Trends in Microbiology

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Review

Two Decades of Studying Functional Amyloids in Microorganisms

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In the past two decades, amyloids, typically associated with human diseases, have been described to play various functional roles in nearly all life forms. The structural and functional diversity of microbial 'functional amyloids' has dramatically increased in recent years, expanding the canonical definition of these assembled molecules. Here, we provide a broad review of the current understanding of microbial functional amyloids and their diverse roles, putting the spotlight on recent discoveries in the field. We discuss their functions as structural scaffolds, surface-tension modulators, adhesion molecules, cell-cycle and gametogenesis regulators, toxins, and mediators of host–pathogen interactions. We outline how noncanonical amyloid morphologies and sophisticated regulatory mechanisms underlie their functional diversity and emphasize their therapeutic and biotechnological implications and applications.

Highlights

This year marks the 20th anniversary of the discovery that amyloids, typically associated with human diseases, can also play beneficial roles essential for normal cellular physiology.

Microbial functional amyloids are extremely diverse and are involved in structural scaffolding and biofilm formation, adhesion, surface-tension modulation, regulation of the cell cycle and gametogenesis, toxicity, host–pathogen interactions, and symbiosis.

Structural and regulatory polymorphism of microbial functional amyloid systems underlies their functional diversity.

A profound understanding of microbial functional amyloid systems could pave the way towards the development of bioinspired materials and anti-infective therapies.

The third decade of functional amyloid research begins with open questions regarding the canonical concept of these assemblies.

Functional Amyloids: A Double-Edged Sword

Most proteins have to fold into a well-defined three-dimensional conformation to function properly. Misfolded proteins can undergo spontaneous **self-assembly** (see Glossary), forming highly stable and insoluble nanostructures known as 'amyloids'. These assemblies demonstrate fibrillar morphology, a characteristic cross-β-sheet structure, and specific dye-binding properties (such as with Thioflavin T (ThT) and Congo red) [1]. Amyloid formation and deposition are the hallmark of Alzheimer's, Parkinson's, and Huntington's diseases, as well as type II diabetes and numerous other conditions [1]. However, in the past two decades it has become increasingly clear that amyloids can also play physiological roles (Figure 1). These 'functional amyloids' are involved in a vast variety of processes, including storage, structural scaffolding, cell signaling, and even memory persistence [2,3].

Although functional amyloids have been identified in nearly all forms of life, they are currently best understood, abundant, and diverse in microorganisms (Figure 1). Structural roles of microbial functional amyloids have been well characterized, including **biofilm** stabilization [4], adhesion [5], and surface-tension modulation [6] (Figure 2). Recent studies have deepened the comprehension of the role that functional amyloids play in these processes and the regulatory mechanisms that underlie them, thus paving the way towards the development of anti-infective and bioinspired materials. Furthermore, it is now clear that functional amyloids contribute to a wider range of biological processes and are more chemically diverse than initially believed. For instance, microbial functional amyloids function as cytolytic toxins [7], antimicrobials [8], and cell-cycle regulators [9,10] (Figure 2). Furthermore, they can obtain noncanonical supramolecular morphologies [7], be composed of relatively short peptide building blocks [8], and assemble in response to environmental fluctuations [11,12]. In this review we outline recent advancements in microbial functional amyloid research, with special emphasis on structurally and functionally unique examples that extend the canonical functional amyloid dogma.

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Functional Amyloids Play Various Structural Roles

Biofilm Formation and Stabilization

Owing to their eminent stability and mechanical robustness, various amyloid-forming proteins have been demonstrated to play pivotal roles in structural scaffolding, especially in biofilm formation (Figure 2). Biofilms increase the availability of essential nutrients and endow the bacterial community with higher resistance to nutritional, mechanical, chemical, and physical stresses, such as antimicrobial agents and the host's immune system [13,14]. Biofilm formation is associated with colonization and host invasion by opportunistic and pathogenic bacteria. It is thus a major concern for human health [15] and a promising target for treating infectious diseases (Box 1). As outlined in Table 1, biofilm-associated functional amyloids have been described in numerous distinct species, yet many of these systems are not completely understood. Here, we outline recent advancements in three of the best-understood ones. These systems hold promise in the engineering of smart bioinspired materials (Box 2).

Curli

The discovery of the amyloid properties of the curli fibrils produced by enterobacteria (*Escherichia coli* and *Salmonella* spp.) [4] has provoked a revolution in amyloid research. Indeed, curli became a model for functional amyloid assembly and has been avidly reviewed throughout the years [16]. The curli biosynthesis machinery has recently been examined at atomic resolution via cryogenic electron microscopy, deciphering the architecture of the secretion complex and its mechanism of substrate recognition [17,18]. The curli system is comprised of seven curli specific genes (*csg*) encoded by two operons (*csgBAC* and *csgDEFG*), with CsgA being the main structural subunit of the amyloid extracellular fibrils [19]. CsgA contains prion-like peptide repeats, homologous to those found in the animal prion protein (PrP) and in the yeast prion Sup35, which are essential for amyloid formation [20]. CsgA is expressed intracellularly as a soluble protein and secreted across the outer membrane via the CsgF–CsgG secretion complex [16,17]. The nonameric secretion complex forms in a 1:1 stoichiometric ratio between CsgF and CsgG, wherein nine CsgF monomers form a funnel-like structure at the extracellularly faced lumen [18]. As recently unraveled, CsgA is recognized by the interaction of a 22-residue domain in its N terminus with CsgG [17]. CsgE reversibly interacts with CsgG and CsgA, thus promoting translocation [17]. In the extracellular matrix, CsgB functions as a nucleator that induces CsgA self-assembly and anchors it to the membrane [19,21]. These novel insights into the molecular organization of the curli apparatus offer intriguing opportunities for the development of novel antibiofilm strategies (Box 1).

