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## DNABII proteins play a central role in UPEC biofilm structure

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### Summary

Most chronic and recurrent bacterial infections involve a biofilm component, the foundation of which is the extracellular polymeric substance (EPS). Extracellular DNA (eDNA) is a conserved and key component of the EPS of pathogenic biofilms. The DNABII protein family includes integration host factor (IHF) and Histone-like protein (HU); both are present in the extracellular milieu. We have shown previously that the DNABII proteins are often found in association with eDNA and are critical for the structural integrity of bacterial communities that utilize eDNA as a matrix component. Here, we demonstrated that Uropathogenic *E. coli* (UPEC) strain UTI89 incorporates eDNA within its biofilm matrix and that the DNABII proteins are not only important for biofilm growth, but are limiting; exogenous addition of these proteins promotes biofilm formation that is dependent on eDNA. In addition, we show that both subunits of IHF, yet only one subunit of HU (HupB), are critical for UPEC biofilm development. We discuss the roles of these proteins in context of the UPEC EPS.

### Keywords

EPS; Biofilm; UPEC; UTI89; IHF and HU

### Introduction

Urinary tract infections (UTIs) are the most common bacterial infection of the developed world, with nearly 50% of all women and approximately 12% of men experiencing at least one urinary tract infection (UTI) during their lifetime (Foxman and Brown, 2003). Most importantly, UTIs are recurrent infections, with at least 25% of women enduring a second UTI within 6–12 months (Foxman et al., 2000). Also, the use of indwelling urinary catheters in hospitals leads to a high incidence of catheter-associated urinary tract infections (CAUTI) that cause significant morbidity and mortality (Jaggi and Sissodia, 2012). According to the most recent national hospital discharge survey in 2007, UTIs account for approximately one half million emergency room visits annually in the US alone (Hall et al., 2010) and this leads to a substantial financial and health burden.

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UTIs can be caused by a number of bacteria including *E. coli*, *Klebsiella*, *Proteus*, *Enterococcus* and *Staphylococcus* spp (Zhang and Foxman, 2003). However a diverse group of *E. coli* strains [collectively termed Uropathogenic *E. coli* (UPEC)] are the causative agents of more than 80% of UTIs (Zhang and Foxman, 2003). Despite the flow of urine in the urinary tract, UPEC adhere to and invade the urinary tract epithelium, wherein they replicate rapidly to form intracellular bacterial communities (IBCs) (Mulvey et al., 1998; Anderson et al., 2003). IBCs are a biofilm-like state that is observed during the course of UPEC infection in animal models and human infections (Mulvey et al., 1998; Anderson et al., 2003; Trautner and Darouiche, 2004). Also, biofilm formation on urinary catheters plays a primary role in the pathogenesis of CAUTI and creates a reservoir for chronic UTIs (Saint and Chenoweth, 2003; Trautner and Darouiche, 2004). Antibiotics are the first-line of treatment for UTIs (Kodner and Thomas Gupton, 2010). According to the Antimicrobial Resistance Epidemiological Survey on cystitis, approximately 48% of *E. coli* isolates that cause cystitis are resistant to ampicillin, 30% are resistant to trimethoprim/sulfamethoxazole, 19% are resistant to nalidixic acid and more than 10% are resistant to at least three different classes of antimicrobial agents (Schito et al., 2009). Also the high incidence of recurrence of UTIs results in repeated antibiotic treatment that negatively impacts the commensal microbiota and, in turn, can result in secondary infections in the gastrointestinal tract and vagina (MacDonald et al., 1993; Dethlefsen et al., 2008). Therefore, there is a critical need for the development of novel ways to undermine UPEC pathogenesis mediated by biofilms.

According to the Centre of Disease Control and Prevention, two-thirds of bacterial infectious processes involve biofilms (Davies, 2003). Growth in a biofilm lifestyle results in a highly organized, sessile but metabolically active, multicellular microbial community enclosed in Extracellular Polymeric Substance (EPS) that is attached to an abiotic or biotic surface. The composition of biofilm EPS is dictated by the microbe and its surrounding environment and is primarily comprised of water, exopolysaccharides, extracellular proteins and extracellular DNA (eDNA) (Flemming and Wingender, 2010). The EPS plays several functional roles including aggregation of bacterial cells, adhesion to biotic and/or abiotic surfaces, cohesion of biofilms, nutrient source, retention of water and providing a protective barrier (Flemming and Wingender, 2010). By acting as a protective barrier, the EPS renders the biofilm recalcitrant to removal by antimicrobial agents and by effectors of both the innate and adaptive immune systems (Slinger et al., 2006; Starner et al., 2008). Since disruption of the biofilm matrix and the release of bacteria resident within the biofilm is sufficient to increase susceptibility to the action of both the immune system and antimicrobials (Flemming and Wingender, 2010; Goodman et al., 2011; Cavaliere et al., 2014), it is essential to understand and elucidate the molecular interactions that contribute to the stability of the biofilm in order to define the key components of the matrix that promote resistance.

Extracellular DNA (eDNA) is common to most bacterial biofilms and serves as a structural component of these biofilms (Steinberger and Holden, 2005; Qin et al., 2007; Izano et al., 2008; Guiton et al., 2009; Flemming and Wingender, 2010; Nur et al., 2013). We have shown, that in biofilms formed *in vivo* by non-typeable *Haemophilus influenzae* (NTHI),

eDNA is organized in an interwoven mesh-like structure (Jurcisek and Bakaletz, 2007). A similar organization of eDNA is also apparent in sputum samples recovered from cystic fibrosis (CF) patients that were culture positive for *Burkholderia cenocepacia* (Novotny et al., 2013), *Pseudomonas aeruginosa*, and *Staphylococci* (Gustave et al., 2013). This organization of eDNA in the biofilm is similar in apparent architecture to the preferred substrates of the DNABII family of proteins (Kamashev and Rouviere-Yaniv, 2000).

The DNABII proteins are a family of proteins that are ubiquitously expressed by all eubacteria and play an integral role in shaping the intracellular bacterial nucleoid structure and function (Browning et al., 2010). DNABII proteins include IHF (integration host factor) and HU (histone-like protein), which bind to DNA as dimers and condense DNA upon binding (Rice et al., 1996). The DNABII proteins have a high affinity (nM,  $K_d$ ) to pre-bent secondary structures of DNA (Kamashev and Rouviere-Yaniv, 2000). Although both IHF and HU bind to and bend DNA, IHF exhibits sequence specificity (WATCAANNNTTTR where W is A or T and R is a purine) while HU binds to DNA in a sequence independent manner (Rice et al., 1996). The DNABII family of proteins is also found in great abundance in the extra-bacterial milieu of Gram-positive bacteria (Winters et al., 1993; Lunsford et al., 1996; Gao, 2000; Boleij et al., 2009; Nur et al., 2013). We have previously shown that not only are the DNABII proteins extra-bacterial, but that they also bind to and stabilize the interwoven mesh-like structure of eDNA in biofilms formed *in vivo* by NTHI (Goodman et al., 2011), *Pseudomonas aeruginosa* (Gustave et al., 2013), *Staphylococci* (Gustave et al., 2013) and *Burkholderia cenocepacia* (Novotny et al., 2013). Nur *et al.*, have also demonstrated that in *S. intermedius*, Histone-like protein (HLP) is found co-localized with eDNA in the extra-bacterial milieu and suggested that this eDNA-HLP complex may play a role in biofilm formation (Nur et al., 2013).

In UPEC strain UTI89, IHF is also found in the matrix of IBCs formed *in vivo* in a female mouse bladder during experimental UTI (Justice et al., 2012). Further, we have shown that in UPEC strain UTI89, both IHFA and IHFB are required for both colonization of the mouse bladder and type I piliation (Justice et al., 2012). We have also demonstrated that antiserum directed against *E. coli* IHF destabilizes the biofilm architecture and results in disruption of biofilms formed by multiple human pathogens *in vitro* (Goodman et al., 2011). Also, immunological targeting of extracellular IHF results in clearance of NTHI biofilms from the middle ear in a chinchilla model of otitis media (Goodman et al., 2011), and dispersal of polymicrobial sputum aggregates recovered from cystic fibrosis patients (Gustave et al., 2013; Novotny et al., 2013). Collectively, these observations suggest that sequestration of DNABII proteins from the biofilm matrix facilitates biofilm disruption *in vitro* (Goodman et al., 2011; Brandstetter et al., 2013; Brockson et al., 2014) and *in vivo* (Goodman et al., 2011; Gustave et al., 2013; Novotny et al., 2013) with subsequent immune mediated clearance that serves to eradicate pre-formed biofilms *in vivo* (Goodman et al., 2011). While our studies to date have demonstrated that DNABII proteins are important in maintaining the structural integrity of bacterial biofilms, how the individual components of the biofilm matrix contribute to overall biofilm architecture has remained unclear.

To determine the contribution of DNABII proteins to the biofilm architecture, we supplemented UPEC strain UTI89 biofilms via the exogenous addition of recombinant

DNABII proteins and also analyzed the ability of DNABII deficient mutants to form biofilms *in vitro*. We found that exogenous addition of DNABII proteins dramatically increased the size of biofilms formed *in vitro* by UPEC strain UTI89. We also found that while both subunits of IHF were critical for UPEC biofilm formation, only one subunit of HU (HupB) was required for *in vitro* biofilm formation. These data suggested that DNABII proteins were limiting and reinforced our hypothesis that they play a critical role in maintaining the structural integrity of UPEC biofilms.

