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Iron sequestration by human lactoferrin stimulates *P. aeruginosa* surface motility and blocks biofilm formation

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Abstract

This paper summarizes a presentation at the 6th International Conference on Lactoferrin Structure and Function. Some of this data has been previously published.

Introduction

Interactions between humans and bacteria range from mutualistic symbiotic relationships that benefit both parties, to acute fulminant infections in which the pathogens rapidly kill. Chronic infections lie somewhere between these extremes. A key factor enabling many of these 'host-pathogen stalemates' is that the bacteria live in biofilms (Costerton *et al.* 1983; Lam *et al.* 1980; Singh *et al.* 2000a).

Biofilms are groups of bacteria, living associated with a surface, encased in a polymeric matrix (Costerton *et al.* 1999). Infections caused by biofilms include endocarditis; osteomyelitis; certain skin, urinary and biliary tract infections; dental caries; medical device infections; and the chronic *P. aeruginosa* airway infections that afflict patients with cystic fibrosis (Costerton *et al.* 1983; Costerton *et al.* 1999; Donlan & Costerton 2002; Gristina *et al.* 1985; Singh *et al.* 2000a). Biofilm infections are extremely difficult to eradicate in spite of the fact that the bacteria are sensitive to killing (when cultured *ex vivo*), and high concentrations of antibiotics reach the site of infection (Geller *et al.* 2002) (Cremieux *et al.* 1989; Joly *et al.* 1987).

Biofilm growth gives pathogens many advantages

Compared to the free-living (planktonic) state, *P. aeruginosa* living in biofilms exhibit significant differences in gene expression and physiology (Sauer *et al.* 2002; Whiteley *et al.* 2001). The most notorious biofilm characteristic is remarkable resistance to killing; biofilms can be up to 1000 times more resistant than the same bacteria in the planktonic state (Stewart 2002). This includes resistance to pharmaceutical antibiotics (Stewart 2002), phagocytic cells (Jensen *et al.* 1992; Jensen *et al.* 1990), as well as nonspecific biocides (Takeo *et al.* 1994). Importantly, this resistance depends upon residence within the biofilm; growing the organisms in the planktonic state restores sensitivity (Stewart 2002). While the mechanisms are not completely understood, resistance likely results from a combination of decreased penetration of the antimicrobial, slow growth by some biofilm bacteria, and other poorly defined physiological changes (Costerton *et al.* 1983; Stewart 2002).

How do normally sterile mucosal surfaces protect against biofilm infections?

The extraordinary resistance of biofilms raises the question as to how the host protects against biofilm infections. Rapid elimination of organisms by mechanisms such as the lung's mucociliary escalator and other innate defenses likely accounts for much of this resistance (Ganz 2001; Travis *et al.* 2001; Welsh & Mason 2001). There are times, however, that bacteria remain on mucosal surfaces for prolonged periods. Even uncomplicated cases of bronchitis, conjunctivitis, urinary and other infections can last days or even weeks.

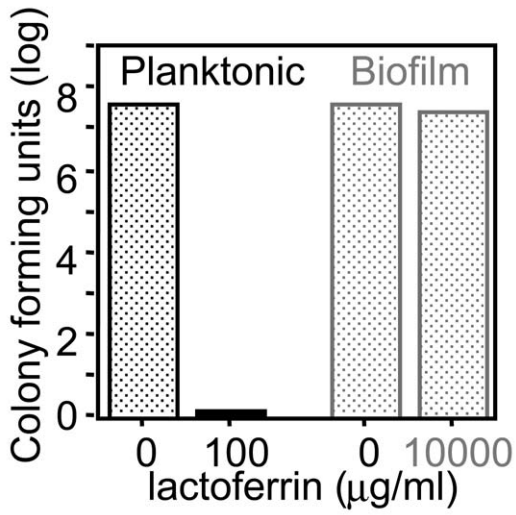

Fig. 1. Susceptibility of planktonic and biofilm P. aeruginosa to killing by human lactoferrin. Biofilms were grown with the rotating disc reactor (see, Singh *et al.*, 2002) that generates uniform biofilms on small removable discs. *P. aeruginosa* biofilms resisted 100 fold higher concentrations of lactoferrin than the same bacteria in the planktonic state.

