

Inhibitory effects of lactoferrin on biofilm formation in clinical isolates of *Pseudomonas aeruginosa*

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Abstract Lactoferrin, a multifunctional protein with antimicrobial activity, is a component of the innate immune system. It may possibly prevent clinical isolates of *Pseudomonas aeruginosa* from developing biofilm, but this hypothesis is yet to be widely accepted. We evaluated the in vitro effects of lactoferrin on biofilm formation by various clinical isolates of *P. aeruginosa* using a modified method of the microtiter plate biofilm assay. Lactoferrin significantly inhibited biofilm formation in these isolates. The effect was the most marked at 2 mg/ml, which suggested that an optimal concentration of lactoferrin might exist. Lactoferrin inhibited biofilm formation in eight of nine clinical isolates after 1 day of incubation; however, the inhibitory effects were maintained until 7 days of incubation in only two of those eight strains. Suppression of biofilm formation may be caused by a mechanism that is independent of the bactericidal effects of lactoferrin because the number of viable bacteria was not influenced by lactoferrin under the experimental conditions. Supplementation of lactoferrin to preformed biofilm demonstrated a reduction in biofilm, which suggests that lactoferrin may have a destructive effect on biofilm. Pretreatment with ferric chloride partially restored biofilm formation, suggesting an iron-chelating action may be involved in the inhibitory mechanism of lactoferrin. These results suggest that lactoferrin provides inhibitory effects on biofilm formation in many clinical isolates of *P. aeruginosa* and that it

may also have destructive effects on preformed biofilm, but further research using multiple clinical strains should be undertaken to clarify if those effects are universal.

Keywords *Pseudomonas aeruginosa* · Biofilm · Lactoferrin

Introduction

Pseudomonas aeruginosa is an important clinical pathogen widely known to have the ability to sense its surrounding environmental conditions. The bacterium forms a biofilm around its cell surface, resulting in the development of an untreatable disease, such as chronic respiratory tract infection, that is resistant and unresponsive to antibiotics [1–3]. This defense mechanism is attained by a quorum sensing system [4, 5], which regulates gene expression so that the bacteria can survive and adjust to the surrounding hostile environment. Specifically, decreased activity of individual bacteria inside the biofilm as well as a thick biofilm to prevent antibiotics from penetrating the cell surface are believed to be related to the occurrence of persistent intractable infections [6–8].

Lactoferrin (LF) is a transferrin family glycoprotein that is present in external secretions of the human body and plays an important role in our innate defense mechanism against innumerable infections via its various antimicrobial properties, such as limiting bacterial growth by sequestering iron and disrupting bacterial membranes by binding lipopolysaccharide [9].

Although the inhibitory effects of LF on biofilm formation of a standard strain of *P. aeruginosa* PAO1 have been reported [10], it is still not fully understood whether the effect can be universally applied to clinical isolates of

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P. aeruginosa. Therefore, in this study, we evaluated the in vitro effects of LF on biofilm formation using clinical isolates of *P. aeruginosa*. We also studied experimental conditions, such as LF concentration and incubation period, to maximize the inhibitory effects of LF. Finally, we examined the influence of pretreatment with ferric chloride (FeCl_3) to assess the mechanism of this effect.

Materials and methods

Bacteria

Pseudomonas aeruginosa IID1052 obtained from the Japan Collection of Microorganisms was used as the standard strain. A total of nine clinical isolates of *P. aeruginosa* were obtained from Tokyo Medical University Hospital; all were derived from bronchial specimens and had a mucoid appearance. All strains were incubated overnight at 37°C in Luria broth (1% casein, 0.5% yeast extract, 0.05% sodium chloride); the initial number of cells forming the biofilm was then adjusted to 1×10^4 colony-forming units (CFU)/ml/well in polyvinylchloride 24-well microtiter plates.

Lactoferrin

Bovine LF was obtained from Morinaga Milk Industry (Tokyo, Japan). LF was dissolved in distilled water at a concentration of 100 or 200 mg/ml; the LF solution was sterilized by passing it through a filter (0.45 μm). The LF solution was added to the broth with the strains at designated concentrations so that the volume of LF solution added would be as little as possible.

Biofilm formation in microtiter plates

The strains were continuously incubated with shaking at 30 rpm at 37°C to allow biofilm formation in the wells of polyvinylchloride 24-well microtiter plates. The plates were tightly wrapped with Saran wrap and placed in a plastic box with distilled water at the bottom to protect them from drying out. The total volume of broth with the strains and LF was adjusted to 1 ml in each well.

