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ORIGINAL PAPER

Identification, cloning, and functional characterization of EmrD-3, a putative multidrug efflux pump of the major facilitator superfamily from *Vibrio cholerae* O395

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Abstract A putative multidrug efflux pump, EmrD-3, belonging to the major facilitator superfamily (MFS) of transporters and sharing homology with the Bcr/CflA subfamily, was identified in *Vibrio cholerae* O395. We cloned the *emrD-3* gene and evaluated its role in antimicrobial efflux in a hypersensitive *Escherichia coli* strain. The efflux activity of this membrane protein resulted in lowering the intracellular concentration of ethidium. The recombinant plasmid carrying *emrD-3* conferred enhanced resistance to several antimicrobials. Among the antimicrobials tested, the highest relative increase in minimum inhibitory concentration (MIC) of 102-fold was observed for linezolid (MIC = 256 μg/ml), followed by an 80.1-fold increase for tetraphenylphosphonium chloride (TPCL) (156.2 μg/ml), 62.5-fold for rifampin (MIC = 50 μg/ml), >30-fold for erythromycin (MIC = 50 μg/ml) and minocycline (MIC = 2 μg/ml), 20-fold for trimethoprim (MIC = 0.12 μg/ml), and 18.7-fold for chloramphenicol (MIC = 18.7 μg/ml). Among the fluorescent DNA-binding dyes, the highest relative increase in MIC of 41.7-fold was observed for ethidium bromide (125 μg/ml) followed by a 17.2-fold increase for rhodamine 6G (100 μg/ml). Thus, we demonstrate that EmrD-3 is a multidrug efflux pump of *V. cholerae*, the homologues of which are present in several *Vibrio* spp., some members of Enterobacteriaceae family, and Gram-positive *Bacillus* spp.

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Introduction

The Gram-negative pathogenic bacterium *Vibrio cholerae*, the causative agent of cholera, has been responsible for eight pandemics and remains a serious public health concern in developing countries (Faruque et al. 1998). As a clinically relevant pathogen, mechanisms of antimicrobial resistance in this bacterium are of interest to researchers and medical professionals alike. Moreover, the current pandemic has witnessed the emergence of *V. cholerae* O1 resistant to antibiotics used in the empiric treatment of cholera (Dalsgaard et al. 2000; Mwansa et al. 2007).

Several mechanisms of bacterial drug resistance have been elucidated, including altered drug targets, antibiotic inactivating enzymes, decreased membrane permeability, and the active efflux of antimicrobials (Hayes and Wolf 1990; Putman et al. 2000). While altered drug targets and antibiotic inactivating enzymes may confer high level but narrow-spectrum drug resistance, efflux systems are capable of providing resistance to a broad spectrum of antibiotics and antimicrobial compounds (Higgins 2007). The whole genome sequencing of several *V. cholerae* strains, including O395, has facilitated identification of putative genes responsible for virulence and antimicrobial resistance. The objective of our study is to better understand the physiology and substrate profile of multidrug efflux proteins through the characterization of one such membrane protein, EmrD-3, of the major facilitator superfamily (MFS). MFS transporters are present in all organisms and comprise the largest family of transporters yet discovered (Maiden et al. 1987; Pao et al. 1998). Energy for transport

is provided by a cation gradient, most commonly, H^+ or Na^+ (Law et al. 2008). Transporters in this family generally have twelve transmembrane helices (Hirai et al. 2003). The MFS transporter proteins are further classified into three major types based on the mechanism of transport: uniporters, symporters, and antiporters (Pao et al. 1998). Uniporters are capable of transporting only one substrate and utilize only the energy generated by the concentration gradient of the substrate itself. Symporters transport two different substrates in the same direction (either into or out of the cell), utilizing the chemical gradient of one of these substrates, usually an ion, for energy. Antiporters transport two substrates in opposite directions; one substrate may enter the cell as the other leaves (Law et al. 2008). EmrD-3 shares homology with the Bcr/CflA subfamily, a group of antiporters shown to confer resistance to chloramphenicol, florfenicol, and bicyclomycin by actively transporting these compounds out of the cell.

The hypothesis of this study is that EmrD-3 is a multidrug efflux pump of the MFS, predicting that EmrD-3 confers reduced antimicrobial susceptibility when introduced into an antimicrobial hypersensitive strain of *Escherichia coli*. The objective of this study is to elucidate the antimicrobial efflux potential of EmrD-3 using a functional cloning strategy. The results of this study can also be extrapolated to other vibrios with homologous transporters.

