TITLE: Functional and Structural Characterization of OXA-935, a Novel OXA-10-family β-

lactamase from *Pseudomonas aeruginosa*

RUNNING TITLE: Characterization of *Pseudomonas aeruginosa* β-lactamase OXA-935

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ABSTRACT

Resistance to antipseudomonal penicillins and cephalosporins is often driven by the

overproduction of the intrinsic β-lactamase AmpC. However, OXA-10-family β-lactamases are a

rich source of resistance in *Pseudomonas aeruginosa*. OXA β-lactamases have a propensity for

mutation leading to extended spectrum cephalosporinase and carbapenemase activity. In this

study, we identified isolates from a subclade of the multidrug-resistant (MDR) high risk clonal

complex CC446 with resistance to ceftazidime. Genomic analysis revealed that these isolates

harbored a plasmid containing a novel allele of bla_{OXA-10}, named $bla_{OXA-935}$, which was predicted

to produce an OXA-10 variant with two amino acid substitutions: an aspartic acid instead of

glycine at position 157 and a serine instead of phenylalanine at position 153. The G157D

mutation, present in OXA-14, is associated with resistance to ceftazidime. Deletion of $bla_{OXA-935}$

restored sensitivity to ceftazidime and susceptibility profiling of *P. aeruginosa* laboratory strains

expressing $bla_{OXA-935}$ revealed that OXA-935 conferred ceftazidime resistance. To better

understand the impact of the variant amino acids, we determined the crystal structures of OXA-

14 and OXA-935. In OXA-14, one of two monomers contained the canonical carbamylated lysine-

70 (K70). In contrast, both monomers of OXA-935 were decarbamylated at K70, and the F153S

mutation conferred increased flexibility to the omega (Ω) loop. Compared to OXA-14, the catalytic

efficiency of OXA-935 for nitrocefin was significantly reduced. Amino acid changes that confer

extended spectrum cephalosporinase activity to OXA-10-family β-lactamases are concerning

given rising reliance on novel β-lactam/β-lactamase inhibitor combinations such as ceftolozane-

tazobactam and ceftazidime-avibactam to treat MDR *P. aeruginosa* infections.

KEYWORDS

Pseudomonas aeruginosa, OXA-beta-lactamase, antimicrobial resistance, ceftazidime, crystal

structure

INTRODUCTION

Infections caused by multidrug-resistant (MDR) and extensively drug-resistant (XDR) organisms are an increasing threat to public health. Leading the way are infections by drug-resistant gram-negative bacteria such as *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and members of the *Enterobacteriaceae* family. Of particular interest is the human pathogen, *P. aeruginosa*, which is responsible for diverse infections including bacteremia, pneumonia, urinary tract and skin and soft tissue infections. At baseline, *P. aeruginosa* harbors a large armamentarium of antimicrobial resistance mechanisms including a chromosomally encoded cephalosporinase, AmpC, and basal and inducible antibiotic efflux pumps. *P. aeruginosa* is notable for the frequent acquisition of exogenous genetic material in the form of plasmids that carry large mobile genetic elements (e.g. integrons, transposons, ICE elements) that can contain series of antimicrobial resistance genes (1, 2). One such element, the class 1 integron in1697, was recently identified as part of a novel antimicrobial resistance (AMR) plasmid discovered in ST298*, a subclade within the globally-distributed *P. aeruginosa* high-risk clonal complex 446 (CC446) (1). Characterization of in1697 revealed the presence of several resistance gene cassettes including genes for sulfonamide, quaternary ammonium compounds and aminoglycoside resistance. In most strains, in1697 also contained the β-lactamase gene, *bla*$_{OXA-10}$.

OXA-10 (PSE-2) is a class D β-lactamase originally described in the late 1970s (3, 4) that confers resistance to cefotaxime and ceftriaxone by not ceftazidime (5). Natural and laboratory-selected variants of OXA-10 have been identified, many of which differ in the spectrum of β-lactams that they hydrolyze (6). One such variant, OXA-14, contains a single amino acid change (glycine to aspartate at position 157, G157D) from the OXA-10 parent. The OXA-14 variant has been implicated in clinically significant resistance to ceftazidime (6, 7). In *P. aeruginosa*, variations in OXA-10-type enzymes are a rich source of expanding AMR, as evidenced by several recently identified members of the class, OXA-40, OXA-198, OXA-655 and OXA-656, that confer resistance to carbapenems (8-11).

Although the isolates within this collection of ST298* *P. aeruginosa* strains were typically

susceptible to ceftazidime, we noted three isolates that were resistant to this antibiotic (1).

Interestingly, these three isolates also contained AMR integrons in which the $bla_{OXA\text{-}10}$ gene

contained two mutations that were predicted to result in a glycine to aspartic acid substitution at

position 157 (similar to OXA-14) and a phenylalanine to serine substitution at position 153. In the

present study, we characterized this novel OXA-10 variant, which we designate "OXA-935". We

determined the crystal structures of both OXA-935 and OXA-14. The structures of OXA-935 and

OXA-14 shared most of the structural features previously described for OXA-10 (12); however,

OXA-935 contained a second amino substitution, phenylalanine to serine at position 153 (F153S),

that increased the flexibility of the Ω-loop resulting in the loss of critical carbamylation at the active

site residue lysine 70 (K70).

RESULTS

Identification of OXA-935, a novel class D, OXA-10 family β-lactamase

In a recent study, we described a prolonged epidemic of ST298* XDR *P. aeruginosa* at a single

academic center (1). Many of these strains possessed sequence aligning to a novel AMR plasmid

(pPABL048) harboring the integron, in1697. This plasmid, originally described in the strain

PABL048, confers resistance to anti-pseudomonal penicillins likely due to the presence of $bla_{OXA\text{-}10}$

in the integron. We noted that three isolates (PS1793, PS1796, and PS1797) showed high

levels of ceftazidime resistance not present elsewhere in the collection. Additionally, they also

showed non-susceptibility to ceftazidime-avibactam and ceftolozane-tazobactam by disc

diffusion. These three isolates were closely related and found to possess single nucleotide

variants (SNVs) in the plasmid-borne $bla_{OXA\text{-}10}$ gene leading to G157D and F153S amino acid

substitutions in OXA-10. These isolates also harbored deletions of amino acids 2-30 in AmpD

(1). AmpD negatively regulates the chromosomally encoded cephalosporinase, AmpC and

mutations in AmpD are linked to overexpression of AmpC and increased resistance to

cephalosporins (13, 14). In this study, we explored the molecular mechanism of ceftazidime

resistance in PS1793, PS1796 and PS1797 with a focus on their OXA-10-variant β-lactamase.

