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MECHANISMS OF RESISTANCE

Contribution of Iron-Transport Systems and β-Lactamases to Cefiderocol Resistance in Clinical Isolates of *Acinetobacter baumannii* Endemic to New York City

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ABSTRACT The development of resistance to cefiderocol among multidrug resistant *Acinetobacter baumannii* has been attributed to downregulation in iron transport systems and a variety of β-lactamases. However, the precise contribution of each in clinical isolates remains to be determined. Sixteen clinical isolates with varying degrees of cefiderocol resistance were investigated. Susceptibility testing was performed with and without the presence of iron and avibactam. Expression of 10 iron transport systems and bla_{ADC} and $bla_{OXA\text{-}51\text{-}type}$ were analyzed by real time RT-PCR. The acquisition of a variety of β-lactamases was also determined. In 2 isolates the impact of silencing the bla_{ADC} gene was achieved using a target specific group II intron. For most resistant isolates, MICS for cefiderocol were similar with or without the presence of iron, and there was an overall decrease in expression of receptors (including *pirA* and *piuA*) involved in ferric uptake. However, expression of the ferrous uptake system (*faoA*) persisted. The addition of avibactam (4 μg/mL) lowered most cefiderocol MICs to 2 to 4 μg/mL. Most isolates possessed ADC-25 or ADC-33. Cefiderocol resistance correlated with over-expression of bla_{ADC}; silencing of this β-lactamase resulted in a ≥ 8-fold decrease in cefiderocol MICs. Over-expression of specific bla_{ADC} subtypes, in a background of generalized repression of ferric uptake systems, were consistent features in clinical isolates of cefiderocol-resistant *A. baumannii*.

KEYWORDS cefiderocol, siderophores

The development of cefiderocol, with its novel catechol siderophore moiety, has been a welcomed addition to our therapeutic armamentarium against multidrug resistant bacteria. Cefiderocol has demonstrated promising activity against a wide range of Gram-negative bacteria, including those resistant to carbapenems (1–5).

While demonstrating consistent activity against multidrug resistant *Enterobacterales*, resistance to cefiderocol has been reported with non-fermenting Gram-negative pathogens. In *Pseudomonas aeruginosa*, the TonB-dependent siderophore receptors PiuA (and its orthologue PiuD) and PirA have been implicated in the uptake of cefiderocol (6). In laboratory-derived deletion mutants of PAO1, cefiderocol MICs increased 16-fold with deletion of *piuA* but were unchanged with deletion of *pirA* (6). However, this finding has not been evident in clinical strains of *P. aeruginosa* with reduced susceptibility to cefiderocol (7). Other TonB-dependent receptors have been identified that are involved in uptake of siderophore-drug conjugates (6). In addition, over-expression of other iron transport systems in *P. aeruginosa* may "outcompete" those involved in uptake of siderophore-antibiotic conjugates and confer resistance (8). Acquisition of β-lactamases, particularly NDM and PER, may contribute to, but are not essential for, reduced susceptibility (1, 3). Alterations in the chromosomal AmpC cephalosporinase have been noted in cefiderocol-resistant isolates (9).

Studies involving mechanisms of siderophore-conjugate resistance in *Acinetobacter baumannii* reflect a similar picture. Deletion of *piuA* or *pirA* in *A. baumannii* ATCC 19606 led to 4 to 8-fold increase in the MICs for 2 monobactam-siderophore conjugates (10). However,

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TABLE 1 Cefiderocol susceptibility results and levels of expression of iron transport genes among isolates of *A. baumannii*

