Effects of extracellular DNA and DNA-binding protein on the development of a

*Streptococcus intermedius* biofilm

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Abstract

Aims: The aim of this study was to clarify the effects of homologous and heterologous extracellular DNAs (eDNAs) and histone-like DNA binding protein (HLP) on Streptococcus intermedius biofilm development and rigidity.

Methods and Results: Formed biofilm mass was measured with 0.1% crystal violet staining method and observed with a scanning electron microscope. The localizations of eDNA and extracellular HLP (eHLP) in formed biofilm were detected by staining with 7-hydoxyl-9H-(1,3-dichloro-9,9-dimethylacridin-2-one) and anti-HLP antibody without fixation, respectively. DNase I treatment (200 U ml⁻¹) markedly decreased biofilm formation and cell density in biofilms. Co-localization of eHLP and eDNA in biofilm was confirmed. The addition of eDNA (up to 1 µg ml⁻¹) purified from S. intermedius, other Gram-positive, -negative bacteria, or human KB cells into the S. intermedius culture increased the biofilm mass of all tested strains of S. intermedius, wild-type, HLP down-regulated strain, and control strains. In contrast, the addition of eDNA (> 1 µg ml⁻¹) decreased the biofilm mass of all S. intermedius strains.

Conclusions: These findings demonstrated that eDNA and eHLP play crucial roles in biofilm development and its rigidity.

Significance and Impact of the Study: eDNA- and HLP-targeting strategies may be applicable to novel treatments for bacterial biofilm-related infectious diseases.
Keywords: Extracellular DNA, extracellular HLP, biofilm development, \textit{S. intermedius}, DNA concentration
Introduction

Bacteria accumulate at the biological interface and form biofilms, which are a community of bacterial cells embedded in a self-produced polymeric matrix. This matrix constitutes about 90% of the biofilm mass and mainly consists of extracellular polysaccharides, proteins, lipids, and nucleic acids (Flemming and Wingender 2010). Polysaccharides and proteins are important components as a critical element of the matrix, and extracellular DNA (eDNA) is a common component among various bacterial constituents of the extracellular polymeric substance (EPS) in the biofilm (Hall-Stoodley et al. 2004). Recently, the role of eDNA has been increasingly recognized in both biofilm structural stability and protection against antimicrobial agents (Mulcahy et al. 2008; Whitchurch et al. 2002; Vilain et al. 2009). Hydroxyl radicals are extremely toxic and readily damage proteins, membrane lipids, and DNA (Farr and Kogoma 1991; Imlay and Linn 1986; Imlay et al. 1988), and H$_2$O$_2$ releases eDNA from S. sanguinis (Kreth et al. 2009). It has also been reported that eDNA serves as an important structural component of S. pneumoniae biofilms and the addition of intact DNA leads to increases in both the biofilm mass and bacterial viability of biofilms (Carollo et al. 2010). A previous report showed that the addition of DNase I into initial inocula at concentrations of 40-400 U ml$^{-1}$ inhibited biofilm formation by S. intermedius, indicating that eDNA may play an important role in the structure of the S. intermedius biofilm (Petersen et al. 2004).

Biofilm development can be divided into three distinct stages: attachment of bacterial cells to a surface, growth of cells into a sessile biofilm, and the detachment of cells from the
biofilm into the surrounding medium. Previous scientific research has focused on the
attachment of planktonic bacterial cells to surfaces and the subsequent growth of the biofilm;
however, the detachment and dispersal of bacterial cells from biofilms has received less
attention (Hall-Stoodley et al. 2004). The dispersal of bacterial cells from the biofilm can
spread, colonize new surfaces, and form biofilms; therefore, this is an essential stage of the
biofilm life cycle (Kaplan 2010). While many bacterial cells can disperse from biofilms by
passive processes, such as the erosion or sloughing of cells from the biofilm caused by fluid
shear (Stoodley et al. 2001), the bacterial biofilm can also periodically undergo active
dispersal events and bacterial cells in sessile, matrix-encased biofilms convert en-masse to
planktonic bacteria (Costerton et al. 1999; Hall-Stoodley et al. 2004).

Bacterial nucleoid-associated proteins have been documented as an accessory
architectural factor in a variety of bacterial cellular processes. The fact that bacterial
histone-like protein (HLP) also exists extracellularly has been known for approximately 30
years (Goodman et al. 2011). Recently, other studies have reported that bacterial HU from
other genera are also present in the extracellular milieu (Paramonova et al. 2009; Menozzi et
al. 1996). Interestingly, Goodman et al. (2011) suggested that the members of HLP, HU
and IHF, significantly contribute to the structural integrity of eDNA.

