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Natural antigenic differences in the functionally equivalent extracellular DNABII proteins of bacterial biofilms provide a means for targeted biofilm therapeutics

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SUMMARY

Bacteria that persist in the oral cavity exist within complex biofilm communities. A hallmark of biofilms is the presence of an extracellular polymeric substance (EPS), which consists of polysaccharides, extracellular DNA (eDNA), and proteins, including the DNABII family of proteins. The removal of DNABII proteins from a biofilm results in the loss of structural integrity of the eDNA and the collapse of the biofilm structure. We examined the role of DNABII proteins in the biofilm structure of the periodontal pathogen *Porphyromonas gingivalis* and the oral commensal *Streptococcus gordonii*. Co-aggregation with oral streptococci is thought to facilitate the establishment of *P. gingivalis* within the biofilm community. We demonstrate that DNABII proteins are present in the EPS of both *S. gordonii* and *P. gingivalis* biofilms, and that these biofilms can be disrupted through the addition of antisera derived against their respective DNABII proteins. We provide evidence that both eDNA and DNABII proteins are limiting in *S. gordonii* but not in *P. gingivalis* biofilms. In addition, these proteins are capable of complementing one another functionally. We also found that while antisera derived against most DNABII proteins are capable of binding a wide variety of DNABII proteins, the *P. gingivalis* DNABII proteins are antigenically distinct. The presence of DNABII proteins in the EPS of these biofilms and the antigenic uniqueness of the *P. gingivalis* proteins provide an opportunity to develop therapies that are targeted to remove *P. gingivalis* and biofilms that contain *P. gingivalis* from the oral cavity.

Keywords

Porphyromonas gingivalis; *Streptococcus gordonii*; oral bacteria; DNABII proteins; antibody

INTRODUCTION

One of the most common chronic infections that affects humans is periodontitis, with nearly 50% of the U.S. population having some form of the disease and upwards of 1 billion people

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(10–15% of the population) worldwide being affected with the most severe form of the disease (Albandar 2005; Eke *et al*. 2012; Petersen and Ogawa 2005). Pathogenesis initiates when the normal, commensal, microbial flora of the oral cavity undergoes a dysbiotic shift to a more virulent composition. The Gram-negative anaerobe *Porphyromonas gingivalis* is one of the major species associated with the onset of periodontitis (Choi *et al*. 1990; Dzink *et al*. 1988; Grossi *et al*. 1994; Lamont and Jenkinson 1998).

Within the oral cavity, *Streptococcus* is the predominant genus and *Porphyromonas* represents a small portion of the bacteria present (Lazarevic *et al*. 2009; Zaura *et al*. 2009). The current working model is that cooperative interactions (co-aggregation and syntrophic metabolism) promote the colonization and persistence of a diverse oral commensal community. One well-characterized binding partner of *P. gingivalis* is *Streptococcus gordonii* with a number of components facilitating interactions between these two bacteria having been identified (Maeda *et al*. 2004; Park *et al*. 2005). Due to the extensive interactions between these two species, *S. gordonii* and *P. gingivalis* are a suitable model system for studying the early stages of a multi-species biofilm community.

One of the defining characteristics of bacterial biofilms is the presence of an extracellular polymeric substance (EPS): a self-formed matrix that acts as a protective barrier for the bacteria present within the biofilm while still allowing for intracellular signaling and communication as well as the exchange of nutrients. The components of EPS include a variety of proteins and carbohydrates and perhaps more universally, nucleic acid, primarily in the form of extracellular DNA (eDNA). The nucleic acid present appears to be primarily prokaryotic in origin, although eDNA may also originate from the release of neutrophil extracellular traps (NETs) by polymorphous neutrophils at sites of infection (Brinkmann *et al*. 2004). eDNA has also been observed to be arranged into a highly organized lattice-like structure (Gustave *et al*. 2013; Jurcisek and Bakaletz 2007; Novotny *et al*. 2013) that appears to rely on critical proteins to maintain its structure (Lappann *et al*. 2010).

We previously established that proteins in the ubiquitous DNABII family not only play important roles in intracellular nucleic acid structure but are also important in maintaining the structure of the eDNA component of the bacterial biofilm EPS. The DNABII family of proteins consists of the ubiquitous histone-like protein HU and integration host factor (IHF), which is only present in α- and γ-proteobacteria (Swinger and Rice 2004). Both proteins can bind and bend double-stranded DNA (dsDNA). The HU protein having a role in DNA repair and recombination, has a higher affinity for pre-bent structures, such as cruciforms or Holliday junctions, as well as nicks, gaps, or other sites of DNA damage (Bonnefoy *et al*. 1994; Kamashev and Rouviere-Yaniv 2000; Pontiggia *et al*. 1993). Like the HU protein, the IHF protein, is able to non-specifically bind DNA, however IHF displays a much higher affinity for the consensus sequence WATCAANNNNTTR (Rice *et al*. 1996). Originally identified for its role in bacteriophage lambda integration, IHF has been shown to play a role in most nucleoprotein reactions such as recombination, replication and transcription.

In the EPS of bacterial biofilms, DNABII proteins have been shown to localize to bent and crossed strands of eDNA, where they are hypothesized to stabilize the architecture (Goodman *et al*. 2011). Our previous work established that antibodies derived against the *E.*

coli IHF protein can target the DNABII proteins from a wide range of bacterial species, removing the proteins from the EPS, resulting in the destabilization of the biofilm matrix and the release of the resident bacteria (Brandstetter *et al*. 2013; Brockson *et al*. 2014; Devaraj *et al*. 2015; Goodman *et al*. 2011; Gustave *et al*. 2013; Novotny *et al*. 2013). This ability to target multiple bacterial species with antisera derived against a single DNABII protein is believed to be a result of the high degree of sequence and structural conservation of DNABII proteins across species.

In our present model, the antibodies can bind and sequester free DNABII proteins from the medium. By doing so there is a shift the protein's equilibrium between the unbound and bound states towards the unbound state. This equilibrium shift results in less protein being associated with the EPS. The corresponding destabilization of the EPS leads to the subsequent release of the biofilm bacteria (Brockson *et al*. 2014).

In this paper, we examine the roles of DNABII proteins in the EPS of *S. gordonii* and *P. gingivalis* biofilms, revealing the antigenic distinctness of the DNABII proteins of *P gingivalis*. Additionally, we demonstrate the presence of these proteins within these biofilms, the importance of these proteins and eDNA to the overall biofilm structure, and the abilities of antibodies derived against these two proteins to disrupt biofilms of their respective bacteria. We also demonstrate the conserved function of these proteins across species by demonstrating that the DNABII proteins of one species can be removed from a biofilm and can be complemented functionally by the DNABII proteins from the other species.

