Studies on the relationship between quorum sensing and biofilm formation of *Eikenella corrodens*

(*Eikenella corrodens* のクオラムセンシングとバイオフィルム形成の関連性に関する研究）

by

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LIST OF ABBREVIATIONS

AHL	Acyl Homoserine Lactone
AI	Autoinducer
AI-2	Autoinducer-2
CFU	Colony Forming Unit
CLSM	Confocal Laser Scanning Microscopy
CRM	Confocal Reflection Microscopy
DMSO	Dimethyl Sulfoxide
DPD	4,5-dihydroxy-2,3- pentanedione
GC-MS	Gas Chromatography-Mass Spectrometry
h	Hour
HA	Hydroxyapatite
MHF	4-hydroxy-5-methyl-3(2*H*)-furanone
Mg	Milligram
µg	Microgram
mL	Milliliter
µL	Microliter
MRD	Modified Robbins Device
QS	Quorum sensing
RP-HPLC	Reverse-Phase High Performance Liquid Chromatography
SEM	Scanning Electron Microscopy
TLC	Thin Layer Chromatography;
TSB	Tryptic Soy Broth
%	Percentage
°C	Degree Celsius

GENERAL INTRODUCTION

Bacteria were for a long time believed to exist as individual cells that sought primarily to find nutrients and multiply. The discovery of intercellular communication among bacteria has led to the realization that bacteria are capable of coordinated activity that was once believed to be restricted to multicellular organisms. The capacity to behave collectively as a group has obvious advantages, for example, the ability to migrate to a more suitable environment/better nutrient supply and to adopt new modes of growth, such as sporulation or biofilm formation, which may afford protection from deleterious environments. The "language" used for this intercellular communication is based on small, self-generated signal molecules called autoinducers (AIs). Through the use of AIs, bacteria can regulate their behavior according to population density (1).

Quorum sensing (QS) refers to the ability of a bacterium to sense information from other cells in the population when they reach a critical concentration (i.e. a *Quorum*). The phenomenon of QS, or cell-to-cell communication, relies on the principle that when a single bacterium releases AIs into the environment, their concentration is too low to be detected. However, when sufficient bacteria are present, AI concentrations reach a threshold level that allows the bacteria to sense a critical cell mass and, in response, to activate or repress target genes (2). It is now known that a significant portion of bacterial genome (4-10%) and proteome (≥20%) can be influenced by QS. This only implies that QS is a mechanism used by pathogenic bacteria not only to modulate virulence factor production but also to adapt to the metabolic demands of living in community (3-7). QS is a generic regulatory mechanism used by many Gram-negative bacteria and Gram-positive bacteria to perceive and respond to factors as varied as changing microbial population density and the expression of specific genes. The

concentration of a signal molecule reflects the density of bacterial cells in a defined environment, and the perception of a threshold level of that signal indicates that the population is "quorate" that is sufficiently dense to make a behavioral group-based decision. QS is thought to afford pathogenic bacteria a mechanism to minimize host immune responses by delaying the production of tissue-damaging virulence factors until sufficient bacteria have amassed and are prepared to overwhelm host defense mechanisms and establish infection (8). QS was originally described in the marine luminescent bacterium *Vibrio fischeri*, where it functions as the control mechanism of light production and numerous other traits (9-12). For years, it was thought that this phenomenon was limited to a few marine organisms but it is now widely recognized that many bacterial species utilize quorum sensing as part of their regulatory machinery (13-16). Of interest, we now know that bacterial virulence is in many cases controlled by QS (13).

At present, there are two major independent types of recognized QS systems in bacteria. Type I QS is a highly specific system and is used for intraspecies communication. In Gram-negative bacteria, the AIs of signal system I (AI-1) have been identified as derivates of an acyl homoserine lactone (acyl-HSL) backbone with species specific substitutions. These molecules diffuse freely in and out of cells. Two genes are crucially involved in QS through acyl-HSL: the synthesis of acyl-HSL is dependent on *luxI*, while *luxR* encodes a transcriptional activator protein that is responsible for the detection of the cognate acyl-HSL and induction of the appropriate output (17-18). Type II QS, most thoroughly characterized for *Vibrio harveyi*, responds to an AI signal (AI-2) that is produced by LuxS enzyme, *S*-ribosyl homocysteine lyase (EC 4.4.1.21), the *luxS* product. LuxS converts *S*-ribosylhomocysteine to 4,5-dihydroxyl-2,3-pentanedione

(DPD), catalyzing AI-2 formation. The AI-2 molecule produced by *V. harveyi* is a furanosyl borate diester (19). In *V. harveyi*, AI-2 binds to a specific receptor/ sensor complex (LuxP/Q), and subsequent phosphorelay via either LuxU or a LuxU homologue leads to modification of the transcriptional activator LuxO (20). Many Gram-positive and Gram-negative bacteria contain highly conserved *luxS* homologues and produce AI molecules that are functionally similar to *V. harveyi* AI-2 (21). The LuxS-dependent QS system has been referred to as an interspecies communication system and may operate as a universal QS system for many bacteria possessing the characteristic *luxS* gene. Several reports have shown the involvement of this type II QS in the regulation of expression of virulence-related factors, motility, secretion systems, regulatory proteins, and polypeptides involved in the acquisition of hemin (21). It was shown that bacterial communication mediated by LuxS is also involved in biofilm formation by *Streptococcus gordonii* (22), *Streptococcus mutans* (23), and *Salmonella enterica* serovar Typhimurium (24).

Biofilms are now considered ubiquitous in the natural world (25). In nature, bacteria are frequently found encased in polysaccharide matrix attached to a solid surface. This mode of growth, referred to as a biofilm, offers protection from environmental agents that would otherwise threaten their planktonic counterparts. Bacterial biofilms have been observed to be extremely heterogeneous, both structurally and with regard to the physiology of the bacterial cells within them. The prevailing conceptual model depicts bacterial biofilms as being made up of microcolonies, which serve as the basic unit of the greater biofilm structure. Microcolonies are hydrated structures consisting of bacterial cells enmeshed in a matrix of exopolymeric substances (EPSs). Bacteria may proliferate on the attachment surface, leading to microcolony expansion. Eventually,

community growth becomes limited by substrate availability due to increased diffusion distances, and the biofilm reaches a steady state. Such mature biofilms often consist of "towers" and "mushrooms" of cells in an EPS matrix. Interstitial voids and channels separate the biofilm structures and facilitate a convective flow in order to transport nutrients to interior parts of the biofilm and remove waste products. Biofilms have become evident in many, if not most, environmental, industrial, and medical bacteria related problems. A recent public announcement from the NIH stated that more than 60% of all microbial infections involve biofilms (26).

Periodontitis and caries are infectious diseases of the oral cavity in which oral biofilms play a causative role. Moreover, oral biofilms are widely studied as model systems for bacterial adhesion, biofilm development, and biofilm resistance to antibiotics, due to their widespread presence and accessibility. Despite descriptions of initial plaque formation on the tooth surface, studies on mature plaque and plaque structure below the gum are limited to landmark studies from the 1970s, without appreciating the breadth of microbial diversity in the plaque (27).

Oral microbial biofilms are three-dimensional structured bacterial communities (28) attached to a solid surface like the enamel of the teeth, the surface of the root or dental implants (29) and are embedded in an exo-polysaccharide matrix (30). Oral biofilms are exemplary and served as a model system for bacterial adhesion (31, 32) and antibiotic resistance (33). Human oral bacteria interact with their environment by attaching to surfaces and establishing mixed-species communities. As each bacterial cell attaches, it forms a new surface to which other cells can adhere. Adherence and community development are spatiotemporal; such order requires communication. The discovery of soluble signals, such as AI-2, that may be exchanged within multispecies communities

to convey information between organisms has emerged as a new research direction (34).

Eikenella corrodens is a periodontopathogen that is a common inhabitant of the oral cavity and the intestinal and genital tracts. It was first isolated by Henriksen in 1948 and was first classified as *Bacteriode corrodens* by Eiken in 1958. In 1972, Jackson and Goodman renamed it *E. corrodens* to avoid mixing it up with *Bacteroides ureolyticus*. Its higher order of taxa is Bacteria; Proteobacteria; Betaproteobacteria; Neisseriales; Neisseriaceae; Eikenella. *E. corrodens* is a Gram negative, facultative and anaerobic, non-motile, non-sporeforming pathogenic bacillus that exists in the form of a straight rod. *E. corrodens* has DNA chromosomes and plasmids but no RNA (35). Its primary ecologic niche within the oral cavity appears to be dental plaque, both in periodontally healthy individuals and in periodontitis patients. However, *E. corrodens* is recognized as an infrequent human pathogen capable of causing extra oral infections, either as the sole infectious agent or as part of a mixed infection and this infection may lead to serious diseases such as osteomyelitis, meningitis, empyema, and endocarditis (36). Periodontobacteria communicate via QS, a communication process that uses secreted chemical signaling molecules called AIs. Bacteria can come together to form colonies via this process. This colonizing ability allows them to control their population size, thus allowing them to adjust the expression of various physiological functions based on the changes in the population density.

In the previous study, *E. corrodens* was found to secrete type 2 signaling molecules, AI-2, which requires the *luxS* gene for synthesis. Though AI-2 was produced by *E. corrodens* during late exponential phase, AI-2 activity was decreased remarkably when it reaches to stationary phase. Azakami *et al*. also reported *luxS* mutant's capacity to colonize and form biofilm on polysterene surface is 1.3-fold greater than the wild type

(37). Thus, they hypothesized that *E. corrodens*'s LuxS-dependent signal plays a key role in the biofilm formation of the oral cavity through QS.

In this study, I performed studies on the relationship between quorum sensing and biofilm formation of *E. corrodens*. Namely, in chapter 1, to know the factor responsible for decreasing AI-2 activity in stationary phase, I purified AI-2 from *E. corrodens*, purified AI-2 inactivating enzyme from *E. corrodens* partially and charecterized it. Moreover, in chapter 2, to know the LuxS dependent signal's role in pathogenicity like biofilm formation, I investigated the effect of purifed AI-2 on biofilm formation of *E. corrodens* and flow cell biofilm study with various microscopies.

Chapter 1: The Periodontopathogenic Bacterium Eikenella corrodens Produces an Autoinducer-2-Inactivating Enzyme

The bacterial signal molecule AI-2 is a product of the LuxS enzyme which is broadly conserved throughout the bacterial world. LuxS enzymes synthesize DPD which undergoes spontaneous rearrangements (34). Importantly, DPD derivatives interconvert and exist in equilibrium. Different bacteria recognize distinct DPD derivatives, and this family of molecules is generically called AI-2. The interconverting nature of these molecules presumably allows bacteria to respond to their own AI-2 and also to AI-2 produced by other bacterial species, giving rise to the idea that AI-2 represents a universal language fostering inter-species bacterial communication (38). Previously, Azakami *et al*. reported that the *E. corrodens* genome has an ortholog of the *luxS* gene, and that *E. corrodens* secretes AI-2 into the culture supernatant. In the supernatant, AI-2 activity was detected using *Vibrio harveyi* BB170 as sensor strain, and no AI-1 activity was detected using either *Chromobacterium violaceum* CV026 or *Agrobacterium*

tumefaciens NTL4 (pZLR4) as sensor strain. Although maximum expression of AI-2 was observed during the mid-exponential growth phase, AI-2 activity rapidly decreased when the bacterium entered the stationary phase (37).

The ability to inactivate AI-1, acylhomoserine lactones (AHLs) enzymatically by AHL lactonase or AHL acylase is shared by diverse bacteria belonging to the α-*Proteobacteria* including *Agrobacterium*, *Sphingomonas*, *Sphingopyxis* and *Bosea*, the β-*Proteobacteria* such as *Variovorax*, *Ralstonia* and *Comamonas*, the γ-*Proteobacteria* including *Pseudomonas* and *Acinetobacter*, Firmicutes such as *Bacillus* and Actinobacteria such as *Rhodococcus* as well as the *Streptomyces* sp (39). In most bacteria, extracellular AI-2 activity reaches a peak in the mid- to late-exponential phases, and declines precipitously during the stationary phase (40). Though AI-2 inactivating enzyme has not been reported to date, a variety of bacterial species have the ability to sequester and process the AI-2 present in the environment, thereby interfering with the cell-to-cell communication of other bacteria. The rapid disappearance of AI-2 in many cases is an effect of the Lsr transport system, which facilitates the uptake of signal molecules into cells, and a kinase that phosphorylates the signal molecule to phospho-DPD (41).

