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Candida albicans biofilm development and its genetic control

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Abstract

The fungus *Candida albicans* is a major source of device-associated infection because of its capacity for biofilm formation. It is part of the natural mucosal flora, and thus has access to available niches that can lead to infection. In this chapter we discuss the major properties of *C. albicans* biofilms, and the insight that has been gleaned from their genetic determinants. Our specific areas of focus include biofilm structure and development, cell morphology and biofilm formation, biofilm-associated gene expression, the cell surface and adherence, the extracellular matrix, biofilm metabolism, and biofilm drug resistance.

The human commensal *Candida albicans* is the leading fungal colonizer of implanted medical devices and a frequent cause of nosocomial infections (1,2). Several *Candida* species, including *C. albicans*, are part of the mucosal flora of most healthy individuals, and reside in the gastrointestinal and genitourinary tracts. These organisms are thus poised to cause infection when a suitable niche becomes available. The use of broad-spectrum antibiotics is an additional risk factor for *Candida* infections, probably because bacterial competitors that are eliminated would otherwise keep fungal populations in check. The extreme resistance of biofilm cells to antifungal therapy is a further complication, and often the infected device has to be removed and replaced to prevent recurrent infection (1). Here, we focus mainly on biofilm formation by *C. albicans*, the most intensively studied of the *Candida* species.

Biofilm structure and development

The first published image of a *Candida* biofilm on an implanted catheter came from the pioneering studies of Marrie and Costerton (3). This and many subsequent reports of *Candida* biofilms on devices prompted Hawser and Douglas to develop an *in vitro* system to study *Candida* biofilm development on catheter material discs (4). Their scanning electron micrographs provided the first glimpse of *C. albicans* biofilm architecture, which has since been studied by confocal imaging as well (see Figure 1). *C. albicans* can grow either as individual oval cells (called yeast cells or blastospores) or as long filamentous cells attached end-to-end (called pseudohyphae or hyphae, distinguished by specifics of cell structure) (5). Biofilms grown *in vitro* under a variety of conditions have a basal substrate-bound layer of yeast cells (Figure 1A,B) that ranges from 20 to 100 microns in depth under many conditions. Filamentous cells project from the basal layer, and can extend for several

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hundred microns (Figure 1A,C). Yeast cells are often found to be produced by the filamentous cells, especially in the apical regions of the biofilm (Figure 1A,C). Amorphous extracellular matrix material is found throughout the biofilm (Figure 1A,B,C), which can appear aggregated (shown here) or dispersed (6), depending on staining and fixation. A three-dimensional reconstruction (Figure 1D) reveals a very dense basal region beneath loosely packed filamentous cells. The loose packing of the upper region may facilitate solvent access to the basal region.

Fungi are nonmotile, and biofilm structure thus reflects the sequence of cell division events that occurs during biofilm development. Chandra *et al.* analyzed time-courses of *C. albicans* biofilm development on two different substrates, and proposed that biofilm development occurs in stages (7). They used a yeast cell inoculum because yeast cells are more likely than long filamentous cells to be able to disseminate to new sites. In the early stage, individual yeast cells adhered to the substrate. They then proliferated as yeast to produce microcolonies, and coalescence of microcolonies yielded the basal layer of the biofilm. Biofilm development then entered an intermediate stage of high metabolic activity along with the emergence of hyphae and production of extracellular matrix material. In the final maturation stage, there was extensive accumulation of extracellular matrix material. The images did not show presence of apical yeast cells, and they may have been obscured by intensely stained matrix. The authors also found that greatly reduced susceptibility to fluconazole, amphotericin B, nystatin, and chlorhexidine was acquired at the time of transition to the intermediate stage, concomitant with the increase in metabolic activity and accumulation of matrix material. This finding is in keeping with more recent studies that reveal that drug binding by extracellular matrix is a major source of biofilm drug resistance (see below).

The final step in biofilm formation can be considered to be the release of cells, permitting colonization of new sites and, unfortunately, disseminated infection (8). Uppuluri *et al*. (9) found that cell dispersion occurs throughout biofilm development and does not represent a temporally distinct stage. Cells released from biofilms were mainly yeast cells, not filaments. Remarkably, the released cells were phenotypically distinct from cells grown planktonically for the same amount of time in the same medium. The released cells displayed higher levels of adhesion to plastic or endothelial cells, probably due to their increased propensity to produce hyphae. In addition, the released cells were more virulent than planktonic cells in a disseminated infection model. Thus biofilm dispersion yields a unique class of yeast cell with increased ability to create new biofilms and cause infection.

