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Effects of short-chain fatty acids on *Actinomyces naeslundii* biofilm formation

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SUMMARY

***Actinomyces naeslundii* is an early colonizer and has important roles in the development of the oral biofilm. Short-chain fatty acids (SCFA) are secreted extracellularly as a product of metabolism by gram-negative anaerobes, e.g. *Porphyromonas gingivalis* and *Fusobacterium nucleatum*; and the SCFA may affect biofilm development with interaction between *A. naeslundii* and gram-negative bacteria. Our aim was to investigate the effects of SCFA on biofilm formation by *A. naeslundii* and to determine the mechanism. We used the biofilm formation assay in 96-well microtiter plates in tryptic soy broth without dextrose and with 0.25% sucrose using safranin stain of the biofilm monitoring 492 nm absorbance. To determine the mechanism by SCFA, the production of chaperones and stress-response proteins (GrpE and GroEL) in biofilm formation was examined using Western blot fluorescence activity with GrpE and GroEL antibodies. Adding butyric acid (6.25 mM) 0, 6 and 10 h after beginning culture significantly increased biofilm formation by *A. naeslundii*, and upregulation was observed at 16 h. Upregulation was also observed using appropriate concentrations of other SCFA. In the upregulated biofilm, production of GrpE and GroEL was higher where membrane-damaged or dead cells were also observed. The upregulated biofilm was significantly reduced by addition of anti-GroEL antibody. The data suggest biofilm formation by *A. naeslundii* was upregulated dependent on the production of stress proteins, and addition of SCFA increased membrane-damaged or dead cells. Production of GroEL may physically play an important role in biofilm development.**

INTRODUCTION

Dental biofilms are produced by more than 700 phylotypes of bacteria (Kroes *et al.*, 1999; Paster *et al.*, 2001; Aas *et al.*, 2005) at high densities (~10^{11} cell g^{-1} wet weight) (Hamilton, 2000). Many of these oral species interact with each other (Kolenbrander *et al.*, 2002) using cell–cell communication between the bacteria. Further, co-adhesion as a result of the interaction between planktonic cells and colonization of surface-attached cells are two important processes in the development of biofilms in the oral cavity (Gibbons & Nygaard, 1970; Ciardi *et al.*, 1987; Kolenbrander *et al.*, 2002; Li *et al.*, 2004). Aggregation of two or more bacteria and the production of extracellular polysaccharides are significant

indicators of biofilm maturation in dental biofilm development (Palmer *et al.*, 2003; Kolenbrander *et al.*, 2006).

Oral streptococci are prominent during the initial stage of biofilm formation on teeth and form a co-aggregation with other species of oral bacteria (Nyvad & Kilian, 1987, 1990; Li *et al.*, 2004**)**. *Actinomyces* spp. as well as streptococci are dominant dental plaque bacteria; they are early colonizers attaching to the saliva-coated pellicle on the tooth surface and possess important roles in the development of the biofilm (Li *et al.*, 2004; Kolenbrander *et al.*, 2005). The bacteria co-interact and provide a foundation for the subsequent attachment and growth of other bacteria that form close metabolic relationships with streptococci (Jenkinson & Lamont, 2005; Kolenbrander *et al.*, 2006). In recent studies, *Actinomyces naeslundii* constituted up to 18% of the microbiota within the first days of dental biofilm formation, with a notable decrease over a 7-day observation period (Al-Ahmad *et al.*, 2007). *Actinomyces naeslundii* was located mainly in the inner part of the multilayered biofilm, indicating that it is one of the species that attaches directly to the pellicle (Dige *et al.*, 2009). The participation of *A. naeslundii* in the initial stages of dental biofilm formation may have important oral ecology consequences.

Periodontal disease is initiated by a microbial biofilm that covers the surface of the teeth at the subgingival sulci in the subgingival pocket where infectious, chronic, destructive disease occurs in the periodontal tissues (Dzink *et al.*, 1988; Moore *et al.*, 1991; Lamont & Yilmaz, 2002). Various studies show *Porphyromonas*, *Prevotella* and *Fusobacterium* spp. are contributing to the pathogenesis of periodontal disease. They form multi-layered bacterial biofilms and spread onto the surface of the teeth from the periodontal pocket. Such biofilms are developed using various supports, e.g. chemically conditioned substances, that allow the bacteria to firmly adhere to the surface and produce macromolecular extracellular substances. The respective metabolisms of these bacteria are important; and are characterized by the production of identifiable fingerprints of short-chain fatty acids (SCFA: butyric acid, propionic acid, valeric acid, acetic acid, etc.) that are the major products of their anaerobic metabolism and are released into the micro-environment (Gorbach *et al.*, 1976). The SCFA species (mostly butyric acid) of extracellularly secreted metabolites from gram-negative bacteria such as *Porphyromonas gingivalis* and *Fusobacterium nucleatum* may be involved in periodontal diseases (Kurita-Ochiai *et al.*, 1995; Socransky *et al.*, 2002; Colombo *et al.*, 2009) where high concentrations of butyric acid (2.6 ± 0.4 mM and 0.2 ± 0.04 mM in severe and mild periodontal disease subjects, respectively), and propionic acid (9.5 ± 1.8 mM and 0.8 ± 0.3 mM in severe and mild periodontal disease subjects, respectively) in the periodontal pockets was demonstrated by Niederman *et al.* (1997). SCFA are at undetectable levels in healthy gingival sulci (Niederman *et al.*, 1997).