In chapter 1, I purified the QS signal molecule AI-2 and tried to know the AI-2 inactivating factor in the stationary phase of *E. corrodens*. I suggest that *E. corrodens* has a novel AI-2 inactivation system and AI-2 might be degraded or converted to another structure by AI-2 inactivating enzyme.

Chapter 2: LuxS affects biofilm maturation and detachment of the periodonto-pathogenic bacterium Eikenella corrodens

Biofilms are sessile, surface-attached communities of microorganisms (42, 43). Most surfaces on this planet teem with microbial biofilms that account for over 99% of microbial life (43-45). Biofilms are spatially structured communities of microbes whose function depends on a complex web of symbiotic interactions (43, 44). High cell density and close proximity of diverse species of microorganisms are typical of life in natural biofilms, where organisms are involved in complex social interactions that occur both within and between species and can be either competitive or cooperative (34, 44, 46-50). Dental plaque is a well-recognized biofilm community characterized by its vast biodiversity (>700 species) and high cell density (10^{11} cells/g wet wt) (34, 47, 51, 52). The high cell density and species diversity within dental biofilms coupled with environmental fluctuations should create an environment that is conducive to inevitable intra and inter-species interactions (47). In modern clinical microbiology, the establishment of bacterial biofilms has been considered an important pathogenic trait in many chronic infections (42, 44, 47-49, 53). It is now known that many bacteria regulate their social activities and physiological processes through a QS mechanism, including symbiosis, formation of spore or fruiting bodies, bacteriocin production, genetic competence, programmed cell death, virulence and biofilm formation (54). In 1998, Greenberg and his colleagues first described the role of the *las* QS in biofilm formation of *Pseudomonas aeruginosa* (55). AI-2 mediated-QS mechanism is widely distributed bacterial species (56-63). AI-2 has also proved important in the development of structured biofilms, especially multi-species biofilms in natural ecosystems (23, 34, 45, 60). The LuxS-dependent biofilm formation and its molecular mechanism have been

demonstrated in clinical isolate of *S. pneumoniae* D39 (61). Many species of bacteria in natural biofilms like dental plaque have been found to have a *luxS* homolog in their genomes (23, 60, 62). Previously, Azakami *et al.* reported that the *E. corrodens* genome has an ortholog of the *luxS* gene, and that *E. corrodens* secretes AI-2 into the culture supernatant. They also reported LuxS mutant's capacity to colonized and form biofilm on polysterene surface is 1.3-fold greater than the wild type *E. corrodens* (37). Thus, they hypothesized that *E. corrodens'*s LuxS-dependent signal plays a key role in the biofilm formation of the oral cavity. To investigate this, in chapter 2, I studied the effect of purified AI-2 on biofilm formation by *E. corrodens* and flow cell biofilm study of wild type and *luxS* mutant *E. corrodens* with various microscopies. I suggested that LuxS dependent signal plays role in biofilm maturation and detachment in *E. corrodens*.

OBJECTIVES

E. corrodens is a facultative anaerobic Gram-negative rod predominantly found in subgingival plaque samples of patients with advanced periodontitis. Periodontobacteria communicate via QS, a communication process that uses secreted chemical signaling molecules called AIs. Previously, Azakami *et al*. reported that the *E. corrodens* genome has an ortholog of the *luxS* gene, and that *E. corrodens* secretes QS signal molecule AI-2 into the culture supernatant. Although maximum expression of AI-2 was observed during the mid-exponential growth phase, AI-2 activity rapidly decreased when the bacterium entered the stationary phase. In modern clinical microbiology, the establishment of bacterial biofilms has been considered an important pathogenic trait in many chronic infections. Azakami *et al*. also reported that LuxS mutant's capacity to colonize and form biofilm on polysterene surface was 1.3-fold greater than the wild type *E. corrodens.* So, they suggested *E. corrodens'*s LuxS-dependent signal plays a key role in the biofilm formation of the oral cavity. However why AI-2 activity is decreased in stationary phase of *E. corrodens* and what`s the role of AI-2 in biofilm formation was remain to be determined. In this study, I studied the relationship between QS and biofilm formation of *E. corrodens.* Therefore, the objectives of this study are summarized as follows:

1. To investigate why AI-2 activity is decreased in stationary phase in *E. corrodens* and is there any enzyme which inactivates AI-2, I purified AI-2 from *E. corrodens* and searched for AI-2 inactivating enzyme.
2. To elucidate the LuxS dependent signal's role in biofilm formation by *E. corrodens*, I investigated the effect of purified AI-2 on biofilm formation and compared the biofilm between wild type and *luxS* mutant of *E. corrodens* using flow cell system.

CHAPTER 1

The Periodontopathogenic Bacterium *Eikenella corrodens* Produces an Autoinducer-2-Inactivating Enzyme

1.1 ABSTRACT

E, corrodens produces AI-2 in the mid log phase, and AI-2 activity decreases dramatically during the stationary phase. Mechanism underlying this decrease in AI-2 activity was investigated. To analyze the mechanism, AI-2 was extracted and purified from the supernatant of mid-log-phase culture. Simultaneously, the stationary-phase culture supernatant was fractionated by ammonium sulfate precipitation. On incubating purified AI-2 and 4-hydroxy-5-methyl-3(2*H*)-furanone (MHF) with each fraction, the 30% fraction decreased both AI-2 and MHF activities. The data suggest that AI-2 and MHF were rendered inactive in the same manner. Heat and/or trypsin treatment of the 30% fraction did not completely arrest AI-2-inactivation, suggesting that partially heat-stable proteins are involved in AI-2 inactivation. An enzyme converted MHF to another form was also observed. This suggests that *E. corrodens* produce an AI-2 inactivating enzyme, and that AI-2 can be degraded or modified by it.

1.2 INTRODUCTION

Bacteria are social entities and are known to use communication to coordinate behavior within a population (63). They produce small chemical signal molecules called AIs that accumulate in the extracellular environment when the bacteria multiply. Detection of these signal molecules by bacterial cells enables them to monitor their population density and adjust their gene regulation accordingly. This process of cell-to-cell communication is known as QS. In this process, a response to signal molecules is initiated only when the signaling molecules reach a threshold concentration (64). The cellular functions regulated by QS include expression of virulence factors, competence for genetic transformation, production of antibiotics and secondary metabolites, and biofilm formation (56, 65, 66).

Many Gram-negative bacteria produce AHL, also known as AI-1. More than a dozen AHL derivatives, which vary in length or substitution at the acyl side chain, have been identified in a range of Gram-negative bacteria. The only QS pathway shared by Gram-positive and Gram-negative bacteria is that involving the LuxS enzyme, *S*-ribosyl homocysteine lyase (EC 4.4.1.21), in the synthesis of a signaling molecule known as AI-2 (64). Conversion of *S*-ribosyl homocysteine to homocysteine by LuxS also yields AI-2 precursor, DPD. The reaction catalyzed by LuxS is an integral part of the activated methyl cycle, an important metabolic pathway that figures in recycling homoserine from *S*-adenosyl methionine to maintain methionine *de novo* biosynthesis. The second product of this reaction, DPD, undergoes spontaneous cyclization to form a mixture of various furanones, including AI-2, which accumulates in the culture supernatant (67, 68).

Dental plaque is a complex biofilm formed of more than 700 bacterial species that

normally exist in commensal harmony with their host (69). *E. corrodens* is a facultative anaerobic Gram-negative rod predominantly found in sub gingival plaque samples of patients with advanced periodontitis (70). *E. corrodens* monoinfection of germ-free or gnotobiotic rats causes severe periodontal disease (71). *E. corrodens* is therefore believed to be a periodontopathogenic bacterium. It is assumed to participate in the early stages of biofilm formation in periodontal pockets (72).

Previously, Azakami *et al*. reported that the *E. corrodens* genome has an ortholog of the *luxS* gene, and that *E. corrodens* secretes AI-2 into the culture supernatant (37). In addition, since the *ΔluxS* mutant of *E. corrodens* shows enhanced biofilm formation, they hypothesized that AI-2 plays a role in biofilm formation by *E. corrodens* (37). In the supernatant, AI-2 activity was detected using *Vibrio harveyi* BB170 as sensor strain, and no AI-1 activity was detected using either *Chromobacterium violaceum* CV026 or *Agrobacterium tumefaciens* NTL4 (pZLR4) as sensor strain. Although maximum expression of AI-2 was observed during the mid-exponential growth phase, AI-2 activity rapidly decreased when the bacterium entered the stationary phase (37). Based on these results, I surmised that AI-2 was degraded or was converted into another structure that could not be detected using *V. harveyi* BB170.

The existence of AHL-degradation enzymes, such as AHL lactonase and AHL acylase, have been reported (73), but to date no AI-2-degradation enzyme has been reported. However, the phosphorylation of AI-2 has been reported to be involved in its degradation in a few bacteria (41). In this study, I investigated the mechanisms of the decrease in AI-2 activity in *E. corrodens* during the stationary phase. The results indicate that *E. corrodens* produces an AI-2 inactivation enzyme that can be obtained from its culture supernatant, and that AI-2 can be degraded or modified by this enzyme.

1.3 MATERIALS AND METHODS

Bacterial strains and growth conditions. *E. corrodens* 1073 was provided by Dr. S. S. Socransky (Forsyth Dental Center, Boston, MA). *E. corrodens* cells were cultured statically at 37°C in tryptic soy broth (TSB) containing 2 mg/mL of KNO_3 and 5 μg/mL of hemin. *V. harveyi* BB170 (provided by Dr. Bonnie L. Bassler, Princeton University) was grown aerobically at 30°C in AB medium containing NaCl, 17.5 g; casamino acids, 2 g; $MgSO_4 \cdot 7H_2O$, 24.6 g; pH 7.5; 1 M potassium phosphate pH 7.0, 10 mL; 0.1 M L-arginine, 10 mL; and 50% glycerol, 20 mL per liter of water.

AI-2 extraction from the culture supernatant. For AI-2 extraction, the log-phase culture of *E. corrodens* 1073 was inoculated in to fresh TSB medium and statically cultured for 15 h at 37°C. Then the bacterial cells were removed by centrifugation (12,000 x *g*, 5 min, 4°C), and the supernatant was filtered by decompression filtration using filter papers of 0.22 μm pore size (Membrane Filters, Advantec, Tokyo, Japan). AI-2 was extracted 3 times by mixing of the filtrated supernatant with ethyl acetate at a ratio of 4 : 3 (supernatant : ethyl acetate). The ethyl acetate phase obtained was dried in a rotary evaporator at 40°C. The resulting sample was dissolved with a small amount of ethyl acetate and then stored at -30°C.

Purification of AI-2 by thin layer chromatography (TLC). A solution of AI-2 in ethyl acetate was applied to a silica gel TLC plate (TLC Silica Gel 60 RP-18F, Merk, Darmstadt, Germany) and developed with 60% methanol. The individual bands that appeared were observed under UV at 254 nm, and a section corresponding to each band was cut out. The compounds obtained from each silica gel section for a specific band

were eluted with ethyl acetate, filtered, and dried under a continuous flow of N_2 gas. The resulting samples were dissolved in dimethyl sulfoxide (DMSO), and AI-2 activity was confirmed by AI-2 assay. 4-Hydroxy-5-methyl-3(2*H*)-furanone (MHF) (Sigma-Aldrich, Steinheim, Germany) was used for comparison with AI-2. The Rf value of each compound was calculated by the formula following: Rf = moving distance of spot / moving distance of solvent. Silica gel from a control (blank) position was also cut out, eluted with ethyl acetate, filtered, and dried under a continuous flow of N_2 gas. This sample was used as the negative control.

