Alanine-501 Mutations in Penicillin-Binding Protein 2 from *Neisseria gonorrhoeae*: Structure, Mechanism, and Effects on Cephalosporin Resistance and Biological Fitness

Joshua Tomberg[a], **Alena Fedarovich**[b], **Leah R. Vincent**[c], **Ann E. Jerse**[c], **Magnus Unemo**[d], **Christopher Davies**[b,*], and **Robert A. Nicholas**[a,e,*]

[a]Department of Pharmacology, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, USA

[b]Department of Biochemistry and Molecular Biology, Medical University of South Carolina, Charleston, South Carolina, USA

[c]Department of Microbiology, Uniformed Services University, Bethesda, Maryland, USA

[d]WHO Collaborating Centre for Gonorrhoea and other STIs, Department of Laboratory Medicine, Microbiology, Örebro University Hospital, Örebro, Sweden

[e]Department of Microbiology and Immunology, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, USA

Abstract

Resistance of *Neisseria gonorrhoeae* to expanded-spectrum cephalosporins such as ceftriaxone and cefixime has increased markedly in the past decade. The primary cephalosporin-resistance determinant is a mutated *penA* gene, which encodes the essential peptidoglycan transpeptidase, penicillin-binding protein 2 (PBP2). Decreased susceptibility and resistance can be conferred by mosaic *penA* alleles containing upwards of 60 amino acid changes relative to wild-type PBP2, or by non-mosaic alleles with relatively few mutations, the most important of which occurs at Ala501 located near the active site of PBP2. Recently, fully cefixime- and ceftriaxone-resistant clinical isolates were identified that harbored a mosaic *penA* allele with an A501P mutation. To examine the potential of mutations at Ala501 to increase resistance to expanded-spectrum cephalosporins, we randomized codon-501 in a mosaic *penA* allele and transformed *N. gonorrhoeae* to increased cefixime resistance. Interestingly, only five substitutions of Ala501 (A501V, A501T, A501P, A501R, A501S) were isolated that increased resistance and preserved essential transpeptidase

To whom correspondence should be addressed: Prof. Robert A. Nicholas, Departments of Pharmacology and Microbiology & Immunology, University of North Carolina at Chapel Hill, CB#7365, 4046 Genetic Medicine Building, 120 Mason Farm Road, Chapel Hill, NC 27599-7365, Telephone: 919-966-6547; FAX: 919-9566-5640; nicholas@med.unc.edu; and Prof. Christopher Davies, Department of Biochemistry and Molecular Biology, Medical University of South Carolina, MSC 509, 173 Ashley Avenue, Charleston, SC 29425, Telephone: 843-876-2302; FAX: 843-792-8568; davies@musc.edu.

function. To understand their structural implications, these mutations were introduced into the non-mosaic PBP2-6140CT, which contains four C-terminal mutations present in PBP2 from the penicillin-resistant strain FA6140. The crystal structure of PBP2-6140CT-A501T was solved and revealed ordering of a loop near the active site and a new hydrogen bond involving Thr501 that connects the loop and the SxxK conserved active-site motif. The structure suggests that increased rigidity in the active site region is a mechanism for cephalosporin resistance mediated by Ala501 mutations in PBP2.

Graphical abstract
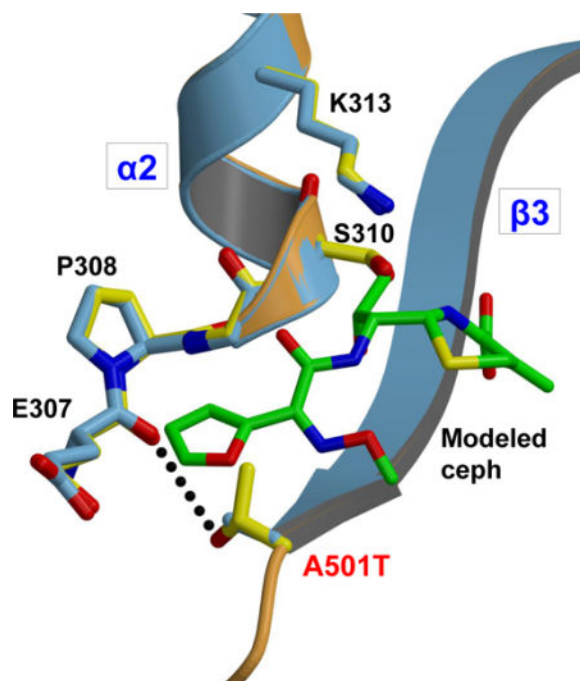
Neisseria gonorrhoeae is the causative agent of the sexually transmitted infection gonorrhea. Gonorrhea is the second most common bacterial sexually transmitted infection worldwide, with an estimated 78 million cases per year in 2012[1]. Gonorrhea is mainly an infection of the lower urogenital tract, although it can also colonize the mucous membranes of the eyes, rectum and pharynx. If left untreated, infections in females can ascend to the upper genital tract and cause pelvic inflammatory disease and the associated increased risks of ectopic pregnancies and infertility. The efficacy of ceftriaxone, hitherto the most effective antibiotic against gonorrhea, is now being threatened by a large increase in infections over the past decade that are caused by cephalosporin intermediate-resistant strains (Ceph[I]), and most recently, by a small number of strains exhibiting high-level cephalosporin resistance (Ceph[R]). This has resulted in verified treatment failures with cefixime in many countries worldwide and sporadic treatment failures with ceftriaxone in Japan, Australia, Sweden, and Slovenia[2–4]. The inexorable rise in ceftriaxone resistance portends a future with no effective antibiotics against gonococcal infections.

N. gonorrhoeae undergoes natural transformation by importing DNA from the extracellular milieu and inserting it into its genome by homologous recombination. The extracellular DNA can come from other Neisserial species (including other strains of *N. gonorrhoeae*) that share the same host niche, and is the predominant means of acquiring resistance to antibiotics. The major determinant mediating resistance to cephalosporins is a mutated *penA* gene encoding penicillin-binding protein 2 (PBP2)[5]. PBP2 is an essential peptidoglycan transpeptidase (TPase) involved in cell division, and the lethal target of β-lactam antibiotics used to treat gonorrhea. β-lactam antibiotics bind to PBPs and form a stable acyl-enzyme complex that inactivates the enzyme and prevents peptidoglycan cross-linking[6]. The role of PBP2 in resistance was first noted in penicillin-resistant *N. gonorrhoeae* strains, which harbored a *penA* allele with both an Asp345a insertion and 4–8 mutations located within the carboxyl terminal domain[7]. In the early 2000s, strains emerged in Japan and the Western Pacific Rim with much higher minimum inhibitory concentrations (MICs) of the expanded-spectrum cephalosporins, ceftriaxone and cefixime, as well as of other oral cephalosporins that were in common use in Japan at that time[2, 5, 8]. These strains contained so-called mosaic *penA* alleles with up to 60 amino acid mutations in the PBP2 coding sequence compared to a wild-type allele[5, 9]. Strains harboring mosaic *penA* alleles often contain the *mtrR* and *penB* resistance determinants, which increase expression of the MtrCDE efflux pump and decrease diffusion of antibiotics across the outer membrane, respectively[10]. The combination of these three determinants markedly increases resistance to ceftriaxone and cefixime[11, 12].

Mutations that confer decreased susceptibility to expanded-spectrum cephalosporins were also identified in non-mosaic mutant alleles of *penA*[13, 14]. These mutations were mostly confined to a single amino acid, Ala501, which is located on the β3-β4 loop of PBP2. In particular, mutations of Ala501 to Val and Thr were associated with increased MICs of expanded-spectrum cephalosporins in non-mosaic alleles, but at the time had not been observed in mosaic *penA* alleles[13–15]. In anticipation of Ala501 mutations appearing in mosaic *penA* alleles, we showed previously that introduction of an A501V mutation into the *penA35* allele of the Ceph[I] strain 35/02 increased the MICs of ceftriaxone and cefixime by ~3-fold. When introduced into FA6140, a penicillin-resistant but cephalosporin-susceptible strain that carries *mtrR* and *penB* mutations, the MIC increased to levels above the resistance breakpoint for the two antibiotics (MIC=0.25 μg ml^{-1} for both antibiotics)[16]. Subsequently, we reported on a clinical isolate from France with a mosaic *penA* allele harboring an A501P mutation that conferred high-level resistance to cefixime and ceftriaxone. This same *penA* allele was reported in two isolates a year later in Spain[17, 18].

To understand the molecular mechanism of how mutations of Ala501 increase resistance, here we identified a set of six mutations that, when incorporated into mosaic and non-mosaic *penA* alleles, increased resistance to cephalosporins while retaining essential transpeptidase activity of PBP2. Structural analysis was initiated with the Ala501 mutants of the non-mosaic PBP2 variant, PBP2-6140CT, and of these, PBP2-6140CT-A501T crystallized and was solved at 2.4 Å. The primary difference of this protein compared to PBP2-6140CT is that the mutation causes the β3-β4 loop to become ordered, whereas the same loop is disordered in both wild-type PBP2 and PBP2-6140CT. These data suggest that increased

rigidity of the β3-β4 loop is the primary mechanism by which these mutations confer resistance to expanded-spectrum cephalosporins.

Experimental Procedures

Strains and Plasmids

N. gonorrhoeae strain FA19, a penicillin- and cephalosporin-susceptible strain, was used for all transformations of mutant *penA* alleles. Gonococci were grown on GC Medium Base (GCB) agar plates containing Supplements I and II[19] at 37°C and 4% CO_2 or in liquid GCB with Supplements I and II, 10 mM $MgCl_2$, and 10 mM sodium bicarbonate in a shaking incubator. *E. coli* strain MC1061 was used for routine cloning experiments and was grown in Lysogeny Broth (LB) or on LB plates. Purified PCR amplification products for all *penA* mutants containing the 10 bp gonococcal uptake sequence[20] on one end of the fragment were used directly in transformation experiments.

