VmeAB, an RND-type multidrug efflux transporter in *Vibrio parahaemolyticus*

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Genes *vmeA* and *vmeB*, encoding a multidrug efflux transporter in the halophilic bacterium *Vibrio parahaemolyticus*, have been cloned using a drug-hypersusceptible *Escherichia coli* strain as the host. Cells of *E. coli* KAM33 (Δ *acrAB* Δ *ydhE*) carrying the *vmeAB* region from *V. parahaemolyticus* conferred much higher MICs for a variety of antimicrobial agents than did control cells. Cells possessing VmeAB under energized conditions maintained very low intracellular concentrations of ethidium. This was as expected for an energy-dependent efflux system, and supports the notion – based on sequence homology – that VmeAB belongs to the resistance nodulation cell division (RND) family of multidrug efflux transporters. It is likely that VmeAB forms functional complexes with the outer-membrane protein TolC in *E. coli*, because introduction of *vmeAB* into cells of *E. coli* KAM43, which lacks the *tolC* gene, failed to elevate the MICs for any of the antimicrobial agents tested. Therefore, a *V. parahaemolyticus* homologue of *tolC* was also cloned, designated *vpoC*, and was introduced together with *vmeAB* into cells of *E. coli* KAM43. The MICs of all agents tested were raised and were comparable to the values observed in *E. coli* KAM33 harbouring a plasmid carrying *vmeAB*. Finally, a *vmeAB*-deficient mutant of *V. parahaemolyticus* was constructed (designated TM3). TM3 showed slightly higher susceptibility than the parental *V. parahaemolyticus* to some antimicrobial agents. Survival rate of the TM3 when exposed to deoxycholate decreased compared with that of the parent.

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INTRODUCTION

Vibrio parahaemolyticus, a slightly halophilic marine bacterium, is a major cause of food poisoning in Japan (Obata *et al.*, 2001; WHO, 1999; Yamazaki *et al.*, 2003) and many other countries (Cabanillas-Beltran *et al.*, 2006; CDC, 2006; Lozano-Leon *et al.*, 2003; McLaughlin *et al.*, 2005; Sen *et al.*, 2007; Su *et al.*, 2005a). The organism has some peculiar physiological characteristics, such as a very fast growth rate, a Na⁺ requirement for growth (Baumann & Schubert, 1984) and an ability to live in diverse

environments including brackish water (Kumazawa & Kato, 1985), on estuarine algae (Kumazawa *et al.*, 1991), and in mammalian hosts, including humans (Yamamoto & Yokota, 1989).

In achieving human infection, *V. parahaemolyticus* survives in the intestine and especially in the duodenum, where bile acids and their conjugates are abundant. Bile acids are anionic detergents and support the digestion of fats in the intestine. In addition, bile has bactericidal effects due to its membrane-solvent property (Provenzano *et al.*, 2000). Thus, bile resistance of enteropathogenic bacteria is thought to be important for their survival in the intestine.

Some enteropathogenic bacteria have been shown to possess systems to protect from the actions of bile. In the case of *Vibrio cholerae*, the outer-membrane component TolC_{VC}, which had already been identified as a component of some multidrug efflux transporters (sometimes called multidrug efflux pumps), was found to contribute to resistance to bile, cholate and deoxycholate (Bina & Mekalanos, 2001). In the case of *Escherichia coli*, energy-dependent efflux of bile salts was observed (Thanassi *et al.*,

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Abbreviations: FLP, flippase; FRT, FLP recombinase recognition target; MATE, multidrug and toxic compound extrusion; RND, resistance nodulation cell division; TPP-Cl, tetraphenylphosphonium chloride.

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1997). Moreover, several multidrug efflux transporters have already been described as having involvement in resistance to deoxycholate and other constituents of bile (Nishino & Yamaguchi, 2001; Nishino *et al.*, 2003; Xu *et al.*, 2003), thus implying that the multidrug efflux transporters themselves play a significant role in resistance to bile, as well as to other antimicrobial agents.

Recently, numerous multidrug efflux transporters have been characterized in micro-organisms. These proteins can be classified into five families, according to their primary structure and energy source for transport. These are (i) RND (resistance nodulation cell division), (ii) MF (major facilitator), (iii) MATE (multidrug and toxic compound extrusion), (iv) SMR (small multidrug resistance) and (v) ABC (ATP-binding cassette) families (Piddock, 2006). From the available databases of genome sequences (Makino *et al.*, 2003), we estimated that there were about 50 multidrug efflux transporters in *V. parahaemolyticus*, according to their primary structure similarities. Therefore, we have set out to clone the multidrug transporter genes from *V. parahaemolyticus*, and to characterize the properties of the transporters.

In previous papers, we have reported on the cloning and properties of two transporters in the MATE family, NorM and VmrA from *V. parahaemolyticus* (Chen *et al.*, 2002; Morita *et al.*, 1998, 2000). Here, we describe cloning of the *vmeA* and *vmeB* genes in *E. coli* KAM3, and characterization of the encoded multidrug efflux transporter VmeAB, which belongs to the RND family. Cells possessing VmeAB conferred much higher MICs than did control cells, of a variety of antimicrobial agents. Moreover, *vmeAB*-deficient *V. parahaemolyticus* showed a reduced survival rate in the presence of deoxycholate. This is believed to be the first description of an RND-type transporter in *V. parahaemolyticus*.

METHODS

Bacterial strains and growth. *V. parahaemolyticus* AQ3334 (Chen *et al.*, 2002; Kuroda *et al.*, 2005; Morita *et al.*, 1998) was used as the source of chromosomal DNA. *E. coli* KAM3 (Δ *acrB*) (Masaoka *et al.*, 2000; Mine *et al.*, 1999), KAM33 (Δ *acrAB* Δ *ydhe*) and KAM43 (Δ *acrAB* Δ *ydhe* Δ *tolC*) were used as hosts for gene cloning and transporter characterization. The constructions of KAM33 and KAM43 are described below. *E. coli* TG1 Δ *tolC* was provided by A. Yamaguchi, Osaka University, Japan, and *E. coli* β 2155 was provided by D. Mazel, Institut Pasteur, France. *V. parahaemolyticus* was grown in LB medium (1% polypeptone, 0.5% yeast extract, 1% NaCl, pH 7.0), and *E. coli* was grown in L medium (1% polypeptone, 0.5% yeast extract, 0.5% NaCl, pH 7.0). Cell growth was monitored by determining the turbidity at 650 nm.

