

**Interspecies Cross-seeding Between Curli Subunits and Curli-dependent Pellicle Biofilm Development by *Escherichia coli***

**By**

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## **Dedication**

This dissertation is dedicated to my parents

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## Chapter 1

### Introduction

#### **Functional amyloids and their roles in biofilm development<sup>1</sup>**

Amyloids are traditionally associated with protein misfolding and neurodegenerative diseases including Alzheimer's disease, Parkinson's disease, and Prion based encephalopathies (1-3). Despite little similarity in primary structure, all amyloids share distinguishing biophysical properties. Amyloid proteins are highly aggregative and can self-assemble into ordered fibers with similar morphological features (4-10 nm widths) (4) and distinct tinctorial properties including the ability to bind dyes such as Thioflavin T and Congo red (5). These fibers are extremely resistant to heat, chemical, and enzymatic denaturation (6). X-ray diffraction indicates amyloid fibers possess cross-beta structures, in which each beta-strand is perpendicular to the fiber axis (4,6,7). The conserved nature of amyloids suggests a common etiology of protein misfolding diseases (5,8).

Recently, a rapidly growing class of "functional amyloids" has been described in all walks of cellular life including bacteria, fungi and mammals (9-13). These functional amyloids exhibit similar structural and biochemical properties to disease-associated amyloids (14-17). However, unlike disease-associated amyloids, functional amyloids are

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<sup>1</sup> Parts of this section are derived from Zhou *et al.*, 2012, Bacterial amyloids. *Methods Mol Biol.* 849:303-20.

assembled by highly regulated biosynthetic pathways to perform physiological tasks (16-23). For instance, the amyloid structure of yeast translational termination factor Sup35 increases stop codon read-through and thus create diverse phenotypes, some of which are beneficial under selective stress (Figure 1.1) (24-26). HET-s filaments generated by *Podospora anserine* facilitate heterokaryon incompatibility (27). In the mammalian organelle melanosome, amyloid fibers composed of Pmel17 accelerate melanin synthesis and sequester cytotoxicity from highly reactive melanin precursors (16). Moreover, many hormone peptides in pituitary secretory granules are found to form amyloid fibers as a storage form (Figure 1.1) (23). Functional amyloids have also been discovered in diverse bacterial species, such as curli produced by enteric bacteria (14), TasA fibers by *Bacillus subtilis* (17), and chaplins by *Streptomyces coelicolor* (28) (Figure 1.1). Most bacterial functional amyloids play important roles in the formation of biofilms (10).

A biofilm is a complex multicellular community of microorganisms held together by an extracellular matrix composed of proteins, exopolysaccharides (EPS), and eDNA (29-34). The biofilm life style of bacteria is commonly found in nature (35,36). Biofilm development inside the host is responsible for a variety of infectious diseases including urinary tract infections (37,38), cystic fibrosis (39), chronic wound infections (40), and catheter-mediated infections (41). Bacteria within biofilms typically exhibit elevated resistance to antimicrobial agents and host immune response (42). In the shipping industry, bacterial biofilm formation leads to biofouling on wetted surfaces, which causes an increase in fuel consumption (43). In agriculture, biofilms can play a positive role in protecting plants from pathogenic infections, and also by providing fixed nitrogen for the plants (44,45).

Amyloids have been found in a wide variety of biofilms under laboratory conditions and in nature (10,46-48). The extraordinarily stable nature and the adhesiveness of bacterial functional amyloid fibers make them ideal materials for the biofilm matrix. This section will detail several well-established bacterial functional amyloids and their roles in biofilm formation.

## **Curli**

Curli were the first described functional amyloid and are produced on the cell surface of many enteric bacteria including *E. coli*, *Salmonella spp.*, *Citrobacter koseri*, and *Enterobacter cloacae* (Figure 1.1A) (14,49-51). Curli mediate bacterial surface attachment (52-54), bacteria-bacteria interactions (55), and host-pathogen interactions (56,57). Curli are also an important component of the biofilm matrix (58-60).

The biogenesis of curli is complex and highly regulated (Figure 1.2) (61). At least seven proteins, encoded by the divergent operons *csgDEFG* and *csgBAC* (*csg*, *curli*-specific genes), are involved in fiber assembly (62). CsgD is a transcriptional regulator required for activation of the *csgBAC* operon (62). The expression of *csgD* is controlled by a complex set of environmental cues, intracellular signals, small RNAs, and regulatory networks (61,63-65). CsgA and CsgB are the major and minor subunits of curli fibers, respectively (19,66). Translocation of CsgA and CsgB to the bacterial surface is mediated by the outer-membrane lipoprotein CsgG (67,68) and the periplasmic chaperone-like proteins CsgE and CsgF (69,70). CsgC is involved in the pore activity of CsgG (71) and may have chaperone activity that discourages CsgA aggregation (Evans, unpublished). Before locating to the cell surface, CsgA and CsgB are highly regulated by molecular chaperones to avoid aggregation in the cytoplasm or periplasm (72). Once secreted to the

bacteria surface, CsgB is proposed to quickly adopt an amyloid fold and is associated with the outer-membrane via the C-terminal domain (73,74). CsgA is templated by CsgB and self-polymerizes into fibers. Curli biogenesis occurs in an extracellular environment with no obvious energy source and dynamic physical conditions (75), yet the assembly process is highly efficient. The resulting fibers are remarkably stable and resistant to most environmental stresses. This combination of properties makes curli-amyloid fibers an ideal constituent of the microbial extracellular matrix.

Under laboratory conditions, *E. coli* and *Salmonella enteric typhimurium* LT2 form pellicle biofilms floating at the air-liquid interface (18,54,59). Curli are indispensable for pellicle biofilm formation of many *E. coli* strains in static liquid culture, as curli defective mutants were unable to develop pellicles (Figure 1.3A) (18,54,59). Pyridone based chemical compounds, curlicides, which inhibit CsgA polymerization and curli biogenesis, also actively prevent pellicle formation of uropathogenic *E. coli* (18). The function of curli in surface-attached biofilm is still elusive. A study on biofilm formation of a curli-overproducing *E. coli* K-12 strain found that curli mediated both the initial attachment and development of multilayered cell clusters on polystyrene surface or Thermanox plastic coverslips (55), while others showed that curli production was not necessarily required for the initial attachment of bacterial to abiotic surfaces (76). Curli are also critical for the development of rugose colony biofilms on solid agar surfaces (Figure 1.3B) (61,77). In an *E. coli* rugose biofilm, bacteria differentiate to at least two sub-populations- curli producing bacteria in the matrix, and the non-matrix producing cells (67,78). Matrix producing cells are thought to protect the whole bacterial community against oxidative stress (78). In addition, curli can be used as common

resource for multispecies biofilm development. *E. coli* and *S. typhimurium* in mixed colony biofilms are able to share curli subunits CsgA and CsgB to assemble heterogeneous curli fibers. These interspecies curli fibers promote surface attachment of both *E. coli* and *S. typhimurium* (54).

In the host, curli provide uropathogenic *E. coli* with a fitness advantage during colonization of the urinary tract in a mouse model (18). Curli also interact with host extracellular matrix fibers such as fibronectin and laminin (19,53), indicating curli may be able to mediate bacteria colonization in the host. Moreover, curli and cellulose are shown to play a synergistic role in bacterial adherence to human colonic HT-29 epithelial cells and to cow colon tissue *in vitro* (79).

### **TasA fibers**

The soil bacterium *B. subtilis* forms biofilms on plant roots, which protect plant from pathogen infections. Under lab conditions, *B. subtilis* forms extremely wrinkly pellicles in static liquid broth and rugose colony biofilms on agar surfaces (80). The primary proteinaceous components of the *B. subtilis* biofilm matrix are TasA fibers (17). TasA fibers were recently reported to possess amyloid-like properties. Purified TasA is able to self-assemble into fibers which are rich in  $\beta$ -sheet structure and bind Congo red (17). These fibers are anchored to the bacterial cell surface by the TasA anchor protein TapA (81).

TasA fibers function together with EPS to support pellicle and rugose colony biofilm development (17). A *tasA*- mutant only formed a flat, fragile pellicle and a rugose colony with a reduced wrinkled morphotype. A *tasA-eps*- mutant completely lost the ability to develop a pellicle or rugose biofilm (17). Therefore, TasA fibers and EPS



provide targets for biofilm inhibition and disassembly. *B. subtilis* releases antibiofilm molecules D-amino acids and norspermidine as the bacterial culture ages (82,83). D-amino acids attack the anchor protein TapA and trigger TasA fiber release from the cell wall (83), while norspermidine, directly interacts with and disrupts the EPS structure (82).

### **Chaplins**

The synergistic role of functional amyloids and polysaccharides in biofilm formation is also demonstrated in *S. coelicolor* (21). *S. coelicolor* is a filamentous bacterium that develops aerial hyphae growing into the air and subsequently form spores. During this process, chaplins are secreted into cell walls of aerial hyphae and assemble into functional amyloid fibers. The amyloidogenic chaplin fibers increase the surface hydrophobicity of aerial hyphae, lower surface tension at the air-water interface, and enable hyphae to raise into the air (28,84). The chaplin family consists of 8 chaplins, ChpA to ChpH. These chaplin proteins are functionally redundant in terms of hyphae formation and rodlet assembly (85). ChpH is identified as the primary determinant for amyloid assembly, which contains two amyloidogenic domains in the N- and C-terminals and can self-assemble into amyloid fibers *in vitro* (86). ChpD, ChpE, ChpF, and ChpG are also able to form amyloid fibers *in vitro* (87).

De Jong *et al.* found that chaplin fibers are anchored to the cell wall by cellulose. Mutation of *chpA-H* or the cellulose synthase gene *csIA* reduced *S. coelicolor* adherent to hydrophobic surfaces. Surface attachment was also interfered with by cellulase and the amyloid inhibitor Congo red (21). Together, chaplins and cellulose synergistically mediate surface attachment of *S. coelicolor*, suggesting these structures are also likely to have roles in *S. coelicolor* biofilm formation.

### **Fibers composed of phenol soluble modulins**

*Staphylococcus aureus* is a human opportunistic pathogen that commonly colonizes the skin and the nose. *S. aureus* biofilms are linked to many chronic infectious diseases (88). Schwartz *et al.* discovered amyloid structures composed of phenol soluble modulins (PSMs) in *S. aureus* biofilms (Figure 1.1B). Unpolymerized PSMs are conventionally thought to play a role in biofilm disassembly (89). The amyloid form of PSMs loses the antibiofilm activity, and instead, promotes biofilm integrity and mediates biofilm resistance to matrix degrading enzymes and mechanical stress (90).

### **Bacterial amyloids in natural environment**

Amyloid-like structures have been widely discovered in natural environments. Studies on biofilms collected from natural habitats found amyloid fibers, indicated by the amyloid-specific dye ThT and conformationally specific antibodies (46,47). Amyloid-producing bacteria were identified within *Proteobacteria*, *Bacteroidetes*, *Chloroflexi* and *Actinobacteria*. These bacteria constituted 5-40% of all prokaryotic populations in biofilms (47). A similar study showed that amyloid-like fibrous structures were abundant in activated sludge collected from different wastewater treatment plants (48). Moreover, an environmental *Pseudomonas* strain UK4 isolated from biofilms developed in non-chlorinated drinking water reservoirs was found to express amyloid fibers composed of FapC. Expression of the *fap* operon led to bacterial aggregation and biofilm formation, suggesting the FapC amyloid fibers may contribute *Pseudomonas* biofilm formation in nature (91).

## **Nucleation dependent polymerization and cross-seeding**

Amyloid polymerization follows a nucleation dependent mechanism, in which amyloid proteins self-polymerize into amyloid fibers with a lag phase, an exponential phase, and a stationary phase (Figure 1.4A) (92-94). In the lag phase, monomers assemble into on-pathway oligomeric nuclei (92-97). Oligomer nucleation is often the rate-limiting step in amyloid formation and is associated with cytotoxicity and prion infections (97,98). Once oligomeric intermediates are formed, they catalyze the fiber elongation process. Preformed amyloid fibers can also promote amyloid polymerization (99-101). The process where nucleators or preformed fibers accelerate the polymerization of amyloid proteins is known as seeding (Figure 1.4B).

### **Stringent seeding specificity of eukaryotic amyloids**

Most amyloid proteins can be cross-seeded by fibers formed from the same protein. The potential of cross-seeding between different amyloids offers a possible pathomechanism for the co-occurrence of diverse amyloid diseases and prion infections (102-106). Cross-species seeding between human prion PrP and mouse PrP is considered as an important mediator of prion transmission and infections (107-109). Cross-seeding between non-transmissible amyloids, Alzheimer's-associated peptide A $\beta$  and islet amyloid polypeptide (IAPP), has also illuminated concerns that one amyloid species influences the folding and amyloidogenesis of heterologous proteins (110).

Despite limited examples of cross-seeding as listed above, seeding is typically highly specific. Cross-seeding among different eukaryotic amyloids occurs with low efficiency or not at all because of species barriers. For example, cross-seeding between A $\beta$ <sub>1-40</sub> and IAPP, polyglutamine or  $\beta$ 2M fibers is more than 90% reduced compared to

self-seeding (110). Fibers formed by A $\beta$  mutants with even a single amino acid mutation abolish the ability to seed wild-type A $\beta$  (110,111). Similarly, a lack of reciprocal seeding ability was observed among immunoglobulin domains and lysozymes from different species (112,113). Cross-seeding is even limited between  $\alpha$ -synuclein and its amyloidogenic homologs (114). Also, strict species barriers commonly exist in mammalian prion amyloids, limiting the cross-species transmission between human PrP and Syrian hamster PrP, and between mouse PrP and Syrian hamster PrP (109). Mutation of a single key amino acid in PrP sequences alters the seeding specificity (115). Moreover, conserved yeast prion domains and closely related Sup35 homologs from *Saccharomyces sensu stricto* group cannot cross-seed (116,117). Remarkably, fibers formed by the same Sup35 protein under different environmental conditions result in two prion species with distinct seeding specificities (118). These studies suggest a highly specific nature of seeding for eukaryotic amyloids.

### **Mechanism of amyloid cross-seeding**

Based on the research on mammalian and fungal prions, several mechanisms have been proposed to explain the stringent seeding specificity. Many studies suggest sequence similarities at the amino acid level is not the major determinant for cross-seeding, as some prion homologs with 90% identity or higher in primary structure cannot cross-seed (109,110,118,119), whereas in other cases fungal prions with 30-40% sequence similarity are able to overcome species barriers (108,120). Instead, compatibility in protein or fiber conformations seems to govern seeding specificity (115,118,121,122). As pointed out above, key amino acid residues at critical regions can influence seeding specificity (109,110). Whether sequence or conformational specificity drives cross-seeding

efficiencies are not mutually exclusive ideas, as small changes in amino acid sequences can dictate conformational variability. One good example is the cross-species propagation of mammalian prion elements (109,115). Vanik *et al.* showed that human PrP can cross-seed with mouse PrP, whereas neither of them can cross-seed with Syrian hamster PrP.(109). The subsequent structural analysis demonstrates the species barrier is mediated by the distinct secondary structure and morphology of prion fibers, as human PrP fibers and mouse PrP fibers adopt highly similar conformation, while hamster prion fibers possess a completely different structure. The structural differences are controlled by two key Ile residues in human PrP, which are mutated into two Met residues in hamster PrP (115). Finally, a study based on surface-bound arrays of yeast prion peptides demonstrates that the specific seeding properties are mediated by recognition elements in the prion domain (123).

Although cross-seeding has been extensively studied between eukaryotic amyloids, seeding specificity of bacterial functional amyloids has not. The interaction between curli subunits CsgA and CsgB represents an example of relaxed seeding specificity (74,124). Both CsgA and CsgB have a C-terminal amyloidogenic domains composed of five imperfect repeating units, with each repeating unit predicted to have a  $\beta$ -strand-loop- $\beta$ -strand motif (74,125). Although CsgA and CsgB are only 30% identical, fibers of CsgB can efficiently promote the polymerization of CsgA (74,124,126). In Chapter 2, I demonstrate that curli homologs from different enteric bacteria are able to cross-seed. This work provides the first direct evidence showing seeding specificity of curli is less stringent (54).

## **Bacterial-derived antibiofilm molecules**

Biofilm development is a dynamic process. A typical life cycle of biofilm development in liquid culture setting includes at least five stages: 1) the initial reversible attachment of planktonic bacteria, 2) the irreversible attachment mediated by adhesive structures, 3) formation of microcolonies, 4) development of microcolonies into a mature biofilm, and 5) biofilm dispersion in which sessile bacteria are released and switch back to the planktonic state (35,127). Changes in environmental cues, such as carbon starvation, oxygen depletion, and waste product accumulation, often prevent biofilm formation or trigger biofilm disassembly (127,128). In addition, many bacteria are able to produce extracellular signaling molecules or effectors to regulate their own biofilm development (127,128).

Quorum sensing molecules are extracellular signals used by bacteria to coordinate gene expression in response to the local cell density (129). Many quorum sensing systems play a positive role in promoting biofilm formation (130,131). However, some of the quorum sensing molecules can prevent biofilm formation and induce dispersion. For example, acyl-homoserine lactones (HSL) and the non-HSL molecule CIA-1 produced by gram-negative bacteria *Vibrio cholerae* and *Vibrio haveyi* activate a quorum sensing pathway that represses EPS biosynthesis and biofilm formation (132,133). In *S. aureus*, a cyclic autoinducing peptide is released into the environment and activates the accessory gene regulatory (*agr*) system. *agr* system promotes the production of extracellular proteases and PSMs that degrade matrix components and lead to biofilm dispersion (134).

Non-quorum sensing signaling molecules are also widely involved in biofilm regulation (82,83,135,136). In nutrient rich environment, *E. coli* produces and secretes

large amounts of indole (136). This feature makes indole a diagnostic marker for *E. coli* identification. Indole and indole derivatives are capable of reducing biofilm formation of *E. coli* K-12 strains (135,137,138). The biofilm-inhibiting effect of indole is thought to be mediated by SdiA, which influences bacterial motility (135). In *B. subtilis*, D-amino acids and norspermidine have been identified as antibiofilm factors (82,83). In an aged biofilm culture, *B. subtilis* releases D-tyrosine, D-leucine, D-methionine and D-tryptophan into the medium. D-amino acids are incorporated into the peptidoglycan and trigger detachment of TasA fibers from the cell wall via the TasA anchor protein, TapA (81). The aged *B. subtilis* culture also produces norspermidine, which binds specifically to and disrupts EPS (82). Thus, D-amino acids and norspermidine function together to facilitate pellicle disassembly (82). Although the mechanism is not clear yet, D-amino acids and norspermidine also exhibit biofilm-inhibiting activity against a range of bacteria including *S. aureus*, *Pseudomonas aeruginosa* and *E. coli* K-12 (82,83,139).

Recently, bacterial polysaccharides have been identified as a novel group of antibiofilm agents (140). Extracellular polysaccharides are traditionally recognized as a major biofilm matrix component that support and stabilize biofilms. However, some of the bacterial-derived polysaccharides have antibiofilm or antimicrobial activity. Group II capsular polysaccharide was the first described antibiofilm EPS produced by extraintestinal *E. coli*. Soluble capsular polysaccharides were found in the culture supernatant of uropathogenic *E. coli* and can effectively abolish biofilm formation of a variety of bacteria including commensal *E. coli*, *P. aeruginosa*, *S. aureus* and *Enterococcus faecalis* (141). Subsequently, *P. aeruginosa*-derived polysaccharide Pel, Psl and alginate, and several novel polysaccharides isolated from cultures of marine

bacteria and lactic bacteria were shown to exhibit antibiofilm activity (142-145). Unlike signaling molecules, which regulate biofilm formation through a specific target, many polysaccharides act by altering physical properties of abiotic surfaces or bacterial cell surfaces (141,144,146). When applied on a glass surface, both capsular polysaccharide and Ec300 anti-adhesion polysaccharide lowered the interfacial energy and increased hydrophilicity of the surface, which interfered with the initial attachment of bacteria (141,146). Polysaccharides can also modify biotic surfaces by reducing bacterial surface hydrophobicity or impairing bacteria-bacteria interactions (141,146). The non-specific action mode makes antibiofilm polysaccharides broad spectrum inhibitors against biofilm formation of diverse bacteria and even fungi.

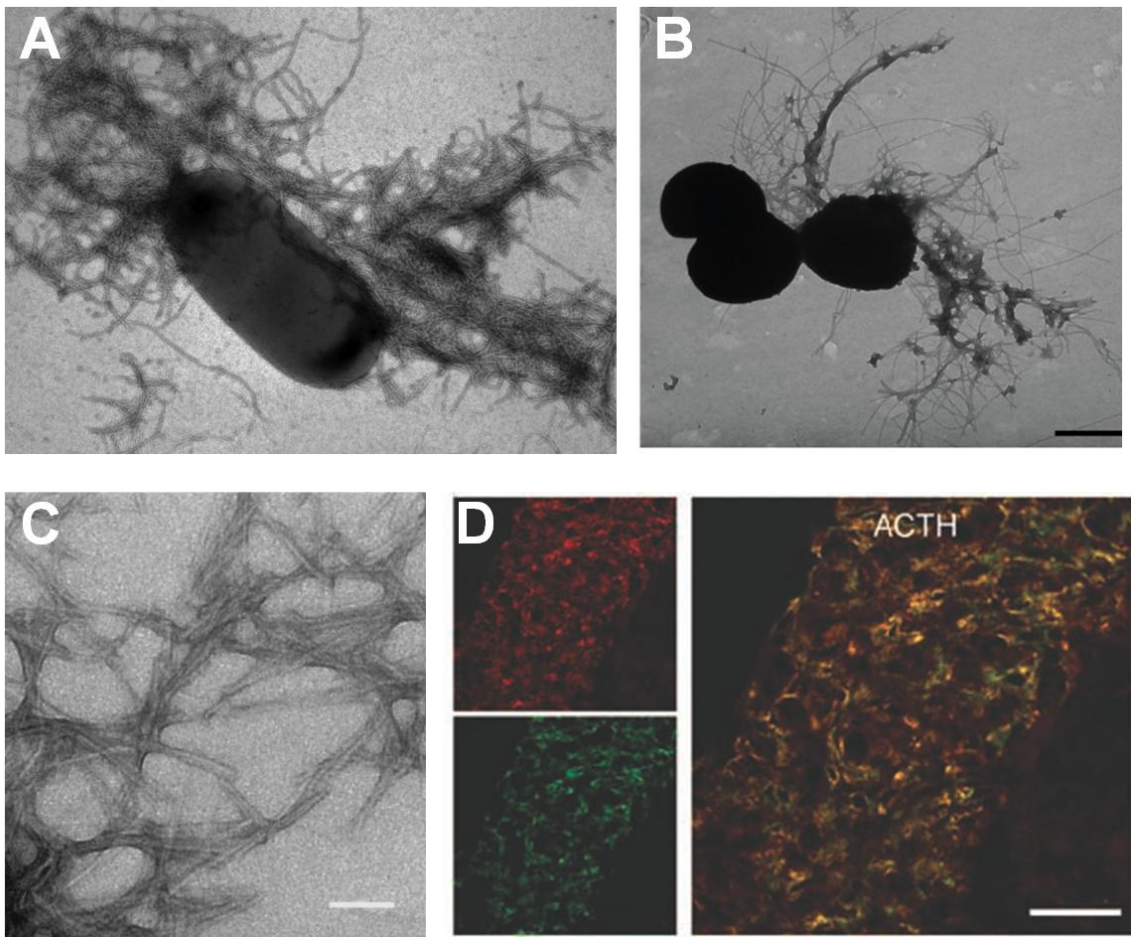
In Chapter 3, I showed that conditioned cultures of uropathogenic *E. coli* contained antibiofilm activity against its own pellicle biofilm formation. The production of the potential antibiofilm factor is mediated by cysteine metabolism.



## Figures

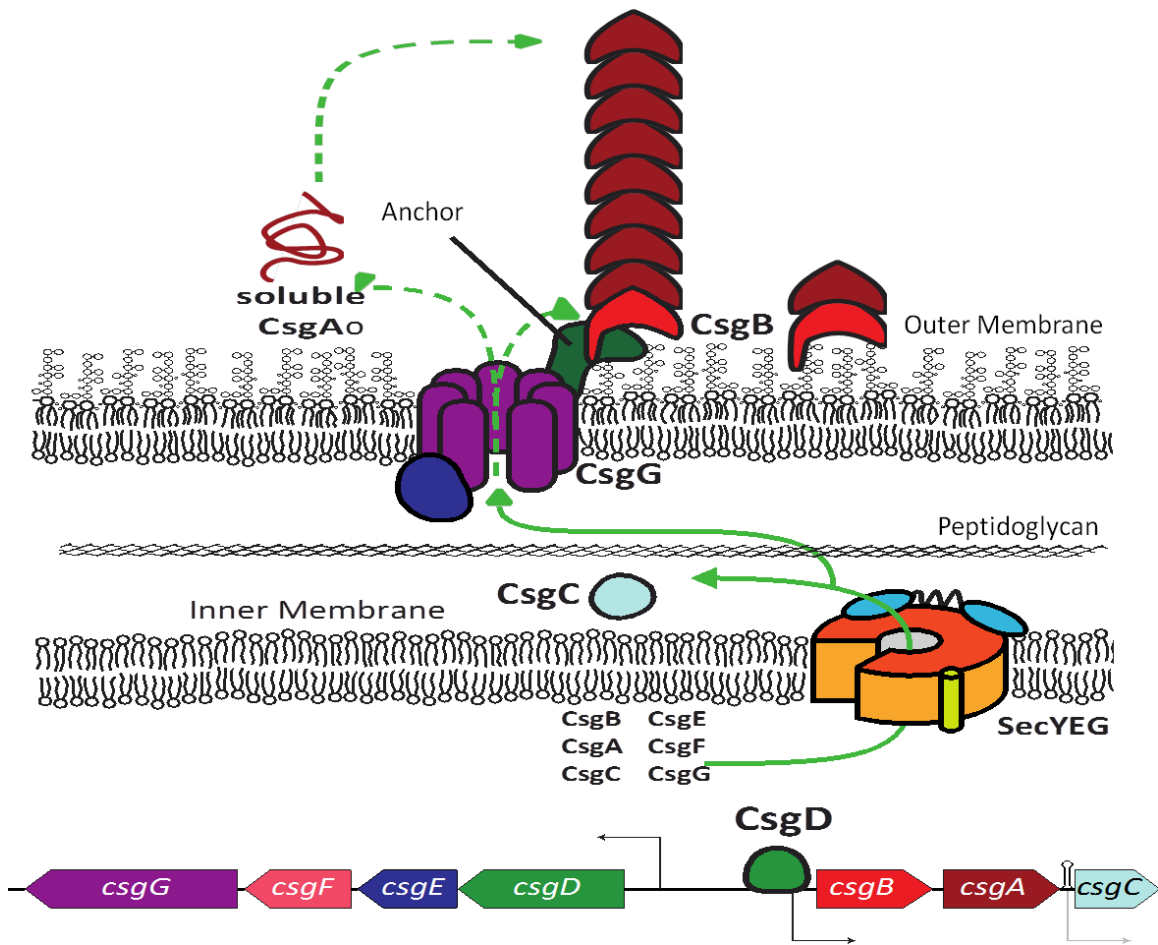
**Figure 1.1. Functional amyloids have been found in all walks of cellular life including bacteria, fungi, and mammalian cells.**

(A) Transmission electron microscopy (TEM) of a curled *E. coli* MC4100 strain. (B) TEM micrograph of *S. aureus* SH1000 producing PSM fibers (Schwartz *et al.*, 2012) (C) TEM of negatively stained fibers formed by the prion domain of yeast prion protein Sup35 (King *et al.*, 1997). (D) Immunohistochemical staining of the mouse pituitary with Thioflavin S and adrenocorticotrophic hormone (ACTH) specific antibody indicates ACTH adopts an amyloid fold (Maji *et al.*, 2009).



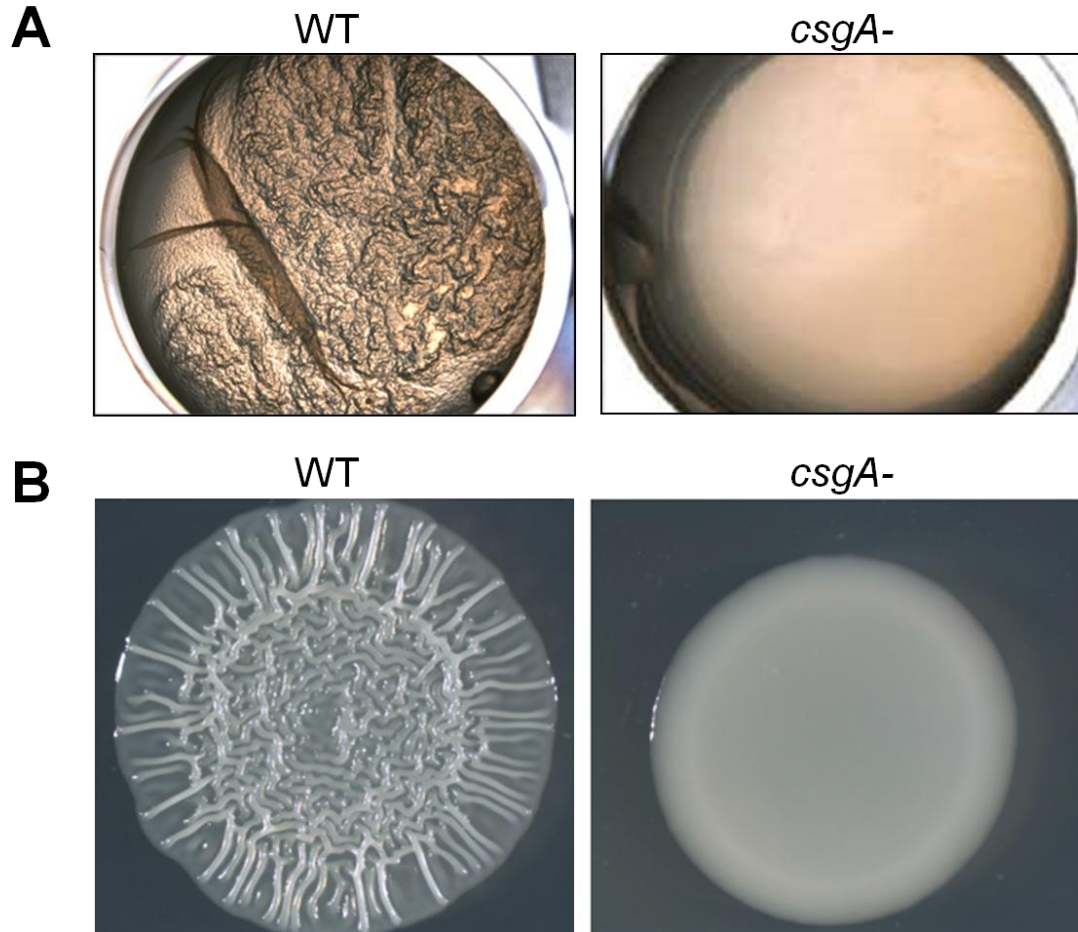
### Figure 1.2. Model of curli biogenesis.

