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Comprehensive analysis of resistance-nodulation-cell division superfamily (RND) efflux pumps from *Serratia marcescens*, Db10

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We investigated the role of the resistance-nodulation-cell division superfamily (RND) efflux system on intrinsic multidrug resistance in *Serratia marcescens*. We identified eight putative RND efflux system genes in the *S. marcescens* Db10 genome that included the previously characterized systems, *sdeXY*, *sdeAB*, and *sdeCDE*. Six out of the eight genes conferred multidrug resistance on KAM32, a drug hypersensitive strain of *Escherichia coli*. Five out of the eight genes conferred resistance to benzalkonium, suggesting the importance of RND efflux systems in biocide resistance in *S. marcescens*. The energy-dependent efflux activities of five of the pumps were examined using a rhodamine 6G efflux assay. When expressed in the *tolC*-deficient strain of *E. coli*, KAM43, none of the genes conferred resistance on *E. coli*. When *hasF*, encoding the *S. marcescens* TolC ortholog, was expressed in KAM43, all of the genes conferred resistance on *E. coli*, suggesting that HasF is a major outer membrane protein that is used by all RND efflux systems in this organism. We constructed a *sdeXY* deletion mutant from a derivative strain of the clinically isolated multidrug-resistant *S. marcescens* strain and found that the *sdeXY* deletion mutant was sensitive to a broad spectrum of antimicrobial agents.

Serratia marcescens, a Gram-negative bacilli, is widely distributed in the environment. Although not initially regarded as a pathogen, *S. marcescens* is associated with occasional hospital-related outbreaks. The treatment of *S. marcescens* infections with antimicrobial agents is becoming more challenging because clinically isolated strains that exhibit elevated resistance against β -lactams, aminoglycosides, and fluoroquinolones have been reported^{1,2}.

Resistance-nodulation-cell division superfamily (RND) efflux systems play a major role in multidrug resistance in Gram-negative bacteria^{3–9}. RND-type efflux systems consist of three components: the inner membrane protein (IMP), periplasmic membrane fusion protein (MFP), and outer membrane protein (OMP). The electrochemical potential of H⁺ across the cell membrane appears to be the driving force for drug efflux associated with RND efflux systems. Three RND efflux systems in *S. marcescens*, *SdeXY*¹⁰, *SdeAB*¹¹, and *SdeCDE*¹¹, have been characterized to date. *SdeXY* was the first multidrug efflux system to be characterized from *S. marcescens*, and showed broad substrate specificity when expressed and characterized in *Escherichia coli*¹⁰. The gene expression of *sdeXY* was up-regulated in a tigecycline-resistant clinically isolated strain of *S. marcescens*¹². The gene inactivation of *sdeXY* from the environmentally isolated *S. marcescens*-type strain, NCTC10211, increased susceptibilities to tigecycline, tetracycline, ciprofloxacin, and ceftiofur. *SdeAB* also showed broad substrate specificity when expressed in *E. coli*¹¹. Gene knockout analyses revealed that *SdeAB* conferred intrinsic multidrug resistance

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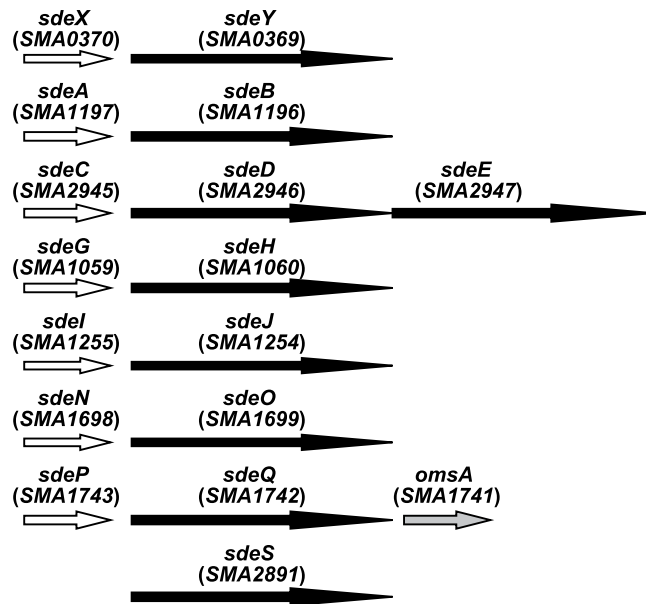


Figure 1. Putative *S. marcescens* RND efflux systems in Db10. White arrow; gene for the periplasmic membrane fusion protein, Black arrow; gene for the inner membrane protein, Gray arrow; gene for the outer membrane protein.

to fluoroquinolones, chloramphenicol, novobiocin, sodium dodecyl sulphate (SDS), and ethidium bromide in *S. marcescens*¹³. In a separate study, a *S. marcescens* cetylpyridinium chloride mutant showed the up-regulated expression of *sdeAB* and also became resistance to fluoroquinolones, tetracycline, chloramphenicol, and benzalkonium chloride¹⁴. In contrast to SdeXY and SdeAB, SdeCDE did not exhibit broad substrate specificity and only conferred novobiocin resistance to *S. marcescens*¹⁵.

