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Microbial Manipulation of the Amyloid Fold

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Abstract

Microbial biofilms are encased in a protein, DNA and polysaccharide matrix that protects the community, promotes interactions with the environment, and helps cells to adhere together. The protein component of these matrices is often a remarkably stable, β -sheet-rich polymer called amyloid. Amyloids form ordered, self-templating fibers that are highly aggregative, making them a valuable biofilm component. Some eukaryotic proteins inappropriately adopt the amyloid fold and these misfolded protein aggregates disrupt normal cellular proteostasis, which can cause significant cytotoxicity. Indeed, until recently amyloids were considered solely the result of protein misfolding. However, research over the past decade has revealed how various organisms have capitalized on the amyloid fold by developing sophisticated biogenesis pathways that coordinate gene expression, protein folding, and secretion so that amyloid-related toxicities are minimized. How microbes manipulate amyloids, by augmenting their advantageous properties and by reducing their undesirable properties, will be the subject of this review.

Keywords

amyloid; biofilm; toxin; fiber; extracellular matrix

Introduction

A biofilm is a community of microbes bound together by an extracellular matrix that includes proteins, polysaccharides, and DNA. Proteins in the extracellular matrix can take on many structures, but one of the most commonly found is amyloid. β -sheet amyloid fibers have long been the hallmark of human neurodegenerative diseases, but amyloids are increasingly appreciated as a functionally diverse protein fold. Amyloids are remarkably stable protein polymers that form β -sheet rich fibers with a diameter of 5–10 nm. The amyloid fold is unique in that it is adopted by a variety of proteins with varying primary sequences.

The first step in amyloid fiber formation is aggregation of monomers into intermediate oligomers, or seeds. Once seeds form, they nucleate rapid fiber elongation, with the final

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amyloid structure being essentially a stack of β -sheet rich monomers, aligned so that each β -strand is perpendicular to the fiber axis (Fig. 1). Dense hydrogen bonding between adjacent β -sheets then provides fiber stability (Harper and Lansbury, 1997, Nelson et al., 2005, O’Nuallain et al., 2004, Scheibel et al., 2001, Serpell et al., 1997, Sunde et al., 1997, Wetzel, 2006). Amyloidogenic proteins can polymerize in the absence of an energy source, so it is not surprising that amyloid fibers are a common component of the microbial extracellular matrix, where energy sources can be scarce and environmental conditions can wreak havoc on lesser protein folds (Chapman et al., 2002, Dueholm et al., 2010, Jordal et al., 2009, Larsen et al., 2007, Romero et al., 2010, Schwartz et al., 2012).

The necessary structural features of an amyloid fiber, β -sheet formation and hydrogen bonding between β -sheets, are partly conferred by the polypeptide backbone and do not require complex amino acids (Chamberlain et al., 2000, Dobson, 2004, Gordon and Meredith, 2003, Kheterpal et al., 2000, Makin et al., 2005). These properties are proposed to lend themselves to the universality of the amyloid fold, and, indeed, there is a hypothesis that the amyloid fold, with its tendency towards elementary amino acid composition and resistance to harsh environmental conditions, was a fundamental structure in the early history of life (Carny and Gazit, 2005). Most proteins can adopt the amyloid fold, although the majority of ‘non-native’ amyloids require exposure to harsh denaturing conditions to destabilize their native structure before they can re-organize into an amyloid (Chiti et al., 1999, Chiti et al., 2000, Ramirez-Alvarado et al., 2000). Conversely, some proteins readily form amyloids. These polypeptides often contain specific regions that promote the amyloid fold (Esteras-Chopo et al., 2005, Pastor et al., 2007, Tenidis et al., 2000). Transfer of amyloidogenic stretches of amino acids to normally non-amyloidogenic proteins can force amyloid formation (Ivanova et al., 2004, Ventura et al., 2004).

Similarly, the amyloid fold confers common biophysical characteristics to proteins that might be dissimilar at the primary sequence level. For instance, a defining characteristic of an amyloid, regardless of primary amino acid sequence, is the ability to bind certain dyes such as Congo red and thioflavin T (Elghetany and Saleem, 1988). Additionally, antibodies have been designed that recognize general epitopes of amyloid fibers or amyloid oligomers (Hrncic et al., 2000, Kaye et al., 2003, Kaye et al., 2007, Larsen et al., 2007, O’Nuallain and Wetzel, 2002), and small molecules have been identified that affect fiber formation of multiple amyloids (Horvath et al., 2012).

Amyloids have been thoroughly studied because of their historical association with neurodegenerative conditions such as Alzheimer’s disease. The Alzheimer’s amyloid β ($A\beta$) peptide was one of the first described disease-associated amyloids and is among the most thoroughly studied (Hardy and Selkoe, 2002). Amyloid formation is also the hallmark of Parkinson’s disease (Breydo et al., 2012), Huntington’s disease (Zuccato et al., 2010), and type II diabetes (Westermarck et al., 2011). Because amyloid fibers were identified in connection with various diseases, it was assumed that amyloid fibrils themselves were toxic (Caughey and Lansbury, 2003). Although new data suggest that mature amyloid fibers are relatively inert, non-cytotoxic, and maybe even protective (Caughey and Lansbury, 2003). Instead, amyloid-related toxicity is likely caused by small oligomers formed as an intermediate step in fiber polymerization (Bucciantini et al., 2002, Caughey and Lansbury, 2003, Glabe and Kaye, 2006, Kaye et al., 2003, Lambert et al., 1998). Indeed, the oligomeric species of various disease causing amyloids have been shown to be toxic (Engel, 2009, Haass and Selkoe, 2007, Volles and Lansbury, 2003).

