Development and regulation of single- and multi-species *Candida albicans* biofilms

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Abstract

Candida albicans is among the most prevalent fungal species of the human microbiota and asymptomatically colonizes healthy individuals. However, it is also an opportunistic pathogen that can cause severe, and often fatal, bloodstream infections. The medical impact of *C. albicans* typically depends on its ability to form biofilms, which are closely packed communities of cells that attach to surfaces, such as tissues and implanted medical devices. In this Review, we provide an overview of the processes involved in the formation of *C. albicans* biofilms and discuss the core transcriptional network that regulates biofilm development. We also consider some of the advantages that biofilms provide to *C. albicans* in comparison with planktonic growth and explore polymicrobial biofilms that are formed by *C. albicans* and certain bacterial species.

Microbial biofilms are communities of cells that adhere to solid surfaces or are present at liquid–air interfaces, and they are considered the most common state of growth for many microbial species[1–3]. Cells within biofilms have properties that are distinct from their planktonic (free-floating) counterparts; for example, they are often more resistant to drugs and to physical perturbations[4]. Microbial biofilms have been observed in aquatic environments, on artificial industrial structures, on implanted medical devices, and on plant and mammalian tissues[1]. Although many microorganisms are capable of forming single-species biofilms, it is much more common to find two or more bacterial and/or fungal species in a biofilm; these polymicrobial biofilms often provide specific advantages to each species when compared with single-species biofilms[5]. In several programme announcements

for funding (PA-03-047 and PA-07-288), the National Institutes of Health (NIH) estimates that biofilms are responsible for a ~80% of microbial infections in humans[1,6].

Candida species are the predominant fungi isolated from infected medical devices and account for ~15% of hospital-acquired cases of sepsis[1]. *Candida albicans* is the most commonly identified *Candida* species in clinical contexts and is one of the leading causes of hospital-acquired infections. However, in healthy humans, *C. albicans* is usually a harmless member of the native microbiota and asymptomatically colonizes many niches, including the gastrointestinal tract, reproductive tract, mouth and skin[7–11]. Disturbances caused by shifts in pH, nutritional alterations, shifts in oxygen levels, antibiotic use, diseases, or immunosuppressant therapy can promote the over-proliferation of *C. albicans* and often lead to severe symptoms. *C. albicans* infections range from superficial mucosal and dermal infections to disseminated bloodstream infections with mortality rates above 40%[12–14]. *C. albicans* infections are particularly serious in immunocompromised individuals, such as patients with AIDS, patients undergoing chemotherapy and individuals receiving immunosuppressant therapies. In addition, individuals who have implanted medical devices are also particularly susceptible[15,16].

C. albicans biofilms are highly structured; they contain yeast-form cells, pseudohyphal cells and hyphal cells surrounded by an extracellular matrix [6,17,18] (FIG. 1). In addition to forming biofilms on implanted medical devices (for example, catheters, pacemakers, heart valves, joint pros-theses and dentures), *C. albicans* biofilms also form on host surfaces, including mucosal surfaces, epithelial cell linings and parenchymal organs[19,20]. Existing antifungal drugs, at concentrations effective against planktonic *C. albicans*, are largely ineffective against *C. albicans* cells in biofilms. Although much higher concentrations can be effective against biofilms, these doses often cause serious side effects to the host (that is, kidney or liver damage). Resistance to antifungal drugs associated with *C. albicans* biofilms and the ability to colonize implanted medical devices have been linked to increased medical costs and negative patient outcomes[19–24]. *C. albicans* biofilms function as reservoirs of drug-resistant cells that can detach, multiply and seed bloodstream infections. If a recalcitrant biofilm infection is suspected, removal of the infected device is typically the standard procedure[25,26]; however, depending on the device, the removal can require invasive and potentially dangerous surgical procedures (for example, the treatment of heart valves is especially problematic). Critically ill patients are often unable to tolerate these procedures, leaving few, if any, available treatment options in these cases[19,26].

In this Review, we provide an overview of the processes involved in the formation of a *C. albicans* biofilm and discuss the transcriptional circuitry that regulates biofilm development. We also consider some of the advantages that biofilms provide to *C. albicans* compared with planktonic growth and explore polymicrobial bio-films that are formed by *C. albicans* and certain bacterial species. Finally, we discuss several recent advances in the field, including the identification of new regulators of biofilm formation, the identification and characterization of biofilm-specific proteolysis, the proteomic characterization of persister cells, the analyses of naturally occurring *C. albicans* strains with differing abilities to form biofilms and the protection that biofilms confer against the host immune system.

C. albicans biofilm formation

The current understanding of *C. albicans* biofilm development is based on *in vitro* experiments, *in vivo* animal models of infection, and examination of biofilms that formed on medical devices in the clinic (BOX 1). On the basis of those observations, *C. albicans* biofilm development has been divided into four stages: adherence, initiation (also referred to as proliferation), maturation and dispersion[17,27–31] (FIG. 1). *In vitro* results suggest that biofilm formation begins with the adherence of yeast-form cells to a solid surface and the formation of a basal layer that functions to anchor the biofilm. *C. albicans* cells adhere to both biotic and abiotic surfaces with consistencies ranging from hard to soft. This feature, coupled with the ability to adhere to other *C. albicans* cells, contributes to the structural integrity of biofilms and is the crucial first step in biofilm formation. Following adherence, these cells proliferate as yeast-form cells and remain attached to the anchor layer. Subsequently, pseudohyphal and hyphal cells begin to form from these dividing yeast-form cells, and they continue to elongate and proliferate throughout the completion of biofilm formation. Under most *in vitro* experimental conditions, the biofilm is considered mature by ~24 h and is characterized by a structured mixture of yeast-form, pseudohyphal and hyphal cells that are surrounded by an extracellular matrix that is composed of proteins, carbohydrates, lipids and nucleic acids. The biofilm matrix functions as a protective physical barrier from the environment and provides structural integrity to the biofilm, and it is critical in the resistance of mature biofilms to mechanical disruption. The presence of the matrix is most pronounced during and after the maturation stage of biofilm development. At those stages, the bio-film has a thick (several hundred micrometres), structured appearance with distinct layers (yeast-form cells towards the solid surface, hyphal and pseudohyphal cells extending away from the surface, and the extracellular matrix encompassing all cells). The hyphae that form in the later stages of *C. albicans* biofilm development form a scaffold that supports the different components of the biofilm, thereby contributing to the overall architectural stability of the biofilm structure. Once fully matured, the biofilm slowly disperses predominantly yeast-form cells that bud off from hyphae, thus contributing to the dissemination of the infection[1,32]. Although the general progression of biofilm development and the properties of the mature structure have been studied primarily *in vitro*, many of the same features (for example, a several-hundred- micrometre-thick biofilm consisting of yeast-form cells at the base, hyphal and pseudohyphal cells throughout the structure, and a thick extracellular matrix) have also been observed *in vivo* in rabbit, mouse and rat catheter models, *in vivo* in rat denture models, and *in vivo* and *ex vivo* in mouse vaginal models. *C. albicans* biofilms have also been extensively observed in clinical settings, for example on catheters and dentures of patients[33–42].

Box 1

Biofilm assays

In vitro biofilm assays typically involve adherence of cells to a solid surface (generally for 1–1.5 h), a wash to remove non-adherent or weakly adherent cells, and a maturation step (typically 24–48 h) with either shaking or continuous flow across the surface. *Candida albicans* biofilms can form under a range of environmental conditions *in vitro*,

such as different surface materials (for example, polystyrene plates or silicone squares), different growth media, different temperatures and different shaking or flow rates[151]. Whereas biofilms can be directly observed using some *in vitro* assays (for example, confocal laser-scanning microscopy and biofilm formation on silicone squares)[46,48], other methods rely on correlated readouts for biofilm formation (for example, dry weight and optical density)[46,61,68,152]. Measurements of cell viability (for example, metabolic reduction of the tetrazolium salt reagent XTT)[153,154] and dye uptake (for example, crystal violet incorporation into the biofilm)[155] are also commonly used as readouts to evaluate biofilm formation *in vitro*.

