

東海大学大学院令和元年度博士論文

Spatial distribution and chemical tolerance of
Streptococcus mutants within dual-species
cariogenic biofilms

(二菌種混合う蝕原性バイオフィルムにお
けるミュータンス菌の空間分布と化学耐性)

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1. Summary

Most bacteria live in multi-species biofilm in natural environment. These bacteria have intra- and interspecies interactions and influence the structural development of biofilms. One of the major culprits in dental carries is the formation of *Streptococcus mutans* (*S. mutans*) biofilm on teeth. The human oral cavity is home to hundreds of different bacteria that form multi-species biofilms. It is also known that cariogenic biofilms are multi-species. However, the relationships between interspecies interaction and biofilm structure remains unclear. We have developed a system to examine multi-species oral biofilms on a hydroxyapatite (HA) substrate using continuous optimizing confocal reflection microscopy (COCRM). COCRM uses reflected light to create 3D images, thus allows for visualization without fluorescent probes. In the present study, to visualize the location of *S. mutans* within multi-species biofilms, we constructed a *S. mutans* strain tagged with a strong fluorescent protein gene under anaerobic condition. *S. mutans* and other oral bacteria, such as *Streptococcus mitis*, *Aggregatibacter actinomycetemcomitans* (*Aa*) and *Lactobacillus casei subsp. casei* were co-cultured on a HA substrate, generating two-species biofilms. We visualized the localization of *S. mutans* in the mixed-species biofilms, using a combination of COCRM and confocal laser scanning microscopy and found that the co-culture of *S. mutans* and *S. mitis* formed double layer biofilms. Visualization of the biofilm architecture and bacterial

localization within the biofilm is essential to understand the bacterial interaction. Here, we showed that the spatial localization of *S. mutans* within dual-species biofilms alters depending on the partner. Furthermore, *S. mutans* with *S. mitis* or *Aa* co-culture biofilm have more tolerance to Chlorhexidine than *S. mutans* mono-culture biofilm. Thus, we revealed that these biofilms influenced the survival of *S. mutans* against disinfectants. This study would provide us basic knowledge on how *S. mutans* interact with other bacteria in multi-species cariogenic biofilms.

Key words: *Streptococcus mutans*, cariogenic biofilm, interspecies interaction, chlorhexidine tolerance, spatial distribution

2. Backgrounds and Introduction

In natural environments, most microbes do not exist individually but live in microbial communities called biofilms, as they are highly advantageous. Biofilms are produced by bacteria. The cells in the biofilms are enclosed in self-produced extracellular polymeric substances (EPS), which comprise various substances such as DNA, proteins, and polysaccharides. Biofilms exhibit increased tolerance to disinfectant chemicals, antibiotics, and host immune systems and are widely recognized as causative agents of infectious diseases and environmental contaminants. Based on previous studies on model mono-species biofilms, our current understanding of biofilm physiology has increased; however, multi-species biofilms, such as those in the cavity are dominant in the natural environment. Interspecies communication shapes the behavior and phenotype of these multi-species biofilms. Recent studies have shown that mono- as well as multi-species biofilms exhibit cell-cell communication through quorum sensing, membrane vesicles, and a variety of other methods. Interspecies communication often consequently influences the three dimensional architecture of the biofilm community, thus affecting bacterial spatial distribution. Imaging of the spatial distribution of cells in multi-species biofilms is important to fully understand the role of interspecies interaction. Oral cavity is the main entry point for food and bacteria into the human body. Among the bacteria residing in the oral cavity, *Streptococcus mutans* is one of the major bacteria that causes dental caries, one of the most prevalent oral infectious diseases caused by oral biofilms. The human oral cavity contains over 700 different bacterial species with more than 100 present in any individual; moreover, each of these bacterial species is known to interact with at least one other species in the oral cavity. It is also known that cariogenic biofilms comprise multiple bacterial species. Interspecies

interactions within the cariogenic biofilms containing multiple bacterial species are considered to influence biofilm properties and architecture. Recently, it was reported that co-culture with other oral bacteria affects gene expression in *S. mutans*. However, the effect of these interspecies interactions on the structure of multi-species biofilms and/or the spatial distribution of *S. mutans* in cariogenic biofilms is unclear.

