Actinomyces naeslundii GroEL-dependent initial attachment and biofilm formation in a flow cell system

（GroEL に依存した *Actinomyces naeslundii* の初期付着とフローセル法による
バイオフィルム形成について）

日本大学大学院松戸歯学研究科歯学専攻 [1]
国立感染症研究所　細菌第 1 部第 6 室 [2]
日本大学歯学部　細菌学講座 [3]

荒井　俊明 [1,2]
落合　邦康 [3]
泉福　英信 [2]

（ 指導：近藤　壽郎　教授 ）

Actinomyces naeslundii **GroEL-dependent initial attachment and biofilm**

formation in a flow cell system

Toshiaki Arai[1,2] Kuniyasu Ochiai[3], Hidenobu Senpuku[1]

[1]Department of Bacteriology I, National Institute of Infectious Diseases, Tokyo, Japan, [2]

Department of Maxillofacial Surgery, Nihon University Graduate School of Dentistry at

Matsudo, Chiba, Japan, [3]Departent of Microbiology, Nihon University, School of

Dentistry, Tokyo, Japan

Running title: Initial attachment and biofilm of *A. naeslundii* in flow cell

Corresponding author: Hidenobu Senpuku, Department of Bacteriology, National

Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan

TEL: 03-5285-1111, FAX: 03-5285-1163, E-mail: hsenpuku@nih.go.jp

Word count = 6266 (including legends and references)

Abstract

Actinomyces naeslundii is an early colonizer with important roles in the development of the oral biofilm. The effects of butyric acid, one of short chain fatty acids in *A. naeslundii* biofilm formation was observed using a flow cell system with Tryptic soy broth without dextrose and with 0.25% sucrose (TSB sucrose). Significant biofilms were established involving live and dead cells in TSB sucrose with 60 mM butyric acid but not in concentrations of 6, 30, 40, and 50 mM. Biofilm formation failed in 60 mM sodium butyrate but biofilm level in 60 mM sodium butyrate (pH 4.7) adjusted with hydrochloric acid as 60 mM butyric media (pH 4.7) was similar to biofilm levels in 60 mM butyric acid. Therefore, butyric acid and low pH are required for significant biofilm formation in the flow cell. To determine the mechanism of biofilm formation, we investigated initial *A. naeslundii* colonization in various conditions and effects of anti-GroEL antibody. The initial colonization was observed in the 60 mM butyric acid condition and anti-GroEL antibody inhibited the initial colonization. In conclusion, we established a new biofilm formation model in which butyric acid induces GroEL-dependent initial colonization of *A. naeslundii* resulting in significant biofilm formation in a flow system.

1. Introduction

Actinomyces naeslundii is an early colonizer of the salivary pellicle – coated tooth surfaces and has important roles as a key bacterium in the development of bacterial aggregation and oral biofilm formation (Jordan and Hammond, 1972; Newman and Socransky, 1977). *A. naeslundii* is principally located in the inner part of the supra and sub-gingival dental biofilm where it is detected in the tooth cervical caries lesion (Bowden et al., 1975; Moore et al., 1987; Kilian et al., 1979; Li et al., 2004; Nyvad and Killian, 1987). Dental biofilms represent one of the most complex biofilm communities known consisting of over 700 bacterial species (Kolenbrabder et al., 2006; Aas et al., 2005). Among species in addition to *A. naeslundii, Veiollonella, Lactobacillus, Bifidobacterium, Propionibacterium, Streptococci*, and *Atopobium*, may also play important roles in poly-microbial biofilm formation (Xu and Gunsolley, 2014). In biofilm formation, *A. naeslundii* contributes to adhesion, colonization and aggregation with other bacterium (Kolenbrander, 1988; Palmer et al., 2003; Syed and Loesche, 1987). The ability of *A. naeslundii* to convert sucrose into extracellular homopolymers of fructose and to catabolize these types of polymers is suspected to be a virulence trait that contributes to the initiation and progression of dental caries and periodontal disease (Allen et al., 1990). *A. naeslundii* produces fructosyltransferases (FTFs), or

levansucrases, which make a levan-type polymer, made primarily of β2 - 6 linkages (Allen et al., 1990; Lori et al., 2000). Levan is an important factor in inducing biological activities and is thought to impact the composition and virulence of oral biofilms (Allen et al., 1990; Van der Hoeven et al., 1976). In addition, levan is thought to serve as a strategic carbohydrate for bacteria to survive in the oral cavity (DaCosta et al., 1968). Therefore, *A. naeslundii* is a key player in poly-microbial habitats on the tooth surface.

Two major fimbrial types have been identified in *A. naeslundii* strains that colonize the oral cavity (Yeung et al., 1999; Mishra et al., 2007; Cisar et al., 1997). Fimbraie type I mediates bacterial adherence to salivary proline-rich-proteins that coat the tooth enamel (Wu et al., 2011). In contrast, type Ⅱ exhibit a lectin activity that was initially detected by the lactose-sensitive coaggregation of *A. naeslundii* strains with several streptococcal strains such as *Streptcoccous oralis* that also colonize teeth. Type Ⅱ fimbriae also mediate bacterial adhesion to various host cells.

Butyric acid is one of the short chain fatty acids (SCFAs). It is secreted extracellularly as a product of metabolism by Gram negative anaerobes e.g., *Porphyromonas gingivalis* and *Fusobacterium nucleatum*. These bacteria contribute to the pathogenesis of periodontal disease where high concentrations of butyric acid (2.6 ± 0.4 mM and 0.2 ± 0.04 mM in severe and mild periodontal disease subjects,

4

respectively) in the periodontal pockets was demonstrated by Niederman *et al* (1997).

SCFAs are at undetectable levels in healthy gingival sulci. The fatty acid-secreting

bacteria are present in lesions of both dental caries and periodontitis and likely

contribute to the biology of oral biofilms. Recently, we reported that one of the SCFAs,

butyric acid increased biofilm formation of *A. naeslundii* in 96 well microtiter plates

using Tryptic soy broth without dextrose (TSB) with 0.25% sucrose (Yoneda et al.,

2013). 6.25 mM butyric acid increased the *A. naeslundii* biofilm formation in 96-well

microtiter plates without flow and GroEL played an important role in biofilm formation.

However, this assay did not control pH in the culture medium during bacterial growth

because *A. naeslundii* produced lactic acid using fermentation of sugar. pH in the

primary culture goes down during culture and the low pH stresses bacterial cells. The

stress may affect biofilm formation. Therefore, to understand biofilm formation using a

dynamic system for *A. naeslundii* culture, a flow cell system was developed that can

control medium pH and provide continuously fresh medium (Heydorn et al., 2000;

Motegi et al., 2006; Yoneda et al., 2013). A conditioning study for biofilm formation

assay using butyric acid was also performed where we found that significant biofilm

was established in 60 mM butyric acid. We studied the mechanisms to determine how

the induction of biofilm occurs; and the induction of initial attachment of *A. naeslundii*

using butyric acid may be associated with biofilm formation. We found that GroEL

contributes to the initial attachment and colonization, and butyric acid and low pH were

important for initial attachment and biofilm formation. These results provide a new

biofilm assay system and a biofilm formation mechanism for *A. naeslundii.*

2. Materials and methods

2.1. Bacterial strains and culture

A. naeslundii X600 was grown in an aerobic atmosphere of 5% CO_2 , 75% N_2,

and 20% O_2 (GasPack CO_2, Becton/Dickinson, Sparks, MD) in Brain Heart Infusion

broth (BHI, Difco Laboratories, Detroit, MI) at 37°C prior to inoculation into the flow

cell system or 6 well plates (Corning Incorporated, New York, NY). Bacterial growth of

A. naeslundii was measured continuously as absorbance at 600 nm at 0 - 28 h after

inoculation into BHI or Tryptic soy broth without dextrose (TSB, Difco Laboratories)

with 0.25% sucrose with or without various concentrations of butyric acid: 0, 6, 10, 20,

30, 40, 50 and 60 mM. Various pH conditions (4.0, 4.5, 5.0, 5.5, 6.0, 6.5 and 7.0) were

adjusted using hydrochloric acid (HCl, Wako chemical, Tokyo, Japan) and were used in

the growth assay in the above medium. Growth was compared adding various

concentrations of butyric acid.

