



Fungal Biofilms: Inside Out

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ABSTRACT We focus this article on turning a biofilm inside out. The “inside” of the biofilm comprises the individual biofilm-related phenotypes, their environmental drivers and genetic determinants, and the coordination of gene functions through transcriptional regulators. Investigators have viewed the inside of the biofilm through diverse approaches, and this article will attempt to capture the essence of many. The ultimate goal is to connect the inside to the “outside,” which we view as biofilm structure, development, pharmacological attributes, and medical impact.

INTRODUCTION

We focus this article on turning a biofilm inside out. The “inside” of the biofilm comprises the individual biofilm-related phenotypes, their environmental drivers and genetic determinants, and the coordination of gene functions through transcriptional regulators. Investigators have viewed the inside of the biofilm through diverse approaches, and this article will attempt to capture the essence of many. The ultimate goal is to connect the inside to the “outside,” which we view as biofilm structure, development, pharmacological attributes, and medical impact.

Biofilms are surface-associated microbial communities, encased in self-produced extracellular material, that exhibit phenotypes distinct from those of planktonic (free-living) cells. Microbes are thought to grow predominantly as biofilms in nature (1). The surfaces with which biofilm cells are associated may be diverse and include solid abiotic materials, tissues and cells, and air-water interfaces. In fact, a colony growing on an agar plate is a biofilm.

Biofilm cells are quite different from the mid-logarithmic-phase planktonic cells that modern microbiologists were trained to study (see reference 2). Biofilm populations are invariably heterogeneous (Fig. 1A). Cells at the periphery of the biofilm are bathed in the external medium; cells at the base of the biofilm may

have limited nutrients and oxygen and are surrounded by their neighbors’ waste products. Cells at the periphery may be exposed to a rapidly fluctuating environment; cells at the base are buffered from many abrupt changes. Thus, a mature biofilm may include cells exposed to a range of nutrients, and the biofilm cell population may be growing at a range of rates.

Our focus on biofilms stems from their central role in infection biology (3). Biofilms are medically relevant in two major contexts: device-associated infection and *in vivo* growth. Device-associated infection is the phenomenon of the presence of an implanted medical device being a significant risk factor for bloodstream or deep-tissue infection. The specific risk factor and the likely types of infecting organisms vary with the kind of device and its location. The connection to biofilm formation was first elucidated by Costerton and colleagues (3), who found biofilms of infecting organisms on the devices that were removed from infected patients. In the vast majority of cases, the devices are sterile when implanted and later become colonized by microbes that enter the bloodstream. The biofilm on a device serves as a reservoir that continually seeds the infection. Unfortunately, as detailed later in this article, biofilm cells are generally recalcitrant to antimicrobials; thus, device removal may be the only therapeutic option. Because the usage of implanted devices continues to increase worldwide, the problem of device-associated infection will only grow in the future.

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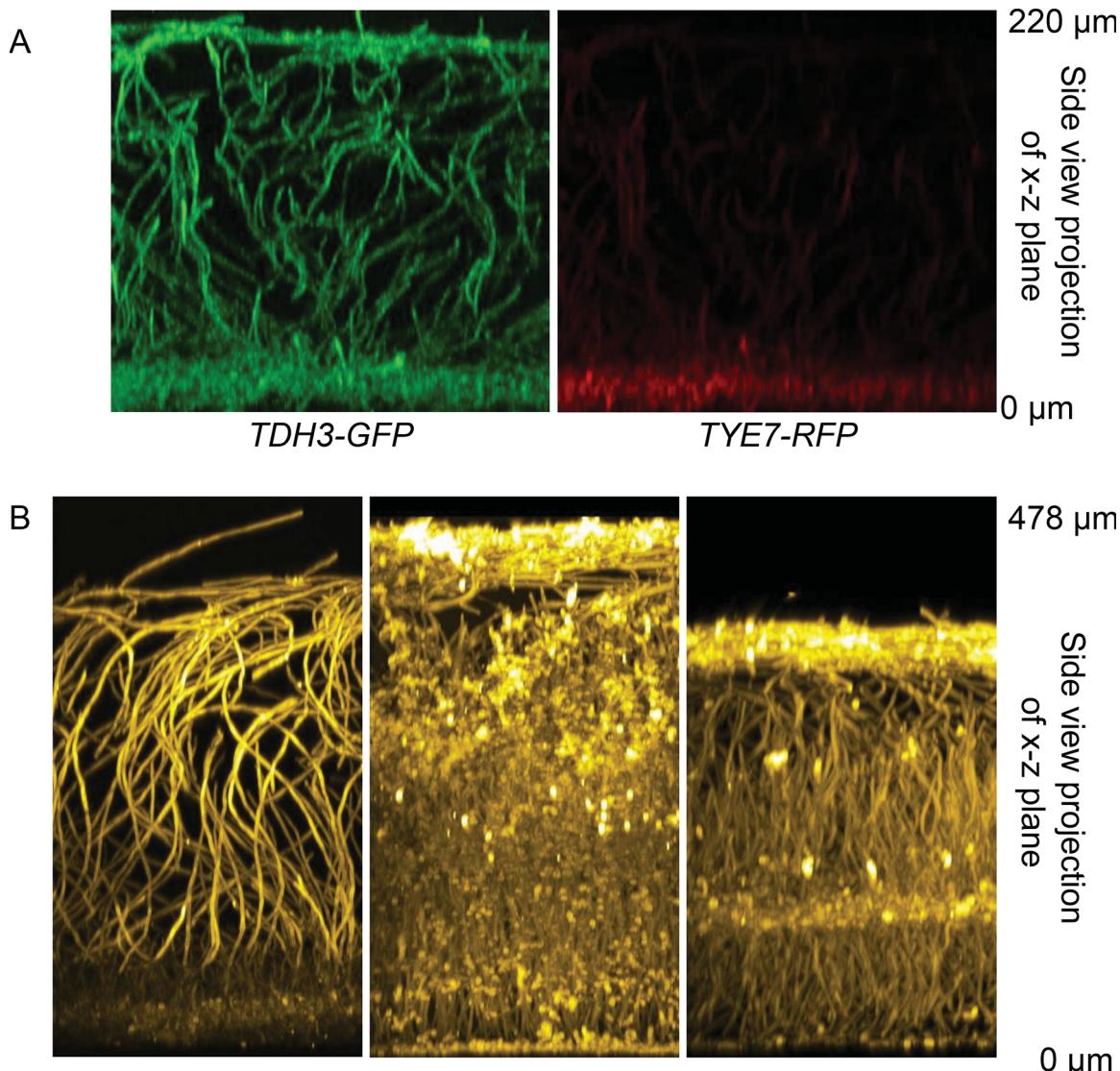