METHODS

Bacterial strains and growth conditions

P. gingivalis 381 was maintained on trypticase soy agar supplemented with 5% defibrinated sheep blood, hemin (5 μg/ml), menadione (1 μg/ml), and 1.5% agar under anaerobic conditions (5% hydrogen 10% carbon dioxide, 85% nitrogen) at 37°C. The *P. gingivalis* ΔHUβ mutant was constructed as described previously (Priyadarshini *et al*. 2013) and selection was maintained with added erythromycin (5 μg/ml). Broth cultures of *P. gingivalis* were grown in Todd Hewitt Broth (THB) supplemented with hemin (5 μg/ml) and menadione (1 μg/ml) (THBHK) under anaerobic conditions at 37°C. *S. gordonii* strain Chalis CH1 (DL1) was maintained on THB agar plates with 1.5% agar at 37°C in an atmosphere of 5% CO_2. Broth cultures of *S. gordonii* were grown in THB at 37°C in an atmosphere of 5% CO_2.

Purification of DNABII proteins

IHF and HU from *E. coli* were purified as described previously (Devaraj *et al*. 2015). *S. gordonii* HU was purified as follows. An *S. gordonii* liquid culture grown overnight in THB was diluted 1 to 100 into 1.5 L of chemically defined medium (CDM) (van de Rijn and Kessler 1980) and grown statically for 16 h at 37°C in an atmosphere of 5% CO_2. Cells were pelleted at 7000 *g* for 10 min and resuspended in 10 mM potassium phosphate, pH 7.0, 200 mM potassium chloride (KCl), 1 mM phenylmethanesulfonyl fluoride (PMSF), and 100 μg/ml DNase I. Cell suspensions were lysed by two passages through a French pressure cell

at 20000 psi. Cell lysates were clarified by centrifugation at 39000 *g* for 35 min followed by filtration through a 0.45μm filter. Clarified lysates were bound to a 1 ml heparin-Sepharose column equilibrated with 10 mM potassium phosphate, pH 7.0, and 200 mM KCl. The protein was then eluted with a 20 column volume linear gradient from 200 to 2 M KCl. Fractions were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and fractions containing purified *S. gordonii* HU were combined and dialyzed overnight against 2 L of 50 mM Tris pH 7.4, 600 mM KCl, 1 mM EDTA, and 10% glycerol, drop frozen over liquid nitrogen and stored at −80°C.

The *P. gingivalis* HUα and HUβ proteins were purified using the Intein Mediated Purification with an Affinity Chitin-binding Tag (IMPACT) kit (New England Biolabs) the following manufacturer's protocols. Briefly, the HUα and HUβ genes from *P. gingivalis* were PCR-amplified and cloned into the *Nde*I and *Sap*I sites of plasmid pTXB1, creating C-terminal fusions with the chitin binding protein. The resulting plasmids were then transformed into the *E. coli* strain ER2566, and the resulting transformants were selected on lysogeny broth (LB) (Bertani 1951; 2004) agar plates supplemented with 100 mg/ml of ampicillin at 37°C. Single colonies were picked and grown overnight in LB broth at 37°C, and the resulting cultures were subcultured 1 to 50 into 1.5 L of LB and grown at 37°C with shaking (200 rpm) until the cultures reached an optical density at 600_{nm} of 0.5 (HUβ) or 0.3 (HUα). The expression of the fusion protein was induced through the addition of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 3 h. Cells were then harvested at 7000 *g* for 10 min and resuspended in chitin binding buffer (20 mM Tris-HCl, pH 8.5, 500 mM NaCl, and 1 mM EDTA). PMSF (1 mM) and deoxyribonuclease I (1 mg/ml) were added to the resuspended cells, and the cell suspensions were lysed by two passages through a French pressure cell at 20000 psi. The cell lysates were clarified by centrifugation at 39000 *g* for 35 min followed by filtration through a 0.45 μm filter. Clarified lysates were bound to 5 ml of chitin beads (New England Biolabs) equilibrated in chitin binding buffer and washed with 20 column volumes of chitin binding buffer. On-column cleavage of the fusion proteins was performed by washing the columns with 3 volumes of chitin binding buffer containing 30 mM DTT at 4°C for 70 h before elution of the purified protein. Fractions were analyzed using SDS-PAGE, and the fractions containing purified *P. gingivalis* HUα or HUβ were combined and dialyzed against 2 L of 50 mM Tris, pH 7.9, 500 mM KCl, 1 mM EDTA, and 10% glycerol overnight at 4°C, drop frozen over liquid nitrogen, and stored at −80 °C.

Western blot analysis

DNABII proteins (200 ng) were resolved using a 16% polyacrylamide SDS-PAGE gel and transferred to nitrocellulose membranes for 50 min at 45 V. After transfer, the membranes were blocked using a solution of TBS-T (20 mM Tris HCl, 1 mM NaCl, and 0.1% Tween) containing 5% Blotto non-fat dry milk overnight at 4°C. The membranes were then washed 3 times with TBS-T and probed with the primary antibody at a dilution of 1:1000 for 1 h at room temperature. The membranes were then washed 3 times with TBS-T and probed with a horseradish peroxidase-linked anti-rabbit IgG antibody (Cell Signaling) at a 1:10000 dilution for 1 h at room temperature. The membranes were then washed 3 times with TBS-T, and developed with the Pierce ECL-2 Western Blotting Substrate (Thermo Fisher) and imaged using a Typhoon FLA 7000 laser scanner (GE Healthcare Life Sciences).

In vitro biofilm analysis

S. gordonii was cultured on THB agar overnight at 37°C in an atmosphere of 5% CO_2. The culture was diluted to an OD_{490nm} of 0.65, diluted 1:4 in THB and grown statically at 37°C until an OD_{490nm} of 0.65 was reached. The culture was then diluted 1:2500 in CDM, with 1% glucose as a carbon source, and 200 μl of this culture was used to inoculate each well of an eight-well chambered glass coverslip (Thermo Scientific). The cultures were grown at 37°C with 5% CO_2 for 24 hours to allow for biofilm formation. *P. gingivalis* cultures were grown anaerobically in THBHK at 37°C for 2 days. The resulting cultures were diluted 1:2 in THBHK, grown for 6 hours, and diluted to an OD_{490nm} of 0.1 in THBHK. Two hundred microliters of this culture were used to inoculate each well of an eight-well chambered glass coverslip. The cultures were grown anaerobically for 40 h at 37°C to allow for biofilm formation.

For the addition of exogenous DNABII proteins, 0.5 μM of protein was added to the cultures at the time of biofilm seeding, and calf thymus DNA (ctDNA) was added at the indicated concentrations at the time of seeding. Antisera derived against DNABII proteins were added at a 1:25 dilution 16 hours after biofilm seeding. *S. gordonii* biofilms were grown for an additional 8 h after antisera addition while *P. gingivalis* biofilms were grown for an additional 24 h after antisera addition.

For DNAII protein complementation DNABII antiserum was diluted 1:50 and 0.5 μM of protein was added 16 h after seeding. *S. gordonii* biofilms were grown for an additional 8 h while *P. gingivalis* biofilms were grown for an additional 24 h. *S. gordonii* biofilms were stained with LIVE/DEAD® stain (Molecular Probes, Eugene OR) according to the manufacturer's protocols, washed once with 200 μl of sterile 0.9% NaCl and fixed with fixative solution (1.6% paraformaldehyde, 0.025% glutaraldehyde, and 4% acetic acid in 0.1 M sodium phosphate buffer, pH 7.4). *P. gingivalis* biofilms were washed twice with sterile 0.9% NaCl, stained with LIVE/DEAD® stain, washed an additional two times and fixed with fixative solution. Biofilms were imaged on a Zeiss 510 Meta-laser scanning confocal microscope (Carl Zeiss) using a 63X water objective. Three-dimensional z-stack images were reconstructed using AxioVision Rel. 4.8 (Carl Zeiss) and the average biofilm thickness and total biofilm biomass parameters were determined using the COMSTAT analysis program running on MatLab software. All biofilm conditions were tested in a minimum of 3 independent experiments, with each experiment performed in duplicate for each condition and 4 images captured and averaged from each well.

