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COMMENTARY

TARGET-MEDIATED RESISTANCE TO β-LACTAM ANTIBIOTICS

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The term intrinsic penicillin resistance refers to a β-lactamase-independent resistance mechanism that primarily involves the production of target-enzymes with a decreased affinity for the drug. The proteins involved, PBPs†, constitute essential enzymes that are functioning at late stages of peptidoglycan assembly. In most cases, they are minor components of the cytoplasmic membrane. As members of the superfamily of bacterial penicilloyl serine transferases that also include β-lactamases, they bear several amino acid motifs that are placed at equivalent positions along the amino acid sequence: SXXK with the active site serine, SXN or YXN, and K(R,H)T(S)G. These "homology boxes" form part of the active cavity according to the known structures of β-lactamases (for a review, see Ref. 1). It is assumed that the crucial penicillin-sensitive reaction is a D,D-transpeptidation involving the C-terminal D-alanyl-D-alanine moiety of the peptidoglycan. Since inhibition of these enzymes by β-lactams is due to acylation (penicilloylation) of an active-site serine, i.e. a critical site of the PBP, remodeling and modification that result in reduced inhibitor binding without interfering (at least not severely) with substrate specific reactions cannot be an easy task for the bacteria.

Two main principles of PBP-mediated resistance are recognizable. In staphylococci and enterococci, resistant strains overproduce a PBP with exceptionally low penicillin affinity, which is not present in sensitive staphylococci or some sensitive enterococci [2–5]. These PBPs have a common modular design [1] and appear to be more related to each other than to other PBPs. Apparently, their (unknown) function enables the cell to survive at high concentrations of β-lactam antibiotics at which all other PBPs are inhibited. This principal mechanism has been the subject of recent review articles and, therefore, is not considered here [6–9]. Resistant *Haemophilus*, *Neisseria*, and *Streptococcus* sp. contain PBPs that are variants of those existing in the sensitive strain with a decreased affinity to β-lactams, often paralleled by an altered mobility of the PBPs on SDS gels [10–13]. Since *Haemophilus* PBPs have not been studied on the molecular level and since the main features about PBP modifications and resistance development in *Neisseria* have been covered extensively [13–15], this article will focus on the situation in pneumococci, which may serve as a paradigm for the evolution of penicillin resistance.

Defining the structural prerequisites of β-lactam/PBP interaction—by determination of the three-dimensional structure of an essential PBP and by determination of the sites in a PBP where modification affects primarily inhibitor-binding—is a prerequisite for molecular modeling and ultimately for designing novel inhibitors effective against low affinity PBPs. These efforts should be paralleled by defining the actual substrates and, thus, the *in vivo* function of PBPs. Since target-mediated penicillin resistance is emerging in several clinical important pathogens, a second main question is related to the epidemiological aspects and concerns the evolutionary pathway of PBP alterations in resistant clinical isolates. Another challenge in the future will be to unravel the role of non-PBP genes in resistance development in view of accumulating evidence that studying PBP genes alone is not sufficient for understanding the action of β-lactam antibiotics, as will be outlined below.

β-Lactam resistance has been investigated in laboratory mutants as well as in clinical isolates of *Streptococcus pneumoniae*, with each system revealing different pathways of resistance development. Six PBPs have been described in *S. pneumoniae* [16]. Five of these PBPs (1a, 1b, 2x, 2a and 2b) belong to the bi(multi)functional high M_r PBPs. The genes encoding PBP1a [17], PBP2x [18], PBP2b [19] and PBP3 ([20]; Krauß J and Hakenbeck R, unpublished results) are known. According to the modular organization, PBP1a is in class A, and PBP2x and PBP2b are in class B [1]. PBP3 is a low M_r PBP with D,D-carboxypeptidase activity *in vitro* [21].

Figure 1 schematically depicts the PBPs in *S. pneumoniae* and their role in β-lactam resistance in clinical isolates and laboratory mutants. Acquisition of resistance in *S. pneumoniae* appears to be a specially complex process with up to four of the five known high M_r PBPs being altered into low affinity variants in highly resistant clinical isolates [22].