Although many amyloids in multicellular organisms are traditionally associated with protein misfolding and disease, the last decade has ushered in the emergence of the functional amyloid – where amyloid formation clearly benefits cellular physiology. Molecular studies

of functional amyloids have revealed elegant systems designed to enhance and fine-tune amyloid formation spatially and temporally within biological systems and to limit amyloid-associated toxicity. While disease-causing amyloids were the first to be described, the rapid discovery of functional amyloids in a variety of organisms, used for a variety of purposes, is forcing us to reconsider which amyloids are the exceptions and which are the rule. This review will detail microbial systems dedicated to functional amyloid formation, focusing on the versatility of the amyloid fold and the functions for which microbes utilize amyloids.

Amyloids as Biofilm Matrix Material

Curli

The first bacterial functional amyloid to be described was curli, extracellular proteinaceous fibers produced by enterics such as *Escherichia coli* and *Salmonella enterica* (Chapman et al., 2002, Collinson et al., 1991, Olsen et al., 1989, Romling et al., 1998). Curli expression is controlled by a variety of environmental signals, with maximum induction typically occurring under low salt, low nutrient conditions (Olsen et al., 1993, Provenance and Curtiss, 1992, Romling et al., 1998). Intriguingly, although CsgA is able to polymerize under a wide variety of environmental conditions, salt concentration affects polymerization kinetics (Dueholm et al., 2011), implying that environmental signals can alter both curli expression and curli aggregation. Curli fibers are important for various biofilm-related phenotypes such as attachment to abiotic surfaces and development of biofilm architecture (Castonguay et al., 2006, Pawar et al., 2005, Vidal et al., 1998). In *E. coli* and *S. enterica*, curli and the extracellular polysaccharide cellulose are co-expressed, as CsgD also activates transcription of a diguanylate cyclase, *adrA*, which functions to initiate cellulose synthesis (Romling et al., 2000, Zogaj et al., 2001). Curli and cellulose can act synergistically, and some phenotypes such as resistance to desiccation and attachment to epithelial cells are dependent on the presence of both (Gualdi et al., 2008, Saldana et al., 2009, White et al., 2006). Other biofilm-related phenotypes are more dependent on one matrix component or the other. For example, cellulose confers greater resistance to bleach and curli are more important for adherence to spinach leaves (Macarasin et al., 2012, White et al., 2006).

TasA

The co-appearance of amyloids and polysaccharides in biofilm matrices is commonly observed. The Gram-positive soil organism, *Bacillus subtilis*, expresses a master biofilm regulator, SinR, that controls expression of matrix components (Kearns et al., 2005). SinR negatively regulates the *epsA-O* operon, whose gene products produce an extracellular polysaccharide (EPS) that is necessary for robust biofilm formation either on a solid surface or at the air/liquid interface of a broth culture (Kearns et al., 2005). Chu *et al.* showed that SinR also represses expression of a three-gene operon *tapA-sipW-tasA* (formerly *yqxM-sipW-tasA*) (Chu et al., 2006), and TasA was shown to be a major protein component of *B. subtilis* biofilms (Branda et al., 2006). Purified TasA readily forms amyloid fibers (Romero et al., 2010), revealing a system in which an amyloidogenic protein and an extracellular polysaccharide are members of the same regulon. EPS and TasA, like cellulose and qcurli in *E. coli* and *S. enterica*, act cooperatively to allow biofilm formation (Romero et al., 2010).

The *B. subtilis* biofilm system also provides an answer to the interesting question of how a cell can escape a self-produced amyloid cage. The first gene in the *tapA-sipW-tasA* operon, TapA, promotes TasA fiber assembly and anchors the amyloid to the cell surface (Branda et al., 2006, Romero et al., 2011). Deletion of *tapA* leads to secretion of smaller amounts of TasA that do not appear to be surface attached (Romero et al., 2011). Interestingly, D-amino acids, which have been described to signal biofilm disassembly (Kolodkin-Gal et al., 2010), act through TapA in order to disassociate *B. subtilis* cells from the amyloid fiber (Romero et

al., 2011). This mechanism circumvents the need to break down an amyloid fiber, a process that would likely be difficult due to the resistance of amyloid fibers to proteases.

Phenol Soluble Modulins

Recently amyloidogenic extracellular fibers composed of small peptides called phenol soluble modulins (PSMs) were identified as biofilm components in *Staphylococcus aureus* (Schwartz et al., 2012). *S. aureus* is a Gram-positive coccus that can colonize the body as a commensal organism in the nasal pharynx and can also cause a variety of illnesses, ranging from minor skin infections to bacteremia and sepsis, many of which involve formation of in-host biofilms (Otto, 2008). The ability of PSMs to form amyloid is particularly novel because soluble PSMs have a variety of reported functions. PSMs, either isolated from *S. aureus* or *S. epidermidis*, have been reported to recruit, activate, and lyse human neutrophils and to kill competing bacteria (Cogen et al., 2010, Cogen et al., 2010, Marchand et al., 2011, Wang et al., 2007). Soluble PSMs are also able to effectively act as a biofilm dissociation factor (Periasamy et al., 2012, Vuong et al., 2000), but upon amyloid fibrillization, PSMs lose that ability (Schwartz et al., 2012). Fibrous PSMs are, however, required for resistance of *S. aureus* biofilms to various dispersion agents such as Dispersin B, DNase I, Protease K, and to mechanical stress (Schwartz et al., 2012), demonstrating functional roles in both the monomeric and fibrous states (Fig. 2). How fiber formation contributes to the non-biofilm roles attributed to PSMs, as well as how each of the seven PSMs, whose genes are encoded on three separate operons throughout the *S. aureus* genome (Janzon et al., 1989, Mehlin et al., 1999, Wang et al., 2007), contributes to fiber formation is unclear. Intriguingly, the recently described *B. subtilis* amyloid TasA may also perform roles as a toxin and as a biofilm stability factor, as prior to its described amyloid properties, TasA was shown to display antimicrobial activity (Stover and Driks, 1999).