Regarding *in vivo* assays, rabbit, mouse and rat catheter models, avascular subcutaneous catheter models, rat denture models and mouse vaginal models have all been used to study biofilm formation in a host[33–35,38,39,42,156,157]. In the future, we can anticipate the results from additional model systems currently in development, such as those involving live imaging of animals and optical scanners[149,150,158].

Regulation of biofilm development

In light of the large number of differences between biofilm cells and planktonic cells, it is not surprising that more than 50 transcriptional regulators have been linked to the formation of *C. albicans* biofilms[1,43–45] (FIG. 2; TABLE 1). Cells in which those genes have been deleted exhibit a range of phenotypes with regard to biofilms, including moderately reduced thickness of the mature biofilm (for example, an *ndt80* deletion mutant), extremely thin biofilms (for example, an *efg1* deletion mutant), abnormally thick biofilms (for example, an *rfx2* deletion mutant), lack of hyphal cells within the biofilm structure (for example, a *bcr1* deletion mutant) and reduced adherence to the solid substrate (for example, an *rfg1* deletion mutant)[46,47]. Genetic screens that were carried out with collections of *C. albicans* deletion mutants identified a 'core' set of nine regulators (Bcr1, Brg1, Efg1, Flo8, Gal4, Ndt80, Rob1, Rfx2 and Tec1) that is required for biofilm development both *in vitro* on polystyrene plates and on silicone squares, and *in vivo* in the rat catheter and denture models[46–48]. Chromatin immunoprecipitation experiments revealed that these nine regulators form a highly interconnected transcriptional network (FIG. 2) in which the individual regulators control each other and, in addition, ~1,000 target genes[46,47]. The complex, highly interconnected structure of this network is similar to those of regulatory networks controlling complex processes such as white–opaque switching in *C. albicans*[49], pseudohyphal growth in *Saccharomyces cerevisiae*[50] and even development of mouse pluripotent embryonic stem cells[51]. Some of the target genes in the biofilm network themselves encode transcriptional regulators, several of which were subsequently shown to also be required for biofilm formation (for example, Grf10 and Sfp1) (TABLE 1). Other regulatory targets are required for specific processes in biofilm formation, such as hyphal growth (for example, Hwp1), the production of the extracellular matrix (for example, Gsc1 and Mnn1) and drug resistance (for example, Cdr1 and Mdr1). However, the functions of most target genes remain to be determined, as most of them lack clear homology with characterized genes from other organisms[46]. Consistent with this observation, orthology mapping suggests that the targets of the *C. albicans* biofilm network are enriched for genes

that evolved more recently, after the *Candida* clade diverged from a non-pathogenic ancestor[46]. Thus, many of the target genes in the biofilm network are 'novel' genes (that is, genes that have rapidly evolved or were acquired by horizontal gene transfer) and their functions remain to be determined. In the following sections, we discuss recent updates to this regulatory circuit that are crucial for the individual steps of *C. albicans* biofilm formation.

Adherence

Although we lack detailed understanding of how *C. albicans* cells adhere to surfaces, current evidence indicates that the nature of the surface, molecules involved in quorum sensing, host hormones and the presence of other interacting microorganisms can influence this initial step of biofilm formation[52–59]. The specific genes involved in adherence, both in the early stages of biofilm formation and in the later maturation phases, thus have important roles in biofilm formation and maintenance. For example, a group of ten genes (including *ALS1*, *ALS2*, *ALS4* and *PGA6*) is upregulated early in biofilm formation, whereas a different set of ten genes (including *IFF4*, *IFF6*, *PGA32* and *PGA55*) is upregulated at later time points[47]. Most of these genes encode proteins that exhibit homology to documented fungal adhesins[52,60] and contain glycosylphosphatidylinositol (GPI) anchors; they were designated 'adhesion genes' based on experiments that established their requirement for adhesion of *C. albicans* in at least one assay measuring cellular adherence (of note, not necessarily in assays of biofilm formation). Of the ten 'early' adhesion genes, five (*ALS1*, *ALS2*, *ALS3*, *EAP1* and *MSB2*) have been shown to be directly required for biofilm formation based on genetic studies in which they have been deleted[46,61–65]. Considerably less is known about the 'late' adhesion genes, as most have not been directly tested for either adhesion or biofilm formation[46,66]. Several models have been proposed to explain the chemical basis for adhesion-mediated adherence to biotic and abiotic surfaces and were recently reviewed[67].

However, the process of adherence involves many more genes than those encoding adhesins. Using a silicone surface in an *in vitro* flow cell, more than 30 transcriptional regulators were shown to be required for adherence[64]. At least four of these regulators (Bcr1, Ace2, Snf5 and Arg81) are also required for adherence to polystyrene, and one of them (Bcr1) is required for biofilm formation under all conditions reported[46,48,64]. The role of Bcr1 is not surprising given its regulatory role in the expression of several cell wall proteins necessary for adherence (for example, Als1, Als3 and Hwp1)[48,61,68,69]. It may seem counterintuitive that so many other transcriptional regulators are also required for the initial adherence step in biofilm formation; however, this observation indicates how little we currently understand about this step in biofilm formation.

Production of the extracellular matrix

A mature *C. albicans* biofilm is encased in an extracellular matrix that is a mixture of glycoproteins (55%), carbohydrates (25%; largely α-mannan and β-1,6-glucan polysaccharides, and, to a lesser extent, β-1,3-glucan), lipids (15%) and nucleic acids (5%)[70]. More than 500 proteins have been identified by mass spectrometry in the matrix, and many of them are enzymes[70]. It has been noted that many of the enzymes found in the matrix lack canonical secretion sequences and are thus unlikely to be secreted enzymes,

which suggests that the matrix contains a small fraction of lysed cells in addition to other components[70]. Although not yet experimentally validated, these extra-cellular enzymes could have several functions, including the digestion of cellular components to provide nutrients for *C. albicans*, promotion of cell dispersion and the protection of fungal cells from destruction by host immune cells[70]. Very little is known about the presence of extra-cellular DNA (eDNA) in the *C. albicans* biofilm matrix, but similar to bacterial biofilms, there is evidence that eDNA contributes to the stability of *C. albicans* biofilms. In agreement with this notion, one study has shown that eDNA is present at all stages of biofilm development and that treatment with DNase decreases biofilm biomass at later time points in biofilm formation[71].

Two transcriptional regulators, Rlm1 and Zap1 (also known as Csr1), have been implicated in matrix production. Deletion of *ZAP1* was shown to increase matrix formation, potentially through the upregulation of Gca1 and Gca2 (REF. 72), which are glucoamylase enzymes that convert long-chain polysaccharides into smaller-chain polysaccharides. Deletion of *RLM1* reduces matrix formation, potentially through downregulation of the Gsc1 (also known as Fks1) β-1,3-glucan synthase subunit[73], which catalyses the formation of β-1,3-glucan. Mutations in genes encoding enzymes that produce polysaccharide components of the matrix (*ALG11*, *MNN4-4*, *MNN9*, *MNN1*, *PMR1*, *VAN1* and *VRG4* involved in mannan synthesis, *BIG1* and *KRE5* involved in β-1,6-glucan synthesis, and *GSC1* involved in β-1,3-glucan synthesis) result in broad defects in matrix synthesis[74], which highlights their crucial role in the production of the matrix and biofilm development.

