

## Overexpression of the MexEF-OprN Multidrug Efflux System Affects Cell-to-Cell Signaling in *Pseudomonas aeruginosa*

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**Intrinsic and acquired antibiotic resistance of the nosocomial pathogen *Pseudomonas aeruginosa* is mediated mainly by the expression of several efflux pumps of broad substrate specificity. Here we report that *nfxC* type mutants, overexpressing the MexEF-OprN efflux system, produce lower levels of extracellular virulence factors than the susceptible wild type. These include pyocyanin, elastase, and rhamnolipids, three factors controlled by the *las* and *rhl* quorum-sensing systems of *P. aeruginosa*. In agreement with these observations are the decreased transcription of the elastase gene *lasB* and the rhamnosyltransferase genes *rhlAB* measured in *nfxC* type mutants. Expression of the *lasR* and *rhlR* regulator genes was not affected in the *nfxC* type mutant. In contrast, transcription of the C4-homoserine lactone (C4-HSL) autoinducer synthase gene *rhlI* was reduced by 50% in the *nfxC* type mutant relative to that in the wild type. This correlates with a similar decrease in C4-HSL levels detected in supernatants of the *nfxC* type mutant. Transcription of an *rhlAB-lacZ* fusion could be partially restored by the addition of synthetic C4-HSL and *Pseudomonas* quinolone signal (PQS). It is proposed that the MexEF-OprN efflux pump affects intracellular PQS levels.**

*Pseudomonas aeruginosa* is an opportunistic pathogen which may cause pneumonia and bacteremia in immunocompromised hosts and is responsible for chronic destructive lung disease in patients suffering from cystic fibrosis. The pathogenicity of *P. aeruginosa* is attributable to an arsenal of virulence factors, some of which are cell associated (pili, nonpilus adhesins, lipopolysaccharide, and alginate) while others are secreted (proteases, rhamnolipids, exotoxin A, exoenzyme S, and pyocyanin). The production of many of these extracellular virulence factors is controlled by two cell-to-cell signaling systems, called *las* and *rhl*, which are both composed of a transcriptional regulator (LasR and RhlR, respectively) and an autoinducer synthase (LasI and RhlI, respectively). LasI and RhlI catalyze the last step in the synthesis of the cell-to-cell signaling molecules 3-oxo-C12-homoserine lactone (3-oxo-C12-HSL) and C4-HSL, respectively; each of these molecules binds to, and activates, its corresponding transcriptional regulator. The systems are connected via a hierarchical cascade (19) and allow coordinated production of extracellular virulence factors, which occurs only when the bacterial cell density has reached a threshold (quorum). Recently, a novel signaling molecule, called PQS, for *Pseudomonas* quinolone signal (39), has been identified. Furthermore, the published genome sequence of PAO1 (53) has revealed a new modulator of cell-to-cell signaling, termed QscR (4). This protein is homologous to both LasR and RhlR and seems to prevent premature transcription of quorum-sensing regulated genes.

Besides its pathogenic capabilities, *P. aeruginosa* is well known for its intrinsic resistance to a wide range of antimicrobial agents and its ability to develop multidrug resistance fol-

lowing antibiotic therapy. Recent investigations in several laboratories have demonstrated that both intrinsic and acquired resistance is caused mainly by active efflux systems which efficiently expel antimicrobial compounds without any apparent structural similarity. So far, four genetically distinct efflux systems have been characterized for *P. aeruginosa*. They are similar in genetic and structural organization but differ in substrate specificity and regulation. The MexAB-OprM system (22, 42) has the broadest substrate spectrum of all bacterial efflux pumps described so far, including quinolones, tetracycline, chloramphenicol (20), trimethoprim (17),  $\beta$ -lactam antibiotics (21),  $\beta$ -lactamase inhibitors (24), and detergents and solvent molecules (23). The transcriptional repressor MexR (43) keeps expression of the *mexAB-oprM* operon at a low constitutive level, but one sufficient to contribute significantly to the elevated intrinsic antibiotic resistance of this organism. A second efflux system, MexCD-OprJ (41), is responsible for efflux of quinolones, erythromycin (29), and cephalosporins (12, 27). Its expression is totally repressed by the transcriptional regulator NfxB (35, 49). The third efflux pump, MexEF-OprN, transports chloramphenicol as well as quinolones, is overexpressed in *nfxC* type mutants (18), and is positively regulated by the transcriptional activator MexT (16). Recently, a fourth efflux system of *P. aeruginosa*, called MexXY, has been cloned into *Escherichia coli*, on which it conferred resistance to quinolones and erythromycin (31). This efflux system was subsequently shown to be involved in the intrinsic resistance of *P. aeruginosa* to aminoglycosides and erythromycin (45).

*nfxC* type mutants were originally isolated from *P. aeruginosa* strain PAO4009 after exposure to the quinolone norfloxacin (9). These mutants displayed cross-resistance to other quinolones but also to nonquinolone antibiotics such as imipenem and chloramphenicol. The *nfxC* locus was mapped to 46 min on the PAO1 chromosome (9), near the *catA* gene, which is located within 15 kb of the *mexEF-oprN* operon (18). Mutations

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TABLE 1. Bacterial strains, plasmids, and bacteriophage used in this study

