

## Overexpression of the Multidrug Efflux Pumps MexCD-OprJ and MexEF-OprN Is Associated with a Reduction of Type III Secretion in *Pseudomonas aeruginosa*

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**The *Pseudomonas aeruginosa* genome contains several different multidrug resistance (MDR) efflux pumps. Overproduction of these pumps reduces susceptibility to a variety of antibiotics. Some recently published works have analyzed the effect of the overproduction of MDR efflux pumps on bacterial virulence. Here we have studied the effect of overproduction of the efflux pumps MexAB-OprM, MexCD-OprJ, MexEF-OprN, and MexXY on type III secretion (T3S) in *P. aeruginosa*. The type III secretion system (T3SS) is used by *P. aeruginosa* to deliver toxins directly into the cytoplasm of the host cell. Our data indicate that overexpression of either MexCD-OprJ or MexEF-OprN is associated with the impairment of T3S in *P. aeruginosa*. No effect on overexpression of either MexAB-OprM or MexXY was detected. The observed defect in T3S was due to a lack of expression of genes belonging to the T3SS regulon. Transcription of this regulon is activated by ExsA in response to environmental signals. Overexpression of this transcriptional regulator complemented the defect in T3S observed in the MexCD-OprJ- and MexEF-OprN-overproducing strains. Taken together, these results suggest that overproduction of either MexCD-OprJ or MexEF-OprN is associated with a reduction in the transcription of the T3SS regulon due to the lack of expression of the *exsA* gene, encoding the master regulator of the system. The relevance of potential metabolic and quorum-sensing imbalances due to overexpression of MDR pumps associated with this phenotype is also discussed.**

*Pseudomonas aeruginosa* is one of the leading pathogens involved in nosocomial infections (39). In addition, *P. aeruginosa* infection is the major cause of morbidity and mortality of cystic fibrosis patients (17) and has an increasing role in AIDS-associated infections (39). One of the reasons for the prevalence of *P. aeruginosa* in hospital settings might be its characteristic low susceptibility to antibiotics. This low susceptibility is mainly the consequence of the presence of several genes encoding multidrug resistance (MDR) efflux pumps in the genome of this bacterial species. Active MDR pumps have been found in *P. aeruginosa* strains associated with clinical or environmental habitats (3). Expression of MDR pumps is usually down-regulated. However, mutants overexpressing those pumps are easily selectable both in vitro (4, 26) and in vivo in clinical posttherapy isolates (22, 38, 51), thus contributing to acquired antibiotic resistance of this bacterial pathogen. Work from our laboratory and others, using simple models such as *Caenorhabditis elegans* (43) or *Dictyostelium discoideum* (8), has previously demonstrated that overproduction of *P. aeruginosa* MDR pumps might reduce bacterial fitness and virulence. *P. aeruginosa* virulence relies on the production of different factors such as siderophores, hemolysins, or toxic compounds

such as pyocyanin or cyanide, among others. The expression of several of these determinants is triggered by quorum-sensing (QS) signals (47). Since MDR pumps can extrude an ample range of molecules, efflux of QS signals by MDR pumps might be one of the reasons for the reduced virulence of *P. aeruginosa* strains overproducing such antibiotic resistance determinants. Indeed, some MDR pumps, such as MexAB-OprM (34), MexEF-OprN (24), or MexGHI-OprM (1), can extrude QS signals, thus reducing the expression of *P. aeruginosa* quorum-sensing-regulated virulence determinants. However, *P. aeruginosa* virulence does not rely only on quorum-sensing-regulated virulence determinants. Upon contact with its target mammalian cell, *P. aeruginosa* injects different exoproteins directly into the cell cytoplasm through a needle-like structure. This secretion mechanism has been termed the type III secretion system (T3SS) and is well conserved in different bacterial pathogens (21). T3SS effectors interfere with different signal pathways in the host eukaryotic cells and have a prominent role in the bacterial pathogenic process. In the case of *P. aeruginosa* (49), it has been described that exoenzyme S (ExoS) and exoenzyme T (ExoT) have ADP-ribosylating activity towards proteins of the Ras family (31). Exoenzyme Y (ExoY) is an adenylate cyclase (50), and exoenzyme U (ExoU) displays lipase activity in vitro (44). Expression of these genes is coordinately regulated by the activator protein ExsA (20, 48), the expression of *exsA* itself being triggered by ExsA in response to different environmental signals, including low calcium and direct contact with tissue culture cells.

Studies on the mechanisms of bacterial virulence have been traditionally done with wild-type antibiotic-susceptible bacte-

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Description	Source or reference
<i>E. coli</i> strains		
CC118( $\lambda$ pir)	CC118 lysogenized with $\lambda$ pir phage	18
TG1	Host for DNA manipulations	42
HB101	<i>supE44 hsdS20(r<sub>B</sub> m<sub>B</sub>) recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1</i>	42
<i>P. aeruginosa</i> strains		
PAO1-V	Clinical isolate	V. de Lorenzo
JFL30	MexAB-OprM-hyperexpressing MDR mutant from PAO1-V	Present work
JFL28	MexCD-OprJ-hyperexpressing MDR mutant from PAO1-V	Present work
JFL10	MexXY-hyperexpressing MDR mutant from PAO1-V	Present work
PAO1-L	Clinical isolate	A. Lazdunski
JFL05	MexEF-OprN-hyperexpressing MDR mutant from PAO1-L	Present work
R-1	<i>lasR</i> mutant derived from PAO1-L	A. Lazdunski
PT462	<i>rhlR</i> mutant derived from PAO1-L	T. Kohler
Plasmids		
pGEMT-Easy	Ap <sup>r</sup> ; vector for cloning of PCR products	Promega
pVLT35	Sm <sup>r</sup> ; RSF1010- <i>lacI</i> <sup>q</sup> / <i>P</i> <sub>tac</sub> hybrid broad-host-range expression vector, multiple cloning site of pUC18	14
pJF03	pVLT35 derivative plasmid that carries the wild-type <i>exxA</i> gene from <i>P. aeruginosa</i> PAO1	Present work
pRK600	Cm <sup>r</sup> <i>oriColE1</i> RK2 <sup>-</sup> Tra <sup>+</sup> , helper plasmid in triparental mating	23

