SmdAB, a Heterodimeric ABC-Type Multidrug Efflux Pump, in *Serratia marcescens* 

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We cloned genes, designated smdAB, that encode a multidrug efflux pump from the chromosomal DNA of clinically isolated *Serratia marcescens* NUSM8906. For cells of the drug-hypersensitive strain *Escherichia coli* KAM32 harboring a recombinant plasmid carrying smdAB, structurally unrelated antimicrobial agents such as norfloxacin, tetracycline, 4',6-diamidino-2-phenylindole (DAPI), and Hoechst 33342 showed elevated MICs. The deduced amino acid sequences of both SmdA and SmdB exhibited similarities to the sequences of ATP-binding cassette (ABC)-type multidrug efflux pumps. The efflux of DAPI and Hoechst 33342 from *E. coli* cells expressing SmdAB was observed, and the efflux activities were inhibited by sodium o-vanadate, which is a well-known ATPase inhibitor. The introduction of *smdA* or *smdB* alone into *E. coli* KAM32 did not elevate the MIC of DAPI; thus, both SmdA and SmdB were required for function. These results indicate that SmdAB is probably a heterodimeric multidrug efflux pump of the ABC family in *S. marcescens*.

Drug resistance in bacteria is a serious problem in the hospital setting. In particular, multidrug resistance causes difficulty in the treatment of infectious diseases. There are several mechanisms by which bacterial cells escape the toxicities of antimicrobial agents. Such mechanisms include the degradation or modification of the drugs, the alteration of targets, the emergence of alternative pathways, and the efflux of drugs out of the cells. Among the drug resistance mechanisms, drug efflux is a major cause of multidrug resistance and has been found to play a major role in the intrinsic resistance of many bacteria (28, 31).

*Serratia marcescens* is a cause of nosocomial and opportunistic infections. It has previously been reported to be associated with respiratory tract infections, urinary tract infections, septicemia, meningitis, and wound infections (9). This organism shows high-level intrinsic resistance to a variety of antimicrobial agents, which makes the treatment of infections with this bacterium very difficult. Previously, we compared the MICs of various antimicrobial agents for several strains of *S. marcescens* with those of *Escherichia coli* and *Pseudomonas aeruginosa* (6). Many antimicrobial agents, such as ampicillin, chloramphenicol, erythromycin, tetracycline, and ethidium bromide, showed higher MICs for *S. marcescens* than for *E. coli*. The levels of drug resistance in *S. marcescens* are roughly comparable to those in *P. aeruginosa*, which shows high-level intrinsic resistance to many antimicrobial agents. Since multidrug efflux pumps have been shown to contribute to the intrinsic resistance of *P. aeruginosa* (19, 20, 28), it may be possible that these pumps are also important for the intrinsic drug resistance of *S. marcescens*.

We have previously reported that we succeeded in the cloning of nine distinct types of genes from the chromosome of *S. marcescens* and that such genes are responsible for drug resistance (6). These genes include *sdeXY* (5), which is a member of the resistance-nodulation-cell division family, and *smfY* (37), which is a member of the major-facilitator superfamily. In addition, Kumar and Worobec have reported the characterization of SdeAB (15). Given the genome sequence of *S. marcescens* Db11, which has been reported by the *S. marcescens* Db11-Sequencing Group at the Sanger Institute (ftp://ftp.sanger.ac.uk/pub/pathogens/sm/), many other multidrug efflux pumps that have not been physiologically characterized are expected to be present.

