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

2 # *Streptococcus intermedius* biofilm

3

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14 Abbreviated running headline: Effect of eDNA on biofilm formation

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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.

1

2 Keywords:

3 Extracellular DNA, extracellular HLP, biofilm development, *S. intermedius*, DNA

4 concentration

5

1 ## Introduction

2

3 Bacteria accumulate at the biological interface and form biofilms, which are a
4 community of bacterial cells embedded in a self-produced polymeric matrix. This matrix
5 constitutes about 90% of the biofilm mass and mainly consists of extracellular
6 polysaccharides, proteins, lipids, and nucleic acids (Flemming and Wingender 2010).
7 Polysaccharides and proteins are important components as a critical element of the matrix,
8 and extracellular DNA (eDNA) is a common component among various bacterial
9 constituents of the extracellular polymeric substance (EPS) in the biofilm (Hall-Stoodley *et*
10 *al*. 2004). Recently, the role of eDNA has been increasingly recognized in both biofilm
11 structural stability and protection against antimicrobial agents (Mulcahy *et al*. 2008;
12 Whitchurch *et al*. 2002; Vilain *et al*. 2009). Hydroxyl radicals are extremely toxic and
13 readily damage proteins, membrane lipids, and DNA (Farr and Kogoma 1991; Imlay and
14 Linn 1986; Imlay *et al*. 1988), and H_2O_2 releases eDNA from *S. sanguinis* (Kreth *et al*.
15 2009). It has also been reported that eDNA serves as an important structural component of
16 *S. pneumoniae* biofilms and the addition of intact DNA leads to increases in both the biofilm
17 mass and bacterial viability of biofilms (Carollo *et al*. 2010). A previous report showed
18 that the addition of DNase I into initial inocula at concentrations of 40-400 U ml^{-1} inhibited
19 biofilm formation by *S. intermedius*, indicating that eDNA may play an important role in the
20 structure of the *S. intermedius* biofilm (Petersen *et al*. 2004).

21 Biofilm development can be divided into three distinct stages: attachment of bacterial
22 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.

1 # Materials and methods

2

3 ## Bacterial strains, human cell line, and culture conditions

4

5 We previously constructed an inducible antisense *Si-hlp* RNA-expressed *S. intermedius*
6 (*Si*-HLP down-regulated *S. intermedius*) strain, BETAHT, by transforming into *S.*
7 *intermedius* ATCC27335 as a wild-type (WT) strain with a Streptococci–*E. coli* shuttle
8 plasmid harboring the tetracycline-regulated antisense *Si-hlp* gene, a control strain, BETT,
9 by transforming a parent plasmid into the WT strain, and another control strain, BETAXT,
10 by transforming into the WT strain with an irrelevant antisense *x* fragment-inserted shuttle
11 plasmid (Liu *et al*. 2008b). The WT, BETAHT, BETT, and BETAXT strains of *S.*
12 *intermedius* were grown in brain heart infusion (BHI) broth (Difco, Detroit, MI, USA) at
13 37°C under anaerobic conditions with AnaeroPack anaerobic atmosphere generation systems
14 (Mitsubishi Gas Chemical Co. Inc., Tokyo, Japan). *S. intermedius* strains transformed with
15 the plasmids were incubated in BHI containing 10 μg ml^{-1} erythromycin as a selective
16 pressure and 20-60 ng ml^{-1} doxycycline for regulating antisense *Si-hlp* gene (Liu *et al*.
17 2008b). *Staphylococcus aureus* 209P was cultured in BHI under aerobic condition at 37°C.
18 *E. coli* strain K12 and *Pseudomonas aeruginosa* PAO1 were cultured in LB broth at 37°C
19 under aerobic conditions.

20 The KB cell line (derived from a human oral epidermoid carcinoma; kindly provided by
21 Dr. T. Okamoto, Hiroshima University School of Dentistry) was cultured in Dulbecco's
22 modified Eagle's medium (DMEM) (Life Technologies, Grand Island, NY, USA)
23 supplemented with 2 mM L-glutamine, 10% (vol/vol) fetal bovine serum (JRH Biosciences,

1 Lenexa, KA, USA), 50 IU ml^{-1} penicillin, and 50 μg ml^{-1} streptomycin at 37°C in a
2 water-saturated atmosphere of 95% air and 5% CO_2.
3
4 **DNA purification from bacteria and human cells**
5
6 *S. intermedius* ATCC27335, *E. coli* K12, *S. aureus* 209P, *P. aeruginosa* PAO1 or KB cells
7 were suspended in TE buffer (10 mM Tris-HCl [pH 8·0], 1 mM EDTA). Only *S.*
8 *intermedius* and *S. aureus* 209P were treated with mutanolysin (final: 250 units ml^{-1}) for 1 h
9 at 37°C. Each cell suspension was then treated with Proteinase K (final 100 μg ml^{-1}) and
10 SDS (final 0.12%) for 2 h at 50°C. DNA purification from cells was performed using
11 phenol/chloroform extraction and ethanol precipitation methods. Briefly, DNA was
12 extracted with an equal amount of 50 mM Tris-HCl (pH 8.0)-saturated phenol and was
13 precipitated with sodium acetate (final: 0.3M) and 2.5 volumes of ethanol. Extracted DNA
14 was treated with RNase A (final: 10 µg ml^{-1}) for 1 h 37°C, followed by re-extraction with
15 phenol-chloroform (1:1) and precipitation with sodium acetate and ethanol. Purified DNA
16 was finally dissolved in TE buffer. The purity and concentration of purified DNA was
17 assessed by measuring the absorbance at 260 and 280 nm and agarose gel electrophoresis.
18
19 **Quantification of the biofilm mass**
20
21 A crystal violet biofilm assay was used to quantify the biofilm mass of *S. intermedius* as
22 previously described (Moscoso *et al*. 2006). Aliquots of a 1:40 dilution of an overnight