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† Abbreviation: PBP, penicillin-binding protein.

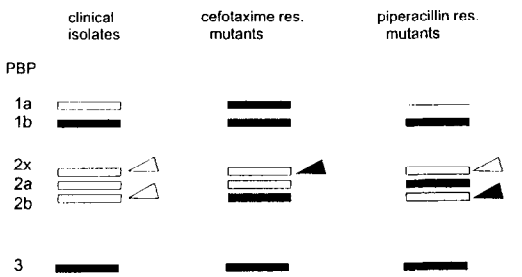

Fig. 1. Penicillin-binding proteins in β-lactam resistant *Streptococcus pneumoniae*. The six PBPs of *S. pneumoniae*, as revealed after labeling with radioactive penicillin, SDS–PAGE and fluorography, are schematically depicted as bars. Three situations are distinguished: resistant clinical isolates, and laboratory mutants resistant to either cefotaxime or piperacillin. White bars indicate PBPs with low affinity for penicillin in high level resistant strains; a thin line represents diminished amounts of PBP1a as seen in some piperacillin-resistant laboratory mutants. The first PBP affected during selection for resistance is PBP2x in the cefotaxime-resistant mutants and PBP2b in piperacillin-resistant mutants (black arrows). Low affinity PBPs conferring resistance upon transformation into a sensitive strain are also indicated by white arrows.

Whereas point mutations in the structural PBP genes are responsible for the altered properties of PBPs in laboratory mutants [23, 24], PBP genes of resistant clinical isolates have a mosaic structure in which parts are replaced by homologous gene fragments diverging by up to 20% or more from those of sensitive strains. This documents that interspecies gene transfer also plays a crucial role in modifying a PBP into a low affinity variant in clinical isolates [22, 25, 26], resulting in an enormous variability of PBP profiles and epitope variants in PBP1a or PBP2b [27]. This extends the concept that originated from DNA sequence analyses of PBP genes in *Neisseria* to another pathogenic species [15]. In no case has instability of the resistance been noted. Thus, once established in a population, resistant strains are easily maintained and can spread, especially during continuous selection pressure. In addition to PBP changes, recent evidence revealed that alterations in non-PBP genes also can be responsible for certain steps in the increase of resistance in laboratory mutants [24, 28].

It is important to realize that different β-lactams may well lead to different evolutionary pathways of resistance development due to differences in their respective targets. Two classes of β-lactam antibiotics can be distinguished on the basis of target PBPs and biological effect. Most β-lactams and all penicillins tested thus far bind to all six PBPs, some of them (e.g. piperacillin) with extremely high affinity. All these antibiotics cause cell lysis. On the other hand, third generation cephalosporins such as cefotaxime, and the monobactam aztreonam, belong to a second class of β-lactams that do not interfere with PBP2b [29]. Curiously, pneumococci treated with these compounds exhibit a tolerant response, i.e. inhibition of growth is not accompanied by rapid cell lysis and the cells are not killed at a substantial rate, indicating that inhibition of PBP2b is a crucial prerequisite for the lytic response [29]. The (trivial) consequence is that resistance to, for example, cefotaxime does not involve PBP2b (since it is not a target for this β-lactam) but primarily PBP2x, whereas penicillin resistance does [30].

Both PBP2x and PBP2b can be considered to be essential PBPs, since each confers β-lactam resistance when introduced in a sensitive strain ([18]; Grebe T and Hakenbeck R, unpublished results). In contrast, a low affinity PBP1a cannot be selected in a sensitive wild-type strain but only after, for example, PBP2x has been changed into a low affinity variant [30]. In agreement with this, attempts to delete PBP2x or PBP2b have been unsuccessful, whereas the penicillin-binding domain of PBP1a could be deleted in a laboratory strain [31, 32]. Although the mutant cells grew more slowly, and penicillin-induced lysis proceeded at a lower rate compared with that of the wild type, they were perfectly viable [31], indicating that PBP1a is not a primary target and that its function is not vital in the wild-type background.