FIGURE 1 Confocal imaging of *C. albicans* biofilms. **(A)** Images show the heterogeneity of biofilms at the level of gene expression using a GFP construct fused to the *TDH3* promoter that is constitutively expressed throughout the biofilm and an RFP construct fused to the *TYE7* promoter showing greater expression at the basal layer of the biofilm, respectively. **(B)** Images show the phenotypic diversity of biofilms based on the growth medium in RPMI, YPD, and RPMI plus 10% serum, respectively. Biofilms were fixed and imaged using a Zeiss DuoScan confocal microscope. Side view projections were generated by reslicing and then z-projecting the stack using ImageJ software from the National Institutes of Health.

The second connection between biofilms and infection has to do with the nature of growth *in vivo*. Some infections are obviously surface-associated growth. Examples include mucosal infections such as thrush and vaginitis. Invasion of the surface may follow initial infection, and the surface itself may change over the course of infection due to inflammation and tissue damage. Other infections are not associated with host surfaces,

but the infecting organisms grow as an aggregate encased in extracellular matrix (ECM) material. The aggregates may be considered self-contained biofilms, analogous to the flocs that form at the end of industrial fermentations (4).

We now look inside fungal biofilms at the major biofilm-relevant phenotypes and, where known, the molecular mechanisms that govern those properties.

We focus primarily on *Candida albicans*, the biofilms of which have been studied extensively, as well as on *Aspergillus fumigatus*.

ADHERENCE

A necessary step in biofilm formation is attachment to a biotic or abiotic surface. For *C. albicans* biofilms, the attachment step is achieved through adhesive proteins expressed on the cell surface that are usually in the category of glycosyl phosphatidyl inositol (GPI)-anchored proteins. This kind of cell wall protein makes up a large portion of the total cell wall protein complement in *C. albicans*. Indeed, 115 putative GPI-anchored proteins have been identified, though more than half of these have no assigned function (5). The general structure of GPI-anchored proteins includes an N-terminal signal sequence, which traffics the protein to the endoplasmic reticulum, and a C-terminal hydrophobic domain site that allows the attachment of a preformed GPI anchor (6). A third structural part of GPI-anchored proteins is the central region, which varies among proteins. Often, the central region contains repeats of conserved, functional units.

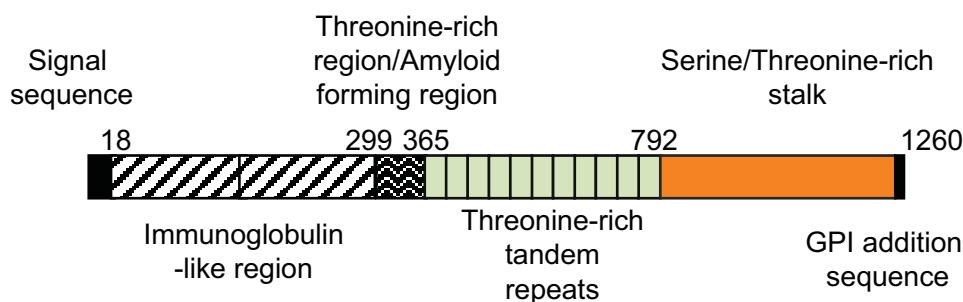
The Als (agglutinin-like sequence) family of adhesins is the most well-known category of GPI-anchored proteins in *C. albicans*. We use Als proteins to illustrate many general principles of fungal adhesins. Mutants defective in expression of *ALS1* and *ALS3* have decreased adherence to host and abiotic substrates (7–9). Due to their cell surface expression and role in virulence, recombinant Als1 and Als3 have been investigated for use as vaccines, with considerable success in mice (10). Als proteins contain the canonical GPI-anchor components along with an immunoglobulin-like region near the N-terminal domain, a conserved threonine-rich region, tandem repeats of a threonine-rich sequence, and a serine- and threonine-rich stalk that is often highly

glycosylated near the C terminus (Fig. 2). Each of these domains has been studied in detail and has been shown to be functionally important (11).

The Als immunoglobulin-like region has two immunoglobulin-like domains and a peptide-binding cavity. This domain is responsible for binding to a broad range of host substrates such as E-cadherins, ECM proteins, or fucose-containing glycans (11–13). Although ligand binding by Als proteins in solution is relatively weak, the loss of a highly expressed Als protein has a very dramatic effect on adherence. This conundrum spurred further structural and biochemical analysis of the N-terminal regions of several Als proteins. Three Als regions are critical for substrate binding: the peptide-binding cavity, the threonine-rich region, and the tandem repeat region (Fig. 2). The peptide-binding cavity interacts directly with ligands (14, 15), and the other regions mediate Als protein aggregation, as discussed below.

