Absence of Individual IHF Subunits

PLOS ONE | www.plosone.org 1 October 2012 | Volume 7 | Issue 10 | e48349

Sheryl S. Justice1,2*, Birong Li1, Jennifer S. Downey3, Shareef M. Dabdoub1, M. Elizabeth Brockson1, G. Duane Probst1, William C. Ray4, Steven D. Goodman3*

1 Center for Microbial Pathogenesis, The Research Institute at Nationwide Children’s Hospital, Columbus, Ohio, United States of America, 2 Department of Pediatrics and Urology, The Ohio State University College of Medicine, Columbus, Ohio, United States of America, 3 Division of Biomedical Sciences, Herman Ostrow School of Dentistry, University of Southern California, Los Angeles, California, United States of America, 4 Battelle Center for Mathematical Medicine, The Research Institute at Nationwide Children’s Hospital, Columbus, Ohio, United States of America

Abstract

Uropathogenic Escherichia coli (UPEC) utilizes a complex community-based developmental pathway for growth within superficial epithelial cells of the bladder during cystitis. Extracellular DNA (eDNA) is a common matrix component of organized bacterial communities. Integration host factor (IHF) is a heterodimeric protein that binds to double-stranded DNA and produces a hairpin bend. IHF-dependent DNA architectural changes act both intrabacterially and extrabacterially to regulate gene expression and community stability, respectively. We demonstrate that both IHF subunits are required for efficient colonization of the bladder, but are dispensable for early colonization of the kidney. The community architecture of the intracellular bacterial communities (IBCs) is quantitatively different in the absence of either IhfA or IhfB in the murine model for human urinary tract infection (UTI). Restoration of Type 1 pili by ectopic production does not restore colonization in the absence of IhfA, but partially compensates in the absence of IhfB. Furthermore, we describe a binding site for IHF that is upstream of the operon that encodes for the P-pilus. Taken together, these data suggest that both IHF and its constituent subunits (independent of the heterodimer), are able to participate in multiple aspects of the UPEC pathogenic lifestyle, and may have utility as a target for treatment of bacterial cystitis.

Citation: Justice SS, Li B, Downey JS, Dabdoub SM, Brockson ME, et al. (2012) Aberrant Community Architecture and Attenuated Persistence of Uropathogenic Escherichia coli in the Absence of Individual IHF Subunits. PLoS ONE 7(10): e48349. doi:10.1371/journal.pone.0048349

Editor: Rajivee Misra, Arizona State University, United States of America

Received May 4, 2012; Accepted September 24, 2012; Published October 25, 2012

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Funding: This work was supported in part by a National Institutes of Health grant (1R01DC011818-01, to SDG). There was no additional external funding received for this study. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: Sheryl.Justice@nationwidechildrens.org (SJ); sgoodman@usc.edu (SDG)

Introduction

Many bacterial species multiply within organized communities as a part or whole of their lifestyles in the environment or in the host. Uropathogenic Escherichia coli (UPEC), the causative agent of up to 80% of all urinary tract infections (UTIs) [1], is one such species. UPEC uses a community-based developmental pathway to propagate within the cytoplasm of urothelial cells during bacterial cystitis [2–4]. The developmental pathway begins with attachment-mediated invasion into the superficial bladder epithelial cells via FimH binding to the mannosylated uroplakin proteins (Zhou et al., 2001) and involves fusiform vesicles [5], cyclic AMP [5], Toll-like receptor-4 (TLR4) [6] and integrins [7]. Within the cytoplasm, bacillary-shaped UPEC multiply within loosely associated intracellular bacterial communities (IBCs) [2]. IBC maturation involves both changes in cell division fidelity and community architecture, which culminates in an organized, globular community architecture composed primarily of UPEC in a coccoid-like morphology. Once the IBC occupies the majority of the cytoplasm, UPEC regain a bacillary shape, become motile and egress from the epithelial cell through disruptions in the cell membrane. The intracellular amplification of UPEC occurs in repetitive cycles through attachment of egressed organisms to naïve superficial epithelial cells and ultimately culminates in the establishment of a latent or chronic infection [8–10]. Evidence for each of these stages is observed in urine samples and bladder biopsies of patients colonized with either UPEC or Klebsiella pneumoniae [11,12], which demonstrates that these events comprise the pathogenic lifestyle of multiple uropathogens during cystitis.

