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A DELETION MUTATION IN *nfxB* OF *IN VITRO*-INDUCED MOXIFLOXACIN-RESISTANT *PSEUDOMONAS AERUGINOSA* CONFERS MULTIDRUG RESISTANCE

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The modulation of efflux pump functions under fluoroquinolone (FQ) exposure is of great concern as it could result in occurrence of multidrug-resistant (MDR) bacterial strains. In this study, MDR mechanism in *Pseudomonas aeruginosa* induced via moxifloxacin (MOX) pressure was investigated. After serial MOX [concentration of 0.5 × the minimum inhibitory concentration (MIC)] exposure, the fully susceptible *P. aeruginosa* ATCC 9027 strain has increased its MIC not only toward MOX (1→128 mg/L) but also to other antibiotics. Furthermore, this MOX-exposed strain did not revert to antibiotic-sensitive phenotype when being cultured in antibiotic-free medium for 12 days. No mutation was observed for FQ-target (*gyrA* and *parC*) or most investigated efflux regulatory genes (*mexT*, *mexR*, and *nalC*) except *nfxB* in which a 100-bp deletion was found. This associated with the elevated expression of multidrug efflux pump operon (*mexCD-oprJ*) which could directly result in MDR phenotype.

Keywords: moxifloxacin, multidrug resistance, *P. aeruginosa*, *nfxB*

Introduction

Pseudomonas aeruginosa is regarded as one of the major causes of nosocomial infections worldwide [1]. Treatment of *P. aeruginosa* infections has now become more challenging due to its remarkable propensity to rapidly acquire resistance to a wide range of antibacterial agents [2].

Fluoroquinolone (FQ) class of antimicrobial agents has been increasingly and extensively used for hospitalized as well as community patients since the late

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1980s due to its potent and broad-spectrum activity against a wide range of Gram-positive and Gram-negative pathogens [3]. In many countries, FQs are the only commonly available oral therapy to treat *P. aeruginosa* infections [4, 5]. FQ resistance can be considered as one of the consequences to widespread FQ consumption together with multidrug resistance (MDR), it has arisen at an alarming rate among *P. aeruginosa* [4].

The major FQ resistance mechanisms found in non-susceptible clinical isolates of *P. aeruginosa* are mutations in the quinolone resistance-determining regions of the genes encoding the FQ-target proteins (*gyrA* and *parC*), and over-expression of specific efflux pumps [6–8]. It has been additionally shown that efflux pump-mediated FQ resistance may also induce a broader, MDR phenotype [7, 8]. However, recent data have questioned the significance of the efflux pump activity in FQ-resistant *P. aeruginosa*. The aim of this study was to investigate the resistance mechanisms that are induced by FQs (and other antimicrobials) in *P. aeruginosa* via prolonged exposure to the FQ, moxifloxacin (MOX).

Materials and Methods

Bacterial strain and MOX exposure in P. aeruginosa

In this study, *P. aeruginosa* ATCC 9027 strain (susceptible to MOX, 1 mg/L) was used as starting strain. This strain was serially exposed to MOX concentrations corresponding to half of the minimum inhibitory concentration (MIC) in Mueller–Hinton Broth (MHB) [9]. Once *P. aeruginosa* ATCC 9027 reached a stable MOX-MIC equilibrium (day 12 of serial exposure), the MOX-resistant strain (designated as MOX-resistant *P. aeruginosa*) was sub-cultured repeatedly onto medium not supplemented with antimicrobials for an additional 12 days in an attempt to obtain a MOX-susceptible strain (designated as MOX-revertant *P. aeruginosa*). The MICs were measured daily. The derived MOX-resistant and MOX-revertant *P. aeruginosa* strains were re-identified as *P. aeruginosa* by standard bacterial identification methods and 16S rRNA sequencing.

Antimicrobial susceptibility testing

The susceptibility test of three bacterial strains (*P. aeruginosa* ATCC 9027, MOX-resistant *P. aeruginosa*, and MOX-revertant *P. aeruginosa*) was determined for nalidixic acid, norfloxacin, ciprofloxacin, levofloxacin, cephalexin, chloramphenicol, lincomycin, erythromycin, and tetracycline using the micro-dilution method in 96-well plates. All tests were performed in duplicate using MHB and

were incubated at 37 °C for 18–24 h. The results were interpreted in accordance with the EUCAST breakpoint tables [10].

