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A Highly Conserved Interaction Involving the Middle Residue of the SXN Active-Site Motif Is Crucial for Function of Class B Penicillin-Binding Proteins: Mutational and Computational Analysis of PBP 2 from *N. gonorrhoeae*

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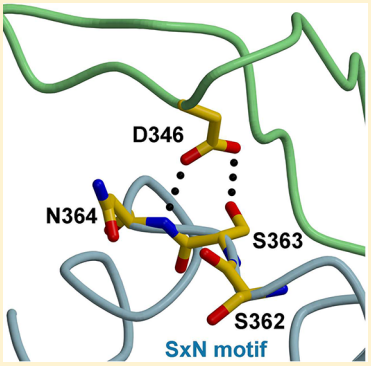
Supporting Information

ABSTRACT: Insertion of an aspartate residue at position 345a in penicillin-binding protein 2 (PBP 2), which lowers the rate of penicillin acylation by ~6-fold, is commonly observed in penicillin-resistant strains of *Neisseria gonorrhoeae*. Here, we show that insertions of other amino acids also lower the penicillin acylation rate of PBP 2, but none supported growth of *N. gonorrhoeae*, indicating loss of essential transpeptidase activity. The Asp345a mutation likely acts by altering the interaction between its adjacent residue, Asp346, in the β2a-β2d hairpin loop and Ser363, the middle residue of the SXN active site motif. Because the adjacent aspartate creates ambiguity in the position of the insertion, we also examined if insertions at position 346a could confer decreased susceptibility to penicillin. However, only aspartate insertions were identified, indicating that only an Asp-Asp couple can confer resistance and retain transpeptidase function. The importance of the Asp346-Ser363 interaction was assessed by mutation of each residue to Ala. Although both mutants lowered the acylation rate of penicillin G by 5-fold, neither could support growth of *N. gonorrhoeae*, again indicating loss of transpeptidase function. Interaction between a residue in the equivalent of the β2a-β2d hairpin loop and the middle residue of the SXN motif is observed in crystal structures of other Class B PBPs, and its importance is also supported by multisequence alignments. Overall, these results suggest that this conserved interaction can be manipulated (e.g., by insertion) to lower the acylation rate by β-lactam antibiotics and increase resistance, but only if essential transpeptidase activity is preserved.



INTRODUCTION

Neisseria gonorrhoeae is an obligate human pathogen that is the etiologic agent of the sexually transmitted infection gonorrhea. In 2007, over 350 000 infections were reported in the U.S.,[1] but the actual number may be twice that. Gonococcal infections in females are often asymptomatic and, if left untreated, can progress to pelvic inflammatory disease. There is a risk of disseminated infections in both sexes, and individuals infected with *N. gonorrhoeae* have an increased likelihood of both transmitting and acquiring HIV.[2,3] Because of the lack of a robust immune response or an effective vaccine, antibiotics are the mainstay for treating gonococcal infections.

Historically, *N. gonorrhoeae* has become resistant to nearly all antibiotics that have been used to treat infections, including penicillin, tetracycline, azithromycin, and fluoroquinolones, leaving the expanded-spectrum cephalosporins, ceftriaxone and cefixime, as the only antibiotics currently recommended by the Centers for Disease Control (CDC). Unfortunately, the emergence of strains over the past decade with decreased susceptibility to these antibiotics,[4] together with the recent isolation of gonococcal strains from Japan[5,6] and France[7] with confirmed MICs for ceftriaxone of 2 μg/mL (>10-fold higher than previous isolates), suggests that the continued effectiveness of these antibiotics is uncertain. The marked decrease in susceptibility to the expanded-spectrum cephalosporins makes it imperative that we understand the molecular mechanisms that lead to resistance and that new antibiotics be developed.

Chromosomally mediated resistance to penicillin and expanded-spectrum cephalosporins is complex and multifaceted. Resistance arises following acquisition of multiple mutant alleles of endogenous genes/loci that work together to increase resistance to the antibiotic.[8] *N. gonorrhoeae* is naturally competent, and thus resistance determinants can be transferred from a resistant isolate to a susceptible strain in a stepwise manner by DNA uptake and homologous recombination.[9,10] The first step is acquisition of an altered *penA* allele encoding penicillin-binding protein 2 (PBP 2), the lethal target for penicillin in the gonococcus. These altered forms, particularly in strains with decreased susceptibility to cephalosporins, have evolved by interspecies recombination with closely related

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pathogenic and commensal Neisseria species, including *N. meningitidis*, *N. flavescens*, *N. cinerea*, and *N. lactamica*.[11] A single altered *penA* allele can arise from multiple recombination events and accumulate >60 mutations. A major goal is to understand how these changes affect β-lactam binding and acylation of PBP 2.

All bacteria are surrounded by peptidoglycan, which confers rigidity and viability to the cell. Peptidoglycan is composed of glycan strands of a repeating disaccharide, *N*-acetylglucosamine−β-1,4-*N*-acetylmuramic acid, in which every muramic acid residue is substituted with a peptide chain.[12,13] PBPs catalyze the cross-linking of the peptide chains during the final steps of peptidoglycan synthesis. A serine nucleophile from the PBP attacks the penultimate D-Ala residue of the D-Ala-D-Ala carboxy terminus of the peptide chain, forming an acyl-enzyme bond that is subsequently attacked by an amino group from another peptide chain, forming a cross-link between two glycan strands (transpeptidation). β-Lactam antibiotics, by virtue of their similarity to acyl-D-Ala-D-Ala, react with PBPs to form stable acyl-enzyme complexes that effectively inhibit activity.[14]

PBPs are generally classified either as high-molecular-mass (HMM) PBPs, which catalyze transpeptidation of peptide chains and are essential for cell viability, or low-molecular-mass (LMM) PBPs, which catalyze carboxypeptidase or endopeptidase activity and are not essential for cell viability.[15] HMM PBPs are divided into two classes: class A PBPs catalyze both a penicillin-insensitive transglycosylase (TGase) activity and penicillin-sensitive transpeptidase (TPase) activity, whereas Class B PBPs catalyze only TPase activity. Further subdivision of each of these classes into A1 to A7 and B1 to B6 + B-like I to B-like III has been proposed based on structural and homology considerations.[16,17] All PBPs contain three conserved sequence motifs that form the catalytic center of the active site: the SxxK tetrad, which contains the serine nucleophile that reacts with peptides and β-lactams, the SXN triad, and the KTG triad. While these conserved motifs are mostly invariant, substitutions nearby or in other regions surrounding the active site collaborate to decrease the acylation rate with β-lactam antibiotics without compromising transpeptidase activity.[18−22]

PBP 2 variants from penicillin-resistant (but cephalosporin-susceptible) strains of *N. gonorrhoeae*, a Class B PBP, commonly contain both an Asp insertion after Arg345 (termed Asp345a) in the β2a-β2d hairpin loop adjacent to the active site and 4−8 mutations in the C-terminus.[23] We recently solved the crystal structure of PBP 2 from a penicillin-susceptible strain of *N. gonorrhoeae* and a PBP 2 variant from a penicillin-resistance strain containing four C-terminal amino acid substitutions.[20] These structures shed light on the mechanisms by which these C-terminal mutations decrease the rate of acylation of the PBP 2 by β-lactam antibiotics, but unfortunately no constructs harboring the Asp345a insertion crystallized, and thus the structural mechanism by which this insertion lowers the rate of acylation remains unclear.