Integration Host Factor (IHF), a member of the ubiquitous DNABII family, acts as a DNA architectural element that can alter the conformation of nucleoprotein interactions inside the cell influencing replication, transcription, recombination and DNA repair [13–15]. IHF is required for regulation of a number of genetic loci associated with virulence. Production of elastase in Vibrio vulnificus [16], cholera toxin in Vibrio cholera [17], and type III secretion effectors in E. coli [18] require IHF. IHF promotes persistence of Legionella pneumophila in the protist host Acanthamoeba castellanii [19]. Regulation of adhesins and capsule is also IHF-dependent in Nisseria [20] and E. coli [21,22].

The DNABII family of proteins is also observed in the extrabacterial milieu [23–25]. The extrabacterial accumulation of the Streptococcus DNABII family member, IpA, elicits a
proinflammatory immune response in macrophages [26] that may contribute to tissue damage associated with infection.

Extrabacterial DNA (eDNA) is a key component of communities formed by many pathogenic bacterial species [27]. The DNA(BII) family is also critical for the integrity of bacterial communities that utilize eDNA as a structural component of the matrix. We demonstrated that antibodies directed against *E. coli* DNA(BII) family members disrupt communities formed by multiple human pathogens under laboratory conditions [28]. Sequestration of extrabacterial DNA(BII) family members from the community matrix also increased bacterial sensitivity to antimicrobials [28]. In addition, vaccination against IHF reduced the duration of established otitis media mediated by non-typeable *Haemophilus influenzae* in a mammalian model of human disease [28]. Therefore, extrabacterial DNA(BII) members, appear to be a plausible target for prevention and/or treatment of community-based infectious diseases.

In this study, we investigate the contribution of one DNA(BII) family member, IHF, to the pathogenic lifestyle of UPEC. Our studies unveil unique phenotypes associated with the presence of individual IHF subunits with regard to the regulation of type 1 pilus, as well as the overall architecture of the intracellular communities and colonization of the urinary tract. In addition, we identify an additional intrabacterial role for IHF in the production of the P-pilus. These studies demonstrate that IHF is essential for UPEC-mediated UTI and establishes functionality for each individual subunit of IHF in the absence of the canonical IHF heterodimer.

**Results**

**Inactivation of *ihfA* and *ihfB* in UPEC**

With our recent observation that IHF (referred to here as IhfAB) functions in the community matrix of a number of organisms including UPEC [28], we were interested to determine the role(s) of IhfAB during bacterial cystitis. IhfAB consists of 2 homologous subunits that form a heterodimer. In contrast to being able to make null alleles in genes encoding both subunits of IhfAB in the laboratory *E. coli* strain MG1655, we were unable to stably inactivate both subunits in our prototypical UPEC strain, UTI89 (Table 1). There were no significant differences in the growth rates of the remainder of the strains used in this study (data not shown). Mutations in either IHF subunit, *ihfA* or *ihfB*, have historically yielded a phenotype equivalent to that of the double mutant in laboratory adapted strains. Hence, the heterodimer has been

| Strain Name | Genotype | Source |
|-------------|----------|--------|
| BW25113     | *rrnB3* *AlocZ4787 hsdR514 A(israBAD567 A(rhaBAD)568 rph-1* | [71] |
| JW1702-1    | BW25113 *AihfA786-kan* | [71] |
| JW0895-3    | BW25113 *AihfB735-kan* | [71] |
| K1141       | *ihfA11::Tn10* E. coli Stock Center |
| MG1655      | *F lambda 1ivGe rfb-50 rph-1* | [72] |
| N99         | *AgaIK* | [73] |
| SGB3        | N99 *ihfB-Cam* | [74] |
| SGB4        | N99 *ihfA82 Tn10* | [75] |
| SJ1000      | UT189 *sunA-kan* | [76] |
| UT189       | *AihfA786::kan* Polar inactivation of region I | [44] |
| ROL607      | MG1665 *ihfB-Cam* *AihfA11::Tn10* | This study |
| UT189       | Cystitis Clinical Isolate | [8] |
| UT189       | *pANT4* | [2] |
| ROL745      | UT189 *ihfA11::Tn10* | This study |
| ROL747      | UT189 *AihfA786::kan* | This study |
| ROL603      | UT189 *ihfB-Cam* | This study |
| ROL748      | UT189 *AihfB735-kan* | This study |

The genotype of the strains and sources are indicated in the table. doi:10.1371/journal.pone.0048349.t001
presumed to be the only active form [29,30]. However, individual subunits readily form functional homodimers in complex with DNA in vitro [29,30], suggesting that homodimers may form upon stoichiometric changes in levels of the individual subunits. The inability to readily inactivate both genes in our clinical isolate suggests that the presence of one subunit may retain some functions that are essential for viability.

