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The Biology of the *Escherichia coli* Extracellular Matrix

David A. Hufnagel¹, William H. DePas², and Matthew R. Chapman^{1,#}

¹Departments of Molecular, Cellular and Developmental Biology

²Departments of Microbiology and Immunology, University of Michigan, Ann Arbor, MI, 48109

Chapter Summary

Escherichia coli (*E. coli*) is one of the world's best-characterized organisms, as it has been extensively studied for over a century. However, most of this work has focused on *E. coli* grown under laboratory conditions that do not faithfully simulate its natural environments. Therefore, the historical perspectives on *E. coli* physiology and life cycle are somewhat skewed toward experimental systems that feature *E. coli* growing logarithmically in a test tube. Typically a commensal bacterium, *E. coli* resides in the lower intestines of a slew of animals. Outside of the lower intestine, *E. coli* can adapt and survive in a very different set of environmental conditions. Biofilm formation allows *E. coli* to survive, and even thrive, in environments that do not support the growth of planktonic populations. *E. coli* can form biofilms virtually everywhere; in the bladder during a urinary tract infection, on in-dwelling medical devices, and outside of the host on plants and in the soil. The *E. coli* extracellular matrix, primarily composed of the protein polymer named curli and the polysaccharide cellulose, promotes adherence to organic and inorganic surfaces, and resistance to desiccation, the host immune system and other antimicrobials. The pathways that govern *E. coli* biofilm formation, cellulose production, and curli biogenesis will be discussed in this book chapter, which concludes with insights into the future of *E. coli* biofilm research and potential therapies.

Basic History, Phyloqeny, and Habitats of *Escherichia coli*

The Enterobacteriaceae bacterial family includes a variety of intestinal symbionts as well as notable pathogens such as *Salmonella enterica*, *Serratia marcescens*, *Klebsiella*, and *Yersinia pestis* (1). Also included among the Enterobacteriaceae family is the most well-documented bacterial species on Earth, *Escherichia coli*. *E. coli* is a fascinatingly diverse bug, featuring a cadre of different strains that have adapted to diverse environmental conditions and lifestyles. While the typical *E. coli* genome contains roughly 4800 genes, only approximately 1700 are shared by every *E. coli* strain (2). In total, there are over 15,000 genes that make up the *E. coli* pangenome (2, 3). The genomic plasticity of various *E. coli* isolates provides *E. coli* the ability to proliferate and survive in an array of environments (4, 5).

[#]To whom correspondence should be addressed: Matthew R. Chapman, Tel.: (734) 764-7592; Fax: (734) 647-0884; chapmanm@umich.edu.

A major niche of *E. coli* is the lower intestinal tract of mammals, birds, and reptiles (6). Indeed, *E. coli* was first isolated by Theodor Escherich from a human stool sample in 1886 (7). Among the first bacteria to colonize the intestinal tract of human infants, *E. coli* establishes a stable population of roughly 10^8 CFUs/g of feces by adulthood (5, 8, 9). The intestinal tract is oxygen limiting environment, and *E. coli* is a facultative anaerobe that can reduce several alternate terminal electron acceptors such as nitrate, fumarate, dimethyl sulfoxide (DMSO), and trimethylamine N-oxide (TMAO) (10). Mice colonization studies have revealed that in the intestine fumarate reductase, nitrate reductase, and *bd* oxidase (high affinity oxygen cytochrome) are particularly important for *E. coli* fitness (11, 12). The physiological flexibility of *E. coli* renders it well-suited for the diverse environments encountered in the intestinal tract.

While the lower digestive tract is the primary habitat of *E. coli*, fecal dissemination leads to the passage of *E. coli* to its secondary environment outside of the host. The host and non-host environments can be dynamic and differ in nutrient availability, temperature, and the number and nature of competitors, among other things. Once *E. coli* is excreted in stool, there is generally a net-negative fitness cost compared to growth in most host environments (9, 13). *E. coli* doubles around every two days in a human host, but outside of the host, instead of dividing, *E. coli* perishes after an average of four days depending on the environment into which it is passaged (9). However, as *E. coli* is constantly being excreted, it has been estimated that half of the *E. coli* cells on Earth exist outside the host (9). Interestingly, studies have found that *E. coli* can grow in soil not only in tropical conditions, but also in colder temperatures (14).

