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Gene Cloning and Characterization of MdeA, a Novel Multidrug Efflux Pump in *Streptococcus mutans*

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Multidrug resistance, especially multidrug efflux mechanisms that extrude structurally unrelated cytotoxic compounds from the cell by multidrug transporters, is a serious problem and one of the main reasons for the failure of therapeutic treatment of infections by pathogenic microorganisms as well as of cancer cells. *Streptococcus mutans* is considered one of the primary causative agents of dental caries and periodontal disease, which comprise the most common oral diseases. A fragment of chromosomal DNA from *S. mutans* KCTC3065 was cloned using *Escherichia coli* KAM32 as host cells lacking major multidrug efflux pumps. Although *E. coli* KAM32 cells were very sensitive to many antimicrobial agents, the transformed cells harboring a recombinant plasmid became resistant to several structurally unrelated antimicrobial agents such as tetracycline, kanamycin, rhodamin 6G, ampicillin, acriflavine, ethidium bromide, and tetraphenylphosphonium chloride. This suggested that the cloned DNA fragment carries a gene encoding a multidrug efflux pump. Among 49 of the multidrug-resistant transformants, we report the functional gene cloning and characterization of the function of one multidrug efflux pump, namely MdeA from *S. mutans*, which was expressed in *E. coli* KAM32. Judging from the structural and biochemical properties, we concluded that MdeA is the first cloned and characterized multidrug efflux pump using the proton motive force as the energy for efflux drugs.

Key words: *Streptococcus mutans*, multidrug resistance, drug efflux pump, gene cloning

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In the last several decades, microorganisms have developed to share resistance to almost every antibiotic that has been developed, often by quite unexpected mechanisms and much more readily than was originally predicted [18]. In fact, the emergence of drug resistance in microorganisms, and its association with serious infectious disease, has increased at an alarming rate over the past several decades [8]. Many bacteria that once appeared to be under control or were believed to be potentially controllable are now causing infections that are increasingly difficult to treat [11]. Antibiotic resistance in the community is thus a growing threat in the world, particularly in developing countries and in high-risk settings in developed countries [12]. Antibiotic-resistant bacteria can live and multiply in the presence of therapeutic levels of antibiotics. They can be resistant in their natural state or they can acquire resistance genes by mutation, expression of a latent chromosomal gene, or exchange of genetic material from other bacteria [13]. To successfully fight the increasing number of drug-resistant and multidrug-resistant (MDR) bacteria, extensive knowledge of the molecular mechanisms underlying microbial antibiotic resistance is required [16].

Analysis of the available genome sequences of various bacteria suggested that there is a large reservoir of resistant genes in bacterial genomes and in extrachromosomal pieces of DNA that encode proteins possibly responsible for different mechanisms of drug resistance, such as enzyme inactivation, antibiotic target alteration, decreased antibiotic uptake, and efflux pumps [6]. Among those drug-resistant mechanisms, multidrug efflux pumps play an important role as a self-defense system in bacteria. Hyperexpression of such multidrug efflux pumps in human pathogens is a serious problem in a clinical setting, because the hyperexpression of a multidrug efflux pump elevates the bacterial resistance

level against various antibiotics; this phenomenon complicates clinical antibiotic-based therapy [14].

Streptococcus mutans is a primary etiologic agent of dental caries that is also known to be resistant to antibiotics, which is known to be effective gram positive bacteria including *S. mutans* [5, 21]. Therefore, understanding the mechanisms of antibiotic resistance in *S. mutans* is important when considering strategies for defense against multidrug-resistant bacteria.

In this study, we report the functional gene cloning and characterization of a noble multidrug efflux pump, MdeA, from *S. mutans*, which was first verified for its multidrug efflux properties experimentally.

Materials and Methods

Bacteria and Growth

S. mutans KCTC 3065 (ATCC 25175) and *Escherichia coli* KAM32 (*ΔacrB, ΔydhE, hsd* negative) [4] were used in this study. *S. mutans* cells were grown in Brain Heart Infusion (BHI) medium (Difco Co., USA) and *E. coli* cells were grown in Luria medium [9] at 37°C. Cell growth was monitored by measuring the optical density of culture broth at 600 nm.

