Recognition of the β-lactam carboxylate triggers acylation of Neisseria gonorrhoeae penicillin-binding protein 2

Received for publication, June 25, 2019, and in revised form, July 25, 2019 Published, Papers in Press, July 30, 2019, DOI 10.1074/jbc.RA119.009942

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Edited by Wolfgang Peti

Resistance of Neisseria gonorrhoeae to extended-spectrum cephalosporins (ESCs) has become a major threat to human health. The primary mechanism by which N. gonorrhoeae becomes resistant to ESCs is by acquiring a mosaic penA allele, encoding penicillin-binding protein 2 (PBP2) variants containing up to 62 mutations compared with WT, of which a subset contribute to resistance. To interpret molecular mechanisms underpinning cephalosporin resistance, it is necessary to know how PBP2 is acylated by ESCs. Here, we report the crystal structures of the transpeptidase domain of WT PBP2 in complex with cefixime and ceftriaxone, along with structures of PBP2 in the apo form and with a phosphate ion bound in the active site at resolutions of 1–7–1.9 Å. These structures reveal that acylation of PBP2 by ESCs is accompanied by rotation of the Thr-498 side chain in the KTG motif to contact the cephalosporin carboxylate of PBP2 by ESCs is accompanied by rotation of the Thr-498 side chain in the KTG motif to contact the cephalosporin carboxylate. Overall, our data suggest that acylation is initiated by conformational changes elicited or trapped by binding of ESCs and that these movements are restricted by mutations associated with resistance against ESCs.

In recent years, the sexually transmitted infection gonorrhea has become increasingly difficult to treat due to antibiotic resistance exhibited by its etiological agent Neisseria gonorrhoeae. Penicillin G was effective for treating gonorrhea for over 40 years, but was discontinued in 1986 as the recommended treatment due to increasing prevalence of resistant strains. Tetracyclines and fluoroquinolones were similarly withdrawn (in 1986 and 2007, respectively), leaving only the extended-spectrum cephalosporins (ESCs), ceftriaxone and cefixime, and the macrolide azithromycin as recommended treatments (1). In 2010, oral cefixime was removed as a recommended treatment, and supplanted with ceftriaxone and azithromycin as a dual therapy (2). Unfortunately, there have been several recent reports of N. gonorrhoeae strains exhibiting resistance to both ceftriaxone and azithromycin, raising fears that untreatable gonorrhea will emerge in the near future (3, 4).

A sentinel strain in the emergence of cephalosporin-resistant (CephR) N. gonorrhoeae was H041, which was isolated in Japan from a pharyngeal infection in a female sex worker (5). The minimum inhibitory concentrations (MICs) of ceftriaxone and cefixime for H041 are 2 and 8 μg/ml, respectively, well above the breakpoints (>0.25 μg/ml) for these antibiotics (6). H041 is also resistant to many other antibiotics, including azithromycin, fluoroquinolones, and tetracycline, and is therefore considered the first pan-resistant strain of N. gonorrhoeae. A second CephR strain termed F89 emerged later in France and again in Spain (7, 8). Similar to H041, this strain also exhibits resistance to several classes of antibiotic in addition to ESCs.

N. gonorrhoeae utilizes a number of mechanisms to elevate resistance to β-lactams, including having mutated alleles of penA, mtrR, penB, and penA, along with an unknown determinant (9–12). All these mechanisms contribute to resistance, but the key difference in CephR strains is acquisition of a mosaic penA allele containing over 60 amino acid mutations compared with penA alleles from either penicillin-resistant (e.g. FA6140) or antibiotic-susceptible strains (e.g. FA19) (6). penA encodes penicillin-binding protein 2 (PBP2), an essential peptidoglycan transpeptidase that catalyzes the formation of peptide cross-links between glycan strands via an acyl-enzyme transpeptidation mechanism during the latter stages of peptidoglycan synthesis (13). ESCs, and indeed all β-lactam antibiotics, mimic the cell-wall peptide and form a long-lived acyl-enzyme complex with the PBP, thus preventing transpeptidation and leading to loss of cell viability. The transpeptidase (TPase) domain in PBP2 contains the archetypal active-site

This work was supported by National Institutes of Health Grants GM066861 (to C. D.) and U19 AI13170 (to R. A. N.). The authors declare that they have no conflicts of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

This article contains Figs. S1–S11 and Table S1.

The atomic coordinates and structure factors (codes 6P52, 6PS3, 6PS4, and 6PS5) have been deposited in the Protein Data Bank (http://wwpdb.org/).

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‡ The abbreviations used are: ESC, extended-spectrum cephalosporin; CephR, intermediate cephalosporin-resistant; CephR, cephalosporin-resistant; MIC, minimum inhibitor concentration; PBP, penicillin-binding protein; TPase, transpeptidase; tPBP2, truncated construct of penicillin-binding protein 2; RMSD, root mean square deviation; CHES, 2-(cyclohexylamino)-ethanesulfonic acid.
motifs for PBPs: SXXX, SXN, and KTG motifs, where X signifies variable residues (for PBP reviews, see Refs. 14 and 15). A key residue in PBP2 is Ser-310 in the SXXX motif because this is the active-site nucleophile that reacts with both peptide substrates and β-lactam antibiotics to form an acyl-enzyme complex.

Previously, we reported that allelic replacement of the penA allele in FA19 with the mosaic penA allele from H041 (penA41) can transform the penicillin- and cephalosporin-susceptible strain FA19 to full resistance for cefixime and ceftriaxone in the absence of any other resistance determinants (16). In addition, we showed that among the 60 or so mutations present in the mosaic PBP2 variants, a subset comprising I312M, V316T, G545S, and N512Y are responsible for much of the elevated cephalosporin resistance in N. gonorrhoeae strain (17). Compared with penA35, penA41 encodes 13 new or different amino acid mutations. Of these, three mutations (A311V, V316P, and T483S) contribute to the increased resistance to ESCs conferred by penA41 above that conferred by penA35 (16). Together, these mutations increase the MICs of cefixime and ceftriaxone for H041 by lowering the rates of acylation of PBP2 by up to 12,000-fold (16) and firmly establish PBP2 as the prime determinant of cephalosporin resistance in N. gonorrhoeae.

Against the background of increasing resistance to ESCs and verified treatment failures of ceftriaxone and cefixime against N. gonorrhoeae strains, it is crucial to understand how PBP2 interacts with these antibiotics and how mutations in PBP2 lower acylation rates while retaining sufficient transpeptidase activity to support viability. Using a construct comprising the TPase domain of PBP2, we present crystal structures of the TPase construct of PBP2 acylated with ceftriaxone or cefixime, as well as structures in the apo form and bound by phosphate in the active site. These structures reveal conformational changes within the active site and the surrounding region elicited by acylation. We propose that twisting of the β3 strand in the active site, along with rolling of the β3–β4 loop, are critical components of acylation, and that mutations associated with cephalosporin resistance hinder these conformational changes.

