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Short Communication

Effects of iron depletion on antimicrobial activities against planktonic and biofilm *Pseudomonas aeruginosa*

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Abstract

Objectives Iron plays an important role in the development of *Pseudomonas aeruginosa* biofilm. Here we evaluated effects of iron depletion on the antimicrobial activity of ceftazidime, tobramycin and ciprofloxacin against planktonic and biofilm *Pseudomonas aeruginosa*.

Methods We tested the sensitivities of wild-type PAO1, type-IV pilus mutant PAO-ΔpilHIJK and the quorum-sensing mutant PAO-JP2 *P. aeruginosa* planktonic cultures and biofilms to antibiotics under iron-depleted conditions.

Key findings In planktonic bacteria, the minimum concentration that inhibited visible growth (MIC) of ciprofloxacin was increased slightly in an iron-depleted environment in all three strains, whereas the MIC of tobramycin was similar in iron-depleted and control environments. The MIC of ceftazidime increased in the PAO-JP2 strain when iron was depleted. Tobramycin achieved the best bactericidal effect in biofilms. Viable counts were reduced by one log under iron-depleted conditions in all three strains when tobramycin reached 4 MIC and when ceftazidime and ciprofloxacin reached 8 MIC.

Conclusions This study suggests that once the biofilm is formed, iron depletion may only slightly promote the bactericidal effect of antibiotics on PAO1, PAO-ΔpilHIJK and PAO-JP2. Although these changes were relatively small, iron as one of the environmental factors should not be ignored when evaluating bactericidal effect of antibiotics. The combination of an iron chelator and antibiotics may have therapeutic value under certain bacterial growth conditions.

Keywords biofilm; ceftazidime; ciprofloxacin; iron; *Pseudomonas aeruginosa*; tobramycin

Introduction

Pseudomonas aeruginosa is an opportunistic pathogen that is frequently isolated from clinical specimens obtained from burns, surface wounds, the urinary tract, ear and eye infections, and the lungs of patients with cystic fibrosis.[1–4] In patients with these underlying diseases, *P. aeruginosa* can cause chronic infections characterised by the formation of biofilms. Biofilms consist of groups of bacteria attached to surfaces and encased in a hydrated polymeric matrix.[5] Biofilms can withstand host immune responses[6] and are much more resistant to antibiotic treatments than their non-attached planktonic counterparts.[7] For these reasons, biofilm infections are often persistent and intractable.

In *P. aeruginosa*, flagella have been shown to be essential for the initial attachment to abiotic surfaces, and twitching motility has been suggested to be necessary for microcolony formation and thus for normal biofilm development.[8] Twitching motility depends on polar type-IV pili, which are known to play a crucial role in mediating adherence to and colonisation of mucosal surfaces. The mechanical basis for this flagellum-independent type of surface motility is believed to be extension and retraction of the type IV pili, which propel the cells along the surface.[9,10] Cell-to-cell communication named the quorum-sensing (QS) system has also been shown to be important for the development of *P. aeruginosa* biofilms.[5,11–15] In *P. aeruginosa*, there are two well-known QS systems termed *las* and *rhl*, with the *las* system situated above the *rhl* system.[16] Rumbaugh *et al*.[17] and Davies *et al*.[18] confirmed that *P. aeruginosa* strains that lack functional QS systems are less virulent than wild-type strains and form flat, undifferentiated biofilms that are less stable than the differentiated biofilms formed by wild-type *P. aeruginosa* as they can be easily disrupted by the detergent sodium dodecyl sulfate.

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Recently, a number of studies have reported that nutritional conditions of bacterial habitat affect biofilm formation and may correlate with QS systems. Free iron concentration is one of these essential nutritional conditions.[19–21] In-vitro experiments have shown that both iron depletion (<1 μmol/l) and excessive iron (>100 μmol/l) can retard biofilm formation.[22] Britigan *et al*.[23] showed that levels of free iron increased (still lower than 100 μmol/l) in airway secretions of patients with cystic fibrosis, suggesting that iron effects are relevant *in vivo*.