Materials and methods

Cloning of *emrD-3*

Bacterial strains and plasmids used in this study are listed in Table 1. The background strain *E. coli* KAM32 lacks the efflux pumps AcrAB and YdhE (Otsuka et al. 2005). Unless noted otherwise, all plasmid containing cells were grown in Luria–Bertani (LB) broth supplemented with 100 μg/ml ampicillin.

Genomic DNA was extracted from *V. cholerae* O395 using the CTAB (cetyl trimethyl ammonium bromide) method (Ausubel et al. 1995). Primers F3Bam (gcgggatccat gaagacgaagccttctctctgg) and R3Xh (gcgctcgagttatggtaga cgggctatgtgac) were designed to contain *Bam*HI and *Xho*I restriction sites (underlined) and used to amplify the 1,140 bp *emrD-3* gene. The PCR product was purified, restriction digested with *Bam*HI and *Xho*I and ligated into similarly digested pSP72 vector (Promega, USA). The ligation mixture was introduced into *E. coli* KAM32 by electroporation to obtain KAM32/pSP72/*emrD-3*. The presence of ligated insert was confirmed by PCR.

Table 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Properties	Reference
E. coli KAM32	Δ*acrAB*, Δ*ydhE*, *hsd*$^-$	Otsuka et al. (2005)
Vibrio cholerae O395	Classical O1 biotype	Rubin et al. (1998)
pSP72	Amp^r	Krieg and Melton (1987)
pSP72/*emrD-3*	Amp^r, contains *emrD-3*	This study

Study of antimicrobial resistance profile

The minimal inhibitory concentrations (MICs) of various antimicrobial compounds were determined for KAM32/pSP72/*emrD-3* and control *E. coli* containing vector alone using CLSI guidelines (CLSI 2006). Initial screening for differences in resistance was performed using *E*-test strips (bioMereieux, Durham, NC, USA) according to the manufacturer's instructions. Final MIC data were determined using the microbroth dilution technique according to CLSI guidelines (CLSI 2006). Each microbroth dilution experiment was repeated four times ($n = 4$). Relative fold increases were calculated by dividing the mean MIC of KAM32/pSP72/*emrD-3* by the mean MIC of vector-alone control cells KAM32/pSP72.

Ethidium accumulation assay

The ethidium accumulation assay was performed as previously described (Minato et al. 2008). To prepare cells for the ethidium accumulation assay, KAM32 cells harboring pSP72/*emrD-3* or pSP72 alone were grown to mid-exponential phase in LB broth supplemented with 100 μg/ml ampicillin and 20 mM potassium lactate at 37°C. Cells were harvested, washed twice with M9 minimal salt solution (pH 7.1), and resuspended in the same medium supplemented with 20 mM potassium lactate to an OD_{625} of about 0.2. This cell suspension was then preincubated for 5 min at 37°C. The natural fluorescence of the cells was measured, and the assay was initiated by the addition of 2.5 μM ethidium bromide. Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was then added at 100 μM to collapse the H^+ gradient across the membrane and inactivate EmrD-3. Fluorescence was measured using an F-2500 fluorescence spectrophotometer (Hitachi High-Technologies, Tokyo, Japan) with an excitation wavelength of 500 nm and an emission wavelength of 580 nm. To test the hypothesis that EmrD-3 is not a Na^+ pump, a separate accumulation assay was performed in sodium-free medium with sodium concentrations ranging from 0 to 154 mM (physiological concentration).

Ethidium efflux assay

The ethidium efflux assay was performed as previously described with minor modifications (Hirata et al. 2004).

Cells were grown to an OD_{625} of approximately 1 in LB broth supplemented with 20 mM potassium lactate and 100 μg/ml of ampicillin. Subsequently, 2 ml of cells was harvested by centrifugation for 1 min at 13,000×*g* and resuspended in 1 ml M9 minimal salt medium containing 5 μM ethidium bromide and 100 μM CCCP. This cell suspension was incubated for 5 min. to load the cells with ethidium and deplete the membrane potential. After incubation, the cells were again harvested by centrifugation for 1 min at 13,000×*g* and resuspended in M9 minimal medium (pH 7.1) containing 5 μM ethidium bromide. The fluorescence of the ethidium-loaded cells was measured; then the proton motive force was reestablished by the addition of potassium lactate (20 mM) to energize the cells and initiate the accumulation assay. The proton gradient was then disrupted by the addition of 100 μM CCCP. Fluorescence was measured using a FL-2500 fluorescence spectrophotometer (Hitachi High-Technologies, Tokyo, Japan) with excitation and emission wavelengths of 500 and 580 nm, respectively.