Do biofilms follow the same developmental steps described above during a true catheter infection? One cannot reason from first principles to reach a conclusion about how representative an *in vitro* model may be. We believe that the simplest approach to validate *in vitro* observations is to use an animal model of biofilm-based infection. There are animal models (10) for venous catheter infection (11,12), urinary catheter infection (13), and denture stomatitis infection (14). (For review, see chapter by Nett and Andes in this volume.) There is also a subcutaneous catheter model that cultures biofilm cells in a host environment, though it may not resemble in detail a device currently in use (10). Finally, there are animal models for both oral and vaginal mucosal infections, which are in essence

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biofilms that form on mucosal tissue (reviewed in (15)). No investigation to our knowledge has validated the detailed observations regarding early, intermediate, and mature *in vitro* biofilms with these models. In addition, the detailed architecture of *in vitro* biofilms is generally not recapitulated in the *in vivo* models. Our perspective is that *in vitro* analysis allows clear documentation and characterization of biofilm alterations caused by genetic, physiological, environmental, or pharmacological perturbations. The selection of *in vivo* models then allows one to validate the key findings from *in vitro* studies, even if some of the details in each model may be different.

Cell morphology and biofilm formation

Under most conditions, both yeast and filamentous cells are required for *C. albicans* biofilm formation. Initial support for this conclusion came from mutants that were locked in either yeast or filamentous growth states (16), though the genetic basis for the mutant phenotypes was uncertain. Each mutant produced an altered biofilm with reduced biomass or cell density. A random insertion mutant screen further substantiated a role of hyphal morphogenesis in biofilm development (17). Mutants with insertions in the genes *NUP85, MDS3, SUV3* and *KEM1* were identified as biofilm-defective, and there was no known molecular or functional connection among them. However, they were all defective in hyphal formation in several media. In addition, in mixed biofilms formed with wild-type cells, each mutant produced only yeast cells. Therefore, the mutations caused defects in filamentation in the context of a biofilm, arguably the most relevant situation to assay. Ramage *et al*. found that two well established hyphal-defective mutant strains, *efg1* / and *efg1* / *cph1* / , were defective in forming biofilms (18). These mutants yielded only sparse substrate-attached cells, not a true basal layer. Remarkably, though, the substrate-attached mutants displayed no susceptibility to fluconazole and only moderate susceptibility to amphotericin B. These findings indicated that surface-bound growth is sufficient to induce the antifungal resistance of biofilm cells, and were consistent with the finding from Chandra *et al*. (7) that resistance increases substantially before a biofilm fully matures.

Why are filamentous growth forms so important for biofilm formation? Insight into the answer came from the transcription factor Bcr1 (19), identified in the first systematic screen of *C. albicans* transcription factor mutants. The *bcr1* / mutant was defective in biofilm formation, and also failed to form hyphae under some conditions. Importantly, though, in mixed biofilms formed with wild-type cells, mutant cells yielded abundant hyphae. Also, the non-adherent cells produced by the mutant under biofilm-inducing conditions included hyphae. These results suggested that the mutant produces hyphae that are defective in a function required for biofilm formation. Transcript profiling and functional analysis pointed to the same conclusion: Bcr1 is required for expression of genes for cell surface adherence proteins (called adhesins), such as *ALS1, ALS3,* and *HWP1.* Many of these genes, including *ALS3* and *HWP1*, are induced strongly during hyphal growth. Importantly, overexpression of adhesin genes *ALS1, ALS3*, or *HWP1* in a *bcr1* / mutant background restored biofilm formation ability, both *in vitro* and in a catheter infection model (20). This study was the first to provide evidence that hyphae are required for biofilm formation because of their cell surface adhesins.

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Biofilm-associated gene expression

If biofilm cells have unique phenotypic properties, one might expect that biofilm cells express a set of genes that are different from planktonic cells. Several studies have characterized the biofilm transcriptome (21-25). Although many different growth conditions and comparison conditions were utilized, there is good overall agreement, especially among many of the most highly induced genes in biofilm formation (22). Most importantly, these transcriptome studies have provided leads for functional analysis. For example, in the first such study, Garcia-Sanchez *et al*. found that amino acid biosynthetic genes were consistently up-regulated in biofilms grown under diverse conditions (21). That observation led them to assay biofilm formation by a *gcn4* / mutant, which is defective in the general control of amino acid biosynthetic genes. The *gcn4* / mutant produced a biofilm, but its overall biomass and metabolic activity was substantially reduced compared to the wild type. These assays were conducted in a rich medium in which planktonic growth of the wild-type and mutant strains were equivalent. Hence the mutant may be defective in retention of cells within the biofilm. Such a mutant phenotype would be difficult to detect in a large *in vitro* screen; the profiling data clearly pointed in a unique direction for functional analysis. In addition, these findings fit well with the observation made repeatedly that ribosome biogenesis genes are up-regulated in biofilm cells compared to planktonic cells. A simple hypothesis is that both amino acid synthesis genes and ribosomal biogenesis genes allow increased protein synthesis in biofilm cells, or perhaps a subset of biofilm cells, that contributes to biofilm stability and cohesion. Given the *gcn4* / mutant phenotype, those protein products may be adhesins or extracellular matrix components that mediate cell-cell adherence.

