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Substitutions in PBP2b from β -Lactam Resistant *Streptococcus pneumoniae* Have Different Effects on Enzymatic Activity and Drug Reactivity

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Running title: Transpeptidase activity and β -lactam reactivity of pneumococcal PBP2b

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Pneumococcus resists β -lactams by expressing variants of its target enzymes, the penicillin-binding proteins (PBPs), with many amino acid substitutions. Up to 10% of the sequence can be modified. These altered PBPs have a much reduced reactivity with the drugs but retain their physiological activity of cross-linking the peptidoglycan, the major constituent of the bacterial cell wall. However, as β -lactams are chemical and structural mimics of the natural substrate, resistance mediated by altered PBPs raises the following paradox: how PBPs that react poorly with the drugs maintain a sufficient level of activity with the physiological substrate? This question is addressed for the first time in this study, which compares the peptidoglycan cross-linking activity of PBP2b from susceptible and resistant strains with their inhibition by different β -lactams. Unexpectedly, the enzymatic activity of the variants did not correlate with their antibiotic reactivity. This finding indicates that some of the numerous amino acid substitutions were selected to restore a viable level of enzymatic activity by a compensatory molecular mechanism.

Penicillin and other β -lactams are arguably the most important drugs ever, having had a global impact on human health in seven decades of continuous use to fend off bacterial infection (1). β -Lactams hamper formation of the peptidoglycan, which is the main constituent of the bacterial cell wall. Peptidoglycan is a giant polymer encasing the cell and consists of chains of tandemly repeated disaccharides cross-linked by peptide bridges. This cross-linking results from a transpeptidation reaction catalysed by enzymes that are inhibited by β -lactams, which are mimics of the donor dipeptide of the transpeptidation reaction. The enzymes responsible for the peptidoglycan assembly are called penicillin-binding proteins or PBPs². They all have a penicillin-binding domain that generally catalyses the transpeptidation, but can also act as a carboxypeptidase or endopeptidase in some cases (2). Some PBPs have an additional transglycosylase domain that catalyse the elongation of the glycan strands. The bi-functional transpeptidase and transglycosylase PBPs constitute the class A, whereas the mono-functional transpeptidase PBPs are of class B.

Resistance to antibiotics is recognized as a major threat to human health (3). Resistance to β -lactams, which are the most widely used

antimicrobials, is particularly worrisome. A case in point is that of pneumococcus, a major human pathogen that causes otitis, pneumonia and meningitis and is estimated to cause over 1.5 million deaths per year (4). Pneumococcus and related oral streptococci resist β -lactams by expression of altered PBPs (5) encoded by mosaic genes that result from multiple events of homologous recombination with genes from close species combined with additional point mutations (6). A similar mechanism of β -lactam resistance operates in *Neisseria* species (7). Mosaic PBPs from resistant strains typically harbour tens of amino acid substitutions amounting to more than 10% of the primary sequence in some instances (5).

Of the six PBPs in *S. pneumoniae*, three are commonly altered in β -lactam resistant strains: the two mono-functional transpeptidase PBP2b and PBP2x, and the bi-functional PBP1a (5). The mechanisms responsible for the diminution of the reactivity with the drugs have been investigated biochemically and structurally to various degrees for the three PBPs. For PBPs from a β -lactam susceptible strain, the common source has been the laboratory strain R6, which has minimal inhibitory concentration (MICs) below 0.016 $\mu\text{g}/\text{mL}$ for penicillin and cefotaxime. The most thoroughly characterized PBPs from resistant pneumococcus are from the highly resistant strain 5204 isolated in France in 1999, with MICs of 6 $\mu\text{g}/\text{mL}$ for penicillin and 12 $\mu\text{g}/\text{mL}$ for cefotaxime (8).

The mono-functional transpeptidase PBP2x is the most studied of these PBPs. The role of important amino acid substitutions within the active site of PBP2x has been established by kinetics and structural studies. For example, a mutation two residues downstream of the active site serine found in highly resistant strains was shown to change the orientation of the hydroxyl group of the serine, thereby diminishing its reactivity with β -lactams (9). Two other substitutions common in resistant strains were shown to destabilize the structure of a loop that lines the active site (10). A comprehensive study of all the substitutions in the transpeptidase domain of PBP2x from the highly resistant strain 5204 determined that only six of the 41 substitutions are important for reducing the reactivity with β -lactams (10).

The other mono-functional class B PBP2b has not been as extensively studied, although the crystal structure has been solved for the variants

from the susceptible laboratory strain R6 and the clinical resistant strain 5204 (11). Like in PBP2x, a loop forming one side or “lip” of the active site is flexible in the variant from the resistant strain. Sequence comparison identified the T446A substitution within the active site as critical for resistance, and an early biochemical study demonstrated that this mutation severely reduces the reactivity with β -lactams although no reaction kinetics could be measured (12).

Likewise, the crystal structure of the transpeptidase domain of the class A PBP1a was solved for the susceptible strain R6 and the resistant strain 5204 (13,14). A mutation adjacent to the active-site serine was found to modify its orientation, and the loop forming a “lip” of the active site was destabilized as in PBP2x and PBP2b by a stretch of four substitutions (14).

β -Lactams owe their tremendous medical success to their structural likeness to the terminal dipeptide dAla-dAla of the donor stem-peptide in the transpeptidation reaction (15). The carbonyl of the β -lactam ring that is attacked by the nucleophilic active site serine is analogous to the carbonyl of the peptide bond that links the two terminal D-alanine residues of the peptidoglycan stem peptide. Due to the similarity between the drugs and physiological substrate, we are facing the following paradox: PBP alterations that affect reaction with β -lactams, as briefly presented above, would be expected to impact negatively on their transpeptidase enzymatic function. To solve this paradox, compensatory mechanisms likely mitigate this problem. Compensation could take place at the cellular level to cope with the effectively reduced enzymatic activity of the PBPs in resistant strains. Alternatively, compensation could occur at the molecular level of the PBPs themselves if the consequences of some substitutions are different on the reactivity with the β -lactams and the transpeptidase activity. The two types of compensatory mechanisms are not mutually exclusive and could operate together.

Investigating cellular mechanisms that could compensate for lower PBP activity is very difficult as the cascade of events that occur in the pneumococcus following β -lactam challenge remains largely mysterious (e. g. (16-18)).

Demonstrating the existence of compensatory mechanism to maintain the enzymatic activity of mosaic PBPs of *S.*

pneumoniae was not possible until recently as there was no assay to evaluate the transpeptidase activity *in vitro*. This hurdle was removed since the proper peptidoglycan precursor is now available. The nature of the stem penta-peptide of the membrane-linked precursor (lipid II) varies slightly in different bacterial species. In the pneumococcus, the second residue is a γ -D-iso-glutamine. The discovery of the amido transferase enzyme (MurT/GatD) that modify the second residue γ -D-glutamate into γ -D-iso-glutamine (19,20) has allowed the preparation of lipid II that can be used by pneumococcal PBPs to synthesize cross-linked peptidoglycan *in vitro* (21).

To investigate the paradox raised by PBP-based resistance and the structural similarity between β -lactams and the natural substrate of PBPs, we present here a comparison of four variants of PBP2b, (i) from the susceptible laboratory strain R6, (ii) from the clinical resistant strain 5204 (11,12), (iii) a hybrid form with the N-terminus from strain 5204 and the C-terminus of strain R6, and (iv) the T446A point mutant (Figs 1 and 2). 5204-PBP2b carries 56 substitutions, of which 43 are within the transpeptidase domain. Hybrid-PBP2b carries 28 substitutions including 15 in the transpeptidase domain and requires further presentation. The hybrid form was not designed but results from the transformation of the R6 strain with the *pbp2b* gene from strain 5204 and selection with piperacillin. *S. pneumoniae* is naturally competent and readily recombine foreign homologous DNA. An allele conferring a resistance can therefore easily be introduced and selected, provided flanking regions are provided to allow recombination. With PBP2b, however, the whole gene could not be introduced as recombination repeatedly occurred within the coding region, resulting in a gene with the 5'-region from the resistant strain and the 3'-region retained from the susceptible strain (12,18,22). Characterization of the resulting Hybrid-PBP2B might shed light on this incomplete incorporation of the 5204-*pbp2b* allele in the R6 strain. The T446A substitution, which is found in both 5204- and Hybrid-PBP2b, is the most commonly found in resistant strains and can confer some resistance alone (12).

For the first time, both the *in vitro* transpeptidase activity and the reactivity with a panel of β -lactams were evaluated for a set of

PBPs. We uncovered a new level of complexity as the impacts of substitutions on the transpeptidase activity and reactivity with β -lactams are not fully correlated, revealing that some substitutions have compensatory effects that restore enzymatic activity. We also discovered that variants of PBP2b can display β -lactamase activity that may contribute to the resistance to some β -lactams.

