Supplemental Materials

	β-lactam resistance determinants							
	MexAB-OprM regulators			MexXY-OprM regulators		Penicillin Binding Proteins		
Isolate	**NalB (MexR)**	**NalC**	**NalD**	**PA5471**	**MexZ**	**PBP1a (PonA)**	**PBP2**	**PBP3 (FtsL)**
PAO1	WT	WT	WT	WT	WT	WT	WT	WT
CLB 24388	WT	G71E, S209R	WT	C40R, L88P, D161G H182Q, V243A	Q103 del	P388S insertion of a P btw A615 and D616	WT	WT
EM 2972704	V126E	G71E, S209R	WT	C40R, L88P, S112N D119E, I237V, V243A	D83E, L138R R187 to F192 del	insertion of a P btw A615 and D616, and V643I	N117S	WT

	Fluoroquinolone resistance determinants				Aminoglycoside resistance determinants	Polymyxin and other resistance determinants				
	QRDRs					Polymyxin				Phenicol, Sulfonamide
Isolate	**GyrA**	**GyrB**	**ParC**	**ParE**	**AMEs**	**PhoP**	**PhoQ**	**PmrA**	**PmrB**	
PAO1	WT	WT	WT	WT		WT	WT	WT	WT	
CLB 24388	T83I	WT	S87L	WT	*aac*(6')-Ib4 *aac*(3)-I family	WT	WT	WT	Y345H	*cat*B7, *sul*1
EM 2972704	T83I	WT	S87L	WT	*aad*A1, *aac*(6')-33, *aac*(6')-Ib4	WT	WT	L71R	S2P, A4T, V15I, G68S, Y345H	*cat*B7, *sul*1

Table S1. Additional resistance determinants

P. aeruginosa PAO1 genome sequence NC_002516.2 was used as the reference genome. Reference comparator amino acid sequences from PAO1 ("WT" - wildtype) listed in Table S1 are as follows: NalB (MexR), NP_249115.1; NalC, NP_252410.1; NalD, NP_2552264.1; PA5471, NP_254158.1; MexZ, AF073776; PBP1A (PonA), NP_253732.1; PBP2, NP_252692.1; PBP3 (FtsL), NP_253109.1; GyrA, NP_251858.1; GyrB, NP_064724.1; ParC, NP_253651.1; ParE, NP_253654.1; PhoP, NP_249870.1; PhoQ, NP_249871.1; PmrA, NP_253464.1; PmrB, NP_253465.1. Abbreviations PBP, penicillin binding protein; QRDR, quinolone resistance-determining region; AME aminoglycoside modifying enzyme.

Variant	1	79	149	178	329	364
PDC-1	G	T	L	V	V	G
PDC-3	G	A	L	V	V	G
PDC-8	G	A	R	V	V	G
PDC-19a	D	A	L	L	I	A

Differences by SANC position between PDC-1, PDC-3, PDC-8, and PDC-19a.

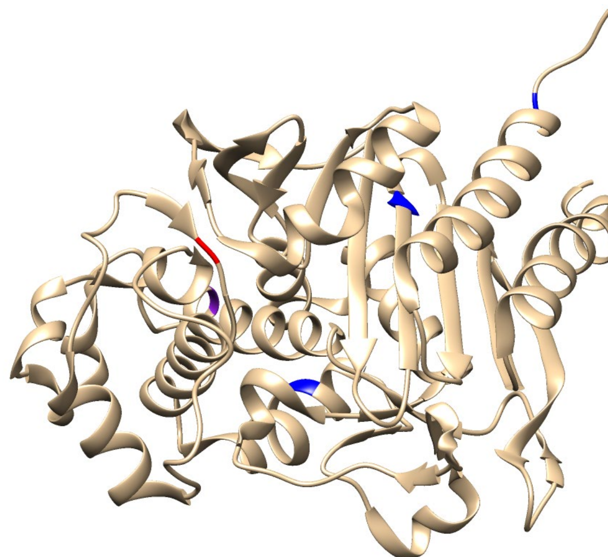

Figure S1. Substitutions present in PDC-8 (red), PDC-19a (blue), or both (purple) compared to PDC-1.

