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Sequence Determinants of Bacterial Amyloid Formation

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Summary

Amyloids are proteinaceous fibers commonly associated with neurodegenerative diseases and prion-based encephalopathies. Many different polypeptides can form amyloid fibers, leading to the suggestion that amyloid is a primitive main-chain-dominated structure. A growing body of evidence suggests that amino acid side chains dramatically influence amyloid formation. The specific role fulfilled by side chains in amyloid formation, especially *in vivo*, remains poorly understood. Here, we determined the role of internally conserved polar and aromatic residues in promoting amyloidogenesis of the functional amyloid protein, CsgA. CsgA is the major protein component of curli fibers assembled by enteric bacteria such as *Escherichia coli* and *Salmonella* spp. *In vivo* CsgA polymerization into an amyloid fiber requires the CsgB nucleator protein. The CsgA amyloid core region is composed of five repeating units, defined by regularly spaced Ser, Gln and Asn residues. The results of a comprehensive alanine scan mutagenesis screen showed that Gln and Asn residues at positions 49, 54, 139 and 144 were critical for curli assembly. Alanine substitution of Q49 or N144 impeded the ability of CsgA to respond to CsgB-mediated heteronucleation, and the ability of CsgA to self-polymerize *in vitro*. However, CsgA proteins harboring these mutations were still seeded by preformed wild-type CsgA fibers *in vitro*. This suggests that CsgA-fibril-mediated seeding and CsgB-mediated heteronucleation have distinguishable mechanisms. Remarkably, Gln residues at positions 49 and 139 could not be replaced by Asn residues without interfering with curli assembly, suggesting that the side chain requirements were especially stringent at these positions. This analysis demonstrates that bacterial amyloid formation is driven by specific side chain contacts and provides a clear illustration of the essential roles of specific side chains in promoting amyloid formation.

Keywords

amyloid; nucleation; polymerization; curli; asparagine and glutamine residues

Introduction

Amyloid formation is readily associated with neurodegenerative diseases and prion-based encephalopathies.¹ Amyloid fibers are 4–10 nm wide, unbranched proteinaceous filaments.¹ Amyloid fibers possess a characteristic cross- β sheet quaternary structure, in which β strands are perpendicular to fibril axis.¹ These fiber structures have specific tinctorial properties, binding to dyes such as Congo red and thioflavin T (ThT).¹ Amyloid toxicity is complex, but a growing body of work suggests that pre-fiber aggregates are cytotoxic, while mature fibers

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are relatively benign.² Therefore, understanding the folding cascades involved in amyloid formation is necessary for developing new therapies to target these processes.

A newly described class of ‘functional’ amyloids suggest that amyloid formation can be an integral part of normal cellular physiology.^{3; 4} Functional amyloids provide a unique perspective on amyloidogenesis because the cell must have coevolved mechanisms to prevent the toxicity normally associated with amyloid formation. One of the best understood functional amyloids is curli, a bacterially produced extracellular fiber required for biofilm formation and other community behaviors.⁵ In *E. coli*, the polymerization of the major curli fiber subunit protein CsgA into an amyloid fiber is dependent on the minor curli subunit protein, CsgB.⁶ CsgA remains soluble until it encounters outer membrane-localized CsgB.⁵ CsgB has also been demonstrated to have amyloid-forming properties and apparently serves as a template for CsgA polymerization.⁷ We previously showed that, like many other amyloids, preformed CsgA fibers could seed soluble CsgA polymerization *in vitro*.⁸ Therefore, we proposed that the growing CsgA fiber on the cell surface could serve as a template promoting soluble CsgA polymerization in a process akin to seeding.⁸ The molecular details of CsgA fiber-mediated seeding and CsgB-mediated heteronucleation remain poorly described. Because nucleation underlies the rate-limiting step of amyloid propagation, understanding the nature of this mechanism will shed light on how cells control amyloid formation.

The primary sequence of CsgA can be divided into three functional domains: an N-terminal Sec signal sequence (cleaved after translocation into the periplasmic space), an N-terminal 22 amino acid segment (N22) that directs CsgA secretion across the outer membrane,⁹ and an amyloid core region (residues 43 to 151) that contains five imperfect repeating units, each 19–23 amino acids in length (Figure 1(a)).⁵ The five repeating units form a protease-resistant structure,¹⁰ which is proposed to be the amyloid core of CsgA.⁸ These repeats are distinguished by the consensus sequence Ser-X5-Gln-X4-Asn-X5-Gln and are linked by 4 or 5 residues.¹⁰ These Ser, Gln and Asn residues are conserved among CsgA homologs of many enteric bacteria.⁸ This high degree of amino acid sequence conservation suggests that these residues may play an important role in curli assembly.