AI-2 assay. The AI-2 assay was conducted by a previously described luminescence assay procedure (74), with *V. harveyi* BB170 as reporter strain. In brief, *V. harveyi* BB170 was grown aerobically for 16 h at 30°C in AB medium and diluted to 1 : 5,000 in fresh AB medium. I added 200 μL of the sample solution (the sample dissolved in 20 μL DMSO and then inserted into 180 μL fresh AB medium) to 1.8 mL of diluted *V. harveyi* BB170 cell culture suspension, and this was incubated for 5 to 6 h at 30°C with shaking. MHF was used as positive control. The resulting light production from each sample was measured with a Tecan GENios Microplate Reader (Tecan, Crailsheim, Germany). To determine the levels of background luminescence, an empty micro-titer plate (Falcon 96-well Microplate, Becton Dickinson, Le Pont De Claix, France) was used as the blank. Induced luminescence was obtained as value relative to the negative control by the following calculation:

Induced luminescence = (luminescence data of each sample - blank) / (luminescence data of negative control - blank).

Gas chromatography-mass spectrometry (GC-MS) analysis. The sample was analyzed using a Shimadzu QP 5050 GC-MS. Column DB-WAX (ϕ0.25 nm × 60 m) was used, and the injector temperature was 240°C and the interference temperature 215°C. The injector temperature was raised from 200°C to 230°C at a rate of 2°C / min.

Fractionation of the AI-2-inactivating compound by ammonium sulfate precipitation. *E. corrodens* culture grown for 18 h showing decreased AI-2 activity was centrifuged (3,000 x *g*, 4°C, 15 min, 2 times) to separate out bacterial cells. Ammonium sulfate was added to the supernatant to 30%, 50%, and 70% salt concentrations, and the mixtures were stirred on ice for 1 h. The solutions were centrifuged at 12,000 x *g* at 4°C for 15 min, the supernatants were inserted into fresh tubes and the precipitate was dissolved in a minimum volume of 10 mM phosphate buffer (pH 7.0). After dialysis to remove salt, the samples were freeze-dried, dissolved in phosphate buffer (pH 7.0), and stored at -30°C.

Assay to monitor AI-2 inactivation. The purified AI-2 and MHF were dissolved in DMSO in a concentration of 1 mg/mL. 20 μL of AI-2 or MHF solution and 80 μL of each sample (1 mg/200 μL) of the 30%, 50%, 70% ammonium sulfate fractions were mixed, and then incubated at 37°C for 5 h. After incubation, the remaining AI-2 activity was measured.

Heat and trypsin treatment of the 30% fraction. The 30% ammonium sulfate fraction was heat and/or trypsin treated. During heat treatment, the sample was boiled at 100°C for 10 min and then immediately transferred to an ice bath. During trypsin

treatment, it was incubated with trypsin at 37°C for 4 h. The enzyme-to-substrate ratios were 1 : 50 and 1 : 20. Trypsin was reconstituted in 25 mM ammonium bicarbonate immediately before insertion of the samples. After treatment, treated 30% fraction was incubated with MHF at 37°C for 5 h and AI-2 activity was measured.

Reverse-phase high performance liquid chromatography (RP-HPLC). The sample was analyzed by RP-HPLC (Shimadzu High Performance Liquid Chromatograph, Shimadzu, Kyoto, Japan). Distilled water with 0.1% trifluoroacetic acid and methanol were used as the mobile phase. The methanol concentration was altered from 0% to 10% over 10 min, and was maintained for 5 min at 10%. It was then altered to 100% and maintained at 100% for 5 min. Finally, it was altered to 0% and maintained at 0% for 25 min.

Statistical analysis. The results of the various series of experiments are shown as mean and standard deviation. The significance of intergroup differences was analyzed by Student's unpaired *t*-test.

1.4 RESULTS

Purification of AI-2 from the culture supernatant

To investigate the decrease in AI-2 activity in *E. corrodens* during the stationary phase, I tried to purify AI-2 from the culture supernatant of *E. corrodens.* Cell-free culture supernatant of *E. corrodens* grown until mid-exponential phase was extracted with ethyl acetate. The extracted samples were separated by silica-gel TLC. As shown in Fig. 1-1B, five bands (1 to 5) were observed. Bands from the silica-gel plate were extracted and analyzed for AI-2 activity. *V. harveyi* BB170 showed high AI-2 activity in band 4 (Fig. 1-2). The data suggest that this fraction contained AI-2 produced by *E. corrodens*. Rf value of this fraction was compared with that of MHF, a commercially available AI-2-like compound, by TLC (Fig. 1-1A). The results indicate that the Rf values of this fraction (0.74) and MHF (0.75) were similar. This might be because AI-2 from *E. corrodens* had a polarity similar to that of MHF. Hence the purity of this fraction was determined by GC-MS. As shown in Fig. 1-3, it was confirmed that the fraction contained highly pure AI-2. Hence it was used as partially purified AI-2 in subsequent experiments. Moreover, since the retention time of the major peak of AI-2 was slightly higher than that of MHF, our results indicate that the polarity of AI-2 was slightly higher than that of MHF.

Mechanism of AI-2 degradation and inactivation

Azakami *et al.* reported that AI-2 activity in *E. corrodens* decreased dramatically during the stationary phase (37). Hence the culture supernatant of *E. corrodens* grown to the stationary phase was used to identify compounds that degrade or inactivate AI-2. The cell-free culture supernatant was fractionated with different percentages of

ammonium sulfate, and the precipitates were incubated with partially purified AI-2. After incubation with the 30% fraction, AI-2 activity decreased remarkably, whereas no significant change was observed after incubation with the 70% fraction (Fig. 1-4A). Next I investigated the effect of the 30% fraction on MHF. It is known that the *V. harveyi* reporter strain detects MHF, as AI-2 does. As shown in Fig. 1-4B, the 30% fraction produced the same effect against MHF as that observed for AI-2. These results suggest that AI-2 and MHF were inactivated in the same manner by this fraction. Hence MHF was used as the substrate to monitor AI-2 inactivation in subsequent experiments.

The 50% fraction also inactivated purified AI-2 partially, but it did not inactivate MHF (Fig. 1-4). Thus the data suggest that the 50% fraction contains a very low amount of the compound responsible for AI-2 inactivation, and that its specificity for AI-2 is higher than that for MHF.

Characterization of the 30% fraction

To determine whether AI-2-inactivation was enzymatic, I investigated the effect of treatment with heat and/or trypsin on the 30% fraction with respect to MHF inactivation. As shown in Fig. 1-5, neither heat treatment nor trypsin treatment had any effect on MHF, but both together resulted in slight inhibition of MHF inactivation by the 30% fraction.

Mechanisms of MHF degradation and modification by the 30% fraction

To determine the mechanism by which the 30% fraction inactivates MHF, I carried out TLC analyses of MHF before and after incubation with the 30% fraction. After incubation at 37°C for 5 h, the spot of MHF was fainter and showed the same R_f value (Fig. 1-6).

To understand the mechanism of inactivation further, RP-HPLC was performed after incubating MHF with the 30% fraction at 37°C for 5 h. After incubation with the 30% fraction, the retention time of MHF in RP-HPLC shifted slightly to the left (an earlier time) (Fig.1- 7).

1.5 DISCUSSION

In this study, I purified AI-2 partially from the culture supernatant of *E. corrodens*. Its Rf value on a TLC plate was almost the same as that of MHF (Fig. 1-1 & Fig. 1-2). Moreover, the GC-MS data suggested that the polarity of AI-2 of *E. corrodens* is slightly higher than that of MHF, and that its molecular weight is 133 (Fig. 1-3). In GC-MS, when a sample's affinity to protonate is high, the molecular weight often appears as +1 with respect to the actual molecular weight. Thus the actual molecular weight might have been 132. In this case, the difference between the molecular weights of AI-2 and MHF is 18, which corresponds to that of H_2O.

AI-2 is widely distributed in the bacterial kingdom, and it controls a variety of traits in different bacteria (21, 59, 75). "AI-2" is the collective term for a group of signal molecules formed from a common precursor, DPD. DPD is generated by many bacteria as a byproduct of the activated methyl cycle in a reaction catalyzed by LuxS. Reactive DPD undergoes spontaneous cyclization to yield two furanone derivatives, (2*S*,4*S*)-dihydroxy-2-methyldihydro-3-furanone (*S*-DHMF) and (2*R*,4*S*)-dihydroxy-2-methyldihydro-3-furanone (*R*-DHMF). In *V. harveyi*, a borate group is added to *S*-DHMF to form (2*S*,4*S*)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran-borate (*S*-THMF-borate). In *Salmonella typhimurium*, *R*-DHMF is hydrated to form (2*R*,4*S*)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran (*R*-THMF). The molecular weight of the derivatives of DPD, *S*-DHMF and *R*-THMF, is 132 in each case (67). My results suggest that AI-2 of *E. corrodens* is one of these compounds, or that it is similar to one of them. This is the first report on the purification and characterization of AI-2 from *E. corrodens*.

Azakami *et al.* have reported that *E. corrodens* produces AI-2 during the mid-exponential phase, and that AI-2 activity decreases rapidly when the culture enters the stationary phase (37). Hence I hypothesized that AI-2 was degraded or converted into another structure, undetectable by *V. harveyi* BB170. My results indicate that there was AI-2-inactivation activity in the 30% ammonium-sulfate precipitation fraction of the supernatant obtained from the stationary phase culture (Fig. 1-4). In most bacteria, extracellular AI-2 activity reaches a peak in the mid- to late-exponential phases, and declines precipitously during the stationary phase (40). A variety of bacterial species have the ability to sequester and process the AI-2 present in the environment, thereby interfering with the cell-to-cell communication of other bacteria. The rapid disappearance of AI-2 in many cases is an effect of the Lsr transport system, which facilitates the uptake of signal molecules into cells, and a kinase that phosphorylates the signal molecule to pospho-DPD (41), but I observed that AI-2 was degraded or converted to another structure even in the absence of bacterial cells (Figs. 1-6 and 1-7). Therefore, I conclude that *E. corrodens* has a novel AI-2-inactivation system.

To determine whether the AI-2 inactivation factor is proteinaceous, I treated the 30% fraction with heat and/or trypsin. Although neither heat nor trypsin treatment had any effect on MHF, both treatments together resulted in a slight inhibition of MHF inactivation by the 30% fraction. However, bioluminescence by MHF did not increase to the level of control after these treatments (Fig. 1-5). This suggests that the AI-2 inactivation factor might be proteinaceous, but might be a heat-stable, trypsin-resistant protein.

Since the 30% fraction also inactivated MHF (Fig. 1-4), my data suggest that AI-2 and MHF were inactivated in the same manner. After incubation with the 30% fraction,

the intensity of the spot of MHF became faint on the TLC plates when observed under UV at 254 nm (Fig. 1-6). This indicates that MHF was degraded or converted to another structure that has lower UV absorption. Moreover, since the retention time of MHF on RP-HPLC was shifted slightly to the left (Fig. 1-7), the data suggest that MHF was converted to another structure that had high polarity after incubation with the 30% fraction, and that the 30% fraction converted both MHF and AI-2 of *E. corrodens* to compounds that were undetectable by *V. harveyi* BB170. The data also suggest that heat-stable and trypsin-resistant proteins in the 30% fraction might be involved in the inactivation of AI-2 in *E. corrodens*. I partially purified the AI-2 inactivating enzyme by ion-exchange chromatography (unpublished results). Hence I conclude that the AI-2 produced by *E. corrodens* can be converted enzymatically to another form.

It is believed that *E. corrodens* participates in the early stages of biofilm formation by specific co-aggregation with many bacteria present in the oral cavity, and that it acts as a middle colonizer linking early colonizers such as *Streptococci* and late colonizers such as *Porphyromonas gingivalis* (72). As mentioned above, AI-2 has many derivative forms and these derivatives are used by diverse bacteria. Although no converted AI-2 was detected using *V. harveyi* BB170, it is not known whether AI-2 has autoinducing activity towards *E. corrodens* or other bacteria. Oral bacteria might use converted AI-2 as their communication signal molecule. Thus my study indicates that *E. corrodens* might act as a translator or converter of AI-2 and also acts as a middle colonizer in the oral environment.