1 bacterial culture (1.0 x 10^7 CFU ml^{-1}) were inoculated into the wells of a 96-well plate and
2 incubated anaerobically at 37°C using the AnaeroPack system for 24 or 48 h. Formed
3 biofilms were washed with PBS without disturbing the adherent biofilm, stained with 50 μl
4 0.1% crystal violet, incubated at room temperature for 15 min, and excess stain was removed
5 by three gentle washes with PBS (pH 7.2). After being dried, the stained biofilm was
6 extracted from the well by adding 50 μl ethanol and was determined by measuring the
7 absorbance of the extract at 540 nm with a microplate reader (model 680; Bio-Rad, Hercules,
8 CA, USA). Sterile BHI broth was substituted for bacterial cultures in control experiments.
9 To determine the inhibitory effect of DNase I on biofilm formation, DNase I (Roche,
10 Mannheim, Germany) was added to the initial inoculum to a final concentration of 200 U
11 ml^{-1} and incubated for 24 and 48 h to form a biofilm. The formed biofilm mass was
12 quantified using the crystal violet biofilm assay as described above. In addition, to
13 determine the effect of eDNA on biofilm stability, the 24-h-cultured *S. intermedius* WT
14 biofilm was treated with DNase I (200 U ml^{-1}) for 24 h and then DNase I-treated biofilm
15 mass was quantified using the crystal violet staining.
16 To investigate the effect of DNA on biofilm formation, various concentrations of DNA
17 purified from *S. intermedius*, *S. aureus*, *E. coli*, *P. aeruginosa* (0.01−100 μg ml^{-1}) or KB
18 cells (0.01−10 μg/ml) were added into 96-wells plate containing 1.0 x 10^7 CFU ml^{-1} of all *S.*
19 *intermedius* strains (WT, BETT, BETAHT and BETAXT) and incubated anaerobically at
20 37°C for 48 h. The 0.1% crystal violet biofilm assay was performed to quantify the biofilm
21 mass as described above.
22 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:

Biofilm removal percentage = (OD_{540nm} before washing − OD_{540nm} after washing) / OD_{540nm} after washing) × 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-hydoxyl-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, Hoechst 33342, DDAO, and anti-*Si*-HLP peptide antibody were used, respectively. The biofilm of *S. intermedius* WT (1.0 x 10^7 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*

1 DNA（1.0 and 10 μg ml^{-1}）purified from the *S. intermedius* WT strain was added into 1.0 x

2 10^7 CFU ml^{-1} of *S. intermedius* WT and incubated anaerobically for 12 h. The growth of *S.*

3 *intermedius* was monitored every 2 h by measuring the absorbance of the culture at OD_{600nm}.

4

5 **Statistical analysis**

6 All statistical analyses were performed using the unpaired Student's *t* test. Differences were

7 considered significant when the probability value was less than 5%.

8

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

1 Ours and other previous studies reported that *Si*-HLP is released outside cells as well as
2 being localized in the intracellular compartment without cell lysis, and the HLPs of
3 *Helicobacter pylori* and *S. pyogenes* have been detected in the culture supernatant and on the
4 bacterial cell surface (Liu *et al*. 2008a; Kim *et al*. 2002; Lei *et al*. 2000; Severin *et al*. 2007).
5 Our recent report further indicated that recombinant *Si*-HLP can bind DNA and alter the
6 structural conformation of DNA *in vitro* (Liu *et al*. 2008b). We next determined the
7 localizations of HLP and eDNA in the *S. intermedius* biofilm using confocal fluorescence
8 microscopy. As shown in Fig. 2, we observed that eDNA and extracellular HLP (eHLP)
9 were present and abundant in a 2-day-old biofilm of *S. intermedius* by immunofluorescent
10 staining without fixation. Some HLP molecules were also co-localized with intracellular
11 DNA and eDNA in the *S. intermedius* biofilm. This observation showing the
12 co-localization of eHLP and eDNA suggests that HLP may form a complex with eDNA and
13 play some roles in biofilm formation and its stability.
14
15 **Effect of *Si*-HLP on biofilm formation by *S. intermedius***
16
17 We previously demonstrated that *Si*-HLP is essential for cell viability and normal growth
18 using gene knockout mutation and *tet*-regulation system-based antisense-mediated gene
19 silencing (Liu *et al*. 2008b). We further determined whether *Si*-HLP could affect biofilm
20 formation by *S. intermedius*. The *Si*-HLP-downregulated strain (BETAHT) formed
21 significantly less biofilm mass than all control strains (WT, BETT and BETAXT) and
22 biofilm mass was dependent on *Si*-HLP expression levels under the control of doxycycline

1 (Fig. 3). This result also suggests that HLP plays a role in biofilm formation.

2

3 **Effect of DNA addition on biofilm formation**

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

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

1 promote biofilm formation and affect the rigidity of the formed biofilm.