RESULTS

Transpeptidase activity—To meaningfully compare their transpeptidase activity, it was necessary to use equivalent amounts of the PBP2b variants. Impurities from the triton X-100 and traces of oxidized DTT prevented the accurate determination of the concentrations of the detergent-solubilized full-length proteins from their UV absorbance. Instead, protein amounts were determined in-gel after electrophoresis by quantifying the fluorescence of Bocillin-labeled PBP2b. The truncated soluble forms of PBP2b were used as standards as their concentration could be determined from their UV absorbance. Care was taken to use a concentration of Bocillin-FL and reaction time sufficient to completely label even the variants with lowest β -lactam reactivity. That this was achieved was shown by the fact that the ratio of fluorescence to subsequent Coomassie-staining was similar for all proteins. As Bocillin-binding implies a functional active-site, this observation suggests that the proportion of active enzyme was the same in all cases.

Peptidoglycan assembly was monitored using the polyacrylamide gel electrophoretic assay used previously and presented by a scheme in Fig. 3 (21). The lipid II peptidoglycan precursor migrates at the front, glycan chains of various length migrate as a smear within the gel, whereas high molecular mass cross-linked peptidoglycan, the product of transpeptidation, remains at the top of the gel. A small amount of dansylated precursor (10%) is incorporated to allow in-gel fluorescence imaging under UV trans-illumination. The amount of fluorescent precursor is kept to a minimum as it cannot function as acceptor in the transpeptidation since the dansyl group is attached to the lysine side chain that normally takes part in the reaction. PBP2b is a mono-functional transpeptidase that does not cross-link lipid II stem-peptides but requires polymerized glycan chains as substrate. Therefore, the S370A-PBP1a point mutant,

which is devoid of transpeptidase activity but retain a functional transglycosylase domain, was included to synthesize glycan chains from lipid II.

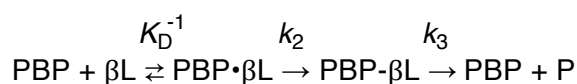
The transpeptidase activity of the PBP2b variants was assessed by comparing the amount of fluorescence present at the top of the electrophoretic gel (Fig. 4). In the end-point experiment shown in Fig. 4A, R6-PBP2b produced more cross-linked peptidoglycan after 16 h of reaction than Hybrid-PBP2b, followed by 5204- and T446A-PBP2b. The PBP2b variants were present in the same amount as checked by SDS-PAGE analysis of the protein mixtures after reaction with Bocillin and subsequent staining with Coomassie blue (Fig. 4B). Note that S370A-PBP1a did not react with Bocillin, as expected from the absence of the active-site serine.

This crude assay does not measure the actual amount of peptide cross-links resulting from transpeptidation, but only the overall amount of cross-linked peptidoglycan. Nevertheless, assuming a uniform density of cross-links and considering the amount of cross-linked peptidoglycan after 16 hours of reaction, the relative activity of the PBP2b variants can be ranked as follows: R6 (100) > Hybrid (22 ± 3) > 5204 (11 ± 2) > T446A (9 ± 2). The numbers represent the amount of cross-linked peptidoglycan relative to that formed by R6-PBP2b. The error is the standard error to the mean of 5 independent experiments. Although the difference between the amount of cross-linked peptidoglycan produced by 5204- and T446A-PBP2b is small, the latter was smaller in all five experiments. A one-tailed paired t-test of the data gave a p value of 0.035, indicating that the small difference is significant. A time course is shown in Fig. 4C. The transpeptidase activity of all PBP2b variants were inhibited with 1 mM penicillin G.

The T446A mutant is the least active, despite having a single substitution that is also present in Hybrid- and 5204-PBP2b. This observation implies that some of the substitutions found in the latter sequences are compensatory and increase the transpeptidase activity that is severely impacted by the T446A mutation. Most interestingly, Hybrid-PBP2b, which is a laboratory selected chimeric sequence, has a better enzymatic activity than its parental clinical strain.

Reaction with Bocillin FL, comparison between full length and soluble truncated PBP2bs—It is easier to produce and manipulate soluble proteins than detergent-solubilized membrane proteins. As we aimed to characterize the acylation of PBP2b by several β -lactams, it was highly desirable to use a truncated soluble form of the proteins in the absence of detergent. However, to ensure the relevance of such measurements, we first compared the reactivity of some detergent-solubilized full length and soluble truncated PBP2bs with Bocillin FL monitored by polyacrylamide gel electrophoresis (Fig. 5).

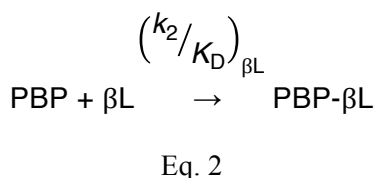
The reaction of PBPs with β -lactams can be described kinetically with the three step model (23) (Eq. 1). A non-covalent complex PBP $\cdot\beta$ L is formed between the enzyme and the inhibitor (β L), with the dissociation constant K_D , from which acylation proceeds to form the covalent complex PBP- β L with the rate k_2 . PBP- β L is finally hydrolyzed with the rate k_3 to regenerate the enzyme and an inactivated product P. The rate described by k_3 is usually negligible on the time scale of a bacterial generation. The second order rate constant k_2/K_D is the efficiency of acylation, which allows calculation of the overall acylation rate at a given concentration of antibiotic. Note that the inhibitory potency of a particular β -lactam for a PBP is given by the c_{50} , which is the antibiotic concentration resulting in the inhibition of half the PBP molecules at 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 $k_3/(k_2/K_D)$.



Eq. 1

Purified recombinant PBP2b variants were incubated with various concentration of Bocillin FL (a fluorescently labeled penicillin V) in large excess. After various time interval, aliquots were withdrawn and the reaction was stopped by the addition of a denaturing solution (0.2% SDS final) and heating at 100°C. Samples were analyzed by polyacrylamide gel electrophoresis which allowed the separation of PBP2b-bound Bocillin from the free form (Fig. 5A). We checked that the inactivation procedure was effective by adding 1 mM Bocillin FL to the denaturing solution. Minimal labeling of PBP2b

occurred in this way (sample Ctl, Fig. 5A). The in-gel fluorescence of PBP2b-bound Bocillin was quantified and normalized with respect to the amount of PBP2b in each sample, as quantified after subsequent Coomassie staining. The second order rate constant k_2/K_D was obtained from global fitting of the time course data at the different drug concentrations using a second order reaction model described in Eq. 2 (Fig. 5B). With R6-PBP2b, which is highly reactive, using low drug concentrations in the same range as that of the protein was necessary to measure sufficiently slow reactions. For this reason, we fitted the data without making the usual first-order approximation that is appropriate when the drug concentration is in large excess and can be considered constant.

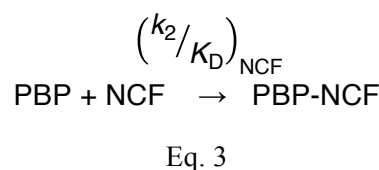


The acylation efficiency of R6-PBP2b by Bocillin FL was found in the range of $10^4 \text{ M}^{-1}\text{s}^{-1}$, which is similar to values reported with PBP3 from *Acinetobacter baumannii* and *Pseudomonas aeruginosa* (24). Reaction with 5204-PBP2b was about 200-fold slower, whereas it was reduced 1000-fold with Hybrid-PBP2b. It is surprising that Hybrid-PBP2b is even less reactive with Bocillin than 5204-PBP2b, despite the fact that it exhibits a better transpeptidase activity.

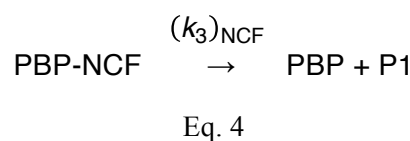
No difference of reactivity with Bocillin was found between the full-length and truncated proteins (Fig. 5C). It was assumed to be true of all β -lactams and further kinetic data were obtained with the soluble forms only.

Reaction with nitrocefin and competition with other β -lactams—In contrast to the other mono-functional PBP2x, the reaction of PBP2b with β -lactams has not been characterized in details, due to the absence of modification of the optical spectra upon acylation by the drugs (12). To palliate this lack of data, we measured the acylation efficiency by several β -lactams by monitoring the chromogenic reaction with nitrocefin. The absorbance spectrum of nitrocefin changes upon opening of the β -lactam ring, and the reactions were followed by the rise in absorbance at 490 nm. With nitrocefin alone,

the data collected at different drug concentrations could be globally fitted to a second order reaction model to extract the acylation efficiency k_2/K_D (Eq. 3).

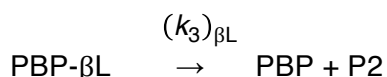


With R6-, Hybrid- and T446A-PBP2b, no further degradation of nitrocefin was detected and reactions reached the expected stoichiometric plateau, unlike what had been observed in a study of *Staphylococcus aureus* PBP2a (25). With 5204-PBP2b, a further linear increase of the absorbance was observed, which was interpreted as a hydrolysis turnover of nitrocefin. Data could be fitted if the deacylation reaction (Eq. 4) was included in the model, which allowed to measure the constant k_3 .