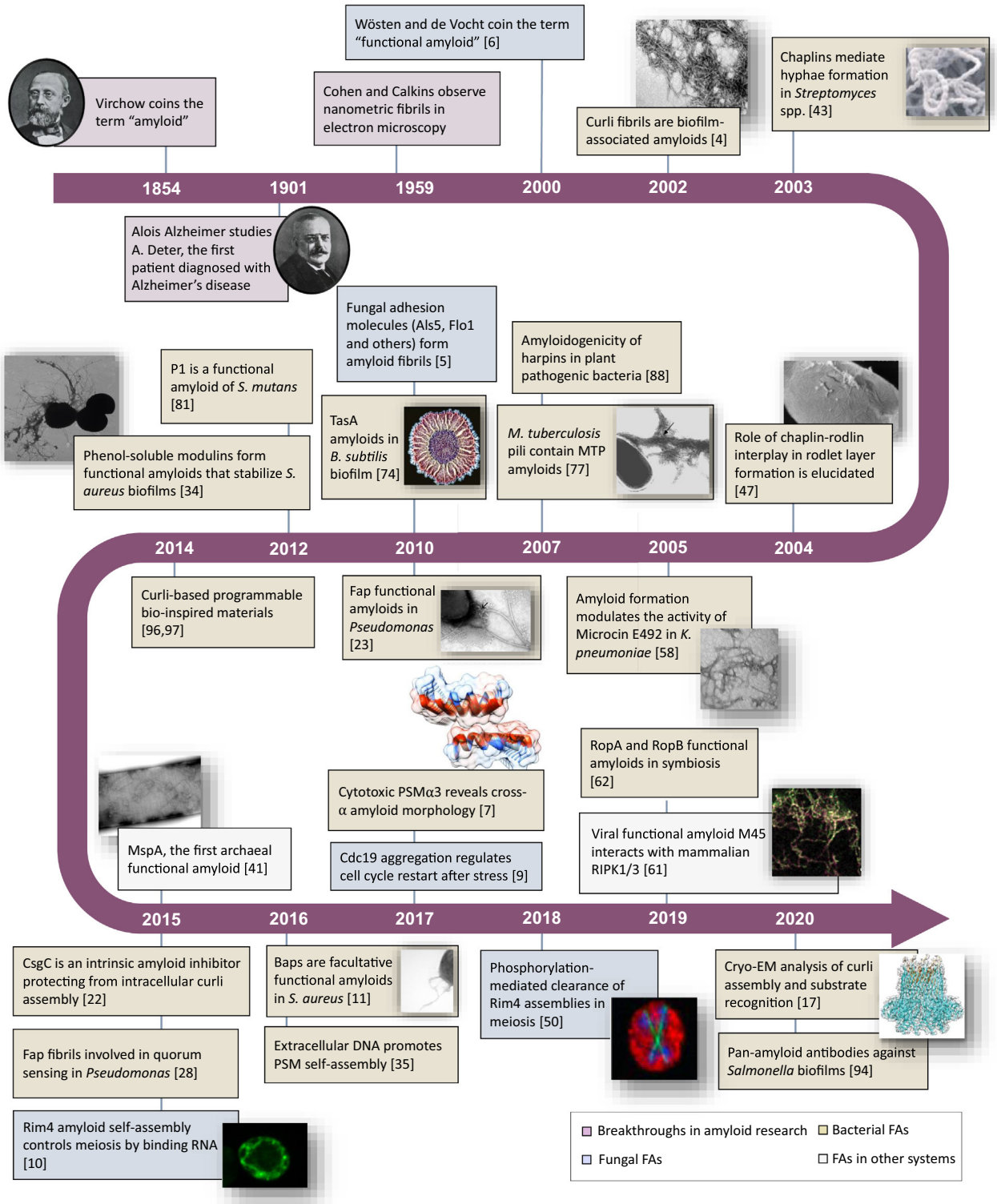
Several sophisticated mechanisms ensure extracellular curli fibril assembly and protect the cells from the potentially deleterious effects of intracellular amyloid formation. First, the self-assembly rate of CsgA is significantly lower in the absence of the CsgB nucleator, which is present only extracellularly [21]. Furthermore, the curli operon encodes the CsgC protein, an 'endogenous' potent amyloid inhibitor that prevents CsgA amyloidogenesis in the periplasm [22].

Fap

The Fap fibrils produced by *Pseudomonas* spp. mediate biofilm formation and increase cell resistance to drying [23,24]. The Fap machinery is encoded by a single operon, *fapABCDEF*. FapC is rich in amyloidogenic domains and functions as the major structural subunit of the amyloid fibrils [25,26]. Like curli, extracellular Fap fibril formation is dependent upon a nucleator protein, FapB [23]. FapF forms a structurally unique secretion system, composed of a trimer of gated β-barrel channels that mediates the secretion of the structural subunits in a mechanism that is dependent upon the FapD protease [27]. Interestingly, the hydrophobic domains of Fap fibrils reversibly bind hydrophobic autoinducers, mediating their trafficking through the biofilm and thus facilitating

Glossary

Aerial hyphae: branching structures wherein several cells are surrounded by a tubular wall that grows above the surface of the medium. In many cases it bears spores, allowing their efficient spreading.
Biofilm: a community of microorganisms that adhere to each other and/or to the surface and are embedded in self-secreted extracellular polymeric substance. The biofilm matrix is composed of proteins, polysaccharides, and extracellular DNA. Amyloids are a major contributor to the great stability and resistance of the biofilm.
Flocculation: a process in which yeast cells adhere to each other, forming multicellular aggregates that sediment in the culture.
Low-complexity region (LCR): a region within proteins that is significantly enriched in one or more amino acids.
Meiosis: a two-stage process in which haploid gametes are produced from diploid progenitors. In the first stage (meiosis I), the homologous chromosomes segregate, followed by separation of the sister chromatids (meiosis II).
Necroptosis: a caspase-independent programmed inflammatory cell death. It can occur in response to viral infection, thus restricting viral spread in the host tissue.
Pan-amyloid epitopes: molecular determinants that are common to several amyloid fibrils, regardless of the monomer that comprises the amyloid.
Polyphenols: mainly natural compounds that contain multiple units of phenols (a phenyl group bonded with a hydroxyl group). They are common in red wine, coffee, and green tea. Examples include tannic acid and epigallocatechin gallate (EGCG).
Quorum sensing: a mechanism for bacterial cell-to-cell communication, allowing the cells to monitor population density and change their physiology accordingly. This mechanism is based on the sensing of small extracellular molecules known as autoinducers that the cells secret during their growth.
Self-assembly: a process in which molecules spontaneously organize into a well-defined three-dimensional supramolecular structure with unique functional properties. It is directed by noncovalent interactions, such as π–π stacking, hydrogen bonds, electrostatic interactions, van der Waals forces, etc.