Amyloids in Environmental Biofilms

To determine if amyloids are a common component of naturally occurring biofilms, Daniel Otzen, Per H. Nielsen and colleagues have utilized the amyloid-specific dye, thioflavin-T, and conformational-specific antibodies to probe environmental biofilm samples. They found that among biofilms from seven different habitats, 5–40% of DAPI-positive bacteria were associated with amyloid adhesions. Among these bacteria were representatives from *Proteobacteria*, *Bacterioidetes*, *Chloroflexi*, and *Actinobacteria* (Larsen et al., 2007). In a follow up study, a *Pseudomonas fluorescens* strain was shown to produce an amyloid in the extracellular matrix of mixed biofilms (Dueholm et al., 2010). The genes necessary for formation of this amyloid were traced to the *fapA-F* operon, which is conserved in many *Pseudomonas* species, including the pathogenic *P. aeruginosa*. Expression of the *fapA-F* operon in *E. coli* resulted in flocculation and biofilm formation (Dueholm et al., 2010). Similar large-scale screening with conformational-specific antibodies demonstrated amyloid production in various members of the *Actinobacteria* and *Firmicutes* phyla (Jordal et al., 2009), further implying that amyloid formation is likely a widespread phenomenon in biofilms.

Amyloids as Shared Biofilm Resources

The abundance of amyloids in natural biofilms could potentially lead to interactions between different types of amyloidogenic proteins. Under curli-inducing conditions, large amounts of monomeric CsgA – the major component of curli fibers – are secreted into the extracellular milieu. Once outside the cell, CsgA encounters the outer-membrane anchored minor fiber component CsgB (Hammar et al., 1996, Loferer et al., 1997). CsgB is able to form amyloid seeds on the bacterial surface that nucleate CsgA fiber formation (Hammer et al., 2007, Hammer et al., 2012) (Fig. 3 A, B). If other bacteria use similar secretion/polymerization

amyloid assembly processes, seeds from one species could nucleate monomers from another species. Indeed, CsgA derived from various enteric bacteria, including *E. coli*, *S. typhimurium* LT2, and *Citrobacter koseri*, as well as from the distantly related *Proteobacteria Shewanella oneidensis*, can cross-seed *in vitro* (Zhou et al., 2012). Moreover, mixed biofilms of an *E. coli* *csgB* mutant (able to secrete soluble CsgA) and a *S. typhimurium* LT2 *csgA* mutant (able to assemble cell-associated CsgB nucleators) formed heterogeneous amyloid fibers (Fig. 3 C, D). Fiber formation between these two species increased surface attachment of the entire mixed-species community, demonstrating functional, inter-species amyloid cross-seeding (Zhou et al., 2012).

Amyloids with Surface Active Properties

Clearly, amyloids can serve as strong, relatively inert, scaffolding material in the extracellular matrix, but amyloids can also be dynamic participants in a variety of cellular processes.

Chaplin amyloids help *Streptomyces coelicolor* form aerial hyphae. *S. coelicolor* is a filamentous, soil-dwelling bacterium whose life cycle is similar to filamentous fungi. The vegetative form of *S. coelicolor*, the feeding submerged mycelium, secretes enzymes onto food substrates from which it derives nutrients. Once starvation signals are sensed, aerial hyphae emerge from the submerged mycelium and ascend towards the surface. Chains of spores develop in these hyphae, and once the soil surface has been breached, the spores are released to colonize elsewhere (Flardh and Buttner, 2009). Vegetative *S. coelicolor* cell surfaces are hydrophilic, so to break the soil/air interface, the cells must first develop a hydrophobic coat. To this end, *S. coelicolor* secretes monomeric chaplin proteins (encoded by *chpA-H*) (Claessen et al., 2003, Elliot et al., 2003). These hydrophobic proteins have been shown to form β -sheet rich amyloid fibers on contact either with air (Claessen et al., 2003) or with hydrophobic Teflon (de Jong et al., 2009). Hyphae protrusion from the soil is dependent on formation of a sheath layer of these hydrophobic chaplin amyloids along with a class of proteins called rodmins (Claessen et al., 2004). Although *S. coelicolor* encodes eight chaplin proteins, a strain producing only *chpC*, *chpE*, and *chpH* is able to produce aerial hyphae. In this minimal strain, ChpH is the major chaplin subunit (Capstick et al., 2011, Di Berardo et al., 2008). To investigate whether the amyloid fold of the chaplins was important in their ability to allow formation of aerial hyphae, Capstick *et. al.* identified and mutagenized two amyloid domains within the ChpH sequence. Mutagenesis of these domains, while not affecting protein stability or localization, prevented amyloidogenesis of ChpH and abolished aerial hyphae formation, indicating that amyloid formation is essential for proper chaplin function (Capstick et al., 2011). In addition to the layered chaplin coat, *S. coelicolor* can also produce chaplin fibers that appear as fimbriae-like appendages instead of the smooth coating associated with aerial hyphae. In this form, the chaplin fibers help attach to hydrophobic surfaces (de Jong et al., 2009), much like curli fibers of *E. coli* help the bacteria attach to polystyrene (Pawar et al., 2005). As with *E. coli* and *B. subtilis*, *S. coelicolor* amyloid fiber formation occurs concurrently with production of an extracellular polysaccharide, in this case cellulose. Interestingly, the cellulose fibers produced by *S. coelicolor* appear to help attach the chaplin fibers to the cell surface (de Jong et al., 2009).

