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Characterization of a Carbapenem-Hydrolyzing Enzyme, PoxB, in *Pseudomonas aeruginosa* PAO1

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***Pseudomonas aeruginosa* is an opportunistic pathogen often associated with severe and life-threatening infections that are highly impervious to treatment. This microbe readily exhibits intrinsic and acquired resistance to varied antimicrobial drugs. Resistance to penicillin-like compounds is commonplace and provided by the chromosomal AmpC β-lactamase. A second, chromosomally encoded β-lactamase, PoxB, has previously been reported in *P. aeruginosa*. In the present work, the contribution of this class D enzyme was investigated using a series of clean in-frame *ampC*, *poxB*, and *oprD* deletions, as well as complementation by expression under the control of an inducible promoter. While *poxB* deletions failed to alter β-lactam sensitivities, expression of *poxB* in *ampC*-deficient backgrounds decreased susceptibility to both meropenem and doripenem but had no effect on imipenem, penicillin, and cephalosporin MICs. However, when expressed in an *ampCpoxB*-deficient background, that additionally lacked the outer membrane porin-encoding gene *oprD*, PoxB significantly increased the imipenem as well as the meropenem and doripenem MICs. Like other class D carbapenem-hydrolyzing β-lactamases, PoxB was only poorly inhibited by class A enzyme inhibitors, but a novel non-β-lactam compound, avibactam, was a slightly better inhibitor of PoxB activity. *In vitro* susceptibility testing with a clinical concentration of avibactam, however, failed to reduce PoxB activity against the carbapenems. In addition, *poxB* was found to be cotranscribed with an upstream open reading frame, *poxA*, which itself was shown to encode a 32-kDa protein of yet unknown function.**

Pseudomonas aeruginosa is a ubiquitous and versatile opportunistic pathogen commonly affecting immunocompromised individuals, such as those with severe burns, AIDS, and cancer (1–6). In addition, it is a significant source of nosocomial infections and the second most common cause of ventilator-associated pneumonia in the hospital setting (7, 8). Most notably, *P. aeruginosa* is the primary pathogen associated with lung deterioration and mortality in patients with cystic fibrosis (CF), a deadly, autosomal recessive genetic disorder affecting about 70,000 individuals worldwide (9, 10). Treatment often proves challenging and ineffective, as the bacterium exhibits innate and acquired resistance to a broad range of antibiotics (11–13). In particular, resistance to the frequently used β-lactam-type antibiotics is common and mediated by the expression and derepression of the chromosomally encoded AmpC β-lactamase (14–17).

The Ambler classification scheme distinguishes four different classes of β-lactamases (classes A, B, C, and D) on the basis of their amino acid sequences (18). All four classes have been reported in *P. aeruginosa* and are often transposon or plasmid borne (19–23). In addition to the acquired β-lactamases, *P. aeruginosa* strains frequently carry the chromosomal and clinically relevant class C AmpC β-lactamase mentioned above (24, 25). Recently, Fajardo et al. identified another naturally occurring β-lactamase, termed PIB-1 (PA5542), conferring intrinsic carbapenem resistance to the clinical isolate 59.20 (26). PIB-1 is ubiquitous in all sequenced *P. aeruginosa* strains but does not appear to be highly expressed in the lab strain PAO1. A third chromosomally encoded β-lactamase, termed PoxB (OXA-50), has been reported in *P. aeruginosa* PAO1 and is the focus of the present study (27, 28).

PoxB belongs to the class D β-lactamases, also termed oxacillinases because of the ability of some members of this class, particularly the earlier reported ones, to degrade isoxazolyl penicillins, such as oxacillin, methicillin, and cloxacillin (29–34). The DBL numbering system is used to correlate homologous residues and signature sequences across class D β-lactamases that may otherwise be located at different amino acid positions (35). Differences in these amino acid signature sequences place PoxB in a new branch of the oxacillinase phylogenetic tree, suggesting that it is only weakly related to other oxacillinases (27, 28). For instance, class A and D enzymes commonly have a serine-threonine-phenylalanine-lysine (STFK) motif at position 70 in the DBL numbering system, where serine, the active-site residue, and lysine are conserved in serine β-lactamases and in penicillin-binding proteins (35, 36). To our knowledge, however, PoxB is only one of two oxacillinases where the traditional STFK motif of class D β-lactamases is replaced by serine-threonine-tyrosine-lysine (STYK) (27, 28), with the other being OXA-62 from *Pandoraea pnomenusa* (37–39). Thus, PoxB appears to be a new kind of oxacillinase, markedly different from other known OXA enzymes. In addition, a previous *in silico* analysis suggested that *poxB* may form a two-gene operon with the upstream open reading frame (ORF) *PA5513*, termed *poxA* (27), which is predicted to encode a putative

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hydrolase or acyltransferase of yet unknown function. The role of *poxA* and its relation to *poxB* have yet to be determined.

In the present work, we examined the role of PoxB and its contribution to β-lactam resistance by generating single and multiple in-frame deletions of the chromosomal β-lactamase-encoding genes *ampC* and *poxB* and of the outer membrane-encoding porin *oprD*. In addition, we studied the activity of avibactam, a novel non-β-lactam β-lactamase inhibitor, and investigated the hypothesis that *poxA* and *poxB* may form an operon.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains, plasmids, and primers used in this study are shown in Table S1 in the supplemental material. *Escherichia coli* and *P. aeruginosa* were cultured routinely in lysogenic broth (LB; 10 g tryptone, 5 g yeast extract, 5 g NaCl per liter). Cation-adjusted Mueller-Hinton (CAMH) broth and agar (BBL, BD) were used for susceptibility testing using the broth microdilution method and Etest, respectively. The following antibiotics were used at the indicated concentrations: ampicillin (Ap) at 100 μg/ml and gentamicin (Gm) at 15 μg/ml for *E. coli* and Gm at 75 μg/ml for *P. aeruginosa*.