Construction of *E. coli* KAM33 and KAM43. *E. coli* KAM33 was constructed by using the gene-deletion method of Datsenko & Wanner (2000). Plasmids (pKD3, pKD46, pCP20) were kindly provided by B. L. Wanner, Purdue University, USA. PCR products were generated by using primers described below. The primers for *acrAB* disruption were *acrANFRT* (5'-atgaacaaaacagagggtt-tacgctctggcggtcagggatgtaacgcactgagaag-3') and *acrBCFRT* (5'-tcaatgatgatgcacagtatggctgtgctgatcatcgtcttgagcgattgttagg-3'). These

primers included homology extensions of those genes and priming sequences for pKD3 as template. KAM32 (Δ *acrB* Δ *ydhe*) (Chen *et al.*, 2002) was used as a parent for the construction of KAM33, and the resulting KAM33 was then used as the parent for construction of KAM43. In the case of the construction of KAM33, *E. coli* KAM32 cells carrying a Red helper plasmid (pKD46) were grown in 5 ml SOB (2% Bacto Tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂) containing 100 µg ampicillin ml⁻¹ and 0.2% L-arabinose at 30 °C until mid-exponential phase. Harvested cells were washed three times with ice-cold distilled water and concentrated 100-fold. PCR products were gel-purified and suspended in distilled water. Electroporation was done by using a Gene-pulser (Bio-Rad) according to the manufacturer's instructions, with 50 µl concentrated cells and 10 ng PCR product. One millilitre of pre-warmed SOC (2% Bacto Tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 0.1% glucose) containing 0.1% L-arabinose was added to the shocked cells, and incubated for 2 h at 37 °C. Cells were spread onto agar and incubated at 30 °C to select the chloramphenicol-resistant (Cm^R) transformants. These candidates were streaked on medium without an antibiotic and incubated at 42 °C. Cm^R and ampicillin-sensitive candidates were selected for a further step.

The Cm^R gene can be eliminated by using a helper plasmid expressing the flippase (FLP) recombinase, which acts on the directly repeated FRT (FLP recognition target) sites flanking the resistance gene (Cherepanov & Wackernagel, 1995; Datsenko & Wanner, 2000). Cm^R candidates were transformed with pCP20, which carried the FLP recombinase gene, and ampicillin-resistant transformants were selected at 30 °C. After non-selective single-colony isolation at 42 °C for 24 h, the isolates were then tested for loss of chloramphenicol and ampicillin resistances. The majority had lost the FRT-flanked resistance gene and the FLP helper plasmid simultaneously. The disruption of genes was confirmed by PCR analysis.

Prior to construction of KAM43 (Δ *acrAB* Δ *ydhe* Δ *tolC*), we constructed KAM42 (Δ *acrB* Δ *ydhe* Δ *tolC*) from KAM32 (Δ *acrB* Δ *ydhe*) (Chen *et al.*, 2002). For disruption of *tolC*, PCR products were amplified with two primers, *tolCFRTN2* (5'-gatcgcgctaa-tactgcttcaccacaaggaatgcaaatgggtgtaggctggagctgcttc-3') and *tolCFRTC2* (5'-gtcatcagttacggaagggttatgaccgttactggtgctggaattagccatggtc-3'), using pKD3 as the template. The PCR products were introduced by electroporation into the parental KAM32 carrying pKD46. Cm^R candidates were selected, and insertion of the Cm^R gene in the *tolC* was confirmed by the PCR method. A region of *tolC*::Cm^R was transferred into KAM33 by P1 transduction, and then elimination of FRT-flanked resistance gene was carried out as described above.

Gene cloning and sequencing. Chromosomal DNA was prepared from the *V. parahaemolyticus* cells by the method of Berns & Thomas (1965). The DNA was partially digested with *Sau3AI*, and 4–10 kb fragments were separated by sucrose density-gradient centrifugation. Vector plasmid pBR322 was digested with *Bam*HI, dephosphorylated with bacterial alkaline phosphatase, and then ligated with the chromosomal DNA fragments by using ligation kit version 2 (TaKaRa BIO). Competent cells of *E. coli* KAM3, prepared by the method of Hanahan *et al.* (1991), were transformed with the recombinant plasmids. The transformed cells were spread onto agar plates containing L medium, 10 µg rhodamine 6G ml⁻¹, 100 µg ampicillin ml⁻¹ and 1.5% agar, on which *E. coli* KAM3 could not grow. The plates were incubated at 37 °C for 48 h. Candidate colonies that appeared on the plate were purified by single-colony isolation, and growth on the plate containing the same drugs as described above was checked. Plasmids were isolated from each of the candidates. Those candidate plasmids were reintroduced into KAM3 cells, and the cells were spread onto an identical plate. Plasmids were isolated from each of these transformants. One of the candidate plasmids was named pRHR2.

To assess the contribution of the cloned resistance determinant to β -lactams, we also constructed pRHR229, in which the *NsiI*–*SphI* fragment of pRHR2 was inserted into the multicloning site of pSTV29 (TaKaRa BIO). Plasmid pSTV29 carries the Cm^R gene, but not the β -lactamase gene. Plasmid pRHR229 had the shortest insert, and transformed cells harbouring this plasmid were resistant to several antimicrobial agents. For sequencing, plasmid pRHR222, which carried the *PvuII*–*SalI* fragment of pRHR2 in pBluescript SK(+) (Stratagene), was constructed. Deletion plasmids for sequencing were constructed using exonuclease III and mung bean nuclease (Henikoff, 1984).

Cloning of *vpoC* (VP0425). Cloning of VP0425 was carried out by PCR. Template DNA was prepared as described above, from *V. parahaemolyticus* RIMD2210633, for which the genome sequence is available (<http://genome.naist.jp/bacteria/vpara/>). The two primers were VP0425-F (5'-tactggatccgcgggaatgaaatgaa-3') and VP0425-R (5'-cgaggatccgcgttttaatttcattga-3'); the PCR product was ligated into pBR322 at the *Bam*HI site. To facilitate the cloning, a *Bam*HI restriction site was introduced at the 5' end of both primers. The resulting plasmid was designated pBVT3.

Drug susceptibility test. The MICs of various antimicrobial agents were determined by the microdilution method according to the recommendations of the Japanese Society of Chemotherapy (1990). Briefly, MICs were determined in Mueller–Hinton broth (Difco) containing each compound in a twofold serial dilution series. The cells were incubated in the test medium at 37 °C for 24 h, and growth was examined visually. The MIC of each compound was defined as the lowest concentration that prevented visible growth.

Ethidium accumulation assay. Ethidium accumulation assays were performed as described previously (Xu *et al.*, 2003).