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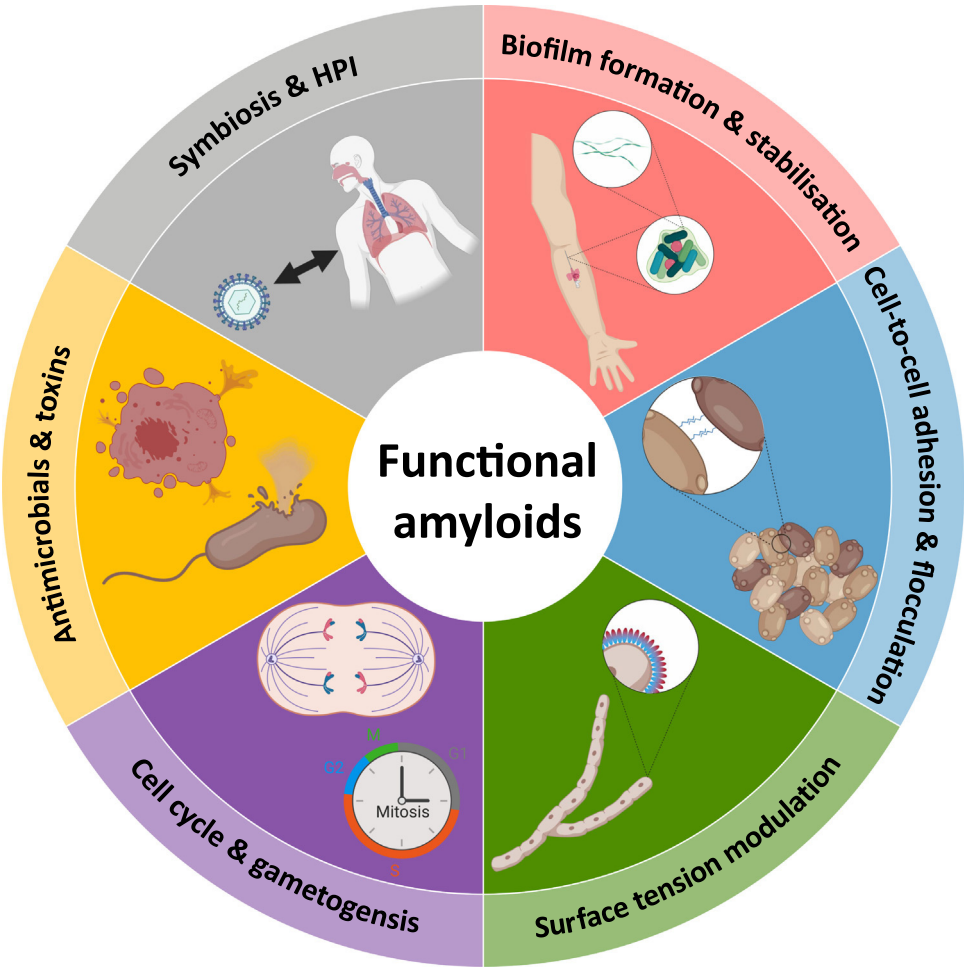

Figure 2. Functional Amyloids Play Diverse Physiological Functions in Microorganisms. The scheme enumerates the six main classes discussed in this review: biofilm formation and stabilization and cell-to-cell adhesion and flocculation (structural scaffolding), surface tension modulation, regulation of cell cycle and gametogenesis, toxicity and antimicrobial activity, and mediation of host–pathogen interactions (HPI) and symbiosis. Created with BioRender.com.

quorum sensing [28]. Furthermore, Fap fibrils have recently been shown to play a role in the retention of diffusible metabolites such as the pyoverdine iron-scavenging molecules. This mechanism limits the loss of essential metabolites to 'cheat' cells that do not pay the metabolic cost of producing them [29].

Notably, although the curli and Fap building blocks share roughly 10% genetic similarity, they manifest a remarkably similar aggregation pattern and functionality of the coding operon. This might imply an independent evolution of the two systems, driven by the significant increase in

Figure 1. Milestones in Microbial Functional Amyloid Research in the Past Two Decades. The field of functional amyloids is a new discipline that has been established more than a century after the initial discovery of amyloids. The milestones are presented in the context of the breakthroughs in amyloid research (purple) and are color-coded according to organisms: fungi (light blue); bacteria (beige); archaea and viruses (white). FAs, functional amyloids; MTP, *M. tuberculosis* pili; PSM, phenol-soluble modulin; *B. subtilis*, *Bacillus subtilis*; *K. pneumoniae*, *Klebsiella pneumoniae*; *M. tuberculosis*, *Mycobacterium tuberculosis*; *S. aureus*, *Staphylococcus aureus*; *S. mutans*, *Streptococcus mutans*. Images reproduced, with permission, from [4,7,10,11,17,23,34,41,43,47,50,58,61,74,77]. Other citations in Figure 1: [5,6,9,22,28,35,62,81,88,94,96,97].

Box 1. Targeting Biofilm Amyloids to Combat Infections

Biofilm formation is considered to be a major risk to human health as it facilitates bacterial colonization, resulting in life-threatening infections [15], and even contributes to the development of autoimmune diseases [89]. Thus, biofilm inhibition emerges as a promising therapeutic alternative on the cusp of the postantibiotic era. Due to the conserved structural and biochemical traits of disease-causing amyloids and microbial amyloids, antiamyloid approaches originally designed to combat neurodegeneration can be repurposed to eradicate bacterial infections and vice versa. These include natural **polyphenols** [90,91], the Aβ-inhibiting protein transthyretin [92], and D-enantiomeric peptides, originally designed to combat Alzheimer's disease [93]. Alongside targeting amyloid formation, the recently elucidated structure of the curli secretion system allows the design of specific peptide inhibitors that inhibit CsgA secretion [17,18].

