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Role of AbeS, a Novel Efflux Pump of the SMR Family of Transporters, in Resistance to Antimicrobial Agents in *Acinetobacter baumannii*▽

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In this study, a chromosomally encoded putative drug efflux pump of the SMR family, named AbeS, from a multidrug-resistant strain of *Acinetobacter baumannii* was characterized to elucidate its role in antimicrobial resistance. Expression of the cloned *abeS* gene in hypersensitive *Escherichia coli* host KAM32 resulted in decreased susceptibility to various classes of antimicrobial agents, detergents, and dyes. Deletion of the *abeS* gene in *A. baumannii* confirmed its role in conferring resistance to these compounds.

Acinetobacter baumannii is an important nosocomial pathogen frequently reported to be associated with a variety of infections, including respiratory tract infections, urinary tract infections, bacteremia, and skin and skin structure infections (3). Reports of the increased isolation of multidrug-resistant *A. baumannii* clinical isolates from different regions of the United States are appearing at a startling rate (1, 4, 10, 25, 30, 33).

Antibiotic resistance in *A. baumannii* has been attributed to either intrinsic or acquired mechanisms (21). The resistance mechanisms in *A. baumannii* are diverse and include enzymatic modification of antibiotics, target gene mutation, altered outer membrane permeability, and upregulated multidrug efflux pump activity (20).

Efflux systems involve transport proteins that function to reduce the concentration of drugs or toxic substances by transporting them across the inner and outer membranes into the external medium (24). These multidrug efflux systems are classified into five different families: ATP-binding cassette (ABC), major facilitator super family (MFS), resistance/nodulation/cell division (RND), multidrug and toxic-compound extrusion (MATE), and the small multidrug resistance (SMR) family of bacterial integral membrane proteins (22). ABC transporters are ATP-driven efflux pumps; MFS, RND, and SMR are proton driven; and MATE transporters consist of an Na^+/H^+ drug antiporter system (22, 23). Genome sequence analyses reveal that, on average, efflux pumps constitute at least 10% of the transporters in bacterial species, and they usually are capable of extruding a broad range of structurally unrelated compounds (18).

Multidrug efflux pumps of the SMR type are made of a transport protein located in the inner membrane (19). The polypeptide chains of SMR efflux pumps, found in the inner membranes of gram-negative bacteria, are 110 amino acid residues in length and contain four transmembrane helices (29). Reports show that 52% of currently sequenced genomes of eubacteria and archaea contain at least one SMR homologue (2). Well-studied examples of SMR efflux pumps include EmrE of *Pseudomonas aeruginosa*, EbrAB of *Bacillus subtilis*, SsmE of *Serratia marcescens*, and EmrE of *Escherichia coli*, which are involved in resistance to a variety of antimicrobial agents and quaternary ammonium compounds (14, 16, 17, 34).

The 3.9-Mb genome of *A. baumannii* AYE is reported to harbor 46 open reading frames (ORFs) encoding putative efflux pumps of different families (8). The efflux systems functionally characterized so far include AdeABC and AdeIJK (RND type), AbeM (MATE type), and CraA (MFS type) (20, 27). Albeit comparative genomics clearly reveal the existence of several chromosomally borne putative efflux transporters (8, 12), apparently the role of the *Acinetobacter* SMR efflux pump was never examined. Therefore, the objective of the present study was to investigate the function of one putative SMR efflux pump from a clinical isolate, *A. baumannii* AC0037.

(Part of this work was presented at the 48th Interscience Conference on Antimicrobial Agents and Chemotherapy, Washington, DC, 2008 [8a].)