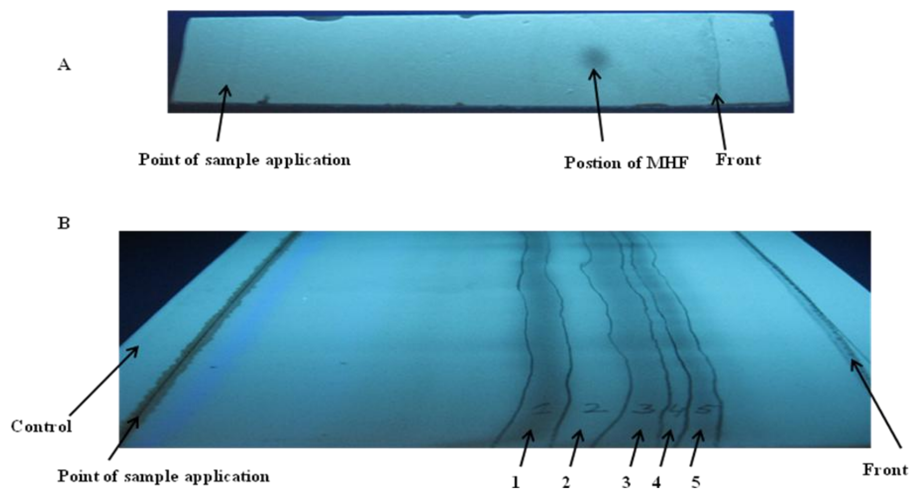

Fig. 1-1. Thin Layer Chromatography (TLC) Analysis of 4-Hydroxy-5-methyl -3(2*H*)-furanone (MHF) (A) and an Extract of the Culture Supernatant of *E. corrodens* (B).

The band fractions (1 to 5) were cut and extracted from the TLC plate.

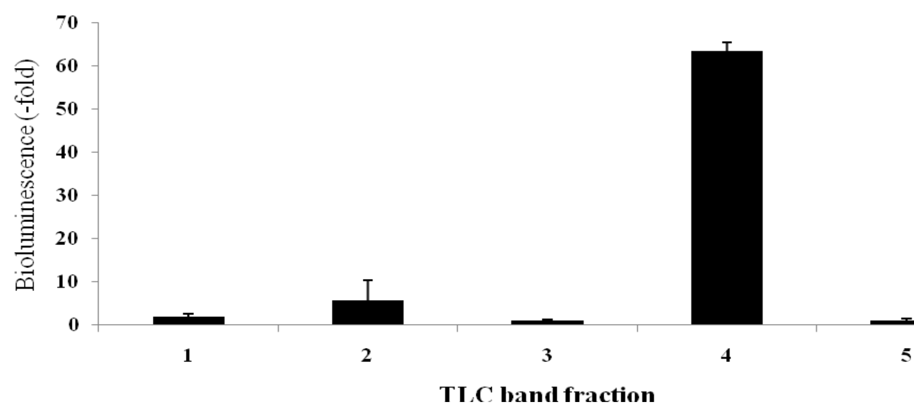

Fig. 1-2. AI-2 Assay of Thin Layer Chromatography Band Fractions (1 to 5).

AI-2 activity in each fraction was measured using *V. harveyi* BB170, as described in "Materials and Methods"

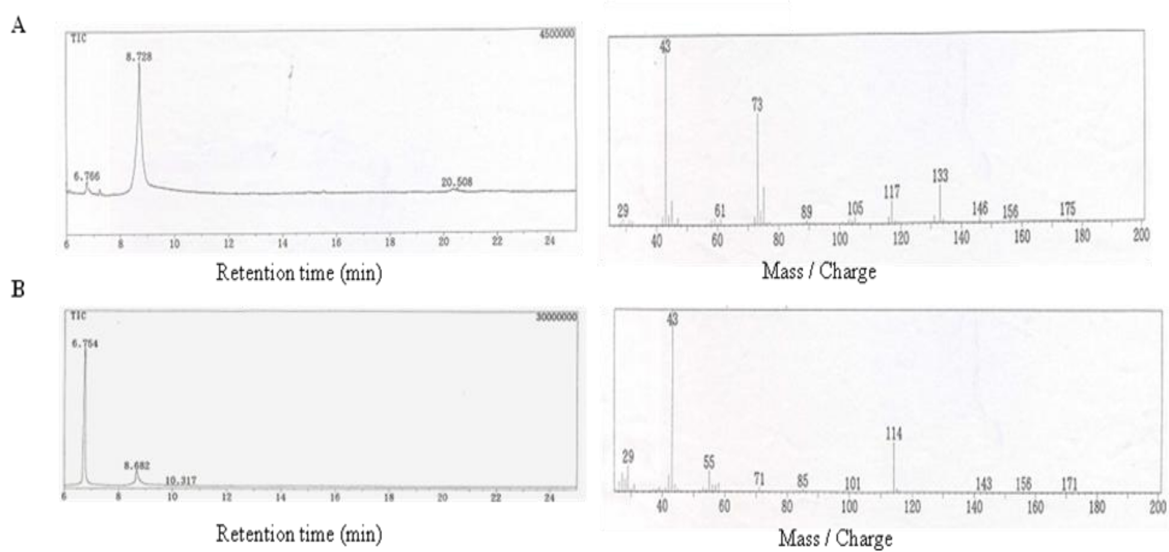

Fig. 1-3. Gas chromatography-mass spectrometry (GC-MS) Analysis of AI-2 from *E. corrodens* (A) and 4-Hydroxy-5-methyl-3(2*H*)-furanone (MHF) (B).

The MHF (molecular weight 114) was used as control.

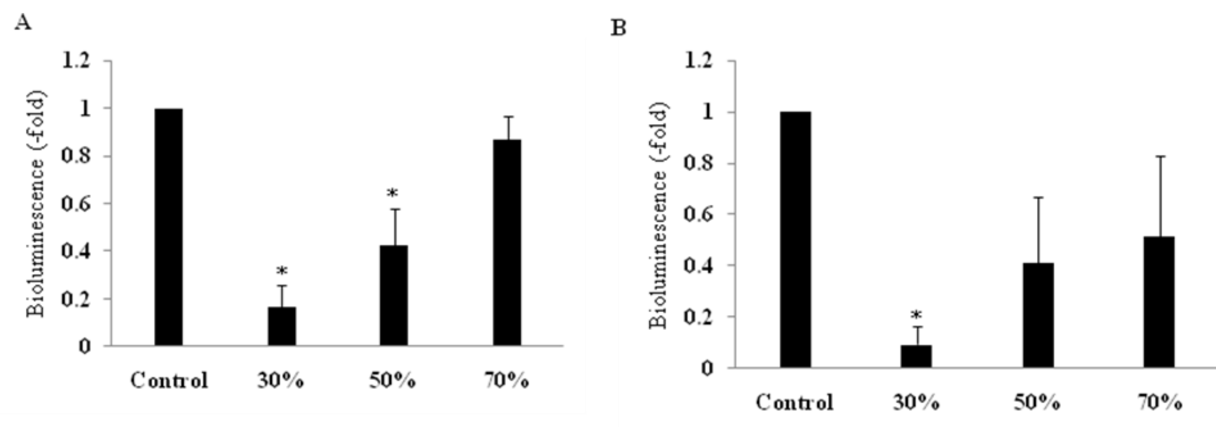

Fig. 1-4. AI-2 Assay after Incubation of Purified AI-2 (A) and 4-Hydroxy-5-methyl-3(2*H*)-furanone (MHF) (B) with Various Ammonium Sulfate Fraction.

AI-2 activity is shown as fold of induced bioluminescence. Means and SDs are for three independent experiments. Asterisks indicate significant difference, $*p < 0.05$ vs. control.

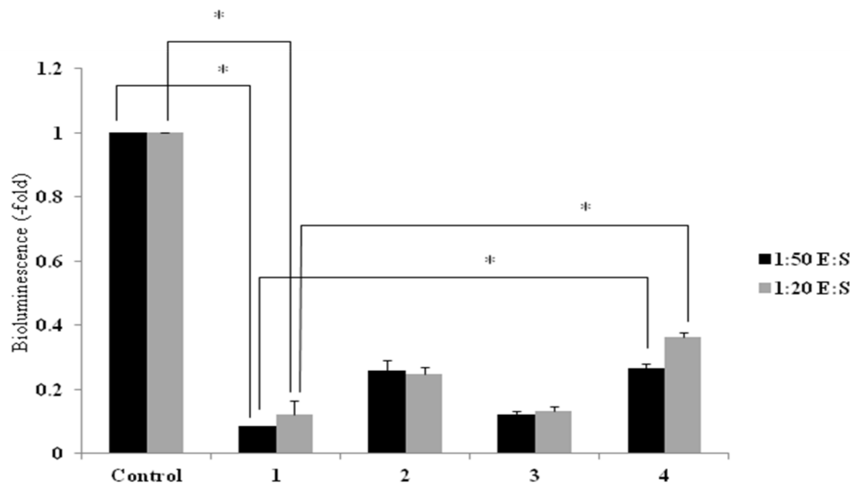

Fig. 1-5. AI-2 Assay after Heat and/or Trypsin Treatment of the 30% Ammonium Sulfate Fraction.

AI-2 activity is shown as fold of induced bioluminescence. (1) Non-treated 30% ammonium sulfate fraction, (2) trypsin-treated 30% ammonium sulfate fraction, (3) heat treated 30% ammonium sulfate fraction, (4) heat and trypsin-treated 30% ammonium sulfate fraction.

Means and SDs are for 3 independent experiments. Asterisks indicate significant difference, $*p < 0.05$.

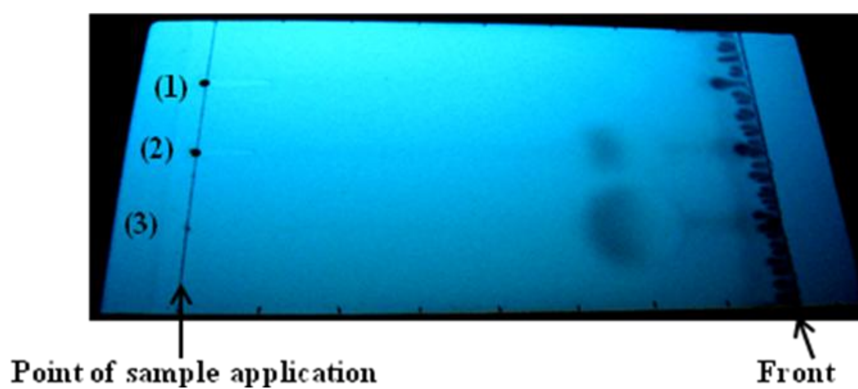

Fig. 1-6. Thin-Layer Chromatography of MHF after Incubation with 30% Ammonium Sulfate Fraction.

(1) 30% ammonium sulfate fraction only, (2) 4-Hydroxy-5-methyl-3(2*H*)-furanone (MHF) after incubation with the 30% ammonium sulfate fraction for 5 h, and (3) MHF alone.

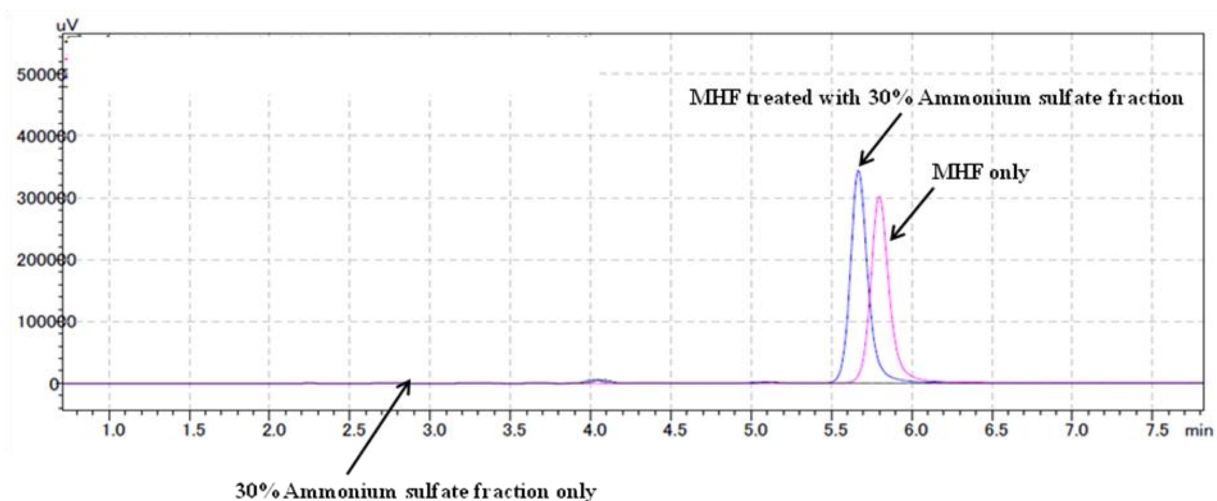

Fig. 1-7. Reverse-Phase High Performance Liquid Chromatography (RP-HPLC) of 4-Hydroxy-5-methyl-3(2*H*)-furanone (MHF) after Incubation with 30% Ammonium Sulfate Fraction.

