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D-Galactose as an autoinducer 2 inhibitor to control the biofilm formation of periodontopathogens

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Autoinducer 2 (AI-2) is a quorum sensing molecule to which bacteria respond to regulate various phenotypes, including virulence and biofilm formation. AI-2 plays an important role in the formation of a subgingival biofilm composed mostly of Gram-negative anaerobes, by which periodontitis is initiated. The aim of this study was to evaluate D-galactose as an inhibitor of AI-2 activity and thus of the biofilm formation of periodontopathogens. In a search for an AI-2 receptor of *Fusobacterium nucleatum*, D-galactose binding protein (Gbp, Gene ID FN1165) showed high sequence similarity with the ribose binding protein (RbsB), a known AI-2 receptor of *Aggregatibacter actinomycetemcomitans*. D-Galactose was evaluated for its inhibitory effect on the AI-2 activity of *Vibrio harveyi* BB152 and *F. nucleatum*, the major coaggregation bridge organism, which connects early colonizing commensals and late pathogenic colonizers in dental biofilms. The inhibitory effect of D-galactose on the biofilm formation of periodontopathogens was assessed by crystal violet staining and confocal laser scanning microscopy in the absence or presence of AI-2 and secreted molecules of *F. nucleatum*. D-Galactose significantly inhibited the AI-2 activity of *V. harveyi* and *F. nucleatum*. In addition, D-galactose markedly inhibited the biofilm formation of *F. nucleatum*, *Porphyromonas gingivalis*, and *Tannerella forsythia* induced by the AI-2 of *F. nucleatum* without affecting bacterial growth. Our results demonstrate that the Gbp may function as an AI-2 receptor and that galactose may be used for prevention of the biofilm formation of periodontopathogens by targeting AI-2 activity.

Keywords: quorum sensing, periodontopathogens, biofilm, AI-2 inhibitor, D-galactose

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Introduction

Quorum sensing (QS) is mediated via small signaling molecules that are secreted from bacteria. QS signaling molecules above a threshold can affect various phenotypes, including bioluminescence, virulence, and biofilm formation (Taga and Bassler, 2003). Autoinducer-2 (AI-2) is a QS molecule secreted from both Gram-negative and Gram-positive bacteria (Rickard *et al.*, 2006). It is synthesized through pathways involving LuxS and released out of cells, and then it reenters bacteria through a transport or two-component system and acts on gene transcription (Schauder *et al.*, 2001; Xavier and Bassler, 2003). LuxP in *Vibrio harveyi*, LsrB in *Salmonella* Typhimurium, and RbsB in *Escherichia coli* are known to be an AI-2 transporter (LuxP) or AI-2 receptors (LsrB/RbsB).

Periodontitis is initiated by multispecies bacteria present in subgingival biofilms. Major periodontopathogens are highly proteolytic and induce immune and inflammatory responses, which result in tissue damage. AI-2 plays a critical role in the biofilm formation and virulence of periodontopathogens. AI-2 can thus be a good target for the control of periodontal infections, and AI-2 inhibitors are ideal for applications that inhibit oral biofilm formation (Kolenbrander *et al.*, 2010; Shao and Demuth, 2010; Jiang and Li, 2013). Two types of AI-2 receptors, LsrB and RbsB, have been reported in *Aggregatibacter actinomycetemcomitans* (James *et al.*, 2006; Shao *et al.*, 2007), but those in other periodontopathogens remain to be identified. AI-2 binds to LsrB and RbsB in the periplasm to transduce intracellular signaling. AI-2 and D-ribose bind competitively to the RbsB of *A. actinomycetemcomitans*, whereby D-ribose can inhibit AI-2-induced intracellular communication. *Fusobacterium nucleatum* plays a critical role in dental biofilm development, which leads to periodontitis by coaggregation with pathogenic bacteria species. Recently, furanone compound and D-ribose have been demonstrated to inhibit the biofilm formation of major periodontopathogens including *F. nucleatum*, *Porphyromonas gingivalis*, *Tannerella forsythia*, and *Treponema denticola* by inhibiting AI-2 (Jang *et al.*, 2013).

