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Two Efflux Systems Expressed Simultaneously in Multidrug-Resistant *Pseudomonas aeruginosa*

LILIAN PUMBWE AND LAURA J. V. PIDDOCK*

Antimicrobial Agents Research Group, Division of Immunity and Infection, University of Birmingham, Birmingham B15 2TT, United Kingdom

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Simultaneous overexpression of MexAB-OprM and MexEF-OprN was demonstrated for a multiply antibiotic-resistant clinical isolate of *Pseudomonas aeruginosa* (G49). G49 also had decreased expression of OprF. No mutations in *mexR* or its upstream promoter region, *mexT*, *oprM*, *oprF*, or *sigX* were revealed, suggesting regulation by a hitherto undescribed locus.

A posttherapy isolate of *Pseudomonas aeruginosa*, G49 selected during therapy in 1986 with enoxacin was multiply resistant to several unrelated classes of antibiotics, including fluoroquinolones, β-lactams, chloramphenicol, and tetracycline (16). G49 lacked OprF and had an altered lipopolysaccharide profile and a point mutation in *gyrA* conferring a substitution of threonine 83 to isoleucine (17, 18). However, these changes did not fully account for the multiple antibiotic resistance phenotype of G49.

OprF is the major outer membrane protein of *P. aeruginosa*; its function and possible role as a porin are still a subject of debate (21). In *Escherichia coli* lack of porin proteins has been associated with decreased antibiotic accumulation and antibiotic susceptibility (5). Antibiotic efflux is a major mechanism of antibiotic resistance in *P. aeruginosa* due to MexAB-OprM, MexCD-OprJ, MexEF-OprN, and possibly a fourth system, MexXY-? (*amRAB*) (6, 9, 18, 19, 24). Mutations in *mexR* (*nalB*) lead to overexpression of the MexAB-OprM system, causing resistance to β-lactams, quinolones, chloramphenicol, tetracycline, trimethoprim, and sulfamethoxazole (18, 19, 22). Mutations in *nfxB* lead to overexpression of the MexCD-OprJ, causing resistance to cefpirome, quinolones, chloramphenicol, erythromycin, and tetracycline (18, 19). Mutations in *nfxC* lead to overexpression of MexEF-OprN, usually with a concomitant decrease in OprD expression, and cause resistance to imipenem, quinolones, and tetracycline (9, 14, 19). Recently, Kohler et al. identified *mexT*, and not *nfxC*, as the regulator of the MexEF-OprN multidrug efflux system (personal communication). Unlike MexCD-OprJ and MexEF-OprN, MexAB-OprM is expressed in wild-type *P. aeruginosa* strains and contributes to intrinsic antibiotic resistance, and it may also be involved in cell-to-cell signalling (13, 15, 18, 19). MexXY has been reported to play a crucial role in the intrinsic resistance of *P. aeruginosa* to aminoglycoside antibiotics (1).

The aim of this study was to investigate the role of efflux and OprF in the multiple antibiotic resistance phenotype of *P. aeruginosa* strain G49.

