Antibacterial and antibiofilm effects of iron chelators against \textit{Prevotella intermedia}

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\textit{Prevotella intermedia}, a major periodontopathogen, has been shown to be resistant to many antibiotics. In the present study, we examined the effect of the FDA-approved iron chelators deferoxamine (DFO) and deferasirox (DFRA) against planktonic and biofilm cells of \textit{P. intermedia} in order to evaluate the possibility of using these iron chelators as alternative control agents against \textit{P. intermedia}. DFRA showed strong antimicrobial activity (MIC and MBC values of 0.16 mg ml\textsuperscript{-1}) against planktonic \textit{P. intermedia}. At subMICs, DFRA partially inhibited the bacterial growth and considerably prolonged the bacterial doubling time. DFO was unable to completely inhibit the bacterial growth in the concentration range tested and was not bactericidal. Crystal violet binding assay for the assessment of biofilm formation by \textit{P. intermedia} showed that DFRA significantly decreased the biofilm-forming activity as well as the biofilm formation, while DFO was less effective. DFRA was chosen for further study. In the ATP-bioluminescent assay, which reflects viable cell counts, subMICs of DFRA significantly decreased the bioactivity of biofilms in a concentration-dependent manner. Under the scanning electron microscope, \textit{P. intermedia} cells in DFRA-treated biofilm were significantly elongated compared to those in untreated biofilm. Further experiments are necessary to show that iron chelators may be used as a therapeutic agent for periodontal disease.

INTRODUCTION

\textit{Prevotella intermedia} is a black-pigmented anaerobic Gram-negative bacterium which is frequently isolated from the periodontal lesions of patients with chronic periodontitis (Petit \textit{et al.}, 1994; Ashimoto \textit{et al.}, 1996; Polson \textit{et al.}, 1997), aggressive periodontitis (Albandar \textit{et al.}, 1997; Kuru \textit{et al.}, 1999; Kamma \textit{et al.}, 2004), puberty-associated gingivitis and acute necrotizing ulcerative gingivitis (Rowland \textit{et al.}, 1993; Novak, 1999). Studies also suggest that this bacterium is associated with various systemic diseases such as acute exacerbation of chronic bronchitis (Brook & Frazier, 2003), pulmonary infection (Shinzato & Saito, 1994) and atherosclerosis (Fiehn \textit{et al.}, 2005). \textit{P. intermedia} has been shown to be resistant to many antibiotics including penicillins, cephalosphorins and tetracyclines (Andrés \textit{et al.}, 1998; Fosse \textit{et al.}, 2002). Furthermore, in a previous study using \textit{P. intermedia} biofilms, the bioactivities of several \textit{P. intermedia} strains including ATCC 49046 were significantly increased when they were treated with tetracyclines, indicating the difficulties involved in designing antibiotic therapy for periodontal disease (Takahashi \textit{et al.}, 2006). Elimination of \textit{P. intermedia} may be critical due to the fact that it can serve as a reservoir of antibiotic resistance (Yu \textit{et al.}, 2007). Accordingly, it is necessary to develop alternative antimicrobial approaches for controlling antibiotic-resistant \textit{P. intermedia}.

**Abbreviations:** BPB, black-pigmented bacteria; DFO, deferoxamine; DFRA, deferasirox; EPS, extracellular polymeric substance.
Iron is indispensable for the growth of most micro-organisms (Shibata et al., 2003) and is intimately connected with the synthesis of several virulence determinants of bacterial and other pathogens (Sritharan, 2006). Deferoxamine (DFO), a Food and Drug Administration (FDA)-approved iron chelator, has been extensively used for chelation therapy in iron-overloaded states (Halliday & Bassett, 1980; Moreau-Marquis et al., 2009). On the one hand, DFO decreases the susceptibility to infections by lowering the iron concentration, but, on the other hand, it increases the virulence of some micro-organisms, such as Yersinia and Klebsiella, because of the ability of the micro-organisms to use the chelator as an iron-sequestering agent for their own metabolism (van Asbeck et al., 1983). However, such an unfavourable effect was not found in orally effective iron chelators such as deferiprone and deferasirox (DFRA) (Lesic et al., 2002; Chan et al., 2009). In the present study, we examined the effect of the FDA-approved iron chelators DFO and DFRA against planktonic and biofilm cells of P. intermedia in order to evaluate the possibility of using these iron chelators as alternative control agents against P. intermedia.