Alongside small molecules and peptides, Tursi *et al.* have recently reported the use of human monoclonal antibodies that target **pan-amyloid epitopes** on curli fibrils as a promising approach for eliminating bacterial biofilms or preventing their formation [94]. Antibody treatment has effectively inhibited *Salmonella* Typhimurium biofilm formation, destabilized preformed biofilms, and increased bacterial phagocytosis by macrophages. Notably, the combination of antibody treatment with antibiotics has resulted in a synergistic effect that effectively eradicated biofilms formed on intravenous catheters *in vivo* [94].

fitness that the biofilm-forming machinery confers [30]. Furthermore, an *in vitro* analysis of the aggregation kinetics of CsgA and FapC has revealed a similar aggregation pattern for both proteins [30]. Compared to the assembly kinetics of disease-associated amyloids, the aggregation mechanism of CsgA and FapC comprises only of primary nucleation and elongation, with no detectable self-replication process, and thus the rate constant for the assembly is much lower. The non-self-replicative nature of CsgA and FapC self-assembly, as compared to pathological amyloids, is postulated to function in controlling the aggregation and restricting it both spatially and temporally [30].

Phenol-Soluble Modulins (PSMs)

PSMs are secreted by *Staphylococcus* species (e.g., *S. aureus*, *S. epidermidis*), which are major contributors to infections of indwelling medical devices and other opportunistic infections [15,31]. PSMs are relatively short peptides, ~20–40 residues in length, known to undergo truncations in nature that contribute to their diversity [32]. Members of this highly diverse family have been implicated in surface attachment and colonization, biofilm formation, antimicrobial activity against competing species, cytolytic activity against human cells, and proinflammatory activity [33]. The first evidence for amyloid-like properties of PSMs was provided by Schwartz *et al.*, demonstrating that several PSMs formed fibrillar structures that stabilize biofilms [34]. Interestingly, the presence of extracellular DNA in the biofilm matrix promotes the self-assembly of the PSM monomers into the amyloid state [35]. The structure of amyloid fibrils formed by the biofilm-associated PSMα1 and PSMα4 has been recently resolved at the atomic level, confirming that the assemblies are β-sheet-rich [8]. Notably, synthetic peptides that were contained within the sequence of the naturally occurring truncated version of the proteins, and corresponded to the computationally predicted amyloidogenic cores of PSMα1 and PSMα4, were sufficient to form canonical cross-β amyloid fibrils [8]. While PSMα1 and PSMα4 facilitate biofilm formation, PSMα3 and its truncated derivatives can form structurally diverse assemblies that have antimicrobial or cytolytic activities [7,8] (see the later section 'Functional Amyloids as Antimicrobials and Toxins').

Environment-Triggered Structural Scaffolding

The monomeric building blocks of functional amyloids discussed so far are expressed as inherently amyloidogenic moieties that self-assemble to form amyloids. Unlike these 'primary' functional amyloids, monomers that comprise 'facultative' functional amyloids adopt the amyloid-forming conformation and self-assemble in response to certain environmental triggers. Their assembly mechanism is not only environment-dependent but also requires processing that exposes or releases the amyloid-forming domain [36]. The facultative functional amyloids

Table 1. Functional Amyloids in Microorganisms (2000–2020)

Functional amyloid	Organism	Amyloid function	Localization	Comments	Refs
Structural scaffolding: biofilm formation					
Curli	*Escherichia coli, Salmonella* spp.	Biofilm stabilization; cell-to-surface adhesion and host invasion	Secreted to the extracellular matrix	First bacterial functional amyloid identified; scaffold for developing bioinspired materials	[4]
Fap (functional amyloids in *Pseudomonas*)	*Pseudomonas* spp.	Biofilm stabilization, cell-to-surface adhesion and host invasion; increases biofilm hydrophobicity; involved in retention of diffusible metabolites and quorum sensing	Secreted to the extracellular matrix		[23,24]
PSM (phenol-soluble modulins)	*Staphylococcus aureus*	PSMα1 and PSMα4 mediate biofilm formation	Secreted	Amyloid formation depends on presence of extracellular DNA	[8,34]
TasA	*Bacillus subtilis*, *Bacillus cereus*	Biofilm stabilization; spore formation; antimicrobial activity	Secreted	Nucleates via a disordered protein (TapA in *B. subtilis* and CalY in *B. cereus*)	[74–76]
MTP (*M. tuberculosis* pili)	*Mycobacterium tuberculosis*	Adhesion, biofilm formation, and host invasion; binds laminin	Secreted	Not required for pathogen survival	[77,78]
Baps (biofilm associated proteins)	*Staphylococcus aureus*	Biofilm stabilization, cell-to-cell and cell-to-surface adhesion, host invasion; monitoring calcium and pH levels	Cell surface	Facultative functional amyloid	[11]
Aap (accumulation-associated protein)	*Staphylococcus epidermidis*	Biofilm formation (Zn^{2+}-dependent)	Cell surface	Facultative functional amyloid	[37]
Esp (enterococcal surface protein)	*Enterococcus faecalis*	Biofilm formation (pH-dependent assembly); metal-dependent nucleation unknown	Cell surface	Facultative functional amyloid	[12]
P1, WapA and Smu_63c	*Streptococcus mutans*	Biofilm formation, host invasion	Secreted	Bacterial colonization causes dental cavities	[79–81]
SuhB	*Staphylococcus aureus*	Might be involved in biofilm scaffolding	Secreted		[82]
Sbp (small basic protein)	*Staphylococcus epidermidis*	Biofilm scaffolding	Secreted		[83]
Bioemulsifier AM1	*Solibacillus silvestris*	Biofilm formation and cell-surface modulation	Secreted		[84]
EF-Tu (elongation factor Tu)	*Gallibacterium anatis*	Cell adhesion and biofilm formation	Secreted		[85]
Structural scaffolding: cell-to-cell adhesion					
Flocculins Flo11p and Flo1p	*Saccharomyces cerevisiae*	Cell-to-cell attachment (flocculation); formation of fungal mats	Cell surface	Aggregation increases in response to mechanical shear	[39,40]
Als5 (agglutinin-like sequence)	*Candida albicans*	Cell-to-cell and cell-to-surface adhesion	Cell surface	Aggregation increases in response to mechanical shear	[40]
Structural scaffolding: other					
MspA (major sheath protein)	*Methanosaeta thermophila, Methanospirillum hungatei* JF-1 (Archaeal methanogens)	Cell-wall scaffolding (tubular sheath), possibly involved in gas vesicle formation	Secreted (extracellular cell wall sheaths)	First archaeal functional amyloid to be identified	[41,42]
GvpA (gas vesicle protein)	*Anabaena flos-aquae*	Scaffolding of the walls of gas vesicles (essential for flotation)	Intracellular gas vesicles		[86]
Surface tension modulation					
Chaplins (*coelicolor* hydrophobic aerial proteins)	*Streptomyces coelicolor*	Modulation of surface tension; aerial hyphae formation (together with rodlins)	Cell surface		[44,45]