We performed long-read sequencing of PS1793 and used this, along with previously-

generated Illumina short reads (1), to assemble a complete genome sequence for PS1793. This

yielded a 7.4 Mb genome, consisting of a 6,868,713 bp circular chromosome and 3 circular

plasmids. Surprisingly, the AMR plasmid pPABL048 described in our previous study aligned to

two separate plasmid sequences in PS1793 (the 318,215 bp PS1793_p1 and 113,189 bp

PS1793_p2) with approximately 19 kb of overlapping sequence present on both plasmids (Fig.

1A-D). No additional sequence was present in these plasmids, suggesting that pPABL048 may,

in fact, be a hybrid plasmid that contains more than one set of replication and partitioning

machinery. This would be similar to other plasmids within this family such as pOZ176 that

possesses both the IncP-2 system and the uncharacterized replication gene described in our

previous study (1, 15). The AMR integron is present in the larger PS1793_p1 plasmid. PS1793

also harbors an additional 69,506 bp plasmid (PS1793_p3) that was not previously described in

PABL048 (Figure 1A-D). However, following a BLAST search, portions of this third plasmid

aligned with the PABL048 chromosome (a 10 kb fragment aligned with 99% identity and a 2.3 kb

fragment aligned with 76% identity) and shared homology with other *Pseudomonas* genus

plasmids. Based on alignments with the PS1793 complete genome, PS1796 was identical (with

0 chromosomal or plasmid SNVs) and PS1797 differed by a single chromosomal SNV (Table S1).

Comparison of the *bla*$_{OXA-10}$ variant seen in PS1793, PS1796 and PS1797 to the NCBI

database revealed that this allele had not been previously described. As such, it was assigned

the name *bla*$_{OXA-935}$ (RefSeq ID WP_141989064.1). When its predicted protein sequence was

compared to other protein sequences within the OXA-10 family, the G157D substitution was

present in multiple homologues, including OXA-11 and OXA-14, which are known to confer

extended spectrum resistance to ceftazidime (7, 16). The F153S substitution was unique to OXA-

935, although OXA-795 possesses a deletion at positions 153 and 154 (Fig. S1). OXA-795 also

confers ceftazidime resistance without an accompanying G157D substitution (17). Phylogenetic

analysis of these OXA-10 family proteins showed that they are divided into two major groups

classified by their first identified and earliest-named members, OXA-7 and OXA-10. OXA-935

belongs to the OXA-10 subgroup and is most closely related to OXA-11, OXA-14, OXA-16, and

OXA-142, although most branches within each group have low bootstrap confidence, likely

secondary to limited sequence variability (Fig. 1E).

We next sought to determine the prevalence of bla_{OXA-10} family genes within the

Pseudomonas genus. By screening 9799 genomes, we found that bla_{OXA-10}-like genes were most

common in *P. aeruginosa* but were also present in other species including *P. stuzeri* and *P. putida*

(Table S2). The bla_{OXA-10} allele was present in a diverse set of species and *P. aeruginosa* STs

while other variants were more likely to be limited to a few or a single ST. Thus far, $bla_{OXA-935}$ has

only been detected in the three ST298* *P. aeruginosa* isolates described in this study (Table S3).

Expression of OXA-935 confers ceftazidime resistance

After identification of PS1793, PS1796, and PS1797 as the only three isolates in our cohort of

ST298* *P. aeruginosa* with resistance to ceftazidime, we sought to characterize the role of the

novel beta-lactamase allele, $bla_{OXA-935,}$ in ceftazidime resistance. Given that OXA-935 shared the

G157D variation with OXA-14 and that expression of OXA-14 has been previously linked to

ceftazidime resistance (6, 7), we hypothesized that the novel beta-lactamase allele, $bla_{OXA-935,}$

conferred ceftazidime resistance on our clinical isolates despite the co-existing truncation of

AmpD. Resistance of PS1793, PS1796, and PS1797 was confirmed using the microbroth dilution

(MBD) method (Table 1). All three isolates demonstrated similarly high levels of resistance to

ceftazidime with MICs of 64 µg/mL. Deletion of $bla_{OXA-935}$ from the AMR plasmid of PS1793,

PS1796 and PS1797 resulted in a reduction in the ceftazidime MIC to 8 µg/mL, 2 µg/mL and 2

µg/mL, respectively, yielding susceptible phenotypes for all three strains. Deletion of $bla_{OXA-935}$

also made the strains susceptible to the 4th generation cephalosporin, cefepime. Previously, when

we deleted the entire pPABL048 plasmid from related strains in CC446, we detected the loss of

resistance to aztreonam and piperacillin/tazobactam (1). Interestingly, deletion of $bla_{OXA-935}$

resulted in minor reductions in the MICs to aztreonam and piperacillin/tazobactam, but did not

restore susceptibility. This suggests that there likely are compensatory mechanisms that govern

resistance to aztreonam and piperacillin/tazobactam. We did not observe any differences in

susceptibility to meropenem, suggesting that OXA-935 is unlikely to contribute to carbapenem

resistance. Other than alterations in MICs to cephalosporins, all three $\Delta bla_{OXA-935}$ deletion strains

maintained resistance to gentamicin and ciprofloxacin and susceptibility to colistin. Taken

together, these results confirm that ceftazidime resistance in PS1793, PS1796 and PS1797 is

driven primarily by the presence of $bla_{OXA-935.}$

To evaluate the impact of the G157D variant in OXA-14 and the G157D, F153S variants

in OXA-935 on β-lactam resistance, $bla_{OXA-10,}$ $bla_{OXA-14,}$ and $bla_{OXA-935}$ were individually cloned into

a plasmid and expressed in *P. aeruginosa* PAO1 and PA14 strain backgrounds. Comparative

MIC data for PAO1 and PA14 expressing OXA-10, OXA-14, and OXA-935 are shown in Table 2.

