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EmmdR, a New Member of the MATE Family of Multidrug Transporters, Extrudes Quinolones from *Enterobacter cloacae*

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Abstract

We cloned a gene, ECL_03329, from the chromosome of *Enterobacter cloacae* ATCC13047, using a drug-hypersensitive *Escherichia coli* KAM32 cell as the host. We show here that this gene, designated *emmdR*, is responsible for multidrug resistance in *E. cloacae*. *E. coli* KAM32 host cells containing the cloned *emmdR* gene (KAM32/pEMMDR28) showed decreased susceptibilities to benzalkonium chloride, norfloxacin, ciprofloxacin, levofloxacin, ethidium bromide, acriflavine, rhodamine6G, and trimethoprim. *emmdR*-deficient *E. cloacae* cells (*EcΔemmdR*) showed increased susceptibilities to several of the antimicrobial agents tested. EmmdR has twelve predicted transmembrane segments and some shared identity with members of the Multidrug and Toxic Compound Extrusion (MATE) family of transporters. Study of the antimicrobial agent efflux activities revealed that EmmdR is an H⁺-drug antiporter but not a Na⁺ driven efflux pump. These results indicate that EmmdR is responsible for multidrug resistance and pumps out quinolones from *E. cloacae*.

Introduction

Enterobacter cloacae is an important nosocomial pathogen that causes a variety of infections, including bacteremia, lower respiratory tract infections, skin and soft-tissue infections, urinary tract infections (UTIs), endocarditis, intra-abdominal infections, septic arthritis, osteomyelitis, and ophthalmic infections (Fraser et al. 2010; Leverstein et al. 2006; Paterson et al. 2006). *E. cloacae* have a global presence in both adult and neonatal Intensive Care Units (ICUs). Recently, multidrug resistant *E. cloacae* have been reported with increasing frequency in clinical and environmental settings (Hidron et al. 2008; Rossi et al. 2006). Also, antimicrobial resistance has been shown to be conferred by several *E. cloacae* determinants, such as *ampC*, *gyrA*, *murA*, *qnrA*, *acrAB*, and *ramA* (Corkill et al. 2005; Diaz et al. 2006; Eschenburg et al. 2005; Giakkoupi et al. 2000; Kaneko et al. 2005; Keeney et al. 2009; Perez et al. 2007; and Weigel et al. 1998). These resistance genes, however, are not sufficient to account for the reported spectra of multidrug resistances, such as resistances to aminoglycosides, quaternary ammonium compounds, trimethoprim, and tetracycline. Thus, it is necessary to study unknown drug resistance mechanisms in this organism.

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Recent studies by Perez et al (2007) and Keeney et al (2007) have demonstrated that, besides AcrAB-TolC, other efflux pumps may contribute to antibiotic resistance in *E. cloacae*. The Multidrug and Toxic Compound Extrusion (MATE) family of transporters represent an important intrinsic multidrug resistance mechanism in many pathogenic bacteria and are responsible for resistances to aminoglycosides, fluoroquinolones, quaternary ammonium compounds (QACs), and dyes (Brown et al. 1999; Kuroda et al. 2009; Levy et al. 2002; Miyamae et al. 2002; Piddock et al. 2006; Poole et al. 2001). According to the genome sequence of *E. cloacae*, a putative MATE family transporter gene, ECL_03329, was found by Ren et al (2010). In this study, we characterized this putative MATE family transporter gene by performing gene cloning, gene expression, gene deletion, and physiological characterization. We conclude that EmmdR from *E. cloacae* is a new member of the MATE family of transporters.