We had previously developed a non-invasive real-time imaging system called continuous optimizing confocal reflection microscopy (COCRM), which allows the visualization of bacteria and the attached surfaces using light reflected from a laser. We used confocal laser scanning microscopy (CLSM) and COCRM to visualize specific oral bacteria and tooth surfaces simultaneously.

S. mutans strains that constitutively express fluorescent proteins were constructed for this purpose. We constructed a strain in which *ZsGreen* was expressed under the control of an *ldh* promoter and was inserted into SMU_1405c locus of the *S. mutans* genome; this enabled the maintenance of constitutive expression of the fluorescent protein without using antibiotics and the successful distinction of *S. mutans* from other bacteria, even in thick biofilm on HA disks. When we inserted the genes of several fluorescent proteins, such as enhanced green fluorescent protein (eGFP), evoglow, and mCherry, in the *S. mutans* genome, we failed to distinguish between *S. mutans* and other oral bacteria because of the weak fluorescence of these proteins and auto-fluorescence from *Streptococci*. eGFP is one of the most widely used fluorescent proteins. Evoglow can develop fluorescence under anaerobic conditions, which can be achieved in the central position of the biofilm. mCherry can develop fluorescence under low pH conditions, for example when *S. mutans* produce mutacin. However, these fluorescent proteins were not useful in this study, which was intended to observe spatial distribution in the biofilm

on the HA disks.

In this study, we investigated the effect of interspecies interactions on the structure of multi-species biofilms and/or the spatial distribution of *S. mutans* in cariogenic biofilms by co-culturing *S. mutans* with *Streptococcus mitis*, *Aggregatibacter actinomycetemcomitans* (Aa), and *Lactobacillus casei* on HA disks and analyzing their growth by microscopy (CLSM and COCRM) and CFU assays. We also studied the tolerance levels of the dual-species biofilms against chlorhexidine gluconate (CHX), a commonly used oral cavity disinfectant.

3. Materials and Methods

Strain construction

Streptococcus mutans YN101:

We chose the smu 1405c locus for the gene insertion site. The smu1405c locus encodes cas9, the deletion of which has no effect on the biofilm formation of *S. mutans*. To terminate the transcription of smu1405, *Bacillus subtilis* rpsD terminator was fused to the region downstream of the 5' region of smu1405. The 5' region of smu1405 and the *B. subtilis* rpsD terminator were PCR-amplified using smu_1405 F1/1405_rpsD-term 2 and 1405_rpsD-term 1/rpsD-term_tetR 2 primers sets, respectively. These two PCR products were ligated by overlap extension PCR using the smu_1405 F2 and rpsD-term_terR 2 primers. The terR-Ptet region was PCR-amplified using the rpsD-tetR 1 and Ptet_nonA 2 primers from pWH353. The nonA-spoVG terminator region was PCR amplified using the Ptet_nonA 1 and spoVG-term_erm 2 primers from *B. subtilis* TAY3203. PCR products of the tetR-Ptet and nonA-spoVG terminator regions were ligated by overlap PCR using the rpsD-term_tetR 1 and spoVG-term_erm primers. The erythromycin resistance gene (*erm*) was PCR amplified using the spoVG-term_erm 1

and erm_1405 2 primers from pJIR418. The 3' region of *smu1405* was PCR amplified using the erm_1405 1 and smu_1405 R1 primers, and the resultant PCR products were ligated by overlap PCR using the spoVG-term_erm 1 and smu_1405 R2 primers. The 5'-*smu1405-rpsD* term, *tetR-Ptet-nonA-spoVG* term, and 3'-*erm-smu1405* regions were ligated by overlap PCR using the smu_1405 F3 and smu_1405 R3 primers, and the PCR product was introduced into the *smu1405* locus of *S. mutans* UA159 to generate *S. mutans* YN101 by natural competence transformation.

