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Antibiofilm effect of warfarin on the biofilm formation of *Escherichia coli* promoted by antimicrobial treatment

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ABSTRACT

Objective : The enhancement of microbial biofilm formation by low antimicrobial doses is a critical problem in the medical field. The objective of our study is to propose a new drug candidate against the promoted biofilm formation by subinhibitory dose of antimicrobials.

Methods : To check the effect on the biofilm formation of *Escherichia coli* cells, the subinhibitory concentration of lactoferrin (LF), a milk protein involved in a large spectrum of biological properties including antimicrobial action, or ampicillin (Amp), a typical antibiotic, was added in the culture of *E. coli* cells using 96-well microtiter plate. On the other hand, warfarin (Waf), an oral anticoagulant, or polymyxin B (PmB), a strong antibiotic for biofilm treatment, was added as an antagonist against the promoted biofilm by LF or Amp.

Results : The amount of biofilm formed at 100 μg ml^{-1} of LF in LB medium was 4 times higher than that in the absence of LF. Meanwhile, it was found that Waf suppressed the LF-promoted biofilm formation to a comparable level with LF-free condition. Waf worked in a similar manner to PmB known as an antibiofilm. Furthermore, Waf also could suppress the promoted biofilm by Amp.

Conclusions : This study suggests that Waf can work as an antibiofilm agent against the promoted biofilm formation by subinhibitory dose of antimicrobials.

INTRODUCTION

The term ‘‘biofilm’’ refers to the microbial consortium located on biotic and abiotic surfaces, including human tissues. Biofilms resist antimicrobial exposure and contribute to bacterial persistence in chronic infections because of their resistant nature, which shelters bacteria from penetration by drugs [1]. The bioavailability of antimicrobials depends on the dose, distribution, elimination, and mode of administration [2, 3]. Therefore, following antimicrobial treatment, bacteria may be exposed to their subinhibitory concentrations. Many studies have warned that low antimicrobial doses conversely promoted biofilm formation [4-6]. It was shown that the subinhibitory concentrations of gentamicin and enrofloxacin induced the formation of *Escherichia coli* and *Pseudomonas aeruginosa* biofilms [7, 8]. Thus, the enhancement of biofilm formation by low antimicrobial doses is a critical problem. A better understanding of the bacterial response against subinhibitory concentrations of antimicrobials may offer clinical potentials in treating bacterial infections.

Lactoferrin (LF) is a milk protein involved in a large spectrum of biological properties including antimicrobial function [9, 10]. Iron–chelating effect has been thought to be the major antibacterial activity of LF. In addition, more complex mechanisms have been presented. LF not only chelates iron, binds to the lipid A of lipopolysaccharide (LPS) on the cell surface and disrupts the cell membrane of bacteria including *E. coli* [11]. A significant reduction on the formation of an *E. coli* cell biofilm was also reported when high amounts of LF were used under non-growth conditions [12, 13]. However, the effect of a lower LF dose on the formation of biofilm under growth conditions has not yet been reported. Meanwhile, warfarin (Waf), a vitamin K antagonist, is the most widely used as an oral anticoagulant agent worldwide; more than

30 million prescriptions were written for this drug in the United States in 2004 [14]. Waf has been established as the oral anticoagulant of choice for many years. Therefore, if Waf has a calming effect on the microbial biofilm formation, it would be beneficial for the clinical treatment of infections caused by biofilms.

In the current study, we report that a subinhibitory LF or Amp dose adversely promotes the biofilm formation of *Escherichia coli*. Furthermore, we demonstrate that the presence of Waf can deteriorate the promoted biofilm formation by subinhibitory dose of antimicrobials.

Materials and Methods

E. coli K-12 BW25113 and MG1655 strains were obtained from the National BioResource Project (National Institute of Genetics (NIG), Mishima, Japan) [15] and American Type Culture Collection (ATCC700926), respectively. *E. coli* cells were cultured in lysogeny broth (LB) medium (10 g l^{-1} Hipolypepton (Wako Pure Chemical Industries, Osaka, Japan), 5 g l^{-1} Bacto-yeast extract and 10 g l^{-1} NaCl).

