Characterization of a new efflux pump, MexGHI-OpmD, from *Pseudomonas aeruginosa* that confers resistance to vanadium

Sverine Aendekerk, Bart Ghysels, Pierre Cornelis and Christine Baysse

Author for correspondence: Pierre Cornelis. Tel: +32 2 3590221. Fax: +32 2 3590399.
e-mail: pcornel@vub.ac.be

Laboratory of Microbial Interactions, Department of Immunology, Parasitology and Ultrastructure, Flanders Interuniversity Institute of Biotechnology, Vrije Universiteit Brussel, Paardenstraat 65, B-1640 Sint Genesius Rode, Belgium

Vanadium has an antibacterial activity against *Pseudomonas aeruginosa*, especially under conditions of iron limitation. Some degree of resistance to V is inducible by prior exposure to the metal. One mutant (VS1) with a higher sensitivity to V was obtained by transposon mutagenesis of *P. aeruginosa* PA 59.20, a clinical isolate. This mutant had an insertion in a non-coding region, upstream of a cluster of four genes. Three of them show similarities to genes corresponding to known *P. aeruginosa* antibiotic efflux systems, including an efflux protein, a membrane fusion protein and an outer-membrane porin. This cluster was named *mexGHI-opmD*. By allelic exchange, three mutants, *ncr* (for non-coding region), *mexI* and *opmD* were constructed in *P. aeruginosa* PAO1. Next to V sensitivity, the *ncr*, *mexI* and *opmD* mutants also showed reduced production of elastase, rhamnolipids, pyocyanine, pyoverdine and had reduced swarming motility, phenotypes that are known to be regulated by quorum sensing. All wild-type phenotypes, including growth in the presence of V, were restored by complementation with the complete cluster. The production of *N*-acyl-homoserine lactones (AHLs) was detected using the *Chromobacter violaceum* bioassay. Total extracts from the three mutants failed to induce the production of violacein by *C. violaceum*, although AHLs were detected by TLC and *C. violaceum* overlay. Violacein production was restored by complementation with *mexGHI-opmD*. The *opmD* mutant grew very slowly in LB or CAA medium, indicating that OpmD has an important physiological function for the cell. In conclusion, it is believed that the MexGHI-OpmD pump is probably involved in AHL homeostasis in *P. aeruginosa*.

Keywords: Mex-type efflux pump, quorum sensing, *N*-acyl-homoserine lactone production

INTRODUCTION

Vanadium exerts a bacteriostatic effect on *Pseudomonas aeruginosa*, especially when the cells are grown under conditions of iron limitation (Baysse *et al.*, 2000). In our previous work, we have shown that V(IV) was complexed by the two *P. aeruginosa* siderophores pyoverdine (Pvd) and pyochelin (Pch), and V-Pch was toxic for the cells (Baysse *et al.*, 2000). Some degree of resistance against V could also be acquired after pre-incubation of the cells with subinhibitory concentrations of VOSO₄

(Baysse *et al.*, 2000). At least one factor, the production of Fe-dependent superoxide dismutase (SodB), seems to contribute to the development of resistance against V in *P. aeruginosa* (Baysse *et al.*, 2000). Indeed, it is known that, at neutral pH, V(IV) can be readily oxidized to V(V), generating the toxic superoxide anion (O₂⁻) (Liochev & Fridovich, 1987). Up to six mechanisms have been recognized by which bacteria can exert resistance against toxic metals, including metal exclusion by permeability barrier, intracellular or extracellular sequestration, detoxification and active transport of the metal out of the cell (reviewed by Nies, 1999; Bruins *et al.*, 2000). So far, no study has been done on the determinants that confer resistance to V. To our knowledge, only one study describes the isolation and characterization of natural isolates of *Escherichia*

Abbreviations: AHL, *N*-acyl-homoserine lactone; BHL, *N*-butanoyl-L-homoserine lactone; OdDHL, *N*-(3-oxododecanoyl)-L-homoserine lactone; Cm, chloramphenicol; Gm, gentamicin; Tc, tetracycline.