Recent work has begun to reveal how the chaplins are anchored to the cell surface. The chaplins can be divided into long chaplins (*chpA-C*) and short chaplins (*chpD-H*), based on the protein product either being roughly 225 or 63 amino acids in length (Claessen et al., 2003, Elliot et al., 2003). The short chaplins are proposed to coat aerial hyphae and act as the amyloidogenic surfactants, but the role of the long chaplins is more ambiguous. Interestingly, the long chaplins all contain sortase signals, and indeed sortase activity is necessary for proper development of aerial hyphae and for cell wall anchoring of ChpC

(Claessen et al., 2003, Duong et al., 2012, Elliot et al., 2003). However, a mutant of all three long chaplin genes was less defective at short chaplin surface assembly than the sortase mutant, indicating an additional, undescribed role for sortases in development of aerial hyphae (Duong et al., 2012).

The *S. coelicolor* chaplins are similar to an extracellular fiber produced by filamentous fungi called class I hydrophobins (Wosten and de Vocht, 2000, Wosten and Willey, 2000). Indeed, hydrophobins were one of the first functional amyloids to be described (Butko et al., 2001, de Vocht et al., 2000, Mackay et al., 2001, Wosten and de Vocht, 2000). Like chaplins, hydrophobins are secreted as soluble, monomeric proteins that polymerize into a layer of amyloid fibers at hydrophobic/hydrophilic interfaces such as those found at the air/soil boundary (Morris et al., 2011, Wosten et al., 1993, Wosten et al., 1994). The growing hyphae can then emerge from the liquid environment into the hydrophilic face of the amyloid coat, facilitating extension into the air (Beever and Dempsey, 1978, Wosten et al., 1993, Wosten and de Vocht, 2000). Structural analysis of a soluble hydrophobin, EAS, reveals a model for how the amyloid fold can be effectively tailored to the formation of an amphipathic coat (Kwan et al., 2006). Six of the eight charged amino acids in the EAS sequence are localized to one face of a central, hydrophobic core, while the opposite surface is composed chiefly of hydrophobic amino acids. Kwan *et al.* proposed a model where the central β -barrel core of each monomer aligns to form a cross- β structure, and the fiber is then divided into a charged face and a hydrophobic face. Lateral alignments of multiple fibers would then produce a biphasic surface layer into which the aerial hyphae could protrude (Kwan et al., 2006). Further work with EAS has identified a particular stretch of residues that is important for amyloid formation. Mutation of residues within this stretch yielded a protein with an increased lag phase, and purified peptides derived from a wild-type version of the predicted amyloid domains readily formed fibers *in vitro* (Macindoe et al., 2012). Furthermore, transfer of this amyloid-forming domain into a class II hydrophobin, which normally will not form amyloid, conferred fiber formation and thioflavin T binding (Macindoe et al., 2012). These examples speak once again to the pliability of the amyloid fold, as a single polypeptide can be engineered to both form an amyloid fiber and to have distinct properties, such as amphipathicity, in the amyloid fold.

Interplay between Microbial Amyloids and the Host

Various interactions between microbial amyloids and host systems have been reported. Curli mediate binding to and internalization by host cells (Gophna et al., 2001, Johansson et al., 2001, Kikuchi et al., 2005), with these attributes being at least partly imbued by curli's fibronectin-binding capacity (Gophna et al., 2002, Olsen et al., 1989). Curli have also been shown to interact with major histocompatibility complex I (MHC-I), but whether or not the amyloid properties of curli fibers affect these interactions is unclear (Olsen et al., 1998).

In contrast, the amyloid properties of curli might impact human amyloidosis. Amyloidosis is a condition in which aberrantly folded amyloids deposit on tissues or organs, disrupting proper function and causing inflammation (Pepys, 2001). AA amyloidosis results from the abnormal deposition of a protein fragment from the typically soluble serum amyloid A (SAA) protein. These aggregates can be seeded by small SAA fragments, and indeed by other amyloid-like fibrils, thus exacerbating the symptoms of amyloidosis (Ganowiak et al., 1994, Kisilevsky et al., 1999, Johan et al., 1998, Niewold et al., 1987). Lundmark *et al.* showed that exogenously added amyloid-proteins including Sup35 from *Saccharomyces cerevisiae*, silk from *Bombyx mori*, and either purified curli or curliated *E. coli* increased the occurrence of SAA aggregates in mice (Lundmark et al., 2005). Further work exploring whether there are direct interactions between SAA and microbial amyloids will help to clarify the role of cross-seeding in amyloidosis.

