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Role of cell wall components of Gram-positive bacteria in bacterial biofilm formation

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정솔민

ABSTRACT

Role of cell wall components of Gram-positive bacteria in bacterial biofilm formation

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Objectives

Streptococcus gordonii and *Enterococcus faecalis* are Gram-positive facultative anaerobes present in the mucosal tissues of the human body such as oral cavity, gastrointestinal tracts, and genital tracts. Recently, these bacteria have been reported to cause inflammatory diseases such as refractory apical periodontitis and endocarditis through opportunistic infections. These oral pathogens can form biofilms in the oral cavity, which contribute to chronic inflammatory diseases by having physical and chemical defenses against antibiotics and host immune system during biofilm formation. Although biofilm formation is important for oral pathology, the role of cell wall components of oral pathogen in biofilm formation

has not been clearly understood. Treatment of biofilm-associated oral diseases by current antibiotics has limitations since biofilms are resistant to antibiotics and immune cells. Therefore, the development of new effective anti-biofilm agents that prevent and suppress the biofilm formation of oral pathogens are needed. Recently, *Lactobacillus* species inhibiting bacterial biofilm formation have been suggested as an anti-biofilm agent. However, there is still a lack of study about what components of *Lactobacillus* species inhibit biofilm formation. Therefore, the aims of this study were (1) to determine the role of cell wall components of *Streptococci* in the biofilm formation, and (2) to evaluate which cell wall components of *Lactobacillus* species inhibit *E. faecalis* biofilm.

Methods

The effects of bacterial cell wall components on biofilm formation were investigated using lipoprotein-deficient strain (Δ*lgt*) and lipoteichoic acid -deficient strain (Δ*ltaS*) of *Streptococcus gordonii*, *Streptococcus mutans*, *Streptococcus pneumoniae*, and *Staphylococcus aureus*. The biofilm formation was examined by a crystal violet assay and the bacterial growth was examined by measuring the optical density at 600 nm (OD_{600}). AI-2 mRNA expression was examined by reverse transcription-polymerase chain reaction (RT-PCR). The effects of *L. plantarum* lipoprotein (Lp.Lpp), lipoteichoic acid (Lp.LTA), and peptidoglycan (Lp.PGN) on biofilm formation were investigated by crystal violet assay, confocal laser scanning microscopy (CLSM) using a LIVE/DEAD viability assay. Biofilm

on human dentin slices were visualized with a scanning electron microscopy (SEM).

Results

Δ*lgt* of *S. gordonii* showed higher biofilm formation compared to wild-type strain. In addition, Δ*lgt* of other Streptococci including *S. mutans* and *S. pneumoniae* showed augmented biofilm formation in common with *S. gordonii*, but *S. aureus* did not. But the growth rate of Δ*lgt* and Δ*ltaS* was not different from that of the wild-type. In addition, *S. gordonii* Δ*lgt* showed upregulation of mRNA expression of *luxS* and increased AI-2 level compared to wild-type. *L. plantarum* culture supernatants containing cell wall components inhibited biofilm formation of *E. faecalis*. Lp.LTA most effectively inhibited biofilm formation of *E. faecalis* in a dose-dependent manner among cell wall components of *L. plantarum* including Lp.LTA, Lp.Lpp, and Lp.PGN. When *E. faecalis* was incubated with Lp.LTA, the inhibitory effect was first observed at 1 h and lasted up to 24 h. Notably, Lp.LTA did not affect bacterial growth. In addition, purified LTA form various *Lactobacillus* species including *Lactobacillus casei*, *Lactobacillus rhamnosus* GG, *Lactobacillus acidophilus* inhibited and disrupted biofilm formation of *E. faecalis*. Among these LTA, Lp.LTA show the most potent inhibition effect on *E. faecalis* biofilm. $_{D}$-Alanine is a key component of Lp.LTA for its inhibitory effects on the biofilm formation of *E. faecalis* based on the result that $_{D}$-alanine-removed Lp.LTA failed to inhibit biofilm formation. Furthermore, Lp.LTA inhibited clinical isolates of *E. faecalis* and *E. faecalis* biofilm on human dentin slices.

Conclusions

Taken together, the results show that lipoprotein-deficient Streptococci form biofilms much potently than the wild-type, which might be related with quorum sensing mechanism including AI-2. Lp.LTA effectively inhibits the biofilm formation of *E. faecalis* and disrupts preformed biofilms implying that LTA from *Lactobacillus* can be used as an anti-biofilm agent to prevent and treat biofilm-associated diseases in oral cavity.

Keywords: Gram-positive bacteria, lipoteichoic acid, lipoprotein, biofilm, *Lactobacillus*

CONTENTS

Chapter I. Introduction

1.1. Biofilm

Many infectious diseases are caused or deteriorated by biofilm formation. Biofilm are defined as 'aggregates of microorganisms in which cells are frequently embedded in a matrix of extracellular polymeric substances (EPS) that are adherent to each other and/or surface' [1]. Microorganisms produce an EPS matrix, which allows easy attachment to surfaces of various tissues, teeth, and medical devices and aggregation with other matrix particles [2]. The life cycle of biofilms can be divided into three stages: attachment, maturation, and dispersion [3]. Through intercellular interaction in conjunction with properties of the matrix, biofilm has distinct properties from free-living bacterial cells [4]. Bacteria in biofilms are more resistant to antimicrobial agents than planktonic cells by inactivating or trapping antimicrobials [5, 6] (Figure 1). Furthermore, biofilm bacteria can avoid phagocytosis and contribute to recurrent or chronic inflammatory diseases [7].

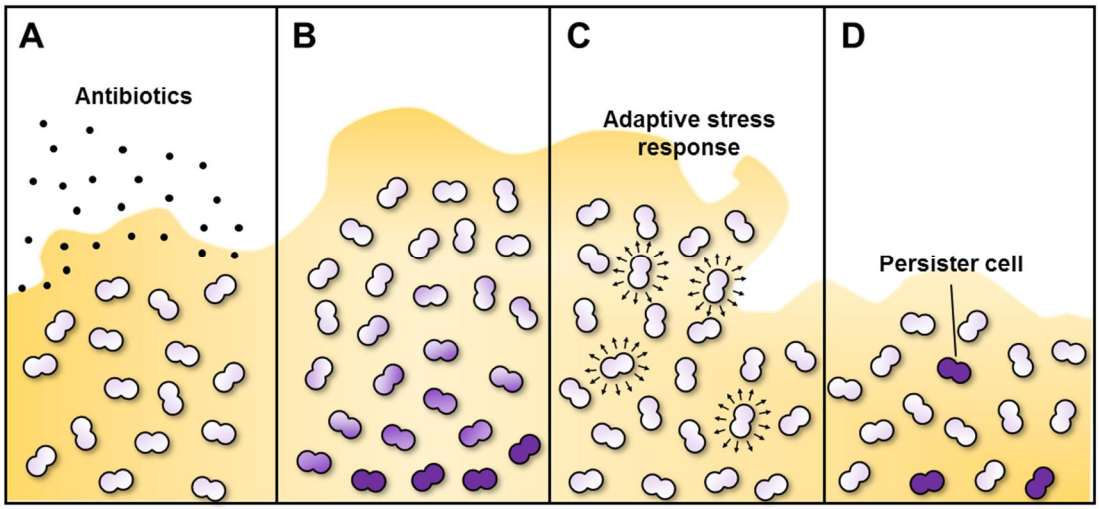

Figure 1. Mechanisms of antibiotic resistance in bacterial biofilm. (A) The extracellular polymeric substance matrix of biofilm interferes with penetration of antibiotics into the biofilm. (B) Nutrients concentration which goes down inside biofilm leads to the attenuation of bacterial growth. (C) Bacterial biofilm demonstrates adaptive resistance in response to antimicrobial stress more effectively than planktonic populations. (D) Part of cells turn into a highly protected persistent state.

1.2. Oral pathogen

Oral infectious diseases occur as a result of interaction between microorganisms and host or its dietary habits, leading to microbial colonization and the establishment of pathogenic biofilm on oral surface [8]. Changes in nutrition enable colonization of a variety of bacterial species, contributing to a complex oral microbiota [9]. Gram-positive facultative anaerobic bacteria such as *Streptococcus* and *Enterococcus* genera, which represent the majority of oral microbiota [10], do not exhibit inflammation under balanced state of host immune response and oral microbiota [11, 12]. However, in susceptible hosts, they can cause inflammatory disease such as refractory apical periodontitis and endocarditis through opportunistic infections [13, 14]. Accumulating reports have shown that biofilm formation of oral pathogen inside dentinal tubules is a crucial factor in the etiology of refractory apical periodontitis [15, 16].

1.3. Cell wall components of Gram-positive bacteria

The bacterial cell wall is an essential structure in the physiology of Gram-positive bacteria. It protects the cell from the surrounding environment, maintains the cell shape, and resists against the intracellular turgor pressure [17, 18]. The cell wall is composed of different components such as peptidoglycan, lipoprotein, wall teichoic acid, and lipoteichoic acid (LTA) [19] (Figure 2). As these cell wall components are located at the outermost of the cell, they interact directly with the surrounding environment or adjacent cells in the biofilm. Lipoproteins are amphipathic molecules composed of a hydrophilic protein moiety and hydrophobic diacylglycerol moiety [20]. In Gram-positive bacteria, lipoprotein synthesis is mediated by lipoprotein diacylglyceryl transferase (Lgt), which is an essential enzyme for formation of covalent bonds between diacylglycerol moieties and cysteine residues in pre-prolipoprotein [21] (Figure 3). Bacterial lipoproteins have many different functions such as nutrient uptake, signal transduction, adhesion, and are involved in antibiotic resistance, transporter system [22-24]. LTA is also an amphipathic molecule composed of a hydrophilic phosphate backbone and a hydrophobic glycolipid anchor [25]. Polymerization of the phosphate-containing groups is mediated by LTA synthase (LtaS), which is an essential enzyme for LTA synthesis [26]. LTA plays important role in various aspects of bacterial physiology such as growth, division, and biofilm formation [27].