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristics*	Reference
Strains		
<i>Pseudomonas aeruginosa</i>		
PA 59.20	Wild-type, clinical isolate	Meyer <i>et al.</i> (1997)
PA 59.20 vs1	Tn5 mutant sensitive to 1.5 mM VOSO ₄	This study
PAO1	Wild-type	ATCC 15692; Meyer <i>et al.</i> (1997)
PAO1 <i>ncr</i>	Mutant with a Gm ^R cassette from pGM inserted in the non-coding region between PA4204 and PA4205 genes	This study
PAO1 <i>mexI</i>	Same as above but with the insertion in the PA4207 gene (<i>mexI</i>)	This study
PAO1 <i>opmD</i>	Same as above but with the insertion in the PA4208 gene (<i>opmD</i>)	This study
<i>Chromobacter violaceum</i> CV026	Mini-Tn5 mutant of ATCC 31532 deficient in AHL and violacein production	McClean <i>et al.</i> (1997)
<i>E. coli</i>		
DH5 α -MCR	Host for subcloning of plasmids [F ⁻ <i>lacZ</i> Δ M15 <i>recA1</i> <i>hsdR17</i> <i>supE44</i> Δ (<i>lacZYA</i> <i>argF</i>)]	BRL
Top10F'	F' [<i>lacI</i> ^q Tn10 (Tet ^R)] <i>mcrA</i> Δ (<i>mrr</i> - <i>hsdRMS</i> - <i>mcrBC</i>) Φ 80 <i>lacZ</i> Δ M15 Δ <i>lacX74</i> <i>recA1</i> <i>araD139</i> Δ (<i>ara-leu</i>) 7697 <i>galU</i> <i>galK</i> <i>rpsL</i> (Str ^R) <i>endA1</i> <i>nupG</i>	Invitrogen
GJ23	JC2692(pGJ28)(R64 <i>drd</i> 11); Km ^R Sm ^R Tc ^R	Van Haute <i>et al.</i> (1983)
S17-1	<i>thi-1 pro</i> <i>hsdR</i> ⁻ <i>hsdM</i> ⁺ <i>recA</i> ::RP4-2-Tcr::Mu; Km ^R	Simon <i>et al.</i> (1983)
Plasmids and constructs		
pGV4692 (miniTnphoA3)	Mini-TnphoA transposon with Gm cassette on plasmid pGV4692, a derivative of pUT; Ap ^R	de Lorenzo <i>et al.</i> (1990); Pattery <i>et al.</i> (1999)
pCR-2.1	TA cloning vector for PCR fragments; Ap ^R Km ^R	Invitrogen
pBBR-1-MCS	Wide-host range vector; Cm ^R <i>lacZ</i> α <i>mo</i> β	Kovach <i>et al.</i> (1994)
pBR325	<i>E. coli</i> cloning vector; Ap ^R Tc ^R Cm ^R	Bolivar (1978)
pGM	Gm cassette cloned into pBBR1-MCS	Kovach <i>et al.</i> (1994); this study
pRG930Cm	Derivative of the cosmid vector pRG930 with the Cm ^R cassette of pBR325; Cm ^R Sm ^R Sp ^R	Van den Eede <i>et al.</i> (1992)
pVS1	11 kb partial <i>Pst</i> I PAO1 genomic DNA fragment (4701616–4712538) cloned in pRG930Cm	This study
pVS2	2.6 kb <i>Bam</i> HI– <i>Sal</i> I fragment (4704443–4707075) from pVS1 subcloned in pRG930Cm	This study
pVS3	3.85 kb <i>Bam</i> HI– <i>Kpn</i> I fragment (4704443–4708308) from pVS1 subcloned in pRG930Cm	This study
pVS4	6.2 kb <i>Bam</i> HI– <i>Pst</i> I fragment (4704443–4710680) from pVS1 subcloned in pRG930Cm	This study

* Ap, ampicillin; Cm, chloramphenicol; Gm, gentamicin; Km, kanamycin; Sm, streptomycin; Sp, spectinomycin; Tc, tetracycline.

hermannii and *Enterobacter cloacae* that accumulate both Ni and V, indicating a possible metal sequestration mechanism (Hernández *et al.*, 1998). Resistance to V is probably multi-factorial, and a molecular approach is necessary to identify the determinants governing its expression. To achieve this, we chose a transposon mutagenesis approach that has been successfully used to detect *P. aeruginosa* and *Pseudomonas fluorescens* mutants impaired in their resistance against Zn and Cd (Hassan *et al.*, 1999; Rossbach *et al.*, 2000a, b).

In this study, we describe one *P. aeruginosa* mutant with an increased susceptibility to V, with an insertion upstream from a previously undescribed efflux pump, with similarity to Mex efflux systems from *P. aeruginosa*

known to contribute to antibiotic resistance. We also show that this new MexGHI-OpmD pump plays a role in the quorum sensing network of *P. aeruginosa*.

METHODS

Strains and transposon mutagenesis. *P. aeruginosa* strain PA 59.20, a type III pyoverdine-producing strain (Meyer *et al.*, 1997) was selected because of the higher stability of Tn5 insertions in this strain, compared to *P. aeruginosa* PAO1 (Cornelis *et al.*, 1992). For mutagenesis, a mini-TnphoA3 transposon construct was used (Pattery *et al.*, 1999). This transposon bears a resistance against gentamicin (Gm) on plasmid pGV4692 (Table 1). The procedures for the conjugational transfer of the transposon and the selection of

Table 2. Oligonucleotide primers used in this study

Primer name	Sequence (5'–3')	Place in the PAO1 genome
Gm1	TGGACCAGTTGCGTGAGCGCATA	
Gm2	TGTCAACTGGGTTCGTGCCTTC	
gent1	ATAAGAATGCGGCCGCACACCGTGGAACGGA	
gent2	ATAAGAATGCGGCCGCATCTCGGCTTGAACGA	
PhoA5	GCGGCAGTCTGATCACCCGTTA	
PhoA4	GCACCGCCGGGTGCAGTAATAT	
4204	AAGACTGCCCACCCCTCTG	4704143–4704161
4205	CACGGGCATGGAGGATCT	4706764–4706781
4205upf	AGTCCCCGAGGCGACGAACA	4706028–4706047
4205upr	AACCAGGATCCGCCACGT	4705533–4705551
4206f	AGAAACCCGTCCTGATCGCCA	4706413–4706433
4206r	ATCCCGTCGCTCAGGTTGATCT	4707430–4707450
4207f	CACCGAGACCGCGCACT	4708334–4708352
4207r	TGTACACGGCGGCCCTGGGT	4708851–4708870
MexI1	AGCGCGTCTCGGTGCGGA	4707437–4707455
MexI2	AAGGCGGCGTCGGAAAGT	4711072–4711090
MexI3	TGTACCGGCGGCCTGGGT	4709112–4709129
OpmD1	AGCGCTGGCACGACAGTTTCGT	4709807–4709827
OpmD2	ATCTCGTTGCGGAAACAACCCTG	4713259–4713282
OpmD3	CGATCTGCTGGTGAACAA	4710599–4710617
OpmD4	AAGTTCCGATTACAGGCCCT	4712082–4712101

transposon mutants were described by Cornelis *et al.* (1992). For the selection of mutants, Casamino acid medium (CAA), containing 100 µg Gm ml⁻¹, was used. The mutants were afterwards transferred to CAA plates containing 1.5 mM VOSO₄ for the detection of V-sensitive clones.