rial strains. However, antibiotic usage has increased the incidence of antibiotic-resistant strains in pathogenic bacterial populations, and therefore the understanding of the relationship between antibiotic resistance and bacterial virulence stands as an important issue (5, 27) that has been addressed in just a few cases. As stated above, our group (43) and others (16, 24) have previously demonstrated that *P. aeruginosa* strains overproducing MDR pumps are defective in the production of quorum-sensing-regulated virulence determinants. To further investigate the relationship between virulence and antibiotic resistance in bacterial pathogens, in the present work we have studied the effect of overproducing the MDR pumps MexAB-OprM (37), MexCD-OprJ (36), MexEF-OprN (25), and MexXY (32) on type III secretion (T3S) in *P. aeruginosa*. Our results show that overexpression of MexEF-OprN and MexCD-OprJ is associated with a reduction in the transcription of the T3SS genes. In contrast, overexpression of either MexAB-OprM or MexXY did not produce any detectable effect on T3S.

#### MATERIALS AND METHODS

**Bacterial strains, plasmids, growth conditions, and DNA methods.** The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* and *P. aeruginosa* strains were routinely grown in Luria-Bertani (LB) broth (6) or on LB agar plates at 37°C. LB broth containing 5 mM EGTA and 20 mM MgCl<sub>2</sub> was used for analyzing type III secretion (12). Antibiotic susceptibilities were tested in Mueller-Hinton agar (6) plates containing different concentrations of antibiotics and with standard inocula of 10<sup>4</sup> cells/ml.

To analyze the mutations present in the transcriptional regulators of MDR pumps, the *mexR*, *nfxB*, *mexT*, and *mexZ* genes were PCR amplified with, respectively, the following pairs of primers: *mexR-1* (5'-CGCCATGGCCCATAT TGAG-3') with *mexR-2* (5'-GGCATTTCGCCAGTAAGCGG-3'), *nfxB-1* (5'-CG ATCCTTCCTATTGCACG-3') with *nfxB-2* (5'-GCCAAGTGCCAGTATCG-3'), *mexT-1* (5'-CGGTTGCGAGCCTCTAGCC-3') with *mexT-2* (5'-CGATTTTC CCGTTGCGACG-3'), and *mexZ-1* (5'-AGCGGCGCGACAGTAGCATA-3') with *mexZ-2* (5'-CCGAGGACGCGCAGGC-3'). The PCR mixture, containing a 0.5  $\mu$ M concentration of each primer, 100 ng of chromosomal DNA, and DNA polymerase gel form (Biotools) was heated at 94°C for 3 min. This was followed by 32 cycles of 30 s at 94°C; 30 s at 53°C (*nfxB*), 55°C (*mexT*), 58°C (*mexR*), or 60°C (*mexZ*); and 60 s at 72°C, with a final 10-min extension step at 72°C. The products of two independent PCRs were sequenced (both strands) for each of the genes.

**Analysis of type III secreted proteins.** T3S was induced as previously described (12). In brief, *P. aeruginosa* was grown in LB broth for 16 h at 37°C with agitation.

Cultures were then diluted to an optical density at 600 nm of 0.1 in calcium-depleted medium (induction condition) containing 5 mM EGTA and 20 mM MgCl<sub>2</sub> and grown again for 4 h. After centrifugation, 40  $\mu$ l of culture supernatants (from cultures containing equivalent numbers of cells) were analyzed directly by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the proteins were visualized with the PlusOne silver staining kit (Amersham Biosciences).

**In-gel digestion of proteins and sample preparation for MALDI-TOF mass spectrometry.** Digestion of proteins in excised gel plugs (in gel) was performed as described previously (45) with minor modifications. The excised gel plugs were washed in water and acetonitrile prior to reduction with 10 mM dithiothreitol and alkylation with 55 mM iodoacetamide and thereafter were dried by vacuum centrifugation. The dried gel pieces were incubated with modified porcine trypsin (10 ng/ $\mu$ l, sequencing grade; Promega, Madison, Wis.) in 50 mM NH<sub>4</sub>HCO<sub>3</sub> for 40 min, after which the supernatant was removed, 20 to 40  $\mu$ l of 50 mM NH<sub>4</sub>HCO<sub>3</sub> was added, and the digestion was continued at 37°C for 12 h. A 0.5- $\mu$ l aliquot of the digestion supernatant was deposited onto the matrix-assisted laser desorption ionization-time-of-flight (MALDI) probe (AnchorChip; Bruker Daltonics) and allowed to dry at room temperature. Then, 0.5  $\mu$ l of matrix solution (saturated  $\alpha$ -cyano-4-hydroxycinnamic acid in 30% [vol/vol] aqueous acetonitrile and 0.1% [vol/vol] trifluoroacetic acid) was added and again allowed to dry at room temperature.

