Available online at www.sciencedirect.com

ScienceDirect

www.elsevier.com/locate/jes

JES JOURNAL OF ENVIRONMENTAL SCIENCES

www.jesc.ac.cn

Low nutrient levels as drinking water conditions can reduce the fitness cost of efflux pump-mediated ciprofloxacin resistance in *Pseudomonas aeruginosa*

Wenfang Lin[1], *Kun Wan*[1,2], *Jie Zeng*[1,2], *Jingjing Li*[1], *Xi Li*[1,2], *Xin Yu*[1,*]

1. *Key Lab of Urban Environment and Health, Institute of Urban Environment, Chinese Academy of Sciences, Xiamen 361021, China*
2. *University of Chinese Academy of Sciences, Beijing 100049, China*

ARTICLE INFO

Article history:
Received 7 November 2018
Revised 26 March 2019
Accepted 26 March 2019
Available online 1 April 2019

Keywords:
Efflux pumps
Antibiotic resistance
Fitness cost
Low nutrient
nfxB mutation
Ciprofloxacin resistance

ABSTRACT

The long-term persistence of antibiotic resistance in the environment, especially in drinking water, is a public health concern. Expression of an efflux pump, an important mechanism of resistance to antibiotics, usually confers a fitness cost in bacteria. In this study, we aimed to determine why antibiotic resistance conferred by overexpression of an efflux pump persisted in low-nutrient environments (TOC < 10 mg/L) such as drinking and source water in which antibiotic selective pressure might be very low or even absent. Competition experiments between wild-type *Pseudomonas aeruginosa* and ciprofloxacin-resistant mutants revealed that the fitness cost of ciprofloxacin resistance significantly decreased ($p < 0.05$) under low-nutrient (0.5 mg/L total organic carbon (TOC)) relative to high-nutrient (500 mg/L TOC) conditions. Mechanisms underlying this fitness cost were analyzed. The *mexD* gene expression in resistant bacteria (cip_3 strain) was significantly lower ($p < 0.05$) in low-nutrient conditions, with 10 mg/L TOC ((8.01 ± 0.82)-fold), than in high-nutrient conditions, with 500 mg/L TOC ((48.89 ± 4.16)-fold). Moreover, *rpoS* gene expression in resistant bacteria ((1.36 ± 0.13)-fold) was significantly lower ($p < 0.05$) than that in the wild-type strain ((2.78 ± 0.29)-fold) under low-nutrient conditions (10 mg/L TOC), suggesting a growth advantage. Furthermore, the difference in metabolic activity between the two competing strains was significantly smaller ($p < 0.05$) in low-nutrient conditions (5 and 0.5 mg/L TOC). These results suggest that nutrient levels are a key factor in determining the persistence of antibiotic resistance conferred by efflux pumps in the natural environment with trace amounts or no antibiotics.

Introduction

Continuing imprudent use of antibiotics has enriched antibiotic-resistant bacteria in the environment, even in source water or drinking water (Lin et al., 2016; Pruden et al., 2006; Su et al., 2018; Xi et al., 2009). The spread of antibiotic-resistant pathogens has become a serious problem for human health around the world. Generally, bacterial antibiotic resistance is achieved through four main mechanisms (Baker-Austin et al., 2006): a reduction in outer membrane impermeability (Delcour, 2009), enzymatic inactivation (Wright, 2005), target alterations (Lambert, 2005), and active

* *Corresponding author. E-mail:* xyu@iue.ac.cn. (Xin Yu).

https://doi.org/10.1016/j.jes.2019.03.022

efflux of antibiotics (Webber and Piddock, 2003). Among these, rapid efflux of antibiotics from cells is thought to play a key role in the intrinsic resistance of clinically important bacteria, including *Pseudomonas aeruginosa*, *Escherichia coli*, and *Salmonella typhimurium* (Poole, 2001; Webber and Piddock, 2003). Besides, active efflux pump is an important mechanism for bacterial resistance to multiple antibiotics, such as quinolones, tetracyclines, chloramphenicol, etc. (Piddock and Laura, 2006; Terzi et al., 2014). To date, five families of bacterial efflux systems have been identified (Hernando-Amado et al., 2016; Webber and Piddock, 2003): the resistance-nodulation-division family (RND), major facilitator (MF) family, multidrug and toxic efflux (MATE) family, small multidrug resistance (SMR) family, and ATP-binding cassette (ABC) family.

Pseudomonas aeruginosa is a ubiquitous opportunistic human pathogen, and often resistant to multiple antibiotics, thus accounting for about 10% of acquired infections in hospital (Peng et al., 2017; Treepong et al., 2018). Frequently, it can also be detected in drinking water system, posing health risk to human health. Besides, *P. aeruginosa* is a commonly used model microorganism, and its molecular and metabolically mechanisms are extensively studied. Four multidrug efflux systems belonging to the RND family have been well characterized in *P. aeruginosa*, including MexAB-OprM, MexCD-OprJ, MexEF-OprN, and MexXY-OprM (Lister et al., 2009; Masuda et al., 2000; Purssell and Poole, 2013). Efflux pump genes are often part of an operon, with a regulatory gene that controls expression. For example, MexCD-OprJ has been shown to confer resistance to fluoroquinolones such as ciprofloxacin. *NfxB* is a transcriptional regulator that inhibits the expression of *MexCD-OprJ* in wild-type strains. Overexpression of efflux pumps can result from mutations in repressor genes. In resistant *P. aeruginosa*, a mutation in *nfxB* leads to derepression of *MexCD-OprJ*, causing high levels of ciprofloxacin resistance (Stickland et al., 2010).

It is generally accepted that the acquisition of antibiotic resistance imposes fitness cost on bacteria (Andersson and Hughes, 2010). Resistance acquired through mutation of elements with important physiological roles imposes a metabolism burden on bacteria. Consequently, wild-type bacteria might outcompete resistant bacteria in the absence of antibiotic selective pressure. For example, *nfxB* mutants have only rarely been detected in a clinical setting since they were first described by Hirai three decades ago (Hirai et al., 1987). An attractive hypothesis is that these bacteria were avirulent because of impaired fitness. Additionally, some previous studies have suggested that *nfxB* mutations in *P. aeruginosa* impair bacterial growth, all forms of motility (swimming, swarming, and twitching), and metabolic products such as siderophores, rhamnolipid, secreted protease, and pyocyanin, or that these mutations have led to specific changes in bacterial physiology (Jeannot et al., 2008; Olivares et al., 2012; Stickland et al., 2010).

The fitness cost of antibiotic resistance is strongly dependent on experimental conditions. For example, some resistance mutations have shown no cost in laboratory medium but a high cost in mice (Björkman et al., 2000). Additionally, environmental factors such as temperature and resource availability affect the fitness cost of rifampicin resistance mutations (Gifford et al., 2016). However, few studies have examined the contributions of efflux pumps to the fitness cost of bacterial antibiotic resistance. Overexpression of efflux pumps is known to consume a lot of energy, which may present a general burden to bacteria in the absence of antibiotics. In addition, bacteria utilize carbon compounds as an important energy source. Thus, we hypothesized that the availability of a carbon source would determine the fitness cost of antibiotic resistance conferred by overexpression of efflux pump in low-nutrient environments such as drinking water (the total organic carbon (TOC) < 5 mg/L) (GB/T5749-2006) or source water (TOC is 2–10 mg/L) (Visco et al., 2005). That is to say, antibiotic resistant bacteria generally grow under such oligotrophic condition (TOC < 10 mg/L), which may influence the persistence of antibiotic resistance.