## Results

### DNABII proteins are limiting in UTI89 biofilms

To determine the contribution of IHF to biofilm stability, we grew biofilms *in vitro* with recombinant *E. coli* IHF (rIHF; to 0.5  $\mu$ M) added exogenously at the time of seeding and maintained throughout the 40 hours of biofilm growth. In preliminary experiments, *E. coli* rIHF was titrated from 5 nM to 1.0  $\mu$ M and we found that 0.5  $\mu$ M *E. coli* rIHF was optimal for *in vitro* biofilm analysis. When compared to the biofilm formed in the absence of exogenous *E. coli* rIHF (Figure 1A), there was a dramatic increase in the size of the biofilm formed when *E. coli* rIHF was added exogenously (Figure 1B). Through COMSTAT analysis of images generated in multiple replicate assays, the measured parameters of biofilm maximum height, average thickness and biomass increased by 53%, 88% and 61%, respectively upon addition of *E. coli* rIHF (Figure 1C, D). Similar increases in biofilm architectural parameters could also be achieved by the addition of 0.5  $\mu$ M recombinant *E. coli* HU (rHU) as well as recombinant HU from the Gram-positive microbe *Streptococcus mutans* (UA159), another member of the DNABII protein family, to UTI89 biofilms at seeding (Figure S1). Importantly, the exogenous addition of DNABII proteins (*E. coli* rIHF or *E. coli* rHU; to 0.5  $\mu$ M) to three additional clinical isolates of UPEC at seeding resulted in an increase in the size of the biofilm formed as evidenced by a significant increase in the measured parameters of maximum biofilm height, average thickness, and biomass (Figure S2). Collectively, our results suggested that DNABII proteins are generally limiting in biofilm formation by UPEC and further, that other matrix components are likely present at sufficient levels to support additional biofilm growth in the presence of an excess of extra-bacterial recombinant DNABII proteins.

### Extracellular DNA (eDNA) is critical for DNABII protein mediated biofilm formation by UPEC strain UTI89

To our knowledge the presence of eDNA in the matrix of biofilms formed by UPEC strain UTI89 has not been documented. To determine whether UTI89 incorporated eDNA within its biofilm matrix, we labeled unfixed *in vitro* preformed UTI89 biofilms with a monoclonal antibody against double stranded DNA (dsDNA) (red). The biofilm biomass was labeled with FilmTracer™ FM® 1–43 (green). The *in vitro* biofilm formed by UPEC strain UTI89 incorporated dsDNA that was uniformly distributed throughout the biofilm matrix as evident from the distribution of red fluorescence observed (Figure 2A). The addition of 1 unit of DNase to the biofilms at seeding dramatically decreased the observed red fluorescence (Figure 2B) and served as a control. Further, in the presence of DNase, the relative abundance of dsDNA as determined by the ratio of the dsDNA (anti-dsDNA labeling) to the

bacteria (FilmTracer™) revealed a statistically significant decrease in the amount of eDNA incorporated within the biofilm matrix compared to the control (Figure S3). To determine the contribution of eDNA to biofilm formation, we grew biofilms *in vitro* with DNase added exogenously at the time of seeding and maintained throughout the 40 hours of biofilm growth. When compared to the control, there was a dramatic decrease in the size of the biofilm formed in the presence of DNase as evidenced by the significant decrease in maximum height (Figure 2C), average thickness (Figure 2D), and biomass (Figure 2D). Biofilm formation in the presence of both DNase and *E. coli* rIHF was significantly decreased compared to the control but was similar to that of biofilms formed in the presence of DNase (Figure 2C, D) suggesting that eDNA is required for DNABII protein-mediated biofilm formation. Moreover, biofilm formation in the presence of calf thymus DNA was not significantly different compared to the control and also the biofilm formation in the presence of both calf thymus DNA and *E. coli* rIHF was not significantly different compared to the biofilms formed in the presence of just *E. coli* rIHF (data not shown) suggesting that eDNA is not limiting in biofilm formation in UPEC strain UTI89. Collectively, these data demonstrated that UTI89 biofilms formed *in vitro* incorporated dsDNA within the biofilm matrix, and that eDNA is not limiting but is nonetheless critical for DNABII protein-mediated biofilm formation.

### Overproduction of *E. coli* rIHF increases biofilm formation by UTI89

The above results demonstrated that exogenous addition of *E. coli* rIHF (or other DNABII family members) can increase biofilm formation and that IHF is limiting in UTI89 biofilms. To confirm this result, we repeated the *in vitro* biofilm formation assay using a merodiploid UPEC strain that overproduced *E. coli* IHF via generation of a mutant wherein one copy of the IHF genes was placed under control of an inducible promoter. When compared to the biofilm formed without induction of *E. coli* rIHF ('-IPTG' lanes in Figure 3A, B), biofilms formed under conditions of overproduction of rIHF ('+IPTG' lanes), showed a significant increase in maximum height, average thickness, and biomass. Since overproduction of *E. coli* rIHF in the intra-bacterial milieu resulted in increased biofilm formation, we reasoned that there was an increase in the accumulation of IHF in the extracellular milieu that mediated the observed biofilm increase. To directly test this, we purified proteins from spent medium of either wild type UPEC strain UTI89 or UPEC that was induced to overproduce *E. coli* rIHF when grown statically (for 6h or 16h, respectively) to compare the extra-bacterial steady state levels of IHF. We could not detect IHF in the spent medium of the wild type (wt) strain at either of the time points tested. However there was an accumulation of HU at 16h (Figure 3C). This outcome suggested that IHF was either limiting or at the detection limit of the assay under the growth conditions employed. However, IHF and relatively more HU accumulated in the spent medium in the strain that overproduced *E. coli* rIHF in comparison to the wild type in each of the conditions tested. These results suggested that there is a coordination of the extra-bacterial and intra-bacterial concentrations of IHF. Overproduction of rIHF also resulted in a significant increase in the abundance of eDNA within *in vitro* preformed biofilms (Figure S3). Taken together with data shown in Figure 1, these findings are supportive of our hypothesis that IHF is part of the extracellular matrix that contributes to the structural integrity of the UPEC strain UTI89 biofilm.

### Both subunits of IHF contribute to *in vitro* biofilm formation in UTI89

To determine the contribution of each of the subunits of IHF to biofilm formation, we employed the wild type (WT) UPEC strain UTI89 or isogenic IHFA deficient strain UTI89 *ihfA11::Tn10* (Table S1) or isogenic IHFB deficient strain UTI89 *ihfB* (Table S1) in our *in vitro* biofilm formation assay. As shown in Figure 4A, B, whereas the WT strain produced a robust biofilm *in vitro*, both UTI89 *ihfA11::Tn10* and UTI89 *ihfB* were less robust as judged by the significant defect in maximum height. UTI89 biofilm deficient in IHFA had a significant defect in average thickness, while the average thickness of UTI89 *ihfB* biofilm was comparable to the WT strain (Figure 4B). The biofilm biomass of UTI89 strains deficient in IHFA and IHFB was comparable to the WT (Figure 4B). These results demonstrated that IHFA and IHFB contribute to *in vitro* biofilm formation in UPEC strain UTI89 and also suggested that the structure of the biofilm is differentially affected as evident from average thickness (Figure 4B) in the absence of either subunit of IHF. This result is in agreement with previous findings (Justice et al., 2012) that there are significant differences in the overall architecture of the biofilm formed by UTI89 *ihfA11::Tn10* and UTI89 *ihfB* *in vivo* in a female mouse bladder. Notably, in the presence of exogenous recombinant *E. coli* DNABII proteins, the maximum height and average thickness of the biofilms formed by UTI89 *ihfA11::Tn10* increased significantly (Figure 4C) and were comparable to that of the WT. However, as shown in Figure 4D, there was no significant increase in the maximum height or average thickness of UTI89 *ihfB* biofilms in the presence of any of the exogenously added recombinant DNABII proteins. These data suggested that IHFA played a major role in the extracellular milieu while IHFB may have additional intracellular functions that cannot be complemented with exogenously added recombinant protein. To determine the contribution of each of the subunits of IHF to the incorporation of eDNA within the biofilm matrix, we determined the distribution and abundance of eDNA in *in vitro* preformed biofilms of UTI89 *ihfA11::Tn10* and UTI89 *ihfB*. We observed no significant difference in the distribution of eDNA by visual inspection (data not shown), and abundance of eDNA compared to the control (Figure S3) within the limits of this assay as presented. These data suggested that the observed defect in biofilm formation by UTI89 strains deficient in IHFA or IHFB could be attributed solely to the absence of each respective subunit of IHF from the extracellular matrix.

### Individual IHF subunits increase *in vitro* biofilm formation by UPEC strain UTI89

The above results demonstrated that both subunits of IHF were involved in biofilm formation. To more thoroughly examine the roles of the individual IHF subunits, we exogenously supplemented *in vitro* formed UTI89 biofilms with 1.0  $\mu$ M recombinant *E. coli* IHFA or recombinant *E. coli* IHFB at the time of seeding and biofilms were grown for 40 hours. When compared to the biofilm formed in the absence of exogenous rIHFA or rIHFB, as shown in Figure 5A, there was a significant increase in biofilm formation in the presence of either rIHFA or rIHFB as judged by maximum height and average thickness. We also observed a significant increase in average thickness and biomass of the *in vitro* formed biofilms when either of these subunits was overproduced (Figure 5B, C). These data reinforced the idea that both subunits of IHF play an integral role in UTI89 biofilm formation and also suggested that individual IHF subunits retain the ability to bind to eDNA.