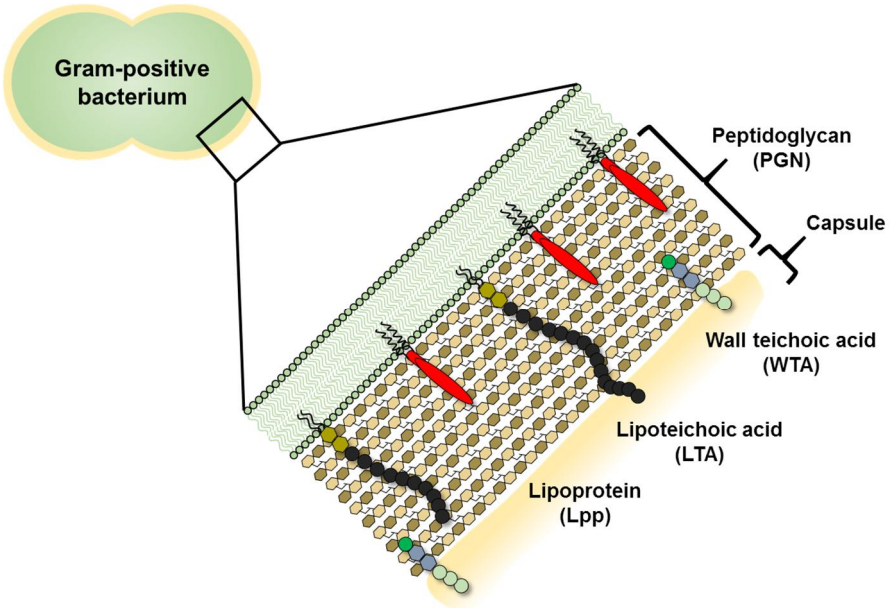

Figure 2. Cell wall structure of Gram-positive bacteria. Gram-positive bacteria have a single lipid bilayer, which is surrounded by a cell wall composed of thick peptidoglycan, wall teichoic acid, lipoteichoic acid, and lipoprotein. Lipoteichoic acid and lipoprotein are anchored to the cell membrane by their diacylglycerol.

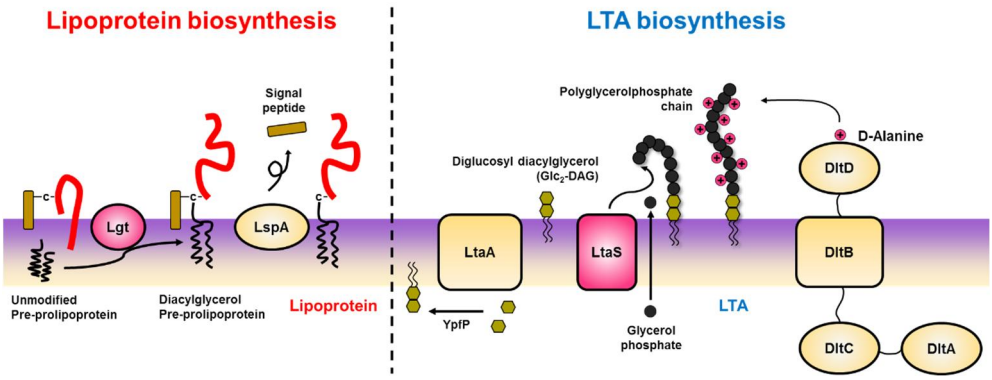

Figure 3. Lipoprotein and LTA biosynthesis of Gram-positive bacteria. In lipoprotein biosynthesis, the Lgt transfers a diacylglycerol moiety to the cysteine residue in lipobox motif after unmodified pre-prolipoproteins are inserted to the membrane by secretion system. Then mature lipoproteins are formed through de04tachment of signal peptide by LspA. In case of LTA biosynthesis, YpfP synthesizes the glycolipid, which occur in the bacterial cytoplasm. The diglucosyl diacylclycerol (Glc_2-DAG) is transferred from inner leaflet of the membrane to the outer leflet by LtaA. In succession, LtaS polymerizes the phosphate chain of LTA. Next, $_D$-alanine is transported and incorporated into the polyglycerolphosphate of LTA by Dlt operon.

1.4. Quorum sensing

Quorum sensing is a bacterial communication process that bacteria share information concerning cell-population density and regulate gene expression via secreted signaling molecules [28]. These molecules are known to regulate collective behaviors such as antibiotic production, bioluminescence, conjugation, virulence factors and biofilm formation [29]. More than 300 different bacterial species are known to exist in the oral cavity, forming mixed species community through plaques on the teeth or mucosal surfaces, which lead to tooth decay and gingivitis [30]. Therefore, the oral cavity is considered an important niche for bacterial communication through quorum sensing. Peptide autoinducer-2 (AI-2) molecules are widespread among gram-negative and gram-positive bacterial species, which are produced by LuxS system. Competence-stimulating peptide (CSP) is produced and sensed by Com system [31]. Streptococci such as *S. gordonii*, *Streptococcus mutans* and *Streptococcus pneumoniae* have two types of quorum sensing system, LuxS/AI-2 and Com systems which have been reported to regulate the biofilm formation [32-34].

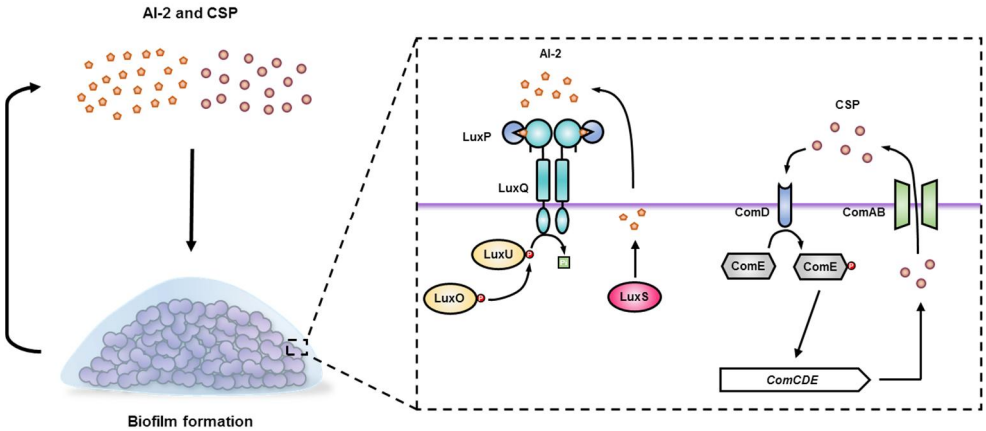

Figure 4. The role of quorum sensing molecules in the biofilm formation of Gram-positive bacteria. AI-2 is synthesized by LuxS. When ComD binds to CSP, it becomes activate to phosphorylates ComE. ComE drives the expression of the *comCDE* operon, which activates CSP production. Then, CSP is secreted through ComAB transporter. These quorum sensing molecules (AI-2 and CSP) act as positive regulators of *S. gordonii* biofilm formation.

1.5. Anti-biofilm property of *Lactobacillus*

Treatment of biofilm-associated infectious diseases using conventional antibiotics has limitation since biofilms are resistant to antibiotics and immune cells through several defense mechanism [35, 36]. Therefore, the development of new effective anti-biofilm agents that prevent and suppress the biofilm formation of oral pathogen are needed. For many decades, *Lactobacillus* has been used as a reliable therapy for treatment of some pathological conditions showing a positive safety profile [37]. Recently studies showed that *Lactobacillus* species have also anti-biofilm property, for example, *Lactobacilllus kefiranofaciens* inhibits bacterial growth and biofilm formation of oral Streptococci through inhibition of adhesion, regulatory protein, and carbohydrate metabolism [38]. It has been reported that supernatants of *Lactobacillus plantarum* and *Lactobacillus fermentum* fine cocoa show inhibitory effects on *S. aureus* growth and its biofilm formation [39], and biosurfactants isolated from *Lactobacillus jensenii* and *Lactobacillus rhamnosus* have anti-adhesive and anti-biofilm abilities against clinical multidrug resistant strains of *Acinetobacter baumanii*, *Escherichia coli*, and *S. aureus* [40]. However, there is still a lack of study about what components of *Lactobacillus* inhibit biofilm formation of oral pathogen.

1.6. Aim of the present study

Little is known about the role of cell wall components of oral pathogen on biofilm formation and it is also important to develop effective anti-biofilm agents that do not induce antibiotic resistance. The aims of present study are (1) to determine the role of cell wall components of oral pathogen on the biofilm formation and (2) to investigate whether cell wall components derived from *Lactobacillus* can inhibit biofilm formation of oral pathogen.

Chapter II. Materials and Methods

2.1. Bacteria, reagents and chemicals

S. gordonii CH1 wild-type, *lgt*-deficient strains (Δ*lgt*), *ltaS*-deficient strains (Δ*ltaS*), *S. pneumoniae* TIGR4 wild-type and Δ*lgt* were provided by Dr. Ho Seong Seo (Korea Atomic Energy Research Institute). *S. mutans* KCTC 3065 was obtained from the Korean Collection for Type Culture (Daejeon, Korea). *S. aureus* RN4220 was provided by Professor Bok Luel Lee (Pusan University, Pusan, Korea). *L. plantarum* KCTC 10887BP, *Lactobacillus casei* KCTC 3260, and *Lactobacillus acidophilus* KACC 12419 were obtained from the Korean Collection for Type Culture and Korean Agricultural Culture Collection (Suwon, Korea), respectively. *Lactobacillus rhamnosus* GG ATCC 53103 and *E. faecalis* ATCC 29212 were obtained from the American Type Culture Collection (VA, USA). *E. faecalis* KCOM 1083, KCOM 1161, and KCOM 1162 were obtained from Korean Collection for Oral Microbiology (Chosun University, Gwangju, Korea). Brain heart infusion (BHI), Tryptic soy broth (TSB), Todd-Hewitt broth (THB), Yeast extract, Bacto™ agar and glucose were purchased from BD Biosciences (San Diego, CA, USA). Lactobacilli MRS broth was purchased from Neogen (Lansing, MI, USA). Crystal violet dye was purchased from Junsei Chemical Co., Ltd (Tokyo, Japan). Triton X-114, aprotinin, leupeptin, lysostaphin, PMSF, octyl-sepharose beads, and glass beads were purchased from Sigma Aldrich Chemical Co. (St. Louis, MO, USA). The LIVE/DEAD BacLight bacterial viability kit was purchased from Thermo Fisher Scientific. RNeasy Mini RNA isolation kit was purchased from Qiagen (Valencia, CA, USA). PCR marker and dNTP mix were purchased

from Elpis Biotech (Daejeon, Korea). M-MLV reverse transcriptase and random primer were purchased from Promega (Madison, WI, USA). EmeraldAmp GT PCR Master Mix was purchased from TaKaRa (Otsu, Shiga, Japan).