In this study, the important opportunistic pathogen and extensively used model microorganism *P. aeruginosa* and its *MexCD-OprJ* efflux pump were focused. The effect of the environmental availability of nutrients (as low as 0.5 and 0.05 mg/L TOC) on the fitness cost of bacterial antibiotic resistance conferred by overexpression of efflux pumps was investigated. We aimed to identify whether *MexCD-OprJ* overexpression imposed a fitness cost on *P. aeruginosa* in different nutrient levels and to analyze the mechanisms underlying fitness cost.

1. Materials and methods

1.1. Strains and growth conditions

Bacterial strains used in this study are described in Table 1. Ciprofloxacin resistance in *P. aeruginosa* PAO1 were induced from the wild-type one by the mutagenic disinfection byproduct dichloroacetonitrile (DCAN) (Lv et al., 2014). Strain cip_1 had a mutation in the *nfx*B gene (L83P). In addition, an insertion mutation at nt 32 occurred in the cip_2 *nfx*B gene. Moreover, a mutation (L14Q) was identified in the *nfx*B gene of the cip_3 strain. These mutants showed increased resistance to ciprofloxacin. The minimal inhibitory concentration (MIC) was determined by broth microdilution method using Luria–Bertani (LB) broth (Reynolds et al., 2003). Briefly, the inoculums were 10^5 CFU/mL of overnight bacterial suspension. Two-fold serial dilutions of compounds were prepared in 96-well plates starting from a stock solution of ciprofloxacin (LKT Laboratories, USA). The MIC of these mutants for ciprofloxacin was 2 μg/mL, whereas the MIC in wild-type *P. aeruginosa* PAO1 was 0.125 μg/mL. Ciprofloxacin resistant strains were grown

Table 1 – Bacterial strains used in this study.

Strain	Mutation in *nfxB*	Amino acid change	Antibiotic resistance	MIC[a] (μg/mL)
cip_1	CTG → CCG	L83P	Ciprofloxacin	2
cip_2	32-nt insert A	–[b]	Ciprofloxacin	2
cip_3	CTG → CAG	L14Q	Ciprofloxacin	2
Wild-type			–[c]	0.125

[a] MIC: minimal inhibitory concentration.
[b] Frameshift mutation.
[c] Antibiotic sensitive strain.

routinely in LB broth, with shaking at 200 r/min, 37°C, and 0.5× MIC. The wild-type ones were cultured in the same condition, but without ciprofloxacin.

1.2. Fitness cost measurement

Fitness costs were determined by competition experiments between ciprofloxacin-resistant mutant and wild-type strains (Sander et al., 2002; Song et al., 2014). Briefly, overnight cultures of resistant and wild-type cells were washed twice with sterile saline solution. Resistant cultures were mixed with wild-type cultures at a ratio of 1:1 (V/V), and 1.5 μL of this mixture was used to inoculate 15 mL of fresh artificial synthetic wastewater (SW) medium (Zhang et al., 2011). The SW medium contained per liter of deionized water: 191 mg NH_4Cl, 56 mg K_2HPO_4, 14 mg $MgSO_4{\cdot}7H_2O$, 11 mg $FeSO_4{\cdot}7H_2O$, 17 mg $CaCl_2{\cdot}H_2O$, and 1 mL trace element solution. The composition of trace element solution was: 1500 mg $FeCl_3{\cdot}6H_2O$, 30 mg $CuSO_4{\cdot}5H_2O$, 30 mg KI, 120 mg $MnCl_2{\cdot}4H_2O$, 60 mg $Na_2MoO_4{\cdot}2H_2O$, 120 mg $ZnSO_4{\cdot}7H_2O$, and 150 mg $CoCl_2{\cdot}6H_2O$ per liter. Filter-sterilized glucose was added to a final concentration of 1.25 g per liter, which was used as the sole carbon source in the SW medium for bacterial growth. The total organic carbon (TOC) of SW medium was determined by TOC analyzer (Shimadzu TOC-V, Japan). Ten-fold dilutions of the SW medium to obtain different nutrient levels: 500, 50, 5, 0.5, and 0.05 mg/L TOC. Thus, the bacteria were cultured under different nutrient conditions in the absence of antibiotics at a starting cell density of 10^5 CFU/mL. Additionally, the SW medium was treated by pasteurization at 70°C for 30 min to avoid potentially damaging components in the medium. Preliminary data revealed that *P. aeruginosa* PAO1 reached the stationary phase after 12 hr in SW medium. Therefore, every 12 hr, 1.5 mL of the cultures were inoculated into 15 mL of fresh SW medium for growth (Olivares et al., 2012).

To determine the number of viable cells, the cultures were serially diluted by 1:10 (V/V) in saline solution, and suitable dilutions were plated every 12 hr on antibiotic-free LB agar to count the total number of colonies. In parallel, plates containing antibiotics (1 mg/L ciprofloxacin) allowed the growth of mutant strains. The number of wild-type cells was calculated as the total number of bacterial cells minus the number of antibiotic-resistant cells. Competition experiments were performed in triplicate. One mutant (cip_3) was selected for application in the following mechanistic analysis.

1.3. Mechanistic analysis

1.3.1. Quantitation of mexD gene expression by Reverse transcription quantitative real-time PCR (RT-qPCR)

To investigate the effect of an efflux pump on the fitness of a resistance mutant under different nutrient conditions, *mexD* gene expression was quantified by RT-qPCR. The Cip_3 strain was grown in LB broth containing ciprofloxacin antibiotic (0.5 × MIC) overnight at 37°C. Afterwards, the cultures were washed twice with saline solution and then re-suspended in 1 mL of the same buffer (optical density at 600 nm, 0.3; ~1 × 10^9 CFU/mL). A 20-μL aliquot of the pre-culture was inoculated into 200 mL of fresh SW medium containing 500 and 10 mg/L TOC (initial cell density, ~10^5 CFU/mL), and shaken at 37°C. Simultaneously, *mexD* gene expression in wild-type *P. aeruginosa* PAO1 was quantitated as a control.

The cells were harvested in the stationary phase (12 hr) by centrifugation (7800 r/min for 20 min). Total RNA was extracted from cell pellets using an RNA isolation kit (TransGene, China) according to the manufacture. After that, RNA was converted into cDNA using a cDNA synthesis kit (TransGene, China) according to the manufacturer's instructions to avoid RNA degradation. Primers used in this study are shown in Appendix A Table S1. Quantitative RT-qPCR of the cDNA was performed on an ABI 7300 detection system (Applied Biosystems, USA) in 20-μL reaction mixtures containing 100 ng isolated RNA, 200 nmol/L each of the two primers, and 10 μL 2× SYBR green PCR mixture. After an initial 2-min incubation at 95°C, the reaction was subjected to 35 cycles of 95°C for 40 sec, 60°C for 30 sec, and 72°C for 40 sec, and then a final 7-min incubation at 72°C. A constitutively expressed gene (16S rRNA) was used as a control to normalize the results, and the amount of each RNA was calculated following the $2^{-\triangle\triangle Ct}$ method (Livak and Schmittgen, 2001). All the experiments were performed in three independent replicates.

