ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, June 1999, p. 1340–1346 Vol. 43, No. 6
0066-4804/99/$04.00+0
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# Use of a Genetic Approach To Evaluate the Consequences of Inhibition of Efflux Pumps in *Pseudomonas aeruginosa*

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Received 11 November 1998/Returned for modification 12 January 1999/Accepted 17 March 1999

**Drug efflux pumps in *Pseudomonas aeruginosa* were evaluated as potential targets for antibacterial therapy. The potential effects of pump inhibition on susceptibility to fluoroquinolone antibiotics were studied with isogenic strains that overexpress or lack individual efflux pumps and that have various combinations of efflux- and target-mediated mutations. Deletions in three efflux pump operons were constructed. As expected, deletion of the MexAB-OprM efflux pump decreased resistance to fluoroquinolones in the wild-type *P. aeruginosa* (16-fold reduction for levofloxacin [LVX]) or in the strain that overexpressed *mexAB-oprM* operon (64-fold reduction for LVX). In addition to that, resistance to LVX was significantly reduced even for the strains carrying target mutations (64-fold for strains for which LVX MICs were >4 μg/ml). We also studied the frequencies of emergence of LVX-resistant variants from different deletion mutants and the wild-type strain. Deletion of individual pumps or pairs of the pumps did not significantly affect the frequency of emergence of resistant variants (at 4× the MIC for the wild-type strain) compared to that for the wild type ($10^{-6}$ to $10^{-7}$). In the case of the strain with a triple deletion, the frequency of spontaneous mutants was undetectable ($<10^{-11}$). In summary, inhibition of drug efflux pumps would (i) significantly decrease the level of intrinsic resistance, (ii) reverse acquired resistance, and (iii) result in a decreased frequency of emergence of *P. aeruginosa* strains highly resistant to fluoroquinolones in clinical settings.**

Decreased intracellular accumulation due to active efflux of antibiotics out of bacterial cells is one of the mechanisms that contributes to the failure of therapy with many currently used antibiotics. Both antibiotic-specific and multidrug-resistant pumps were identified. The latter class of transporter proteins can extrude out of the cell a large variety of structurally unrelated compounds with different modes of action. Many of them are currently used antibiotics (15–17, 24–27).

*Pseudomonas aeruginosa* is an important opportunistic pathogen in which three multicomponent, multidrug-resistant efflux pumps have been identified, namely, Mex-AB-OprM (30, 31), MexCD-OprJ (29), and MexEF-OprN (11). Of the known multidrug-resistant pumps in *P. aeruginosa*, only MexAB-OprM is expressed at a level sufficient to confer intrinsic multidrug resistance in wild-type cells. Deletion of the *mexA*, *mexB*, or *oprM* gene renders *P. aeruginosa* more susceptible to multiple antibiotics (6, 31, 38). Multidrug-resistant mutants with increased expression of any of the pumps can easily be isolated and manipulated under laboratory conditions (8, 19, 20, 32).

Fluoroquinolones, primary therapeutic antibiotics for *P. aeruginosa*, are effluxed by all the known Mex pumps. Mutants with elevated levels of expression of the pumps, which confer increased resistance to fluoroquinolones, have been identified among clinical strains: *nalB* mutants that overproduce the MexAB-OprM pump (2), *nfxB* mutants that overproduce MexCD-OprJ (10, 40), and *nfxC* mutants that overproduce the MexEF-OprN efflux pump (5). This resistance to fluoroquinolones through the overproduction of efflux pumps is distinct from the resistance to fluoroquinolone antibiotics through the mutation of quinolone resistance-determining regions (QRDRs) (9, 28, 39) in DNA gyrase and topoisomerase IV, which are encoded by *gyrAB* and *parCE* genes, respectively (9), in many organisms (28) including *P. aeruginosa* (14, 21).

In this report we show that deletion of efflux pumps reduces the level of resistance to fluoroquinolones even in highly resistant strains with multiple target mutations. We also show that deletion of all three described pumps significantly reduces the frequency of emergence of fluoroquinolone-resistant mutant strains. These results demonstrate the potential effects of inhibition of efflux pumps on the susceptibility to fluoroquinolones.

## MATERIALS AND METHODS

**Bacterial strains and media.** The bacterial strains used in this study are listed in Table 1. Bacterial cells were grown in Luria (L) broth (1% [wt/vol] tryptone, 0.5% [wt/vol] yeast extract, 0.5% [wt/vol] NaCl) or L agar (L broth plus 1.5% agar) at 37°C. The following antibiotics were added to the media at the indicated concentrations: tetracycline, 20 μg/ml for *Escherichia coli* and 100 to 150 μg/ml for *P. aeruginosa*; chloramphenicol, 20 μg/ml for *E. coli* and 100 μg/ml for *P. aeruginosa*; gentamicin, 15 μg/ml for both *E. coli* and *P. aeruginosa*; $HgCl_2$, 15 μg/ml for both *E. coli* and *P. aeruginosa*; ampicillin, 100 μg/ml for *E. coli*; and kanamycin, 50 μg/ml for *E. coli*. L agar was supplemented with 5% (wt/vol) sucrose as required. Levofloxacin (LVX) was synthesized at Daiichi Pharmaceutical Co., Ltd. (Tokyo, Japan). All other antibiotics were purchased from Sigma Chemical Co. (St. Louis, Mo.).

**Selection of multidrug-resistant mutants of *P. aeruginosa*.** Selection of multidrug-resistant mutants of *P. aeruginosa* was performed as described previously (20). The frequency of resistance was determined as the ratio of the numbers of CFU per milliliter that appeared after overnight incubation on antibiotic-containing L agar plates versus the numbers that appeared after overnight incubation on antibiotic-free L agar plates.