### Effect of disruption of HU expression on UTI89 biofilm formation

Since exogenous addition of recombinant HU from *E. coli* or *S. mutans* increased *in vitro* biofilm formation (Figure S1) and HU accumulated in the spent medium of UTI89 (Figure 3C), we were interested in the role of the two subunits of HU in biofilm formation. To this end, we employed the wild type (WT) UPEC strain UTI89 or the isogenic strain deficient in HupA (UTI89 *hupA::cam*) or the isogenic strain deficient in HupB (UTI89 *hupB::kan*) in our *in vitro* biofilm formation assay. As shown in Figure 6A, B the UTI89 strain deficient in HupA did not exhibit a defect in biofilm formation in comparison to the WT strain when grown under the same conditions. However, as shown in Figure 6C, the biofilms formed by the HupB deficient strain exhibited a significant defect in maximum height and average thickness in comparison to the WT strain. The biomass of the biofilm formed by UTI89 *hupB::kan* strain was comparable to that of the WT strain (Figure 6D). Remarkably, the height and average thickness of the biofilms formed by UTI89 *hupA::cam* and UTI89 *hupB::kan* increased significantly in the presence of exogenous DNABII proteins (Figure 6A, C). We have also demonstrated that the qualitative distribution (data not shown) and abundance of eDNA in the biofilms formed by UPEC strains deficient in HupA or HupB was similar to that of the control (Figure S3) within the limits of this assay as presented, suggesting that the observed defect in biofilm formation in UTI89 *hupB::kan* was solely due to the lack of HupB. Taken together, these results suggested that HupB was directly involved in UTI89 biofilm formation.

### UPEC strain UTI89 partitions towards the biofilm state in the presence of *E. coli* rIHF

As COMSTAT analysis demonstrated that there was a significant increase in biofilm biomass upon incubation with *E. coli* rIHF (Figure 1B, D), and since the growth rate of UTI89 is not affected by exogenous addition of *E. coli* rIHF (data not shown), we reasoned that the presence of added rIHF was inducing partitioning of bacteria to the biofilm/adherent state from the planktonic state. To determine whether there was a relative increase in adherent (i.e. biofilm) bacteria upon incubation with *E. coli* rIHF, we enumerated the total number of bacteria present within the chamber slide, both free (planktonic, in the spent medium) and adherent (part of the biofilm). As shown in Figure 7, there was a significant increase in the adherent bacteria and a corresponding significant decrease in the planktonic bacteria in the presence of *E. coli* rIHF. These results agree well with the COMSTAT analysis (Figure 1D) and reinforce our hypothesis that in the presence of *E. coli* rIHF, more bacteria partition towards the biofilm state.

### UPEC strain UTI89 biofilm formed in the presence of *E. coli* rIHF is functionally similar to that formed with no added rIHF

Since the addition of recombinant DNABII proteins resulted in a bigger biofilm as judged by maximum height, biomass and average thickness, it was imperative to determine if the structures formed in the presence of exogenously added recombinant DNABII proteins were similar to native biofilms in terms of several selected biological phenotypes. To address this, first we determined the Minimum Inhibitory Concentration (MIC<sub>100</sub>) of ciprofloxacin (CIP) for UTI89 grown either planktonically or as a biofilm in either the presence or absence of exogenously added *E. coli* rIHF. As shown in Table 1, the MIC of CIP for UTI89 biofilm in

the presence of rIHF was indistinguishable from that of the native UTI89 biofilm. Consistent with previous observations of increased resistance of bacterial biofilms to antimicrobial agents (Slinger et al., 2006; Kaji et al., 2008; Starner et al., 2008), the MIC of CIP was more than 2500 times greater than that of UTI89 in the planktonic state. Second, we exposed the planktonic and biofilm state UTI89 that had been grown in the presence or absence of exogenous *E. coli* rIHF to hydrogen peroxide for 30 min and then enumerated the bacteria that survived the challenge. As shown in Figure 8A, while none of the UTI89 grown in the planktonic state (with or without added exogenous *E. coli* rIHF) survived exposure to 0.2% hydrogen peroxide, the biofilm state (irrespective of the presence or absence of exogenous rIHF) was resistant to incubation with 0.8% hydrogen peroxide as demonstrated by  $\sim 10^7$  viable CFU/ml. Lastly, we measured the relative expression of Antigen 43 (Ag43), a UPEC protein that is specifically expressed in the biofilm state when cultured in minimal medium (Schembri et al., 2003; Biscola et al., 2011) by RTPCR analysis and Western blot. As shown in Figure 8B, and C, while there was no expression of Ag43 by UPEC when grown in the planktonic state, there was similar expression of this bacterial protein when grown in the biofilm state either with or without added exogenous *E. coli* rIHF. Collectively, these results demonstrated that the biofilm formed with exogenous *E. coli* rIHF retains several functional attributes of a native biofilm. Together, our data suggested that the presence of *E. coli* DNABII proteins during biofilm development was limiting and that additional protein promoted the formation of the biofilms that were indistinguishable from native biofilms in specific biological functions tested here.

## Discussion

In this study, we have demonstrated that DNABII proteins are critical components of the UPEC strain UTI89 biofilm matrix and further, that these proteins effectively directly or indirectly facilitate partitioning of bacteria to the biofilm state when added exogenously. The observed increase in biofilm maximum height, average thickness and biomass in response to the exogenous addition of recombinant DNABII proteins (Figure 1, S1, S2) suggested that these proteins are limiting in biofilms formed by multiple clinical isolates of UPEC. In this regard, it is interesting that such a critical component of the matrix is limiting, given that biofilms are the preferred state of growth for most bacteria. We speculate that there is a delicate balance between IHF bound to eDNA within the biofilm matrix and free IHF in the extracellular milieu such that there is sufficient IHF in the EPS in order to maintain the structural integrity of the biofilm yet an insufficient excess to elicit an adaptive immune response. Indeed, it is evident from the resolved structure of IHF bound to DNA, as elucidated by x-ray crystallography, that a large portion of the protein is occluded by bound DNA, namely specific regions of the N and C terminus and  $\beta$ -ribbons that contain the contact points for DNA (Rice et al., 1996). We have also shown that while immunization of chinchillas with *E. coli* rIHF resulted in a protective adaptive immune response, immunization with *E. coli* rIHF pre-bound to an excess of DNA failed to induce an effective immune response in a chinchilla model of experimental otitis media (Goodman et al., 2011).

The DNABII family is a group within a class of proteins called Nucleoid Associated Proteins (NAPs) where members, while not related in either primary or secondary structure, all affect the global conformation of bacterial chromatin. In *E. coli* the NAP protein family



includes at least 11 other proteins in addition to IHF and HU (Ali Azam et al., 1999). Interestingly, two NAPs Fis and H-NS that are highly expressed in the exponential phase (Ali Azam et al., 1999), have a predominant role in DNA structure intra-bacterially (Shiraishi et al., 2007; Cendra Mdel et al., 2013; Flatten and Skarstad, 2013; Ohniwa et al., 2013; Sato et al., 2013), yet have no effect on biofilm formation when added exogenously within the limits of the assays as presented here (Figure S4). This outcome suggested that the two tested NAPs do not have a dominant extracellular role in biofilm formation in this UPEC strain, under these growth conditions.

Extracellular DNA (eDNA) has been identified as a major component of the biofilm matrix of several Gram-positive and Gram-negative bacteria (Steinberger and Holden, 2005; Qin et al., 2007; Izano et al., 2008; Guiton et al., 2009; Flemming and Wingender, 2010). In this study, we have demonstrated that UPEC strain UTI89 incorporated dsDNA within its biofilm matrix and that the eDNA was distributed throughout the biofilm matrix (Figure 2A, B). We have previously shown that the biofilm formed by NTHI during experimental otitis media and sputum samples recovered from cystic fibrosis patients that were culture positive for *Burkholderia cenocepacia* included an abundant amount of double stranded, extra-bacterial DNA that assembled into an intricate lattice-like network that resembled cruciform DNA structures (Goodman et al., 2011). *E. coli* HU has been shown to bind to DNA with bent architectures and to branch-like DNA structures (Kamashev and Rouviere-Yaniv, 2000). It has also been shown that *E. coli* HU has a high affinity to cruciform DNA structures (Pontiggia et al., 1993). Although the nature of eDNA has yet to be determined, if the interwoven mesh-like structures that have been observed is a derivative of the cruciform structure, these data suggest that the DNABII family of proteins likely play a predominant role in maintaining the structural integrity of eDNA in a biofilm through high affinity binding and DNA secondary structure stabilization (Tjokro et al., 2014). In support of this hypothesis, we have shown that the exogenous addition of DNase or of DNase and *E. coli* rIHF in combination to UTI89 biofilms resulted in a significant decrease in the size of the biofilm compared to the control (Figure 2C, D), suggesting that DNABII proteins mediate biofilm formation likely by stabilizing the secondary structure of eDNA.