Samples were measured on a Reflex III MALDI-TOF mass spectrometer (Bruker-Franzen Analytic GmbH, Bremen, Germany) equipped with the SCOUT source in positive-ion reflector mode. The ion acceleration voltage was 20 kV. The equipment was first externally calibrated by employing protonated mass signals from a peptide mixture covering the 1,000- to 4,000-*m/z* range, and thereafter every spectrum was internally calibrated by using signals arising from trypsin autolysis.

**RT-PCR.** Bacterial cells growing in conditions that induce T3S (see above) were collected, spun down at 4°C, and frozen in dry ice. Total RNA was extracted with the phenol-guanidine thiocyanate mix Tri Reagent LS (Molecular Research Center, Inc.). Residual DNA was removed by treatment with the DNA-Free kit (Ambion). Reverse transcriptase PCR (RT-PCR) assays were performed with Ready-To-Go RT-PCR beads (Amersham Biosciences) as indicated by the manufacturer. The amplifications were performed with primers specific for the selected genes (listed in Table 2) and two serial 10-fold dilutions of the RNA (1 and 0.1  $\mu$ g). To ascertain that no residual DNA was present in the RNA preparations, PCRs were performed with the same primers and overall conditions, except that no reverse transcriptase was added. Expression of the *rpsL* gene was measured as an internal control that ensured that equal amounts of RNA were used in all of the RT-PCRs done. The reaction mixtures were incubated for 30 min at 42°C, followed by 10 min at 95°C. This was followed by 30 cycles of 30 s at 95°C; 30 s at 55°C (for *mexC* and *mexE*), 57°C (for *mexA*), 60°C (for *exoT*, *rpsL*, *exsA*, *exsD*, and *mexX*), 63°C (for *aceB* and *exoS*), or 65°C (for *perV*); and 1 min at 72°C, prior to the final 7-min elongation at 72°C.

**Cloning of *exxA*.** The *exxA* gene was amplified by using the primers *exxA-3* (5'-ATAAAATCGACTCCGTGTCA-3') and *exxA-4* (5'-TTCTACTCATGCA

TABLE 2. Primers used for RT-PCR

Primer	Sequence
<i>mexA-1</i> .....	5'-CCTGCTGGTCGCGATTTCCGG-3'
<i>mexA-2</i> .....	5'-CCAGCAGCTTGTAGCGCTGG-3'
<i>mexC-1</i> .....	5'-TTGGCTATGGCCATCGCGTT-3'
<i>mexC-2</i> .....	5'-ATCGAAGTCCTGCTGGCTGA-3'
<i>mexE-1</i> .....	5'-ATCCCACTTCTCCTGGCGCT-3'
<i>mexE-2</i> .....	5'-GGTCGCCTTTCTTACCAGT-3'
<i>mexX-1</i> .....	5'-GCGATGCGGATTGCGGAACA-3'
<i>mexX-2</i> .....	5'-TGGTCGCCCTATTCTCGT-3'
<i>rpsL-1</i> .....	5'-GCAACTATCAACCAGCTG-3'
<i>rpsL-2</i> .....	5'-GCTGTGCTCTTGCAGGTTGTG-3'
<i>exsA-1</i> .....	5'-CGCTGTGATGTCGGTCTCT-3'
<i>exsA-2</i> .....	5'-GCCGTGCTGATGTCGGTCTGA-3'
<i>exoT-1</i> .....	5'-CATCGAGTGGCTGGGCAAAC-3'
<i>exoT-2</i> .....	5'-TCTCCGCTGTCAAAGTCGCC-3'
<i>exoS-1</i> .....	5'-AAGCGGTAGAGAGCGAGGTC-3'
<i>exoS-2</i> .....	5'-CCGCTTCCACTGCTGCTG-3'
<i>pcrV-1</i> .....	5'-CAAGCAGGGCATCAGGATCG-3'
<i>pcrV-2</i> .....	5'-TGTCGTTGAGCAGGTTGGTC-3'
<i>exsD-1</i> .....	5'-GGAGCAGGAAGACGATAAGC-3'
<i>exsD-2</i> .....	5'-GCCAGTTCGGAGCACCAGC-3'
<i>aceB-1</i> .....	5'-CTCTCGACCACCTGCTCCTG-3'
<i>aceB-2</i> .....	5'-GCCGAAACACCAACTGGACCG-3'

GCCGCTA-3'). The PCR mixture, containing a 0.5  $\mu$ M concentration of each primer, 100 ng of chromosomal DNA, and DNA polymerase gel form (Biotools), was heated at 94°C for 3 min; this was followed by 32 cycles of 30 s at 94°C, 30 s at 60°C, and 60 s at 72°C, with a final 10-min extension step at 72°C. The PCR product obtained was purified with the Qiaquick purification PCR kit (Qiagen), cloned in the pGEMT-Easy vector (Promega), and recovered as an EcoRI fragment. The EcoRI fragment containing the *exsA* gene was cloned under the control of the *Ptac* promoter in the EcoRI site of the plasmid pVLT35, rendering plasmid pJF03. The insert was sequenced to ensure that it did not contain any mutation, and its orientation in the vector was confirmed by PCR with the primers *Ptac* (5'-GACAATTAATCATCGGCTCG-3') and *exsA-2*. Plasmid pJF03 was then introduced in *P. aeruginosa* strains JFL28 and JFL05 by conjugation in triparental matings as described previously (15), using plasmid pRK600 as a helper for the transfer functions. Exconjugants were selected in minimal salts M9 (6) agar plates containing citrate (0.2%, wt/vol) and streptomycin (50  $\mu$ g/ml). Expression of *exsA* from *Ptac* was induced with 3 mM IPTG (isopropyl- $\beta$ -D-thiogalactopyranoside).

