

## Negative Regulation of the *Pseudomonas aeruginosa* Outer Membrane Porin OprD Selective for Imipenem and Basic Amino Acids

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***Pseudomonas aeruginosa* OprD is a specific porin which facilitates the uptake of basic amino acids and imipenem, a carbapenem antibiotic. Resistance to imipenem due to the loss of OprD is an important mechanism for the loss of clinical effectiveness. To investigate the negative regulatory mechanisms influencing *oprD* expression, a gene upstream of the coregulated *mexEF-oprN* efflux operon, designated *mexT*, was cloned. The predicted 304-amino-acid mature MexT protein showed strong homology to LysR-type regulators. When overexpressed it induced the expression of the *mexEF-oprN* efflux operon while decreasing the level of expression of OprD. The use of an *oprD::xylE* transcriptional fusion indicated that it acted by repressing the transcription of *oprD*. Salicylate, a weak aromatic acid known to reduce porin expression and induce low levels of multiple antibiotic resistance in *Escherichia coli*, was able to induce imipenem resistance and reduce the expression of OprD but not multiple antibiotic resistance or OprN expression in *P. aeruginosa*. This was also demonstrated to occur at the level of transcription. Acetyl salicylate and benzoate, but not catechol, were also able to reduce the levels of OprD in the *P. aeruginosa* outer membranes. These OprD-suppressing compounds increased imipenem resistance even in a *mexT*-overexpressing and *nfxC* mutant backgrounds, suggesting that such resistance is independent of the MexT repressor and that *oprD* is influenced by more than a single mechanism of repression.**

*Pseudomonas aeruginosa* infections are a major clinical problem and can be one of the more difficult infections to treat, due to the organisms's high intrinsic resistance to many of the most commonly used antibiotics (12). This phenomenon is partly due to its relatively low outer membrane permeability, in conjunction with secondary resistance mechanisms like  $\beta$ -lactamase and active efflux (11, 22). Most small hydrophilic molecules, including antibiotics, enter the periplasm by diffusion through the channels of nonspecific porins that are present in the outer membrane, and it has been shown that this pathway in *P. aeruginosa* is 10- to 100-fold less efficient than, e.g., that in *Escherichia coli*. In contrast, it has been shown that the basic amino acid-specific OprD porin of *P. aeruginosa* mediates the uptake of the  $\beta$ -lactam antibiotic imipenem (13, 35).

Imipenem, a carbapenem  $\beta$ -lactam, is of particular interest because of its high potency, broad spectrum, and general lack of microbial cross-resistance to other  $\beta$ -lactam antibiotics (26). At least two types of imipenem-resistant mutants are observed in clinics. The major type involves the loss by mutation of OprD, and such mutants are resistant only to zwitterionic carbapenem antibiotics (17). Genetic analysis of laboratory-derived mutants has shown that the loss of OprD expression is due to deletions in the *oprD* coding region and the upstream promoter region (36). A second common type of resistance observed involves multiple antibiotic resistance to both imipenem and other unrelated classes of antibiotics (4, 9). Such mutants are of the *nfxC* class (8), which show an increased resistance to chloramphenicol, quinolones, and imipenem. They tend to show increased susceptibility towards most  $\beta$ -lac-

tam antibiotics (except imipenem) and aminoglycoside antibiotics. There are two changes in the outer membrane profile of *nfxC* mutants, including a strong reduction in the expression of OprD and new expression of a 51-kDa protein designated OprN (20). Kohler et al. (15) recently cloned the operon responsible for the *nfxC*-type phenotype. It consisted of three open reading frames (ORFs), which encoded the cytoplasmic membrane protein MexF (115 kDa), the membrane fusion protein MexE (45 kDa), and the outer membrane protein OprN (51 kDa). MexE, MexF, and OprN showed high similarity to components of the major efflux operon involved in intrinsic antibiotic resistance in *Pseudomonas*, namely, MexA, MexB, and OprM, respectively. Kohler et al. (15) reported the existence (but not the sequence) of an ORF upstream of this operon, which encoded a putative LysR-type transcriptional activator. The overexpression of this fragment resulted in a multiple-drug-resistant phenotype including resistance to imipenem. Recently we have found that OprD expression is positively regulated in a variety of different ways, including arginine-responsive activation mediated by the AraC-type regulator ArgR (24). A prior study in our laboratory had indicated that a protein termed OpdE was also involved in the regulation of OprD (14). Therefore, we set out to determine the roles of OpdE and the putative transcriptional regulator defined by Kohler et al. (15) and in particular to determine whether they regulate *oprD* at the transcriptional level. It had been previously shown that salicylate caused reduced porin expression, induced low levels of multiple antibiotic resistance in *E. coli* (7, 27), and was also able to reduce the expression of a 45-kDa outer membrane protein in *P. aeruginosa* (34). Therefore, we examined if salicylate exerted its effect through MexT. We have demonstrated here that our previous conclusions as to the function of OpdE were incorrect and that MexT and

