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MECHANISMS OF RESISTANCE

Cefiderocol Resistance in *Acinetobacter baumannii*: Roles of β-Lactamases, Siderophore Receptors, and Penicillin Binding Protein 3

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ABSTRACT Cefiderocol is a siderophore cephalosporin active against many multidrug-resistant (MDR) Gram-negative pathogens. We examined the resistance mechanisms in 12 *Acinetobacter baumannii* strains with cefiderocol MICs ranging from ≤0.03 to >32 μg/ml. Cefiderocol resistance could not be explained by β-lactamase activity. Cefiderocol resistance was associated with reduced expression of the siderophore receptor gene *pirA*. Mutations involving PBP3 may have contributed to resistance in one strain. Additional studies are needed to assess the role of other siderophore receptors.

KEYWORDS *Acinetobacter*, siderophore

Because of its propensity to acquire and develop resistance to multiple classes of antibiotics, multidrug-resistant *Acinetobacter baumannii* has been labeled as a "critical" pathogen by the World Health Organization (1). Carbapenem-resistant *A. baumannii* is especially problematic, with very few therapeutic options currently available (2). Cefiderocol is a novel siderophore cephalosporin with a broad spectrum of activity against Gram-negative pathogens (3–5). Cefiderocol maintains activity against pathogens possessing a wide variety of β-lactamases and carbapenemases (2–4). Against *A. baumannii*, reduced susceptibility has been suggested in isolates possessing OXA carbapenemases and extended-spectrum β-lactamases (ESBLs) (3, 5), although overt resistance has been unusual. For *A. baumannii*, mechanisms leading to high-level resistance to cefiderocol remain undefined.

TonB-dependent receptors allow uptake of specific siderophore-iron complexes across the bacterial outer membrane (6–8). These iron transporters have been associated with cellular entry of cefiderocol into *Pseudomonas aeruginosa* (9). Downregulation of specific iron transport receptors in *Pseudomonas aeruginosa* have correlated with resistance to an investigational siderophore antibiotic (10). Similarly, downregulation of two TonB-dependent receptors in *A. baumannii* have been associated with resistance to investigational siderophore-drug conjugates (11). In this report, we investigate the roles of β-lactamases, two siderophore receptors, and PBP3 in cefiderocol resistance in *A. baumannii*.

Susceptibility studies. Twelve clinical isolates of *A. baumannii*, gathered from prior surveillance studies (5), were examined. Isolates were selected based on varying susceptibility rates for cefiderocol and other β-lactam antibiotics. Cefiderocol MICs were performed in iron-depleted cation-adjusted Mueller-Hinton broth. MICs for the remaining antibiotics were performed by the agar dilution method with Mueller-Hinton agar according to established CLSI methods (12). Control strains included *Escherichia coli* ATCC 25922 and ATCC 35218 and *P. aeruginosa* ATCC 27853. Doubling times were determined in Mueller-Hinton broth; a 1:1,000 dilution of an overnight growth was

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TABLE 1 Primers and probes used in the PCR studies

Primer or probe name	Sequence (5′–3′)[a]
Primers used for amplification and sequencing study target genes	
PirAfor	AGCAGGGCAGCCATTGCCATT
PirArev	GTTCTGGTGCGGCAGGTGGG
PiuAfor	TGCATCAAAGTCTGGGCGTGCT
PiuArev	TGCAGGTTCAGTTGGTGGCAGC
PBP3afor	GATAAGCGAACAAAGCAAACAC
PBP3arev	TCAACTGCATTTCCAAGCC
PBP3bfor	ATCTTGATGAACTTGCCGAC
PBP3brev	GAACGCTTACCAAAACCAAC
PBP3cfor	GCTTTGACCGTAAGTGGTG
PBP3crev	AAACAGTCATAGCTTACCTGC
Primers and probes for the real-time RT-PCR studies	
RTAbRibofor	GTAGCGGTGAAATGCGTAG
RTAbRiborev	CTTTCGTACCTCAGCGTCAG
RTAbRiboprobe	[DFAM] CGAAGGCAGCCATCTGGCCT [DTAM]
RTAbAmpCfor	TGCTATTTCAAAGGAACCTTCA
RTAbAmpCrev	TTAATGCGCTCTTCATTTGG
RTAbAmpCprobe	[DFAM] TGGCTCAACTAACGGTTTCGGAAC [DTAM]
RTAbOxa51for	GGAAGTGAAGCGTGTTGGTT
RTAbOxa51rev	TAAAGGACCCACCAGCCAAA
RTAbOxa51probe	[DFAM] ACTTGGGTACCGATATCTGCATTGCCA [DTAM]
RTPirAfor	AAGCCACTTCGCGTTTAGAA
RTPirArev	CGCCATAACCTGAACCACTG
RTPirAprobe	[DFAM] ACTCTTCGCTTTAACGGCGAGGC [DTAM]
RTPiuAfor	TGTTTGCTGTACTCTGCTCCT
RTPiuArev	TGTCTGGACAAACCCAGATGA
RTPiuAprobe	[DFAM] TGCCAAACAAGACCTTTGCCGACA [DTAM]