Initial biofilm formation was set up as reported in our previous paper with some modifications [16]. Prior to inoculation, all test cultures were warmed in LB medium for 14 h at 37°C, and then diluted in fresh LB medium to reach optical density at 660 nm (OD_{660}) = 0.01. The diluted suspension in fresh LB medium (200 μl) was transferred to a 96-well microtiter plate made from polyvinyl chloride (PVC) (Corning Inc., Corning, NY, USA). After initial biofilm formation at 37 ^{o}C for 16 h, the culture broth containing planktonic cells was removed and fresh medium with antibiotics were added into each well. Bovine lactoferin (LF) and ampicillin (Amp), purchased from Wako Pure Chemical Industries (Osaka, Japan), were employed as model antimicrobials. When necessary, warfarin (Waf, Wako Pure Chemical Industries) was added together with the antimicrobials as an anticoagulant [17]. Polymyxin B (PmB), obtained from Tokyo Chemical Industry Co., Ltd., was also used as a typical antibiofilm agent [18].

After culturing for another 24 h at 37°C the culture broth containing planktonic cells was harvested and the cell growth was recorded by measuring OD_{660}. For the quantitative evaluation of biofilm formation, the cells adhering to the well surface were stained by incubating them with 200 µl of 50 mg l^{-1} safranin solution for 20 min at room temperature, followed by washing 2 times with water. The dye pigmenting cells on the well surface were solubilized by adding 200 µl of 20 % (v/v) acetone in ethanol. The solubilized dye sample was condensed from 4 wells under a given condition to obtain a sufficient value of measurement. The index of biofilm cells was indicated by the absorbance of the dye solution measured at 492 nm by a microtitre plate reader (Chromate-4300, Awareness Technology, Palm City, FL, USA).

Results and Discussion

To study the physiological response, *E. coli* BW25113 cells were incubated in the LB medium containing LF at 0-200 µg ml^{-1}. These concentrations of LF did not change the OD_{660} values of the culture broth, suggesting that these were subinhibitory levels against the planktonic cells of *E. coli* (Fig. 1A). Despite this lack of inhibition, the range of subinhibitory LF concentrations enhanced the biofilm formation (Fig. 1A, B). The biofilm formation was significantly enhanced in the presence of 12.5 µg ml^{-1} of LF, and slightly increased afterward. The amount of biofilm at 100 µg ml^{-1} of LF was the highest and 4 times greater than that in the absence of LF. Similarly, MG1655 strain also formed considerably more biofilm in the presence of LF (Fig. 1C). The amount of biofilm showed a dose-dependent increase with LF concentration, and it was approximately 6 times larger at 100 μg ml^{-1} of LF than that under the LF-free condition. Thus, the biofilm formation was strongly promoted by the subinhibitory concentration of LF regardless of *E. coli* strain.

Subsequently, the effect of War on the LF-promoted biofilm formation was examined. Waf could be a candidate of antibiofilm drug since its safety has been proven as the oral anticoagulant for many years. If Waf has an antibiofilm activity, it would be beneficial for the clinical treatment of biofilm in the case of such catheter-associated urinary tract infection. Figure 2 shows the dose dependent effect of Waf on the biofilm formation of *E. coli* with or without 100 μg ml^{-1} of LF. In the absence of LF, Waf did not significantly influence the biofilm formation within the range of less than 5 mM. At 7.5 mM Waf, the formation of the biofilm significantly decreased by 40% compared with that without Waf. In the presence of LF, Waf did not significantly change the biofilm formation within the range of less than 2.5 mM (Fig. 2). However, with 5.0 mM of Waf, biofilm formation was decreased by 50% comparing to the data without Waf. Furthermore, 7.5 mM Waf restored the level of biofilm formation to that in the absence of LF. Though the antibiofilm effect of Waf has not been reported yet, our results demonstrated that the LF-promoted biofilm formation could be suppressed by Waf. Next, PmB was added against the LF-promoted biofilm formation since PmB has been known as a strong antibiotic for the treatment of biofilm formation caused by Gram-negative bacteria [18]. PmB addition also showed the dose-dependent suppression against LF-promoted biofilm formation. At 10 μg ml^{-1} of PmB, biofilm formation was suppressed to a comparable level with that in the absence of LF. Thus, it was clarified that anticoagulant Waf had the inhibitory effect on the biofilm formation in a similar manner to a strong antibiofilm PmB.

In addition to LF, ampicillin (Amp), an inhibitor of cell wall synthesis, was chosen as a typical antibiotic to induce the promoted biofilm formation by the subinhibitory dose. Then, the versatility of the function of Waf on the promoted biofilm formation was examined. As a result, a subinhibitory level of Amp (2.5 μg ml^{-1}) also increased the biofilm formation of *E. coli* cells (Fig. 3). The value of A_{492} was two times higher than that in the absence of Amp. In contrast, the addition of Waf notably decreased the biofilm formation promoted by Amp. The value was comparable with that in the absence of Amp, as seen in the case of LF effect.

