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Short Communication

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

Yoshihiro Ojima[a,*], Shota Nunogami[b], Masahito Taya[b]

[a] *Department of Applied Chemistry and Bioengineering, Graduate School of Engineering, Osaka City University, 3-3-138 Sugimoto, Sumiyoshi-ku, Osaka-shi, Osaka 558-8585, Japan*
[b] *Division of Chemical Engineering, Graduate School of Engineering Science, Osaka University, 1-3 Machikaneyama-cho, Toyonaka, Osaka 560-8531, Japan*

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ABSTRACT

Enhancement of microbial biofilm formation by low antimicrobial doses is a critical problem in the medical field. The objective of this study was to propose a new drug candidate against the biofilm formation promoted by subinhibitory dose of antimicrobials. To determine the effect on biofilm formation of *Escherichia coli*, a subinhibitory concentration of lactoferrin (LF), a milk protein involved in a broad range of biological properties including antimicrobial action, or ampicillin (AMP), a typical antibiotic, was added to an *E. coli* cell culture in a 96-well microtiter plate. On the other hand, warfarin (WARF), an oral anticoagulant, or polymyxin B (PMB), a strong antibiotic for biofilm treatment, was added as an antagonist against the biofilm promoted by LF or AMP. The amount of biofilm formed at 100 μg/mL LF in lysogeny broth medium was four times higher than in the absence of LF. Meanwhile, it was found that WARF suppressed the LF-promoted biofilm formation to a level comparable with the LF-free condition. WARF worked in a similar manner to PMB, which is known as an antibiofilm agent. Furthermore, WARF could also suppress the biofilm promoted by AMP. In conclusion, this study suggests that WARF can work as an antibiofilm agent against the biofilm formation promoted by subinhibitory dose of antimicrobials.

1. 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 subinhibitory antimicrobial concentrations. Many studies have warned that low antimicrobial doses conversely promoted biofilm formation [4–6]. It was shown that subinhibitory concentrations of gentamicin and enrofloxacin induced the formation of *Escherichia coli* and *Pseudomonas aeruginosa* biofilms [7,8]. Thus, 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 potential in treating bacterial infections.

Lactoferrin (LF) is a milk protein involved in a broad range of biological properties, including antimicrobial function [9,10]. Its 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, it binds to the lipid A of lipopolysaccharide on the cell surface and disrupts the cell membrane of bacteria, including *E. coli* [11]. A significant reduction in the formation of *E. coli* 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 (WARF), a vitamin K antagonist, is the most widely used oral anticoagulant agent worldwide; more than 30 million prescriptions were written for this drug in the USA in 2004 [14]. WARF has been established as an oral anticoagulant of choice for many years. Therefore, if WARF has a diminishing effect on microbial biofilm formation, it would be beneficial for clinical treatment of infections caused by biofilms.

* Corresponding author. Fax: +81 6 6605 3092.
E-mail addresses: ojima@bioa.eng.osaka-cu.ac.jp, yo_ojima@hotmail.com (Y. Ojima).

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In the current study, we report that a subinhibitory dose of LF or ampicillin (AMP) adversely promotes biofilm formation of *E. coli*. Furthermore, we demonstrate that the presence of WARF can diminish the biofilm formation promoted by subinhibitory dose of antimicrobials.

2. Materials and methods

E. coli K-12 BW25113 and MG1655 strains were obtained from the National BioResource Project (National Institute of Genetics, Mishima, Japan) [15] and the American Type Culture Collection (ATCC 700926), respectively. *E. coli* cells were cultured in lysogeny broth (LB) medium [10 g/L Hipolypepton (Wako Pure Chemical Industries, Osaka, Japan), 5 g/L Bacto™ yeast extract and 10 g/L 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 were then diluted in fresh LB medium to reach an optical density at 660 nm (OD_{660}) of 0.01. Then, 200 μL of the diluted suspension in fresh LB medium was transferred to a 96-well polyvinyl chloride microtiter plate (Corning Inc., Corning, NY). After initial biofilm formation at 37 °C for 16 h, the culture broth containing planktonic cells was removed and fresh medium with antibiotics was added into each well. Bovine LF and AMP, purchased from Wako Pure Chemical Industries, were used as model antimicrobials. Where required, WARF (Wako Pure Chemical Industries) was added together with the antimicrobials as an anticoagulant [17]. Polymyxin B (PMB) (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) 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 cell growth was recorded by measuring the OD_{660}. For quantitative evaluation of biofilm formation, cells adhering to the well surface were stained by incubation with 200 μL of 50 mg/L safranin solution for 20 min at room temperature, followed by washing twice with water. The dye staining cells on the well surface was solubilised by adding 200 μL of 20% (v/v) acetone in ethanol. The solubilised dye sample was condensed from four wells under a given condition to obtain a sufficient measurement. The index of biofilm cells was indicated by absorbance of the dye solution measured at 492 nm (A_{492}) using a microtiter plate reader (Chromate-4300; Awareness Technology, Palm City, FL).