S. mutans YN102:

Using the *S. mutans* YN101 strain constructed above, we changed the P_{tet} promoter to the constitutive promoter P_{ldh} and *nonA* to *ZsGgreen*. The *ldh* gene is the most highly expressed constitutive gene. The 5' region of *smu1405*, P_{ldh} region, and 3' region of *smu1405* were PCR amplified using the smu_1405 F 1/*rspDterm_Pldh_2*, *rspDterm_Pldh_1/Pldh-ZsGreen-2*, and *ZsGreen-erm-1/smu_1405 R 3* primers sets, respectively. The *ZsGreen* fragments were PCR amplified using the *Pldh-ZsGreen-1* and *ZsGreen-erm-2* primers from a pZsGreen Vector (Takara Biotechnology Co., Ltd., Shiga, Japan). These four PCR products were ligated by overlap PCR using smu_1405 F 2 / smu_1405 R 2 primers. The resultant PCR product was introduced into the *smu1405* locus of the *S. mutans* UA159 to generate *S. mutans* YN102 by natural competence transformation.

Briefly, *S. mutans* stored in -80°C deep freezer was inoculated in brain heart infusion (BHI) (Becton Dickinson, Sparks, MD, USA) agar under anaerobic conditions at 37°C for 24 h and incubated in BHI medium in aerobic and static conditions. The liquid culture was diluted 1:20 in fresh BHI medium with 10% vol/vol horse serum and incubated for 2 h in anaerobic and static conditions at 37°C. After 2 h incubation, 100

μL medium was incubated with PCR production in a 96-well plate (IWAKI non-treated microplate flat bottom, polystyrene) for 2.5 h in anaerobic and static conditions at 37°C. This medium with PCR production was inoculated in BHI agar with erythromycin and incubated for 24 h at 37°C in anaerobic conditions. Incubated colonies were picked up, re-inoculated, and incubated on BHI agar plate with erythromycin in anaerobic conditions. A single colony was picked up and then checked by PCR. Additionally, using microscopy, we observed the constitutive fluorescence exhibited.

Bacterial strains

To examine the multi-species biofilms, *Streptococcus mitis* TU003, *Aggregatibacter actinomycetemcomitans* ATCC 43718, and *Lactobacillus casei* subsp. *casei* JCM 8129 were chosen for co-inoculation with *S. mutans*. *S. mitis* is a pioneer colonizer of tooth surfaces in the human oral cavity and its colonization is important for other oral bacteria to adhere to the tooth surfaces and build dental plaque biofilms. *S. mitis* belongs to the group of early colonizing bacteria. *Aa* is anaerobic bacteria and causes localized aggressive periodontitis. *Aggregatibacter* spp. are co-aggregated with *Streptococcus* spp. in human plaque. Additionally, quorum sensing in *S. mutans* is activated by *Aa*, suggesting that these bacteria interact with each other in oral biofilms *in vivo*. *Aa* belongs to group of late colonizing bacteria. *L. casei* is known to enhance the demineralization of tooth enamel when co-cultured with *S. mutans*. *L. casei* is an early colonizing bacterium. The constructed *comE* and *comC* mutant *S. mutans* strains used in this study were procured from Narisawa Naoki, Ph. D.

Culture conditions and biofilm formation

Each bacterial stock stored at -80°C was thawed, inoculated onto BHI agar, and incubated at 37°C for 24 h in anaerobic conditions to isolate single colony. These

isolated colonies were grown in liquid BHI overnight at 37°C in static aerobic conditions. Each overnight culture was diluted till OD₆₀₀ of 0.01 was obtained in fresh medium and then incubated anaerobically at 37°C for 24 h in polystyrene microtiter plates containing BHI medium on human saliva-coated HA disc (diameter, 7 mm; Clarkson Chromatography Products Inc., South Williamsport, PA, USA) in a 24-well plate (IWAKI non-treated microplate flat bottom, polystyrene). Briefly, we collected the human saliva from individuals who had not eaten for at least 2 h. Collected human saliva was filter sterilized using 0.22 µm low protein binding filter. Autoclaved HA disks were dipped into filter-sterilized whole saliva for more than 2 h under shaking at 37°C to simulate the environment in the oral cavity. We measured the OD₆₀₀ of the overnight culture and diluted it so that it would give a final OD₆₀₀ of 0.01 in the fresh medium. We also confirmed the accuracy of the inoculation volume by repeating the same dilution and measuring the CFU.