In the present study, we aimed to identify uncharacterized *S. marcescens* RND efflux systems that have the potential to render *S. marcescens* with multidrug resistance. To achieve this, we examined putative *S. marcescens* RND efflux system genes from *S. marcescens* Db10 and characterized their substrate specificities in drug-hypersensitive *E. coli* strain KAM32¹⁶. We identified an additional three uncharacterized RND efflux systems with broad substrate specificities. A gene deletion analysis revealed that SdeXY conferred intrinsic multidrug resistance to *S. marcescens*.

Results

Cloning of putative RND-type efflux pumps from *S. marcescens*. When we initiated this study, the *S. marcescens* Db11 genomic sequence database (<http://www.sanger.ac.uk/resources/downloads/bacteria/serratia-marcescens.html>) was the only publicly available resource for the genomic sequence of this bacterium. Using the *S. marcescens* Db11 genomic sequence, we searched for RND-type efflux systems in the *S. marcescens* Db11 genome and identified eight RND-type efflux systems (Fig. 1). These included three characterized *S. marcescens* RND efflux systems, SdeXY (SMA0370-0369)¹⁰, SdeAB (SMA1197-1196)¹¹, and SdeCDE (SMA2945-2946-2947)¹¹, and five putative RND-type efflux systems. We designated these putative RND efflux systems as shown in Fig. 1. The putative outer membrane protein (OMP) gene, *omsA*, was located adjacent to *sdePQ*. The other RND efflux systems did not contain the adjacent OMP gene. All of the RND efflux system genes contained the periplasmic membrane fusion protein (MFP) gene, except for *sdeS*. SdeCDE contained the two inner membrane protein (IMP) genes, *sdeD* and *sdeE*.

We performed a dendrogram analysis of entire sequences of IMPs from *S. marcescens*, *E. coli*, *Vibrio parahaemolyticus*, *Vibrio cholerae*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa*, and revealed that the IMPs from these organisms were divided into five groups (Fig. 2). In contrast to *E. coli* that does not have IMP in Group 2 or 3, each group contained at least one *S. marcescens* IMP, indicating that *S. marcescens* has a wide variety of RND efflux systems.

We cloned all *S. marcescens* RND efflux system genes from the *S. marcescens* Db10 strain, the parental strain of Db11, and expressed them in the drug-hypersensitive *E. coli* strain, KAM32, for further characterization.

Substrate specificities of *S. marcescens* RND efflux systems. To assess the substrate specificity of each RND efflux system, we measured the MICs of various antimicrobial agents using strains expressing each RND efflux system gene(s) in *E. coli* KAM32 (Table 1).

Many of the RND efflux systems in Group 1 play a major role in intrinsic multidrug resistance due to their broad substrate specificities^{3,10,17–25}. Group 1 also contains the RND efflux system genes that are not in an operon with an MFP gene (e.g. *acrD* from *E. coli*). This type of RND efflux system generally exhibits narrow substrate specificities and SdeS only conferred resistance to erythromycin, novobiocin, SDS, and deoxycholate. Consistent with our previous findings¹⁰, the KAM32 strain expressing *sdeXY* conferred resistance to a broad spectrum of