1.3.2. Quantitation of rpoS gene expression by RT-qPCR

To quantify *rpoS* gene expression, the bacterial strain (cip_3) was grown to the early stationary phase (9 hr) in SW medium supplemented with 500 and 10 mg/L TOC and then harvested by centrifugation. Total RNA extraction and RT-qPCR were performed as described in Section 1.3.1.

1.3.3. Specific oxygen uptake rate (SOUR) measurement

SOUR was measured to evaluate differences in metabolic activity between resistant and wild-type cells under different nutrient conditions. One mutant (cip_3) was selected as an example. The bacteria were cultured in LB broth at 37°C overnight and washed twice with saline solution by centrifugation at 7800 r/min for 10 min. The SW media containing 500, 5, and 0.5 mg/L TOC was aerated until it was saturated with dissolved oxygen (DO), and then the cell pellets were resuspended. The cell density was approximately 10^{10} CFU/mL, and the oxygen concentration decreased with respiration of the bacteria. DO concentrations were recorded using a Multi 3420 DO meter (WTW Weilheim, Germany). The slope in the DO concentration versus time was used to obtain SOUR values. All SOUR assays were conducted in triplicate.

1.4. Data analysis

1.4.1. Determination of bacterial fitness

The fitness cost of an efflux pump in a resistance mutant was evaluated by determining two values (Lin et al., 2018): the ratios (u) of the competing strains and their fitness at time t (fit_t). u is a denary logarithmic transformation of the ratios of the numbers of ciprofloxacin-resistant and wild-type cells at time t. To eliminate the effect of initial cell density, ratios of

the cells numbers at time t were normalized to that at time t_0 as Eq. (1):

$$u = \log 10\left(\frac{Nr_t/(Nv_t-Nr_t)}{Nr_0/(Nv_0-Nr_0)}\right) \quad (1)$$

where, Nr_t and Nr_0 represent the absolute number of ciprofloxacin-resistant cells at time t and time zero (t_0), Nv_t and Nv_0 represent the total bacterial cells including the ciprofloxacin-resistant and wild-type cells at time t and t_0. Therefore, $(Nv_t - Nr_t)$ and $(Nv_0 - Nr_0)$ denote the absolute number of wild-type cells at time t and t_0, respectively. u is equal to 0 if there is no difference in fitness cost between the competing strains, u is positive if resistance reduces the bacterial fitness cost, and u is negative if resistance increases the bacterial fitness cost.

In addition, the bacterial fitness (fit_t) of the two competing strains was calculated from the quotient of the growth rates of the competing strains at time t and the preceding time point $t - 1$. This quotient was standardized with the exponent $1/n$. n is the number of generations of bacterial growth from $t - 1$ to t (Eq. (2)). fit_t is 1 plus the natural logarithmic transformation of the quotient (Eq. (3)) using the following function (Sander et al., 2002):

$$n = \log 2\frac{N_t}{N_{t-1}} \quad (2)$$

$$fit_t = 1 + \ln\left[\left(\frac{Nr_t/(Nv_t-Nr_t)}{Nr_{t-1}/(Nv_{t-1}-Nr_{t-1})}\right)^{1/n}\right] \quad (3)$$

$$\overline{fit} = \frac{\sum_{i=1}^{t} fit_i}{t}, \quad i = 1, 2, \ldots, t \quad (4)$$

Furthermore, $\overline{fit}$ denotes the average fitness (fit_t) (Eq. (4)). fit_t or $\overline{fit}$ is equal to 1 if there is no difference in fitness cost between the competing strains, fit_t or $\overline{fit}$ is above 1 if resistance reduces the bacterial fitness cost, and fit_t or $\overline{fit}$ is below 1 if resistance increases the bacterial fitness cost.

1.4.2. RT-qPCR

The relative expressions of *mexD* and *rpoS* gene were estimated by RT-qPCR. Additionally, the $2^{-\triangle\triangle Ct}$ method was used to calculated the qPCR data as follows:

$$\Delta Ct = Ct_{(Target)} - Ct_{(Reference)}$$
$$\Delta\Delta Ct = \Delta Ct_{(Treated)} - \Delta Ct_{(Control)}$$
$$fc(\text{fold change}) = 2^{-\Delta\Delta Ct}$$

where, Ct means the cycle threshold value. ΔCt is the difference in Ct values between the target gene (*mexD* or *rpoS*) and the endogenous reference gene (16S rRNA) for each sample, and ΔΔCt is the difference in the ΔCt values for the two samples (treated and control). The treated sample is the bacterial strain grown in low-nutrient medium (5 mg/L TOC), and the control samples is the bacterial strain grown in high-nutrient medium (500 mg/L). The fold change in relative expression levels of target genes for resistant bacteria and wild-type ones between low-nutrient and high-nutrient conditions was calculated using the $2^{-\triangle\triangle Ct}$ method. Genes expression levels were considered elevated in the treatment groups when the fold change was >1.

1.5. Statistical analysis

Analysis of variance (ANOVA) and Kruskal–Wallis tests were used to determine significant differences in bacterial fitness (fit_t) and SOUR under different nutrient conditions. Additionally, differences in *mexD* and *rpoS* gene expression levels under high and low-nutrient conditions were analyzed using Student's t-test. Results were significant at the 95% level ($p < 0.05$).

2. Results and discussion

2.1. Bacterial growth under different nutrient conditions

The wild-type strain showed different bacterial growth than the ciprofloxacin-resistant strains (Appendix A Fig. S1). As shown in Appendix A Fig. S1, the wild-type strain grew to approximately 10^7–10^8 CFU/mL after 24 hr inoculation in the medium containing 500, 50, and 5 mg/L TOC. However, bacterial growth became unstable under low-nutrient conditions. It fluctuated around 10^6 CFU/mL in the medium containing 0.5 mg/L TOC and at about 10^5–10^6 CFU/mL in the medium supplemented with 0.05 mg/L TOC. The cell concentrations depended on the available nutrients in the medium because the carbon source provided energy for microbial metabolism.

In contrast, the concentrations of the ciprofloxacin-resistant strains showed a downward trend under high-nutrient conditions (Fig. 1). The cell density reached 10^6–10^7 CFU/mL 24 hr after inoculation in the medium containing 50 mg/L TOC, but it decreased rapidly to 10^5 CFU/mL (cip_1) and 10^1 CFU/mL (cip_3). Similarly, the cell density decreased from 10^6 to 10^2 CFU/mL (cip_1) and 10^1 CFU/mL (cip_3) under high-nutrient conditions with 500 mg/L TOC (Fig. 1).

