The Extracellular DNA Lattice of Bacterial Biofilms is Structurally Related to Holliday Junction Recombination Intermediates

Aishwarya Devaraj[1], John R. Buzzo[1], Lauren Mashburn-Warren[1], Erin S. Gloag[2], Laura A. Novotny[1], Paul Stoodley[2,3,4], Lauren O. Bakaletz[1], and Steven D. Goodman[1]*.

[1] Center for Microbial Pathogenesis, Abigail Wexner Research Institute at Nationwide Children's Hospital, and The Ohio State University College of Medicine, Columbus, Ohio 43205

[2] Department of Microbial Infection and Immunity, The Ohio State University, Columbus, Ohio, 43210

[3] Department of Orthopedics, The Ohio State University, Columbus, Ohio, 43210

[4] National Centre for Advanced Tribology at Southampton, University of Southampton, Southampton, SO17 1BJ, UK

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*Corresponding author:

Steven D. Goodman, Ph.D., Abigail Wexner Research Institute at Nationwide Children's Hospital, Center for Microbial Pathogenesis, The Ohio State University College of Medicine, Department of Pediatrics, 700 Children's Drive, W492, Columbus, OH 43205-2696 USA, Phone: (614) 355-2761, Fax: (614) 722-2818, E-Mail: Steven.Goodman@NationwideChildrens.org

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Significance

Most chronic and recurrent bacterial infections are the result of biofilms. Extracellular DNA (eDNA) is a ubiquitous and pivotal structural component of biofilms that protects the resident bacteria from the host immune system and antimicrobial agents. It is of the highest priority to characterize the structure of the eDNA to understand the development of bacterial biofilm communities. Here, we employed the prototypic Holliday junction-specific (HJ) DNA binding protein RuvA and demonstrated that eDNA within biofilms formed by three human pathogens, uropathogenic *Escherichia coli* (UPEC), nontypeable *Haemophilus influenzae* (NTHI) and *Staphylococcus epidermidis* was structurally related to HJ recombination intermediates and further demonstrated that this HJ-like structure was critical to the structural and mechanical integrity of the bacterial biofilm matrix.

Abstract

Extracellular DNA (eDNA) is a critical component of the extracellular matrix of bacterial biofilms that protects the resident bacteria from environmental hazards which includes imparting significantly greater resistance to antibiotics and host immune effectors. eDNA is organized into a lattice-like structure, stabilized by the DNABII family of proteins, known to have high affinity and specificity for HJs. Accordingly, we demonstrated that the branched eDNA structures present within the biofilms formed by NTHI in the middle ear of the chinchilla in an experimental otitis media model, and in sputum samples that contain multiple mixed bacterial species and were recovered from cystic fibrosis (CF) patients possess a HJ-like configuration. Next, we showed that the prototypic *E. coli* HJ-specific DNA-binding protein RuvA could be functionally exchanged for DNABII proteins in the

stabilization of biofilms formed by three diverse human pathogens, UPEC, NTHI and *Staphylococcus epidermidis*. Importantly, while replacement of DNABII proteins within the NTHI biofilm matrix with RuvA was shown to retain similar mechanical properties when compared to the control NTHI biofilm structure, we also demonstrated that biofilm eDNA matrices stabilized by RuvA could be subsequently undermined upon addition of the HJ resolvase complex, RuvABC, which resulted in significant biofilm disruption. Collectively, our data suggested that nature has recapitulated a functional equivalent of the HJ recombination intermediate to maintain the structural integrity of bacterial biofilms.

Introduction

Most bacteria in natural ecosystems prefer a biofilm lifestyle. Biofilm bacteria are encased within a self-produced extracellular matrix (extracellular polymeric substances or EPS) comprised of eDNA, proteins, lipids, and exopolysaccharides (1). The biofilm EPS provides structural integrity, protects resident bacteria against physical, chemical and environmental stresses that includes host effectors and antimicrobial therapies, affects gene regulation and nutrient adsorption [reviewed in (2)]. Hence, it is of utmost importance to characterize not only the EPS components, but their subsequent structure to gain insight into the development of bacterial biofilm communities and consequently, for pathogenic biofilms, identify the means to undermine them.

eDNA is a key structural component of the EPS and therefore an attractive target for the control of bacterial biofilms. Although the importance of eDNA in the biofilm matrix has been established, the structure of the eDNA itself has not been well characterized. We have previously shown that eDNA in biofilms formed by NTHI within a chinchilla

middle ear (3), and by *Pseudomonas aeruginosa* in a murine lung model (4), as well as biofilms in pediatric sputum and otorrhea samples that were culture positive for multiple mixed bacterial species (5-7), was present in a lattice structure. In addition, we have revealed that the DNABII family of proteins (integration host factor, IHF and histone-like protein, HU) bind to and stabilize the eDNA lattice structure and are fundamental to the structural stability of bacterial biofilms (5, 7-14).

DNABII proteins that are localized at the vertices of the eDNA lattice within bacterial biofilms (5-8) have high affinity for branched DNA structures which include HJ DNA (15, 16). HJs are single-strand crossover intermediates of homologous recombination and are common across both eukaryotes and prokaryotes (17). HJs appear as cross-like or cruciform structures with four double-stranded DNA arms. In most eubacteria, the resolution of homologous recombination occurs through the association of HJ DNA with RuvA, RuvB and RuvC, where RuvA binds to HJ DNA with high affinity in a structure-specific, but sequence independent manner (18). RuvA then recruits RuvB to the HJ, and the RuvAB complex drives translocation of the junction that expands the heteroduplex region in an ATP-dependent fashion (19). Lastly, the endonuclease RuvC binds the RuvAB complex, which results in cleavage of HJ DNA and resolution to yield two nicked duplexes (20). RusA, a resolvase of lambdoid phage origin, binds HJ in a sequence-independent manner and cleaves the phosphodiester bond 5' of CC dinucleotides to resolve HJ into nicked duplexes (21).