Cloning of a putative SMR efflux pump gene from *A. baumannii*. *A. baumannii* AC0037 used in this study (isolated from the respiratory system of an infected patient in The Ohio State University Medical Center) is a multidrug clinical strain (31) with the following MICs for antibiotics: amikacin, >128 μg/ml; ceftazidime, 32 μg/ml; cefepime, >16 μg/ml; ciprofloxacin, >72 μg/ml; imipenem, 4 μg/ml; and meropenem, 7.5 μg/ml. The MICs were determined using the broth dilution method, and interpretation was done per the criteria approved by the Clinical and Laboratory Standards Institute (CLSI) (7). The drug-hypersusceptible *E. coli* KAM32 (generously provided by Tomofusa Tsuchiya) that lacks the major multidrug efflux pumps AcrAB and YdhE was used as a host (5).

The protein encoded by ORF ABAYE1181 in the AYE genome is annotated as a putative multidrug efflux protein. It exhibits 51.8% identity (E value of 1e−25 and score of 108) to *E. coli* EmrE (GenBank accession number NC_013364), a well-characterized member of the SMR family (34). Thus, the ABAYE1181 ORF was selected as the putative SMR-type-pump gene to be investigated in this study. The genomic DNA

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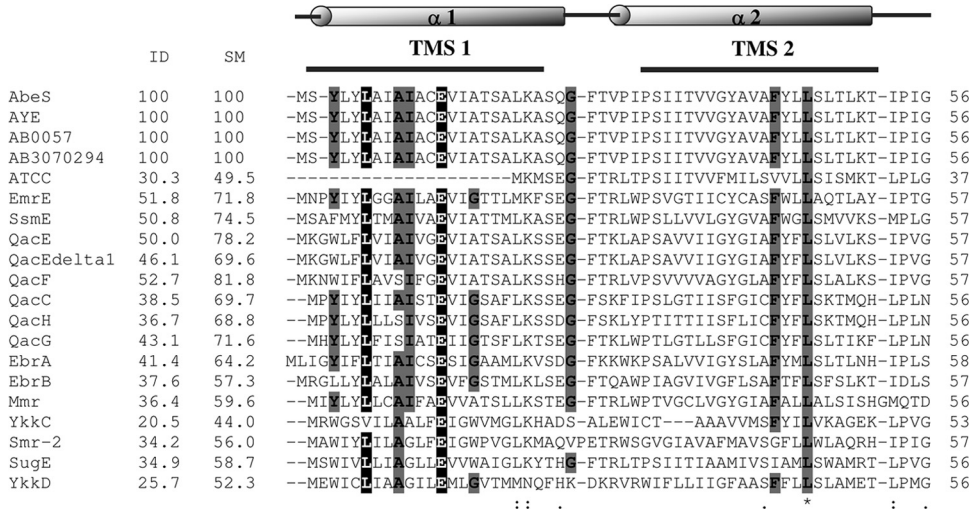

FIG. 1. Multiple sequence alignment of AbeS and related SMR efflux proteins. Multiple sequence alignment of AbeS and related homologues was generated using ClustalW (32). Secondary structure elements indicated on top correspond to those observed in the *E. coli* EmrE protein (Protein Data Bank identification no. 3b5d). Four probable transmembrane segments (TMS) are indicated with horizontal lines above the alignments. Asterisks indicate fully conserved residues, colons indicate strongly similar residues, and dots indicate weakly similar residues. Dashes represent gaps. Essential residues (as shown in EmrE [29]) are highlighted in black. Conserved residues are highlighted in gray. The identities (ID) and similarities (SM) of AbeS with the corresponding SMR transporter are shown in their respective column. Sequences were obtained from the GenBank database by the use of the following (protein) accession numbers: AbeS, *A. baumannii* AC0037 (FJ843079); *A. baumannii* AYE (YP_001713109.1); AB0057 (YP_002319996.1); AB307-0294 (YP_002325052.1); ATCC 17978 (YP_001085323.1); EmrE, *E. coli* (P23895.1); SsmE, *S. marcescens* (BAF80121.1); QacE, *K. aerogenes* (P0AGD0.1); QacEdelta1, *Klebsiella pneumoniae* (ABF48386.1); QacF, *Enterobacter aerogenes* (Q9X2N9.1); QacC, *Staphylococcus aureus* (AAM94143.1); QacH, *Staphylococcus saprophyticus* (O87868.1); QacG, *Staphylococcus* sp. strain ST94 (O87866.1); EbrA, *Bacillus subtilis* (O31792.1); EbrB, *B. subtilis* (O31791.1); Mmr, *Mycobacterium tuberculosis* (P95094); YkkC, *B. subtilis* (Q65KV1.1); Smr-2, *Pseudomonas aeruginosa* (CAH04647.1); SugE, *E. coli* (AAQ16658.1); and YkkD, *B. subtilis* (Q65KV0.1).