**Stepwise selection of LVX resistance.** Wild-type strain PAM1020 (LVX MIC, 0.25 μg/ml) was plated on LBA plates with LVX at 4× the MIC. The first-generation spontaneous mutants were selected at a frequency of $10^{-6}$ to $10^{-7}$. The same procedure was repeated several times for subsequent generations of mutants, each time with higher concentrations of LVX but still at 4× the MIC. During the next four steps of selection, spontaneous mutants were isolated at a frequency of ca. $10^{-8}$. The highest MIC achieved after five selection steps was 128 μg/ml.

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TABLE 1. Bacterial strains and plasmids used in this study

| Strain group and strain | Genotype or description[a] | Source or reference |
|---|---|---|
| *P. aeruginosa* strains that lack or overexpress individual pumps or combinations of the pumps | | |
| PAM1020 | PAO1 prototroph | This study |
| PAM1032 | *nalB* (*mexAB-oprM* is overexpressed) | This study |
| PAM1033 | *nfxB* (*mexCD-oprJ* is overexpressed) | This study |
| PAM1034 | *nfxC* (*mexEF-oprN* is overexpressed) | This study |
| K590 | *met-9011 amiE200 rpsL pvd-9 mexA::*Tc | 30 |
| K613 | *met-9011 amiE200 rpsL pvd-9 oprM*::ΩHg | 31 |
| PAM1106 | *mexA::*Tc | This study |
| PAM1154 | *oprM*::ΩHg | This study |
| PAM1177 | *nfxB oprM*::ΩHg | This study |
| PAM1187 | *nfxC oprM*::ΩHg | This study |
| PAM1360 | *mexA::*Tc Δ*mexCD-oprJ::*Gm | This study |
| PAM1409 | Δ*mexCD-oprJ::*Gm | This study |
| PAM1610 | *nalB* Δ*mexEF-oprN*::ΩHg | This study |
| PAM1623 | Δ*mexEF-oprN*::ΩHg | This study |
| PAM1536 | *nfxB* Δ*mexAB-oprM::*Cm | This study |
| PAM1554 | Δ*mexAB-oprM::*Cm | This study |
| PAM1561 | Δ*mexAB-oprM::*Cm Δ*mexCD-oprJ::*Gm | This study |
| PAM1624 | Δ*mexCD-oprJ::*Gm Δ*mexEF-oprN::*ΩHg | This study |
| PAM1625 | Δ*mexAB-oprM::*Cm Δ*mexEF-oprN::*ΩHg | This study |
| PAM1626 | Δ*mexAB-oprM::*Cm Δ*mexEF-oprN::*ΩHg Δ*mexCD-oprJ::*Gm | This study |
| Strains with different levels of *mexAB-oprM* operon expression containing target-based mutations | | |
| *P. aeruginosa* | | |
| PAM1548 | *gyrA* (Thr83→Ile) | This study |
| PAM1324 | *gyrA* (Asp87→Tyr) | This study |
| PAM1572 | *nalB gyrA* (Thr83→Ile) | This study |
| PAM1573 | *nalB gyrA* (Thr83→Ile) | This study |
| PAM1481 | *nalB gyrA* (Asp87→Tyr) | This study |
| PAM1569 | *nfxB gyrA* (Thr83→Ile) | This study |
| PAM1482 | *nfxB gyrA* (Asp87→Tyr) | This study |
| PAM1570 | *nfxC gyrA* (Thr83→Ile) | This study |
| PAM1491 | *nfxC gyrA* (Asp87→Tyr) | This study |
| PAM1582 | *nalB gyrA* (Thr83→Ile) *parC* (Ser87→Leu) | This study |
| PAM1609 | *nalB gyrA* (Thr83→Ile) *parC* (Ser87→Leu) *gyrA* (Asp87→Tyr) | This study |
| PAM1064 | *mexA-phoA::*Tc | This study |
| PAM1667 | *gyrA* (Thr83→Ile) *parC* (Ser87→Leu) *mexA-phoA::*Tc | This study |
| PAM1669 | *gyrA* (Thr83→Ile) *parC* (Ser87→Leu) *gyrA* (Asp87→Tyr) *mexA-phoA::*Tc | This study |
| PAM1665 | *gyrA* (Thr83→Ile) *oprM::*ΩHg | This study |
| PAM1600 | *gyrA* (Thr83→Ile) *parC* (Ser87→Leu) *oprM::*ΩHg | This study |
| PAM1640 | *gyrA* (Thr83→Ile) *parC* (Ser87→Leu) *gyrA* (Asp87→Tyr) *oprM::*ΩHg | This study |
| *E. coli* | | |
| DH5α | *endA hsdR17 supE44 thi-1 recA1 gyrA relA1* Δ(*lacZYA-argF*)*U169 deoR* [φ*80dlac*Δ(*lacZ*)]*M15* | 1 |
| S17-1 | *thi pro hsdR recA* Tra | 36 |
| Plasmids | | |
| pX1918-GT | Ap$^r$ Gm$^r$; contains the selectable Gm$^r$ marker downstream from the *xylE* reporter gene | 35 |
| pNOT19 | Ap$^r$; pUC19 with 10-bp *Nde*I-*Not*I adaptor in *Nde*I site | 34 |
| pMOB3 | Km$^r$ Cm$^r$ *sacB oriT* | 34 |
| pHP45ΩHg | Ap$^r$, $HgCl_2^r$; derivative of pHP45Ω with Ω interposon containing the *mer* operon from Tn*501* | 4 |
| pRS14 | Tc$^r$; contains a 4.3-kb *Hin*dIII fragment carrying the *mexAB-oprM* operon with a 4.1-kb internal deletion | 37 |
| pSUP202-*mexA-phoA* | Tc$^r$ Cb$^r$ Cm$^r$; pSUP202 carrying the 5′ upstream region of *mexA* fused to promoterless *phoA* gene | K. Poole |
| pX1918-Cm | Ap$^r$ Cm$^r$; pX1918GT in which *Bam*HI fragment with Gm$^r$ *xylE* cassette replaced by *Bam*HI fragment with Cm$^r$ from pMOB3 | This study |
| pMOB3-Gm | Km$^r$ Gm$^r$; pMOB3 in which *Bam*HI fragment with Cm$^r$ replaced by *Bam*HI fragment with Gm$^r$ *xylE* cassette from pX1918-GT | This study |
| pAL219 | Ap$^r$; pNOT19 without *Sal*I site (removed by Klenow treatment and religation) | This study |
| pAL225 | Ap$^r$; pAL219 with 4.3-kb *Hin*dIII (contains Δ*mexAB-oprM*) from pRS14 in *Hin*dIII | This study |
| pAL231 | Ap$^r$ Cm$^r$; pAL225 with *Sal*I Cm$^r$ fragment from pX1918-Cm in *Sal*I (located in *mexAB-oprM* region) | This study |
| pAL232 | Ap$^r$ Cm$^r$ Gm$^r$; pAL231 with 6.9-kb *Not*I fragment from pMOB3-Gm with *oriT*, *sacB*, and Gm$^r$ cloned in *Not*I located in insert portion by *Not*I partial digest | This study |
| pAL234 | Ap$^r$; pNOT19 with 0.65-kb *Eco*RI-*Bam*HI PCR fragment with part of *mexE* in *Eco*RI-*Bam*HI | This study |
| pAL237 | Ap$^r$; pAL234 with 0.98-kb *Bam*HI-*Hin*dIII PCR fragment with part of *oprN* in *Bam*HI-*Hin*dIII | This study |
| pAL239 | Ap$^r$ Hg$^r$; pAL237 with 5.5-kb *Bam*HI fragment with Hg$^r$ from pHP45ΩHg in *Bam*HI | This study |
| pAL241 | Ap$^r$ Gm$^r$; pAL239 with 6.7-kb *Not*I fragment from pMOB3 with *oriT*, *sacB*, and Gm$^r$ in *Not*I located in vector portion by *Not*I partial digest | This study |
| pAL215 | Ap$^r$; pNOT19 with 0.95-kb *Eco*RI-*Bam*HI PCR fragment with *nfxB* and part of *mexC* and 0.97-kb *Bam*HI-*Hin*dIII PCR fragment with part of *oprJ* in *Eco*RI-*Hin*dIII | This study |
| pAL217 | Ap$^r$ Gm$^r$; pAL215 with 2.4-kb *Bam*HI Gm$^r$ fragment from pX1918-GT in *Bam*HI | This study |
| pAL224 | Ap$^r$ Cm$^r$ Gm$^r$; pAL217 with 5.3-kb *Not*I fragment from pMOB3 with *SacB*, *oriT*, and Cm$^r$ in *Not*I located in vector portion by *Not*I partial digest | This study |