Immunofluorescence of DNABII proteins within *in vitro* biofilms

Biofilms were grown and stained as described above and fixed for 1 h before being washed twice with 200 μl of Tris buffered saline, pH 7.4 (TBS). The primary antibody (α-DNABII) was added at a 1:150 dilution in TBS and incubated for 1 hour at room temperature. The biofilms were washed two times with 200 μl of TBS and incubated with the ECL-Plex Goat-α-Rabbit IgG Cy5 (GE Healthcare Life Sciences) secondary antibody at a 1:250 dilution for 30 min. The biofilms were then washed twice with 200 μl of TBS and imaged on a Zeiss 510 Meta-laser scanning confocal microscope using a 63X water objective. Three-dimensional z-stack images were reconstructed using AxioVision Rel. 4.8.

RESULTS

The DNABII proteins of *S. gordonii* and *P. gingivalis* are antigenically distinct

We found previously that multiple species of bacteria take advantage of extracellular DNABII proteins to maintain the structural integrity of the eDNA-dependent EPS of their biofilm (Devaraj *et al.* 2015; Goodman *et al.* 2011). Antisera directed against these extracellular DNABII proteins titrate free protein away from the EPS, shifting the equilibrium from an eDNA-bound state to an unbound state, causing the catastrophic collapse of the biofilm and the release of the resident bacteria (Brockson *et al.* 2014). Previous work demonstrated that polyclonal antisera directed against the *E. coli* IHF protein (αIHF_{Ec}) have the ability to recognize DNABII proteins from a wide variety of organisms (Goodman *et al.* 2011). Indeed, αIHF_{Ec} is able to recognize a variety of DNABII proteins including DNABII proteins from the oral microbes *S. gordonii* and *Streptococcus mutans*, as judged by Western blot analysis (Figure 1). Similarly, antiserum derived against the HU protein of *S. gordonii* (αHU_{Sg}) is capable of recognizing other HU-like DNABII proteins. These results are consistent with the conservation of the secondary structure of the DNABII family beyond the modest primary sequence identity/similarity. Intriguingly, αIHF_{Ec} and αHU_{Sg} fail to recognize the DNABII proteins HUα and HUβ of *P. gingivalis* ($\alpha HU\alpha_{Pg}$ and $\alpha HU\beta_{Pg}$, respectively) (Figure 1). This observation is particularly striking because we have shown that $HU\beta_{Pg}$ can complement *E. coli* HU (Priyadarshini *et al.* 2013). To date, this finding represents the first instance that antisera from one DNABII protein failed to cross-react with DNABII proteins of any other species. Additionally, antisera derived against $\alpha HU\alpha_{Pg}$ and $\alpha HU\beta_{Pg}$ display no cross-reactivity with other DNABII proteins tested to date, instead recognizing only the proteins against which they were originally generated against (Figure 1). This result suggests that the DNABII proteins of *P. gingivalis* are antigenically distinct from other DNABII proteins, providing the opportunity to discern species-specific extracellular DNABII proteins within a bacterial community or biofilm.

DNABII proteins are present in *S. gordonii* and *P. gingivalis* biofilms

We showed previously that the ability of αIHF_{Ec} to disperse biofilms requires the titration of DNABII proteins from the EPS (Brandstetter *et al.* 2013; Brockson *et al.* 2014; Devaraj *et al.* 2015; Goodman *et al.* 2011; Gustave *et al.* 2013; Novotny *et al.* 2013). Hence, we first wanted to determine if both *S. gordonii* and *P. gingivalis* biofilms possess extracellular DNABII proteins. Immunofluorescence microscopy was performed on mono-species biofilms of *S. gordonii* and *P. gingivalis* to detect the presence of the DNABII proteins of each species within the biofilm EPS. Biofilms of *S. gordonii* and *P. gingivalis* were grown *in vitro* and then probed with αHU_{Sg}, $\alpha HU\alpha_{Pg}$, or $\alpha HU\beta_{Pg}$ followed by a goat anti-rabbit secondary antibody conjugated to a fluorophore. Confocal laser scanning microscopy revealed that the *S. gordonii* biofilms were extensively labeled when the biofilms were probed with αHU_{Sg} (Figure 2A) but not when the biofilms were probed with antisera raised against *P. gingivalis* $HU\beta_{Pg}$ (Figure 2B). The biofilms of *P. gingivalis* had the greatest signal when probed with $\alpha HU\beta_{Pg}$ (Figure 2C), but no fluorescence was observed when αHU_{Sg} was used (Figure 2D). The use of $\alpha HU\alpha_{Pg}$ resulted in no signal when either *S. gordonii* or *P. gingivalis* biofilms were probed (data not shown). Taken together, these results suggest that DNABII proteins are present in the EPS of biofilms of both *S. gordonii* and *P.*

gingivalis, with *P. gingivalis* biofilms relying on the HUβ$_{Pg}$ protein alone, without an important role for HUα$_{Pg}$.

Antibodies directed against the DNABII proteins of *S. gordonii* and *P. gingivalis* have differing effects on biofilms

Previous work indicated that αIHF$_{Ec}$ has the ability to disrupt biofilms of a wide range of bacterial species (Brandstetter *et al.* 2013; Brockson *et al.* 2014; Devaraj *et al.* 2015; Goodman *et al.* 2011; Gustave *et al.* 2013; Novotny *et al.* 2013). As αIHF$_{Ec}$ exhibited a differing ability to recognize DNABII proteins from *S. gordonii* or *E. coli* and *P. gingivalis* (Figure 1), experiments were performed to determine if the antigenic distinctiveness of those proteins resulted in differing abilities to disrupt biofilms in these two species. COMSTAT analysis of *in vitro* grown biofilms revealed that the addition of αIHF$_{Ec}$ at a 1:25 dilution resulted in a significant decrease in the measured parameters of average thickness and total biomass of *S. gordonii* biofilms (Table 1). However, the antibody had no significant effects on *P. gingivalis* biofilms. Additionally, the same parameters of *S. gordonii* biofilms were also reduced by the addition of the αHU$_{Sg}$ antibody, and as observed with the αIHF$_{Ec}$ antibody, there was no effect on biofilms of *P. gingivalis* (Table 1). Conversely, when biofilms were treated with α-HUβ$_{Pg}$ only *P. gingivalis* biofilms were diminished, and *S. gordonii* biofilms were unaffected. To demonstrate the specificity of the αHUβ$_{Pg}$ antisera, biofilms of the *P. gingivalis* HUβ deletion mutant (PgΔHUβ) were examined. Importantly, these mutants were biofilm-deficient with ~75% reductions in average thickness and biomass relative to the wild-type strain (data not shown), indicating that the absence of the HUβ protein causes a deficiency in biofilm formation. Moreover, αHUβ$_{Pg}$ antisera had no effect on PgΔHUβ biofilms, suggesting that the ability of the antisera to disrupt biofilms is dependent on the ability to recognize the HUβ$_{Pg}$ protein. In addition, antisera derived against the HUα protein of *P. gingivalis* had no effect on either *P. gingivalis* or *S. gordonii* biofilms (Table 1). Taken together, these results are consistent with our working model that the HU protein from *S. gordonii* and the HUβ protein from *P. gingivalis* maintain the structural integrity of the eDNA-based EPS, while the HUα protein from *P. gingivalis* fails to play a similar extracellular role.