To obtain the acylation efficiency for other non-chromogenic β -lactams we performed competition experiments where each drug was added to PBP2b together with nitrocefin. Absorbance data, which measure the disappearance of nitrocefin, were fitted with a model that includes the two competing reactions described in Equations 2 and 3, while using the value of k_2/K_D for nitrocefin measured before. This was the case for R6- and T446A-PBP2b with amoxicillin, penicillin G, piperacillin and cefotaxime, as well as Hybrid-PBP2b with penicillin. No reaction of 5204- and Hybrid-PBP2b was observed with cefotaxime.

Following the first phase of the reaction with 5204-PBP2, a linear increase of the absorbance was observed with amoxicillin, penicillin G and piperacillin, which was greater than with nitrocefin alone, indicating an additional hydrolysis turnover of these β -lactams. Data could be fitted with a model including the four reactions described in Equations 2 to 5, while introducing the k_2/K_D and k_3 measured independently for nitrocefin.



Eq. 5

In the cases of amoxicillin and piperacillin with Hybrid-PBP2b, a turnover of these antibiotics was also detected and the absorbance data were fitted with a model that included the three reactions described by Eqs 2, 3 and 5 introducing the known k_2/K_D for nitrocefin.

Examples of the data and regression curves are given in Fig. 6. Note that in such competition assays between two reactants, of which only one can be monitored (such as nitrocefin in this case), the amplitude of the signal decreases and the apparent acylation rate increases with increasing concentrations of the invisible competitor (25). Values of acylation efficiencies k_2/K_D , and deacylation rate constant k_3 are given in Table 1. Errors in Table 1 are standard errors from the regression, which do not include errors on the concentrations of drugs and proteins. Concentration of the β -lactams was calculated from the weighted mass of powder used to prepare stock solutions. Protein concentration was determined from the UV absorbance. Assuming an error of 10% on the concentration of reactants, the accuracy of the measured acylation efficiencies is around 20%. With PBP2b, nitrocefin and Bocillin FL are the most reactive drug of our panel, followed by amoxicillin, piperacillin, and penicillin G. Cefotaxime is much less reactive. R6-PBP2b has an efficiency of acylation by penicillin G of $930 \text{ M}^{-1}\text{s}^{-1}$, which is 100-fold slower than the other mono-functional PBP2x (9). With cefotaxime, R6-PBP2b is $4 \cdot 10^7$ -fold less reactive than PBP2x (9). Qualitatively, the reactivities measured *in vitro* here and in other studies for R6 PBPs (9,10,14) are in agreement with the acylations observed *in vivo* (18,26).

The single T446A substitution accounts for most of the diminution in acylation efficiency with a 170- to 8-fold reduction with nitrocefin or piperacillin respectively. The additional 55 substitutions in 5204-PBP2b collectively only modestly affect the reactivity, whereas removal of the 27 C-terminal substitutions, as in Hybrid-PBP2b, produces a further two-fold reduction (Table 1).

Amoxicillin turnover evidenced by further reaction with Bocillin FL—Given the possible

significance for resistance of the deacylation of 5204-PBP2b with several β -lactams and the indirect way it was measured with nitrocefin, we conducted an alternative time-course experiments with amoxicillin. Full length R6- and 5204-PBP2b were first acylated by incubation with amoxicillin at a concentration sufficient to insure full acylation. Then, at the start of the deacylation time course, an large excess of fluorescent Bocillin FL was added, at a concentration at which reaction can be considered instantaneous on the time scale of the experiment. Amoxicillin was still present but its concentration much lower than that of Bocillin FL. Reacylation by amoxicillin was therefore negligible and time course of labeling by Bocillin FL reflects the deacylation rate of the PBP2b-amoxicillin complex.

A very slow deacylation of R6-PBP2b was detected. After 80 min, less than 25% of the enzyme had been regenerated and able to react with Bocillin FL (Fig. 7A). The data did not allow the determination of a reliable k_3 , but implied a value smaller than $6 \cdot 10^{-5} \text{ s}^{-1}$, or a half-life of the acyl-enzyme greater than 3 h. A comparable value of $3.8 \cdot 10^{-5} \text{ s}^{-1}$ has been reported for the deacylation of a radioactive penicillin G acyl-enzyme with a soluble form of R6-PBP2b (12). Such rates are negligible on the bacterial generation time scale.

In contrast, a significant deacylation was measured with 5204-PBP2b (Figs 7B and C). Nearly complete turnover was observed after 4 min, which yielded the deacylation rate $k_3 = (10 \pm 1) \cdot 10^{-3} \text{ s}^{-1}$ and a half-life of the inhibitory complex of 70 s. These values are clearly significant for growing bacteria. The value of k_3 measured for full length 5204-PBP2b by the time course of reacylation with Bocillin FL is in good agreement with the value of $6.2 \cdot 10^{-3} \text{ s}^{-1}$ measured by competition with nitrocefin for the soluble form of the protein (Table 1).

Thermofluor assay—In order to gain insight on the interactions between the PBP2b variants and the various β -lactams that may account for the different kinetic behavior, we investigated the effect of the acylation on the thermal stability of the soluble proteins. The thermofluor assay measure the fluorescence emitted by a probe (SYPRO® Orange) that binds to hydrophobic surfaces exposed during protein unfolding induced by rising the temperature (27,28). Fluorescence of the probe is enhanced in the hydrophobic environment.

Thermofluor data without or with penicillin or piperacillin are shown in Fig. 8. Three observations can be made from the melting temperatures (T_m) given in Table 2. First, acylation of R6-PBP2b by penams such as amoxicillin, penicillin G and piperacillin stabilizes the protein, whereas acylation by the cephalosporin cefotaxime is destabilizing. Second, in the absence of bound antibiotic, R6-PBP2b is thermally less stable than its variants from resistant strains. 5204- and Hybrid-PBP2b have the same higher T_m . Third, on the contrary, variants from the resistant strains in their acylated form are more stable than acylated R6-PBP2b. This effect is more important for 5204- than for Hybrid-PBP2b. Note that this does not apply with cefotaxime, which presumably did not acylate the resistance variants at the concentration used (1 mM).

DISCUSSION

The properties of the PBP2b variants measured *in vitro*, both the transpeptidase activity and the reactivity with β -lactams, are consistent with the impossibility to introduce the full PBP2b sequence from a clinical resistant strain (5204) into the susceptible strain R6. Indeed, the Hybrid-PBP2b resulting from the splicing of the N-terminal part of 5204-PBP2b and the C-terminal part of R6-PBP2b has better enzymatic activity and lower drug reactivity than the full 5204-PBP2b. The biochemical properties of Hybrid-PBP2b explain why it is repeatedly selected by β -lactams in laboratory transformation experiments (12,18,22). Note that if the c_{50} is considered, 5204-PBP2b is slightly less susceptible to β -lactam than Hybrid-PBP2b. The level of transpeptidase activity would therefore be the main factor favoring the selection of Hybrid-PBP2b instead of 5204-PBP2b by β -lactams.

Examination of the modeled structure of Hybrid-PBP2b shows that the missing substitutions compared to 5204-PBP2b are distributed in the “upper lip” of the active site and at the “back” of the protein (Fig. 2). The “upper lip” substitutions A619G, D625G, Q628E and T630N are the most likely to cause the severe diminution of transpeptidase activity of 5204-PBP2b due to their proximity to the active site residues at the entrance of the cleft. The region spanning these substitutions forms a loop connecting strands β 3 and β 4 that is mobile and not visible in the crystal structure of 5204-

PBP2b (11). However, the role of these substitutions in reducing the reactivity with the drugs is likely limited, contrary to what was thought previously (11), since Hybrid-PBP2b with a probable “stiff upper lip” is even less reactive with β -lactams than the more “relaxed” 5204-PBP2b. In contrast, substitutions in the “lower lip” of the active site S412P, N422Y, T426Q and Q427L likely contribute the most to the decrease in reactivity with the drugs. Interestingly and in agreement with the PBP2b observations, substitutions in the “lower” lip of PBP2x were found to reduce β -lactam reactivity, whereas substitutions in the β 3- β 4 “upper” lip had no effect or even increased the reactivity with the drug (10). Note that the β 3- β 4 “upper” lip in PBP2x protrudes in the solvent and makes no contact with a bound cefuroxime molecule (29). The “lower” lip on the contrary is held tightly against the active site and contacts the bound antibiotic. Unfortunately, no crystal structure of PBP2b with bound antibiotic and no data on the transpeptidase activity of PBP2x variants are available to further compare both proteins.