Bioinformatic analysis

The *emrD-3* gene and associated promoter sequences were identified in the NCBI database by searching for sequences with homology to known MFS transporters. The deduced amino acid sequence of EmrD-3 was compared to all other known proteins in the NCBI database by BLASTP analysis (Altschul et al. 1997). The two-dimensional structure was determined by using the TMHMM sever (Transmembrane helix prediction based on hidden Markov models), the results of which were analyzed using the TMRpres2d (Transmembrane Re-presentation in 2-dimensions). Multiple sequence alignments were conducted using the CLUSTAL W program (Higgins et al. 1994). The phylogenetic tree was constructed using twenty-three proteins closely related to EmrD-3 using the neighbor-joining method in CLUSTALX2, with 10,000 iterations of bootstrapping, and with LacY as an out-group (Varela and Wilson 1996). The tree was then visualized using TreeViewX.

Results

Identification and analysis of EmrD-3

An 1,140 bp *emrD-3* gene was identified in the genome of *V. cholerae* O395 corresponding to the coordinates 283,612 to 284,751 on the second chromosome (GenBank accession no. CP001236). The *emrD-3* determinant encodes a protein product of 379 amino acid residues with a calculated molecular mass of 40.5 kDa and an isoelectric point (pI) of 9.83. Secondary structure analysis revealed 12 transmembrane helices supporting our hypothesis that EmrD-3 is an intrinsic membrane protein (Fig. 1). BLAST and multiple amino acid sequence alignment analyses revealed that protein homologues of EmrD-3 are widely distributed among the Gram-positive and -negative bacteria. EmrD-3 is 80% similar and 65% identical with a multidrug protein of *V. harveyi*, *V. alginolyticus*, *V. parahemolyticus*, *V. fischeri*, and *V. vulnificus* (Fig. 2). The whole genome sequences of other Gram-negative bacteria such as *Proteus penneri*, *Aeromonas hydrophila*, *Citrobacter youngae*, *Serratia proteamaculans*, and *Pseudomonas fluorescens* also have protein sequences bearing 65% similarity and 50% identity with EmrD-3. Among the Gram-positive bacteria, sequences homologous to EmrD-3 are found in *Bacillus cereus*, *Lysinibacillus sphaericus*, *B. anthracis*, *B. weihenstephanensis*, and *B. thuringiensis* with 38% similarity and 61% identity.