Many studies have investigated how enterohemorrhagic *E. coli* (EHEC) strains, particularly EHEC O157:H7, make their way from environmental reservoirs to humans (15). *E. coli* is transmitted from host to host by the fecal-oral route. The ability to survive outside of the host therefore facilitates the host-to-host transmission of all *E. coli* variants, including pathogenic strains. EHEC strains cause diarrhea, abdominal cramps, and in certain cases the life threatening hemolytic-uremic syndrome (16). The intestinal tract of domesticated cattle serves as the primary reservoir for EHEC in the United States and EHEC can contaminate meat during the slaughter process (17, 18). Additionally, EHEC is shed in feces and can survive in manure for months (17, 19–21), making contact with animal feces a risk factor for EHEC infections. Even using untreated manure as fertilizer can result in contaminated produce products (15, 22–24). While contaminated manure or water can foul the surface of plants, there is also evidence that EHEC can invade plant tissue (23–25). *E. coli*'s ability to survive outside of the intestinal tract is integral to EHEC's ability to cause outbreaks.

Extraintestinal pathogenic *E. coli* (ExPEC) are strains that cause disease outside of the intestinal tract of animals, including uropathogenic or 'UPEC' strains. Urinary tract infections (11) are one of the most common bacterial infections, costing over 3 billion dollars to health care in the United States alone (26). UPEC can live in the intestine without causing disease, and recent work has shown that UPEC has no fitness defects in the gut environment when compared with commensal *E. coli* strains (27). How ExPEC strains like UPEC transmit from the intestine to the site of disease is not completely understood, but at least some strains can spread via the fecal-oral route (28). In fact, the primary source of

UPEC that colonizes the urethra is a patient's own intestinal tract (29). ExPEC, including UPEC strains, can also be found on food products, and clonal UPEC outbreaks have been reported (30–32). Taken together these data suggest that UPEC are at least partly dependent on survival outside of the host before they can recolonize a second host and cause disease.

UPEC have specifically adapted to cause disease in the urinary tract (26). UPEC ascend the urinary tract to the bladder and cause infection (26). UPEC invades bladder epithelia cells in a type 1 pili-dependent mechanism, where they form a tight knit and aggregated intracellular bacterial communities called IBC's (33, 34). IBCs are drug resistant and can evade the host immune responses allowing for cells within the IBC to proliferate and to further infect additional bladder epithelial cells (35, 36).

Introduction to Biofilms and the Extracellular Matrix

Biofilm formation can increase bacterial fitness in both host and non-host environments (37, 38). In this review we will use the general definition of a biofilm as a group of surface-associated bacteria enveloped in a self-produced extracellular matrix (39). The *E. coli* extracellular matrix contains a major protein polymer called curli and the carbohydrate polymer, cellulose (40–42). Although curli and cellulose are typically the most abundant biofilm constituents, the extracellular matrix of *E. coli* can also include type 1 pili, flagella, antigen 43, DNA, β -1,6-N-acetylglucosamine (β -1,6-GlcNAc), capsule sugars, and colonic acid (43). Most pathogenic strains of *E. coli* form robust biofilms, however, some laboratory strains of *E. coli* are attenuated in their ability to produce biofilms. The K12 strain of *E. coli* was first isolated from Stanford in 1922, and was subsequently passaged for more than 50 years (44). This passaging led to evolutionary adaptation to the laboratory growth conditions, and to the loss of certain traits that influence biofilms (45). K12 *E. coli* therefore requires extended periods of time to adhere to surfaces and form biofilms (40). On the other hand, a host of pathogenic, environmental, and commensal *E. coli* isolates readily form biofilms in the laboratory and therefore make great model organisms for biofilm formation studies (46–50).

Biofilm formation correlates with resistance to a variety of environmental stresses, including antibiotics, the immune system, and predation (38). Resistance is conferred through at least two distinct mechanisms. First, the extracellular matrix forms a physical barrier that can resist shear stress and recognition and phagocytosis by immune cells (38). Secondly, bacteria within biofilms often assemble into subpopulations that have distinct physiological characteristics (46, 51, 52).

Subpopulation development can be triggered by mutations, stochastic gene expression, or chemical gradients that develop during biofilm formation (37, 52–54). For instance, bacteria at the biofilm surface are exposed to more oxygen, stimulating a higher rate of aerobic respiration (37, 55, 56). Metabolic changes often coincide with resistance to different stresses (54, 57). A biofilm community with multiple subpopulations, each resistant to different stresses, therefore demonstrates resistance to a broader range of environmental pressures to the biofilm community as a whole (37, 54, 57).