**Cytotoxicity assays.** The J774 macrophage cell line (10) was grown in Dulbecco's modified Eagle medium supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (Gibco). The macrophages were seeded in 96-well culture plates 20 h before infection at  $10^4$  cells/well. On the day of the experiment, the cells were carefully washed and infected with the *P. aeruginosa* strains at a multiplicity of infection of 50. Eight wells were infected with each bacterial strain. After 360 min of infection, cytotoxicity was evaluated with a cytotoxicity detection kit (LDH) in accordance with the instructions of the manufacturer (Roche). One hundred percent cytotoxicity was estimated by lysing noninfected cells with 2% (vol/vol) Triton X-100. Means and standard deviations were calculated with Microsoft Excel.

## RESULTS AND DISCUSSION

**Obtainment of *P. aeruginosa* strains overexpressing MDR pumps.** To analyze the effect of overexpression of MDR pumps on bacterial physiology, we needed to mimic as much as possible the type of mutants encountered in clinical settings after antibiotic treatment. To that end, single-step mutants hyperexpressing MexAB-OprM, MexCD-OprJ, MexEF-OprN, and MexXY were selected on antibiotic selective plates. Two wild-type *P. aeruginosa* PAO1 strains were used, namely, PAO1-L (from the laboratory of Andrée Lazdunsky) and PAO1-V (from the laboratory of Víctor de Lorenzo). In the course of our studies, we realized that strain PAO1-V has an

internal deletion in the *mexT* gene, leading to a truncated version of MexT, the transcriptional activator of *mexEF-oprN* (our unpublished results). Due to this deletion, *mexEF-oprN* cannot be up-regulated in the PAO1-V strain. It has been described that *mexEF-oprN* overproduction might interfere with analysis of the effect of MDR pumps on the *P. aeruginosa* QS response (24). Therefore, the genetic background of PAO1-V is suitable for avoiding those problems and was used for obtaining mutants that overexpressed MexAB-OprM, MexCD-OprJ, and MexXY. The PAO1-L strain was used for obtaining the MexEF-OprN-overexpressing mutant. Strain JFL30 was selected with 20  $\mu$ g of tetracycline per ml (4). As shown in Table 3, this mutant strain has a lower susceptibility to quinolones, aztreonam, and ceftiofime but not to imipenem, a phenotype that is compatible with overexpression of MexAB-OprM. The mutant strain JFL28 was selected with 8  $\mu$ g of norfloxacin per ml and 500  $\mu$ g of erythromycin per ml (26) and has a decreased susceptibility to ceftiofime and quinolones but not to aztreonam (Table 3). This phenotype is compatible with MexCD-OprJ overexpression. The mutant strain JFL05 was selected with 600  $\mu$ g of chloramphenicol per ml (24) and was less susceptible to imipenem, chloramphenicol, and quinolones (Table 3), a phenotype compatible with MexEF-OprN overexpression. Finally, strain JFL10 was selected on Mueller-Hinton agar plates containing 4  $\mu$ g of gentamicin per ml and 1  $\mu$ g of ofloxacin per ml (29). This mutant strain was less susceptible to quinolones and gentamicin (Table 3), a phenotype compatible with MexXY overproduction. The overexpression of the corresponding MDR system was confirmed by semiquantitative RT-PCR with primers specific for the *mexA*, *mexC*, *mexE*, and *mexX* genes as described in Materials and Methods. As shown in Fig. 1, each of the mutant strains overexpressed just the MDR determinant predicted to be hyperexpressed on the basis of the aforementioned phenotypes of antibiotic susceptibility. To further characterize these mutant strains, the local regulator of each MDR pump was PCR amplified and sequenced. Consistent with the observed phenotypes, *mexR* (repressor of *mexAB-oprM*) from strain JFL30 carries a 1-bp deletion at the position 81, resulting in an out-of-frame sequence. *nfxB* (repressor of *mexCD-oprJ*) from strain JFL28 has a 1-bp deletion at the position 47, resulting in an out-of-frame sequence. *mexT* (activator of *mexEF-oprN*) from strain JFL05 contains an 8-bp deletion at the position 104 that renders an active form of MexT (28). Mutations in the *mexZ* gene (local repressor of *mexXY-oprM*) are not frequently found in MexXY-OprM-

TABLE 3. Antibiotic susceptibilities of *P. aeruginosa* strains

Strain	MIC ( $\mu$ g/ml) <sup>a</sup>					
	CIP	GEN	CHL	IMI	AZT	CEF
PAO1-V	0.25	2	64	2	4	4
JLF30	1	2	512	2	32	16
JFL28	4	2	128	1	1	32
JFL10	4	8	256	2	4	32
PAO1-L	0.25	2	64	2	4	4
JFL05	1	2	1,024	4	4	16

<sup>a</sup> CIP, ciprofloxacin; GEN, gentamicin; CHL, chloramphenicol; IMI, Imipenem; AZT, aztreonam; CEF, ceftiofime.



