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Intrinsic Resistance to Inhibitors of Fatty Acid Biosynthesis in *Pseudomonas aeruginosa* Is Due to Efflux: Application of a Novel Technique for Generation of Unmarked Chromosomal Mutations for the Study of Efflux Systems

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Many strains of *Pseudomonas aeruginosa* are resistant to the antibiotics cerulenin and thiolactomycin, potent inhibitors of bacterial fatty acid biosynthesis. A novel yeast Flp recombinase-based technique was used to isolate an unmarked *mexAB-oprM* deletion encoding an efflux system mediating resistance to multiple antibiotics in *P. aeruginosa*. The experiments showed that the MexAB-OprM system is responsible for the intrinsic resistance of this bacterium to cerulenin and thiolactomycin. Whereas thiolactomycin was not a substrate of the MexCD-OprJ pump expressed in a Δ(*mexAB-oprM*) *nfxB* mutant, cerulenin was efficiently effluxed by the MexCD-OprJ system. It was also found that the MexAB-OprM system is capable of efflux of irgasan, a broad-spectrum antimicrobial compound used in media selective for *Pseudomonas*.

Clinical isolates of *Pseudomonas aeruginosa* are characterized by their frequent resistance to antibiotics, including cross-resistance to chemically unrelated compounds. Recent studies showed that the major cause of cross-resistance is the existence of multidrug efflux pumps (19, 21, 24, 26, 30). These pumps can pump out a wide range of compounds, and it is often difficult to discern any common structural features among the substrates. To date, three such efflux systems have been described for *P. aeruginosa*, MexA-MexB-OprM (hereafter, MexAB-OprM), MexC-MexD-OprJ (hereafter, MexCD-OprJ), and MexE-MexF-OprN, respectively (for reviews, see references 24 and 26). These three systems are members of the RND-type family of multidrug efflux systems in gram-negative bacteria. This family is characterized by a transporter (e.g., MexB), a linker protein (e.g., MexA), and a putative outer membrane channel (e.g., OprM). The MexAB-OprM system appears to mediate efflux of a very wide range of compounds (tetracycline, chloramphenicol, fluoroquinolones, β-lactams [except carbapenems], novobiocin, erythromycin, fusidic acid, rifampin, etc.). The system is overexpressed in many carbenicillin-resistant (Cb^r) clinical isolates of *P. aeruginosa*, and the multidrug-resistant phenotype of many such isolates can now be explained as a consequence of the expression of this system. The additional MexAB-OprM homologs are very similar in structure and function, but there are significant differences in the specificities of the systems. For example, whereas the MexCD-OprJ system efficiently extrudes "fourth-generation" cephems, it does not pump carbenicillin or carbapenems.

Emerging resistance to existing antibiotics has prompted investigations into the hitherto unexplored *P. aeruginosa* fatty acid biosynthetic (Fab) pathway for potential antimicrobial targets. The hypothesis is that this pathway is an excellent candidate for targeting antimicrobial agents since it plays a pivotal role in providing metabolic precursors for several important cellular functions, including cell wall biogenesis (phospholipids, lipopolysaccharide, and lipoproteins) (4), rhamnolipid synthesis (25), and synthesis of the acylated homoserine lactones required for virulence factor gene expression (23, 27, 28, 32).

Several of the Fab proteins are the targets of inhibitors of fatty acid synthesis (FAS), including cerulenin (Cer) and thiolactomycin (TLM) (Fig. 1), which specifically target the fatty acid synthases (for a review, see reference 4). Cer is a fungal product that irreversibly inhibits at least two of the three *Escherichia coli* FASs, as well as yeast and mammalian FASs (5, 40). Unlike Cer, TLM specifically inhibits dissociated or type II bacterial and plant FASs but not multifunctional or type I yeast and mammalian FASs (9, 10, 16, 38). Thus, only TLM is of therapeutic interest.

Kawahara et al. (17) reported that *P. aeruginosa* is intrinsically resistant to Cer. Cer-susceptible mutants that simultaneously became hypersusceptible to several other antibiotics, especially carbenicillin and tetracycline, could be isolated. Growth of the susceptible mutant was severely inhibited by 50 μg of Cer per ml.

