

Exploiting amyloid: how and why bacteria use cross- β fibrils

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Abstract

Many bacteria produce protein fibrils that are structurally analogous to those associated with protein misfolding diseases such as Alzheimer's disease. However, unlike fibrils associated with disease, bacterial amyloids have beneficial functions including conferring stability to biofilms, regulating development or imparting virulence. In the present review, we consider what makes amyloid fibrils so suitable for these roles and discuss recent developments in the study of bacterial amyloids, in particular the chaplins from *Streptomyces coelicolor*. We also consider the broader impact of the study of bacterial amyloids on our understanding of infection and disease and on developments in nanotechnology.

Introduction

The association of amyloid fibrils with numerous diseases [1–3] has led to considerable interest in elucidating their structures, the mechanisms by which they form and the molecular basis of their toxicity. These investigations have benefitted greatly from insights provided by the study of amyloidogenic proteins from bacteria. Although amyloid fibrils from bacteria are structurally indistinguishable from those found in patients suffering from amyloidoses, rather than inducing cell damage or death in the host, they often confer favourable properties such as mechanical strength or virulence [4].

In the present review, we first consider why amyloid fibrils are such suitable candidates for roles in the extracellular environment and discuss the importance of sequence in determining the propensities of proteins to form fibrils. We then present an overview of the roles in which amyloid fibrils are exploited by bacteria and focus on the process of morphological differentiation in *Streptomyces coelicolor*, which is mediated by a family of amyloidogenic proteins called the chaplins. We close by considering how studies of bacterial amyloid fibrils with positive functions may inform our understanding of disease and lead to the development of new materials for nanotechnology.

Overview of amyloid fibril structure and formation

The native state of a protein is the result of a large number of weak interactions between residues that may be far apart in the primary sequence. The folding pathways of most

proteins, especially those with more than 100 residues, are transiently populated by metastable intermediates in which hydrophobic amino acids and regions of unstructured polypeptide backbone that would normally be buried in the native state may be exposed [5]. Such intermediates may associate with one another to form aggregated structures that are further stabilized by intermolecular interactions. The resulting aggregates may be amorphous structures or highly ordered fibrillar species called amyloid fibrils, which comprise stacks of β -sheets aligned perpendicular to the fibril axis (Figure 1) [6].

Under physiological conditions, the formation of amyloid fibrils appears to be restricted to a subset of proteins. However, under mildly denaturing conditions, many other proteins can be induced to adopt this thermodynamically stable conformation. Thus the ability to form amyloid fibrils is considered to be an inherent property of the polypeptide chain, rather than the special property of any given sequence elements [3]. There is little sequence conservation between the amyloid domains of different proteins, but many amyloid domains share common characteristics, such as the presence of glutamine/asparagine- or hydrophobic-rich sequences [7,8]. Amyloid fibril formation is often promoted by short contiguous sequences or 'amyloid stretches' within the protein, which are often sufficient for fibril formation and can be used to seed fibril formation of the full-length protein [9,10].

Proteins spontaneously adopt the conformation of lowest free energy that is kinetically accessible to them. Outside the cell, where proteins may encounter a range of destabilizing conditions, such as the presence of hydrophobic or denaturing compounds, the cross- β fibrillar (amyloid) conformation is readily accessible. Therefore bacteria appear to have evolved proteins that assemble into amyloid fibrils for a range of extracellular functions, including the formation of biofilms [11] and microbial cell coats [4].

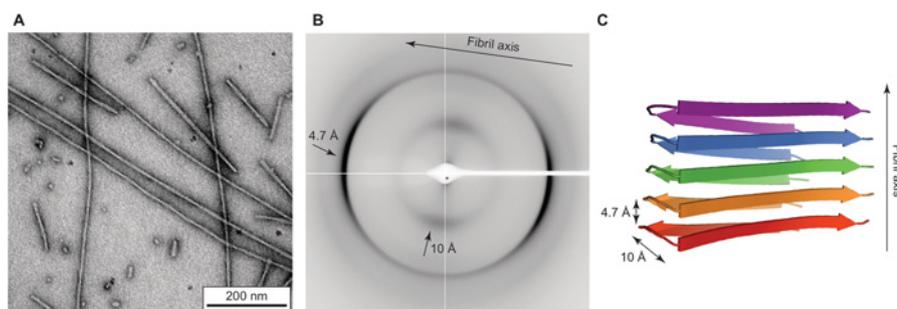
Key words: biofilm, chaplin, curli, fimbria, functional amyloid, *Streptomyces coelicolor*.