In searching for AI-2 receptors in periodontopathogens and for AI-2 inhibitors, we investigated the galactose-binding protein with a molecular weight of 36 kDa (Gbp) of *F. nucleatum*, which showed high sequence similarity with the RbsB of *A. actinomycetemcomitans* and *E. coli*. A galactose-binding adhesin (30 kDa) of *F. nucleatum*'s surface has been demonstrated to be involved in coaggregation with other periodontopathogens, including *P. gingivalis* and *A. actinomycetemcomitans* (Shaniztki *et al.*, 1997; Rosen and Sela, 2006). However, the roles of *F. nucleatum* galactose binding protein as an AI-2 receptor and galactose as an AI-2 in-

hibitor have not been assessed.

In this study, we tested whether D-galactose can act as an AI-2 inhibitor by inhibiting AI-2 activity and the biofilm formation of periodontopathogens. We demonstrated that D-galactose showed high inhibitory activity against the AI-2 activity of *F. nucleatum* and *V. harveyi* and the biofilm formation of *F. nucleatum*, *P. gingivalis*, and *T. forsythia* in the presence of semi-purified AI-2 or secreted molecules of *F. nucleatum*.

Materials and Methods

Identification of RbsB-like proteins

To identify an AI-2 binding protein in *F. nucleatum*, we used LuxP of *V. harveyi*, LsrB of *S.* Typhimurium, LsrB and RbsB of *A. actinomycetemcomitans*, and RbsB of *E. coli* as query sequences to perform a BLAST search against protein sequences of *F. nucleatum*.

Bacterial culture

F. nucleatum (ATCC 25586) and *P. gingivalis* (ATCC 33277) were cultured anaerobically (10% H_2, 10% CO_2, 80% N_2) in brain heart infusion broth supplemented with hemin (10 μg/ml) and vitamin K (0.2 μg/ml) at 37°C. *T. forsythia* (ATCC 43037) was cultured anaerobically in new oral spirochete broth (ATCC medium 1494) supplemented with N-acetylmuramic acid (0.01 μg/ml) and vitamin K (0.2 μg/ml). AI-2 reporter strain *V. harveyi* BB170 (ATCC BAA-1117) and AI-2 producing strain *V. harveyi* BB152 (ATCC BAA-1119) were aerobically cultured in autoinducer bioassay (AB) medium, which consisted of 0.3 M sodium chloride, 0.05 M magnesium sulfate, 0.2% casamino acids, 10 mM potassium phosphate (pH 7.0), 1 mM L-arginine, and 2% glycerol at 30°C with shaking.

Purification of *F. nucleatum* AI-2

The AI-2 of *F. nucleatum* was partially purified as described previously (Jang *et al.*, 2013). Briefly, bacteria from an overnight culture of *F. nucleatum* and *V. harveyi* BB152 were diluted 1:20 with fresh culture medium and cultured at 37°C and 30°C, respectively, until the late exponential phase (optical density at 660 nm = 0.7). The culture supernatants were collected by centrifugation at 10,000 × *g* at 4°C. After the culture supernatants were passed through 0.2-μm pore-size membrane filters (Sartorius Stedium Biotech), the filtrate was subsequently passed through a Centricon YM-3 3-kDa exclusion filter (Millipore) and then chromatographed on a C18 Sep-Pak reverse-phase column (Waters Co.) according to the manufacturer's instructions.

Determination of AI-2-mediated bioluminescence and AI-2 inhibitory activity of D-galactose

V. harveyi BB170 was diluted to a concentration of 1×10^6 bacteria/ml in fresh AB medium. Then, the bacterial suspension was mixed with 10% (vol/vol) partially purified AI-2 of *F. nucleatum* or *V. harveyi* BB152 in the presence or absence of D-ribose and D-galactose, and incubated for 1–12 h at 30°C under aerobic conditions. The bioluminescence was measured using a luminometer (GloMax-Multi Detection System, Promega).