Formation of hyphal cells

C. albicans can form hyphal cells both in planktonic cultures and during the maturation step of biofilm formation[1,32,75]. Many of the same gene products, including transcriptional regulators and structural proteins, are important for hyphal formation in both of these contexts. For example, several of the previously mentioned core regulators of biofilm formation (for example, Efg1, Ndt80, Rob1 and Tec1) are also involved in the regulation of hyphal growth in planktonic cells; deletion of any one of these genes reduces or even eliminates the formation of hyphae under planktonic conditions[46,76,77]. Further support for the link between hyphal growth in planktonic cells and biofilms comes from a screen of kinase deletion mutants; those with defects in hyphal formation in suspension cultures were also defective in biofilm formation[78]. Furthermore, transcriptional profiling studies of *C. albicans* clinical isolates with varying abilities to form biofilms *in vitro* revealed that strains that formed thicker biofilms had increased expression of hyphal-specific genes (for example, *HWP1*) compared with the strains that formed thinner biofilms[79]. Finally, although the core biofilm regulator Bcr1 is not required for hyphal formation per se, it is required for hyphae to adhere to one another[48], a critical property for maintaining the strength of the biofilm. Consistent with this idea, deletion of either *BCR1* or *HWP1* resulted in reduced retention of cells within a biofilm[61,68]. Thus, hyphae in the *C. albicans* biofilm seem to provide a scaffold for other cells and the matrix, resulting in a resilient structure that is several hundred micrometres thick. The extensive hyphal networks observed in mature *C. albicans* biofilms are a hallmark that distinguish *C. albicans* biofilms from those of closely related species such as *Candida parapsilosis*, which tend to have fewer and shorter hyphae[80]; this

difference may result in the increased strength of *C. albicans* biofilms relative to those of other species[1].

Dispersion

The dispersal of *C. albicans* into the surrounding environment (primarily as round, yeast-form cells) occurs throughout biofilm formation, with greater numbers of cells being dispersed once the biofilm reaches maturity[28,81]. Dispersed cells can initiate the formation of new biofilms, lead to systemic infections in the bloodstream of the host, or disseminate into host tissues, thus spreading the infection. The transcriptional regulators Ume6, Nrg1 and Pes1 (also known as Nop7) have all been linked to dispersal; overexpression of *UME6* reduces dispersal, whereas overexpression of either *NRG1* or *PES1* increases the number of cells released from the biofilm[28,82]. Set3, a component of a chromatin-modifying complex, is also required for dispersal, probably by being recruited to specific genes by the transcriptional regulator Nrg1 (REFS 81,83). In addition, deletion of the molecular chaperone Hsp90 and the cell wall protein Ywp1 reduced dispersal[84–86]. Moreover, although both nutrient limitation and nutrient excess have been hypothesized to influence dispersion[87], recent evidence indicates that more cells are dispersed in nutrient-rich medium than in nutrient-poor medium[28,88]. However, future studies are required to determine how these environmental changes lead to changes in dispersion.

Specific properties of biofilm cells

Cells in *C. albicans* biofilms have several properties that are distinct from those of planktonic cells. A combination of genetic screens, genome-wide transcriptional profiling and proteomics has been used to study *C. albicans* bio-film development[46–48,64,70,89–91], and these approaches have uncovered major differences between cells of biofilms and those of exponentially growing suspension cultures. For example, RNA-sequencing studies identified ~1,600 genes that were upregulated and ~600 genes that were downregulated in cells in biofilms compared with cells grown in suspension cultures[46], accounting for approximately 35% of the protein-coding genes in the haploid genome. When gene expression patterns were compared between biofilms and suspension-cultured cells in the stationary phase, additional differences were uncovered. For example, some metabolic pathways that are down-regulated in cells during stationary growth (compared with exponentially growing cells) have been reported to be further downregulated in cells of biofilms. However, other pathways that are downregulated in stationary growth (compared with exponential growth) are highly active in biofilm cells[47,92]. The differences between bio-film and planktonic cells extend beyond their metabolic activities; for example, several secreted proteases are upregulated in biofilm cells but not in planktonic cells, which results in distinct proteolytic cleavage profiles for each growth state[91]. Moreover, differential gene expression profiles have also been noted for specific stages of biofilm formation; for example, ~250 genes were upregulated and ~150 genes were downregulated in cells that had just adhered to a surface compared with the cells that are not adhered in the same medium[47]. Finally, when compared with 'standard' planktonic cells, the yeast-form cells that disperse from mature biofilms seem to be more virulent and to have increased abilities to adhere to surfaces to form new

biofilms[28]. In the following section, we discuss two of these differences, increased drug resistance and altered detection by the host immune system.

Antifungal drug resistance

C. albicans biofilms are resistant to currently available antifungal drugs, although this resistance is less pronounced for the echinocandin class of antifungals[93,94]. This resistance is due to a combination of factors, including increased expression of drug efflux pumps (even in the absence of drugs), protective features of the extracellular matrix, and the existence of 'persister' cells in the biofilm (see below) (FIG. 3). Drug resistance is a serious issue for treating biofilm-based infections; in fact, the azole class of drugs, a common initial therapeutic for *C. albicans* infections, is largely ineffective against biofilms (BOX 2). Given the limited options, when a device-localized biofilm infection is suspected, removal of the medical device is typically recommended instead of antifungal treatments[95].

Box 2

Antifungal drugs

Four major classes of antifungal drugs are in common use to treat invasive fungal infections[25,100]. The oldest class of antifungal drugs is the polyenes (for example, amphotericin B), which are fungicidal against *Candida albicans*. Polyenes interact with ergosterol in the fungal cell membrane, forming pores that destabilize the membrane and induce lethal leakage of the cytoplasm. Azoles (for example, fluconazole and voriconazole) inhibit a demethylase enzyme (Erg11 in *C. albicans*) in the ergosterol biosynthesis pathway, which results in the depletion of ergosterol. Azoles also lead to the accumulation of toxic methylated sterol intermediates. Azoles, the most commonly used class of antifungals, have a fungistatic effect on *C. albicans*. Antifungals of the newest class, the echinocandins (for example, caspofungin), have a fungicidal effect on *C. albicans*. They inhibit the synthesis of β-1,3-glucan, which is important for cell wall crosslinking; this inhibition affects cell wall integrity and ultimately causes osmotic lysis of the cell. The final, less commonly used class of antifungal drugs is made up of the pyrimidine or nucleoside analogues, of which flucytosine (5-FC) is a common example. 5-FC is converted by the fungal cell into compounds that mimic nucleotides that become incorporated into DNA or RNA, interfering with fungal DNA replication and protein synthesis.

Common mechanisms of azole antifungal drug resistance include upregulation of (or point mutations within) genes whose products are directly inhibited by antifungal drugs (for example, Erg11), compensatory mutations or regulatory changes elsewhere in targeted pathways (for example, Erg3 in the ergosterol biosynthesis pathway), and upregulation of efflux pumps that export drugs from the cell (for example, Cdr1 and Mdr1)[100].

C. albicans has two main classes of efflux pumps, the major facilitator (MF) and ATP binding cassette (ABC) transporter superfamilies[96–99]. Several efflux pumps are upregulated in the presence of antifungal drugs in planktonic cells, and mutations that lead to the

constitutive upregulation of efflux pumps such as mutations in the promoters of the genes encoding Cdr1, Cdr2 or Mdr1 (or in transcriptional regulators that control their expression) are frequently associated with increased resistance to antifungal drugs[100]. The upregulation of genes encoding efflux pumps (even in the absence of drugs) seems to be a core part of the biofilm transcriptional programme[46,47]; several efflux pumps are upregulated early during the biofilm developmental process, within 6 h of adherence. Some of these efflux pumps are only transiently upregulated (for example, Nag3 and Nag4)[47], whereas others (for example, Cdr1, Cdr2 and Mdr1) remain upregulated in mature biofilms[46,98,99,101–103].