Disruption of *vmeAB* in *V. parahaemolyticus*. The plasmid pRHR228 was constructed using the same procedure as for pRHR229 (described above), but using pSTV28 (TaKaRa BIO) instead of pSTV29. It also carries the whole *vmeAB* region. There are two *HpaI* sites in the *vmeAB* region, one in *vmeA* and another in *vmeB*. Plasmid pRHR228 was digested with *HpaI*, and the *HpaI* fragment covering a large portion of *vmeAB* was removed by self-ligation. A *SalI*–*XbaI* fragment carrying the incomplete *vmeAB* region lacking the *HpaI* fragment was cut out and ligated to the suicide vector pXAC623 (Kuroda *et al.*, 2005), which had been digested with *SalI* and *XbaI*. The resulting plasmid, pXAC1092, carries the incomplete *vmeAB*, the *cat* gene and the *sacB* gene.

Plasmid pXAC1092 was introduced into cells of *E. coli* donor strain β 2155 (Herz *et al.*, 2003). Mating between donor cells of *E. coli* β 2155/pXAC1092 and recipient cells of *V. parahaemolyticus* AQ3334 was carried out as described previously (Kuroda *et al.*, 2005). Briefly, donor cells were grown in LB medium supplemented with 0.5 mM diaminopimelic acid and 20 μg chloramphenicol ml^{-1} , and recipient cells were grown in LB medium. Both cells were harvested at the early exponential phase of growth, mixed gently, and trapped on a membrane filter (0.2 μm pore size, ADVANTEC Toyo). After washing, the filter was put on an LB medium agar plate (1.5 % agar) and incubated at 37 °C for 3 h. The cells were suspended in LB medium, and shaken at 37 °C for 1 h. Candidate colonies of *V. parahaemolyticus*, into the genome of which pXAC1092 had been integrated, could be obtained on agar plates containing 5 μg chloramphenicol ml^{-1} and 100 μg ampicillin ml^{-1} , since *V. parahaemolyticus* AQ3334 is resistant to ampicillin at this concentration but *E. coli* β 2155 is susceptible. The candidates were cultured in LB medium without antibiotics, spread on VDS-broth agar plates (1 % polypeptone, 0.5 % yeast extract, 30 mM NaCl, 55 mM KCl, 10 % sucrose, pH 7.0, 2.5 % agar) and incubated at 25 °C. Occurrence

of a second recombination should yield cells resistant to sucrose but susceptible to chloramphenicol, and several such candidates were identified. After checking deletion of the *vmeAB* region by PCR, we picked a single isolate for further use and designated it TM3.

Transformation of *V. parahaemolyticus* was carried out by the method of Hamashima *et al.* (1995). *V. parahaemolyticus* cells were cultured in LB medium until mid-exponential phase was reached. Harvested cells were washed with 20 ml ice-cold EP buffer (272 mM sucrose, 1 mM MgCl_2 , 7 mM $\text{KH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$, pH 7.4) and the pellet was resuspended in 2 ml of the same buffer. One microgram of pSTV29 or pRHR229 was added into 200 μl of the cell suspension. The mixture was placed on ice for 15 min and transferred to a chilled cuvette with a distance of 0.2 cm between electrodes, and pulsed with a time constant of 25 ms (25 μF capacitance, 1000 Ω) at 1.4 kV by using a Gene-pulser (Bio-Rad) according to the manufacturer's instructions. Shocked cells were added into 800 μl pre-warmed LB medium, and incubated at 37 °C for 1 h. Transformants were selected on an LB plate containing 10 μg chloramphenicol ml^{-1} (2.5 % agar).

Effect of sodium deoxycholate on survival rate of *V. parahaemolyticus* strains. Approximately 2×10^8 cells of *V. parahaemolyticus* were suspended in 1 ml of 0.5 M NaCl-PB (0.1 M phosphate buffer, pH 7.2) including 1 mg sodium deoxycholate ml^{-1} . After incubation at 37 °C for 10 min, cells diluted 10^{-4} with 0.5 M NaCl-PB were spread onto an LB agar plate (2.5 % agar). Following incubation at 37 °C for 12 h, c.f.u. were counted.

RESULTS

Cloning of multidrug resistance gene

Using *E. coli* KAM3 as the host, we cloned a DNA fragment that enabled KAM3 cells to grow in the presence of 10 μg rhodamine 6G ml^{-1} . *E. coli* KAM3 could not grow on this plate. After incubation at 37 °C for 48 h, some candidate colonies appeared. Plasmids isolated from the candidates were reintroduced into KAM3 cells, and the cells could grow on the same medium. Since rhodamine 6G was shown to be a good substrate for some multidrug transporters (Chen *et al.*, 2003; Huda *et al.*, 2003; Nishino & Yamaguchi, 2001; Sekiya *et al.*, 2003; Su *et al.*, 2005b), multidrug transporter genes were suggested to be included in these candidate plasmids. One of the candidate plasmids was named pRHR2.

Sequencing

Upon sequencing of the plasmid insert that conferred elevated MICs of several antimicrobial agents, we found two ORFs. Both of these were required for the elevated MICs and we designated them *vmeA* and *vmeB*. Comparison of the new sequences with the published genome sequence of *V. parahaemolyticus* (Makino *et al.*, 2003) showed *vmeA* to correspond to VP1091, and *vmeB* to VP1092. Fourteen differences of nucleotide sequence were identified between *vmeA* and VP1091, and 37 differences between *vmeB* and VP1092. All these differences were translationally silent, except two: changing amino acid residue Gly33 in VP1091 to Ala33 in VmeA, and changing Ala1046 in VP1092 to Asp1046 in VmeB. A BLAST search

for protein sequence similarities (NCBI) showed VmeA to share, respectively, 49, 49, 47 and 46 % identities with AcrA and AcrE of *E. coli* (Klein *et al.*, 1991; Ma *et al.*, 1993), SdeX of *Serratia marcescens* (Chen *et al.*, 2003) and MexA of *Pseudomonas aeruginosa* (Poole *et al.*, 1993). All of these are so-called membrane fusion proteins (MFPs) of RND-type multidrug efflux transporters. The analysis also showed VmeB to share 63, 63, 62, 62 and 61 % identities, respectively, with AcrB, AcrD and AcrF of *E. coli* (Klein *et al.*, 1991; Ma *et al.*, 1993, 1994), SdeY of *S. marcescens* (Chen *et al.*, 2003) and MexB of *P. aeruginosa* (Poole *et al.*, 1993). These are inner-membrane proteins of RND-type multidrug efflux transporters. Thus, VmeAB seems to be a solid member of the RND family of multidrug efflux transporters. Multidrug efflux transporters of the RND family have been shown to play an important role in intrinsic resistance to various antimicrobial agents in various Gram-negative bacteria, such as *E. coli* and *P. aeruginosa* (Li *et al.*, 1995; Morita *et al.*, 2001; Okusu *et al.*, 1996; Poole *et al.*, 1993; Tseng *et al.*, 1999). We then further analysed VmeAB.