β-Lactam resistance in laboratory mutants

Independent lineages of laboratory mutants were selected on stepwise increasing concentrations of either cefotaxime (resistance not involving PBP2b) or piperacillin (which binds to all PBPs already at very low concentrations) [33]. In such mutant families, the genes and mutations involved must be identified for each step of resistance increase in order to comprehend the genetic flexibility of resistance development. The results revealed that, indeed, the degree of flexibility is such that it is simply impossible to deduce the evolutionary history retrospectively from the genotype of higher level resistant mutants without the availability of the other parental mutants, neither on the level of the genes involved, nor on the level of point mutations introduced into one particular gene. In other words, there is neither a fixed succession of genes that are altered with increasing resistance, nor is there a specific order of mutations that are introduced into a target PBP.

In addition, cefotaxime-resistant mutants (C-mutants) differ from piperacillin-resistant mutants (P-mutants) by the set of PBP and non-PBP genes affected during selection of resistance. The first PBP gene (but not necessarily the first gene) affected is *pbp2x* in the case of the C-mutants, whereas in the P-mutants it is *pbp2b*. However, in some C-mutant families, and in all P-mutant families, a non-PBP gene confers the first change in β-lactam susceptibility (and in some other selection steps) ([24]; van der Linden M, Krauß J and Hakenbeck R, unpublished results). The nature of these non-PBP genes also depends on the selective β-lactam: in one set of C-mutants a two-component signal transducing system *cia* is involved [28], whereas resistance increase in some P-mutants is due to alterations in a gene distinct from the *cia* operon (Grebe T and Hakenbeck R, unpublished results). Selection with either cefotaxime or piperacillin results in resistant mutants that are also defective in genetic transformation, indicating that both pathways of resistance

development feed into a regulatory circuit that controls competence in the pneumococcus [23, 24, 34].

In all five lineages of cefotaxime-resistant mutants studied, PBP2x has a low affinity phenotype, and mutations in its structural gene can be responsible for the first steps of resistance. Thus, the *pbp2x* gene of higher resistant mutants encoding a low affinity PBP2x can easily be detected in a transformation assay using a sensitive recipient strain and cefotaxime resistance for selection of transformants, and this holds true for *pbp2x* of resistant clinical isolates also [18, 22].

Although each C-mutant contained a distinct set of mutations in PBP2x, two regions were primarily altered in four independently obtained mutant lineages and are thus considered to be important for β-lactam interaction: (i) In three cases, PBP2x contained (among other mutations) a Thr-550 to Ala substitution located directly adjacent to the KSG homology box; and (ii) in all four PBP2x, one or two mutations at the end of the penicillin-binding domain between amino acid positions 596 and 601 were found [23]. The first position in PBP2x to be altered during selection for resistance can be in either one of these regions. Completely different positions in PBP2x were affected in another C-mutant family that resulted in a different phenotype, i.e. an apparent total loss of penicillin-binding capacity. This shows that different mutational pathways exist for remodeling a PBP into a low affinity variant that apparently still allows for sufficient functional integrity of the enzyme.

The two regions close to the KT(S)G triad and the end of the penicillin-binding domain are also modified in PBP2b and PBP2x in P-mutants, i.e. after selection with a different class of β-lactam [24]. This indicates that both sites are involved in a more general way in the interaction of β-lactam antibiotics. Also, in clinical isolates, these regions in PBP2x and PBP2b are fairly variable and may also, at least in some cases, contribute to a decrease in affinity [18]. Mutations at homologous sites confer cefalexin resistance in *Escherichia coli*, confirming that this region is generally important [35]. Even a low affinity form of the D,D-carboxypeptidase PBP3 of a C-mutant (the only strain known thus far where PBP3 appears to be linked to resistance) contains a mutation directly adjacent to the KTG site (Krauß J and Hakenbeck R, unpublished results), extending this principle to the class of low M_r PBPs, and mutations in β-lactamases causing changes in substrate profile are also located in this region [36].