There is strong evidence that the cross- β structure of curli fibers interferes with host blood clotting mechanisms (Gebblink et al., 2005). Fibrin, a major component of blood clots, displays a cross β -sheet structure and other amyloid-like characteristics (Kranenburg et al., 2002). Fibrin activates the mammalian tissue-type plasminogen activator (tPA) by promoting contact between tPA and its substrate, plasminogen. tPA can then cleave plasminogen into the active serine protease plasmin, which degrades fibrin and results in blood clot dissolution. (Nieuwenhuizen, 2001). Kranenburg *et al.* showed that the cross- β structure specifically allows for tPA activation (Kranenburg et al., 2002). Indeed, various other amyloid-like proteins, such as A β and IAPP (the amyloid associated with β -cell toxicity in type II diabetes) have been shown to effectively activate tPA and trigger the degradation either of themselves or of fibrin (Gebblink, 2011, Kranenburg et al., 2002). Curli, either purified or on the surface of *E. coli* or *S. enterica*, is both able to sequester tPA and plasminogen from plasma, and both of these proteins are functional when bound to curliated bacteria (Sjoberg et al., 1994).

Curliated *E. coli* and *Salmonella spp.* are also able to interfere with the host contact system, which is an enzymatic cascade triggered when blood contacts surface material (Gebblink et al., 2005). As part of the contact cascade, the activated form of plasma zymogen Factor XII (FXII) can cleave another plasma protein, high-molecular weight kininogen (HK). Cleavage of HK results in the release of the peptide hormone bradykinin (BK), which leads to an inflammatory response. Curliated *E. coli* or *S. typhimurium* can bind and sequester various contact phase proteins such as FXII, HK, and fibrinogen, resulting in release of the pro-inflammatory signals BK and fibrinopeptides (BenNasr et al., 1996, Herwald et al., 1998, Persson et al., 2000, Persson et al., 2003). Intriguingly, although the *in vivo* activators of FXII are not completely understood, Maas *et al.* demonstrated that various unfolded proteins, including pre-fibrillar A β , are able to induce FXII autoactivation (Maas et al., 2008), leading to the question of the folding state of CsgA upon binding to FXII. Concurrent with an increase in inflammation, curliated bacteria reduced clotting in isolated plasma as well as in an *in vivo* mouse model, likely due to curli-mediated sequestration of blood clotting elements such as fibrinogen (Herwald et al., 1998, Persson et al., 2000, Persson et al., 2003). Finally, Bian *et al.* demonstrated that serum from patients diagnosed with *E. coli* bacteremia contained anti-CsgA antibodies, while no such antibodies were present in sera from healthy patients (Bian et al., 2000). Collectively, these results allude to the interesting hypothesis that bacteria could use curli, and potentially other amyloids, as a mimetic of host amyloids in order to trigger specific pathways.

The importance of amyloid fibers in microbial pathogenesis is further cemented by reports of interactions between amyloid fibers and the immune system. Human macrophages were shown to produce increased levels of pro-inflammatory cytokines, including interleukin-8 (IL-8), when exposed to curliated *E. coli* compared to non-curliated *E. coli* (Bian et al., 2000). Likewise, mice injected with *E. coli* showed curli-dependent increases in expression of nitric oxide synthase, increases in nitric oxide levels, and decreased blood pressure (Bian et al., 2001). In studies with *S. typhimurium*, curli-mediated induction of nitric oxide and IL-8 in HEK293 cells was shown to be dependent on the host toll-like receptor 2 (TLR2) (Tukel et al., 2005, Tukel et al., 2009). Different bacterial structures are recognized by varying TLRs, either alone or in combination. For example, TLR2 can also bind lipoprotein, lipoteichoic acid, and peptidoglycan (Aliprantis et al., 1999, Schwandner et al., 1999). TLR4 recognizes LPS (Politorak et al., 1998), and TLR5 recognizes flagellin (Hayashi et al., 2001). Once bound, a signaling cascade is triggered that results in an immune response. TLR5 is specific for flagellin, and cannot be activated by fibrous flagella (Smith et al., 2003). In contrast, only CsgA curli polymers fully stimulate TLR2 induction of HEK293 cells, while monomeric CsgA has little activity (Tukel et al., 2009). Interestingly, another amyloid, A β fibers, is able to induce IL-8 production in human macrophage-like (THP-1) cells in a

TLR2-dependent-manner (Tukel et al., 2009). The same pattern was observed with Nos2 mRNA production in microglia cells (Tukel et al., 2009). TLR2 has independently been shown to recognize A β fibers as well as SAA, indicating that it may function partly as a general amyloid receptor (Chen et al., 2010, Cheng et al., 2008, He et al., 2009, Jana et al., 2008, Udan et al., 2008). However, curli fiber recognition additionally requires TLR1 signaling, while A β recognition also involves TLR4 and TLR6, indicating that the interactions between amyloids and the immune system are likely complex (Liu et al., 2012, Tukel et al., 2010, Udan et al., 2008).

Similarly, *S. aureus* PSMs are recognized by the neutrophil-expressed formyl peptide receptor 2 (FPR2) (Kretschmer et al., 2010, Rautenberg et al., 2011). FPRs trigger neutrophil chemotaxis in response to peptides of bacterial origin (Fu et al., 2006), both formylated and nonformylated (Kretschmer et al., 2012). As with TLR2, FPR2 has also been demonstrated to recognize A β and SAA (Liang et al., 2000, Tiffany et al., 2001), although whether amyloid formation of these agonists is necessary for stimulation is unknown.

