J Antimicrob Chemother 2010
doi:10.1093/jac/dkq194
Advance publication 4 June 2010

Inactivation of *nfuA* enhances susceptibility of *Pseudomonas aeruginosa* to fluoroquinolone antibiotics

Jintana Daung-nkern[1], Paiboon Vattanaviboon[2–4] and Skorn Mongkolsuk[1,2,4,5]*

[1]*Department of Biotechnology, Faculty of Science, Mahidol University, Bangkok, Thailand;* [2]*Laboratory of Biotechnology, Chulabhorn Research Institute, Bangkok, Thailand;* [3]*Program in Applied Biological Science: Environmental Health, Chulabhorn Graduate Institute, Bangkok, Thailand;* [4]*Center of Excellence on Environmental Health, Toxicology and Management of Chemicals, Bangkok, Thailand;* [5]*Center for Emerging Bacterial Infections, Faculty of Science, Mahidol University, Bangkok, Thailand*

*Corresponding author. Laboratory of Biotechnology, Chulabhorn Research Institute, Lak Si, Bangkok 10210, Thailand. Tel: +662-574-0622, ext. 3816; Fax: +662-574-2027; E-mail: scsmk@mahidol.ac.th

Keywords: fluoroquinolones, iron-sulphur clusters, *nfuA*, *P. aeruginosa*

Sir,

Pseudomonas aeruginosa is a common cause of nosocomial infections. The presence of highly antibiotic-resistant strains has complicated the treatment of bacterial infections. During growth, *P. aeruginosa* generally encounters oxidative stress generated from the aerobic metabolism of phagocytic cells during infection and from the action of some antibiotics.[1] Genomewide screening of *P. aeruginosa* mutants has identified several genetic loci that alter antibiotic resistance levels.[2]

Iron-sulphur clusters ([Fe-S]) are important co-factors for a number of proteins with biologically relevant functions, mostly in various redox reactions. To date, three pathways have been identified for the assembly of [Fe-S] in bacteria: Isc (iron-sulphur cluster); Suf (sulphur formation); and Nif (nitrogen fixation) systems.[3] The BLASTP algorithm (http://blast.ncbi.nlm.nih.gov), and *Escherichia coli* Isc and Suf, and *Azotobacter vinelandii* Nif as the query sequences were used to search the *P. aeruginosa* (PAO1) genome[4] for [Fe-S] assembly systems. This unveiled the presence of a putative *isc* gene cluster, comprising *iscR* (PA3815), *iscS* (PA3814), *iscU* (PA3813) and *iscA* (PA3812) encoding a transcriptional regulator, cysteine desulphurase, U-type [Fe-S] scaffold protein and A-type [Fe-S] scaffold protein, respectively, as well as a *suf* gene cluster, composed of *sufS* (PA3667, cysteine desulphurase) and *sufE* (PA3668,[Fe-S] scaffold protein).[3] The deduced amino acid sequences of these putative genes share >40% identity with the query sequences.

In addition to the *isc* and *suf* pathways, PAO1 also possesses an open reading frame (PA1847) homologous to *nfuA*. NfuA represents a novel class of [Fe-S] scaffold protein involved in the chaperone and maturation of [Fe-S] proteins, such as aconitase.[5,6] The deduced amino acid sequence of PAO1 NfuA shares 82% and 52% identity with *A. vinelandii* and *E. coli* NfuA, respectively. PAO1 NfuA, a 194 amino acid protein, contains two functionally related domains: an A-type [Fe-S] synthesis domain at the N terminus; and a NifU-like domain for a U-type scaffold protein at the C terminus. The CXXC motif of the NifU domain, essential for the *in vitro* assembly of [Fe-S],[5] is conserved.