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TABLE 1. Bacterial strains and plasmids relevant to this study

Strain or plasmid	Description	Source or reference
<i>P. aeruginosa</i>		
H103	Wild-type PAO1	10
H673	H103 <i>opdE</i> ::Tn501	14
H847	H103 <i>opdE</i> :: <i>xyIE</i> -Gm <sup>r</sup>	This study
H846	H103 <i>oprD</i> :: <i>xyIE</i> -Gm <sup>r</sup>	This study
H729	H103 <i>oprD</i> ::Km <sup>r</sup>	13
1008	Clinical isolate	20
1008OIR01	1008 <i>nfxC</i> phenotype	20
<i>E. coli</i> S17-1		
	<i>thi pro hsdR recA</i> Tra <sup>+</sup>	33
Plasmids		
pBK19R	ColE1-derived plasmid, Ap <sup>r</sup> <i>plac oprD</i>	14
pX1918GT	pUC-based plasmid containing <i>xyIE</i> -Gm <sup>r</sup> cassette flanked by MCS of pUC19, Ap <sup>r</sup>	32
pEX100T	Ap <sup>r</sup> <i>oriV sacB</i> ; gene replacement vector	32
pD2-45	<i>oriV mob</i> <sup>+</sup> <i>ORF2 opdE opdR</i> Tc <sup>r</sup>	14
pUCP19	<i>Escherichia-Pseudomonas</i> shuttle vector; Ap <sup>r</sup> <i>plac</i>	31
pMC2	pUCP19 <i>mexT</i>	This study
pET28a	Expression vector, Km <sup>r</sup> , pT7, <i>lac</i> operator, <i>lacI</i>	Novagen, Inc.
pMC5	pET28a <i>mexT</i>	This study

salicylate independently repress the synthesis of OprD at the level of transcription.

#### MATERIALS AND METHODS

**Strains and growth conditions.** Bacterial strains and plasmids used in this study are listed in Table 1. All medium components were purchased from Difco Laboratories (Detroit, Mich.). *E. coli* strains were routinely grown in Luria-Bertani broth (1.0% tryptone, 0.5% yeast extract, 0.5% NaCl) containing 0.5% glucose; to obtain a solid medium, agar was added to 1.5% (wt/vol). *P. aeruginosa* strains were routinely grown in Mueller-Hinton broth; to obtain a solid medium, agar was added to 1.5% (wt/vol). BM2 minimal glucose medium was made as previously described (10). Solutions of acetyl salicylate, salicylate, benzoate, and catechol were prepared fresh daily, and the pH was adjusted to 7 with NaOH as required. For selection purposes, 100 µg of ampicillin per ml and 10 µg of gentamicin per ml were used for *E. coli*, whereas for *P. aeruginosa*, carbenicillin at 400 µg/ml, norfloxacin at 1 µg/ml, and gentamicin at 10 µg/ml were used.

**Reagents.** Gentamicin and carbenicillin were purchased from ICN Biomedicals Inc. (Aurora, Ohio), imipenem was obtained from Merck, Sharp and Dohme (West Point, Pa.), meropenem was obtained from ICI Pharmaceuticals (Macclesfield, U.K.), cefpirome was purchased from Roussel (Paris, France), and ciprofloxacin was supplied by Bayer (Elberfeld, Germany). All other chemical reagents used in this study were purchased from Sigma Chemical (St. Louis, Mo.). The meganuclease I-SceI was purchased from Boehringer Mannheim (Laval, Quebec, Canada). Restriction endonucleases, DNA modifying enzymes, and other molecular biology reagents were purchased from Gibco BRL (Burlington, Ontario, Canada) and New England Biolabs (Mississauga, Ontario, Canada). Mouse anti-OprD (YD11), mouse anti-OprM (TM01), mouse anti-OprJ (HJ004), and mouse anti-OprN (TN009) monoclonal antibodies (MAbs) were a kind gift from Naomasa Gotoh (Kyoto Pharmaceutical University, Kyoto, Japan). Alkaline phosphatase-conjugated goat anti-mouse antibodies were purchased from Bio-Rad (Richmond, Calif.).