Peaks obtained for MHF alone, MHF incubated with the 30% fraction, and the 30 % fraction alone are shown by arrowheads.

CHAPTER 2

LuxS Affects Biofilm Maturation and Detachment of the Periodontopathogenic Bacterium *Eikenella corrodens*

2.1 ABSTRACT

Previously, Azakami *et al.* reported that biofilm formation of *E. corrodens* is regulated by AI-2, based on observations that biofilm-forming efficiency of Δ*luxS* mutant was greater than that of the wild type (J. Biosci. Bioeng., 102, 110-117 (2006)). To determine whether the AI-2 molecule affects biofilm formation directly, purified AI-2 was added to *luxS* mutant and wild-type *E. corrodens* and compared biofilm formations by using a static assay. Results indicated that biofilm formation in *E. corrodens* was enhanced by the addition of AI-2. Biofilms formed by flow cell system were also compared for the *luxS* mutant and the wild type by using scanning electron microscopy and confocal laser scanning microscopy. The number of viable bacteria in the *luxS* mutant biofilm was dramatically reduced and more sparsely distributed than that of the wild type, which suggested that AI-2 might enhance the mature biofilm. Conversely, further analysis by modified confocal reflection microscopy indicated that the wild-type biofilm was matured earlier than that of the *luxS* mutant, and became thinner and more sparsely distributed with time. These data suggest that LuxS may facilitate the maturation and detachment of biofilm in *E. corrodens.*

2.2 INTRODUCTION

Biofilms are sessile, surface-attached communities of microorganisms (42, 43). Microorganisms undergo profound morphological changes during the transition from planktonic organisms to cells that constitute complex, surface-attached communities. These changes may occur in response to a variety of environmental signals and are reflected in the new phenotypic characteristics of the biofilm bacteria (76, 77). Many bacterial infections are characterized by important factors such as formation of sessile communities and the inherent resistance of these communities to antimicrobial agents (78). Biofilm formation is a complex developmental process requiring a series of discrete and well-organized steps (79). The early stage in biofilm formation involves the attachment of active cells to a solid surface, followed by immobilization on that surface, while the late-stage involves cell adhesion, cell-to-cell interactions, microcolony formation, and formation of a multi-layered architecture of the mature biofilm (80, 81).

Human oral bacteria are highly interactive organisms that exist within multi-species dental plaque biofilms (82). Dental plaque is a complex and dynamic microbial community that forms as a biofilm on the surfaces of teeth and oral tissues. It is composed of over 700 species of bacteria (47, 83) and is the prime etiological agent of 3 common human oral diseases: dental caries, gingivitis, and periodontal disease (82, 84, 85). For inter-species communication to occur effectively within dental plaque biofilms, cell-cell association and cell-cell signaling are required (86, 87).

QS is a widespread system, used by bacteria for cell-to-cell communication, which regulates expression of multiple genes in a cell density-dependent manner (56, 88). QS has been shown to control cell density-dependent behaviors, such as the expression of virulence factors, biofilm formation, and iron acquisition, in a variety of organisms (56,

66, 89, 90). Thus, QS has been thought to allow bacteria in a biofilm to react concertedly, like a multicellular organism, to changes in the external environment. The unique QS system shared by Gram-positive and Gram-negative bacteria is mediated by AI-2 (75), which is a signaling molecule synthesized by the *luxS* gene (91).

E. corrodens, a facultative anaerobic Gram-negative rod, is predominantly found in sub-gingival plaque samples of patients with advanced periodontitis (70). Previous research has revealed that the monoinfection of germ-free or gnotobiotic rats by *E. corrodens* can cause severe periodontal disease (71), which suggests that *E. corrodens* a periodontopathogenic bacterium. Additionally, since *E. corrodens* has been detected in tooth plaque (72), this bacterium may participate in the early stages of biofilm formation in the periodontal pockets.

Previously, Azakami *et al*. reported that *E. corrodens* includes an ortholog of the *luxS* gene on its genome, and secretes AI-2 in its culture supernatant. Moreover, the mutation Δ*luxS* was found to enhance biofilm formation in *E. corrodens* (37), which suggests that AI-2 signal plays a role in biofilm formation by *E. corrodens*. In fact, a number of studies have described the role of AI-2 in biofilm formation. For example, synthesized AI-2 directly stimulates *Escherichia coli* biofilm formation and controls biofilm architecture by stimulating bacterial motility (92). Additionally, several studies have indicated that AI-2 controls biofilm formation (61, 93). Conversely, other studies have shown that the addition of AI-2 failed to restore the biofilm phenotype of the parental strain (94-100), due to either the central metabolic effect of LuxS [*S*-ribosyl homocysteine lyase (EC 4.4.1.21)] or the difficulty in complementation of AI-2 (101). AI-2 is produced by the LuxS enzyme, which converts *S*-ribosyl homocysteine into DPD, and DPD is subsequently converted into AI-2. The LuxS-catalyzed reaction is

also an integral part of the activated methyl cycle, which is an important metabolic pathway that serves to recycle homoserine from *S*-adenosyl methionine to maintain methionine biosynthesis. Therefore, whether enhanced biofilm formation by the Δ*luxS* mutation in *E. corrodens* is responsible for either effect is not clear.

In this study, I investigated the direct effect of AI-2 on biofilm formation. Moreover, I compared the structure and mechanism of biofilm formation between the *luxS* mutant and wild type. The results of this study suggest that LuxS may facilitate the biofilm maturation and detachment in *E. corrodens*.

2.3 MATERIALS AND METHODS

Bacterial strains and growth conditions. *E. corrodens* 1073 was provided by S. S. Socransky (Forsyth Dental Center, Boston, MA, USA). *E. corrodens* cells were statically cultured at 37°C in TSB containing 2 mg/mL of KNO_3 and 5 µg/mL of hemin. The *luxS*-mutant cells (37) of *E. corrodens* were cultured in a medium supplemented with 50 µg/mL kanamycin. An *E. corrodens* strain harboring a plasmid pLESluxS, which contains the *luxS* gene, was cultured in a medium supplemented with 30 µg/mL carbenicillin.

Adherence assay for the quantitation of biofilm production by *E. corrodens* strains (static assay). *E. corrodens* strains formed a macroscopically visible biofilm that was firmly attached to the wells of tissue culture plates (96-well cell culture plate, nontreated polystyrene, flat-bottom with lid; BD Bioscience, San Jose, CA, USA), and the biofilm production was determined by a previously described assay (102). This assay is used to measure the degree of primary attachment and accumulation of multilayered cell clusters and the subsequent biofilm production on a polystyrene surface. Briefly, following growth of the biofilm in the TSB medium for 36 h at 37°C, the plates were gently washed 4 times with distilled water and the adherent bacterial cells were stained with crystal violet. The stain was then dissolved by ethanol and optical density was measured at 595 nm (OD_{595}) using a spectrophotometer (Tecan GENios Microplate Reader, Männedorf, Switzerland).

AI-2 purification from culture supernatant. Purification of AI-2 from culture supernatant of *E. corrodens* was performed as described previously (103). For AI-2

extraction, the log-phase culture of *E. corrodens* 1073 was inoculated in fresh TSB medium and then statically cultured for 15 h at 37°C. After that, the bacterial cells were removed by centrifugation (12,000 ×*g*, 5 min, 4°C), and the supernatant was filtered by performing decompression filtration using filter papers with 0.22 mm pore size (Membrane Filters, ADVANTEC, Japan). AI-2 was extracted 3 times by mixing the filtrated supernatant with ethyl acetate at a ratio of 4:3 (supernatant:ethyl acetate). The obtained ethyl acetate phase was dried in a rotary evaporator at 40°C. The resulting sample was dissolved with a small amount of ethyl acetate and then stored at -30°C. A solution of AI-2 in ethyl acetate was applied to a silica gel TLC plate (TLC Silica gel 60 RP-18F, Merk Ltd. Germany) and developed with 60% methanol. Individual bands that appeared were observed under UV at 254 nm, and the section corresponding with each band was cut out. The compounds obtained from each silica gel section for a specific band were eluted using ethyl acetate, filtered, and dried under a continuous flow of N_2 gas. The resulting samples were dissolved in DMSO, and AI-2 activity was confirmed by performing the AI-2 assay using *Vibrio harveyi* BB170 as a sensor strain (74).

Biofilm formation of the *E. corrodens* in flow cell biofilm model using modified Robbins device (MRD). The *E. corrodens* biofilm formation in flow cell model using MRD and hydroxyapatite (HA) disks was used as described previously (104) with some modifications. Four separate MRDs and HA disks (10 disks/MRD), processed with saliva for 8 h, were prepared for each strain. *E. corrodens* biofilms were formed by anaerobic perfusion of culture medium containing bacterial cells for 7 days. Culture medium was perfused through 3 separate MRDs for a 7-day period. HA disks were removed aseptically from each MRD. HA disks were observed by scanning electron

microscopy (SEM) and the number of viable bacterial cells was counted. HA disks were washed and ultrasonicated in 1.5 mL distilled water at 4°C for 30 min to remove the *E. corrodens* biofilms. The collected bacteria were diluted and spread over sheep-blood agar plates. After culturing at 37°C for 2 days, CFUs were counted.

For SEM examination, HA disks were first washed 3 times with 0.1 M cacodylate buffer and fixed with 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 30 min at room temperature, and then subsequently washed 3 times in 0.1 M cacodylate buffer. After fixation, all samples were dehydrated in a graded series of aqueous ethanol, dried, ion coated with platinum, and examined by SEM (JSM-6390LV; Jeol, Japan) as previously described (105).

Three-dimensional observations of *E. corrodens* flow cell biofilms. To observe the *E. corrodens* biofilms three-dimensionally, flow cell biofilm samples on celluloid disks (Celltight; Sumitomo Bakelite Co., Tokyo, Japan) were prepared using an MRD as described above (106). Biofilm samples were stained with the Live/Dead BacLight bacterial viability kit L7007 (Invitrogen, Carlsbad, CA) for 15 min at room temperature. The samples were observed by confocal laser scanning microscopy (CLSM; LSM 510; Carl Zeiss, Oberkochen, Germany). Images were processed by image analysis software (Imaris; Bitplane AG, Zurich, Switzerland).

Observation of biofilm by confocal reflection microscopy (CRM). For biofilm flow cultures, biofilms were maintained using the flow-reactor method, as previously described (107). Briefly, stationary-phase cultures were diluted to an OD_{660} of 0.1 with TSB media, and 300 μL of the cell suspension was injected into a flow-cell (Stovall Life

Science, Greensboro, NC, USA) with channel dimensions of 1 ×4 × 40 mm (H × W × L). After static incubation for 1 h, a flow of 1/10× TSB medium was initiated at a rate of 0.2 mL/min. Biofilms were grown at 37°C for 48 h.

A Carl Zeiss LSM510 META (Carl Zeiss, Jena, Germany) was used to acquire confocal micrographs. For the CRM observation, cells were illuminated with a 514-nm argon laser, and the reflected light was collected through a 505–530 nm band-pass filter to avoid the influence of autofluorescence. An NT 80/20 half mirror (Carl Zeiss) was used as a beam splitter. Confocal images were analyzed using the Carl Zeiss LSM 5 PASCAL Software (Version 3.5, Carl Zeiss). The thickness of each image stack was 0.8 µm. For bio-volume quantification, confocal images were analyzed using the COMSTAT computer program, which functions as a script in MATLAB software (Mathworks, Natick, MA, USA).

Statistical analysis. The results of each series of experiments are shown as Mean (standard deviation). The significance of intergroup differences was analyzed using Student's *t*-test (unpaired *t* test).

2.4 RESULTS AND DISCUSSION

AI-2 enhances biofilm formation directly in *E. corrodens*.