Abbreviations used: ECM, extracellular matrix; FTIR, Fourier-transform infrared.

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Figure 1 | Amyloid fibril structure

(A) Transmission electron microscope (TEM) image of amyloid fibrils formed by the chaplin protein ChpE, negatively stained with 2% phosphotungstic acid. (B) X-ray fibre diffraction of partially aligned amyloid fibrils, shown for ChpE, gives rise to anisotropic reflections at 4.7 and 10 Å (indicated with arrows), corresponding to the interstrand and intersheet spacings of the cross- β core. The direction of the long fibril axis is indicated. Reproduced from Sawyer, E.B., Claessen, D., Haas, M., Hurgobin, B. and Gras, S.L. (2011) The assembly of individual chaplin peptides from *Streptomyces coelicolor* into functional amyloid fibrils. PLoS ONE 6, e18839 with permission. (C) Structure of the cross- β core of amyloid fibrils, derived from NMR data for the Alzheimer's peptide, A β . Generated with Mac PyMol using PDB code 2BEG.

**Diverse roles of bacterial amyloids**

Amyloid fibrils have been found to play diverse physiological roles in many organisms, from bacteria to humans [12]. In bacteria, these fibrils are mostly assembled and act extracellularly, whereas intracellular amyloid fibrils with positive functions have been described in higher organisms. In this section, we will explore the diversity of roles fulfilled by amyloid fibrils in bacteria (summarized in Figure 2) and discuss in detail the role of the chaplins as regulators of *Streptomyces* development.

One of the major roles of bacterial amyloids is in the formation of biofilms, as a significant proportion of the organisms found in biofilms produce fibrils with amyloid-like properties [11]. A biofilm is a multicellular community in which microbes adhere to one another forming a layer on a solid or liquid surface. Adhesion is mediated by the ECM (extracellular matrix), which comprises extracellular DNA, polysaccharides and proteins. Growth within a biofilm not only offers bacteria some protection against environmental factors, such as the presence of detergents and antibiotics, but also enables bacteria to co-operate and interact with one another and facilitates lateral gene transfer. Biofilms can grow on biological or non-biological surfaces, such as hospital equipment and cannulae, where they present a serious threat of infections such as ventilator-associated pneumonia, which accounts for approximately 60% of all healthcare-associated infections [13] and is the most common fatal infection contracted in intensive care [14].

