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Community Behavior and Amyloid-associated Phenotypes among a Panel of Uropathogenic *E. coli*

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Abstract

Uropathogenic *E. coli* (UPEC) are the major causative agents of urinary tract infection and engage in a coordinated genetic and molecular cascade to colonize the urinary tract. Disrupting the assembly and/or function of virulence factors and bacterial biofilms has emerged as an attractive target for the development of new therapeutic strategies to prevent and treat urinary tract infection, particularly in the era of increasing antibiotic resistance among human pathogens. UPEC vary widely in their genetic and molecular phenotypes and more data are needed to understand the features that distinguish isolates as more or less virulent and as more robust biofilm formers or poor biofilm formers. Curli are extracellular functional amyloid fibers produced by *E. coli* that contribute to pathogenesis and influence the host response during urinary tract infection (UTI). We have examined the production of curli and curli-associated phenotypes including biofilm formation among a specific panel of human clinical UPEC that has been studied extensively in the mouse model of UTI. Motility, curli production, and curli-associated biofilm formation attached to plastic were the most prevalent behaviors, shared by most clinical isolates. We discuss these results in the context on the previously reported behavior and phenotypes of these isolates in the murine cystitis model *in vivo*.

Keywords

curli; functional amyloid; biofilm; amyloid; Congo red; uropathogenic *E. coli*

INTRODUCTION

Uropathogenic *E. coli* (UPEC) are the major causative agents of urinary tract infection and are associated with acute infections as well as chronic and recurrent infections that require long-term antibiotic therapy and are often associated with life-threatening sequelae that can include antibiotic resistance and sepsis [1,2,3,4]. UPEC engage a coordinated and regulated genetic and molecular cascade to assemble type 1 and P pili, which are key virulence factors associated with infections of the bladder and kidney, respectively [5,6,7,8,9]. In the bladder, type 1 pili mediate attachment to mannosylated cell-surface glycoproteins expressed on host bladder-epithelial (urothelial) cells [5,10]. *E. coli* can invade urothelial cells and initiate

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a pathogenic cascade, involving several distinct stages as examined in the mouse cystitis model and in human UTI [11,12,13]. Within urothelial cells, bacteria can replicate to form dense biofilm-like intracellular bacterial communities (IBCs). UPEC eventually detach and then disperse, or flux, from the IBC to initiate new rounds of attachment and invasion in other cells. Some fluxing bacteria become filamentous and evade neutrophil phagocytosis, facilitating bacterial survival [6,12]. Even after acute infection is resolved, bacteria can remain within the bladder for many days to weeks, regardless of standard antibiotic treatments [14]. UPEC also colonize abiotic surfaces, including plastic and stainless steel and are associated with catheter-associated UTI.

Targeting adhesion and biofilm formation with molecular inhibitors has emerged as an attractive anti-virulence approach to prevent and treat urinary tract infections. Among the challenges to developing effective anti-virulence compounds, however, is the elucidation of the roles of various virulence factors and mechanisms that contribute to pathogenesis. The type 1 pilus is perhaps the most well-studied virulence factor associated with UPEC infections of the bladder [5,6,7,8,9]. Type 1 pili are essential to bladder colonization. However, clinical isolates differ tremendously in their phenotypes *in vitro* and *in vivo* due to the myriad of other molecular features that differentiate them and their interactions with the host [15,16]. Therefore, much research is needed to uncover the contributions and interplay of other genetic and molecular contributions to UPEC physiology and pathogenesis.

Curli are functional amyloid fibers that have been studied for their role in community behavior, adhesion, and biofilm formation in *E. coli* and *Salmonella*. They are among a growing list of functional amyloids that are produced by bacteria and implicated in adhesion and biofilm formation [17,18,19,20,21,22]. Curli production is prevalent among UPEC isolates and influences pathogenesis *in vivo* [23,24,25,26,27]. Recent *in vivo* studies suggest that curli are produced in the host and provide a fitness advantage to UPEC as reflected in bladder and kidney bacterial titers in the murine UTI model [25]. Similarly, the expression of curli and cellulose has been demonstrated to modulate the immune response [26], and curli were identified in human patient urine samples by electron microscopy and by antibody reactivity, indicating that UPEC express curli within the human bladder [26]. Curli-expressing *E. coli* are recognized by the immune system and activate the production of proinflammatory cytokines (tumor necrosis factor- α , interleukin-6 and -8) in human sepsis and may contribute to inflammation during UTI [23]. Curli also contribute to the immune response by activating the Toll-like receptors (TLR1 and TLR2) and the CD14 complex [28,29]. A recent study examining isolates from UTI in children found that the co-expression of curli and cellulose was most prevalent in isolates associated with severe UTI, particularly pyelonephritis [27]. In the laboratory, the coproduction of curli and cellulose enables the elaboration of bacterial biofilms formed on agar [30], characterized by the hallmark wrinkled colony morphology, as well as biofilms at the air-liquid interface (pellicle), and biofilms attached to plastic (*e.g.* PVC) [31,32].