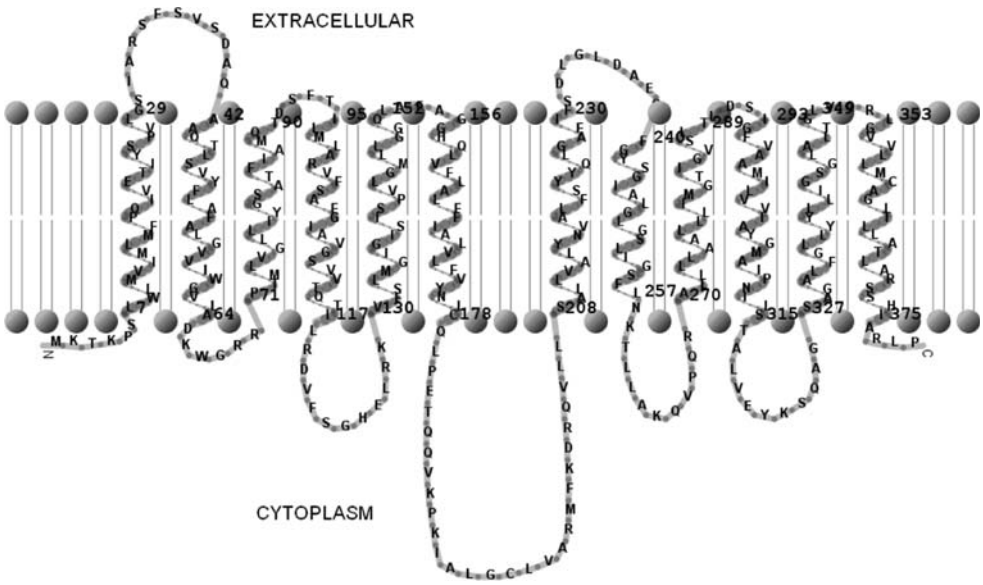

Fig. 1 Predicted two-dimensional structure of EmrD-3

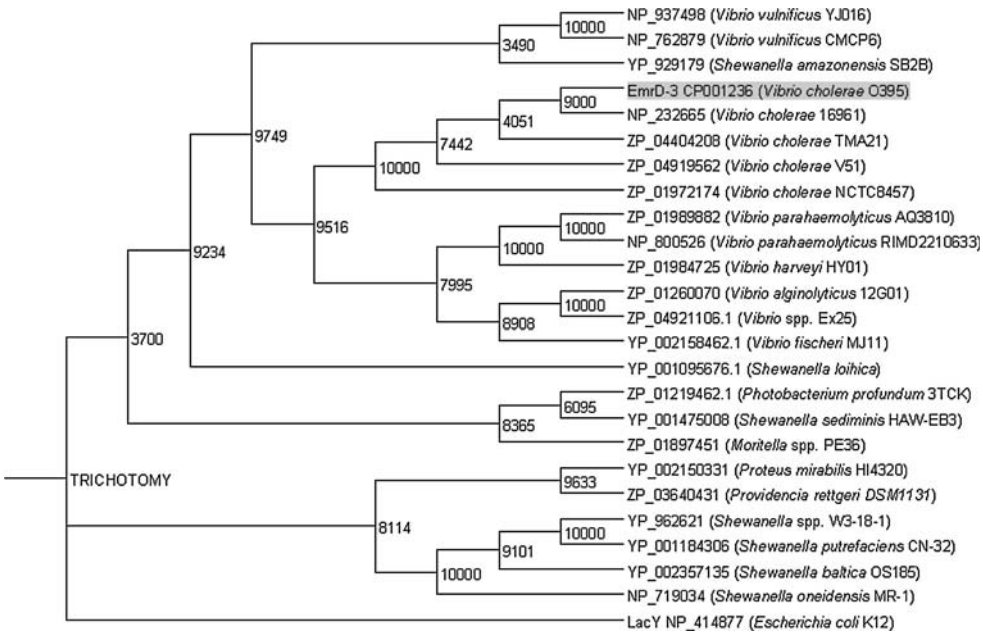

Fig. 2 Phylogenetic tree showing proteins highly related to EmrD-3 (*shown highlighted*)

A multiple sequence alignment of EmrD-3 with related membrane proteins from *V. harveyi*, *P. fluorescens*, and *B. cereus* shows relatively high N-terminal sequence similarity (Fig. 3).

Ethidium accumulation assay

The ethidium accumulation assay was performed to test the hypothesis that EmrD-3 is a true efflux pump. Since ethidium fluoresces when bound to DNA, the accumulation of ethidium in cells can easily be measured. Fig. 4 shows the difference in ethidium accumulation between *E. coli* KAM32/pSP72/*emrD-3* and *E. coli* KAM32/pSP72 alone.

Upon the addition of ethidium at the time point indicated in Fig. 4, compared to control, KAM32/pSP72/*emrD-3* showed less of an increase in fluorescence as the EmrD-3 protein actively extrudes ethidium, preventing it from binding DNA. When a protonophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was added, a dramatic increase in the fluorescence was observed due to the disruption of the H^+ gradient across the membrane resulting in the entry of ethidium bromide back into the cell and binding to the DNA. *E. coli* KAM32/pSP72 showed a large increase in intracellular ethidium immediately after its addition. The accumulation of ethidium is not significantly changed by the addition of CCCP, indicating that negligible H^+-dependent efflux of ethidium occurs in KAM32/pSP72. Similar accumulation activity was observed for both control and experimental cells in sodium-free medium. The fluorescence intensity did not change in response to increasing sodium concentrations (Data not shown).

Ethidium efflux assay

The ethidium efflux assay provided evidence for the efflux of ethidium from cells mediated by EmrD-3 (Fig. 5). The initial fluorescence values for de-energized cells of KAM32/pSP72 and KAM32/pSP72/*emrD-3* were very similar. Upon energization by the addition of potassium lactate, the experimental cells showed nearly a 50% reduction in fluorescence whereas control cells showed only about a 15% reduction. The addition of the uncoupler CCCP resulted in near total recovery of initial fluorescence in both the experimental and control cells (Fig. 5).