Amyloids have also been reported to interact with host-derived antimicrobial peptides (AMPs). LL-37 is a human AMP important for resistance to bacterial infection of the urinary tract (Chromek et al., 2006), and curli was shown to be expressed on 59% of *E. coli* urinary tract infection (UTI) isolates (Kai-Larsen et al., 2010). Expression of curli increased the ability of an *E. coli* isolate to survive LL-37 exposure in broth culture. Additionally, LL-37 was shown to bind to curli fibers and monomers by surface plasmon resonance and was demonstrated to inhibit *in vitro* polymerization of CsgA (Kai-Larsen et al., 2010), implying a dynamic interplay between LL-37 and CsgA within the host. *Staphylococcus epidermidis* PSMs have also been demonstrated to directly interact with LL-37 (Cogen et al., 2010). Interestingly, LL-37 itself has been shown to form fibrous structures that induce CR birefringence when exposed to liposomes (Sood et al., 2008).

The Weaponization of the Amyloid Fold

A common characteristic of amyloids is passage through a toxic oligomer stage en route to mature fibrils. The structurally dynamic oligomeric species can disrupt lipid membranes (Demuro et al., 2005, Kaye et al., 2004, Kourie et al., 2002, Lashuel and Lansbury, 2006, Valincius et al., 2008). Microbes have managed to harness the inherent toxicity of amyloid oligomers to kill surrounding cells. The small hydrophobic microcin E492 (MccE492) protein produced by *Klebsiella pneumoniae* exemplifies this idea. Like many bacteriocins, MccE492 exerts toxicity by forming pores in lipid membranes (de Lorenzo and Pugsley, 1985, Destoumieux-Garzon et al., 2003, Lagos et al., 1993). This mode of action would seem to confer toxicity to a broad range of target microbes, however MccE492 only has demonstrable antimicrobial activity against a small range of enteric bacteria (de Lorenzo et al., 1984, Destoumieux-Garzon et al., 2003). The explanation for this phenomenon lies in the fact that MccE492 is post-translationally modified with a salmochelin-like molecule (Nolan et al., 2007, Thomas et al., 2004). Salmochelin is a siderophore produced by many enteric bacteria that is recognized and imported by the FepA/Fiu/Cir outer membrane complex (Hantke et al., 2003, Mercado et al., 2008). The toxicity of MccE492 relies on the target bacteria expressing a functional copy of this receptor complex, in order for MccE492 to be transported across the outer membrane and have access to the susceptible inner membrane, (Patzner et al., 2003, Pugsley et al., 1986, Strahsburger et al., 2005) where it can disrupt membrane stability (Destoumieux-Garzon et al., 2006, Lagos et al., 2009). This targeted toxicity is made all the more interesting by the fact that MccE492 readily forms amyloid fibers *in vitro* and *in vivo* (Arranz et al., 2012, Bieler et al., 2005). Fiber formation by MccE492 completely ablates toxicity to susceptible target cells, likely due to the concurrent loss of the toxic oligomer species that would permeate membranes (Bieler et al.,

2005). Further studies with MccE492 revealed that the amyloid form could be destabilized under particular conditions, including high pH or low ionic strength, and destabilization correlated both with release of oligomeric species and with an increase in toxicity. Moreover, changing conditions back to those that favor fiber formation could reverse the presence of oligomers and once again increase the number of fibers (Shahnawaz and Soto, 2012). These results indicate that amyloid formation by MccE492 is a dynamic process and imply the potential for environmental triggering of toxic oligomer release from amyloid reservoirs (Shahnawaz and Soto, 2012).

Changes in environmental pH also affect the aggregation of the listeriolysin O (LLO) protein of *Listeria monocytogenes*. Among other functions, LLO forms pores in the phagolysosome, allowing *L. monocytogenes* to escape into the cytoplasm during infection (Hamon et al., 2012). Bavdek *et al.* showed that under alkaline pH and high temperatures, LLO readily aggregated into fibrous structures that bind to the amyloid dyes thioflavin T and Congo red. As with MccE492, LLO did not demonstrate pore-forming capabilities when present in the fibrous form (Bavdek et al., 2012).

Plant pathogens have also been reported to employ the pore-forming capabilities of amyloidogenic proteins (Oh et al., 2007). Harpins are heat-stable, type III-secreted proteins that plant pathogens inject into host cells and that induce a hypersensitive response (Kim et al., 2003, Wei et al., 1992). Although the mode of harpin toxicity is not completely understood, various harpins have been shown to interact with synthetic lipid membranes (Lee et al., 2001) and to depolarize plant cell membranes (Ahmad et al., 2001, Pike et al., 1998). Oh *et al.* demonstrated that the harpin HpaG produced by *Xanthomonas axonopodis* pv. *Glycines 8ra* readily converts from a soluble α -helix monomer to β -sheet rich amyloid fibers (Oh et al., 2007). Mutants unable to convert to the amyloid fiber were unable to elicit a hypersensitive response in host cells, indicating that the ability to form amyloids is likely necessary for HpaG function. The authors went on to investigate the amyloidogenicity of other harpins, XopA from *Xanthomonas campestris* pv. *Vesicatoria*, HrpN from *E. amylovora*, and HrpZ from *P. syringae* pv. *Syringae*. Unmodified HrpN and HrpZ readily formed amyloids, while XopA did not. XopA may be the most informative of these, as a WT version of this protein is unable to cause a hypersensitive response. A mutant version of XopA that does elicit such a response, however, was able to form amyloid fibers (Oh et al., 2007).