Isolate	ST	MICs (μg/mL)		Relative expression									
ATCC 19606		IDB[a] ≤ 0.03	MHB[b] ND[c]	*pirA* 1	*piuA* 1	gene2581 (*bauA*) 1	gene1679 1	gene1633 1	gene120 1	gene1761 1	gene3537 1	gene3521 1	gene265 (*faoA*) 1
D13	250	0.25	0.25	↔	↔	↓	↔	↔	↓	↓	↔	↓	↔
D1	250	1	2	↔	↔	↓	↓	↓	↓	↑	↓	↓	↔
S15	229	4	32	↔	↔	↓	↔	↔	↔	↑	↓	↓	↔
K47	229	16	32	↓	↓	↓	↓	↓	↓	↓	↔	↓	↔
S6	229	16	16	↓	↓	↓	↓	↓	↓	↓	↓	↓	↔
W7	229	16	16	↓	↓	↓	↔	↓	↔	↓	↔	↓	↔
W315	229	64	32	↔	↓	↓	↓	↓	↔	↓	↓	↓	↔
D103	229	> 64	64	↔	↓	↓	↓	↓	↓	↓	↓	↓	↔
E27	2	2	2	↓	↑	↓	↓	↔	↓	↔	↓	↓	↓
A53	2	4	4	↔	↔	↓	↑	↑	↔	↔	↔	↔	↔
L301	2	16	32	↔	↔	↓	↓	↓	↓	↔	↓	↓	↔
U20	2	16	16	↓	↔	↓	↔	↔	↔	↔	↓	↓	↔
D501	2	32	16	↔	↔	↓	↑	↔	↔	↔	↓	↓	↔
C120	2	32	32	↔	↓	↓	↓	↓	↔	↔	↓	↓	↔
H13	2	64	64	↓	↓	↓	↔	↔	↓	↔	↓	↓	↔
A8	2	> 64	> 64	↓	↓	↓	↔	↔	↓	↔	↓	↓	↔

[a]Iron deficient Mueller-Hinton broth.
[b]Mueller-Hinton broth.
[c]Not determined.

downregulation of these genes was not consistently seen in clinical isolates resistant to cefiderocol (11). The acquisition of β-lactamases, often belonging to the OXA, PER, NDM, or SHV classes of enzymes, can contribute to cefiderocol resistance (1–4, 12, 13). The development of overt cefiderocol resistance for *A. baumannii* may be multifactorial, involving specific β-lactamases with downregulation of iron transport systems (13).

In this report we searched for common themes in the expression of iron transport systems and presence of β-lactamases among a panel of *A. baumannii* clinical isolates with varying degrees of susceptibility to cefiderocol.

RESULTS

From surveillance studies conducted in New York City from 1999 to 2009, 39 cefiderocol-resistant *A. baumannii* isolates underwent sequence typing. Of the 39 isolates, 25 belonged to ST2 and 10 belonged to ST229. Fourteen representative isolates from these 2 sequence types, as well as 2 susceptible isolates (D1 and D13) from ST250 gathered from a 2013 surveillance study, were selected for further analysis.

Iron transport studies. To examine the effect of iron-limiting conditions on the MICs of cefiderocol, susceptibility studies were performed in standard cation-supplemented (MHB) and iron-deficient Mueller-Hinton broth (IDB) (Table 1). The MICs of cefiderocol were generally within 1 dilution when performed in MHB and IDB. In general, reduced expression of *pirA* and *piuA* correlated with cefiderocol MICs $\geq$8 μg/mL. For most isolates with elevated MICs to cefiderocol, there was a general repression in receptors for ferric substrates; however, expression of gene265 (*faoA*), involved in the uptake of ferrous iron, remained present in nearly all isolates. Compared to *A. baumannii* ATCC19606, none of the isolates had alterations in ExbD3 and all possessed a Thr226Ser substitution in TonB3.

***β*-lactamase studies.** The characterization of the β-lactamases in the isolates, and the effect on cefiderocol MICs by the addition of 4 μg/mL of avibactam are noted in Table 2. While most highly resistant isolates possessed an SHV or OXA-type β-lactamase, a susceptible isolate (D1) possessed both SHV and OXA enzymes, and 1 resistant isolate (U20) possessed neither, suggesting they are not essential contributors to cefiderocol resistance. Similarly, increased expression of $bla_{\text{OXA-51-type}}$ was evident in most isolates including an isolate (D13) fully susceptible to cefiderocol.

***bla*$_{\text{ADC}}$ studies.** In contrast, a consistent finding was elevated expression of bla_{ADC} in cefiderocol-resistant isolates (but not in the 2 susceptible isolates D13 and D1). Most

TABLE 2 Susceptibility testing and β-lactamase characterization in clinical isolates of *A. baumannii*

