Promiscuous Cross-seeding between Bacterial Amyloids Promotes Interspecies Biofilms*1,2

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Background: Seeding of eukaryotic amyloids is highly specific.

Results: Curli subunits from different bacteria can cross-seed and such interspecies interactions restore surface attachment and biofilm formation.

Conclusion: Curli cross-seeding is relaxed, which promotes interspecies biofilms.

Significance: This is the first study on cross-seeding of bacterial amyloids and will help define the roles of bacterial amyloids in multispecies biofilms.

Amyloids are highly aggregated proteinaceous fibers historically associated with neurodegenerative conditions including Alzheimers, Parkinsons, 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 Escherichia 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 was secreted from E. coli csgB—mutants assembled into fibers on adjacent S. typhimurium that presented CsgB on its surfaces. Similarly, CsgA was 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.

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Amyloids are β-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, Parkinson, 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-β-sheet structures, with each β-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 tincorial ability of binding the dyes Congo red and thioflavin T (ThT)3 (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 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-polymORIZATION 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-associated peptide Aβ and islet amyloid polypeptide (25), as well as between Aβ and human prion element PrP (24). Additionally, Aβ(1–42) fibers have been reported to induce the formation of tau-containing filaments in vivo (26), and in vitro preformed Aβ(1–42) oligomers

3 The abbreviations used are: ThT, thioflavin T; Aβ, amyloid β; TEM, transmission electron microscopy; HFIP, 1,1,1,3,3,3-hexafluoro-2-propanol.
can induce the conversion of tau monomers to β-sheet-rich, toxic oligomers (27). Also, functional amyloids curli and Sup35 can promote amyloid protein A amyloidosis, suggesting that interactions between heterologous 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).

Although 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. 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 both abiotic and biotic 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, the polymerization and membrane-localization of CsgA 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 β-helix structures (12). The in vitro fibrillation 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 enterica serovar 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/liter of yeast extract, 10 g/liter of casamino acids, and 20 g/liter of agar) or YESCA-CR (50 μg/ml of Congo red and 1 μg/ml of Coomassie Blue) at 26 °C for 48 h. To induce pellicle biofilm formation, bacteria were inoculated in 4 ml of static LB-no salt broth (10 g/liter of tryptone and 5 g/liter of yeast extract) at 26 °C for 3 days. Antibiotics were added at the following concentration: kanamycin, 50 μg/ml, and ampicillin, 100 μg/ml.

#### Strains and Plasmid—Strains and plasmids used in this study are listed in supplemental Tables S1 and S2. Primer sequences are listed in supplemental Table S3. S. typhimurium curli mutants were constructed according to the methods described by Datsenko and Wanner (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 Celgelski et al. (57) and Wang et al. (58). Briefly, expression of C-terminal His₆-tagged CsgA or CsgB homologs without the Sec signal sequence in NEB3016 was induced at A₆₀₀ 0.9 by 0.5 mM isopropyl-1-thio-β-D-galactopyranoside at 37 °C for 1 h. Bacteria were lysed in 8 M guanidine hydrochloride in 50 mM potassium phosphate buffer (Pi) at room temperature for 1 h and then loaded onto a disposable polystyrene column (Thermo). Proteins were eluted into 50 mM potassium Pi solution containing 125 mM imidazole. To get monomeric CsgA fractions with the target protein were combined and loaded onto a 30-kDa centrifugal filter units (Thermo) to remove dimers and other oligomers.

#### AB₁₋₄₂ Disaggregation—100% Trifluoroacetic acid (TFA) was added to AB₁₋₄₂ 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 h. Residual TFA was removed by dissolving the pellet in 500 μl of 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) and SpeedVac drying for 1 h; the process was repeated 3 times. The peptides were dissolved in 2 mM cold potassium hydroxide to 62.5 μM and cold 5× potassium Pi buffer was immediately added. Samples were centrifuged at 70,000 × g at 4 °C for 3 h and the top solution was taken carefully for the polymerization assay.