**Genetic manipulations.** General molecular biology protocols were performed according to the method of Sambrook et al. (28). DNA fragments were isolated by using the Gene Clean kit (Bio 101 Inc., Vista, Calif.). Sticky-end ligations were performed at 23°C for 30 to 60 min; blunt-end ligations were done overnight in a thermocycler programmed to continuously cycle between 10 and 30°C for 30 s at each temperature. DNA sequencing was done with an ABI 373 automated DNA sequencer (Applied Biosystems, Foster City, Calif.) and the ABI Prism dye terminator sequencing kit according to the manufacturer's directions. Plasmid DNA was prepared for sequencing with Qiagen columns (Qiagen, Inc., Chatsworth, Calif.), and plasmid DNA was quantified with a Hoefer TKO 100 minispectrofluorimeter. Oligonucleotide sequencing primers were synthesized as needed on an ABI 392 oligonucleotide synthesizer according to the manufactur-

er's directions. Nucleotide and deduced amino acid sequences were analyzed with the PC Gene software package (Intelligenetics Inc., Mountain View, Calif.). The DNA sequence was used for homology searches of the National Center for Biotechnology Information nonredundant database with the BLASTX program (21a) by using the default settings for all searches. The DNA sequence was also used for homology searches of the *P. aeruginosa* genome sequencing project with the BLASTN program (21a). Plasmids were transferred into *P. aeruginosa* by transformation (25).

**Cloning of the *mexT* gene.** According to Kohler et al. (15), a gene encoding a putative regulator of *mexEF-oprN*, named here the *mexT* gene consistent with the GenBank entry accession no. AJ007825, was directly upstream and in the same orientation as the *mexE* gene. The sequence of *mexE* was retrieved from the National Center for Biotechnology Information data bank (accession no. X99514) and used to search the contents of the *P. aeruginosa* genome sequencing project (21a), and contig 1523 was retrieved and found to contain the 5' end of *mexE* and one other upstream ORF. The sequence of this contig was used to design primers for PCR amplification of the *mexT* gene from strain H103 by using Vent polymerase and primers with overhangs containing a *Bam*HI site and an *Eco*RI site to permit easy cloning. To ensure the expression of *mexT*, the 1.2-kb *Bam*HI/*Eco*RI fragment containing the *mexT* gene was cloned into pUCP19 to form the plasmid pMC2, so that the direction of expression of the *mexT* gene was in the same orientation as the *lac* promoter. It was then transformed into *P. aeruginosa* H103 and H846 (*oprD*::*xyIE*-Gm<sup>r</sup>). The *mexT* gene was also amplified by PCR by using primers with overhangs containing a *Bam*HI site and an *Nde*I site, to permit cloning into the *Bam*HI/*Nde*I sites of expression vector pET28a (Novagen, Madison, Wis.), creating pMC5.

**Construction of *oprD*::*xyIE*-Gm<sup>r</sup> and *opdE*::*xyIE*-Gm<sup>r</sup> mutants.** A 2.1-kb *Sst*I/*Hind*III fragment containing the *oprD* gene was excised from plasmid pBK19R, treated with Klenow polymerase, and cloned into the *Sma*I site of the gene replacement vector pEX100T. The *oprD* gene was disrupted by inserting a 2.5-kb *Eco*RI fragment containing the *xyIE*-Gm<sup>r</sup> cassette of plasmid pX1918GT into the *Eco*RI site of *oprD*. The orientation of the *xyIE*-Gm<sup>r</sup> cassette was determined by restriction analysis, and a plasmid carrying an *oprD*::*xyIE* transcriptional fusion was transformed into the mobilizing *E. coli* strain S17-1 and subsequently transferred into *P. aeruginosa* H103 by biparental mating. Gentamicin- and carbenicillin-resistant exconjugants were isolated, which had integrated the entire plasmid into the chromosome. Vector pEX100T carries also the *sacB* gene from *Bacillus subtilis*, allowing sucrose-inducible expression, which in turn is lethal to *P. aeruginosa*. Consequently, a positive selection of true mutants was permitted by streaking the exconjugants on a medium containing 10% sucrose and gentamicin. Single colonies were isolated, and the loss of vector sequences was confirmed by testing for carbenicillin sensitivity. The isolated *oprD*::*xyIE*-Gm<sup>r</sup> mutant was resistant to imipenem as expected, and the insertion of the *xyIE*-Gm<sup>r</sup> cassette in the *oprD* gene was confirmed by Southern blot analysis of chromosomal DNA. This strain was named H846. For the inactivation of the *opdE* gene the following cloning steps were performed: a 1,680-bp *Pst*I/*Sst*I fragment containing the *opdE* gene was excised from plasmid pD2-45, treated with Klenow polymerase, and cloned into the *Sma*I site of vector pUCSce (32). The 2.5-kb *xyIE*-Gm<sup>r</sup> cassette from plasmid pX1918GT was excised with *Sph*I and cloned into the *Sph*I site of the *opdE* gene, disrupting the gene. The *Sma*I site of pUCSce was flanked by restriction sites for the meganuclease I-SceI, allowing the excision of the 4.2-kb DNA fragment containing the *opdE*::*xyIE*-Gm<sup>r</sup> DNA and cloning into the I-SceI sites of the gene replacement vector pEX100T. The *opdE*::*xyIE*-Gm<sup>r</sup> mutant strain H847 was constructed from H103 as described above.