Here, we profiled curli production among a panel of UPEC human clinical isolates that has been examined *in vivo* in the murine cystitis model [16] in order to examine the prevalence and importance of curli to bacterial biofilm formation in laboratory conditions. Each isolate in the panel, except Pyelo 3, forms biofilm-like IBCs in murine bladder epithelial cells [16], a hallmark feature of the UPEC pathogenic cascade that is documented also in human UTI [13]. We implemented several curli quantification and curli-associated biofilm assays and discovered a strong propensity for curli-associated phenotypes among these organisms.

MATERIALS AND METHODS

Bacterial strains and standard growth conditions

19 UPEC clinical isolates were examined [16]. The curli and type 1 pilus mutants were used as controls: UTI89 Δ *csgBG*, UTI89 Δ *csgA*, UTI89 Δ *fimA*, UTI89 Δ *fimH*; and the laboratory strain MC4100. Colony biofilm formation was initiated by spotting 10 μ l of overnight bacterial culture onto YESCA (0.5 g/mL yeast extract, 10 g/mL casamino acid) agar plates with or without Congo Red (CR, SIGMA #C6277) or Calcofluor (SIGMA #F3543, Fluorescent Brightener 28). Final concentrations of CR and Calcofluor were 20 μ g/mL and 10 μ g/mL, respectively. The associated colony morphology for each strain was observed after 48 hours of growth at 26 or 34°C. Colonies on agar plates containing Calcofluor were visualized and photographed over a UV light table.

Western blot analysis

The major curli subunit protein, CsgA, was examined by immunoblot assays as described [25]. For the detection of CsgA and the type 1 pilus major subunit protein, FimA, in the PVC-associated biomass assay in Figure 2b, formic acid was used to rinse the wells of 6 replicate wells, combining them into one 100 μ L sample that was processed as described [25].

Motility assay

Single colonies from bacterial cells grown on YESCA agar were stabbed into 0.3% YESCA agar and incubated at 26 or 34°C for 12 h, after which the diameter of the motility halo was measured.

Crystal-violet-based biofilm assay

Biofilm formation in YESCA or LB broth attached to plastic (polyvinyl chloride, PVC) was assessed using the Kolter crystal violet assay to quantify the attached biomass as described [33].

Pellicle biofilm assay

Pellicle formation was initiated by inoculating 2 μ L of an overnight bacterial culture grown in YESCA broth in 2 mL YESCA broth in 24-well-plate wells and incubated at 26°C. Pellicle formation was inspected visually and assessed by perturbation with a pipet tip after 48 hours of growth.

Agglutination assay

Agglutination assays with dimethyl sulfoxide (DMSO) were performed as described previously [30]. DMSO has been demonstrated to increase curli expression and to induce an agglutination phenotype [30]. All UPEC clinical isolates were grown in 4 mL of YESCA broth in the presence of 4% (DMSO) with 200 rpm shaking. The agglutination phenotype was evaluated after 24 hours.

RESULTS

Colony morphology, dye binding, curli expression, and motility on agar

Curliated bacteria bind the amyloid dye Congo red (CR) [34] and dye uptake is often used as an indicator of curli expression. However, curli-independent variability in the CR phenotype among strains has been noted [35], attributed to factors such as the ability of CR to bind to cellulose[34], and should not be the only assay used to score bacterial amyloid production.