#### In Vitro Polymerization and Seeding Assay—100 μl 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 μM.

#### Stability Assay—22 μg Fibers were spun down and resuspended in 50 mM potassium Pi, or 50 (v/v), 70, 80, 90, or 100% HFIP for 5 min and immediately dried on a SpeedVac for 3 h.
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The resulting pellets were boiled in 2× 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 μL of 1:1(v/v) mixture of 0.4 M 1-ethyl-3-(3-dimethylpropyl)-carbodiimide and 0.1 M N-hydroxysuccinimide at a flow rate of 5 μL/min. Mature CsgA or CsgB fibers were sonicated (Sonicator XL2020, Misonix) with three 15-s bursts at power 2 and 50-s pauses between. These seeds were then diluted into acetate buffer to final concentrations of 3.5 μM for CsgB fibers and 2.6 μM for CsgA fibers. 55 μL of sonicated fibers were injected at a flow rate of 5 μL/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 μL of 1 M ethanolamine-HCl, pH 8.5, and the sensor chip was primed with 50 μM potassium Pi, pH 7.2. 40 μL of 0.25 μM monomeric E. coli CsgA or S. typhimurium CsgA was injected over the sensor chip surface at a flow rate of 50 μL/min and the response was recorded in resonance units. Elution of a mock purification from NEB3013 harboring an empty vector pET11d and monomeric AB1-42 with a concentration higher than 1 μM 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 h were performed as described previously (45, 58). For whole cell lysates and bacteria with the underlying agar (plug) were controls. Blank flow cells on the same chip were used as negative controls. After ligand immobilization, excessive reactive groups were deactivated with 35 μL of 1 M ethanolamine-HCl, pH 8.5, and the sensor chip was primed with 50 μM potassium Pi, pH 7.2. 40 μL of 0.25 μM monomeric E. coli CsgA or S. typhimurium CsgA was injected over the sensor chip surface at a flow rate of 50 μL/min and the response was recorded in resonance units. Elution of a mock purification from NEB3013 harboring an empty vector pET11d and monomeric AB1-42 with a concentration higher than 1 μM 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 h were suspended in 50 mM potassium Pi, pH 7.2, normalized by optical density at 600 nm (A600), and pelleted down. The pellets were pre-treated with or without 70 μL of HFIP immediately followed by SpeedVac centrifugation for 30 min at 45 °C. For the plug, overnight cultures of bacteria were normalized by A600 and 4 μL of culture were spotted on thin YESCA agar, incubated at 26 °C for 48 h. 8-Millimeter circular plugs including bacteria colonies and the underlying agar were collected, treated with or without 150 μL of 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 antisera raised against purified E. coli curli fibers (Proteintech, Chicago, IL).

Transmission Electron Microscopy—A Philips CM100 transmission electron microscope was used to visualize bacteria colonies and plates that were prepared as previously described (58).

Mixed Colony Biofilms—Interspecies curli assembly assay in mixed colony biofilms was adapted from Chapman et al. (7) and White et al. (59). Briefly, overnight cultures of E. coli and S. typhimurium curli mutants were normalized by A600 and mixed at 1:1 (v/v) ratio. 4 μL of each sample were spotted on YESCA agar and incubated at 26 °C for 3 days. 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 A600 and were mixed at 1:1 (v/v) ratio. Bacterial cultures were spread on 3 ml of YESCA agar on 12-well plates and incubated at 26 °C for 3 days. 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. Nonadherent bacteria in PBS were removed and the cell densities were determined by A600. Adherent bacteria were suspended in another 1 ml of PBS buffer with an inoculation loop and the A600 was measured. The percentage of adherent bacteria was calculated. To determine the adhesiveness of S. typhimurium, two approaches were used. S. typhimurium WT 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). Nonadherent 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, nonfluorescence 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 WT, csgA− and csgBA− mutants were diluted by 1:1,000 (v/v) into 4 ml of 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 days. 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 S. typhimurium and C. koseri (~70% identity) and a distant homolog from S. 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 CsgAEC (CsgA from E. coli), CsgASt (S. enterica serovar Typhimurium), CsgAck (C. koseri), and CsgAng (S. oneidensis).