2.2. Bacterial strains and culture condition

S. gordonii CH1 wild-type, Δ*lgt*, Δ*ltaS* were grown in THB with 0.5% yeast extract (THY) at 37°C to mid-log phase. *S. mutans* KCTC 3065 wild-type and Δ*lgt* were grown in BHI at 37°C to mid-log phase. *S. pneumoniae* TIGR4 wild-type and Δ*lgt* were grown in THY at 37°C to mid-log phase. *S. aureus* RN4220 wild-type and Δ*lgt* were grown in TSB at 37°C to mid-log phase. *E. faecalis* ATCC 29212, KCOM 1083, KCOM 1161, and KCOM 1162 were grown BHI at 37°C to mid-log phase. *L. plantarum* KCTC 10887BP, *L. casei* KCTC 3260, *L. rhamnosus* GG ATCC 53103, and *L. acidophilus* KACC 12419 were grown on Lactobacilli MRS broth at 37°C to mid-log phase.

2.3. Preparation of human dentin slices

Experiments with human dentin slices were approved by the Institutional Review Board of Seoul National University Dental Hospital, Seoul, Korea (CRI 17010). Human dentin slices were prepared as previously described (Ahn et al., 2018a). Briefly, the surfaces of single-rooted premolars were cleaned using an ultrasonic scaler before producing crosssections of 500 μm thickness with an Isomet precision saw (Isomet). The dentin slices were treated with 17% EDTA for 5 min and then

treated with 2.5% sodium hypochlorite for 5 min. After neutralizing with 5% sodium thiosulfate, the dentin slices were autoclaved at 121°C for 15 min.

2.4. Preparation of supernatants from *L. plantarum*

L. plantarum was grown in Lactobacilli MRS broth at 37°C to mid-log phase. The culture was centrifuged at 10000 rpm for 10 min at 4°C. The culture supernatants were transferred and filtered through a 0.2 μm membrane filter to remove the remaining bacteria and debris.

2.5. Purification of LTA

LTA was prepared from *L. plantarum*, *L. casei*, *L. rhamnosus* GG, and *L. acidophilus* [41]. *Lactobacillus* were grown in Lactobacilli MRS at 37°C to mid-log-phase. Bacterial pellets were harvested and suspended in 0.1 M sodium citrate (pH 4.7) and sonicated to break bacterial cell wall followed by n-butanol extraction for 30 min. After centrifugation, aqueous phase was lyophilized. It was dissolved in distilled water and dialyzed against pyrogen-free distilled water and equilibrated with 0.1 M sodium acetate buffer containing 15% 1-propanol (pH 4.7) that was subjected to hydrophobic interaction chromatography on an octyl-sepharose CL-4B. The column was eluted with 35% 1-propanol in 0.1 M of the sodium acetate buffer. The fractions containing LTA were collected by phosphate assay, and the pool was subjected to DEAE-sepharose ion-exchange chromatography. The fractions containing LTA were collected by phosphate assay, and the pool was subjected to

dialysis against pyrogen-free distilled water. Biologically-active molecules such as endotoxins, nucleic acids, or proteins were not detected in the purified LTA. To prepare $_{D}$-alanine-removed Lp.LTA, Lp.LTA was incubated with 0.1 M Tris-HCl at pH 8.5 for 24 with shacking. It is dialyzed in non-pyrogenic water for 2 days and lyophilized for 1 day. Finally, lyophilized LTA was re-suspended with 1 ml of non-pyrogenic water [42]. Then, $_{D}$-alanine and acyl chain moieties of Lp.LTA were detected by 1% ninhydrin solution and 5% phosphomolybdic acid, respectively.

2.6. Isolation of lipoprotein

Purified lipoproteins were isolated from *L. plantarum* [43]. Briefly, bacterial pellets were harvested and suspended in Tris-buffered saline (TBS) containing proteinase inhibitors. After sonication, bacterial lysates were inverted with 2% Triton X-114 at 4°C for 2 h. After centrifugation, the supernatant was transferred and incubated at 37°C for 15 min and centrifuged to separate into aqueous phase and Triton X-114 phase. The aqueous phase was discarded and the equal volume of TBS was added into the Triton X-114 phase. After centrifugation, the Triton X-114 phase was mixed with methanol and incubated at -20°C for overnight. The precipitated *L. plantarum* lipoproteins were dissolved in 10 mM Octyl β-D-glucopyranoside.

2.7. Purification of peptidoglycan (PGN)

Purified PGNs were prepared from *L. plantarum* [44]. Briefly, the bacterial pellets were washed three times with 20 mM sodium citrate buffer (pH 5.0). The bacteria were disrupted with glass beads in 20 mM sodium citrate buffer containing 0.5 M

for 1 min. After centrifugation, the bacterial pellets were washed with PBS, followed by incubation with 0.5% SDS in PBS at 60°C for 30 min. After washed with 20 mM sodium citrate buffer (pH 5.0), insoluble PGN was treated with 1 M Tris-HCl (pH 7.0) containing trypsin and 10 mM $CaCl_2$ at 37°C for 24 h. The insoluble PGN were solubilized by incubating with lysozyme and lysostaphin at 37°C for 1 h.

2.8. Examination of bacterial growth

S. gordonii CH1 wild-type, Δ*lgt*, and Δ*ltaS* were cultured in THY under static condition for 1, 3, 6, 9, 12, 15, 18, 21, or 24 h. *S. mutans* KCTC 3065 wild-type and Δ*lgt* were cultured in BHI under shaking condition for 1, 2, 3, 4, 5, 6, 7, 8, 9, 12, or 24 h. *S. pneumoniae* TIGR4 wild-type and Δ*lgt* were cultured in THY under static condition for 1, 2, 3, 4, 5, 6, 7, 8, 9, 12, or 24 h. *S. aureus* RN4220 wild-type and Δ*lgt* were cultured in TSB under shaking condition for 1, 2, 3, 4, 5, 6, 12, or 24 h. *E. faecalis* was cultured in BHI media under shaking condition for 1, 3, 6, 12, 24, or 48 h in the presence or absence of Lp.LTA (30 μg/ml). The bacterial growth was determined by measuring the optical density at 600 nm (OD_{600}).

2.9. Crystal violet assay

Bacteria were grown overnight in proper media at 37°C. The culture was diluted 1:100 in media and incubated at 37°C to mid-log phase. The bacteria were incubated in 96-well cell culture plates at 37°C for 24 h. The amounts of biofilms

were analyzed by crystal violet assay [45]. Biofilms were washed with 100 µl of phosphate-buffered saline (PBS) and stained with 1% crystal violet solution for 30 min. The wells were washed with PBS, and the crystal violet-stained biofilms were solubilized in 100 µl of dissociation buffer (95% ethanol and 0.1% acetic acid in water). The optical density at 600 nm was determined using a microplate reader (Molecular Devices).

2.10. Reverse transcription-polymerase chain reaction (RT-PCR)

S. gordonii CH1 wild-type, Δ*lgt*, and Δ*ltaS* were cultured in THY at 37°C to mid-log phase. The bacterial pellets (5×10^9 CFU) were suspended in Buffer RLT and transferred safe-lock tube containing 0.1 mm acid-washed glass beads. The cells were disrupted in bead-beater for 3 min at maximum speed. Total RNA was extracted from bacterial lysates using a RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized from 1 µg of total RNA with random primer and reverse transcriptase. Amplification of cDNA using PCR was in 20 µl containing EmeraldAmp GT PCR Master Mix and 10 pmole of primers specific for ScaR (forward primer: 5'-GTCCACCATCTAGACTATACCAC-3', reverse primer: 5'-CTTGCGGATCCACTTCAAGAGA-3'), LuxS (forward primer: 5'-TTTCGAGCTTGATCACACCA-3', reverse primer: 5'-TCCTTGGCAGAAAAGAGGCT-3'), ComD (forward primer: 5'-AAATGCACATCTTAATAGCTTTGCTAGT-3', reverse primer: 5'-CATATTGTTCACGAGCAGACTTCAG-3'), and ComE (forward primer: 5'-

TTGAGTCAGACGAGGTAAATCAACTT-3', reverse primer: 5'-GCATAGGGATTATGTTGGCGTATA-3'). Amplified PCR products (after 25 cycles for scaR, 28 cycles for LuxS, ComD, ComD) were separated on 1.5% agarose gels and visualized by staining with safe view. The data were analyzed an image analyzer (Gel Documentation system; Vilber Lourmat, Torcy, France)

2.11. Autoinduer-2 (AI-2) measurement

The AI-2 reporter assay was performed according to previously described [46]. *S. gordonii* was grown at 37°C for 24 h, then culture supernatants were obtained by centrifugation and filtered with 0.2 μm pore size filter. *S. gordonii* culture supernatants (20 ul) were added into 96-well plates. To examine bioluminescence, overnight culture of *V. harveyi* BB170 was diluted 1:5,000 in AB medium and added to each sample. Luminescence was measured by a microplate reader (Spark 10M; Tecan, Mannedorf, Switzerland).

2.12. Confocal laser scanning microscopy

E. faecalis was grown in coverglass bottom dishes at 37°C for 24 h in the presence of Lp.LTA (30 μg/ml). The biofilm was washed with PBS and stained with the LIVE/DEAD BacLight Bacterial Viability Kit containing SYTO9 and propidium iodide according to the manufacturer's instructions. Images were visualized under a confocal laser scanning microscope (LSM 800; Carl Zeiss MicroImaging GmbH).