The susceptibility of all strains to all antimicrobial agents, dyes, detergents, and disinfectants was determined as described previously (20, 23). In general, the posttherapy isolate G49 was four to eight times more resistant to multiple antibiotics than the pretherapy isolate (G48) and G1 (NCTC 10662). The exceptions were imipenem, erythromycin, and gentamicin, for which no difference between G49 and G48 was observed. The phenotype of G49 was similar to both a *nalB* and/or an *nfxC* mutant, being resistant to tetracycline, chloramphenicol, quinolones, and β-lactams (similar to a *nalB* mutant) and resistant to chloramphenicol and quinolones (similar to an *nfxC* mutant) (Table 1). Restoration of OprF in G49 (by plasmid pRW5) had no effect upon MICs, whereas wild-type *gyrA* increased the susceptibility of G49 to fluoroquinolone antibiotics twofold but had no effect on other antibiotic classes (20). Accumulation of one example of each class of antimicrobial—ciprofloxacin (10 μg/ml; 25.9 μM), [^{3}H]chloramphenicol (1.6 μg/ml; 5 μM), [^{3}H]tetracycline (2.4 μg/ml; 5 μM), and [^{4}C]cephalothin (100 μg/ml; 239 μM)—was measured as described previously (10, 12). Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was used at a final concentration of 500 μM. Clavulanic acid was added to a final concentration of 25 μg/ml (105.37 μM) for 15 min prior to the addition of cephalothin; this was sufficient to inactivate the low level of AmpC β-lactamase (17). Accumulation of ciprofloxacin was not affected by clavulanic acid (data not shown). G49 accumulated approximately half the concentration of ciprofloxacin, chloramphenicol, and tetracycline accumulated by G48 (Fig. 1). The addition of CCCP significantly increased the steady-state concentrations of ciprofloxacin, chloramphenicol, and tetracycline accumulated by G49 ($P < 0.05$; $n = 5$) to concentrations comparable, although not identical, to those of G48. In addition, after the addition of CCCP, the accumulation of ciprofloxacin, chloramphenicol, and tetracycline by G48 attained equilibrium by the end of the assay (5 min), whereas accumulation by G49 continued to increase and did not reach equilibrium. Despite the eightfold difference in the MICs of cephalothin for G48 and G49, there was little difference between the concentrations accumulated by both strains and no effect of CCCP on [^{14}C]cephalothin accumulation by G49 compared with that of G48 ($P > 0.05$; $n = 5$) (Fig. 1). Expression of OprF (pRW5) by G49 had no significant effect ($P > 0.05$; $n = 5$) upon the concentration of ciprofloxacin, chloramphenicol, and tetracycline accumulated by G49 (Fig. 2).

Western blotting (6) showed that G1, G48, and G49 all expressed OprM. The intensity of the G49 band was similar to that of the *nalB* mutant positive control, whereas G1 and G48 gave fainter bands. Northern blotting was performed with RNA (20 μg) extracted with Trizol (Gibco BRL) and as described in the Amersham Gene Images kit insert. Sample-to-sample RNA uniformity was determined by examining 16S rRNA expression in parallel. For quality control reverse tran-

* Corresponding author. Mailing address: Antimicrobial Agents Research Group, Division of Immunity and Infection, University of Birmingham, Birmingham B15 2TT, United Kingdom. Phone: 0121-414-6969. Fax: 0121-414-6966. E-mail: l.j.v.piddock@bham.ac.uk.

TABLE 1. Antibiotic susceptibilities of G1, G48, and G49 compared with efflux mutants

Class and compound	MIC (μg/ml) for:					
	G1	G48	G49	Control *nalB* mutant (K766)[a]	Control *nfxB* mutant (G385)[a]	Control *nfxC* mutant (KG4001)[b]
β-Lactams						
Aztreonam	4	4	8	16	4	4
Carbenicillin	16	16	64	64	16	16
Cefotaxime	0.25	0.25	4	4	0.25	0.25
Cefoperazone	2	2	32	32	2	2
Cefpirome	2	2	8	8	8	2
Ceftazidime	1	1	8	8	1	1
Cephalothin	4	4	32	32	4	4
Imipenem	0.5	0.5	0.5	0.5	0.5	4
Meropenem	0.5	0.5	2	2	0.5	0.5
Quinolones						
Ciprofloxacin	0.5	0.5	4	2	4	2
Clinafloxacin	0.25	0.25	2	1	ND[c]	ND
Enoxacin	0.5	0.5	4	4	4	2
Fleroxacin	4	4	32	16	16	8
Gatifloxacin	1	1	8	4	ND	ND
Grepafloxacin	1	1	4	4	ND	ND
Levofloxacin	1	1	4	4	ND	ND
Moxifloxacin	1	1	4	4	ND	ND
Nalidixic acid	128	128	>256	>256	>256	>256
Norfloxacin	0.25	0.25	8	4	2	2
Pefloxacin	0.5	0.5	4	4	4	2
Trovafloxacin	1	1	8	4	ND	ND
Other agents						
Chloramphenicol	4	4	64	64	64	128
Erythromycin	32	32	32	32	>256	32
Gentamicin	0.5	0.5	0.5	0.5	0.25	0.5
Sulfamethoxazole	32	32	64	64	32	32
Tetracycline	16	16	64	64	64	16
Trimethoprim	64	64	128	128	64	64