Following the N terminus, there is a threonine-rich region (T). One function of this region is to promote secretion, though the mechanistic basis for this role is not understood (13). The second function is related to a highly conserved amyloid-forming domain within this region that mediates Als protein association, yielding polyvalent Als protein aggregates. This domain has been explored in detail by the Lipke group (16). Using atomic force microscopy and amyloid binding dyes such as thioflavin T, amyloid formation was observed with surface-expressed Als5. Following the discovery of amyloid formation in the Als proteins, Chan and Lipke made the connection that the adherence of fungal cells to surfaces is mediated through a process called “catch-bonding” (17). The process of catch-bonding seems to be broadly utilized by other organisms to mediate adhesion, because it has been described to occur with other systems such as mammalian selectins and bacterial adhesins (18). Chan and Lipke showed that the

FIGURE 2 Als1 structure. Domains of Als proteins that are discussed in the text are depicted with amino acid coordinates of Als1 indicated.



domains are activated through sheer force. The force causes nanodomains to form, comprising Als protein aggregates. This mechanism accounts for the strong adherence exhibited by *C. albicans* when it adheres and forms a biofilm compared to the relatively weak binding of adhesins to specific ligands in solution (17, 19).

The threonine-rich amyloid-forming region is followed by threonine-rich tandem repeats (TR). These repeats have been shown to be heavily glycosylated (13) and are predicted to form antiparallel β -sheets (20). Expression of Als5 derivatives that differed in the numbers of TR repeats in the heterologous host *Saccharomyces cerevisiae* has shown that they mediate cell-cell aggregation (13). This cell-cell aggregation function was independent of the immunoglobulin-like domain (13). Several modeling approaches argue that aggregation of the TR is mediated primarily by hydrophobic interactions (20). In addition, the TR region increases the affinity of the Als5 immunoglobulin-like region for at least one substrate, fibronectin (13). Spectroscopic analysis argues that this TR function is mediated by an effect on the folding of the immunoglobulin-like domain (13). It is remarkable that a region of such simple sequence composition can have two distinct roles in Als5 activity. It would be interesting to know whether the two TR functions are separated genetically and if they are both critical for biofilm formation.

There is a serine- and threonine-rich region adjacent to the TR region. Mutations that reduce the length of this region prevent surface exposure of *S. cerevisiae*-expressed Als1 (21). These results are consistent with detailed work on the *Candida glabrata* adhesin Epa1 (22), which revealed that lack of the corresponding region caused the adhesin to be embedded within the cell wall rather than surface-exposed (22). The serine- and threonine-rich regions of several other *C. albicans* adhesins, Eap1 and members of the Hyr/Iff family, are also required for surface exposure (23, 24). Thus, the function of this region, to enable surface exposure of an N-terminal ligand-binding domain, seems to be similar among many adhesin-like proteins. This region is sometimes called the stalk, a name that seems to reflect its function quite well.

At the very C terminus of Als proteins (and many other cell wall proteins) is a short hydrophobic peptide that serves as a GPI-anchor addition signal. The GPI anchor, which is added in the endoplasmic reticulum, possesses a lipid tail that serves as a membrane tether. Upon trafficking to the cell surface, some GPI-anchored proteins remain tethered to the outer face of the plasma membrane, whereas others are transferred to become

covalently linked to cell wall β -1,6 glucan (25). In *C. albicans* (26), as in *S. cerevisiae* (27), amino acid residues neighboring the GPI-anchor addition site determine whether the protein becomes localized primarily to the plasma membrane or cell wall. Beyond this generic function for the GPI-anchor addition signal, Ahmad et al. have provided evidence that this region influences the affinity of Als5 for its ligands (28). However, the study used recombinant proteins, and it will be exciting if this function can be demonstrated in live fungal cells. In general, the most broadly documented function for the GPI-anchor addition signal is to mediate cell wall attachment of adhesins and other mannoproteins (25).

How can you determine if a protein functions as an adhesin? Various approaches have been used to answer this question. One way to show adhesive function is to express the protein in a nonadherent organism. Fanning and colleagues utilized a *bcr1Δ/Δ* mutant strain of *C. albicans* that is defective in expression of most known adhesins, including Als1 and Hwp1 (29). Therefore, this strain does not adhere to surfaces under a variety of conditions. By overexpressing various genes of interest, Fanning et al. showed that several known adhesins (Als1, Als4, Als2) restored adherence in a *bcr1Δ/Δ* mutant.

The model yeast *S. cerevisiae*, like the *C. albicans* *bcr1Δ/Δ* mutant, does not adhere well to many surfaces. Therefore, it has been widely used as a heterologous expression system for assessing adhesive protein function. Als1, Als3, Eap1, Hwp1, and Rbt1 have each been heterologously expressed in *S. cerevisiae* and shown to be functional adhesins (30–32). The *S. cerevisiae* cells expressing these proteins attached and formed biofilms on abiotic and cellular substrates, albeit to varying degrees (30–32). Using *S. cerevisiae* to express *C. albicans* proteins is an effective way to reduce the redundancy of adhesins and other adherence mechanisms that are utilized by *C. albicans* cells. However, it is important to note that *C. albicans* translates the CUG codon as serine, while most other organisms including *S. cerevisiae* translate CUG as leucine (33). This coding difference may cause unintended structural consequences if the protein is not codon-optimized.