2.13. Scanning electron microscope (SEM) analysis

E. faecalis was grown on human dentin slices in the presence or absence of Lp.LTA and was visualized using a scanning electron microscope as previously described (Velusamy et al., 2016; Ahn et al., 2018a). Briefly, biofilms on human dentin slices were pre-fixed with a PBS solution containing 2.5% glutaraldehyde and 2% paraformaldehyde and washed with PBS. The biofilms were subsequently fixed with 1% osmium tetroxide for 90 min. After washing three times with distilled water, the biofilms were dehydrated by replacing the buffer with increasing concentrations of ethanol (70%, 80%, 90%, 95%, and 100% for 15 min each). The samples were dried with hexamethyl disilazane and coated with gold sputter. The image was visualized under a scanning electron microscope (S-4700, Hitachi). The images of *E. faecalis* biofilms were analyzed to determine the area of bacterial aggregates by using ImageJ software (National Institutes of Health).

2.14. Statistical analysis

All experiments were performed at least three times. The mean value ± standard deviation (S.D.) was obtained from triplicate samples. Statistical significance was examined with a *t*-test. Asterisks (*) indicate treatment groups that were significantly different from the control group at $P < 0.05$.

Chapter III. Results

3.1. Lipoprotein-deficient *S. gordonii* forms biofilms much potently in comparison with the wild-type bacteria

To examine the role of bacterial cell wall components in *S. gordonii* biofilm formation, the biofilm-forming abilities of *S. gordonii* wild-type, Δ*lgt*, and Δ*ltaS* were compared. *S. gordonii* wild-type, Δ*lgt*, and Δ*ltaS* (1×10^4, 10^5, or 10^6 CFU/ml) were grown in polystyrene plates at 37°C for 24 h. Analysis of biofilm formation using crystal violet showed that biofilm formation of *S. gordonii* Δ*lgt* was 1.5-fold higher than wild-type and Δ*ltaS* was not (Figure 5A). To determine if the increase in biofilm formation of *S. gordonii* Δ*lgt* is related to the bacterial growth rate, bacterial growth was examined. But the growth rate of *S. gordonii* Δ*lgt* and Δ*ltaS* was not different from the wild-type (Figure 5B). These results suggest that membrane-anchored lipoproteins on *S. gordonii* would negatively affect the bacterial biofilm formation.

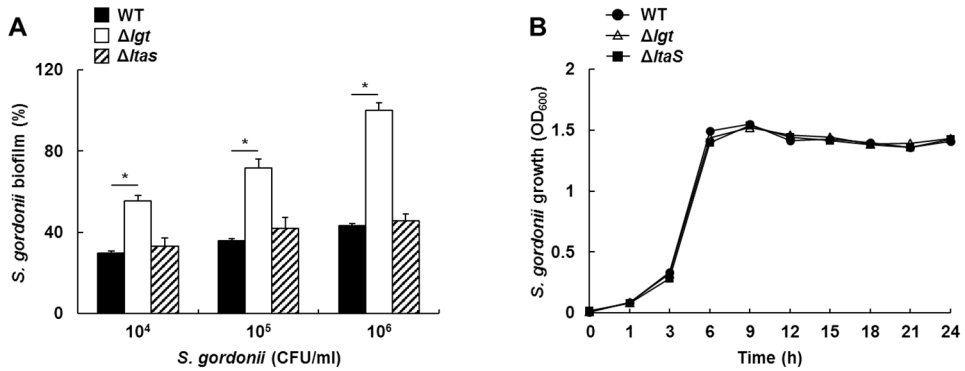

Figure 5. Lipoprotein-deficient *S. gordonii* forms biofilms much potently in comparison with wild-type bacteria without affecting the bacterial growth. (A) *S. gordonii* wild-type, Δ*lgt*, and Δ*ltaS* (1×10^4, 10^5, or 10^6 CFU/ml) were grown in polystyrene plates at 37°C for 24 h. The biofilm formation was determined by a crystal violet assay. (B) *S. gordonii* wild-type, Δ*lgt*, and Δ*ltaS* (1×10^7 CFU/ml) were grown under static condition for 1, 3, 6, 9, 12, 15, 18, 21, or 24 h. The bacterial growth was determined by measuring the optical density at 600 nm (OD_{600}). Data are the mean values ± S.D. of triplicate samples. Asterisks indicate significant induction at $P < 0.05$ compared with wild-type.

3.2. Lipoprotein-deficient *S. mutans* and *S. pneumoniae* form biofilms more potently than the wild-type bacteria

In order to confirm that the regulation of biofilm formation by lipoprotein on bacteria is common phenomenon in Streptococci, the biofilm-forming abilities of *S. mutans* Δ*lgt* and *S. pneumoniae* Δ*lgt* were compared to those of their wild-types, respectively. Analysis of biofilm formation using crystal violet showed that biofilm formation of *S. mutans* and *S. pneumoniae* Δ*lgt* were higher than those of wild-types (Figure 6A, B). To determine if the increase in biofilm formation of *S. mutans* and *S. pneumoniae* Δ*lgt* is related to the bacterial growth rate, the bacterial growth rate was examined. But the growth rate of *S. mutans* and *S. pneumoniae* Δ*lgt* was not different from that of the wild-types, respectively (Figure 6C, D). These results suggest that membrane-anchored lipoproteins on Streptococci suppress its own biofilm formation.

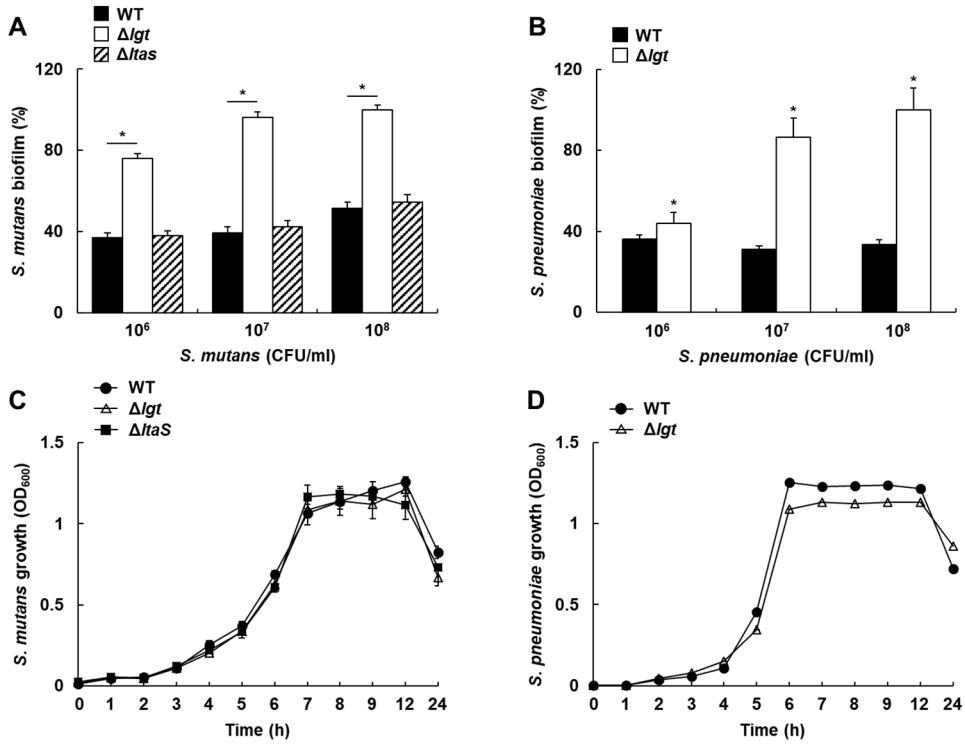

Figure 6. Lipoprotein-deficient *S. mutans* and *S. pneumoniae* form biofilms much potently in comparison with wild-type bacteria without affecting the bacterial growth. (A) *S. mutans*, and (B) *S. pneumoniae* wild-type and Δ*lgt* (1×10^6, 10^7, or 10^8 CFU/ml) were grown in polystyrene plates at 37°C for 24 h. The biofilm formation was determined by a crystal violet assay. (C) *S. mutans* wild-type and Δ*lgt* (1×10^6 CFU/ml), and (D) *S. pneumoniae* wild-type and Δ*lgt* (1×10^6 CFU/ml) were grown under shaking condition for 1, 2, 4, 5, 6, 7, 8, 9, 12, or 24 h. The bacterial growth was determined by measuring the optical density at 600 nm (OD_{600}). Data are the mean values ± S.D. of triplicate samples. Asterisks indicate significant induction at $P < 0.05$ compared with wild-type.

3.3. Lipoprotein-deficient *S. aureus* shows the same degree of biofilm formation compared to wild-type bacteria

To investigate whether lipoprotein-deficient bacteria also form enhanced biofilms in other Gram-positive bacteria, the role of cell wall components in *S. aureus* biofilm formation was examined. The biofilm-forming abilities of *S. aureus* Δ*lgt* and wild-type strain were compared. *S. aureus* wild-type and Δ*lgt* (1×10^4, 10^5, or 10^6 CFU/ml) were grown in polystyrene plates at 37°C for 24 h. Analysis of biofilm formation using crystal violet showed that there is no difference in biofilm formation and growth rate between each strain (Figure 7). These results suggest that membrane-anchored lipoproteins were only involved in biofilm formation of Streptococci, but not in *S. aureus*.

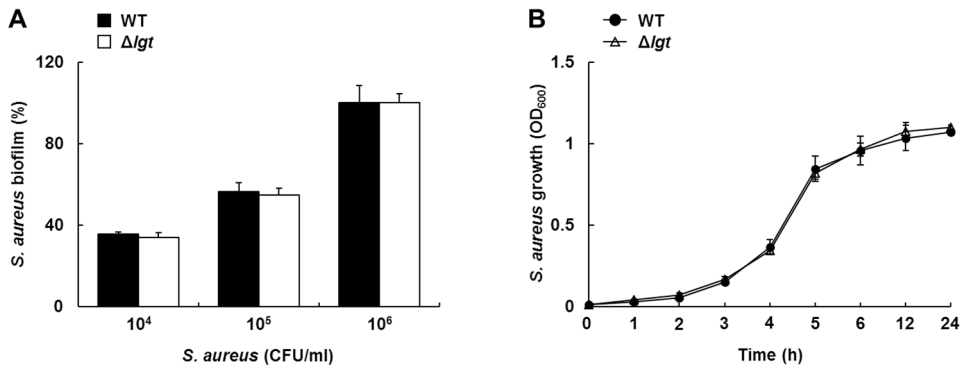

Figure 7. Lipoprotein-deficient strain of *S. aureus* shows the similar biofilm forming abilities to the wild-type. (A) *S. aureus* wild-type and Δ*lgt* (1×10^4, 10^5, or 10^6 CFU/ml) were grown in polystyrene plates at 37℃ for 24 h. The biofilm formation was determined by a crystal violet assay. (B) *S. aureus* wild-type and Δ*lgt* (1×10^6 CFU/ml) were grown under shaking condition for 1, 2, 3, 4, 5, 6, 12, or 24 h. The bacterial growth was determined by measuring the optical density at 600 nm (OD_{600}). Data are the mean values ± S.D. of triplicate samples. Asterisks indicate significant induction at $P < 0.05$ compared with wild-type.