2

3 **SEM observations**

4

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

16

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

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

22

Discussion

This study successfully demonstrated that eDNA plays roles in *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 *S. intermedius* biofilms and degraded eDNA in the matrix of the mature biofilm (Fig. 1). These results indicate that eDNA plays essential roles in *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 *P. aeruginosa* biofilm (Whitchurch *et al*. 2002) and another report suggesting that eDNA is important for the development of an *S. intermedius* biofilm (Petersen *et al*. 2004).

Regarding the regulation of biofilm formation, it has been recently reported that *E. coli* H-NS, the histone-like nucleoid structuring protein, plays important roles in regulating biofilm formation (Dalai *et al*. 2009). We previously demonstrated that *Si*-HLP could be released from bacteria without cell lysis as well as being localized in the intracellular compartment (Liu *et al*. 2008a). Here, we observed that abundant *Si*-HLP and DNA were co-localized in the matrix of the 24-h cultured biofilm (Fig. 2). This finding indicates that *Si*-HLP and DNA are the important components of the matrix in a biofilm and suggests that *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 *Si-hlp*, we constructed an

1 inducible antisense *Si-hlp* RNA-expressed *S. intermedius* strain (BETAHT) by transforming
2 into the WT strain with a *Streptococci–E. coli* shuttle plasmid harbouring the inserted *Si-hlp*
3 gene between the tetR/O promoter and Ω fragment in an antisense orientation and
4 demonstrated that doxycycline-induced *Si-hlp* antisense RNA expression specifically
5 inhibited *Si*-HLP protein expression driven by the chromosomal *Si-hlp* locus (Liu *et al.*
6 2008b). Regarding this *tet*-regulation system-based antisense-mediated gene silencing,
7 base pairing between sense mRNA and complementary antisense RNA has been considered
8 to passively block the processing or translation of mRNA, or result in the recruitment of
9 nucleases that promote mRNA destruction (Brantl 2002; Huntzinger *et al.*, 2005). Using
10 these doxycycline-regulated antisense RNA expression techniques, we demonstrated that the
11 *Si*-HLP-downregulated strain (BETAHT) formed significantly less biofilm mass and biofilm
12 mass was dependent on *Si*-HLP expression levels (Fig. 3). This result indicates that HLP as
13 well as eDNA plays an important role in biofilm formation.

14 By adding *S. intermedius* DNA at a low concentration (up to 1 μg ml^{-1}) as exogenous
15 DNA supplementation, the biofilm mass of all tested strains was increased in a
16 dose-dependent manner and the cell density in the formed biofilms of both *S. intermedius*
17 WT and *Si*-HLP-downregulated strains at 1 μg ml^{-1} of DNA was also increased (Figs. 4 and
18 5). Our results correspond with a previous report showing that DNA addition enhanced *S.*
19 *pneumoniae* biofilm mass in a dose-dependent manner and suggest that eDNA is essential
20 for the enhancement of biofilm growth and has an important role in biofilm architecture.
21 (Carrolo *et al.* 2010). Interestingly, we demonstrated that the biofilm mass of the
22 *Si*-HLP-downregulated strain was also increased by the addition of purified DNA, but was

1 still significantly less than other WT and control strains (Fig. 4). Our previous report has
2 shown that the *Si*-HLP-downregulated strain grows significantly more slowly with
3 prolonged lag and logarithmic phases than WT and control strains, and this growth inhibition
4 results from the induction of antisense *Si-hlp* RNA expression controlled with the
5 tetR/O-inducible promoter (Liu *et al*. 2008b). Moreover, we found that the ATP assay and
6 cell numbers counted as CFU showed that the doxycycline-induced *Si*-HLP-downregulated
7 strain displayed lower amounts of intracellular ATP and lower numbers of living cells than
8 those of control strains when their culture reached the same value of OD_{600}, respectively.
9 Therefore, growth inhibition of the *Si*-HLP-downregulated strain may be one of the reasons
10 for the lower ability of this strain to form a normal biofilm.

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

16 In contrast to the effect of eDNA supplementation at lower concentrations (up to 1 μg
17 ml^{-1}), the addition of DNA at higher concentrations (10 and 100 μg ml^{-1}) significantly
18 decreased *S. intermedius* biofilm mass in a dose-dependent manner and the cell density in
19 the formed biofilm with the addition of 100 μg ml^{-1} was markedly decreased (Figs. 4 and 5).
20 We suggested that this opposite effect may be due to the growth inhibitory properties of
21 higher concentrations of DNA because we found that the addition of 10 μg ml^{-1} *S.*
22 *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 μg 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 μg 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