The deacylation rate (k_3), which may also contribute to the resistance, is particularly elevated with 5204-PBP2b. It is possible that the flexibility of the “upper lip” and the replacement of Ala619 by a glycine very close to the active site Ser386 allow easier access of water to the acyl-enzyme bond.

The T446A substitution is the most important to reduce the acylation by β -lactams (Table 1). Collectively, the 55 other substitutions in 5204-PBP2b cause only a modest additional reduction of the acylation rate (Table 1). Among these, the 27 substitutions at the C-terminus must collectively have an opposite effect on the reactivity with β -lactams, since their absence in Hybrid-PBP2b further decreases the reactivity (Table 1). The T446A mutation, however, is very detrimental to the transpeptidase activity, the point mutant being the less active of the four variants (Fig. 3).

Therefore, among the many substitutions found in mosaic PBPs from resistant strains, some substitutions play key roles in reducing the reactivity towards β -lactams, such as the T446A in PBP2b or the six substitutions identified among 41 in the transpeptidase domain of 5204-PBP2x (10)). Other substitutions are likely neutral and present solely by virtue of the “hitch-hiking” effect resulting from the homologous

recombination of long stretches of DNA. What is revealed in the present study, is that some substitutions are compensatory and participate to the resistance not by restricting the reaction with the drugs, but by restoring an acceptable level of physiological enzymatic activity, that would otherwise be severely impacted by mutations that hamper the reaction with β -lactams.

The findings above raise the question: why is 5204-PBP2b present in a clinical resistant strain, since a sequence with fewer substitutions appears biochemically superior? A clue can be found in a laboratory study of the transfer of β -lactam resistance from *Streptococcus mitis* to *S. pneumoniae* (22). The full sequence of PBP2b from a resistant *S. mitis* strain, including the 16 substitutions at the C-terminus following position 590, could be transformed and selected by benzylpenicillin in *S. pneumoniae*, only if the mosaic variant of the *murM* gene from the *S. mitis* was also introduced.

The *murMN* operon encodes two cytoplasmic enzymes responsible for the “branching” of the stem-peptides of the peptidoglycan (17). The precursor of the peptidoglycan is synthesized with a pentapeptide, the third residue of which is a lysine in pneumococcus. The free amine of the lysine side-chain of the donor peptide forms the peptide bond with the fourth residue (d-Ala) carboxyl group of the donor peptide to cross-link glycan chains. Alternatively, MurM and MurN can add successively two residues onto the lysine side chain to produce “branched” stem-peptides with additional Ser-Ala or Ala-Ala dipeptides. It is then the N-terminus of the dipeptide on the acceptor that form the cross-linking peptide bond with the donor stem-peptide. Strain R6 and its parental strain R36A were found to have about 45 and 36% “branched” peptides, respectively (30,31). In clinical resistant strains that express a MurM variant from a mosaic allele, the proportion of “branched” stem-peptide is increased up to 85% (31), due to a greater enzymatic activity of the MurM variant (32). In the absence of MurM, no “branched” peptides are detected, and the resistance is abolished despite the presence of altered PBPs (31).

To explain the special relationship between PBP2b and MurM in β -lactam resistance, two plausible explanations can be considered. More “branched” peptides may have a compensatory

role (i) on the enzymatic activity of PBP2b or (ii) on the cell wall metabolism.

(i) “Branched” stem-peptides may be better substrates for altered PBP2bs than the linear form, either as donor or acceptor, thus compensating the decreased transpeptidase activity. It is highly desirable to test this hypothesis *in vitro*, but suitable substrates are unfortunately not available in sufficient amounts. However, the fact that in the absence of antibiotic challenge, a strain devoid of MurM and “branched”-peptide grows normally with altered PBPs (31) argues strongly against PBP2b simply discriminating between different peptide substrates.

(ii) PBP2b participates to the elongation of the ovoid pneumococcal cell (33). Addition of peptidoglycan material to the cell wall during elongation requires concomitant opening of the existing peptidoglycan to permit insertion. A peptidoglycan lytic transglycosylase has recently been identified that likely works in concert with PBP2b during elongation of the pneumococcus (34). A decreased transpeptidase activity of PBP2b might create an imbalance with the degradative activity of its associated lytic transglycosylase that may trigger full lysis with the participation of other peptidoglycan hydrolases such as the major autolysin LytA. The greater proportion of “branched”-peptides due to mosaic MurM may protect against lysis. Indeed, elevated level of “branched”-peptides due to expression of a mosaic hyper-active MurM also protects against cell lysis normally induced by non- β -lactam antibiotics (17). Also, gradual depletion of PBP2b was found to be tolerated to a large extent due to an increased level of “branched”-peptides. In the absence of MurM, and consequently of “branched”-peptides, depletion of PBP2b was much less tolerated (35).

In conclusion, we propose that the deleterious effect of mutations that greatly diminish the reactivity of PBP2b with β -lactams, such as the T446A, is compensated by a combination of compensatory substitutions within PBP2b that maintain a minimal necessary transpeptidase activity, together with an alteration of the peptidoglycan composition that compensate for the reduced enzymatic activity.

The reactivity with Bocillin FL of the extracellular domain of PBP2b was found to be the same as that of the full length membrane proteins (Fig. 5). In contrast, the transpeptidase activity was severely impacted by the truncation

of the trans-membrane segment. Residual activity was detected only with soluble R6-PBP2b (not shown). An influence of the membrane anchor on the active site cannot be ruled out, but is unlikely in the light of the reaction with β -lactams. Rather, the glycan chains and the full length enzymes may be brought into close proximity by their common anchoring into detergent micelles. This efficient concentration effect is lost by truncation of the trans-membrane segment. In any case, the *in vitro* transpeptidase activities reported here and previously are extremely weak and far from realistic physiologic rates. In the future, it will be necessary to study mono-functional PBPs in a membrane environment and/or in the presence of their protein partners (36), in particular SEDS proteins that act as transglycosylase (37).

Additional comments can be made by comparing of the reaction rates with different β -lactams. Cefotaxime does not react significantly with PBP2b, as reported previously (12,26). This property is not general to cephalosporins, however, as nitrocefin was the most reactive drug of our panel. Examination of the structure of the different cephalosporins that fail to react with PBP2b (26,38) suggests that the nature of the R2 substituent is important. Bulky and planar substituent in position R2 appear to prevent recognition by PBP2b, whereas smaller and tetrahedral substituent seem compatible. The unfavorable interaction between cefotaxime and PBP2b seems to persist once the β -lactam ring is open and the acyl-enzyme complex is formed, as the thermal stability of the protein is diminished (Table 2).

It is remarkable that substitutions that lead to the thermal stabilization of the apo-form of 5204- and Hybrid-PBP2b compared to the apo-form of R6-PBP2b, have the opposite effect on the acylated forms (Table 2). In the variants from the resistant strains, the covalently bound drugs must make unfavorable interactions with the proteins. Although the “open” forms of the antibiotics that are bound in the active site are different from the original β -lactams, they likely retain interactions that also occur in the pre-acylation complex or the transition state, and may be relevant to the acylation and deacylation kinetics.

Beside nitrocefin, which is not in clinical use, the most important diminution of reactivity measured for 5204-PBP2b compared to R6-PBP2b was with amoxicillin. It is also with

amoxicillin that the greatest β -lactamase activity was measured for 5204-PBP2b (Table 1). Amoxicillin has long been a widely prescribed treatment for otitis media and sore throat, even when bacterial infection is not clearly diagnosed (39,40). The nasopharyngeal flora, including *S. pneumoniae* has therefore been subjected to a severe selection pressure by amoxicillin and it is possible that the substitutions found in 5204-PBP2b reflects the clinical practice. Strain 5204 exhibits the highest level of amoxicillin resistance (MIC $6 \mu\text{g mL}^{-1}$) in a panel of French clinical isolates, together with isolate 5268, which has a similar PBP2b sequence (8).

The surprising finding that a laboratory selected PBP2b (Hybrid) performs better *in vitro* than its clinical parental variant (5204) indicates that β -lactam resistance in pneumococcus is a complex process that is not fully described by biochemical reconstitution experiments. Resistance to β -lactams must be considered as a complete physiological response with compensatory mechanisms at play. Such mechanisms should be studied in the future as they that may offer novel therapeutic options.

EXPERIMENTAL PROCEDURES

Chemicals—Lyophilized nitrocefin was purchased from Oxoid (ref: SR0112C) and reconstituted at 2 mM in DMSO and stored at -20°C . Bocillin FL was from ThermoFischer Scientific (ref: B13233). Stock solutions were prepared in 50 mM HEPES, pH 7.5, 100 mM NaCl, 10 mM MgCl_2 at 50mM and stored at -20°C . Amoxicillin (ref: A8523), cefotaxime (ref: C7912), penicillin G (ref: P3032) and piperacillin (ref: P8396) were from Sigma and solubilized at 100 mM in the same buffer and stored at -20°C , except amoxicillin, which was solubilized with the addition of 1% NH_4OH and kept for a maximum of 15 days at -80°C .