Freshly purified CsgAEC monomers spontaneously assemble into fibers with identifiable lag, exponential and stationary phases that can be followed by thioflavin T fluorescence in real time (55). Addition of preformed CsgAEC fiber seeds completely eliminated the lag phase (Fig. 1A), which was consistent with previous observations (55). In the presence of 5% (w/w) preformed CsgASt or CsgAck seeds, CsgAEC fibrillated with no lag phase, indicating fibers of CsgAEC or CsgASt could be cross-seeded by seeds of CsgAEC efficiently.
or CsgAEC (data not shown). Interestingly, the polymerization of CsgAEC was also seeded efficiently by a distant CsgA homolog from *S. oneidensis*. CsgASO spontaneously assembled into amyloid-like fibers that were morphologically similar to *E. coli* curli fibers (supplemental Fig. S1A) and were rich in /H9252 sheet secondary structure (supplemental Fig. S1B). The addition of preformed CsgASO fibers effectively eliminated the lag phase of CsgAEC polymerization (Fig. 1C). Taken together, these results suggest that CsgA homologs from different bacteria efficiently cross-seed in vitro.

CsgAEC polymerization can also be seeded by *E. coli* CsgB (CsgBEC) fibers in vitro (Fig. 1D). CsgBEC is proposed to quickly adopt an amyloid-fold and to template CsgAEC fiber formation in vivo (51). The amino acid sequences of CsgAEC and CsgBEC 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, CsgBEC and CsgB homologs from *S. typhimurium* (CsgBST) were expressed and purified. 5% (w/w) CsgBST fibers efficiently promoted the polymerization of CsgAEC (Fig. 1D). Similarly, 5% (w/w) CsgBEC seeds cross-seeded the polymerization of CsgAST and CsgACK (Fig. 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 CsgAEC in the presence of various seeds in terms of 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 (supplemental Fig. S2). 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 /H9252 (1–42) (Aβ1–42) 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 Aβ1–42 nor Sup35 NM was able to seed CsgAEC, nor could the fibrillization of Aβ1–42 or Sup35 NM be seeded by CsgBEC fibers (supplemental Fig. S3). The addition of CsgAEC seeds slightly increased the fibrillization of Sup35 NM, although to much lower levels than Sup35 NM seeded by its own fibers (supplemental Fig. S3). Therefore,
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although cross-seeding occurs between curli homologs, curli cannot cross-seed with unrelated amyloidogenic proteins.