[a] ΩHg, Hg resistance derivative of interposon Ω; Ap$^r$, ampicillin resistance; Cm$^r$, chloramphenicol resistance; Gm$^r$, gentamicin resistance; Tc$^r$, tetracycline resistance; Km$^r$, kanamycin resistance; Cb$^r$, carbenicillin resistance; *oriT*, origin of transfer from RP4; *sacB*, *sacB* locus from *Bacillus subtilis*.

**Transductions.** Transductions in *P. aeruginosa* were performed with phage F116L by a previously described protocol (13).

**MIC determinations.** MICs were determined in 96-well microtiter plates by a standard broth microdilution method (22) in Muller-Hinton broth (Difco). The inoculum was $10^4$ to $10^5$ cells/ml.

**DNA manipulations.** Plasmid DNA was purified with the RPM Spin Kit (Bio 101, Inc., Vista, Calif.). Chromosomal DNA was prepared by using the Qiagen Blood and Cell Culture Mini Kit (Qiagen Inc., Valencea, Calif.). DNA fragments were gel purified and extracted with the Qiagen Gel Purification Kit or the Bio 101 GeneClean Kit. Restriction enzymes were obtained from New England Biolabs (Beverly, Mass.), and AmpliTaq was obtained from Perkin-Elmer (Branchburg, N.J.). Plasmid DNA was introduced into *E. coli* strains by electroporation (Bio-Rad Laboratories, Mississauga, Ontario, Canada). All molecular biology techniques were performed according to the manufacturer's instructions or as described by Sambrook et al. (33). PCR was carried out in a Perkin-Elmer GeneAmp 9600 thermal cycler. Typically, 30 cycles of denaturation (30 s at 95°C), annealing (30 s at 55°C), and extension (1 min at 72°C) were used to amplify the DNA used in the construction of operon deletions. Analysis of the sequences of the QRDRs was performed directly with PCR-amplified genomic DNA segments. Cycle sequencing was carried out with the ABI-PRISM fluorescent dye terminator kit (Applied Biosystems Inc., Foster City, Calif.). The QRDR of the *gyrA* (14) gene was amplified with primers TTATGCCATGAGC GAGCTGGGCAACGACT (29mer) and TTCCGTTGACCAGCAGGTTGGG AATCTT (28mer). The QRDR of the *parC* (21) gene was amplified with primers ATCTGAGCCTGGAAG (15mer) and AGCAGCACCTCGGAATAG (18mer).