Polymerase chain reaction (PCR) amplification and sequencing of FQ-target and efflux regulatory genes

To assess the antibiotic resistance mechanism of the induced, non-reversible, MOX-resistant phenotype, genomic DNA from wild-type *P. aeruginosa* ATCC 9027 and the two MOX-resistant strains (post-12 days' MOX sub-culture and post-12 days' non-supplemented sub-culture) was extracted. These DNA extractions were used as templates to amplify the genes encoding the FQ-target proteins (*gyrA* and *parC*) and efflux regulatory genes (*mexT*, *mexR*, *nalC*, and *nfxB*). Each of PCR reaction contained a volume of 50 μL using TopTaq Master Mix Kit (Qiagen, Hilden, Germany). The amplification conditions are as previously described [6, 8, 11, 12] and the primers are shown in Table I.

Amplification profile of *nfxB* was as follows: 4 min at 94 °C, followed by 30 cycles (1 min at 94 °C, 45s at 49.3 °C, and 2 min at 72 °C) and a final 5 min elongation at 72 °C. The PCR products were finally electrophoresed and subjected to sequencing. The *nfxB* sequence of *P. aeruginosa* ATCC 9027, MOX-resistant *P. aeruginosa*, and MOX-revertant *P. aeruginosa* in this study have been submitted to GenBank under accession nos. KR673324, KR673325, and KR673326.

Reverse transcription-PCR (RT-PCR) analysis

To test the effect of the deletion in the *nfxB* gene, total RNA extraction and cDNA synthesis were performed for *P. aeruginosa* ATCC 9027, MOX-resistant

Table I. List of primers used in this study for PCR and RT-PCR

Gene	Forward (5′→3′)	Reverse (5′→3′)	Reference
Primers for PCR			
gyrA	GTGTGCTTTATGCCATGAG	GGTTTCCTTTTCCAGGTC	[6]
parC	CATCGTCTACGCCATGAG	AGCAGCACCTCGGAATAG	[6]
mexT	GTAGTAGACGCTGGCCTCCAC	GTGAATTCGTCCCACTCGTTC	[7]
mexR	CATTAGGTTTACTCGGCCAAACC	CGCCAGTAAGCGGATACCTG	[11]
nalC	TCAACCCTAACGAGAAACGCT	TCCACCTCACCGAACTGC	[12]
nfxB	CGATCCTTCCTATTGCACGC	AGGGTGATGAACAGTTCGGT	This study
Primers for RT-PCR			
mexC	AGCCAGCAGGACTTCGATACC	ACGTCGGCGAACTGCAAC	[13]
rpsL	GCAACTATCAACCAGCTGGTG	GCTGTGCTCTTGCAGGTTGTG	[13]
oprJ	GTTCCGGGCCTGAATGCCGCTGC	TCGCGGCTGACCAGGGTCTGACG	[14]

P. aeruginosa strain, and MOX-revertant *P. aeruginosa* strain. RT-PCR was performed to determine the expression of *mexC* and *oprJ*. All of the reagents for these experiments were purchased from Qiagen. The amplification profile of RT-PCR included denaturation at 95 °C for 5 min and followed by 40-cycle steps, including denaturation for 30 s at 95 °C, annealing for 30 s at 55 °C, elongation for 30 s at 72 °C, and a final elongation at 72 °C for 10 min. *rpsL* was used as an internal control in all RT-PCR experiments [13]. The band intensities were quantified in relation to the *rpsL* band using ImageJ software (Wayne Rasband, National Institutes of Health, Bethesda, MD, USA).

Results and Discussion

Serial exposure of P. aeruginosa *to MOX*

In this study, over the period of sub-culture, there was a sequential increase in MIC against MOX. After 12 days, a MOX-resistant *P. aeruginosa* was derived and had plateaued at an MIC of 128 mg/L; wild-type MIC 1 mg/L (Figure 1). Notably, our attempt to derive a MOX-susceptible revertant strain was unsuccessful, as the MOX-resistant *P. aeruginosa* did not revert to a susceptible phenotype despite 12 days of sub-culture in non-supplemented growth media.

