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Effect of MexXY Overexpression on Ceftobiprole Susceptibility in *Pseudomonas aeruginosa*[▽]

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Ceftobiprole, an anti-methicillin-resistant *Staphylococcus aureus* broad-spectrum cephalosporin, has activity (MIC for 50% of strains tested, ≤4 μg/ml) against many *Pseudomonas aeruginosa* strains. A common mechanism of *P. aeruginosa* resistance to β-lactams, including cefepime and ceftazidime, is efflux via increased expression of Mex pumps, especially MexAB. MexXY has differential substrate specificity, recognizing cefepime but not ceftazidime. In ceftobiprole clinical studies, paired isolates of *P. aeruginosa* from four subjects demonstrated ceftobiprole MICs of 2 to 4 μg/ml at baseline but 16 μg/ml posttreatment, unrelated to β-lactamase levels. Within each pair, the level of *mexXY* RNA, but not *mexAB*, *mexCD*, and *mexEF*, increased by an average of 50-fold from baseline to posttreatment isolates. Sequencing of the negative regulatory gene *mexZ* indicated that each posttreatment isolate contained a mutation not present at baseline. *mexXY* expression as a primary ceftobiprole and cefepime resistance mechanism was further examined in isogenic pairs by using cloned *mexXY* and *mexZ*. Expression of cloned *mexXY* in strain PAO1 or in a baseline isolate increased the ceftobiprole MIC to that for the posttreatment isolate. In contrast, in posttreatment isolates, lowering *mexXY* expression via introduction of cloned *mexZ* decreased the ceftobiprole MIC to that for the baseline isolates. Similar changes were observed for cefepime. A spontaneous mutant selectively overexpressing *mexXY* displayed a fourfold elevation in its ceftobiprole MIC, while overexpression of *mexAB*, *-CD*, and *-EF* had a minimal effect. These data indicate that ceftobiprole, like cefepime, is an atypical β-lactam that is a substrate for the MexXY efflux pump in *P. aeruginosa*.

Ceftobiprole is a broad-spectrum, anti-methicillin-resistant *Staphylococcus aureus* cephalosporin with in vitro microbiological activity against many strains of *Pseudomonas aeruginosa* (MIC for 50% of strains tested, ≤4 μg/ml) (4, 22). Resistance to antipseudomonal cephalosporins can be due to overexpression of the chromosomal AmpC cephalosporinase (17); however, decreased susceptibility of *P. aeruginosa* to a wide variety of antibiotics can also be attributed to drug efflux (17, 21). In particular, basal expression of the Mex pumps of the resistance nodulation division family appears to be responsible for the intrinsic resistance of *P. aeruginosa* to multiple antibiotics and for enhanced resistance upon overexpression (21, 23). The efflux pumps MexAB-OprM, MexCD-OprJ, MexEF-OprN, and MexXY-OprM have each been shown to be clinically relevant factors for decreased antibiotic susceptibility, with expression of the operons encoding these pumps subject to regulation (21). Pump overexpression may be due to an assortment of mutations that affect regulation of transcription from the efflux operon, such as mutations in the cognate repressor or in a global regulatory gene, or upstream of the operon, including but not limited to its promoter (21).

The MexAB-OprM efflux pump is constitutively expressed in *P. aeruginosa* and contributes to intrinsic resistance to a wide variety of antibiotics, including most β-lactams (except imipenem), chloramphenicol, fluoroquinolones, macrolides, novobiocin, sulfonamides, tetracycline, and trimethoprim (15, 23). In isogenic strains either constitutively expressing MexAB or with MexAB deleted, all of 13 tested cephalosporins were substrates for this efflux pump, including ceftazidime and cefepime. However, ceftazidime and cefepime appeared to be relatively poor substrates for MexAB, with MIC reductions of 8- and 32-fold, respectively, in the deletion strain. In contrast, cefotaxime, cefuroxime, and cefoxitin exhibited a >1,000-fold MIC reduction upon deletion of MexAB (15).