We found no ORF in the downstream region of *vmeB* that might code for an outer-membrane component of this RND-type efflux transporter.

Substrate specificity of VmeAB

To characterize VmeAB, we determined the MICs of various antimicrobial agents using *E. coli* strain KAM33, made to be drug-hypersusceptible by deletion of the major multidrug efflux transporter, AcrAB. After plasmid pRHR229, carrying *vmeAB*, was introduced into KAM33, the resulting strain KAM33/pRHR229 displayed elevated MICs of rhodamine 6G, cloxacillin, oxacillin, erythromycin, tetracycline, norfloxacin, novobiocin, trimethoprim, benzalkonium chloride, acriflavine, crystal violet, ethidium bromide, sodium deoxycholate, SDS and tetraphenylphosphonium chloride (TPP-Cl), as shown in Table 1, compared with the control (*E. coli* KAM33/pSTV29). Since plasmid pSTV29 carries the *Cm^R* gene, we also investigated the MIC of chloramphenicol for KAM33/pRHR2. In addition to the antimicrobial agents described above, KAM33/pRHR2 showed four times higher MICs of chloramphenicol than KAM33/pBR322 (data not shown). Clearly, VmeAB contributes to multidrug resistance, when expressed in cells of *E. coli*, and its resistance spectrum is very similar to that of the AcrAB transporter in *E. coli* (Nishino & Yamaguchi, 2001). There was no change in the MICs of aztreonam, carbenicillin, cefotaxime, imipenem, kanamycin or chlorhexidine gluconate (data not shown).

Ethidium extrusion via VmeAB

In order to demonstrate the transport activity of VmeAB directly, we measured intracellular ethidium levels of *E. coli* KAM33/pRHR229 exposed to 40 μ M ethidium bromide. Accumulation of ethidium in cells of KAM33/pRHR229

Table 1. MICs of various antimicrobial agents for *E. coli* KAM33/pRHR229

Antimicrobial agent	MIC (μ g ml ⁻¹)	
	KAM33/pSTV29 (control)	KAM33/pRHR229 (<i>vmeAB</i>)
Rhodamine 6G	8	128
Cloxacillin	1	64
Oxacillin	0.5	128
Erythromycin	4	16
Tetracycline	0.5	1
Norfloxacin	0.03	0.13
Novobiocin	2	128
Trimethoprim	0.06	0.13
Benzalkonium chloride	4	16
Acriflavine	2	32
Crystal violet	4	16
Ethidium bromide	4	256
Sodium deoxycholate	1000	>32 000
SDS	100	>51 200
TPP-Cl	8	128

(Fig. 1, line B) under energized conditions was much lower than that in cells of KAM33/pSTV29 (Fig. 1, line A). However, after addition of the H⁺ conductor, carbonyl-cyanide-*m*-chlorophenylhydrazone (CCCP), intracellular ethidium reached the same levels in both types of cells, demonstrating the energy-dependent nature of ethidium extrusion via VmeAB.

Because two other multidrug efflux transporters from *V. parahaemolyticus*, NorM and VmrA, had proven to be Na⁺-stimulated (Na⁺-coupled), we also tested the effect

Fig. 1. Ethidium extrusion activity in cells of *E. coli* KAM33/pSTV29 (control, trace A) and KAM33/pRHR229 (carrying *vmeAB*, trace B). Ethidium bromide (EtBr) was added to cell suspensions at a final concentration of 40 μ M at the indicated time point. Accumulation of ethidium in the cells was monitored continuously by measuring the fluorescence of ethidium with excitation at 500 nm and emission at 580 nm. Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was added at a final concentration of 100 μ M to de-energize the cells at the indicated time point.

Table 2. Complementation of *tolC* deficiency with *vpoC* of *V. parahaemolyticus*

Antimicrobial agent	MIC ($\mu\text{g ml}^{-1}$)	
	TG1 Δ <i>tolC</i> /pBR322 (control)	TG1 Δ <i>tolC</i> /pBVT3 (<i>vpoC</i>)
Erythromycin	2	16
Norfloxacin	0.015	0.06
Novobiocin	0.25	4
Acriflavine	2	8
Ethidium bromide	2	64
Rhodamine 6G	4	16
Sodium deoxycholate	125	4000
SDS	25	400
TPP-Cl	4	64

of Na^+ on VmeAB-mediated ethidium extrusion. However, addition of 10 mM NaCl hardly affected ethidium extrusion activity via VmeAB (data not shown). We also tested whether Na^+ movement via VmeAB was observed or not using an Na^+ -electrode as reported previously (Chen *et al.*, 2002; Morita *et al.*, 2000). However, we observed no detectable Na^+ movement after the addition of ethidium to the assay mixture (data not shown). Thus, VmeAB is not likely to be a Na^+ -coupled transporter.

Requirement of TolC for the function of VmeAB

Although RND-type efflux transporters require an outer-membrane component to function in multidrug efflux (Piddock, 2006; Putman *et al.*, 2000), the downstream region of *vmeAB* in the plasmid pRHR229 contained no gene that might encode an outer-membrane protein; and no such ORF exists, either, in the vicinity of the

VP1091-VP1092 loci of the *V. parahaemolyticus* genome sequence. In *E. coli*, RND-type efflux transporters require TolC as their outer-membrane component (Nagakubo *et al.*, 2002; Nishino *et al.*, 2003). We then investigated the possibility that TolC of *E. coli* could be utilized by expressed VmeAB, by simply introducing the *vmeAB* plasmid into the cells of *E. coli* KAM43 lacking *tolC*. The resulting cells showed MICs of all of the antimicrobial agents tested to be lower than KAM33 possessing *tolC* and expressing *vmeAB* (data not shown). This complementation of VmeAB by the *E. coli tolC* gene strongly implicates TolC as a functional protein partner of heterologously expressed VmeAB.