1 development and determine their expression levels are needed and are currently under
2 investigation.
3 It has recently been reported that autolysins (bacterial murein hydrolases) of
4 Gram-positive bacteria, such as *Enterococcus faecalis* and *Staphylococcus epidermidis*, are
5 implicated in biofilm formation, apparently through the mediation of bacterial lysis with the
6 subsequent eDNA release (Guiton *et al*. 2009; Qin *et al*. 2007; Thomas *et al*. 2008, 2009).
7 It has been demonstrated that DNA release displayed in the stationary phase of liquid
8 cultures of pneumococcal cells can occur through cell lysis, rather than through a specific
9 mechanism of secretion, and this release depends on the major autolytic *N*-acetylmuramyl-
10 L-alanine amidase, LytA and the autolytic lysozyme, LytC, and it has also been shown that
11 the competence-dependent release of DNA occurred by autolysis (Tomasz *et al*. 1988,
12 Steinmoen *et al*. 2002; Moscoso and Claverys 2004). Moreover, recent interesting reports
13 have shown that *S. pneumoniae* biofilm formation is influenced by the presence of eDNA,
14 LytA mutants have a decreased capacity to form biofilms, and LytA-induced pneumococcal
15 lysis may be related to biofilm formation through the release of eDNA (Hall-Stoodley *et al*.
16 2008; Moscoso *et al*. 2006). Previous studies have reported that eDNA present in bacterial
17 biofilms results from cell lysis (Perry *et al*. 2009; Kreth *et al*. 2009; Farr and Kogoma 1991;
18 Imlay and Lin 1986; Imlay *et al*. 1988) or is a product of direct secretion from intact cells
19 (Whitchurch *et al*. 2002). However, the origin of eDNA in the *S. intermedius* biofilm is
20 still unclear and is currently under investigation.
21 This is the first report to demonstrate that both homologous and heterologous DNA
22 addition directly affects *S. intermedius* biofilm development and its rigidity, and suggests

1 that all kinds of DNA present at infection sites can increase bacterial biofilm formation.
2 Moreover, our present results clearly show that bacterial histone-like DNA binding protein
3 (HLP) also plays crucial roles in biofilm development by forming a complex with eDNA.
4 Regarding the recognition of bacterial infection, it has been known that the bacterial DNA,
5 one of pathogen-associated molecular patterns, activates transcription factors, including
6 NF-κB and interferon regulatory factors via TLR9-mediated and TLR-independent signaling
7 pathways and induces the production of pro-inflammatory cytokines and type I interferon
8 (Hemmi *et al*. 2000, Takeuchi and Akira 2007.). In addition, we recently reported that
9 bacterial HLP initiates and exacerbates pro-inflammatory reactions during bacterial infection,
10 as well as its physiological role in bacterial growth through DNA binding (Liu *et al*. 2008a).
11 Considering these immunological findings and biofilm as a pathogenic factor, the contents of
12 eDNA and HLP in bacterial biofilms can be used as indicators of the severity of infection.
13 Furthermore, targeting HLP and eDNA may create a novel strategy to fight
14 microorganism-caused infectious diseases, especially those related to biofilm formation.
15
16

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10

1 **Figure legends**

2

3 **Figure 1** Effect of the DNase I treatment on *S. intermedius* ATCC27335 biofilm formation
4 (a, b) and its stability (c, d). (a, b) DNase I (200 U ml^{-1}) was added to the initial *S.*
5 *intermedius* WT inoculum and incubated for 24 and 48 h to form a biofilm. After staining
6 with crystal violet, the formed biofilm mass was quantified by measuring the absorbance at
7 540 nm as shown on the *y* axis (a). Scanning electron microscopy images of *S. intermedius*
8 WT cells after the 48-h incubation with DNase I (b). (c) The 24-h-cultured *S. intermedius*
9 WT biofilm was treated with DNase I (200 U ml^{-1}) for 24 h and then biofilm mass was
10 quantified by measuring the absorbance at 540 nm as shown on the *y* axis after staining with
11 crystal violet. (d) *S. intermedius* cells were disrupted by mixing with glass beads (Φ =
12 100 μm) for 10 min and followed by staining with DDAO. Non-disrupted cells were used
13 as a control. After washing, non-disrupted cells (I) and glass beads in the cell lysate (II)
14 were observed by confocal fluorescence microscopy. The 24-h-cultured biofilm of *S.*
15 *intermedius* WT was treated with DNase I (200 U ml^{-1}) for 24 h and then stained with
16 DDAO (IV). Non-treated cells were used as a control (III). After washing, the *S.*
17 *intermedius* WT biofilm was observed by confocal fluorescence microscopy. The results
18 are representative of four different experiments demonstrating similar results. Data are the
19 mean and SD of four independent experiments. Asterisks show significant differences
20 between the indicated groups (*, $P < 0.01$).

21

22 **Figure 2** Fluorescence microscopic observations of genomic DNA, extracellular DNA,