**Determination of catechol 2,3-dioxygenase activity.** *P. aeruginosa* strains containing the chromosomal *oprD*::*xyIE* transcriptional fusion and either no plasmid, pUCP19, or pMC2 were harvested in mid-logarithmic growth phase (optical density at 600 nm of 0.5), and cell extracts were assayed for activity of catechol 2,3-dioxygenase, the *xyIE* gene product, as described previously (16). To ensure that salicylate or acetyl salicylate did not influence the dioxygenase activity, cell extracts from cultures grown in Mueller-Hinton broth only were incubated with various concentrations of salicylate or acetyl salicylate prior to the addition of catechol. We did not observe any substantial influence of acetyl salicylate or salicylate on the dioxygenase activities.

**Outer membrane isolation and immunodetection.** Outer membranes were prepared from cultures grown to an optical density at 600 nm of 1.0 by sucrose gradient density centrifugation (10). Analysis of protein profiles was done by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10). Immunoblotting procedures were performed as described previously (21).

**MIC determination.** The MIC was determined in Mueller-Hinton broth by the broth microdilution method (1). Microtiter plates were inoculated with a final concentration of  $2 \times 10^4$  to  $2 \times 10^5$  CFU/ml and incubated for 18 h at 37°C.

#### RESULTS

**Characterization of OpdE.** Previously we isolated a Tn501 mutant of *P. aeruginosa* H103, named H673, which was resistant to imipenem and deficient in OprD (14). The DNA surrounding the Tn501 insertion site was cloned and sequenced,

and the gene carrying Tn501 was named *opdE* for the putative OprD expression regulator (14). A recent comparison to protein sequence databases revealed that OpdE was most homologous to two proteins, f451 and AraJ, from *E. coli* and several cytoplasmic membrane proteins from gram-positive bacteria that were linked to chloramphenicol resistance, and analysis of the OpdE protein sequence indicated that it was a cytoplasmic membrane protein. Subsequent experiments showed that H673 was resistant not only to imipenem but also to chloramphenicol and ciprofloxacin, as well as hypersusceptible to carbenicillin. This led us to reexamine here the role of OpdE in the regulation of *oprD*. An *opdE* interposon mutant was constructed, which showed wild-type susceptibility to all antibiotics tested (imipenem, chloramphenicol, ciprofloxacin, carbenicillin, and tetracycline), and there was no change in OprD expression. Similarly, *opdE* provided on a plasmid failed to alter the antibiotic susceptibility of the wild type, H103. The original complementation experiment with strain H673 (14) could not be repeated and probably was due to a reversion mutation. Therefore, we conclude that the *opdE::Tn501* mutation was not the cause of the imipenem resistance and OprD deficiency phenotypes and furthermore that this protein is not involved in chloramphenicol resistance as in its gram-positive counterparts. Preliminary data indicated overexpression of OprN in strain H673, indicating along with the characteristic pattern of antibiotic susceptibilities and OprD deficiency that strain H673 carried a separate *nfxC* mutation.

**Characterization of the *mexT* gene.** The putative regulatory gene located upstream of the *mexE* gene (15) was amplified by PCR in separate reactions from *P. aeruginosa* H103. The PCR products were cloned into pUCP19 and pET28a and sequenced. Compared to the recent GenBank entry by T. Kohler and coworkers (accession no. AJ007825), which designated this gene *mexT*, the sequences of our PCR products contained four base pair changes, of which only one resulted in a conservative amino acid change, L26V. The overexpression of the *mexT* gene, with the pET28a-based expression system, yielded a major protein band with an  $M_r$  of approximately 33 kDa (data not shown). There were two potential ATG start codons at the predicted 5' end of the *mexT* gene, although the largest ORF was likely to be correct, based on the alignments of the largest deduced MexT product with other bacterial proteins (see below).

The deduced MexT protein sequence was compared with sequences in the GenBank database by using the BLAST algorithm. The search for amino acid sequences similar to MexT identified 14 proteins with similarity scores higher than 115. Thirteen of the first 14 were NodD proteins from various species of *Rhizobium*. The protein with the highest similarity was NahR, a LysR-type transcriptional regulator that regulates the production of enzymes for the metabolism of naphthalene and salicylate as sole carbon sources in *Pseudomonas putida* (37). An alignment of the two sequences (MexT, 304 amino acids, and NahR, 300 amino acids) revealed a total of 33% identical residues and 14.5% conserved residue changes. The regions of similarity were concentrated somewhat at the N terminus. This region of NahR contains the DNA binding helix-turn-helix domain of the protein. A number of very highly conserved residues in NahR and other LysR-type regulators essential for DNA binding (Ala 27, Pro 35, and Arg 43) (30) were also conserved in MexT. Analysis of the region of NahR which binds salicylate as a coinducer (5) and the residues important for binding showed only 18% sequence identity and no conservation of key residues in the same region of MexT, consistent with results observed for other LysR-type transcriptional regulators which show little similarity in this region.