In *V. parahaemolyticus* itself, ORF VP0425 codes for a protein showing high sequence identity (44%) and similarity (88%) with TolC of *E. coli*, but VP0425 is located at a distant chromosomal site remote from *vmeAB*. We therefore cloned out VP0425 (designated *vpoC*) by PCR and expressed it in *E. coli* TG1 Δ *tolC*, having the native *tolC* gene deleted. TolC plays a crucial role in resistance to many antimicrobial agents in *E. coli* (Fralick, 1996), and TG1 Δ *tolC* proved hypersusceptible to those antimicrobial agents (Nagakubo *et al.*, 2002; Nishino *et al.*, 2003). However, when *vpoC* was expressed in TG1 Δ *tolC*, the transformed strain (TG1 Δ *tolC*/pBVT3) showed much higher MICs of the tested agents (Table 2). It became clear that VpoC was functional when expressed in *E. coli* cells and that VpoC could function with some efflux transporters of *E. coli*. Next, in order to investigate the effect of VpoC on VmeAB activity, we co-expressed *vpoC* together with *vmeAB* in *E. coli* KAM43, also lacking *tolC* (see above). Expression of VpoC in KAM43 hardly affected the MICs, possibly because VpoC could function only with AcrAB and/or YdhE in *E. coli* (Table 3, columns 1 and 2). Additional expression of VmeAB in KAM43 expressing VpoC showed much higher MICs of the tested antimicrobial

Table 3. VmeAB requires VpoC for function

Antimicrobial agent	MIC ($\mu\text{g ml}^{-1}$)			
	Host: Plasmid: <i>vpoC</i> : <i>vmeAB</i> :	KAM43 pSTV29/pBR322	KAM43 pSTV29/pBVT3	KAM43 pRHR229/pBR322 KAM43 pRHR229/pBVT3
Erythromycin	2	2	2	32
Norfloxacin	0.03	0.03	0.03	0.5
Novobiocin	0.5	1	0.5	64
Acriflavine	2	2	2	16
Ethidium bromide	2	2	2	128
Hoechst 33342	0.25	0.25	0.25	32
Rhodamine 6G	8	8	8	64
Sodium deoxycholate	125	125	125	4000
SDS	50	50	50	6400
TPP-Cl	4	4	4	128

agents, as compared with the control, KAM43 expressing only VpoC (Table 3). Because the substrate specificity for VmeAB expressed in *E. coli* KAM43 harbouring both pRHR229 and pBVT3 (Table 3) was very similar to that observed in *E. coli* KAM33 harbouring pRHR229 (Table 1), it seems safe to conclude that VpoC of *V. parahaemolyticus* does in fact form the third structural element of a tripartite RND-type multidrug efflux transporter based on VmeAB.

Properties of a *vmeAB*-deficient mutant

In order to examine the role of VmeAB in *V. parahaemolyticus* itself, we disrupted the native *vmeAB* genes, yielding strain TM3. This mutant strain was more sensitive (lower MICs) to Hoechst 33342; and it was slightly more sensitive (slightly lower MICs) to oxacillin, acriflavine and ethidium bromide (Table 4). The MICs of erythromycin, rhodamine 6G, TPP-Cl, SDS and sodium deoxycholate, however, were not changed by the gene disruption, even though these agents proved to be good substrates for VmeAB expressed in *E. coli*.

We then carried out a survival test in the presence of sodium deoxycholate in order to investigate the bactericidal effect. The survival rate of TM3 was lower than that of AQ3334 (Fig. 2). The survival rate was restored by the introduction of plasmid pRHR229 carrying *vmeAB* into the TM3 strain. This indicates that one of the functions of VmeAB might be to protect the organism from the bactericidal effects of sodium deoxycholate.

DISCUSSION

From its amino acid sequence and its functional characteristics upon expression in *E. coli*, the *V. parahaemolyticus* multidrug transporter VmeAB – which we have now isolated and characterized – is clearly that organism's orthologue of AcrAB in *E. coli*.

Such RND-type efflux transporters, especially AcrAB in *E. coli*, and MexAB in *P. aeruginosa*, contribute strongly to the

intrinsic multidrug resistance of these bacteria (Li *et al.*, 1995; Morita *et al.*, 2001; Okusu *et al.*, 1996; Poole *et al.*, 1993). However, the *vmeAB*-deficient mutant *V. parahaemolyticus* (TM3) showed only modestly lowered MICs of a variety of antimicrobial agents. To explain this phenomenon, there are three possibilities: (1) VmeAB is not expressed in cells of *V. parahaemolyticus*; (2) VmeAB does not function as an efflux transporter in *V. parahaemolyticus*; and (3) the transport activity of other efflux transporter(s) is stronger than that of VmeAB, and the contribution of VmeAB is masked. mRNA of *vmeAB* was detected at a considerable level in *V. parahaemolyticus* by RT-PCR analysis (data not shown), and *V. parahaemolyticus* TM3 showed reduced survival in the presence of sodium deoxycholate (Fig. 2). Thus, the third possibility seems to be likely. VmeAB is probably not a singular multidrug efflux transporter in *V. parahaemolyticus*, at least under ordinary laboratory conditions. Judging from the genome sequence (Makino *et al.*, 2003), indeed, 11 more putative RND-type efflux transporter genes should be present in cells of *V. parahaemolyticus*, along with nearly 40 other drug efflux transporter genes in different structural classes, which are certainly important for the survival and growth of *V. parahaemolyticus* as a human intestinal parasite.

Interestingly, we did not observe a significant difference between the growth of cells of the parental strain and that of the *vmeAB*-deleted mutant TM3 in the presence of sodium deoxycholate, but significant differences were observed in their survival rates when exposed to sodium deoxycholate. A possible explanation for this discrepancy is that VmeAB is involved in the escape from the instantaneous bactericidal effect of sodium deoxycholate, followed by induction of other gene(s) responsible for growth of *V. parahaemolyticus* in the presence of sodium deoxycholate. Since we observed that *vmeAB* was expressed constitutively, and no induction by deoxycholate was seen (data not shown), it seems possible that expression of some efflux transporters other than VmeAB was induced, which were involved in the resistance to deoxycholate. Namely, VmeAB could be involved in the escape from the instantaneous bactericidal effect of deoxycholate, and efflux transporters other than VmeAB might be involved in the growth in the presence of deoxycholate. In fact, expression of *vexAB* and *vexCD*, which encode RND-type efflux transporters in *V. cholerae*, has been shown to be induced by bile salts (Bina *et al.*, 2006). Our results suggest that other multidrug transporter(s) which is (are) able to extrude deoxycholate is (are) present in *V. parahaemolyticus*.