The MexXY-OprM efflux pump also extrudes a diverse assortment of antibiotics, including aminoglycosides, erythromycin, tetracycline, and fluoroquinolones (23). Cefepime, but not ceftazidime, is also subject to efflux by MexXY-OprM (8, 15). The OprM subunit of the MexXY pump is constitutively transcribed from the *mexAB-oprM* operon (2, 18); overexpresson of the *mexXY* operon is frequently due to mutation of the coding region of the MexZ protein, a negative regulator of the *mexXY* gene (7, 8, 14). Mutations outside of *mexZ*, termed *agrW*, have also been postulated to occur; however, the exact location and identity of such mutations are unclear (14).

In the present study, paired isolates of *P. aeruginosa* taken from four individual subjects before and after ceftobiprole treatment in phase III clinical trials displayed a greater-than-fourfold increase in ceftobiprole MICs after treatment. For these isolates, AmpC cephalosporinase activity and expression of *mex* efflux genes were investigated as possible ceftobiprole resistance mechanisms. Additionally, in isogenic pairs, the effects of overexpression of individual *mex* genes, and of cloned

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mexXY and *mexZ*, on cephalosporin susceptibility were examined.

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MATERIALS AND METHODS

Isolates and strains. Clinical isolates of *P. aeruginosa* were obtained from ceftobiprole phase III clinical trials.

MICs were determined by CLSI broth microdilution assays (3).

Pulsed-field gel electrophoresis (PFGE). Agarose-embedded genomic DNA was prepared with a CHEF Bacterial Genomic DNA Plug kit (Bio-Rad, Hercules, CA) as described by the manufacturer. DNA was digested with SpeI (New England BioLabs, Beverly, MA) and separated with a CHEF DR III apparatus (Bio-Rad). PFGE patterns of isolates from the same subject were compared on the basis of the criteria described by Tenover et al. (30). Isolates with exactly the same PFGE pattern were considered identical, and isolates with a one- to three-band difference between their PFGE patterns were considered closely related.

β-Lactamase assays. Freeze-thaw lysates of log-phase cultures were tested for β-lactamase activity by measuring initial hydrolysis rates with nitrocefin (100 μM) as the substrate (26). β-Lactamase activity was expressed in micromoles of nitrocefin hydrolyzed per minute per milligram of protein.

Isolation of RNA and quantitative real-time PCR (RT-PCR). Total RNA was purified from late-log-phase cultures of *P. aeruginosa* grown in Mueller-Hinton broth with a Qiagen RNeasy Mini kit (Qiagen, Valencia, CA). After DNase treatment (DNA free; Ambion, Inc., Austin, TX), first-strand cDNA was synthesized from ~3 μg total RNA with the Promega Improm-II reverse transcription system (Promega, Madison, WI). Quantitative RT-PCR was performed as described previously (20), with the Qiagen QuantiTect SYBR green PCR kit to quantify the expression level of each gene compared to that of *rpsL* with a LightCycler instrument (Roche, Indianapolis, IN). Primers for *mexX* (5′-AGCT GTTCAAGCAGACCCAGAACA-3′ and 5′-AGGGTGTCGAAGATGTCGCT GAT-3′), *mexY* (5′-GGACCACGCCGAAACCGAACG-3′ and 5′-CGCCGCA ACTGACCCGCTACA-3′), and *oprJ* (5′-CGATCGACACGCTGGAAG-3′ and 5′-TGGCCACTTCCGAGCGATTG-3′) were designed with Primerquest (www .IDTdna.com) or were as previously described (*mexA*, *-C*, *-E*, and *-X*, reference 16; *mexB* and *-D*, reference 6; *oprM*, reference 28; *oprN*, reference 13). A set of specific primers was designed with Primerquest to hybridize only to recombinant (plasmid-encoded), and not to chromosomal, *mexXY* transcripts. The Light Cycler was programmed to perform a denaturation step (95°C, 15 min), followed by 45 cycles at 94°C (15 s), 58 to 62°C (primer set dependent, 10 s), and 72°C (15 s). For each pair, the RNA level of the posttreatment isolate was normalized to that of the corresponding baseline isolate, which was assigned an expression value
of 1.