Construction of *poxA*, *poxB*, and *oprD* deletion mutants. A single in-frame deletion of *poxA* (*PA5513*) was constructed using overlap extension PCR and homologous recombination as previously described (40). Briefly, sequences upstream (816 bp) and downstream (773 bp) of the target deletion were amplified using primer pairs CA*poxA*UF1-CA*poxA*UR1 and CA*poxA*DF2-CA*poxA*DR2, respectively (see Table S1 in the supplemental material). The PCR products were then ligated through another round of PCR, cloned into the suicide vector pEXG2 (41), and moved into *P. aeruginosa* PAO1 for homologous recombination with the genome. Double-crossover recombinants were selected for by screening for Gm sensitivity and sucrose resistance. The deletion was confirmed by PCR amplification of the deletion product from the genome and sequencing. The strain is henceforth referred to as PAOΔ*poxA*. In-frame deletions of *poxB* (*PA5514*) and *oprD* (*PAO958*) were generated in a similar manner using primer pairs DB*poxB*UF1-DB*poxB*UR1 and DB*poxB*DF2-DB*poxB*DR2 and primer pairs DZ*oprD*UF1-DZ*oprD*UR1 and DZ*oprD*DF2-DZ*oprD*DR2, respectively (see Table S1 in the supplemental material). The *poxB* and *oprD* deletions were also introduced into a previously generated *ampC* deletion strain (PAOΔ*ampC*) (42) to create strains PAOΔ*ampC*Δ*poxB*, PAOΔ*ampC*Δ*oprD*, and PAOΔ*ampC*Δ*poxB*Δ*oprD*.

PCR amplification and cloning of *poxA*, *poxB*, and *ampC*. The *P. aeruginosa* PAO1 genome was used as the template for PCR amplification of the *poxA* (*PA5513*), *poxB* (*PA5514*), and *ampC* (*PA4110*) ORFs with primer pairs DZ*poxA*F-DZ*poxA*R, DZ*poxB*F-DZ*poxB*R, and DZ*ampC*F-DZ*ampC*R, respectively (see Table S1 in the supplemental material). The amplicons were independently cloned into the pCR2.1-TOPO vector and sequenced. Each insert was then moved into the EcoRI-BamHI sites of the broad-host-range expression vector pMMB67EH-Gm (43) and propagated in *E. coli* TOP10 or DH5α cells. The plasmid carrying *ampC*, pAmpC, was subsequently introduced by electroporation into PAOΔ*ampC*Δ*poxB*, while the plasmid carrying *poxB*, pPoxB, was introduced into PAOΔ*poxB*, PAOΔ*ampC*, PAOΔ*ampC*Δ*poxB*, and PAOΔ*ampC*Δ*poxB*Δ*oprD*. The pPoxA plasmid was introduced into PAO1::P_{pox}-*lacZ*.

For protein expression in *E. coli* BL21(DE3), the *poxA* ORF was amplified using primers DZ*poxA*Ford4 and DZ*poxA*Rev4, cloned into the pGEM-T Easy vector (Promega, Madison, WI), and sequenced. The 891-bp fragment was then subcloned into the XhoI and BamHI sites of the broad-host-range shuttle vector pET15bVP (38) and propagated in *E. coli* TOP10 cells (Life Technologies, Grand Island, NY). The resultant plasmid was then introduced into *E. coli* BL21(DE3) cells (44) for expression studies.

Protein expression and one-dimensional gel electrophoresis. To determine if *poxA* was translated, the ORF was cloned into the expression vector pET15bVP and introduced into *E. coli* BL21(DE3) cells as described above. Stationary-phase cultures were diluted to an optical density at 600 nm (OD_{600}) of 0.02 in 25 ml of LB and incubated with shaking at 37°C until the culture density reached an OD_{600} of 0.5. The cells were then induced with 1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) and reincubated at 37°C. One-milliliter samples were taken at the time of induction and every hour for a total of 4 h for both IPTG-induced and noninduced samples. The cells were recovered by centrifugation, resuspended in 100 μl of 4× SDS protein sample buffer, and boiled for 10 min. Proteins were separated on a 12% SDS-polyacrylamide gel and stained with Coomassie brilliant blue.

Analysis of PAO1 mRNA. To determine if *poxA* and *poxB* are cotranscribed, RNA was extracted from the wild-type *P. aeruginosa* PAO1 strain using an RNeasy minikit (Qiagen). cDNA was synthesized with SuperScript III reverse transcriptase (Invitrogen) and an $(NS)_5$ random primer using standard methods (45). Two sets of primers were used to amplify the intergenic region between *poxA* and *poxB* plus 24 bp corresponding to the 3′ end of *poxA* and 53 or 486 bp from the 5′ end of *poxB*. Primers RT*poxA*Ford2 and RT*poxA*Rev1 were designed to amplify a 126-bp product, while amplification with primers RT*poxA*Ford2 and RT*poxA*Rev2 yielded a 559-bp product. RNA samples not treated with SuperScript III were also tested to ensure that no carryover DNA contamination resulted from the RNA isolation procedure.

qPCR analysis of *ampC* and *poxB* mRNAs. Total RNA was extracted from strain PAO1 in the presence and absence of the β-lactam inducer (0.15 μg/ml of imipenem) with the RNeasy minikit (Qiagen). cDNA was synthesized using SuperScript III (Invitrogen) and the $(NS)_5$ random primer as previously described (45). Quantitative PCR (qPCR) was performed with an ABI 7500 cycler (Applied Biosystems) using Power SYBR green PCR master mix with carboxy-X-rhodamine (Applied Biosystems). Primer pairs qRT_*ampC*F-qRT_*ampC*R and RT*poxB*Ford1-RT*poxB*Rev1 were used for the real-time amplification of *ampC* and *poxB*, respectively. The readings were normalized to the expression of the housekeeping gene *clpX* (*PA1802*).