Strain, plasmid, or bacteriophage	Relevant characteristics <sup>a</sup>	Source or reference
<b>Strains</b>		
<i>E. coli</i>		
MC1061	F <sup>-</sup> <i>araD139</i> Δ( <i>ara-leu</i> )7696 <i>galE15 galK16</i> Δ( <i>lac</i> )X74 <i>rpsL thi</i>	Laboratory collection
S17-1	<i>thi pro hsdR recA</i> chr::RP4-2	51
DH5α	<i>endA1 hsdR17</i> (r <sub>k</sub> <sup>-</sup> m <sub>k</sub> <sup>+</sup> ) <i>glmV44 thi-1 recA1 gyrA relA1</i> Δ( <i>lacIZYA-argF</i> )U169	Laboratory collection
MG4λI <sub>4</sub>	λ lysogen carrying a chromosomal <i>lasI::lacZ</i> fusion	48
<i>P. aeruginosa</i>		
PAO-BI	PAO1 wild type	10
PAO-R1	PAO-BIΔ <i>lasR</i> Tc <sup>r</sup>	10
JP2	PDO100Δ <i>lasI</i> Tc <sup>r</sup> Hg <sup>r</sup>	37
JP3	PDO111Δ <i>lasR</i> Tc <sup>r</sup> Hg <sup>r</sup>	37
PT5	PAO1 wild type	Laboratory collection
PT121	PT5 <i>mexE::Ω</i> Hg (formerly PAO <i>mexE</i> ) Hg <sup>r</sup>	18
PT149	PT5 <i>nfxC</i> (formerly PAO-7H); overproduces MexEF-OprN	18
PT466	PT5Δ <i>lasI</i> Tc <sup>r</sup>	15
PT498	PT5Δ <i>lasR</i> Tc <sup>r</sup>	15
PT454	PT5Δ <i>rhlI::Tn501</i> Hg <sup>r</sup>	15
PT462	PT5 <i>rhlR::Tn501</i> Hg <sup>r</sup>	15
PT531	PT5 <i>rhlR::Tn501</i> Δ <i>lasRI</i> Tc <sup>r</sup> Hg <sup>r</sup>	This study
PT469	PT149Δ <i>lasI</i> ; transduced from PAO-JP1 Tc <sup>r</sup>	This study
PT456	PT149Δ <i>rhlI::Tn501</i> ; transduced from PDO100 Hg <sup>r</sup>	This study
PT464	PT149 <i>rhlR::Tn501</i> ; transduced from PAO-JP3 Hg <sup>r</sup>	This study
PT500	PT149 <i>rhlR::Tn501</i> ; Δ <i>lasR</i> Tc <sup>r</sup> Hg <sup>r</sup>	This study
PT509	PT149Δ <i>lasR</i> ; transduced from PAO-JP3 Tc <sup>r</sup>	This study
PT637	PT149 <i>mexE::Ω</i> Hg; transduced from PT121 Hg <sup>r</sup>	This study
<b>Plasmids</b>		
pTS400	<i>lasB::lacZ</i> on pSW205; Ap <sup>r</sup>	36
pECP60	<i>rhlAB::lacZ</i> on pSW205; Ap <sup>r</sup>	40
pPCS1001	<i>lasR::lacZ</i> on pLP170; Ap <sup>r</sup>	40
pPCS223	<i>lasI::lacZ</i> on pLP170; Ap <sup>r</sup>	56
pPCS1002	<i>rhlR::lacZ</i> on pLP170; Ap <sup>r</sup>	40
pMAL-I	<i>rhlI::lacZ</i> on pMP220; Tc <sup>r</sup>	19
pMP220	Promoterless <i>lacZ</i> fusion vector; Tc <sup>r</sup>	52
pECP61.5	<i>rhlAB::lacZ</i> , <i>ptac::rhlR</i> ; Ap <sup>r</sup>	37
pLP170	Promoterless <i>lacZ</i> fusion vector; Ap <sup>r</sup>	44
pSW205	Promoterless <i>lacZ</i> fusion vector; Ap <sup>r</sup>	11
pEZ5	<i>mexE::lacZ</i> on pSW205; Ap <sup>r</sup>	This study
Bacteriophage E79tv2	Transducing phage	32

<sup>a</sup> Resistance phenotypes: Hg, mercury; Tc, tetracycline; Ap, ampicillin.

which lead to overexpression of the MexEF-OprN pump have recently been shown to result from variations in the transcriptional activator gene *mexT* (26). NfxC is therefore to be considered a phenotype, since overexpression of the MexEF-OprN pump might result from mutations which are not necessarily linked to *mexT* (26) (T. Köhler and J. L. Dumas, unpublished data).

We previously showed that an *nfxC* type mutant which overexpressed the MexEF-OprN efflux operon produces about 20 times less pyocyanin than the isogenic wild-type strain (18). Since pyocyanin is a typical secondary metabolite whose production is controlled by the *rhl* cell-to-cell signaling system (2), we decided to investigate the production of other virulence factors in *nfxC* type mutants. Our results show that overexpression of the MexEF-OprN efflux pump is correlated with a decrease in production of extracellular virulence factors, par-

ticularly those controlled by the *rhl* system. Evidence is presented that the PQS (39) is involved in this response.

#### MATERIALS AND METHODS

**Bacteria, media, and growth conditions.** Bacterial strains and plasmids are listed in Table 1. *E. coli* and *P. aeruginosa* were routinely grown in Luria-Bertani (LB) broth supplemented when necessary with antibiotics at the following concentrations, in milligrams per liter: gentamicin, 15; ampicillin (for *E. coli*), 100; carbenicillin, 250 (for *P. aeruginosa*); tetracycline, 10 (for *E. coli*) or 50 (for *P. aeruginosa*); mercury chloride, 12.5. For analysis of exoproducts and autoinducers in culture supernatants and for *lacZ* fusion experiments, *P. aeruginosa* strains were grown as follows. Strains to be tested were streaked from -80°C glycerol stocks on selective LB agar plates. Single colonies were inoculated into 5 ml of PB (2% Bacto Peptone [select peptone 140; Gibco-BRL], 1.4 g of MgCl<sub>2</sub>/liter, 10 g of K<sub>2</sub>SO<sub>4</sub>/liter) (6) supplemented with antibiotics where appropriate. Cultures were grown overnight at 37°C with agitation in 50-ml flasks. One milliliter of this overnight culture was centrifuged and resuspended in 1 ml of fresh PB. From this suspension, 25 ml of prewarmed PB without antibiotics was inoculated 1:100 and grown in 250-ml flasks with agitation. Defined media were based on

TABLE 2. Exoproduct assays and resistance profiles in *P. aeruginosa* multidrug efflux and quorum-sensing mutants

Strain	Genotype	Exoproduct activity <sup>a</sup>		MIC of:		
		Pyocyanin	Elastase	Ciprofloxacin	Chloramphenicol	Imipenem
PT5	wt <sup>b</sup>	100	100	0.125	32	1
PT149	<i>nfxC</i>	5	42	1	1,024	4
PT454	<i>rhlI</i>	3	ND	0.125	32	1
PT462	<i>rhlR</i>	<1	4	0.125	32	1
PT466	<i>lasI</i>	80	ND	0.125	64	1
PT498	<i>lasR</i>	45	6	0.125	32	1
PT531	<i>rhlR lasR</i>	<1	4	0.125	32	1
PT637	<i>nfxC mexE</i>	ND	88	0.125	32	4
PAO-BI	<i>nfxC</i>	ND	45	1	1,024	4
PAO-RI	<i>nfxC lasR</i>	ND	<1	1	1,024	4

<sup>a</sup> Expressed as a percentage of the activity in the wild-type strain PT5. Pyocyanin activities were determined in supernatants of cultures grown for 18 h in PB medium. ECR assays were performed on supernatants of cultures grown for 10 h in PB medium. At this point the cultures were in stationary phase, and the OD<sub>600</sub>s of the cultures were 5 ± 0.5. ND, not determined.

<sup>b</sup> wt, wild type.

M9 salts (25) supplemented with 2 mM MgSO<sub>4</sub> and 0.4% glucose. For phage transductions, donor strains were grown in LB broth and recipient strains were resuspended in TNM medium (10 mM Tris-HCl [pH 7.4], 150 mM NaCl, 10 mM MgSO<sub>4</sub>).

