Curli-Containing Enteric Biofilms Inside and Out: Matrix Composition, Immune Recognition, and Disease Implications

Sarah A. Tursi,^a Çağla Tükel^a

^aDepartment of Microbiology and Immunology, Lewis Katz School of Medicine, Temple University, Philadelphia, Pennsylvania, USA

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SUMMARY Biofilms of enteric bacteria are highly complex, with multiple components that interact to fortify the biofilm matrix. Within biofilms of enteric bacteria such as *Escherichia coli* and *Salmonella* species, the main component of the biofilm is amyloid curli. Other constituents include cellulose, extracellular DNA, O antigen, and various surface proteins, including BapA. Only recently, the roles of these components in the formation of the enteric biofilm individually and in consortium have been evaluated. In addition to enhancing the stability and strength of the matrix, the components of the enteric biofilm influence bacterial virulence and transmission. Most notably, certain components of the matrix are recognized as pathogen-associated molecular patterns. Systemic recognition of enteric biofilms leads to the activation of several proinflammatory innate immune receptors, including the Toll-like receptor 2 (TLR2)/TLR1/CD14 heterocomplex, TLR9, and NLRP3. In the model of *Salmonella enterica* serovar Typhimurium, the immune response to curli is site specific. Although a proinflammatory response is generated upon systemic presentation

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Address correspondence to Çağla Tükel, ctukel@temple.edu.

of curli, oral administration of curli ameliorates the damaged intestinal epithelial barrier and reduces the severity of colitis. Furthermore, curli (and extracellular DNA) of enteric biofilms potentiate the autoimmune disease systemic lupus erythematosus (SLE) and promote the fibrillization of the pathogenic amyloid α -synuclein, which is implicated in Parkinson's disease. Homologues of curli-encoding genes are found in four additional bacterial phyla, suggesting that the biomedical implications involved with enteric biofilms are applicable to numerous bacterial species.

KEYWORDS *Enterobacteriaceae*, biofilms, extracellular DNA, amyloid, curli, Toll-like receptors, innate immunity, autoimmunity, neurodegenerative diseases, *Escherichia coli*, *Salmonella enterica* serovar Typhimurium, extracellular matrix

INTRODUCTION

One of the first recorded observations of bacterial biofilms dates back to the 18th century. Antoine van Leeuwenhoek observed bacterial aggregates from tooth plaque, later discovered to be a multispecies biofilm, using a rudimentary microscope. He noted: "from whence I conclude, that the vinegar with which I wash't my teeth kill'd only those animals which were on the outside of the scruf, but did not pass thro the whole substance of it" (1). It was from this observation that the recognition of biofilms originated. Due to his astute observation, Van Leeuwenhoek is credited with the discovery of bacterial biofilms in addition to the title as the father of modern microbiology. Centuries later, in the early 1930s, a more defined observation regarding biofilms in water was made by Arthur Henrici, who stated, "it is quite evident that for the most part bacteria are not free floating organisms, but grow upon surfaces" (2). J. W. Costerton continued to explore the ecology of biofilms and established that it was through biofilms that bacteria were able to adhere to surfaces (3). Costerton and colleagues were principal driving forces in defining our understating of biofilms though the use of microscopy (4).

The complex nature of the extracellular matrix of biofilms, the unique biology of bacteria within the biofilm, and the interaction of bacteria in the biofilm community have continued to capture the attention of scientists across the globe. Not only do bacterial biofilms colonize surfaces within the environment, but biofilms are also medically relevant. Biofilms frequently colonize medical indwelling devices, including catheters and stents, and adhere to biological surfaces within and on the body. Research into the biomedical impact of biofilms was further warranted after the link between biofilms and infections was established. As various biofilm components have been shown to be conserved pathogen-associated molecular patterns (PAMPs) that are recognized by the immune system, researchers attempt to understand the mechanisms by which biofilm components are recognized by the innate immune system, and these studies have further established the relationship between biofilms and disease pathogenesis. Today, due to our growing understanding of the impact of the microbial communities, referred to as the microbiome, on health and disease, biofilm research has been propelled to the forefront of biomedical research. Moreover, the biofilms of enteric bacteria have been linked recently with promotion of a human autoimmune disease, systemic lupus erythematosus (SLE) (5), and the neurodegenerative disease Parkinson's disease (6). This review specifically focuses on the biofilm composition, innate immune recognition, and disease pathogenesis of curli-containing enteric biofilms.

COMPOSITION OF ENTERIC BIOFILMS

A commonly accepted definition of bacterial biofilms is a group of bacteria that are encapsulated in a three-dimensional, self-produced extracellular matrix that is adhered to an abiotic or biotic surface (1, 7). Bacterial biofilms can be composed of a single species of bacteria or a consortium of multiple species of bacteria. The biofilm composition and the construction of the biofilm depend both on the bacteria within the biofilm and the environment in which the biofilm is located. The matrix that encapsulates the bacteria is often referred to as the extracellular polymeric substance (EPS). The

EPS accounts for approximately 90% of the biofilm biomass; only 10% of the biomass is bacterial cells (8), thus emphasizing the importance of the EPS. Although the EPS varies by species, biofilms of enteric bacteria, which are the focus of this review, are composed mainly of amyloid, polysaccharide, DNA, and surface protein components. As curli is the major proteinaceous component of the enteric biofilm, for the purposes of this review we define enteric biofilms as curli-producing bacteria, excluding bacteria that do not produce curli, such as *Klebsiella* species, *Shigella* species, and enteroinvasive *Escherichia coli* (9, 10).