In this study, we investigated the mechanism by which Asp345a insertions alter the acylation rate of penicillin for PBP 2. We focused on the interaction of Asp346 (the amino acid adjacent to Asp345a) with Ser363 (the X in the SXN motif), which is the most likely interaction affected by Asp345a.[20] We show that this interaction is essential for TPase function (but not β-lactam-binding) and, further, that all Class B PBPs have a conserved interaction between the middle residue of the SXN motif and a residue in the loop that precedes the motif.

MATERIALS AND METHODS

Strains and Plasmids. FA19 is a penicillin- and cephalosporin-susceptible wild-type strain that served as the recipient strain for all of the transformation experiments.[24] FA19 was grown on GC Broth (GCB) agar plates containing supplements I and II[25] in a 4% CO_2/96% air atmosphere at 37 °C. In most cases, the wild-type *penA* gene from FA19 was used as the template for mutations and 345a/346a insertions, but in some cases, i.e., those where the mutations or insertions did not decrease the k_2/K_S acylation rate constant more than 3-fold, the *penA35* gene from strain 35/02 (a cephalosporin intermediate-resistant or Ceph^I strain[26]) was used as template (see below). For transformation experiments, the *penA* genes being transformed were either cloned into pUC18us, which is pUC18 containing a 10-bp gonococcal uptake sequence,[27] or were constructed by PCR and transformed directly into FA19. In the latter case, one of the primers for PCR amplification contained the uptake sequence appended to its 5′ end. The plasmid for overexpression and purification of PBP 2 constructs (a derivative of pMAL-C2; New England Biolabs, Beverly, MA) was described in earlier publications.[20,28] *E. coli* strain MC1061 was used for routine subcloning, while GW6011 (MC4100 *lon-146::Tn10*) was used for protein purification.

Mutagenesis. Mutations and insertions were incorporated into the *penA* or *penA35* gene by overlap-extension PCR.[29] The templates for the reactions were pUC18us-*penA* or pUC18us-*penA35*.[21] For the 346a insertion library, the 3′ internal gene-specific primer was complementary to the *penA* sequence from codons 340−346, contained three random bases, and then had complementary sequence from codons 347−353, while the 5′ internal primer consisted of codons 347−353. Following amplification of the two halves of the *penA* gene, the individual amplification products were purified, mixed back together, and amplified with only the outside vector-specific primers. The assembled large fragment was digested and cloned back into pUC18us, the ligation was transformed into MC1061, and the library of plasmid clones was purified. Sequencing of the library confirmed the presence of all four bases in essentially equal amounts at the 3 positions of codon 346a.

Assessment of TPase Activity of PBP 2 Mutants by Transformation. There is no *in vitro* assay capable of quantifying TPase activity of Class B PBPs, as these PBPs do not react with model substrates or cross-link linear peptidoglycan. However, since PBP 2 is essential for the growth and viability of *N. gonorrhoeae*,[23,30] we assessed TPase function of the PBP 2 mutants by determining whether they were capable of transforming *N. gonorrhoeae*. Previous experiments showed that resistant colonies could be readily selected when FA19 was transformed with *penA* DNA encoding PBP 2 mutants with ≥3-fold decrease in the acylation rate constant and plated at penicillin concentrations just above its minimal inhibitory concentration (MIC) (see below).[20] Anything below this threshold was difficult to select without obtaining significant numbers of untransformed colonies.

For those mutations that did not decrease the k_2/K_S (see below) of penicillin G below 3-fold, we incorporated the mutations into PBP $2^{35/02}$, which confers a 100-fold increase in the MIC of cefixime when expressed in FA19.[31] While it is possible that the 345a/346a insertions might affect activity of PBP $2^{35/02}$ differently than wild-type PBP 2 (due to the presence of 58 amino acid changes), PBP $2^{35/02}$-D345a was functional and capable of transforming FA19, and thus there

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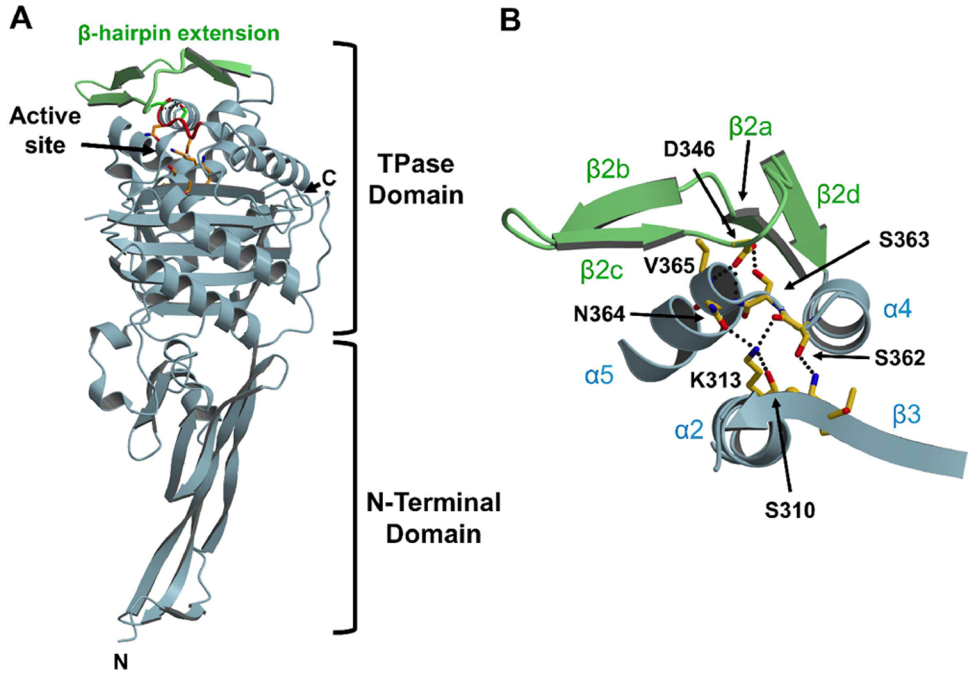

Figure 1. Interaction of Asp346 in the β-hairpin extension of NgPBP 2 with the middle Ser in the SSN active-site motif. (A) The structure of NgPBP 2, with the β-hairpin extension (also referred to as the β2a−β2d loop) shown in green. The side chains of the residues within the three active site motifs are shown as orange bonds, and the backbone of the SxN motif is colored red. The active site, N- and C-termini, and the penicillin-binding/TPase and N-terminal domains are highlighted. (B) A closer view of the active site, showing hydrogen-bonding interactions between residues in the interaction of Asp346 in the β2a−β2d loop with Ser363 of the SSN motif. Putative hydrogen bonds are shown as dashed lines, and structural elements are labeled.

appear to be an absence of negative interactions between the insertion and the other alterations in PBP 2[35/02].

