ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Apr. 2004, p. 1320–1328 Vol. 48, No. 4
0066-4804/04/$08.00+0 DOI: 10.1128/AAC.48.4.1320–1328.2004

Enhancement of the *mexAB-oprM* Efflux Pump Expression by a Quorum-Sensing Autoinducer and Its Cancellation by a Regulator, MexT, of the *mexEF-oprN* Efflux Pump Operon in *Pseudomonas aeruginosa*

Hideaki Maseda,[1]* Isao Sawada,[2] Kohjiro Saito,[1] Hiroo Uchiyama,[2] Taiji Nakae,[1] and Nobuhiko Nomura[2]*

Institute of Applied Biochemistry, University of Tsukuba, Tsukuba, Ibaraki 305-8572,[2] and Department of Molecular Life Science, Tokai University School of Medicine, Isehara, Kanagawa 259-1193,[1] Japan

Received 16 May 2003/Returned for modification 9 August 2003/Accepted 14 December 2003

***nfxC*-type cells of *Pseudomonas aeruginosa* that produce the MexEF-OprN efflux pump exhibit resistance to fluoroquinolones and chloramphenicol and hypersusceptibility to most classical β-lactam antibiotics. We investigated the molecular mechanism of how the *nfxC* mutation causes β-lactam hypersusceptibility. The MexAB-OprM extrusion pump transports and confers resistance to β-lactam antibiotics. Interestingly, expression of the *mexAB-oprM* operon reached the highest level during the mid-stationary growth phase in both wild-type and *nfxC*-type mutant strains, suggesting that expression of the *mexAB-oprM* operon may be controlled by cell density-dependent regulation such as quorum sensing. This assumption was verified by demonstrating that exogenous addition of the quorum-sensing autoinducer *N*-butyryl-L-homoserine lactone (C4-HSL) enhanced the expression of MexAB-OprM, whereas *N*-(3-oxododecanoyl)-L-homoserine lactone had only a slight effect. Furthermore, this C4-HSL-mediated enhancement of *mexAB-oprM* expression was repressed by MexT, a positive regulator of the *mexEF-oprN* operon. It was concluded that β-lactam hypersusceptibility in *nfxC*-type mutant cells is caused by MexT-mediated cancellation of C4-HSL-mediated enhancement of MexAB-OprM expression.**

Pseudomonas aeruginosa is an opportunistic pathogen that causes infections in immunocompromised hosts and colonizes the lungs of individuals with cystic fibrosis. This organism shows broad resistance to structurally and functionally dissimilar antibiotics. This type of multidrug resistance is attributable mainly to the expression of the xenobiotic extrusion transporter MexAB-OprM coupled with tight outer membrane permeability (21, 23, 29). The *mexAB-oprM* operon encodes three protein subunits (21, 26, 29): the intrinsic inner membrane protein MexB (9), the inner membrane-associated periplasmic lipoprotein MexA (45), and the outer membrane lipoprotein OprM (13, 22, 44). The *mexAB-oprM* operon is negatively regulated by the product of the *mexR* gene, which is located upstream of the *mexAB-oprM* genes and is divergently transcribed (1, 5, 14, 34, 37). *nalB*-type mutants caused by the *mexR* mutation derepress MexAB-OprM production and are highly resistant to fluoroquinolones, chloramphenicol, and most classical β-lactam antibiotics (30, 32, 35, 42).

Recently, it was reported that the MexAB-OprM transporter exports quorum-sensing mediators, acylhomoserine lactones (AHSLs), which induce the production of cell density-dependent virulence factors, including proteases, rhamnolipids, exotoxin A, exoenzyme S, and pyocyanin (24, 25). The AHSLs control at least two quorum-sensing systems in *P. aeruginosa*, namely, LasR-LasI and RhlR-RhlI. LasI and RhlI catalyze the last steps in the syntheses of *N*-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL) and *N*-butyryl-L-homoserine lactone (C4-HSL), respectively. LasR and RhlR are specifically activated by the diffusible signaling molecules 3-oxo-C12-HSL and C4-HSL, respectively.

Four resistant-nodulation-division efflux pumps (MexAB-OprM, MexCD-OprJ, MexEF-OprN, and MexXY) have been identified in *P. aeruginosa*, and they are derepressed in *nalB*, *nfxB*, *nfxC*, and N135 mutant cells, respectively (10, 18–21, 27, 28, 32). Transcription of the *mexAB-oprM*, *mexCD-oprJ*, and *mexXY* operons is derepressed by a mutation of the repressors *mexR*, *nfxB*, and an unidentified gene, respectively (2, 3, 28, 40). On the other hand, transcription of the *mexEF-oprN* operon is dually regulated by a positive regulator, MexT, and a putative negative regulator, MexS (10, 17). Both *nfxB* and *nfxC* mutants exhibit resistance to structurally diverse antibiotics and hypersusceptibility to most classical β-lactam antibiotics (12), suggesting the presence of a common mechanism in which pump protein expression is coupled with β-lactam hypersusceptibility.

This paper reports the mechanism of *nfxC* mutation-mediated β-lactam hypersusceptibility and its connection to quorum sensing and growth phase-dependent expression of MexAB-OprM.

* Corresponding authors. Mailing address for Hideaki Maseda: Gene Research Center, University of Tsukuba, Tsukuba, Ibaraki 305-8577, Japan. Phone: 81-298-53-7792. Fax: 81-298-53-7723. E-mail: maseda@sakura.cc.tsukuba.ac.jp. Mailing address for Nobuhiko Nomura: Institute of Applied Biochemistry, University of Tsukuba, Tsukuba, Ibaraki 305-8572, Japan. Phone: 81-298-53-6627. Fax: 81-298-53-6627. E-mail: nomunobu@sakura.cc.tsukuba.ac.jp.

TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Relevant properties	Source or reference
Strains		
P. aeruginosa		
PAO4290	*leu-10 argF10 aph-9004*; FP^-	18
TNP090	PAO4290 carrying a chromosomal *mexB*::*xylE* fusion; Km^r	This study
TNP091	TNP090 Δ*lasI*	This study
TNP092	TNP090 Δ*rhlI*	This study
TNP093	TNP090 Δ*lasI* Δ*rhlI*	This study
TNP099	TNP090 Δ*mexEF-oprN*	This study
PAO1S	$lacI^q$ *lacZ*Δ*M15*; Tc^r	33
PAO1SC	*nfxC*-type mutant of PAO1S designated PAO1S-Lac#1	16
OCR-Lac4	*nalB*-type mutant of PAO1S	34
E. coli		
DH5α	*E. coli* strain for transformation	TaKaRa
S17-1	Mobilizer strain	41
Plasmids		
pMMB67EH	Broad-host-range vector; Amp^r IncQ	8
pMEXEF-OPRN1	pMMB67EH derivative carrying the *mexE, mexF,* and *oprN* genes	18
pMEXT8380	pMMB67EH derivative carrying the *mexT* gene from *P. aeruginosa* 8380	17
X1918	pUC1918 derivative carrying an *xylE* cassette; Amp^r	39
pMOB3	pHSS21 derivative carrying a MOB cassette; Cm^r Km^r	38
pME4510	Broad-host-range promoter-probe vector carrying a *lacZ* cassette; Gm^r	33
pME4510-mexOP	pME4510 derivative carrying the *mexAB-oprM* operator-promoter region	34
pG19II	pK19mobsac derivative carrying the Gm^r gene as a marker	This study

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used are listed in Table 1. *Escherichia coli* DH5α was used as the host in DNA manipulations.