Many polypeptides, if not all, can assemble into an amyloid fiber *in vitro* even though amyloid-forming proteins do not necessarily share amino acid similarities.¹ Therefore, it has been proposed that amyloid formation is an inherent property of polypeptide main chains.¹ However, specific residues likely play a role in promoting both disease-associated and functional amyloid formation. Yeast prion protein Sup35p has a Gln/Asn rich domain at N-terminus that has been implicated in prion propagation.^{11; 12; 13} Moreover, the specific sequences in this Gln/Asn rich domain govern self-recognition and species-specific seeding activity.¹⁴ Aromatic residues in the islet amyloid polypeptide fragment positively contribute to its polymerization into amyloid fibers *in vitro*.¹⁵ Structural analysis of A β 40 and A β 42 revealed that two β sheets form a parallel β -sandwich stabilized by specific intermolecular side chain contacts and changes of these side chains resulted in defective fiber assembly.^{16; 17} Therefore, it is clear that side chains can influence amyloid polymerization, but the contribution of side chains to *in vivo* amyloidogenesis and the exact roles of amino acid side chain contacts remain poorly understood. Here, we performed a comprehensive mutagenesis study on CsgA and identified the residues that promote CsgA amyloidogenesis. We showed that CsgA amyloidogenesis is driven by the side chain contacts of four Gln and Asn residues in N- and C-terminal repeats. These Gln and Asn residues play essential roles in the response to CsgB-mediated heteronucleation and the initiation of efficient self-assembly *in vitro*. Strikingly, these four Gln and Asn residues are not required for CsgA seeding suggesting CsgA seeding and CsgB-mediated heteronucleation have distinct mechanisms.

Results

Ala scan mutagenesis of internally conserved polar residues

The amyloid core of CsgA is composed of five repeating units, defined by internally conserved and regularly spaced Ser, Gln and Asn residues conserved among many enteric bacteria (Figure 1(a)).⁸ We performed an Ala scan mutagenesis on these 20 polar residues to test their importance in directing bacterial amyloid formation. Amyloid formation of each CsgA mutant was initially assessed by growing bacteria on plates amended with Congo red, as cells expressing wild-type CsgA will stain a deep red color on this medium (Figure 1(b)). Only two mutations, Q49A and N144A, significantly reduced Congo red binding relative to wild-type CsgA (Figure 1(b)). Transmission electron microscopy (TEM) was used to validate the Congo red binding phenotypes. *csgA* cells (LSR10) transformed with pLR5 (encoding CsgA) produced curli fibers that were indistinguishable from those assembled by wild-type strain MC4100 by TEM. Cells expressing CsgA^{Q49A} or CsgA^{N144A} assembled fewer fibers than cells expressing wild-type CsgA observed by TEM (Figure 1(c)).

CsgA polymerization into an amyloid fiber *in vivo* can also be monitored by its ability to migrate as a monomer on SDS PAGE gels after dissociation by a strong acid, formic acid (FA).¹⁸ For example, CsgA produced by wild-type cells is whole cell-associated and SDS insoluble.¹⁹ Brief treatment with FA liberates CsgA monomers from curli fibers produced by wild-type strain MC4100.⁶ Similar to the wild-type strain, CsgA produced by *csgA*/pLR5 was SDS insoluble and required brief pretreatment with FA to migrate into the gel (Figure 2(a), lanes 1 and 2). All of the Ser mutants (S43A, S66A, S88A, S111A and S133A) were also SDS insoluble and associated with the whole cell fraction (Figure 2(a), lanes 5, 6, 13, 14, 21, 22, 29, 30, 37 and 38). However, 13 of 15 Ala substitution mutants of Gln/Asn residues (Q49A, N54A, Q60A, Q72A, N77A, Q94A, N99A, Q105A, Q117A, N122A, Q128A, Q139A and N144A) showed different levels of SDS solubility by whole-cell western analysis, suggesting these polar residues help stabilize the amyloid structure (Figure 2(a)). CsgA^{Q49A} and CsgA^{N144A} were unable to complement Congo red binding to a *csgA* mutant (Figure 1(b)), and very little of these mutant proteins could be recovered from whole cell lysates scraped off YESCA plates (Figure 2(a), lanes 7, 8, 41 and 42).

To test the possibility that CsgA^{Q49A} and CsgA^{N144A} were secreted away from the cell as soluble proteins, cells and the underlying agar were collected and analyzed by western blotting. In these samples, called 'plugs', both CsgA^{Q49A} and CsgA^{N144A} were readily detected and SDS soluble, demonstrating that CsgA^{Q49A} and CsgA^{N144A} were stable, secreted to the cell surface and unpolymerized (Figure 2(b), lanes 2, 3, 11 and 12). CsgA^{N54A} and CsgA^{Q139A} were also significantly different from other mutants in the whole cell SDS solubility assays. CsgA^{N54A} was completely SDS soluble (Figure 2(a), lanes 9 and 10) and CsgA^{Q139A} was not predominately cell associated (Figure 2(a), lanes 39 and 40). CsgA^{N54A} and CsgA^{Q139A} were SDS soluble detected by western analysis of cells and the underlying agar (Figure 2(b), lanes 4, 5, 8 and 9), suggesting CsgA^{N54A} and CsgA^{Q139A} were not assembled into wild-type like fibers *in vivo*. Collectively, Q49A and N144A were the most defective mutants in curli formation of the 20 mutants examined. In addition, the N54A and Q139A mutants were also significantly defective in curli assembly as measured by western analysis.