DNABII proteins and dsDNA are limiting in *S. gordonii* but not *P. gingivalis* biofilms

Prior work in our laboratory indicated that in uropathogenic *E. coli* (UPEC), extracellular DNABII proteins can be a limiting constituent in biofilm formation, while other EPS components, such as eDNA, are not (Devaraj *et al.* 2015). It was also demonstrated that the addition of exogenous DNABII proteins resulted in an increase in the measured parameters of UPEC biofilms (Devaraj *et al.* 2015). We explored a similar line of investigation here to determine whether the DNABII and eDNA components of the EPS are limiting in *S. gordonii* and *P. gingivalis* biofilms. Mono-culture biofilms were grown *in vitro* and supplemented at the time of seeding with 0.5 μM of DNABII protein from various homologous and heterologous sources. The addition of exogenous DNABII proteins from *S. gordonii* or *E. coli* to *S. gordonii* biofilms resulted in increases of 50–260% for both the measured biomass and average thickness of the biofilm (Table 2). Addition of HUβ$_{Pg}$ to *S. gordonii* also resulted in increases in biofilm average thickness and biomass, albeit to a lesser degree (40–45%) compared to HU$_{Sg}$ and IHF$_{Ec}$. In contrast, the HUα$_{Pg}$ protein had

no effect on biofilms of *S. gordonii*. In the recriprocal experiment, the addition of DNABII proteins had no effect on *P gingivalis*, or the *P. gingivalis* ΔHUβ mutant biofilms. The observed absence of any measurable effect on the PgΔHUβ strain is significant because the deletion of the gene results in a decrease in biofilm formation (data not shown).

The titration of exogenous dsDNA in the form of calf thymus DNA at concentrations ranging from 1 to 10 μg/ml resulted in increases in *S. gordonii* biofilm biomass and average thickness when added at a concentration of 5 μg/ml (Table 3). Both higher and lower concentrations had smaller effects on biofilm size, but those effects were not statistically significant. As observed after the addition of DNABII proteins, the addition of dsDNA to biofilms of both *P gingivalis* and the PgΔHUβ mutant biofilms resulted in no significant changes in the size of the biofilms. According to our model, DNABII proteins and eDNA should work in concert, with the DNABII proteins facilitating the formation of and stabilizing the resulting meshwork of eDNA to protect and support the resident bacterial cells within the biofilm (Brockson *et al.* 2014; Goodman *et al.* 2011). Indeed, increasing the extracellular levels of IHF_{Ec}, $HU\beta_{Pg}$ and HU_{Sg} but not $HU\alpha_{Pg}$ (0.5 μM) and calf thymus DNA (5 μg/ml) in *S. gordonii* biofilms resulted in 390 to 845% increases in the biomass and average thickness of the biofilms (Table 4). Interestingly, even the addition of any of the DNABII proteins in combination with and DNA had no effect on the biofilm parameters of *P. gingivalis* or its isogenic PgΔHUβ mutant, indicating that DNABII and eDNA are not in and of themselves limiting factors within the biofilm EPS for this microbe.

DNABII proteins from *S. gordonii* and *P. gingivalis* can complement one another functionally

Having demonstrated the presence of DNABII proteins in the EPS of *S. gordonii* and *P. gingivalis* biofilms *in vitro* (Figure 2) and the importance of those proteins for the maintenance of the biofilm structure (Table 1), we performed experiments to determine the ability of these DNABII proteins to complement one another functionally within a biofilm structure. To examine this question, biofilms of *S. gordonii* were treated with αHU_{Sg} to deplete the native *S. gordonii* DNABII proteins and simultaneously supplemented with the antigenically distinct $HU\beta_{Pg}$ protein (Figure 3AB&F). Likewise, *P. gingivalis* biofilms were treated with $\alpha HU\beta_{Pg}$ and simultaneously supplemented with the HU_{Sg} protein (Figure 3CD&E). If DNABII proteins are functionally equivalent within the EPS of different species, then the addition of the second heterologous DNABII protein should replace the protein that was removed from the EPS via depletion with species-specific antisera. Indeed, the addition of 0.5 μM $HU\beta_{Pg}$ to *S. gordonii* biofilms treated with a 1:50 dilution of αHU_{Sg} eliminated the observed reductions in biofilm average thickness and total biomass (Figure 3E). Immunofluorescence microscopy probing for the $HU\beta_{Pg}$ protein revealed the extensive presence of the protein within biofilms treated with both αSgHU antisera and the $HU\beta_{Pg}$ protein (Figure 3B), but no protein could be detected in biofilms treated with naïve serum with no addition of exogenous $HU\beta_{Pg}$ (Figure 3A). Conversely, the addition of 0.5 μM HU_{Sg} to *P. gingivalis* biofilms treated with a 1:50 dilution of $\alpha HU\beta_{Pg}$ also resolved the effects of the antiserum on *P. gingivalis* biofilms (Figure 3F). Immunofluorescence microscopy of the *P. gingivalis* biofilms indicated the presence of HU_{Sg} in the biofilms (Figure 3D), but no protein was present in biofilms treated with naïve serum without the addition of the HU_{Sg}

protein (Figure 3C). It should be noted in the z-stack side view in Figure 3D that as the cell density of *P. gingivalis* increased towards the bottom of the biofilm there was a decrease (~2.5 fold) in the DNABII detected (data not shown). This suggests either an inability of the antibodies to sufficiently penetrate the biofilm and bind to the protein or an actual decrease in incorporation of the HU_{Sg} protein within the biofilm. The observed effects were specific to the presence of the specific DNABII proteins, as a heterologous nucleoid-associated protein, (the non-typeable *Haemophilus influenzae* DNA-binding protein H-NS) had no ability to restore the thickness and biomass of *S. gordonii* or *P. gingivalis* biofilms (Figure 3E & F). Additional immunofluorescence microscopy experiments revealed minimal detection of the HU_{Sg} or $HU\beta_{Pg}$ proteins in biofilms treated with antibodies and complemented with a second DNABII protein, indicating that the immunofluorescence signal detected within the biofilm was specific for the DNABII protein added to the biofilm (Figure S1 A & C). The addition of the $HU\alpha_{Pg}$ protein did not affect the biofilm and the protein could not be detected within the biofilm structure by immunofluorescence, again indicating that it is not likely that the protein plays a significant role in the biofilm EPS. As a whole, these data indicated that while the HU_{Sg} and $HU\beta_{Pg}$ proteins are antigenically distinct, they are capable of fulfilling equivalent roles within the EPS of both *S. gordonii* and *P. gingivalis* biofilms.