Identification of transposon insertions. Genomic DNA from V-sensitive mutants was isolated using the procedure described by Wilson (1990). After restriction with *Sma*I, the DNA was self-ligated under conditions that favour the formation of circular molecules. The ligated DNA was further used as template for a PCR amplification using primers Gm1 and PhoA5 (Table 2) for 30 cycles and the following parameters: 50 s at 94 °C, 30 s at 55 °C and 4 min at 72 °C using ExTakara polymerase (Biowhitaker).

After the first PCR reaction, a nested PCR reaction was performed using primers Gm2 and PhoA4 (Table 2). Fragments were further cloned in the pCR2.1 vector of the TA cloning kit (Invitrogen). *E. coli* DH5α and *E. coli* Top10F' (Invitrogen) (Table 1) were used as hosts for cloning.

DNA sequencing was performed by Eurogentec, using universal primers. Sequences obtained were compared to sequences present in the database of the *Pseudomonas* genome (<http://www.pseudomonas.com>) using the BLASTX algorithm.

Mutagenesis by allelic exchange. From *P. aeruginosa* strain PAO1 (Stover *et al.*, 2000) a 2.6 kb sequence between genes PA4204 and PA4205 was amplified by PCR using primers 4204 and 4205 (Table 2). This fragment was ligated to pCR2.1 using the TA cloning kit (Invitrogen). The recombinant plasmid was further restricted with *Sac*II and the ends polished by treatment with Klenow polymerase. The Gm cassette from the pBBR-1-MCS vector (Kovach *et al.*, 1994) was amplified with *Taq* polymerase (Gibco-BRL) using the primers gent1 and

gent2 (Table 2), and cloned into the pCR2.1 vector, resulting in plasmid pGM. The Gm cassette was removed from plasmid pGM by *Not*I restriction, the fragment was blunt-ended and then ligated to the *Sac*II-digested plasmid. After transformation, the resulting plasmid containing the Gm-interrupted DNA was isolated from a recombinant cloned and digested by *Hind*III and *Bcl*I to liberate the DNA fragment containing the Gm cassette. This fragment was in turn ligated to *Hind*III/*Bam*HI-restricted pBR325 DNA (Bolivar, 1978) before transformation of *E. coli* DH5α or S17-1. Transformed *E. coli* S17-1 was used to mobilize the suicide plasmid to *P. aeruginosa* PAO1 (Simon *et al.*, 1983). Selection for double recombination events was done by plating the recombinants on CAA plates containing Gm (100 µg ml⁻¹), and Gm plus chloramphenicol (Cm) (200 µg ml⁻¹), and selecting the clones that were sensitive to Cm. Confirmation of the double recombination event in these clones was done by PCR amplification using primers 4205upf and 4205upr (Table 2), since double recombination should result in the amplification of a unique band of 1.3 kb.

The *P. aeruginosa* PAO1 *opmD* gene (PA4208) was amplified by PCR using primers opmD1 and opmD2 (Table 2). The resulting fragment was ligated into vector pCR2.1 (Invitrogen).

After restriction with *Eco*N1, the Gm cassette was ligated into the *opmD* gene. The complete DNA fragment with the inserted Gm cassette was liberated by restriction with *Eco*RI and ligated to *Eco*RI-restricted pBR325. Similarly, the *mexI* gene (PA4207) was amplified using primers mexI1 and mexI2 (Table 2). After *Stu*I restriction, the Gm cassette was ligated into *mexI*. The complete fragment was liberated from pCR2.1 by restriction with *Hind*III and *Bcl*I, and ligated to *Hind*III/*Bam*HI-restricted pBR325. After transformation of *E. coli* GJ23, the plasmids were transferred by conjugation to *P. aeruginosa* PAO1. Transconjugants showing the loss of Cm

Fig. 1. (A) Organization of the *mexGH-opmD* cluster, corresponding to genes PA4205–PA4208 (<http://www.pseudomonas.com>). The place of the *TnphoA3* insertion in strain PA 59.20 is indicated by a black triangle, while the place of the insertion of the Gm cassette in strain PAO1 is indicated by an arrow. The hatched squares represent the *lux* boxes identified upstream of the cluster and in the intergenic region between *phzM* and *phzA* (Mavrodi *et al.*, 2001; Pattery *et al.*, 2001). The thick black lines below correspond to the inserts of PAO1 genomic DNA in pRG930 described in Table 1. Short arrows represent the primers used in the RT-PCR analysis. (B) Sequence of the non-coding region between gene PA4204 and *mexG*. The two underlined sequences correspond to putative *lux* boxes as defined by Whiteley & Greenberg (2001). The sequence CCGCGG in bold and italic type corresponds to the *SacI* restriction site used to insert the Gm cassette, to realize an allelic exchange in strain PAO1. The ATG in bold and italic corresponds to the predicted translation initiation codon of *mexG* (<http://www.pseudomonas.com>). The ATG in bold and the sequence GGAGG in bold, upstream of the ATG, correspond to the newly predicted initiation codon of *mexG* (this work). The place of the *TnphoA3* insertion in strain PA 59.20 is indicated by an arrow.

resistance ($200 \mu\text{g ml}^{-1}$) were selected and the double recombination confirmed by PCR using the above described primers.