Effect of D-galactose on biofilm formation of periodontopathogens

Biofilm formation assays were performed by staining with crystal violet and confocal laser scanning microscopy as described previously (Jang *et al.*, 2013). Briefly, bacterial suspensions were added to 24-well plates with round glass slips (12-mm radii) in the presence of *F. nucleatum* AI-2 (10% vol/vol) and/or sugars at various concentrations and were incubated for 24–48 h under anaerobic conditions. The initial numbers of *F. nucleatum*, *P. gingivalis*, and *T. forsythia* were 2×10^7/ml, 2×10^8/ml, and 2×10^8/ml, respectively. After the glass slips were washed three times with phosphate buffered saline (PBS), the biofilms that had formed on the glass slips were stained with 1% crystal violet for 10 min, washed three times with PBS, and destained with 1 ml of acetone–alcohol (20:80, vol/vol). The absorbance at 590 nm of the destaining solution containing crystal violet was measured using a microplate reader (Wallac Victor3 microtiter, PerkinElmer Life Sciences).

The biofilms formed on the cover glasses were stained using the Live/Dead-BacLight bacterial viability kit (Invitrogen), observed using a confocal laser scanning microscope (Carl Zeiss LSM 700, Gena) at a magnification of 1,000 ×, and quantified by measuring the mean intensity values of green (SYTO9) and red (propidium iodide) fluorescence.

Effects of co-culture of *F. nucleatum* and other periodontopathogens on biofilm formation using a transwell system

A two-compartment separated co-culture system was used to evaluate the effects of secreted molecules of *F. nucleatum* on the biofilm growth of *P. gingivalis* and *T. forsythia*. *P. gingivalis* (1×10^7 /ml) or *T. forsythia* (1×10^7 /ml) cells were suspended in coculture medium and placed in the bottom wells of transwell plates (BD Falcon™), and *F. nucleatum* (1×10^7 /ml) cells were placed in the upper wells, which were separated from the bottom wells by a membrane with a 0.4-μm pore size. The coculture medium was composed of one part of *F. nucleatum* culture medium and one part of the culture medium of the other partner bacterium. The bacterial growth for each species in the co-culture medium was confirmed to be indistinguishable from the growth observed in their own culture media. After anaerobic incubation at 37°C for 72 h, the biofilms that had formed on the glass slips in the bottom wells were stained with crystal violet and measured as described above.

Effect of D-galactose on planktonic bacterial growth

F. nucleatum were grown anaerobically in the presence or absence of various concentrations of D-galactose and D-ribose. Bacterial growth was monitored for up to 48 h by measuring the absorbance at 600 nm using a spectrophotometer.

Statistical analysis

Statistical analyses were performed using Student's *t*-test.

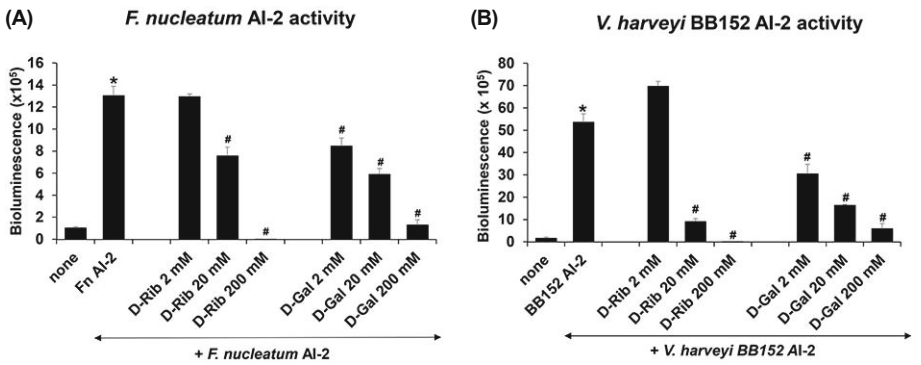

Fig. 1. Inhibitory effect of D-galactose on AI-2 activity. *V. harveyi* BB170 (1×10^6 bacteria/ml) was incubated for 6 h with 10% partially purified AI-2 of *F. nucleatum* (A) and *V. harveyi* BB152 (B) in the presence or absence of D-galactose (D-Gal) and D-ribose (D-Rib). The bioluminescence was measured using a luminometer. The experiments were performed three times in triplicates, and representative data are shown. $^*P < 0.05$ compared to the untreated control value; $^{\#}P < 0.05$ compared to the *F. nucleatum* AI-2 or *V. harveyi* AI-2-treated value.

Statistically significant differences between the control and AI-2 groups or between the AI-2 and sugar-treated groups were analyzed. A *p*-value less than 0.05 was considered statistically significant.