P. aeruginosa wild-type PAO1, the type-IV pilus mutant PAO-ΔpilHIJK and the QS mutant PAO-JP2 are generally used in biofilm studies. However, there has been no systematic study of the effects of iron on their sensitivities to antibiotics. Here we evaluated the effects of iron depletion on the antimicrobial activity of ceftazidime, tobramycin and ciprofloxacin against planktonic and biofilm *P. aeruginosa*. Our study focused on the effect of iron depletion because the concentration of free iron *in vivo* does not exceed 100 μmol/l. The results will provide useful information on biofilm control conducted on these strains and the clinical control of biofilm infections.

Materials and Methods

Bacterial strains and growth conditions

Wild-type PAO1,[24] type-IV pilus mutant PAO-ΔpilHIJK[24] and the *lasR* and *rhlR* mutant PAO-JP2 *P. aeruginosa* strains were used in this study. Strains were cultured in Mueller-Hinton (M-H) broth (Difco, Detroit, MI, USA).

Drugs

Ceftazidime, tobramycin and ciprofloxacin were from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Antibiotic powders were used to prepare stock solutions at concentrations of 2048 μg/ml as recommended by the Clinical and Laboratory Standards Institute (CLSI). Stock solutions were stored at 4°C.

Determination of dipyridyl concentration

Iron depletion in the medium was achieved by the addition of the iron-specific chelator 2,2-dipyridyl (DPD, Sigma Aldrich, Shanghai, China). To determine the appropriate concentration of DPD, PAO1, PAO-ΔpilHIJK and PAO-JP2 from 24 h growth plates were suspended in M-H broth and the turbidity adjusted to a 0.5 McFarland standard, which was about 1.5×10^8 cfu/ml, by spectrophotometric methods. After two 10-fold dilutions, 1.5×10^6 cfu/ml of each bacterial suspension was obtained and 1 ml of the bacterial suspension was added to 9 ml M-H broth plus different concentrations of DPD. The bacterial cultures were placed on a shaker at 250 rpm and incubated at 37°C. At 0, 2, 4, 8, 12 and 24 h of culturing, 100 μl aliquots were removed from each sample. After serial 10-fold dilutions, a 50 μl aliquot from each dilution was streaked in triplicate onto M-H agar plates without DPD for determination of the colony count. The number of colony-forming units on each plate after 24 h incubation at 37°C was determined. The highest concentration of DPD that did not affect the growth of the three planktonic strains was used in tests of drug effects.

Static biofilm formation assay

Static biofilms were prepared as described by Favre-Bonté and colleagues,[25] with some modifications. Briefly, PAO1, PAO-ΔpilHIJK and PAO-JP2 were grown in M-H broth for 6 h with agitation at 37°C. Silica gel chips (1 cm^2) were then immersed in the bacterial culture medium without agitation, to allow bacteria to adhere and biofilm to form, for 7 days at 37°C. The culture medium was replaced every 48 h.

Determination of MIC

The minimum inhibitory concentration (MIC) was defined as the lowest drug concentration that inhibited visible growth. The MIC of each drug was assessed by a microdilution method derived from the standardised procedure established by the CLSI for broth microdilution antimicrobial susceptibility testing. Briefly, the initial concentration of bacterial suspension was 1.5×10^5 cfu/ml and the final concentrations of ceftazidime, tobramycin or ciprofloxacin ranged from 0.012 to 16 μg/ml. Two-fold dilutions of each drug were tested. Plates were read after 24-h incubation at 37°C.

Bactericidal effects on biofilm strains

After 7 days' cultivation, the silica gel chips were rinsed in physiological saline and then placed in 24-well plates with different antibiotics, alone or in combination with DPD. Antibiotic concentrations ranged from 1 to 8 MIC as determined by the microdilution method. After incubation at 37°C for 24 h, the silica gel chips were rinsed and then put into vials with 1 ml M-H broth, sonicated for 10 min and vortexed for 3 min. Aliquots of 100 μl were removed from each vial. After serial 10-fold dilution, a 50 μl aliquot from each dilution was streaked in triplicate onto M-H agar plates for determination of colony count. The number of colony-forming units on each plate after 24-h incubation at 37°C was determined.