METHODS

Bacteria and culture conditions. Prevotella intermedia ATCC 49046 was obtained from the American Type Culture Collection (Manassas, VA, USA). It was grown either on brucella agar (Difco) containing 5 % laked sheep blood, 5 μg haemin ml\(^{-1}\) and 1 μg vitamin K\(_1\) ml\(^{-1}\), or in brucella broth (Difco) containing 5 μg haemin ml\(^{-1}\) and 1 μg vitamin K\(_1\) ml\(^{-1}\) (B-HK), at 37 °C under an atmosphere of 85 % N\(_2\), 10 % H\(_2\) and 5 % CO\(_2\).

**Determination of MIC and MBC.** The iron chelators DFO (Novartis Pharma Stein) and DFRA (Exjade; Novartis) were dissolved in water and DMSO, respectively, and were diluted with B-HK. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined as described in our previous study (Moon et al., 2011b). The MIC was defined as the lowest concentration that inhibited the bacterial growth on brucella blood agar according to CLSI guidelines (CLSI, 2007). The MBC was defined as the lowest concentration at which >99.9 % of the cells were killed after 24 h incubation (NCCLS, 1999).

**Bacterial total growth and doubling time.** To examine effect of the iron chelators on bacterial total growth (biofilm cells attached to the bottom and the wall of a well and planktonic cells free-floating in the well), a 24 h culture of P. intermedia was adjusted to OD\(_{600}\) 0.1, dispensed (100 μl per well) into triplicate wells of polystyrene 96-well plates containing various concentrations of the iron chelators in B-HK (100 μl), and then incubated for 24 h at 37 °C anaerobically. Planktonic cells were aspirated, and the wells were washed twice with physiological saline, followed by staining of the biofilms with 0.1 % crystal violet for 10 min. After the plates had been washed twice with water and air-dried, the bound dye was solubilized in 200 μl 95 % ethanol, and A\(_{600}\) was read. Biofilm-forming activity was calculated by normalizing dye binding (A\(_{600}\)) to total bacterial growth (biofilm cells + planktonic cells) determined in parallel identical wells by measuring OD\(_{600}\).

**Viscosity of spent culture medium.** P. intermedia ATCC 49046 cells were grown in B-HK containing either of the test agents at subMICs. After a 24 h incubation, the culture medium was centrifuged to remove cells, and then the viscosity of the spent culture medium was measured at 25 °C using an SV-10 vibroviscometer (A&D Company).

**Determination of ATP content in biofilm.** The viability of the biofilms was determined by ATP-bioluminescence quantification. Briefly, 200 μl bacterial inoculum (1–2 × 10\(^8\) cells ml\(^{-1}\)) in B-HK was dispensed into each well of a 96-well plate and incubated at 37 °C anaerobically. After 24 h incubation, the established biofilms were washed once with physiological saline, and then 200 μl B-HK containing the test agents was dispensed in triplicate into each well. After incubation for 24 h, the wells were washed twice with physiological saline, and the bioactivity of the biofilms was determined by ATP-bioluminescence quantification using the BacTiter-Glo Microbial Cell Viability Assay kit (Promega) according to the manufacturer’s instructions.