Mutagenesis and Transformation

A library of *penA35* mutants in which the codon for Ala501 was randomized was constructed by overlap-extension PCR[21]. Briefly, the first fragment was amplified with a 5′-primer complementary to codons 42–48 (and with the gonococcal uptake sequence GCCGTCTGAA at its 5′ end) and a 3′-primer complementary to codons 495 to 500, three random bases, and codons 502–508. The second fragment was amplified with a 5′-primer comprising codons 502–508 and a 3′-primer that hybridizes ~200 bp downstream of the *penA* stop codon. A plasmid with *penA35* cloned into pUC18us (pUC18 containing the gonococcal uptake sequence) was used as template. The resulting fragments were isolated, combined, and a second amplification was carried out with the 5′-primer of the first reaction and the 3′-primer from the second reaction. The resulting PCR product was sequenced to confirm randomization of codon 501, and then used to transform FA19 directly. Overlap-extension mutagenesis with primers encoding A501G, A501K, A501L, and A501I mutations was used in later experiments to insert specific mutations of Ala501 into the *penA35* allele.

Transformations were carried out exactly as previously described[16]. Pilot experiments revealed that transformants of FA19 with *penA35* could be selected at concentrations of cefixime between 0.02 and 0.08 μg ml^{-1}, but decreased to nearly zero transformants at higher concentrations. Thus, FA19 bacteria transformed with the randomized PCR product were plated on GCB agar containing 0.1 μg ml^{-1} cefixime in the first set of experiments, and 0.2 μg ml^{-1} cefixime in later experiments to identify alleles conferring the highest levels of resistance. Transformants with increased resistance were isolated, and bacteria were boiled in water to provide template for PCR reactions using the two outside primers from above. DNA fragments were purified with a PCR purification kit (Bio Basic, Amherst, NY) and sequenced (Genewiz, South Plainfield, NJ).

For the crystallization experiments described below, we introduced the five Ala501 mutations identified in the randomized screen into PBP2-6140CT, which is a construct of PBP2 containing four mutations (relative to wild-type) in the C-terminal region that decrease the acylation rate of the protein with penicillin G by 5-fold[22]. The same PCR protocol was

used as described above, except that primers with the specific codons were used in the PCR reactions.

MIC determinations, growth curves, and competitive co-culture

FA19 transformants harboring *penA* alleles with mutations at Ala501 were grown overnight on GCB plates, resuspended in GCB broth with supplements I and II, 10 mM $MgCl_2$, and 20 mM sodium bicarbonate at a final OD_{600} of 0.018 (~1×10^7 colony forming units (CFU) ml^{-1}). Aliquots (5 μl) of each strain were spotted onto GCB plates containing antibiotics at <2-fold dilutions. The MIC was defined as the lowest concentration of antibiotic on which fewer than 5 colonies appeared after 24 hrs of incubation.

For assessment of growth kinetics, bacterial colonies with a non-piliated morphology were harvested from GCB agar plates after 18–20 hours incubation and inoculated into 35 ml of supplemented GC broth with 5 mM sodium bicarbonate at an initial optical density at 600 nm (OD_{600}) of 0.08. Bacteria were grown in untreated T75 tissue culture flasks for 8 hours shaking at 220 rpm and 37°C. The OD_{600} values were recorded every hour, and every two hours aliquots were removed for determination of viable bacteria (CFU ml^{-1}). To avoid potential problems with clumping and to obtain more consistent CFU counts, bacteria were diluted in GCB + 0.05% saponin prior to plating.

For competitive co-culture experiments, similar numbers of FA19 bacteria and FA19 bacteria harboring a *penA35*-A501 mutant allele were inoculated into GC broth as above and aliquots quantitatively cultured every two hours on non-selective GC agar (total number of CFU ml^{-1}) and GC agar with either 0.016 μg ml^{-1} or 0.031 μg ml^{-1} of ceftriaxone for selection (number of mutant CFU ml^{-1}). The higher ceftriaxone concentration was used to select for FA19 bacteria having an A501P or A501R mutation. The competitive index was determined at each time point, which is the ratio of mutant to wild-type bacteria divided by the ratio of mutant to wild-type bacteria in the initial inoculation. All growth experiments were repeated at least twice and gave similar results.

Acylation Rate Constants

The second order rate constants (k_2/K_S) of acylation with [^{14}C]penicillin G (Moravek, Brea, CA) for PBP-35/02 with and without four Ala501 mutations (the A501S mutant was not assessed) were determined using purified protein, as described previously[22–24]. The k_2/K_S values for ceftriaxone and cefixime were determined indirectly by determining the concentrations of the cephalosporins that inhibited the binding of [^{14}C]penicillin G by 50% and using the following equation[24]:

$$(k_2/Ks)_{\text{ceph}} = (k_2/Ks)_{\text{penG}} \left(\frac{[\text{penG}]}{[\text{ceph}]_{0.5}} \right)$$

Protein purification and crystallization

The constructs encoding PBP2-6140CT with A501 mutations comprise residues 44–581 of PBP2 fused in-frame to the C-terminal end of His$_6$-tagged maltose-binding protein (MBP)

with an intervening tobacco etch virus (TEV) protease site. The protein was expressed at 18°C, the cells lysed, and the fusion protein was purified from the soluble fraction on a Ni^{2+}-NTA column (GE Healthcare, Piscataway, NJ). The peak fractions were pooled and dialyzed at 4 °C at a 10:1 molar ratio of MBP-PBP2 to His$_6$-TEV, which resulted in nearly complete digestion of the fusion protein. The mutant PBP2 variants were then rerun over a Ni^{2+}-NTA column to remove His$_6$-MBP, uncleaved fusion protein, and His$_6$-TEV. The purified proteins were concentrated to ~20 mg ml^{-1} in 20 mM Tris•HCl, pH 8.0, 500 mM NaCl, 10% glycerol and stored at −80°C until ready for use.

Crystals of PBP2-6140CT-A501T were grown by vapor diffusion at 24°C in hanging drops, where the reservoir contained 2.2 M ammonium sulfate buffered with 100 mM Tris pH 8.25. Crystals were cryo-protected by passage through mother liquor to which glycerol had been added to a final concentration of 30% (v/v), followed by flash freezing in liquid nitrogen.

Data collection and refinement

Data were collected on the SER-CAT beam line ID22 in 1° oscillations, with a crystal-to-plate distance of 200 mm and an exposure time of 3 sec at 40% transmission. Data were collected for 180° and processed using HKL2000[25]. The space group and unit dimensions are the same as for wild-type PBP2[22] and there are two molecules in the asymmetric unit. The beginning model for refinement was the crystal structure of PBP2-6140CT (PDB code 3EQV). The model was improved by iterative rounds of manual building using the program O[26] and REFMAC refinement[27]. |Fo|-|Fc| difference maps were used to build the β3-β4 loops that are ordered in this structure. The final model was refined to 2.4 Å and contains 39 water molecules, 3 glycerols and 12 sulfates (Table 1). In both molecules of the asymmetric unit, positive electron density next to the side chain hydroxyl indicates that Thr465 is phosphorylated. This was also observed in both wild-type and 6140CT structures of PBP2 and is presumed to be an artifact resulting from protein expression in the cytoplasm of *E. coli*[22].

Circular dichroism

PBP2, PBP2-6140CT and four PBP2-6140CT mutants (A501R, A501P, A501V, A501T) were each diluted to a concentration of 0.5–1 μM in a buffer containing 10 mM Tris-HCl, pH 8.0, 250 mM NaCl and 10% glycerol. Melting curves for each protein were measured in an AVIV Biomedical, Inc. (Lakewood, NJ) Model 400 circular dichroism (CD) spectrophotometer at 206 nm, which corresponds to the minimum in a 260 to 195 nm wavelength scan. Data were recorded every degree across the temperature range of 25 to 75°C with 30 seconds of temperature equilibration time at each step. For each protein, at least 3 experiments were performed using a new sample each time and then averaged. All spectra were corrected against a control spectrum collected from buffer only. The molar ellipticity (ME) was calculated using AVIV software. The melting temperature (T_m) for each construct was calculated as the midpoint of the slope between the ME values where the protein was folded and unfolded.

Results

Random selection of Ala501 mutants

To investigate the mechanism(s) by which mutations at Ala501 of PBP2 confer resistance to expanded-spectrum cephalosporins and to define the types of amino acids that confer increased resistance, we randomized codon-501 in the mosaic *penA*35 allele, transformed the library of randomized PCR products into the wild-type strain FA19, and selected for transformants with increased resistance to cefixime. We initially used 0.1 μg ml^{-1} cefixime for selection, and obtained hundreds of transformants with increased resistance; of these, we sequenced the *penA* genes from 65 strains (Table 2). Three mutations (A501R, A501V, and A501T) comprised 90% of the sequenced clones and were obtained with similar frequency, whereas two other mutations, A501S and A501P, were isolated at much lower frequencies. The identification of three of these mutations (A501V, A501T, and A501P) was expected, as these mutations have been observed in either non-mosaic *penA* alleles from strains with decreased susceptibility to cephalosporins (A501V and A501T)[13, 28–31], or in a mosaic *penA* allele from ceftriaxone-resistant strains (A501P)[17, 18]. The isolation of just one A501P mutation in our screen was surprising given its prior emergence in the *penA* alleles of the ceftriaxone-resistant F89[17] and the Spanish gonococcal isolates[18]. In contrast, the appearance of both A501R and A501S was novel, as these mutations have not yet been reported in a *penA* gene from any gonococcal clinical isolate.

To increase the probability of isolating transformants with Ala501 mutations that confer higher levels of resistance, we increased the stringency of selection by plating transformants at 0.2 μg ml^{-1} cefixime, which preliminary MIC data suggested would eliminate A501S, A501T, and A501V mutations, but select for A501R and A501P and any other mutations conferring higher levels of resistance. Under these selection conditions, only A501R mutations were obtained in 24 isolates sequenced (Table 2).

