Mutations in penicillin-binding protein 2 from cephalosporin-resistant *Neisseria gonorrhoeae* hinder ceftriaxone acylation by restricting protein dynamics

The global incidence of the sexually transmitted disease gonorrhea is expected to rise due to the spread of *Neisseria gonorrhoeae* strains with decreased susceptibility to extended-spectrum cephalosporins (ESCs). ESC resistance is conferred by mosaic variants of penicillin-binding protein 2 (PBP2) that have diminished capacity to form acylated adducts with cephalosporins. To elucidate the molecular mechanisms of ESC resistance, we conducted a biochemical and high-resolution structural analysis of PBP2 variants derived from the decreased-susceptibility *N. gonorrhoeae* strain 35/02 and ESC-resistant strain H041. Our data reveal that mutations in PBP2 diminish affinity for ceftriaxone and restrict conformational changes that normally accompany acylation. Specifically, we observe that a G545S substitution hinders rotation of the β3 strand necessary to form the oxanoyl bond for acylation and also traps ceftriaxone in a noncanonical configuration. In addition, F504L and N512Y substitutions appear to prevent bending of the β3–β4 loop that is required to contact the R1 group of ceftriaxone in the active site. Other mutations also appear to act by reducing flexibility in the protein. Overall, our findings reveal that restriction of protein dynamics in PBP2 underpins the ESC resistance of *N. gonorrhoeae*.

Gonorrhea, a sexually-transmitted infection caused by *Neisseria gonorrhoeae*, is increasingly difficult to treat due to emergence of strains exhibiting decreased susceptibility or resistance to the extended-spectrum cephalosporins (ESCs) and ceftriaxone or cefixime, which are the last remaining options for monotherapy of gonorrhea. As a result, the Centers for the Disease Control and Prevention in the United States and many other public health agencies worldwide have revised their treatment guidelines for gonorrhea from monotherapy with ceftriaxone or cefixime to dual therapy with ceftriaxone and azithromycin (1–4). An important event that led to this change was the isolation in Japan of the first *N. gonorrhoeae* strain (H041) exhibiting high-level resistance to cefixime and ceftriaxone (5). The minimum inhibitory concentrations (MICs) of ceftriaxone and cefixime for H041 are 2 and 8 μg/ml, respectively, and well-above the EUCAST breakpoints (>0.125 μg/ml) for these antibiotics (6). A second ESC-resistant (ESC<sup>R</sup>) strain (F89) was subsequently isolated in France and Spain (7, 8). Consistent with the spread of ESC<sup>R</sup> strains, there have now been several reports of treatment failures with ESCs (9–12), and the first international spread of a ceftriaxone-resistant strain has been verified (2). In 2018, the first strain with ceftriaxone resistance in combination with high-level azithromycin resistance was identified (13, 14).

A number of resistance determinants contribute to β-lactam resistance to *N. gonorrhoeae*, including *mtrR*, *penB*, and *pomA* (15–19), but the primary determinant in the emergence of ESC<sup>R</sup> strains in *N. gonorrhoeae* is acquisition of so-called mosaic alleles of the *penA* gene through allelic replacement (20). The *penA* gene encodes penicillin-binding protein 2 (PBP2), an essential transpeptidase (TPase) that catalyzes the formation of cross-links between peptides emanating from parallel glycan strands during the latter stages of peptidoglycan synthesis (21). PBP2 is a two-domain protein composed of a TPase catalytic domain and an N-terminal domain of unknown function (22). This latter domain may act as a pedestal to project the active site toward peptidoglycan or could mediate protein–protein interactions with other components of the peptidoglycan machinery. Although evidence has uncovered an allosteric site in the N-terminal domain of *Staphylococcus* TPase, transpeptidase; TPBP2, truncated construct of penicillin-binding protein 2; RMDSD, root-mean-square deviation; ITC, isothermal titration calorimetry; CHES, 2-(cyclohexylamino)ethanesulfonic acid; TEV, tobacco etch virus.

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This article contains Figs. S1–S5, Table S1, and supporting Ref. 1.

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3 The abbreviations used are: ESC, extended-spectrum cephalosporin; MIC, minimum inhibitory concentration; PBP, penicillin-binding protein;
**Crystal structures of PBP2 from ESC-resistant N. gonorrhoeae**

*PBP2a* (23), there is no indication of a similar site in PBP2. PBP2 recognizes and reacts with the d-Ala–d-Ala C terminus of the peptide chain to form an acyl–enzyme complex with the penultimate d-Ala, and then it catalyzes the transfer of the acylated peptide to the amino group of *meso*-diaminopimelic acid from an adjacent peptide strand to form an amide cross-link. All β-lactam antibiotics, including ceftriaxone and cefixime, are mimics of the peptide d-Ala–d-Ala C terminus and react with PBPs to form a long-lived acylated complex that inhibits the enzyme and leads to loss of TPase activity required for proper peptidoglycan synthesis (for PBP reviews, see Refs. 24–26).

The active sites of PBPs contain three signature motifs: SXXK, SXN, and KTG, where X is a variable amino acid. In PBP2, Ser-310 of the SXXK motif is the site of acylation by both peptide substrate and β-lactams. PBP2 variants from ESCR strains contain upwards of 60 amino acid mutations compared with PBP2 from the penicillin- and cephalosporin-susceptible strain FA19 (7, 20, 27). These variants have arisen through homologous recombination of DNA segments from commensal *Neisseria* species and are therefore referred to as mosaic *penA* alleles. Prior investigation of PBP2 from the ESC-decreased susceptibility strain 35/02 showed that out of the 57 mutations compared with FA19, three (I312M, V316T, and G545S) were responsible for the majority of resistance to ESCs and that mutations present in the N-terminal domain do not contribute (28). Strikingly, the *penA* gene from H041 (*penA41*) can transform FA19 to full resistance for cefixime and ceftriaxone in the absence of other resistance determinants (29). Its PBP2 contains 61 amino acid mutations compared with FA19 (20), and 13 of these are new or different compared with 35/02. Three of the mutations (A311V, V316P, and T483S) significantly increase the MICs of ceftriaxone and cefixime when introduced into PBP2 from ESC-resistant *N. gonorrhoeae* mapped onto the crystal structure of apoPBP2WT (29). The structures of the mutations (A311V, V316P, and T483S) significantly increase the MICs of ceftriaxone and cefixime when introduced into PBP2 (25-02) (29). Two additional mutations, N512Y and F504L, are present on the β3–β4 loop after the KTG motif (28). Only the A311V and I312M mutations are within a conserved sequence motif; the rest generally surround the active site (Fig. 1). An alignment of the relevant PBP2 sequences is shown in Fig. S1. Together, the mutations lower the second-order rates of acylation of PBP2 by ESCs by up to 12,000-fold (29).