Lys-containing lipid II and dansylated lipid II were prepared as described previously (41,42). Amidation of lipid II and subsequent purification were performed as previously (21).

Structure modeling—The structural model of Hybrid-PBP2b was constructed by superposing the crystal structures of R6-PBP2b (PDB# 2WAF) from residue 518 to 682 onto that of 5204-PBP2b (PDB# 2WAD) using LSQ Superpose in COOT (43) from the CCP4 program suite (44). The model structure was

then created by replacing residues 518-682 in 5204-PBP2b by those from R6-PBP2b. As examination of the model found no clash or other anomaly, a simple structure idealization was performed with REFMAC (45).

Expression plasmids—Full-length R6-PBP2b with a C-terminal Strep-tag was expressed from the modified pET-30 plasmid described before (21). The gene encoding Hybrid-PBP2b was PCR amplified from genomic DNA from laboratory strain R6 Δ *lytA pbp2b5* (18) using primers Nde2b and 2bStopNheSpeBam (21). The gene coding for 5204-PBP2b was amplified from DNA of the clinical strain 5204 (8) using primers Nde2b and 2b5204Bam (GGTCGACGGATCCATTCATTG GATGGTGTGG). A *NdeI* internal site was silently mutated by PCR using primers 2b5204mutNdeFW (GTATAAATTGGCGTAT GGATCTTTTC) and 2b5204mutNdeRV (GA AAAGATCCATACGCCAATTTATAC). The resulting PBP2b coding genes were introduced as *NdeI*/*BamHI* fragments into a modified pET-30 plasmid, which encodes a C-terminal Strep-tag.

N-terminally truncated versions of the three PBP2b variants starting at residue M39 were sub-cloned in the same modified pET-30 expression plasmid following PCR amplification from the parent plasmids encoding the full-length proteins, using primers Nde2bstar (GAGAATTCATATGCAGGTTTTGAACAA GGATTTTACG) and 2bStopNheSpeBam or 2b5204bam. All final coding sequences were checked.

Protein expression and purification—Full-length S370A-PBP1a with an N-terminal His-tag was produced and purified as described (46).

The preparation of PBP2bs were as detailed in the supporting information of previous work (21). Briefly, all pneumococcal proteins were produced in *Escherichia coli* BL21 (DE3) star. Full-length variants were purified from Triton X-100 solubilized membranes whereas soluble truncated forms were purified from the soluble fraction of cell lysates. All variants harboring a C-terminal Strep-tag were purified by Strep-Tactin affinity chromatography in 50 mM HEPES, pH 7.5, 150 mM NaCl, 10 mM MgCl₂. Proteins were eluted with 2.5 mM desthiobiotin. Full-length protein solutions also contained 0.02%

Triton X-100 and 1 mM DTT as the PBP2bs contain a cysteine residue near the N-terminus.

Peptidoglycan synthesis—Unlabelled isoglutamine-containing lipid II and dansylated lipid II in CHCl₃:methanol (1:1) were mixed in a 10:1 ratio and dried under nitrogen flow. The dried lipid II mix was then redissolved to a final concentration of 55 μ M in the reaction mix typically containing 50 mM HEPES, pH 7.5, 150 mM NaCl, 10 mM MgCl₂, 25% (v/v) DMSO, and 0.02% (w/v) Triton X-100, and the PBP2bs investigated. The concentrations of buffer, salts, and particularly of detergent contributed by the protein stocks were taken into account. The concentration of S370A-PBP1a and PBP2bs were 0.3 and 1 μ M, respectively. Penicillin G (1 mM) was included as required. Reactions were left to proceed overnight at 30 °C unless otherwise stated. For time course experiments, aliquots were withdrawn after various time intervals, and the reaction was stopped by the addition of penicillin G (1 mM) and moenomycin (Flavomycin, Hoechst, 0.5 mM). Samples were analysed by SDS-PAGE (21) and visualized with blue trans-illumination using the GelDoc ChemiDoc MP imager (BioRad). Quantification was performed on unsaturated images using the Image Lab software (BioRad).

Kinetics of PBP2b reaction with Bocillin FL—Full-length and truncated soluble variants of PBP2b were incubated in the presence of various concentrations of Bocillin FL at 20°C in 50 mM HEPES, pH 7.5, 150 mM NaCl, 10 mM MgCl₂, and 0.02% (w/v) Triton X-100 for the full length proteins. The ranges of Bocillin FL concentrations were from 5 to 20 μ M for R6-PBP2bs or 0.25 to 1 mM for Hybrid- and 5204-PBP2bs. Aliquots were withdrawn after various time intervals, and the reaction was stopped by the addition of loading buffer (0.2% (w/v) SDS final concentration) and a heat shock (100°C, 5 min). The fluorescence of Bocillin-PBP2b complexes was recorded under blue trans-illumination. Fluorescence data were corrected for the relative amount of PBP2b in each sample after subsequent quantification of Coomassie-stained protein bands. Imaging and quantification were performed using a GelDoc ChemiDoc MP imager and the Image Lab software (BioRad). Data were fitted globally with the DynaFit software (47) (Biokine) using a second order reaction model.

Amoxicillin deacylation kinetics—Full-length R6- or 5204-PBP2b were incubated at 1 μ M for 15 min at 20°C with 100 μ M amoxicillin in 50 mM HEPES, pH 7.5, 150 mM NaCl, 10 mM MgCl₂, and 0.02% (w/v) Triton X-100, prior to addition of 1 mM Bocillin FL at time zero of the deacylation time course. After various time intervals, the reaction was stopped and samples were analyzed as above. Data for the fraction (f) of Bocillin-labeled protein were fitted to a first-order kinetic ($f = 1 - \exp(-k_3t)$) using the Kaleidagraph software (Synergy).

Kinetics of PBP2b reaction with nitrocefin and other β -lactams—The different soluble PBP2b variants were incubated with either various concentrations of nitrocefin, or a fixed concentration nitrocefin and varying concentrations of other β -lactams, in 50 mM HEPES, pH 7.5, 150 mM NaCl, 10 mM MgCl₂. Absorbance at 490 nm was recorded at 25°C in an OPTIMA FLUOstar plate reader where 150 μ L of protein solution were automatically injected onto 100 μ L of antibiotic solution in a Greiner 96-wells plate. PBP2b and drug concentrations were as follows. With 18 μ M R6-PBP2b, nitrocefin was varied alone from 20 to 100 μ M, whereas it was kept at 10 μ M while amoxicillin ranged from 0 to 25 μ M, penicillin G from 0 to 800 μ M, piperacillin from 0 to 80 μ M and cefotaxime from 0 to 5 mM. With 20 μ M 5204-PBP2b, nitrocefin was varied alone

from 100 to 500 μ M, whereas it was kept at 150 μ M while amoxicillin and penicillin G ranged from 0 to 2.5 mM, and piperacillin from 0 to 800 μ M. With 30 μ M Hybrid-PBP2b, nitrocefin was varied alone from 200 to 800 μ M, whereas it was kept at 70 μ M while amoxicillin ranged from 0 to 1.2 mM, penicillin G from 0 to 2 mM and piperacillin from 0 to 150 μ M. With 20 μ M T446A-PBP2b, nitrocefin was varied alone from 70 to 200 μ M, whereas it was kept at 35 μ M while amoxicillin ranged from 0 to 625 μ M, penicillin G from 0 to 2.4 mM, piperacillin from 0 to 300 μ M, and cefotaxime from 0 to 4.5 mM.

For each PBP2b variant, the data for a range of β -lactam concentrations were fitted globally with the DynaFit software (47) (Biokine) using second order reaction models for the acylations and first order model for the deacylation, as described in the Results section.

Thermofluor assay—Soluble PBP2b variants at a final concentration of 0.5 mg/mL (about 6 μ M) were incubated with or without 1 mM of amoxicillin, penicillin G, piperacillin or cefotaxime, in 50 mM HEPES, pH 7.5, 150 mM NaCl, 10 mM MgCl₂ for 5 min at room temperature prior to addition of SYPRO® Orange (12x final) on ice. Fluorescence melting curves were recorded in the SYBR channel in a CFX Connect instrument (Biorad) with a 20 to 100°C temperature ramp (0.5°C steps of 7.2 s) and analyzed with the CFX Manager software.

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Conflict of Interest

The authors declare that they have no conflicts of interest with the contents of this article.

Author Contributions

P. C. and A. Z. designed and conducted most of the experiments, and analyzed the results. E. B. synthesized lipid II. D. I. R. Purified PBP1a. M. D. performed the thermal shift assays. C. C.-M. modeled the protein structure. A. Z. wrote the manuscript.

FOOTNOTES

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²The abbreviations used are: AMX, amoxicillin; BCN, Bocillin FL; CTX; cefotaxime; PBP, penicillin-binding protein; PEN, penicillin G, PIP, piperacillin; NCF, nitrocefin.

