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Role of the drug efflux pump in the intrinsic cefiderocol resistance of *Pseudomonas aeruginosa*

Running title: Role of drug efflux pump in cefiderocol resistance

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Abstract

Cefiderocol is a novel siderophore cephalosporin antibiotic exhibiting activities against carbapenem-resistant Gram-negative bacteria including *Pseudomonas aeruginosa* and *Enterobacteriaceae*. Drug efflux pumps are reportedly involved in both intrinsic and acquired drug resistance, although their role in bacterial cefiderocol susceptibility remains poorly understood. In this study, we investigated how drug efflux pumps contribute to bacterial cefiderocol susceptibility using the efflux pump(s)-deficient and overexpressing strains of *P. aeruginosa*, *Escherichia coli*, and *Salmonella enterica*. We observed that the *mexAB-oprM*-deficient *P. aeruginosa* mutant displayed increased cefiderocol susceptibility compared to the wild-type strain. The overexpression of *mexAB-oprM* or *mexXY-oprM* in the *mexAB-oprM*-deficient mutant increased the MIC value of cefiderocol. Furthermore, the pump inhibitor phenylalanine-arginine β-naphthylamide increased cefiderocol susceptibility in wild-type *P. aeruginosa* whereas it did not affect the susceptibility of the *mexAB-oprM*-deficient mutant. These data indicate that the MexAB-OprM drug efflux system contributes to the intrinsic cefiderocol resistance of *P. aeruginosa*. In addition, MexXY-OprM partially complemented the function of MexAB-OprM in the cefiderocol susceptibility, when expressed.

Keywords

cefiderocol, drug efflux pump, MexAB-OprM, MexXY-OprM, *Pseudomonas aeruginosa*

Introduction

The emergence of drug-resistant bacteria is a current global problem. WHO has published a list of drug-resistant organisms that represent a serious threat to human health, and called for the development of new antimicrobial agents against them. *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacteriaceae* have been listed in the highest priority category as organisms that acquired resistance against various antimicrobial agents, including carbapenems and third-generation cephalosporins (1). The resistance mechanisms against carbapenems include hydrolysis of antimicrobials by carbapenemases, reduced porin expression or mutation of porins, and efflux pump overexpression (2).

Cefiderocol is a novel siderophore cephalosporin antibacterial agent that effectively crosses the outer membrane of Gram-negative bacteria, including that of multidrug-resistant bacteria (3). Cefiderocol is the only drug so far that exhibits antimicrobial activity against carbapenem-resistant *A. baumannii*, *P. aeruginosa*, and *Enterobacteriaceae* (4). In addition, cefiderocol, as a siderophore, possesses an iron-binding structure that allows its active bacterial uptake through iron uptake mechanisms, and it could effectively inhibit cell wall synthesis in the periplasm. Cefiderocol was suggested to be unaffected by the three carbapenem resistance acquisition-associated mechanisms: mutated porin channel-related reduced membrane permeability, β-lactamase-mediated inactivation, and efflux pump overproduction (5, 6). Previous studies examined the antibacterial activity of cefiderocol against various drug-resistant bacteria and showed its higher potency against β-lactamase-possessing Gram-negative bacteria compared to carbapenems, including ESBL and carbapenemases. The presence or absence of porins, related to carbapenem susceptibility, has also been suggested to leave cefiderocol activity unaffected (7). Moreover, the iron transporters reportedly affect cefiderocol activity (3). However,

68 the role of drug efflux pumps in bacterial cefiderocol susceptibility remains elusive.

69 Previous studies described the presence of numerous drug efflux pumps in Gram-negative
70 bacteria including *E. coli*, *Salmonella*, and *P. aeruginosa*. Among them, the drug efflux system of
71 the resistance-nodulation division (RND) superfamily plays a particularly important role, such as
72 AcrAB-TolC in *Salmonella* and *E. coli* or MexAB-OprM in *P. aeruginosa*, recognizing and
73 exporting a wide variety of structurally unrelated antimicrobial agents (8–11).