Broader surveys of mutants defective in biofilm-induced genes have not always yielded many genes that clearly function in biofilm formation, based on mutant phenotype (26). One reason for the limited correlation may be functional redundancy of biofilm-associated genes, for which examples are well known (20,27,28). A second reason may be the limited spectrum of biofilm properties that have often been assayed. Desai *et al*. used a panel of assays to explore defects in mutants with insertions in biofilm-induced genes, including adherence, azole tolerance, overall biofilm integrity, and quorum-sensing responses (22). They found that the majority of mutants had a significant phenotypic alteration in at least one assay, though many were not obviously deficient in overall biofilm formation ability. Because many processes contribute to the overall structure of a biofilm, it seems reasonable that functional understanding of many biofilm-induced genes may require assays of several phenotypic parameters.

Because a biofilm is a complex and heterogeneous environment, one might expect that some biofilm-induced genes may be part of response pathways that have little impact on biofilm phenotypes *per se*. Thus many investigations have sought to prioritize biofilm-induced genes for functional analysis. Perhaps the most elegant prioritization approach was undertaken by Nobile *et al*. (29), who extended the transcription factor mutant screen (19) to identify six biofilm regulators. They combined genome-wide expression profiling of the transcription factor mutants with chromatin immunoprecipitation assays to define the transcription factors' direct targets. There were over ~1000 target genes in the overall

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biofilm network, but only 23 genes were bound by all six regulators. These shared targets may be highly enriched for biofilm-related functions. A second prioritization approach is to focus on genes that are biofilm-induced under diverse conditions, as the d'Enfert group did with a panel of growth conditions (21,26) or as Desai *et al*. (22) did by employing two different *C. albicans* isolates to define common biofilm-induced genes.

The cell surface and adherence

The cell wall is the cellular structure that interacts most directly with the substratum or another cell. The *C. albicans* cell wall is primarily made of carbohydrates and glycoproteins (30). Carbohydrates such as β-glucan and chitin form an inner core of cell wall, responsible for its mechanical strength, mannoproteins that include adhesins form an outer fibrillar layer (30). Adhesins are defined by their ability to mediate adherence directly or their structural similarity to proteins that do so (31). Other cell wall or cell surface proteins may affect adhesin levels, processing, or exposure at cell surface, and thus affect adherence indirectly.

Many adhesins of *C. albicans* have a C-terminal sequence that is used for covalent attachment of a glycophosphatidylinositol (GPI) anchor (32). This GPI anchor initially tethers the protein on the outer face of the plasma membrane. The GPI anchor is then cleaved; the protein and anchor remnant are transferred to β-1,6-glucan and remain attached to the cell wall (32). Adhesins of this class include members of the Als (**A**gglutinin **L**ike **S**equence) family (33), Eap1 (**E**nhanced **A**dherence to **P**olystyrene 1) (34), Hwp1 (**H**yphal **W**all **P**rotein 1) (35,36), and Rbt1 (**R**epressed **B**y **T**UP1) (37), all of which are expressed at much higher levels in hyphal cells than in yeast cells. There is an adhesin-like protein expressed at highest levels in yeast cells, Ywp1 (**Y**east **W**all **P**rotein 1), but it seems to function as an anti-adhesin (38). There are also proteins that may function as adhesins but lack a GPI anchor, including Mp65 (**M**anno**p**rotein of **65**kDa) (39), Csh1 (**C**ell **S**urface **H**ydrophobicity) (40) and Pra1 (**p**H **r**egulated **a**ntigen) (32).

Early approaches to identify adhesins involved analysis of cell wall components that adhered to a surface after the adherent cells were washed away (41,42). However, the first studies to define *C. albicans* adhesins functionally relied upon heterologous expression in *Saccharomyces cerevisiae* (43,44). Als1 was identified in a screen of a *C. albicans* expression library in *S. cerevisiae* for clones that improved *S. cerevisiae* adherence to epithelial and endothelial cells (44). Als5 was identified though a similar approach: its expression in *S. cerevisiae* improved adherence to beads coated with fibronectin, laminin, and collagen (43). Adhesins from this *ALS* gene family have since been studied in detail (31,33). They are organized into four major regions: (1) an N-terminal immunoglobulin-like domain, (2) a threonine-rich region, (3) a series of 36 amino acid tandem repeats, and (4) a highly glycosylated stalk region (31,45). (All Als proteins have N-terminal signal sequences as well, allowing their entry into the secretion pathway.) Initial adherence has been proposed to be mediated by the N-terminal module, which is capable of ligand binding (46-48). These ligands include a broad range of denatured peptides, reflecting the broad specificity of Als proteins (48). The threonine-rich region and the tandem repeat region are required for cell-cell adherence, as demonstrated through heterologous expression of domain deletion mutants in *S. cerevisiae* (49). The eight different Als proteins seem to have redundant

