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Review

Portrait of *Candida* Species Biofilm Regulatory Network Genes

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Most cases of candidiasis have been attributed to *Candida albicans*, but *Candida glabrata*, *Candida parapsilosis* and *Candida tropicalis*, designated as non-*C. albicans Candida* (NCAC), have been identified as frequent human pathogens. Moreover, *Candida* biofilms are an escalating clinical problem associated with significant rates of mortality. Biofilms have distinct developmental phases, including adhesion/colonisation, maturation and dispersal, controlled by complex regulatory networks. This review discusses recent advances regarding *Candida* species biofilm regulatory network genes, which are key components for candidiasis.

Trends

Candida albicans is the main cause of candidiasis, however, non-*C. albicans Candida* species are now frequently identified as potential human pathogens.

Biofilm formation is a potent virulence factor for a number of *Candida* species, as it confers significant resistance to traditional antifungal therapy.

There are common genetic requirements for biofilm formation; however, much work is needed to complete the picture of biofilm regulatory network genes.

Candida: Both a Commensal and a Pathogen

Of the fungi regarded as human pathogens, members of the genus *Candida* are the most frequently recovered from fungal infections and these *Candida* infections are collectively referred to as candidiasis. The genus *Candida* is an extremely heterogeneous group of over 150 species, but it is well established that only a few of these are implicated in human infectious diseases [1]. Furthermore, it is clear that fungal infections have emerged as important public health problems and candidiasis has been associated with high morbidity and mortality [1].

Candida species normally exist as commensals, but they are opportunistic pathogens with the ability to cause superficial and systemic infections [2]. The prevalence of opportunistic *Candida* infections has dramatically increased over recent decades, and this is particularly evident in immunocompromised individuals [3]. Although most of the cases of candidiasis have been attributed to *C. albicans*, in recent decades improved diagnostic methods and higher levels of resistance to certain antifungals [4] have led to the appearance of NCAC species, particularly *C. glabrata*, *C. parapsilosis*, and *C. tropicalis* [5]. Moreover, the pathogenicity of *Candida* species is facilitated by a number of virulence factors, including dimorphism, secretion of hydrolytic enzymes (such as proteases, lipases, and haemolysins) and the ability to adhere and form biofilm on medical devices and/or the host mucosal epithelium [6].

It is assumed that one of the major contributions to *Candida* species virulence is its versatility to adapt to a variety of different habitats and the formation of surface-attached microbial communities, known as biofilms [5]. Biofilm development by *Candida* species is a fascinatingly intricate process involving fine alterations in gene expression, requiring complex and well coordinated regulation to accomplish the process with high efficiency, both spatially and temporally. Thus, this review examines recent advances about the regulators of biofilm network genes in *Candida* species that are key components of candidiasis.

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Biofilm Formation by *Candida* Species

Biofilms are communities of microorganisms properly organized and embedded in an extracellular matrix [7]. This mode of growth is a potent virulence factor for all *Candida* species. Moreover, *C. albicans*, *C. parapsilosis*, *C. tropicalis* and *C. glabrata* isolates are good biofilm formers, and the presence of biofilms during infection has been related to higher morbidity and mortality rates compared to isolates incapable of forming biofilms [8].

Biofilm formation is a sequential phenomenon which involves attachment, maturation, and detachment [9], as illustrated in Figure 1. Attachment and colonisation of yeast cells to an abiotic or/and biotic surface is the first step of biofilm development (Figure 1A). Initial attachment of *Candida* cells is followed by cell division, this proliferation leading to the formation of a basal layer of anchoring microcolonies [9,10] (Figure 1B), and then subsequent biofilm maturation (Figure 1C). The biofilm maturation is, generally, characterized by the presence of filamentous