Another family of multidrug efflux pumps is the ATP-binding cassette (ABC) family. The ABC-type multidrug efflux pumps in eukaryotes have been well characterized and have previously been shown to be involved in tolerance to various cytotoxic agents (22). The human P-glycoprotein is a representative of the eukaryotic ABC-type multidrug efflux pumps (4, 41). The ABC-type multidrug efflux pumps utilize ATP as the energy source and are thus primary transporters. Meanwhile, most of the prokaryotic multidrug transporters are secondary transporters. Some ABC-type drug efflux pumps in prokaryotes, especially in gram-positive bacteria, have been characterized in detail previously (23, 25, 35, 38, 39, 42). These include *LmrCD of Lactococcus lactis*, which has been shown to be involved in intrinsic resistance to some antimicrobial agents (21). In addition, three ABC-type drug efflux pumps in gram-negative bacteria have been reported previously: MacAB, MsbA, and VcaM. MacAB is a macrolide-specific efflux pump and has been identified in several gram-negative bacteria, such as *E. coli*, *Salmonella enterica* serovar Typhimurium, and *Neisseria gonorrhoeae* (14, 29, 30, 34). MacA belongs to the membrane fusion protein family, and MacB is an integral mem-
brane protein with a nucleotide-binding domain. MacAB seems to form a tripartite complex together with TolC, which is a multifunctional outer membrane protein in E. coli (13). MsbA is an essential ABC-type pump in E. coli and is involved in the transport of lipopolysaccharides and phospholipids (43). Furthermore, Reuter et al. demonstrated that MsbA confers multidrug resistance upon E. coli and mediates the transport of ethidium from cells (33). We have previously cloned and characterized a multidrug efflux pump, VcaM, from non-O1 Vibrio cholerae (10). MsbA and VcaM probably function as homo- or heterodimers, similar to LmrA of L. lactis.

Here, we report the properties of an ABC-type multidrug efflux pump, SmdAB, of S. marcescens. This pump rendered host E. coli cells resistant to various antimicrobial agents. Moreover, SmdAB probably functions as a heterodimer. To our knowledge, this is the first report of an ABC-type pump functioning as a heterodimer in gram-negative bacteria.

MATERIALS AND METHODS

Bacterial strains and growth. A clinically isolated S. marcescens strain, NUSM8906, was used as the source of chromosomal DNA (6). E. coli KAM32 (ΔacrB ΔydhE hsdS ΔL), which lacks the major multidrug efflux pumps AcrAB and YdhE, is hypersusceptible to many antimicrobial agents (7). E. coli KAM232 (ΔacrB ΔydhE hsdS ΔLcoI), a ΔtolC-deficient strain derived from KAM32, was constructed as described previously (32). Cells were grown in Luria (L) broth (18) at 37°C under aerobic conditions.

An environmentally isolated strain, S. marcescens Db10, was a kind gift from Jonathan Ewbank of the Centre d’Immunologie de Marseille Luminy, France. CLoning, sequencing, and gene manipulation. Genes responsible for resistance to antimicrobial agents were cloned from the chromosome of S. marcescens (6). Briefly, chromosomal DNA was prepared from S. marcescens NUSM8906 by the method of Berns and Thomas (2). The DNA was partially digested with Sau3AI, and the fragments from 4 to 10 kb were separated by sucrose density gradient centrifugation. Plasmid pSTV28 (TaKaRa BIO Inc.) was used as a cloning vector. This vector carries cat, the chloramphenicol acetyltransferase gene. Plasmid pSTV28 was digested with BamHI dephosphorylated with bacterial alkaline phosphatase, and then ligated with the chromosomal DNA fragments by using a ligation kit (version 2; TaKaRa BIO Inc.). Competent cells of E. coli KAM32 were transformed with the recombinant plasmids and were spread on 1.5% agar plates containing L broth, 20 μg of chloramphenicol/ml, and 0.5 μg of 4,5-diamidino-2-phenylindole (DAPI)/ml. The plates were incubated at 37°C for 24 h. We obtained eight candidate hybrid plasmids and selected one of them, named pSD6.

The DNA insert in plasmid pSD6 was digested with several restriction endonucleases and subcloned into pSTV26. The resulting plasmids, which had shorter inserts than the original pSD6 plasmid, were introduced into E. coli KAM32 cells, and all transformants were tested for their susceptibilities to DAPI. Of the plasmids that conferred resistance to DAPI upon E. coli KAM32, pSD6c64l carried the shortest insert and was used for further analysis.