Gene Cloning and Sequencing

Chromosomal DNA was prepared from *S. mutans* cells by the method of Berns and Thomas [2]. The DNA was partially digested with *Sau*3AI, and the fragments from 4 to 10 kbp were separated by gel purify kit (SolGent Co., Korea). Plasmid pSTV28 DNA was digested with *Bam*HI, dephosphorylated with alkaline phosphatase (TaKaRa Co., Japan), and then ligated to the chromosomal DNA fragments by using a ligation kit, ver. 2 (TaKaRa Co., Japan). Competent cells of *E. coli* KAM32 were transformed with the recombinant plasmids and were spread onto agar plates containing Luria broth, 20 μg/ml chloramphenicol with 4 μg/ml kanamaycin, 1 μg/ml tetracycline, 20 μg/ml ampicillin, 8 μg/ml acriflavine, 16 μg/ml ethidium bromide, 32 μg/ml rhodamine 6G, and 8 μg/ml tetraphenyl-phosphonium chloride (TPP·Cl) and 1.5% agar. The plates were incubated at 37°C for 24 h. Candidate colonies were replica plated, and plasmids were isolated from each of the candidates. The nucleotide sequence of the gene was determined by the dideoxy chain termination method [18] with an automated DNA sequencer (3730XL, Macrogen Co.). The nucleotide sequence data were analyzed with Genetyx-win (ver. 5.1) and compared with the genome sequence data base of *S. mutans* UA159 (http://www.ncbi.nlm.nih.gov/genome/856?project_id=57947).

Drug Susceptibility Test

The minimal inhibitory concentrations (MICs) of various drugs were determined in Muller–Hinton broth (Difco Co.) by the standard 2-fold dilution method [7]. Cells were incubated in the test medium at 37°C for 24 h and the growth was examined by visual inspection.

Fluorescent Drugs Accumulation Assay in the Cells

KAM32 cells harboring recombinant plasmids were grown in Luria broth at 37°C until the exponential phase of growth. The cells were harvested and washed with phosphate, buffered saline (PBS, pH 7.4). Cells were resuspended in PBS (pH 7.4). Then, 2 ml of the cell suspension supplemented with 20 mM glucose was incubated at 37°C in a cuvette for 5 min and thereafter 10 μM ethidium bromide or 4.2 μM acriflavine was added to the cell suspension. When the accumulation apparently ceased, 40 μM carbonyl cyanidem-chlorophenylhydrazone (CCCP) was added in order to prepare energy-starved cells. The relative amount of ethidium and acriflavine retained in cells was measured indirectly by detecting fluorescence from the cells using a Hitachi F4500 fluorescence spectrophotometer. The excitation and emission wavelengths used for each of the fluorescent compounds are as follows; ethidium, 500 and 580 nm; and acriflavine, 468 and 499 nm, respectively.

Results

Functional Cloning and Sequencing of the *mdeA* Drug Resistance Gene

For cloning of the gene responsible for multidrug resistance from *S. mutans*, partially digested chromosomal DNA fragments from *S. mutans* were ligated into the cloning vector pSTV28, and transformed into *E. coli* KAM32 (*ΔacrB, ΔydhE*). We obtained 49 recombinant plasmids using kanamaycin (4 μg/ml), tetracycline (1 μg/ml), ampicillin (20 μg/ml), acriflavine (8 μg/ml), ethidium bromide (16 μg/ml), rhodamine 6G (32 μg/ml), and TPP·Cl (8 μg/ml) in the growth media as antimicrobial agents for selection. Each of the transformant was tested for measuring MIC against various drugs to identify their multiple drug resistance. Recombinant plasmid pMDE29, which conferred to *E. coli*

Table 1. MIC of various drugs of *mdeA*-expressing KAM32 cells.

Drugs	MIC (ug/ml)		
	E. coli KAM32/ pSTV28	*E. coli* KAM32 /pMDE29	Relative resistance[a]
Antibiotics			
Kanamycin	1.0	4.0	4
Tetracycline	0.5	2.0	4
Ampicillin	4.0	128.0	32
Oxacillin	2.0	8.0	4
Ciprofloxacin	0.005	0.1	20
Nalidixic acid	1.0	8.0	8
Other antimicrobials			
Acriflavine	2.0	8.0	4
Ethidium bromide	8.0	16.0	2
Rhodamine 6G	8.0	32.0	4
TPP·Cl[b]	4.0	8.0	2

[a]Relative resistance was calculated by comparison of KAM32/pSTV28 and KAM32/pMDE29.

[b]TPPCl, tetraphenlyphosphonium chloride.

The experiment was repeated three times with similar results, and a representative result is shown.

KAM32 the strongest drug resistance against multiple drugs, was selected as a candidate for further analysis (Table 1). The agarose electrophoresis analysis revealed that the insert size of pMDE29 was about 2.2 kbp long (data not shown).