Previously, Azakami *et al.* reported that the biofilm-forming efficiency of the *ΔluxS* mutant was approximately 1.3-fold greater than that of wild type and suggested that AI-2 signal plays a role in the biofilm formation by *E. corrodens* (37). Although the LuxS enzyme catalyzes the reaction that leads to the synthesis of the AI-2 precursor, DPD, this reaction is also a part of the activated methyl cycle. Therefore, whether the enhancement of biofilm formation observed in the *ΔluxS* mutant was due to the direct effect of AI-2 deletion or a defect of the C1 metabolism remains unclear. To evaluate this, it was necessary to add purified AI-2 into the culture of *E. corrodens*, and compare the biofilm-forming efficiency with and without AI-2. Partially purified AI-2 was collected from the culture supernatant of *E. corrodens*, grown until mid-exponential phase, when the amount of secreted AI-2 reached a maximum level (103). The high-purity AI-2 was obtained by extraction from the culture supernatant with ethyl acetate and separation by thin-layer chromatography (TLC) (103). The band exhibiting AI-2 activity on the TLC plates was extracted (103) and used as partially purified AI-2 in the subsequent experiment.

As shown in Fig. 2-1, the addition of 0.1 μg/mL of the partially purified AI-2 resulted in enhanced biofilm formation in the *ΔluxS* mutant strain (~1.3-fold), while the addition of AI-2 did not affect biofilm formation in the wild-type strain. Previously, Azakami *et al.* have reported that the *ΔluxS* mutant strain can produce no AI-2 (37) and that the purified AI-2 has AI-2 activity using *V. harveyi* BB170 (103). Thus, these results suggest that the addition of AI-2 enhanced biofilm formation directly in *E. corrodens*. However, this result contradicts our previous result that the biofilm-forming efficiency

of the *ΔluxS* mutant was greater than that of the wild type. From their previous results, they assumed that AI-2 reduces biofilm formation in *E. corrodens.* In the current study, the biofilm of wild type appeared to be detached from the bottoms of the microtiter plate wells, whereas the biofilm of the *luxS* mutant remained firmly attached. This observed detachment may reflect a problem with the static biofilm assay, which requires multiple cycles of solution exchange and washing. Therefore, results of the static assay may not reflect actual biofilm-forming efficiency.

***ΔluxS* mutation decreases bacterial viability in *E. corrodens* biofilm.**

The static assay using microtiter plate is widely used biofilm assay. However, it is difficult to analyze the biofilm formation over time owing to batch cultivation. To investigate the actual biofilm, continuous biofilm formation in a flow cell system was observed. The biofilms were formed on hydroxyl apatite disks fixed in MRD using flow cell system. Following biofilm formation, the bacterial cells were removed by sonication. The number of viable bacteria in biofilm was determined by calculating CFU. As shown in Fig. 2-2, total bacterial CFU in the *ΔluxS* mutant biofilm was lower than that from wild type, but this difference was restored by the complementation of the *luxS* gene. These results indicate a reduction in the number of viable bacteria in the *ΔluxS* mutant biofilm.

As described above, the reaction by LuxS enzyme is also a part of the activated methyl cycle, which plays role to maintain methionine biosynthesis by recycling homoserine from *S*-adenosyl methionine. It has been reported that *luxS* mutation affects cellular levels of fermentation products, fatty acids, nucleic acids, and amino acids (108). Prior research has suggested a relationship between *luxS* mutation and bacterial

viability in biofilm. For example, Maria *et al.* reported that low levels of *luxS* expression seem to provide an advantage for bacterial survival during infections by the group A streptococci (109). Moreover, Ahmed *et al.* reported that the level of viability in the *luxS* mutant biofilms is lower than that in the wild-type biofilm of *Streptococcus intermedius* (110). Therefore, I hypothesized that decreasing metabolic levels for C1 compounds in the *ΔluxS* mutant might lead to a reduction in bacterial viability in the biofilm.

AI-2 facilitates biofilm maturation in *E. corrodens*.

To compare wild-type and *ΔluxS* mutant biofilms in more detail, Biofilm formations on hydroxyl apatite disks was observed by using MRD by SEM. Results of this analysis revealed that wild-type *E. corrodens* formed dense biofilms, whereas the *ΔluxS* mutant formed sparse biofilms (Fig. 2-3). Studies of several bacterial species indicate that both the initial adherence stage and the later stages, including biofilm maturation, are affected, when quorum-sensing pathways are inhibited (80, 81). Thus, biofilm maturation in the *ΔluxS* mutant might be inhibited by the lack of AI-2, and conversely, AI-2 might enhance the maturation of *E. corrodens* biofilm.

Biofilms of both strains formed on celluloid disks by CLSM (Fig. 2-4) were also observed. Similar to what was observed during the SEM analysis, the wild-type strain was again observed to form a dense biofilm, whereas the *luxS* mutant formed a sparse biofilm, although the thicknesses of both biofilms were nearly equivalent (30 μm). These results support my hypothesis that AI-2 can enhance the maturation of *E. corrodens* biofilm.

Results of live/dead staining showed that live (green) bacteria dominated the wild-type biofilm, whereas dead (red) bacteria were the dominant component in the *luxS* mutant biofilm (Fig. 2-4), which supports my suggestion that viable bacteria decreased in *luxS* mutant biofilm.

AI-2 facilitates biofilm maturation and detachment in *E. corrodens*.

The SEM technique requires dehydration of the sample prior to analysis, which leads to major distortions and artifacts in the actual images in the case of wet biological samples. In CLSM observation, fluorescence staining is required after biofilm formation. Therefore, CRM was observed to analyze the difference of intact biofilm structures between the 2 strains (Fig. 2-5). It was observed over time that wild-type biofilm masses were increasingly projected outward into the surrounding medium, where they gradually became thinner. This growth projection was not observed in the *ΔluxS* mutant biofilm, and the biofilm continued to mature without detachment at 48 h. Thus, the results suggest that in wild-type strain, AI-2 facilitated both the maturation and the detachment of biofilm.

Biofilm development can be divided into 3 distinct stages: attachment of cells to a surface, growth of the cells into a sessile biofilm colony, and detachment of cells from the colony into the surrounding environment (111). The final stage of biofilm development, which is the detachment of cells from the biofilm colony and their dispersal into the environment, is an essential stage of the biofilm life cycle that contributes to biological dispersal, bacterial survival, and disease transmission. Like other stages of biofilm development, dispersal can be a complex process that involves numerous environmental signals, signal-transduction pathways, and effectors (112).

Relative to this, it has been reported that AI-2 regulates biofilm maturation and dispersal in some bacteria. For example, in *Vibrio cholerae,* AI-2 mutants form thicker biofilms than wild-type strains on glass coverslips (113), and are also deficient in biofilm detachment (114).

My study suggested that LuxS might facilitate the final stage of biofilm development, maturation, and detachment. Since, biofilm detachment plays a key role in the communicable transmission of many pathogens, AI-2 in *E. corrodens* might facilitate not only biofilm maturation but also transmission of periodontal disease.

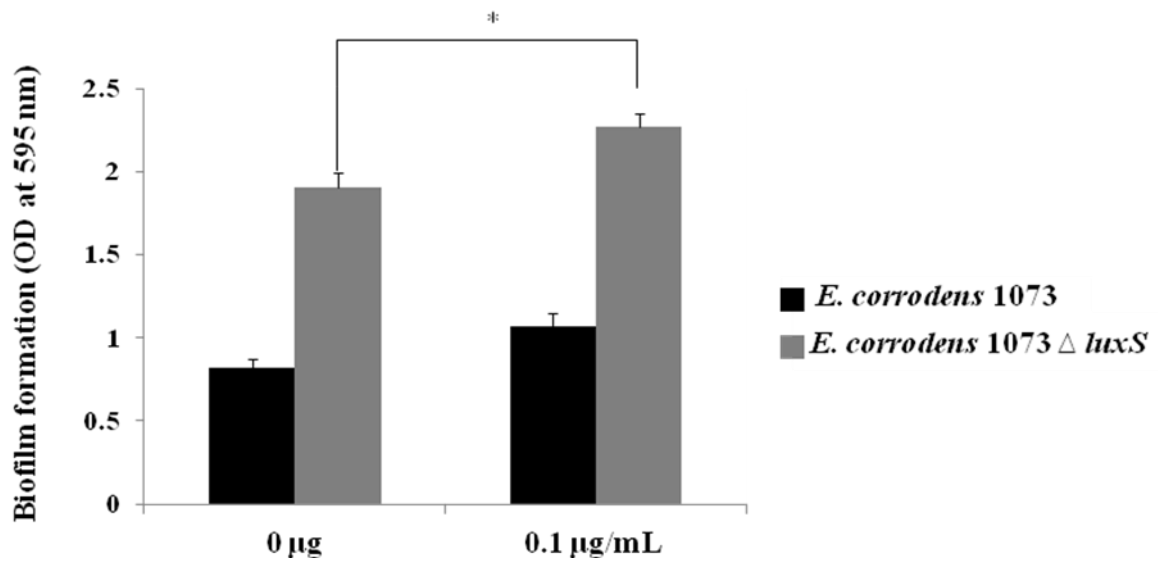

Fig. 2-1. Effect of purified AI-2 on biofilm formation by *E. corrodens.*

Purified AI-2 was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 0.1 µg/mL. DMSO was used as a negative control. Biofilm formation with or without the purified AI-2 was assayed using the static method. Assays were performed in at least eight wells; mean values and standard deviations have been indicated. Asterisk indicates significant difference, $*p < 0.05$.

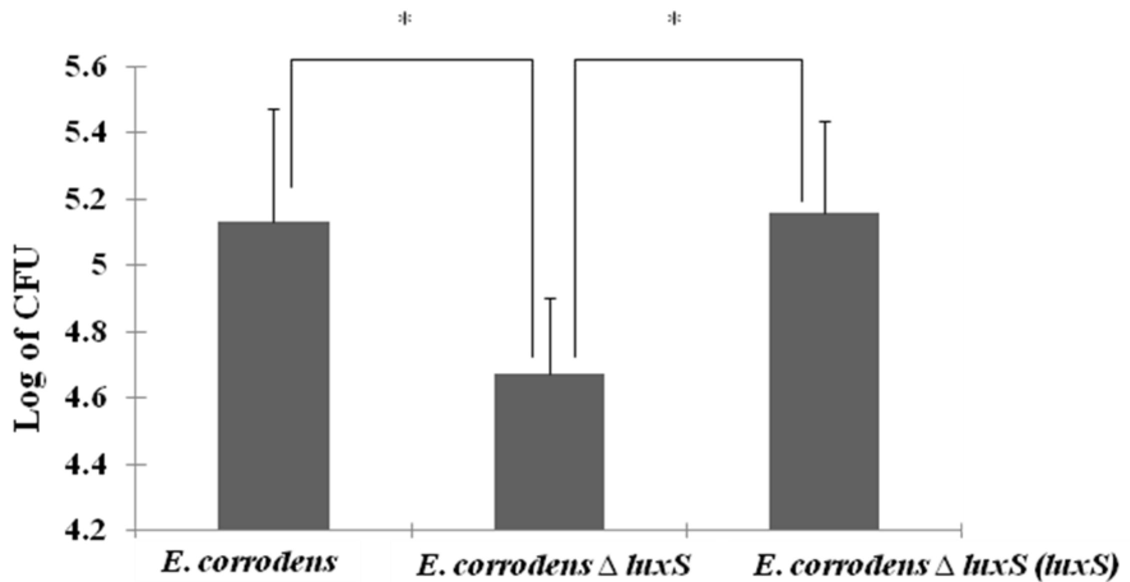

Fig. 2-2. Enumeration of viable bacteria in biofilm.

Counting was performed in at least 9 wells; log of mean values and standard deviations have been indicated. Asterisk indicates significant difference, $*p < 0.05$. Δ*luxS* = Δ*luxS* mutant strain of *E. corrodens* 1073; Δ*luxS* (*luxS*) = Δ*luxS* mutant strain complemented by the *luxS* gene.