Because HJ DNA is necessarily bent, they serve as excellent substrates for the DNABII family that bind bent DNA with high affinity (15, 16). We therefore hypothesized that the structure of eDNA at the vertices was comprised of HJs. To test this hypothesis,

we employed antibodies that are highly specific for HJ DNA as well as proteins that bind to and resolve HJ DNA. First, we demonstrated that the lattice structure found within the EPS of biofilms formed *in vivo* by NTHI (in the chinchilla middle ear during experimental otitis media), and in polymicrobial sputum samples recovered from CF patients was recognized by the these highly specific HJ-directed antibodies. Further, we took advantage of the proteins involved in the resolution of HJ DNA, RuvABC complex and RusA to demonstrate that the HJ DNA binding protein RuvA functionally complemented DNABII proteins within the EPS and stabilized biofilms formed by UPEC, NTHI and *S. epidermidis in vitro.* We further showed that NTHI biofilms stabilized by RuvA were biophysically indistinguishable from the control NTHI biofilms as measured by mechanical axial indentation. Finally, we also showed that the HJ resolvases RuvABC and RusA efficiently disrupted biofilms and directly targeted these HJ structures within the EPS of NTHI biofilms to inhibit the formation of the eDNA lattice structure. Collectively, our data suggested that eDNA lattice within bacterial biofilms is structurally related to HJ structures and is critical for the stability of the bacterial biofilm matrix.

Results

Bacterial biofilms formed by NTHI within the middle ear of the chinchilla and polymicrobial sputum samples recovered from CF patients contained HJ-like DNA structure

We previously demonstrated that eDNA within the biofilm EPS formed by multiple single (3, 5, 8) and mixed bacterial species (6, 7) is organized into an interwoven web-like structure that is stabilized by DNABII proteins positioned at the vertices of each of the

crossed strands of eDNA. Since these DNABII proteins have a high affinity for HJ DNA (K_D ~ nM) (15, 16), we hypothesized that these branched structures were related to HJ recombination intermediates. Toward this goal, we assessed whether the HJ-like structure was found within bacterial biofilms that had formed *in vivo* with a monoclonal antibody specific for cruciform DNA that exclusively binds to the elbow region of a HJ DNA structure (22). Immunohistochemistry analysis of middle ear sections from chinchilla infected with NTHI, and sputum solids from CF patients that contained multiple mixed bacterial species revealed a complex lattice-like eDNA structure as indicated in green, with punctate labeling for cruciform DNA in white, at the majority of the crossed strands of the eDNA (Fig. 1). Given the specificity of the monoclonal antibody against cruciform DNA (*SI Appendix*, Fig. S1) (22), these data confirmed the presence of HJ DNA within the EPS of single and multi-species biofilms *in vivo*.

The prototypic HJ DNA binding protein RuvA compensated for the removal of DNABII proteins in structural stabilization of UPEC, NTHI and *S. epidermidis* biofilms

To further confirm the presence of HJ-like structure within the EPS of bacterial biofilms, we employed three opportunistic pathogens: UPEC, NTHI and *S. epidermidis*, all of which are known to persist in a biofilm lifestyle, that is disrupted upon depletion of DNABII proteins. We depleted the DNABII proteins within the EPS of biofilms that were established *in vitro*, by the addition of a hyperimmune polyclonal antibody directed against *E. coli* IHF [(α-IHF), which recognizes both IHF and HU with variable avidities (*SI Appendix*, Fig. S1)], and simultaneously supplemented with purified recombinant *E. coli*

RuvA, the prototypical HJ binding protein in bacteria, to determine if RuvA could functionally replace DNABII proteins to stabilize the biofilm EPS. Biofilms were then stained with LIVE/DEAD®, visualized via confocal laser scanning microscopy (CLSM), and total biomass and average thickness were quantified by COMSTAT analysis (23). It was evident that α-IHF-mediated disruption of biofilms formed by each bacterial species, which included UPEC (Fig. 2A, B), NTHI (Fig. 2C) and *S. epidermidis* (Fig. 2D) was prevented by the addition of RuvA. Addition of H-NS, a nonspecific DNA-binding protein was unable to compensate for the loss of DNABII proteins within the biofilm matrix and thus served as a negative control (Fig. 2). This result was consistent with our previous findings that H-NS is not required for the structural integrity of biofilms formed by UPEC and NTHI (10, 11).

Next, we used immunofluorescence to detect DNABII proteins and RuvA within bacterial biofilms and observed the depletion of DNABII proteins within the extracellular matrix of biofilms formed by UPEC upon treatment with α-IHF in the presence of RuvA (Figs. 3A, B) and the concomitant incorporation of RuvA within the biofilm matrix (Figs. 3C, D). While RuvA labeling was observed throughout the depth of the biofilms (see orthogonal projections in the bottom row of Figs. 3C and D) treated with naive or α-IHF IgG, RuvA was much more densely accumulated at the bottommost portions of those treated with α-IHF. We are further investigating the mechanism(s) of this spaciotemporal labeling pattern, as α–RuvA antibody was confirmed to neither adhere to the substratum nor to planktonic UPEC or NTHI cells (*SI Appendix*, Fig. S2). However, these results suggested that the observed distribution of RuvA was likely characteristic of UPEC biofilms. The relative abundance of IHF and RuvA within the biofilm EPS, was determined

