Iron-binding compounds impair *Pseudomonas aeruginosa* biofilm formation, especially under anaerobic conditions

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The success of *Pseudomonas aeruginosa* in cystic fibrosis (CF) and other chronic infections is largely attributed to its ability to grow in antibiotic-resistant biofilm communities. This study investigated the effects of limiting iron levels as a strategy for preventing/disrupting *P. aeruginosa* biofilms. A range of synthetic and naturally occurring iron-chelating agents were examined. Biofilm development by *P. aeruginosa* strain PAO1 and CF sputum isolates from chronically infected individuals was significantly decreased by iron removal under aerobic atmospheres. CF strains formed poor biofilms under anaerobic conditions. Strain PAO1 was also tested under anaerobic conditions. Biofilm formation by this model strain was almost totally prevented by several of the chelators tested. The ability of synthetic chelators to impair biofilm formation could be reversed by iron addition to cultures, providing evidence that these effective chelating compounds functioned by directly reducing availability of iron to *P. aeruginosa*. In contrast, the biological chelator lactoferrin demonstrated enhanced anti-biofilm effects as iron supplementation increased. Hence biofilm inhibition by lactoferrin appeared to occur through more complex mechanisms to those of the synthetic chelators. Overall, our results demonstrate the importance of iron availability to biofilms and that iron chelators have potential as adjunct therapies for preventing biofilm development, especially under low oxygen conditions such as encountered in the chronically infected CF lung.

INTRODUCTION

Cystic fibrosis (CF) individuals are highly susceptible to chronic lung infection by the opportunistic environmental bacterium *Pseudomonas aeruginosa* (Lyczak et al., 2002). This organism is thought to persist by forming biofilms within the hypoxic mucus of the CF lung. Bacteria growing in biofilms exhibit increased resistance to antimicrobials and the host immune response compared to their free-living, planktonic counterparts. Once established, biofilm-dwelling bacteria are virtually impossible to eradicate with existing therapies (Ceri et al., 1999; Singh et al., 2000; Worlitzsch et al., 2002). To improve the quality of life for CF individuals, novel strategies for preventing or delaying *P. aeruginosa* differentiation into the biofilm growth mode are urgently needed.

Reducing iron availability has been proposed as a potential means to impair *P. aeruginosa* biofilm development (Banin et al., 2006; Musk et al., 2005). We recently demonstrated a strong positive correlation between sputum iron content and quantitative load of *P. aeruginosa* infection. Moreover, we found that the iron content of airway secretions in CF is elevated in patients from whom *P. aeruginosa* has not been isolated, suggesting that increased iron in the CF lung may be a critical factor in facilitating initial acquisition and then chronicity (Reid et al., 2007). These *in vivo* observations support the hypothesis that limiting bacterial iron availability in the CF lung may be a strategy that could be used to disrupt the success of *P. aeruginosa*.

The recent publication by Moreau-Marquis et al. (2008) demonstrating that cultured CF airway epithelial cells promote *P. aeruginosa* biofilm formation because of an inherent problem with epithelial cell iron sequestration and luminal loss support our avenue of investigation into iron chelation. There are also emerging reports on the importance of iron to *P. aeruginosa* biofilm formation,

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Abbreviations: 2DP, 2,2'-dipyridyl; CF, cystic fibrosis; DM, deferoxamine mesylate; DTPA, diethylenetriaminepentaacetic acid; EDDA, ethylenediamine-N,N,N',N'-diacetic acid.
although the findings from recent studies that have looked at the biofilm effects of limiting iron or adding large amounts of iron have provided conflicting results (Moreau-Marquis et al., 2008; Musk et al., 2005; Musk & Hergenrother, 2008; Patriquin et al., 2008; Yang et al., 2007). A factor that has been poorly considered by nearly all studies that have recently looked at strategies to interfere with P. aeruginosa iron homeostasis and inhibit biofilm formation is the environment in which such treatments may be deployed, i.e. iron replete and either microaerobic or frankly anaerobic (Reid et al., 2007; Worlitzsch et al., 2002).

In this study, we therefore evaluated a range of synthetic and biological iron chelators for their abilities to affect P. aeruginosa model strain PAO1 (originally isolated from a burn wound and widely used to model biofilms in CF lung disease) growth and biofilm development. Experiments were conducted in a variety of biofilm models with or without supplementary iron and under aerobic and anaerobic atmospheres to assess effectiveness under atmospheric conditions similar to those found in the CF lung. Clinical CF strains were also examined, but our previous published data demonstrate that the short-term tube model is not ideal for assessing biofilm formation under anaerobic conditions because CF strains are slow-growing in this setting (O'May et al., 2006). Clinical CF strains were therefore only examined under aerobic conditions.