Amyloid Curli

Identification of amyloid curli in enteric biofilms. Amyloids are proteins with a beta-sheet structure with fibers ranging from 4 to 10 nm in width, where the beta-sheet strands are oriented perpendicular to the axis of the fiber (11, 12). The first observation of amyloid proteins dates back to 1854, when Rudolph Karl Virchow, a German physician, stained liver and kidney autopsy samples with iodine. Amyloids were initially believed to be starches; this led to the name amyloid (Latin for *amylum*, which means starch) (reviewed in reference 13). Subsequent analyses conducted by Friedreich and Kekule in 1859 revealed that amyloids are proteinaceous due to their high nitrogen content and fibrillar structure (13–15). Although amyloid proteins are commonly associated with human diseases, amyloids are produced by bacteria as well and are associated with the biofilm EPS (16–19). The presence of amyloids in bacterial biofilms was initially observed by Chapman and colleagues in *E. coli* (20). Curli, one of the most well studied and characterized bacterial amyloids, is produced by certain enteric bacteria. Curli is the major proteinaceous component of enteric biofilms of *E. coli* and *Salmonella* (9, 21) and serves many functions within the biofilm.

To identify the presence of amyloids within biofilms, various amyloid-specific dyes, including Congo red and thioflavin T, can be employed (16, 20). The beta-sheet structure can be deduced through the polarization of light via circular dichroism, and amyloids can be readily visualized through electron microscopy (20). Cegelski and colleagues have pioneered novel solid-state nuclear magnetic resonance (NMR) approaches to study the interactions of extracellular matrix components at an atomic level. This pioneering technique has allowed investigation of the extracellular matrix and interactions between cellulose and curli from undisrupted, intact *E. coli* biofilms (22). In this “sum-of-all-parts” method, extracellular baskets (the biofilm matrix) that contain cellulose and curli can be removed from the bacteria and then can be examined by NMR spectroscopy (22).

Genetic regulation of curli. The production of curli is a highly regulated process. Dedicated machinery produces the amyloid and prevents fibrillization within the cell. Curli expression is triggered when enteric bacteria are grown under stressful environmental conditions that favor biofilm formation over planktonic cell growth. Stress factors include temperature, osmolarity, and oxygen or nutrient availability (23–25). In the laboratory, curli is expressed by enteric bacteria when grown at low temperatures (28°C) in Luria-Bertani (LB) broth with no sodium chloride or in various low-nutrient broths, including T medium or YESCA (yeast extract supplemented with Casamino Acids) broth (26). Depending on the medium used, curli expression within the bacterial population begins within the first 24 to 48 h of the change in environmental conditions (5).

Amyloid curli production is expressed by the bidirectional *csgBAC* and *csgDEFG* (curli-specific gene) operons (20). CsgD is key to the regulating the transition from motile to sessile behavior. In addition to the environmental stimuli that regulate CsgD expression, cyclic (5' to 3')-di-GMP (c-di-GMP) also plays a key role in dictating the switch to sessile behavior (27). In studies of *E. coli* and *Salmonella enterica* serovar Typhimurium (*S. Typhimurium*), increasing levels of c-di-GMP were found to promote biofilm formation by blocking the activity of flagellar motor protein YcgR (28) and activating RpoS (29–31) and, indirectly, MlrA (31), which in turn activate the expression of *csgD*, the master regulator of curli expression.

The biogenesis of curli is a complicated process that requires an orchestrated symphony of multiple proteins that result in the production of a functional fiber. Proper timing and production of curli are essential, and each protein produced from the *csg* operons plays a significant role in the assembly of the curli fiber. The *csgDEFG* operon encodes regulatory proteins that assist in the regulation, export, and assembly of the curli fiber, whereas the *csgBAC* operon encodes proteins that make up the curli fiber and that inhibit polymerization of subunits within the cell (32).

CsgD is the master bidirectional regulator of curli biogenesis. Global transcriptional regulators such as RpoS, OmpR, H-NS, and integration host factor (IHF) regulate the transcription of *csgD* in *S. Typhimurium* (29). CsgD binds to both the *csgBAC* and *csgDEFG* operons. To initiate the transcription of the curli genes, unphosphorylated CsgD binds to the *csg* promoter sequence 5'-CGGGKGAKNKA-3' (where K is G or T and N is any nucleotide) (33, 34). A single point mutation in the *csgD* gene of *S. Typhimurium* can decrease the production of curli by 3-fold (35). Upon binding of CsgD to the promoter of the *csgBAC* operon, CsgB, CsgA, and CsgC are produced. CsgA and CsgB are the two principal structural elements of curli; CsgA is termed the major curli subunit, and CsgB is termed the minor curli subunit. CsgA, a 13-kDa protein, is a soluble monomer that is secreted across the cell membrane. CsgA is characterized by five imperfect repeating domains (R) of 19 to 23 amino acids that contain conserved glutamine and arginine residues that form the amyloidogenic core domain of CsgA (36). These conserved glutamine and arginine residues are critical for formation of the beta-sheet structure of curli (37, 38). Each repeat of CsgA folds with a different efficiency. The first and the fifth repeats have the greatest tendencies to form the beta-sheet structure and promote seeding of CsgA monomers (37).

CsgB, the minor curli subunit, is required for nucleating CsgA into the curli fiber. Like CsgA, CsgB is composed of five imperfect repeating domains capable of forming beta-sheet structures. CsgB adheres to the cell wall at the C-terminal domain and binds to CsgA, enhancing the formation of the curli fiber (38). CsgB is capable of nucleating and promoting the polymerization of purified CsgA when added exogenously to a bacterial supernatant that contains CsgA (37).

Recently, Evans and colleagues showed that CsgC prevents polymerization of CsgA within the periplasmic space (39). Polymerization of CsgA within the cell has deleterious outcomes that could lead to cell death. CsgC stabilizes the intermediate form of CsgA that is initially produced intracellularly through binding interactions between CsgC and the first and fifth repeats of CsgA. Various models of CsgC inhibition have been proposed. Evans et al. proposed that CsgC induces a structural change in CsgA that prevents the fibrillization of CsgA (39). Second, Evans et al. proposed that CsgC binds to a pool of oligomeric CsgA, preventing CsgA from fibrillizing (39). Additional investigations of CsgC inhibition of CsgA showed that the inhibitory effect of CsgC on CsgA is in part due to electrostatic interactions between the two proteins (40).