Compared to strains expressing a vector control, the expression of OXA-14 resulted in a 16-fold

increase in the ceftazidime MIC in PAO1 and an 8-fold increase in PA14, consistent with previous

reports (6, 7, 17). Expression of OXA-935 resulted in a 16-fold increase in ceftazidime MIC in

PAO1 and a 32-fold increase in PA14, providing evidence that both OXA-14 and OXA-935 confer

resistance to ceftazidime, but that expression of OXA-935 has a greater impact on MIC. The

detailed molecular mechanism of the differential impact of OXA-14 and OXA-935 expression on

ceftazidime resistance in various PA strain backgrounds requires more study. Compared to

strains containing an empty vector, expression of OXA-10 resulted in an 8-fold increase in the

piperacillin-tazobactam MIC in PAO1 and a 16-fold increase in PA14. Expression of OXA-14

resulted in a 4-fold increase the MIC to piperacillin-tazobactam in both strains while expression

of OXA-935 resulted in no increase in the MIC to piperacillin-tazobactam. The impact on cefepime

susceptibility was more uniform in that all three proteins, OXA-10, OXA-14, and OXA-935,

resulted in a 4-fold increase in MIC when expressed in both strain backgrounds. These results

confirm previous findings indicating that OXA-10 has activity against piperacillin-tazobactam but

not ceftazidime. In contrast, OXA-935 is not appreciably active against piperacillin-tazobactam in

these strain backgrounds but has substantial activity against ceftazidime. OXA-14 has an

intermediate resistance phenotype.

The amino acid change F153S introduced high flexibility into the Ω-loop of OXA-935

OXA-935 differs from OXA-14 in a single amino acid substitution in the protein's Ω-loop at position

153 (Fig. 1A). Changes in this loop in multiple classes of β-lactamases are known to increase its

flexibility and therefore increase substrate access to the active site (12, 18, 19), which could

explain increased activity against bulky substrates such as ceftazidime. Since structures of OXA-

14 and OXA-935 were unavailable to corroborate this hypothesis, we determined the crystal

structure of OXA-14 and OXA-935. We were able to determine apo structures for both enzymes

(Fig. 2, Table S4), but not in complex with ceftazidime. Both protein structures contained two

chains in the asymmetric unit, corresponding to a dimer as previously described for OXA-10 (12,

20). OXA-935 (PDB code: 7L5V) belonged to the space group $P2_1$, and OXA-14 (PDB code:

7L5R) belonged to the space group $P2_12_12_1$ (Tables S5, S6). Structural alignment of these

revealed a root mean square deviation (r.m.s.d.) of 0.65 driven primarily by a different

conformation of the Ω-loop, where the F153S mutation was located (Fig. 3A-C, Fig. S3). In chain

A of OXA-14, the Ω-loop was closer to the active site and the indole group from W154 interacted

with the carbamylated K70 (3.2 Å) as was seen in OXA-10 (Fig. 3D). The interaction between K70

and W154 is critical for both the activity and the stability of OXA-10 (21). In the chain B of OXA-

14, K70 was decarbamylated, causing an open confirmation, consistent with what was observed

for OXA-10. In contrast, the Ω-loops of both chains in the crystal structure of OXA-935 had open

conformations, the hydrogen bond between K70 and the indole group of W154 was lost (16.4 Å),

and both K70 residues were decarbamylated (Fig. 3E, Fig. S3). This more open confirmation of

the Ω-loop led to a larger and more positively charged active site cavity, which may allow it to

accommodate bulkier and more negatively charged substrates such as ceftazidime (Fig. 3F,G).

An additional structure of OXA-935 determined (PDB code: 7N1M) from different crystallization

conditions and a different space group, $P2_12_12_1$, also revealed that both monomers of OXA-935

had decarbamylated K70 residues and disordered Ω-loops supporting the observations that S153

conferred significant flexibility to the Ω-loop.

The carbamylation of K70 in other class D β-lactamases is favored at more basic pH (22,

23), whereas OXA-935 showed decarbamylated K70 even at a pH of 8.3. We hypothesized that

the F153S substitution conferred enough flexibility to the Ω-loop to destabilize the carbamylated

state of K70 even at basic pH. To corroborate this hypothesis, we used nitrocefin hydrolysis to

examine the activity of OXA-14 and OXA-935 across a range of pH values (pH 7.0 - 8.5) in a

saturated sodium bicarbonate buffer. Sodium bicarbonate stimulated the activity of both enzymes

across the tested pH range (Fig. S2). We observed an increase in nitrocefin hydrolysis for OXA-

14 as pH values increased suggesting that K70 was more stably carbamylated at higher pH.

Conversely, we observed little increase from the baseline in nitrocefin hydrolysis by OXA-935 as

pH increased. Thus, unlike OXA-10, OXA-14, and other related β-lactamases, it is likely that K70

of OXA-935 remains decarbamylated *in vitro* despite increases in pH.

Nitrocefin hydrolysis by OXA-935 is slower than OXA-14

The enzymatic activities of OXA-14 and OXA-935 were tested using nitrocefin as a substrate.

Despite the fact that both proteins harbor the G157D mutation and both proteins are responsible

for ceftazidime resistance, there were significant differences in their kinetic parameters (Table 3).

Consistent with previous reports, the enzyme-substrate binding efficiency of OXA-14 showed

cooperativity even at low enzyme concentrations (2.5 nM) and the $K_{0.5}$, the concentration of

nitrocefin at which OXA-14 was at half maximum velocity, was 10.1 ± 0.5 µM (7, 20). Contrary to

OXA-14, OXA-935 exhibited Michaelis-Menten kinetics and its K_m for nitrocefin (20.8 ± 1.7 µM)

was almost double the $K_{0.5}$ of OXA-14, suggesting that the affinity of OXA-935 for nitrocefin was

approximately half that of OXA-14. When comparing OXA-935 to OXA-14, we saw an even more

dramatic effect on the substrate turnover rate. OXA-935 had a 4.5-fold lower k_{cat} than the V_{max} of

OXA-14 for nitrocefin, suggesting that the F135S mutation present in OXA-935 had a dramatic

impact on the ability of OXA-935 to hydrolyze nitrocefin (Table 3). The crystal structure showed

that the F135S mutation of OXA-935 led to increased Ω-loop conformational flexibility and a lack

of carbamylation at K70 (Fig. 3E) (22). It is conceivable that the lack of carbamylation at the K70

residue led to the poor nitrocefin hydrolysis observed in our biochemical assay. Despite the fact

that the nitrocefin hydrolysis kinetics for OXA-935 were slower than for OXA-14, MBD testing

confirmed that expression of OXA-935 was sufficient for significant resistance to ceftazidime

(Table 2). Additional studies are needed to understand the dynamics of the activation of OXA-

935 in the context of our broth dilution assay.