Transformation of *penA* mutant constructs into FA19 was accomplished as described previously.[32] Transformants were selected on GCB plates containing 0.02 μg/mL penicillin G, which is 2-fold higher than its MIC.[32] To verify correct recombination, transformants were passaged on GCB plates, colonies were boiled in 25 μL of water, spun briefly to pellet cell debris, and the supernatants were used as templates in PCR reactions with the appropriate *penA* primers. PCR products were sequenced by the UNC sequencing facility or by Eton Bioscience Inc. (Research Triangle Park, NC). Transformations of mutants in the *penA*35 allele from strain 35/02 were plated on GCB plates containing 0.01 μg/mL cefixime (~3-fold higher than its MIC).[21,31]

Purification of PBP 2 Variants. PBP 2 variants were purified as described previously.[20] Briefly, the genes encoding each variant were cloned into a derivative of pMAL-C2 (New England Biolabs, Beverly, MA), pMAL-C2KV, which fuses His_6-maltose-binding protein and an intervening tobacco etch virus (TEV) protease site to amino acid 44 of PBP 2, and the proteins were expressed in *E. coli* (this construct removes the N-terminal transmembrane anchor and greatly increases the expression levels and solubility of the PBP). The fusion proteins were purified on a Ni^{2+}-NTA column (GE Healthcare, Piscataway, NJ), cleaved with His_6-tagged TEV protease, and the digests were rechromatographed over a Ni^{2+}-NTA column. The purified proteins were eluted in TNG buffer (20 mM Tris, 500 mM NaCl, 10% glycerol, pH 8.0) containing 15 mM imidazole, while uncleaved protein, His_6-TEV, and His_6-maltose-binding protein were eluted with TNG buffer containing 250 mM imidazole. The proteins were dialyzed in TNG buffer to remove imidazole, concentrated to ~6 mg/mL and frozen at −80 °C.

k_2/K_S Measurements of the Rate of Acylation by β-Lactam Antibiotics. The reaction of β-lactam antibiotics with PBP 2 is denoted by the equation

$$E + S \overset{K_S}{\leftrightarrow} E{\cdot}S \overset{k_2}{\rightarrow} E{-}S' \overset{k_3}{\rightarrow} E + P$$

where E·S is the noncovalent enzyme−antibiotic complex, E−S′ is the acyl-enzyme complex, and P is the hydrolyzed antibiotic. k_2/K_S constants, which are a direct measure of the ability of an antibiotic to inhibit a PBP,[33] were calculated from first-order rates of acylation of purified, soluble PBP 2 variants by [^{14}C]penicillin G (Moravek, Brea, CA), as previously described.[20,34] Graphs of PBP 2-[^{14}C]penicillin G complex formation versus time were obtained by incubating 27 μg of protein with 0.5−1.0 μM [^{14}C]penicillin G, and aliquots of ~4 μg were removed at 15 s intervals, precipitated with 5% trichloroacetic acid, filtered over Whatman GC-A filters, and the filters were submitted to scintillation counting.

Bioinformatics. A Blast search using *N. gonorrhoeae* PBP 2 as a query was carried out using the Swiss Institute for Bioinformatics Blast program[35] at www.expasy.org. The top 1000 sequences were selected, and those with >90% similarity were culled by the Blast program. Because there were far fewer sequences with SDN and SCN motifs than SSN motifs in the original search, the Blast search and culling was repeated using either PBP 2a from *S. aureus* (SDN motif) or PBPA from *M. tuberculosis* (SCN motif) as the query sequence. The three groups of sequences were then aligned separately using the multisequence alignment program, MUSCLE.[36] The region comprising the equivalent of the β2a−β2d hairpin extension loop through the SXN motif of the different alignments was

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extracted and used to create Sequence Logos[37] through the Web-based application, WebLogo[38] (weblogo.berkeley.edu).

Proteolytic Sensitivity of PBP 2 Variants. Purified PBPs (100 μg) were diluted to 56 μL with TNG buffer, and 10 μL was removed and added to 2.5 μL of 5X SDS-PAGE sample buffer (0 time point). Trypsin (1 μL of a 50 ng/μL solution) was added to the remainder of the protein at room temperature, and 10 μL of the digest was removed at 5, 10, 30, and 60 min and denatured as above. The samples were heated at 95 °C for 5 min after denaturation, and 10 μL of each sample was separated on a 10% SDS–polyacrylamide gel. The proteins were stained with Coomassie Brilliant Blue R-250, and the gel was scanned on a Typhoon 9400 Variable Mode Imager (GE Healthcare, Piscataway, NJ) using the red 633 nm laser for fluorescence excitation.

RESULTS

Random 346a Codon Insertions. As shown by the crystal structure of PBP 2,[20] the Asp345a insertion is located after Arg345 on the β2a–β2d loop that lies above the active site in close proximity to the SXN active-site motif (Figure 1A). The residue following the insertion site, Asp346, is involved in an intimate hydrogen-bonding network in which the carboxylate of Asp346 contacts Ser363 (the X in the SXN motif) and two amide nitrogens at the N-terminal end of the α5 helix (Figure 1B), and we have hypothesized that the aspartate insertion exerts its effect by altering this interaction.[20] One of the curious aspects of the Asp345a insertion in PBP 2 is that aspartate is the only amino acid observed in clinical isolates, and selection of transformants of a laboratory strain with a library of *penA* genes containing random codon insertions at position 345a revealed only aspartate codon insertions, leading to the conclusion that aspartate is the only amino acid insertion capable of decreasing susceptibility to penicillin.[39] However, because position 346 is also an aspartate, the assignment of the insertion position is ambiguous; i.e., it could be Asp345a or Asp346a.