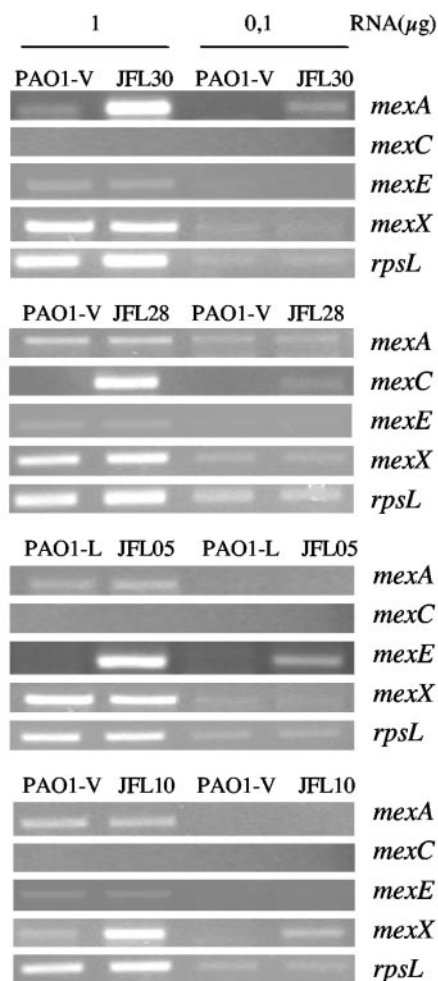


FIG. 1. Expression of MDR genes by single-step multidrug-resistant *P. aeruginosa* mutants. The amounts of mRNAs corresponding to the first genes (*mexA*, *mexE*, *mexC*, or *mexX*) of the four best-characterized MDR pumps of *P. aeruginosa* were measured by RT-PCR in strains PAO1-L (wild type) and its derivative JFL05 and in PAO1-V (wild type) and its derivatives JFL30, JFL28, and JFL10. Different amounts of RNA (1 or 0.1 µg) were used. Expression of the *rpsL* gene was used as a control to ensure that the amounts of RNAs from wild-type and mutant strains used for the RT-PCR assays were comparable.

overproducing mutants (46). In agreement with this, we did not detect mutations in *mexZ* in strain JFL10.

**Effect of overexpression of MDR pumps on type III secretion in *P. aeruginosa*.** Bacteria were grown under conditions that stimulate T3S (12), and the proteins secreted by the wild-type and the MDR mutant strains were analyzed by SDS-PAGE. A few changes could be observed upon comparison of the SDS-PAGE profiles. The experiment was repeated three times, and the bands whose intensities consistently changed in the three experiments were analyzed. The bands of interest were excised from the gel and digested with trypsin, and the masses of the generated peptides were determined by MALDI-TOF mass spectrometry to obtain the protein's peptide mass fingerprint. The peptide fingerprint obtained for each band was matched against those derived from a virtual digestion of each protein encoded by *P. aeruginosa* PAO1 ([www.pseudomonas.com](http://www.pseudomonas.com)). This approach allowed us to find that the bands with the most rel-

evant modifications in intensity corresponded to the T3S proteins ExoT, ExoS, and PcrV. As shown in Fig. 2, expression of these three proteins was significantly reduced in mutant strains JFL05 (overexpresses MexEF-OprN) and JFL28 (overexpresses MexCD-OprJ). This reduction was not observed in the strains overexpressing MexAB-OprM or MexXY. Since strain PAO-V, from which JFL28 was obtained, has a deletion in *mexT* (see above), the effect on T3S of MexCD-OprJ overexpression cannot be attributed to an undesired overexpression of MexEF-OprN.

**Type III secretion is not affected by quorum sensing at the exponential growth phase.** It has been described previously that overexpression of the MDR efflux pumps MexAB-OprM, MexCD-OprJ, and MexEF-OprN reduces the expression of QS-regulated virulence factors (16, 24, 43). In fact, MexAB-OprM (34) and MexEF-OprN (24) can extrude QS signals, thus contributing to regulate QS homeostasis. Although the supernatants used for measuring T3S were obtained from exponential-phase cultures in which QS signaling is unlikely, it might still be conceivable that the effect of MDR efflux pump overproduction in T3S could be mediated by quorum-sensing. In fact, a recent work has stated that T3S is down-regulated in *P. aeruginosa* biofilms and that the expression of *exoS* in stationary phase might be regulated by quorum sensing (19). QS in *P. aeruginosa* is mediated mainly by two different, yet interconnected, circuits. One is the *las* pathway, controlled by the