2.2. Biofilm formation in the flow cell system

Biofilm formation of *A. naeslundii* was assayed using the method described previously (Motegi et al., 2006) with individual channel dimensions of $1 \times 4 \times 40$ mm. A three channel flow-cell system (Stoval Howcell; Stovall Life Science Inc., Greensboro, NC) was assembled and prepared. To supply the cell suspension to the substratum consisting of a microscope glass coverslip and the flow cells, an overnight culture of *A. naeslundii* in BHI was washed with sterile PBS, diluted and adjusted to A_{600} OD=0.4 using fresh TSB with 0.25% sucrose. The diluted cell suspension (350 µl) was applied to the overturned flow cell and cultured without flow for 3 h at 37°C in an aerobic atmosphere. The overturned flow cells were returned and flow began using TSB with 0.25% sucrose or TSB with 0.25% sucrose with or without butyric acid (30, 40, 50 and 60 mM). The medium was pumped through the flow cells at a constant rate of 3 ml/h for 48 h using a peristaltic pump (Ismatec; IDEX Corp-Glattbrugg-Zürich, Switzerland). As a control medium, low pH culture medium was prepared using HCl adjusted to the same pH in TSB with 0.25% sucrose and added with 30, 40, 50 and 60 mM butyric acid.

2.3. Assessment of biofilms

Confocal laser scanning microscopy (LSM510 or LSM700, CLSM, Zeiss, Jena, Germany) was used to assess biofilm formation levels of live and dead cells. Biofilms were labeled in situ using two fluorescent probes; LIVE/DEAD BacLight Bacterial viability kit (Molecular Probes, Eugene, OR, USA) consisting of a two nucleic acid-binding stain mixture: Syto 9 and propidium iodide. Syto 9 stains all viable bacteria green, while propidium iodide stains bacteria red whose membranes were damaged (non-viable bacteria). After 48 hours of bacterial colonization, the non-adhering cells were removed by washing once with distilled water. A mixed solution of Syto 9 and propidium iodide was injected into the flow cell chambers and incubated with the biofilm in the dark for 30 min. The biofilm cells were washed using sterile distilled water and assessed using CLSM. Confocal images were photographed with a 63 × immersion oil objective and ZEN software (Zeiss). Confocal microscopy acquisition parameters (pinhole, detector and amplifier gain, amplifier offset filters) were set using reference samples and were kept constant in the acquisition of all the remaining images. CLSM images were acquired using an argon laser at 488 and a HeNe-G laser at 555 nm. Experiments were performed in duplicate. Five fields of view were analyzed in each sample.

The biofilm was photographed using 2 - 5 image stacks taken at different locations in the biofilm in 0.38-μm z-step increments. Images were digitally reconstructed with ZEN. Biofilm structure was quantified using the confocal z stacks and the image analysis software package COMSTAT (Technical University of Denmark, Lyngby, Denmark).

2.4. Cell initial attachment and micro colonization on the culture plate

Overnight cultured cells of *A. naeslundii* in BHI were washed with sterile PBS, diluted and adjusted at A_{600} to OD = 0.4 using fresh TSB with 0.25% sucrose. Three hundred fifty microliter of the *A. naeslundii* cell suspension was mixed into 2650 μl of TSB with 0.25% sucrose, and applied into a six well plate. This culture was incubated at 37°C for 3 h in an aerobic atmosphere of 5% CO_2, 75% N_2, and 20% O_2. After 3 h cultivation, the supernatant was removed and the wells were rinsed once with sterile PBS. Then, we added 3 ml of fresh medium with 60 mM butyric acid, 6 mM butyric acid (pH 6.7), 60 mM butyrate, 60 mM butyrate (pH 4.7), HCl (pH 4.7) or 6 mM butyric acid + lactic acid (pH 4.7), respectively. After 1 h culture in an aerobic atmosphere of 5% CO_2, 75% N_2, and 20% O_2, we removed the culture supernatant; the attached and colonized cells were rinsed with PBS to remove non-adherent cells. To

9

assess the level of attached and colonized cells, the cells were stained with the LIVE/DEAD BacLight Bacterial viability kit. The cell attachment and colonization were visually observed by CLSM images. Parameter set was performed in the same method as a biofilm analysis. Confocal images were photographed with a 10 × objective and ZEN. Experiments were performed in duplicate. Five fields of view were analyzed in each sample.

To determine the mechanism of attachment and colonization, we added diluted 1/2000, 1/3000, 1/4000, 1/8000 and 1/10000 anti-GroEL polyclonal rabbit antibodies (MBL: Medical & Biological Laboratories Co., Ltd., Nagoya, Japan) to both the first culture medium and second culture medium. Anti-glucan binding protein C (gbpC) polyclonal rabbit antibody was used as a control (provided by Dr. Yutaka Sato, Tokyo Dental University). Further, the fructanase mixture (Megazyme, Wicklow, Ireland) that included exo-inulinase and endo-inulinase, was added to both the first culture medium and second culture medium. We therefore determined the effects of FruA on the digestion of fructan to fructose, since FruA digests sucrose to glucose and fructose (Ogawa et al., 2011). The effects of antibodies and FruA were assessed through staining with the LIVE/DEAD BacLight Bacterial viability kit and visual observation using CLSM.

2.5. Statistical analysis

Comparison of the biomass using COMSTAT among concentrations of butyric acid in TSB with 0.25% sucrose was performed using the student's *t*-test using Stat View (version 4.5).

3. Results

3.1. Butyric acid effects on growth

To determine if butyric acid affects bacterial growth, BHI or TSB with 0.25% sucrose and with various concentrations of butyric acid were used in the *A. naeslundii* growth assay. As a control, the medium adjusted with HCl to various low pHs was also used as a primary culture medium for the growth assay. Because low pH gives stress to biofilm cells it may trigger stress response in biofilm cells. Butyric acid inhibited the growth of planktonic cells in a dose-dependent manner in BHI (Fig. 1A). The growth was completely inhibited in 30, 40, 50 and 60 mM butyric acid. In the control growth assay using low pH, the growth was also inhibited in a low pH-dependent manner and completely inhibited at pH 5.0, 4.5 and 4.0 (Fig. 1B). The medium with 30 mM butyric acid at pH 5.5 grew about 40% of the cells to control (Fig. 1). Butyric acid may exert

11

other effects other than lowering the pH. Similar data were obtained in TSB with 0.25%

sucrose (data not shown).

3.2. Butyric acid effects on biofilm formation

TSB with 0.25% sucrose containing 60 mM butyric acid (60 mM butyric acid

medium; pH 4.7) induced significant biofilm (Fig. 2A). To determine the contribution of

low pH in biofilm formation, TSB with 0.25% sucrose containing 60 mM butyrate (60

mM butyrate medium, pH 7.0) and 60 mM butyrate medium adjusted from pH 7.0 to

pH 4.7 using HCl were used for biofilm formation assay. The 60 mM butyrate medium

at pH 7.0 did not form a biofilm but did when the pH was adjusted to pH 4.7 (Fig. 2B

and C). In contrast, the 60 mM butyric acid medium adjusted to pH 7.0 using NaOH (60

mM butyric acid medium, pH 7.0) induced sparse biofilm (Fig. 2D). No butyric acid

and 6 mM butyric acid were used as controls in the assay whereas no significant biofilm

formation was induced (data not shown).

We next compared biofilm formation levels visualizing live and dead cells

using 60 mM butyric acid medium and 60 mM butyrate medium (pH 4.7). Cell

populations with high levels of live cells were observed in both 60 mM butyric acid

medium and 60 mM butyrate (pH 4.7) (Fig. 3B, D). However, there were more dead

cells in the 60 mM butyric acid medium than in the 60 mM butyrate medium (pH 4.7) (Fig. 3A, C). The proportion of live and dead cells in 60 mM butyric acid medium was slightly different compared to the 60 mM butyrate medium, pH 4.7.

To determine the pH biofilm formation effects with butyric acid and low pH, mediums were adjusted using HCl and compared. At pHs 5.5, 5.0, 4.9, and 4.7, biofilm formation was identical in butyric acid media at 30, 40, 50 and 60 mM, respectively. To measure quantitatively the volume (biomass) of the live and dead cells in the biofilms, a COMSTAT analysis was performed. The amounts of live and dead cells were significantly larger in 60 mM butyric acid than other concentrations of butyric acid (Fig. 4). In contrast, the amount of live cells slightly increased at pH 5.0 and pH 4.9 in comparison to pH 5.5 but decreased in the pH 4.7 medium. A higher level was kept in the pH 4.7 medium as compared with pH 5.5. The amount of dead cells slightly increased in a low pH − dependent manner. The amount of live cells compared to dead cells was larger in 60 mM butyric acid mediums than other conditions. Therefore, both butyric acid and low pH are required for significant *A. naeslundii* biofilm formation consisting of both live and dead cells.