*Streptococcus intermedius* is a commensal bacterium and a member of the *Streptococcus
anginosus* group (SAG). Among the SAG species, *S. intermedius* is the most common
pathogen that is often isolated from oral infectious lesions, such as periodontitis, and fatal
purulent infections in internal organs, especially brain and liver abscesses (Wagner et al.
2006; Erne et al. 2010). In addition, some clinical case reports also presented its ability in
causing various kinds of infections such as infective endocarditis (Cunha et al. 2009). *S. intermedius* often causes chronic and/or recurrent infectious diseases depending on the biofilm life cycle.

Besides eDNA resulting from lysed or autolysed resident bacterial cells, eDNA within the biofilm can also originate from polymorphonuclear neutrophils, which release DNA (Brinkmann et al. 2004). In diseases with a biofilm component, biofilms formed *in vivo* are likely to be composed of eDNA of both host and bacterial origins (Goodman et al. 2011). *In vivo* studies showed that eDNA levels in the human lung are abundant (100-200 µg ml$^{-1}$), even under normal physiological conditions, and that levels reach as high as 4 mg ml$^{-1}$ in cystic fibrosis patients (Potter et al. 1969). Since *S. intermedius* has also been reported to have been isolated from a patient suffering from cystic fibrosis (Olson et al. 2010; Sibley et al. 2010; Grinwis et al. 2010), eDNA levels in the *S. intermedius* biofilm may also reach such high concentrations. Furthermore, since the habitat of *S. intermedius* is in the mouth and gastrointestinal tract, we assume that DNA derived from host cells in the oropharynx may also have an effect on *S. intermedius* biofilm development. To date, there have been no reports to demonstrate whether the external addition of homologous or heterologous DNA into the bacterial culture medium and HLP could affect the biofilm mass of *S. intermedius*.

In this study, we examined the effects of the exogenous DNA (final concentration of 0.01 - 100 µg ml$^{-1}$) of *S. intermedius*, *Escherichia coli*, and human KB cells, a human carcinoma cell line of the oropharynx, and Si-HLP on the development of the *S. intermedius* biofilm.
Materials and methods

Bacterial strains, human cell line, and culture conditions

We previously constructed an inducible antisense *S. intermedius* (Si-HLP down-regulated *S. intermedius*) strain, BETAHT, by transforming into *S. intermedius* ATCC27335 as a wild-type (WT) strain with a Streptococci–*E. coli* shuttle plasmid harboring the tetracycline-regulated antisense *Si-hlp* gene, a control strain, BETT, by transforming a parent plasmid into the WT strain, and another control strain, BETAXT, by transforming into the WT strain with an irrelevant antisense *x* fragment-inserted shuttle plasmid (Liu *et al.* 2008b). The WT, BETAHT, BETT, and BETAXT strains of *S. intermedius* were grown in brain heart infusion (BHI) broth (Difco, Detroit, MI, USA) at 37°C under anaerobic conditions with AnaeroPack anaerobic atmosphere generation systems (Mitsubishi Gas Chemical Co. Inc., Tokyo, Japan). *S. intermedius* strains transformed with the plasmids were incubated in BHI containing 10 μg ml⁻¹ erythromycin as a selective pressure and 20-60 ng ml⁻¹ doxycycline for regulating antisense *Si-hlp* gene (Liu *et al.* 2008b). *Staphylococcus aureus* 209P was cultured in BHI under aerobic condition at 37°C. *E. coli* strain K12 and *Pseudomonas aeruginosa* PAO1 were cultured in LB broth at 37°C under aerobic conditions.

The KB cell line (derived from a human oral epidermoid carcinoma; kindly provided by Dr. T. Okamoto, Hiroshima University School of Dentistry) was cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Life Technologies, Grand Island, NY, USA) supplemented with 2 mM L-glutamine, 10% (vol/vol) fetal bovine serum (JRH Biosciences,
Lenexa, KA, USA), 50 IU ml\(^{-1}\) penicillin, and 50 μg ml\(^{-1}\) streptomycin at 37°C in a water-saturated atmosphere of 95% air and 5% CO\(_2\).

**DNA purification from bacteria and human cells**

*S. intermedius* ATCC27335, *E. coli* K12, *S. aureus* 209P, *P. aeruginosa* PAO1 or KB cells were suspended in TE buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA). Only *S. intermedius* and *S. aureus* 209P were treated with mutanolysin (final: 250 units ml\(^{-1}\)) for 1 h at 37°C. Each cell suspension was then treated with Proteinase K (final 100 μg ml\(^{-1}\)) and SDS (final 0.12%) for 2 h at 50°C. DNA purification from cells was performed using phenol/chloroform extraction and ethanol precipitation methods. Briefly, DNA was extracted with an equal amount of 50 mM Tris-HCl (pH 8.0)-saturated phenol and was precipitated with sodium acetate (final: 0.3M) and 2.5 volumes of ethanol. Extracted DNA was treated with RNase A (final: 10 μg ml\(^{-1}\)) for 1 h 37°C, followed by re-extraction with phenol-chloroform (1:1) and precipitation with sodium acetate and ethanol. Purified DNA was finally dissolved in TE buffer. The purity and concentration of purified DNA was assessed by measuring the absorbance at 260 and 280 nm and agarose gel electrophoresis.