In conclusion, this study is the first report indicating that the promoted biofilm formation by subinhibitory dose of LF or Amp could be suppressed by War. Further examination will be conducted to elucidate a detailed mechanism of this suppression by War.

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FIGURE LEGENDS

Fig. 1 (A) Effect of LF on the cell growth and biofilm formation of *E. coli* BW25113 strain. (B) Photographs showing LF-induced biofilm formation of *E. coli* BW25113 strains cultured on PVC surface visualized by safranin staining. (C) Effect of LF on the biofilm formation of *E. coli* MG1655 strain. In the graphs (A) and (C), the data were determined from more than three independent experiments. The vertical bars indicate standard deviation. The asterisks show the statistical significance against the data without LF (p<0.05).

Fig. 2 (A) Effect of Waf on the biofilm formation of *E. coli* BW25113 strain under conditions with or without 100 μg ml^{-1} LF. (B) Effect of PmB on the biofilm formation of *E. coli* BW25113 strain under conditions with or without 100 μg ml^{-1} LF. In the both graphs, the data were determined from more than three independent experiments. The vertical bars indicate standard deviation. The single asterisk show the statistical significance against the data without Waf or PmB in the absence of LF (p<0.05). The double asterisks show the statistical significance against the data without Waf or PmB in the presence of LF (p<0.05).

Fig. 3 Biofilm formation of *E. coli* BW25113 strain in the presence of 2.5 μg ml^{-1} Amp and/or 5 mM Waf. The data were determined from more than three independent experiments. The vertical bars indicate standard deviation. The asterisk shows the statistical significance against the data of without Amp and Waf (p<0.05).

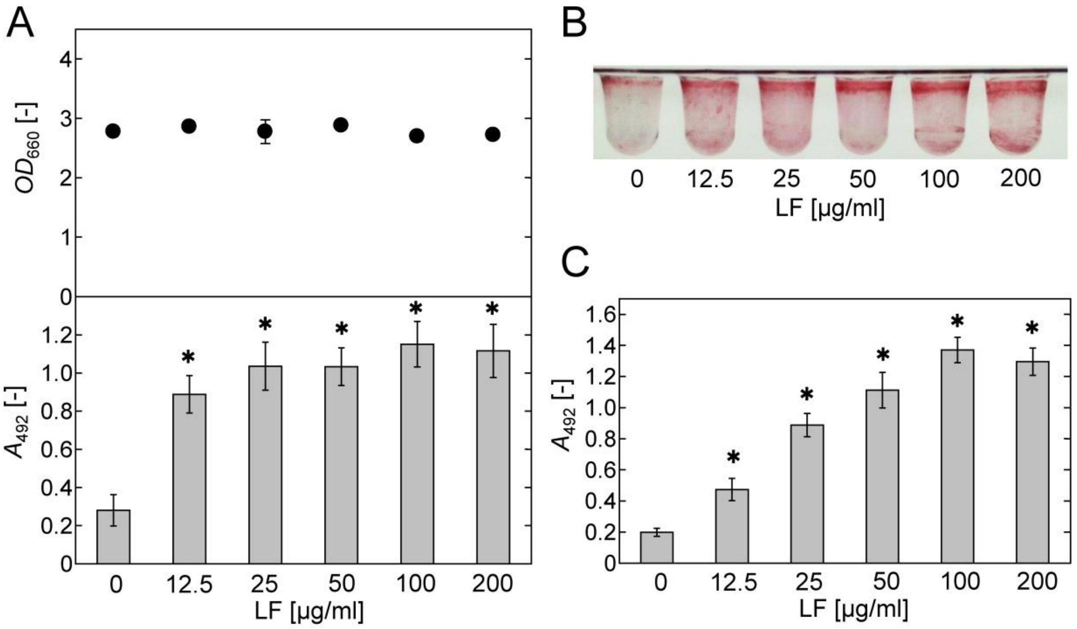
A
B
C
OD_{660} [-]
A_{492} [-]
LF [μg/ml]
0
12.5
25
50
100
200
*