Results

Structure of the TPase domain of two forms of PBP2

We reported previously that PBP2 lacking its N-terminal domain and 15 residues from a protruding loop (hereafter referred to as tPBP2) exhibits essentially identical $k_e/k_s$ acylation rates for penicillin G and other antibiotics as the full-length protein (18). To establish whether any structural changes occur when the TPase domain of PBP2 is expressed independently of the N-terminal domain and for the purposes of comparison with acylated structures, we determined the crystal structure of tPBP2. Crystals were obtained in two crystal forms in the P2₁ space group, termed tPBP2APO and tPBP2PO₄, depending on whether phosphate ions were observed in the structure (the former form was obtained from tPBP2 prepared in Tris buffer, the latter from protein prepared in phosphate buffer; see “Experimental procedures”). Both forms crystallized with two molecules in the asymmetric unit, but with slightly different cell dimensions. The two molecules in the asymmetric unit of the tPBP2APO structure superimpose on each other with a root mean square deviation (RMSD) of 1.0 Å between common Cα atoms (Fig. S1A), whereas the two molecules of the tPBP2PO₄ structure superimpose on each other with RMSD of 0.94 Å (Fig. S1B). The data collection and model refinement statistics are shown in Table 1.

As expected, the structure of tPBP2APO superimposes closely with the TPase domain of full-length PBP2 (19), including the active-site residues (Fig. S2, A and B). The RMSD is 0.69 and 1.07 Å for molecules A and B of tPBP2APO, respectively, for common Cα atoms with the corresponding molecules of full-length PBP2, and differences between the two structures are minimal. The only major difference is that there is electron density corresponding to the β3–β4 loop for molecule B of tPBP2APO, whereas density for this region is lacking in both molecules of full-length PBP2.

The tPBP2PO₄ structure is so termed because it contains a phosphate ion bound in the active site of the enzyme. The phosphate lies between α2 (containing the SXXX motif) and β3 (containing the KTG motif) and replaces a water molecule observed at this position in the tPBP2APO structure (Fig. 1A) (for nomenclature of secondary structure in PBP2, see Ref. 19). The phosphate is within hydrogen-bonding distance of the side chain hydroxyls of Ser-310, Ser-362, Thr-498, and Thr-500, with an additional hydrogen bond to the main chain amide of Gly-546 via a bridging water oxygen (Fig. 1B). A sulfate occupying a similar position as the phosphate has been observed in the crystal structure of Streptococcus pneumoniae PBP2x (20) and also a sulfonic acid group (as part of a MES molecule) in the structure of PBP1a, also from S. pneumoniae (21).

A phosphate bound in the active site elicits a conformational change in β3

The tPBP2APO and tPBP2PO₄ structures superimpose upon each other with an RMSD of 0.95 Å between A molecules (Fig. S3A) and 0.66 Å between B molecules (Fig. S3B), indicative of overall similar structures, but with changes evident in the β-hairpin region (β2a–β2d) and the α1–β1 and β5–α11 loops. In contrast to tPBP2APO, the β3–β4 loop is disordered in both molecules of the asymmetric unit of tPBP2PO₄. The comparison also shows that in the active site of tPBP2PO₄, the side chain of Thr-498 (the second residue of the KTG motif) has rotated away from the main chain amide of Gly-546 to form a new hydrogen bond with the phosphate (Fig. 1C). In tandem, the KTG motif of β3 has twisted toward the active site and there is a similar movement of β4 alongside, albeit of lesser magnitude. The different rotamers for Thr-498 in the two structures are clearly supported by unbiased difference electron density (Fig. S4). Far from being an anomaly, the conformational change elicited by the phosphate has implications for the mechanism of acylation, as will be discussed later.

Acylation structures of tPBP2 with extended-spectrum cephalosporins

tPBP2 acylated by cefixime—The extended-spectrum cephalosporin cefixime is composed of a β-lactam ring fused with a
Crystal structures of PBP2 acylated by cephalosporins

Table 1
Data collection and crystallographic refinement statistics for tPBP2 structures

|                | PBP2<sup>APSO</sup> | PBP2<sup>POS</sup> | PBP2<sup>CFX</sup> | PBP2<sup>CRO</sup> |
|----------------|----------------------|---------------------|---------------------|---------------------|
| Data collection|                      |                     |                     |                     |
| Space group    | P<sub>2</sub>        | P<sub>2</sub>        | P<sub>2</sub>        | P<sub>2</sub>        |
| Cell dimensions: a, b, c (Å) and β (°) | 45.3, 77.3, 88.0, 91.8 | 42.0, 76.9, 86.5, 92.3 | 44.5, 78.0, 86.9, 90.5 | 43.2, 78.5, 87.6, 91.3 |
| Resolution range (Å) | 36.09–1.92 | 38.35–1.83 | 44.49–1.74 | 35.01–1.83 |
| R<sub>merge</sub> (%) | 5.2 (41.3) | 7.1 (93.2) | 8.5 (38.3) | 8.7 (64.3) |
| Completeness (%) | 90.8 (97.3) | 99.6 (99.2) | 99.9 (99.0) | 98.8 (97.2) |
| Redundancy | 6.3 (5.4) | 6.8 (6.5) | 7.5 (6.7) | 5.2 (3.9) |
| No. of non-hydrogen protein atoms | 4,913 | 4,788 | 5,005 | 4,993 |
| No. of water/phosphate/PEG molecules | 218/1/0 | 166/2/0 | 373/3/3 | 210/0/1 |
| No. of unique reflections | 42,141 (2,272) | 48,725 (2,394) | 60,961 (3,014) | 50,936 (2,469) |
| Refinement |                      |                     |                     |                     |
| Resolution (Å) | 36.09–1.92 | 38.35–1.83 | 44.49–1.74 | 35.01–1.83 |
| No. of non-hydrogen protein atoms | 4,913 | 4,788 | 5,005 | 4,993 |
| No. of water/phosphate/PEG molecules | 218/1/0 | 166/2/0 | 373/3/3 | 210/0/1 |
| No. of unique reflections | 42,141 (2,272) | 48,725 (2,394) | 60,961 (3,014) | 50,936 (2,469) |
| R<sub>merge</sub> (%) | 0.184/0.229 | 0.197/0.233 | 0.161/0.188 | 0.174/0.210 |
| Mean B factor (main chain) | 32.3 | 36.0 | 28.9 | 31.6 |
| Mean B factor (side chains and waters) | 36.7 | 40.6 | 34.2 | 37.6 |
| Distribution of residues in the Ramachandran plot (%) | 92.6 | 92.8 | 94.6 | 92.6 |
| Most favored region | 6.8 | 6.9 | 5.0 | 6.8 |
| Generously allowed region (%) | 0.2 | 0.0 | 0.0 | 0.2 |
| Disallowed region | 0.4 | 0.4 | 0.4 | 0.4 |
| PDB code | 6P53 | 6P52 | 6P55 | 6P54 |

Contact made by cefixime with tPBP2 comprise a number of hydrogen-bonding interactions (Fig. 2, B and C). One of these is the carbonyl oxygen of the cefixime ester and the main chain amides of Ser-310 and Thr-500, which together comprise the oxanion hole formed during the reaction with both penicillin (24, 25), but the electron density in molecule A indicates that the leaving group has remained attached even though acylation has completed (as indicated by the covalent bond between Ser-310 and cefixime). This suggests that an intermediate stage has been trapped in molecule A of the asymmetric unit, whereas the reaction has proceeded to completion in molecule B. The C3 dioxo-triazine ring packs between Tyr-544 and Tyr-543 (Fig. 3B), with an imperfect stacking arrangement with Tyr-544. The retention of the leaving group in molecule A may result from a symmetry contact, where Arg-258 from a symmetry-related molecule in the crystal lattice interacts with one of the oxygens of pyridine ring (Fig. S5), and such trapping may have impeded its elimination during acylation. This symmetry interaction is not observed in the B molecule. To the best of our knowledge, this is the first time the leaving group of a cephalosporin has been observed in an acylated-PBP structure.