**Quantification of the biofilm mass**

A crystal violet biofilm assay was used to quantify the biofilm mass of *S. intermedius* as previously described (Moscoso *et al*. 2006). Aliquots of a 1:40 dilution of an overnight
bacterial culture (1.0 x 10^7 CFU ml⁻¹) were inoculated into the wells of a 96-well plate and incubated anaerobically at 37°C using the AnaeroPack system for 24 or 48 h. Formed biofilms were washed with PBS without disturbing the adherent biofilm, stained with 50 µl 0.1% crystal violet, incubated at room temperature for 15 min, and excess stain was removed by three gentle washes with PBS (pH 7.2). After being dried, the stained biofilm was extracted from the well by adding 50 µl ethanol and was determined by measuring the absorbance of the extract at 540 nm with a microplate reader (model 680; Bio-Rad, Hercules, CA, USA). Sterile BHI broth was substituted for bacterial cultures in control experiments.

To determine the inhibitory effect of DNase I on biofilm formation, DNase I (Roche, Mannheim, Germany) was added to the initial inoculum to a final concentration of 200 U ml⁻¹ and incubated for 24 and 48 h to form a biofilm. The formed biofilm mass was quantified using the crystal violet biofilm assay as described above. In addition, to determine the effect of eDNA on biofilm stability, the 24-h-cultured *S. intermedius* WT biofilm was treated with DNase I (200 U ml⁻¹) for 24 h and then DNase I-treated biofilm mass was quantified using the crystal violet staining.

To investigate the effect of DNA on biofilm formation, various concentrations of DNA purified from *S. intermedius*, *S. aureus*, *E. coli*, *P. aeruginosa* (0.01–100 µg ml⁻¹) or KB cells (0.01–10 µg/ml) were added into 96-wells plate containing 1.0 x 10^7 CFU ml⁻¹ of all *S. intermedius* strains (WT, BETT, BETAHT and BETAXT) and incubated anaerobically at 37°C for 48 h. The 0.1% crystal violet biofilm assay was performed to quantify the biofilm mass as described above.

To investigate the rigidity of biofilm, formed biofilm mass before washing and retained
biofilm mass after washing were quantified by the 0.1% crystal violet biofilm assay. The biofilm removal percentage was calculated using the following equations:

\[
\text{Biofilm removal percentage} = \left( \frac{\text{OD}_{540\text{nm}} \text{ before washing} - \text{OD}_{540\text{nm}} \text{ after washing}}{\text{OD}_{540\text{nm}} \text{ after washing}} \right) \times 100%.
\]

Scanning Electron Microscopy (SEM) observation

The *S. intermedius* suspension (1.0 x 10^7 CFU ml^-1) was added to each well of a 24-well culture plate with a type I collagen coating coverslip (Celldesk LF1; Sumitomo Bakelite Co., Tokyo, Japan) and was anaerobically incubated for 48 h at 37°C. After incubation, the coverslips were removed, rinsed with distilled water, and fixed with 2.5% glutaraldehyde solution for 1 h at room temperature. Samples were then rinsed three times with distilled water and were dehydrated through a graded series of ethanol solutions to 100% ethanol. All samples were air-dried and were coated with Au ions for SEM analysis. SEM was carried out with a Miniscope TM-1000 (Hitachi High-Technologies Corp., Tokyo, Japan).

eDNA staining

7-hydroxyl-9H-(1,3-dichloro-9,9-dimethylacridin-2-one) (DDAO; Invitrogen, Carlsbad, CA, USA) was used to stain eDNA. Before using DDAO to stain the *S. intermedius* biofilm, we first confirmed the effectiveness of DDAO in staining eDNA. *S. intermedius* cells were disrupted by mixing with glass beads (Φ = 100 µm) for 10 min and non-disrupted cells were
used as a control. DDAO was added into the cell sample at a concentration of 2 µM and the sample was incubated for 30 min. The sample was then observed using a BIOREVO BZ-9000 microscope (KEYENCE Co., Osaka, Japan). After confirmation of the effectiveness of DDAO, we determined the effect of DNase I treatment on eDNA in the S. intermedius biofilm by staining with DDAO. The 24-h-cultured biofilm of S. intermedius WT was treated with DNase I (200 U ml⁻¹) for 24 h at 37°C. Without fixation, the biofilm was then stained with DDAO and observed using a confocal fluorescence microscope as described above.