DISCUSSION

In this study, we characterized a novel OXA-10-family β-lactamase, OXA-935, found in

three XDR ST-298* *P. aeruginosa* isolates unique with respect to their resistance to ceftazidime.

OXA-935 differs from OXA-10 by two amino acid substitutions, G157D which it shares with OXA-

14 and F153S which is novel. We showed that deletion of $bla_{OXA-935}$ from our three isolates

restored susceptibility to ceftazidime and that expression of $bla_{OXA-935}$ in laboratory *P. aeruginosa*

strains was sufficient for resistance to ceftazidime. We also determined the crystal structures of

both OXA-14 and OXA-935. In the crystal structure of OXA-935, the critical active site K70

residue was decarbamylated in both monomers and the F153S substitution resulted in significant

flexibility of OXA-935's Ω-loop. These structural changes likely impact the ability of OXA-935 to

hydrolyze bulkier substrates such as ceftazidime.

The most well characterized pathway of antimicrobial resistance to 3rd generation

cephalosporins, such as ceftazidime and the newer β-lactam/β-lactamase combination

antimicrobials, is the accumulation of chromosomal mutations leading to the overproduction of

the intrinsic cephalosporinase AmpC (19). Overexpression of AmpC is often secondary to

mutations in the AmpC repressor, AmpD. AmpD inactivation in *P. aeruginosa* results in both

moderate basal-level expression and hyperinducible expression of AmpC (14) and is known to

lead to extended spectrum beta-lactam resistance. Genome sequencing of our three isolates

revealed a mutation in AmpD, resulting in a truncated N-terminus (Δaa 2-30). Despite this AmpD

truncation, deletion of *bla*$_{OXA-935}$ restored ceftazidime susceptibility and its ectopic expression

produced resistance in laboratory strains of *P. aeruginosa.* This suggests that the resistance

phenotype observed in our strains was largely driven by OXA-935 expression and less impacted

by the co-existing AmpD truncation. Interestingly, the G157D and F153S substitutions had a more

unpredictable impact on the MICs to additional β-lactam compounds other than ceftazidime.

When compared to OXA-10, expression of both OXA-14 and OXA-935 resulted in decreased

MICs to aztreonam and piperacillin-tazobactam. This suggests that these substitutions, while

extending the spectrum of activity against ceftazidime, may negatively impact the ability of OXA-

935 to hydrolyze other β-lactam compounds and that there is likely a trade-off to mutations that

affect the confirmation of the active site and the flexibility of the Ω-loop.

In *P. aeruginosa* there is growing concern for the increasing spectrum of antimicrobial

resistance among OXA-10-family β-lactamases. OXA-10 has been subject to significant

mutational pressure both in the laboratory where ceftazidime (6), carbapenem (10, 11, 24),

ceftolozane-tazobactam and ceftazidime-avibactam (17, 25, 26) resistant mutants have been

selected, and clinically, as evidenced by the sheer diversity of described mutations leading to

clinically-significant AMR (7, 16, 27-29). Class D β-lactamases share the same general structure

with conserved motifs including an active-site serine 67, a carbamylated lysine (K70), and a

stabilizing and invariant tryptophan residue (W154) within the Ω-loop (Fig. S4) (21). In order to

better understand the impact of the OXA-935 amino acid substitutions on enzymatic function, we

determined the crystal structures of OXA-14 and OXA-935. OXA-14 crystallized as a dimer where

only one of the two K70 residues was carbamylated (22, 23). The Ω-loop conformation was

maintained and the hydrogen bond between the indole group of W154 and K70 was present. The

F153S variant found within OXA-935 yielded a distinctly different structure of the Ω-loop whereby

W154 was oriented away from the active site cavity, carbamylation of K70 was not observed, and

the active site groove was more positively charged. W154, in addition to stabilizing K70, helps

orient K70 so that it has optimal interactions with other residues in the active site (21). Critically,

mutations in W154 (W154C), deletion of F153-W154, and duplications of negatively charged

residues in this region have been identified in *P. aeruginosa* clinical isolates resistant to

ceftazidime-avibactam and ceftolozane-tazobactam (17, 30). In addition, the Ω-loop of OXA-935

was considerably more flexible than in OXA-14. This suggests that both F153 and its neighboring

residue W154 may be critical sites of mutation that allow for increased substrate accessibility of

the active site.

In assessing functional kinetics, purified OXA-935 hydrolyzed nitrocefin at a slower rate

than OXA-14; however, expression during MIC testing was sufficient for ceftazidime resistance.

It is possible that decarbamylation of K70 is contributing to this reduced activity against nitrocefin

in our biochemical assay. While the lack of carbamylation may offer an explanation for poor

nitrocefin hydrolysis, it is unclear what the impact is of this lost post-translational modification on

ceftazidime hydrolysis. Several possible explanations exist for why OXA-935 hydrolyzes

ceftazidime efficiently despite poor nitrocefin hydrolysis. First, it is possible that OXA-935 may be

more highly expressed than OXA-14, more efficiency transported to the periplasm, or more stable

once it arrives there. Alternatively, it is possible that carbamylation of K70 is favored under

conditions that were disrupted during purification and crystallization. In this case, nitrocefin

hydrolysis may be a poor substitute for the kinetics of ceftazidime hydrolysis. Attempts to

crystalize OXA-14 or OXA-935 with bound ceftazidime were unsuccessful which may be due to

rapid hydrolysis by both enzymes and a lack of K70 carbamylation in OXA-935. Regardless, the

new structures of OXA-14 and OXA-935 provide structural insights into the interplay between the

Ω-loop and the active site which governs the broad spectrum of activity for this family of enzymes.