The pore-forming protein CsgG facilitates the secretion of CsgA and CsgB monomeric units into the extracellular space. CsgG is a lipoprotein that is exported from the periplasmic space to the outer membrane, where it forms a pore (41, 42). Nucleation of CsgA and the formation of curli occurs in close proximity to the CsgG pore complex (43). When CsgG reaches a high concentration, CsgA and CsgB are released, thus promoting curli formation. Without CsgG, CsgA and CsgB undergo proteolytic degradation within the periplasmic space (42). The remaining proteins encoded by the *csgDEFG* operon, CsgE and CsgF, aid in the transport of CsgA and CsgB through the CsgG pore. CsgF chaperones CsgB through the pore and ensures that CsgB is tethered to the cell wall (44). CsgE prevents premature fibrillization of CsgA within the cell, prevents its degradation by periplasmic proteases, and also chaperones CsgA through the CsgG pore (45).

Role of curli within the enteric biofilm. Amyloid curli is the major proteinaceous component of the biofilm (46). Curli is responsible for the development of the overall biofilm architecture (46–48). In comparison to the formation of mature biofilms characterized by three-dimensional mushroom clustering of bacteria (7, 49), *E. coli* lacking

the ability to express curli does not form mature three-dimensional biofilms; these curli-deficient *E. coli* cells grow only in a single layer (46, 47). In addition to serving as an architectural component, curli mediates initial surface attachment. Small molecules such as the ring-fused 2-pyridones FN075 and BibC6 are considered curlicides, as they inhibit biofilm formation by preventing matrix adherence to surfaces (50).

Amyloids are highly resistant to chemical, proteolytic, and enzymatic degradation. Exposure to harsh chemicals such as 90% formic acid or hexafluoroisopropanol is required to depolymerize the fibrils into monomeric subunits (51). Purified curli fibers remain intact even during sodium dodecyl sulfate (SDS) treatment or enzymatic exposure to proteinase K (52). The ability to withstand chemical and enzymatic degradation enhances the strength and durability of biofilms, allowing them to withstand harsh environmental conditions (53).

***In vivo* expression of curli: a heated debate.** The expression of curli *in vivo* has been debated for many years. Curli expression *in vivo* was doubted, as curli expression is triggered in the laboratory at temperatures below that of body conditions and under nutrient-limiting conditions (26). Supporting this speculation, the thermosensor protein Crl induces curli expression only at low temperatures and not at 37°C (31, 54).

Multiple lines of evidence indicate that curli is expressed *in vivo*, however. As *E. coli* is the Gram-negative bacterium most frequently involved in sepsis pathogenesis (55) blood cultures from patients suffering from sepsis, as well as healthy volunteers, were collected and analyzed for the presence of curli expression. Of 46 septic patient serum samples analyzed, 24 were positive for anti-CsgA antibodies (56).

Expression of curli-associated genes is detected systemically in mice orally infected with *S. Typhimurium*. Using bioluminescent *csgD* *S. Typhimurium* strains, luminescence was detected in the gastrointestinal tract and fecal pellets, as well as the liver and spleen (57). Consistent with this study, Humphries and colleagues observed seroconversion to various fimbrial antigens, including CsgA, following infection with *S. Typhimurium* (58). In a study of 31 clinical isolates of uropathogenic *E. coli* (UPEC) from patients with reoccurring urinary tract infections (UTIs), all clinically isolated strains were positive for *csgA* expression (59). The authors determined that curli is an integral factor involved in cell adhesion during UTI and during UPEC-induced cystitis (59).

Systemic infection by curli is attributed to the ability of to bind fibronectin, plasminogen, and tissue type plasminogen activator (60, 61). Activated plasminogen degrades tissue (62), which suggests a mechanism facilitating bacterial dissemination. Curli also binds to host fibrinogen and bradykinin, proteins that enhance transmission through blood vessels and promote vasodilation (63, 64).

In refutation of *in vivo* expression of curli, in clinical isolates of *E. coli* O157:H7, several mutations have been identified that prevent expression of curli *in vivo*. In a screen of *E. coli* O157:H7 serotypes, prophage insertions in the *mlrA* gene and mutations in the *rsc* phosphorelay system were associated with low Congo red binding, indicative of little curli expression (65). Both MrlA and Rsc are DNA binding proteins that directly impact CsgD expression: MrlA serves as a CsgD enhancer, and Rsc serves as a CsgD repressor (66, 67). Mutations in the *csgD* gene have also been linked with clinical isolates of *E. coli* O157:H7 that do not express curli *in vivo* (35, 68, 69). It should be noted that a single point mutations in the *csgD* promoter of *E. coli* O157:H7 is associated with a lack of curli expression, and this mutation was detected in the majority of screened clinical isolates, suggesting that *in vivo* expression of curli is uncommon in *E. coli* O157:H7 (68). Thus, the debate continues.

Cellulose

Genetic regulation of cellulose. The second major component of the enteric biofilm matrix is the polysaccharide cellulose, defined by 1 to 4 β -linked linear glucose chains (reviewed in reference 70). Cellulose within the biofilms of enteric bacteria is best characterized in the context of *E. coli* or *Salmonella* species; however, cellulose production has been examined in other enteric bacteria, including *Citrobacter*, *Enterobacter*, and *Proteus* species (9). The production of cellulose is tightly coupled with the

production of curli. The *bcs* (bacterial cellulose synthesis) operon includes four genes necessary for the biosynthesis and secretion of cellulose, *bcsA*, *bcsB*, *bcsC*, and *bcsD*. In addition to activation by c-di-GMP (71), curli-dependent genes can also activate cellulose production. Upon production of the transcriptional regulator CsgD, *adrA* is transcribed, and AdrA synthesis ultimately leads to the transcription of the cellulose genes (72).