**Determination of biofilm MBCs.** The broth recovery-based biofilm MBC method was performed as described previously (Qu et al., 2010) with some modifications. Briefly, established biofilms in the wells of a 96-well microplate were exposed to the test agents for 24 h, as described above. After washing the wells twice with physiological saline, fresh medium (200 μl) was dispensed into the wells. The plate was incubated for a further 24 h with shaking at 100 r.p.m. The supernatants were carefully transferred to a new 96-well microplate, and the turbidity of the contents was visually assessed. The minimum concentration of the test agent that resulted in no visible bacterial growth in the suspensions was defined as the biofilm MBC.

**Morphological observations by scanning electron microscopy (SEM).** Biofilms of P. intermedia ATCC 49046 were developed on a polystyrene 48-well plate as described above, and then treated with the test agents at 1/4 × the biofilm MBC for 24 h. After washing with physiological saline, the remaining biofilm cells were fixed with 2.5 % glutaraldehyde in 0.1 M phosphate buffer for 1 h and 1 % Os\(_2\)O\(_4\) in 0.1 M phosphate buffer for 1 h at 4 °C, and dehydrated through a graded ethanol series (Nakao et al., 2006; Yamanaka et al., 2009). The samples were dried by critical-point drying, coated with gold using a sputter-coater (IB-3, Eiko), and observed by SEM (S2300, Hitachi, Tokyo, Japan) at an accelerating voltage of 15 kV. Images were analysed using ImageJ (NIH) for cell length measurement.

**Statistical analyses.** All values were expressed as the mean±SD. Statistical analysis was conducted using Student’s t-test or the Mann–Whitney U test, when appropriate. P-values <0.05 were considered significant.

**RESULTS**

**MIC and MBC of iron chelators**

The colony formation of P. intermedia ATCC 49046 was completely inhibited on brucella blood agar containing plates containing various concentrations of the iron chelators in B-HK (100 μl), and then incubated for 24 h at 37 °C anaerobically. Planktonic cells were aspirated, and the wells were washed twice with physiological saline, followed by staining of the biofilms with 0.1 % crystal violet for 10 min. After the plates had been washed twice with water and air-dried, the bound dye was solubilized in 200 μl 95 % ethanol, and A\(_{600}\) was read. Biofilm-forming activity was calculated by normalizing dye binding (A\(_{600}\)) to total bacterial growth (biofilm cells + planktonic cells) determined in parallel identical wells by measuring OD\(_{600}\).

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0.16 mg DFRA ml⁻¹. In contrast, the MIC of DFO could not be determined: colony formation by the bacterium was observed on brucella blood agar containing DFO at concentrations up to 0.64 mg ml⁻¹. DFRA exhibited an MBC equal to its MIC value of 0.16 mg ml⁻¹.

**Effect of iron chelators on total bacterial growth**

Antibacterial activity of the iron chelators was further monitored in liquid medium by determining OD₆₀₀. To examine the effect of the iron chelators on total bacterial growth (biofilm cells + planktonic cells), *P. intermedia* was incubated with different concentrations of DFRA and DFO in B-HK. As shown in Fig. 1, DFRA inhibited the total growth of the bacterium in a dose-dependent manner as reflected by a decrease of OD₆₀₀. The total growth of the bacterium was significantly inhibited by DFRA and DFO in the concentration range of 0.01–0.32 and 0.08–0.32 mg ml⁻¹, respectively. We calculated the doubling time of *P. intermedia* grown with either DFRA or DFO. Both DFRA (0.01–0.04 mg ml⁻¹) and DFO (0.08 mg ml⁻¹) prolonged the bacterial doubling time (Table 1). Supplementary ferric citrate partially attenuated the inhibitory effect of the iron chelators (Fig. 1, Table 1).