Materials and Methods

We cloned the DNA encoding an open reading frame, ECL_03329 (http://www.genome.jp/dbget-bin/www_bget?enc:ECL_03329) by the polymerase chain reaction (PCR) method using the chromosomal DNA from *E. cloacae* ATCC13047 as the PCR template. The chromosomal DNA was prepared by using Qiagen Genomic Tip 500/G (QIAGEN Inc, USA). The primers used were P1-F, 5'-GGAATCGTTGTCGACGCTTA-3' (forward, containing a *Sal* I site) and P1-R, 5'-TTGTCAAAGGCAGAATTCAG-3' (reverse, containing an *Eco*R I site). The P1-F primer carries a putative promoter region for this gene. We designated the ECL_03329 gene as *emmdR*. Vector plasmid pSTV28 DNA was digested with *Sal* I and *Eco*R I (New England BioLabs, Inc, USA), dephosphorylated with calf intestinal alkaline phosphatase (Promega Co, USA), and then ligated the vector DNA to the 1681 bp PCR fragment containing *emmdR* using T4 DNA ligase (New England BioLabs, Inc, USA). The resulting hybrid plasmid harboring the *emmdR* gene was designated pEMMDR28. *E. coli* strain KAM32 was used as a host cell, which is a drug hypersensitive strain that lacks the efflux pump genes *acrB* and *ydhE* (Chen et al. 2002). All of these cells were grown aerobically in L-broth at 37 °C. Growth of cells was monitored turbidimetrically at 650 nm. Competent cells of *E. coli* KAM32 were transformed with the recombinant plasmids and spread onto agar plates containing L-broth, 20 µg/ml chloramphenicol, 1.5 % agar, and 4 µg/ml ethidium bromide. We chose ethidium bromide as the screening drug because it is a substrate for many transporters belonging to the MATE family. The plates were incubated aerobically at 37 °C for 24 h. Candidate colonies were replica-plated, and the plasmids were isolated from each of the candidates. The plasmids were then reintroduced into competent cells by transformation and spread onto the same type of plate as described above. The clone strain was designated *E. coli* KAM32/pEMMDR28. The *emmdR* gene was confirmed by PCR using the plasmid isolated from *E. coli* KAM32/pEMMDR28 as the template. The *emmdR* region is inverted toward the *lac* promoter of pSTV28, suggesting that the native promoter of the *emmdR* gene is functional in *E. coli* KAM32. The *emmdR* gene of *E. cloacae* was predicted to encode a protein of 462 amino acids. TCDB hydropathy analysis and GenomeNet TBLASTN suggested that *emmdR* encodes a hydrophobic protein with 12 transmembrane segments. We compared EmmdR with members of the MATE family of transporters by using a ClustalW search on GenomeNet. EmmdR showed 100% identity with ECL_03329 of *E. cloacae* ATCC13047, 55% identity and 64% similarity with NorMI of *Brucella melitensis* (Braibant et al. 2002), 39% identity and 54% similarity with MepA of *Staphylococcus aureus* (McAleese et al. 2005), 36% identity and 50% similarity with PmpM of *Pseudomonas aeruginosa* (He et al. 2004), and 37% identity and 82% similarity with HmrM of *Haemophilus influenzae* (Xu et al. 2004).

Deletion of *emmdR* by gene disruption was performed using an Flp-*FRT* recombination system, as previously reported (He et al. 2004; Hoang et al. 1998). To construct the *emmdR*-

deficient *E. cloacae* cell, a 1998 bp region (amplicon A) between positions 2766 and 768 upstream of *emmdR* and a 1192 bp region (amplicon B) between positions 784 and 1976 downstream of *emmdR* were amplified using chromosomal DNA from *E. cloacae* ATCC13047 as the template DNA. Primers were designed by using the OligoPerfect primer design tool from Invitrogen. P2-F: 5'-AACTCCCTTTTCCCGGGCAA -3' (forward, containing a *Sma* I site) and P2-R: 5'-CGCGTTGAGGATTCTCTGTT -3' (reverse, containing a *Bam* HI site) were used for amplicon A. P3-F: 5'-GGTAGAACCCGGGACTGTTA -3' (forward, containing a *Bam* HI site) and P3-R: 5'-TCGGAACCCGGGATGAAA-3' (reverse, containing a *Sma* I site) were used for amplicon B. The digested PCR fragments were ligated with the *Bam* HI-Gentamicin resistance cassette (G) obtained with the pPS858 vector (He et al. 2004). The constructed AGB fragment was inserted into a *Sma* I-digested plasmid pEX100T, which contains a *sacB* gene (Hoang et al. 1998), resulting in the plasmid pAGB. The *emmdR* gene was removed from the chromosome of *E. cloacae* ATCC13047 by replacing the *emmdR* region with the corresponding deleted region of pAGB by an FLP-*FRT* recombination system, as reported previously (Hoang et al. 1998). Inactivation of the *emmdR* gene by insertion of pAGB was confirmed by PCR amplification, and the gene-deleted *E. cloacae* mutant was designated *EcΔemmdR*.