Function of cellulose in the enteric biofilm. As cellulose and curli expression are linked, the role of cellulose in the enteric biofilm has been difficult to dissect. In biofilms of clinically relevant UPEC isolates, NMR spectroscopy revealed that the enteric biofilm had a curli-to-cellulose mass ratio of 6:1 (22). In biofilms of *E. coli*, cellulose is chemically modified through phosphoethanolamine transferase, which results in phosphoethanolamine cellulose (73). The modified phosphoethanolamine cellulose structurally and functionally strengthens enteric biofilms. Modified cellulose creates long, thick filaments that form nanoparticles with curli that span the surface of the colony (65). The dense networks formed between the phosphoethanolamine cellulose and curli impart characteristics of cohesion and the ability to resist shear force stress (73). These curli-cellulose complexes create hydrophobic networks that tightly pack bacterial cells in a rigid matrix (74). The honeycomb-like or basket-like structures formed by curli and cellulose stabilize the biofilm, aid in surface adherence (75), and confer properties of elasticity (9, 46, 73).

Phenotypic detection of cellulose and curli in the enteric biofilm. Biofilm cellulose and curli phenotypes can be analyzed by growing biofilms on low-nutrient agar supplemented with Congo red and Coomassie blue. Curliated bacteria expressing cellulose produce a rough, dry, and red colony morphology. Curli mutants expressing cellulose produce pink, dry, and rough colonies. Cellulose mutants expressing curli produce brown, dry, and rough colonies. Curli-cellulose double mutants have a white, smooth colony morphology (51, 76, 77).

Colanic Acid

An additional polysaccharide component of the enteric biofilm is colanic acid. Colanic acid was initially described in 1963 by Goebel, who examined *E. coli* and identified a mucoid and serologically active polysaccharide (78). Colanic acid is a negatively charged polysaccharide polymer composed of repeating units of glucose, fructose, and glucuronic acid (reviewed in reference 79). Colanic acid is structurally similar to group I capsules that are encoded by the *wca* (also known as *cps*) gene cluster (80, 81). Shifts in temperature and osmolarity leads to the expression of *wca* genes (reviewed in references 79 and 82). Recent reports have shown that upon exposure to a subset of β -lactam antibiotics, the *wca* genes are upregulated, leading to the production of colanic acid and subsequent biofilm formation (83). Studies conducted by Danese and colleagues have identified a role for colanic acid in *E. coli* biofilm formation. In this model, colanic acid was not necessary for surface attachment but was essential for formation of the three-dimensional biofilm structure (84). These results were confirmed by additional studies where it was found that curli was necessary for initial attachment for the biofilm and that colanic acid was essential for formation of the mature biofilm (85).

Extracellular DNA

Another integral component of enteric biofilms is extracellular DNA (eDNA). Until recently, the importance of DNA in biofilms was underestimated. The first account of eDNA in biofilms was published in 2002 by Whitchurch and colleagues (86). eDNA can be observed within biofilms by staining with the nucleic acid stains such as propidium iodide, Toto-1, or Hoechst stain. General enzymatic protocols, including incubation with *N*-glycanase (a hydrolase that degrades glycoproteins), dispersin B (a biofilm-dispersing glycoside hydrolase), and proteinase K (a protein hydrolase), have been developed to extract eDNA from biofilms (87). Studies suggest that the primary sequence of secreted eDNA is the same as that of intracellular DNA, indicating that DNA is secreted directly

FIG 1 Curli-extracellular DNA complexes in biofilms of *S. Typhimurium*. (A) *P_{csgBA::gfp}* *S. Typhimurium* biofilms stained with Congo red (amyloid) and TOTO-1 iodide (nucleic acid stain) at 72 h. Biofilms were imaged using a Leica SP5 confocal microscope. (B) Zoomed-in ($\times 3$) images of panel A highlighting the curli-eDNA complex within the *S. Typhimurium* biofilm. Arrows indicate curli-DNA complexes. (C) Curli fibers purified from biofilms of *S. Typhimurium* stained with the amyloid dye thioflavin T (green, left panel) and the nucleic acid dye Hoechst 33258 (blue, center panel) contain amyloid-eDNA complexes (merged, right panel).

into the biofilm (88, 89). DNA released upon cell death can also contribute to the concentration of eDNA within the biofilm matrix. As an active mechanism of DNA release into the biofilm, in *E. coli*, the HipBA toxin-antitoxin system induces bacterial lysis leading to the release of genomic DNA into the biofilm (90). The mechanism by which DNA is released into the biofilm by enteric bacteria and whether it is an active or passive process are not fully established, and further investigations are required.

Curli-eDNA Complexes: an Interaction That Strengthens the Biofilm

One of the primary functions of eDNA within biofilms is to provide stability, but it also influences the architecture of the biofilm. In biofilms such as that of *Staphylococcus aureus* that do not contain curli, endogenous and exogenous DNases reduce the biomass of biofilm (91, 92). In comparison to treatment of biofilms that contain curli, DNase treatment is less effective (5). Recent work has demonstrated that eDNA and curli form irreversible complexes (5). eDNA can be detected within purified curli isolated from biofilms of *S. Typhimurium* and from the biofilm itself (Fig. 1) (5). Even when curli fibers are isolated using a protocol that calls for numerous rounds of DNase I and RNase H treatment and boiling in sodium dodecyl sulfate, nucleic acids are detected in tight association with curli (52). The tight interactions between curli and eDNA may explain why treatment of most biofilms with DNase results in only a partial reduction of the biomass, as the eDNA is embedded within the curli fibers, preventing the DNase enzyme from having access to the DNA.