DISCUSSION

Here, we have demonstrated that the DNABII proteins of *P. gingivalis* are antigenically distinct from those of other bacterial species, particularly *S. gordonii*, which is a known co-aggregation partner within the oral cavity (Figure 1). This report represents the first time that this phenomenon has been observed between members of the DNABII family. It is not entirely surprising that the DNABII proteins of *P. gingivalis* are somewhat unique, as previous biochemical analysis of $HU\beta_{Pg}$ revealed significant differences from other HU-like proteins, such as a low discrimination between various pre-formed DNA structures and a degree of sequence-specific binding that was not observed with other HU proteins (Tjokro *et al.* 2014). It was hypothesized that these differences may be the result of an evolutionary divergence in the DNABII family of proteins; if this hypothesis is true, there should be other DNABII proteins with unique antigenic properties that have yet to be studied. Additionally, antisera derived against the HU_{Sg} protein also displayed a certain degree of specificity, recognizing only HU and not any IHF proteins from the other organisms tested (Figure 1). These initial examples of antigenic specificity suggest that it may be possible to differentiate and target different bacterial species within multispecies biofilms through their DNABII proteins.

The antigenic distinctness of the *P. gingivalis* DNABII proteins is somewhat surprising being that most DNABII proteins share a high degree of sequence similarity, HU_{Sg} and $HU\beta_{Pg}$ share 55% identity and 63% similarity while $HU\alpha_{Pg}$ is somewhat less similar to HU_{Sg} with 34% identity and 54% similarity (Figure S2). All three proteins share a higher degree of similarity in the β-sheet DNA-binding domain of the protein (approximately amino acids 48–83) while having a much more variable dimerization domain comprised of α-helix. Our previous work has identified the DNA-binding domain of the protein as being the most immunoreactive domain of the protein as well as being critical for the biofilm dispersal

activity of anti-DNABII antibodies (Brockson *et al*. 2014). Accordingly, amino acid differences in the DNA-binding domain of the proteins could play a large role in the observed antigenic differences, as well as the observed differences in the abilities of these proteins to promote increases in biofilm. Of particular interest is the isoleucine in the β2' sheet of $HU\beta_{Pg}$ (position 62 in Figure S2) a variation that has not been observed in any DNABII protein studied to date. Additionally, differences in the loop region between the β2' and β3' regions may be of particular importance as this is the loop containing the conserved proline that intercalates between base pairs and induces DNA bending. The lack of this conserved proline could be one of the reasons that $HU\alpha_{Pg}$ does not have any effect on *P. gingivalis* or *S. gordonii* biofilms.

We have also demonstrated that DNABII proteins are present within the EPS of *P. gingivalis* and *S. gordonii in vitro* biofilms, with only HUβ detected in *P. gingivalis* biofilms. These proteins are critical for maintaining the structural stability of the biofilm, as treatment with antisera raised against the DNABII proteins resulted in a collapse of the biofilm structure and a decrease in measured biomass and average thickness. Interestingly, the addition of exogenous DNABII proteins and/or dsDNA to *S. gordonii* resulted in large increases in the measured parameters of average thickness and biomass but had no effect on *P. gingivalis* biofilms. These results suggest that the presence of extracellular DNABII proteins and DNA are limiting factors in *S. gordonii* biofilm formation and growth but are not limiting in the case of *P. gingivalis*. However, the ability to disperse biofilms of both species with antisera derived against their respective DNABII proteins indicates that these proteins are integral to the structural integrity of both species' biofilm EPS. It should also be noted that the biofilm defect of the *P. gingivalis* ΔHUβ strain was not recovered by the addition of exogenous DNABII proteins or DNA. Previous work indicated that $HU\beta_{Pg}$ affects global gene expression in *P. gingivalis* (Priyadarshini *et al*. 2013), and the deletion of this gene appears to result not only in a defect in biofilm formation but also in additional effects that are not recovered through the addition of extracellular protein.

Additionally, we demonstrated that DNABII proteins from different species have the ability to increase the thickness and total biomass of *S. gordonii* biofilms, providing evidence that DNABII proteins play a structural role in the EPS, independent of the source organism. These results also show that DNABII proteins have the ability to complement one another functionally. Functional complementation by DNABII proteins of different species was also demonstrated through the simultaneous addition of antibodies to remove the native DNABII proteins of *S. gordonii* and *P. gingivalis* and supplementation with the DNABII proteins of the other species. The ability of DNABII proteins to complement one another functionally suggests that although *S. gordonii* may be limited for DNA and DNABII proteins *in vitro*, when growing within the complex microbial community of the oral cavity, these bacteria may be capable of sharing portions of the EPS with other species rather than solely relying on their own DNABII proteins and eDNA. Indeed, we have recently shown that uropathogenic *E. coli* (UPEC) is limited for DNABII proteins; the addition of DNABII proteins, regardless of their source, increased the thickness and biomass of UPEC biofilms, driving more planktonic bacteria into the biofilm (Devaraj *et al*. 2015). Thus, it would appear that some bacteria are limited and can scavenge for EPS materials while other bacteria are sufficiently self-supplied.

In our present model, DNABII proteins and eDNA work together as a structural scaffold for the bacteria within the biofilm, irrespective of the original source of the eDNA or DNABII protein present, as most DNABII proteins bind DNA without regard to sequence or source. In fact, limiting the amount of DNABII protein present in the extracellular environment and instead using DNABII proteins and eDNA from other sources may allow for the bacteria to scavenge enough protein and DNA to maintain the structure of the EPS without expending significant energy or resources and to identify the locations of extant biofilms. Limiting extracellular DNABII proteins may also provide an advantage to the bacteria in avoiding the host adaptive immune response. Our previous work with a chinchilla model of otitis media indicated that while immunization with the *E. coli* IHF protein resulted in a robust immune response, immunization with IHF pre-bound to DNA did not have the same effect (Goodman *et al.* 2011). Lowering the levels of extracellular DNABII protein should result in a decrease in unbound protein, reducing the risk of inducing a host immune response to these proteins.

It should be noted that the observed differences in the effects of exogenously added eDNA and DNABII proteins between *S. gordonii* and *P. gingivalis* may also reflect differing strategies and approaches to the initiation and formation of biofilms. The EPS structure is a complex matrix of constituents in which eDNA and DNABII proteins both play a part. Additional proteins and various polysaccharides also play important roles in EPS formation, as do additional host and environmental factors. Bacteria employ a myriad of strategies to form the EPS, and these strategies can vary depending upon the conditions under which the biofilm is formed. The limiting nature of eDNA and DNABII proteins for *S. gordonii* biofilms should be investigated in a more native setting to determine if the obtained results reflect an actual strategy employed by the bacteria when colonizing the oral cavity or if the results are merely a consequence of the physiological conditions under which these experiments were performed. It is possible that changes in the nutritional composition of the growth medium and in environmental conditions, such as temperature, pH, or atmospheric conditions (% CO_2 or O_2), would alter the effects of exogenous eDNA and DNABII proteins on biofilm formation.