**eDNA and HLP co-localization**

To observe the distribution of genomic DNA, eDNA, and Si-HLP in the S. intermedius biofilm, Hoechist 33342, DDAO, and anti-Si-HLP peptide antibody were used, respectively. The biofilm of S. intermedius WT (1.0 x 10⁷ CFU ml⁻¹) was formed as described above. The formed biofilm was blocked with PBS-BSA (1.5%) without fixation and then reacted with rabbit anti-Si-HLP peptide antibody for 1 h at room temperature. After washing with PBS, the biofilm was reacted with Alexa fluor 488-labeled anti-rabbit IgG (Invitrogen) for 1 h, followed by eDNA staining with 2 µM DDAO for 30 min and continued by genomic DNA staining with 10 µg ml⁻¹ Hoechst 33324 for 30 min. The biofilm was then observed using a confocal fluorescence microscope (model BZ-9000).

**Growth of S. intermedius**
DNA (1.0 and 10 µg ml⁻¹) purified from the *S. intermedius* WT strain was added into 1.0 x 10⁷ CFU ml⁻¹ of *S. intermedius* WT and incubated anaerobically for 12 h. The growth of *S. intermedius* was monitored every 2 h by measuring the absorbance of the culture at OD₆₀₀nm.

**Statistical analysis**

All statistical analyses were performed using the unpaired Student's *t* test. Differences were considered significant when the probability value was less than 5%.
Results

Effect of DNase I treatment on biofilm formation and its stability

To determine the effect of DNase I on biofilm formation, DNase I (200 U ml\(^{-1}\)) was added to the initial *S. intermedius* WT inoculum and incubated for 24 and 48 h to form a biofilm. As shown in Fig. 1(a), *S. intermedius* WT biofilm mass with the DNase I treatment was significantly lower in both the 24- and 48-h cultures than in the untreated control. SEM observations also showed that cell density with the DNase I treatment was markedly lower in the biofilm than in the untreated control (Fig. 1b). Next, to determine the effect of eDNA on biofilm stability, the 24-h-cultured *S. intermedius* WT biofilm was treated with DNase I (200 U ml\(^{-1}\)) for 24 h. As shown in Fig. 1(c), biofilm mass with the DNase I treatment was significantly lower in the 24-h-cultured *S. intermedius* WT biofilm than in the untreated control. Before using DDAO to stain eDNA in the *S. intermedius* WT biofilm, we first confirmed the usefulness of DDAO for eDNA-specific staining in a planktonic *S. intermedius* WT culture (Fig. 1d: I and II). We observed that eDNA stained with DDAO in the 24-h-cultured *S. intermedius* WT biofilm was markedly decreased by the DNase I treatment (Fig. 1d: III and IV). These results suggest that eDNA may induce biofilm formation and plays an important role in the stability of the formed biofilm.

eDNA and HLP co-localization in the biofilm
Ours and other previous studies reported that Si-HLP is released outside cells as well as being localized in the intracellular compartment without cell lysis, and the HLPs of *Helicobacter pylori* and *S. pyogenes* have been detected in the culture supernatant and on the bacterial cell surface (Liu *et al.* 2008a; Kim *et al.* 2002; Lei *et al.* 2000; Severin *et al.* 2007). Our recent report further indicated that recombinant Si-HLP can bind DNA and alter the structural conformation of DNA *in vitro* (Liu *et al.* 2008b). We next determined the localizations of HLP and eDNA in the *S. intermedius* biofilm using confocal fluorescence microscopy. As shown in Fig. 2, we observed that eDNA and extracellular HLP (eHLP) were present and abundant in a 2-day-old biofilm of *S. intermedius* by immunofluorescent staining without fixation. Some HLP molecules were also co-localized with intracellular DNA and eDNA in the *S. intermedius* biofilm. This observation showing the co-localization of eHLP and eDNA suggests that HLP may form a complex with eDNA and play some roles in biofilm formation and its stability.

Effect of Si-HLP on biofilm formation by *S. intermedius*

We previously demonstrated that Si-HLP is essential for cell viability and normal growth using gene knockout mutation and *tet*-regulation system-based antisense-mediated gene silencing (Liu *et al.* 2008b). We further determined whether Si-HLP could affect biofilm formation by *S. intermedius*. The Si-HLP-downregulated strain (BETAHT) formed significantly less biofilm mass than all control strains (WT, BETT and BETAXT) and biofilm mass was dependent on Si-HLP expression levels under the control of doxycycline.
Effect of DNA addition on biofilm formation

To determine the role of eDNA in *S. intermedius* WT biofilm formation, *S. intermedius* WT was incubated for 48 h to form a biofilm with purified *S. intermedius* DNA at various concentrations. Moreover, to investigate the rigidity of biofilm, formed biofilm mass before washing and retained biofilm mass after washing were quantified and the biofilm removal percentage was calculated. *S. intermedius* DNA increased the retained biofilm mass of *S. intermedius* WT strain in a dose-dependent manner up to 1.0 µg ml⁻¹ by crystal violet biofilm assay after washing. In contrast, the higher concentrations of *S. intermedius* DNA at 10 and 100 µg ml⁻¹ decreased the biofilm mass of *S. intermedius* WT strain (Fig. 4a). Interestingly, this decreased biofilm mass at higher concentrations of DNA may be causally related to its fragile structure because we observed that the adherent biofilm was broken off during washing and the biofilm removal percentage at 10 and 100 µg ml⁻¹ was extremely high (Fig. 4b).