Recently, the molecular mechanism underpinning acylation of *N. gonorrhoeae* PBP2 by ESCs has begun to be understood (30). Acylation of a PBP2 construct comprising the TPase domain by ceftriaxone or cefixime is associated with structural changes within and near the active site (Fig. S2). One is twisting of the β3 strand, which contains the KTG motif, to form the oxyanion hole that stabilizes the tetrahedral intermediate/transition state of acylation, and the other is movement of the β3–β4 loop toward the antibiotic to form a cluster of interactions around the aminothiazole group of the R1 side chain of the cephalosporins. Rotation of the side chain of Thr-498 on β3 to form a hydrogen bond with the β-lactam carboxylate appears to be the trigger for twisting of the β3 strand, because in a crystal structure of PBP2 in complex with a phosphate occupying the same position as the β-lactam carboxylate, a similar hydrogen bond has formed, and β3 has also twisted.

This recent new understanding of how PBP2 is acylated by ESCs sets the stage to investigate how this process is impaired in the presence of mutations associated with ESC resistance. Elucidation of the molecular mechanisms of these mutations will both inform treatment and enable design of new antimicrobials that circumvent such mechanisms. Toward this goal, we present crystal structures of the TPase domain of apoPBP2 (35-02), derived from the ESC-decreased susceptibility strain 35/02 in apo-form, and of PBP2 (30), derived from the ESC-resistant strain H041, in both apo-form and acylated by ceftriaxone. The structures strongly suggest that mutations present in PBP2 from resistant strains of *N. gonorrhoeae*, notably the G545S mutation on the β5–α11 loop and F504L and N512Y mutations on the β3–β4 loop, hinder acylation by restricting conformational flexibility of the protein.

**Results**

*ESCR* mutations lower affinity of PBP2

For our studies of PBP2, we used a construct comprising only the TPase domain of the protein, referred to here as tPBP2 (where “t” denotes truncated). Such constructs retain identical acylation rates as the full-length protein for a range of antibiotics (31) and are therefore valid systems to examine the cephalosporin resistance of PBP2.

The kinetics by which PBPs react with β-lactam antibiotics or peptide substrate are as shown in Equation 1.

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4 J. Tomberg, R. A. Nicholas, and C. Davies, unpublished data.
Crystal structures of PBP2 from ESC-resistant N. gonorrhoeae

Figure 2. Mutations conferring ESC resistance affect both affinity and the rate of deacylation of tPBP2. A, isothermal titration calorimetry data for the interaction between ceftriaxone and S310A mutants of tPBP2. Upper panels show the binding isotherms for the wildtype (WT), 35/02, and H041 variants of tPBP2, and the lower panel shows the fit of the data for tPBP2WT by nonlinear regression with a single-site isotherm using NanoAnalyze software (TA Instruments). Three technical replicates were performed under identical conditions using 100 μM protein and 1000 μM ceftriaxone. B, deacylation rates (k_d) for tPBP2WT and tPBP2H041–acylated complexes. WT and H041 tPBP2 constructs were incubated with Bocillin-FL for an hour, after which ceftriaxone was added to a concentration of 3 mM (r = 0). Aliquots were removed at time intervals and separated by SDS-PAGE, and the amount of Bocillin-FL remaining bound was detected by UV illumination and quantified by densitometric scanning. The data were normalized for protein levels using Coomassie staining. Data were fit to first-order kinetics to derive the Bocillin-FL deacylation constant, k_d. Experiments were performed in triplicate using protein samples from the same purification. For both experiments, the errors reported are the standard error of the mean.

\[
E + S \xrightleftharpoons[k_{-1}]{k_1} E\cdot S \xrightarrow{k_2} E\cdot S' \xrightarrow{k_3} E + P \quad (\text{Eq. 1})
\]

where formation of the noncovalent Michaelis-Menten complex, ES, is described by the dissociation constant \(K_i\) (i.e. \(k_{-1}/k_1\); \(k_2\) is the rate of formation of the covalent acyl–enzyme complex, \(E\cdot S'\); and \(k_3\) is the rate constant for deacylation that releases the product P (32, 33).

Mutations in PBP2 that contribute to ESC resistance could in principle alter kinetics by the following: (a) reducing affinity for the antibiotic (\(K_i\)); (b) lowering the rate of acylation (\(k_2\)); (c) increasing the rate of deacylation (\(k_3\)); or (d) by any combination of these. The covalent nature of the PBP reaction means that \(K_i\) cannot be measured independently from \(k_{-2}\); therefore, the second-order rate of acylation (\(k_2/K_i\)) is widely used as a measure of inhibition by \(\beta\)-lactam antibiotics (34–37). Only by separating \(K_i\) and \(k_2\) can it be determined whether mutations impact affinity, the rate of acylation, or both. To address this, we mutated Ser-310 to alanine in tPBP2WT, tPBP235/02, and tPBP2H041 and used isothermal titration calorimetry (ITC) to measure the dissociation constants (\(K_i\)) for ceftriaxone (traditionally, \(K_i\) has been used to denote the binding affinity of \(\beta\)-lactam antibiotics, but it is identical to \(K_{i0}\)). A crystal structure of an S310A mutant in the background of tPBP2WT was solved at 1.7 Å resolution and shows that mutation of Ser-310 does not alter the architecture of the protein, including in the active site (Fig. S3 and supplemental Table 1).