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functions in biofilm formation for the most part, because high-level expression of any *ALS* gene in a biofilm-defective *als1 / als3 /* mutant restores biofilm formation *in vitro* and *in vivo* in the rat venous catheter model (50). Thus our current understanding is that the Als proteins function as a set of interchangeable adhesins to promote biofilm formation.

Recent studies have addressed a long-standing mystery about the Als proteins and other adhesins: how can proteins with such weak affinities for their ligands mediate stable binding? The answer lies in the ability of the threonine-rich region to form multi-protein aggregates, or amyloids (45). When amyloid formation is initiated (by tugging an Als in an atomic force microscope, for example), it spreads across the cell surface to create a nanodomain. The Als aggregate becomes in essence a multivalent adhesin. Thus even weakly bound ligands are rebound rapidly after they are released (45). Such amyloid-forming regions are found in many other cell surface adhesins, so amyloid formation may be a common mechanism to stabilize ligand-binding interactions.

Several other GPI-linked cell wall proteins function as biofilm adhesins, including Eap1, Hwp1, and Rbt1. Eap1 was identified as a *C. albicans* library clone that enabled adherence to plastic by otherwise nonadherent *S. cerevisiae* strain (34). Like the Als adhesins, Eap1 has an N-terminal ligand-binding domain followed by serine- and threonine-rich repeats that permit the N-terminal domain to project beyond the cell wall glucan (51). Eap1 is required for biofilm formation, because an *eap1 /* mutant is defective in biofilm formation *in vitro* and *in vivo* in the rat venous catheter model (52).

Hwp1 is structurally distinct from the Als proteins and Eap1. It is in essence a set of short peptide repeats followed by a GPI anchor addition site. Its role in host cell binding is remarkable: it is a substrate for host transglutaminases, which link it covalently to epithelial cell surfaces (35). Although it may also serve as a transglutaminase substrate during biofilm formation *in vivo*, it must function differently in biofilms formed *in vitro* because *C. albicans* does not make its own transglutaminases (35). An *hwp1 /* mutant has a moderate-to-severe biofilm defect *in vitro* and *in vivo* (36). Two observations argue that Hwp1 has a distinct and complementary role to that of the Als adhesins in biofilm formation (50). First, overexpression of *HWP1* does not allow biofilm formation by the *als1 / als3 /* mutant, in contrast to overexpression of any *ALS* gene. Second, a mixture of biofilm-defective *als1 / als3 /* cells and biofilm-defective *hwp1 /* cells is able to form a biofilm. The mechanism seems likely to be that Hwp1 and Als1/Als3 can interact on cell surfaces to mediate cell-cell binding. This inference comes from the fact that heterologous expression of *HWP1* in *S. cerevisiae* improves its adherence wild-type *C. albicans* cells, and not to *als1 / als3 /* mutant cells (50). Hwp1 and Als1/Als3 may thus function analogously to mating agglutinins of *S. cerevisiae* that permit binding of *MAT***a** and *MAT*α cells (31).

Rbt1 is in the same adhesin family as Hwp1 (37,53). An *rbt1 /* mutant has a mild biofilm defect *in vitro*, but shows additive effects with mutations in family members *HWP1* and *HWP2* (53). Its N-terminal region promotes surface hydrophobicity and mediates adherence to polystyrene (37). A central domain is predicted to have high aggregation potential, and amyloid-inhibitor experiments similar to those carried out with Als5 support such a function (37). Although Rbt1 is normally expressed only on hyphal cells, Monniot et al. could create

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a constitutive *RBT1* allele through fusion to the *TEF1* promoter. Interestingly, this consitutively expressed protein could be recognized by anti-epitope antibodies only on hyphal cell surfaces. Recognition on yeast cell surfaces required mild digestion of the cell wall with zymolyase (37). These observations suggest that there is a fundamental structural difference between yeast and hyphal cell walls that affects the exposure of Rbt1 and, potentially, many other adhesins.

One interesting GPI-anchor containing protein, Ywp1, functions to reduce adherence (38,54). *YWP1* is expressed at much higher levels in yeast cells than in hyphae, so it is possible that Ywp1 is critical for dispersion of yeast cells from a biofilm. It is yet not known how Ywp1 exerts its anti-adhesive effects; it may interact with specific adhesins, or it may alter the cell surface to deny access to adhesins. In that context, it would be interesting to see if Ywp1 is required for the inhibition of Rbt1 epitope access on yeast cells observed by Monniot *et al*. (37).