by the ratio of the protein (α-IHF/α–RuvA labeled) to total DNA (DAPI) and revealed a statistically significant decrease in DNABII proteins, which corresponded with a statistically significant increase in RuvA compared to the control (indicated by naive serum + RuvA; Fig. 3E). Given the skewed distribution of the fluorescence signal at the bottommost portion of the biofilm, we determined that even after digitally removing the three-micron section from the bottom, we still observed a statistically significant decrease in DNABII proteins within the remainder of the biofilm, an observation that corresponded with a correlating and statistically significant increase in RuvA compared to the control (*SI Appendix*, Fig. S3). These results suggested that the observed distribution of RuvA was characteristic of UPEC biofilms. The specificities of α-IHF and α-RuvA were determined by Western blot analysis and were confirmed to be highly specific for their target protein (*SI Appendix*, Fig. S1). We and others have shown that RuvA specifically binds to HJ DNA with high affinity (*SI Appendix*, Fig. S4A) (24). Given the high affinity and specificity of RuvA to HJ DNA, these results suggested that RuvA compensated for the loss of DNABII proteins and thus stabilized the eDNA structure by selectively binding to HJ DNA structures that were vacated by DNABII proteins as a result of DNABII protein depletion with α-IHF.

NTHI biofilms stabilized by DNABII proteins and DNABII-depleted biofilms stabilized by RuvA exhibited similar mechanical properties

Rheological analysis was performed on NTHI biofilms to determine how DNABII depletion, and complementation of this depletion by addition of RuvA, induced any changes to the bulk biofilm mechanical properties. Axial mechanical indentation was

184 performed on control (naive IgG) and DNABII-depleted biofilms (α-IHF) in the presence or
185 absence of RuvA. Indentation has been commonly used to assess the impact of EPS
186 components on biofilm mechanical stability (25, 26). An 8-mm geometry was lowered
187 onto the biofilm, and the force required to compress the biofilm was determined. NTHI
188 biofilms displayed a characteristic “J-shaped” stress-strain response (Fig. 4A), which
189 indicated that as the biofilms were compressed, they progressively became stiffer, which
190 is typical of viscoelastic biological materials (27). It was evident from the differences in the
191 stress-strain curves particularly at the lower strains that the different treatments
192 influenced the stiffness of NTHI biofilms (Fig. 4A). To quantify these differences, the
193 Young’s modulus (*E*) was calculated from the lower linear portion of the curve (Fig. 4A;
194 inset) using equation 1. The Young’s modulus is a measurement of how stiff a material is,
195 i.e. how much a material deforms (measured as strain) in response to an applied normal
196 force (i.e. force that is applied perpendicular to a material) (28). The Young’s modulus of
197 DNABII depleted biofilms was significantly reduced compared to control (naïve IgG; no
198 DNABII depletion) (Fig. 4B). This result suggested that the DNABII depletion, and
199 subsequent disruption of the eDNA lattice network resulted in NTHI biofilms that were
200 mechanically less rigid than control biofilms. However, DNABII-depleted biofilms that had
201 been complemented with RuvA exhibited a Young’s modulus similar to the control (Fig.
202 4B). These data suggested that complementation with RuvA mechanically compensated
203 for the loss of DNABII proteins and restored biofilms to their normal stiffer phenotype.
204

Bacterial biofilm matrix stabilized by RuvA was disrupted upon treatment with HJ-specific endonuclease complex RuvABC

Since RuvA readily and effectively replaced DNABII proteins to maintain the structural stability of biofilms formed by UPEC, NTHI and *S. epidermidis*, we hypothesized that the biofilm matrix stabilized by RuvA is susceptible to disruption by the HJ-specific endonuclease complex RuvABC. To test this, established UPEC, NTHI and *S. epidermidis* biofilms wherein the DNABII proteins had been experimentally replaced with RuvA (Figs. 2&3) were further incubated with RuvB and RuvC proteins at a concentration that has no effect on planktonic growth (*SI Appendix*, Fig. S5), so as to create the RuvABC complex followed by the addition of LIVE/DEAD® stain, visualization with CLSM and quantification with COMSTAT (23). Strikingly, as evident from Fig. 5, the addition of RuvABC complex to biofilms in which the EPS was stabilized by RuvA (indicated by α-IHF IgG + RuvABC), induced a significant reduction in biofilm biomass compared to control biofilms wherein the matrix was stabilized by DNABII proteins (indicated by naive IgG + RuvABC) in UPEC (Fig. 5A), NTHI (Fig. 5B) and *S. epidermidis* (Fig. 5C). Also, established UPEC and NTHI biofilms wherein the EPS was stabilized by DNABII proteins (no depletion) were only modestly disrupted by the addition of RuvABC (Fig. 5A, B). With no depletion of DNABII proteins, RuvABC was ineffective at disruption of *S. epidermidis* biofilms (Fig. 5C). We have previously shown that DNABII proteins are limited in UPEC (11) (e.g. a situation wherein addition of exogenous DNABII proteins partitions bacteria from the planktonic to the biofilm state), however they are not limited in NTHI (*SI Appendix*, Fig. S6), nonetheless, exogenously added DNABII proteins do incorporate within their respective EPSs (10). These data suggested the presence of at least