**Construction of a recombinant plasmid for deletion of the *mexAB-oprM* operon.** Plasmid pAL232 was constructed to replace a part of the sequence of the *mexAB-oprM* operon with the chloramphenicol resistance [*cat* (Cm$^r$)] gene in the chromosome of *P. aeruginosa*. Construction was performed as follows. First, we created auxiliary plasmids pAL219, pX1918-Cm, and pMOB3-Gm. pAL219 is a derivative of pNOT19 (34) whose *Sal*I site was removed by the Klenow treatment and religation. pX1918-Cm is a derivative of pX1918-GT (35) in which a *Bam*HI fragment carrying a gentamicin resistance [*aacC1* (Gm$^r$)] gene was replaced with a *Bam*HI fragment carrying the *cat* gene. The *cat* gene was obtained from plasmid pMOB3 (34). pMOB3-Gm is a derivative of pMOB3 in which a *Bam*HI fragment carrying a *cat* gene was replaced with a *Bam*HI fragment carrying an *aacC1* gene. The *aacC1* gene was obtained from plasmid pX1918-GT. Second, plasmid pAL225 was constructed from pAL219 and pRS14. Plasmid pRS14, obtained from K. Poole (37), contains a 4.3-kb *Hin*dIII fragment carrying the *mexAB-oprM* operon with a 4.1-kb internal deletion (obtained by *Sac*II digestion and religation). Plasmid pAL225 was created by cloning the 4.3-kb *Hin*dIII fragment from pRS14 into the *Hin*dIII site of pAL219. Third, plasmid pAL231 was created by inserting a 1.6-kb *Sal*I fragment with a *cat* gene (isolated from plasmid pX1918-Cm) into the unique *Sal*I site of pAL225 located in the *oprM* gene, close to the remaining *Sac*II site. A final construct, plasmid pAL232, was obtained by ligating the 6.9-kb *Not*I fragment from pMOB3-Gm into the *Not*I site of pAL231. Besides the *mexAB-oprM* sequence being partially replaced with the *cat* gene, this plasmid also contained *sacB* and *oriT*.

**Construction of recombinant plasmids for deletion of *mexEF-oprN* operon.** Plasmid pAL241 was constructed to replace a part of the sequence of the *mexEF-oprN* operon with the mercury resistance [*mer* (Hg$^r$)] operon in the chromosome of *P. aeruginosa*. Construction was performed as follows. A 0.65-kb portion of the *mexE* gene was amplified from the chromosome and was subsequently ligated into the *Eco*RI and *Bam*HI sites of pNOT19 to create pAL234. Primers MexE-*Eco*RI (GCTGAACGAGTGGGACGAATTCAC) and MexE-*Bam*HI (CAGGATCCGGTTGACCTGGTTGTCGA) were used. A 0.98-kb portion of the *oprN* gene was amplified from the chromosome with primers OprN-*Bam*HI (CGGGATCCAACGATCGCTTCCCGGT) and OprN-*Hin*dIII (CTCAAGCTTGGTGCCTTCGCGGTACGGAT). The resulting PCR fragment was ligated into the *Bam*HI and *Hin*dIII sites of pAL234 to create pAL237. The 5.5-kb *Bam*HI fragment of pHP45ΩHg (4) containing the mercury resistance determinant was ligated into the *Bam*HI site of pAL237 to create plasmid pAL239. The mercury resistance determinant therefore separates the two gene fragments. The final construct, pAL241, was obtained by ligating the 6.7-kb *Not*I fragment of pMOB3 containing the *sacB*, *oriT*, and *aacC1* genes into pAL239.

**Construction of a recombinant plasmid for deletion of the *mexCD-oprJ* operon.** Plasmid pAL224 was constructed to replace a part of the sequence of the *mexCD-oprJ* operon with the gentamicin resistance gene in the chromosome of *P. aeruginosa*. Construction was performed as follows. First, we constructed plasmid pAL215. The 0.95-kb *Eco*RI-*Bam*HI fragment that contains the gene *nfxB* and the 5′ end of the gene *mexC* and the 0.97-kb *Bam*HI-*Hin*dIII fragment containing part of the gene *oprJ* were inserted into pNOT19 to obtain pAL215. The *nfxB-mexC* fragment was obtained by chromosomal PCR with the primers NfxB-*Eco*RI (TTTGAATTCGCCAAGTGCCAGTATCG) and NfxB-*Bam*HI (TTTGGATCCCGATCCTTCCTATTGCACG); the *oprJ* fragment was obtained by chromosomal PCR with the primers OprJ-*Bam*HI (GGGGGAT CCGAGTACGAACTGGACCTC) and OprJ-*Hin*dIII (CCCAAGCTTTAGC ACCGTTTCCCACAC). Second, a 2.4-kb *Bam*HI fragment from pX1918-GT containing a gentamicin marker was inserted between the *nfxB* gene fragment and the *oprJ* gene fragment to create pAL217. The final construct, pAL224, was created by ligation of a 5.3-kb *Not*I fragment obtained from pMOB3 containing *sacB*, *oriT*, and a *cat* gene into pAL217.