TLM exhibits broad antibacterial action (9, 22, 38), with some exceptions (1, 8). Hayashi and coworkers (10) reported that some strains of *P. aeruginosa* were hypersusceptible to TLM (they were completely inhibited by TLM at ~0.5 μg/ml) and that this hypersusceptibility coincided with that to several other antibiotics, including carbenicillin and tetracycline. Although these findings indicated the feasibility of using TLM as an anti-*Pseudomonas* drug, subsequent studies on the antibacterial action of TLM focused on *E. coli* (10, 16, 39), mainly since until very recently nothing was known about the FAS system in pseudomonads and since the mechanism(s) of TLM resistance remained not understood.

Mutational analyses of the multidrug resistance (MDR) efflux systems are hampered by the fact that mutations in these systems generally lead to drug hypersensitivity (20, 30). This precludes application of conventional mutational strategies that usually include tagging of plasmid-borne genes with antibiotic resistance markers, followed by their return into the chromosome (35, 36). In the case of efflux systems, subsequent analyses of their roles in MDR make it desirable to have mutants devoid of any drug markers. Although the previously

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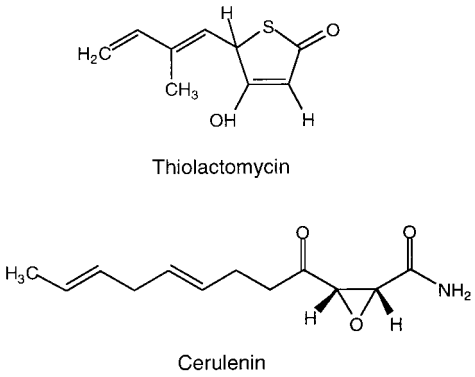

FIG. 1. Structures of the fatty acid synthase inhibitors TLM and Cer.

described *sacB*-based technique has proven to be very useful for the introduction of marked and unmarked mutations into the chromosome (13, 36), despite repeated attempts, I was unable to use this technique to return the unmarked K337 Δ(*mexAB-oprM*) mutation (29) into the PAO1 chromosome. I therefore decided to isolate the desired Δ(*mexAB-oprM*) mutation by tagging it with a selectable antibiotic marker followed by its in vivo excision (3, 14, 18).

In this paper, I describe the application of a novel gene replacement method for investigations of the role of efflux in resistance to the FAS inhibitors Cer and TLM.

MATERIALS AND METHODS

Bacterial strains and growth media. The *P. aeruginosa* strains used in this study are listed in Table 1 and were derived as follows. Among the PAO1 derivatives, PAO196 is PAO1 with Δ(*mexAB-oprM*)::Gm^r-GFP (gentamicin resistant [Gm^r] and expressing green fluorescent protein [GFP] from the gentamicin gene promoter) (this study) and PAO200 contains an unmarked Δ(*mexAB-oprM*) and is derived from PAO196 by Flp-mediated excision of the Gm^r-GFP markers (this study). Among the K337 derivatives, K337 Δ(*mexAB-oprM*) (29) and K337 Δ(*mexAB-oprM*) *nfxB* (29) were previously derived from K337 (30). The Δ(*mexAB-oprM*) deletion in K337 is slightly smaller than the one described in this study and was derived by deletion of an internal 4,103-bp *Sac*II fragment, followed by integration of the resulting unmarked deletion into the K337 chromosome (29). For growth of *P. aeruginosa* strains, the rich media used were Luria-Bertani (LB) agar (31), RB (rich broth) agar (11), or *Pseudomonas* isolation agar (PIA; Difco, Detroit, Mich.), and VBMM (Vogel-Bonner minimal medium) (33) was used as the minimal medium. *E. coli* strains were grown on LB medium. Unless indicated otherwise, antibiotics were used in the selection media at the following concentrations (per milliliter): for *E. coli*, ampicillin (Sigma, St. Louis, Mo.) was used at 100 μg and gentamicin (ICN, Costa Mesa, Calif.) was used at 10 μg; for *P. aeruginosa*, carbenicillin (Foothills Hospital Pharmacy, Calgary, Alberta, Canada) was used at 500 μg and gentamicin was used at 200 μg. Cer (Sigma) and TLM (synthesized as described previously [38] in the Mycobacteriology Research Laboratories at Colorado State University) were used at the concentrations specified in Table 2 and the legend to Fig. 3.