transiently free HJ DNA sites within the EPS of these biofilms, wherein RuvA could be incorporated. This outcome was confirmed by immunofluorescence, which revealed the incorporation of a modest amount of RuvA within the EPS of UPEC biofilms in the presence of naive serum, which does not deplete DNABII proteins (Fig. 3C) and was also in line with the modest disruption of UPEC and NTHI biofilms (Figs. 5A and B) in the absence of depletion of DNABII proteins. DNABII proteins were not limited in *S. epidermidis* biofilms (*SI Appendix*, Fig. S6), which suggested the absence of free HJ within the biofilm EPS, and therefore was consistent with a lack of disruption of *S. epidermidis* biofilms by RuvABC (Fig. 5C). However, depletion of DNABII proteins with α-IHF, allowed more HJ sites to be vacated within the biofilm EPS of UPEC, NTHI and *S. epidermidis*, and as a result significant amount of RuvA was incorporated within UPEC biofilm EPS (Fig. 3). Once RuvA was stably in place, the addition of RuvB and RuvC significantly disrupted UPEC, NTHI and *S. epidermidis* biofilms (Fig. 5). These data implied that the observed significant disruption of biofilms by RuvABC was due to the incorporation of additional RuvA on the HJ DNA sites that were vacated by DNABII proteins as a result of depletion with α-IHF. In the absence of the endonuclease RuvC (indicated by, Naïve IgG + RuvAB and α-IHF IgG + RuvAB), no significant disruption was observed in biofilms formed by UPEC, NTHI and *S. epidermidis*. The RuvAB complex drives branch migration of the HJ in an ATP-dependent manner (19). This result suggested three possibilities: 1) that the HJs were immobile and could be in an antiparallel configuration, 2) that there was insufficient complementarity beyond the HJ and or 3) that there are other mitigating factors that impeded branch migration. Further, we confirmed the resolvase activity of the RuvABC complex on synthetic HJ DNA pre-

incubated in the presence and absence of DNABII protein (*SI Appendix*, Fig. S4B). Collectively, these data indicated that the eDNA lattice structure within these biofilms contained HJ DNA structure that served a critical structural role in the stability of the bacterial biofilm EPS.

Bacterial biofilms were disrupted upon treatment with another HJ-specific resolvase, RusA

To further validate the presence of HJ DNA structure within the bacterial biofilm EPS, biofilms formed by UPEC, NTHI or *S. epidermidis* were incubated with varying concentrations of RusA, a HJ-specific endonuclease. Biofilms were then stained with LIVE/DEAD®, visualized via CLSM and quantified by COMSTAT analysis (23) to determine total biofilm biomass and average thickness. The addition of RusA at concentrations that have no effect on planktonic growth (*SI Appendix*, Fig. S5) destabilized the biofilm matrix and induced a significant dose-dependent reduction in UPEC (Fig. 6A), NTHI (Fig. 6B) and *S. epidermidis* (Fig. 6C) biofilm biomass compared to control. Although RusA bound with very high affinity to HJ and Y-DNA (*SI Appendix*, Fig. S7A), it only selectively cleaved HJ-DNA to nicked duplex DNA (*SI Appendix*, Fig. S7B). Also, RusA efficiently cleaved synthetic HJ prebound to HU (*SI Appendix*, Fig. S7C). Given the cleavage specificity of RusA for HJ DNA, these data further confirmed the presence of HJ DNA within the biofilm EPS and demonstrated that it was crucial for the structural integrity of bacterial biofilms.

RuvABC and RusA targeted HJ DNA within the biofilm extracellular matrix and prevented the formation of the eDNA lattice-like network within an NTHI biofilm

Since treatment of biofilms formed by multiple bacteria with HJ-specific endonucleases disrupted biofilms, we reasoned that these endonucleases specifically targeted HJ DNA structure within the biofilm EPS to mediate biofilm disruption. To demonstrate this, immunofluorescence was used to visualize eDNA and evaluate the effect of RuvABC and RusA on the eDNA lattice structure of NTHI biofilms (used here as a representative model bacterial biofilm) formed in the absence or presence of RuvABC or RusA. Unfixed NTHI biofilms were then labeled with a monoclonal antibody against double stranded DNA to visualize the eDNA. While the eDNA was organized into a complex web-like structure in the absence of HJ-specific endonucleases (indicated by control, Fig. 7), the eDNA lattice structure was radically diminished with a few eDNA strands in the presence of RuvABC or RusA (Fig. 7). Upon addition of higher concentrations of RusA, either at initiation of biofilms or when added to established biofilms, a highly diminished lattice structure with fewer eDNA strands was observed (*SI Appendix*, Fig. S8). These results suggested that the remaining eDNA strands were either inaccessible to RusA, or perhaps that other branched structures of eDNA were present within the biofilm matrix that could not be cleaved by RusA. In addition, biofilms were probed with a monoclonal antibody specific for cruciform DNA (*SI Appendix*, Fig. S1) (22) to directly visualize HJs within the EPS of biofilms formed by NTHI. In the absence of RusA, HJs were particularly visible in the lower, denser part of the biofilm as evidenced by the relative distribution of the yellow fluorescence within the biofilm matrix (Fig. 7E), whereas no fluorescence signal was detected when biofilms were incubated with naive

