An RND-type multidrug efflux pump SdeXY from *Serratia marcescens*

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**Objectives:** *Serratia marcescens*, an important cause of nosocomial infections, shows intrinsic resistance to a wide variety of antimicrobial agents (multidrug resistance). Multidrug efflux pumps are often involved in the multidrug resistance in many bacteria. A study was undertaken to characterize the multidrug efflux pumps in *S. marcescens*.

**Methods:** The genes responsible for the multidrug resistance phenotype in *S. marcescens* were cloned into *Escherichia coli* KAM32, a drug-hypersusceptible strain, for further analysis.

**Results:** We cloned *sdeXY* genes and determined the nucleotide sequence. Clones that carried the *sdeXY* genes displayed reduced susceptibility to several antimicrobial agents including erythromycin, tetracycline, norfloxacin, benzalkonium chloride, ethidium bromide, acriflavine and rhodamine 6G. A protein similarity search using GenBank revealed that SdeY is a member of the resistance nodulation cell-division (RND) family of multidrug efflux proteins and SdeX is a member of the membrane fusion proteins. Introduction of *sdeXY* into *E. coli* cells possessing *tolC*, but not in cells lacking *tolC*, resulted in multidrug resistance. We observed energy-dependent ethidium efflux in cells of *E. coli* KAM32 possessing *sdeXY* and *tolC*.

**Conclusions:** *SdeXY* is the first RND-type multidrug efflux pump to be characterized in multidrug-resistant *S. marcescens*.

**Keywords:** SdeXY, multidrug efflux pump, RND family, *S. marcescens*

**Introduction**

Chemotherapeutic agents are very useful for the treatment of infectious diseases. The discovery of antibiotics and synthesis of chemotherapeutic agents are extremely valuable for human beings. Many infectious diseases once considered incurable have become curable owing to chemotherapeutic agents, including antibiotics. However, the emergence and spread of drug-resistant bacteria, especially multidrug-resistant bacteria, and their association with serious infectious diseases have recently increased. It is becoming difficult to treat patients who have infectious diseases caused by drug-resistant bacteria using chemotherapeutic agents. Several mechanisms confer drug resistance in bacterial cells, including degradation or modification of drugs, alteration of targets, emergence of alternative pathways and efflux of drugs out of cells. Among such drug resistance mechanisms, multidrug efflux is a major cause of multidrug resistance. Once a bacterium acquires a gene(s) for a certain multidrug efflux pump(s), or if a silent or weak gene(s) for a multidrug efflux pump is activated, then the cell instantly becomes resistant to many antimicrobial agents. Large numbers of multidrug efflux pumps have so far been reported, in numerous bacteria. Thus, it is very important to investigate multidrug efflux pumps in bacteria to gain insight into multidrug resistance and to overcome the problem of multidrug-resistant bacteria. Gene cloning, expression and biochemical characterization are very useful approaches to the understanding of multidrug efflux pumps.

*Serratia marcescens* is a cause of nosocomial and opportunistic infections. It has been reported that *S. marcescens* is associated with respiratory tract infections, urinary tract infections, septicaemia, meningitis and wound infections. This microorganism shows intrinsic resistance to many antimicrobial agents. Previously, we compared the MICs of various antimicrobial agents in several strains of *S. marcescens* with those in *Escherichia coli* and *Pseudomonas aeruginosa*. *S. marcescens* showed higher resistance than *E. coli* to many antimicrobial agents, such as ampicillin, chloramphenicol, erythromycin, norfloxacin, tetracycline, acriflavine and ethidium bromide. The levels of drug resistances in *S. marcescens* were roughly comparable to those in *P. aeruginosa*, which shows intrinsic resistance to many antimicrobial agents. We have also reported on the cloning of genes responsible for drug resistance from the chromosomal region of *S. marcescens*. Here we report on genes responsible for a multidrug efflux pump and on properties of the pump, which belongs to the resistance nodulation cell-division (RND) family.

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SdeXY multidrug efflux pump of S. marcescens

<table>
<thead>
<tr>
<th>Restriction Site</th>
<th>KAM32/pSEC3</th>
<th>KAM32/pSEC31</th>
<th>KAM32/pSEC32</th>
<th>KAM32/pSEC34</th>
<th>KAM32/pSEC37</th>
<th>KAM32/pSEC38</th>
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<tr>
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<td>SndI</td>
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<td></td>
<td></td>
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<tr>
<td>NotI</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>PvuII</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>SalI</td>
<td></td>
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<tr>
<td>Sse83I</td>
<td></td>
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</tbody>
</table>

Ethidium bromide resistance

+  
-  
+  
+  
-  
+  
+  

Figure 1. Plasmids and restriction maps of cloned S. marcescens DNA containing sdeXY genes. Physical maps of the DNA inserts derived from the S. marcescens chromosome in pSEC3 and its derivatives are shown. The growth capabilities of E. coli KAM32 cells harbouring each plasmid in L medium containing 10 mg/L of ethidium bromide are shown on the right. The ‘+’ sign indicates that cells grew, and the ‘-’ sign indicates that cells did not grow. The position and direction of the sdeXY genes revealed by sequencing are shown at the bottom.

