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Review

We find them here, we find them there: Functional bacterial amyloid

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We seek them here, we seek them there,
We scientists seek them everywhere:
Are they our heaven, are they our hell?
Those damned elusive strands in that fibril!

Adapted from *The Scarlet Pimpernel* by Baroness d'Orczy

Abstract. Protein amyloid is often deposited in connection with neurodegenerative diseases. Such deposits generally possess three principal drawbacks: cytotoxicity, lack of spatial control in their deposition and structural polymorphism. These are typical features of biologically non-optimized systems which have not been exposed to evolutionary pressure. Nevertheless, Nature uses the cross-β self-organizing principle in many structural contexts where a strong but pliable material is needed. Functional amyloid is found in humans, invertebrates, fungi and, not least, bacteria, in which amyloid may be the rule rather than the exception. Detailed case studies reveal how directed nucleation can use tailor-made proteins optimized to assume a specific amyloid conformation, leading to remarkably robust assemblies. This makes it highly challenging to purify and analyze the products formed *in vivo*. We contrast pathogenic and *in-vitro*-formed amyloid with functional amyloid, paying particular reference to bacterial amyloid, and discuss challenges and perspectives in identifying and characterizing this class of protein.

Keywords. Protein, bacteria, aggregate, CsgA, functional amyloid, cytotoxicity, polymorphism.

Introduction: a historical preamble

It is an abiding passion of human beings to attempt to read and re-view history as an orderly progression of events running according to a well-coordinated and rational script. Such views generally do not hold up to closer inspection. Nevertheless, in the experimental sciences, there is a steady development in technical abilities which engage in intense dialogue with scientific theories and views to generate some broad fronts along which many of the most exciting events take place. Viewed retrospectively, a perception of these fronts may make it easier to appreciate the current

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research climate and perhaps even gain an inkling of the scientific activities just over the horizon.

Functional amyloid: from folding to misfolding and back

The case of functional bacterial amyloid lends itself well to this historical perspective. Functional amyloid (FA), as we will detail below, is in essence a biologically useful example of what was originally thought to be a regrettable lapse in Mother Nature's painstaking attempts to fold proteins properly. As such, the intellectual origins of FA are intimately tied to the understanding of a protein as an autonomously folding biological entity, that is, a molecule whose amino acid sequence intrinsically encodes a single biologically active structure that is also the thermodynamically most stable state. This stunning concept was introduced by Anfinsen [1] and colleagues in the late 1950s, sparking off a flurry of activities over the next two to three decades that led to the presentation of the Levinthal paradox [2] and the search for protein intermediates that could explain how proteins could reach the folded state on a biologically meaningful time scale [3]. Thanks to the advent of protein engineering in the early 1980s, it then became possible to map out protein-folding pathways, including the structures of both intermediates and more elusive transition states, at residue- and even atomic-scale resolution using Fersht's ϕ-value approach [4, 5]. This made the decade from the late 1980s to the late 1990s a hotbed for the study of 'proper' *in vitro* protein folding, focusing on the process leading from the denatured state to the native state, generating sophisticated models for protein folding [6] which could be backed up by *in silico* simulations [7]. Most of these studies used simple single-domain model proteins that folded rapidly and reversibly but were not representative of the large and lumbering multi-domain proteins making up most of the cell. Such proteins were often found to be difficult to persuade to jump blithely from a chemically denatured state to the native state, but tended to collapse in an undignified heap as misfolded aggregates, although the nature of these aggregates was generally subjected to closer scrutiny (Fig. 1). In parallel, it became apparent that living organisms had developed a whole arsenal of proteins to ensure that folding of such proteins *in vivo* happened in an orderly and controlled fashion in the cell, namely the molecular chaperones [8]. Concomitantly, proteins were identified which formed cellular aggregates in neurodegenerative diseases such as Parkinson's disease [9]. In the late 1990s, attention thus shifted from folding to misfolding, the latter sparkling with the glitter of medical relevance. A bold generalization by C. M. Dobson, F. Chiti and their colleagues on the generic ability of proteins to fibrillate to amyloid has helped to make the study of protein misfolding as amyloid a very prominent topic in protein science in the last decade and has established interesting predictive models of aggregation. While much remains to be discovered within dysfunctional amyloid formation, it is also becoming increasingly clear that such well-ordered aggregates serve a useful purpose in Nature in an immense number of circumstances, making FA not a curiosity but almost a bedrock of biological structures. This will undoubtedly make studies of FA formation a very active front in the coming decade. In a sense, therefore, we have returned to the 'proper' folding studies of the 1980s and 1990s with the twist that these studies involve intermolecular rather than just intramolecular associations.

In this review, we will begin by reviewing some basic tenets of amyloid structure, formation and pathogenicity, as well as some attempts by researchers to harness these properties, before we lift the curtain on how Nature itself has exploited amyloid for its own ends and avoided the pitfalls associated with the uncontrolled formation of this state. We will also highlight aspects of the purification and identification of these components which, by their very nature, are difficult to mobilize into an aqueous phase. We also refer the reader to a number of excellent more general or more specialized reviews of pathological and FA which have recently appeared [10–17].

We will not discuss the rich and complex field of prion proteins, which deserves a review in its own right. Prion proteins are defined as being able to exist in two different stable monomeric states, one of which is able to actively convert the other to its own form. If the complex is stable, it can form the basis for a growing aggregate. This conformational imprinting is analogous to that of amyloid, except that conventional amyloid usually relies on a oligomeric structure to convert and incorporate monomers; furthermore, the oligomeric structure does not usually accumulate to the same extent as the infectious prion state. With the possible exception of the CPEB protein from *Aplysia,* there is no evidence for a functional use of the prion aggregate, although the loss-of-function associated with aggregation can serve purposes summarized in Table 1.

General amyloid features

Amyloid structure

Amyloid can be defined as orderly repeats of protein molecules arranged as a fiber of indefinite length in a cross-β structure, in which the β strands are perpen-

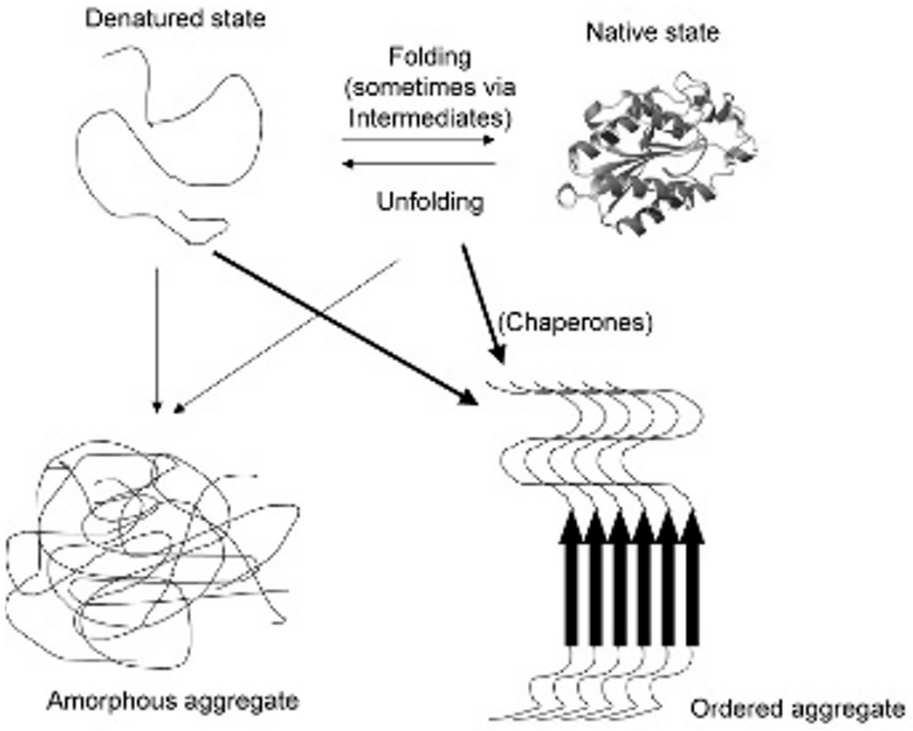

Figure 1. Different pathways in the folding of a protein molecule. In addition to folding to the native state (*in casu* the *F. solani* pisi cutinase, PDB code 1CEX), the protein can aggregate to a state devoid of persistent structure (amorphous aggregate) or form well-ordered but unwanted assemblies rich in β-sheet structure. Chaperones can guide the protein to form such β-sheet structures fulfilling certain biological functions, as described in this review.