The nucleotide sequence was determined by the dideoxy chain termination method (36) using a DNA sequencer (ALF Express; Pharmacia Biotech). Nucleotide sequence data were analyzed with GENETYX sequence analysis software (Software Development Co.).

Drug susceptibility tests. The MICs of various antimicrobial agents were determined by using the microdilution method according to the recommendations of the Japanese Society of Chemotherapy (12). Briefly, MICs were determined in Mueller-Hinton broth (Difco) containing each compound in a twofold serial dilution series. The cells were incubated in the test medium at 37°C for 24 h, and growth was examined visually. The MIC of each compound was defined as the lowest concentration that prevented visible growth.

Efflux assays. The DAPI efflux assay was carried out as described previously (16). Briefly, cells of E. coli KAM32 harboring control or recombinant plasmids were grown in 20 ml of L broth containing 20 μg of chloramphenicol/ml and 0.5 mM isoprropyl-β-D-thiogalactopyranoside (IPTG) until the optical density at 650 nm reached 0.7 units. After the cells were harvested, they were washed with modified Tanaka buffer (27, 40) and were then resuspended in the same buffer containing 5 μM DAPI and 1 mM 2,4-dinitrophenol (DNP) and incubated at 37°C for 10 h. DNP, which is a well-known conductor of protons across the cytoplasmic membrane (1), was used to de-energize the cells. The cells were washed with modified Tanaka buffer and then resuspended in the same buffer to obtain an optical density at 650 nm of 0.4 units. The fluorescence of DAPI was measured at excitation and emission wavelengths of 355 and 457 nm, respectively, with a fluorescence spectrophotometer, model F-2000 (Hitachi). The fluorescence intensity of DAPI is higher when DAPI binds to DNA molecules. Thus, the efflux of DAPI from the cell can be monitored by the detection of a decrease in the level of fluorescence over time. The cell suspension was incubated at 37°C for 5 min, and then glucose at 20 mM was added as an energy source to monitor the efflux of DAPI.

The assay for the efflux of Hoechst 33342 was carried out as described previously (10). Briefly, cells were cultured and washed as described above. Washed cells were resuspended in modified Tanaka buffer containing 1 μM Hoechst 33342 and 1 mM DNP and incubated at 37°C for 10 h. The cells were washed with 100 mM 3-morpholinopropanesulfonic acid-tetramethylammonium hydroxide (MOPS-TMAH) containing 1 μM Hoechst 33342 and then resuspended in the same buffer to obtain an optical density at 650 nm of 0.4 units. The fluorescence of Hoechst 33342 was measured at excitation and emission wavelengths of 355 and 457 nm, respectively.

To evaluate the effects of sodium o-vanadate on the efflux of DAPI or Hoechst 33342, cell suspensions were prepared in the same way as described above. The cell suspensions were preincubated for 5 min at 37°C with different concentrations of sodium o-vanadate (0 to 3 mM) prior to the addition of glucose.

RT-PCR analysis. Total RNA from cells of S. marcescens NUSM8906 and Db10 that were grown in L broth until the exponential growth phase was extracted by using the QIAGEN RNaseay mini kit. For efficient RNA extraction, the cell suspensions were kept on dry ice. The cells were washed with PBS and then resuspended in Roswell Park Memorial Institute (RPMI) medium. Total RNA was then extracted from the suspension by using TRIzol reagent (Life Technologies). The extracted total RNA was used for reverse transcription-PCR (RT-PCR) with the QIAGEN one-step RT-PCR kit. RT-PCR without the RT reaction was performed to confirm the lack of detectable DNA contamination. RT-PCR products were analyzed by 3% agarose 21 gel (Nippon Geno Co.) electrophoresis.

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper have been deposited in the DDBJ, EMBL, and GenBank nucleotide sequence databases with the accession no. AB560548.