A PAO1 *ΔnfuA* mutant was constructed using an allelic exchange technique, with a *cre-lox* for antibiotic marker recycling as previously described.[7] Briefly, the 1 kb upstream region of *nfuA* was PCR amplified from PAO1 genomic DNA with BT3076 (5′-AGCGAACCTACTACGGGC-3′) and BT3077 (5′-GAATTCGGCCAG ATACGC-3′) primers. The product, cut with EcoRI, was cloned into pBluescript KS(−) (Stratagene), yielding pKSUp. The 1 kb downstream region of *nfuA* amplified with BT3096 (5′-AAG CTTCGGGATGGTCGA-3′) and BT3097 (5′-GACTGGCGATTTCCTCTG-3′) primers was cloned into pKSUp, forming pKSUpDown. Next, the *lox*-flanked gentamicin resistance (Gm^r) cassette from pCM351[7] was cloned into pKSUpDown digested with EcoRV–EcoRI, generating pKSUpGmDown. The recombinant plasmid was transferred into PAO1 and the *ΔnfuA* mutant was selected from carbenicillin-susceptible (Cb^s) and Gm^r phenotypes. Deletion of Gm^r from the *ΔnfuA* mutant was achieved through Cre-Lox recombination, as previously described.[7] The *ΔnfuA* mutant was confirmed by PCR with two primers flanking the deletion site. The *ΔnfuA* mutant showed no growth defect in rich media.

Since proteins containing [Fe-S] have been proposed as one of the key players in the hydroxyl radical-mediated cell death induced by fluoroquinolone antibiotics,[8,9] we tested whether *ΔnfuA* altered the level of antibiotic susceptibility. The results of the Kirby–Bauer disc diffusion assay of PAO1, the *ΔnfuA* mutant and the complemented strain (*ΔnfuA* mutant harbouring the pNfuA plasmid for ectopic expression of *nfuA*) illustrated that the *nfuA* mutant was more susceptible to fluoroquinolone antibiotics (ciprofloxacin and norfloxacin) than PAO1, as judged by wider growth inhibition zones (Figure 1). The enhanced susceptibility to antibiotics of the *nfuA* mutant was fully restored to the wild-type level by the expression of *nfuA* from the pNfuA plasmid (Figure 1). The MICs of ciprofloxacin and norfloxacin were determined using the broth dilution method with Mueller–Hinton medium. The *ΔnfuA* mutant showed reduced MICs (0.05 mg/L for ciprofloxacin and 0.5 mg/L for norfloxacin) compared with PAO1 (0.2 mg/L for ciprofloxacin and 2.0 mg/L for norfloxacin). Expression of *nfuA* from the pNfuA plasmid restored the reduced MIC values of the *ΔnfuA* mutant to PAO1 levels. The data confirmed that the altered susceptibility levels to ciprofloxacin and norfloxacin were due to the loss of *nfuA* function. Fluoroquinolone antibiotics exert their bactericidal activity by blocking the replication forks through binding DNA gyrases (topoisomerase II) and/or topoisomerase IV.[9] Recently, a new pathway has been proposed for gyrase-inhibiting antibiotics to modulate cell death via the generation of the hydroxyl radical.[1,8] The treatment of *E. coli* with norfloxacin causes an increased production of superoxide anions and results in an up-regulation of genes involved in superoxide stress response, the assembly of [Fe-S] and iron homeostasis, respectively.[8,9] Thus, gyrase inhibition by antibiotics promotes

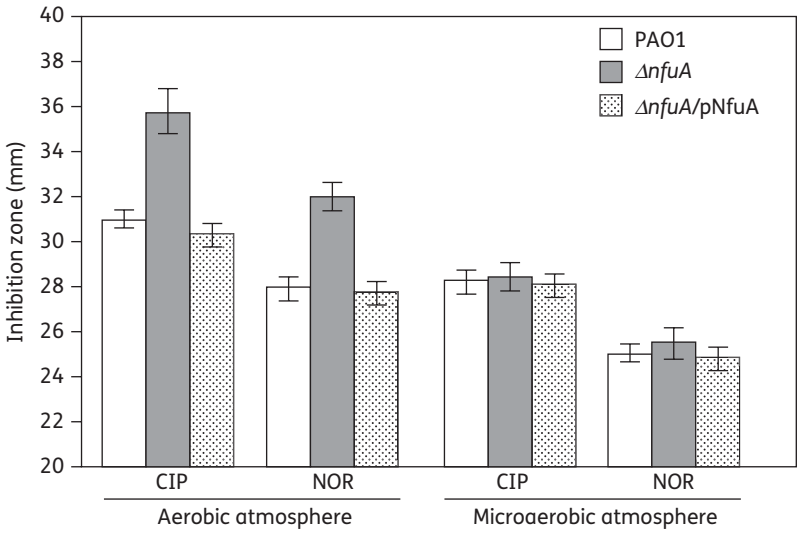

Figure 1. Susceptibility of *P. aeruginosa* strains to fluoroquinolone antibiotics. Susceptibility of the wild-type (PAO1), *ΔnfuA* mutant and complemented strain (*ΔnfuA*/pNfuA) to ciprofloxacin (CIP) and norfloxacin (NOR) was determined using the disc diffusion method. Plates were incubated in aerobic and microaerobic (5% O_2) atmospheres.