DNA sequence analysis. The *mexZ* gene from multiple independent isolates was amplified by PCR from boiled cell lysates with the Invitrogen Platinum Supermix High Fidelity kit (Invitrogen, Carlsbad, CA) and the following program: an initial denaturation step of 95°C for 5 min, followed by 30 cycles of 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min and a final extension of 72°C for 5 min. The PCR primers used were MexZ-F (5′-ATTGGATGTGCATGGGT G-3′) and MexZ-R (5′-TGGAGATCGAAGGCAGC-3′) (24). The direct and complementary strands were sequenced (ACGT, Inc., Wheeling, IL) with the PCR primers.

Cloning of recombinant *mexXY* and *mexZ* into expression vector pJAK16. Sequences for strain PAO1 were obtained from the *Pseudomonas* Genome Database (www.pseudomonas.com). The wild-type *mexZ* gene (promoter and coding region) was amplified by PCR with primers MexZ-F2 (5′-TAATGAAT TCAGCGAGCCGGTCCATTGGAT-3′) and MexZ-R2 (5′-ATTAGGATCCC GTGGAGATCGAAGGCAGCC-3′), which contain a 5′ EcoRI and a 3′ BamHI site, respectively. The *mexXY* coding region was synthesized (Genscript Corp., Piscataway, NJ) after optimization for *P. aeruginosa* codon usage. The native noncoding *mexXY* intergenic region, an EcoRI site upstream of the *mexX* start codon, and a BamHI site downstream of the *mexY* stop codon were included in the synthetic product. The *mexZ* PCR product and the synthetic *mexXY* genes were cleaved with EcoRI and BamHI and separately cloned into the corresponding sites of chloramphenicol resistance-encoding vector pJAK16 (ATCC 37622) under *tac* promoter control (isopropyl-β-D-thiogalactopyranoside [IPTG] inducible) (5). Plasmids (200 ng) were electroporated into electrocompetent *P. aeruginosa* with a Bio-Rad Gene Pulser Xcell Electroporator with the preset conditions for *P. aeruginosa* (2,500 V, 25-μF capacitance, 200-Ω resistance) in 0.2-cm-gap cuvettes. Fresh electrocompetent cells were prepared as described previously (31), except that overnight cultures in Mueller-Hinton broth were used and cell pellets were washed twice with ice-cold 2% sucrose containing 1 mM $MgCl_2$. Transformants were selected on Mueller-Hinton plates containing chloramphenicol (200 or 400 μg/ml, depending on the chloramphenicol MIC for the strain to be transformed). For MIC determinations and RNA preparation from transformed strains, liquid cultures were maintained with 200 μg/ml chloramphenicol unless otherwise stated. IPTG, when present, was added to 1 mM.

Isolation of spontaneous mutants of PAO1 overexpressing Mex pumps. Mutants selectively overexpressing only a single efflux pump were selected in the *P. aeruginosa* PAO1 background on Mueller-Hinton agar containing the following agents: for MexAB-OprM (PAO-AB_{up}), ciprofloxacin (0.25 μg/ml) and cefoperazone (8 μg/ml) (29); for MexCD-OprJ (PAO-CD_{up}), ciprofloxacin (0.25 μg/ml) and cefepime (2 μg/ml) (27); for MexEF-OprN (PAO-EF_{up}), chloramphenicol (500 μg/ml) (12); and for MexXY-OprM (PAO-XY_{up}), gentamicin (8 μg/ml). Overexpression of a single pump was confirmed through RT-PCR analysis to quantify the RNA levels for each gene in the four major pump systems and was at least 10-fold higher in mutants than in the parental PAO1 strain.