Ala scan mutagenesis of the aromatic residues

It was reported that aromatic residues may play an important role during amyloidogenesis.^{15;}²⁰ We tested the contribution of aromatic residues in the CsgA amyloid core region by Ala scan mutagenesis. Except for CsgA^{Y151A}, Ala substitutions in the aromatic residues resulted in proteins that were phenotypically identical to wild-type CsgA as detected by Congo red binding and whole-cell western analysis (Figure 3(a) and 3(b)). CsgA^{Y151A} was undetectable

by western analysis of cells and the underlying agar, indicating that Y151A was unstable (Figure 3(c), lanes 5 and 6). When Tyr¹⁵¹ was changed to Phe, Trp, Lys or Asp, curli formation was restored as evidenced by Congo red binding and whole-cell western analysis, suggesting Tyr residue at position 151 is not absolutely required (Figure 3(d) and data not shown).

CsgA^{Q49A} and CsgA^{N144A} are defective in self-polymerization *in vitro*

To further characterize the most defective CsgA mutant proteins, we purified CsgA^{Q49A} and CsgA^{N144A} and compared their polymerization to wild-type CsgA using the ThT assay described in our previous work.⁸ Wild-type CsgA assembles into an amyloid fiber *in vitro* at concentrations above 2.0 μ M in the absence of CsgB.⁸ Two parameters were used to compare the polymerization kinetics of CsgA and its mutant analogues. The first kinetic parameter was the time period preceding rapid fiber growth, called lag phase or T₀. The second parameter was the time period encompassing the fiber growth phase from initiation of rapid polymerization to its completion, called conversion time (T_c).¹¹ At a concentration of 40 μ M, the T₀ of CsgA^{Q49A} was similar to that N_{144A} of CsgA, while the T_c was much greater than that of CsgA (Figure 4(a)). CsgA polymerization had much greater T₀ and T_c than those of CsgA, suggesting the amido group of Asn at position 144 is critical for aggregation (Figure 4(a)). After 120 hrs, both CsgA^{Q49A} and CsgA^{N144A} had assembled into amyloid fibers with similar fiber morphology to wild-type CsgA fibers (Figure 4(b), 4(c) and 4(d)).

CsgA^{Q49A} and CsgA^{N144A} are defective in heteronucleation response

Even though CsgA^{Q49A} and CsgA^{N144A} were defective in self-polymerization, in the presence of wild-type CsgA seeds they polymerized with efficiency similar to wild-type CsgA (data not shown). To test of the ability of CsgA^{Q49A} and CsgA^{N144A} to respond to CsgB-mediated heteronucleation, two different approaches were employed. The first was an overlay assay using freshly purified CsgA or CsgA mutant proteins and cells expressing the CsgB nucleator protein.²¹ In a CsgB-dependent manner, soluble wild-type CsgA was converted into an amyloid fiber within 1 minute of the overlay as evidenced by Congo red staining, or by TEM.²¹ Various concentrations of CsgA, CsgA^{Q49A} and CsgA^{N144A} were overlaid on *csgA* cells (CsgB⁺). At a concentration of 10 μ M or higher, CsgA polymerized into an amyloid fiber in a CsgB-dependent fashion detected by Congo red staining (Figure 5(a)). However, 10 μ M CsgA^{Q49A} and CsgA^{N144A} did not polymerize into amyloid-like fibers under the same conditions (Figure 5(a)). When the concentration of CsgA^{Q49A} and CsgA^{N144A} was increased to 40 μ M, a Congo red-binding structure on the surface of CsgB-expressing cells was detected (Figure 5(a)).

Curli fibers are proposed to assemble after CsgA is secreted to the extracellular space. This can be illustrated by interbacterial complementation where a *csgA* mutant strain (CsgB⁺) is grown in close proximity to a *csgB* mutant strain (CsgA⁺). CsgA molecules secreted by *csgB* donor cells polymerize into amyloid fibers on the *csgA* acceptor cells, as shown in Figure 5(b).⁶ We performed an interbacterial complementation assay to test CsgB-mediated heteronucleation responsiveness of CsgA, CsgA^{Q49A} and CsgA^{N144A} *in vivo*. CsgA secreted from *csgBA*/pLR5 (donor) responded to the heteronucleation of CsgB on the *csgA* cells (acceptor), and formed an amyloid fiber on the surface of *csgA* cells as demonstrated by Congo red (Figure 5(b)).⁶ There was reduced Congo red binding on the surface of *csgA* cells when *csgBA* cells containing plasmid encoding CsgA^{Q49A} or CsgA^{N144A} were grown adjacently to *csgA* cells (Figure 5(b)). The protein level of CsgA, CsgA^{Q49A} and CsgA^{N144A} produced in *csgBA* genetic background is similar as detected by western analysis of cells and the underlying agar (data not shown). These data collectively demonstrate that the polymerization of CsgA^{Q49A} and CsgA^{N144A} were defective in responding to the heteronucleator, CsgB heterogeneous nucleator CsgB.