Statistical analysis

Determination of DPD concentration and bactericidal effects of antibiotics on biofilm strains were performed independently in triplicate. Data were analysed using the Mann–Whitney *U* test (SPSS statistical software).

Results

Optimal dipyridyl concentration

DPD at 500 μmol/l was chosen as the appropriate concentration. To ensure that the activity of DPD was related only to its iron-chelating property, we used the highest concentration of DPD that did not affect the growth of planktonic cells. The effects of DPD on growth of PAO1, PAO-ΔpilHIJK and PAO-JP2 are shown in Figure 1a–c. Growth of all three strains were greatly inhibited when DPD reached 1000 μmol/l. Growth of PAO-ΔpilHIJK and PAO-JP2 was partially inhibited at 750 μmol/l DPD, while PAO1 was not affected. DPD concentrations of 500 μmol/l and lower did not affect the growth of any strain, it being similar to that in cultures without DPD. Thus, 500 μmol/l DPD was chosen as the concentration to achieve iron-depletion in subsequent experiments.

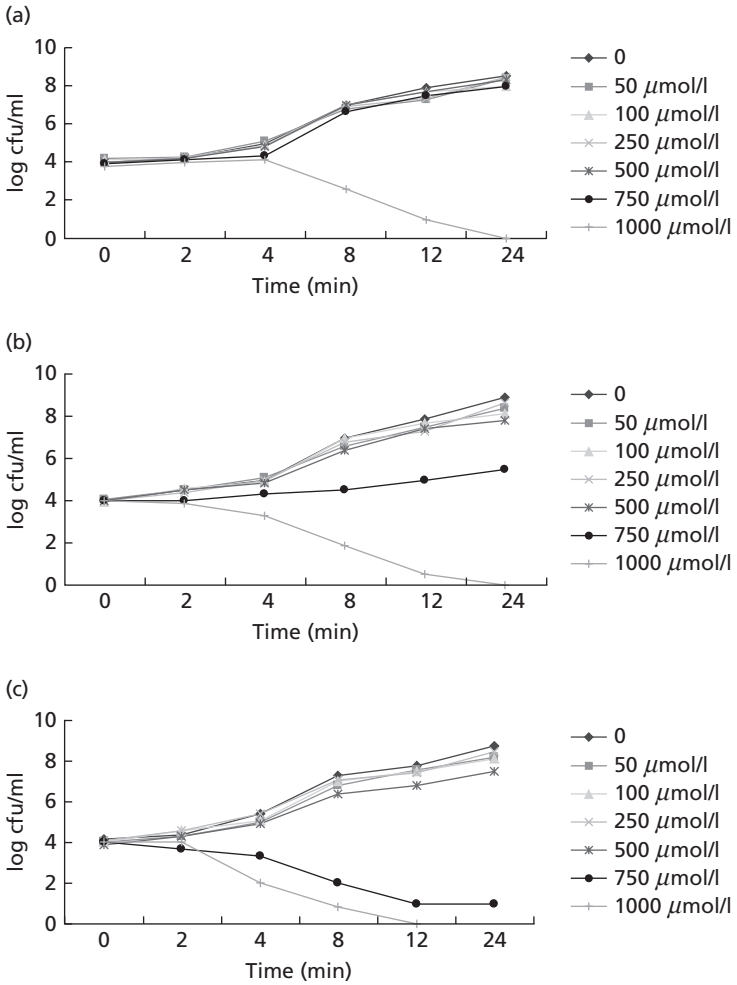

Figure 1 Growth of planktonic *Pseudomonas aeruginosa* strains in various concentrations of 2,2-dipyridyl: (a) PAO1, (b) PAO-ΔpilHIJK, (c) PAO-JP2. Data are means from triplicate samples.