Table 1. (continued)

Functional amyloid	Organism	Amyloid function	Localization	Comments	Refs
Rodlins	*Streptomyces coelicolor*	Modulation of surface tension; aerial hyphae formation (together with chaplins)	Cell surface		[46]
Hydrophobins	Filamentous fungi (e.g., *Neurospora crassa*)	Modulates cell-surface tension; aerial hyphae formation; surface adhesion	Cell surface	First functional amyloid identified	[6,48]
Repellent (Rep1-1)	*Ustilago maydis*	Modulates cell-surface tension; aerial hyphae formation; surface adhesion	Cell surface		[87]
Metabolic and cell-cycle regulation					
Cdc19	*Saccharomyces cerevisiae*	Cell-cycle regulation (amyloid form is inactive)	Cytoplasmic granules		[9]
Rim4	*Saccharomyces cerevisiae*	Amyloid form is a translation repressor, regulates meiosis	Cytoplasmic granules		[10,50]
Toxins and antimicrobials					
PSMα3	*Staphylococcus aureus*	Full-length protein is cytolytic; derived peptides have antimicrobial activity	Secreted	Full-length protein adopts cross-α morphology	[7,8,54]
Harpins	*Xanthomonas axonopodis* (plant pathogenic bacteria)	Membrane pore-forming activity, induce hypersensitivity response	Secreted		[88]
Microcin E492	*Klebsiella pneumoniae*	Loss-of-function in the amyloid form; soluble form is a membrane pore-forming peptide (microbial competition)	Secreted		[57,58]
Host–pathogen interactions and symbiosis					
M45	*Cytomegalovirus*	Coassembles with RIPK1/RIPK3 and inhibits necroptosis	Encoded by the viral genome	Sequence related to mammalian RIP1/RIP3	[61]
RopA and RopB	*Rhizobium leguminosarum*	Likely involved in bacteria–plant symbiosis in legume root nodules	Outer cell membrane		[62]

discovered so far are biofilm-associated; they thus have a dual function as both structural scaffolds and sensors for monitoring environmental fluctuations.

The first member of this group to have been discovered is the biofilm-associated protein (Bap) produced by *S. aureus*. Bap is displayed as a full-length extracellular membrane protein and undergoes processing that releases its N-terminal amyloidogenic fragment. The N-terminal fragments undergo conformational rearrangements, giving rise to a transient, unstable, 'molten globule-like' state which can self-assemble into amyloid fibrils. However, amyloid formation occurs only at acidic pH and at a low calcium concentration [11]. When calcium levels increase, the divalent cations bind and stabilize the disordered monomers, hence halting amyloid formation. Interestingly, the optimal pH range for Bap aggregation is close to that present in human host niches (such as skin, nasal cavity, vagina, etc.), and thus this system appears to have adapted for optimal function in its host [11].

The amyloid-forming properties of the Bap ortholog Esp, produced by *Enterococcus faecalis*, have recently been reported. Amyloid formation by its amyloidogenic N terminus is dependent upon cleavage and acidification, just as with Bap. However, Esp lacks the calcium-binding domain, leaving possible metal-dependent aggregation an unresolved matter [12].

Box 2. Biotechnological Applications of Functional Amyloids

As we have extensively discussed, microorganisms exploit the unique properties of amyloids for various functional purposes. In recent years these evolutionarily optimized nanoassemblies have served as a basis for the bioinspired engineering of smart materials [95]. For example, by fusing different peptide domains to the CsgA protein, Nguyen *et al.* engineered programmable biofilm-based materials that retain the function of the fused peptide and confer it on the entire biofilm [96]. Upon expression, the CsgA–peptide fusion constructs are secreted and self-assembled in the extracellular matrix, generating biofilms with diverse properties, such as surface-binding and protein display [96]. A similar methodology was applied to combine the mechanical robustness and rapid assembly time of the curli fibrils with the remarkable underwater adhesion properties of mussel-foot proteins [97]. By genetically conjugating the two proteins, Zhong *et al.* demonstrated the bioinspired fabrication of a strong underwater adhesive. Furthermore, the nucleation-seeding mechanism of the curli fibril formation has recently been coupled to DNA origami technologies, allowing the generation of a spatially oriented nanoscale fibrillar network. Specifically, CsgB was coupled to a DNA origami scaffold, whose orientation directed the fibrilization of CsgA, eventually controlling the spatial properties of the resulting fibrillar network [98].

Other technologies take advantage of the ability of hydrophobins to self-assemble at hydrophobic–hydrophilic interfaces [99]. The hydrophobin Vmh2 from *Pleutorus ostreatus*, for instance, has been used to coat glass surfaces, allowing them to efficiently adsorb various proteins (e.g., IgG antibodies) [100] and nanomaterials (quantum dots, graphene oxide) [101], thus rendering the biofunctionality of the surface. Additionally, the amphiphilic nature of hydrophobins offers intriguing opportunities for drug delivery via self-assembled bioinspired nanoparticles [102]. These hydrophobin-based nanoparticles encapsulate drugs and can increase their bioavailability. Thus, the unique properties of these naturally occurring proteins could lead to the development of biodegradable, environment-friendly, and cost-effective composite biomaterials for a vast variety of applications.