How is adherence regulated? As mentioned above, many of the major known adhesins are expressed at highest levels on hyphal cells. Their expression is regulated by transcription factors that also govern hyphal development (29,55). In addition, the adherence of yeast cells, which is thought to be the initial step in biofilm formation, appears to be under complex control. Finkel *et al*. screened for transcription factor mutants with altered adherence to silicone (56), and uncovered 30 transcription factors that are required for adherence. Expression of all known and predicted cell wall protein genes was assayed in the mutants, which allowed provisional assignment of both regulators and cell wall protein genes to pathways. The value of this approach was supported by positive overexpression-rescue tests of several new pathway relationships. For example, the findings indicated that Snf5 and Ace2 lie in a pathway that governs adherence, biofilm formation, and cell wall integrity (56). In addition, the findings argued that the protein kinase Cbk1 and transcription factor Bcr1 act in the same pathway, and contemporaneous studies revealed that Cbk1 phosphorylates Bcr1 (57). A simple interpretation is that a large number of transcriptional regulatory pathways govern adherence, but they ultimately impact a small number of response mechanisms. Interestingly, several of the transcription factors were not required for biofilm formation in an *in vitro* system, but were required in the rat catheter *in vivo* model (56). This finding emphasizes the limitations of *in vitro* biofilm models, and the potential that our reliance on *in vitro* models may cause us to overlook critical functions that act *in vivo* during infection.

Several upstream regulators that govern adhesin expression have also been identified, thus paving the way to define the actual molecular or physiological signals that govern biofilm formation. As mentioned above, the protein kinase Cbk1 phosphorylates and activates Bcr1, perhaps ensuring that hyphal adhesins are only expressed when Cbk1-dependent cell polarity functions are active (57). In addition, the Tor1 kinase, a central regulator of ribosome biogenesis and starvation responses, is a negative regulator of adhesin genes *ALS1, ALS3*, and *HWP1* (58). This relationship may reflect a role for starvation in promoting adherence and biofilm formation. Recent studies have revealed that the stress-responsive MAP Kinase Hog1 mediates this effect of Tor1, and that transcription factor Brg1 may be the direct target of this pathway (59). Because Hog1 is activated by high osmolarity as well

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as oxidative stress (60), these signals may also influence the ability to adhere and form a biofilm. Finally, we note that the cyclic AMP-dependent protein kinase catalytic subunit Tpk1 functions as a negative regulator of adherence and *ALS1* expression (61), perhaps through effects on the cyclic AMP pathway target transcription factor Efg1 (60). This pathway governs hyphal morphogenesis, so it seems possible that the response can modulate the adhesin levels on hyphae in a biofilm. Clearly these novel pathway relationships will whet our appetites for dissection of signals and responses in biofilm formation for some time to come.

Many genes that have broad effects on cell wall biogenesis or integrity also affect adherence or biofilm formation. For example, *GAL102* and the *PMT* (**P**rotein **M**annosyl **T**ransferase) gene family govern protein mannosylation (62,63). The impact of respective mutations on biofilm formation may result from altered adhesin glycosylation. Other cell wall proteins that govern adherence but may not be adhesins are Sun41 and Pga1, both of which have roles in cell wall integrity (64-67). However, the fact that a cell wall protein affects cell wall integrity does not rule out the possibility that it is an adhesin. The Als adhesins in particular are famous as multifunctional proteins. Als3 is the best example, with roles in adherence to numerous substrates, host receptor binding, host cell invasion, and iron acquisition (68). Als2 is a possible bridge between cell wall integrity and adhesin function: it seems to be essential for viability, and changes in *ALS2* gene dosage have profound effects on cell wall depth and sensitivity to call wall perturbing agents (61,69). Thus a known adhesin seems to have a role in overall cell wall architecture and integrity.

Might the cell wall have a sensory function? The transcription of many genes (including adhesin genes) is induced rapidly after the initial adherence step (24). Perhaps surface binding generates a signal that switches the cell growth program from planktonic to biofilm. In fact, several groups have studied contact sensing phenomena and their regulation (70-73). The transmembrane protein Dfi1, through calmodulin binding, regulates the activity of a MAP kinase Cek1. The MAP kinase Mkc1 is also activated after cells interact with semisolid surfaces (71,72,74). Both Cek1 and Mkc1 have roles in biofilm formation (71,75). Thus, while the evidence now is fragmentary, a fascinating possibility is that physical changes in the cell wall occur upon substrate binding that activate Cek1 and Mkc1 to promote biofilm formation.