[a]DFAM, 6-carboxyfluorescein; DTAM, 6-carboxytetramethylrhodamine.

performed, and colony counts were determined every 30 min from 1 to 2.5 h of incubation.

PCR studies. Isolates were screened for the presence of β-lactamases belonging to the following groups as previously described (5): TEM, SHV, KPC, IMP, VIM, NDM, OXA-23, OXA-24, and OXA-58. RNA was extracted (RNeasy; Qiagen, Inc.) from cultures in the early log phase of growth and treated with DNase. For the real-time reverse transcriptase PCR (RT-PCR) studies, expression of the β-lactamases *ampC* and bla_{OXA-51} was performed as previously described (13). A 1,037-bp segment of *pirA* and an 856-bp segment of *piuA* were amplified using the primers listed in Table 1. For the expression of *pirA* and *piuA*, primers and probes were designed based on conserved sequences of amplified genes. Primer and probe concentrations were adjusted to give replicative efficiencies of 90 to 110%, and 25 ng of RNA was used as the template. All studies were performed in triplicate. Expression of target genes was normalized to that of a housekeeping ribosomal gene, and an isolate susceptible to cefiderocol served as the calibrator (*A. baumannii* ATCC 19606 for the β-lactamases and BV1 for the siderophore receptor genes). For the RT-PCR studies (Table 2), the following primer and probe concentrations were used: ribosomal housekeeping gene primers, 100 nM, and probe, 100 nM; RTAb*ampC* primers, 400 nM, and probe, 100 nM; RTAb*oxa51* primers, 200 nM, and probe, 200 nM; amd RT*pirA* and RT*piuA* primers, 400 nM, and probe, 200 nM. Finally, *pbp3* was amplified and sequenced using the primers listed in Table 1. Multilocus sequence typing (MLST) was performed using the Pasteur scheme for *A. baumannii* (http://pubmlst.org/abaumannii/).

Twelve clinical isolates, including six susceptible and six resistant to cefiderocol, were selected for further study. There was no clear relationship between resistance to cefiderocol and the presence of acquired β-lactamases (TEM-1, SHV-5 or SHV-12, or OXA-23) or with the expression of the chromosomal β-lactamases (bla_{ampC} and bla_{OXA-51}; Table 2).

TABLE 2 Characteristics of clinical isolates examined

Isolate	ST[a]	MIC (μg/ml)			β-lactamase(s)	Relative expression				Protein sequence	
		Cefiderocol	Ceftazidime	Meropenem		bla_{ampC}	bla_{OXA51}	*pirA*	*piuA*	PirA	PiuA
BV1	2	0.12	4	>16	TEM-1	0.84	54	1	1	WT[b]	WT
KB343	2	≤0.03	16	0.5		0.03	0.21	1.37	NA[c]	WT	NA
BD30	1142	0.06	8	16	OXA-23	0.02	1	0.41	NA	WT	NA
KB4	990	0.12	8	>16	OXA-23; SHV-12	53	17	2.0	0.81	WT	WT
KB316	1434	0.5	>32	>16		69	72	0.51	3.04	NA	V216G; N381S
BD6	2	2	>32	16	SHV-12	15	28	0.67	1.23	WT	WT
QU20	2	32	>32	>16		64	3.42	NA	NA	WT	WT
VM306	2	32	>32	>16	SHV-5	132	73	0.23	NA	WT	WT
MA28	2	>32	>32	>16	SHV-5; TEM-1	72	10	NA	NA	WT	WT
BD503	229	>32	>32	>16	SHV-5; TEM	91	135	NA	NA	NA	NA
BD5	250	>32	>32	>16	OXA-23	0.95	0.14	0.39	4.1	L275F; I277V	V216G; T280S; N381S; S412T
BD13	250	>32	>32	>16	OXA-23	0.18	0.24	0.17	1.7	L275F; I277V	V216G; T280S; N381S; S412T

[a]ST, sequence type.
[b]WT, wild type.
[c]NA, not amplifiable.