[a] Strain kindly donated by K. Poole.
[b] Strain kindly donated by N. Gotoh.
[c] ND, not determined.

scriptase PCR (QC-RT-PCR), total cellular RNA (5 μg) was reverse transcribed into cDNA as previously described (4). The banding intensities were determined using IPLab Spectrum software, which gave band intensities as peak values, or Adobe PhotoShop version 4.0 software, which gave band intensities as pixel area (means ± standard deviations). Northern blotting for *oprM* RNA with a 1,351-bp probe (nucleotides 29 to 1380) and QC-RT-PCR revealed that G49 had increased expression of this gene. A threefold increase in *oprM* mRNA expression compared to that for G48 was shown for G49 (12.34 fg/μg versus 4.11 fg/μg of total cellular RNA). DNA sequencing of 80% of *oprM* (nucleotides 38 to 1268) revealed no mutations. Southern blotting of chromosomal DNA digested with *Hin*dIII for *oprM* also revealed no altered gene copy numbers or rearrangements. G49 also expressed greater levels of *mexB* compared with G1 and G48, confirming increased expression of this pump. However, DNA sequencing of *mexR* and its upstream promoter region (35) revealed no mutation.

Western blotting (8) of G1, G48, G49, and an *nfxB* positive control with OprJ monoclonal antibody gave no band for G1, G48, or G49. However, G49 had a C-to-A transversion at bp 254 of *nfxB*, giving rise to the replacement of threonine by asparagine in NfxB. Western blotting (9) showed that G1 and G48 did not react with the OprN monoclonal antibody, whereas G49 reacted to a similar level as the OprN positive control. Northern blotting and QC-RT-PCR showed that G49 expressed *oprN* mRNA. However, no concomitant decrease in OprD expression was observed by Northern blotting, and the MIC of imipenem was similar to that for G48. DNA sequencing of 80% of *mexT* (nucleotides 5 to 1256) revealed no mutations. Southern blotting for *oprN* also revealed no altered gene copy numbers or arrangements.

Previous work in this laboratory revealed no OprF upon sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting (17, 20). Northern analysis of *oprF* expression by G49 gave a faint band, suggesting that G49 produced very small amounts of *oprF* mRNA. QC-RT-PCR showed at least an 80-fold reduction in *oprF* mRNA expression by G49 compared with G48. DNA sequencing of the entire *oprF* structural gene, its upstream region, and a putative extracytoplasmic function sigma factor (*sigX*) (3) revealed no mutation(s).

Efflux in multiple-antibiotic-resistant strains of *P. aeruginosa* has been reported to result from overexpression of one of three efflux systems, MexAB-OprM, MexCD-OprJ, or MexEF-OprN, and possibly the newly identified MexXY-?. The present study showed that G49 expresses two of these previously described efflux systems, MexAB-OprM and MexEF-OprN. However, unlike previous studies no mutation was detected in *oprM* or the regulatory gene *mexR* or its promoter region, suggesting that G49 is not a *nalB* mutant. Likewise, no mutation in *nfxC* or *mexT* was demonstrated. Confirmation that G49 was not an *nfxC* mutant is provided by no concomi-