3.4. Lipoprotein-deficient *S. gordonii* shows upregulation of *luxS* expression and enhanced AI-2 release

Quorum sensing systems are largely involved in bacterial biofilm formation [47]. In case of Streptococci, both AI-2 and CSP are known to enhance biofilm formation of *S. gordonii*, *S. mutans*, and *S. pneumoniae* [32, 34, 48]. In order to test whether the regulation of *S. gordonii* biofilm formation by lipoprotein is associated with its quorum sensing system, the biofilm-associated gene expression such as *scaR*, *luxS*, *comD*, and *comE* was examined in *S. gordonii* wild-type, Δ*lgt*, and Δ*ltaS*. *S. gordonii* Δ*lgt* showed upregulation of mRNA expression of *luxS* compared to wild-type (Figure 8A). To determine extracellular AI-2 release of *S. gordonii* wild-type, Δ*lgt*, and Δ*ltaS*, AI-2 from *S. gordonii* culture supernatants was measured by using a bioluminescent bacterial reporter strain, *V. harveyi* BB170. *S. gordonii* Δ*lgt* showed enhanced extracellular AI-2 level compared to wild-type (Figure 8B). When *S. gordonii* was treated with $_{D}$-ribose, an AI-2 antagonist, *S. gordonii* Δ*lgt* showed similar degree of biofilm formation as wild-type (Figure 8C). These results suggest that membrane-anchored lipoproteins of *S. gorodnii* would suppress the expression of *luxS*, resulting in a decrease of AI-2 production which regulates biofilm formation.

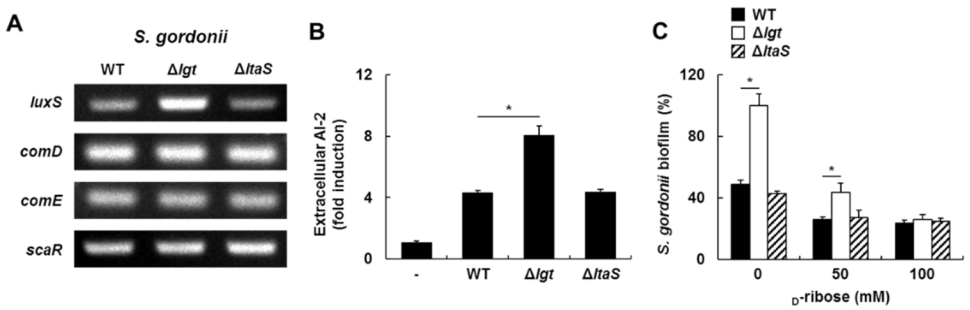

Figure 8. Expression of *luxS* is upregulated in lipoprotein-deficient strain of *S. gordonii*. (A) *S. gordonii* wild-type, Δ*lgt*, and Δ*ltaS* (1×10^7 CFU/ml) were cultured in THY to mid-log phase. Total RNA was isolated from *S. gordonii* and the mRNA expression of *luxS*, *comD*, *comE*, or *scaR* was examined by RT-PCR. The data were analyzed with an image analyzer. (B) *S. gordonii* wild-type, Δ*lgt*, and Δ*ltaS* (1×10^6 CFU/ml) were grown on polystyrene plates at 37°C for 24 h. The culture supernatants were collected to determine AI-2 release using a bioluminescent bacterial reporter strain, *V. harveyi* BB170. Luminescence was measured by a microplate reader. (C) *S. gordonii* wild-type, Δ*lgt*, and Δ*ltaS* (1×10^6 CFU/ml) were grown in polystyrene plates at 37°C for 24 h in the presence of $_D$-ribose (50 or 100 mM). The biofilm formation was determined by a crystal violet assay. Data are the mean values ± S.D. of triplicate samples. Asterisks indicate significant induction at $P < 0.05$ compared with wild-type.

3.5. LTA inhibits *E. faecalis* biofilm formation most effectively among cell wall components of *L. plantarum*

Next, the role of extracellular cell wall components in biofilm formation was examined. Previous reports have demonstrated that whole cells and supernatant of *Lactobacillus* inhibit oral pathogen biofilms effectively [49]. However, it has not been clearly understood which cell wall components of *Lactobacillus* play a role in biofilm inhibition. To determine which cell wall components of *Lactobacillus* inhibit *E. faecalis* biofilm, first, the effects of *L. plantarum* culture supernatant (Lp.sup) on *E. faecalis* biofilm formation were examined. *E. faecalis* were grown in polystyrene plates at 37°C for 24 h in the presence of the Lp.sup. Lp.sup inhibited biofilm formation in a dose-dependent manner and *E. faecalis* biofilms were inhibited up to 54.1% (Figure 9A). To characterize molecules that might be involved in Lp.sup-mediated inhibition of *E. faecalis* biofilm formation, *E. faecalis* was treated with various cell wall components from *L. plantarum*. As shown in Figure 9B, *L. plantarum* LTA (Lp.LTA) most effectively inhibited biofilm formation of *E. faecalis* up to 69.4%, *L. plantarum* lipoprotein (Lp.Lpp) showed inhibition effects up to 23.9%, but there was no effects on biofilm formation by treatment of *L. plantarum* peptidoglycan (Lp.PGN). These results suggest that Lp.LTA might be a key molecule in Lp.sup responsible for inhibition of *E. faecalis* biofilm formation.

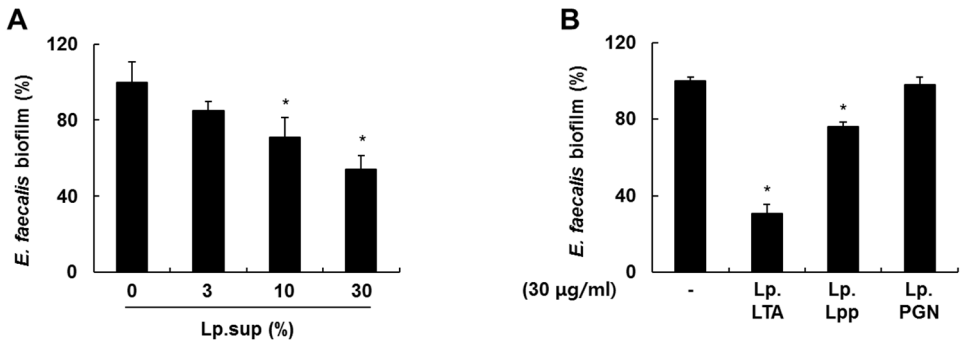

Figure 9. Lp.LTA inhibits *E. faecalis* biofilm formation most effectively among cell wall components of *Lactobacillus plantarum*. (A) *E. faecalis* (1.4×10^9 CFU/ml) was grown in polystyrene plates at 37°C for 24 h in the presence of the culture supernatants of *Lactobacillus plantarum* (3, 10, or 30% in BHI). (B) *E. faecalis* (1.4×10^9 CFU/ml) was grown in polystyrene plates at 37°C for 24 h in the presence of Lp.LTA, Lp.Lpp and Lp.PGN (30 μg/ml). The biofilm formation was determined by a crystal violet assay. Data are the mean values ± S.D. of triplicate samples. Asterisks indicate significant induction at $P < 0.05$ compared with non-treatment group.

3.6. *L. plantarum* LTA inhibits *E. faecalis* biofilm formation in a dose-dependent manner

To examine the effect of Lp.LTA on *E. faecalis* biofilm formation, *E. faecalis* formed in the presence of Lp.LTA was evaluated quantitatively and visually using crystal violet staining and confocal microscopy. *E. faecalis* (1.4×10^9 CFU/ml) was grown in polystyrene plates and confocal dish at 37°C for 24 h in the presence of Lp.LTA (3, 10, or 30 μg/ml) respectively. The biofilms were measured by crystal violet staining and SYTO9/propidium iodide staining were performed. Crystal violet staining demonstrated that *E. faecalis* biofilm was decreased in a dose-dependent manner when incubated with Lp.LTA (Figure 10A). SYTO9/propidium iodide staining also visualized that Lp.LTA inhibited *E. faecalis* biofilm formation (Figure 10B). These results indicate that Lp.LTA inhibits *E. faecalis* biofilm formation.

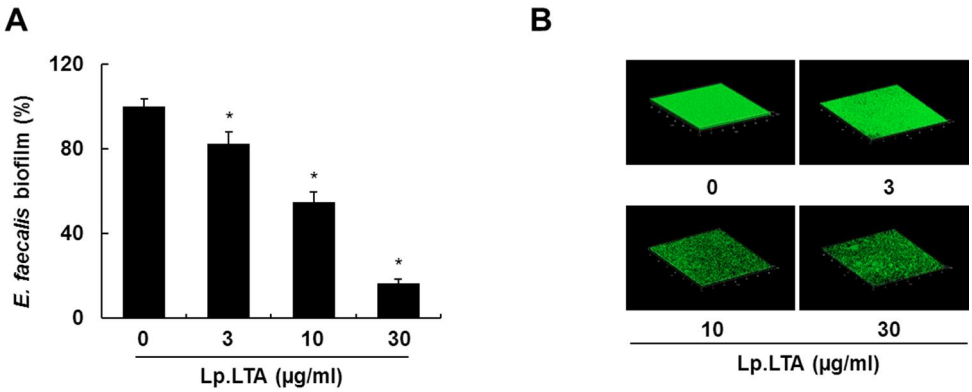

Figure 10. *L. plantarum* LTA inhibits *E. faecalis* biofilm formation in a dose-dependent manner. (A) *E. faecalis* (1.4×10^9 CFU/ml) was grown in polystyrene plates at 37°C for 24 h in the presence of Lp.LTA (3, 10, or 30 μg/ml). The biofilm formation was determined by a crystal violet assay. (B) *E. faecalis* (1.4×10^9 CFU/ml) was grown in confocal dishes at 37°C for 24 h in the presence of Lp.LTA (3, 10, or 30 μg/ml). The biofilms were visualized under a confocal laser scanning microscope: Green (SYTO9; live cells), Red (propidium iodide; dead cells). Data are the mean values ± S.D. of triplicate samples. Asterisks indicate significant induction at $P < 0.05$ compared with non-treatment group.