Materials and methods

Bacterial strains and growth conditions

A clinically isolated S. marcescens strain NUSM8906 was used as the source of chromosomal DNA. E. coli KAM32 (ΔacrB, ΔydhE, ΔhsdD5), which lacks the major multidrug efflux pumps AcrAB and YdhE, is hypersusceptible to many antimicrobial agents. E. coli N43 (ΔacrA) and N43 tolC:TN10 (acrA1, ΔtolC) were also used. Cells were grown in L broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl, pH 7) at 37°C.

Gene manipulation

Genes responsible for resistance to antimicrobial agents were cloned from the chromosome of S. marcescens. We obtained 28 candidate hybrid plasmids and selected one of them, named pSEC3, which conferred resistance to a wide range of antimicrobial agents in E. coli KAM32, and characterized this plasmid further. The DNA insert in plasmid pSEC3 was digested with several restriction endonucleases, and subcloned into pSTV28 (a vector plasmid carrying a chloramphenicol resistance marker; from Takara Co., Kyoto, Japan). The resulting hybrid plasmids were introduced into KAM32 cells, and all transformants were analysed for their susceptibility to ethidium bromide. Transformants harbouring plasmids pSEC31, pSEC32 and pSEC38 displayed reduced susceptibility to ethidium bromide.

The nucleotide sequence was determined by the dideoxy chain termination method using a DNA sequencer (ALF express; Pharmacia Biotech). The nucleotide sequence data reported in this paper has been deposited in the DDBJ/EMBL/GenBank nucleotide sequence database with the accession number AB104882.

Sequence data were analysed with GENETYX sequence analysis software (Software Development Co., Tokyo, Japan). The SwissProt and GenBank databases were screened for sequence similarities.

Drug susceptibility testing

MICs of various drugs were determined as reported previously. For growth testing, cells in L broth (10⁷ cells/5 mL) containing each of the drugs were incubated at 37°C for 24 h, and the growth was judged by visual observation.

Ethidium bromide efflux assay in cells

E. coli KAM32 cells harbouring plasmid were grown in L broth supplemented with 20 mM potassium lactate under aerobic conditions at 37°C. The cells were harvested at the late exponential phase of growth, washed twice with a minimal medium, and suspended in the same medium to an OD650 of ~0.2. Ethidium bromide was added to cell suspensions of E. coli KAM32/pSEC38 and KAM32/pSTV28 to a final concentration of 20 µM. Accumulation of ethidium bromide was continuously monitored by measuring the fluorescence of ethidium bromide in cells, at excitation and emission wavelengths of 500 and 580 nm, respectively. An H⁺ conductor carboxylcyanide m-chlorophenylhydrazone (CCCP) was added to the suspensions to give a final concentration of 30 µM during the assay to assess energy-dependent efflux.

Results and discussion

Sequence analysis

Plasmid pSEC3, which carries a DNA insert ~7 kbp long, was derived from the chromosome of S. marcescens and conferred resistance to several antimicrobial agents in E. coli KAM32 cells. We constructed a series of deletion plasmids carrying various portions of the DNA insert in pSEC3, and tested resistance to ethidium bromide in cells of E. coli KAM32 harbouring each plasmid (Figure 1). Plasmids pSEC32 and pSEC38 showed ethidium resistance. Thus, we localized the gene(s) responsible for ethidium resistance to a short DNA region. Sequencing of this region revealed two open reading frames, respectively. SdeY and SdeX proteins consist of 393 and 1051 amino acid residues, respectively. SdeY is very rich in hydrophobic residues and possesses many putative transmembrane domains, suggesting that SdeX is an integral membrane protein. SdeX is a hydrophilic protein with one hydrophobic region.

Similarity searches for SdeXY in the GenBank database revealed that SdeY is a member of the RND family of proteins, and SdeX is a member of the membrane fusion proteins. Thus, it is very likely that the SdeXY is an RND-type multidrug efflux pump.

It seemed curious that plasmid pSEC32, which carries only the sdeY gene, conferred drug resistance on E. coli KAM32 (Figure 1). Originally we thought that E. coli KAM32 and its parent KAM3 lacked both acrA and acrB. We checked the deletion in the acrAB region of KAM3 and KAM32, and found that the acrA region was complete and ~200 nucleotides were missing in the acrB region (data not shown). Thus, it is very likely that AcrA and SdeY, perhaps...
chloramphenicol (Table 1). Thus, TolC is also required for resistance.