six-membered dihydrothiazine ring, an allyl group at the C3 position, a carboxylate at the C4 position, and an R1 group at the C7 position comprising an aminothiazole ring and carboxymethyl oxime side chain (Fig. 2A). The crystal structure of tPBP2 acylated by cefixime (tPBP2<sup>CFX</sup>) was determined at 1.7 Å resolution by soaking crystals of tPBP2<sup>APSO</sup> with the antibiotic (Table 1). An unbiased difference electron density map clearly identifies the covalent bond between C8 of cefixime and Ser-310 in both molecules of the asymmetric unit (Fig. 2A). The crystal structure of tPBP2 acylated with ceftriaxone (tPBP2<sup>CRO</sup>) was solved at 1.8 Å resolution by soaking crystals of tPBP2<sup>APSO</sup> (Table 1). Like the cefixime-acylated structure, unbiased electron density shows ceftriaxone covalently bound to Ser-310 in both molecules of the asymmetric unit (Fig. 3, B and C). The electron density for ceftriaxone is generally similar in both molecules of the asymmetric unit, except for being slightly weaker overall in molecule B.

When comparing molecules of the asymmetric unit, a striking difference in the electron density for ceftriaxone is the presence of the C3 dioxo-triazine ring (R2 group) in molecule A. For ceftriaxone, this group is normally eliminated during acylation (24, 25), but the electron density in molecule A indicates that the leaving group has remained attached even though acylation has completed (as indicated by the covalent bond between Ser-310 and ceftriaxone). This suggests that an intermediate stage has been trapped in molecule A of the asymmetric unit, whereas the reaction has proceeded to completion in molecule B. The C3 dioxo-triazine ring packs between Tyr-544 and Tyr-543 (Fig. 3B), with an imperfect stacking arrangement with Tyr-544. The retention of the leaving group in molecule A may result from a symmetry contact, where Arg-258 from a symmetry-related molecule in the crystal lattice interacts with one of the oxygens of pyridine ring (Fig. S5), and such trapping may have impeded its elimination during acylation. This symmetry interaction is not observed in the B molecule. To the best of our knowledge, this is the first time the leaving group of a cephalosporin has been observed in an acylated-PBP structure.
A consequence of the C3 leaving group of ceftriaxone remaining in molecule A of the structure is a difference in the configuration of the dihydrothiazine ring. In molecule A, the dihydrothiazine ring remains in an endocyclic methylene form, but in molecule B, where the C3 leaving group has been eliminated, the ring has rearranged to the exocyclic form (Fig. 3).

Figure 1. The structures of \(N. \) gonorrhoeae tPBP2\(^{Apo}\) and tPBP2\(^{PO4}\). \(A\), the overall structure of tPBP2\(^{Apo}\) (light blue) where secondary structures are labeled according to Powell et al. (19). A water molecule in the active site is shown as a red sphere. The inset shows a close-up of the active site region and electron density for the water molecule. \(B\), the overall structure of tPBP2\(^{PO4}\) (green) containing a phosphate bound to a similar position as the water molecule in the apo structure. The inset shows the electron density for the phosphate and for a bridging water molecule between the phosphate and the main chain amide of Gly-546. For \(A\) and \(B\), unbiased electron density maps for phosphate and waters are contoured at 3.0 \(\sigma\). Unbiased here and elsewhere signifies maps calculated before inclusion of ligand atoms or water molecules in the model. \(C\), superimposition of the tPBP2\(^{Apo}\) and tPBP2\(^{PO4}\) structures, showing conformational changes elicited by the phosphate, including twisting of \(\beta3\) and alteration of the Thr-498 rotamer. Potential hydrogen bonds whose distance is in the range of 2.6 –3.2 \(\AA\) are shown as dashed lines.

Figure 2. The structure of tPBP2 acylated by cefixime. \(A\), chemical structure of cefixime (CFX) in which the R1 and R2 groups are labeled. Note the absence of a large R2 substituent. \(B\) and \(C\), interactions made by cefixime (orange bonds) in the active site of tPBP2 for molecule A (panel B) and molecule B (panel C) of the asymmetric unit. \(\left| F_o \right| - \left| F_c \right|\) unbiased electron density maps corresponding to CFX and a water molecule are contoured at 2.8 \(\sigma\). The protein is colored cyan and potential hydrogen bonded interactions are shown as dotted lines. \(D\), superimposition of CFX from molecules A and B of the asymmetric unit.

A consequence of the C3 leaving group of ceftriaxone remaining in molecule A of the structure is a difference in the configuration of the dihydrothiazine ring. In molecule A, the dihydrothiazine ring remains in an endocyclic methylene form, but in molecule B, where the C3 leaving group has been eliminated, the ring has rearranged to the exocyclic form (Fig. 3D).
For molecule A, the R2 leaving group is interactions made by ceftriaxone in the active site of tPBP2 for molecule A (panel B, purple bonds) and molecule B (panel C, pink bonds) of the asymmetric unit. For molecule A, the R2 leaving group is circled in red. (PDB 3–H9251) unbiased electron density maps corresponding to CRO and a water molecule are contoured at 3.0σ. The protein is colored yellow and potential hydrogen bond interactions are shown as dotted lines. D, superimposition of CRO from molecules A and B of the asymmetric unit showing endocyclic and exocyclic forms of the dihydrothiazine ring according to the molecule.

Figure 3. The structure of tPBP2 acylated by ceftriaxone. A, chemical structure of ceftriaxone (CRO) in which the R1 and R2 groups are labeled. B and C, interactions made by ceftriaxone in the active site of tPBP2 for molecule A (panel B, purple bonds) and molecule B (panel C, pink bonds) of the asymmetric unit showing. For molecule A, the R2 leaving group is absent in molecule A of both structures. Across most of the molecules A and B, respectively. The higher RMSD for the B molecule superimposition reflects the large structural changes in the β3–β4 loop, which is mostly ordered in molecule B (Fig. S6), but absent in molecule A of both structures. Across most of the PBP2 molecule, conformational changes elicited by acylation with cefixime are relatively few and most occur in the active-site region (Fig. 4A). The most notable change is rearrangement of the β3–β4 loop in the acylated structure, comprising both twisting of the loop and rolling toward the active site (Fig. 4B). A major consequence of this shift is a striking movement of Tyr-509 from the apex of this loop distant from the active site in the apo structure to form part of a cluster of side chains near the R1 group of cefixime (Fig. 4C). This amounts to a 12-Å shift of its Ca atom and 22 Å for its hydroxyl group. In its new position, the side chain hydroxyl of Tyr-509 is within hydrogen bonding distance of Glu-307, which resides on the loop preceding the SXXK motif. Tyr-422 is also part of this cluster and has shifted from forming a hydrogen bond with Thr-500 in the tPBP2apo structure to contacting Arg-502. By shifting, it avoids a steric clash with the R1 group of cefixime and in its new position, stacks against the aminothiazole ring of cefixime. Asp-511 also moves over 10 Å to contact Arg-502. Overall, the grouping of Tyr-422, Tyr-509, Asp-511, Glu-307, and Arg-502 appears to occlude the active site when cefixime is bound.