of *A. baumannii* was extracted using the DNeasy tissue kit (Qiagen, Valencia, CA). The putative efflux gene from AC0037 was amplified by PCR as described previously (28), with minor modifications. The primers used were P1 and P2 (5′-TAGAGAATTCATGTCTTATCTTTATTTAGC-3′ [EcoRI sequence underlined] and 5′-CGCTCTGCAGTTATAGATGGGTGTTTTTAG-3′ [PstI sequence underlined]). The PCR product was purified using the QiaQuick PCR purification kit (Qiagen, Valencia, CA). The amplicon was digested with EcoRI and PstI and subsequently ligated with vector pUC18 (New England Biolabs, MA) to obtain recombinant plasmid pVBS1. At least two independently generated recombinant plasmids were sequenced bidirectionally using the CEQ 8000 capillary electrophoresis system (Beckman Coulter Instruments, Inc., Palo Alto, CA) to rule out mutations introduced during PCR. Analysis revealed the presence of one ORF that was designated *abeS* (*A. baumannii* efflux pump of SMR family). Further analysis revealed that *abeS* was a 330-bp gene encoding a 109-amino-acid protein with a calculated mass of 11.14 kDa. AbeS exhibited 100% identity to the product of ORF ABAYE1181 found in the *A. baumannii* AYE genome (8). AbeS exhibited different degrees of identity with other SMR transporters from various gram-negative and gram-positive species (Fig. 1).

Antimicrobial susceptibility of *E. coli* expressing *abeS*. To evaluate the role of *abeS* in antimicrobial resistance, the MICs of various antibiotics, detergents, and dyes (Sigma, St. Louis, MO) were determined in triplicate in Mueller-Hinton broth (Difco, Sparks, MD) by the broth dilution method, according to the guidelines of CLSI (7).

The SMR efflux pump-expressing cells exhibited a higher MIC for erythromycin (sixfold) as well as novobiocin (fivefold). Minor increases (twofold) were observed with MICs of aminoglycosides (amikacin), quinolones (ciprofloxacin, norfloxacin), tetracycline, and trimethoprim. No significant differences in MICs were found for chloramphenicol, nalidixic acid, and rifampin (rifampicin) (Table 1). KAM32/pVBS1 also showed increased levels of resistance to detergents, such as deoxycholate (25-fold) and sodium dodecyl sulfate (16-fold), and dyes, such as acridine orange (fivefold), acriflavine (eightfold), and benzalkonium chloride (sixfold). A nearly fourfold increase in resistance was observed for 4′,6-diamidino-2-phenylindole (DAPI), ethidium bromide (EtBr), and rhodamine 123. A marginal decrease in susceptibility toward chlorhexidine, pyronin Y, and tetraphenylphosphonium chloride was also noted (Table 1).

Overall, results demonstrated that AbeS could decrease susceptibility to some antibiotics, disinfectants, dyes, and detergents. The broad substrate specificity displayed by AbeS was very similar to those of other SMR transporters reported previously (6, 14, 16, 17, 34).

To confirm the role of AbeS as an efflux pump, the MICs of substrates with strong specificity were monitored in the presence of 25 μg/ml efflux pump inhibitor carbonyl cyanide 3-chlorophenylhydrazone (CCCP; an uncoupler of oxidative phosphorylation which disrupts the proton gradient on the membrane) (31). Results were consistent with the notion that the *abeS* gene product conferred resistance to various antimicrobial compounds through an efflux mechanism (Table 1).