RESULTS AND DISCUSSION

Clinical isolates of *P. aeruginosa* with elevated ceftobiprole MICs. During the course of ceftobiprole clinical studies, four subjects were identified with *P. aeruginosa* isolates displaying ceftobiprole MICs of 2 to 4 μg/ml at baseline but 16 μg/ml posttreatment (Table 1). Despite the observation of elevated ceftobiprole MICs, two of the four subjects (subjects 3 and 4) experienced clinical cures (Table 1). The MICs of cefepime, but not ceftazidime, also increased (two- to fourfold) from baseline to posttreatment isolates. The ciprofloxacin MICs were similar when baseline and posttreatment isolates were compared, except for pair 4, which exhibited a fourfold increase; gentamicin MICs were similar within pairs 3 and 4 but increased two- or fourfold (pairs 2 and 1, respectively). For each pair, as determined by PFGE, the baseline and posttreatment isolates were either identical (pairs 1 and 2) or closely related (pairs 3 and 4), suggesting that the increased ceftobiprole MIC for the posttreatment isolate was not due to acquisition of a different organism by the subject (Fig. 1).

Characterization of resistance determinants. To determine whether an increase in β-lactamase activity in each posttreatment isolate compared to its corresponding baseline isolate could be responsible for the increase in the ceftobiprole MIC, the β-lactamase activity of the isolates was examined (Table 1). Within each pair, the amount of β-lactamase activity was similar, indicating that increased β-lactamase activity was not the cause of the increase in the ceftobiprole MIC.

To examine possible changes in the expression of efflux pumps in comparing baseline and posttreatment isolates, the amounts of RNA for MexAB-OprM, MexCD-OprJ, MexEF-OprN, and MexXY-OprM were quantified by RT-PCR (Table 1). Within each pair, a significant increase in *mexXY* RNA in the posttreatment isolate (9- to 140-fold) compared to that in its corresponding baseline isolate was observed. OprM, a component of the MexXY efflux pump, is encoded by the MexAB-OprM operon (2, 18); the abundance of the *oprM* transcript was unchanged in the posttreatment isolates (Table 1), consistent with other cases of *mexXY* overexpression (9). In addition to *mexXY* overexpression, pair 2 (but not pairs 1, 3, and 4) showed an increase in *mexEF-oprN* expression (4- to 16-fold,

TABLE 1. MICs, β-lactamase activities, and *mex* expression of pairs of *P. aeruginosa* isolates from four subjects either prior to treatment or posttreatment with ceftobiprole

Pair	Isolate	MIC (μg/ml)[a]					β-Lactamase activity[b]	*n*-Fold change in RNA level[c]	
		BPR	CAZ	FEP	GEN	CIP		*mexX*	*mexY*
1	OC14805 (B)[f]	2	2	2	2	2	0.12		
1	OC14812 (PT)[g]	16	4	8	8	1	0.09	37	140
2	OC16827 (B)	4	2	2	4	0.5	0.11		
2	OC16830 (PT)	16	2	8	8	0.5	0.03	24	53
3[d]	OC17349 (B)	4	4	4	4	4	0.31		
3	OC17355 (PT)	16	4	8	4	4	0.17	57	65
4[e]	OC14552 (B)	4	4	4	4	0.12	0.06		
4	OC14551 (PT)	16	4	8	4	0.5	0.09	11	9

[a] Abbreviations: BPR, ceftobiprole; CAZ, ceftazidime; FEP, cefepime; GEN, gentamicin; CIP, ciprofloxacin.
[b] Micromoles of nitrocefin hydrolyzed per minute per milligram of protein; PAO1 β-lactamase activity was ≤0.1 μg/min/mg.
[c] Posttreatment/baseline RNA levels are shown. For *mexAB*, *oprM*, *mexCD*, *oprJ*, *mexEF*, and *oprN*, the change was generally ≤2-fold, except for pair 2 (*mexE*, 16-fold; *mexF*, 4-fold; *oprN*, 12-fold).
[d] Persistent cure.
[e] Presumed cure.
[f] B, baseline.
[g] PT, posttreatment.

Table 1). Since only *mexXY* was consistently elevated in each of the four pairs, it was likely to be associated with the increase in ceftobiprole MICs.

