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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 for *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-

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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 homodimers, 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.

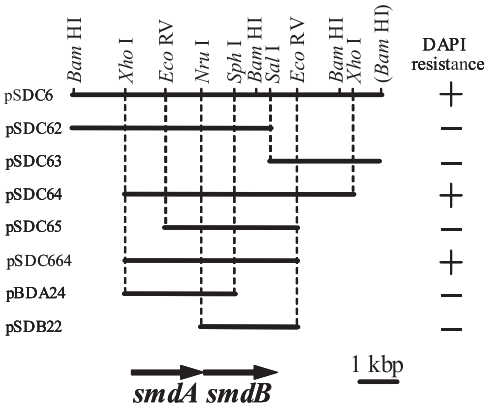

FIG. 1. Restriction map of pSDC6 and its derivatives. DNA regions derived from *S. marcescens* chromosomal DNA and carried by each plasmid are shown. The ability of *E. coli* KAM32 cells harboring each plasmid to grow in L broth containing 0.5 μg of DAPI/ml is indicated on the right; plus signs indicate that cells grew, and minus signs indicate that cells did not grow. The positions and directions of the *smdAB* genes as revealed by sequencing are shown at the bottom.

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 (Δ*acrB* Δ*ydhE hsd*Δ*5*), which lacks the major multidrug efflux pumps AcrAB and YdhE, is hypersusceptible to many antimicrobial agents (7). *E. coli* KAM42 (Δ*acrB* Δ*ydhE hsd*Δ*5* Δ*tolC*), a *tolC*-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 kbp 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 onto 1.5% agar plates containing L broth, 20 μg of chloramphenicol/ml, and 0.5 μg of 4′,6-diamidino-2-phenyindole (DAPI)/ml. The plates were incubated at 37°C for 24 h. We obtained eight candidate hybrid plasmids and selected one of them, named pSDC6.

The DNA insert in plasmid pSDC6 was digested with several restriction endonucleases and subcloned into pSTV28. The resulting plasmids, which had shorter inserts than the original pSDC6 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, pSDC664 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). 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 isopropyl 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 RNeasy mini kit. For efficient RNA extraction, the cells were well broken using a QIAshredder (QIAGEN Inc.) prior to the extraction of RNA. The extracted total RNA was used for reverse transcription-PCR (RT-PCR) with the QIAGEN one-step RT-PCR kit. 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 Gene 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. AB360548.

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* NUSU8906 and found that such genes are responsible for drug resistances (6).