dicular to the fiber axis. Amyloid was first described as insoluble deposits in 1854 by Virchow, who considered it amylose- or cellulose-like due to its ability to stain with iodine [18]. Friedrich and Kekulé [19] identified protein components 5 years later, but the name has stuck. Although 'amyloid' is somewhat misleading, the name is a salutary reminder of some structural properties it shares with cellulose and amylose, namely a repetitive sequence of units which form an organized structure with regular binding pockets for various dyes. Thus, amyloid in human tissue is traditionally visualized by pathologists using the sulfonated azo dye Congo Red, which lends a distinct apple-green birefringence to amyloid deposits under cross-polarized filters. Similarly, the fluorescent dye Thioflavin T (ThT) undergoes a spectral shift upon binding. Despite their availability and ease of handling, none of these dyes has absolute specificity for amyloid [20] and they are best used against isolated proteins; *in vivo* it is more appropriate to use conformationally specific monoclonal antibodies [21, 22]. These antibodies, originally developed against Aβ fibrils, bind to all hitherto tested amyloid fibrils, and illustrate that all fibrillated proteins share an underlying structural motif [23].

Indeed, the ability to make a large group of completely unrelated proteins fibrillate to the same overall end structure has led to the suggestion that essentially all proteins can form such structures under appropriate conditions [24]. This is confirmed by atomic-level structural details about amyloid which are emerging at a breathtaking pace. Conventional amyloid is non-crystalline and insoluble, and therefore does not lend itself to X-ray crystallography or solution-state nuclear magnetic resonance (NMR). However, solid-state NMR is currently making very impressive advances and has already been used to reveal structures of both peptides [25–27] and, in less detail, full-length proteins [28]. In parallel, the recent availability of highly focused synchrotron X-ray beamlines (Fig. 2) has made it possible to elucidate the structure of microcrystals of a large number of different fibrillating peptides [29, 30]. Although all the peptides in this highly impressive work are organized as standard β sheets with β strands perpendicular to the fibril axis, the individual β strands contact each other in different ways for different peptides. This has led to a unified structural scheme with eight different types of interface or zipper to which all amyloid structures, including the functional ones, are likely to adhere [29, 30].

The current challenge is to link such atomic-level details with the overall fibrillar architecture. Here the emerging method of choice is synchrotron-based small-angle X-ray scattering, a solution technique which makes it possible to monitor the overall structure of different aggregative states which decay and accumulate during the fibrillation process [31]. The technique was put to impressive use in the case of insulin, allowing the authors to identify the coarse

Table 1. Overview of functional amyloid.

Organism	Protein	Purpose	Ancillary components	References
Eukaryotic amyloid				
Humans	Mα (component of Pmel17)	by fibrillating, Mα forms a scaffold for binding and cross-linking to the thioflavin T-like substance melanin, thus protecting against DNA-damaging UV light	furin (cleavage and subsequent liberation of Mα from the cell membrane)	65–67
Silk moth and fish	chorion proteins with conserved central amyloid-forming domain	forms a protective coating for the oocyte and embryo	unknown; difficult to identify individual proteins	137–139
Spiders	spidroins	spider silk for webs; stored up to 50 % as liquid crystals in specific glands; assembled by passage through spinning duct and extraction of water and salt	spinning duct organ	140
Aspergillus and other mycelial fungi	hydrophobins	form an amphipathic amyloid membrane at the air-water surface to reduce surface tension and allow hyphal growth out of water; provide spores and appressoria with a hydrophobic coat for host attachment at the cuticle surface as well as evasion of host macrophages	unknown	10, 89
Prion proteins[1]				
Mammals	ECTO-NOX	involved in both [NAD(P)H] oxidation and protein disulfide-thiol interchange; oscillates between these two states in a synchronized (entrained) fashion, via structural changes (α helix to β sheet and vice versa); ECTO-NOX can, like the other prions, fibrillate and bind copper ions; mice lacking the PrP protein have problems with their circadian rhythm	probably spontaneous	141
Aplysia (sea slug)	CPEB (cytoplasmic polyadenylation element-binding protein)	homologous to yeast prions and behaves like prions when expressed in yeast; the prion form may function as a kind of memory storage, leading to long-term synaptic changes	probably spontaneous	142
Saccharomyces cerevisiae (baker's yeast)	Ure2p	the native state is involved in nitrogen catabolism; aggregation of the prion state leads to upregulation of the Dal5 ureidosuccinate and allantoate transporter	probably spontaneous	143
S. cerevisiae	Sup35	Sup35 is a termination factor in the native state; aggregation of Sup35 occurs spontaneously in 10^{-7} to 10^{-5} of all yeast cells, leading to stop codon read-through and (random) emergence of new properties	spontaneous aggregation	144
Podospora anserina (filamentous fungus)	HET-s	the native state serves to form and maintain barriers between heterokaryons of different prion strains (HET-S versus HET-s)	probably spontaneous	145
Bacterial amyloid				
Escherichia coli and other *Enterobacteriaceae*	CsgA	CsgA monomers form curli amyloid fibrils; these are involved in attachment to surfaces, biofilm formation and invasion into host cells	CsgB (nucleator), CsgG (membrane pore for export of CsgA and CsgB)	68, 69, 79
Streptomycetes coelicolor and other streptomycetes	ChpA–H	chaplins modulate the surface tension of water and the surface of streptomycetes, allowing production of aerial hyphae; they are organized in rodlets at the surface of aerial hyphae and spores	self-assemble to amyloid fibrils; ChpA–C are probably anchored to the cell wall; unknown nucleator; rodlins help form rodlets	73

Table 1 *(Continued)*

Organism	Protein	Purpose	Ancillary components	References
Mycobacterium tuberculosis	MTP	pili have adhesive properties and are antigens for infection and recognized by IgG from patients		74
Xanthomonas spp. and other plant pathogens	HpaG	cytotoxin; harpin causes a hypersensitive response in plant cells, leading to cell death	forms amyloid fibrils under plant apoplast-like conditions	76
Klebsiella pneumoniae	Microcin E492	cytotoxin; microcin kills *Enterobacteriaceae* bacteria by forming pores in the cytoplasmic membrane		103

[1] Note that in the case of prion proteins, the functionality appears mainly to be derived from loss-of-function associated with aggregation.

structure not only of the starting monomer and end-point fibrils, but also of the otherwise elusive oligomeric state [32]. Let us therefore now briefly turn to the actual mechanism of fibrillation in which this important state also features.