Susceptibility of P. aeruginosa *strains*

With regard to susceptibility tests, we observed a significant increase in MIC between the wild-type and the MOX-resistant isolate against all antimicrobials:

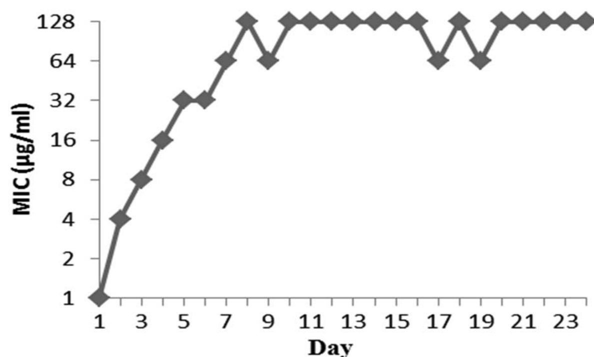

Figure 1. MOX exposure to *P. aeruginosa* ATCC 9027 strain. *P. aeruginosa* ATCC 9027 was exposed to MOX for 12 days to obtain MOX-resistant *P. aeruginosa*. This strain was continuously sub-cultured for another 12 days in antibiotic-free medium with daily examination of MIC (MOX-revertant strain)

nalidixic acid (16→128 mg/L), norfloxacin (0.125→16 and 8 mg/L for MOX-resistant and MOX-revertant strains), ciprofloxacin (0.0625→8 mg/L), levofloxacin (0.25→8 mg/L), cephalexin (2→16 mg/L), chloramphenicol (2→16 mg/L), lincomycin (1→4 mg/L), erythromycin (2→8 mg/L), and tetracycline (1→32 mg/L). Notably, there was no difference between the MICs in the primary MOX-resistant isolate and the secondary MOX-resistant isolate (MOX-revertant strain) after 12 days on non-selective growth media.

These data indicated that after being exposed to MOX, susceptible *P. aeruginosa* strain could turn into multidrug-resistant phenotype not only to other FQs but also to other antibiotics of unrelated groups.

Sequencing results of gyrA*,* parC*,* mexT*,* mexR*,* nalC*, and* nfxB

The most common and extensively studied mechanism of FQ resistance is the alteration of the FQ targets resulting from mutation in one or more of the genes encoding for gyrase and topoisomerase IV [15]. These enzymes are both tetrameric with pairs of two different sub-units: GyrA and GyrB for gyrase and ParC and ParE for topoisomerase IV. The genes encoding for these sub-units are chromosomally encoded and named *gyrA*, *gyrB*, *parC*, and *parE*, respectively. Until recently, the acquisition of FQ resistance in *P. aeruginosa* is known to be mainly due to the mutations in target genes, *gyrA* and *parC* [6]. However, after successful amplification and DNA sequencing of amplicons, we found that neither of the novel strains contained mutations in *gyrA* and *parC* in comparison with wild-type *P. aeruginosa* ATCC 9027.

The phenomenon of enhanced expression of endogenous efflux systems that restrict the accumulation of drugs inside bacterial cell is well described in *P. aeruginosa* [16]. It is common that organisms with enhanced efflux activity have an MDR phenotype [17]. In FQ-resistant *P. aeruginosa*, mutations are often found in regulators that can have a dramatic effect on drug efflux pump activity [7, 8]. To test this efflux pump activity hypothesis, we amplified the genes *mexT*, *mexR*, *nalC*, and *nfxB* in the three strains and sequenced the resulting amplicons. No modifications indicative of changed regulation in *mexT*, *mexR*, or *nalC* have been found.