As *P. gingivalis* is one of the best characterized periodontal pathogens, significant efforts have been put forth to develop methods to prevent it from establishing itself in a biofilm community. Most of this work has specifically focused on preventing *P. gingivalis* attachment to and outgrowth on *S. gordonii*. Preliminary results have been observed by using small peptides to block the ability of the minor fimbriae of *P gingivalis*, Mfa1, from interacting with streptococcal antigen I/II, SspB (Daep *et al.* 2011), as well as through the use of 2-aminoimidazole and 2-aminobenzimidiazole based small molecule inhibitors to reduce expression of *P. gingivalis* fimbrial genes (Wright *et al.* 2014). Additional work indicated that other small molecules like the sugar alcohol erythritol, as well as gallium and silver ions could inhibit *S. gordonii* and *P. gingivalis* heterotypic biofilm development (Hashino *et al.* 2013; Valappil *et al.* 2012). While these approaches have made progress in preventing *P. gingivalis* from *entering* a biofilm, we believe that the approach presented here will prove more effective in the *removal* of biofilms that have already formed in the oral cavity.

This work provides a framework for examining the interactions of *S. gordonii* and *P. gingivalis* during growth in a mixed biofilm and will allow us to address questions concerning how these bacteria integrate their EPS. This work also provides a framework for examining the interactions of the complex microbial communities that are present in oral biofilms. Additionally, the antigenic differences between the DNABII proteins of these two organisms may allow us to use one of these antibodies, namely $\alpha HU\beta_{Pg}$ to either specifically target biofilms containing *P. gingivalis* for removal (dual-species biofilms between *P. gingivalis* and *S. gondonii* could rely so heavily on *P. gingivalis* $HU\beta_{Pg}$ that they may become vulnerable to $\alpha HU\beta_{Pg}$, allowing dispersal of only *P. gingivalis* rich biofilms) or prevent *P. gingivalis* from entering a biofilm. Indeed, we have previously shown (Justice *et al*. 2012) that preincubation with αIHF_{Ec} inhibits uropathogenic *E. coli* from attaching to epithelial cells suggesting that extracellular DNABII protein is sufficiently plentiful even on planktonic cells to be a target to prevent binding to native surface. Based on this result we predict that αHU_{Pg} could act similarly to prevent *P. gingivalis* from entering a pre-formed biofilm consisting of *S. gordonii*. While the initial studies described here could introduce a new strategy for specifically targeting and removing a pathogen while leaving healthy commensals minimally disturbed within a mixed biofilm population more work needs to be done to fully understand the roles each species DNABII proteins play within mixed-species biofilms. In particular, further study into the extent to which DNABII proteins can functionally complement one another within the EPS of mixed-species biofilms is needed.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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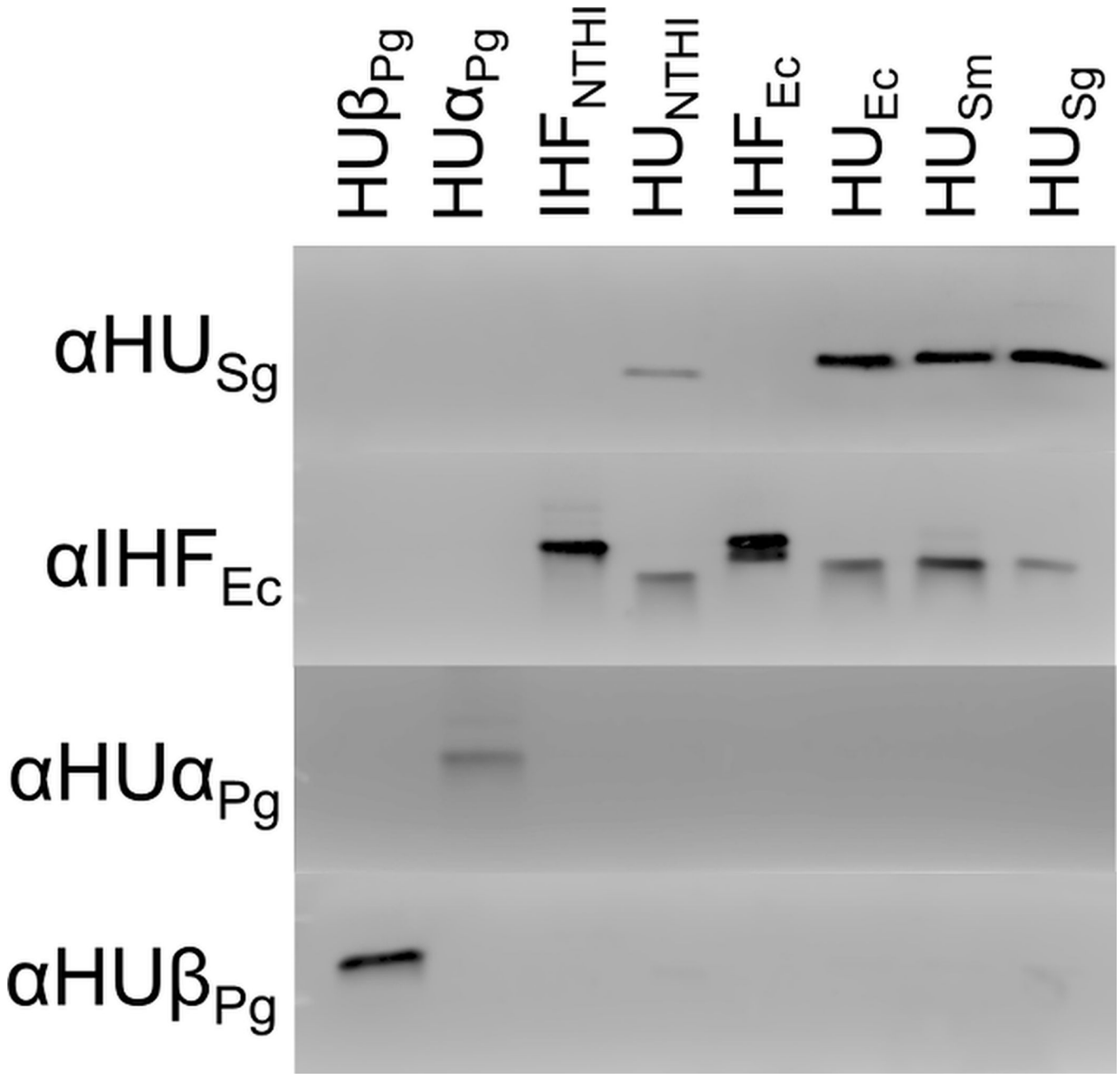

Fig. 1.