In addition to contributing to the structural features of the biofilm itself, the extracellular matrix is thought to sequester antifungal drugs and provide a physical barrier. Both eDNA and polysaccharides in the matrix have been found to contribute to antifungal resistance; compromising either of these components (for example by treatment with DNase I or β-1,3-glucanase, or by deletion of genes that encode enzymes involved in the synthesis of mannan, β-1,6-glucan or β-1,3-glucan) were shown to increase sensitivity to antifungal drugs[74,104–106]. Likewise, the addition of exogenous β-1,3-glucan to medium was found to increase the resistance of planktonic cells to fluconazole[104]. Moreover, the matrix component β-1,3-glucan has also been shown to bind to and sequester the antifungal drug amphotericin B[107].

During *C. albicans* biofilm development, but not during planktonic growth, persister cells develop stochastically by an unknown mechanism[108]. These persister cells can revert back to metabolically active cells and contribute to the reformation of the biofilm once the antifungal drug has been removed[109]. Indeed, following treatment of a *C. albicans* biofilm with high doses of amphotericin B, a fraction of cells remained viable at drug concentrations well above those that killed most cells in the biofilm[108,110,111]. Based on extrapolations from persister cells in other microbial species, two hypotheses have been advanced: *C. albicans* persister cells exhibit low metabolic activity, and persister cells are stochastically upregulated for the stress response at the time the drug is added. Consistent with both hypotheses, a recent proteomics study found that the protein levels of many metabolic enzymes (including several enzymes involved in ergosterol biosynthesis, which is the target of azole class of drugs) were downregulated in persister cells compared with control cells in a biofilm, while the levels of proteins involved in the stress response and proteins maintaining the integrity of the cell wall were highly upregulated[92]. The fact that a number of genes and metabolic pathways involved in glycolysis, the electron transport chain and the tricarboxylic acid cycle are downregulated in biofilm cells, even below the levels observed in planktonic cells growing in the stationary phase, is also consistent with a link between low rates of metabolism and drug resistance in biofilms[47]. Finally, a recent study found that levels of the alkyl hydroperoxide reductase Ahp1 (a cell wall peroxidase involved in the response to oxidative stress) directly correlated with the fraction of persister cells that survived exposure to amphotericin B[112], a result consistent with the persister cells expressing higher levels of the gene.

Evasion of host immunity and detection

A range of host immune cells, including epithelial cells, neutrophils, macrophages and dendritic cells, have key roles in the recognition of and elimination of *C. albicans* (Supplementary information S1 (box)). *C. albicans* uses several strategies to evade the host immune response, many of which are linked to biofilms. For example, the hyphal cells in the top layer of mature biofilms 'mask' their β-glucans (a cell wall feature recognized by the immune system), have the ability to penetrate epithelial cell layers during invasive growth, and mediate escape from phagocytic cells by physically piercing the phagocytic cell[113]. The differential expression of genes in cells in biofilms compared with planktonic cells has been linked to immune evasion mechanisms of the former. The activation of the host complement system is blocked by *C. albicans* Pra1 (an antigenic, zinc-binding cell surface protein) and Gpd2 (a glycerol-3-phosphate dehydrogenase that is found at the cell surface), as well as members of the secreted aspartyl protease (Sap) family[114]; these proteins are all highly expressed during biofilm formation. Moreover, Msb2, a mucin that functions as a cell wall damage sensor and is also highly expressed in biofilms, protects *C. albicans* from host-secreted antimicrobial peptides[115,116].

Although neutrophils may surround biofilms *in vivo*, they often fail to engulf biofilm cells, possibly because of the protective matrix[117]. In the presence of biofilm cells, neutrophils also fail to trigger a reactive oxygen species response, although the mechanisms for this are unknown[118]. Building on this observation, it has also been shown that the extracellular matrix of *C. albicans* biofilms helps to block the release of neutrophil extracellular traps (NETs), thus reducing neutrophil- mediated killing[119]. In support of this, decreased production of the extracellular matrix leads to increased NET levels and decreased fungal proliferation[119].

Mature *C. albicans* biofilms consist of a mixture of distinct cell types (including yeast-form, hyphal and pseudo-hyphal cells; FIG. 1) and distinct microenvironments (for example, hypoxic niches within the depths of the biofilm and aerobic niches near the biofilm surface); this heterogeneity also extends to the transcriptomes and proteomes of individual cells. This broad cellular diversity may be a key aspect that contributes to the resistance of biofilms to antifungal agents and the host immune system because any threat to the biofilm must address multiple targets rather than just a single type of cell. Coupled with the extracellular matrix and the physical architecture of the biofilm itself, given that its large, interconnected nature functions as a barrier to innate immune cells, it is not surprising that the biofilm state is the preferred state of *C. albicans* in stressful environments, such as in the presence of antifungal drugs or the host immune response.

Polymicrobial biofilms

The human microbiota includes bacteria, archaea and fungi, providing numerous opportunities for physical and chemical interactions between different species and even different kingdoms. Dental caries (oral cavities), periodontitis (gum disease), otitis media (ear infections), diabetic wound infections, chronic pulmonary infections (for example, cystic fibrosis), urinary tract infections and medical device infections are among the many ailments that are caused by polymicrobial biofilm formation[120] (FIG. 4a). Polymicrobial

infections are observed at an even higher frequency in immunocompromised patients[32,120,121].

C. albicans is the fungal pathogen most frequently isolated from mixed bacterial–fungal infections (FIG. 4a). For example, *Staphylococcus aureus* and *C. albicans* are often co-isolated from neonatal polymicrobial bloodstream infections[122]. Biofilms containing *C. albicans* and *Streptococcus mutans* or *Streptococcus gordonii* have been isolated from denture stomatitis and dental carries[123–126], whereas *C. albicans* and *Pseudomonas aeruginosa* are commonly co-isolated from skin and lung infections[127]. Scanning electron microscopy studies of dual-species biofilms has revealed that cells from species such as *Staphylococcus epidermidis*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and various *Streptococcus* species bind to *C. albicans* hyphal cells present in a biofilm[121,128] (FIG. 4b). Other studies have demonstrated that *C. albicans* adhesion proteins (for example, Als1, Als2 and Als3), hyphal wall proteins (for example, Hwp1) and transcription regulators (for example, Bcr1 and Tec1) play crucial roles in the interactions between *C. albicans* and bacterial species such as *S. epidermidis*, *S. gordonii* and *S. aureus*[129–131]; deletion of these proteins results in fewer bacteria bound to *C. albicans* as well as weaker adhesion of interacting bacteria. Several of these *in vitro* findings have also been observed in a murine model of oral candidiasis[132].

Some relationships between *C. albicans* and bacterial species are synergistic, providing protection to one or both species in the context of dual-species biofilms[133] (FIG. 4c). For example, when *C. albicans* and methicillin-resistant *S. aureus* (MRSA) strains were grown together in a biofilm, the presence of *C. albicans* seemed to protect the MRSA strain from elimination by vancomycin, which is an antibiotic that would normally be effective against MRSA[134,135]. A recent study linked this protection to the *C. albicans* extracellular matrix; both increased β-1,3-glucan production and exogenous β-1,3-glucan supplementation increased the survival of MRSA in the presence of vancomycin, and this protective effect was reduced if β-1,3-glucan was disrupted or if its synthesis was blocked[136]. In another study, dual-species biofilms were formed between *C. albicans* and either *Bacteroides fragilis* or *Clostridium perfringens*, which are two anaerobic bacterial species that are present in the human gut. *C. albicans* biofilms provided a protective hypoxic microenvironment that enabled the growth of these strictly anaerobic bacteria within the biofilm, even though the external environment was aerobic[137] (FIG. 4c). Moreover, when cultured together under planktonic conditions in the presence of ambient oxygen, these anaerobic bacteria induced *C. albicans* to form 'mini-biofilms', which are free-floating cellular aggregates resembling miniature biofilms, that incorporated the bacteria within these structures, enabling *B. fragilis* and *C. perfringens* to proliferate under otherwise toxic conditions[137]. Thus, the presence of a *C. albicans* biofilm enables the proliferation of anaerobic pathogens in otherwise-hostile oxygen-rich environments; moreover, the bacteria seem to induce the formation of these protective structures. However, *C. albicans*–bacteria interactions are not confined to cooperation; for example, *P. aeruginosa* has been shown to kill *C. albicans* cells after attachment to *C. albicans* hyphae[138], although the underlying mechanism is not understood in detail.

