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Diversity, biogenesis and function of microbial amyloids

Luz P. Blanco, Margery L. Evans, Daniel R. Smith, Matthew P. Badtke, and Matthew R. Chapman

Department of Molecular Cellular and Developmental Biology, University of Michigan, Ann Arbor, MI 48109, USA

Abstract

Amyloid is a distinct β -sheet-rich fold that many proteins can acquire. Frequently associated with neurodegenerative diseases in humans, including Alzheimer's, Parkinson's and Huntington's, amyloids are traditionally considered the product of protein misfolding. However, the amyloid fold is now recognized as a ubiquitous part of normal cellular biology. 'Functional' amyloids have been identified in nearly all facets of cellular life, with microbial functional amyloids leading the way. Unlike disease-associated amyloids, functional amyloids are assembled by dedicated, directed pathways and ultimately perform a physiological function that benefits the organism. The evolved amyloid assembly and disassembly pathways of microbes have provided novel insights into how cells have harnessed the amyloid assembly process for productive means. Understanding functional amyloid biogenesis promises to provide a fresh perspective on the molecular events that underlie disease-associated amyloidogenesis. Here, we review functional microbial amyloids with an emphasis on curli fibers and their role in promoting biofilm formation and other community behaviors.

Functional amyloids

Amyloid fibers are ordered protein aggregates generally associated with neurodegenerative diseases such as Alzheimer's, Parkinson's and the various prion diseases. Despite great variation in primary sequence, many proteins can assemble into amyloid fibers [1]. However, all amyloids share several distinguishing biophysical properties. Proteins or peptides that adopt the amyloid fold assemble into β -sheet-rich fibers, where the individual β -strands are oriented perpendicular to the fiber axis [2,3]. Amyloid fibers are highly resistant to protease digestion and denaturation, requiring treatment with harsh denaturants, such as formic acid (FA) or hexafluoro-2-propanol (HFIP), to liberate monomers from the fiber [4,5]. Amyloids can bind to specific dyes such as Congo red (CR), resulting in a red shift and green birefringence under polarized light, and thioflavin T (ThT), leading to increased fluorescence at certain wavelengths [6]. Colorimetric and biochemical analyses of amyloids are often coupled as CR and ThT can bind other molecules, such as cellulose and amorphous aggregates, respectively [7].

Whereas amyloid formation is historically thought to arise from protein misfolding, newer research is identifying organisms that utilize the amyloid fold to fulfill important physiological functions. These 'functional' amyloids share many of the characteristics of

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Corresponding author: Chapman, M.R. (chapmanm@umich.edu).

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classical disease-associated amyloids including oligomeric intermediates in the amyloid-folding pathway [8,9]. Amyloid fibers and their folding intermediates are highly cytotoxic and can cause disruptions in membrane integrity. To deal with this toxicity, dedicated and highly controlled pathways often assemble functional amyloids. By avoiding cellular toxicity, functional amyloids provide the cell with a stable protein structure that can be utilized in many different ways. Functional amyloids have now been identified in all kingdoms of life from bacteria to humans [10].

In microbes, amyloid fibers often mediate self and non-self interactions during the formation of biofilms, communities of bacteria encased in an extracellular matrix of proteins and polysaccharides. In fact, amyloids are the major proteinaceous component in many biofilms [11]. Within biofilms, amyloid fibers are well suited for the role of protein scaffolds, they are remarkably strong and aid in adherence and resistance to a variety of environmental insults [12]. The assembly of amyloid fibers also does not require energy because amyloid proteins can ‘seed’ their own oligomerization [13].

The use of amyloid fibers in biofilms is not the only role of functional amyloids. Functional amyloids often aid in adhesion to surfaces and tissues and in changing surface properties. Amyloids can also act epigenetically by sequestering and interfering with the function or activity of specific proteins. Finally, some functional amyloids act as cytotoxins, killing susceptible neighboring cells and providing an interesting link to the classical disease-associated amyloids. Throughout this review we will discuss the assembly of microbial amyloids and how these molecules participate in biofilm formation, persistence and other cellular functions. We will also illustrate, with an emphasis on curli, the many mechanisms that have evolved in functional amyloid systems that discourage potential amyloid-induced cytotoxicity.