Six of the 12 isolates belonged to the international strain ST2. The three susceptible isolates all had detectable expression of *pirA*; the three resistant isolates had diminished or absent expression of this gene (Table 2). The three resistant ST2 isolates also had absent expression of *piuA*; however, absent expression was also noted in one susceptible isolate. Four other isolates belonging to other STs (990, 1142, 1434, and 229) also followed the same pattern. For the resistant isolate belonging to ST229, attempts to amplify the 1,037-bp product of *pirA* and the 856 bp of *piuA* were unsuccessful, suggesting deletions of these receptor genes. Taken together, the data suggest that loss of *pirA*, possibly in combination with loss of *piuA*, was important for the development of resistance to cefiderocol.

Two isolates belonged to ST250, and both were resistant to cefiderocol (Table 2). Unlike the other four resistant isolates, these isolates had measurable expression of both *pirA* and *piuA* (Table 2). However, both isolates had two mutations (Leu275Phe and Ile277Val) in the midst of a beta strand of PirA. The change from a hydrophobic to an aromatic amino acid at location 275 would be predicted to have moderate likelihood of affecting the function of the protein (14). Therefore, posttranslational alterations may have diminished the functionality of this receptor protein.

Eleven of the 12 isolates possessed wild-type (corresponding to *A. baumannii* ATCC 19606) PBP3. One resistant isolate (VM306), belonging to ST2, had mutations leading to Ile236Asn and His370Tyr alterations. The alteration of the hydrophobic isoleucine to the charged asparagine at position 236 would be predicted to have a moderate likelihood of affecting the functionality of PBP3.

Doubling times were determined for seven isolates, including four cefiderocol-susceptible (BD6, BV1, KB4, KB343) and three resistant (MA28, QU20, VM306) isolates. Doubling times for the susceptible isolates were similar to those for the resistant isolates (23.6 $\pm$ 3.4 versus 24.0 $\pm$ 4.4 min, respectively).

Consistent with other reports (3, 15), the β-lactamases that we tested did not explain cefiderocol resistance in our isolates of *A. baumannii*. Although some studies have found that SHV- and PER-type β-lactamases may be associated with higher cefiderocol MICs (5, 16), clearly other mechanisms are involved for overt resistance. Other frequently identified mechanisms of antibiotic resistance, including efflux systems (e.g., *adeB* and *abeM*) or porin deficiencies, also do not appear to contribute to cefiderocol resistance (5, 17). Because of its unique ability to gain entry into the periplasmic space of Gram-negative bacteria and its stability against a wide variety of β-lactamases, cefiderocol is able to evade typical resistance mechanisms.

TonB-dependent cytoplasmic membrane receptor proteins are membrane-spanning barrels that allow selective uptake of siderophore-ferric complexes (6–8). The develop-

ment of an antibiotic-siderophore complex allows efficient entry of the antibiotic into the bacterial cell ("Trojan horse" strategy). The ideal antibiotic-siderophore complex would utilize several different TonB-dependent receptors in order to minimize the chances of the development of resistance. In this study, with clinical isolates of *A. baumannii*, we report the importance of expression of the PirA receptor for maintaining susceptibility of cefiderocol. This finding was especially evident for strains belonging to the clone ST2, an internationally recognized multidrug-resistant pathogen. However, isolates belonging to ST250 did not have markedly reduced expression of PirA. Additional studies will be needed to determine if the mutations that we identified in this gene affected posttranslational function of the receptor protein in these isolates. Alternatively, other TonB-dependent receptors not studied in this report may be involved. Examination of a larger number of isolates and expression of other TonB-dependent siderophore receptors will be needed.

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