A typical mechanism of overexpression of the MexXY-OprM efflux pump involves mutation of the coding region of the *mexZ* repressor, which is a negative regulator of the *mexXY* operon (7, 8, 14). To determine if mutations in *mexZ* could be responsible for *mexXY* overexpression in the posttreatment isolates, the genomic sequence of *mexZ* was determined for the four pairs of isolates and compared to that of *P. aeruginosa* PAO1 (Table 2). Each of the posttreatment isolates contained a distinct mutation that was not present in its corresponding baseline isolate: pair 1, T_{86}→G (Val_{29}→Gly); pair 2, deletion of T_{301}; pair 3, insertion of GC at C_{238}; pair 4, C_{415}→T (Arg_{139}→Trp). Similar point mutations, and insertions and deletions causing frameshifts, have been characterized previously in the *mexZ* coding region and are associated with increases in both *mexXY* expression and cefepime MICs (7, 8, 14).

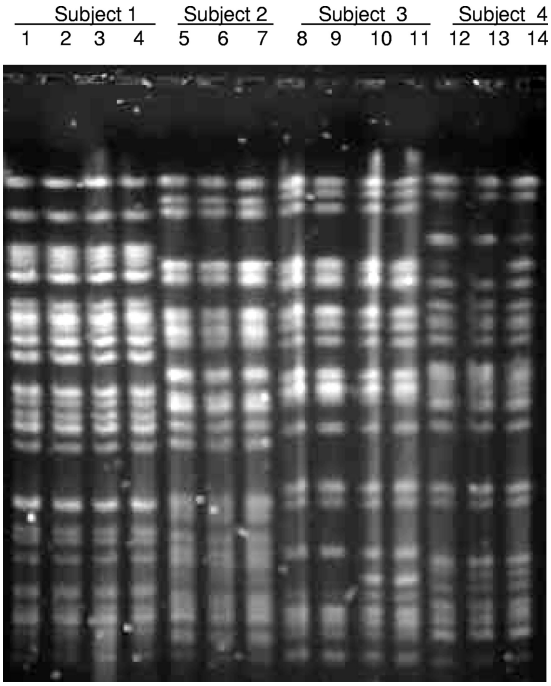

FIG. 1. PFGE patterns after SpeI digestion of *P. aeruginosa* isolates matched by subjects. Subject 1: lane 1, OC14805; lane 2, OC14806; lane 3, OC14809; lane 4, 14812. Subject 2: lane 5, OC16827; lane 6, OC16829; lane 7, OC16830. Subject 3: lane 8, OC17349; lane 9, OC17350; lane 10, OC17353; lane 11, OC17355. Subject 4: lane 12, OC14552; lane 13, OC14436; lane 14, OC14551.

Additional isolates that had been collected from these four subjects at intermediate days between baseline and posttreatment sample collection days were available for analysis, making it possible to determine whether a correlation could be found between the onset of *mexXY* overexpression and the increase in the ceftobiprole MIC from the baseline value (Table 2). For each subject, the increase in the ceftobiprole MIC first occurred in the same isolate in which *mexXY* overexpression was first detected (Table 2). Moreover, the same point mutation, deletion, or insertion in *mexZ* that had been found in the posttreatment isolate was also present in the intermediate isolates in which increased *mexXY* expression and increased ceftobiprole MIC were observed (Table 2).

Cephalosporin susceptibility of spontaneous mutants overexpressing Mex efflux pumps. To determine whether ceftobiprole could serve as a substrate for the individual Mex efflux pumps, a panel of strains was constructed from strain PAO1, each of which overexpressed either MexAB-OprM, MexCD-OprJ, MexEF-OprN, or MexXY-OprM. Strain PAO1-XY_{up} overexpressed *mexXY* by 40- to 60-fold and exhibited a 4-fold increase in its ceftobiprole MIC, which was the largest difference in ceftobiprole susceptibility observed within the panel of efflux mutants. DNA sequence analysis of *mexZ* in strain PAO1-XY_{up} revealed no mutation in that gene, consistent with *mexXY* overexpression in the absence of *mexZ* mutation reported in other studies (14).