Laboratory biofilm models

Laboratory biofilm models have been used to determine most of the spatiotemporal and molecular characteristics of biofilms (Figure 1). *E. coli* forms at least two distinct types of biofilm in static liquid cultures—both form at the air liquid interface and both require production of extracellular polymers (41, 43, 47, 58, 59) (Figure 1A, Figure 1B, and Figure 1C). The accumulation of biomass at the air-exposed edges of polyvinyl chloride wells or glass culture tubes with cultures grown in lysogeny broth (LB) media is reliant on type-1-pili, poly- β -1,6-GlcNAc, and flagella (47, 58, 60) (Figure 1A top image). The crystal violet stained biofilm rings can be visualized in the top image in Figure 1A, whereas the flagella mutant (*fliC::kan*) grown in the tube shown in the bottom image did not form rings on the glass culture tube (Figure 1A).

E. coli can also form a pellicle biofilm that floats at the air liquid interface. Pellicles are films of curli/cellulose-encased cells that span the entire air-liquid interface of a single well (47, 59, 61). Pellicle formation can be quantified by crystal violet staining of the biofilm biomass (62, 63) (Figure 1B). The culture in the top well in Figure 1B formed a robust pellicle at the air-liquid interface of the culture, whereas the culture in the bottom well did not.

Pellicle biofilm formation relies on the biofilm master regulator, CsgD. CsgD regulates expression of the matrix components, curli and cellulose (41, 64). CsgD-mediated biofilms can be inhibited by the presence of glucose, temperatures greater than 30°C, and high osmolarity, because all of these conditions repress CsgD activity (47, 65–67). When *E. coli* is grown in conditions that are conducive for *csgD* expression, curli and cellulose-dependent biofilms can manifest in a variety of ways. The culture grown in the bottom image of Figure 1B is a *csgD* mutant that is unable to produce curli or cellulose (Figure 1B). The pellicle architecture at the air-biofilm interface can be highlighted by growing the static culture in media amended with the diazo dye Congo red (61, 68) (Figure 1C). Congo red stains both curli and cellulose, allowing for visualization of the pellicle biofilms and the ornate wrinkled morphology at the surface of the culture (Top image Figure 1C).

Confocal laser scanning microscopy (CLSM) and electron microscopy (EM) analysis revealed that two separate populations exist at the pellicle biofilm air-liquid interface (59, 61, 69). At the air-biofilm interface cells are encased in a thick fibrous extracellular matrix, whereas cells at the liquid interface are more evenly spaced and often not surrounded by a fibrous extracellular matrix (59). While curli and cellulose are the chief components of pellicle biofilms, pili are involved in pellicle development and maturation, as deletion of type-1-pili leads to a less-robust pellicle (59). Flagella are also required for pellicle development, since motile cells are necessary for colonization at the top of the static culture (47, 59).

The CsgD-induced matrix components curli and cellulose are also required for colony biofilm formation in *E. coli* and *Salmonella* spp. A variety of bacterial species, including *E. coli*, produce wrinkled colony biofilms on agar plates (Figure 1D). The nomenclature for the wrinkled colony phenotype varies between species, but some common names are rugose

biofilms, wrinkled colony biofilms, and red dry and rough (rdar) biofilms. Here, we will use the term rugose biofilms to describe all wrinkled colony biofilms on agar plates. The mechanics of rugose biofilm formation have been studied extensively in multiple species including *E. coli*, *Vibrio cholera*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, and *S. enterica* serovar Typhimurium (41, 70–78).

When grown under CsgD-inducing conditions on agar plates, *E. coli* will form rugose colony biofilms (41, 79) (Left image Figure 1D). Curli and cellulose are required for rugose development, and are the major extracellular structures in the rugose biofilm matrix (41, 46, 80). Curli and cellulose mutant strains form non-spreading and unwrinkled colonies that do not bind Congo red (Right image Figure 1D). While flagella are not required for the rugose colony morphotype, including colony spreading, the interior cells in a rugose colony are heavily flagellated (51). Since *E. coli* K12 strains have acquired a mutation that prevents cellulose production, they do not form rugose biofilms unless the cellulose synthesis defect is repaired (81). Indeed, repeated culturing tends to select for genetic suppressors of biofilm formation in a variety of bacteria (45). However, more recently isolated commensal *E. coli*, ExPEC, and IPEC are able to form rugose biofilms (62, 82, 83) (Figure 1D). Curli, cellulose, and rugose biofilms are not only produced in *E. coli* and *Salmonella* spp., but also in other Enterobacteriaceae, such as *Citrobacter* spp. (46, 84). As curli and cellulose are required for rugose biofilm development, the environmental signals leading to rugose development coincide with those affecting CsgD expression (78, 85).

