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Resistant penicillin-binding proteins

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Abstract. Low-affinity penicillin-binding proteins (PBPs), which participate in the β-lactam resistance of several pathogenic bacteria, have different origins. Natural transformation and recombination events with DNA acquired from neighbouring intrinsically resistant organisms are responsible for the appearance of mosaic genes encoding two or three low-affinity PBPs in highly resistant strains of transformable microorganisms such as *Neisseria* and *Streptococcus pneumoniae*. Methicillin-resistant *Staphylococcus aureus* and coagulase-negative staphylococcal strains possess the *mecA* determinant gene, which probably evolved within the *Staphylococcus* genus from a closely related and physiologically functional gene that was modified by point mutations. The expression of *mecA* is either inducible or constitutive. A stable high-level resistant phenotype requires the synthesis of a normally constituted peptidoglycan. Enterococci have a natural low susceptibility to β-lactams related to the presence of an intrinsic low-affinity PBP. Highly resistant enterococcal strains overexpress this PBP and/or reduce its affinity.

Key words. Penicillin-binding protein; penicillin resistance; mosaic genes; *Streptococcus pneumoniae*; *Neisseria gonorrhoeae*; *Staphylococcus aureus*; *Enterococcus faecium*; *Enterococcus hirae*.

Alterations in PBPs with decreased affinity for β-lactam compounds

Bacteria have evolved a variety of mechanisms to escape the action of antibiotics: permeability changes causing restricted entry into the periplasm, enhanced export of a drug via specific transport proteins, production of enzymes that modify or hydrolyse and thereby inactivate the antibiotic, and modification of the target itself. The latter mechanism contributes to β-lactam resistance in a variety of bacterial species. Investigations on such evolutionary pathways have revealed an astounding versatility in respect to alterations in penicillin-binding proteins (PBPs), the essential target enzymes for β-lactam antibiotics.

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Penicillin-binding proteins are minor membrane components functioning in the late steps of murein biosynthesis. They are multimodule proteins as described elsewhere in this issue by Nguyen-Distèche et al. The catalytic penicillin-binding (PB) module occurs also as part of penicillin sensor-transducers such as *Staphylococcus aureus* MecR and *Bacillus licheniformis* BlaR [1]. Each bacterial species has at least three and up to more than eight PBPs, whose function in vivo is, in most cases, not known. *Escherichia coli* PBPs comprise two high molecular mass (hmm) class A PBPs 1a and 1b acting as penicillin-sensitive transpeptidases and -insensitive transglycosylases, two hmm class B PBPs 2 and 3 exhibiting transpeptidase activity, and five low molecular mass (lmm) PBPs 4, 5, 6, 6b and 7 functioning as DD-carboxypeptidases, endopeptidases and/or model

transpeptidases. This group of enzymes reveals features that are important for understanding PBP-related resistance mechanisms [2–4]. Some *E. coli* PBPs appear essential (the class B hmm PBPs), whereas others are dispensable and can be deleted, at least under laboratory conditions (the lmm PBPs). There is also a pair of PBPs (the class A hmm PBPs) where either one, but not both, can be deleted, and this has been interpreted as either one being able to 'replace' the other one sufficiently to allow cellular growth. According to sequence comparisons, each bacterial species has homologues of the *E. coli* PBPs, probably with similar physiological roles.

The deletion (absence) of a PBP has been equated to the in vivo situation in which a PBP is completely inhibited by penicillin (nonfunctional PBP) [4–6]. Therefore, those PBPs that formed in vivo penicilloyl complexes at or far below the minimal inhibitory concentration (MIC) of the respective β-lactam were regarded as unimportant for development of resistance. There are, however, indications that this correlation is not so simple. The lmm PBP 3 of *Streptococcus pneumoniae*, for example, is saturated by β-lactams at concentrations far below their respective MIC values [7] and can be deleted [8], but the mutants have severe defects in septum formation and murein structure [8, 9]. It has also been suggested that, in non-β-lactamase-producing organisms, only PBP alterations and, in Gram-negative bacteria, also permeability changes contribute to penicillin resistance. There are, however, clear cases where other mutational pathways can be used to establish β-lactam resistance, examples being mecillinam resistance in *E. coli* [10, 11] and β-lactam resistance in *S. pneumoniae* [12, 13].