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FIGURE LEGENDS

FIGURE 1. Sequence alignment of PBP2b variants. The yellow band indicate the extent of the transpeptidase domain. The scissors indicate the site of truncation to produce the soluble extracellular domain. Conserved active site motifs are denoted by asterisks and corresponding residues are boxed in red, or green for the active site serine that undergoes acylation. Other boxed residues indicate positions that are substituted in 5204-PBP2b. The important T446A mutation is in orange and denoted by a triangle. The sequence from 5204-PBP2b is in grey, that of R6-PBP2b is in blue. The sequence of hybrid-PBP2b is colored according to its two sequences of origin, with the junction positioned arbitrarily midway between unambiguous positions. Residues forming the “upper lip” of the active site (619-629) are in a darker shade.

FIGURE 2. Structural model of the extracellular domain of Hybrid-PBP2b. The model was constructed by combining the x-ray structures of R6- and 5204-PBP2b (PDB entries 2WAF and 2WAD). The positions of the active site residues and substitutions are shown by spheres. The color scheme is as in Fig. 1. Therefore, the substitutions shown in blue are present in 5204-PBP2b and absent in Hybrid-PBP2b.

FIGURE 3. Scheme of the in vitro reaction of peptidoglycan synthesis performed and the gel system used for analysis. Transpeptidase inactivated S370A-PBP1a was used to elongate glycan chains by transglycosylation (TG) and PBP2b cross-linked chains by transpeptidation (TP). A small amount of fluorescent lipid II (denoted by a star) was incorporated to visualize the reagents and products after polyacrylamide gel electrophoresis.

FIGURE 4. In vitro transpeptidase activity of PBP2b variants. Reactions were carried out with 0.3 μ M S370A-PBP1a to polymerize the glycan chains, without (Ctl) or with 1 μ M of the different PBP2b variants, at pH 7.5, 30°C and the presence of 0.02% Triton X-100, with 50 μ M lipid II and 5 μ M dansylated lipid II. The assay is depicted schematically in Fig. 3. (A) SDS-PAGE analysis of peptidoglycan synthesis after overnight reaction. (B) SDS-PAGE analysis of the protein mixes used in (A) after reaction with Bocillin. The PBP2b-bound Bocillin was revealed by UV trans-illumination (bottom panel), prior to Coomassie staining (top panel). (C) Time course of peptidoglycan synthesis reactions. Reactions were stopped after various time interval by the addition of penicillin and moenomycin. The graph shows the quantification of the cross-linked peptidoglycan resulting from R6- (\bullet), Hybrid- (\blacksquare), 5204- (\blacktriangle), and T446A-PBP2b (\blacklozenge) activity, as the fraction of fluorescent material remaining at the top of the gel, after subtraction of the amount measured in the control in the absence of PBP2b (Ctl).

FIGURE 5. Reaction of full-length and soluble PBP2b variants with Bocillin FL at pH 7.5 and 25°C. (A) Time course of the reaction of 1 μ M Hybrid-PBP2b with 1 mM of Bocillin FL. The reaction was stopped after various time intervals by the addition of SDS-containing loading buffer and analyzed by SDS-PAGE. Ctl is the control of PBP2b inactivation by SDS where 1mM of Bocillin FL was added to the loading buffer. BCN indicates the fluorescence of Bocillin FL and CB the Coomassie-stained protein. (B) Time course of the reaction of 1 μ M R6-PBP2b with 5 (\blacklozenge), 10 (\blacktriangle), 15 (\blacksquare) and 20 μ M (\bullet) of Bocillin FL. The procedure was as in (A). The fluorescence of Bocillin FL weighted by the Coomassie protein signal of each sample was plotted versus time. The regression curves were calculated globally using a second order reaction model. (C) Comparison of the acylation efficiencies of full length and soluble truncated forms of PBP2b, measured as in (A) and (B). Error bars are the standard errors from the regressions.

FIGURE 6. Reaction of soluble PBP2b variants with nitrocefin, alone or in competition with amoxicillin, at pH 7.5 and 25 °C. The change in absorbance that occurs upon opening of the β -lactam ring of nitrocefin was recorded at 490 nm. A fraction of the data points is shown (between 5 and 20%). The solid lines are global fits to the data using the reaction models described in the text. Rate constants are given in Table 1. (A) 18 μ M of R6-PBP2b was incubated in the presence of 20 (\bullet), 40 (\blacksquare), 60 (\blacktriangle), 80 (\blacklozenge) and 100 (\blacktriangledown) μ M of nitrocefin. (B) 20 μ M of T446A-PBP2b was incubated in the presence of 35 (\bullet), 50 (\blacksquare), 70 (\blacktriangle), 85 (\blacklozenge) and 100 (\blacktriangledown) μ M of nitrocefin. (C) 30 μ M of Hybrid-PBP2b and 70 μ M of nitrocefin were incubated in the presence of 0 (\blacktriangledown), 150 (\blacklozenge), 300 (\blacktriangle), 600 (\blacksquare) and 1200 (\bullet) μ M of amoxicillin. (D) 20 μ M of 5204-PBP2b and 50 μ M of nitrocefin were incubated with of 0 (\blacktriangledown), 75 (\blacklozenge), 150 (\blacktriangle), 300 (\blacksquare) and 600 (\bullet) μ M of amoxicillin.

FIGURE 7. Deacylation of the PBP2b-amoxicillin complex. Full-length R6- (A) and 5204-PBP2b (B) were incubated at 1 μ M for 15 min with 100 μ M amoxicillin prior to the addition at time zero of 1 mM of Bocillin FL. After various time intervals, the reaction was stopped by the addition of denaturing loading buffer and samples were analyzed by SDS-PAGE. Fully Bocillin-labeled samples were prepared without amoxicillin (Ctl). Bocillin-labeled PBP2b was imaged and quantified under UV illumination. (C) Data for the labeling of 5204-PBP2B were fitted to a first-order kinetic to extract the amoxicillin deacylation constant $k_3 = (10 \pm 1) 10^{-3} \text{ s}^{-1}$. The error is the standard error from the regression.

FIGURE 8. Thermal denaturation of PBP2b variants in apo and acylated forms (Thermofluor). Soluble truncated R6- (A), Hybrid- (B) and 5204-PBP2b (C) were incubated without (continuous line) or with 1 mM penicillin (wide-dashed line) or piperacillin (short-dashed line) prior to thermal denaturation in the presence of the fluorescent probe SYPRO® Orange. The first derivative of the fluorescence as a function of increasing temperature is shown.

TABLE 1**Kinetic parameters of the reaction between β -lactams and PBP2 variants**

NCF, AMX, PEN, PIP and CTX results are from a continuous colorimetric assay following absorbance increase at 490 nm resulting from the reaction of nitrocefin. BCN results are from SDS-PAGE time course experiments. Errors are the standard errors from the regressions.

		R6	T446A	5204	Hybrid
	k_2/K_D ($M^{-1}s^{-1}$)	12700 \pm 350	74 \pm 13	230 \pm 2	120 \pm 6
NCF	k_3 (s^{-1})	ND	ND	(8 \pm 2) 10^{-5}	ND
	c_{50} (M)			3.5 10^{-7}	
	k_2/K_D ($M^{-1}s^{-1}$)	3000 \pm 130	222 \pm 10	70 \pm 2	40 \pm 1
AMX	k_3 (s^{-1})	ND	ND	(6.2 \pm 0.1) 10^{-3}	(1.0 \pm 0.2) 10^{-4}
	c_{50} (M)			8.9 10^{-5}	2.5 10^{-5}
	k_2/K_D ($M^{-1}s^{-1}$)	930 \pm 25	33 \pm 1	35 \pm 1	19 \pm 0.3
PEN	k_3 (s^{-1})	ND	ND	(2.0 \pm 0.05) 10^{-3}	ND
	c_{50} (M)			5.7 10^{-5}	
	k_2/K_D ($M^{-1}s^{-1}$)	2400 \pm 94	280 \pm 8	190 \pm 6	80 \pm 2
PIP	k_3 (s^{-1})	ND	ND	(4.0 \pm 0.2) 10^{-4}	(7 \pm 2) 10^{-5}
	c_{50} (M)			2.1 10^{-6}	9 10^{-7}
	k_2/K_D ($M^{-1}s^{-1}$)	(5 \pm 2) 10^{-3}	(1.0 \pm 0.1) 10^{-3}	< 10^{-3}	< 10^{-3}
CTX	k_3 (s^{-1})	ND	ND	ND	ND
BCN	k_2/K_D ($M^{-1}s^{-1}$)	13700 \pm 111	ND	68 \pm 11	12 \pm 1

TABLE 2**Melting temperatures (T_m) of PBP2b in the absence or presence of 1 mM β -lactam**

