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Bacterial amyloids

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Abstract:

Amyloids are supramolecular protein assemblies based on fibrillar arrangements of β -sheets that were first found as linked to neurodegenerative and systemic human diseases. However, there is now overwhelming evidence on alternative roles of amyloids as functional assemblies and as epigenetic determinants of beneficial traits, both in Fungi and Metazoa. Bacteria also use amyloids as functional devices, mainly as extracellular scaffolds in biofilms, but there is increasing evidence for functional roles of amyloids in the bacterial cytosol, and these have enabled to engineer minimal models of a 'generic' amyloid disease. Amyloids are thus key players in the physiology of bacteria and versatile building blocks in Synthetic Biology.

Key words: amyloid; prion; bacteria; biofilm; sRNA regulation; Hfq; plasmid replication control; microcin E492; RepA-WH1

Key Concepts:

- Besides determining human diseases and functional traits in yeast, amyloids also play functional roles in bacteria.
- Secreted bacterial amyloidogenic proteins scaffold the extracellular matrix in biofilms, bacterial consortia key for biofouling and antibiotic resistance.
- Intracellular amyloid assemblies control regulation of gene expression by small RNAs, transcription termination and plasmid DNA replication in bacteria.
- The assembly of antimicrobial peptides as amyloids counteracts their toxicity, while functional amyloids can be turned into cytotoxic models of disease.
- Bacterial amyloids, due to their modularity and high stability, are excellent blocks for the design of synthetic supramolecular assemblies.

Introduction

The amyloid fold is made from the assembly of multiple protein molecules, each providing at least a β -strand, thus forming β -sheet fibrils of indefinite length (see section B for an structural outline; Eisenberg and Sawaya, 2017). It can be adopted by short peptides, intrinsically disordered or properly folded protein domains, provided that the latter become unstable due to mutations, post-translational modifications (proteolysis included) or allosteric (ligand-selected) conformational changes (Eichner and Radford, 2011). Up to the turn of this century, interest on amyloids were always linked to their association with human disease, either neurodegenerative (Alzheimer's, Parkinson's or Huntington's, among others) or systemic (type II diabetes, serum amyloidosis) diseases (Chiti and Dobson, 2017; see also: DOI: 10.1002/9780470015902.a0024459 and DOI: 10.1002/9780470015902.a0002146.pub3). In parallel, amyloids were shown to be behind the aggregated form of some glutamine/asparagine-rich domains in the yeast *Saccharomyces cerevisiae* that were epigenetic determinants (i.e., cytoplasmic-inherited through non-Mendelian genetics) of selectable beneficial phenotypic traits (for comprehensive reviews on functional amyloids, see Si, 2015; Hafner-Bratkovic, 2017).

The discovery that the curli fibres contributing to scaffold biofilms of the bacterium *Escherichia coli* were, in fact, amyloids (Chapman *et al.*, 2002) opened the realm of functional amyloids to prokaryotes. Since then, many other secreted proteins across the whole *Bacteria* Life Domain have been found to use the amyloid fold to assemble extracellular matrixes (see section A.1.1). Later on, intracellular functional amyloids have been also found in bacteria, involved either in plasmid DNA replication or in the pre or post-transcriptional control of RNAs (see section A.1.2). Furthermore, these intracellular amyloids can be engineered to become cytotoxic, providing clues on the essential minimal pathways that determine an amyloid disease (see section A.2). Since amyloids are the most stable protein assemblies described so far, they are valuable building blocks for protein engineering in Synthetic Biology (Wang *et al.*, 2018).