3.3. Butyric acid effects on initial attachment and colonization

The initial attachment and colonization of *A. naeslundii* are very important for biofilm formation in the flow cell system because planktonic cells are lost in the flow. The initial incubation for 3 h without flow and 1 h after flow began was used for the assay of attachment and colonization. To observe the initial attachment and the colonization of *A. naeslundii*, the assay without flow was performed because flow is not a big contributor in reducing the pH during the first 3 h of culture without flow and 1 hour after flow starts. This was easily observed using the six well plates. To observe if butyric acid affects attachment and the colonization of *A. naeslundii*, various media containing butyric acid and butyrate were used. The initial attachment and colonization of *A. naeslundii* in 60 mM butyric acid were observed at the edge of the bottom of the six well plates (Fig. 5). However, 60 mM butyric acid, pH 7.0 and pH 4.7 medium adjusted by HCl slightly induced the attachment and initial colonization. Butyric acid at 6 mM and sodium butyrate (pH 7.0) failed to show initial attachment and colonization. Lactic acid pH 4.7 was used as another acid and induced slight attachment and colonization (Fig. 5). A mixture of 6 mM butyric acid and lactic acid, pH 4.7 induced significant initial attachment and colonization but showed lower levels than 60 mM butyric acid alone.

We previously reported GroEL contributed to adhesion of *A. naeslundii* and biofilm formation (Yoneda et al., 2013). Anti-GroEL antibody was applied in the flow assay at 1/2000, 1/3000 and 1/4000 wherein anti-GroEL antibody inhibited cell attachment and colonization in comparison with control (Fig. 6). Control; anti-gbpC antibody did not inhibit initial attachment and colonization (data not shown). FruA digests fructan to fructose and sucrose to glucose and fructose. After treatment by FruA in TSB with sucrose, sucrose doesn't function as a substrate to produce polysaccharide. FruA was applied in the assay and cell attachment and colonization were observed. However, FruA slightly increased attachment and colonization rather than inhibition of attachment and colonization (Fig. 6).

4. Discussion

In our previous study, 6 mM butyric acid stimulated biofilm formation with *A. naesludii* without flow using 96 well micro titer plates (Yoneda et al., 2013). However, using flow and 6 mM butyric acid failed to produce biofilm formation. We speculated that the pH, with using flow was maintained at 6.7. In contrast, without flow, lactic acid is produced when sugar is metabolized. After the incubation, the pH was less than 5.0. Therefore, low pH is important for biofilm formation. Media containing 60 mM butyric

acid at pH 4.7 in the primary culture medium showed significant biofilm formation in the flow cell system (Fig. 2A). However, unconditioned media at pH 4.7 adjusted using HCl did not show similar levels of biofilm formation with 60 mM butyric acid in the media conditioned to 4.7 (Fig. 4). Further, the pH 4.7 medium with 60 mM butyrate adjusted using HCl showed significant biofilm but biofilm formation in the 60 mM butyric acid culture disappeared by adding NaOH adjusting to pH 7.0 in the primary medium (Fig. 2). Taken together, the data suggests that both butyric acid and low pH are required to form significant biofilms in the flow cell system.

Previous investigators used 25% saliva solution in the flow cell system to observe single and mix species biofilm formation with early oral colonizers (Foster et al., 2004; Periasamy et al., 2009). This method shows usual attachment, aggregation and biofilm formation without extra nutrients such as sugar. Using a single inoculation of *A. naeslundii* showed significant bio-volume of biofilm formation after 14 or 18 h incubation in flow cell experiments. Here we found that a nutrient rich medium including sucrose was used as a substrate to produce levan type polysaccharides and activate biofilm formation (Bergeron et al., 2000). Levan is an important factor to induce the composition and virulence of oral biofilms (Allen et al., 1990; Hoeven et al., 1976). However, in control conditions without butyric acid, butyrate, or pH adjustment,

significant biofilm formation was not observed. After 48 h, more aggregated cells were observed inside the flow cells and were removed by washing with DW. Therefore, the aggregated cells produced by levan may not attach without biofilm formation. In the 6 well plate assay, treatment with FruA in the medium digests sucrose, which supports for the initial attachment, colonization and biofilm formation, to both glucose and fructose. This suggests that the synthesis of levan is not required for the initial attachment and colonization but may play a later role for cell to cell communication in biofilm formation.

Ho et al. (2013) report low pH increased biofilm formation in group B *Streptococcus* (GBS) in nutrient-limited medium. Other investigators report GBS produced a greater amount of biofilm at low pH (Yang et al., 2012; D'Urzo et al., 2014). Biofilm formation with *Streptococcus intermedius* and *Streptococcus pyogenes* was also enhanced at low pH (Ahmed et al., 2008; Manetti et al., 2012). Therefore it is commonly recognized that low pH is a stimulator of biofilm cells and increased biofilm formation in various streptococci. *A. naeslundii* may be similar to streptococci in terms of the effects of low pH on biofilm formation. Low pH modulates the expression of a large number of proteins in GBS including the proteins associated with surface adhesion (Park et al., 2012) and stress protein family members (Yang et al., 2012). Paddick *et al.*

reported that 76 proteins including adhesins, chaperones, and stress-response proteins were significantly up-regulated in biofilm cells relative to planktonic cells of *A. naeslundii* (Paddick et al., 2006). D'Urzo *et al.* suggest an acidic pH may be important in unmasking surface-associated proteins that promote adhesion by biofilm-forming bacterial strain (D'Urzo et al., 2014). These reports suggest that low pH is key for enhanced expression of adhesins on the cells and biofilm formation. However, with *A. naeslundii*, butyric acid is required for biofilm formation in addition to low pH because the contribution to biofilm formation was higher in the butyric acid than with HCl. Other unknown effects of butyric acid may induce the cell status potential for biofilm formation.

In the flow cell system, early adhesion and colony formation are important using pre-culture without flow for 3 h. The initial attachment and colonization of *A. naeslundii* in 60 mM butyric acid were observed at the edge bottom of the six well plates. In the flow cell system, we believe that *A. naeslundii* adherence and colonization in the pre-culture are a trigger for biofilm formation. At 60 mM butyric acid inhibited cell growth but induced initial attachment, colonization and biofilm formation. The attached and colonized cells have resistance to low pH where butyric acid and low pH induced more adhesion and colony formation with the bacterial cells.

We found that *A. naeslundii* GroEL contributed to biofilm formation in a previous study (Yoneda et al., 2013). Anti-GroEL antibody clearly inhibited initial attachment and colonization. Therefore, GroEL with butyric acid and low pH-dependent cell attachment stimulates biofilm formation. In previous reports, the GroEL homologue, Hsp60, an essential heat shock protein, is expressed on the surface of virulent *Legionella pneumophila* (Garduńo et al., 1998). The GroEL of *Haemophylus ducreyi* is responsible for the attachment of *H. ducreyi* to carbohydrate receptors (Pantzar et al., 2006). Therefore, the associated chaperon GroEL concentration during stress conditions act as an adhesin (Garduńo et al., 1998; Hennequin et al., 2001). A previous report suggests that chaperones and stress proteins may act as adhesins when expressed at the cell surface (Lewthwaite et al., 1998). Recently, it was reported that the biofilm supernatants of *Lactobacillus* sp. contained large amounts of GroEL (Rieu et al., 2014). The authors suggest that the secreted GroEL is involved in enhanced pathogenic adhesion activities. These reports support the contribution of GroEL for biofilm formation of *A. naeslundii.* We speculate that butyric acid may be used to express GroEL on the cell surface.

In the supra-gingival plaque at a critical pH of 5.5 or less oral bacteria form biofilms to survive in acidic conditions. The reduced pH involving acidic metabolites

e.g., lactic acid is produced by bacteria in the oral biofilm. According to the ecological plaque hypothesis, if the low pH remains below the "critical pH" for demineralization at 5.5 for extended periods of time, a shift in the bacterial populations to more cariogenic organisms that are acid-producing and acid-tolerant occurs. In contrast, in sub-gingival plaque, it is possible that this environment is caused by butyric acid and lactic acid that increase biofim formation on the tooth surface in various local sub-gingival areas. Then, *A. naeslundii* may form biofilms using the metabolic products from other oral bacteria in the gingival pocket.