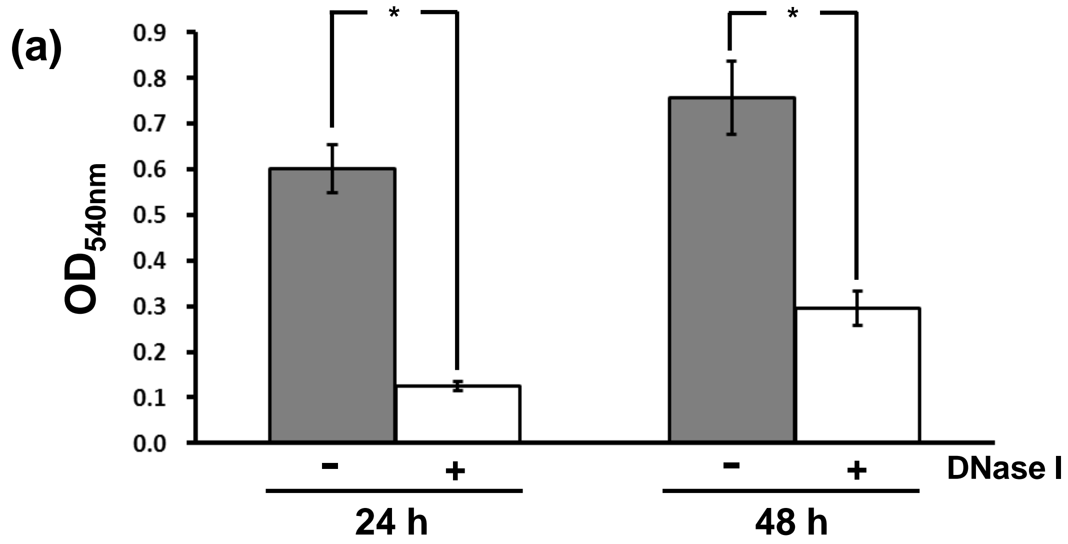
1 and *Si*-HLP in *S. intermedius* biofilms. A total of 1.0 x 10^7 CFU ml^{-1} *S. intermedius*
2 ATCC27335 was incubated in BHI broth for 48 h at 37°C. The formed biofilm was
3 blocked with PBS-BSA (1.5%) without fixation and then reacted with rabbit anti-*Si*-HLP
4 antibody for 1 h at room temperature. After washing with PBS, the biofilm was reacted
5 with Alexa fluor 488 anti-rabbit IgG as the secondary antibody for 1 h, followed by eDNA
6 staining with 2 μM DDAO for 30 min and continued by genomic intracellular DNA staining
7 with 10 μg ml^{-1} Hoechst 33324. The stained biofilm was then observed using a confocal
8 fluorescence microscope. A2 and A3 show the images of Y- and X-axis cross sections,
9 respectively. All images show that *Si*-HLP, eDNA and intracellular DNA are stained green,
10 red and blue, respectively. White arrows and white arrowheads show the co-localizations
11 of HLP with eDNA and genomic intracellular DNA, respectively.
12
13 **Figure 3** Effect of *Si*-HLP on biofilm formation by *S. intermedius*. The *Si*-HLP
14 down-regulated strain (BETAHT), *S. intermedius* wild-type (WT), and 2 control
15 transformant strains (BETT and BETAXT) were anaerobically cultured in BHI medium
16 containing 10 μg ml^{-1} erythromycin and 20-60 ng ml^{-1} doxycycline for 24 and 48 h. The
17 formed biofilm mass was quantified by measuring the absorbance at 540 nm as shown on the
18 *y* axis after staining with crystal violet. Asterisks show significant differences from WT
19 and control transformant strains ($*P < 0.01$)
20
21 **Figure 4** Effect of DNA addition on biofilm rigidity (a. b) and formation (c) by *S.*
22 *intermedius*. (a, b) Various concentrations of *S. intermedius* DNA was added into 1.0 x

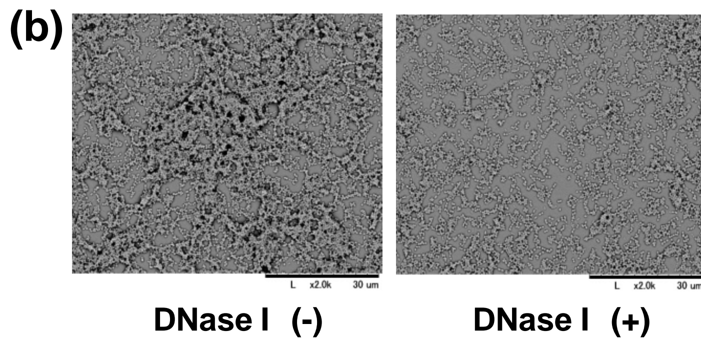
1 10^7 CFU ml^{-1} of *S. intermedius* WT strain. After the 48-h incubation, formed biofilm mass
2 before washing and retained biofilm mass after washing were quantified by the 0.1% crystal
3 violet biofilm assay. Symbols (* and #) indicate significant differences versus the control
4 ($P < 0.01$). (c) Various concentrations of exogeneous DNA purified from *S. aureus* 209P,
5 *E. coli* K12, *P. aeruginosa* PAO1 and KB cells as well as homologous *S. intermedius* DNA
6 were added into 1.0 x 10^7 CFU ml^{-1} of *S. intermedius* WT, BETT, BETAHT, and BETAXT
7 strains and all *S. intermedius* strains were anaerobically cultured in BHI medium containing
8 10 μg ml^{-1} erythromycin and 40 ng ml^{-1} doxycycline. The crystal violet biofilm assay was
9 performed to quantify the formed biofilm mass after the 48-h incubation. Asterisks show
10 significant differences from WT and control transformant strains ($*P < 0.01$).
11
12 **Figure 5** Scanning electron microscopy images of the *S. intermedius* biofilm formed in the
13 absence or presence of *S. intermedius* DNA. DNA（1.0 and 100 μg ml^{-1}）purified from the
14 *S. intermedius* WT strain was added into 1.0 x 10^7 CFU ml^{-1} of *S. intermedius* WT and
15 *Si*-HLP down-regulated BETAHT strains that were then incubated anaerobically in BHI
16 medium containing 40 ng ml^{-1} doxycycline for 48 h. The formed biofilms were then
17 observed under SEM. Images are representative of each biofilm.
18
19 **Figure 6** Effect of DNA addition on the growth of *S. intermedius*. DNA（1.0 and 10 μg
20 ml^{-1}） purified from the *S. intermedius* WT strain was added into 1.0 x 10^7 CFU ml^{-1} of *S.*
21 *intermedius* WT that was then incubated anaerobically for 12 h. The growth of *S.*
22 *intermedius* was monitored every 2 h by measuring the absorbance of the culture at OD_{600nm}.

