JOURNAL OF BACTERIOLOGY, Oct. 1999, p. 6300–6305 Vol. 181, No. 20
0021-9193/99/$04.00+0

Characterization of MexT, the Regulator of the MexE-MexF-OprN Multidrug Efflux System of *Pseudomonas aeruginosa*

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Received 20 April 1999/Accepted 5 August 1999

We investigated the regulation of the MexEF-OprN multidrug efflux system of *Pseudomonas aeruginosa*, which is overexpressed in *nfxC*-type mutants and confers resistance to quinolones, chloramphenicol and trimethoprim. Sequencing of the DNA region upstream of the *mexEF-oprN* operon revealed the presence of an open reading frame (ORF) of 304 amino acids encoding a LysR-type transcriptional activator, termed MexT. By using T7-polymerase, a 34-kDa protein was expressed in *Escherichia coli* from a plasmid carrying the *mexT* gene. Expression of a *mexE*::*lacZ* fusion was 10-fold higher in *nfxC*-type mutants than in the wild-type strain; however, transcription of *mexT* as well as the *mexT* DNA region was unchanged. Located adjacent to *mexT* but transcribed in opposite direction, the beginning of an ORF termed *qrh* (quinone oxidoreductase homologue) was identified. Expression of a *qrh*::*lacZ* fusion was also found to be activated by MexT. Further, we present evidence for coregulation at the transcriptional and the posttranscriptional level between the MexEF-OprN efflux system and the OprD porin responsible for cross-resistance of *nfxC*-type mutants to carbapenem antibiotics.

Pseudomonas aeruginosa is a leading cause of hospital acquired infections. Its high intrinsic antibiotic resistance and the ability to develop multidrug resistance pose serious therapeutic problems. For a long time it has been assumed that this elevated intrinsic resistance was mainly due to the low outer membrane permeability of *P. aeruginosa* which was correlated to the appearance of outer membrane proteins with sizes in the range of 50 kDa. These proteins (OprM, OprJ, and OprN) have now been shown to be part of multidrug efflux systems with broad specificity (13, 25, 26) which catalyze the energy-dependent extrusion of antibiotics such as β-lactams, quinolones, tetracycline, chloramphenicol, macrolides, and trimethoprim. The three efflux systems of *P. aeruginosa* have similar patterns of genetic organization. The first gene of each operon encodes a periplasmic fusion protein (MexA, MexC, or MexE), the second encodes a cytoplasmic membrane protein (MexB, MexD, or MexF) thought to be the actual efflux pump, and the third gene encodes an outer membrane protein (OprM, OprJ, or OprN). The three proteins are believed to form a channel across the inner and outer membranes. The *mexAB-oprM* operon is expressed constitutively and contributes to the intrinsic resistance of *P. aeruginosa* to a variety of toxic substances (14, 15). Transcription of the *mexAB-oprM* operon is increased in *nalB*-type mutants (30) due to mutations in the repressor protein MexR (27, 43). The *mexCD-oprJ* operon (25) is not expressed constitutively but is overexpressed in mutants displaying mutations in *nfxB*, the gene coding for the transcriptional repressor of this efflux system (24, 35).

The third efflux operon, *mexEF-oprN*, which confers resistance to quinolones, chloramphenicol, and trimethoprim, is overexpressed in *nfxC*-type mutants of *P. aeruginosa* (13). NfxC-type mutants (7) are also cross-resistant to the carbapenem imipenem (3, 8, 18), since they show decreased expression of OprD, an outer membrane protein facilitating the diffusion of basic amino acids, small peptides (40), and several carbapenem antibiotics (39). The *mexEF-oprN* efflux operon differs from the other efflux systems in that it is positively regulated by a protein belonging to the LysR family of transcriptional activators (13). In the present study, we characterize this activator, called MexT, and show that it is required for the expression of the MexEF-OprN efflux pump. We also demonstrate the involvement of MexT in the regulation of an open reading frame (ORF) adjacent to *mexT* and present evidence for coregulation at the transcriptional and posttranscriptional levels between the OprD porin and the MexEF-OprN efflux system.

(The MexT sequence and the regulation of the *mexEF-oprN* operon were presented at the *Pseudomonas* meeting in Madrid, Spain, in September 1997.)