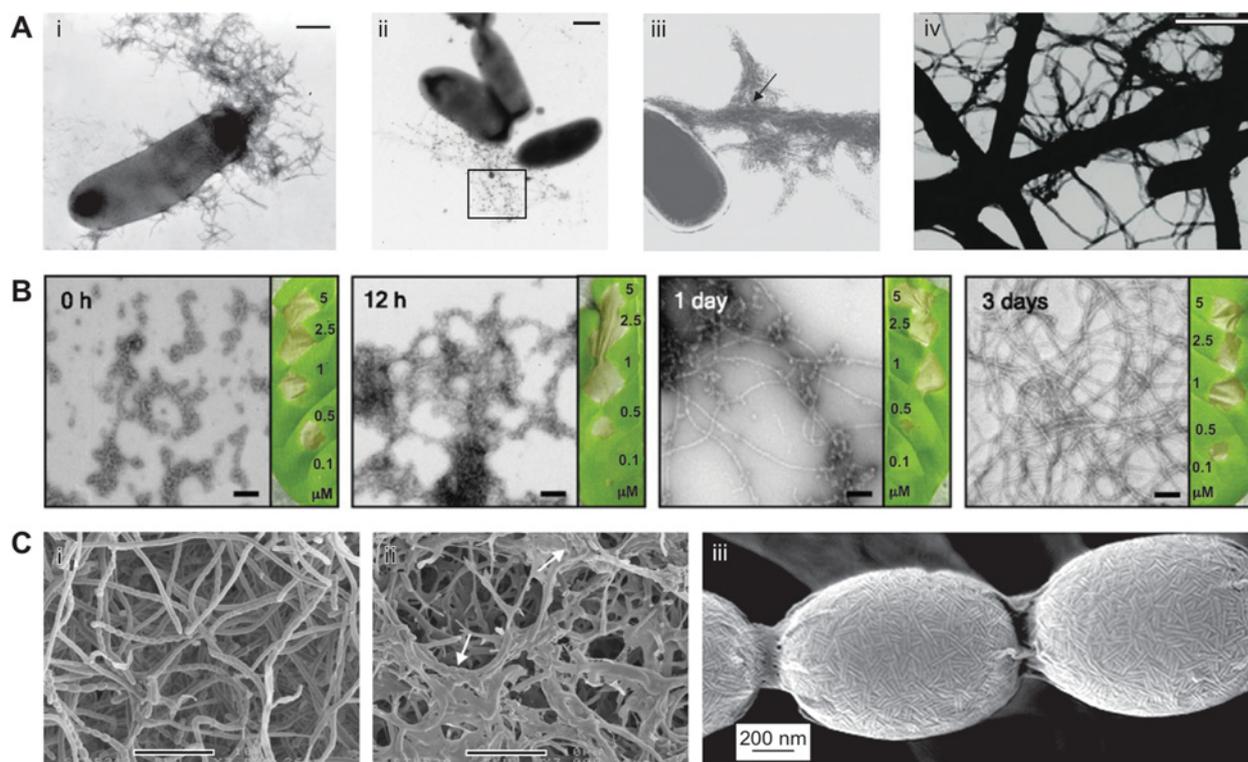
Biofilms are stabilized by the ECM, a major component of which is fibrous protein. The first of these fibrous proteins to be identified was CsgA, which forms fimbriae known as curli in *Escherichia coli* [15]. Fimbriae (or pili) are hair-like proteinaceous structures extending from the bacterial cell surface that are often involved in cell adhesion (Figure 2A). Curli are one of the types of fimbriae produced by *E. coli* (Figure 2Ai) and have a range of functions

in addition to biofilm formation, including mediation of host cell adhesion and invasion. Curli assembly requires the expression of two divergently transcribed operons, *csgBA*, which encodes the protein subunits that make up curli, CsgA and CsgB, and *csgDEFG*, which encodes non-structural proteins required for the expression, secretion and assembly of CsgA and CsgB [16]. CsgE, CsgF and CsgG form a complex at the outer membrane; CsgA and CsgB secretion across the outer membrane is CsgG-dependent. Following secretion, CsgB remains associated with the outer membrane, where it templates the conversion of CsgA into the amyloid conformation. Amyloid CsgA then further templates conversion of monomeric CsgA, such that protein subunits are added to the growing fibre tip [17,18]. Previous advances have led to the identification of similar fibrous proteins in biofilms (Figure 2 and Table 1), such as those formed by *Bacillus subtilis* [19], *Pseudomonas* [20] and many mycolata species [21] including the pathogen *Mycobacterium tuberculosis* [22].

Some bacteria are able to harness the toxicity of amyloid fibrils and employ them as virulence factors. Amyloidogenic proteins called harpins are produced by plant pathogenic species of *Xanthomonas*, *Erwinia* and *Pseudomonas*. Harpins cause cell death in a localized region of plant tissue, a phenomenon known as a hypersensitive response (Figure 2B). Harpins from several species have been found to form amyloid fibrils *in vitro* and studies of HpaG, a harpin from *Xanthomonas*, have revealed a direct correlation between amyloid formation and the hypersensitive response [23]. The process of fibril formation of HpaG closely resembles that of the Alzheimer's disease peptide, amyloid β -peptide₄₀, with the formation of spherical oligomers and protofibril intermediates preceding the mature fibrils [24]. The amylospheroid form of amyloid β -peptide₄₀ is more neurotoxic than the mature fibrillar form [25]. In contrast, protofibrils and mature fibrils of HpaG induce hypersensitive responses of equal severity (Figure 2B and Table 1). The