It is not well understood as to how the DNABII proteins that do not contain a signal sequence for export exit the bacterial cell into the extracellular milieu. In this study we have shown that overproduction of *E. coli* rIHF in UTI89 resulted in accumulation of IHF in the spent medium (Figure 3) and an increased amount of eDNA in the biofilm matrix (Figure S3), suggesting that the release of IHF and DNA is likely a stochastic process. It has recently been demonstrated in *Burkholderia cenocepacia* strain K56-2, that the Type VI secretion system (T6SS) contains a binding site for IHF directly upstream of the coding sequence and that the export of IHF into *in vitro* formed biofilms was dependent on an active T6SS suggesting that IHF could possibly regulate its own export via the T6SS in *B. cenocepacia* (K56-2) (Novotny et al., 2013). Such an active mechanism of export of DNABII proteins has not been described in UPEC strain UTI89. We envision that these proteins and DNA gain access to the extracellular milieu by passive or active cell lysis or autolysis (Claverys and Havarstein, 2007; Rice and Bayles, 2008). It is intriguing that HU is indispensable for the signal recognition particle complex (SRP) in Gram-positive bacteria

(Nakamura et al., 1999); recently it was shown that HU may even associate with the 4.5s RNA of the SRP of *E. coli* (Macvanin et al., 2012). It is possible that HU could exit the bacterial cell via this system. Each of these mechanisms is being further investigated.

IHF is crucial for regulation of a number of genes that contribute to virulence including production of elastase in *Vibrio vulnificus* (Jeong et al., 2010), cholera toxin in *Vibrio cholera* (Stonehouse et al., 2008), and type III secretion effectors in *E. coli* (Li et al., 2004). Regulation of type I pilus and P- pilus in UTI89 (Justice et al., 2012), adhesins and capsule in *Neisseria* (Hill et al., 2002) and *E. coli* (Rowe et al., 2000; Corcoran and Dorman, 2009) are also dependent on expression of IHF. In this study we have shown that both subunits of IHF play a role in UTI89 biofilm formation. However, exogenous *E. coli* rIHF could only restore biofilm formation in the absence of IHFA but not IHFB, suggesting that IHFB likely plays a more critical role in the intracellular milieu. It is conceivable that IHFB indirectly affects biofilm formation through the Type I pilus. In support of this hypothesis, we have previously shown that while both subunits of IHF are required for type I piliation in UPEC strain UTI89, only IHFB maintains the type I pilus promoter in the 'ON' state. Further, the ectopic production of Type I pilus in UTI89 *ihfB* fully restored virulence, which suggests that the major defect in the absence of IHFB is the lack of expression of Type I pilus (Justice et al., 2012). Since the Type I pilus of UPEC has been linked to virulence and biofilm formation (Hultgren et al., 1985; Pratt and Kolter, 1998; Martinez et al., 2000; Bahrani-Mougeot et al., 2002; Justice et al., 2006; Snyder et al., 2006; Ulett et al., 2007; Wright et al., 2007; Crepin et al., 2012; Hung et al., 2013), we envision that IHFB affects biofilm formation via the expression of Type I pilus. Also, the ability of other components of the EPS to support biofilm growth in the absence of IHFB remains to be evaluated.

The characterization of biofilms formed by UTI89 *ihfA11::Tn10* and UTI89 *ihfB* suggested that the two subunits of IHF have a differential effect on the structure of the biofilm as judged by the average thickness (Figure 4B). This may indicate that individual homodimers of IHF have independent and unique functions, which is in sharp contrast to the null phenotype observed in laboratory *E. coli* strains that lack either subunit of IHF (Werner et al., 1994; Zulianello et al., 1994). To support our data, we have previously demonstrated that while both IHFA and IHFB are required for colonization of the mouse bladder, the community architecture of UTI89 that could not express IHFA was strikingly different from that of the UTI89 mutant that could not express IHFB. In the absence of IHFA, the IBCs formed by UTI89 exhibited a more globular architecture while in the absence of IHFB the IBCs were more open and elongated suggesting that the homodimers of IHF have unique functions (Justice et al., 2012). Alas, we were previously unable to construct a mutant that contained a deletion of both *ihf* alleles in UTI89 to test if there would be, as we might expect, an even stronger biofilm phenotype in a *ihfA ihfB* double mutant (Justice et al., 2012). Other studies have shown that individual IHF subunits form homodimers *in vitro* in complex with DNA and that either subunit can support Lambda site-specific recombination (Werner et al., 1994; Zulianello et al., 1994) *in vitro*, suggesting that the homodimers of IHF are functional. Together, these data reinforced our hypothesis that individual IHF homodimers have independent functions and it is likely that homodimers and heterodimers of IHF play unique roles in biofilm formation by UTI89.

We have yet to determine if the roles of IHF and HU in eDNA dependent biofilm EPS are different, similar or redundant. IHF and HU are both highly expressed in the early and late stationary phases (Ali Azam et al., 1999) and an increased accumulation of these proteins in the spent medium is evident as shown in Figure 3C. IHF and HU have some redundant functions in terms of DNA binding and it is possible that these proteins act independently, compensate or substitute for the lack of the other in order to maintain the structural integrity of eDNA. It is also conceivable that these proteins coordinate with each other to support biofilm formation by UTI89 and likely other strains. In support of this hypothesis, Bonnefoy et al., have shown that homo and hetero dimers of HU modulate the DNA binding activity of IHF to random linear dsDNA sequences (Bonnefoy and Rouviere-Yaniv, 1991). One could also imagine possible interaction of IHF monomers with HU monomers to form mixed heterodimers that modulate the structural integrity of eDNA. To further investigate this, we determined the distribution of eDNA within *in vitro* preformed UPEC biofilms and showed that in the presence of homodimers of IHF (strains UTI89 *ihfA11::Tn10* and UTI89 *ihfB*) or homodimers of HU (strains UTI89 *hupA::cam* and UTI89 *hupB::kan*), the qualitative distribution (data not shown) and abundance of eDNA (Figure S3) within the biofilm matrix was similar to that of the wild type UPEC biofilm within the limits of the assay as presented here which suggested that these proteins could compensate or substitute for the lack of the other in order to maintain the distribution and likely structural integrity of eDNA. The fine structural analysis of eDNA in the presence of homodimers of IHF or HU remains to be further evaluated. Also, the biofilms formed in the presence of homodimers of IHF or HU (same strains as above) were not more susceptible to hydrogen peroxide compared to the wild type UPEC biofilm (data not shown). The susceptibility of these biofilms to antimicrobial peptides remains to be evaluated. Hence it remains an open question as to how redundant each DNABII protein is with respect to one another in terms of biological functions performed within the extracellular matrix.

Most intriguing is that HU from the Gram-positive bacterium *S. mutans*, when added exogenously to UPEC strain UTI89, resulted in a dramatic increase in the size of the biofilm (Figure S1). This result suggested that the DNABII proteins are likely interchangeable between various bacterial genera and, in turn, implies a similar eDNA structure that is compatible with a consortia of heterologous bacteria within a biofilm. In support of this hypothesis, we have previously shown that antiserum directed against *E. coli* rIHF destabilizes the biofilm architecture and notably disrupts biofilms formed by multiple Gram-positive and Gram-negative human pathogens *in vitro* (Goodman et al., 2011). We envision that the structural arrangement of eDNA in a biofilm and the proteins that stabilize the secondary structure of the eDNA have evolved to support a compatible EPS such that most of the constituent bacterial species within a given ecological niche could co-exist and intermingle.

This study identifies key constituents of the EPS that contribute to the integrity of the biofilm matrix formed by UPEC strain UTI89. Given that IHF affects biofilm formation *in vitro* and community architecture of UTI89 *in vivo* and also regulates expression of a number of genes associated with virulence such as Type I pilus, P pilus and capsule (Rowe et al., 2000; Justice et al., 2012) suggests that a therapeutic approach that targets DNABII

proteins would affect both intracellular and extracellular targets of UPEC pathogenesis and thereby would severely impair the virulence of UTI89. Although this study is focused on UTI89 biofilms, these components of the EPS (i.e. DNA, DNABII proteins) are common to many pathogenic bacteria (Flemming and Wingender, 2010; Goodman et al., 2011). Hence, these observations will likely aid in the development of novel therapies to treat and/or prevent acute and chronic bacterial infection during the course of UTI that could likely be applicable to a multitude of biofilm-mediated diseases.

## Experimental Procedures

### Bacteria strains and plasmids

The strains and plasmids used in this study are shown in supplementary Table 1. The *hupA::cam* and *hupB::kan* mutations were independently introduced into UTI89. The *hupA::cam* and *hupB::kan* mutations were transduced from MG1655 *hupA::cam* and MG1655 *hupB::kan* into UTI89 respectively using standard P1 transduction (Slihavy, 1984) and transductants were selected on LB agar (Fisher Scientific) containing 25 µg/ml chloramphenicol (Fisher Scientific) and 25 µg/ml kanamycin sulfate (Fisher Scientific), respectively. Ectopic production of either or both IHF subunits was accomplished using pHN $\alpha$  (Granston and Nash, 1993), pHN $\beta$  (kindly provided by Howard Nash), or pHN $\alpha\beta$  (Lee et al., 1992). Ectopic production of *E. coli* HU was accomplished using pRLM118 (kindly provided by Roger McMacken).