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* DH10B was used for cloning experiments and plasmid propagation. Plasmids pUC119 and pIC20H were used for subcloning and sequencing. Plasmids were conjugated from *E. coli* S17-1 (36) or by triparental mating with the helper plasmid pRK2013. Transconjugants were selected on M9 minimal medium (16) supplemented with 40 mM citrate as a carbon source or on Luria-Bertani (LB) medium supplemented with ampicillin at 40 μg/ml to counterselect against *E. coli*. MICs in Mueller-Hinton broth were determined by the microdilution method (11).

DNA sequencing and protein analysis. DNA sequences were determined from double-stranded templates according to the dideoxy chain termination method (32) with an automatic sequencer (Applied Biosystems model 373A). DNA sequences were processed and analyzed with the PCGENE program (Intelligenetics Inc., Mountain View, Calif.) and the BLAST algorithm (1). Protein alignments were generated by the program CLUSTAL.

Strain and plasmid constructions. The *mexT* mutant was constructed by inserting a 2.2-kbp *Bam*HI fragment of pOPN4 (13) carrying the entire *mexT* gene into the suicide vector pJQ200mp18 (28), yielding pJQN3. MexT was inactivated by inserting the Hg^r determinant as a 4.8-kbp *Bam*HI fragment of pHP45ΩHg (6) into the unique *Bgl*II site of pJQN3. The resulting construct, called pJQCΩ, was mobilized from strain S17-1 into PAO1. Hg^r and Gm^s colonies were recovered after counterselection on sucrose-containing plates. Three independent colonies were analyzed by Southern blot analysis with a digoxigenin-labeled DNA fragment. All three showed the same banding pattern, in agreement with an integration of the ΩHg cassette into the *mexT* structural gene. The *mexT*::ΩHg mutation was then transduced by phage E79*tv2* (22) into the *nfxC*-type mutant PT149. The *mexE*::*lacZ* fusion plasmid pNFZ4 was constructed by cloning a 0.5-kbp *Bgl*II-*Eco*RI fragment of cosmid pOPN4 (13) containing the 3′ end

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristic(s)[a]	Reference or source
Strains		
E. coli		
MC1061	F$^-$ *araD139* Δ(*ara-leu*)*7696 galE15 galK16* Δ(*lac*)*X74 rpsL thi*	Laboratory collection
S17-1	*thi pro hsdR recA chr*::RP4-2	36
DH10B	F$^-$ *araD139* Δ(*ara-leu*)*7696 galU galK* Δ(*lac*)*X74 mcr*Δ(*mrr-hsdRMS-mcrBC*) *deoR* Φ80d*lacZ* ΔM15 *endA1 nupG recA1 rpsL*	Laboratory collection
P. aeruginosa		
PAO1	Wild-type	Laboratory collection
PT149	PAO-7H, PAO1 overproducing MexEF-OprN, selected on ciprofloxacin	13
PT579	PAO1 *mexT*::ΩHg	This study
Plasmids		
pUC119	High-copy-number cloning vector, Ap^r	41
pIC20H	High-copy-number cloning vector, Ap^r	17
pJQ200mp18	Mobilizable suicide vector, *sacB*, Gm^r	28
pWSK29	Low-copy-number T7 expression vector, Ap^r	42
pWNC5	pWSK29 with 1.5-kbp *Kpn*I-*Eco*RI fragment expressing *mexT* under T7 control, Ap^r	This study
pOPN4	pLAFR3-based cosmid containing 27-kb insert harboring *mexE-mexF-oprN*, Tc^r	13
pME6001	Broad-host-range vector, Gm^r	D. Haas
pMEXT	pNFC8, 1.5-kbp *Hin*dIII-*Eco*RI fragment of pOPN4 cloned in *Hin*dIII-*Eco*RI-cleaved pME6001, Gm^r	13
pMP220	Promoterless *lacZ* fusion vector, IncP, Tc^r	37
pNFZ4	*mexE*::*lacZ* fusion on pMP220, Tc^r	This study
pTZ4	*mexT*::*lacZ* fusion on pMP220, Tc^r	This study
pQRZ4	*qrh*::*lacZ* fusion on pMP220, Tc^r	This study
pSE45	*oprD*::*lacZ* transcriptional fusion on pMP220, Tc^r	This study
pSE50	*oprD*::*phoA* translational fusion on pMP220, Tc^r	This study