The kinetics of amyloid formation

Most proteins only fibrillate if they are exposed to conditions such as extremes of pH, elevated temperature or intermediate concentrations of denaturants [33]. This typically induces partially structured states which remain flexible enough to form contacts with similar protein molecules but not so unstructured as to make ordered contacts unfeasible. Proteins which fibrillate under physiological conditions typically do so because they have either suffered a destabilizing mutation [34] and/or have been proteolytically cleaved [35]. Both of these events can make them more disposed to form such a flexible state. A typical time profile for fibrillation (Fig. 3), monitored by the change in ThT fluorescence which reports on the accumulation of fibrillar structures, will contain a lag phase of minutes to days (characterized by the lag time t_{lag}), in which there is no formation of amyloid. The lag phase is followed by the elongation phase, in which aggregation rapidly commences, and within a short time (relative to t_{lag}) most of the monomer population is incorporated into fibrils, precluding further growth and leading to a plateau in ThT fluorescence. The lag phase is conventionally ascribed to the build-up of a fibril nucleus [36] although alternative models are also available [37]. The nucleus is typically thermodynamically unstable, because it requires many interactions to be formed cooperatively between a number of different protein molecules (anywhere between 2 and 40). This leads to an apparent activation energy for the fibrillation reaction. However, once formed, it can act as a template for the subsequent deposition of additional protein molecules to form the mature fibril. There is still no consensus about how the nuclei are elongated to form fibrils and how multi-stranded fibrils form. Usually it is supposed that monomers

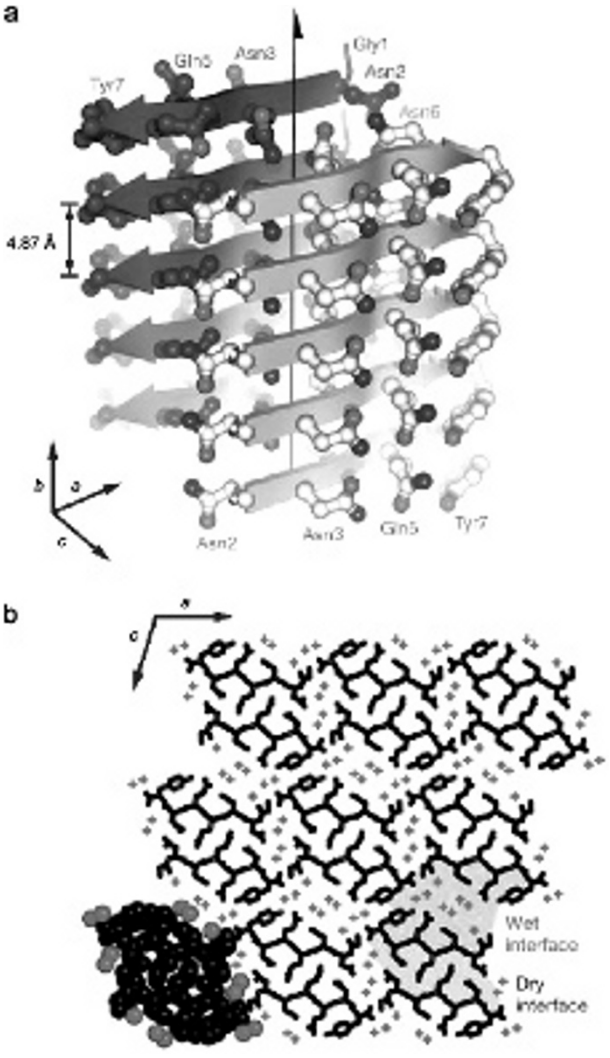

Figure 2. Atomic structure of the GNNQQNY peptide which fibrillates below 1 mM but forms diffracting microcrystals at 10–100 mM. (*a*) The figure emphasizes the regular hydrogen bonding between individual β-strands which are in perfect register and perpendicular to the long axis. (*b*) The crystal is stabilized by close side chain contacts between each sheet. Reproduced with permission from Nelson et al. [29].

simply add on to the growing ends of the fibrils [38], as can be seen directly by seeding fibrils with monomers [39]. However, there is also evidence that oligomers can join up directly [32, 40] to form mature fibrils. Alternative models of lateral association of fibrils have also been proposed [41].

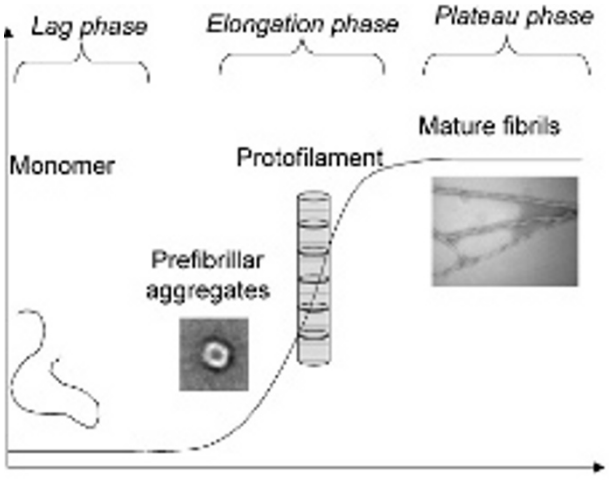

Figure 3. Stages in protein fibrillation *in vitro*. The monomer assembles into a prefibrillar aggregate such as an oligomer (in this case α-synuclein visualized by electron microscopy [104]) which are then assumed to be elongated to protofilaments that mature to thicker fibrils consisting of interwoven bundles of protofilaments (in this case fibrils of glucagon visualized by electron microscopy [C. B. Andersen, G. Christiansen and D. E. Otzen, unpublished results].

Multiple prefibrillar stages and fibrillar polymorphism

In practice, a protein will often aggregate in several stages, and in this process, oligomers of various sizes can accumulate. This is not least due to the fact that these intermolecular interactions are – at least in the pathological state – not evolutionarily optimized, and therefore a number of different states with similar but not identical structure and stability will coexist in a dynamic equilibrium, some of them closer to the fibrillar state than others. However, it is mechanistically simplest to lump them all together into a 'prefibrillar aggregate' class with an overall activation energy. In some cases, these states have very distinctive kinetics and structural properties, and thus it is possible to separate individual steps such as initial formation of amorphous aggregates, followed by rearrangements to crystalline or other ordered states and then finally the fibrillar state [42, 43].

The fact that most amyloid (apart from the functional kind) is not biologically optimized means that we cannot expect that each aggregate is necessarily a well-defined species which is well separated from other aggregates in terms of structure and stability. This goes for both prefibrillar aggregates and mature fibres. We call this phenomenon 'fibrillar polymorphism.' For example, the peptide hormone glucagon can form different fibrils with remarkably different structural and thermodynamic properties depending on solute composition, temperature and concentration [44–46], and fibrils formed by the Aβ peptide involved in Alzheimer's disease vary significantly depending on whether they are shaken or not during the fibrillation process [47]. Thus, in the absence of biological optimization, it is left to the fibrillating protein itself to undergo an *in situ* evolutionary process in which the most stable aggregate under the prevailing conditions is selected [48].