74 In this study, we first measured and compared the cefiderocol MIC values of wild-type
75 *E. coli*, *S. enterica*, and *P. aeruginosa* strains and multiple drug efflux pump-deleted mutants
76 using iron-limited media. Moreover, we examined the changes in susceptibility when
77 *mexAB-oprM* or *mexXY-oprM* was expressed in *P. aeruginosa mexAB-oprM*-deleted mutants.
78 Furthermore, to investigate how pump inhibition affects the cefiderocol susceptibility of
79 *P. aeruginosa*, we used phenylalanine-arginine β-naphthylamide to monitor the changes in
80 susceptibility. Based on the obtained results, our study clarifies the role of the drug efflux pump
81 in the intrinsic cefiderocol resistance of *P. aeruginosa*.

82

83 **Results**

84 **Effect of efflux pump deletions on the cefiderocol susceptibility of *E. coli*, *S. enterica*, and**
85 ***P. aeruginosa***

86 In order to investigate the contribution of drug efflux pumps to cefiderocol susceptibility, we
87 measured cefiderocol MIC values against the wild-type and efflux pump-deleted mutants of
88 *E. coli*, *S. enterica*, and *P. aeruginosa* (Table 1). We measured the MIC values both in
89 cation-adjusted and iron-depleted cation-adjusted Muller-Hinton broths ((CAMHB and
90 ID-CAMHB, respectively). We detected no significant difference in the cefiderocol susceptibility

between the wild-type *E. coli* strain and the mutant lacking five RND efflux systems (Table 1). Furthermore, we observed no difference either in the susceptibility of the wild-type *S. enterica* strain and that of the mutant lacking nine efflux pumps. The number of drug efflux pumps, including AcrAB, AcrD, MdtABC, MdtEF, AcrEF, MdsAB, EmrAB, and MacAB, can use the TolC outer membrane protein for drug efflux in both *E. coli* and *S. enterica* (12,13). We also measured the cefiderocol susceptibility of the *tolC*-deleted *E. coli* and *S. enterica* mutants. However, the MIC values against these mutants were the same as in the case of the wild-type strains. In contrast to the results obtained in *E. coli* and *S. enterica*, we observed a difference in the cefiderocol susceptibility between the wild-type *P. aeruginosa* and the efflux pump-deleted mutants. For example, the mutant lacking five drug efflux systems, including *mexAB-oprM*, *mexXY*, *mexCD-oprJ*, *mexEF-oprN*, and *mexHI-opmD*, was 32-fold more susceptible to cefiderocol than the wild-type in ID-CAMHB. The cefiderocol MIC values against the *P. aeruginosa* strains in CAMHB were higher than those in ID-CAMHB. Furthermore, smaller differences could be observed between the wild-type and the efflux pump-deleted strains. This was probably due to the good cefiderocol uptake by *P. aeruginosa* under iron limitation. In such a situation, the presence or absence of a drug efflux pump might have affected more the drug accumulation, observed as a difference in susceptibility. The *mexAB* or *mexAB-oprM* genes were deleted in all the mutants susceptible to cefiderocol, suggesting that the MexAB-OprM efflux system is involved in the intrinsic cefiderocol resistance under iron-limited conditions. In fact, the *mexAB-oprM*-deleted mutant was 16-fold more susceptible to cefiderocol than the wild-type strain. *P. aeruginosa* growth in ID-CAMHB was slower than in CAMHB, although no significant difference could be detected between the wild-type strain and the *mexAB-oprM*-deleted mutant under both conditions (Fig. 1), suggesting that the difference in cefiderocol susceptibility

between them is probably due to the MexAB-OprM-mediated cefiderocol efflux.