**Strain and plasmid constructions.** Mutations in the cell-to-cell signaling regulator genes were transferred into the PAO1 wild-type strain PT5 and the *nfxC* mutant PT149 using the transducing phage E79n2 (32). The genotype of the strains was verified by Southern hybridization as described previously (15). The *mexE* gene was inactivated in strain PT149 by using bacteriophage E79n2 (32) to transduce the *mexE::ΩHg* mutation from strain PT121 (called PAO*mexE* in reference 18). In all of the 12 transductants analyzed, wild-type antibiotic susceptibility to the efflux pump substrates was restored. However, 4 of the 12 transductants remained imipenem resistant. One of these strains, called PT637, was shown by sequencing to contain a full-length *mexT* open reading frame (ORF) (see Results). The *mexE-lacZ* fusion pEZ5 was constructed by ligating a 1.8-kbp *BglII-EcoRV* fragment from plasmid pNFZ4 (16) into *BamHI-EcoRV*-cleaved pSW205 (11).

**DNA-manipulations.** Plasmids were introduced into *P. aeruginosa* by electroporation or by triparental mating using pRK2013 as a helper plasmid (8). Genomic DNA was isolated as described previously (1). PCR amplification was performed by using 100 ng of genomic DNA as a template. PCR mixtures contained primers at 0.1 μmol, 2.5 mM deoxynucleoside triphosphates, and 2 U of *Taq* polymerase (Appligene, Illkirch, France) in a total volume of 50 μl. Reaction mixtures were subjected to an initial 1-min denaturation step at 95°C, followed by 25 cycles of 30 s at 95°C, 30 s at 55°C, and 2 min at 72°C, with a final 5-min elongation at 72°C. Amplification of *rhlI* with primers RhlI-P30 (5'-CCA TCATCTGAGCATCTCCAGAGAGC-3') and RhlI-M6 (5'-GGAATGACTT CGGCATGGCGACTCC-3') yielded a 1,074-bp fragment, and amplification of *rhlR* with primers RhlR-P4 (5'-CGGCGTTTCAATGGAATTGTCAACCC-3') and RhlR-M5 (5'-GGCGGCATCCCTACCCTGATACTCCC-3') yielded a 1,109-bp fragment. PCR products were run on Tris-acetate-EDTA gels (1.2% agarose) and then purified using a Qiagen gel extraction kit. The *mexT* DNA region was amplified using primers *nfxC*-P1 (5'-TCTCGCACGCAAGGCTTG ACG-3') and *nfxC*-M2 (5'-TCCCCTCGTTCAGCGGTTGTTC-3'). PCR conditions were as follows: 1 min at 95°C, followed by 25 cycles of 30 s at 95°C, 20 s at 52°C, and 2 min at 72°C, with a final 5-min elongation at 72°C. DNA sequences were determined from double-stranded templates according to the dideoxy chain termination method (47) using an automatic sequencer (model 377A; Applied Biosystems).

**Qualitative plate assays.** Rhamnolipid production was estimated by inoculating strains on M9-based agar plates supplemented with 0.2% glucose (vol/vol), 2 mM MgSO<sub>4</sub>, trace elements, 0.05% (vol/vol) glutamate (unless otherwise stated) instead of NH<sub>4</sub>Cl as an N source, 0.0005% (vol/vol) methylene blue, and 0.02% (vol/vol) cetyltrimethylammonium bromide (50). Plates were incubated first at 37°C for 24 h and then for at least 48 h at room temperature until a blue halo appeared around the colony. Swarm plates were prepared and inoculated as described previously (15). Incubation was carried out for 18 h at 37°C.

**Quantitative exoproduct assays on culture supernatants.** Samples of 0.5 ml were taken at various time points during growth in PB, centrifuged (at 8,000 × g for 5 min), and filtered (pore size, 0.22 μm). Filtrates were immediately frozen and kept at -80°C. Elastolytic activity was determined by the elastin Congo red

(ECR) method (56). Five milligrams of ECR (Elastin Products Company, Owensville, Mo.) was used per assay. Triplicate samples were analyzed for each time point. Pyocyanin was determined in culture supernatants as described previously (6).

**β-Galactosidase assays.** Cultures were grown at 37°C with agitation as described above. Triplicate 100-μl samples were taken to determine the optical density at 600 nm (OD<sub>600</sub>) and β-galactosidase activity (30). For complementation assays with autoinducers, strains were grown overnight in PB medium. Cells were resuspended in M9-based medium supplemented with 0.2% glucose (vol/vol), 2 mM MgSO<sub>4</sub>, trace elements, and 0.05% (vol/vol) tryptophan instead of NH<sub>4</sub>Cl as an N source. C4-HSL was added from a dimethyl sulfoxide stock solution. PQS was synthesized as described previously (39) and dissolved in dimethyl sulfoxide.

**Autoinducer bioassays.** Aliquots (3 ml) were taken at different time points during growth in PB medium and centrifuged, and the supernatants were filtered (pore size, 0.22 μm). Aliquots (2 ml) were extracted twice with 2 ml of ethyl acetate (containing 0.01% acetic acid). The extracts were kept at -20°C. Aliquots of the ethyl acetate extract were evaporated, and the dried residue was resuspended directly in 1 ml of the bioassay strain culture. The *E. coli* bioassay strain was grown in M9 glucose medium supplemented with 0.001% thiamine, 1% LB medium, 50 μg of ampicillin/ml, and 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) when required. The *P. aeruginosa* bioassay strain JP2 (pECP61.5) was grown in LB medium. Incubation and β-galactosidase determinations were performed as described above.

## RESULTS

**Production of extracellular virulence factors is affected in *nfxC* type mutants.** We previously observed that the *nfxC* type mutant PT149 produced 20-fold less pyocyanin than the wild-type strain PT5 (18). We therefore tested the production of other extracellular virulence factors in these strains and compared their levels to those of isogenic *lasR*, *lasI*, *rhlR*, and *rhlI* mutants. As expected, the *nfxC* type mutant and the two *rhl* mutants showed drastically reduced production of pyocyanin, while the *lasR* and *lasI* mutants still secreted substantial amounts of pyocyanin (Table 2). Elastase activity was reduced by more than 50% in the *nfxC* mutant, while the *rhlR* and *lasR* mutants showed only marginal activity in culture supernatants after 10 h of growth in PB medium (Table 2).