Effect of EmrD-3 on antibiotic tolerance

The MICs of 29 antimicrobial compounds were determined for KAM32/pSP72/*emrD-3*. Expression of EmrD-3 in *E. coli* KAM32 conferred enhanced resistances to fourteen antimicrobials (Table 2). Among the antimicrobials tested, the highest relative increase of 102-fold was observed for linezolid (MIC = 256 μg/ml), followed by an 80.1-fold increase for tetraphenylphosphonium chloride (TPCL) (156.2 μg/ml), 62.5-fold for rifampin (MIC = 50 μg/ml), 33.3-fold for minocycline (MIC = 2 μg/ml), 31.3-fold for erythromycin (MIC = 50 μg/ml), 20-fold for trimethoprim (MIC = 0.12 μg/ml), and 18.7-fold for chloramphenicol (MIC = 18.7 μg/ml). A 7.8-fold increase was found for oxytetracycline (MIC = 6.2 μg/ml) and tetracycline (MIC = 6.2 μg/ml), and a 5-fold increase for nalidixic acid (MIC = 2 μg/ml); while a 4.9-fold increase in the MIC was observed for florfenicol (MIC = 4.6 μg/ml), compared to the control cells. Among the fluorescent DNA-binding

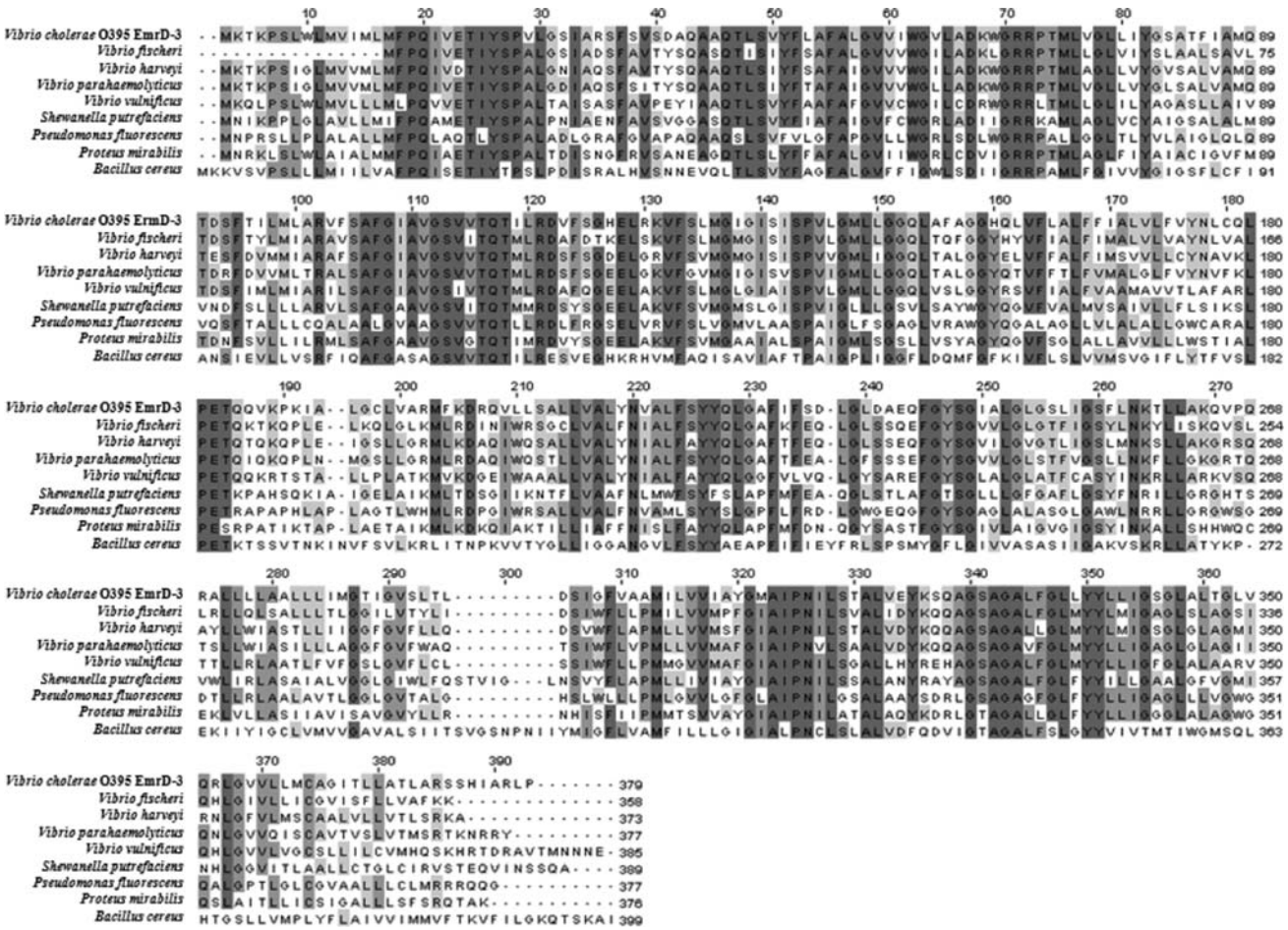

Fig. 3 Multiple sequence alignment comparing efflux proteins to EmrD-3 of *V. cholerae* O395. The putative proteins of *Pseudomonas fluorescens* (YP_261166), *Bacillus cereus* (YP_002452428), *V. vulnificus* (NP_762879), *V. parahaemolyticus* (NP_800526), *Proteus mirabilis* (YP_002150331), *V. fischeri* (YP_002158462), *Shewanella putrefaciens* (YP_001184306), and *V. harveyi* (ZP_01984725) were compared to EmrD-3 of *V. cholerae* O395 (ACP11144)