A. The Biology of bacterial amyloids

1. Functional bacterial amyloids

1.1. Extracellular bacterial amyloids

The first report of a bacterial functional amyloid was in 1989, corresponding to the ***E. coli* curli fibers** described from a cow mastitis infection (Olsen *et al.*, 1989). These fibers are extracellular polymers produced by different strains of *Escherichia* and *Salmonella*, which participate in adhesion to animal and plant tissues, in cell invasion and pathogenesis, in the immune recognition by the host, and in providing a fitness advantage in the environment (Chapman *et al.*, 2002; Gophna *et al.* 2002; Gallo *et al.* 2015; Tursi *et al.* 2017). These fibers are formed after the polymerization of the amyloidogenic protein CsgA, in a process induced by the membrane-anchored nucleator protein CsgB (Hammer *et al.* 2007; Shu *et al.* 2012). Its production also requires several other proteins including transcriptional regulators, chaperones that prevent the uncontrolled aggregation, and molecular channels allowing the secretion of the curli subunits (Nenninger *et al.* 2011, Evans *et al.* 2015). After the discovery of the curli fibers, several other examples of bacterial extracellular amyloids have been described, most of them sharing two common features: 1) they act as adhesion factors and structural components of the extracellular matrix, and 2) they play some role in

pathogenesis or host colonization. Among them, the **Fap proteins in *Pseudomonas*** were involved in biofilm formation (see also DOI: 10.1002/9780470015902.a0000342.pub2), auto-aggregation, and attachment to biotic and abiotic surfaces, enhancing the mechanical robustness, stiffness and resistance to drying of biofilms (Dueholm *et al.*, 2010; Dueholm *et al.*, 2013a; Zeng *et al.*, 2015). The production and assembly of the Fap fibers require the activity of several proteins encoded in the *fapAF* operon, where FapC corresponds to the main amyloid component and FapB would function as nucleator protein. The rest of the proteins are required for secretion. The role of Fap fibers as virulence factors in *Pseudomonas* is supported by the attenuated phenotype observed for the *fapC* deletion mutant in different infection models, as well as by the transcriptional upregulation of the *fap* operon during infection, compared to laboratory conditions (Wiehlmann *et al.*, 2007; Turner *et al.*, 2014). The *fap* operon can also be found in other members of Gammaproteobacteria including strains from *Aeromonas*, *Vibrio*, and *Shewanella*, as well as in some species of Betaproteobacteria and Deltaproteobacteria (Dueholm *et al.*, 2013b).

Extracellular functional amyloids are also present in Gram-positive bacteria. A well-studied case is the **TasA protein** produced by species of *Bacillus*, which is able to polymerize forming fibrils both *in vivo* and *in vitro*, being a major component of the extracellular matrix and essential for biofilm formation and repression of the immune response during infection of plant tissues (Romero *et al.* 2010; Lakshmanan *et al.* 2012). Such fibrils have been reported as amyloid-like (Diehl *et al.* 2018), but substantially maintaining the native 3D-fold of the protein (Erskine *et al.*, 2018). Besides, the **phenol-soluble modulins** (PSMs) from *Staphylococcus* are key components of the extracellular matrix and possess antimicrobial and immunomodulatory properties that are exploited during host infection (Wang *et al.* 2007; Cogen *et al.* 2010; Periasamy *et al.* 2012; Schwartz *et al.* 2012; Da *et al.* 2017). Other functional amyloids found in *Staphylococcus* are the fibers formed by the **biofilm-associated protein Bap**, which play a role in the colonization *in vitro* and during infection *in vivo* (Cucarella *et al.*, 2001). Another case corresponds to **chaplins**, proteins found on the hyphae surface of *Streptomyces coelicolor*, which assemble into amyloid fibers that reduces the tension at the air-water interface, allowing previously submerged hyphae to emerge into the air (Claessen *et al.* 2003). The variety of examples described above points out that the amyloid fold is a widespread feature in the bacterial world, which can be exploited through different ways to dispose functional tools or weapons in the extracellular milieu of the microbial cells.