In addition to the β3–β4 loop, β3 itself also twists toward cefixime and in so doing, repositions the KTG motif (Fig. 4D). This brings the amide nitrogen of Thr-500 to within hydrogen bonding distance (3.1 Å) of the cefixime carboxyl, an interaction that is necessary for correct formation of the oxyanion hole that stabilizes the negative charge of the oxygen during acylation. Thr-498 has also moved toward cefixime by rotating ~35 degrees about the β3 axis and in tandem, its side chain has also rotated. The new rotamer position results in loss of a hydrogen bond with Gly-546 and gain of hydrogen bonds with the cefixime carboxylate and with Thr-483 on the α10–β3 loop. A further consequence of the reorientation of Thr-498 is displacement of a water molecule, which now hydrogen bonds with the hydroxyl groups of Thr-498 and Thr-483, as well as the side chain amino group of Lys-497 and the main chain carbonyl of Met-359 (Fig. S7).

Another structural difference in the tPBP2apo structure compared with its apo equivalent is movement of Tyr-543 and Tyr-544, both of which reside on the β5–α11 loop. In the acylated structure, both tyrosines move slightly closer to the active site (Fig. 4B). The movement of the Tyr-544 side chain is more pronounced when comparing the B molecules of the asymmetric unit rather than molecule A (Fig. S8). Additionally, the electron density for this part of the α10–β3 loop is weaker in the apo structure rather than when acylated by cefixime.
Ceftriaxone elicits conformational changes in tPBP2apo similar to those by cefixime

The RMSD values for superimpositions of the tPBP2apo structure with tPBP2apo are 0.7 and 1.7 Å for molecules A and B, respectively. Similar to tPBP2cfx, molecule B has a higher RMSD value due to changes in the β3–β4 loop. This superimposition paints a very similar view as the tPBP2apo versus tPBP2cfx comparison, because essentially the same conformational changes are observed with cefixime (Fig. 5). Thus, the same striking movement in β3–β4 toward the active site occurs to form the cluster of residues around the aminothiazole ring of cefixime, β3 twists to reposition the KTG motif to form the oxyanion hole, and the rotamer of Thr-498 is altered. Tyrosines 543 and 544 also move in a similar way as in the cefixime-acylated structure. Again, the movement of Tyr-544 is more pronounced in molecule B of the asymmetric unit and occupies a position slightly closer to the antibiotic compared with cefixime (Fig. S8). Finally, the two acylated structures superimpose very closely with each other (RMSD values are 0.3 and 0.5 Å for molecules A and B, respectively), indicating that they both converge to essentially the same conformational state after acylation (Fig. 6).

Binding of phosphate alone elicits the acylated conformational state

When comparing the structure of tPBP2po4 with the two acylated structures, it is clear that all three overlap very closely with RMSD values of ~1.0 Å between structures (Fig. 7). The convergence of these structures shows that the phosphate alone can elicit the same conformational changes associated with the acylated state, including twisting of β3 to form the oxyanion hole, rotation of the Thr-498 side chain, and movement of the β5–α11 loop. Excluded from these changes is rearrangement of the β3–β4 loop because this is disordered in both molecules of the tPBP2po4 structure. The spatial overlap of the phosphate with the β-lactam carboxylates of both cefixime and ceftriaxone (Fig. 7, inset) strongly suggests that recognition of the cephalosporin carboxylate is primarily responsible for the conformational changes in the active site that precede acylation. The carboxylate group is a common feature of all β-lactams and its negative charge is likely responsible for the shift.

tPBP2-T498A exhibits markedly lower reactivity with Bocillin-FL

The change in side chain rotamer and hydrogen bonding interactions as a result of acylation led us to test whether Thr-498 is necessary for acylation using the fluorescent penicillin, Bocillin-FL (28). SDS-PAGE gels show that a T498A mutant of tPBP2 is labeled only weakly by Bocillin-FL compared with the WT construct (Fig. S9). Moreover, the T498A mutant was crystallized and its structure does not exhibit any changes aside from the absence of the threonine side chain (Fig. S9 and Table S1). Hence, it appears that Thr-498 is required for efficient...
acylation, as would be expected from its key involvement in the conformational changes in the active site.

**Discussion**

The emergence of isolates of *N. gonorrhoeae* exhibiting resistance to ESCs is a major threat to public health and addressing the problem requires an understanding of the molecular mechanisms underpinning resistance. To reveal how mutations confer resistance, however, it is first necessary to understand how PBP2 recognizes and reacts with ESCs. We have taken a major step toward this goal by solving crystal structures of PBP2 with the two cephalosporins used to treat gonorrhea and using these structures to understand the molecular mechanism of acylation. Our data suggest that ligand-induced conformational changes are required for PBP2 to undergo efficient acylation and also suggest how specific mutations confer resistance. Mapping conformational changes that result from acylation provides the first indication of a molecular mechanism. We find that a number of important conformational changes occur in the active site of PBP2 as a result of acylation by ceftriaxone or cefixime. The most important of these appears to be twisting of β3 toward the active site. This orients the KTG motif such that the amide nitrogen of Thr-500 is positioned properly to form the oxyanion hole along with the amide of Ser-310. The geometry of the oxyanion hole, including the distance to the tetrahedral oxyanion, is critical for rate enhancement and, although the transition state cannot be seen in the post-covalent state, the amides are within hydrogen bonding distance of the ester linkage carbonyl of ceftriaxone and cefixime in the crystal structures. In the absence of β3 twisting, the geometry of the transition state would be suboptimal, with a negative impact on the rate of acylation. The β3 strand also undergoes transitions after acylation by antibiotics in other PBP structures, including *Staphylococcus aureus* PBP2a (29, 30), *Enterococcus faecalis* PBP4 (31), and the *Actinomadura* R39 DD-peptidase (32). In some of these, β3 appears to twist outwards from the active site when acylated by β-lactams rather than inward (30, 31). By creating an energetic barrier against acylation, the twisting of β3 in *S. aureus* PBP2a has been suggested to be a mechanism for lowering reactivity with β-lactams that contributes to resistance (29). However, the observation of similar transitions occurring in nonmutated PBPs from susceptible organisms suggests it is more likely a facet of the PBP reaction. The twisting of β3 to bring the KTG motif toward the active site of PBP2 begs the question as to how binding of cephalosporin elicits this conformational change because, without knowing its trigger, it will be difficult to understand the effect of resistance mutations. The fortuitous binding of a phosphate ion in the active site of tPBP2PO4 suggests that recognition of the β-lactam carboxylate is the key event. There are two lines of evidence to support this assertion. First, the conformational changes observed in the active site of the phosphate-bound structure (tPBP2PO4) are essentially the same as those seen in...
both the cefixime- and ceftriaxone-acylated structures when compared with tPBP2APO, showing that the tPBP2PO4 structure is representative of an acylated conformation. Second, the phosphate binds at a position that overlaps closely with the carboxylate of the β-lactams (Fig. 7). Taken together, these data are consistent with recognition of the carboxylate being the primary signal for conformational change in the KTG motif when PBPs bind and react with β-lactam antibiotics. This can be thought of as an induced-fit mechanism, but it is also possible that binding of phosphate selects the acylation-competent state from a population of two or more states.