This fact led us to hypothesize that mucosal surfaces might possess defenses that can kill biofilm bacteria. A number of potent innate antimicrobial factors are present on mucosal surfaces. These include lactoferrin, lysozyme, defensins, surfactant proteins and others (Bals *et al.* 1998; Cole *et al.* 1999; Coonrod 1986; Ganz 2001; Singh *et al.* 1998; Singh *et al.* 2000b). We chose to evaluate lactoferrin because it is among the most abundant of these antimicrobial factors and it is present in most every mucosal secretion (Abe *et al.* 1999; Bard *et al.* 2003; Hirai *et al.* 1990; Thompson *et al.* 1990). Furthermore, it is known to have pleotrophic host defense effects including inhibiting the growth of microorganisms by sequestering iron, killing bacteria by iron independent mechanisms, and anti-inflammatory actions (Vorland 1999).

To examine whether lactoferrin killed *P. aeruginosa* biofilms we utilized a growth reactor that generates uniform biofilms for susceptibility testing (we have previously described methods in (Singh *et al.* 2002)). Figure 1 shows in biofilms, *P. aeruginosa* were more than a hundred times more resistant to killing by lactoferrin as compared to the same bacteria in the planktonic state. Thus, the extraordinary resistance of biofilms extends to a key mucosal defense factor as well as pharmaceutical antibiotics.

This finding suggested another possibility, that innate antimicrobial factors like lactoferrin may function to prevent biofilm formation by bacteria that escape initial killing. To test this hypothesis, we examined the effect of a subinhibitory concentration of lactoferrin (20 μg/ml) on biofilm development. This concentration of lactoferrin did not affect the growth rate of *P. aeruginosa* in the medium used for these experiments.

To evaluate the effect of lactoferrin on biofilm formation, we grew *P. aeruginosa* expressing green fluorescent protein (gfp) in continuous culture flow cells and followed biofilm development over time (methods described in (Singh *et al.* 2002)). In medium without lactoferrin, the prototypical stages of biofilm development were observed. The bacteria attached to the surface and subsequently formed microcolonies. Microcolonies are clusters of attached bacteria that form in the early stages of biofilm development. In the absence of lactoferrin, microcolonies subsequently matured into towering pillar and mushroom shaped biofilms (Figure 2A). In the presence of subinhibitory concentrations of lactoferrin (20 μg/ml), the bacteria attached and multiplied, but they failed to form microcolonies or differentiated biofilm structures (Figure 2B). Importantly, this anti-biofilm action occurred at concentrations of lactoferrin well below those required for killing or inhibiting the growth of these organisms.

To investigate the mechanism of lactoferrin's anti-biofilm action, we examined the behavior of the bacteria during biofilm development using time-lapse microscopy. The analysis of these movies revealed that lactoferrin stimulated a type of bacterial surface locomotion known as twitching motility. This stimulated motility had a dramatic effect on biofilm development. In twitching motility, bacteria attach to a surface using a specialized appendage, the type IV pilus (Mattick 2002). Retraction of the pilus results in locomotion over surfaces (Skerker & Berg 2001). In the absence of lactoferrin, the daughter cells of attached bacteria remained localized near the point of parent cell division. When the daughter cells subsequently divided, their progeny also remained near the point of the original cell division (represented in Figure 3). Through this process microcolonies, the precursors to mature biofilms, began to form. In the presence of lactoferrin, daughter cells moved away from the point of parental cell division, hence microcolonies were unable to form. Interestingly, iron-saturated lactoferrin did not prevent *P. aeruginosa* biofilm formation indic-