We then compared the production of rhamnolipids using a standard plate assay. As a reference, we included the PAO1 wild-type strain (PAO-BI) (10) and its *lasR* derivative, PAO-R1 (10). The *nfxC* mutant PT149 showed strongly reduced rhamnolipid production (Fig. 1). Surprisingly the wild-type strain PAO-BI also showed reduced rhamnolipid produc-

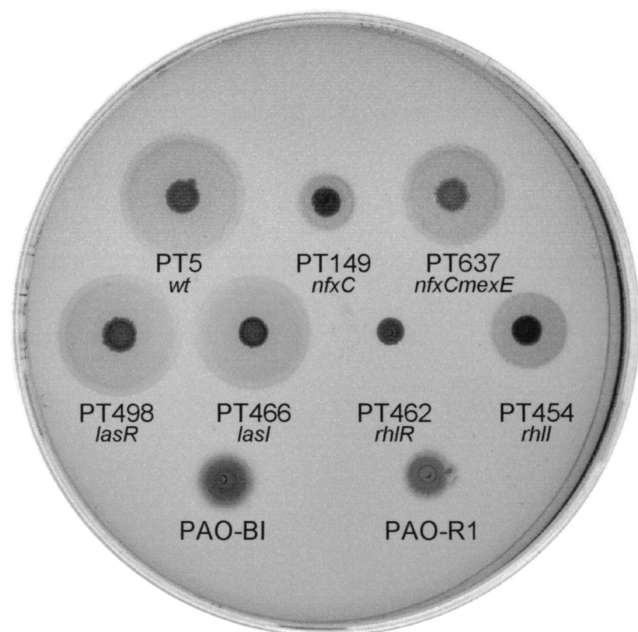


FIG. 1. Rhamnolipid plate assay. Strains to be tested were grown for 8 h in LB medium, and 2  $\mu$ l was spotted on the plate. Tyrosine at a final concentration of 0.05% was used as the nitrogen source. Incubation was carried out for 24 h at 37°C and then for 72 h at room temperature. The presence of a dark halo around the colony indicates production of rhamnolipids.

tion. Unlike PAO-R1, the *lasR* mutant PT498 constructed in the PT5 background was still able to produce rhamnolipids. As expected, the *rhlR* mutant PT462 (Fig. 1) was completely deficient in rhamnolipid production as shown previously in other wild-type backgrounds (2, 33). We therefore verified the resistance profiles of PAO-BI and PAO-R1. Indeed, both strains were resistant to chloramphenicol, ciprofloxacin, and imipenem, a phenotype reminiscent of our *nfxC* mutant PT149 (Table 2). Furthermore  $\beta$ -galactosidase levels expressed from a *mexE-lacZ* fusion were similar in PAO-BI and the *nfxC* mutant PT149 (data available upon request). This strongly suggests that strains PAO-BI and PAO-R1 are *nfxC* mutants (see also below).

As expected, the *rhlR lasR* double mutant PT531 was completely deficient in production of all exoproducts tested (Table 2).

To rule out the possibility that a particular mutation in the *nfxC* mutant strain PT149, which had been selected previously on ciprofloxacin (strain PAO-7H in reference 18), was responsible for the decrease in virulence factor production, new *nfxC* type mutants were selected by plating the wild-type strain PT5 on LB agar plates containing chloramphenicol at 600  $\mu$ g/ml, a condition which exclusively selects *nfxC* type mutants (T. Köhler, unpublished data). Fifty spontaneous independent  $\text{Cm}^r$  colonies were analyzed. All of them were cross-resistant to quinolones and imipenem, as expected for *nfxC* type mutants. All 50 colonies showed drastically decreased production of rhamnolipids in the plate assay, demonstrating the link between the *nfxC* phenotype and exoproduct synthesis.

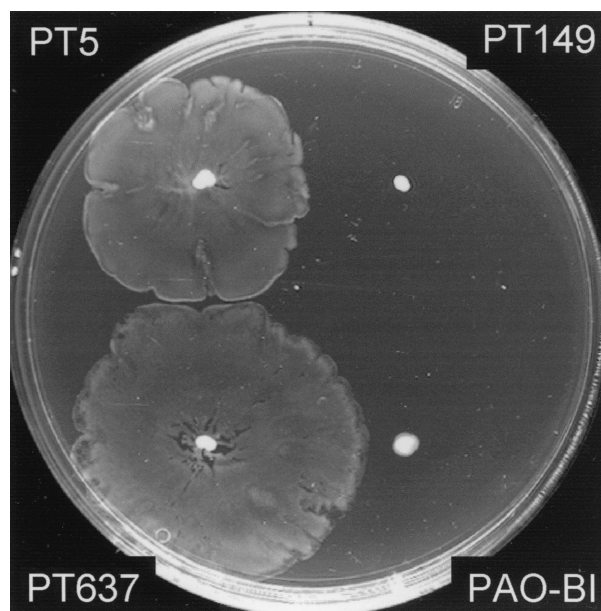


FIG. 2. Swarming was tested on M8-based minimal medium (M9 medium without  $\text{NH}_4\text{Cl}$ ), supplemented with 0.2% glucose and 0.05% glutamate as the sole nitrogen source and solidified with agar to a final concentration of 0.6%. Strains were inoculated by using a toothpick, and plates were incubated at 37°C for 18 h.

***nfxC* mutants are deficient in swarming.** We (15) and others (46) recently demonstrated the swarming motility of *P. aeruginosa* on semisolid agar plates. Swarming was shown to depend on rhamnolipids as biosurfactants (15). We therefore tested the *nfxC* mutant PT149 on swarm plates. While the wild type showed normal swarming behavior, both the *nfxC* mutant and strain PAO-BI were unable to swarm (Fig. 2). This is in agreement with the finding that rhamnolipid production was decreased in these strains.

**Efflux pump overexpression is responsible for decreased virulence factor production.** MexEF-OprN is positively regulated by the transcriptional activator MexT (16), located upstream of the efflux operon. In agreement with the findings of Maseda et al. (26), we recently found that the *nfxC* mutant PT149 contains a full-length *mexT* ORF, while in the wild-type strain PT5, the *mexT* ORF is interrupted by an 8-bp insert (CGGCCAGC), resulting in a truncated MexT protein (Fig. 3). This means that wild-type strains which do not express the MexEF-OprN efflux pump may encode an inactive *mexT* gene, while *nfxC* type mutants express a functional *mexT* gene. As expected from the phenotype, we found that strain PAO-BI encodes a functional *mexT* gene, whose complete sequence is identical to that of the *mexT* gene in our *nfxC* mutant PT149 (data available on request).