Western blot analysis of the recognition of DNABII proteins by DNABII antisera. Antisera derived against *P. gingivalis* DNABII proteins ($\alpha HU\alpha_{Pg}$, $\alpha HU\beta_{Pg}$) displays no cross reactivity, while antisera derived against *E. coli* IHF (αIHF_{Ec}) and *S. gordonii* HU (αHU_{Sg}) display wide cross-reactivity. (Ec; *Escherichia coli*, NTHI; nontypeable *Haemophilus influenzae*, Pg; *Porphyromonas gingivalis*, Sg; *Streptococcus gordonii*, Sm; *Streptococcus mutans*)

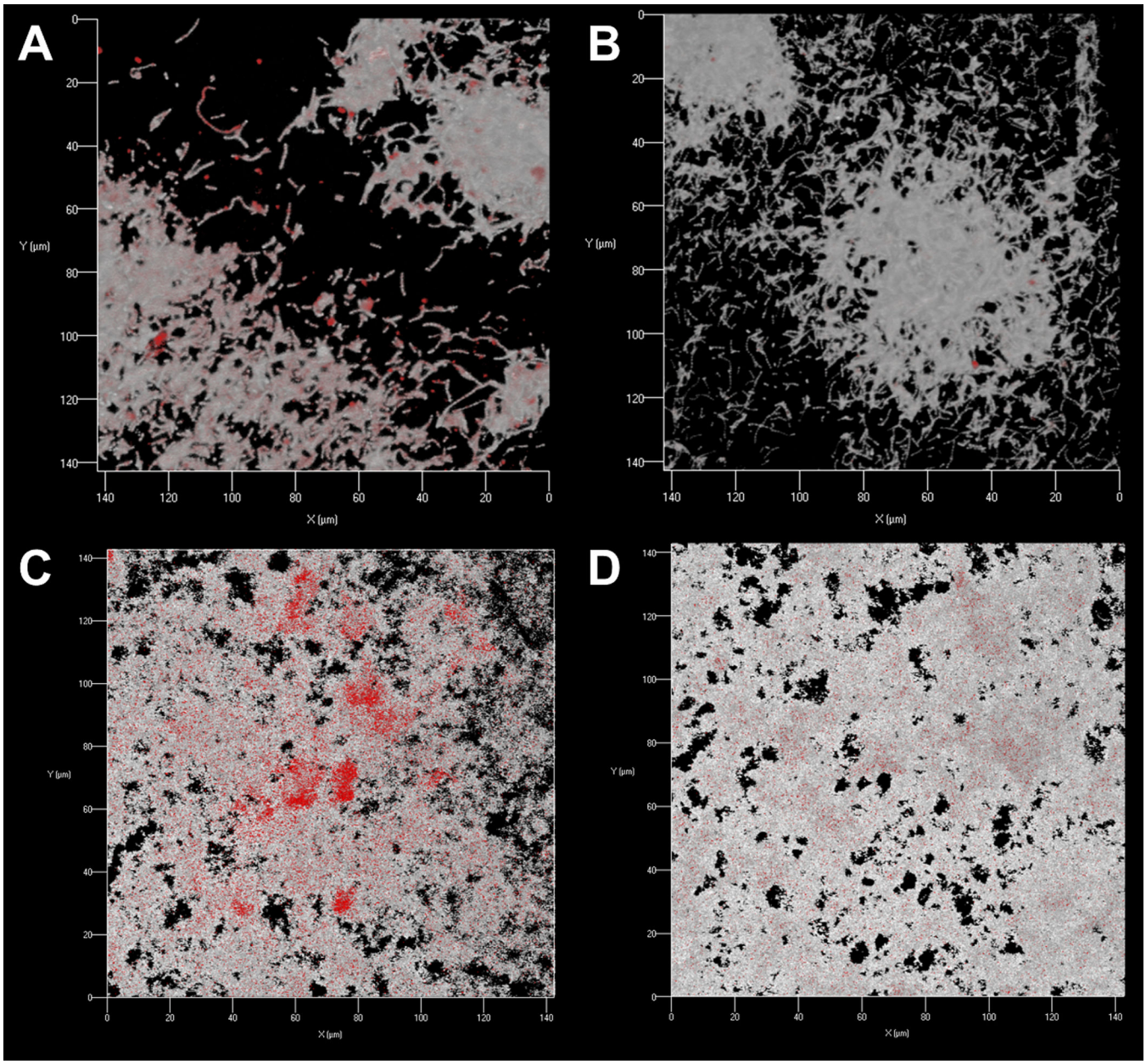

Fig. 2.

Immunofluorescence microscopy of *S. gordonii* biofilms probed with αHU_{Sg} (A) or $\alpha HU\beta_{Pg}$ (B) and *P. gingivalis* biofilms probed with $\alpha HU\beta_{Pg}$ (C) and αHU_{Sg} (D). Biofilms were grown for 24 h in THBHK for *P. gingivalis* and CDM for *S. gordonii*. cells were stained with SYTO-9, which is shown in white, and bound antibodies were labeled with a secondary antibody conjugated to Cy5, which is shown in red.

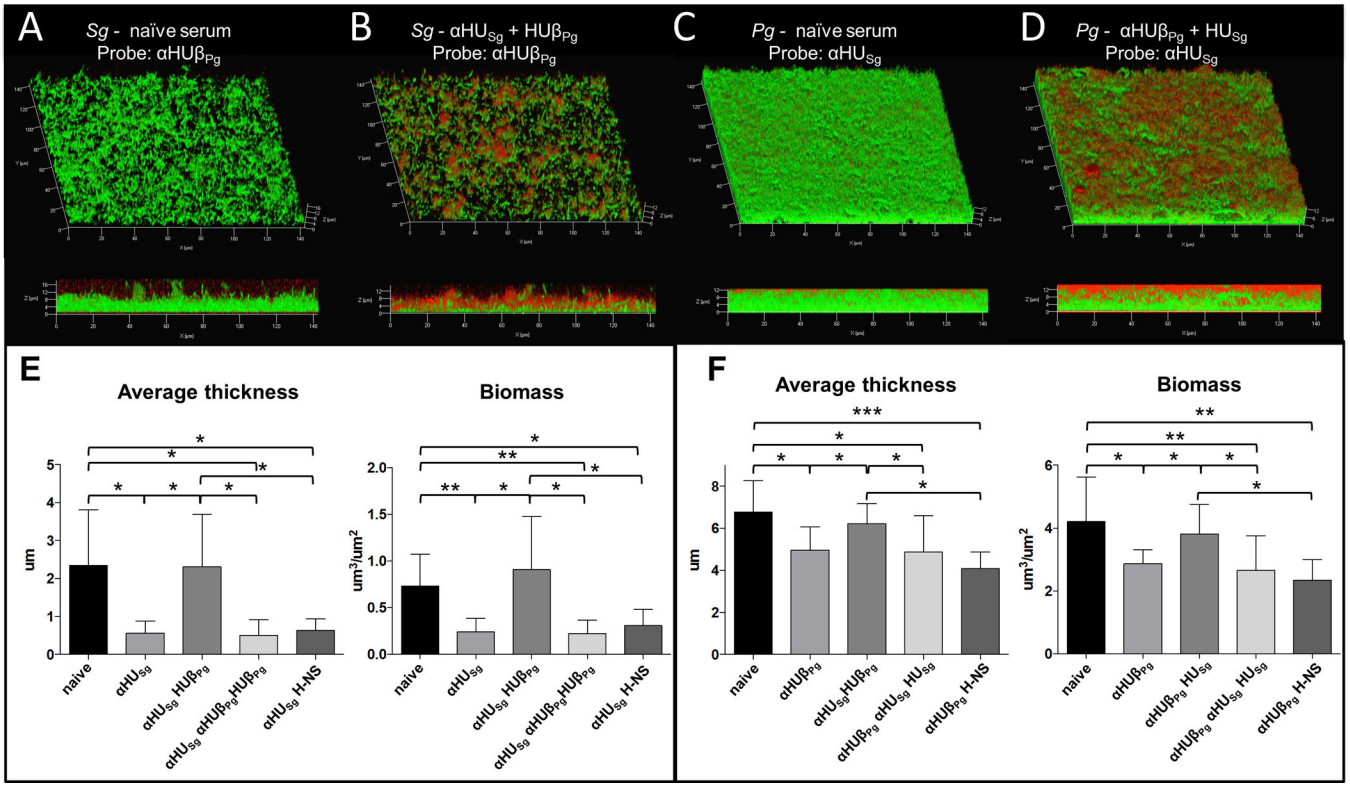

Fig. 3.