METHODS

Bacterial strains and growth conditions. P. aeruginosa strain PAO1, originally isolated from a burn wound, was provided by Dr R. Alm from the Centre for Molecular and Cellular Biology, Australia (Holloway, 1955). CF clinical strains were isolated from the sputum samples of adult CF individuals with chronic infection, as described previously (O'May et al., 2006). All CF strains (3A, 18A, 50A, 75A) were mucoid on first isolation, as is typical with chronic infection. As discussed, our previous published experiments with CF clinical strains demonstrate that they are relatively slow-growing and poor at forming biofilms in short-term models, especially under anaerobic conditions. In this study, we therefore concentrated on strain PAO1 to test our hypotheses as a proof of concept. However, to examine the potential clinical effectiveness of chelation therapy, we assessed the previously well-characterized CF strains 3A, 18A, 50A and 75A in the borosilicate tube method under aerobic conditions (O'May et al., 2006).

Strains were routinely grown from storage on tryptone soy agar (1.2%, w/v) supplemented with 0.6% yeast extract (TSAY Oxoid) for 16–18 h under aerobic conditions. Growth and biofilm assays were conducted in minimal medium with succinate (MMS; Meyer & Abdallah, 1978). To permit anaerobic growth of the strains, 1% KNO₃ was added to all culture media as nitrate provides a terminal electron acceptor for anaerobic respiration (Worlitzsch et al., 2002). Iron (FeCl₃) was supplemented as indicated. Experiments in the continuous culture flow-through model were conducted in M9 minimal medium (Miller, 1972). Anaerobic incubation was undertaken in anaerobe jars or bags using anaerogen compacts (Oxoid). Bacterial growth experiments and short-term biofilm assays were undertaken in MMS.

The synthetic iron chelators used were: 2,2'-dipyridyl (2DP), diethylentriaminepentacetic acid (DTPA), EDTA, deroxamine mesylate (DM) and ethylenediamine-N,N'-diacetic acid (EDDA). Experiments were conducted over a range of chelator concentrations (0–2500 μM). Unless otherwise stated, experiments were conducted in MMS medium supplemented with 10 μM FeCl₃.

Biofilm assays. Biofilm formation was examined in a borosilicate glass tube screening assay and selected strains were also tested in a glass coverslip model, as described below (O’Toole et al., 1999). For the tube model, overnight P. aeruginosa cultures were diluted 1:100 into fresh LB broth plus nitrate (LBN) medium. Aliquots of each culture dilution were dispensed into three borosilicate glass tubes (300 μl) and incubated (37°C, 24 h) under aerobic and anaerobic conditions. Excess broth was removed and used to quantify planktonic growth, and biofilms were washed and stained with 1% (w/v) crystal violet (violet crystallisate, Reactifs R-24) for 15 min at room temperature. After vigorous washing with water, the stained biofilms were dissolved in 100% ethanol and the A₅₇₀ of 200 μl aliquots was determined in a microplate reader (Bio-Rad). Aliquots of broth culture prior to staining were used as an indicator of the level of planktonic growth.

For the anaerobic glass coverslip model, aliquots (500 μl) of ~10⁶ bacteria in LBN from overnight cultures standardized as per the previous published data demonstrate that the short-term tube model is not ideal for assessing biofilm formation under anaerobic conditions because CF strains are slow-growing in this setting (O’May et al., 2006). Clinical CF strains were therefore only examined under aerobic conditions.

Continuous culture flow-through model. Biofilms were cultivated at room temperature (22°C) in three-channel glass flow-cells with individual channel dimensions of 0.3 x 4 x 40 mm as described elsewhere (Kirov et al., 2007; Webb et al., 2003). Flow-cell medium was M9 (glucose as carbon source). For inoculation of bacteria, medium flow was arrested and the inoculum (0.5 ml of overnight grown broth culture in MMS medium) was inoculated into the flow-cell. After inoculation, flow-cells were inverted (2 h) to allow bacterial adherence. The flow-cell was then returned upright and media flow was started at 0.4 mm s⁻¹ (1 h) to wash through any non-adherent bacteria. Flow was then maintained at a velocity of 0.2 mm s⁻¹. To determine the effects of the chelator 2DP on established biofilms, the chelator was incorporated into the flow-cell medium at either day 2 or day 3 and the biofilms were subsequently analysed at days 4 and 7, respectively.