Measurement of bacterial biofilm

The total volume of biofilm was measured using a modified method of the microtiter plate biofilm assay reported by O'Toole et al. [11]. To measure the entire biofilm that had been formed in the wells (Fig. 1), the sample in each well was stained with 0.1% crystal violet, left at room temperature for 10 min, then passed through a filter (Cell Strainer 40 μm ; BD Japan, Tokyo, Japan). The sample on the filter

was washed with distilled water, air dried, and 125 μl 95% ethanol was then added. The optical density of the extracted solution at 580 nm (OD_{580}) was measured with a microplate reader. The number of viable bacteria in each well at various time point was determined by the colony counting method. Each measurement was performed using three different samples.

Statistical analysis

Data are expressed as the mean \pm standard deviation (SD) and were compared using unpaired *t* tests for means. *P* values <0.05 were considered to indicate statistical significance.

Results

Comparison of biofilm formation between *Pseudomonas aeruginosa* IID1052 and *P. aeruginosa* TMUH-PA1

To select mucoid strains from among the clinical isolates, we first compared the morphological appearance of the colonies of the strains. *P. aeruginosa* TMUH-PA1 was selected as the representative strain from among the clinical isolates examined because of its high level of biofilm formation. Although *P. aeruginosa* TMUH-PA1 and *P. aeruginosa* IID1052 showed the same growth curve until 7 days of incubation (Fig. 2a), biofilm formation by *P. aeruginosa* TMUH-PA1 was significantly increased compared with that of *P. aeruginosa* IID1052 (Fig. 2b). This result agreed with our expectations and suggested that measuring the total volume of biofilm produced in each well using our modified method was an appropriate way to quantify biofilm formation.



Fig. 1 The biofilm that formed in the wells was a mucoid-like material

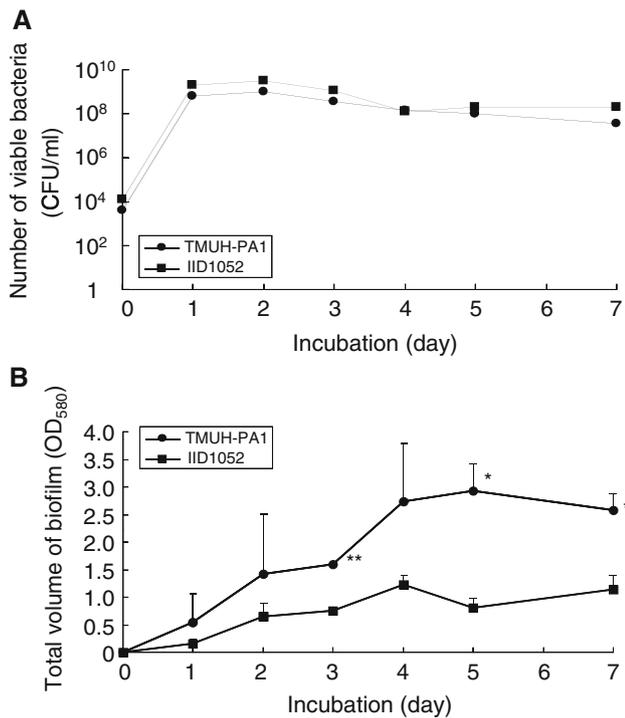


Fig. 2 Bacterial growth curves and biofilm formation of *Pseudomonas aeruginosa* IID1052 and *P. aeruginosa* TMUH-PA1. **a** There was no significant difference in viable bacterial count between *P. aeruginosa* IID1052 and *P. aeruginosa* TMUH-PA1. **b** However, *P. aeruginosa* TMUH-PA1 developed significantly more biofilm and exhibited a progressive increase during the course of cultivation until measurement at 4 days. * $P < 0.05$ compared with the control (*P. aeruginosa* IID1052) at 5 and 7 days; ** $P < 0.001$ compared with the control (*P. aeruginosa* IID1052) at 3 days. CFU colony-forming units

Inhibitory effects of LF on biofilm formation

P. aeruginosa TMUH-PA1 was incubated with various concentrations of LF to determine whether LF affected biofilm formation by the clinical strain of *P. aeruginosa*. Because the minimum inhibitory concentration (MIC) of LF for *P. aeruginosa* TMUH-PA1 was 16 mg/ml, the concentrations of LF used in this experiment were all less than the MIC. The volume of biofilm was measured after 4 days of incubation; we found that LF inhibited biofilm formation in a dose-dependent manner up to a concentration of 2 mg/ml (Fig. 3). Interestingly, the inhibitory effect was diminished at 4 mg/ml, which suggests that an optimal concentration of LF may exist with respect to the inhibition of biofilm formation.