Biofilm integrity probably depends on a balance of cell-substrate adherence and cell-cell adherence (sometimes called cohesion [34]). The importance of this balance was emphasized in the studies by Cabral et al. of a panel of *C. albicans* strains that overexpressed 531 different individual genes, including many cell wall protein genes (35). They made the perplexing observation that overexpression of certain cell wall proteins affected

a strain's ability to form biofilms differentially, based on whether the biofilm was a mono-culture or multistain biofilm (35). For example, when cell wall genes were overexpressed in a pooled biofilm assay of 531 strains, the PGA22-overexpressing strain had increased abundance compared to other strains. However, when the strain was grown in a single-strain biofilm assay, the PGA22-overexpressing strain showed decreased biomass. The study found that overexpression of PGA22 in *C. albicans* or a PGA22-derived hybrid gene in *S. cerevisiae* augmented cell-cell adhesion, and the resulting clumps of cells had increased sensitivity to shear force (35). Therefore, the flow conditions under which these biofilm assays were conducted could remove exposed cell aggregates in single-strain biofilms but could not dislodge the PGA22-overexpressing cells that were shielded by their 531 cousins in mixed-strain biofilms. This imaginative study illustrates the importance of a balance of adhesion to cells and substrates, as well as that cell-cell interactions within a biofilm can allow increased cell diversity.

The mold pathogen *A. fumigatus* also forms adhesive biofilms, though many of its properties are quite different from *C. albicans*. Biofilm formation in *A. fumigatus* occurs *in vitro* when grown under static aerial conditions or when grown attached to human bronchial epithelial cells. The biofilm is characterized by a mat of hyphae encased in ECM and shows increased resistance to antifungal cells compared to planktonic cells (36, 37). For biofilms to form, these infections begin with inhaled conidia that adhere to bronchial epithelial cells or macrophages. *A. fumigatus* RodA was discovered as a hydrophobin—a surface protein that confers hydrophobicity—that is expressed on conidia (38). A rodA mutant was shown to have decreased adhesion but was dispensable for virulence in a mouse model of systemic infection (38). Similarly, CalA, a predicted adhesin, was shown to bind mouse lung cells and laminin (39). However, its role in virulence or biofilm formation remains to be determined. Current understanding of *A. fumigatus* biofilms suggests that an ECM component, discussed in the next section, may have a more significant role in biofilm formation than any cell wall proteins.

ECM MATERIAL

One of the hallmarks of all biofilms is the presence of ECM (3, 40). The ECM can have diverse functions, including surface adherence, cell-cell aggregation, and nutrient storage (41). Of special relevance to biofilm

infections, ECM can present a barrier that buffers biofilm cells from antimicrobial agents as well as host defenses (42, 43).

ECM has been characterized from biofilms of several *Candida* species (summarized in reference 44). We focus here on two species, *C. albicans* and *A. fumigatus*, whose ECM has been studied in detail. We note that ECM abundance and composition can vary considerably with growth conditions (45), as does the biofilm structure itself (Fig. 1B), so some conclusions may differ between studies because of the use of distinct media.

Initial characterization of the *C. albicans* ECM through chemical analysis revealed the presence of glucose, hexosamine, protein, extracellular DNA (eDNA), and a few other components (46). This analysis was complemented through functional assays for biofilm integrity and adherence after enzymatic hydrolysis of ECM components (47). Overall biofilm integrity was found to depend on multiple ECM components, including β -1,3 glucan, chitin, protein, and eDNA.

Are there unique proteins in the ECM? A combination of electrophoretic separation and mass spectrometry identified abundant *C. albicans* biofilm matrix proteins and compared them to planktonic culture supernatant proteins (48). There was good correspondence between the samples, which included both conventionally secreted proteins, which have an N-terminal signal sequence, and unconventionally secreted proteins, which lack a signal sequence (49). No biofilm-specific ECM proteins were identified. Therefore, if any ECM proteins have unique functions in biofilm properties, these functions must arise in the context of ECM, perhaps as a result of high concentration or association with other ECM components.

The composition of *C. albicans* ECM has recently been defined comprehensively through a chemical and biochemical tour de force (50). The major components were proteins, carbohydrates, lipids, and eDNA. Most importantly, an abundant mannan-glucan complex called MGCx (sometimes pronounced “magic X”) was identified. It comprised branched β -1,6 mannan complexed with linear β -1,6 glucan. Because this complex is present at low levels if at all in cell wall preparations, it cannot arise from inadvertent contamination of ECM with cell wall fragments. In fact, MGCx may be the first *C. albicans* biofilm-specific marker.

How might an ECM-specific marker like MGCx be assembled? A fascinating answer comes from a study in which candidate genes were used to make mutants defective in individual ECM carbohydrate components (51). One surprising result was that accumulation of

three ECM carbohydrates—mannan, β -1,3 glucan, and β -1,6 glucan—is coordinated: mutations that reduce accumulation of one component generally reduce accumulation of all three. The most intriguing finding was that mixed biofilms comprising mutants blocked in production of different carbohydrate components displayed extracellular complementation, accumulating all components at levels as great or greater than the wild type. These findings indicate that ECM is assembled extracellularly (51). Perhaps the high local concentrations of substrates and enzymes for ECM assembly in the biofilm enable the reactions to proceed more rapidly than in the relatively dilute supernatant of a planktonic culture.

A second important finding from the comprehensive ECM analysis came from proteomic studies of biofilms formed *in vivo* in animal infection models (50). Specifically, although ECM from *in vitro* biofilms had 565 *C. albicans* proteins, ECM from *in vivo* biofilm models (venous catheter, urinary catheter, and denture) had only 16 *C. albicans* proteins. The vast majority of ECM proteins found *in vivo* were from the host. Therefore, host proteins may make unique functional contributions to the ECM from infecting biofilms. Interestingly, Nett et al. found a group of 14 host proteins in common among ECM samples from the three *in vivo* biofilm models (52), a very manageable number for functional assessments. The significance of the host protein contribution is illustrated by the fact that an inhibitor of *C. albicans*-fibronectin interaction blocks biofilm formation *in vivo* in a catheter infection model (53). These exciting findings reinforce the importance of testing conclusions from *in vitro* studies in an *in vivo* biofilm model to validate their potential clinical significance.