A particular and well-studied amyloidogenic protein is the **pore-forming bacteriocin microcin E492** (MccE492) produced by some strains of *Klebsiella pneumoniae*. MccE492 exhibits remarkable properties: it induces apoptosis in malignant cell lines, and forms amyloid fibers as a mean to control its antibacterial activity (reviewed in Lagos *et al.*, 2009), being its formation kinetically dependent on the production of unmodified MccE492 in higher proportion (Marcoleta *et al.*, 2013). To be active, MccE492 has to undergo post-translational modification by the covalent binding of salmochelin-like molecules (glycosylated enterochelin derivatives from the catechol-siderophore family) to MccE492 C-terminal mediated by the proteins MceIJ. This process is regulated by iron availability (Marcoleta *et al.*, 2018), because the expression of *mceIJ* is controlled by the Fur repressor. A negative regulator of *mceA* (the structural gene of MccE49) expression is also controlled by Fur, consequently, at low iron availability, the production of enterochelin as well as the maturation proteins MceIJ will be optimal for producing highly modified MccE492 (with a high antibacterial activity) disfavoring amyloid formation. On

the contrary, at high iron availability mostly unmodified MccE492 would be produced (low antibacterial activity), and this favors amyloid fibers formation (Marcoleta *et al.*, 2013; Marcoleta *et al.*, 2018; see Fig 1).

MccE492 capacity to form intracellular amyloids *in vivo* has been assessed through two specific amyloidophilic fluorescent dyes (Aguilera *et al.*, 2016). Using this detection assay a pro-amyloidogenic region was identified (54-63) including residues P57 and P59 that control aggregation (termed gatekeepers). A deletion mutant of this region presents a hypo-amyloidogenic behavior, while the mutants P57A and P59A are hyper-amyloidogenic (Aguilera *et al.*, 2016). The physiological implication of MccE492 intracellular amyloid formation is probably to sequester the toxic form when this bacteriocin is produced in large quantities inside the cell, as observed for the extracellular MccE492 amyloids.

1.2. Intracellular bacterial amyloids

Intracellular functional amyloids were reported in bacteria later than the extracellular ones. The first report on one of such functional amyloids was on the **DNA replication protein RepA** of the *Pseudomonas savastanoi* plasmid pPS10 (reviewed in Giraldo and Fernández-Tresguerres, 2004; see also: DOI: 10.1002/9780470015902.a0000468.pub2). RepA is a multifunctional protein that, as a dimer, represses transcription of its own gene by binding to an inverted repeat operator sequence overlapping with its promoter. As a monomer, RepA binds to four directly repeated sequences (iterons) found at the plasmid origin of replication to initiate DNA synthesis by attracting the components of the replication machinery of the host cell. RepA dimerization depends on a N-terminal 'winged-helix' domain (WH1), whereas the C-terminal domain (WH2) provides the main DNA-binding interface. Interestingly, upon replication completion RepA remains attached to the iteron sequences where WH1 bridges together two or more copies of plasmids through protein-protein interactions. These complexes inhibit premature re-replication rounds through steric hindrance at the origin of replication. These inhibitory RepA-RepA complexes are built on a WH1 amyloid oligomer, which clusters plasmids at the bacterial nucleoid (Molina-García *et al.*, 2016) (Fig. 2a).

A proteome-wide search of proteins sharing in *Bacteria* a sequence composition similar to yeast prions indicated that the N-terminal domain in the *Clostridium botulinum* **transcriptional terminator CbRho** (see also: DOI: 10.1002/9780470015902.a0000858.pub2) assembles as an amyloid *in vitro* (Pallarés *et al.*, 2016). These studies were followed by *in vivo* testing of chimeras between such prion-like domain and two heterologous proteins: Sup35 C-terminal domain in yeast, showing its ability to control the aggregation-dependent overcoming of translation termination; and *E. coli* Rho, which controlled transcriptional termination of a reporter gene in this bacterium (Yuan and Hochschild, 2017) (Fig. 2b).