In the first part, we will give an overview of penicillin resistance in *Streptococcus* and *Neisseria*, both being species where alterations of the chromosomally encoded structural PBP genes are observed in penicillin-resistant clinical isolates. The second part will summarize the situation in *Staphylococcus* and *Enterococcus*, where resistance is associated with overproduction of a PBP that is absent or produced only in low amounts in susceptible strains.

Development of non-β-lactamase mediated penicillin resistance in *N. gonorrhoeae and S. pneumoniae*

Non-β-lactamase-mediated penicillin resistance in both *Neisseria* and *Streptococcus* sp., with MIC values of 1 to 2 μg/ml compared with MICs close to 0.02 μg/ml for benzylpenicillin in susceptible isolates, became apparent in the early 1980s. In all cases, resistance is accompanied by the appearance of low-affinity PBP variants.

Neisseria has three PBPs, 1, 2 and 3. Lower-level-resistance isolates contain a low-affinity PBP2, and increased resistance is accompanied by alteration in PBP1 as well as in two further non-PBP genes, possibly leading to permeability changes due to alterations in outer membrane proteins [14]. Only PBP2, the primary target responsible for low resistance levels, has been analysed in penicillin-resistant isolates at the molecular level.

S. pneumoniae has six PBPs: the class A hmm PBPs 1a, 1b and 2a, class B hmm PBP 2x and 2b, and the lmm PBP3. All six PBPs can occur as low-affinity variants in β-lactam-resistant laboratory mutants [15], clinical isolates [16–18] or transformants obtained with chromosomal DNA of resistant *Streptococcus* sp. ([19, 19a], and unpublished results). The combination of low-affinity PBPs expressed in a resistant strain varies, perhaps reflecting different selective conditions and indicating a flexibility of the resistance development pathway.

PBP2x and PBP2b are primary targets for β-lactams. When transformed in a sensitive recipient strain, *pbp2x* or *pbp2b* genes from resistant clinical isolates or laboratory mutants confer low resistance levels [15, 20–22]. Since PBP2b does not interact with third-generation cephalosporins, it is not required for resistance to these drugs [23]. A low-affinity PBP1a considerably increases the resistance in a strain with low-affinity PBP2x and/or PBP2b [24–26], and many resistant isolates are modified in these three PBPs only.

Mosaic genes in clinical isolates

Comparison of PBP genes from sensitive and resistant isolates has revealed that the low-affinity PBP2 of *Neisseria* contains homologous sequences that diverged from those in sensitive isolates with from five to over 20% of the nucleotide sequence being altered; most probably, the result of transfer of homologous genes followed by recombinational events [27]. The PBP alleles are apparently accessible to a variety of related species. *N. flavescens* and *N. cinerea*, both intrinsically more resistant to penicillin, were identified as 'donor' species, and *N. lactamica*, *N. mucosa*, *N. polysaccharaea* and the pathogenic species *N. gonorrhoeae* and *N. meningitidis* all participate in the exchange of PBP variants [28, 29].

PBP2b, 2x and 1a of 3*S. pneumoniae* of resistant clinical isolates are also encoded by mosaic genes [18, 30, 31] that can be transferred between *Streptococcus sanguis*, *S. oralis*, *S. mitis*, *S. pneumoniae* and other still unidentified streptococcal species [19, 32–34]. There

are no reports of commensal species that are truly penicillin-resistant. The penicillin-susceptible strains *S. oralis* M3 and *S. mitis* NCTC10712 each contain a *pbp2x* gene that is closely related to the mosaic blocks in penicillin-resistant *S. pneumoniae* [35]. One of them has a mosaic structure, i.e. this feature is not exclusively associated with penicillin resistance. Gene transfer involving these *pbp2x* genes can also occur. Also, the *pbp2b* gene of *S. mitis* NCTC10712 strain (and that of other sensitive strains) is closely related to the mosaic *pbp2b* genes of *S. pneumoniae* [36].

Point mutations in PBPs

So far, mutations have been identified only in the class B hmm PBPs that represent primary target enzymes. PBP2 in resistant *N. gonorrhoeae* strains contains an additional amino acid which represents a major resistance factor [37]. In *S. pneumoniae* PBP2b, a succession of seven altered amino acids has been linked to penicillin resistance in clinical isolates [38]. In both cases, these changes are located between the active site serine and the SXN motif. Another site where the affinity of PBP2b can be modified is revealed by the Thr446 to Ala substitution directly after the SSN box [36, 38], and this mutation alone is sufficient to confer a selectable resistance in wild-type cells [21]. Even more interesting is the fact that this mutation causes a second phenotype which is especially important in a clinical setting, i.e. cells with this mutation, and particularly with a low-affinity PBP2b, lyse much more slowly, if at all, even at high β-lactam concentrations, allowing a much better survival during treatment with these drugs and under other conditions as well [19, 21].