Butyric acid is known to decrease virulence gene expression and invasion of *Salmonella* in epithelial cells *in vitro* (Lawhon *et al.*, 2002; Van Immerseel *et al.*, 2004). Butyric acid inhibits germination of *Candida albicans* (Noverr & Huffnagle, 2004). Therefore, the fatty acid in the local environment can influence the activities of microorganisms. In particular, in subgingival plaque formation around the periodontal pocket from patients with periodontal disease, SCFA may stimulate re-colonization and aggregation of oral bacteria. The local fatty acid environment in the oral cavity that influences the initial colonizers such as *Streptococci* and *A. naeslundii* to form biofilm has not been examined. Therefore, our aim was to investigate the effects of SCFA on biofilm formation of initial colonizers and to define the mechanisms. We found increased *A. naeslundii* biofilm formation inducing membrane-damaged cells and more production of GrpE and GroEL in biofilms upregulated by SCFA. These results may help to understand biofilm development in subgingival plaque and pathogenic formation conditions in the oral cavity.

METHODS

Bacterial strains and culture

We used *Streptococcus gordonii* ATCC 10558, *Streptococcus sobrinus* AHT, *Streptococcus mutans* ATCC 25175 and UA159, *Streptococcus mitis* ATCC 6249, *Streptococcus anginosus* ATCC 33397, *Streptococcus salivarius* HT9R, and *Actinomyces naeslundii* X600 and WVU398A. All bacteria were 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 before inoculation into 96-well microtitre plates. *Actinomyces naeslundii* X600 bacterial growth was measured as absorbance at 600 nm at 0, 4, 8, 12, 16 and 20 h after inoculation into tryptic soy broth (TSB without dextrose, Difco Laboratories) with 0.25% sucrose with or without various concentrations of butyric acid.

Biofilm formation assay

Biofilm formation using each strain was assayed using the method described previously (Motegi *et al.*, 2006; Tamura *et al.*, 2009). To evaluate the effects of SCFA, a 20-μl suspension of *A. naeslundii* X600 (4×10^5 colony-forming units ml^{-1}) was mixed with 20 μl SCFA [diluted using sterilized phosphate-buffered saline (PBS) at various concentrations] and 160 μl of TSB with 0.25% sucrose. The biofilm formation assay was performed (37°C, 5% CO_2 in an aerobic atmosphere) and measured at 0, 2, 4, 6, 8, 10, 12, 14, 18 and 20 h. To determine the time points for upregulation using SCFA, 6.25 mM butyric acid was added to the *A. naeslundii* X600 suspension in TSB with 0.25% sucrose at 0, 6, 10 and 14 h after starting incubation. For the inhibition assay in the biofilm formation, antibodies diluted to 1/8000 and 1/16,000 were added into the cell suspensions of *A. naeslundii* X600. The biofilm formation assay was performed at 37°C after 16 h of incubation using 5% CO_2 in an aerobic atmosphere. After the plates were incubated, the liquid medium with planktonic cells was removed and the wells were rinsed twice with sterile distilled water. The plates were air-dried and stained with 0.25% safranin/0.5% ethanol/distilled water for 15 min. After staining, the plates were rinsed with sterile distilled water to remove the excess dye and then air dried. The biofilm mass was dissolved with 70% ethanol and measured using a microplate reader (OD_{492}) (Thermo Bioanalysis Japan, Tokyo, Japan). Quantification of the stained biofilm was performed measuring the absorbance at 492 nm.

SDS–PAGE and Western blot analysis

Actinomyces naeslundii X600 (4×10^4 colony-forming units) was mixed with SCFA (3.125 or 6.25 mM butyric acid, 1.56 or 3.125 mM valeric acid and 3.125, 6.25 or 12.5 mM acetic acid) in 1 ml TSB with 0.25% sucrose using six-well culture plates and incubated at 37°C for 16 h using 5% CO_2 in an aerobic atmosphere. The liquid medium was removed and the wells were rinsed twice with distilled water. Biofilm cells were scraped off and washed twice with PBS. Biofilm cells were suspended in 200 μl PBS and homogenized by cell destruction using beads and Tissue Lyser (QIAGEN GmbH, Hilden, Germany). Debris and destroyed cells were removed by centrifugation at 6000 ***g*** and the precipitates after 20,000 ***g*** centrifugation were used for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blot. For SDS–PAGE, the precipitates were re-suspended and boiled at 100°C for 5 min in SDS loading buffer including 5% 2-mercaptoethanol; and separated using 12.5% PAGE (e-PAGEL; ATTO Corp, Tokyo, Japan). When electrophoresis was complete, proteins in the gel were stained with Coomassie Brilliant Blue (Bio-Safe™ Coomassie; Bio-Rad, Hercules, CA). For Western blot, the gels were transferred to polyvinylidene difluoride membranes (Atto, Tokyo, Japan). After incubating the membrane in Tris-buffered saline (TBS) containing 0.5% skim milk for 1 h and washing, the membrane was incubated with a 1/1000 dilution of anti-GrpE (MBL: Medical & Biological Laboratories Co., Ltd., Nagoya, Japan) or GroEL anti-serum (MBL) at 37°C for 1 h. The membrane was washed with TBS containing 0.05% Tween-20 (TBST); and incubated with goat anti-rabbit peroxidase-conjugated immunoglobulin G (IgG) secondary antibody (1/10,000 dilution) (Sigma-Aldrich, St Louis, MO) for 1 h. The membrane was washed with TBST and exposed to chemiluminescent detection reagents (ECL Plus, GE Healthcare, Buckinghamshire, UK).

Microscopy for membrane-damaged or dead cells and the production of GroEL

Biofilm cells from *A. naeslundii* X600 were cultured in TSB with 0.25% sucrose using six-well culture plates with 3.125 or 6.25 mM butyric acid at 37°C for 16 h using 5% CO_2 in an aerobic atmosphere. The liquid medium was removed and the wells were rinsed twice with distilled water. To observe membrane-damaged or dead cells in the biofilm, biofilm cells on the six-well plates were treated with a LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit solution (Molecular Probes, Leiden, the Netherlands) according to the manufacturer's instructions. After washing with PBS

three times, a cover glass was placed on the dye-stained biofilm. The stained biofilm was observed using a fluorescence microscope (BX50; Olympus, Tokyo, Japan). The excitation/emission wavelengths of the dyes in this kit solution were 480/530 nm for SYTO 9 and 520/580 nm for propidium iodide.