To determine whether possible pleiotropic effects of the functional MexT protein or overexpression of the MexEF-OprN efflux pump per se was responsible for the decrease in virulence factor production, the MexEF-OprN pump was inactivated in the *nfxC* type mutant PT149 by insertion of an  $\Omega$ Hg cassette into the *mexE* gene. The resulting strain, called PT637, expressed a full-length *mexT* ORF and was susceptible to the pump substrates chloramphenicol and ciprofloxacin but

	Met	
PT5	TGGAACGAGGAACGCC <b>ATGA</b> ACCGAAACGACCTGCGCCGCGT <b>CGAT</b> CTG	33
PT149	TGGAACGAGGAACGCC <b>ATGA</b> ACCGAAACGACCTGCGCCGCGT <b>CGAT</b> CTG	33
PAO-BI	TGGAACGAGGAACGCC <b>ATGA</b> ACCGAAACGACCTGCGCCGCGT <b>CGAT</b> CTG	33
*****		
PT5	AACCTGCTGATCGTGTTCGAGACCCTGATGCACGAACGCAGCGTGACCCG	88
PT149	AACCTGCTGATCGTGTTCGAGACCCTGATGCACGAACGCAGCGTGACCCG	88
PAO-BI	AACCTGCTGATCGTGTTCGAGACCCTGATGCACGAACGCAGCGTGACCCG	88
*****		
PT5	CGCCGCAGAGAAACTGTTCT <b>CGGCCAGC</b> CGGCCAGCCGGCCATCAGCGC . . .	143
PT149	CGCCGCAGAGAAACTGTTCT-----CGGCCAGCCGGCCATCAGCGC . . .	135
PAO-BI	CGCCGCAGAGAAACTGTTCT-----CGGCCAGCCGGCCATCAGCGC . . .	135
*****		

FIG. 3. Alignment of a partial DNA sequence of *mexT* from the wild-type strain PT5, the *nfxC* mutant PT149, and strain PAO-BI. The 8-bp insert inactivating the *mexT* ORF is boldfaced. The entire *mexT* gene was sequenced for all strains, and no other mutation was found.

remained resistant to imipenem. This is in agreement with previous observations demonstrating that imipenem resistance is independent of MexEF-OprN overexpression (see the last row of Table 1 in reference 18) but results from decreased expression of the porin OprD. Strain PT637 was used in subsequent experiments as a means of distinguishing between phenotypes related to MexEF-OprN pump overexpression and those related to other MexT-mediated effects. Indeed, LasB activities, as measured by elastase production (Fig. 4A), were restored to wild-type levels in strain PT637, and both rhamnolipid production and swarming ability were comparable to those of the wild type (Fig. 1 and 2). We therefore concluded that overexpression of the MexEF-OprN efflux pump is solely responsible for the decrease in virulence factor production in the *nfxC* type mutant PT149.

**Mutations in the cell-to-cell signaling regulators do not affect expression of the MexEF-OprN efflux system in the *nfxC* type mutant.** Since virulence factor production is affected in *nfxC* mutants, we asked whether the cell-to-cell signaling regulators were required for expression of the MexEF-OprN efflux system in the *nfxC* mutant PT149. For this purpose *lasI*, *lasR*, *rhlI*, and *rhlR* knockout mutations were transduced into PT149. The resistance profiles of the resultant mutants PT469 (*nfxC lasI*), PT509 (*nfxC lasR*), PT456 (*nfxC rhlI*), PT464 (*nfxC rhlR*), and PT500 (*nfxC rhlR lasR*) were compared with those of PT5 and PT149 on antibiotic gradient plates. All of the cell-to-cell signaling mutants showed the same susceptibilities to pefloxacin, chloramphenicol, and imipenem as the parental strain PT149 (data not shown). We further introduced plasmid pEZ5, carrying a *mexE-lacZ* fusion, into the *nfxC* type mutants PT149, PT464, PT500, and PT509 and measured  $\beta$ -galactosidase activities during exponential growth. In all four strains similar  $\beta$ -galactosidase activities ( $170 \pm 25$  Miller units) were obtained. This clearly establishes the quorum-sensing-independent regulation of the *mexEF-oprN* operon.

**Expression of the elastase (*lasB*) and rhamnosyltransferase (*rhlAB*) genes is affected in *nfxC* type mutants.** To further analyze the mechanism of extracellular virulence factor production of the *nfxC* type mutant PT149, LasB elastase activity was recorded over time and compared to the expression of a plasmid-encoded *lasB-lacZ* fusion. In both the wild type and

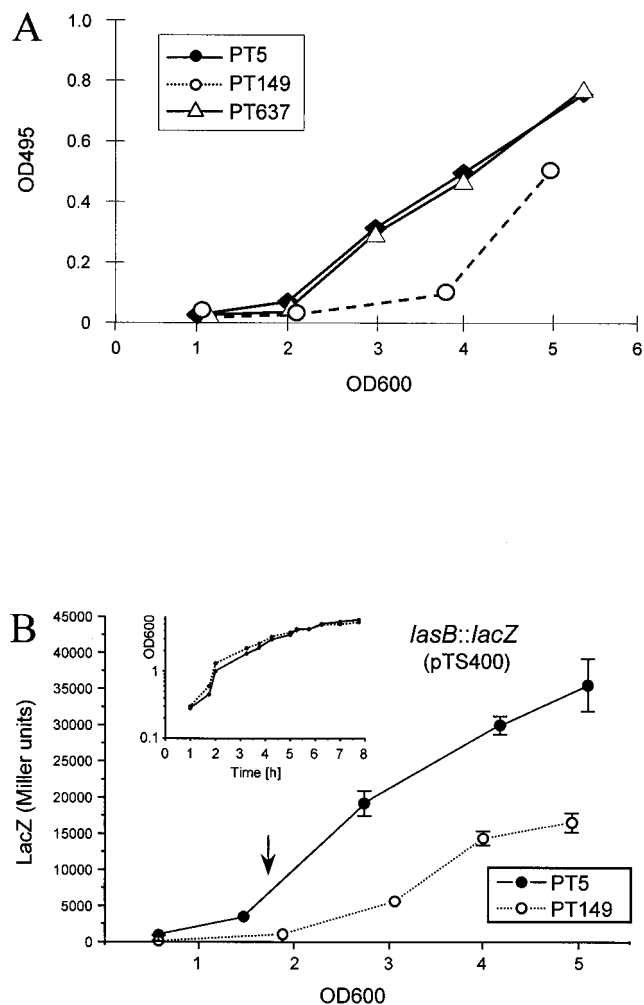


FIG. 4. (A) ECR assay. Elastase production was determined on filtered culture supernatants of strains grown in PB. Determinations were performed on three different occasions. Results from one typical experiment are shown. (B) Expression of the *lasB* gene was monitored during growth in PB using a *lasB::lacZ* fusion carried on plasmid pTS400. Growth, expressed as the OD<sub>600</sub>, was monitored (inset).

the *nfxC* type mutant, LasB activity and *lasB* expression started to appear at an OD<sub>600</sub> of 2. However, LasB activity and *lasB* expression continued to increase at a lower rate in the *nfxC* type mutant, although growth was comparable to that of the wild type (Fig. 4). Hence, the reduced elastase production in strain PT149 results from decreased expression of the *lasB* gene.