Type 1 piliation in the absence of IhfA or IhfB

Previous studies have demonstrated that the IhfA and IhfB heterodimer (IhfAB) participates in recombination events associated with promoter orientation of the fim operon (encoding type 1 pilus) in laboratory strains [22]. The type 1 pilus is essential for both bladder colonization and internalization [31–40]. Therefore, we wanted to carefully examine type 1 piliation phenotype of either ihfA or ihfB inactivation in our prototypical cystitis UPEC strain, UTI89. We examined each of the ihf mutations currently available (Table 2) to validate that the observed phenotypes were not allele specific. Type 1 piliation in UPEC strains defective in both ihfA and ihfB allele (Table 2, Figure 1), suggesting that IhfB may retain sufficient activity to initiate promoter conversion to the “on” orientation but is unable to support full piliation.

Both IhfA and IhfB are required for colonization of the urinary bladder

We have previously demonstrated that extrabacterial IhfAB (eIhfAB) contributes to the stability of UPEC communities grown on glass surfaces in vitro [28]. We next investigated the contribution of the individual subunits to the intracellular community development observed during cystitis [2]. Female mice were transurethrally inoculated with UTI89, UTI89 ΔihfA (ROL745), UTI89 ΔihfB (ROL603) or the complemented mutant strains to determine the ability of each strain to persist in the urinary tract. Inactivation of either Ihf subunit resulted in a 3 log decrease in colonization of the bladder as early as 6 hours post infection (Figure 2, p = 0.0017). The attenuation of colonization was more severe in the bladder at 48 hours post infection, as evidenced by at least a 4 order of magnitude decrease in bacterial burden (p<0.0006) in both the ΔihfA and ΔihfB mutants compared to wild type UTI89 (Figure 2). There was a statistically significant increase in the persistence of UPEC when either IhfA or IhfB were produced in trans in the strains devoid of chromosomal ihfA or ihfB, respectively (Figure 2). There was a bimodal distribution of bacterial burden, approximately 45% of the bladders infected with either mutant strain exhibited UPEC colonization that was indistinguishable from that of the parental strain. In the bladders where the bacterial burden was not fully restored, the level was still

### Table 2. Type 1 pilus phenotypes.

| Genotype | Piliation | Promoter Orientation |
|----------|-----------|----------------------|
| MG1655   | ++        | mix (off)            |
| MG1665 ihfB::Cam ihfA11::Tn10 | -- | not done |
| UTI89    | ++        | mix (even)           |
| UTI89/pANT4 | ++    | mix (even)           |
| UTI89/pMMB66 | +++ | N.D. |
| UTI89 ihfA11::Tn10 | -- | off |
| UTI89 ihfA11::Tn10/pHNx | ++ | on |
| UTI89 ihfA11::Tn10/pHNj | -- | off |
| UTI89 ihfA11::Tn10/pMMB66 | +++ | N.D. |
| UTI89 ΔihfA786::kan | + | mix (off) |
| UTI89 ΔihfA786::kan/pHNx | +++ | on/off |
| UTI89 ΔihfA786::kan/pHNj | + | mix (off) |
| UTI89 ihfB::Cam | -- | on |
| UTI89 ihfB::Cam/pHNx | -- | on |
| UTI89 ihfB::Cam/pHNj | +++ | mix (even) |
| UTI89 ΔihfB735::kan | -- | on |
| UTI89 ΔihfB735::kan/pHNx | -- | on |
| UTI89 ΔihfB735::kan/pHNj | +++ | mix (even) |
| UTI89 ΔihfB735::kan/pMMB66 | +++ | N.D. |

The relative level of type 1 piliation is indicated by the number of “+” signs. The orientation of the fimS promoter sequence is indicated. In cases where the promoter orientation was mixed in the culture, the predominant orientation is indicated in parentheses. N.D., not determined.
one or two orders of magnitude greater than in the absence of complementation (Figure 2), indicating that IhfAB participates in functions that are critical for persistence in the host.