CsgD-mediated control of the extracellular matrix

CsgD is a FixJ/LuxR/UhpA type response regulator that contains a typical C-terminal helix-turn-helix DNA-binding domain (64, 86). The N-terminus of CsgD is thought to process environmental signals that affect the protein's ability to bind DNA and regulate transcription (86–88). The N-termini of FixJ type response regulators are typically modified by phosphorylation on highly conserved aspartic acid residues (89, 90). However, CsgD is missing the conserved phosphorylation sites, which has confused the role that phosphorylation plays in regulating CsgD (88, 91). However, acetyl phosphate can phosphorylate CsgD *in vitro* (89), which reduces the ability of CsgD to bind particular promoters, suggesting that post-translational phosphorylation regulation occurs (89).

The expression of *csgD* is controlled by a large number of transcriptional regulators and small RNAs (92, 93). In general, low salt, low temperature, and low glucose conditions trigger *csgD* expression (64, 66, 92, 94, 95). Additionally, *csgD* expression requires the stationary phase sigma factor RpoS (67, 77), and CsgD activity is regulated by the small molecule, cyclic-di-GMP (96). Diguanylate cyclases contain a GGDEF domain that promotes cyclic-di-GMP production and phosphodiesterases contain an EAL domain promotes the breakdown of c-di-GMP (96). *E. coli* and *S. enterica* encode a number of diguanylate cyclases and phosphodiesterases to regulate cytoplasmic levels of cyclic-di-GMP (97–99). The GGDEF and EAL containing proteins, STM2123 (YegE) and STM3388, are both required for WT CsgD protein levels in *Salmonella enterica* serovar Typhimurium (99). Alternatively, the diguanylate cyclases, YdaM and YfiN, are required for WT CsgD protein levels in *E. coli* (68, 98). The mechanism by which these diguanylate cyclases

control CsgD protein levels is unknown. C-di-GMP can be bound by RNA (riboswitches) and by multiple binding motifs in proteins (100). In addition the diguanylate cyclases themselves can have secondary function outside of c-di-GMP production, leading to a wide variety of potential causes of CsgD protein level suppression (100).

CsgD controls a modest regulon of roughly 13 genes/operons (64, 101–103). Included in the regulon of genes that CsgD upregulates is *iraP*, which leads to a relay system where the IraP protein stabilizes RpoS, which results in more CsgD expression (102). CsgD directly represses the flagella biosynthesis genes *fliE* and *fliF* (103), and induces expression of curli by directly binding to the *csgBAC* promoter (64). CsgD induces cellulose production indirectly by positively regulating the diguanylate cyclase AdrA (41) (Figure 2). Cyclic-di-GMP produced by AdrA activates the cellulose synthase and promotes cellulose production (41) (Figure 2). The complex regulation of CsgD and the promotion of curli and cellulose go hand-in-hand (Figure 2), suggesting that only under particular conditions is it advantageous for the cell to decrease motility and produce an extracellular matrix.

History of Curli

Curli were first described in 1989 when Stafan Normark and colleagues were investigating Bovine mastitis isolates for the ability to bind to host cell matrix components (94). The authors noticed that half of the isolates bound fibronectin when grown under conditions that we now know to favor curli production (94). Electron microscopy revealed that the fibronectin binding isolates produced fibrous coiled surface structures that they called ‘curli’ (94). The presence of curli fibers in *Salmonella* was discovered a few years later by Collinson *et al.* (104). Originally termed thin aggregative fimbriae or Tafi, the authors did not initially think that these fibers were related to curli (104) because *Salmonella* produced Tafi fibers when grown at both 30°C and 37°C (104). However, Arnqvist *et al.* found that both *S. enteritidis* tafi and *E. coli* curli are the same fiber and that they were both primarily composed of the protein monomer, CsgA (105).