T_m ($^{\circ}$ C) were determined as the minimum of the first derivative of the melting curves. The mean of two experiments is given. Standard deviation was at most 0.5 $^{\circ}$ C.

	none	AMX	PEN	PIP	CTX
R6-PBP2b	49.5	59.0	57.5	59.5	46.0
5204-PBP2b	52.0	53.0	45.5	51.5	52.0
Hybrid-PBP2b	52.0	56.0	49.0	54.5	52.5

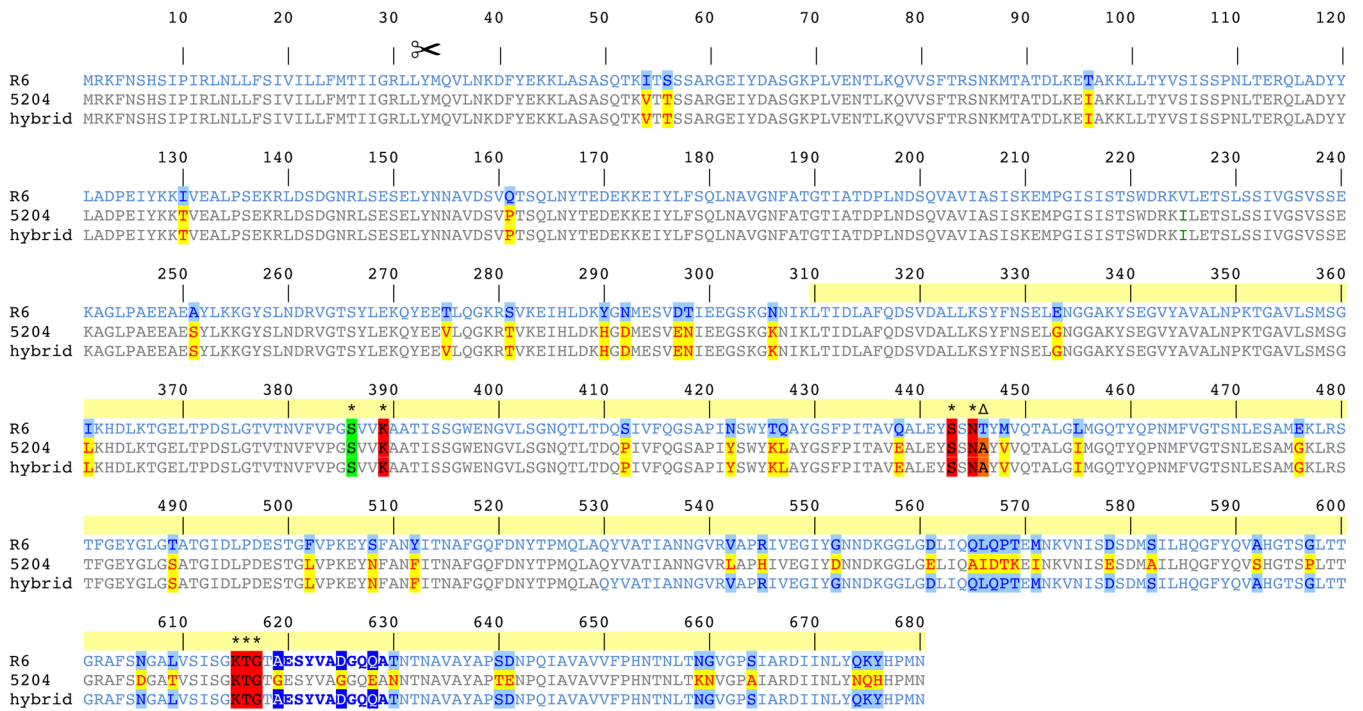


FIGURE 1

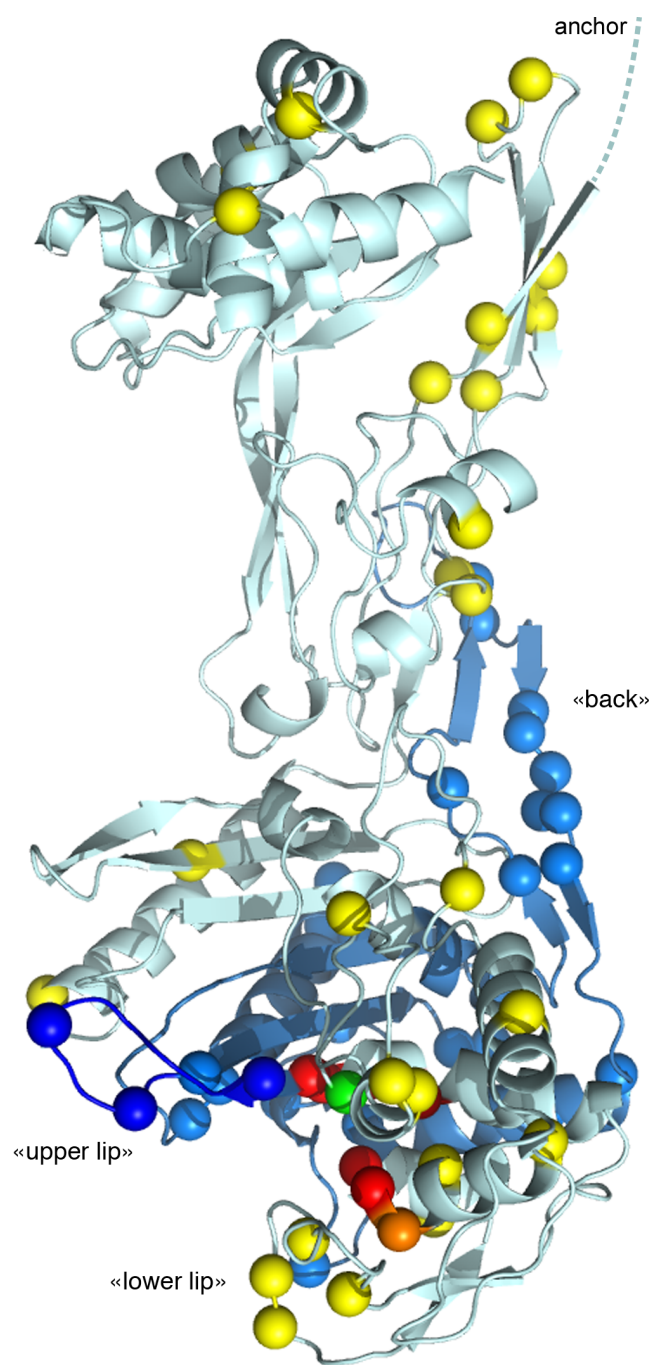


FIGURE 2

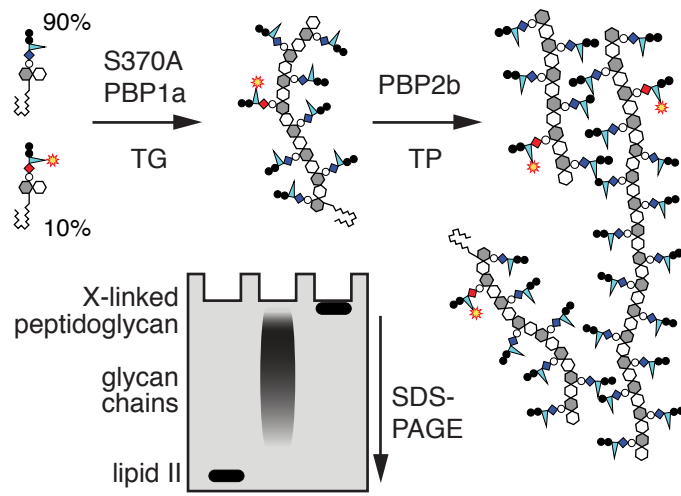