However, growth of the resistant mutants was more stable under low-nutrient conditions. For instance, cell concentrations were maintained at 10^5–10^6 CFU/mL in the presence of 0.5 mg/L TOC (cip_1). Similarly, the density of resistant strains was maintained at approximately 10^4–10^5 CFU/mL in the medium supplemented with 0.05 mg/L TOC (cip_1). Additionally, the cell density of cip_3 decreased in all nutrient conditions (500, 50, 5, and 0.5 mg/L TOC), but it showed a slower decrease at lower nutrient levels. Moreover, the cip_2 resistance mutant remained at 10^6 CFU/mL in the medium supplemented with 0.5 mg/L TOC and fluctuated under low-nutrient conditions (0.05 mg/L TOC), although it was reduced only minimally, from 10^7 to 10^6 CFU/mL, in high-nutrient concentrations (500 and 50 mg/L TOC). These results revealed that nutrient levels affected the growth of ciprofloxacin resistance mutant cells.

2.2. Ratio of cell numbers and fitness costs for the two competing strains

The ratios of two competing strains (u) decreased under high-nutrient conditions (Fig. 2). Using cip_2 as an example (Fig. 2b), the value of u decreased to −2 in the medium containing 500 and 50 mg/L TOC. This result indicated that the wild-type strain outcompeted the *nfxB* mutant that was resistant to

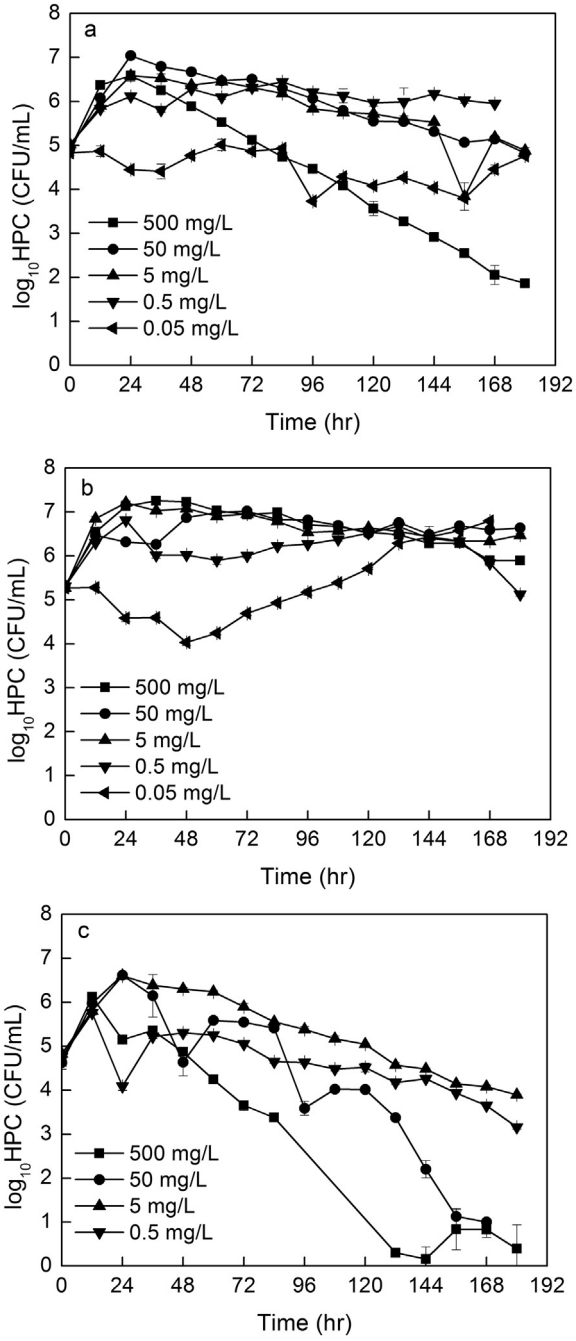

Fig. 1 – Bacterial growth of ciprofloxacin-resistant *P. aeruginosa* PAO1 during a competition experiment. (a) cip_1, (b) cip_2, and (c) cip_3. The competition tests were conducted in triplicates, and the results were reported as the mean and standard deviation (SD) of the mean.

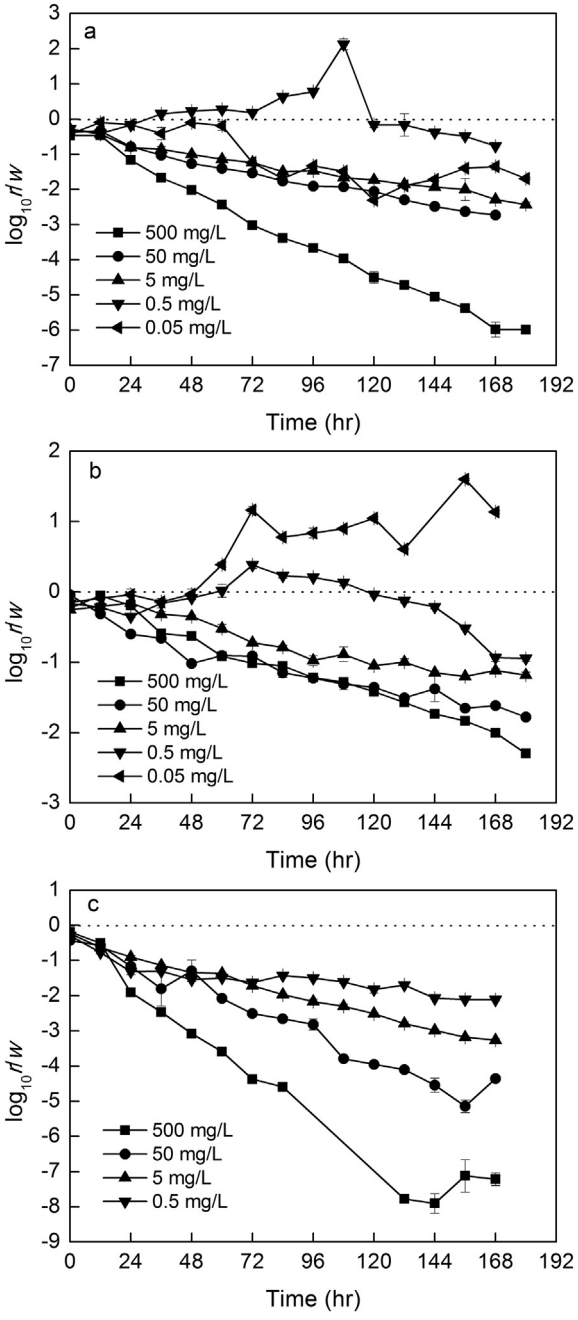

Fig. 2 – Change in the ratio of the number of ciprofloxacin-resistant *P. aeruginosa* PAO1 cells and cells of the wild-type strain. (a) cip_1, (b) cip_2, and (c) cip_3. The competition tests were conducted in triplicates, and the results were reported as the mean and standard deviation (SD) of the mean. *r/w* is the ratio of cells numbers between ciprofloxacin-resistant and wild-type strains.

ciprofloxacin. The acquisition of antibiotic resistance is generally assumed to represent an extra metabolic burden that affects bacterial fitness (Andersson, 2006). However, the value of *u* gradually decreased to −1 at a nutrient level of 5 mg/L TOC. Additionally, this value fluctuated around 0 in the medium supplemented with 0.5 mg/L TOC. Furthermore, this value increased to 1 at 0.05 mg/L TOC. Similar trends were also observed for cip_1 and cip_3. These data indicate that the number of ciprofloxacin-resistant mutant cells was reduced less or even outcompeted by the wild-type strain under low-nutrient conditions.