Fluorometric efflux assay of *E. coli* expressing AbeS. EtBr is highly fluorescent when bound to DNA. Upon being extruded

TABLE 1. MICs of various antimicrobial agents for *E. coli* (KAM32/pUC18 and KAM32/pVBS1) and *A. baumannii* (AC0037, AC0037Δ*abeS*, and AC0037Δ*abeS*Ω*abeS*) strains used in this study

Compound	MIC (μg/ml) for *E. coli* strain[d]:		Fold change[a]	MIC (μg/ml) for *A. baumannii* strain:		Fold change[c]	MIC (μg/ml) for *A. baumannii* AC0037Δ*abeS*Ω*abeS*
	pUC18/KAM32	pVBS1/KAM32		AC0037	AC0037Δ*abeS*[b]		
Amikacin	1 (0.15)	2 (0.15)	2	256	256	1	256
Chloramphenicol	0.2	0.2	1	256	64	4	128
Ciprofloxacin	0.002 (0.0005)	0.004 (0.0005)	2	120	40	3	>60
Erythromycin	4 (<0.05)	24 (0.25)	6	64	8	8	32
Nalidixic acid	1	1	1	256	128	2	256
Norfloxacin	0.06 (0.001)	0.12 (0.001)	2	512	256	2	>128
Novobiocin	4 (<0.5)	20 (<0.5)	5	120	40	3	84
Rifampin	2	2	1	2	2	1	2
Tetracycline	0.5	1	2	64	64	1	64
Trimethoprim	0.125	0.2	2	64	64	1	64
Acridine orange	16	80	5	200	50	4	125
Acriflavine	2	16	8	128	16	8	64
Benzalkonium chloride	1 (0.25)	6 (0.25)	6	48	12	4	24
Chlorhexidine	2	4	2	32	16	2	32
DAPI	0.5	2	4	2	0.5	4	2
Deoxycholate	125 (<0.25)	3,125 (5)	25	>2,048	128	>16	512
EtBr	2	8	4	1,024	128	8	>512
Methyl viologen	64 (<2.5)	128 (<2.5)	2	800	400	2	800
Pyronin Y	4	8	2	4	2	2	4
Rhodamine 123	8	32	4	128	64	2	128
Sodium dodecyl sulfate	50 (<5)	800 (<5)	16	>256	16	>16	128
Tetraphenylphosphonium chloride	8 (0.25)	16 (0.25)	2	1,500	300	5	>500

[a] Ratio of MIC for pVBS1 to MIC for pUC18.
[b] AC0037Δ*abeS* did not show any difference in susceptibility toward imipenem, meropenem, and ceftazidime.
[c] Ratio of MIC for AC0037 to MIC for AC0037Δ*abeS*.
[d] Values in parentheses are MICs in the presence of the efflux pump inhibitor CCCP at 25 μg/ml.

from the cell through an efflux pump, the fluorescence decreases, and this difference can be measured with a spectrofluorometer (13, 17). To confirm the role of AbeS as an efflux pump, we compared the rates of EtBr accumulation in *E. coli* KAM32 cells carrying either pUC18 or recombinant plasmid pVBS1. We found that the intracellular level of EtBr in *E. coli* KAM32/pVBS1 (carrying *abeS*) was significantly lower than that of the control strain *E. coli* KAM32/pUC18 under energized conditions (in the absence of CCCP) (Fig. 2A). After the addition of CCCP, at 25 μg/ml, there was a rise in the intracellular accumulation level of EtBr in *E. coli* KAM32/pVBS1, which eventually reached a plateau equal to that of the control strain *E. coli* KAM32/pUC18 (Fig. 2A). These results showed that AbeS is an energy-dependent active efflux pump. Subsequently, we tested the influence of monovalent cations, such as Na^+ and Li^+, in the efflux process because the activities of several multidrug efflux pumps are coupled with monovalent cations (9). The addition of either NaCl or LiCl caused no difference in the efflux activity from that of the control strain (data not shown). Thus, results demonstrated that AbeS is an efflux pump and the active extrusion is proton (H^+) driven.

