Curli Biogenesis: Bacterial Amyloid Assembly by the Type VIII Secretion Pathway

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ABSTRACT In 1989, Normark and coworkers reported on fibrous surface structures called curli on strains of *Escherichia coli* that were suspected of causing bovine mastitis. Subsequent work by many groups has revealed an elegant and highly regulated curli biogenesis pathway also referred to as the type VIII secretion system. Curli biogenesis is governed by two divergently transcribed operons, *csgBAC* and *csgDEFG*. The *csgBAC* operon encodes the structural subunits of curli, CsgA and CsgB, along with a chaperone-like protein, CsgC. The *csgDEFG* operon encodes the accessory proteins required for efficient transcription, secretion, and assembly of the curli fiber. CsgA and CsgB are secreted as largely unstructured proteins and transition to β-rich structures that aggregate into regular fibers at the cell surface. Since both of these proteins have been shown to be amyloidogenic in nature, the correct spatiotemporal synthesis of the curli fiber is of paramount importance for proper functioning and viability. Gram-negative bacteria have evolved an elegant machinery for the safe handling, secretion, and extracellular assembly of these amyloidogenic proteins.

Curli are extracellular proteinaceous fibers made by Gram-negative bacteria. Curli-specific genes (*csg*) are primarily found in *Proteobacteria* and *Bacteroidetes* (1–3). The main function of curli fibers is associated with a sedimentary lifestyle and multicellular behavior in biofilms, as they form scaffolds that provide adhesive and structural support to the community (4–8). In certain pathogenic bacteria, curli have also been implicated in host colonization, innate response activation, and cell invasion (9–13).

Curli appear as rigid, 2- to 5-nm-thick coiled fibers that entangle into dense congregates that surround the cell (Fig. 1A and B) (1, 7, 14). Curli fibers form via a nucleation-dependent self-assembly process, and they adopt an amyloid fold as their native structure (15, 16). Curli are the product of a dedicated secretion-assembly pathway known as the nucleation-precipitation pathway, or type VIII secretion system (T8SS) (3, 17, 18). In *Escherichia coli*, seven curli-
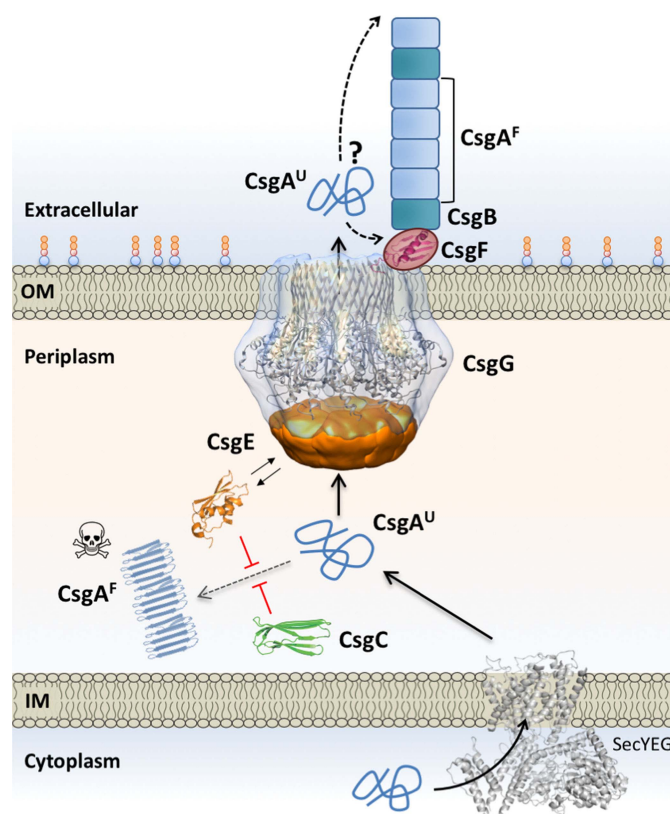
Figure 1 Curli composition and structure. (A and B) Transmission electron micrographs of individual *E. coli* cells producing curli fibers **(A)** and curli-like fibers grown *in vitro* from purified CsgA **(B)**. Scale bars: 500 nm and 200 nm, respectively. **(C)** Schematic organization of the *csgDEFG* and *csgBAC* curli gene clusters and architecture of the curli subunits CsgA (blue) and CsgB (dark blue). Subunits comprise an N-terminal signal sequence (SEC) that is cleaved upon export into the periplasm. The mature proteins contain curlin pseudorepeat regions (N22, R1 to R5) that guide substrate specificity in the secretion pathway and form the amyloidogenic core of the curli subunits. Repeats that efficiently self-polymerize *in vitro* are underscored. **(D)** Theoretical model of CsgA predicted based on amino acid covariation analysis ([42](#)). The predictions point to a right- or left-handed β-helix made up from stacked curlin repeats (labeled R1 to R5). **(E)** Representation of typical *in vitro* CsgA polymerization profiles in the absence (red) or presence (blue) of preformed fibers or the CsgB nucleator. In the presence of CsgE (1:1 ratio) or CsgC (1:500 ratio), no CsgA polymerization is observed (black curve).

