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Autoinducer 2 of *Fusobacterium nucleatum* as a target molecule to inhibit biofilm formation of periodontopathogens

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ABSTRACT

Periodontitis is initiated by bacteria in subgingival biofilms, which are composed mostly of Gram-negative anaerobes. Autoinducer 2 (AI-2) is a universal quorum sensing (QS) molecule that mediates intergeneric signalling in multispecies bacterial communities and may induce biofilm formation. As *Fusobacterium nucleatum* is the major coaggregation bridge organism that links early colonising commensals and late pathogenic colonisers in dental biofilms via the accretion of periodontopathogens, we hypothesised that AI-2 of *F. nucleatum* contributes to this interspecies interaction, and interruption of this signalling could result in the inhibition of biofilm formation of periodontopathogens. To test this hypothesis, we evaluated the effect of partially purified *F. nucleatum* AI-2 on monospecies biofilm as well as mutualistic interactions between *F. nucleatum* and the so-called 'red complex' (*Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia*). Then we tested the effect of two QS inhibitors (QSIs), (5Z)-4-bromo-5-(bromomethylene)-2(5H)-furanone (furanone compound) and D-ribose, on AI-2-induced biofilm formation and coaggregation. *F. nucleatum* AI-2 remarkably induced biofilm growth of single and dual species and coaggregation between *F. nucleatum* and each species of the 'red complex', all of which were inhibited by the QSIs. *F. nucleatum* AI-2 induced the expression of the representative adhesion molecules of the periodontopathogens, which were inhibited by the QSIs. Our results demonstrate that *F. nucleatum* AI-2 plays an important role in inter- and intraspecies interactions between periodontopathogens via enhanced expression of adhesion molecules and may be a target for the inhibition of pathogenic dental biofilm formation.

1. Introduction

Periodontitis is a chronic inflammation caused by subgingival biofilms, which are composed of mostly Gram-negative and highly proteolytic anaerobes. *Porphyromonas gingivalis*, *Tannerella forsythia*, and *Treponema denticola* are potent periodontopathogens that degrade periodontal tissue and are directly associated with gingival epithelium. These bacteria, referred to collectively as the 'red complex', are late colonisers in dental biofilm formation,[1] and *Fusobacterium nucleatum* is the major coaggregation bridge organism linking early and late colonisers.[2]

Quorum sensing (QS) is mediated by a cell-to-cell signalling system comprising small signalling molecules secreted by various microbial species.[3,4] When the number of QS signalling molecules reaches a threshold, they are either transported back into the cells through free diffusion or transporters or

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they are sensed by a two-component phosphorylation system that triggers gene expression, affecting various phenotypes including bioluminescence, virulence, sporulation, adhesion, and biofilm formation. These phenotypes are beneficial for bacterial survival in challenging environments. As QS inhibitors (QSIs) hinder biofilm formation and reduce bacterial virulence in the biofilm state, they are ideal for applications that inhibit oral biofilm formation, providing a good target for the control of bacterial infection.[5,6] Because QS and biofilm formations are directly related, the formation of bacterial biofilms has been shown to be weakened by QSIs.[5,7–10] QSIs can act as structural analogues in the production of QS signalling molecules, QS receptors, or signalling molecules themselves. An ideal QSI should significantly reduce the expression of QS-controlled genes without having a toxic effect on the bacteria or host. Bromated furanones from the marine red alga *Delisea pulchra* were the first natural QSIs described, and they have served as a prototype for the design of synthetic inhibitors.[11]

Autoinducer-2 (AI-2) is a universal quorum-sensing molecule that mediates intergeneric signalling in multispecies bacterial communities.[12,13] It is formed from spontaneous rearrangement of 4,5-dihydroxy-2,3-pentanedione, which is the product of the catabolism of S-adenosylhomocysteine by the enzyme LuxS,[6,14] and is the primary QS molecule produced by oral bacteria.[15] According to the hypothesis suggested by Kolenbrander,[16] commensal bacteria produce and receive AI-2 signals at picomolar concentrations, while pathogens produce and receive AI-2 at much higher concentrations. High levels of AI-2 accelerate the growth of pathogens and reduce the growth of commensal bacteria, contributing to subgingival plaque formation and maturation, leading to periodontitis.