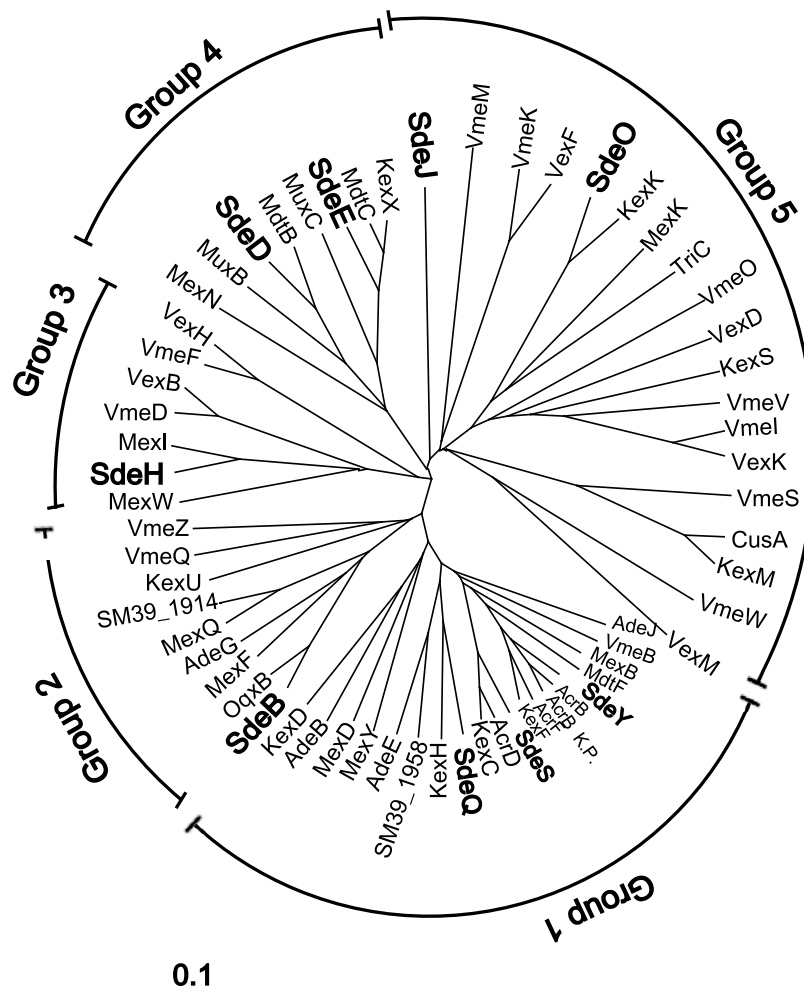


Figure 2. Unrooted phylogenetic tree of the inner membrane protein of RND efflux pumps. The phylogenetic tree was obtained using CLUSTALW (<https://clustalw.ddbj.nig.ac.jp>). *E. coli*: AcrB, AcrD, AcrF, MdtB, MdtC, MdtF, CusA^{22,33,45,46}. *V. parahaemolyticus*: VmeB, VmeD, VmeF, VmeI, VmeK, VmeM, VmeO, VmeQ, VmeS, VmeW, VmeV, VmeZ^{3–5}. *V. cholerae*: VexB, VexD, VexF, VexH, VexK, VexM^{29,31}. *S. marcescens*: SdeB, SdeD, SdeE, SdeY, SdeH, SdeJ, SdeO, SdeQ, SdeS, SM39_1914, SM39_1958^{10–15}. *A. baumannii*: AdeB, AdeE, AdeG, AdeJ^{17,18,20,26}. *P. aeruginosa*: MexD, MexF, MexI, MexK, MexN, MexQ, MexW, MexY, MuxB, MuxC, TriC^{21,24,27,28,30,32,34–36}. *K. pneumoniae*: AcrB(K.P.), KexD, OqxB, KexC(KPN_RS15040), KexF(KPN_RS19875), KexH(KPN_RS21805), KexK(KPN_RS11560), KexM(KPN_RS25535), KexS(KPN_RS04245), KexU(KPN_RS03035), KexW(KPN_RS13595), KexX(KPN_RS13600)^{19,23,47}.

antimicrobial agents (Table 1). When *sdePQ* was expressed in the KAM32 strain with the adjacent OMP gene, *omsA*, multidrug resistance against several antimicrobial agents was conferred.

The RND efflux systems categorized into Group 2 have relatively broad substrate specificities and generally confer acquired resistance^{4,11,13,14,26–28}. SdeB was categorized into this group and conferred multidrug resistance to *E. coli* KAM32; however, the substrate specificity of SdeAB was not as broad as those of SdePQ-OmsA and SdeXY (Table 1). The amino acid sequence of SdeB was similar to the *P. aeruginosa* RND efflux pump, MexF. Similar to MexEF²⁷, fluoroquinolone and chloramphenicol were good substrates for SdeAB. Consistent with previous findings¹¹, the KAM32 strain expressing *sdeAB* was resistant to the quaternary ammonium compound benzalkonium chloride.

The RND efflux systems in Group 3 have relatively broad substrate specificities and also confer acquired resistance^{4,29–32}. Among *S. marcescens* RND IMPs, only SdeH was categorized into this group. The expression of *sdeGH* in *E. coli* KAM32 conferred multidrug resistance. The substrate specificity of SdeGH was broader than that of SdeAB, but was not similar to that of SdeXY or SdePQ-OmsA. Although SdeH showed amino acid sequence similarities to MexI and MexW, the substrate specificity of SdeGH was not similar to that of MexHI³² or MexVW³⁰.

sdeCDE contained two IMP genes within its operon and SdeD and SdeE were both categorized into Group 4. As previously described¹⁵, novobiocin was the only substrate for SdeCDE (Table 1). To establish whether both of these IMP genes are required for this system, we constructed plasmids that expressed *sdeCD* or *sdeCE* and found that neither of these plasmids conferred novobiocin resistance in KAM32 (Table 1). This result indicated