In addition, the relative fitness (fit_t) of the bacteria was also calculated (Appendix A Fig. S2 and Fig. 3). The values of fit_t were found to be below 1 more frequently at higher nutrient levels (500 and 50 mg/L TOC). However, the fit_t values were

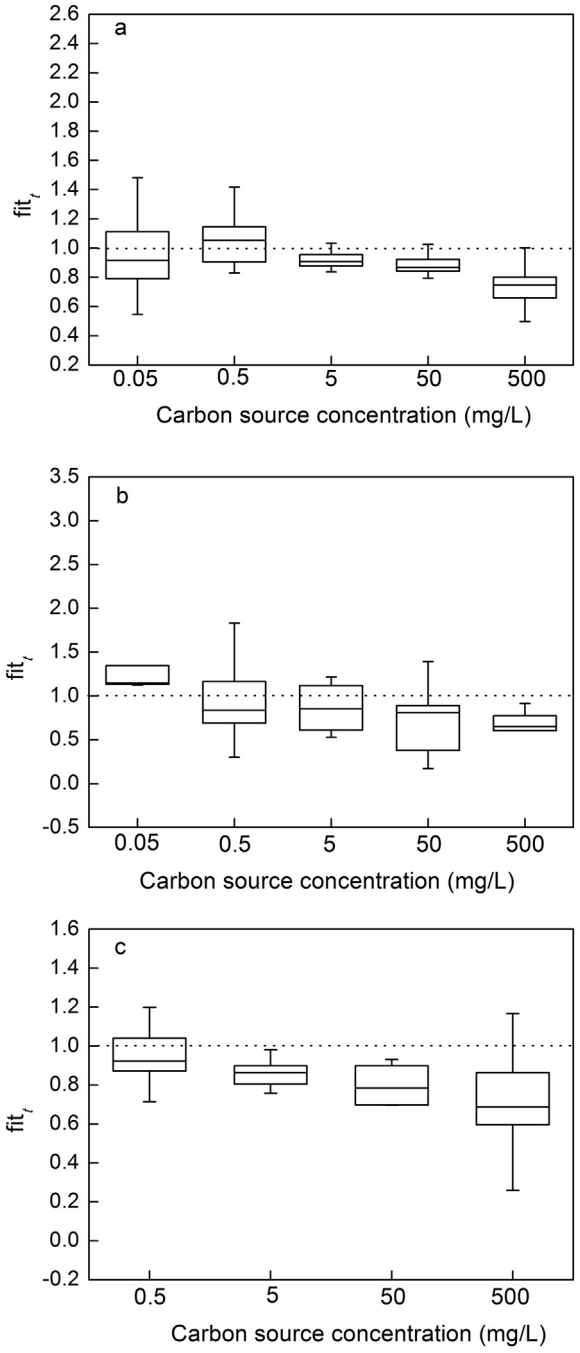

Fig. 3 – Box plots showing change in bacterial relative fitness (fit_t) at different nutrient levels, (a) cip_1, (b) cip_2, and (c) cip_3. Symbols indicate the following: box, 25th to 75th percentile; horizontal line, median; and whiskers, 10th and 90th percentile. The data represent the average fitness at all sampling time for each nutrient level.

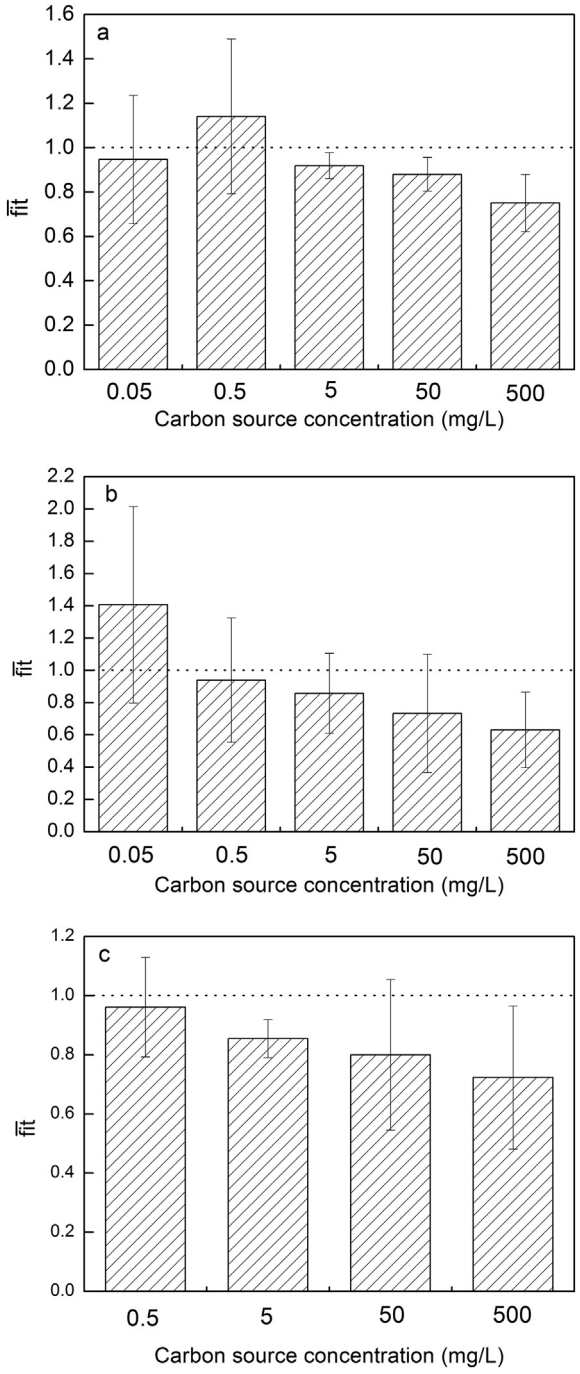

Fig. 4 – Change in the average bacterial fitness ($\overline{fit}$) at different nutrient levels. (a) cip_1, (b) cip_2, and (c) cip_3. The data represent the average fitness at all sampling time for each nutrient level.

close to or above 1 for strains grown in medium with low nutrient levels (0.5 and 0.05 mg/L TOC). Next, the average fitness ($\overline{fit}$) was calculated to more directly compare the fitness cost of resistant strains under different nutrient levels (Fig. 4). The average fitness ($\overline{fit}$) of the four resistant mutants (cip_1, cip_2, and cip_3) were all below 1 under higher nutrient conditions (50 and 500 mg/L TOC), suggesting considerable fitness costs in the resistant bacteria. This result was in line with those of previous studies, in which *nfxB* mutants conferring resistance to ciprofloxacin were shown to impair fitness, expressed as a reduced growth rate or altered virulence and metabolite production (Jeannot et al., 2008; Stickland et al., 2010).