**Deletions of efflux pump operons in chromosome of *P. aeruginosa*.** Plasmids pAL224, pAL232, and pAL241 were transformed into *E. coli* S-17 (36) and were subsequently mobilized into various strains of *P. aeruginosa* via conjugation. Conjugation was performed as described elsewhere (30). Subsequent sucrose selection rendered strains PAM1360, PAM1536, and PAM1610, which were then used as sources of the *mexCD-oprJ::*Gm, *mexAB-oprM::*Cm, and *mexEF-oprN::*ΩHg deletions, respectively.

**Gene replacement.** Strains PAM1106 (PAM1020 *mexA::*Tc) and PAM1154 (PAM1020 *oprM::*ΩHg) were obtained by transducing tetracycline (Tc) or Hg resistance markers from strains K590 (30) or K613 (31), respectively, which were kindly provided by K. Poole. Strain PAM1064 (PAM1020 *mexA-phoA::*Tc) was constructed as follows. Plasmid pSUP202-*mexA-phoA* (a gift from K. Poole) contains the *mexA-phoA* transcriptional fusion inserted into vector pSUP202 (which confers the Tc$^r$ Cb$^r$ Cm$^r$ phenotype), which cannot replicate in *P. aeruginosa* but which does contain the *mob* (mobilization) site. This plasmid was mobilized into *P. aeruginosa* PAM1020. One of the transconjugants, PAM1064, was confirmed by PCR and its antibiotic susceptibility profile to contain a chromosomal *mexA-phoA* fusion, an intact and functional *mexAB-oprM* operon, and closely linked plasmid-encoded Tc$^r$ and Cb$^r$ markers.

**SDS-PAGE and Western immunoblotting.** Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed by a previously described protocol (6) with 10% (wt/vol) acrylamide in the running gel. Proteins separated by SDS-PAGE were electrophoretically transferred to a nitrocellulose membrane (BA85; Schleicher & Schuell) as described previously (7), with the exception that SDS (0.1% [wt/vol]) was included in the buffer and transfer was carried out at 100 mA for 90 min. The membranes were processed as described previously (6) with murine monoclonal antibodies specific for the OprM, OprJ, or OprN protein (obtained from N. Gotoh) as the primary antibodies and alkaline phosphatase-conjugated goat antibodies to mouse immunoglobulin G as the secondary antibodies (Bio-Rad). The blots were developed with the AP Conjugate Substrate Kit (Bio-Rad) by the manufacturer's protocol.

## RESULTS

**Creation of isogenic strains overexpressing individual efflux pumps.** Strain PAO1(PAM1020) was chosen as a parent strain for all subsequent selection and construction procedures (Table 1). Two types of selections were used: PAM1020 was plated either on LBA plates with levofloxacin at 4× the MIC (1 μg/ml) or on plates with combinations of antibiotics. The latter procedure was based on the previously reported susceptibility profiles for the mutants overexpressing individual efflux pumps (19, 20). Each mutant was profiled with a panel of antibiotics. Mutants that did not show the multidrug-resistant phenotype were tested for the presence of *gyrA* mutations (QRDRs were PCR amplified and sequenced). Two types of *gyrA* mutants were isolated: *gyrA* (Asp87→Tyr) and *gyrA* (Thr83→Ile), as exemplified by strains PAM1324 and PAM1548, respectively. Mutants with mutations in *nalB* (resulting in overexpression of MexAB-OprM) and *nfxB* (resulting in overexpression of MexCD-OprJ) were isolated at a frequency of $10^{-6}$ to $10^{-7}$, *gyrA* mutants were isolated at a frequency $10^{-8}$, and *nfxC* mutants (resulting in overexpression of the MexEF-OprN pump) were isolated at a frequency of $10^{-9}$ to $10^{-10}$. Overexpression of individual efflux pumps in multidrug-resistant mutants (*nalB*, *nfxB*, and *nfxC*) was confirmed with monoclonal antibodies (obtained from N. Gotoh) that were raised against the OprM, OprJ, or OprN protein (data not shown). LVX MICs (1 to 2 μg/ml) were comparable for *gyrA* and pump-overexpressing mutants.

**Creation and characterization of isogenic mutants lacking individual efflux pumps and combinations of pumps.** In order to model the effects of inhibition of multiple pumps, we have constructed the strain that lacks all three known efflux pumps and strains that lack one or two efflux pumps in various combinations. Strains with deletions of individual operons and the MexAB-OprM/MexCD-OprJ double knockout were reported previously (6, 11, 29–31, 38), and their viabilities were not impaired. Neither the double-deletion mutants nor the triple-deletion mutant that were constructed in the course of our work had detectable growth defects under laboratory conditions (data not shown). These data suggest that a drug that

TABLE 2. Effect of target mutations in strains overexpressing various efflux pumps[a]

| Strain | Pump status | *gyrA* status | LVX MIC (μg/ml) |
|---|---|---|---|
| PAM1020 | WT[b] | WT | 0.25 |
| PAM1548 | WT | *gyrA* (Thr83→Ile) | 2 |
| PAM1324 | WT | *gyrA* (Asp87→Tyr) | 1 |
| PAM1032 | *nalB* | WT | 1 |
| PAM1572 | *nalB* | *gyrA* (Thr83→Ile) | 8 |
| PAM1481 | *nalB* | *gyrA* (Asp87→Tyr) | 4 |
| PAM1033 | *nfxB* | WT | 2 |
| PAM1569 | *nfxB* | *gyrA* (Thr83→Ile) | 16 |
| PAM1482 | *nfxB* | *gyrA* (Asp87→Tyr) | 8 |
| PAM1034 | *nfxC* | WT | 4 |
| PAM1570 | *nfxC* | *gyrA* (Thr83→Ile) | 32 |
| PAM1491 | *nfxC* | *gyrA* (Asp87→Tyr) | 16 |

[a] The *nalB*, *nfxB*, or *nfxC* mutation was transduced from strain PAM1032, PAM1033, or PAM1034, respectively, into PAM1548 [*gyrA*(Thr83→Ile)] or PAM1324 [*gyrA*(Asp87→Tyr)]. All transductants were selected on various combinations of antibiotics in accordance with the specificity of each particular efflux pump. LVX was not used for the selection.