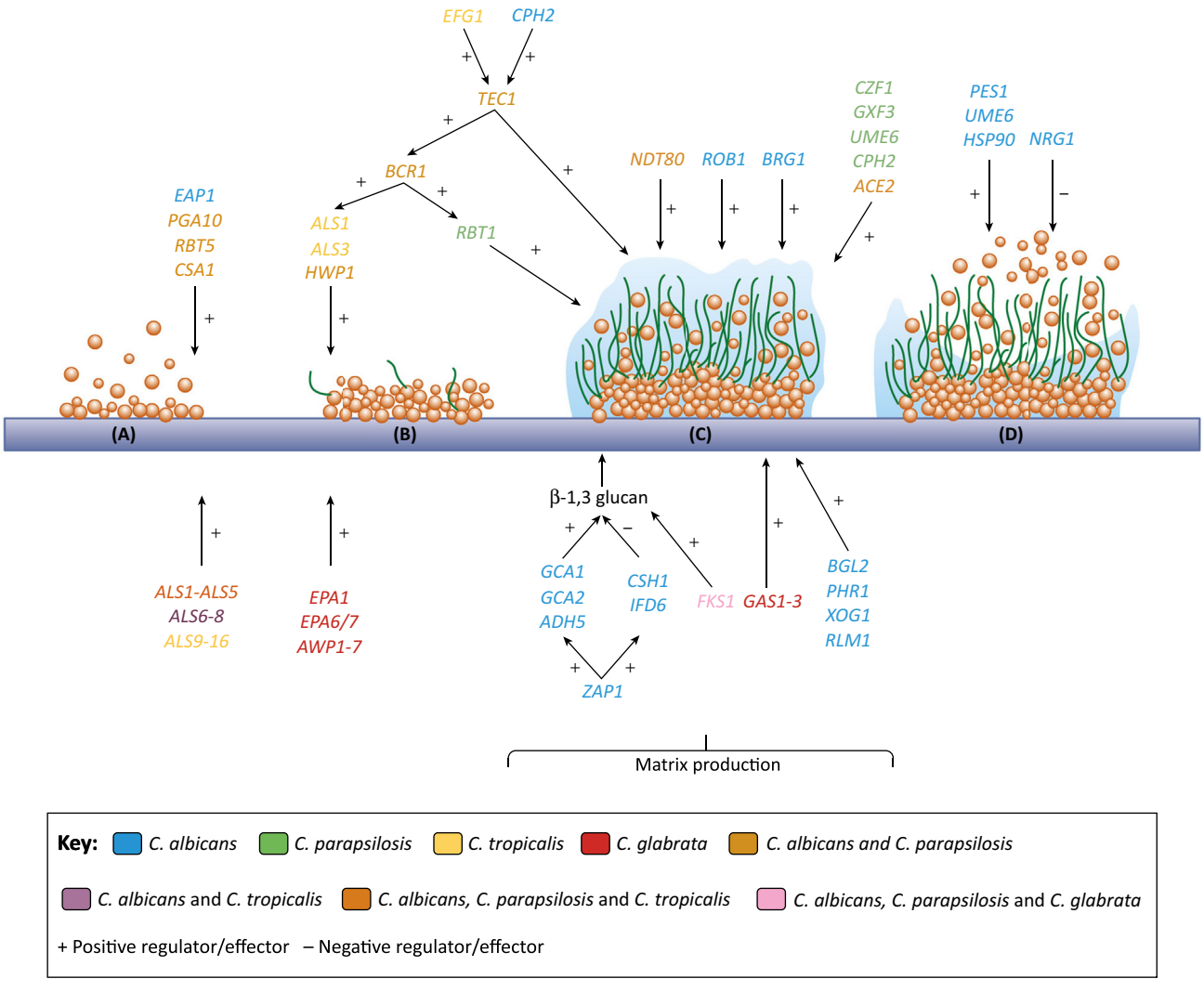

Figure 1. Regulatory Network Genes for the Different Biofilm Stages of *Candida albicans*, *Candida parapsilosis*, *Candida tropicalis*, and *Candida glabrata*. (A) Initial adhesion. (B) Formation of basal microcolony layers. (C) Mature biofilm constituted by cells with diverse morphologies and extracellular matrix. (D) Biofilm detachment and dispersion.

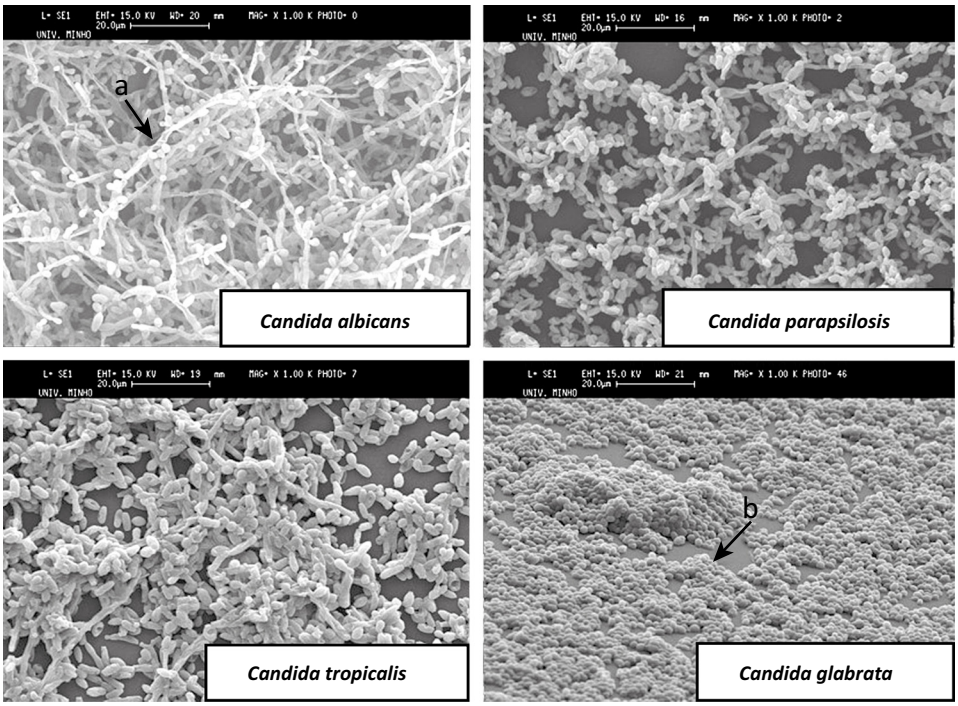

Figure 2. Biofilm Structure of *Candida albicans*, *Candida parapsilosis*, *Candida tropicalis*, and *Candida glabrata*. Filamentous forms (hyphae or pseudohyphae). Blastospores. Images obtained with scanning electron microscopy after 24 h of biofilm growth.