RT-PCR analysis. RNA was extracted (High Pure RNA Isolation Kit; Roche Diagnostics) from *P. aeruginosa* PA 59.20 wild-type, PA 59.20 vs1, PAO1 wild-type and PAO1 *ncr* mutants grown in LB and harvested in the middle of the exponential phase. cDNA, synthesized using the First-Strand cDNA Synthesis Kit (Amersham Pharmacia) was done using two sets of primers: *mexI3* and *mexI1*, and *opmD3* and *opmD4* (Table 2). The relative position of the primers is shown in Fig. 1(A). As a control for RNA contamination by DNA, the PCR reaction was done on the same samples, without first strand cDNA synthesis.

Physiological studies. Growth parameters were measured using a Bioscreen Apparatus (Life Technologies), using the following parameters: shaking for 10 s every 3 min; reading every 20 min; temperature 37°C ; volume of the culture, $300 \mu\text{l}$. As inoculum, an overnight culture of PAO1 in CAA was diluted to achieve a final OD_{600} of 0.001. Each culture was realized in triplicate and each experiment was repeated three times.

Vanadyl oxydo sulfate ($\text{VOSO}_4 \cdot 5\text{H}_2\text{O}$) was prepared as a 100 mM stock solution and kept at 4°C . Growth curves were also followed in the presence of increasing concentrations (0.5, 1, 1.5, 2 and 2.5 mM) of different metal salts in CAA medium at 37°C : CoCl_2 , ZnSO_4 , NiCl_2 , CuSO_4 , CrCl_3 and $\text{Bi}(\text{NO}_3)_3$. CdCl_2 was used at 0.2 mM , final concentration.

Antibiotic susceptibility. Antimicrobial susceptibility tests were performed by the disk diffusion method on Mueller–Hinton agar plates. The antibiotics tested (Sanofi Diagnostic Pasteur) included amikacin ($30 \mu\text{g}$), ticarcillin-clavulanate ($75/10 \mu\text{g}$), tobramycin ($10 \mu\text{g}$), tetracycline (Tc; 30 UI), imipenem ($10 \mu\text{g}$), netilmicin ($5 \mu\text{g}$), ciprofloxacin ($5 \mu\text{g}$) and colistin ($10 \mu\text{g}$). Zones of inhibition were measured after incubation at 37°C for 24 h.

Production of elastase. Overnight LB-grown cultures were centrifuged and the supernatants collected. One-hundred microlitres of culture supernatant were added to glass test tubes containing 10 mg elastin Congo Red ml^{-1} in 0.9 M Tris/HCl. After 6 h incubation at 37°C , the tubes were centrifuged and the OD_{495} of the supernatants were measured (Rust *et al.*, 1994). All measurements were repeated three times.

Production of rhamnolipids. M9-glutamate minimal medium agar plates containing 0.2 g cetyltrimethylammoniumbromide and 5 mg methylene blue l⁻¹ were inoculated with 10 µl of an overnight LB culture of *P. aeruginosa* strains. After an overnight incubation at 37 °C, the diameter of the clearing zone around the bacterial spots was measured as evidence of rhamnolipid production (Siegmond & Wagner, 1991).

Swarming motility. Two microlitres from overnight agitated cultures in LB (5 ml) were spotted on LB plates containing 1.3 % agarose. The plates were incubated overnight at 37 °C and photographed. Spreading bacteria indicated swarming from the inoculation spot.

Measurement of siderophore and pyocyanine production. The pyoverdine content in the supernatant was determined by measuring A₄₀₀ or by spectrofluorimetry (excitation at 405 nm, emission at 460 nm, using a Shimadzu spectrofluorimeter) and normalized by a biomass unit expressed as OD₆₀₀ of the culture (Höfte *et al.*, 1993). Measurements are means of three independent experiments.

Pyocyanine production was visualized by plating the bacteria on Pseudomonas P agar (Difco), followed by 48 h incubation. Pyocyanine production caused the medium to be coloured deep blue. Pyocyanine was measured in culture supernatants according to Mavrodi *et al.* (2001).

Bio-assay for acyl-homoserine lactone (AHL) production. As indicator strain, a *Chromobacter violaceum* CV026 AHL-deficient mutant was used as described by McClean *et al.* (1997). TLC was used to separate the AHLs and overlaid with the CV026 indicator strain as described by McClean *et al.* (1997). Total culture supernatants from overnight-agitated cultures grown at 37 °C in LB were extracted with dichloromethane as described by McClean *et al.* (1997) to extract the AHLs.

Complementation in trans with wild-type DNA. A genomic bank of PAO1 DNA was constructed as described previously (Lim *et al.*, 1997) in the wide-host range cosmid pRG930Cm (van den Eede *et al.*, 1992), using *Pst*I to partially digest the genomic DNA. The bank was screened by colony blotting using the 2.6 kb PCR fragment amplified using primers 4204 and 4205 as probe. The Roche Diagnostics non-radioactive Dig detection system was used, in combination with the chemiluminescent substrate CPD-star (Roche Diagnostics). Positive clones were confirmed by PCR analysis using the following primers: opmD3 and opmD4, 4207f and 4207r, 4206f and 4206r, and 4205upf and 4205upr (Table 2).

RESULTS

Screening of the transposon mutant bank

After mutagenesis with mini-Tn*phoA*3, 3000 separate clones were screened by replica-plating on CAA medium and on CAA containing 1.5 mM VOSO₄, a concentration at which the wild-type PA 59.20 could grow. One mutant, vs1, could not grow in the presence of 1 mM VOSO₄. These results were confirmed in liquid medium (results not shown).