However, the fitness values increased with decreasing nutrient levels. Specifically, this fitness value (1.41 ± 0.61) was

above 1 for cip_2 grown in medium containing 0.05 mg/L TOC. Additionally, the value of $\overline{fit}$ (1.14 ± 0.35) for cip_1 reached nearly 1 at 0.5 mg/L TOC. These results suggested that the fitness cost of the *nfxB* mutants was reduced in low-nutrient conditions. Furthermore, the values of $\overline{fit}$ for cip_1, cip_2, and cip_3 under low-nutrient conditions (0.5 mg/L) were significantly larger ($p < 0.05$) than under high-nutrient conditions (500 mg/L TOC). Furthermore, this value for cip_2 was also significantly larger ($p < 0.05$) at low nutrient levels (0.05 mg/L TOC) than at high nutrient levels (500 mg/L TOC). Consequently, the increased numbers of ciprofloxacin-resistant cells under low-nutrient conditions (Fig. 1) can be explained by a decreasing fitness cost, and possible underlying mechanisms were explored in the following experiments.

2.3. Mechanisms analysis

In general, antibiotic resistance mechanism, i.e., overexpression of efflux pump *mexCD-oprJ* induced by mutational *nfxB* gene, is associated with a lot of energy consumption and has an impact on normal physiological metabolism, thus affecting bacterial fitness cost. Besides, sigma factor (*rpoS* gene) as a regulator for stress response would also involve in fitness cost under low-nutrient condition. Therefore, three mechanisms were targeted in this study: (1) the expression of *mexD* gene that is directly related to efflux pump; (2) the regulation of stress-related factor (*rpoS* gene); (3) the comparison of metabolic activity in high and relative low nutrient levels.

2.3.1. Efflux pump mexD gene expression

As mentioned above, mutations in *nfx*B (L83P and L14Q) caused ciprofloxacin resistance in the cip_1 and cip_3 strains, respectively. Additionally, an insertion mutation at nt 32 was present in *nfx*B in the cip_2 strain. It was generally considered that the expressions of efflux pump conferred antibiotic resistance often led to a large energy consumption and metabolism burden, which was associated with bacterial fitness cost (Bhargava and Collins, 2015; Martínez and Rojo, 2011). Besides, efflux pump MexCD-OprJ is comprised of a cytoplasmic membrane spanning drug–proton antiporter (MexD), an outer membrane spanning channel (OprJ), and a periplasmic component that joins the other two (MexC) (Purssell et al., 2015). Thereby, the *mexD* gene expressions were targeted in this study. As shown in Fig. 5, *mexD* gene expression was much higher in the mutant strains than in the wild-type strain, regardless of whether they were grown in high- or low-nutrient conditions. According to Purssell and Poole (2013), the MexCD-OprJ efflux pump is quiescent in wild-type *P. aeruginosa* cells and does not contribute to intrinsic antibiotic resistance under standard laboratory conditions. However, mutations in the *nfxB* repressor lead to hyperexpression of the MexCD-OprJ efflux pump. Moreover, *mexD* gene expression was significantly higher ($p < 0.05$) under high-nutrient conditions containing (500 mg/L TOC; (48.89 ± 4.16)-fold) than low-nutrient conditions (10 mg/L TOC; (8.01 ± 0.82)-fold). Thus, less energy was needed for expression of the MexCD-OprJ efflux pump in *nfxB* mutants under low-nutrient conditions, which might have contributed to the reduction in fitness cost due to antibiotic resistance (Schweizer, 2003).

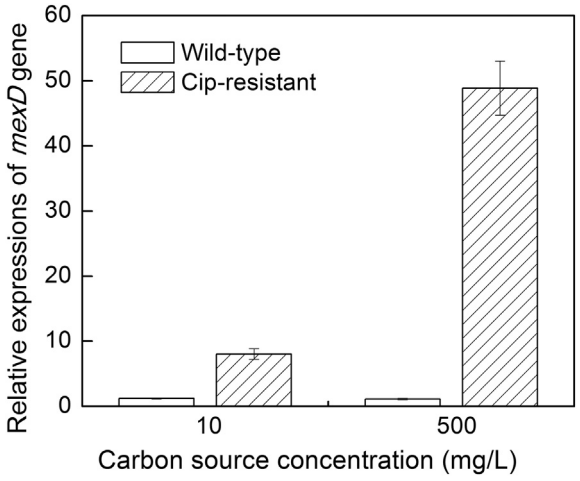

Fig. 5 – Relative amount of *mexD* gene expression under high (500 mg/L TOC) and low (10 mg/L TOC) nutrient conditions by RT-qPCR. Levels of mRNA were normalized to that of the wild-type strain under low-nutrient conditions (set to 1.0). The experiments were conducted in triplicates, and the results were reported as the mean and standard deviation (SD) of the mean.

2.3.2. rpoS gene regulation

Sigma factor (*rpoS*) is known to regulate the expression of hundreds of genes involved in adaption of bacteria in the stationary phase (Hengge-Aronis, 1993) and in osmotic conditions (Shiroda et al., 2014) and other stress environments (O'Neal et al., 1994). Therefore, in wild-type cells, the *rpoS* gene is induced in low-nutrient conditions, which inhibit their growth. As shown in Fig. 6, expression of the *rpoS* gene in the wild-type strain was significantly higher ($p < .05$) with 10 mg/L TOC ((2.78 ± 0.29)-fold) than with 500 mg/L TOC ((1.02 ± 0.02)-fold).

However, at a low nutrient level (10 mg/L TOC), *rpoS* gene expression in the resistant mutants ((1.36 ± 0.13)-fold) was significantly lower ($p < 0.05$) than that in the wild-type strain ((2.78 ± 0.29)-fold). Therefore, the inhibitory effect was less. The resistance mutants might show an advantage when competing with the wild-type strain. Similarly, in a previous study, Paulander et al. (2009) found that the streptomycin resistance mutations *K42N* and *P90S* in ribosomal protein S12 impaired bacterial growth in a nutrient-rich medium, but that the mutants grew faster in poor nutrient conditions than the wild-type strain because the *rpoS* gene was not induced. Consequently, *rpoS* gene regulation might contribute to the reduced fitness cost of ciprofloxacin resistance under low-nutrient conditions.

2.3.3. Metabolic activity comparison

SOUR is an important indicator of microbial metabolism activity. In this study, the SOURs of ciprofloxacin-resistant strains were significantly lower ($p < 0.05$) than that of the wild-type strain at all nutrient levels (500, 5, and 0.5 mg/L TOC) (Fig. 7). For example, the SOURs of wild-type *P. aeruginosa* PAO1 were $(7.19 \pm 0.12) \times 10^{-12}$, $(3.16 \pm 0.09) \times 10^{-12}$, and $(2.39 \pm 0.07) \times 10^{-12}$ mg O_2/(cells·hr) in the

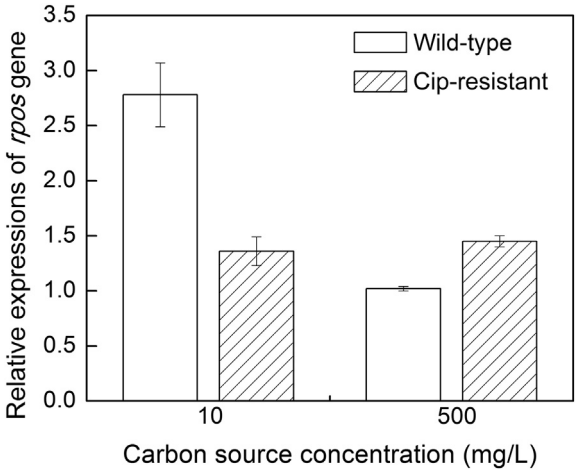

Fig. 6 – Relative amount of *rpoS* gene expression in *P. aeruginosa* PAO1 under high (500 mg/L TOC) and low (10 mg/L TOC) nutrient conditions by RT-qPCR. Levels of mRNA were normalized to that of the wild-type strain under nutrient-rich conditions (set to 1.0). The experiments were conducted in triplicates, and the results were reported as the mean and standard deviation (SD) of the mean.