Community architecture in the absence of IhfAB during cystitis

Previous microscopic evaluation of UPEC community development revealed important populations that are essential for persistence [2,41,42] in the bladder. Furthermore, the use of UPEC strains defective in specific bacterial traits results in variations in community development [43]. However, the regulatory mechanisms that control UPEC development are not understood. To gain more insight into the potential mechanisms that underlie the persistence defect by exploitation of the pleiotropic phenotypes associated with loss of IhfA or IhfB, bladders of mice infected with \(^{\text{ihfA11}}\) or \(^{\text{ihfB}}\) deficient strains were analyzed by fluorescence microscopy. Introduction of the pANT4 plasmid for constitutive production of green fluorescent protein had no effect on the production of Type 1 pili or the orientation of the promoter (data not shown). Communities were evaluated at 6 hours post infection, when the transition from the rod to coccoid shape generally occurs [2,33,41]. In contrast to the potential for 100 s of communities formed with the parental strain, and in accordance with the observed bacterial burden, in the absence of either IhfA or IhfB only a few epithelial cells (≤2) contained communities but unlike the parent strain, their architecture was aberrant (Figure 3). Consistent with the increase in the bacterial burden (Figure 2), robust IBC development was fully restored by complementation of the gene of interest in \(^{\text{trans}}\) (Figure 4). The lack of visible clusters of bacteria within the epithelial cells indicates that the growth of an organized community is severely crippled in the absence of either subunit. The observation that the developmental cycle of intracellular communities was restored in the presence of the gene in \(^{\text{trans}}\) suggests that the defects in community development are due to the absence of the subunit in question. The defect in community development in the absence of proteins with pleiotropic phenotypes has been observed with other UPEC mutants that display normal growth rates under laboratory conditions [33,39,40,44,45].

In the few cases where intracellular clusters were observed, there were measurable visual differences as compared with prototypical IBCs (Figure 5). Although the bacterial burden of the UTB89 \(^{\text{ihfA11::Tn10}}\) and UTB89 \(^{\text{AihfB}}\) strains was similar (Figure 2), the overall organization of the intracellular clusters is unique for each strain (Figures 3, 5). In the absence of capsule, the intracellular bacterial clusters have distinctive changes in both community architecture as well as bacterial morphology (Figure3, 5) [44]. In contrast to UPEC \(^{\text{AihfB}}\), there was no significant difference in the morphology of the individual bacteria deficient in either IHF subunit (Figure 5A), suggesting that the phenotypic changes associated with intracellular clusters of UTB89 \(^{\text{ihfA11::Tn10}}\) or UTB89 \(^{\text{AihfB}}\) are not related to a defect in capsule synthesis. UTB89 \(^{\text{ihfA11::Tn10}}\) retains the ability to form the more
globular-shaped community indicative of the middle stage community \[2\] as observed with UTI89 and UPEC \(D_{\text{surA}}\) (ratio of width to height \(\sim 1.2\)) (Figure 5B). However, there was a decrease in the area occupied by the bacterial cluster, suggesting that robust intracellular growth may require IhfA. Moreover, the conversion to the more coccoid-shaped individuals was not observed, suggesting that targets of IhfAB contribute to the morphological changes associated with intracellular community maturation. In contrast, the shape and density of the community formed by UTI89 \(D_{\text{kps}}\) was more open and elongated, resembling that of UPEC \(D_{\text{kps}}\) (ratio of width to height \(\sim 3\)) (Figure 5B). The increase in interbacterial distance and shape of the cluster suggests that IhfB is important in coordination of the community matrix or production of a surface structure that mediates bacterial interaction with the matrix. The differences in the architecture of the intracellular clusters formed in the absence of either subunit further suggests that the individual subunits retain functionality and that, although homologous, retain unique functions.

**Type 1 piliation does not restore virulence in the absence of IhfA**

It is well established that the presence of the Type 1 pilus is an essential trait for both the initial adhesion to bladder epithelial cells and intracellular community \[31–40\]. The absence of piliation for strains defective in either Ihf subunit could account for the colonization defects observed. To bypass the requirement for IhfAB in the production of the Type 1 pilus and determine additional roles for the IhfAB subunits during pathogenesis, an episomal copy of the \(\text{fim}\) operon under control of the \(\text{P}_{\text{trc}}\) promoter was introduced into the mutant strains. The capacity to agglutinate yeast was fully restored in the mutant strains (Table 2). In the absence of IhfA, there was at least a 2 log decrease in bacterial burden, despite the presence of abundant Type 1 piliation \(p<0.002\) (Figure 6). There were no detectible bacteria in the majority of the bladders \(4\) of \(7\), suggesting that IhfA participates in additional processes during cystitis. There was approximately a 1 log decrease in the bacterial burden in the absence of IhfB that did not reach statistical significance (Figure 6). The recovery of virulence in the absence of IhfB suggests that the major defect is associated with loss of this subunit is the absence of Type 1 pili. These results suggest that there are unique functions for each of the Ihf subunits and that at least IhfA participates in functions in addition to piliation.