Recombinant DNA techniques. We manipulated recombinant DNA by standard procedures described previously (36). PCR amplification of chromosomal DNA was carried out by using the LA *Taq* kit (TaKaRa Shuzo, Osaka, Japan) according to the manufacturer's instructions.

Construction of conjugative plasmid pG19II. To construct a conjugative plasmid with a gentamicin resistance gene as a marker, pK19mobsac was treated with BglII and NcoI and then ligated with Gm^r gene cassette, which was treated by BamHI and NcoI after amplification with Fgm (5′-CGGGATCCCGAATTGACATAAGCCTGTTCG-3′) and Rgm (5′-ACCCATGGACGAATTGTTAGGTGGCGGTACTT-3′), a primer pair containing a newly added cutting site (underlined) for restriction nucleases from pME4510. The resulting plasmid was named pG19II (Fig. 1A).

Insertion of an *xylE* gene cassette into the chromosomal *mexAB-oprM* operon. The *mexAB-oprM* reporter with an *xylE* fusion was constructed as follows (Fig. 1B). The *xylE* gene from pX1918 was inserted in the EcoRI site of pBluescript SK(+). The resulting plasmid was treated with PstI and ligated with a PstI-treated kanamycin resistance gene (Pharmacia) to yield the pXyl-Km/SK(+) plasmid. The 2.8-kbp *xyl*-Km cassette from pXyl-Km/SK(+) was inserted into BamHI-treated pMex(sac/Hind)/ΔRI-pNOT19 plasmid, which carries the *mexAB-oprM* operon. The resulting plasmid, pMex::Xyl, was treated with NotI and ligated with a NotI-treated Mob cassette from pMOB3 to yield the suicide plasmid pMex::Xyl-MOB. This plasmid was introduced into the mobilizer strain *E. coli* S17-1 and then was transferred to *P. aeruginosa* PAO4290 by conjugation as reported earlier (18). The insertion of the *xyl*-Km cassette in the chromosomal *mexAB-oprM* operon was confirmed by Southern hybridization (36) and PCR analyses. This constructed strain was designated *P. aeruginosa* TNP090.

Deletion of the chromosomal *lasI*, *rhlI*, or *mexEF-oprN* genes. To construct a series of isogenic mutants lacking the *lasI*, *rhlI*, or *lasI* and *rhlI* genes, PCR primers for amplification of regions containing the *lasI* or *rhlI* gene were synthesized based on nucleotide sequences from the *Pseudomonas* genome sequencing project database. After amplification of ca. 2.7-kb DNA fragments on PAO1 chromosomal DNA as a template with Flas1 (5′-CGGGATCCGCTGGAACGCTCAAGTGGAAAATTGGAG-3′) and Rlas2 (5′-CCCAAGCTTTCTGCGAAGGCCTGGAGAACCTTGC-3′) or with Frhl1 (5′-CGAGCTCGCATAACAGATAGGGTTGCCATG-3′) and Rrhl2(5′-GGGAAGCTTGGACCAGGCACCAGGATGG-3′), the amplified DNA fragments were ligated into the BamHI-HindIII site or SacI-HindIII site in a multicloning site of pHSG398 to yield pHSG-las12 or rhl12, respectively. Next, 600 bp of *lasI* or 67 bp of *rhlI* in these DNA fragments was deleted by inverted PCR with the primer pair Flas3 (5′-GGAAGATCTCTTCACTTCCTCCAAATAGGAAGCTGAAG-3′) and Rlas4 (5′-GGAAGATCTCGGGGACCTGTCGGCTCGC-3′) or Frhl3 (5′-GGTCTAGACTTCATCGCCAGCTGCGGATCGTCC-3′) and Rrhl4 (5′-GCTCTAGACGGCCATGGAGCGCTATTTCGTTCGC-3′), respectively, and both amplified fragments were self-ligated in the BglII or XbaI site to yield pHSG-dellas or -delrhl, respectively. After reinsertion of the insert fragments of pHSG-dellas into pG19II, the resulting plasmid was named pG19-dellas. After amplification of the 2-kb DNA fragment by using the primer pair delrhl1 (5′-GGGAAGCTTATCCGGCGATCCTCAACGGCCTGC-3′) and delrhl2 (5′-GGGAAGCTTGCCTTGCCGTCGACGATCTGCTGG-3′) from pHSG-delrhl, the amplified DNA fragment was inserted into pG19II to yield pG19-delrhl. For disruption of the chromosomal *mexEF-oprN* genes, pUC19-*mexEFN* (18) was treated with SphI and then self-ligated, yielding pUC19-delEFN. An EcoRI-HindIII DNA fragment from pUC19-delEFN was subcloned into pG19II, yielding pG19II-delEFN. pG19-dellas, pG19-delrhl, and pG19-delEFN were mobilized from *E. coli* S17-1 to *P. aeruginosa* TNP090 to introduce a deletion of the *lasI* region, the *rhlI* region, or the *mexEF-oprN* region into the recipient chromosomes by allelic exchange as above.

Thus, we constructed mutants lacking the *lasI* gene, the *rhlI* gene, the *lasI* and *rhlI* genes, and the *mexEF-oprN* genes from TNP090, and we designated them TNP091, TNP092, TNP093, and TNP099, respectively. The deletions of the *lasI*, *rhlI*, and *mexEF-oprN* genes were confirmed by PCR analyses and phenotypes of the mutants (data not shown).

Experiment to test the effect of C4-HSL on expression of MexAB-OprM. Cells were grown aerobically in Luria-Bertani (LB) medium and diluted with 4 ml of fresh LB medium to an A_{600} of ca. 0.025. After incubation for 3 h at 37°C, an appropriate concentration of C4-HSL was added and the cultures were incubated for 3 h at 37°C. At an A_{600} of 1.3 to 1.8, samples were collected and catechol 2,3-dioxygenase activity was determined as reported previously (39).

Other techniques. Western blot analysis has been described previously (18). The MICs of antibiotics were determined by the agar dilution method with Mueller-Hinton agar II (Becton Dickinson Microbiology Systems, Cockeysville, Md.). Protein was quantified by the method of Lowry et al. (15). β-Galactosidase activity was assayed by the method of Miller as described by Sambrook et al. (36). For assay of the activity of catechol 2,3-dioxygenase (the *xylE* gene product), cells