Construction of an *abeS* null mutant in *A. baumannii*. To evaluate the role of AbeS in *A. baumannii* AC0037, an *abeS* deletion mutant was generated. The vector pUC18, not capable of replicating in *A. baumannii*, was used as a suicide vector (15). To construct the *abeS* deletion mutant, the 548-bp upstream region (amplicon A) and 542-bp downstream region (amplicon B) of the efflux gene were amplified using specific primers. The primers for amplicon A were P3 (5′-TGATTCTAGATGTGATATGTGCTTACCAGAATGC-3′, with an XbaI linker) and P4 (5′-TCATCTGCAGAGTAAAACCTTGGATGCTTT-3′, with a PstI linker). The primers for amplicon B were P5 (5′-GATACTGCAGTGCATTGGTTTAGCTTTA-3′, with a PstI linker) and P6 (5′-ACAGGGATCCTCTGAGGCGGAAGAACGTGCAC-3′, with a BamHI linker) (enzyme sequences are underlined). PCR products were generated from the genome of AC0037. The digested fragments were ligated with the PstI-kanamycin resistance cassette (K) obtained with the pUC-4K vector (Pharmacia). The generated AKD fragment was inserted into the XbaI- and BamHI-digested plasmid pUC18, resulting in the pabeS-Kan plasmid. It was bidirectionally sequenced to ensure the presence of the kanamycin resistance cassette and to confirm authenticity. The plasmid pabeS-Kan was then introduced into *A. baumannii* AC0037 for the double homologous recombination event to occur by electroporation as described previously (15).

Selection of transformants was made on 50 μg/ml kanamycin- and 80 μg/ml ticarcillin-containing plates. Inactivation of the *abeS* gene by insertion of pabeS-Kan was confirmed by PCR amplification and DNA sequencing, and the null mutant was designated AC0037Δ*abeS*.

The primers used for confirmation were the gene-specific primers P1 and P2, as well as the flanking chromosomal region primers P11 (5′-GCTTATTCAGCGAGTTAAATATG-3′) and P12 (5′-CACATGACAGTACTGGAAAATGT-3′).

Functional characterization of *abeS* in *A. baumannii*. Following the confirmation of the *abeS* deletion mutant, the role of *abeS* in *A. baumannii* was evaluated. Susceptibility testing data showed that deletion of *abeS* resulted in increased susceptibility to various antimicrobial compounds (Table 1).