Predicting amyloid formation

Significant efforts have been made to model the kinetics of aggregation computationally based on simple physico-chemical properties such as secondary-structure propensities, hydrophobicity, charge and patterns of amphiphilicity [49, 50]. Current models [49, 51, 52] are able to predict with reasonable success not only the effect of various mutations on the *change* in the growth rate of fibrils but also the absolute rates of elongation. Like most pioneering efforts, these models are not without limitations; they presuppose that aggregation occurs from the denatured state rather than partially structured states with various idiosyncratic features, they cannot take into account the pervasive but elusive phenomenon of fibrillar polymorphism and they do not predict the lag time of fibrillation. This is not surprising, since lag times are very sensitive to a simple experimental variable such as agitation which is very difficult to model quantitatively or vary in a simple and systematic fashion. However, it is somewhat ironic that the lag phase is predictively the most elusive parameter, since it is also biologically the most important.

Why is amyloid toxic?

Accumulation of mature fibrils can be detrimental if it occurs on a large scale in the systemic amyloidoses where kilograms of protein can accumulate and ultimately lead to organ failure and death by simple physical blockage [53]. However, biologically, the fibrillated state is relatively inert and does not appear to play a major role in neurodegenerative diseases; instead it is the prefibrillar aggregates which have been shown to be cytotoxic [54–56]. The reasons for this are hotly contested and an intense focus of current investigation but are likely to be related one way or the other to the accumulation of 'unwanted protein surface,' which would otherwise be sequestered in the core of the correctly folded protein. Much evidence

has been marshalled to support the hypothesis that the prefibrillar aggregates interact with cell membranes, leading to e.g. pore formation and uncontrolled ion efflux [57–59] or generally increased membrane permeabilization [60]. Such pore formation has even been suggested to have a physiological role in assisting Ca^{2+} homeostatis in bone tissue [61]. It has also been suggested that oligomers could react with metabolites and proteins in unpredictable ways, leading to undesirable compounds [55].

Amyloids in Nature

Repetitive elements in Nature

Nature provides us with many examples of how to exploit repetitive protein elements in structural contexts. These include collagen, fibrin, keratin and myosin, to name but a few. As if to highlight our own present inadequacy in the molecular-assembly business, each case provides an example of how important it is to be able to control proper alignment of these units. Thus, for actin, monomer- and fiber-binding proteins regulate the monomer pool, orchestrate the formation of fibers, organize fibers into arrays and depolymerize fibers for monomer recycling [62]. The three polypeptide chains forming the collagen helix are aligned through helical extensions which are cleaved off before assembly [63]. Tropoelastin (the soluble precursor of mature elastin) assembles on a preformed template of fibrillin-rich microfibrils [64] and is subsequently cross-linked enzymatically.

Using amyloid with a purpose: FAs and the lessons they teach

In view of the widespread use of such repetitive elements in living organisms and the universal ability of proteins to aggregate under appropriate conditions, it should come as no surprise that Nature has also found widespread use of the cross-β structure. In order to form a biologically optimized system, however, Nature must overcome three weaknesses observed for amyloid formed *in vitro* and in deposition diseases: pathogenicity, uncontrolled formation (i.e. random deposition) and polymorphism. In all cases where FA has been studied in molecular detail, it has emerged that the pitfalls can be avoided by the use of ancillary proteins and/or the spatial separation provided by different cellular compartments. Let us look at a few instructive examples.

The protein Mα, which is the main component of human melanosomes, is so aggregation-prone that, left to its own devices, it aggregates within a few seconds even in the presence of 5 M guanidinium chloride [65]. Yet *in vivo*, Mα is synthesized as the lumen-exposed component of the membrane protein Pmel17, and this association suppresses its aggregative behavior until it is released in a post-Golgi compartment by furin cleavage [66]. A disulfide bond between Mα and the membrane-bound moiety Mβ, may further regulate against fibrillation until Mβ is degraded, after which Mα can presumably aggregate rapidly [67].

The protein CsgA is the main component in *Escherichia coli* curli structures, but although it aggregates readily by itself upon incubation as a purified one-component solution *in vitro* [68], *in vivo* it is secreted out of *E. coli* and only starts to form curli fibrils if exposed to the protein CsgB, which remains attached to the surface of the outer membrane where it may form a nucleating structure [69]. Furthermore, *in vitro* studies suggest that when CsgA aggregates by itself, the fibrillation nucleus is a dimer [70] which by virtue of its small size is presumably not cytotoxic. This suggests that *E. coli* may even have two complementary systems to prevent formation of cytotoxic aggregates.

Undoubtedly many more exquisite mechanisms will be revealed as we uncover details of the formation of other FAs. In the following sections, we mainly address bacterial FA, but for overview purposes we have also included in Table 1, a list of amyloid occurring in eukaryotic tissue.

Prokaryotic amyloids: an overview

Among the prokaryotes, amyloids were not detected before the late 1980 s, where the Gram-negative *E. coli* was shown to produce amyloid fimbriae called curli [71]. Since then, several other enteric bacteria such as *Salmonella* spp. have also been shown to produce amyloid fimbriae, known as tafi [72]. These filamentous structures were thought to be involved in adhesion to surfaces, cell aggregation and biofilm formation and have been studied in detail in terms of amyloid structure, involved genes and their regulation, and their possible function (see below). In 2003, another family of bacterial amyloids, known as chaplins, were found in the Gram-positive filamentous *Streptomyces coelicolor* by Claessen et al. [73]. These amyloids are very different from curli and tafi in amino acid composition, structure and function and are involved in production of aerial hyphae and dispersal of spores. Within the past 3 years, other bacterial FAs have been described: pili from *Mycobacterium tuberculosis* [74], Microcin 492 from *Klebsiella pneumoniae* [75], and harpins from *Xanthomonas axonopodis* [76], the last two acting as cytotoxins.

Table 2. Methods used to isolate functional amyloid.

Organism	Protein	Method	Reference
S. coelicolor	chaplins	removal of other components by boiling 2 % SDS and extraction with TFA	73
M. tuberculosis	MTP pili	mechanical shearing, differential centrifugation and extraction to remove lipids	74
P. fluorescens	25-kDa protein	homogenization and freeze-thaw cycles, followed by removal of contaminating macromolecules by 2 % boiling SDS	M.S. Dueholm et al., unpublished results
E. coli	CsgA	*E. coli* cells blended to free curli, followed by pelleting of curli by centrifugation and purification by SDS-PAGE	68

However, amyloids are much more widely distributed among bacteria than those isolated discoveries suggest. Our recent study of biofilm samples from a variety of different habitats, including freshwater lakes, brackish water, drinking reservoirs and waste water treatment plants, shows that amyloids are present in all habitats and are often associated with a large fraction of the bacteria [22]. Using amyloid-specific antibodies and dyes, we have found that amyloids are expressed by 5–40 % of all bacteria in these habitats. The list embraces bacteria from several phyla, including *Proteobacteria*, *Actinobacteria*, *Firmicutes*, *Chloroflexi* and *Bacteriodetes* [22]. Furthermore, several earlier reports of pili and other cell appendages and cell envelope components are likely to be amyloid, although it was often not realized in the original studies. Some of these results are also briefly reviewed below. The methods used for their isolation are summarized in Table 2.

Bacterial amyloids seem to be generally present in most bacteria with multiple functions, and we have just scratched the surface to reveal the huge diversity in structure and function of amyloid in Nature.