Yarawsky *et al.* have recently described the amyloidogenic properties of the Aap protein from *S. epidermidis*. Similar to Bap, Aap is proteolytically cleaved (either by bacterial or human protease) in its N terminal, exposing the amyloidogenic domain and initiating bacterial clumping [37]. Unlike curli and Fap mechanisms, that depend on the presence of an accessory nucleator protein, Aap aggregation is heavily dependent on the presence of zinc-ion nucleators [37]. This metal-ion-dependent nucleation is especially interesting in light of the well established connection between metal ions and self-assembly of pathological amyloids [38].

Cell-to-Cell Adhesion and Flocculation

Saccharomyces cerevisiae expresses adhesins, known as flocculins, that mediate **flocculation** and the formation of biofilm-like mats (Figure 2). Flo1 and Flo11, key proteins in the process, have been demonstrated to form amyloid fibrils [5]. The amyloid domains of flocculin monomers at the cell surface are believed to interact, mediating the formation of multimeric adhesive flocculin bundles. Flocculin bundling results in an increase in the local adhesins' concentration, and thus elevated avidity to flocculins presented on adjacent cells. Thus, the amyloid-mediated flocculin clustering increases the intercellular binding strength and mediates flocculation [5]. Similarly, the opportunistic pathogen *Candida albicans* produces the amyloidogenic protein Als5 that mediates adhesion and biofilm formation, although it does not share sequence similarity with the flocculins [39,40]. Interestingly, flocculin and Als5-mediated adhesion are increased upon shear force, which could be an indicator of stress in nature. The transformation into a more robust multicellular state might be a mechanism of adaptation to harsh environments [39].

Support and Shielding by Crystalline Sheaths

Members of the Archaea are among the most adaptive and widespread forms of life and are known for their extreme resistance to various chemical and physical conditions. As in several recently discovered cases, this remarkable stability and adaptivity is conferred by functional amyloids. For instance, the methanogen *Methanosaeta thermophila* PT frequently grows and divides within tubular crystalline sheaths. These structures encase the cells, provide mechanical support and shielding, and make them resistant to various toxins and chemical stressors, while simultaneously allowing the diffusion of essential small molecules [41]. The encasing sheath is composed of MspA as a single major sheath protein which acquires an amyloid

supramolecular arrangement [41]. A distant homolog of MspA has been recently identified as a functional amyloid component in the sheath of another archaea species, *Methanospirillum hungatei* JF-1 [42].

Surface Tension Modulation

Many filamentous fungi and bacteria can form branching structures known as **aerial hyphae** that grow above the humid surface (Figure 2). In order to prevent back-growth into the hydrophilic soil, microorganisms render the surface of aerial hyphae hydrophobic, thus lowering surface tension at the water–air interface [43]. In *Streptomyces* filamentous bacteria, the outer layer of the aerial hyphae, termed the rodlet layer, is composed of eight chaplins (ChpA-H) and two rodlins (RdlA and RdlB). Many members of these groups have been demonstrated to form amyloid fibrils that coat the surface of the aerial hyphae. The hydrophobic nature of the amyloid fibrils lowers the water's surface tension in the surrounding aqueous environment, allowing the penetration of the aerial hyphae at the water–air (hydrophilic–hydrophobic) interface [44–46]. The chaplin–rodlin system is a unique functional amyloid system as it involves several amyloidogenic proteins from two distinct groups, unlike other functional amyloid systems where only one component is amyloidogenic [47].

The fungal analogs of the bacterial chaplin–rodlin system are hydrophobins, a wide class of amphipathic amyloid-forming proteins expressed by filamentous fungi (e.g., DewA of *Aspergillus nidulans*, Eas of *Neurospora crassa*, and SC3 of *Schizophyllum commune*). They function in a similar manner to their bacterial counterparts to allow aerial hyphae formation. Alongside modulating the surface tension at water–air interfaces, the hydrophobic nature of the chaplins, rodlins, and hydrophobins mediates microbial attachment to surfaces, thus facilitating colonization [6,44,48]. These surface tension-modulating functional amyloids can thus also be viewed as functional amyloids with a structural role. Notably, hydrophobins were the first proteins to be identified as functional amyloids two decades ago (Figure 1); their discovery marked a milestone in amyloid research [6], and their remarkable properties make them promising candidates for the development of bioinspired materials (Box 2).

Cell-Cycle Regulation and Gametogenesis

Cell-Cycle Restart after Stress

The pyruvate kinase Cdc19 is a central regulator of cellular metabolism and cell growth and is essential for progression through G1. Saad *et al.* have recently discovered that, upon glucose starvation or heat shock in *S. cerevisiae*, Cdc19 can form amyloid-like aggregates that are recruited to stress granules (Figure 2). The transition from a monomeric to an amyloid state and the consequent compartmentalization protects the protein from stress-induced degradation and is accompanied by its temporary inactivation [9]. When stress conditions are reversed, the Cdc19 foci are rapidly resolubilized, allowing fast and efficient reinitiation of cell growth without the need for *de novo* protein synthesis [9].

The aggregation of Cdc19 is mediated and regulated by a **low-complexity region** (**LCR**) within the protein. During logarithmic growth, Cdc19 aggregation is inhibited by phosphorylation in the LCR region or by the formation of tetrameric protein complexes that bury the aggregative LCR. Upon metabolic stress, Cdc19 tetramers dissociate and the LCR is dephosphorylated, allowing aggregation and amyloid formation [9]. Interestingly, the mammalian pyruvate kinase PKM2 was also found to contain a short LCR and several predicted amyloidogenic domains, suggesting the possibility that reversible protein aggregation is an evolutionarily conserved mechanism for storing proteins for instantaneous utilization post-stress [49].

