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VmrA, a Member of a Novel Class of Na\(^+\)-Coupled Multidrug Efflux Pumps from *Vibrio parahaemolyticus*

Jing Chen, Yuji Morita, M. Nazmul Huda, Teruo Kuroda, Tohru Mizushima, and Tomofusa Tsuchiya*

Department of Microbiology, Faculty of Pharmaceutical Sciences, Okayama University, Tsushima, Okayama 700-8530, Japan

Received 26 July 2001/Accepted 18 October 2001

Gene *vmrA*, cloned from *Vibrio parahaemolyticus*, made *Escherichia coli* resistant to 4',6-diamino-2-phenylindol, tetraphenylphosphonium chloride, acriflavine, and ethidium bromide. VmrA belongs to the DinF branch of MATE family efflux transporters. VmrA catalyzed acriflavine efflux and showed Na\(^+\)/drug transporter activity because the addition of tetraphenylphosphonium to Na\(^+\)-loaded cells caused Na\(^+\) efflux.

Drug resistance in bacterial cells is currently a serious clinical problem. In particular, it is extremely difficult to treat patients infected with multidrug-resistant bacteria. There are several mechanisms of drug resistance in bacterial cells, including degradation or modification of the drug, alteration of the target, or efflux of the drug from the cells. However, the presence of multidrug efflux pumps in bacterial cells is a major cause of multidrug resistance. Large numbers of multidrug efflux pumps have been reported in numerous bacteria (19). Once a bacterium acquires certain multidrug efflux pump(s), or if a silent or weak multidrug efflux pump is activated, then the cell instantly becomes resistant to many antimicrobial agents. Thus, it is important to investigate multidrug efflux pumps in bacteria to gain insight into multidrug resistance in bacteria. Gene cloning, expression, and biochemical characterization are useful approaches to the understanding of multidrug efflux pumps.

*V. parahaemolyticus* is a slightly halophilic marine bacterium and is one of the major causes of food poisoning in Japan and many other countries (14). This microorganism requires Na\(^+\) for its growth (2). Energy metabolism and energy coupling in membranes of this microorganism are unique (21, 26). Cells of *V. parahaemolyticus* utilize an electrochemical potential of Na\(^+\) across the membrane as one of the major driving forces for energy-dependent membrane processes (1, 26). Interestingly, cells of *V. parahaemolyticus* show some natural resistance to some (or many) antimicrobial agents (unpublished observation). Thus, we were interested in multidrug efflux pumps of *V. parahaemolyticus*.

Previously, we reported the gene cloning and characterization of NorM, a member of new class of a multidrug efflux pump, from *V. parahaemolyticus* (16), and we reported that NorM is a Na\(^+\)-driven Na\(^+\)/drug antiporter (15). Here we report the gene cloning and characterization of a new multidrug efflux pump, VmrA, from *V. parahaemolyticus* and that VmrA is a member of a novel class of Na\(^+\)/drug antiporters.

**Host strain *E. coli* KAM32 and gene cloning.** It has been reported or suggested that *E. coli* cells possess many multidrug efflux pumps and putative multidrug efflux pumps (19). The major multidrug efflux pump in *E. coli* is the AcrAB system (10). We previously constructed a mutant strain of *E. coli*, KAM3, which lacks AcrAB and a restriction system (Δhsd [16]). The KAM3 strain was shown to be very useful for the cloning of multidrug efflux pumps from other bacteria (12, 13, 16). We also reported that YdhE from *E. coli* was a multidrug efflux pump and a homologue of NorM from *V. parahaemolyticus* (16). We later found that NorM was a Na\(^+\)-driven Na\(^+\)/drug antiporter (15). Also, we found that YdhE was a Na\(^+\)/drug antiporter (unpublished results). Most multidrug efflux pumps in *E. coli* are H\(^+\)-driven H\(^+\)/drug antiporters. Thus, for the analysis within *E. coli* cells of Na\(^+\)-dependent multidrug efflux pumps derived from other bacteria, it is desirable to use an *E. coli* mutant lacking YdhE, in addition to the AcrAB, as a host cell. Thus, we tried to construct a mutant lacking YdhE from KAM3. Previously, we cloned the ydhE gene into pBR322 and obtained pMEC2 (16). An Ncol fragment was removed from the ydhE gene in pMEC2 by NcoI digestion and self-ligation, and pMDEC2 was obtained. The disrupted ydhE region was transferred to a delivery vector pKO3 (9) and pKOEY2 was obtained. The pKOEY2 plasmid was introduced into KAM3 cells possessing the chromosomal ydhE gene. Chromosomal ydhE gene was replaced with the disrupted ydhE gene by homologous recombination (9), and strain KAM3 was obtained. Disruption of the ydhE gene in the chromosome of KAM3 was confirmed by the Southern blot hybridization method (24) (data not shown). We observed a slight reduction in the MICs of norfloxacin, kanamycin, and streptomycin in KAM32 cells compared with KAM3 cells (data not shown). These results were consistent with our previous results that introduction of the ydhE gene into KAM3 cells slightly increased the MICs of norfloxacin, kanamycin, and streptomycin (16). This indicated that KAM32 was a useful...
host for the cloning of multidrug efflux pumps from other organisms.

