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An H^+-Coupled Multidrug Efflux Pump, PmpM, a Member of the MATE Family of Transporters, from *Pseudomonas aeruginosa*

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We cloned the gene PA1361 (we designated the gene *pmpM*), which seemed to encode a multidrug efflux pump belonging to the MATE family, of *Pseudomonas aeruginosa* by the PCR method using the drug-hypersensitive *Escherichia coli* KAM32 strain as a host. Cells of *E. coli* possessing the *pmpM* gene showed elevated resistance to several antimicrobial agents. We observed energy-dependent efflux of ethidium from cells possessing the *pmpM* gene. We found that PmpM is an H^+-drug antiporter, and this finding is the first reported case of an H^+-coupled efflux pump in the MATE family. Disruption and reintroduction of the *pmpM* gene in *P. aeruginosa* revealed that PmpM is functional and that benzalkonium chloride, fluoroquinolones, ethidium bromide, acriflavine, and tetraphenylphosphonium chloride are substrates for PmpM in this microorganism.

Pseudomonas aeruginosa is an opportunistic pathogen and a leading cause of nosocomial infections. A major problem in the treatment of patients infected with *P. aeruginosa* is that this bacterium shows intrinsic and acquired resistance against many antibiotics and disinfectants, including most β-lactams, fluoroquinolones, tetracycline, chloramphenicol, erythromycin, and benzalkonium chloride (4, 7, 11, 12, 13, 15, 20, 27). Several mechanisms for drug resistance are known, such as (i) inactivation of drugs by degradation or modification, (ii) alteration of the target, (iii) emergence of an alternative pathway, and (iv) active efflux of the drug. Among these mechanisms, active efflux, especially multidrug efflux, has been recognized as a major mechanism for multidrug resistance. Once a bacterium acquires a gene for a certain multidrug efflux pump or if a silent or weak gene for a multidrug efflux pump is activated, the cell instantly becomes resistant to many antimicrobial agents because multidrug efflux pumps extrude many structurally unrelated antimicrobial agents from cells. Thus, multidrug efflux pumps play important roles in multidrug resistance in bacteria. Multidrug resistance in *P. aeruginosa* has been attributed mainly to the activity of several multidrug efflux pumps. MexAB-OprM (11, 15), MexCD-OprJ (20, 23), MexEF-OprN (7), MexXY-OprM (15, 16), MexJK-OprM (4), MexHI-OpmD (27), MexVW-OprM (13), and EmrE (12) have been characterized and are potent multidrug efflux pumps in *P. aeruginosa*. MexAB-OprM is the only constitutive Mex pump in wild-type *P. aeruginosa* (11, 15). MexCD-OprJ (20, 23) and MexXY-OprM (15, 16) are inducible pumps, and MexEF-OprN (7), MexJK-OprM (4), MexHI-OpmD (27), and MexVW-OprM (13) are silent pumps in the wild-type strain.

The genome sequence of *P. aeruginosa* (29; http://www.Pseudomonas.com) suggests the presence of about 34 multidrug efflux pumps in this microorganism. Twenty of these pumps belong to the major facilitator superfamily, 10 or 12 belong to the resistance nodulation cell division family, 6 belong to the small multidrug resistance (SMR) family (12), 2 or 3 belong to the multidrug and toxic compound extrusion (MATE) family, and 1 belongs to the ATP binding cassette superfamily. Multidrug efflux pumps of the major facilitator, resistance nodulation cell division, and SMR families utilize an electrochemical potential of H^+ across the cytoplasmic membrane as the driving force for drug extrusion. Pumps of the ATP binding cassette superfamily utilize ATP as an energy source. The driving force in the MATE family is unique. Pumps of this family utilize an electrochemical potential of Na^+ across the cytoplasmic membrane as the driving force (2, 19, 21, 22).

We are especially interested in multidrug efflux pumps belonging to the MATE family because of its unique energy coupling. So far, we have characterized several MATE family pumps, such as NorM (18) and VmrA (2) of *Vibrio parahaemolyticus*, YdhE of *Escherichia coli* (18), and VcmA (22) and VcrM (21) of *Vibrio cholerae* non-O1. We have shown that all of them utilize an electrochemical potential of Na^+ across membranes as the driving force (2, 18, 21, 22). Here, we report that PmpM (a product of the PA1361 gene) (http://www.Pseudomonas.com) of *P. aeruginosa* is a unique multidrug efflux pump belonging to the MATE family that utilizes H^+, but not Na^+, as the coupling ion for drug extrusion.