β-Lactamase assay. The β-lactamase assay was modified from a previously published protocol (46). Briefly, stationary-phase cultures were diluted in 25 ml of LB to an OD_{600} of 0.02 and incubated with shaking at 37°C. At an OD_{600} of 0.6, cells were induced with 50 μg/ml of benzylpenicillin, while cells containing the expression plasmid (pPoxB) were induced with 1 mM IPTG. In both cases, induction was carried out for an hour. Ten milliliters of cells was harvested by centrifugation and resuspended in 1,000 μl of 1× BugBuster solution (Novagen, EMD Millipore, Billerica, MA) containing 1 μl of Benzonase (Novagen) and 1 kU of rLysozyme (Novagen). The lysate was rotated for 20 min at room temperature before centrifugation at 4°C for 15 min to collect the β-lactamase-containing supernatant. A 2-μl aliquot of cell lysate was added to nitrocefin (final concentration, 100 μM) in 250 μl of assay buffer. The reaction mixture was incubated at 37°C for 20 min, and the hydrolysis of nitrocefin was measured spectrophotometrically at 482 nm. The total protein concentration in the same supernatant was determined by the Bradford method. The activity was expressed as the number of nanomoles of nitrocefin degraded per minute per microgram of total protein.

MIC. MICs were determined by use of the Etest system according to the manufacturer's instructions (bioMérieux, Marcy l'Etoile, France) or by the broth microdilution method according to standard protocols (47, 48). Etest values are often reported as a range or as two independent values to illustrate the variation seen in biological and technical replicates.

RESULTS AND DISCUSSION

P. aeruginosa infections are commonly treated with a combination of β-lactams and aminoglycosides. Resistance to the β-lactam antibiotics is frequently observed in the clinical setting and is commonly due to the expression of enzymes that can hydrolyze these compounds, namely, β-lactamases (13, 49–52). *P. aeruginosa* clinical isolates often harbor acquired β-lactamases, particularly those of the oxacillinase type (class D) and the metallo-β-lactamase IMP

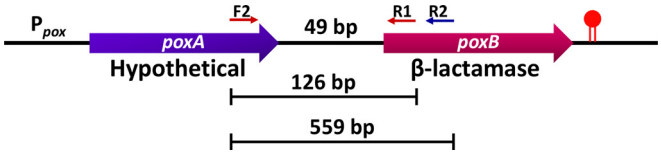

FIG 1 The *poxAB* operon. The *pox* genes and the approximate locations of primers used to show that *PA5513* and *PA5514* form an operon are shown. Two sets of primers were used to amplify the intergenic region as well as the 3′ end of *poxA* and the 5′ end of *poxB* from wild-type cDNA. Primer pair RT-*poxA*Ford2 and RT*poxA*Rev1, denoted F2 and R1, respectively, was used for amplification of a 126-bp product, while primer pair RT*poxA*Ford2 and RT-*poxA*Rev2, denoted F2 and R2, respectively, was used to amplify a 559-bp region. A red hairpin structure downstream of *poxB* denotes the location of a potential Rho-independent terminator.

and VIM types (class B) (23, 25, 53–58). Chromosomally, *P. aeruginosa* carries a cephalosporinase, AmpC, that provides intrinsic resistance and that can be expressed at high levels in the presence of β-lactams (24, 59, 60) and a carbapenemase, PIB-1, that is reported to be ubiquitous but expressed at low levels in strain PAO1 (26). The aim of this study was to examine the role of a third chromosomally encoded class D β-lactamase, PoxB (OXA-50), in *P. aeruginosa* PAO1.

***poxA* and *poxB* form an operon.** A previous *in silico* analysis revealed that the oxacillinase-encoding gene (*PA5514*) is located 49 bp downstream of a putative ORF annotated *PA5513* (Fig. 1). Given the close proximity between *PA5513* and *PA5514*, the absence of promoter-like sequences in the intergenic region, and the presence of a potential ρ-independent terminator downstream of *PA5514*, we previously hypothesized that *PA5513*, termed *poxA*, and *poxB* form a two-gene operon (27). To determine if, in fact, these two genes are cotranscribed, RNA was extracted from the prototypic *P. aeruginosa* strain PAO1, cDNA was synthesized, and two sets of primers (RT*poxA*Ford2-RT*poxA*Rev1 and RT*poxA*Ford2-RT*poxA*Rev2) were used to amplify the intergenic region plus small portions of the 3′ end of *poxA* and the 5′ end of *poxB* (Fig. 1). As expected, the first set of primers yielded a 126-bp amplicon with both genomic DNA and cDNA templates (see Fig. S1, lanes 1 and 2, in the supplemental material), with the second set yielding a 559-bp product with the same templates (see Fig. S1, lanes 5 and 6, in the supplemental material). In the absence of template DNA or reverse transcriptase, no amplification was detected with either primer set (see Fig. S1, lanes 3, 4, 7, and 8, in the supplemental material). This work confirms that these two genes are cotranscribed and that they form an operon.