Photographs of a representative set of nine isolates illustrate the range of variations in colony morphology and dye binding exhibited by the isolates. The complete set of results for all isolates is provided in Table 1. In addition to simple dye binding, bacteria expressing curli and cellulose typically form colonies with a red, dry, and rough (RDAR) morphology when grown on CR-supplemented agar. Both CR and Calcofluor binding were observed in each case where the RDAR morphology was displayed, consistent with the production of both curli and cellulose. However, there were five isolates (Acute-1, ASB-3, rUTI-2, rUTI-3, and rUTI-5) which bound CR and Calcofluor, but did not exhibit the RDAR phenotype. Western blot analysis was performed to confirm and quantify the relative production of curli among the isolates. ASB-3 was the only strain from the five isolates above that did not make curli, thus the other four isolates are making curli but do not exhibit the RDAR morphology. In total, there were 15 isolates that bound CR at 26 °C (13 exhibited CR and Calcofluor binding; 2 exhibited only CR binding). Of these, only ASB-3 did not produce curli as determined by Western blot analysis. Thus, the CR binding phenotype was fairly predictive of curli production, but not perfect, as is generally appreciated [32, 33].

Motility has been shown to be required for *E. coli* biofilm formation in LB broth, promoting initial interactions with and movement along a surface [36]. Motility was observed in 14 of the 19 isolates. With the exception of Pyelo-4, the lack of motility was always correlated with lack of the RDAR phenotype.

Biofilm formation attached to plastic

UPEC can form biofilms attached to plastic that require either curli or type 1 pili, depending on whether cells are grown in YESCA or LB broth, respectively [25]. Thus, the panel was evaluated for the ability to form biofilms attached to PVC plastic in YESCA and LB. For all isolates that formed appreciable biofilm ($OD_{600} > 0.1$ in Figure 2), except rUTI-5 which was a relatively poor biofilm former, the measured biomass was larger in the YESCA grown cells, as reflected in the highest crystal violet OD_{600} values. Furthermore, only 5 of the 19 isolates formed an appreciable biofilm in LB, whereas 13 of the 19 isolates formed a PVC-associated biofilm when grown in YESCA, the condition that requires curli production. Thus, biofilm formation was significantly more prevalent in YESCA broth and a greater amount of attached biomass was associated with these curli-dependent biofilms (Figure 2).

We also present the results from nine representative isolates in further molecular detail to illustrate the range of possible differences we observed in biofilm formation among UPEC. Figure 2b reveals that when grown in LB, type 1 pili were, of course, produced by the two strong biofilm formers, UTI89 and ASB-1, yet UTI89 also produced curli in their biofilm, although curli are not required for biofilm formation in LB. Among the biofilm formers in YESCA, some cells produced only curli, while some produce both curli and type 1 pili.

Broth-grown curli expression and DMSO-induced agglutination

Some strains can produce curli during growth in nutrient broth even while shaking [25,30] and curli expression is upregulated when grown in the presence of 4% DMSO, resulting in a readily detectable agglutination phenotype in which cells agglutinate and fall out of solution [30]. At 26 °C, all isolates except rUTI-1 that produced curli also exhibited increased agglutination in the presence of DMSO (Supplemental Figure 1a). Furthermore, some isolates that did not express curli or produced very low levels of curli, including Acute-1, ASB-3, Pyelo-1, and rUTI-5, exhibited agglutination when growing in the presence of 4% DMSO, consistent with an increase in curli gene transcription and curli production [30]. The agglutination phenotype was observed less frequently at 34 °C.

Pellicle formation

UTI89 can form a biofilm at the air-liquid interface, referred to as a pellicle, when growing static in YESCA broth [25]. We examined the ability of the UPEC isolates to form pellicles in YESCA broth at 26 and 34 °C after 72 hours (Supplemental Figure 1b). Pellicles require the production of curli and were only observed among isolates that were determined to produce curli in broth as described above [25]. Nevertheless, not all curli producers could assemble a pellicle. Thus, while curli are required, they are not sufficient for pellicle formation.

DISCUSSION

Blocking the assembly and/or function of virulence factors has emerged as an attractive target for the development of new therapeutic strategies to prevent and treat UTI, particularly in the era of increasing antibiotic resistance among human pathogens. Such anti-virulence strategies could complement existing antibacterial therapies that target cell viability. Still, we need to learn a great deal more about UPEC virulence and the genetic and molecular features that distinguish isolates as more or less virulent and as robust biofilm formers or poor biofilm formers [13,30,31,37,38]. The production of curli by many clinical isolates and the production of curli *in vivo* emphasizes the need to examine curli contributions to bacterial physiology and community behavior and, ultimately, to understand their role in the pathogenesis of urinary tract infection [21,26].