Ala substitutions of Q49, N54, Q139 and N144 result in the dramatic loss of heteronucleation response to CsgB

In order to abolish the four critical Gln/Asn residues identified by our Ala scan mutagenesis, we constructed the mutant Q49A/N54A/Q139A/N144A. *In vivo* CsgA^{Q49A/N54A/Q139A/N144A} (CsgA^{slowgo}) completely lost the ability to assemble into amyloid fibers as detected by Congo red binding, TEM and whole-cell western analysis (data not shown). We tested the competence of CsgA^{slowgo} to respond to CsgB-mediated heteronucleation by overlay assay. Purified homogeneous CsgA^{slowgo} could not be promoted to form amyloid fibers by cell-associated CsgB as evidenced by the lack of Congo red binding in the overlay assay even at a concentration of 100 μ M (Figure 6(a)). There was no Congo red binding on the surface of *csgA* cells when *csgBA* cells containing plasmid encoding CsgA^{slowgo} were grown adjacently to *csgA* cells shown in Figure 6(b), suggesting that CsgA^{slowgo} completely lost the response to CsgB-mediated heteronucleation in the interbacterial complementation. The protein level of CsgA^{slowgo} was similar to wild-type CsgA in this genetic background (data not shown). Collectively, these results demonstrate that CsgA^{slowgo} remains unpolymerized even in the presence of the heteronucleator CsgB, suggesting these four polar residues are required in CsgB-mediated heteronucleation.

Q49, N54, Q139 and N144 are required for efficient self-polymerization and CsgB_{trunc} cross-seeding not for CsgA seeding

CsgA^{slowgo} was purified and its polymerization kinetics were analyzed *in vitro*. The T_0 and T_c of CsgA^{slowgo} were much higher than CsgA (Figure 7(a)), suggesting that Q49, N54, Q139 and N144 play critical roles in self-polymerization *in vitro*. Self-assembled CsgA^{slowgo} fibers were similar to CsgA fibers shown by TEM (Figure 7(b)). Interestingly, wild-type CsgA seeds efficiently eliminated the lag phase of the polymerization of CsgA^{slowgo} as detected by ThT assay (Figure 7(c)). In the presence of CsgA seeds, the polymerization rate CsgA^{slowgo} is very similar to wild-type CsgA⁸ and ThT signal reached stationary phase within 5 hours (Figure 7(c)). Polymerized CsgA^{slowgo} fibers promoted by wild-type CsgA were similar to CsgA fibers shown by TEM (Figure 7(d)). A nucleation-competent CsgB truncation mutant (called CsgB_{trunc}) was purified as previously described.⁷ CsgB_{trunc} has been demonstrated to efficiently seed the polymerization of wild-type CsgA.⁷ Unlike CsgA seeds, CsgB_{trunc} seeds did not efficiently promote the polymerization of CsgA^{slowgo} under the same condition shown in Figure 7(c). Self-assembled CsgA^{slowgo} fibers were able to seed wild-type CsgA polymerization, suggesting CsgA^{slowgo} fibers are similar to wild-type CsgA fibers in terms of seeding specificity (data not shown).

Conservative Q/N substitutions

It was proposed that *in vitro* amyloid formation is strongly influenced by simple physicochemical factors of polypeptides such as hydrophobicity, charge and secondary structure propensity.^{1; 22} Ala substitutions at positions 49, 54, 139 and 144 decreased CsgA polymerization *in vivo*. To test the stringency of the amino acid present at these critical positions, we constructed four mutants (Q49N, N54Q, Q139N and N144Q) that contained relatively conservative amino acid changes. Gln and Asn residues have similar physicochemical features, including the same amido group. Surprisingly, even though the side chain of Asn is only one carbon distance shorter than that of Gln, CsgA^{Q49N} had dramatically reduced Congo red binding and was almost undetectable in lysates of whole cells scraped off YESCA plate (Figure 8(a) and 8(b), lanes 7 and 8). CsgA^{Q49N} was stable and secreted into the agar as detected by western analysis of cells and the underlying agar (Figure 8(c), lanes 7 and 8). In addition, whole-cell western analysis showed that Q139N (Figure 8(b), lanes 15 and 16) was as defective as Q139A (Figure 2(a), lanes 39 and 40). The sequence requirements at positions 49 and 139 appear extremely stringent during fiber assembly since either Ala or Asn