Fig. 1

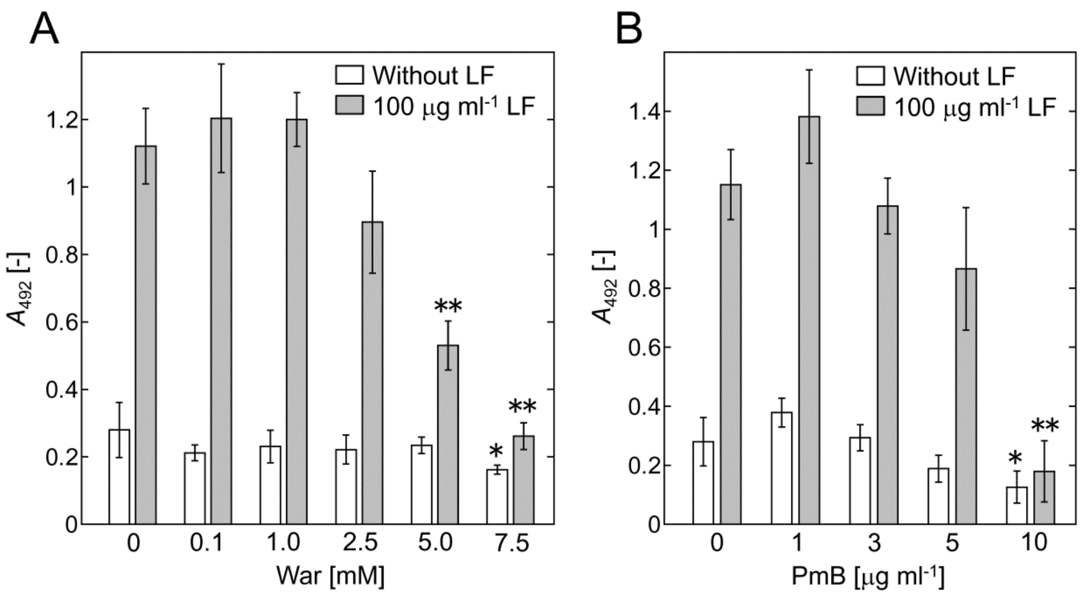
A
B
Without LF
100 μg ml-1 LF
A_{492} [-]
War [mM]
0
0.1
1.0
2.5
5.0
7.5
PmB [μg ml-1]
0
1
3
5
10
*
**

Fig. 2

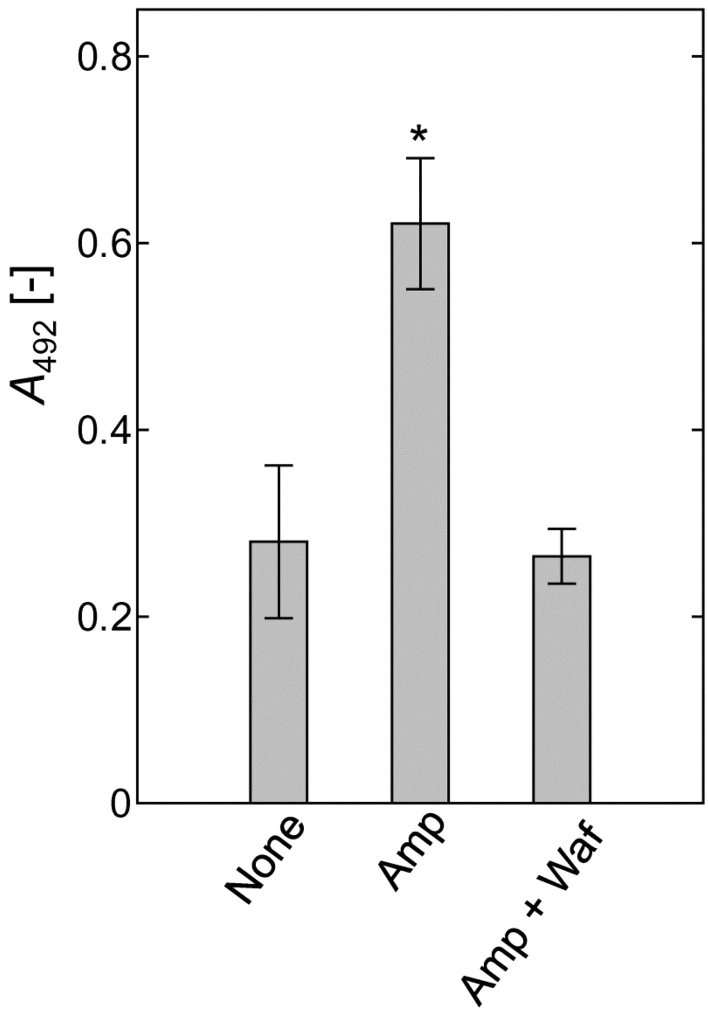

Fig. 3