[a] Hg, mercury; Tc, tetracycline; Gm, gentamicin; Ap, ampicillin.

of *mexT* into the promoter probing vector pMP220 (37). To generate the *qrh*::*lacZ* fusion plasmid pQRZ4, a 0.5-kbp DNA fragment was amplified from genomic DNA of strain PAO1 with primers procxP1 (5′-CTGCTCGGGGGCCA GGTTCTGAC-3′) and procxM3 (5′-GGTGGGCTCCATGCTGCGTC-3′) by using Pwo polymerase (Boehringer Mannheim). The blunt-ended fragment was cloned into the *Eco*RV-cleaved vector pIC20H. From the resulting plasmid pPRO1, a *Bgl*II-*Eco*RI fragment was cloned into *Bgl*II-*Eco*RI-cleaved pMP220. The *mexT*::*lacZ* fusion plasmid was obtained after *Hin*dIII digestion of pQRZ4 and religation. A clone in which the *Hin*dIII fragment was in the opposite orientation with respect to that of the *lacZ* gene as in pQRZ4 was called pTZ4. The transcriptional *oprD*::*lacZ* fusion pSE45 was constructed by inserting a 1.3-kbp PCR fragment containing 735 bp upstream of the *oprD* initiation codon as a *Bam*HI-*Xba*I fragment into *Bgl*II-*Xba*I-cleaved pMP220. The translational *oprD*::*phoA* fusion was constructed by inserting a 941-bp *Bam*HI-*Hin*dIII fragment containing the same 735 bp upstream of OprD as in pSE45 and 206 bp of the N-terminal sequence encoding OprD into *Bam*HI-*Hin*dIII-cleaved pPHOK102 (31) to yield pSE48. Plasmid pSE50 was obtained by inserting a 3-kbp *Bam*HI-*Pst*I fragment of pSE48 into *Bgl*II-*Pst*I-cleaved pMP220. To clone the DNA region upstream of *mexT*, chromosomal DNA of a *P. aeruginosa* strain carrying the Gm^r suicide plasmid pJQ200mp18 (28) integrated into the *oprN* gene (unpublished results) was digested with *Sac*I and religated. The ligation mix was used to transform *E. coli* DH10B, and Gm^r clones were selected. The restriction profiles of plasmids from three transformants were analyzed and found to contain the same piece of chromosomal DNA upstream of the *mexT* gene.

MexT expression with T7 polymerase. An *E. coli* strain harboring the T7 polymerase gene on plasmid pGP1-2 was transformed with the control plasmid pWSK29 or the *mexT*-carrying plasmid pWNC5. Single transformants were grown at 30°C in LB medium supplemented with the appropriate antibiotics. At an optical density at 600 nm of approximately 0.4, the cultures were shifted for 20 min to 42°C followed by a further 60-min incubation at 37°C. One-milliliter aliquots were centrifuged, and the pellets were resuspended in 1 ml of M9 minimal medium supplemented with all 20 amino acids except methionine and cysteine. ^{35}S-labeled methionine and cysteine (10 μCi) were added, and the mixture was incubated for 2 min in the presence of rifampin at 400 μg/ml. The suspensions were centrifuged, and the pellets were resuspended in 100 μl of sodium dodecyl sulfate loading buffer. The samples were boiled, and 10 μl of each was loaded onto a 12% acrylamide minigel. After migration, the gel was dried and exposed to X-ray film for approximately 18 h.

Determination of β-galactosidase and alkaline phosphatase activities. Strains were inoculated from −70°C glycerol stocks in LB supplemented with the appropriate antibiotics and grown overnight at 37°C. Cultures were diluted 1:100 in fresh LB without antibiotics. When strains carried two plasmids, antibiotics for the marker on each plasmid were added (gentamicin at 15 μg/ml and tetracycline at 50 μg/ml). β-Galactosidase (21) and alkaline phosphatase (4) activities were determined in triplicate samples at various times during growth.

Nucleotide sequence accession number. The DNA sequence of *mexT* and its upstream region have been deposited in the EMBL databank and assigned accession number AJ007825.