_CsgA Monomers Physically Bind Preformed Curli Fibers—To determine whether 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 was performed to monitor interactions between CsgA monomers and preformed fibers in real time. Preformed CsgA_EC fibers were immobilized on a sensor chip and 0.25 μM freshly purified CsgA_EC or CsgA_ST 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_EC monomers and CsgA_EC seeds, and between CsgA_ST and CsgA_EC seeds (Fig. 2A). The same response was detected when monomeric CsgA_EC or CsgA_ST was flowed over CsgA_ST seeds, showing a strong association of polymeric CsgA_ST with CsgA_ST or CsgA_EC monomers (Fig. 2B). Similarly, both freshly purified CsgA_EC and CsgA_ST bound to polymeric CsgB_EC or CsgB_ST (Fig. 2, C and D). As the control, BSA or mock purification from cells harboring an empty vector was flowed over polymeric CsgA_EC, CsgA_ST, CsgB_EC, or CsgB_ST and no significant interaction was observed (Fig. 2 and data not shown). Finally, consistent with results shown in supplemental Fig. S3B, Aβ1–42 only weakly interacted with CsgA_EC seeds and quickly dissociated after injection (supplemental Fig. S4A). Aβ1–42 did not interact with CsgB_EC seeds at all (supplemental Fig. S4B). 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 β-strand-loop-β-strand repeating units to Ala result in a slow polymerizing variant of CsgB (58, 63). Mutations of Gln and Asn residues in these conserved Gln and Asn residues in CsgA and other CsgA homologs. The polymerization of freshly purified CsgA slowgo was not efficiently seeded by fibers of CsgA_ST, CsgA_CK, or CsgA_SO (Fig. 3 and data not shown). Consistent with previous results, 5% _E. coli_ CsgA fibers completely eliminated the lag phase and promoted the polymerization of CsgA slowgo (Fig. 3). 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) (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_EC cross-seeds CsgA homologs under physiological conditions, we expressed csgA_SP, csgA_CK, and csgA_SO 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_EC 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_EC on bacterial surfaces. Colonies formed by an _E. coli_ csgA− mutant expressing CsgA_EC, CsgA_ST, or CsgA_CK stained red on YESCA-CR plates, whereas the csgA− mutant with the vector control appeared white (Fig. 4A). Bacteria-associated CsgA_EC, CsgA_ST, and CsgA_CK fibers were detected in whole cell lysates by Western blot after treatment with HFIP. CsgA_ST and CsgA_CK 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 (Fig. 4C). Curli-like fibers were observed on the _E. coli_ csgA− mutant expressing CsgA_ST and CsgA_CK by electron microscopy (Fig. 4D). Thus, CsgA_ST and CsgA_CK complemented curli assembly in an _E. coli_ csgA− mutant. Moreover, the complementation was CsgB dependent. csgBA−/pcsgA_ST or csgBA−/pcsgA_CK did not bind Congo red or produce fibers on the cell surface (Fig. 4, B and E), and no cell-associated CsgA_ST/CsgA_CK was detected by Western blot. Instead, SDS-soluble CsgA_ST/CsgA_CK was found in agar blocks underneath the colonies (Fig. 4C), indicating that CsgA homologs were secreted without polymerizing into fibers. Collectively, CsgA_ST and CsgA_CK can be seeded by CsgB_EC on the cell surface. Additionally, an _E. coli_ csgB− mutant could be complemented by expression of CsgB_EC and CsgB_CK (supplemental Fig. S5). Thus, CsgA_EC could also be seeded by CsgB homologs from different bacteria. CsgA_SO and CsgB_SO were unable to complement _E. coli_ csgA− or _E. coli_ csgB− mutants (data not shown), possibly indicating that CsgA_SO and CsgB_SO are not properly expressed or localized in _E. coli_. Consistent with this we were
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FIGURE 3. Conserved Gln and Asn residues were required for cross-seeding between curli subunits. ThT fluorescence of 30 μM freshly purified E. coli CsgA<sup>Δ<sub>slow</sub></sup> alone ( ), or in the presence of 5% CsgA<sub>sc</sub> seeds ( ), CsgA<sub>ST</sub> seeds ( ), or CsgA<sub>SK</sub> seeds ( ).