Cloning of *pmpM*. We cloned the open reading frame PA1361 (http://www.Pseudomonas.com) by the PCR method using chromosomal DNA of *P. aeruginosa* PAO1 as a template. Chromosomal DNA was prepared by the procedure described by Chen (3). The primers used were forward primer 1 (F1), 5′-CTACGGAATTCCCCTGCCCAGACAAGGAC-3′ (containing an *Eco*RI site), and reverse primer 1 (R1), 5′-TCCTCGCCTCGGTCGACACTACCCTCAG-3′ (containing a *Sal*I site). The primer F1 carries the Shine-Dalgarno sequence of the *pmpM* gene but not a putative promoter of the gene. We designated the gene *pmpM* (*Pseudomonas* MATE family efflux pump). The vectors used were pSTV28 and pUCP20T, and the

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TABLE 1. MICs of various antimicrobial agents for *E. coli* KAM32/pSTV28 and *E. coli* KAM32/pPBE2

Drug	MIC (μg/ml) for:	
	E. coli KAM32/ pSTV28	*E. coli* KAM32/ pPBE2
Norfloxacin	0.015	0.06
Ciprofloxacin	0.002	0.015
Ofloxacin	0.008	0.03
Fradiomycin	0.5	2
Streptomycin	1	1
Kanamycin	0.5	0.5
Erythromycin	2	2
Ampicillin	1	1
Tetracycline	0.5	0.5
Benzalkonium chloride	1.2	37.5
Chlorhexidine gluconate	4.5	18
Triclosan	2	2
Ethidium bromide	2	16
TPPCl	4	128
Rhodamine 6G	4	64
Acriflavine	1	2

resulting hybrid plasmids, pPBE2 and pUPBE2T, respectively, carry the *pmpM* gene but not its original promoter. The cloned *pmpM* gene is located downstream from the lactose promoter in the two plasmids. The addition of an inducer of the lactose operon (isopropyl β-D-thiogalactopyranoside) did not have a significant effect on the level of drug resistance when cells were grown in L broth, which may contain a natural inducer. Thus, we investigated drug resistance without the addition of an inducer.

Drug susceptibility. To investigate the role of PmpM in drug resistance, plasmid pPBE2, carrying the *pmpM* gene, was introduced into cells of drug-hypersensitive *E. coli* KAM32 (Δ*acrB* Δ*ydhE* Hsd$^-$) (2). We compared the MICs of various antimicrobial agents for *E. coli* KAM32/pPBE2 and *E. coli* KAM32/pSTV28 (control) (Table 1). Elevated MICs of fluoroquinolones, fradiomycin, benzalkonium chloride, chlorhexidine gluconate, ethidium bromide, tetraphenylphosphonium chloride (TPPCl), and rhodamine 6G were observed for KAM32/pPBE2. Thus, the *pmpM* gene is responsible for conferring multidrug resistance. It seems that the disinfectant benzalkonium chloride is a good substrate for PmpM when expressed in *E. coli* cells.

Drug transport via PmpM and cation coupling. PmpM is a member of the MATE family of efflux pumps. Members of this family, such as NorM (18, 19) and VmrA (2) of *V. parahaemolyticus* and YdhE of *E. coli* (18), are Na^+-coupled efflux pumps. Thus, it was anticipated that PmpM would also be a Na^+-coupled efflux pump. If this were the case, then ethidium efflux would be very weak in the absence of Na^+ and greatly enhanced by the addition of Na^+ to the assay mixture. Thus, we tested the effect of NaCl on ethidium efflux. First, we prepared energy-starved and ethidium-loaded cells (17, 22). Then, lactate was added to energize the cells, and ethidium efflux was measured in the absence or presence of NaCl. Ethidium efflux energized by lactate was observed with cells of *E. coli* KAM32/pPBE2 but not with cells of KAM32/pSTV28 (Fig. 1). Addition of NaCl resulted in no significant change in the efflux. We added various concentrations of NaCl ranging