was not tolerated at these positions. The Congo red binding of CsgA^{N144Q} and CsgA^{N54Q} was similar to that of wild-type CsgA (Figure 8(a)). In addition, CsgA^{N144Q} and CsgA^{N54Q} were predominately SDS insoluble as shown by whole-cell western analysis (Figure 8(b), lanes 11, 12, 13 and 14). The SDS insolubility of CsgA^{N144Q} and CsgA^{N54Q} was similar to wild-type CsgA as detected by western analysis of cells and the underlying agar (Figure 8(c), lanes 11, 12, 15 and 16). We observed wild-type like fibers assembled by the *csgA* strain that contained plasmid encoding CsgA^{N144Q} by TEM, while relatively few fibers were observed on the *csgA* strain expressing CsgA^{Q49N} (Figure 8(d)). Collectively, *in vivo* Gln residues at positions 49 and 139 could not be replaced by Asn, while Asn residues at position 54 and 144 could be replaced by Gln and function like wild-type CsgA. *In vitro* CsgA^{Q49N} was as defective as CsgA^{Q49A} in both heteronucleation responsiveness and self-polymerization. CsgA^{Q49N} polymerization had greater T_c than that of CsgA at the same concentration measured by ThT assay (Figure 8(e)). CsgA^{Q49N} did not respond to CsgB nucleation at 10 μ M as detected by overlay assay (Figure 8(e) insert). It has been proposed that amino acid content can be an important factor in promoting amyloid formation.^{23; 24} To test whether N144Q can suppress the polymerization defect of Q49N, we constructed the mutant Q49N/N144Q. Like Q49N, Q49N/N144Q had significantly reduced Congo red binding relative to wild-type CsgA (Figure 8(f)). Collectively, these findings suggest that CsgB-mediated heteronucleation and CsgA self-polymerization require spatially constrained contacts between certain amino acid side chains.

Discussion

Curli function as part of the extracellular matrix produced by many Gram-negative enteric bacteria. Curli assembly is a precisely coordinated process that is highly amenable to study because of the sophisticated genetic and biochemical tools afforded by *E. coli*. Here, we elucidated the CsgA sequence determinants that drive amyloid formation. We found that Gln and Asn residues at the N- and C- terminal repeats are critical for curli assembly. These Gln and Asn residues are necessary for CsgA to respond to CsgB-mediated heteronucleation and to initiate efficient CsgA self-assembly *in vitro*.

The contribution of amino acid side chains to functional amyloid formation

Unlike amorphous aggregates formed by nonspecific hydrophobic interactions, amyloid fibers are highly ordered proteinaceous polymers that are stabilized by specific interactions. The side chains can clearly influence amyloidogenesis.¹ But how specific sequences influence amyloid formation *in vivo* remains poorly understood. We demonstrated that four internally conserved Gln and Asn residues were required to respond to CsgB-mediated heteronucleation. When these four residues are changed to Ala, the resulting protein, CsgA^{slowgo}, cannot efficiently initiate self-assembly *in vitro* as indicated by an extremely long lag phase before polymerization (Figure 7(a)). CsgA^{slowgo} also cannot efficiently respond to CsgB-mediated heteronucleation (Figure 6). These results suggest that the specific side chain interactions are the driving forces to efficiently initiate bacterial amyloid formation *in vivo* and *in vitro*. Interestingly, we found that these Gln/Asn residues required for CsgB-mediated heteronucleation are not necessary for CsgA fiber-mediated seeding, suggesting that CsgA seeding and CsgB-mediated heteronucleation may have somewhat distinct mechanisms (Figure 7(c)). CsgA^{slowgo} is completely defective in amyloid formation *in vivo*, although it can inefficiently assemble into amyloid fibers *in vitro* (Figure 7(a) and 7(b)). This distinction between *in vitro* and *in vivo* behaviors suggests that *in vivo* amyloid formation has highly stringent requirements that limit initiation of amyloid formation to CsgB-mediated heteronucleation. For example, CsgA remains soluble in the extracellular milieu in the absence of nucleator CsgB even though CsgA is amyloidogenic *in vitro*.⁸ This indicates that CsgA may diffuse into the extracellular milieu if it cannot participate in efficient nucleation. Curli also provides an elegant example how amyloidogenicity can be temporally and spatially controlled.

It is possible that an evolved functional amyloid such as curli capitalizes on specific side-chain interactions to tightly regulate and control amyloidogenesis.

The diverse sequence determinants of amyloid formation

The sequence-specific determinants of amyloid formation have been most studied *in vitro*. Clearly, different amyloid proteins employ various interactions such as hydrogen bonding, hydrophobic and electrostatic interactions to promote intra- and inter-molecular associations. For example, the hydrophobic stretches in A β 42, rather than specific side chains, are sufficient to promote aggregation.²⁵ Yeast prion proteins Sup35p, Ure2p and Rnq1p all have Gln/Asn rich domains that are essential for prion propagation *in vivo* and *in vitro*.^{11; 26; 27; 28} In Huntington's disease, polyglutamine peptides play an essential role in molecular etiology. In addition, the length of polyglutamine peptides is positively correlated to *in vitro* aggregation.²⁹ Gln/Asn rich domains are proposed to have a high potential to be amyloidogenic because they can form intramolecular hydrogen bonds.²⁶ Here, we showed that the internally conserved Gln and Asn residues at the N- and C- terminal repeats are critical for CsgA fiber formation. The initiation of CsgA polymerization both *in vitro* and *in vivo* appears to be dependent on hydrogen bonds formed by these Gln and Asn residues.