Figure 2 | Examples of functional amyloids from bacteria

(A) Fimbriae composed of amyloid fibrils in different bacterial systems. TEM images of: (i) *E. coli* curli fimbriae formed from CsgA (scale bar, 500 nm); (ii) *B. subtilis* fimbriae formed from TasA (scale bar, 500 nm); (iii) *M. tuberculosis* fimbriae formed from Mtp (magnification $\times 45000$); (iv) *S. coelicolor* fimbriae (thinner fibrils) formed from chaplins associated with vegetative hyphae (thicker structures) (scale bar, 2.5 μm). (B) *Xanthomonas axonopodis* harpin HpaG is a virulence factor. TEM images of fibril formation by HpaG (all scale bars, 200 nm) and photographs of tobacco leaves following injection of various amounts of HpaG aggregates (as indicated to the side of each image). Pale patches indicate plant cell death due to a hypersensitive response. (C) *S. coelicolor* development is regulated by the chaplins which promote growth of aerial hyphae. (i) Wild-type cells produce a robust aerial mycelium that differentiates into spores; (ii) a $\Delta\text{chpABCDEFHG}$ strain (lacking all eight chaplin genes) produces hyphae that clump together and collapse at the colony surface (scale bar, 10 μm); (iii) scanning electron micrograph of the rodlet structures present on the surface of *S. coelicolor* spores (scale bar, 200 nm). (A, i) Reproduced from Hammer, N.D., Schmidt, J.C. and Chapman, M.R. (2007) The curli nucleator protein, CsgB, contains an amyloidogenic domain that directs CsgA polymerization. *Proc. Natl. Acad. Sci. U.S.A.* **104**, 12494–12499, with permission. (A, ii) Reproduced from Romero, D., Aguilar, C., Losick, R. and Kolter, R. (2010) Amyloid fibers provide structural integrity to *Bacillus subtilis* biofilms. *Proc. Natl. Acad. Sci. U.S.A.* **107**, 2230–2234 with permission. (A, iii) Reproduced from Alteri, C.J., Xicohtencatl-Cortes, J., Hess, S., Caballero-Olin, G., Giron, J.A. and Friedman, R.L. (2007) *Mycobacterium tuberculosis* produces pili during human infection. *Proc. Natl. Acad. Sci. U.S.A.* **104**, 5145–5150 with permission. (A, iv) Reproduced from De Jong, W., Wösten, H.A.B., Dijkhuizen, L. and Claessen, D. (2009) Attachment of *Streptomyces coelicolor* is mediated by amyloid fimbriae that are anchored to the cell surface via cellulose. *Mol. Microbiol.*, **73**, 1128–1140 © 2009 The Authors; Journal compilation © 2009 Blackwell Publishing Ltd. (B) Reproduced from Oh, J., Kim, J.-G., Jeon, E., Yoo, C.-H., Moon, J.S., Rhee, S. and Hwang, I. (2007) Amyloidogenesis of type III-dependent harpins from plant pathogenic bacteria. *J. Biol. Chem.* **282**, 13601–13609 with permission. © 2007 The American Society for Biochemistry and Molecular Biology. (C, i and ii) Reproduced from Claessen, D., Stokroos, I., Deelstra, H., Penninga, N., Bormann, C., Salas, J., Dijkhuizen, L. and Wösten, H.A.B. (2004) The formation of the rodlet layer of streptomycetes is the result of the interplay between rodlines and chaplins. *Mol. Microbiol.* **53**, 433–443 ©2004 Blackwell Publishing Ltd. (C, iii) Reproduced from Di Berardo, C., Capstick, D.S., Bibb, M.J., Findlay, K.C., Buttner, M.J. and Elliot, M.A., Function and redundancy of the chaplin cell surface proteins in aerial hypha formation, rodlet assembly, and viability in *Streptomyces coelicolor*, *J. Bacteriol.* (2008) **190**, 5879–5889, doi:10.1128/JB.00685–08, with permission from American Society for Microbiology.



mechanism of induction of hypersensitive response by harpins is not well understood, but observation of the interaction of harpins with plant cell walls [26] and synthetic

membranes, leading to increased cation permeability [27] suggests that membrane destabilization is likely to play an important role.