Extracellular matrix material

A mature biofilm shows complex architecture with heterogeneous cell types enmeshed in extracellular matrix. Biofilm matrix was first characterized by the Douglas group (76). They found presence of carbohydrate, protein, hexosamine, phosphorus and uronic acid. Additionally, they observed that treatment with enzymes such as β-1,3-glucanase, proteinase K, DNase I, chitinase and β-N-acetylglucosaminidase compromised biofilm cohesion (76). A good portion of the glucose initially detected by the Douglas group is found in soluble β-glucan (77), which Nett and colleagues have shown to be a key matrix determinant of antifungal drug resistance (see below). Thus the *C. albicans* biofilm matrix functions in both biofilm integrity and drug resistance.

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Matrix production can vary considerably with growth conditions. For example, there is less matrix production when biofilms are grown statically than with shaking (78). Also, matrix production is greater in RPMI medium than in Spider medium (79), both of which are commonly used by many investigators. A further complication is that matrix composition has not been dissected under these varied growth conditions. Given the broad functional roles of matrix components, it may be useful to develop some standardized procedures for analysis of biofilm properties.

The most well understood role of a matrix component is the function of β-1,3 glucan in biofilm azole resistance. Nett *et al*. manipulated the essential *FKS1* gene, which is responsible for cell wall β-1,3 glucan synthesis (80). They showed that decreased or increased *FKS1* expression or activity results in a corresponding change in amount of biofilm matrix (soluble) β-1,3 glucan. Hence matrix β-1,3 glucan follows the same biosynthetic pathway as cell wall β-1,3 glucan. Remarkably, the strains with reduced *FKS1* activity produced biofilms *in vitro* and *in vivo* that were exquisitely sensitive to fluconazole, while during planktonic growth there was no change in fluconazole sensitivity (80). These observations showed that β-1,3 glucan synthesis is required for a biofilm-specific drug resistance mechanism. In fact, addition of isolated biofilm matrix to planktonic cells conferred fluconazole resistance. Direct binding assays were used to show that drug sequestration is the mechanism by which β-1,3 glucan confers biofilm fluconazole resistance (80). In order to understand the biogenesis of matrix β-1,3 glucan, Taff *et al*. created null mutant strains in candidate glucan modification genes that were up-regulated *in vivo* during biofilm development (28). They found three genes, two that encode glucan transferases Bgl2 and Phr1, and one that encodes exoglucanase Xog1, to affect matrix β-1,3 glucan production and fluconazole susceptibility. Because the glucan modification pathway is extracellular, it seems like an excellent target for anti-biofilm therapeutics.

Proteins and DNA also constitute an integral part of the matrix material. The protein component has been characterized through a proteomic approach by Lopez-Ribot and colleagues (81). Many of the most abundant proteins found in matrix were similar to the proteins found in supernatants of planktonic cultures. In addition, a large proportion of the matrix proteins are annotated as cytoplasmic. DNA is also a functional matrix component, as indicated by the finding that DNase I treatment compromises biofilm integrity (76). Moreover, addition of DNA improves biofilm formation as indicated by increased biomass (82). It seems possible that cell lysis may be a major source of the cytoplasmic proteins and DNA in the biofilm matrix.

There have been several approaches to identify the regulators of biofilm matrix production. An unusual biofilm morphology led Nobile *et al*. to identify the zinc acquisition regulator Zap1 as a negative regulator of matrix β-1,3 glucan (27). Transcriptomic and ChIP assays followed by functional analysis revealed the key Zap1 targets to include two glucoamylases (Gca1 and Gca2) and three alcohol dehydrogenases (Csh1, Ifd6 and Adh5) (27). Although Gca1 and Gca2 may act directly on matrix polysaccharides, it seems likely that Csh1, Ifd6 and Adh5 act indirectly, perhaps through effects on quorum sensing molecule production (83). Recently, the Soll lab identified a role for Bcr1 in regulating the impenetrability of MTL-heterozygous biofilms to dyes and polymorphonuclear leukocytes, which are likely to

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be matrix-associated traits. Through a series of Bcr1 target gene overexpression assays, they found that the extracellular CFEM (**C**ommon in several **F**ungal **E**xtracellular **M**embrane proteins) proteins promote this matrix function (79). The CFEM proteins were shown previously to be required for biofilm formation, but their role in matrix properties was not anticipated (84). It remains to be determined whether the CFEM proteins are themselves matrix components, or if they act more indirectly through effects on signaling or nutrient acquisition (85).