AcrB (*E. coli*) and MexB (*P. aeruginosa*) are known as H⁺-coupled transporters (Murakami *et al.*, 2002; Ocaktan *et al.*, 1997; Thanassi *et al.*, 1997), and three characteristic charged residues in the transmembrane domains of these two proteins have been suggested to be part of the proton-translocating pathway (Murakami *et al.*, 2002). Since these residues are also conserved in VmeB of *V. parahaemolyticus*

Table 4. Involvement of VmeAB in intrinsic resistance in *V. parahaemolyticus*

Antimicrobial agent	MIC (µg ml ⁻¹)	
	AQ3334 (parent)	TM3 (Δ <i>vmeAB</i>)
Hoechst 33342	2	0.25
Oxacillin	128	64
Acriflavine	16	8
Ethidium bromide	64	32
Erythromycin	4	4
Norfloxacin	0.0625	0.0625
Rhodamine 6G	128	128
TPP-Cl	128	128
Sodium deoxycholate	8000	8000

(K342, E346, D407, D408, K937), this protein is also likely to be energized by proton coupling, a conclusion that is supported by the finding, reported above, that Na^+ levels and fluxes are unrelated to VmeAB-mediated transport.

It was shown that VmeAB could form a functional complex with outer-membrane component TolC of *E. coli*. Some RND-type efflux transporters have been reported to utilize TolC of *E. coli* when expressed heterologously in *E. coli* (Chen *et al.*, 2003; Hansen *et al.*, 2004; Kumar & Worobec, 2005a; Mine *et al.*, 1999; Srikumar *et al.*, 1998). They are MexCD and MexXY of *P. aeruginosa*, SdeAB and SdeXY of *S. marcescens*, and OqxAB of *E. coli*. Disulfide cross-linking data suggested that residues of AcrB interacted directly with TolC (Tamura *et al.*, 2005). However, only G796 was conserved in VmeB among the five deduced residues. The interaction of VmeB or AcrB with TolC may require some other residues.

VpoC of *V. parahaemolyticus* was shown to be a functional orthologue of TolC of *E. coli*. We showed that introduction of *vpoC* into *E. coli* TG1Δ*tolC* conferred elevated resistance to several antimicrobial agents (Table 2). Similar results have been shown with TolC_{Vc} of *V. cholerae* (Bina & Mekalanos, 2001) and HasF of *S. marcescens* (Kumar & Worobec, 2005b). On the other hand, the introduction of *vpoC* into *E. coli* KAM43 caused an increase in MIC values for only novobiocin (Table 3). Since both *acrAB* and *ydhE* were deleted in KAM43, it is likely that VpoC functions with AcrAB and/or YdhE, and not with any other efflux transporters in *E. coli*. As with TolC in *E. coli*, VpoC might be important in intrinsic resistance to antimicrobial agents in *V. parahaemolyticus*.

According to the published genome sequence, several RND-type efflux transporters seem to be present among the *Vibrionaceae*, such as *V. cholerae*, *Vibrio vulnificus* and *Vibrio fischeri* (Heidelberg *et al.*, 2000; Makino *et al.*, 2003;

Ruby *et al.*, 2005). In fact, two RND-type efflux transporters (VexAB and VexCD) in *V. cholerae* have been characterized (Bina *et al.*, 2006). VmeAB shared high sequence similarity with AcrAB and MexAB, but to a lesser extent with VexAB and VexCD. We could not find such proteins, so-called 'orthologues' among the *Vibrionaceae*, indicating that VmeAB is not common among *Vibrionaceae*. The BLAST search showed that the most similar homologue was SO4692-4693 in *Shewanella oneidensis* [59 % identity (VmeA versus SO4693), 72 % identity (VmeB versus SO4692)] (Heidelberg *et al.*, 2002).

As described above, 11 more genes for the putative RND-type efflux transporters are present in the chromosome of *V. parahaemolyticus*. Our present results suggested the contribution of other efflux transporters to resistance against bile acids and antimicrobial agents. We are currently trying to characterize other efflux transporters in *V. parahaemolyticus*.

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REFERENCES

- Baumann, P. & Schubert, R. H. W. (1984). Family II. *Vibrionaceae*. In *Bergey's Manual of Systematic Bacteriology*, vol. 1, pp. 516–550. Edited by N. R. Krieg & J. G. Holt. Baltimore: Williams & Wilkins.
- Berns, K. I. & Thomas, C. A., Jr (1965). Isolation of high molecular weight DNA from *Haemophilus influenzae*. *J Mol Biol* **11**, 476–490.
- Bina, J. E. & Mekalanos, J. J. (2001). *Vibrio cholerae* *tolC* is required for bile resistance and colonization. *Infect Immun* **69**, 4681–4685.
- Bina, J. E., Provenzano, D., Wang, C., Bina, X. R. & Mekalanos, J. J. (2006). Characterization of the *Vibrio cholerae* *vexAB* and *vexCD* efflux systems. *Arch Microbiol* **186**, 171–181.
- Cabanillas-Beltran, H., Llausás-Magaña, E., Romero, R., Espinoza, A., Garcia-Gasca, A., Nishibuchi, M., Ishibashi, M. & Gomez-Gil, B. (2006). Outbreak of gastroenteritis caused by the pandemic *Vibrio parahaemolyticus* O3:K6 in Mexico. *FEMS Microbiol Lett* **265**, 76–80.
- CDC (2006). *Vibrio parahaemolyticus* infections associated with consumption of raw shellfish – three states, 2006. *MMWR Morb Mortal Wkly Rep* **55**, 854–856.
- Chen, J., Morita, Y., Huda, M. N., Kuroda, T., Mizushima, T. & Tsuchiya, T. (2002). VmrA, a member of a novel class of Na^+ -coupled multidrug efflux pumps from *Vibrio parahaemolyticus*. *J Bacteriol* **184**, 572–576.
- Chen, J., Kuroda, T., Huda, M. N., Mizushima, T. & Tsuchiya, T. (2003). An RND-type multidrug efflux pump SdeXY from *Serratia marcescens*. *J Antimicrob Chemother* **52**, 176–179.
- Cherepanov, P. P. & Wackernagel, W. (1995). Gene disruption in *Escherichia coli*: Tc^R and Km^R cassettes with the option of Flp-catalyzed excision of the antibiotic-resistance determinant. *Gene* **158**, 9–14.

Fig. 2. Effect of sodium deoxycholate on the survival rate of parent and *vmeAB*-deleted *V. parahaemolyticus*. After exposure of *V. parahaemolyticus* cells to 1 mg sodium deoxycholate ml^{-1} , c.f.u. were determined. Error bars represent standard deviations for results from triplicate samples. A representative of two experiments is shown. Similar results were obtained in the other experiment.