forms, pseudohyphae and/or hyphae, and by the production of extracellular matrix [10,11]. The role of the matrix is to protect *Candida* cells from phagocytic cells and to act as a barrier to drugs and toxic substances [12,13]. Moreover, this matrix allows the maintenance of nutrients within it to reach biofilm cells [12,13]. Finally, mature biofilms have the ability to initiate detachment and dispersion (Figure 1D) on their own. Furthermore, this release of cells from the original biofilm community is a step forward in generating novel communities at new locations [14].

It is important to address the fact that biofilms are variable in their structure and matrix composition, differing between species and strains [15] (Figure 2 and Table 1). In the case

Table 1. Biofilm Characteristics of *Candida albicans*, *Candida parapsilosis*, *Candida tropicalis*, and *Candida glabrata*

Species	Biofilm Structure	Matrix Composition	Refs
C. albicans	Bilayer structure with yeast, hyphae, and pseudohyphae	Mainly composed by carbohydrates, proteins, phosphorus, and hexosamines	[3,11,12,14,73,74]
C. parapsilosis	Thin biofilm consisting of aggregated blastospores with yeast cells and pseudohyphae	High quantities of carbohydrates and low levels of proteins	[4,74–77]
C. tropicalis	Dense network of yeast cells with evident filamentous morphologies	Low levels of carbohydrates and proteins	[4,7,11,74]
C. glabrata	Compact monolayer or multilayer of only blastospores	High levels of carbohydrates and proteins; mainly composed of hexosamine	[5,7,11,74]

of *C. albicans*, the biofilm structure normally consists of two layers, a basal deposit of blastospores covered by a thick matrix film with hyphal forms (Figure 2). Furthermore, biofilm formation in this species is related to the transition from yeast to hyphal growth, as mentioned above [14]. Compared to *C. albicans*, biofilms of *C. parapsilosis* are much less thick, consisting of aggregated blastospores with yeast cells and pseudohyphae [4] (Figure 2). In the case of *C. tropicalis*, mature biofilms are usually characterised by a dense network of yeast cells with evident filamentous morphologies. In contrast to this species (Figure 2), *C. glabrata* biofilms are characterised by a compact monolayer or multilayer with only blastospores, since this species is unable to form filamentous forms (Figure 2) [5].

Table 1 summarizes the general characteristics of the four most important pathogenic *Candida* species. Carbohydrates, proteins, phosphorus, and hexosamines are the major constituents of the biofilm matrix of *C. albicans* [12,16]. In the case of *C. parapsilosis*, Silva *et al.* [7] reported that the extracellular matrices contain high quantities of carbohydrates; however, the quantity of protein is lower compared with that of other species. The matrices of *C. glabrata* and *C. tropicalis* are also composed of proteins and carbohydrates [5], but *C. glabrata* has higher levels as compared to *C. tropicalis* [7].

Regulatory Network Genes for the Different Biofilm Phases of *Candida* Species

Mucosal infections could be associated with biofilms in that the pathogen adheres to a surface and produces an extracellular matrix [17]. This relationship has prompted investigations to test the hypothesis that genes required for biofilm formation *in vitro* may be required for mucosal infection as well. Findings from these studies have underscored the utility of this perspective in that there are several common genetic requirements for the formation and development of biofilms on abiotic and mucosal surfaces [9]. The distinct developmental phases of biofilms (adhesion/colonisation, maturation, and dispersal) are directed by complex molecular events. Biofilm formation is strongly dependent on environmental conditions, which makes the comparison of regulatory genetic alterations among *Candida* species not easy. It should also be noted that the whole genome is only known for *C. albicans* and *C. glabrata*. Despite that, the currently known regulatory network of genes involved in biofilm formation for *C. albicans*, *C. parapsilosis*, *C. glabrata*, and *C. tropicalis* are described and compared below and summarized in Table 2.

Adherence and/or Colonisation

Adherence of *Candida* cells to mucosal surfaces and/or synthetic material is an early step leading to proliferation and consequently biofilm formation and infection (Figure 1) [4]. The adhesion mechanism is based on interaction between the cell wall of the microorganism and cell surfaces of the host. Thereby, adherence is mediated by host cells, fungal cells, and environmental conditions [18]. Modulation of the adhesion process can be achieved by microbial adhesins or host cell receptors, or by physical and chemical manipulations [19].