RESULTS

Nucleotide sequence of the *mexT* gene. The *mexE-mexF-oprN* operon was previously shown to be located on pOPN4, a pLAFR3-based cosmid clone (13). The DNA region upstream of *mexE* was sequenced and a single ORF of 912 nucleotides (nt) was identified. This ORF, called *mexT*, starts 112 nt from the end of the insert of the cosmid and is transcribed in the same direction as the *mexE-mexF-oprN* operon. A Shine-Dalgarno sequence (GAGGA) was located 6 nt upstream of the initiation codon. The 304-amino-acid sequence of the putative MexT polypeptide was compared to the entries in the Swissprot and EMBL databases by using the BLASTP program (1). Significant homology was found with several members of the LysR family of transcriptional activators (34). The highest amino acid identity (32%) was to NahR (33), the activator of the plasmid-encoded *nah* operon, specifying genes for the degradation of naphthalene in *Pseudomonas putida*. The second best alignment (30% amino acid identity) was obtained with NodD, one of the transcriptional activators required for nodulation in *Rhizobium* spp. Homology was most pronounced toward the N termini of the proteins containing the helix-turn-helix motif.

Mutations leading to overexpression of the *mexAB-oprM* and the *mexCD-oprJ* efflux systems have been located in the corresponding regulator genes, *mexR* (27, 43) and *nfxB* (24),

respectively. The *mexT* gene was therefore a likely candidate to harbor a mutation responsible for overexpression of the *mexEF-oprN* operon. The *mexT* region, encompassing the structural gene and the regulator region, was amplified by PCR from strain PT149 (formerly PAO-7H) overexpressing the MexEF-OprN efflux system and from its parental strain PAO1. However, sequence analysis of these PCR fragments showed no nucleotide changes. The *mexT* region of two other spontaneous *nfxC*-type mutants was sequenced. Again, neither of them displayed any nucleotide changes, suggesting that the mutation responsible for the *nfxC* phenotype was not located in *mexT* or its regulatory region.

Cloning of the DNA region upstream of *mexT*. To further investigate the regulation of the *mexEF-oprN* efflux operon, the DNA region upstream of *mexT* was cloned by plasmid rescue as described in Materials and Methods. The restriction pattern of three recovered clones was analyzed and found to contain about 10 kbp of chromosomal DNA. The DNA sequence upstream of *mexT* was determined from one of the plasmids and found to contain the beginning of an ORF transcribed divergently from *mexT* and located 221 bp from the *mexT* start codon. The deduced amino acid sequence of the ORF was homologous to a family of quinone oxidoreductases from *E. coli* (38) and *P. aeruginosa* (EMBL accession number X85015) as well as to eucaryotic homologues. The intergenic region between *mexT* and this ORF, called tentatively *qrh* (quinone oxidoreductase homologue), contained two putative promoter sequences located on the two different DNA strands and overlapping at their −10 regions. The putative *mexT* (CTGACA-18 bp-GATAAT) and *qrh* (CTGACA-15 bp-AATAAC) promoter sequences were very similar to the *E. coli* σ^{70} consensus sequence (TTGACA-15 to 17 bp-TATAAT).

Examination of the previously sequenced *mexE* promoter region (13) did not reveal significant homology to the *E. coli* σ^{70} consensus sequence. However, the sequence GTATCAC TGTTCGTGATAATCAAAATCTCGTCGTTCGATTAGT was found 58 bp upstream of the *mexE* start codon. This sequence showed a striking similarity to the sequence of the *nod* box (9) (NYATCCAYNNYRYRGATGNNNNYNATC NAAACAATCGATTTTA) located upstream of genes regulated by NodD in a *Rhizobium* spp. MexT is therefore likely to activate *mexEF-oprN* transcription by binding to the *nod*-box-like sequence.

Analysis of the *mexT* gene product and phenotype of a *mexT* mutant. T7 polymerase-directed expression of *mexT* in *E. coli* revealed a protein band with a size of 34 kDa (Fig. 1). This size is close to the molecular mass of 33,418 deduced from the nucleotide sequence of *mexT*, and the labeled protein is therefore likely to correspond to the *mexT* gene product.