The Curli Biogenesis Pathway

Curli fibers were the first described extracellular fibers to polymerize by the nucleation-precipitation mechanism, also known as the type VII secretion system (106–110). Curli fiber subunits and their assembly machinery are encoded on two divergently transcribed operons, *csgDEFG* and *csgBAC* (Figure 2) (64). The *csgBAC* operon encodes the major and minor curli subunits, CsgA and CsgB, respectively. Both CsgA and CsgB are secreted across the outer membrane. On the cell surface, CsgB associates with the outer membrane and provides a template that promotes CsgA to adopt an amyloid fold and form cell-associated curli fibers (111) (Figure 2). Electron microscopy analysis has led to the estimate that 30% of *E. coli* cells produce curli under curli inducing conditions (112). A *csgB* mutant secretes unfolded CsgA that can polymerize on the surface of a *csgA* mutant cell (which presents a surface-associated CsgB), in a process called ‘interbacterial complementation’ (106, 108). Curli subunits from *E. coli*, *Salmonella enterica* serovar Typhimurium, and *Citrobacter* can cross-seed and polymerize into amyloid fibers *in vitro* (113). Furthermore, interbacterial complementation between *E. coli* and *Salmonella enterica* serovar Typhimurium has been

observed *in vivo*. Interspecies curli production restores agar adherence to the mixed species bacterial communities (113), supporting the idea that curli subunits may be a ‘community resource’ that can be utilized indiscriminately by all cells in a localized community.

The *csgDEFG* operon encodes the CsgD regulator and three curli assembly accessory proteins that are each required for WT levels of curli production (Figure 2) (64). CsgG forms a nonomeric pore in the outer membrane that facilitates curlin secretion (Figure 2) (112, 114–116). CsgG has an approximately 2 nm central pore that spans the outer membrane (112, 116). During curli assembly CsgG forms discrete puncta that associate with two other curli accessory proteins, CsgE and CsgF (Figure 2) (114). Electron microscopy analysis revealed that CsgG, CsgE, and CsgF cluster together around curli fibers (112). CsgE is a periplasmic protein that provides substrate specificity to CsgG mediated secretion and also has chaperone-like activity as it can prevent CsgA from assembling amyloid fibers *in vitro* (108, 117). CsgE caps the secretion vestibule on the periplasmic side of CsgG, which facilitates unidirectional migration of substrates to the cell surface (116). CsgE anti-amyloid activity can arrest CsgA fiber formation at different stages of CsgA polymerization, although it cannot disassemble preformed fibers (118). When CsgE is added exogenously to the media, pellicle biofilm formation is interrupted (118). Finally, CsgF is necessary for both cell surface association of curli and CsgA polymerization into fibers *in vivo* (108, 111) (Figure 2).

Chemical and protein modulation of CsgA amyloid formation

Curli are part of a growing class of β -rich, ordered, protein fibers called amyloid. Amyloids have a villainous history, as the amyloid fold is associated with type-2 diabetes, Alzheimer’s disease, and Parkinson’s disease, among many others (119). The signature and well-studied role of amyloid formation in human diseases has led to the search for drugs that can prevent amyloid formation. However, progress in the amyloid formation field has been somewhat hampered by lack of robust, reproducible and tractable cellular model systems. The curli biogenesis system in *E. coli* is ideally situated as a platform for screening drugs that might interrogate the amyloid formation process. The curli system in *E. coli* provides sophisticated genetics and a suite of different assays to measure *in vivo* amyloid formation, including curli-dependent biofilm formation and Congo red binding. We have used the curli system to begin screening and characterizing a library of designer peptidomimetic compounds called 2-pyridones (61, 118, 120). Pyridone compounds were initially recognized as anti-amyloid compounds in a screen of small organic molecules testing for binding strength to the amyloid associated with Alzheimer’s disease, $A\beta^{1-42}$ (121). The 2-pyridone compounds have a rigid bicyclic structure, which maintains the compounds in a straight conformation that mimics a β -strand (118). Altered 2-pyridone compounds were constructed that had increased solubility and were used to inhibit the type 1 pilus assembly in UTI89 via interruption of the chaperone-usheer interaction (122, 123).

The CF_3 -phenyl substituted 2-pyridone, FN075, is a potent amyloid inhibitor (61, 120, 123). FN075 inhibits CsgA amyloid polymerization *in vitro*, and it also blocks biofilm formation by preventing curli formation and inhibiting type 1 pilus assembly (61). FN075 inhibits CsgA amyloid fiber formation by directing CsgA into soluble oligomers that are not on

pathway to the amyloid fiber (120). Interestingly, the CF₃-phenyl substitution does not abrogate 2-pyridone activity against the type 1 pilus. Thus, FN075 functions as a dual-function pilus-inhibitor (pilicide) and curli-inhibitor (curlicide). FN075 co-inoculation with UPEC in a mouse UTI-model caused decreases in initial titers of bacteria and IBC formation (61). Recently, FN075 was used as a scaffold for the production of new, chemically distinct compounds (118). Compounds from this library are being screened for their ability to inhibit CsgA amyloid formation and several interesting leads have been identified (118). Two of these compounds were found to accelerate CsgA amyloid formation (118). Interestingly, acceleration of amyloid formation may be one route to prevent amyloid-related cellular toxicity, as conformationally-dynamic amyloid intermediates are hypothesized to be the root of amyloid toxicity (124). Therefore, small molecule ‘accelerators’ should facilitate the conversion of dynamic oligomers into the more stable and less cytotoxic amyloid state, and thus have promising therapeutic value.