A. fumigatus engages in biofilm growth during infection of the lung, where it clearly forms a cohesive multicellular aggregate enmeshed in ECM (54, 55). (*A. fumigatus* is also linked to some medical device-associated infections [56]). The *A. fumigatus* ECM comprises galactomannan, β -1,3 glucan, monosaccharides, polyols, melanin, and a small amount of protein (54). Importantly, *A. fumigatus* aggregates, called aspergillomas, resected from the lungs of patients contain both galactomannan and β -1,3 glucan, thus emphasizing the relevance to infection of these ECM components (55).

One of the major *A. fumigatus* ECM components is a polymer made of galactose and N-acetyl-galactosamine (galactosaminogalactan [GAG]). Comparison to *Aspergillus nidulans*, which makes a GAG that is low in N-acetyl-galactosamine, argues that *A. fumigatus* GAG has a major role in promoting biofilm formation (57).

GAG is localized to *A. fumigatus* germ tubes and mycelia (58), and it mediates a multitude of virulence phenotypes. For example, it promotes adherence to abiotic surfaces through hydrophobic interactions and has been shown to interact with and bind to host cells such as pneumocytes (58). GAG also interacts with the immune system and is necessary for resistance to killing by neutrophil extracellular traps (57). There is growing evidence that *C. albicans* ECM also functions in immune evasion and manipulation of the host response (59).

A. fumigatus ECM formed *in vitro* has some eDNA content (60, 61). Rajendran et al. found that chitinase activity accumulates in mature biofilms (60), and the association of chitinase with autolysis (62) suggests that autolysis leads to DNA release into the ECM. In support of this hypothesis, addition of a chitinase inhibitor reduced ECM eDNA content (60). DNase treatment disrupts *A. fumigatus* biofilm integrity, thus indicating that eDNA plays a structural role in this species' biofilm (60).

DISPERSED CELLS

Dispersion is an important step in the developmental cycle of biofilm formation. The propagation of new biofilms necessitates the release and dispersal of new cells, because fungi are nonmotile. Uppuluri et al. measured the kinetics of dispersion of *C. albicans* cells using a flow biofilm model to mimic *in vivo* conditions where blood or other fluids create flow over implanted medical devices (63). They showed that even at early time points in biofilm formation, cells were being dispersed from the biofilm. Logically, the released cells were not composed of true hyphae, but the vast majority of cells were polarized yeast-form cells. Although dispersed cells were predominantly yeast, the cells showed unique phenotypic properties such as increased adhesion compared to planktonic yeast cells. The dispersed cells also displayed increased virulence in a mouse model of disseminated candidiasis. This suggests that the dispersed cells represent a distinct class of cell type in *C. albicans*.

Environmental signals can impact dispersion. Uppuluri showed that the amount of cell dispersion positively correlated with the amount of glucose in the media (63). Understanding the circumstances under which cells are most likely to be released from a biofilm and enter a patient's bloodstream could be important for clinical applications. Similarly, preventing dispersal of these highly virulent cells could be a way to combat systemic fungal infections caused by biofilms on implanted medical devices. In that context, promoter shut-off experi-

ments have identified four regulators—Ume6, Pes1, Nrg1, and Hsp90—that govern cell dispersion from preformed biofilms (56, 63, 64). In addition, defects in a histone deacetylase complex encoded by *HOS2*, *SIF2*, *SNT1*, and *SET2* reduce the abundance of dispersed cells (65). These regulators and the genes they control may be useful pharmacological targets for limiting biofilm accumulation.

DRUG RESISTANCE AND PERSISTENCE

All biofilms are notorious for their resistance to antimicrobial drugs and antiseptics, and *C. albicans* biofilms are no exception. One of the most careful comparisons of resistance of *C. albicans* biofilm and planktonic cells, in the same assay format, comes from the pioneering studies of Hawser and Douglas (66). They observed that biofilm growth conferred 5- to 7-fold greater tolerance to amphotericin B, flucytosine, or several azoles. Time-course studies by Chandra et al. revealed that *C. albicans* biofilms develop resistance to amphotericin B, fluconazole, and other treatments rapidly—between 2 and 12 h after initiation of biofilm formation (67). How does biofilm resistance arise? Our current understanding is that several mechanisms contribute to this phenotype, which makes it robust and quite consistent among different *C. albicans* isolates and *Candida* species (66).

One factor that contributes to resistance is the up-regulation of drug efflux pump genes *CDR1*, *CDR2*, and *MDR1* early in biofilm development (68, 69). Mutant analysis indicates that these genes have measurable impact on fluconazole resistance early in biofilm formation. As biofilms mature, though, other mechanisms make more prominent contributions to resistance.

A second factor is the presence of extracellular matrix material. One of the functional matrix constituents is DNA: it contributes to resistance to amphotericin B and caspofungin, as shown by the increased susceptibility of biofilm cells after DNase treatment (70). Interestingly, the treatment did not affect fluconazole susceptibility, thus indicating that overall biofilm properties were not disrupted by the treatment. The mechanism by which matrix extracellular DNA confers resistance is not known. Soluble β-1,3 glucan is a second functional matrix constituent; it has a major role in biofilm azole resistance, as shown by Nett and colleagues through engineering altered expression of the β-1,3 glucan synthase gene *FKS1* (71). They observed that changes in *FKS1* expression were accompanied by corresponding changes in soluble β-1,3 glucan in the matrix.

The phenotypic impact was dramatic: reductions in *FKS1* expression yielded biofilms that were extremely fluconazole-sensitive. The effect was biofilm-specific, in that the same strains were unaffected in fluconazole sensitivity during planktonic growth (71). Direct binding assays revealed that soluble β-1,3 glucan binds directly to fluconazole, thus indicating that drug sequestration is the mechanistic basis for biofilm fluconazole resistance (71). A follow-up study of candidate genes showed that three genes—*BGL2*, *PHR1*, and *XOG1*—also govern fluconazole susceptibility and matrix β-1,3 glucan production (72). These three genes specify extracellular glucan modification enzymes, making them inviting targets for antibiofilm therapeutics that might be combined with fluconazole.

