positions 574–577 belong to a loop between strands β3 and β4, which form the side of a tunnel at the entrance of the catalytic cleft. This wall has a hydrophobic character conferred by Phe577, which is certainly changed in the mutant (148). Reversion of this set of substitutions decreased the additional resistance conferred by PBP1a (151). 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 (151). Much remains to be learnt 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 *S. 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 *S. mitis* to a laboratory strain of *S. pneumoniae* was shown to require transfer of the genes encoding the five high molecular weight PBPs (152). A point mutation in the low molecular weight PBP3 was found to contribute to the resistance of a strain selected on cefotaxime in the laboratory (109). In contrast to these laboratory experiments, examination of the PBPs from clinical isolates failed to reveal significant modification of PBP1b or PBP3 (125, 153). 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 (97, 104). Also, transfer in the laboratory of resistance from a *S. mitis* strain to *S. pneumoniae* involved modification of PBP2x, PBP2b, PBP1a, and PBP2a, but not of PBP1b and PBP3 (116). Various combinations of point mutations, including silent ones, were observed in some PBP2a sequences, suggesting events of intraspecies recombination (154). The role of PBP2a in β-lactam resistance is now firmly established in at least one instance (155). 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 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 *S. pneumoniae*, which is consistent with the selection of variants of these proteins by β-lactams (146). PBP1b and PBP3 are not essential (156, 157), which again is 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 (156, 158). 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 (159).

A puzzling discovery was made, which is directly related to PBP-based β-lactam resistance. Clinical resistant isolates have an abnormal peptidoglycan structure with an elevated proportion of cross-bridges that involve branched stem-peptides (160). 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) (161). The genes responsible for the synthesis of branched precursors were found to constitute the *murMN* operon (162), also known as the *fibAB* operon (163). Mosaic *murM* genes often increase the resistance level conferred by a set of mosaic *pbp* genes (130, 162). A naïve explanation is that mosaic PBPs prefer branched substrates. However, deletion of *murM* abolishes the resistance but does not have impact on the growth rate in the absence of antibiotic challenge (162), demonstrating that mosaic PBPs can efficiently use linear precursors. The situation is reminiscent of the role of *femAB* operon in *S. 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* (52, 53). 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 (162).

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 (154).

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

4.4 Neisseria

Neisseria meningitidis and *Neisseria gonorrhoeae* are pathogens that have acquired reduced susceptibility to penicillin via two routes. The modification of at least one chromosomally encoded PBP will be discussed below. Alternatively, production of a plasmid-encoded β-lactamase is common in *N. gonorrhoeae* (e.g., (164)), while it is rare in *N. meningitidis* (165).

Neisseria species contain only three PBPs called PBP1, PBP2, and PBP3, which are respectively class A, class B, and a low molecular weight carboxypeptidase. Gonococcal strains with reduced susceptibility to β-lactams that do not express a β-lactamase were found to exhibit reduced labeling of PBP2 and PBP1 with radiolabeled penicillin (166). Reduced labeling of PBP2 was observed in meningococci (167). PBP2 is encoded by the *penA* gene, which is mosaic in resistant strains of *N. gonorrhoeae* (168) and *N. meningitidis* (169). The mechanism of acquisition of non-plasmidic resistance in *Neisseria* is therefore similar to that of *S. pneumoniae*.

Like pneumococcus, *Neisseria* species are naturally competent organisms and horizontal gene transfers are common (170). The origin of the foreign sequence fragments that are found in the *penA* gene of resistant 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 to *penA* genes from resistant strains (169, 171–173). *N. flavescens* isolates recovered from the preantibiotic era have relatively high penicillin MICs and a PBP2 with an intrinsic low affinity for penicillin (171). Transfer in the laboratory of the *penA* gene from such *N. flavescens* isolates could indeed confer some resistance to *N. meningitidis* (171). In contrast, *N. cinerea* is not naturally resistant, and accordingly, no resistance was achieved in *N. meningitidis* upon transfer of the *penA* gene from this species (171). It was found that the PBP2 sequences of *N. cinerea* origin found in resistant meningococci have an additional aspartic acid following D345, which is not present in the susceptible *N. cinerea* strains (171). This insertion was also found in mosaic PBP2 sequences from most resistant gonococcal strains. Site-directed mutagenesis has demonstrated that this insertion is sufficient to decrease the reactivity of PBP2 for β-lactams and to confer some resistance to *N. gonorrhoeae* (174). A clinical resistant strain was later discovered that only has this additional aspartic acid (175). The sequence identity between *Neisseria* PBP2 and PBPs of known structures is too low to obtain 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 to the SXN motif. This region was found to be destabilized in a low-affinity PBP2x of *S. pneumoniae* (140).

Thus, it appears that *penA* alleles that confer 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. When, how often, and in which species these recombination and mutation events have occurred are difficult questions. As commensal *Neisseria* species readily exchange genetic material, the *penA* alleles conferring resistance may be considered as forming a common gene pool, which is shared by several species (176, 177).