**Effect of iron chelators on biofilm formation**

We investigated the effect of the iron chelators on biofilm formation of *P. intermedia* by the crystal violet staining method using microtitre plates and compared the effect of the iron chelators to that of metronidazole, which is regarded as highly effective against black-pigmented *Prevotella* spp. (Ardila et al., 2010). In a preliminary study, we determined the metronidazole MIC (0.0035 mg ml⁻¹) for *P. intermedia* ATCC 49046 by the agar dilution method according to CLSI guidelines. *P. intermedia* ATCC 49046 cells were consistently able to form biofilm on the polystyrene surface of the microtitre plates over the 24 h incubation period. Metronidazole at 0.0004–0.0035 mg ml⁻¹ (1/8–1 × MIC) decreased the amount of *P. intermedia* biofilm to 82.5–10.4 % of the control (Fig. 2a). The amount of bacterial biofilm was also significantly decreased in the presence of DFRA. In the concentration range tested (0.01–0.16 mg ml⁻¹, corresponding to 1/16–1 × MIC), the decrease in the amount of the biofilm by DFRA was greater than that by metronidazole. Although DFO (0.08–0.32 mg ml⁻¹) led to a statistically significant reduction in the biomass of *P. intermedia* biofilm as compared to the control, the reduction at higher concentrations was much less than that by metronidazole and DFRA. Biofilm-forming activity was analysed by normalizing dye binding (A₆₀₀) to total bacterial growth (biofilm cells + planktonic cells). This showed that DFRA and metronidazole significantly decreased the biofilm-forming activity at 1 × their respective MICs while DFO showed no effect, even at 0.32 mg ml⁻¹ (Fig. 2b).

**Effect of iron chelators on production of EPS in culture**

Extracellular polymeric substance (EPS) is the primary matrix material of biofilm, and viscosity of culture supernatant is an indication of the relative amount of EPS production in culture (Denny et al., 1988; Donlan, 2002). To identify whether the iron chelators affect EPS production by *P. intermedia*, we measured the viscosity of the spent culture medium of the bacterium. A significant increase in the viscosity of the spent culture medium was observed as compared to that of the control B-HK medium after 24 h incubation (Fig. 3). The viscosity of the spent culture medium of the bacterial cells grown with subMICs of DFO, DFRA and metronidazole was statistically significantly increased as compared to that of the cells grown without the test agents.

**Effects of DFRA against pre-formed biofilms**

Bacteria that live inside biofilm are strongly resistant to antimicrobials (Stewart & Costerton, 2001; Donlan & Costerton, 2002; Fux et al., 2003; Smith, 2005; Eckert et al., 2006; Dong et al., 2012). High levels of antibiotic resistance were found in *P. intermedia* ATCC 49046 biofilms (Takahashi et al., 2006). To identify whether DFRA also has inhibitory activity against already established *P. intermedia* ATCC 49046 biofilms in vitro, we measured the biofilm formation of *P. intermedia* ATCC 49046 biofilms with 1–2×10⁸ cells ml⁻¹ were incubated anaerobically in the presence of DFO or DFRA at the indicated concentrations. The total growth of *P. intermedia* cells (planktonic + biofilm cells) was measured as OD₆₀₀ after a 24 h incubation. Results are expressed as mean ± SD of two independent experiments performed in triplicate. Supplementary ferric citrate at 0.05 mg ml⁻¹ is indicated as ‘+ Fe’. *, P<0.05 versus control; †, P<0.05 versus without ferric citrate.
viability by quantification of ATP bioluminescence, which reflects viable cell counts (Takahashi et al., 2006), and compared the inhibitory activity of DFRA against the biofilm to that of metronidazole. As shown in Fig. 4, metronidazole decreased the viability of P. intermedia biofilms in the concentration range 0.32–1.28 mg ml\(^{-1}\), while the biofilm viability was not decreased but rather significantly increased up to 2.56-fold in the presence of metronidazole at and below 0.16 mg ml\(^{-1}\). On the other hand, DFRA (0.02–1.28 mg ml\(^{-1}\)) significantly suppressed the biofilm viability in a concentration-dependent manner regardless of the presence or absence of ferric citrate. The broth-recovery-based biofilm MBC of both DFRA and metronidazole was 0.64 mg ml\(^{-1}\).