To investigate whether amino acid insertions (other than aspartate) at position 346a are capable of decreasing susceptibility to penicillin, we constructed a library of *penA* genes containing a randomized codon insertion at position 346a and used this to transform FA19 (a penicillin-susceptible wild-type strain of *N. gonorrhoeae*), selecting for transformants with decreased susceptibility to penicillin G. The *penA* genes from 15 transformants were amplified and sequenced, but in each instance, only an aspartate codon (either GAC or GAT) at position 346a was recovered. These data indicate that an Asp-Asp couple at this position in PBP 2 is requisite for decreasing penicillin susceptibility of *N. gonorrhoeae*.

Characterization of Individual 345a and 346a Insertion Mutants of PBP 2. There are two potential explanations for the exclusive selection of an Asp-Asp couple: (1) insertions of other amino acids do not decrease the rate of acylation, and thus would not be selected by transformation of penicillin-susceptible recipient strains, or (2) insertion of amino acids other than aspartate decreases acylation, but also disrupts the essential TPase activity of PBP 2 and therefore cannot support growth. To distinguish between these two possibilities, we used site-directed mutagenesis to insert a variety of amino acids at positions 345a and 346a, purified the mutant proteins, and determined their rates of acylation with [^{14}C]penicillin G. As shown in Table 1, nearly all of the insertions decreased the acylation rate with [^{14}C]penicillin G (the lone exception being His346a, which slightly increased the acylation rate), with one insertion, Glu345a, having a greater effect on acylation than the Asp345a insertion. However, none of these individual *penA* mutants except Asp345a or Asp346a were capable of transforming FA19 to higher resistance, whereas transformants with decreased susceptibility to penicillin G were readily obtained using a *penA*-P551S/A516G double mutant, which has a k_2/K_S that is 2.9-fold lower than wild-type.[20] As described in Materials and Methods, transformants of *N. gonorrhoeae* could be readily selected using DNA encoding PBP 2 mutants with at least a 3-fold decrease in the k_2/K_S constant of penicillin compared to wild-type, provided that the mutant was a functional TPase.

Table 1. Kinetic Constants of PBP 2 Mutants[a]

PBP 2 variants	k_2/K_S (M^{-1} s^{-1})	fold change[b]	TPase activity[c]
PBP 2 WT	74700 ± 5000 (n = 8)		+
PBP 2-D345a or D346a	12200 ± 2400 (n = 16)	6.1	+
PBP 2-P551S/A516G	25900 ± 2400 (n = 8)	2.9	+
PBP 2-E345a	19900 ± 900 (n = 4)	3.8	–
PBP 2-N345a	31200 ± 1800 (n = 4)	2.4	–
PBP 2-Q345a	45400 ± 1400 (n = 4)	1.6	–
PBP 2-A345a	19100 ± 900 (n = 4)	3.9	–
PBP 2-E346a	5100 ± 400 (n = 4)	14.6	–
PBP 2-N346a	11800 ± 100 (n = 5)	6.3	–
PBP 2-Q346a	20700 ± 1400 (n = 4)	3.6	–
PBP 2-K346a	16300 ± 1200 (n = 4)	4.6	–
PBP 2-H346a	114500 ± 6300 (n = 5)	0.7	–
PBP 2-D346A	14600 ± 800 (n = 8)	5.1	–
PBP 2-S363A	16400 ± 3800 (n = 8)	4.6	–
PBP 2-S363C	51300 ± 2100 (n = 8)	1.5	+
PBP 2-S363D	10400 ± 2300 (n = 8)	7.2	–

[a]The k_2/K_S constants of [^{14}C]penicillin G for the indicated PBP 2 variants were determined using purified protein as described in Materials and Methods. Insertions are denoted by a small "a" following the amino acid, whereas mutations are denoted in capitals (e.g., PBP 2-D346a is an insertion after amino acid 346, while PBP 2-D346A is a mutation of D346 to alanine). [b]Fold change in k_2/K_S relative to PBP 2 WT. [c]TPase activity is defined as the capacity of the PBP 2 variant to support cell growth (see Materials and Methods for details).

Because the fold decreases in k_2/K_S values for PBP 2-H346a, -N345a, and -Q345a mutants were below the 3-fold threshold for selection of transformants on penicillin (Table 1), we could not demonstrate unambiguously that these insertions were functional. Therefore, we took advantage of a *penA* allele (*penA*35) from the penicillin-resistant, cephalosporin-intermediate resistant strain 35/02, which confers a 100-fold increase in cefixime resistance to FA19.[31] The *penA*35 allele contains 58 mutations compared to wild-type *penA* but does not have the Asp345a insertion;[26] therefore, if the insertion disrupts TPase activity, no transformants containing the insertion should be recovered. The D345a, N345a, Q345a, and H346a insertions were introduced into *penA*35, and the resulting clones were used to transform FA19 to increased cefixime resistance. Only the *penA*35-D345a construct resulted in the growth of resistant colonies, strongly suggesting that the insertion of histidine, asparagine, or glutamine at position 345a results in the loss of the essential TPase function. Taken together, these data are consistent with the conclusion that only an Asp insertion at position 345a or 346a is capable of both

decreasing the rate of acylation of penicillin G and retaining TPase activity.

Role of Asp346 and Ser363 in TPase Activity of PBP 2. The interaction of Asp346 and Ser363 shown in Figure 1B suggests that insertion of Asp345a may impact enzyme activity by altering or disrupting the hydrogen bonds between Asp346 and Ser363, and therefore we employed a mutagenesis approach to investigate the roles of these residues in the activity of PBP 2. Asp346 and Ser363 were mutated individually to Ala, and each mutant PBP 2 variant was examined for its rate of acylation with penicillin G and its ability to support growth of *N. gonorrhoeae* (as a measure of its TPase activity). The acylation rates of PBP 2-D346A and PBP 2-S363A were both decreased around 5-fold, about the same as PBP2-D345a or PBP 2-6140CT,[20] compared to wild-type PBP 2 (Table 1). Despite the 5-fold decrease in the k_2/K_S of penicillin G, neither *penA*-D346A nor *penA*-S363A was capable of transforming FA19 to decreased susceptibility (i.e., neither supported growth of *N. gonorrhoeae*), whereas hundreds of transformants were obtained with *penA* constructs encoding PBP 2-D345a or PBP2-6140CT. These data strongly suggest that although the D346A and S363A mutations decrease the rate of acylation with penicillin G, they also render the enzyme nonfunctional as a TPase, highlighting the importance of the interaction between these two amino acids for catalyzing TPase activity.