The **small RNA binding protein Hfq** is a pleiotropic regulator that notably regulates translation efficiency and RNA decay in Gram-negative bacteria, usually via its interaction with small regulatory noncoding RNA (Vogel *et al.*, 2011; see also DOI: 10.1002/9780470015902.a0000970.pub3). Hfq, precisely its C-terminal region, forms amyloid assemblies that integrate in the bacterial membrane (Malabirade *et al.*, 2017). Based on these findings it seems likely that Hfq promotes the export of entire small nucleic acid molecules through the bacterial membrane by forming pores. This would

allow direct genetic material exchange into the extracellular environment. This plausible hypothesis is based on the evidence of noncoding RNA found in the extracellular space (Malabirade *et al.*, 2018). Besides these RNA-related functions, Hfq has also been described as one of the nucleoid associated proteins shaping the bacterial chromosome (Azam *et al.*, 2000; Cech *et al.*, 2016}. *E. coli* Hfq has been shown to have a strong propensity to compact DNA, thanks to its amyloid C-terminal region and, conversely, DNA induces amyloidogenesis of this region (Malabirade *et al.*, 2017; Malabirade *et al.*, 2018). Nevertheless, DNA seems to be a constituent of the amyloid fibril (Malabirade *et al.*, 2018) (Fig. 2c). Strikingly, as RepA, Hfq was shown to bridge together two plasmid DNA molecules through an amyloid oligomeric structure, and both proteins influence plasmid copy number *in vivo* (Molina-García *et al.*, 2016; Cech *et al.*, 2014).

Other intracellular bacterial proteins, such as CarD (Kaur *et al.*, 2018) and RavA (Chan *et al.*, 2016), have been found to assemble as amyloids *in vitro*. However, if this behaviour has any relevance for its function *in vivo* remains to be determined.

2. Cytotoxicity of bacterial amyloids

As discussed above, bacteria usually secrete amyloidogenic proteins out of the cells, in passing a safe way to avoid the presence of cytotoxic aggregates at the cytosol. The formation of inclusion bodies (IBs), which are made of heat/acid/redox-stressed, or recombinant over-expressed proteins and have an amyloid component (Carrio *et al.*, 2005) and a discrete impact on bacterial fitness (Lindner *et al.*, 2008), is an efficient mechanism to minimize the damage to bacteria of protein aggregation: IBs can be segregated asymmetrically to one of the daughter cells upon cell division, in such a way that the other (and its descendants) is freed from this burden (Govers *et al.*, 2018).

A common feature of human amyloidoses is the formation of protein aggregates, albeit at different tissue locations and built by distinct proteins. This fact has inspired the search for simpler models of amyloid diseases by expressing human amyloidogenic proteins in organisms such as yeast, worms, flies or mice (Narayan *et al.*, 2014), but still the outcome complexity is mind-blowing. For the reasons expressed above, and due to their genetic distance to humans and to their much simpler cellular organization, bacteria do not seem suitable model organisms for amyloidoses.

The conformational activation of the WH1 domain in RepA (see section A.1.2) implies not only dimer dissociation, but also a complex conformational change that exposes an amyloidogenic sequence, thus driving the assembly of amyloid fibres by the isolated WH1 domain (Giraldo, 2007; Torreira *et al.*, 2015). The expression of RepA-WH1 in *E. coli* (Fig. 3) results in cytotoxicity for bacteria (Fernández-Tresguerres *et al.*, 2010), with the formation of two alternative aggregate types, similar to prion strains, with distinct phenotypes (Gasset-Rosa *et al.*, 2014): either multiple, globular, with a high affinity for an amyloidotropic fluorogenic agent, low tropism to the IBs small chaperone marker IbpA, and acute cytotoxicity; or a single, fluidized elongated particle, with reduced affinity for that fluorophore, and reduced toxicity. DnaK, the Hsp70 chaperone of *E. coli* cytosol (see also: DOI: 10.1002/9780470015902.a0023188), participates in the conversion of the former into the latter. The mechanisms for RepA-WH1 cytotoxicity in bacteria, outlined by Systems Biology comparisons of the transcriptome and aggregated proteome of *E. coli* expressing either IBs or the prion-like RepA-WH1 (Molina-García *et al.*, 2017), include the formation of pores at the bacterial inner membrane (Fernández *et al.*, 2016), which reduces ATP synthesis and metabolite transport, and induction of the