Curli as a model for bacterial functional amyloid assembly

Enteric bacteria are a major cause of human diseases. Many Enterobacteriaceae produce curli: extracellular proteinaceous fibers that contribute to biofilm formation, host colonization, immune activation and cell invasion [14]. Curli are the major proteinaceous component of the extracellular matrix produced by *Escherichia coli* and *Salmonella enterica* serovar Typhimurium [14]. Curli share all of the biophysical properties of amyloids. Curli are ordered β -sheet-rich fibers that are able to resist proteases and other harsh denaturants and bind the dyes CR and ThT [15]. In contrast to disease-associated amyloids, curli assembly is not the result of protein misfolding. Instead, curli are the product of a dedicated biogenesis pathway providing a paradigm for understanding controlled amyloidogenesis (Figure 1).

Curli assembly is guided by the products of seven curli specific genes (*csg*) encoded on two divergently transcribed operons, *csgDEFG* and *csgBAC* (Figure 1). CsgD is the master regulator of curli biogenesis and is required for transcription of the *csgBAC* operon. The remaining Sec-tagged curli proteins are secreted into the periplasm by the Sec–translocon system [14,16,17]. Once in the periplasm, the lipoprotein CsgG is transported by the LOL (lipoprotein outer-membrane localization) transport system to the outer membrane where it oligomerizes into a pore-like structure that is required for secretion of the major subunit CsgA and the minor subunit CsgB into the extracellular milieu [18–20]. At the cell surface, CsgA is nucleated into an amyloid fiber by cell surface associated CsgB [21]. Nucleation of CsgA occurs at a few discrete locations on the cell surface that are dictated by clustering of CsgG [19,21].

The secretion and localization of curli fiber subunits through CsgG is mediated by three additional proteins: CsgC, CsgE and CsgF [22–24]. The surface exposed, membrane-

associated protein CsgF is required for CsgB surface exposure and is crucial for efficient CsgA polymerization [15,19,21, 23]. CsgE, a small periplasmic protein, is proposed to direct CsgA to the CsgG secretion complex and helps mediate secretion specificity via an N-terminal sequence in the mature CsgA [22]. CsgE also inhibits CsgA polymerization *in vitro*, and hence CsgE could be considered a CsgA-specific chaperone [22]. The role of CsgC in curli biogenesis is less well understood. Recently, the structure of CsgC was solved and it was predicted to have oxidoreductase activity. Within the periplasm, CsgC may regulate CsgG outer membrane assembly and pore activity through modification of C230 in CsgG [24]. Although these accessory proteins play integral roles in efficient and localized curli biogenesis, the mechanistic details of their individual contributions and interactions are not completely understood.

The curli fiber subunits CsgA and CsgB are both composed of five imperfect repeats. Each repeat is individually composed of a strand-loop-strand motif that is predicted to stack into two parallel β -sheets [25,26] (Figure 1). However, despite much research, the exact structure of curli fibers has not yet been completely elucidated. Shewmaker *et al.* compared the physical properties of CsgA fibers with pathological amyloid fibers and concluded that the essential cross- β fold of amyloids is present in curli fibers, probably as a β -helix structure [3]. Our understanding of the molecular features of curli subunits and their roles in governing subunit interactions has added details to our current model of curli assembly. In CsgA, the first (R1) and fifth (R5) repeating units are proximal to the N- and C-termini, respectively. Unlike the other repeating units, both R1 and R5 are required for efficient self-polymerization and CsgB-mediated nucleation. Specific residues in the internal repeats (R2, R3 and R4) instead play key roles in regulating the kinetics of CsgA polymerization and the toxicity of the curli fibers *in vivo* [27].

Upon secretion to the cell surface, CsgA is present in a mostly unstructured state [14]. However, when CsgA interacts with CsgB or a growing fiber tip, it transitions into a cross- β fold [8,21]. The *in vitro* polymerization of CsgA into an amyloid involves three distinct phases: a lag phase, a fiber elongation phase (growth) and a plateau or stationary phase (Figure 2). Formation of a transient oligomeric species (or nucleus) is seen in disease-associated amyloid formation [28,29]. Charles Glabe and colleagues demonstrated that the oligomeric intermediate could be experimentally probed for by using a conformational specific antibody raised against A β , the amyloid subunit associated with Alzheimer's disease, in the oligomeric state [30]. The anti-oligomer antibody also transiently recognizes other amyloidogenic proteins, including CsgA, demonstrating that CsgA polymerization *in vitro* also involves a structural intermediate analogous to that formed by disease-associated amyloids but nontoxic to the host bacteria [8,29]. More recently, the mechanism of CsgA *in vitro* polymerization has been characterized under different conditions [9]. Interestingly, a needle-like protofibril intermediary is formed that precedes the formation of the mature CsgA amyloid [9].