Localization of the place of transposon insertion in PA 59.20 vs1

DNA from *P. aeruginosa* PA 59.20 vs1 was isolated and the sequence of the DNA flanking the transposon insertion was determined as described in Methods. After

the nested PCR reaction, a fragment of about 600 bp was obtained, which was cloned in pCR2.1 and sequenced. The sequence analysis revealed 94% identity for a 171 bp sequence with a PAO1 sequence (<http://www.pseudomonas.com>) found between genes PA4204 and PA4205 (Fig. 1A, B). According to the PAO1 genome annotation, gene PA4205 encodes a hypothetical integral membrane protein with four predicted transmembrane helices. The predicted ATG start codon for this protein is at position 4705955, but is not preceded by a convincing ribosome-binding site (AAGGA, Fig. 1B). An alternative ATG start codon is present, 128 nt downstream, in the same frame, just after a ribosome-binding site closer to the consensus (GGAGG, Fig. 1B). From this new start codon, an ORF extends that encodes a protein of 105 aa containing three transmembrane domains (10–28, 35–55, 64–85) (Zhai & Saier, 2001). Gene PA4206 encodes a putative RND efflux protein of 370 residues (Tseng *et al.*, 1999), while gene PA4207 encodes a protein with highest similarity to *P. aeruginosa* MexF, a member of the family of membrane fusion proteins (Zgurskaya & Nikaido, 2000). The last protein, encoded by gene PA4208, is most similar to the *P. aeruginosa* efflux porin OprN (Köhler *et al.*, 1997). After this gene cluster, and transcribed in the opposite orientation, is gene PA4209 that has recently been shown to correspond to the *phzM* gene encoding an O-methyltransferase necessary for the biosynthesis of pyocyanine (Mavrodi *et al.*, 2001; Pattery *et al.*, 2001). This gene cluster, with the exception of gene PA4205, is very similar to the already described complete tripartite gene clusters that encode the MexABOprM, MexCDOprJ and MexEFOprN systems, which function in drug efflux in *P. aeruginosa* (Poole, 2001a, b).

Generation of new mutants by allelic exchange

Since the transposon was inserted in a non-coding region where its presence could eventually have positively influenced the transcription of downstream genes, it was decided to create a mutant in *P. aeruginosa* PAO1 with a Gm cassette insertion in the same locus, but downstream of the original Tn5 mutation. This was done by insertion of a Gm cassette into the unique *Sac*II site (Fig. 1B). The double recombinant PAO1 *ncr* mutant had exactly the same phenotype as the original vs1 mutant in strain PA 59.20. This mutant could not grow in CAA or in LB medium in the presence of 1.5 mM VOSO₄, and showed reduced growth in the presence of 1 mM VOSO₄ (Fig. 2).

The growth of the mutant in the presence of Co, Cr, Ni, Cd, Cu, Bi or Zn was unaffected (results not shown). Given the high similarity of this operon with similar operons encoding efflux systems, we decided to name the genes, *mexG-mexH-mexI-opmD* (the name *opmD* for this gene has already been given to this protein; see <http://www.cmdr.ubc.ca/bobh/OprMfamily.html>). Similarly, we obtained two other mutants, one with the Gm cassette inserted in the *mexI* gene, the other one with the insertion in the *opmD* gene. Both mutants were also sensitive to 1 mM VOSO₄ (Fig. 2). Mutant *opmD*

Fig. 2. Growth, in CAA plus 1 mM VOSO_4 , of wild-type *P. aeruginosa* PAO1 (●), mutant PAO1 *ncr* (○), mutant PAO1 *mexI* (△), mutant PAO1 *opmD* (□) and *opmD* complemented with *mexGHI-opmD* in trans (+). Growth was measured in the Bioscreen as described in Methods. Each growth curve is the mean of three separate experiments.

grew very slowly in liquid CAA medium and could not form isolated colonies on LB-agar plates (results not shown).

Complementation of the PAO1 *ncr*, *mexI* and *opmD* mutants

A PAO1 DNA genomic bank was constructed by cloning partially *Pst*I-digested DNA into the wide host-range cosmid vector pRG930Cm (van den Eede *et al.*, 1992; S. Matthijs, unpublished results). About 3500 colonies were screened by colony hybridization using as probe the 2.5 kb PCR fragment amplified with primers 4204 and 4205. One positive clone was selected and was confirmed by PCR amplifications with primers designed to amplify the *mexG*, *mexH*, *mexI* and *opmD* genes. Based on the results of these amplifications, we confirmed that the insert contained the genes PA4200–4209 (Table 1 and Fig. 1A). Subclones were obtained that contained only *mexG*, or *mexG-mexH*, or *mexG-mexH-mexI* (Table 1 and Fig. 1A). These subclones, as well as the original cosmid, were transferred by conjugation to PAO1 *ncr*, *mexI* or *opmD* and the transconjugants were tested for growth in the presence of VOSO_4 . From the results, it was clear that only recombinants containing *mexG-mexH-mexI-opmD* could grow in the presence of 1 mM V, indicating that the complete pump is needed to confer resistance to the metal (results shown only for *opmD* complemented with *mexG-mexH-mexI-opmD* in Fig. 2).

RT-PCR analysis of the *mexGHI-opmD* transcript

To confirm the operon organization of the cluster and to evaluate the effect of the two mutations in the intergenic region (the original transposon insertion in strain PA 59.20 and the PAO1 *ncr* mutation) on transcription efficiency, we did an RT-PCR analysis on mRNAs extracted from wild-type PA 59.20, PA 59.20 vs1, PAO1 and PAO1 *ncr*. Different primer combinations were

used (Fig. 1A). Transcripts were clearly less abundant in the PA 59.20 vs1 transposon mutant and in the PAO1 *ncr* mutant compared to the wild-type (see Fig. 3a for *mexI* primers, Fig. 3b for *opmD* primers). When amplifications were performed using the *opmD* primer combinations, almost no transcript could be amplified from the two mutant mRNA preparations.