Expression of the *rhlAB* operon encoding rhamnolytransferase was assayed using the translational *rhlAB-lacZ* fusion carried by plasmid pECP60. *rhlAB* expression was determined after 18 h of incubation in M8 medium supplemented with 0.2% glucose and 0.05% glutamate as the sole nitrogen source. While the wild-type PT5 yielded  $1,976 \pm 10$  Miller units, the *nfxC* type mutant PT149 and the *rhlR* mutant PT462 yielded  $269 \pm 11$  and  $59 \pm 2$  Miller units, respectively. These results suggest that the drastically reduced rhamnolipid production in strain PT149 (Fig. 1) is caused by a strong reduction in *rhlAB* transcription.

**Expression of cell-to-cell signaling regulator genes in the *nfxC* mutant.** Both *lasB* and *rhlAB* are controlled by the *las* and *rhl* cell-to-cell signaling systems. We therefore introduced *lacZ* fusions carried on plasmids to the *lasR*, *lasI*, *rhlR*, and *rhlI* genes into PT5 and PT149 in order to determine whether their expression was altered, which could account for the decreased elastase and rhamnolipid production in the *nfxC* mutant. Both *lasR*(pPCS1001) and *lasI*(pPCS223) expression reached similar levels in the wild type and the *nfxC* mutant (Fig. 5). The expression of *lasR* increased in both strains during early stationary phase, as previously reported with the pPCS1001 *lasR::lacZ* fusion in strain PAO1 (40). In contrast, the expression of *lasI* was constant and even decreased slightly in both strains when stationary phase was reached (Fig. 5B). This surprising expression profile is very likely due to the absence on pPCS223 of the *rsaL* gene, encoding the recently described inhibitor of *lasI* expression (5). In the absence of multiple copies of the RsaL repressor, expression of *lasI* is already at a maximum during the exponential-growth phase and therefore does not display an induction profile typical of other genes regulated by the cell-to-cell signaling system. Importantly, the *nfxC* mutation did not affect the expression of *lasI* compared to that in the wild type strain.

Expression of the regulator gene *rhlR*(pPCS1002) also increased after the exponential-growth phase in both the wild type and the *nfxC* mutant and remained comparable even during stationary phase (Fig. 6A). Surprisingly, a significant difference was found when expression of *rhlI*(pMAL-I) was determined. Indeed, in the *nfxC* type mutant PT149, *rhlI* transcription was drastically decreased and reached only 35% of wild-type levels in stationary phase (Fig. 6B). We subsequently sequenced the *rhlR-rhlI* region in strains PT5 and PT149. However, no differences were found between the two strains, suggesting that the observed effect on *rhlI* expression in the *nfxC* type mutant PT149 does not result from mutations in the *rhlR-rhlI* regulatory region.

**Autoinducer production in culture supernatants.** Since the transcription of the autoinducer synthase gene *rhlI* was affected by the *mexEF-oprN* expression level, production of the C4-HSL autoinducer was determined in culture supernatants of PT5 and PT149 and compared to that in the *rhlI* mutant PT454. While the wild-type supernatants reached a C4-HSL

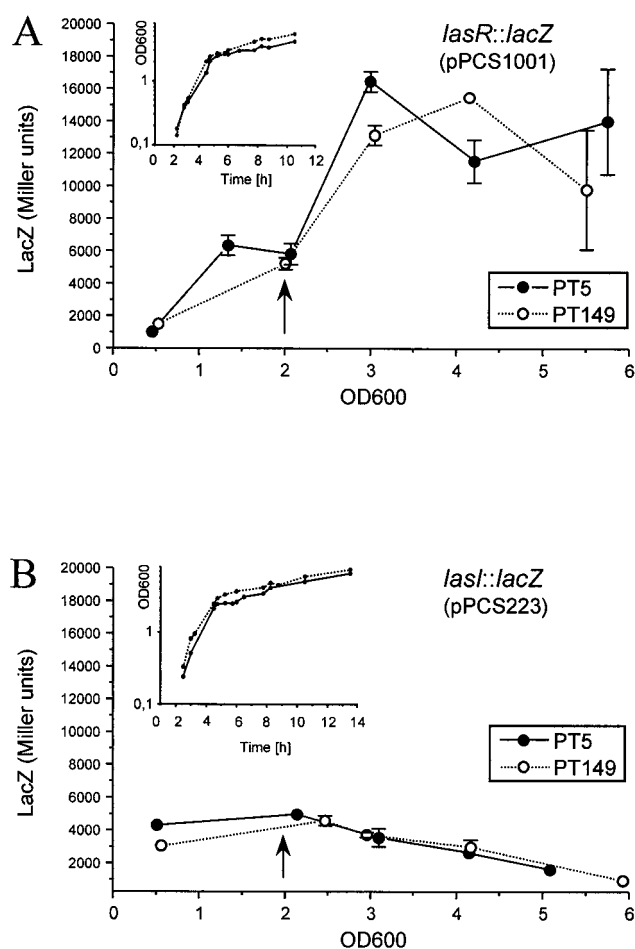


FIG. 5.  $\beta$ -Galactosidase activities expressed from *lasR::lacZ* (A) and *lasI::lacZ* (B) fusions were determined during growth in PB. Growth, expressed as the OD<sub>600</sub>, was monitored (inset). Experiments were repeated on three different occasions. Error bars represent standard deviations of triplicate LacZ determinations for one typical experiment. Where error bars are not shown, the standard deviation was within the size of the symbol. Arrows indicate the end of exponential growth. The antibiotic phenotypes of the strains at the end of the experiment were determined on pefloxacin-containing gradient plates. The LacZ activities of control plasmids pSW205 and pLP170 were approximately 3 and 400 Miller units, respectively, and remained fairly constant during growth.

concentration of  $10 \pm 0.6 \mu\text{M}$ , the PT149 supernatants contained  $3.5 \pm 0.7 \mu\text{M}$  C4-HSL (Fig. 7A). As expected, no C4-HSL was detectable in the *rhlI* mutant PT454 (data not shown). C4-HSL levels determined in supernatants of strain PT637 (*nfxC mexE*) were comparable to those for the wild type (data available on request), suggesting again that MexEF-OprN overexpression is solely responsible for the decreased amounts of C4-HSL in the *nfxC* type mutants. When the concentrations of the 3-oxo-C12-HSL autoinducer were determined, we found increased concentrations in supernatants of strain PT149 at ODs above 4 (Fig. 7B). This suggests that MexEF-OprN may contribute to the secretion of the hydrophobic 3-oxo-C12-HSL molecule, as was previously shown for the MexAB-OprM pump (7, 38).