Susceptibility studies

The MIC data for each strain of *P. aeruginosa* in normal and iron-depleted environments (500 μmol/l DPD) are summarised in Table 1. The MICs for tobramycin were the same under iron-depleted condition and normal growth conditions in all three strains. The MIC of ceftazidime against PAO-JP2 in the iron-depleted condition was double that in the normal growth condition, whereas the MICs against PAO1 and PAO-ΔpilHIJK were similar in the iron-depleted and normal growth conditions. An iron-depleted environment increased the MIC of ciprofloxacin 2-fold in PAO1 and PAO-JP2, and 3-fold in PAO-ΔpilHIJK.

Bactericidal activities of antibiotics on biofilm strains

The bactericidal activities of the three antibiotics at 1–8 MIC in normal and iron-depleted conditions (DPD 500 μmol/l) are shown in Figure 2. When the ceftazidime concentration

Table 1 Susceptibilities of planktonic PAO1, PAO-ΔpilHIJK and PAO-JP2 to ceftazidime, tobramycin and ciprofloxacin under normal and iron-depleted conditions (500 μmol/l 2,2-dipyridyl)

	PAO1		PAO-ΔpilHIJK		PAO-JP2	
	Normal	Iron-depleted	Normal	Iron-depleted	Normal	Iron-depleted
Ceftazidime	2	2	0.5	0.5	0.03125	0.0625
Tobramycin	1	1	4	4	4	1
Ciprofloxacin	0.5	1	0.0625	0.25	0.125	0.25

Values are minimum inhibitory concentration in mg/l.