[b] WT, wild type.

inhibits Mex pumps, singularly or in multiples, will have no antibacterial effect by itself.

As expected, deletion of the *mexAB-oprM* operon (strain PAM1554) resulted in a dramatic reduction in intrinsic resistance to fluoroquinolones and other antibiotics (data not shown). Deletion of both MexCD-OprJ and MexEF-OprN pumps did not have an additional effect on the intrinsic resistance even when the *mexAB-oprM* operon was deleted (data not shown). The MIC of LVX for triple-deletion strain PAM1626 was 0.015 μg/ml.

It was previously shown that overexpression of the MexCD-OprJ efflux pump compensated for the lack of the MexAB-OprM pump for antibiotics which are substrates of MexCD-OprJ (7). We have shown here that the same is true for the MexEF-OprN efflux pump. The susceptibility of PAM1034 (in which MexEF-OprN is overexpressed) to antibiotics that are the substrates for MexEF-OprN (data not shown) was nearly the same as that of PAM1187 (PAM1034 *oprM*::ΩHg).

**Effect of overexpression of various efflux pumps on strains with *gyrA* mutations.** We studied the effects of overexpression of various efflux pumps on strains containing mutations in the target genes. A series of strains with various *gyrA* mutations that also overexpress efflux pumps was constructed. To do so, we transduced *nalB*, *nfxB*, and *nfxC* mutations from strains PAM1032, PAM1033, and PAM1034, respectively, into PAM1324 with *gyrA* (Asp87→Tyr) or PAM1548 with *gyrA* (Thr83→Ile) mutations. Our results (Table 2) indicate that when both *gyrA* and efflux pump-overexpression mutations are present in the same strain, the MIC of LVX is increased above the MIC for either mutant alone. The *gyrA* mutation (Asp87→Tyr) increased the LVX MIC fourfold for the strain in which efflux pumps were not overexpressed (compare PAM1020 and PAM1324), while the *gyrA* mutation (Thr83→Ile) resulted in an eightfold increase in the MIC (compare PAM1020 and PAM1548). The same four- or eightfold increase in the MIC due to these mutations was also observed in strains which overexpressed any of these three efflux pumps (Table 2). Since various efflux pumps confer slightly different levels of resistance to LVX to begin with, the MICs of this antibiotic for the resulting transductants were also different.

**Effect of *mexAB-oprM* operon on strains with multiple target mutations.** To establish further the contribution of efflux pumps to acquired resistance to fluoroquinolones, we investigated the effects that an efflux pump(s) would have on the strains with multiple target mutations. To obtain such mutants, we used stepwise selection by increasing the concentrations of LVX in the medium. After the first step of selection we obtained both efflux and target-based mutant strains, and all of them had comparable susceptibilities to LVX (MICs, 1 to 2 μg/ml). It is noteworthy that efflux mutants arose at a higher frequency (see above). The stepwise mutants were obtained in the following order: PAM1020 (wild type) > PAM1032 (*nalB*) > PAM1573 (*nalB gyrA* [Thr83→Ile]) > PAM1582 (*nalB gyrA* [Thr83→Ile] *parC* [Ser87→Leu] *gyrA* [Asp87→Tyr]). For quadruple mutant PAM1609 the LVX MIC was 128 μg/ml (Table 3).

In order to elucidate the role of efflux pumps (in this case, the MexAB-OprM pump) in strains with multiple target mutations, we constructed two other series of mutants. First, we constructed strains with the same target mutations but with the wild-type level of expression of the *mexAB-oprM* operon (Table 3). To construct these strains, the $Tc^r$ marker from PAM1064 was transduced into the strains obtained from the stepwise selection process. Second, the MexAB-OprM efflux pump was inactivated by deletion of the *oprM* gene from the mutants obtained in the course of the stepwise selection (Table 3).

Our results indicate that the same target mutations afford different degrees of LVX resistance depending on the status of the efflux pumps. Overproduction of the MexAB-OprM efflux pump (due to the presence of the *nalB* mutation) always increased the LVX MIC eightfold, regardless of the presence of

TABLE 3. Effect of *mexAB-oprM* operon on LVX susceptibility of strains with multiple target mutations

| *gyrA* or *parC* mutation | MIC (μg/ml) for indicated strain with the following pump status: | | |
|---|---|---|---|
| | *nalB*[a] | WT[b] | *oprM*::ΩHg[c] |
| None | 2 (PAM1032) | 0.25 (PAM1020) | 0.015 (PAM1154) |
| *gyrA* (Thr83→Ile) | 8 (PAM1573) | 2 (PAM1548) | 0.125 (PAM1665) |
| *gyrA* (Thr83→Ile) *parC* (Ser87→Leu) | 32 (PAM1582) | 4 (PAM1667) | 0.5 (PAM1600) |
| *gyrA* (Thr83→Ile) *parC* (Ser87→Leu) *gyrA* (Asp87→Tyr) | 128 (PAM1609) | 16 (PAM1669) | 2 (PAM1640) |

[a] The strains were obtained by stepwise selection with increasing concentrations of LVX. Strain PAM1032 was selected from wild-type strain PAM1020. The order of the strains in the column corresponds to the order in which the strains were selected, so that for example, PAM1032 is a parent of PAM1573. All mutant selections were performed with LVX at 4× the MIC for the corresponding parent.