As CsgA and presumably other functional amyloids can adopt the toxic oligomeric forms, there must be a means to limit the inherent cytotoxicity associated with preamyloid oligomers. Mechanisms exist for minimizing amyloid-related toxicity in cells that assemble functional amyloids: (i) by employing chaperones to discourage inappropriate aggregation; (ii) through localization of amyloidogenic proteins to specific regions in or around the cell; or (iii) through temporal control to minimize toxic oligomeric intermediates. The curli biogenesis system has elements of each mechanism. We hypothesize that during secretion, CsgA interacts with molecular chaperones that maintain it in a soluble state, thus preventing CsgA from making inappropriate interactions with itself and cell membranes. Furthermore, the outer membrane secretion machinery is localized to discrete places around the cell, which creates high local concentrations of CsgA on the cell surface [19]. Finally, soluble

CsgA, which upon secretion can interact with CsgB or the growing curli fiber tip, can rapidly transition into a mature amyloid polymer. Thereby, the rapid conversion of CsgA from a soluble monomer to a mature fiber in specific locations on the cell surface minimizes the chance that CsgA could sample the toxic oligomer state.

Functional amyloids in biofilms and flocs

A variety of microbes including bacteria and fungi are capable of biofilm formation. Gram-negative bacteria such as *Salmonella* and *E. coli* produce a complex extracellular matrix that contributes to the formation of biofilms [7,31]. This matrix includes cellulose, exopolysaccharide such as colonic acid, and curli [7]. Curli are critical determinants of biofilm formation as they can mediate initial surface attachment and also provide a scaffold that contributes to biofilm integrity [14,31–34]. Accordingly, curliated bacteria better colonize plant tissues, stainless steel and glass [34–37]. Rationally designed, small, peptidomimetic molecules that are able to interfere with curli assembly also prevent biofilm formation, supporting a key role for curli in biofilm formation [38].

Amyloids have been identified in the natural environment in biofilms produced by *Proteobacteria*, *Bacteroidetes*, *Chloroflexi* and *Actinobacteria* [11]. Amyloids have also been identified in organisms involved in water treatment processes, where the crucial bioremediation step is mediated by biofilm-like structures called flocs: aggregative clusters of microbes in a network of polysaccharides and protein. Amyloids have been characterized in the matrix of activated sludge-derived flocs containing a broad variety of microbes [39]. In this context, amyloids probably play diverse roles in adhesion and as a scaffold important for biofilm formation, integrity and persistence. However, amyloids may also help to act as physical barriers, scavengers of toxins, moisture regulators or as toxins that kill other microbial species. Specific examples of these possible amyloid functions during biofilm development are discussed below.

Pseudomonas fluorescens and *Bacillus subtilis* produce identifiable functional amyloids in their biofilm matrix [14,40,41]. FapC, originally characterized in *P. fluorescens*, is a highly conserved amyloid protein found in many *Pseudomonas* species. FapC has three repeat motifs and forms an extracellular amyloid matrix that contributes to the aggregative properties of *Pseudomonas* species [40]. Biofilm integrity and persistence in *B. subtilis* are also influenced by the amyloid-forming protein TasA [42]. TasA forms extracellular amyloid fibers that are anchored to the peptidoglycan layer of the cell wall by TapA [41]. When biofilms age or D-amino acids are elevated, TapA is shed from the membrane-releasing TasA fibers from the cell surface and allowing for dispersal from the biofilm [41]. Although unrelated at the primary sequence level, TasA and FapC together with CsgA serve similar functions in biofilm formation demonstrating a common function for amyloids in biofilms.

Functional amyloids as adhesins

Amyloids also function as adhesins, as is the case with *Mycobacterium tuberculosis* [43]. Electron microscopic analysis of the *M. tuberculosis* pili (MTP) shows fibers that are strikingly similar to curli fibers. These fibers have also been reported in aged and carbon starved *M. tuberculosis* cultures [44,45]. Additionally, MTP are sodium dodecyl sulfate (SDS)-insoluble and bind CR, suggesting that MTP are in fact amyloid fibers. MTP fibers have recently been shown to contain the protein product of the Rv3312a reading frame [43]. The significance of MTP fibers for the pathogenesis of *M. tuberculosis* is unclear; however, MTP has been shown to bind laminin, a human extracellular matrix protein. MTP are conserved only in pathogenic mycobacterial species; a recent study showed that tuberculosis

patients produce serum antibodies against MTP [43]. These data suggest that MTP are amyloid fibers that may be involved in *M. tuberculosis* cellular adherence and pathogenesis.