Additionally, it was demonstrated that DNA accelerates the fibrillization of curli (5). This phenomenon was observed when curli monomers were incubated with synthetic or bacterial genomic DNA or with salmon sperm DNA, suggesting that DNA promotes the formation of amyloid fibers and that the DNA does not have to be of bacterial origin

(5). Overall, it is likely that the interactions between curli and eDNA lead to a stronger matrix that is resistant to enzymatic degradation (53).

Biofilm-Associated Proteins

In addition to curli and cellulose, another protein commonly found in biofilms of enteric bacteria is Bap (biofilm-associated protein). Bap is a surface protein that exhibits amyloid-like behavior (93). Bap was initially studied in *Staphylococcus aureus* biofilms. Bacteria with the *bap* mutation lack the ability to form mature biofilms and adhere to surfaces (94). In a genome database screen of *S. enterica* for sequence homologues of *S. aureus* Bap, it was found that BapA is also expressed by *S. enterica* (93). Similar to the case for the cellulose *bcs* genes, the production of BapA is regulated by CsgD (93). The overexpression of curli, but not cellulose, can counteract the lack of BapA production in *S. enterica* biofilms, as BapA is also involved in formation of the bacterial pellicle (93). Although the role of BapA in the enteric biofilm matrix has not been completely defined, it has been suggested that BapA plays a role in surface adhesion (93).

INNATE IMMUNE RESPONSES TO ENTERIC BIOFILMS

Systemic Recognition of Curli

Recognition of curli by TLR2. The innate immune response to curli has been thoroughly investigated over the past decade. Curli is an ideal PAMP, as it is secreted into the extracellular space and thus can be readily recognized by innate immune cells. Curli binds to and activates Toll-like receptor 2 (TLR2), leading to the production of proinflammatory cytokines and chemokines such as interleukin-8 (IL-8), IL-6, and tumor necrosis factor alpha (TNF- α), as well as nitric oxide (95–97) (Fig. 2). TLR1 is also required to facilitate the recognition of curli, suggesting cooperativity between TLR1 and TLR2 in elicitation of the proinflammatory response to biofilms (98). Cluster of differentiation 14 (CD14) was identified as an adaptor molecule that enhances the recognition of amyloid curli by the TLR2/TLR1 heterocomplex (99).

Examination of the structural basis for the interaction between curli and TLR2 revealed that activation of TLR2 by curli is dependent on the beta-sheet structure of the amyloid. Amino acid mutagenesis at position 122 of CsgA caused a marked loss of the beta-sheet structure of curli and a reduction of TLR2 activation (97). Subsequent experiments identified that TLR2 recognizes the conserved beta-sheet structures of two distinct amyloids of host and microbial origin, β -amyloid 1–42 and curli fibrils, respectively. Although these two amyloids do not share any sequence similarity on the amino acid level, both amyloids stimulate the innate immune responses through a TLR2-dependent mechanism. This activity was abrogated when the amyloid conformation was disrupted, suggesting that amyloids of different origins are recognized by the same receptor because they share a similar quaternary structure (95). These findings suggest that TLR2 likely recognizes amyloids produced by other bacterial species as well.

Endosomal escape of curli and cytosolic NLRP3 activation. After TLR2 is activated by PAMPs at the cell surface, TLR2 is engulfed into an endosome within the cell (100, 101). Subsequent activation of TLR2 by curli leads to the activation of the NLRP3 inflammasome. The NLRP3 inflammasome is one of the best characterized of the intracellular sensors that reside within the cytosolic space (102). Upon stimulation of macrophages by curli, activation of NLRP3 leads to the production of IL-1 β (103). All three components of the NLRP3 inflammasome (NLRP3, caspase-1, and the adaptor ASC) are required to mount an IL-1 β -driven response against curli (103). NLRP3 activation is dependent on TLR2, as production of IL-1 β is abrogated in TLR2-deficient macrophages (103). This suggests a series of events where first TLR2 must be activated, leading to the endocytosis of curli, and subsequently the NLRP3 inflammasome is activated (Fig. 2).

Although the ability of amyloid curli to activate NLRP3 has been defined, the mechanism by which curli escapes the TLR2-containing endosome to gain access to the cytosolic NLRP3 has yet to be determined. It has been suggested that upon amyloid beta stimulation of microglia, the amyloid beta induces lysosomal swelling and rupture,

FIG 2 Activation of innate immune receptors by curli-extracellular DNA complexes. Curli binds to and activates the TLR2/TLR1/CD14 heterocomplex and is internalized. The curli-eDNA-containing endosome fuses to a TLR9-containing endosome. eDNA activates TLR9 to generate a type I interferon response. By unknown mechanisms, curli can escape, gain cytosolic access, and activate the NLRP3 inflammasome, cleaving pro-IL-1 β to mature IL-1 β . Whether curli can activate cytosolic DNA sensors, including STING, is under investigation.

thus allowing the release of amyloid beta into the cytosol (104). As amyloids from bacterial and host origins share similar features (reviewed in reference 105), curli may cause similar lysosomal dysfunction, leading to the release of curli into the cytosolic space. Further research is required to investigate this hypothesis.

Site Specificity Impacts the Response to Curli: Intestine-Associated Curli Reinforce the Epithelial Barrier and Ameliorate Colitis

Within the context of systemic infection, the innate immune system responds to curli by generating a proinflammatory response whose hallmark is the production of proinflammatory cytokines and chemokines to recruit other immune cells to clear the infection. However, in the intestine, exposure to curli promotes an anti-inflammatory response and serves to strengthen damaged intestinal epithelium (Fig. 3). Toll-like receptors are pivotal in maintaining gut homeostasis. Specifically, TLR2 activation by microbial products promotes overall gut health (106–109). A *Bacteroides fragilis* polysaccharide, termed PSA, was the first bacterial component shown to educate the immune system (110) and suppress intestinal inflammation (111) by serving as a TLR2 agonist (112). Most bacteria that inhabit the intestine do not produce PSA, whereas amyloid fibers are found in the environmental biofilms of *Bacteroidia*, *Clostridia*, and *Proteobacteria*, the major phyla inhabiting the intestine, which makes them good candidates for a conserved microbe-associated molecular pattern detected by the innate immune system to indicate the presence of gut microbes (16, 113).