The cell wall of strains 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 (178).

Early studies hinted at the possibility that PBP1, the class A PBP, also had decreased reactivity for penicillin in gonococci (166), but subsequent studies failed to uncover mosaicity in the *ponA* gene encoding PBP1. 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 (179). This substitution is 40 residues N-terminal to the catalytic S461. The sequence identity with the only PBP of class A of known struture (*S. pneumoniae* PBP1b, (180)) is too low to determine the location of the L421P substitution. Nevertheless, the L421P substitution was shown in vitro to diminish about fourfold the acylation efficiency of PBP1 by various β-lactams (179).

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 (181), while *penB* codes for a porin (182). The nature of the third locus *penC*, which is required to allow phenotypic expression of the *ponA* mutation, remains undetermined (179).

4.5 Other Pathogens

Modified PBPs as a means to resist β-lactams has been documented in a few other pathogens, including the species where the most frequently encountered mode of resistance is the production of a β-lactamase. Some examples will be briefly presented below.

Most resistant clinical isolates of *Haemophilus influenza* evade the action of β-lactams by producing a β-lactamase. However, the number of β-lactamase-negative ampicillin-resistant (BLNAR) strains is rising, particularly in Japan (183). First documented in 1980 (184), BLNAR strains were found to express PBPs with a reduced reactivity towards penicillin (185). Early studies that monitored the PBPs by reaction with radiolabeled penicillin found modifications in PBP2, PBP3, PBP4, PBP5, and PBP6, depending on the resistant strain (186, 187). Further scrutiny and gene sequencing confirmed only the role of modifications in PBP3, the division specific class B PBP (188–190). Truncation of PBP4, a low molecular weight PBP, was found in some BLNAR strains, but this anomaly was not correlated with resistance (191). Another study failed to find significant substitutions in the high molecular weight PBPs: PBP1a, PBP1b, and PBP2 (192).

Sequencing of the gene fragment encoding the transpeptidase domain of PBP3 revealed in excess of twenty mutation patterns, with a number of mutations per sequence ranging from one to nine, affecting 21 different positions (191–193). These PBP3 sequences are not mosaic but show an accumulation of point mutations. Various classification schemes have been proposed (191, 193, 194). Some sequences are characterized by the presence of an R517H substitution (group I), while others have the N526K mutation (groups II and III). 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, which is also mutated in a group of PBP2x sequences from *S. pneumoniae* (120). Sequences that contain the N526K substitution can also possess the three additional mutations M377I, S385T, and L389F surrounding the second SSN381 catalytic motif (group III). Site-directed mutagenesis and transformation experiments have shown that S385T and L389F increase the resistance conferred by N526K. M377I does not and may be a neutral mutation linked to the S385T substitution (194). Modeling of the structure of *H. influenza* PBP3 on that of *S. pneumoniae* PBP2x showed that residues 517, 526, 377, 385/ and 389 are probably lining the active-site cavity (191). It has been noted that the PBP3 sequence of group III, found only in Japan, is association with a high resistance to cefotaxime, whereas group I and group II sequences confer only weak resistance to this cephalosporin (193).

The affinity for penicillin of a few *H. influenza* PBP3 variants has been measured in vitro (192). PBP3 of group II, including one variant that has only the N526K mutation had lower affinity than a PBP3 of group I, in agreement with the resistance level of the originating strains. Surprisingly a PBP3 with only the R517H substitution, the mutation defining group I sequences, had the same high affinity as a wild-type PBP3. This substitution in isolation therefore cannot confer resistance.

BLNAR strains with high level of resistance can combine mechanisms that involve alteration of PBP3 and an efflux pump (192), must be added. In addition, it is now evident that a low-affinity PBP3 can also be found in strains expressing a β-lactamase, and that both mechanisms can cooperate to increase the resistance sense combinations of β-lactams and β-lactamase inhibitors such as the widely used amoxicillin/clavulanate formulations (193, 195).

The genome of *Helicobacter pylori* encodes three recognizable PBPs. These are the homologues of the class B PBP2 and PBP3, and of the class A PBP1a from *E. coli*. Using a fluorescein-labeled penicillin, a fourth low molecular weight penicillin-binding protein was identified (196). Its sequence shows no homology with proteins of the ASPRE family and the catalytic motifs cannot be recognized in their usual positions. The status of this protein with respect to the subject of this review is therefore uncertain.

Clinical amoxicillin-resistant *H. pylori* strains have been isolated that lose their resistance following storage as frozen samples (197). This type of unstable resistance may be related to the transient loss of expression of the fourth mysterious penicillin-binding protein (198).

The isolation of a few stable amoxicillin-resistant strains was also reported (199–202). In one strain, the resistance was shown to result entirely from the single point mutation S414R in PBP1a, although another substitution was also present (202). Two other stable resistant strains were found to have the three substitutions T556S, N562Y, and T593A as well as the insertion of a Glu after residue 464 (201). One strain had ten substitutions, all of them in the second half of the transpeptidase domain, including the T556S and N562Y mutations (203). It may be noteworthy that the T556S is within the third catalytic motif KTG. In vitro selection on amoxicillin also yielded strains with modified PBP1a (204). The PBP1a of one such strain had four substitutions, including the S414R mutation (205).