By using strain KAM32 as a host, plasmid pBR322 as a vector, and V. parahaemolyticus AQ3334 (26) as a source of chromosomal DNA, a gene responsible for ethidium bromide resistance was cloned from V. parahaemolyticus as follows. Cells of V. parahaemolyticus were grown in Luria-Bertani medium (11). Chromosomal DNA was prepared from cells of V. parahaemolyticus by the method of Berns and Thomas (3). The DNA was partially digested with Sau3A1, and fragments of 4 to 10 kbp were separated by sucrose density gradient centrifugation. The DNA fragments were ligated into pBR322 (which had been digested with BamHI and dephosphorylated with bacterial alkaline phosphatase) by using T4 DNA ligase. Competent cells (6) of E. coli KAM3 were transformed with the ligated hybrid plasmids and were spread onto agar plates containing L broth (8), 10 g/ml of ethidium bromide/ml, 60 g of ampicillin/ml, and 1.5% agar. The plates were incubated at 37°C for 24 h, and the clones formed were picked up. Plasmids contained in the transformants were isolated, reintroduced into KAM3 cells, and spread onto the same plates again. The plates were incubated at 37°C for 24 h. Plasmids contained in the retransformants were prepared. Restriction patterns of selected 24 plasmids were compared, and they were classified into four groups. KAM32 cells harboring one of the groups formed the largest colonies. We further analyzed this group of plasmids (10 plasmids). It seemed that the resistance system encoded by the gene carried by this group of plasmids was the major system for ethidium resistance, as judged based on the size of colonies formed on plates containing ethidium bromide. We picked a plasmid carrying the shortest DNA insert, pVCJ6.

### Drug specificity

We tested drug specificity with KAM32 cells harboring plasmid pVCJ6. Table 1 shows that KAM32/pVCJ6 is more resistant not only against ethidium bromide but also against 4,6-diamino-2-phenylindole (DAPI), tetraphenylphosphonium chloride (TPPCl), and acriflavine compared with the KAM32 control. The structures of these compounds are different. Thus, it seems that plasmid pVCJ6 carries a gene(s) responsible for multidrug resistance. On the other hand, cells of KAM32 and KAM32/pVCJ6 showed indistinguishable susceptibilities to other antimicrobial agents tested, such as norfloxacin, tetracycline, erythromycin, streptomycin, chloramphenicol, and so on (Table 1).

### Sequence analysis

We constructed a series of deletion plasmids carrying various portions of the DNA insert in plasmid pVCJ6. The DNA insert in plasmid pVCJ6 was digested with several restriction endonucleases and subcloned into pSTV28 (a vector plasmid carrying chloramphenicol resistance marker [TaKaRa Co.]). The resulting hybrid plasmids were introduced into KAM32 cells, and the transformants were tested for sensitivity or resistance to ethidium bromide. Thus, plasmids pVCJ6, pVCJ60, pVCJ61, pVCJ62, pVCJ63, pVCJ64, pVCJ69, and pVCJ7 were obtained (Fig. 1). The presence of DNA inserts in the plasmids was confirmed by single or double digestion with restriction enzymes. We tested the drug susceptibility in cells of KAM32 harboring each plasmid (Fig. 1). Thus, we localized the gene responsible for ethidium resistance to a short DNA region. Next, we determined the nucleotide sequence (22) of this region by using a DNA sequencer (ALF Express; Pharmacia Biotech).

The nucleotide sequence data reported in this study has been deposited in the DDBJ, EMBL, and GenBank nucleotide sequence databases under accession number AB063282.