TABLE 2. Influence of various additives to the growth medium on the MIC of different antibiotics

<i>P. aeruginosa</i> strain	Additive <sup>b</sup>	MIC (μg/ml) <sup>a</sup>				
		IPM	CHL	NOR	TET	CFP
H103	None	0.5	25	0.1	3.1	1
	Salicylate	8	25	0.1	1.6	1
	Acetyl salicylate	4	25	0.2	1.6	1
	Benzoate	4	25	0.1	3.1	1
H103/pUCP19	None	0.5	25	0.2	1.6	1
	Salicylate	8	25	0.1	3.1	2
H103/pMC2:: <i>mexT</i>	None	2	>400	1.6	6.3	1
	Salicylate	8	>400	1.6	3.1	2
H729 <i>oprD::Km<sup>r</sup></i>	None	8	25	0.1	1.6	1
	Salicylate	8	25	0.2	1.6	1
1008	None	1	50	0.1	3.1	1
	Salicylate	8	50	0.2	1.6	2
1008OIR01 <i>nfxC</i>	None	4	>400	3.1	6.3	1
	Salicylate	8	>400	3.1	3.1	1

<sup>a</sup> IPM, imipenem; CHL, chloramphenicol; NOR, norfloxacin; TET, tetracycline; CFP, ceftiofur. Results are the mean MICs from four to eight determinations.

<sup>b</sup> All additives were used at a concentration of 16 mM.

**Expression of the *mexT* gene in *P. aeruginosa*.** To determine the effect of overexpressing *mexT* in *P. aeruginosa*, plasmid pMC2, in which *mexT* was cloned in the same orientation as the *lac* promoter, was transformed into a *P. aeruginosa* wild-type strain, H103. The antimicrobial susceptibility and outer membrane profiles of this strain were examined. In comparison to the wild-type strain with and without the vector, the overexpression of *mexT* resulted in a two- to fourfold increase in resistance to imipenem, a 32-fold increase in resistance to chloramphenicol, and an 8- to 16-fold increase in resistance to norfloxacin (Table 2). There were no significant changes in the susceptibilities to gentamicin, ceftiofur, and tetracycline. The antibiotic susceptibility profile when *mexT* was overexpressed was consistent with that of the *nfxC*-type mutant 1008OIR01 compared to its parent 1008 (Table 2) (20). The characteristic increase in susceptibility to carbenicillin seen in *nfxC*-type mutants could not be confirmed, due to the plasmid-encoded carbenicillin resistance marker in the strains containing pUCP19 and pMC2.

Consistent with the findings of Kohler et al. (15), analysis of the outer membrane proteins showed substantially reduced expression of a 45-kDa protein (Fig. 1A, lane 3) in the *mexT*-overexpressing strain compared to that of the control strains (Fig. 1A, lanes 1 and 2). This underexpressed protein was confirmed to be OprD by Western blot analysis with an OprD-specific MAb (Fig. 1B). This reduction of OprD in the outer membrane was consistent with the results for the *nfxC* mutant (Fig. 1A, lane 5). Outer membrane preparations were further analyzed by Western blotting with anti-OprM, -OprJ, and -OprN specific MAbs. OprN was immunodetected only in the *mexT*-overexpressing strain H103/pMC2 and the *nfxC* mutant (Fig. 1C, lanes 3 and 5). No cross-reactive bands were seen with the OprJ antibody, while the OprM MAb revealed only wild-type levels of OprM in all strains (data not shown). No other changes in the outer membrane profiles were observed. These results confirm that the overexpression of *mexT* is itself sufficient to reproduce an *nfxC*-type phenotype.

**Reduction of transcription of *oprD* by MexT.** To directly test whether MexT exerted its effect at the transcriptional level, plasmid pMC2 was transformed into *P. aeruginosa* H846, which had an *oprD::xylE* transcriptional fusion in the chromosome.