Amyloids produced by fungi also play a role in adhesion. *Candida albicans* produces Als adhesins, which are involved in host tissue adherence and self-aggregation [46–50]. Als5p was shown to be important in surface changes that resulted in yeast aggregation when expressed heterologously in *Saccharomyces cerevisiae* or in *C. albicans* [51]. Als5p was shown to undergo changes in protein secondary structure and bind to CR. Short peptides from Als were also shown to form amyloids *in vitro*, demonstrated by CR binding, ThT fluorescence and electron microscopy [48]. Intriguingly, oral infections in humans occur more prevalently with *C. albicans* when there is a higher amount of ferritin in the oral cavity. This observation led to the discovery that Als3 adhesin expression in the hyphae morphotype of *C. albicans* allows binding and utilization of iron sequestered in ferritin [52]. Hence, in this yeast, expression of functional amyloid leads to key events for fungal survival and virulence.

Functional amyloids as surface property modifiers

There are several examples of amyloids modifying the surface properties of microbes. One example, chaplins, are produced by the soil-derived bacteria *Streptomyces coelicolor*. *S. coelicolor* produce aerial hyphae to disperse spores. While underground, the hyphae are hydrophilic, whereas the aerial hyphae and spores are hydrophobic [53], and the formation of aerial hyphae requires a change in the surface hydrophobicity. This change in surface property is concomitant with the formation of an SDS-insoluble surface layer in the aerial hyphae and spores. The formation of this layer requires chaplin proteins, which are encoded by *chpA-H* [54]. Chaplin proteins assemble into a network of amyloid fibers and cellulose on the surface of the aerial hyphae [55]. The chaplins also self-assemble into β -sheet-rich fibers *in vitro* and bind CR and ThT [54]. The amyloid properties of chaplins provide the dramatic hydrophobicity change to the surface layer that results in decreased surface tension at the air–water interface. The hyphae are then freed from the soil and continue to rise into the air [56].

The individual contributions of the eight chaplin proteins to the amyloid network are starting to be elucidated. Recently, five of them (ChpD–H) have been shown to form amyloid fibers *in vitro* [57]. Remarkably, addition of crude cell extracts containing chaplins restores formation of aerial hyphae to mutant *S. coelicolor* lacking some of the *chp* genes. This observation is similar to the interbacterial complementation phenotype of *E. coli csgA* and *csgB* mutants where the individual strains are unable to produce curli, but when grown in close proximity to each other *csgA* and *csgB* mutants are able to make curli [21,54]. Furthermore, there may be temporal regulation influencing the expression of the chaplin genes suggesting that a coordinated biosynthesis pathway is at work in chaplin amyloid assembly [54]. How chaplin assembly is controlled on the molecular level remains undefined. Several of the *chp* genes are not essential for fiber formation and could function as accessory factors for preventing inappropriate amyloidogenesis [57,58]. In addition, the importance of cellulose in chaplin formation could be another control mechanism, preventing chaplin fiber formation until the subunits contact cellulose on the cell surface [55].

Hydrophobins are another class of small, secreted proteins of approximately 100 amino acids that are produced by fungi. Hydrophobins play a similar role to that of chaplins in spore and fruiting body formation, but also play a role in attachment to host surfaces and protection against the host innate immune response during infection [59,60]. Hydrophobins can be divided into Classes I and II. The better-characterized Class I hydrophobins are

functionally related and can complement each other. Hydrophobins self-assemble at the hydrophilic–hydrophobic interface (reviewed in [59]). Strikingly, the hydrophobin polymerization process is most efficient over interfaces with high surface tension, whereas agents that reduce this property slow down the rate of hydrophobin polymerization *in vitro* [61]. The layer that forms from Class I hydrophobins is SDS-insoluble and can only be disassembled with FA treatment [62]. Amino acid sequences among the hydrophobins are diverse, but they all have eight conserved cysteine residues. The SC3 hydrophobin is β -sheet-rich and forms a helical intermediate at the hydrophilic–hydrophobic interface [63]. This β -sheet-rich structure forms stable 5–12 nm wide rodlets (rod-shaped structures) that fluoresce when mixed with ThT and can bind CR. Therefore, the rodlets appear to share many properties with amyloids.