Finally, OXA-10-family β-lactamases are often found in mobile transmissible elements

such as plasmids (17). OXA-935 is no exception in that it is located within a plasmid-borne

integron that harbors additional AMR elements (1). The mobility of OXA-10-family β-lactamases

coupled with their propensity for mutation in the face of antibiotic pressure make this enzymatic

class one of growing concern. The discovery of yet another variant of OXA-10, OXA-935, that

confers ceftazidime resistance, and non-susceptibility to ceftazidime-avibactam and ceftolozane-

tazobactam prior to the clinical introduction of the latter two antimicrobial agents is worrisome.

Minimal amino acid changes in OXA-10 lead to clinically significant extended-spectrum β-

lactamase activity, especially when located in the Ω-loop. Most concerning is the development

of resistance to ceftolozane-tazobactam and ceftazidime-avibactam which are often used as last-

resort therapy to treat MDR *P. aeruginosa* infections. Ultimately, our findings emphasize the

importance of continued molecular surveillance of multidrug-resistant *P. aeruginosa* and

increased recognition of the contribution of OXA-10-family β-lactamases to AMR phenotypes.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

P. aeruginosa PS1793, PS1796, and PS1797 are clinical strains from the respiratory tract of patients at Northwestern Memorial Hospital (NMH) isolated between 2005 and 2007 and PABL048 is a clinical strain from the bloodstream of a patient at NMH isolated in 2001 (1). PA14 and PAO1 are commonly used laboratory strains (31, 32). Relevant characteristics of these strains are listed in Table S7.

Escherichia coli strain TOP-10 (Invitrogen) was used for cloning and *E. coli* strains S17.1 λpir (33) and SM10 λpir were used to introduce plasmids into *P. aeruginosa*. *E. coli* BL21(DE3) with and without the pMagic plasmid (34) were used for protein expression. Bacterial strains were streaked from frozen cultures onto LB agar and, unless otherwise stated, grown at 37˚C in LB.

Antibiotics were used at the following concentrations: irgasan 5 µg/mL (irg), hygromycin 500 µg /mL (hyg), and gentamicin 100 µg/mL (gent) for *P. aeruginosa*, and gentamicin 15 µg/mL, hygromycin 100 µg/mL, kanamycin 50 µg/mL (kan), and ampicillin 200 µg/mL (amp) for *E. coli*. Further details on the strains and plasmids used in this study can be found in Tables S7 and S8.

Hybrid assembly of PS1793 and comparison to PABL048

For short-read and long-read sequencing, genomic DNA was extracted from an overnight culture of PS1793 using a Promega Maxwell Cell DNA Purification Kit (Promega Corp., Madison, WI). For short-read sequencing, a sequencing library was prepared using a Nextera XT kit (Illumina, San Diego, CA) and sequenced using an Illumina MiSeq instrument and a v3 flow cell, yielding 2 x 301 bp paired-end reads for a total of 1,174 Mbp of sequence, with an approximate coverage of 160-fold. For long-read sequencing, genomic DNA from PS1793 was used to create a sequencing library using ligation sequencing kit SQK-LSK109 (Oxford Nanopore, UK, catalogue number SQK-LSK109) and sequenced on the Oxford Nanopore MinION platform using a FLO-MIN106 flow cell. Base calling with default quality score filtering and demultiplexing of sequenced

reads was performed using Guppy (v3.4.5) yielding 31,050 reads totaling 297 Mbp of sequence

and approximate coverage of 41-fold. Hybrid genome assembly was performed using Unicyler

(v0.4.8) (35) with default settings to generate a single circular 6,868,713 bp chromosome and 3

circular plasmids totaling 318,215, 113,189, and 69,506 bp respectively. The PS1793 complete

genome was annotated using the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) (v5.2)

(36, 37) and is available through NCBI BioSample SAMN12162657 and GenBank locus

accessions CP083366-CP083369. PS1793 plasmid contigs and pPABL048 were aligned to each

other and visualized using BRIG (v0.95) (38).

Sequencing of PS1796 and PS1797 and comparison to PS1793

For short-read sequencing of PS1796 and PS1797, genomic DNA was extracted from an

overnight culture as described previously. Sequencing libraries were prepared using a Nextera

XT kit (Illumina, San Diego, CA) and sequenced using an Illumina MiSeq instrument and v3 flow

cell as described previously. Reads were quality trimmed and adapter sequences were removed

using Trimmomatic (v0.36) (39). Trimmed reads were aligned to the PS1793 complete genome

(chromosome and plasmids) using BWA (v0.7.15) (40) (https://arxiv.org/abs/1303.3997v2) and

sorted and indexed using samtools (v0.1.19-44428cd) (41). SNV sites were then identified as

described previously (1).

Alignment and phylogenetic comparison of OXA-10-family β-lactamases

For alignment visualization, multiple alignment of all OXA-10 family β-lactamase amino acid

sequences was performed using Qiagen CLC Sequence Viewer (v8.0) with default parameters.

For phylogenetic analysis, we used all OXA-10 family β-lactamase amino acid sequences and

included OXA-5, a relatively closely related class D β-lactamase, as an outgroup protein (42).

Sequences were aligned using MUSCLE (v3.8.31) (43). A maximum likelihood phylogenetic tree

was constructed based on this alignment using RAxML (v8.2.11) (44) with automatic protein

model selection and gamma model of rate heterogeneity (-m PROTGAMMAAUTO) and 1000

rapid bootstraps to assess support (-f a -N 1000). The resulting tree was plotted using iTOL v.6

(45).

Genus-wide screen for OXA-10-family β-lactamase prevalence

OXA-10 family β-lactamase sequences were obtained from the NCBI Pathogen Detection

Reference Gene Catalog using "OXA-10" as the search query in January 2021. OXA-16 was

additionally included based on literature review (27). *Pseudomonas* genus genomes available in

the *Pseudomonas* Genome Database (46), along with accompanying metadata (species, MLST)

were obtained on November 10th, 2020. After the exclusion of one genome which failed to

download, this yielded a total of 9799 genomes (including both complete and draft genomes).