Cellulose production

The other major polymer present in the *E. coli* extracellular matrix is cellulose, a linear chain of β -(1,4)-linked glucose monomers (41, 80). Bacterial cellulose production was first described in 1887, and in recent years *Gluconacetobacter xylinus* has been the model organism for studies of bacterial cellulose synthesis (125–127). Cellulose production by the Enterobacteriaceae family was first described in 2001 (41), which initiated the discovery of cellulose production in many additional *E. coli* strains (41, 46, 62, 84, 128). Most non-K12 *E. coli* strains can produce cellulose as a component of the biofilm matrix, while K12 strains do not (41, 128). In the case of K12 strains W3110 and MG1655, the cellulose defect is due to a single nucleotide polymorphism (SNP) in *bcsQ* that results in a pre-mature stop codon (81). Repairing this SNP in *bcsQ* restores cellulose synthesis in *E. coli* W3110 and induces rugose colony formation (81).

Expression of the cellulose synthesis genes is usually dependent on the master biofilm regulator, CsgD (41, 46). CsgD modulates cellulose synthesis by activating transcription of *adrA* (41, 46, 85, 128) (Figure 2). AdrA contains a GGDEF domain typical of diguanylate cyclases (85, 97), and AdrA-produced cyclic-di-GMP binds to the PilZ domain of the cellulose synthase, BcsA, which stimulates cellulose synthesis (129, 130) (Figure 2). BcsA covalently links UDP-D-glucose monomers into a growing glucan chain (127, 131) (Figure 2). AdrA is not the only diguanylate cyclase that can produce cyclic-di-GMP to drive BcsA activation. *E. coli* 1094 can produce cellulose independently of CsgD, and does so via the diguanylate cyclase YedQ that works in place of AdrA (128). Furthermore, mutants in the disulfide bonding system (DSB) produce cellulose independently of CsgD in the uropathogenic strain UTI89 (68). The YfiN negative regulator protein, YfiR, is unstable and degraded in DSB mutants and can no longer repress activation of the diguanylate cyclase, YfiN (68, 132). Constitutive activation of YfiN leads to accumulation of cyclic-di-GMP and activation of the cellulose synthase in the presence of high salt, high temperature and glucose, conditions where CsgD is normally repressed (68). Additionally, YfiN and cellulose production are activated by reducing conditions, suggesting that under these conditions cellulose can be produced independently of curli (68).

The biophysics of BcsA activation and cellulose production have been recently elucidated (131, 133, 134). The BcsA glycosyl transferase domain is on the cytoplasmic C-terminus located next to the PilZ domain (133). In *Rhodobacter sphaeroides*, a salt bridge between an arginine of the PilZ domain and a glutamine in the gating loop is broken once c-di-GMP is bound, which allows the glycosyl transferase domain to engage UDP-glucose (133, 134). Mutation of the glutamine in the gating loop results in constitutive cellulose expression, as the inhibitory salt bridge is not formed (134). The UDP-binding site allows space for a single addition to the nascent glucan chain (133). A glucan chain of approximately 10 glucose monomers aligns on the periplasmic side of BcsA, perpendicular to the axis of the BcsA β -barrel (133). Additionally, a by-product of cellulose formation, UDP, acts as a competitive feedback inhibitor of the glycosyl transferase (131, 134). *E. coli* and *Rhodobacter sphaeroides* BcsA and BcsB are sufficient for production of cellulose *in vitro*, but the reaction requires c-di-GMP, UDP-glucose, and both BcsA and BcsB to be co-localized in the same liposome to function (131).