The PBP2x of a resistant *S. pneumoniae* isolate may differ from a PBP2x of a sensitive *Streptococcus* species by only 6 to 10 amino acid residues (fig. 1). Two of these sites known to contribute to resistance are shown by the substitution Thr338 to Ala or to Pro immediately after the active site Ser337 (J. Krauß and R. Hakenbeck, unpublished results), and by the Gln552 to Glu mutation [39]. Furthermore, the His394 to Tyr mutation was identified in a cefotaxime-resistant mutant [22] and occurs also in a PBP2x of a resistant *S. pneumoniae*. The Thr550 to Ala change that is found in cefotaxime-resistant laboratory mutants has also been identified as a major resistance factor in a high-level cephalosporin-resistant *S. pneumoniae* isolate [40]. This latter mutation is especially noteworthy since, while it only confers resistance to third-generation cephalosporins, it also induces hypersensitivity to oxacillin [22, 40]. In contrast, a Thr550 to Gly substitution still enhances the cefotaxime resistance, but abolishes the hypersensitivity to oxacillin [21]. In addition to these sites, all of which are close to the active-site motifs, the residues 596/597 and 600/601 at the beginning of helix 11 are mutated in different ways in cefotaxime- and piperacillin-resistant laboratory mutants [41, 42]. There are still other sites within the penicillin-binding domain that clearly contribute to cefotaxime resistance in laboratory mutants, some conferring resistance in a temperature-dependent manner [22].

Depending on the mutation, one single amino acid change in PBP2x can confer different levels of resistance to cefotaxime at between 0.06 to 0.6 μg/ml compared with 0.02 μg/ml of sensitive strains, and a low-affinity PBP2b changes the piperacillin MIC from 0.04 μg/ml to 0.08 μg/ml [21, 22]. The fact that a single mutation in a primary target PBP results only in a small MIC increase in sensitive wild-type strains is often overlooked in view of the fact that three multiply mutated PBPs from clinical isolates, transferred successively into a sensitive genetic background, confer MIC increases of at least 100- or even 1000-fold.

Does analysis of the mutations in PBP2x provide clues as to which regions are generally important for β-lactam interactions in PBPs? The answer is clearly yes – as far as mutations in the penicillin-binding domain are concerned, which according to the three-dimensional structure is related to the single domain β-lactamases and the R61 enzyme [43]. Mutations at or close to the KS/TG sequence are important parameters for β-lactam interactions, e.g. the change KTG to KTA in the *S. pneumoniae* class B hmm PBP2b of a piperacillin-resistant mutant [42], the change KTGT to KTGI in the *S.*

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                333333333444444555555556666 6
                833667899022556122556999000 0
                989491948326685263025679011 45

R6              MTMLAISHVLGRVQFRTVTQLSGQLGGAN
C/P             T------Y-FDC-K-WS-A--LD-WVE--
53139/72        -P----------A------ET--P---T-
29044           -ALFVTL-------L--I--S-------T
M3              ----------------L--S------S-
10712           --------I--------R--S--------
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Figure 1. Amino acid substitutions in the penicillin-binding domain of PBP2x of penicillin-resistant *S. pneumoniae* compared with that of sensitive strains. The position of the amino acid residue is indicated by the three vertical figures. Penicillin-sensitive strains: *S. pneumoniae* R6, *S. oralis* M3 [25] and *S. mitis* NCTC10712. C/P, mutations in cefotaxime- and piperacillin-resistant mutants [31, 33, 34]. PBPs 2x of the resistant *S. pneumoniae* 29044 and 53139/72 have been described [8]. The active-site motifs in PBP2x are S337TMK, S395SN, and K547SG.

pneumoniae lmm PBP3 of a cefotaxime-resistant mutant [44], and the KTGA to KTGT change in β-lactamases of different substrate profiles [45]. The positioning of a Thr instead of an Ala directly after the triad affects the interaction with third-generation cephalosporins in other enzymes as well, including β-lactamases, due to a crucial hydrogen bonding involving the Thr residue with this specific class of β-lactams [46].