The biogenesis of curli is complex and highly regulated. At least seven proteins, encoded by divergent operons *csgDEFG* and *csgBAC* (*csg*, curli-specific genes), are involved in fiber assembly. CsgD is a transcriptional regulator required for activation of the *csgBAC* operon and the regulation of CsgD is controlled by a complex set of environmental cues. CsgA and CsgB are the major and minor subunits of curli fibers, respectively. CsgB is associated with the outer-membrane via the C-terminal domain. The translocation of CsgA and CsgB to the bacterial surface is mediated by the outer-membrane lipoprotein CsgG and the periplasmic chaperone-like proteins CsgE and CsgF. CsgC is involved in the pore activity of CsgG. The model is adapted from Blanco *et al.*, 2012



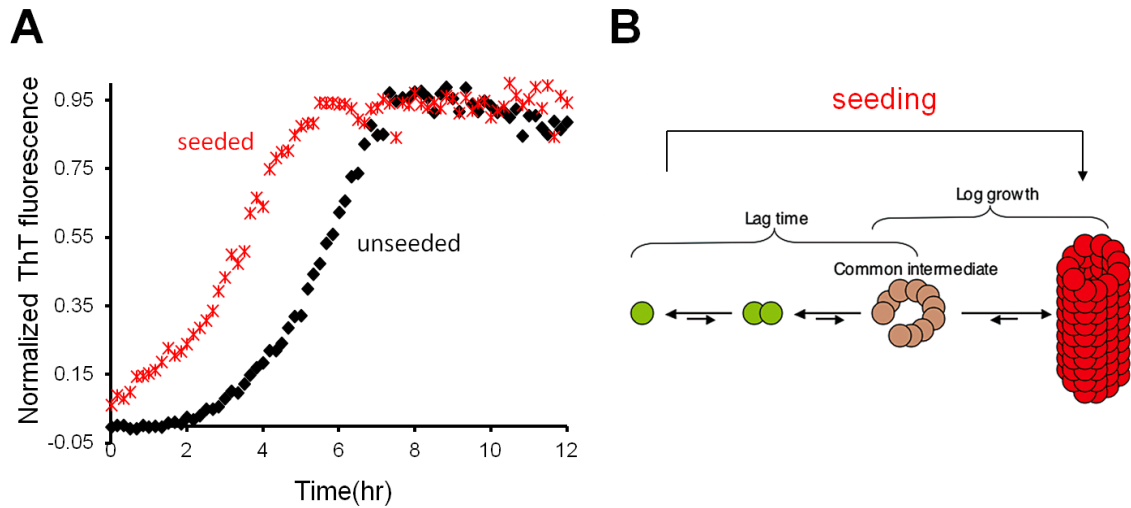
**Figure 1.3. Curli mediate the formation of rugose colony biofilms and pellicle biofilms under lab conditions.**

(A) In a static liquid YESCA medium, uropathogenic *E. coli* UTI89 formed a pellicle-like biofilm floating at the air-liquid interface. An UTI89 *csgA*- mutant was defective in pellicle formation. (B) On solid agar surface, UTI89 developed a rugose colony biofilm. UTI89 *csgA*- mutant formed a flat and smooth colony with no wrinkly morphotype.



**Figure 1.4. The nucleation dependent polymerization and seeding.**

(A) Amyloid formation of the curli major subunit CsgA followed a characteristic kinetic with a lag phase, an exponential phase and a stationary phase (black). Preformed CsgA fibers can act as templates that accelerate the polymerization of monomeric CsgA and eliminate the lag phase (red). Amyloid polymerization was monitored by Thioflavin T fluorescence. (B) A schematic of the nucleation dependent polymerization adapted from Epstein *et al.*, 2008. Monomers assemble into oligomeric nuclei. The formation of oligomeric intermediates is proposed to be the rate-limiting step. The oligomeric nucleus acts as a template that catalyzes the fiber formation in a process known as seeding. Preformed fibers can also seed the polymerization of monomers.



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## Chapter 2

### Cross-seeding of curli promotes interspecies biofilm formation<sup>2</sup>

#### Abstract

Amyloids are highly aggregated proteinaceous fibers historically associated with neurodegenerative conditions including Alzheimer's, Parkinson's and prion-based encephalopathies. Polymerization of amyloidogenic proteins into ordered fibers can be accelerated by preformed amyloid aggregates derived from the same protein in a process called seeding. Seeding of disease-associated amyloids and prions is highly specific and cross-seeding is usually limited or prevented. Here we describe the first study on the cross-seeding potential of bacterial functional amyloids. Curli are produced on the surface of many gram-negative bacteria where they facilitate surface attachment and biofilm development. Curli fibers are composed of the major subunit CsgA and the nucleator CsgB, which templates CsgA into fibers. Our results showed that curli subunit homologs from *Escherichia coli*, *Salmonella typhimurium* LT2 and *Citrobacter koseri* were able to cross-seed *in vitro*. The polymerization of *E. coli* CsgA was also accelerated by fibers derived from a distant homolog in *Shewanella oneidensis* that shares less than 30% identity in primary sequence. Cross-seeding of curli proteins was also observed in mixed colony biofilms with *E. coli* and *S. typhimurium*. CsgA secreted from *E. coli* *csgB*-

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<sup>2</sup> A version of this chapter has been published as Zhou *et al.*, 2012, *J Bio Chem*, (42):35092-10

mutants assembled into fibers on adjacent *S. typhimurium* that presented CsgB on its surfaces. Similarly, CsgA secreted by *S. typhimurium csgB*- mutants formed curli on CsgB-presenting *E. coli*. This interspecies curli assembly enhanced bacterial attachment to agar surfaces and supported pellicle biofilm formation. Collectively, this work suggests that the seeding specificity among curli homologs is relaxed and that heterogeneous curli fibers can facilitate multispecies biofilm development.

## **Introduction**

Amyloids are  $\beta$ -sheet rich proteinaceous fibrils traditionally associated with protein misfolding and cytotoxicity (1-3). Amyloid formation is the hallmark of many neurodegenerative diseases such as Alzheimer's, Parkinson's and prion based diseases. Recently a rapidly growing class of "functional amyloids" suggests that the amyloid fold can be utilized to facilitate non-degenerative physiological tasks (4-6). A number of functional amyloids have been described, such as curli produced by *Escherichia coli* (7), TasA by *Bacillus subtilis* (8) and Pmel17 by mammalian cells (9). Despite having little similarity in primary structure, amyloids share biochemical and structural propensities. Amyloid fibers are characterized by cross  $\beta$ -sheet structures, with each  $\beta$ -strand perpendicular to the fiber axis (1,10-12). These fibers are extraordinarily stable, resistant to most denaturation treatments and protease K digestion (7,13), and possess the distinct tinctorial ability of binding the dyes Congo red (CR) and Thioflavin T (ThT) (7,14). Another common feature of amyloids is the nucleation-dependent kinetics of assembly, in which amyloid proteins polymerize into fibers after a lag phase that is followed by an exponential growth (15-17). Formation of an oligomeric nucleus or seeds is rate limiting

and is associated with amyloid toxicity (18,19). The self-polymerization of amyloid proteins can be accelerated by the presence of preformed fibers or nucleators in a process called seeding (20,21).

Most amyloidogenic proteins can be seeded by fibers derived from the same protein. In rare cases, one amyloidogenic protein can be cross-seeded by different amyloid fibers. Cross-seeding is considered a possible mechanism for diverse pathologies of amyloid diseases and prion infections (22-25). Cross-seeding was observed between the Alzheimer's-associated peptide A $\beta$  and islet amyloid polypeptide (IAPP) (25), as well as between A $\beta$  and human prion element PrP (24). Additionally, A $\beta_{1-42}$  fibers have been reported to induce the formation of tau-containing filaments *in vivo* (26), and *in vitro* preformed A $\beta_{1-42}$  oligomers can induce the conversion of tau monomers to  $\beta$ -sheet rich, toxic oligomers (27). Also, functional amyloids curli and Sup35 can promote Amyloid protein A amyloidosis, suggesting that interactions between heterogeneous amyloid proteins may be a risk factor for accelerating the onset of amyloid diseases (23). Cross-seeding is also observed between some of the mammalian and yeast prion species, providing a mechanism for prion transmission and prion-based disease infection (28,29).

While limited cross-seeding among diverse amyloids has been reported, these interactions typically occur with reduced efficiency or are often completely prevented by species barriers. Seeding and cross-species transmission of most mammalian prion and yeast prion proteins are highly specific (29-31). Strict species barriers are present among conserved yeast prion domains including closely related Sup35 homologs from the *Saccharomyces sensu stricto* group (32,33). A single amino acid mutation can alter the seeding specificity of Sup35 (34). Even the same Sup35 protein polymerizing at different

temperatures forms fibers with distinct seeding specificity (35). Cross-seeding is also inefficient among mammalian prions (29), closely related synuclein homologs (36), different immunoglobulin domains (37) and lysozymes from different species (38).

Functional amyloids have been widely described in bacteria including *E. coli*, *Salmonella spp.*, *B. subtilis*, *Streptomyces coelicolor* and *Pseudomonas fluorescens* (7,8,39-41). Although cross-seeding among amyloid proteins has been extensively studied in disease-associated amyloids and prions, the seeding specificity of bacterial amyloids has not been investigated. In order to assess cross-seeding among functional bacterial amyloids, as well as the resultant biological consequences, we utilized the well-studied bacterial functional amyloid called curli. Curli are amyloid fibers produced on the cell surface of *E. coli* and other enteric bacteria that facilitate adherence to biotic and abiotic surfaces (42,43), biofilm development (39,43-45) and pathogen-host interactions (46-48). Unlike disease-associated amyloids, curli assembly is highly regulated by dedicated pathways (49,50). At least seven proteins, encoded by the *csgBAC* and *csgDEFG* operons (curli specific gene), are involved in curli biogenesis (4,49). The major subunit of curli is CsgA. *In vivo*, CsgA's polymerization and membrane-localization is dependent on the nucleator protein CsgB (51). The secretion of both CsgA and CsgB requires the outer-membrane pore-forming protein CsgG (52) and chaperone proteins CsgE and CsgF (53,54). *In vitro* CsgA and CsgB self-assemble into amyloid fibers (51,55) with  $\beta$ -helix structures (12). The *in vitro* fibrillization of CsgA can be seeded by its own fibers or by fibers of the nucleator protein CsgB (51,55).

We report here that CsgA from *E. coli*, *Salmonella typhimurium* LT2, *Citrobacter koseri* and even a distant CsgA homolog from *Shewanella oneidensis* MR-1 are able to

cross-seed *in vitro*. *In vivo*, both *S. typhimurium* and *E. coli* share curli subunits as building blocks to assemble functional fibers in colony biofilms and such interspecies interactions of curli subunits aid in bacterial adherence to abiotic surfaces and restore biofilm formation. Our results suggest that seeding between curli homologs is relaxed and that cross-seeding between different bacteria has an impact on multispecies communities.

## **Experimental Procedures**

### **Bacterial growth**

Bacteria were grown in LB at 37°C with overnight shaking. To induce curli expression, bacteria were grown on YESCA agar (1 g/L yeast extract, 10 g/L Casamino acids and 20 g/L agar) or YESCA-CR (50 µg/ml Congo red and 1 µg/ml Coomassie blue) at 26°C for 48 hr. To induce pellicle biofilm formation, bacteria were inoculated in 4 ml static LB-no salt broth (10 g/L tryptone and 5 g/L yeast extract) at 26°C for 3 d. Antibiotics were added at the following concentration: kanamycin 50 µg/ml, ampicillin 100 µg/ml.

### **Strains and plasmid**

Strains and plasmids used in this study are listed in Table 2.1 and Table 2.2. Primer sequences are listed in Table 2.3. *S. typhimurium* curli mutants were constructed according to the methods described by Datsenko and Wanner *et al.* (56). All the *S. typhimurium* curli mutants can be complemented by expressing *S. typhimurium* CsgA or CsgB under the control of *S. typhimurium* *csgBA* promoter from the plasmid pACYC177.

### **Protein purification**

Purification of CsgA/CsgB homologs was adapted from Cegelski *et al.* and Wang *et al.* (57,58). Briefly, expression of C-terminal His<sub>6</sub>-tagged CsgA or CsgB homologs without the Sec signal sequence in NEB3016 was induced at OD<sub>600</sub> 0.9 by 0.5 mM IPTG at 37°C for 1 hr. Bacteria were lysed in 8 M guanidine hydrochloride in 50 mM potassium phosphate buffer (KPi) overnight. After centrifugation at 10,000 xg for 20 min the supernatant was incubated with NiNTA resin (Sigma) at room temperature for 1 hr and was then loaded onto a disposable polypropylene column (Thermo). Proteins were eluted into 50 mM KPi containing 125 mM imidazole. To get monomeric CsgA, fractions with the target protein were combined and loaded onto a 30 kD centrifugal filter units (Thermo) to remove dimers and other oligomers.

### **A $\beta$ <sub>1-42</sub> disaggregation**

100% trifluoroacetic acid (TFA) was added to A $\beta$ <sub>1-42</sub> peptides to a 1 mg/ml ratio and bath sonicated at room temperature for 10 min. TFA was removed by a SpeedVac at room temperature for 1 hr. Residual TFA was removed by dissolving the pellet in 500  $\mu$ l 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP) and SpeedVac drying for 1 hr; the process was repeated 3 times. The peptides were dissolved in 2 mM cold potassium hydroxide to 62.5  $\mu$ M and cold 5X KPi buffer was immediately added. Samples were centrifuged at 70,000 xg at 4°C for 3 hr and the top solution was taken carefully for the polymerization assay.

### ***In Vitro* polymerization and seeding Assay**

100  $\mu$ l of freshly purified CsgA homologs were loaded on a 96-well opaque plate and polymerization kinetics were monitored by ThT fluorescence with excitation wavelength at 438 nm and emission at 495 nm (bandwidth 20 nm) on a Tecan plate



reader. ThT fluorescence was normalized as described (55). Seeds were prepared by sonication of preformed fibers with three 15 s bursts on ice. ThT was added to a final concentration of 20  $\mu\text{M}$ .

### **Stability assay**

22  $\mu\text{g}$  fibers were spun down and resuspended in 50 mM KPi or 50% (v/v), 70%, 80%, 90% or 100% HFIP for 5 min and were immediately dried on a SpeedVac for 3 hrs. The resulting pellets were boiled in 2x SDS sample buffer for 5 min before being loaded on 15% SDS-PAGE gels.

### **Biacore binding assay**

A BIAcore3000 (GE healthcare) was used to monitor real-time interactions between monomers and fibers. A CM5 sensor chip was activated with 35  $\mu\text{l}$  1:1(v/v) mixture of 0.4 M 1-ethyl-3-(3-dimethylpropyl)-carbodiimide (EDC) and 0.1 M N-hydroxysuccinimide (NHS) at a flow rate of 5  $\mu\text{l}/\text{min}$ . Mature CsgA or CsgB fibers were sonicated (Sonicator XL2020, Misonix) with three 15 s bursts at power 2 and 50 s pauses in between. These seeds were then diluted into acetate buffer to final concentrations of 3.5  $\mu\text{M}$  for CsgB fibers and 2.6  $\mu\text{M}$  for CsgA fibers. 55  $\mu\text{l}$  of sonicated fibers were injected at a flow rate of 5  $\mu\text{l}/\text{min}$  to allow the immobilization of 2500-3500 resonance units. Blank flow cells on the same chip were used as negative controls. After ligand immobilization, excessive reactive groups were deactivated with 35  $\mu\text{l}$  of 1 M ethanolamine-HCl, pH 8.5 and the sensor chip was primed with 50  $\mu\text{M}$  KPi, pH 7.4. 40  $\mu\text{l}$  of 0.25  $\mu\text{M}$  monomeric *E. coli* CsgA or *S. typhimurium* CsgA was injected over the sensor chip surface at a flow rate of 50  $\mu\text{l}/\text{min}$  and the response was recorded in resonance units. Elution of a mock purification from NEB3013 harboring an empty

vector pET11d and monomeric A $\beta$ <sub>1-42</sub> with concentration higher than 1  $\mu$ M were used as negative controls.

### **Western blot analysis**

Western blot analysis of the whole cell lysates and bacteria with the underlying agar (plug) were performed as described previously (45,58). For whole cell lysate, bacteria grown on YESCA agar at 26°C for 48 hr were suspended in 50 mM KPi, pH 7.2, normalized by optical density at 600nm (OD<sub>600</sub>) and pelleted down. The pellets were pre-treated with or without 70  $\mu$ l HFIP immediately followed by SpeedVac centrifugation for 30 min at 45°C. For the plug, overnight cultures of bacteria were normalized by OD<sub>600</sub> and 4  $\mu$ l of culture were spotted on thin YESCA agar, incubated at 26°C for 48 hr. 8-mm circular plugs including bacteria colonies and the underlying agar were collected, treated with or without 150  $\mu$ l HFIP and dried immediately by a SpeedVac. Proteins in whole cell lysates or plugs were separated by electrophoresis and transferred onto a polyvinylidene difluoride membrane. CsgA was probed by antiserum raised against purified *E. coli* curli fibers (Proteintech, Chicago, IL).

### **Transmission electron microscopy**

a Philips CM100 transmission electron microscope was used to visualize bacteria samples that were prepared as previously described (58).

### **Mixed colony biofilms**

Interspecies curli assembly assay in mixed colony biofilms was adapted from Chapman *et al.* and White *et al.* (7,59). Briefly, overnight cultures of *E. coli* and *S. typhimurium* curli mutants were normalized by OD<sub>600</sub> and mixed at 1:1 (v/v) ratio. 4  $\mu$ l of

each sample were spotted on YESCA agar and incubated at 26°C for 3 d. Curli formation was analyzed by western blot and transmission electron microscopy.

### **Measurement of bacterial adhesiveness**

Overnight cultures of *E. coli* and *S. typhimurium* were normalized by OD<sub>600</sub> and were mixed at 1:1 (v/v) ratio. Bacterial cultures were spread on 3 ml YESCA agar on 12-well plates and incubated at 26°C for 3 d. To determine the bacterial adhesiveness, 1 ml of phosphate buffered saline (PBS) was added into each well, and plates were rocked vigorously on an orbital titer plate shaker at speed 5 for 30 min at room temperature. Non-adherent bacteria in PBS were removed and the cell densities were determined by OD<sub>600</sub>. Adherent bacteria were suspended in another 1 ml PBS buffer with an inoculation loop and the OD<sub>600</sub> was measured. The percentage of adherent bacteria was calculated. To determine the adhesiveness of *S. typhimurium*, two approaches were used. *S. typhimurium* wild-type or mutants were transformed with mCherry-expressing plasmid pAH9 and *E. coli* cells were transformed with YFP-expressing plasmid pAH16. Both mCherry and YFP were driven by *Staphylococcus aureus sarA* P1 promoter which was constitutively on in *E. coli* (60). Non-adherent *S. typhimurium* and adherent *S. typhimurium* were determined by mCherry signal with the excitation wavelength at 600±9 nm and the emission wavelength at 630±20 nm. Alternatively, non-fluorescence labeled *E. coli* and *S. typhimurium* were used for adhesiveness as described above. Bacteria collected before and after washing with PBS were diluted and plated out on YESCA-CR plates. *E. coli* curli mutants formed white colonies whereas *S. typhimurium* curli mutants formed pink colonies. Colony forming units of pink colonies before and

after washing were counted and the percentage of adhesive *S. typhimurium* was calculated.

### **Pellicle biofilm assay**

Overnight cultures of *S. typhimurium* wild-type, *csgA*- and *csgBA*- mutants were diluted by 1:1,000 (v/v) into 4 ml liquid LB-no salt medium. Freshly purified *E. coli* CsgA, sonicated *E. coli* CsgA fibers, or mock purification from cells harboring an empty vector was added. Bacteria were incubated at 26°C without shaking for 3 d. The liquid was removed carefully and pellicles were stained with 0.1% crystal violet for visualization.

## **Results**

### **Curli subunits of different bacteria cross-seed *in vitro***

To investigate the seeding specificity of curli amyloid, closely related homologs of *E. coli* CsgA from *Salmonella enterica* serovar Typhimurium LT2 and *Citrobacter koseri* (~ 70% identity) and a distant homolog from *Shewanella oneidensis* MR-1 (28% identity) were purified and tested for their ability to cross-seed *E. coli* CsgA polymerization *in vitro*. For simplicity, we refer to the CsgA homologs from different organisms as CsgA<sub>EC</sub> (CsgA from *E. coli*), CsgA<sub>ST</sub> (*Salmonella enterica* serovar Typhimurium), CsgA<sub>CK</sub> (*Citrobacter koseri*), and CsgA<sub>SO</sub> (*Shewanella oneidensis*).

Freshly purified CsgA<sub>EC</sub> monomers spontaneously assemble into fibers with identifiable lag, exponential and stationary phases which can be followed by Thioflavin T fluorescence in real time (55). Addition of preformed CsgA<sub>EC</sub> fiber seeds completely eliminated the lag phase (Figure 2.1A), which was consistent with previous observations

(55). In the presence of 5% (w/w) preformed CsgA<sub>ST</sub> or CsgA<sub>CK</sub> seeds, CsgA<sub>EC</sub> fibrillated with no lag phase, indicating fibers of CsgA<sub>ST</sub> or CsgA<sub>CK</sub> efficiently cross-seeded CsgA<sub>EC</sub> polymerization (Figure 2.1A). Like CsgA<sub>EC</sub>, monomeric CsgA<sub>ST</sub> assembled into fibers with an approximately four-hour lag phase. The polymerization was accelerated by 5% (w/w) of its own fibers, CsgA<sub>EC</sub> fibers or CsgA<sub>CK</sub> fibers (Figure 2.1B). The decrease in ThT fluorescence after the exponential phase was possibly due to the adhesion of fiber aggregates to the wall of wells, a phenomenon we often observe. CsgA<sub>CK</sub> also self-polymerized and could be cross-seeded by seeds of CsgA<sub>EC</sub> or CsgA<sub>ST</sub> (data not shown). Interestingly, the polymerization of CsgA<sub>EC</sub> was also seeded efficiently by a distant CsgA homolog from *S. oneidensis*. CsgA<sub>SO</sub> spontaneously assembled into amyloid-like fibers which were morphologically similar to *E. coli* curli fibers (Figure 2.2A) and were rich in  $\beta$ -sheet secondary structure (Figure 2.2B). The addition of preformed CsgA<sub>SO</sub> fibers effectively eliminated the lag phase of CsgA<sub>EC</sub> polymerization (Figure 2.1C). Taken together, these results suggest that CsgA homologs from different bacteria efficiently cross-seed *in vitro*.

CsgA<sub>EC</sub> polymerization can also be seeded by *E. coli* CsgB (CsgB<sub>EC</sub>) fibers *in vitro* (Figure 2.1D). CsgB<sub>EC</sub> is proposed to quickly adopt an amyloid fold and to template CsgA<sub>EC</sub> fiber formation *in vivo* (51). The amino acid sequences of CsgA<sub>EC</sub> and CsgB<sub>EC</sub> are less than 30% identical. Therefore, the interaction between CsgA and CsgB in *E. coli* represents a unique example of cross-seeding among amyloids (51,61). It is unknown if CsgB has species-specific seeding determinants, or if CsgB can seed CsgA homologs from other bacterial species. To determine the seeding specificity of CsgB, CsgB<sub>EC</sub> and CsgB homologs from *S. typhimurium* (CsgB<sub>ST</sub>) were expressed and purified. 5% (w/w)

CsgB<sub>ST</sub> fibers efficiently promoted the polymerization of CsgA<sub>EC</sub> (Figure 2.1D). Similarly, 5% (w/w) CsgB<sub>EC</sub> seeds cross-seeded the polymerization of CsgA<sub>ST</sub> and CsgA<sub>CK</sub> (Figure 2.1E and data not shown), suggesting that CsgB can cross-seed CsgA of a different bacteria.

We also measured the stability of fibers formed by CsgA homologs, CsgB homologs or fibers formed by CsgA<sub>EC</sub> in the presence of various seeds in terms of hexafluoro-2-propanol (HFIP) resistance. HFIP is a strong denaturant that dissociates curli fibers into SDS-soluble monomers that migrate at 17 kDa on a SDS-PAGE gel (57). These fibers showed similar resistance to HFIP treatment: fibers were mostly resistant to 50%, 70% or 80% HFIP treatment, and were largely dissociated with 90% or 100% HFIP treatment (Figure 2.3). This result suggests that CsgA/CsgB fibers or those formed in the cross-seeding reaction may adopt similar conformations.

We further tested whether curli subunits can cross-seed with unrelated amyloidogenic peptides and proteins. The peptide amyloid  $\beta$  1-42 ( $A\beta_{1-42}$ ) and the prion domain of yeast prion element Sup35 (Sup35 NM, which includes the N-terminal and the middle domain) were analyzed for their ability to cross-seed with *E. coli* CsgA. Neither  $A\beta_{1-42}$  nor Sup35 NM was able to seed CsgA<sub>EC</sub>, nor could the fibrillization of  $A\beta_{1-42}$  or Sup35 NM be seeded by CsgB<sub>EC</sub> fibers (Figure 2.4). The addition of CsgA<sub>EC</sub> seeds slightly increased the fibrillization of Sup35 NM, although to much lower levels than Sup35 NM seeded by its own fibers (Figure 2.4). Therefore, although cross-seeding occurs between curli homologs, curli cannot cross-seed with unrelated amyloidogenic proteins.

### **CsgA monomers physically bind preformed curli fibers**

To determine if monomeric CsgA can physically interact with heterogeneous seeds or whether seed addition allows for adoption of a new fold without direct binding, surface plasmon resonance (SPR) was performed to monitor interactions between CsgA monomers and preformed fibers in real time. Preformed CsgA<sub>EC</sub> fibers were immobilized on a sensor chip and 0.25  $\mu$ M of freshly purified CsgA<sub>EC</sub> or CsgA<sub>ST</sub> was injected over the chip. An increase in resonance units with no obvious signal decay was observed after the injection, suggesting a strong interaction between CsgA<sub>EC</sub> monomers and CsgA<sub>EC</sub> seeds, and between CsgA<sub>ST</sub> and CsgA<sub>EC</sub> seeds (Figure 2.5A). The same response was detected when monomeric CsgA<sub>EC</sub> or CsgA<sub>ST</sub> was flowed over CsgA<sub>ST</sub> seeds, showing a strong association of polymeric CsgA<sub>ST</sub> with CsgA<sub>ST</sub> or CsgA<sub>EC</sub> monomers (Figure 2.5B). Similarly, both freshly purified CsgA<sub>EC</sub> and CsgA<sub>ST</sub> bound to polymeric CsgB<sub>EC</sub> or CsgB<sub>ST</sub> (Figure 2.5C, D). As the control, BSA or mock purification from cells harboring an empty vector was flowed over polymeric CsgA<sub>EC</sub>, CsgA<sub>ST</sub>, CsgB<sub>EC</sub>, or CsgB<sub>ST</sub> and no significant interaction was observed (Figure 2.5 and data not shown). Finally, consistent with results shown in Figure 2.4B, A $\beta$ <sub>1-42</sub> only weakly interacted with CsgA<sub>EC</sub> seeds and quickly disassociated after injection (Figure 2.6A). A $\beta$ <sub>1-42</sub> did not interact with CsgB<sub>EC</sub> seeds at all (Figure 2.6B). Together, these results suggest monomeric CsgA homologs directly bind fibers during cross-seeding reactions.

### **Mutations of conserved Gln/Asn residues abolish the cross-seeding of curli**

All the CsgA or CsgB homologs contain a C-terminal domain predicted to have imperfect  $\beta$ -strand-loop- $\beta$ -strand repeating units and conserved Gln and Asn stacks (62). The first and last repeating units of *E. coli* CsgA are required for self-seeding and

interactions between CsgA and CsgB (58,63). Mutations of Gln and Asn residues in these repeating units to Ala result in a slow polymerizing variant of CsgA named CsgA<sup>slowgo</sup> (CsgA<sup>Q49A, N54A, Q139A, N144A</sup>) which can be seeded by *E. coli* CsgA fibers, but cannot respond efficiently to CsgB-mediated heteronucleation (64), suggesting that CsgA self-seeding and heteronucleation may be mediated by distinct mechanisms. Interestingly, we found the conserved Gln and Asn residues were also necessary for cross-seeding between *E. coli* CsgA and other CsgA homologs. The polymerization of freshly purified CsgA<sup>slowgo</sup> was not efficiently seeded by fibers of CsgA<sub>ST</sub>, CsgA<sub>CK</sub> or CsgA<sub>SO</sub> (Figure 2.7 and data not shown). Consistent with previous results, 5% *E. coli* CsgA fibers completely eliminated the lag phase and promoted the polymerization of CsgA<sup>slowgo</sup> (Figure 2.7). These results indicated that the conserved Gln and Asn residues in *E. coli* CsgA help mediate cross-seeding.

### **Interspecies cross-seeding *in vivo***

Like *E. coli*, *S. typhimurium*, *C. koseri* and *S. oneidensis* all harbor the *csgDEFG* and *csgBA* operons required for curli biogenesis, and curli fibers were detected on the surface of *S. typhimurium* and *C. koseri* ((65) and data not shown). The relaxed seeding of curli subunits *in vitro* led us to ask if cross-seeding of curli also occurred *in vivo* between different bacterial species.

To test if CsgB<sub>EC</sub> cross-seeds CsgA homologs under physiological conditions, we expressed *csgA<sub>ST</sub>*, *csgA<sub>CK</sub>* and *csgA<sub>SO</sub>* driven by the *E. coli csgBA* promoter from a low copy plasmid in an *E. coli csgA*- mutant. As a control, we cloned the gene encoding CsgA<sub>EC</sub> into the same plasmid. If expression of CsgA homologs complements curli formation in an *E. coli csgA*- mutant, it would suggest that these homologs can be cross-



seeded by CsgB<sub>EC</sub> on bacterial surfaces. Colonies formed by an *E. coli csgA*- mutant expressing CsgA<sub>EC</sub>, CsgA<sub>ST</sub>, or CsgA<sub>CK</sub> stained red on YESCA-CR plates while the *csgA*- mutant with the vector control appeared white (Figure 2.8A). Bacteria-associated CsgA<sub>EC</sub>, CsgA<sub>ST</sub> and CsgA<sub>CK</sub> fibers were detected in whole cell lysates by western blot after treatment with HFIP. CsgA<sub>ST</sub> and CsgA<sub>CK</sub> in the whole cell lysates were mostly SDS-insoluble, indicating that these CsgA homologs were incorporated into SDS resistant curli fibers that could not migrate into SDS-PAGE (Figure 2.8C). Curli-like fibers were observed on the *E. coli csgA*- mutant expressing CsgA<sub>ST</sub> and CsgA<sub>CK</sub> by electron microscopy (Figure 2.8D). Thus, CsgA<sub>ST</sub> and CsgA<sub>CK</sub> complemented curli assembly in an *E. coli csgA*- mutant. Moreover, the complementation was CsgB dependent. *csgBA*-/pCsgA<sub>ST</sub> or *csgBA*-/pCsgA<sub>CK</sub> did not bind CR or produce fibers on the cell surface (Figure 2.8B, E), and no cell-associated CsgA<sub>ST</sub>/CsgA<sub>CK</sub> was detected by Western blot. Instead, SDS-soluble CsgA<sub>ST</sub>/CsgA<sub>CK</sub> was found in agar blocks underneath the colonies (Figure 2.8C), indicating that CsgA homologs were secreted without polymerizing into fibers. Collectively, CsgA<sub>ST</sub> and CsgA<sub>CK</sub> can be seeded by CsgB<sub>EC</sub> on the cell surface. Additionally, an *E. coli csgB*- mutant could be complemented by expression of CsgB<sub>ST</sub> and CsgB<sub>CK</sub> (Figure 2.9). Thus, CsgA<sub>EC</sub> could also be seeded by CsgB homologs from different bacteria. CsgA<sub>SO</sub> and CsgB<sub>SO</sub> were unable to complement *E. coli csgA*- or *E. coli csgB*- mutants (data not shown), possibly indicating that CsgA<sub>SO</sub> and CsgB<sub>SO</sub> are not properly expressed or localized in *E. coli*. Consistent with this we were unable to detect expression of his-tagged CsgA<sub>SO</sub> in *E. coli* (data not shown).