Extracellular factors in pathogenic biofilm cell metabolism and the stress response play roles for survival in the interaction between initial colonizers and other bacteria. Our data show a biofilm formation mechanism with butyric acid for the development of pathogenic biofilm formation in a nutrient-rich condition. To our knowledge, this is the first report that shows the role of stress proteins in initial attachment, colonization, and establishes biofilm formation with *A. naeslundii* in a flow cell system. This new model is useful for the future studies of effects by preventive agents on the biofilm formation and the molecular effects by butyric acid on the expression of surface proteins and other acids that may aid understanding cell to cell communication, in bacterial oral flora.

5. Conclusion

Both butyric acid and low pH are required to form significant biofilms of *A. naeslundii* in the flow cell system. Metabolic products such as butyric acid from Gram negative anaerobes induce GroEL-dependent initial attachment and colonization of *A. naeslundii* resulting in significant biofilm formation at low pH in a flow system. This is the first report that shows the role of stress proteins in initial attachment, and colonization, and establishes biofilm formation with *A. naeslundii* in a flow cell system, and a useful new model for investigation of effects by preventive agents on the biofilm formation, and cell to cell communication in bacterial oral flora involving *A. naeslundii*.

References

Aas, J.A., Griffen, A.L., Dardis, S.R., Lee, A.M., Olsen, I., Dewhirst, F.E., Leys, E.J., Paster B.J., 2008. Bacteria of dental caries in primary and permanent teeth in children and young adults. J. Clin. Microbiol. 46, 1407-1417.

Ahmed, N.A., Petersen, F.C., Scheie, A.A., 2008. Biofilm formation and autoinducer-2 signaling in *Streptococcus intermedius*: role of thermal and pH factors. Oral Microbiol. Immunol. 23, 492-497.

Allen, P.Z., Bowen W.H., 1990. Immunochemical studies on levans from several strains of *Actinomyces viscosus*. Arch. Oral Biol. 35, 55–62.

Bergeron, L.J., Morou-Bermudez, E., Burne, A.B., 2000. Characterization of the Fructosyltransferase Gene of *Actinomyces naeslundii* WVU45. J. Bacteriol. 182, 3649-3654

Bowden, G.H.W., Ellwood, D.C., Hamilton, I.R., 1975. Microbial ecology of the oral cavity. In: Alexander, M. (ed.), Advances in microbial ecology. Plenum Press, New York, pp. 135-217.

Cisar, J.O., Takahashi, Y., Ruhl, S., Donkersloot, J.A., Sandberg, A.L., 1997. Specific Inhibitors of Bacterial Adhesion: Observations From the Study of Gram-Positive Bacteria that Initiate Biofilm Formation on the Tooth Surface. Adv. Dent. Res. 11, 1168-1175.

DaCosta, T., Gibbons, R.J., 1968. Hydrolysis of levan by human plaque streptococci. Arch. Oral Biol. 13, 609-617.

D'Urzo, N., Martinelli, M., Pezzicoli, A., De Cesare, V., Pinto, V., Margarit, I., Telford, J.L., Maione, D., Members of the DEVANI Study Group., 2014. Acidic pH strongly enhances *in vitro* biofilm formation by a subset of hypervirulent ST-17 *Streptococcus agalactiae* strains. Appl. Environ. Microbiol. 80, 2176-2185.

Foster, J.S., Kolenbrander, P.E., 2004. Development of a multispecies oral bacterial community in a saliva-conditioned flow cell. Appl. Envirol. Microbiol. 70, 4340-4348.

Garduńo, R.A., Garduńo, E., Hoffman, P.S., 1998. Surface-associated HSP60 chaperonin of *Legionella pneumophila* mediates invasion in a Hela cell model. Infect. Immun. 66, 4602-4610.

Garduńo, R.A., Faulknex, G., Trevors, M.A., Vats, N., Hoffman, P.S., 1998. Immunolocalization of Hsp60 in *Legionella pneumophila*. J. Bacteriol. 180, 505-513.

Hennequin, C., Porcheray, F., Wallgora-Dupriet, A.J. Collignon, A., Barc, M., Bourlioux, P., Karjalainen, T., 2001. GroEL (Hsp 60) of *Clostridium difficile* is involved in cell adherence. Microbiology 147, 87-96.

Heydorn, A., Ersbøll, B. K., Hentzer, M., Parsek, M.R., Givskov, M., Molin, S., 2000. Experimental reproducibility in flow-chamber biofilms. Microbiology 146, 2409-2415.

Heydorn, A., Nielsen, A. T., Hentzer, M., Sternberg, C., Givskov, M., Ersbøll, B.K., Molin, S., 2000. Quantification of biofilm structures by the novel computer program COMSTAT. Microbiology 146, 2395-2407.

Ho, Y.R., Li, C.M., Yu, C.H., Lin, Y.J., Wu, C.M., Harn, I.C., Tang, M.J., Chen, Y.T., Shen, F.C., Lu, C.Y., Tsai, T.C., Wu, J.J., 2013. The enhancement of biofilm formation in group B streptococcal isolates at vaginal pH. Med. Microbiol. Immunol. 202, 105–115.

Jordan, H.V., Hammond, B.F., 1972. Filamentous bacteria isolated from human root surface caries. Arch. Oral Biol. 17, 1333-1342.

Kilian, M., Larsan, M. J., Fejerskov, O., Thylstrup., 1979. Effects of fluoride on the initial colonization of teeth in vivo. Caries Res. 13, 319-329.

Kolenbrander, P.E., 1988. Intergeneric co-aggregation among human oral bacteria and ecology of dental plaque. Annu. Rev. Microbiol. 42, 627-636.

Kolenbrander, P.E., Andersen, R.N., and Moore, L.V., 1990. Intrageneric coaggregation among strains of human oral bacteria: potential role in primary colonization of the tooth surface. Appl. Environ. Microbiol. 56, 3890-3894.

Kolenbrander, P.E., Palmer, R.J.Jr., Rickard, A.H., Jakubovics, N.S., Chalmers, N.I., Diaz, P.I., 2006. Bacterial interactions and successions during plaque development. Periodontal. 2000. 42, 47-79.

Lewthwaite, J., Skinner, A., Henderson, B., 1998. Are molecular chaperones microbial virulence factors? Trends Microbiol. 6, 426-428.

Li, J., Heimerhorst, E.J., Leone, C.W., Yaskell, T., Haffajee, A.D., Socransky, S.S., Oppenheim, F.G., 2004. Identification of early microbial colonizers in human dental biofilm. J. Appl. Microbiol. 97, 1311-1318.

Manetti, A.G., Koller, T., Becherelli, M., Buccato, S., Kreikemeyer, B., Podbielski, A., Grandi, G., Margarit, I., 2010. Environmental acidification drives *S. pyogenes* pilus expression and microcolony formation on epithelial cells in a FCT-dependent manner. PLoS One. 5, e13864.

Mishra, A., Das, A., Cisar, J.O., Ton-That, H., 2007. Sortase-catalyzed assembly of distinct heteromeric fimbriae in *Actinomyces naeslundii*. J Bacteriol. 189, 3156-3165.

Moore, W. E., 1987. Microbiology of periodontal diseases. J. Periodont. Res. 22, 335-341.

Motegi, M., Takagi, Y., Yonezawa, H., Hanada, N., Terajima, J., Watanabe, H., Senpuku, H., 2006. Assessment of genes associated with *Streptococcus mutans* biofilm morphology. Appl. Environ. Microbiol. 72, 6277-6287.

Newman, M.G. and Socransky, S.S., 1977 Predominant cultivable microbiota in periodontosic. J. Periodont. Res. 12**,** 120-128.

Niederman, R., Buyle-Bodin, Y., Lu, B.Y., Robinson, P., Naleway, C., 1997. Short-chain carboxylic acid concentration in human gingival crevicular fluid. J. Dent. Res. 76, 575-679.

Nybad, B., and Killian, M., 1987. Microbiology of the early colonization of human enamel and root surfaces in vivo. Scand. J. Dent. Res. 95, 369-380.

