

AbeM, an H⁺-Coupled *Acinetobacter baumannii* Multidrug Efflux Pump Belonging to the MATE Family of Transporters

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We cloned a gene, *abeM*, for a multidrug efflux pump from *Acinetobacter baumannii* using *Escherichia coli* as the host. Sequence analysis revealed that AbeM is a member of the MATE family of pumps. AbeM was found to be an H⁺-coupled multidrug efflux pump and a unique member of the MATE family.

Acinetobacter spp. are emerging as a major cause of nosocomial infections or in outbreaks of cross-infection, particularly in intensive care units, where the use of antimicrobial agents is greatest and the host is most susceptible. *Acinetobacter baumannii* is one of the *Acinetobacter* spp. that is the most frequently isolated from human infections (3). Certain strains of *A. baumannii* are now resistant to many commonly prescribed antibiotics (including imipenem and fluoroquinolones), and the multiple-drug resistance is often responsible for the failure of antibiotic therapy.

Among the drug resistance mechanisms in *A. baumannii*, degradation of drugs seems to be a major cause because of the production of relevant enzymes in this organism (1, 13, 18, 24). Modification of drugs is also common in this organism due to the synthesis of specific modifying enzymes (19, 20). Alterations of the drug target due to mutations in *gyrA* and *parC* have been associated with high levels of resistance to fluoroquinolones (21, 22). Recently, decreased expression of the outer membrane proteins has been reported to be involved in resistance to some drugs (6, 7, 12). Studies of drug resistance mediated by an efflux pump(s) are very few; only one efflux system, AdeABC, that mediates multidrug resistance has been identified and studied in detail in this organism (9, 14). Here we report on the gene cloning and biochemical characterization of a multidrug efflux pump from *A. baumannii*.

Gene cloning. *A. baumannii* ATCC 19606 (generously provided by Shigeo Yamamoto, Okayama University) and *Escherichia coli* KAM32 which lacks the major multidrug efflux pumps AcrAB and YdhE (5) were used in this study. To identify a putative multidrug efflux transporter(s) in *A. baumannii*, we tried to clone the gene(s) responsible for multidrug resistance. Fragments of chromosomal DNA from strain ATCC 19606 were prepared and ligated to a vector plasmid, pBR322. The resulting recombinant plasmids that were obtained (five candidates) enabled *E. coli* KAM32 cells to grow in the presence of 10 µg/ml of ethidium bromide. We chose one of the plasmids, pABE6, and constructed several deletion plas-

mids. The shortest deletion plasmid that conferred resistance to ethidium bromide, pABE618, was further analyzed. As a result of DNA sequencing, we found one open reading frame, designated *abeM*. Several promoter-like sequences and a Shine-Dalgarno sequence preceded the *abeM* gene. The deduced AbeM protein consists of 448 amino acid residues, is very rich in hydrophobic amino acid residues, and possesses 12 hydrophobic regions.

Recently, the complete genome sequence of *Acinetobacter* ADP1 was published (2). The AbeM protein showed sequence homology (79% identity, 94% similarity) with the sequence of ACIAD0429, which was estimated to be a NorM homolog in this organism. A search for protein sequence similarity by using the BLAST program (National Center for Biotechnology Information) showed that AbeM shared 39, 39, 37, 37, and 34% identities and 77, 76, 76, 75, and 75% similarities with PmpM of *Pseudomonas aeruginosa* PAO1 (8), VcmA of *Vibrio cholerae* non-O1 (11), YdhE of *E. coli*, NorM of *Vibrio parahaemolyticus* (17), and HmrM of *Haemophilus influenzae* (23), respectively. Thus, it is highly likely that AbeM is a member of the MATE family of multidrug efflux pumps (4).