We next asked if different bacteria grown in the same community could exchange curli subunits to assembled interbacterial curli fibers. Here we used *E. coli* and *S.*

*typhimurium* because curli biogenesis is well understood in these two organisms and they are proposed to frequently share the same ecological niche (65-67). During normal curli assembly in *E. coli*, CsgA is secreted unpolymerized into the extracellular environment prior to incorporation into fibers (7,55). Soluble CsgA produced by an *E. coli csgB*- mutant ( $A^+B^-$ ) can assemble into fibers on an adjacent *E. coli csgA*- mutant ( $A^-B^+$ ) (7). In *Salmonella enterica* serovar Enteritidis, such interbacterial curli assembly was detected only when the lipopolysaccharide O polysaccharide synthesis gene was mutated (59). To determine if interspecies curli assembly occurred between *E. coli* and *S. typhimurium* (schematic in Figure 2.10A), *E. coli* and *S. typhimurium csgA*-/*csgB*- mutants were mixed at 1:1 ( $OD_{600}/OD_{600}$ ) ratio and grown into mixed colonies. In a mixed colony with *E. coli csgB*- ( $A^+B^-$ ) and *S. typhimurium csgA*- ( $A^-B^+$ ), bacteria-associated, SDS-insoluble CsgA was readily detected by western blot (Figure 2.10B) and curli fibers were observed by TEM (Figure 2.10G). Similar interbacterial curli assembly was detected in a mixed colony with *E. coli csgA*- ( $A^-B^+$ ) and *S. typhimurium csgB*- ( $A^+B^-$ ) by Western analysis (Figure 2.10B) and by TEM (Figure 2.10K). It is interesting to notice that less curli were formed between *E. coli csgA*- ( $A^-B^+$ ) and *S. typhimurium csgB*- ( $A^+B^-$ ) than between *E. coli csgB*- ( $A^+B^-$ ) and *S. typhimurium csgA*- ( $A^-B^+$ ). This was probably because there were less *E. coli csgA*- ( $A^-B^+$ ) cells to template CsgA<sub>ST</sub> into fibers. *S. typhimurium csgB*- ( $A^+B^-$ ) outgrew *E. coli csgA*- ( $A^-B^+$ ) by a ratio of 4:1 in mixed colonies, while *S. typhimurium csgA*- ( $A^-B^+$ ) did not outcompete *E. coli csgB*- ( $A^+B^-$ ) (Figure 2.11). Consistent with this notion, more SDS-soluble CsgA was detected in agar underneath the colony with *E. coli csgA*- ( $A^-B^+$ ) and *S. typhimurium csgB*- ( $A^+B^-$ ) (Figure 2.11),

suggesting that some CsgA<sub>ST</sub> subunits were not incorporated into fibers and were secreted into the agar.

As controls, none of the single mutants produced curli on their own (Figure 2.10B and TEM (Figure 2.10D, E, I, J), and curli were not detected in a mixture of *E. coli csgB*- (A<sup>+</sup>B<sup>-</sup>) and *S. typhimurium csgBA*- (A<sup>-</sup>B<sup>-</sup>) or of *S. typhimurium csgB*- (A<sup>+</sup>B<sup>-</sup>) and *E. coli csgBA*- (A<sup>-</sup>B<sup>-</sup>), demonstrating that the nucleator CsgB was required to mediate interspecies curli assembly (date not shown). Consistent with previous reports (7,59), interbacterial curli formed between *E. coli csgA*- and *E. coli csgB*- mutants (Figure 2.10B, F) but not between *S. typhimurium csgA*- and *csgB*- mutants (Figure 2.10B, L). However, curli formed by the two *E. coli* mutants were less resistant to SDS, suggesting either that those fibers may adopt a distinct conformation or that polymerization was less efficient. Together, both *E. coli* and *S. typhimurium* are capable of sharing curli subunits to build interspecies curli.

### **Interbacterial complementation between *E. coli* curli mutants and *S. typhimurium* curli mutants restores bacterial surface attachment**

The ability of *E. coli* and *S. typhimurium* to share curli subunits (Figure 2.10) led us to further investigate the impact of such interspecies interactions on multispecies communities. Curli mediate surface attachment (42,43) and bacteria-bacteria interactions (44). Wild-type *E. coli* formed a colony biofilm that tightly adhered to the agar surface even after vigorous shaking in phosphate buffered saline (PBS) (Figure 2.12A). The adhesiveness was dependent on curli production. Colonies of an *E. coli csgA*- mutant (A<sup>-</sup>B<sup>+</sup>) or an *E. coli csgB*- mutant (A<sup>+</sup>B<sup>-</sup>) did not strongly adhere to the agar surface; less than 40% of the curli-defective bacteria remained on agar after washing compared with

95% of wild-type (Figure 2.12A). A *csgA*- mutant ( $A^-B^+$ ) or a *csgB*- mutant ( $A^+B^-$ ) of *S. typhimurium* also showed low adherence to the agar surface (Figure 2.12A). Wild-type *S. typhimurium* has low surface adherence as well (Figure 2.12A and B), possibly due to the production of cellulose and other extracellular polysaccharides that counteract curli-mediated adherence (59,68). However, over 80% of a mixed colony with *E. coli csgB*- ( $A^+B^-$ ) and *S. typhimurium csgA*- ( $A^-B^+$ ) stayed attached to the agar surface after vigorous washing with PBS (Figure 2.12A). The increase in surface attachment was dependent on interspecies curli assembly, as mixed colonies with *E. coli csgB*- ( $A^+B^-$ ) and *S. typhimurium csgBA*- ( $A^-B^-$ ), or *S. typhimurium csgA*- ( $A^-B^+$ ) and *E. coli csgBA*- ( $A^-B^-$ ) mutants did not attach to the surface well (Figure 2.13A). Consistent with the low level of interspecies curli production, we did not observe a significant increase in adherence when *E. coli csgA*- ( $A^-B^+$ ) and *S. typhimurium csgB*- ( $A^+B^-$ ) was mixed (Figure 2.12A). Collectively, these results demonstrate that curli assembled between *E. coli* and *S. typhimurium* are effective in restoring bacterial surface attachment.

Because *S. typhimurium* did not adhere to the agar surface as well as *E. coli*, we modified the assay to determine whether interspecies curli assembly between *E. coli* and *S. typhimurium* also facilitated surface attachment of *S. typhimurium*. In order to differentiate between the two species, a *S. typhimurium* strain carrying a plasmid that constitutively expresses mCherry was used. The adhesiveness of *S. typhimurium* was determined by mCherry fluorescence before and after the PBS wash. When mixed with *E. coli csgB*- ( $A^+B^-$ ) mutant, the percentage of *S. typhimurium csgA*- ( $A^-B^+$ ) mutant adhering to the agar surface increased by more than 60% (Figure 2.12B). The increase in attachment was dependent on the nucleator CsgB; an *E. coli csgBA*- ( $A^-B^-$ ) did not

promote *S. typhimurium* adherence (Figure 2.12B). These results were confirmed by the colony forming units (CFU) of *S. typhimurium* in the mixed colony (Figure 2.13B).

Together, these results demonstrate that interspecies curli assembly between *E. coli* and *S. typhimurium* restores bacterial adhesiveness of the whole population and also promotes the attachment of *S. typhimurium* to agar.

### **Cross-seeding of curli restores pellicle biofilm formation of *S. typhimurium***

Curli are important for the development of a pellicle biofilm, a type of biofilm that grows at the air-liquid interface (8,45,57). In static LB-no salt medium, *S. typhimurium* formed a pellicle biofilm (Figure 2.14). The development of pellicle biofilms requires curli production (45), and indeed *S. typhimurium csgA-* or *csgBA-* mutants were unable to make pellicles (Figure 2.14). We asked if *S. typhimurium csgA-* could utilize and incorporate CsgA<sub>EC</sub> into fibers to support a pellicle biofilm. The addition of freshly purified CsgA<sub>EC</sub> monomers restored the pellicle development of the *S. typhimurium csgA-* mutant (Figure 2.14). However, a *S. typhimurium csgBA-* mutant mixed with *E. coli* CsgA monomers did not form a pellicle (Figure 2.14). Furthermore, sonicated, preformed *E. coli* CsgA seeds did not restore pellicle formation of the *S. typhimurium csgA-* mutant. This is probably because exogenously added fibers were not associated with cells and thus could not function as a scaffold to support pellicle biofilm formation. Thus, the cross-seeding of CsgA<sub>EC</sub> by CsgB<sub>ST</sub> on *S. typhimurium* cell surfaces facilitates pellicle biofilm development.

## Discussion

Amyloid proteins polymerize into fibers with nucleation dependent kinetics. The conversion of monomers to an oligomeric nucleus contributes to the lag phase, but subsequently preformed fibers or nucleus can act as templates that accelerate amyloid formation. This process is known as seeding. Seeding is a critical step of amyloid propagation and disease development (1,69). The work presented here demonstrates that bacterial functional amyloids have relatively relaxed seeding specificities. Amyloid cross-seeding of curli subunits was observed both *in vitro* between *E. coli*, *S. typhimurium*, *C. koseri* and *S. oneidensis* as well as *in vivo* between *E. coli* and *S. typhimurium*. Remarkably this interspecies interaction facilitated bacterial surface attachment and biofilm development.

Amyloid seeding is typically highly specific. For instance, lack of cross-seeding is reported between the Parkinson's Disease-associated amyloid,  $\alpha$ -synuclein, and closely related amyloidogenic homologs with more than 78% similarity (36). Species barriers are also commonly observed between different mammalian prion species, and between Sup35 homologs from different yeast species with up to 95% identity (29,30,32,33). Even a single amino acid mutation could alter the seeding specificity of A $\beta$ 1-42 and prions (25,29). However, our results suggest that the cross-seeding specificity between curli subunits is relaxed. Efficient seeding was observed *in vitro* between curli subunits from a range of bacteria including *E. coli*, *S. typhimurium*, *C. koseri* and *S. oneidensi*. Strikingly, although the CsgA<sub>SO</sub> is only 28% identical to *E. coli* CsgA in primary structure, it efficiently seeded CsgA<sub>EC</sub> amyloid formation with only 2% (w/w) seed concentration

(Figure 2.1C). We have also shown that during seeding and cross-seeding reactions, monomers physically interact with and bind fibers, as indicated by SPR (Figure 2.5).

Curli assembly on bacterial surfaces is mediated by the nucleator protein CsgB. A longstanding model of curli biogenesis suggests that, once secreted, CsgB quickly adopts an amyloid fold which serves as a template to direct the assembly of CsgA into amyloid polymers (61,70). The interaction between CsgA and CsgB in *E. coli* represents an example of heterogeneous seeding of amyloid proteins since *E. coli* CsgA and CsgB are less than 30% identical in amino acid sequence (51,71). *In vitro*, both CsgA<sub>EC</sub> and CsgB<sub>EC</sub> spontaneously polymerize into amyloid fibers with a similar structure measured by CD and EM (51,55). Shewmaker *et al.* have also shown that both CsgA and CsgB adopt similar beta-helix structure (12). Thus we hypothesize that CsgB-mediated templating is analogous to CsgA-mediated templating. CsgB-dependent heteronucleation could be mediated either by species-specific recognition sequences or by a promiscuous seeding mechanism. We have shown both *in vitro* and *in vivo* that CsgB<sub>EC</sub> can cross-seed CsgA<sub>ST</sub> and CsgA<sub>CK</sub>, and in turn CsgB<sub>ST</sub> can cross-seed CsgA<sub>EC</sub>. (Figure 2.1, Figure 2.5, Figure 2.8, Figure 2.9). Although cross-seeding between CsgA<sub>EC</sub> and other CsgB species was not tested due to the technical challenges in protein purification, curli formation by an *E. coli* *csgB*- mutant can be complemented by expressing CsgB<sub>CK</sub> in trans (Figure 2.9), suggesting CsgB<sub>CK</sub> can also cross-seed the fibrillization of CsgA<sub>EC</sub>. Therefore, CsgB-mediated seeding is likely to be a result of relaxed seeding of curli subunits.

Despite low sequence identity, both *E. coli* CsgA<sub>EC</sub> and CsgB<sub>EC</sub> are composed of an N-terminal Sec signaling sequence and a C-terminal domain with five 19-24 amino acid imperfect repeating units. Each repeating unit is predicted to adopt a  $\beta$ -strand-loop-

$\beta$ -strand motif and contains conserved Gln and Asn which are critical for amyloid fiber formation (72). The same primary structure arrangement with regularly spaced Gln and Asn residues was also found in CsgA and CsgB homologs from other gram-negative bacteria (62). Our previous results have shown that Gln and Asn residues in the N- and C- terminal repeating units (Q49, N54, Q139, N144) of *E. coli* CsgA are essential for efficient amyloid formation and interaction with *E. coli* CsgB, as a CsgA<sup>slowgo</sup> mutant with those four Gln/Asn residues mutated cannot be cross-seeded by CsgB (72). In this study we further demonstrated that this mutant was also defective in cross-seeding with other CsgA homologs. Therefore these conserved Gln and Asn residues are important sequence determinants that mediate cross-seeding. An emerging consensus from studies of prion seeding specificity suggests that compatibility in protein or fiber conformations governs seeding specificity (35,73-76). Not surprisingly, small changes in amino acid sequences can dictate conformational variability; therefore seeding specificity is tightly correlated with primary sequence (29,72,77). Specific side-chain interactions also play an important role in amyloid formation and seeding (78). Gln and Asn residues in amyloid proteins form intra-molecular hydrogen bonds and help to stabilize the cross- $\beta$  structure (78,79). It is possible that the hydrogen bonds formed between Gln and Asn mediate interaction and seeding between different curli proteins. CsgA<sup>slowgo</sup> seeding with *E. coli* CsgA may be facilitated by other strain-specific side chain interactions.

The spatial arrangement of Gln and Asn within an amyloidogenic protein is proposed to play an important role in amyloid assembly (72). This could explain why the yeast prion Sup35, another Gln/Asn rich amyloid protein, did not cross-seed with *E. coli* CsgA (Figure 2.4), as the spacing between Gln and Asp in CsgA and in Sup35 are



different. Moreover, circular dichroism spectra revealed that the secondary structures of CsgA fibers and Sup35-NM fibers are different- NM fibers have more random coil structure ((7,80), and data not shown), indicating that CsgA and Sup35 fibers may adopt incompatible conformations which limit the cross-seeding efficiency.

Although no cross-seeding was observed between curli and Sup35 or curli and A $\beta$ <sub>1-42</sub> (Figure 2.4), other eukaryotic amyloidogenic peptides including the human antimicrobial peptide LL-37 and amyloid protein A (AA) have been suggested to interact with curli directly or indirectly (23,68). LL-37 is produced by epithelial cells of the urinary track to protect against infections with uropathogenic *E. coli* (81,82). It is recently shown that LL-37 strongly bound to polymeric CsgA fibers (68), suggesting that curli may have the potential to promote the aggregation of LL-37 and bacteria might use cross-seeding as a strategy to counteract the antimicrobial effects. Amyloid protein A deposition is a result of chronic inflammations (83). Lundmark *et al.* has shown that AA deposition can be accelerated by amyloids including curli (23). Thus, cross-seeding may provide a mechanism for amyloidogenesis.

The highly specific seeding between disease-associated amyloids and prions is proposed to be a mechanism to prevent amyloid disease propagation and transmission. As a class of functional amyloids, the relaxed seeding propensity of curli may have an impact on biological events. Curli are an important component of enteric bacterial biofilm communities; mediating surface attachment and cell-cell interactions (43,44,57). Cross-seeding of curli subunits was observed *in vivo* in a mixed-species community, as interspecies curli assembly was found between *E. coli* and *S. typhimurium* curli mutants (Figure 2.10). Moreover, interspecies assembled curli fibers were biologically functional.

Intercellular curli assembly between *E. coli* and *S. typhimurium* curli mutants restored bacterial adherence to agar surfaces (Figure 2.12).

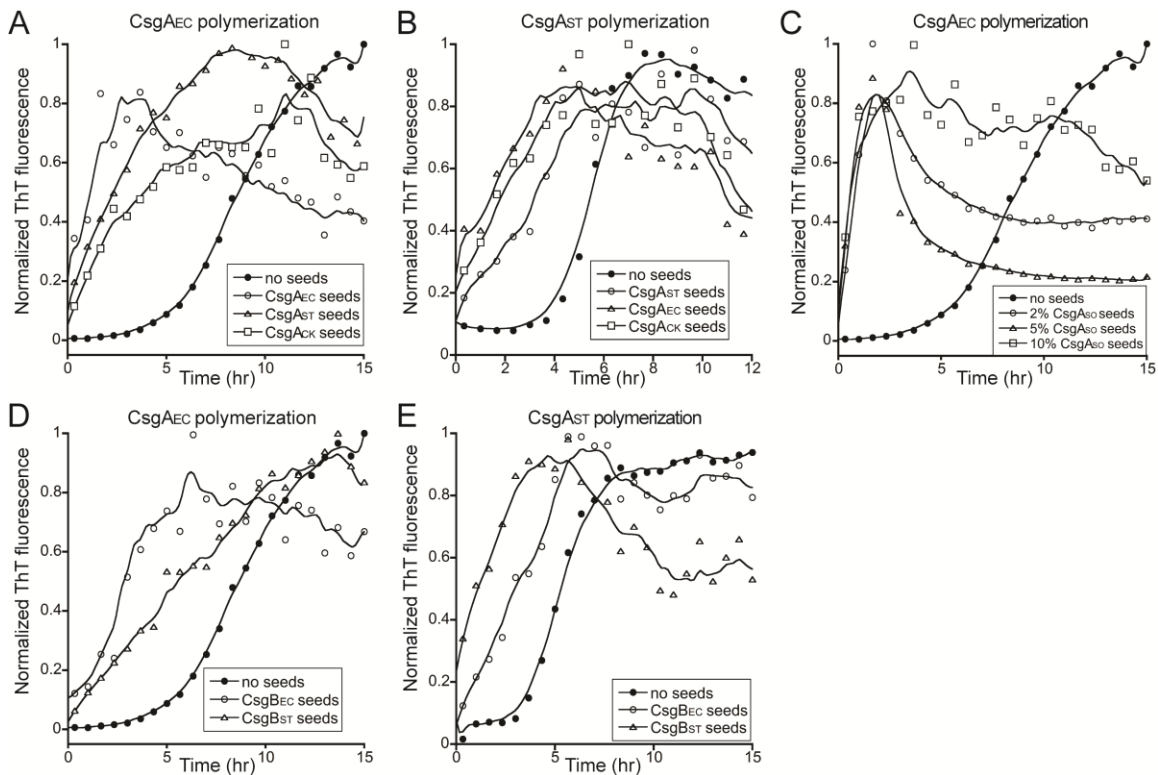
Curli are also required for pellicle biofilm formation (57). A *S. typhimurium csgA*-mutant that cannot make a pellicle biofilm was able to utilize *E. coli* CsgA monomers to form a robust pellicle (Figure 2.14). Romero *et al* recently showed that addition of *B.subtilis* amyloids TasA fibers restored the pellicle development of a *B.subtilis tasA* mutant (8). The addition of polymerized *E. coli* CsgA fibers did not restore pellicle formation by *S. typhimurium csgA*-, indicating that *S. typhimurium* cannot simply utilize curli fibers as a scaffold to support pellicle formation (Figure 2.14). *E. coli* CsgA monomers or fibers were also unable to restore pellicle formation of a *S. typhimurium csgBA*- mutant that lacks the nucleator protein on its surface. Thus, the complementation of *S. typhimurium* pellicle formation by *E. coli* CsgA is dependent on the nucleation process.

In nature, bacterial communities are composed of multiple species (84,85), and bacterial amyloids are abundant in natural biofilms (86). Because *E. coli* and *S. typhimurium* are proposed to share ecological niches, it is likely that these two species interact in biofilm environments (65,67). Since curli subunits are secreted into the extracellular environment prior to assembly into polymers, and since, as an amyloid, curli can be cross-seeded, it is plausible that *E. coli* and *S. typhimurium* coexisting in natural communities can share curli subunits to build a heterogeneous matrix. Curli-like structures are also found in *Enterobacter spp.* and *Citrobacter spp.* (65,80), and curli homologs are prevalent among *Pseudomonas spp* and *Shewanella spp*. Thus the promiscuous seeding of curli may have a broad impact on the multispecies communities.

## Figure and Tables

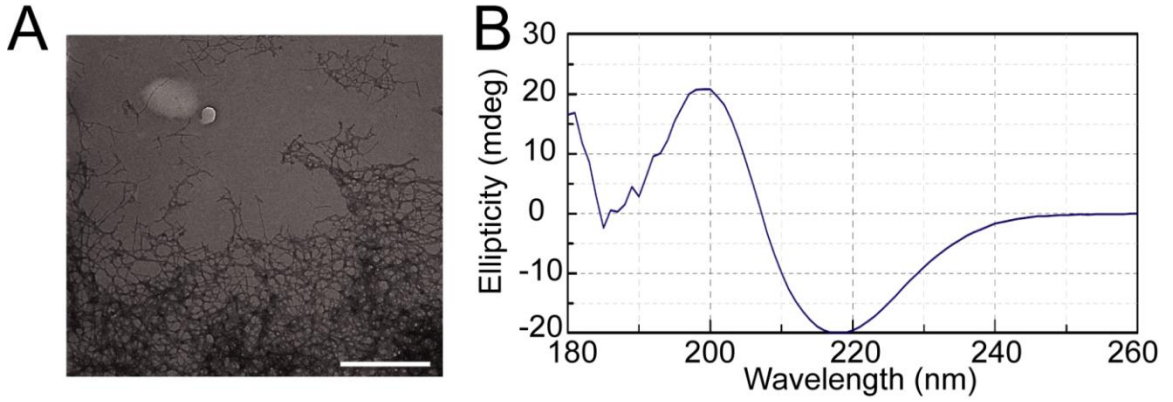
**Figure 2.1. Curli subunits cross-seeded *in vitro*.**

(A) Normalized ThT fluorescence monitoring the polymerization kinetics of 10  $\mu\text{M}$  *E. coli* CsgA (CsgA<sub>EC</sub>) alone (●), or in the presence of 5% (w/w) sonicated CsgA<sub>EC</sub> seeds (○), *S. typhimurium* CsgA (CsgA<sub>ST</sub>) seeds (Δ) or *C. koseri* CsgA (CsgA<sub>CK</sub>) seeds (□). (B) 10  $\mu\text{M}$  freshly purified CsgA<sub>ST</sub> polymerized with no seeds (●), or in the presence of 5% sonicated CsgA<sub>ST</sub> seeds (○), CsgA<sub>EC</sub> seeds (Δ) or CsgA<sub>CK</sub> seeds (□). (C) 10  $\mu\text{M}$  CsgA<sub>EC</sub> polymerized alone (●), or in the presence of 2% (○), 5% (Δ) or 10% (□) *S. oneidensis* CsgA (CsgA<sub>SO</sub>) seeds. (D) 10  $\mu\text{M}$  CsgA<sub>EC</sub> polymerized alone (●), with 5% CsgB<sub>EC</sub> seeds (○), or with 5% CsgB<sub>ST</sub> seeds (Δ). (E) 10  $\mu\text{M}$  CsgA<sub>ST</sub> polymerized alone (●), with 5% CsgB<sub>EC</sub> seeds (○), or with 5% CsgB<sub>ST</sub> seeds (Δ).



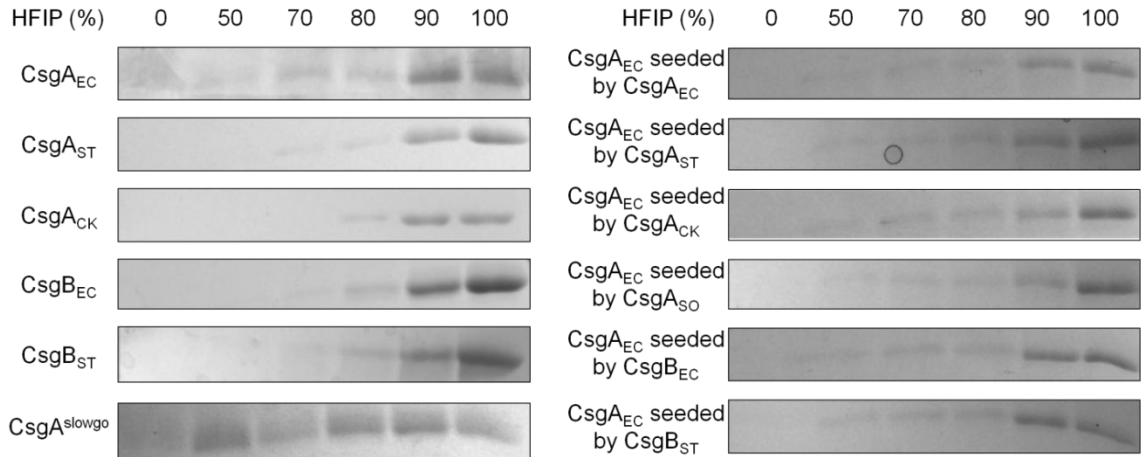
**Figure 2.2. A CsgA homolog from *S. oneidensis* MR-1 (CsgA<sub>SO</sub>) formed amyloid fibers *in vitro*.**

A CsgA homolog from *S. oneidensis* MR-1 (CsgA<sub>SO</sub>) formed amyloid fibers *in vitro*. (A) TEM of fibers formed by freshly purified CsgA<sub>SO</sub> after incubated at room temperature for 24 h. The scale bar equals 500 nm. (B) Circular dichroism (CD) analysis of CsgA<sub>SO</sub> fibers.



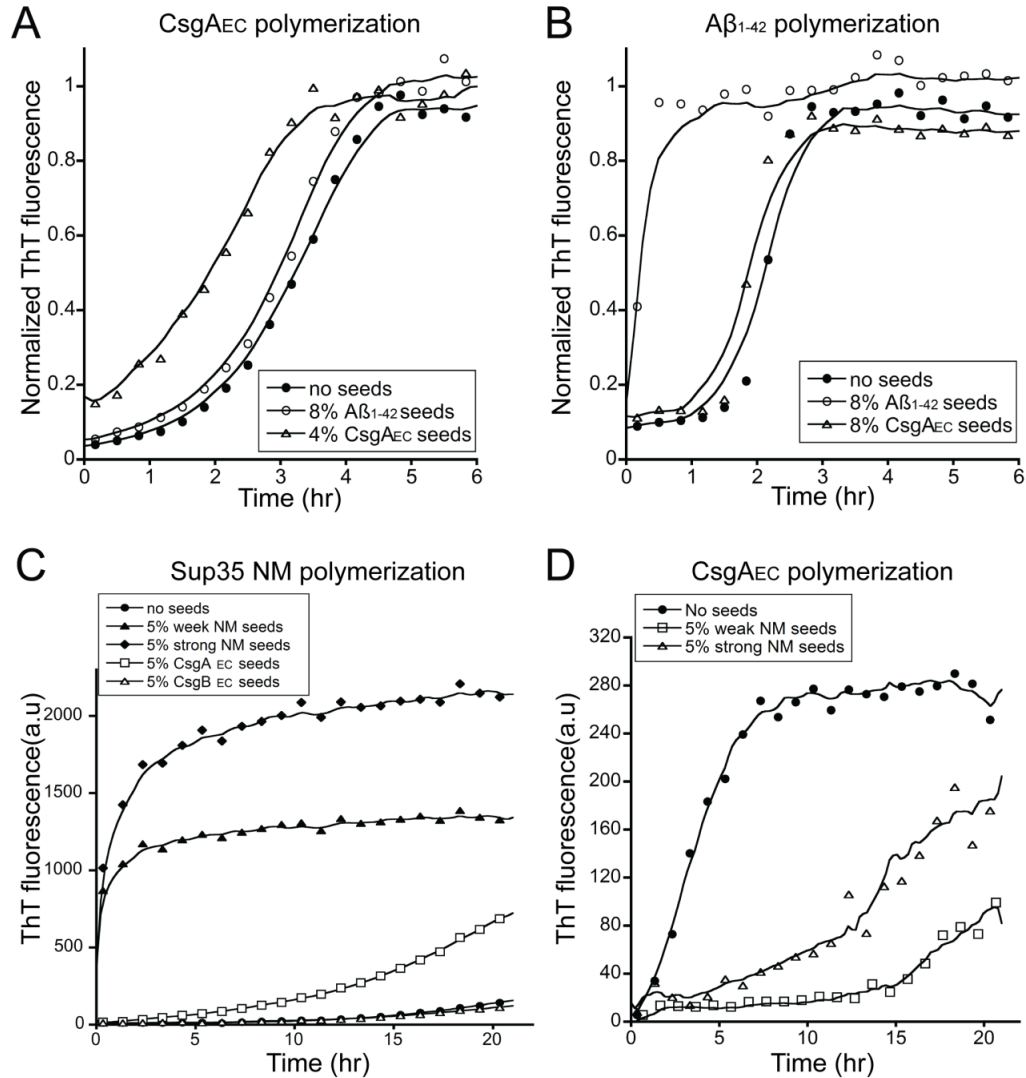
**Figure 2.3. Fibers formed by CsgA homologs, CsgB homologs or fibers formed by CsgA<sub>EC</sub> in the presence of various seeds had similar stability.**

Fibers formed by CsgA homologs, CsgB homologs or fibers formed by CsgA<sub>EC</sub> in the presence of various seeds had similar stability. CsgA<sup>slowgo</sup> was more sensitive to HFIP treatment. 22 µg fibers as indicated were treated with no HFIP or with 50% (v/v), 70%, 80%, 90% or 100%. Samples were immediately dried on a SpeedVac. Samples were boiled in 2x SDS-loading dye for 5 min and loaded on 15% SDS-PAGE gels. Monomers dissociated from fibers by HFIP treatment migrated into the gel at 17 kDa.



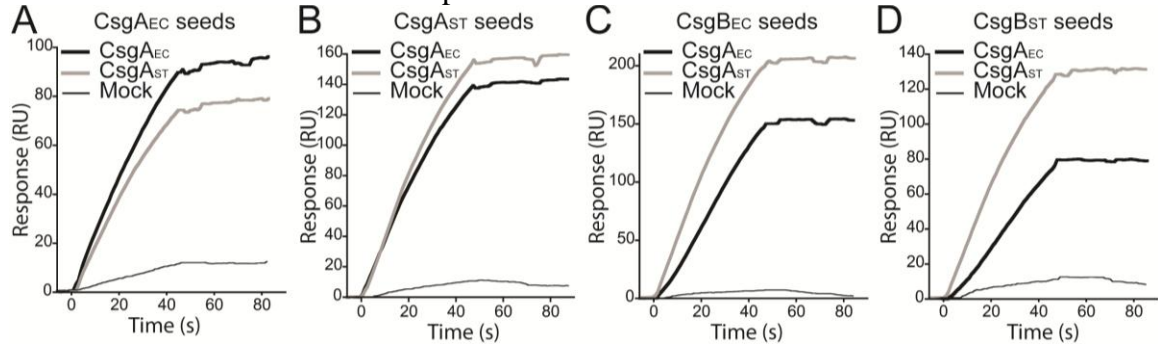
**Figure 2.4. Curli subunits did not cross-seed with A $\beta$ <sub>1-42</sub> or Sup35-NM.**

Curli subunits did not cross-seed with A $\beta$ <sub>1-42</sub> or Sup35-NM. (A) ThT fluorescence monitoring the polymerization kinetics of 40  $\mu$ M freshly purified CsgA<sub>EC</sub> alone ( $\bullet$ ), in the presence of 8% (w/w) A $\beta$ <sub>1-42</sub> seeds ( $\circ$ ) or 4% CsgA<sub>EC</sub> seeds ( $\Delta$ ). (B) Polymerization kinetics of 25  $\mu$ M freshly purified A $\beta$ <sub>1-42</sub> alone ( $\bullet$ ), in the presence of 8% A $\beta$ <sub>1-42</sub> seeds ( $\circ$ ) or 8% CsgA<sub>EC</sub> seeds ( $\Delta$ ). (C) 10  $\mu$ M monomeric Sup35 NM polymerized on its own ( $\bullet$ ), or in the presence of 5% weak Sup35 NM seeds ( $\blacktriangle$ ), strong Sup35 NM seeds ( $\blacklozenge$ ), CsgA<sub>EC</sub> seeds ( $\square$ ) or CsgB<sub>EC</sub> seeds ( $\Delta$ ). (D) 10  $\mu$ M freshly purified CsgA polymerized with no seeds ( $\bullet$ ), with 5% weak Sup35 NM seeds ( $\square$ ) or 5% strong Sup35 NM seeds ( $\Delta$ ).