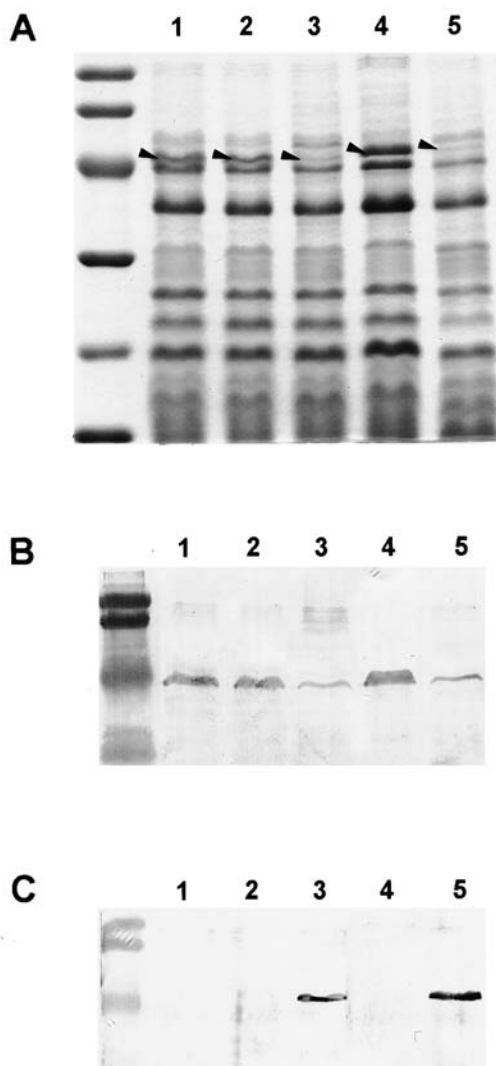


FIG. 1. Expression of the cloned *mexT* gene in *P. aeruginosa*. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of outer membrane proteins (A) and Western immunoblots of outer membrane proteins with an anti-OprD MAb (B) and an anti-OprN MAb (C). Lanes 1, H103; lanes 2, H103/pUCP19; lanes 3, H103/pMC2::*mexT*; lanes 4, 1008; lanes 5, 1008OIR01 (*nfxC*). The positions of OprD in panel A are labelled with arrowheads. Molecular weight standards in the left-hand lane of panel A are, from top to bottom, 94,000, 67,000, 43,000, 30,000, 20,100, and 14,400. Molecular weight standards in the left-hand lanes of panels B and C are 104,000, 81,000, 47,700, and 34,600.

The overexpression of *mexT* in this strain resulted in a 2.5-fold reduction in activity of catechol 2,3-dioxygenase, the *xylE* gene product (Table 3), whereas the vector control plasmid had no significant effect.

**Influence of organic acids on *oprD* expression.** In *P. aeruginosa*, it has been shown that salicylate reduces the expression of an outer membrane protein with a molecular mass of 45 kDa (34). Salicylate has also been reported to induce the expression of a 50-kDa protein thought to be OprN (20). For this reason we were interested in investigating the possibility that salicylate or other structurally related compounds may be inducers of the *mexEF-oprN* operon and a repressor of *oprD*, by acting as a cofactor of the MexT protein.

The antibiotic susceptibilities of *P. aeruginosa* H103 were tested in the presence of salicylate and a number of related

TABLE 3. Effects of *mexT*, salicylate, and acetyl salicylate on the catechol 2,3-dioxygenase activity of the chromosomal *oprD::xylE* transcriptional fusion in *P. aeruginosa* H846

Plasmid	Growth additive	Catechol 2,3-dioxygenase activity (pmol/min/ $\mu$ g of extract) (%) <sup>a</sup>
None	None	61 $\pm$ 5 (115)
pUCP19	None	55 $\pm$ 12 (104)
pMC2:: <i>mexT</i>	None	22 $\pm$ 5 (42)
None	None	53 $\pm$ 14 (100)
None	16 mM salicylate	16 $\pm$ 6 (30)
None	16 mM acetyl salicylate	29 $\pm$ 10 (55)

<sup>a</sup> Results are means  $\pm$  standard deviations of at least three independent experiments. The second and fourth rows present data for independent control experiments performed at separate times for the two experiments below each control. The value 53 was used as the 100% activity to calculate the percentages in the third column.

compounds known to induce multiple antibiotic resistance in *E. coli* (Table 2). Salicylate induced a 16-fold increase in resistance to imipenem but did not lead to significant changes in resistance to chloramphenicol, norfloxacin, tetracycline, or the control  $\beta$ -lactam antibiotic cefpirome (Table 2). Similarly, the only significant change in MIC with benzoate or acetyl salicylate was an eightfold increase in resistance to imipenem (Table 2). Catechol, dinitrophenol, naphthalene, or naphthaquinone, as well as acetate, induced no significant changes in susceptibility to any of the antibiotics tested.

All compounds were tested for their abilities to influence the antibiotic susceptibilities of strains H103/pUCP19, the *mexT*-overexpressing clone H103/pMC2, the OprD-deficient mutant H729, the *nfxC* mutant 1008OIR01, and its parent strain 1008. For clarity, only the data for salicylate are presented in Table 2. In the *oprD::Km<sup>r</sup>* mutant H729, there were no significant changes in MIC of any antibiotic in the presence of salicylate (Table 2), benzoate, acetyl salicylate, or catechol, indicating that the imipenem resistance induced in the isogenic parent strain H103 was entirely due to *oprD* repression. In contrast, all agents able to influence imipenem resistance in H103 had similar effects on the other strains. Maximal levels of imipenem resistance induced in the *mexT*-overexpressing clone and the *nfxC* mutant were to a MIC of 8  $\mu$ g/ml, which was the same MIC observed for the OprD-deficient mutant H729.