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 are proposed to frequently share the same ecological niche (65–67). During normal colony formation 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 S. enterica serovar Enteritidis, such interbacterial curli assembly was detected only when the lipopolysaccharide O polysaccharide synthesis gene was mutated (59). To determine whether interspecies curli assembly occurred between E. coli and S. typhimurium (schematic in Fig. 5A), E. coli and S. typhimurium csgA−/csgB− mutants were mixed at a 1:1 (A<sub>600</sub>/A<sub>600</sub>) 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 (Fig. 5B) and curli fibers were observed by TEM (Fig. 5G). 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 (Fig. 5B) and TEM (Fig. 5K). 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, whereas S. typhimurium csgA− (A−B−) did not outcompete E. coli csgB− (A−B−) (supplemental Fig. S6A). 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−) (supplemental Fig. S6B), 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 (Fig. 4B), and TEM (Fig. 5, D, E, I, and J) and curli were not detected in a mixture of E. coli csgB− (A−B−) and S. typhimurium csgBA− (A−B+) or S. typhimurium csgB− (A−B−) and E. coli csgBA− (A−B−), 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 (Fig. 5, B and F) but not between S. typhimurium csgA− and csgB− mutants (Fig. 5, B and J). However, curli formed by the two E. coli mutants was 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 Restore Bacterial Surface Attachment—The ability of E. coli and S. typhimurium to share curli subunits (Fig. 5) 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 PBS (Fig. 6A). The adhesiveness was dependent on curli production. Colonies of an E. coli csgA− mutant (A−B−) or an E. coli csgB− mutant (A−B−) 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 WT (Fig. 6A). A csgA− mutant (A+ B−) or a csgB− mutant (A− B+) of S. typhimurium also showed low adherence to the agar surface (Fig. 6A). Wild-type S. typhimurium has low surface adherence as well (Fig. 6, A 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 (Fig. 6A). 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 (supplemental Fig. S7A). 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 (Fig. 6A). 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. 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% (Fig. 6B). The increase in attachment was dependent on the nucleator CsgB; an *E. coli csgBA* (A⁻ B⁻) did not promote *S. typhimurium* adherence (Fig. 6B). These results were confirmed by the colony forming units of *S. typhimurium* in the mixed colony (supplemental Fig. S7B). 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 (Fig. 7). The development of pellicle biofilms requires curli production (45), and indeed *S. typhimurium csgA* or *csgBA* mutants were unable to make pellicle biofilms (Fig. 7). These results were confirmed by the colony forming units of *S. typhimurium* in the mixed colony (supplemental Fig. S7B). 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.

**FIGURE 4.** 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 Congo red binding of an *E. coli csgA* mutant. *E. coli csgA* harboring an empty vector control or plasmids encoding CsgAEC (pCsgAEC), CsgAST (pCsgAST), or CsgACK (pCsgACK) were grown on YESCA-CR plates at 26 °C for 48 h. CsgB was required for the complementation of Congo red binding. *E. coli csgBA* mutant harboring an empty vector or plasmid pCsgBAEC, pCsgAEC, pCsgAST, or pCsgACK were grown on a YESCA-CR agar at 26 °C for 48 h. C. Western blot of whole cell lysates (top panel) and plugs (bottom panel) of an *E. coli csgA* mutant transformed with pCsgAEC (lanes 1 and 2), the vector control (lanes 3 and 4), pCsgAEC (lanes 5 and 6), or pCsgACK (lanes 7 and 8), and an *E. coli csgBA* mutant with pCsgAEC (lanes 9 and 10), pCsgAEC (lanes 11 and 12), or pCsgACK (lanes 13 and 14) grown on YESCA agar plates at 26 °C for 48 h. Samples were treated with (+) or without (−) HFIP before electrophoresis and analyzed by a CsgA antibody. D, TEM of *E. coli csgA* or *csgBA* mutant transformed with pCsgAEC, pCsgAST, or pCsgACK. Scale bars equal 500 nm.
FIGURE 5. Interbacterial curli assembly between E. coli and S. typhimurium curli mutants. A, a schematic describing the interaction between an E. coli csgB− mutant (A− B+) and S. typhimurium csgA− mutant (A− B−). Unpolymerized E. coli CsgA secreted by E. coli csgB− (A− B+) is templated by CsgB on the surface of S. typhimurium csgA− (A− B+) B. Western blots of HFIP-treated (+) or nontreated (−) whole cell lysates of the indicated strains or strain mixtures. The blots were probed with anCsgA antibody. The bottom panel is a longer exposure of the same blot to visualize the faint bands. C–L, TEM of E. coli WT (C), E. coli csgA− (A− B+) (D), E. coli csgB− (A− B+) (E), S. typhimurium WT (F), S. typhimurium csgA− (A− B−) (G), S. typhimurium WT (H), S. typhimurium csgA− (A− B−) (I), a mixture of E. coli csgA− (A− B+) and S. typhimurium csgB− (A− B+) (J), and a mixture of S. typhimurium csgA− (A− B−) and S. typhimurium csgB− (A− B+) (L). Curli fibers are indicated by red arrows and flagella are indicated by black arrows. Scale bars equal to 500 nm.