Structural Comparison of *N. gonorrhoeae* PBP 2 with Other Class B PBPs. Another well-studied Class B PBP whose structure is known, PBP 2x from *S. pneumoniae* (SpPBP 2x), also contains an SSN active-site motif.[40] While these two PBPs share only 20% sequence identity, inspection of the crystal structure of SpPBP 2x revealed that Ser396 (the middle serine in the SSN motif) and Asp373 in the equivalent of the β2a-β2d loop in PBP 2x form a nearly identical interaction as Asp346 and Ser363 in NgPBP 2 (Figure 2A,B). Indeed, superimposition of the two structures shows that there is almost complete overlap of the two residues (Figure 1, Supporting Information), suggesting a broad conservation of structure and function of this interaction, at least in PBPs harboring an SSN motif.

We recently reported the structure of the Class B-like PBP, PBPA from *M. tuberculosis* (MtPBPA),[41] which shows an unusual disulfide bond between a Cys residue in the X position of the SXN motif and a Cys residue in the equivalent of the β2a–β2d loop (Figure 2C). The architecture of this covalent bond is very reminiscent of the Asp-Ser interaction in NgPBP 2 and SpPBP 2x. An additional Class B PBP whose structure is known, PBP 2a from methicillin-resistant *Staphylococcus aureus* (SaPBP 2a),[42] contains an SDN motif in which the Asp carboxylate forms an electrostatic interaction with an Arg residue in the loop equivalent to the β2a–β2d loop in NgPBP 2 (Figure 2d). Thus, in four different Class B PBPs, an interaction between the middle residue of the SXN motif and a residue within the equivalent of the β2a–β2d loop is observed. These data prompted a bioinformatics analysis to determine the extent of this apparently conserved interaction in Class B PBPs from a wide range of bacterial genera and species.

Multiple Sequence Alignment of Class B PBPs. We identified the top 1000 sequences in a Blast search using NgPBP 2 as a query (see Materials and Methods for more details) and culled highly similar (>90%) sequences to decrease redundancy. Following multisequence alignment, three major groups (which we denote as groups 1–3) of Class B PBPs were evident: group 1 PBPs contained an SSN motif, group 2 PBPs contained an SCN motif, and group 3 PBPs contained an SDN motif. To identify as many group members as possible, BLAST searches for group 2 and group 3 PBPs were undertaken using MtPBPA and SaPBP 2a, respectively, as query sequences, and again redundant sequences from each of the three groups were culled by eliminating those with >90% sequence identity. The sequences in each group were then aligned using the multialignment program MUSCLE.[36] Below, we summarize the major findings of these analyses.

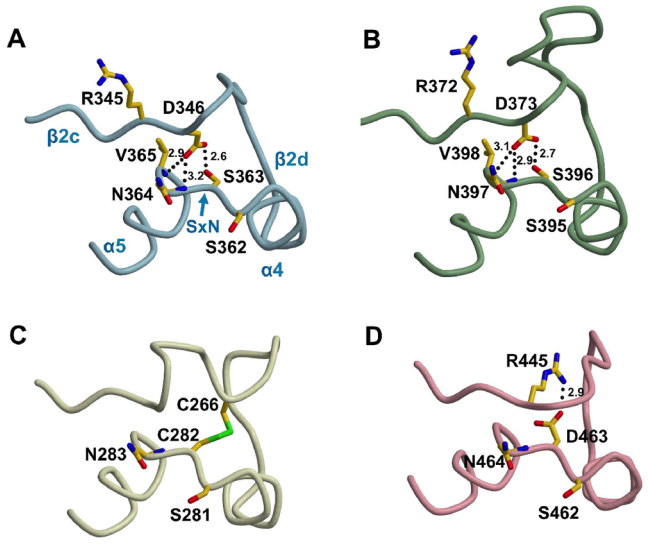

Figure 2. Conservation of the interaction between the X residue of the SXN motif with a residue in the preceding loop in several Class B PBPs. (A) PBP 2 from *N. gonorrhoeae* (PDB code 3EQU[20]), showing the interaction between Asp346 and Ser363. Structural elements and the SXN motif are as shown. (B) PBP 2x from *S. pneumoniae* (1QME[40]), showing the interaction between Asp373 and Ser396. (C) *M. tuberculosis* PBPA (3LO7[41]), showing a disulfide bond between Cys266 and Cys282. (D) PBP 2a from methicillin-resistant *S. aureus* (1VQQ[42]), showing the interaction between Arg445 and Asp463. Hydrogen bonds are depicted by the dotted lines, and the numbers next to the lines refer to the lengths of the bonds in Å. The orientation of the PBPs in (A)–(D) is the same as in Figure 1B.

SSN-Containing PBPs (Group 1). This is the largest group, with 364 PBPs included in the alignment (Supporting Information Figure 2). Strikingly, in 95% of the sequences, a conserved Asp residue is observed between 16 and 24 residues N-terminal to the SSN motif (Figure 3). The remaining 5% contain either an asparagine or serine residue at this position of the alignment, both of which are hydrogen-bonding acceptors/donors that presumably could substitute for the aspartate in linking the β2a–β2d loop to the SSN motif. We also observed that nearly 10% of the PBPs (30/364) had alternative residues, e.g., aspartate, glutamine, and cysteine, at the third position of the SSN motif.

SCN-Containing PBPs (Group 2). This group comprised 135 sequences (Supporting Information Figure 3), which could be further divided into two subgroups (2a and 2b). Subgroup 2a sequences (110 members) contained a conserved cysteine 11–16 residues N-terminal to the SCN motif (Figure 3 and Supporting Information Figure 3A), while in subgroup 2b sequences (25 members), the conserved cysteine was located farther N-terminal to the motif (24–42 residues) (Figure 3 and Supporting Information Figure 3B). None of the subgroup 2a PBPs and only 20% of the subgroup 2b PBPs harbored an