Effects of DFRA on cell morphology
Sessile cells of P. intermedia in the biofilms exhibited normal rod-shaped morphology with a cell length of \(~1.02\ \mu m\) (Fig. 5a). Treatment of the biofilm with metronidazole at 1/4 biofilm MBC (0.16 mg ml\(^{-1}\)) for 24 h did not cause any detectable change in biofilm cell shape (Fig. 5b). In contrast, sessile cells of P. intermedia in the biofilm treated with DFRA at 0.16 mg ml\(^{-1}\) were elongated (Fig. 5c) compared to control cells. Software-aided cell length measurement clearly demonstrated that the cells were significantly elongated (\(~1.4\ \mu m\), Fig. 5e). Cell elongation also appeared in the P. intermedia biofilm treated with DFRA even after the addition of ferric citrate (0.05 mg ml\(^{-1}\)) to the culture (Fig. 5d).

### Table 1. Doubling times of P. intermedia ATCC 49046 grown with or without iron chelators

Results are expressed as mean ± SD of two independent experiments performed in triplicate. Supplementary ferric citrate at 0.05 mg ml\(^{-1}\) is indicated as ’+ Fe’. ND, Not determined. *, P<0.05, †, P<0.02, versus control (0 mg ml\(^{-1}\)). ‡, P<0.05 versus without ferric citrate.

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<td>DFRA</td>
<td>5.7 ± 0.45</td>
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<td>6.28 ± 0.73</td>
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<td>DFRA + Fe</td>
<td>5.65 ± 0.74</td>
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<td>DFO + Fe</td>
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**Fig. 2.** Analyses of biofilm biomass and biofilm-forming activity of P. intermedia ATCC 49046 in the presence of iron chelators. P. intermedia cells were incubated in 96-well plates with DFO (○), DFRA (□) or metronidazole (■) at the indicated concentrations for 24 h, then biofilm biomass was quantified by crystal violet staining (a). Biofilm-forming activity was calculated as crystal violet staining normalized to total bacterial growth (b). Data are mean ± SD of two independent experiments performed in triplicate. The concentration of 1×MIC corresponds to 0.0035, 0.16 and 0.32 mg ml\(^{-1}\) for metronidazole, DFRA and DFO, respectively. Since DFO at concentrations up to 0.64 mg ml\(^{-1}\) was unable to inhibit the growth of P. intermedia on brucella blood agar according to CLSI guidelines, its MIC could not be determined. Therefore, the MIC of DFO used for the biofilm inhibition assay was arbitrarily chosen within the concentration range employed for the other assays. *, P<0.05 versus control; **, P<0.02 versus control.
Effects of iron chelators against P. intermedia

DISCUSSION

Black-pigmented Gram-negative anaerobic rods such as Porphyromonas gingivalis and Prevotella intermedia, referred to as BPB, are among the organisms that have been detected in subgingival plaque and have been implicated as the principal aetiologial agents of periodontitis (Clark et al., 1991; Tompkins et al., 1997). To survive in the diseased periodontal pocket the pathogens must be able to escape host defences and acquire sufficient nutrients to sustain their growth (Lewis, 2010). One such nutrient required for the growth of both species is iron, preferentially in the form of haemoprotein-derived haemin (iron protoporphyrin IX) (Gibbons & MacDonald, 1960; Tompkins et al., 1997; Leung et al., 1998; Lewis et al., 1999; Lewis, 2010). Black pigment on the cell surface of the species is believed to result from accumulation of haemin (Smalley et al., 1998a; Lewis et al., 1999; Smalley et al., 2003; Soukos et al., 2005). The redox potential of haemin, required as a prosthetic group of cytochrome b, allows it to mediate electron transfer with generation of cellular energy (Smalley et al., 1998b; Lewis et al., 1999).