FIGURE 3

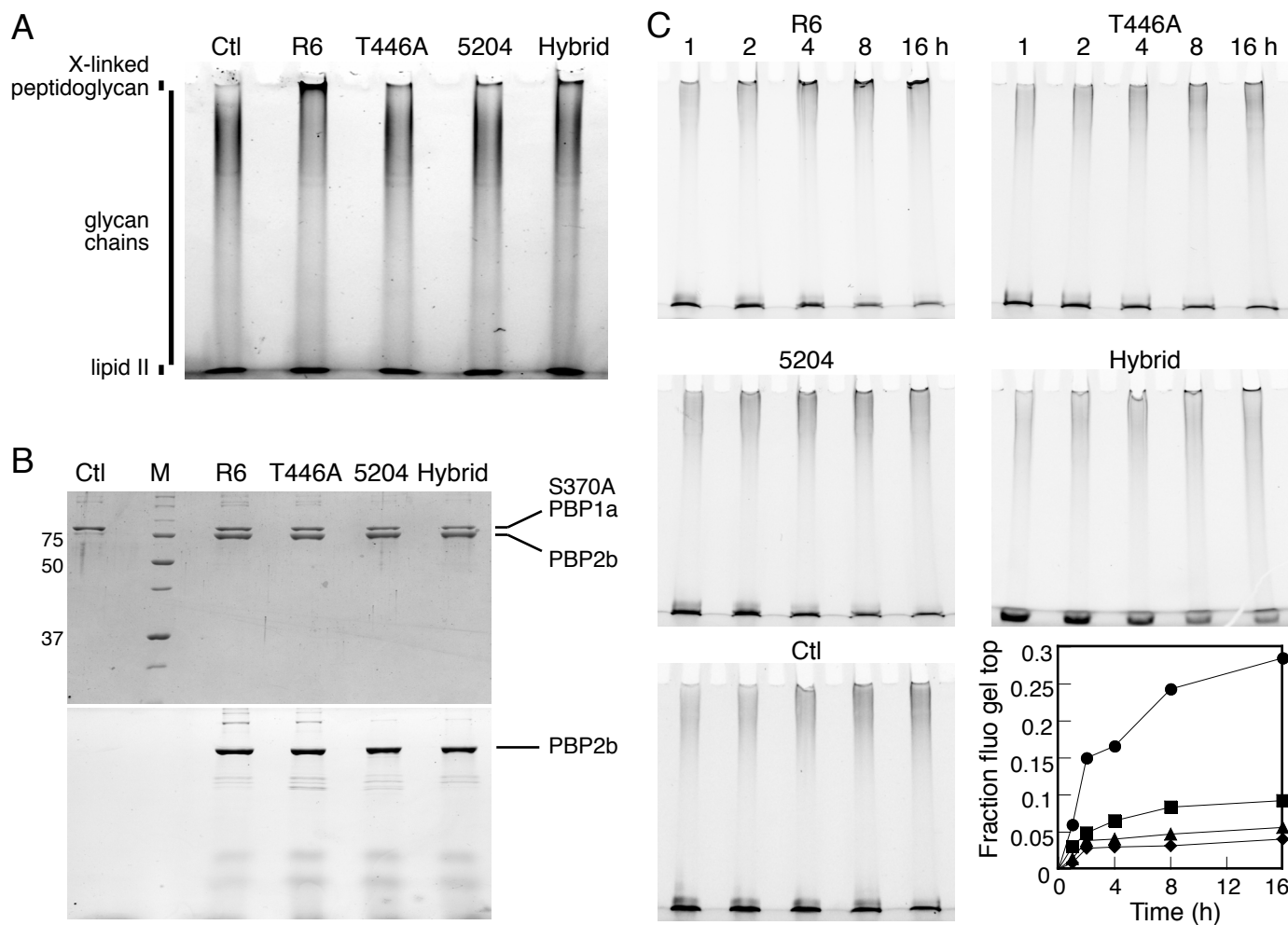


FIGURE 4

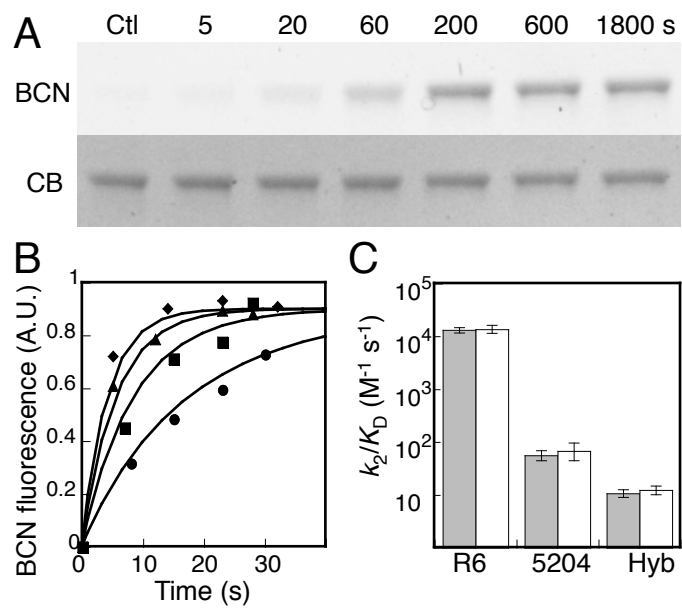


FIGURE 5

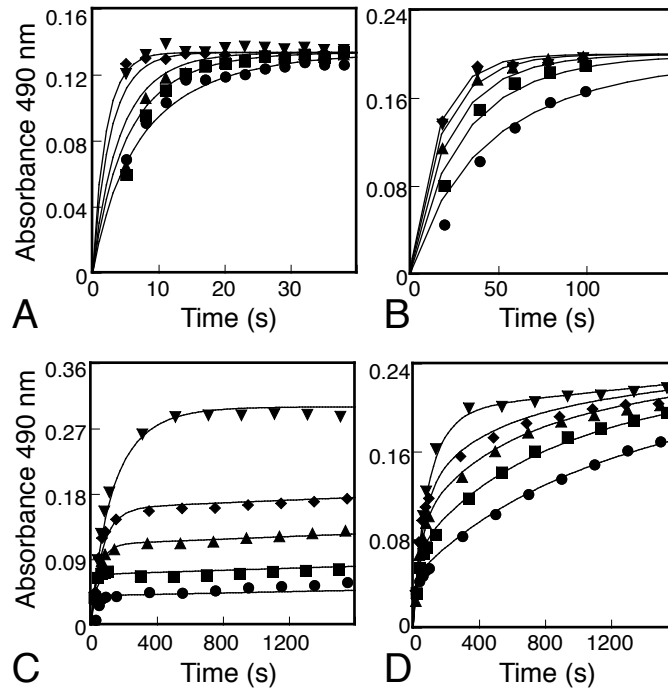


FIGURE 6

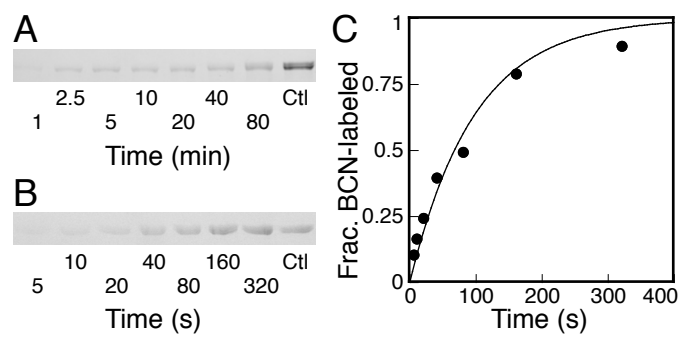


FIGURE 7

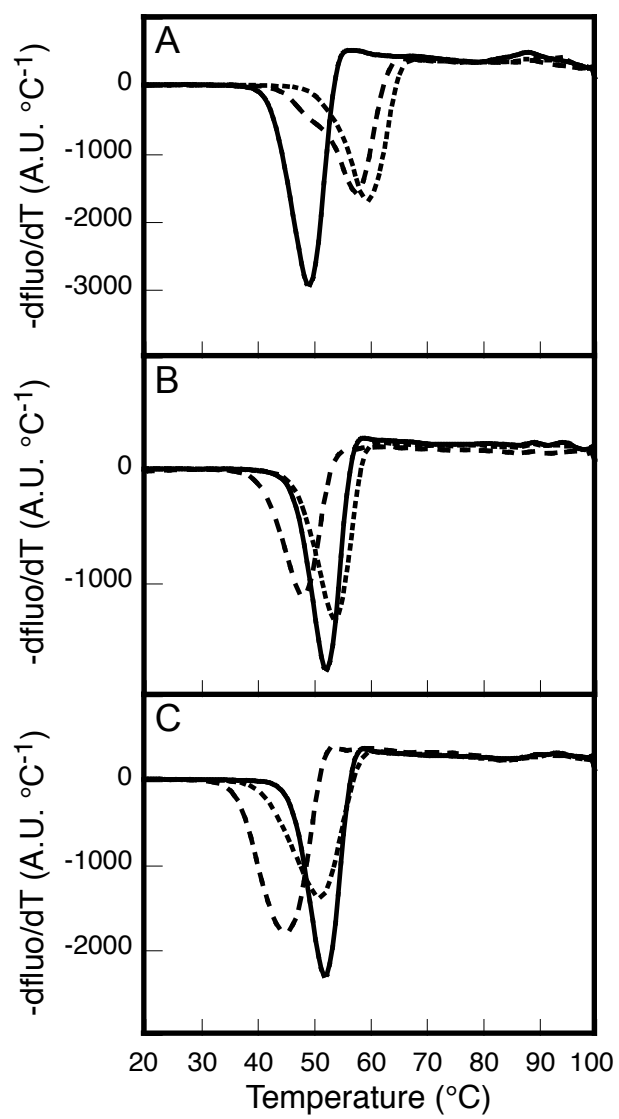


FIGURE 8

Substitutions in PBP2b from β -lactam resistant *Streptococcus pneumoniae* have different effects on enzymatic activity and drug reactivity

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