RESULTS

Cloning of smdAB and sequence analysis. To understand the role of multidrug efflux pumps in the intrinsic multidrug resistance of S. marcescens, it is important to identify multidrug efflux pump genes. We previously cloned nine distinct types of genes from the chromosome of S. marcescens NUSM8906 and found that such genes are responsible for drug resistances (6).
One of the recombinant plasmids, pSDC6, rendered E. coli KAM32 cells resistant to DAPI, norfloxacin, and tetracycline. Judging from the spectrum of drug resistance, it seemed that the plasmid pSDC6 carried other genes different from sdeXY and smfY, which we had already reported (5, 37). Thus, we analyzed pSDC6 further.

Plasmid pSDC6 carries a DNA insert about 8 kbp long. We constructed a series of deletion plasmids carrying various portions of the DNA insert in pSDC6 and tested whether those plasmids conferred DAPI resistance upon E. coli cells (Fig. 1).

Plasmid pSDC664 carried the shortest DNA insert that conferred DAPI resistance. The sequencing of this insert revealed two open reading frames (ORFs). We designated the ORFs smdA and smdB (for Serratia multi drug resistance). The putative gene products were estimated to comprise 591 and 592 amino acid residues, respectively. Only the smdA gene has a promoter-like sequence in its upstream region, and both genes have ribosome-binding sequences (Shine-Dalgarno sequences), each of which is followed by a start codon. The smdB gene is followed by a transcription terminator-like (inverted repeat) sequence. The two ORFs overlap by 5 nucleotides. Hydropathy analysis by the method of Eisenberg et al. (8) suggested that both SmdA and SmdB possess six putative transmembrane segments followed by hydrophilic segments (data not shown).

The comparison of our sequence with the genome sequence of S. marcescens Db11 (a streptomycin-resistant mutant of Db10) (ftp://ftp.sanger.ac.uk/pub/pathogens/sm/) showed that...
TABLE 1. Sequence similarities to SmdA and SmdB

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<th>Transporter</th>
<th>Organism</th>
<th>% Identity to SmdA</th>
<th>% Similarity to SmdA</th>
<th>% Identity to SmdB</th>
<th>% Similarity to SmdB</th>
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* N, N-terminal half; C, C-terminal half.

Eighty differences in the nucleotide sequences of smdA and SMA0354 and 83 differences between those of smdB and SMA0355 were identified. Almost all of these differences were translationally silent, but nine and six amino acid residues were different, respectively. In addition, the ORF of smdB was 132 nucleotides shorter than that of SMA0355. SmdA and SmdB showed 27% identity and 70% similarity to each other. A BLAST search of the NCBI database for protein sequence similarities showed SmdA to be 79% identical to MdlA and SmdB to be 79% identical to MdlB of E. coli. The search also showed both SmdA and SmdB to have nearly 30% identity to other ABC-type multidrug efflux pumps (Table 1). The levels of similarity in the nucleotide-binding domains were much higher than those in the transmembrane domains.

We also investigated the expression of smdAB in S. marcescens NUSM8906 (a clinical isolate) and Db10 (an environmental isolate). RT-PCR analysis showed that smdAB was expressed to similar extents in the two strains (data not shown).

Drug susceptibility. To investigate the contribution of SmdAB to drug resistance, we measured the MICs of various antimicrobial agents for E. coli KAM32 cells to which the plasmid carrying smdAB had been introduced. The MICs for E. coli KAM32 cells harboring pSDC664 (which carries smdAB) or pSTV28 (control) are shown in Table 2. For E. coli KAM32 cells, the introduction of the plasmid pSDC664 elevated the MICs of several structurally unrelated drugs: norfloxacin, tetracycline, Hoechst 33342, and tetraphenylphosphonium chloride (TPPCI), in addition to DAPI. Therefore, we concluded that SmdAB conferred multidrug resistance upon E. coli KAM32.