Results

Structural similarities of galactose-binding protein and RbsB

Because there have been no previously identified AI-2 receptors of *F. nucleatum*, we searched for a protein in *F. nucleatum* that had a sequence similarity with known AI-2 receptors in other bacteria. We found that the RbsB of *E. coli* and *A. actinomycetemcomitans* has a high level of sequence similarity with periplasmic D-galactose-binding protein (341 amino acid residues, Mw 36 kDa, Gbp) of *F. nucleatum* ATCC 25586 (gene ID FN1165 in EnsemblBacteria and KEGG), with e-values of 5×10^{-8} and 2×10^{-11}. No significant hits were found for the remaining AI-2 receptors. Significant amino acid sequence similarity between the RbsB and *F. nucleatum* Gbp suggests that these proteins share a common function. In particular, the result implies that although *F. nucleatum* Gbp is simply known as the galactose binding protein ATP-binding cassette (ABC) transporter in the periplasm, it may have a second function of being a quorum sensing AI-2 receptor. Therefore, we evaluated D-galactose for the competitive binding of AI-2 to Gbp and thus its feasibility as a quorum sensing inhibitor for further analysis.

Inhibitory effect of D-galactose on AI-2 activity

In a previous report, we demonstrated that D-ribose inhibited the AI-2 activity of *V. harveyi* BB152, an AI-2 producing strain, and that of *F. nucleatum* (Jang *et al.*, 2013). In this study, we evaluated the inhibitory effect of D-galactose on AI-2 activity and compared it with that of D-ribose. As shown in Fig. 1, D-galactose significantly inhibited the AI-2 activity of *V. harveyi* BB152 and *F. nucleatum* in a dose-dependent manner. The inhibitory activity of D-galactose against the AI-2 of *F. nucleatum* and *V. harveyi* was comparable to that of D-ribose at 20 and 200 mM. However, the AI-2 inhibitory activity of D-galactose at 2 mM was superior to that of D-ribose. D-Ribose did not show an inhibitory effect at 2 mM.

Inhibitory effect of D-galactose on biofilm formation of periodontopathogens induced by *F. nucleatum* AI-2

Next, we tested whether D-galactose inhibited the *F. nucleatum* AI-2-induced biofilm formation of three species of periodontopathogens. As semi-purified *F. nucleatum* AI-2 induced the biofilm formation of periodontopathogens (Jang *et al.*, 2013), we assessed the inhibitory effects of D-galactose and D-ribose on the biofilm formation of major periodontopathogens in the presence of *F. nucleatum* AI-2 or secreted

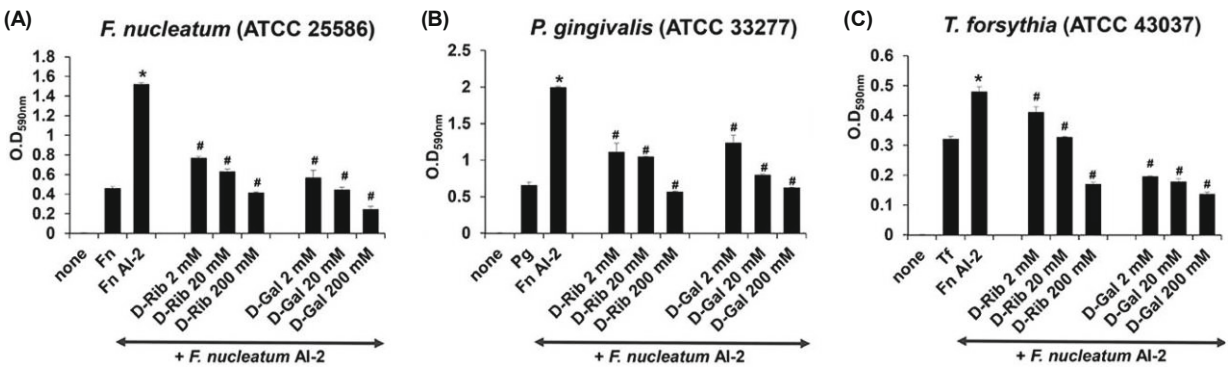

Fig. 2. Inhibitory effect of D-galactose on the biofilm formation of periodontopathogens. (A) *F. nucleatum* (Fn, 2×10^7/ml), (B) *P. gingivalis* (Pg, 2×10^8/ml), and (C) *T. forsythia* (Tf, 2×10^8/ml) were cultured with 10% partially purified *F. nucleatum* AI-2 in the presence of D-galactose (D-Gal) or D-ribose (D-Rib) for 48 h under anaerobic conditions at 37°C. Biofilm formation on glass slips was assessed by crystal violet staining. $^*P < 0.05$ compared to the untreated control value; $^{\#}P < 0.05$ compared to the biofilm formation of the *F. nucleatum* AI-2- treated value in the absence of the quorum sensing inhibitors.