IgG (Fig. 7D). The addition of RusA to biofilms at initiation of the biofilm significantly decreased the observed yellow fluorescence (Fig. 7F). Further, in the presence of RusA, the relative abundance of HJ DNA as determined by the ratio of the HJ DNA (α-cruciform labeled) to the bacteria (FilmTracer™) revealed a statistically significant decrease in the amount of HJ DNA within the biofilm matrix compared to the control (Fig. 7G). Next, we co-localized cruciform DNA and dsDNA within the EPS of established *in vitro*-formed biofilms in the absence (*SI Appendix*, Fig. S9A) and presence of RusA (*SI Appendix*, Fig. S9B) or RuvABC (*SI Appendix*, Fig. S9C), and observed a complex lattice-like eDNA structure in the control (as indicated in green), with punctate labeling for cruciform DNA (in white) at the majority of the crossed strands of eDNA. Further, in the presence of RusA or RuvABC, the eDNA lattice structure and the cruciform DNA were significantly reduced as compared to the control (*SI Appendix*, Fig. S9). Finally, we wanted to determine if the HJ structures exclusively co-localized with DNABII as our hypothesis suggests. Our hypothesis was supported by the fact that we were unable to co-localize the DNABII proteins and cruciform DNA within the NTHI biofilm EPS (*SI Appendix*, Fig. S10A) with specific antibodies when these were added simultaneously, a result that suggested that one antibody was blocking the other from also finding its target due to the shared physical location of their specific binding sites. Thereby, we used the alternative approach wherein we added the specific antibodies sequentially to determine if the failed ability to co-localize the DNABII proteins and the HJs at the same time was perhaps due to occlusion of each antibody to the same physical structure i.e. DNABII bound HJs. To demonstrate this likely occlusion, we sequentially labeled first the DNABII proteins then the cruciform DNA (and *vice versa*) and observed that the labeling of either DNABII

proteins or cruciform DNA occluded the labeling of the other (*SI Appendix*, Fig. S10B, C). Addition of H-NS, a nonspecific DNA-binding protein had no effect on the labeling of cruciform DNA (*SI Appendix*, Fig. S10D, E). These data provided additional support for our hypothesis that the DNABII proteins and cruciform DNA likely co-localize within the NTHI biofilm matrix. Collectively, these data further proved the presence and critical significance of HJ DNA to the stability of the bacterial biofilm EPS.

Discussion

Our overarching hypothesis is that in a multi-species biofilm with co-aggregating partners, the DNABII proteins in conjunction with eDNA assemble a common nucleoprotein complex that creates an inclusive EPS infrastructure within the means of all eubacteria which is permissive for bacteria to enter into a community biofilm architecture. Multiple human pathogens, which include NTHI, UPEC, *Neisseria gonorrhoeae*, *P. aeruginosa*, *S. epidermidis*, *Staphylococcus aureus*, *Streptocoocus pneumoniae*, *Enterococcus faecalis*, *Helicobater pylori*, and *Campylobacter jejuni* incorporate eDNA into their biofilms [reviewed in (29)]. Bacteria not only release their own DNA in a multitude of ways, but also secrete toxins that induce lysis of host cells by apoptosis and necrosis wherein the released host DNA now facilitates biofilm development (30). Neutrophil extracellular traps (NETs), a host defense mechanism wherein neutrophils release nuclear DNA associated with histones and cytoplasmic granules to combat pathogens, also serves as a source of eDNA. In particular, *P. aeruginosa* exhibits an enhanced biofilm formation in the presence of neutrophils (31), an outcome that suggests that the eDNA within bacterial biofilms is likely comprised of both host-derived and

bacterial-derived DNA. eDNA was first shown to be critical for biofilm formation of *P. aeruginosa* (32) and several other studies revealed its structural role within the biofilm EPS of Gram-positive and Gram-negative bacteria in natural, industrial and medical ecosystems (32-35). Now, it is known that eDNA is a common component in bacterial biofilms, however the structural configuration of eDNA within biofilms has not been well characterized.

A filamentous network of eDNA has been previously described for *Reinheimera* sp. F8 and *Pseudomonoas* sp. FW1 isolated from freshwater stream (33). A similar structural organization of eDNA is also evident in NTHI, *Myxococcus xanthus*, *E. faecalis*, and *Streptococcus mutans* (3, 36-38) biofilms. We have previously shown that eDNA in single species biofilms formed by NTHI and *P. aeruginosa* in a chinchilla experimental otitis media and murine lung infection model respectively; in pediatric sputum samples that were culture positive for *Burkholderia cenocepacia*, *P. aeruginosa* and *Staphylococci,* as well as in pediatric otorrhea samples that were culture positive for *Haemophilus influenzae*, methicillin-resistant *S. aureus*, *S. pneumoniae, Moraxella catarrhalis* and *P. aeruginosa* is arranged into an interwoven lattice structure that is stabilized by the DNABII family of proteins (4-8).

The DNABII family of proteins condense DNA upon binding and in doing so, play a critical role in intracellular bacterial nucleoid structure and function (39). Members of the DNABII protein family exhibit high affinity towards pre-bent secondary structures of DNA that includes HJ DNA (15, 16). The DNABII family of proteins are also found within the extracellular matrix of various single and multi-species biofilms and serve as lynchpin proteins in stabilization of the lattice-like structure of the eDNA (4-8). The universal

conservation of DNABII in eubacteria and the presence of eDNA in bacterial biofilms, combined with the observation of the DNABII protein-stabilized lattice-like arrangement of eDNA in the biofilms formed by multiple bacterial species and under various conditions indicated the likely universality of this organization of eDNA in bacterial biofilms. Given the preference of DNABII proteins for branched DNA structures that include HJ DNA, and the positioning of DNABII proteins at the each of the vertices of the crossed-strands of the eDNA, we hypothesized that the eDNA lattice in bacterial biofilms was structurally related to HJ DNA, and that other HJ DNA-binding proteins would provide similar structural integrity. Accordingly, RuvA, the prototypic HJ DNA-binding protein stabilized the bacterial biofilm structure upon the depletion of the DNABII proteins and thus functionally replaced DNABII proteins within the EPS of bacterial biofilms. Since RuvA exclusively binds to HJ DNA (24), these data strongly implied that HJ DNA was a significant component within the EPS of these biofilms.