Isolate	ST	MICs (μg/mL)				Acquired β-lactamases	ADC type	Relative expression	
		Ceftazidime	Meropenem	Cefiderocol	Cefiderocol + avibactam (4 μg/mL)			bla_{ADC}	$bla_{oxa-51-type}$
ATCC 19606		8	1	≤ 0.03	≤ 0.03			1	1
D13	250	8	> 16	0.25	0.25	TEM-1	NA[a]	↓	↑
D1	250	> 32	> 16	1	1	SHV-12, OXA-23	ADC25	↔	↔
S15	229	> 32	> 16	4	2	OXA-24	ADC33	↑	↑
K47	229	> 32	> 16	16	8	SHV-5	ADC25	↑	↑
S6	229	> 32	> 16	16	2	SHV-5	ADC25	↑	↑
W7	229	> 32	> 16	16	4	SHV-5	ADC25	↑	↑
W315	229	> 32	8	64	2	SHV-5	ADC25	↑	↑
D103	229	> 32	16	> 64	4	SHV-5	NA	↑	↑
E27	2	> 32	> 16	2	0.25	TEM-1, SHV-12	ADC30	↑	↑
A53	2	> 32	> 16	8	4	OXA-24	ADC33	↑	↑
L301	2	> 32	16	16	2	TEM-1, SHV-5	ADC25	↑	↑
U20	2	> 32	16	16	4	None	ADC33	↑	↑
D501	2	> 32	8	32	4	TEM-1, SHV-5	ADC33	↑	↑
C120	2	> 32	> 16	32	4	TEM-1, SHV-5	ADC25	↑	↑
H13	2	> 32	> 16	64	1	SHV-5	ADC25	↑	↑
A8	2	> 32	> 16	> 64	4	OXA-23	ADC33	↑	↑

[a]No amplification.

isolates possessed ADC-25 or ADC-33. Two cefiderocol-resistant, kanamycin susceptible isolates were selected for bla_{ADC} gene silencing using a site-specific intron (Table 3). Both isolates belonged to ST2 and possessed ADC-25. For 1 isolate (D501), which also possessed SHV-5 and TEM-1, silencing of bla_{ADC} resulted in a decrease in cefiderocol MIC from 32 to 4 μg/mL. For the second isolate (KH111), which did not possess any identified acquired β-lactamase, gene silencing led to a decrease in the MIC of cefiderocol from 24 to 0.25 μg/mL. For both isolates with the disrupted bla_{ADC} gene, the addition of avibactam did not appreciably affect the MICs for cefiderocol or meropenem. For isolate KH111, the ceftazidime MIC fell from > 32 to 16 μg/mL. For isolate D510, which possessed bla_{SHV-5}, there was no change in ceftazidime MICs with gene silencing. Lastly, silencing of the bla_{ADC} gene in *A. baumannii* ATCC 19606, which had a cefiderocol MIC of ≤0.03 μg/mL, had no effect on the MICs to ceftazidime and meropenem (data not shown).

DISCUSSION

With its siderophore side chain, cefiderocol can circumvent most mechanisms associated with antimicrobial resistance. Cefiderocol has retained activity against a wide range of pathogens, including those with a variety of cephalosporinases and carbapenemases. While the overall clinical experience with this agent has been promising, higher mortality and microbiological failure rates have been observed in treated patients harboring infections with *A. baumannii* (14, 15). Understanding the mechanisms leading to cefiderocol resistance in *A. baumannii* will be important in strategizing effective therapeutic regimens.

Cellular uptake of cefiderocol has been linked to specific iron transport systems (16). For example, in laboratory-derived strains of *P. aeruginosa*, disruption of the TonB-dependent siderophore receptors PiuA or its orthologue PiuD increased cefiderocol MICs from 0.5 to 8 and from 0.06 to 2 μg/mL, respectively (6). In our investigation of 2 endemic strains of

TABLE 3 Effect of silencing bla_{ADC} on MICs of cefiderocol, ceftazidime, and meropenem

Isolate	Cefiderocol MICs (μg/mL)	Cefiderocol + avibactam 4 μg/mL	Ceftazidime	Meropenem
D501	32	4	> 32	8
D501 Δ bla_{ADC}	4	4	> 32	8
KH111	24	0.25	> 32	> 32
KH111 Δ bla_{ADC}	0.25	0.094	16	> 32

A. baumannii, a reduction in *piuA* expression was evident in most highly resistant isolates. Our resistant isolates also tended to have reduced expression of other ferric ion transport systems, including those involving acinetobactin (*bauA*) and heme. In contrast, there was continued expression of the ferrous ion uptake complex. Taken together, it appears in cefiderocol-resistant isolates, there is reliance of ferrous uptake and an overall downregulation of ferric transport systems. Finally, these findings were not explained by mutations involving the TonB3-ExbB3-ExbD3 complex that were observed in laboratory-derived isolates resistant to monobactam siderophore agents (10).