Partial sequencing of the DNA insert in plasmid pMDE29 revealed that it contained one complete open reading frame (ORF) and two incomplete ORFs, according to the genome sequence information for *S. mutans* UA159 (www.ncbi.nlm.nih.gov) [1]. Specifically, one complete ORF, SMU 2109, was predicted as a multidrug resistant (MDR) permease included in the major facilitator superfamily (MFS), a large transporter group of drug efflux pump, predicting 12 transmembrane domains, which might encode a multidrug resistance protein [1]. We renamed this gene as *mdeA*, meaning for multidrug efflux pump of *S. mutans*.

The nucleotide sequence of the whole *mdeA* region in plasmid pMDE29 was determined, because it was cloned from *S. mutans* ATCC 25175, not from *S. mutans* UA159. The DNA sequence of the *mdeA* gene was exactly the same as that of *S. mutans* UA159. The *mdeA* gene, 1,380 bp in length, specifies a putative 459-amino acid protein with a calculated molecular mass of 49.4 kDa. In the amino acid composition of MdeA, hydrophobic residues were 64%, showing a unique property of membrane protein, followed by 24% of neutral residues, and 12% of hydrophilic residues. The deduced amino acid sequence of the MdeA showed 98%, 56%, 56%, 43%, and 41% identities and 99%, 73%, 75%, 64%, and 59% similarities with putative multidrug efflux proteins of *S. mutans* NN2025, *Scardovia inopinata* F030, *Lactobacillus oris* F0423, *Bacillus cereus* H3081.97, and *Staphylococcus*