Interspecies interactions are not limited to direct physical contact; some rely on the secretion and diffusion of signalling molecules and others on local environmental changes (for example, alterations in pH, oxygen or carbon dioxide levels) to influence the behaviour of one species towards the other[32,133] (FIG. 4d,e). For example, studies on the vaginal microbiota have revealed that *Lactobacillus* species lower the local pH (by releasing lactic acid), which results in the inhibition of *C. albicans* initial adherence to the vaginal mucosal surface[139] (FIG. 4e). Extensive research on the crosstalk between *P. aeruginosa* and *C. albicans* has revealed that each species secretes a similarly structured quorum sensing molecule that can be detected by the other species. For example, the 12-carbon acyl homoserine lactone, which is secreted by *P. aeruginosa*, inhibits *C. albicans* hyphal growth[140,141] (FIG. 4d). Similarly, *Enterococcus faecalis* secretes the bacteriocin EntV, which inhibits *C. albicans* hyphal and biofilm formation and disrupts mature *C. albicans* bio-films[142]. Finally, the LuxS quorum sensing system in *S. gordonii* is needed for the increase in biomass observed when *S. gordonii* and *C. albicans* form a dual-species bio-film[125]. The *S. gordonii luxS* mutant strain is less capable of inducing *C. albicans* hyphal growth than the wild-type *S. gordonii* strain and causes an overall reduction in biomass of the dual-species biofilm formed. This reduction in biomass is not due to a general biofilm defect in the *luxS* strain; the single-species biofilms formed by the *luxS* and wild-type strains are similar in biomass[125]. Other fascinating, but less explored, interactions include the sharing of nutrients and the exchange of electrons between microbial species during biofilm formation[143,144] (FIG. 4f). The latter interaction is particularly intriguing in the context of polymicrobial biofilms, as it enables microorganisms of different species to gain energy from a series of reactions that a single species might lack. This strategy has been well documented for methane-producing and anaerobic polymicrobial biofilms found in the environment[145]. To our knowledge, the positive feedback loop between phenazine production in *P. aeruginosa* and ethanol production in *C. albicans* is the only instance in which such a strategy has been documented for polymicrobial biofilms that are relevant to human health[146,147]. Exposure to sub-toxic concentrations of phenazines results in the increased production of fermentation products, such as ethanol, by *C. albicans*. In response to the resulting increased ethanol levels, *P. aeruginosa* increases production of phenazine and promotes its own biofilm formation. This interaction has been shown to increase *P. aeruginosa* biofilm formation on bronchial epithelial cells and could, in principle, lead to increased virulence for both species in the host[146,147].

Other interactions between *C. albicans* and bacterial partners can also affect virulence during an infection[133]. For example, when *C. albicans* and specific oral *Streptococcus* species, such as *Streptococcus oralis*, *S. sanguinis*, or *S. gordonii*, were placed in a flow-cell-based oral-cavity infection model, the two species demonstrated a cooperative interaction that increased tissue invasion and colonization compared with monotypic infections[148]. Similar synergies in virulence have been observed for other *C. albicans*–bacterial dual-species interactions; for example, higher host mortality was observed when *S. aureus* and *C. albicans* were introduced together at sub-lethal doses in a mouse peritonitis infection model compared with infections with either species alone[123].

The broad range of synergies that have been observed between two or more microorganisms has implications for the treatment of infections. For example, treatment of a polymicrobial

biofilm in the catheter of a patient is particularly difficult, often leading to the removal of the device instead. Further studies are needed to understand the complexity of polymicrobial biofilm infections and interspecies interactions to guide the development of new therapeutic strategies.

Conclusion

The medical impact of *C. albicans* on its human host depends on its ability to form biofilms, which are densely packed communities of cells that adhere to a range of biotic and abiotic surfaces. The specific characteristics of biofilms, especially their resistance to existing antifungal drugs, their ability to evade components of the host immune response, their stability to mechanical forces, and their ability to seed new infections, make them an important clinical problem. Work to date has identified numerous potential starting points for more focused examinations of specific stages of the biofilm life cycle. More targeted studies of the initial adhesion of *C. albicans* cells to solid surfaces, the subsequent development of mature biofilms, the formation of the extracellular matrix, the formation of persister cells, and the mechanism of dispersion have identified numerous gene products required for each of these steps[28,47,70,92], although the function of many of them is unknown. Recent work on dual-species biofilms and the protection that they can provide for each species has revealed fascinating ways in which distantly related microorganisms work closely together.

Despite these recent advances, we still lack a clear, comprehensive understanding of the processes involved in the development of a biofilm, even in the simplest case of a pure *C. albicans* biofilm forming under controlled, *in vitro* conditions. For example, we do not know how environmental signals and proteins other than transcriptional regulators (such as kinases and cell wall proteins) feed into the biofilm regulatory circuitry. Likewise, more information is needed about the temporal regulation of biofilms, particularly how cells 'decide' to advance to later stages of the life cycle. We also do not understand how the different cell types in a biofilm are produced and organized in a biofilm. Another intriguing question concerns whether biofilm cells have a 'cell memory' and whether it persists in cells that are dispersed from bio-films. Finally, and perhaps most importantly, the roles of the great majority of genes upregulated during biofilm formation are simply not known.

Although not strictly a developmental process, bio-film formation could be profitably studied in the future using many of the tools used in developmental biology, such as lineage tracers and distinct fluorescent makers for each cell type. Widespread use of recently developed assays to track biofilm development in live rodent hosts in real time will undoubtedly challenge and change much of our current understanding of biofilms[149,150]. Finally, the study of mixed *C. albicans*–bacterial biofilms, although only beginning, has already revealed unanticipated synergies that further complicate the treatment of biofilms in the clinic. In the end, it is hoped that these studies will lead to new therapies to prevent, disrupt and otherwise render harmless the peculiar ability of *C. albicans* to form biofilms on almost any surface in the human body.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Glossary

Yeast-form cells

Spherical fungal cells that form daughter cells, which bud off from the parent cell.

Pseudohyphal cells

Ovoid chains of fungal cells that contain constrictions (rather than septa) at the cell junctions.

Hyphal cells

Elongated, cylindrical fungal cells that contain complete septa at the cell junctions.

Extracellular matrix

A protective physical barrier that surrounds cells in a biofilm and is composed of proteins, carbohydrates, lipids and nucleic acids.

Persister cells

Non-dividing fungal cells with decreased metabolic activity that are resistant to antimicrobial agents.

White–opaque switching

The ability for *Candida albicans* cells to switch between the 'white' and 'opaque' phenotypic cell types. The switch occurs epigenetically; that is, without a change in the primary DNA sequence of the genome.

Horizontal gene transfer

The process through which genetic material is transferred between microorganisms through mechanisms such as transformation, conjugation and transduction. This process is distinct from vertical gene transfer, in which genetic material is transferred from mother cells to daughter cells.

Quorum sensing

A method of communication that allows microorganisms to sense cell density and microbial community composition and respond as a group. The process involves the production and detection of soluble quorum sensing molecules.