We further determined whether heterologous eDNA could also induce biofilm formation by the addition of DNA purified from other bacteria, such as *S. aureus*, *E. coli*, and *P. aeruginosa*, or KB cells as well as homologous *S. intermedius* DNA. All tested DNA increased the biofilm mass of all *S. intermedius* strains, including the Si-HLP down-regulated strain BETAHT, in a dose-dependent manner up to 1.0 µg ml⁻¹, but the higher concentrations of all tested DNA decreased the biofilm mass of all *S. intermedius* strains (Fig. 4a). This result suggests that eDNA, regardless of the origin of DNA, may
promote biofilm formation and affect the rigidity of the formed biofilm.

**SEM observations**

As shown in Fig. 5, we also observed that the addition of 1.0 µg ml\(^{-1}\) of *S. intermedius* DNA dramatically increased the biofilm mass of both *S. intermedius* WT and *S. intermedius* Si-HLP down-regulated BETAHT strains and *S. intermedius* cell density in both biofilms. However, the addition of 100 µg ml\(^{-1}\) of *S. intermedius* DNA markedly decreased the biofilm mass of both strains and cell density in both biofilms, indicating that biofilms formed at higher concentrations of eDNA become structurally weakened. These observations of structural changes to the biofilm formed in the presence of DNA correlated with the results of biofilm mass quantification formed in a culture with eDNA and after the DNase I treatment (Figs. 1 and 4). We further observed similar images for biofilms formed by the addition of DNA purified from *E. coli* (data not shown). These results also suggest that eDNA, regardless of the origin of DNA, may affect biofilm formation and the rigidity of the formed biofilm.

**Effect of DNA addition on the growth of *S. intermedius***

We finally determined whether the addition of DNA could affect the growth rate of *S. intermedius*. Fig. 6 shows that the presence of DNA at a high concentration (10 µg ml\(^{-1}\)) inhibited the growth of *S. intermedius* whereas 1 µg ml\(^{-1}\) DNA had no effect on *S. intermedius* growth.
Discussion

This study successfully demonstrated that eDNA plays roles in \textit{S. intermedius} biofilm formation and the rigidity of the formed biofilm. Our first results show that DNase I treatment markedly decreased biofilm formation as well as cell density in \textit{S. intermedius} biofilms and degraded eDNA in the matrix of the mature biofilm (Fig. 1). These results indicate that eDNA plays essential roles in \textit{S. intermedius} biofilm formation and its structural strength. Our findings are in agreement with the first report showing that eDNA is required for the initial establishment of a \textit{P. aeruginosa} biofilm (Whitchurch \textit{et al}. 2002) and another report suggesting that eDNA is important for the development of an \textit{S. intermedius} biofilm (Petersen \textit{et al}. 2004).

Regarding the regulation of biofilm formation, it has been recently reported that \textit{E. coli} H-NS, the histone-like nucleoid structuring protein, plays important roles in regulating biofilm formation (Dalai \textit{et al}. 2009). We previously demonstrated that \textit{Si}-HLP could be released from bacteria without cell lysis as well as being localized in the intracellular compartment (Liu \textit{et al}. 2008a). Here, we observed that abundant \textit{Si}-HLP and DNA were co-localized in the matrix of the 24-h cultured biofilm (Fig. 2). This finding indicates that \textit{Si}-HLP and DNA are the important components of the matrix in a biofilm and suggests that \textit{Si}-HLP may bind to eDNA and form an eDNA-eHLP complex. Therefore, this eDNA-eHLP complex may play roles in biofilm formation and its structural strength.

To date, the role or function of bacterial HLP in biofilm formation has not been investigated. In our previous study to verify the essentiality of \textit{Si}-\textit{hlp}, we constructed an
inducible antisense Si-hlp RNA-expressed S. intermedius strain (BETAHT) by transforming
into the WT strain with a Streptococci–E. coli shuttle plasmid harbouring the inserted Si-hlp
gene between the tetR/O promoter and Ω fragment in an antisense orientation and
demonstrated that doxycycline-induced Si-hlp antisense RNA expression specifically
inhibited Si-HLP protein expression driven by the chromosomal Si-hlp locus (Liu et al.
2008b). Regarding this tet-regulation system-based antisense-mediated gene silencing,
base pairing between sense mRNA and complementary antisense RNA has been considered
to passively block the processing or translation of mRNA, or result in the recruitment of
nucleases that promote mRNA destruction (Brantl 2002; Huntzinger et al., 2005). Using
these doxycycline-regulated antisense RNA expression techniques, we demonstrated that the
Si-HLP-downregulated strain (BETAHT) formed significantly less biofilm mass and biofilm
mass was dependent on Si-HLP expression levels (Fig. 3). This result indicates that HLP as
well as eDNA plays an important role in biofilm formation.