Effect of MexAB-OprM and MexXY-OprM expression on the cefiderocol susceptibility of *P. aeruginosa*

As described above, the *mexAB-oprM*-deleted *P. aeruginosa* mutant was more susceptible to cefiderocol than the wild-type strain. To see the potential complementary effect of the *mexAB-oprM* expression, we transformed a plasmid carrying *mexAB-oprM* into the *mexAB-oprM*-deleted mutant and measured MIC. The transformation with the *mexAB-oprM* expression plasmid restored cefiderocol susceptibility to the level of the wild-type strain (Table 2). Similarly, the deletion of *mexAB-oprM* also reduced the MICs of antimicrobial agents other than imipenem, and they got restored upon the *mexAB-oprM* expression plasmid transformation (Table 2). Regarding imipenem, we observed no change in MIC in the presence or absence of *mexAB-oprM* (Table 2), consistently with a previous study reporting that imipenem was not affected by the efflux pump but the OprD expression level (14).

As MexXY is also known to form a functional complex with OprM (15) and it is also an important efflux system expressed in multidrug-resistant *P. aeruginosa* strains (16,17), we also investigated the complementary effect of *mexXY-oprM* expression. When we transformed the plasmid carrying *mexXY-oprM* into the Δ*mexAB-oprM* mutant, we observed a four-fold increase in the cefiderocol MIC value, similar to ceftazidime, meropenem, and chloramphenicol (Table 3). Therefore, we could conclude that MexXY-OprM is involved in exporting cefiderocol, meropenem, ceftazidime, and chloramphenicol in a manner that partially complements the function of MexAB-OprM. In addition, *mexXY-oprM* expression increased cefepime and norfloxacin resistance beyond the wild-type level. Therefore, MexXY-OprM is considered to be

the efflux system that can efficiently recognize and export cefepime and norfloxacin.

Effect of the efflux pump inhibitor on the cefiderocol susceptibility of *P. aeruginosa*

The aforementioned results indicate that MexAB-OprM is involved in the intrinsic cefiderocol resistance in *P. aeruginosa*, suggesting that cefiderocol might exhibit its bactericidal function more effectively against *P. aeruginosa* upon functional MexAB-OprM inhibition. We thus evaluated the synergistic effects of the efflux pump inhibitor phenylalanine-arginine β-naphthylamide (PAβN) and cefiderocol against *P. aeruginosa* using the checkerboard method (Fig. 2). In the wild-type strain, we observed a PAβN concentration-dependent increase in cefiderocol susceptibility with a 16-fold increase in the presence of 32 mg/L of PAβN (Fig. 2). However, we observed no change in cefiderocol sensitivity with Δ*mexAB-oprM* in the presence of PAβN (Fig. 2). These results indicated that the intrinsic cefiderocol resistance of *P. aeruginosa* could be suppressed by inhibiting the function of the drug efflux pump.

Discussion

This study demonstrates the involvement of the MexAB-OprM drug efflux system in the intrinsic cefiderocol resistance of *Pseudomonas aeruginosa*. MexXY-OprM is also reportedly involved in cefiderocol efflux in a manner that partially complements the function of MexAB-OprM. Probably because MexAB-OprM masks MexXY function or the *mexXY* expression level is low in the wild-type strain, the *mexXY* deletion itself did not alter cefiderocol susceptibility. As MexXY is reportedly induced in the presence of certain drugs (18), if a MexXY-OprM is expressed in a strain in which MexAB-OprM is no longer functional, this efflux system is suggested to be also involved in intrinsic cefiderocol resistance.

As for the MexCD-OprJ and MexEF-OprN efflux systems, we observed no change in the cefiderocol susceptibility of *P. aeruginosa* when these genes were deleted from the *mexXY*-deficient strain, suggesting that neither of these efflux systems is involved in intrinsic cefiderocol resistance in the PAO1 strain. In *E. coli* and *S. enterica*, no changes could be detected in cefiderocol susceptibility between the wild-type and multiple drug efflux gene-lacking strains, suggesting that the drug efflux systems of these two bacterial species are not related to the intrinsic cefiderocol resistance. MexAB-OprM plays a significant role in the intrinsic resistance of *P. aeruginosa*, probably due to its high cefiderocol efflux activity.