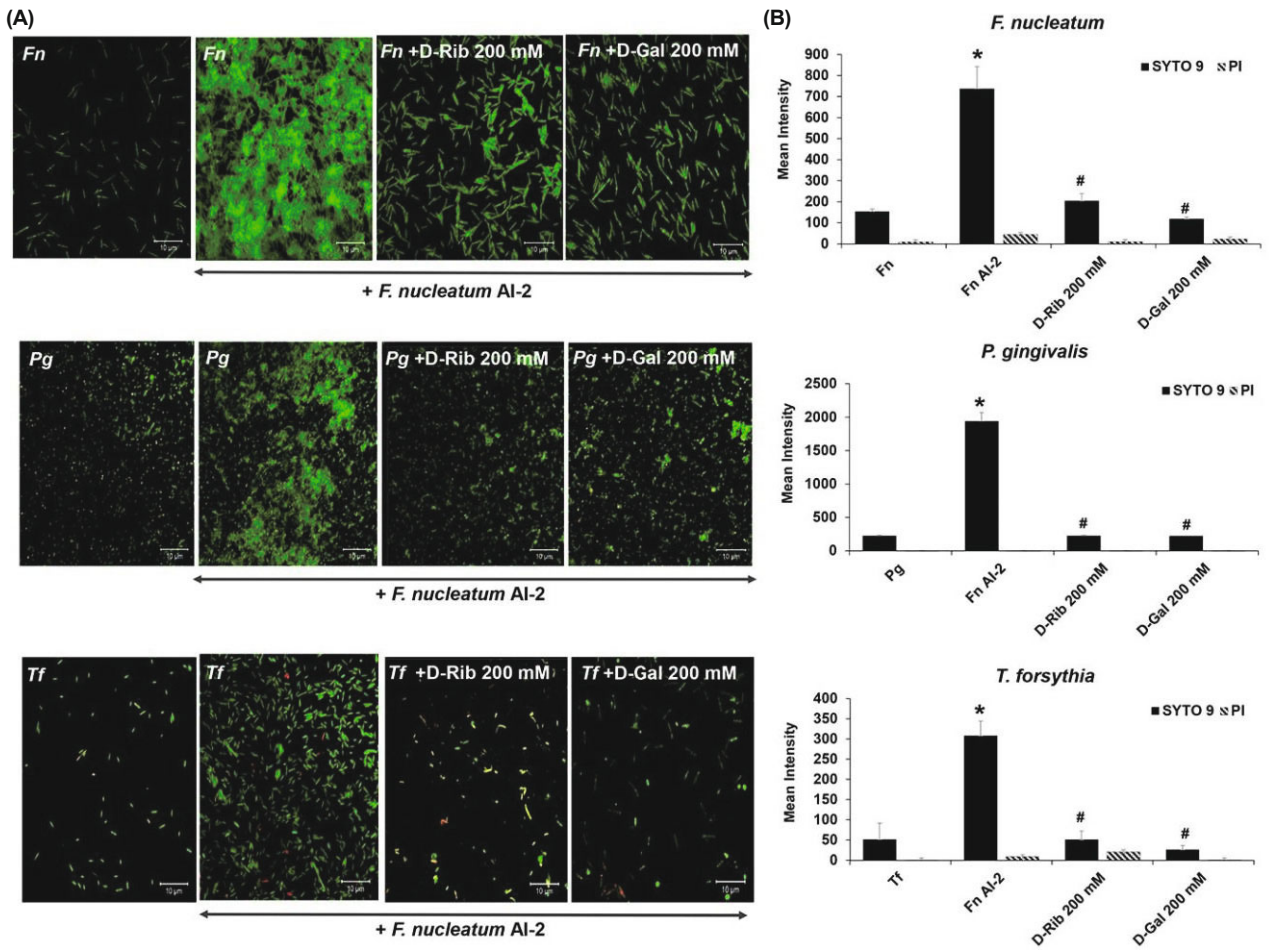

Fig. 3. Confocal images showing the inhibitory effect of D-galactose on the biofilm formation of periodontopathogens. *F. nucleatum* (Fn, 2×10^7/ml), *P. gingivalis* (Pg, 2×10^8/ml), and *T. forsythia* (Tf, 2×10^8/ml) were cultured with 10% partially purified *F. nucleatum* AI-2 in the presence of D-galactose (D-Gal) or D-ribose (D-Rib) for 48 h under anaerobic conditions at 37°C. The biofilms formed on cover glasses were stained using a Live/Dead-BacLight bacterial viability kit and observed using a confocal laser scanning microscope at a magnification of 1,000 × (A) and quantified by measuring the mean intensity values of green (SYTO 9) and red (propidium iodide) fluorescence (B). $^*P < 0.05$ compared to the untreated control value; $^{\#}P < 0.05$ compared to the biofilm formation of the *F. nucleatum* AI-2-treated value in the absence of the quorum sensing inhibitors.

molecules of this bacterium by crystal violet staining and confocal microcopy. As shown in Fig. 2, D-galactose inhibited the biofilm formation of *F. nucleatum*, *P. gingivalis*, and *T. forsythia* in the presence of *F. nucleatum* AI-2 in a dose-dependent manner when measured by crystal violet staining. Its inhibitory effect was comparable to or better than that of D-ribose at the same concentrations. Although D-ribose did not inhibit AI-2 activity at 2 mM during 6 h of treatment (Fig. 1), a biofilm formed over 48 h was inhibited by D-ribose at this concentration (Fig. 2).