To observe GroEL on the cells, the biofilm cells were treated with anti-GroEL rabbit antiserum (MBL) in pH 8.0 PBS for 30 min at room temperature. After washing with PBS three times, the anti-GroEL-treated biofilms were treated with anti-rabbit antibody (from goat) conjugated with fluorescein isothiocyanate (Sigma-Aldrich Japan, Tokyo, Japan) in pH 8.0 PBS for 30 min in the dark at room temperature. After washing with PBS three times, the immunostained biofilms were observed using a fluorescence microscope (B×50). The excitation/emission wavelengths of the fluorescence were 480/530 nm.

Statistics

Comparison of biofilm formation levels among various cultures treated with butyric acid and un-treated cultures were performed using analysis of variation. A P value of 0.05 or less was considered to be statistically significant.

RESULTS

Effects of SCFA on biofilm formation

To assess the effects of SCFA on biofilm formation, butyric acid, propionic acid and valeric acid were added to the biofilm formation assay with *S. gordonii* or *A. naeslundii.* In *S. gordonii*, all SCFA induced slight upregulation of biofilm formation but did not show significant biofilm formation level increase compared with the control: no addition of SCFA (Fig. 1A–C). Similar results were observed using other streptococci (data not shown). In contrast, using the *A. naeslundii* X600 biofilm formation assay, butyric acid led to significantly increased biofilm formation increasing from 1.56 to 6.25 mM; however the level decreased at a concentration of more than 6.25 mM (Fig. 1D). Similar effects were also observed using other SCFA; an increase shown at 3.13 mM with

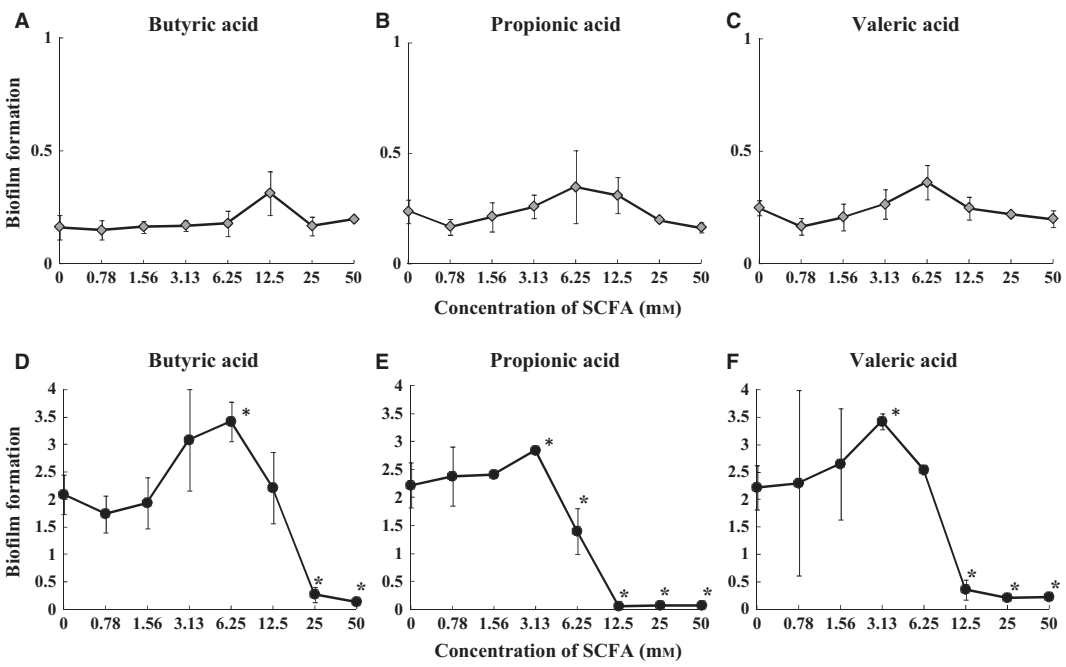

Figure 1 Biofilm formation of *Actinomyces naeslundii* increased by short-chain fatty acids. Butyric acid, propionic acid and valeric acid were added at various concentrations to cell suspensions of *Streptococcus gordonii* ATCC 10558 (A–C) and *A. naeslundii* X600 (D–F). Cell suspensions were incubated for biofilm formation in a 96-well microtiter plate; and the biofilms were assessed after 16 h of incubation. The results are expressed as the mean ± SD of absorbance (OD_{492}) obtained in triplicate assays. Representative data from three independent experiments are presented where similar results were obtained from each experiment. The asterisks denote significantly different biofilm formation comparing the SCFA treatment and the control ($P < 0.05$).