specific genes (*csg*) are clustered in two divergently described operons, *csgBAC* and *csgDEFG* ([17](#)) ([Fig. 1C](#)), where *csgA* and *csgB* are structural components of the fiber ([1](#), [16](#)), *csgC* is a periplasmic chaperone ([19](#)), *csgE*, *csgF*, and *csgG* form the secretion-assembly machinery ([20](#)–[22](#)), and *csgD* is a transcriptional activator of the *csgBAC* operon ([23](#)). We review curli biogenesis and the T8SS using the *E. coli* components as a representative system.

THE SECRETION-ASSEMBLY MACHINERY

In Gram-negative bacteria, assembly of surface appendages requires the passage of two lipid bilayers, the cytoplasmic or inner membrane and the outer membrane. The curli subunit proteins CsgA and CsgB cross the inner membrane via the Sec general secretory pathway, after which CsgE, CsgF, and CsgG orchestrate translocation of CsgA and CsgB across the outer membrane ([20](#)–[22](#)), where they assemble into curli polymers ([21](#)). CsgG is a

262-residue lipoprotein that forms the curli translocation channel in the outer membrane ([20]). It forms a nonameric complex with a 36-stranded β-barrel that inserts into the outer membrane and is connected to the periplasm via a cage-like vestibule with an inner diameter of approximately 35 Å ([24], [25]) ([Fig. 2]). The transmembrane and periplasmic domains are separated by a 9-Å-wide channel constriction that would be compatible with the passage of unfolded polypeptides. Single-channel current recordings and *in vivo* bile salt sensitivity assays show a constitutively open channel, which can be gated in the presence of the periplasmic accessory factor CsgE ([24]). The latter is a soluble 107-residue protein that can oligomerize into a nonameric cap-like structure that binds the periplasmic entry to the CsgG secretion channel ([24]) ([Fig. 2]). Although at elevated concentrations CsgG can facilitate a nonselective leakage of periplasmic polypeptides, under native conditions secretion is specific for curli subunits and requires CsgE ([22]). *In vitro*, CsgE inhibits CsgA self-assembly when present at a 1:1 stoichiometric ratio, demonstrating that it can directly interact with curli subunits. The prevailing model is for CsgE to act as specificity factor by binding periplasmic CsgA and targeting it to the CsgG secretion channel. *In vitro* the CsgE:CsgG complex is in a reversible equilibrium ([24]), suggesting that CsgE may cycle between a periplasmic form and a CsgG-bound form. The nuclear magnetic resonance (NMR) structure of the CsgE monomer shows a compact alpha/beta sandwich protein with a flexible C-terminal tail ([26]) ([Fig. 2]). Two regions of CsgE involved in the CsgE-CsgA interaction were determined: a head comprising a positively charged patch centered around R47 and a stem comprising a negatively charged patch containing E31 and E85 ([27]). R47 was found to mediate an indispensable charge-charge interaction with CsgA, while mutations in the negatively charged neck region retain CsgA binding capacity but impact CsgE-dependent secretion of CsgA. The exact binding epitopes in CsgA are currently unknown, although the first pseudorepeat in the curli subunits (in *E. coli* CsgA referred to as N22) is sufficient to target polypeptides for secretion through the curli pathway ([20], [22]). Secretion of nonnative sequences was found to have a size constraint in the case of folded fusion proteins, which is likely to reflect the width of the CsgG constriction ([28]). The dimensions of the channel constriction suggest that the native substrate navigates the CsgG channel in an extended conformation ([24], [25]). In agreement with this, in strains with curli assembly defects, extracellular CsgA is found as an unfolded chain ([16], [29]). The transition of secreted CsgA to the amyloid state requires the activity of CsgF and the nucleator subunit CsgB ([15], [21], [29]–[31]).