Construction of recombinant plasmids. Restriction enzymes and T4 DNA ligase were used as recommended by the supplier (Gibco-BRL, Gaithersburg, Md.). DNA fragments were blunt-ended with T4 DNA polymerase in the presence of 100 μM deoxynucleoside triphosphates (31). Small-scale isolations of plasmid DNA from *E. coli* and DNA transformations were done as described previously (37). Restriction fragments were eluted from agarose gels by using the Geneclean procedure (Bio 101, Inc., San Diego, Calif.). Plasmid pPS951 was derived in several steps. First, a 1.8-kb *Hin*dIII-*Kpn*I fragment from pRSP14 (29) containing the N-terminal 172 *mexA* codons and codons 104 to 395 of *oprM* (GenBank accession no. L11616) was subcloned between the same sites of pUC18 (41) to form pPS807. Next, two oligonucleotides were designed to introduce unique *Eco*RV sites at positions corresponding to codon 165 of *mexA* and codon 128 of *oprM*. These primers were used in a reverse PCR to prime synthesis from pPS807 DNA in a 50-μl reaction mixture containing 1× *Taq*$^+$ buffer (Stratagene, La Jolla, Calif.), 200 μM (each) deoxynucleoside triphosphate, 10 pmol of each primer, ~10 pmol of pPS807 DNA, and 5 U of *Taq*$^+$ (Stratagene). The reaction mixtures were subjected to the following cycles: 1 cycle at 96°C for 5 min; 35 cycles of 95°C for 1 min, 57°C for 20 s, and 72°C for 4.5 min; and a final extension at 72°C for 5 min. The 4.5-kb PCR product was eluted from an agarose gel. After digestion with *Eco*RV and gel purification, the resulting fragment was ligated to an 1,812-bp GFP-containing and Gm^r-conferring *Sac*I fragment from pPS858 (14) to yield plasmid pPS809 [Δ(*mexAB-oprM*)::Gm^r-GFP]. The mutated region was then subcloned as a blunt-ended *Hin*dIII-*Kpn*I fragment into the *Sma*I site of the gene replacement vector pEX100T (35) to form pPS951. Plasmid pPS952 was constructed by ligating a blunt-ended *mexA*$^+$-*mexB*$^+$-*oprM*$^+$

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant properties	Reference or origin
P. aeruginosa		
PAO1	Prototroph	15
PAO196	PAO1 with Δ(*mexAB-oprM*)::Gm^r-GFP	This study
PAO200	PAO1 with unmarked Δ(*mexAB-oprM*)	This study
K337[a]	Same as ML5087 (*ilv-220 thr-9001 leu-9001 met-9011 pur-67 aphA*)	30
E. coli SM10	Km^r; mobilizer strain (*thi-1 thr leu tonA lacY supE recA*::RP4-2Tc::Mu)	6
Plasmids[b]		
pAK1900	Ap^r; broad-host-range cloning vector	30
pEX100T	Ap^r; *sacB*$^+$ *oriT*$^+$; gene replacement vector	35
pFLP	Ap^r; source of yeast Flp recombinase	14
pUC18	Ap^r; general purpose cloning and expression vector	41
pUCP21T	Ap^r; mobilizable broad-host-range cloning vector	36
pRSP01	Ap^r; *mexA*$^+$ *mexB*$^+$ *oprM*$^+$ (8.5-kb chromosomal *Hin*dIII fragment cloned into pAK1900)	29
pRSP14	Ap^r; Δ(*mexAB-oprM*) (pRSP01 with 4.1-kb *Sac*II deletion)	29
pPS807	Ap^r; Δ(*mexAB-oprM*) (1.8-kb *Hin*dIII-*Kpn*I fragment from pRSP14 cloned between the same sites of pUC18)	This study
pPS809	Ap^r; PCR-amplified 4.5-kb fragment from pPS807 ligated to 1.8-kb blunt-ended Gm^r-GFP fragment from pPS858)	This study
pPS858	Ap^r Gm^r; source of Gm^r-GFP Gm^r-conferring fragment flanked by *FRT* sites	14
pPS951	Ap^r Gm^r; subcloning of a 5.9-kb blunt-ended *Hin*dIII-*Kpn*I fragment from pPS809 into the *Sma*I site of pEX100T	This study
pPS952	Ap^r; *mexA*$^+$ *mexB*$^+$ *oprM*$^+$ (8.5-kb chromosomal *Hin*dIII fragment from pRSP01 cloned into the same site of pUCPT21T)	This study

[a] For a detailed description of other K337 derivatives used in this study, see Materials and Methods.
[b] Details on the construction of recombinant plasmids are presented in Materials and Methods.