**IhfAB is present in extrabacterial milieu of IBCs**

We have previously demonstrated that addition of antibody directed against IhfAB will disrupt the integrity of communities of UPEC grown on glass surfaces \[28\], suggesting that eIhfAB is important for the stability of interbacterial interactions. In addition, little is known regarding the presence and nature of the IBC matrix. To determine whether IhfAB could be detected in...
the extrabacterial milieu of IBCs as an indicator for the presence of extrabacterial DNA, immunofluorescence microscopy was performed on infected, splayed whole mount mouse bladders. The epithelial cells were permeabilized under conditions that do not affect bacterial cell integrity (Methods and Materials). Evaluation of IBCs of UTI89 (green) at 16 hours post infection revealed the presence of eIhfAB (red) throughout the community architecture of established IBCs (Figure 7). In addition, eIhfAB is detected associated with UPEC that egress from infected epithelial cells (Figure 7). The punctate staining was not observed when naive serum was used under the same experimental conditions. This evidence suggests that IhfAB is a component of the IBC matrix and that some of the phenotypes associated with the aberrant architecture may be associated with defects in the composition or deposition of eIhfAB in the IBC matrix.

Antibody against DNABII family of proteins reduces UPEC binding to bladder epithelia

To further validate the presence of eIhfAB associated with UPEC, we determined the ability of antisera to prevent binding of UPEC to bladder epithelial cells. We hypothesized that the steric hindrance imposed by antibody binding to eIhfAB would preclude UPEC type 1-mediated attachment to cultured human bladder epithelial cells. UTI89 was exposed to antisera directed against IhfAB prior to introduction to a monolayer of bladder epithelial cells. The presence of the antisera directed against IhfAB reduced the attachment of UTI89 (Figure 8) further supporting the observation of IhfAB on the bacterial surface.

IhfA and IhfB are dispensable for early colonization of the kidney

In contrast to the bladder, where the IhfA and IhfB subunits are required for early stages of colonization, the absence of either the IhfA or IhfB subunit does not dramatically affect the colonization of the kidney at 6 hours post infection (Figure 2). Although statistical analysis demonstrated a significant difference, the biological relevance for less than a half-log difference in bacterial burden at this time point is unclear. The initial slight differences in colonization of the kidney may be indicative of the statistically significant two to four log decline in persistence observed with both mutant strains at 48 hours post infection (Figure 2; p<0.0006). As observed in the bladder tissue, addition of the gene in trans significantly increases the bacterial burden (Figure 2). The absence of either subunit was better tolerated in the kidney at 48 hours when these same mutants were essentially cleared from the bladder. IhfA alone supports more robust colonization of the kidney than does IhfB alone, suggesting that the subunits can at least function in the absence of the heterodimer in the kidney. The decreased persistence is likely not due to effects on motility as infection with strains defective in flagellum synthesis did not display a colonization defect under similar infection conditions [46] and Type 1 piliation was unable to restore the colonization of the kidney (p<0.0002) (Figure 6). The P-pilus is known to bind to the globo-series glycoproteins on kidney epithelial cells [47]. Although the role of the P-pilus in adherence to the mouse kidney is unclear, the defect in colonization of the kidney and the predicted DNA bend in the promoter region of the pap operon [48] led us to evaluate the production of the P-pilus in our mutants. In contrast to the parental strain, neither mutant strain demonstrated hemagglutination following growth on tryptic soy agar (data not shown), suggesting that P-pilus (important for colonization of the kidney) also requires IhfAB.

**IhfAB protects the promoter region upstream of papB**

As a first approach to determine whether IhfAB participates in regulation of the P-pilus, potential binding sites for IhfAB were evaluated in the promoter region of the operon encoding the genes for the P-pilus (pap). Using Regulatory Sequence Analysis DNA pattern matching Tools we found six sites with potential homology to the IhfAB consensus sequence (data not shown) in the 404 bp region between the divergent start sites of papB and papI. After closer examination of the critical residues for IHF-DNA associ-
ination [49], we restricted our evaluation to two regions, 88 bp (AATCAATATTATAC) and 48 bp (TATCAACGGAAAG) from the transcriptional start of *papB* and *papI*, respectively. The site identified upstream of *papI* does not appear to serve as an IhfAB binding site based upon DNase I footprint analysis (data not shown). However, the sequence upstream of *papB*, was protected by the inclusion of IhfAB in the reaction, indicating that IhfAB binds to the promoter region of *papB* which encompasses the −35 region and −10 regions (Figure 9) [50,51]. Immediately upstream of both the −35 and −10 region we observed sites of enhanced DNase I digestion. The enhanced sensitivity to digestion is often the result of local DNA bending which allows greater access of DNase I to the phosphodiester backbone and is indicative of induced structure of IhfAB [52].