E. coli and *Salmonella typhimurium* curli: attachment cables

E. coli, S. typhimurium and other *Enterobacteriaceae* produce thin fimbriae called curli or tafi (thin aggregative fimbriae) [12, 68, 72, 77], which can be visualized as extracellular material by atomic force microscopy (AFM) and light microscopy [78]. They are 3–12 nm wide and may be up to a few micrometers long. Although the major protein component in the final curli fibril is CsgA, at least six proteins are involved in the formation of curli. Of these, CsgA forms a central outer membrane pore in complex with CsgE and CsgF [79], which probably plays a role in the export of CsgA and CsgB to the surface. The important contribution of CsgB has been described above. Interestingly, the CsgA core amyloid-forming domain (i.e. the part integrated into the amyloid structure) can be divided into five repeating units; peptides representing three of these repeating units also form amyloid [70], indicating that CsgA is optimized for fibrillation.

The operons in *E. coli* (*csg*BA and *csg*DEFG) have homologous operons in *Salmonella* (*agf*BA and *agf*DEFG) and the fimbriae are biochemical and functional analogs, so we will mainly focus on curli from *E. coli.* Together with cellulose fibers, which are also produced by many of theses bacteria, curli (or tafi) are of key importance for adhesion to surfaces and biofilm formation and thus in pathogenesis by attachment, invasion of host cells, interaction with host proteins and activation of the immune system [10, 12, 80]. Curli can also bind to the cytokine-releasing factor flagellin in intestinal epithelial cells and thus elicit an immune response [81]. Firm adhesion to the surface only takes place after 8–16 h when curli are expressed, so curli are believed to be important for aggregation and for making strong attachment to the surfaces [78]. Thus, curli promote binding to a variety of other surfaces, including plant cells [82, 83], stainless steel [84, 85], glass and plastics [85, 86], and can substantially enhance the resistance to chlorine [84].

Chaplins from Gram-positive *S. coelicolor:* formation of hydrophobic surfaces

The filamentous bacterium *S. coelicolor* and many other streptomycetes undergo complex morphological differentiation, forming a submerged mycelium that may grow into the air and septate into spores. *S. coelicolor* produces chaplins which are a class of eight hydrophobic proteins (ChpA–H) that spontaneously self-assemble into amyloid fibrils forming an amphipathic membrane [73]. This hydrophobic membrane mediates attachment to other hydrophobic surfaces, facilitates penetration of the liquid-air interface by lowering surface tension and thus allows formation of aerial hyphae. The chaplins are also responsible for making the spores hydrophobic, which probably facilitates wind dispersal. Three groups of protein are responsible for formation of aerial filaments and spores: the spore-associated protein SapB, chaplins and rodlins [73, 87, 88]. The chaplin genes have only been discovered in sporulating *Actinomyces*, a group

of Gram-positive Actinobacteria. In function, the chaplins are very similar to the hydrophobins produced by many filamentous fungi [89]. The proteins ChpA–C are around 225 amino acids and CHpD–H are up to 63 amino acids in length. They are only known from *Streptomyces*, except for the aerial mycelium-forming *Termobifida fusca*, which has a single *chp* gene [90]. Chaplins assemble in solution when seeded with the assembled form of the protein. Chaplin fibrils are organized in rodlets at the surface of aerial hyphae and on spores from *S. coelicolor.* The presence of rodlin proteins may help organize the chaplin fibrils in a rodlet layer which consists of paired rods 8–12 nm wide and up to 450 nm long [91, 92]. It is still not clear whether the chaplins assemble primarily on the surface of the filaments or at the water-air interphase after release from the cells. Claessen and coworkers [73, 91] suggested an initial release to the water during submerged growth where they lower the surface tension by assembling at the water-air interface. This allows the bacteria to form aerial hyphae. The amphipathic membrane is then formed at the cell wall-air interface and may induce the rodlet layer. However, new AFM studies [93] on the surface of living *S. coelicolor* and *S. avermitilis* indicate that the chaplins can also be assembled to form amyloid fiber directly on the cell surface during submerged vegetative growth. These studies also showed that aerial growth is characterized by a thick fibrous layer consisting of chaplin fibrils and rodlins. The long chaplins (ChpA–C) are probably anchored to the cell wall, because they contain a sequence which is specifically recognized by sortase enzymes [73, 94]. In contrast, the short chaplins, encoded by genes expressed 10 to 25-fold more than those for the long chaplins [92] are not recognized, making them less stable and more easily sheared off from growing aereal hyphae. It is not yet known whether *Streptomycetes* form nucleation proteins as is known for *E. coli*, but it is possible that a smaller degree of biological control is required to form two-dimensional films at the water-air interface, as opposed to long fibrils growing from a bacterial surface.

M. tuberculosis pili: adherence via pili

The Gram-positive *M. tuberculosis* causes tuberculosis in humans. The bacterium produces thin pili (MTP, 2–3 nm in width) that are antigens for infection and recognized by IgG from patients [74]. MTP are assumed to be critical for adherence, colonization and infection of the host. They bind to the human extracellular matrix protein laminin and share a number of properties with curli, although their amyloid nature is not yet conclusively proven. The purified pili were clearly fibrillar by transmission electron microscopy but, like curli, could not be solubilized sufficiently to run on SDS-PAGE. Instead, LC-MS was used to identify a peptide sequence and hence the gene *mtp* (H37Rv ORF Rv3312A), which contains a putative transmembrane (TM) sequence (aa 10–30). Mutants without this gene demonstrated reduced laminin-binding capabilities, consistent with the fact that pili mediate specific recognition of host cell receptors facilitating close contact and tissue colonization [95]. *M. tuberculosis* preferentially attaches to and invades damaged areas of the human respiratory mucosa in an organ culture system [96]. In these instances of tissue damage, extracellular matrix proteins such as laminin may be more highly exposed than in healthy tissue.

The *mtp* gene is present in only a few mycobacteria and seems to be limited to the pathogenic species, underlining its role in cell invasion. An investigation by Dahl [97] reported surface projections in the form of extracellular fibrils from carbon-starved *M. tuberculosis* cells. The fibrils could branch and adhere between cells and to surfaces, forming meshes and clumping. The fibrils did not detach from the cell and were clustered, suggesting a cellular source. They are very similar to fibrils seen in *M. avium* subsp. *paratuberculosis* [98] and might well be amyloid. Appendages termed pili are present on many bacteria that cause disease in the human respiratory tract including the Gram-positive pathogens group B *Streptococcus* and *Corynebacterium diphtheriae* [99, 100]. Future studies will show whether some of these also are amyloid.

Amyloids in Gram-positive bacteria

Among the Gram-positive bacteria, only the amyloids of *S. coelicolor,* a few other streptomycetes and *M. tuberculosis* have been studied in detail. However, beside these and the Mycolata, other Gram-positive bacteria belonging to the phyla *Actinobacteria* and *Fircimutes* produce amyloids. Phylogenetically distant non-Mycolata actinobacteria (from genera *Actinospica, Geodermatophilus* and *Streptosporangium)* produce amyloid surface structures [P. L. Jensen, P. H. Nielsen and D. Otzen, unpublished results]. Among *Firmicutes*, bacteria from the genera *Bacillus*, *Paenibacillus* and *Enterococcus* produce amyloid [22; P. L. Jensen, P. H. Nielsen and D. Otzen, unpublished results]. Several *Bacillus* species (e.g. *Bacillus cereus*) often form biofilms at the air-liquid surface when releasing spores to the air [101], so this could indicate a streptomycetes-like function of the amyloids on the cell-water surfaces by these species. Furthermore, spores from *B. atrophaeus* are covered with rodlets which are structurally very similar to amyloid fibrils [102], so it seems that the spores can also be covered