The three constructs exhibited dramatic differences in their affinities for ceftriaxone (Fig. 2A and Table 1). tPBP2WT has a \(K_d\) of 2.3 ± 0.6 μM, whereas for tPBP235/02 and tPBP2H041, the binding isotherms were essentially flat, making it impossible to derive \(K_d\) values. The curve for tPBP2WT is unimodal, indicative of 1:1 stoichiometry. These data demonstrate that PBP2 mutations associated with ESC resistance have a significant impact on affinity for ceftriaxone. Using the \(K_d\) value of 2.3 μM determined from ITC, we estimate that ceftriaxone acylates tPBP2WT at a rate of 3.9 s\(^{-1}\), based on the previously measured second-order acylation constant of ceftriaxone for WT PBP2 of 1,710,000 M\(^{-1}\) s\(^{-1}\) (29). Without corresponding \(K_d\) values, \(k_2\) values could not be derived for tPBP235/02 or tPBP2H041.

**ESC**\(^r\) mutations increase the rate of deacylation of Bocillin-FL–tPBP2 complex

We next investigated whether mutations present in PBP2 from H041 alter the rate of deacylation using the fluorescent penicillin, Bocillin-FL, as a reporter molecule (38). Acylated complexes of tPBP2WT and tPBP2H041 were formed by incubation with equimolar amounts of Bocillin-FL, and deacylation was measured after dilution into 300-fold molar excess of ceftriaxone by quantifying the decrease in fluorescence of enzyme-bound Bocillin-FL over
Crystal structures of PBP2 from ESC-resistant N. gonorrhoeae

Table 1

| Strain | MIC for ceftriaxone (μg/ml) | Kₜ of S310A tPBP2 mutant (μM) | Kₑ/Kₜ for acylation of PBP2 (M⁻¹ s⁻¹) | kₑ of tPBP2 (s⁻¹) | kₑ of Bocillin-FL–tPBP2 complex (tₑ/h) |
|--------|-----------------------------|-------------------------------|--------------------------------------|-------------------|---------------------------------------|
| FA19   | 0.001 (69)                  | 2.34 ± 0.56                  | 1,710,000 ± 90,000                  | 3.9               | 8.1 × 10⁻⁵ ± 1.2 × 10⁻⁶ (69 ± 29)    |
| 35/02  | 0.094 (70)                  | ND                           | 11,300 ± 400                        | ND                | ND                                    |
| H041   | 2–4 (20)                    | ND                           | 741 ± 28                            | ND                | 2.8 × 10⁻⁶ ± 1.7 × 10⁻⁶ (2.4 ± 0.1)  |

*ND means not determined.

Structures of tPBP235/02 and tPBP2H041 are essentially identical to one another but differ from tPBP2WT by the location of the β3–β4 loop

The structures of tPBP235/02 and tPBP2H041 were solved at resolutions of 1.93 and 1.55 Å, respectively, from crystals obtained in space group P2₁2₁2₁ with one molecule in the asymmetric unit (Table 2). For both structures, the majority of the protein is visible in the electron density map, including the β3–β4 loop, and only residues Tyr-543 and Tyr-544 occupy relatively weak density. The two structures are highly similar to each other, with a root mean square deviation (RMSD) of 0.21 Å for 313 common Ca atoms, and the superimposition shows essentially no differences in the architecture of the proteins other than a very slight alteration in the α10–β3 loop (Fig. 3). Having established that the structures of tPBP235/02 and tPBP2H041 are highly similar, we have mostly limited our comparisons below to tPBP2H041 and tPBP2WT for clarity.

The single molecule in the asymmetric unit of tPBP2H041 superimposes onto the two molecules of tPBP2WT (30) with RMSDs of 1.0 Å for molecule A (303 common Ca atoms) and 1.5 Å for molecule B (310 common Ca atoms). The higher RMSD for molecule B of tPBP2WT reflects the different positions of the β3–β4 loop, whereas this loop could not be modeled in molecule A. For this reason, molecule B of the tPBP2WT structure was used for comparison with that of tPBP2H041.

Overall, the tPBP2H041 structure overlaps closely with the tPBP2WT structure, but there is a major difference in the β3–β4 loop and minor changes in the β2–β2c hairpin (Fig. 4A). In particular, the β3–β4 loop in tPBP2H041 occupies a conformation in which the loop has bent away (“outbent”) from the active site compared with its extended conformation in tPBP2WT. The difference in the position of the β3–β4 loop in tPBP2H041 compared with that of tPBP2WT is important because when tPBP2WT is acylated by ceftriaxone or cefixime, this loop transitions from an extended conformation toward the active site to form a cluster of interactions around the R1 aminothiazole group (Fig. S2) (30). Its displaced conformation in tPBP2H041 would appear to make the distance that must be traversed to reach the R1 group considerably larger.

Another difference between the structures of tPBP2WT and tPBP2H041 occurs in the active site. Although the positions of residues in the active site are to a large extent unchanged in tPBP2H041 compared with the WT structure, the side-chain rotamer of Ser-362 (the S of the SXN motif) has altered such that its hydroxyl side chain is no longer hydrogen-bonded with either Ser-310 or Lys-313 of the SXXK motif and now only contacts Lys-497 of the KTG motif (Fig. 4B). Interestingly, this alteration is not observed in tPBP235/02, and this may be related to a T483S mutation present in tPBP2H041 but not tPBP235/02 (discussed below).

Most Cephr® mutations have minimal impact on apo structures

The structures of tPBP235/02 and tPBP2H041 in apo-form allow an examination of the seven mutations implicated in ESC resistance in the pre-covalent states (Fig. 5A). Both PBP235/02 and PBP2H041 have mutations located on helix α2: I312M and V316T for tPBP235/02, and A311V, I312M, and V316P for tPBP2H041. This helix is an important part of the structure because the serine nucleophile, Ser-310, is located at its N terminus. Helix α2 is mostly buried within the protein and surrounded by hydrophobic residues. The mutations do not significantly alter the conformation of the helix in either tPBP235/02 (Fig. 5B) or tPBP2H041 (Fig. 5D) and tPBP235/02 (Fig. S4). It is nevertheless interesting to note that the A311V and I312M mutations involve hydrophobic residues and increase the size of the side chain. These mutations would therefore be expected to increase the degree of hydrophobic packing around α2: the valine side chain at position 311 in tPBP2H041 packs against Ile-519 of β3, and the methionine at position 312 packs against hydrophobic residues on α6, as well as Phe-315 on α2. The situation at position 316 is slightly different because this residue is relatively less engaged in hydrophobic packing. In tPBP235/02, this is a threonine, which could form a weak hydrogen bond (3.4 Å) with the hydroxyl group of Tyr-383 from helix α6, and in tPBP2H041 it is replaced by a proline.