Antimicrobial agent	MIC ($\mu\text{g/mL}$)											
	KAM32/ pUC19 (control)	Group 1				Group 2	Group 3	Group 4			Group 5	
		KAM32/ pURS2 (<i>sdeXY</i>)	KAM32/ pURS8 (<i>sdePQ-omsA</i>)	KAM32/ pURS2 (<i>sdePQ</i>)	KAM32/ pURS9 (<i>sdeS</i>)	KAM32/ pURS3 (<i>sdeAB</i>)	KAM32/ pURS5 (<i>sdeGH</i>)	KAM32/ pURS4 (<i>sdeCDE</i>)	KAM32/ pURS44 (<i>sdeCD</i>)	KAM32/ pURS45 (<i>sdeCE</i>)	KAM32/ pURS6 (<i>sdeIJ</i>)	KAM32/ pURS7 (<i>sdeNO</i>)
Norfloxacin	0.016	0.06	0.13	0.06	0.016	0.06	0.13	0.03	0.03	0.03	0.016	0.016
Erythromycin	4	64	8	8	8	4	4	4	4	4	4	4
Chloramphenicol	1	8	1	2	1	8	1	1	1	1	1	1
Tetracycline	0.5	4	0.25	0.25	0.5	0.5	0.5	0.5	0.25	0.25	0.5	0.5
Benzalkonium Cl	4	32	8	4	4	16	32	4	4	4	32	4
Triclosan	0.5	2	0.25	0.13	0.5	4	8	0.5	N.D.	N.D.	0.5	0.5
Novobiocin	2	>128	16	16	8	4	4	8	2	2	2	2
SDS	128	>8200	>8200	256	>8200	256	1020	256	256	256	128	512
Deoxycholate	2050	32800	>32800	>32800	16400	2050	32800	2050	2050	2050	2050	2050
Ethidium bromide	4	>128	16	8	4	128	128	4	4	4	4	4
Rhodamine 6G	8	>128	>128	128	8	64	>128	8	8	8	>128	8

Table 1. MICs of antimicrobial agents in *E. coli* KAM32 harboring each RND-type efflux pump. N.D.; not determined.

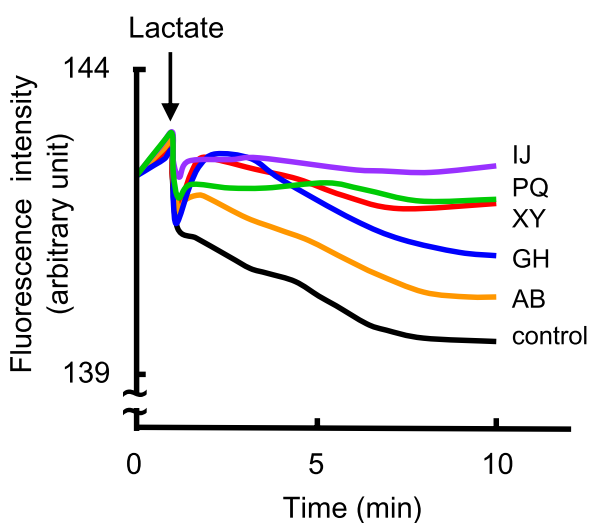


Figure 3. Rhodamine 6G efflux assay. Energy-starved cells of *E. coli* KAM32 strains that express *S. marcescens* RND efflux systems were prepared as described in the Materials and Methods. Energy-starved cells were resuspended in PBS containing 5 mM MgSO_4 and 1 μM rhodamine 6G. At the time point indicated by the arrow, 20 mM potassium lactate (K-Lactate) was added to energize cells. Intracellular rhodamine 6G levels were monitored continuously by measuring the fluorescence of rhodamine 6G at excitation and emission wavelengths of 529 and 553 nm, respectively. IJ; *E. coli* KAM32/pURS6 (*sdeIJ*), PQ; *E. coli* KAM32/pURS8 (*sdePQ-omsA*), XY; *E. coli* KAM32/pURS2 (*sdeXY*), GH; *E. coli* KAM32/pURS5 (*sdeGH*), AB; *E. coli* KAM32/pURS3 (*sdeAB*), control; *E. coli* KAM32/pUC19.

that SdeD and SdeE are both required for novobiocin resistance. This phenotype is similar to other RND efflux systems categorized into Group 4, such as MdtABCD from *E. coli*³³ and MuxABC from *P. aeruginosa*³⁴.

SdeJ and SdeO were categorized into Group 5. Although Group 5 contains many of the *Vibrio* RND efflux systems that show relatively wide substrate specificities^{4,5,29,31}, the expression of *sdeIJ* conferred resistance to only benzalkonium and rhodamine 6G, while *sdeNO* expression conferred resistance solely to SDS. TriC and MexK of *P. aeruginosa* were reported to contribute to the resistance of triclosan^{35,36}. However, introduction of *sdeIJ* and *sdeNO* didn't render triclosan resistance to host *E. coli*.

We measured the efflux of rhodamine 6G to evaluate the activity of each RND efflux system because rhodamine 6G is a good substrate for most of the *S. marcescens* RND efflux systems (SdeXY, SdeAB, SdeGH, SdeIJ, and SdePQ-OmsA) (Fig. 3). All of these five efflux systems showed higher rhodamine 6G efflux activities when lactate was provided as the energy source, indicating that rhodamine 6G efflux systems are energy-dependent.