DFO was discovered in the actinomycete Streptomyces pilosus, which produced a molecule that bound extracellular iron and was used for iron acquisition (Liu & Hider, 2002). DFO is a hexadentate siderophore from the family of hydroxamic acids, whose tris-hydroxymate groups bind Fe\(^{3+}\) with high affinity and specificity (Richardson & Ponka, 1998). DFRA is a tridentate chelator (Weiss et al., 2006) from the family of hydroxyphenyl triazoles that bind Fe\(^{3+}\) with high affinity and specificity with their phenolate oxygens and one triazole nitrogen atom (Heinz et al., 1999). The basic difference between the two chelators is that the hydrophilic DFO is a biologically derived product with large size [a partition coefficient (log \(P\)) value of −2.2, and molecular mass of 656.8 Da], whereas DFRA is a synthetic small molecule with high lipophilicity (log \(P\)-value of 3.8 and molecular mass of 373.4 Da) (Liu & Hider, 2002; Nick et al., 2002; Glickstein et al., 2005). The large size and hydrophilic nature of DFO impede its accessibility to iron within cells (Richardson et al., 1994; Cable & Lloyd, 1999), while the small size of the DFRA molecule allows its accessibility to iron within cells (Nick et al., 2002; Glickstein et al., 2005; Weiss et al., 2006). In the present study, DFRA was demonstrated to be bactericidal against P. intermedia, and even at subMICs, strongly interfered with total bacterial growth and biofilm formation of the bacterium. In contrast, DFO showed less effectiveness against the bacterial cells. DFO considerably prolonged the doubling time of P. intermedia cells but was not bactericidal at concentrations up to 0.64 mg ml\(^{-1}\).

Fig. 3. Viscosities of spent culture media obtained from P. intermedia ATCC 49046 in the presence of iron chelators. B-HK, control medium without bacterial inoculation; non-treated, bacterium grown with 1/4 MIC DFO; DFO, bacterium grown with 0.08 mg DFO ml\(^{-1}\); DFRA, bacterium grown with 1/4 MIC DFRA; MET, bacterium grown with 1/4 MIC metronidazole. Results are expressed as mean \(\pm\) SD of two independent cultures performed in triplicate. *, \(P<0.05\); **, \(P<0.02\).

Fig. 4. Effect of DFRA and metronidazole on the bioactivity of pre-formed P. intermedia biofilm. Established biofilms of P. intermedia ATCC 49046 were treated with metronidazole (MET) and DFRA at the indicated concentrations with or without 0.05 mg ferric citrate ml\(^{-1}\) (Fe). After 24 h incubation, the biofilm bioactivity was measured by ATP-based luminescence quantification, expressed in relative light units (RLU). The results are expressed as the mean \(\pm\) SD of two independent experiments performed in triplicate. *, \(P<0.05\); **, \(P<0.02\), versus control.
(Table 1), which is similar to the results observed with Porphyromonas gingivalis (Moon et al., 2011a). It is likely that the better antibacterial activity of DFRA against P. intermedia in comparison to DFO may be at least partly related to the small molecular size and high lipophilic character of DFRA, facilitating its entry into the bacterial cells and causing subsequent intracellular iron deprivation. Previously, we observed a significant decrease in the surface accumulation of haemin by P. gingivalis cells when the bacterial cells were exposed to DFO (Moon et al., 2011a). Accordingly, the colour of the bacterial cells grown on blood agar supplemented with haemin appeared brighter in the presence of DFO as compared to that of control, while the colour of the bacterial cells grown with DFRA was similar to that of control (our unpublished observation). All these findings may suggest that DFO mainly exerts antibacterial effect against BPB through iron/haemin chelation in the extracellular milieu because of its poor cell permeability. It should be noted that enhanced ability of lipophilic chelators to permeate membranes may render them potentially more toxic. Although available data on limited toxicity of DFRA in experimental animals are encouraging (Hershko et al., 2001), further studies examining its possible toxic side-effects in the clinical setting are still required.