Table 1 | Characterization of bacterial amyloids with positive functions

Amyloidogenic proteins from a range of bacteria have been characterized using the following techniques: CR, Congo Red binding; EM, electron microscopy (negative or immunogold staining); ssNMR, solid-state NMR spectroscopy; ThT, Thioflavin T fluorescence assay; XRD, X-ray fibre diffraction.

Organism	Protein(s)	Function	Method of characterization	Reference(s)
<i>Escherichia coli</i>	CsgA	Fimbriae (curli) formation	CD, CR, EM, ssNMR, ThT, XRD,	[42,43,50]
<i>Bacillus subtilis</i>	TasA	Fimbriae formation	CR, EM, ThT	[19]
<i>Mycobacterium tuberculosis</i>	Mtp	Fimbriae formation	CR, EM	[22]
<i>Pseudomonas fluorescens</i>	FapC	Fimbriae formation	CD, EM, FTIR, ThT	[20]
<i>Xanthomonas axonopodis</i>	HpgA	Virulence factor	CD, CR, EM	[23]
<i>Streptomyces coelicolor</i>	ChpD-H	Regulation of differentiation; fimbriae formation	CD, CR, EM, FTIR ThT, XRD	[36,38,39]

A third role of amyloid fibrils in bacteria is as developmental regulators. In the following section, we focus on the chaplins, which are a family of amyloidogenic proteins from *S. coelicolor*, the model organism for this genus. The chaplins are the best characterized example of amyloids that regulate morphological differentiation (Figure 2C and Table 1).

The chaplins: regulators of *Streptomyces* development

Streptomycetes are filamentous soil bacteria that grow in colonies that closely resemble fungi. They produce many pharmaceutically important secondary metabolites, most notably antibiotics, and have a remarkably complex developmental life cycle involving both physiological and morphological differentiation of several types of cells. Following spore germination, cells produce vegetative hyphae, which give rise to mycelial networks that explore the substrate for available nutrients. Over the next few days, bacteria within the colony differentiate to produce a multicellular consortium in which cells in different parts of the colony play different roles. Cells at the periphery continue to grow and acquire nutrients, while hyphae nearer the middle of the colony start synthesizing pigmented antibiotics. Simultaneously, aerial hyphae are formed on top of the colony surface [28].

The regulation of aerial hyphae formation is complex and involves several regulatory pathways including the *bld* cascade and *sky* pathway, which both control the synthesis of structural proteins involved in aerial hyphae formation. (For an in-depth discussion of these regulatory networks, see reviews by Claessen et al. [29] and McCormick and Flårdh [30].) The vegetative mycelium is sessile, while the dispersion of cells to colonize the wider environment occurs via spores, which are formed on reproductive aerial hyphae. Both aerial hyphae and spores are coated with a hydrophobic, fibrous sheath consisting of paired rods (or rodlets) 8–12 nm in width and up to 450 nm in length [31] (Figure 2C).

Rodlet formation requires the expression of two groups of proteins: the chaplins [32,33] and the rodlins [34], which together constitute the rodlet layer [35]. *S. coelicolor* produces eight chaplin proteins, designated ChpA–ChpH, which share significant sequence identity, including a highly conserved hydrophobic ‘chaplin’ domain of approximately 40 residues.

ChpA–ChpC have two N-terminal chaplin domains and a C-terminal sorting signal that targets them for sortase-mediated covalent attachment to the *S. coelicolor* cell wall, whereas ChpD–ChpH are smaller proteins (5–6 kDa) comprising an N-terminal secretion signal peptide and a single chaplin domain [32,33]. With the exception of ChpE, all chaplin domains contain two highly conserved cysteine residues that may form intramolecular [33] or intermolecular [36] disulfide bonds. All sporulating actinomycetes possess genes for both long and short chaplins, although the numbers of these genes may vary, with ChpC, ChpE and ChpH representing the smallest group of components required to form a chaplin apparatus [32].