alternative dehydrogenase NdhII, which increases the generation of reactive oxygen species (see also: DOI: 10.1002/9780470015902.a0001376.pub3). Titration of key factors for the defence of the host, such as catalases and the transcription factors for oxidative and envelope stresses, leaves bacteria disarmed against RepA-WH1. Interestingly, this scenario has points in common with mitochondrial routes for amyloidosis in, e.g. Parkinson's (Haelterman *et al.*, 2014; see also: DOI: 10.1002/9780470015902.a0006031.pub2), which makes sense since mitochondria have a bacterial symbiotic origin, and provides a proof of concept of the potential of bacteria as minimal models of amyloid disease.

B. The structure of bacterial amyloids

The determination of high-resolution structures of amyloid fibrils is challenging. Although this has been very recently achieved for fibrils assembled by proteins involved in human disease (Eisenberg and Sawaya, 2017), three-dimensional structures of bacterial amyloids are limited to a few protein precursors in their native state (Fig. 4).

For X-ray crystallography, short peptides are required (Moshe *et al.* 2016; see also: DOI: 10.1038/npg.els.0002721). These peptides assemble 4.7– 4.8 Å stacked β -strands (Fig. 5a), the structural hallmark of amyloids. Alternatively, micro-electron diffraction can be used, enabling structural determination using submicron crystals (Guenther *et al.*, 2018). Solid-state nuclear magnetic resonance (ssNMR) spectroscopy may also be used to determine amyloid structures (Martial *et al.*, 2018). ssNMR reveals molecular distances and dihedral angles of fibril subunits, which combined allow the determination of 3D structures. However, ssNMR is an expensive technique due to the requirement of isotopic labelling, restricting the use to small protein fragments. Recently, cryo-electron microscopy (cryo-EM) has demonstrated its power to solve near atomic resolution structures of amyloid fibrils made from longer peptide stretches (see below). As a complement to high-resolution studies, other biophysical techniques are used to analyse bacterial amyloids at lower resolutions, prior to time-consuming NMR, X-ray diffraction or cryo-EM.

1. Analysis of amyloid self-assembly by molecular imaging

Straight amyloid self-assembly observation can be performed by microscopy techniques, including fluorescence microscopy, transmission electron microscopy (TEM) and atomic force microscopy (AFM). Thioflavin T (ThT) is commonly used to visualize amyloids. When bound to aggregated β -sheets, the dye displays an enhanced red-shifted fluorescence (Reinke and Gestwicki, 2011). Even if the dye fluorescence is not completely specific, it is a good clue for amyloid structure formation. Although ThT is widely used, it has to be taken with caution because ThT can also be an inhibitor of fibrillation (Stains *et al.*, 2007). Other dyes may alternatively be used, such as Congo red or poly-thiophenes. Super-resolution microscopy (such as direct stochastic optical reconstruction microscopy dSTORM, or photo-activated localization microscopy PALM) may also be used to study the kinetics of addition of labelled monomers to fibrils, giving information about their growth polarity (Pinotsi *et al.*, 2014).

Atomic force microscopy is a scanning probe technique that explores the sample surface via a tip at the end of a cantilever, allowing topographic images at nanometer resolution. Nevertheless, AFM measurements have poor lateral (XY plane) resolution compared to TEM (see below). One of the interesting possibilities of AFM is that it can

operate either on dried samples or in solution (Kad *et al.*, 2003); but to study fibrillar protein structures with AFM, it is essential to adsorb them on a flat surface, like mica or a lipid bilayer, that may also interact with protein fibrils (Malabirade *et al.*, 2017) (Fig. 5b). Recently, an innovative tool that combines infrared spectroscopy (IR; see below) and AFM, allowed identification of secondary structure elements (Dazzi and Prater, 2017).