CsgF is a 119-residue protein that is found to be surface exposed in a CsgG-dependent manner and is speculated to be in close proximity or direct contact with the CsgG channel ([Fig. 2]) ([21]). Structural data obtained by NMR spectroscopy revealed that the CsgF monomer consists of

Figure 2 Integrated model for curli subunit secretion. Curli subunits enter the periplasm via the SecYEG translocon, from where they progress to the cell surface as unfolded polypeptides via the curli transporter CsgG. Premature folding and polymerization of CsgA in the periplasm (right dotted line) are inhibited by CsgE and CsgC. CsgE binds and targets subunits to the secretion channel, while CsgC provides a safeguard against runaway polymerization, likely by the binding and neutralization of early assembly intermediates and/or nascent fibers. CsgG forms a nonameric complex that acts as a peptide diffusion channel and cooperates with the periplasmic factor CsgE, which binds the channel and forms a capping structure to the secretion complex. Recruitment and (partial) enclosure of CsgA in the secretion complex are proposed to create an entropy gradient over the channel that favors CsgA's outward diffusion as an unfolded, soluble polypeptide. Once secreted, curli fiber formation and elongation are templated by CsgB, in a CsgF-dependent manner. CsgF is likely to be in contact or close proximity to the CsgG channel. The exact role of CsgF and whether fibers extend from the proximal or distal end (dashed arrows) are presently unknown. Abbreviations: IM, inner membrane; OM, outer membrane.

three independent elements: an N-terminal unstructured region, a 21-residue α-helix, and a C-terminal antiparallel β-sheet made of 4 strands ([32]). The absence of tertiary structure and the presence of exposed hydrophobic surfaces may correspond to putative interaction sides with CsgG or the secretion substrates CsgA and CsgB. Although CsgF appears to be dispensable for secretion of curli subunits, it is essential for curli fiber formation ([21]). When *csgF* is deleted, CsgB is no longer associated with the cell surface and is not able to exert its nucleating function. These observations implicate CsgF as a coupling factor between CsgA secretion and extracellular polymerization into curli fibers by coordinating or chaperoning the nucleating activity of the CsgB minor subunit ([21]).

A striking conundrum in curli assembly is the mode of protein translocation across the outer membrane. Protein transport is an energy-dependent process, in which the driving force is generally drawn from ATP or GTP hydrolysis, protein synthesis, or an electrochemical potential such as the proton motive force ([33]). However, the periplasmic space is devoid of known hydrolyzable energy carriers and the semiporous outer membrane does not support the buildup of solute or ion gradients. In Gram-negative secretion pathways, this hurdle is overcome by means of cell envelope-spanning complexes such as type I, II, III, and IV secretion systems ([34]) or by drawing energy from substrate folding and assembly such as seen for the chaperone-usher assembly and type V secretion pathways ([35], [36]). Available structural data indicate that CsgG operates as a peptide diffusion channel ([24], [25]). The height of the CsgG constriction is compatible with the simultaneous binding of 4 or 5 residues along the length of an extended polypeptide chain, so that passage of the full protein implies stepwise Brownian diffusion along the length of the polypeptide chain. The rectifying force that ensures a net forward Brownian diffusion is presently unclear. The transition from the extended preassembly state to the amyloid state upon secretion and incorporation into curli fibers may provide such a driving force. However, in *csgB* deletion mutants, CsgA does not assemble into curli and can be found as disordered polypeptide in the cell medium, indicating that fiber assembly is not a prerequisite for secretion ([21], [29]). In an alternative hypothesis, secretion is driven by an entropy gradient across the outer membrane and the channel in a process that involves the secretion factor CsgE ([24], [37]). It is speculated that the local high concentration and conformational confinement of curli subunits in the CsgG vestibule would raise an entropic free-energy gradient over the translocation channel.

Under physiological conditions, the entropy potential of CsgA capture at the translocation channel and the folding energy released from the disorder-order transition of subunits incorporating into surface-associated fibers may cooperate to ensure forward diffusion across the CsgG channel.

FIBER COMPONENTS AND SELF-ASSEMBLY

Curli fibers are linear, noncovalent polymers of the major and minor subunits CsgA and CsgB, respectively ([1], [16]). The major curlin subunit, CsgA, is a 151-amino-acid-long peptide with five imperfect repeat units, R1 to R5 ([Fig. 1C]). The repeating units in CsgA are predicted to form β-strand–loop–β-strand motifs that constitute the core of the β-sheet-rich curli amyloid fiber ([3], [16], [38]–[42]) ([Fig. 1D]). *In vitro*, CsgA amyloid formation can be monitored using the amyloid binding dye thioflavin T (ThT) ([43]). CsgA amyloid aggregation displays canonical sigmodal polymerization kinetics typical for amyloid proteins, with a lag phase, a growth phase, and a plateau phase ([44]) ([Fig. 1E]). The lag phase represents the time required for the buildup of enough primary nuclei to lead to the rapid incorporation of remaining free monomers into the nascent fibers during the growth phase ([38], [44], [45]). Recent *in vitro* studies suggest that the nucleation of CsgA into the amyloid template is a single-step process and does not pass through one or more oligomeric aggregated states as often seen in pathological amyloids ([45]). The phase transitions of the curli repeats from an unfolded to a folded β-strand–loop–β-strand motif, in the CsgA subunit itself or in a minimal oligomer such as a CsgA dimer, serve as the "nucleus" in this model ([45]). Once formed, CsgA fibers display distinctive structural and biophysical properties that are shared by all amyloids ([16], [44], [45]).