The presence of specific cell-wall proteins, designated normally as adhesins, is a trigger in the modulation of the adhesion process [5]. In *C. albicans*, the adhesion is mediated by agglutinin-like sequence (Als) proteins (Figure 1) [6]. The family of Als adhesins in *C. albicans* consists in eight members, namely Als1–7 and 9, and all proteins have a similar structure containing an N-terminal secretory signal sequence [6]. Specifically, Als1 and Als3 proteins are involved in biofilm surface attachment; however, their expression differs depending on *C. albicans* cell morphology [20]. In the case of *ALS1*, expression is detectable in both yeast and hyphal cell morphology [21], but *ALS3* is expressed only in the hyphal lifestyle [22]. Of the eight *ALS* genes, *ALS1*, *ALS3*, and *ALS5* are reported to be involved in the adhesion of *C. albicans* to a variety of biotic substrates [23].

Table 2. Genes Involved in Genetic Control of Adherence and Biofilm Formation in *Candida albicans*, *Candida parapsilosis*, and *Candida glabrata* Species

Species		Systematic Name	Gene Name	Description	Refs
C. albicans	Adhesion and/or colonisation	Orf19.5741/ Orf19.1816	*ALS1/ ALS3*	Cell-wall protein adhesin; involved in adherence to vascular endothelial cells and oral epithelial cells	[35,78,79]
		Orf19.5736	*ALS5*	Cell-wall protein involved in adherence process	[78,79]
		Orf19.1321	*HWP1*	Cell-wall adhesin; involved in adhesion step: promotes physical contact between epithelial cells and the fungal cells	[25,27,80]
		Orf19.1401	*EAP1*	GPI-linked cell wall protein; involved in the cell–cell adhesion	[10,21, 24,73]
		Orf19.5674	*PGA10*	GPI membrane protein; involved in full adherence and essential for biofilm development	[10,29,81]
		Orf19.6274	*PBR1*	White cell ∝ factor-induced gene; full adherence	[10,28,82]
		Orf.19.4477	*CSH1*	White cell ∝ factor-induced gene; full adherence of biofilm	[10,28,82]
		Orf19.3127	*CZF1*	Transcription factor; required for yeast adherence to silicone	[50]
	Maturation	Orf19.1321	*HWP1*	Hyphal cell-wall mannoprotein; required for hyphal formation	[23,25,27, 80,83,84]
		Orf19.723	*BCR1*	Transcription factor required for biofilm formation; involved in the early adhesion stage	[17,38, 73,76]
		Orf19.610	*EFG1*	Transcription factor of biofilm formation; Involved in cell surface, adhesion and hyphal formation	[9,38,43, 85–87]
		Orf19.5908	*TEC1*	Transcription factor required for biofilm formation; required for hyphal formation	[38,40,44, 46,73,82]
		Orf19.2119	*NDT80*	Transcription factor of biofilm formation; involved in hyphal development	[9,40]
		Orf19.4998	*ROB1*	Transcription factor of biofilm formation	[6,9]
		Orf19.4056	*BRG1*	Transcription factor of biofilm formation	[6,9]
		Orf19.1822	*UME6*	Transcription regulator of yeast–filament transition	[40,72,88]
		Orf19.1187	*CPH2*	Transcription factor; promotes hyphal growth	[51]
		Orf19.6124	*ACE2*	Involved in regulation of biofilm development	[48]
		Orf19.3794	*ZAP1*	Negative regulator of biofilm matrix production; high levels of β-1,3 glucan both *in vitro* as *in vivo* in *Δzap1/zap1*	[6,10, 56,89]

Table 2. (continued)

Species		Systematic Name	Gene Name	Description	Refs
		Orf19.5101	*CCR4*	Negative regulator of biofilm matrix production; over-production of extracellular matrix in *Δccr4/ccr4*	[61]
		Orf.19.2929	*FKS1*	β-1,3 glucan synthase; biofilm resistance to fluconazole via a role in β-1,3 glucan levels in the extracellular matrix	[10,54, 55,57]
		Orf.19.4477	*CSH1*	Cell-matrix adhesion; negative regulator of matrix production when *ZAP1* is activated	[10,56]
		Orf.19.1048	*IFD6*	Negative regulator of matrix production when *ZAP1* is activated	[10,56]
		Orf.19.4899	*GCA1*	Positive regulator of matrix production when *ZAP1* is activated	[10,56]
		Orf.19.999	*GCA2*	Positive regulator of matrix production when *ZAP1* is activated	[10,56]
		Orf.19.2608	*ADH5*	Positive regulator of matrix production when *ZAP1* is activated	[10,56]
		Orf.19.4662	*RLM1*	Putative transcription factor; positive regulator of matrix production	[57,89]
		Orf.19.4565	*BGL2*	Cell wall 1,3-beta-glucanosyltransferase; involved in matrix delivery	[6,55,60]
		Orf.19.3829	*PHR1*	Glucanosyltransferase; involved in matrix delivery	[55,59]
		Orf.19.2990	*XOG1*	β-1,3 exoglucanase involved in matrix delivery	[6,55,58]
	Dispersion	Orf19.7150	*NRG1*	Transcription factor; overexpression increases released cells; negative regulator of filamentation	[10,44, 69–72,88]
		Orf19.4093	*PES1*	Responsible for reverse morphological transition (from hyphae to yeast): overexpression increases released cells	[10,67, 69,90]
		Orf19.1822	*UME6*	Transcription regulator of yeast-filament transition; overexpression reduces released cells	[67,72,88, 90,91]
		Orf19.6515	*HSP90*	Biofilm azole resistance; key regulator in biofilm dispersal	[6,67, 89,92]
C. parapsilosis	Attachment	CPAR2_403520	*HWP1*	Cell adhesion; involved in attachment step and consequently in biofilm formation	[25]
			ALS1-5	Putative adhesins involved in adhesion step	[31]