- Datsenko, K. A. & Wanner, B. L. (2000). One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci U S A* **97**, 6640–6645.
- Fralick, J. A. (1996). Evidence that TolC is required for functioning of the Mar/AcrAB efflux pump of *Escherichia coli*. *J Bacteriol* **178**, 5803–5805.
- Hamashima, H., Iwasaki, M. & Arai, T. (1995). A simple and rapid method for transformation of *Vibrio* species by electroporation. *Methods Mol Biol* **47**, 155–160.
- Hanahan, D., Jessee, J. & Bloom, F. R. (1991). Plasmid transformation of *Escherichia coli* and other bacteria. *Methods Enzymol* **204**, 63–113.
- Hansen, L. H., Johannesen, E., Burmolle, M., Sorensen, A. H. & Sorensen, S. J. (2004). Plasmid-encoded multidrug efflux pump conferring resistance to olaquinox in *Escherichia coli*. *Antimicrob Agents Chemother* **48**, 3332–3337.
- Heidelberg, J. F., Eisen, J. A., Nelson, W. C., Clayton, R. A., Gwinn, M. L., Dodson, R. J., Haft, D. H., Hickey, E. K., Peterson, J. D. & other authors (2000). DNA sequence of both chromosomes of the cholera pathogen *Vibrio cholerae*. *Nature* **406**, 477–483.
- Heidelberg, J. F., Paulsen, I. T., Nelson, K. E., Gaidos, E. J., Nelson, W. C., Read, T. D., Eisen, J. A., Seshadri, R., Ward, N. & other authors (2002). Genome sequence of the dissimilatory metal ion-reducing bacterium *Shewanella oneidensis*. *Nat Biotechnol* **20**, 1118–1123.
- Henikoff, S. (1984). Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing. *Gene* **28**, 351–359.
- Herz, K., Vimont, S., Padan, E. & Berche, P. (2003). Roles of NhaA, NhaB, and NhaD Na⁺/H⁺ antiporters in survival of *Vibrio cholerae* in a saline environment. *J Bacteriol* **185**, 1236–1244.
- Huda, M. N., Chen, J., Morita, Y., Kuroda, T., Mizushima, T. & Tsuchiya, T. (2003). Gene cloning and characterization of VcrM, a Na⁺-coupled multidrug efflux pump, from *Vibrio cholerae* non-O1. *Microbiol Immunol* **47**, 419–427.
- Japanese Society of Chemotherapy (1990). Microbroth dilution methods for determination of minimum inhibitory concentrations. *Chemotherapy* **38**, 102–105.
- Klein, J. R., Henrich, B. & Plapp, R. (1991). Molecular analysis and nucleotide sequence of the *envCD* operon of *Escherichia coli*. *Mol Gen Genet* **230**, 230–240.
- Kumar, A. & Worobec, E. A. (2005a). Cloning, sequencing, and characterization of the SdeAB multidrug efflux pump of *Serratia marcescens*. *Antimicrob Agents Chemother* **49**, 1495–1501.
- Kumar, A. & Worobec, E. A. (2005b). HasF, a TolC-homolog of *Serratia marcescens*, is involved in energy-dependent efflux. *Can J Microbiol* **51**, 497–500.
- Kumazawa, N. H. & Kato, E. (1985). Survival of Kanagawa-positive strains of *Vibrio parahaemolyticus* in a brackish-water area. *J Hyg (Lond)* **95**, 299–307.
- Kumazawa, N. H., Fukuma, N. & Komoda, Y. (1991). Attachment of *Vibrio parahaemolyticus* strains to estuarine algae. *J Vet Med Sci* **53**, 201–205.
- Kuroda, T., Mizushima, T. & Tsuchiya, T. (2005). Physiological roles of three Na⁺/H⁺ antiporters in the halophilic bacterium *Vibrio parahaemolyticus*. *Microbiol Immunol* **49**, 711–719.
- Li, X. Z., Nikaido, H. & Poole, K. (1995). Role of *mexA-mexB-oprM* in antibiotic efflux in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* **39**, 1948–1953.
- Lozano-Leon, A., Torres, J., Osorio, C. R. & Martinez-Urtaza, J. (2003). Identification of *tdh*-positive *Vibrio parahaemolyticus* from an outbreak associated with raw oyster consumption in Spain. *FEMS Microbiol Lett* **226**, 281–284.
- Ma, D., Cook, D. N., Alberti, M., Pon, N. G., Nikaido, H. & Hearst, J. E. (1993). Molecular cloning and characterization of *acrA* and *acrE* genes of *Escherichia coli*. *J Bacteriol* **175**, 6299–6313.
- Ma, D., Cook, D. N., Hearst, J. E. & Nikaido, H. (1994). Efflux pumps and drug resistance in gram-negative bacteria. *Trends Microbiol* **2**, 489–493.
- Makino, K., Oshima, K., Kurokawa, K., Yokoyama, K., Uda, T., Tagomori, K., Iijima, Y., Najima, M., Nakano, M. & other authors (2003). Genome sequence of *Vibrio parahaemolyticus*: a pathogenic mechanism distinct from that of *V. cholerae*. *Lancet* **361**, 743–749.
- Masaoka, Y., Ueno, Y., Morita, Y., Kuroda, T., Mizushima, T. & Tsuchiya, T. (2000). A two-component multidrug efflux pump, EbrAB, in *Bacillus subtilis*. *J Bacteriol* **182**, 2307–2310.
- McLaughlin, J. B., DePaola, A., Bopp, C. A., Martinek, K. A., Napolilli, N. P., Allison, C. G., Murray, S. L., Thompson, E. C., Bird, M. M. & Middaugh, J. P. (2005). Outbreak of *Vibrio parahaemolyticus* gastroenteritis associated with Alaskan oysters. *N Engl J Med* **353**, 1463–1470.
- Mine, T., Morita, Y., Kataoka, A., Mizushima, T. & Tsuchiya, T. (1999). Expression in *Escherichia coli* of a new multidrug efflux pump, MexXY, from *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* **43**, 415–417.
- Morita, Y., Kodama, K., Shiota, S., Mine, T., Kataoka, A., Mizushima, T. & Tsuchiya, T. (1998). NorM, a putative multidrug efflux protein, of *Vibrio parahaemolyticus* and its homolog in *Escherichia coli*. *Antimicrob Agents Chemother* **42**, 1778–1782.
- Morita, Y., Kataoka, A., Shiota, S., Mizushima, T. & Tsuchiya, T. (2000). NorM of *Vibrio parahaemolyticus* is an Na⁺-driven multidrug efflux pump. *J Bacteriol* **182**, 6694–6697.
- Morita, Y., Komori, Y., Mima, T., Kuroda, T., Mizushima, T. & Tsuchiya, T. (2001). Construction of a series of mutants lacking all of the four major *mex* operons for multidrug efflux pumps or possessing each one of the operons from *Pseudomonas aeruginosa* PAO1: MexCD-OprJ is an inducible pump. *FEMS Microbiol Lett* **202**, 139–143.
- Murakami, S., Nakashima, R., Yamashita, E. & Yamaguchi, A. (2002). Crystal structure of bacterial multidrug efflux transporter AcrB. *Nature* **419**, 587–593.
- Nagakubo, S., Nishino, K., Hirata, T. & Yamaguchi, A. (2002). The putative response regulator BaeR stimulates multidrug resistance of *Escherichia coli* via a novel multidrug exporter system, MdtABC. *J Bacteriol* **184**, 4161–4167.
- Nishino, K. & Yamaguchi, A. (2001). Analysis of a complete library of putative drug transporter genes in *Escherichia coli*. *J Bacteriol* **183**, 5803–5812.
- Nishino, K., Yamada, J., Hirakawa, H., Hirata, T. & Yamaguchi, A. (2003). Roles of TolC-dependent multidrug transporters of *Escherichia coli* in resistance to β -lactams. *Antimicrob Agents Chemother* **47**, 3030–3033.
- Obata, H., Kai, A. & Morozumi, S. (2001). The trends of *Vibrio parahaemolyticus* foodborne outbreaks in Tokyo: 1989–2000. *Kansenshogaku Zasshi* **75**, 485–489.
- Ocaktan, A., Yoneyama, H. & Nakae, T. (1997). Use of fluorescence probes to monitor function of the subunit proteins of the MexA-MexB-OprM drug extrusion machinery in *Pseudomonas aeruginosa*. *J Biol Chem* **272**, 21964–21969.
- Okusu, H., Ma, D. & Nikaido, H. (1996). AcrAB efflux pump plays a major role in the antibiotic resistance phenotype of *Escherichia coli* multiple-antibiotic-resistance (Mar) mutants. *J Bacteriol* **178**, 306–308.
- Piddock, L. J. (2006). Multidrug-resistance efflux pumps – not just for resistance. *Nat Rev Microbiol* **4**, 629–636.