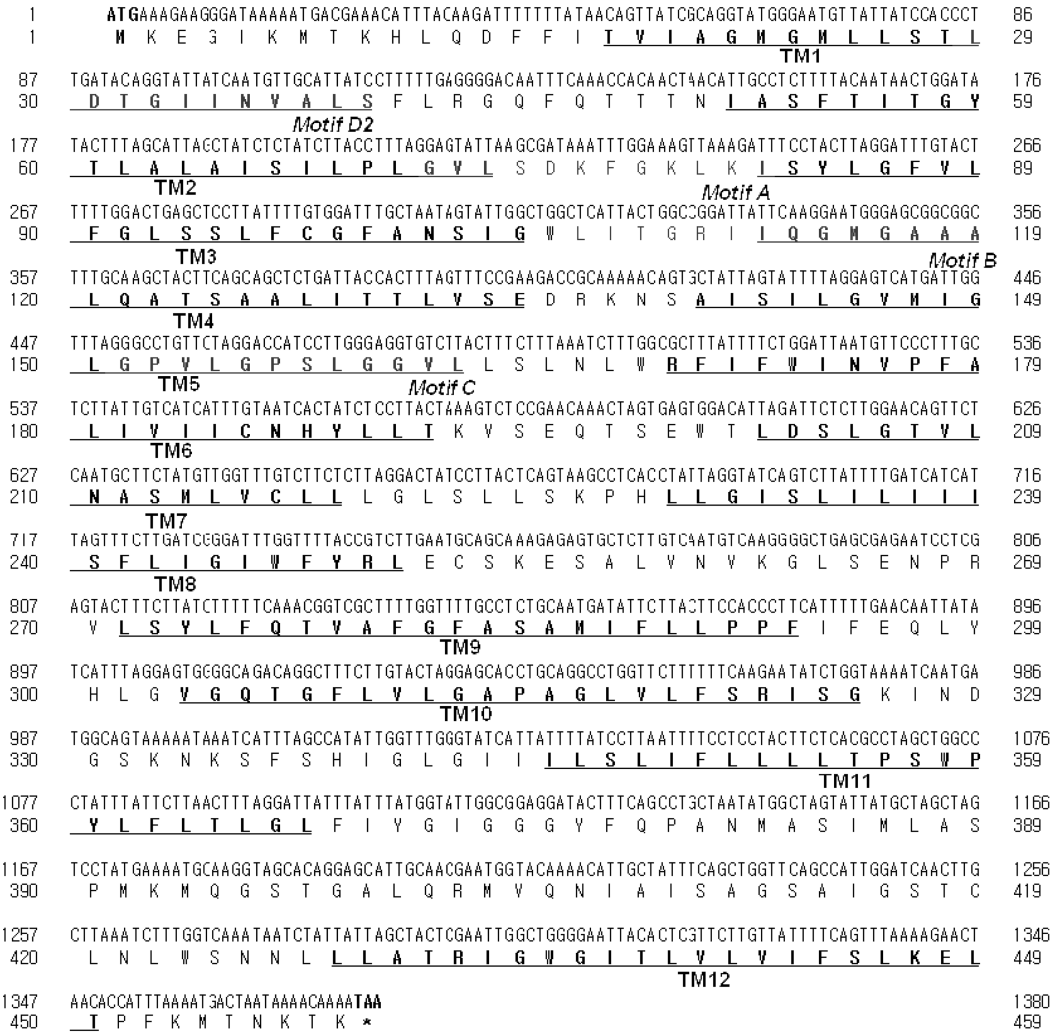

Fig. 1. Nucleotide sequence of the sense of *mdeA* gene.
The deduced amino acid sequences are displayed below of the nucleotide sequences. Twelve transmembrane domains (TM1 to TM12) are shown by underlining the respective amino acid residues. Shadowed gray boxes represent the reserved motif sequences, motifs A, B, C, and D2 of the MFS family [17]. TMs and motifs were analyzed through the web site software SOSUI (http://sosui.proteome.bio.tuat.ac.jp/sosuimenu0.html) and MOTIF (http://motif.genome.ac.jp), respectively.

Table 2. Homologs of MdeA drug efflux pump.

Organisms	Length	Function	% Identity	% Similarity	Accession No.
Streptococcus mutans UA159	459	Multidrug efflux	100	100	NP_722397 (this study)
Streptococcus mutans NN2025	459	Multidrug efflux	98	99	YP_003485765
Scardovia inopinata F030	456	Multidrug efflux	56	73	ZP_06756393
Lactobacillus oris F0423	450	Multidrug efflux	56	75	EGS36279
Bacillus cereus H3081.97	463	Multidrug efflux	43	64	ZP_03237572
Staphylococcus epidermidis	459	Multidrug efflux	41	59	ZP_06285501
Clostridium acetobutylicum	487	Multidrug efflux	30	52	NP_348080
Weissella koreensis KACC 1551	476	Multidrug efflux	27	49	YP_004726583
Enterococcus gallinarum EG2	480	Multidrug efflux	26	49	ZP_05650735

epidermidis, respectively (Table 2). Additionally other known important Gram-positive strains, including *Clostridium* sp., *Weisselaa* sp., and *Enterococcus* sp. also showed significant similarity with MdeA of *S. mutans*. All of these transporters are from Gram-positive bacteria and included in the major facilitator superfamily, which is same family with MdeA in this study.

Hydropathy analysis of MdeA predicted a highly hydrophobic protein with 12 membrane-spanning regions and hydrophilic sequence at both the $-NH_2$ and –COOH termini of the protein (Fig. 2).

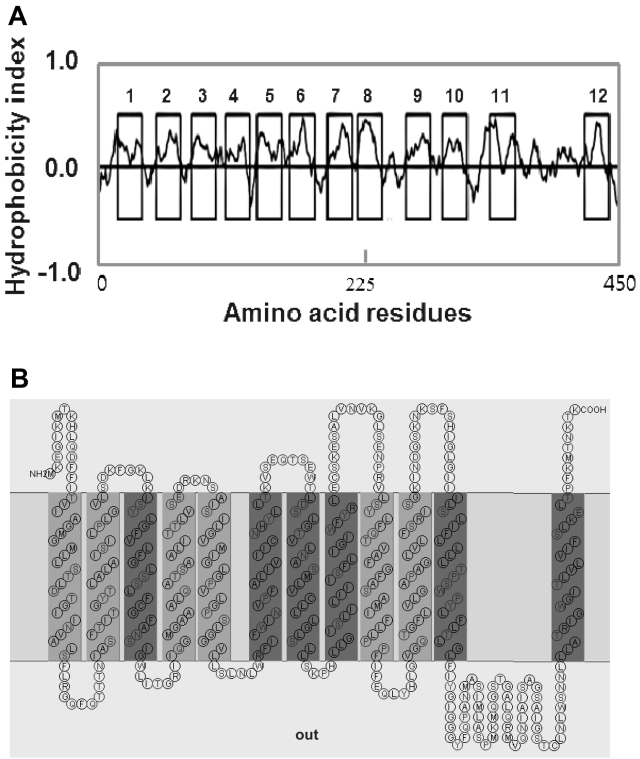

Fig. 2. Hydropathy pattern (**A**) and predicted secondary structure (**B**) of MdeA of *S. mutans*, showing a typical membrane protein having 12 transmembrane helices.
The plus digit of hydrophobicity index of (**A**) means the hydrophobic residues.