The utilization of amyloids is not limited to cellular life, as influenza A virus PB1-F2 protein is one of three reported viral amyloids (Chevalier et al., 2010, Freire et al., 2008, Wetzler et al., 2007). PB1-F2 is necessary for WT virulence in a mouse model, induces apoptosis in macrophages and monocytes, and has been shown to form pores in planar lipid membranes (Krumbholz et al., 2011). When exposed to non-polar solvents or membrane-mimicking agents, PB1-F2 was further demonstrated to form amyloids (Chevalier et al., 2010). Again, the ability of PB1-F2 to puncture membranes was lost upon fiber formation, indicating that the toxic activity is limited to pre-fibrillar aggregates (Chevalier et al., 2010).

Recent work with antimicrobial peptides (AMPs) derived from higher organisms further implicates membrane disruption as a common characteristic of amyloidogenesis (Kagan et al., 2012). In addition to the potential amyloidogenesis of LL-37, human antimicrobial peptide Protegrin-1 forms thioflavin T-binding fibers with similar morphology to A β after contact with a hydrophilic surface (Jang et al., 2011). Interestingly, atomic force microscopy revealed that Protegrin-1 formed small oligomeric species instead of full-length fibers upon contact with lipid bilayers (Jang et al., 2011). Various AMPs isolated from frog skin have also shown the ability to form amyloid fibers (Auvynet et al., 2008, Gossler-Schofberger et al., 2009, Gossler-Schofberger et al., 2012). Moreover, recent reports have demonstrated

that the amyloid component of Alzheimer's Disease plaques, A β , displays antimicrobial activity (Harris et al., 2012, Papareddy et al., 2012, Soscia et al., 2010), calling into question the native function of A β and potentially blurring the line between what is seen as a functional vs. a disease-associated amyloid. Altogether these results support the hypothesis that during polymerization, an amyloidogenic protein passes through a toxic oligomeric stage, at which time it is able to disrupt lipid membranes (Kagan et al., 2012). Evidence is mounting that biological systems utilize the generic pore-forming capabilities of the amyloid fold to generate toxins.

How Microbes Avoid Self-induced Amyloid Toxicity

Because of the common features that the amyloid fold confers on divergent proteins, the usefulness of microbial amyloids as a model for amyloidogenesis during human disease is apparent. As the ultimate goal from this model would be development of therapeutics to negate the harmful effects of amyloid-associated diseases, the most pertinent research question becomes "How do microbes prevent amyloid-associated toxicity?" It turns out there are a variety of answers to this question as microbes utilize various chaperones, nucleators, trafficking systems, and the protein sequence of the amyloid itself to promote innocuous amyloid formation.

Since small oligomers are widely considered to be the toxic species in amyloid formation, one mechanism for avoiding toxicity has been hypothesized to be rapid passage through the oligomeric stage. In agreement with this hypothesis, *E. coli* uses a nucleator protein, CsgB, to seed rapid polymerization of the major curli component, CsgA, on the cell surface. CsgB itself can form amyloid fibers *in vitro* and the addition of CsgB seeds allows CsgA to bypass the characteristic lag phase associated with amyloid fiber formation (Hammer et al., 2007, Hammer et al., 2012). Specific amino acids in CsgA mediate interaction between these two fiber subunits, and mutation of these residues results in a CsgA protein that is secreted from the cell in a soluble state (Wang and Chapman, 2008, Wang et al., 2008). The main functions of CsgB *in vivo* then seem to be templating extracellular CsgA amyloid formation and, along with the surface-exposed CsgF protein, anchoring CsgA fibers to the cell (Hammer et al., 2007, Nenninger et al., 2009), outlining a model where CsgA monomers are kept unfolded and soluble until they are transported across the outer-membrane where they encounter CsgB seeds. CsgA then rapidly folds into an amyloid fiber, potentially bypassing the toxic oligomeric stage.

Perhaps the most direct mechanism for avoiding amyloid toxicity is through manipulation of the amyloid protein's primary amino acid sequence. The chief component of curli fibers, CsgA, is able to polymerize into amyloid fibers in a wide variety of environmental conditions (Dueholm et al., 2011). Similarly, the *S. coelicolor* chaplins are able to polymerize in a wide variety of pHs (Sawyer et al., 2011). These results contrast with non-native amyloids that need to be exposed to specific, destabilizing conditions in order to aggregate (Chiti et al., 1999, Chiti et al., 2000, Marcon et al., 2005). Investigations into the primary sequence of CsgA have revealed that particular residues play crucial roles in the polymerization of this amyloid-by-design. The amyloidogenic domain of CsgA is composed of 5 imperfect repeats (R1-R5) that share 30% amino acid identity to each other (Collinson et al., 1999, Wang and Chapman, 2008). The two terminal repeating subunits, R1 and R5, readily form amyloid fibers *in vitro*, while R2 and R4 do not form fibers and R3 forms short, fibrous aggregates (Wang et al., 2007). Wang *et. al.* demonstrated that R2, R3, and R4 contain 'gatekeeper' residues that inhibit the aggregative propensity of these peptides and therefore the entire CsgA protein (Wang et al., 2010). A CsgA mutant in which the gatekeeper residues were substituted with amino acids that bolster amyloid formation, CsgA*, polymerized more rapidly than WT CsgA *in vitro* and formed mislocalized fibers

without the need for CsgB *in vivo*. Overexpression of CsgA* was significantly more toxic to the cell than overexpression of WT CsgA (Wang et al., 2010). These data indicate that the CsgA protein sequence has evolved specific traits to allow for manageable amyloid formation by the cell. Additionally, both curli fiber components, CsgA and CsgB, indeed the majority of secreted proteins, are composed of a disproportionate amount of inexpensive amino acids (Smith and Chapman, 2010), indicating that the CsgA protein is designed for both safe amyloid formation and for cost-effectiveness.