Ogawa, A., Furukawa, S., Fujita, S., Mitobe, J., Kawarai, T., Narisawa, N., Sekizuka, T., Kuroda, M., Ochiai, K., Ogihara, H., Kosono, S., Yoneda, S., Watanabe, H., Morinaga, Y.,Uematsu, H., Senpuku, H., 2011. Inhibition of *Streptococcus mutans* biofilm formation by *Streptococcus salivarius* FruA. Appl. Environ. Microbiol. 77, 1572-1580.

Paddick, J.S., Brailsford, S.R., Rao, S., Soares, R.F., Kidd, E.A., Beighton, D., Homer, K.A., 2006. Effect of biofilm growth on expression of surface proteins of *Actinomyces naeslundii* genospecies 2. Appl. Exp. Microbiol. 72, 3774-3779.

Palmer, R.J.Jr., Gordon, S.M., Cisar, J.O., Kolenbrander, P.E., 2003. Coaggregation-mediated interactions of streptococci and actinomyces detected in initial human dental plaque. J. Bacteriol. 185, 3400-3409.

Pantzar, M., Teneberg, S., Lagergård, T., 2006. Binding of *Haemophilus ducreyi* to carbohydrate receptors is mediated by the 58.5-kDa GroEL heat shock protein. Microbes Infect. 8, 2452-2458.

Park, S.E., Jiang, S., Wessels, M.R., 2012. CsrRS and environmental pH regulate group B *Streptococcus* adherence to human epithelial cells and extracellular matrix. Infect. Immun. 80, 3975–3984.

Periasamy, S., Chalmers, N. I., Du-Thumm, L., Kolenbrander, P.E., 2009. *Fusobacterium nucleatum* ATCC 10953 requires *Actinomyces naeslundii* ATCC 43146 for growth on saliva in a three-species community that includes *Streptococcus oralis* 34. Appl. Environ. Microbiol. 75, 3250-7.

Rieu, A., Aoudia, N., Jego, G., Chluba, J., Yousfi, N., Briandet, R., Deschamps, J., Gasquet, B., Monedero, V., Garrido, C., Guzzo, J., 2014. The biofilm mode of life

boosts the anti-inflammatory properties of *Lactobacillus*. Cell. Microbiol. 16, 1836-1853.

Syed, S.A., Loesche, W.J., 1978. Bacteriology of human experimental gingivitis effect of plaque age. Infect. Immun**.** 21, 821-829.

Van der Hoeven, J. S., Vogels, G. D., Bekkers, M. F., 1976. A levansucrase from *Actinomyces viscosus*. Caries Res. 10, 33-48.

Wu, C., Mishra, A., Yang, J., Cisar, J.O., Das, A., Ton-That, H., 2011. Dual function of a tip fimbrillin of *Actinomyces* in fimbrial assembly and receptor binding. J. Bacteriol. 193, 3197-3206.

Xu, P., Gunsolley, J., 2014 Application of metagenomics in understanding oral health and disease. Virulence 5, 424-432.

Yang, Q., Porter, A.J., Zhang, M., Harrington, D.J., Black, G.W., Sutcliffe, I.C., 2012. The impact of pH and nutrient stress on the growth and survival of *Streptococcus agalactiae*. Antonie Van Leeuwenhoek 102, 277–287.

Yeung, M.K., 1999. Molecular and genetic analyses of *Actinomyces* spp. Crit. Rev. Oral Biol. Med. 10, 120-138.

Yoneda, S., Kawarai, T., Narisawa, N., Tuna, E.B., Sato, N., Tsugane, T., Saeki, Y., Ochiai, K., Senpuku, H., 2013. Effects of short-chain fatty acids on *Actinomyces naeslundii* biofilm formation. Mol. Oral Microbiol. 28, 354-365.

Figure Legends

Figure 1.　Effects of butyric acid and low pH on growth of *A. naeslundii*

A. naeslundii was inoculated in BHI supplemented with various butyric acid concentrations (0, 6, 10, 20, 30, 40, 50 and 60 mM) and incubated. Various pH conditions (4.0, 4.5, 5.0, 5.5, 6.0, 6.5 and 7.0) were prepared with HCl and used for the growth assay as control. Cell growth levels were assessed by absorbance at 600 nm and presented as butyric acid (A) and low pH (B). The data are expressed as the mean ± SD of OD in three independent assays.

Figure 2. Effects of butyric acid on biofilm formation in the flow cell system.

CLSM Images (3D images) of biofilms formed in the presence of 60 mM butyric acid (A), 60mM sodium butyrate pH 4.7 adjusted using HCl (B), 60 mM sodium butyrate pH 7.0 (C) and 60 mM butyric acid pH 7.0 adjusted using NaOH (D) were used for the biofilm formation assay in the flow cell system. Photographs were analyzed

and presented using a confocal laser microscope LSM510. Live and dead cell staining of the biofilm was performed and photographed. CLSM images were captured at 63 × oil objective. Representative data from three independent experiments are presented where similar results were obtained from each experiment. Scale bars are all 25 μm.

Figure 3.   Assessment of biofilm formation levels with live and dead cells.

Comparison of intensities in biofilm live and dead cells stained LIVE/DEAD stain (green: live cells. red: dead cells) was performed with 60 mM butyric acid (A: dead cells, B: live cells) and 60 mM butyrate pH4.7 (C: dead cells, D: live cells). Photographs were analyzed and presented using a confocal laser microscope LSM510. Representative data from three independent experiments are presented where similar results were obtained from each experiment.

Figure 4. Comparison of biofilm biomass between butyric acid media and low pH media.

The biofilm biomass of live cells and dead cells were compared in various concentrations of butyric acid (B) and various pH media adjusted using HCl (A). The butyric acid concentration was adjusted to 30, 40, 50 and 60 mM. pH 4.7, 4.9, 5.0 and

5.5 adjusted using HCl was identical with 30, 40, 50 and 60 mM butyric acid medium, respectively. Photographs were analyzed and presented using a confocal laser microscope LSM700. The biomass was assessed using COMSTAT. The data are expressed as the mean ± SD of the biomass in three (all data excepting 50 mM butyric acid) and four (50 mM butyric acid) independent assays. The asterisks note significantly different biomasses in the biofilm (*: $p < 0.01$, **: <0.05, ***: <0.01 vs 30 mM, 40 mM and 50 mM).

Figure 5. Effects of butyric acid on the initial attachment and colonization.

CLSM Images of initial attachment and colonization were observed in the presence of 6 mM butyric acid, 60 mM butyric acid, 60 mM pH 7.0 adjusted using NaOH, pH 4.7 medium adjusted using HCl, sodium butyrate pH 7.0, sodium butyrate pH 4.7 adjusted using HCl, lactic acid pH 4.7, or 6.0 mM butyric acid pH 4.7 adjusted using lactic acid. Initial attachment and colonization were observed at the edge portion of the bottom of the six well plates. Photographs were analyzed and presented using a confocal laser microscope LSM700. CLSM images were captured at 10 × objective. Representative data from three independent experiments are presented where similar results were obtained from each experiment. Scale bars are all 200 μm.

Figure 6. Effects of anti-GroEL antibody and FruA on initial attachment and colonization.

CLSM Images of initial attachment and colonization were observed in the presence of 60 mM butyric acid medium supplemented with 1/2000, 1/3000 and 1/4000 anti-GroEL antibody, and FruA at 1 and 10 units in six well plates. Initial attachment and colonization were observed at the edge portion of the bottom of the six well plates. Photographs were analyzed and presented using a confocal laser microscope LSM700. CLSM images were captured at 10 × objective. Representative data from three independent experiments are presented where similar results were obtained from each experiment. Scale bars are all 200 μm.