Periodontal diseases are complex polymicrobial biofilm infections that involve interactions between a large subset of oral bacteria and the host (Palmer et al., 2011). It has become clear that biofilm-grown cells express properties distinct from planktonic cells, one of which is an increased resistance to antimicrobial agents (Mah & O'Toole, 2001). For some antibiotics, the concentration required to kill sessile bacteria in biofilm may be more than a thousand times that required to kill planktonic bacteria of the same strain (Olson et al., 2002). Biofilm-producing bacteria exhibit resistance to antimicrobial agents by various methods: restricted penetration of antimicrobial agents through the matrix of EPS they have produced; decreased metabolic activity; or expression of resistant genes (Rice, 2006; Tenover, 2006; Frank et al., 2007; Hassan et al., 2011). In the present work, P. intermedia ATCC 49046 cells grown in B-HK produced EPS, increasing the viscosity of the culture broth, and formed biofilm on the polystyrene surface of microtitre plates. A statistically significant increase was observed in EPS production by the bacterial cells grown with the iron chelators as well as metronidazole at subMICs (Fig. 3). As documented for the biofilm-forming bacterium Pseudomonas cepacia, in which exopolysaccharide synthesis was significantly increased in the presence of subMICs of ciprofloxacin (McKenney et al., 1994), the increased production of EPS in Prevotella intermedia exposed to subMICs of antimicrobial agents. As shown in Fig. 2(b), when the bacterial cells were treated with non-bactericidal DFO or bactericidal DFRA at subMICs (MBCs), no significant decrease was seen in the biofilm-forming activity (i.e. biofilm biomass normalized against total bacterial growth at each concentration), which may be attributed to the increased EPS-cell aggregation under the effect of the chelators at subMICs. However, DFRA at 1 × MIC significantly decreased the biofilm-forming activity. Moreover, treatment of the bacterial cells with subMICs (0.01–0.08 mg ml⁻¹, corresponding to 1/16–1/2 × MIC) of DFRA caused significant reduction of biofilm biomass to 62.3–7.6 % of the untreated control (Fig. 2a), which may be due to the decrease in the total

Fig. 5. DFRA-induced cell elongation of P. intermedia ATCC 49046. (a–d) SEM images of untreated P. intermedia biofilm (a) and biofilm treated with 0.16 mg metronidazole ml⁻¹ (b), 0.16 mg DFRA ml⁻¹ (c), or 0.16 mg DFRA ml⁻¹ and ferric citrate (d). Bars, 2 μm. (e) Mean cell length was determined from 15–20 randomly chosen cells per SEM image; mean ± SD is shown. *, P<0.01 vs control cells.
number of bacterial cells exposed to DFRA. Despite the adaptive response of *P. intermedia* cells, it is likely that DFRA still exerts an inhibitory effect on the total bacterial growth as well as the biofilm formation even at subMICs.

The biofilm MBC represents the lowest concentration that kills most of the biofilm cells and thus inhibits visually apparent bacterial growth in the suspensions. The biofilm MBC has been proposed to be used to cure biofilm-related infections at a chronic stage (Zhang & Mah, 2008; Qu et al. 2010). In the present study, the metronidazole MBC for the biofilm of *P. intermedia* ATCC 49046 was 0.64 mg ml\(^{-1}\), more than 180 times that for the planktonic cultures. Notably, the viability of the *P. intermedia* biofilm was not decreased but rather significantly increased up to 2.56-fold in the presence of metronidazole at and below 46 \(\mu\)g ml\(^{-1}\). Our finding is in agreement with a recently published study (Takahashi et al., 2006), in which although tetracyclines exhibited a strong effect against planktonic cells of *P. intermedia*, the antibiotics rather increased the bioactivities of biofilms formed by *P. intermedia* strains (ATCC 15032, ATCC 15033 and ATCC 49046, but not ATCC 25611). Especially, the bioactivities of *P. intermedia* ATCC 49046 biofilms treated with the tetracyclines even at 100 \(\times\) MIC increased about twice more than those of control groups. On the other hand, ofloxacin, which showed moderate effectiveness against planktonic *P. intermedia* cells, suppressed the biofilm bioactivities of *P. intermedia* ATCC 15032 and ATCC 15033 but not ATCC 49046 (Takahashi et al., 2006). These results illustrate the difficulty in treating *P. intermedia* biofilm-related infections. In this respect, the fact that DFRA is a drug with a long half-life (Cappellini, 2007) and exerts antibacterial effect against biofilm as well as planktonic *P. intermedia* cells even at subMICs may indicate its potential clinical advantages. As haemin mediates electron transfer with generation of cellular energy (Smalley et al., 1998b; Lewis et al., 1999), DFRA-induced haemin starvation in *P. intermedia* may be the direct cause of decrease of ATP in the bacterium (Fig. 4). The exact molecular mechanism underlying an increase in the viability of *P. intermedia* biofilm exposed to certain antibiotics, including metronidazole and tetracycline, has yet to be determined.

