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FEMS Microbiology Letters 143 (1996) 25–28

FEMS MICROBIOLOGY LETTERS

Role of *gyrA* mutation and loss of OprF in the multiple antibiotic resistance phenotype of *Pseudomonas aeruginosa* G49

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Received 6 April 1996; revised 17 June 1996; accepted 28 June 1996

Abstract

A clinical isolate of *Pseudomonas aeruginosa* G48, became resistant during fluoroquinolone treatment giving rise to the post-therapy isolate, G49. To determine whether mutation in *gyrA* gave rise to fluoroquinolone resistance, G49 was transformed with a plasmid encoding *gyrA* (pNJR3-2); this reduced the MIC of fluoroquinolones for G49 two-fold. DNA sequencing of *gyrA* of G49 demonstrated a mutation at Thr-83, substituting with isoleucine. The outer membrane of G49 was shown to lack OprF, suggesting that loss of this protein may be involved in the multiple antibiotic resistance phenotype; however, when G49 was transformed with a plasmid encoding *oprF* (pRW5), expression of *oprF* was shown to have no effect upon the phenotype.

Keywords: *gyrA*; OprF; *Pseudomonas aeruginosa*; Multiple antibiotic resistance

1. Introduction

Pseudomonas aeruginosa usually colonises individuals whose immune status is compromised by the nature of their disease or by prolonged chemotherapy [1,2]. In the past, the vast majority of isolates of *P. aeruginosa* were generally sensitive to most antibiotics, but with increasing use of these agents a predominant percentage of more resistant strains have been recognised [1]. A resistant isolate of *P. aeruginosa* (strain G49) emerged during enoxacin therapy and is multiply resistant to antibiotics such as β-lactams, fluoroquinolones, chloramphenicol and tetracycline [3]. G49 has several changes in its cell envelope compared to the isogenic pre-therapy isolate G48, in particular the complete loss of the outer membrane protein OprF [3]. In the present study, the role of mutation in *gyrA* to fluoroquinolone resistance, and of OprF in the multiple antibiotic resistance phenotype was determined.

2. Materials and methods

2.1. Bacterial strains and plasmids

P. aeruginosa strains G48 and G49 are a pair of enoxacin pre- and post-therapy isolates and have been previously described [3]. The control strain

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PII S0378-1097(96)00287-X

was *P. aeruginosa* NCTC 10662 (G1). Plasmid pUCP19 is a derivative of the cloning vector pUC19 containing a stability factor which allows maintenance of the plasmid within *Pseudomonas*. Plasmid pUCP19 3.6 is pUCP19 containing the cloned *P. aeruginosa* wild-type *gyrA* gene obtained from J. Diver [4]. Plasmid pRW5 is pUCP19 containing the cloned *oprF* gene [5]. All strains were maintained on infusion agar slopes and at −20°C on Protect™ beads, except for G49 which was stored at 4°C due to its outer membrane defect.

2.2. Transformation of P. aeruginosa G48 and G49 with pUCP19, pUCP19 3.6 and pRW5

Plasmids were prepared using the Qiagen plasmid midi kit. Competent cells of G48 and G49 were prepared and transformed with the plasmids by electroporation [6]. Transformants were selected on LB agar containing carbenicillin at 500–1000 μg ml^{-1}. The presence of plasmid in the transformants was verified by extracting plasmid DNA by the alkaline lysis method [7], restriction digestion with *Hin*dIII and agarose gel electrophoresis.

2.3. Antibiotics and determination of susceptibility

All antibiotics used in this study were supplied and used according to the manufacturers' instructions: carbenicillin, chloramphenicol, tetracycline, norfloxacin and nalidixic acid (Sigma); ciprofloxacin (Bayer); and enoxacin (Park-Davies Warner Lambert). The minimum inhibitory concentrations (MIC) of each agent for all strains and transformants were determined in Isosensitest broth (Unipath) using a microtitre tray doubling dilution method, final inocula of 10^5 CFU ml^{-1}, and incubation at 37°C for 18 h. The MIC of each antibiotic was defined as the lowest concentration at which no growth was observed.

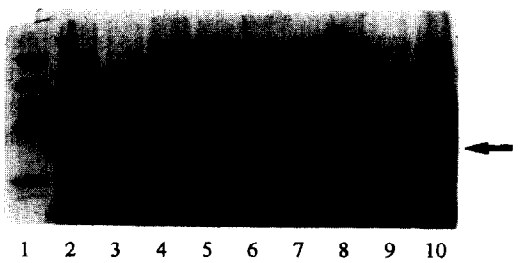

Fig. 1. SDS-polyacrylamide gel electrophoretogram of outer membrane proteins from G1 and pre- and post-transformed G48 and G49. Lanes 1 = molecular size marker, lane 2 = G1, lane 3 = G48, lane 4 = G49, lane 5 = G48 + pUCP19, lane 6 = G49 + pUCP19, lane 7 = G48 = pUCP19 3.6, lane 8 = G49 + pUCP19 3.6, lane 9 = G49 + pRW5, lane 10 = G48 + pRW5. The arrow indicates OprF.

2.4. Outer membrane protein analysis

Outer membranes were isolated from all strains and transformants as described previously [3]. Proteins were separated by electrophoresis on 14% SDS-PAGE gels and detected by Coomassie blue staining.