Herein, we also analyzed the mechanical properties of NTHI biofilms using axial indentation. All analyzed NTHI biofilms displayed a J-shaped stress-strain response, which has been observed for *S. mutans* (40, 41) and *P. aeruginosa* biofilms (26), as well as in mixed biofilms of *P. aeruginosa*, *Pseudomonas fluorescens, Klebsiella pneumoniae* and *Stenotrophomonas maltophilia* (42) under shear and compression. It therefore appears that this classic J-shape response to applied forces is a common property of bacterial biofilms. Furthermore, the Young's modulus of the NTHI biofilms determined here, is greater than that previously determined for both *S. mutans* biofilms [20-40 kPa; (40, 41)] and for mixed biofilms (0.04 kPa; (42)). However, these values are on the same order as those calculated for wild type *P. aeruginosa* biofilms [>100 kPa; (26)].

How does a HJ configuration figure into the eDNA dependent EPS of biofilms? HJ, a universal intermediate formed during repair and homologous recombination events, consists of a branched structure with four double-helical arms that extend from the center. HJ adopts two configurations dependent on the local concentration of cations and interaction with HJ DNA-binding proteins: an open-X form wherein the four double helical arms are extended in a square planar geometry is the preferred configuration at low ionic strength and a stacked-X form wherein the arms coaxially pair and stack into a more compact structure is favored at high ionic strength [reviewed in (43)]. The DNA strands in the stacked-X configuration align either parallel or antiparallel to each other. While the stacked-X configuration with parallel strands can migrate along the DNA strands ('branch migration'), the junction with antiparallel DNA strands is topologically incapable of branch migration (44). DNABII proteins, IHF and HU recognize and bind to stacked-X HJ. While HU locks the HJ in stacked-X configuration, IHF induces the junction to adopt the open-X configuration (45-47). RuvA on the other hand binds and stabilizes the open-X configuration (48). Although the binding preference of each of these HJ DNA binding proteins are different, the fact that RuvA complemented the loss of DNABII proteins implied that the eDNA lattice within bacterial biofilms was comprised of a structure sufficiently similar to a *bona fide* HJ that complementation was possible.

The second HJ binding protein we utilized was the resolvase, RusA, that efficiently cleaved synthetic HJ prebound to HU and also significantly disrupted biofilms formed by UPEC, NTHI and *S. epidermidis* in a dose-dependent manner. RusA binds and stabilizes stacked-X HJ (49). In this configuration, the faces of the junction are distinct such that one side of the HJ exhibits minor groove characteristics and the other side exhibits the

411 characteristics of the major groove (50). Hence, the four angles in the junction are
412 sterochemically distinct. Several junction-specific endonucleases that include T4
413 Endonuclease VII, yeast endonuclease X2 and Calf thymus junction-specific
414 endonuclease bind to the 120° angle of the HJ DNA on the minor groove side and cleave
415 HJ DNA. DNABII protein HU failed to inhibit T4 Endonuclease VII activity and therefore it
416 was proposed that HU bound to the 60° angle of the HJ DNA (15). In our study RusA was
417 effective at disruption of biofilm EPS wherein the eDNA was stabilized by DNABII proteins
418 suggested that RusA bound to a site on the HJ DNA that was distinct from the site bound
419 by DNABII proteins to mediate cleavage of HJ DNA.
420 While our data suggested that HJ DNA was present within single and mixed
421 species biofilms *in vitro* and *in vivo*, there is the potential for variability in the number of
422 HJ DNA sites and endogenous steady state levels of DNABII that stabilize these sites in
423 each biofilm, which likely contributes to the differences in the efficiency of biofilm
424 disruption by the HJ resolvases. Also, the possibility of variable proportions of the
425 respective HJ topologies, which likely depends on the microbial species from which they
426 are derived, could not be excluded. Since the DNABII family of proteins bind to a variety
427 of other DNA structures which include double stranded (ds) DNA, ds-DNA with nicks,
428 gaps and overhangs, single strand fork, double strand fork and three-way junction with
429 nicks (16), the presence of these specific structures within the EPS of bacterial biofilms
430 cannot be excluded and remains to be investigated.
431 Finally, while cleavage/removal of these HJ structures was coincident with biofilm
432 disruption, it is unclear why other nucleases fail to likewise disrupt extant biofilms (32). In
433 accordance with our model, DNABII proteins bound to HJs and stabilized the eDNA

lattice. Disruption of extant biofilms either by sequestration of the DNABII proteins or competition for the HJs by HJ resolvases demonstrated the importance of these structures. However, independent of these HJ structures, the remaining eDNA enters into a nuclease resistant state as various DNases prevent bacterial biofilm formation, but fail to affect mature biofilms (5, 8, 32). Future work will explore the nature of this nuclease recalcitrant state and the capacity of the resident bacteria to create a formidable eDNA dependent extracellular matrix.

Methods

Bacteria strains

NTHI strain 86-028NP isolated from the nasopharynx of a child with chronic otitis media at Nationwide Children's Hospital was used in this study. This strain has been sequenced (51) and well characterized (52). UPEC strain UTI89 was isolated from a patient with cystitis (53). *S. epidermidis* strain #1618 was originally isolated from a child with serous otitis media in 1987 and has been maintained at low passage number in liquid nitrogen since its isolation.