The studies of Zap1 and Bcr1 seem to have defined pathways that do not affect *FKS1* regulation (see (28) in particular). However, a candidate gene approach based on *S. cerevisiae* ortholog function identified Smi1 as a regulator that acts upstream of *FKS1* (86). Specifically, a *smi1* / mutant had decreased biofilm fluconazole resistance, β-glucan production, and *FKS1* RNA accumulation. Moreover, increased expression of *FKS1* caused increased fluconazole resistance in the *smi1* / mutant. Current evidence indicates that Smi1 acts through the transcription factor Rlm1 to govern *FKS1* expression (86). In addition, the chaperone Hsp90 is required for matrix β-glucan production (87). This role for Hsp90 is independent of its regulatory interactions with the known client proteins calcineurin and Mkc1. Hsp90 may affect *FKS1* expression or activity, perhaps through the Smi1-Rlm1 pathway.

Biofilm metabolism

A central theme that has emerged from transcriptome studies is that the mature *C. albicans* biofilm presents a hypoxic environment. The first general indication of biofilm hypoxia came from the observation that glycolytic genes are up-regulated in biofilms (21,26). This response might be expected if energy from hexoses in biofilms derives from fermentative reactions, which are much less efficient than respiration. Indeed, the Butler group set out to do a comparison of gene expression during biofilm growth and during hypoxia with the species *C. parapsilosis* (88). A set of 60 genes was common to the two responses, representing mainly genes involved in glycolysis or in synthesis of fatty acids and ergosterol. In addition, a recent metabolomic comparison of biofilm and planktonic cells revealed that biofilms accumulate lower levels of succinate, fumarate, citrate and malate (89). This outcome probably reflects diminished flux through the tricarboxylic cycle, as expected if respiration rates are lower in biofilm cells than in planktonic cells. The overall hypoxic metabolism of biofilm cells is functionally significant, based on properties of the transcription factor Tye7. This transcription factor is an activator of glycolytic genes, and its function is critical for growth when respiration is blocked (90). Bonhomme *et al*. found that a *tye7* / mutant had greatly reduced ability to form a biofilm, in keeping with the hypothesis that the biofilm environment is hypoxic) In addition, the mutant biofilm contained an excess of filamentous cells, and observations with metabolic inhibitors argued that hyperfilamentation was a result of decreased glycolytic flux and ATP synthesis (26). This study leads to two interesting conclusions. First, hypoxic or fermentative carbon metabolism is critical for biofilm formation. Second, it is generally appreciated that biofilm growth leads to abundant hyphal formation in media that induce planktonic hyphae poorly (see (7,17) for example); it seems possible that hypoxia may be the signal that induces hyphal formation during biofilm growth.

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The transcription factor Efg1, a central regulator of biofilm formation and hyphal formation (1,60), may have a pivotal role in coordinating hyphal formation and hypoxic metabolism. Stichternoth and Ernst explored this connection through examination of Efg1-responsive genes under hypoxic conditions (91). Interestingly, many of the same genes that were activated rapidly by Efg1 corresponded to metabolic genes activated during biofilm formation. In fact, the *TYE7* gene is a direct target of Efg1 (29). Therefore, the metabolic genes that respond to Efg1 during hypoxia may do so through their activation by Tye7.

If fermentation is necessary for biofilm physiology, one might expect biofilms to accumulate increased levels of fermentation products such as ethanol compared to planktonic cells. However, ethanol is not more abundant in biofilms (89,92), and in fact inhibition of ethanol production leads to increased biofilm formation (92). These observations can be reconciled with the metabolic inferences discussed above if *C. albicans* uses alternate electron acceptors, thus yielding reduced products other than ethanol. For example, hypoxic growth induces the genes involved in sulfur assimilation and methionine and cysteine biosynthesis (91). These genes were also found to be up-regulated in biofilms (21,23-25,91). It is possible that, when oxygen is scarce such as in biofilms, the sulfur assimilation pathway, with its multiple reduction involving steps, provides additional means to balance the reducing equivalents arising from glycolysis.

Many metabolic products have impact on *C. albicans* cell properties that affect the structure or integrity of the biofilm. The most intensively studied example is the quorum sensing molecule farnesol, which functions as an inhibitor of hyphal morphogenesis and of biofilm formation (93,94) through its action on the Ras1-cyclic AMP pathway (95). Additionally, farnesol has recently been shown to block Nrg1 degradation (96), and Nrg1 can promote cell dispersion from biofilms (96). Although the biofilm environment may modify responses to quorum sensing molecules (83), the simplest generally accepted model at this time is that farnesol and other quorum sensing molecules promote release of yeast cells from mature biofilms.