Drug Susceptibility Studies in *E. coli* KAM32

To define the contribution of MdeA towards mediating drug resistance, the protein was expressed in *E. coli* KAM32, which is hypersensitive to many drugs owing to deficiencies of the major multidrug efflux pumps AcrB and YdhE [4]. The drug susceptibility in cells of KAM32/pMDE29 (carrying *mdeA*) and KAM32/pSTV28 (vector control) are shown in Table 1. Plasmid pMDE29 in KAM32 cells conferred resistance to several structurally unrelated drugs compared with the vector control KAM32/pSTV28. The MICs of kanamycin, tetracycline, ampicillin, oxacillin, ciprofloxacin, nalidixic acid, acriflavine, ethidium bromide, rhodamine 6G, and TPP·Cl in *E. coli* KAM32/pMDE29 were 4-, 4-, 32-, 4-, 20-, 8-, 4-, 2-, 4-, and 2-fold higher, respectively, than the vector control. MdeA expressed from a single gene conferred resistance to KAM32 against various antimicrobial drugs. Thus, we concluded that the MdeA is a protein conferring multidrug resistance, perhaps a multidrug efflux pump.

Drug Accumulation Assay

As mentioned above, sequence similarity with other MFS family multidrug efflux proteins suggested that the putative MdeA is also a multidrug efflux pump, which can translocate drugs outside from inside of the cells. To confirm this suggestion, we tested this possibility by measuring the amount of ethidium and acriflavine accumulated in the cells. Ethidium binds to double-stranded DNA, resulting in a substantial increase in fluorescence, whereas acriflavine binds to DNA to result in fluorescence quenching [4]. As shown in Fig. 3, we observed a clear increase in ethidium fluorescence when CCCP, a proton conductor, was added to a cell suspension of KAM32/pMDE29, indicating that the accumulation of ethidium took place after the addition of CCCP. On the other hand, we observed little change in fluorescence caused by the addition of CCCP with control cells of KAM32/pSTV28. The final levels of the fluorescence intensities after the addition of CCCP became similar in

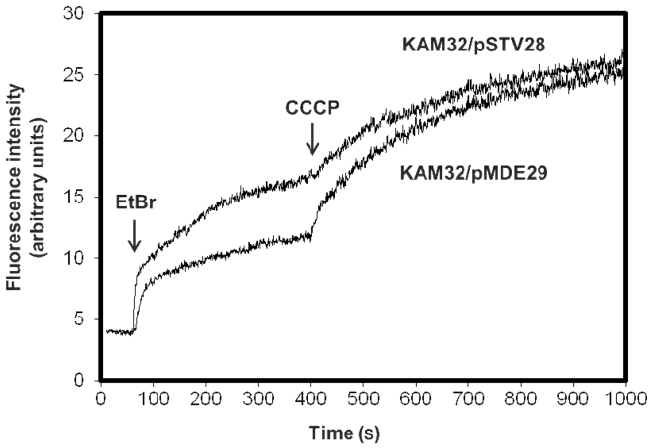

Fig. 3. Drug accumulation assays of ethidium bromide showing the less accumulation of drug in the MdeA-expressing cells.
Ethidium bromide (10 μM) was added to initiate the drug accumulation and then the proton-conductor CCCP (40 μM) was added at the time point indicated by a downward arrow to de-energize the membrane. The increase of fluorescence indicates accumulation of ethidium into the cells.

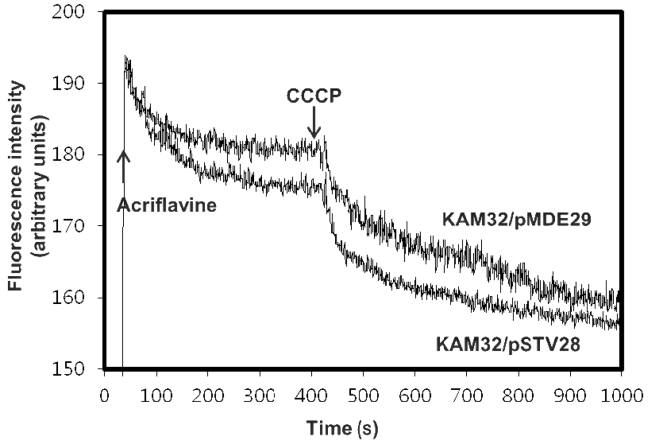

Fig. 4. Drug accumulation assays of acriflavine showing less accumulation of drug in the MdeA-expressing cells.
Acriflavine (4.2 μM) was added to initiate the drug accumulation and then the proton-conductor CCCP (40 μM) was added at the time point indicated by a downward arrow to de-energize the membrane. The decrease of fluorescence indicates accumulation of acriflavine into the cells.