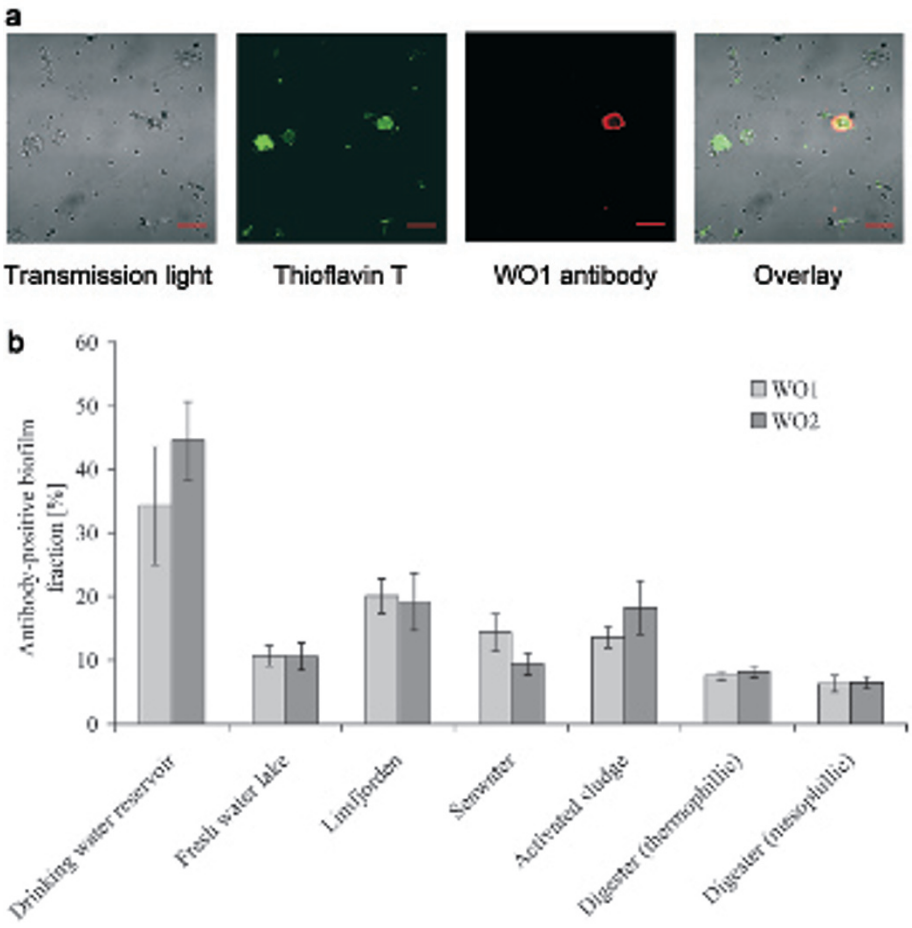

Figure 4. (*a*) Simultaneous staining of microcolonies with Thioflavin T and conformationally specific amyloid antibody [from ref. 22]. Bar represents 20 μm. (*b*) Fraction of microorganisms from different natural biofilms that contain amyloid as determined using the amyloid-specific antibodies WO1 and WO2 [reproduced with permission from ref. 22].

by amyloids and this will explain their extreme resistance. These results strongly indicate that production of amyloids is widely distributed among most Gram-positive bacteria, and they form an integrated part of the cellular and spore surface.

Amyloids in natural biofilms

We have recently demonstrated the widespread distribution of amyloids among microorganisms growing in biofilms from a lake, seawater, drinking water and waste water treatment systems [22]. We initially detected surface-associated amyloids by ThT staining, following this up by two conformationally specific antibodies targeting amyloid directly (Fig. 4a). The detection methods were each combined with fluorescence *in situ* hybridization using fluorescently labeled oligonucleotide probes in order to link phenotype with identity of uncultured microorganisms. Representatives from *Proteobacteria* (*Alpha-, Beta-, Gamma-* and *Deltaproteobacteria*), *Bacteriodetes*, *Chloroflexi* and *Actinobacteria*, and most likely also from other phyla, produced amyloids in these natural biofilms. Quantification of the microorganisms producing amyloid adhesins showed that they constituted at least 5–40 % of all prokaryotes present in the biofilms (Fig. 4b). Most of the bacteria formed monospecies microcolonies within the biofilms and the amyloids could be visualized on the cell surfaces and in the extracellular matrix between the cells. Interestingly, some filamentous bacteria, e.g. from the phylum *Chloroflexi*, also expressed amyloids as a part of the sheath or close to the septum between the individual cells in the filaments. Production of amyloids was confirmed by environmental isolates belonging to the *Gammaproteobacteria*, *Bacteriodetes*, *Firmicutes* and *Actinobacteria.*

Some of these have been investigated in more detail. A 25-kDa protein constituting amyloid fimbriae could be purified from an isolate closely related to *Pseudomonans fluorescens* (Fig. 5a, Table 2) [M. S. Dueholm, P. Larsen, J. L. Nielsen, P. H. Nielsen and D. E. Otzen, unpublished results]. Fibrillation could be reproduced *in vitro.* The amyloid-like structure was verified by Fourier-transform infrared spectroscopy, circular dichroism and ThT fluorescence (Fig. 5b). Partial sequencing by MS/MS revealed that the fimbrin contained at least one repeated motif. This motif was different from those previously found for other known

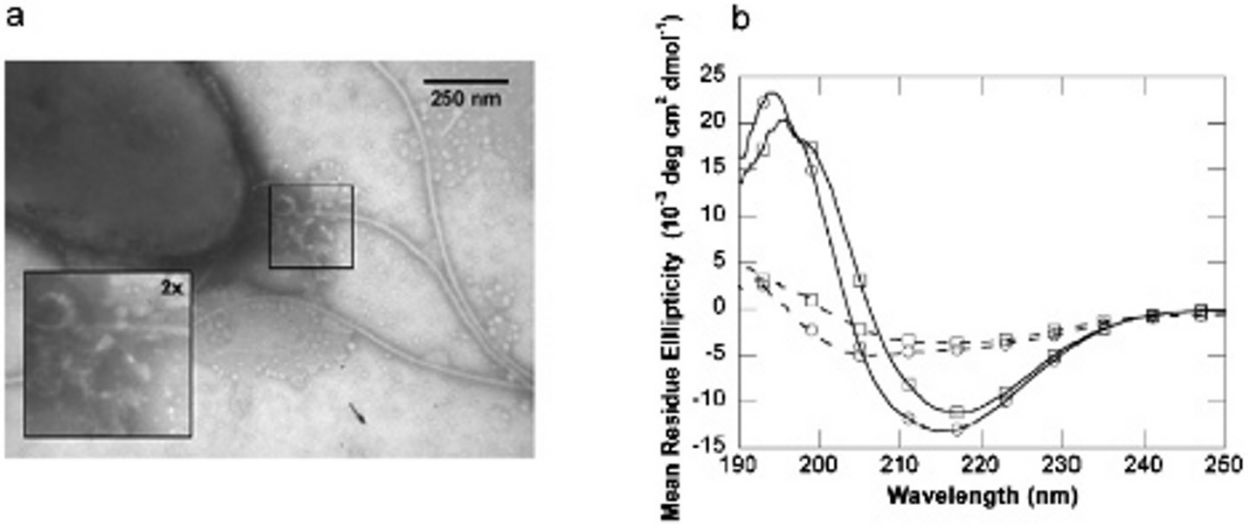

Figure 5. (*a*) Transmission electron microscopy picture of amyloid produced by *Pseudomonas* species [Dueholm et al., unpublished data]. (*b*) Circular dichroism spectra of amyloid produced by *Psedomonas* sp. (squares) and CsgA from *E. coli* (circles) resuspended in PBS buffer (full lines) and dissolved in 80% formic acid (dashed lines) [Dueholm et al., unpublished data].

amyloids, but it was not possible to find the corresponding gene in genomes from various pseudomonads. The diversity of species and morphologies expressing amyloids in natural biofilms suggest that their function is very diverse and not only directly related to adhesion and biofilm formation.