One metabolite with enigmatic biological impact is glycerol. It is familiar to most yeast biologists as a major osmoprotectant and net output of the HOG pathway (97). Glycerol levels are considerably elevated in biofilm cells compared to planktonic cells (22,89), and the glycerol biosynthetic genes are up-regulated in biofilms (21,22,24,25). Deletion of the glycerol biosynthetic gene *RHR2* causes a severe biofilm defect *in vitro* and *in vivo* in a rat catheter model (22,26). Unexpectedly, the reduced glycerol levels cause decreased expression of biofilm adhesin genes (including *ALS1, ALS3*, and *HWP1*), and expression of any of these adhesins at elevated levels restores biofilm formation by the *rhr2* / mutant *in vitro* and *in vivo* (22). It is not clear why glycerol and biofilm formation should be so intimately coupled; fermentation of a hexose to glycerol does not allow ATP production, though it could be used to consume reducing equivalents generated under hypoxic conditions. The glycerol-adhesin regulatory relationship may reflect the role of glycerol in synthesis of GPI anchors, or perhaps a coupling of biofilm formation and turgor-requiring tissue invasion in natural contexts. This example illustrates that biofilm metabolites may have impact that extends far beyond metabolism.

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Biofilm drug resistance

C. albicans biofilm cells are much more resistant than planktonic cells to a spectrum of antifungal drugs. As described above, drug sequestration by matrix β-1,3 glucan is one major resistance mechanism (98). However, the, extracellular DNA of biofilm matrix contributes to resistance to amphotericin B, as DNase treatment increases antifungal susceptibility of biofilm cells (99). Several additional processes further contribute to drug resistance. For example, the drug efflux pump genes *CDR1, CDR2* and *MDR1* are upregulated in biofilms, and contribute to fluconazole resistance of early, though not mature, biofilms (100,101). A decrease in ergosterol levels is observed in intermediate and mature biofilms, so there is potentially less target available for amphotericin B (100). Additionally, persister cells have been observed for *C. albicans* biofilms as they have for bacterial biofilms (102). LaFleur *et al*. identified these phenotypic variants from biofilms as survivors after amphotericin B treatment (102). There has been exciting progress recently in defining the genetic determinants of persister cell formation: The Thevissen group has shown that reactive oxygen species generated by miconazole treatment induce expression of the superoxide dismutase (*SOD*) gene family. They linked this response to generation of persisters by showing that chemical superoxide dismutase inhibition, or a genetic deletion affecting the major cell surface family members Sod4 and Sod5, causes a severe reduction in the level of persisters (103). The authors note that this mechanism may be specific to miconazole. The challenge in analysis of persisters reflects in part a broader knowledge gap: we do not understand at this time the extent of heterogeneity among fungal biofilm cells (104), nor have we developed the tools to dissect subpopulations. In any case, it is clear that biofilm drug resistance is a multifactorial phenomenon. The most effective therapies may prevent biofilms from forming, rather than trying to eliminate them once they are present.

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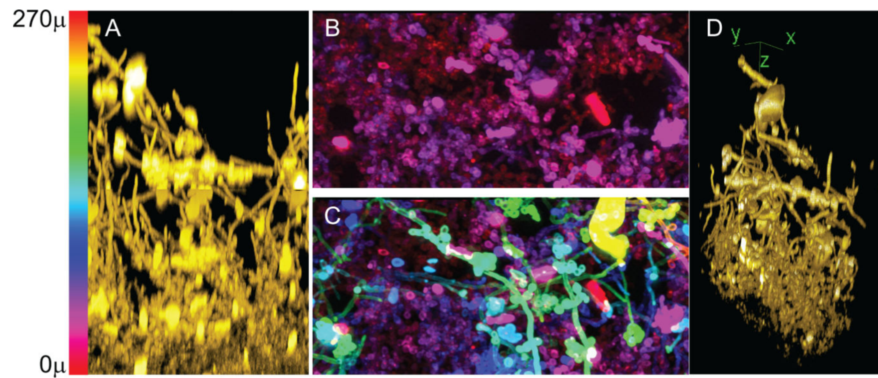

Figure 1.

Confocal micrographic images of a *Candida albicans* biofilm. These images present a biofilm grown in vitro in YPD medium at 37°C. The sample was prepared by embedding and staining with Alexafluor 594-conjugated Concanavalin A, using a procedure modified from reference (83). (A) Side projection view. Hyphae are clearly visible in the upper portion of the biofilm, as are aggregates of brightly stained extracellular material. A color scale bar represents the 270 micron depth, and indicates the pseudocolor scale used for apical projections. (B) Apical projection of basal (substrate-proximal) 50 micron region. A yeast cell layer is evident from the substrate level (red) to 50 microns above the substrate (blue). A few hyphae or pseudohyphae are visible as well. Some amorphous extracellular material is apparent. (C) Apical projection of entire biofilm. Hyphae are visible above the basal layer, extending from ~150 microns (green) to 270 microns (red) above the substrate. Yeast cells are seen in clusters at the ends of hyphae. (D) Three-dimensional reconstruction of the biofilm sample. Hyphae at the top of the biofilm are readily visible above the dense basal region.