Table 2. (continued)

Species		Systematic Name	Gene Name	Description	Refs
	Maturation	CPAR2_205990	*BCR1*	Transcription factor required for biofilm formation; when the ortholog is deleted, the biofilm formed is thinner	[38,40,41, 73,76,93]
		CPAR2_701620	*EFG1*	Transcription factor involved in biofilm formation and hyphal growth	[40,43]
		CPAR2_213640	*NDT80*	Transcription factor activity involved in biofilm formation	[40,43]
		CPAR2_403510	*RBT1*	Required for biofilm development; regulated by *BCR1*	[76,94]
		CPAR2_501290	*CZF1*	Transcription factor of biofilm formation	[40]
		CPAR2_800210	*GZF3*	Transcription factor of biofilm formation	[40]
		CPAR2_803820	*UME6*	Transcription factor of biofilm formation	[40]
		CPAR2_603440	*CPH2*	Transcription factor of biofilm formation	[40]
		CPAR2_204370	*ACE2*	Transcription factor of biofilm formation	[40]
		CPAR2_106400	*FKS1*	Maturation of biofilm in presence of glucose	[62]
C. tropicalis	Attachment		*ALS1-16*	Putative adhesins involved in adhesion step of biofilm formation	[31]
	Maturation		*EFG1*	Filamentation, biofilm formation and white-opaque switching	[45]
C. glabrata	Attachment	CAGL0E06644g	*EPA1*	Cell adhesion molecule; epithelial adhesin involved in adherence to host surface	[5,23, 32–34]
		CAGL0C00110g/ CAGL0C05643g	*EPA6/ EPA7*	Epithelial adhesin involved in adherence to host surface	[32,35,95]
		CAGL0J02508g/ CAGL0K00110g	*AWP1/ AWP2*	Adhesin-like wall protein	[36,37,96]
		CAGL0J11891g	*AWP3*	Putative adhesin-like wall protein	[36,37,96]
		CAGL0J11990g	*AWP4*	Adhesin-like wall protein	[36,37,96]
		CAGL0K13024g/ CAGL0G10175g	*AWP5/ AWP6*	Adhesin-like wall protein	[37,96]
		CAGL0C00209g	*AWP7*	Putative adhesin-like wall protein	[37]
	Maturation	CAGL0G01034g	*FKS1*	β-1,3 glucan synthase involved in cell wall and extracellular matrix	[55,63,64]
		CAGL0G00286g/ CAGL0M13849g/ CAGL0F01287g	*GAS1/ GAS2/ GAS5*	β-1,3 glucan synthase	[36,65,66]

Eap1 is a GPI-linked (glycosylphosphatidylinositol-dependent) cell-wall protein which is involved in cell–cell adhesion in *C. albicans* (Figure 1) [24]. This protein mediates surface binding; its synthesis is regulated by the transcription factor *EFG1* [21].

Hyphal wall protein I (Hwp1) is a fungal cell-wall mannoprotein that promotes attachment of *Candida* cells to the host surface [23]; it was also the first cell-surface protein described as involved in *in vivo C. albicans* biofilm formation (Figure 1) [25]. In the other instances, Orsi *et al.* [26] demonstrated that this gene is involved in the formation of germ tubes and hyphal forms and thus promotes physical contact between epithelial cells and the fungi, concluding that Hwp1 is an important effector of *C. albicans* pathogenicity [27]. This gene was extensively studied in *C. albicans*; however, in the case of NCAC species little is known [25]. Despite that, Wan Harun *et al.* [25] demonstrated that *HWP1* mRNA was also expressed in *C. parapsilosis* and *C. tropicalis*, but not detected in *C. glabrata*. The fact that *HWP1* is positively expressed in these three species during the process of adhesion and biofilm formation indicates its involvement in producing adhesins covalently linked to the cell-wall glucan [25].

The cell-surface protein Pga10 and the secreted Pbr1 protein are also described as important for the full adherence of *C. albicans* biofilms (Figure 1) [28]. Pga10, also known as *RBT51*, is a member of CFEM (common in several fungal extracellular membranes) proteins and has a role in adhesion in *C. albicans* [29]. Pérez *et al.* [29] demonstrated that Δ*pga10* forms a less consistent biofilm and contributes to early detachment from the plastic substrate, when compared to the parental strain. Additionally, Sahni et *al.* [28] showed that *C. albicans* adhesion to blood cells was lower when the *PBR1* gene was deleted.