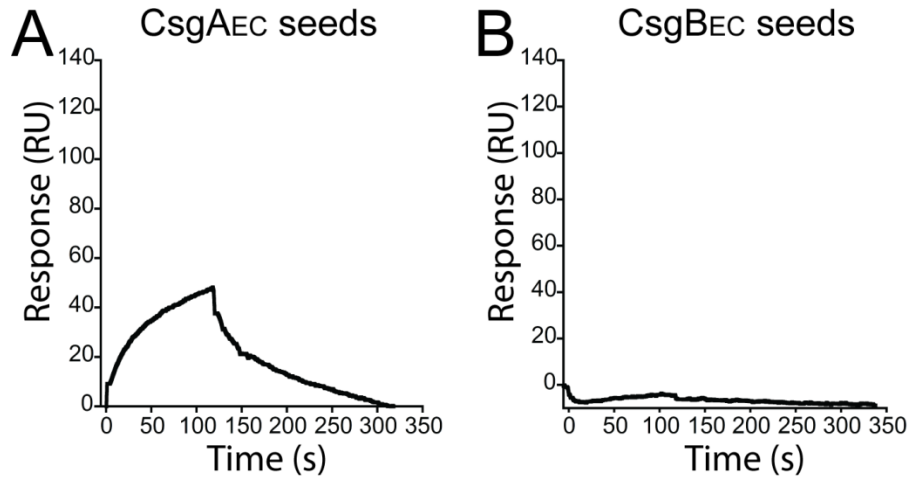
**Figure 2.5. Freshly purified CsgA bound preformed CsgA or CsgB seeds.**

Freshly purified CsgA bound preformed CsgA or CsgB seeds. SPR sensorgrams of 0.25  $\mu$ M freshly purified CsgA<sub>EC</sub> (black line) and CsgA<sub>ST</sub> (gray line) or products from a mock CsgA purification from strains harboring an empty vector (thin black line) were injected over CsgA<sub>EC</sub> seeds (A), CsgA<sub>ST</sub> seeds (B), CsgB<sub>EC</sub> seeds (C) or CsgB<sub>ST</sub> (D) that were immobilized on a CM5 sensor chip.



**Figure 2.6. A $\beta_{1-42}$  did not bind curli fibers efficiently.**

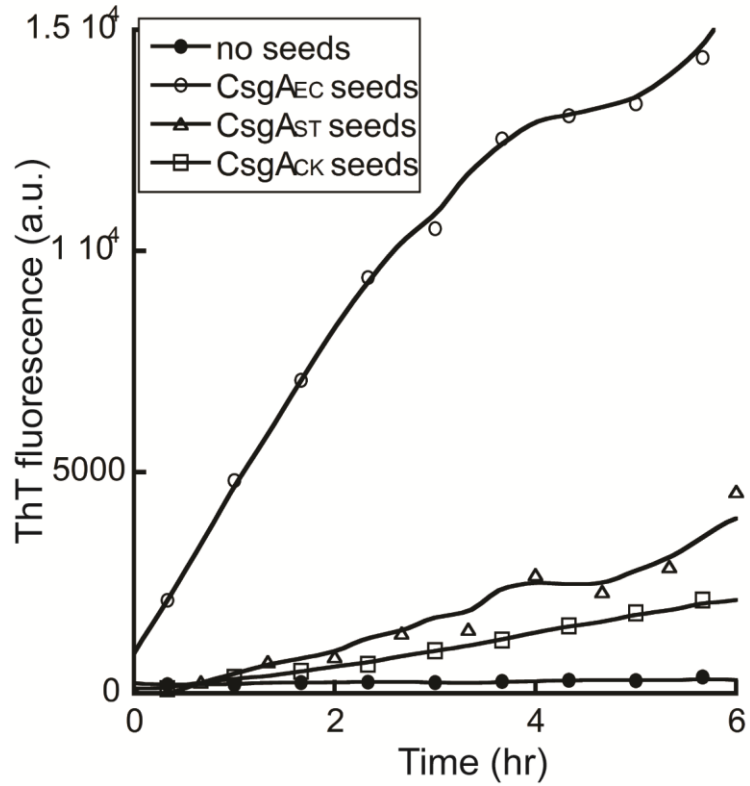
A $\beta_{1-42}$  did not bind curli fibers efficiently. Sensorgrams of interactions between over 1  $\mu$ M freshly prepared A $\beta_{1-42}$  monomers and CsgA<sub>EC</sub> seeds (A) or CsgB<sub>EC</sub> seeds (B).





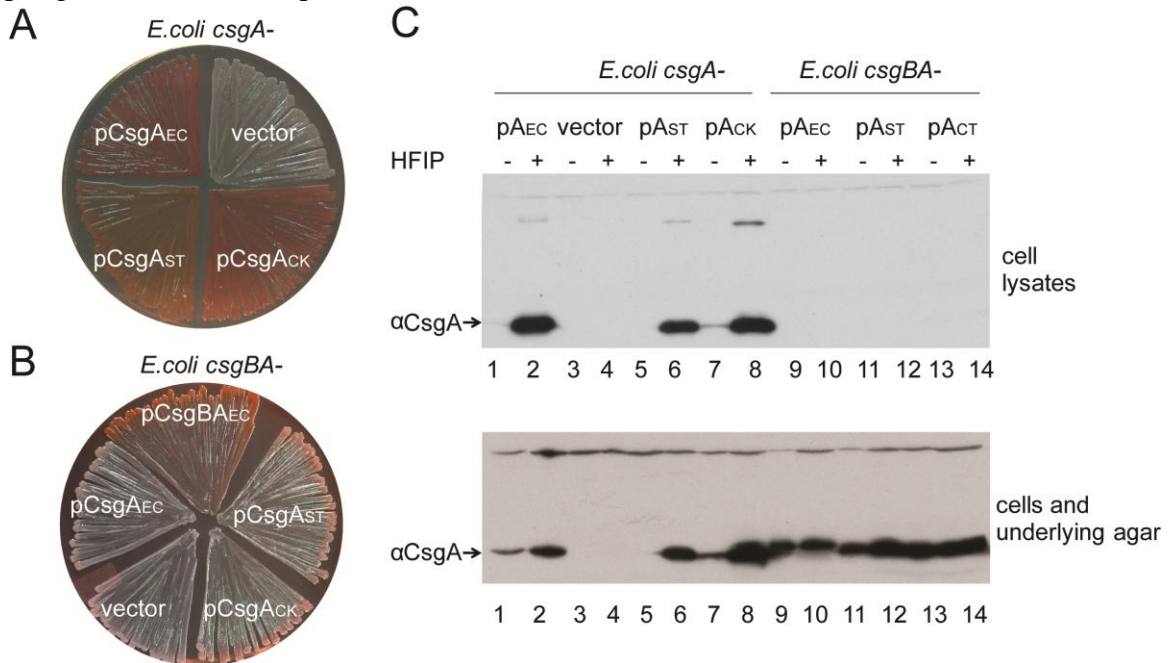
**Figure 2.7. Conserved Gln and Asn residues were required for cross-seeding between curli subunits.**

ThT fluorescence of 30  $\mu\text{M}$  freshly purified *E. coli* CsgA<sup>slowgo</sup> alone ( $\bullet$ ), or in the presence of 5% CsgA<sub>EC</sub> seeds ( $\circ$ ), CsgA<sub>ST</sub> seeds ( $\Delta$ ) or CsgA<sub>CK</sub> seeds ( $\square$ ).



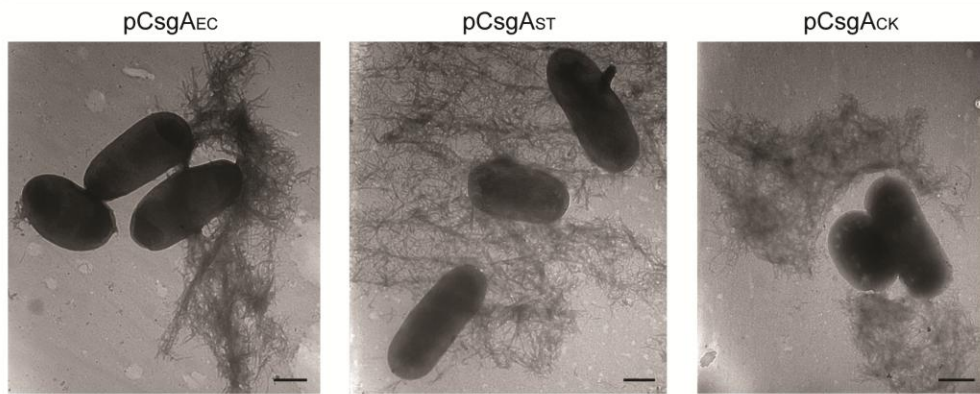
**Figure 2.8. An *E. coli csgA*- mutant was complemented by CsgA homologs from *S. typhimurium* or *C. koseri* in a CsgB dependent manner.**

An *E. coli csgA*- mutant was complemented by CsgA homologs from *S. typhimurium* or *C. koseri* in a CsgB dependent manner. (A) The expression of CsgA homologs complemented the CR binding of an *E. coli csgA*- mutant. *E. coli csgA*- harboring an empty vector control or plasmids encoding CsgA<sub>EC</sub> (pCsgA<sub>EC</sub>), CsgA<sub>ST</sub> (pCsgA<sub>ST</sub>) or CsgA<sub>CK</sub> (pCsgA<sub>CK</sub>) was grown on YESCA-CR plates at 26°C for 48 hr. (B) CsgB was required for the complementation of CR binding. *E. coli csgBA*- mutant harboring an empty vector or plasmid pCsgBA<sub>EC</sub>, pCsgA<sub>EC</sub>, pCsgA<sub>ST</sub>, or pCsgA<sub>CK</sub> were grown on a YESCA-CR agar at 26°C for 48 hr. (C) Western blot of whole cell lysates (top panel) and plugs (bottom panel) of an *E. coli csgA*- mutant transformed with pCsgA<sub>EC</sub> (lane 1, 2), the vector control (lane 3, 4), pCsgA<sub>ST</sub> (lane 5, 6), or pCsgA<sub>CK</sub> (lane 7, 8), and an *E. coli csgBA*- mutant with pCsgA<sub>EC</sub> (lane 9, 10), pCsgA<sub>ST</sub> (lane 11, 12) or pCsgA<sub>CK</sub> (lane 13, 14) grown on YESCA agar plates at 26°C for 48 hr. Samples were treated with (+) or without (-) HFIP before electrophoresis and were analyzed by αCsgA antibody. (D) TEM of *E. coli csgA*- or *csgBA*- mutant transformed with pCsgA<sub>EC</sub>, pCsgA<sub>ST</sub> or pCsgA<sub>CK</sub>. Scale bars equal 500 nm.



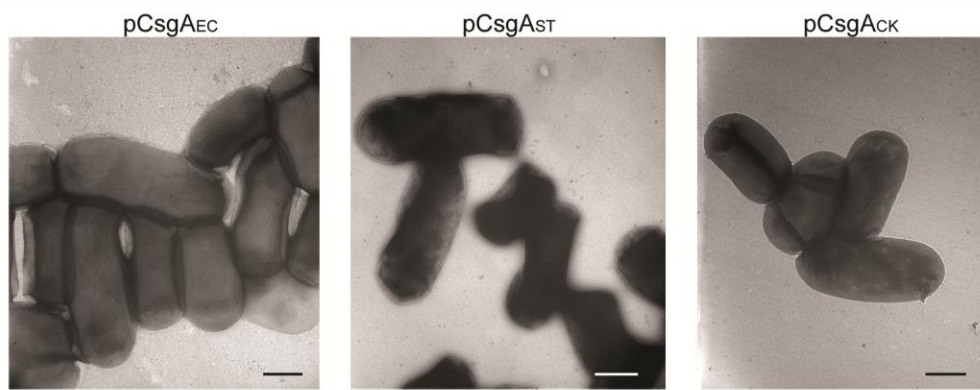
D

*E. coli csgA-*



E

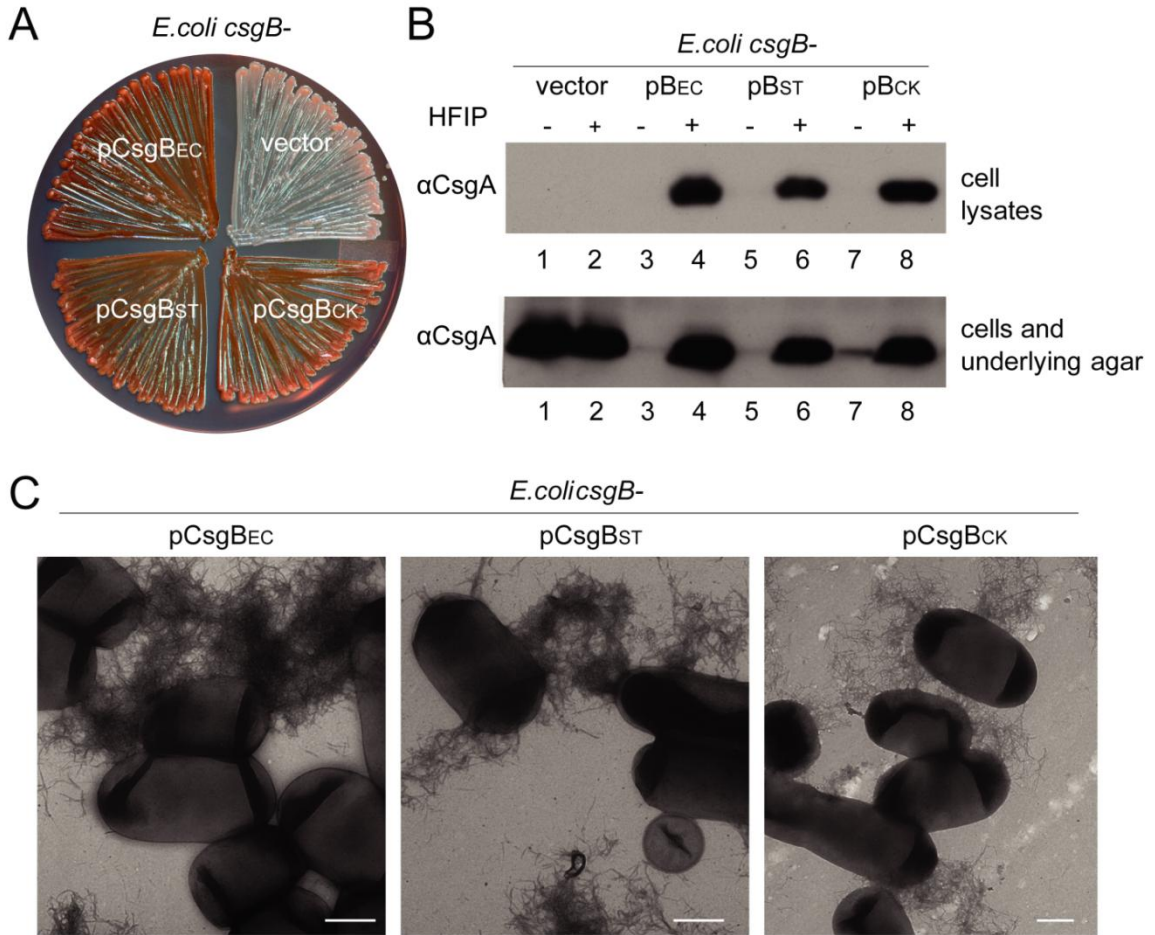
*E. coli csgBA-*



Scale bars= 500nm

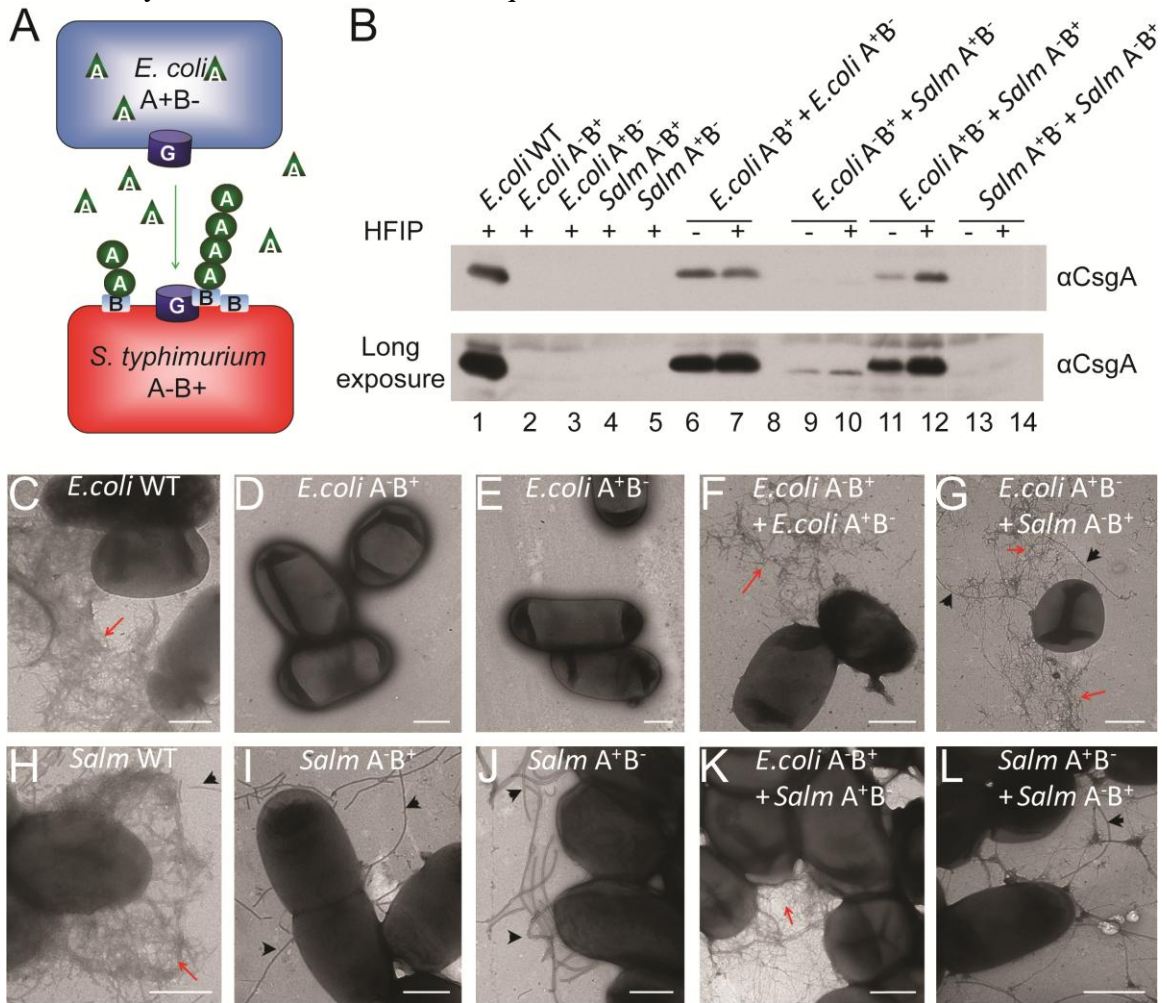
**Figure 2.9. *E. coli csgB*- was complemented by CsgB homologs from *S. typhimurium* (pCsgB<sub>ST</sub>) or *C. koseri* (pCsgB<sub>CK</sub>).**

*E. coli csgB*- was complemented by CsgB homologs from *S. typhimurium* (pCsgB<sub>ST</sub>) or *C. koseri* (pCsgB<sub>CK</sub>). (A) YESCA-CR agar plate with *E. coli csgB*- harboring an empty vector, pCsgB<sub>EC</sub>, pCsgB<sub>ST</sub> or pCsgB<sub>CK</sub> after 48 hr incubation at 26°C. (B) Western analysis of the whole cell lysates and bacteria with underlying agar of *E. coli csgB*- harboring the empty vector, pCsgB<sub>EC</sub>, pCsgB<sub>ST</sub> or pCsgB<sub>CK</sub> after grown on YESCA agar at 26°C for 48 hr. Samples were pre-treated with (+) or without (-) HFIP before electrophoresis. (C) TEM of *E. coli csgB*- mutant complemented with pCsgB<sub>EC</sub>, pCsgB<sub>ST</sub> or pCsgB<sub>CK</sub> after grown on YESCA agar at 26°C for 48 hr. Scale bars equal to 500 nm.



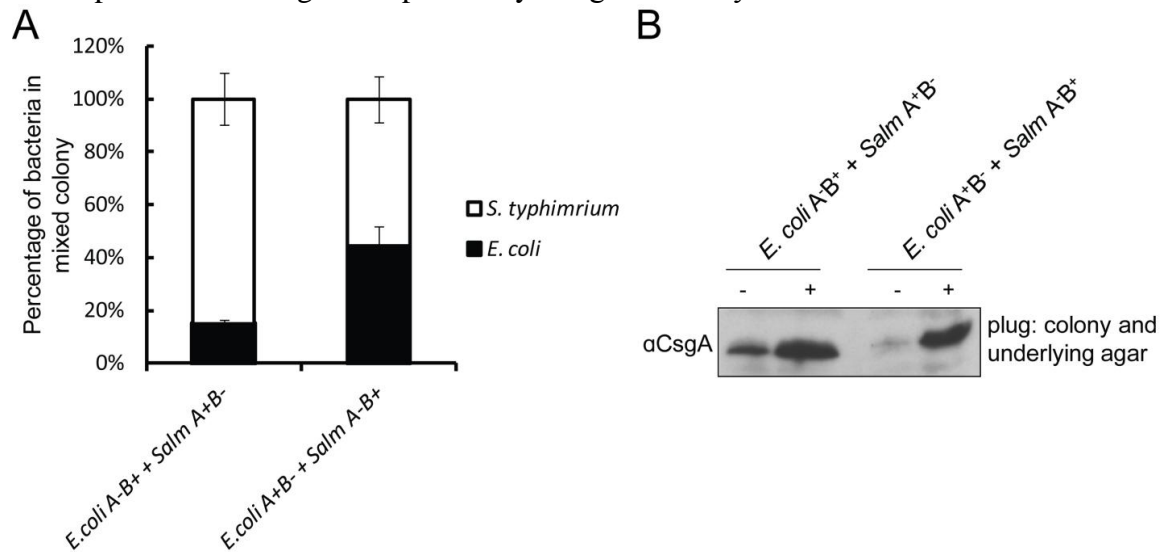
**Figure 2.10. Interbacterial curli assembly between *E. coli* and *S. typhimurium* curli mutants.**

Interbacterial curli assembly between *E. coli* and *S. typhimurium* curli mutants. (A) A schematic describing the interaction between an *E. coli* *csgB*<sup>-</sup> mutant ( $A^+B^-$ ) and *S. typhimurium* *csgA*<sup>-</sup> mutant ( $A^-B^+$ ). Unpolymerized *E. coli* CsgA secreted by *E. coli* *csgB*<sup>-</sup> ( $A^+B^-$ ) is templated by CsgB on the surface of *S. typhimurium* *csgA*<sup>-</sup> ( $A^-B^+$ ). (B) Western blots of HFIP-treated (+) or non-treated (-) whole cell lysates of the indicated strains or strain mixtures. The blots were probed with  $\alpha$ CsgA antibody. The bottom panel is a longer exposure of the same blot to visualize the faint bands. (C)-(L), TEM of *E. coli* wild-type (C), *E. coli* *csgA*<sup>-</sup> ( $A^-B^+$ ) (D), *E. coli* *csgB*<sup>-</sup> ( $A^+B^-$ ) (E), a mixed colony with *E. coli* *csgA*<sup>-</sup> ( $A^-B^+$ ) and *E. coli* *csgB*<sup>-</sup> ( $A^+B^-$ ) (F), a mixture of *S. typhimurium* *csgA*<sup>-</sup> ( $A^-B^+$ ) and *E. coli* *csgB*<sup>-</sup> ( $A^+B^-$ ) (G), *S. typhimurium* wild-type (H), *S. typhimurium* *csgA*<sup>-</sup> ( $A^-B^+$ ) (I), *S. typhimurium* *csgB*<sup>-</sup> ( $A^+B^-$ ) (J), a mixture of *E. coli* *csgA*<sup>-</sup> ( $A^-B^+$ ) and *S. typhimurium* *csgB*<sup>-</sup> ( $A^+B^-$ ) (K) and a mixture of *S. typhimurium* *csgA*<sup>-</sup> ( $A^-B^+$ ) and *S. typhimurium* *csgB*<sup>-</sup> ( $A^+B^-$ ) (L). Curli fibers are indicated by red arrows and flagella are indicated by black arrows. Scale bars equal to 500 nm.



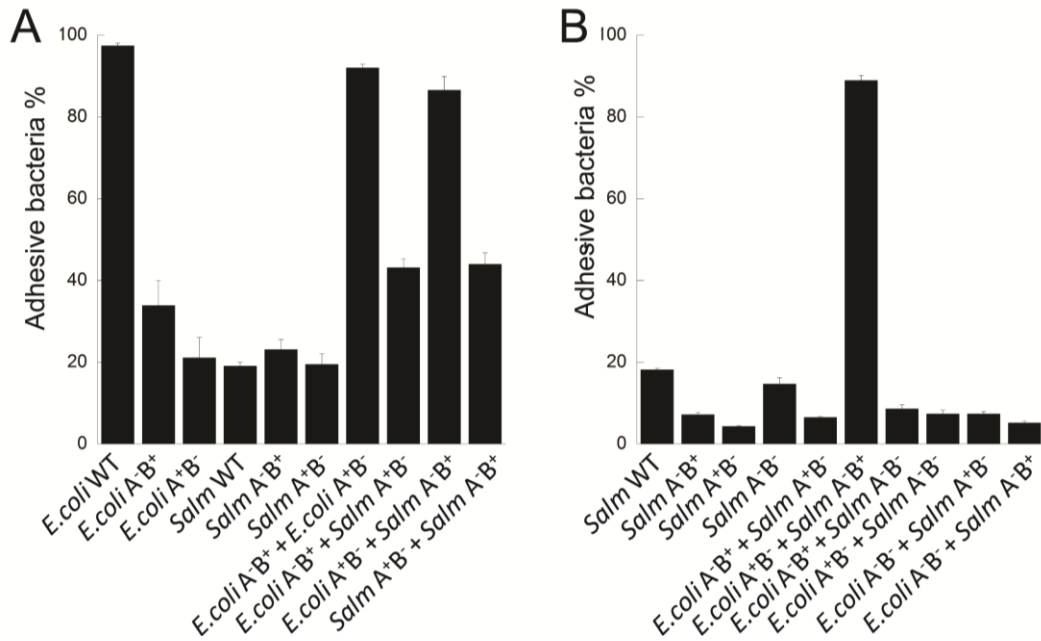
**Figure 2.11. *S. typhimurium* csgB<sup>-</sup> (A+B<sup>-</sup>) outgrew *E. coli* csgA<sup>-</sup> (A-B<sup>+</sup>) in the mixed colony.**

*S. typhimurium* csgB<sup>-</sup> (A<sup>+</sup>B<sup>-</sup>) outgrew *E. coli* csgA<sup>-</sup> (A<sup>-</sup>B<sup>+</sup>) in the mixed colony. (A) The percentage of *S. typhimurium* or *E. coli* curli mutants in the mixed colony. Overnight cultures of *E. coli* and *S. typhimurium* mutants were normalized by OD<sub>600</sub>, mixed at 1:1 (v/v) ratio and spotted on YESCA plates. After 3 days, mixed colonies were suspended in PBS, series diluted and plated on YESCA-CR plates. *S. typhimurium* curli mutants formed pink colonies on YESCA-CR agar while *E. coli* curli mutants formed white colonies. Colony forming units (CFU) of pink or white colonies were measured and plotted as 100% stacked columns. (B) Western analysis bacteria with underlying agar of *E. coli* A<sup>-</sup>B<sup>+</sup> / *S. typhimurium* A<sup>+</sup>B<sup>-</sup> mixed colonies or *E. coli* A<sup>+</sup>B<sup>-</sup> / *S. typhimurium* A<sup>-</sup>B<sup>+</sup> mixed colonies. Samples were pre-treated with (+) or without (-) HFIP before electrophoresis and CsgA was probed by αCsgA antibody.



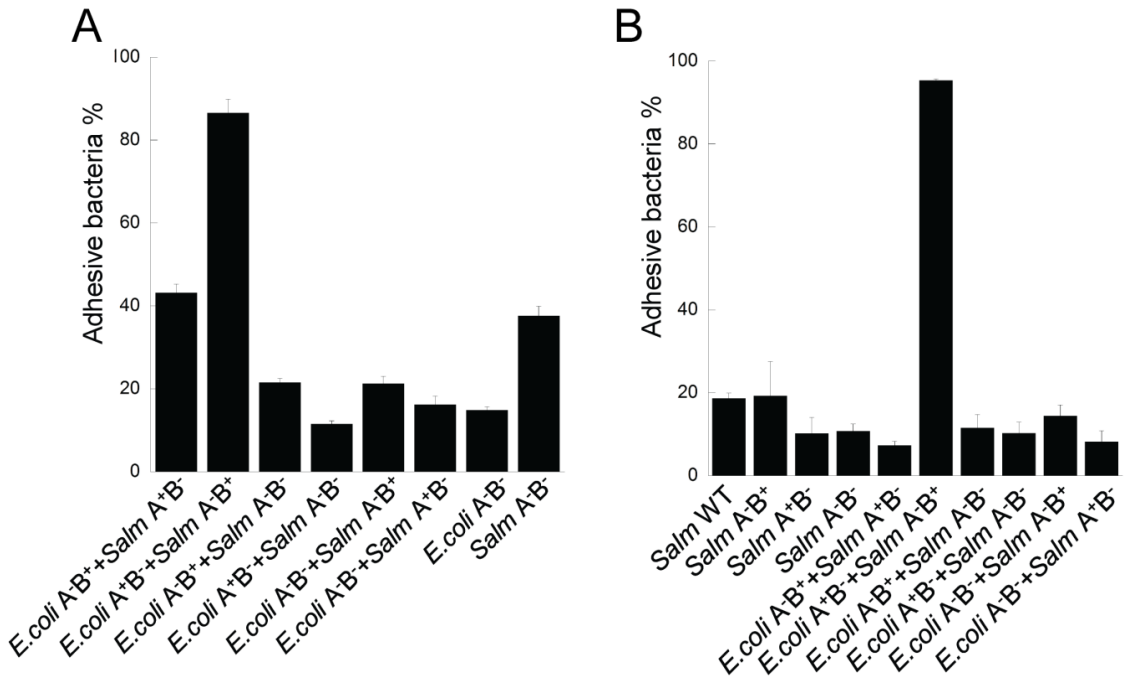
**Figure 2.12. Interbacterial curli formation between *E. coli* and *S. typhimurium* curli mutants restored bacteria adherence to the agar surface.**

Interbacterial curli formation between *E. coli* and *S. typhimurium* curli mutants restored bacteria adherence to agar surface. (A) Overnight cultures of *E. coli*, *S. typhimurium*, or a 1:1 (OD<sub>600</sub>/OD<sub>600</sub>) mixture of *E. coli* and *S. typhimurium* curli mutants were spread on YESCA agar in 12-well tissue culture plates, then incubated at 26°C for 3 d. Bacterial adhesiveness was determined by the percentage of adhered bacteria after vigorous washing as described in the Experimental Procedures. (B) Overnight cultures of *E. coli* constitutively expressing YFP from plasmid pAH16, *S. typhimurium* expressing constitutively expressing mCherry from pAH9 (60), or the mixed culture of *E. coli*/pAH16 and *S. typhimurium*/pAH9 were spread on YESCA agar on 12-well tissue culture plates, incubated at 26°C for 3 d and washed in PBS with vigorous shaking. Percentage of adhesive *S. typhimurium* was determined by mCherry signal with the excitation wavelength at 600±9 nm and the emission wavelength at 630±20 nm.