Non-β-lactamase-mediated penicillin resistance in *Staphylococcus*

The β-lactam targets in susceptible staphylococci (penicillin MIC values as low as 0.03 μg/ml) are 3 hmm and 1 lmm PBPs, whose precise functions in the assembly of the cell wall peptidoglcyan have not yet been clearly identified [47]. PBP1 belongs to the class B PBPs. It plays a key role in cell growth, most probably in cell division, as its encoding gene was recently identified in a gene cluster analogous to the *d*ivision and *c*ell *w*all (*dcw*) cluster found in *B. subtilis* and *E. coli* [48–50]. The two other hmm PBPs, 2 and 3, were considered to have secondary functions, as their simultaneous specific inhibition did not induce bacteriolysis [48, 49]. PBP2 has, however, a primary structure similar to those of class A PBPs and consequently should function as a transglycosylase-transpeptidase enzyme [51]. Information concerning PBP3 is lacking. Finally, the lmm PBP4 acts as a transpeptidase-carboxypeptidase needed for the secondary cross-linking of peptidoglycan [52, 53].

During the preantibiotic era, the vast majority of the staphylococcal strains were susceptible to β-lactams. A few years after benzylpenicillin was introduced as a therapeutic agent, resistant staphylococcal strains were selected. They were able to produce penicillinases, the genes of which were plasmid-borne and could easily be transferred horizontally. Now 50 to 80% of staphylococci produce a β-lactamase [54].

Methicillin-resistant *S. aureus* (MRSA), with intrinsic resistance to all β-lactams (MIC values up to 1600 μg/ml), were isolated shortly after methicillin was introduced to inhibit resistant *S. aureus* strains that produced penicillinases. They are generally found in hospital settings, probably because of the high antibiotic pressure they are subjected to. Frequently, they possess several additional resistance genes that enable them to resist a large variety of antibiotics [54].

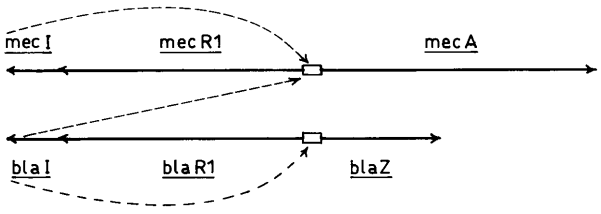

Figure 2. Schematic representation of the *mec* and *bla* loci involved in the synthesis of PBP2′ and β-lactamase BlaZ of *S. aureus*, respectively. The thick and thin arrows indicate the direction of the gene transcription and the repression sites, respectively.

Methicillin resistance in MRSA is due to the *mec* determinant borne on a 30- to 40-kb element of unknown origin that is integrated in a specific site of the *S. aureus* chromosome [55, 56]. MRSA strains synthesize an additional penicillin-binding protein, termed PBP2′ or PBP2a, which is determined by the *mecA* gene [57, 58] and has a lower affinity for penicillin than the natural PBPs of susceptible strains [59–62]. It behaves as a transpeptidase, enabling the MRSA to survive when penicillin concentrations are sufficient to inhibit the other PBPs [54, 59–62]. Some MRSA strains have two regulatory genes (*mecR1* and *mecI*) upstream of *mecA* (fig. 2) that are transcribed in a direction opposite to that of *mecA* [63, 64]. MecR1 is a transmembrane β-lactam sensor needed for induction, and MecI is the *mecA* repressor [65]. When β-lactams are absent, *mecA* is repressed and PBP2′ is not synthesized. However, many MRSA produce PBP2′ constitutively due to a complete lack of *mecI* or mutations in the sensor gene *mecR1* [66]. The regulatory *blaR1* and *blaI* genes of the staphylococcal BlaZ penicillinase are very similar to *mecR1* and *mecI*, and are able to control not only *blaZ* but also *mecA* (fig. 2) [64, 65].

Phenotypic expression of methicillin resistance is largely dependent on environmental factors such as temperature, osmolarity, chelating agents and divalent cations, pH and anaerobiosis [62, 67–70]. Genetic mechanisms involved in the control of the intrinsic resistance by these factors are still not identified.