In contrast to MexXY, overexpression of MexAB, MexCD, or MexEF had little effect on ceftobiprole MICs, resulting in,

TABLE 2. Correlation of ceftobiprole MIC, *mexXY* expression, and *mexZ* mutation

Subject	Isolate	Visit	Ceftobiprole MIC (μg/ml)	*n*-Fold change in *mexXY* RNA level	*mexZ* mutation
1	OC14805	Baseline	2	1	None
1	OC14806	Day 4	4	0.4	None
1	OC14809	7–14 days after EOT[a]	16	140	T_{86}→G (Val_{29}→Gly)
1	OC14812	LFU[b]	16	91	T_{86}→G (Val_{29}→Gly)
2	OC16827	Baseline	2	1	None
2	OC16829	Day 14	16	100	T_{301} deletion
2	OC16830	EOT	16	190	T_{301} deletion
3	OC17349	Baseline	4	1	None
3	OC17350	Day 4	16	79	GC insertion at C_{238}
3	OC17353	EOT	16	52	GC insertion at C_{238}
3	OC17355	7–14 days after EOT	16	45	GC insertion at C_{238}
4	OC14552	Baseline	4	1	None
4	OC14436	Day 4	4	0.4	None
4	OC14551	Day 8	16	10	C_{415}→T (Arg_{139}→Trp)

[a] EOT, end of treatment.
[b] LFU, long-term follow-up.

at most, a twofold increase in susceptibility (data not shown). However, it should be noted that the twofold ceftobiprole MIC increase observed upon *mexAB* overexpression is the same as that shown by cefepime and ceftazidime, which are known to be subject to efflux by MexAB-OprM (15). We therefore cannot exclude the possibility that ceftobiprole is also a substrate for the MexAB-OprM efflux pump, although this has not yet been observed among the limited number of available clinical isolates that displayed elevated ceftobiprole MICs. Cefepime additionally appeared to be a substrate for MexCD, displaying a fourfold MIC increase upon *mexCD* overexpression (data not shown), consistent with previous observations (10, 15). The minor (twofold) ceftobiprole MIC increases in strains PAO-AB_{up} and PAO-CD_{up} suggest that the compound may retain microbiological activity upon MexAB or MexCD overexpression. More efficient efflux of ceftobiprole by MexXY than by other efflux pumps of *P. aeruginosa* is supported by in vitro resistance studies in which ceftobiprole-selected isolates overexpressed MexXY (25).

Effect of cloned *mexXY* or *mexZ* on cephalosporin susceptibility. To further investigate the effect of *mexXY* overexpression on cephalosporin susceptibility, two complementary approaches were taken. One approach was to increase *mexXY* expression in *P. aeruginosa* by introduction of the cloned gene on a plasmid, under the control of the IPTG-inducible *tac* promoter. The other approach was to reduce *mexXY* expression by introducing cloned *mexZ* on a plasmid.

Plasmid-encoded recombinant *mexXY* was introduced into *P. aeruginosa* strain PAO1 and baseline clinical isolate OC16827. Untransformed strains PAO1 and OC16827 both exhibited a chloramphenicol MIC of 128 μg/ml; accordingly, chloramphenicol (200 μg/ml) was present throughout these experiments to maintain the plasmid. Although it has been reported that chromosomal *mexXY* expression can be upregulated by chloramphenicol (11), RT-PCR experiments with transformed cells harboring either control vector pJAK16 or pMexXY had similar levels of RNA from chromosomal *mexXY*, with or without chloramphenicol (or IPTG) present (data not shown). The amount of plasmid-encoded *mexXY* RNA upon IPTG induction was approximately 100-fold higher than that of the chromosomally encoded *mexXY* RNA (Table 3).