A PAO1 derivative, called PT579 and inactivated by the insertion of an ΩHg cassette in the coding region of *mexT*, was constructed. As expected, MICs of the *mexT* mutant were indistinguishable from those of the wild-type strain (data not shown). The *mexT*::ΩHg mutation was transferred by transduction into the multidrug-resistant *nfxC*-type mutant PT149. All of the tested transductants recovered the antibiotic susceptibility profile of the wild-type strain, demonstrating that a functional *mexT* gene is required for expression of the multidrug resistance phenotype in *nfxC*-type mutants.

MexT is required for selection of the *nfxC* phenotype. Selection of the *nfxC* phenotype can easily be achieved by plating wild-type PAO1 cells on LB agar containing at least 500 μg of chloramphenicol per ml. To establish the role of MexT in selection of the *nfxC* phenotype, PAO1 and the *mexT*::ΩHg mutant PT579 were plated on LB agar plates containing chloramphenicol at 600 μg/ml. While resistant colonies of PAO1 appeared at a frequency of about 10^{-8}, no colonies ($<10^{-10}$) were obtained with the *mexT* mutant. Among the 10 colonies derived from PAO1, all presented the *nfxC* phenotype (data not shown). These results demonstrate the absolute requirement of *mexT* for the selection of the *nfxC* antibiotic resistance phenotype. Indeed, PAO1 strains unable to yield *nfxC* mutants carry mutations in *mexT* (unpublished result).

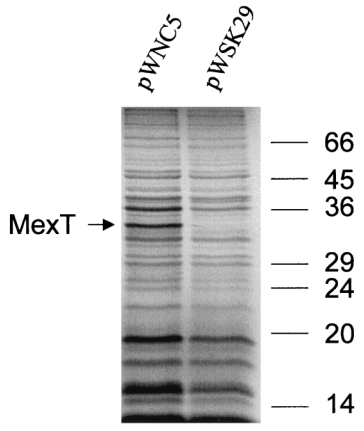

FIG. 1. Labeling of *E. coli* cells with [^{35}S]Met and [^{35}S]Cys carrying either the control T7 expression vector pWSK29 or the *mexT*-carrying plasmid pWNC5. The molecular masses (in kilodaltons) of standard protein markers are indicated on the right.

Expression of *mexE*, *mexT*, and *qrh*. To further study the regulatory circuits of the *qrh-mexT-mexEF-oprN* DNA region, transcriptional *lacZ* fusions to *mexE* (pNFZ4), *mexT* (pTZ4), and *qrh* (pQRZ4) were constructed with the low-copy-number IncP derivative pMP220. In PAO1, β-galactosidase activities expressed from the *mexE*::*lacZ* fusion were always comparable to those of the control vector pMP220. However, in the *nfxC*-type mutant PT149 expression of the *mexE*::*lacZ* fusion was already higher in the lag phase and further increased during exponential and stationary growth (Fig. 2A). A similar increase in *mexE*::*lacZ* expression was found when MexT was introduced in *trans* on the multicopy plasmid pMEXT (data not shown). These results clearly show a correlation in the level of expression of the *mexEF-oprN* operon with the antibiotic resistance phenotype. They also suggest that the amount of MexT might be critical to the regulation of the *mexEF-oprN* operon. Therefore, the *mexT*::*lacZ* fusion plasmid pTZ4 was introduced into the wild type and the *nfxC* mutant PT149. In both strains, the fusion showed similar levels of elevated constitutive expression of β-galactosidase during growth in LB (data not shown), in agreement with a gene displaying a σ^{70} consensus promoter sequence (Fig. 2B). This result suggests that overexpression of MexEF-OprN in the *nfxC*-type mutant PT149 was not due to increased levels of *mexT* transcription.

Genes controlled by LysR-type activators are often located adjacent to the regulator and transcribed in the opposite direction. We therefore analyzed expression of the *qrh*::*lacZ* fusion plasmid pQRZ4. Like that for *mexT*, *qrh* expression was found to be constitutive; however, four- to fivefold-higher levels of β-galactosidase were measured in strains PT149 and PAO1 (pMEXT) compared to the wild type (Fig. 2B), suggesting that the *qrh* gene is also positively regulated by MexT.