[b] WT, wild type. PAM1548 was obtained as a spontaneous LVX-resistant mutant selected from strain PAM1020. PAM1667 and PAM1669 were constructed by transduction of the *mexAB-oprM* operon with the wild-type level of expression (no *nalB* mutation) from strain PAM1064 into PAM1582 and PAM1609, respectively, as described in Materials and Methods.

[c] Strains PAM1665, PAM1600, and PAM1640 were constructed by transduction of the Hg resistance from strain PAM1154 (PAM1020 *oprM*::ΩHg) into strains PAM1573, PAM1582, and PAM1609, respectively.

TABLE 4. Frequency of LVX-resistant mutants in strains with deletions of the efflux pump operons

| Strain | Pump status | LVX MIC (μg/ml) | Frequency of LVX-resistant mutants[a] |
|---|---|---|---|
| PAM1020 | WT[b] | 0.25 | $2 \times 10^{-7}$–$4 \times 10^{-7}$ |
| PAM1554 | Δ*mexAB-oprM*::Cm | 0.015 | $2 \times 10^{-7}$–$4 \times 10^{-7}$ |
| PAM1409 | Δ*mexCD-oprJ*::Gm | 0.25 | $2 \times 10^{-7}$–$4 \times 10^{-7}$ |
| PAM1623 | Δ*mexEF-oprN*::ΩHg | 0.25 | $2 \times 10^{-7}$–$4 \times 10^{-7}$ |
| PAM1625 | Δ*mexAB-oprM*::Cm Δ*mexEF-oprN*::ΩHg | 0.015 | $2 \times 10^{-7}$–$10^{-7}$ |
| PAM1624 | Δ*mexCD-oprJ*::Gm Δ*mexEF-oprN*::ΩHg | 0.25 | $2 \times 10^{-6}$ |
| PAM1561 | Δ*mexAB-oprM*::Cm Δ*mexCD-oprJ*::Gm | 0.015 | $1 \times 10^{-9}$ |
| PAM1626 | Δ*mexAB-oprM*::Cm Δ*mexCD-oprJ*::Gm Δ*mexEF-oprN*::ΩHg | 0.015 | $<1 \times 10^{-11}$ |

[a] The frequency of resistance to LVX was determined by plating 100 μl of an overnight culture of the corresponding mutant strain onto LBA containing LVX (1 μg/ml). Frequencies were determined as ratios between the number of colonies that grew on LBA plates containing LVX (expressed as numbers of CFU per milliliter to the number of colonies appearing on drug-free LBA plates after overnight growth.

[b] WT, wild type.

the target mutations. Remarkably, inactivation of the MexAB-OprM efflux pump resulted in a consistent 64-fold decrease in resistance to LVX (in strains which overexpressed this efflux pump), also regardless of the presence of additional target mutations in the same strain.

**Effect of deleting efflux pump operons on the emergence of clinically relevant resistance to fluoroquinolones.** Since overexpression of any of the efflux pumps will lead to increased resistance to LVX, one can hypothesize that the frequency of emergence of resistant variants will be decreased if efflux pumps are inactive. Various deletion mutants were used to test this hypothesis. Selection was performed at 1 μg/ml (4× the MIC for the wild type). The results are presented in Table 4. Deletion of only individual efflux pumps did not alter the frequency of emergence of resistant mutants compared to that for the wild-type strain (despite the low level of resistance of the Δ*mexAB-oprM* mutant PAM1554, for which the MIC was 0.015 μg/ml). The mutants isolated in this experiment were shown to overexpress the MexCD-OprJ efflux pump (data not shown). Two of the strains that lacked two efflux pumps, either Δ*mexAB-oprM* Δ*mexEF-oprN* (PAM1625 [MIC, 0.015 μg/ml]) or Δ*mexCD-oprJ* Δ*mexEF-oprN* (PAM1624 [MIC, 0.25 μg/ml]) also demonstrated no alteration in frequency. Mutants overexpressing MexCD-OprJ or MexAB-OprM were isolated from the double-knockout strains (data not shown). When the Δ*mexAB-oprM* Δ*mexCD-oprJ* double mutant was used in the selection (PAM1561 [MIC, 0.015 μg/ml]), the frequency was detectable but was significantly decreased. Mutants obtained from PAM1561 were confirmed to overexpress the MexEF-OprN efflux pump (data not shown). However, the frequency of emergence of LVX-resistant mutants was undetectable when the triple-deletion mutant PAM1626 (Δ*mexAB-oprM* Δ*mexCD-oprJ* Δ*mexEF-oprN* [MIC, 0.015 μg/ml]) was used in the selection experiments with LVX at 1 μg/ml). Importantly, no target-based mutations were isolated under these selective conditions. Mutants with a low level of LVX resistance were isolated at a frequency of $10^{-8}$ to $10^{-9}$ when selection was performed with LVX at 4× the MIC (0.05 μg/ml) for the triple-deletion mutant. This frequency is in good accordance with that expected for target-based mutations.