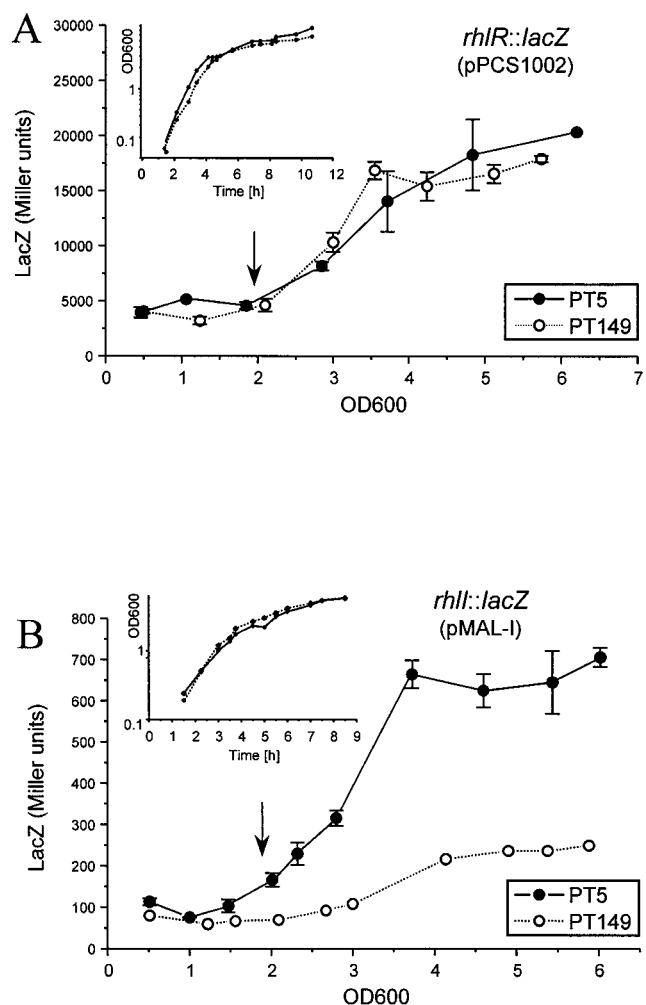


FIG. 6.  $\beta$ -Galactosidase activities expressed from *rhIR::lacZ* (A) and *rhII::lacZ* (B) fusions were determined during growth in PB. Growth, expressed as the  $OD_{600}$ , was monitored (inset). Error bars represent standard deviations of triplicate LacZ determinations for one typical experiment. Where error bars are not shown, the standard deviation was within the size of the symbol. Arrows indicate the end of exponential growth.

**Complementation of the *nfxC* mutant with exogenous auto-inducers.** The results described above show an effect of MexEF-OprN overexpression on *rhII* transcription and hence on the levels of C4-HSL produced. For full induction, the *rhI* system requires the 3-oxo-C12-HSL autoinducer and a recently identified novel regulator molecule, PQS (39). We therefore tested the effects of 3-oxo-C12-HSL, C4-HSL, and PQS (kindly synthesized by the group of U. Burger, Faculty of Chemistry, University of Geneva, Geneva, Switzerland) on rhamnolipid production using the plate assay. Addition of 3-oxo-C12-HSL alone or in combination with either PQS or C4-HSL did not affect rhamnolipid production. In contrast, addition of either C4-HSL alone or C4-HSL and PQS increased rhamnolipid production (data not shown). Hence, we tested the effects of the three signaling molecules on expression of the *rhIAB-lacZ* fusion in strain PT5, in the *nfxC* mutant PT149, and in the *rhI nfxC* double mutant PT464. PQS alone at a final concentration

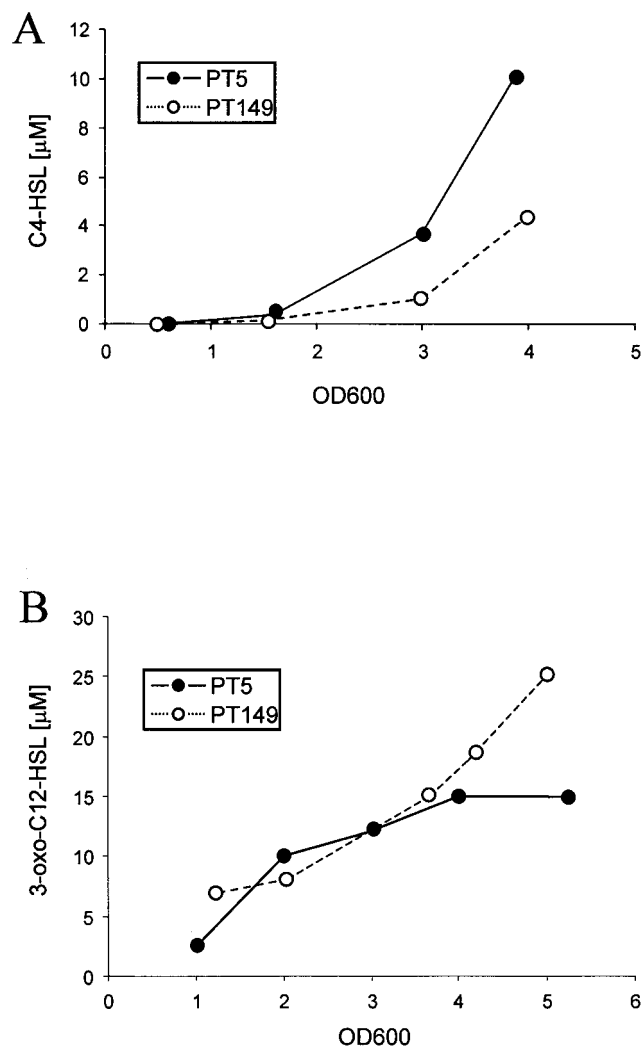


FIG. 7. Autoinducer concentrations determined in culture supernatants. C4-HSL (A) and 3-oxo-C12-HSL (B) were determined using the bioassay strains JP2(pECP61.5) and MG4 $\lambda$ I,4, respectively.

of 50  $\mu$ M had no significant effect on the transcription of the *rhIAB* fusion in any of the three strains (Table 3). In contrast, the presence of 10  $\mu$ M C4-HSL alone increased the transcription of *rhIAB* about sixfold, but only in the *nfxC* mutant. Sur-

TABLE 3. Complementation of the *rhIAB::lacZ* fusion with C4-HSL and PQS

Signal added	$\beta$ -Gal activities <sup>a</sup> (Miller units)		
	PT5 (pECP60)	PT149 (pECP60)	PT464 (pECP60)
None	23,298 $\pm$ 4,318	268 $\pm$ 10	168 $\pm$ 45
C4-HSL (10 $\mu$ M)	21,512 $\pm$ 274	1,716 $\pm$ 484	133 $\pm$ 24
PQS (50 $\mu$ M)	16,258 $\pm$ 3,453	249 $\pm$ 4	103 $\pm$ 11
C4-HSL (10 $\mu$ M) + PQS (50 $\mu$ M)	21,058 $\pm$ 4,674	4,073 $\pm$ 146	129 $\pm$ 24

<sup>a</sup> Cultures were grown for 3 h in the absence of signal; after signal was added, cultures were incubated for 18 h and  $\beta$ -galactosidase ( $\beta$ -Gal) activity was determined. Values are means from two independent experiments done in triplicate.

prisingly, when both molecules were present at these concentrations, the transcription increased 15-fold in the *nfxC* mutant. When 3-oxo-C12-HSL (final concentration, 5  $\mu$ M) was added to the two other signaling molecules, the expression of *rhlAB* did not increase further. These results strongly suggest that the levels of both C4-HSL and PQS are affected by *mexEF-oprN* overexpression. Since *rhlAB* transcription in the *rhlR nfxC* mutant was not influenced by the addition of autoinducers, it can be concluded that the partial complementation observed in the *nfxC* mutant requires the presence of the RhlR regulator.