CsgA is secreted by the T8SS to the cell surface as an unstructured protein and remains in a nonamyloid form unless it interacts with the CsgB "nucleator" protein. CsgB shares 30% sequence identity with CsgA and is also predicted to form five imperfect β-strand–loop–β-strand repeats, R1 to R5 ([31]) ([Fig. 1C]). The C-terminal R5 repeat of CsgB has been shown to be necessary for anchoring CsgB to the cell surface ([30], [31]). While the precise mechanism of *in vivo* nucleation has not been elucidated, it is tempting to postulate that the R1 to R4 repeats of membrane-anchored CsgB assume a β-solenoid structure that provides a template for recognition by either R1 or R5 of CsgA ([38], [41]). The R1 and R5 repeats in CsgA are

suggested to mediate interaction with CsgB, but also self-association by providing a template for secreted CsgA monomers to add on to the growing fiber tip (38, 46). A distinctive characteristic of curli assembly is that CsgA and CsgB can be independently secreted as unstructured subunits, which can then self-assemble into extracellular fibers when they contact each other on the cell surface (15, 29).

Interestingly, the R2, R3, and R4 repeating units of CsgA contain conserved Asp and Gly "gatekeeper" residues (47). The positions of these gatekeeper residues are conserved in most CsgA homologs, and certain gatekeeper residues modulate the amyloidogenic nature of CsgA by inhibiting its intrinsic aggregation propensity (47). The gatekeeper residues allow CsgA to remain in an unstructured form before being secreted to the cell surface and nucleated by CsgB (15, 30, 31, 38, 47). Interestingly, a CsgA mutant called CsgA* that lacks the gatekeeper residues polymerized *in vivo* in the absence of CsgB. However, the expression of CsgA* is toxic to cells, underlying the importance of controlled amyloid formation afforded by the T8SS system (47). In this respect, the T8SS evolved an intricate ability to maintain amyloidogenic proteins unstructured until secretion across the outer membrane.

PATHWAY CONTROL AND CHEMICAL INHIBITION

An intriguing issue in functional amyloid assembly pathways such as curli biogenesis is the avoidance of cytotoxic effects that are associated with amyloid formation in human and animal protein aggregation disorders. In addition to elaborate regulation at the transcriptional level (3, 17, 48–52), several adaptations within the pathway components ensure safe secretion of the amyloidogenic subunits and controlled fibril formation at the cell surface. A recent study found that unlike most pathological amyloid depositions, curli nucleation is a single-step process that does not involve intermediary oligomeric aggregates, which are often considered the more toxic species (45, 53). This suggests that curli subunits are evolutionarily optimized to have a sharp and direct transition from an intrinsically disordered conformation to the relatively inert amyloid conformation. In addition, by the provision of the major subunit CsgA and the minor subunit CsgB, the pathway has segregated efficient fiber elongation and fiber nucleation, providing spatiotemporal control over curli deposition. On top of that, the T8SS evolved a further safeguard to quell premature CsgA amyloid formation by having a dedicated periplasmic chaperone (19). CsgC

serves as a potent chaperone-like protein to prevent the runaway polymerization of CsgA in the periplasmic space (19) (Fig. 2). *In vitro*, CsgC inhibits CsgA polymerization down to substoichiometric molar ratios of 1:500 (19). The exact mechanism by which CsgC inhibits CsgA aggregation is not completely understood. The observed substoichiometric molar ratio may suggest that CsgC transiently interacts with a soluble oligomeric pool of CsgA to prevent seed formation and subsequent polymerization of CsgA into amyloid fibers (19, 54). Another study, however, found that CsgC does not bind unfolded CsgA and instead acts at the growth poles of nuclei and curli fibers (45). Either way, a conserved patch of positively charged residues on the surface-exposed β-strand (β4-β5 edge) of CsgC appears to mediate an electrostatic interaction between CsgA and CsgC that drives the inhibitory effect (54).

Finally, because functional amyloids like curli are important for biofilm formation and also have been shown to be responsible for host colonization, virulence, and eliciting host immune response (45), chemical inhibitors of curli assembly have been developed (55, 56). A number of small-molecule inhibitors effectively inhibit curli formation at low micromolar to nanomolar concentrations, although these have yet to be evaluated in *in vivo* disease models. In recent years, at least 10 distinct bacterial amyloid systems have been described (9–13). Among these, the *Pseudomonas* Fap pathway is noticeable in encompassing a dedicated secretion-assembly pathway (57). Although nonhomologous to the type VIII secretion pathway, Fap assembly involves a dedicated outer membrane channel (FapF) and a major, polymerizing (FapC) and minor, nucleating (FapB) fiber subunit, reminiscent of curli assembly (58, 59).

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