The differential expression of chaplins throughout the *S. coelicolor* life cycle suggests that these proteins play different roles *in vivo*. Analysis of RNA transcripts from cell cultures at different stages of morphological development revealed that *chpE* and *chpH* are expressed at high levels during both the vegetative and aerial mycelial phases, whereas the other *chp* genes are only expressed during aerial hyphae formation [33]. The roles of individual chaplins have been addressed at the microbiological level by the construction of a ‘minimal strain’ and systematic re-introduction of genes. Results indicate that expression of both long and short chaplins, with at least one short chaplin containing the conserved cysteine motif, is vital for the development of a robust aerial mycelium, but that there is otherwise some degree of redundancy among the chaplins [37]. Two recent biophysical studies have shed further light on the role of the chaplins in the formation of the hydrophobic coat [36,38].

We recently demonstrated that each of the five short chaplins forms fibrils *in vitro* that closely resemble the fibrils found on the surfaces of *S. coelicolor* aerial hyphae and spores [36]. The fibrils have a β -sheet-rich secondary structure, as demonstrated by CD and FTIR (Fourier-transform infrared) spectroscopy, and give rise to X-ray diffraction patterns with characteristic anisotropic reflections at 4.7 and ~ 10 Å (1 Å = 0.1 nm), indicating the presence of cross- β structure within the fibril core (Figure 1); this is the first evidence that the structures formed by the chaplins are true amyloid fibrils. The chaplins can form fibrils under reducing and non-reducing conditions, further suggesting that the cysteine residues conserved in all chaplin domains except ChpE are not essential for fibril formation. Furthermore, we demonstrated

the ability of synthetic peptides corresponding to the short chaplins to restore aerial growth to the $\Delta chpABCDEH$ strain (lacking six chaplin genes) within 16 h, providing evidence that peptide assembly on or near the bacterial surface is sufficient to enable functionality.

As discussed above, amyloid fibril formation is often driven by short 'amyloid stretches' within protein sequences [9]. A recent study by Capstick et al. [38] identified two amyloidogenic regions in ChpH that contribute differentially to the assembly of chaplin fibrils. Computational analysis led to the identification of a five residue SVIGL motif in the C-terminus of ChpH that was predicted to be highly aggregation-prone. However, only when this motif was present in a 16-residue peptide (corresponding to the sequence between the two conserved cysteine residues) was fibril formation observed. This 16-residue peptide was able to seed fibril formation of full-length ChpH. Mutating the SVIGL motif resulted in a reduced ability to form amyloid fibrils *in vitro* and the inability to restore a wild-type phenotype to a $\Delta chpH$ mutant strain of *S. coelicolor*. A second putative 'amyloid stretch' was also identified in the N-terminal region of ChpH. Similar to the C-terminal peptide, a 17-residue peptide corresponding to this amyloid domain formed fibrils *in vitro*. Mutational studies revealed that both N- and C-terminal amyloid domains of ChpH are required for formation of aerial hyphae, although the N-terminal domain is dispensable for assembly of the rodlet ultrastructure, suggesting that the two amyloid domains within ChpH play subtly different roles.

A further role of chaplins

In addition to their role in regulating morphological differentiation, the chaplins are also involved in the formation of fimbriae that mediate cell attachment [39]. MS and mutational studies have confirmed that these fimbriae are composed of chaplins. The fimbriae are anchored to the bacterial cell wall at spike-shaped protrusions. In contrast with the fimbriae of Gram-negative bacteria, fimbriae from Gram-positive bacteria are covalently attached to the cell wall via sortases. The long chaplins, ChpA–ChpC are putative sortase substrates, but unlike typical Gram-positive pilins they are not located close to any of the seven sortase homologues on the *S. coelicolor* chromosome [40] and strains lacking the long chaplin genes are still able to produce chaplin fimbriae [39]. Therefore the *S. coelicolor* fimbriae appear to be atypical Gram-positive pili that are polymerized in a sortase-independent manner, thus resembling the pili in *M. tuberculosis* [22].