3.7. *L. plantarum* LTA inhibits biofilm formation at early and late stages, but not bacterial growth

To examined if the inhibitory effects of Lp.LTA occurs at early or late phases of biofilm development, the *E. faecalis* biofilms were measured at different time points after treated with Lp.LTA. *E. faecalis* (1.4×10^9 CFU/ml) was grown in polystyrene plates at 37°C for 1, 3, 6, 9, 12, 15, 18, 21, or 24 h in the presence of Lp.LTA (30 μg/ml). Analysis of biofilm formation was determined by crystal violet assay at each time points. When *E. faecalis* was incubated with Lp.LTA, the inhibitory effect was first observed at 1 h and lasted up to 24 h (Figure. 11A). *E. faecalis* was grown under shaking condition for 1, 3, 6, 12, 24, or 48 h in the presence or absence of Lp.LTA (30 μg/ml) and the bacterial growth was measured at each time points after treated with Lp.LTA. Notably, Lp.LTA did not affect bacterial growth (Figure. 11B), indicating that the inhibitory effect of Lp.LTA on *E. faecalis* biofilm formation was not due to the bacterial growth interference.

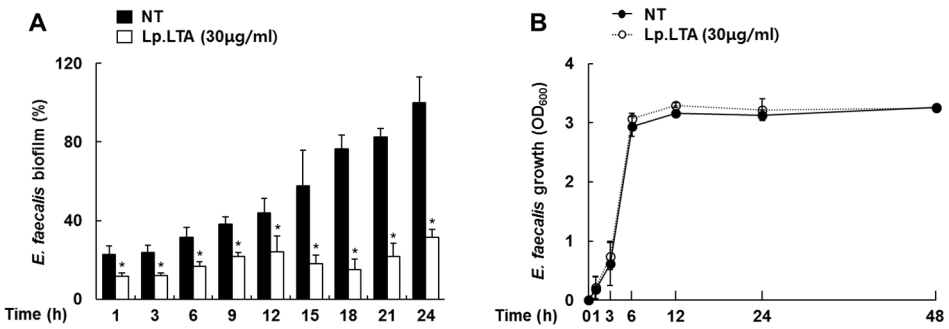

Figure 11. *L. plantarum* LTA inhibits biofilm formation at early and late stages, but not bacterial growth. (A) *E. faecalis* (1.4×10^9 CFU/ml) was grown in polystyrene plates at 37°C for 1, 3, 6, 9, 12, 15, 18, 21, or 24 h in the presence or absence of Lp.LTA (30 μg/ml). The biofilm formation was determined by a crystal violet assay. (B) *E. faecalis* was grown under shaking condition for 1, 3, 6, 12, 24, or 48 h in the presence or absence of Lp.LTA (30 μg/ml). The bacterial growth was determined by measuring the optical density at 600 nm (OD_{600}). Data are the mean values ± S.D. of triplicate samples. Asterisks indicate significant induction at $P < 0.05$ compared with non-treatment group.

3.8. LTAs from various *Lactobacillus* species inhibit biofilm formation and disrupt preformed biofilm

To investigate whether an inhibitory effect on biofilm formation of *E. faecalis* is a common characteristic of LTA from *Lactobacillus* species, LTA was purified from various *Lactobacillus* species including such as *L. casei*, *L. rhamnosus* GG, *L. acidophilus*. *E. faecalis* (1.4×10^9 CFU/ml) was grown in polystyrene plates at 37°C for 24 h in the presence of 10 or 30 μg/ml of LTA from *L. casei* (Lc.LTA), *L. plantarum* (Lp.LTA), *L. rhamnosus GG* (Lr.LTA), or *L. acidophilus* (La.LTA). All *Lactobacillus* species LTA tested in this experiment inhibited *E. faecalis* biofilm formation, while Lp.LTA showed most effective inhibition (Figure 12A). Next, the disruptive effects of Lp.LTA on preformed biofilm of *E. faecalis* were determined. Pre-formed biofilm (24 h) of *E. faecalis* was treated with *Lactobacillus* species LTA including Lc.LTA, Lp.LTA, Lr,LTA, or La.LTA for 6 h and the degree of destruction of the pre-formed biofilm was examined. As shown in Figure 12B, all *Lactobacillus* species LTA tested in this experiment disrupted preformed biofilm, while Lp.LTA showed most effective inhibition. These results indicate that inhibition effects on *E. faecalis* biofilm formation is common characteristic of LTAs from *Lactobacillus* species, furthermore these LTA can disrupt preformed biofilms.

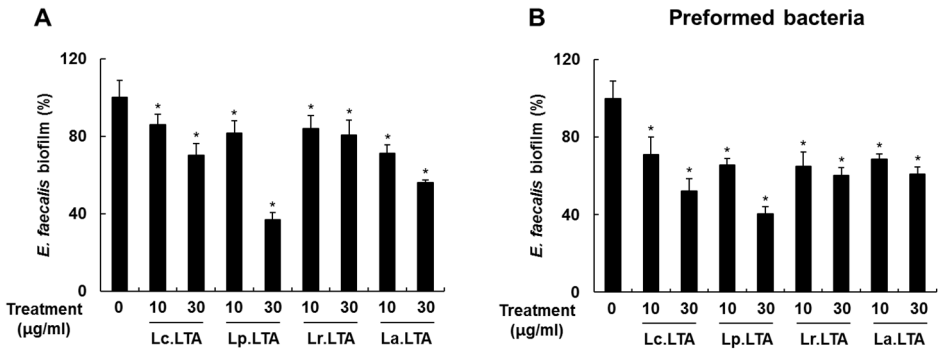

Figure 12. *Lactobacillus* species LTA inhibits biofilm formation and disrupts preformed biofilm. (A) *E. faecalis* (1.4×10^9 CFU/ml) was grown in polystyrene plates at 37°C for 24 h in the presence of 10 or 30 μg/ml of LTA from *L. casei* (Lc.LTA), *L. plantarum* (Lp.LTA), *L. rhamnosus* GG (Lr.LTA), or *L. acidophilus* (La.LTA). (B) *E. faecalis* (1.4×10^9 CFU/ml) was grown in polystyrene plates at 37°C for 24 h, and then supernatant containing planktonic bacteria was removed. Preformed biofilm was treated with 10 or 30 μg/ml of Lc.LTA, Lp.LTA, Lr.LTA, or La.LTA and further incubated at 37°C for 6 h. The biofilm formation was determined by a crystal violet assay. Data are the mean values ± S.D. of triplicate samples. Asterisks indicate significant induction at $P < 0.05$ compared with non-treatment group.

3.9. $_{D}$-Alanine moieties of *L. plantarum* LTA are important for the inhibitory effect on *E. faecalis* biofilm formation

Lp.LTA has $_{D}$-alanine moiety which gives positive charge to the negative phosphate backbone of LTA and molecules with positive charge are known to be related to the prevention of biofilm formation [50]. Previous reports have suggested that $_{D}$-alanine moiety of Lp.LTA plays an essential role in the inhibitory effect on biofilm formation of *S. aureus* and *S. mutans* [45, 51]. To examine the functional moieties responsible for the inhibitory effects of Lp.LTA on biofilm formation of *E. faecalis*, $_{D}$-alanine-removed Lp.LTA (Deala-Lp.LTA) was prepared by treatment with HCl (pH 8.5) [42]. *E. faecalis* (1.4×10^9 CFU/ml) was grown in polystyrene plates at 37°C for 24 h in the presence of Lp.LTA or Deala-Lp.LTA (10 or 30 μg/ml) respectively. The biofilm formation was determined by a crystal violet assay. As shown in Figure 13, Lp.LTA inhibited biofilm formation of *E. faecalis* in a dose-dependent manner, whereas Deala-Lp.LTA failed to do so. These results suggest that $_{D}$-alanine is a key component of Lp.LTA for its inhibitory effects on the biofilm formation of *E. faecalis*.

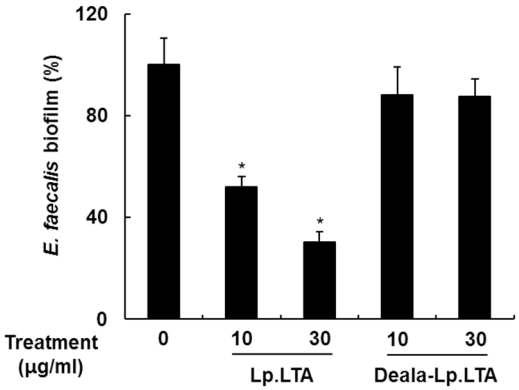

Figure 13. D-Alanine moieties of *L. plantarum* LTA are important for the inhibitory effect on *E. faecalis* biofilm formation. *E. faecalis* (1.4×10^9 CFU/ml) was grown in polystyrene plates at 37°C for 24 h in the presence of 10 or 30 μg/ml of Lp.LTA or Deala-Lp.LTA. The biofilm formation was determined by a crystal violet assay. Data are the mean values ± S.D. of triplicate samples. Asterisks indicate significant induction at $P < 0.05$ compared with non-treatment group.

3.10. *L. plantarum* LTA inhibits the biofilm formation of *E. faecalis* clinical isolates

In order to determine whether Lp.LTA inhibits both laboratory and clinical strains, the biofilm formation of *E. faecalis* KCOM 1083, KCOM 1161, and KCOM 1162, which have been isolated from human dental plaque, was examined with Lp.LTA. *E. faecalis* KCOM 1083, KCOM 1161, and KCOM 1162 (1.4×10^9 CFU/ml) were grown in polystyrene plates at 37°C for 24 h in the presence of Lp.LTA (10 or 30 μg/ml). The biofilm formation was determined by a crystal violet assay. As shown in Figure 14, Lp.LTA inhibited the biofilm formation of all *E. faecalis* clinical strains used in the study.