The chemical structure of cefiderocol is similar to both ceftazidime and cefepime, the major difference is the addition of a chlorocatechol group on the end of the C-3 side chain, conferring the siderophore activity (7). When we compared ceftazidime, cefepime, and cefiderocol for their MIC reduction in the lack of *mexAB-oprM*, this reduction was higher for cefiderocol (ceftazidime and cefepime: 2-fold decrease, cefiderocol: 16-fold). Therefore, the unique siderophore structure of cefiderocol could facilitate its recognition by MexAB-OprM.

Previous studies have shown that mutations in *mexR* and *nalD*, resulting in the increased expression of *mexAB-oprM*, led to a 2-fold increase in the cefiderocol MIC value. This is a less potent MIC increase than that related to other agents such as ceftazidime, aztreonam, and ciprofloxacin, the authors thus concluded the lack of significant impact on the cefiderocol susceptibility along with MexAB-OprM (6). Our study revealed that under the iron-limiting conditions, the loss of MexAB-OprM significantly affected the cefiderocol susceptibility of *P. aeruginosa*, indicating the importance of MexAB-OprM in the intrinsic cefiderocol resistance of this organism.

In this study, we showed that the intrinsic cefiderocol resistance of *P. aeruginosa* can be

suppressed by the combination with the efflux inhibitor, suggesting that the inhibition of the MexAB-OprM drug efflux pump can suppress the intrinsic cefiderocol resistance of *P. aeruginosa*. If efflux inhibitors applicable in clinical practice would be developed, a more effective cefiderocol treatment might be possible when used in combination. Finally, it is also possible that the drug efflux overexpression-related future emergence of cefiderocol-resistant strains could be effectively treated in combination with efflux inhibitors.

Materials and Methods

Bacterial strains, plasmids, and growth conditions

Table 3 contains all the bacterial strains and plasmids that we used in this study. The *E. coli*, *S. enterica* serovar Typhimurium, and *P. aeruginosa* strains used in this study were derived from the wild-type strains MG1655 (19), ATCC14028s (20), and PAO1 (21), respectively. The bacterial strains were grown at 37°C in Lysogeny broth, CAMHB, or iron-depleted cation-adjusted Mueller-Hinton broth (ID-CAMHB). Ampicillin was added to the growth media at a final concentration of 100 mg/L for plasmid maintenance. The plasmids pMMB67HE, p*mexAB-oprM*, and p*mexXY-oprM* were kindly provided by Dr. Taiji Nakae (17).

Construction of gene deletion mutants

To construct the gene deletion mutants of *E. coli* and *S. enterica*, gene disruption was performed as described by Datsenko and Wanner (8,22,23). The chloramphenicol resistance *cat* gene and the kanamycin resistance *aph* gene, flanked by Flp recognition sites, were PCR-amplified, and the products were transformed into the recipient MG1655 or ATCC14028s strain harboring plasmid pKD46, expressing the Red recombinase. The mutated loci were PCR-verified and *cat*

and *aph* were eliminated using plasmid pCP20. *P. aeruginosa* PAO1-derived strains lacking genes encoding the MexAB-OprM, MexCD-OprJ, MexEF-OprN, MexXY, and MexHI-OpmD drug efflux systems were kindly provided by Dr. Yuji Morita and Dr. Tomofusa Tsuchiya (21,24).

Susceptibility test

MIC was measured using the broth microdilution method. We added 5 μL of antimicrobials into 96-well plates with 95 μL of bacteria-containing medium and incubated them at 37°C for 18 hrs. Antimicrobials were prepared according to the Clinical and Laboratory Standards Institute (CLSI) (25). Bacteria were cultured in CAMHB or ID-CAMHB overnight at 37°C with shaking and finally tested at 5×10^4 CFU/well after preparation to McFarland standard turbidity of 1 (± 0.1). For the combination of PAβN and cefiderocol, the checkerboard method (26) was used. Five μL each of cefiderocol and PAβN were added into a 96-well plate with 90 μL of the bacteria-containing medium and incubated at 37°C for 18 hrs. Twofold dilutions of each compound were added into 96-well plates at various concentrations as indicated (Fig. 2). Bacteria were tested at 5×10^4 CFU/well as MIC measurement.