Moreover, we showed that 13 of 15 Ala substitution mutants of Gln/Asn residues (Q49A, N54A, Q60A, Q72A, N77A, Q94A, N99A, Q105A, Q117A, N122A, Q128A, Q139A and N144A) showed somewhat more sensitive to SDS relative to wild-type CsgA (Figure 2(a)), suggesting these polar residues help stabilize the amyloid structure. Interestingly, the polar amides of these internally conserved Gln and Asn residues were proposed to form hydrogen bonds with backbones to stabilize its fibril structure in a CsgA predicted structural model.¹⁰ It is possible that these critical Gln/Asn residues not only play an important role to initiate fiber assembly but also stabilize the fiber structure when fibers are formed.

It was proposed that interactions among aromatic residues by π -stacking possibly play a critical role in self-assembly of some amyloid fibrils such as islet amyloid polypeptide.¹⁵ To assess the role of aromatic residues in guiding *in vivo* CsgA amyloidogenesis, we individually replaced each aromatic residue in the amyloid core of CsgA, revealing that aromatic residues do not significantly contribute to curli assembly (Figure 3).

Bacterial amyloid formation is influenced by the spatial arrangement of Q/N residues

It has been suggested that amyloid formation is determined by simple physicochemical factors of polypeptides such as hydrophobicity, charge and secondary structure propensity.²² Furthermore, the amyloidogenesis of yeast prions Ure2p and Sup35p appears to be encoded by a particular amino acid composition, rather than specific sequences.^{23; 24} In contrast, our work demonstrates that CsgA amyloidogenesis, especially *in vivo*, is highly dependent on its specific sequence rather than on its overall amino acid composition or the physicochemical properties of polypeptide chains. The Gln residue at positions 49 and 139 of CsgA could not be replaced by Asn residues without interfering with curli assembly (Figure 8), even though Asn shares many physicochemical features with Gln. CsgA^{Q49N-N144Q} and wild-type CsgA share an identical composition of amino acids, but CsgA^{Q49N-N144Q} is dramatically defective in curli assembly (Figure 8(f)). Additionally, although CsgA^{Q49A}, CsgA^{Q60A}, CsgA^{Q94A}, CsgA^{Q105A}, CsgA^{Q117A}, CsgA^{Q128A}, CsgA^{Q150A} have the identical composition of amino acids, CsgA^{Q49A} is dramatically defective than other Ala mutants in fiber assembly (Figure 1 (b) and 2(a)). We previously showed that N- and C-terminal CsgA repeats drive curli assembly.²¹ Our current study clearly suggests the position of Gln or Asn residues plays an equally important role for amyloid assembly.

Globular protein folding depends on specific side chain contacts. Whether amyloid formation, especially *in vivo*, depends on specific side chain contacts is just beginning to be tested. Our work demonstrates that the initiation of CsgA amyloid formation requires specific amino acid side chains. Specific side chain contacts are likely to facilitate the spatial and temporal control over the polymerization of functional amyloids.

Materials and Methods

Bacterial Growth

To induce curli production, bacteria were grown on YESCA plates (1.0 g yeast extract, 10 g casamino acids and 20 g agar per liter) at 26°C for 48 h.¹⁸ Antibiotics were added to plates at the following concentrations: kanamycin 50 µg/ml, chloramphenicol 25 µg/ml, or ampicillin 100 µg/ml. Curli production was monitored by using Congo red-YESCA (CR-YESCA) plate.¹⁸

Strains and Plasmids

Strains LSR10 (MC4100::*csgA*), LSR12 (C600::*csgBAC* and *csgDEFG*), LSR13 (MC4100::*csgBA*) and MHR261 (MC4100::*csgB*) and plasmids pLR5 (encoding CsgA), pLR2 (vector control) and pMC1 (encoding CsgG) were previously constructed.^{6; 7; 9; 18} Plasmids containing CsgA mutations were constructed by site-specific mutagenesis using standard overlapping PCR extension. PCR products containing relevant mutations and NcoI/BamHI restriction endonuclease sites at 5'/3' ends were cloned downstream of the *csgBA* promoter into NcoI/BamHI sites of vector pLR2. The expression vector pMC3 was generated previously.¹⁸ To express and purify CsgA mutant proteins, PCR amplified mutant sequences including C-terminal hexahistidine tag were cloned into pMC3, replacing the sequence encoding CsgA-his. Sequences of constructs were verified by DNA sequencing.