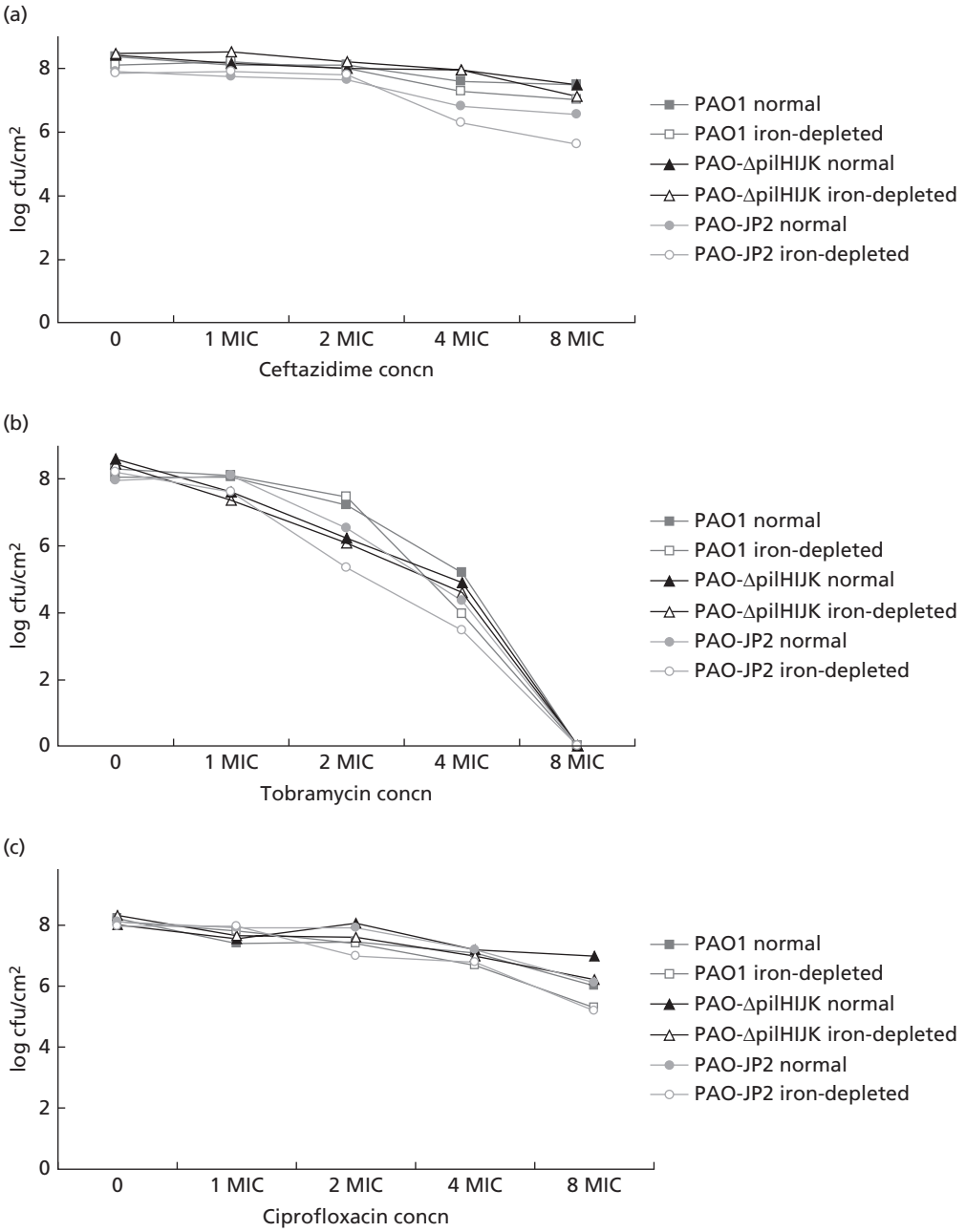

Figure 2 Bactericidal activity of (a) ceftazidime, (b) tobramycin and (c) ciprofloxacin on biofilm strains in normal and iron-depleted conditions. Data are means from triplicate experiments. Standard errors are reported in Table 2.

was below 4 MIC, there was little difference in viable counts between normal and iron-depleted conditions for all three strains (Figure 2a). When ceftazidime reached 8 MIC, viable counts of PAO-JP2 biofilm in the iron-depleted condition were reduced by one log compared with the normal condition ($P < 0.05$), whereas PAO1 and PAO-ΔpilHIJK showed some decrease (less than one-log reduction; not significant) under iron depletion compared with the normal condition (Figure 2a, Table 2). Tobramycin at 4 MIC significantly reduced both PAO1 and PAO-JP2 growth by one log ($P < 0.05$) under the iron-depleted condition (Figure 2b, Table 2). Ciprofloxacin had similar effects to ceftazidime when the concentration was lower than 4 MIC, but at 8 MIC viable counts were significantly reduced by one-log under the iron-depleted condition in all three strains (Figure 2c, Table 2).

Discussion

β-Lactam antibiotics such as ceftazidime, aminoglycoside antibiotics such as tobramycin, and fluoroquinolones such as ciprofloxacin are commonly used to treat *P. aeruginosa* infections in clinical practice. In this study, we investigated the effect of these antibiotics on planktonic and biofilm bacteria. We found that the susceptibility of all three

Table 2 Bactericidal activity of ceftazidime, tobramycin and ciprofloxacin on biofilm strains in normal and iron-depleted conditions (500 μmol/l 2,2-dipyridyl).

	PAO1		PAO-ΔpilHIJK		PAO-JP2	
	Normal	Iron-depleted	Normal	Iron-depleted	Normal	Iron-depleted
Ceftazidime (8 MIC)	7.48 ± 0.66	7.03 ± 0.46	7.47 ± 0.32	7.14 ± 0.86	6.57 ± 0.89	5.64 ± 0.56*
Tobramycin (4 MIC)	5.19 ± 0.73	3.98 ± 0.26*	4.88 ± 0.67	4.59 ± 0.59	4.35 ± 0.23	3.47 ± 0.68*
Ciprofloxacin (8 MIC)	5.96 ± 0.43	5.26 ± 0.78*	6.97 ± 0.54	6.17 ± 0.53*	6.06 ± 0.43	5.17 ± 0.79*

Values are means ± SE of log cfu/cm^2 from triplicate experiments. *$P < 0.05$ vs same strain in normal conditions (Mann–Whitney U test). MIC, minimum inhibitory concentration in mg/l.