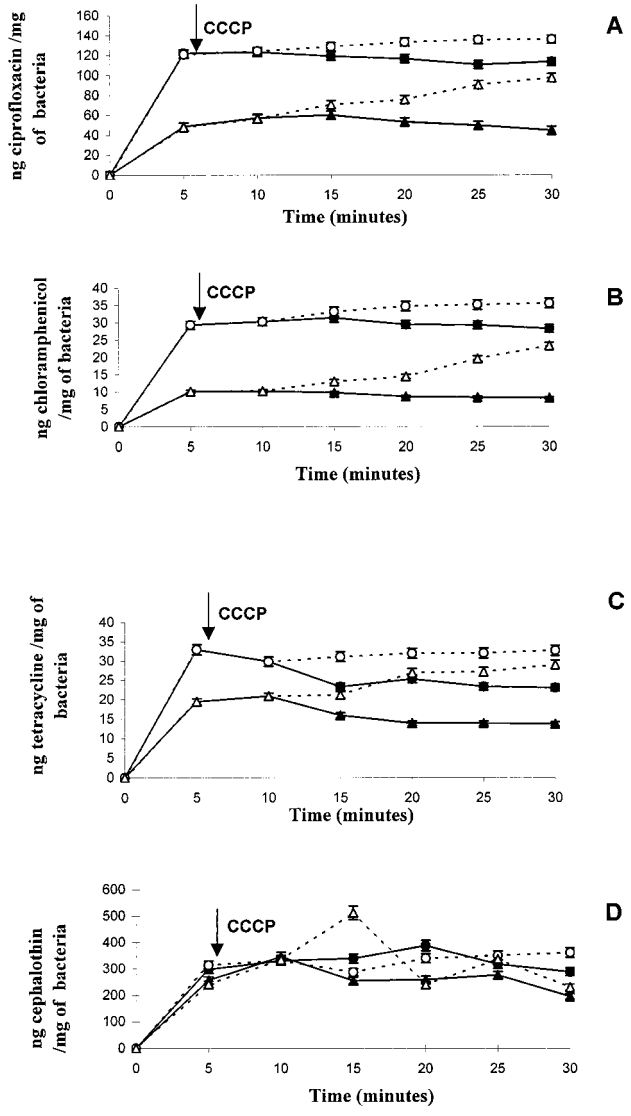

FIG. 1. Accumulation of antibiotics by G48 in the presence (○) and absence (●) of 500 μM CCCP compared with G49 with CCCP (△); ▲, no CCCP. (A) Ciprofloxacin; (B) chloramphenicol; (C) tetracycline; (D) cephalothin. Data points are the means ± standard deviations from five separate experiments.