The G545S mutation is present in both tPBP2H041 and tPBP235/02 and is located on the β5–α11 loop (Fig. 5C). This loop is immediately adjacent to the KTG motif on β3 and projects toward the active site. An obvious consequence of the G545S mutation is that the serine side chain forms a hydrogen bond with the side chain of Thr-498, thus forming a new connection to β3. This is expected to have a direct impact on the rate of acylation, as discussed later.

One mutation that is found in tPBP2H041 but not tPBP235/02 is the T483S mutation. We have shown previously that T483S is
a major contributor to the increased resistance conferred by the penA allele from H041 compared with that conferred by the penA allele from 35/02 (29). Thr-483 is present on the H9251–H9252 loop, above the KTG motif as viewed in Fig. 5C. Two other mutations present in ESC-decreased susceptibility or ESCR variants of PBP2, P480A and T485I, also occur on this loop, but these do not appear to contribute to ESC resistance (29). The conformation of the loop is similar in both structures, and the impact of the T483S mutation is difficult to ascertain. The most obvious difference is that in tPBP2H041, Ser-483 is slightly shifted with respect to Thr-483 in tPBP2WT, and it is interesting to note that the side-chain rotamer of Ser-362 in the SXN motif has altered to point toward the serine. This change may be elicited by the absence of the methyl group in Ser-483 (tPBP2H041) compared with Thr-483 (tPBP2WT).

Finally, the F504L and N512Y mutations, which are present in both tPBP235/02 and tPBP2H041, are located on the H9252–H9253 loop (Fig. 5D). When PBP2 is acylated by ceftriaxone or cefixime, this loop moves toward the active site to form a cluster of interactions around the R1 side chain of the antibiotic (30). As noted above, this loop is bent away from the active site in tPBP2H041 and tPBP235/02, whereas it is largely extended in tPBP2WT. As discussed below, these mutations may impede the conformational change in the H9252–H9253 loop that occurs when ESCs bind and acylate PBP2.

Acylated structure of tPBP2H041

The “outbent” conformation observed for the H9252–H9253 loop in the structures of tPBP235/02 and tPBP2H041 poses the question whether this loop can move as a result of acylation by ESCs to form the cluster of interactions around the R1 aminothiazole group as occurs in tPBP2WT (30). To address this, the crystal structure of tPBP2H041 acylated by ceftriaxone was determined at 1.8 Å resolution, obtained by soaking crystals of tPBP2H041 with the antibiotic (Table 2). An unbiased Fo – Fc difference electron density map identifies ceftriaxone covalently bound to Ser-310 (Fig. 6). Electron density is observed for the major part of the ceftriaxone molecule with the exception of the aminothiazole ring and the C3 position on the dihydrothia-

![Figure 3. Structures of tPBP235/02 and tPBP2H041 are highly similar. Shown is an overlay of the two backbones where tPBP235/02 is orange, and tPBP2H041 is green. The secondary structure nomenclature is the same as in Powell et al. (22).](image-url)

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Table 2

| Data collection | tPBP235/02 | tPBP2H041 | tPBP2H041–CRO complex |
|----------------|-----------|-----------|----------------------|
| Space group    | P2₁,2₁,2₁ | P2₁,2₁,2₁ | P2₁,2₁,2₁            |
| Cell dimensions: |           |           |                      |
| a, b, c (Å)    | 50.6, 61.1, 109.1 | 50.4, 61.3, 108.9 | 50.3, 60.7, 109.3 |
| Resolution range (Å) | 31.72–1.93 | 31.69–1.55 | 40.62–1.80 |
| Rmerge (%)     | 13.7 (97.9) | 11.8 (62.5) | 10.5 (52.8) |
| Rfree (%)      | 4.9 (105.6) | 4.7 (26.8) | 3.0 (15.7) |
| CC₁/₂          | 0.997 (0.658) | 0.994 (0.851) | 0.995 (0.917) |
| Completeness (%)| 99.5 (99.8) | 98.3 (97.2) | 100.0 (99.9) |
| Redundancy     | 8.7 (9.0) | 6.8 (6.1) | 13.3 (12.0) |
| No. of unique reflections | 26,212 (1,285) | 48,525 (2,372) | 31,680 (1,560) |
| Refinement     |           |           |                      |
| Resolution (Å) | 31.72–1.93 | 31.69–1.55 | 40.62–1.80 |
| No. of non-hydrogen protein atoms | 2,461 | 2,504 | 2,495 |
| No. of antibiotic atoms | 0 | 0 | 26 |
| No. of waters/glycerol | 100 | 153 | 122/1 |
| Rwork/Rfree (%) | 18.4/20.5 | 17.9/20.0 | 17.2/19.4 |
| RMSDs from ideal stereochemistry |           |           |                      |
| Bond lengths (Å) | 0.007 | 0.007 | 0.008 |
| Bond angles (°) | 1.2 | 1.3 | 1.4 |
| B-factors: | Mean B-factor (main chain) (Å²) | 33.3 | 16.0 | 27.5 |
| RMSD in main-chain B-factors (Å²) | 1.0 | 0.6 | 1.1 |
| Mean B-factor (side chains and waters) (Å²) | 38.5 | 19.7 | 33.3 |
| RMSD in side-chain B-factors (Å²) | 1.8 | 1.0 | 1.9 |
| Ramachandran plot: | Residues in most favored region (%) | 94.2 | 93.4 | 93.8 |
| Residues in generously allowed region (%) | 5.8 | 6.6 | 6.2 |
| Residues in additional allowed region | 0.0 | 0.0 | 0.0 |
| Residues in disallowed region | 0.0 | 0.0 | 0.0 |
| PDB code | 6VBL | 6VBC | 6VBD |
zine ring. Weaker density of the aminothiazole is consistent with flexibility of this group, and only the more dominant occupancy is modeled. This contrasts with ceftriaxone binding to tPBP2WT, where the R1 group is ordered (30). No density was observed for the C3 leaving group, although this group was unexpectedly observed in one of molecules of the asymmetric unit in the structure of tPBP2WT acylated by ceftriaxone. The weakness in density at C3 is consistent with a mixture of C3 endo and exo forms of the dihydrothiazine ring.