MICs of strains harboring penA35-A501 mutant alleles

The MICs of cefixime, ceftriaxone and penicillin G for FA19 strains containing the *penA35* allele with each of the five Ala501 mutations were determined (Fig. 1). For the cephalosporin MICs, these strains fell into two groups. The fold-increases in the MICs for FA19 *penA35*-A501V, -A501T, and -A501S relative to that of FA19 *penA35* were very similar, with increases of 2- to 3-fold for cefixime and ~2-fold for ceftriaxone. In contrast, the MICs for FA19 *penA35*-A501R and -A501P were higher, with increases of ~5-fold for both antibiotics compared to that of FA19 *penA35*. These data are consistent with the increased resistance of strains such as F89 that harbor a mosaic *penA* allele with an A501P mutation[17], but also suggest that A501R mutations would be just as effective at increasing resistance if they appear in clinical isolates in the future. In contrast to the MICs of the extended-spectrum cephalosporins, the Ala501 mutations decreased the MIC of penicillin compared to the parental *penA35* allele. Four of the mutants (A501S, A501T, A501V and A501R) conferred a 2-fold decrease in the MIC, while the A501P mutant conferred a 10-fold decrease in the MIC.

The isolation of only five mutations of Ala501 in the random screen suggested that only a small subset of mutations confers increased resistance to cefixime while retaining essential TPase activity. This result prompted us to investigate why other amino acids were not isolated in the screen. Two reasons seemed likely: 1) the mutant PBP2 proteins are functional as TPases but do not increase the MIC of cefixime to levels high enough to be selected above background, or 2) they are unable to catalyze sufficient levels of TPase activity. To distinguish between these possibilities, we generated four new Ala501 mutations: A501I, A501L, A501K and A501G. Ile and Leu were chosen because they are most similar in size and hydrophobicity to Val, Lys because of its charge similarity to Arg, and Gly because of its smaller size relative to Ala.

FA19 was transformed with pUC18-*penA35*-A501I, -A501L, -A501K, or –A501G and resistant transformants were selected on 0.04 μg ml^{-1} cefixime, a concentration ~3-fold lower than the MIC of transformants harboring *penA35* without Ala501 mutations, but high enough to ensure that FA19 did not grow. The transformations with *penA35*-A501L and -A501G yielded no colonies, suggesting that these mutations disrupt essential TPase activity of PBP2. In contrast, transformants were obtained with *penA35*-A501I and –A501K, and subsequent PCR and sequencing verified that the mutations were present.

When the MICs of cefixime were determined, FA19 *penA35*-A501K was ~2-fold higher than FA19 *penA35*, similar to FA19 *penA35*-A501T, whereas FA19 *penA35*-A501I was essentially identical to FA19 *penA35* (Fig. 2A). The inability of a *penA35*-A501I mutant allele to increase the MIC of cefixime above that conferred by *penA35* is consistent with the absence of such transformants arising from the random screen. It was not evident, however, why A501K mutations were not identified in the screen. One consistent observation from these transformations was that while transformants of FA19 with *penA35*-A501K could be selected, the number of colonies obtained was markedly lower than that from transformations with other mosaic *penA* alleles.

To examine this phenomenon in more detail, we transformed FA19 with equivalent amounts of PCR products of six *penA35*-Ala501 mutants (*penA35*-A501T, -A501R, -A501I, - A501K, -A501G, and -A501L) and quantified the number of resistant colonies obtained on plates containing 0.01 μg ml^{-1} cefixime, which is 10-fold lower than the MIC of FA19 *penA35* (Fig. 2B). Compared to *penA35*-A501T, the number of transformants with *penA35*- A501R, -A501I and –A501P ranged from 45% to 15% of that obtained with *penA35*-A501T. In contrast, the transformation efficiency of *penA35*-A501K was 2.3% of *penA35*-A501T. Because the six DNAs differed at most by 3 nucleotides (i.e. the mutations at codon-501), our data suggest that the wide variation in the transformation efficiency of the different Ala501 mutants represents the viability of the transformant, and therefore most likely reflects the capacity of each PBP2 mutant to catalyze essential TPase activity. This is an important factor when considering the likelihood that these mutations will emerge in mosaic *penA* alleles in nature.

In previous work, we have shown that some mutations, while critical for increasing resistance to β-lactam antibiotics, require other mutations (called epistatic mutations) that allow the critical mutations to increase resistance[16]. We therefore asked whether a single

A501P mutation in the wild-type *penA* background could confer an increase in the MIC of cefixime. FA19 was transformed with *penA*-A501P and sequence-verified transformants could be obtained at 0.01 μg/ml cefixime (4-fold higher than its MIC for FA19). Consistent with our MIC data for penicillin G (Fig. 1), no transformants of FA19 *penA*-A501P were obtained when selected on a concentration of penicillin G 2-fold higher than the MIC for FA19. These data indicate that the A501P mutation is capable of conferring cefixime resistance without any additional mutations present in the *penA35* allele.

Acylation rate constants of mutant PBPs

We next assessed the *in vitro* acylation rates for cefixime, ceftriaxone, and penicillin G. These 2nd order rate constants (defined as k_2/K_S) describe both the acylation rate constant (k_2) and the binding affinity of the antibiotic (K_S)[24]. As shown in Table 3, the k_2/K_S constants of the PBP2-35/02-Ala501 mutants identified in the screen were mostly consistent with the MICs of strains containing these variants, with the exception of the rate constants of ceftriaxone, which actually increased in the A501T and A501V mutant constructs compared to PBP-35/02. Consistent with the decreased MICs of penicillin G for FA19 harboring the Ala501 mutants (Fig. 1), the k_2/K_S values for all of the PBP2 variants were higher than that for PBP2$^{35/02}$. However, the k_2/K_S values and the MIC did not show a linear or proportional correlation. For example, the k_2/K_S values for penicillin G of PBP2$^{35/02}$-A501R and – A501P were very similar and ~1.5- to 2-fold lower than the –A501V and -A501T variants, but while FA19 *penA35*-A501R had a 2-fold lower MIC than FA19 *penA35*, FA19 *penA35*-A501P had a 10-fold lower MIC. Likewise, the k_2/K_S values of PBP2$^{35/02}$-A501P for ceftriaxone and cefixime were ~20-fold lower than PBP2$^{35/02}$-A501R, but the MICs of the two antibiotics for FA19 harboring the different alleles were about the same. As discussed later, these data suggest that, in addition to rates of acylation, other factors can influence the MIC.

Effects of Ala501 mutations on bacterial fitness

Antibiotic-resistance determinants, especially those that are altered versions of an essential enzyme, can decrease growth and/or fitness of the bacteria[32]. To assess the fitness cost of the Ala501 mutations in the *penA35* background, we measured the *in vitro* growth rate of strains cultured alone or in competition with the parental strain, FA19. The presence of the mutant *penA35* allele either with or without Ala501 mutations negatively impacted growth of the bacteria compared to FA19, causing a shorter logarithmic phase as measured by OD_{600} and a decrease in the number of viable bacteria (CFU ml^{-1}) recovered over time (Fig. 3A, B). When measured in co-culture experiments, all of the resistant strains were out-competed by FA19, with FA19 *penA35*-A501R and FA19 *penA35*-A501P showing the smallest competitive index (Fig. 3C). These data indicate that the *penA35* allele itself negatively impacts growth of a wild-type strain, particularly at later time points, and that mutation of Ala501 to Arg or Pro, but not Val, Thr, or Ser, further impacts growth.

Effects of Ala501 mutations in a non-mosaic PBP2 background

We next incorporated the Ala501 mutations into PBP2-6140CT (PBP2 containing the four point mutations, but not the Asp346a insertion, found in PBP2 from FA6140;[22]) for structural studies. This was done for several reasons: 1) we had already solved the structure

of PBP2-6140CT, which increased the probability of obtaining crystals in this background compared to the PBP2[35/02] background (as yet, PBP2[35/02] constructs have not crystallized); 2) Ala501 mutants originally emerged in non-mosaic alleles of *penA*, making this background clinically relevant[13–15]; and 3) interpretation of the effects of the Ala501 mutations would be much less complicated in a non-mosaic PBP2 background.

To ascertain the effects of the Ala501 mutations in a non-mosaic background, we determined the MICs of cefixime and ceftriaxone of FA19 *penA*-6140CT alone or with one of four Ala501 mutations (Fig. 4). Unlike with penicillin G, the mutations in PBP2-6140CT have no effect on the MICs of cefixime or ceftriaxone. Incorporation of the A501V mutation into PBP2-6140CT conferred a ~2-fold increase in the MIC, whereas incorporation of A501T or A501R increased the MIC ~3-fold. In contrast, incorporation of an A501P mutation into PBP2-6140CT increased the MICs of cefixime and ceftriaxone by 14- and 9-fold, respectively.

Crystal structure description of PBP-6140CT-A501T

The PBP2-6140CT variant containing an A501T mutation crystallized under similar conditions as PBP2 and PBP2-6140CT with two molecules in the asymmetric unit and the structure was solved to 2.4 Å resolution (none of the other PBP2 variants yielded crystals that diffracted to high resolution). Inspection of |Fo|-|Fc| difference electron density maps revealed that the β3-β4 loops of both molecules in the asymmetric unit could be modeled. This is in marked contrast to the structures of PBP2 and PBP2-6140CT, in which residues 501–512 lacked electron density in both molecules of the asymmetric unit. Although the electron density for the side chains in this loop is comparatively weak in places, especially at its apex, that for the main chain is mostly complete, thus establishing the overall structure of the loop.

Two observations suggest that the ordering of the β3-β4 loop is a direct consequence of the A501T mutation and not crystal packing interactions. Firstly, being of the same crystal form, the A501T mutant of PBP2-6140CT has the same packing arrangement as the wild-type structure. Secondly, the structures of the β3-β4 loops in both molecules of the asymmetric unit of PBP2-6140CT-A501T are very similar and yet each has different packing environments. In molecule B, the β3-β4 loop packs against the β2a-β2b loop of a neighboring molecule and against β7n of the N terminal region of another neighboring molecule, whereas the β3-β4 loop of molecule A is exposed to solvent.

The structures of wild-type PBP2 and PBP2-6140CT-A501T superimpose very closely, with an RMS deviation between all common main chain atoms of 0.30 Å for molecule A and 0.36 Å for molecule B. The only significant difference between the structures is the ordering of the β3-β4 loop in the A501T mutant (Fig. 5). Since PBP2 and PBP2-6140CT have essentially the same structures, a superimposition of the A501T mutant with PBP2-6140CT produces the same outcome.