The use of classical **Transmission Electron Microscopy** to observe amyloid fibrils has a long background. A quick way to check the presence and structural characteristics of fibrils (length, diameter, homogeneity, interweaving) is negative staining (Fig. 3c). The sample is first adsorbed on a carbon-coated grid, and then the contrast of the adsorbed sample can be enhanced with uranyl acetate or gadolinium salt solutions. Images are acquired, usually with a sCCD camera, at $\sim 40,000\times$ nominal magnification. However, negative staining implies the dehydration of the sample, which can lead to artefacts. Therefore, when high-resolution ultra-structural studies are required, samples should be observed in hydrated conditions, which is possible by cryo-transmission electron microscopy (cryo-TEM) (Fig. 5c). Cryo-TEM of amyloid fibres allows structure determinations at mid-scale and more recently at atomic resolution ($\leq 4\text{\AA}$), often used in combination with ssNMR and molecular modelling (Guenther *et al.*, 2018).

2. Analysis of amyloid secondary and supersecondary structure

Fourier transform infrared (FTIR) spectroscopy detects vibrations of molecules and can be used to analyse protein secondary structure. Precisely, FTIR measurements focus on peptide bonds, which adopt specific geometries in the different secondary structures. The assignment of a peak at a wavenumber to a given secondary structure can be done, e.g.: a peak near 1655 cm^{-1} is indicative of α -helix, and at 1630 and 1675 cm^{-1} of β -sheet (Tatulian, 2013). Stronger hydrogen bonding results in a shift to lower wavenumbers and, therefore, amyloid fibrils often have β -sheet peaks below 1625 cm^{-1} (Zandomeneghi *et al.*, 2004) (Fig. 5d). Quantitative evaluation of secondary structure can be achieved upon peak curve-fitting.

Circular dichroism (CD) is a sensitive absorption spectroscopy technique for studying biological samples. Circular left and right polarized light are differentially absorbed by proteins, due to the excitation of the $n\text{-}\pi$ and $\pi\text{-}\pi^*$ electronic transition of the peptide bonds. Calibrated and standardized CD spectra are deconvoluted and used for protein secondary structure determination. Synchrotron radiation circular dichroism (SRCD) allows for the extension of the spectral range down to 168 nm in aqueous solution, with very good signal to noise ratios (Fig. 5e). Especially for the weak CD signals of proteins with high β -sheet or disordered content, SRCD has improved the spectral analysis substantially (Wallace 2009). Many algorithms exist for the estimation of the secondary structure composition from CD spectra. For the special case of β -sheet-rich proteins, a recent publicly accessible algorithm BestSel allows the determination of secondary structure contents, including parallel and antiparallel β -sheets and distinction of β -sheet twisting (Micsonai *et al.* 2015, Micsonai *et al.* 2018). In practical terms, $10\text{-}50\text{ mM NaCl}$ allows spectral acquisitions down to 185 nm and ideally, phosphate buffer is used because its stability because of its transparency in deep-UV. All spectra are normalized to the mean residue weight ellipticity (Θ_{MRW} , $\text{deg.cm}^2.\text{dmol}^{-1}$). As a general rule of thumb, a negative peak centred around 220 nm is indicative for amyloids, specially if the native unfolded protein spectra is about 200 nm .

Electron paramagnetic resonance (EPR) spectroscopy, which detects the properties of unpaired electrons, may also be used to analyse the secondary structure of amyloids (Der-Sarkissian et al., 2003). Since there are no unpaired electrons in proteins, a free radical must be incorporated via site-directed spin labelling. The EPR spectrum is very sensitive to the environment and gives information about side chain mobility, accessibility and molecular distances. EPR spectra allow to distinguish between loop, surface, or buried sites, while the periodicity in mobility gives information about secondary structure.

Finally, **small angle X-ray scattering** (SAXS) is based on dispersion of an incoming X-ray wave by the electrons of the sample. The resulting signal is a scattering pattern that depends on the sample internal symmetry, is proportional to the number of particles within the sample and directly results from their size, shape and internal structure (Fig. 5f). Practically, when identical units are regularly spaced, typically > 1 nm, diffraction is observed. In amyloid assemblies, the β -strands are perpendicular to the protofibril main axis. Using synchrotron radiation (see also: DOI: 10.1002/9780470015902.a0003109.pub2) for these studies is an absolute requirement due to weak signal given by cross- β structures. Peaks corresponding to a cross- β can be seen at ~ 0.7 and $\sim 1.35 \text{ \AA}^{-1}$, corresponding to inter-sheet and inter-strand distances (d) of 8-10 $\text{\AA}$ and 4.7 $\text{\AA}$, respectively ($d(\text{\AA})=2\pi/q(\text{\AA}^{-1})$).