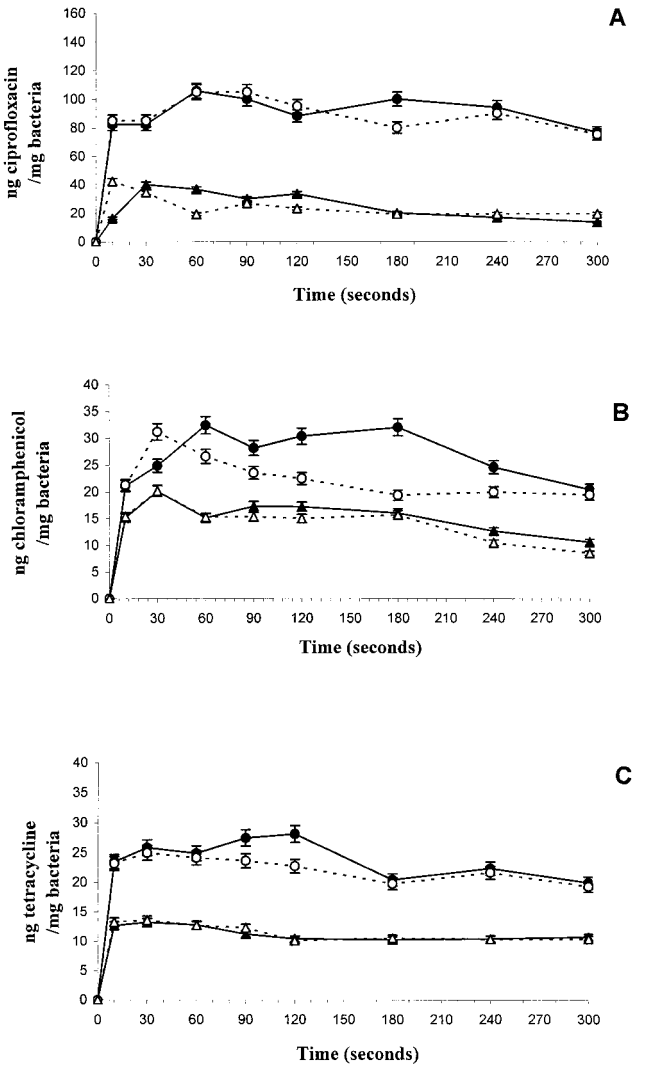

FIG. 2. Effect of OprF expression on antibiotic accumulation by G48 (●), G48 plus OprF (○), G49 (▲), and G49 plus OprF (△). (A) Ciprofloxacin; (B) chloramphenicol; (C) tetracycline. Data points are the means ± standard deviations from five separate experiments.

tant expression of *oprD* or imipenem resistance. To date, there is no evidence that OprN can function independently of MexE and MexF. However, two studies have shown that OprM can be expressed independently of MexAB and interplay with other pumps (25). However, increased expression of *mexB* was shown, confirming expression of the operon. These data clearly show that G49 coexpresses two efflux systems. Although G49 has virtually lost OprF, no mutations were detected in the *oprF* structural gene or its upstream region. In addition, a role for OprF in the multiple antibiotic resistance phenotype of G49 was difficult to identify, but this may be due to the simultaneous overexpression of two efflux systems, which counteracted any role of OprF. Gotoh et al. (7) have demonstrated coexpression of two Mex systems by laboratory mutants, but no clinical isolate of *P. aeruginosa* has been previously reported to concomitantly overexpress two Mex efflux systems and underexpress a major outer membrane protein, such as we observed with G49. Since targeting of efflux systems is an area of new drug development (11), this finding is both interesting and clinically important, as the phenotype of G49 makes antimicrobial therapy difficult. Although this study does not fully characterize the efflux system interactions in one strain, it provides new evidence that two (or even more) Mex efflux systems can be overexpressed and function together in one bacterium.

It is known that in *E. coli* mutations in the *marRAB* or *soxRS* loci can give rise to multiple antibiotic resistance due to enhanced expression of an efflux pump plus down-regulation of the outer membrane protein, OmpF (5). Since G49 had no mutations in the individual structural or regulatory genes of the Mex efflux pumps that could account for its phenotype, a mutation(s) at another locus, possibly a global regulator, analogous to MarA or SoxS may play a role. Data from early studies with G49 isolated a revertant, strain G49R2. This strain showed a concomitant restoration of antibiotic susceptibility and OprF expression and an increase in enoxacin accumulation (affected by CCCP) to levels similar to those of G48 (17).

These data suggest that the multiple phenotypic changes (altered expression of efflux systems and outer membrane proteins) were genetically linked.

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ERRATA

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LILIAN PUMBWE AND LAURA J. V. PIDDOCK

Antimicrobial Agents Research Group, Division of Immunity and Infection, University of Birmingham, Birmingham B15 2TT, United Kingdom

Volume 44, no. 10, p. 2861–2864, 2000. Page 2861, column 2, last line: "quality control" should read "quantitative competitive."

Letter to the Editor: Isolation of an SHV-12 β-Lactamase-Producing *Escherichia coli* Strain from a Dog with Recurrent Urinary Tract Infections

Volume 44, no. 12, p. 3483–3484, 2000. Page 3484: Names of authors should read as follows.

Tirushet Teshager
Lucas Domínguez
Miguel A. Moreno
Departamento de Patología Animal I (Sanidad Animal)
Facultad de Veterinaria
Universidad Complutense
Madrid, Spain

Yolanda Saénz
Myriam Zarazaga
Carmen Torres
Área de Bioquímica y Biología Molecular
Universidad de La Rioja
Logroño, Spain

Susana Cardeñosa
Servicio de Microbiología y Parasitología
Hospital Veterinario
Facultad de Veterinaria
Universidad Complutense
Madrid, Spain