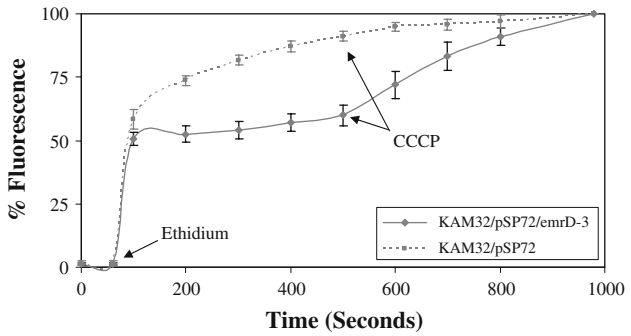

Fig. 4 Accumulation of ethidium bromide in *E. coli* cells containing cloned *emrD-3*/pSP72 and plasmid vector alone averaged over three trials. The *arrows* indicate the points at which 2.5 μM ethidium bromide or 100 μM CCCP was added to the cell suspension. *Error bars* indicate one standard deviation from the mean

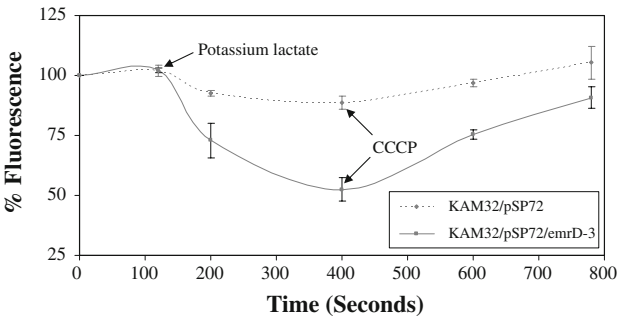

Fig. 5 Efflux of ethidium from preloaded cells. Percent fluorescence was averaged over three separate trials. *Arrows* indicate the points at which 20 mM potassium lactate or 100 μM CCCP was added to the cell suspension preloaded with 5 μM ethidium bromide. *Error bars* indicate one standard deviation from the mean

dyes, the highest relative increase in MIC of 41.7-fold was observed for ethidium bromide (125 μg/ml) followed by 17.2-fold increase for rhodamine 6G (100 μg/ml). EmrD-3 did not confer increased resistance to fifteen other antimicrobials including acriflavine, amikacin, cefotaxime, ciprofloxacin, fosfomycin, gatifloxacin, gentamicin, imipenem, levofloxacin, mercury, norfloxacin, ofloxacin, rose bengal, thioridazine, and tigecycline.