### Purification of DNABII proteins

IHF was purified from *E. coli* strain HN880 (Nash et al., 1987). The cells were grown at 30°C to an Optical Density (OD) of 0.4–0.6 at 600 nm. At mid-log phase, the growth temperature was shifted to 42°C for 4h to induce expression of IHF. Bacterial cells were pelleted at 15000g for 5 min and resuspended in lysis buffer (20 mM Tris pH 7.4, 1 mM EDTA, 10% glycerol, 20 mM NaCl, 1 mM PMSF, 5 mM MgCl<sub>2</sub>, 5 mM CaCl<sub>2</sub>). The suspension was treated with DNase (100 µg/ml) for 30 min on ice followed by cell lysis by three passes in a French press at 20,000 psi. The cell lysate was clarified at 25,000g for 30 min at 4°C and passed through a 0.45 µm filter. The proteins were precipitated with ammonium sulphate (50% saturation) and the cell lysate was clarified further at 25,000g for 15 min at 4°C. Ammonium sulphate was added to 80% saturation; the proteins were pelleted and resuspended in binding buffer (10 mM Phosphate buffer pH 7, 500 mM NaCl). This IHF enriched protein fraction was dialyzed (3000 MWCO) in binding buffer overnight at 4°C. The dialyzed fraction was bound to heparin-Sepharose column equilibrated in binding buffer. The column was washed and the bound IHF was eluted with 10 column volumes of linear gradient of elution buffer (10 mM Phosphate buffer pH 7, 2M NaCl). The collected fractions were analyzed by SDS PAGE, pooled and dialyzed in 10 mM Tris pH 7.4, 50 mM KCl. The fraction was further purified by a Co-Talon column (GE Healthcare) and pure IHF was eluted with 10 mM Tris pH 7.4, 300 mM KCl. The protein was concentrated in a centrifugal filter (3000 MWCO) and dialyzed in storage buffer (50 mM Tris pH 7.4, 600 mM KCl, 1 mM EDTA, 10% glycerol) and stored at –80°C.

HU was purified from *E. coli* strain N99 *himA sma him ::cat* made by D. Friedman that lacks both subunits of IHF (Granston et al., 1988) and overproduces HU from an inducible plasmid. HU was purified using the same procedure as IHF except that the binding buffer with 200 mM NaCl and the elution buffer for Co-Talon column with 200 mM KCl was used.

IHFA and IHFB were purified from *E. coli* strains containing the plasmid pHN $\alpha$  and pHN $\beta$ , respectively. Both proteins were purified using the same procedure as HU except that the proteins were eluted with 10 column volumes of linear gradient of elution buffer (10 mM Phosphate buffer pH 7, 800 mM NaCl) from the heparin-Sepharose column.

### ***In vitro* biofilm analysis**

For *in vitro* biofilm analysis, all strains were cultured on LB agar for 18 - 20 h at 37°C, in a humidified atmosphere containing 5% CO<sub>2</sub>, and then suspended in LB broth to an OD of 0.65 at 490 nm. Cultures were diluted (1:6) in LB broth, then incubated statically at 37°C, 5% CO<sub>2</sub> until reaching an OD<sub>490nm</sub> of 0.65. The cultures were diluted 1:2500 in LB, and 200  $\mu$ l of this suspension was inoculated into each well of an eight-well chamber slide (Fisher Scientific). After 16h of incubation at 37°C, 5% CO<sub>2</sub>, the medium was replaced with fresh medium. After an additional 8h incubation period, the medium was replaced again as described above and the chamber slides were incubated for an additional 16 h as before. For exogenous addition of DNABII proteins or DNase, the appropriate protein was added at 0.5  $\mu$ M and RNase-free DNase (Promega) was added at 1 unit at the time of seeding. Also, proteins and RNase-free DNase were added at 0.5  $\mu$ M every time the medium was replaced. For all bacterial isolates, after the final incubation (40 h total), the medium was removed and the biofilms were washed twice with saline (0.9% NaCl) and stained with LIVE/DEAD® stain (Molecular probes, Eugene, OR) according to manufacturer's instructions. Biofilms were then washed twice in saline and fixed with 1.6% paraformaldehyde, 0.025% glutaraldehyde and 4% acetic acid in 0.1M phosphate buffer at pH 7.4. The biofilms were imaged using a x63 objective on a Zeiss 510 Meta-laser scanning confocal microscope (Carl Zeiss, Thornwood, NY). Three-dimensional images were reconstructed with AxioVision Rel. 4.8 (Carl Zeiss) and maximum biofilm height, average thickness and biomass were determined via COMSTAT analysis (Heydorn et al., 2000). For overexpression of DNABII proteins, IPTG was used at 1 mM to induce the expression of IHFA, IHFB or IHFAB at the time of seeding and throughout biofilm growth for 40h. All *in vitro* biofilm assays were repeated a minimum of three times on separate days, and all individual biofilm assays were done in duplicates on each assay day. Data are presented as mean values  $\pm$  SEM.

### **Distribution of DNA within a UTI89 biofilm**

The distribution of dsDNA within a UTI89 biofilm was determined as previously described (Novotny et al., 2013). Briefly, unfixed 40 hour UTI89 biofilms were labeled with mouse anti-dsDNA monoclonal antibody (Abcam, Inc.), which was revealed with goat anti-mouse IgG conjugated to AlexaFluor 647 (Molecular Probes). Bacterial cells were labeled with FilmTracer™ FM® 1–43 (Molecular Probes) according to manufacturer's instructions. The biofilms were imaged using a x63 objective on a Zeiss 510 Meta-laser scanning confocal microscope (Carl Zeiss, Thornwood, NY). Three-dimensional images were reconstructed with AxioVision Rel. 4.8 (Carl Zeiss). Zeiss image acquisition software was employed to

determine the relative fluorescence of anti-dsDNA labeling to FilmTracer™. The relative abundance of dsDNA was determined by the ratio of the dsDNA (anti-dsDNA labeling) to the bacteria (FilmTracer™).

### Number of bacteria in the biofilm or planktonic state

Biofilm formation was initiated in the presence or absence of *E. coli* rIHF at 0.5 µM in a chamber slide for 40 h at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. The biofilms were cultured in either medium (LB) alone or in medium containing *E. coli* rIHF (0.5 µM) at 16 h and 24 h to maintain viability. At the end of 40 h, the contents of the well was resuspended, serially diluted and plated on LB agar to calculate the total number of bacteria. For planktonic bacteria, the spent medium from chamber slides was carefully removed. For the remaining adhered bacteria, biofilms were washed twice with sterile PBS and the washes were combined with the bacteria from the spent medium as the source of bacteria in the planktonic state. After the washes, the biofilm was resuspended in 200 µl of sterile PBS. Both planktonic and biofilm bacteria were serially diluted and plated on LB to calculate the number of bacteria (colony forming units). The assay was repeated a minimum of three times on separate days and all individual assays were done in duplicates on each assay day. Statistical significance was assessed by unpaired t-test (GraphPad Prism version 6.0). A *p* value < 0.05 was considered significant.

### Purification of proteins from spent medium

UTI89 was cultured in M9 medium (modified M9) supplemented with 0.1 mg/ml thiamine, 4 mM cysteine and vitamins (Desai et al., 2012) at 37°C, 200rpm for 16h. The cells were removed by spinning at 15000g for 5 min at 4°C and then by passing the spent medium through a 0.45 µm filter. PMSF (1 mM) was added to the spent medium to prevent protein degradation. Proteins in the spent medium were precipitated with ammonium sulphate (80% saturation) at 4°C for 3h. The spent medium was centrifuged at 15000g, 4°C for 15 min to pellet the proteins. The proteins were resuspended in 10 mM Tris pH 7.4, 200 mM KCl; dialyzed (3 KDa MWCO) in the same buffer overnight at 4°C and then concentrated with a centrifugal filter (3 KDa MWCO). The proteins were resolved by SDS-PAGE and probed with polyclonal antiserum from rabbits directed against *E. coli* rIHF in a western blot analysis. Briefly, 2 µl protein suspension was separated on a 16% resolving, 5% stacking gel in Tris/Glycine/SDS buffer, then transferred on to nitrocellulose membrane (Invitrogen). Membrane was blocked overnight at 4°C in 5% Blotto in Tris-buffered saline with 0.5% Tween 20 (Fisher Scientific) prior to incubation with rabbit anti-IHF followed by anti-rabbit IgG-HRP (Invitrogen). Blots were developed with ECL plus substrate kit (Pierce) and images were captured with Typhoon scanner (GE Healthcare).

### Hydrogen peroxide survival assay

Biofilm formation was initiated in the presence or absence of *E. coli* rIHF at 0.5 µM in a chamber slide for 24 h at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. The biofilms were cultured in either medium (LB) alone or in medium containing *E. coli* rIHF (0.5 µM) at 16 h to maintain viability. At the end of 24 h, the biofilms were incubated with 0 – 0.8% hydrogen peroxide for 30 min at 37°C. Then hydrogen peroxide was removed and



the biofilms were resuspended in sterile PBS, diluted and plated on LB agar to calculate the number of bacteria that survived the challenge. Planktonic state cells were obtained by suspending UPEC strain UTI89 in LB broth to an OD of 0.65 at 490 nm. Cultures were diluted (1:6) in LB broth, then incubated statically at 37°C, 5% CO<sub>2</sub> until an OD<sub>490nm</sub> of 0.65. 100 µl of this culture was used for the hydrogen peroxide challenge. All assays were repeated a minimum of three times on separate days. Data are presented as mean values ± SEM.