Nucleotide BLAST alignments were performed using each OXA-10 family β-lactamase as the

query sequence and each genome as the subject sequence. In order to identify which genomes

contained known OXA-10 family β-lactamase genes, BLAST results were parsed to identify

alignments with 100% sequence identity and coverage. In order to identify genomes containing

any OXA-10 family β-lactamase gene, BLAST results were parsed to identify alignments with 90%

sequence identity and coverage for bla_{OXA-10}.

Generation of pEX18HygB and bla_{OXA-14} and $bla_{OXA-935}$ expression plasmids

The hygromycin B resistance gene (*hygR*) and associated promoter (*ampR* promoter) were

amplified from the pFLP_hyg (47) plasmid using primers TT115 and TT116, creating a 1,185 bp

amplicon. Inverse PCR with primers TT113 and TT114 were used to amplify the backbone of the

pEX18Ap plasmid (48), excluding the ampicillin resistance gene and associated promoter,

resulting in a 4,644 bp amplicon. Both amplicons were designed with matching overhangs to allow

for annealing through Gibson assembly. The *hygR* insert and the inverse PCR product of the

pEX18Ap backbone were mixed at a 5:1 ratio and ligated for 30 minutes at 50°C with New

England Biolabs (NEB) Gibson Assembly ® Cloning Kit to create pEX18HygB. The resulting

assembled product was transformed into chemically competent *E. coli* TOP10 and plated on LB

agar plates supplemented with 100 µg/mL hygromycin B (GoldBio, USA). An individual colony

was picked and transferred to 5 mL of LB medium supplemented with 100 µg/mL hygromycin B

and incubated overnight at 37 °C. After 14 h, pEX18HygB was isolated from *E. coli* TOP10 using

a QIAprep Spin Miniprep Kit (Qiagen, Germany) and was verified by sequencing (using 9 pairs of

overlapping primers, TT117 – TT125) at the NuSeq facility at Northwestern University. The

resulting sequences confirmed the creation of the 5,829 bp plasmid, pEX18HygB.

For the creation of OXA β-lactamase expression vectors for protein purification, both full

length (FL) and mature sequence (trunc, Δaa1-20) bla_{OXA-14} (NCBI Reference Sequence

WP_064056056.1) and $bla_{OXA-935}$ (NCBI Reference Sequence WP_141989064.1) were codon

optimized (SmartGene®) for expression in *E. coli*, synthesized (Twist Bioscience) and cloned into

the pMCSG53 vector (49), which contains a tobacco etch virus (TEV) cleavable N-terminal 6x

His-tag, ampicillin resistance and genes for rare codons.

For the creation of plasmids with isopropyl β-d-1-thiogalactopyranoside (IPTG)-inducible

expression of bla_{OXA-10}, bla_{OXA-14} and $bla_{OXA-935}$, the full length (fl) and mature (tr) sequences

without their native promoters were amplified from PABL048 (for bla_{OXA-10}), from pMCSG53-*oxa14*

FL (for bla_{OXA-14}), and from PS1793 (for $bla_{OXA-935}$) using the primers pPSV37_OXA_F_Gibs,

pPSV37_OXA_trunc_F_Gibs and pPSV37_OXA_R_Gibs, respectively.   HindIII-digested

pPSV37 (50) was mixed at a 1:3 ratio with each OXA β-lactamase gene product and ligated using

the NEB Gibson Assembly ® Cloning Kit. The resulting vectors, pPSV37-tr-*oxa10*, pPSV37-fl-

oxa10, pPSV37-tr-*oxa14*, pPSV37-fl-*oxa14* and pPSV37-tr-*oxa935,* pPSV37-fl-*oxa935* were

verified by sequencing using primers SeqFwPr and SeqRevPr PSV37 and they, along with

pPSV37 vector control, were transformed into electrocompetent (51) PAO1 and PA14. All

recombinant methods including introduction of plasmids expressing OXA-10, OXA-14 and OXA-

935 in PAO1 and PA14 were reviewed and approved by the Northwestern University Institutional

Biosafety Committee. All primer sequences are listed in Supplementary Table S8.

Generation of $\Delta bla_{OXA-935}$ *P. aeruginosa* PS1793, PS1796, and PS1797

Upstream and downstream fragments surrounding the $bla_{OXA-935}$ gene were amplified from

PS1793 genomic DNA using the following primers: oxa10 5-1-HindIII, oxa10 5-2, oxa10 3-1, and

oxa10 3-2-HindIII where oxa10 5-2 and oxa10 3-1 contain a 24-bp overlapping linker sequence

(*TTCAGCATGCTTGCGGCTCGAGTT*) to generate an in-frame deletion of the $bla_{OXA-935}$ gene

(Table S8). The resultant upstream and downstream fragments were used as templates for

overlap extension PCR and amplified with oxa10 5-1-HindIII and oxa10 3-2-HindIII to create a

single linear fragment for insertion. The integration proficient vector, pEX18HygB, was cut with

HindIII and the plasmid and insertion fragment were ligated using the NEB Gibson Assembly ®

Cloning Kit. The resulting vector, pEX18HygB-$\Delta bla_{OXA-935}$, was verified by sequencing at the

NuSeq facility at Northwestern University and transformed into *E. coli* SM10 λpir. Following

conjugation and allelic exchange with PS1793, PS1796 and PS1797 whole-genome sequencing

was performed on all mutant strains to confirm the mutation. Briefly, genomic DNA was isolated

from an overnight culture of each parental strain and its corresponding mutant, sequencing

libraries were prepared using a Nextera XT kit, and sequencing performed using an Illumina

MiSeq instrument and a v2 flow cell, yielding 2 x 251 bp paired end reads. Reads were quality

trimmed and aligned to the PS1793 complete genome as previously described. The site of the

deletion was examined using Tablet v1.21.02.08 (52).

Protein production and purification

Gene sequences of the mature sequence from OXA-14 and OXA-953 were cloned as described

above. The plasmids were transformed into *E. coli* BL21(DE3)(Magic) cells (53) and

transformants were cultured in Terrific Broth (TB) medium supplemented with 200 µg/ml ampicillin

and 50 µg/ml kanamycin. The expression of the protein was induced by addition of 0.5 mM IPTG

when cultures reached OD_{600}=1.8-2.0; cultures were further incubated at 25 °C at 200 rpm for 14

h (53). The cells were harvested by centrifugation, resuspended in lysis buffer (50 mM Tris pH

8.3, 0.5 mM NaCl, 10% glycerol, 0.1% IGEPAL CA-630) and frozen at -30°C until purification.