By adding S. intermedius DNA at a low concentration (up to 1 µg ml⁻¹) as exogenous
DNA supplementation, the biofilm mass of all tested strains was increased in a
dose-dependent manner and the cell density in the formed biofilms of both S. intermedius
WT and Si-HLP-downregulated strains at 1 µg ml⁻¹ of DNA was also increased (Figs. 4 and
5). Our results correspond with a previous report showing that DNA addition enhanced S.
pneumoniae biofilm mass in a dose-dependent manner and suggest that eDNA is essential
for the enhancement of biofilm growth and has an important role in biofilm architecture.
(Carrolo et al. 2010). Interestingly, we demonstrated that the biofilm mass of the
Si-HLP-downregulated strain was also increased by the addition of purified DNA, but was
still significantly less than other WT and control strains (Fig. 4). Our previous report has shown that the Si-HLP-downregulated strain grows significantly more slowly with prolonged lag and logarithmic phases than WT and control strains, and this growth inhibition results from the induction of antisense Si-hlp RNA expression controlled with the tetR/O-inducible promoter (Liu et al. 2008b). Moreover, we found that the ATP assay and cell numbers counted as CFU showed that the doxycycline-induced Si-HLP-downregulated strain displayed lower amounts of intracellular ATP and lower numbers of living cells than those of control strains when their culture reached the same value of OD_{600}, respectively. Therefore, growth inhibition of the Si-HLP-downregulated strain may be one of the reasons for the lower ability of this strain to form a normal biofilm.

Intriguingly, we further demonstrated that the addition of heterologous DNAs (up to 1µg ml\(^{-1}\)) led to more robust biofilm formation in all tested S. intermedius strains in a dose-dependent manner (Fig. 4). These results suggest that enhancements in biofilm formation may not be dependent on homologous DNA or be species-specific and that all kinds of DNA may increase the biofilm mass formed in a dose-dependent manner.

In contrast to the effect of eDNA supplementation at lower concentrations (up to 1 µg ml\(^{-1}\)), the addition of DNA at higher concentrations (10 and 100 µg ml\(^{-1}\)) significantly decreased S. intermedius biofilm mass in a dose-dependent manner and the cell density in the formed biofilm with the addition of 100 µg ml\(^{-1}\) was markedly decreased (Figs. 4 and 5). We suggested that this opposite effect may be due to the growth inhibitory properties of higher concentrations of DNA because we found that the addition of 10 µg ml\(^{-1}\) S. intermedius DNA led to a 20% inhibition in the S. intermedius growth rate (Fig. 6). In
accordance with this result, a previous study showed that high concentrations of DNA (5 mg ml\(^{-1}\) or more) had a toxic effect on the growth of \(P.\ aeruginosa\) by acting as a cation chelator and subsequently induced cell lysis (Mulcahy et al. 2008). Although recent reports have shown the effect of exogenous DNA on enhancing biofilm formation, we here suggest that different DNA concentrations may have had different effects on biofilm formation and this may be part of the potential of bacteria to survive in unfavorable environments. Paramonova et al. (2009) reported that the increase in eDNA contents in the \(Candida\) \(albicans\) biofilm led to a decrease in biofilm strength and the formed biofilm could be more easily removed. Our present data also showed that the biofilm removal percentage at 10 and 100 \(\mu\)g ml\(^{-1}\) was extremely high because the adherent biofilm was broken off during washing (Fig. 4b). Therefore, a similar mechanism may have occurred in the \(S.\ intermedius\) biofilm and it could be considered that the biofilm formed with a high concentration of DNA (> 10 \(\mu\)g ml\(^{-1}\)) has low rigidity and is therefore easy to remove by fluid shear stress of washing and the dispersed bacterial cells then attach to another site.

Our previous report also showed that the \(Si\)-HLP-downregulated strain, BETAHT, largely lost its surface hydrophobicity as a result of alterations in cell surface components and \(luxS\) gene expression was downregulated in the BETAHT strain (Liu et al. 2008b). It has been reported that LuxS plays an important role in biofilm formation by \(S.\ intermedius\) (Ahmed et al. 2008, 2009). Considering these findings, we proposed that \(Si\)-hlp encoding histone-like DNA binding protein may be involved in the biofilm development of \(S.\ intermedius\) by regulating the expression of bacterial surface components and bacterial quorum sensing communication. Therefore, further studies to identify the genes involved in biofilm
development and determine their expression levels are needed and are currently under investigation.