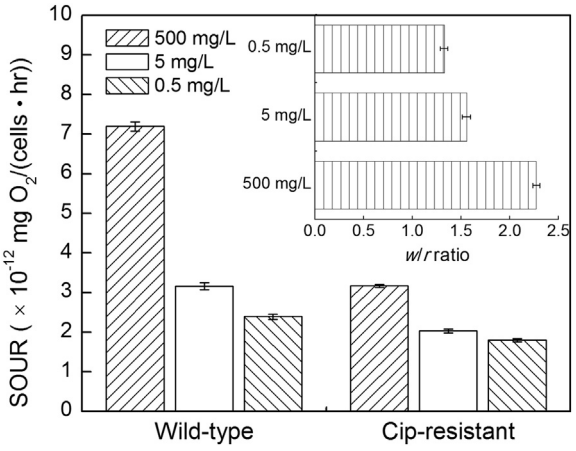

Fig. 7 – Respiratory rate of wild-type and ciprofloxacin resistant *P. aeruginosa* PAO1 at different nutrient levels (0.5, 5, and 500 mg/L TOC) by specific oxygen uptake rate (SOUR) test. The data represent the average of three repeated independent experiments. The insets at the right top corner represent the ratios (*w*/*r*) of SOURs between the wild-type and resistant strains.

medium containing 500, 5, and 0.5 mg/L TOC, respectively. However, lower SOURs, i. e., $(3.17 \pm 0.05) \times 10^{-12}$, $(2.03 \pm 0.05) \times 10^{-12}$, and $(1.80 \pm 0.04) \times 10^{-12}$ mg O_2/(cells·hr), were observed for ciprofloxacin-resistant *P. aeruginosa* PAO1 at corresponding nutrient levels. These results indicated that the *nfxB* mutants had defects in metabolism. It is generally accepted that the acquisition of resistance is metabolically costly for bacteria (Bhargava and Collins, 2015). For example, Stickland et al. (2010) found that a mutation in *nfxB* upregulated MexCD-OprJ expression, leading to global changes in *P. aeruginosa* PAO1 metabolism.

Additionally, the difference in SOURs between the two competing strains was reduced with decreasing nutrient levels (Fig. 5). The differences in SOURs under low-nutrient conditions (5 mg/L TOC, (1.56 ± 0.04)-fold; 0.5 mg/L TOC, (1.33 ± 0.04)-fold) were significantly less ($p < 0.05$) than those at 500 mg/L TOC ((2.27 ± 0.03)-fold). This might be an explanation for the reduction in fitness cost at low nutrient levels (0.5 and 5 mg/L TOC).

3. Conclusions

Antibiotic resistance in environmental bacteria is becoming a public health problem. Efflux pumps play an important role in bacteria resistant to one or more antibiotics. This study shown that the ratio of the number of cells of two competing strains decreased and the average fitness of resistant mutants increased under low-nutrient conditions (0.05, 0.5, and 5 mg/L TOC), suggesting a reduction in fitness cost in the *nfxB* mutants in these cases. Mechanisms analysis found that *mexD* gene expression decreased in low-nutrient medium, leading to lower energy consumption. In addition, *rpoS* gene expression levels were lower in the resistant mutants than in the wild-type strain in low-nutrient conditions, reducing the inhibitory effect of the gene product. Furthermore, the difference in SOURs between the two competing strains was reduced with decreasing nutrient levels. Therefore, low nutrient levels can reduce the fitness cost of ciprofloxacin resistance mediated by an efflux pump. In most natural environments such as source water, nutrient levels are low or even extremely low. Resistant bacteria can persist longer in these environments than in laboratory or clinical conditions; thus, antibiotic-resistant strains of bacteria in the environment are a reason for concern.

Conflict of interest

The authors declare no competing financial interests.

Acknowledgments

This study was supported by the National Natural Science Foundation of China (Nos. 51708534, 51678551, and 51478450), the National Key Research and Development Program of China-International collaborative project from the Ministry of Science and Technology (No. 2017YFE0107300), the Xiamen Major Science and Technology Project (No. 3502Z20171003), and K.C. Wong Education Foundation.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jes.2019.03.022.

REFERENCES

Andersson, D.I., 2006. The biological cost of mutational antibiotic resistance: any practical conclusions? Curr. Opin. Microbiol. 9, 461–465.

Andersson, D.I., Hughes, D., 2010. Antibiotic resistance and its cost: is it possible to reverse resistance? Nat. Rev. Microbiol. 8, 260–271.

Baker-Austin, C., Wright, M.S., Stepanauskas, R., McArthur, J.V., 2006. Co-selection of antibiotic and metal resistance. Trends Microbiol. 14, 176–182.

Bhargava, P., Collins, J., 2015. Boosting bacterial metabolism to combat antibiotic resistance. Cell Metab. 21, 154–155.

Björkman, J., Nagaev, I., Berg, O., Hughes, D., Andersson, D.I., 2000. Effects of environment on compensatory mutations to ameliorate costs of antibiotic resistance. Science 287, 1479–1482.

Delcour, A.H., 2009. Outer membrane permeability and antibiotic resistance. BBA Proteins Proteom. 1794, 808–816.

Gifford, D.R., Moss, E., MacLean, R.C., 2016. Environmental variation alters the fitness effects of rifampicin resistance mutations in *Pseudomonas aeruginosa*. Evolution 70, 725–730.

Hengge-Aronis, R., 1993. Survival of hunger and stress: the role of *rpoS* in early stationary phase gene regulation in *E. coli*. Cell 72, 165–168.

Hernando-Amado, S., Blanco, P., Alcalde-Rico, M., Corona, F., Reales-Calderón, J.A., Sánchez, M.B., et al., 2016. Multidrug efflux pumps as main players in intrinsic and acquired resistance to antimicrobials. Drug Resist. Updates 28, 13–27.

Hirai, K., Suzue, S., Irikura, T., Iyobe, S., Mitsuhashi, S., 1987. Mutations producing resistance to norfloxacion in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. 31, 582–586.

Jeannot, K., Elsen, S., Köhler, T., Attree, I., Van Delden, C., Plésiat, P., 2008. Resistance and virulence of *Pseudomonas aeruginosa* clinical strains overproducing the *MexCD-OprJ* efflux pump. Antimicrob. Agents Chemother. 52, 2455–2462.

Lambert, P.A., 2005. Bacterial resistance to antibiotics: modified target sites. Adv. Drug Deliv. Rev. 57, 1471–1485.