Ustilago maydis is a fungal plant pathogen that produces proteins called repellents, which are involved in aerial hyphae formation and attachment to host cells. In *U. maydis*, these repellents perform a similar function to hydrophobins; however, these two classes of proteins are unrelated at the amino acid level. The *rep1* gene encodes a prepropeptide that is processed into the ten peptides that are collectively referred to as repellents. Teertstra *et al.* have shown that the peptide Rep1-1 is able to form filaments both *in vitro* and *in vivo* with amyloid characteristics [64]. Microarray analysis conducted comparing a *rep1* mutant with a wild-type strain found induction of a small subset of genes encoding secreted cysteine-rich proteins that are predicted to form amyloids [65,66]. Thus, these amyloids may be functionally redundant to ensure that proper fungal development occurs. This redundancy supports an essential role of functional amyloids in fungi.

Functional amyloids as toxins

One way many functional amyloids bypass the toxicity associated with amyloid formation is through separation of nucleator and major subunit functionalities. However, some functional amyloids capitalize on the inherent cytotoxicity of the amyloid fold. Microcin E492 is a bactericidal peptide produced by *Klebsiella pneumoniae* that assembles into oligomeric pores within the cytoplasmic membrane of neighboring bacteria, particularly Enterobacteriaceae species [67]. Other microcins have also been described with similar antimicrobial functions [68]. Microcin E492 is imported across the bacterial outer membrane in a receptor-mediated manner [69] and has been shown to form voltage-independent ion channels in planar lipid bilayers [67]. The toxicity of the peptide changes during the growth cycle of the *K. pneumoniae*. The toxicity is greatest during exponential phase and lowest during stationary phase, and this change in microcin E492 toxicity is associated with the formation of mature amyloid fibers [70]. The assembly of amyloid fibers can also be observed *in vitro* and corresponds to a loss of toxicity [70]. Therefore, the toxic pore-forming microcin E492 is presumably a transient preamyloid oligomer intermediate. It has been speculated that the toxic microcin E492 species may have structural similarity with the transient oligomeric species formed by disease-associated amyloids. Microcin E492 has been shown to trigger apoptosis in some human cell lines, reminiscent of the toxic effect observed for disease-associated preamyloid oligomers [71]. Microcin E492 is currently being explored as a method to eliminate malignant cells [72]. The mechanisms for promoting oligomeric pore formation, but preventing aggregation into fibers, requires further study.

Harpins are proteins secreted by bacteria that elicit a hypersensitive immune response in plants, some of which might include the toxic aspect of amyloid formation intermediates. The hypersensitive response is characterized by localized cell death in plant tissue, which is a mechanism to prevent the spread of plant pathogens. Several pathogens including *Xanthomonas*, *Erwinia* and *Pseudomonas* species secrete harpins as virulence factors to

assist in movement through plant hosts [73,74]. Harpins from several bacterial species form amyloid-like fibers *in vitro* [75]. The amyloid forming ability of HpaG, a harpin from *Xanthomonas*, directly correlates with hypersensitive response. A mutant form of HpaG that is unable to form amyloid also fails to elicit the hypersensitive response. Interestingly, size exclusion chromatography and visualization by electron microscopy suggests that a tetrameric oligomer is present when a hypersensitive response is observed [75]. These results raise intriguing questions about how the bacteria avoid the toxicity associated with oligomer formation.

Functional amyloids in epigenetic inheritance

Prions have been shown to cause many diseases, including transmissible spongiform encephalopathies (reviewed in [76]). The cause of the prion diseases is a normally soluble protein PrP^C that misfolds and forms an amyloid aggregate, PrP^{Sc}. The prion form can then convert other soluble PrP^C proteins into insoluble aggregates that propagate the misfolded protein conformation and cause cellular damage [77]. However, several prions can be categorized as 'functional' in yeast because a definable advantage is gained upon their formation [78]. In addition, many of these proteins form a cytoplasmic amyloid that is passed on to progeny during cellular division. These prions are examples of an epigenetic, non-Mendelian type of inheritance. Sup35p is the best-studied example of how particular protein folds can impart heritable changes. Sup35 is a translation termination factor that prevents protein synthesis past stop codons in *S. cerevisiae*. This regulation is lost when Sup35p assembles into an amyloid in [PSI⁺] *S. cerevisiae* leading to C-terminal extensions on some proteins and therefore greater protein diversity [79]. Similarly, through aggregation, Ure2p can regulate Gln3p activity. Gln3p is a transcription factor that regulates expression of *DAL5*, which encodes a ureidosuccinate and allantoate transporter. Aggregation of Ure2p in [URE3⁺] strains of yeast causes Gln3p to be constitutively active and *DAL5* expression is upregulated. This effectively eliminates nitrogen catabolite repression and enables yeast to utilize ureidosuccinate in the presence of ammonium [80]. Rnq1p, a glutamine/asparagine-rich protein with unknown function in *S. cerevisiae*, can also polymerize into the prion form [PIN⁺] and has been shown to promote the formation of [PSI⁺], another prion phenotype [81,82]. Collectively, the transition to and from the amyloid form of these multidomain proteins impacts cellular physiology.