Fig. 1

A

B

25μm

25μm

C

D

25μm

25μm
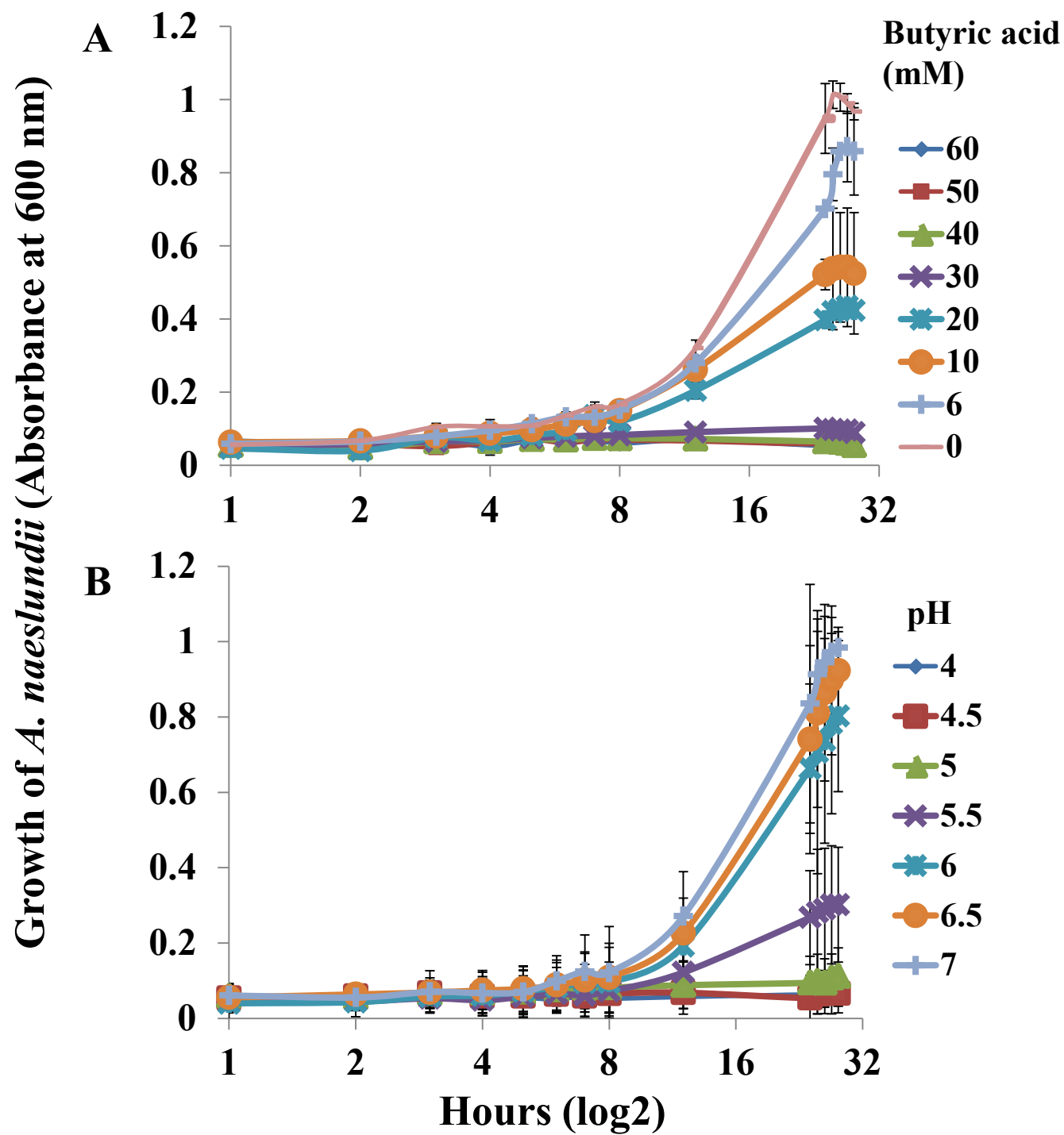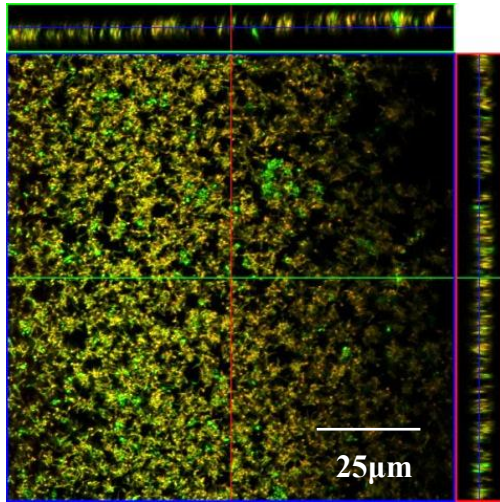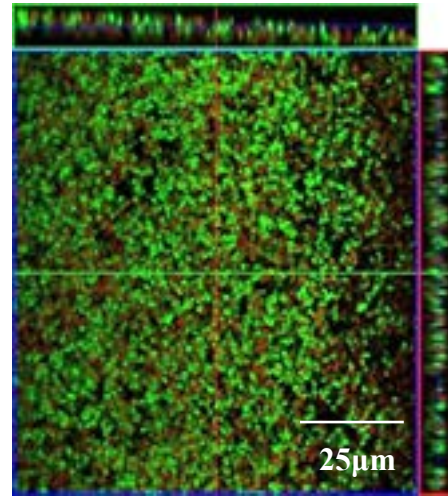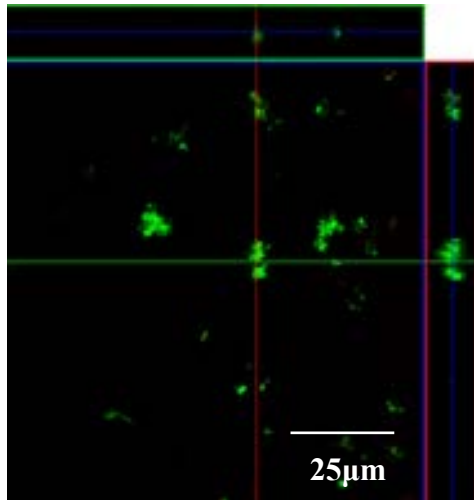