Image analysis of biofilm formation. Image stacks of biofilms were acquired using a confocal laser scanning microscope equipped with a krypton–argon laser containing filters to monitor the green fluorescence of SYTO 9 (excitation 480 nm, emission 500 nm) and red fluorescence of propidium iodide (excitation 490 nm, emission 635 nm) used for viability staining. Three-dimensional images were acquired using the f900e software version 1.6.0 and collated to allow analysis with the software program Image J 1.33u (Wayne Rasband, National Institutes of Health, USA). Measurements undertaken included biomass (the overall volume) of the biofilm, mean thickness and surface area coverage. Identical threshold values were used for all analyses.

Growth assay. Aliquots (500 μl) of ~10⁶ bacteria in MMS (as per the coverslip biofilm assay) were inoculated into 24 borosilicate glass tubes (12 x 75 mm). Half of these were incubated under aerobic conditions and half under anaerobic conditions (37°C, shaking).
Three tubes for each strain were removed at intervals (8, 16, 24 and 48 h) and vortexed with glass beads (1 min) to remove any glass-adherent bacteria. The $A_{570}$ of the culture was then determined and used as an indicator of the level of growth.

**Statistical analysis.** Microsoft Excel X 2001 was used to calculate the means and standard deviations of the results. The significance of the results was determined using the two-tailed Student’s $t$ test and a $P$-value of less than 0.05 was considered significant.

**RESULTS AND DISCUSSION**

**Iron requirements for growth and biofilm formation were higher under anaerobic conditions**

The influence of iron on *P. aeruginosa* strain PAO1 aerobic and anaerobic growth was determined in MMS broth supplemented with 0, 10, 100 or 1000 $\mu$M FeCl$_3$. Under both atmospheres, the level of growth increased as the iron concentration increased (Fig. 1a). *P. aeruginosa* demonstrated a higher requirement for iron under anaerobic conditions than under aerobic conditions. Thus, while moderate growth levels were seen aerobically even in the absence of supplementary iron, strain PAO1 was unable to grow in an anaerobic atmosphere without additional iron (Fig. 1a). Furthermore, anaerobic growth was much slower than aerobic growth at equivalent iron concentrations (Fig. 1a).

The effect of iron levels on strain PAO1 aerobic and anaerobic biofilm formation was determined in the borosilicate glass tube assay. Under aerobic conditions, biofilms formed regardless of the level of iron in the medium (24 h; Fig. 1b). It would appear that in these assays there is enough residual iron present in the MMS to satisfy the organism’s growth requirements (MMS medium <1 $\mu$M FeCl$_3$) (Takase et al., 2000). In contrast, under anaerobiosis, strain PAO1 required iron supplementation to form biofilms at 24 h (Fig. 1a) and also at 48 h (data not shown). This finding was consistent with the growth rates observed in these cultures (Fig. 1a). Furthermore, the robust biofilms that formed anaerobically at 10 and 100 $\mu$M FeCl$_3$ were significantly reduced ($P<0.05$) with the addition of 1000 $\mu$M FeCl$_3$, implying that anaerobically too much iron is detrimental to biofilm formation (but not growth). Similarly, this anaerobic biofilm requirement for iron supplementation was demonstrated in the glass coverslip model (data not shown).

These results demonstrate that *P. aeruginosa* has a higher iron requirement for growth and biofilm development under anaerobic conditions than under aerobic conditions. This result was unexpected as under anaerobic conditions iron exists in the soluble and easily assimilable ferrous (Fe$^{2+}$) form (Vasil & Ochsner, 1999). Intuitively, one would expect *P. aeruginosa* growth to be promoted under anaerobic conditions, but there was a threshold level for...
iron below which *P. aeruginosa* would not grow. The explanation may lie in the additional requirement for Fe$^{3+}$ as a terminal electron acceptor in the process of denitrification during anaerobic respiration. It may also take time anaerobically for *P. aeruginosa* to convert the supplementary Fe$^{3+}$ used in our experiments to the generally usable Fe$^{2+}$ form (Nealson & Saffarini, 1994). Consistent with this, green pigment indicative of pyoverdine production was only observed under aerobic growth conditions (data not shown), and there are in fact very few data on *P. aeruginosa* iron acquisition under anaerobic conditions, but this needs further examination (Vasil, 2007; Vasil & Ochsner, 1999).