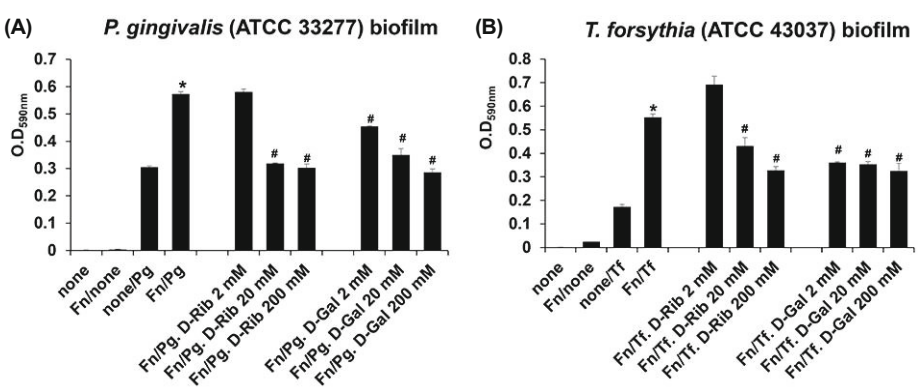

Fig. 4. Inhibitory effect of D-galactose on the biofilm formation of *P. gingivalis* and *T. forsythia* by secreted molecules of *F. nucleatum* using a transwell system. Using a transwell system, *F. nucleatum* (1×10^7/ml) was cultured in the upper well, while (A) *P. gingivalis* (1×10^7/ml) or (B) *T. forsythia* (1×10^7/ml) was cultured in the bottom well in the presence of D-ribose (D-Rib) or D-galactose (D-Ggal) for 72 h under anaerobic conditions at 37°C. Biofilm formation on glass slips was assessed by crystal violet staining. $^*P < 0.05$ compared to the untreated control value; $^{\#}P < 0.05$ compared to the biofilm formation in the absence of the quorum sensing inhibitors.

The biofilm formed was also visualized by confocal image analysis. As shown in Fig. 3, D-galactose markedly reduced the biofilm formation of *F. nucleatum*, *P. gingivalis*, and *T. forsythia* in the presence of *F. nucleatum* AI-2. Quantitative analyses of the fluorescences of the biofilms revealed that D-galactose significantly reduced the biofilm formation of *F. nucleatum*, *P. gingivalis*, and *T. forsythia*. D-Galactose reduced the fluorescence of the biofilms to a similar degree as that observed with D-ribose at 200 mM.