We examined a panel of human clinical UPEC isolates with a molecular-level focus on curli. These isolates have been well studied in the mouse cystitis model, but without considering curli production and curli-associated phenotypes. We observed a strong positive correlation for isolates to exhibit: (i) the RDAR morphology on agar, (ii) motility, and (iii) curli-dependent biofilm formation on plastic (summarized in Supplemental Table 1). For each case where the RDAR morphology was observed, bacteria also bound the dyes CR and Calcofluor, exhibited motility, and definitively expressed curli as determined by Western blot analysis. However, there were four isolates which exhibited CR binding, curli expression, and Calcofluor binding, but did not exhibit the RDAR phenotype. These strains illustrate that bacteria can produce curli and some polysaccharide that stains with Calcofluor, but are not competent for biofilm formation.

Indeed, although Calcofluor is used as a qualitative dye to quickly screen isolates for potential cellulose production, dye binding is not entirely specific for cellulose and, when used in indicator plates, does not serve as a quantitative measure of how much cellulose could be present. Thus, these isolates could be producing lower amounts of cellulose that are not sufficient to exhibit the RDAR morphology. Alternatively, the isolates may be making both curli and cellulose at wild-type levels, but not displaying the wrinkled morphology typically associated with biofilm formation due to the absence of another yet-to-be-identified biofilm determinant. Thus, these strains will be of value in ongoing efforts to understand the complete set of molecular determinants for biofilm formation, including, for example, metabolic redox activities and oxygen accessibility [39].

Pellicle formation by UTI89 does require curli [25,40] and small molecule inhibitors of curli can disrupt pellicle formation [25,41], whereas moderate concentrations of DMSO or ethanol can increase pellicle formation in UTI89 [30]. We found that pellicle formation is a phenotype that is shared by only a small number of clinical isolates, even though most do produce curli. Thus, the attributes for bacterial colonization and film formation at the air-liquid interface appear to be more complex than biofilm formation on a solid surface such as agar or plastic. Thus, the colony morphology and surface-associated biofilm formation assays better parallel the expected curli-associated phenotypes based on curli production

among the isolates. As was recently demonstrated by Hadjifrangiskou, Hultgren and coworkers, curli are required but not sufficient for pellicle formation, and it is of interest to identify the other requisite factors for this more selective mode of biofilm formation [40].

The extent of biofilm formation and attached biomass on plastic when formed in curli-dependent biofilm conditions (YESCA broth) was nearly always higher than in type 1 pili-dependent biofilm conditions (LB broth). We also discovered that, although biofilms grown in LB are type 1 pili-dependent, curli are expressed in the biofilms formed by UTI89 and ASB-4, which are also the two strains that exhibited the most robust wrinkled morphology on agar and the most robust pellicles. Similarly, although type 1 pili are not required for biofilm formation in YESCA, several isolates were found to co-express curli and type 1 pili in these biofilms. In the murine cystitis model, only Pyelo-3 is not competent for IBC formation in single-strain infections, whereas all other isolates can assemble IBCs. Thus, neither the LB nor the YESCA conditions completely recapitulate the necessary environment to yield biofilm formation phenotypes that match the *in vivo* observations, although the curli-dependent biofilms formed in YESCA permitted biofilm formation among most isolates. Thus, although the inability to completely recapitulate environmental conditions that mimic *in vivo* niches is a general and grand challenge in studying biofilm formation and is one reason why many models of biofilm formation are often employed, we found that the curli-dependent biofilm forming conditions allow for biofilm formation among the highest number of isolates.

The high prevalence of UPEC to express curli and to participate in curli-integrated biofilm formation under many conditions underscores the need to further consider and study the role of curli in host pathogenesis. The ability to form biofilms under specific conditions that are either dependent on curli or type 1 pili are valuable because they allow us to interrogate behaviors that are correlated with the two fibers separately, yet we found that even under curli- or pili-dependent biofilm conditions, strains could produce the other fiber. Thus, while certain surface components are required for biofilm formation in different conditions, the complexity of multiple and sometimes redundant adhesive features clearly influence biofilm function and inspire the development of multi-pronged biofilm inhibitors. Furthermore, since the discovery of curli as amyloid in 2002, we now appreciate that many bacteria harbor dedicated genetic and molecular machinery to assemble amyloid fibers for function and our work inspires the need to consider amyloid production in biofilms formed by other microorganisms.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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HIGHLIGHTS

- Curli production is prevalent among uropathogenic *E. coli* clinical isolates
- Curli-dependent biofilms have more biomass than pili-dependent biofilms
- Biofilm formation in YESCA is more prevalent than biofilm formation in LB.
- Biofilms differ depending on growth conditions and production of curli and/or pili.
- Targeting biofilm formation will require a multipronged approach.