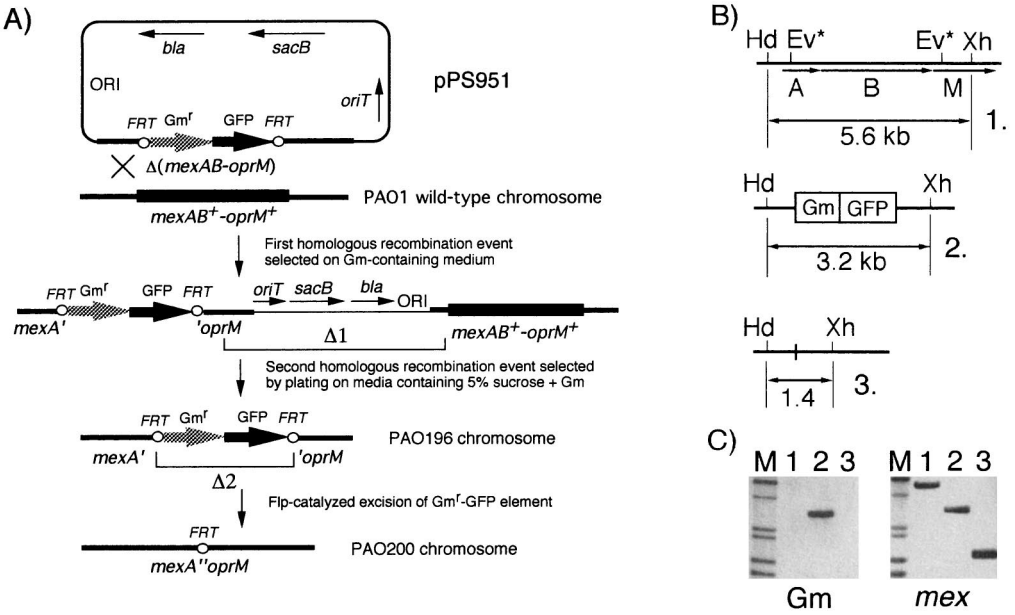

FIG. 2. Strategy for isolation of an unmarked Δ(*mexAB-oprM*) mutation. (A) For gene replacement, the previously described *sacB*-based strategy (35) was used, as detailed in Materials and Methods. Colonies having undergone the deletion marked Δ1 were screened as sucrose resistant, Gm^r, and Cb^s. The unmarked Δ(*mexAB-oprM*) mutant was then derived from the Gm^r-GFP integrant by Flp-catalyzed excision (Δ2) of the Gm^r-GFP markers. Abbreviations: *bla*, β-lactamase structural gene; GFP, green fluorescent protein structural gene; ORI, ColE1-derived origin of replication; *oriT*, origin of transfer; *sacB*, levansucrase structural gene. (B) Genomic organization of the PAO1 *mexA*$^+$-*mexB*$^+$-*oprM*$^+$ region (B-1) and of Δ(*mexAB-oprM*) mutants PAO196 (B-2) and PAO200 (B-3). Ev* mark the positions of the artificially generated *Eco*RV sites used for deletion of the intervening 4.2 kb of DNA and replacement with a 1.8-kb Gm^r-GFP fragment. The relative positions of *Hin*dIII (Hd) and *Xho*I (Xh) sites, as well as the lengths of *Hin*dIII-*Xho*I fragments expected after digestion of the respective chromosomal DNAs, are shown. (C) Genomic Southern analysis. Nylon membranes containing electrophoretically separated genomic DNA fragments from the isolates depicted in panel B were probed either with a biotinylated DNA fragment from pPS807 (panel labeled *mex*) or with a Gm^r fragment (panel labeled Gm), as described in Materials and Methods. The DNAs in lanes 1, 2, and 3 correspond to the *Hin*dIII-*Xho*I fragments from the strains 1, 2, and 3, respectively, described for panel B. Lane M contained (top to bottom) biotinylated λ *Hin*dIII fragments (6.3, 4.3, 2.4, and 2.04 kb) and biotinylated ϕX *Hae*III fragments (1.35 and 1.08 kb).