**Figure 2.13. The increase in bacterial adherence was CsgB-dependent.**

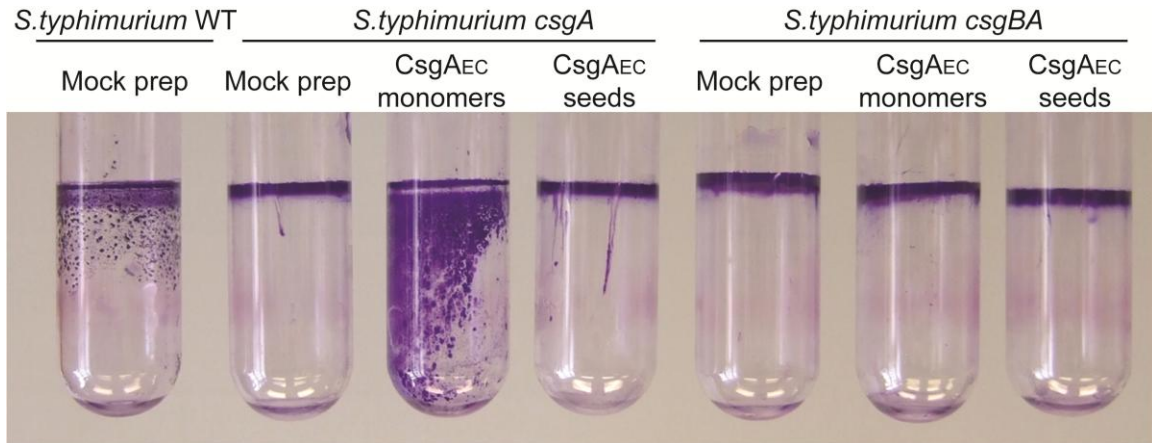
The increase in bacterial adherence was CsgB-dependent. (A) Overnight culture of *E. coli*, *S. typhimurium* or 1:1 (OD<sub>600</sub> ratio) mixture of both species as indicated in the figure were spread on YESCA and incubated at 26°C for 3 d. Bacteria were washed in 1ml PBS with vigorous shaking for 30 min. OD<sub>600</sub> of non-adhesive bacteria (both *E. coli* and *S. typhimurium*) and bacteria adhere to the agar after washing were measured as described in the Experimental Procedures. The percentage of adhered bacteria in total population was graphed. (B) *E. coli*, *S. typhimurium* or the mixed culture grown on YESCA agar for 3 d were washed in PBS with vigorous shaking. Bacteria washed off into PBS or adhere to the agar were series diluted and spread on YESCA-CR plates. *E. coli* curli mutants stained white and *S. typhimurium* curli mutant stained pink on YESCA-CR agar. Percentage of adhesive *S. typhimurium* was determined by CFU of pink colonies.





**Figure 2.14. Pellicle formation of *S. typhimurium* *csgA*- was complemented by exogenous *E. coli* CsgA subunits.**

Pellicle formation of *S. typhimurium* *csgA*- was complemented by exogenous *E. coli* CsgA subunits. *S. typhimurium* wild-type, *csgA*- or *csgBA*- mutants were incubated in static LB-no salt for 3 d at 26°C in glass tubes. 10 µg/ml of freshly purified CsgA<sub>EC</sub> or CsgA<sub>EC</sub> seeds, or products from a mock CsgA purification from strains harboring an empty vector was added to each tube. Pellicles were stained with crystal violet (CV) for visualization.



**Table 2.1. Strains used in this study**

Strains	Relevant characteristics	References
MC4100	F- <i>araD139</i> $\Delta$ ( <i>argF-lac</i> ) U169 <i>rpsL150(strR)</i> <i>relA1 fibB5301 deoC1 ptsF25 rbsB</i>	(87)
<i>E. coli csgA-</i> (LSR10)	MC4100 $\Delta$ <i>csgA</i>	(7)
<i>E. coli csgB-</i> (MHR261)	MC4100 $\Delta$ <i>csgB</i>	(88)
<i>E. coli csgBA-</i> (LSR13)	MC4100 $\Delta$ <i>csgBA</i>	(63)
<i>S. typhimurium</i> LT2	<i>Salmonella enterica</i> subsp. <i>enterica</i> serovar Typhimurium str. LT2	From Dr. Blaise Boles
<i>S. typhimurium csgA-</i>	<i>S. typhimurium</i> LT2 <i>csgA::kan</i>	This study
<i>S. typhimurium csgB-</i>	<i>S. typhimurium</i> LT2 <i>csgB::kan</i>	This study
<i>S. typhimurium csgBA-</i>	<i>S. typhimurium</i> LT2 <i>csgBA::kan</i>	This study
NEB 3016	<i>fhuA2 lacZ::T7 gene1 [lon] ompT gal sulA11</i> <i>R(mcr-73::miniTn10--Tet<sup>S</sup>)2 [dcm] R(zgb-</i> <i>210::Tn10--Tet<sup>S</sup>) endA1 <math>\Delta</math>(mcrC-</i> <i>mrr)114::IS10 [mini-lacI<sup>f</sup> (Cam<sup>R</sup>)]</i>	NEB

**Table 2.2. Plasmids used in this study**

Plasmids	Relevant characteristics	References
pLR2	Vector with <i>csgBA</i> promoter	(52)
pCsgA <sub>EC</sub>	<i>E. coli csgA</i> cloned into pLR2. Previously named as pLR5.	(63)
pCsgA <sub>ST</sub>	<i>S. typhimurium csgA</i> cloned into pLR2.	This study
pCsgA <sub>CK</sub>	<i>C. koseri csgA</i> cloned into pLR2.	This study
pCsgA <sub>SO</sub>	<i>S. oneidensis csgA</i> cloned into pLR2	This study
pCsgBA <sub>EC</sub>	<i>E. coli csgBA</i> cloned into pLR2, previously named as pLR30.	This study
pCsgB <sub>EC</sub>	<i>E. coli csgB</i> cloned into pLR2. Previously named as pLR8.	(51)
pCsgB <sub>ST</sub>	<i>S. typhimurium csgB</i> cloned into pLR2.	This study
pCsgB <sub>CK</sub>	<i>C. koseri csgB</i> cloned into pLR2.	This study
pCsgB <sub>SO</sub>	<i>S. oneidensis csgB</i> cloned into pLR2.	This study
pET11d	Expression vector	NEB
pNH11	C-terminal His <sub>6</sub> tagged <i>E. coli csgA</i> cloned into pET11d	(89)
pET11d-CsgA <sub>ST</sub>	C-terminal His <sub>6</sub> tagged <i>S. typhimurium csgA</i> cloned into pET11d	This study
pET11d-CsgA <sub>CK</sub>	C-terminal His <sub>6</sub> tagged <i>C. koseri csgA</i> cloned into pET11d	This study
pET11d-CsgA <sub>SO</sub>	C-terminal His <sub>6</sub> tagged <i>S. oneidensis csgA</i> cloned into pET11d	This study
pAN1	C-terminal His <sub>6</sub> tagged <i>E. coli csgB</i> cloned into pET11d	(89)
pET11d-CsgB <sub>ST</sub>	C-terminal His <sub>6</sub> tagged <i>S. typhimurium csgB</i> cloned into pET11d	This study
pAH9	Plasmid for mCherry expression	(60)
pAH16	Plasmid for YFP expression	(60)

**Table 2.3. Primers used in this study**

Primer	Primer Sequence	Constructs
WD1	5'-CACGACCCATGGCGAACTTTTAAAAGTGG CAG-3'	pCsgA <sub>ST</sub> , pCsgA <sub>CK</sub>
WD2	5'-CAGCTTGGATCCTTAATACTGGTTAGCCGT GG-3'	pCsgA <sub>ST</sub> , pCsgA <sub>CK</sub>
YZ15-f	5'-GCGTTTCCATGGGCGTCGTTCCACAATGGG GCGG-3'	pET11d-CsgA <sub>ST</sub>
YZ15-r	5'-GTTTAAAGCTTGGATCCTTAGTGATGGTGA TGGTGAT GATACTGGTTAGCCGTGGCGTTG-3'	pET11d-CsgA <sub>ST</sub> pET11d-CsgA <sub>CK</sub>
YZ16-f	5'-GCGTTTCCATGGGTGTTGTTCCGCAGTGGG GCGGT-3'	pET11d-CsgA <sub>CK</sub>
YZ17-f	5'-GCGCCATGGCGAAAAACAAATTGTTATTTA TG-3'	pCsgB <sub>ST</sub> , pCsgB <sub>CK</sub>
YZ21-r	5'-GCGGGATCCTTAACGTTGCGTAACGCG-3'	pCsgB <sub>CK</sub>
YZ22-r	5'-GCGGATCCTTAGCGTTGGGTGACGCGAAT AG-3'	pCsgB <sub>ST</sub>
YZ22-f	5'-GCTACCATGGCGACAAATTATGATCTG-3'	pET11d-CsgB <sub>ST</sub>
YZ22-r	5'-GCGGATCCTTAGTGATGGTATGGTGATGGC GTTGGGT GACGCGAATAG-3'	pET11d-CsgB <sub>ST</sub>
YZ25-f	5'-GCGTTTCCATGGCAAGTACGATCAACGAAA TC	pET11d-CsgA <sub>SO</sub>
YZ25-r	5'-GCGGATCCTTAGTGATGGTATGGTGATGG TATTGCA CTACAGTCG-3'	pET11d-CsgA <sub>SO</sub>
YZ47	5'-CAACGCTAATACCGTTACGACTTTTAAATCA ATCCGATGGGGTTTTACCCACCAAACACCCCC CAAACC-3'	<i>S. typhimurium</i> csgA-
YZ48	5'-CAGGGCTTATGCCCTGTTTTTTTATTAGCGC AGACGCT AAACACACAACCACACCACACCAC-3'	<i>S. typhimurium</i> csgA- and csgBA-
YZ50	5'-CAAGGTAATAGATAATTTTCGCTATGTACG ACCAGGTCCAGGGTGACAGCCACCAAACACCCCC AAAACC-3'	<i>S. typhimurium</i> csgB- and csgBA-
YZ51	5'-AAGTTTCATGGTAAAACCCCATCGGATTG ATTTAAAAGTCGTAACGGTACACACAACCACA CCACACCAC-3'	<i>S. typhimurium</i> csgB-

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## Chapter 3 <sup>3</sup>

### UTI89 pellicle biofilm formation and a self-produced biofilm inhibitor

#### Abstract

Uropathogenic *E. coli* (UPEC), the major cause of urinary tract infections, develops distinct biofilm types under different environmental conditions. Outside the host in static cultures, UPEC strain UTI89 produces a pellicle biofilm that floats at the air-liquid interface. Several extracellular structures including curli, flagellum, and cellulose are required for robust pellicle formation. Biofilm development is a dynamic process. Bacteria have been shown to produce biofilm inhibitors as a culture ages. In this study, we found that a bacteria-free, conditioned medium from a static culture of UTI89 impeded UTI89 pellicle formation. The inhibitory effect was not linked to changes in pH, low nutrient availability, or altered cell growth. Biochemical analyses indicated the potential antibiofilm factor was heat-stable and proteinase resistant. A candidate approach further revealed that the antibiofilm effect of the conditioned medium was dependent on cysteine metabolism. The cysteine biosynthesis defective mutants,  $\Delta cysE$  and  $\Delta cysK\Delta cysM$  were unable to produce the antibiofilm factor. The antibiofilm activity

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<sup>3</sup> Parts of this section are derived from Hung C, **Zhou Y**, Cusumano CK, Pinkner JS, Dodson KW, Roth R, Walker J, Crowley JR, Heuser J, Chapman MC, Henderson JP, Hultgren SJ. Formation of Uropathogenic *E. coli* Pellicle Biofilm Involves Extensive Fibrous Structures and Complex Components in the Extracellular Matrix. *Manuscript in prep for J Bacteriology*.

can be restored specifically by supplementing  $\Delta cysE$  or  $\Delta cysK\Delta cysM$  culture with exogenous cysteine or cystine, but not other thiols such as D-cysteine or glutathione. The deficient pellicle formation in the conditioned medium coincided with reduced *csgD* level and curli production.

## **Introduction**

Many bacteria live as members of complex communities called biofilms (1-3). Bacteria living within biofilms are encased in an extracellular matrix composed of proteins, exopolysaccharides, and extracellular DNA (4). Biofilms are linked with a variety of chronic infections such as lung infection in cystic fibrosis, wound infections, and urinary tract infections (UTIs), among others (3,5-7). Biofilm bacteria are typically more resistance to antimicrobial agents and environmental stress. The antibiotic resistance is mediated by various characteristics of the biofilm, including the distinct sessile life style of bacteria, induced stress responses, diffusive small signaling molecules, and the physical barriers established by biofilm matrix (8-11).

*E. coli* causes 70-95% of UTIs. A clinical uropathogenic *E. coli* (UPEC) isolate, UTI89, forms biofilm-like intracellular bacteria communities in bladder epithelium cells which protect UTI89 from immune clearance (6,12). In a laboratory setting, UTI89 develops rugose colony biofilms on solid agar surfaces and pellicle biofilms floating at the air-liquid interface in static cultures (13,14). The development of colony and pellicle biofilms requires expression of curli and cellulose (14,15). Both curli and cellulose were found in the pellicle matrix ((14) and Figure 3.1)), and expression is controlled by the master regulator CsgD (16-18).

Curli are functional amyloid fibers produced on the surface of many enteric bacteria (19). The adhesiveness and stable nature of curli fibers makes them perfect structures for facilitating both bacteria-surface and bacteria-bacteria interactions (20). Bacteria unable to assemble curli fibers were defective in pellicle formation (Figure 3.2 and (14,21)). A cellulose synthase mutant, *ΔbcsA*, formed fragile pellicles that were frequently collapsed or partially submerged (Figure 3.2). In addition to curli and cellulose, flagella and type 1 pili are also involved in pellicle formation. Deletion of the type 1 pilus subunit gene *fimA* resulted in a strain that exhibited a delayed pellicle formation phenotype and loss of the typical wrinkled morphotype (Figure 3.2). Similarly, flagellum mutants, *ΔfliC* and *ΔflhDC*, formed small floating "rosette-like" microcolonies (Figure 3.2). These microcolonies were unable to merge into an intact pellicle biofilm. Once a mature pellicle was formed, flagella were detected only in planktonic bacteria underneath the pellicle, but not within pellicles (Figure 3.2), suggesting that flagella are required for initial biofilm formation and are shed off by bacteria in later biofilm development stages.

As a biofilm culture ages, carbon and nitrogen are depleted and metabolic wastes accumulate in the culture. Bacteria underneath the pellicles are under oxygen limitation (22,23). Many bacteria are capable of producing metabolic intermediates or structural components that prevent their own biofilm formation or inhibit biofilm development of other species (24-30). For instance, *Staphylococcus aureus* is capable of using the accessory gene regulator (*agr*) system, which can be activated by a cyclic autoinducing peptide secreted to the environment, to mediate biofilm dispersion (24). *Bacillus subtilis* releases D-amino acids and norspermidine as the culture ages, which not only disperse its own pellicle biofilm, but can also prevent biofilm formation of a broad spectrum of

bacteria species (25,26). The gram-negative bacterium *Vibrio cholerae* uses quorum sensing molecules which repress biofilm matrix production at high cell density (31,32). In *E. coli*, self-produced indole and capsular polysaccharides were reported to have antibiofilm activity (27,29,30).

In this study, we found an aged biofilm culture of UTI89 demonstrated antibiofilm activity against its own pellicle formation. Molecular analysis suggests that the potential biofilm inhibitor is heat-stable and therefore not protein-based. Genetic studies revealed that the antibiofilm activity of the conditioned medium is dependent on cysteine metabolism.

## **Experimental Procedures**

### **Bacteria strains and growth condition**

A fully sequenced and well-characterized clinical UPEC isolate, UTI89, was used in this study (33,34). UTI89 mutants were generated by red-swap as described (35) or kindly provided by the Hutgren lab. These mutants are listed in Table 3.1. Strains used in this study Primers used to construct these mutants are listed in Table 3.2. Primers used in this study Unless otherwise stated, bacteria were typically grown in LB at 37°C with shaking.

### **Pellicle biofilm**

UTI89 pellicle cultures were grown in YESCA media (1% casamino acid and 0.12% yeast extract) in 24-well plastic plates (TPP, Switzerland). Briefly, bacteria were grown in 3 mL LB broth from single colonies overnight. Subsequently, bacteria were diluted 1,000-fold into 2 mL/well YESCA media to initiate pellicle biofilm culture.

Unless specifically indicated, pellicle biofilm cultures were incubated at 26 °C for 32-48 hours before analyses were performed. For better visualization, each well was stained with 2 mL of 0.1% crystal violet and washed with PBS twice.

To prepare conditioned media, the overnight culture of UTI89 was diluted by 1:1,000 fold into 3-6 mL YESCA media in a glass tube and incubated statically at 26°C for 4 d. Bacteria-free conditioned media were made by passing the 4 d old cultures through a 0.2 mm syringe filter and stored at 4°C for no longer than 2 weeks.

### **Western blot analysis**

Bacteria from UTI89 pellicle cultures were subjected to Western blot analysis to determine the presence of bacterial proteins. To test protein levels in a mature biofilm, pellicle and planktonic bacterial populations from the same biofilm cultures were harvested after 72 hr static growth. Bacteria in the pellicles were mechanically separated by brief homogenization for 20 seconds to ensure viability of bacteria. Cell densities of both homogenized pellicle and planktonic populations were normalized based on optical density at 600 nm. To test protein expression in UTI89 grown in conditioned media or the fresh medium, bacteria grown at 26°C for 24 hr were collected, including biofilm cells and planktonic cells. Collected bacteria were homogenized for 20 seconds to separate bacteria in the pellicle.

For each Western blot analysis, 150 µL of normalized bacterial suspension was processed further according to the protein of interest. Western blot analysis of CsgA was performed as previously described (36). Briefly, bacterial pellets from pellicle or planktonic cultures were treated with or without 70 µL HFIP to depolymerize curli fibers. HFIP treated samples were then dried in a Speedvac at 45 °C for 35 min and resuspended

in 150  $\mu$ L 2X SDS loading buffer. Bacterial pellets not treated with HFIP were resuspended directly in 150  $\mu$ L 2X SDS loading buffer. Non-HFIP treated samples were also used for detection of FliC. For Western analysis of type 1 pili, bacterial lysates in 150  $\mu$ L SDS loading dye was acidified with 3  $\mu$ L 1M HCl, boiled for 5 min and then neutralized with 3  $\mu$ L 1M NaOH (37). Normal cell lysate in 2X SDS loading buffer was used for Western analysis of FliC, RpoS and CsgD. 7  $\mu$ L of each sample was resolved on 15% SDS-PAGE gels. Subsequent to electrophoresis, proteins were transferred onto PVDF membrane by semidry blotting apparatus (FisherBiotech, Fisher Scientific, Pittsburgh, PA) at 10V for 20 min. For CsgD and RpoS, wet transfer was carried out as described (38). Blots were probed with primary antisera (against CsgA, FimA, FimCH complex, CsgD, RpoS or FliC) followed by HRP-conjugated goat anti-rabbit or anti-mouse secondary antibodies (Sigma, St. Louis, MO), or IRDye secondary antibody (LICOR Biosciences, Lincoln, NE). Rabbit anti-CsgA antiserum was custom-generated against purified CsgA proteins (Proteintech Group, Inc., Chicago, IL). Rabbit antiserum raised against type 1 pili and FimCH complexes were custom-generated against purified type 1 pili and FimCH protein complexes, respectively (Sigma Genosys, St. Louis, MO). Anti-FliC antiserum was kindly provided by Dr. Harry Mobley (39). Rabbit anti-CsgD antibody was provided by Dr. Ute Romling.

### **Growth curve measurements**

UTI89 wild-type or mutants were inoculated in LB medium at 37°C overnight and diluted by 1:1000 folds into 200  $\mu$ L YESCA, YESCA mixed with H<sub>2</sub>O or YESCA mixed with the conditioned medium in wells on a 96-well microtiter plate. The plate was sealed with a transparent sticker. OD<sub>600</sub> was measured by a plate reader (Molecular Devices,

Sunnyvale, CA) every hour in a 48 hr period of time. The fresh YESCA medium with no bacteria was used as the control.

## **Results**

### **Conditioned UTI89 cultures impeded its own biofilm formation**

To investigate if UTI89 produce anti-biofilm molecules during growth, we collected filter-sterilized UTI89 biofilm culture media after biofilms had grown at 26°C for 3-5 d. Conditioned media was mixed with fresh YESCA media at a 1:1 (v/v) ratio, which was then used in pellicle biofilm assays. A deficient biofilm formation phenotype was observed for UTI89 grown in conditioned media (Figure 3.3A). UTI89 was only able to form a thin, fragile pellicle in conditioned media collected from 3 d old cultures, and was completely unable to form a pellicle in conditioned media collected from 4 d or 5 d old culture after 48 hr of incubation (Figure 3.3A). As a control, UTI89 formed robust pellicles in fresh YESCA or fresh YESCA mixed 1:1 v/v with H<sub>2</sub>O, indicating that the deficient pellicle formation in conditioned media is not due to nutrient limitation (Figure 3.3A). Additionally, no substantial growth defects were observed for UTI89 grown in the conditioned medium in comparison to that in the fresh medium or diluted fresh medium (Figure 3.3B).

### **Molecular characterization of the conditioned medium**

Environmental factors such as pH play a role in biofilm development, sometimes by regulating curli production. Indeed, the pH of UTI89 static cultures increased from 6.6 to 8.1 after 4 d of incubation. To test if an elevated pH contributes to deficient biofilm formation, we adjusted the pH of the fresh medium to 8.1 by sodium hydroxide and

found that pellicle formation was not affected (Figure 3.4A). Consistent with this result, UTI89 was unable to form a pellicle in the conditioned medium buffered to pH 6.6 (Figure 3.4A). Thus, the inhibitory effect of the conditioned medium does not result from changes in pH.

To further characterize the potential antibiofilm factor in the conditioned medium, we boiled the conditioned medium at 95 °C for 60 min or pretreated the conditioned medium with proteinase K. The conditioned medium that was boiled or treated with proteinase K maintained the ability to inhibit pellicle formation (Figure 3.4B), suggesting that the antibiofilm factor is not likely to be a protein. It is, however, still possible that polysaccharides or heat-stable small molecules are the antibiofilm factor. Moreover, conditioned media collected from pellicle defective *ΔcsgD* and *ΔcsgBA* mutants also inhibited pellicle formation of UTI89 (Figure 3.4C), suggesting the antibiofilm activity is not solely produced by bacteria in the pellicle. Instead, the predominant antibiofilm factor is produced by planktonic cells. Further separation by size exclusion spin units showed that neither the retention nor the effluent exhibited antibiofilm activity (Figure 3.5). It is possible that the antibiofilm agent was trapped by the cellulose membrane or lost activity.

### **The antibiofilm effect of the conditioned medium was not due to known biofilm inhibitors**

In order to identify the potential antibiofilm component in the conditioned medium, a candidate approach was utilized. *E. coli* K-12 is able to produce large amount of indole in a rich medium, which in turn decreases its biofilm formation (29,30). To test if indole is the antibiofilm agent in conditioned media, we added indole exogenously into the fresh media inoculated with UTI89. Addition of indole had no effect on pellicle



development even at relatively high concentrations (Figure 3.6A). Moreover, conditioned media collected from 4 d old culture of a tryptophanase mutant *ΔtnaA*, which is unable to produce indole, still exhibited antibiofilm activity (Figure 3.6A), suggesting the biofilm-inhibiting effect is not a result of indole or its downstream products.

Uropathogenic *E. coli* also produces and releases soluble capsular polysaccharides that prevent surface attachment and biofilm formation of a broad spectrum of Gram-negative and Gram-positive bacteria. To test if capsular polysaccharides contribute to the antibiofilm activity, wild-type UTI89 was inoculated in conditioned media from capsular polysaccharide synthesis mutants *ΔkpsF* and *ΔkpsS*. Conditioned media from these mutants efficiently inhibited pellicle formation of UTI89 (Figure 3.6B), suggesting that capsular polysaccharides are not likely to be the antibiofilm agent in the conditioned medium.

Additionally, we analyzed the ability of norspermidine and D-amino acids to inhibit pellicle biofilm formation. Norspermidine and D-amino acids are antibiofilm metabolites produced by *B. subtilis* (25,26). Norspermidine is also reported to effectively inhibit biofilm formation by *E. coli* (25). However, under the conditions tested, supplementation of UTI89 cultures with norspermidine, D-amino acids or the combination of the two agents had no inhibitory effect on pellicle development (Figure 3.6C).

### **The inhibitory effect of the conditioned medium was dependent on cysteine biosynthesis**

Cysteine and the precursor O-acetyl-serine (OAS) have been shown to modestly inhibit biofilm formation of *S. aureus* and *E. coli* MG1655 (40,41). Interestingly,

conditioned media harvested from UTI89  $\Delta cysE$  and  $\Delta cysK\Delta cysM$ , mutants, cysteine auxotrophs, supported pellicle formation of UTI89. CysE is a serine acetyltransferase that converts serine to OAS (Figure 3.7A) (42). The OAS sulfhydrylases CysK and CysM further convert OAS to cysteine (Figure 3.7A) (43-45). Restoration of pellicle formation in conditioned media from  $\Delta cysE$  and  $\Delta cysK\Delta cysM$  mutants was not because of an increase of bacteria growth or higher cell density, as the growth rate of UTI89 in the  $\Delta cysE$  conditioned medium was almost identical to that in the wild-type conditioned medium (Figure 3.8). The antibiofilm effect was restored by expressing *cysE* or *cysK* *in trans* from a plasmid in a  $\Delta cysE$  mutant or a  $\Delta cysK\Delta cysM$  mutant, respectively (Figure 3.7B).

To investigate if the antibiofilm activity of the conditioned medium is cysteine-dependent, cultures of  $\Delta cysE$  or  $\Delta cysK\Delta cysM$  mutants were supplemented with exogenous cysteine at various concentrations from 20  $\mu\text{M}$  to 200  $\mu\text{M}$ , grown for 4 d, and the resulting conditioned media were collected. Conditioned media from the  $\Delta cysE$  or  $\Delta cysK\Delta cysM$  supplemented with cysteine regained the antibiofilm activity (Figure 3.7C). Remarkably, incubation of the  $\Delta cysE$  or  $\Delta cysK\Delta cysM$  culture with 20  $\mu\text{M}$  cysteine was sufficient to restore the antibiofilm activity of the resulting conditioned medium. Supplementation of  $\Delta cysE$  or  $\Delta cysK\Delta cysM$  mutants with cysteine also restored the antibiofilm activity of the conditioned media collected from these two mutants (Figure 3.7C). Together, these results demonstrate that the antibiofilm activity of the UIT89 conditioned medium is dependent on cysteine/cystine metabolism.

Cysteine itself did not directly function as a biofilm inhibitor, as pellicle formation was not prevented in the fresh medium supplemented with cysteine (Figure

3.9A). Consistent with this result, post-addition of cysteine to conditioned media collected from a  $\Delta cysE$  and  $\Delta cysK\Delta cysM$  cultures did not complement the antibiofilm effect (Figure 3.9B). Moreover, the precursor OAS also showed no inhibitory impact on pellicle formation in the fresh medium (Figure 3.9C).

Cysteine is a strong reducing agent involved in intracellular redox balance (46,47). Addition of the strong reducing agent dithiothreitol (DTT) did not affect pellicle formation (Figure 3.10A). Other thiols including glutathione (GSH) and D-cysteine also had no effect on pellicle formation in the fresh medium (Figure 3.10A). Conditioned media from glutathione mutants,  $\Delta gshA$  and  $\Delta gshB$ , still maintained antibiofilm activity like that from wild-type (Figure 3.10B). In addition, incubation of  $\Delta cysE$  or  $\Delta cysK\Delta cysM$  mutants with DTT, glutathione, or D-cysteine did not restore the antibiofilm activity of the resulting conditioned culture (Figure 3.10C and data not shown). Collectively, these results suggest the antibiofilm activity is specifically dependent on cysteine/cystine and is not due to alteration of redox state.

### **Deficient biofilm formation in the conditioned medium coincided with reduced curli production**

Pellicle development is dependent on curli production. We asked if curli expression in UTI89 grown in the conditioned medium was reduced. Curli production in UTI89 grown in the fresh medium, the diluted fresh medium, or the conditioned medium collected from 4 d old wild-type UTI89 culture was analyzed by western blot. UTI89 grown in the fresh medium or the diluted fresh medium for 24 hr produced bacteria associated CsgA, whereas no CsgA was detected from UTI89 grown in the wild-type conditioned medium (Figure 3.11A). Interestingly, curli production was partially restored

in UTI89 grown in conditioned media collected from a  $\Delta cysE$  culture and a  $\Delta cysK\Delta cysM$  culture (Figure 3.11A), suggesting curli production is negatively correlated with the antibiofilm activity of conditioned media. Similarly, CsgD protein level was significantly reduced in UTI89 grown in the wild-type conditioned medium compared to that in fresh medium. Bacteria grown in the  $\Delta cysE$  conditioned medium or the  $\Delta cysK\Delta cysM$  conditioned medium showed higher CsgD level than those in the wild-type conditioned medium (Figure 3.11A). Bacteria grown under these medium conditions showed no differences in RpoS level (Figure 3.11B). Collectively, the defective pellicle formation in the conditioned medium was accompanied with a decrease in curli expression.

## Discussion

As a bacterial culture ages, many bacteria are capable of producing extracellular signals, such as autoinducing peptides, D-amino acids and indole, to trigger biofilm disassembly or prevent biofilm formation (25-30). We found that UTI89 released an antibiofilm factor into the environment as the culture aged (Figure 3.3). The antibiofilm activity of conditioned medium was observed among several commensal *E. coli* strains grown in different types of media (Figure 3.12), suggesting it may be a common mechanism utilized by *E. coli* to prevent planktonic bacteria from switching to a sessile state.