To confirm that plasmid-encoded MexT was sufficient to activate transcription, the *lacZ* fusions were analyzed in *E. coli*. In the presence of plasmid pMEXT, expression levels of the *mexE*::*lacZ* fusion were increased about 40-fold, while those of the *qrh*::*lacZ* fusion were increased fivefold. Plasmid pMEXT

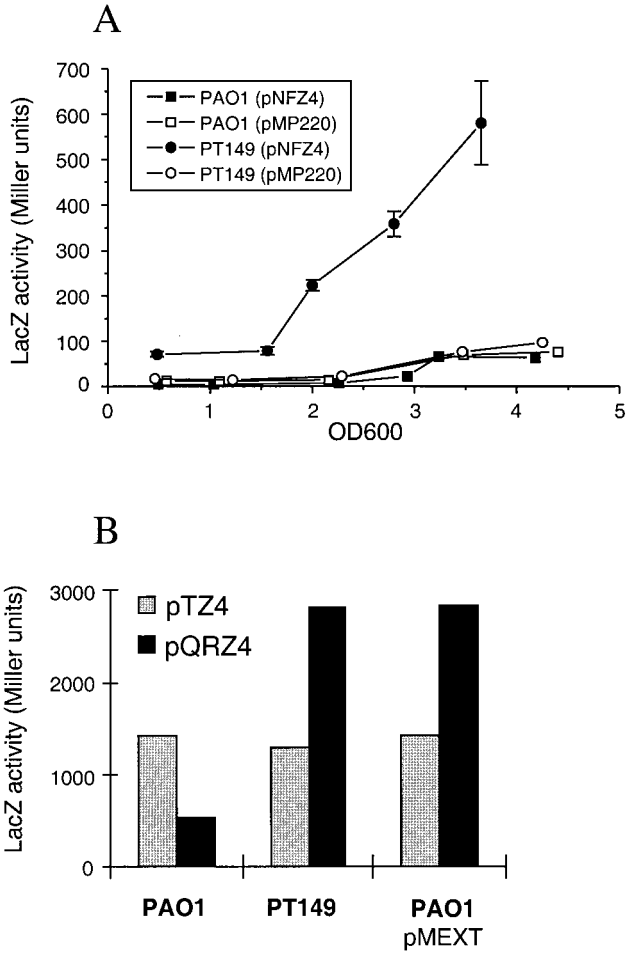

FIG. 2. (A) β-Galactosidase activity in *P. aeruginosa* wild-type strain PAO1 and *nfxC*-type mutant PT149 carrying either the control plasmid pMP220 or the *mexE*::*lacZ* fusion plasmid pNFZ4. Data are the results from one representative experiment. Error bars indicate standard deviations for triplicate determinations of LacZ activity. (B) Expression of *mexT*::*lacZ* and *qrh*::*lacZ* fusions in wild-type strain PAO1 in the absence or presence of plasmid-encoded copies of MexT and in the *nfxC*-type mutant PT149. Samples were taken during the mid-exponential growth phase.

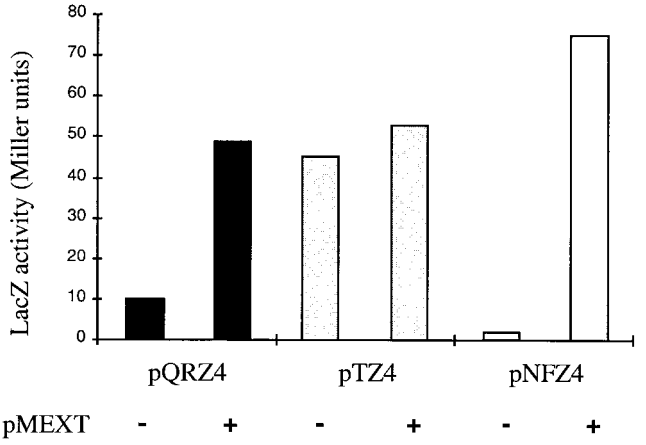

FIG. 3. Effect of *mexT* on expression of *mexE*::*lacZ* (pNFZ4), *mexT*::*lacZ* (pTZ4), and *qrh*::*lacZ* (pQRZ4) fusions in *E. coli* MC1061. The presence and absence of the *mexT*-carrying plasmid pMEXT are indicated by + and − symbols, respectively.

had no significant effect on expression of the *mexT*::*lacZ* fusion (Fig. 3). These results are in agreement with those found for *P. aeruginosa*, where plasmid-encoded MexT had a greater effect on *mexE* than on *qrh* transcription.