*Hin*dIII fragment from pRSP14 (29, 30) into the blunt-ended *Pst*I site of pUCP21T (36), resulting in transcription of the *mexA*$^+$-*mexB*$^+$-*oprM*$^+$ operon from its own promoter.

Gene replacement. For gene replacement, the previously described *sacB*-based strategy (35) was used, selecting Gm^r colonies on VBMM-gentamicin medium after conjugal transfer of pPS951 from *E. coli* SM10 (6). Sucrose-resistant colonies were obtained on LB medium containing 5% sucrose and 15 μg of gentamicin per ml. Deletion of the chromosomally integrated Gm^r-GFP markers by Flp recombinase-catalyzed excision was achieved by conjugally transferring Flp-expressing, nonreplicative pFLP (14) from *E. coli* SM10 into the Gm^r-GFP strain and plating dilutions of the recipient cells at 42°C on VBMM plates. The cells growing on these plates were then tested for the loss of the Gm^r marker.

Genomic Southern analyses. Chromosomal DNA was isolated by a miniprep procedure (2). Nylon membranes containing electrophoretically separated genomic DNA fragments were probed with biotinylated DNA by previously described procedures (12). The gentamicin probe was derived by labelling a 850-bp gel-purified fragment from pUCGM (34), and the *mex* probe was obtained by labelling the 1.8-kb insert of pPS807 (this study) containing the N-terminal *mexA*-coding sequence and sequences internal to *oprM*.

Antibiotic susceptibility studies. Susceptibilities to antimicrobial agents were tested as described previously (30), with minor modifications. One-milliliter cultures of LB medium containing 100, 75, 50, 25, 10, 5, 2.5, 1, and 0 μg of each antimicrobial agent were inoculated with 5×10^6 logarithmically (absorbance at 540 nm, ~0.8 to 1.0) growing organisms. Growth was assessed visually after 18 h of incubation at 37°C. The MIC was defined as the lowest concentration of antimicrobial agent that inhibited visible growth. Plasmid-containing strains were pregrown in LB medium containing 200 μg of carbenicillin per ml, and susceptibilities to antimicrobial agents were then tested in the absence of carbenicillin.

RESULTS AND DISCUSSION

Construction of an unmarked Δ(*mexAB-oprM*) mutant. A defined pPS951-borne Δ(*mexAB-oprM*) mutation was constructed as described in Materials and Methods, and the deletion was returned to the *P. aeruginosa* chromosome as illustrated in Fig. 2A. After conjugal transfer of the nonreplicative pPS951 from *E. coli* SM10 into PAO1, merodiploids were obtained by selecting for Gm^r. From these, colonies having undergone the deletion marked with Δ1 in Fig. 2A were selected as sucrose resistant, Gm^r, and Cb^s. The unmarked Δ(*mexAB-oprM*) mutant PAO200 was then derived from the Gm^r-GFP integrant PAO196 by Flp-catalyzed excision of the Gm^r-GFP markers. During its transient expression in the recipient, Flp recombinase acted at the Flp recombination target (*FRT*) sites to catalyze excision of the Gm^r-GFP element (marked with Δ2 in Fig. 2A) at low but detectable frequencies (0.1 to 0.5%), leaving behind a short *FRT*-containing sequence (3, 14). It should be noted that although the GFP marker has proven to be useful for monitoring the loss of the entire Gm^r-GFP cassette in *E. coli*, it did not prove to be useful during these particular experiments due to the intense fluorescence of *P. aeruginosa* cells grown on VBMM.