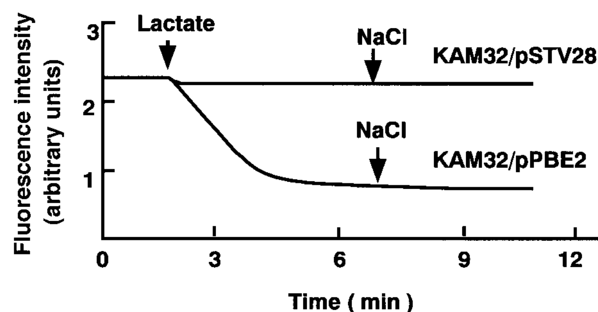

FIG. 1. Ethidium transport assays. Cells (*E. coli* KAM32/pSTV28 and KAM32/pPBE2) were grown in L broth (10) under aerobic conditions at 37°C. The cells were harvested, washed twice with 0.1 M MOPS (morpholinepropanesulfonic acid)–tetramethylammonium hydroxide (pH 7.0) containing 2 mM $MgSO_4$, and suspended in the same medium containing 25 μM ethidium bromide and 40 μM carbonyl cyanide *m*-chlorophenylhydrazone (16, 21). The cells were incubated at 37°C for 1 h to starve cellular energy and to load with ethidium (16, 21). The cells were washed three times with the same medium (no carbonyl cyanide *m*-chlorophenylhydrazone) and resuspended in the same medium. This cell suspension was preincubated at 37°C for 5 min, and lactic acid (the pH was adjusted to 7.0 with tetramethylammonium hydroxide) was added to the assay mixture at 40 mM. The change in the fluorescence intensity was measured. Where indicated, NaCl was added at 10 mM to test the effect of NaCl.

from 1 to 100 mM and observed no significant effect (data not shown). Addition of NaCl prior to the addition of lactic acid gave no significant effect. We also tested the effect of LiCl, because Li^+ can replace Na^+ as a coupling cation in the case of NorM (18). However, no significant effect of LiCl was observed (data not shown).

If the coupling ion is Na^+, we should be able to observe Na^+ efflux from cells elicited by influx of substrate, as observed with NorM (18, 19) and VmrA (2). We measured Na^+ flux by using a Na^+ electrode. No Na^+ flux was detected when substrates of the PmpM pump were added to a suspension of Na^+-loaded cells of *E. coli* KAM32/pPBE2, although Na^+ efflux was observed with cells of *E. coli* KAM32/pMVP36 (data not shown). pMVP36 carries *norM*, the gene for a Na^+-coupled multidrug efflux pump, NorM, belonging to the MATE family (18, 19). Thus, it is highly likely that H^+ instead of Na^+ is the coupling cation for PmpM.

If the coupling cation in PmpM is an H^+, then it must be an H^+-substrate antiporter. One convenient method to test this possibility is to measure the flux of H^+ caused by a substrate of the pump involved. A previous study has reported evidence for H^+-chloramphenicol antiport in a multidrug efflux pump, Cmr (MdfA), by measuring fluorescence quenching of quinacrine (17). Therefore, we measured fluorescence quenching in everted membrane vesicles prepared from cells of *E. coli* KAM32/pSTV28 and KAM32/pPBE2 (Fig. 2). Fluorescence quenching due to inwardly directed H^+ transport by the respiratory chain was elicited by the addition of lactate in both types of everted membrane vesicles. We chose benzalkonium chloride as the substrate for the assay because some other substrates of PmpM had some unfavorable effects on the fluorescence of quinacrine. Addition of benzalkonium chloride to the assay mixture caused efflux of H^+, indicating that H^+-benza-