MacAB is the sole pump in E. coli characterized as an ABC-type drug efflux pump (14). MacAB has previously been shown to require the outer membrane component TolC for function. We therefore investigated whether TolC was required for the function of SmdAB. E. coli KAM42 is a tolC-lacking strain derived from strain KAM32. The introduction of the plasmid pSDC64 into cells of E. coli KAM42 resulted in elevated MICs of various antimicrobial agents, similar to those for E. coli KAM32 (data not shown). Thus, we conclude that TolC is not necessary for the function of SmdAB.

To test whether both SmdA and SmdB are necessary for the pump function, we constructed plasmids carrying each one of the corresponding genes. Plasmid pBDA24 carried smdA, while plasmid pSDB22 carried smdB. Both plasmids were introduced into E. coli KAM32 cells. The smdA gene was located under the control of the tet promoter in the pBR322 vector, and smdB was located under the control of the lac promoter in the pSTV28 vector (Fig. 1). Since vectors pBR322 and pSTV28 were compatible, both plasmids could be retained simultaneously. The drugs tested did not show elevated MICs for either the KAM32 transformant harboring pBDA24 (carrying smdA) or the KAM32 transformant harboring pSDB22 (carrying smdB) (Table 3). On the other hand, norfloxacin, tetracycline, TPPCI, and Hoechst 33342 showed elevated MICs for the KAM32 transformant harboring both pBDA24 and pSDB22, similar to those for KAM32 harboring pSDC644 (carrying smdB). Thus, we conclude that both SmdA and SmdB are necessary for resistance.

TABLE 2. MICs of various antimicrobial agents for E. coli KAM32/pSTV28 and KAM32/pSDC664

<table>
<thead>
<tr>
<th>Antimicrobial agent</th>
<th>MIC (µg/ml) for:</th>
<th>Increase (n-fold) in MIC*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>KAM32/pSTV28</td>
<td>KAM32/pSDC664</td>
</tr>
<tr>
<td>DAPI</td>
<td>0.25</td>
<td>8</td>
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<tr>
<td>Norfloxacin</td>
<td>0.016</td>
<td>0.125</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>0.002</td>
<td>0.004</td>
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<td>Tetracycline</td>
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<td>Streptomycin</td>
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</tr>
<tr>
<td>Erythromycin</td>
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<td>Ampicillin</td>
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</tr>
<tr>
<td>Hoechst 33342</td>
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<td>TPPCI</td>
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<td>Acriflavine</td>
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* Increase in MIC for E. coli KAM32/pSDC664 compared to that for E. coli KAM32/pSTV28.
**Table 3. MICs of various antimicrobial agents for *E. coli* KAM32 cells carrying smdA and/or smdB**

<table>
<thead>
<tr>
<th>Antimicrobial agent</th>
<th>KAM32/pSTV28 (negative control)</th>
<th>KAM32/pBDA24 (carrying smdA)</th>
<th>KAM32/pSDB22 (carrying smdB)</th>
<th>KAM32/pBDA24/pSDB22 (carrying smdA and smdB)</th>
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<tbody>
<tr>
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<td>0.25</td>
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<td>Norfloxacin</td>
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<tr>
<td>Hoechst 33342</td>
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<td>0.25</td>
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<tr>
<td>TPPCl</td>
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**Eflux of DAPI and Hoechst 33342.** In order to show that SmdAB is a multidrug efflux pump, we measured the efflux of DAPI and Hoechst 33342. Cells of *E. coli* KAM32 harboring either pSTV28 (control) or pSTV28 (control) were de-energized and preloaded with DAPI. The addition of glucose as an energy source caused the rapid extrusion of DAPI from KAM32 cells harboring pSDD64 compared with that from KAM32 cells harboring pSTV28 (Fig. 3A). When we measured the efflux of Hoechst 33342, we obtained a similar result (Fig. 3B). The addition of lactate instead of glucose as an energy source caused similar levels of extrusion (data not shown). These results indicate that SmdAB is an energy-dependent multidrug efflux pump.