propionic acid and valeric acid (Fig. 1E,F), and at 12.5 mM with acetic acid (data not shown). These increasing effects were also observed with the other strain of *A. naeslundii*, WVU398A. Butyric acid was previously investigated for various activities in bacterial cells and biological cells such as lymphocytes (Kurita-Ochiai *et al*., 1997, 2001; Lawhon *et al*., 2002; Van Immerseel *et al*., 2004). To study whether the increased biofilm was induced by the specific reactivity of SCFA, butyric acid was selected as a typical component for SCFA activities. Various pH conditions were controlled adding HCl or NaOH in TSB with 0.25% sucrose, compared with TSB with 0.25% sucrose with sodium butyrate and used in the biofilm formation assay. TSB with 0.25% sucrose not including and including 6.25 mM butyric acid was respectively pH 7.12 and pH 6.73 in the initial culture condition before inoculation of *A. naeslundii.* To observe the effects of slightly lowering the pH in the primary culture, before *A. naeslundii* cultivation in TSB with 0.25% sucrose, the pH was adjusted using 500 mM HCl from pH 7.12 to pH 6.73. In contrast, the pH was adjusted using 500 mM NaOH from pH 6.73 to pH 7.12 in TSB with 0.25% sucrose and 6.25 mM butyric acid; and then the biofilm formation assay was performed after 16 h of incubation. In addition, we substituted 6.25 mM sodium butyrate instead of 6.25 mM butyric acid (pH 7.11) in the primary medium and this was used for the biofilm formation assay. The effects of butyric acid were compared with other SCFA using these pH conditions and with butyrate. Except for butyric acid, the biofilm formation levels were not affected by pH changes and sodium butyrate in comparison with the controls (Fig. 2). Therefore, the upregulation of the biofilm was specifically induced by butyric acid, involving a slightly lower pH in the primary culture.

To determine when the biofilm formation is upregulated by butyric acid, the biofilm levels were evaluated at various time points during the culture of *A. naeslundii* X600 with or without addition of 6.25 mM butyric acid. The biofilm formation was upregulated by addition of butyric acid after 14 h of incubation in comparison to no addition of butyric acid (Fig. 3A). However, there was no significant difference in growth between *A. naeslundii* with and without addition of 6.25 mM butyric acid (Fig. 3B). Therefore, the upregulation effects of biofilm were observed in the mid phase of biofilm cell growth and not dependent on cell growth. To observe whether additional time with butyric acid in the culture affects the biofilm formation, butyric acid was added at 0, 6, 10 and 14 h after the beginning of the culture in TSB with 0.25% sucrose and the biofilm formation levels were observed every 2 h after beginning the culture. The biofilm formation levels were compared with the culture without butyric acid. The biofilm was upregulated by the addition of 6.25 mM butyric acid at 0, 6 and 10 h after beginning culture but not at 14 h (Fig. 4A). However, there were no significant differences in growth between *A. naeslundii* with and without addition of 6.25 mM butyric at the different times (Fig. 4B). Therefore, 16 h from 10 h (early-mid phase) after addition of butyric acid were required for the upregulation of the biofilm and occurred in the mid phase.

Mechanism for upregulation by butyric acid in biofilm formation

We speculated on the mechanism of how butyric acid upregulated biofilm formation. A search of the

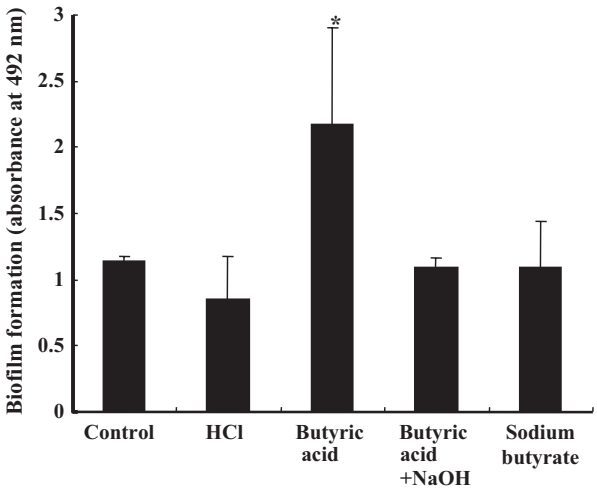

Figure 2 Biofilm formation of *Actinomyces naeslundii* in differing pH level including butyric acid. The primary culture pH of the cell suspension of *A. naeslundii* was adjusted using 500 mM HCl and the primary pH including 6.25 mM butyric acid was adjusted using 500 mM NaOH. The culture pH levels were adjusted including 6.25 mM butyric acid or 6.25 mM sodium butyrate; and were performed before beginning the culture. Biofilm formation levels were assessed after 16 h of incubation. The results are expressed as the mean ± SD of absorbance (OD_{492}) obtained in triplicate assays. Representative data from three independent experiments are presented where similar results were obtained from each experiment. The asterisks denote significantly different biofilm formation comparing the SCFA treatment and the control ($P < 0.05$).

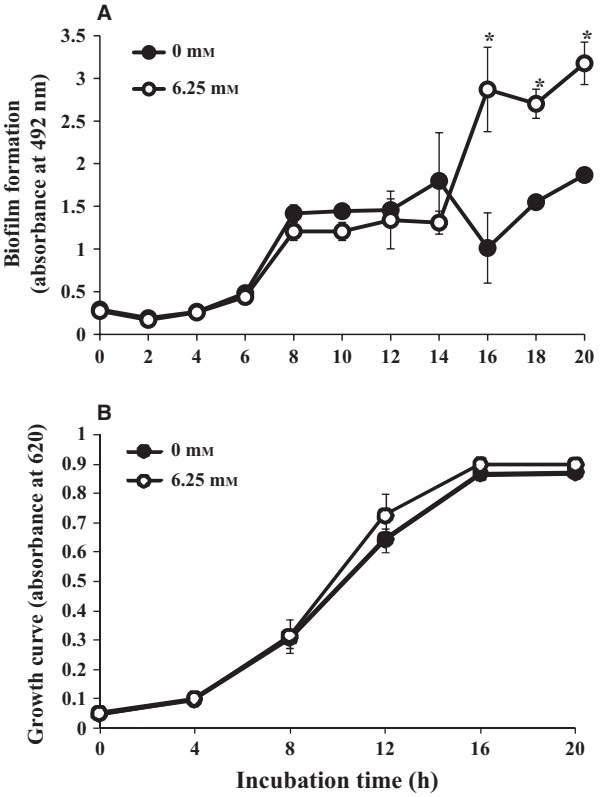

Figure 3 Time–course of biofilm formation and growth of *Actinomcyes naeslundii* treated with butyric acid. Biofilm formation and growth of *A. naeslundii* X600 treated with 6.25 mM butyric acid was compared with the control (no addition) at various time points. The results are expressed as the mean ± SD absorbance (OD_{492}) obtained in triplicate assays. Representative data from three independent experiments are presented where similar results were obtained from each experiment. The asterisks denote significantly different biofilm formation comparing the butyric acid treatment and the control ($P < 0.05$).