Expression of recombinant MexXY in *P. aeruginosa* strain PAO1 and in baseline strain OC16827 led to a four- to eight-

TABLE 3. Susceptibility of *P. aeruginosa* to ceftobiprole and comparators following introduction of recombinant, plasmid-encoded *mexXY* or *mexZ*

Strain and plasmid	Amt of *mexXY* RNA	MIC (μg/ml)[b]				
		BPR	FEP	CAZ	GEN	CIP
PAO1						
pJAK16	1.0	4	2	2	4	0.25
pMexXY	100[a]	32	8	1	ND	1
pMexZ	0.1	1	1	1	0.25	0.12
Baseline OC16827						
pJAK16	1.0	4	2	2	4	0.25
pMexXY	100[a]	16	8	2	ND	0.5
pMexZ	0.2	1	2	2	0.5	0.12
Posttreatment OC16830						
pJAK16	1.0	16	8	2	8	0.5
pMexZ	0.004	1	4	2	0.5	0.12
Posttreatment OC14812						
pJAK16	1.0	16	8	4	8	1
pMexZ	0.008	2	2	4	2	0.5
Posttreatment OC17355						
pJAK16	1.0	8	8	4	16	4
pMexZ	0.003	2	2	4	0.25	1
Posttreatment OC14551						
pJAK16	1.0	16	16	4	8	0.25
pMexZ	0.06	4	4	4	4	0.25

[a] Amount of plasmid-encoded (relative to chromosomal) *mexXY* RNA.
[b] Cultures were grown with 200 μM chloramphenicol to maintain plasmids and 1 mM IPTG to induce expression of the cloned gene. Abbreviations: BPR, ceftobiprole; FEP, cefepime; CAZ, ceftazidime; GEN, gentamicin; CIP, ciprofloxacin; ND, not determined.

fold increase in the MICs of ceftobiprole and cefepime (Table 3). Thus, expression of plasmid-encoded *mexXY* in these strains reduced susceptibility to these cephalosporins, increasing the MIC for PAO1 and baseline strain OC16827 to the level seen for the posttreatment isolate OC16830. The MICs of ceftazidime, which is not subject to efflux by MexXY-OprM (8, 15), were not increased in the presence of plasmid-encoded *mexXY*.

Introduction of plasmid-borne *mexZ* (repressor of *mexXY* expression) decreased the amount of *mexXY* RNA approximately 20- to 300-fold in posttreatment isolates OC16830, OC14812, OC17355, and OC14551 relative to that in the control (pJAK16-containing) strains (Table 3). The presence of the cloned *mexZ* repressor (but not control plasmid pJAK16) rendered posttreatment strains OC16830, OC14812, OC17355, and OC14551 as susceptible as the corresponding baseline strains to ceftobiprole, reducing the baseline MICs by 4- to 16-fold, from 16 μg/ml (Table 1) to 1 to 4 μg/ml (Table 3), strongly suggesting that elevated *mexXY* levels were responsible for the elevated ceftobiprole MICs observed in the posttreatment strains. The MICs of cefepime declined by two- to fourfold, from ≥8 to ≤4 μg/ml, in posttreatment strains containing the *mexZ* plasmid. As expected, the presence of the *mexZ* plasmid did not affect ceftazidime MICs but lowered gentamicin MICs (Table 3). In strain PAO1 and in baseline isolate OC16827, the *mexZ* plasmid reduced the level of *mexXY* RNA by 5- to 10-fold and reduced the ceftobiprole MICs by 4-fold (from 4 to 1 μg/ml, Table 3), consistent with decreased cephalosporin MICs observed in PAO1 with *mexXY* deleted (15).

Efflux of antibiotics, resulting in resistance, is a well-documented phenomenon in *P. aeruginosa* (1, 21, 23). Given that most β-lactams, but not ceftobiprole, are subject to efficient efflux by MexAB-OprM, it is possible that ceftobiprole may prove useful in combination with other drugs that are subject to efflux by MexAB-OprM but not by MexXY-OprM (since the latter would cause the efflux of ceftobiprole). For example, certain fluoroquinolones or aminoglycosides appear to be only moderately subject to efflux by MexXY-OprM (15, 19, 32). In a small retrospective study of patients receiving combination therapy with an antipseudomonal β-lactam or fluoroquinolone together with an aminoglycoside, multidrug-resistant, efflux-overproducing mutants were not observed (1, 33). Such combinations of antibiotics that together avoid the major efflux pumps of *P. aeruginosa* may lead to unexpected synergies between antibiotics and provide more effective treatment for pseudomonal infections.

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