In order to verify that the observed operon expression was not a singularity of the particular *nfxC*-type strain PT149, the three *lacZ* fusions were introduced into four other *nfxC* type strains as well as into strains overexpressing either the *mexAB-oprM* (*nalB*) or the *mexCD-oprJ* (*nfxB*) operon. All four *nfxC*-type strains showed increased expression of the *mexE*::*lacZ* fusion (10- to 20-fold) and of the *qrh*::*lacZ* fusion (fivefold). In the strains overexpressing either of the two other efflux operons, all three fusions were expressed at levels comparable to those of the wild type (data not shown).

MexT coregulates *oprD* expression. Several reports have clearly established that in *nfxC*-type mutants, overexpression of the *mexEF-oprN* operon is linked to decreased amounts of the OprD porin in the outer membrane (18, 19). Since OprD is the port of entry of the carbapenem imipenem, *nfxC*-type mutants are cross-resistant to imipenem (3, 8, 18). Whether this cross-resistance is due to transcriptional repression of the *oprD* gene by MexT was investigated by constructing a pMP220-based *lacZ* fusion to the *oprD* gene (pSE45). Twofold-lower LacZ activity was found in the *nfxC*-type mutant PT149 compared to the wild type (Table 2). Since this weak effect could probably not account for the dramatic decrease in OprD expression in *nfxC* mutants as determined previously by Western blot analysis (13, 18, 19), we further examined the possibility of posttranscriptional regulation by MexT. A plasmid-encoded *oprD*::*phoA* translational fusion (pSE50) was constructed and introduced into PAO1 and PT149. Compared to the wild type, a fivefold decrease was observed in the *nfxC*-type mutant PT149 (Table 2). Furthermore, a similar fivefold decrease in alkaline phosphatase expression was found when plasmid pMEXT was introduced into PAO1 carrying the *oprD*::*phoA* fusion. These results strongly suggest that MexT downregulates *oprD* expression also at the posttranscriptional level.

DISCUSSION

Our results clearly establish MexT as the transcriptional activator of the *mexEF-oprN* efflux operon and demonstrate its requirement for the expression of the *nfxC* multidrug resistance phenotype. Furthermore, we found that when the gene is expressed from a multicopy plasmid, *mexT* on its own is able to activate transcription of a *mexE*::*lacZ* fusion in both *P. aeruginosa* and *E. coli*. This finding is in agreement with the previous observation that the *mexT*-carrying plasmid pMEXT (pNFC8) is sufficient to confer a *nfxC* resistance phenotype to a suscep-

TABLE 2. Effects of *nfxC* mutation and *mexT* on transcriptional *oprD*::*lacZ* and translational *oprD*::*phoA* fusions in *P. aeruginosa*

Strain	Plasmid	LacZ or PhoA activity[a]
PAO1	pSE45 (*oprD*::*lacZ*)	2,086 ± 373
PT149(*nfxC*)	pSE45	1,012 ± 31
PAO1	pSE50 (*oprD*::*phoA*)	10.4 ± 2.1
PT149(*nfxC*)	pSE50	1.8 ± 0.1
PAO1	pSE50, pME6001 (control plasmid)	8.7 ± 0.4
PAO1	pSE50, pMEXT (pME6001::*mexT*)	1.8 ± 0.1

[a] Results are the means ± standard deviations of triplicate determinations. Samples were taken during the mid-exponential growth phase. Enzymatic activities are expressed in Miller units (LacZ) or alkaline phosphatase units (PhoA).

tible PAO1 wild type strain (13). We also show that the *mexT* DNA region is unchanged in the *nfxC*-type mutant PT149 and that *mexT* transcription levels are comparable to those of the wild type. How then can one account for the increased *mexEF-oprN* transcription in the *nfxC* mutants? The majority of the LysR-type regulators are synthesized in a nonactive form and become activated upon binding of a cognate effector molecule(s) (34). We therefore assume that in the *nfxC*-type mutants, the effector molecule of MexT is produced constitutively or in larger amounts than in the wild type, thereby causing permanent activation of MexT and hence overexpression of the MexEF-OprN efflux system. The fact that introduction of additional plasmid-encoded copies of MexT into a wild-type strain also causes increased *mexEF-oprN* expression can be explained by a shift in the equilibrium between the inactive and active forms of MexT. Such a mechanism has been suggested for the XylS regulator protein of the TOL plasmid pWW0 from *P. putida* (29), a member of the AraC family of transcriptional activators. Therefore, even in the absence of MexT effector molecules, plasmid-encoded MexT is able to activate *mexEF-oprN* transcription and to confer a *nfxC* multidrug resistance phenotype on the susceptible wild type strain.