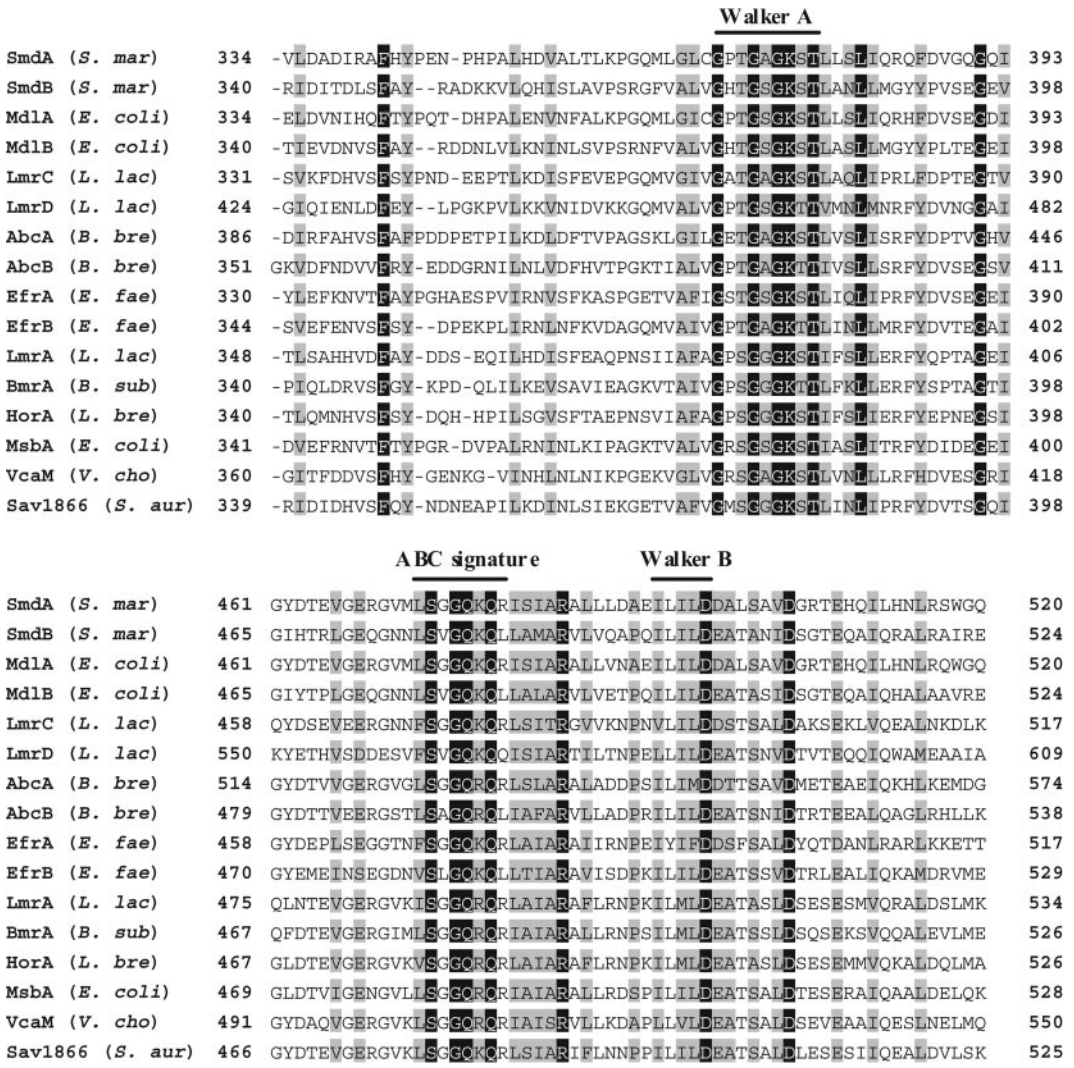

FIG. 2. Multiple-sequence alignments of SmdA, SmdB, and similar or putative proteins. The amino acid sequence alignments of the Walker A motif, the Walker B motif, and the ABC signature sequences are shown. Identical and similar residues are indicated with black and gray backgrounds, respectively. Gaps in the alignment are indicated by hyphens. The numbers on the left and right of the sequences indicate the beginning and ending positions of each sequence, respectively. The sequences were aligned by using the EMBL ClustalW program (available at http://decypher.stanford.edu/decypher/algo-cw/cw_ax.shtml). The names of the organisms from which the proteins are derived are shown in Table 1. *S. mar*, *S. marcescens*; *L. lac*, *L. lactis*; *B. bre*, *Bifidobacterium breve*; *E. fae*, *Enterococcus faecalis*; *B. sub*, *Bacillus subtilis*; *L. bre*, *Lactobacillus brevis*; *V. cho*, *V. cholerae*; *S. aur*, *Staphylococcus aureus*.

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 S*erratia m*ulti*d*rug 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 hydrophilic segments of both SmdA and SmdB contain putative nucleotide-binding domains, Walker A and Walker B motifs, and ABC signature sequences (11) (Fig. 2).

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

Transporter	Organism	% Identity to SmdA	% Similarity to SmdA	% Identity to SmdB	% Similarity to SmdB	Reference or accession no.
SmdA	*S. marcescens*			27	70	This study
SmdB	*S. marcescens*	27	70			This study
MdlA	*E. coli*	79	96	26	68	P77265
MdlB	*E. coli*	26	70	79	96	P0AAG5
LmrC	*L. lactis*	26	68	22	67	AAK04408
LmrD	*L. lactis*	26	71	24	64	AAK04409
EfrA	*Enterococcus faecalis*	26	70	24	69	NP_816538
EfrB	*Enterococcus faecalis*	27	66	28	70	NP_816537
AbcA	*Bifidobacterium breve*	17	33	27	67	DQ486860
AbcB	*Bifidobacterium breve*	28	70	29	71	DQ486860
BmrA	*Bacillus subtilis*	28	68	27	69	D70031
HorA	*Lactobacillus brevis*	27	68	24	62	AB005752
LmrA	*L. lactis*	26	70	26	64	U63741
MsbA	*E. coli*	28	69	27	68	P27299
VcaM	*V. cholerae*	27	68	26	68	AB073220
MDR1 (N)[a]	*Homo sapiens*	23	56	23	58	P08183
MDR1 (C)[a]	*Homo sapiens*	26	67	25	66	P08183