#### **Determination of Minimum Inhibitory Concentration (MIC) of ciprofloxacin (CIP) for UPEC strain UTI89**

Biofilm formation was initiated in the presence or absence of *E. coli* rIHF at 0.5 µM in a chamber slide for 24 h at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. The biofilms were cultured in either medium (LB) alone or in medium containing *E. coli* rIHF (0.5 µM) at 16 h to maintain viability. At the end of 24 h, the biofilms were treated with 0 – 2000 µg ml<sup>-1</sup> CIP for 16 h at 37°C. The CIP was removed and the biofilms were washed twice with sterile PBS, resuspended in sterile PBS, diluted and spotted on LB agar. MIC was defined as the lowest concentration of CIP at which no growth was observed (Andrews, 2001). Planktonic state cells were obtained by suspending UTI89 in LB broth to an OD of 0.65 at 490 nm. Cultures were diluted (1:6) in LB broth, then incubated statically at 37°C, 5% CO<sub>2</sub> until an OD<sub>490nm</sub> of 0.65. 100 µl of this culture was used for the CIP treatment. All assays were repeated a minimum of three times on separate days. Data are presented as mean values ± SEM.

#### **Detection of autotransporter protein antigen43 (Ag43) by RT-PCR**

Biofilm formation was initiated in the presence or absence of *E. coli* rIHF at 0.5 µM in a chamber slide for 16 h at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub> in modified M9 medium. At the end of 16 h, biofilms were resuspended and centrifuged at 5000g for 10 min. RNA extraction was performed with the RNeasy mini kit (Qiagen) according to the manufacturer's instructions. RNase-free DNase (Qiagen) was used according to the manufacturer's specifications to remove DNA. As a planktonic control, exponentially growing UTI89 in the same medium was used. To obtain cDNA, Superscript III (Life technologies) was used according to the manufacturer's specifications. Primers for 16S ribosomal RNA (Levertson and Kaper, 2005) were used as an internal control for the PCR, and the assay was then carried out using specific primers for Ag43 5'-atgacaaccagattgcctcggtac-3' and 5'-ggcatctccgtagacggtct- 3'.

#### **Statistical evaluation**

Significance in maximum height, mean thickness and biomass was assessed by unpaired t-test (GraphPad Prism version 6.0). A  $p < 0.05$  was represented as \* and a  $p < 0.01$  was represented by \*\*.

#### **Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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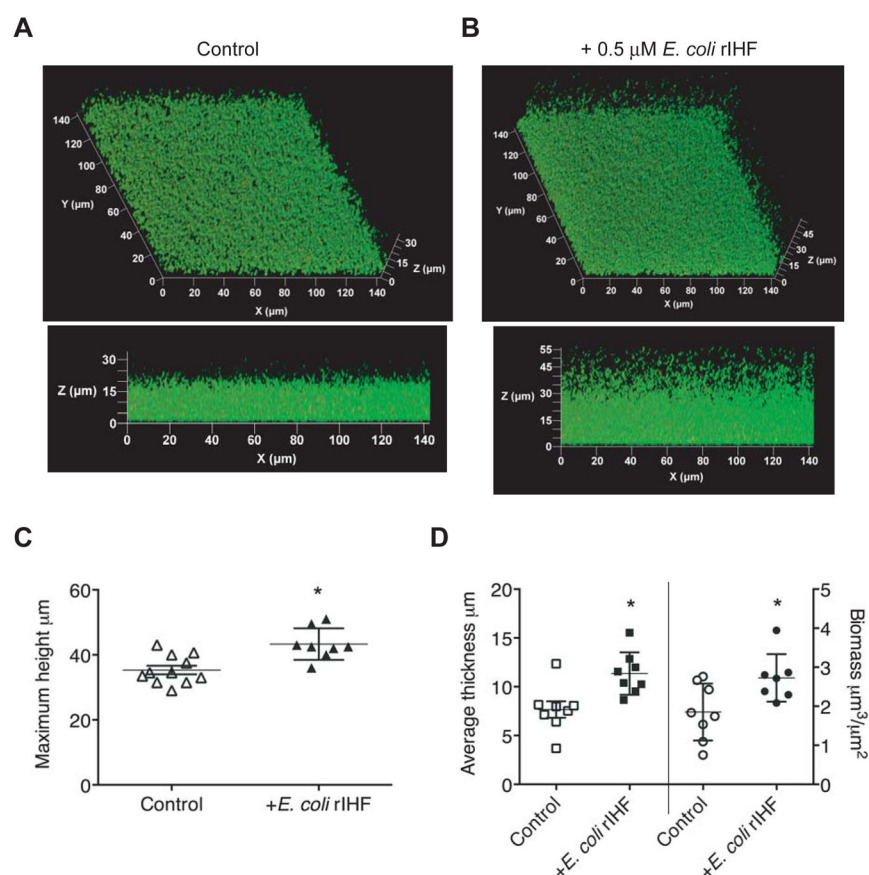
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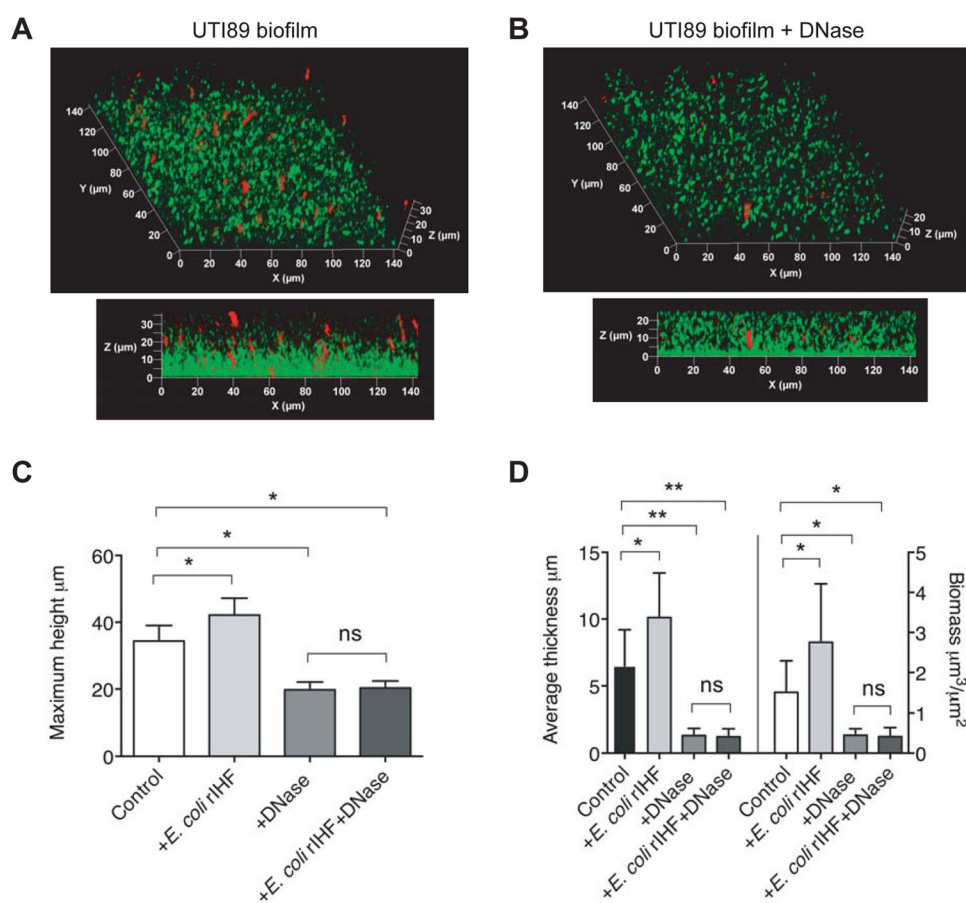
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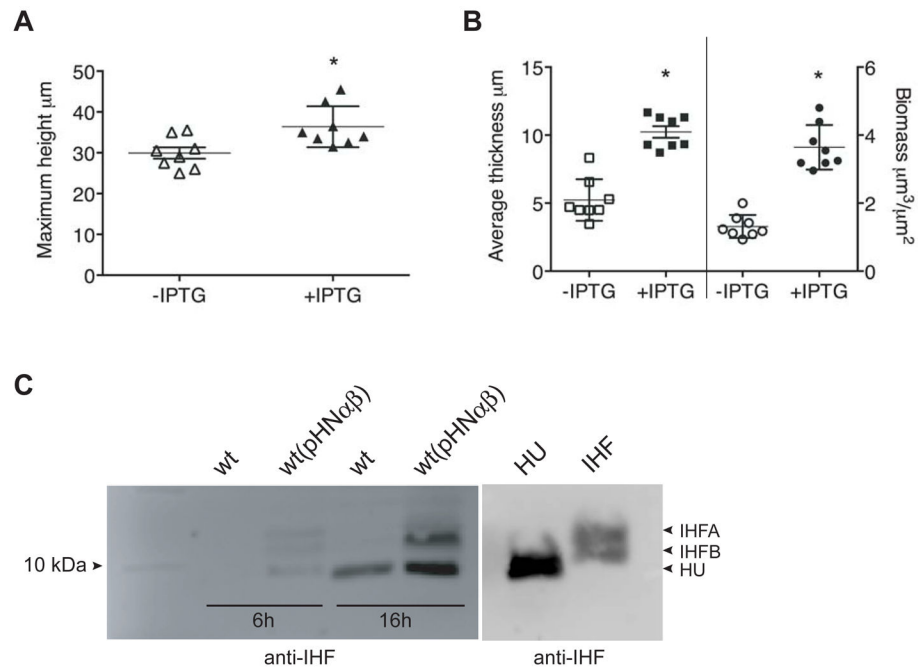


**Figure 1. Effect of exogenous addition of *E. coli* rHf on UPEC strain UTI89 biofilm formation** (A, B) Biofilm growth was initiated then maintained in the absence (A) or presence (B) of exogenous *E. coli* rHf ( $0.5 \mu\text{M}$ ) for 40 hours. (C, D) Images were analyzed by COMSTAT to calculate maximum biofilm height, average thickness and biomass. Asterisks indicate statistical significance ( $p < 0.05$ ) compared to control. All images were captured with a 63X objective.