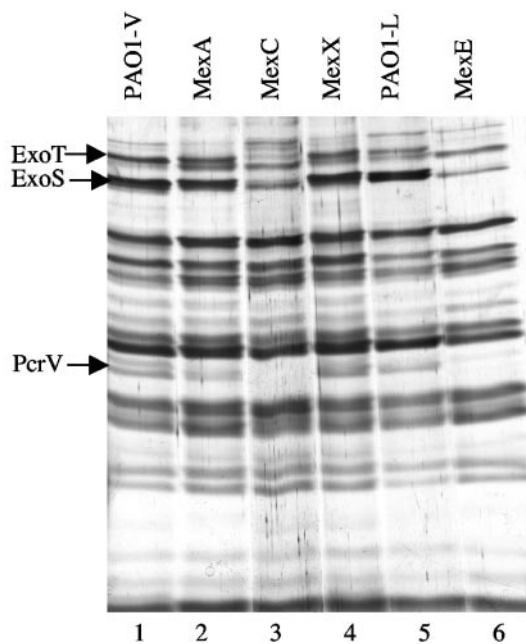


FIG. 2. Analysis of the proteins secreted by *P. aeruginosa* strains that overproduce MDR efflux pumps. The proteins secreted by the indicated strains while growing exponentially under conditions that stimulate T3S (see Materials and Methods) were analyzed by SDS-PAGE. Lane 1, wild-type strain PAO1-V; lane 2, single-step mutant overexpressing MexAB-OprM; lane 3, single-step mutant overexpressing MexCD-OprJ; lane 4, single-step mutant overexpressing MexXY-OprM; lane 5, wild-type strain PAO1-L; lane 6, single-step mutant overexpressing MexEF-OprN. Proteins expressed at lower levels in the strains that overproduce MexCD-OprJ and MexEF-OprN, identified by MALDI-TOF mass spectrometry (see text), are indicated with arrows.

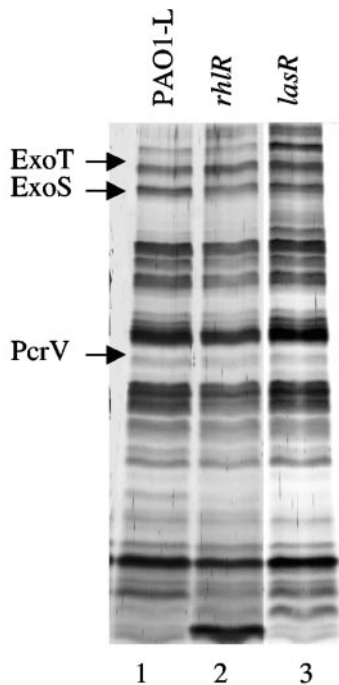


FIG. 3. Proteins secreted by strains having an impaired quorum-sensing signaling pathway. The effect of mutations in QS control on the profile of secreted proteins was evaluated by using *P. aeruginosa* mutants with mutations in the *rhl* and *las* QS pathways. Lane 1, PAO1-L (wild-type strain); lane 2, R-1 (*rhlR* derivative of PAO1-L); lane 3, PT462 (*lasR* derivative of PAO1-L). The arrows indicate the positions of the proteins whose expression decreased upon overexpression of the MexCD-OprJ and MexEF-OprN MDR pumps.

LasR transcriptional regulator (33). The other is the *rhl* pathway, which is transcriptionally regulated by RhlR (7). Both systems are connected through a third intercellular signaling element, the *P. aeruginosa* quinolone signal (35). We analyzed proteins secreted under conditions that stimulate T3S in *lasR* and *rhlR* mutant strains that are defective in the *las* and *rhl* QS pathways, respectively. As shown in Fig. 3, no differences were detected in the bands corresponding to ExoT, ExoS, and PcrV when comparing wild-type with QS mutant strains. This indicates that the defect in T3S detected in the MexEF-OprN and MexCD-OprJ mutants cannot be attributed to a putative defect in QS signaling in these strains.

**Overexpression of MDR pumps challenges type III secretion at the transcriptional level.** The defect in T3S observed in the mutant strains overproducing MexCD-OprJ and MexEF-OprN could be due either to a specific reduction in the secretion of some proteins or to a reduced transcription of the genes encoding T3S proteins. To analyze this possibility, RNAs were obtained from strains PAO1-V (wild type), JFL28 (overexpressing MexCD-OprJ), PAO1-L (wild type), and JFL05 (overexpressing MexEF-OprN) grown under conditions that stimulate T3S, and the expression of genes encoding T3S factors was measured by semiquantitative RT-PCR. Expression of *rpsL* (constitutive expression) (46) and either *mexC* or *mexE* (overexpressed) was also measured as a control. As shown in Fig. 4, expression of the genes *exoT*, *exoS*, and *pcrV*, encoding T3S proteins, was clearly lower in the strain overproducing MexEF-OprN than in its wild-type isogenic parental strain. Expression

of these genes was also decreased, although to a lesser extent, in the strain overexpressing MexCD-OprJ. Control reactions performed in parallel with the same RNA preparations showed that expression of the *rpsL* gene was similar in all cases and that expression of the MDR genes was increased in the mutant strains (Fig. 4). Therefore, the results presented suggest that the effect on T3S associated with MDR overexpression in *P. aeruginosa* occurs at the transcriptional level. As stated above, expression of the T3S regulon is triggered by the ExsA activator. Thus, we compared *exsA* transcription in the strains overexpressing MexCD-OprJ or MexEF-OprN and their isogenic wild-type strains. Semiquantitative RT-PCR analyses (Fig. 3) showed that *exsA* expression was lower in the strains overexpressing the MDR pumps than in the wild-type strains. This suggests that the strains overexpressing MexCD-OprJ and MexEF-OprN are unable to trigger *exsA* expression in response to the signal that activates T3S.