FIG 3 The response to curli varies depending on the site of recognition. (A) Curli recognition in the gut leads to an anti-inflammatory response and repair of damaged intestinal epithelium. Curli binds to TLR2 on the epithelium, strengthening the intestinal epithelium and preventing bacterial dissemination. Binding of curli to intestinal macrophages leads to the production of IL-6 and IL-23, which act on CD4 T cells and $\gamma\delta$ T cells to produce IL-22 and IL-17. Those cytokines act on the intestinal epithelium to thus promote gut health. (B) Systemic recognition of curli leads to a proinflammatory response. Recognition of curli by systemic macrophages and dendritic cells by the TLR2/TLR1/CD14 heterocomplex, as well as NLRP3, leads to the induction of proinflammatory cytokines and cleavage of pro-IL-1 β to IL-1 β . DNA within the curli-DNA complex is also recognized by TLR9, leading to the production of type I interferons that are hallmarks of the autoimmune disease systemic lupus erythematosus.

It is important to note that the curli proteins expressed by both commensal and pathogenic bacteria are homologous and that curli does not function as a virulence factor (32, 48). Recent studies showed that epithelial cells directly respond to curli fibers, leading to the reinforcement of the barrier and resulting in a reduction of bacterial translocation (114). Across the barrier, other immune cells respond to curli, resulting in the expression of IL-17 and IL-22, which has been shown to regulate the barrier function, in a TLR2-dependent manner (115). In a 2,4,6-trinitrobenzenesulfonic acid-induced colitis mouse model, a common model for inflammatory bowel disease (IBD), it was determined that a single oral administration of curli fibers ameliorates the disease pathology (116). The outcome with the oral curli treatment was comparable to that with the current IBD treatment, anti-TNF- α antibody treatment (116). Further analysis suggested that oral administration of curli fibers not only promoted intestinal barrier function but also enhanced the anti-inflammatory immune response. In a TLR2-dependent fashion, hallmarks of oral treatment of curli included the upregulation of anti-inflammatory markers, including *il10*, in the small intestine (114) (Fig. 3). Despite this progress, the mechanisms by which bacterial biofilms are recognized in the gut and promote epithelial barrier function remain unclear. As immune responses generated against systemic and intestinal responses to curli drastically vary, this principle may be exploited to provide novel treatment strategies to combat systemic infections and to develop therapeutics to combat intestinal diseases.

Curli-eDNA Complexes Enhance Immune Recognition of Enteric Biofilms

The immune response generated against curli-eDNA complexes highlights the complexity of enteric biofilms. Initial reports revealed that both intraperitoneal injection of curli-eDNA complexes into mice and stimulation of wild-type bone marrow-derived macrophages cause an upregulation of type I interferon genes *ifn β* , *irf7*, *isg15*, and *cxcl0* (5). The generation of a type I interferon response suggests that receptors other than TLR2 are involved in the recognition of the curli-eDNA complex. It was

hypothesized that TLR9 is activated by the eDNA component of the complex, leading to the type I interferon response. TLR9 is an endosome-bound TLR that is classically activated by unmethylated bacterial DNA sequences (117). Upon stimulation of wild-type, TLR2-deficient, TLR9-deficient, or doubly TLR2- and TLR9-deficient macrophages with curli-eDNA complexes, a significant decrease in levels of the transcripts encoding various type I interferons was observed, implicating TLR9 in generation of the type I interferon response to biofilms (118). Reduction of the type I interferon response was additionally decreased in wild-type macrophages pretreated with cytochalasin D prior to stimulation with curli-eDNA complexes, suggesting that TLR2 serves as a carrier to bring the curli-eDNA complex into the cell (118) (Fig. 2). After internalization of TLR2, the endosome containing the curli-eDNA complex fuses with a TLR9-containing endosome, allowing recognition of the eDNA by TLR9 (118) (Fig. 2).

Examination of the curli-eDNA complex using synchrotron small-angle X-ray scattering (SAXS) analysis shed light upon the manner in which eDNA interacts with curli. The inter-DNA spacing of curli and eDNA is 4.16 nm, with a sharp diffraction peak at $0.151 \text{ \AA}^{-1}$. This inter-DNA spacing is within the range reported to sterically optimize the activation of TLR9 (3.0 nm to 4.0 nm), suggesting that the eDNA of the curli-eDNA complex is organized into a lattice that optimally activates TLR9 (118).

TLR9 is not the sole innate immune receptor responsible for recognizing foreign DNA within the cell from a pathogenic source. Other cytosolic innate immune receptors that recognize nucleic acids from viral or microbial sources include STING and AIM2 (119, 120). The ability of eDNA from curli-eDNA complexes to activate other cytosolic DNA sensors has yet to be explored.

Components of Enteric Biofilms Allow for Immune Evasion and Virulence

Enteric bacteria have evolved numerous mechanisms to evade assaults by the host immune system. Although the innate immune system can recognize various components of the enteric biofilm as PAMPs, some components aid in immune evasion and thus promote bacterial virulence. Although curli is recognized as a PAMP, curli can protect *E. coli* against complement-mediated phagocytosis (121). Complement is a branch of the innate immune system that aids in the phagocytosis of bacteria by macrophages and dendritic cells. Curli enhances survival of *E. coli* within the blood, as curli binds to and blocks the actions of C1q, promoting the overall systemic virulence of *E. coli* (121).