Lastly, fungal hyphae from adjacent colonies can fuse to form heterokaryons, genetically different nuclei coexisting in a common cytoplasm, and this process is mediated by the specific interactions that occur between amyloidogenic proteins. A well-characterized protein named HET-s regulates heterokaryon formation in *Podospora anserina* by prion formation. The prion form of HET-s results in heterokaryon incompatibility of genetically similar fungi [83]. This is a currently expanding field, with several new examples of functional yeast prions reported including Swi1p, a part of the SWI/SNF chromatin-remodeling complex that is involved in regulation of 6% of the genes in *S. cerevisiae*. Aggregation of Swi1p alters the regulation of the complex and is transmitted to offspring by the cytoplasm [84,85]. Therefore, amyloid aggregation can significantly impact fungal physiology, while also imparting inheritable changes.

Concluding remarks

Because amyloid formation is the hallmark of many notable human diseases, a somewhat unfriendly nomenclature has historically surrounded the amyloid field. Descriptions of or about amyloids invariably contained words such as misfolded, aberrant aggregation, sporadic, toxic and disease. Even to the aficionado, the word 'amyloid' itself conjured up images of mangled piles of protein debris that were intimately associated with the cause of

Alzheimer's disease [86,87]. However, the bad rap of amyloid is rapidly brightening. For starters, mature amyloid fibers are not probably the cause of Alzheimer's disease [28,29,88]. An even more important factor in reshaping the image of amyloid has been the discovery of functional amyloids. The functional amyloids have taught us that the amyloid fold can be an important part of cellular physiology. As our appreciation for amyloid biology continues to grow, it will be important to fully vet the mechanisms that cellular systems have evolved to assemble, disassemble and control amyloid formation. The mature amyloid fold found in the fibrils does not appear to be inherently toxic, if handled appropriately. However, similar to many things, amyloid formation at the wrong time, the wrong place or with the wrong constituents can be catastrophic. The functional amyloids provide a how-to guide for coordinating ordered protein aggregation in ways such that other cellular processes are not negatively impacted.

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Figure 1.

Model of curli biogenesis. Excluding CsgD, the master curli regulator, all Csg proteins have Sec-dependent signal sequences allowing their secretion into the periplasm. The lipoprotein CsgG forms a pore-like structure in the outer membrane. The major subunit protein CsgA and the nucleator CsgB are secreted to the cell surface in a CsgG- and CsgE-dependent manner. CsgF associates with the outer membrane and is required for cell association of the minor curli fiber subunit CsgB. Situated at the cell surface, CsgB nucleates soluble, unstructured CsgA into a highly ordered amyloid fiber. Curli production can be visualized by CR binding which is absent in a *csgA* mutant and by transmission electron microscopy

(left inserts). Also shown are two CsgA subunits interacting in a cross- β conformation with the R1–R5 interaction depicted.

Figure 2.

Schematic representation of amyloid polymerization. **(a)** Typical ThT fluorescence kinetics of soluble purified CsgA monomers polymerizing into curli [15]. The lag, growth and stationary phases are indicated. The blue arrow indicates the end of the lag phase. The insert shows a transmission electron micrograph of CsgA fibers formed when purified CsgA is allowed to polymerize *in vitro* as described by Wang *et al.* [8]. Scale bar: 500 nm. **(b)** The transition from soluble, monomeric proteins into polymeric and insoluble amyloid fibers is characterized by distinguishable steps that result in loss- or gain-of-function properties for the protein. In the lag phase, soluble protein assembles into a common intermediate or nucleus that is proposed to be toxic to membranes [29,88]. The formation of the

intermediate is proposed to be the rate-limiting step of amyloidogenesis. Once the nucleus is formed, monomers are templated into growing amyloid fibers causing an increase in ThT fluorescence. When the monomer population is depleted, elongation stops and enters the stationary phase. The green boxes below the schematic highlight properties of some of the functional amyloids, whereas the red boxes highlight some general properties of disease-associated amyloids.