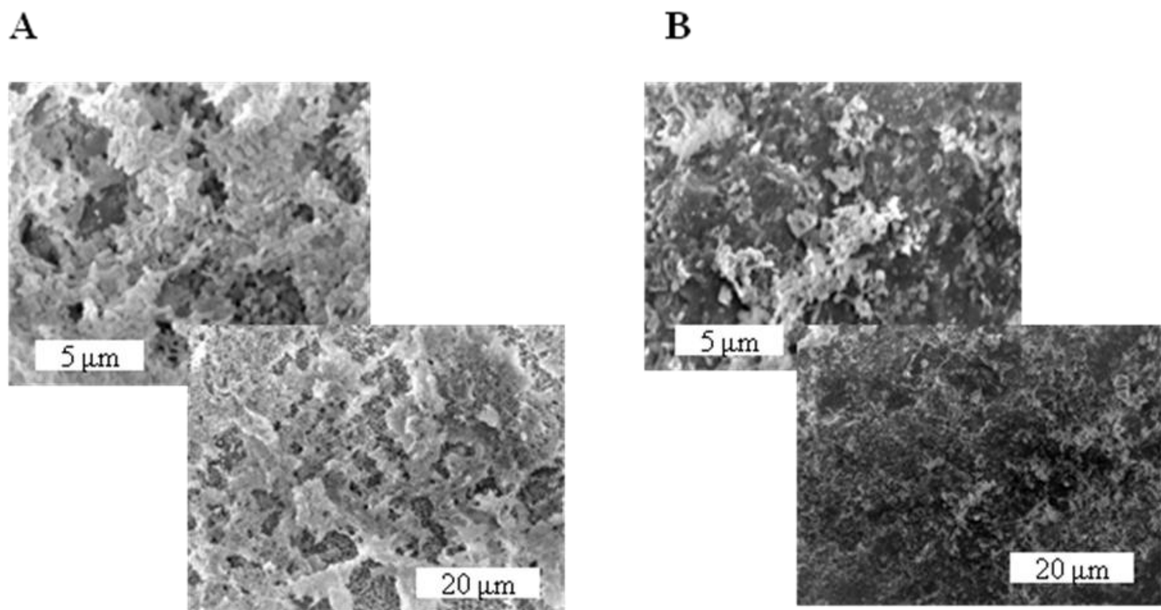

Fig. 2-3. Scanning electron microscopy (SEM) images of biofilm formation formed by *E. corrodens* 1073 (A) and *E. corrodens* 1073 Δ*luxS* (B).

Scale bars are 5 μm (upper) and 20 μm (lower), respectively. A single example from the 5 images is presented.

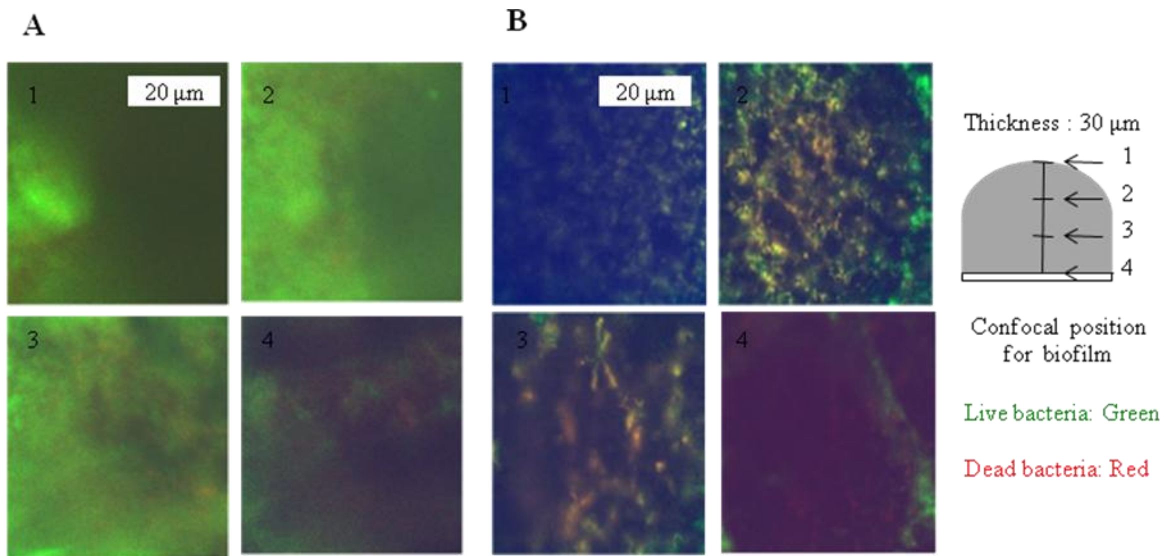

Fig. 2-4. Confocal laser scanning microscopy (CLSM) of biofilm formation on celluloid disks by *E. corrodens* 1073 (A) and *E. corrodens* 1073 Δ*luxS* (B).

Live cells and dead cells are stained green and red, respectively. Panels 1–4 correspond to confocal positions in the biofilm (30, 20, 10, and 0 μm from the surface of disks, respectively). A single example from the 5 images is presented.

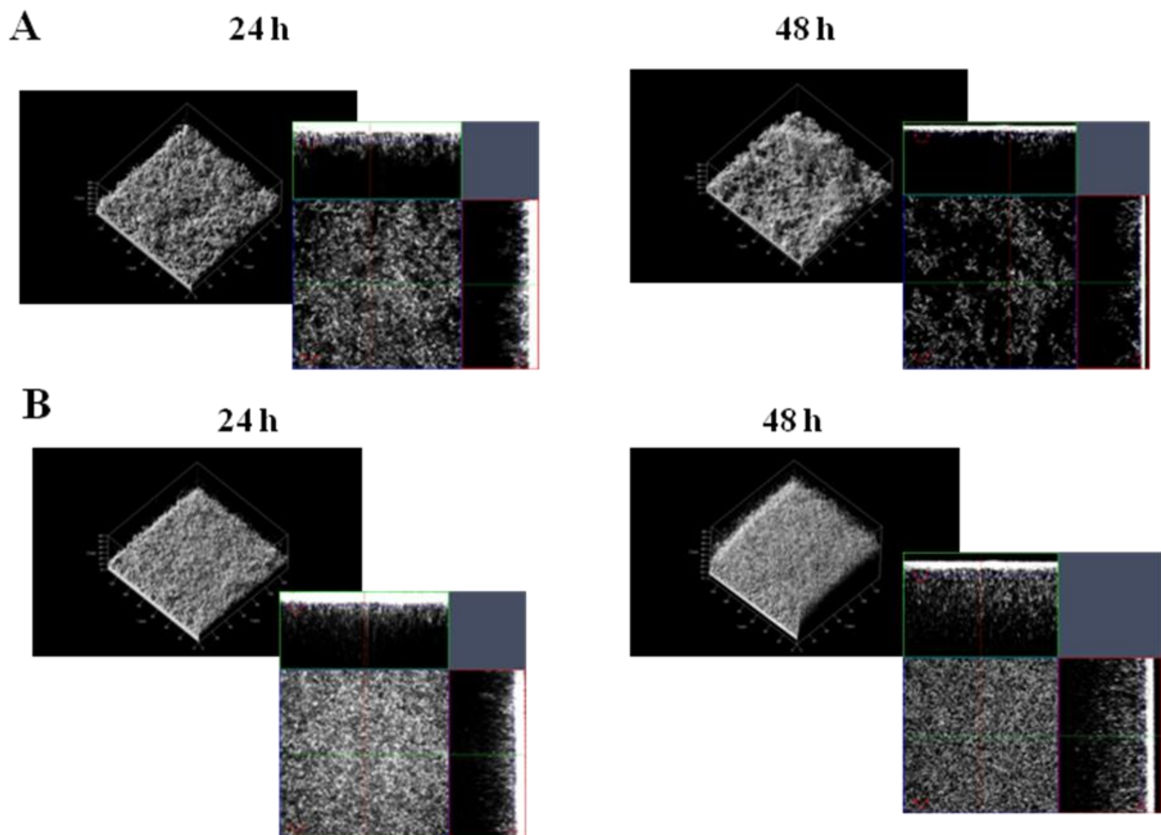

Fig. 2-5. Confocal reflection microscopy (CRM) of biofilm formation by *E. corrodens* 1073 (A) and *E. corrodens* 1073 Δ*luxS* (B) at 24 h and 48 h.

A field of 140 × 140 μm (x, y) is shown, as indicated in the panels. A single example from the 5 images is presented.

GENERAL CONCLUSION

QS refers to the ability of a bacterium to sense information from other cells and communicate in the population when they reach a critical concentration (2). The cellular functions regulated by QS include expression of virulence factors, competence for genetic transformation, production of antibiotics and secondary metabolites, and biofilm formation (56, 65, 66). The only QS pathway shared by Gram-positive and Gram-negative bacteria is that involving the LuxS enzyme, *S*-ribosyl homocysteine lyase, in the synthesis of a signaling molecule known as AI-2 (64). The LuxS-dependent QS system has been referred to as an interspecies communication system and may operate as a universal QS system for many bacteria possessing the characteristic *luxS* gene (21). Biofilms are sessile, surface-attached communities of microorganisms (42, 43). Human oral bacteria are highly interactive organisms that exist within multi-species dental plaque biofilms (82). Dental plaque is a complex and dynamic microbial community that forms as a biofilm on the surfaces of teeth and oral tissues (47, 83). *E. corrodens*, a facultative anaerobic Gram-negative rod, is predominantly found in sub-gingival plaque samples of patients with advanced periodontitis (70). Previous research has revealed that the monoinfection of germ-free or gnotobiotic rats by *E. corrodens* can cause severe periodontal disease, which suggests that *E. corrodens* is a periodontopathogenic bacterium (71). Additionally, since *E. corrodens* has been detected in tooth plaque, this bacterium may participate in the early stages of biofilm formation in the periodontal pockets (72). Previously, Azakami *et al*. reported that the *E. corrodens* genome has an ortholog of the *luxS* gene, and that *E. corrodens* secretes quorum sensing signal molecule AI-2 into the culture supernatant (37). Although maximum expression of AI-2 was observed during the mid-exponential growth phase,

AI-2 activity rapidly decreased when the bacterium entered the stationary phase (37). They also reported LuxS mutant's capacity to colonize and form biofilm on polysterene surface is 1.3-fold greater than the wild type *E. corrodens* (37). So, they suggested *E. corrodens*'s LuxS-dependent signal plays a key role in the biofilm formation of the oral cavity. However why AI-2 activity is decreased in stationary phase and what`s the role of AI-2 in biofilm formation was remain to be determined. In this study, I studied the relationship between QS and biofilm formation of *E. corrodens.*

In this study, I found that *E. corrodens* has a novel AI-2 inactivation system, in which it produce AI-2 inactivating enzyme and AI-2 is converted to another structure by this enzyme. From these results, I made two hypotheses against the biological role of this enzyme as shown in Fig. 3-1. At first, I proposed the model in which *E. corrodens* serves as "translator of AI-2" in oral environment. In oral cavity, some bacteria might use the converted AI-2 by *E. corrodens* as their communication signal molecule. Thus, my study suggested that *E. corrodens* might act as a translator of AI-2 signal, functioning as a middle colonizer in the oral environment. Moreover, I proposed another model in which *E. corrodens* uses both intact AI-2 and converted AI-2 to regulate their functions at multiple stage. In this model, they use intact AI-2 during the exponential phase, whereas they use another signal converted by the enzyme after stationary phase. Thus, *E. corrodens* might make it possible to regulate more intricately by multiple QS.

My study also suggested that LuxS enzyme might facilitate the final stage of biofilm development including maturation and detachment. I proposed the role of LuxS enzyme in pathogenicity of *E. corrodens* as shown in Fig. 3-2. Since biofilm detachment plays a key role in the communicable transmission of many pathogens, AI-2 in *E. corrodens* might facilitate not only biofilm maturation but also transmission of periodontal disease.

From these investigations, I suggested that QS is intimately involved in periodontopathogenesis of *E. corrodens*. Targeting the QS system might lead to clinical application for new treatment of periodontal disease.