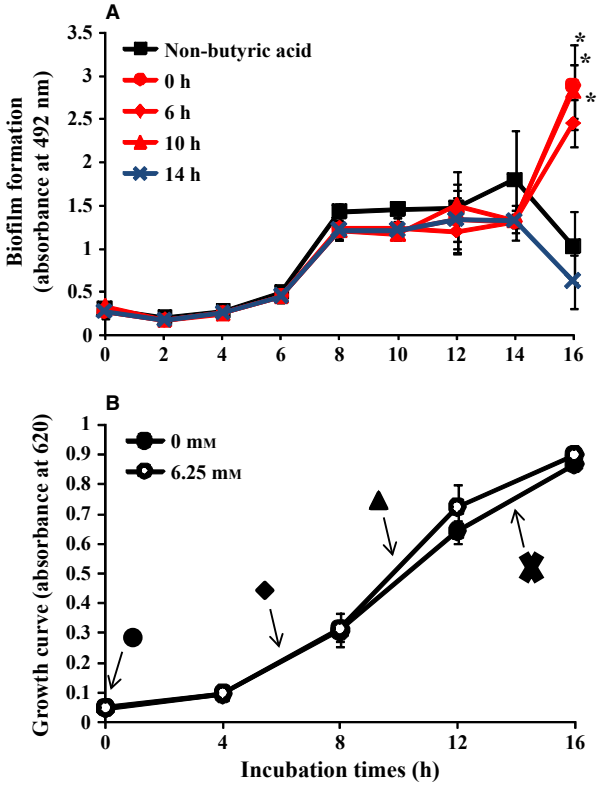

Figure 4 Biofilm formation and growth of *Actinomyces naeslundii* with 6.25 mM butyric acid at various time points. Biofilm formation (A) and growth (B) of *A. naeslundii* X600 with 6.25 mM butyric acid at the time points shown were compared with control: no butyric acid. Butyric acid (6.25 mM) was added at different times [0 h (●), 6 h (♦), 10 h (▲) and 14 h (**x**)] after initiation of the culture. Red lines indicate upregulation of biofilm in comparison with control: non-butyric acid. The growth data were presented with the addition of 6.25 mM butyric acid at 0 h after the incubation. The addition of butyric acid at different times did not affect the growth (B). The results are expressed as the mean ± SD of absorbance (OD_{492}) obtained in triplicate assays. Representative data from three independent experiments are presented where similar results were obtained from each experiment. The asterisks denote significantly different biofilm formation comparing the butyric acid treatment and control ($P < 0.05$).

literature shows several possibilities. Paddick *et al.* (2006) reported 76 proteins including adhesins, chaperones and stress-response proteins that were significantly upregulated in biofilm cells relative to planktonic cells. Of these biofilm-associated proteins, the stress-response proteins played important roles in the development of biofilm formation. To show the production of the GrpE protein, cell protein was extracted using physical destruction and centrifugation; and used for SDS–PAGE with anti-GrpE antibody Western blot. The expression levels and concentration of protein bands were similar in samples with the same conditions in SDS–PAGE gels stained with Comassie Brilliant Blue. In Western blot, the GrpE protein was strongly present in biofilm cells treated with 6.25 mM butyric acid at 16 h after incubation in comparison with the control without butyric acid (Fig. 5A). However, 3.125 mM butyric acid did not stimulate production of GrpE in the biofilm cells. GroEL is an important cell-associated protein in *Legionella pneumophilia*, and *Clostridium difficile* (Garduño *et al.*, 1998a,b; Hennequin *et al.*, 2001), and may act as an adhesin in the development of the *A. naeslundii* biofilm. Therefore, we determined the production of GroEL. GroEL was highly produced in biofilm cells in 3.125 mM butyric acid at 16 h after

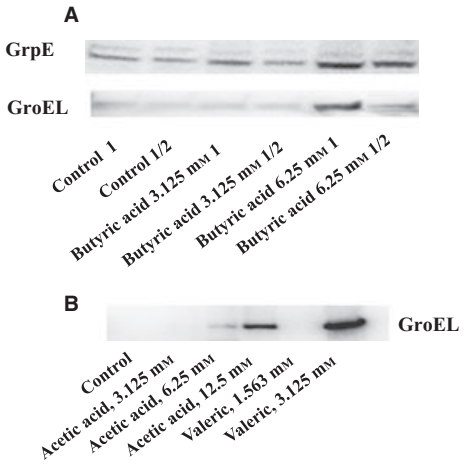

Figure 5 Production of GrpE and GroEL in the biofilms of *Actinomyces naeslundii*. Expression of GrpE and GroEL was assessed in the biofilms of *A. naesulundii* treated with 0, 3.125 and 6.25 mM butyric acid, 1.56 and 3.125 mM valeric acid and 3.125, 6.25 and 12.5 mM acetic acid. Samples from the treatment of butyric acid were undiluted or diluted 1 : 2 and applied to sodium dodecyl sulfate–polyacrylamide gel electrophoresis transferred to Western blot membranes and assayed using anti-GrpE and anti-GroEL antisera. Representative data from three independent experiments are presented where similar results were obtained from each experiment.