Drug susceptibility tests were performed by the broth microdilution method as recommended by the Clinical and Laboratory Standards Institute (CLSI, <http://www.clsi.org>). The cells were incubated at 37 °C for 24 h in cation-adjusted Muller-Hinton broth (Difco, USA) using different concentrations of antimicrobial agents (Sigma-Aldrich, USA) (Huda et al. 2003; Levy et al. 2002). The substrate specificity profile of EmmdR was investigated by comparing the susceptibilities to various antimicrobial agents of KAM32/pSTV28 (control) with those of KAM32/pEMMDR28 (encodes the *emmdR* gene) and *EcΔemmdR* (*emmdR* gene deleted mutant). The MIC assays were repeated 3 times under the same growth conditions.

Ethidium efflux was measured using the efflux-accumulation method described by Mine et al. (1998). In order to establish that EmmdR has the ability to extrude drugs from bacterial cells, we measured the efflux of ethidium from within preloaded cells. We selected ethidium as a substrate, because it can bind to double-stranded DNA, resulting in a substantial increase in fluorescence. KAM32/pSTV28 (control) and KAM32/pEMMDR28 cells (contains the *emmdR* gene) were grown in L broth containing 20 mM potassium lactate and 20 µg/ml chloramphenicol under aerobic conditions at 37 °C. The cells were harvested at the late exponential phase of growth, washed twice with Tanaka buffer in which sodium salts were replaced with potassium salts and supplemented with 0.3 mM Mg₂SO₄ (Mine et al. 1998). Potassium was used in the reaction buffer, instead of sodium, because if EmmdR had been a Na⁺/drug antiporter, then the sodium cation would have affected the efflux assay. Finally, the cells were suspended in the same buffer to an optical density of 0.25 at 650 nm. Potassium lactate (final concentration 20 mM) was added to the cell suspension as an energy source and incubated at 37°C for 5 min (He et al. 2004; Mine et al. 1998). With gentle stirring, ethidium bromide was added to the cell suspension at a final concentration of 30 µM, to initiate the assay. Where specified, an H⁺-conductor, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), was added to a final concentration of 100 µM, to dissipate the electrochemical potential of protons across the membrane. Accumulation of ethidium in cells was monitored by measuring the fluorescence intensity at excitation and emission wavelengths of 500 nm and 580 nm, respectively, using a Hitachi F2500 fluorescence spectrophotometer (Mine et al. 1998).

H⁺-drug antiport assays were performed using the quinacrine fluorescence quenching method described by Mine et al. (1998) with some modifications. Efflux pumps drive drug

extrusion through coupling with a proton, a sodium cation, or ATP hydrolysis. In order to understand the energy mode that EmmdR uses to drive drug efflux, we tested first whether H^+ is the coupling cation by measuring H^+ -drug antiport activity. The H^+ -drug antiport activity was examined by observing fluorescence quenching of quinacrine in everted membrane vesicles prepared from cells of KAM32/pEMMDR28 and KAM32/pSTV28. Cells of KAM32/pSTV28 and KAM32/pEMMDR28 were grown in L broth supplemented with 20 mM potassium lactate and 20 μ g/ml chloramphenicol under aerobic conditions at 37 °C until the late exponential phase of growth (Mine et al. 1998). The cells were harvested, washed twice with Tris chloride buffer (10mM Tris, 140mM choline chloride, 5mM $MgSO_4$, 10% Glycerol, pH 7.5) and suspended in the same buffer. The cells were disrupted by passing through an ice-chilled Microfluidizer at 12000 p.s.i. three times; the membranes were collected by centrifugation at 30,000 rpm for one hour and stored at -80 °C (Krulwich et al. 2002; Mine et al. 1998). The protein concentration of the everted membrane vesicles was determined with the BCA™ Protein Assay Kit (Pierce Biotechnology, Inc) (He et al. 2004; Mine et al. 1998). Fluorescence quenching due to an inwardly directed H^+ transport by the respiratory chain was caused by the addition of potassium lactate in the assay mixture. Norfloxacin and ciprofloxacin were chosen as substrates for the assay because several other substrates of EmmdR had unfavorable effects on the fluorescence of quinacrine. The assay mixture was made of 10 mM MOPS-KOH (pH 7.0), 140 mM KCl, 1 μ M quinacrine, and 50 μ g/ml protein of membrane vesicles. Potassium lactate was added at 5 mM to initiate respiration. The quinacrine fluorescence quenching due to an electrochemical proton potential was measured at excitation and emission wavelengths of 420 nm and 500 nm, respectively (Chen et al. 2002; He et al. 2004; Mine et al. 1998), using a Hitachi F2500 fluorescence spectrophotometer. Triton X-100 was used to break down the H^+ -gradient, where indicated.