Table 2 MICs of various antimicrobial compounds to *E. coli* KAM32 harboring cloned *emrD-3* and the vector-alone control *E. coli* KAM32 determined by the microbroth dilution technique

Substrate	MIC values (μg/ml)		Relative increase
	KAM32/pSP72	KAM32/pSP72/*emrD-3*	
Linezolid	2.5	256	102.4
TPCL	2.0	156.2	80.1
Rifampin	0.8	50	62.5
Ethidium bromide	3.0	125	41.7
Minocycline	0.06	2.0	33.3
Erythromycin	1.6	50	31.3
Trimethoprim	0.006	0.12	20.0
Chloramphenicol	1.0	18.7	18.7
Rhodamine 6G	5.8	100	17.2
Oxytetracycline	0.8	6.2	7.8
Tetracycline	0.8	6.2	7.8
Nalidixic acid	0.4	2.0	5.0
Florfenicol	0.93	4.6	4.9
SDS	7.3	19.5	1.9

Discussion

Several multidrug efflux pumps belonging to the RND (resistance-nodulation-cell division) and the MATE (multidrug and toxic compound extrusion) families of membrane proteins have been described in *V. cholerae* O1 and non-O1 (Begum et al. 2005; Woolley et al. 2005; Rahman et al. 2007; Bina et al. 2008). Here, we identified and characterized the multidrug efflux protein EmrD-3 of the MFS from the *V. cholerae* O395 whole genome sequence using a functional cloning strategy. BLAST analysis revealed that proteins similar to EmrD-3 are widely present in the whole genomes of all *Vibrio* species, some members of the Enterobacteriaceae and *Bacillus* spp. Fig. 2 shows the comparison of EmrD-3 with 23 other closely related putative membrane proteins encoded in the whole genome sequences of other Gram-negative bacteria, the majority of which are of marine origin. Significantly, EmrD-3 homologues are not found in *E. coli*, *Salmonella*, *Campylobacter*, *Mycobacterium,* and *Staphylococcus aureus*. Comparison of EmrD-3 with sequence homologues from *V. fischeri*, *P. fluorescens,* and *B. cereus* shows sequence conservations across these diverse species (Fig. 3). The 40.5 kDa EmrD-3 with 12 transmembrane domains bears homology with the Bcr/CflA subfamily of membrane proteins (Figs. 1 and 2). Members of this family with known activity include Bcr (bicyclomycin resistance protein) in *E. coli*, Flor (chloramphenicol and florfenicol resistance) in *Salmonella typhimurium* DT104 and CmlA (chloramphenicol resistance) in *Pseudomonas*. A highly conserved amino acid sequence motif G-[RKPATY]-L-[GAS]-[DN]-[RK]-[FY]-G-R-[RK]-[RKP]-[LIVGST]-[LIM] is present between TMS-2 and TMS-3 in all 17 families of the MFS proteins (Griffith et al. 1992; Pao et al. 1998). In EmrD-3, this conserved motif GVLADKWGRRPTM corresponds well with the motif except for W at position 7 otherwise represented by F/Y. EmrD-3 harbors elements of the antiporter motif (motif C), $G(X_6)G(X_3)GP(X_2)GP(X_2)G$, shown to be important for drug/H^+ antiport activities (Ginn et al. 2000; Jin et al. 2002; Pasrija et al. 2007; Varela et al. 1995). Consistent with the predicted topology, EmrD-3 actively extrudes DNA-binding fluorescent compounds such as ethidium bromide and TPCL. Addition of ethidium bromide resulted in high accumulation of this dye in control cells compared to KAM32/pSP72/*emrD-3* demonstrating efflux activity of EmrD-3 (Fig. 4). Further, the addition of a membrane de-energizer CCCP at the time point indicated in Fig. 4 resulted in disruption of ethidium bromide efflux suggesting that EmrD-3-mediated efflux is potentiated by a proton gradient across the membrane, characteristic of proton-dependent bacterial transporters (Padan and Schuldiner 1994; Putman et al. 2000).

Though structurally diverse molecules are substrates for MFS multidrug efflux pumps, substrate profile elucidation helps in transporter classification (Lewis 1994; Grkovic et al. 2002; Van Veen and Konings 1998). We tested a broad range of antimicrobial compounds to determine whether reduced susceptibilities were conferred by EmrD-3 as would be evidenced by increased MICs of those compounds. Though the antibiotics used here do not represent all of the antibiotics or their classes, we used many antibiotics relevant in cholera treatment, such as ciprofloxacin, erythromycin, tetracycline, and trimethoprim. Our results suggest that EmrD-3 actively extrudes diverse antimicrobials from *E. coli* KAM32 (Table 2). Among the DNA-binding fluorescent dyes tested, the MIC of KAM32/pSP72/*emrD-3* to ethidium bromide was 41.7-fold higher than the control strain KAM32/pSP72 containing cloning vector alone, suggesting that EmrD-3 pumps ethidium bromide efficiently. The ethidium accumulation assay further demonstrated that the accumulation of this dye in control cells took place much more rapidly than in cells expressing EmrD-3 (Fig. 4). The similar fluorescence values observed during a sodium-free accumulation assay suggest that EmrD-3 is not a sodium-dependent efflux pump (Data not shown). The hypothesis that EmrD-3 is an efflux pump is further supported by our observation that ethidium efflux in cells harboring EmrD-3 occurs much more effectively than in control cells (Fig. 5). Though we tested a limited number of antibiotics as substrates for EmrD-3, extrusion of these agents was evident from increased MICs of cells containing EmrD-3. Of these antibiotics tested, linezolid was implicated