**Synthetic iron-binding compounds impaired biofilm formation, especially under anaerobic conditions**

The chelators 2DP, DTPA and EDTA significantly impaired biofilm formation (MMS + 10 μM FeCl$_3$) under both aerobic and anaerobic atmospheres at this iron concentration ($P < 0.05$) (Fig. 2). Overall, these chelators were most effective at impairing biofilms under anaerobic conditions and the inhibition occurred at much lower chelator concentrations than were required for the same effect aerobically. Indeed, some biofilm formation still occurred under aerobic conditions despite high concentrations of iron chelators (Fig. 2). Significant impairment was also seen when FeSO$_4$ (FeII) was used as the iron source instead of FeCl$_3$ (FeIII) (data not shown). This is consistent with the increased iron requirements observed for *P. aeruginosa* growth under anaerobic conditions. Overall, the chelators DM and EDDA did not affect aerobic or anaerobic biofilm formation at the concentrations tested ($P > 0.05$) (Fig. 2) and we therefore did not investigate these compounds further.

In parallel, iron chelator effects on growth levels were also determined (arrows, Fig. 2). For 2DP and DTPA, the concentrations that impaired the level of biofilm formation also inhibited bacterial growth (Fig. 2). EDTA similarly impaired growth, but unlike 2DP and DTPA, lower concentrations of EDTA prevented biofilm development independently of direct inhibition of *P. aeruginosa* growth (Fig. 2).

The different effects on biofilm formation probably relate to the chemical structure and iron-binding coefficients of the individual synthetic chelators. The synthetic chelator DM, for instance, has a low affinity for Fe$^{2+}$ (7.2) compared to 2DP, DTPA or EDTA, and the ability of *P. aeruginosa* siderophores and proteases to acquire iron from DM is well described (Brock et al., 1988; Liu & Hider, 2002). The efficiency of synthetic chelators will, therefore, depend on their ability to out-compete *P. aeruginosa* for iron. Other recently published work also supports the finding that interference with iron acquisition prevents biofilms. Kaneko et al. (2007) demonstrated that addition of gallium (structurally similar to iron) to cultures was able to inhibit biofilms.

**Effects of biological iron-binding compounds on biofilm development**

The biological chelators lactoferrin, apo-transferrin and conalbumin were also assessed as above. Due to differences in molecular mass of these compounds compared to the synthetic compounds, they were evaluated at lower concentrations. Of the transferrin-like molecules tested, lactoferrin was the only compound to significantly impair biofilm formation (Fig. 3). As observed with the synthetic chelators, biofilm inhibition with lactoferrin was more pronounced under anaerobic conditions. However, at the concentrations tested, lactoferrin was relatively ineffective at impairing biofilm formation and the inhibition was quite variable (Fig. 3). In contrast to the synthetic chelators, lactoferrin did not impair bacterial growth levels at the concentrations tested.

**Anaerobic biofilm impairment using the glass coverslip assay**

The synthetic chelators that had been most effective in the borosilicate glass tube assay (2DP, DTPA and EDTA) and the biological chelator lactoferrin were tested under anaerobic conditions in the coverslip assay. In the absence of a chelator, *P. aeruginosa* formed a thick biofilm with little exposed substratum, but when either 2DP, DTPA or EDTA was present, the resultant surface growth consisted of only a few bacteria attached to the surface (Fig. 4). Subsequently, when the surface grown cultures were analysed with COMSTAT, those grown in the presence of chelators showed significant reduction in biomass, mean thickness and surface area coverage compared to cultures grown without a chelator present ($P < 0.05$) (Table 1). With lactoferrin, despite similar trends, there remained areas of thick biofilm coverage on the coverslip and the overall reduction in biomass, mean thickness and surface area coverage was not significant (Table 1). Overall, however, these data confirmed the inhibitory effect of synthetic iron chelators on anaerobic biofilm formation using an alternative model.