The MexT homologues NahR and NodD are activated upon binding of salicylate (33) and phenolic plant-derived compounds (20), respectively. Interestingly, MexT not only has significant amino acid similarity to NodD, but the regulatory region of the *mexEF-oprN* operon also contains a *nod* box element (9) found upstream of NodD-regulated genes in *Rhizobium* species. However, neither NodD effectors (flavone, trigonellin, naringenin, and vanilline, etc.) provided at a final concentration of 2 mM nor the NahR inducer salicylate at concentrations of up to 50 mM showed any significant effect on *mexEF-oprN* transcription (unpublished results). Furthermore, the addition of the MexEF-OprN substrate molecules chloramphenicol, norfloxacin, or trimethoprim at subinhibitory concentrations had no effect on the expression of the *mexE*::*lacZ* fusion. Supposing that effector molecules are also substrates of the efflux pump, these results are further evidence that antibiotics are not the natural substrates of the MexEF-OprN efflux pump.

We found that MexT activates transcription not only of the *mexEF-oprN* efflux operon but also of an adjacent ORF, which we tentatively called *qrh*. The protein encoded by *qrh* shows homology to a family of quinone oxidoreductases of eucaryotic and procaryotic origins (38). Whether this gene is involved in the expression of the *nfxC* phenotype remains to be determined.

Numerous reports have demonstrated a decrease in OprD expression in *nfxC*-type mutants (12, 13, 18). OprD is a porin which facilitates diffusion of basic amino acids and small peptides and is also the port of entry of carbapenem antibiotics. In addition to MexEF-OprN substrates, *nfxC*-type strains are therefore cross-resistant to imipenem. Our results with *oprD*::*lacZ* and *oprD*::*phoA* fusion experiments suggest that the coregulation between *oprD* and the *mexEF-oprN* operon is exerted both at the transcriptional and posttranscriptional levels. A 2.5-fold decrease in the expression of a transcriptional *oprD*::*xylE* fusion in the presence of MexT has been reported recently by Ochs et al. (23). This result is in agreement with the twofold decrease in expression of our transcriptional *oprD*::*lacZ* fusion observed in a *nfxC* mutant. However, this modest effect seems unlikely to account solely for the almost complete absence of OprD in outer membranes of *nfxC* mutants (10, 13, 18). Therefore, the observed posttranscriptional effect on *oprD* expression offers a further explanation. A similar type of coregulation is found in *E. coli mar* mutants in which increased expression of the AcrAB efflux system is correlated with decreased expression of the porin OmpF (5). This effect is mediated by the antisense *micF* RNA (2). Alternatively, one can assume that transport of OprD across the cytoplasmic membrane is decreased in *nfxC*-type mutants by the overexpression of the MexEF-OprN efflux system.

Among the three multidrug efflux systems characterized so far, only the *mexAB-oprM* system (14) is constitutively expressed. It also displays the broadest substrate specificity and might therefore represent a natural defense mechanism against a variety of harmful substances. The fact that the other two efflux systems are not expressed under normal laboratory conditions and are tightly regulated by their respective regulator protein could suggest a role in more specific tasks, for example in the secretion of cellular metabolites. Identification of their substrates should help to elucidate the physiological role of these efflux pumps.

ACKNOWLEDGMENTS

We thank Mehri Michéa-Hamzehpour and Patrick Plésiat for helpful discussions and Colette Rossier for performing the sequencing. We thank R. Fellay (Novartis, Nyon, Switzerland) for pointing out the homology to the *nod* box of *Rhizobium* sp. and for providing chemicals. We are grateful to D. Haas (University of Lausanne, Lausanne, Switzerland) for providing plasmid pME6001.

This work was supported by a grant to T.K. from the Fonds National Suisse pour la Recherche Scientifique.

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