Time course of biofilm formation and growth curve of a clinical strain

We investigated the time course of biofilm formation and the growth curve of *P. aeruginosa* TMUH-PA1.

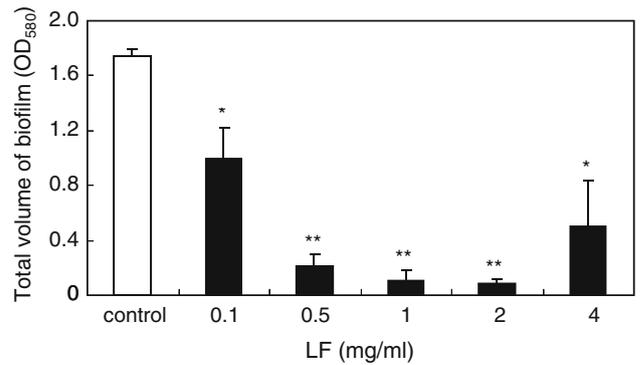


Fig. 3 Inhibitory effects of lactoferrin (LF) at various concentrations. Volume of biofilm was measured after 4 days of incubation of *P. aeruginosa* TMUH-PA1 with various concentrations of LF. LF inhibited biofilm formation in a dose-dependent manner up to 2 mg/ml; however, the inhibitory effects declined when the concentration exceeded 4 mg/ml. * $P < 0.05$ compared with the control (no LF); ** $P < 0.001$ compared with the control (no LF); open column, control; closed columns, supplementation with various concentrations of LF

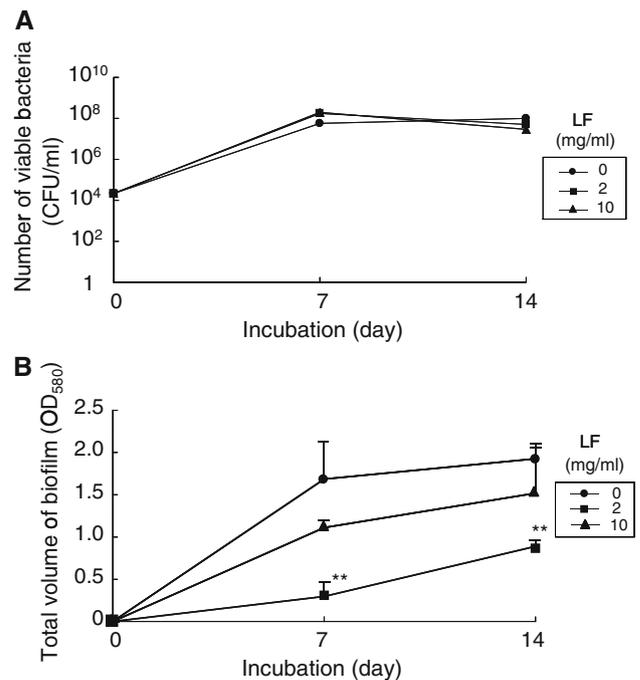


Fig. 4 Inhibitory effects of LF on biofilm formation and growth curve of *P. aeruginosa* TMUH-PA1 along the time course. **a** Although there was no significant difference in viable bacterial counts with or without LF, LF significantly inhibited biofilm formation. **b** The inhibitory effects were maintained until 14 days of incubation and were more marked at 2 mg/ml than at 10 mg/ml. ** $P < 0.001$ compared with the control (no LF)

P. aeruginosa TMUH-PA1 was incubated with two different concentrations of LF and the volume of biofilm was measured after 7 and 14 days of incubation. LF significantly inhibited biofilm formation without any influence on the number of viable bacteria (Fig. 4a). The inhibitory