In *C. parapsilosis*, adhesion to epithelial and acrylic surfaces was associated with cell-surface hydrophobicity. Moreover, *C. parapsilosis* has a greater ability than *C. albicans* to adhere to buccal epithelial cells [30]. Butler *et al.* [31] identified five members of Als and six members of Pga30 cell-wall proteins, though there has been no further work to understand the role of these proteins in the process of adhesion in *C. parapsilosis* (Figure 1) [4,31]. In the case of *C. tropicalis*, Butler *et al.* [31] identified 16 members of the *ALS* family in the genome; however, additional confirmative research is needed on their involvement in biofilm development.

Similar to *C. albicans*, adherence of *C. glabrata* is mediated by epithelial adhesins (Epa) that have a similar structure to the Als proteins [5]. The family of *EPA* genes are composed of 17–23 genes, depending on the strain; however, *EPA1*, *EPA6*, and *EPA7* are the most important adhesins (Figure 1) [32]. Deletion of the *EPA1* gene reduces adherence *in vitro* to host epithelial cells [33], and adherence of this adhesin is inhibited in the presence of lactose [34]. Regarding the Epa6 adhesin, *C. glabrata* does not normally express *EPA6 in vitro*; however, it is expressed during urinary infection due to low levels of nicotinic acid [35]. De Groot *et al.* [36] identified another family of adhesins involved in the first stage of *C. glabrata* biofilm development, namely Awp adhesins (Figure 1). Initially, four Awp adhesins (Awp1–4) were identified using liquid chromatography tandem mass spectrometry [36]. A subsequent study revealed the gene expression profile of the seven Awp adhesins (Awp1–7) [37]. Expression of these adhesins is significantly higher in biofilms when compared to planktonic cells in two different media [37].

Likewise, for *C. albicans*, NCAC species adhesion mechanisms indicate that the cell wall likely plays a crucial role for colonisation and subsequent biofilm formation.

Biofilm Maturation

Initial attachment of *Candida* cells is followed by cell division and proliferation, known as biofilm development (Figure 1). The biofilm formation in *Candida* species is regulated by several transcription factors that play a fundamental role in various pathways, and they have an

important potential in the regulation of other genes involved in biofilm formation (Table 2) [38]. Nobile *et al.* [9] investigated the transcriptional network and identified a set of six transcription factors in *C. albicans* that play an important role in the regulation of biofilm formation, namely *BCR1*, *EFG1, TEC1, NDT80, ROB1*, and *BRG1* (Figure 1) [9]. Furthermore, *C. albicans* biofilms are defective when any of these regulators are deleted [9]. However, in the case of NCAC species little is known about the influence of these genes in biofilm formation.

BCR1 is a C_2H_2 zinc-finger protein essential for biofilm formation in *C. albicans* and in *C. parapsilosis*. Moreover, this gene is essential for the expression of several cell-wall proteins in *C. albicans* [38], namely Als1, Als3, and Hwp1 [23,39]. Nobile *et al.* [38] described that the expression of *ALS3* and *HWP1* is reduced in a Δ*bcr1/bcr1* strain, which was decreased after adherence to plastic, endothelial and epithelial cells [35,38]. Another study by Nobile *et al.* [20] highlighted the complementary surface function between Hwp1 and Als1/3 adhesins for *in vitro* and *in vivo* biofilm formation. Moreover, Nobile *et al.* [39] also demonstrated that *BCR1* is a regulator of adherence but it is not involved in hyphal formation. In *C. parapsilosis*, the ortholog of *BCR1* is also required for biofilm formation [40], and when this gene is deleted, the biofilm formed by *C. parapsilosis* is thinner with scant layer cells compared to a biofilm formed in normal conditions, showing that *BCR1* is also required for *in vivo* biofilm formation (Figure 1) [41]. The CFEM family of proteins are targets of *BCR1* in both species and can act as cell-surface receptors or as adhesins [42]. However, the CFEM family has just been described in *C. albicans* as having a role in biofilm development [41], namely *RBT5*, *PGA10*, and *CSA1* (Figure 1) [29]. In *C. parapsilosis*, this family consists of seven members, which include the three members of *C. albicans* involved in biofilm formation [41]. In contrast to *C. albicans* and *C. parapsilosis*, in *C. glabrata* the function of *BCR1* is unknown at the moment.

EFG1 is another transcription factor required for biofilm formation that regulates the cell surface and hyphal formation [43]. Holland *et al.* [40] demonstrated similar reduction in biofilm for *C. albicans* and *C. parapsilosis* when *EFG1* is eliminated (Figure 1). This study confirmed the results obtained by Nobile *et al.* [9] for *EFG1* in *C. albicans.* Connolly *et al.* [43] also verified that deleting *EFG1* reduces biofilm formation in *C. parapsilosis*. Moreover, Δ *efg1/efg1* strains were unable to form hyphae in *C. albicans*, even when grown under hypha-inducing conditions [44]. Recently, Mancera *et al.* [45] identified the *C. tropicalis EFG1* gene and confirmed that this transcriptional factor possessed a similar role in *C. albicans* (Figure 1). This gene is involved in the regulation of filamentation and biofilm formation, since deletion of both alleles is critical for these factors [45]. Furthermore, an ortholog of *EFG1* was identified in the genome of *C. tropicalis*; however, its position is completely different to that of *EFG1* in *C. albicans* [45]. Again in the case of *C. glabrata*, the role of this gene is unknown. It is likely that *C. glabrata* does not possess a gene with a similar function in its genome since they are not polymorphic microorganisms.