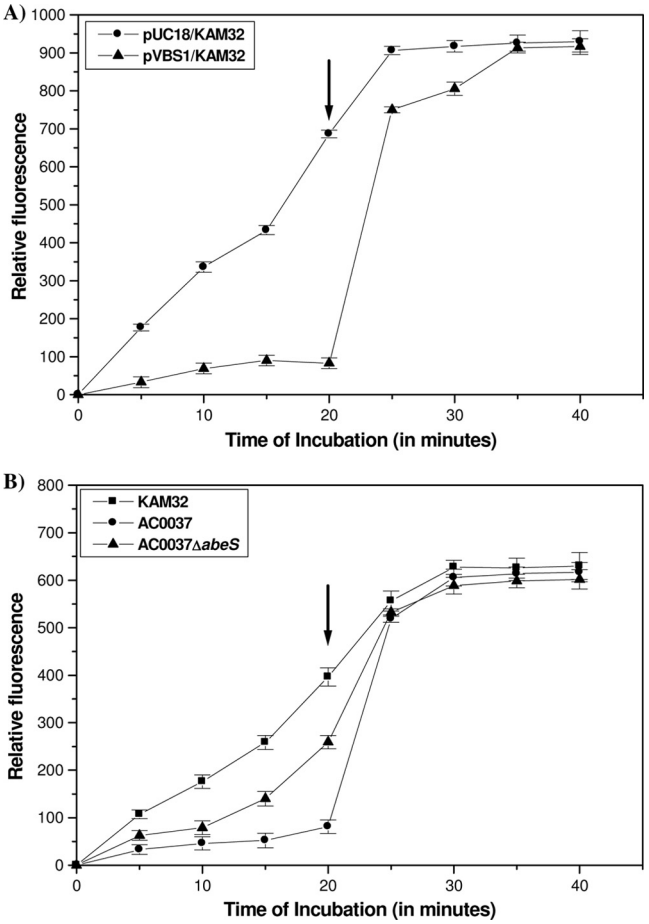

FIG. 2. Fluorometric accumulation assays. The accumulation study was done as described previously (31). (A) Accumulation studies using 10 μg/ml of EtBr with KAM32/pVBS1 and KAM32/pUC18. (B) Accumulation studies using 5 μg/ml of EtBr with *A. baumannii* AC0037, the *A. baumannii abeS* null mutant (AC0037Δ*abeS*), and *E. coli* KAM32 (lacking AcrAB and YdhE). Instead of sodium phosphate buffer, phosphate-buffered saline (0.136 M NaCl, 0.0026 M KCl, 0.01 M Na_2HPO_4, 0.00176 M KH_2PO_4, pH 7.0) was used, and cell lysis was carried out for 6 h. The fluorescence of the supernatant was measured at excitation at 530 nm and emission at 600 nm, using a SpectraMax M2 spectrofluorometer (Molecular Devices Corporation, Sunnyvale, CA). Where indicated (after 20 min of incubation with the substrate), the proton motive force uncoupler CCCP was added to a final concentration of 25 μg/ml. Relative fluorescence intensity on the *y* axis represents the levels of accumulated EtBr in the bacterial cells. The graph reflects the difference in the fluorescence shown by the bacterial cell in the presence and absence of the inhibitor CCCP. Each data point represents the mean plus the standard deviation of three independent experiments.

The EtBr accumulation experiments demonstrated that the efflux was more efficient in the wild-type AC0037 strain, whereas the efflux was significantly less efficient (increased accumulation of >3-fold for EtBr) in the AC0037Δ*abeS* deletion mutant. Addition of efflux inhibitor CCCP increased the intracellular accumulation of EtBr in AC0037Δ*abeS* (Fig. 2B).

The role of the *A. baumannii abeS* gene was confirmed by gene complementation experiments. Briefly, a DNA fragment containing a functional *aadA1* gene was amplified from an AC0019 clinical isolate (GenBank accession number EU977568.1) (26, 31) by PCR using primers P7 (5′-TAC AGATATCATGAGGGAAGCGGTGATC-3′ [EcoRV linker underlined]) and P8 (5′-TACGGTCGACTTATTTGCCTAC TACCTTGGTGA-3′ [SalI linker underlined]). The cassette was ligated into the *E. coli-Acinetobacter* shuttle vector pWH1266 (11). The resulting plasmid, pWH-Spc, was modified by cloning a PCR-amplified wild-type *abeS* gene with the primer pair P1/P2 into the PstI site of the plasmid, yielding a recombinant plasmid, pWH-abeS. Electroporation of the recombinant plasmid pWH-abeS into the *A. baumannii* AC0037Δ*abeS* mutant resulted in strain AC0037Δ*abeS* Ω*abeS*. Selection of the AC0037Δ*abeS*Ω*abeS* mutant was made on 200 μg/ml streptomycin, 50 μg/ml kanamycin, and 80 μg/ml of ticarcillin-containing plates. Complementing the mutant with the wild-type *abeS* gene nearly restored reduced susceptibility to antimicrobial agents, detergents, and dyes, similar to that of the parental strain AC0037 (Table 1). These findings confirmed the role of *abeS* in mediating resistance also in *A. baumannii*.

In conclusion, this study demonstrated for the first time the role of the SMR efflux pump AbeS in mediating resistance to some antibiotics, hydrophobic compounds, detergents, and disinfectants in *A. baumannii* clinical strain AC0037.

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper have been deposited in the GenBank nucleotide sequence database under the accession number FJ843079.

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