When the environment demands it, bacteria can accelerate or delay cell division and septation, thereby creating shorter or longer cells, respectively (Young, 2006). Studies also suggest that various bacteria respond to nutrient deprivation by filamentation (Webb, 1953; Pine & Boone, 1967; Wills & Chan, 1978; Rao et al., 2008), which increases their total surface area without an appreciable increase in the surface-to-volume ratio (Young, 2006). In the present study, unlike the short rods observed in the untreated (control) biofilms, *P. intermedia* cells in the DFRA-treated biofilms became elongated (Fig. 5). This may be due to iron limitation, resulting in division delay, as also demonstrated by the increased doubling times of planktonic cells exposed to DFRA (Table 1). It is noteworthy that ferric citrate abolished the effects of DFRA against planktonic *P. intermedia*, while the decreased viability and the elongated morphology of the bacterial cells in the DFRA-treated biofilms were not restored to the control state by ferric citrate (Figs 4 and 5). The simplest explanation for these results may be sequestration of ferric ion by the polyanionic polysaccharides constituting EPS (Donlan, 2002; Yamanaka et al., 2011).

Collectively, our results show that DFRA is an effective antimicrobial agent (MIC and MBC values of 0.16 mg ml\(^{-1}\)) against planktonic *P. intermedia*. DFRA also significantly decreased the biofilm formation of the bacterial cells and the bioactivity of pre-formed biofilms even at subMICs. By contrast, DFO showed less effectiveness against the bacterial cells. These results indicate that DFRA may be an alternative antibacterial agent for controlling *P. intermedia*. It is noteworthy that DFO was reported to enhance polymorphonuclear leukocyte function (van Asbeck et al., 1984) and was effective in tissue protection and anti-inflammation (Lauzon et al., 2006; Hanson et al., 2009). As periodontitis is a disease in which comprehensive treatment should ideally combine control of inflammation and periodontal destruction with tissue repair promotion (Escartin et al., 2003), combined use of DFO and DFRA for controlling bacterial infection and preventing tissue damage seems a fascinating strategy in the prevention and treatment of periodontal disease. However, the use of DFO and DFRA is not recommended by the drug manufacturers in patients with serum ferritin levels lower than 0.5 mg l\(^{-1}\) because of possible toxicity implications (Kontoghiorghes et al., 2010). In fact, the risk of several complications in non-iron-overloaded patients (no excessive accumulation of iron in the body) systemically receiving iron-chelating agents has been described (Weinberg, 1990; Bentur et al., 1991). These negative attributes of the iron chelators may limit their utility as systemic agents for periodontal disease, but their clinical usefulness as local agents for the disease would not be limited. The future use of iron chelators will require careful studies on both efficacy and toxic effects. Further research is also needed to study whether the iron chelators exert inhibitory effects against multi-species oral biofilms and to evaluate the efficacy of DFRA in combination with DFO in patients with periodontitis.

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**REFERENCES**


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