Quantitative real time reverse transcriptase polymerase chain reaction (RT-PCR) was performed using the method described by Li et al. (2006) with some modifications. The expression levels of *emmdR* were measured in the multidrug resistant isolate *E. cloacae* DFW9, *E. cloacae* ATCC23355, and *E. cloacae* ATCC13047. *E. cloacae* DFW9, a multidrug resistant isolate from dairy farm waste water, was identified using BD BBL Crystal Rapid Identification System and confirmed by 16S rRNA gene sequencing (He et al. unpublished data). The expression levels were normalized using the house-keeping gene, *rpoB*. Primers used for *rpoB* were P4-F: 5'-CAGCCGCGAACAGGTTGACTACA-3' (forward) and P4-R: 5'-GACGCACCGACGGATACCACCTG-3' (reverse); and for *emmdR*, the primers were P1-F and P1 – R. The total RNA was isolated with the Qiagen RNeasy Mini Kit (Qiagen Inc, USA), according to the manufacturer's instructions and treated with RNase-free DNase I (New England BioLabs, USA). The concentration of RNA was determined with a UV-spectrophotometer. DNase-treated total RNA (1 μ g) was used as a template for RT-PCR. cDNA synthesis and subsequent amplification were carried out with an iScript select cDNA synthesis kit, according the manufacturer's instructions (Bio-Rad). Quantitative real-time PCR (qRT-PCR) was performed with an iQ5 real-time PCR (RT-PCR) detection system (Bio-Rad) using Invitrogen's SYBR greenER QPCR SuperMix for iCycler. cDNA synthesized from the house-keeping gene *rpoB* was used as a control. Melting curve analysis was performed on all reactions to rule out secondary products and primer dimer formation (Li et al. 2006). The expression levels were analyzed using threshold cycle (C_T) values.

Results and Discussion

Drug susceptibility tests revealed cells harboring EmmdR showed decreased susceptibilities to benzalkonium chloride (4x), ethidium bromide (4x), acriflavine (8x), norfloxacin (16x), ciprofloxacin (16x), levofloxacin (4x), rhodamine6G (32x), and trimethoprim (8x); whereas

no significant changes were observed in the MICs of the drugs tested for KAM32/pSTV28 (control). The antimicrobial susceptibilities of *E. cloacae* ATCC13047 were compared with its *emmdR* gene-deficient mutant *EcΔemmdR*. As shown in Table 1, the *EcΔemmdR* strain had increased susceptibilities to acriflavine (0.25x), norfloxacin (0.25x), ethidium bromide (0.25x), rhodamine6G (0.125x), ciprofloxacin (0.25x), trimethoprim (0.125x), and benzalkonium chloride (0.5x) compared to the ATCC13047 strain. No such increases in susceptibilities to the other agents examined were observed during the MIC testing of the *EcΔemmdR* strain.

During the ethidium efflux assay, we observed a clear increase in ethidium fluorescence when a proton conductor, CCCP, was added to the assay mixture of KAM32/pEMMDR28 (Fig. 1). This suggested that accumulation of ethidium occurred after the addition of CCCP. On the other hand, only a slight change in fluorescence was caused by the addition of CCCP in the control cells of KAM32/pSTV28. These results indicate that cells of *E. coli* KAM32/pEMMDR28 possess an energy-dependent ethidium efflux activity.

Also, *EmmdR* is an H^+ -drug antiporter. As shown in Fig. 2, the addition of ciprofloxacin or norfloxacin to the assay mixture elevated quinacrine de-quenching, due to proton flux. This indicated that H^+ -drug antiport occurred across the everted membrane vesicles that were prepared from cells of KAM32/pEMMDR28. We also investigated whether H^+ -drug antiport would be observed across everted membrane vesicles prepared from the control cells of KAM32/pSTV28. No significant quinacrine de-quenching was observed in the everted membrane vesicles from KAM32/pSTV28. Thus, we believe that the coupling cation for transport by *EmmdR* is an H^+ .