**Expression of ExsA from a heterologous promoter suppresses the effect of MDR overexpression on type III secretion.** Our data indicate that the defect in secretion detected in the *P. aeruginosa* strains overexpressing the MexCD-OprJ or MexCD-OprN pump might be due to a low-level expression of *exsA* compared to that in the wild-type parental strains. To confirm this possibility, the *exsA* gene was PCR amplified and cloned in the vector pVLT35 to render pJF03. In this plasmid, expression of the cloned *exsA* gene is under control of the heterologous *Ptac* promoter. The strains overexpressing MexCD-OprJ and MexEF-OprN were transformed with the *exsA* expression plasmid pJF03, and T3S was compared to that in the parental strains. As shown in Fig. 5, secretion of ExoT, ExoS, and PcrV was restored to the normal levels in the mutant strains when *exsA* was expressed from the *Ptac* promoter. This result strongly supports the hypothesis that the lack of

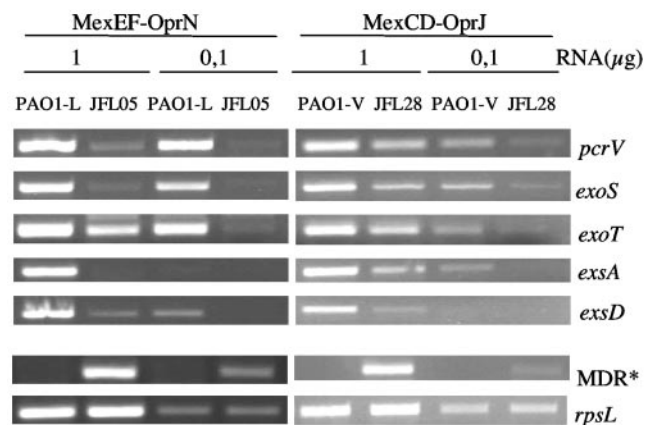


FIG. 4. Expression of T3SS genes by strains that overproduce MexCD-OprJ and MexEF-OprN. The amounts of mRNAs corresponding to different genes of the T3SS regulon (*exoT*, *exoS*, *pcrV*, *exsA*, or *exsD*) were measured by RT-PCR in strains PAO1-L (wild type), JFL05 (derivative of PAO1-L overexpressing MexEF-OprN), PAO1-V (wild-type), and JFL28 (derivative of PAO1-V overexpressing MexCD-OprJ). Different amounts of RNA (1 or 0.1 μg) were used. Expression of the *rpsL* gene was used as a control to ensure that the amounts of RNAs from wild-type and mutant strains used for the RT-PCR assays were comparable. Expression of the first gene of each MDR pump operon (either *mexA* or *mexE*) was also analyzed by RT-PCR (indicated as MDR\*).

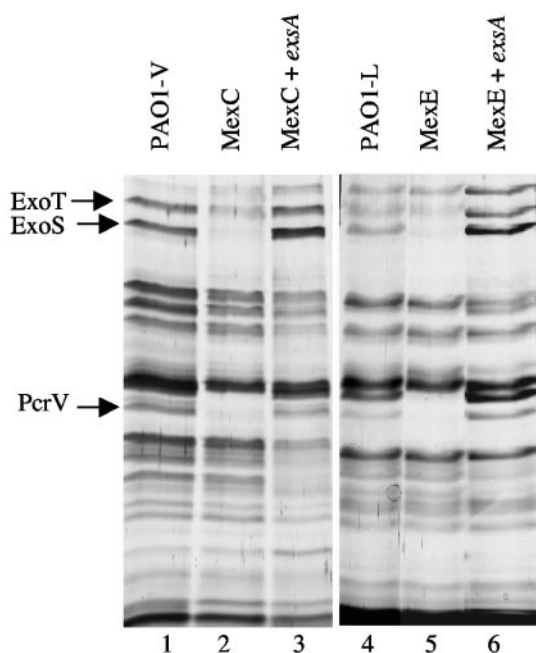


FIG. 5. Effect of ExsA expression on the proteins secreted by strains overexpressing the MDR pumps MexCD-OprJ and MexEF-OprN. The proteins secreted by cells grown under conditions that stimulate T3S were resolved by SDS-PAGE. Lane 1, PAO1-V (wild type); lane 2, JFL28 (derivative of PAO1-V overexpressing MexCD-OprJ); lane 3, JFL28 containing plasmid pJF03 (overexpresses *exsA*) grown in the presence of 3 mM IPTG; lane 4, PAO1-L (wild type); lane 5, JFL05 (derivative of PAO1-L overexpressing MexEF-OprN); lane 6, JFL05 containing plasmid pJF03 (overexpresses *exsA*) grown in the presence of 3 mM IPTG. The bands corresponding to the T3S proteins ExoT, ExoS, and PcrV are indicated.

*exsA* expression is responsible for the effect of MexCD-OprJ and MexEF-OprN on T3S in *P. aeruginosa*. To ensure that this defect was not due to secondary mutations present in *exsA*, the genes from strains JFL05 and JFL28 were PCR cloned and sequenced. No mutation was found in the *exsA* sequence in either of the strains. T3S is triggered *in vivo* by contact with the target mammalian cell and *in vitro* by growing bacteria in media with low calcium and high magnesium levels. In both cases ExsA is the master transcriptional activator of the system. Nevertheless, the mechanism by which the extracellular calcium concentration (or the contact with host cell) is translated into the activation of *exsA* transcription is unknown. It has been described that ExsD, a negative regulator of the T3S regulon, blocks the activity of ExsA (30). We thus measured *exsD* expression to determine whether it is increased in the MDR mutants compared to the wild-type strains, causing the low *exsA* expression. As shown in Fig. 4, *exsD* is expressed at lower levels in the MDR mutants than in the wild-type strains. Thus, a role of this protein in the observed decrease in T3S seems unlikely.