Observation of the biofilms on the HA disks by microscopy

After incubation, the HA discs with biofilms were dipped in phosphate buffered saline (PBS) or 0.05% CHX for 5 min. These dipped HA disks with biofilms were washed with PBS twice. The HA disks with biofilms were stained with 0.75 µM propidium iodide (PI) (Thermo Fisher Scientific, Waltham, MA, USA) and 5 µM SYTO® 59 (Thermo Fisher Scientific, Waltham, MA, USA) for 30 min, protected from light. The HA disks with biofilms were washed in PBS and the biofilm on the HA disks were observed in PBS. Confocal microscopy images were acquired using an upright confocal laser scanning microscope LSM880 (Carl Zeiss, Oberkochen, Germany) equipped with a W N-Achroplan 40× water-dipping objective lens with 0.80 numerical aperture (Carl Zeiss). ZsGreen was excited by a two-photon laser (880 nm) and detected with a 499–

545 nm band-pass filter, whereas PI was excited by an argon laser (514 nm) and detected with a 590–655 nm band-pass filter. HA disc surfaces were illuminated with a HeNe633 laser (633 nm), and the reflected light was collected through a 627–639 nm band-pass filter. SYTO59 was excited by a HeNe633 laser (633 nm) and detected with a 646–740 nm band-pass filter. We observed the biofilms in at least three independent experiments.

Chlorhexidine (CHX) tolerance test and CFU count

Here, we tested the tolerance of the mono- and dual-species biofilms against CHX, which is frequently used for oral disinfection and is approved by the Food and Drug Administration. CHX, the most commonly used disinfectant in the oral cavity, was used to investigate the tolerance of these biofilms. Incubated biofilms on the HA disks were dipped in 0.05% CHX or a PBS for 5 min. Later, the whole biofilms on the HA disks were imaged using microscopy or washed with PBS twice and then dipped into the liquid BHI medium. The biofilms were removed completely from the HA disc by pipetting and were transferred to the liquid BHI medium. The biofilm can be removed by pipetting and that was confirmed by safranin staining of the HA disk. The medium containing the removed biofilm was diluted and planted on BHI agar with and without erythromycin. They were incubated in anaerobic conditions at 37°C for 24 h. We counted the colonies in every agar plate and performed at least three independent experiments.

Selection of bacterial strains

S. mutans constructed in this study has erythromycin resistance gene, so we can select the *S. mutans* in dual-species biofilm using BHI agar plate with erythromycin. We found that the *S. mitis*, *Aa*, and *L. casei* strains used in this study were killed by

erythromycin. Penicillin G did not have any effect on *Aa* cells in BHI agar as observed by the same number of CFU in media with and without the antibiotic. This might be attributed to the fact that *Aa* is a gram-negative rod. We also found that penicillin G can kill *S. mutans* (even if it has the erythromycin resistance gene), *S. mitis*, and *L. casei*. Hence, we used BHI agar with 10 µg/mL erythromycin or 0.1 µg/mL penicillin-G to select *S. mutans* or *Aa*.

Co-culture of comC or comE mutant *S. mutans* strain with *S. mitis*

We speculated that the *S. mutans* biofilms increases its tolerance to CHX through bacteriocin or quorum-sensing system. *S. mutans* bacteriocin is called as mutacin and its genes are regulated by *comE*. *S. mutans* quorum-sensing system is mediated by competence stimulating peptide (CSP) and CSP is encoded by *comC*.

S. mutans strains mutant for these genes were co-cultured with *S. mitis* and the bilayer structure of the biofilm was analyzed by microscopy.

4. Results

We confirmed that the *ldh* promoter was a constitutive promoter by western blotting; this constructed strain showed no major change in growth at log phase in the liquid culture (data not shown). The CLSM image of ZsGreen-tagged *S. mutans* stained with nucleic acid-staining dye SYTO 59 completely merged with the image showing the green fluorescence of the cells, indicating that virtually the entire population of *S. mutans* expressed the desired fluorescent protein in the bottom cells of the thick biofilm on the HA disks. Mono-culture of *S. mutans* exhibited thin biofilm formation on the HA disks under the present experimental conditions. A few dead cells stained by PI (red signals) were observed in the biofilm aggregates. In this mono-culture biofilm, viable cells were 9.6×10^7 CFU. Many cells in the *S. mutans* mono-species biofilm were alive