Growth curve measurement

Cultures of PAO1 and Δ*mexAB-oprM* were incubated overnight at 37°C with shaking, then diluted and added to CAMHB and ID-CAMHB to obtain an OD_{600} of 0.1. Then they were incubated with shaking at 37°C using Infinite M200 Pro (Tecan), and the absorbance in OD_{600} was measured over time.

Chemicals

Cefiderocol was obtained from MedKoo Biosciences, Inc., (USA, Morrisville). Ceftazidime hydrate, cefepime hydrate, norfloxacin, Chloramphenicol, and PAβN were obtained from Sigma-Aldrich Co. LLC. (USA, St. Louis). Meropenem was obtained from LKT Labs, Inc. (USA, St. Paul). Imipenem was obtained from Banyu Pharmaceutical Co.Ltd. (Japan, Tokyo).

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Transparency declarations

None to declare.

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Table 1. Effects of the drug efflux pump deletion on cefiderocol susceptibility

	MIC (mg/L)	
	Cefiderocol	
Organism	CAMHB	ID-CAMHB
E.coli		
Wild-type	0.5	0.25
Δ*tolC*	0.25	0.25
Δ*acrB acrC mdtABC mdtEF acrEF*	0.25	0.25
Salmonella		
Wild-type	0.25	0.25
Δ*tolC*	0.25	0.25
Δ*acrAB acrEF acrD mdtABC mdsABC emrAB mdfA mdtK macAB*	0.12	0.25
P. aeruginosa		
Wild-type	1	0.5
Δ*mexXY*	1	0.5

Δ*mexAB*	0.5	0.062
Δ*mexAB-oprM*	0.5	0.03
Δ*mexCD-oprJ*	1	0.5
Δ*mexXY mexAB*	0.5	0.062
Δ*mexXY mexAB-oprM*	0.5	0.062
Δ*mexXY mexCD-oprJ mexEF-oprN*	2	0.5
Δ*mexAB mexCD-oprJ mexEF-oprN*	0.5	0.062
Δ*mexAB-oprM mexXY mexCD-oprJ*	1	0.016
Δ*mexAB-oprM mexXY mexEF-oprN*	1	0.008
Δ*mexAB mexXY mexCD-oprJ mexEF-oprN*	1	0.03
Δ*mexAB-oprM mexXY mexCD-oprJ mexEF-oprN*	1	0.03
Δ*mexAB-oprM mexXY mexCD-oprJ mexEF-oprN mexHI-opmD*	0.5	0.016

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Table 2. Effect of *mexAB-oprM* or *mexXY-oprM* on *P. aeruginosa* drug susceptibility

Organism	MIC (mg/L)							
	Cefiderocol		Ceftazidime	Cefepime	Meropenem	Imipenem	Norfloxacin	Chloramphenicol
	CAMHB	ID-CAMHB						
P. aeruginosa								
Wild-type	1	0.5	2	2	0.5	1	0.5	64
Δ*mexAB*	0.5	0.062	1	1	0.12	1	0.25	16
Δ*mexAB-oprM*	0.5	0.03	1	0.25	0.12	1	0.062	4
Δ*mexAB-oprM*/vector(pMMB67HE)	0.5	0.03	1	0.5	0.12	1	0.12	4
Δ*mexAB-oprM*/p*mexAB-oprM*	1	0.5	2	2	0.5	1	0.5	128
Δ*mexAB-oprM*/p*mexXY-oprM*	1	0.12	1	8	0.25	1	2	16

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Table 3. Bacterial strains and plasmids used in this study.