Western analysis

The immunoblotting of CsgA in whole-cells and plugs was performed as previously described.¹⁸ Briefly, bacterial cells grown on YESCA plate for 48 h were scraped off and normalized by optical density at 600 nm. Cell pellets were resuspended in 2X SDS loading buffer either with or without prior formic acid (FA) treatment as previously described.¹⁸ Alternatively, 8 mm circular plugs including cells and underlying agar were resuspended in 2X SDS loading buffer either with or without prior FA treatment. Samples were electrophoresed on 15% sodium dodecyl sulfate (SDS)-polyacrylamide and transferred onto polyvinylidene difluoride membrane using standard techniques. Western blots were probed and developed as previously described.⁷

Transmission Electron Microscopy

A Philips CM10 Transmission Electron Microscope was used to visualize the cell samples and protein fiber aggregates. Samples (10 µl) were placed on Formvar-coated copper grids (Ernest F. Fullam, Inc., Latham, NY) for 2 min, washed with deionized water, and negatively stained with 2% uranyl acetate for 90 sec.

In vitro polymerization and nucleation assay

CsgA or CsgA mutant proteins and CsgG were co-overexpressed by induction with isopropyl β-D-1-thiogalactopyranoside (IPTG) in LSR12 (C600::*csgBAC* and *csgDEFG*) strain. CsgA or CsgA mutant proteins were secreted to the supernatant and purified as previously described.⁸ After removal of imidazole by a desalting column, purified proteins were passed through a 30 kD cutoff filter (Amicon® Ultra, MA) to remove possible aggregates or seeds that might alter polymerization kinetics. Purified homogeneous proteins were loaded on 96-well opaque

plate and ThT was added to a concentration of 20 μ M. ThT fluorescence was measured every 10 min after shaking 5 sec by a Spectramax M2 plate reader (Molecular Devices, Sunnyvale, CA) set to 438 nm excitation and 495 nm emission with a 475 nm cutoff. Alternatively, samples were kept at room temperature in the presence of 0.02% NaN₃. At the indicated times, aliquots were removed, ThT was added at a concentration of 20 μ M and fluorescence intensity was measured as described above. For seeding reactions, CsgA fiber seeds, prepared as previously described,⁸ were added to freshly purified CsgA mutant proteins immediately before the start of ThT fluorescence assay.

Overlay assay

10 μ l freshly purified proteins were spotted onto a lawn of *csgA* (LSR10) or *csgBA* (LSR13) cells grown on YESCA plates at 26°C for 48 h. Samples were incubated on cells for 10 min at room temperature, stained with a 0.5 mg/ml Congo red in 50 mM potassium phosphate (pH 7.2) for 5 min, then washed with potassium phosphate buffer and photographed.

Interbacterial complementation

Cells that secrete CsgA or CsgA mutant proteins were streaked adjacently to *csgA* cells on a CR-YESCA plate. The plates were photographed after 48 h growth at 26°C.

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Abbreviations used are

ThT	thioflavin T
FA	formic acid
TEM	Transmission Electron Microscopy

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Figure 1. Effect of Ala substitutions of conserved Ser, Gln and Asn of CsgA on curli assembly
 (a) Schematic of CsgA including an N-terminal Sec signal sequence and the N-terminal 22 residues that precede the five repeating units. The regularly-spaced Ser, Gln and Asn residues are indicated in red color and the aromatic residues are indicated in blue color. The position of highlighted residues is indicated above the residue. (b) CR-YESCA plate with *csgA* cells transformed with the plasmids encoding CsgA with Ala substitution after 48 h of growth at 26°C. *csgA*/pLR5 and *csgA*/pLR2 were used as the positive control (PC) and vector control (VC), respectively. The specific Ala substitution is indicated. (c) Negative-stain TEM micrographs of *csgA* cells containing vector control (pLR2) or plasmids encoding wild-type CsgA (pLR5), CsgA^{Q49A} and CsgA^{N144A}. Scale bars are equal to 500 nm.

Figure 2. Western analysis of CsgA mutants with Ala substitutions of internally conserved Ser, Gln and Asn

(a) Western blots of whole-cell lysates from *csgA* cells containing vector control or plasmids encoding wild-type CsgA or indicated Ala substituted mutants. (b) Western blot of whole-cells and underlying agar (agar plugs) from *csgA* strains containing plasmids encoding CsgA, CsgA^{Q49A}, CsgA^{N54A}, CsgA^{Q139A} or CsgA^{N144A}. Samples were treated with (+) or without (-) FA. The blots were probed with anti-CsgA antibody.

Figure 3. Ala scan mutagenesis of aromatic residues in CsgA amyloid core regions

(a) CR-YESCA plate with *csgA* cells transformed with the plasmids encoding CsgA, CsgA^{Y48A}, CsgA^{Y50A}, CsgA^{F97A}, CsgA^{W106A}, CsgA^{F118A}, CsgA^{F142A} or CsgA^{Y151A}. (b) Western blots of whole-cell lysates from *csgB* cells and *csgA* cells containing plasmids encoding wild-type CsgA or indicated Ala mutants. (c) Western blot of whole-cells and underlying agar (agar plugs) from *csgB* cells and *csgA* strains containing plasmids encoding CsgA or CsgA^{Y151A}. (d) The CR-YESCA plate with *csgA* cells transformed with the plasmids encoding CsgA, CsgA^{Y151F}, CsgA^{Y151W}, CsgA^{F151D} or CsgA^{Y151K}.