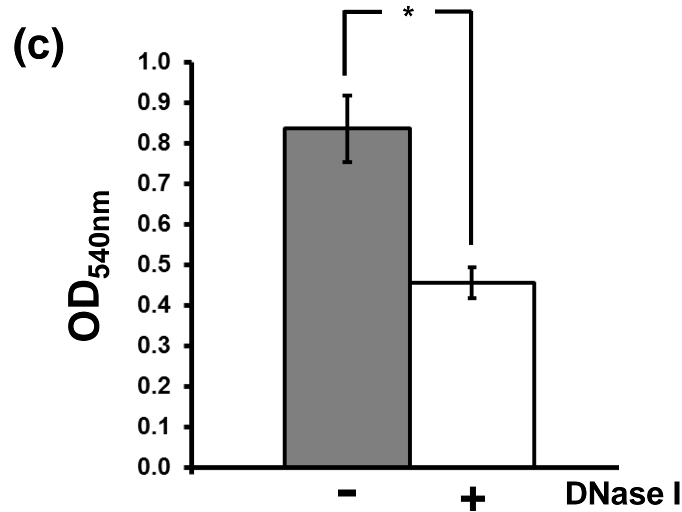
1 Asterisks show significant differences from control and 1.0 μg/ml groups (*$p < 0.01$).

2

Figure 1a-c. Asikin Nur et al.

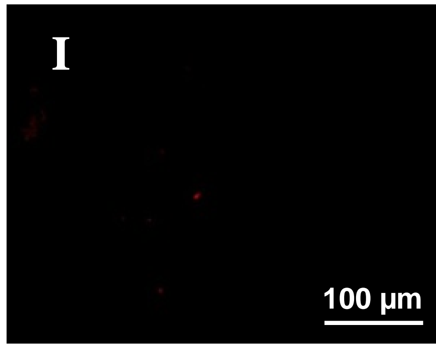




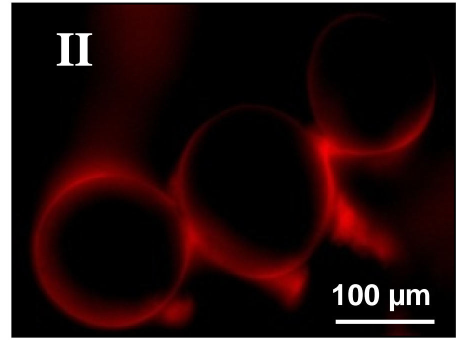


(d)

Planktonik Cells

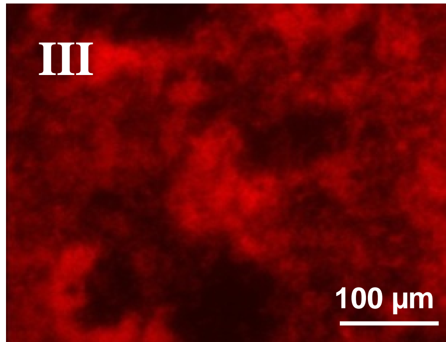


Non-disrupted cells

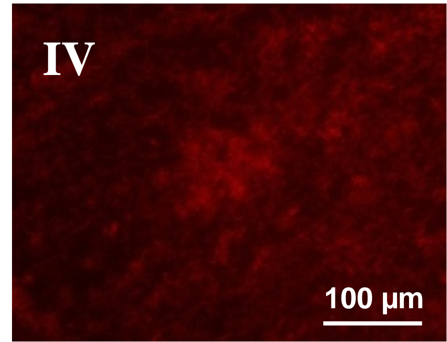


Cell lysate

Biofilm



DNase I (-)



DNase I (+)

Figure 2. Asikin Nur et al.