***poxA* is translated into a 32.4-kDa protein.** Since our present work indicates that *poxA* and *poxB* are cotranscribed, it is also expected that *poxA* mRNA is translated into a protein with a function potentially related to that of PoxB. To confirm our prediction, the *poxA* ORF was amplified and cloned into the expression vector pET15bVP (38) and expressed in *E. coli* BL21(DE3) cells (44). Whole-cell extracts were obtained from cells carrying the *poxA*-overexpressing plasmid in the presence and absence of IPTG. Proteins were visualized in an SDS-polyacrylamide gel. A protein band corresponding to PoxA was detected at about 32 kDa an hour after induction with IPTG and thereafter (see Fig. S2, lanes 3 to 6, in the supplemental material). The same band was also present, but its amount was reduced in whole-cell extracts from uninduced cells that had been grown for 4 h after induction (see Fig. S2, lane 7, in the supplemental material). PoxA was absent under the uninduced and induced conditions at the zero time point (see Fig. S2, lanes 1 and 2, in the supplemental material), as well as from cells containing only the empty vector (see Fig. S2, lanes 8 and 9, in the supplemental material). Thus, the *poxA* ORF codes for a protein.

Since both *poxA* and *poxB* are part of a single operon, they could potentially have related functions. *In silico* analysis revealed that the nucleotide sequences composing this operon are not found anywhere else except in *P. aeruginosa*. Sequences showing a high degree of similarity to the *poxA* ORF (73 to 82%) and the PoxA protein (66 to 78%) were also identified in other *Pseudomonas* species, such as *Pseudomonas denitrificans*, *Pseudomonas entomophila*, *Pseudomonas monteilii*, and *Pseudomonas putida*, but were unlinked to any β-lactamase-encoding gene. Like PoxA, these hypothetical proteins are classified as putative hydrolases or acyltransferases of the α/β hydrolase superfamily. The α/β hydrolase fold is present in a varied number of proteins that share little in terms of function or sequence homology but show structural similarity (61, 62). Proteins carrying the canonical α/β fold include lipases, esterases, proteases, peroxidases, as well as transporters and hormone precursors. Bioinformatics analysis thus failed to pinpoint a clear role for PoxA and its relation to PoxB. From expression studies, however, we do know that *poxA* does not encode a β-lactamase.

Since the role of PoxA was unclear, we investigated whether PoxA could autoregulate the *pox* operon. Autogenous regulation is a common regulatory mechanism in which a gene product regulates expression of the very gene that encodes it (63). Autoregulation, especially negative autoregulation, is a common theme among transcriptional factors and regulators, with over 40% of *E. coli* transcriptional factors regulating their own synthesis (63–65). Autoregulation, however, also occurs in nonregulatory structural genes, including those coding for enzymes involved in a myriad of metabolic processes, often with the first gene regulating the expression of the rest of the genes in that operon (63, 66).

In order to determine if PoxA could autoregulate its own promoter, *poxA* was expressed in a low-copy plasmid under the control of an IPTG-inducible promoter and introduced into strain PAO1 carrying a chromosomal P_{pox}-*lacZ* fusion. In the absence of induction, the *pox* promoter exhibited high basal, constitutive levels of expression in both the presence and absence of *poxA* in *trans* (see Fig. S3 in the supplemental material). After 1 h of induction with IPTG, a small but significant decrease in P_{pox} activity was observed in the presence of *poxA*, suggesting that at high levels PoxA can negatively regulate its own transcription. Although the trend was no longer statistically significant, it was also observed 2 h after IPTG induction. PoxA may thus contribute to maintaining low levels of PoxB. Indeed, *poxB* mRNA levels were relatively low and for the most part appeared to be uninducible in the wild-type strain (Fig. 2). In conclusion, these results suggest that PoxA, a putative hydrolase of yet uncharacterized function, may autoregulate its own promoter.

***poxA* and *poxB* deletions do not alter β-lactamase activity or β-lactam susceptibility.** To investigate the role of *pox* genes in *P. aeruginosa*, single in-frame deletions of *poxA* and *poxB* were generated in the parent strain PAO1, and the β-lactamase and β-lactam susceptibility profiles of the strains were examined. A single deletion of *poxA* or *poxB* did not alter the β-lactamase or susceptibility profiles of the strains compared to those of parent strain PAO1 (Table 1 and Fig. 3). Since the AmpC β-lactamase was pres-

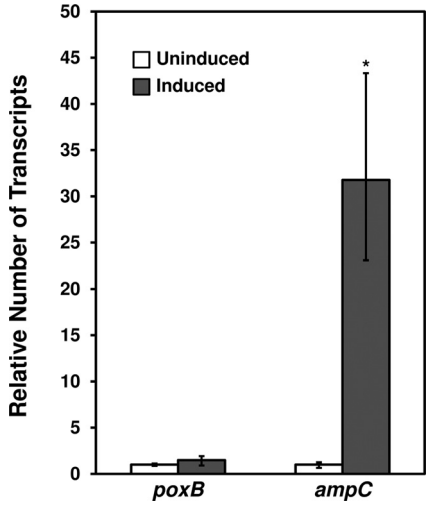

FIG 2 Expression of *poxB* and *ampC* in *Pseudomonas aeruginosa* PAO1. RNA was isolated from PAO1 in the presence (gray bars) and absence (white bars) of β-lactams, reversed transcribed to cDNA, and tested by qPCR with *poxB*- and *ampC*-specific primers, as described in Materials and Methods. Values were normalized to the level of expression of the housekeeping gene *clpX* (PA1802) and represent the means ± SDs from two experiments conducted in triplicate. *, $P = 0.0191$ for *ampC* transcript levels under the induced versus the uninduced condition, as determined by Student's *t* test.