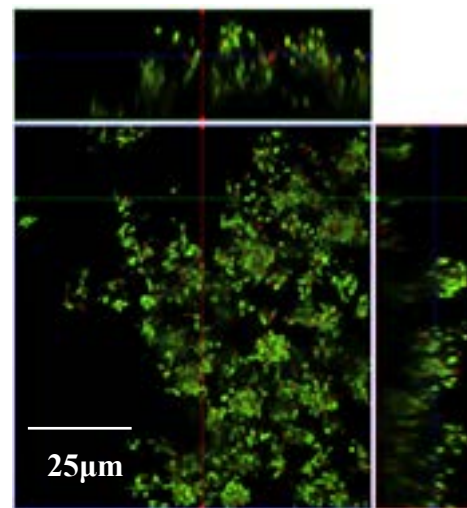
Fig. 2
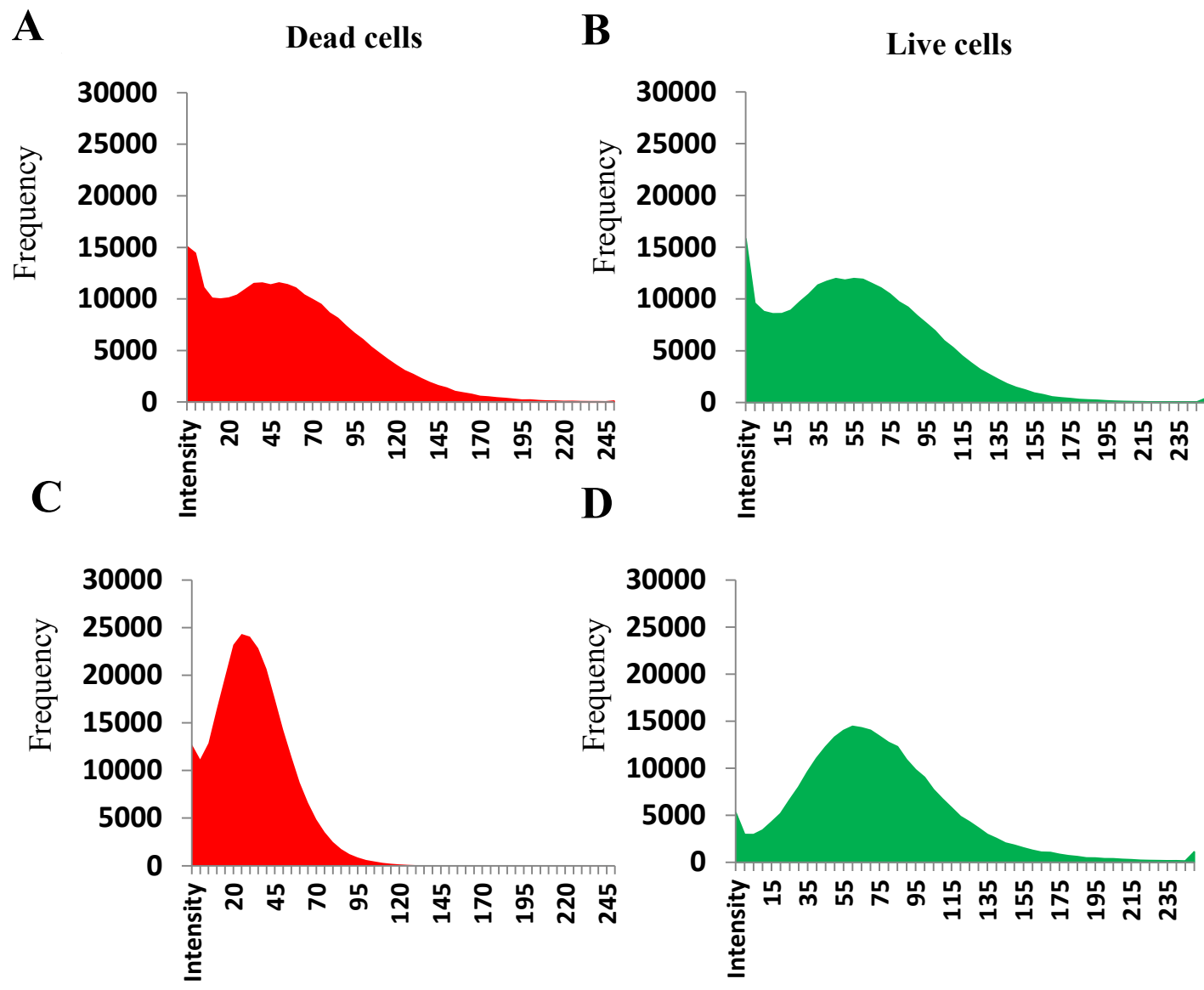
Fig. 3
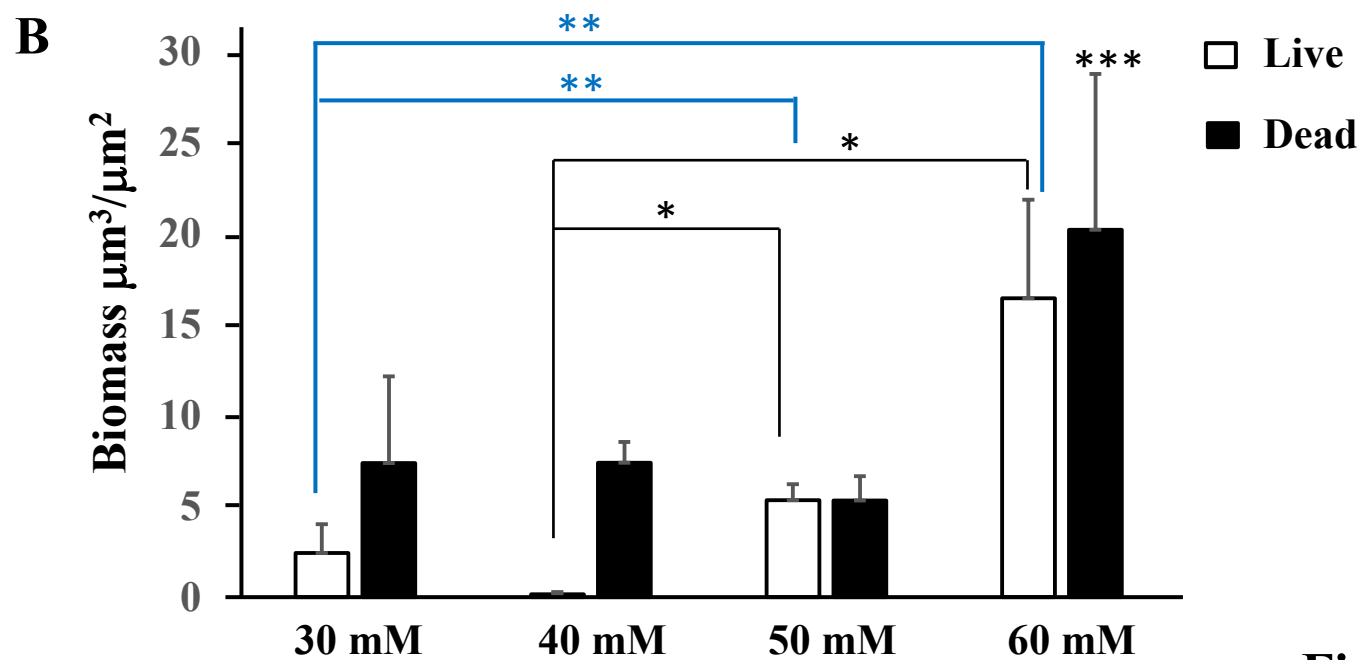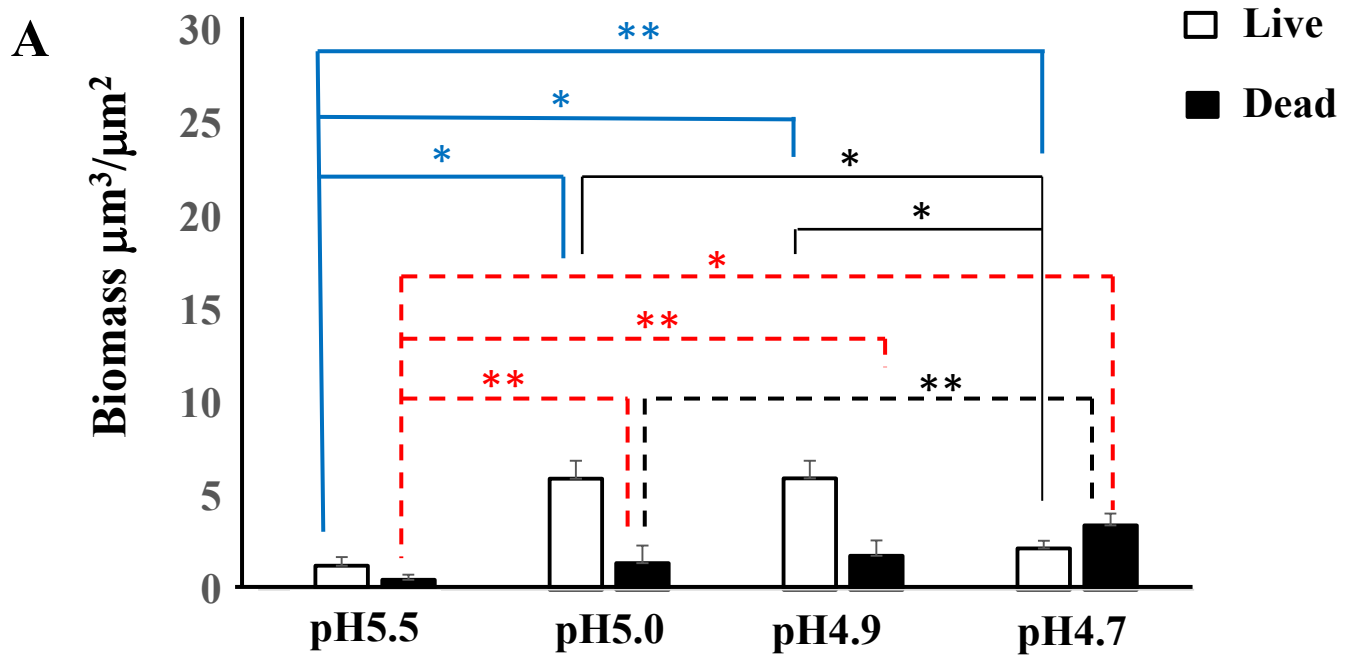
Fig. 4