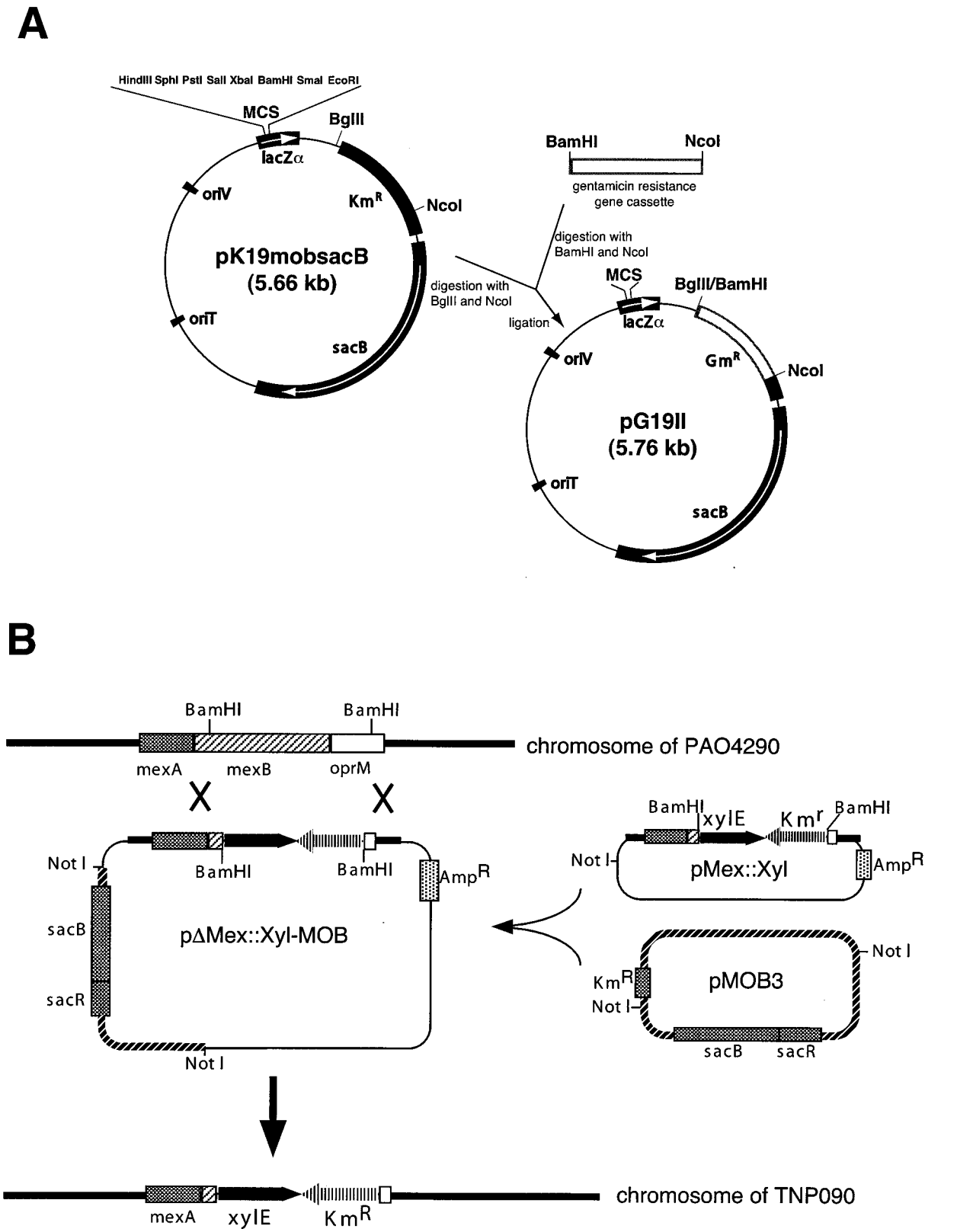

FIG. 1. Schematic representation of the procedure for deletion of the chromosome. (A) Construction of a conjugative plasmid with a gentamicin resistance gene. The open box represents the gentamicin resistance gene region amplified by PCR. MCS, multiple cloning site. (B) Procedure used to insert the *xylE* reporter cassette into the chromosomal *mexAB-oprM* operon. NotI-treated pMex::Xyl was ligated to the NotI site of MOB3, and the resulting pΔMex::Xyl-MOB was inserted into the chromosomal *mexAB-oprM* operon by homologous recombination. The transconjugants were Km^r and sucrose sensitive. The unwanted DNA fragment was excised by selecting for sucrose resistance.

TABLE 2. Antibiotic susceptibilities of the strains used to test the effect of *mexT*

Strain	Plasmid	Pump component(s) expressed[a]	MIC (μg/ml)[b]								
			NFLX	CP	CAZ	CPZ	CPR	CZOP	KM	GM	IPM
PAO1S		ABM	0.78	100	1.56	6.25	3.13	0.78	100	3.13	0.78
OCR-Lac4		ABM^{++c}	25	800	6.25	25	6.25	3.13	100	3.13	0.78
PAO1SC		ABM, EFN	25	≥1,600	0.78	3.13	0.78	0.39	50	1.56	3.13
PAO1S	pMMB67EH	ABM	0.78	100	1.56	12.5	3.13	1.56	100	3.13	0.78
	pMEXEF-OPRN1	ABM, EFN	25	≥1,600	1.56	12.5	1.56	0.78	50–100	3.13	0.78
	pMEXT8380	ABM, EFN	25	≥1,600	1.56	6.25	1.56	0.78	50	1.56	3.13
PAO4290	pMMB67EH	ABM	0.78	100	3.13	50	6.25	1.56	6.25	3.13	1.56
	pMEXEF-OPRN1	ABM, EFN	25	≥1,600	1.56	25	3.13	1.56–0.78	3.13	3.13	1.56
	pMEXT8380	ABM, EFN	12.5	≥1,600	1.56	12.5	3.13	0.78	3.13	1.56	6.25

[a] Pump components are shown as combinations of the following subunits: A, MexA; B, MexB; E, MexE; F, MexF; M, OprM; N, OprN.
[b] Abbreviations: NFLX, norfloxacin; CP, chloramphenicol; CAZ, ceftazidime; CPZ, cefoperazone; CPR, cefpirome; CZOP, cefozopram; KM, kanamycin; GM, gentamicin; IPM, imipenem.
[c] ++, overexpression.

were suspended in 990 μl of assay buffer. The reaction was initiated by adding 10 μl of 100 mM catechol dissolved in water, and the A_{375} was recorded at 25°C. Specific activity was defined as nanomoles of product formed per minute per milligram of protein ($\varepsilon^{1\,cm}_{375\,nm} = 4.4 \times 10^4$) (39).

RESULTS

Linkage between the *nfxC* mutation and MexAB-OprM expression. To characterize the antibiotic susceptibility of the *nfxC*-type cells used in this experiment, we determined MICs of antibiotics. The *nfxC*-type mutant PAO1SC, which produces the MexEF-OprN efflux pump, exhibited resistance to fluoroquinolones, tetracycline, chloramphenicol, and imipenem and hypersusceptibility to β-lactam antibiotics, confirming previous results (7, 12) (Table 2). In contrast, the *nalB* mutant OCR-Lac4, which produces a derepressed level of the MexAB-OprM efflux pump, showed resistance to most β-lactam agents. Because the *nfxC*-type PAO1SC cells exhibited hypersusceptibility to the substrates of the MexAB-OprM efflux pump, a possible linkage between the *nfxC* mutation and the level of MexAB-OprM expression was suggested. In fact, we and other investigators observed reduced expression of OprM and MexA in the *nfxC*-type cells compared with the wild-type cells (12, 21).

Linkage between growth phase-dependent quorum sensing and *mexAB-oprM* expression. To assess the connection between the overall growth phase of *P. aeruginosa* cells and the expression of the *mexAB-oprM* operon, a reporter plasmid, pME4510-mexOP, carrying the *mexAB-oprM* operator-promoter region, was introduced into the wild-type strain PAO1S. With this strain, the expression of the *mexAB-oprM* operon was monitored by measuring β-galactosidase activity at several different growth points. The fully grown cells were diluted 200-fold with prewarmed fresh medium, and the β-galactosidase

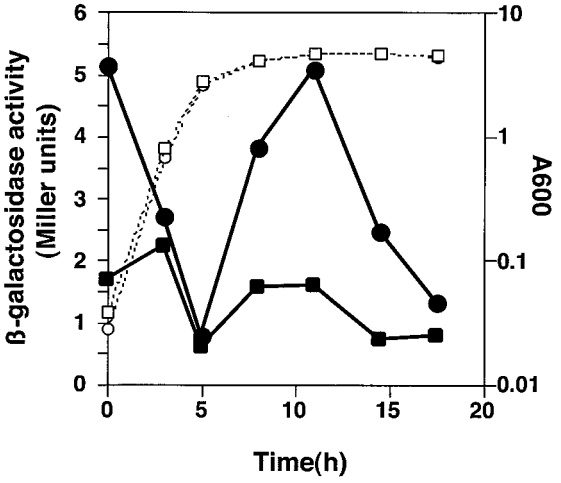

FIG. 2. Expression of the *mexA*::*lacZ* transcriptional fusion at various times during growth of the wild-type strain. Cells were grown at 37°C in LB medium with rotation at 200 rpm and harvested at the time points indicated, and the β-galactosidase activity was determined. Open and closed symbols represent turbidity at A_{600} and β-galactosidase activity, respectively. Circles, PAO1S(pME4510-mexOP); squares, PAO1S(pME4510). Three independent experiments were done, and representative data are shown.