Indole is one of the major metabolites produced by *E. coli* that displays antibiofilm activity (29,30). In rich medium, *E. coli* produces high concentrations of extracellular indole as a stationary phase signal (30). Indole is able to decrease biofilm formation of *E. coli* K12 strains through the homoserine lactone transcriptional regulator,

SdiA (29,48). Although indole and indole derivatives have been reported to efficiently decrease biofilm formation of various *E. coli* strains (29), they do not seem to affect pellicle biofilm formation of UTI89, as robust pellicles were formed in the presence of millimolar concentrations of indole (Figure 3.6A). Moreover, the fact that the indole synthase mutant *AtnaA* was still able to produce antibiofilm activity suggests indole or its derivatives are not pellicle inhibitors (Figure 3.6A). Additionally, a *ΔsdiA* mutant was unable to form a pellicle in the conditioned medium (data not shown). Thus, the antibiofilm activity is not mediated by SdiA. Other than indole, UPEC-derived group II capsular polysaccharide was not responsible for the antibiofilm activity against pellicle (Figure 3.6B). Group II capsular polysaccharide inhibits biofilm formation by remodeling the physicochemical properties of abiotic surfaces and reducing bacterial attachment (27). Pellicle development is more likely to be mediated by bacterial-bacterial interactions instead of bacterial-surface interactions. Thus, it is not surprising that capsular polysaccharides did not affect pellicle development.

Here we revealed the antibiofilm activity of the conditioned medium was a cysteine/cystine dependent product (Figure 3.7). High concentrations of sulfhydryl compounds including cysteine were reported to inhibit biofilm formation in *S. aureus* by reducing biosynthesis of polysaccharide intercellular adhesin (40). *E. coli* exports cysteine and its precursor OAS (47,49,50), and genes involved in cysteine biosynthesis play a role in modulating biofilm development (51,52). Sturgill *et al.* showed that an *E. coli* MG1655 *ΔcysE* mutant, which is unable to catalyze the conversion of serine to OAS, exhibits accelerated biofilm formation with increased biomass, suggesting that cysteine or OAS may act as a biofilm inhibitor intracellularly or extracellularly(41). We have

demonstrated that the conditioned medium from neither  $\Delta cysE$  nor  $\Delta cysK\Delta cysM$  was able to inhibit pellicle biofilm development, and the antibiofilm activity can be restored efficiently by supplementing the mutant culture with cysteine or cystine (Figure 3.7), suggesting that the antibiofilm factor in the wild-type conditioned medium is dependent on cysteine/cystine metabolism. The antibiofilm activity seems to be specially mediated by cysteine/cystine, as other thiols including GSH, D-cysteine and DTT were unable to restore the antibiofilm activity (Figure 3.10). We also noticed that the  $\Delta cysE$  mutant forms a more robust pellicle than wild-type. Therefore, the enhanced biofilm formation phenotype of UTI89 and MG1655 cysteine mutants can be explained by the lack of the extracellular cysteine/cystine dependent signal.

It is unclear how cysteine/cystine serves to prevent pellicle development. Cysteine or the precursor OAS did not impair pellicle development when added directly to fresh cultures even at millimolar concentrations (Figure 3.9). Consistent with this result, we did not detect a significant difference of extracellular cysteine concentration between the fresh medium and the conditioned medium quantified by the method of Gaitonde (53)(data not shown). Also, the inhibitory effect is not likely a result of altered redox balance, as addition of other reducing agents such as DTT and glutathione to  $\Delta cysE$  or  $\Delta cysK\Delta cysM$  cultures did not restore biofilm inhibition (Figure 3.10).

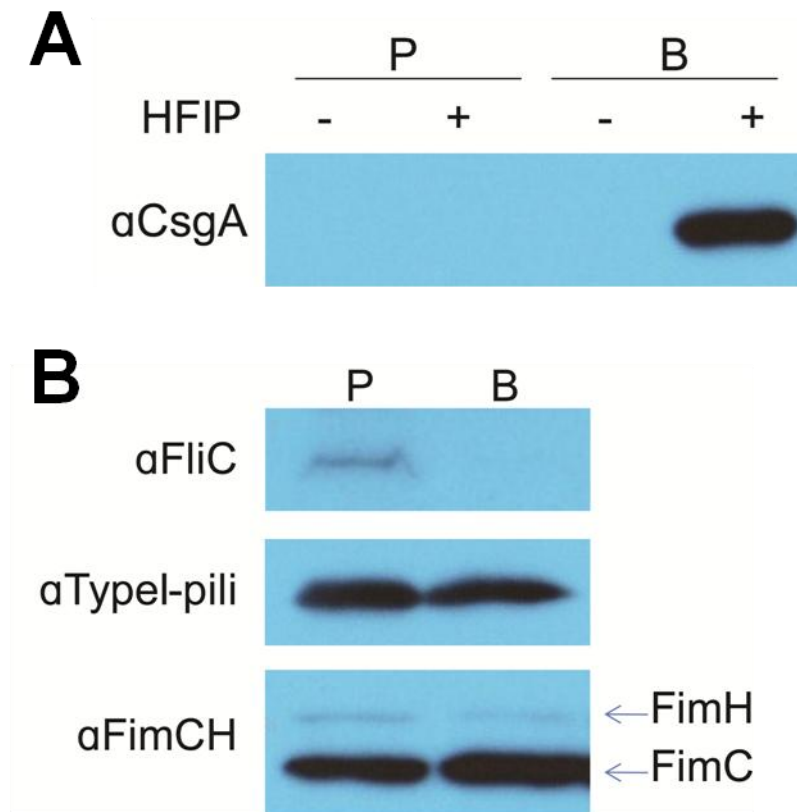
There are several possible mechanisms of how cysteine mediates the production of antibiofilm activity in the conditioned culture. One possibility is, as the UTI89 culture ages, cysteine downstream products are exported and accumulate in the culture medium. These products may play a role in biofilm inhibition. In *E. coli*, cysteine can be converted to glutathione, ammonia, hydrogen sulfide and pyruvate (44,54-57). Although none of

these compounds were found to inhibit pellicle formation (Figure 3.10 and data not shown), we cannot rule out the possibility that accumulation of these metabolites have additive effects against biofilm formation. It is also possible that a novel cysteine-dependent extracellular signal contributes directly to the antibiofilm activity. Alternatively, cysteine metabolism in the aged culture may lead to alteration of other metabolic pathways. Cysteine is linked to homocysteine and methionine biosynthesis and other sulfur metabolism (58). Imbalance of intracellular cysteine may also affect the metabolic network. Biofilm development is sensitive to subtle changes in environmental cues including nutrient composition, levels of phosphate, and osmolarity (4). Thus, subtle changes in extracellular amino acids level or sulfur metabolites may result in deficient pellicle formation.

## Figures and Tables

### Figure 3.1. Expression of extracellular organelles in pellicle and planktonic cells.

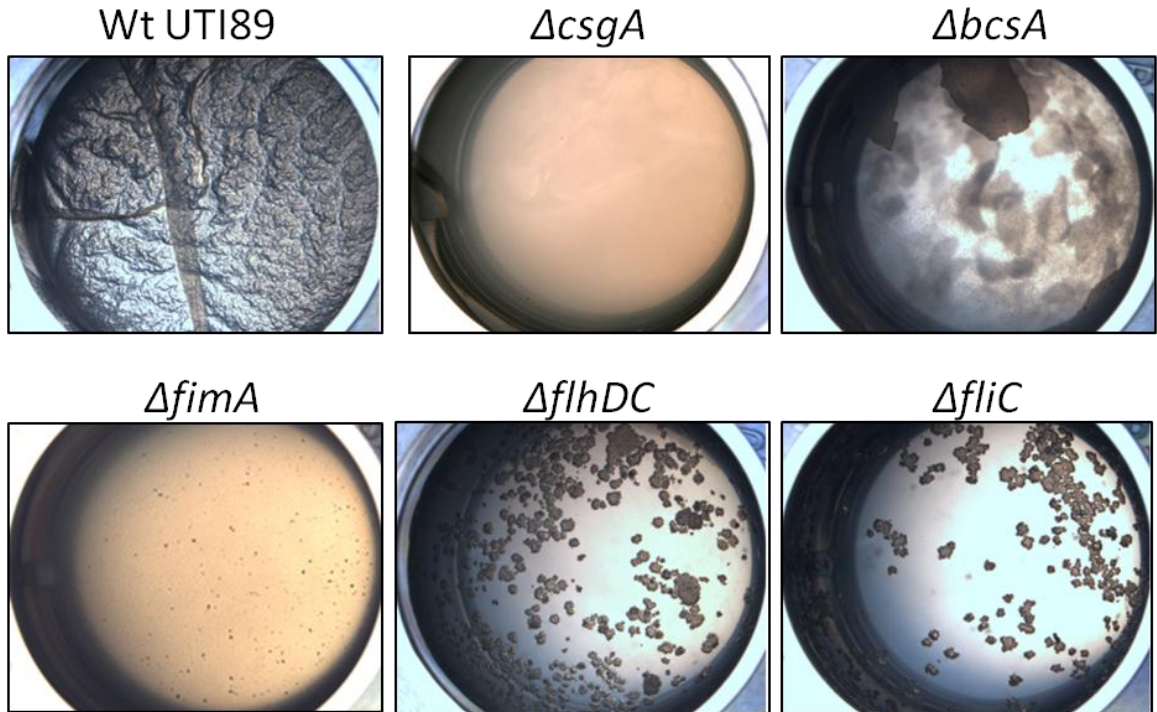
The expression of curli, type 1 pili and flagella in 72 hr old pellicles were examined by Western blot analysis. (A) Western blot analysis with anti-CsgA antiserum revealed the presence of CsgA only in pellicles, not planktonic population. SDS-insoluble CsgA was detected after HFIP treatment (+), which dissociates monomeric CsgA from curli fibers. No CsgA was detected in planktonic bacteria either with (+) or without (-) HFIP treatment. (B) Western blot analysis was performed on planktonic and pellicle bacteria to determine the biosynthesis of flagella (FliC) and type 1 pili (FimA and FimCH) in these bacterial populations. The major pilin subunit, FimA, the periplasmic chaperone, FimC, and the adhesin, FimH, of type 1 pili were all detected in both planktonic and pellicle bacteria. The major flagellin subunit, FliC, was only found in the planktonic population.





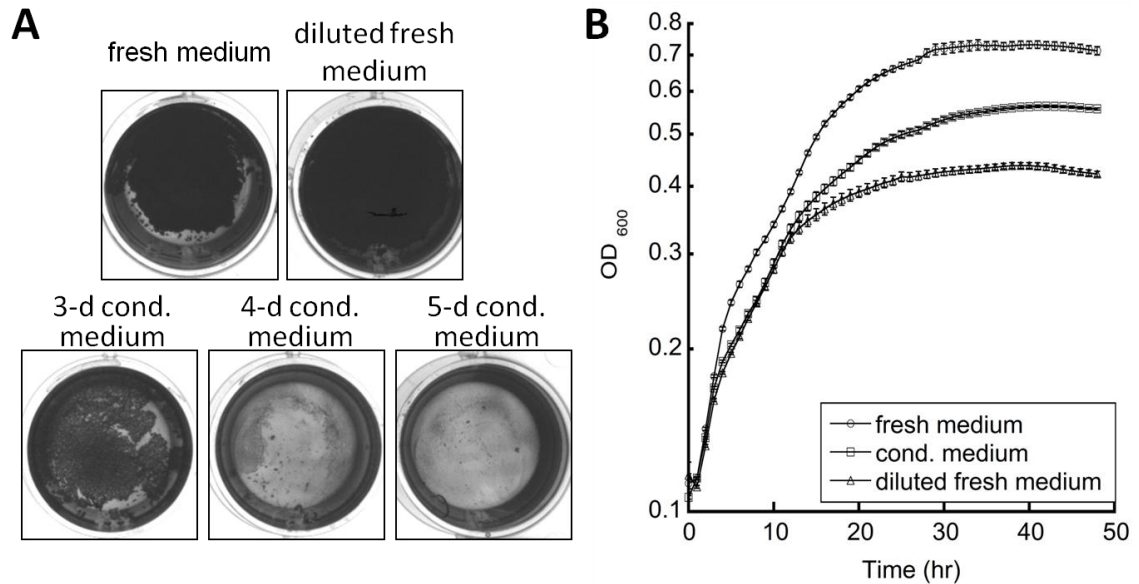
**Figure 3.2. Effect of curli, cellulose, type 1 pili and flagella mutations on pellicle integrity.**

Macroscopic images of UTI89 wild-type,  $\Delta csgA$ ,  $\Delta bcsA$ ,  $\Delta fimA$ ,  $\Delta flhDC$ , and  $\Delta fliC$  grown in YESCA media at 26°C for 48 hr. The  $\Delta csgA$  mutant was able to form a pellicle at all. Without cellulose ( $\Delta csgA$ ), the pellicle broke into pieces and fell to the bottom of the well.  $\Delta fimA$  mutant did not exhibit the typical wrinkled morphology as wild-type. Flagella mutants ( $\Delta flhDC$ , and  $\Delta fliC$ ) were only able to form rosette-like bacterial communities.



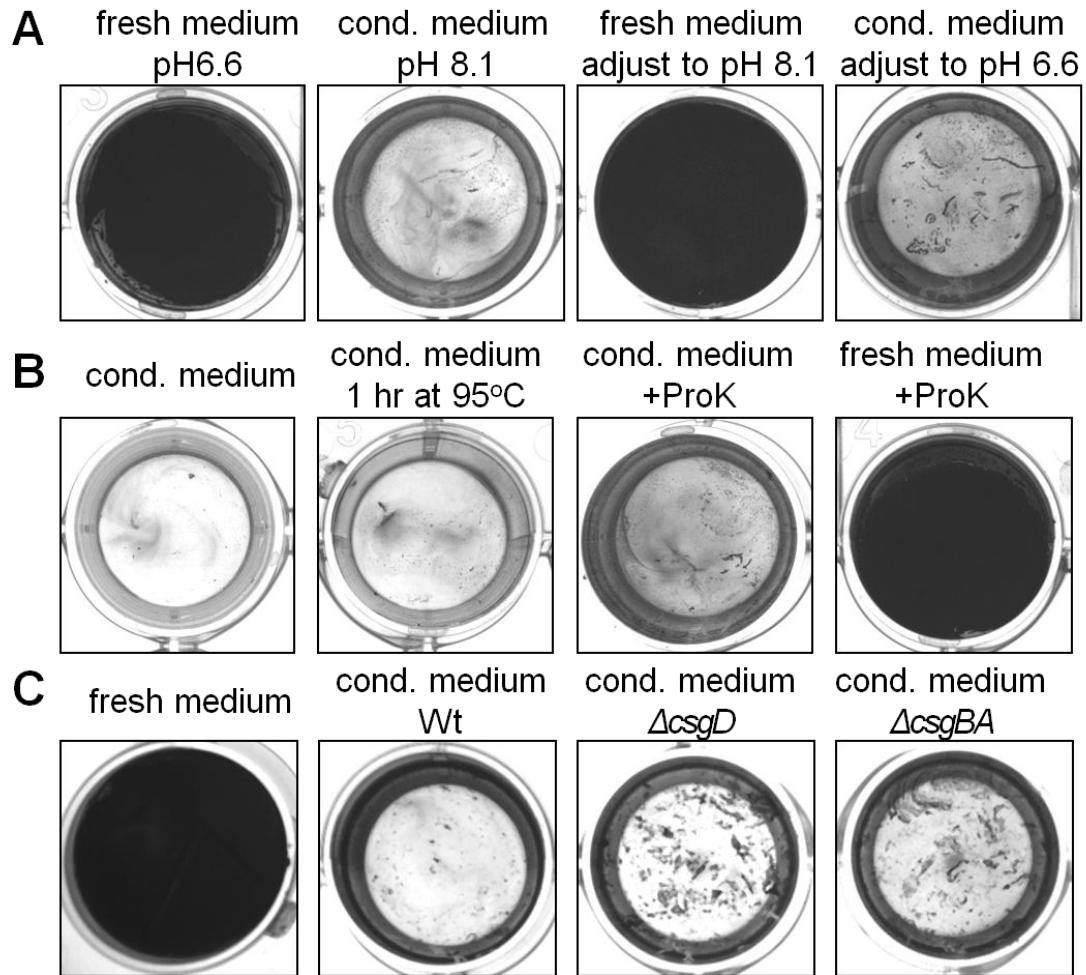
**Figure 3.3. Conditioned media collected from UTI89 cultures impeded UTI89 pellicle formation.**

(A) UTI89 was grown statistically at 26°C in the fresh YESCA medium, the diluted fresh medium, or conditioned media collected from 3-5 d old cultures. Bacteria were grown on a 24 well tissue culture plate for 48 hr. Each well was stained with crystal violet for a better visualization. (B) Growth curves of UTI89 in the fresh YESCA medium (circle), the conditioned medium (square), and the diluted fresh medium (triangle) on a 96-well plate. Bacteria were grown at 26°C statistically for 48 hr. Optical density was measured at 600 nm by a plate reader. Each experiment was measured with 6 replicates.



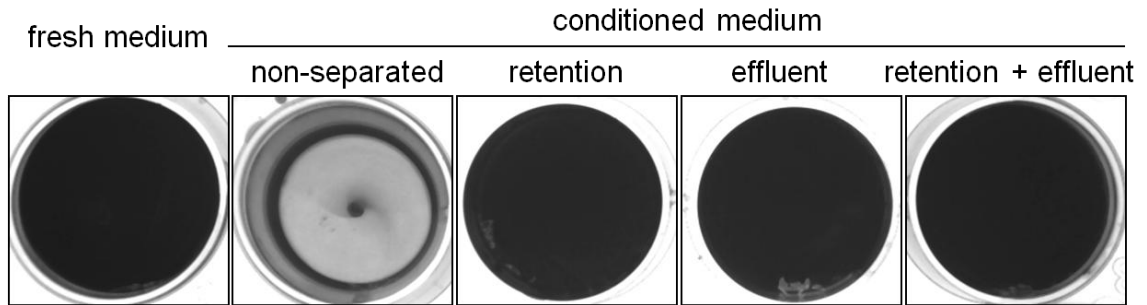
**Figure 3.4. Molecular characterization of the conditioned medium.**

(A) Increase of pH in the conditioned medium was not responsible for the antibiofilm activity. UTI89 grown statically at 26°C for 48 hr in fresh medium (pH6.6), conditioned medium (pH 8.1), fresh medium with pH adjusted to 8.1, or conditioned medium buffered to pH6.6. (B) The potential inhibitor in the conditioned medium was heat stable and was resistant to proteinase K treatment. UTI89 was grown at 26°C for 48 hr in conditioned medium, conditioned medium boiled at 95°C for 1 hr, conditioned medium treated with 0.2 µg/mL proteinase K, or fresh medium treated with proteinase K for 3 hr. Prior to inoculation, proteinase K in the medium was deactivated by boiling at 95 °C for 1 hr. (C) Pellicle defective bacteria were able to produce the potential biofilm inhibitor. UTI89 was grown in fresh medium, or conditioned media collected from wild-type, *ΔcsgD* or *ΔcsgBA* at 26°C for 48 hr. Wells were stained with crystal violet.



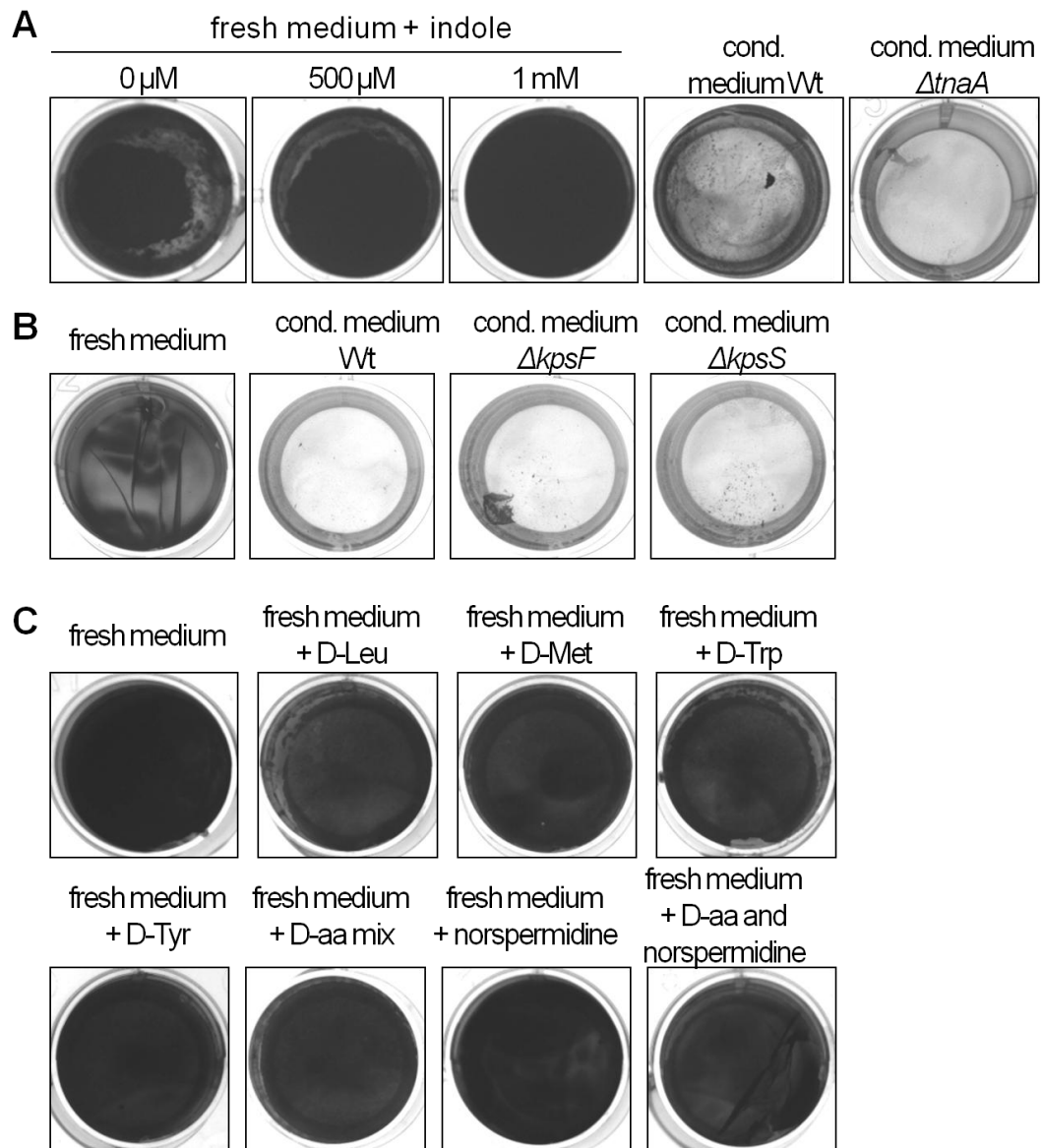
**Figure 3.5. The antibiofilm activity was lost after size exclusion filtration.**

4 mL of the conditioned medium collected from 4 d old UTI89 culture was separated by a 10 kDa cut-off spin column. The retention and the effluent were collected, and the volume of the retention was brought back to 4 mL by water. To test the antibiofilm activity of the fractions, UTI89 was grown in the fresh medium, the non-separated conditioned medium, the retention (> 10 kDa), the effluent (<10 kDa) and a combination of the retention and the effluent mixed by 1:1 (v:v). Each well was stained with crystal violet.



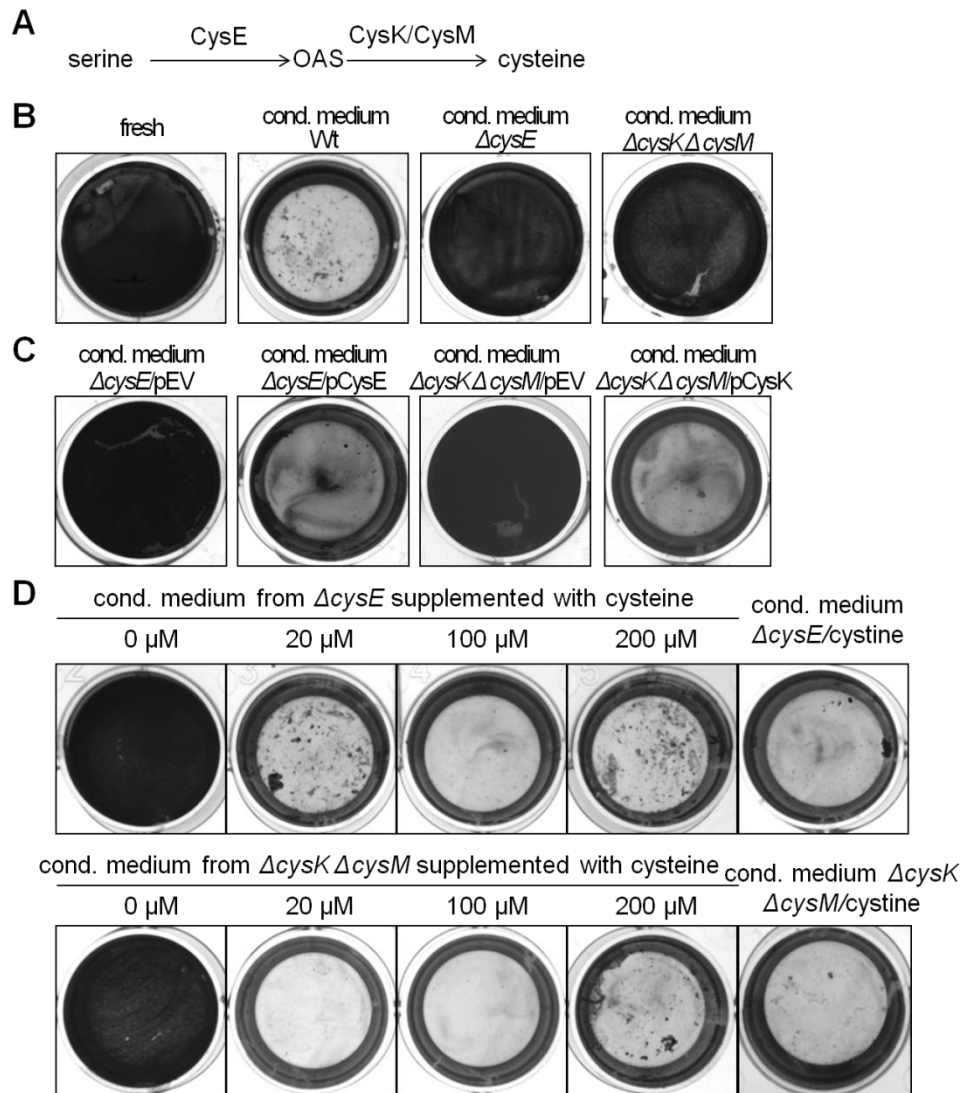
**Figure 3.6. The antibiofilm activity was not due to reported biofilm inhibitors including indole, capsular polysaccharides, D-amino acids and norspermidine.**

(A) UTI89 grown at 26°C for 48 hr in fresh YESCA medium added with 0 μM, 500 μM, or 1 mM indole, or conditioned medium from 4 d old cultures of wild-type UTI89 or a *ΔtnaA* mutant. (B) UTI89 grown at 26°C for 48 hr in the fresh medium, or conditioned media collected from 4-d old cultures of wild-type, *ΔkpsF* mutant or *ΔkpsS* mutant. (C) UTI89 grown at 26°C for 48 hr in fresh medium, or fresh medium supplemented with 1 mM D-Leucine, 1 mM D-Methionine, 1 mM D-Tryptophan, 1 mM D-Tyrosine or 1 mM D-amino acid mix (D-Leu, D-Met, D-Trp, D-Tyr), 100 μM norspermidine, or a combination of 100 μM D-amino acids and 100 μM norspermidine. Bacteria were grown in wells on 24 well tissue culture plates, and each well was stained with crystal violet for better visualization.



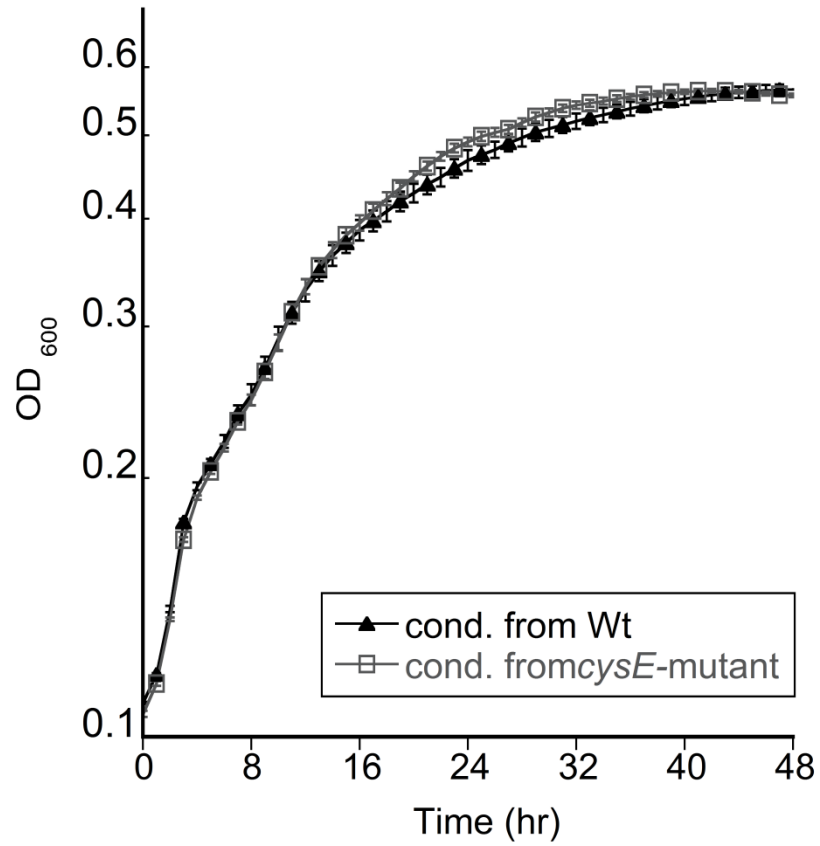
**Figure 3.7. The biofilm-inhibitory effect of the conditioned medium was dependent on L-cysteine metabolism.**

A schematic of cysteine biosynthesis mediated by the serine acetyltransferase CysE and OAS sulfhydrylases CysK and CysM. **(B)** Conditioned media collected from  $\Delta cysE$  and  $\Delta cysK\Delta cysM$  mutants lost biofilm inhibitory effect. UTI89 grown at 26°C for 48 hr in fresh medium, or conditioned media collected from 4 d old cultures of wild-type,  $\Delta cysE$ ,  $\Delta cysK\Delta cysM$ ,  $\Delta cysE/pCysE$ , or  $\Delta cysK\Delta cysM/pCysK$ . **(C)** Supplementation of  $cysE$  and  $cysKcysM$  cultures with cysteine restored the antibiofilm activity of the conditioned medium. UTI89 wild-type,  $\Delta cysE$  and  $\Delta cysK\Delta cysM$  were grown statically in YESCA medium supplemented with 20  $\mu$ M, 100  $\mu$ M, or 200  $\mu$ M cysteine, or 100  $\mu$ M cystine. After 4 d of growth at 26°C, corresponding conditioned media were collected and tested for the ability to support pellicle formation of UTI89 wild-type at 26°C for 48 hr. Wells were stained with crystal violet.