Contribution to the resistance of genes other than those encoding PBPs has been suggested since in some laboratory mutants an increase in resistance did not correlate with apparent PBP changes or mutations in *pbp2x* or *pbp2b* genes. Another phenotype that surprisingly correlates with higher levels of resistance is reduction or even complete loss of genetic competence [34]. The two properties, resistance and competence deficiency, are mediated in one C-mutant by a point mutation in a histidine kinase CiaH. *ciaH* and the preceding gene *ciaR* encode proteins that are members of the bacterial two-component signal transducing proteins that are involved in a variety of environmental signal recognitions [28]. The Cia proteins belong to the subfamily that constitutes the prototypes of these proteins including the osmoregulation system in *E. coli* EnvZ/OmpR, or the *Enterococcus faecium* VanS/VanR proteins required for induction of vancomycin resistance [37–39]. The link between the CiaH mutation and penicillin resistance is not understood. It is possible that the mutation affects the phosphorylation state of the histidine kinase CiaH and hence the activity of the response regulator CiaR. CiaR may act as a transcriptional regulator of genes important for competence development, some of which may indirectly control PBP activity, but a clear answer has to await further experiments.

Non-PBP genes distinct from *cia* are also affected in other C-mutants and P-mutants as well [34]. Some appear to be linked to competence development, or indirectly to PBP activity. In summary it appears that complex regulatory pathways are involved in resistance development that include PBP and non-PBP genes, and that different routes are taken depending on whether resistance involves PBP2b as the target protein or not.

Clinical isolates

Several features need to be considered before trying to understand resistance development in clinical isolates of *S. pneumoniae*. First, although clinical strains cover a wide range of resistance levels, there are no lineages of successively descending isolates, and thus the evolutionary history for any of the resistant strains cannot be deduced. Second, the fact that all PBP genes that contribute to resistance have a mosaic structure (as far as we know) shows that it is not the wild-type pneumococcal gene that has evolved into a resistance determinant by accumulation of point mutations, but that gene transfer events are an important prerequisite for the establishment of resistance in clinical strains. Thus, although, in principle, also PBP mediated, major aspects of resistance development in clinical isolates differ from the situation in laboratory strains.

Resistant clinical isolates contain a peptidoglycan that is biochemically distinct from that of sensitive strains, and it has been suggested that this is due to PBP alterations [40, 41]. Analysis of the cell wall of genetically defined transformants obtained with various resistance determinants from clinical isolates, but also from laboratory mutants in which the cell wall chemistry has not been studied yet, will be of major importance in order to link this phenomenon to distinct PBPs. It is conceivable that modulation of PBP genes in clinical isolates (the mosaic gene structure that consequently results in a mosaic protein) may also alter the *in vivo* substrate profile or affect possible interactions with other proteins, PBP functions that may not be related to penicillin resistance but may contribute to better survival in the human host. Also, at this point it cannot be excluded that non-PBP genes important for resistance development may contribute to the cell wall architecture, as has been demonstrated in methicillin-resistant *Staphylococcus aureus* [42–44].

Understanding the evolution of mosaic genes requires that the gene sequences of both partners,