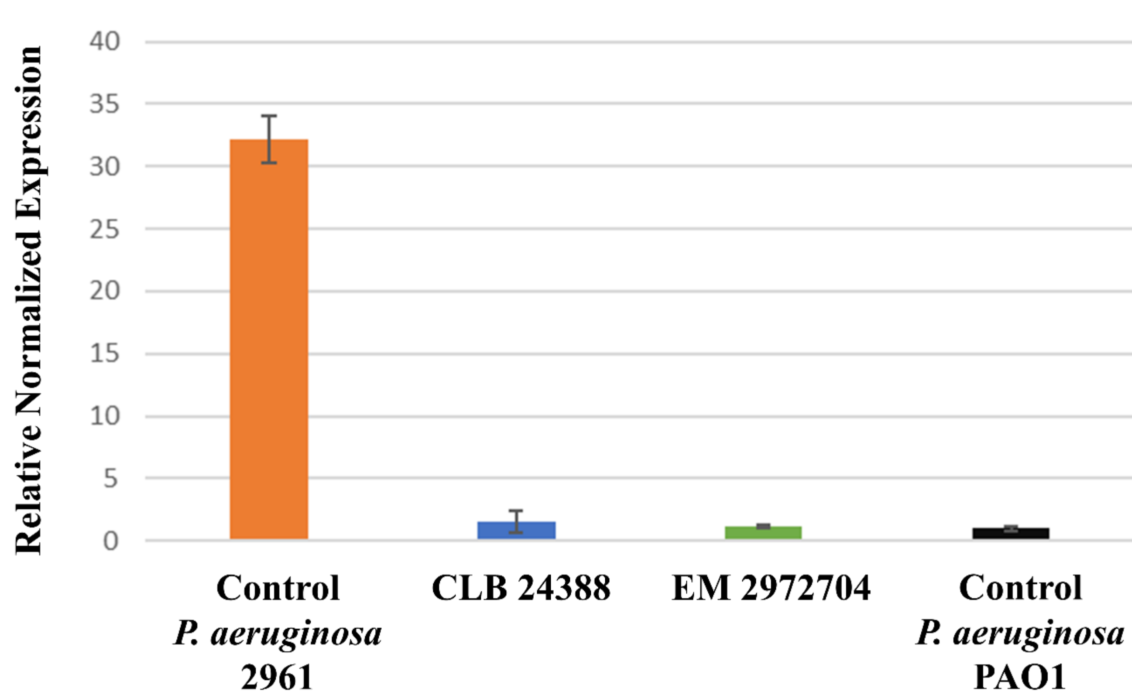

Figure S2. Normalized expression of AmpC (PDC-8 in CLB 24388 and PDC-19a in EM 2972704) in the two study isolates was not significantly different than in the *P. aeruginosa* control strain PAO1 (PDC-1). However, expression of AmpC in isolates CLB 24388 and EM 2972704 was 22-fold and 27-fold less, respectively, than in the *P. aeruginosa* control strain 2961 which is known to overexpress its AmpC (PDC-16) due to a D135N mutation in AmpR. This demonstrates that the PDCs in CLB 24388 and EM 2972704 are not overexpressed. Expression of PDC was set at 1.0 in PA01 and relative expression of variant genes is presented as the mean ± standard deviations.