Regulation of Gametogenesis in Meiosis

Gametogenesis by **meiosis** is essential for sexual reproduction and is thus responsible for the genetic variation in nature. One of the meiotic regulators in *S. cerevisiae* is the B-type cyclin Clb3, which is translationally repressed throughout meiosis I and reactivated at the onset of meiosis II. Inadequate *CLB3* repression during meiosis I fails chromosomal segregation [10]. As recently deciphered, the translational repression of *CLB3* is facilitated by the amyloid aggregates of the RNA-binding protein Rim4 [10] (Figure 2). Specifically, the amyloid form of Rim4 binds the mRNA encoding *CLB3*, repressing its translation. At the onset of meiosis II, the Ime2 protein kinase phosphorylates Rim4 at its intrinsically disordered region, reducing its amyloidogenicity and making it accessible to molecular chaperones. This leads to the disassembly of Rim4 amyloids into smaller oligomers that undergo proteasomal degradation, permitting the reactivation of *CLB3* and allowing meiosis to proceed [50]. Although Rim4 and Cdc19 regulate two unrelated processes, phosphorylation of the monomeric protein is a common mechanism to repress amyloid formation. Interestingly, the functional homolog of Rim4 in humans and mice, DAZL, forms sodium dodecylsulfate (SDS)-resistant aggregates in the testes, supporting the notion that amyloid aggregation might be an evolutionarily conserved mechanism for regulating gametogenesis [10].

Functional Amyloids as Antimicrobials and Toxins

Human disease-associated amyloids are known to exert their cytotoxic effect by forming membrane pores [51,52]. Several functional amyloid toxins 'exploit' this inherent toxicity mechanism of the amyloid fold (Figure 2).

As outlined previously (section 'Biofilm Formation and Stabilization'), PSMα1 and PSMα4 can form amyloids that play essential roles in biofilm scaffolding [34]. The third member of the group, PSMα3, functions as either a cytolytic toxin or an antimicrobial agent, depending on the supramolecular organization of the amyloid that is determined by the length of its peptide building blocks. Specifically, PSMα3-derived hexapeptides and heptapeptides form amyloids that display antibacterial activity against *Micrococcus luteus* and *Staphylococcus hominis* but are nontoxic to *S. aureus* itself [8]. Contrastingly, fibrils formed by PSMα1 and PSMα4 do not exhibit such activity. This exclusive antimicrobial activity could be attributed to the unique structure of the amyloid fibrils formed by these peptides, where the β-strands are arranged in a hexameric configuration [8].

While PSMα3-derived peptides are antimicrobial, full-length PSMα3 is cytolytic. Fibrils formed by the full-length protein bind ThT and display internal fluorescence and nucleation-seeding kinetics [7]. However, an analysis of the crystal structure has concluded that PSMα3 obtains a noncanonical cross-α-helical supramolecular conformation, where the monomers are composed of α-helices rather than β-strands. These unique fibrils displayed robust toxicity to human cell lines. It was also reported that mutated proteins that failed to form fibrils were less toxic [7]. The underlying mechanism of toxicity appears to include dynamic coaggregation of monomers and fibrils with the cell membrane that results in its rupturing, rather than an interaction with a specific protein [53,54]. However, the role of fibril formation in PSMα3 toxicity remains debatable [53,55,56].

While the pore-forming activity of PSMα3 and PSMα3-derived peptides correlates with fibril formation, this is not the case for Microcin E492. This protein, secreted by *Klebsiella pneumoniae*, forms pores in the cell membranes of closely related bacterial species, thus promoting survival in competitive environments [57]. Interestingly, the active membrane-poring form of the protein is produced only during the exponential growth phase of the bacteria and halts at the stationary phase, a loss-of-function that is attributed to the self-assembly of the protein and formation of amyloid fibrils [58].

Host–Pathogen Interaction and Symbiosis

As we have discussed, functional amyloids serve as structural scaffolds for biofilms formed by opportunistic and pathogenic bacteria and fungi, and are thus involved in host invasion and colonization. In some cases, as outlined herein, functional amyloid-mediated host–pathogen interaction is independent of biofilms (Figure 2). In both cases, in the context of host–pathogen interaction, functional amyloids are only 'functional' for the pathogen, not for the host.

Supramolecular Coevolution of Host and Virus Functional Amyloids

'Cellular suicide' by **necroptosis** is one of the first lines of defense against viral infections in mammals as it prevents viral spread in the tissues [59]. Necroptosis is mediated by the association of the receptor-interacting protein kinases 1 and 3 (RIPK1 and RIPK3) via a ~20 amino acid RIP homotypic interaction motif (RHIM) [59]. Li *et al.* have previously discovered that the RIPK1–RIPK3 complex is a heterogeneous functional amyloid complex whose formation is essential for downstream signaling in the necroptosis cascade [60]. Remarkably, it appears that viruses have coevolved functional amyloids that act to inhibit the RIPK1–RIPK3 association at the supramolecular level and thus counteract the host's defense mechanism. Specifically, the M45 protein of the murine cytomegalovirus contains a similar RHIM motif and can form amyloid-like structures and coaggregate with the RIPK1 and RIPK3 proteins of the host. This heterodimeric assembly of host and viral functional amyloids interferes with necroptosis activation, allowing the virus to overcome the host's defense mechanism [61].

Root Nodule Symbiosis

We have outlined many examples of the role that functional amyloids play in pathogenesis, yet only recently have functional amyloids been described in the context of a beneficial symbiosis. The bacterium *Rhizobium leguminosarum* colonizes the root nodules of legumes where it fixes atmospheric nitrogen, converting it to ammonia. This symbiosis is one of the best-characterized plant–bacteria relationships in nature and is of great agricultural and economic significance. Kosolapova *et al.* have recently described the formation of amyloid-like fibrils by RopA and RopB expressed at the outer membrane of *R. leguminosarum* [62]. The expression of both genes is upregulated at the early stages of nodulation and is consequently downregulated upon transition to the nitrogen-fixing form. The changes in the expression levels of *ropA* and *ropB* genes imply the importance of these functional amyloids for the establishment of the symbiotic relationships, yet the exact role of the soluble proteins and the amyloid structures they form is yet to be elucidated [62].

'Functional Metabolite Amyloids': Do They Exist?

The structural and functional diversity and complexity of identified functional amyloids have been dramatically expanded in recent years, especially in microbial systems. It is now clear that not only large proteins and protein complexes, but also short peptides (e.g., truncated PSMs), can self-assemble into biologically active functional amyloids. These considerable advances in the field raise elementary questions regarding the minimal building blocks required for the formation of a functional amyloid system.