Based on the structural data for PBP2PO4 and the two acylated structures, we propose that recognition of the carboxylate being the key event responsible for the twisting of β3. In PBP2APO, the side chain of Thr-498 projects away from the active site and forms a hydrogen bond with the amide nitrogen of Gly-546 (Fig. 1A). When a cephalosporin binds PBP2, the side chain of Thr-498 rotates, so that the hydrogen bond with Gly-546 is broken and a new bond is formed with the cephalosporin carboxylate (Figs. 2 and 3). Although the temporal sequence of events cannot be ascertained unambiguously in the post-covalent state, it is reasonable to speculate that the new hydrogen bond between Thr-498 and the β-lactam carboxylate triggers rotation of β3 and subsequent movement of the KTG toward the active site (or if the mechanism is conformational selection, traps β3 when twisted toward the active site). Consistent with its central role in the conformational change, a side chain hydroxyl (i.e., threonine or serine) is invariant at this position in all active-site serine-based PBPs. Moreover, tPBP2 containing a T498A mutation reacts much slower with Bocillin-FL than its WT counterpart and a crystal structure of the mutant shows no structural perturbations other than the loss of the side chain (Fig. S9).

In addition to the KTG motif, there are other conformational changes associated with acylation in PBP2. The most significant of these is rolling of the β3–β4 loop from a solvent-exposed extended conformation in PBP2APO to a position where the loop appears to occlude the active site. This transition occurs in both acylated structures. This is the first time the β3–β4 loop has been observed in a PBP occupying two distinct conformations where both states are ordered in the crystal structure. In its new closed position in the acylated structures, the loop forms a network of interactions around the R1 group of the cephalosporin involving Asp-511, Tyr-509, Arg-502, Glu-307, and Tyr-422 (Fig. 4). These interactions may contribute to the specificity for cephalosporins (33), but it is also possible that occlusion of the active site promotes the acylation mechanism.

In tPBP2, the transition in the β3–β4 loop seems to be elicited by acylation because it occurs in both ceftriaxone- and ceftriaxone-acylated structures. However, in the structure of Acinetobacter baumannii PBP3, the β3–β4 loop adopts a conformation similar to acylated tPBP2 even though the enzyme is in apo form (34). This suggests that the β3–β4 loop can occupy both open and closed states when the PBP is in the apo form and that the closed form predominates after acylation. This is in concordance with the inherent flexibility of the β3–β4 loop that is a common feature of PBPs, including PBP2. For instance, in the crystal structure of PBP2 derived from the penicillin-resistant strain FA6140, the β3–β4 loop is ordered in one molecule of the asymmetric unit, but disordered in the other (18).

Similar variations in degree of order and conformation of this loop are also observed in other PBPs, such as PBP1a, PBP2b, and PBP2x of S. pneumoniae, PBP3 of P. aeruginosa, and PBP1a of A. baumannii (21, 34–37). Given the involvement of crystal packing interactions in this region, interpretation of changes in the β3–β4 loop can be uncertain, especially when interpreting the impact of mutations implicated in antibiotic resistance. Nevertheless, plasticity of the β3–β4 loop is consistent with the apparent role it plays in acylation of PBP2 by cephalosporins, as seen in the tPBP2CFX and tPBP2CRO structures.

Another interesting facet of the tPBP2 structures is the mobility of the β5–α11 loop, which is generally closer to the active site in both acylated structures compared with the apo structure. In molecule A of the tPBP2CRO structure, Tyr-544 stacks against the dioxytriazine leaving group of ceftriaxone, which has remained attached to the antibiotic. These two tyrosines are highly conserved in PBPs from Gram-negative
bacteria that are involved in cell division (FtsI homologs), alongside two conserved glycines (Fig. S10). Similar flexibility of this region has also been observed in other PBP structures (26, 27, 34, 38). In P. aeruginosa PBP3 (a homolog of N. gonorrhoeae PBP2), the equivalent residues (Tyr-532 and Phe-533) along with Phe-503, have been characterized as an aromatic “wall” that accommodates the gem-dimethyl group of cefazidime (26). Such a group is lacking in both ceftriaxone and cefixime, however. It is interesting to note that Tyr-544 also moves in the PBP2\textsuperscript{CFX} structure, even though cefixime lacks a C3 leaving group. Hence, mobility of this region may be associated with transpeptidase activity rather than being an important factor in the reaction with cephosphorins.

Whether the conformational changes elicited by ESCs also occur during the natural peptidoglycan cross-linking reaction is an interesting question to consider. Of the three major shifts observed, the most likely to occur during transpeptidation is twisting of β3 because the β-lactam carboxylate that elicits this change mimics the C-terminal carboxylate of the peptide substrate. For the reason given above, the shift of the β5–α11 loop may also occur during peptidoglycan cross-linking, and given its proximity with the R2 side of the cephosphorin, it seems more likely this would involve the acceptor peptide, rather than the donor. Finally, the situation with the β3–β4 loop is less clear. The interactions this loop makes with the R1 region of ESCs appear highly specific and yet it is very likely that inherent flexibility of the loop is important for transpeptidase activity. Clarity on these questions must await structural information showing PBPs in complex with peptide substrates.

The structures of PBP2 in complex with ceftriaxone and cefixime afford the first interpretation of mutations associated with Ceph\textsuperscript{R} in the context of acylated structures. There are 61 mutations in PBP2 from the Ceph\textsuperscript{R} strain H041 and it has been established previously that at least six of these are important for conferring resistance. Three mutations were identified in the penA35 allele from the Ceph\textsuperscript{R} strain 35/02 (17) and an additional three were identified in penA from H041 (16). Mapping these mutations onto the structure of PBP2 acylated by ceftriaxone shows that they are distributed around the binding site, although none make direct contact with the antibiotic (Fig. 8). Three mutations (V316P, I312M, and A311V) are found on β2, which contains Ser-310 at its N-terminal end, but none are particularly near ceftriaxone. The side chain of Val-311 is closest, but lies on the opposite side of the helix to Ser-310.

The T483S mutation resides on the α10–β3 loop but again, does not contact ceftriaxone directly. However, it occupies a position where it can hydrogen bond with Thr-498 via its side chain hydroxyl and, given the important role that Thr-498 appears to play in the mechanism of acylation, could impact reactivity with cephalosporins. In the acylated state, Thr-498 also hydrogen bonds with Ser-362 of the SXN motif and Lys-497 of the KTG motif. However, it is worth noting that Thr-483 is a serine in PBP2 from H041 and thus should also be capable of forming the same hydrogen bond with Thr-498. This makes the role of this residue in cephalosporin resistance unclear at the present time.

Another mutation known to contribute to cephalosporin resistance is N512Y, which is present on the β3–β4 loop (17). As noted above, this region undergoes a major transition between apo and acylated states and it is possible that mutation of Asn-512 to tyrosine hinders the conformational change. Some PBPs contain mutations in the β3–β4 loop that are associated with resistance (39, 40) and if similar transitions occur when these proteins are acylated by β-lactams, they may act in a similar way.