- Poole, K., Krebs, K., McNally, C. & Neshat, S. (1993). Multiple antibiotic resistance in *Pseudomonas aeruginosa*: evidence for involvement of an efflux operon. *J Bacteriol* **175**, 7363–7372.
- Provenzano, D., Schuhmacher, D. A., Barker, J. L. & Klose, K. E. (2000). The virulence regulatory protein ToxR mediates enhanced bile resistance in *Vibrio cholerae* and other pathogenic *Vibrio* species. *Infect Immun* **68**, 1491–1497.
- Putman, M., van Veen, H. W. & Konings, W. N. (2000). Molecular properties of bacterial multidrug transporters. *Microbiol Mol Biol Rev* **64**, 672–693.
- Ruby, E. G., Urbanowski, M., Campbell, J., Dunn, A., Faini, M., Gunsalus, R., Lostroh, P., Lupp, C., McCann, J. & other authors (2005). Complete genome sequence of *Vibrio fischeri*: a symbiotic bacterium with pathogenic congeners. *Proc Natl Acad Sci U S A* **102**, 3004–3009.
- Sekiya, H., Mima, T., Morita, Y., Kuroda, T., Mizushima, T. & Tsuchiya, T. (2003). Functional cloning and characterization of a multidrug efflux pump, *mexHI-opmD*, from a *Pseudomonas aeruginosa* mutant. *Antimicrob Agents Chemother* **47**, 2990–2992.
- Sen, B., Dutta, B., Chatterjee, S., Bhattacharya, M. K., Nandy, R. K., Mukhopadhyay, A. K., Gangopadhyay, D. N., Bhattacharya, S. K. & Ramamurthy, T. (2007). The first outbreak of acute diarrhea due to a pandemic strain of *Vibrio parahaemolyticus* O3:K6 in Kolkata, India. *Int J Infect Dis* **11**, 185–187.
- Srikumar, R., Kon, T., Gotoh, N. & Poole, K. (1998). Expression of *Pseudomonas aeruginosa* multidrug efflux pumps MexA-MexB-OprM and MexC-MexD-OprJ in a multidrug-sensitive *Escherichia coli* strain. *Antimicrob Agents Chemother* **42**, 65–71.
- Su, H. P., Chiu, S. I., Tsai, J. L., Lee, C. L. & Pan, T. M. (2005a). Bacterial food-borne illness outbreaks in northern Taiwan, 1995–2001. *J Infect Chemother* **11**, 146–151.
- Su, X. Z., Chen, J., Mizushima, T., Kuroda, T. & Tsuchiya, T. (2005b). AbeM, an H⁺-coupled *Acinetobacter baumannii* multidrug efflux pump belonging to the MATE family of transporters. *Antimicrob Agents Chemother* **49**, 4362–4364.
- Tamura, N., Murakami, S., Oyama, Y., Ishiguro, M. & Yamaguchi, A. (2005). Direct interaction of multidrug efflux transporter AcrB and outer membrane channel TolC detected via site-directed disulfide cross-linking. *Biochemistry* **44**, 11115–11121.
- Thanassi, D. G., Cheng, L. W. & Nikaido, H. (1997). Active efflux of bile salts by *Escherichia coli*. *J Bacteriol* **179**, 2512–2518.
- Tseng, T. T., Gratwick, K. S., Kollman, J., Park, D., Nies, D. H., Goffeau, A. & Saier, M. H., Jr (1999). The RND permease superfamily: an ancient, ubiquitous and diverse family that includes human disease and development proteins. *J Mol Microbiol Biotechnol* **1**, 107–125.
- WHO (1999). *Vibrio parahaemolyticus*, Japan, 1996–1998. *Wkly Epidemiol Rec* **74**, 361–363.
- Xu, X. J., Su, X. Z., Morita, Y., Kuroda, T., Mizushima, T. & Tsuchiya, T. (2003). Molecular cloning and characterization of the HmrM multidrug efflux pump from *Haemophilus influenzae* Rd. *Microbiol Immunol* **47**, 937–943.
- Yamamoto, T. & Yokota, T. (1989). Adherence targets of *Vibrio parahaemolyticus* in human small intestines. *Infect Immun* **57**, 2410–2419.
- Yamazaki, M., Inuzuka, K., Matsumoto, M., Miwa, Y., Hiramatsu, R., Matsui, H., Sakae, K., Suzuki, Y. & Miyazaki, Y. (2003). Epidemiological study of outbreaks and sporadic cases due to *Vibrio parahaemolyticus* – serotype O3:K6 in Aichi Prefecture, Japan, during 1988 and 2001. *Kansenshogaku Zasshi* **77**, 1015–1023.

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