Table 1 Inhibitory effects of LF on biofilm formation in nine clinical isolates

Strain	Lactoferrin (LF) (mg/ml)	Day 1	Day 4	Day 7
TMUH-PA1	0	0.422 ± 0.206	1.741 ± 0.058	1.597 ± 0.159
	2	0.018 ± 0.008*	0.085 ± 0.035**	0.443 ± 0.090**
601	0	0.411 ± 0.041	1.028 ± 0.074	0.765 ± 0.106
	2	0.048 ± 0.011**	0.264 ± 0.083**	0.735 ± 0.114
603	0	0.154 ± 0.012	1.025 ± 0.156	1.056 ± 0.112
	2	0.118 ± 0.021*	0.787 ± 0.073*	0.583 ± 0.091*
791	0	0.207 ± 0.010	1.308 ± 0.510	1.211 ± 0.213
	2	0.173 ± 0.052	0.555 ± 0.068	0.223 ± 0.059**
804	0	0.432 ± 0.036	1.598 ± 0.161	1.311 ± 0.108
	2	0.028 ± 0.006*	1.377 ± 0.042*	1.388 ± 0.345
811	0	0.150 ± 0.006	1.131 ± 0.408	1.080 ± 0.142
	2	0.073 ± 0.022*	1.387 ± 0.138	1.585 ± 0.090
813	0	0.542 ± 0.025	0.901 ± 0.160	0.969 ± 0.021
	2	0.081 ± 0.013**	0.782 ± 0.127	0.988 ± 0.166
903	0	0.291 ± 0.047	0.791 ± 0.129	0.930 ± 0.152
	2	0.102 ± 0.024*	0.800 ± 0.101	1.373 ± 0.325
D4	0	0.117 ± 0.013	0.536 ± 0.110	1.033 ± 0.348
	2	0.034 ± 0.004**	0.688 ± 0.109	1.503 ± 0.084

* $P < 0.05$ compared with the control of each strain (no LF) at each time of incubation

** $P < 0.001$ compared with the control of each strain (no LF) at each time of incubation

effect was maintained until 14 days of incubation and was more marked at 2 mg/ml than at 10 mg/ml (Fig. 4b), which also suggests that there is an optimal concentration of LF for the inhibition of biofilm formation.

Effects of LF on biofilm formation in other clinical isolates

We investigated whether the inhibitory effects of LF on biofilm formation were observed among other clinical isolates of *P. aeruginosa*. LF showed significant inhibitory effects in eight of nine clinical isolates after 1 day of incubation. However, the inhibitory effects of LF were maintained until 7 days of incubation in only two of those eight strains (Table 1). These results suggest that inhibition of biofilm formation by LF can be demonstrated in most of the clinical isolates at a short time after incubation, but the inhibitory effects of LF on biofilm formation may be lost on further incubation. The difference in the inhibitory effects of LF on biofilm formation among strains could not be explained by their morphological differences because all were basically similar, being mucoid-type strains.

Reduction of preformed biofilm by LF

We further investigated whether LF has any effect on preformed biofilm in *P. aeruginosa*. Various concentrations of LF were added after 4 days of incubation of *P. aeruginosa* TMUH-PA1 and the volume of biofilm was

measured 3 days later for a total incubation time of 7 days. LF significantly inhibited biofilm formation at a concentration of 2 mg/ml. Furthermore, the volume of biofilm was reduced to below the preformed level (Fig. 5). These results suggest that LF not only inhibits biofilm formation but also breaks down existing biofilm.

Mechanism of inhibitory effect of LF on biofilm formation

Because one of the major biochemical properties of LF is an iron-chelating action, we hypothesized that this may be the mechanism of its inhibitory effects on biofilm formation. The inhibitory effects of LF on biofilm formation by *P. aeruginosa* TMUH-PA1 were significantly reduced with pretreatment by both 200 and 2,000 μM FeCl_3 (Fig. 6), suggesting that iron chelation may be involved in the inhibitory mechanism of LF.

Discussion

In this study, we have demonstrated, using a total of nine clinical isolates, that LF has an inhibitory effect on biofilm formation by *P. aeruginosa*. Microtiter plate biofilm assay has been used so far to measure the volume of bacterial biofilm in vitro [11]; however, we noticed that portions of samples containing biofilm attached to the surface of the well were partially flushed away by the process of washing.

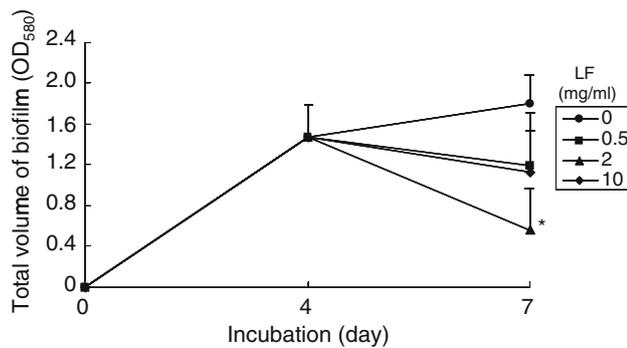


Fig. 5 Destructive effects of LF on preformed biofilm were investigated using *P. aeruginosa* TMUH-PA1. LF significantly inhibited biofilm formation, and the volume of biofilm was reduced even below the level that existed when LF was added. The effect of LF was the most marked at 2 mg/ml. * $P < 0.05$ compared with both the control after 7 days of incubation (no LF) and before LF supplementation after 4 days of incubation (preformed or existing biofilm)