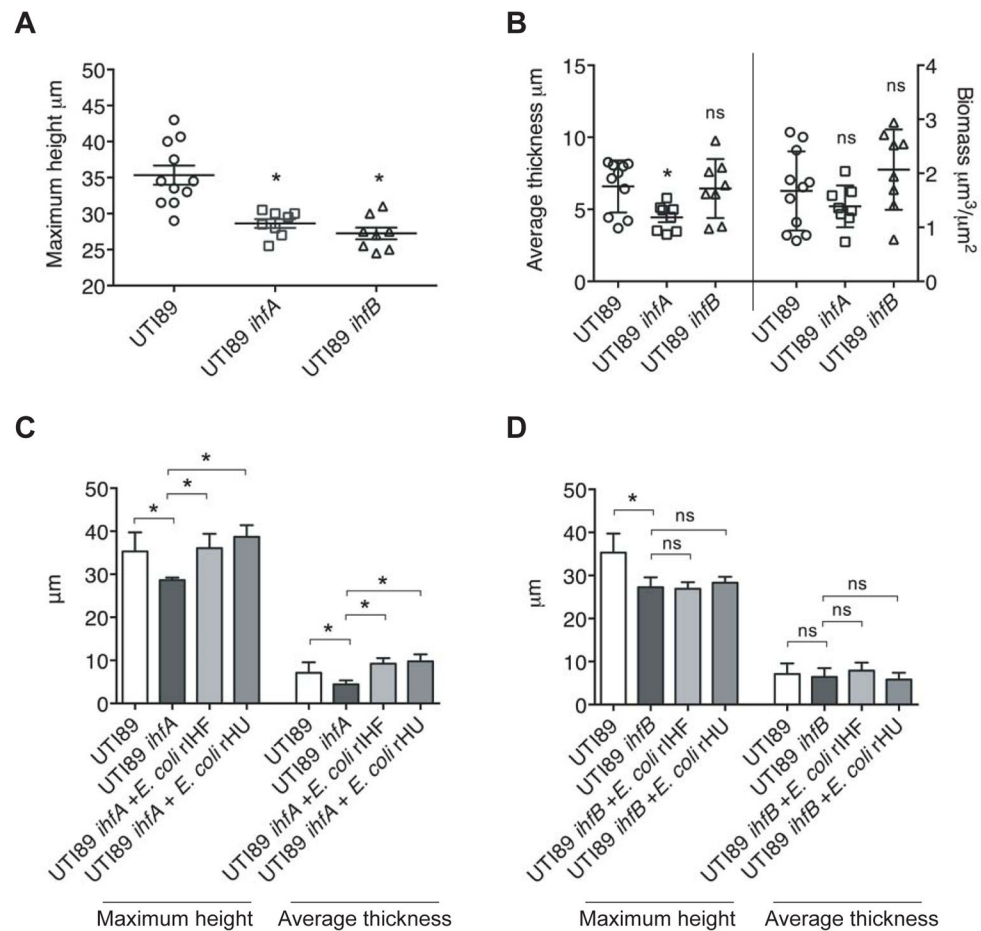
**Figure 2. Distribution of DNA in a UTI89 biofilm and the effect of addition of DNase on biofilms formed by UPEC strain UTI89**

(A, B) Biofilm growth was initiated then maintained in the absence (A) or presence (B) of DNase (1 unit) for 40 hours. Unfixed 40 hour biofilm was labeled with anti-dsDNA monoclonal antibody (DNA - red) and FilmTracer™ FM® 1–43 (biomass – green). All images were captured with a 63X objective. (C, D) Biofilm growth was initiated then maintained in the absence (control) or presence of DNase (+DNase) or DNase and exogenous *E. coli* rHF (+*E. coli* rHF + DNase) for 40 hours. Biofilms were stained with LIVE/DEAD® stain and the images were analyzed by COMSTAT to calculate maximum biofilm height (C), average thickness (D), and biomass (D). A  $p < 0.05$  was represented by \* and a  $p < 0.01$  was represented by \*\*.

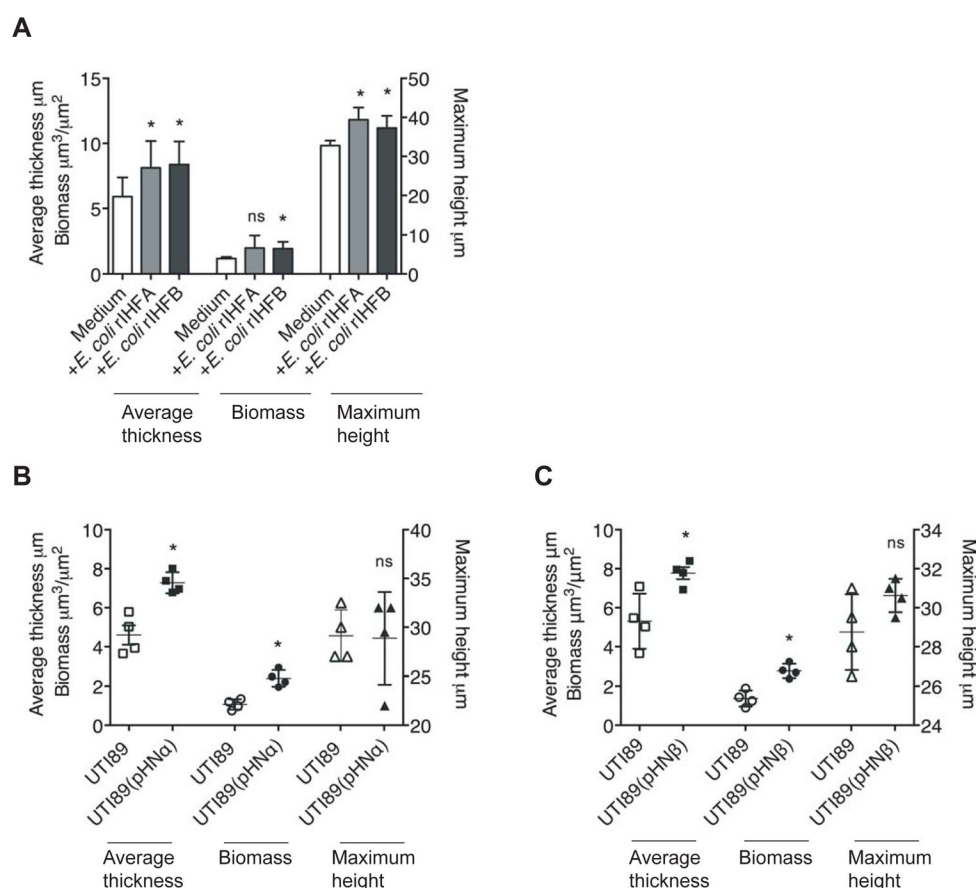


**Figure 3. Effect of overproduction of IHF on UPEC strain UT189 biofilm formation**

(A, B) Ectopic expression of *E. coli* rIHF was accomplished by IPTG induction of plasmid pHNαβ. Biofilm growth was initiated in the presence or absence of IPTG for 40 hours. Images were analyzed by COMSTAT to calculate biofilm maximum height (A), average thickness and biomass (B). Left Y-axis in panel B corresponds to average thickness and right Y-axis corresponds to biomass. (C) Western blot analysis of the spent medium from wild type or wild type overproducing *E. coli* rIHF [wt(pHNαβ)] using rabbit anti-IHF antibody directed against *E. coli* rIHF. Anti-IHF antibody specifically recognizes the two DNABII proteins IHF and HU. Asterisks indicate statistical significance ( $p < 0.05$ ) compared to -IPTG lane.