The qRT-PCR data indicated the expression level of *emmdR* in *E. cloacae* DFW9 is lower than in *E. cloacae* ATCC13047 and ATCC23355, in which the mean C_T value of *emmdR* in *E. cloacae* DFW9 was 20.8; in *E. cloacae* ATCC13047 the mean C_T value was 18.6; and in ATCC 23355 the mean C_T value was 18.3.

Proteins of the MATE family usually consist of 420 to 470 amino acid residues and are predicted to form 12 transmembrane segments (Brown et al. 1999; Guelfo et al. 2010; Kuroda et al. 2009; Morida et al. 1998). To date, several MATE family members have been identified, such as NorM of *Vibrio parahaemolyticus* (Morita et al. 1998), NorMI of *B. melitensis* (Braibant et al. 2002), HmrM of *H. influenzae* (Xu et al. 2003), PmpM of *P. aeruginosa* (He et al. 2004), VcrM of *Vibrio cholerae* (Huda et al. 2002), AbeM of *Acinetobacter baumannii* (Su et al. 2005), NorM of *Neisseria gonorrhoeae* (Rouquette-Loughlin C et al. 2003), CdeA of *Clostridium difficile* (Dridi et al. 2004), BexA of *Bacteroides thetaiotaomicron* (Miyamae et al. 2002), MepA of *S. aureus* (McAleese et al. 2005), and NorM of *E. coli* (Guelfo et al. 2010). These MATE family members were found to be encoded on the chromosome and are responsible for resistance to fluoroquinolones, disinfectants, H_2O_2 , and dyes (Guelfo et al. 2010). Since *EmmdR* has 12 transmembrane segments and has substrates similar to other MATE family members, we thus compared *EmmdR* with the above described MATE family members. We found that over-expression of *EmmdR* mediated high level resistances to norfloxacin, ciprofloxacin, rhodamine6G, benzalkonium chloride, acriflavine, trimethoprim and low level resistances to ethidium bromide and levofloxacin. *E. cloacae* cells lacking this protein (*EcΔemmdR*) resulted in increased susceptibilities to fluoroquinolones, trimethoprim, disinfectants, and dyes. We prepared a phylogenetic relationship tree (data not shown) for a comparison of *EmmdR* with reported MATE family members, using a multiple sequence alignment generated by the ClustalW search algorithm (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>). The phylogenetic relationship tree indicated that *EmmdR* of *E. cloacae* and HmrM of *H. influenzae* are in the

same root. EmmdR appears to be a new member of the MATE family of transporters according to the phylogenetic tree.

In conclusion, EmmdR from *E. cloacae* is a new member of the MATE family of multidrug transporters. We have provided physiological evidence for the transport of ethidium bromide, norfloxacin, and ciprofloxacin to indicate that EmmdR mediates resistances to these antimicrobial agents. EmmdR is thus an important multidrug resistance mechanism in *E. cloacae* which extrudes quinolones. Also, our data indicated that EmmdR is an H⁺/drug antiporter. The drug susceptibility profile of the *emmdR*-deficient mutant has confirmed that EmmdR confers resistances to quinolones, dyes, and benzalkonium chloride in *E. cloacae*.