To determine whether the increased imipenem resistance was due to decreased expression of OprD and/or one of the efflux operons, we examined the outer membrane profiles of *P. aeruginosa* when grown in the presence of 32 mM salicylate or 16 mM benzoate. The presence of salicylate or benzoate led to reduced levels of OprD, as confirmed by Western immunoblotting with an anti-OprD MAb (Fig. 2A). Wild-type levels of OprM were detected in all samples by using an anti-OprM MAb (Fig. 2C), and no cross-reacting bands were revealed with the OprN antibody (Fig. 2B) or the OprJ antibody (data not shown). The same results were obtained in the presence of 16 mM acetyl salicylate, and we also detected no salicylate-mediated changes in the levels of OprN when *mexT* was expressed from plasmid pMC2 (data not shown).

**Influence of organic acids on *oprD* gene transcription.** To further examine the mechanism through which salicylate and acetyl salicylate reduced OprD levels in the outer membrane, we assessed the expression of the *oprD::xylE* transcriptional fusion in strain H846 (Table 3). The presence of 16 or 32 mM salicylate led to a 3.3-fold reduction of catechol 2,3-dioxygenase activity, and a similar but somewhat lesser reduction was observed with acetyl salicylate. This indicated that salicylate

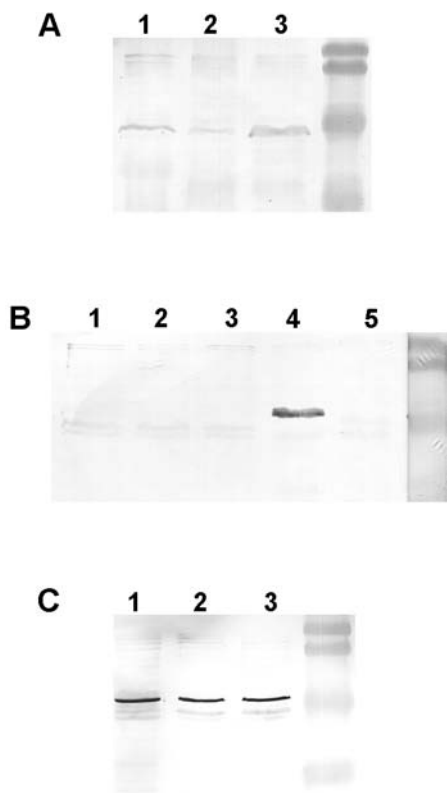


FIG. 2. The effects of addition of salicylate and benzoate to the growth medium on the expression of OprD, OprM, and OprN. Western immunoblots of outer membrane proteins of strain H103 grown in Mueller-Hinton medium with 16 mM benzoate (lane 1) or 32 mM salicylate (lane 2) or without any additives (lane 3). In lanes 4 and 5 of panel B, strains 1008OIR01 (*nfxC*) and 1008, respectively, were grown without any additives. Proteins were detected with anti-OprD (A), anti-OprN (B), and anti-OprM (C) MAbs, respectively. Molecular weight standards in the right-hand lanes are, from top to bottom, 104,000, 81,000, 47,700, and 34,600.

and acetyl salicylate led to reduced levels of OprD by causing reduction of *oprD* transcription. We could not do these same studies in the presence of benzoate, because these cultures had a strong increase in yellow coloration due presumably to the bioconversion of benzoate to catechol. We also tested acetate and observed a slight increase of catechol 2,3-dioxygenase activity (data not shown).

## DISCUSSION

The existence of the LysR-type regulator MexT was predicted by Kohler et al. (15), and the *P. aeruginosa* genome sequencing project proved to be very useful in designing primers that allowed PCR amplification of the *mexT* structural gene from the genome of *P. aeruginosa* PAO1. The overexpression of the cloned *mexT* gene in *P. aeruginosa* confirmed the previous notion (15) that it is sufficient to reproduce the multiple-drug-resistant *nfxC* phenotype. Outer membrane profiles were also changed by *mexT* overexpression, with a reduction of OprD and expression of the outer membrane component of the *mexEF-oprN* efflux operon, OprN. Significantly, by using a chromosomal *oprD::xylE* transcriptional fusion, MexT was shown to cause reduced levels of transcription of the *oprD* gene. Thus, it appears that MexT is a regulator that can presumably activate the efflux operon while repressing OprD. While unusual, this is not the first example of a LysR-type

regulator acting both as a repressor and an activator. The AmpR proteins from *Citrobacter freundii* and *Enterobacter cloacae* repress or activate the expression of *ampC* depending on the conditions. The level of expression of *ampC* is two- to threefold higher in an *ampR*-negative situation than in the presence of AmpR under noninducing conditions (2). However, although it seems unlikely, we cannot exclude the possibility that the repression of *oprD* by MexT is mediated by an unknown regulatory mechanism, and there was no obvious consensus region in the upstream regions of these two genes that would be a candidate for the MexT binding region.