E. coli and S. marcescens MexXY of function as drug resistance machinery. Previously we reported that drug efflux pump when expressed in sdeY possesses –. Cells of sufficient, and either SdeX or AcrA is required for the drug resistance. Cells of sdeX did not grow when pSEC32 was introduced. Thus, SdeY alone is not sufficient, and either SdeX or AcrA is required for the drug resistance. Cells of SdeX or AcrA in addition to acrA, did not grow in the presence of ethidium bromide or erythromycin when pSEC38 or pSEC32 carrying sdeY was introduced. On the other hand, cells of E. coli N43, which lack acrA, did not grow when pSEC32 was introduced, although the cells grew when pSEC38 was introduced. Thus, SdeY alone is not sufficient, and either SdeX or AcrA is required for the drug resistance. Cells of SdeX or AcrA in addition to acrA, did not grow in the presence of ethidium bromide or erythromycin when pSEC38 or pSEC32 were introduced. The presence of plasmid in the cells was confirmed by growth in the presence of chloramphenicol (Table 1). Thus, TolC is also required for resistance. It seems that SdeX–SdeY–TolC or AcrA–SdeY–TolC complexes function as drug resistance machinery. Previously we reported that MexXY of P. aeruginosa required TolC for its function as a multi-drug efflux pump when expressed in E. coli cells. Thus, SdeY of S. marcescens and MexY of P. aeruginosa can utilize TolC as an outer membrane channel component for drug efflux. Recently, the three-dimensional structure of AcrB of E. coli was reported. According to the three-dimensional structure, AcrB directly binds to TolC. It seems that the structures of the binding regions of AcrB, SdeY and MexY are very similar.

Ethidium bromide efflux activity

As mentioned above, sequence similarity with other RND family multidrug efflux proteins suggested that SdeXY is a multidrug efflux pump. We tested this possibility by measuring ethidium bromide efflux. We observed a much lower level of ethidium bromide in cells of KAM32/pSEC38 (possessing sdeXY) than in cells of KAM32/pSTV28 (control) (Figure 2). Addition of an H+ conductor CCCP to the assay mixture increased cellular ethidium bromide levels in both types of cells. The final levels of intracellular ethidium bromide after the addition of CCCP were almost same in the two strains, indicating that the accumulation levels of ethidium bromide in both strains are the same under de-energized conditions. An important point is that the ethidium bromide accumulation level in cells of KAM32/pSEC38 was much lower than that in cells of KAM32/pSTV28 before the addition of CCCP. This indicates that cells of KAM32/pSEC38 possess energy-dependent ethidium bromide efflux activity. Thus, we conclude that SdeXY is an energy-dependent drug efflux pump.

Drug specificity of SdeXY

To investigate the drug specificity of SdeXY, we measured the MICs of many antimicrobial agents for E. coli KAM32/pSEC38 and KAM32/pSTV28 (control) (Table 2). KAM32/pSEC38 showed increased resistance to erythromycin, tetracycline, benzalkonium chloride, norfloxacin, acriflavine, ethidium bromide, rhodamine 6G, Hoechst 33342 and triclosan. A small increase in the MIC was observed with ampicillin and chlorhexidine gluconate. No increase in resistance to streptomycin was observed. Thus, SdeXY has a wide range of antimicrobial agent substrates.

SdeXY is the first multidrug efflux pump belonging to the RND family to be found in S. marcescens. It is likely that RND-type multi-

<table>
<thead>
<tr>
<th>Host (deleted gene)</th>
<th>Plasmid (carried gene)</th>
<th>ethidium bromide (10 mg/L)</th>
<th>erythromycin (10 mg/L)</th>
<th>chloramphenicol (20 mg/L)</th>
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</thead>
<tbody>
<tr>
<td>E. coli KAM32 (ΔacrB)</td>
<td>pSTV28</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>E. coli N43 (acrA1)</td>
<td>pSEC38 (sdeXY)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>E. coli N43 tolC::Tn10 (acrA1, ΔtolC)</td>
<td>pSEC38 (sdeXY)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Figure 2. Accumulation of ethidium in cells. E. coli KAM32/pSTV28 and KAM32/pSEC38 cells were grown in L broth supplemented with 20 mM potassium lactate. Ethidium bromide was added to cell suspensions of KAM32/pSTV28 and KAM32/pSEC38 at a final concentration of 20 µM. Accumulation of ethidium was monitored continuously by measuring the fluorescence of ethidium in cells. After 6.7 min, CCCP was added to the suspensions at a final concentration of 30 µM.
drug efflux pumps play an important role in multidrug resistance in this organism, as in *E. coli* and *P. aeruginosa*.

**Acknowledgements**

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**References**


**Table 2. MICs of various antimicrobial agents for *E. coli* KAM32 harbouring pSTV28 (control) or pSEC38 (carrying sdeXY)**

<table>
<thead>
<tr>
<th>Drug</th>
<th>MIC (mg/L)</th>
<th>KAM32/pSTV28</th>
<th>KAM32/pSEC38</th>
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<tr>
<td>Tetracycline</td>
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<tr>
<td>Benzalkonium chloride</td>
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<tr>
<td>Chlorhexidine gluconate</td>
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<tr>
<td>Triclosan</td>
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<tr>
<td>Acriflavine</td>
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<tr>
<td>Ethidium bromide</td>
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<td>&gt;128</td>
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<tr>
<td>Rhodamine 6G</td>
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<tr>
<td>Hoechst 33342</td>
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**References**