Requirement of OMP. Except for *sdePQ-omsA*, the other eight *S. marcescens* RND efflux systems did not contain the adjacent OMP gene. Thus, these RND efflux systems must rely on *E. coli* OMP(s) when expressed in

Antimicrobial agent	MIC (µg/mL)										
			Group 1						Group 2		
	KAM43/pUC19	KAM43/pUC19/pSOS2	KAM43/pURS2	KAM43/pURS2/pSOS2	KAM43/pURS82	KAM43/pURS82/pSOS2	KAM43/pURS8	KAM43/pURS9	KAM43/pURS9/pSOS2	KAM43/pURS3	KAM43/pURS3/pSOS2
	(control)	(<i>hasF</i>)	(<i>sdeXY</i>)	(<i>sdeXY</i>)	(<i>sdePQ</i>)	(<i>sdePQ</i>)	(<i>sdePQ</i>)	(<i>sdeS</i>)	(<i>sdeS</i>)	(<i>sdeAB</i>)	(<i>sdeAB</i>)
Norfloxacin	0.016	0.032	0.016	0.064	0.016	0.016	0.032	0.016	0.016	0.016	0.032
Erythromycin	4	4	4	128	4	4	32	2	4	2	2
Benzalkonium	2	2	4	8	4	2	8	2	2	4	4
Novobiocin	0.25	4	0.5	32	0.5	4	32	0.5	2	0.5	2
SDS	32	64	32	>512	32	128	>512	32	128	32	128
Ethidium bromide	1	2	2	128	1	4	32	1	2	1	1
Rhodamine 6 G	4	8	4	>128	4	32	256	4	4	4	64

Antimicrobial agent	MIC (µg/mL)										
			Group 3			Group 4		Group 5			
	KAM43/pUC19	KAM43/pUC19/pSOS2	KAM43/pURS5	KAM43/pURS5/pSOS2	KAM43/pURS4	KAM43/pURS4/pSOS2	KAM43/pURS6	KAM43/pURS6/pSOS2	KAM43/pURS7	KAM43/pURS7/pSOS2	
	(control)	(<i>hasF</i>)	(<i>sdeGH</i>)	(<i>sdeGH</i>)	(<i>sdeCDE</i>)	(<i>sdeCDE</i>)	(<i>sdeIJ</i>)	(<i>sdeIJ</i>)	(<i>sdeNO</i>)	(sdeNO)	
Norfloxacin	0.016	0.032	0.016	0.128	0.016	0.016	0.016	0.032	0.016	0.032	
Erythromycin	4	4	4	8	2	4	4	8	4	4	
Benzalkonium	2	2	4	16	2	4	4	4	2	4	
Novobiocin	0.25	4	0.5	4	0.5	8	0.5	4	0.5	4	
SDS	32	64	16	>512	32	64	32	256	32	256	
Ethidium bromide	1	2	2	64	2	4	1	16	1	32	
Rhodamine 6 G	4	8	4	>128	4	4	4	32	4	32	

Table 2. Effects of HasF on antimicrobial sensitivity.

E. coli. TolC is a major OMP in *E. coli* and all of the *E. coli* RND efflux systems require TolC for their activities³⁷. To clarify whether *S. marcescens* RND efflux systems utilize TolC when expressed in *E. coli*, we introduced *S. marcescens* RND efflux system genes into *E. coli* KAM43, a *tolC*-deficient strain, and tested antimicrobial susceptibilities by measuring MICs. Except for *sdePQ-omsA*, none of the *S. marcescens* RND efflux systems showed increases in MICs when expressed in KAM43, indicating that *S. marcescens* RND efflux systems require TolC for their activities when expressed in *E. coli* (Table 2).

We then investigated whether SdePQ utilizes TolC as an OMP. We subcloned *sdePQ* and expressed it in *E. coli* KAM 32 and KAM43. When expressed in *E. coli* KAM32, *sdePQ* increased MICs for several antimicrobial agents, similar to *E. coli* KAM32 expressing *sdePQ-omsA*, except for SDS (Table 1). However, when expressed in KAM43, *sdePQ* didn't increase MIC for any agents. These results indicated that SdePQ utilizes TolC when *omsA* is absent; however, this interaction may be weaker than that with *OmsA* (Table 2).