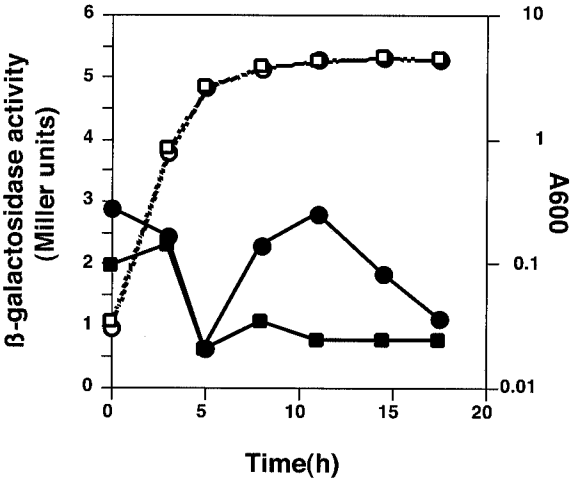

FIG. 3. Expression of the *mexA*::*lacZ* transcriptional fusion at various times during growth of the *nfxC*-type mutant. Experimental procedures and symbols are the same as described in the legend to Fig. 2. Three independent experiments were done, and representative data are shown.

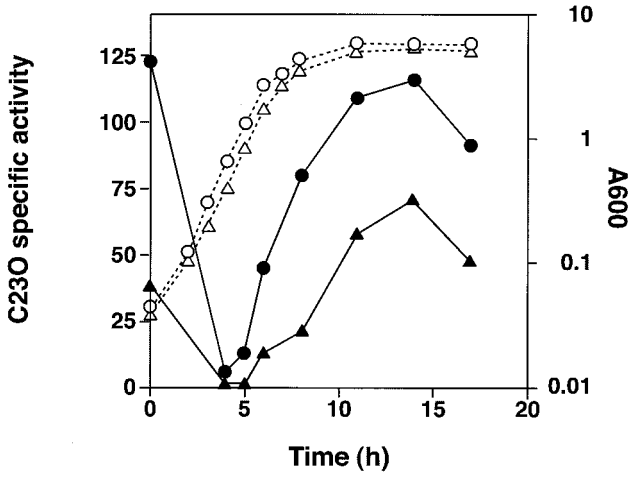

FIG. 4. Expression of the *mexB*::*xylE* chromosomal fusion at various times during growth of TNP090 cells. Open and closed symbols represent A_{600} and catechol 2,3-dioxygenase (C23O) activity, respectively. Circles and triangles represent TNP090(pMMB67EH) and TNP090(pMEXT8380), respectively. Three independent experiments were done, and representative data are shown.

activity dropped dramatically at 3 to 5 h after the dilution. This result was interpreted to mean that fully expressed MexAB-OprM in the high-cell-density preculture was repressed to a low level as the culture was diluted to a low cell density. When the cell growth reached stationary phase and incubation was continued for 3 to 5 h, β-galactosidase activity again reached a high level, and thereafter it gradually declined (Fig. 2). This was interpreted to mean that the cells sensed a high population density in the stationary phase and induced MexAB-OprM expression.

To verify that the regulation of the chromosomal *mexAB-oprM* operon is similar to that of the plasmid-borne operon, we constructed a chromosomal fusion of the *xylE* gene downstream of *mexA*, generating strain TNP090. With this strain, the transcription of the chromosomal *mexAB-oprM* operon was monitored. The results showed that the catechol 2,3-dioxygenase activity at several growth points was close to the β-galactosidase activity profile (data not shown). This is consistent with a recent report by Sanchez et al. which demonstrated that *mexA* expression was triggered at the onset of the stationary growth phase (37).

MexT-mediated regulation of *mexAB-oprM* expression. To ascertain whether the decreased expression of the MexAB-OprM efflux pump in the *nfxC*-type mutant was under the control of the MexEF-OprN regulator MexT, pME4510-mexOP, encoding *mexA*::*lacZ*, was introduced into PAO1SC, which carries a functional *mexT* gene. As the cells entered the stationary growth phase, expression of the *mexAB-oprM* operon increased, and it reached the highest level at mid-stationary phase; this profile was comparable to that of the parent strain, PAO1S (Fig. 3). However, the level of gene expression in PAO1SC at most time points was half as high as that in PAO1S carrying an impaired chromosomal *mexT* gene (compare Fig. 2 and 3). These data are fully consistent with the antibiotic susceptibility profiles of these strains (Table 2).

To ascertain whether the decreased transcription of the *mexAB-oprM* operon in *nfxC* cells is attributable to the presence of a functional *mexT* gene, we introduced pMEXT8380, which carries a functional *mexT* gene, in *trans*, into the parent

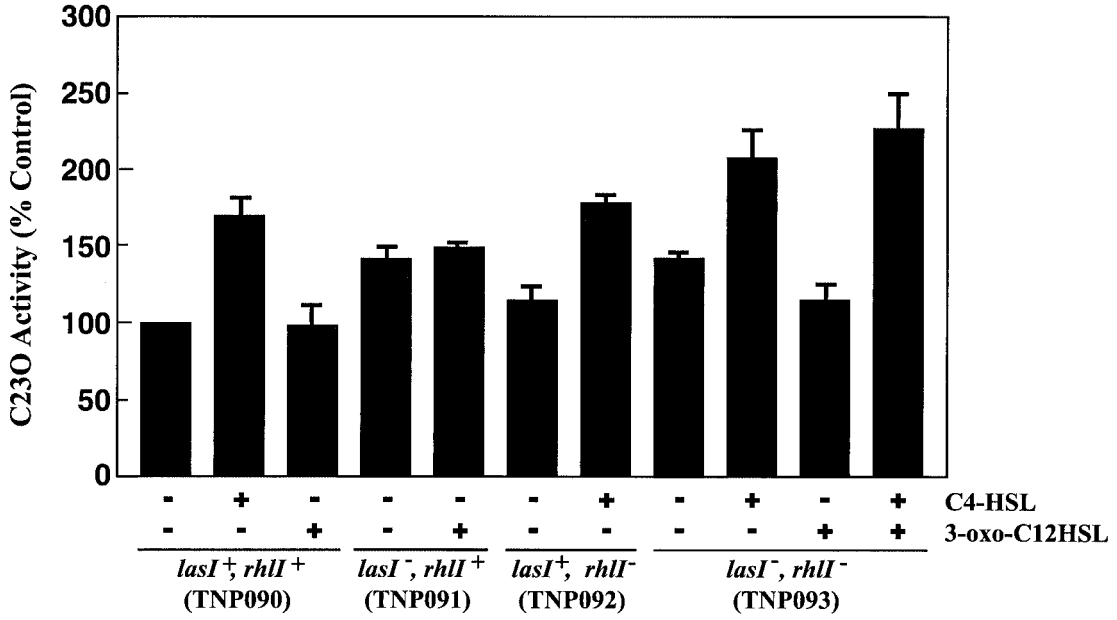

FIG. 5. Effect of exogenous C4-HSL and 3-oxo-C12-HSL on expression of the *mexAB-oprM* operon. Catechol 2, 3-dioxygenase (C23O) activities are expressed as values relative to the activity (ca. 50) of C23O in *P. aeruginosa* TNP090 without additions. Cultures were grown for 3 h in the absence of autoinducers; after addition of 50 μM (final concentration) autoinducer(s) (+, addition; −, no addition), cultures were incubated for 3 h. The final optical densities at 600 nm were 1.3 to 1.8. The C23O activities were then determined. Data are means ± standard deviations from three independent experiments.