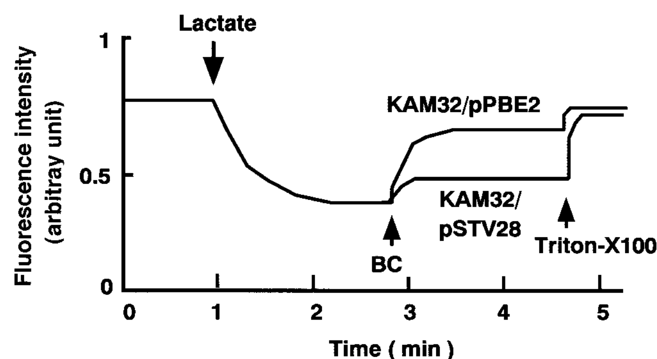

FIG. 2. Benzalkonium chloride-H^+ antiport activity in membrane vesicles. Cells (*E. coli* KAM32 and KAM32/pPBE2) were grown in L broth supplemented with 20 mM potassium lactate, and everted membrane vesicles were prepared by passing cells through a French press (9). Benzalkonium chloride-H^+ antiport was measured by quinacrine fluorescence quenching (the final concentration of quinacrine was 0.5 μM). At the time point indicated by the arrow, potassium lactate (5 mM) was added to initiate respiration. After the fluorescence quenching reached a steady state, benzalkonium chloride (BC; the final concentration was 0.0004%) 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 collapse the H^+ gradient.

lkonium chloride antiport took place. Thus, we conclude that the coupling ion in PmpM is H^+ but not Na^+.

We compared the amino acid sequences of Na^+-coupled pumps (NorM, YdhE, VmrA, VcmA, and VcrM) and H^+-coupled PmpM and tried to find residues or regions important for recognition of ions. Unfortunately, we have not succeeded in locating such residues or regions, so far. It would be interesting to isolate or construct mutant-type PmpMs (or NorMs) which show differences in ion recognition in order to gain an insight into the mechanism of ion recognition. Previously, other studies identified residues important for ion recognition in the melibiose transport protein by a similar strategy (5, 8).

We prepared a dendrogram using representatives of the MATE family of efflux pumps or putative pumps (data not shown). There are several subfamilies within the MATE family. NorM of *V. parahaemolyticus* (18), YdhE of *E. coli* (19), and VcmA of *V. cholerae* (22) belong to one subfamily. It seems that PmpM belongs to this subfamily but is a little apart from these three. VmrA of *V. parahaemolyticus* (2) and VcrM of *V. cholerae* (21) belong to another subfamily. All of the MATE family pumps so far characterized are Na^+-coupled efflux pumps. Since PmpM is an H^+-coupled pump, it is likely that there are other H^+-coupled pumps in the MATE family. It seems that PA5294 of *P. aeruginosa* is a member of the first subfamily, although PA5294 has not previously been recognized as a drug efflux pump. Thus, PA5294 may be a multidrug efflux pump and may be an H^+-coupled pump because it is apart from the Na^+-coupled NorM, YdhE, and VcmA pumps in the dendrogram.

Deletion of the *pmpM* gene and role of the PmpM pump in *P. aeruginosa*. So far, several *P. aeruginosa* mutants lacking genes for Mex multidrug efflux pumps have been constructed (20, 27). *P. aeruginosa* YM64 lacks four major Mex pumps, MexAB-OprM, MexCD-OprJ, MexEF-OprN, and MexXY (20). Recently, a mutant PMX52 lacking an additional pump,

TABLE 2. MICs of various antimicrobial agents for *P. aeruginosa* PMX52, PMX6, and PMX6/pUPBE2T

Drug	MIC (μg/ml) for:		
	PMX52	PMX6	PMX6/ pUPBE2T
Norfloxacin	0.03	0.03	0.12
Ciprofloxacin	0.06	0.06	0.24
Ofloxacin	0.06	0.06	0.12
Kanamycin	16	16	16
Fradiomycin	4	4	4
Tetracycline	0.25	0.25	0.25
Benzalkonium chloride	20	5	40
Chlorhexidine gluconate	5	5	5
Ethidium bromide	8	1	16
Acriflavine	2	0.25	2
TPPCl	32	8	64
Rhodamine 6G	16	16	16

MeHI-OpmD, was constructed from YM64 (27). The *pmpM* gene from the chromosome of PMX52 was deleted.