Ceftriaxone forms a number of hydrogen-bonding interactions with tPBP2H041 (Fig. 6). These include bonds between the ester carbonyl oxygen and the main-chain amides of Ser-310 and Thr-500, which together form the oxyanion hole, and between the carbonyl oxygen preceding the aminothiazole ring (R1 group) and the side chain of Asn-364 from the SXN motif. In addition, a water molecule bridges the β-lactam carboxylate and amide of Gly-546. The β-lactam carboxylate forms hydrogen bonds with the main-chain amides and side-chain hydroxyls of both Thr-500 on β3 and Ser-545 from the β5-α11 loop. As noted later, these latter interactions are different compared to when ceftriaxone binds to tPBP2WT.

Acylation of tPBP2H041 elicits only minimal changes to the protein

The structure of acylated tPBP2H041 superimposes onto that of apo-tPBP2H041 with an RMSD of 0.27 Å (319 Cα atoms), indicative of the closely overlapping structures (Fig. 7A). This contrasts sharply with the significant changes observed when PBP2WT is acylated by ceftriaxone (30). Hence, rather than moving toward the active site, the β3–β4 loop remains in the same “outbent” conformation present in the structure of apo-tPBP2H041 (Fig. 7B), and β3 does not twist, suggesting it may be more difficult to form the oxyanion hole in PBP2 from H041. Although the apo and acylated structures are generally highly similar, there are some small-scale differences in the active site. One is alteration of the Ser-362 side chain to adopt a position where it forms hydrogen bonds with Ser-310 and Lys-313, as well as Lys-497 (Fig. 7C). There are also slight differences in the positions of the side chains of lysines 497 and 313.

Acylation structures of tPBP2WT and tPBP2H041 are different

Given the similarity in the apo tPBP2WT and tPBP2H041 structures, and the large changes between apo and acylated tPBP2WT structures, differences between the acylated forms of tPBP2WT and tPBP2H041 were expected. Indeed, the structures superimpose with an RMSD of 1.7 Å, which is considerably higher than apo versus acylated tPBP2H041 (Fig. 8A). The most obvious difference is in the position of the β3–β4 loop. As noted earlier, this loop moves when tPBP2WT is acylated by ceftriaxone to form a cluster of interactions around the R1 group of the antibiotic, but in acylated PBP2H041, the loop remains in the same “outbent” conformation observed in the apo structure. In tPBP2H041, this loop contains two mutations (N512Y and F504L) that are located at the approximate right-hand angle of the bend (Fig. 8B). The β-hairpin region (β2b–β2c) is also shifted between the two structures, although the significance of this difference is unclear. Finally, the β5–α11 loop containing the G545S mutation is slightly closer to the active site in acylated tPBP2WT compared with tPBP2H041.
bonding interactions it makes are also different. In tPBP2WT, the β-lactam carboxylate contacts only Thr-498, but in tPBP2H041 this bond is absent, and instead the carboxylate forms hydrogen bonds with Thr-500 and Ser-545. The G545S mutation may be a major determinant of the altered binding of ceftriaxone in tPBP2H041 because it seems to act as a buttress to prevent direct binding between Thr-498 and the ceftriaxone carboxylate group (Fig. 9B), and the hydrogen bond made by

Figure 5. Impact of ESC resistance mutations on the structure of tPBP2H041. A, superimposition of tPBP2WT (gray) and tPBP2H041 (green) structures showing the positions of the seven critical mutations present in the active-site region of PBP2. Side chains of mutated residues are shown for both structures, and their Ca positions are indicated by red spheres for the tPBP2H041 structure. B–D show close-up views of the mutations. B, three mutations in helix α2. C, G545S on the β5–α11 loop and the T483S mutation on the α10–β3 loop. Note Ser-545 forms a potential hydrogen bond with Thr-498 in the structure of tPBP2H041 as indicated by a dashed line. D, F504L and N512Y mutations in the β3–β4 loop. For all panels, relevant side chains are shown in stick form and are colored according to structure. Mutations associated with ESC resistance are colored red and labeled in the same color. Mutations present in PBP2 from H041 but not directly implicated in ESC resistance are underlined.
Ser-545 with the carboxylate may be responsible for the relative shift of ceftriaxone toward β3 and α11.

Discussion

The goal of this work was to understand how mutations in PBP2 contribute to ESC resistance of \textit{N. gonorrhoeae}. We find that structures of PBP2 derived from the reduced susceptibility strain 35/02 and ESC-resistant strain H041 are nearly identical to each other, but differ from WT PBP2 (derived from the susceptible FA19 strain) in the β3–β4 loop. We also find that the β3–β4 loop occupies the same position when tPBP2\textsuperscript{H041} is acylated by ceftriaxone, whereas this loop moves a considerable distance when tPBP2\textsuperscript{WT} is acylated by ESCs. We also show that ceftriaxone binds to a nonacylating S310A mutant of tPBP2\textsuperscript{WT} with micromolar affinity, but binding affinities for the S310A variants of tPBP2\textsuperscript{H041} and tPBP2\textsuperscript{35/02} are below detection. Finally, we observe that ceftriaxone occupies a markedly different position in the active site of tPBP2\textsuperscript{H041} compared with tPBP2\textsuperscript{WT}.