Next, we tested the inhibitory effects of D-galactose on the biofilm formation of periodontopathogens in a transwell culture system, where secreted molecules including AI-2 of *F. nucleatum* in the upper well could affect the biofilm formation of periodontopathogens in the bottom well without physical contact between the bacterial pairs. As shown in Fig. 4, secreted molecules from *F. nucleatum* in the upper well significantly increased the biofilm formation of *P. gingivalis* and *T. forsythia* in the bottom well, and D-galactose significantly inhibited their biofilm formation at all of the concentrations used, as measured by crystal violet staining.

Effect of D-galactose on the growth of *F. nucleatum*

As *F. nucleatum* plays the central role in the biofilm formation of periodontopathogens, we tested whether the inhibitory effect of D-galactose and D-ribose on biofilm formation could be attributed to the growth inhibition of *F. nucleatum*. As shown in Fig. 5, D-galactose did not affect the planktonic bacterial growth for 48 h incubation at the concentrations of 2 and 20 mM, while the enhanced growth was observed at 48 h of incubation with 200 mM of D-galactose. D-Ribose did not affect the planktonic bacterial growth for 48 h incubation at the concentrations of 2 and 20 mM, but reduced growth was observed at 24 h incubation with 200 mM D-ribose.

Discussion

This study demonstrated that D-galactose can be used as an AI-2 inhibitor to prevent the biofilm growth of periodontopathogens, possibly by binding to the Gbp. As QS enhances biofilm formation and virulence expression, it has emerged as a potential target against bacterial infection (Jiang and Li, 2013; Sepahi *et al.*, 2015; Zhu *et al.*, 2015). Periodontopathogens including *F. nucleatum*, *P. gingivalis*, *Prevotella intermedia*, and *A. actinomycetemcomitans* produce AI-2 (Frias *et al.*, 2001; Novak *et al.*, 2010). AI-2 of *F. nucleatum* has been demonstrated to induce biofilm formation and surface adhesins of the so-called 'red complex': *P. gingivalis*, *T. denticola*, and *T. forsythia* (Socransky *et al.*, 1998; Jang *et al.*, 2013). As AI-2 plays an important role in the biofilm formation of periodontopathogens (Novak *et al.*, 2010; Shao and Demuth, 2010), the antagonists of AI-2 receptors can be good candidates to inhibit biofilm formation.

AI-2 receptors or transporters have been reported in several species of bacteria: LsrB and LsrR in *E. coli* and *S. typhimurium*; AgrC in *S. aureus*; TlpB in *H. pylori*; and LuxP/Q in *Vibrio* species (Guo *et al.*, 2013). Although periodontopathogens are known to produce AI-2, AI-2 receptors have been reported only in *A. actinomycetemcomitans* (James *et al.*, 2006; Shao *et al.*, 2007). *A. actinomycetemcomitans* expresses two periplasmic proteins, RbsB and LsrB, which can interact with AI-2. *F. nucleatum* connects early colonizers and late pathogenic colonizers including *P. gingivalis* and *T. forsythia*, which are highly proteolytic and are present in subgingival biofilms adjacent to pocket epithelium (Socransky and Haffajee, 2005; Kolenbrander *et al.*, 2010). Thus, the identification of an AI-2 receptor of *F. nucleatum* and the blocking of that receptor would be a way to prevent the development of a biofilm composed of periodontal pathogens. *F. nucleatum* Gbp with the predicted molecular weight of approximately 36 kDa, which shows a high structural similarity to the RbsB of *A. actinomycetemcomitans* and *E. coli*, seems to be an AI-2 receptor, because D-galactose inhibited the AI-2 activity of *F. nucleatum* and *V. harveyi*.