DNA within biofilms of enteric bacteria also enhances the virulence of enteric bacteria. In *S. Typhimurium*, eDNA within the biofilm induces the expression of the *pmr* antimicrobial resistance operon through mechanisms of cation chelation. (Expression of the *pmr* operon is significantly higher in biofilms than in planktonic bacteria, highlighting the importance of the biofilm in recalcitrance against antibiotics [122].) Activation of the *pmr* operon protects biofilm-associated *S. Typhimurium* from the actions of aminoglycosides, antimicrobial peptides, and ciprofloxacin (122).

ENTERIC BIOFILM AND HUMAN DISEASE

Biofilms of *S. Typhimurium* Accelerate the Autoimmune Disease Systemic Lupus Erythematosus

The ability of *S. Typhimurium* biofilms, specifically the curli-eDNA complex, to potentiate the autoimmune disease systemic lupus erythematosus (SLE) has been explored. SLE is a disease in which etiologic triggers, environmental and genetic, induce the production of autoantibodies that accumulate systemically, leading to complications, including nephritis (123). Characteristic to SLE is the production of type I interferons, including IFN- β , IRF7, and ISG15 (124). Interestingly, bacterial infections are a trigger of lupus flares, and infections with enteric bacteria are the most common bacterial infections diagnosed in SLE patients (125, 126); specifically, bloodstream infections and urinary tract infections caused by *Salmonella enterica* serovars Typhimurium and Enteritidis as well as *E. coli* are common (127). Not only do curli-eDNA

complexes induce the upregulation of type I interferon transcripts, but infection with curli-competent bacteria also leads to the generation of anti-double-stranded DNA and antichromatin autoantibodies in SLE patients (5). The dominant subclasses of autoantibodies produced in SLE patients were found to be IgG2a and IgG2b, indicating that curli-DNA complexes are a pathogenic antigen reservoir (5).

The mechanisms by which curli-eDNA complexes promote SLE have been only partially explored. Induction of autoantibodies is reliant on both TLR2 and TLR9 (118). It is speculated that curli-eDNA complexes result in induction of neutrophil extracellular traps (NETs). NETs are strands of DNA embedded with antimicrobial histones and myeloperoxidase that are secreted by neutrophils in an effort to eliminate pathogenic foes (128). One of the modalities of SLE pathogenesis is the inability to degrade NETs, potentially due to a lack of serum DNase (129). It is believed that the NETs that remain intact serve as a reservoir of antigens leading to production of autoantibodies to DNA and chromatin (129). It has also been speculated that DNA of bacterial origin may become complexed within NETs, contributing to the reservoir of autoantibody antigens (129).

Although most people encounter multiple infections with enteric bacteria over the course of their lifetimes, not all people develop autoimmune diseases. It is speculated that biofilm-associated infections trigger a transient autoimmune response due to activation of TLR2 and TLR9 by bacterial amyloid-eDNA complexes. However, in individuals with a genetic predisposition to SLE, exposure to curli-eDNA complexes may lead to an autoantibody phenotype that is sustained and detrimental.

Enteric Biofilm Amyloids and Neurodegenerative Diseases

In humans, there are over 30 pathogenic-amyloid deposition-associated diseases, where each disease has a unique amyloid pathogen (12). These amyloids can induce neurodegenerative and systemic inflammatory diseases such as Alzheimer's disease, Parkinson's disease, prion diseases, and type 2 diabetes (12). In each of these diseases, amyloids accumulate within the brain or systemically, resulting in local inflammation that contributes to tissue injury (130, 131). Although human and bacterial amyloids do not share conserved amino acid sequences, numerous features are shared. Bacterial and host amyloid precursor peptides fold into highly conserved beta-sheet structures due to the presence of conserved glutamine and asparagine residues, and amyloids from both origins fibrillize from monomeric subunits (37, 132). Both human β -amyloid 1-42 and bacterial curli fibrils elicit proinflammatory immune responses generated through TLR2 (88, 95, 98). Cross seeding between various amyloid species has been demonstrated. The coexistence of combinations of α -synuclein, tau, prion protein, and beta-amyloid has been observed in amyloid deposits in human patients (133). More significantly, cross seeding of human α -synuclein (the pathogenic amyloid involved in Parkinson's disease) and the curli subunit CsgE has been reported (6). CsgE interacts with α -synuclein in a manner, which causes the release of the intraprotein interactions between the C-terminal domain and the non-amyloid- β component (NAC) region of α -synuclein (6). The NAC region spans residues 60 to 95 in the α -synuclein primary sequence (134). As it is established that the interaction between the C-terminal domain and NAC region prevents the fibrillization of α -synuclein, disruption of this interaction and exposure of the NAC domain promote amyloid fibrillization (6). In contrast, however, interactions between α -synuclein and CsgC were found to prevent amyloid polymerization (6). The authors suggested that interactions between CsgC and α -synuclein enhanced intraprotein interactions, shielding NAC from exposure and thus preventing fibrillization of α -synuclein (135). Furthermore, Westermarck and colleagues showed that curli fibers enhance the fibrillization of amyloid protein A, which is implicated in the pathogenesis of systemic amyloidosis (136). These data suggest that the subunits of curli enhance the progression of amyloid diseases.

Although the spatial interactions between bacterial and host amyloids that would allow for cross seeding are still unknown, ideas of how cross seeding occur have been proposed. In a model of CsgE-mediated acceleration of α -synuclein fibrillization, curli

expressed in the gastrointestinal system can leak from the compromised gut and spread to the peripheral nervous system, where it influences the progression of Parkinson's disease (137). This notion, along with other lines of evidence, has spurred great interest in the recently termed "gut-brain axis." Further investigations are clearly required.