3. Results and discussion

To study the physiological response, *E. coli* BW25113 cells were incubated in LB medium containing LF at 0–200 μg/mL. 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 biofilm formation (Fig. 1A and B). Biofilm formation was significantly enhanced in the presence of 12.5 μg/mL LF, and slightly increased thereafter. The amount of biofilm at 100 μg/mL LF was the highest and was four 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 ca. six times higher at 100 μg/mL LF than that under the LF-free condition. Thus, biofilm formation was strongly promoted by a subinhibitory concentration of LF regardless of the *E. coli* strain.

Subsequently, the effect of WARF on the LF-promoted biofilm formation was examined. WARF may be a candidate antibiofilm drug since its safety has been proven as an oral anticoagulant for many years. If WARF has antibiofilm activity, it would be beneficial for the clinical treatment of biofilm in the cases such as catheter-associated urinary tract infection. Fig. 2 shows the dose-dependent effect of WARF on the biofilm formation of *E. coli* with or without 100 μg/mL LF. In the absence of LF, WARF did not significantly influence biofilm formation within the range of ≤5 mM. At 7.5 mM WARF, biofilm formation significantly decreased by 40% compared with that without WARF. In the presence of LF, WARF did not significantly change biofilm formation within the range of ≤2.5 mM (Fig. 2). However, with 5.0 mM WARF, biofilm formation was decreased by 50% compared with the data without WARF. Furthermore, 7.5 mM WARF reduced the level of biofilm formation to that in the absence of LF. Although the antibiofilm effect of WARF has not yet been reported, the current results demonstrated that the LF-promoted biofilm formation could be suppressed by WARF.

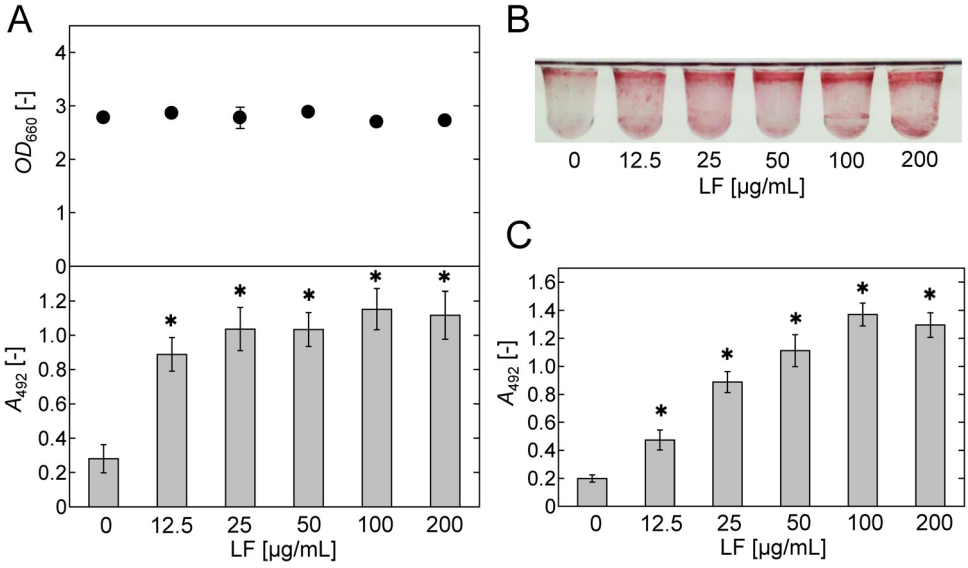

Fig. 1. (A) Effect of lactoferrin (LF) on cell growth and biofilm formation of *Escherichia coli* BW25113 strain. (B) Photographs showing LF-induced biofilm formation of *E. coli* BW25113 strain cultured on a polyvinyl chloride surface visualised by safranin staining. (C) Effect of LF on biofilm formation of *E. coli* MG1655 strain. In graphs (A) and (C), the data were determined from more than three independent experiments. Vertical bars indicate the standard deviation. *Statistically significant difference compared with the data without LF ($P < 0.05$). OD_{660}, optical density at 660 nm; A_{492}, absorbance at 492 nm.