Visualization of eDNA and cruciform DNA within biofilms formed *in vivo*

Middle ear sections from chinchilla infected with NTHI strain 86-028NP were prepared as previously described (8). Sputum samples were collected after receipt of written informed consent and under a protocol (IRB11-00790) approved by Nationwide Children's Hospital Institutional Review Board. Samples were then de-identified and sectioned as described in (7). Sections were air-dried for 15 minutes at room temperature

and fixed in cold acetone for 10 minutes. Sections were then equilibrated in wash buffer that contained 0.05 M Tris-HCl pH 7.4, 0.15 M NaCl and 0.05% Tween 20 at room temperature for 5 minutes in a humidified chamber. Image-iT FX signal enhancer (Molecular probes) was added to the sections and incubated at room temperature for 30 minutes. The sections were then washed three times with wash buffer. The sections were incubated with SuperBlock (Thermo Fisher Scientific) at room temperature for 10 minutes. Zenon™ Alexa Fluor® 488 mouse IgG_{2a} labeling kit (Thermo Fisher Scientific) was used to label the monoclonal antibody against dsDNA as per manufacturer's instructions. Sections were then incubated with 1.5 μg of monoclonal antibody against dsDNA conjugated to Alexa Fluor® 488 and 1.5 μg of monoclonal antibody against cruciform DNA at room temperature for 1 hour. The sections were incubated with naive IgG as a negative control. The sections were fixed with 4% formaldehyde at room temperature for 10 minutes. The sections were then rinsed three times in wash buffer and incubated with goat anti-mouse IgG1 conjugated to Alexa Fluor® 594 (Molecular Probes) for 30 minutes at room temperature. The sections were cover-slipped with ProLong™ Gold antifade mountant (Molecular Probes). Sections were imaged with a x63 objective on a Zeiss 800 laser scanning confocal microscope (Zeiss).

Mechanical indentation of NTHI biofilms

Mechanical indentation was performed using a TA Instruments Discovery Hybrid Rheometer-2 (HR-2) with the Peltier plate connected to a heat exchanger (TA Instruments). The rheometer was fitted with 8mm-sand blasted Smart Swap parallel plate geometry. Rheology measurements were performed at 25°C. TRIOS v4 (TA instruments)

480 software was used for data collection. Biofilms formed by NTHI strain 86-028NP were
481 established in 35mm FluoroDishes (World Precision Instruments) for 16 h as described
482 above in section '*Stabilization of bacterial biofilm structure*'. After 16 h of incubation at
483 37°C, 5% CO_2, the medium was replaced with fresh medium that contained one of the
484 following: naive IgG (1000 nM), α-IHF IgG (1000 nM), naive IgG + RuvA (450 nM), or α-
485 IHF + RuvA (450 nM). After an additional 8h incubation period, the medium was replaced
486 again as described above and the biofilms were incubated for an additional 16 h. Prior to
487 rheological analysis, biofilms were washed twice with sterile PBS and the dishes were
488 filled with 3 ml PBS. Dishes were transferred to the Peltier plate, and mechanical
489 indentation was performed using an approach rate of 1 μm/s, with a termination step set
490 to 8N. For data interpretation, the force-displacement curves were converted to stress-
491 strain curves. Force (*F*) was converted to normal stress (σ) by dividing by the area of the
492 geometry ($\sigma = F/\pi r^2$). Displacement was converted to strain (γ) by dividing the resultant
493 change in thickness by the original thickness ($\gamma = \Delta L/L$). The Young's modulus (*E*) was
494 calculated using the force-displacement relationship previously described (28):

$$E = \frac{slope \cdot (1 - v^2)}{2r}$$

495 where the slope is of the force-displacement curve (N/m), *r* was the radius of the
496 geometry (*r* = 0.004m) and *v was* the assumed Poisson's ratio of a biofilm (*v* = 0.5) (40).
497 The slope of the lower, linear, portion of the force-displacement curve was measured,
498 which corresponded to 0-40% strain. Two biological replicates were analyzed, with
499 duplicate biofilms analyzed per biological replicate and two technical replicates per
500 biofilm.
501

Statistical evaluation

Statistical significance was assessed by unpaired or paired t-test (GraphPad Prism version 6.0). A $p \leq 0.05$ was represented as *, a $p \leq 0.01$ was represented by **, and a $p \leq 0.001$ was represented by ***.

Detailed materials and methods can be found in *SI Appendix*.

Data Availability

Raw data files are available from the corresponding author upon fair request.

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Figure Legends

Figure 1. Labeling of dsDNA and cruciform DNA within the chinchilla middle ear infected with NTHI and within sputum collected from a CF patient. (A) Representative images of an OCT-embedded section of the chinchilla middle ear infected with NTHI labeled for the presence of dsDNA (green) and cruciform DNA (white). (B) Representative images of an OCT-embedded section of sputum sample recovered from a

CF patient. Scale bar represents 10 μm. Note the complex lattice structure of eDNA and the punctate labeling of cruciform DNA at the vertices (yellow arrows) formed by the crossed strands of eDNA.

Figure 2. Holliday junction (HJ)-specific DNA binding protein RuvA stabilized bacterial biofilm structure even when DNABII proteins were depleted. (A) Representative images of a UPEC biofilm. (B) 16-hour UPEC and (C) 16-hour NTHI biofilms were incubated with the indicated protein and/or antibody for 24 hours. (D) 24-hour *S. epidermidis* biofilm was incubated with the indicated protein and/or antibody for 16 hours. Biofilms were stained with LIVE/DEAD® stain and visualized via CLSM. Images were analyzed by COMSTAT to calculate average thickness and biomass. Percent change in biomass compared to control was plotted. Bars represent the standard error of the mean (SEM). Statistical significance compared to control was assessed with unpaired t-tests, *$p<0.05$, **$p<0.01$, ***$p<0.001$. Note that RuvA prevented α-IHF-mediated disruption of the biofilm structure of UPEC, NTHI and *S. epidermidis* and thus confirmed the presence of HJ DNA within the biofilm matrix.