**Disruption of mature biofilms by 2DP**

Continuous-culture, flow-cell model experiments tested the ability of 2DP (2500 μM) to inhibit biofilm formation, 2DP being the most effective biofilm inhibitory compound identified in previous experiments. Bacterial attachment and biofilm development in the flow-cell initially occurs under aerobic conditions, but as biofilms mature, micro-colony interiors become anaerobic (Stewart & Franklin, 2008). Biofilms were grown for 2 or 3 days before flow-cell medium was supplemented with 2DP. Biofilms were subsequently analysed 2 and 4 days later, respectively (i.e. 4 days and 7 days from the start of the experiment). Strain PAO1 formed a thick biofilm that covered the entire
The surface of the flow-cell coverslip when no chelator was present. In contrast, the addition of 2DP resulted in biofilms that were significantly thinner than controls at both 4 and 7 days (*P*, 0.05) (Fig. 4). No significant differences were observed in the surface area coverage of these biofilms. This demonstrates that in the presence of 2DP, *P. aeruginosa* was still able to attach and form an adherent uniform monolayer on the surface, but was unable to form a robust three-dimensional biofilm.

**CF lung isolates were susceptible to chelator-induced biofilm inhibition**

The effects of chelators 2DP, DTPA (both 1250 μM) and lactoferrin (6 μM) on CF strain biofilm formation were only investigated under aerobic conditions (borosilicate glass tube assay), based on our previous observations of their decreased abilities to form biofilms in short-term models (O’May *et al.*, 2006). To counteract the slower
growth of CF isolates (strains 3A, 18A, 50A and 75A), biofilm measurements were taken at 48 h.

For CF isolates 3A, 18A and 75A, biofilm formation was significantly decreased by at least two of the three chelators (Fig. 5). Interestingly, the effectiveness of each individual chelator differed depending on the particular isolate assessed. Biofilm development by strain 50A appeared particularly susceptible to chelator inhibition, irrespective of the compound assessed. As reported with strain PAO1, 2DP and DTPA significantly impaired bacterial growth ($P < 0.05$), but lactoferrin did not impair the growth of any of the clinical isolates (data not shown).

**Synthetic chelators were less effective at impairing anaerobic biofilms as iron levels increased**

To determine whether the chelators were decreasing strain PAO1 anaerobic biofilm formation through direct iron chelation, efficacy was retested in the presence of added iron in the borosilicate glass tube assay. The effective chelators [2DP, DTPA, EDTA (all 1250 μM)] were examined in the presence of MMS supplemented with 10, 25, 50, 100 and 500 μM FeCl$_3$.

The chelators (2DP, DTPA and EDTA) became less effective at impairing anaerobic biofilm development as iron supplementation was increased (Fig. 6a). At high iron levels, the chelators 2DP and DTPA did not affect biofilm formation at all. In contrast, EDTA was still able to significantly inhibit biofilm formation at these high iron levels ($P < 0.05$), but it was still less effective than under iron limitation. This is in agreement with data presented in Fig. 1, where EDTA prevented biofilm formation at lower concentrations compared to the inhibition seen with 2DP and DTPA.

Lactoferrin appeared to modulate anaerobic biofilm formation in the presence of high iron by a different mechanism to that seen with the synthetic chelators. At low iron levels, lactoferrin was ineffective at inhibiting biofilms, yet as iron levels increased, lactoferrin effectiveness increased rather than decreased. Indeed, at iron levels of 250 and 500 μM FeCl$_3$, biofilms were completely inhibited (Fig. 6a) in spite of the fact that overall bacterial growth...
**Fig. 4.** Effect of 2DP on long-term aerobic biofilm formation. Biofilms were grown in the flow-cell model (4 and 7 days) without or with 2DP (2500 μM). (a) Representative images of the biofilms. (b) Mean thickness and surface area coverage of the biofilms grown without (black columns) and with (grey columns) 2DP. Values shown represent the mean ± SD of triplicate experiments carried out in separate channels at the same time, with five images analysed per channel. *Denotes significantly poorer thickness of the biofilms grown with 2DP compared to biofilms grown without 2DP (P < 0.05).