Glycosylphosphatidylinositol (GPI) anchors

Post-translational modifications of proteins in which a glycolipid is covalently attached and anchors the protein in the plasma membrane.

Flow cell
A light microscopy method for observing biofilm formation *in vitro* under laminar flow conditions.

Glucoamylase
An enzyme that catalyses the hydrolysis of glucosidic linkages in starch, which releases glucose.

Glucan synthase
A glucosyltransferase enzyme that catalyses the synthesis of glucans, which are critical polysaccharide components of the fungal cell wall and the extracellular matrix.

Chromatin-modifying complex
A protein complex that alters the chromatin structure.

Complement system
A group of proteins that, when activated, mediate the innate immune response and inflammatory response to a pathogen.

Mucin
A glycosylated protein that is the major component of mucus.

Ergosterol
A sterol component of the fungal cell membrane necessary for membrane fluidity.

Bacteriocin
A pore-forming peptide produced by some bacterial and archaeal species that is toxic to other microorganisms.

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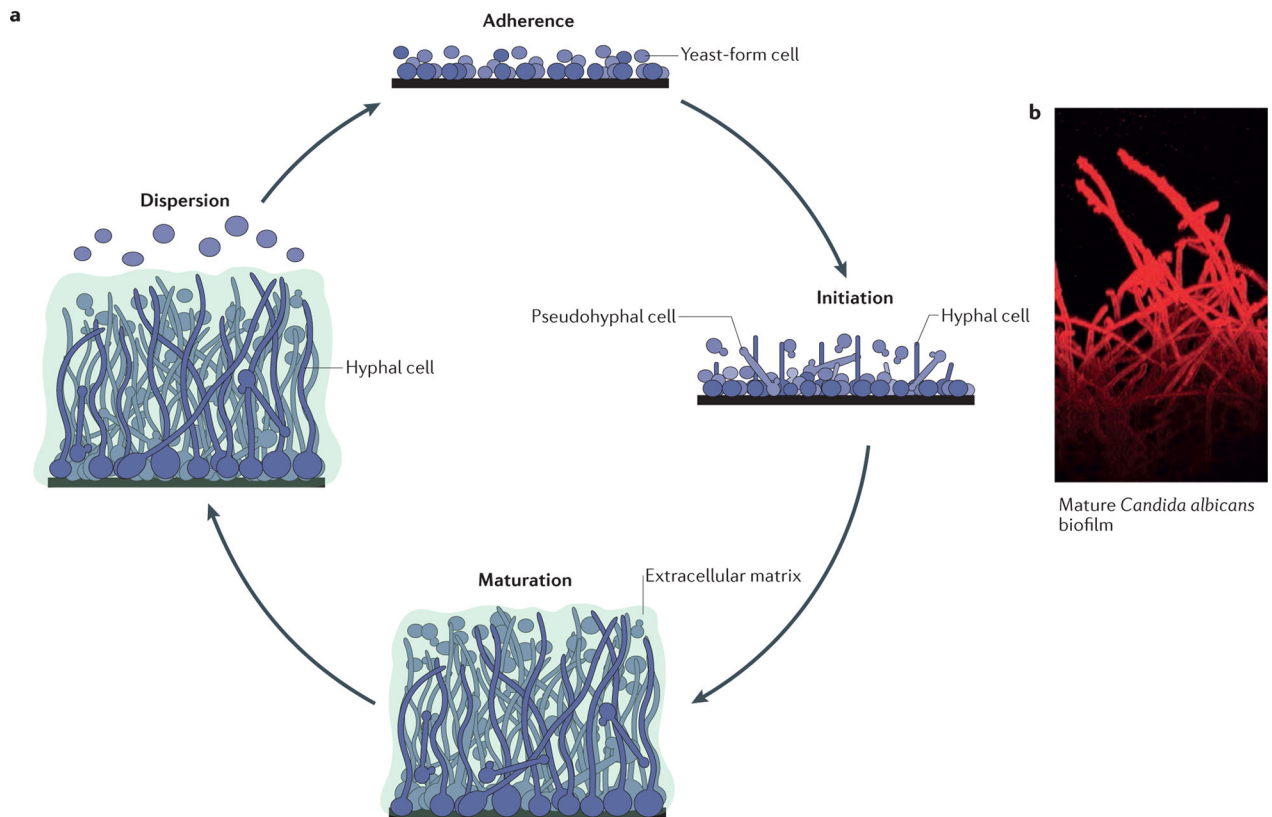
Figure 1. Formation of *Candida albicans* biofilms
a | The formation of *Candida albicans* biofilms has been divided into four major stages: adherence of round yeast-form cells to a surface; initiation of biofilm formation, during which the cells adhered to the surface form a basal layer that contains yeast-form, pseudohyphal and hyphal cells (also known as the proliferation stage); maturation into a complex, structured biofilm, in which cells are encased in the extracellular matrix; and dispersion of yeast-form cells from the biofilm to seed new sites. **b** | A confocal laser-scanning microscopy image of the side view of a mature *C. albicans* biofilm is shown, with the long hyphal cells clearly visible. The dye (concanavalin A–Alexa Fluor 594 conjugate) used for imaging does not penetrate to the bottom of the biofilm; hence, the yeast-form cells attached to the solid surface are not readily visible. The extracellular matrix is also not visible, as it does not bind the dye. Part **a** modified with permission from the Annual Review of Microbiology, Volume 69 © 2015 by Annual Reviews, http://www.annualreviews.org.
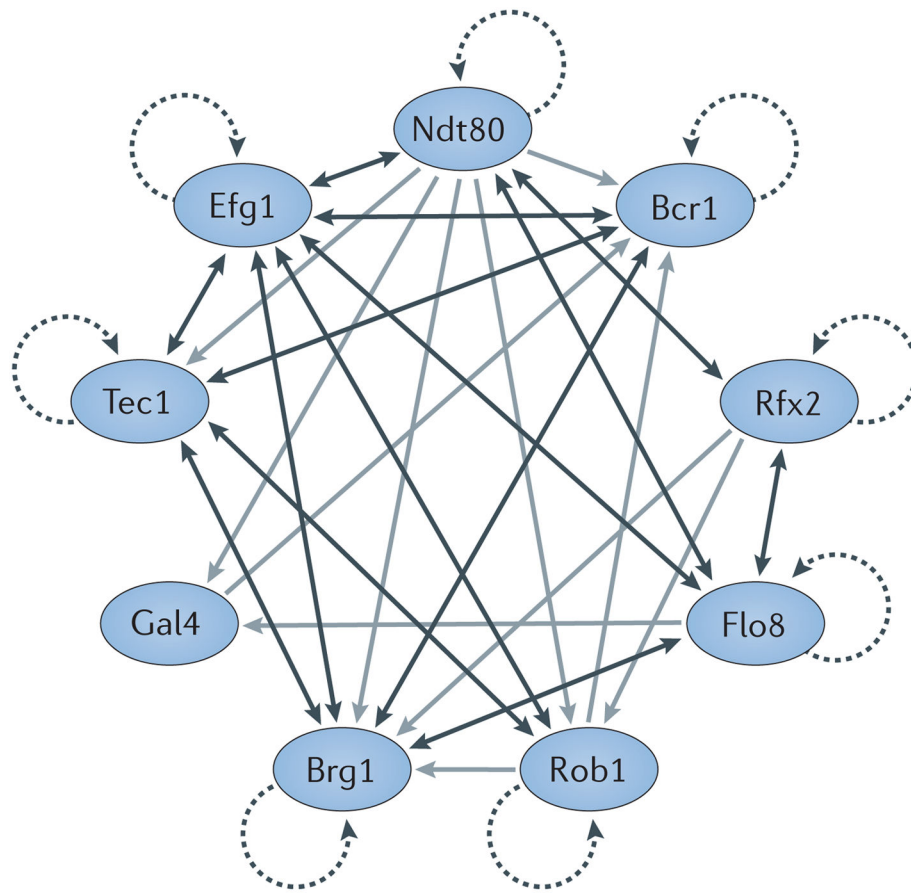
Figure 2. The core transcriptional network controlling biofilm formation in *Candida albicans*
More than 50 transcriptional regulators have been linked to the formation of *Candida albicans* biofilms. The proteins depicted are a 'core' set of nine regulators (Ndt80, Bcr1, Rfx2, Flo8, Rob1, Brg1, Gal4, Tec1 and Efg1) that is required for biofilm development. Autoregulation is indicated by dotted arrows, direct binding interactions between two regulators that each regulate the activity of the other are indicated by double-headed dark grey arrows, and direct binding interactions where one regulator controls another regulator are indicated by single-headed light grey arrows. The indicated interactions are based on previously reported chromatin immunoprecipitation data[46,47].
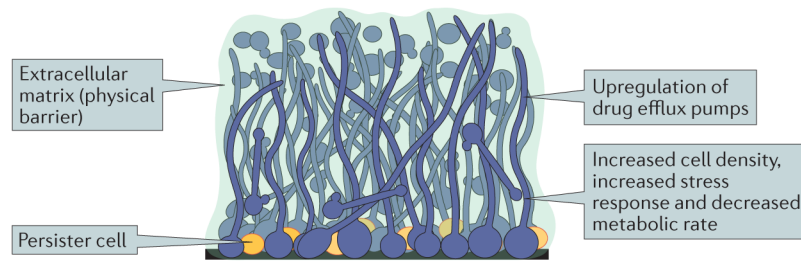
Figure 3. Overview of *Candida albicans* biofilm antifungal drug resistance
Characteristics of *Candida albicans* biofilms that contribute to resistance to antifungal drugs. *C. albicans* biofilms are complex structures that contain round, yeast-form cells, pseudohyphal cells and hyphal cells (shown in blue) that are encased in an extracellular matrix (shown in green). The extracellular matrix functions as a physical barrier to antifungal drugs. Cells within the biofilm also exhibit increased cell density, increased stress response and decreased metabolic activity, which all contribute to antifungal drug resistance. A minority cell population, called 'persister' cells (shown in orange), can exist in the basal layer of the biofilm. Persister cells are non-dividing cells with decreased metabolic activities, making them highly resistant to antimicrobial drugs and likely to seed new biofilm infections after drug treatment. Efflux pumps export drug molecules from the inside the cell to the environment. Expression of these efflux pumps is highly upregulated in *C. albicans* biofilms, even in the absence of antifungal drugs, which contributes to the overall drug-resistant nature of biofilms.
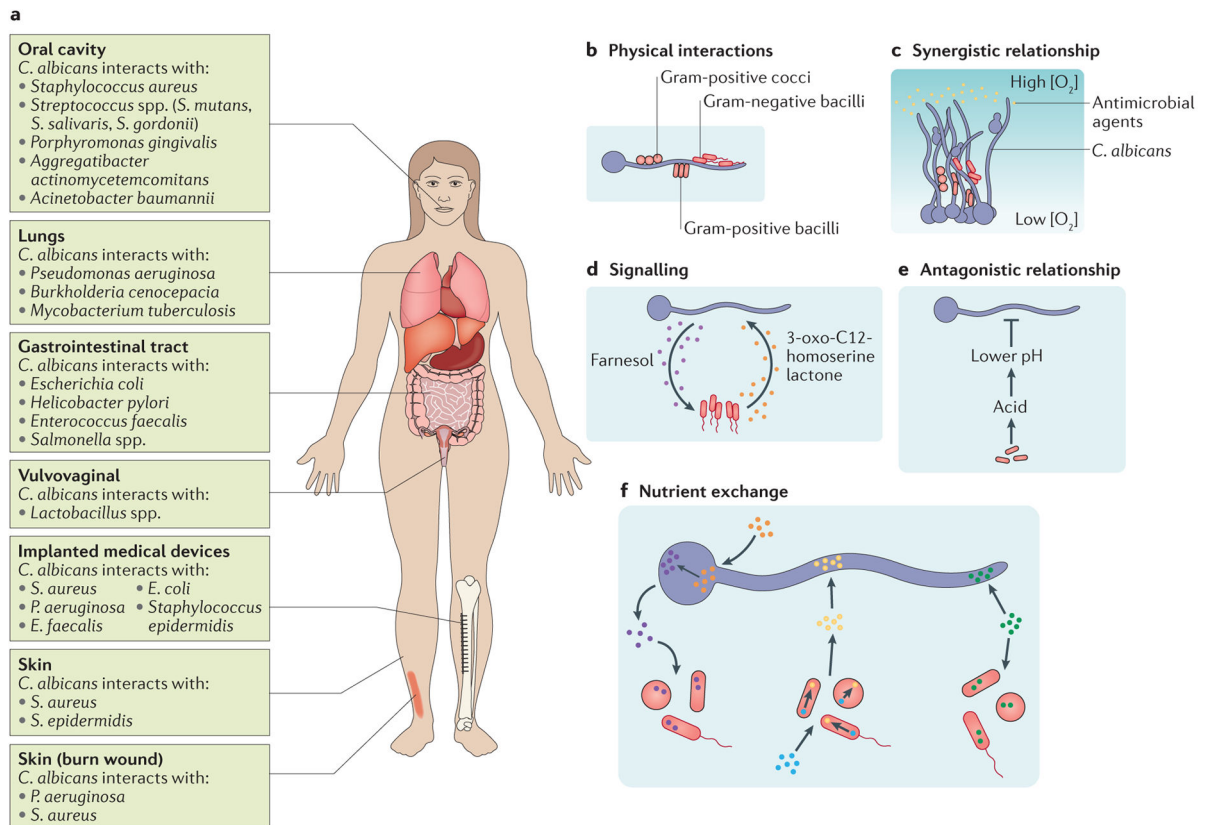
Figure 4. Multi-species biofilm formation