planktonic strains to tobramycin did not change under the iron-depleted condition. The PAO-JP2 strain, but not other strains, showed a 2-fold increase in resistance to ceftazidime when iron deprived. The MIC for ciprofloxacin was increased 1–3-fold in all three strains in the iron-depleted condition compared with the normal condition. The slightly reduced sensitivities to ceftazidime and ciprofloxacin, which did not reach drug-resistant standard, might be partially due to reduced metabolism by the bacteria. The iron-depleted environment was not optimal for bacterial growth, and therefore these strains might enter a slower growth stage. Accordingly, their sensitivities to ceftazidime and ciprofloxacin, which are known to be more effective during exponential growth, decreased. Tobramycin, a good bactericide for growth-arrested bacteria, has similar effects in normal and iron-depleted conditions.

Our study showed that among the three antibiotics tested, tobramycin had the best bactericidal effect on biofilm strains. Ceftazidime and ciprofloxacin even at 8 MIC could not kill all the strains in biofilms whereas tobramycin could in the normal non-iron-depleted condition. Measurements of antibiotic penetration in *P. aeruginosa* biofilms showed that fluoroquinolones such as ofloxacin and ciprofloxacin readily penetrate *P. aeruginosa* biofilms, whereas aminoglycosides such as tobramycin and gentamicin show retarded delivery.[26,27] Aminoglycosides diffuse more slowly because they bind to extracellular polymers such as alginate.[28] However, contrary to other reports,[26–28] our study showed that tobramycin had the best bactericidal effect among the three antibiotics tested, suggesting that slow diffusion may not be the most important factor when evaluating bactericidal effects on biofilm strains. The effect of tobramycin, which is bactericidal during the rest period, on biofilm cultures suggests that such biofilm bacteria may be entering a resting period and/or have slower metabolism.

Once biofilms are formed, iron depletion had some effect on their sensitivity to antibiotics. For PAO1 and PAO-JP2 strains treated with ceftazidime or ciprofloxacin at 8 MIC or tobramycin at 4 MIC, viable counts in biofilms decreased by one log under iron-depleted conditions compared with normal conditions. For PAO-ΔpilHIJK, ciprofloxacin at 8 MIC also achieved 1-log reduction compared with normal conditions. Banin *et al.*[29] reported that exposure of *P. aeruginosa* biofilms to EDTA killed *P. aeruginosa* cells and triggered detachment of cells from biofilms. The addition of either calcium or iron, but not magnesium, completely blocked EDTA-induced detachment, suggesting that iron and calcium might be involved in maintenance of biofilms. Iron salts may be potent crosslinkers of the biofilm matrix because iron cations can increase the viscosity of a bacterial biofilm suspension.[30] In an iron-depleted environment, adhesion of bacteria decreases along with a decrease in hydrophobicity.[31] Thus, iron appears to be important in the formation and maintenance of stable biofilm communities, principally by promoting cell adhesion. Our results basically supported those from previous studies. However, unlike the other studies, in our study DPD was added after the biofilms were completely formed, and DPD might have caused certain destruction only to the outer superficial layer of the biofilm. Therefore, only slight increases in the bactericidal effect of the antibiotics were observed in biofilms.

In summary, this study demonstrated that both planktonic and biofilm PAO1, PAO-ΔpilHIJK and PAO-JP2 have similar antibiotic sensitivities to iron depletion. Sensitivities of planktonic cells to different antibiotics decreased a little, while sensitivities of biofilm cells to antibiotics increased slightly. Although these changes were relatively small, they suggest that iron levels should be considered when evaluating the bactericidal effect of antibiotics. Because iron depletion can destroy the biofilm structure to a certain extent, the combination of a chelator (such as deferoxamine, a detoxicant used in the clinic) and antibiotics may have therapeutic utility. Further experiments aimed at understanding changes in iron conditions *in vivo*, particularly in relation to its exact time and site of usage, may help find a new way to control *P. aeruginosa* biofilm infections.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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