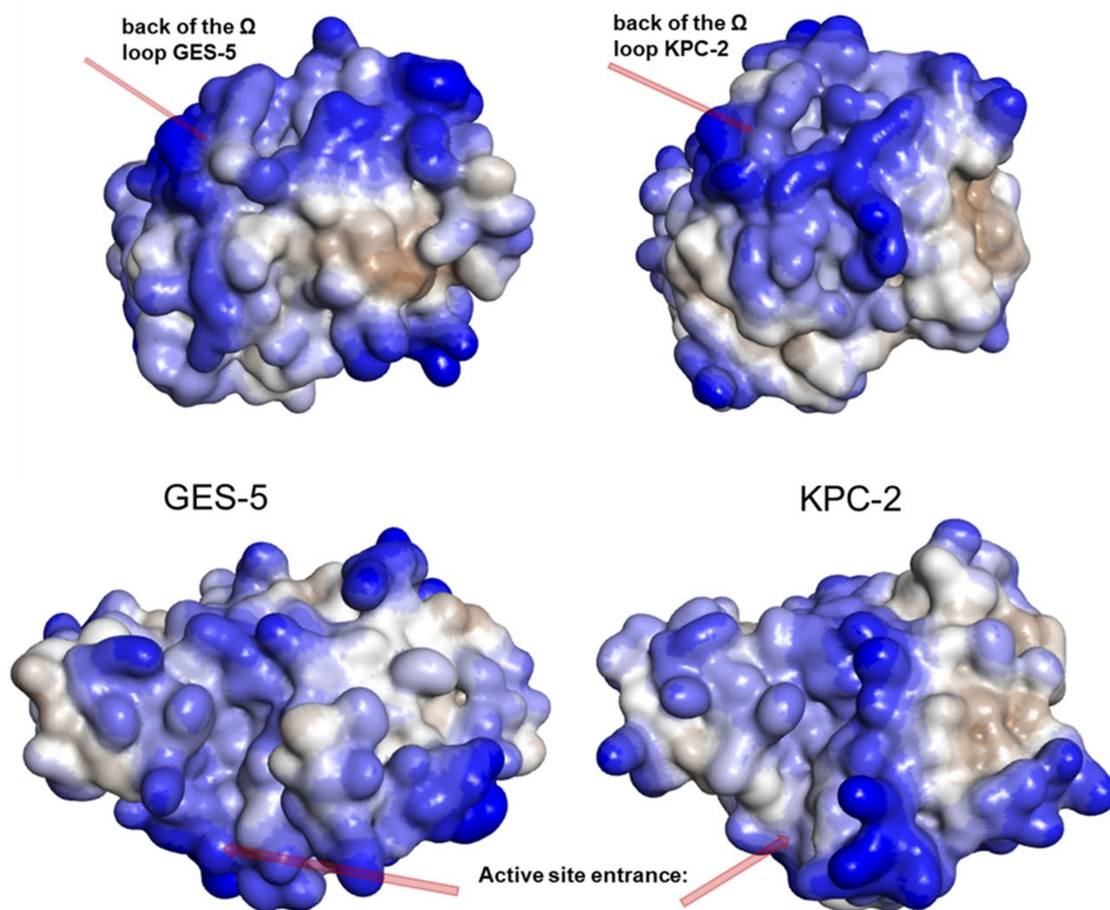

Figure S3. Connolly surface of the active site entrance: change in the shape and hydrophobicity. In KPC-2 the active site entrance is more hydrophobic, and the active site is larger and shallower. Whereas in GES-20 (GES-5), the active site entrance is more hydrophilic, and the active site is deeper.

Supplemental Results and Discussion of Other Resistance Determinants

An analysis of other non-β-lactam resistance determinants that play a role in the XDR phenotype of these isolates were analyzed and differences found in comparison to the orthologous genes in PAO1 WT are shown in **Table S1**. Both isolates possessed substitutions in their quinolone resistance-determining regions (QRDRs), specifically the previously described T83I in GyrA and S87L in ParC (1-4). The sequences of GyrB and ParE were identical to PAO1. The T83I substitution in GyrA and the S87L in ParC, found in both isolates, are known mechanisms of resistance to the fluoroquinolone antibiotics ciprofloxacin and levofloxacin (1-4).

Aminoglycoside modifying enzyme (AME) genes were also present: *aac*(6')-Ib4 and an *aac*(3)-I family gene were found in CLB 24388, and *aad*A1, *aac*(6')-33, and *aac*(6')-Ib4 in EM 2972704 (**Table S1**). AME genes were also present: *aac*(6')-Ib4 in both isolates, with the addition of *aad*A1and *aac*(6')-33 in isolate EM 2972704, and an *aac*(3)-I family gene in CLB 24388. These genes are known to encode aminoglycoside modifying enzymes that are responsible for aminoglycoside resistance and contribute to the amikacin and gentamicin resistant phenotypes (5).