inoculation in comparison to the control (Fig. 5A). However, 3.125 mM butyric acid did not stimulate production of GroEL. Therefore, addition of SCFA may exert more stress on *A. naeslundii* cells than the control medium. Other SCFA, acetic acid and valeric acid, were also added in the biofilm formation assay and significant production of GroEL was observed (Fig. 5B). At 3.125 mM valeric acid and 12.5 mM acetic acid, production of GroEL was stimulated in the biofilm cells compared with the control without valeric and acetic acid. Stress proteins, e.g. GrpE and GroEL, were induced by the addition of SCFA. This excess stress may induce membrane-damage during the development of the biofilm. To determine if there were membrane-damaged cells and live cells in the biofilm, the biofilm cells were stained using SYTO 9 and propidium iodide. Some membrane-damaged cells were observed in the biofilms with 3.125 mM butyric acid (Fig. 6H,K); many were observed in biofilms with 6.25 mM butyric acid (Fig. 6I,L); the control had few damaged cells (Fig. 6G,J). From these data we conclude the increase in membrane-damaged cells appeared when butyric acid was present; and this may be associated with the upregulation of the *A. naeslundii* biofilm.

Significant expression of GroEL occurred during biofilm formation treated with 6.25 mM butyric acid. This was shown after 16 h of incubation but not after 14 h of incubation with 6.25 mM and 3.125 mM (Fig. 7). Therefore, the expression of GroEL on the surface of biofilm cells may be associated with the enhancement of biofilm formation. To determine whether the expression of GroEL was associated with the upregulation of biofilm formation, antibody to GroEL was added to a primary culture of *A. naeslundii* and we observed the effects of the antibody on the biofilm formation treated with 6.25 mM butyric acid after 16 h of incubation. Anti-GroEL antibody (1/8000 and 1/16,000) significantly inhibited biofilm formation upregulation by butyric acid (Fig. 8). However, the antibody did not affect the biofilm without butyric acid. Interestingly, the biofilm levels using the antibody were lower in the butyric acid and valeric acid treatments than biofilm levels in the control: no treatment (Fig. 8).

DISCUSSION

Actinomcyes naeslundii is considered a commensal microorganism in the oral cavity. Evidence suggests that colonization with *A. naeslundii* may exclude pathogenic bacteria and protect against other diseases (Tada *et al.*, 2006). However, *A. naeslundii* may play a key role in binding *S. gordonii* and its coaggregate, *Fusobacterium nucleatum*, in primary colonization and later in the biofilm (Kolenbrander *et al.*, 2005, 2006). As a result, *A. naeslundii* interacts with other bacteria and may indirectly stabilize the environment for virulent pathogens in the oral cavity. Our study suggests that biofilm formation produced by *A. naeslundii* was upregulated by SCFA such as butyric acid, propionic acid and valeric acid, which are produced by periodontopathic gram-negative anaerobes such as *P. gingivalis* and *F. nucleatum.*

Short-chain fatty acids are specifically required for the upregulation of biofilm formation by *A. naeslundii* because the same pH (6.73), prepared using HCl in TSB with 0.25% sucrose, as the pH of TSB with 0.25% sucrose and 6.25 mM butyric acid before inoculation of bacteria did not affect the biofilm formation (Fig. 2). Moreover, the culture pH with 6.25 mM sodium butyrate or the culture pH with 6.25 mM butyric acid adjusted to pH 7.12 using NaOH did not affect the biofilm formation (Fig. 2). Weak acids such as the butyric acid used here lower the pH in the

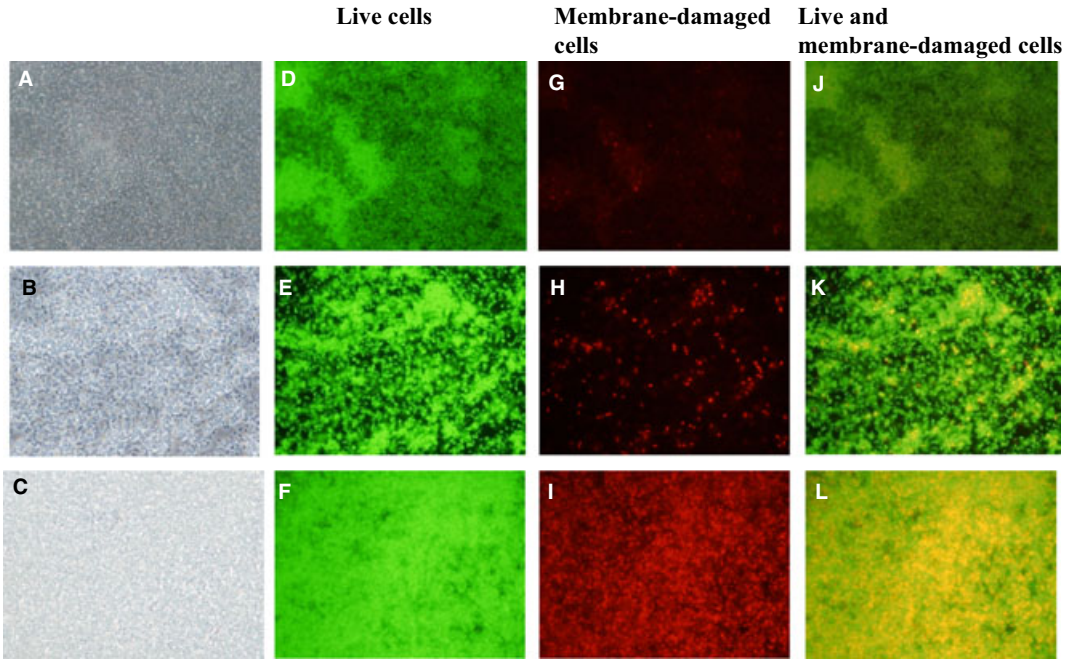

Figure 6 Live and membrane-damaged cells in the biofilm of *Actinomyces naeslundii* treated with butyric acid. Live and membrane-damaged cells were visually assessed in the biofilm formation of *A. naeslundii* X600 treated with 0 (A, D, G and J), 3.125 mM (B, E, H and K) and 6.25 mM (C, F, I and L) butyric acid. Photographs of biofilms (A–C), live cells (D–F), membrane-damaged cells (G–I) and photographs merged comparing live cells and membrane-damaged cells (J–L) were photographed observed using a laser microscope after staining with the Live/Dead kit (Molecular Probes). Representative data from three independent experiments are presented where similar results were obtained from each experiment.