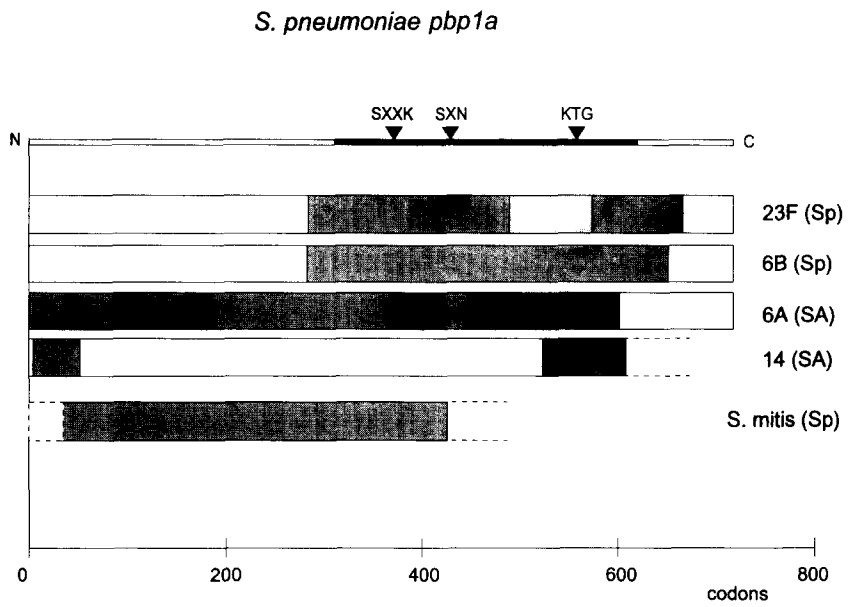

Fig. 2. Mosaic structure of *pbp1a* genes from penicillin-resistant clinical isolates of *S. pneumoniae* and an *S. mitis* strain. The sequence blocks diverging from corresponding regions in the gene from the sensitive strain (white) identified in various isolates are schematically drawn in different shadings of grey. Sequences that are > 97% identical are indicated by identical shading. The *pbp1a* gene with its three homology boxes is shown on top with the penicillin binding domain drawn in black. Shown are sequences from isolates of different serotypes, as indicated on the right, that were isolated either in Spain (Sp) or South Africa (SA) (modified from Ref. 26). The 23F clone predominant in Spain has also been recovered in South Africa [45]. The *S. mitis* gene has been determined in a low level resistant isolate from Spain [46]. The light and dark grey secondary blocks have only tentatively been assigned to some genes, since the respective donor sequences are not identified. Therefore, it is not known whether the short sequence regions shared by more than two of the genes represent regions conserved in the various homologous genes, or whether they reflect a complex mosaic structure.

the recipient and the donor strain, are known. Only then can the mosaic structure *per se* be fully comprehended, and mutations on the nucleotide and amino acid level possibly be recognized. The problem is shown in Fig. 2, where possible relationships between mosaic *pbp1a* genes are outlined.

Mosaic PBP genes can be very complex, containing more than one mosaic block, or more than one potential donor species (as deduced from comparison with other related genes). Genetic exchange of penicillin resistance determinants between *S. mitis* and *S. pneumoniae* has been documented [47, 48]. *pbp2b* sequences of *S. pneumoniae* were found in penicillin-resistant *S. oralis* and *S. sanguis* isolates [49, 50]. Two streptococcal species have been identified to contain PBP genes homologous to the entire mosaic block in the PBP genes of resistant pneumococci. One class of *pbp2b* genes [51] and one class of *pbp2x* genes (König A and Hakenbeck R, unpublished results) appear to be derived from *S. mitis*, and another class of *pbp2x* genes contains sequences highly related to an *S. oralis* strain [52]. The main point of these findings is the fact that the streptococci involved are not naturally resistant commensal species (as has been shown in the case of resistant *Neisseria* sp. [13]), but that *S. mitis* as well as *S. oralis* are perfectly sensitive, with minimum inhibitory concentration (MIC) values in the same range as *S. pneumoniae* (between 0.015 and 0.03 μg/mL for cefotaxime).

The genes of the sensitive species do not confer high resistance levels. However, in the case of the *pbp2x* gene, transfer of DNA including *pbp2x* sequences from *S. mitis* as well as from *S. oralis* into *S. pneumoniae* could be demonstrated using a cefotaxime concentration for selecting transformants close to the MIC values of either one of the parental strains [52]. No mutations could be found in the *pbp2x* genes of the transformants, demonstrating that the mosaic gene structure *per se*, or the introduction of a PBP gene into a heterologous genetic background, is sufficient for a selectable susceptibility shift. This experiment showed that genetic exchange between sensitive strains is, in principle, possible, and does not only occur if one of the partners is highly resistant, as shown previously in several cases. That this may have happened indeed outside the laboratory is suggested by the fact that the sensitive *S. mitis* strain analysed (NCTC 10712) contains a mosaic *pbp2x*-related gene compared with the *S. oralis* strain [52].