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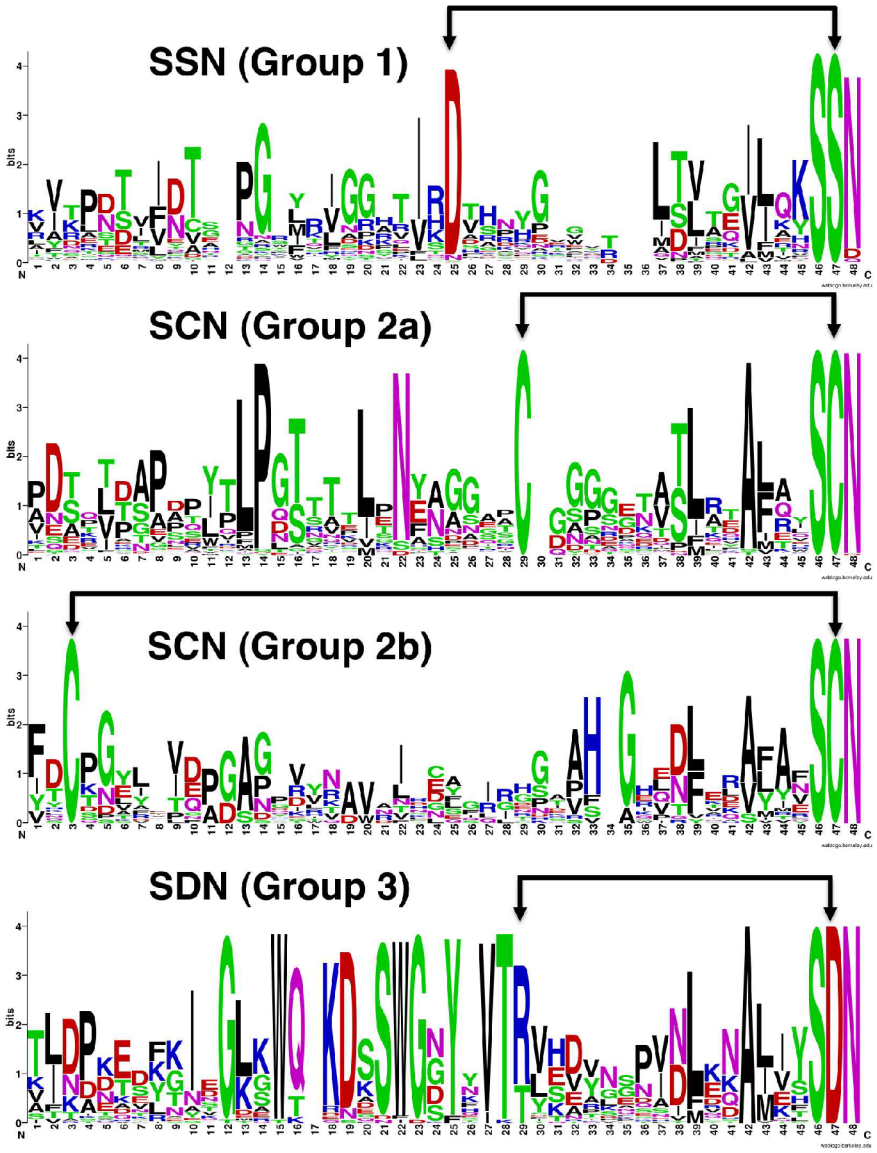

Figure 3. Conservation of an amino acid in the β2a–β2d loop that interacts with the X residue in the SXN motif in each of the three groups of PBPs. The conservation of a residue in the loop preceding the SXN motif and the X residue of that motif in each PBP Group as shown by a WebLogo plot[37,38] of multiple-sequence alignments. The connected arrows in each plot point to the two interacting residues in each group of PBPs. See text for details.

additional cysteine residue in the region between the SXXK and SCN motifs, but, if present, none were conserved.

SDN-Containing PBPs (Group 3). Group 3 PBPs were the smallest group, with only 45 representatives (Figure 3 and Supporting Information Figure 4). Thirty-four of these sequences (75%) contained a conserved arginine in the same relative position as in SaPBP 2a, while the remaining 11 (25%) contained a threonine residue at that position, which, as posited above, could substitute in the interaction of the loop with Asp463 in the SDN motif (this interaction would be the reverse of that in NgPBP 2 and SpPBP 2x). Taken together, it appears that an interaction between the X residue of the SXN motif and a residue within the equivalent of the β2a–β2d loop is highly conserved in Class B PBPs.

Analysis of NgPBP 2 with Cysteine and Aspartate Substitutions at Ser363. To determine if either a cysteine or aspartate residue could substitute for Ser363 in the SXN motif of NgPBP 2, we incorporated these mutations into the protein and determined the k_2/K_S constants for [^{14}C]penicillin G (Table 1). Whereas the S363D mutation lowered the acylation rate of NgPBP 2 by 7-fold, the S363C mutation had only a minor effect on acylation (1.5-fold decrease). Transformation experiments to assess function revealed that neither of mutant genes was capable of transforming FA19 to higher resistance, whereas in the same experiment the *penA*-D345a gene generated hundreds of transformants. Because substitution of Ser363 with Asp would likely disrupt interaction with Asp346 from the β2a-β2d loop, we also mutated Asp346 in *penA*-S363D to Arg to mimic the interaction in SaPBP 2a, but while the acylation rate remained low, no transformants with reduced susceptibility could be obtained.

While these results strongly suggested that the S363D mutation abolishes the TPase activity of NgPBP 2, the inability to select transformants containing the S363C mutation could have been due to its minimal effects on acylation. Therefore, as described above, we incorporated the S363C mutation into the

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highly resistant *penA35* allele and transformed the *penA35*-S363C mutant into FA19. Transformants were selected for increased resistance to cefixime, and sequencing of the *penA* gene from these colonies confirmed the presence of the S363C mutation. Thus, cysteine is capable of substituting for Ser363 without compromising the TPase activity of NgPBP 2, presumably by preserving the interaction between the β2a–β2d loop and the SXN motif.

Flexibility of the β2a–β2d Loop in PBP2-Asp345a Insertion Mutant. In SpPBP 2x from the cefotaxime-resistant strains 5204 and 328 (PBP 2x sequences from these strains are nearly identical), two mutations involved in decreasing the rate of acylation of cefotaxime, I371T and R384G, are located in the equivalent of the β2a–β2d loop of NgPBP 2. Interestingly, the structure of SpPBP 2x from strain 328 revealed disordering and displacement of ~30 residues encompassing the equivalent of the β2a–β2d loop up to the SSN motif of the active site[43] and increased susceptibility of this loop to proteolytic digestion of purified PBP 2x from strain 5204 provided corroborating evidence of this flexibility in solution.[44] The flexibility of this loop has been posited to underlie the effects of the substitutions on the acylation rate and decreased MICs.