**Discussion**

Evaluation of multiple phenotypes associated with UPEC persistence during urinary tract infection demonstrates that the inactivation of *ihfA* and *ihfB* are not equivalent. Consistent with previous observations that homodimer species are active *in vitro* [29,30], our data suggests that there may be independent functions for the homodimers under specific environmental conditions. For example, *ihfA* expression follows LexA-dependent activation by the SOS response [53]. We have demonstrated that the SOS response is activated in intracellular UPEC during bacterial cystitis [41,45] and we would expect increased production of IhfA in the presence of host immune responses that include DNA damaging agents (i.e. reactive oxygen and nitrogen species) during infection. This increased production of IhfA could result in the presence of both IhfAB heterodimers and IhfA homodimers within the same bacterium during infection. The potential requirement for additional IhfA during the SOS response may partially explain the decreased persistence of UTB99 *ihfA11::Tn10* during cystitis. IhfA is unstable under conditions of overproduction [54] and could contribute to the decreased persistence observed in the bladder. As we have demonstrated that individual subunits retain novel properties with regards to community development, it is possible that mixed homodimer and heterodimer populations may provide unique roles during the pathogenic lifestyle.

**Figure 6. Type 1 piliation does not restore bladder colonization in the absence of IhfA.** Each symbol represents single infected murine bladder or combined kidney pair. Female mice were infected with wild type UTI89/pMMB66 (black filled squares), ROL745/pMMB66 (UTI89 *ihfA11*; gray filled circles), or ROL603/pMMB66 (UTI89 *ΔihfB*; gray filled triangles) for 6 hours post inoculation. Bacterial burden of bladders and kidneys was enumerated as colony forming units ( CFUs). Statistical significance determined using non-parametric Mann Whitney (***<0.002, **=0.0006). doi:10.1371/journal.pone.0048349.g006

**Figure 7. IhfAB is extrabacterial within the IBC milieu.** Bladders were removed at 16 hours following transurethral introduction of UTI89/pANT4 (green). At 16 hours post introduction of bacteria, the bladders were harvested, bisected, fixed, host cells permeabilized and eIhfAB was observed with antibody (red) and Hoescht counterstained (blue). (A, D) No staining is observed when naïve rabbit serum is used as primary antibody. (B, E) eIhfAB is observed between the bacteria within the epithelial cell. (C, F) eIhfAB remains associated with UPEC following egress onto the surface of the bladder. Scale bar = 10 μm. doi:10.1371/journal.pone.0048349.g007
IhfAB is known to participate in the transcription of type 1 pili and capsule [21,22]. Strains defective in production of the type 1 pilus demonstrate a reduction in the number of infected cells, and in the few cases where intracellular bacteria are detected, there is an absence of interbacterial interactions resulting in evenly distributed bacteria within the superficial bladder epithelial cell [32]. We demonstrated that replacement of Type 1 piliation did not restore virulence in the absence of IhfA. This is in contrast to recent reports where restoration of Type 1 piliation led to a recovery of virulence with other pleiotropic mutants [39,40] when tested independently. In addition, we compared the intracellular phenotypes of the *ihfA* and *ihfB* mutants with strains deficient in type 1 pili and K1 capsule, where aberrant architecture of intracellular clusters was previously described [32,33,44]. UTI89 *ihfA11::Tn* retains interbacterial interactions during intracellular growth (Figure 3) and the more globular architecture of the prototypical IBC, but appear to have reduced growth and morphological changes as compared with the parent (Figure 5). Due to the few infected cells observed with the mutant strain, we were unable to determine the contribution of IhfA to the later developmental stages. However, the significant decrease in bacterial burden (Figure 2) suggests that the later stages of development are also compromised.

The phenotype of the UTI89 Δ*ihfB* included the reduction in the number of intracellular clusters as well as a unique appearance in interbacterial interactions (Figure 3). The interbacterial spacing resembles that of UTI89 *surA* (defective in type 1 pili and outer membrane beta barrel proteins) [33,55–57] but the overall shape of the cluster is different (Figure 5). The clusters formed by UTI89 Δ*ihfB* are similar in overall shape with those of the UTI89 Δ*kpaF* strain (defective in the K1 capsule) (Figures 3, 5) [44], but lack the morphological changes associated with deficiencies in capsule production. Comparison of the architectural and growth characteristics of each UPEC mutant provides additional insight into the specific defects rather than just evaluation of bacterial burden alone.