Butyric acid 6 mM
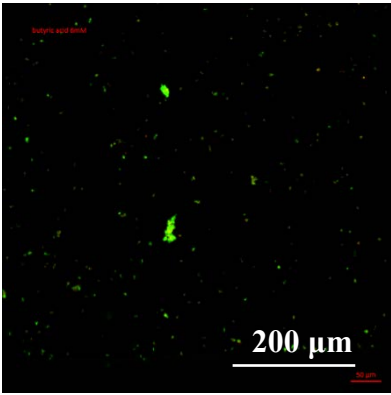
Butyric acid 60 mM
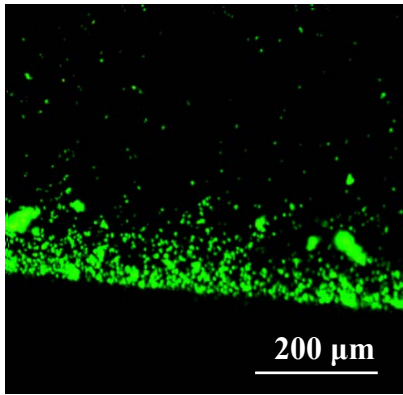
Butyric acid 60 mM + NaOH (pH7.0)
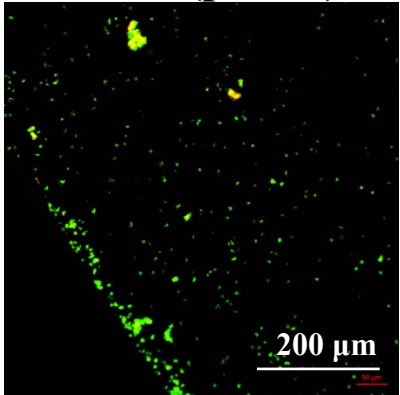
HCl (pH4.7)
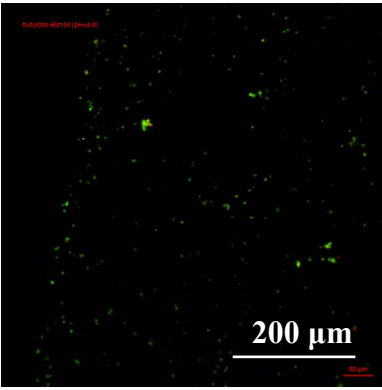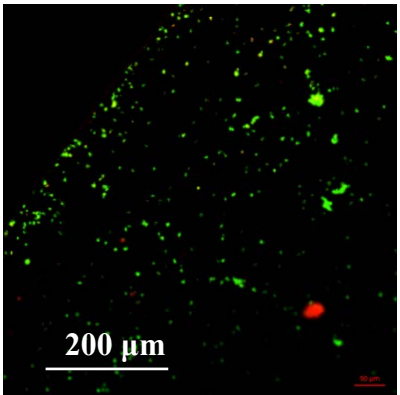
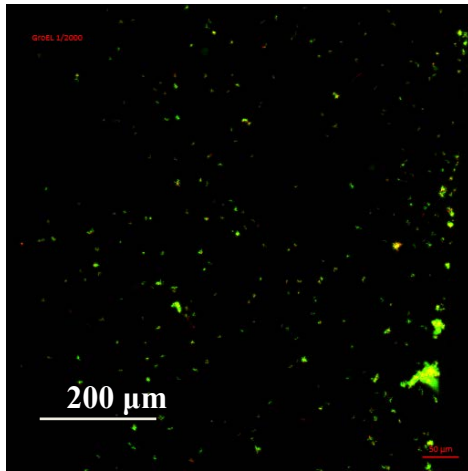**Sodium butyrate 60 mM (pH7.0)**
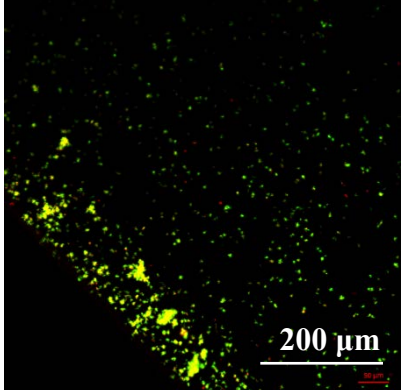
Sodium butyrate 60 mM (pH4.7)
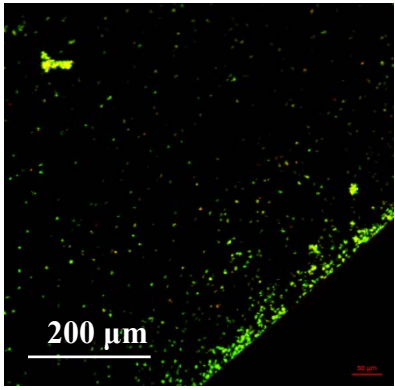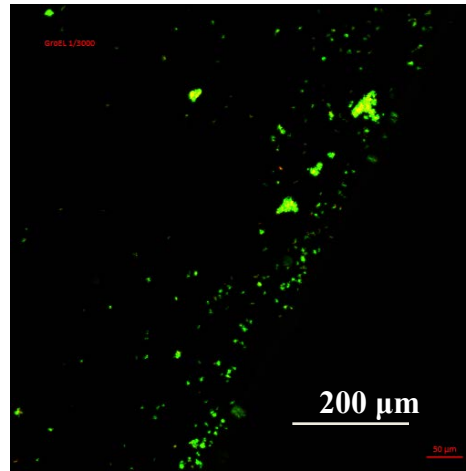
Lactic acid (pH4.7)
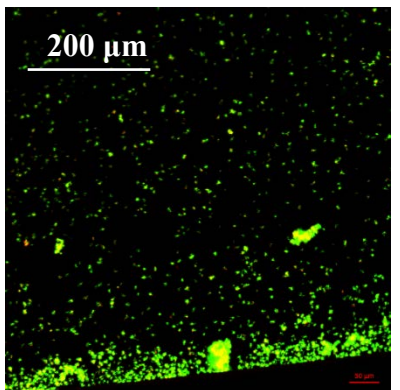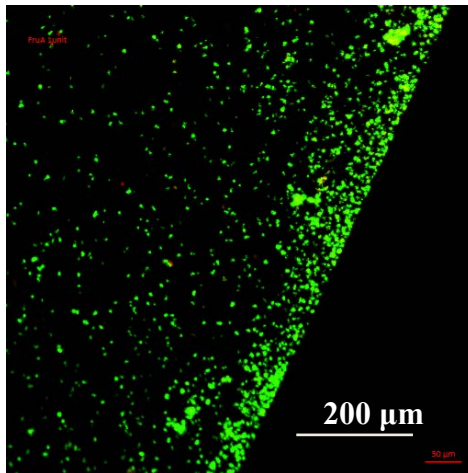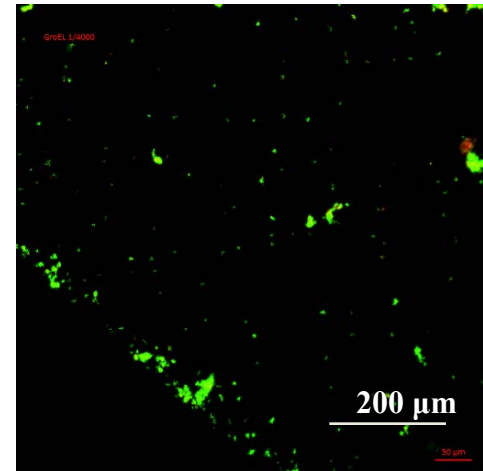
6.0 mM Butyric acid + Lactic acid (pH4.7)
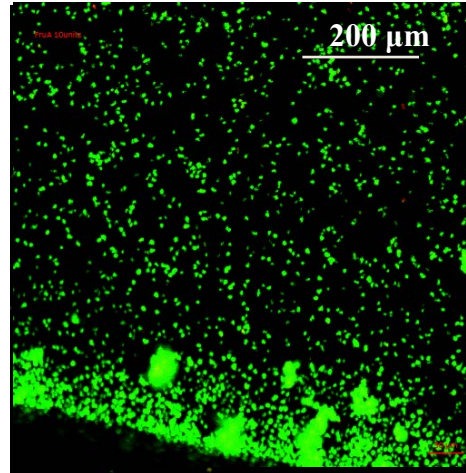
Fig. 5.

1/2000 anti-GroEL antibody

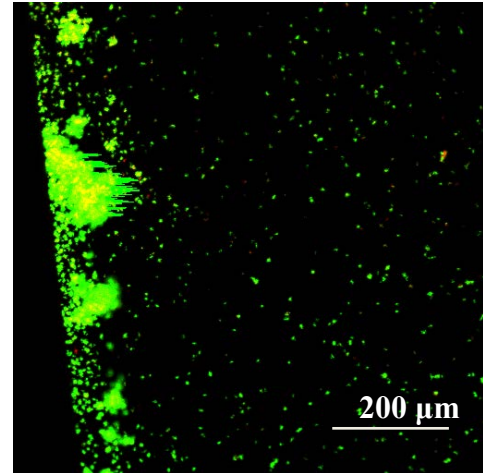**1/3000 anti-GroEL antibody**

1/4000 anti-GroEL antibody

Butyric acid 60 mM + FruA 1 unit

Butyric acid 60 mM + FruA 10 unit

Butyric acid 60 mM

Fig. 6