Chromosomal DNA from *P. aeruginosa* PAO1 was used as a template for PCR. We cloned a longer DNA region containing the *pmpM* gene for disruption of the gene, because longer fringe regions are better for recombination to take place. The primers used were forward primer 2 (F2), 5′-AGATAATTCA CCGGGCTCTTCG-3′ (containing an *Eco*RI site), and reverse primer 2 (R2), 5′-CCTTGCCCGGTACCCTGGAAAT GG-3′ (containing a *Kpn*I site). The PCR product with the length of 2.2-kbp was digested with *Eco*RI and *Kpn*I, the *Eco*RI-*Kpn*I fragment containing the *pmpM* gene was ligated into vector pSTV29 (TaKaRa Co., Kyoto, Japan), and the resulting pPBEA29 plasmid was obtained. The pPBEA29 plasmid was digested with *Stu*I, and a 1.9-kbp *Sma*I-*Sma*I fragment from pPS858 (6) containing a gentamicin-resistance marker sandwiched by two FRT sites was ligated to the *Stu*I-*Stu*I sites, which are present in the *pmpM* gene of pPBEA29. The resulting recombinant plasmid was designated pPBEA29G. Plasmid pPBEA29G contains a disrupted *pmpM* gene. The length of the deleted *Stu*I fragment is 796 bp. Finally, a 4.7-kbp *Ssp*I-*Ssp*I fragment from plasmid pPBEA29G was ligated to the *Sma*I site of pEX100T (30), which contains a *sacB* gene (6), to construct plasmid pPBEA29GS. The *pmpM* gene was removed from the chromosomal DNA of strain PMX52 by replacing the *pmpM* region with the corresponding deleted region of pPBEA29GS by a Flp-*FRT* recombination system, as previously reported (1, 20, 24, 25, 26, 28), to obtain strain PMX6. Disruption of the *pmpM* gene in PMX6 was confirmed by PCR methods.

We compared the MICs of various antimicrobial agents for *P. aeruginosa* PMX52, PMX6, and PMX6/pUPBE2T in order to evaluate the role of the PmpM pump in *P. aeruginosa* (Table 2). Deletion of the *pmpM* gene from the chromosome of PMX52 resulted in a decrease in the MICs of benzalkonium chloride, ethidium bromide, acriflavine, and TPPCl. Thus, we conclude that PmpM is functional in the parental cell PMX52. Since PMX52 is a deletion derivative of wild-type PAO1, it seems that PmpM is also functional in wild-type PAO1.

Seven Mex multidrug efflux pumps (MexAB-OprM, MexCD-OprJ, MexEF-OprN, MexXY-OprM, MexJK-OprM, MexHI-OpmD, and MexVW-OprM) (4, 7, 11, 12, 13, 15, 16,

20, 23, 27) and one SMR pump (12) have been characterized in *P. aeruginosa* so far. Benzalkonium chloride is an inducer and substrate of MexCD-OprJ (20). It has been reported that cells of *P. aeruginosa* that were adapted to benzalkonium chloride showed resistance to other membrane-active agents (14). One reason for this adaptive resistance might be that the MexCD-OprJ multidrug efflux pump is induced by benzalkonium chloride. It is also possible that another membrane-related mechanism(s) is involved in this adaptive resistance (14). The PmpM pump is also responsible for resistance to benzalkonium chloride. The MIC of benzalkonium chloride for *P. aeruginosa* PAO1 was 64 μg/ml. Deletion of the *mexCD-oprJ* operon reduced the MIC to 20 μg/ml. Further deletion of the *pmpM* gene reduced the value to 5 μg/ml (Table 2). It seems that these two pumps are major systems for extrusion of benzalkonium chloride in *P. aeruginosa*.

The MICs of fluoroquinolones, fradiomycin, chlorhexidine, and rhodamine 6G, which are thought to be substrates of PmpM in *E. coli* cells (Table 1), were not changed by *pmpM* gene disruption in *P. aeruginosa*. On the other hand, introduction of the *pmpM* gene into the *pmpM*-deleted PMX6 cell resulted in an increase in the MICs not only of benzalkonium chloride, ethidium bromide, acriflavine, and TPPCl but also of fluoroquinolones (norfloxacin, ciprofloxacin, and ofloxacin). The observed MICs of benzalkonium chloride, ethidium bromide, and TPPCl for PMX6/pUPBE2T were about twofold higher than those for PMX52, perhaps due to a gene dosage effect. The increase in the MICs of fluoroquinolones for PMX6/pUPBE2T may also be due to the gene dosage effect.