A galactose-binding adhesin (30 kDa) in the outer membrane of *F. nucleatum* PK1594 has been reported to coaggregate with periodontopathogens, including *P. gingivalis* and *A. actinomycetemcomitans*, and to mediate hemagglutination (Shaniztki *et al.*, 1997; Weiss *et al.*, 2000; Rosen and Sela, 2006; Rosen *et al.*, 2008). It has also been shown to mediate the interaction of *F. nucleatum* with epithelial cells, fibroblasts, lymphocytes, and erythrocytes. These interactions were inhibited by D-galactose. However, it is not likely that the galactose-binding adhesin refers to the Gbp identified in our study. In addition to having a different molecular weight, the subcellular locations of these two molecules are also different, so that this galactose-binding adhesin is in the bacterial surface (Shaniztki *et al.*, 1997), while the Gbp is predicted to localize in the periplasm. According to the sequence homology, the Gbp is the galactose/glucose ABC transporter. *F. nucleatum* can utilize sugars and amino acids as carbon and energy sources, whereby amino acids are the preferred energy

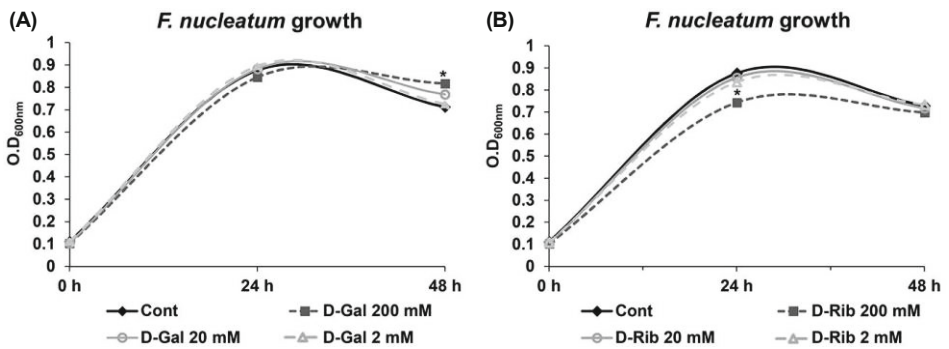

Fig. 5. Effect of D-galactose on planktonic bacterial growth. *F. nucleatum* was grown in brain-heart infusion medium for 48 h under anaerobic conditions at 37°C in the presence of D-galactose (D-gal) or D-ribose (D-rib). Bacterial growth was monitored by measuring the absorbance at 600 nm using a spectrophotometer. $^*P < 0.05$ compared to the untreated control value

source (Kapatral *et al*., 2002). *F. nucleatum* takes up galactose by a galactose/glucose ABC transporter, which is a member of the pentose/hexose sugar-binding protein family of the type I periplasmic binding protein superfamily in various bacterial species. The ABC transporter is involved in chemotaxis towards galactose and glucose, and in active transport of the sugars. *F. nucleatum* possesses genes encoding the enzymes involved in galactose utilization (Kapatral *et al*., 2002). Galactose is converted to glucose-6-phosphate which can enter into a pathway of glycolysis. In our study, D-galactose did not affect the bacterial growth at the concentrations used for the experiments, suggesting its role as an AI-2 inhibitor rather than energy source. *F. nucleatum* is known to use sugars including glucose, galactose, and fructose for synthesis of intracellular polymers which can be utilized to survive under conditions of amino acid deprivation (Bolstad *et al*., 1996).

The inhibition of biofilm formation by D-galactose could be solely attributed to coaggregation inhibition by the competitive binding of galactose to the galactose-binding adhesin rather than the inhibition of AI-2 activity. However, D-galactose inhibited the AI-2 activity of *F. nucleatum* and *V. harveyi* as indicated by bioluminescence assay, indicating its function as an AI-2 inhibitor. In addition, semi-purified AI-2 or secreted molecules including AI-2 of *F. nucleatum* increased the biofilm formation of periodontopathogens, which was significantly reduced by D-galactose. These results indicate that D-galactose inhibits the biofilm formation of periodontopathogens by competitive binding to the Gbp with AI-2. In addition to the direct effect of D-galactose on the Gbp, it is possible that D-galactose may inhibit other adhesins of periodontopathogens induced by AI-2, as do other AI-2 inhibitors including D-ribose and furanone compound, resulting in the further inhibition of biofilm formation (Jang *et al*., 2013). However, this hypothesis remains to be investigated. In the future, construction of a *F. nucleatum* mutant strain defective in the Gbp may allow us to verify the role of the Gbp in the QS. In conclusion, the Gbp of *F. nucleatum* may be an AI-2 receptor, and D-galactose is a good candidate to inhibit biofilm formation of periodontopathogens effectively, thus contributing to the control of periodontitis.

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