Successful execution of the steps labelled Δ1 and Δ2 in Fig. 2A was monitored by colony PCR analysis with primers specific for the gentamicin resistance gene (data not shown) and by genomic Southern analysis (Fig. 2B and C). From the results presented in Fig. 2C it is evident that both deletion events produced the desired restriction patterns. Probing with a *mexA*- and *oprM*-specific probe (panel labeled *mex* in Fig. 2C) revealed deletion of a 4.2-kb region from the PAO1 wild-type chromosome in both the Δ(*mexAB-oprM*)::Gm^r-GFP insertion mutant PAO196 (lane 2) and the Δ(*mexAB-oprM*) mutant PAO200 (lane 3). The size of the 5.6-kb *Hin*dIII-*Xho*I fragment observed in wild-type PAO1 (lane 1) was reduced to 3.2 kb (5.6 kb minus 4.2 kb of genomic DNA plus 1.8 kb of the Gm^r-GFP fragment) (lane 2) in the insertion mutant PAO196 and to 1.4 kb (lane 3) in the unmarked deletion mutant PAO200. Probing with a probe specific for the gentamicin

TABLE 2. Susceptibilities of *P. aeruginosa* strains to selected antimicrobial agents

Strain	MIC (μg/ml)[a]				
	Cb	Tc	Gm	Cer	TLM
PAO1	75	7.5	1–5	>100	>100
PAO200	0.75	<0.5	0.5	7.5	25–50[b]
PAO200/pUCP21T	ND[c]	<0.5	ND	10	25
PAO200/pPS952	ND	10	ND	>100	>100
K337 Δ(*mexAB-oprM*)	0.5	<0.5	ND	50	25
K337 Δ(*mexAB-oprM*) *nfxB*[d]	0.5	25	ND	>100	25

[a] Susceptibilities to antimicrobial agents were tested as described in Materials and Methods. Cb, carbenicillin; Tc, tetracycline; Gm, gentamicin.
[b] Inhibition levels were somewhat dependent with different batches of TLM.
[c] ND, not determined.
[d] This *nfxB* strain expresses the MexCD-OprJ efflux system.

resistance gene (panel labeled Gm in Fig. 2C) revealed the presence of the 1.8-kb Gm^r-FRT cassette only in the Δ(*mexAB-oprM*)::Gm^r-GFP insertion mutant PAO196 on a 3.2-kb *Hin*dIII-*Xho*I fragment (lane 2). As expected, the sequences encoding gentamicin resistance were absent from wild-type PAO1 genomic DNA (lane 1), and they were deleted from the excision mutant PAO200 (lane 3).

The results suggest that the experimental strategy described herein will facilitate studies aimed at elucidation of the modes of action of the efflux systems of *P. aeruginosa* and other pathogenic bacteria. More generally, unmarked efflux pump mutants will enable the search and design of new antimicrobial agents that are no longer substrates of the efflux systems, while maintaining their inhibitory effects.

Susceptibility of efflux pump mutants to FAS inhibitors. Susceptibility studies revealed that PAO200 was hypersusceptible to all of the antibiotics tested except gentamicin; the pattern of susceptibility to gentamicin was marginally altered (Table 2). Transformation with the *mexA*$^+$-*mexB*$^+$-*oprM*$^+$ plasmid pPS952 restored resistance to tetracycline, Cer, and TLM to the levels found in PAO1 (Table 2 and Fig. 3). These experiments demonstrated that the MexAB-OprM efflux system was indeed responsible for the previously observed intrinsic resistance of some *P. aeruginosa* strains to Cer and TLM, as well as the concomitant cross-resistance to other antibiotics.

Preliminary experiments with K337 Δ(*mexAB-oprM*) *nfxB*, i.e., a strain expressing the MexCD-OprJ pump, indicated that whereas Cer is a substrate of the MexCD-OprJ pump, TLM is not effluxed by this system (Table 2). As observed with PAO200 (Fig. 3B), growth of K337 Δ(*mexAB-oprM*) *nfxB* in RB medium was completely inhibited by 50 μg of TLM per ml. In contrast to PAO200, which showed no visible growth in medium containing 50 μg of Cer per ml (Fig. 3A), K337 Δ(*mexAB-oprM*) was intrinsically more resistant to this antimicrobial agent (levels of growth inhibition were 53 and 80% with 50 and 100 μg of Cer per ml, respectively). Since strain K337 Δ(*mexAB-oprM*) *nfxB* was resistant to >100 μg Cer per ml, it can be concluded that this antimicrobial agent is efficiently extruded by the MexCD-OprJ pump. Clearly, since the PAO1 and K337 strain backgrounds display different levels of intrinsic resistance, the experiments described above will have to be repeated with a PAO200 *nfxB* strain.