Another biofilm feature that contributes to the resilience of biofilms against antifungals is the presence of persister cells. Persister cells are a distinct portion of dormant cells in a genetically identical population that can survive concurrent lethal rounds of antimicrobial treatments (73). *C. albicans* persister cells are unique in that they form only when grown in a biofilm (74); bacterial persister cells have been shown to form during both stationary phase and biofilm growth. This implies a distinct mechanism underlying persistence formation for fungal cells. When treated with amphotericin B or chlorhexidine, *C. albicans* cells grown to stationary or exponential phase are killed efficiently, but when the cells are grown as a biofilm they show a distinct biphasic curve of killing. Interestingly, *efg1Δ/Δ*, *cph1Δ/Δ*, and *mkc1Δ/Δ* mutants, which are defective in biofilm formation, still formed persister cells at consistent levels. This suggests that the mechanism of persistence formation is independent of formation of a normal biofilm; evidence points to the idea that attachment to a surface may be sufficient to induce persister formation (74). Additionally, the phenomenon of persister cells in *Candida* infections was shown to be clinically relevant through the screening of 22 patients that presented with long-term *Candida* infections. Perplexingly, the *C. glabrata* strains that were recovered showed low levels of persister formation. On the other hand, the *C. albicans* isolates formed high levels of persister cells (75).

One clue to the genetic basis of persister cells in *C. albicans* comes from Bink et al. (76), who showed that SOD (superoxide dismutase) mutants generate lower percentages of persister cells when treated with miconazole. SODs function by converting ROS (reactive oxygen species) to O₂⁻, thereby detoxifying the ROS. The reduction in persister cell formation was only true when using the antifungal miconazole, which had been

shown to raise ROS levels in cells. However, these mutants did not completely eradicate the persister cells, so undoubtedly there is still research to be done to understand the genetic regulation of this phenomenon.

GENE EXPRESSION

There has been long-standing interest in the identification of genes that are induced during biofilm formation. Such genes were thought to be—and have proven to be—enriched for genes that contribute to biofilm formation, and they can be informative in terms of broader biological processes that are biofilm-relevant. As such, biofilm-associated genes can reveal the identities of major regulatory systems and environmental signals that act during biofilm formation. Below we call these genes “biofilm-associated genes” because they have been defined through many kinds of comparisons. Any one particular comparison may yield a distinctive set of genes that are upregulated in biofilm cells.

Is there a fundamental biofilm-associated gene expression pattern? There have been a few different approaches to this problem. García-Sánchez et al. profiled biofilm cells grown under diverse conditions in comparison to planktonic cells also grown under diverse conditions (77). They arrived at a set of 325 genes (out of only 2,002 represented on their microarrays) whose differential expression was characteristic of biofilm cells from multiple comparisons; 214 genes were upregulated in biofilms. These 214 genes were enriched in transcription and translation genes as well as biosynthetic genes for amino acids, polyamines, nucleotides, and lipids. Upregulation of numerous translation-associated genes in biofilm cells was also observed in a comparison that looked for shared gene expression features among two wild-type strains, SC5314 and WO-1 (78). These findings argue that biofilm growth enables cellular biogenesis to occur over a prolonged period compared to planktonic growth in the same medium. A simple explanation is that a biofilm may capture excreted metabolites, thus facilitating their utilization when preferred metabolites are exhausted.

Many studies have reported that hyphal genes are upregulated in biofilms compared to planktonic cells (79–81). García-Sánchez et al. filtered those genes out by design through inclusion of a nonfilamentous mutant grown under biofilm and planktonic conditions (77); it was discovered that the mutant had the novel ability to form a biofilm on a glass surface. However, in the RNA-Seq biofilm-planktonic comparison of both SC5314 and WO-1, downregulation of hyphal genes was consistently

observed in biofilm cells (78). The biofilm time-course analysis of Fox et al. (82) may explain these disparate results: at early times, hyphal genes were upregulated in biofilm cells, but at later times, they were downregulated, relative to planktonic hyphae. Perhaps sufficiently high concentrations of quorum sensing molecules such as farnesol accumulate in mature biofilms (83, 84), inhibiting hyphal growth and hyphal gene expression at late times.

How do gene expression features compare between biofilms grown *in vitro* and *in vivo*? Profiling of *C. albicans* catheter biofilms by Nett et al. (85) revealed some consistent responses. Specifically, amino acid biosynthetic genes and transcription/translation genes were upregulated in biofilm cells *in vivo*. In addition, numerous transporters were upregulated, suggesting that biofilm cells may be limited for nutrients. The study compared biofilm cells to planktonic hyphae, a likely basis for the failure to detect upregulation of hypha-associated genes in biofilm cells. Overall, then, the correlations among broad trends in gene expression indicate that *in vitro*-grown biofilms serve as a useful model for catheter biofilms that form *in vivo*.