primary culture and are effective for biofilm upregulation with *A. naeslundii* during metabolism and growth of *A. naeslundii*. The *A. naeslundii* reduced the extracellular pH more slowly compared with other streptococci conditioned with 0.4% glucose (Schlafer *et al.*, 2011). However, this subtle difference in the biological activities of *A. naeslundii* may be critical for the upregulation to the mature biofilm development and additional effects of butyric acid on terminal low pH conditions after metabolism of carbohydrate (Fig. 3). Therefore, the appropriate concentration of SCFA induces *A. naeslundii* to increase membrane-damaged or dead cells involving the stress proteins and biofilm development. The concentration of butyric acid in the periodontal pockets from patients with periodontal disease is 2.6 mM and lower than the appropriate concentration (6.25 mM) in the *in vitro* assay. At 3.125 mM, a value approximating physiological concentrations, butyric acid induced increased biofilm (Fig. 1). Therefore, considering the difference between *in vitro* and *in vivo* conditions, range; 2.6–6.25 mM may be appropriate concentrations for increasing activity of the biofilm formation *in vitro* and *in vivo*. It is also considered that appropriate concentrations (range; 3.125–9.5 mM) of propionic acid provide a similar relationship between *in vitro* and *in vivo* concentrations.

Biofilm formation is a major component in the extracytoplasmic stress response for bacteria. Heat-shock proteins (Hsp) are molecular chaperones that play important roles for bacterial stress tolerance. Heat-shock proteins provide major protection against cytoplasmic protein damage. The Hsp include HrcA, GrpE, DnaK and GroEL and are molecular chaperones and proteases synthesized in elevated amounts during heat and other stress conditions. They prevent mis-folding and aggregation of proteins; and promote refolding and proper assembly under normal and stress conditions (Craig, 1985; Checa & Viale, 1997; Diamant & Goloubinoff, 1998). The Hsp bind and release hydrophobic segments of the unfolded polypeptide chain using an ATP-dependent hydrolytic reaction (Walter & Buchner, 2002). From our data we speculate that the production of GroEL is associated with upregulation of biofilm formation in *A. naeslundii* at appropriate concentrations before a sublethal

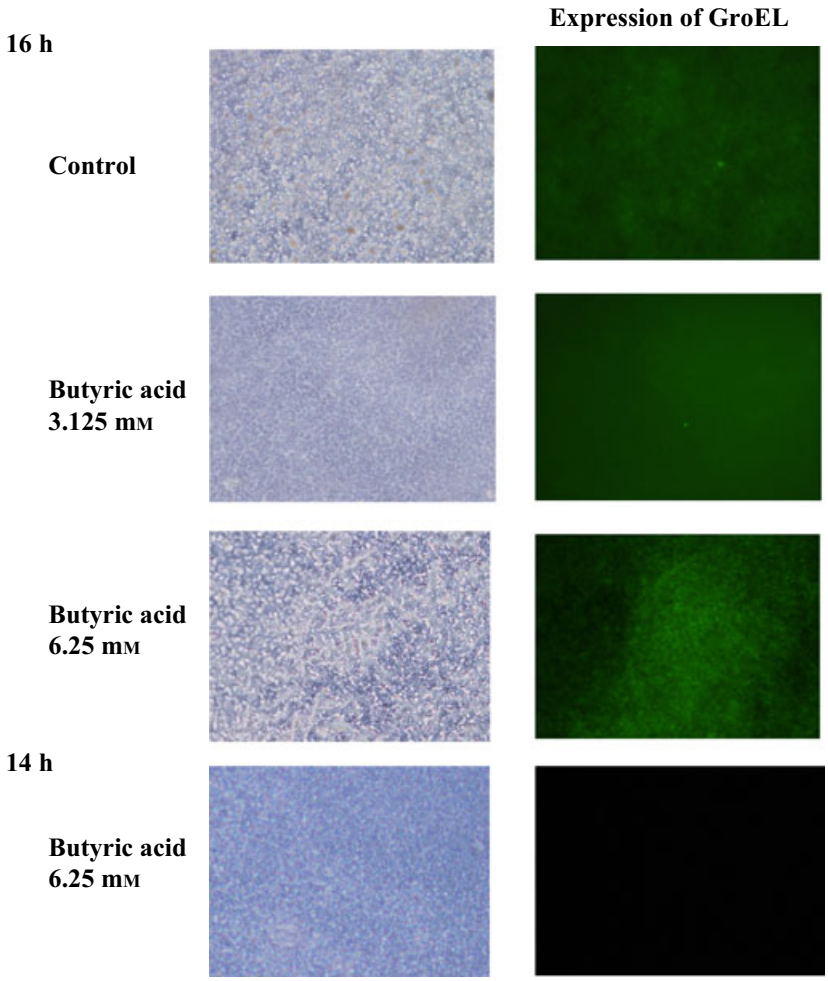

Figure 7 Expression of GroEL in various biofilms of *Actinomyces. naeslundii*. The *A. naeslundii* X600 cell suspensions were incubated with 0, 3.125 or 6.25 mM butyric acid after 16 h of culture and with 6.25 mM butyric acid added at 14 h. After incubation, expression of GroEL in the biofilm cells was assessed using immunofluorescent microscopy. Representative data from three independent experiments are presented where similar results were obtained from each experiment.