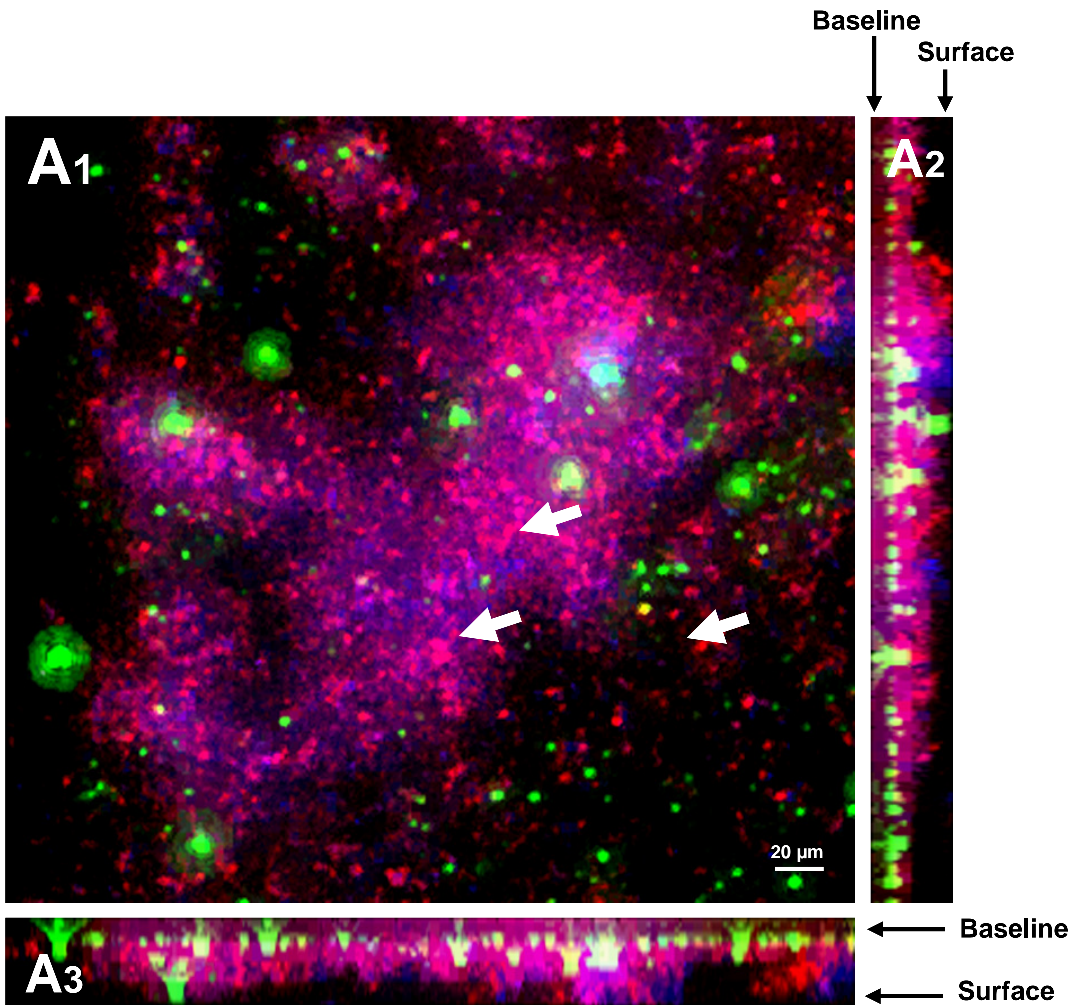


Figure 3. Asikin Nur et al.

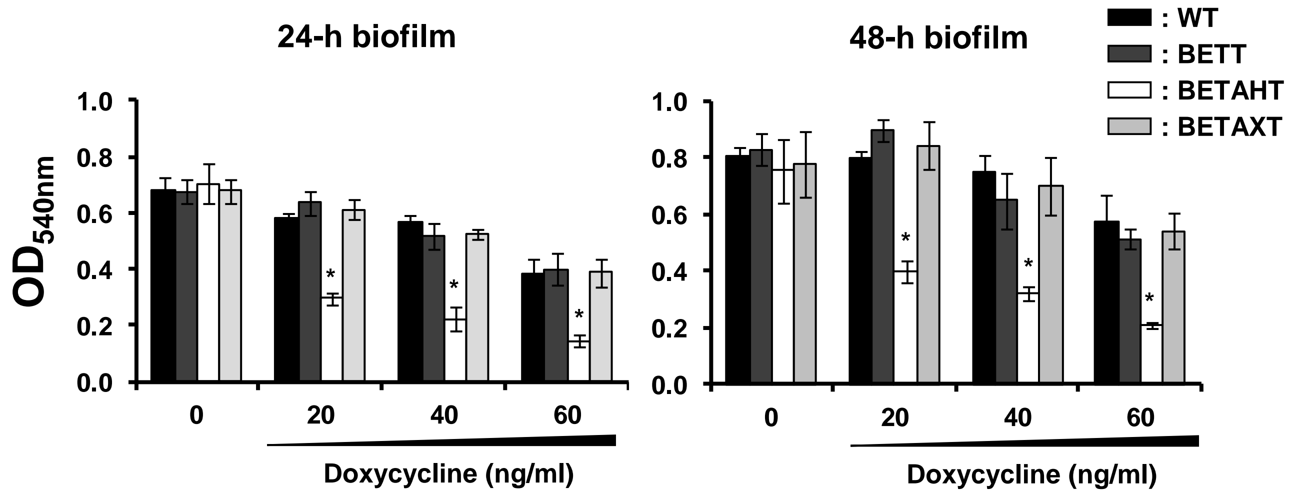
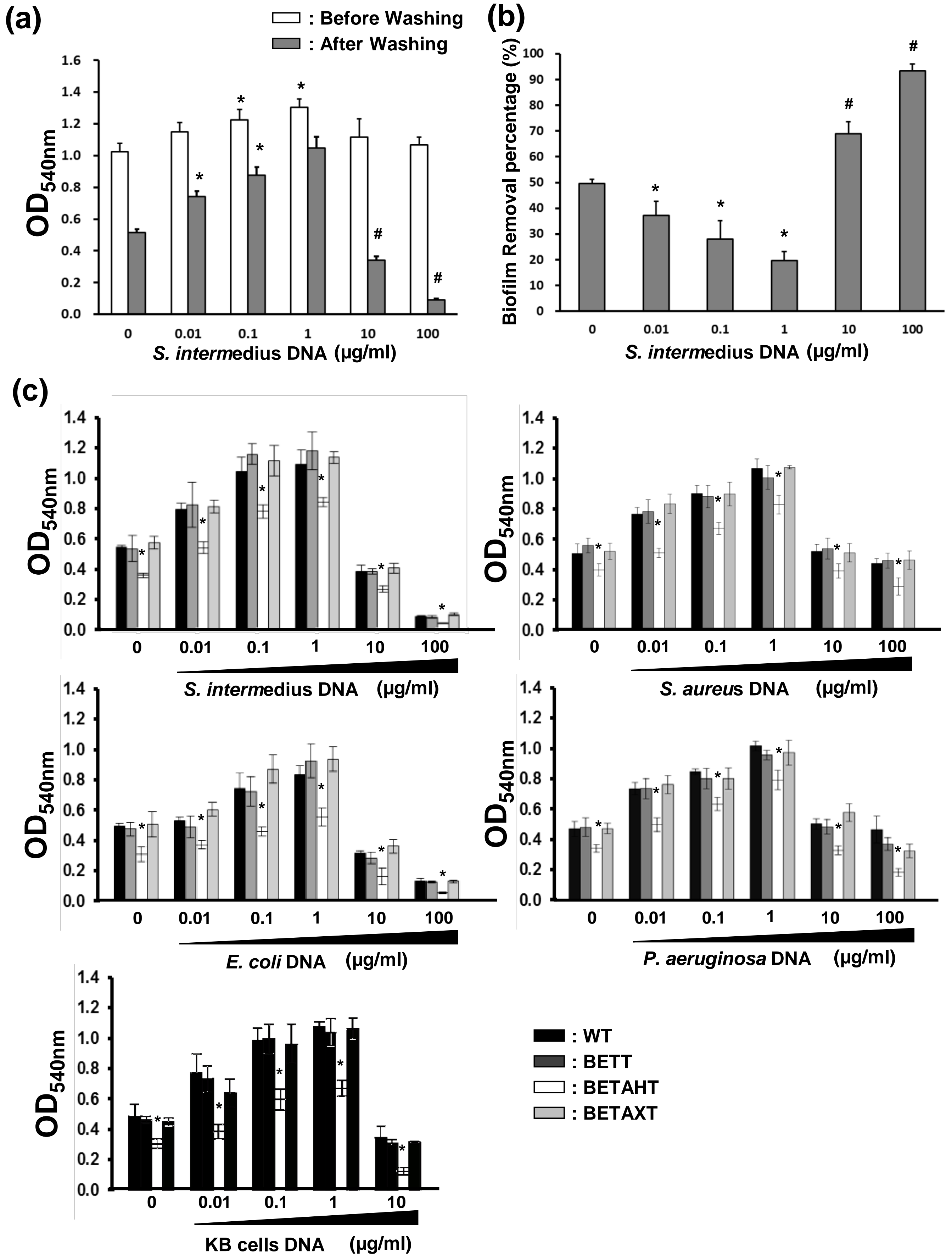