Although they synthesize PBP2′, MRSA strains may vary widely in their methicillin resistance, with MIC values ranging from as low as 2 up to more than 1000 μg/ml. Some strains have a homogeneous resistant phenotype (i.e. all the cells in the population have the same high resistance). Others have a heterogeneous phenotype with a few cells within a culture (10^{-4} to 10^{-7} ratio) that express a high-level resistance (MIC over 250 μg/ml) and the majority of cells that have a comparatively low resistance corresponding to the MIC of the strain [10]. The homogeneous phenotype is stable, and highly resistant subclones isolated from a heterogeneous population generally do not revert readily to the original heterogeneous phenotype [67]. No satisfying genetic or physiological model for this drug-resistance heterogeneity has yet been proposed. It is supposed that one or more genes coding for additional factors are respons-

ible for the homogeneous resistance. They are located on the chromosome and not on the *mec* determinant [61, 71]. Various chromosomal auxiliary genes (e.g. the *fem* genes) independent of the *mec* locus are implicated in the expression of methicillin-resistance (fig. 3). The *femAB* operon codes for enzymes involved in the synthesis of the pentaglycine side chain of the peptidoglycan precursors. Inactivation of *femAB* leads to monoglycine side chains, reduced cross-linking and β-lactam hypersusceptibility [72–76]. Mutants in *femC* have fewer isoglutamine and more isoglutamic acid residues in the peptidoglycan due to a shortage of cytoplasmic glutamine [77]. Mutants in *femD* and *femF* are defective in cytoplasmic steps of peptidoglycan precursor synthesis [78, 79]. Other genes such as *llm* [80] and *fmt* [81] can affect methicillin resistance without changing PBP2′ production. The impact of these different factors on methicillin resistance in clinical isolates has not yet been measured.

Clinical low-level or borderline methicillin-resistant mutants were also identified which were *mec*-independent. Their resistance was linked to a reduced affinity of the natural PBP2 and PBP4. These isolates resemble laboratory mutants whose resistance could be related to an overproduction and/or a reduced affinity of PBP2 and PBP4 [82–84]. These low-level resistant mutants exhibit, however, a progressively slower growth rate as the level of resistance increases [85].

The *mecA* gene, the *femAB* operon and presumably the other auxiliary genes are not restricted to MRSA. They were described in coagulase-negative staphylococci causing serious infections, whose phenotypes were similar to those of MRSA strains [86–88].

Finally, it has recently been proposed that the *mecA* gene originated within the genus of *Staphylococcus* from a natural closely related gene that could perform a normal physiological function. To become a resistance determinant this *mecA* precursor must have undergone modifications probably due to mutations [89].

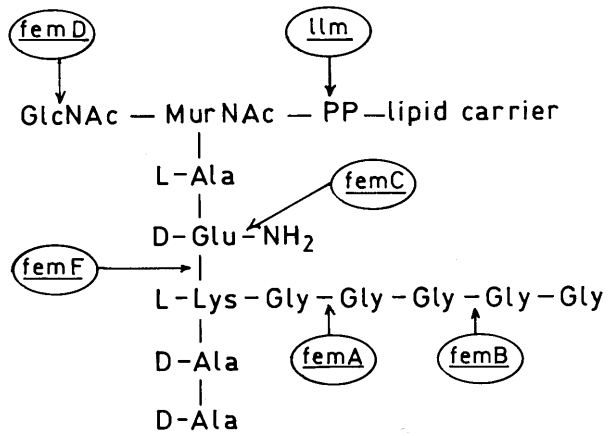

Figure 3. Chemical structure of the lipid-bound disaccharide peptide involved in the synthesis of *S. aureus* peptidoglycan. The arrows indicate the sites affected by the *fem* or *llm* mutations.

Non-β-lactamase mediated penicillin resistance in *Enterococcus*

Enterococci do not produce β-lactamases except for a few strains of *Enterococcus faecalis* and *E. faecium* which secrete the BlaZ enzyme after acquisition of the *blaZ* gene from *S. aureus* [90, 91]. Because they are devoid of the *blaI* repressor gene, these strains produce the enzyme constitutively [90].

Relative to streptococci, enterococci have a greatly reduced susceptibility to β-lactams. *E. faecalis*, *E. faecium*, *E. durans*, *E. raffinosus* and *E. avium* represent 90% of the enterococcal clinical isolates, originating essentially from nosocomial infections. *E. faecalis*, the most commonly isolated species, is less resistant to penicillin (MIC usually smaller than 4 μg/ml) than *E. faecium* and *E. raffinosus* (MIC usually higher than 4 μg/ml) [92, 93].