a | Bacterial species most frequently isolated with *Candida albicans* from specific niches of the human body, including the oral cavity, lungs, gastrointestinal tract, vulvovaginal region and skin are listed. Infections resulting from the presence of a burn wound or an implanted medical device are often sites for bacterial–fungal infections; bacterial species most commonly isolated from these infections are listed. **b–f** | Common ways in which *C. albicans* interacts with various bacterial species in the context of a biofilm. **b** | Bacterial cells (Gram-positive cocci and bacilli are shown in red with a black outline; Gram-negative bacilli are shown in red with a red outline) can directly bind to *C. albicans* hyphal cells (shown in blue). **c** | *C. albicans*–bacterial interactions can be synergistic; for example, a *C. albicans* biofilm can physically protect bacteria from antimicrobial agents (shown in yellow) or can protect anaerobic bacteria from high oxygen concentrations by providing a low oxygen niche within the depths of the biofilm. **d** | Signalling molecules produced by bacterial species and *C. albicans* enable inter-kingdom communication within mixed-species biofilms. For example, in *C. albicans*–*Pseudomonas aeruginosa* mixed-species biofilms, the *P. aeruginosa*-secreted signalling molecule 3-oxo-C12-homoserine lactone (shown in orange) can influence the behaviour of *C. albicans*, and the *C. albicans* secreted signalling molecule farnesol (shown in purple) can influence the behaviour of *P. aeruginosa*. **e** | *C. albicans*–bacterial interactions can be antagonistic; for example, acid produced by *Lactobacillus* spp. results in a lower local pH, which inhibits *C. albicans* hyphal formation. **f** | Cells within mixed-species biofilms can also exchange nutrients. For example, cells can share nutrients (shown in green), or one species can utilize available nutrients (blue and orange) and in turn

produce nutrients (yellow and purple) needed by the other species. *S. gordonii*, *Streptococcus gordonii*; *S. mutans*, *Streptococcus mutans*; *S. salivaris*, *Streptococcus salivaris*.