Other phenotypes of the PAO *ncr*, *mexI* and *opmD* mutants

We first observed that the PAO *ncr* mutant produced less pyocyanine on P agar medium and in liquid medium (50% less compared to the wild-type; results not shown). Pyocyanine production is known to be under the control of the RhlR activator, together with the AHL cell-to-cell signal molecule C4-AHL, the production of which is in turn controlled by the 3-oxo-C12-AHL molecule, together with the activator LasR (Latifi *et al.*, 1996; Pesci & Iglewski, 1997). We therefore decided to compare the PAO1 wild-type, the *ncr*, *mexI* and *opmD* mutants and the complemented mutants for different quorum-sensing-regulated traits. These include the production of the LasB elastase, controlled by LasR, and RhlR rhamnolipids, controlled by RhlR (reviewed by de Kievit & Iglewski, 2000), pyoverdine, controlled by LasR (Stintzi *et al.*, 1998), and the capacity to swarm (Köhler *et al.*, 2000; Reimann *et al.*, 2002). Table 3 shows the results for the measurement of elastase activity, production of rhamnolipids and the siderophore pyoverdine. In the case of elastase, the *ncr*, *mexI* and *opmD* mutants showed a drastic reduction of activity, while the *ncr* and *opmD* mutants complemented by the *mexGHI-opmD* cluster displayed wild-type elastase activity. The production of rhamnolipids, as judged by the diameter of the clearing zone around the inoculated spot, was also reduced in the three mutants, but less severely in the case of the *ncr* mutant. Again in this case, complementation by the complete gene cluster restored wild-type levels of rhamnolipid production.

Fig. 3. RT-PCR analysis of abundance of transcripts in *P. aeruginosa* PA 59.20 and PA 59.20 vs1 mutant, and in PAO1 and PAO1 *ncr* mutant. (a) Amplification with primers *mexI1* and *mexI3*. Lanes: 1, PA 59.20 vs1; 2, PA 59.20 wild-type; 3, PAO1 *ncr*; 4, PAO1 wild-type; 5, markers. (b) Amplification with primers *opmD3* and *opmD4*. Lanes: 1, markers; 2, PA 59.20 vs1; 3, PA 59.20 wild-type; 4, PAO1 *ncr*; 5, PAO1 wild-type.

Finally, pyoverdine production also decreased to about 50% of the level of the wild-type in the case of the *mexI* and the *opmD* mutants, while the two complemented

Fig. 4. Swarming motility assay. Clockwise from the top right: PAO1, PAO1 *ncr*, PAO1 *mexI*, PAO1 *opmD* and PAO1 *opmD* complemented with *mexGHI-opmD*.

mutants produced as much pyoverdine as the wild-type. Fig. 4 shows that the three mutants have a much reduced swarming capacity, compared to the wild-type. This is particularly true for the *mexI* mutant, which does not swarm at all. Interestingly, we regularly observed that the *opmD* mutant produced sectors around the inoculation zone, an indication of the selection of phenotypic revertants. In this case, similar to what was observed for the other traits, the *ncr* mutant was the least affected. Complementation of the *opmD* mutant resulted in restoration of normal swarming behaviour.

Production of AHLs

Because of the down-regulation of several typical quorum-sensing-regulated traits, we decided to look for the production of signalling AHL molecules by wild-type and mutants without and with complementation. *C. violaceum* CV026 was used as a biosensor. This strain is unable to produce the violet pigment violacein because of a Tn5 insertion in the *cvil* gene, which encodes a homoserine lactone synthase (McClean *et al.*, 1997). In this strain, violacein production is inducible by

Table 3. Production of elastase, rhamnolipids and pyoverdine by wild-type PAO1, *ncr*, *mexI*, *opmD* mutants and complemented *ncr* and *opmD* mutants

Strain	Elastase*	Rhamnolipids†	Pyoverdine‡
PAO1	0.28 ± 0.03	1.6 ± 0.1	785 ± 9
<i>ncr</i>	0.03 ± 0.01	1.1 ± 0.1	564 ± 12
<i>mexI</i>	0.03 ± 0.01	0.7 ± 0.1	405 ± 3
<i>opmD</i>	0.03 ± 0.01	0.6 ± 0.1	386 ± 14
<i>ncr</i> (<i>mexGHI-opmD</i>)	0.30 ± 0.01	1.7 ± 0.1	793 ± 5
<i>opmD</i> (<i>mexGHI-opmD</i>)	0.34 ± 0.02	1.7 ± 0.1	751 ± 9

* OD measured at 495 nm (Rust *et al.*, 1994); mean of three replicates.

† Diameter of the clearing zone around the bacterial spots in cm.

‡ Excitation at 405 nm, emission at 460 nm, normalized by a biomass unit expressed as OD₆₀₀ of the culture (Höfte *et al.*, 1993).

Fig. 5. (a) Bioassay of violacein production by *C. violaceum* CV026 as an indication of the presence of short AHLs. Top: left, total AHLs from *P. aeruginosa* PAO1; middle, total AHLs from PAO1 *ncr*; right, total AHLs from PAO1 *mexI*. Bottom: left, total AHLs from PAO1 *opmD*; middle, total AHLs from PAO1 *ncr* complemented with *mexGHI-opmD*; right, total AHLs from PAO1 *opmD* complemented with *mexGHI-opmD*. (b) TLC separation of AHLs from PAO1 (lane 1), PAO1 *ncr* (lane 2), PAO1 *mexI* (lane 3), PAO1 *opmD* complemented with *mexGHI-opmD* (lane 4) and PAO1 *opmD* (lane 5). The TLC plates were dried and overlaid with *C. violaceum* CV026 as described by McClean *et al.* (1997).