Figure 1.

Colony morphology of UPEC clinical isolates. Bacteria were grown on YESCA agar (left panel), and YESCA agar supplemented with Congo Red (center panel) or Calcofluor (right panel). Photographs of Calcofluor-treated samples were taken under UV light. The designations of I through IV represent the phenotypic groups that strains were assigned to based on all measured phenotypes as summarized in Supplemental Table 1.

Figure 2. Biofilm formation of UPEC clinical isolates on PVC plastic. (a) Comparison of the biofilm-forming ability of bacteria as a function of nutrient medium and temperature. (b) Relative biofilm-forming ability of strains compared to UTI89 and expression levels of CsgA (curli major subunit) and FimA (type 1 pilus major subunit) proteins extracted from the plastic-associated biomass, as determined by western blot analysis. (–), no protein expression; (+), protein expression; (nd), not determined due to lack of attached biomass.

Table 1

Phenotypes of UPEC clinical isolates in agar-based assays

Strains	RDAR morphology ^a		Congo Red binding		Calcofluor binding		Curli expression		Motility	
	26	34	26	34	26	34	26	34	26	34
UTI89	+	+	+	+	+	+	+	+	+	+
Acute-1	-	-	+	+	+	+	+	+	-	-
Acute-2	+	+	+	+	+	+	+	+	+	+
Acute-3	+	-	+	+	+	+	+	+	+	+
Acute-4	-	-	+	+	-	-	+	+	+	+
ASB-1	+	+	+	+	+	+	+	+	+	+
ASB-2	-	-	-	-	-	-	-	-	+	+
ASB-3	-	-	+	-	+	-	-	-	+	+
ASB-4	+	+	+	+	+	+	+	+	+	+
ASB-5	-	-	-	-	-	-	-	-	+	+
Pyelo-1	-	-	-	-	-	-	-	-	+	+
Pyelo-2	+	+	+	+	+	+	+	+	-	-
Pyelo-3	-	-	-	-	-	-	-	-	+	+
Pyelo-4	+	+	+	+	+	+	+	+	-	-
rUTI-1 ^b	+	-	+	+	+	+	+	+	+	+
rUTI-2	-	-	+	+	+	+	+	+	-	-
rUTI-3	-	-	+	+	+	+	+	+	+	+
rUTI-4	-	-	+	-	-	-	+	-	-	-
rUTI-5	-	-	+	-	+	+	+	-	+	+

^a Red, Dry, and Rough morphology of colony biofilm evaluated after 48 hours^b RDAR phenotype after 3–4 days

Table 2

Phenotypes of UPEC clinical isolates in broth-based assays

Strains	Curli expression ^a		Agglutination ^b		Pellicle ^c	
	26	34	26	34	26	34
UTI89	+	+	+	-	P	P
Acute-1	-	-	+	-	N	N
Acute-2	+	+	+++	++	P	P
Acute-3	+	+	+++	++	SP	SP
Acute-4	-	-	-	-	N	N
ASB-1	+	+	++	-	P	P
ASB-2	-	-	-	-	N	N
ASB-3	-	-	++	-	N	SP
ASB-4	+	+	+++	++	P	SP
ASB-5	-	-	-	-	N	SP
Pyelo-1	-	-	+	-	N	N
Pyelo-2	+	+	+++	++	P	P
Pyelo-3	-	-	-	-	N	N
Pyelo-4	-	+	-	-	SP	SP
rUTI-1	+	+	-	-	N	P
rUTI-2	+	+	++	-	N	N
rUTI-3	+	-	+++	++	N	SP
rUTI-4	-	-	-	-	N	N
rUTI-5	-	-	+	-	SP	SP

^a Curli expression level measured using Western blot analysis after 48 hours^b Aggregation phenotype with 4% DMSO (v/v) evaluated after 24 hours^c Pellicle formation evaluated after 72 hours. P, Pellicle formation; SP, Surface Partial Pellicle formation; N, No-pellicle formation.