Microcin E492 from *K. pneumoniae* and harpins from plant pathogens

Some bacteria purposely produce cytotoxic proteins, some of which have recently been shown to have amyloid properties. Microcin E492 is a small peptide bacteriocin naturally produced by *K. pneumoniae* that kills *Enterobacteriaceae* bacteria by forming pores in the cytoplasmic membrane [103]. Microcin activity is highest during the exponential phase and disappears in the stationary phase although production continues [103]: this is probably because it assembles into amyloid-like fibrils, as shown to occur *in vitro* [75]. The fibrils have the same structural, morphological, tinctorial and biochemical properties as the aggregates observed in the disease conditions. Aggregation is seeded by preformed aggregates. Amyloid formation also occurs *in vivo*, where it is associated with a loss of toxicity of the protein. The finding that microcin E492 naturally exists both as functional toxic pores and as harmless fibrils suggests that protein aggregation into amyloid fibrils can be used to 'park' potentially explosive proteins when their aggressive properties are no longer needed. Interestingly, small annular Aβ and α-synuclein protofibrils [104, 105] resemble the cytolytic β barrel pore-forming toxins from bacteria such as *Clostridium perfringens* [106], suggesting that a common structure could be responsible for toxic membrane permeabilization.

In many Gram-negative plant pathogens, the *hrp* (hypersensitive response and pathogenicity) genes encode proteins involved in the secretion of harpins. Harpins are heat-stable glycine-rich type III-secreted proteins which elicit a hypersensitive response in plants, i.e. an early defense response restricting growth of plant pathogens by causing cell death (similar to apoptosis) [76]. Mutants of these bacteria unable to produce harpins lose their plant pathogenicity [107]. Many *Xanthomonas* species produce harpins and the protein HpaG from *X. axonopodis* pv. *glycines* 8ra has recently been shown to form amyloid under plant apoplast conditions [76]. When expressed in *E. coli*, both oligomers, protofibrils and fibrils lead to a hypersensitive response in the plant cells (Fig. 6), which is unexpected in view of the conventional wisdom that only the oligomers are the cytotoxic species [76]. It is possible that this may be related to the fact that the harpin sequence has a region which is homologous to the yeast prion protein [108], and the prion toxicity mechanism is not clearly understood. Harpins from other plant pathogens, such as *Erwinia amylovora* and *P. syringae,* also form amyloid-like protofibrils [76]. It is presently not clear whether the formation of amyloid takes place on the surface of the bacteria or whether the monomers only or primarily self-assemble in the plant cells. To our knowledge, on the surface of these bacteria has not yet been investigated whether amyloidic appendages are present, and we hope that future studies will address these issues.

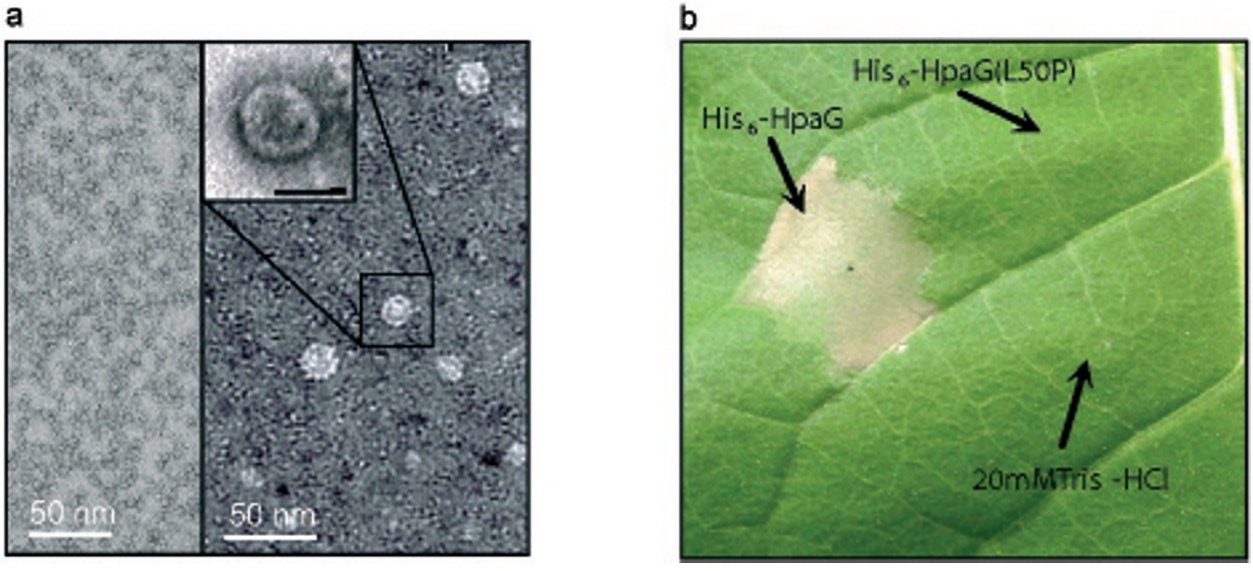

Figure 6. (*a*) Formation of oligomer structures by the harpin protein HpaG overexpressed with a His6 tag in *E. coli.* (*b*) Ability of recombinant HpaG to induce a hypersensitive response in tobacco leaves. An inactive variant L50P is included for comparison. [Reproduced with permission from ref. 76.]

Model studies of the aggregation of bacterial proteins

There are cases where aggregation of bacterial proteins under physiological conditions may be an unwanted spin-off of an otherwise useful propensity to associate. Thus, the five *Salmonella* flagellar rod proteins normally associate in α-helical coiled coils to form a straight rod structure [109]. However, overexpression of these proteins in *E. coli* leads four of them to self- or cross-aggregate under physiological conditions, rather than cross-reacting with flagellal complex [109]. A large part of these proteins is made up of natively unfolded regions. The chaperone FliJ appears to prevent unwanted aggregation by binding to the unfolded regions [110].

There exist reports of the aggregation of numerous other bacterial proteins such as HypF-N [111] and S6 [112], but these studies have taken place in the presence of organic solvent or extreme pH, and have served more to decipher the biophysical process of aggregation *per se.* In the same vein, several studies have focused on the fibrillation of peptides derived from soluble bacterial proteins, e.g. CsgB [113] and OspA [114]. In general, the behavior of isolated sequences which form part of an otherwise globular protein may bear little resemblance to the properties of the globular protein, making such studies of questionable biological relevance (despite fascinating properties in their own right) unless the sequence can be considered representative for the protein as such. Thus, already in 1992, Jarrett and Lansbury [115] studied the *E. coli* protein OsmB which mainly consists of repeat motifs showing a resemblance to the Aβ peptide; a 17-residue peptide representing such a motif fibrillated readily *in vitro* in a nucleation-dependent manner, similar to the fibrillar nature of the repeat motifs in CsgA [116]. In view of our discovery of the widespread role of amyloid in bacterial biofilm [22], it is interesting that OsmB is linked to capsular polysaccharide synthesis which plays a role in the later stages of biofilm development [117]. Despite their lack of homology, both OsmB and the *bona fide* bacterial amyloid protein CsgA consist of multiple repeats of a simple repeat [70].