Finally, the Ceph\textsuperscript{R} mutation that appears to have the most obvious consequence is G545S. In WT PBP2, Gly-545 lies on the β5–α11 loop close to Thr-498 of the KTG motif. Given the central role that Thr-498 likely plays in the mechanism of acylation, it is probable that the hydroxyl group introduced by the G545S mutation will form a hydrogen bond with the hydroxyl of Thr-498, thus hindering rotation of the latter’s side chain after binding of the cephalosporin. In turn, this may make rotation of β3 more difficult and impede formation of the oxyanion
hinder the acylation process by increasing the energetic cost required for formation of the transition state.

The probable mechanism of the G545S mutation is reminiscent of mutations at position 501 in PBP2 that are associated with cephalosporin resistance (41–43). This position constitutes the transition point between β3 and the β3–β4 loop. The crystal structure of an A501T mutant has been solved and, similar to G545S, the side chain hydroxyl of Thr-501 forms a new hydrogen bond (with Glu-307 prior to the SXXK motif) that would be expected to reduce flexibility in this region (44). Such similarity in mechanisms at different positions in the protein may be the beginning of a consistent pattern emerging to explain cephalosporin resistance mediated by mosaic variants of PBP2.

The key role of the β-lactam carboxylate in the acylation mechanism of PBP2 has implications for drug design of new antimicrobials that target PBP2 and potentially other PBPs. As noted, the cephalosporin carboxylate appears responsible for the twisting of β3 to form the oxyanion hole in PBP2 and the presence of a carboxylate or sulfate is a common feature of β-lactams and also of some new antimicrobials under development, including diazabicyclooctanes (45, 46). Similarly, other compounds under development include boronate groups at the equivalent position (47). The apparent impairment of β3 twisting in PBP2 by the G545S mutation suggests that the efficacy of an antimicrobial drug candidate containing a carboxylate or similar group may be compromised by the same resistance mechanism. This would also apply to other PBPs if twisting of β3 is also a requirement for efficient acylation. This being the case, then new antimicrobials directed against resistant strains should avoid inclusion of a carboxylate or equivalent. It is interesting to note that neutral β-lactams lacking the carboxylate hole required for acylation. Such loss of flexibility in β3 would hinder the acylation process by increasing the energetic cost required for formation of the transition state.

Crystal structures of PBP2 acylated by cephalosporins

Previously, we have reported the cloning of a truncated construct of PBP2 (here termed tPBP2) comprising only the TPase domain of WT PBP2 (18) derived from FA19, a penicillin- and cephalosporin-susceptible strain of N. gonorrhoeae (49). This construct has essentially identical $k_c/K_s$ acylation constants as full-length PBP2 for a range of antibiotics. tPBP2 is expressed as a fusion with maltose-binding protein, separated by a tobacco etch virus protease site and with a His$_6$ tag at its N terminus. Nucleotides encoding amino acids 283–297 were excluded from the construct, because in the crystal structure of full-length PBP2, these form a loop that projects from the TPase domain (19) and was predicted to hinder crystallization. Residues Pro-282 and Arg-298 are connected with a nonnative glycine linker.

After transformation of the WT construct into E. coli BL21 (DE3) cells, 2 liters of cell culture were grown at 37 °C. Protein expression was induced by addition of 0.3 mM isopropyl-β-D-thiogalactoside, followed by overnight incubation at 20 °C. After centrifugation, cells were lysed in 20 mM Tris-HCl, pH 8.0, 500 mM NaCl, and 10% glycerol (TNG buffer) and the supernatant was passed over a 5-ml HisTrap™ FF Ni$^{2+}$-affinity column (GE Healthcare). After elution with 0–500 mM imidazole gradient in TNG, the fusion protein was concentrated and digested with His$_6$-tagged tobacco etch virus protease at a molar ratio of 50:1, followed by overnight dialysis at 4 °C against TNG. The digested protein was then passed over a 5-ml HisTrap™ HP Ni$^{2+}$-affinity column, washed with TNG, and tPBP2 was eluted by a TNG, 15 mM imidazole step gradient. The protein was then dialyzed overnight against 50 mM phosphate, pH 7.1, with 10% glycerol and loaded onto 5-ml HiTrap™ SP FF ion exchange column (GE Healthcare) equilibrated in the same buffer. The protein was eluted by a 0–500
Crystal structures of PBP2 acylated by cephalosporins

mm NaCl linear gradient in the same buffer and tPBP2-containing fractions were pooled. The protein was concentrated to 37 mg/ml using a 10-kDa cut-off Amicon concentrator (Millipore, Sigma) at 4 °C. The concentrated protein was flash frozen in liquid nitrogen and stored at −80 °C.

Crystallization

Prior to crystallization trials, tPBP2 was diluted to 13 mg/ml in two buffers: TNG or PNG (50 mM phosphate, pH 7.1, 10% glycerol, 180 mM NaCl). Crystallization trials were performed in 96-well sitting drop format using a Gryphon liquid dispensing system (Art Robbins, Sunnyvale, CA) in which 200 nl of protein was mixed with 200 nl of well solution. The protein was screened against a number of sparse matrix screens, including JCSG Cores I-III and Suite + (Qiagen) with incubation at 21 °C. A number of crystallization hits were obtained and all were in solutions containing polyethylene glycol (PEG). After optimization, diffracting crystals of tPBP2 (diluted in either TNG or PNG buffers) were obtained after 3–4 weeks at 18 °C in 35–40% PEG 600 and 0.1 M CHES buffered at pH range 9.0–9.7 in both hanging and sitting drop configuration.

Apo- and phosphate-bound structures

Crystals of tPBP2 were flash-frozen in liquid nitrogen and data were collected at a wavelength of 1.0 Å using the SER-CAT 22-ID beamline at the Advanced Photon Source (APS), Argonne National Laboratory. 360 degrees of data were collected on a Rayonix MX300 HS detector in 0.5-degree oscillations with 0.3-s exposures with a crystal-to-plate distance of 160 mm. Data were processed and scaled with HKL2000 (50). tPBP2APO was solved by molecular replacement using PHASER (51) in which the search model was the crystal structure of the Tpase domain of PBP2 derived from the penicillin-resistant strain FA6140 (PDB 4U3T) (18). Data were also collected from crystals grown using protein diluted in phosphate-containing PNG buffer using the SER-CAT 22-BM beamline. 360 degrees of data were collected using a MAR225 imaging plate detector in 1-degree oscillations with an exposure time of 3 s and a crystal-to-plate distance of 180 mm. This structure (tPBP2PO4) was solved by refining the tPBP2APO structure. Both models were refined with iterative rounds of model building and refinement using the programs COOT (52) and REFMAC (53). The stereochemistry of the models was analyzed with PROCHECK (54).

A nonnative alanine arising from cloning was added to the N termini of both the A and B molecules of the tPBP2APO structure, and residues 504–512, which comprise the β3–β4 loop, could not be modeled in molecule A due to weak or absent electron density. The tPBP2PO4 model begins with Leu-237 in both molecules A and B, and similarly, residues 503–513 (molecule A) and 501–511 (molecule B) of the β3–β4 loop could not be modeled. Leu-447 occupies a disallowed region of the Ramachandran plot in both structures. The electron density for this residue is unambiguous and therefore is a biological outlier.