The characterization of bacterial elements that are critical in the development of intracellular communities has been hampered by the severe bottleneck in the number of infectious organisms required to establish disease [38]. The use of microscopic evaluation of the intracellular clusters formed in the absence of SurA (another pleiotropic mutation)[33], led to the identification of Type 1 pili and OmpA as critical complexes required for intracellular development [32,59]. Along these lines, we herein describe a new binding site for IhfAB that contributes to regulation of the *pap* operon. Moreover, the observation that the defects in UPEC development are quantitatively different in the absence of either subunit using our newly developed software package [60] provides a platform for the identification of additional bacterial traits that are required for UPEC development.

The observation that IHF simultaneously participates in the virulence of UPEC by regulating transcription of key virulence
traits intra-bacteriaally as well as contributing to the composition of the matrix extra-bacteriaally suggests that the use of therapeutics that target IHF function would severely cripple UPEC through inactivation of a number of traits that are critical for pathogenesis (i.e. type 1 pilus, P pilus, capsule, community integrity). Such a therapy could potentially affect both extrabacterial IhfAB and intrabacterial IhfAB targets of UPEC pathogenesis simultaneously and significantly reduce the probability for accumulation of resistant strains. Thus, further characterization of the bacterial traits influenced by IhfAB could lead to novel approaches to control UTIs.

Materials and Methods

Bacteria strains and plasmids

The strains and plasmids used in this study are indicated in Table 1. Constitutive synthesis of green fluorescent protein was performed using pANT4 (previously designated pCOMGFP) [2,61]. Production of either or both IHF subunits was accomplished using pHNz [62], pHNB (kindly provided by Howard Nash), or pHN6p [63]. Ectopic production of the Type 1 pilus was accomplished using pMMB66 [64].

Media and growth

Bacteria were grown at 37°C in Luria-Bertani (LB; Fisher Scientific, Pittsburg, PA) broth in the absence of aeration unless otherwise indicated (planktonic). For assessment of community development in vitro, E. coli strains indicated were prepared as described [28]. When appropriate, antibiotics were used at the following concentrations: 30 µg/ml kanamycin, 25 µg/ml chloramphenicol, 25 µg/ml tetracycline hydrochloride, or 100 µg/ml ampicillin (Fisher Scientific, Pittsburg, PA).

The fimS orientation

Twenty-five microliters of overnight liquid cultures as well as in vitro community cultures [28] of various growth phases of planktonic and biofilms were lysed, and the supernatants containing chromosomal DNA were used for amplification of the fimS region. The amplification products were digested with BsuUI (New England Biolabs) and visualized following separation on a 2% agarose gel as described previously [65] to determine the orientation (‘‘off’’ = 539 & 187 bp, while ‘‘on’’ = 433 & 293 bp) of the promoter.

Inactivation of ihfA and ihfB in MG1655 & UTI89

Laboratory strains of E. coli that carry the individual mutations were obtained from various sources (Table 1). The mutations were introduced into the prototypical UPEC strain, UTI89, and a laboratory adapted strain, MG1655, from the laboratory strains by P1 transduction [66]. To facilitate P1 binding to UPEC, cultures were grown at room temperature to reduce production of the K1 capsule [67]. In all cases, at least three independent transductants were tested in all laboratory-based assay.

in vitro yeast agglutination assay

The mannose-sensitive Yeast Agglutination (YA) assay (type 1 pilation) or the hemagglutination assay (P pilation) was performed as previously described [45,68].

Mouse infections

UTI89, ROL745 (UTI89 ΔhfaI::Tn10) and ROL603 (UTI89 ΔhfbI) were grown in LB broth in the absence of aeration for 16 hours to an OD600 ~1.2 at 37°C. Inoculum of each strain was normalized such that ~10⁷ bacteria were transurethrally introduced directly to the bladders of female C3H/HeNshd mice (Figure 2: Harlan Sprague Laboratory; Indianapolis, IN) (Figure 6: Charles River Laboratory, Wilmington, MA) as previously described [2,3,69]. Tissues were harvested at times indicated post-inoculation. Enumeration of bacterial burden was performed as previously described [33,41]. Statistical significance was determined using a two-tailed Mann Whitney test of the wild type UTI89 for the comparison with each UPEC mutant individually. The experiments were performed in cohorts of 5 mice and repeated on separate occasions. All data points are presented. Maintenance of all mice was in strict accordance of the Institutional Animal Care and Use Committee (IACUC) rules and regulations. All animals are housed in accordance with USDA guidelines for the care and housing of laboratory animals, and USDA officials routinely inspect the facilities. The experiments presented in this manuscript are approved (AR06-00119) by The Research Institute at Nationwide Children’s Hospital Institutional Laboratory Animal Care and Use Committee (Welfare Assurance Number A3544-01).