Point mutations in *nfxB* were frequently found in clinical *P. aeruginosa* isolates and involved in the reduction of the susceptibility to FQs, especially for ciprofloxacin [18–20]. In this study, we identified a 100-bp deletion in *nfxB* genes in the MOX-resistant and MOX-revertant strains from nucleotide position 196→296 (GenBank accession no. KR673324). This seemed strongly related to a high level of MOX resistance as no significant mutations on principal genes involving in drug resistance were found. This finding supported Monti et al.'s [21]

study which showed that *nfxB* mutations including substitution, 1-bp deletion and insertion, >1-bp deletion, and duplication mutations were also linked to FQ (ciprofloxacin) resistance in *P. aeruginosa*.

Disruption of nfxB *resulting in elevated expression of multidrug efflux pumps*

nfxB is a repressor of *mexCD-oprJ* a multidrug efflux operon of *P. aeruginosa* [22, 23]. Loss of function mutations in *nfxB* is typically associated with an over-expression of *mexCD-oprJ* [9, 24]. However, occurrence of mexCD-oprJ-expressing mutants was very rare in a clinical setting [20, 25, 26]. To assess whether the 100 bp within *nfxB* was influencing *mexCD-oprJ* expression, we determined the expression of *mexC* and *oprJ* by RT-PCR. As predicted, the *nfxB* deletion in the MOX-resistant and MOX-revertant *P. aeruginosa* strains induced an increase in expression of both *mexC* and *oprJ* (Figure 2).

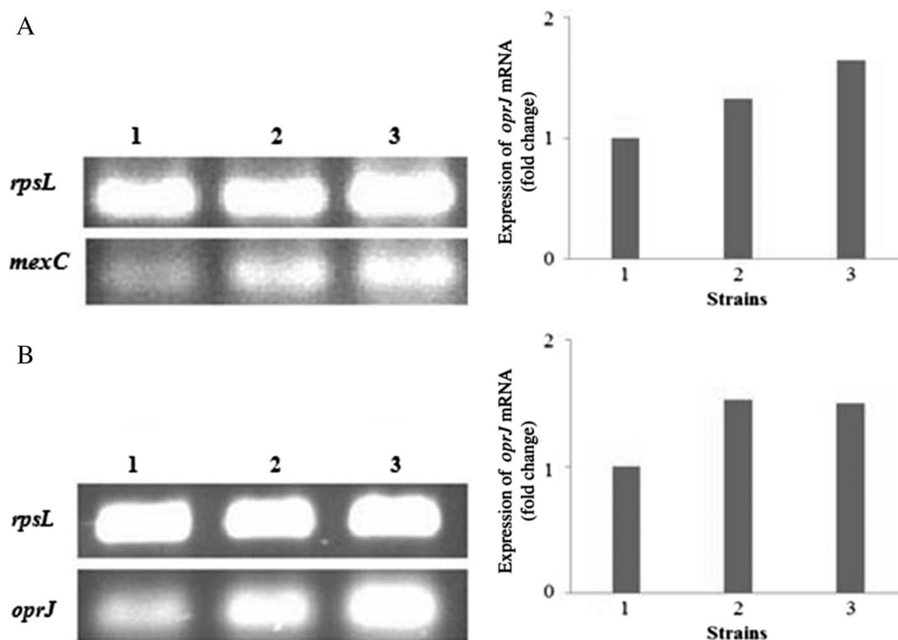

Figure 2. Comparison of *mexC* and *oprJ* expression between initial *P. aeruginosa* ATCC 9027 and MOX-exposed strains. Expression of *mexC* (A) and *oprJ* (B) was assessed in initial *P. aeruginosa* (1), MOX-resistant *P. aeruginosa* (2), and MOX-revertant *P. aeruginosa* (3). *rpsL* was used as internal control. The band intensities were quantified by ImageJ software

Conclusion

In conclusion, we report that disruption of *nfxB* gene plays an important role in the development of FQ resistance and an MDR phenotype in *P. aeruginosa*. In this study, *P. aeruginosa* can gain resistance to FQs and other antibiotics via mutations in intrinsic regulatory pathways. Furthermore, the type of gene regulation containing this disruption seems not reversible resulting in stable MDR phenotype. The mechanism underlying the resistance to antibiotics of other classes is currently unknown but could be of clinical significance in an era of diminishing antibiotic treatment options for *P. aeruginosa* infection.

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Conflict of Interest

The authors declare no conflict of interest.

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