S. marcescens is known to possess the functional ortholog of TolC, HasF. Previous studies showed that SdeAB and SdeXY utilized HasF as their OMP component^{12,13}. Since TolC may be utilized by all *S. marcescens* RND efflux systems, we hypothesized that the other *S. marcescens* RND efflux systems also utilize HasF as the OMP. To examine this, the *hasF* gene was cloned and expressed with *S. marcescens* RND efflux systems in the KAM43 strain. Consistent with previous findings, *sdeXY* and *sdeAB* both increased MICs when expressed with *hasF* in *E. coli* KAM43 (Table 2). When expressed with *hasF*, all of the *S. marcescens* RND efflux systems showed increased MICs, indicating that they also utilized HasF as the OMP. Compared Table 1 with Table 2, SdeAB-HasF and SdeNO-HasF showed higher MICs than SdeAB-TolC and SdeNO-TolC, whereas SdeIJ-HasF showed lower MICs than SdeIJ-TolC. These results indicated that compatibility between IMP and/or MFP and OMP is important.

Introduction of *sdeS* in KAM43 or KAM43/pSOS2 didn't render the increase for any tested antimicrobial agents (Table 2), but in KAM32, increase of MICs for novobiocin, SDS, deoxycholate was observed (Table 1). Since no increase of MICs was observed in KAM33, an *acrA* disruptant of KAM32, SdeS would utilize AcrA as MFP in *E. coli* (Supplementary Table S1).

The *sdeXY* deletion mutant of *S. marcescens* is susceptible to a broad range of antimicrobial agents.

Since the present results indicated that SdeXY has the broadest substrate specificity among the characterized *S. marcescens* efflux pumps¹⁰, we constructed a *sdeXY* mutant strain from *S. marcescens*. We attempted to construct the deletion strain from Db10, but were unsuccessful for an unknown reason. Therefore, we used another strain KS24, a derivative of the clinically isolated strain of *S. marcescens* SM39³⁸. The *sdeXY* deletion strain of KS24 became more sensitive to a broad spectrum of antimicrobial agents than the parental strain (Table 3). The *sdeXY* mutant strain also showed the decreased energy-dependent efflux of ethidium (Fig. 4). These results indicated that SdeXY is a major RND efflux pump that confers intrinsic resistance to *S. marcescens* against multiple antimicrobial agents.

Antimicrobial agent	MIC ($\mu\text{g/mL}$)			
	KS24	KS24 ΔsdeXY	KS24 $\Delta\text{sdeXY}/\text{pSTV28}$	KS24 $\Delta\text{sdeXY}/\text{pSMXY}$
Cloxacillin	512	16	0.5	>512
Oxacillin	512	16	0.5	512
Erythromycin	256	2	2	256
Tetracycline	8	1	1	8
Chloramphenicol	8	1	N.D.	N.D.
Novobiocin	64	1	1	64
Norfloxacin	16	1	1	16
Ofloxacin	4	0.25	0.25	8
Ciprofloxacin	4	0.25	0.25	4
Benzalkonium chloride	32	2	2	32
Chlorhexidine gluconate	16	2	2	16
Triclosan	>1024	256	256	>1024
Acriflavine	128	16	16	256
Ethidium bromide	512	1	2	512
Hoechst33342	16	0.13	0.063	16
Rhodamine6G	1024	4	4	1024
TPP	4096	4	4	4096
Sodium cholate	>51200	3200	800	>51200
Sodium deoxycholate	3200	800	400	3200
SDS	51200	50	<25	51200

Table 3. MICs of antimicrobial agents in *S. marcescens*. N.D.; not determined, TPP; tetraphenylphosphonium chloride.

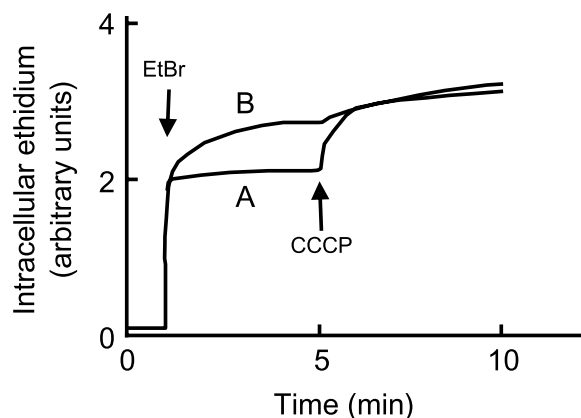


Figure 4. Ethidium efflux activity in *S. marcescens* cells. The cells of the *S. marcescens* KS24 strain (A) and its KS24 ΔsdeXY (B) were prepared as described in the Materials and Methods. Ethidium bromide was added to cell suspensions at a final concentration of $10\mu\text{M}$ at the time point indicated by the first downward arrow. Intracellular ethidium levels were monitored continuously by measuring the fluorescence of ethidium at excitation and emission wavelengths of 500 and 580 nm, respectively. At the second downward arrow, CCCP was added to the suspensions at a final concentration of $100\mu\text{M}$. Assays were performed at 37°C .