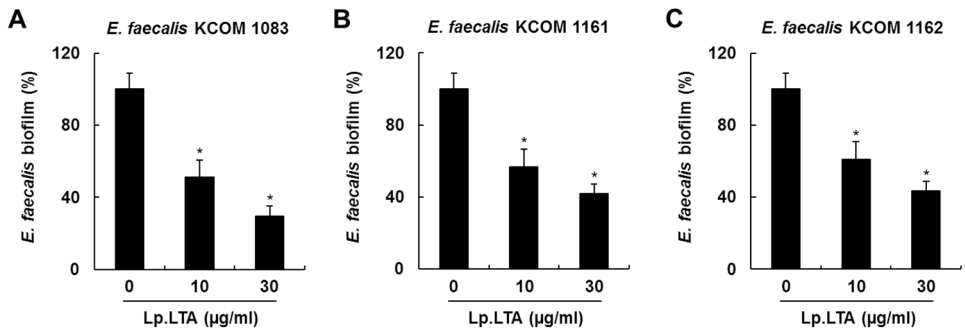

Figure 14. *L. plantarum* LTA inhibits the biofilm formation of *E. faecalis* clinical isolates. (A) *E. faecalis* KCOM 1083, (B) *E. faecalis* KCOM 1161, or (C) *E. faecalis* KCOM 1162 were grown in polystyrene plates at 37°C for 24 h in the presence of Lp.LTA (10, or 30 μg/ml). The biofilm formation was determined by a crystal violet assay. Data are the mean values ± S.D. of triplicate samples. Asterisks indicate significant induction at $P < 0.05$ compared with non-treatment group.

3.11. *L. plantarum* LTA inhibits the biofilm formation on human dentin slices

To evaluate the applicability of Lp.LTA on biofilm-associated oral disease, the inhibitory capacity of Lp.LTA on biofilms formed on human dentin slices was examined. *E. faecalis* (1.4×10^9 CFU/ml) was grown on human dentin slices at 37°C for 24 h in the presence or absence of Lp.LTA (30 μg/ml). After fixed and coated, *E. faecalis* biofilms on human dentin slices were visualized using a scanning electron microscope (SEM). As shown in Figure 15, Lp.LTA significantly inhibited *E. faecalis* biofilm formation and disrupted the biofilm preformed on human dentin slices. These results indicate that LTA derived from *Lactobacillus* species could be a therapeutic candidate for blocking *E. faecalis* biofilm.

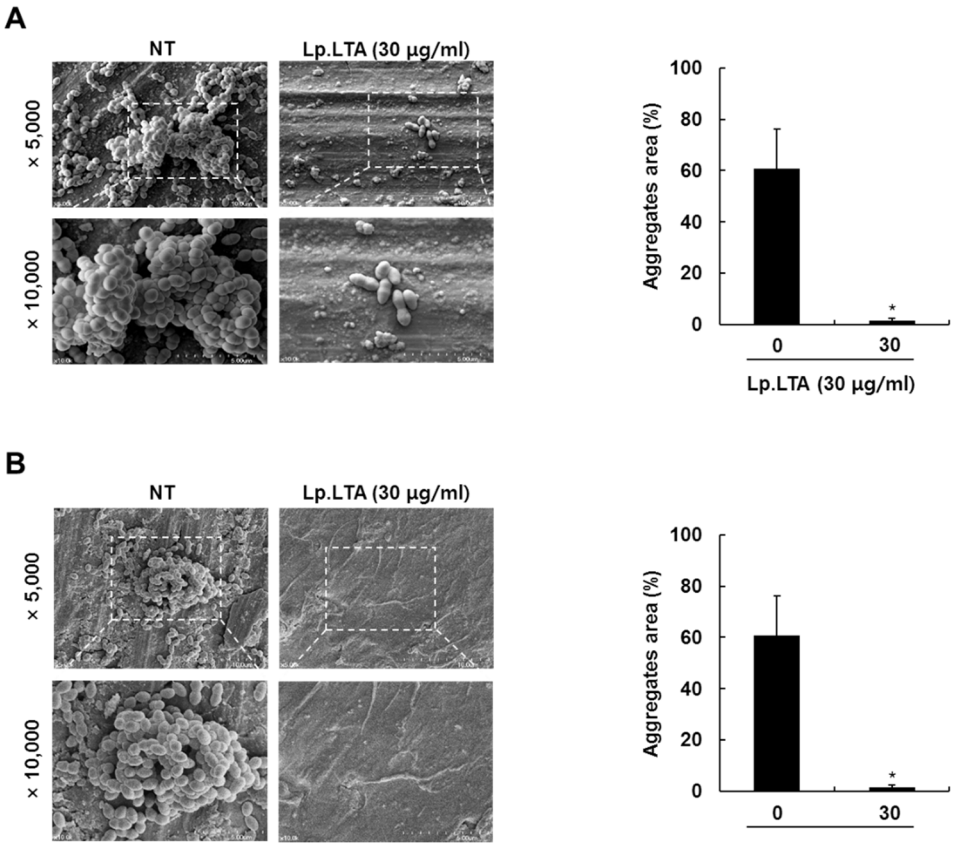

Figure 15. *L. plantarum* LTA inhibits the biofilm formation on human dentin slices. (A) *E. faecalis* (1.4×10^9 CFU/ml) was grown on sterile human dentin slices at 37°C for 24 h in the presence or absence of Lp.LTA (30 μg/ml). (B) *E. faecalis* (1.4×10^9 CFU/ml) was grown on sterile human dentin slices at 37°C for 24 h, and then supernatant containing planktonic bacteria was removed. Preformed biofilm was treated with Lp.LTA (30 μg/ml). The biofilm was analyzed with scanning electron microscope (magnification: ×5,000 and ×10,000). The area of bacterial aggregates was analyzed by ImageJ software. Data are the mean values ± S.D. of triplicate samples. Asterisks indicate significant induction at $P < 0.05$ compared with non-treatment group.

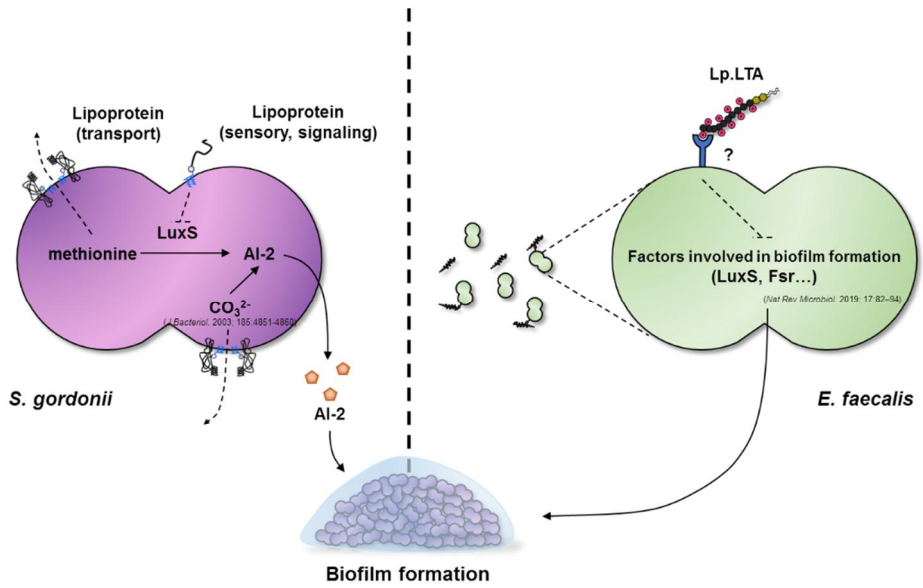

Figure 15. Schematic illustration of the proposed role of cell wall components of Gram-positive bacteria in bacterial biofilm. Membrane-anchored lipoproteins of Streptococci have the potential to suppress its biofilm formation by modulating AI-2 system through downregulation of *luxS* expression. Lp.LTA inhibits the biofilm formation of *E. faecalis* and disrupts preformed biofilms. $_{D}$-Alanine act as crucial functional moieties of LTA for inhibitory effect against *E. faecalis* biofilm formation.

Chapter IV. Discussion

The biofilm has been involved in more than half of human microbial infections [52]. Especially in oral cavity, there are over 1,000 different bacterial species which form mixed species community through biofilm formation on the teeth or mucosal surfaces, which lead to tooth decay, furthermore apical periodontitis [30, 53]. In addition, it has been known that oral pathogen induces systemic infection such as bacteremia, cardiovascular disease, and infective endocarditis through dissemination to distant body sites [54]. Although biofilm formation is important for oral pathology, the role of cell wall components of oral pathogen on biofilm formation has not been clearly understood.

In this study, lipoprotein-deficient strain (Δ*lgt*) of *S. gordonii* showed higher biofilm formation compared to wild-type strain. In addition, other Streptococci Δ*lgt* including *S. mutans* and *S. pneumoniae* showed augmented biofilm formation in common with *S. gordonii*, but *S. aureus* did not. There is no difference in the bacterial growth rate and morphology between *S. gordonii* wild-type and Δ*lgt* [55]. These results indicate that membrane-anchored lipoprotein on Streptococci would negatively regulate its biofilm formation. On the other hand, lipoprotein VacJ-deficient strain of *Actinobacillus pleuropneumoniae* showed more susceptibility to antibiotics, deformed or swelled cell morphology, and reduced biofilm formation. It suggests that VacJ lipoprotein is essential in maintaining outer membrane integrity of *A. pleuropneumoniae* [56] and *Bacillus subtilis* lipoprotein, PrsA, are associated with the bacterial growth [57]. These conflicting results can be explained as follows, the biological roles of lipoproteins or its proportion are quite

different within a variety of bacterial species and many are still unknown [58]. For example, a large proportion of the staphylococcal lipoprotein is involved in the uptake of essential ions and nutrients. But, in general, *S. pneumoniae* has a smaller number of ABC transporters in comparison with an average *S. aureus* strain [58]. According to these results, membrane-anchored lipoproteins of Streptococci may be expected to have more involved in other parts such as transport system or signaling system than structural roles.