Acylated structures

Crystals of tPBP2 prepared from protein diluted in TNG buffer were soaked for 5–7 min in 50 mM cefixime or ceftriaxone (Sigma), followed by cryo-freezing as above. For the ceftriaxone complex, data were collected on an EIGER 16M detector at the SER CAT 22-ID beamline. 180 degrees of data were collected in 0.25-degree increments with an exposure time of 0.25 s/frame and a crystal-to-plane distance of 240 mm. For the cefixime complex, data were collected on a Rayonix MX300 HS detector at the SER CAT 22-BM beamline. 180 degrees of data were collected in 1-degree increments with an exposure time of 6 s/frame and the crystal-to-plane distance was 260 mm. In both cases, the starting model for refinement was the structure of tPBP2APO. Antibiotics were modeled based on $|F_o| - |F_c|$ difference electron density maps, followed by iterative cycles of model building and refinement as above.

A number of nonnative residues were modeled at the N termini in both the cefixime- (tPBP2CFX) and ceftriaxone-bound (tPBP2CRO) structures. Gly-235 and Ala-236 were modeled in both molecules of tPBP2CFX, whereas molecule B also includes Gly-234. Similarly, Ala-236 was modeled in both molecules of tPBP2CRO, whereas molecule B has an additional residue, Gly-235. Except for molecule A in the structure of tPBP2CFX, residues 505–507 exhibit comparatively weak density in the acylated structures but are included in the models. Three phosphate molecules are included in the tPBP2CFX model. These are presumed to result from the phosphate buffer used for ion exchange chromatography. None are present in the active site. Equivalent phosphates were not observed in the tPBP2CRO structure. Three molecules of PEG were modeled in the tPBP2CFX structure and one in the tPBP2CRO structure. A molecule of unreacted ceftriaxone was observed the tPBP2CRO structure between molecule B and its symmetry-related counterpart (Fig. S11). The $|F_o| - |F_c|$ electron density map reveals most of the molecule, although density for R2 group is less clear. This binding at a symmetry contact is considered an artifact. As in the tPBP2APO and tPBP2CPO4 structures, Leu-447 is an outlier in the Ramachandran plot. The data collection and final refinement statistics for all structures are given in Table 1.

Author contributions—R. A. N. and C. D. conceptualization; A. S. and C. D. formal analysis; R. A. N. and C. D. supervision; R. A. N. and C. D. funding acquisition; A. S. and C. D. validation; A. S., J. T., R. A. N., and C. D. investigation; A. S. visualization; A. S. and J. T. methodology; A. S. and C. D. writing—original draft; R. A. N., and C. D. project administration; A. S., R. A. N., and C. D. writing—review and editing.

Acknowledgments—Use of the Advanced Photon Source was supported by the United States Department of Energy, Office of Science, Office of Basic Energy Sciences, under Contract W-31-109-ENG-38. Data were collected at Southeast Regional Collaborative Access Team (SER-CAT) 22-ID beamline at the Advanced Photon Source, Argonne National Laboratory. Supporting institutions may be found at www.ser-cat.org/members.html.3 The X-ray crystallography facility used for this work is supported by the Medical University of South Carolina’s Research Resource Facilities program. The liquid handling robot used for crystallization was obtained via an NIH Shared Instrumentation Award S10 RR027139. The authors thank Dr. Shaun Olsen for assistance with the preparation of figures.

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15. Sauvage, E., Kerff, F., Terrak, M., Ayala, J. A., and Charlier, P. (2008) The
penicillin-binding proteins of Neisseria gonorrhoeae strain H041. Antimicrob. Agents Chemother. 57, 3029–3036 CrossRef Medline

14. Macheboeuf, P., Contreras-Martel, C., Job, V., Dideberg, O., and Dessen,
M. G., and Strynadka, N. C. (2012) Structural insights into the anti-meth-
cillin-resistant Staphylococcus aureus (MRSA) activity of cefitobiprole. J.
Biol. Chem. 287, 32096–32102 CrossRef Medline

13. Nicola, G., Peddi, S., Stefanova, M., Nicholas, R. A., Gutheil, W. G., and
Davies, C. (2005) Crystal structure of Escherichia coli penicillin-binding
protein 5 bound to a tripeptide boronic acid inhibitor: a role for Ser-110 in
deacetylation. Biochemistry 44, 477–485 CrossRef Medline

12. Ropp, P. A., Hu, M., Olesky, M., and Nicholas, R. A. (2002) Identification and
analysis of amino acid mutations in porin IB that mediate intermediate-level
resistance to penicillin and tetracycline in Neisseria gonorrhoeae. Antimi-
crob. Agents Chemother. 46, 2811–2820 CrossRef Medline

11. Olesky, M., Hobbs, M., and Nicholas, R. A. (2002) Identification of
mutation in loop 3 of the porin encoded at the penB locus. Antimicrob. Agents
Chemother. 46, 2799–2803 CrossRef Medline

10. Gill, M. J., Simjee, S., Al-Hattawi, K., Robertson, B. D., Easmon, C. S., and
Jison, C. A. (1998) Gonococcal resistance to beta-lactams and tetracycline
involves mutation in loop 3 of the porin encoded at the penB locus. Antimi-
crob. Agents Chemother. 42, 2799–2803 CrossRef Medline

9. Spratt, B. G. (1988) Hybrid penicillin-binding proteins in penicillin-resistant
strains of Neisseria gonorrhoeae. Nature 332, 173–176 CrossRef Medline

8. Cámara, J., Serra, J., Ayats, J., Bastida, T., Carnicer-Pont, D., Andreu, A.,
and Ardanuy, C. (2012) Molecular characterization of two high-level
ceftriaxone-resistant Neisseria gonorrhoeae isolates detected in Catalonia,
Spain. J. Antimicrob. Chemother. 67, 1858–1860 CrossRef Medline

7. Ohnishi, M., Golparian, D., Shimuta, K., Saika, T., Hoshia, S., Iwasaku, K.,
Nakayama, S., and Kitawaki, J., and Unemo, M. (2011) Is Neisseria gonorrhoeae
initiating a future era of untreatable gonorrhoea?: detailed characterization of
the first strain with high-level resistance to ceftriaxone. Antimicrob. Agents
Chemother. 55, 3538–3545 CrossRef Medline

6. Ohnishi, M., Golparian, D., Nicholas, R., Ohnishi, M., Gallay, A., and Sed-
nouai, P. (2012) High-level cefixime- and ceftriaxone-resistant Neisseria
gonorrhoeae in France: novel penA mosaic allele in a successful interna-
tional clone causes treatment failure. Antimicrob. Agents Chemother. 56, 1273–1280 CrossRef Medline

5. Ohnishi, M., Golparian, D., Shimuta, K., Saika, T., Hoshia, S., Iwasaku, K.,
Nakayama, S., Kitawaki, J., and Unemo, M. (2011) Is Neisseria gonorrhoeae
resistant to both ceftriaxone and high-level azithromycin. Lancet Infect. Dis. 18, 717–718 CrossRef Medline