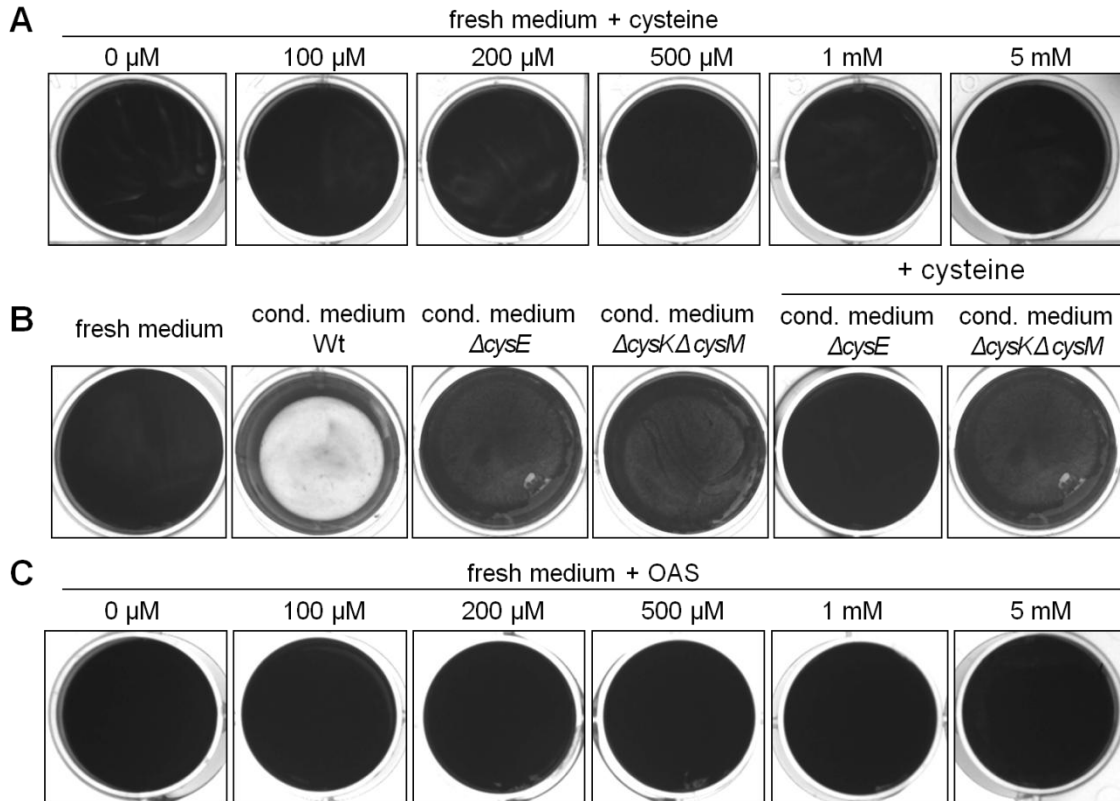
**Figure 3.8. Growth curves of UTI89 grown in the conditioned medium collected from 4 d old cultures of wild-type or a *ΔcysE* mutant.**

Optical density was measured at 600 nm by a plate reader. Each sample had 6 replicates.



**Figure 3.9. Cysteine did not directly inhibit pellicle formation.**

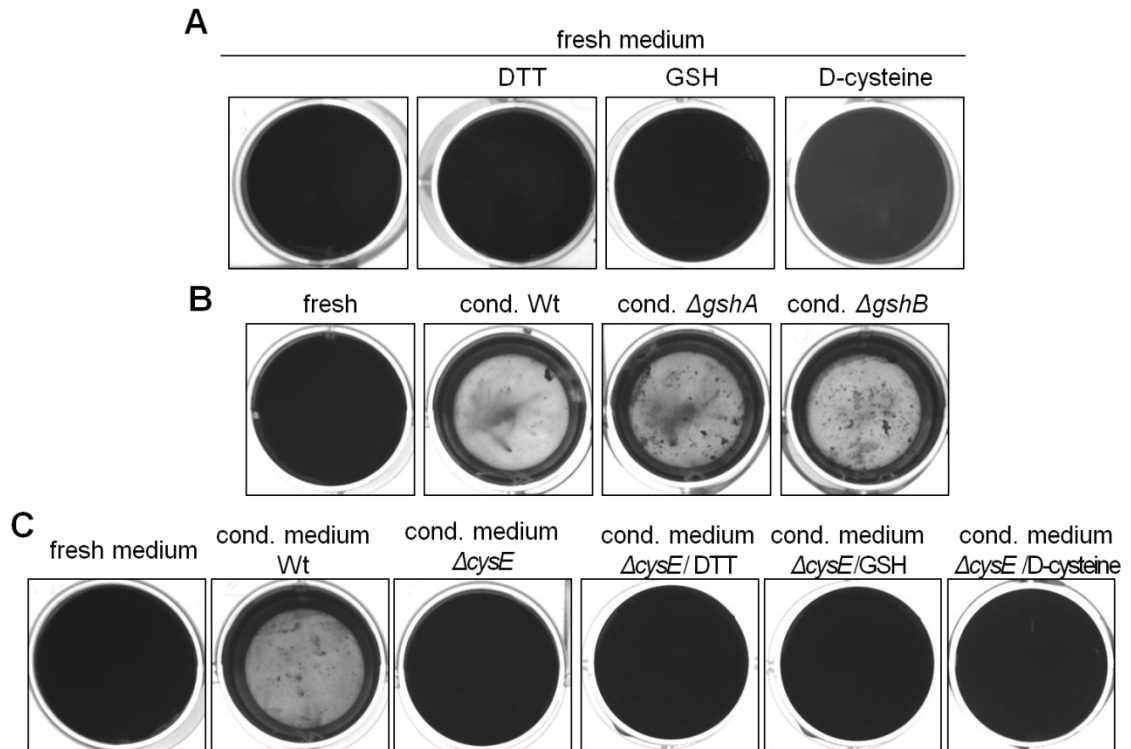
(A) UTI89 was grown in fresh medium supplemented with 100  $\mu$ M or 1 mM cysteine at 26°C for 48 hr. (B) UTI89 was grown at 26°C for 48 hr in fresh medium, wild-type conditioned medium,  $\Delta$ *cysE* conditioned medium,  $\Delta$ *cysK* $\Delta$ *cysM* conditioned medium, or  $\Delta$ *cysE* and  $\Delta$ *cysK* $\Delta$ *cysM* conditioned media post-supplemented with 100  $\mu$ M cysteine. Wells were stained with crystal violet.





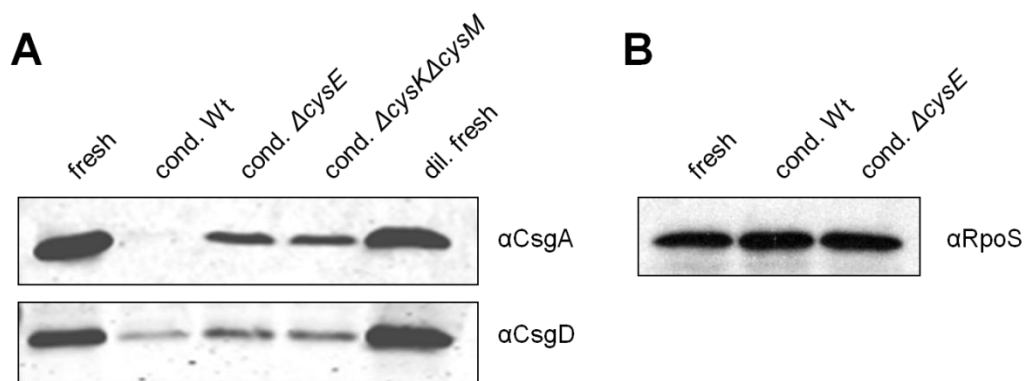
**Figure 3.10. DTT, glutathione (GSH) or D-cysteine did not contribute to the antibiofilm activity of the conditioned medium.**

(A) UTI89 grown at 26°C for 48 hr in fresh medium in the presence of 1 mM DTT, GSH or D-cysteine. (B) UTI89 grown in fresh medium or conditioned media collected from 4 d old cultures of wild-type,  $\Delta gshA$  or  $\Delta gshB$ . (C) UTI89 grown in fresh medium, conditioned media from wild-type or  $\Delta cysE$  cultures, or conditioned media from  $\Delta cysE$  cultures supplemented with GSH or D-cysteine.



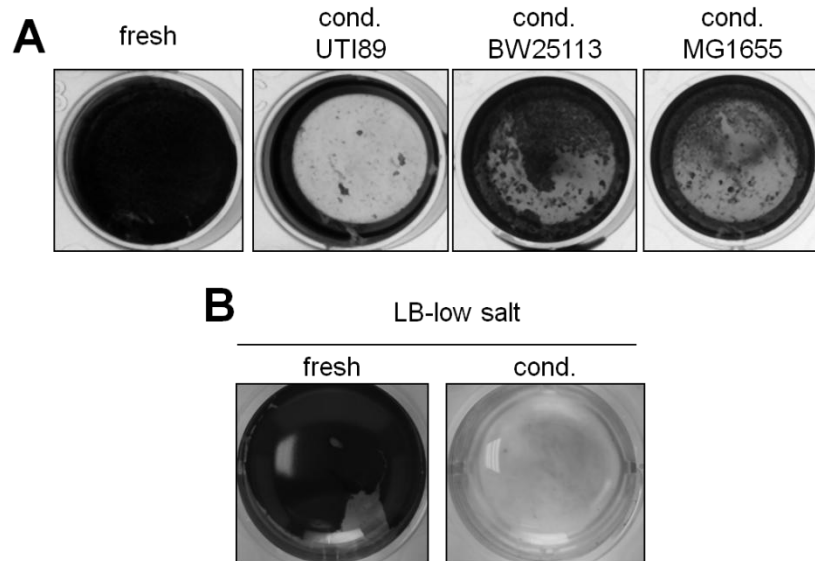
**Figure 3.11. Curli production was reduced in UTI89 grown in the conditioned medium.**

(A) UTI89 was grown at 26°C in fresh YESCA, diluted fresh YESCA, or conditioned media collected from cultures of wild-type,  $\Delta cysE$  mutant or  $\Delta cysK\Delta cysM$  mutant. After 24 hr of growth when biofilm just started to form, all the bacteria in the well including the pellicle and planktonic cells were collected for Western blot analysis. To detect curli production, cell lysates were pre-treated with HFIP. Cell lysates without HFIP treatment were used for the CsgD blot. The blots were probed with  $\alpha$ CsgA or  $\alpha$ CsgD antibody, respectively. (B) UTI89 was grown at 26°C for 24 hr in fresh YESCA, diluted fresh YESCA, or the conditioned medium collected from wild-type UTI89 culture. Bacteria from the pellicle and the planktonic bacteria were collected for western blot. The blot was probed by  $\alpha$ RpoS antibody.



**Figure 3.12. The antibiofilm activity is produced in other *E. coli* strains that we tested .**

(A) UTI89 was grown in a fresh medium or conditioned media collected from 4-d old cultures of UTI89, BW25113 or MG1655. (B) UTI89 was grown in fresh LB-low salt medium or a corresponding conditioned medium. The conditioned medium was collected from a 3 d old culture of UTI89 grown in LB-low salt medium at 26°C. Biofilms were grown for 48 hr at 26°C and wells were stained with crystal violet.



**Table 3.1. Strains used in this study**

<b>Bacterial Strains</b>	<b>Relevant genotype and features</b>	<b>Resistances</b>	<b>References</b>
wild-type UTI89	Wild type clinical UPEC	None	(34)
UTI89 $\Delta csgA$	Deletion of the major curlin gene, <i>csgA</i> , in UTI89 Abolishment of curli expression.	None	(14)
UTI89 $\Delta cysE$	Deletion of the serine acetyltransferase <i>cysE</i>	None	This study
UTI89 $\Delta cysK \Delta cysM$	Deletion of cysteine synthesases <i>cysK</i> and <i>cysM</i>	None	This study
UTI89 $\Delta csgBA$	Deletion of the curli operon <i>csgBA</i>	None	This study
UTI89 $\Delta csgD$	Deletion of the curli and cellulose regulator <i>csgD</i>	None	This study
UTI89 $\Delta tnaA$	Deletion of the tryptophanase <i>tnaA</i>	None	This study
UTI89 $\Delta gshA$	Deletion of $\gamma$ -Glutamate-cysteine ligase <i>gshA</i>	Kanamycin	This study
UTI89 $\Delta gshB$	Deletion of the glutathione synthetase <i>gshB</i>	Kanamycin	This study
UTI89 $\Delta kpsS$	Deletion of the polysialic acid capsule synthesis gene <i>kpsS</i>	None	From the Hultgren lab
UTI89 $\Delta kpsF$	Deletion of the polysialic acid capsule synthesis gene <i>kpsF</i>	None	From the Hultgren lab

**Table 3.2. Primers used in this study**

Primer	Primer Sequence	Constructs
Cyse rsF	5'-GCCCGCGCAGAACGGGTCGGTCATTATCTTA TCGTGTGGAGTAAGCAATGCATATGAATATCCTCC TTAG -3'	UTI89 $\Delta$ <i>cysE</i>
cysersR	5' - CACGCCGCATCCGGCACGATCACAGAATG TCAGATCCCATCACCATACTCGTGTAGGCTGGAGC TGCTTC-3'	UTI89 $\Delta$ <i>cysE</i>
csymrsf	5'-AGACGCGTAAGCGTCGCATCAGGCAACACC ACGTATGGACAGAGATCGTGGTGTAGGCTGGAGCT GCTTC -3'	UTI89 $\Delta$ <i>cysK</i> $\Delta$ <i>cysM</i>
cysmrsr	5'-ACGGATAAAACGGTGCCTGCGCAATAATCT TAAATCCCCGCCCTGGCTCATATGAATATCCTCC TTAG -3'	UTI89 $\Delta$ <i>cysK</i> $\Delta$ <i>cysM</i>
cyskrsf	5'-GGTATGCTACCTGTTGTATCCCAATTTTCATA CAGTTAAGGACAGGCCATGGTGTAGGCTGGAGCTG CTT-3'	UTI89 $\Delta$ <i>cysK</i> $\Delta$ <i>cysM</i>
cyskrsf	5'-CTTTTTTACGCATTTTTTACAAGCTGGCATT ACTGTTGCAGTTCTTTCTCCATATGAATATCCTCCT TAG -3'	UTI89 $\Delta$ <i>cysK</i> $\Delta$ <i>cysM</i>
csgD RS	5'-CAATCCAGCGTAAATAACGTTTCATGGCTTT ATCGCCTGAGGTTATCGTTCATATGAATATCCT CCTTA-3'	UTI89 $\Delta$ <i>csgD</i>
csgD RS	5'-GAGGCAGCTGTCAGGTGTGCGATCAATAAA AAAAGCGGGTTCATCATGGTGTAGGCTGGA GCTGCTTC-3'	UTI89 $\Delta$ <i>csgD</i>
csgD RS seq	5'-TGTAATGGCTAGATTGAAATCAGATG-3'	UTI89 $\Delta$ <i>csgD</i>
csgD RS seq	5'-TGGGCCTTTCATTAATCGTT-3'	UTI89 $\Delta$ <i>csgD</i>
csgBA RS	5'-AAATACAGGTTGCGTTAACAACCAAGTTGA AATGATTTAATTTCTTAAGTGTGTAGGCTGGAGCTG CTT-3'	UTI89 $\Delta$ <i>csgBA</i>
csgBA RS	5'-CGAAAAAAAAACAGGGCTTGCGCCCTGTTTC TTTAATACAGAGGATGTATATGAATATCCTCCTTAG -3'	UTI89 $\Delta$ <i>csgBA</i>
csgBA seq	5'-CGCAGACATACTTTCCATCG-3'	UTI89 $\Delta$ <i>csgBA</i>
csgBA seq	5'-GAAAGTGCCGCAAGGAGTAA-3'	UTI89 $\Delta$ <i>csgBA</i>
tnaA rs	5'-AATATTCACAGGGATCACTGTAATTTAAAT AAATGAAGGATTATGTAATGGTGTAGGCTGGAGCT GCTTC-3'	UTI89 $\Delta$ <i>tnaA</i>
tnaA rs	5'-ACATCCTTATAGCCACTCTGAGTGTTTTAAT TAAACTTCTTTCAGTTTTGCCATATGAATATCC TCCTTAG-3'	UTI89 $\Delta$ <i>tnaA</i>

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## Chapter 4

### Conclusions and Future Directions

#### Relaxed seeding specificity of curli

The nucleation-dependent mechanism of amyloid assembly underlies diverse protein misfolding diseases such as Alzheimer's disease and prion transmission (1,2). The fibrillization process of amyloid proteins can be accelerated by nucleators or amyloid fibers formed from the same protein in a process known as seeding (3,4). Seeding is a highly specific process. Cross-species seeding is typically limited by species barriers (5-10). Stringent seeding specificity limits prion transmission and reduces co-occurrence of diverse amyloid diseases. (8,11).

Although cross-seeding has been intensively studied in eukaryotic amyloids, seeding specificity of bacterial functional amyloids has not. Bacterial functional amyloids are found in a broad range of bacteria species (12,13). In Chapter 2, I investigated the seeding specificity of curli amyloids. No species barrier was observed among curli homologs from a spectrum of bacteria including *E. coli*, *S. typhimurium*, *C. koseri* and *S. oneidensis* (Figure 2.1, Figure 2.5, Figure 2.8, Figure 2.9). Remarkably, a distantly related CsgA homolog from *S. oneidensis* also efficiently cross-seeded with *E. coli* CsgA (Figure 2.1). Moreover, *E. coli* and *S. typhimurium* curli mutants in mixed colonies were

able to cross-seed curli subunits from each other and build heterogeneous curli fibers (Figure 2.9). Taken together, these results demonstrate that seeding of curli is relaxed.

### **Structural basis of cross-seeding**

Research on mammalian and yeast prions suggests that species barriers are determined by key amino acid residues and recognition peptide sequences. Mutations of these sequence determinants can alter seeding specificities (8,14). The primary structure of a protein further dictates its dominant conformations. Recent structural studies demonstrate that conformational compatibility between monomers and seeds is the key mechanism for cross-seeding (15). Amyloids with highly similar conformations tend to cross the species barrier, whereas amyloids with distinct conformations usually don't cross-seed (15).

All CsgA and CsgB homologs have similar amino acid arrangement in the C-terminal amyloidogenic domains, which contain several imperfect repeating units predicted to adopt a  $\beta$ -strand-loop- $\beta$ -strand motif and conserved stacks of Gln and Asn residues (Figure 4.1). In Chapter 2, I have shown that conserved Gln and Asn residues in the first and last repeating units of *E. coli* CsgA facilitated cross-seeding (Figure 2.7). Mutating these residues yielded a protein (*E. coli* CsgA<sup>Q49A, N54A, Q139A, N144A</sup>) that maintained its ability to self-seed but cannot be cross-seeded by other curli homologs. Gln and Asn residues have been suggested to form intra-molecular hydrogen bonds and stabilize the amyloid fold (16-18). In this study, we showed that these residues also mediate intermolecular interactions between *E. coli* CsgA monomers and curli homologs. Interestingly, seeds formed from *E. coli* CsgA<sup>Q49A, N54A, Q139A, N144A</sup> can still efficiently cross-seed CsgA monomers from *S. typhimurium* and *C. koseri* (Figure 4.2). The lack of

reciprocal seeding has been observed in other amyloids and the mechanism is not clear (8,11). One explanation is that the conserved Gln and Asn in the first and last repeating units of *E. coli* CsgA are important for monomers to efficiently interact with the seeds and fold into a compatible conformation; whereas for seeds the general conformation and Gln/Asn in other repeating units can facilitate the interactions with monomers.

The amyloidogenic domains of *E. coli* CsgA has been well-studied. The first and last repeating units of *E. coli* CsgA are required for CsgA self-polymerization and interaction with CsgB (19). However, the sequence determinants of other CsgA homologs and CsgB homologs are still elusive. For instance, the first repeating unit of *E. coli* CsgB is dispensable for curli assembly both *in vitro* and *in vivo* (20). Moreover, Mutations of Gln and Asn in the first repeat of CsgB did not affect curli assembly *in vivo*. Instead, mutations of Gln and Asn in repeat 2, 3 or 4 disrupted the localization of CsgB to the cell surface and completely abolished curli assembly (Figure 4.3), indicating the mechanism for CsgB folding is more complex. In addition, the structural basis for curli assembly and seeding has not been reported in *S. typhimurium*, *C. koseri*, and *S. oneidensis*. Therefore, it is possible that seeding specificities of these CsgA and CsgB homologs are mediated by completely different amino acid residues.

The conserved primary structural arrangement of CsgA and CsgB homologs indicates that fibers formed from these curli homologs may have similar conformations. The conformation of curli fibers were not directly analyzed in this study due to technical challenges. Curli fibers tend to tangle into large aggregates, which make it difficult for atomic force microscopy analysis. Alternatively, fiber stability was measured by HFIP resistance (Figure 2.3). Fibers formed by CsgA homologs and fibers formed from the

cross-seeding reactions all exhibit highly similar resistance to HFIP, suggesting they may adopt similar conformations. Further structure analysis such as FTIR (21) and EPR spectra (22) will provide more details about the fiber conformations.

### **Cross-seeding between wild-type bacteria**

Surface anchored curli fibers are critical structures for adherence and biofilm formation (23-25). In curli biogenesis, CsgA molecules are secreted, diffuse into the extracellular environment, and then are templated by CsgB into fibers. In Chapter 2, I have demonstrated that *E. coli* curli mutants and *S. typhimurium* curli mutants were able to share curli subunits to build heterogeneous matrix. In a mixed colony, CsgA secreted from an *E. coli csgB*- mutant can be cross-seeded into fibers by adjacent *S. typhimurium* that presented CsgB on its surfaces. Similarly, an *E. coli csgA*- mutant was able to template CsgA secreted from the neighboring *S. typhimurium csgB*- mutant (Figure 2.10). Therefore, curli subunits are used as common structural units in a mixed species community.

One remaining question is, does cross-seeding occur between wild-type *E. coli* and *S. typhimurium* or any bacteria producing both CsgA and CsgB (Figure 4.4)? *E. coli* and *S. typhimurium* are found in the same ecological niches (26,27). Thus, it is likely that *E. coli* and *S. typhimurium* can coexist in the same multispecies community and may be able to share curli subunits to build a heterogeneous matrix. Moreover, curli-like structures are also found in *Enterobacter spp.* and *Citrobacter spp.* (27,28), and curli homologs are prevalent among *Pseudomonas spp.* and *Shewanella spp.* Phylogenetic analysis (<http://string-db.org/>) of different bacteria species containing curli homologs shows that these bacteria harbor both *csgA* and *csgB* genes. Therefore, it is less likely that

bacteria produce only CsgA or CsgB under natural environment. It is more biologically relevant to ask if two wild-type strains share curli subunits or if they selectively utilize their own curli subunits.

To detect the potential cross-seeding between wild-type *E. coli* and *S. typhimurium* is technically challenging. Immunofluorescence and immunogold labeling techniques failed to detect potential heterogeneous curli fibers composed of both *S. typhimurium* and *E. coli* CsgA. To distinguish *E. coli* CsgA and *S. typhimurium* CsgA, a Myc tag was inserted between the Sec signaling sequence and the first 22 amino acid of *E. coli* CsgA, and a His-tag was added at the C-terminal of *S. typhimurium* CsgA. *E. coli* *csgA*-/pMycCsgA and *S. typhimurium* *csgA*-/pCsgA-His were grown to mixed colonies. Bacteria collected from colonies were collected and probed with the monoclonal anti-Myc antibody and polyclonal anti-His primary antibody followed by Alexa secondary antibodies or gold-conjugated secondary antibodies. Ideally, heterogeneous curli fibers composed of both *E. coli* CsgA and *S. typhimurium* CsgA are expected to be labeled with two different fluorophores or be coated with gold particles of different sizes. However, due to the adhesive nature of curli, both anti-MycA and anti-His antibodies showed high level of non-specific binding even when the blocking and probing conditions were optimized. Thus, it is difficult to distinguish heterogeneous fibers from false non-specific binding.

Some novel labeling techniques provide alternative approaches to detect cross-seeding between wild-type bacteria. Protein labeling with fluorescein derivatives such as fluorescein isothiocyanate (FITC) and NHS-Fluorescein has been widely used to label proteins with free thiols (29). Neither *E. coli* CsgA nor *S. typhimurium* CsgA contains

cysteine in the primary structure. A cysteine residue can be introduced to CsgA sequence to facilitate fluorescein labeling. Indeed, previous unpublished data show that replacing Q150 with Cys in *E. coli* CsgA doesn't affect curli assembly *in vivo*. Thus, *E. coli* CsgA Q150C is a good candidate for fluorescein labeling. Other than fluorescein, metal-chelation based labeling method has also been successfully used. A biarsenical dye FLAsH can specifically bind to an artificial peptide tag CCPGCC with high affinity (30). Additionally, a number of fluorescent non-natural amino acids have been developed for labeling of proteins and cell walls (31). Non-natural amino acids can be incorporated into specific sites of proteins expressed in *E. coli* by non-natural codons and aminoacyl-tRNA synthetase/tRNA pair (32).

### **Interspecies interactions of curli in polymicrobial communities**

Many curli-producing bacteria were found in the same niches (33), indicating they may live as a polymicrobial community. How does cross-seeding affect bacteria in a polymicrobial community? In Chapter 2, I explored the biological consequences of cross-seeding between *E. coli* and *S. typhimurium* in a mixed colony biofilm. Heterogeneous curli formation between an *E. coli* *csgB*- mutant and a *S. typhimurium* *csgA*- mutant promoted surface attachment of the total population and also significantly increased the adhesiveness of *S. typhimurium* to the agar surface (Figure 2.12).

In a mixed colony, *S. typhimurium* normally outcompetes against *E. coli*. The mechanism of how *S. typhimurium* outgrows *E. coli* is not clear. Interestingly, a *S. typhimurium* *csgA*- mutant didn't outgrow *E. coli* *csgB*- mutant (Figure 2.11) in a mixed colony; the ratio of *S. typhimurium* population to *E. coli* population is about 1:1. It is possible that cross-seeding of curli turns the relationship between *E. coli* and *S.*

*typhimurium* from competition to cooperation, so that *S. typhimurium* can utilize *E. coli* CsgA for curli assembly.

Many amyloids display antimicrobial activities, including PSM fibers produced by *S. aureus* (34), TasA fibers by *B. subtilis* (35), microcin E492 by *Klebsiella pneumonia* (36), human antimicrobial peptides LL-37 (37), and even A $\beta$  (38). The antimicrobial activities are generally associated with amyloid precursors including oligomeric intermediates or pre-fibrillar aggregates, which disrupt the lipid membranes and cause toxicity (39-41). Seeding is considered as a mechanism to promote rapid conversion of toxic species to stable, non-toxic amyloid fibers. Although it is not clear if CsgA monomers or oligomers have any antimicrobial activities, oligomeric intermediates are detected in CsgA polymerization and a CsgA variant polymerizes independent of CsgB causes cytotoxicity to *E. coli*. Therefore, it is reasonable to speculate that bacteria may employ cross-seeding as a mechanism to reduce potential toxic intermediate species produced by the neighbors.

We noticed that some curli-producing bacteria do not share curli subunits with neighboring bacteria. For instance, UTI89 CsgA is able to cross-seed other curli homologs *in vitro*. *In vivo*, however, cross-seeding was not detected between UTI89 curli mutants and *E. coli* K12 or between two UTI89 curli mutants. Moreover, although *S. typhimurium* is able to share curli subunits with *E. coli* K12 curli mutants, cross-seeding was not detected between a *S. typhimurium csgA*- mutant and a *S. typhimurium csgB*- mutant. One possible explanation is other extracellular structures such as capsules, cellulose, and LPS function as physical barriers that reduce diffusion of CsgA molecules from and into the extracellular environment. White *et al.* showed that mutation of LPS

production restored intercellular curli production in *Salmonella enterica* Serovar *Enteritidis* (42).

Other than cross-seeding, curli fibers also interact with other extracellular fibrillar structures. Quick-freeze, deep-etch, and high-resolution electron microscopy analysis of pellicle and colony biofilms of UTI89 reveals that bacteria are encased within a dense networks of fibers composed of curli and cellulose (unpublished data). Also, curli and cellulose have been shown to play synergistic role in surface attachment and biofilm formation (43). Therefore, interspecies interactions between curli fibers and between curli and cellulose may also play a role in a polymicrobial community development.

### **Cross-seeding between curli and non-curli amyloids**

It is unknown if curli can cross-seeding non-curli amyloid fibers. Other than curli, an increasing number of bacterial functional amyloids have been discovered in other species, like TasA fibers (44), chaplin by *S. coelicolor* (45) and FapA by *P. fluorescens* (46). There is an enticing potential that curli may interact with other functional bacterial amyloids and play a role in multispecies biofilm development.

In Chapter 2, I observed a species barrier between curli and A $\beta$ <sub>1-42</sub>, and between curli and yeast prion Sup35 (Figure 2.4). However, these limited examples do not exclude the possibility that curli can cross-seed with eukaryotic amyloids. Recent studies indicate that curli may have the potential to interact with non-curli eukaryotic amyloids. LL-37 is a human antimicrobial peptide with amyloid properties produced by epithelial cells of the urinary track to protect against infections with uropathogenic *E. coli* (47,48). It is reported that LL-37 can strongly bind to CsgA fibers and also interacting with CsgA monomers (49). If curli can cross-seed the polymerization of LL-37, bacteria might



utilize cross-seeding as a strategy to promote aggregation of LL-37 and counteract the antimicrobial effects. Amyloid protein A (AA) forms amyloids in secondary amyloidosis caused by chronic inflammatory diseases and chronic infections (50). Lundmark *et al.* has shown that injection of curli accelerated AA deposition in a murine model of AA amyloidosis, indicating that curli may serve as seeds to promote AA polymerization and promote amyloidosis.

### **Alternative functions of curli subunits**

The curli major subunit CsgA has been intensively studied as a building block of extracellular fibers (13,51,52). However, the function of the non-amyloid fold of CsgA has not been investigated. Many amyloid precursors have alternative physiological functions. For instance, the non-amyloid fold of *S. aureus* PSM peptides promotes biofilm disassembly and display antimicrobial activities (34). The native fold of Sup35 acts as a translational termination factor in *S. cerevisiae* (53-55). We commonly detect SDS-soluble CsgA in the agar underneath *E. coli* colonies, indicating CsgA may exist in an oligomeric form or non-amyloid fold. We speculate that CsgA may have alternative functions other than structural components. Studies in UTI89 colony biofilm in the lab showed that the colonies of wild-type UTI89 spread more than that of a *csgA*- or *csgB*-mutant, suggesting CsgA could facilitate bacteria motion (unpublished data). Moreover, many amyloid proteins have antimicrobial activities (34-38). The oligomeric form of CsgA exhibits cytotoxicity to neuroblast cells (unpublished data). It is possible that oligomeric CsgA can also act as an antimicrobial agent. In addition, CsgA might even serve as a signal to regulate curli expression from outside the cell. It would also be interesting to test if bacteria can sense and respond to extracellular CsgA or curli fibers.

## **Cysteine-dependent metabolite inhibits UPEC pellicle biofilm**

Biofilm development is regulated by structural components, intracellular regulatory networks, and extracellular signaling molecules. An uropathogenic *E. coli* strain UTI89 is able to form a pellicle biofilm at the air-liquid interface of static liquid culture. Robust pellicle formation requires the expression of curli, cellulose, and flagellum (Figure 3.2). In Chapter 3, I found that UTI89 produced antibiofilm activity that inhibited its own pellicle formation. Deficient pellicle formation was observed for UTI89 grown in a bacteria-free conditioned medium collected from an aged UTI89 culture (Figure 3.3). By a candidate approach, I identified that antibiofilm activity was dependent on cysteine production, as cysteine auxotroph lost the ability to produce the biofilm inhibitor, and this phenotype could be complemented by addition of cysteine or cystine into mutant cultures (Figure 3.7). Interestingly, the antibiofilm activity seems to depend on cysteine and cystine specifically, as addition of other thiols did not restore the biofilm inhibitor production (Figure 3.10). This result also explains a previous observation that a cysteine auxotroph, *cysE*<sup>-</sup>, exhibited enhanced biofilm formation, which can be abolished by exogenous cysteine (56). Taken together, the cysteine-dependent metabolite is likely to act as an extracellular signal that controls biofilm development. As a culture ages, nutrient resources in the local environment are depleted and matrix production could be burdensome. The cysteine-dependent extracellular signal provides a strategy to prevent further biofilm formation.

