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. 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 reported1,2. Resistance-nodulation-cell division superfamily (RND) efflux systems play a major role in multidrug resistance in Gram-negative bacteria3–9. 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, SdeXY10, SdeAB11, and SdeCDE11, have been characterized to date. SdeXY was the first multidrug efflux system to be characterized from S. marcescens10. 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.

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to fluoroquinolones, chloramphenicol, novobiocin, sodium dodecyl sulphate (SDS), and ethidium bromide in *S. marcescens*.

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 chloride. In contrast to SdeXY and SdeAB, SdeCDE did not exhibit broad substrate specificity and only conferred novobiocin resistance to *S. marcescens*.

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 KAM32. 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), SdeAB (SMA1197-1196), and SdeCDE (SMA2945-2946-2947), 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. 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 findings, the KAM32 strain expressing *sdeXY* conferred resistance to a broad spectrum of agents.

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**Table 1.** MICs of antimicrobial agents for strains expressing each RND efflux system gene(s) in E. coli KAM32.

| RND Efflux System | MIC (μg/mL) |
|-------------------|-------------|
| SdeXY             | 16          |
| SdeAB             | 4           |
| SdeCDE            | 1           |
| SdeE              | 4           |
| SdeF              | 1           |
| SdeG              | 2           |
| SdeH              | 8           |
| SdeI              | 16          |
| SdeJ              | 8           |
| SdeK              | 16          |
| SdeL              | 16          |
| SdeM              | 16          |
| SdeN              | 8           |
| SdeO              | 16          |
| SdeP              | 16          |
| SdeQ              | 16          |
| SdeR              | 16          |
| SdeS              | 16          |
| SdeT              | 16          |
| SdeU              | 16          |
| SdeV              | 16          |
| SdeW              | 16          |
| SdeX              | 16          |
| SdeY              | 16          |
| SdeZ              | 16          |

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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.
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 resistance4,11,13,14,26–28. 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 resistance4,29–32. 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 MexX, the substrate specificity of SdeGH was not similar to that of MexHI30 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
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 6 G, 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 6 G to evaluate the activity of each RND efflux system because rhodamine 6 G 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 6 G efflux activities when lactate was provided as the energy source, indicating that rhodamine 6 G 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 | Group 3 | Group 4 | Group 5 |
|---------------------|------------|---------|---------|---------|---------|---------|
| Norfloxacin         | 0.016      | 0.06    | 0.13    | 0.06    | 0.016   | 0.016   |
| Erythromycin        | 4          | 64      | 8       | 8       | 4       | 4       |
| Chloramphenicol     | 1          | 8       | 1       | 2       | 1       | 1       |
| Tetracycline        | 0.5        | 4       | 0.25    | 0.25    | 0.5     | 0.5     |
| Benzalkonium Cl     | 4          | 32      | 8       | 4       | 16      | 32      |
| Triclosan           | 0.5        | 2       | 0.25    | 0.13    | 0.5     | 0.5     |
| Novobiocin          | 2          | >128    | 16      | 16      | 8       | 4       |
| SDS                 | 128        | >8200   | >8200   | 256     | >8200   | 256     |
| Deoxycholate        | 2050       | >32800  | >32800  | >32800  | 16400   | 2050    |
| Ethidium bromide    | 4          | >128    | 16      | 8       | 4       | 128     |
| Rhodamine 6G        | 8          | >128    | 128     | 8       | 64      | >128    |

| Antimicrobial agent | MIC (µg/mL) | KAM32/ pUC19 (control) | KAM32/ pURS2 (sdeXY) | KAM32/ pURS8 (sdePQ-omsA) | KAM32/ pURS9 (sdeIJ) | KAM32/ pURS3 (sdePQ) | KAM32/ pURS5 (sdeGH) | KAM32/ pURS6 (sdeCDE) | KAM32/ pURS4 (sdeCD) | KAM32/ pURS44 (sdeCE) | KAM32/ pURS45 (sdeNO) |
|---------------------|------------|------------------------|----------------------|--------------------------|---------------------|---------------------|----------------------|----------------------|---------------------|----------------------|----------------------|
| Norfloxacin         | 0.016      | 0.06                   | 0.13                 | 0.06                     | 0.016               | 0.016               | 0.13                 | 0.03                 | 0.03                | 0.03                 | 0.03                 |
| Erythromycin        | 4          | 64                     | 8                    | 8                        | 4                   | 4                   | 4                    | 1                    | 1                   | 1                    | 1                    |
| Chloramphenicol     | 1          | 8                      | 1                    | 2                        | 1                   | 1                   | 1                    | 1                    | 1                   | 1                    |
| Tetracycline        | 0.5        | 4                      | 0.25                 | 0.25                     | 0.5                 | 0.5                 | 0.5                  | 0.25                 | 0.25                | 0.5                  |
| Benzalkonium Cl     | 4          | 32                     | 8                    | 4                        | 16                  | 32                  | 4                    | 4                    | 4                   | 32                   | 4                    |
| Triclosan           | 0.5        | 2                      | 0.25                 | 0.13                     | 0.5                 | 0.5                 | 0.5                  | 0.25                 | 0.25                | 0.5                  |
| Novobiocin          | 2          | >128                   | 16                   | 16                       | 8                   | 4                   | 8                    | 5                    | N.D.                | N.D.                 |
| SDS                 | 128        | >8200                  | >8200                | 256                      | >8200               | 256                 | 256                  | 256                 | 256                 | 128                 |
| Deoxycholate        | 2050       | >32800                 | >32800               | >32800                   | 16400               | 2050                | 2050                 | 2050                | 2050                | 2050                |
| Ethidium bromide    | 4          | >128                   | 16                   | 8                        | 4                   | 128                 | 128                  | 4                    | 4                   | 4                    |
| Rhodamine 6G        | 8          | >128                   | 128                  | 8                        | 64                  | >128                | 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 6 G 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 MgSO4 and 1 µM rhodamine 6 G. At the time point indicated by the arrow, 20 mM potassium lactate (K-Lactate) was added to energize cells. Intracellular rhodamine 6 G levels were monitored continuously by measuring the fluorescence of rhodamine 6 G 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.
Table 2. Effects of HasF on antimicrobial sensitivity.