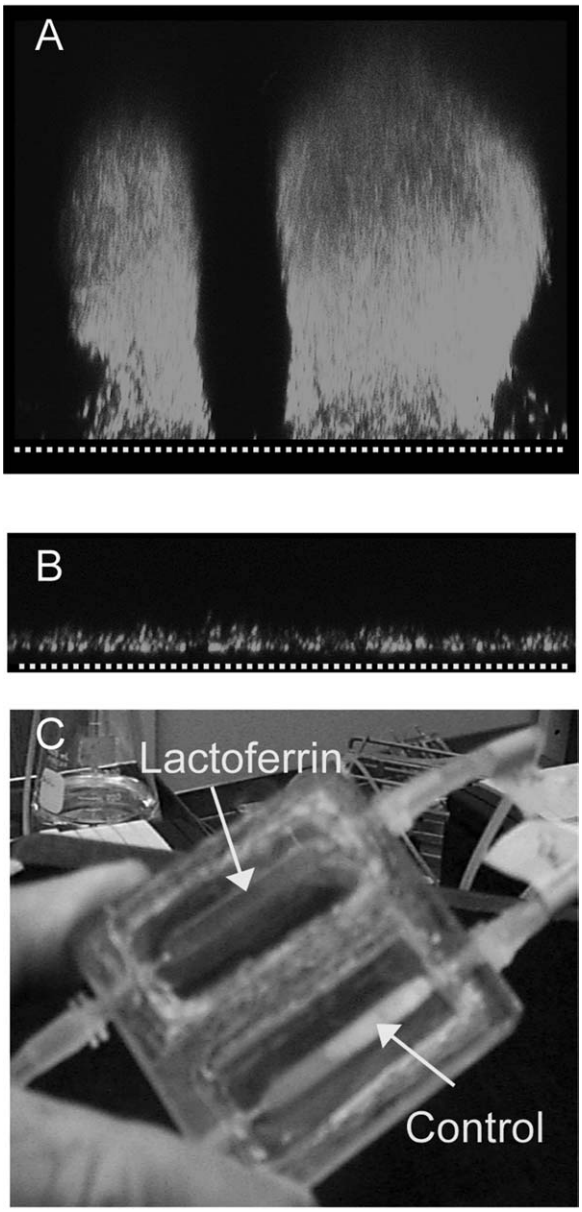

Fig. 2. Effects of lactoferrin on biofilm formation by *P. aeruginosa*. Gfp expressing *P. aeruginosa* were grown in continuous culture flow cells in the presence and absence of lactoferrin (20 μg/ml) and examined after 5 days using confocal microscopy. In the absence of lactoferrin (A), tower-shaped biofilms developed that were markedly resistant to antibiotics. In the presence of a sub-inhibitory concentration of lactoferrin (B) biofilm formation (and biofilm-mediated antibiotic resistance) was prevented. (C) shows a photograph of the continuous culture flow cells. The bottom channel was perfused with media lacking lactoferrin; biofilm formation can be seen with the naked eye (greenish material filling channel). The bottom channel was perfused with lactoferrin (20 μg/ml); no biofilm formation is seen.

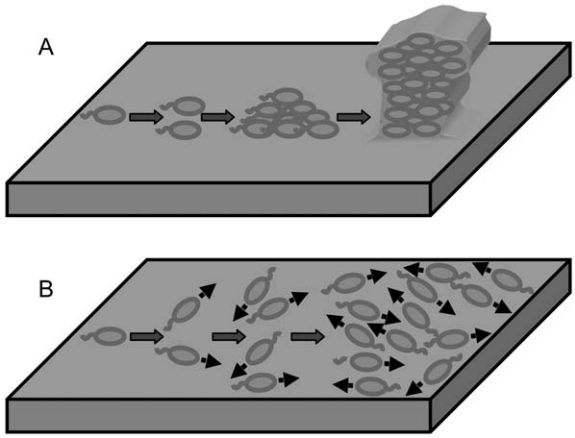

Fig. 3. Schematic representation of the mechanism of lactoferrin's action. In the absence of lactoferrin (A), *P. aeruginosa* attach to the growth surface and multiply. The daughter cells of attached bacteria join developing microcolonies that eventually mature into biofilms. In the presence of lactoferrin (B), bacterial attachment and multiplication occur normally, however lactoferrin stimulated bacterial surface motility cause the daughter cells to move off in different directions. This action prevents the formation of biofilms.

ating that the iron sequestrating ability of lactoferrin was required for this anti-biofilm action.

From these data insights can be gleaned about the process of biofilm development and, perhaps, the function of lactoferrin on mucosal surfaces. The fact that stimulated surface motility prevented the formation of *P. aeruginosa* biofilms indicates that under the conditions tested, microcolonies develop largely from the division of attached cells, rather than from active aggregation of bacteria. These data also indicate that interfering with the early steps in development may be a useful strategy to disrupt the formation of biofilms and prevent biofilm-mediated antibiotic resistance.

From the perspective of host defense, the anti-biofilm action of lactoferrin may serve as a fail-safe mechanism that prevents the bacteria that survive initial killing from assuming the intractable biofilm state. This may allow time for adaptive responses to be recruited. Interestingly, the anti-biofilm effect may be an example of a host defense taking advantage of a self-serving bacterial response. Because iron is among the most difficult nutrients for microorganisms to acquire, stimulated motility under these conditions may benefit the bacteria by protecting them from constructing complex, durable biofilm structures in locations where this critical nutrient is in short supply.

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