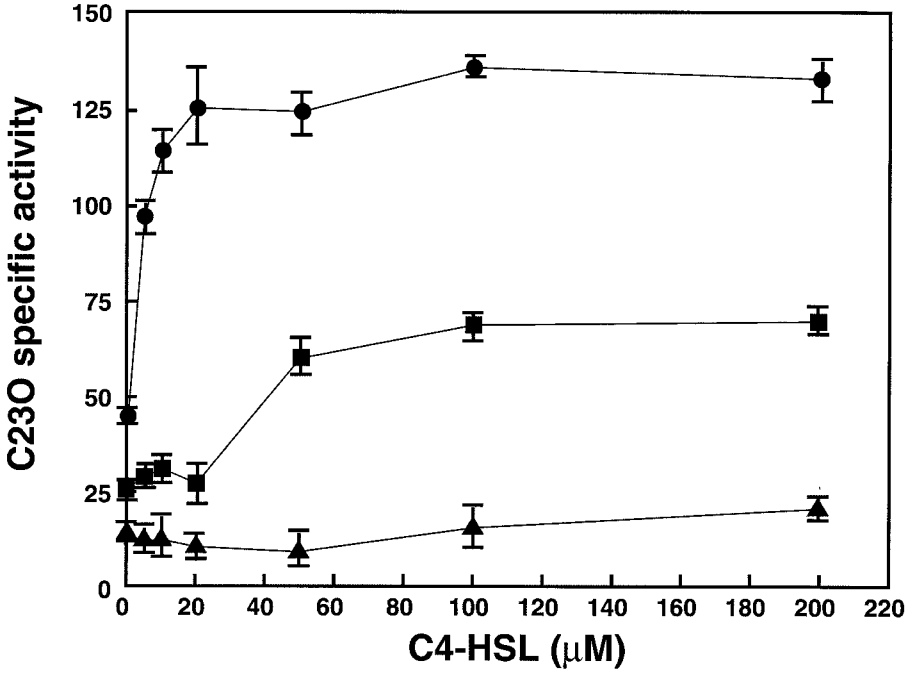

FIG. 6. Effect of C4-HSL concentration on expression of the *mexB*::*xylE* reporter in TNP090 cells producing MexEF-OprN under different conditions. Experimental procedures are described in Materials and Methods. Circles, TNP090(pMMB67EH); squares, TNP090(pMEXEF-OPRN1); triangles, TNP090(pMEXT8380). C23O, catechol 2,3-dioxygenase. Data are means ± standard deviations from three independent experiments.

cells with the *mexB*::*xylE* fusion (TNP090) and investigated whether the transcriptional expression of the *mexAB-oprM* operon was modulated. As shown in Fig. 4, catechol 2,3-dioxygenase activity in TNP090(pMEXT8380) was about half of that of TNP090 carrying the vector only. The result is fully consistent with the above-mentioned data and firmly establishes that expression of MexAB-OprM in the *nfxC*-type cells is down regulated in the presence of functional MexT. In fact, both PAO4290 and PAO1S with an intact *mexT* gene (harboring pMEXT8380) exhibit an elevated level of β-lactam susceptibility compared with cells harboring the vector only (Table 2).

Growth phase-dependent and C4-HSL-mediated regulation of *mexAB-oprM* expression. *P. aeruginosa* produces two major AHSLs, 3-oxo-C12-HSL and C4-HSL (6, 24), one of which, 3-oxo-C12-HSL, was reported to be unrelated to growth phase-dependent regulation of the *mexAB-oprM* operon (6). An earlier paper reported that production of C4-HSL was nearly undetectable in culture supernatants at cell densities below an A_{600} of ca. 2 but was quantitatively detectable in cultures with an A_{600} of over 2 (11). This growth phase-dependent accumulation of C4-HSL appears to be analogous to the *mexAB-oprM* transcription profile (Fig. 2 and 3). This observation suggests that the growth phase-dependent transcription of *mexAB-oprM* might be linked to the accumulation of AHSLs in the stationary phase. We therefore tested the effects of AHSLs on the expression of *mexAB-oprM* by adding 3-oxo-C12-HSL and/or C4-HSL (final concentration, 50 μM) during the logarithmic growth phase to cultures of TNP090 and its derivatives lacking the *lasI* and/or *rhlI* genes. Addition of C4-HSL enhanced transcription of *mexAB-oprM* in TNP090 and derivatives of TNP090. In contrast, addition of 3-oxo-C12-HSL only slightly affected transcription of *mexAB-oprM* on these strains compared to C4-HSL (Fig. 5), suggesting that especially the enhancement of *mexAB-oprM* expression in the stationary growth phase can be controlled by the concentration of C4-HSL. Hence, we additionally tested the effects of C4-HSL on the expression of *mexAB-oprM* by adding various concentrations of C4-HSL to TNP090 harboring vector only, pMEXEF-OPRN1 (carrying the *mexEF-oprN* genes), or pMEXT8380 (carrying an unimpaired *mexT* gene). For strain TNP090 with the *mexB*::*xylE* reporter, catechol 2,3-dioxygenase activity sharply increased as the extracellular concentration of C4-HSL was raised to approximately 20 μM and thereafter showed a steady expression (Fig. 6). This result clearly indicates that transcription of the *mexAB-oprM* operon is regulated by C4-HSL and suggests that the increased transcription of *mexAB-oprM* in

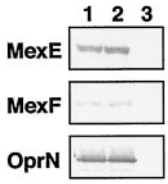

FIG. 7. Western blot analysis of the production of MexE, MexF, and OprN in TNP090 cells producing MexEF-OprN under different conditions. Total cell lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the protein bands were visualized by immunoblotting with antibodies against MexE, MexF, or OprN. The amounts of protein applied per lane were 10, 40, and 20 μg for MexE, MexF, and OprN, respectively. Lanes: 1, TNP090(pMEXEF-OPRN1); 2, TNP090(pMEXT8380); 3, TNP090 (pMMB67EH).