the production of superoxide anions that could attack [Fe-S] in proteins, causing a release of free ferrous ions (Fe^{2+}). Free cytoplasmic Fe^{2+} then rapidly reacts with intracellular H_2O_2 via a Fenton's reaction to generate the hydroxyl radical, which could cause cell death.[8,9]

We further investigated whether the increased susceptibility to fluoroquinolone antibiotics in the *ΔnfuA* mutant involved antibiotic-induced reactive oxygen species (ROS) by testing antibiotic susceptibility levels under microaerobic conditions. Ciprofloxacin treatment caused a 2-fold induction of *nfuA* expression.[10] We hypothesized that if the antibiotic killing of the bacteria involved the generation of an ROS-reduced overall oxygen level, then a condition known to reduce intracellular ROS levels should negate the phenotype. The results showed that under reduced oxygen atmospheric conditions, both the PAO1 and the *ΔnfuA* mutant displayed similar zones of growth inhibition against ciprofloxacin and norfloxacin antibiotics (Figure 1). The data suggest that *nfuA* may be involved in the oxidative stress protection pathway, which is responsible for the protection against ROS killing by fluoroquinolones, but further experimentation is required to elucidate the precise roles of NfuA. No significant changes in the drug susceptibility levels of the *ΔnfuA* mutant compared with PAO1 were observed with other antibiotic families, even in those antibiotic families (aminoglycoside and β-lactam) known to generate hydroxyl radicals,[1] suggesting that *nfuA* is not a part of the killing pathways of these antibiotics.

Funding

This work was supported by grants from the National Center for Genetic Engineering and Biotechnology (BT-B-01-PG-14-5112) and from Mahidol University. J. D. was supported by the 60th Year Supreme Reign of His Majesty King Bhumibol Adulyadej Scholarship from Mahidol University.

Transparency declarations

Parts of this work are from the thesis of J. D. submitted for the M.Sc. degree from Mahidol University. J. D., P. V. and S. M. have no financial conflicts of interest.

References

1 Kohanski MA, Dwyer DJ, Hayete B *et al*. A common mechanism of cellular death induced by bactericidal antibiotics. *Cell* 2007; **130**: 797–810.

2 Dotsch A, Becker T, Pommerenke C *et al*. Genomewide identification of genetic determinants of antimicrobial drug resistance in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* 2009; **53**: 2522–31.

3 Ayala-Castro C, Saini A, Outten FW. Fe-S cluster assembly pathways in bacteria. *Microbiol Mol Biol Rev* 2008; **72**: 110–25.

4 Stover CK, Pham XQ, Erwin AL *et al*. Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. *Nature* 2000; **406**: 959–64.

5 Bandyopadhyay S, Naik SG, O'Carroll IP *et al*. A proposed role for the *Azotobacter vinelandii* NfuA protein as an intermediate iron-sulfur cluster carrier. *J Biol Chem* 2008; **283**: 14092–9.

6 Angelini S, Gerez C, Ollagnier-de Choudens S *et al*. NfuA, a new factor required for maturing Fe/S proteins in *Escherichia coli* under oxidative stress and iron starvation conditions. *J Biol Chem* 2008; **283**: 14084–91.

7 Marx CJ, Lidstrom ME. Broad-host-range cre-lox system for antibiotic marker recycling in gram-negative bacteria. *Biotechniques* 2002; **33**: 1062–7.

8 Dwyer DJ, Kohanski MA, Hayete B *et al*. Gyrase inhibitors induce an oxidative damage cellular death pathway in *Escherichia coli*. *Mol Syst Biol* 2007; **3**: 91.

9 Dwyer DJ, Kohanski MA, Collins JJ. Role of reactive oxygen species in antibiotic action and resistance. *Curr Opin Microbiol* 2009; **12**: 482–9.

10 Cirz RT, O'Neill BM, Hammond JA *et al*. Defining the *Pseudomonas aeruginosa* SOS response and its role in the global response to the antibiotic ciprofloxacin. *J Bacteriol* 2006; **188**: 7101–10.