We found an open reading frame preceded by a Shine-Dalgarno sequence (23) in this region and designated the open reading frame vmrA (vibrio’s multidrug resistance). Several promoter-like sequences (18, 20) were present upstream from vmrA. The vmrA gene consisted of 1,341 nucleotides, with a deduced polypeptide (VmrA) consisting of 447 amino acid

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**TABLE 1. Drug specificity in VmrA**

<table>
<thead>
<tr>
<th>Drug</th>
<th>MIC (µg/ml)</th>
<th>KAM32</th>
<th>KAM32/pVCJ6</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAPI</td>
<td>0.25</td>
<td>128</td>
<td></td>
</tr>
<tr>
<td>TPPCl</td>
<td>8</td>
<td>128</td>
<td></td>
</tr>
<tr>
<td>Acriflavine</td>
<td>2</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>Ethidium bromide</td>
<td>4</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>0.5</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>Norfloxacin</td>
<td>0.03</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td>Rhodamine 6G</td>
<td>8</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Tetracycline</td>
<td>0.5</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>Erythromycin</td>
<td>4</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Streptomycin</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Sodium deoxycholate</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Sodium dodecyl sulfate</td>
<td>0.1</td>
<td>0.1</td>
<td></td>
</tr>
</tbody>
</table>

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**FIG. 1.** Plasmids and restriction maps of cloned V. parahaemolyticus DNA containing the vmrA gene. Physical maps of the DNA inserts derived from the V. parahaemolyticus chromosome in pVCJ6 and its derivatives are shown. Restriction sites determined in pVCJ6 are shown. The growth capabilities of E. coli KAM32 cells harboring each plasmid in L medium containing 10 µg of ethidium bromide/ml are shown on the right. +, Cells grew; −, cells did not grow. The position and direction of the vmrA gene revealed by sequencing are shown at the bottom.

Cells of V. parahaemolyticus AQ3334 showed considerable resistance against ethidium bromide, DAPI, TPPCl, and acriflavine (MICs of 16 to 256 µg/ml) and susceptibility to norfloxacin, tetracycline, and erythromycin (MICs of 0.12 to 0.5 µg/ml). Thus, VmrA may be functional in cells of V. parahaemolyticus AQ3334.
residues with a calculated molecular mass of 49 kDa. VmrA was very rich in hydrophobic residues, indicating that the protein was an integral membrane protein.

Hydropathy analysis by the method of Eisenberg et al. (5) revealed that VmrA possessed 12 hydrophobic regions that may be transmembrane domains (data not shown).

We searched for amino acid sequence homology between VmrA and the reported sequences in a protein sequence database (SwissProt). We found one hypothetical protein (VC0650) suggested from the genome sequence of V. cholerae, which showed high sequence similarity (identity, 63%; similarity, 77%) with VmrA (7). Judging from the overall sequence similarity, VmrA is classified as a member of MATE family drug efflux pumps (4). Brown et al. classified the MATE family into three subfamilies (clusters) (4). Figure 2 shows a dendrogram for representatives of the three subfamilies of the MATE family. Subfamily 1 includes NorM, subfamily 2 includes ERC1, and subfamily 3 includes DinF (4). Judging from the dendrogram, we believe that the VmrA is a member of subfamily 3 (DinF subfamily), which has not so far been shown to contain drug efflux transporters.

Acriflavine efflux. We tested whether VmrA is really a drug efflux pump. Some fluorescent antimicrobial agents such as ethidium bromide are commonly used for the measurement of efflux via multidrug efflux pumps (15). We tried to investigate whether VmrA is really a drug efflux pump by using fluorescent substrates. Although ethidium seemed to be a substrate for the VmrA system as described above, we were able to detect only small changes in fluorescence intensity of ethidium when an energy inhibitor such as CCCP (carbonyl cyanide m-chlorophenylhydrazone) was added to the assay mixture (data not shown). However, our results with ethidium clearly indicated that VmrA was a drug efflux pump. It was hard to characterize VmrA with ethidium as a substrate because of the low efflux activity. Thereafter, we tried to use DAPI and acriflavine as fluorescent probes, both of which seemed to be fairly good substrates of VmrA (Table 1). We found it difficult, however, to clearly measure accumulation and efflux of DAPI, which seemed to be the best substrate for VmrA (Table 1). On the other hand, the use of acriflavine gave us good results, as shown below.