It has recently been reported that autolysins (bacterial murein hydrolases) of Gram-positive bacteria, such as *Enterococcus faecalis* and *Staphylococcus epidermidis*, are implicated in biofilm formation, apparently through the mediation of bacterial lysis with the subsequent eDNA release (Guiton *et al.* 2009; Qin *et al.* 2007; Thomas *et al.* 2008, 2009). It has been demonstrated that DNA release displayed in the stationary phase of liquid cultures of pneumococcal cells can occur through cell lysis, rather than through a specific mechanism of secretion, and this release depends on the major autolytic N-acetylmuramyl-L-alanine amidase, LytA and the autolytic lysozyme, LytC, and it has also been shown that the competence-dependent release of DNA occurred by autolysis (Tomasz *et al.* 1988, Steinmoen *et al.* 2002; Moscoso and Claverys 2004). Moreover, recent interesting reports have shown that *S. pneumoniae* biofilm formation is influenced by the presence of eDNA, LytA mutants have a decreased capacity to form biofilms, and LytA-induced pneumococcal lysis may be related to biofilm formation through the release of eDNA (Hall-Stoodley *et al.* 2008; Moscoso *et al.* 2006). Previous studies have reported that eDNA present in bacterial biofilms results from cell lysis (Perry *et al.* 2009; Kreth *et al.* 2009; Farr and Kogoma 1991; Imlay and Lin 1986; Imlay *et al.* 1988) or is a product of direct secretion from intact cells (Whitchurch *et al.* 2002). However, the origin of eDNA in the *S. intermedius* biofilm is still unclear and is currently under investigation.

This is the first report to demonstrate that both homologous and heterologous DNA addition directly affects *S. intermedius* biofilm development and its rigidity, and suggests
that all kinds of DNA present at infection sites can increase bacterial biofilm formation.

Moreover, our present results clearly show that bacterial histone-like DNA binding protein (HLP) also plays crucial roles in biofilm development by forming a complex with eDNA. Regarding the recognition of bacterial infection, it has been known that the bacterial DNA, one of pathogen-associated molecular patterns, activates transcription factors, including NF-κB and interferon regulatory factors via TLR9-mediated and TLR-independent signaling pathways and induces the production of pro-inflammatory cytokines and type I interferon (Hemmi et al. 2000, Takeuchi and Akira 2007.). In addition, we recently reported that bacterial HLP initiates and exacerbates pro-inflammatory reactions during bacterial infection, as well as its physiological role in bacterial growth through DNA binding (Liu et al. 2008a).

Considering these immunological findings and biofilm as a pathogenic factor, the contents of eDNA and HLP in bacterial biofilms can be used as indicators of the severity of infection. Furthermore, targeting HLP and eDNA may create a novel strategy to fight microorganism-caused infectious diseases, especially those related to biofilm formation.
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Figure legends

Figure 1  Effect of the DNase I treatment on *S. intermedius* ATCC27335 biofilm formation (a, b) and its stability (c, d).  (a, b) DNase I (200 U ml\(^{-1}\)) was added to the initial *S. intermedius* WT inoculum and incubated for 24 and 48 h to form a biofilm.  After staining with crystal violet, the formed biofilm mass was quantified by measuring the absorbance at 540 nm as shown on the y axis (a).  Scanning electron microscopy images of *S. intermedius* WT cells after the 48-h incubation with DNase I (b).  (c) The 24-h-cultured *S. intermedius* WT biofilm was treated with DNase I (200 U ml\(^{-1}\)) for 24 h and then biofilm mass was quantified by measuring the absorbance at 540 nm as shown on the y axis after staining with crystal violet.  (d) *S. intermedius* cells were disrupted by mixing with glass beads (\(\Phi = 100 \mu \text{m}\)) for 10 min and followed by staining with DDAO.  Non-disrupted cells were used as a control.  After washing, non-disrupted cells (I) and glass beads in the cell lysate (II) were observed by confocal fluorescence microscopy.  The 24-h-cultured biofilm of *S. intermedius* WT was treated with DNase I (200 U ml\(^{-1}\)) for 24 h and then stained with DDAO (IV).  Non-treated cells were used as a control (III).  After washing, the *S. intermedius* WT biofilm was observed by confocal fluorescence microscopy.  The results are representative of four different experiments demonstrating similar results.  Data are the mean and SD of four independent experiments.  Asterisks show significant differences between the indicated groups (*, \(P < 0.01\)).

Figure 2  Fluorescence microscopic observations of genomic DNA, extracellular DNA,
and Si-HLP in *S. intermedius* biofilms. A total of 1.0 x 10^7 CFU ml⁻¹ *S. intermedius* ATCC27335 was incubated in BHI broth for 48 h at 37°C. The formed biofilm was blocked with PBS-BSA (1.5%) without fixation and then reacted with rabbit anti-Si-HLP antibody for 1 h at room temperature. After washing with PBS, the biofilm was reacted with Alexa fluor 488 anti-rabbit IgG as the secondary antibody for 1 h, followed by eDNA staining with 2 µM DDAO for 30 min and continued by genomic intracellular DNA staining with 10 µg ml⁻¹ Hoechst 33324. The stained biofilm was then observed using a confocal fluorescence microscope. A2 and A3 show the images of Y- and X-axis cross sections, respectively. All images show that Si-HLP, eDNA and intracellular DNA are stained green, red and blue, respectively. White arrows and white arrowheads show the co-localizations of HLP with eDNA and genomic intracellular DNA, respectively.