Figure 4. Asikin Nur et al.



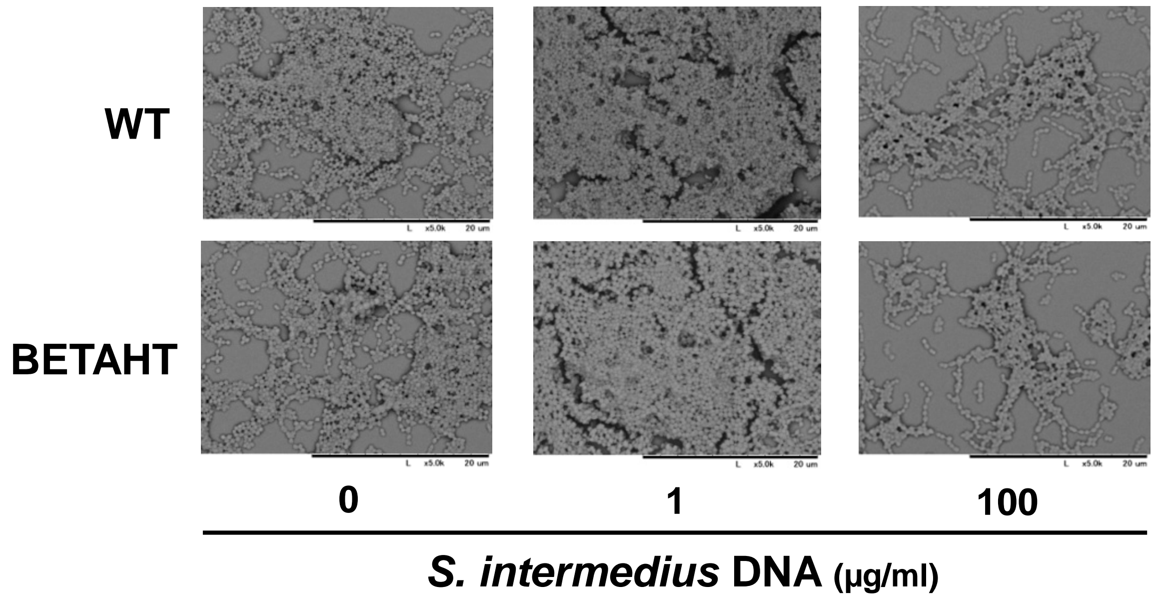

WT
BETAHT
0
1
100
S. intermedius DNA (μg/ml)

Figure 6. Asikin Nur et al.