Thr501 forms a hydrogen bond near Ser310

The site of the mutation is at the end of β3, where its threonine side chain packs closely between Val515 on β4 and Tyr422 at the C-terminal end of α8. A further consequence of the

A501T mutation is that the side-chain hydroxyl of Thr501 is within hydrogen bonding distance of the main-chain carbonyl of Glu307, thus forming a new connection between β3 and the loop immediately prior to Ser310 (Fig. 6). The nearly exact overlap of wild-type and A501T mutant structures of PBP2 in this region shows that the new hydrogen bond does not alter the architecture, but it may nevertheless be close enough to impact nucleophilic activation of Ser310.

Thermal stability of 501 mutants

The ordering of the β3-β4 loop of PBP2 in the PBP2-6140CT-A501T structure suggests a mechanism of antibiotic resistance involving increased rigidity of the protein. To evaluate the impact of the mutations at position 501 on the stability of the protein, we collected irreversible thermal denaturation curves for the Thr, Val, Pro and Arg mutants of PBP2-6140CT using circular dichroism (CD) spectroscopy. Spectra were also recorded for wild-type PBP2 and for PBP2-6140CT. Surprisingly, the T_m of all the mutants was significantly lower than that of wild-type PBP2, suggesting an overall destabilizing effect of the mutations (Fig. 7). Furthermore, all of the Ala501 mutants exhibit a lower T_m than PBP2-6140CT, with A501R and A501P mutations lowering T_m the most. The contrast between observed ordering of the β3-β4 loop and lowered thermal stability of the mutant proteins is discussed below.

Discussion

In this study, we identified five amino acids (Thr, Val, Ser, Arg, and Pro) in a random screen for mutations at Ala501 of PBP2, plus an additional amino acid (Lys) by site-directed mutagenesis, that increase resistance of *N. gonorrhoeae* to expanded-spectrum cephalosporins over that conferred by the parental mosaic PBP, PBP2-35/02. Three of these amino acids, Thr, Val, and Pro, have been identified previously as mutations at Ala501 in *penA* alleles from gonococcal clinical isolates, while Ser, Arg and Lys have not. Of these, both A501R and A501P mutations have a more pronounced effect on the MIC (~5-fold) and appear to cause a larger fitness deficit compared to the other mutations. The structure of PBP2-6140CT-A501T reveals that the primary impact of the A501T mutation is to promote ordering of the β3-β4 loop.

Antibiotic resistance determinants often have a negative effect on fitness. This is particularly true with mosaic PBP2 variants as these likely have sub-optimal TPase activity. Unfortunately, in the absence of a biochemical assay of TPase activity using purified PBPs (particularly for the class B PBPs such as PBP2)[6], it is not possible to quantify directly the impact of resistance mutations on the TPase function of PBP2. That said, our *in vitro* growth experiments suggest that bacteria expressing a mosaic *penA* allele (*penA35*) grow less well than wild-type bacteria. Moreover, the two Ala501 mutations that increase the MICs of cefixime and ceftriaxone the most (i.e. A501P and A501R) are the least fit in competitive co-culture experiments, which would be expected if these mutations have a more deleterious effect on TPase activity than the other mutations. It seems likely, therefore, that dissemination of any of these alleles containing Ala501 mutations would require compensatory mutations in the strain that increase fitness. Indeed, compensatory mutations

that help abrogate the fitness defect of resistance alleles have been identified in other bacteria, and for an *mtrA* mutant and an *mtrR*/*gyrA*/*parC* triple mutant in *N. gonorrhoeae*[33, 34].

Surprisingly, nearly 50% of the total transformants sequenced in the random screen harbored an A501R mutation, compared to the identification of a single transformant containing an A501P mutation. The high number of transformants obtained containing A501R mutations compared to A501P mutations was particularly unexpected, because in the clinical setting, three temporally and geographically distinct cefixime-resistant isolates harbored a mosaic *penA* allele with an A501P mutation[17, 18], but no isolates with an A501R mutation have been observed to date. We investigated this apparent anomaly by comparing the transformation frequencies of identical quantities of *penA35* PCR fragments containing different mutations at Ala501, and observed large differences in the capacities of these DNAs to transform competent gonococci. One explanation for these differences is that they reflect the degree to which transpeptidase activity is compromised by the mutation, although this does not explain why only A501P mutations have occurred in nature. Irrespective, these results highlight the fine line that *N. gonorrhoeae* must navigate to increase resistance to antibiotics by remodeling PBP2 whilst preserving essential TPase activity.

An interesting observation from these studies is that there is not a clear correlation between the acylation rates of purified proteins and the MICs of β-lactam antibiotics for FA19 harboring the corresponding *penA* alleles. The MIC of a β-lactam antibiotic for *N. gonorrhoeae* is determined by three main characteristics: i) the acylation rate of PBP2, ii) the capacity to serve as a substrate for efflux pumps, and iii) the rate of influx through porin channels. An additional factor is the degree to which the PBP2 variant needs to be inhibited to cause cell death. For example, the TPase activity of wt PBP2 may need to be inhibited by 80–90% to cause cell death, whereas that of the PBP2$^{35/02}$-A501P variant, which our data suggest is markedly impaired, may only need to be inhibited by 30–40% before the level falls below the threshold that supports cell growth. The lack of correlation observed in this study between the acylation rates and MICs of the different antibiotics is most evident for the A501P mutant, and is consistent with the fitness data and transformation frequencies.

We crystallized PBP2-6140CT containing the A501T mutation and solved its structure to 2.4 Å. The most striking difference compared to the crystal structure of PBP2-6140CT is significant ordering of the β3-β4 loop. This same loop in other PBPs has also been associated with resistance to β-lactams. A Q556E mutation in *Streptococcus pneumoniae* PBP2x, which is implicated in cephalosporin resistance, lies in a very similar position as Ala501 at the C-terminal end of β3[35]. Similarly, for *S. pneumoniae* PBP1a, there is a clustering of four mutations on the β3-β4 loop that lowers the rates of acylation for cefotaxime and penicillin G[36]. Interestingly, whether the β3-β4 loop is ordered or disordered varies in relation to resistance. In the crystal structure of PBP1a from the R6 (susceptible) strain of *S. pneumoniae*, the β3-β4 loop is ordered, but is disordered in the protein derived from the cephalosporin-resistant strain 5204[37]. Opposite to that and in alignment with *N. gonorrhoeae* PBP2, the crystal structure of PBP2a from *S. pneumoniae* shows a disordered β3-β4 loop in the protein derived from the susceptible strain and an

ordered loop in the protein from the resistant strain[38]. These observations have led to the idea that flexibility in this region is a key factor in antibiotic resistance[38].

A key question is how mutations at position 501 confer increased resistance to cephalosporins. One simple mechanism is that larger side chains at position 501 sterically impede the binding of antibiotic. Given the close proximity of position 501 to the core of the active site, direct clashes between side chains at position 501 and the cephalosporin can be envisaged (see Fig. 5). These clashes would block binding of antibiotic (i.e. increase K_S) and thereby lower the rate of acylation, while in some way retaining the capacity to recognize the peptide C-terminus of peptidoglycan during transpeptidation. In a similar vein, the negative charge introduced by the Q552E mutation in *S. pneumoniae* PBP2x is believed to impede binding of antibiotic in a direct manner[35]. This mechanism is consistent with the mutations all introducing larger side chains at position 501 and the proportionally higher increase of MICs for the largest side chain, Arg. It does not explain, however, why mutation of Ala501 to Ile, which also introduces a larger side chain, has no effect on resistance, suggesting that steric hindrance alone does not explain the changes in resistance in PBP2.

Perhaps the most compelling mechanism, and consistent with changes in the crystal structure, is that resistance results from increased rigidity of the active site and its immediate environs. As revealed by the structure of PBP2-6140CT-A501T, the β3-β4 loop exhibits significantly increased order, compared to the otherwise identical protein construct lacking the A501T mutation. One possible reason for this is the new hydrogen bond between Thr501 and Glu307 near the SxxK motif that tethers the beginning of β3 with a loop preceding the SxxK motif. The other mutations at position 501 also have the potential to lower flexibility. A serine would likely form the same hydrogen bond with Glu307 as threonine. A valine cannot mediate a hydrogen bond, but could form hydrophobic interactions with Val515 and Tyr422. An arginine could form a new electrostatic interaction with the side chain of Glu307 and introduction of proline would clearly restrict conformational freedom.

At first sight, the idea of increased rigidity causing resistance to cephalosporins appears at odds with the circular dichroism results. These data show that mutations at Ala501 lower the thermal stability of the protein and thus appear more consistent with a mechanism in which resistance mutations *destabilize* the enzyme. However, the apparent destabilization of the protein detected by a lowered T_m may result from entropic compensation elsewhere in the protein framework. This situation has been described as a rigidity-flexibility equilibrium, where lowered entropy due to a stabilizing mutation at one site is compensated by increased flexibility in remote regions[39]. The reverse can also happen when the mutation is destabilizing. Relationships between mutations, protein stability and ligand binding are generally protein specific and specific mutations can reduce ligand affinity by lowering or increasing the stability of proteins[40]. In the case of PBP2, it appears that mutations at position 501 reduce reactivity for β-lactams via increasing localized ordering in the active site region and the entropic penalty is paid by increased flexibility elsewhere in the framework that is detected by circular dichroism.

Increased rigidity in the active site could lower rates of acylation by cephalosporins in two ways: by preventing requisite conformational changes necessary for binding of the

antibiotic, or by hindering formation of the ideal geometry of the transition state for acylation (i.e. by lowering k_2). The first of these mechanisms infers that binding of cephalosporins by PBP2 requires movement of the protein to bind the antibiotic in a productive orientation prior to acylation. Due to the increased rigidity conferred by the mutations at position 501, the energetic barrier against such conformational changes is made higher and thus cephalosporins are less likely to acylate the enzyme successfully. This mechanism may act synergistically with the simple steric hindrance described above. A similar mechanism was proposed for *S. aureus* PBP2a in which mutations associated with β-lactam resistance were hypothesized to restrict movement of β3[41].