The first question we addressed is what component of the catalytic reaction is impacted by mutations: the noncovalent binding affinity (\(K_c\)), the rate of acylation (\(k_a\)), or the rate of deacylation (\(k_d\))? We demonstrate that all three are altered in the tPBP2\textsuperscript{H041} variant relative to tPBP2\textsuperscript{WT}. ITC measurements of the interaction of ceftriaxone with S310A mutants of tPBP2 variants showed that mutations in tPBP2\textsuperscript{35/02} and tPBP2\textsuperscript{H041} have a marked effect on affinity, such that noncovalent binding of ceftriaxone was undetectable at the same concentrations used to demonstrate binding to tPBP2\textsuperscript{WT}. An unavoidable caveat to our experimental approach is that we used S310A mutants to measure binding (in order to prevent acylation, which confounds the analysis), but aside from the absence of the Ser-310 nucleophile, the mutation did not significantly alter the architecture of tPBP2\textsuperscript{WT} (Fig. S3).

One of the more surprising findings from our investigation is that mutations conferring resistance to ESCs in \textit{N. gonorrhoeae} also decrease the half-life (\(t_{1/2}\)) of the ceftriaxone-acylated complex by 28-fold compared with tPBP2\textsuperscript{WT}. Typically, \(t_{1/2}\) values for β-lactam–acylated complexes of high-molecular mass PBPs are on the order of hours-to-days (42–44) and are considered too low to have any impact on resistance. The \(t_{1/2}\) of 69 h for the acylated ceftriaxone complex of tPBP2\textsuperscript{WT} is on the same scale as these other PBPs, but the \(t_{1/2}\) of 2.4 h for the deacylation of tPBP2\textsuperscript{H041} is markedly faster, raising the question of whether increased hydrolysis of the acylated complex could contribute to resistance. The doubling time for \textit{N. gonorrhoeae} in culture of \(\sim 1\) h would appear to make this decrease outside the range of physiological relevance, but it remains possible that within the genital tract or other host niches where \textit{N. gonorrhoeae} colonizes, growth rates may be considerably slower. The molecular basis for the increased rate of deacylation is unclear, but it may result from the noncanonical binding mode for ceftriaxone in tPBP2\textsuperscript{H041} compared with tPBP2\textsuperscript{WT}.

Of the seven mutations known to contribute directly to the ESC resistance of \textit{N. gonorrhoeae} H041, a clear mechanism of action can be inferred for some, and less so for others. The three mutations occurring on helix α2 (A311V, I312M, and V316T/V316P) fall into the latter category because they do not visibly alter the structure of α2. However, the most likely effect of these mutations is to increase rigidity, with the A311V and I312M mutations increasing hydrophobic packing around α2, and the V316T/V316P mutations introducing either a hydrogen bond (35/02) or a conformationally-restricted side chain (H041). It has been suggested previously that the N terminus of helix α2 undergoes conformational change to promote acylation in PBP2a of methicillin-resistant \textit{S. aureus} (45). Although this helix does not shift in the crystal structures when either WT or mutated PBP2 is acylated by ESCs, it is tempting to speculate that flexibility in α2 undetected by crystallography promotes acylation, and this is reduced in the presence of the mutations in tPBP2\textsuperscript{35/02} and tPBP2\textsuperscript{H041}.

Another mutation whose mechanism is not immediately clear is T483S. This is an important mutation for conferring elevated ESC resistance in H041 compared with 35/02 (29). It lies on the α10–β3 loop, which moves only very slightly when comparing the apo and acylated structures of both tPBP2\textsuperscript{WT} and tPBP2\textsuperscript{H041}. In the structure of tPBP2\textsuperscript{WT} acylated by ESCs, Thr-483 appears important for acylation because it forms a hydrogen bond with Thr-498, which in turn contacts the cephalosporin carboxylate. Even though Ser-483 in tPBP2H041 also possesses a hydroxyl group, the hydrogen bond with Thr-498 is absent in the structure, and its loss may impede rotation of β3. In addition, the loss of the methyl group caused by the T483S mutation could alter packing interactions with the SXN motif, thus accounting for the altered side-chain rotamer for Ser-360 and Lys-313 in a way that impairs acylation by ESCs.
By contrast, the mechanisms by which the F504L and N512Y mutations in tPBP2^{H041} and tPBP2^{H041} reduce the rate of acylation are more evident. Whereas the F504L and N512Y mutations initially appear too distant from the active site to have an obvious effect on reactivity with ESCs, their roles can be understood in the context of the movement of the β3–β4 loop toward the active site observed in the acylated structures of PBP2^{WT} (30). The N512Y mutation resides on β4 at the precise location where the β3–β4 loop in PBP2^{H041} bends away from the active site, thereby acting as a hinge (Fig. 8B). Likewise, the F504L mutation, which is found in tPBP2^{H041}, tPBP2^{H041}, and the penicillin-resistant strain FA6140 (22, 46), occupies the equivalent “hinge” position on the β3 side of the loop. Together, both mutations appear to impede bending of β3–β4, thus preventing formation of the cluster of residues around the R1 group of the antibiotic with subsequent impact on the reaction with ESCs. In support of this, the β3–β4 loop occupies the same “outbent” conformation in both apo and acylated structures of tPBP2^{H041} (green versus light blue). Residues that are mutated in tPBP2^{H041} are labeled.

Figure 7. Acylation of tPBP2^{H041} by ceftriaxone does not elicit conformational changes in the protein. A, superimposition of acylated (light blue) and apo (green) structures of tPBP2^{H041}. B, detail of ceftriaxone (CRO) bound in the active-site region of tPBP2^{H041}, where ceftriaxone is shown as magenta bonds. C, superimposition of active-site residues showing the difference in position of the Ser-362 side-chain rotamer between structures. For clarity, ceftriaxone is not included in this view. B and C, potential hydrogen bonds are indicated by dashed lines, and in C the distances in Ångstroms are indicated.

Figure 8. Structural differences between acylated structures of tPBP2^{WT} and tPBP2^{H041}. A, superimposition of acylated tPBP2^{H041} (light blue) and acylated tPBP2^{WT} (lilac). Note how the β3–β4 loop in tPBP2^{H041} adopts an “outbent” conformation. The ceftriaxone (CRO) molecules are shown as blue bonds for tPBP2^{H041}, and magenta bonds for tPBP2^{WT}. B, detailed view of the β3–β4 loop showing its two conformations in the apo and acylated state of tPBP2^{WT} (gray versus lilac) and the “outbent” conformation observed in both apo and acylated structures of tPBP2^{H041} (green versus light blue). Residues that are mutated in tPBP2^{H041} are labeled.
and therefore appears incapable of movement toward the active site.