Figure 3. RuvA incorporated into the bacterial biofilm matrix when DNABII proteins were depleted. UPEC biofilms were formed for 16 hours, then incubated with naïve IgG (1000 nM) and RuvA (450 nM) [A, C] or α-IHF (1000 nM) and RuvA (450 nM) [B, D] for 24 hours. Immunofluorescence was performed on unfixed biofilms wherein the biofilms were incubated with either α-IHF antiserum (1:200 dilution) (A, B) or α-RuvA antiserum (1:200 dilution) (C, D) then incubated with goat anti-rabbit IgG conjugated to Alexa Fluor® 594. eDNA was stained with DAPI (gray). Biofilms were visualized via CLSM. Images

represent the top and side view of biofilms. (E) The relative abundance of DNABII proteins or RuvA was determined by the ratio of the respective protein (α-IHF/ α-RuvA labeled) to total DNA (DAPI). Statistical significance was assessed with paired t-tests, *p<0.05. Note the depletion of DNABII proteins and the concomitant incorporation of RuvA within the UPEC biofilm matrix.

Figure 4. RuvA mechanically compensated for the depletion of DNABII proteins to structurally stabilize NTHI biofilms. (A) NTHI biofilms were formed for 16 h, and incubated with either: naïve IgG (1000 nM), α-IHF (1000 nM) naïve IgG and RuvA (450 nM) or α-IHF and RuvA (450 nM) for a further 24 hours. Mechanical indentation analysis was depicted as stress-strain curves. The inset depicts a closer view of 0-40% strain (γ) portion of the curve. (B) Young's modulus calculated from the lower linear portion of the curve, depicted in the inset in (A). Data presented as mean ± SD; n = 4. Significance determined using a one-way ANOVA, * p<0.05, *** p<0.001, ns; not significant. Note that the Young's modulus of DNABII depleted biofilms stabilized by RuvA was comparable to control biofilms and thus confirmed that RuvA functionally and mechanically complemented for the depletion of the DNABII proteins within the NTHI biofilm EPS.

Figure 5. Disruption of bacterial biofilm structure by the Holliday junction (HJ)-specific endonuclease complex, RuvABC. (A) UPEC and (B) NTHI biofilms were established for 16 hours, then incubated with the indicated antibody (1000 nM) and RuvA (450 nM) for 24 hours (total 40 hours). Biofilms were incubated with RuvB (1130 nM) and RuvC (90 nM) in the final 16 hours. (C) *S. epidermidis* biofilm was established for 24 hours, then incubated with the indicated protein and/or antibody for 16 hours. Biofilms

were stained with LIVE/DEAD® stain and visualized via CLSM. Images were analyzed by COMSTAT to calculate biomass. Bars represent the SEM. Statistical significance compared to control was assessed with unpaired t-tests, *p<0.05, **p<0.01, ****p<0.0001. Note that upon replacement of DNABII proteins with RuvA within the biofilm matrix, biofilms were susceptible to HJ-specific endonuclease RuvC that resulted in the statistically significant collapse of UPEC, NTHI and *S. epidermidis* biofilm structure, consistent with the critical structural role of the HJ DNA in the stability of the bacterial biofilm extracellular matrix.

Figure 6. Dose-dependent disruption of bacterial biofilms by the Holliday junction-specific resolvase, RusA. 24-hour (A) UPEC, (B) NTHI and (C) *S. epidermidis* were incubated with varied concentrations of RusA (1, 5, and 10 μg/ml for UPEC and NTHI; 10 and 20 μg/ml for *S. epidermidis*) for 16 hours. Biofilms were stained with LIVE/DEAD® stain and visualized via CLSM. Images were analyzed by COMSTAT to calculate biomass. Bars represent the SEM. Statistical significance compared to control was assessed with unpaired t-tests, *p<0.05, **p<0.01, ***p<0.001. Note that RusA disrupted UPEC, NTHI and *S. epidermidis* biofilms in a statistically significant, dose-dependent manner.

Figure 7. Holliday junction-specific resolvases targeted HJ DNA within the NTHI biofilm extracellular matrix to disrupt the lattice-like eDNA network. NTHI biofilm growth was initiated in the absence (A) or presence (B) of RusA or (C) RuvABC for 16 hours. Unfixed biofilms were incubated with α-dsDNA monoclonal antibody then incubated with goat anti-mouse IgG conjugated to Alexa Fluor® 488. NTHI biofilm growth was initiated in the absence (D, E) or presence of RusA (10 μg/ml) (F) for 16 hours.

Unfixed biofilms were incubated with α-cruciform DNA monoclonal antibody then incubated with goat anti-mouse IgG conjugated to Alexa Fluor® 488 (yellow). NTHI were stained with FilmTracer FM™ 4-64 (gray). Biofilms were visualized via CLSM. (G) The relative intensity of cruciform DNA was determined by the ratio of cruciform DNA (yellow) to NTHI (gray). Bars represent the SEM. Statistical significance compared to control was assessed with paired t-tests, *$p<0.05$. Scale bar represents 10 μm. Note the complex web-like structure of eDNA in the control and the loss of this eDNA structure in the presence of RusA or RuvABC. Also, note the distribution of cruciform DNA throughout the biofilm matrix, particularly visible in the lower, denser part of the biofilm within an NTHI biofilm (E) and the loss of cruciform DNA in the presence of RusA (F).