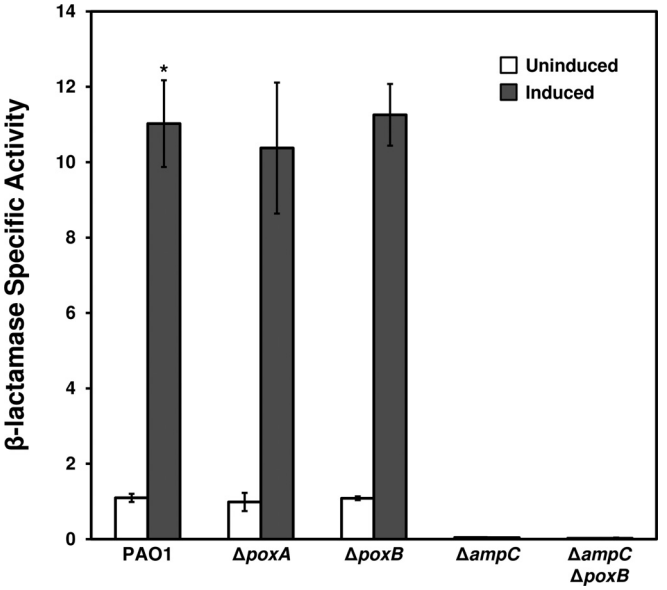

FIG 3 β-Lactamase activity of *pox* and *ampC* deletion mutants. The β-lactamase activities of the PAOΔ*poxA*, PAOΔ*poxB*, PAOΔ*ampC*, and PAOΔ*ampC*Δ*poxB* strains were quantified in the presence (gray bars) and absence (white bars) of β-lactams. Assays were carried out on cell lysates with 100 μM nitrocefin as the chromogenic substrate. One milliunit of β-lactamase activity was defined as 1 nanomole of nitrocefin hydrolyzed per minute per microgram of protein. *, $P < 0.005$ for the β-lactamase activity of induced PAO1 cells versus the activity of uninduced PAO1 cells, as determined by Student's *t* test.

ent in these backgrounds and potentially obscured the role of PoxB, susceptibility and β-lactamase activity were also examined in strains lacking *ampC* (PAOΔ*ampC*) and both *ampC* and *poxB* (PAOΔ*ampC*Δ*poxB*).

Little to no β-lactamase was detected in PAOΔ*ampC* or PAOΔ*ampC*Δ*poxB*, suggesting that AmpC is responsible for the activity observed in the wild type and in the *poxA* and *poxB* deletion strains (Fig. 3). The lack of β-lactamase production seen in PAOΔ*ampC* under the induced condition also shows that PoxB does not respond to induction by β-lactams, in this case, benzylpenicillin. Indeed, further induction studies carried out in PAOΔ*ampC* failed to show induction of PoxB by various other β-lactams, including amoxicillin, carbenicillin, ampicillin, cefoxitin, imipenem, and meropenem (data not shown).

In terms of susceptibility, a single *ampC* deletion significantly increased sensitivity to the aminopenicillins (ampicillin and amoxicillin) and to imipenem but led to only negligible MIC changes for the cephalosporins and for the rest of the penicillins and carbapenems tested (Table 1). When overexpressed, however, AmpC fully restored the phenotype against the aminopenicillins and significantly increased the MICs of the other penicillins and cephalosporins (Table 2), in most cases past clinical breakpoints, while having little to no effect on carbapenem susceptibility. These results are in agreement with the known hydrolysis spectrum of AmpC, which includes penicillins and cephalosporins but rarely carbapenems (67–70).

TABLE 1 Susceptibility profiles of *poxA*, *poxB*, and *ampC* deletion mutants

β-Lactam	MIC[a] (μg/ml)				
	PAO1	PAOΔ*poxA*	PAOΔ*poxB*	PAOΔ*ampC*	PAOΔ*ampC*Δ*poxB*
Ampicillin	>256	>256	>256	16–24	24
Ampicillin-sulbactam	>256	>256	>256	16–32	16–32
Amoxicillin	>256	>256	>256	8–12	16–24
Amoxicillin-clavulanate	>256	>256	>256	8	8
Ticarcillin-clavulanate	8–12	12	8–12	8	12
Piperacillin-tazobactam	3–4	3	3	3	3–4
Piperacillin	4–6	4	4	4	4–6
Aztreonam	1.5–3	2	2	1.5–3	2–3
Cefepime	1–1.5	1–1.5	1	0.75–2	1
Cefotaxime	4–6	6	6	4–6	4
Ceftazidime	1–1.5	1.5	1.5	1.5	1
Imipenem	1–1.5	1.5	1.5	0.25–0.5	0.38–0.5
Meropenem	0.25–0.5	0.38–0.50	0.38	0.25–0.75	0.25–0.38
Doripenem	0.125–0.38	0.38	0.38	0.094	0.094–0.125

[a] MICs were determined by Etest.

TABLE 2 MICs for AmpC- and PoxB-overexpressing strains

β-Lactam	MIC[a] (μg/ml)					
	PAO1(vector)	PAOΔ*ampC* (vector)	PAOΔ*ampC* (pPoxB)	PAOΔ*ampC*Δ*poxB* (vector)	PAOΔ*ampC*Δ*poxB*(pPoxB)	PAOΔ*ampC*Δ*poxB*(pAmpC)
Ampicillin	>256	24	24	32	64	>256
Ampicillin-sulbactam	>256	12	16	12–16	16	>256
Amoxicillin	>256	8–16	16	8–16	24	>256
Amoxicillin-clavulanic acid	>256	8–12	12	8–12	16	>256
Ticarcillin-clavulanic acid	12	6–12	8	8	12	64
Piperacillin-tazobactam	3	2–3	1.5– 2	3	3	64
Piperacillin	3	3–4	4	4	3	>256
Cefepime	1–1.5	1.5	1–1.5	1–1.5	1.5	4
Cefotaxime	6	6	6	8	8	>256
Ceftazidime	1.5	1	1	1	1–1.5	12–16
Imipenem	1.5	0.5	0.5– 0.75	0.5–0.75	0.5– 0.75	0.5
Meropenem	0.38	0.25–0.38	1.0– 1.5	0.25–0.38	1.5–2	0.5
Doripenem	0.38	0.094	1.5	0.094–0.125	1.5–2	0.75

[a] MICs were determined by Etest. The vector used to construct pPoxB and pAmpC was pMMB67EH-Gm.