While downregulation of iron transport systems appears to provide a level of baseline resistance, β-lactamase activity appears to be the driving force for high-level cefiderocol resistance. A variety of β-lactamases have been associated with cefiderocol-resistant *A. baumannii* (1–4, 12). In our collection, the serine SHV and OXA-type β-lactamases were common in cefiderocol-resistant isolates. However, they were present in a susceptible isolate, and absent in a resistant isolate, suggesting they are not essential for cefiderocol resistance. Similarly, elevated expression of $bla_{\text{OXA-51-type}}$ was observed in a susceptible isolate.

In 1 report involving cefiderocol-resistant *A. baumannii* belonging to a variety of sequence types (ST455, ST473, and ST787), the addition of avibactam lowered cefiderocol MICs approximately 4 to 8-fold (17). In that report, most isolates harbored TEM-1, OXA-23, ADC-30, and OXA-66 β-lactamases (17). In our report, involving isolates belonging to ST229 and the international clone ST2, the presence of avibactam also lowered the cefiderocol MICs $\sim$ 4 to 8-fold. The only common theme among our resistant isolates was increased expression of the AmpC β-lactamase ADC. All the ADC β-lactamases were ADC-25, -30, or -33. For 2 resistant isolates, gene silencing of bla_{ADC} resulted in a $\geq$ 8-fold reduction in cefiderocol MICs, highlighting the importance of this β-lactamase.

The ADC-25, -30, or -33 β-lactamases share Gly242Asp and Arg342Gly alterations. The Gly242Asp change is located in the Ω loop and the Arg342Gly change is near the active site and conserved KTG motif; these alterations have been shown to increase the MICs of both ceftazidime and cefotaxime (18). Mutations affecting other AmpC β-lactamases have correlated with reduced susceptibility to cefiderocol. In *P. aeruginosa*, a Glu247Lys substitution in the Ω loop resulted in reduced susceptibility to cefiderocol, ceftazidime-avibactam, and ceftolozane-avibactam (9). In *Enterobacter cloacae*, an Ala-Pro deletion in the R2 loop provided structural changes allowing increased hydrolysis of cefiderocol (19).

For the clinical isolates examined in this investigation, the driving force for cefiderocol resistance was over-expression of particular bla_{ADC} subtypes in a background of globally reduced ferric iron uptake. Restoring cefiderocol susceptibility will require effective inhibition of these ADC β-lactamases.

MATERIALS AND METHODS

Isolates of *A. baumannii*, gathered from surveillance studies conducted from 1999 to 2009 in medical centers in New York City, were screened for resistance to cefiderocol. Isolates with previously known cefiderocol MICs from a study conducted in 2013 were also considered. Susceptibility testing was performed according to CLSI standards (20). Iron-deficient Mueller-Hinton broth was prepared using Chelex (Sigma-Aldrich) as previously described (6). Multilocus sequence typing (MLST) was performed using the Pasteur scheme (http://pubmlst.org/abaumannii/).

Real time RT-PCR studies. RNA was extracted (RNeasy, Qiagen, Inc.) from cultures, grown in iron-deficient Mueller-Hinton broth, in the early log phase of growth and treated with DNase. Primers and probes were selected based on conserved regions of the genes. (Table 4). Primer and probe concentrations were adjusted to give replicative efficiencies of 90 to 110%, and $\sim$ 25ng of RNA was used as the template. All studies were performed in triplicate. Expressions of target genes were normalized to that of a housekeeping ribosomal gene and calibrated to *A. baumannii* ATCC 19606. Relative expression values of $<$0.2 were considered decreased, values of 0.2 to 1.5 as baseline, and $>$1.5 as increased for the iron transport genes. Isolates were considered to have increased expression of bla_{ADC} or $bla_{\text{OXA-51-like}}$ if relative expression levels were $>$ 10.

Iron transport studies. Expression of the siderophore uptake receptor genes *pirA* and *piuA* were examined as previously described (11). To determine if upregulation of competing iron transport genes may be involved in cefiderocol-resistant strains, additional iron transport complexes were analyzed (21). Gene numbers are based on the *A. baumannii* ACICU genome (https://www.ncbi.nlm.nih.gov/nucleotide/CP000863.1). Three TonB-dependent receptor genes prevalent in most strains of *A. baumannii* were studied: gene 2581 (*bauA*, involved in acinetobactin transport), gene 1633 (involved in heme uptake), and gene 1679 (involved in hydroxamate siderophore transport). In addition, 4 other genes involved in iron transport were included: genes 120, 1761, 3521, and 3537. Finally, gene 265 (*faoA*, involved in ferrous uptake) was also included. All 8 genes were