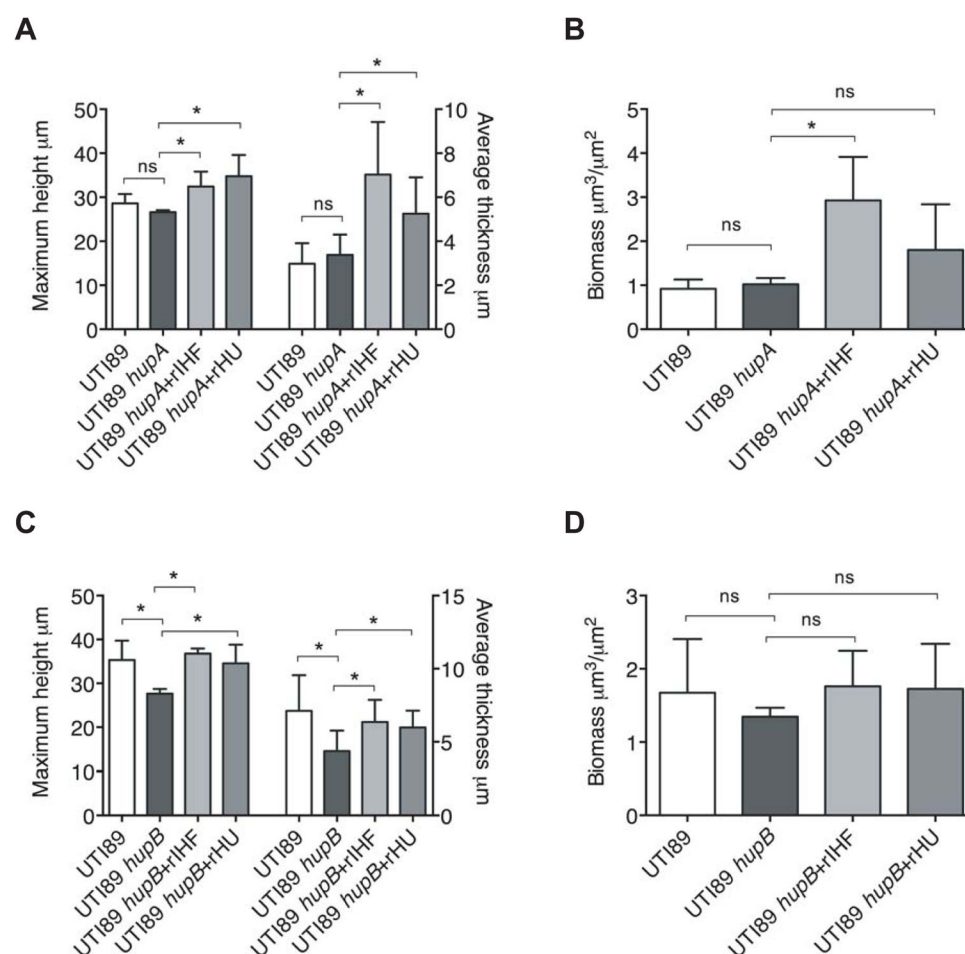


**Figure 4. Relative ability of UPEC strain UT189 IHF mutants to form biofilms *in vitro***  
 UT189, UT189 *ihfA11::Tn10* or UT189 *ihfB* biofilms were grown for 40 hours *in vitro* in a chamber slide. Biofilms were stained with Live/Dead stain and visualized by a confocal microscope to acquire 3-dimensional datasets. Images were analyzed by COMSTAT to calculate biofilm height (A) and average thickness and biomass (B). (C) Biofilm growth of UT189 *ihfA11::Tn10* was initiated in the presence or absence of exogenous *E. coli* rIHF (0.5 μM) for 40 hours and the images were analyzed by COMSTAT to measure maximum height and average thickness. (D) Biofilm growth of UT189 *ihfB* was initiated in the presence or absence of exogenous *E. coli* rIHF (0.5 μM) for 40 hours and the images were analyzed by COMSTAT to measure maximum height and average thickness. A *p*-value = 0.05 was considered significant.



**Figure 5. Effect of the addition of individual recombinant IHF subunits on UPEC strain UT189 biofilm formation**

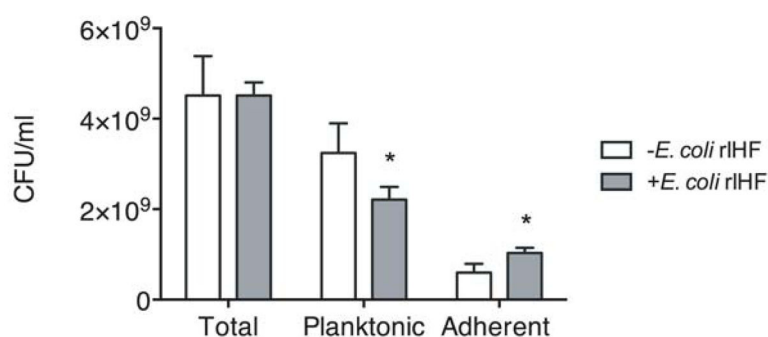
(A) Biofilm growth of UT189 was initiated in the presence (+*E. coli* rIHFA or +rIHFB) or absence (medium) of exogenous *E. coli* rIHFA or rIHFB ( $\sim 1.0 \mu\text{M}$ ) for 40 hours. Images were analyzed by COMSTAT to calculate biofilm maximum height, average thickness and biomass. (B) Ectopic expression of *E. coli* rIHFA was accomplished by IPTG induction of plasmid pHN $\alpha$ . Biofilm growth was initiated in the presence or absence of IPTG for 40 hours. Images were analyzed by COMSTAT to calculate maximum biofilm height, average thickness and biomass. (C) Ectopic expression of *E. coli* rIHFB was accomplished by IPTG induction of plasmid pHN $\beta$ . Biofilm growth was initiated in the presence or absence of IPTG for 40 hours. Images were analyzed by COMSTAT to calculate biofilm maximum height, average thickness and biomass. Left Y-axis in panels A, B and C correspond to average thickness and biomass and right Y-axis corresponds to maximum height. A *p*-value 0.05 was considered significant.



**Figure 6. Effect of HU deficiency on biofilm formation by UPEC strain UTI89**

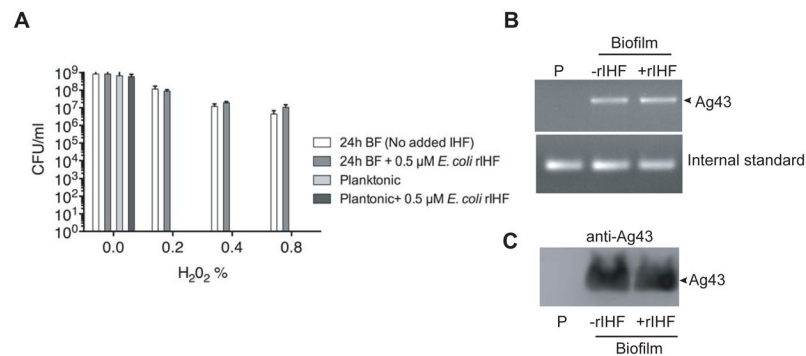
(A, B) Biofilm growth of UTI89 *hupA*::*cam* was initiated in the presence or absence of exogenous *E. coli* rIHF or *E. coli* rHU (0.5 μM) for 40 hours and the images were analyzed by COMSTAT to measure maximum height (A), average thickness (A) and biomass (B). (C, D) Biofilm growth of UTI89 *hupB*::*kan* was initiated in the presence or absence of exogenous *E. coli* rIHF or *E. coli* rHU (0.5 μM) for 40 hours and the images were analyzed by COMSTAT to measure maximum height (C), average thickness (C) and biomass (D). A *p*-value 0.05 was considered significant.





**Figure 7. Partitioning of UPEC strain UTI89 in a biofilm in the presence of *E. coli* rIHF**

Biofilm formation was initiated and then maintained in the presence or absence of *E. coli* rIHF (0.5  $\mu$ M) in a chamber slide for 40 hours. The contents of a well were re-suspended in the spent medium and plated on LB agar to calculate total CFU/ml. To calculate planktonic CFU/ml, the spent medium was carefully removed from the biofilm and the wells were washed twice with sterile PBS. The washes were combined with the spent medium, diluted and plated on LB agar. To calculate adherent CFU/ml, the biofilm (after two washes) was resuspended in sterile PBS, diluted and plated on LB agar. A *p*-value  $\leq 0.05$  was considered significant.



**Figure 8. *E. coli* rIHF treated UTI89 biofilms maintain sensitivity to hydrogen peroxide and express Ag43**

(A) Effect of hydrogen peroxide on UPEC strain UTI89 biofilm. Biofilm formation was initiated then maintained in the presence or absence of *E. coli* rIHF (0.5  $\mu$ M) in a chamber slide for 24 hours. The biofilms were then treated with the indicated concentration of hydrogen peroxide for 30 min. The plot represents the CFU/ml of UPEC that survived the hydrogen peroxide challenge for 30 min. (B) Representative RT-PCR analysis for identification of antigen43 (Ag43) from cDNA of planktonic (P) and biofilm (in the presence or absence of 0.5  $\mu$ M *E. coli* rIHF) state UTI89.

**Table 1**Minimum Inhibitory Concentration (MIC) for Ciprofloxacin MIC ( $\mu\text{g ml}^{-1}$ )

UTI89	No added <i>E. coli</i> IHF	+ <i>E. coli</i> rIHF 0.5 $\mu\text{M}$
Planktonic ( $\sim 10^8$ CFU/ml)	0.8	0.8
24h Biofilm ( $\sim 10^8$ CFU/ml)	>2000	>2000