**Inhibition of SmdAB-mediated DAPI efflux by vanadate.** From the primary amino acid sequence, SmdAB was categorized within the ABC family of multidrug efflux pumps. The activities of some ABC-type multidrug efflux pumps have previously been reported to be inhibited by sodium o-vanadate, an inhibitor of some ATPases (10, 17, 23, 25, 29, 33, 35, 38, 39, 42). We investigated the effect of sodium o-vanadate on the DAPI efflux activity of SmdAB. As shown in Fig. 4, sodium o-vanadate inhibited the activity in a concentration-dependent manner. The concentration causing 50% inhibition was approximately 1.1 mM. This 50% inhibitory concentration of sodium o-vanadate is similar to those for other ABC-type multidrug efflux pumps (10, 17). Meanwhile, Hoechst 33342 efflux activity was also inhibited by sodium o-vanadate (data not shown). Thus, it seems that SmdAB is an ATP-dependent multidrug efflux pump of *S. marcescens*.

**DISCUSSION**

We previously cloned genes that conferred multidrug resistance upon drug-hypersusceptible *E. coli* cells (6). We designated the genes *smdAB* and characterized the properties of SmdAB. SmdAB was categorized into the ABC family of multidrug efflux pumps according to the primary structure. Both SmdA and SmdB were found to contain putative nucleotide-binding domains, Walker A and Walker B motifs, and ABC signature sequences (11). We observed the elevation of the MICs of several antimicrobial agents for cells into which *smdAB* was introduced and detected energy-dependent efflux of DAPI and Hoechst 33342 in these cells. The efflux of DAPI and Hoechst 33342 mediated by SmdAB was inhibited by sodium o-vanadate, which is a known ATPase inhibitor. Thus, we conclude that SmdAB is an ABC-type multidrug efflux pump. We found that both SmdA and SmdB were necessary for pump function. To our knowledge, SmdAB is the first example of a probably heterodimeric ABC-type multidrug efflux pump in gram-negative bacteria.

Several ABC-type multidrug efflux pumps have been cloned from gram-positive bacteria and characterized previously (10, 14, 17, 23, 25, 29, 33, 35, 38, 39, 42). Among them, LmrCD in *L. lactis* has been demonstrated to be a heterodimeric ABC-type multidrug efflux pump and to contain two structurally and functionally distinct nucleotide-binding domains (24). In LmrD, a canonical glutamate residue following the Walker B motif, which has been postulated to fulfill a critical catalytic role in the hydrolysis of ATP (3), is conserved, but in LmrC,
this residue is replaced with a noncanonical aspartate residue. In each pair of heterodimeric transporters shown in Fig. 2, including SmdAB, one polypeptide contains a canonical glutamate residue and the other polypeptide contains a noncanonical aspartate residue instead of a glutamate residue. This pattern may be a feature of heterodimeric ABC-type transporters.

By searching with the BLAST system, we found homologues of SmdAB in other microorganisms, such as E. coli, Yersinia pestis, Shigella flexneri, Salmonella enterica serovar Typhimurium, and Vibrio parahaemolyticus. In all cases, two ORFs were located in tandem, and many of the genes seemed to encode the multidrug resistance ABC-type proteins. Among them, only mdlAB in E. coli has been cloned (30). However, MdIAB did not confer any drug resistance upon E. coli host cells even if expressed from the high-copy-number plasmid pUC119 (30). We cloned SmdAB into a medium-copy-number plasmid. Thus, it seems that the differences in copy number did not cause the phenotypic differences. It is possible that MdIAB by itself does not possess drug efflux activity; another possibility is that the expression of MdIAB is repressed by an unknown mechanism at the transcriptional or translational steps.

It has been reported previously that ABC-type efflux pumps have some roles aside from drug resistance (22). Since homologues of SmdAB are widely distributed in gram-negative bacteria, it may be possible that SmdAB-type ABC pumps have some important physiological roles. Further analyses of SmdAB and its homologues should be necessary to understand such unknown roles.

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