In *E. coli*, the plant-derived compound salicylate has been shown to reduce the expression of outer membrane porins as well as to induce low-level resistance to some antibiotics (7, 27). Salicylate does this in part by antagonizing the repressor activity of the MarR protein, which negatively regulates the *marRAB* locus. By reducing the binding of MarR to the promoter region of the *marRAB* genes, salicylate relieves the repression of MarA, which in turn results in changes in the expression of several unlinked target genes (19). Interestingly, the *P. aeruginosa* genome has no obvious homologs to the *marA* or *marB* gene (data not shown), and it was reported that salicylate can also act in *E. coli* by a *mar*-independent pathway (7). Salicylate is a membrane-permeative weak acid and is known to also suppress the synthesis of certain outer membrane proteins in a number of gram-negative bacteria (3, 29), although the mechanism by which this occurs has not been elucidated to date. Given the observed apparent homology between the transcriptional regulators MexT and NahR and the previous reports that salicylate, one of the natural cofactors of NahR, reduced the expression of a 45-kDa outer membrane protein in *P. aeruginosa* (20, 34), we were intrigued with the possibility that salicylate reduced *oprD* expression via the MexT protein. In the presence of 16 or 32 mM salicylate, the levels of OprD protein in the outer membrane and the susceptibility of *P. aeruginosa* to the carbapenem imipenem were reduced. This was consistent with previous reports, but we demonstrated by Western immunoblotting that the 45-kDa protein with reduced expression was indeed OprD (an important point given the 17 OprD homologs of similar molecular weight in the genome). By using a chromosomally encoded *oprD::xylE* fusion, it was determined that the presence of salicylate caused decreased *oprD* expression at the level of transcription by 3.3-fold, a level similar to that seen when *mexT* was overexpressed.

Masuda et al. (20) reported that increased resistance to ofloxacin and the induction of an outer membrane protein of approximately 50 kDa occurred in the presence of 32 mM salicylate. This 50-kDa protein was not heat modifiable and was therefore thought to be OprN rather than OprM. In the publication by Sumita and Fukasawa (34), however, no change in the MIC of quinolones was seen and no expression of a non-heat-modifiable protein was noted in the presence of 32 mM salicylate. Our results showed some minor variations of MICs, which were not considered significant enough to suspect a meaningful induction of an efflux operon. An examination of outer membrane profiles showed that only OprD expression was changed and that there were wild-type levels of expression of OprM and the usual lack of expression of either OprJ or OprN. These data indicate that salicylate alone does not increase the expression of the *mexEF-oprN* operon. Similarly, acetyl salicylate and benzoate reduced the levels of OprD, and we could demonstrate in the former case that this was mediated at the level of transcription. In contrast, the structurally related compounds naphthalene, naphthaquinone, catechol, and dinitrophenol, the latter of which strongly induced the

*marRAB* operon in *E. coli* (7), resulted in no significant increase in resistance to any of the antibiotics tested. We also observed that the weak membrane-soluble acid acetate had no effect on the transcription of *OprD*, indicating that salicylate and related compounds are not acting as weak membrane-soluble acids perturbing the pH gradient across the cytoplasmic membrane. Furthermore, it seems that only molecules with an ionizable carboxyl group mediated the repression of *oprD*. We propose that there is at least one more negative regulatory mechanism acting on *oprD*, independently of MexT. Recent data suggested that the transcription of *oprD* decreased in stationary phase (23). In addition we have demonstrated that ArgR can act as an activator of *oprD* expression, that independent glutamate-mediated activation occurs, and that catabolite repression by succinate and probably positive regulation mediated by nitrogen sources are operative (24). Thus, *OprD* appears to be a highly regulated protein. While this is probably related in part to the function of *OprD* in the uptake of basic amino acids and peptides and to the ability to grow on these as the sole carbon and nitrogen source, the physiological purpose of coregulation with a broad specificity antibiotic efflux operon through MexT is not as easy to understand. Possibly MexT serves a function in *P. aeruginosa* analogous to that of the *E. coli mar* operon which also upregulates the efflux of antibiotics (18) and downregulates through *micF* the major porin *OmpF* (6). In this regard, it should be noted that *OprD*, while demonstrating specificity for imipenem and basic amino acids, also has a nonspecific role in the uptake of small compounds like gluconate (13).

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