Table 4. Resistance of PAO1 (WT) and PAO1 *ncr* to antibiotics

All data are given as the diameter of the inhibition zone in mm.

Antibiotic	WT	<i>ncr</i>	<i>ncr</i> (<i>mexGHI-opmD</i>)
Tobramycin	20	21	20
Amikacin	21	22	20
Imipenem	27	25	27
Colistin	15	16	15
Tetracycline	10	0	10
Netilmicin	21	14	13
Ticarcillin + clavulanic acid	21	12	0
Ciprofloxacin	32	31	29

AHLs with *N*-acyl side chains from C₄ to C₈ in length; on the other hand, the presence of C₁₀–C₁₄ AHLs inhibits the production of violacein when mixed with the stimulatory shorter chain AHLs (McClean *et al.*, 1997). As seen in Fig. 5(a), the production of violacein is stimulated by total AHLs from *P. aeruginosa*, but not by extracts from *mexI* or *opmD* mutants, while a faint pigment production can be visualized around the well

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mexG1  A A C T C G C C C C G G C A G G A G G T
mexG2  A C C T C A A C T T A A C T T G A G G T
qscI02 A A C T G C C C G G A A G G G C A G G T
rsaL   A A C T A G C A A A T G A G A T A G A T
lasI   A T C T A T C T C A T T T G C T A G T T
qscI17 C A C T G C C A G A T C T G G C A G T T
phzA   A C C T A C C A G A T C T T G T A G T T
lasB   A C C T G C C A G T T C T G G C A G G G
hcnA   A C C T A C C A G A A T T G G C A G G G

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Fig. 6. Comparison of the two putative *lux* boxes found upstream of *mexG* (*mexG1* and *mexG2*) and other promoter-specific elements found upstream of *P. aeruginosa* quorum-sensing-controlled genes (after Whiteley & Greenberg, 2001).

containing extracts from the *ncr* mutant. Extracts from *ncr* and *opmD* mutants complemented with *mexGHI-opmD* were in turn strongly stimulatory. Although little or non-stimulatory, extracts from *ncr* and *opmD* contain at least one faint AHL spot as detected by TLC and *C. violaceum* overlay (Fig. 5b). In the case of the *opmD* mutant, this AHL spot could not be detected. When the *opmD* mutant was complemented with the *mexGHI-opmD* genes, one strong AHL spot could be detected in TLC, corresponding to one of the two spots detected in PAO1 extracts.

Susceptibility of the PAO *ncr* mutant to antibiotics

Since the *mexGHI-opmD* gene cluster shows high similarity to described efflux pumps that are known to confer multiple drug resistance to *P. aeruginosa*, we decided to test the susceptibility of the *ncr* mutant to different antibiotics. Unexpectedly, we repeatedly found that this mutant is fully resistant to Tc while being less sensitive to netilmicin and ticarcillin plus clavulanic acid (Table 4). Interestingly, complementation with the full cluster restored sensitivity to Tc and complete resistance to ticarcillin plus clavulanic acid.

In silico analysis of the non-coding region upstream of the gene cluster

Analysis of the non-coding DNA between PA4204 and PA4205 allowed identification of two sequences with the NNCT-(N₁₂)-AGNN motif (Fig. 1B) that is conserved in so-called *lux* box elements that are generally found about –40 bp upstream of some *P. aeruginosa* quorum-sensing-regulated genes (Whiteley & Greenberg, 2001). Fig. 6 shows the comparison of the motifs upstream of *mexG* and other motifs described by Whiteley & Greenberg (2001).

DISCUSSION

Only two reports, so far, have established that V can be toxic for bacteria, in the case of *Streptococcus pneumoniae* (Fukuda & Yamase, 1997) and, by our group in the case of *P. aeruginosa* (Baysse *et al.*, 2000). As mentioned by Nies (1999) in his review on microbial heavy-metal resistance, nothing is known about the

mechanisms of resistance against vanadyl- or vanadate ions. In their study, Hernández *et al.* (1998) described that *E. hermannii* cells grown in the presence of V accumulate the metal and show the induction of a 45 kDa outer-membrane protein, which could be indicative of the induction of an efflux system porin. This study is a first attempt to elucidate the mechanisms of bacterial resistance against V. Using a transposon mutagenesis strategy, we isolated a mutant that showed an increased susceptibility to V. By choosing a mini-Tn ϕ oA translational fusion reporter transposon, we hoped to discover some in-frame periplasmic fusions that would respond to V, an approach that was used successfully to detect *lacZ* fusions responding to Zn and Cd (Rossbach *et al.*, 2000a, b). However, after screening of more than 3000 independent clones containing a transposon insertion, no clear and specific V-responding fusion was found. Yet, we succeeded in isolating one V-sensitive mutant that could not grow at a concentration of 1.5 mM VOSO₄. It was found to have an insertion in a non-coding region upstream of a gene cluster with a likely operon structure and bearing resemblance to known antibiotic efflux systems already described in *P. aeruginosa* (Poole, 2001b). It is known that active efflux plays an important role in the development of resistance against some toxic metals (Rosen, 1996; Nies, 1999). The best known examples are the plasmid-borne *czc*, *cnr* and *ncc* systems of *Ralstonia metallidurans* (Hassan *et al.*, 1999; Silver & Phung, 1996). In *P. aeruginosa*, similar metal efflux systems have been described for cadmium and zinc, and also identified in *P. fluorescens* (Hassan *et al.*, 1999; Rossbach *et al.*, 2000a, b). It is however the first time, to our knowledge, that an operon with clear similarity to antibiotic efflux determinants has been shown to be involved in resistance against a metal. It is interesting to note that the expression of the pump only contributes to resistance against V, and not to other metals. That the efflux pump itself is needed for V-resistance has been confirmed by our complementation experiments.