Enterococci possess at least five and sometimes more than nine different membrane-bound PBPs which, on SDS-polyacrylamide gel electrophoresis (PAGE), form a typical pattern that can be used for taxonomic purposes [94]. Very little information is available concerning the physiological or morphological roles of these PBPs. Inactivation of PBP2 and PBP3 by temperature-sensitive mutations indicated that both were essential for growth of *E. hirae* but had different functions [95]. Specific inhibition of PBP2 by cefoxitin does not affect cell morphology. In contrast, specific binding of cefotaxime to PBP3 blocked cell division and induced cell elongation, particularly under optimal growth conditions [96–98].

The intrinsic low susceptibility of enterococci to β-lactams is due to the presence of a natural low-affinity hmm PBP which provides most if not all the functions of the other PBPs when they are inactivated either by temperature-sensitive mutations [95] or by β-lactams [99, 100]. Inactivation of the low-affinity PBP (PBP5) of *E. hirae* by point mutations yields clones the susceptibility of which is increased 20 to 50 times (MIC for penicillin: 0.01 to 0.02 μg/ml) [101; O. Dardenne and J. Coyette, unpublished results]. These results indicate also that, under normal growth conditions, the low-affinity PBP5 of *E. hirae* is dispensable.

Occasionally, two low-affinity PBPs may be present, as observed in *E. hirae* S185 isolated from pig intestine. In addition to the natural PBP5, that strain produces another low-affinity PBP, designated PBP3r, the apparent size (as estimated by SDS-PAGE) of which is equivalent to that of the high-affinity PBP3 [102].

Several genes determining the synthesis of low-affinity PBPs of different enterococcal species or strains were cloned and sequenced [103–107]. All are chromosomal genes except that coding for PBP3r in *E. hirae* S185, which is borne on a large-size plasmid next to a transposon-like element [108].

Comparison of the primary structures of low-affinity PBPs of *E. hirae* [103, 104], *E. faecalis* [105] and *E. faecium* [106, 107] with those of PBP2′ of *S. aureus* [57] and *S. epidermidis* [88] indicates that they form a subgroup of class B PBPs. All share specific features such as specific conserved motifs in the C-terminal PB module and a 110-amino acid polypeptide inserted between the N-terminal peptide anchor and the non-PB module [107]. The role and function of these elements are still unknown, but one may hypothesize that some determine the binding characteristics of these PBPs.

In *E. hirae* ATCC9790 and S185, the expression of the PBP5-encoding gene (*pbp5*) is under the control of a repressor gene (*psr*) located immediately upstream from *pbp5* [109]. Recent reexamination of sequencing data revealed that the *psr* gene was significantly longer than previously reported (O. Massidda et al., unpublished results). Inactivation of the *psr* gene by a deletion or a point mutation leads to increased synthesis of PBP5 that results in a higher β-lactam resistance of the mutants (O. Dardenne and J. Coyette, unpublished results).

Similar observations were made on laboratory and clinical strains of *E. faecium* susceptible or resistant to penicillins [107, 110, 111]. However, analysis of these strains showed that overproduction of PBP5 is not the only mechanism of β-lactam resistance in *E. faecium*. Most highly resistant clinical isolates synthesize normal amounts of PBP5 whose affinity is further reduced with respect to that of the wild-type PBP5. The reduction in affinity is related to amino acid substitutions in the C-terminal module resulting from point mutations [106, 107, 111, 112].

The lack of Psr and the consequent higher amount of PBP5 in resistant mutants of *E. hirae* does not seem to induce significant modifications of the general composition and structure of peptidoglycan (S. Bossrez and J. Coyette, unpublished results). The Psr protein seems, however, to participate in the regulation of several additional surface-related properties. In the absence of Psr, *E. hirae* cells appear to be more sensitive to lysozyme, autolyse more rapidly in buffer and, in contrast to cells that possess a functional Psr, retain those characteristics after they enter the stationary growth phase [113]. These pleiotropic effects of *psr* suggest that it is part of a global regulatory system the extent and functioning of which are still unknown. It is interesting to note that the Psr regulator shows some similarity to other proteins thought to play a regulatory role in exo- or capsular polysaccharide synthesis or cell wall metabolism [114–117].

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