[a] N, N-terminal half; C, C-terminal half.

smdA corresponded to SMA0354 and *smdB* to SMA0355. 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 (TPPCl), 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 pSDC664 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, TPPCl, and Hoechst 33342 showed elevated MICs for the KAM32 transformant harboring both pBDA24 and pSDB22, similar to those for KAM32 harboring pSDC664 (carrying *smdAB*). 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

Antimicrobial agent	MIC (μg/ml) for:		Increase (*n*-fold) in MIC[a]
	KAM32/pSTV28	KAM32/pSDC664	
DAPI	0.25	8	32
Norfloxacin	0.016	0.125	8
Ciprofloxacin	0.002	0.004	2
Ofloxacin	0.016	0.016	1
Nalidixic acid	1	1	1
Tetracycline	0.25	2	8
Streptomycin	2	2	1
Erythromycin	4	4	1
Ampicillin	2	2	1
Hoechst 33342	0.25	4	16
TPPCl	4	16	4
Acriflavine	2	2	1

[a] 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*

Antimicrobial agent	MIC (μg/ml) for:			
	KAM32/pSTV28 (negative control)	KAM32/pBDA24 (carrying *smdA*)	KAM32/pSDB22 (carrying *smdB*)	KAM32/pBDA24/pSDB22 (carrying *smdA* and *smdB*)
DAPI	0.25	0.25	0.25	8
Norfloxacin	0.016	0.016	0.016	0.125
Tetracycline	0.25	0.25	0.25	2
Hoechst 33342	0.25	0.25	0.25	4
TPPCl	4	4	4	16

Efflux 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 pSDC664 (carrying *smdAB*) 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 pSDC664 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, 26, 35, 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) (Fig. 2). 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,

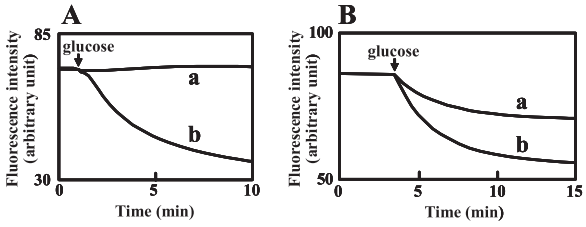

FIG. 3. Efflux of DAPI and Hoechst 33342 via SmdAB. Energy-starved *E. coli* KAM32 cells harboring pSDC664 (carrying *smdAB*) (curves b) or KAM32 cells harboring pSTV28 (control) (curves a) were loaded with 5 μM DAPI (panel A) or 1 μM Hoechst 33342 (panel B). At the time point indicated by the arrow, glucose (final concentration, 20 mM) was added to energize the cells. The fluorescence of dyes at 37°C over time was monitored with a fluorescence spectrophotometer. The downward deflection indicates the efflux of DAPI or Hoechst 33342 from the cells.

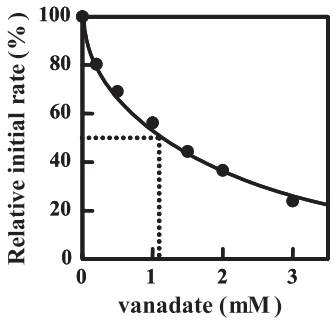

FIG. 4. Inhibition of DAPI efflux activity by sodium *o*-vanadate. Various concentrations of sodium *o*-vanadate were added to the assay mixture, and the mixture was preincubated with the cells for 5 min. Glucose (final concentration, 20 mM) was added to initiate the assay. The relative initial velocity of DAPI efflux was measured. The initial velocity observed in the absence of an inhibitor was set at 100%. Dotted lines indicate IC_{50} (approximately 1.1 mM).

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*, etc. 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, MdlAB 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 middle-copy-number plasmid. Thus, it seems that the differences in copy number did not cause the phenotypic differences. It is possible that MdlAB by itself does not possess drug efflux activity; another possibility is that the expression of MdlAB 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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