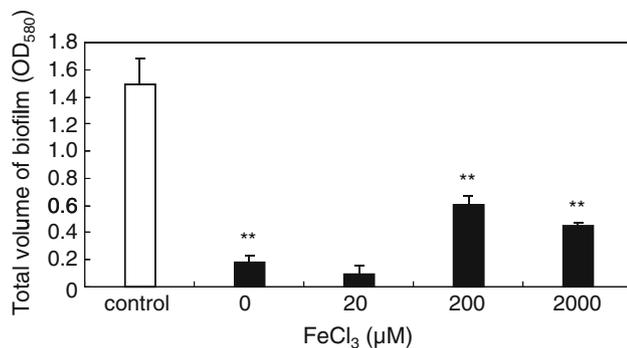


Fig. 6 Influence of iron on inhibitory effects of LF on biofilm formation. LF was treated with FeCl₃ at room temperature for 30 min before initiating cultivation of *P. aeruginosa* TMUH-PA1. FeCl₃ was added at concentrations of 20, 200, and 2000 µM; volume of biofilm was measured after 7 days of incubation. Biofilm formation was significantly inhibited with no pretreatment; in contrast, the inhibitory effects of LF on biofilm formation were significantly reduced with pretreatment by both 200 and 2000 µM FeCl₃. ** $P < 0.001$ compared with both control and no FeCl₃; open column, control; closed columns, FeCl₃ at various concentrations

Thus, we modified the method for biofilm measurement as described in the “Materials and methods.” We believe this modified method is useful for measuring the total volume of biofilm produced in the wells, although the results can be affected by a slight discrepancy between the measurements, which may explain the difference of biofilm formation by *P. aeruginosa* TMUH-PA1 between Fig. 2b and Table 1.

LF inhibited biofilm formation in a dose-dependent manner at concentrations of 0.1–2 mg/ml, but concentrations exceeding 4 mg/ml showed less inhibition. These results indicate that an optimal concentration of LF may exist with respect to the inhibition of biofilm formation. We believe that excessive levels of LF may be utilized as

an energy source by the bacteria because the number of viable bacteria in the wells increased with higher concentrations of LF compared with lower concentrations (data not shown).

An inhibitory effect of LF on biofilm formation was detected in all nine clinical isolates in the present study, the duration of the effects being somewhat different between strains. These results may be explained based on the same mechanism reported in previous studies, or in other words, that LF is degraded by the protease produced by *Porphyromonas gingivalis* and *Prevotella intermedia* [12, 13], and the effects of LF appear to be weak or absent after a longer period of incubation [14]. These findings suggest that the inhibitory effects of LF on biofilm formation can be obtained at short time points, such as 1 day after incubation, in many clinical isolates of *Pseudomonas aeruginosa*, and that these effects can also be maintained up to long time points, such as 7 days after incubation, in some strains.

Iron is indispensable for bacterial growth, and antimicrobial activity of LF can be obtained via its iron-chelating action [15]. Therefore, the mechanism of inhibition of biofilm formation by *P. aeruginosa* may also be related to the iron-chelating properties of LF [16, 17]. The results of the present study support this hypothesis, because we demonstrated that pretreatment with FeCl₃ partially restored the biofilm formation, even in cultures containing LF. However, another inhibitory mechanism may also be involved because biofilm formation had not fully recovered after incubation with a sufficient concentration of FeCl₃.

Furthermore, we were able to demonstrate that the addition of LF after 4 days of incubation reduced the volume of biofilm in the well. This finding suggests that LF had a destructive effect on the biofilm, even after a sufficient volume of biofilm was already present in the well [18]. In summary, all these findings suggest that LF has in vitro inhibitory effects on biofilm formation by *P. aeruginosa* clinical isolates and that it may also have destructive effects on a preformed biofilm. However, further research is required to establish the practical application of LF to treat intractable respiratory infections caused by biofilm formation by *P. aeruginosa* because some data suggest that ingested LF is generally not absorbed in the blood [19].

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