Lastly, deletion of *poxB* from an already *ampC*-deficient background did not further alter susceptibility, suggesting either that PoxB plays no role in β-lactam resistance or that it is not significantly expressed under the tested experimental conditions (Table 1). Indeed, the levels of *poxB* transcript appeared to be low compared with those of *ampC* in the presence of induction (Fig. 2), suggesting that expression of PoxB in the wild-type strain is not sufficient to produce significant and detectable hydrolysis of β-lactams.

PoxB is a carbapenemase. To determine the hydrolysis spectrum of PoxB in *P. aeruginosa*, we overexpressed the ORF in a low-copy plasmid under the control of an IPTG-inducible promoter in both PAOΔ*ampC* and PAOΔ*ampC*Δ*poxB*. Overexpression produced a 20-fold induction of β-lactamase activity in the presence of IPTG (Fig. 4) but provided little to no resistance to the penicillins and the cephalosporins (Table 2), suggesting that these β-lactams are not good substrates of PoxB. A significant decrease in susceptibility (an increase in MIC) to the carbapenems meropenem and doripenem was observed, however, as determined by the Etest (Table 2). A 4-fold increase in the MIC of meropenem (not determined for doripenem) was also observed using the broth microdilution method (Fig. 5). PoxB, however, did not appear to alter susceptibility to imipenem in an *ampC* or *ampCpoxB*-deficient background, suggesting that PoxB might not be able to hydrolyze this carbapenem.

In carbapenems, the signature β-lactam ring, composed of one nitrogen and three carbons, is fused to an unsaturated five-atom ring with a sulfur substituent at position C-2. The basic structural differences between the carbapenems (imipenem, doripenem, and meropenem) are imparted by the various side chains extending from the sulfur at C-2. Meropenem and doripenem have bulk-

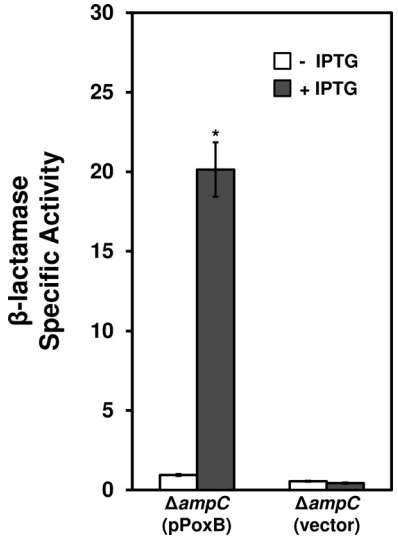

FIG 4 β-Lactamase activity of a PoxB-overexpressing clone. The *poxB* ORF was cloned into the low-copy plasmid pMMB67EH-Gm and introduced into the *ampC*-deficient strain PAOΔ*ampC*. The β-lactamase activity was quantified in the presence and absence of 1 mM IPTG. *, $P < 0.0001$ for the activity in induced PAOΔ*ampC*(pPox) cells versus the activity in uninduced PAOΔ*ampC*(pPox) cells, as determined by Student's *t* test.

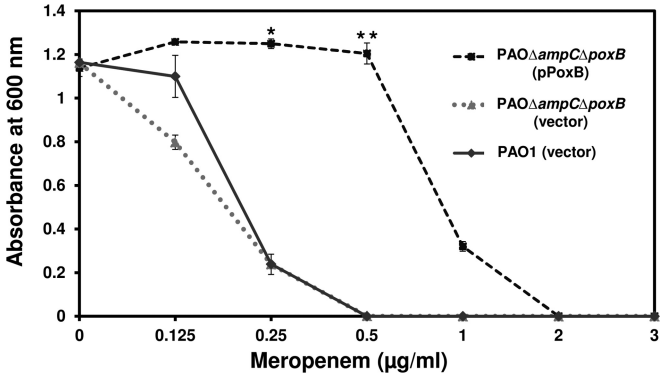

FIG 5 Meropenem MIC in a PoxB-overexpressing background. The meropenem MICs of PAOΔ*ampC*Δ*poxB*(pPoxB), PAOΔ*ampC*Δ*poxB*(vector), and PAO1(vector) were determined by the broth microdilution method. Cells were grown overnight in the presence of 1 mM IPTG with increasing concentrations of meropenem (0 to 3 μg/ml). The final cell density measured at an OD_{600} after 18 h of growth is reported. *, $P < 0.0001$ for the OD_{600} of PAOΔ*ampC*Δ*poxB*(pPoxB) versus the optical density of PAOΔ*ampC*Δ*poxB*(vector) with 0.25 μg/ml meropenem; **, $P = 0.0005$ for the OD_{600} of PAOΔ*ampC*Δ*poxB*(pPoxB) versus the optical density of PAOΔ*ampC*Δ*poxB*(vector) with 0.5 μg/ml meropenem, as determined by unpaired *t* test.