The second mechanism is derived from the mechanistic relationship between PBPs and serine proteases. It is well established that acylation requires the formation of a tetrahedral intermediate in which the negative charge of the intermediate is stabilized by an oxyanion hole, which in PBP2 comprises the amide nitrogens of Ser310 and Thr500. The latter, of course, is immediately adjacent to Ala501. A productive transition state requires precise geometry, and increased rigidity in the structure elicited by mutations at position 501 may create a barrier to successfully achieving this state with the kinetic consequence of lowered rates of acylation.

Of course, in both of these mechanisms, the peptidoglycan substrate of PBP2 must be less affected than antibiotic, and how this is achieved is a matter of significant interest. One possibility is that the larger peptide substrate overcomes the energetic barrier imposed by the Ala501 mutations by making more contacts with the enzyme. That said, it seems inevitable that the efficiency by which the acyl-enzyme complex forms with peptide substrates must be compromised to some degree, and this is consistent with the larger fitness deficit of bacteria harboring *penA* alleles with Ala501 mutations that increase the MIC the most (Arg and Pro), as revealed in the competitive co-culture experiments.

The opposite effect of the A501P mutation on penicillin G MICs and k_2/K_S compared to cefixime and ceftriaxone is interesting because the mechanisms that discriminate against cephalosporins must somehow favor reactivity with penicillin. How this happens mechanistically is unclear, but perhaps the precise active-site geometry that is formed by this mutation is ideal for the binding or acylation of penicillin G. There are key chemical differences between cephalosporins and penicillins that can affect binding and/or reactivity with a PBP, including the 6-membered dihydrothiazine ring of cephalosporins rather than 5-membered thiazolidine ring of penicillins, the presence of a C3 substituent in cephalosporins, and the different groups comprising the R1 side chains.

In this study, we have shown that specific mutations of Ala501 in PBP2 can cause cephalosporin resistance in *N. gonorrhoeae* while retaining sufficient essential transpeptidase function necessary for viability. Substitutions to amino acids with significantly different physicochemical properties compared to Ala confer the biggest increases on MICs. A crystal structure of PBP2 containing an A501T mutation shows dramatic ordering of the β3-β4 loop and suggests that increased rigidity in the active site correlates with resistance. These results are important for predicting future emergence of

Ala501 mutations that may threaten the clinical utility of cephalosporins against gonorrhea and for understanding the underlying molecular mechanism of resistance.

Acknowledgments

Abbreviations and Textual Footnotes

PBP	Penicillin-Binding Protein
TPase	transpeptidase
MIC	minimum inhibitory concentration
Ceph^I	cephalosporin-intermediate resistant
Ceph^R	cephalosporin-resistant
GCB	GC Medium Base
LB	lysogeny broth
CFU	colony forming units

References

1. Newman L, Rowley J, Vander Hoorn S, Wijesooriya NS, Unemo M, Low N, Stevens G, Gottlieb S, Kiarie J, Temmerman M. Global Estimates of the Prevalence and Incidence of Four Curable Sexually Transmitted Infections in 2012 Based on Systematic Review and Global Reporting. PLoS One. 2015; 10:e0143304. [PubMed: 26646541]

2. Unemo M, Nicholas RA. Emergence of multidrug-resistant, extensively drug-resistant and untreatable gonorrhea. Future Microbiology. 2012; 7:1401–1422. [PubMed: 23231489]

3. Unemo M, Shafer WM. Antimicrobial resistance in *Neisseria gonorrhoeae* in the 21st century: past, evolution, and future. Clin Microbiol Rev. 2014; 27:587–613. [PubMed: 24982323]

4. Unemo M. Current and future antimicrobial treatment of gonorrhoea - the rapidly evolving *Neisseria gonorrhoeae* continues to challenge. BMC Infect Dis. 2015; 15:364. [PubMed: 26293005]

5. Ito M, Deguchi T, Mizutani KS, Yasuda M, Yokoi S, Ito S, Takahashi Y, Ishihara S, Kawamura Y, Ezaki T. Emergence and spread of *Neisseria gonorrhoeae* clinical isolates harboring mosaic-like structure of penicillin-binding protein 2 in Central Japan. Antimicrob Agents Chemother. 2005; 49:137–143. [PubMed: 15616287]

6. Nicholas, RA., Davies, C. Structural mechanisms of β-lactam antibiotic resistance in penicillin-binding proteins. In: Dougherty, TJ., Pucci, MJ., editors. Antibiotic Discovery and Development. Springer; New York: 2012. p. 397-425.

7. Spratt BG. Hybrid penicillin-binding proteins in penicillin-resistant strains of *Neisseria gonorrhoeae*. Nature. 1988; 332:173–176. [PubMed: 3126399]

8. Ito M, Yasuda M, Yokoi S, Ito S, Takahashi Y, Ishihara S, Maeda S, Deguchi T. Remarkable increase in central Japan in 2001–2002 of *Neisseria gonorrhoeae* isolates with decreased susceptibility to penicillin, tetracycline, oral cephalosporins, and fluoroquinolones. Antimicrob Agents Chemother. 2004; 48:3185–3187. [PubMed: 15273147]

9. Ochiai S, Sekiguchi S, Hayashi A, Shimadzu M, Ishiko H, Matsushima-Nishiwaki R, Kozawa O, Yasuda M, Deguchi T. Decreased affinity of mosaic-structure recombinant penicillin-binding protein 2 for oral cephalosporins in *Neisseria gonorrhoeae*. J Antimicrob Chemother. 2007; 60:54–60. [PubMed: 17540669]

10. Unemo, M., Nicholas, RA., Jerse, AE., Davies, C., Shafer, WM. Molecular mechanisms of antibiotic resistance expressed by the pathogenic *Neisseria*. In: Davies, JK., Kahler, CM., editors. Pathogenic Neisseria. 2nd. Caister Academic Press; Norfolk, UK: 2014. p. 161-192.

11. Zhao S, Duncan M, Tomberg J, Davies C, Unemo M, Nicholas RA. Genetics of chromosomally mediated intermediate resistance to ceftriaxone and cefixime in *Neisseria gonorrhoeae*. Antimicrob Agents Chemother. 2009; 53:3744–3751. [PubMed: 19528266]

12. Lindberg R, Fredlund H, Nicholas RA, Unemo M. *Neisseria gonorrhoeae* isolates with reduced susceptibility to cefixime and ceftriaxone: association with genetic polymorphisms in *penA, mtrR, porB1b*, and *ponA*. Antimicrob Agents Chemother. 2007; 51:2117–2122. [PubMed: 17420216]

13. Whiley DM, Limnios EA, Ray S, Sloots TP, Tapsall JW. Diversity of *penA* alterations and subtypes in *Neisseria gonorrhoeae* strains from Sydney, Australia, that are less susceptible to ceftriaxone. Antimicrob Agents Chemother. 2007; 51:3111–3116. [PubMed: 17591846]

14. Osaka K, Takakura T, Narukawa K, Takahata M, Endo K, Kiyota H, Onodera S. Analysis of amino acid sequences of penicillin-binding protein 2 in clinical isolates of *Neisseria gonorrhoeae* with reduced susceptibility to cefixime and ceftriaxone. J Infect Chemother. 2008; 14:195–203. [PubMed: 18574654]

15. Lee SG, Lee H, Jeong SH, Yong D, Chung GT, Lee YS, Chong Y, Lee K. Various *penA* mutations together with *mtrR, porB* and *ponA* mutations in *Neisseria gonorrhoeae* isolates with reduced susceptibility to cefixime or ceftriaxone. J Antimicrob Chemother. 2010; 65:669–675. [PubMed: 20093260]

16. Tomberg J, Unemo M, Davies C, Nicholas RA. Molecular and structural analysis of mosaic variants of penicillin-binding protein 2 conferring decreased susceptibility to expanded-spectrum cephalosporins in *Neisseria gonorrhoeae*: role of epistatic mutations. Biochemistry. 2010; 49:8062–8070. [PubMed: 20704258]

17. Unemo M, Golparian D, Nicholas R, Ohnishi M, Gallay A, Sednaoui P. High-level cefixime- and ceftriaxone-resistant *N. gonorrhoeae* in France: novel *penA* mosaic allele in a successful international clone causes treatment failure. Antimicrob Agents Chemother. 2011; 56:1273–1280. [PubMed: 22155830]

18. Camara J, Serra J, Ayats J, Bastida T, Carnicer-Pont D, Andreu A, Ardanuy C. Molecular characterization of two high-level ceftriaxone-resistant *Neisseria gonorrhoeae* isolates detected in Catalonia, Spain. J Antimicrob Chemother. 2012; 67:1858–1860. [PubMed: 22566592]

19. Kellogg DS, Peacock WL, Deacon WE, Browh L, Perkle CI. *Neisseria gonorrhoeae*. I. Virulence genetically linked to colonial variation. J Bacteriol. 1963; 85:1274–1279. [PubMed: 14047217]

20. Elkins C, Thomas CE, Seifert HS, Sparling PF. Species-specific uptake of DNA by gonococci is mediated by a 10-base-pair sequence. J Bacteriol. 1991; 173:3911–3913. [PubMed: 1904861]

21. Ho SN, Hunt HD, Horton RM, Pullen JK, Pease LR. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. Gene. 1989; 77:51–59. [PubMed: 2744487]

22. Powell AJ, Tomberg J, Deacon AM, Nicholas RA, Davies C. Crystal structures of penicillin-binding protein 2 from penicillin-susceptible and -resistant strains of *Neisseria gonorrhoeae* reveal an unexpectedly subtle mechanism for antibiotic resistance. J Biol Chem. 2009; 284:1202–1212. [PubMed: 18986991]

23. Stefanova ME, Tomberg J, Olesky M, Holtje JV, Gutheil WG, Nicholas RA. *Neisseria gonorrhoeae* penicillin-binding protein 3 exhibits exceptionally high carboxypeptidase and β-lactam binding activities. Biochemistry. 2003; 42:14614–14625. [PubMed: 14661974]

24. Frere, JM., Nguyen-Disteche, M., Coyette, J., Joris, B. The chemistry of β-lactams. Chapman & Hall; Glasgow: 1992. Mode of action: Interaction with the penicillin binding proteins; p. 148-196.

25. Otwinowski Z, Minor W. Processing of X-ray diffraction data collected in oscillation mode. Methods Enzymol. 1997; 276:307–326.