To our knowledge, no clinical isolates of *Escherichia coli* were found to resist through the expression of modified PBPs. However, as a laboratory workhorse, *E. coli* was used to demonstrate that β-lactam pressure can select altered PBPs (206). Several point mutations in PBP3 were found to confer resistance to cephalexin and other cephalosporins. Note that *E. coli* PBP3 is the class B PBP dedicated to division. Interestingly, the substitution T308A, next to the active site S307, is analogous to the PBP2x T338A and PBP1a T471A that confer resistance to *S. pneumoniae* (207, 208). Another mutation was found in the second catalytic motif, changing SSN361 into SSS361 (207).

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 (*209) and *Pseudomonas aeruginosa* (210). A cefsulodin-resistant clinical isolate of *P. aeruginosa* also had one PBP with reduced affinity, although not the same as the imipenem-resistant isolate (211). Overexpression of

PBP3, in addition to decreased outer-membrane permeability, was found in a highly resistant strain of *Salmonella muenchen* (212). 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 (213). In the laboratory, imipenem could select a resistant clone of *Acinetobacter baumanii* with an altered PBP (214). Alterations of PBP3 or PBP2 were selected in laboratory mutants of *Listeria monocytogenes* (215, 216). Altered PBPs were also found in laboratory resistant mutants of the *Bacteroides fragilis* group (217) and of *Rhodococcus equi* (218).

Pathogens have been submitted to severe antibiotic pressure over the past five decades, leading to the emergence of resistant strains. In a natural setting, β-lactam-producing bacteria need to be protected against drugs of their own making. Two examples have been documented, which involve low-affinity PBPs. Expression of a particular PBP is responsible in part for the resistance of β-lactam-producing *Streptomyces clavuligenus* (219). 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 (220).

5 Are the PBPs Sustainable Targets?

The PBPs involved in the β-lactam resistance of the major pathogens are summarized in Table 2. The use of β-lactams to treat staphylococcal, enterococcal, and pneumococcal infections is already largely compromised. The isolation of strains with modified PBPs from species that usually resist by producing β-lactamases is worrying. The long-term efficacy of β-lactams may thus be compromised even in the advent of efficient β-lactamase inhibitors. It is therefore reasonable to ask whether PBPs are still valid targets for future antimicrobial therapies.

Half a century of β-lactam therapy has largely validated the targeting of PBPs. The uniquely eubacterial synthesis of peptidoglycan is a good predictor of the near absence of negative secondary effects in vertebrates. These two reasons justify the continued effort to target the PBPs. In which direction should the research effort be headed?

The main lesson from detailed kinetic studies of the reaction between PBPs and β-lactams is that these antibiotics are a poor fit to the enzyme-active site. The high dissociation constant of the noncovalent complex guarantees the broad specificity of the β-lactams, but also hints that attempts to improve their affinities may be misguided. Moreover, crystal structures of PBPs complexed covalently to various antibiotics can only suggest what might be the interactions taking place in the preacylation complexes. The structure of a preacylation complex would help to understand both the noncovalent affinity (K_d) and the acylation rate (k_2), the latter being most affected in altered PBPs.

Instead of focusing on the reaction between PBPs and β-lactams, research should be directed towards what may be PBPs' Achilles' heel: their physiological reaction of transpeptidation. Indeed, the remarkable feature of the low-affinity PBPs is their retained capacity to catalyze peptidoglycan cross-linking, even though the acylation chemistry is expected

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	PBP1b	PBP2a	PBPC(B)	PBP2b	PBP5 ↗
	ponA	pbpZ	pbpF	pbpB	pbpA	
Streptococcus pneumoniae	PBP1a	PBP1b	PBP2a	PBP2x	PBP2b	
Neisseria	PBP1			PBP2		
	ponA			penA		
Haemophilus influenzae	PBP1a	PBP1b		PBP3	PBP2	
				ftsI		
Helicobacter pylori	PBP1a			PBP3	PBP2	

Low-affinity PBPs are boxed. *Hatched borders* indicate an intrinsic low affinity. An *arrow* indicate overexpression. *No shading* indicates point mutations, *light shading* indicates mosaicity, and *dark shading* indicates acquisition of exogenous origin. Alternative gene names are given below their respective product

to be similar to β-lactams and D-Ala-D-Ala-containing substrates. Understanding how the natural PBP substrates maintain the reactivity of the catalytic serine even in PBPs from resistant bacteria should help the design of novel compounds. Such new drugs could react with all PBPs, regardless of their reactivity with β-lactams (221). Alternatively new molecules might serve as adjuvant to restore or maintain the reactivity of all PBPs towards traditional β-lactams.

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