A third mechanism to control amyloid formation is the use of cellular chaperones. In curli biogenesis, the role of CsgB as an outer-membrane nucleator implies that CsgA must be maintained in a soluble, unfolded state as it is trafficked through the cytoplasm and periplasm. Consistent with this hypothesis, various *E. coli* chaperones, including cytoplasmic DnaK and Hsp33 and periplasmic Spy, were demonstrated to inhibit CsgA aggregation (Evans et al., 2011). Once in the periplasm, CsgA encounters a specificity factor for curli secretion, CsgE. In addition to trafficking unfolded CsgA to the outer-membrane pore CsgG during secretion, CsgE has been demonstrated to inhibit CsgA polymerization *in vitro* (Nenninger et al., 2011). These data are consistent with a model of CsgA maintaining an unfolded, unstructured state on its voyage through the cytoplasm and periplasm, and it appears that various cellular chaperones are able to inhibit inappropriate CsgA aggregation before secretion.

Chaperones also play a major role in amyloid formation of the fungal prions, a fascinating family of intracellular amyloids that have diverse roles in gene regulation and heterokaryon formation. The central principle of fungal prions is that a soluble cytoplasmic protein has a particular function that is altered when the protein takes on an amyloid fold. Once in the amyloid fold, the fiber serves as a sink for soluble monomers of the same protein, effectively diminishing the intracellular concentration, and therefore functional capacity, of the soluble protein. Moreover, the amyloidogenic form of a prion protein acts as an epigenetic element, i.e. it can be passed on to progeny cells or introduced as a functional unit into a prion-negative cell. The prion field has been extensively described in recent reviews (Tuite et al., 2011, Wickner et al., 2007), and will only be briefly mentioned here in regards to amyloid-chaperone interactions. As intracellular amyloids, yeast prions serve as fantastic examples of how amyloid formation can occur safely in the crowded cytoplasmic environment. A complex of chaperones, including those from the Hsp104, Hsp70, and Hsp40 families are involved in ameliorating amyloid related toxicities in human cells as well as yeast (Douglas et al., 2009). The yeast prion [RNQ+] has no known function except to influence the conformation state of other prion proteins such as Sup35 (Stein and True, 2011). However, overexpression of Rnq1 in a [RNQ+] strain is toxic to the cell. This toxicity can be tempered by overexpression of the Hsp40 chaperone Sis1 (Douglas et al., 2008). Sis1, along with Hsp104, is necessary for the propagation of the [RNQ+] state (Sondheimer and Lindquist, 2000, Sondheimer et al., 2001). Douglas *et al.* demonstrated that [RNQ+] toxicity was not due to sequestration of Sis1, but was instead caused by accumulation of SDS-soluble Rnq1 aggregates. Overexpression of Sis1 leads to a depletion of these aggregates and an increase in SDS-insoluble [RNQ+] amyloids, indicating that an Rnq1 toxic oligomer was likely responsible for the cell death and that Sis1 promotes formation of innocuous [RNQ+] amyloids (Douglas et al., 2008). Altogether these results indicate that microbes have evolved intricate systems in order to avoid amyloid-derived toxicity.

Concluding Remarks

The diversity and ubiquitous nature of the amyloid fold is now easily appreciated. Amyloids can be utilized as structural material, toxins, surface-active fibers, genetic material, adhesins, host mimetics, and likely much more. While amyloid represents a conserved, versatile, and

important protein fold, the use of amyloids requires dedicated systems in order to limit toxic amyloid formation in the wrong place and/or at the wrong time. A detailed understanding of these systems will no doubt yield insights into both protein misfolding diseases and microbial physiology.

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Figure 1.

General schematic for how amyloids form. Unfolded monomers form intermediate oligomers, some of which are toxic, during the lag phase of amyloid assembly. Once the on-pathway oligomers, or seeds, have formed rapid fiber assembly ensues. The lag phase can be bypassed by addition of pre-formed seeds to monomers.

Figure 2.

Structure-function dynamics of *Staphylococcal* phenol-soluble modulins (PSMs). Soluble PSMs are able to mediate *S. aureus* biofilm disassembly, to recruit and activate neutrophils, and to lyse neutrophils and niche bacteria such as group A *Streptococcus*. Once polymerized, PSMs lose the ability to disrupt *S. aureus* biofilms. Fibrous PSMs stabilize biofilms and confer resistance to proteinase K, dispersin B, and DNase I.

Figure 3.

Secretion/polymerization curli fiber assembly. (A) WT *E. coli* secretes soluble CsgA proteins into the extracellular milieu. Once outside the cell, CsgA encounters surface-attached CsgB seeds that nucleate CsgA amyloid formation. (B) Transmission electron microscopy (TEM) image of curliated *E. coli*. (C) Inter-bacterial complementation between a CsgA expressing *E. coli csgB* mutant and a CsgB expressing *S. typhimurium csgA* mutant. *E. coli* secretes monomeric CsgA that can interact with CsgB nucleators on the surface of *S. typhimurium*. (D) TEM images of an *E. coli csgB* mutant, a *S. typhimurium csgA* mutant (producing flagella), and a mixed culture biofilm where curli fibers can be seen.