**Table 1.** Analysis of *P. aeruginosa* biofilms grown on glass coverslips (anaerobic atmosphere, 24 h, 37 °C)

<table>
<thead>
<tr>
<th></th>
<th>No chelator</th>
<th>DTPA (1250 μM)</th>
<th>2DP (1250 μM)</th>
<th>EDTA (1250 μM)</th>
<th>Lactoferrin (6 μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biomass (μm³ μm⁻²)</td>
<td>15.14 ± 5.32</td>
<td>0.65 ± 0.42 *</td>
<td>0.66 ± 0.33 *</td>
<td>0.26 ± 0.07 *</td>
<td>12.35 ± 4.46</td>
</tr>
<tr>
<td>Mean thickness (μm)</td>
<td>11.66 ± 4.50</td>
<td>0.68 ± 0.46 *</td>
<td>0.77 ± 0.45 *</td>
<td>0.17 ± 0.04 *</td>
<td>10.55 ± 4.62</td>
</tr>
<tr>
<td>Surface area (μm²)</td>
<td>1.05 × 10⁶ ± 5.19 × 10⁵</td>
<td>9.61 × 10⁵ ± 5.98 × 10⁴ + 1.44 × 10⁵ ± 6.61 × 10⁴</td>
<td>7.32 × 10⁵ ± 1.31 × 10⁴</td>
<td>6.69 × 10⁵ ± 1.73 × 10⁵</td>
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*Denotes significantly poorer values in comparison to that seen when no chelator was present (P < 0.05).

**Fig. 5.** Effect of iron chelation on aerobic biofilm formation by CF isolates. Level of aerobic biofilm formation by CF isolates (3A, 18A, 50A and 75A) without and with different iron-chelating compounds (48 h). Values shown represent the mean level of biofilm formation ± SD of two experiments, with three replicate tubes per experiment. *Denotes significantly poorer biofilms in the presence of the chelator than with no chelator (P < 0.05).
was promoted (Fig. 6b). This anti-biofilm effect was not related to increased iron levels per se because biofilms were still able to form at high iron levels in the absence of any chelator (Fig. 6a). These data confirm previous reports of lactoferrin possessing anti-biofilm activity. Yet our results suggested that the mechanism of this inhibition is not simply by iron removal as it can for the synthetic chelators (Singh et al., 2002; Weinberg, 2004). Our control experiments demonstrated that biofilm formation under aerobic conditions was minimally affected by increasing iron concentrations. In contrast, published work has suggested that very high iron concentrations can themselves inhibit biofilm formation (Musk et al., 2005); however the iron concentrations used in these studies would likely be detrimental if administered to lungs already subject to increased levels of iron and a high burden of oxidative stress. Under anaerobic conditions, there was less promotion of biofilm formation with 1000 μM FeCl₃ compared to lower concentrations, but even at this level of supplementation, iron was still clearly facilitating robust biofilm development.

Taken overall, the results indicate that iron-chelation therapy may be a particularly effective anti-
P. aeruginosa biofilm strategy in the CF lung where environmental conditions of low oxygen and high iron content are well described (Costerton, 2002; Reid et al., 2004). A range of synthetic iron-chelating molecules and the biologically occurring chelator lactoferrin were able to substantially impair biofilm formation accompanied by variable inhibition of bacterial growth under such conditions. Synthetic and biological chelators appeared to work by very different mechanisms, which may suggest potentially complementary actions, but this needs further investigation. Furthermore, future treatment would need to be tailored according to whether the aim was to either prevent initial establishment of infection or target chronic infections. It was possible to overcome the inhibitory effects of the synthetic chelators with increasing the amount of supplementary iron, and before iron chelators can be considered as a potential therapy in CF, the possibility that the increased levels of iron reported in the CF airway (17–134 μM) will limit efficacy in vivo needs to be explored (Reid et al., 2004; Stites et al., 1998).

In addition, clinical strains responded differently to different chelators. It may be that combinations of iron chelators would prove most effective clinically. Further research into the mechanisms and efficacy of chelation is clearly warranted to develop it as an effective adjunct therapy in CF.

Fig. 6. Effect of iron chelators on biofilm formation in different iron levels under anaerobic conditions. Biofilm levels by effective iron-binding compounds in MMS supplemented with different concentrations of exogenous iron (FeCl₃). (a) Columns represent the level of biofilm formation that occurred at the different iron levels without a chelator (black columns) and with the chelator (grey columns). (b) Planktonic growth levels in the presence of lactoferrin and different concentrations of exogenous iron (FeCl₃). Values shown represent the mean level of biofilm formation or growth ± SD of three experiments, with three replicate tubes per experiment. *Denotes significantly poorer biofilm levels than that seen in MMS + 10 μM FeCl₃ without chelator (P < 0.05). #Denotes significantly higher growth levels than that seen in MMS + 10 μM FeCl₃ without chelator (P < 0.05).
ACKNOWLEDGEMENTS

This work was supported by the National Health and Medical Research Council (NH&MRRC), Australian Cystic Fibrosis Research Trust (ACFRT), Royal Hobart Hospital Research Foundation (RHHRF) and University of Tasmania Institutional Research Grant Scheme (IRGS).

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