To examine whether the Asp345a insertion in NgPBP 2 increases flexibility of the β2a–β2d loop in a manner similar to the aforementioned mutations in SpPBP 2x, we carried out proteolytic susceptibility experiments for PBP 2 and PBP 2-Asp345a. As shown in Figures 4A and 4B, these two proteins

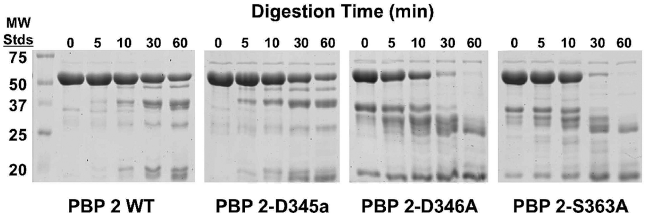

Figure 4. Trypsin sensitivity of wild-type and mutant PBP 2 variants. PBP 2 variants (83 μg) were incubated with 50 ng trypsin in 20 mM Tris, pH 8.0, 500 mM NaCl, and 10% glycerol at room temperature. At the indicated times, samples were withdrawn, added to SDS-PAGE loading buffer, and separated on a 10% gel. The gels were stained with Coomassie Brilliant Blue R-250. The molecular weights of protein standards are indicated on the side of the first gel.

had identical sensitivities to trypsin in solution. The lack of trypsin sensitivity of PBP 2-D345a is not due to an absence of cleavage sites, as the β2a–β2d loop contains three potential trypsin sites, following Arg331, Lys338, and Arg345. We also examined whether mutation of either D346 or S363 to alanine, both of which abolish TPase activity (as indicated by failure to transform *N. gonorrhoeae*), disrupts the tethering of the β2a–β2d loop to the SSN motif, thereby inducing flexibility and increasing proteolytic sensitivity of the mutant enzymes. As shown in Figures 4C and 4D, both mutations markedly increased proteolytic sensitivity of PBP 2 compared to the wild-type or Asp345a insertion mutant. These data show that loss of TPase function is likely due to an increased flexibility of the β2a–β2d loop and suggests that the Asp345a insertion acts by a specific mechanism to lower the rate of acylation and not by increasing flexibility of the β2a–β2d loop.

DISCUSSION

Asp345a insertions in PBP 2 are nearly ubiquitous in penicillin-resistant strains of *N. gonorrhoeae*, but the mechanism by which the Asp345a insertion lowers the acylation rate for penicillin while preserving TPase function is unknown. In the absence of structural information, we employed mutagenesis to address this question and identified an interaction between the middle residue of the SXN motif (Ser363 in NgPBP 2) and a residue in a loop that precedes the motif (Asp346 in NgPBP 2) that is both required for TPase activity and important (but not essential) for penicillin binding. Mutation of either of these residues mediating this interaction to Ala lowers the k_2/K_S acylation rate constant by about the same fold as an Asp345a insertion, but also renders PBP 2 inactive as a TPase (based on its inability to support growth). Bioinformatics analyses revealed that an interaction between the SXN motif and the loop above the active site is a highly conserved feature of all Class B PBPs. The implications of these findings for the transpeptidase function of PBPs, as well as resistance to β-lactams, are discussed below.

Because there is no *in vitro* assay to quantify TPase activity, we assessed the capacity of PBP 2 mutants to transform *N. gonorrhoeae* to decreased penicillin susceptibility as a measure of TPase function. As long as the mutation decreased the k_2/K_S of penicillin G ≥3-fold (a threshold at which resistant colonies could be readily obtained), transformation of FA19 to increased penicillin resistance was a straightforward and reliable method because PBP 2 is essential and loss of TPase activity would not support bacterial growth. While this method is qualitative, it is clinically relevant, as those mutations that lower the TPase activity below the level that supports growth would never be observed in clinical isolates. Hence, the transformation assay is a useful and informative method to determine the effects of mutations on TPase activity of essential PBPs, especially in naturally competent organisms such as *N. gonorrhoeae*.

The observation that only an aspartate insertion at position 345a or 346a decreases the rate of penicillin binding of PBP 2 without compromising TPase activity, whereas insertions of other amino acids, including those that are similar to aspartate (e.g., glutamate or asparagine), disrupt TPase activity, is intriguing. These data indicate that only an Asp-Asp couple can lower k_2/K_S while also preserving TPase function. We also show that both PBP 2-D346A and PBP 2-S363A (neither of which catalyze TPase activity) are more susceptible to proteolysis compared to wild-type, suggesting that once the tether between the β-hairpin loop and the SXN motif is disrupted, the loop becomes more flexible. In contrast, the D345a insertion does not increase sensitivity to proteolysis, suggesting that the loop remains tethered. Hence, the Asp insertion lowers reactivity with penicillin in a very specific way, most likely through perturbation of the interaction of Asp346 in the β2a–β2d loop with Ser363 of the SSN motif. One possibility to explain the apparent requirement of an Asp-Asp couple is that one of the two aspartates is needed to retain the required interaction of the loop with Ser363, while the other aspartate alters that interaction in a subtle way that decreases the rate of acylation of penicillin G.

One of the most surprising outcomes of this study was the essentially absolute conservation of an interaction of a residue in the loop with the middle residue of the SXN motif in Class B PBPs that was independent of the physical nature of the interaction. When bioinformatics and structural data are

considered together with our mutational and biochemical analyses of NgPBP 2, it is clear that all Class B PBPs have a crucial and highly conserved connection between the β-hairpin loop above the active site and the X residue in the SXN motif (Figure 3), suggesting that this connection is necessary for TPase activity of the PBP.

Inspection of the crystal structures of three bifunctional Class A PBPs, PBPs 1a and 1b from *S. pneumoniae* and PBP 2 from *S. aureus*, suggests that a similar connection also exists in other HMM PBPs. SpPBP 1a contains an SRN motif, and the arginine (Arg429) of the motif interacts with Asp412, which is located on the equivalent of the β2a–β2d hairpin extension loop that precedes the SRN motif (Figure 5A).[45] SpPBP 1b

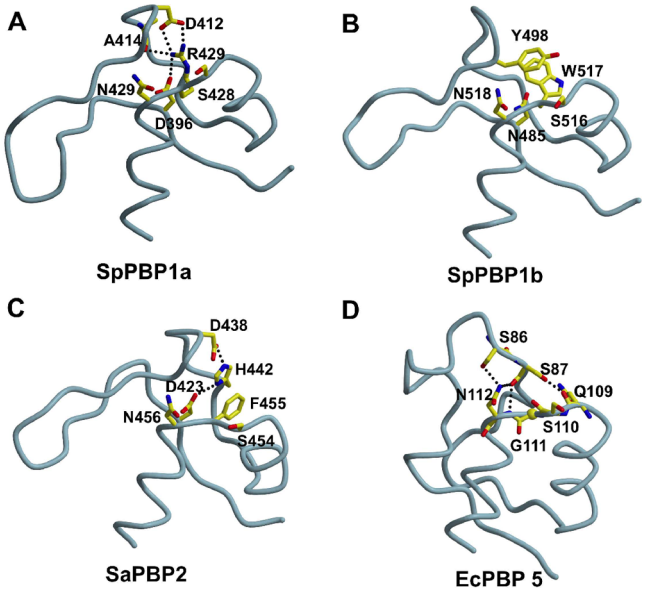

Figure 5. Interaction between amino acids in the SXN motif and within the β-hairpin loop is conserved in other non-Class B PBPs. (A) PBP 1a from *S. pneumoniae* (PDB code 2C6W[45]), a bifunctional Class A PBP. (B) PBP 1b from *S. pneumoniae* (2FFF[46,47]), a Class A PBP. (C) PBP 2 from *S. aureus* (2OLU[48]), a Class A PBP. (D) PBP 5, a LMM CPase from *E. coli* (1NZO[50]). Hydrogen bonds are shown as dashed lines. The orientation of the PBPs in (A)–(D) is the same as in Figures 1B and 2.