Enterobacteriaceae Extracellular Matrix in the Host and Disease

Despite the fact that many *E. coli* strains express curli and cellulose maximally at 26°C, as might be the case outside of the host, both components are expressed in the host environment and play a role in commensal and disease interactions. Both *E. coli* and *Salmonella* clinical isolates express CsgD, curli, and cellulose at 37°C (62, 77). UPEC curli production is part of early stage bladder colonization in a mouse model, as curli mutants have decreased bladder titers (61). Curli promote interactions with epithelial cells, and they can inhibit the polymerization of the antimicrobial factor LL-37, which plays a major role in host defense of the urinary tract (135). Untreated UPEC infections can lead to dissemination and infections of the bloodstream, also known as bacteremia (136). Interestingly, UPEC strains isolated from bacteremic patients produce more curli at 37°C than UPEC strains isolated from non-bacteremic patients (137). Additionally, over 50% of *E. coli* isolates from patients with sepsis produce curli at 37°C (138). Serum samples from patients that had sepsis had antibodies against the major curli subunit, CsgA, whereas control patients did not (138). Because curli and cellulose are expressed by clinical isolates under host-colonization conditions, it is imperative to understand how these matrix components shape the host-pathogen interaction and the molecular pathways that induce their production in these non-laboratory conditions.

In addition to curli and cellulose, the extracellular matrix components type-1-pili, K-antigen, and antigen 43 also have roles in promoting UPEC bladder colonization and biofilm formation. Antigen 43 is an outer-membrane protein required for biofilm formation in glucose minimal media, but not LB media (139). Antigen 43 drives aggregation and cell-surface and cell-cell interactions of *E. coli* biofilms via antigen 43-antigen 43 interaction (139, 140). Antigen 43 production increases interspecies biofilm formation between *E. coli* and *Pseudomonas fluorescens* (140). IBCs that form in the superficial facet cells that line the bladder epithelium are composed of densely compact and coccoid shaped cells. Type 1 pili mutant strains have cells that are very dispersed and retain their rod shape (34). The polysaccharide K 1 capsule is produced in IBCs and helps UPEC evasion of neutrophils in

the host (141). K 1 capsule mutants form dispersed and disordered intracellular bacterial populations that have neutrophils within the confines of their communities (141).

Beyond causing disease in the urinary tract, most *E. coli* in the human body persists and associates within the lower intestinal tract by interacting with gut and colon epithelia (9, 13). Curli and cellulose are required for proper attaching and effacing of *E. coli* to host colon cancer cells and to bovine cow colon explants (142). Curli also increase the intestinal colon cell internalization of *E. coli* commensal isolates (143).

The immune system and host cells interact with the extracellular matrix components produced by *E. coli* and *Salmonella*. Colon cells have increased IL-8 production in the presence of both flagellated and curliated *E. coli*, while macrophages have increased nitrous oxide and IL-6 production in the presence of purified *Salmonella* curli fibers (143, 144). Host cells recognize amyloids and also *Salmonella* curli fibers through TLR1/2 heterodimer (145). CD14 on the host cell surface complexes with TLR1/2 and increases the colon cell response to curli fibers (144). Polarized epithelial cells recognize curli and decrease the permeability of junctions between cells, thus decreasing dissemination of *Salmonella* past gut cells (146). The permeability of host gut epithelial cells were visualized via the migration of labelled beads through a polarized cell epithelia monolayer in the presence and absence of curli (146). In agreement with these findings, infection with curli positive *Salmonella in vivo* also led to increased barrier formation in gut epithelial cells, and fewer curli positive *Salmonella* were recovered from extraintestinal tissues in a mouse model (146).

Enterobacteriaceae Extracellular Matrix Outside of the Host

E. coli and *Salmonella* also utilize biofilms in their lifecycle outside of the host. The strategy of gut microbes outside of the host shifts from growth and nutrient acquisition to a lifestyle of attachment, aerobiosis, and prolonged survival (13). Conditions associated with life outside of the host such as low temperature, low glucose and nutrients, and oxidative stress induce extracellular matrix production (46, 147–149). Curli and cellulose are generally co-expressed due to their mutual dependence on the transcriptional regulator CsgD (41, 64, 95). The extracellular matrix components cellulose and curli contribute to the survival of *Salmonella enterica* serovar Typhimurium to both desiccation and bleach stress (150). Expression of both curli fibers and cellulose in the extracellular matrix fraction of rugose biofilms correlates with H₂O₂ resistance (46, 150). Conditions outside of the host induce curli and cellulose, which protects Enterobacteriaceae from these harsh conditions.

The extracellular matrix helps bacteria remain in close proximity with one another and also facilitates attachment to the surfaces colonized in the extra-host environment (40, 151). EHEC curli expression increases attachment to produce and abiotic surfaces (152, 153). Curli are important for *E. coli* attachment to alfalfa sprouts and seed coats after 3 days of coinubation (154). *E. coli* O157:H7 expressing curli strains are more hydrophobic and have increased binding to produce surfaces (155). Interaction with the lettuce rhizosphere upregulates a curli regulator, Crl, and attachment to the rhizosphere is decreased in a curli mutant (156).