One of the first obvious phenotypes of our V-sensitive mutants was the clearly reduced production of the phenazine pigment pyocyanine. One possibility is that pyocyanine excretion is directly mediated by this pump. In this regard, it is worth noting that *phzM*, a gene recently demonstrated to be necessary for the biosynthesis of pyocyanine, is transcribed just downstream of *opmD*, in the opposite orientation (Mavrodi *et al.*, 2001; Pattery *et al.*, 2001). Another more likely explanation for the decreased pyocyanine production is that decreased extracellular release of AHLs by the pump mutants results in decreased transcription of the *phz* phenazine biosynthesis cluster. Indeed, we observed that different, well known, quorum-sensing-regulated traits were affected in our different *ncr*, *mexI* and *opmD* mutants, while the presence of the pump *in trans* restored the wild-type phenotype in all instances. *P. aeruginosa* is known to produce two major AHL signal molecules, one long-chain, N-(3-oxododecanoyl)-L-homoserine lactone (OdDHL), and one short-chain,

N-butanoyl-L-homoserine lactone (BHL). The *C. violaceum* assay is known to detect short-chains AHLs and, conversely, to be inhibited by long-chain homoserine lactones, including OdDHL (McClellan *et al.*, 1997). The fact that total AHL fractions from the *mexI* and *opmD* mutants failed to stimulate the production of violacein by *C. violaceum* may be due to an imbalance in the BHL/OdDHL ratio. If the proportion of long-chain AHLs is higher in these mutant extracts, an inhibition of the biosensor is likely to occur (McClellan *et al.*, 1997). Supporting this hypothesis is the fact that one stimulating AHL can be detected in the extracts of these mutants when separated first by TLC. This AHL spot is the same as one that is detected by TLC separation of wild-type PAO1 extracts and is likely to be BHL. However, only a more thorough analysis of AHL production in these mutants by HPLC will definitively answer this question. It is interesting to note that the *opmD* mutant is also affected in its growth in CAA medium. Its morphology is also different on LB plates since it does not form isolated colonies but grows in patches (results not shown). Also, this mutant aggregates in liquid cultures (results not shown). These observations indicate that OpmD is an important outer-membrane porin for *P. aeruginosa* and that it cannot be replaced by another member of the OprM porin family (<http://www.cmdr.ubc.ca/bobh/OprMfamily.html>). Recently, Köhler *et al.* (2001) showed that overproduction of the MexEF-OprN efflux pump results in decreased production of virulence factors dependent on RhlI-RhlR, including pyocyanine and rhamnolipids. Two groups, Evans *et al.* (1998) and Pearson *et al.* (1999), reached opposite conclusions concerning the influence of the MexAB-OprM pump in regard to quorum sensing. The first group found that strains overexpressing *mexAB-oprM* excrete less OdDHL while the opposite was observed by Pearson and co-workers. The difference between the two observations could be explained by the fact that Evans and co-workers worked with a strain that is a *nalB* mutant overexpressing *mexAB-oprM*, while Pearson and co-workers worked with a PAO1 strain that was not mutated for *nalB*. Köhler *et al.* (2001) propose that another quorum sensing molecule, 2-heptyl-3-hydroxy-4-quinolone, termed PQS (Pesci *et al.*, 1999; Holden *et al.*, 2000), could be the substrate of MexEF-OprN. PQS is indeed known to positively regulate the *rhl* system (McKnight *et al.*, 2000). Interestingly, Whiteley *et al.* (1999) previously identified the *mexGHI-opmD* gene cluster (called *qsc133*) as being regulated by quorum sensing, in response to both BHL and OdDHL. With this in mind, we looked for putative *lux* boxes in the intergenic region between genes PA4204 and PA4205. Interestingly, two sequences that match the consensus sequence NNCT-(N₁₂)-AGNN, as defined by Whiteley & Greenberg (2001), were found. Although no conclusions can be taken at this stage concerning their localization compared to the transcription start point, it is tempting to assume that one would be a site for binding of LasR while the other would be a sequence where RhlR would bind. In their analysis, Whiteley *et al.* (1999) did not find a putative *lux* box upstream of

qsc133, but they did not, at that time, take the PA4205 gene into account.

Curiously, the V-sensitive mutants did not display a decreased resistance to any of the antibiotics that we tested. Conversely, we observed an increased resistance towards Tc, ticarcillin plus clavulanic acid, and netilmicin. It has been shown that loss of *mexAB-oprM* expression correlates with an increased expression of *mexEF-oprN* and *mexCD-oprJ* (Li *et al.*, 2000). It is therefore possible that decreased expression of *mexGHI-opmD* could be compensated by increased expression of other pumps, such as *mexCD-oprJ* that is known to confer resistance to Tc, Cm and some cepheims (Poole *et al.*, 1996). The presence of the full *mexGHI-opmD* in *trans* results in increased resistance to ticarcillin and clavulanic acid. One possible explanation for this phenomenon is that clavulanic acid (itself a lactone) is excreted by the pump. This, however, will need experimental confirmation. It will therefore be necessary in the future to study the expression of *mexGHI-opmD* and its interplay with other efflux systems in *P. aeruginosa*.

While this paper was being reviewed, an article was published (Diggle *et al.*, 2002) where a Tn5 insertion in *mexI* is described as causing a reduction of a *lecA::lacZ* fusion expression. The *lecA* gene encodes a lectin that is regulated positively by the RhII–RhIR system (Winzer *et al.*, 2000).

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