the two strains, indicating that the accumulation levels of ethidium in both strains are similar under de-energized conditions by CCCP addition. An important point is that the ethidium accumulation level in cells of KAM32/pMDE29 was much lower than in cells of KAM32/pSTV28 before CCCP was added (Fig. 3). This indicates that cells of KAM32/pMDE29, but not of KAM32/pSTV28, possess energy-dependent ethidium efflux activity.

In the case of acriflavine assay, although the assay results represented the opposite phenomenon due to the chemical property of this drug described above, the MdeA-mediated acriflavine efflux was revealed (Fig. 4). Therefore, we conclude that MdeA is an energy-dependent drug efflux pump.

DISCUSSION

The genome sequences of the entire chromosome and plasmid DNA of *S. mutans* UA159 and NN2025 have been completed and are available on the web [1, 10]. They predicted the presence of a putative multidrug efflux pump gene (GenBank Accession No. NP_722397 and YP_003485765, respectively) in the chromosomal DNA of each *S. mutans*, through *in silico* analyses based on the other published bacterial efflux pumps. These genes have not been cloned, expressed, characterized, and functionally analyzed yet. In this study, the *mdeA* gene from *S. mutans* ATCC 25175 was first cloned and its functional properties were verified experimentally, not just by prediction. The experimental method of a particular gene cloning or gene deletion mutation followed by expression and characterization is a good way to verify whether the gene is actually functional or not, and the importance of the gene in the aspect of drug resistance of a bacterial strain.

A number of multidrug efflux pumps have been reported in various bacteria. These pumps are classified into five families based on structure and coupling energy: resistance nodulation cell division (RND), small multidrug resistance (SMR), multidrug and toxic compounds extrusion (MATE), major facilitator superfamily (MFS), and ATP binding cassette (ABC) [20]. Most bacterial MDRs belong to the MFS [3]. The MDR proteins of this family utilize the proton motive force (PMF) of the transmembrane electrochemical proton gradient for exporting drugs from the cells [15]. The drug efflux activity of MdeA using fluorescent substrates was clearly inhibited by the addition of CCCP, a proton conductor, which disrupts the proton gradient across the cytoplasmic membrane of bacteria. These results indicated that MdeA used the PMF to efflux drugs outside of the cell membrane. We clearly observed an energy-dependent efflux of drugs medicated by MdeA, which is the first experimental verification in this study.

Fluorescent antimicrobial agents, ethidium bromide and acriflavine, are often used as a substrate during the fluorometric assay of multidrug efflux pump activities. In the case of MdeA, it seemed that fluorescent acriflavine was a much better substrate, based on the MIC results (Table 1). However, the results of accumulation assay presented bigger difference by ethidium bromide than by acriflavine between MdeA expressed cell and vector control cell (Figs. 3 and 4). This difference might come from the fluorescence intensity and diffraction scattering in the buffer.

The analysis of the amino acid sequence of MdeA on the web site of SOSUI (http://sosui.proteome.bio.tuat.ac.jp/

sosuimenu0.html) predicted that MdeA consisted of 12 transmembrane segments (TMS) like the other MFS homologs (Fig. 2B). Moreover, four conserved sequences of the 12 TMS of the MFS, motifs A, B, C, and D2 [17], were found in the MdeA sequence at the corresponding positions through the analysis using MOTIF (http://motif.genome.ac.jp). Thereafter, the amino acid sequences of MdeA were aligned with its homologs classified into the MFS using the CLUSTAL W program on the web site of EMBnet (http://www.ch.embnet.org/software/ClustalW.html) and using the software Genetyx-Win (ver. 5.1). MdeA of *S. mutans*, a putative multidrug efflux protein of *L. oris, B. cereus, S. capitis*, and *E. gallinarum* aligned with each other.

Future studies are needed to evaluate the molecular and biochemical studies by the overexpression, drug binding site analysis, deletion mutation, and point mutation of MdeA. In addition, screening for efflux pump inhibitors from the natural compounds and/or chemical library will be an alternative solution to overcome the multidrug-resistant pathogenic bacteria.

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