Colanic acid is an extracellular polysaccharide that has varying uses in different *E. coli* strain and biofilms. Colanic acid is upregulated in conditions that occur outside of the host (157). In K12, colanic acid does not contribute to surface adherence, but is necessary for development of three dimensional architecture in biofilms on glass slides and for robust LB biofilm development in static culture (158, 159). In contrast to other surfaces, colonic acid does increase adherence of *E. coli* to alfalfa sprouts and to certain plastic surfaces (160).

Conclusions and thoughts for future studies

The fantastic understanding we have of *E. coli* physiology, combined with the wealth of genetic tools afforded by *E. coli*, provides an exciting platform for understanding biofilm biology. We must better define the role that curli and cellulose play in the life cycle of enteric bacteria like *E. coli*. To date, only a few studies have attempted to assess the prevalence of curli and cellulose in environmental soil and water samples. Initial studies have estimated that some 5–40% of environmental biofilms contain microbial curli-like amyloids, highlighting the prevalence of amyloids in natural systems. Furthermore, *csg* homologues have been found in a wide-array of bacterial species inside and outside of Enterobacteriaceae (161, 162). Fully understanding the range of curli in natural environments will allow a greater appreciation of the importance of the ECM to Earth's microbiota.

We must also better understand the role that the ECM plays in mediating interactions with plants and animals. Mounting evidence suggests that curli are required for interactions with plants and curliated *E. coli* adheres robustly to the rhizosphere and the leaves of various plants (152, 155, 156). Advancing the biochemical knowledge of the curli and plant interaction could foster various treatments for the prevention and removal of contaminating bacteria on fresh produce.

How the ECM shapes the infectious cycle of important human pathogens has yet to be defined. Antibodies are produced against curli in patients suffering from sepsis, so the host is clearly exposed to curli during an infection (138, 146). Curli have been shown to augment epithelial cell barriers, and to decrease titers in extraintestinal tissue of mice, suggesting that maybe curliated bacteria in the intestinal tract lead to a commensal relationship between resident flora and mammalian gut cells (146). An important part of this equation will be to determine how certain isolates of *E. coli* and *Salmonella* produce cellulose and curli at human body temperature, while other strains only produce curli in temperatures less than 30°C. Study of various ECM activation pathways of *E. coli* at human body temperatures could lead to potential powerful therapeutic molecular targets for ECM related *E. coli* infections.

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Figure 1. Laboratory *E. coli* biofilm models

A) Ring biofilm stained by crystal violet. Cultures were grown in LB media in glass tubes at 26°C for 48 hours. Liquid culture was removed and tube was stained with .1% (w/v) crystal violet (CV) for 5 minutes. Tubes were subsequently washed with water. The top image is a WT strain and the lower image is a flagella mutant (*fliC::kan*) **B)** Pellicle biofilms grown in 24 well plate for 48 hours at 26°C. Liquid media was removed followed by 5 minutes of staining with .1% CV. Stained pellicles were washed 3x with water prior to imaging. The top image is a CV stained WT UTI89 pellicle, whereas the lower picture was a culture of a *csgD* mutant that did not produce a pellicle. **C)** Pellicle biofilms grown in 1:7500 (Congo red:YESCA) media in a 24 well dish for 48 hours at 26°C. The top image shows a WT UTI89 culture that produced a pellicle, whereas the lower image is a culture of a *csgD*

mutant that did not form a pellicle. **D)** 4μL spots of 1-OD₆₀₀ *E. coli* were grown at 26°C for 48 hours on YESCA CR plates. The colony on the left is UTI89 WT on the right is a *csgD* mutant colony.

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Figure 2. ECM production model

CsgD is the master regulator of the biofilm extracellular matrix. CsgD transcriptionally upregulates the *csgB* and *csgA* genes, which encode the minor and major curli fiber subunits, respectively. CsgA and CsgB are secreted through an outer membrane pore formed by CsgG. CsgE is thought to facilitate translocation of curli subunits across the outer membrane by capping the periplasmic side of the secretion vestibule so that movement in the channel is unidirectional. CsgB associates with the cell surface and templates amyloid polymerization of CsgA. CsgD also transcriptionally upregulates *adrA*. AdrA is an inner membrane

diguanylate cyclase, which produces the secondary messenger, c-di-GMP. c-di-GMP binds and activates BcsA, which then produces cellulose fibers via the building block UDP-glucose. C-di-GMP that activates BcsA can also be produced via YedQ and YfiN.

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