The colistin resistance-associated genes *pho*P, *pho*Q, *pmr*A, and *pmr*B were next examined. The translated PhoP and PhoQ were identical to PAO1 in both isolates, as was PmrA in isolate CLB 24388. Differences in amino acid sequence were observed for PmrB, relative to that of PAO1, in both isolates. PmrB in isolate CLB 24388 had a Y345H substitution, and isolate EM 2972704 had S2P, A4T, V15I, G68S, Y345H substitutions. The differences in amino acid sequence observed for PmrB, relative to that of PAO1, in both isolates are notable, as all of the reported mutations have been associated with colistin resistant *Pseudomonas* isolates (6-10). However, their exact role in the colistin resistant phenotype is not known. Other resistance genes found in both isolates were *cat*B7 and *sul*1 conferring chloramphenicol and sulfonamide

resistance, respectively. Regulators of the main efflux pumps were also analyzed, differences were found between their sequence and that of orthologous sequences in PAO1, but those differences have not been described previously as affecting efflux and their exact role is not known.

Supplemental Methods

PCR amplification, cloning, and sequencing

PCR amplification of *opr*D in EM 2972704 was performed using the primers and methodology described by Cabot et al. (11). PCR amplification of the stop gap region and adjacent sequence in CLB 24388 was performed using primers GESATG 5’ ATGCGCTTCATTCACGCA 3’ and OXA-5R 5’ TTAGCCACCAATGATG 3’. The amplified integron fragment was then cloned into the Invitrogen pCR-XL-TOPO vector (Thermo Fisher) according to the manufacturer’s protocol and direct sequencing of the plasmid preparation was performed. The positive amplicon and plasmid preparation were sequenced at a commercial sequencing facility (MCLAB). DNA sequence analysis was performed using Lasergene 7.2 (DNASTAR,). The final amino acid sequences were determined using the ExPASy Translate tool and sequences were compared with BLAST online software (http://blast.ncbi.nlm.nih.gov), using the protein blast algorithm.

qRT-PCR

The *P. aeruginosa* strains were grown to mid-log phase at 37°C and treated with Qiagen RNAprotect Cell Reagent (Qiagen). Cells were harvested and total mRNA was extracted using the Qiagen RNeasy Mini Kit including the on-column DNase digestion according to the manufacturer's protocol. To confirm the absence of genomic DNA, negative control reactions were performed without RNA reverse transcriptase (no-RT controls). qRT-PCR was performed on an CFX96 Touch Real-Time PCR detection system (Bio-Rad) using the iTaq™ Universal SYBR® Green One-Step Kit (Bio-Rad) according to the manufacturer’s instructions; 2 µl total mRNA

(62.5 ng/ul) was used in each reaction. The *rpl*S gene coding for the 50S ribosomal protein L19 was used as an internal control for quantitation of mRNA levels (12) (*amp*C: 5' GATACCAGATTCCCCTGC 3', 5' GGTTCTCCTTTCAGGCTG 3'; *rpl*S: 5' ACCCGAGGTGTCCAGCGAACC 3', 5' GCTGCAAAACTGCCCGCAACG 3'. Amplification was undertaken in 20 µL final volume using the following protocol: reverse transcription 50°C for 10 min, 95°C for 1 min, followed by 40 cycles consisting of 10 sec at 95°C, 35 sec at 60°C. Experiments were conducted in triplicate. AmpC expression in all samples was normalized to *rpl*S coding for the 50S ribosomal protein L19, which was used as the internal control for quantitation of mRNA levels. Expression of AmpC for PAO1 was set at 1.0, and relative expression of variant genes presented as means ± standard deviations. Significance was defined as ≥ 2-fold difference in expression.

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