Have biofilm-associated genes been validated functionally? In other words, is gene regulation a predictor of gene function? One approach is to use biofilm-associated gene properties to deduce biofilm-relevant regulatory pathways. The d'Enfert lab implemented this approach in their initial study of biofilm gene expression (77). Because many amino acid biosynthetic genes were upregulated in biofilm cells, they inferred that the general amino acid control regulator, Gcn4, may be critical for biofilm formation. Indeed, they observed that a *gcn4Δ/Δ* mutant produced a defective biofilm, one with reduced biomass compared to the wild-type strain (77). Another illustration of this kind of thinking comes from the observation that biofilm-associated gene expression resembles the response to hypoxia (86), an observation first reported for *Candida parapsilosis*. That observation suggests that a mutant defective in hypoxic regulation may be defective in biofilm formation. Indeed, Bonhomme et al. found that Tye7, a transcription factor required for the hypoxic response (86), is required for many features of *C. albicans* biofilms (87). A third example comes from the point raised above that hypha-associated genes have often been observed to be upregulated in biofilms (depending on the specific biofilm-planktonic comparison). That observation makes sense because hyphae are a prominent feature of *C. albicans* biofilms grown under almost any condition, and it suggests that mutants defective in

expression of hypha-associated genes will be defective in biofilm formation. That prediction has turned out to be correct time and again (80, 88–90). Therefore, inferences from biofilm profiling data about biofilm-relevant regulatory pathways have proven successful in terms of functional validation.

The second approach to functional validation is to determine whether each specific biofilm-associated gene has a measurable function in biofilm formation. Here the results have been mixed, though there may be some useful lessons for the future. The initial study of this kind, from the d'Enfert group, examined deletion mutants of 38 biofilm-associated genes for defects in biofilm biomass or hyphal morphogenesis (87). The genes had been chosen based on the magnitude of their upregulation in biofilms after elimination of likely essential genes and members of gene families. Eight of the 38 mutants produced biofilms with moderately reduced biomass but had no planktonic growth defect; one mutant had severely reduced biofilm biomass and a severe planktonic growth defect. On its face, the results were slightly disappointing because the yield of biofilm-defective mutants was low and because the biofilm-defective mutants identified had mild defects. In a related approach, Desai et al. focused on genes that were upregulated in biofilms of two clinical *C. albicans* isolates (78). Of the 62 most highly upregulated genes, viable insertion mutants could be isolated for 25 genes. The mutants were screened for a panel of biofilm-related phenotypes, including biofilm formation, drug tolerance, quorum-sensing signaling, and others. Most of these mutants, 20 of 25, had significant defects in at least one biofilm-related phenotype. The high yield of biofilm-relevant phenotypes in this study may have reflected both the gene selection criteria and the range and sensitivity of the phenotypic assays.

The validation approaches and results presented above apply to *in vitro*-grown biofilms. However, the specific *in vitro* conditions used can have dramatic effects on biofilm properties (34, 91), and some mutations cause a biofilm defect only under a subset of *in vitro* conditions (92). In fact, one theme that has emerged repeatedly in all aspects of infection biology is that *in vitro* conditions can be poor mimics of *in vivo* conditions. Hence, the key validation approach in our view is to determine whether a biofilm-associated gene can be shown to function in an animal model of biofilm infection. The most frequently used model is a rat venous catheter biofilm model (93). Several mutant strains have similar biofilm defects *in vitro* and *in vivo* (7, 56, 72, 78, 80, 94–96). However, there are examples in which the severity of a mutant defect is much greater *in vitro* than

in a catheter model *in vivo*, and vice versa (78, 80, 97, 98). It would accelerate biofilm research considerably if there existed an *in vitro* model that could accurately predict *in vivo* outcomes.

BIOFILM GENE REGULATION

For three main reasons, it has proven useful to identify biofilm regulators. First, because a single transcription factor often controls many functionally related target genes, a single biofilm-defective transcription factor mutant can lead to discovery of many biofilm-relevant genes among the target genes that it regulates. One of the initial illustrations of this principle came from studies of the *S. cerevisiae* mating type locus, which specifies master regulators whose target genes confer individual cell type-specific properties (99). Second, there are many examples in which a transcription factor mutant has a more prominent phenotype than mutants defective in individual target genes of the transcription factor. A simple illustration comes again from studies of *S. cerevisiae*, this time studies of meiosis and spore formation: many mutations that caused a prominent sporulation defect affected meiotic regulators rather than the machinery that mediated specific meiotic events (100). This outcome could reasonably be considered a result of the first principle. Third, some biofilm regulators identified in *C. albicans* have orthologs in other *Candida* species that also govern biofilm formation (discussed below). Hence, a biofilm regulator can provide an entry point for definition of biofilm-relevant genes and biological processes in many organisms.

The transcription factors that control biofilm formation have been identified through several approaches. One approach is deductive logic based on expression profiling or other biofilm features, as discussed above (87–90). A second approach is to screen a set of transcription factor mutants for defects in biofilm formation or related phenotypes. For example, panels of both insertion and deletion mutants have been screened for failure to form biofilms (80, 82, 101). An elegant variation on this approach was to assay mutants at several time points in biofilm formation (82). Another related screen was for mutants that were defective in adherence to a silicone substrate (97). Overall, 51 transcription factor genes have been shown to affect biofilm-related properties in these large-scale screens as well as more focused studies (88, 102).

These studies have revealed that there is a major biofilm regulatory network, sometimes called the “core network” (102), comprising transcriptional regulators

of hyphal morphogenesis and hyphal genes. These transcription factors include Flo8, Rfx2, Gal4 (82), Brg1, Bcr1, Rob1, Efg1, Ndt80, and Tec1 (80, 90, 101). They are functionally interconnected, in that each binds to at least one other network regulator's upstream region and regulates its expression (80, 82). Rfx2 and Gal4 are negative regulators of biofilm formation; the respective null mutations cause increased biofilm formation (82), and for Rfx2, an increase in expression of many hyphal genes (103). The other transcription factors in this network are positive regulators of biofilm formation; null mutations cause reduced biofilm formation and reduced expression of hyphal genes (80, 82, 90, 101). For many of the positive regulators in this network, current evidence indicates that adhesin genes *ALS1*, *ALS3*, and *HWP1* are critical downstream target genes, because overexpression of one of these genes can restore considerable biofilm formation (7, 9, 80). However, there are likely to be additional network target genes that contribute to biofilm formation, because phenotypic rescue by adhesin overexpression is only partial in some cases (80). There are 21 transcription factor genes whose 5' regions are bound by one or more network regulators and that govern biofilm-relevant phenotypes (*AHR1*, *BPR1*, *CAS5*, *CRZ2*, *CZF1*, *FCR3*, *GCN4*, *GRF10*, *GZF3*, *MSS11*, *NRG1*, *RFG1*, *RIM101*, *TRY4*, *TRY5*, *TRY6*, *TYE7*, *UME6*, *ZAP1*, *ZCF31*, and *ZCF8*) (88, 102). Hence, this regulatory network controls adhesins and, potentially, many additional diverse biofilm properties.