as per the results of the microscopic and CFU analysis. *S. mitis* single biofilm appeared thick and had uniform overall density. Almost no cells stained by PI were observed. *S. mitis* viable cells were 4.7×10^7 CFU. *S. mutans* mono-culture biofilm on the HA disks had more number of viable cells than *S. mitis* mono-culture biofilm, even though *S. mutans* biofilm was thinner. *Aa* formed the thinnest biofilm in single culture biofilm and some dead cells were shown to exist in this biofilm by microscopy. *Aa* mono-culture biofilm had 6.1×10^6 CFU and *Aa* biofilm had the fewest viable cells in a single culture biofilm. *L. casei* mono-culture also exhibited thick and flat biofilm formation similar to that of *S. mitis*. Only a few cells were stained by PI in this biofilm using microscopy. This biofilm had 1.1×10^8 alive cells. Among the single culture biofilms of this experiment grown under the above-mentioned conditions, *L. casei* mono-culture biofilm had the highest number of living cells.

When *S. mutans* was co-cultured with *S. mitis*, the dual-species biofilm was flat and had increased in thickness. Moreover, cross-sectional view of the dual-species biofilm revealed that *S. mutans* occupied the bottom layer of the biofilm. In contrast, PI-stained cells were observed in the upper layer of the biofilm. This indicates that cells in the upper layer of the biofilm were dead cells. Further, the biofilms on HA discs were subjected to a CFU assay. Biofilms grown in BHI were disrupted thorough pipetting and incubated anaerobically on BHI agar plates. As an erythromycin resistant gene was inserted into the *S. mutans* genome together with *ZsGreen*, erythromycin (10 µg/mL) was added to distinguish *S. mutans* from *S. mitis* and was compared with BHI plates without erythromycin. The plates were incubated at 37°C in anaerobic conditions for 48 h. This assay confirmed that most of the living cells in the biofilm were *S. mutans*. Thus,

S. mutans and *S. mitis* dual-species biofilm showed a layered structure and live *S. mutans* cells were completely covered by dead cells. When *S. mutans* was co-cultured with *Aa*, there was little effect on the biofilm thickness. The population of dead cells slightly increased when compared with those observed in the *S. mutans* mono-biofilm. Although co-culture with *S. mutans* drastically (10^{-5}) decreased the CFU of *Aa* cells compared with the monoculture of *Aa*, few *Aa* survived in this dual-species biofilm; thus, it can be considered that *S. mutans* coexists with *Aa* in the dual-species biofilm. Co-culture of *S. mutans* with *L. casei* showed decreased cell density in the biofilm aggregates, compared with that of the mono-species biofilm. Our results demonstrated that *S. mutans* cells were the most viable cells in all the combinations of dual-species biofilms and the localization of *S. mutans* within the biofilms were altered depending on the combination.

Next, we investigated whether the co-culture of the oral bacteria affects the phenotypic properties of *S. mutans*, such as chemical tolerance. Confocal images of biofilms and CFU assays both showed that most of the cells in *S. mutans* mono-biofilm were dead after CHX treatment and only a few *S. mutans* cells could survive. *S. mitis* and *L. casei* mono-biofilms which were dipped in CHX had more dead cells (stained by PI) as observed by microscopy. However, *S. mitis* and *L. casei* mono-biofilms had almost the same number of living cells in the CFU assay. *S. mitis* and *L. casei* mono-biofilms had strong tolerance to CHX. *S. mitis* and *L. casei* mono-species biofilms were highly tolerant to CHX, which was consistent with the results of a previous study. When *Aa* mono-biofilm was dipped in CHX, almost all the cells were dead. *Aa* cells that could survive in CHX were rare. In contrast, cells in the bottom layer of the *S. mutans*-*S. mitis* dual-species biofilm remained alive after CHX treatment. Notably, dual-species biofilm

co-cultured with *S. mitis* significantly increased *S. mutans* viability after CHX treatment. Similarly, CHX tolerance was significantly increased in the *S. mutans*-*Aa* dual-species biofilm. Co-culture with *L. casei* did not affect the CHX tolerance of *S. mutans*. *ComE* mutant *S. mutans* -*S. mitis* co-cultured biofilm showed bilayered structure like *S. mutans* -*S. mitis* co-culture biofilm. Then, *comC* mutant *S. mutans* -*S. mitis* co-cultured biofilm showed that live and dead cells were co-aggregated in the biofilm.