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References

- Braibant L, Guilloteau L, Zygmunt MS. Functional Characterization of *Brucella melitensis* NorMI, an Efflux Pump Belonging to the Multidrug and Toxic Compound. *Antimicrob Agents Chemother.* 2002; 46:3050–3053. [PubMed: 12183269]
- Brown MH, Paulsen IT, Skurray RA. The multidrug efflux protein, NorM is a prototype of a new family of transporters. *Mol Microbiol.* 1999; 31:394–395. [PubMed: 9987140]
- Chen J, Morita Y, Huda MN, Kuroda T, Mizushima T, Tsuchiya T. VmrA, a member of a novel class of Na⁺-coupled multidrug efflux pumps from *Vibrio parahaemolyticus*. *J Bacteriol.* 2002; 184:572–576. [PubMed: 11751837]
- Corkill JE, Anson JJ, Hart CA. High prevalence of the plasmid-mediated quinolone resistance determinant *qnrA* in multidrug-resistant Enterobacteriaceae from blood cultures in Liverpool, UK. *J Antimicrob Chemother.* 2005; 56:1115–1117. [PubMed: 16260446]
- Diaz MA, Cooper RK, Cloeckaert A, Siebeling RJ. Plasmid-mediated high-level gentamicin resistance among enteric bacteria isolated from pet turtles in Louisiana. *Appl Environ Microbiol.* 2006; 72:306–312. [PubMed: 16391058]
- Dridi L, Tankovic J, Petit JC. CdeA of *Clostridium difficile*, a new multidrug efflux transporter of the MATE family. *Microb Drug Resist.* 2004; 10:191–6. [PubMed: 15383161]
- Eschenburg S, Priestman M, Schönbrunn E. Evidence that the fosfomycin target Cys115 in UDP-N-acetylglucosamine enolpyruvyl transferase (MurA) is essential for product release. *J Biol Chem.* 2005; 280(5):3757–63. [PubMed: 15531591]
- Fraser, S.; Anette, M.; Sinave, CP. *Enterobacter Infections.* 2010. <http://emedicine.medscape.com/article/216845-overview>
- Giakkoupi P, Tzouveleakis LS, Tsakris A, Loukova V, Sofianou D, Tzelepi E. IBC-1, a novel integron-associated class A beta-lactamase with extended-spectrum properties produced by an *Enterobacter cloacae* clinical strain. *Antimicrob Agents Chemother.* 2000; 44:2247–2253. [PubMed: 10952563]
- Guelfo JR, Rodríguez-Rojas A, Matic I, Blázquez J. A MATE-Family Efflux Pump Rescues the *Escherichia coli* 8-Oxoguanine-Repair-Deficient Mutator Phenotype and Protects Against H₂O₂ Killing. *PLoS Genet.* 2010; 6(5):e1000931. [PubMed: 20463878]
- He GX, Kuroda T, Mima T, Morita Y, Mizushima T, Tsuchiya T. An H(+)-coupled multidrug efflux pump, PmpM, a member of the MATE family of transporters, from *Pseudomonas aeruginosa*. *J Bacteriol.* 2004; 186:262–265. [PubMed: 14679249]
- Hidron AI, Edwards JR, Patel J, Horan TC, Sievert DM, Pollock DA. NHSN annual update: antimicrobial-resistant pathogens associated with healthcare-associated infections: annual summary of data reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention (2006–2007). *Infect Control Hosp Epidemiol.* Nov; 2008 29(11):996–1011. [PubMed: 18947320]