A reductionist approach for amyloid formation has allowed re-examination of the canonical amyloid hypothesis, with the discovery that simple metabolites, such as monomeric amino acids, nucleobases, and various other metabolites can form amyloid-like structures. These assemblies demonstrate quintessential amyloid properties, including fibrillar morphology, ThT and Congo Red binding, significant cytotoxicity, and inhibition by generic amyloid polyphenolic inhibitors [63–68]. Furthermore, metabolite amyloid-like structures interact with model membranes similarly to classical amyloids [69], seed the formation of proteinaceous amyloids [66], and function as

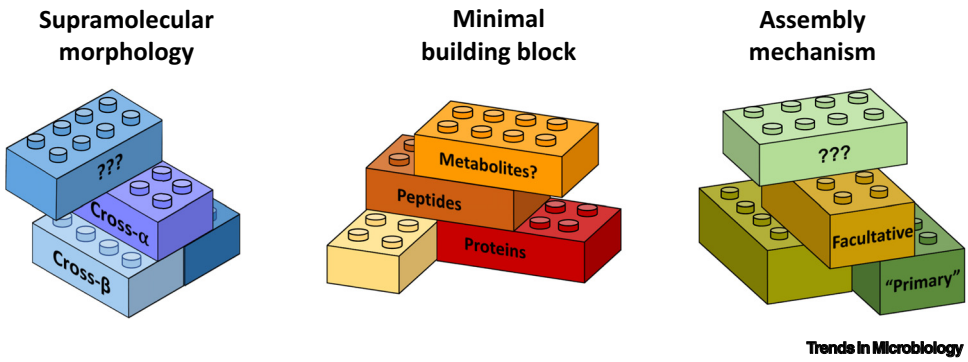

Figure 3. Extension of the Canonical Functional Amyloid Dogma. Recent studies have demonstrated that microbial functional amyloids are more structurally and functionally diverse, with respect to their supramolecular morphology (cross-α, rather than only cross-β [see section Functional Amyloids as Antimicrobials and Toxins]), the minimal building block that self-assembles to form the fibril (short peptides, and not only large proteins [see sections Biofilm Formation and Stabilization, and 'Functional Metabolite Amyloids': Do They Exist?]), and their assembly mechanism as either primary (inherently amyloidogenic building blocks) or facultative (environment-triggered amyloidogenicity [see section Environment-triggered Structural Scaffolding]). Future research might further extend these concepts, such as the discovery of functional metabolite amyloids *in vivo*.

distinct immunogenic entities [67,70]. The biological significance of metabolite amyloid-like structures has been recently validated *in vivo* at the whole-organism level, demonstrating the accumulation and self-assembly of adenine amyloid-like fibrils in a genetically engineered yeast model [71].

Can metabolite amyloid-like structures have functional roles in organisms? Makam *et al*. have recently demonstrated that phenylalanine can coordinate with zinc ions, forming robust metallosupramolecular amyloid-like structures that exhibited remarkable carbonic anhydrase-like catalytic activity *in vitro* [72]. With this being the first example of a biologically relevant role that metabolite amyloid-like structures play *in vitro*, functional metabolite assemblies (nonamyloid) have been extensively investigated and characterized in photonic crystal systems found in animals. For instance, guanine assemblies are involved in the color change of the panther chameleon, and uric acid composes the reflecting layers in the scarab beetle's cuticle [73]. Nevertheless, whether functional metabolite assemblies exist in microorganisms, and whether functional metabolite amyloids exist *in vivo* remain open questions (Figure 3). We hypothesize that these putative assemblies could act together with 'classical' functional amyloids in a wide range of biological processes such as structural scaffolding, metabolic regulation, and toxicity. Functional metabolite assemblies composed of ubiquitous minimal building blocks, like amino acids and nucleobases, could be highly conserved among all domains of life, from microorganisms to humans.

Concluding Remarks and Future Perspectives

The year 2020 marks the 20th anniversary of the discovery that amyloids can also play functional roles (Figure 1). Although functional amyloids have been described in nearly all forms of life, microbial functional amyloids remain the best-understood ones. In recent years, the canonical dogma for the functional and structural characteristics of these structures has been extended, demonstrating that they are much more diverse than previously believed. In this review, we have discussed recent advancements in microbial functional amyloid research and described newly discovered examples. We have outlined discoveries that deepen the understanding of 'classical' functional amyloid biology and provided proofs that extend the canonical dogma for the functional and structural characteristics of functional amyloids. These include environment-triggered

Outstanding Questions

What other physiological roles do functional amyloids play, aside from those discovered so far?

Why are functional amyloids not toxic to the cells that express them?

Are there additional cases of coevolution of host and pathogen functional amyloids?

How do all the components of functional amyloid operons (e.g., *cgs* and *fap*) interact and function harmonically?

Have microbial functional amyloids from genetically distinct species evolved independently?

What other noncanonical morphologies, in addition to those acquired by PSMs, can functional amyloids obtain? What is their structure–function relationship?

Do additional facultative functional amyloids exist, and what other environmental factors affect their self-assembly?

What is the minimal peptide building block that makes up functional amyloids?

Do functional metabolite amyloids exist? What is their physiological role? Do they crosstalk with proteinaceous functional amyloids?

Can microbial functional amyloids produced by pathogenic bacteria accelerate the onset of amyloid-associated neurodegenerations?

What are the future uses of microbial functional amyloids in the design and fabrication of bioinspired materials and technologies?

In what novel approaches can we target functional amyloids produced by pathogenic and opportunistic bacteria to treat infections?

amyloid self-assembly, roles in cell cycle regulation and gametogenesis, and paradigm-changing discoveries on noncanonical amyloid morphologies (Figures 1 and 3). In light of these advancements, we have discussed the current understanding of the diverse roles that functional amyloids play in microorganisms (Figure 2, Table 1). We have not touched upon microbial prions, which are amyloids that function as information carriers, as this rapidly developing field deserves a separate review.

The next decade of functional amyloid research begins with the realization that they are much more structurally and functionally diverse than previously believed, bringing about many open questions that are yet to be elucidated (see Outstanding Questions and Figure 3). Gaining a better understanding of these widespread systems could pave the way towards the development of bioinspired materials and more effective therapies.

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