Strains and plasmids as in text	Original name	Characteristics	Reference
E. coli			
Wild-type	MG1655	*Escherichia coli* Wild-type	19
Δ*tolC*	NKE95	Δ*tolC*::Cm	23
Δ*acrB acrD mdtABC mdtEF acrEF*	NKE1329	Δ*acrB* Δ*acrD* Δ*mdtABC* Δ*mdtEF* Δ*acrEF*::km	23
Salmonella			
Wild-type	ATCC 14028s	*Salmonella enterica serovar Typhimurium* Wild-type	20

Δ*tolC*	NKS174	Δ*tolC*	8
Δ*acrAB acrEF acrD mdtABC mdsABC emrAB mdfA mdtK macAB*	NKS196	Δ*acrAB* Δ*acrEF* Δ*acrD* Δ*mdtABC* Δ*mdsABC* Δ*emrAB* Δ*mdfA* Δ*mdtK*::cm Δ*macAB*::km	8
P. aeruginosa			
Wild-type	PAO1	*Pseudomonas aeruginosa* Wild-type	21
Δ*mexXY*	YM2	Δ*mexXY*	21
Δ*mexAB*	YM3	Δ*mexAB*	21
Δ*mexAB-oprM*	YM5	Δ*mexAB-oprM*	21
Δ*mexCD-oprJ*	YM7	Δ*mexCD-oprJ*	21
Δ*mexXY mexAB*	YM4	Δ*mexXY* Δ*mexAB*	21
Δ*mexXY mexAB-oprM*	YM6	Δ*mexXY* Δ*mexAB-oprM*	21

Δ*mexXY mexCD-oprJ mexEF-oprN*	YM24	Δ*mexXY* Δ*mexCD-oprJ* Δ*mexEF-oprN*	21
Δ*mexAB mexCD-oprJ mexEF-oprN*	YM34	Δ*mexAB* Δ*mexCD-oprJ* Δ*mexEF-oprN*	21
Δ*mexAB-oprM mexXY mexCD-oprJ*	YM62	Δ*mexAB-oprM* Δ*mexXY* Δ*mexCD-oprJ*	21
Δ*mexAB-oprM mexXY mexEF-oprN*	YM63	Δ*mexAB-oprM* Δ*mexXY* Δ*mexEF-oprN*	21
Δ*mexAB mexXY mexCD-oprJ mexEF-oprN*	YM44	Δ*mexAB* Δ*mexXY* Δ*mexCD-oprJ* Δ*mexEF-oprN*	21
Δ*mexAB-oprM mexXY mexCD-oprJ mexEF-oprN*	YM64	Δ*mexAB-oprM* Δ*mexXY* Δ*mexCD-oprJ* Δ*mexEF-oprN*	21
Δ*mexAB-oprM mexXY mexCD-oprJ mexEF-oprN mexHI-opmD*	PMX52	Δ*mexAB-oprM* Δ*mexXY* Δ*mexCD-oprJ* Δ*mexEF-oprN* Δ*mexHI-opmD*	24
Δ*mexAB-oprM*/vector(pMMB67HE)	NKP32	Δ*mexAB-oprM*/vector(pMMB67HE)::Amp	this study
Δ*mexAB-oprM*/p*mexAB-oprM*	NKP26	Δ*mexAB-oprM*/p*mexAB-oprM*::Amp	this study

Δ*mexAB-oprM*/p*mexXY-oprM*	NKP33	Δ*mexAB-oprM*/p*mexXY-oprM*::Amp	this study
Vector			
pMMB67HE			17
Plasmid			
p*mexAB-oprM*		*mexAB-oprM* genes cloned into pMMB67HE	17
p*mexXY-oprM*		*mexXY-oprM* genes cloned into pMMB67HE	17

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Figure legends

Figure 1. Growth of *P. aeruginosa* PAO1 wild-type and Δ*mexAB-oprM* strains in cation-adjusted Mueller-Hinton broth (CAMHB) (left column) or iron-depleted cation-adjusted Mueller-Hinton broth (ID-CAMHB) (right column). The growth of PAO1 and YM5 was measured as described in the Materials and Methods.

Figure 2. Synergistic effect of cefiderocol and the efflux inhibitor PAβN against *P. aeruginosa*. To evaluate the effect of the combination of cefiderocol and PAβN against PAO1 (wild-type) and Δ*mexAB-oprM* (YM5), we performed the checkerboard assay as described in the Materials and Methods.