It is still elusive how cysteine or cystine mediates the antibiofilm activity. One possibility is that, a cysteine breakdown product or multiple products may have additive inhibitory effects (Figure 4.5). To keep intracellular cysteine balance, bacteria degrade

cysteine into hydrogen sulfide, ammonia and pyruvate (57-61). The effects of these metabolic intermediates on pellicle formation can be tested by exogenous addition of the agents or by analyzing the desulhydrases mutants (57-61). Alternatively, the antibiofilm signal may come from the methionine and homocysteine pathway (Figure 4.5). Cysteine is linked with methionine metabolism (62). Interestingly, methionine can be converted to S-adenosyl-methionine (SAM), which is the precursor of quorum sensing molecules such as AI-2. Therefore, manipulation of cysteine biosynthesis may affect the production of SAM and potential downstream signaling molecules. It is also possible that the antibiofilm activity is due to the imbalance of extracellular amino acid composition or an unknown signaling molecule (Figure 4.5).

In addition to the candidate screening approach, chemical fractionation followed by NMR and GC/MS is commonly used for identification of extracellular signaling molecules such as CAI-1, D-amino acids, and norpermidine (63-65). Moreover, large scale screening for mutants defective in biofilm inhibitor production was also reported for identification of antibiofilm polysaccharides (66,67). These two approaches will not only provide clues for the cysteine-dependent signals, but will also reveal a secondary antibiofilm agent in the conditioned medium.

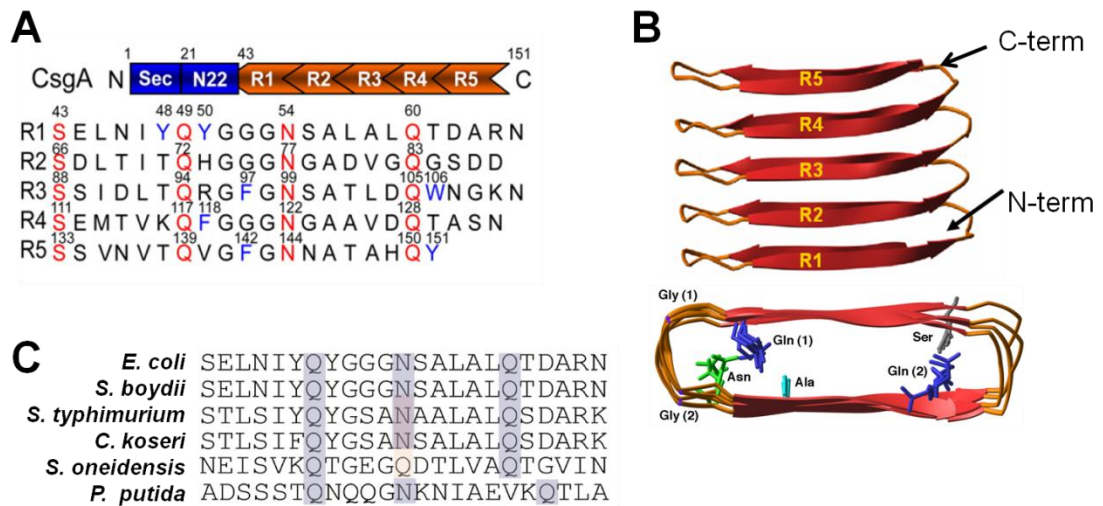
Interesting, our preliminary result demonstrated that conditioned medium from an aged UTI89 culture also inhibited biofilm formation of *S. aureus* (Figure 4.6). It is unclear if the inhibiting effect is mediated by cysteine or by other mechanisms. Recently, a polysaccharide isolated from *E. coli* biofilm extract, not the planktonic cell culture, was reported to have anti-adhesion activity against *S. aureus* and mediate rapid exclusion of *S. aureus* in the mixed culture (67). Many other extracellular molecules such as surfactin,

siderophore, and phenol soluble modulins have also been reported to mediate bacteria competition and colony invasion (68,69). Thus, the antibiofilm agent produced by UTI89 may also play a role in microbial competition against *S. aureus*.

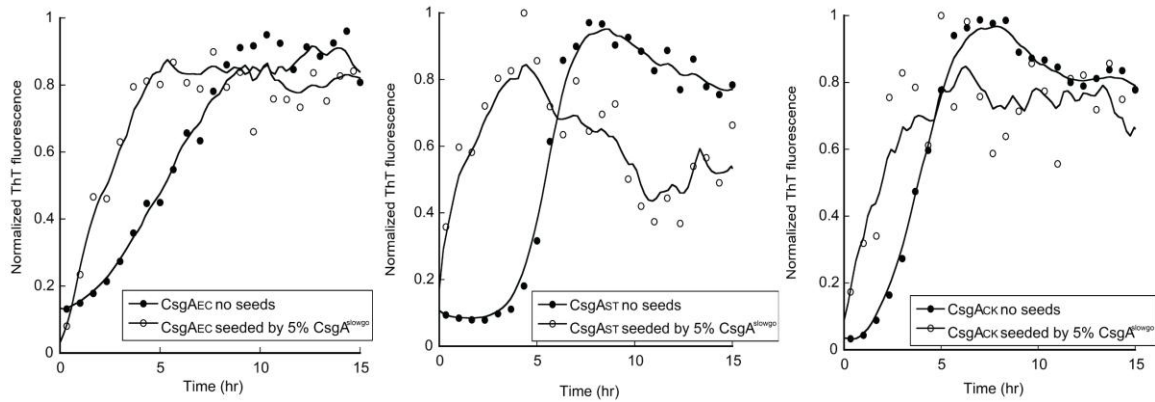
## Figures

### Figure 4.1. Schematics of the C-terminal amyloidogenic domain of CsgA and sequence alignment of CsgA homologs.

*E. coli* CsgA is composed of a N-terminal Sec secretion sequence, a 22-amino acid sequence and an amyloidogenic C-terminal domain with five repeating units. (B) Predicted structure of CsgA (Collinson *et al.*, 1999, *J Mol Biol*). Each repeating unit is predicted to adopt a  $\beta$ -strand-loop- $\beta$ -strand motif. (C) Sequence alignment of the first repeating units of amyloidogenic domains of CsgA homologs. All of them contain the conserved Gln and Asn residues.

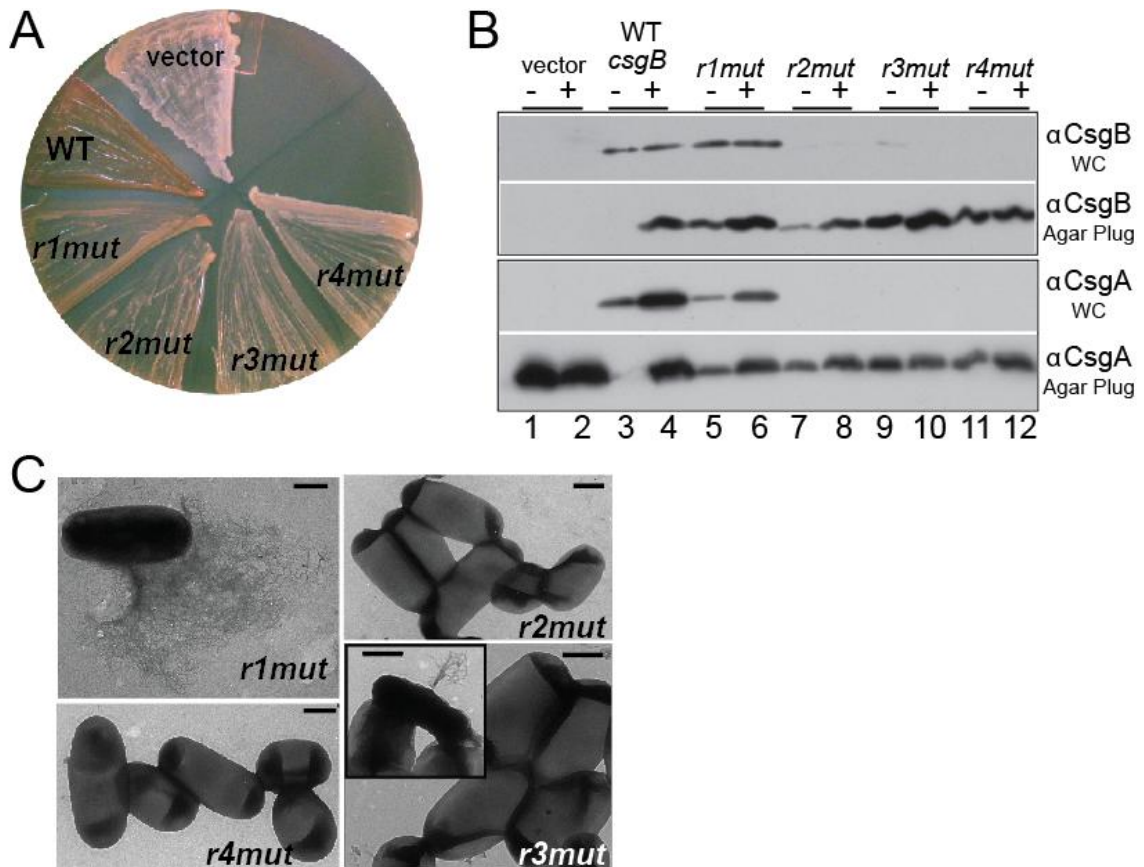


**Figure 4.2. Fibers of CsgA<sup>Q49A, N54A, Q139A, N144A</sup> cross-seeded CsgA from *E. coli*, *S. typhimurium* and *C. koseri*.**  
5% seeds were added into 10  $\mu$ M freshly purified CsgA. Polymerization kinetics were monitored by Thioflavin T fluorescence.

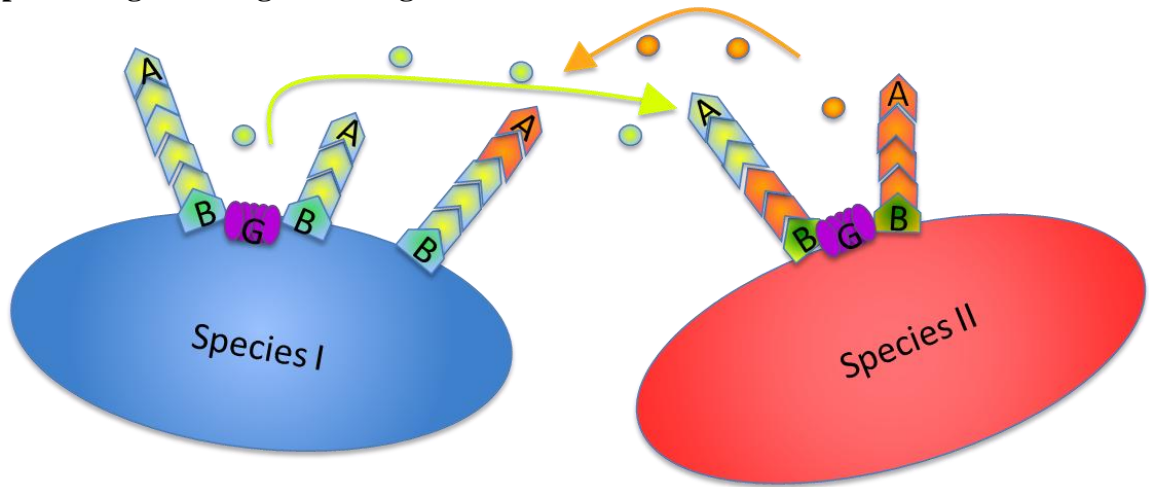


**Figure 4.3. *In vivo* functional analysis of the conserved glutamine and asparagine residues in the amyloidogenic repeating units of CsgB.**

(A) Congo red binding phenotype of MC4100 (WT) or a *csgB* mutant strain harboring vector control, WT *csgB*, *csgB r1mut* (*csgB*<sup>Q51A N56A</sup>), *csgB r2mut* (*csgB*<sup>Q73A N78A</sup>), *csgB r3mut* (*csgB*<sup>Q95A N100A</sup>), or *csgB r4mut* (*csgB*<sup>Q117A N122A</sup>). (B) Western blot analysis of a *csgB* strain harboring a vector control (vector lanes 1 and 2), WT *csgB* (lanes 3 and 4), *csgB r1mut* (lanes 5 and 6), *csgB r2mut* (lanes 7 and 8), *csgB r3mut* (lanes 9 and 10), or *csgB r4mut* (lanes 11 and 12). Samples were resuspended in SDS loading buffer with (+) or without (-) prior treatment with formic acid (FA). Top two panels were blotted with anti-CsgB antibody. Bottom two panels were blotted with anti-CsgA antibody. Whole cell samples are represented in the first and third panel, while samples containing whole cells and the underlying agar are represented in the second and fourth panel. (C) TEM of a *csgB* strain harboring *csgB r1mut*, *csgB r2mut*, *csgB r3mut*, or *csgB r4mut*. Cells were collected after 48 hours of growth under curli inducing-conditions. The scale bar represents 500nm.

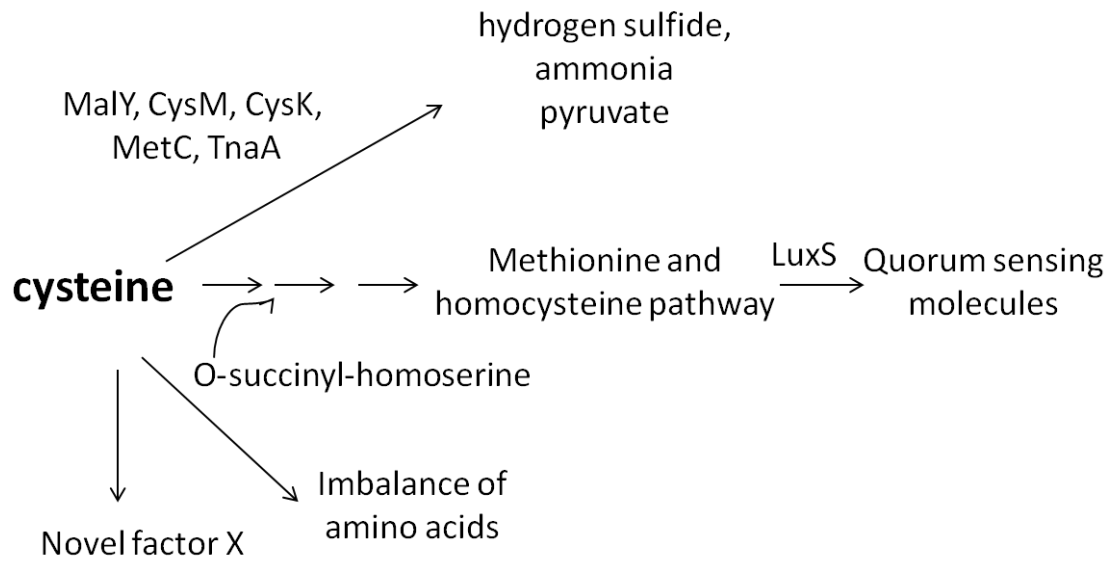


**Figure 4.4. A schematic of cross-seeding between different bacteria species producing both CsgA and CsgB.**





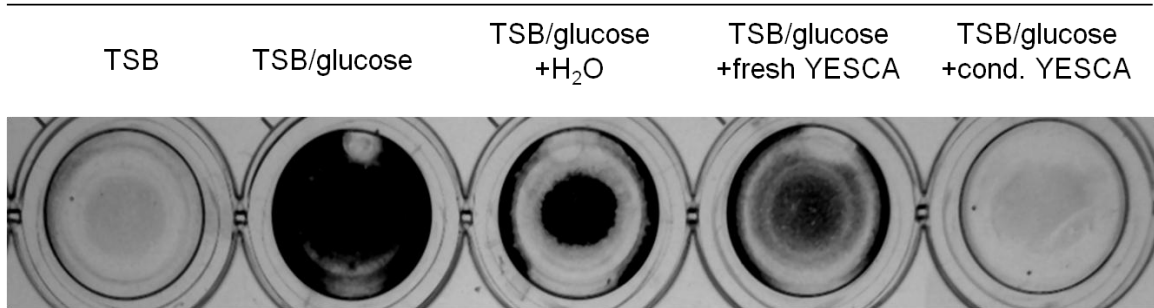
**Figure 4.5. Potential mechanisms of cysteine-dependent antibiofilm effect.**



**Figure 4.6. Biofilm formation of *S. aureus* was inhibited by a conditioned medium from UTI89 culture.**

*S. aureus* was grown in tryptic soy broth (TSB), TSB supplemented with glucose, TSB/glucose mixed with water (1:1), TSB/glucose mixed with fresh YESCA, or TSB/glucose mixed with conditioned medium collected from 4-d old UTI89 culture in YESCA. Bacteria were grown in wells of a 96-well plate at 37°C with agitation for 14 hr. After incubation, bacteria supernatants were removed and biofilms were stained with 0.1% crystal violet.

Biofilm of *Staphylococcus aureus*



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## **Appendices**

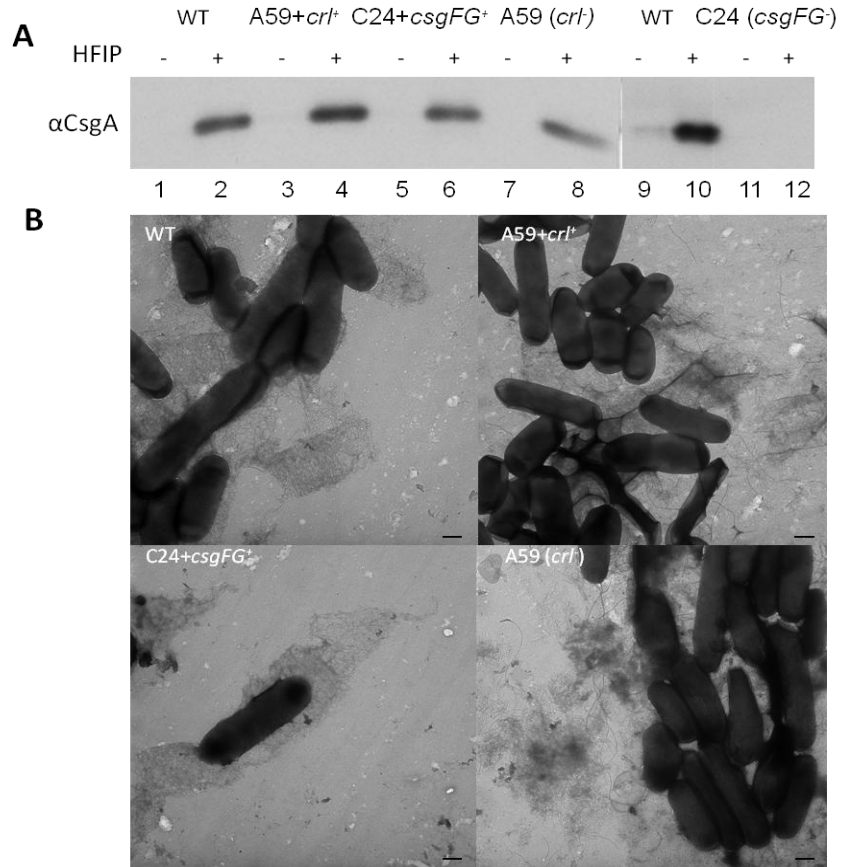
## **Appendix A**

### **Curli expression during pellicle formation of Enteropathogenic *E. coli* clinical isolate O55:H7<sup>4</sup>**

Enteropathogenic *E. coli* (EHEC) is an important cause of diarrheal. Dr. Sela's lab found that a clinical EHEC isolate O55:H7 formed a dense pellicle and developed rugose colony biofilm. From a transposon mutagenesis screening looking for biofilm deficient mutants, they identified two mutants, with inactivated *csgFG* gene and *crl* gene, respectively. CsgF and CsgG are components required for curli biogenesis. Crl is a thermosensitive regulator that controls downstream curli expression. In collaboration with Dr. Sela, we verified that curli was expressed during pellicle formation of wild-type O55:H7. Curli production was abolished in the *csgFG*- (C24) mutant and was reduced in the *crl*- (A59) mutant by western and electron microscopy.

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<sup>4</sup> This result is published in Weiss-Muszkat M, Shakh D, **Zhou Y**, Pinto R, Belausov E, Chapman MR, Sela S. 2007, Biofilm formation by and multicellular behavior of *Escherichia coli* O55:H7, an atypical enteropathogenic strain. *Appl Environ Microbiol.* 76(5):1545-54



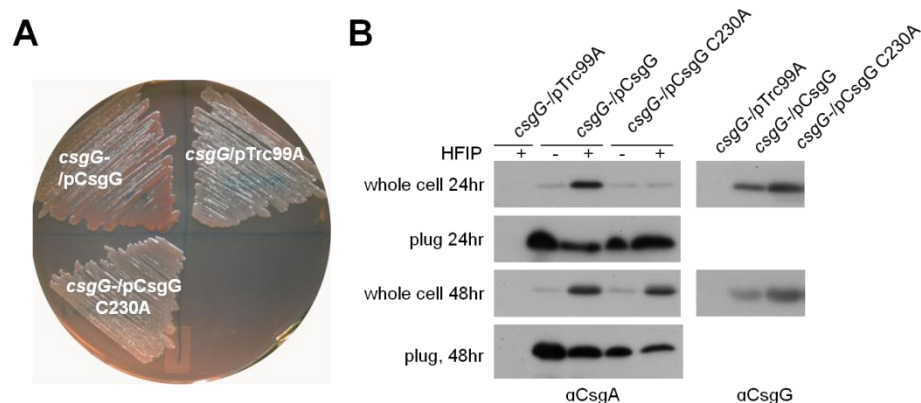
A) Western blots of wild-type (lane 1 and 2), A59+*crl*<sup>+</sup> (lane 3 and 4), C24+*csgFG*<sup>+</sup> (lane 5 and 6) and A59 (*crl*<sup>-</sup>) (lane 7 and 8). Since C24 (*csgFG*<sup>-</sup>) mutant did not form a pellicle, planktonic bacteria from the liquid culture and aggregates at the bottom were collected for western analysis (lane 11 and 12). A mixture of pellicle, planktonic cells and aggregates of the wild-type strain were used as the control (lane 9 and 10). Samples were pre-treated with (+) or without (-) HFIP before loading onto SDS-PAGE and probed with CsgA antibody. B) Transmission electron microscopy of wild-type, A59+*crl*<sup>+</sup>, C24+*csgFG*<sup>+</sup>, and A59 (*crl*<sup>-</sup>) collected from pellicles. Bacteria were grown in LB broth without salt for 3 days at 26 °C. Scale bars equal to 500nm.



## Appendix B

### Functional analysis of CsgG C230A<sup>5</sup>

According to the structural model of the CsgG transmembrane region established by Dr. Steve Matthew's group, the conserved cysteine residue (C230) would project into the lumen of the CsgG pore at the periplasmic entrance. Thus, C230 could play an important role in CsgG pore activity (Taylor, *et al.*, *Structure*, 2011). We found that a *csgG*- mutant expressing CsgG C230A variant had delayed curli formation phenotype. *csgG*-pCsgG C230A was unable to bind Congo Red after 24 hr growth on YESCA plate under curli inducing condition, while *csgG*- expressing wild-type CsgG stained red on YESCA-Congo red plate. (Panel A). In agreement with the Congo red result, western blot analysis showed that *csgG*-CsgG C230A mutant produced very little bacteria-associated CsgA after 24 hr growth on YESCA plate, while majority of the CsgA proteins were secreted into the curli in an unpolymerized, SDS-soluble form (Panel B). After 48 hr of incubation, curli formation in *csgG*-pCsgG C230A caught up, indicated by the wild-type level of bacteria-associated, SDS-insoluble CsgA detected by western (Panel B). The delayed curli assembly phenotype is not likely due to amount of CsgA secret, as similar amount of SDS-soluble CsgA was detected in the underlying agar (the plug) for *csgG*-pCsgG and *csgG*-pCsgG C230A. Thus, mutation of C230 caused a nucleation defect of CsgA, possibly by mishandling of the curli subunits.



<sup>5</sup> This result is published in Taylor JD, **Zhou Y**, Salgado PS, Patwardhan A, McGuffie M, Pape T, Grabe G, Ashman E, Constable SC, Simpson PJ, Lee WC, Cota E, Chapman MR, Matthews SJ. 2011, Atomic resolution insights into curli fiber biogenesis. *Structure*. 19(9):1307-16.

(A) MC4100*csgG*- expressing CsgG C230A was deficient in growth on bile salt agar. *csgG*- harbouring pTrc99A (the empty vector), pCsgG and pCsgG C230A were inoculated in LBNS, normalised and spotted on LB-no salt agar plates or LB-no salt agar with 2%(w/v) bile salt, incubated for 24 hours. (B) Western blots of whole-cell lysates from a *csgG*- mutant containing plasmid pCsgG, pCsgG C230A or the empty vector grown on YESCA agar for 24 h or 48 h at 26°C. Bacteria were treated with (+) or without (-) HFIP before electrophoresis as indicated and probed with  $\alpha$ CsgA antibody. Blots with non-HFIP treated samples were also probed with  $\alpha$ CsgG antibody.

## Appendix C

### Functional analysis of CsgC<sup>6</sup>

CsgC is a component in curli biogenesis system. While the functions of other curli subunits have been intensively studied, the role of csgC remains elusive. A study on the CsgC homolog from *Salmonella enteritidis*, AgfC, showed that loss of *agfC* resulted in aberrant fibers with increased diameters and cell surface hydrophobicity (Gibson *et al.*, *Microbiology*, 2007). Dr. Steve Matthew's group purified CsgC and solved the crystal structure of CsgC. They reported that CsgC adopts an immunoglobulin-like folds with similarity to the N-terminal domain of DsbD. CsgC also contains a CXC motif. They proposed that CsgC may have redox activity and CsgG with a cysteine in the transmembrane domain may be a substrate for CsgC.

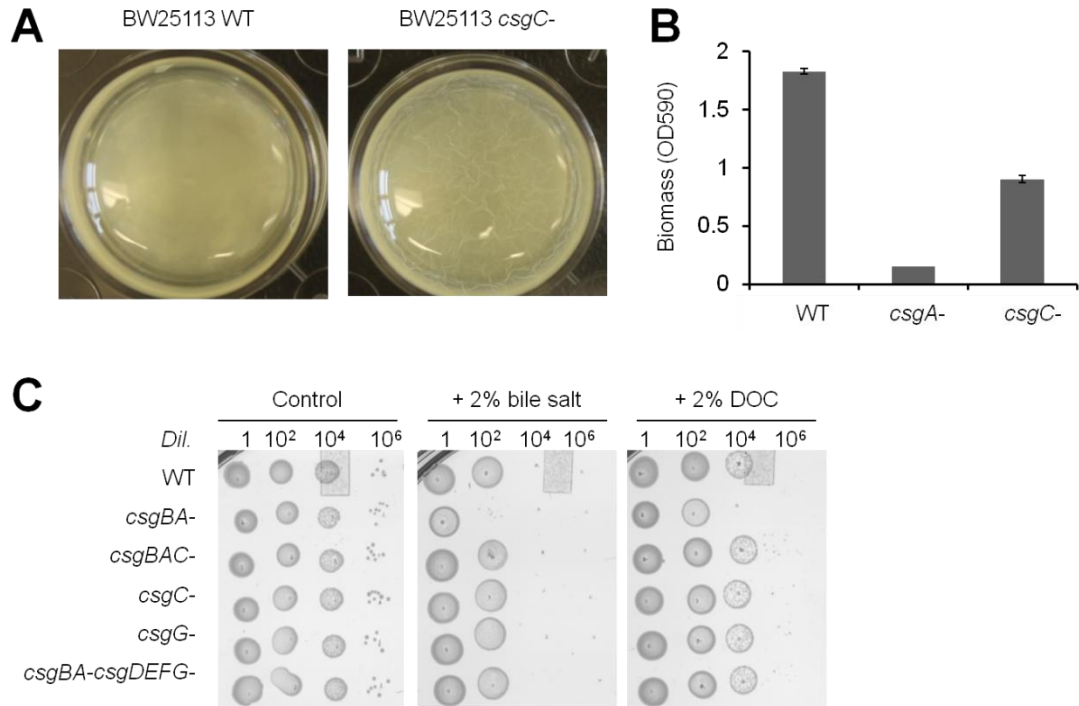
In the collaborative study with Dr. Matthew's group, we investigated the function of CsgC in curli biogenesis and curli-dependent pellicle biofilm formation. We found a *csgC*- mutant in *E. coli* K-12 BW25113 background formed morphologically distinct pellicle biofilm (Panel A). BW25113 typically developed a flat, fragile pellicle, whereas the pellicle formed by the *csgC*- mutant was heavily wrinkled. In MC4100 strain background, both wild-type and *csgC*- mutant develop a ring-shaped biofilm attached to the polystyrene surface of wells. However, mutation of *csgC* lead to a ~50% decrease in biomass (Panel B). The altered biofilm characteristic may be explained by previous observation that *S. enteritidis agfC*- mutant assemble curli fibers with aberrant structure and modified surface properties (Gibson *et al.*, *Microbiology*, 2007).

We also found CsgC was involved in bile salt sensitivity. *E. coli* MC4100 possesses some degree of resistance to bile salts or the active component of bile salt, deoxycholate, possibly through active export mechanisms (Thanassi *et al.*, *J Bacteriol*, 1997). In the absence of curli formation, a MC4100 *csgBA*- mutant was sensitive to bile salt and deoxycholate (Panel C). Bile salt resistance was restored to wild-type level when *csgC* was also knocked out in the *csgBA*- mutant. A logical explanation for this observation is, without the efflux of curli subunits and cell-associated polymerization in *csgBA*- mutant, the CsgG pore was more accessible to bile salts and resulted in increased sensitivity to bile salts. The absence of *csgC* in the *csgBA*- background caused the pore closed to bile salt influx. Consistent with this notion, a *csgG*- mutant or the complete curli deletion mutant were resistant to bile salt (Panel C). Moreover, a curli mutant overexpressing CsgG exhibited a growth defects in liquid culture with deoxycholate, while co-expression of CsgC with CsgG restored the deoxycholate resistance.

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<sup>6</sup> This result is published in Taylor JD, **Zhou Y**, Salgado PS, Patwardhan A, McGuffie M, Pape T, Grabe G, Ashman E, Constable SC, Simpson PJ, Lee WC, Cota E, Chapman MR, Matthews SJ. 2011, Atomic resolution insights into curli fiber biogenesis. *Structure*. 19(9):1307-16.

Collectively, these results suggest that CsgC is involved in CsgG pore activity by increasing the flux of macromolecules.



(A) Pellicle biofilm formation of BW25113 wild-type and *csgC*<sup>-</sup> mutant. Strains were incubated in liquid LB-no slat at 26°C statically for 3 days. BW25113 formed a flat pellicle whereas *csgC*<sup>-</sup> formed a wrinkled pellicle. (B) Biofilm quantification of MC4100 wild-type, *csgC*<sup>-</sup> and *csgA*<sup>-</sup> mutants. Strains were incubated in liquid YESCA at 26°C statically for 7 days and the biomass was quantified by CV staining at OD<sub>595</sub>. (C) Bile salt sensitivity assays. Liquid *E. coli* cultures were inoculated at the dilutions (*Dil.*) indicated onto low salt LB agar plates with or agar with 2% (w/v) bile salt or 2% (w/v) deoxycholate and incubated at 26 °C for 24 hrs.