**MDR overexpression is associated with a reduction of *P. aeruginosa* cytotoxicity.** T3S has a relevant role in *P. aeruginosa* cytotoxicity (9, 12, 13) To validate our *in vitro* data with a physiologically relevant model, we measured the cytotoxicities of the MexCD-OprJ- and MexEF-OprN-overproducing mutants in comparison with the wild-type strains by using the macrophage cell line J774 (10). Under the conditions tested, strain PAO1-V produced  $78\% \pm 7\%$  cell lysis and the MexCD-

OprJ-overproducing isogenic mutant strain JFL28 was clearly less cytotoxic ( $39\% \pm 6\%$ ). Similarly, the MexEF-OprN-overproducing strain JFL05 was less cytotoxic ( $7\% \pm 3\%$ ) than its isogenic wild-type strain PAO1-L ( $44\% \pm 4\%$ ). Expression of *exsA* from a heterologous promoter (see above) in strains JFL28 and JFL05 increased cytotoxicity to  $91\% \pm 9\%$  and  $83\% \pm 12\%$ , respectively, indicating that *exsA* can complement both *in vitro* (see above) and *in vivo* the defects on T3S and cytotoxicity associated with overexpression of the MexCD-OprJ or MexEF-OprN efflux pump. Clinical *P. aeruginosa* isolates are either cytotoxic or cytoinvasive. However, it has been found that noncytotoxic cystic fibrosis isolates can be converted to cytotoxic isolates simply by expressing the *exsA* gene from a heterologous promoter (10). Since mutants overproducing MDR pumps can be selected by antibiotic treatment, it would be interesting to know whether there is an association between MDR overproduction and reduced cytotoxicity in *P. aeruginosa* cystic fibrosis isolates.

**Metabolic imbalance and T3S in *P. aeruginosa* MDR-overproducing mutants.** Two recent articles have reported that *P. aeruginosa* T3S can be affected by mutations in metabolic genes. A search of new genes involved in T3SS-dependent cytotoxicity toward human polymorphonuclear neutrophils demonstrated that the T3SS requires an intact *aceAB* operon (11), which encodes the E1 and E2 subunits of pyruvate dehydrogenase (PDH). Those authors suggested that the most likely explanation is that the E1 or E2 subunit of PDH could play a role in regulation of T3S gene expression. A role of PDH in transcriptional regulation was first demonstrated in *Azotobacter vinelandii*, in which the E1 subunit of PDH is an activator of NADPH:ferredoxin reductase transcription in response to oxidative stress (40). Our group has previously shown that overexpression of MDR pumps can challenge bacterial metabolism (2). It might thus be possible that *aceAB* expression could be altered in the MexCD-OprJ- and MexEF-OprN-overproducing mutants, and these changes could be the reason for the lack of expression of T3SS in them. We have compared the expression of *aceB* in the MDR mutants and wild-type parental strains by semiquantitative RT-PCR. As shown in Fig. 6, the

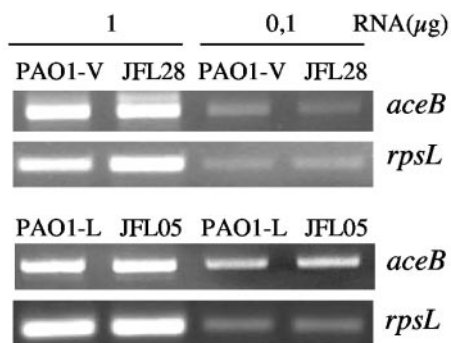


FIG. 6. Expression of the *aceAB* operon in strains that overproduce MexCD-OprJ and MexEF-OprN. The expression of the *aceB* gene in strains PAO1-V (wild type), JFL28 (derivative of PAO1-V overexpressing MexCD-OprJ), PAO1-L (wild type), and JFL05 (derivative of PAO1-L overexpressing MexEF-OprN) was measured by RT-PCR. Two different amounts of RNA (1 or 0.1 µg) were used. Expression of the *rpsL* gene was used as a control to ensure that the amounts of RNAs from the strains compared were similar.



levels of *aceAB* RNAs were the same in the wild-type strains and in the strains overproducing MexCD-OprJ or MexEF-OprN. Thus, the defect in T3SS transcription is not due to a reduced expression of *aceAB* in these mutant strains.

A different search for mutations affecting T3S in *P. aeruginosa* showed that overproduction of *hutT*, a gene encoding a histidine transporter, is associated with a decrease in the expression of the T3S gene *exoS* (41). The mutant strain was also impaired in growth at the expense of histidine as the sole carbon source. We have determined that the growth of the strains overproducing MexCD-OprJ or MexEF-OprN at the expense of histidine is not impaired (data not shown). Consequently, a role of *hutT* in the defect of T3SS transcription in our mutant strains is unlikely.

In summary, the results presented here indicate that the transcriptional activation of the T3S regulon is reduced in mutants overexpressing either the MexCD-OprJ or MexEF-OprN MDR pump, making these mutants less cytotoxic. This phenotype is associated with low expression of *exsA* under conditions that trigger T3S. Since MDR pumps can extrude a wide range of compounds belonging to different structural families, it might be possible that MexCD-OprJ and MexEF-OprN are extruding the intracellular signal that allows ExsA to activate transcription of the T3SS genes. Identification of this signal has so far remained elusive. Alternatively, T3S impairment in these mutant strains could be due to the metabolic challenge associated with MDR overexpression or to a direct effect of the MDR regulators.

The results presented here, together with the observed interference of the overexpression of MDR pumps in quorum-sensing regulation (16, 24, 43), emphasizes the importance of a proper regulation of these efflux systems so as not to compromise *P. aeruginosa* fitness and virulence.

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