Frozen suspensions of bacteria were thawed and sonicated at 50% amplitude, in 5 s x 10 s cycle

for 20 min at 4°C. The lysate was centrifugated at 39,000 x *g* for 40 min at 4°C, the supernatant

was collected, and the protein was purified as previously described with some modifications (22,

54). The supernatant was loaded onto a His-Trap FF (Ni-NTA) column using a GE Healthcare

ÅKTA Pure system in loading buffer (50 mM Na+/Phosphate buffer pH 7.8, 0.5 M NaCl, 1 mM

Tris(2-carboxyethyl) phosphine (TCEP) and 5% glycerol). The column was washed with 10

column volumes (cv) of loading buffer followed by 10 cv of wash buffer (50 mM Na+/phosphate

buffer pH 7.8, 1 M NaCl, 1 mM Tris(2-carboxyethyl) phosphine (TCEP) and 5% glycerol, 25 mM

imidazole), and was eluted with elution buffer (50 mM Na+/phosphate buffer pH 7.8, 0.5 M NaCl,

1 mM Tris(2-carboxyethyl) phosphine (TCEP) and 5% glycerol, 1 M imidazole). The protein was

loaded onto a Superdex 200 26/600 column and run with loading buffer. The peak fraction was

collected, mixed with TEV protease (1:20 protease:protein) and incubated overnight at room

temperature to remove the 6xHis-tag. The cleaved protein was separated from TEV and the tag

by affinity chromatography (Ni-NTA) and dialyzed in crystallization buffer (20 mM K^+/Na^+ pH 7.8)

for 2 h, concentrated to 8-9 mg/ml and set up for crystallization immediately or flash-frozen and

stored at -80°C for further use.

Crystallization and data collection

Purified OXA-14 or OXA-935 proteins were set up as 2 µl crystallization drops (1µl protein:1µl

reservoir solution) in 96-well plates (Corning) using commercially available Classics II, Anions

and Ammonium sulfate suites (Qiagen). Diffraction quality crystals of OXA-14 apo-form (PDB

code 7L5R) grew from 0.1 M bicine pH 9.0, 2.4 M ammonium sulfate and were cryoprotected

using 2 M lithium sulfate prior to freezing. Crystals of OXA-935 (PDB code 7L5V) grew from 0.2

M ammonium acetate, 0.1 M Tris pH 8.5, 25% PEG 3350 and crystals for the second OXA-935

structure (PDB code: 7N1M) grew from 0.2 M ammonium iodide, 2.2 M ammonium sulfate (Tables

S4, S5) and were similarly cryoprotected.

The data sets were collected at the beam lines 21ID-D and 21ID-F of the Life Sciences-

Collaborative Access Team (LS-CAT) at the Advanced Photon Source (APS), Argonne National

Laboratory. Images were indexed, integrated and scaled using HKL-3000 (55).

Structure solution and refinement

The OXA-14 and OXA-935 structures were solved by Molecular Replacement with Phaser (55)

from the CCP4 Suite (56) using the crystal structure of the OXA-10 (PDB code 1E3U) as a search

model. Initial solutions went through several rounds of refinement in REFMAC v. 5.8.0258 (57)

and manual model corrections using Coot (58). The water molecules were automatically

generated using ARP/wARP (59) and ligands were manually fit into electron density maps. The

Translation–Libration–Screw (TLS) groups were created by the TLSMD server (60)

(http://skuldbmsc.washington.edu/~tlsmd/) and TLS corrections were applied during the final

stages of refinement. MolProbity (61) (http://molprobity.biochem.duke.edu/) was used for

monitoring the quality of the model during refinement and for the final validation of the structure.

Final model and diffraction data were deposited to the Protein Data Bank (https://www.rcsb.org/)

with the assigned PDB codes: 7L5R (OXA-14), 7L5V (OXA-935) and 7N1M (OXA-935 #2) (Table

S6). Structures were visualized using PyMOL v2.4., Schrodinger, LLC®. Composite omit maps

were created in CCP4 at the sigma level (62).

Structural and sequence alignment

The primary amino acid sequences of OXA-10, OXA-14 and OXA-935 were aligned in Clustal

Omega (63). The alignment was used to produce the secondary structure depiction using OXA-

935 (PDB code 7L5V) as template using ESPript 3.0 server (64). Additional structure alignments

were performed using the POSA (65) and FATCAT (66) servers.

Minimal Inhibitory Concentrations

Minimal inhibitory concentrations (MICs) for *P. aeruginosa* strains were determined in triplicate

using the MBD protocol described by Weigand, et al. (67). The following antibiotics were prepared

from commercially available sources and were used to assess MICs: piperacillin/tazobactam,

cefepime, ceftazidime, ciprofloxacin, meropenem, gentamicin, colistin and aztreonam. For *P.*

aeruginosa strains containing expression vectors, 1 mM of IPTG was added to the MIC assay

plate to induce OXA-β-lactamase expression.

Kinetic Assays

The enzyme kinetic parameters of purified OXA-14 and OXA-935 β-lactamases were determined

measuring the initial hydrolysis of nitrocefin (λ = 490 nm, and $\Delta\varepsilon$ = 17,400 M^{-1}cm^{-1}) over time using

different concentrations of nitrocefin dissolved in 100 mM sodium phosphate with 50 mM sodium

bicarbonate, pH 7.0. The reactions were performed using clear bottom 96-well plates (Grenier) at

30˚C and measured on the Tecan Safire2 spectrophotometer. The protein concentrations of

OXA-14 and OXA-935 were 2.5 nM and 10 nM, respectively. For OXA-935, the k_{cat} (turnover rate)

and K_m (Michaelis constant) values were obtained through nonlinear regression of the data by the

Michaelis-Menten equation, using the plot V/[E] versus [S] (where V is the initial velocity, and [E]

and [S] are the enzyme and substrate concentration, respectively). For OXA-14, the initial velocity

was plotted against the concentrations of nitrocefin but fitted using the Hill equation to determine

$K_{0.5}$ and V_{max}. All plots and curve fitting were generated using Prism software (GraphPad v.9.1.2).