Discussion

Previous studies suggested that RND efflux systems play a major role in multidrug resistance in *S. marcescens*^{10–14}. Since Gram-negative bacteria have been suggested to possess ‘multiple’ and ‘active’ RND efflux systems^{3–9}, we hypothesized that *S. marcescens* has other ‘active’ RND efflux systems. To investigate this, we cloned all of the putative RND efflux systems from the *S. marcescens* Db10 strain, characterized them in *E. coli*, and further identified “active” *S. marcescens* RND efflux systems with broad substrate specificities.

A previous study suggested that SdeAB is the primary RND efflux system in *S. marcescens*, with the *sdeB* mutant becoming hypersensitive to multiple antimicrobial agents, similar to the *hasF* mutant¹³. However, the present study showed that SdeAB had narrower substrate specificities than SdeXY, SdePQ, and SdeGH. We also found that the *sdeXY* mutant became hypersensitive to a broad spectrum of antimicrobial agents. Furthermore, an independent study indicated that *sdeAB* was not expressed in the wild-type strain of *S. marcescens* and the

expression of *sdeAB* was induced by the biocide, cetylpyridinium chloride¹⁴. Thus, SdeAB may play a primary role in multidrug resistance only in specific strains of *S. marcescens* and/or a strain that is exposed to a specific biocide.

We identified two previously uncharacterized *S. marcescens* RND efflux systems, SdePQ-OmsA and SdeGH, which exhibit broad substrate specificities. Our reverse transcription-PCR analyses on the *S. marcescens* Db10 and KS24 strains under normal growth conditions revealed that *sdeQ* and *sdeH* gene expression was not detected, while *sdeY* gene expression was observed in both strains (data not shown). When some mutations were occurred which caused the expression of SdePQ-OmsA and SdeGH systems, these pumps would contribute to the acquired resistance in *S. marcescens*.

All of the *S. marcescens* RND efflux systems utilized TolC and HasF when expressed in *E. coli*. However, some RND pumps when expressed with TolC of *E. coli* or HasF of *S. marcescens* showed different substrate specificities. SdeNO expressed with HasF in KAM43 showed higher MICs than that expressed in KAM32 with TolC, whereas SdeIJ and SdeS showed lower MICs. These results suggest that compatibility between outer membrane proteins and other components in RND pumps is important for its efflux activity. As our group reported previously, only VmeAB in *V. parahaemolyticus* showed high MICs expressed with TolC of *E. coli*, while other RND pumps in *V. parahaemolyticus* had markedly higher MICs when expressed with VpoC, an orthologue of TolC⁴. This result may be important for understanding the interaction between outer membrane proteins and other components.

In summary, the present study revealed that *S. marcescens* has multiple RND efflux systems that have the potential to confer multidrug resistance. Among these systems, SdeXY plays a major role in intrinsic multidrug resistance in *S. marcescens*. SdePQ-OmsA and SdeGH showed broad substrate specificities similar to SdeXY; however, these systems appear to be inducible and do not play major roles in the intrinsic multidrug resistance of *S. marcescens*.

Materials and Methods

Bacterial strains and growth conditions. The bacterial strains and plasmids used in the present study are listed in Supplementary Table S2. Unless otherwise noted, bacterial cells were grown in Luria (L) medium (1% polypeptone, 0.5% yeast extract, 0.5% NaCl, pH 7) at 37 °C under aerobic conditions. Antibiotics were supplemented when required as follows: ampicillin, 100 µg/ml; chloramphenicol, 20 µg/ml.

Phylogenetic tree of IMPs. Entire sequences of inner membrane protein were obtained from several database. The phylogenetic tree was obtained using CLUSTALW (<https://clustalw.ddbj.nig.ac.jp>).

Cloning, sequencing, and gene manipulation. We identified putative *S. marcescens* RND efflux system genes using the *S. marcescens* Db11 genomic sequence database (<http://www.sanger.ac.uk/resources/downloads/bacteria/serratia-marcescens.html>). DNA fragments, which contained the open reading frames (ORFs) of *S. marcescens* RND efflux system genes or *S. marcescens* OMP, *hasF* (SMA3509), were amplified by PCR using the chromosomal DNA of *S. marcescens* Db10, the parent strain of Db11³⁹, as a template. Primers used for cloning are listed in Supplementary Table S3. Each primer included a restriction enzyme recognition site (underlined). The PCR products obtained were digested with the indicated restriction enzymes, gel-purified, and then ligated into the same restriction enzyme sites of the vector pUC18, pUC19, or pSTV28 (for *hasF*), which were located downstream of the *lac* promoter of the vector plasmid. Since PCR products did not include the promoter region of the genes, gene expression was controlled by the *lac* promoter.