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REFERENCES

1. **Barekzi, N., K. Beinlich, T. T. Hoang, X.-Q. Pham, R. Karkhoff-Schweizer, and H. P. Schweizer.** 2000. High-frequency Flp recombinase-mediated inversions of the *oriC*-containing region of the *Pseudomonas aeruginosa* genome. J. Bacteriol. **182:**7070–7074.
2. **Chen, J., Y. Morita, M. Nazmul Huda, T. Kuroda, T. Mizushima, and T. Tsuchiya.** 2002. VmrA, a member of a novel class of Na^+-coupled multidrug efflux pumps from *Vibrio parahaemolyticus*. J. Bacteriol. **184:**572–576.
3. **Chen, W. P., and T. T. Kuo.** 1993. A simple and rapid method for the preparation of gram-negative bacterial genomic DNA. Nucleic Acids Res. **21:**2260.
4. **Chuanchuen, R., C. T. Narasaki, and H. P. Schweizer.** 2002. The MexJK efflux pump of *Pseudomonas aeruginosa* requires OprM for antibiotic efflux but not for efflux of triclosan. J. Bacteriol. **184:**5036–5044.
5. **Ding, P. Z., and T. H. Wilson.** 2001. The effect of modifications of the charged residues in the transmembrane helices on the transport activity of the melibiose carrier of *Escherichia coli*. Biochem. Biophys. Res. Commun. **285:**348–354.
6. **Hoang, T. T., R. R. Karkhoff-Schwiezer, A. J. Kutchman, and H. P. Schweizer.** 1998. 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 **212:**77–86.
7. **Kohler, T., M. Michea-Hamzehpour, U. Henze, N. Gotoh, L. K. Curty, and J. C. Pechere.** 1997. Characterization of MexE-MexF-OprN, a positively regulated multidrug efflux system of *Pseudomonas aeruginosa*. Mol. Microbiol. **23:**345–354.
8. **Kuroda, M., T. H. Wilson, and T. Tsuchiya.** 2001. Regulation of galactoside transport by the PTS. J. Mol. Microbiol. Biotechnol. **3:**381–384.
9. **Kuroda, T., T. Shimamoto, K. Inaba, M. Tsuda, and T. Tsuchiya.** 1994. Properties and sequences of the NhaA Na^+/H^+ antiporter of *Vibrio parahaemolyticus*. J. Biochem. **116:**1030–1038.
10. **Lennox, E. S.** 1995. Transduction of linked genetic characters of host by bacteriophage P1. Virology **1:**190–206.
11. **Li, X. Z., H. Nikaido, and K. Poole.** 1995. Role of *mexA-mexB-oprM* in antibiotic efflux in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **39:**1948–1953.
12. **Li, X. Z., K. Poole, and H. Nikaido.** 2003. Contributions of MexAB-OprM and an EmrE homolog to intrinsic resistance of *Pseudomonas aeruginosa* to aminoglycosides and dyes. Antimicrob. Agents Chemother. **47:**27–33.
13. **Li, Y., T. Mima, Y. Komori, Y. Morita, T. Kuroda, T. Mizushima, and T. Tsuchiya.** 2003. A new member of the tripartite multidrug efflux pumps, MexVW-OprM, in *Pseudomonas aeruginosa*. J. Antimicrob. Chemother. **52:**572–575.
14. **Loughlin, M. F., M. V. Jones, and P. A. Lambert.** 2002. *Pseudomonas aeruginosa* cells adapted to benzalkonium chloride show resistance to other membrane-active agents but not to clinically relevant antibiotics. J. Antimicrob. Chemother. **49:**631–639.
15. **Masuda, N., E. Sakagawa, S. Ohya, N. Gotoh, H. Tsujimoto, and T. Nishino.** 2000. Substrate specificities of MexAB-OprM, MexCD-OprJ, and MexXY-OprM efflux pumps in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **44:**3322–3327.
16. **Mine, T., Y. Morita, A. Kataoka, T. Mizushima, and T. Tsuchiya.** 1999. Expression in *Escherichia coli* of a new multidrug efflux pump, MexXY, from *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **43:**415–417.