TEC1 is a gene required for hyphal formation and virulence in *C. albicans* [39] and is a member of the TEA/ATTS transcription factor family [46]. The DNA-binding region is contained in 66–76 conserved amino acids in the N-terminus [47]. This gene is regulated by *EFG1* [2], which is involved in regulation of filamentous growth. In *C. parapsilosis*, *TEC1*, seems not to play the same role as in *C. albicans* [39], once Holland *et al.* [40] did not observe a dramatic reduction in biofilm formation in the case of *C. parapsilosis* mutant for this gene (Figure 1). It is unclear whether *TEC1* is involved in *C. glabrata* biofilm formation.

Other transcription factors described by Nobile *et al.* [9] in *C. albicans* biofilm development, *NDT80*, *BRG1*, and *ROB1*, were studied also by Holland *et al.* [40] in *C. parapsilosis* (Figure 1). *NDT80* is a key factor in response to different environmental conditions and is involved in hyphal development and virulence in *C. albicans* [40]. In *C. parapsilosis*, *NDT80* deletion results in a

significant growth defect in biofilm formation. Furthermore, deleting *BRG1* does not dramatically reduce biofilms in *C. parapsilosis* [40], indicating that this gene is not involved in biofilm formation in this species. In *C. parapsilosis* there is no ortholog of the *ROB1* gene [40].

Biofilm development in *C. parapsilosis* is regulated by further transcription factors, namely *CZF1*, *GZF3*, *UME6*, *CPH2*, and *ACE2* (Figure 1) [40]. Biofilm formation is dramatically reduced when *ACE2* is deleted [40]. However, this gene is also involved in regulation of biofilm development in *C. albicans*, controlling the adherence of this species and cell division (namely, M and early G1 phases of the cell cycle) [48]. However, when *ACE2* was inactivated in *C. glabrata*, its ability to cause disease increased significantly [49]. Wätchler *et al.* [50] described *CZF1* as a gene that contributes to yeast adhesion in *C. albicans*, leading to invasion and damage in the oral cavity. The *GZF3* gene is a GATA-type transcription factor [40]; however, according to Nobile *et al.* [9] it is not a key gene in biofilm development in *C. albicans*. In this species, *CPH2* promotes hyphal growth [51] and regulates the expression of *TEC1*, which is a transcription factor of biofilm formation as described above [52].

The production of extracellular matrix is another important feature in biofilm maturation [10]. As described above, the composition of the matrix varies according to species, strains, and environmental conditions, and it is well known that the main components of the matrix are carbohydrates and proteins [11]. One of the carbohydrates present in *C. albicans* matrix is β-1,3 glucan [53]. However, a recent study by Zarnowski *et al.* [16] demonstrated that β-1,6 glucan is also an important matrix component, and that it is highly dependent on the environmental conditions used. The gene responsible for glucan synthase is *FKS1* (Figure 1), more commonly designated *GSC1*, and it has been implicated in *C. albicans* biofilm resistance to fluconazole [53]. The susceptibility to fluconazole is the result of *FKS1* disruption which reduces the deposition of β-1,3 glucan in the biofilm matrix [54]. Furthermore, the increase in *FKS1* transcription is coupled with a reduction in the delivery of glucan to matrix [55]. In *C. albicans*, *RLM1* and *ZAP1* are two other regulators involved in matrix production in biofilms (Figure 1). The transcription factor *ZAP1* is a negative regulator of biofilm matrix production, and Δ*zap1/zap1* produces a biofilm with high levels of β-1,3 glucan both *in vitro* as *in vivo* [56]. Some target genes of *ZAP1* are *CSH1, IFD6, GCA1, GCA2*, and *ADH5*, which modulate levels of β-1,3 glucan in the biofilm matrix (Figure 1) [56]. In the case of *CSH1* and *IFD6*, when *ZAP1* activates the expression of these genes, the production of β-1,3 glucan decreases and therefore these genes are considered as negative regulators of matrix production [56]. However, *GCA1*, *GCA2*, and *ADH5* are positive regulators since there is an increase in β-1,3 glucan when these genes are activated by the *ZAP1* gene [56]. Another regulator of matrix production is *RLM1*, a positive regulator, whose deletion promoted a reduction in its matrix levels [57]. Taff *et al.* [55] described a role for *BGL2*, *PHR1*, and *XOG1* (Figure 1) as glucan-modifying genes involved in glucan delivery and matrix incorporation. The *BGL2* and *PHR1* genes encode glucanosyltransferases, and *XOG1* is a β-1,3 exoglucanase [58–60]. Recently, Verma-Gaur *et al.* [61] identified another gene regulator of biofilm matrix production in *C. albicans*, *CCR4*. The authors [61] identified in Δ*ccr41/ccr4 C. albicans* biofilms several structural modifications with morphological changes and overproduction of extracellular matrix. Therefore, as in the case of *ZAP1*, the *CCR4* gene is another negative regulator [61].