Functional complementation of DNABII proteins in *S. gordonii* and *P. gingivalis* biofilms. *S. gordonii* biofilms were treated with either naïve serum alone (A) or αHU_{Sg} antiserum and the $HU\beta_{Pg}$ protein (B) and then probed with $\alpha HU\beta_{Pg}$ antisera. *P. gingivalis* biofilms were treated with naïve serum alone (C) or both $\alpha HU\beta_{Pg}$ antiserum and the HU_{Sg} protein (D) and probed with αHU_{Sg} antisera. The biofilms were stained with SYTO-9 (shown in green), and DNABII-bound antibodies were labeled with a secondary antibody conjugated to Cy-5 (shown in red). The changes in the average thickness and biomass are plotted for both *S. gordonii* (E) and *P. gingivalis* (F).

Table 1

Effects of antisera on biofilms of *P. gingivalis* and *S. gordonii*

P. gingivalis biofilms were grown for 40 h in THB supplemented with hemin (5μg/ml) and menadione (1 μg/ml) in the presence of antisera. *S. gordonii* was grown for 24 h in a chemically defined medium in the presence of antisera.

	Species/Strain		
Percent reduction after addition of:	***P. gingivalis***	***P. gingivalis*ΔHUβ**	***S. gordonii***
αIHF$_{Ec}$			
Avg. thickness (μm)	<15%	<15%	**−65%** **
Biomass (μm^3/μm^2)	<15%	<15%	**−60%** *
αHuβ$_{Pg}$			
Avg. thickness (μm)	**−45%** ***	+ 30%	<15%
Biomass (μm^3/μm^2)	**−45%** ***	+ 30%	<15%
αHUα$_{Pg}$			
Avg. thickness (μm)	<15%	<15%	<15%
Biomass (μm^3/μm^2)	<15%	<15%	<15%
αHU$_{Sg}$			
Avg. thickness (μm)	<15%	<15%	**−85%** **
Biomass (μm^3/μm^2)	<15%	<15%	**−80%** *

P- values are indicated by asterisks:

* $P \leq 0.05$,

** $P \leq 0.01$,

*** $P \leq 0.001$.

Table 2

Effect of the addition of exogenous DNABII proteins on *P. gingivalis* and *S. gordonii* biofilms

P. gingivalis biofilms were grown for 40 h in THB supplemented with hemin (5μg/ml) and menadione (1 μg/ml) in the presence of 0.5 μM protein. *S. gordonii* was grown for 24 h in a chemically defined medium in the presence of 0.5 μM protein.

	Species/Strain		
Percent increase after the addition of:	***P. gingivalis***	***P. gingivalis*ΔHUβ**	***S. gordonii***
IHF_{Ec}			
Avg. thickness (μm)	+25%	<15%	**+195%** **
Biomass ($\mu m^3/\mu m^2$)	+25%	<15%	**+260%** **
HU_{Sg}			
Avg. thickness (μm)	<15%	<15%	**+50%** *
Biomass ($\mu m^3/\mu m^2$)	<15%	<15%	**+90%** *
$HU\alpha_{Pg}$			
Avg. thickness (μm)	<15%	−45%	<15%
Biomass ($\mu m^3/\mu m^2$)	<15%	−45%	<15%
$HU\beta_{Pg}$			
Avg. thickness (μm)	<15%	−30%	**+40%** *
Biomass ($\mu m^3/\mu m^2$)	<15%	−25 %	**+45%** *

P- values are indicated by asterisks:

* $P \leq 0.05$,

** $P \leq 0.01$.

Table 3

Effect of the addition of exogenous dsDNA on *P. gingivalis* and *S. gordonii* biofilms

P. gingivalis biofilms were grown for 40 h in THB supplemented with hemin (5μg/ml) and menadione (1 μg/ml) in the presence of the indicated amounts of dsDNA. *S. gordonii* was grown for 24 h in a chemically defined medium in the presence of the indicated amounts of dsDNA.

Percent increase after the addition of dsDNA	Amount of dsDNA Added			
	1 μg/ml	5 μg/ml	10 μg/ml	15 μg/ml
S. gordonii				
Avg. thickness (μm)	<15%	**+55%** *	<15%	25%
Biomass ($\mu m^3/\mu m^2$)	<15%	**+ 55%** *	<15%	35%
P. gingivalis				
Avg. thickness (μm)	<15%	<15%	−25%	<15%
Biomass ($\mu m^3/\mu m^2$)	<15%	−20%	−25%	<15%
***P. gingivalis* Δ*HU*β**				
Avg. thickness (μm)	<15%	−20%	<15%	−30%
Biomass ($\mu m^3/\mu m^2$)	<15%	−20%	<15%	−30%

P- values are indicated by asterisks:

* $P \leq 0.05$.

Table 4

Effect of the addition of exogenous DNABII proteins and dsDNA on *P. gingivalis* and *S. gordonii* biofilms

P. gingivalis biofilms were grown for 40 h in THB supplemented with hemin (5μg/ml) and menadione (1 μg/ml) in the presence of 0.5 μM protein and 5 μg/ml dsDNA. *S. gordonii* was grown for 24 h in a chemically defined medium in the presence of 0.5 μM protein and 5 μg/ml dsDNA.

	Species/Strain		
Percent increase after the addition of dsDNA and:	***P. gingivalis***	***P. gingivalis*ΔHUβ**	***S. gordonii***
IHF_{Ec}			
Avg. thickness (μm)	<15%	+35%	**+390%** ***
Biomass ($\mu m^3/\mu m^2$)	<15%	+40%	**+495%** ***
HU_{Sg}			
Avg. thickness (μm)	<15%	<15%	**+685%** ****
Biomass ($\mu m^3/\mu m^2$)	<15%	<15%	**+845%** ****
$HU\alpha_{Pg}$			
Avg. thickness (μm)	<15%	<15%	<15%
Biomass ($\mu m^3/\mu m^2$)	−20%	<15%	<15%
$HU\beta_{Pg}$			
Avg. thickness (μm)	<15%	<15%	**+390%** **
Biomass ($\mu m^3/\mu m^2$)	<15%	<15%	**+485%** **

P- values are indicated by asterisks:

** $P \leq 0.01$,

*** $P \leq 0.001$,

**** $P \leq 0.0001$.