Biofilms and Gallstone Chronic Carriage: Vi Capsule and O Antigen

S. enterica serovar Typhi causes typhoid fever in humans. Three to five percent of infected individuals become chronic carriers with the gallbladder being a site of persistence, and these individuals serve as a critical reservoir to further spread the disease (138–141). Vi antigen is a surface-associated capsular polysaccharide produced by *S. Typhi* that mediates protection against complement-mediated bacterial killing (142) and helps bacteria evade the innate immune recognition, thus allowing for the establishment of a systemic infection (143, 144). *S. Typhi* favors the gallbladder (145), and bile facilitates the expression of the EPS. If gallstones exist in the gallbladder, *S. Typhi* forms mature biofilms on gallstones (146–149). Vi antigen, cellulose, and colonic acid are indispensable for the establishment of *Salmonella* biofilms on gallstones (150). O-antigen capsule (*yihU-yshA* and *yihV-yihW*) also plays a critical role in the formation of biofilms in the presence of bile (139, 151). Although curli has been shown to play a role in the colonization of gallbladder epithelial cells *in vitro* (152), no studies have yet elucidated the expression of curli on gallstone biofilms recovered from typhoid fever patients. Therefore, the implications of possible curli presence in biofilms formed by typhoidal *Salmonella* serotypes still remain unsolved.

Beyond Enteric Biofilms: Potential Implications for Other Amyloid-Based Diseases

Not only are amyloids found in enteric biofilms, but homologues of the *csg* genes have been identified in four other phyla, i.e., *Bacteroidetes*, *Proteobacteria*, *Firmicutes*, and *Thermodesulfobacteria* (113). Amyloids produced in bacteria other than enteric bacteria include TasA secreted by *Bacillus subtilis*, functional amyloid protein (Fap) secreted by *Pseudomonas aeruginosa*, and phenol-soluble modulins (PSMs) secreted by *Staphylococcus aureus* (153–155). PSMs have been rigorously studied. PSMs are small, amphipathic, alpha-helical peptides of 15 to 20 amino acids in length (155). Two operons, α psm and β psm, regulate the expression of PSMs, and four variants of PSM α (PSM α 1 to PSM α 4) and two variants of PSM β (Psm β 1 and PSM β 2) are produced (155, 156). Similar to curli, PSMs are resistant to treatment with detergents, including SDS, and can be degraded only by treatment with formic acid or hexafluoroisopropanol (155). Like curli, PSMs bind both Congo red and thioflavin T (155). Within biofilms, it has been shown that *S. aureus* PSM α and PSM β mutants produce biofilms that have decreased matrix integrities relative to those of wild-type biofilms. *S. aureus* PSM mutants are susceptible to matrix degradation with enzymes, including proteinase K, DNase I, and dispersin B, and to mechanical stress, including biofilm disruption by vortexing (155). Therefore, similar to the case for curli, PSMs are considered to be functional amyloids that stabilize the biofilm matrix (155).

One of the main characteristics of PSMs is their ability to lyse both eukaryotic and prokaryotic cells due to their surfactant-like properties. These properties allow the PSMs to intercalate into and disrupt membranes (157–159). PSMs can cause release of eDNA into the biofilm, which further promotes the integrity and the strength of the matrix (160). As is the case with *S. Typhimurium*, eDNA in *S. aureus* biofilms promotes the fibrillization of PSMs (161). When autolysin genes that are responsible for the release of DNA into the biofilm matrix are mutated, the fibrillization of PSMs is halted (161). As PSMs are amyloidogenic and are in close contact with eDNA within the biofilm matrix, PSMs likely form complexes with DNA, similar to curli-eDNA complexes. It has been proposed that PSMs activate TLR2 (162); thus, immunogenic PSM-DNA complexes may potentially induce the production of interferon-stimulated genes through TLR9. Additionally, it has been shown that PSMs recruit neutrophils, leading to both the generation of NETs, the reservoir for antigenic DNA (129), and lysis of neutrophils due to

cytolytic properties of PSMs. Taken together, these findings lead to the hypothesis that amyloid-DNA complexes from nonenteric biofilms can impact the pathogenesis of autoimmune diseases such as SLE.

CONCLUDING REMARKS

A significant amount is known regarding the complex composition and interactions of the components of curli-containing enteric biofilms, as well as the functions of these components within the biofilm. The main components of enteric biofilms are the amyloid curli, cellulose, extracellular DNA, and various surface proteins, including BapA. Various components of the enteric biofilm are recognized as PAMPs by the innate immune system to alert the body of an infection. In a highly orchestrated symphony, curli is recognized by TLR2, leading to the endocytosis within an intracellular endosome. Once in the endosome, the eDNA of the curli-eDNA complex activates TLR9. By mechanisms that are still unknown, curli then escapes into the cytosol to activate the NLRP3 inflammasome. Although curli-eDNA complexes are pathogenic when given systemically, within the intestine curli strengthens the intestinal epithelial barrier and has the ability to ameliorate colitis. Additionally, eDNA and cellulose have been shown to increase virulence by enabling evasion of immune responses or by enhancing bacterial transmission.

Enteric biofilms have gained attention in the field of biomedical research because they potentiate the autoimmune disease SLE and can accelerate amyloid-based neurodegenerative diseases. The relevance of bacterial amyloids to disease goes well beyond the scope of enteric biofilms. Even though curli production is most often studied in the *Enterobacteriales*, genes homologous to those necessary for the production of the bacterial amyloid curli have been detected in bacteria from the phyla *Bacteroidetes*, *Proteobacteria*, *Firmicutes*, and *Thermodesulfobacteria* (48, 113). Although our understanding of bacterial biofilms has progressed significantly since their initial observation in the 18th century, much is unknown. We predict that continued research into the roles of biofilms in immunity and infection will lead to the development of novel antibiofilm strategies as well as life-saving therapeutics.

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