4. Whiley, D. M., Jennison, A., Pearson, J., and Lahra, M. M. (2018) Genetic
caracterisation of Neisseria gonorrhoeae resistant to both ceftriaxone and
azithromycin. Lancet Infect. Dis. 18, 717–718 CrossRef Medline

3. Eyre, D. W., Sanderson, N. D., Lord, E., Begisford-Reimmer, N., Chau, K.,
Barker, L., Morgan, M., Newham, R., Golparian, D., Unemo, M., Crook,
D. W., Peto, T. E., Hughes, G., Cole, M. J., Flier, H., Edwards, A., and
Andersson, M. I. (2018) Gonorrhoea treatment failure caused by a Neis-
seria gonorrhoeae strain with combined ceftriaxone and high-level azi-
thromycin resistance, England, February 2018. Euro Surveill. 23, 10.2807/
1560-7917.ES.2018.23.27.1800323 CrossRef Medline

2. Centers for Disease Control and Prevention (CDC) (2012) Update to
CDC’s sexually transmitted diseases treatment guidelines, 2010: oral
cephalosporins no longer recommended for treatment of gonococcal infec-
tions. MMWR Morb. Mortal. Wkly. Rep. 61, 590–594 Medline

1. Centers for Disease Control and Prevention (CDC) (2007) Update to
CDC’s sexually transmitted diseases treatment guidelines, 2006: fluoro-
quinolones no longer recommended for treatment of gonococcal infec-
tions. MMWR Morb. Mortal. Wkly. Rep. 56, 352–356 Medline

References
Crystal structures of PBP2 acylated by cephalosporins

33. Josephine, H. R., Kumar, I., and Pratt, R. F. (2004) The perfect penicillin? Inhibition of a bacterial DD-peptidase by peptidoglycan-mimetic β-lactams. J. Am. Chem. Soc. 126, 8122–8123 CrossRef Medline

34. Han, S., Caspers, N., Zaniewski, R. P., Lacey, B. M., Tomaras, A. P., Feng, X., Geoghegan, K. F., and Shanmugasundaram, V. (2011) Distinctive attributes of beta-lactam target proteins in Acinetobacter baumannii relevant to development of new antibiotics. J. Am. Chem. Soc. 133, 20536–20545 CrossRef Medline

35. Conterras-Martel, C., Dahout-Gonzalez, C., Martins Ados, S., Kotnik, M., and Dessen, A. (2009) PBP active site flexibility as the key mechanism for β-lactam resistance in pneumococci. J. Mol. Biol. 387, 899–909 CrossRef Medline

36. Yamada, M., Watanabe, T., Miyara, T., Baba, N., Saito, I., Takeuchi, Y., and Ohsawa, F. (2007) Crystal structure of cefditoren complexed with Streptococcus pneumoniae penicillin-binding protein 2X: structural basis for its high antimicrobial activity. Antimicrob. Agents Chemother. 51, 3902–3907 CrossRef Medline

37. Sainsbury, S., Bird, L., Rao, V., Shepherd, S. M., Stuart, D. I., Hunter, W. N., Owens, R. J., and Ren, J. (2011) Crystal structures of penicillin-binding protein 3 from Pseudomonas aeruginosa: comparison of native and antibiotic-bound forms. J. Mol. Biol. 405, 173–184 CrossRef Medline

38. Fedarovich, A., Nicholas, R. A., and Davies, C. (2012) The role of the β5-α11 loop in the active-site dynamics of acylated penicillin-binding protein A from Mycobacterium tuberculosis. J. Mol. Biol. 418, 316–330 CrossRef Medline

39. Pernot, L., Chesnel, L., Le Gouellec, A., Croizé, J., Vernet, T., Dideberg, O., and Dessen, A. (2004) A PBP2x from a clinical isolate of Streptococcus pneumoniae exhibits an alternative mechanism for reduction of susceptibility to β-lactam antibiotics. J. Biol. Chem. 279, 16463–16470 CrossRef Medline

40. Conterras-Martel, C., Job, V., Di Guilmi, A. M., Vernet, T., Dideberg, O., and Dessen, A. (2006) Crystal structure of penicillin-binding protein 1a (PBP1a) reveals a mutational hotspot implicated in β-lactam resistance in Streptococcus pneumoniae. J. Mol. Biol. 355, 684–696 CrossRef Medline

41. Whiley, D. M., Limnios, E. A., Ray, S., Sloots, T. P., and Tapsall, J. W. (2007) Diversity of penA alterations and subtypes in Neisseria gonorrhoeae strains from Sydney, Australia, that are less susceptible to ceftriaxone. J. Infect. Chemother. 13, 195–203 CrossRef Medline

42. Lee, S. G., Lee, H., Jeong, S. H., Yong, D., Chung, G. T., Lee, Y. S., Chong, Y., and Lee, K. (2010) Various penA mutations together with mtrR, porB and penA mutations in Neisseria gonorrhoeae isolates with reduced susceptibility to cefixime or ceftriaxone. J. Antimicrob. Chemother. 65, 669–675 CrossRef Medline

43. Levy, N., Bruneau, J. M., Le Rouzic, E., Bonnard, D., Le Strat, F., Caravano, A., Chevreuil, F., Barbion, J., Chasset, S., Ledoussal, B., Moreau, F., and Ruff, M. (2019) Structural basis for E. coli penicillin-binding protein (PBP) 2 inhibition, a platform for drug design. J. Med. Chem. 62, 4742–4754 CrossRef Medline

44. King, A. M., King, D. T., French, S., Brouillette, E., Ashi, A., Alexander, J. A., Vuckovic, M., Maiti, S. N., Parr, T. R., Jr., Brown, E. D., Malouin, F., Strynadka, N. C., and Wright, G. D. (2016) Structural and kinetic characterization of diazabicyclooctanes as dual inhibitors of both serine-β-lactamases and penicillin-binding proteins. ACS Chem. Biol. 11, 864–868 CrossRef Medline

45. Maness, M. J., and Sparling, P. F. (1973) Multiple antibiotic resistance due to development of new drugs for an old target: the penicillin binding proteins. Molecules 17, 12478–12505 CrossRef Medline

46. McCoy, A. J., Grosse-Kunstleve, R. W., Adams, P. D., Winn, M. D., Stothard, P., Caffrey, M., MacArthur, M. W., Peternell, T., Moriarty, N. W., and Read, R. J. (2007) Phaseology: an automated system for phase determination. J. Appl. Crystallogr. 40, 658–674 CrossRef Medline

47. Emsley, P., Lohkamp, B., Scott, W. G., and Cowtan, K. (2010) Features and development of Coot. Acta Crystallogr. D Biol. Crystallogr. 66, 486–501 CrossRef Medline

48. Murshudov, G. N., Vagin, A. A., and Dodson, E. J. (1997) Refinement of macromolecular structures by the maximum-likelihood method. Acta Crystallogr. D Biol. Crystallogr. 53, 240–255 CrossRef Medline

49. Laskowski, R. A., MacArthur, M. W., Moss, D. S., and Thornton, J. M. (1993) PROCHECK: a program to check the stereochemical quality of protein structures. J. Appl. Cryst. 26, 283–291 CrossRef Medline