In addition, in *C. parapsilosis*, the gene *FKS1* is involved in maturation of biofilms in the presence of glucose (Figure 1) [55]. Pereira *et al.* [62] demonstrated that the upregulation of *FKS1* is induced by high levels of glucose, leading to an increase in β-1,3 glucan synthesis. This glucan is accumulated in the matrix, forming a dense and structured biofilm [62]. Little is known about the composition of the biofilm matrix of *C. glabrata*; however, it is known that it is composed of β-1,3 glucan [4]. As in the previous species, the *FKS1* gene is responsible for production of β-1,3 glucan in *C. glabrata* (Figure 1) [55], and echinocandins inhibit β-1,3 glucan synthase by

targeting *FKS* subunits [63,64]. The *GAS* gene family is another regulator in the production of β-1,3 glucan in this species [36]. Similarly to *Saccharomyces cerevisiae*, *GAS1*, *GAS2*, and *GAS5* are a glycosylphosphatidylinositol (GPI)-anchored cell-surface proteins [65] which are involved in the production of β-1,3 glucan in *C. glabrata* (Figure 1) [66].

Biofilm Detachment and Dispersion

The last step is characterized by dispersal of yeast cells and/or pieces of the biofilm from its mature form; this allows the organism to colonize new sites for further adherence and colonization [9], completing the biofilm life cycle (Figure 1). Biofilm dispersion occurs in response to environmental changes, such as a decrease in, or lack of, nutrients or other modifications in the growth media composition [67]. Furthermore, the dispersion of biofilm cells can lead to a development of infections in deep organs due to the ability to invade the bloodstream [67]. In the past decade, early events associated with *Candida* biofilm formation have received considerable attention. However, very little is known about *Candida* biofilm dispersion or the mechanisms and genes that trigger it.

Recent studies on *C. albicans* biofilms have reported that the majority of dispersed cells are yeast cells and that there are three regulatory genes in this step, namely, *PES1, UME6*, and *NRG1* (Figure 1) [10]. Uppuluri *et al.* [67] demonstrated that the major yeast cells dispersed from biofilm were released from the upper hyphal layers. Furthermore, overexpression of *PES1* results in an increase of yeast growth when cells were grown on medium without doxycycline (DOX) [68]. By contrast, when *NRG1* was overexpressed, in the absence of DOX, the biofilm contained only a monolayer of yeast and pseudohyphae cells [69] since *NRG1* is a negative regulator of filamentation [70,71]. *UME6* is a transcription regulator of yeast–filament transition in *C. albicans*, and more precisely it is required for hyphal extension (Figure 1) [72]. When *UME6* was deleted, there was a little reduction in the *C. albicans* biofilm [40], which means that this gene has a minor role in biofilm development.

Summarizing, despite all *Candida* species having some similar regulatory genes in each stage of biofilm formation, there is a lack of information concerning NCAC species, which makes a comparison among them difficult. However, it is possible to stress that the *ALS* genes are involved in the adhesion process of the three *Candida* species (*C. albicans*, *C. parapsilosis*, and *C. tropicalis*), and that in *C. glabrata* this phenomenon is regulated by the Epas, which have a similar structure to the Als proteins. Additionally, some transcription factors described as involved in *C. albicans* biofilm formation (*BCR1, EFG1*, and *HWP1*) are the same as those implicated in *C. parapsilosis* biofilms. *C. glabrata* is the species that presents more contrasts in relation to the other *Candida* species, reflecting its genetic distance.

However, despite all our knowledge about *C. albicans* biofilm regulators, little is known about their involvement in other NCAC species, and thus much more research must be conducted in order to increase our knowledge in this area.

Concluding Remarks

Biofilms are communities of microorganisms embedded in an extracellular matrix, and biofilms are assumed to be the most important virulence factors for pathogenicity in *Candida* species. These species utilize several genes that are confirmed to play an important role in the different stages of biofilm development. Indeed, additional and comparative genomic (genome sequencing) and transcriptomic approaches (RNA seq and/or microarrays) are needed to deepen our knowledge about the real biofilm regulatory network genes, specifically in the case of NCAC species (see Outstanding Questions). Therefore, further studies in this area will contribute towards the identification of new targets to be used to design new nanodrugs against these emerging pathogens.

Outstanding Questions

Are biofilm regulatory genes from NCAC species similar to the known biofilm regulatory genes for *C. albicans*?

What are the signal transduction pathways that regulate biofilm formation in *Candida* species?

Given the variability of the *Candida* species genome, what effect does this exert on biofilm formation?

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