Immunofluorescence microscopy of whole mount tissue

Visualization of UPEC colonization was performed as previously described [33,41]. Bladders were harvested and stretched followed by 4% PFA with 0.1% Triton X-100 in PBS overnight to permeabilize the epithelial plasma membranes. Bacterial and host DNA were visualized by the addition of Hoechst 34580 (Invitrogen; Carlsbad, CA) for 10 minutes. Bladders were stained as indicated below and mounted with ProLong Gold antifade reagent (Invitrogen, Carlsbad, CA). Images were acquired using an Axiovert 200 M inverted epifluorescence microscope equipped with a motorized stage, an Axioscan MRM CCD camera and the Apotome component to improve fluorescence resolution (Carl Zeiss, INC, Thornwood, NY). The levels of the fluorescent images were adjusted to all pixels within the image using Adobe Photoshop (Adobe Systems Incorporated; San Jose, CA). Quantitation of bacterial morphologies was performed using Image J (developed at the National Institutes of Health, http://rsbweb.nih.gov/ij/) and ProkaryMetrics [60].

Visualization of UPEC

Intracellular UPEC were visualized the addition of Rabbit Anti-E.coli polyclonal antisera (PAb) (USBiological, MA) diluted 1:200 in PBS with 0.1% Triton X-100 at 37°C for 1 hour. Splayed bladders were washed by PBS for 3 times before the addition of Goat anti Rabbit secondary IgG conjugated to Alexa-594 (Life Technologies) 1:200 with 0.1% Triton X-100 at 37°C for 1 hour. Residual antibodies were removed by washing with PBS for 3 times before stained by Hoechst 34580 (Life Technologies, Carlsbad, CA).

Visualization of extrabacterial UPEC in IBCs

Antisera raised against purified recombinant IHF [62] or naive rabbit serum was diluted 1:100 in PBS and applied to mounted bladder tissues for 1 hour. The presence of specific antibody was visualized using Alexa 594-conjugated anti-IgG antibodies (Life Technologies, Carlsbad, CA).

Binding to bladder epithelium in the presence or absence of antisera directed against IhfAB

Overnight cultures of UTI89 was diluted to ~1×10⁵ colony forming units (CFU)/ml in minimal essential medium (ATCC; Manassas, VA). A 1:50 dilution of antisera raised against IhfAB
Identification of IHF recognition sites

Using available Regulatory Sequence Analysis DNA pattern matching tools (RSAT, http://rsat.ulb.ac.be/rsat/) we analyzed the divergent *papB* and *papI* promoter region for the presence of IHF recognition sites (WATCAANNNNTTR, where W = A or T, R = G or A, N = any nucleotide) [19].

DNaSel Footprinting Assay

To label the DNA substrates 1 µM oSG759 (5′-ATGTTTCCCCCCTTCATG3′) was labeled at the 5′-end with 0.85 µM of [γ-32P] ATP (10 mCi/ml, New Life Science Products) using T4 polynucleotide kinase (Promega). To amplify the promoter region of *papB* (141 bp) labeled oSG759 was used along with the unlabeled primer oSG760 (5′-TCTGTTTGGTTTGGTTGG3′) in a PCR reaction using purified E. coli UTB189 chromosomal DNA. Identification of residues protected by IHF binding was performed as previously described [70]. Briedly, the labeled DNA substrate was incubated with 50 nM purified recombinant IhfAB [54] for 30 min at room temperature in footprinting buffer (25 mM Tris-HCl, pH 7.5, 50 mM KCl, 6.25 mM MgCl2, 10% glycerol, 1 mM DTT) before the addition of 2.5 mM CaCl2 and 5 mM MgCl2. Partial digestion using DnaseI (Promega, Madison, Wisconsin) was allowed for 1 min. DNA was extracted with phenolchloroform, ethanol-precipitated and resuspended in Sequitherm EXCEL II DNA sequencing stop buffer (Epicentre Biotechnologies). A sequence ladder for the *papB* fragments was generated using labeled oSG759, the *papB* promoter fragment and the Sequitherm EXCEL II DNA sequencing kit (Epicentre Biotechnologies) following the manufacturer’s instructions. All reactions were separated on a 6% sequencing gel run at 40 V/cm for 1.5 hours. The gel was then dried and scanned with the Typhoon imaging system (GE Healthcare).

Author Contributions

Conceived and designed the experiments: SSJ SDG. Performed the experiments: BL JSD MEB SMD GDP. Contributed reagents/materials/analysis tools: SMB WCR. Wrote the paper: SSJ SDG.

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