26. Jones TA, Zhou JY, Cowan SW, Kjeldgaard M. Improved methods for building protein models in electron density maps and the location of errors in those models. Acta Crystallographica A47. 1991:110–119.

27. Murshudov GN, Vagin AA, Dodson EJ. Refinement of macromolecular structures by the maximum-likelihood method. Acta Crystallogr D Biol Crystallogr. 1997; 53:240–255. [PubMed: 15299926]

28. Serra-Pladevall J, Barbera MJ, Rodriguez S, Bartolome-Comas R, Roig G, Juve R, Andreu A. *Neisseria gonorrhoeae* antimicrobial susceptibility in Barcelona: *penA, ponA, mtrR,* and *porB* mutations and NG-MAST sequence types associated with decreased susceptibility to cephalosporins. Eur J Clin Microbiol Infect Dis. 2016; 35:1549–1556. [PubMed: 27255221]

29. Chen SC, Yin YP, Dai XQ, Unemo M, Chen XS. Antimicrobial resistance, genetic resistance determinants for ceftriaxone and molecular epidemiology of *Neisseria gonorrhoeae* isolates in Nanjing, China. J Antimicrob Chemother. 2014; 69:2959–2965. [PubMed: 25011655]

30. Olsen B, Pham TL, Golparian D, Johansson E, Tran HK, Unemo M. Antimicrobial susceptibility and genetic characteristics of *Neisseria gonorrhoeae* isolates from Vietnam, 2011. BMC Infect Dis. 2013; 13:40. [PubMed: 23351067]

31. Whiley DM, Goire N, Lambert SB, Ray S, Limnios EA, Nissen MD, Sloots TP, Tapsall JW. Reduced susceptibility to ceftriaxone in *Neisseria gonorrhoeae* is associated with mutations G542S, 551S and 551L in the gonococcal penicillin-binding protein 2. J Antimicrob Chemother. 2010; 65:1615–1618. [PubMed: 20511367]

32. Andersson DI. The biological cost of mutational antibiotic resistance: any practical conclusions? Curr Opin Microbiol. 2006; 9:461–465. [PubMed: 16890008]

33. Kunz AN, Begum AA, Wu H, D'Ambrozio JA, Robinson JM, Shafer WM, Bash MC, Jerse AE. Impact of fluoroquinolone resistance mutations on gonococcal fitness and in vivo selection for compensatory mutations. J Infect Dis. 2012; 205:1821–1829. [PubMed: 22492860]

34. Warner DM, Folster JP, Shafer WM, Jerse AE. Regulation of the MtrC-MtrD-MtrE efflux-pump system modulates the in vivo fitness of *Neisseria gonorrhoeae*. J Infect Dis. 2007; 196:1804–1812. [PubMed: 18190261]

35. Mouz N, Di Guilmi AM, Gordon E, Hakenbeck R, Dideberg O, Vernet T. Mutations in the active site of penicillin-binding protein PBP2x from *Streptococcus pneumoniae. Role in the specificity for β-lactam antibiotics*. J Biol Chem. 1999; 274:19175–19180. [PubMed: 10383423]

36. Contreras-Martel C, Job V, Di Guilmi AM, Vernet T, Dideberg O, Dessen A. Crystal structure of penicillin-binding protein 1a (BP1a) reveals a mutational hotspot implicated in β-lactam resistance in *Streptococcus pneumoniae*. J Mol Biol. 2006; 355:684–696. [PubMed: 16316661]

37. Job V, Carapito R, Vernet T, Dessen A, Zapun A. Common alterations in BP1a from resistant *Streptococcus pneumoniae* decrease its reactivity toward β-lactams: structural insights. J Biol Chem. 2008; 283:4886–4894. [PubMed: 18055459]

38. Contreras-Martel C, Dahout-Gonzalez C, Martins Ados S, Kotnik M, Dessen A. PBP active site flexibility as the key mechanism for beta-lactam resistance in pneumococci. J Mol Biol. 2009; 387:899–909. [PubMed: 19233207]

39. Li T, Tracka MB, Uddin S, Casas-Finet J, Jacobs DJ, Livesay DR. Redistribution of flexibility in stabilizing antibody fragment mutants follows Le Chatelier's principle. PLoS One. 2014; 9:e92870. [PubMed: 24671209]

40. Teilum K, Olsen JG, Kragelund BB. Protein stability, flexibility and function. Biochim Biophys Acta. 2011; 1814:969–976. [PubMed: 21094283]

41. Lim D, Strynadka NC. Structural basis for the β-lactam resistance of BP2a from methicillin-resistant *Staphylococcus aureus*. Nat Struct Biol. 2002; 9:870–876. [PubMed: 12389036]
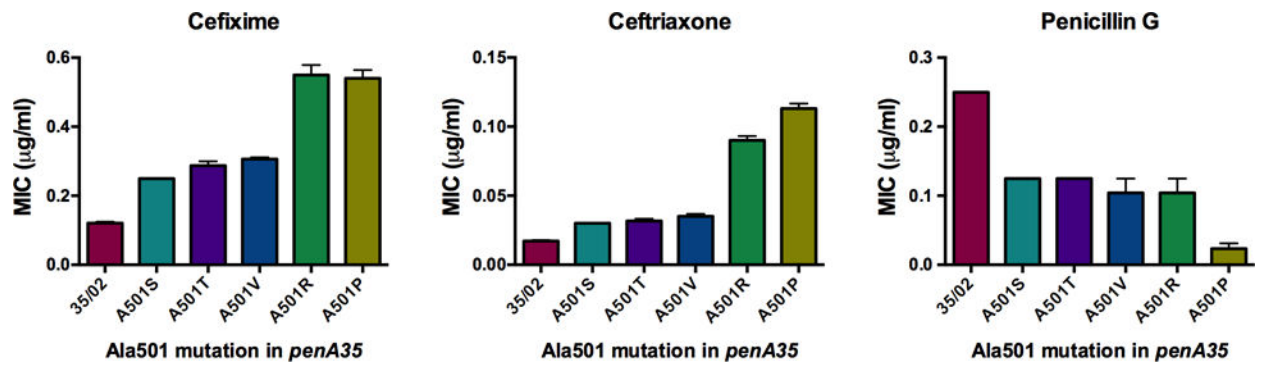
Figure 1. MICs of cefixime, ceftriaxone, and penicillin G for *Neisseria gonorrhoeae* strain FA19 harboring *penA35*-Ala501 mutants

Strains were generated by transformation with the indicated *penA* allele, and the MICs of the indicated antibiotics were determined as described in Material and Methods. The MICs reported are the averages ± standard deviation determined from a minimum of 3 independent experiments using 3–6 sequence-verified transformants of each strain.
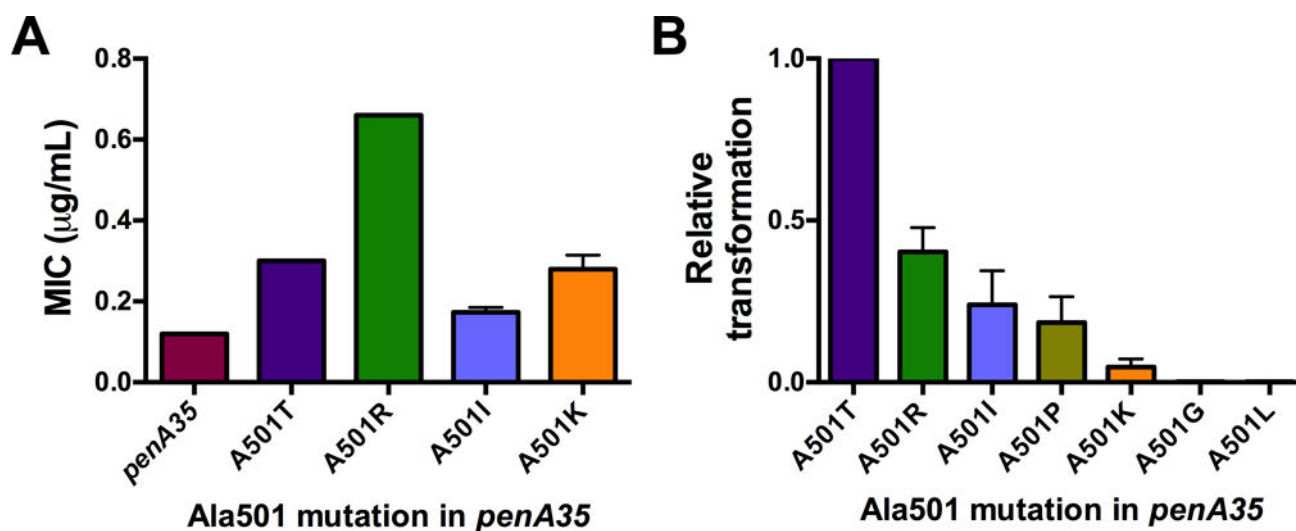
Figure 2. MICs and relative transformation efficiencies of *Neisseria gonorrhoeae* strain FA19 harboring *penA35*-Ala501 mutants