In contrast to what we observed with ethidium, the addition of DNA to an acriflavine solution decreased the fluorescence intensity (Fig. 3). This suggests that the binding of acriflavine to DNA decreases the fluorescence intensity of acriflavine. There was a good correlation between DNA concentration (exponential) and the decrease in fluorescence intensity. Thus, it is anticipated that the accumulation of acriflavine in cells elicits the binding of acriflavine to DNA and results in a decrease in fluorescence. Efflux of acriflavine from cells decreases intracellular acriflavine and will cause dissociation of acriflavine from DNA and will result in an increase in the fluorescence.

As shown in Fig. 4, we observed a clear decrease in acriflavine fluorescence when CCCP was added to a cell suspension of KAM32/pVCJ6, indicating that the accumulation of acriflavine took place after the addition of CCCP. On the other hand, we observed little change fluorescence caused by the addition of CCCP with cells of KAM32 (Fig. 4). The final levels of the fluorescence intensities after the addition of CCCP were similar in the two strains, indicating that the accumulation levels of acriflavine in both strains are similar under deenergized conditions. An important point is that the acriflavine accumulation level in cells of KAM32/pVCJ6 was much lower than that in cells of KAM32. This indicates that cells of KAM32/pVCJ6, but not of KAM32, possess energy-dependent acriflavine efflux activity. Therefore, we conclude that VmrA is an energy-dependent drug efflux pump.

We tested whether the observed energy-dependent efflux of acriflavine from cells via VmrA was stimulated with Na⁺. NaCl stimulated the efflux activity and increasing the concentrations of NaCl up to 15 mM resulted in increasing efflux activity (data not shown). The efflux activity decreased at concentrations of NaCl higher than 20 mM. Efflux activity of VmrA was very weak in the absence of NaCl. The addition of LiCl (15 mM) instead of NaCl to the assay mixture resulted in some stimulation of the efflux activity, and the addition of KCl (15 mM) resulted in no stimulation (data not shown). Thus, we conclude that VmrA is an Na⁺/drug antiporter.

**VmrA is an Na⁺/drug antiporter.** We reported previously that NorM of *V. parahaemolyticus* is an Na⁺/drug antiporter.
YdhE. Thus, it is likely that there is a weak Na\(^+/\)H\(^{+}\) able effect on the Na\(^{+}\) that other substrates for the VmrA than TPP\(^{+}\) gave unfavorable effect which was the first example of this in the biological world (15). We tested whether VmrA is also an Na\(^+/\)/drug antiporter. First, we loaded cells with Na\(^{+}\) to test this possibility. E. coli cells possess the inducible Na\(^+/\)/melibiose symporter, MelB (17, 27). Cells of E. coli KAM32/pVCJ7 (possessing vmrA) and of KAM32/pSTV28 (control) were grown in the presence of melibiose to induce MelB expression. Methyl-\(d\)~-galactopyranoside (M\(\beta\)Gal), a substrate of MelB (28), was added to the cell suspension to elicit accumulation of Na\(^{+}\) in the cells (Fig. 5). After a plateau level of Na\(^{+}\) accumulation was attained due to the symport of M\(\beta\)Gal and Na\(^{+}\), TPP\(^{+}\) (a substrate of VmrA) was added to the cell suspension. Strong efflux of Na\(^{+}\) was elicited in KAM32/pVCJ7 cells, and a slight efflux was observed with KAM32/pSTV28 cells (Fig. 5). These results indicate that VmrA is a Na\(^+/\)/drug antiporter. It is suggested from these results that cells of E. coli KAM32 possess slight Na\(^{+}\)/TPP\(^{+}\) antiport activity, which is due to neither AcrAB nor YdhE. Thus, it is likely that there is a weak Na\(^+/\)-coupled drug efflux pump(s) in E. coli other than YdhE. It should be noted that other substrates for the VmrA than TPP\(^{+}\) gave unfavorable effect on the Na\(^{+}\)-electrode.

The drug efflux pumps described thus far utilize either an electrochemical potential of H\(^{+}\) across cell membrane (H\(^{+}\)/drug antiporter) or ATP as energy sources (19). NorM of V. parahaemolyticus was found to be an Na\(^+/\)/drug antiporter, which was the first example of this in the biological world (15). VmrA is the second example of an Na\(^+/\)/drug antiporter.

Recently, we cloned a gene very similar to the vmrA gene from chromosomal DNA of V. cholerae O1 El Tor (7), a nonhalophilic bacterium. Thus, it seems that the Na\(^{+}\)-driven multidrug efflux pump VmrA is not specific for halophilic V. parahaemolyticus.

The present study is the first report that shows that a member of subfamily 3 (DiInF subfamily) of the MATE family is a drug efflux transporter.

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REFERENCES