- Hoang T, RoxAnn R, Schweizer K, Kutchma A, Schweizer H. A broad-host-range Flp-FRT recombination system for site-specific excision of chromosomally-located DNA sequences: application for isolation of unmarked *Pseudomonas aeruginosa* mutants. *Gene*. 1998; 212:77–86. [PubMed: 9661666]
- Huda MN, Chen J, Morita Y, Kuroda T, Mizushima T, Tsuchiya T. Gene cloning and characterization of VcrM, a Na⁺-coupled multidrug efflux pump, from *Vibrio cholerae* non-O1. *Microbiol Immunol*. 2003; 47(6):419–27. [PubMed: 12906102]
- Kaneko K, Okamoto R, Nakano R, Kawakami S, Inoue M. Gene mutations responsible for overexpression of AmpC beta-lactamase in some clinical isolates of *Enterobacter cloacae*. *J Clin Microbiol*. 2005; 43:2995–2998.
- Keeney D, Ruzin A, Bradford PA. RamA, a transcriptional regulator, and AcrAB, an RND-type efflux pump, are associated with decreased susceptibility to tigecycline in *Enterobacter cloacae*. *Microb Drug Resist*. 2007; 13(1):1–6. [PubMed: 17536927]
- Krulwich T, Guffanti A, Wei Y, Rood S. An antiporter mechanism for a member of the cation diffusion facilitator family: divalent cations efflux in exchange for K⁺ and H⁺ *Mol Microbiol*. 2002; 45:145–153. [PubMed: 12100555]
- Kuroda T, Tsuchiya T. Multidrug efflux transporters in the MATE family. *BBA - Proteins & Proteomics*. 2009; 5:763–768. [PubMed: 19100867]
- Levy SB. Active efflux, a common mechanism for biocide and antibiotic resistance. *J Appl Microbiol*. 2002; 92:65S–71S. [PubMed: 12000614]
- Leverstein-van, Hall MA.; Blok, HE.; Paauw, A.; Fluit, AC.; Troelstra, A.; Mascini, EM.; Bonten, MJ.; Verhoef, J. Extensive hospital-wide spread of a multidrug-resistant *Enterobacter cloacae* clone, with late detection due to a variable antibiogram and frequent patient transfer. *J Clin Microbiol*. 2006; 44:518–524. [PubMed: 16455907]
- Li W, Hartung JS, Levy L. Quantitative real-time PCR for detection and identification of *Candidatus Liberibacter* species associated with citrus huanglongbing. *J Microbiol Meth*. 2006; 66:104–115.
- McAleese F, Petersen P, Ruzin A, Dunman PM, Murphy E, Projan SJ, Bradford PA. A Novel MATE Family Efflux Pump Contributes to the Reduced Susceptibility of Laboratory-Derived *Staphylococcus aureus* Mutants to Tigecycline. *Antimicrob Agents Chemother*. 2005; 49:1865–1871. [PubMed: 15855508]
- Miyamae S, Ueda O, Yoshimura F, Hwang J, Tanaka Y, Nikaido H. A MATE family multidrug efflux transporter pumps out fluoroquinolones in *Bacteroides fragilis*. *Antimicrob Agents Chemother*. 2002; 45:3341–3346. [PubMed: 11709306]
- Mine T, Morita Y, Kataoka A, Mizushima T, Tsuchiya T. Evidence for chloramphenicol/H⁺ antiporter in Cmr (MdfA) system of *Escherichia coli* and properties of the antiporter. *J Biochem (Tokyo)*. 1998; 124:187–193. [PubMed: 9644262]
- Morita Y, Kodama K, Shiota S, Mine T, Kataoka A, Mizushima T, Tsuchiya T. NorM, a putative multidrug efflux protein, of *Vibrio parahaemolyticus*, and its homolog in *Escherichia coli*. *Antimicrob Agents Chemother*. 1998; 42:1778–1782. [PubMed: 9661020]
- Paterson DL. Resistance in Gram-negative bacteria: Enterobacteriaceae. *The Am J Med*. 2006; 119:S20–28.
- Pérez A, Canle D, Latasa C, Poza M, Beceiro A, Tomás Mdel M, Fernández A, Mallo S, Pérez S, Molina F, Villanueva R, Lasa I, Bou G. Cloning, nucleotide sequencing, and analysis of the AcrAB-TolC efflux pump of *Enterobacter cloacae* and determination of its involvement in antibiotic resistance in a clinical isolate. *Antimicrob Agents Chemother*. 2007; 51(9):3247–53. [PubMed: 17638702]
- Piddock L. Multidrug-resistance efflux pumps ? not just for resistance. *Nature Reviews Microbiology*. 2006; 4:629–636.
- Poole K. Multidrug efflux pumps and antimicrobial resistance in *Pseudomonas aeruginosa* and related organisms. *J Mol Microbiol Biotechnol*. 2001; 3:255–264. [PubMed: 11321581]
- Ren Y, Ren Y, Zhou Z, Guo X, Li Y, Feng L, Wang L. Complete Genome Sequence of *Enterobacter cloacae* subsp. *cloacae* Type Strain ATCC 13047. *J Bacteriol*. 2010; 192(9):2463–2464. [PubMed: 20207761]

- Rossi F, Baquero F, Hsueh PR. In vitro susceptibilities of aerobic and facultatively anaerobic Gram-negative bacilli isolated from patients with intra-abdominal infections worldwide: (2004) results from SMART (Study for Monitoring Antimicrobial Resistance Trends). *J Antimicrob Chemother.* 2006; 58(1):205–210. [PubMed: 16717055]
- Rouquette-Loughlin C, Dunham SA, Kuhn M, Balthazar JT, Shafer WM. The NorM Efflux Pump of *Neisseria gonorrhoeae* and *Neisseria meningitidis* Recognizes Antimicrobial Cationic Compounds. *J Bacteriol.* 2003; 185:1101–1106. [PubMed: 12533487]
- Su XZ, Chen J, Mizushima T, Kuroda T, Tsuchiya T. AbeM, an H⁺-Coupled *Acinetobacter baumannii* Multidrug Efflux Pump Belonging to the MATE Family of Transporters. *Antimicrob Agent Chemother.* 2005; 49:4362–4364.
- Weigel LM, Steward CD, Tenover FC. *gyrA* mutations associated with fluoroquinolone resistance in Eight species of Enterobacteriaceae. *Antimicrob Agents Chemother.* 1998; 42:2661–2667. [PubMed: 9756773]
- Xu XJ, Su XZ, Morita Y, Kuroda T, Mizushima T, Tsuchiya T. Molecular cloning and characterization of HmrM multidrug efflux pump from *Haemophilus influenzae* Rd. *Microbiol Immunol.* 2003; 47:937–943. [PubMed: 14695443]