A, MICs of cefixime for the indicated strains; the data were generated and graphed as described in the legend to Fig. 1. *B*, Normalized transformation frequencies of the indicated *penA35*-A501 variants. Equal amounts of DNA were used to transform FA19, and transformants were selected on 0.01 μg ml^{-1} of cefixime. For each experiment, the relative transformation frequency for each strain was determined as a ratio of that obtained with *penA35*-A501T. The experiment was repeated three times and the ratios were averaged ± standard deviation.
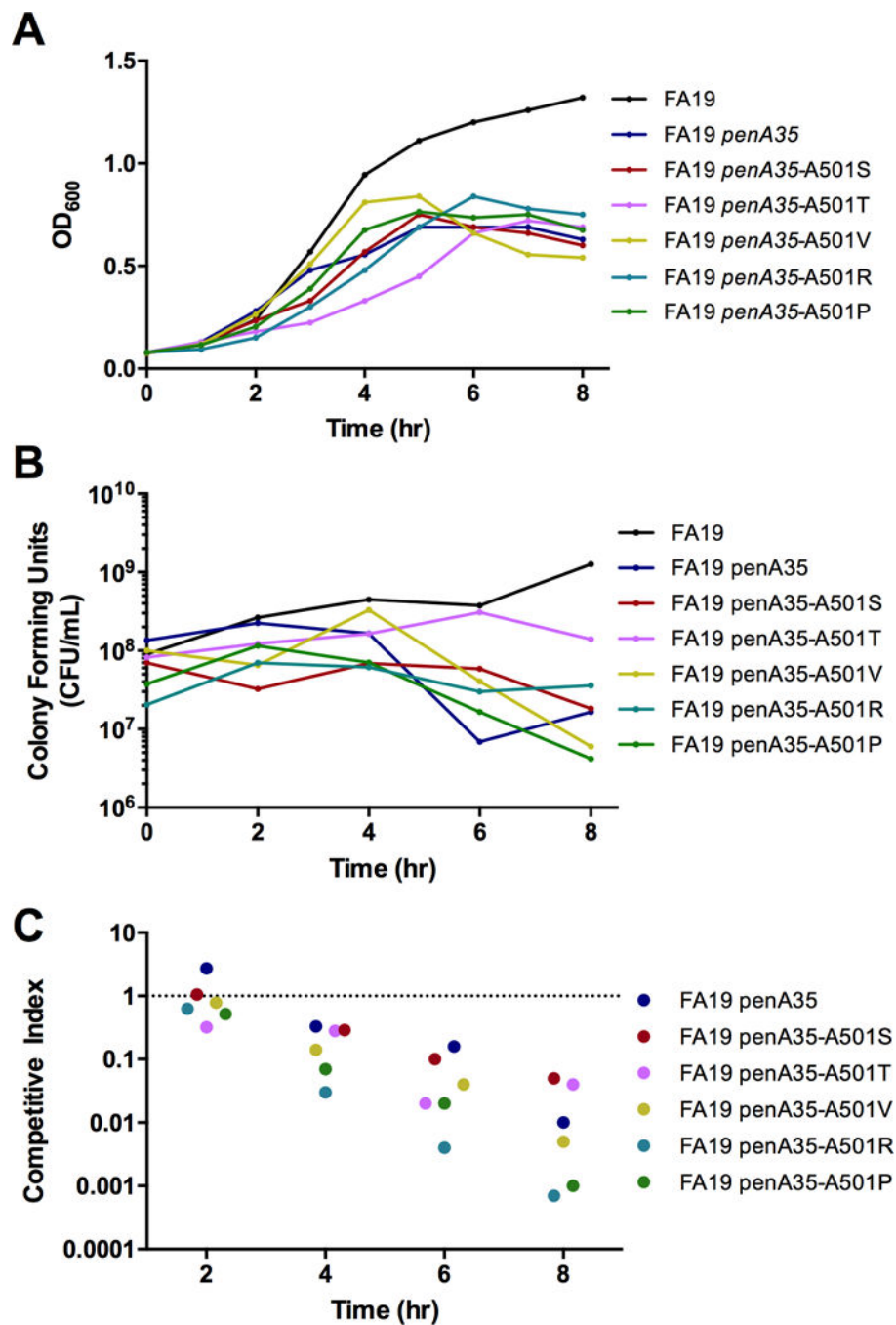
Figure 3. *In vitro* **fitness of** *Neisseria gonorrhoeae* **strain FA19 harboring** *penA35***-Ala501 mutant alleles**

Growth of FA19 harboring wild-type *penA*, the mosaic *penA35*, or *penA35*-Ala501 mutant alleles cultured individually was monitored in liquid GCB medium by quantifying the OD_{600} (*A*) and number of colony-forming units (CFU) per ml (*B*). *C*, Competitive co-cultures of FA19 versus either FA19 *penA35* or individual FA9 *penA35-Ala501* mutants. Results are expressed as a Competitive Index (CI) as described in Materials and Methods. CIs less than 1 indicate reduced fitness relative to parent FA19. The experiments were repeated twice with similar results (results from a single experiment are shown).
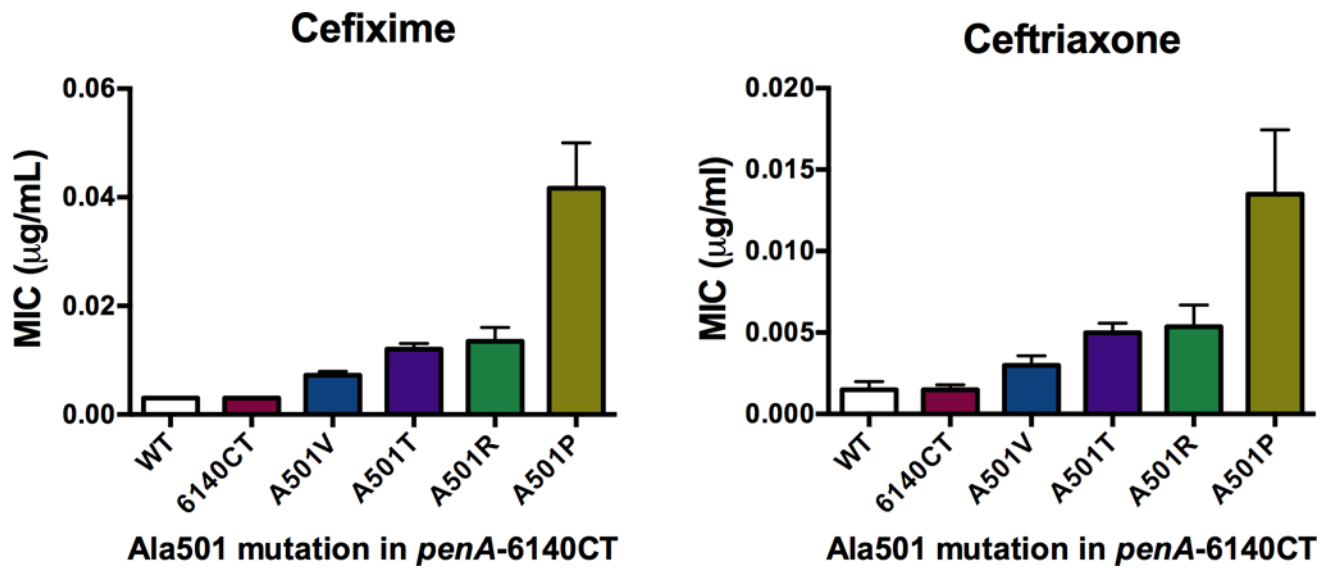
Figure 4. MICs of cefixime and ceftriaxone for FA19 alone or FA19 harboring *penA6140CT-Ala501 mutant alleles*

The MICs were determined by agar dilution as described in Materials and Methods and represent the averages (± standard deviation) determined from a minimum of 3 independent experiments using 3–6 sequence-verified transformants of each strain.
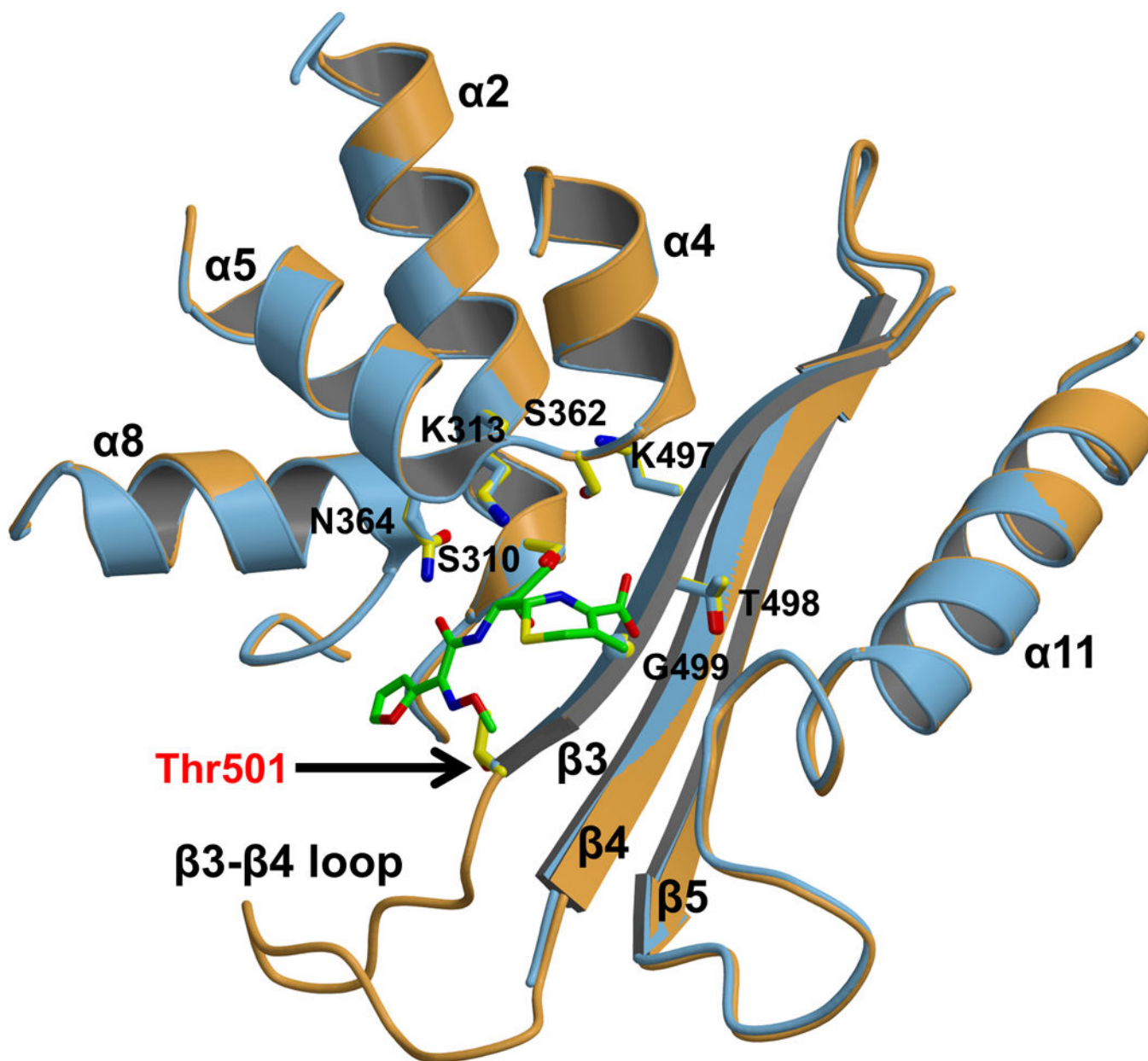
Figure 5. The β3-β4 loop is ordered in the PBP2 A501T mutant