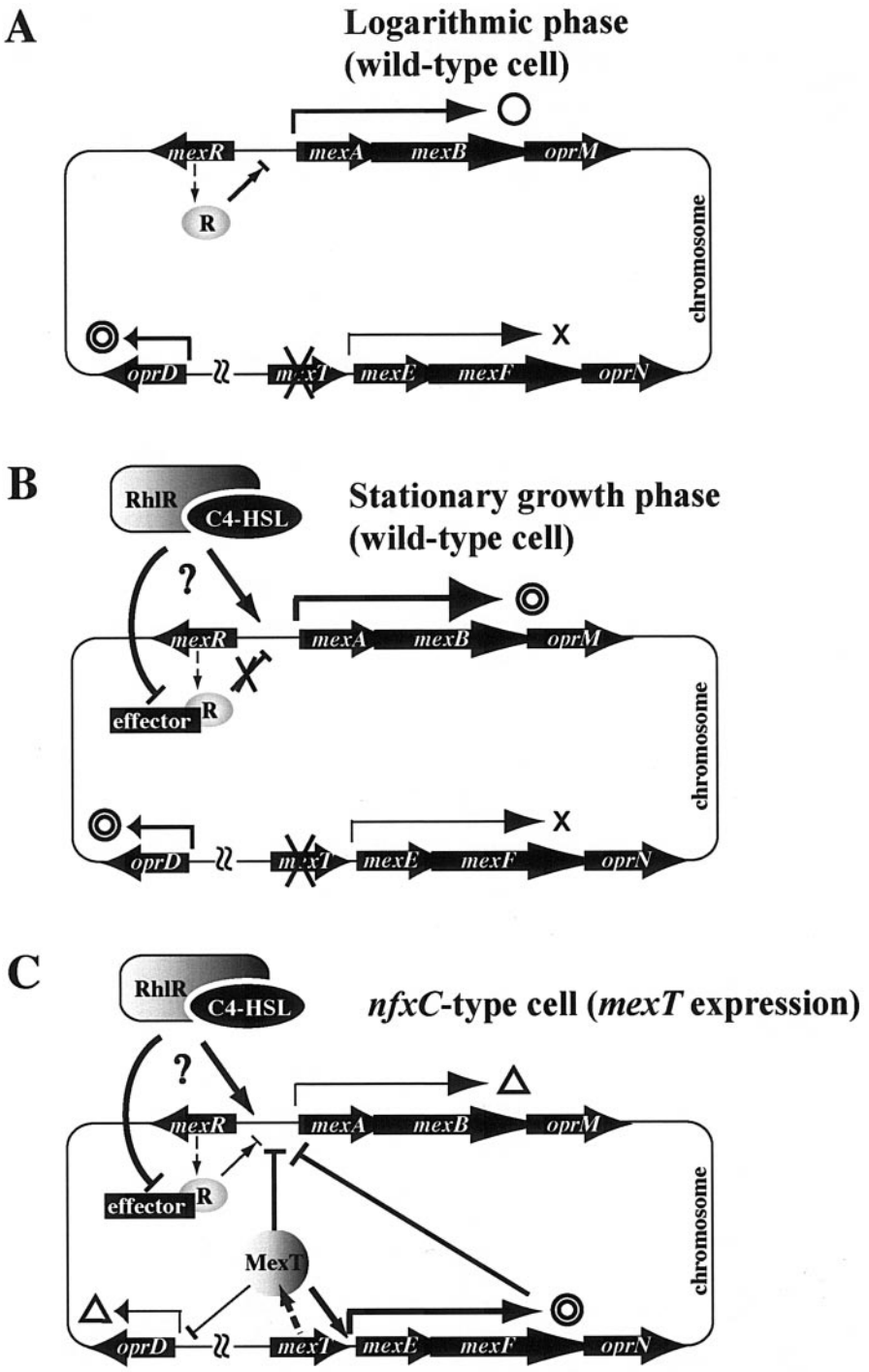

FIG. 8. Schematic representation of *mexAB-oprM* operon regulation by C4-HSL, MexR (R), and MexT. Symbols: double circles, high-level expression; open circles, low-level expression; open triangles, very low-level expression; ×, no expression or no function; ⊢, repression. Thick and thin arrows represent high and low expression or effect, respectively. Arrows with question marks indicate deduced regulatory pathways.

stationary phase is most likely dependent on the concentration of C4-HSL. Furthermore, we found that the transcription levels of *mexAB-oprM* in stationary growth phase in an *rhlI* mutant (TNP092) were almost lower than those in TNP090 (data not shown). In contrast, transcription of the *mexAB-oprM* operon in TNP090 harboring pMEXT8380 (carrying an unimpaired *mexT* gene) was barely affected by C4-HSL (Fig. 6), suggesting that MexT canceled the C4-HSL-mediated enhancement of *mexAB-oprM* expression. However, the growth phase-dependent expression of *mexAB-oprM* was observed even in the strains carrying the *mexT* gene (Fig. 2 and 3). Therefore, it seemed that the expression of *mexAB-oprM* is

regulated by another, unknown mechanism(s) as well as C4-HSL.

One may ask whether MexEF-OprN is involved in extrusion of C4-HSL. This activity would result in a decrease in the cellular concentration of C4-HSL. When the transcription of *mexAB-oprM* in TNP090 cells harboring pMEXEF-OPRN1 or with the vector only was compared, we observed a lower level of transcription of *mexAB-oprM* in cells harboring pMEXEF-OPRN1 than in cells with the vector only (Fig. 6). These data suggest but do not prove that the MexEF-OprN efflux pump might participate in export of C4-HSL and consequently decrease the transcription of *mexAB-oprM*. Needless to say, the activity and expression level of MexEF-OprN were similar in cells carrying pMEXEF-OPRN1 or pMEXT8380 (Table 2 and Fig. 7).

DISCUSSION

We demonstrated in this investigation that the *mexAB-oprM* operon was actively transcribed in the stationary growth phase and that this observation could be linked with the report that the quorum-sensing system was switched on in the stationary phase (4, 31, 43). In fact, transcription of the *mexAB-oprM* operon in wild-type cells was increased by the extracellular addition of C4-HSL (Fig. 5 and 6). These results suggest that the enhanced transcription of *mexAB-oprM* in the stationary phase depends on quorum-sensing autoinducer accumulation in the medium. How would one summarize the complicated regulation of the *mexAB-oprM* expression? We reported recently that the MexR protein negatively regulates transcription of *mexAB-oprM* operon (34), and we interpret the available data as follows. In the logarithmic growth phase, the MexR repressor negatively regulates *mexAB-oprM* expression by binding at the MexR-MexAB-OprM operator-promoter region, as reported recently (Fig. 8A) (5, 34). As the cells enter the stationary growth phase, they sense a high population density and turn on a quorum-sensing switch producing an autoinducer, C4-HSL (Fig. 8B). We considered two mechanisms: (i) C4-HSL induces the expression of *mexAB-oprM* operon directly and not through participation of the *mexR* gene, or (ii) C4-HSL inactivates the MexR repressor or represses the expression of the *mexR* gene by an unknown mechanism and consequently enhances the transcription of *mexAB-oprM* operon. We explain in the final paragraph of Discussion that probably C4-HSL directly induces the expression of *mexAB-oprM.*

Wild-type cells of *P. aeruginosa* have the *mexEF-oprN* operon, but it is not transcribed under normal growth conditions due to the mutation in the positive regulator gene *mexT* (17). The *nfxC*-type mutant produces active MexT that promotes transcription of the *mexEF-oprN* operon, simultaneously lowering the growth phase-dependent enhancement of *mexAB-oprM* transcription (Fig. 3 and 4) and rendering the cells hypersusceptible to β-lactam antibiotics (Table 2). Both the growth phase-dependent and C4-HSL-mediated increases in the production of MexAB-OprM were totally repressed by overdose of the plasmid-borne MexT (Fig. 6). It is likely, therefore, that β-lactam hypersusceptibility in the *nfxC*-type mutant is caused by the activation of MexT, consequently decreasing the C4-HSL effect. Thus, all the data presented in this report