Each experiment was performed in triplicate.

The effect of carbamylation on OXA-14 and OXA-935 was tested using purified protein

(OXA-14 and OXA-935) at 10 nM, incubated with 50 mM of nitrocefin prepared in 100 mM of

sodium phosphate buffer at the following pH values: 7.0, 7.5, 8.0, 8.5 with or without 50 mM of

sodium bicarbonate as indicated. Solutions were prepared in a 96-well plate with a total volume

of 100 µL. Nitrocefin hydrolysis was measured as described above. Each condition was assayed

in triplicate. Velocity was determined using the following equation: nitrocefin (µM/min) = ((slope

(abs/min)/path length (cm)) x $\Delta\varepsilon$) x 1,000,000. Linear regression of the initial velocity (nitrocefin

(µM/min)), was determined and plotted according the pH.

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FIGURES, TABLES and LEGENDS

FIGURE 1 Comparison of the plasmids found in *P. aeruginosa* PS1793 with pPABL048 and

phylogenetic relationships between OXA-10-like family serine β-lactamases. (A) Alignment

of pPABL048 (blue) with the three plasmids present in PS1793, p1 (teal), p2 (magenta) and p3

(green). **(B, C)** Alignment revealed that two separate plasmids, p1 and p2 shared substantial

sequence overlap with pPABL048. PS1793_p1 and p2 also share an overlapping 19 kb region.

(D) The 69 kb PS1793_p3 plasmid did not share substantial sequence with the previously

characterized pPABL048 plasmid. **(E)** Maximum likelihood phylogenetic tree of OXA-10-family

serine β-lactamases. Tree is rooted at OXA-5, which was included as an outgroup. Bootstrap

confidence is indicated by color. Analysis revealed two major families with OXA-10 and OXA-7

as their earliest-identified members.

TABLE 1 Minimal Inhibitory Concentrations (MICs)

	MICs (μg/mL)							
Strain	**Gent**	**Cipro**	**Col**	**Mero**	**Pip/Tazo**	**Az**	**Cep**	**Ctz**
PS1793	>128 – ns	32 – ns	1	16 – ns	64 – ns	16 - ns	16 - ns	64 - ns
PS1793 Δ*bla*_{oxa935}	>128 – ns	32 – ns	0.5	16 – ns	32 – ns	16 - ns	8	8
PS1796	>128 – ns	32 – ns	1	16 – ns	64 – ns	16 - ns	16 - ns	64 - ns
PS1796 Δ*bla*_{oxa935}	>128 – ns	16 – ns	0.25	8 – ns	32 – ns	16 – ns	4	2
PS1797	>128 – ns	32 – ns	1	16 – ns	128 - ns	16 – ns	32 - ns	64 – ns
PS1797 Δ*bla*_{oxa935}	>128 – ns	16 – ns	0.25	8 – ns	32 – ns	16 – ns	4	2

771 Gent: gentamicin, Cipro: ciprofloxacin, Col: Colistin, Mero: Meropenem, Pip/Tazo: Piperacillin-Tazobactam,
772 Az: Aztreonam, Cep: Cefepime, Ctz: Ceftazidime
773 ns: non-susceptible (intermediate and resistant); Clinical Laboratory Standards Institute, MIC Interpretive
774 Standards (μg/mL), 2018.

TABLE 2 MICs for PAO1 and PA14 producing OXA-10, OXA-14, and OXA-935

Strain	MICs (μg/mL)			
	Pip/Tazo	Az	Cep	Ctz
PAO1 + pPSV37	2	4	1	1
PAO1 + pPSV37-*oxa10*	16	8	4	1
PAO1 + pPSV37-*oxa14*	8	8	4	16 – ns
PAO1 + pPSV37-*oxa935*	2	4	4	16 – ns
PA14 + pPSV37	2	4	1	2
PA14 + pPSV37-*oxa10*	32 – ns	8	4	4
PA14 + pPSV37-*oxa14*	8	8	4	16 – ns
PA14 + pPSV37-*oxa935*	2	8	4	64 - ns

776 Pip/Tazo: Piperacillin-Tazobactam, Az: Aztreonam, Cep: Cefepime, Ctz: Ceftazidime
777 ns: non-susceptible (intermediate and resistant); Clinical Laboratory Standards
778 Institute, MIC Interpretive Standards (μg/mL), 2018.
779
780

781
782 **FIGURE 2 Differences in the structures of OXA-935 and OXA-14. (A)** Sequence alignment of

783 the Ω-loop of OXA-935, OXA-14 and OXA-10 indicating in orange (F) and green (S) the

784 changes in the residue 153**.** Cartoon representation of the asymmetric dimeric structures of **(B)**

785 OXA-935 and **(C)** OXA-14 showing the Ω-loop in green for OXA-935 and orange for OXA-14.

FIGURE 3 The F153S substitution disrupts the interactions of W154 in the Ω-loop of OXA-

935 with the catalytic residue K70. **(A)** Structural alignment of OXA-14 (blue) and OXA-935

(pink) highlighting the Ω-loop in orange and green, respectively. Zoomed in view of the Ω-loop

of **(B)** OXA-14 and **(C)** OXA-935. Position of the Ω-loop and interactions of W154 and the

catalytic residue K70 in **(D)** OXA-14 and **(E)** OXA-935   Dashed lines represent hydrogen bond

792   interactions. The continuous black line shows the distance between K70 and W154 in OXA-

793   935. Surface charge representation of the Ω-loop and the active site of **(F)** OXA-14 and **(G)**

794   OXA-935.

TABLE 3 Steady-state kinetic constants of nitrocefin hydrolysis for OXA-14 and OXA-935[a]

	bla_{OXA-14} (G157D)		$bla_{OXA-935}$ (G157D, F153S)	
	v_{max} (s^{-1})	$K_{0.5}$(μM)	k_{cat} (s^{-1})	K_m (μM)
Nitrocefin	18.5 ± 0.5	10.1 ± 0.5	4.0 ± 0.169	20.8 ± 1.7

797
798   [a]The data represent the means $\pm$ standard deviations from three independent experiments.
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