Superimposition of molecule A of wild-type PBP 2 (3EQU; slate blue) with molecule A of PBP2-6140CT-A501T (orange). Cefuroxime has been docked into the wild-type structure based on the structure of the acylated PBP2x from *S. pneumoniae* (1QMF). Active site residues of the SxxK, SxN, and KTG motifs are shown in stick form.
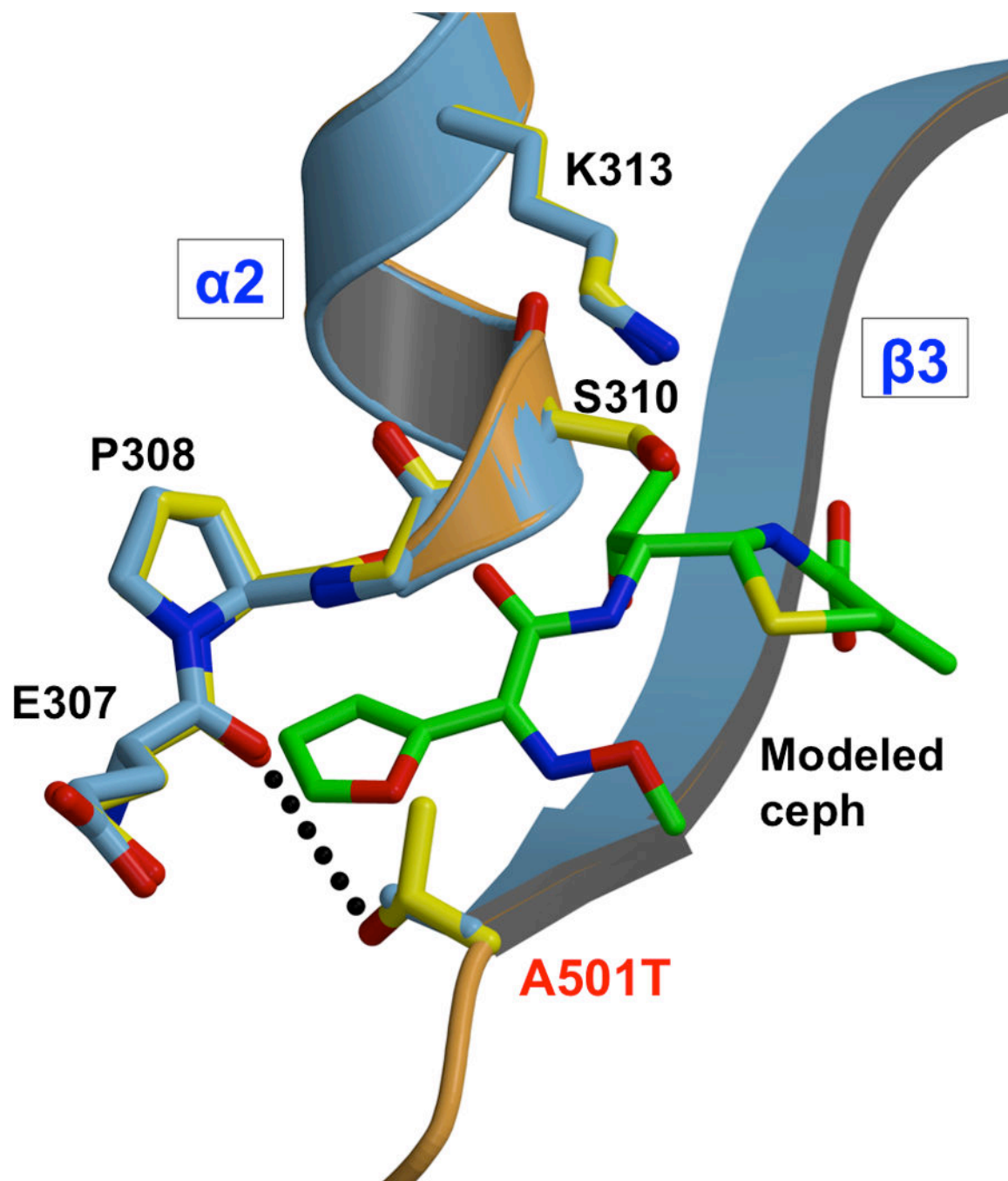
Figure 6. Close-up view of the PBP2 region near the A501T mutation
Superimposition of molecule B of wild-type PBP2 (3EQU; blue) and molecule B of
PBP2-6140CT-A501T (orange). Cefuroxime has been docked into the wild-type structure
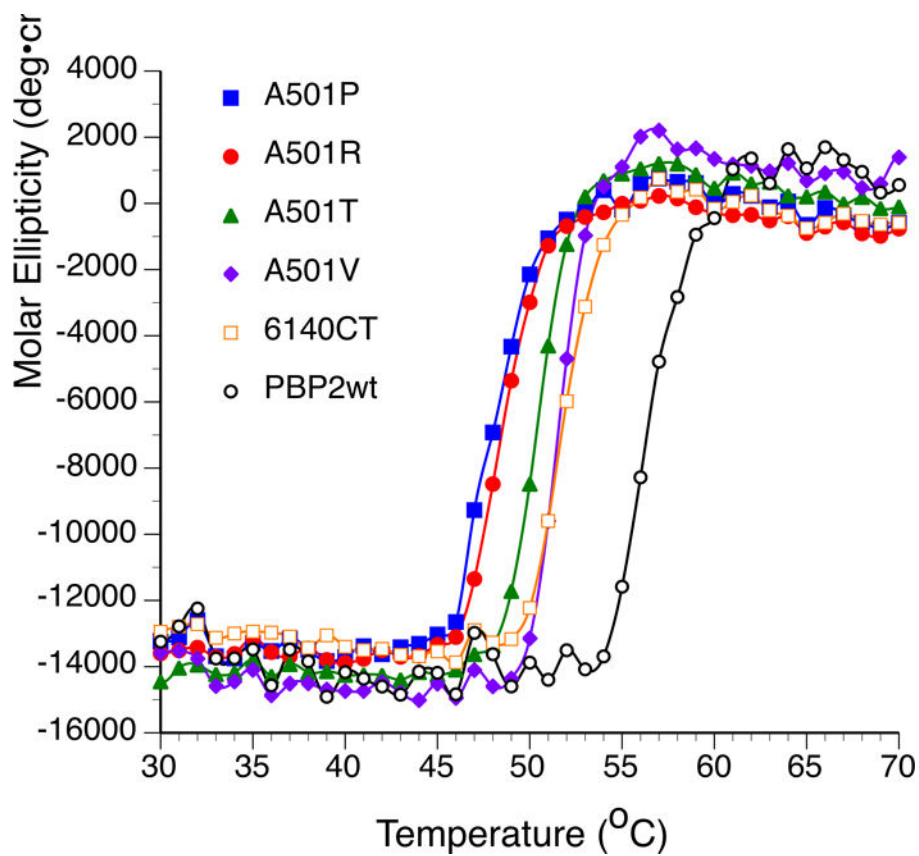based on the structure of the acylated PBP2x from *S. pneumoniae* (1QMF).

Figure 7. Melting temperatures of PBP2, PBP2-6140CT, and PBP2-6140CT-A501 mutants
The increase in molar ellipticity at 208 nm of the indicated PBP variants was quantified at different temperatures by circular dichroism.

Table 1

X-ray diffraction data and refinement statistics

$R_{merge}=\Sigma|I_i - I_m|/\Sigma I_i$, where I_i is the intensity of the measured reflection, and I_m is the mean intensity for all observations of that reflection. Numbers within parentheses are for the outer resolution shell of data.

Data collection:	
Cell dimensions: a, b, c (Å)	57.6, 137.2, 229.8
Resolution range (Å)	39.0 – 2.40 (2.44–2.40)
R_{merge}* (%)	10.7 (99.8)
Completeness (%)	98.9 (97.6)
Redundancy	5.9 (5.5)
<I>/<σI>	26.0 (2.5)
No. of unique reflections	71,874 (3,435)
Refinement:	
Resolution (Å)	39.0 – 2.4
No. of sulfates	12
No. of glycerols	3
No. of water molecules	39
R_{cryst}/R_{free} (%)	0.218/0.244
RMS deviations from ideal stereochemistry:-	
bond lengths (Å)	0.013
bond angles (°)	1.72
Ramachandran plot:	
Residues in most favored region (%)	92.2
Residues in disallowed region (%)	7.8
Residues in generously allowed regions	0.0
Residues in disallowed regions	0.0
PDB code	5KSH

Table 2

Random mutagenesis of Ala501 in PBP2 revealed 5 amino acids that increase resistance to expanded-spectrum cephalosporins

Transformants were selected at the indicated concentrations of cefixime, and the *penA* genes were amplified and sequenced. Data are from three independent transformations.

Cefixime concentration used for selection	Ala501 mutations	Number of isolates
0.1 μg ml⁻¹	Arg	21
	Ser	5
	Thr	20
	Val	18
	Pro	1
0.2 μg ml⁻¹	Arg	24

Table 3

k_2/K_s second order rate constants for acylation

The values represent the mean ± standard deviation for 3 independent determinations.

PBP2 Variant	k_2/K_S (M^{-1}s^{-1})		
	Cefixime	Ceftriaxone	Penicillin G
PBP2	1480000 ± 22000	1710000 ± 90000	75700 ± 2300
PBP2$^{35/02}$	7170 ± 280	11300 ± 440	510 ± 90
PBP2$^{35/02}$-A501T	5550 ± 160	30400 ± 300	1860 ± 130
PBP2$^{35/02}$-A501V	3150 ± 120	20000 ± 390	1370 ± 140
PBP2$^{35/02}$-A501R	820 ± 40	6460 ± 70	960 ± 100
PBP2$^{35/02}$-A501P	40 ± 1	250 ± 20	810 ± 40