This scenario has several implications. First, it almost proves that it is a sensitive species whose gene has evolved into a resistance determinant. In agreement with this hypothesis, a very high ratio of amino acid alterations per nucleotide substitution is

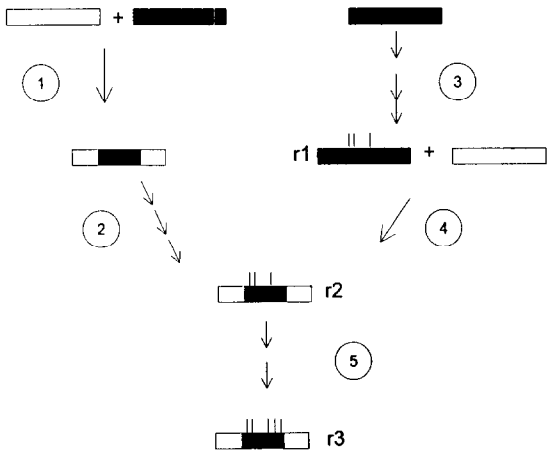

Fig. 3. Models for the evolution of a PBP gene into a mosaic resistance determinant. r1–r3 indicate PBP genes that confer resistance. Gene transfer resulting in a mosaic gene structure can precede introduction of point mutation (steps 1 and 2) as opposed to the route via steps 3 and 4. A mosaic gene conferring low levels of resistance, r2, can further evolve into a high level resistance determinant, r3, upon further modifications (step 5).

seen comparing the *pbp2x* sequence of the sensitive *S. oralis* to the highly related sequences in the mosaic genes of resistant pneumococci [52]. However, it will not be easy (if not impossible) to clarify whether genetic exchange producing a mosaic PBP gene has first occurred between sensitive strains and then evolved into a resistance determinant secondarily, or whether the evolution into a resistance determinant preceded genetic exchange (Fig. 3), especially since both mutation and genetic exchange can occur repeatedly.

Analysis of the mosaic structure of the PBP genes in the transformants that were the result of recombination between homologous but divergent genes revealed further problems in trying to understand the evolution of such genes. Even if different sequences are involved, identical recombination sites may become apparent in the mosaic product, and one transformation event may result in complex mosaic structures [52]. This aggravates (if not excludes) the possibility of determining the transfer route of these genes, in other words whether one mosaic gene is derived from another or whether the two mosaic genes are the product of two distinct transfer events with the same donor DNA.

The amino acid changes responsible for the reduction in penicillin affinity have been identified as a Thr-445 to Ala change in the *S. pneumoniae pbp2b* gene [51]. Also in the case of *Neisseria gonorrhoeae*, only one amino acid change appears to be crucial for affinity decrease [53]. In contrast, amino acids important for resistance development in PBP2x or PBP1a have not yet been assigned. The sites may not necessarily correspond to those identified in laboratory mutants, first since the principle of flexibility will hold true also for the mosaic structures, and second since mosaic blocks important for resistance development have been found that cover the active site serine, but not the KTG site or further C-terminal regions known to be important in the laboratory mutant PBP2x [52]. In many PBP2x, the amino acid sequence STMK with the active site serine is altered into SAMK, a change that may well contribute to altered penicillin binding if not even to altered substrate specificity ([22]; Reichmann P, König A and Hakenbeck R, unpublished results).

Investigating the mode of resistance development under defined conditions in the laboratory with well-characterized strains will be helpful for tracing evolutionary mechanisms that, in principle, participate in the response to β-lactam antibiotics. Comparative sequence analysis using genetically defined clones will further help to deduce the history of evolution of resistant clinical isolates.

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