FIGURE 6. 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 (A600/A600) 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 days. Bacterial adhesiveness was determined by the percentage of adhered bacteria after vigorous washing as described under “Experimental Procedures.” B, overnight cultures of E. coli constitutively expressing YFP from plasmid pAH16, S. typhimurium expressing constitutively expressing mCherry from plasmid 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 days, 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.
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pellicles (Fig. 7). We asked if S. typhimurium csgA− could utilize and incorporate CsgAEC into fibers to support a pellicle biofilm. The addition of freshly purified CsgAEC monomers restored the pellicle development of the S. typhimurium csgA− mutant (Fig. 7). However, a S. typhimurium csgBA− mutant mixed with E. coli CsgA monomers did not form a pellicle (Fig. 7). 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 CsgAEC by CsgBST 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 disease-associated amyloid, α-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 Aβ1–42 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. oneidensis. Strikingly, although the CsgASO is only 28% identical to E. coli CsgA in primary structure, it efficiently seeded CsgAEC amyloid formation with only 2% (w/w) seed concentration (Fig. 1C). We have also shown that during seeding and cross-seeding reactions, monomers physically interact with and bind fibers, as indicated by surface plasmon resonance (Fig. 2).

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 that 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 because E. coli CsgA and CsgB are less than 30% identical in amino acid sequence (51, 71). In vitro, both CsgAEC and CsgBEC spontaneously polymerize into amyloid fibers with a similar structure measured by CD and EM (51, 55). Shewmaker et al. (12) have also shown that both CsgA and CsgB adopt similar β-helix structure. 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 CsgBEC can cross-seed CsgAEC and CsgACK, and in turn CsgBEC can cross-seed CsgAEC (Figs. 1, 2, and 4, and supplemental Fig. S5). Although cross-seeding between CsgAEC 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 CsgBCK in trans (supplemental Fig. S5), suggesting CsgBCK can also cross-seed the fibrillation of CsgAEC. Therefore, CsgB-mediated seeding is likely to be a result of relaxed seeding of curli subunits.

Despite low sequence identity, both E. coli CsgAEC and CsgBEC 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 β-strand-loop-β-strand motif and contains conserved Gln and Asn, which are critical for amyloid fiber formation (64). 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 (Gln-49, Asn-54, Gln-139, Asn-144) of E. coli CsgA are essential for efficient amyloid formation and interac-

FIGURE 7. Pellicle formation of S. typhimurium csgA− was complemented by exogenous E. coli CsgA subunits. S. typhimurium WT, csgA−, or csgBA− mutants were incubated in static LB-no salt for 3 days at 26 °C in glass tubes. 10 μg/ml of freshly purified CsgAEC or CsgBEC seeds, or products from a mock CsgA purification from strains harboring an empty vector were added to each tube. The liquid culture underneath pellicles was removed and tubes were stained with crystal violet. When the liquid media was removed, pellicles fell against the wall of the glass tubes.
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Moreover, interspecies assembled curli fibers were biologically significant. 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 (Fig. 5). 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 (Fig. 6).

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 (Fig. 7). Romero et al. (8) recently showed that addition of *B. subtilis* amyloids TasA fibers restored the pellicle development of a *B. subtilis* *tasA* mutant. The addition of polymerized *E. coli* CsgA fibers did not restore pellicle formation by *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 (83, 84), and bacterial amyloids are abundant in natural biofilms (85). 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). Because curli subunits are secreted into the extracellular environment prior to assembly into polymers, and because, 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, 79), 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.

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