Table 1

Transcriptional regulators involved in *Candida albicans* biofilm formation

ORF	Gene	Transcript levels in biofilm versus planktonic cells*	Core regulators binding to the control region of the gene‡	Biofilm-related processes affected‡	Refs
orf19.6124	*ACE2*	1.15	None detected	Adherence, initiation and hyphal formation	64,159
orf19.2331	*ADA2*	0.14	None detected	Adherence and initiation	64
orf19.7381	*AHR1*	1.15	Bcr1, Brg1, Efg1, Ndt80, Tec1	Adherence and initiation	160
orf19.4766	*ARG81*	−0.16	None detected	Adherence and initiation	64
orf19.723	*BCR1*	1.26	Bcr1, Brg1, Efg1, Gal4, Ndt80, Rob1, Tec1§	Adherence and initiation	46,48
orf19.6874	*BPR1*	−0.26	Bcr1, Brg1, Efg1, Ndt80, Rob1, Tec1	Adherence and initiation	47
orf19.4056	*BRG1*	2.02	Bcr1, Brg1, Efg1, Flo8, Ndt80, Rob1, Rfx2, Tec1§	Adherence and initiation	46
orf19.4670	*CAS5*	−0.82	Efg1, Ndt80	Adherence and initiation	64
orf19.2356	*CRZ2*	1.16	Bcr1, Brg1, Efg1, Ndt80, Rob1, Tec1	Adherence and initiation	64
orf19.3127	*CZF1*	1.33	Bcr1, Brg1, Efg1, Ndt80, Tec1	Adherence, initiation and hyphal formation	64
orf19.3252	*DAL81*	0.62	None detected	Adherence and initiation	64
orf19.610	*EFG1*	−0.87	Bcr1, Brg1, Efg1, Flo8, Ndt80, Rob1, Tec1§	Adherence, initiation and hyphal formation	46,76
orf19.3193	*FCR3*	0.03	Bcr1, Brg1, Ndt80	Adherence and initiation	64
orf19.6680	*FGR27*	1.08	None detected	Adherence and initiation	64
orf19.1093	*FLO8*	1.04	Brg1, Efg1, Flo8, Ndt80, Rfx2§	Adherence, initiation and hyphal formation	47
orf19.5338	*GAL4*	0.07	Flo8, Ndt80§	Adherence and initiation	47
orf19.1358	*GCN4*	1.89	Ndt80	Adherence and initiation	161
orf19.4000	*GRF10*	1.07	Bcr1, Brg1, Efg1, Ndt80, Tec1	Adherence, initiation and hyphal formation	44
orf19.2842	*GZF3*	0.07	Ndt80	Adherence and initiation	47
orf19.4225	*LEU3*	0.18	None detected	Adherence and initiation	64
orf19.5312	*MET4*	0.18	None detected	Adherence and initiation	64
orf19.6309	*MSS11*	−0.10	Rob1	Adherence, initiation and hyphal formation	162
orf19.2119	*NDT80*	0.49	Efg1, Flo8, Ndt80, Rfx2§	Adherence, initiation and hyphal formation	46,163
orf19.2012	*NOT3*	−0.68	None detected	Adherence and initiation	64
orf19.7150	*NRG1*	−0.82	Bcr1, Brg1, Efg1, Ndt80, Rob1, Tec1	Dispersion and hyphal formation	82

ORF	Gene	Transcript levels in biofilm versus planktonic cells[*]	Core regulators binding to the control region of the gene[‡]	Biofilm-related processes affected[‡]	Refs
orf19.4093	PES1	0.12	None detected	Dispersion	28
orf19.2823	RFG1	0.14	Bcr1, Brg1, Efg1, Ndt80	Adherence and initiation	47
orf19.4590	RFX2	1.07	Flo8, Ndt80, Rfx2[§]	Adherence and initiation	47
orf19.7247	RIM101	1.18	Bcr1, Brg1, Efg1, Tec1	Adherence, initiation and hyphal formation	47
orf19.4662	RLM1	0.60	None detected	Production of the extracellular matrix	73
orf19.4998	ROB1	0.01	Efg1, Ndt80, Rob1, Rfx2, Tec1[§]	Adherence, initiation and hyphal formation	46
orf19.5953	SFP1	0.21	Brg1, Ndt80	Adherence and initiation	45
orf19.5871	SNF5	1.24	None detected	Adherence and initiation	64
orf19.7319	SUC1	0.34	None detected	Adherence and initiation	64
orf19.798	TAF14	−0.52	None detected	Adherence and initiation	64
orf19.5908	TEC1	1.28	Bcr1, Brg1, Efg1, Ndt80, Rob1, Tec1[§]	Adherence, initiation and hyphal formation	46,48
orf19.4062	TRY2	−0.14	None detected	Adherence and initiation	64
orf19.1971	TRY3	−0.15	None detected	Adherence and initiation	64
orf19.5975	TRY4	1.38	Ndt80	Adherence and initiation	64
orf19.3434	TRY5	0.00	Bcr1, Brg1, Ndt80	Adherence and initiation	64
orf19.6824	TRY6	1.17	Ndt80	Adherence and initiation	64
orf19.4941	TYE7	0.14	Bcr1, Brg1, Efg1, Ndt80	Hyphal formation	164
orf19.7317	UGA33	1.86	None detected	Adherence and initiation	64
orf19.1822	UME6	1.20	Bcr1, Brg1, Efg1, Ndt80, Tec1	Dispersion and hyphal formation	28,82
orf19.1035	WAR1	0.72	None detected	Adherence and initiation	64
orf19.3794	ZAP1	1.43	Bcr1, Brg1, Efg1	Production of the extracellular matrix	64,72,165
orf19.4767	ZCF28	0.75	None detected	Adherence and initiation	64
orf19.5924	ZCF31	−0.11	Bcr1, Brg1, Efg1, Ndt80, Tec1	Adherence and initiation	64
orf19.5940	ZCF32	−0.53	None detected	Adherence, initiation and hyphal formation	43
orf19.6182	ZCF34	−0.25	None detected	Adherence and initiation	64
orf19.7583	ZCF39	−0.48	None detected	Adherence and initiation	64
orf19.1718	ZCF8	0.04	Brg1, Efg1, Ndt80	Adherence and initiation	64
orf19.6781	ZFU2	1.36	None detected	Adherence and initiation	64

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ORF	Gene	Transcript levels in biofilm versus planktonic cells[*]	Core regulators binding to the control region of the gene[‡]	Biofilm-related processes affected	Refs
orf19.3187	ZNC1	0.34	None detected	Adherence and initiation	64

[*]Transcriptional expression levels comparing biofilm and planktonic cells (as determined by microarrays) are indicated; values are on a log2 scale and are taken from previous reports[46]. Positive values indicate genes whose expression is upregulated in biofilms and negative values indicate genes whose expression is downregulated in biofilms.

[‡]Based on previously published studies of genome-wide chromatin immunoprecipitation followed by DNA microarray (ChIP–chip) data for Bcr1, Brg1, Efg1, Ndt80, Rob1, Tec1 (REF. 46) and chromatin immunoprecipitation coupled with quantitative PCR (ChIP–qPCR) data for Flo8, Gal4, Rfx2 (REF. 47).

[§]Binding by Flo8, Gal4 and Rfx2 has been evaluated only for the control regions of the nine core regulators; all other genes have been evaluated for binding by Bcr1, Brg1, Efg1, Ndt80, Rob1 and Tec1.