Figure 4. *In vitro* self-polymerization of CsgA^{Q49A} and CsgA^{N144A} are defective
 (a) The fluorescence of 40 μ M CsgA (solid circle), CsgA^{Q49A} (open circle) and CsgA^{N144A} (open square) mixed with 20 μ M ThT was measured in 10-minute intervals at 495nm after excitation at 438nm. *a.u.*, arbitrary units. (b–d) Negative-stain EM micrographs of *in vitro* self-polymerized fibers of CsgA (b), CsgA^{Q49A} (c) and CsgA^{N144A} (d). Scale bars are equal to 200 nm.

Figure 5. CsgA^{Q49A} and CsgA^{N144A} are defective in response to CsgB-mediated heteronucleation
 (a) A schematic presentation of the overlay assay in which soluble CsgA is spotted onto cells (*csgA*) expressing the nucleator protein, CsgB, where CsgA polymerization is catalyzed by CsgB (top). Freshly purified CsgA, CsgA^{Q49A} or CsgA^{N144A} at the indicated concentrations was spotted onto on *csgA* cells. After 10 min incubation, cells were stained with 0.5 mg/ml Congo red solution and washed by potassium phosphate buffer (bottom). (b) A schematic presentation of the interbacterial complementation assay in which soluble CsgA molecules secreted from *csgB* cells interact with CsgB on the surface of *csgA* cells to form fibers (top). *csgBA* cells containing plasmids encoding CsgA, CsgA^{Q49A} or CsgA^{N144A} were grown adjacently to *csgA* cells on a CR-YESCA plate for 48 h at 26°C (bottom).

Figure 6. The heteronucleation responsiveness of CsgA^{slowgo} (CsgA^{Q49A/N54A/Q139A/N144A})
 (a) Freshly purified homogeneous CsgA^{slowgo} at the indicated concentrations was spotted onto *csgA* or *csgAB* cells. After 10 min incubation, cells were stained with 0.5 mg/ml Congo red solution and washed by potassium phosphate buffer. (b) *csgAB* cells containing plasmids encoding CsgA or CsgA^{slowgo} were grown adjacently to *csgA* cells on a CR-YESCA plate for 48 h at 26°C.

Figure 7. *In vitro* self-polymerization and seeding responsiveness of CsgA^{slowgo}

(a) ThT fluorescence intensity of 40 μ M CsgA^{slowgo} incubated for the indicated time intervals. *a.u.*, arbitrary units. (b) Negative stain TEM micrograph of *in vitro* self-polymerized aggregates of CsgA^{slowgo}. Scale bars are equal to 200 nm. (c) 5% CsgA seeds, CsgB_{trunc} seeds by weight or buffer vehicle were added to 60 μ M CsgA^{slowgo} and the polymerization was measured by ThT fluorescence assay. The fluorescence intensity of nucleated CsgA^{slowgo} samples were shown at the indicated time intervals. The contribution to fluorescence intensity by seeds was subtracted. (d) Negative-stain TEM micrograph of *in vitro* polymerized aggregates of CsgA^{slowgo} promoted by CsgA. Scale bars are equal to 200 nm.

Figure 8. Conservative Q/N substitutions at positions 49, 54, 139 and 144

(a) CR-YESCA plate with *csgA* transformed with the plasmids encoding CsgA, CsgA^{Q49A}, CsgA^{Q49N}, CsgA^{N54A}, CsgA^{N54Q}, CsgA^{Q139A}, CsgA^{Q139N}, CsgA^{N144A} or CsgA^{N144Q}. (b) Western blots of whole-cell lysates from *csgA* cells containing plasmids encoding wild-type CsgA, CsgA^{Q49A}, CsgA^{Q49N}, CsgA^{N54Q}, CsgA^{Q139N}, CsgA^{N144A} or CsgA^{N144Q}. (c) Western blot of whole-cells and underlying agar (agar plugs) from *csgA* strains containing plasmids encoding CsgA, CsgA^{Q49A}, CsgA^{Q49N}, CsgA^{N54A}, CsgA^{N54Q}, CsgA^{N144A} or CsgA^{N144Q}. Samples were treated with (+) or without (-) FA. The blots were probed with anti-CsgA antibody. (d) Negative-stain TEM micrographs of *csgA* cells containing plasmids encoding CsgA^{Q49N} and CsgA^{N144Q}. Scale bars are equal to 500 nm. (e) The polymerization of 56 μ M CsgA^{Q49N} was measured by ThT fluorescence. Congo red staining of *csgA* cells overlaid with CsgA^{Q49N} at various concentrations is shown in the graph inset. (f) CR-YESCA plate of a *csgA* strain transformed with the control vector pLR2 or the plasmids encoding CsgA, CsgA^{Q49N} or CsgA^{Q49N/N144Q}.