Although the results indicate a major role of efflux systems in resistance to FAS inhibitors, additional resistance mechanisms, i.e., target alterations, probably exist, and their contributions to resistance, if any, will need to be further evaluated. In *E. coli*, the two known mechanisms contributing to TLM^r are efflux via the major facilitator-type EmrAB system (7) and FAS I (FabB) target overproduction (39).

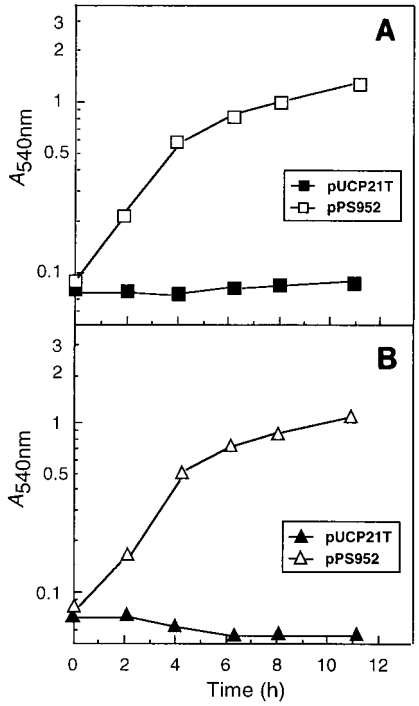

FIG. 3. Complementation of PAO200 with a cloned *mexA*$^+$-*mexB*$^+$-*oprM*$^+$ operon. Cells of strain PAO200 containing the vector pUCP21T (closed symbols) or pPS952 (open symbols) were grown overnight in RB medium (11) supplemented with 200 μg of carbenicillin per ml and were inoculated into carbenicillin-free RB medium to an initial absorbance at 540 nm of 0.05 to 0.08 (time zero). Cultures were shaken at 37°C. At the indicated times, samples were withdrawn and the absorbance at 540 nm was recorded. Cultures contained either 50 μg of Cer per ml (A) or 50 μg of TLM per ml (B).

MexAB-OprM-mediated efflux is required for growth of wild-type *P. aeruginosa* on *Pseudomonas* isolation agar. In the course of the studies on FAS inhibitors it was discovered that strains PAO196 and PAO200 were no longer able to grow on PIA. Growth on medium with the same formulation except that it contained no irgasan indicated that this was due to the susceptibility of the mutants to the broad-spectrum antimicrobial irgasan (also known as triclosan) present in PIA, which in wild-type *P. aeruginosa* is apparently pumped out of the cell via the MexAB-OprM efflux system. Transformation with the *mexA*$^+$-*mexB*$^+$-*oprM*$^+$ plasmid pPS952 restored the ability of PAO200 to grow on PIA. By streaking the same strain on PIA, irgasan-resistant mutants could be isolated at high frequencies. Since the MIC of tetracycline (~0.5 μg/ml) for these mutants was still low, none of the other known efflux systems seems to be responsible for the irgasan resistance in these mutant strains. In contrast to PAO200, strain K337 Δ(*mexAB-oprM*) grew normally on PIA, again indicating a hitherto unknown mechanism besides efflux contributing to irgasan resistance. In light of these observations, the usefulness of this medium for the differentiation of *Pseudomonas* strains may be limited to MexAB-OprM efflux system-expressing organisms, while MexAB-OprM efflux pump-negative or MexAB-OprM-nonexpressing mutants may be missed when this medium is used for screening. As with the FAS inhibitors, the role of other *P. aeruginosa* efflux pumps in irgasan resistance, if any, has yet to be elucidated. In light of the present findings, the general use of PIA medium for differentiation purposes should therefore be reevaluated. In contrast, wild-type and efflux pump mutants grew equally well on agar base selective for *Pseudomonas* (also

called cetrimide agar) (Difco), whose active ingredient, cetyltrimethylammonium bromide, did not seem to be a substrate for the MexAB-OprM pump. These results would indicate that cetrimide agar is the more reliable choice for use as a selective medium for *Pseudomonas*.

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