TABLE 3 Susceptibility profiles of *oprD* mutants

Carbapenem	IPTG concn (mM)	MIC[a] (μg/ml) PAO1 (vector)	PAOΔ*oprD* (vector)	PAOΔ*ampC*Δ*oprD* (vector)	PAOΔ*ampC*Δ*poxB*Δ*oprD* (vector)	PAOΔ*ampC*Δ*poxB*Δ*oprD*(pPoxB)
Imipenem	0	1.5	8–12	0.75–1	0.75–1.5	0.75–1.5
	1	1.5	8–16	1	0.75–1	4–8
Meropenem	0	0.38– 0.75	6	2–4	4	6
	1	0.50– 0.75	6	2–4	6	16–32
Doripenem	0	0.38	0.75–1	0.75	1–1.5	1–1.5
	1	0.38	1.5	0.5–1	1	8–12

[a] MICs were determined by Etest.

ier side chains than imipenem. Additionally, meropenem and doripenem have a methyl group at C-1 that is absent from imipenem and which could be the basis for the lack of activity of PoxB against imipenem. However, beyond structural differences, resistance/susceptibility to carbapenems in *P. aeruginosa* must also take into account the role of the outer membrane porin OprD, as well as that of efflux pumps.

OprD is a substrate-specific porin that mediates the diffusion of basic amino acids and most carbapenems into the cell (39, 71, 72). Imipenem remains a viable and powerful treatment option against *P. aeruginosa*; however, mutations in or loss of *oprD* and subsequent resistance to this β-lactam are often selected for with imipenem treatment (73–75). The loss of OprD increases the MICs of doripenem and meropenem to some extent. However, in the absence of OprD, the MIC of imipenem reaches resistance levels of ~8 to 16 μg/ml, as the antibiotic cannot penetrate the cell as fast as it can in the wild type (74, 76, 77). Imipenem still enters the cell, most likely through other pores and porins, albeit at a much lower rate. This lower rate of diffusion makes imipenem a better substrate for the AmpC β-lactamase, which is active in the periplasm of the cell. Thus, in an OprD-deficient background, the activity of imipenem is determined by the expression of AmpC and potentially that of PoxB but not by the Mex efflux system, as imipenem, unlike meropenem and doripenem, is not subject to efflux (78–80). We surmised that if AmpC is also removed from the equation, the activity of imipenem should be determined by PoxB. Previous studies have shown that PoxB has some affinity for imipenem (28); however, PoxB activity on imipenem has not been examined in an OprD-deficient background. We thus set out to test the ability of PoxB to provide resistance to imipenem in the absence of OprD.

Our work corroborates the established literature on OprD-based imipenem resistance. Specifically, in our study, deletion of *oprD* alone significantly decreased susceptibility to the carbapenems, with the greatest increase in MIC being observed for meropenem, followed by that for imipenem (Table 3). AmpC clearly provided resistance to imipenem in this background, since in its absence the strain became very susceptible to this carbapenem. Thus, as observed in previous studies, AmpC afforded greater protection against imipenem in a less permeable background, i.e., with the loss of OprD (Table 3). In our study, resistance to meropenem and doripenem was found to be affected less by AmpC than by the loss of OprD, suggesting that these two carbapenems are not good substrates for AmpC and/or they are efficiently efluxed. In agreement with these results, our single *ampC* deletion mutant also showed a slight increase in susceptibility to imipenem but no change in susceptibility to meropenem (Table 1).

A further deletion of *poxB* from an *ampC* and *oprD* deletion mutant did not alter susceptibility (Table 3). However, overexpression of PoxB in an *oprDampCpoxB*-deficient background significantly increased the MICs of all the carbapenems, including imipenem (Table 3), showing that, in fact, PoxB can hydrolyze all of these β-lactams. In particular, the increase in MIC observed for meropenem reached the CLSI clinical resistance breakpoint for this carbapenem (≥8 μg/ml) (48). Determination of MICs by the broth microdilution method corroborated the Etest results (see Fig. S4A to D in the supplemental material).

Previous biochemical studies showed that the supposed preferred substrates of oxacillinases, namely, oxacillin and cloxacillin, were hydrolyzed very poorly, if at all, by PoxB (28). Additionally, PoxB exhibited very low affinity toward most of the substrates tested, which included benzylpenicillin, ampicillin, piperacillin, and cephalothin. PoxB, however, did exhibit the highest affinity toward imipenem, although its ability to hydrolyze it was low (28). Against meropenem, PoxB exhibited low affinity and weak hydrolysis, while overexpression *in trans* did afford the cells a decrease in susceptibility (increase in MIC) against meropenem but not imipenem (28). Similarly, our work shows that PoxB does not significantly hydrolyze penicillin and cephalosporin β-lactams. In con-

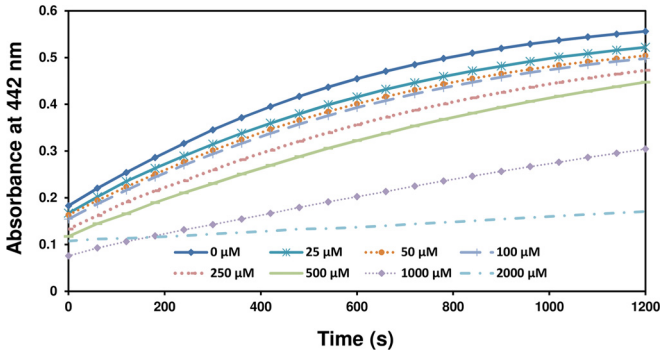

FIG 6 Timed inhibition of PoxB with increasing concentrations of avibactam. Cell lysate from IPTG-induced (1 mM) PoxB-overexpressing cells was incubated with various concentrations of avibactam. The absorbance at 442 nm was measured to examine inhibition of chromacef hydrolysis and, thus, inactivation of PoxB by avibactam.