TABLE 4 Primers and probes used in the real time RT-PCR and PCR studies

Primers and probes for the real-time RT-PCR studies (5′–3′)[a]	DNA sequence
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]
RTGene2581for (*bauA*)	TTGCAGCAACTGATCCATCG
RTGene2581rev	TAACAAAGCGGAGGGACCTT
RTGene2581probe	[DFAM] TCAACACCTCAACGCGGCCA [DTAM]
RTGene265for (*faoA*)	GCGACCATCACCAAAGTGAA
RTGene265rev	TTCCAAACGAATTGCGACCA
RTGene265probe	[DFAM] TGCTGCTCGGACTCTACTTCTATCCGA [DTAM]
RTGene1679for	AGACGTGGCGAATAAATGGC
RTGene1679rev	GCAAATGCGGCTAATTCACG
RTGene1679probe	[DFAM] ACGGTTGCTCCAGTGAAAGGAACCC [DTAM]
RTGene1633for (heme uptake)	GAGCCTTGGGTGCCAATAAG
RTGene1633rev	CGGTCTAACATTGCGAGTGG
RTGene1633probe	[DFAM] TGCATTTGCAGCTAATCGACGGCCA [DTAM]
RTGene120for	ACCTACGTGCCAATACCCAA
RTGene120rev	GCGTTCACTGCTGACATGAT
RTGene120probe	[DFAM] AGTCTGGAGCATACCGCGCCT [DTAM]
RTGene1761for	CGGTCGACTACAGTCCTTCA
RTGene1761rev	AATCGGTATCACGGCTTTGC
RTGene1761probe	[DFAM] ACGATCGCCAGGGCTTTCCGA [DTAM]
RTGene3537for	TGGGACTCCGCTACGATTAC
RTGene3537rev	CCATTTGTCCACCAAACGGT
RTGene3537probe	[DFAM] TGCCATACCAAGCCAACATTTGGGC [DTAM]
RTGene3521for	CAAGATTGGGCACAAACCCA
RTGene3521rev	AGCTCACATAAGGTGCCACA
RTGene3521probe	[DFAM] TGACAGAAGCACTGCCCGTAAATGCA [DTAM]
Primers used for the bla_{ADC}-specific intron	
IBS	AAAAAAGCTTATAATTATCCTTAGGTATCGCTGTGGTGCGCCCAGATAGGGTG
EBS1d	CAGATTGTACAAATGTGGTGATAACAGATAAGTCGCTGTGGGTAACTTACCTTTCTTTGT
EBS2	TGAACGCAAGTTTCTAATTTCGATTATACCTCGATAGAGGAAAGTGTC
Universal intron template	CGAAATTAGAAACTTGCGTTCAGTAAAC
Primers for amplification of the iron transporter regulatory genes	
exbD3for	CACCATGTTGCAGCAAGACTC
exbD3rev	TACTGCAACTTTGCAATTCCACC
tonB3for	TCGCGATAATGCGGAATGTT
tonB3rev	AAGGTTAGCCGTTGGGACTC

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

identified in the *A. baumannii* 19606 genome and in 2 representative isolates of the sequence types included in this study (ST2 and ST250). To search for previously described frameshift mutations (10), the regulatory genes *exbD3* and *tonB3* were amplified and sequenced using the PCR primers noted in Table 4.

***β*-lactamase studies.** Isolates were screened by PCR for the following β-lactamases, using previously reported primers and conditions: TEM, SHV, KPC, OXA-type, CTX-M, metallo- β-lactamases (IMP, VIM, SIM, GIM, SPM, and NDM), VEB, and PER (22–26). Identification of the chromosomal cephalosporinase bla_{ADC} was performed using previously described primers (27). Expression of bla_{ADC} and $bla_{OXA\text{-}51\text{-}type}$ was performed as previously described (11).

***bla*$_{ADC}$ studies.** Disruption of the bla_{ADC} gene was achieved by insertion of group II intron (Targetron Gene Knockout System, Sigma-Aldrich). The gene-specific intron was created using proofreading DNA polymerase and the primers listed in Table 4. The intron was inserted into a Zero Blunt TOPO plasmid (Invitrogen)

and introduced into chemically competent isolates of *A. baumannii* ATCC 19606 and 2 cefiderocol-resistant, kanamycin-susceptible isolates of *A. baumannii*. Transformants were selected on LB agar with 50 μg/mL of kanamycin. The presence of the intron was confirmed by sequencing using M13 primers in the transformed isolates. Cefiderocol MICs of the parent and transformed isolates were performed using antibiotic test strips (Liofilchem).

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