In conclusion, our work provides evidence that overexpression of the MexEF-OprN multidrug efflux pump reduces the production of virulence factors controlled mainly by the *rhl* cell-to-cell signaling system (pyocyanin and rhamnolipids) and that this reduction results from decreased *rhlI* transcription and decreased C4-HSL autoinducer production.

## DISCUSSION

*P. aeruginosa* is known for its ability to develop resistance to a number of structurally unrelated antibiotics, a phenomenon which can now be attributed predominantly to chromosomal mutations leading to overexpression of multidrug efflux systems. *P. aeruginosa* also produces a series of exoproducts, several of which, such as elastase, alkaline protease, exotoxins, and pyocyanin, have been shown to be virulence factors (3, 54, 55). In this study, we show a link between the active efflux system MexEF-OprN and the production of virulence factors regulated by the *las* (10, 11, 19) and *rhl* (2, 33) cell-to-cell signaling systems. This important finding suggests that *P. aeruginosa* strains becoming resistant to multiple antibiotics by overexpression of MexEF-OprN are likely to be less virulent. Indeed, we recently found that *nfxC* mutants exhibit significantly reduced virulence both in a nonmammalian system and in a rat model of acute pneumonia (P. Cosson et al., submitted for publication).

The connection between multidrug resistance and virulence factor production was previously suggested in a study comparing 18 multidrug-resistant *P. aeruginosa* clinical samples collected in a Japanese hospital. All multidrug-resistant strains were deficient in production of pyoverdine, pyocyanin, elastase, hemolysin, and casein protease, while at least 8 out of 13 antibiotic-susceptible strains from the same ward were positive for these virulence factors (34).

Furthermore, two recent reports established a link between the expression of efflux pumps and the quorum-sensing system in *P. aeruginosa*. Evans et al. (7) showed that strains overexpressing the MexAB-OprM system secrete less 3-oxo-C12-HSL. Furthermore, these investigators found reduced production of pyocyanin, elastase, and casein protease compared to that in the wild type. However, several strains used by Evans et al. were derived from PAO-BI, which we show here to be an *nfxC* mutant. We sequenced the *mexT* gene in these strains and confirmed that all express a functional *mexT* gene and are therefore *nfxC* mutants. Thus, it is not clear whether the effects on virulence factor production observed by Evans et al. are due to indirect effects of MexAB-OprM overexpression on the expression of MexEF-OprN. In obvious contradiction of these results, Pearson et al. (38) found that in a non-*nfxC* background, overexpression of the MexAB-OprM pump increased

secretion of 3-oxo-C12-HSL, while deletion of *mexAB-oprM* resulted in decreased release of this autoinducer, suggesting that this hydrophobic molecule is actively secreted by the MexAB-OprM efflux pump.

Our finding that strain PAO-BI is an *nfxC* type mutant also allows us to explain discrepancies between several laboratories working in the field of quorum sensing. While it was observed that the *lasR* mutant PAO-R1 was deficient in rhamnolipid production (37), several investigators showed substantial rhamnolipid production in *lasR* mutants constructed in other strain backgrounds (2, 33). Our findings suggest that the *las* system has only a marginal effect on rhamnolipid production. Therefore, the strongly reduced rhamnolipid production in strain PAO-R1 is mainly due to its *NfxC* phenotype.

How can we explain the effect of MexEF-OprN overexpression on the *rhl* quorum system? One possibility is that autoinducers are substrates of the MexEF-OprN efflux pump, in which case the overexpression of this pump could lead to decreased intracellular autoinducer concentrations and hence diminished production of virulence factors. In the *nfxC* type mutant, we observed increased amounts of 3-oxo-C12-HSL at OD<sub>600</sub> values above 4, suggesting that 3-oxo-C12-HSL could also be a substrate for the MexEF-OprN efflux pump, as suggested for MexAB-OprM (7, 38). On the other hand, supernatants of the *nfxC* type mutant contained about 60% less of the second autoinducer, C4-HSL, than those of the wild-type strain. Short-chain autoinducers like 3-oxo-C6-HSL of *Photobacterium fischeri* (14) and C4-HSL of *P. aeruginosa* apparently (38) diffuse freely across the bacterial cell membrane. It is therefore unlikely that an efflux system, such as the MexEF-OprN pump, is involved in active export of C4-HSL. Our data support the conclusion that the reduced amounts of C4-HSL produced by the *nfxC* type mutant are the result of altered *rhlI* expression. Indeed, *rhlI* transcription levels in PT149 were reduced to 50% of those in the wild type, while *rhlR* transcription levels were unaffected. Since sequencing of the *rhlR-rhlI* DNA region obtained from the *nfxC* type mutant did not reveal any mutation, it seems likely that altered expression or activity of another regulatory element required for *rhlI* expression might be involved in the *nfxC* type mutant. The existence of such a regulator of the *rhl* operon has already been suggested (19). Furthermore, a novel signaling molecule, called PQS (39), has been identified and shown to positively regulate the transcription of *lasB* and also of *rhlI* (28). We propose that the MexEF-OprN pump decreases intracellular PQS levels, which could result either from the transport of PQS by the pump or from efflux of a precursor required for PQS biosynthesis, like, for example, tryptophan (13). This would explain the observed decrease in *rhlI* transcription and the concomitant decrease in C4-HSL levels. The combined decrease in PQS and C4-HSL levels could therefore be responsible for the diminished exoproduct synthesis in the *nfxC* type mutant. In agreement with this hypothesis is the observation that PQS in combination with C4-HSL is able to partially restore *rhlAB* transcription in the *nfxC* type mutant. PQS has a quinolone structure to which a 7-carbon-atom acyl side chain is attached. This confers a hydrophobic character on the molecule which probably prevents diffusion through the membrane, as in the case of 3-oxo-C12-HSL. The results presented here, together with our observation on the reduced virulence of the *nfxC*



mutant PT149 (Cosson et al., submitted), demonstrate that antibiotic resistance can have dramatic effects on the virulence properties of a strain without necessarily affecting its overall fitness.

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