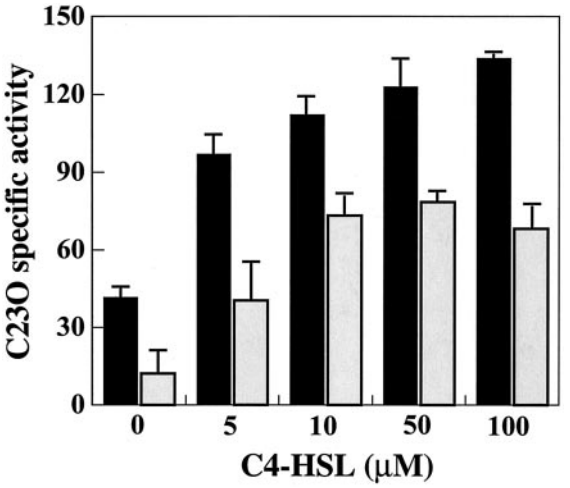

FIG. 9. Effect of C4-HSL concentration on expression of the *mexB*::*xylE* reporter in TNP099 cells with a defect in the *mexEF-oprN* operon under different conditions. Experimental procedures are described in Materials and Methods. Black bars, TNP099(pMMB67EH); gray bars, TNP099(pMEXT8380). Data are means ± standard deviations from three independent experiments.

suggest that MexT may regulate the expression of the *mexAB-oprM* operon in mediating the quorum-sensing system. We carried out a further experiment to determine whether it is MexT itself or MexEF-OprN which affects the expression of *mexAB-oprM*. We constructed a *mexEF-oprN* disruption mutant of TNP090, named TNP099, and then observed the induction level of *mexAB-oprM* in TNP099 with or without MexT (pMEXT8380) after adding exogenous C4-HSL (Fig. 9). The expression of MexAB-OprM in the *mexEF-oprN* disruption mutant (TNP099) was further decreased in the presence of MexT (pMEXT8380) compared to the level in the absence of MexT, indicating that MexT also possesses the regulatory function required for the decrease of the expression of *mexAB-oprM* not through expression of MexEF-OprN. Moreover, MexT is involved in expression of various genes, including *mexEF-oprN*, *oprD*, *lasB* (encoding elastase), *rhlAB*, and swarming and pyocyanin genes (10, 11). Hence, the MexT protein may function as a global regulator. On the other hand, we observed a lower level of transcription of *mexAB-oprM* in TNP090 cells harboring pMEXEF-OPRN1 than that of vector only (Fig. 6). Therefore, it is concluded that the MexT-mediated down regulation of the transcription of the *mexAB-oprM* operon is caused by the regulatory function of MexT itself and the MexEF-OprN efflux pump expressed by MexT (Fig. 8C).

MexT may bind either the MexR binding site or a putative second repressor binding site (34) at the *mexR-mexAB-oprM* operator-promoter region and repress the transcription of the *mexAB-oprM* operon in a MexR-independent manner (Fig. 8C). If this was the case, the *nfxC*-type cells would lower the transcription of *mexAB-oprM* even in the absence of MexR. In fact, the *mexR nfxC* double mutant showed a 50% lower MIC of aztreonam, which is one of the β-lactam agents and is extruded effectively by the MexAB-OprM efflux pump, than the cells with a *mexR* single mutation. Needless to say, the former cells showed a MIC of nolfloxacin that is eight times higher than that for the latter cells (H. Maseda, unpublished

results). To substantiate such a possibility, the transcriptional expression of *mexR* and *mexT* at different growth phases needs to be tested. Such experiments are in progress in our laboratory.

ACKNOWLEDGMENT

This study was partially supported by a grant to N.N. from Industrial Technology Research Grant Program '01 of the New Energy and Industrial Technology Development Organization (NEDO) of Japan.