A potential caveat here is the possibility that the conformation of β3–β4 loop is the product of crystal packing interactions, especially because the structures of tPBP2WT and tPBP2H041 were solved in a different crystal system compared with tPBP2WT. As shown in Fig. S5, there are some symmetry contacts involving the β3–β4 loop. Although it is possible these contacts have trapped the β3–β4 into the characteristic “out-bent” conformation observed, they could equally result from the loop adopting this conformation in solution prior to crystallization. In this regard, it is important to note that the space between the β3–β4 loop and where ceftriaxone binds in the active site is unoccupied by symmetry-related molecules, suggesting there is sufficient space for the loop to adopt a conformation closer to the active site in the crystal when ceftriaxone binds.

Among the mutations that decrease the acylation rate by ESCs, the impact of the G545S mutation present on the β5–α11 loop is perhaps the easiest to understand. New hydrogen bonds introduced by the replacement of glycine with serine have two potential outcomes that may affect both binding and the rate of acylation by ceftriaxone. One is a “locking” mechanism whereby the hydrogen bond formed between Ser-545 and Thr-498 prevents side-chain rotation of Thr-498 and subsequent rotation of β3, thus hindering formation of the oxyanion hole required for acylation. In this context, Ser-545 also acts as a wedge to prevent contact between Thr-498 and the β-lactam carboxylate. The other outcome may result from the hydrogen bond between Ser-545 and the β-lactam carboxylate. With Thr-498 locked into position by Ser-545, the hydrogen bonding requirement of the β-lactam carboxylate is now satisfied by a direct hydrogen bond with Ser-545, and this may be responsible for ceftriaxone occupying a position that is relatively closer to the β5–α11 loop in tPBP2H041 compared with the structure of acylated tPBP2WT. This could be regarded as an induced-fit mechanism where ceftriaxone moves after initial binding, but to a conformation that is considerably less favorable for acyla-
tion. This binding mode may also explain why tPBP2_Ho41 exhibits a higher rate of deacylation of ceftriaxone compared with tPBP2_WT.

Mutations in several PBPs have been implicated in resistance to β-lactams, and it is important to consider whether mechanisms are shared or distinct across species. The seven mutations in PBP2 known to contribute to ESC resistance occur in three loop regions (α10–β3, β3–β4, and β5–α11) and helix α2, and in general, mutations are also observed in these regions in PBPs from other bacterial species (47). A known hot spot is the β3–β4 loop because mutations in this loop associated with β-lactam resistance are observed in a number of PBPs, including Streptococcus pneumoniae PBP2x, PBP1a, and PBP2b and Haemophilus influenzae PBP3 (44, 47–49). How such mutations work has been unclear, but given the movement of the β3–β4 loop observed in N. gonorrhoeae PBP2 acylated by ESCs (30), it is possible they function in a similar way as the F504L and N512Y mutations in PBP2. Similarly, mutations in the β5–α11 loop are observed in Pseudomonas aeruginosa PBP3 (47), and of these, G352D/G352E mutations could affect interactions with the KTG motif. Finally, helix α2 harbors mutations in several PBPs, including those close to the serine nucleophile similar to A311V in N. gonorrhoeae PBP2 (48, 50–53), although again, their contribution to resistance remains unclear.

The overall picture emerging from our studies of PBP2 is that mutations implicated in resistance both lower affinity and restrict motions important for acylation by ESCs. By locking the side-chain rotamer of Thr-498, the G545S mutation may also restrict rotation of the glycan strand. We hypothesize that the larger number of contacts with the substrate helps overcome the energetic barrier created by the ESC resistance mutations and allows TPase activity to proceed. That said, while it is not possible to measure TPase activity directly without a suitable biochemical assay, it is clear that the activity of both tPBP2_35/02 and tPBP2_441 is lower than WT, which manifests in a slower growth rate for strains harboring the mosaic penA alleles (60, 61). These data suggest that the energetic barrier is not entirely overcome, but nevertheless sufficient activity remains to support cell growth. Testing this hypothesis must await a detailed understanding of PBP2–peptidoglycan interactions and examination of the dynamic behavior of PBP2 from ESC-resistant strains of N. gonorrhoeae.

Experimental procedures

Cloning and expression of transpeptidase domain constructs

We previously reported the cloning of a TPase domain construct for WT PBP2 derived from FA19 (referred to here as tPBP2_WT) (31) that retained identical acylation rates as the full-length protein for a range of antibiotics. For this study, we made equivalent constructs for PBP2 from 35/02 and Ho41. Briefly, nucleotides 709–1746 of penA35 from 35/02 (tPBP2_35/02) or penA41 from Ho41 (tPBP2_Ho41), both encoding amino acids 237–582, were cloned into pMALC2KV, which expresses tPBP2 as an N-terminal hexa-histidine–tagged fusion with maltose-binding protein, separated by a tobacco etch virus (TEV) protease site. Nucleotides encoding amino acids 283–297 were not included in the construct to promote the chances of crystallization, as these form a loop that projects from the TPase domain (22, 31). A non-native glycine was included to connect residues Pro-282 and Arg-298.

The constructs were transformed into Escherichia coli BL21 (DE3) cells for expression. Two litres of culture were grown at 37°C, and protein expression was induced by the addition of 0.3 mm isopropyl β-D-thiogalactoside, followed by overnight growth at 20°C. The harvested cells were lysed in 20 mm Tris·HCl, pH 8.0, 500 mm NaCl, and 10% glycerol (TNG), and the solution was passed over a 5-ml HiTrap™ FF Ni²⁺-nitrilotriacetic acid affinity column (GE Healthcare) equilibrated in TNG buffer. After elution of the protein by a TNG/15–500 mm imidazole gradient, His₆-tagged TEV protease was used to digest the fusion protein at a molar ratio of 50:1 (fusion protein/TEV), followed by overnight dialysis at 4°C against buffer containing 20 mm Tris, pH 7.0, 10% glycerol (TG). The digested protein was then passed over a 5-ml HiTrap™ SP FF ion-ex-
Crystal structures of PBP2 from ESC-resistant N. gonorrhoeae

change column (GE Healthcare) pre-equilibrated with TG buffer, and the protein was eluted by a 0–500 mM NaCl linear gradient in the same buffer, and fractions were pooled.