2.5. PCR and direct sequencing of oprF and gyrA

A 469 bp fragment of *gyrA*, covering the region analogous to the QRDR of *E. coli gyrA*, was amplified by the polymerase chain reaction (PCR) from *P. aeruginosa* G1, G48 and G49, using primers pagyrA1 (TGGGCAACGACTGGAACAAG) and pagyrA2 (ACTGCATCAGCTCATCGACG), as previously described [8]. PCR products were purified using Qiaquick spin columns (Qiagen) the nucleotide sequence determined directly using primer pagyrA1 and Sequenase 2.0 polymerase (US Biochemicals). Similarly, a 511 bp PCR product containing the 5′-end and upstream promoter region of *oprF* was generated using primers paoprF1 (CAGATGCGACCGAAACATAG) and 5′-biotinylated paoprF2 (CTGTCGCTGTTGATGTTGGT). PCR products were purified by binding to streptavidin-coated magnetic beads (Dynal) and the first 439 bp sequenced by solid-phase technology using primer paoprF1 and Sequenase 2.0. A 1052 bp PCR product encompassing the entire *oprF* gene was amplified using primers no. FP1 (TTAGGCGTTGTCATCGGCTCG) and no. 3 (TTACTTGGCTTCGGCTTCTAC).

3. Results

3.1. Contribution of mutation in gyrA to fluoroquinolone resistance

Sequencing of the amplified *gyrA* QRDR from

G48 and G49 revealed a point mutation in G49 at codon 83 (ACC to ATC), resulting in the putative substitution of threonine for isoleucine. To determine the contribution of this mutation to the resistance phenotype, G48 and G49 were transformed with plasmid pUCP19.3.6, containing the wild-type *P. aeruginosa gyrA* gene cloned into pUCP19 [4]. Transformation of G49 with pUCP19 3.6 resulted in a reproducible 2–4-fold decrease in the MICs of all 16 tested quinolones but did not alter the susceptibility to non-quinolone antibiotics (Table 1). However, the G49 transformant still required higher concentrations of fluoroquinolone for inhibition than G49, suggesting that mutation in *gyrA* may be only partially responsible for the quinolone resistance phenotype of this isolate. Transformation of G48 with pUCP19 3.6 or either strain with pUCP19 had no effect on the susceptibility of the transformants to any antibiotic tested.

3.2. *Contribution of loss of OprF to multiple antibiotic resistance*

Outer membrane profiles of G49 show several changes compared to G48, most noticeably complete loss of the outer membrane protein OprF [3]. To determine whether loss of this protein contributed to the multiple antibiotic resistance phenotype of G49, both G48 and G49 were transformed with plasmid pRW5, containing *oprF* cloned into pUCP19. Expression of *oprF* in the G49 transformants was verified by SDS-PAGE analysis of outer membrane extracts (Fig. 1), however, no changes were observed in the MICs of any of the antibiotics tested, suggesting that OprF is not involved in the multiple antibiotic resistance phenotype.

Loss of OprF in G49 was demonstrated not to be due to deletion of the structural gene *oprF* by amplification of a PCR product encompassing the entire gene. Sequencing of the promoter and first 400 nucleotides of *oprF* from G48 and G49 revealed no changes between the two strains.

4. Discussion

In the previous study conventional mechanisms of resistance to β-lactams and chloramphenicol, but not to fluoroquinolones, had been excluded [3]. In this study mutation in *gyrA* was shown to contribute, in part, to fluoroquinolone resistance, although complementation with wild-type *gyrA* did not restore susceptibility to pre-therapy levels. Therefore, another mechanism is required to account for the multiple antibiotic resistance in G49, including the non-*gyrA* mediated fluoroquinolone resistance. Despite the multiple antibiotic resistance phenotype there are several differences between G48 and G49, such as poor growth, brown pigmentation and outer membrane changes in the latter. Most noticeably, the outer membrane of G49 completely lacks OprF, and it was hypothesised whether this could be responsible for multiple antibiotic resistance. In a recent study, lack of OprF in a laboratory-generated multiple antibiotic resistant mutant was restored by transformation with cloned *oprF* [5] and expression nulled the multiple antibiotic resistance phenotype; however, in the present study, expression of OprF had no effect

Table 1
In vitro activity of carbenicillin, cefotaxime, chloramphenicol and quinolones on G1 and pre- and post-transformed G48 and G49 (μg ml^{-1})

Agent	Strain								
	G1	G48	G49	G48 pUCP19	G48 pUCP19 3.6	G48 pRW5	G49 pUCP19	G49 pUCP19 3.6	G49 pRW5
Carbenicillin	2	2	64	> 512	> 512	> 512	> 512	> 512	
Cefotaxime	0.25	0.25	4	0.25	0.25	0.25	4	4	4
Chloramphenicol	16	16	64	16	16	16	64	64	64
Tetracycline	16	16	32	16	16	16	32	32	32
Ciprofloxacin	1	1	4	1	1	1	4	4	2
Enoxacin	0.5	0.5	2	0.5	0.5	0.5	2	2	1
Nalidixic acid	8	8	32	8	8	8	32	32	16

upon the susceptibility of G49. The lack of expression of OprF in G49 is perplexing, as G49 contains an intact structural gene for this protein, including the promoter region. No evidence exists to suggest that *oprF* is under regulatory control; however, it is possible that G49 contains a mutation in a genetic locus analogous to the *marRAB* operon in *E.coli* [9] affecting expression of several unlinked genes, including those giving rise to multiple antibiotic resistance, and also expression of *oprF*. Current work on G49 is focussing upon the role of antibiotic efflux [10–13].

Acknowledgments

M.J.E. is a Lilly Industries Research Fellow, and R.E.W.H. receives support from the Canadian Cystic Fibrosis Foundation.

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