REFERENCES

1. **Adewoye, L., A. Sutherland, R. Srikumar, and K. Poole.** 2002. The *mexR* repressor of the *mexAB-oprM* multidrug efflux operon in *Pseudomonas aeruginosa*: characterization of mutations compromising activity. J. Bacteriol. **184:**4308–4312.
2. **Aires, J. R., T. Köhler, H. Nikaido, and P. Plesiat.** 1999. Involvement of an active efflux system in the natural resistance of *Pseudomonas aeruginosa* to aminoglycosides. Antimicrob. Agents Chemother. **43:**2624–2628.
3. **Beinlich, K. L., R. Chuanchuen, and H. P. Schweizer.** 2001. Contribution of multidrug efflux pumps to multiple antibiotic resistance in veterinary clinical isolates of *Pseudomonas aeruginosa.* FEMS Microbiol. Lett. **198:**129–134.
4. **de Kievit, T. R., and B. H. Iglewski.** 2000. Bacterial quorum sensing in pathogenic relationships. Infect. Immun. **68:**4839–4849.
5. **Evans, K., L. Adewoye, and K. Poole.** 2001. MexR repressor of the *mexAB-oprM* multidrug efflux operon of *Pseudomonas aeruginosa*: identification of MexR binding sites in the *mexA-mexR* intergenic region. J. Bacteriol. **183:** 807–812.
6. **Evans, K., and K. Poole.** 1999. The MexA-MexB-OprM multidrug efflux system of *Pseudomonas aeruginosa* is growth-phase regulated. FEMS Microbiol. Lett. **173:**35–39.
7. **Fukuda, H., M. Hosaka, K. Hirai, and S. Iyobe.** 1990. New norfloxacin resistance gene in *Pseudomonas aeruginosa* PAO. Antimicrob. Agents Chemother. **34:**1757–1761.
8. **Fürste, J. P., W. Pansegrau, R. Frank, H. Blocker, P. Scholz, M. Bagdasarian, and E. Lanka.** 1986. Molecular cloning of the plasmid RP4 primase region in a multi-host-range *tacP* expression vector. Gene **48:**119–131.
9. **Guan, L., M. Ehrmann, H. Yoneyama, and T. Nakae.** 1999. Membrane topology of the xenobiotic-exporting subunit, MexB, of the MexA, B-OprM extrusion pump in *Pseudomonas aeruginosa.* J. Biol. Chem. **274:**10517–10522.
10. **Köhler, T., S. F. Epp, L. K. Curty, and J. C. Pechére.** 1999. Characterization of MexT, the regulator of the MexE-MexF-OprN multidrug efflux system of *Pseudomonas aeruginosa.* J. Bacteriol. **181:**6300–6305.
11. **Köhler, T., C. van Delden, L. K. Curty, M. M. Hamzehpour, and J. C. Pechére.** 2001. Overexpression of the MexEF-OprN multidrug efflux system affects cell-to-cell signaling in *Pseudomonas aeruginosa.* J. Bacteriol. **183:** 5213–5222.
12. **Li, X. Z., N. Barre, and K. Poole.** 2000. Influence of the MexA-MexB-OprM multidrug efflux system on expression of the MexC-MexD-OprJ and MexE-MexF-OprN multidrug efflux systems in *Pseudomonas aeruginosa.* J. Antimicrob. Chemother. **46:**885–893.
13. **Li, X. Z., and K. Poole.** 2001. Mutational analysis of the OprM outer membrane component of the MexA-MexB-OprM multidrug efflux system of *Pseudomonas aeruginosa.* J. Bacteriol. **183:**12–27.
14. **Lim, D., K. Poole, and N. C. Strynadka.** 2002. Crystal structure of the MexR repressor of the *mexRAB-oprM* multidrug efflux operon of *Pseudomonas aeruginosa.* J. Biol. Chem. **277:**29253–29259.
15. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
16. **Maseda, H., M. Kitao, S. Eda, E. Yoshihara, and T. Nakae.** 2002. A novel assembly process of the multicomponent xenobiotic efflux pump in *Pseudomonas aeruginosa.* Mol. Microbiol. **46:**677–686.
17. **Maseda, H., K. Saito, A. Nakajima, and T. Nakae.** 2000. Variation of the *mexT* gene, a regulator of the MexEF-oprN efflux pump expression in wild-type strains of *Pseudomonas aeruginosa.* FEMS Microbiol. Lett. **192:**107–112.
18. **Maseda, H., H. Yoneyama, and T. Nakae.** 2000. Assignment of the substrate-selective subunits of the MexEF-OprN multidrug efflux pump of *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **44:**658–664.
19. **Masuda, N., E. Sakagawa, S. Ohya, N. Gotoh, H. Tsujimoto, and T. Nishino.** 2000. Contribution of the MexX-MexY-OprM efflux system to intrinsic resistance in *Pseudomonas aeruginosa.* Antimicrob. Agents Chemother. **44:** 2242–2246.
20. **Mine, T., Y. Morita, A. Kataoka, T. Mizushima, and T. Tsuchiya.** 1999. Expression in *Escherichia coli* of a new multidrug efflux pump, MexXY, from *Pseudomonas aeruginosa.* Antimicrob. Agents Chemother. **43:**415–417.
21. **Morshed, S. R., Y. Lei, H. Yoneyama, and T. Nakae.** 1995. Expression of genes associated with antibiotic extrusion in *Pseudomonas aeruginosa.* Biochem. Biophys. Res. Commun. **210:**356–362.
22. **Nakajima, A., Y. Sugimoto, H. Yoneyama, and T. Nakae.** 2000. Localization of the outer membrane subunit OprM of resistance-nodulation-cell division family multicomponent efflux pump in *Pseudomonas aeruginosa.* J. Biol. Chem. **275:**30064–30068.
23. **Nikaido, H.** 1989. Outer membrane barrier as a mechanism of antimicrobial resistance. Antimicrob. Agents Chemother. **33:**1831–1836.
24. **Parsek, M. R., and E. P. Greenberg.** 2000. Acyl-homoserine lactone quorum sensing in gram-negative bacteria: a signaling mechanism involved in associations with higher organisms. Proc. Natl. Acad. Sci. USA **97:**8789–8793.
25. **Pearson, J. P., C. Van Delden, and B. H. Iglewski.** 1999. Active efflux and diffusion are involved in transport of *Pseudomonas aeruginosa* cell-to-cell signals. J. Bacteriol. **181:**1203–1210.
26. **Poole, K.** 2000. Efflux-mediated resistance to fluoroquinolones in gram-negative bacteria. Antimicrob. Agents Chemother. **44:**2233–22241.
27. **Poole, K.** 2001. Multidrug efflux pumps and antimicrobial resistance in *Pseudomonas aeruginosa* and related organisms. J. Mol. Microbiol. Biotechnol. **3:**255–264.
28. **Poole, K., N. Gotoh, H. Tsujimoto, Q. Zhao, A. Wada, T. Yamasaki, S. Neshat, J. Yamagishi, X. Z. Li, and T. Nishino.** 1996. Overexpression of the *mexC-mexD-oprJ* efflux operon in *nfxB*-type multidrug-resistant strains of *Pseudomonas aeruginosa.* Mol. Microbiol. **21:**713–724.
29. **Poole, K., K. Krebes, C. McNally, and S. Neshat.** 1993. Multiple antibiotic resistance in *Pseudomonas aeruginosa*: evidence for involvement of an efflux operon. J. Bacteriol. **175:**7363–7372.
30. **Poole, K., K. Tetro, Q. Zhao, S. Neshat, D. E. Heinrichs, and N. Bianco.** 1996. Expression of the multidrug resistance operon *mexA-mexB-oprM* in *Pseudomonas aeruginosa*: *mexR* encodes a regulator of operon expression. Antimicrob. Agents Chemother. **40:**2021–2028.
31. **Rahmati, S., S. Yang, A. L. Davidson, and E. L. Zechiedrich.** 2002. Control of the AcrAB multidrug efflux pump by quorum-sensing regulator SdiA. Mol. Microbiol. **43:**677–685.
32. **Rella, M., and D. Haas.** 1982. Resistance of *Pseudomonas aeruginosa* PAO to nalidixic acid and low levels of beta-lactam antibiotics: mapping of chromosomal genes. Antimicrob. Agents Chemother. **22:**242–249.
33. **Rist, M., and M. A. Kertesz.** 1998. Construction of improved plasmid vectors for promoter characterization in *Pseudomonas aeruginosa* and other gram-negative bacteria. FEMS Microbiol. Lett. **169:**179–183.
34. **Saito, K., S. Eda, H. Maseda, and T. Nakae.** 2001. Molecular mechanism of MexR-mediated regulation of MexAB-OprM efflux pump expression in *Pseudomonas aeruginosa.* FEMS Microbiol. Lett. **195:**23–28.
35. **Saito, K., H. Yoneyama, and T. Nakae.** 1999. *nalB*-type mutations causing the overexpression of the MexAB-OprM efflux pump are located in the *mexR* gene of the *Pseudomonas aeruginosa* chromosome. FEMS Microbiol. Lett. **179:**67–72.
36. **Sambrook, J., E. Fritsch, F., and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
37. **Sanchez, P., F. Rojo, and J. L. Martinez.** 2002. Transcriptional regulation of *mexR*, the repressor of *Pseudomonas aeruginosa mexAB-oprM* multidrug efflux pump. FEMS Microbiol. Lett. **207:**63–68.
38. **Schweizer, H. P.** 1992. Allelic exchange in *Pseudomonas aeruginosa* using novel ColE1-type vectors and a family of cassettes containing a portable *oriT* and the counter-selectable *Bacillus subtilis sacB* marker. Mol. Microbiol. **6:**1195–1204.
39. **Schweizer, H. P.** 1993. Two plasmids, X1918 and Z1918, for easy recovery of the *xylE* and *lacZ* reporter genes. Gene **134:**89–91.
40. **Shiba, T., K. Ishiguro, N. Takemoto, H. Koibuchi, and K. Sugimoto.** 1995. Purification and characterization of the *Pseudomonas aeruginosa* NfxB protein, the negative regulator of the *nfxB* gene. J. Bacteriol. **177:**5872–5877.
41. **Simon, R., M. O'Connell, M. Labes, and A. Pühler.** 1986. Plasmid vector for the genetic analysis and manipulation of rhizobia and other gram-negative bacteria. Methods Enzymol. **118:**640–659.
42. **Srikumar, R., C. J. Paul, and K. Poole.** 2000. Influence of mutations in the *mexR* repressor gene on expression of the MexA-MexB-OprM multidrug efflux system of *Pseudomonas aeruginosa.* J. Bacteriol. **182:**1410–1414.
43. **Whitehead, N. A., A. M. Barnard, H. Slater, N. J. Simpson, and G. P. Salmond.** 2001. Quorum-sensing in Gram-negative bacteria. FEMS Microbiol. Rev. **25:**365–404.
44. **Wong, K. K., F. S. Brinkman, R. S. Benz, and R. E. Hancock.** 2001. Evaluation of a structural model of *Pseudomonas aeruginosa* outer membrane protein OprM, an efflux component involved in intrinsic antibiotic resistance. J. Bacteriol. **183:**367–374.
45. **Yoneyama, H., H. Maseda, H. Kamiguchi, and T. Nakae.** 2000. Function of the membrane fusion protein, MexA, of the MexA, B-OprM efflux pump in *Pseudomonas aeruginosa* without an anchoring membrane. J. Biol. Chem. **275:**4628–4634.