to be pumped very efficiently from the cells as suggested by a sharp increase in the MIC to 256 μg/ml. This corresponded to a 102-fold increase in the MIC compared to *E. coli* KAM32 with vector alone. Other antibiotics actively extruded, as implicated by significant increases in MICs, were rifampin, erythromycin, and chloramphenicol. Efflux-mediated resistance to chloramphenicol was described in *E. coli* (McMurry et al. 1994; Edgar and Bibi 1997; Mine et al. 1998; Moreira et al. 2004). The multidrug efflux pump AcrAB confers chloramphenicol resistance in *E. coli* and *Enterobacter cloacae* (Okuso et al. 1996; Moreira et al. 2004). Active efflux is an important mechanism of macrolide resistance (Zhong and Shortridge 2000). At least 14 such genes of the MFS family or ATP transporters have macrolide, lincosamides, streptogramins, ketolides, and oxazolidinones efflux activities in various Gram-positive and -negative bacteria (Roberts 2008). In *Pseudomonas*, MexXY confers elevated resistance to erythromycin, fluoroquinolones, tetracycline, chloramphenicol, and kanamycin (Mine et al. 1999). However, comparison of the amino acid sequence of EmrD-3 with previously reported chloramphenicol, erythromycin, and rifampin efflux proteins did not show any similarity. Thus, EmrD-3 identified here is distinct from known proteins responsible for efflux of erythromycin, chloramphenicol, and rifampin.

Though several antibiotics are efflux substrates for EmrD-3, the antibiotic linezolid is very effectively pumped by EmrD-3. Linezolid belongs to the oxazolidinone class of drugs used to treat Gram-positive bacterial infections by *Streptococcus* spp., vancomycin-resistant *Enterococcus faecium*, and methicillin-resistant *Staphylococcus aureus* (MRSA) (Zurenko et al. 1996). The antibacterial action of linezolid is due its interaction with the 50S ribosomal subunit resulting in the inhibition of protein synthesis by preventing the formation of the initiation complex (Swaney et al. 1998). Resistance to linezolid was first reported in *Enterococcus* followed by MRSA, *E. coli*, and many other bacteria (Gonzales et al. 2001; Tsiodras et al. 2001; Mutnick et al. 2003). However, the resistance mechanism is via modification of the target site which involves a G to A substitution at position 2,032 in the peptidyl transferase center of 23S rRNA and resulting in reduced affinity of linezolid to the 50S subunit (Xiong et al. 2000). This and other sites of mutations (e.g. T2500A in *S. aureus*), confirm the mechanism of action of oxazolidinones (Meka et al. 2004). In addition, one report described a non-ribosomal mechanism of resistance in *Mycobacterium smegmatis* (Sander et al. 2002). Ribosomes isolated from these strains behaved essentially like wild-type ribosomes in the presence of drug. It is speculated that the resistance may arise from decreased uptake or increased efflux of the drug (Slatter et al. 2001). The genome of a linezolid-resistant *Streptococcus* strain sequenced recently revealed novel efflux mechanisms responsible for the resistance phenotype (Feng et al. 2009). Inactivation of AcrAB, an RND-type efflux pump, has been shown to make it more susceptible to linezolid, suggesting the role of efflux pumps in linezolid resistance of Gram-negative bacteria (Buysse et al. 1996; Bohnert and Kern 2005). The study of *emrD-3* expression in clinical isolates of multidrug resistant *V. cholerae* could provide clues to the ecological distribution of this determinant as well as to its role in antimicrobial resistance or virulence. Furthermore, our results strongly suggest that EmrD-3-mediated efflux has physiological relevance, and our work will help to identify and characterize homologous efflux proteins in other Gram-negative and -positive bacteria.

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