Perspectives

Once infamous because their involvement in human disease, amyloids have recently expanded their biological relevance to multiple physiological processes, in which protein aggregation has a central role in the expression of phenotype variability and its epigenetic transmission. Such functional amyloids, first discovered in yeast, are ubiquitous in bacteria, where they are central partners in scaffolding bacterial consortia through biofilms, but also in counteracting the activity of bacterial cytotoxic peptides. More recently, intracellular functional amyloids have been found involved in regulating plasmid DNA replication and RNA biology in bacteria and, in at least one instance, have enabled the development of a minimal model of amyloid disease. From the point of view of structural biology, the core differences between functional and pathogenic amyloids are the existence of a major, defined 3D structure with fast assembly kinetics for the former vs. the coexistence of an ensemble of conformations (polymorphisms) with slow kinetics for the latter. Bacterial amyloids are well positioned to explore and exploit such differences in the expanding field of nanotechnology, providing biomaterials with outstanding physical properties.

Glossary

Amyloidosis. Diseases (studied mainly in mammals) that result from the accumulation or toxic effect of amyloid aggregates in different tissues. Some of them include Alzheimer's, Parkinson's and Huntington's and Prion Diseases, and Type II Diabetes.

Cross-beta (β) structure. A typical folding pattern displayed by proteins forming amyloid fibrils. It is composed of arrays or β -strands, contributed either by different segments in a protein or from distinct molecules of the same protein, disposed perpendicular to the long axis of the fibrils.

Extracellular matrix. A three-dimensional network (matrix) formed by extracellular macromolecules secreted by cells embedded in it, mainly carbohydrates and proteins.

For bacterial cells, the production of extracellular matrix normally leads to the formation of a biofilm, often attached to a surface.

Microcin. Antimicrobial peptides with a molecular mass less than 10 000 Da produced by Gram-negative bacteria. Normally, they are toxic against bacterial species closely related to the producer.

Nucleator protein. A protein that interacts with the molecules of another, or the same (amyloidogenic), protein to enable (templating), accelerate and/or anchor the formation of amyloid fibrils.

Prion. A particular kind of protein aggregate (normally with amyloid structure) which displays infective properties, propagating the amyloid conformation and its effects when transferred to a new biological system or organism.

Protein folding. A process, occurring inside any cell, in which a polypeptide (protein) acquires a particular, functional tridimensional structure (native state). The folding can occur spontaneously or with the aid of specialised proteins named chaperones, which act favouring or accelerating the process.

Resolution. In the field of microscopy and spectroscopy, it means the smallest interval measurable by a scientific instrument or technique. In the context of this article, it indicates the minimum distance allowing to differentiate two or more atoms inside a macromolecule (normally measured in Angstroms, Å).

Siderophore. Molecules secreted by bacteria to scavenge iron in environments where it is scarce. They can have different chemical nature and structures.

Synchrotron. A kind of particle accelerator used to enhance the resolution of some spectroscopic techniques by generating strong electromagnetic radiation with a broad, tunable range of energies/wavelengths.