Lin, W., Li, S., Zhang, S., Yu, X., 2016. Reduction in horizontal transfer of conjugative plasmid by UV irradiation and low-level chlorination. Water Res. 91, 331–338.

Lin, W., Zeng, J., Wan, K., Lv, L., Guo, L., Li, X., et al., 2018. Reduction of the fitness cost of antibiotic resistance caused by chromosomal mutations under poor nutrient conditions. Environ. Int. 120, 63–71.

Lister, P.D., Wolter, D.J., Hanson, N.D., 2009. Antibacterial-resistant *Pseudomonas aeruginosa*: clinical impact and complex regulation of chromosomally encoded resistance mechanisms. Clin. Microbiol. Rev. 22, 582–610.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. Methods 25, 402–408.

Lv, L., Jiang, T., Zhang, S.H., Yu, X., 2014. Exposure to mutagenic disinfection byproducts leads to increase of antibiotic resistance in *Pseudomonas aeruginosa*. Environ. Sci. Technol. 48, 8188–8195.

Martínez, J.L., Rojo, F., 2011. Metabolic regulation of antibiotic resistance. FEMS Microbiol. Rev. 35, 768–789.

Masuda, N., Sakagawa, E., Ohya, S., Gotoh, N., Tsujimoto, H., Nishino, T., 2000. Substrate specificities of MexAB-OprM, MexCD-OprJ, and MexXY-OprM efflux pumps in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. 44, 3322–3327.

Olivares, J., Alvarez-Ortega, C., Linares, J.F., Rojo, F., Köhler, T., Martínez, J.L., 2012. Overproduction of the multidrug efflux pump *MexEF-OprN* does not impair *Pseudomonas aeruginosa* fitness in competition tests, but produces specific changes in bacterial regulatory networks. Environ. Microbiol. 14, 1968–1981.

O'Neal, C.R., Gabriel, W.M., Turk, A.K., Libby, S.J., Fang, F.C., Spector, M.P., 1994. RpoS is necessary for both the positive and negative regulation of starvation survival genes during phosphate, carbon, and nitrogen starvation in *Salmonella typhimurium*. J. Bacteriol. 176, 4610–4616.

Paulander, W., Maisnier-Patin, S., Andersson, D.I., 2009. The fitness cost of streptomycin resistance depends on *rpsL* mutation, carbon source and *RpoS* (σ^S). Genetics 183, 539–546.

Peng, J., Cao, J., Ng, F.M., Hill, J., 2017. *Pseudomonas aeruginosa* develops ciprofloxacin resistance from low to high level with distinctive proteome changes. J. Proteome 152, 75–87.

Piddock, L.J., Laura, J.V., 2006. Multidrug-resistance efflux pumps-not just for resistance. Nat. Rev. Microbiol. 4, 629–636.

Poole, K., 2001. Multidrug efflux pumps and antimicrobial resistance in *Pseudomonas aeruginosa* and related organisms. J. Mol. Microbiol. Biotechnol. 3, 255–264.

Pruden, A., Pei, R.T., Storteboom, H., Carlson, K.H., 2006. Antibiotic resistance genes as emerging contaminants: studies in northern Colorado. Environ. Sci. Technol. 40, 7445–7450.

Purssell, A., Poole, K., 2013. Functional characterization of the *NfxB* repressor of the *mexCD–oprJ* multidrug efflux operon of *Pseudomonas aeruginosa*. Microbiology 159, 2058–2073.

Purssell, A., Fruci, M., Mikalauskas, A., Gilmour, C., Poole, K., 2015. EsrC, an envelope stress-regulated repressor of the *mexCD-oprJ* multidrug efflux operon in *Pseudomonas aeruginosa*. Environ. Microbiol. 17, 186–198.

Reynolds, R., Shackcloth, J., Felmingham, D., Macgowan, A., 2003. Comparison of BSAC agar dilution and NCCLS broth microdilution MIC methods for in vitro susceptibility testing of *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Moraxella catarrhalis*: the BSAC respiratory resistance surveillance programme. J. Antimicrob. Chemother. 52, 925–930.

Sander, P., Springer, B., Prammananan, T., Sturmfels, A., Kappler, M., Pletschette, M., et al., 2002. Fitness cost of chromosomal drug resistance-conferring mutations. Antimicrob. Agents Chemother. 46, 1204–1211.

Schweizer, H.P., 2003. Efflux as a mechanism of resistance to antimicrobials in *Pseudomonas aeruginosa* and related bacteria: unanswered questions. Genet. Mol. Res. 2, 48–62.

Shiroda, M., Pratt, Z.L., Wong, A.C., Kaspar, C.W., 2014. *RpoS* impacts the lag phase of *Salmonella enterica* during osmotic stress. FEMS Microbiol. Lett. 357, 195–200.

Song, T., Park, Y., Shamputa, I.C., Seo, S., Lee, S.Y., Jeon, H.S., et al., 2014. Fitness costs of rifampicin resistance in *Mycobacterium tuberculosis* are amplified under conditions of nutrient starvation and compensated by mutation in the β' subunit of RNA polymerase. Mol. Microbiol. 91, 1106–1119.

Stickland, H.G., Davenport, P.W., Lilley, K.S., Griffin, J.L., Welch, M., 2010. Mutation of *nfxB* causes global changes in the physiology and metabolism of *Pseudomonas aeruginosa*. J. Proteome Res. 9, 2957–2967.

Su, H.C., Liu, Y.S., Pan, C.G., Chen, J., He, L.Y., Ying, G.G., 2018. Persistence of antibiotic resistance genes and bacterial community changes in drinking water treatment system: from drinking water source to tap water. Sci. Total Environ. 616–617, 453–461.

Terzi, H.A., Kulah, C., Ciftci, I.H., 2014. The effects of active efflux pumps on antibiotic resistance in *Pseudomonas aeruginosa*. World J. Microbiol. Biotechnol. 30, 2681–2687.

Treepong, P., Kos, V.N., Guyeux, C., Blanc, D.S., Bertrand, X., Valot, B., et al., 2018. Global emergence of the widespread *Pseudomonas aeruginosa* ST235 clone. Clin. Microbiol. Infec. 24, 258–266.

Visco, G., Campanella, L., Nobili, V., 2005. Organic carbons and TOC in waters: an overview of the international norm for its measurements. Microchem. J. 79, 185–191.

Webber, M.A., Piddock, L.J., 2003. The importance of efflux pumps in bacterial antibiotic resistance. J. Antimicrob. Chemother. 51, 9.

Wright, G.D., 2005. Bacterial resistance to antibiotics: enzymatic degradation and modification. Adv. Drug Deliv. Rev. 57, 1451–1470.

Xi, C.W., Zhang, Y.L., Marrs, C.F., Ye, W., Simon, C., Foxman, B., et al., 2009. Prevalence of antibiotic resistance in drinking water treatment and distribution systems. Appl. Environ. Microbiol. 75, 5714–5718.

Zhang, S.H., Yu, X., Guo, F., Wu, Z.Y., 2011. Effect of interspecies quorum sensing on the formation of aerobic granular sludge. Water Sci. Technol. 64, 1284–1290.