Figure 1. Ethidium bromide accumulation in cells

Ethidium bromide accumulation in cells of *E. coli* KAM32/pSTV28 (control) and *E. coli* KAM32/pEMMDR28 (carrying *emmDR*) was monitored continuously by a Hitachi F-2500. ^aEthidium bromide (ETB; final concentration was 20 μ M) was added to the assay mixture to initiate the assay, and ^bcarbonyl cyanide-m-chlorophenylhydrazone (CCCP; final concentration was 50 μ M) was added to de-energize the cells at the time point indicated. A.U. = arbitrary units of fluorescence quenching.

Figure 2. H^+ -drug antiport assay in everted membrane vesicles

Cells of *E. coli* KAM32/pSTV28^{b,d} (control) and *E. coli* KAM32/pEMMDR28^{a,c} (carrying *emmdR*) were grown in L-broth supplemented with 20 mM potassium lactate, and everted membrane vesicles were prepared by passing the cells through a French press. H^+ -drug antiport was measured by quinacrine fluorescence quenching (the final concentration of quinacrine was 1.0 mM). At the time point indicated by the arrow, potassium lactate (5mM) was added to initiate respiration. After the fluorescence quenching reached a steady state, ciprofloxacin (40 μ M) or norfloxacin (40 μ M) was added to the assay mixture. Finally, at the time point indicated by the arrow, Triton X-100 was added at a concentration of 0.0125% to break down the H^+ gradient. ^{a,c} indicated the fluorescence dequenching which occurred after adding the ciprofloxacin or norfloxacin (40 μ M) to the reaction mixture containing everted membrane vesicles from *E. coli* KAM32/pEMMDR28; ^{b,d} indicated that there was no significant fluorescence intensity change having occurred after adding drugs in the everted membrane vesicles prepared from KAM 32/pSTV28.

Table 1Antimicrobial Susceptibilities of *E. cloacae* and *E. coli*.

Drug ^a	MIC(μg/ml)			
	<i>E. cloacae</i>		<i>E. coli</i>	
	ATCC13047	<i>EcΔemmR</i> ^b	KAM32/pSTV28	KAM32/pEMMR28 ^c
ACR	>128	32 (0.25) ^d	2	16 (8) ^d
ETB	32	8 (0.25)	2	8 (4)
RHO	256	32 (0.125)	4	128 (32)
NOR	0.032	0.008 (0.25)	0.016	0.256 (16)
CIP	0.064	0.016 (0.25)	0.004	0.064 (16)
LEV	0.032	0.008 (0.25)	0.002	0.008 (4)
SXT	4	2 (0.5)	0.12	0.96 (8)
BEN	4	4	0.5	2
TPPCI	64	64	4	8
SDS	256	256	32	32
GEN	0.25	0.25	2	2
KAN	1	1	1	1
AMC	32	32	4	4
AMP	2	2	1	1
CHL	64	64	24	24
ERY	32	32	2	4
FOS	16	16	2	4
TET	2	2	0.5	0.5
TRI	N	N	1	1

^a AMC, amoxicillin; AMP, ampicillin; GEN, gentamicin; KAN, kanamycin; SDS, sodium dodecyl sulfate; NOR, norfloxacin; CIP, ciprofloxacin; LEV, levofloxacin; CHL, chloramphenicol; ERY, erythromycin; FOS, fosfomycin; TET, tetracycline; ACR, acriflavine; BEN, benzalkonium chloride; TRI, triclosan; SXT, trimethoprim; ETB, ethidium bromide; RHO, rhodamine6G; TPPCI, tetraphenylphosphonium chloride.

^b *emmR* gene deleted *E. cloacae* ATCC13047.

^c *E. coli* KAM32 strains carrying the *emmR* gene.

^d Relative resistance