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Further Reading

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Figures

Figure 1. Steps involved in microcin E492 production, antibacterial activity and amyloid formation. (1) MccE492 peptide precursor is synthesized from its coding gene *mceA* and must be co-expressed with the immunity protein encoded by *mceB*. Besides, the MceC adds sugar moieties to the enterochelin siderophore, produced by the enzymes encoded in the *ent* genes (2), giving salmochelin. (3) The MceJI proteins catalyze the attachment of salmochelin to MccE492 peptide, originating mature (active) MccE492 (MccE492*). A mixture of unmodified (non-active) and modified forms is secreted to the extracellular space through the dedicated exporter formed by MceHG and the outer membrane protein TolC, where the N-terminal leader peptide carried by the MccE492 precursor is cleaved. Both enterochelin production and MccE492 maturation are induced in response to low iron availability. In this situation, a high proportion of modified MccE492 is exported, which can enter the target cells through the catechol siderophore receptors, resulting in a high antibacterial activity. The production of a high amount of modified MccE492, disfavors its amyloid aggregation, preventing toxin inactivation. At high iron availability, low amounts of enterochelin and salmochelin are produced, generating a low amount of MccE492*. Unmodified MccE492 accumulation favors its aggregation into amyloid fibers that lack antibacterial activity.

Figure 2. A sketch with the three intracellular bacterial functional amyloids characterized so far. (a) RepA, inhibiting premature DNA replication firing by sterically bringing together the replication origins of plasmids (green). (b) CbRho as an hexamer finishes mRNA (pink) transcription, while as an amyloid oligomer allows transcription to proceed further, generating longer mRNA molecules. (c) Hfq, a hexamer that can also assemble amyloid oligomers, is a global regulator of sRNA (magenta) stability as well as of genome packing, while it can also assemble pores at the bacterial membrane. In all cases, amyloidogenic modules (domains) have been depicted in blue, whereas functional ones were coloured yellow (RepA C-terminal DNA binding domain) or cyan (CbRho and Hfq RNA binding modules).

Figure 3. The cytotoxic prion-like protein RepA-WH1. (a) TEM section through an *E. coli* cell showing the amyloidogenic precursors of RepA-WH1 assembled at the nucleoid (yellow sector), as revealed by an antibody specific of the amyloidogenic conformation (arrows: gold particles). (b) At the membrane, RepA-WH1 assembles as pores (EM 2D-projection, bottom). These pores can be reconstituted in lipid vesicles (red: RepA-WH1-mCherry; green: confined calcein label). (c) In mature intracellular aggregates (electron-dense areas), RepA-WH1 monomers would assemble head-to-tail ($\beta 1$ - $\beta 2$) as helical tubules (3D-EM, left) with a diameter section alike that of the pores (right).

Figure 4. The three-dimensional structures of the amyloidogenic modules of curli-like fibrils (modelled as 2NNT; Ferguson *et al.*, 2006; a), PSM α 3 (PDB 5I55; Tayed-Fligelman *et al.*, 2017; b), TasA (PDB 5OF1; Diehl *et al.*, 2018; c) and RepA-WH1 (PDB 1HKQ; Giraldo *et al.*, 2003; d). Distinct folds, either all- β (a), all- α (b) or α + β (c and d), can be used to build amyloid fibres (fibril axes depicted as dotted lines in a and b), but implying conformational transformations into β -sheet rich fibres (c and d), or the assembly of unaltered α -helical building blocks into cross- α fibres that match the overall shape of bona-fide amyloids (c).

Figure 5. Techniques to characterise the structure of amyloid self-assemblies. (a) The hallmark cross- β amyloid assembly consists in inter-strand and inter-sheet distances of 4.7 Å and 8-10 Å, respectively. Molecular imaging of amyloid fibrils is enabled by (b) AFM and (c) cryo-TEM. Secondary and super-secondary structures are addressed by (d) FTIR and (e) SRCD. (f) *Left*: Experimental setting for small angle X-ray scattering (SAXS). The electrons in the fibril sample scatter X-rays, which are registered by a detector as the amyloid signature reflection arches. *Right*: SAXS curves. The cross- β reflections are seen at ~ 0.7 and ~ 1.35 Å⁻¹, corresponding to inter-sheet and inter-strand distances (see *left* and a). (b) Source: Figure courtesy Marisela Vélez; (c) Source: Figure courtesy Sylvain Trepout; (d) Source: Figure courtesy Frederic Geinguenaud; (f) Source: Figure courtesy Thomas Bizien.