Are all biofilm properties under the control of this core network? It is not clear as of yet. There are 21 transcriptional regulatory genes that control biofilm formation or biofilm-relevant properties, including *ACE2*, *ADA2*, *ARG81*, *DAL81*, *FGR27*, *LEU3*, *MET4*, *NOT3*, *RLM1*, *SNF5*, *SUC1*, *TAF14*, *TRY2*, *TRY3*, *UGA33*, *WAR1*, *ZCF28*, *ZCF34*, *ZCF39*, *ZFU2*, and *ZNC1* (102). These regulators govern such properties as the level of ECM (104) or the adherence of yeast-form cells (97). These features could conceivably be regulated independently of hyphal adhesins. Whether they are regulated through the core network is an interesting question for future studies.

Have we found all of the biofilm regulators? One could reasonably argue that 51 regulators are enough. We suspect that there are others, though, and that they are worth studying because of the *in vitro-in vivo* differences mentioned earlier in this article. Specifically, our lab has identified transcription factors that are required for biofilm formation *in vivo* but not *in vitro* (97). Thus, we hypothesize that the systematic screens used

so far may have overlooked other transcription factors with these properties. Perhaps they could be identified through application of the elegant mutant pool screens used by Noble (105) to assay *in vivo* biofilm formation assays.

CONSERVED FEATURES

Are there conserved features among biofilms of different *Candida* species? There are certainly some conserved features, even though the biofilm architecture of each species has been shown to vary. Several researchers have investigated this question of conservation by comparing *C. albicans* and *C. parapsilosis* biofilms. Evidence suggests that the major regulators of biofilm formation are conserved by both species. This may come as a surprise, because *C. parapsilosis* does not form true hyphae. Ding et al. (106) showed that the transcription factor Bcr1 is a major regulator of biofilm formation in *C. parapsilosis*, comparable to *C. albicans*. Also, some downstream targets of Bcr1 are conserved between the two species. Specifically, Bcr1 was shown to regulate genes encoding a family of proteins called CFEM (Common in Fungal Extracellular Membrane) in both species. The CFEM proteins have been implicated in biofilm integrity (107, 108) in *C. albicans*, but Ding found no evidence that CFEM mutants in *C. parapsilosis* are defective in biofilm formation *in vitro* (106). Several CFEM proteins also function in heme iron acquisition (109), and their regulation by Bcr1 is consistent with the iron utilization defect of the *C. albicans* *bcr1Δ/Δ* mutant (106). However, the specific Bcr1-regulated genes that are required for *C. parapsilosis* biofilm formation remain to be identified.

Holland et al. were the first to make an extensive deletion collection in *C. parapsilosis*, which included homozygous deletions of 100 different genes, including many transcription factor genes (110). From this study, many of the phenotypes were shown to be conserved between *C. albicans* and *C. parapsilosis*. In the roles of biofilm formation, Efg1, Bcr1, and Ace2 have all been confirmed as major regulators in both *Candida* species. However, Holland found several other transcription factors (Cph2, Czf1, Gzf3, and Ume6) that played a role only in *C. parapsilosis*. Upon sequencing of the biofilm transcriptome from each species, Holland found that similarities between the two species clustered around genes involved with metabolism (110). Similarly, when biofilms of both species were grown under hypoxic conditions, there was good correlation between the two transcriptional profiles (86). For ex-

ample, Upc2 is required in both species for regulation of ergosterol biosynthesis genes during hypoxia (111). It seems that the transcriptional landscape of biofilm formation in *C. albicans* and *C. parapsilosis* may be broadly conserved, but with some distinct divergences among regulators.

CONCLUSION

Inspection of the inside-out biofilm leads to a simple question: Will a biofilm turn out to be simply the sum of the parts? It is almost certainly not the sum of the parts that we know at this time. We still have limited understanding of the drivers and determinants of cell heterogeneity, as well as the flip side of heterogeneity, which is cell-cell cooperation. Biofilms, like tissues, present a considerable systems-level challenge. We often conceive of the collection of genes that govern biofilm formation as a network that acts within a single cell, much like the systems-level view of *S. cerevisiae*. However, consideration of cell heterogeneity makes a compelling case to us that many different networks are active among the diverse cells of a biofilm.

An additional challenge that underlies the sum-of-the-parts question is that our biofilm parts list is incomplete. One reason it is incomplete is because our *in vitro* biofilm models fail to recapitulate many features of *in vivo*-grown biofilms; hence, mutant phenotypes differ *in vitro* and *in vivo*. Each kind of biofilm—venous catheter, mucosal, etc.—is likely to reflect a distinct balance of biofilm phenotypes and regulatory inputs. Going forward, it seems imperative to validate experimental results with *in vivo* models to see which biofilm determinants are most relevant to infection biology.

Despite those challenges, a brief reflection on the history of fungal biofilm research underscores our optimism and excitement for the future. It was only about 20 years ago that Julia Douglas' lab first viewed *C. albicans* biology through the lens of the biofilm paradigm. Investigators have since elucidated the genetic and biochemical determinants of the many biofilm features discussed in this chapter. We now have a solid foundation from which to build our understanding of a fungal biofilm from the inside out.

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