## DISCUSSION

We have chosen *P. aeruginosa* and fluoroquinolone antibiotics to evaluate the consequences of inhibition of efflux pumps in this organism. One obvious expectation from inhibition of the efflux pumps became apparent after several groups reported that the MexAB-OprM efflux pump significantly contributes to the high intrinsic resistance in *P. aeruginosa* (6, 31, 38). It is clear that inhibition of the MexAB-OprM efflux pump alone should decrease the intrinsic resistance of the wild-type strains of *P. aeruginosa* to many clinically relevant antibiotics that are the substrates of this pump. For example, as we have shown in this report, the susceptibility of the *mexAB-oprM* deletion mutant to LVX was increased eightfold compared to that of the wild-type strain. It is equally obvious that inhibition of multiple efflux pumps should reverse the acquired fluoroquinolone resistance associated with efflux pump overexpression. Indeed, susceptibility to LVX was increased 64-fold in the mutant that lacks three known efflux pumps (which would be the maximal expected effect of pump inhibition) compared to those for the strains that overexpress efflux pumps. However, the unqualified efficacy of efflux pump inhibitors for use in conjunction with fluoroquinolones may be argued, since efflux is not the sole mechanism of fluoroquinolone resistance and target modification mutations (in gyrase and topoisomerase IV) have been recognized to confer resistance to fluoroquinolones. To assess the relative contributions of the efflux pumps and the target modification in the acquisition of resistance to fluoroquinolones by *P. aeruginosa*, isogenic strains with various combinations of efflux and target mutations were used.

With these strains, it was demonstrated that overexpression of the *mexAB-oprM* operon due to a particular *nalB* mutation resulted in the same relative (eightfold) increase in resistance to LVX whether or not multiple target-based mutations were present in the same strain. This indicates that efflux contributes equally to fluoroquinolone resistance over a wide range of fluoroquinolone concentrations. Deletion of the MexAB-OprM efflux pump from the strain in which this pump was overexpressed resulted in a 64-fold reduction in the LVX MIC, independent of the presence of additional resistance mechanisms. These results indicate that, depending on the level of expression of efflux pumps, inhibition of the efflux pumps should result in 8- to 64-fold reductions in LVX MIC even for strains with target mutations. Analysis of isogenic mutant strains also showed that individual efflux- and target-based mutations resulted in comparable four- to eightfold increases in the LVX MIC.

An important observation that we have made, which is in a good agreement with previously reported results (12), is that frequencies of occurrence of mutants due to pump overexpression are ca. 10-fold higher compared with those due to target-based mutations, at least in the case of the MexAB-OprM and MexCD-OprJ pumps. Therefore, it is conceivable that a high proportion of mutants present among both moderately and highly resistant clinical strains of *P. aeruginosa* are efflux mediated. Indeed, recently, several laboratories have reported the

presence of multiple resistance mechanisms, including efflux, in a single bacterial strain isolated from the clinic (3, 40). These observations further support the notion that an inhibitor of multiple efflux pumps will serve as a good LVX-potentiating agent.

Another important beneficial consequence of inhibition of multiple efflux pumps demonstrated in this report is the decreased frequency of emergence of *P. aeruginosa* strains with clinically relevant levels of resistance to fluoroquinolones. Specifically, the emergence of clinically relevant resistant mutants for which the LVX MIC is 1 μg/ml was nondetectable ($<10^{-11}$) for the *mexAB-oprM mexCD-oprJ mexEF-oprN* triple-deletion strain (MIC, 0.015 μg/ml). While inhibition of the efflux pumps should prevent the appearance of efflux-mediated mutants, we also did not obtain strains with increased resistance due to target-based mutations. As we have shown here, in order for the bacteria without efflux pumps to grow under the selective conditions used (LVX at 1 μg/ml), such bacteria are required to acquire simultaneously at least three target-based mutations to attain the necessary level of resistance (Table 3, PAM1640). Multiple target-based mutations are required since, as we have shown in this report, a single target-based mutation provides only a four- to eightfold increase in LVX resistance. Furthermore, the simultaneous acquisition of multiple mutations in a single experiment is an extremely rare event. It is also noteworthy that no additional efflux-based mutants conferring an increase in LVX resistance like that provided by three known efflux-based pumps were selected from the triple-deletion strain. Similar effects of inhibition of efflux pumps on the frequency of emergence of resistance were obtained in experiments with *Staphylococcus aureus*. When selection for norfloxacin resistance was performed in the presence of the NorA efflux pump inhibitor reserpine (23), a significant decrease in the frequency of emergence of resistance was observed (18).

In conclusion, we have demonstrated that efflux pumps contribute significantly to LVX resistance in *P. aeruginosa*. Inhibition of efflux pumps will (i) decrease intrinsic resistance, (ii) significantly reverse acquired resistance, and (iii) result in a decreased frequency of emergence of *P. aeruginosa* strains highly resistant to fluoroquinolones. These results occur only with simultaneous inhibition of multiple efflux pumps in *P. aeruginosa*. The benefits of broad-spectrum bacterium efflux pump inhibitors for the control of LVX resistance in *P. aeruginosa* warrant vigorous searches for such inhibitors.

## ACKNOWLEDGMENTS

We thank K. Poole for providing numerous strains and plasmids and H. Schweizer for the plasmids used in gene disruption experiments. We thank N. Gotoh for monoclonal antibodies against OprM, OprJ, and OprN proteins. We are grateful to G. Miller, M. Schmidt, D. Biek, P. Nakane, M. Dudley, and K. Sato for reading the manuscript and providing insightful comments. We are thankful to T. Akasaka for sharing with us the sequence of the *parC* gene of *P. aeruginosa* prior to publication.

This work was supported by Daiichi Pharmaceutical Co., Ltd., and Microcide Pharmaceuticals, Inc.

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