Substrate specificity of AbeM. The substrate specificity of AbeM was investigated by comparing the susceptibilities of *E. coli* KAM32/pABE618 (which carries *abeM*) and *E. coli* KAM32/pUC18 (control) to various antimicrobial agents. As shown in Table 1, significant increases (more than fourfold) in the MICs were observed for norfloxacin, ofloxacin, ciprofloxacin, gentamicin, 4',6-diamino-2-phenylindol (DAPI), triclosan, acriflavine, Hoechst 33342, daunorubicin, doxorubicin, rhodamine 6G, and ethidium bromide, whereas reproducible twofold increases were observed for kanamycin, erythromycin, chloramphenicol, tetraphenylphosphonium chloride (TPPCI), and trimethoprim. The hydrophilic fluoroquinolones, such as norfloxacin and ciprofloxacin, appeared to be better substrates for the AbeM pump than the hydrophobic fluoroquinolone such as ofloxacin.

Efflux of ethidium. To confirm that AbeM is really a drug efflux pump, we measured the transport of drug in cells of *E. coli* KAM32/pABE618 and *E. coli* KAM32/pUC18. We measured the levels of accumulation of ethidium bromide as described previously (23) and observed that the level of accumulation of ethidium bromide in cells of *E. coli* KAM32/pABE618 was remarkably less than that in cells of KAM32/pUC18 under

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TABLE 1. Susceptibilities of transformants to antimicrobial agents

Antimicrobial class and antimicrobial agent	MIC ($\mu\text{g/ml}$)		Relative resistance ^a
	<i>E. coli</i> KAM32/pUC18	<i>E. coli</i> KAM32/pABE618	
Aminoglycosides			
Kanamycin	1	2	2
Gentamicin	0.25	1	4
Quinolones			
Norfloxacin	0.03	1	32
Ofloxacin	0.015	0.06	4
Ciprofloxacin	<0.008	0.125	16
β -Lactam, imipenem	0.25	0.25	1
Erythromycin	4	8	2
Tetracycline	0.5	0.5	1
Chloramphenicol	1	2	2
Others			
SDS ^b	>128	>128	1
DAPI	0.5	8	16
TPPCI	8	16	2
Triclosan	0.5	2	4
Acriflavine	2	32	16
Hoechst 33342	0.5	32	64
Daunorubicin	4	64	16
Doxorubicin	1	128	128
Trimethoprim	0.125	0.25	2
Rhodamine 6G	8	64	8
Ethidium bromide	4	128	32

^a Relative resistance: MIC in *E. coli* harboring pABE618/MIC in *E. coli* harboring pUC18.

^b SDS, sodium dodecyl sulfate.

energized conditions (Fig. 1). With the addition of a protonophore, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), the levels of accumulation of ethidium in the cells became higher and reached a plateau. The plateau levels in the two strains were the same. These results indicate that energy-dependent ethidium efflux activity in cells of *E. coli* KAM32/pABE618 is much stronger than that in cells of *E. coli* KAM32/pUC18.

It seemed that AbeM is a member of the MATE family of transporters. An electrochemical potential of Na^+ is the driving force for the MATE family of transporters, such as NorM, YdhE, VmrA, VcmA, VcrM, and HmrM (5, 10, 11, 16, 17, 23). On the other hand, a recent study in our laboratory revealed that a multidrug efflux pump, PmpM, from *Pseudomonas aeruginosa* belongs to the MATE family and is an H^+ -coupled efflux pump (8). Thus, we investigated whether AbeM is either an Na^+ -coupled pump or an H^+ -coupled pump. First, we tested the effects of NaCl, LiCl, and KCl (control) on ethidium efflux. However, addition of NaCl or LiCl to the assay mixture at 1 to 100 mM did not give any significant effect (data not shown). These results suggest that AbeM is not an Na^+ -driven pump but is an H^+ -driven drug efflux pump.