Site-directed mutagenesis

The S310A mutants for tPBP2WT, tPBP235/02, and tPBP2H041 were generated using the QuikChange Lightning kit (Agilent, Santa Clara, CA). The DpnI-treated amplified PCR product was used to transform E. coli BL21 (DE3) cells. All mutants were confirmed by sequencing.

ITC

Measurements of heat exchange to quantify the interaction between ceftriaxone and the S310A mutants of tPBP2WT, tPBP235/02, and tPBP2H041 were conducted on a Nano ITC instrument (TA instruments, New Castle, DE). Purified proteins were dialyzed against 50 mM sodium phosphate, 180 mM NaCl, 10% glycerol, pH 7.1, and concentrated to 100 μM. Measurements were carried out at 25 °C, with a 300-rpm stirring rate, and a total of 18 injections of 2.7 μl of ceftriaxone (1 mM stock) was added to the protein solution (initial volume 300 μl). Three technical replicates were performed, using a fresh sample of protein derived from the same purification. The binding parameters ΔH, Kd, and n (stoichiometry) were calculated using the Nano analyzer software using nonlinear regression for a single-site isotherm (62). Heats of reaction between the ligand and buffer only were subtracted from the measured values.

Derivation of Kd

Using Kd values obtained from ITC measurements and previously measured values of the second-order rate constant (k2/K3) of ceftriaxone for full-length WT PBP2 (29), estimated values for the rate of acylation (k2) of tPBP2WT for ceftriaxone were calculated using the formula k2 = Ks(k2/K3), where Ks is equivalent to Kd (32, 33).

Measurement of deacylation rate

The deacylation rate constant (k3) values were determined using Bocillin-FL as a reporter molecule. Purified tPBP2 protein at a concentration of 20 μM was incubated with 20 μM Bocillin-FL for 1 h at 22 °C in 50 mM phosphate, 10% glycerol, 180 mM NaCl, pH 7.1, at which time the final concentration of protein and Bocillin-FL was adjusted to 10 μM, and ceftriaxone was added at a final concentration of 3 mM. The amount of Bocillin-FL covalently bound to PBP2 was determined by removing aliquots at various times, adding to SDS sample buffer, and boiling at 95 °C for 3 min. After separation by SDS-PAGE and scanning using an electrophoresis analysis system (ESAS 290, Kodak Inc., Rochester, NY), the relative amounts of Bocillin-FL–labeled tPBP2 remaining over time were quantified by densitometric analysis using ImageJ software (National Institutes of Health). The deacylation constants (k3) were calculated by plotting the ln of % Bocillin-FL bound (PBP2/PBP2) versus time, with the slope of the line equal to −k3, using Equation 2,

\[-k_3 = \ln(PBP_2/PBP_2)/t\]  
(Eq. 2)

where PBP2 is the Bocillin-FL–PBP concentration at time t, and PBP2 is the initial concentration of the Bocillin-FL–PBP complex. The half-life (t1/2) of the acylated complex was then calculated using the formula \(t_{1/2} = \ln2/k_3\).

Crystallization

tPBP2 variants were concentrated to 13 mg/ml, and crystallization trials were performed using a Gryphon liquid-dispensing system (Art Robbins, Sunnyvale, CA) in a 96-well sitting-drop format in which 200 nl of protein solution was mixed with 200 nl of well solution. The proteins were screened against a number of sparse matrix screens, including JCSG Cores I-IV and Suite+ (Qiagen, Germantown, MD), with incubation at 18 °C. A number of hits were obtained for tPBP235/02 and tPBP2H041 in solutions containing PEG 600. After optimization, the best crystals were obtained at 18 °C over wells containing 37–40% PEG 600, buffered with 0.1 M CHES at pH 9.1–9.3 for tPBP2H041 and pH 9.6 for tPBP235/02. Crystals appeared after 3–4 days and exhibited a plate-like morphology.

X-ray data collection and model refinement

Crystals of tPBP235/02 and tPBP2H041 were flash-frozen without adding cryo-protectant, and diffraction data were collected at a wavelength of 1.00 Å on an Eiger 16M detector at the SER-CAT 22-ID beamline at the Advanced Photon Source in Argonne, IL (Table 2). For crystals of tPBP2H041, 360° of data in 0.25° oscillations were collected, with an exposure time of 0.2 s/frame and a crystal-to-plate distance of 240 mm. For crystals of tPBP235/02, 360° of data in 0.2° oscillations were collected, with an exposure time of 0.25 s/frame and a crystal-to-plate distance of 200 mm. Both datasets were processed and scaled with HKL2000 (63). Structures were solved by molecular replacement using PHASER (64). To generate the tPBP235/02 structure, the tPBP2WT structure (30) was used as a search model, whereas the structure of tPBP2H041 was solved by molecular replacement using an earlier structure of tPBP2H041 determined at low pH. A4 All models were refined with iterative rounds of model building and automated refinement, using the graphics programs O (65) or COOT (66) and REFMAC (67). The stereochemistry of models was analyzed with PROCHECK (68).

To generate an acylated complex of tPBP2H041, crystals were soaked in a super-saturated solution of ceftriaxone (Sigma) for 2–3 h at room temperature, followed by flash-freezing without additional cryo-protectant. Diffraction data were collected at a wavelength of 1.00 Å on a MX300–HS detector at the SER-CAT 22-BM beamline. 360° of data were collected in 1° oscillations with an exposure time of 6 s/frame and a crystal-to-plate distance of 240 mm and were processed using HKL2000. The structure was solved by refinement of the tPBP2H041 structure. Ceftriaxone was modeled using the 

\(|F_o| - |F_c|\) difference electron density map, followed by iterative cycles of model building and refinement.

Data availability

Coordinates and structure factors have been deposited with the Protein Data Bank with PDB codes as follows: 6VBL,
Crystal structures of PBP2 from ESC-resistant N. gonorrhoeae

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