5. Discussion

We have reported that the majority of the cells that survived were those of *S. mutans* in each co-culture biofilm on the HA disk. Few other cells were also alive in the co-culture as we could observe few cells of different nature on CFU agar. We also observed that *S. mutans* acquired higher tolerance to CHX by co-existing with *S. mitis* or *Aa*. A possible explanation for this increased tolerance of *S. mutans* co-cultured with *S. mitis* is the layered architecture of the dual-species biofilms; the dead cells in the upper layer of the biofilm protect *S. mutans* in the bottom layer from chemical disinfectants. The cells in the upper layer might have been killed due to bacteriocins and/or H₂O₂, known to be produced by *S. mutans* and *S. mitis* group (e.g., *S. mitis*, *S. sanguinis*), respectively. *S. mutans* produces a bacteriocin called mutacin, which is not produced in sufficient amounts to kill other bacteria when in nutrient-rich conditions. The bacteria belonging to the Mitis group produce H₂O₂. Additionally, *S. sanguinis* cannot produce enough H₂O₂ to inhibit the growth of *S. mutans* when in nutrient-rich conditions. We assume the increased tolerance is the result of this phenomenon. Initially, *S. mutans* and *S. mitis* grow together in nutrient-rich conditions. Later, *S. mutans* forms the biofilm and nutrients become limited within the biofilm. Because of the limited nutrition, *S. mutans* produces mutacin and kills *S. mitis* faster as the rate at which it produces mutacin is

faster than the rate at which *S. mitis* produces H₂O₂. We speculate that in nutrient-limited condition, *S. mutans* produces mutacin and forms bilayer structured biofilm with upper layer containing dead cells. We assumed that bacteriocin produced by *S. mutans* inhibited the growth of *S. mitis* and impeded its early colonization. In mono-culture biofilm, *S. mitis* produced more EPS and exhibited higher tolerance to CHX. *S. mutans* present in the bottom layer of the co-culture is protected by *S. mitis* present in the upper layer, which has tolerance to CHX. This is consistent with the previous reports, which states that peripheral cells in a biofilm protects the interior cells from external stresses. We speculated that the dead cells present in the upper layer were not *S. mutans*. Furthermore, we were unable to confirm that the upper layer was *S. mitis* as we could not identify the dead cells; however, *S. mutans* could fluoresce even after death. In addition, other mechanisms that increase the CHX tolerance of *S. mutans* in the dual-species biofilm may exist since the *S. mutans*-*Aa* biofilm did not show such a bilayered structure. *S. mutans* quorum sensing is activated by *Aa*. *S. mutans* and *Aa* dual-species biofilm has twice the biomass of that of single species biofilms. We assumed that this was involved in increasing the tolerance of *S. mutans* and *Aa* dual-species biofilm. The number of viable *Aa* cells was much lower than that of *S. mutans* cells (10^3 vs. 10^7). However, we considered that not all the other bacteria had died and a few cells had survived. These results suggest that *S. mutans* and *Aa*, which are known causative agents of periodontal disease, act synergistically in mixed-species biofilms leading to tooth decay and aggressive periodontal disease. In this study, we also co-cultured *comE* or *comC* mutant *S. mutans* with *S. mitis*. From the results, we suggested that CSP is involved in bilayered structure biofilm. *S. mutans* has two quorum sensing pathways that regulate competence. The first gene to function in the

pathway is *comC*. *ComAB*, *comD*, *comE*, *comR*, and *comS* genes participate in these two quorum sensing pathways. Thus, we predicted one or more of these genes are involved in this characteristic bilayer-structure biofilm formation.

6. Conclusion

Our results suggest that the spatial distribution of bacteria within the multi-species biofilms differs depending on the combination of the bacteria. Moreover, multi-species biofilms largely impact the tolerance of *S. mutans* against disinfectants. Previous studies have reported that living cells in biofilms are important for understanding infectious diseases. We have shown that all bacteria in the biofilm are important regardless of whether they are living or dead cells. As per our results, both bacterial combination and their spatial distribution in the biofilms are important in understanding the infectious diseases. We believe that this knowledge will be useful in advancing the research on biofilm infections. Understanding cariogenic biofilm formation will be helpful in the development of efficient tooth preservation and treatment approaches. We also believe that this information is important and necessary for the treatment of all biofilm infections in this era of multidrug-resistant bacteria.