17. **Mine, T., Y. Morita, A. Kataoka, T. Mizushima, and T. Tsuchiya.** 1998. Evidence for chloramphenicol/H^+ antiport in Cmr (MdfA) system of *Escherichia coli* and properties of the antiporter. J. Biochem. **124:**187–193.
18. **Morita, Y., A. Kataoka, S. Shiota, T. Mizushima, and T. Tsuchiya.** 2000. NorM of *Vibrio parahaemolyticus* is an Na^+-driven multidrug efflux pump. J. Bacteriol. **182:**6694–6697.
19. **Morita, Y., K. Kodama, S. Shiota, T. Mine, A. Kataoka, T. Mizushima, and T. Tsuchiya.** 1998. NorM, a putative multidrug efflux protein, of *Vibrio parahaemolyticus* and its homolog in *Escherichia coli*. Antimicrob. Agents Chemother. **42:**1778–1782.
20. **Morita, Y., Y. Komori, T. Mima, T. Kuroda, T. Mizushima, and T. Tsuchiya.** 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.
21. **Nazmul Huda, M., J. Chen, Y. Morita, T. Kuroda, T. Mizushima, and T. Tsuchiya.** 2003. Gene cloning and characterization of VcrM, a Na^+-coupled multidrug efflux pump, from *Vibrio cholerae* non-O1. Microbiol. Immunol. **47:**419–427.
22. **Nazmul Huda, M., Y. Morita, T. Kuroda, T. Mizushima, and T. Tsuchiya.** 2001. Na^+-driven multidrug efflux pump VcmA from *Vibrio cholera* non-O1, a non-halophilic bacterium. FEMS Microbiol. Lett. **203:**235–239.
23. **Poole, K., K. Tetro, Q. Zhao, A. Wada, T. Yamasaki, S. Neshat, J. Yamagishi, X. Z. Li, and T. Nishino.** 1996. Overexpression of the *mexC-mexD-0prJ* efflux operon in *nfxB*-type multidrug resistant strains of *Pseudomonas aeruginosa*. Mol. Microbiol. **21:**713–724.
24. **Schweizer, H. P., and T. T. Hoang.** 1995. An improved system for gene replacement and *xylE* fusion analysis in *Pseudomonas aeruginosa*. Gene **158:**15–22.
25. **Schweizer, H. P.** 1994. A method for construction of bacterial hosts for *lac*-based cloning and expression vector: alpha-complementation and regulated expression. BioTechniques **17:**452–456.
26. **Schweizer, H. P.** 1991. Improved broad-host-range *lac*-based plasmid vectors for the isolation and characterization of protein fusions in *Pseudomonas aeruginosa*. Gene **103:**87–92.
27. **Sekiya, H., T. Mima, Y. Morita, T. Kuroda, T. Mizushima, and T. Tsuchiya.** 2003. Functional cloning and characterization of a multidrug efflux pump, MexHI-OpmD, from a mutant of *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **47:**2990–2992.
28. **Simon, R., U. Priefer, and A. Puhler.** 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in gram-negative bacteria. Bio/Technology **1:**784–791.
29. **Stover, C. K., X. Q. Pham, A. L. Erwin, S. D. Mizoguchi, P. Warrener, M. J. Hickyey, F. S. L. Brinkman, W. O. Hufnagle, D. J. Kowalik, M. Lagrou, R. L. Garber, L. Goltry, E. Tolentino, S. Westbrock-Wadman, Y. Yuan, L. L. Brody, S. N. Coulter, K. R. Folger, A. Kas, K. Larbig, R. Lim, K. Smith, D. Spencer, G. K. S. Wong, Z. Wu, I. T. Paulsen, J. Reizer, M. H. Saler, R. E. W. Hancock, S. Lory, and M. V. Olson.** 2000. Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. Nature **406:**959–964.
30. **West, S. E. H., H. P. Schweizer, C. Dall, A. K. Sample, and L. J. Runyen-Janecky.** 1994. Construction of improved *Escherichia-Pseudomonas* shuttle vectors derived from pUC18/19 and sequence of the region required for their replication in *Pseudomonas aeruginosa*. Gene **128:**81–86.