De Jong et al. [39] found that cellulose was tightly associated with the fimbriae, but not required for their formation. This is consistent with previous work which showed that cellulose is a common component of the extracellular matrices of several enteric bacteria [41]. Enzymatic treatment of the cells with cellulase resulted in detachment of fimbriae from the cell surface, indicating that cellulose plays an important role in fimbrial anchoring.

Emerging themes and variations

Studying amyloidogenic proteins of different function from different species of bacteria reveals some interesting features. First, the number of amyloidogenic components varies considerably between systems: *B. subtilis*, *E. coli* and *Pseudomonas* species have only one major amyloidogenic component, which is expressed alongside accessory proteins that facilitate secretion or assembly. In contrast, *S. coelicolor* produces eight amyloidogenic proteins, in addition to accessory proteins. The reasons for producing such an array of highly similar amyloidogenic proteins have not yet been uncovered, but the differential expression of chaplins throughout the *Streptomyces* developmental cycle suggests that they are likely to play subtly different roles.

A comparison of the structures of bacterial amyloidogenic proteins prior to fibril formation reveals further interesting differences. Although the chaplins are predicted to be largely unstructured, the CD spectra of ChpG and ChpH before fibril formation are indicative of the presence of some α -helical secondary structure [36]. This is consistent with the finding that incubation with Teflon induces α -helix formation in a solution of mixed chaplins extracted from the cell wall [39]. Monomeric CsgA is largely unstructured [42], whereas even in the fibrillar form of TasA some random coil and α -helix could be detected by CD [19].

Thirdly, the presence of multiple 'amyloid stretches' within the sequence appears to be a common feature of bacterial amyloids. The *E. coli* curli protein CsgA contains several amyloidogenic domains; at least three of these domains can form amyloid fibrils *in vitro*, but only two are critical for amyloid fibril formation *in vivo* [42,43]. ChpH has two amyloid domains, one near the N-terminus and the other near the C-terminus. The two domains have different assembly kinetics and both domains are necessary for aerial hyphae formation, but the C-terminal domain appears to be more important for rodlet assembly than the N-terminal domain [38].

What can be learned from the study of bacterial amyloids?

As we have shown, bacterial amyloids play diverse physiological roles, from forming key components of the ECM in biofilms, to mediating toxicity, to enabling the development of aerial hyphae and spores. Not only are these processes interesting in their own right, they are also highly relevant to some of the challenges of modern life, including combating hospital superbugs and producing pathogen-resistant crops. The extraordinary wealth of antibiotics produced by *Streptomyces* species makes these organisms pharmaceutically very important. Thus there is considerable interest in understanding the molecular mechanisms underlying their biology. In particular, there is a desire to understand how their development is regulated and the mechanisms by which they achieve biofilm formation, both of which are processes in which the chaplins play a key role.

Bacterial amyloids with positive functions are remarkably similar to those associated with diseases such as Alzheimer's. Despite extensive research, relatively little is understood about the factors that govern the formation of amyloid fibrils, neither is it clear exactly what determines whether or not amyloid fibrils will be toxic. By close investigation of naturally occurring systems in which amyloid fibrils confer positive functions, our understanding of protein misfolding diseases will also be enhanced.

Finally, the study of naturally occurring amyloid fibrils with positive functions may inspire the design of new materials for nanotechnology. Amyloid fibrils have a number of features that make them attractive targets for nanotechnology: they self-assemble from their component peptides/proteins, are incredibly strong and robust, and the physicochemical properties of the fibril core and surface can be controlled with relative ease by changing the amino acid sequence of the peptides [44–46]. This enables the display of biomarkers such as metalloporphyrins [47], fluorophores or other functional groups [48]. The hydrophobins, a group of fungal proteins similar to the chaplins, have been tested for their suitability as rheological agents to increase the stability of air bubbles during ice cream manufacture [49]. Thus, just as amyloid fibrils are found in a wide range of biological systems, similarly they may be suitable for use in a wide range of technological applications.

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