TABLE 4 Effect of avibactam on carbapenem MICs

Carbapenem	CAMH agar supplement	MIC[a] (μg/ml) PAOΔ*ampC*Δ*poxB* Δ*oprD*(vector)	MIC[a] (μg/ml) PAOΔ*ampC*Δ*poxB* Δ*oprD*(pPoxB)
Imipenem	Alone	0.25	0.75
	IPTG[b]	1.5	6
	IPTG + AV[c]	1.5	4, 6
Meropenem	Alone	6	4
	IPTG	4, 6	32
	IPTG + AV	6, 4	32
Doripenem	Alone	0.75–1	0.75–1
	IPTG	0.75–1	8
	IPTG + AV	1–1.5	8

[a] MICs were determined by Etest.
[b] IPTG was used at a concentration of 1 mM.
[c] AV, avibactam. Avibactam was used at a concentration of 4 μg/ml.

trast to the findings of previous work, however, we show that PoxB, when overexpressed, is capable of hydrolyzing not only meropenem but also imipenem and doripenem, producing a decrease in susceptibility toward these β-lactams. Importantly, our work here also includes strains that lack the outer membrane porin OprD, which we know plays an important role in carbapenem uptake. In short, PoxB is a carbapenemase with a narrow spectrum of hydrolysis.

Avibactam is a weak inhibitor of PoxB β-lactamase. The currently available β-lactamase inhibitors, tazobactam, sulbactam, and clavulanic acid, have traditionally been considered class A enzyme inhibitors, as they generally lack activity against the class B, C, and D β-lactamases (81–85). PoxB is not the exception, and like most class D enzymes, it is only poorly inhibited by these compounds (28). A new reversible β-lactamase inhibitor, termed avibactam, with a broader spectrum of activity that includes class A, some class C, and even some class D enzymes, has recently been approved for clinical use in combination with ceftazidime (86–88). We have examined the ability of avibactam to inhibit PoxB activity in cell lysates from PAOΔ*ampC*Δ*poxB*(pPoxB) by quantifying the hydrolysis of the chromogenic substrate chromacef in the presence of avibactam.

A general downward trend of inhibition was observed with increasing concentrations of avibactam (Fig. 6; see also Fig. S5D in the supplemental material). Avibactam did appear to be a more effective PoxB inhibitor than tazobactam, sulbactam, and clavulanic acid, but even very high concentrations of avibactam failed to completely abolish β-lactamase activity, suggesting that avibactam is only a weak inhibitor (see Fig. S5 in the supplemental material). Specifically, a concentration of 1,000 μM was needed to significantly reduce the PoxB-mediated hydrolysis of chromacef (Fig. 6; see also Fig. S5D in the supplemental material), whereas PoxB activity was decreased by more than half (see Fig. S5D in the supplemental material) at a concentration of 2,000 μM (530.488 μg/ml), which is 75-fold higher than that of the highest concentration of avibactam used in clinical studies (0.5 to 7 μg/ml) (89–91).

PoxB inhibition was also tested by growing PoxB-expressing strains in CAMH agar with IPTG in the presence of avibactam at a clinically relevant concentration (4 μg/ml), which has previously been shown to significantly increase susceptibility and/or eliminate resistance to various β-lactams (92–94). Given that PoxB expression increases the MICs of imipenem, meropenem, and doripenem (Table 3), inhibition of PoxB by avibactam should produce a decrease in the MICs of these carbapenems. Our results show, however, that the carbapenem MICs for PoxB-expressing cells were not altered in the presence of this inhibitor (Table 4), suggesting that avibactam does not significantly inhibit PoxB activity.

Although avibactam has shown variable activity against class D enzymes, it remains a more potent and effective inhibitor of class A (87, 89–91, 95) and C (87, 88, 90, 95) enzymes, including the problematic class A *Klebsiella pneumoniae* carbapenemases, or KPCs (96, 97). OXA-48 appears to be just one of a few class D enzymes against which avibactam is active (89, 98, 99). However, a kinetics study found the rates of reaction of avibactam with OXA-48 and OXA-10 to be much lower than those of the other tested class A and C enzymes (100). Although the structures and hydrolytic activities of oxacillinases vary widely, the emerging trend suggests that avibactam is not a very good inhibitor of class D enzymes. It is thus not surprising that avibactam is only a weak inhibitor of PoxB (OXA-50).

Concluding remarks. The PoxB β-lactamase is a naturally occurring and uninducible enzyme that is expressed at low and constitutive levels in *P. aeruginosa*. The naturally low levels of expression do not appear to contribute to the intrinsic β-lactam resistance of the lab strain PAO1. Its narrow hydrolysis spectrum, however, consisting of carbapenems, was evident upon expression in *trans*. Although the carbapenem-hydrolyzing phenotype was particularly pronounced in the absence of the carbapenem-specific outer membrane porin OprD, the relevance of PoxB in the clinical setting is uncertain.

Mutational overexpression of PoxB has not been previously reported, but the introduction and use of the newly developed class A/C enzyme inhibitors, such as avibactam and relebactam, are likely to act as selective pressure for expression of the naturally encoding carbapenemases in *P. aeruginosa*, namely, PoxB (OXA-50) and the recently reported metallo-β-lactamase PIB-1 (26). Relebactam, currently in clinical development in conjunction with imipenem-cilastatin, and its closely related counterpart, avibactam, can both inactivate *P. aeruginosa* AmpC but generally do not improve the activity of β-lactams against most class B and D enzymes (87, 93, 101–104). The concern about the use of such inhibitors is that inactivation of AmpC by avibactam- or relebactam-like compounds in the presence of carbapenems could select for carbapenem-nonsusceptible strains that overproduce a carbapenemase.

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