We also examined whether drug influx could elicit Na^+ efflux via the AbeM pump using an Na^+ electrode (16). How-

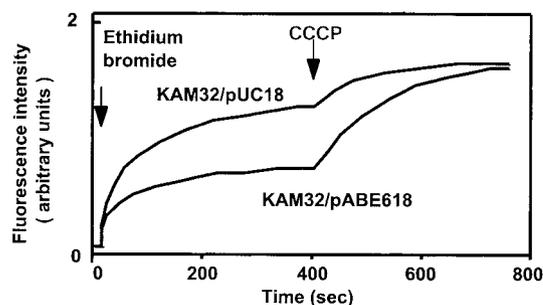


FIG. 1. Transport of ethidium bromide via the AbeM pump. The accumulation of ethidium bromide in cells is shown. Cells of *E. coli* KAM32/pUC18 (control) or *E. coli* KAM32/pABE618 (carrying *abeM*) were grown in Luria-Bertani medium containing 100 $\mu\text{g/ml}$ of ampicillin and suspended in modified Tanaka medium (sodium salts were replaced with potassium salts). After preincubation at 37°C for 5 min, ethidium bromide (final concentration, 10 μM) was added to initiate drug accumulation, and then a proton conductor (CCCP; final concentration, 100 μM) was added at the time point indicated by the arrow to deenergize the membrane. The increase in fluorescence indicates the accumulation of ethidium bromide in the cells.

ever, we were unable to detect Na^+ efflux (data not shown). Thus, we conclude that AbeM is not an Na^+ -coupled pump.

H^+ -drug antiport mediated by AbeM. If the coupling cation in AbeM is an H^+ , then it must be an H^+ -drug antiporter. One convenient method that can be used to test for this possibility is to measure the flux of H^+ caused by the addition of a substrate of the pump. We measured H^+ flux by measuring the changes in fluorescence quenching in everted membrane vesicles, as described previously (15). Addition of norfloxacin to the assay mixture containing vesicles from *E. coli* KAM32/pABE618 elicited a much larger efflux of H^+ (dequenching of the fluorescence) compared with that from vesicles from *E. coli* KAM32/pUC18 (Fig. 2). This indicates that H^+ -norfloxacin

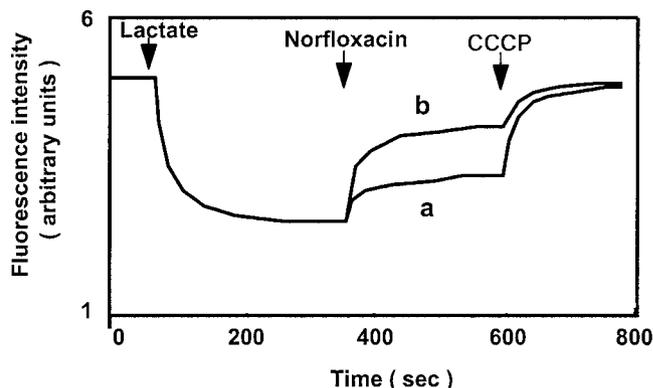


FIG. 2. H^+ -norfloxacin antiport via AbeM. H^+ -norfloxacin antiport was measured in everted membrane vesicles prepared from cells of *E. coli* KAM32/pUC18 (control) (curve a) and *E. coli* KAM32/pABE618 (carrying *abeM*) (curve b). The formation and dissipation of the H^+ gradient across vesicle membrane, which reflect the influx and the efflux of H^+ , respectively, were monitored by measuring the changes in fluorescence intensity of quinacrine. At the time points indicated by arrows, potassium D,L-lactate (final concentration, 5 mM) was added to initiate respiration. After the fluorescence quenching reached a steady-state level, norfloxacin (final concentration, 5 μM) was added to the assay mixture, and then CCCP (final 50 μM) was added to collapse the H^+ gradient.

antiport via AbeM took place. Thus, we conclude that the coupling ion in the AbeM pump is H⁺ and not Na⁺. It should be pointed out that some H⁺-norfloxacin antiport activity is present in vesicles of *E. coli* KAM32/pUC18.

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper have been deposited in the DDBJ/EMBL/GenBank nucleotide sequence databases under accession number AB204810.

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