The cloning of *sdeCDE* (SMA2945-2946-2947) was performed in two steps. The 5' half fragment was amplified with two primers (SMA2945-2947F fw EcoRI and SMA2945-2947F re BamHI), and the 3' half was then amplified with two primers (SMA2945-2947B fw EcoRI and SMA2945-2947B re BamHI). After digestion with *EcoRI* and *BamHI*, each fragment was individually inserted into pUC18. The resultant plasmids were designated as pURS4F and pURS4B. After the digestion of pURS4B with *MluI* and *BamHI*, the fragment was inserted into pURS4F at the same sites. It was named pURS4. The plasmid pURS4 carrying *sdeC* and *sdeD*, but not *sdeE* was also constructed. The plasmid pURS4 carrying *sdeC-sdeE* was digested with *NcoI* and self-ligated. Similarly, the plasmid pURS45 carrying *sdeC* and *sdeE*, but not *sdeD* was constructed. The plasmid pURS4 was digested with *Tth1111*, blunted, and self-ligated.

To evaluate the function of OmsA, the gene of which is located downstream of *sdePQ* (SM1743-1742), two types of plasmids were constructed. The fragment including *sdePQ-omsA* was amplified with PCR using SMA1743-1741 fw *XbaI* and SMA1743-1741 re *EcoRI*. After digestion with *XbaI* and *EcoRI*, the fragment was inserted at the same site in pUC19, and the resultant plasmid was named pURS8. To construct pURS82 carrying incomplete *omsA*, pURS8 was digested with *HpaI* and self-ligated.

Since *S. marcescens* showed higher β -lactam resistance, we were unable to utilize pURS2 carrying *sdeXY*. To complement *sdeXY*, we constructed another plasmid pSMXY. PCR was initially performed with two primers SMA0370-0369 fw *EcoRI* and SMA0370-0369 re *XbaI* using the Db10 genome as a template. PCR was then performed with two different primers, SMA0370-0369 fw *EcoRI* and SMA0370-0369 re *BglII*, to create the *BglII* site instead of the *XbaI* site. The PCR products obtained were digested with *EcoRI* and *BglII*, gel-purified, and then ligated into the *EcoRI-BamHI* sites of the vector pSTV28.

Minimum inhibitory concentrations (MICs). The MICs of various antimicrobial agents were assessed in Muller-Hinton broth (Difco) using the standard two-fold dilution method as previously described⁴⁰.

Construction of the *sdeXY* deletion strain. *S. marcescens* KS24, a plasmid pSMC2-cured derivative of *S. marcescens* KS3⁴¹ was used to construct a *sdeXY* deletion strain. The *sdeXY* deletion strain of *S. marcescens* KS24 was constructed by homologous recombination using the lambda Red recombinase system⁴². Two-step PCR of the gentamicin cassette flanked by long (1000 nt) homologous extensions of the target gene were essentially performed as previously described⁴³ using pBRFRTGM and the genomic DNA of *S. marcescens* KS24 as the template

and the primers listed in Supplementary Table S3. The resulting PCR product was separated on an agarose gel and purified using GENE CLEAN II KIT (MP Biomedicals Inc.). *sdeXY* mutant strains were generated by electroporation of the purified PCR product into *S. marcescens* KS24/pKD46 as described previously⁴². The deletion of *sdeXY* in the mutant strain was verified by PCR.

Measurements of rhodamine 6 G and ethidium efflux activities. The efflux of rhodamine 6 G and ethidium was evaluated as previously described^{3,40}. In the rhodamine6G efflux assay, *E. coli* KAM32 strains were grown in L media until O.D.₆₅₀ = 0.7. *E. coli* cells were harvested by centrifugation, washed twice using Potassium Phosphate Buffer (PPB) containing 5 mM MgSO₄, and resuspended in the same buffer that contained 1 μM rhodamine 6 G and 40 μM carbonyl cyanide-*m*-chlorophenylhydrazone (CCCP). The cell suspension was incubated at 37 °C for one hour to de-energize cells, washed twice using the same buffer that did not contain CCCP, and then resuspended in the same buffer. The resultant cell suspension was incubated on ice for two hours and used in the efflux assay.

In the ethidium efflux assay, the *S. marcescens* KS24 and *S. marcescens* KS24Δ*sdeXY* strains were grown in L media until O.D.₆₅₀ = 0.7. *S. marcescens* cells were harvested by centrifugation, washed twice using modified Tanaka Buffer⁴⁴, and resuspended in the same assay. The resultant cell suspension was incubated at 37 °C in the presence of 20 mM lactate-tetramethylammonium hydroxide (pH 7.0) for 5 min and used in the efflux assay.

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Author Contributions

S.T., Y.M., W.O., T.T. and T.K. planned this project. Y.M. and T.K. wrote the main manuscript. S.T., Y.M., Y.K. and D.M. constructed all plasmids and transformants. S.M. and N.G. cured plasmids from *S. marcescens*. S.T. and Y.M. constructed the *sdeXY* deletion strain, and evaluated R6G efflux activity. S.T., Y.M., Y.K., K.H., S.K. and D.M. evaluated MICs. Y.M., W.O. and T.K. prepared Figures 1–2. Y.M., W.O., T.K. and Y.M. had critical discussions with T.K. All authors reviewed the manuscript.

Additional Information

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