| Antimicrobial agent | MIC (µg/mL) | Group 1 | Group 2 |
|---------------------|-------------|---------|---------|
|                     | KAM43/pUC19 | KAM43/pUC19 | KAM43/pUC19 | KAM43/pUC19 |
|                     | (control)   | (sdeXY)  | (sdeXY)  | (sdeXY)  |
| Norfloxacin         | 0.016       | 0.016    | 0.016    | 0.016    |
| Erythromycin        | 4           | 4        | 4        | 4        |
| Benzalkonium        | 2           | 2        | 2        | 2        |
| Novobiocin          | 0.25        | 0.25     | 0.25     | 0.25     |
| SDS                 | 32          | 32       | 32       | 32       |
| Ethidium bromide    | 1           | 1        | 1        | 1        |
| Rhodamine 6G        | 4           | 4        | 4        | 4        |

| Antimicrobial agent | MIC (µg/mL) | Group 3 | Group 4 | Group 5 |
|---------------------|-------------|---------|---------|---------|
|                     | KAM43/pUC19 | KAM43/pUC19 | KAM43/pUC19 | KAM43/pUC19 |
|                     | (control)   | (sdeGH)  | (sdeGH)  | (sdeGH)  |
| Norfloxacin         | 0.016       | 0.016    | 0.016    | 0.016    |
| Erythromycin        | 4           | 4        | 4        | 4        |
| Benzalkonium        | 2           | 2        | 2        | 2        |
| Novobiocin          | 0.25        | 0.25     | 0.25     | 0.25     |
| SDS                 | 32          | 32       | 32       | 32       |
| Ethidium bromide    | 1           | 1        | 1        | 1        |
| Rhodamine 6G        | 4           | 4        | 4        | 4        |

E. coli. TolC is a major OMP in E. coli and all of the E. coli RND efflux systems require TolC for their activities. 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 KAM32 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. 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 pumps, 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 SM39. 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.
Discussion

Previous studies suggested that RND efflux systems play a major role in multidrug resistance in *S. marcescens*. Since Gram-negative bacteria have been suggested to possess 'multiple' and 'active' RND efflux systems, 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 mutant. 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

| Antimicrobial agent | MIC (µg/mL) | KS24 | KS24ΔsdeXY | KS24ΔsdeXY/pSTV28 | KS24ΔsdeXY/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 KS24ΔsdeXY (B) were prepared as described in the Materials and Methods. Ethidium bromide was added to cell suspensions at a final concentration of 10 µM 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 µM. Assays were performed at 37°C.
expression of sdeAB was induced by the biocide, cetylpolyridinium chloride. 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 with TolC of E. coli, while other RND pumps in V. parahaemolyticus had markedly higher MICs when expressed with VpoC, an orthologue of TolC. 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 databases. 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 (SMA2945-2946F fw EcoRI and SMA2945-2947F re BamHI), and the 3′ half was then amplified with two primers (SMA2946-2947B 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 was 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.

To evaluate the function of OmsA, the gene of which is located downstream of sdePO (SM1743-1742), two types of plasmids were constructed. The fragment including sdePO-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 pUC18, and the resultant plasmid was named pURS8. To construct pURS8 carrying incomplete omsA, pURS8 was digested with Hpal 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 pSMX. 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 described.

Construction of the sdeXY deletion strain. S. marcescens KS24, a plasmid pSMC2-cured derivative of S. marcescens KS34 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 recombination system. Two-step PCR of the gentamicin cassette flanked by long (1000 nt) homologous extensions of the target gene were essentially performed as previously described using pBRFRGTGM 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 GENECLEAN II KIT (MP Biomedicals Inc.). sdeXY mutant strains were generated by electroporation of the purified PCR product into S. marcescens KS24/pKD46 as described previously⁴⁴. 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 described⁴⁴. In the rhodamine6G efflux assay, E. coli KAM32 strains were grown in L media until OD₆₅₀ = 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 carbonylcyanide-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 KS24/sdeXY strains were grown in L media until OD₆₅₀ = 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.

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