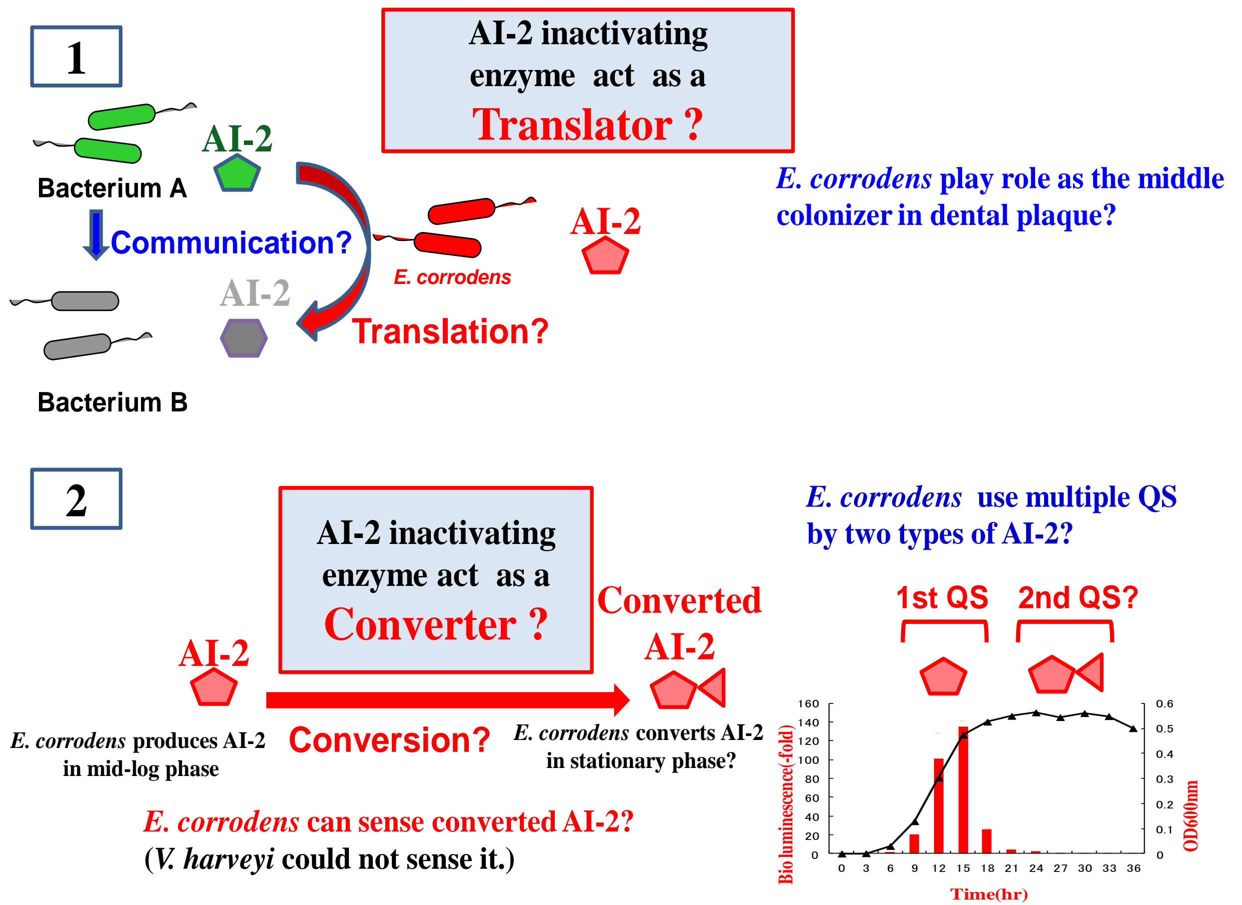

Fig. 3-1. Proposed biological role of AI-2 inactivating enzyme in *Eiekenlla corrodens*

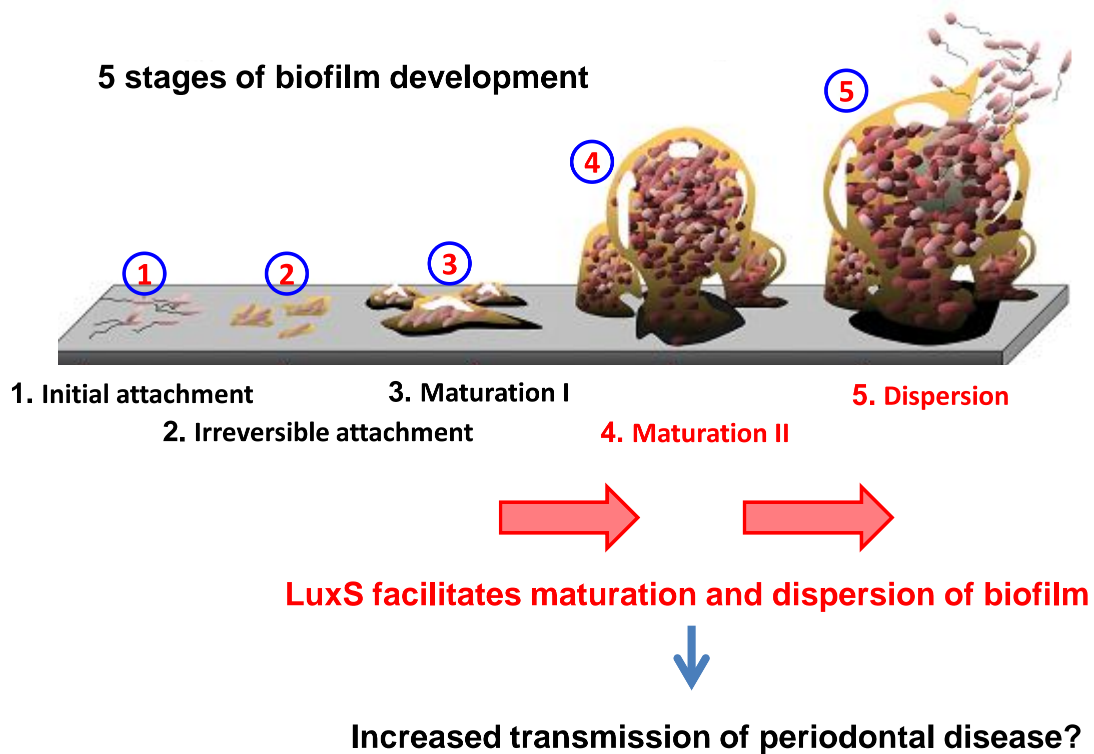

Fig. 3-2. Proposed role of LuxS enzyme in pathogenicity of *Eikenella corrodens*.

(Figure modified from Monroe D (2007) Looking for Chinks in the Armor of Bacterial Biofilms. *Public Library of Science Biology; 5(11): e307.*)

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SUMMARY

Studies on the relationship between quorum sensing and biofilm formation of *Eikenella corrodens*.

In this study, I investigated the relationship between quorum sensing and biofilm formation of *E. corrodens*. To identify the factor responsible for decreasing AI-2 activity in stationary phase, I purified AI-2 inactivating enzyme from *E. corrodens* partially and characterized it. Moreover, to clarify the LuxS dependent signal's role in pathogenicity including biofilm formation, I investigated the effect of purifed AI-2 on biofilm formation of *E. corrodens*. Furthermore, I compared the biofilm formation between wild type strain and *luxS* mutant strain using flow cell system and various microscopic technics.

In chapter 1, I investigated the mechanism underlying decrease in AI-2 activity in stationary phase of *E. corrodens.* To analyze the mechanism, I extracted and purified AI-2 from the supernatant of mid-log-phase culture. Simultaneously, the stationary-phase culture supernatant was fractionated by ammonium sulfate precipitation. On incubating purified AI-2 and MHF with each fraction, the 30% fraction decreased both AI-2 and MHF activities. The data suggest that AI-2 and MHF were rendered inactive in the same manner. Heat and/or trypsin treatment of the 30% fraction did not completely arrest AI-2-inactivation, suggesting that partially heat-stable proteins are involved in AI-2 inactivation. I observed that an enzyme converted MHF to another form. This suggests that *E. corrodens* produce an AI-2 inactivating enzyme, and that AI-2 can be degraded or modified by it.

In chapter 2, I investigated the role of AI-2 in biofilm formation of *E. corrodens*. To determine whether the AI-2 molecule affects biofilm formation directly, I added purified AI-2 to *luxS* mutant and wild-type *E. corrodens* and compared biofilm formations by using a static assay. Results indicated that biofilm formation in *E. corrodens* was enhanced by the addition of AI-2. I also compared the biofilms formed by flow cell system for the *luxS* mutant and the wild type by using scanning electron microscopy and confocal laser scanning microscopy. The number of viable bacteria in the *luxS* mutant biofilm was dramatically reduced and more sparsely distributed than that of the wild type, which suggested that AI-2 might enhance the mature biofilm. Conversely, further analysis by modified confocal reflection microscopy indicated that the wild-type biofilm was matured earlier than that of the *luxS* mutant, and became thinner and more sparsely distributed with time. These data suggest that LuxS may facilitate the maturation and detachment of biofilm in *E. corrodens*.

SUMMARY (IN JAPANESE)

Eikenella corrodens のクオラムセンシングと

バイオフィルム形成の関連性に関する研究

本研究は、*E. corrodens* のクオラムセンシングとバイオフィルム形成との関わりを調べたものである。定常期での AI-2 活性の減少に関与する因子を同定するために、*E. corrodens* から AI-2 を精製し、その性質を調べた。また、バイオフィルム形成を含めた病原性に及ぼす LuxS 依存的シグナルの役割を明らかにするために、*E. corrodens* のバイオフィルム形成に対する精製 AI-2 の効果を調べた。さらに、フローセルシステムや種々の顕微鏡技術を使って、野生株と luxS 欠損株のバイオフィルム性状を比較した。

第 1 章では、*E. corrodens* の定常期における AI-2 活性の減少のメカニズムについて研究を行った。このメカニズムを調べるために、対数増殖期中期の培養上清から AI-2 を抽出、精製した。また同時に、定常期の培養上清を硫安沈殿により分画した。それぞれの画分を精製した AI-2 または MHF とインキュベーションしたところ、30%硫安画分により AI-2 活性、MHF 活性ともに減少した。このことは、AI-2 と MHF が同じ機構で不活性化されたことを示唆している。30%画分の熱処理およびトリプシン処理を行ったところ、この画分による AI-2 の不活化を完全に停止させることはできなかった。したがって、部分的に熱安定なタンパク質が AI-2 の不活化に関与することが示唆された。さらに、この酵素が MHF を別の形に変換することを観察した。これらの結果から、*E. corrodens* は AI-2 の不活化酵素を生産し、AI-2 はこれによって分解または修飾されることが示唆された。

第 2 章では、*E. corrodens* のバイオフィルム形成における AI-2 の役割を調べた。AI-2 分子がバイオフィルム形成に直接影響を与えるかどうかを調べるために、精製した AI-2 を *E. corrodens* の *luxS* 変異株と野生株に添加し、静置系のバイオフィルムアッセイによりバイオフィルム形成を比較した。その結果、AI-2 の添加により、*E. corrodens* のバイオフィルム形成が増加した。さらに、走査型電子顕微鏡や共焦点レーザー顕微鏡を用いて、*luxS* 変異株と野生株がフローセルシステムで形成したバイオフィルムを比較した。*luxS* 変異株のバイオフィルムでは野生株のものと比較して、生菌数が著しく減少し、疎らな構造が見られた。このことから、AI-2 がバイオフィルムの成熟に関与することが示唆された。一方、共焦点反射顕微鏡を用いた解析では、野生株のバイオフィルムは *luxS* 変異株のものと比べてより早く成熟し、時間とともに薄く疎らになっていくことが示された。これらの結果から、LuxS 酵素は *E. corrodens* においてバイオフィルムの成熟と剥離を促進することが示唆された。

LIST OF PUBLICATIONS

1. **Mohammad Minnatul KARIM**, Ayako NAGAO, Fariha Jasin MANSUR, Tetsuro MATSUNAGA, Yoshihiko AKAKABE, Yuichiro NOIRI, Shigeyuki EBISU, Akio KATO, and Hiroyuki AZAKAMI.

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   (CHAPTER 1)

2. **Mohammad Minnatul Karim**, Tatsunori Hisamoto, Tetsuro Matsunaga, Yoko Asahi, Yuichiro Noiri, Shigeyuki Ebisu, Akio Kato, and Hiroyuki Azakami.

   LuxS Affects Biofilm Maturation and Detachment of the Periodontopathogenic Bacterium *Eikenella corrodens.*

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   (CHAPTER 2)

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