butyric acid concentration occurs (Figs 1, 5, 8). In a previous report, the GroEL homologue, Hsp60, an essential Hsp, is expressed on the surface of virulent *Legionella pneumophila* (Garduño *et al.*, 1998a,b). Other reports found that the 58.5-kDa GroEL Hsp of *Haemophilus ducreyi* is responsible for the attachment of *H. ducreyi* to carbohydrate receptors and is mediated by the 58.5-kDa GroEL Hsp (Pantzar *et al.*, 2006). Therefore, the associated chaperone GroEL concentration during stress conditions is not enough to kill, but is enough to act as an adhesin (Garduño *et al.*, 1998a,b; Hennequin *et al.*, 2001). A previous report suggests that chaperones and stress proteins may act as microbial virulence factors when expressed at the cell surface and may function as bacterial adhesins (Lewthwaite *et al.*, 1998). These reports support our observations that GroEL was produced in the biofilm cells and we confirmed the role of GroEL because the upregulated biofilm was inhibited by anti-GroEL antibody in *A. naeslundii* biofilm treated with butyric acid (Fig. 8). The biofilm formation levels after the addition of anti-GroEL antibody were lower in the presence of SCFA than those in control without SCFA. The SCFA may change the conformation of the cell surface expressed GroEL that contributes to biofilm formation by *A. naeslundii*. The rate of groEL-dependency in total biofilm formation level increases and exceeds the rate compared with the control biofilm formation. Therefore, the biofilm formation level was inhibited by addition of the antibody

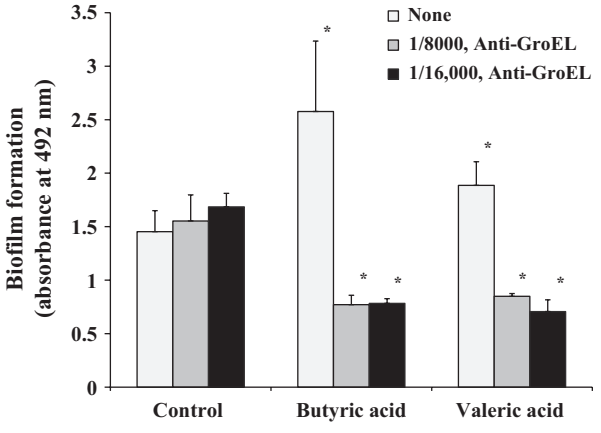

Figure 8 Effects of anti-GroEL antibody on the biofilm of *Actinomyces naeslundii* treated with butyric acid. Biofilm formation was assessed in various conditions treated with 6.25 mM butyric acid and 3.125 mM valeric acid in the presence and absence of anti-GroEL antiserum diluted 1/8000 and 1/16,000. The results are expressed as the mean ± SD of absorbance obtained in triplicate assays. Representative data from three independent experiments are presented where similar results were obtained from each experiment.

and was at lower levels compared with the control biofilm formation.

The acid tolerance response is an important means of surviving exposures to the weak acids, such as butyric acid, prevalent in the gastrointestinal environment. There are distinct acid tolerance response systems induced in exponential and stationary phase cells (Foster & Hall, 1990; Lee *et al*., 1994, 1995). Likewise, we found that the upregulation of the biofilm with butyric acid was also observed in the mid phase (Fig. 3). However, we found that upregulation required at least 6 h of culture (from 10 to 16 h after the start of culture) with 6.25 mM butyric acid in the early-mid phase (Fig. 4). These proteins may be stimulated using butyric acid in the early-mid phase and contribute to the upregulation of the biofilm in the mid phase. In the exponential phase, the acid tolerance response involves the induction of at least 50 proteins called acid-shock proteins (Foster, 1991; Lee *et al*., 1995). The molecular genetics and expression of these conserved Hsp have been extensively studied in *Escherichia coli* and *Bacillus subtilis*. In *E. coli*, the expression of the Hsp is regulated by the alternative sigma factor 32 (α^{32}) that is encoded by the *rpoH* gene (Lee *et al*., 1995; Mogk *et al*., 1999; Yura *et al*., 2000). The alternative sigma factor clearly plays an important role in protecting against the lethal effects of weak acids (Baik *et al*., 1996). Although the presence of α^{32} has not been observed in *A. naeslundii*, a similar protein to this sigma factor may be stimulated by weak acids (such as butyric acid) and regulate the expression process of Hsp that are associated with the upregulation of *A. naeslundii* biofilm formation. Therefore, definitive conclusions regarding the molecular mechanisms of expressed Hsp on the cell surface to enhance biofilm formation require further investigation to determine how they interact and are sensed with SCFA on *A. naeslundii*.

Our data suggest that membrane-damaged cells and expression of GroEL from *A. naeslundii* may increase the local SCFA environment produced by various bacteria in the subgingival plaque around the periodontal pocket. Membrane-damaged or dead cells may change the conformation and charge on the cell surface compared with intact cells. The membrane-damaged or dead cells presenting GroEL may help cell-to-cell aggregation and adherence to the surface, where other bacteria attach to the tooth and gingival surfaces. Therefore, after oral hygiene by brushing, when *A. naeslundii* re-colonize on the tooth surface, bacteria producing SCFA may support the re-colonization, aggregation and biofilm formation of *A. naeslundii* around periodontal pockets. Taken together, we propose that this is the role of increased *A. naeslundii* biofilm formation in the environment involving SCFA-producing bacteria.

Extracellular factors in pathogenic biofilm cell metabolism and the stress response aid in biofilm formation with commensal and other bacteria. This suggests that they have acquired the abilities to survive in poor nutrient, stressed environments. Our data reinforce a biofilm formation mechanism using SCFA in mechanisms for the development of pathogenic biofilm formation. To our knowledge, this is the first report that shows the role of stress proteins in biofilm formation with *A. naeslundii*.

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