Heterologous overexpression of protein in bacteria in many cases leads to its accumulation in insoluble inclusion bodies. Interestingly, proteins in these inclusion bodies are not simply amorphous collections of unfolded proteins but possess genuine amyloid structure, as seen by their high β sheet content as well as their ability to seed deposition of soluble versions of the same protein and bind Congo Red and Thioflavin TCR/ThT [118]. Furthermore, aggregates from inclusion bodies can be cytotoxic to mammalian cells, in contrast to thermal aggregates of the same protein [118]. The presence of amyloid structures in inclusion bodies could stem from the generic ability of proteins to form amyloid when forced to undergo folding through partially structured intermediates at high concentrations. They also illustrate yet again how Nature can use amyloid as a storage state for surplus unwanted protein.

Detecting, purifying and characterizing bacterial amyloid

It is clear from the many previous sections that much has yet to be discovered about the formation and action of bacterial amyloid. Central to these efforts is an ability to identify, purify and analyse these proteins, and we will therefore in the following section focus on how this may best be accomplished.

Detection of bacterial amyloids in pure culture studies is usually based on the binding of the dye Congo Red to the β sheets of the proteins [119]. In this way, bacteria that produce amyloids are generally easily identified by red colonies when growing on agar plates containing Congo Red [120]. Amyloid fibrils can also be visualized by Congo Red and Sirius Red with bright-field or polarized light microscopy [121], while staining with fluorescent dyes can be carried out with Congo Red [122], ThT [123], 2-(4′-methylaminophenyl) or benzothiazole (BTA-1) [124]. ThT and Congo Red are among the most frequently used dyes for amyloid staining and ThT is considered to be the most sensitive [121]. The specificity of ThT and Congo Red is not as good as the antibodies, because other organic molecules, such as DNA and cellulose, can also be stained by ThT [125, 126] and Congo Red [127]. This kind of dye promiscuity reflects a profound truth about the nature of their binding: although there is little direct structural information on the nature of the dye binding [128], these types of dyes probably target grooves provided by regularly repeating structures, which may also allow them to dimerize and thus enhance their spectroscopic properties [129]. In addition, Congo Red can also react specifically with certain non-amyloid proteins [20].

We have found antibodies to be more reliable for detection of amyloid [22, 121, 130]. The use of conformationally specific antibodies (WO1 and WO2) is particularly efficient, because they specifically target the fibril structure described above, without binding to monomers of the same proteins [21]. Furthermore, antibodies can be combined with fluorescence *in situ* hybridization with oligonucleotide probes for detection of microbes directly in mixed biofilms [22]. Nevertheless, antibodies are not perfect, either: due to their large size, they may find it difficult to access amyloid fibrils if they are covered and masked by other extracellular polymers, leading to false-negative results. We have found that saponification of Gram-positive bacteria by prolonged exposure to an alcoholic alkali solution at elevated temperatures can improve antibody binding without destroying the overall cellular architecture [P. L. Jensen, P. H. Nielsen and D. Otzen, unpublished results].

The real proof for the presence of bacterial amyloids is usually regarded as transmission electron microscopy images and purification in hot/boiling SDS, with a verification of the structure using Fourier-transform infrared spectroscopy, circular dichroism and ThT fluorescence. Furthermore, treatment with strong acid such as 90–100% formic acid should release monomers that can be further purified and characterized by SDS-PAGE and various spectroscopic and microscopic techniques and sequenced by MS/MS. This requires pure cultures, vigorous expression of amyloids – which typically only takes place under certain growth conditions – and an efficient purification protocol, which in our experience needs optimization for each species due to the very robust – not to say recalcitrant – amyloid structures involved (cf. Table 2).

In view of these difficulties, we believe that amyloids have in many former studies been overlooked simply because the purification and detection methods were not available or suitable. In future studies there will be a great challenge to refine and develop the above procedures to reveal the true structure of these cell envelopes.

Bacterial amyloids and diseases

Bacterial amyloid may be associated with diseases in numerous ways. As previously mentioned, curli, pili and other amyloid states are implicated in the ability of bacteria to attach to and invade human cells, with detrimental effects for the host cell, while harpins induce plant cell apoptosis – though the latter may be viewed as a defense mechanism by the plant to prevent bacteria spreading to other parts of the organism. Other bacteria, e.g., the spirochete *Borrelia burgdorferi*, induce Alzheimer disease-like symptoms (formation of hyperphosphorylated tau and amyloid deposits of the Aβ peptide as well as upregulation of the Aβ precursor protein) in mammalian cells [131]. The mechanism is unclear, but may involve exposure to the outer membrane's lipopolysaccharide [131], possibly even in a 'templating' process where the plaque forms around the spirochete [132]. *Borrelia* is not known to form amyloid by itself. However, in other cases, it is possible that bacterial amyloid may play a direct role in disease induction. *Nocardia otitidiscaviarum* can induce Parkinson's disease symptoms in the brain [133]. Broxmeyer [134] hypothesized that several bacteria in the Mycolata (*Nocardia* and *Mycobacterium*) may induce amyloidosis, as they are often correlated with Parkinson's. As we have mentioned, all these bacteria produce amyloid, which could be envisaged to seed amyloid formation in the brain. This type of mechanism is supported by the work of Lundmark et al. [135], who injected curli fibrils into mice, leading to accelerated formation of the disease-associated amyloid protein AA. However, it was not directly demonstrated that amyloid fibrils

can act as direct templates for further amyloid formation. If this is the case, amyloid-producing microbes may constitute a rich potential source – either at infections or as environmental amyloids that may affect human health. The same research group also demonstrated that amyloid fibrils added to the drinking water of a mouse model for amyloidosis decreased the lag time to onset of 'disease' [136], suggesting that the added amyloid can penetrate the gut and act as a seed for amyloid growth and disease induction. Thus, amyloid-producing microbes constitute a source of environmental amyloids which might potentially act as seed for formation of amyloid in humans.

Perspectives

This review has provided a glimpse of the great variety of bacterial amyloids which play central roles in maintaining structural integrity of individual cells as well as whole bacterial communities. Apart from some very general guidelines, nothing indicates that there is any simple unifying principle in the biological build-up and consolidation of amyloid; rather, its formation reflects the immediate functional demand of the amyloid-producing organism. There is likely to be just as much variation among bacterial amyloid assembly as between bacterial and eukaryotic amyloid. In this sense, bacterial amyloid nicely illustrates how the principle of (more or less) self-organized assembly can be tailored to meet specific requirements. In the future, we expect that insights into the molecular assemblies of FA will be very important to uncover more of these biological principles. Some aspects that are of particular relevance include the following:

- the existence of sequence motifs for amyloid formation (e.g. repetitive elements and the physico-chemical properties of the sequence)
- the details of the nucleation mechanism (for example, what determines whether it involves one or several proteins)
- fibril growth (directionality and polarity of growth, branching properties, rate of growth under different conditions)
- the molecular composition of the amyloid (single component or hybrid and how this affects material properties such as pliability and mechanical strength)
- the molecular structure of the cross-β elements that constitute the core of the amyloid
- the ultrastructure of the *in vivo* amyloid (fibril quaternary structure, diameter, extent of coiling).

All these questions will ensure that FA research will be an active and fertile research area for many years to come.

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