**Figure 3** Effect of Si-HLP on biofilm formation by *S. intermedius*. The Si-HLP down-regulated strain (BETAHT), *S. intermedius* wild-type (WT), and 2 control transformant strains (BETT and BETAXT) were anaerobically cultured in BHI medium containing 10 µg ml⁻¹ erythromycin and 20-60 ng ml⁻¹ doxycycline for 24 and 48 h. The formed biofilm mass was quantified by measuring the absorbance at 540 nm as shown on the y axis after staining with crystal violet. Asterisks show significant differences from WT and control transformant strains (*P* < 0.01)

**Figure 4** Effect of DNA addition on biofilm rigidity (a, b) and formation (c) by *S. intermedius*. (a, b) Various concentrations of *S. intermedius* DNA was added into 1.0 x
10^7 CFU ml\(^{-1}\) of \emph{S. intermedius} WT strain. After the 48-h incubation, formed biofilm mass before washing and retained biofilm mass after washing were quantified by the 0.1% crystal violet biofilm assay. Symbols (*) and (#) indicate significant differences versus the control \((P < 0.01)\). (c) Various concentrations of exogeneous DNA purified from \emph{S. aureus} 209P, \emph{E. coli} K12, \emph{P. aeruginosa} PAO1 and KB cells as well as homologous \emph{S. intermedius} DNA were added into 1.0 x 10^7 CFU ml\(^{-1}\) of \emph{S. intermedius} WT, BETT, BETAHT, and BETAXT strains and all \emph{S. intermedius} strains were anaerobically cultured in BHI medium containing 10 \(\mu\)g ml\(^{-1}\) erythromycin and 40 ng ml\(^{-1}\) doxycycline. The crystal violet biofilm assay was performed to quantify the formed biofilm mass after the 48-h incubation. Asterisks show significant differences from WT and control transformant strains (*\(P < 0.01\)).

**Figure 5** Scanning electron microscopy images of the \emph{S. intermedius} biofilm formed in the absence or presence of \emph{S. intermedius} DNA. DNA (1.0 and 100 \(\mu\)g ml\(^{-1}\)) purified from the \emph{S. intermedius} WT strain was added into 1.0 x 10^7 CFU ml\(^{-1}\) of \emph{S. intermedius} WT and \emph{Si-HLP} down-regulated BETAHT strains that were then incubated anaerobically in BHI medium containing 40 ng ml\(^{-1}\) doxycycline for 48 h. The formed biofilms were then observed under SEM. Images are representative of each biofilm.

**Figure 6** Effect of DNA addition on the growth of \emph{S. intermedius}. DNA (1.0 and 10 \(\mu\)g ml\(^{-1}\)) purified from the \emph{S. intermedius} WT strain was added into 1.0 x 10^7 CFU ml\(^{-1}\) of \emph{S. intermedius} WT that was then incubated anaerobically for 12 h. The growth of \emph{S. intermedius} was monitored every 2 h by measuring the absorbance of the culture at OD\(_{600\text{nm}}\).
1 Asterisks show significant differences from control and 1.0 µg/ml groups (*$p < 0.01$).

2
Figure 1a-c. Asikin Nur et al.

(a) OD_{540nm} over time with and without DNase I treatment.

(b) Microscopic images showing cell morphology with and without DNase I treatment.

(c) OD_{540nm} readings at different DNase I concentrations.
Figure 1d. Asikin Nur et al.

(d)

Planktonik Cells

Non-disrupted cells

Cell lysate

Biofilm

DNase I (-)

DNase I (+)
Figure 3. Asikin Nur et al.

24-h biofilm

48-h biofilm

OD$_{540\text{nm}}$

Doxycycline (ng/ml)

- **WT**
- **BETT**
- **BETAHT**
- **BETAXT**

* Indicates significance.
Figure 4. Asikin Nur et al.

(a) S. intermedius DNA (µg/ml)

(b) Biofilm Removal percentage (%)

(c) S. intermedius DNA (µg/ml)

S. aureus DNA (µg/ml)

E. coli DNA (µg/ml)

P. aeruginosa DNA (µg/ml)

KB cells DNA (µg/ml)

- : Before Washing
- : After Washing

- : WT
- : BETT
- : BETAHT
- : BETAXT

OD540nm

0.010 0.1 1 10 100 (µg/ml)
Figure 5. Asikin Nur et al.

S. intermedius DNA (µg/ml)

WT

BETAHT

0  1  100
Figure 6. Asikin Nur et al.

S. intermedius DNA

- 0 µg/ml (control)
- 1 µg/ml
- 10 µg/ml

OD$_{600nm}$ vs. Time (h)