As *F. nucleatum* plays an important role in subgingival biofilm formation and maturation, we hypothesised that AI-2 produced by this bacterium induces biofilm formation and coaggregation of periodontopathogens and that QSIs reduce AI-2-induced biofilm formation/coaggregation. To test this hypothesis, we evaluated the effect of partially purified *F. nucleatum* AI-2 on intraspecies interactions, including biofilm formation and coaggregation, between *F. nucleatum* and the 'red complex', and the effect of two QSIs, (5Z)-4-bromo-5-(bromomethylene)-2(5H)-furanone (furanone compound) and D-ribose, on AI-2-induced biofilm formation/coaggregation. We also evaluated the effect of *F. nucleatum* AI-2 on the expression of adhesion molecules of periodontopathogens.

2. Materials and methods

2.1. Bacteria culture

F. nucleatum (ATCC 25586) was cultured in peptic medium (6 g of peptic digest of animal tissue, 6 g of desiccated beef extract, 5 g of sodium chloride, 14.5 g of pancreatic digest of casein, and 2.5 g of sodium phosphate), and *P. gingivalis* (ATCC 33277) was cultured in brain heart infusion medium supplemented with hemin (10 μg/ml) and vitamin K (0.2 μg/ml). *T. forsythia* (ATCC 43037) was cultured in new oral spirochete (NOS) broth (ATCC medium 1494) supplemented with vitamin K (0.2 μg/ml) and N-acetylmuramic acid (0.01 μg/ml). *T. denticola* (ATCC 33521) was cultured in NOS broth. The bacteria were incubated under anaerobic conditions (5% H_2, 10% CO_2 and 85% N_2) at 37 °C for 2–4 days. *Pseudomonas aeruginosa* was cultured in Luria Bertani (LB) broth.

The AI-2 reporter strain *Vibrio harveyi* BB170 (sensor 1^-, sensor 2^+) and the AI-2 producing strain *V. harveyi* BB152 (AI-1^-, AI-2^+) were cultured in autoinducer bioassay (AB) medium overnight at 30 °C until $OD_{660\ nm} = 0.7$ with aeration. AB medium was prepared as described previously.[17] *V. harveyi* BB170 and *V. harveyi* BB152 were a gift from Dr. S.H. Choi (Seoul National University, Korea).

2.2. Partial purification of F. nucleatum AI-2

AI-2 of *F. nucleatum* and *V. harveyi* BB152 was partially purified as described previously.[18,19] Briefly, cells from an overnight culture of *F. nucleatum* were diluted 1:20 with fresh medium and cultured at 37 °C until the late exponential phase ($OD_{660\ nm} = 0.7$). The culture supernatants were collected by centrifugation at 10,000 × *g* and then passed through 0.2 μm-pore-size membrane filters and subsequently through a Centricon YM-3 3-kDa exclusion filter (Millipore, Bedford, MA). The filtrate was then chromatographed on a C18 Sep-Pak reverse-phase column (Waters Co., Milford, MA) according to the manufacturer's instructions. The same procedure was applied for preparation of a fraction of conditioned medium from *P. aeruginosa*, which is known to produce acyl homoserine lactones (AHLs), to be used as a negative control.[20]

2.3. QS inhibitors

D-Ribose and (5Z)-4-bromo-5-(bromomethylene)-2(5H)-furanone (furanone compound) were used as AI-2 QSIs. D-Ribose was purchased from Tokyo Chemical Industry Co. (Tokyo, Japan). The furanone compound was synthesised according to the method of Manny et al.[21] The compound was verified by ^1H and ^{13}C NMR spectroscopy.

2.4. Determination of AI-2-mediated bioluminescence

Bioluminescence mediated by AI-2 was determined using the AI-2 reporter strain *V. harveyi* BB170. Quorum-sensing molecules can stimulate the luciferase operon (*lux* genes) to express luciferase. *V. harveyi* BB170 was cultured in AB medium overnight at 30 °C until $OD_{660\ nm} = 0.7$. The bacteria were washed with fresh AB medium and diluted to a concentration of 1×10^6 cells/ml. The bacterial suspension (5 ml) was mixed with the spent culture medium of *F. nucleatum* or a partially purified *F. nucleatum* AI-2 fraction at a final concentration of 1% (vol/vol) and incubated for 1–8 h at 30 °C. One percent of *F. nucleatum* AI-2 is corresponding to the bacterial number of approximately 1.9×10^7. As a positive control, the spent culture medium and partially purified AI-2 of *V. harveyi* BB152 were used at a final concentration of 1%. *P. aeruginosa* fraction and bacterial media used for culture of *F. nucleatum*, *V. harveyi*, and *P. aeruginosa* were included as negative controls. The bioluminescence was measured with a luminometer (GloMax®-Multi detection System, Promega, Madison, WI).

QSIs of various concentrations were evaluated for their ability to inhibit AI-2 activity using the AI-2 reporter strain