Interestingly, *S. gordonii* Δ*lgt* showed upregulation of *luxS* gene expression, which is one of the key factors for AI-2 production [59]. AI-2 is known to be crucial for biofilm formation in Streptococci, for example, *S. gordonii luxS* mutant that cannot produce AI-2 formed altered biofilm structure [32] and LuxS/AI-2 quorum-sensing system is necessary for biofilm formation and the ear infection *in vivo* [48]. In Streptococci, lipoproteins mainly function as transport system, which may develop genetic competence by sensing the surrounding environment [60, 61]. It has been reported that lipoproteins LpqB of *Mycobacterium* regulate multidrug resistance and cell wall homeostasis by modulating MtrAB two-component system [62]. Therefore, membrane-anchored lipoproteins of Streptococci may have the potential to suppress its biofilm formation by modulating AI-2 system through downregulation of *luxS* expression.

This study shows that there is no difference in biofilm formation or cell growth between LTA-deficient strain (Δ*ltaS*) of *S. gordonii*, *S. mutans*, and *S. pneumoniae* and their wild-types. In addition, previous study showed that *S. gordonii* Δ*ltaS*

showed the same bacterial size and morphology with its wild-type [55]. Although it has been known that membrane-anchored LTA is important for bacterial growth and biofilm formation in *B. subtilis*, *Listeria monocytogenes*, and *Parvimonas micra* [63-65], these results indicate that membrane-anchored LTA of Streptococci would not be involved in cell morphology, size, as well as its biofilm formation.

In the present study, *L. plantarum* supernatant (Lp.sup) inhibited biofilm formation, in which Lp.LTA is a key molecule for inhibition of *E. faecalis* biofilm formation. In addition, LTA from various *Lactobacillus* species inhibits *E. faecalis* biofilm formation. Lp.LTA has the highest inhibitory effect on *E. faecalis* biofilm formation among various *Lactobacillus species* including *L. casei*, *L. rhamonosus* GG, and *L. acidophilus*. Concordantly, *L. fermentum* strains have been shown to inhibit biofilm formation caused by antibiotic-resistant strains of *Pseudomonas aeruginosa* [66]. It is also previously reported that Lp.LTA inhibits the biofilm formation of *S. aureus*, a pathogenic bacterium causing serious infections including pneumonia, sepsis, and osteoarthritis [45], and *S. mutans*, one of the major pathogens causing dental caries [51]. Thus, Lp.LTA might be a potential biofilm blocker inhibiting biofilm formation of oral pathogens.

Interestingly, Lp.LTA not only inhibited biofilm formation but disrupted preformed biofilm. Previously it has been reported that lactobacilli and their culture supernatant disrupted the preformed biofilms of pathogens such as *Vibrio* species and *Aggregatibacter actinomycetemcomitans* [67, 68]. These results are concordant with the previous report showing the inhibitory effect of Lp.LTA on preformed

biofilm for *S. aureus* by mediating AI-2 production [45]. It has been known that biosurfactant rhamnolipid disrupts preformed biofilm through detachment mechanism [69]. Furthermore, production of biosurfactant proteins is regulated by quorum sensing molecules in *P. aeruginosa* [70]. Therefore, Lp.LTA may have potential to induce biosurfactant proteins that disrupt preformed biofilm.

$_{D}$-Alanine moieties play an essential role for inhibitory effects of Lp.LTA on *E. faecalis* biofilm formation in the present study. Consistent with these results, $_{D}$-amino acid such as $_{D}$-leucine and $_{D}$-tyrosine induce biofilm disassembly in *B. subtilis*, *S. aureus*, *Staphylococcus epidermidis*, and *P. aeruginosa* [71]. Lp.LTA has $_{D}$-alanine moiety giving a positive charge to the negative phosphate backbone of LTA. Positive charge has been used for the prevention of biofilm formation. For instance, the increase of *N*-deacetylation degrees in chitosan-streptomycin conjugates enhance its anti-biofilm ability [72]. It supports that positive charge of $_{D}$-alanine is a key factor for the inhibitory activity of Lp.LTA.

Accumulating reports suggest that LTA, as a major cell wall component of Gram-positive bacteria, can induce innate and inflammatory responses [73]. LTA efficiently induces the expression of nitric oxide, chemokines, and cytokines [41, 74-76]. Likewise, previous studies have mainly focused on the immunostimulatory function of LTA. Recently, however, LTA has been investigated for its role as a regulator of immune response [77, 78]. Staphylococcal LTA attenuates lipopolysaccharide (LPS)-induced B cell proliferation [79]. Lp.LTA inhibits lipoprotein-induced IL-8 expression in human intestinal epithelial cells [78] and the

expression of TNF-α in LPS induced endotoxin shock mice [80]. In our study, Lp.LTA efficiently inhibited the *E. faecalis* biofilm formation. Therefore, I suggest that bacteria-derived LTA could be an attractive therapeutic target for modulating immune responses and treating bacteria-mediated diseases.

In conclusion, the results from the present study demonstrated that Lp.LTA inhibits *E. faecalis* biofilm formation and disrupts the preformed biofilm. Furthermore, Lp.LTA efficiently inhibits biofilm formation at early stage and keeps the inhibitory effect ever afterward. These results provide evidence that LTA derived from *Lactobacillus* species could be a useful biofilm blocker to eliminate oral pathogen including *E. faecalis* and contribute to treatment of refractory apical periodontitis.

Chapter V. References

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국문초록

그람 양성균 세포벽 성분이 바이오필름 형성에 미치는 영향

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1. 목 적

*Enterococcus faecalis*와 *Streptococcus gordonii*는 일반적으로 구강, 위장관, 생식기와 같은 인체 내 점막조직에 정상세균총으로 존재하는 통성혐기성 그람 양성균으로 알려져 있지만, 최근 병원 내에서 기회감염을 통하여 난치성 치근단 치주염과 심내막염과 같은 염증질환을 유발하는 대표적인 원인균으로 보고되고 있다. 이들은 구강 내에서 바이오필름을 형성하는 대표적인 균으로 알려져 있으며, 바이오필름 형성 시 항생제와 대식작용에 대해 물리적, 화학적인 방어벽을 가지게 되어 만성적인 염증 질환에 기여하는 것으로 알려져 있다. 하지만

현재까지 이러한 균들이 바이오필름을 형성에 관여하는 핵심 세포벽 인자와, 이를 제어하는 방법에 대한 연구는 미비한 상태이다. 따라서 본 연구에서는 균의 바이오필름 형성에 관여하는 핵심 세포벽 성분 및 작용기전을 연구하고, 이를 제어할 수 있는 유산균 유래 물질 및 조절 기전을 규명하는 연구를 진행하였다.

2. 방 법

균의 표면에서 세포벽 성분이 바이오필름 형성에 미치는 영향을 확인하기 위해 *S. gordonii*, *Streptococcus mutans*, *Streptococcus pneumoniae*, 그리고 *Staphylococcus aureus* 정상균주와 지질단백질 결여균주 및 리포테이코익산 결여균주를 사용하였으며 크리스탈 바이올렛 염색법을 이용하여 바이오필름 형성 정도를 확인하였다. 유산균의 세포벽 성분의 바이오필름 형성 억제 능력을 평가하기 위해 *E. faecalis*를 *Lactobacillus plantarum*의 정제된 리포테이코익산, 지질단백질, 펩티도글리칸을 처리한 후 형성된 바이오필름을 크리스탈 바이올렛 염색법과 LIVE/DEAD 박테리아 염색법을 통해서 확인하였다. 유산균 리포테이코익산이 박테리아의 성장에 미치는 영향을 확인하기 위해 *E. faecalis*에 유산균 리포테이코익산을 처리한 뒤 분광광도계를 이용하여 흡광도를 측정하였다. 또한 실제로 치아에 형성된 바이오필름에 대해서도 유산균 리포테이코익산이 억제능을 보일지 확인하기 위해 사람의 상아질 절편 상에서 형성된 바이오필름은 주사 전자 현미경을 통해서 관찰되었다.

3. 결 과

S. gordonii 지질단백질 결여균주는 정상균주와 리포테이코익산 결여균주에 비해 더 많은 바이오필름을 형성하여, 균의 지질단백질이 바이오필름 형성을 매개한다는 것을 확인하였다. 또한 지질단백질 결여균주가 정상균주보다 더 많은 바이오필름을 형성하는 현상은 Streptococci에서 공통적으로 나타나는 현상이었지만 *S. aureus*에서는 차이가 나타나지 않았다. 그리고 *S. gordonii* 지질단백질 결여균주는 더 높은 *luxS* 발현량과, AI-2 생성을 보였다. 리포테이코익산을 포함한

유산균 배양액은 *E. faecalis*의 바이오필름 형성을 억제하였다. 각각의 유산균 세포벽 성분을 정제하여 *E. faecalis*에 처리한 결과 *L. plantarum* 리포테이코익산이 가장 큰 억제효과를 나타냈다. 이러한 *L. plantarum* 리포테이코익산 처리에 의해 바이오필름 형성은 초기 단계인 1시간 이후부터 떨어졌으며, 균의 성장속도에는 영향을 미치지 않았다. 또한 *L. plantarum* 뿐 아니라 다양한 유산균 리포테이코익산이 바이오필름을 억제한다는 것을 확인하였다. 이러한 바이오필름 억제 효과에는 리포테이코익산의 $_{D}$-alanine 잔기가 필수적이라는 것을 밝혔으며, 리포테이코익산이 사람의 상아질 상에서 형성된 바이오필름과, 환자의 상아질 절편에서 분리한 균에 대해서도 억제효과를 보인다는 것을 보였다.

4. 결 론

이상의 연구결과들로부터 다음과 같은 결론을 얻을 수 있었다. *S. gordonii*의 지질단백질은 쿼럼인식인자인 AI-2의 발현을 억제하여 바이오필름을 조절하고 있으며, 유산균 리포테이코익산은 *E. faecalis*의 바이오필름의 형성을 효과적으로 억제하고 형성된 바이오필름을 무너뜨린다. 이를 통해 유산균 리포테이코익산은 구강내의 바이오필름 연관 질병을 예방하고 치료하는 항바이오필름 물질로 활용될 수 있음을 보였다.

주요어 : 그람양성균, 리포테이코익산, 지질단백질, 바이오필름, 유산균

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