harbors a tryptophan (W517) in the middle position of the SXN motif, and this aromatic amino acid forms two interactions with residues in the loop: (1) a π–π interaction with the phenolic side chain of Tyr498 and (2) a carboxamide–π interaction with Asn485 (Figure 5B).[46,47] Lastly, SaPBP 2 has an SFN motif where Phe455 forms a π–π interaction with the loop residue His442, which is held in place by interactions of the imidazolium nitrogens with two neighboring aspartate residues (Figure 5C).[48] Although a comprehensive analysis has not been carried out, it appears that Class A PBPs also contain a conserved interaction between the loop that resides above the active site and the middle residue of the SXN motif.

The results from this study are highly reminiscent of those with *E. coli* PBP 5, a LMM PBP/D-Ala carboxypeptidase (CPase) with a relatively high β-lactamase activity ($t_{1/2}$ of the penicilloyl–enzyme complex ≈10 min).[49] This PBP contains a loop (referred to as the 74–90 loop) that precedes the SXN motif ($S^{110}GN^{112}$ in EcPBP 5) in a similar position in the primary sequence as the β2a–β2d hairpin extension loop in NgPBP 2. Two consecutive Ser residues on this loop (Ser86 and Ser87) interact with side chain and main chain atoms of residues within or adjacent to the SGN motif (Figure 5D).[50] Although these interactions are fundamentally different than those discussed above for Class B PBPs, there is still a strong similarity in that the 74–90 loop of PBP 5 connects to the SGN motif through hydrogen-bonding interactions.

We have studied a PBP 5 point mutant (PBP 5-G105D; also called PBP 5′), which has a 5-fold decrease in acylation rate (relative to wild-type) with penicillin G but is deficient in CPase activity.[49,51] The crystal structure of PBP 5′ revealed that while all of the active site motifs were intact, the 74–90 loop was disordered, suggesting that an ordered loop is essential for carboxypeptidase activity but not β-lactam binding.[52] Subsequent biochemical and mutational experiments, in which the 74–90 loop was deleted in the wild-type background or the two Ser residues were mutated to Ala, showed that disordering of the loop was the primary reason for the loss of CPase activity and reduced penicillin binding in the mutant enzyme.[50] Given the equivalence of the 74–90 loop to the β2a–β2d loop in NgPBP 2, these results are strikingly similar to our observations in the PBP 2-D346A and -S363A mutants (i.e., a decrease but not loss of penicillin binding and loss of functional TPase/CPase activity).

These results all point to a critical role of the β2a–β2d loop (and its equivalents) in TPase/CPase activity of PBPs. One explanation for the importance of this interaction in TPase activity is that the loop may be critical for binding and orienting the natural peptide substrate; this view is supported by the crystal structure of *E. coli* PBP 6 in complex with a peptidoglycan fragment containing a pentapeptide in which there are hydrogen bonds between the equivalent loop and the L-Lys of the peptide.[53] Alteration of the structure of the loop by insertions other than aspartate would likely prevent such interactions and therefore compromise TPase activity. By comparison, β-lactam antibiotics are much smaller and interactions with the β2a–β2d loop are less important for penicillin-binding activity; consequently, acylation by β-lactams is less affected by the Asp345a insertion.

Our study suggests that mutations or insertions into the loop preceding the SXN motif can be exploited for increasing resistance, provided that the PBPs remain functional TPases. Indeed, two other PBPs, SpPBP 2x and PBP5fm from penicillin-resistant strains of *S. pneumoniae* and *Enterococcus faecium*, respectively, have acquired mutations or an insertion in the loop that have been shown to confer decreased susceptibility to β-lactam antibiotics. PBP5fm, a group 3 PBP with an SDN motif and conserved arginine in the loop, harbors an insertion of a serine (Ser466′) that, when present, increases the MIC of penicillin 2-fold.[54,55] In SpPBP 2x from strain 5204, reversion of two mutations in the loop, I371T and R384G, back to wild-type either individually or together and subsequent recombination back into *S. pneumoniae* resulted in a 2–3-fold decrease in the MIC of cefotaxime.[44] As mentioned above, I371T and R384G in *S. pneumoniae* strains 328 and 5204 increase flexibility of the equivalent of the β2a–β2d loop, as evidenced by increased proteolytic degradation of the enzyme and disordering of the loop in the crystal structure.[43,44] Our data with NgPBP 2, however, point to an entirely different and more specific mechanism because the Asp345a insertion does not increase the proteolytic susceptibility of the protein. Indeed, the increased proteolytic sensitivity of the nonfunctional PBP 2-D346A and -S363A mutants suggests that

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disruption of the loop−SXN motif interaction induces disorder in this loop and is responsible for loss of essential TPase activity.

In conclusion, insertion of an aspartate, but no other amino acid, in the β2a−β2d loop of NgPBP 2 at position 345a or 346a lowers the rate of acylation by penicillin G, while preserving essential TPase function. This, together with the requirement of an Asp-Asp couple and the absence of flexibility in the β2a−β2d resulting from the insertion, suggests a very precise mechanism underlying this mechanism of penicillin resistance involving perturbation but not disruption of an important interaction between the β2a−β2d loop and the SxN active site motif. The importance of this interaction is highlighted by its conservation in Class B and other PBPs. Overall, these findings hint at the delicate balancing act that occurs during the evolution of resistance, in which mutations lower the susceptibility of the target for one ligand (the antibiotic) without compromising the natural function of the enzyme with its peptide substrate.

■ ASSOCIATED CONTENT

Supporting Information

Figure 1S: superimposition of NgPBP 2 and SpPBP 2x in the region around the SXN active-site motif; Figure 2S: multisequence alignment of group 1 (SSN motif) PBPs; Figure 3S: multisequence alignment of group 2 (SCN motif) PBPs; and Figure 4S: multisequence alignment of group 3 (SDN motif) PBPs. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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