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Minireview

MINIREVIEW

Biofilm formation in *Candida glabrata*: What have we learnt from functional genomics approaches?

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One sentence summary: This review summarizes how functional genomics approaches have contributed recent progresses in our understanding of the molecular mechanisms of biofilm formation by the fungal pathogen *Candida glabrata*.

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ABSTRACT

Biofilms are a source of therapeutic failures because of their intrinsic tolerance to antimicrobials. *Candida glabrata* is one of the pathogenic yeasts that is responsible for life-threatening disseminated infections and able to form biofilms on medical devices such as vascular and urinary catheters. Recent progresses in the functional genomics of *C. glabrata* have been applied to the study of biofilm formation, revealing the contribution of an array of genes to this process. In particular, the Yak1 kinase and the Swi/Snf chromatin remodeling complex have been shown to relieve the repression exerted by subtelomeric silencing on the expression of the *EPA6* and *EPA7* genes, thus allowing the encoded adhesins to exert their key roles in biofilm formation. This provides a framework to evaluate the contribution of other genes that have been genetically linked to biofilm development and, based on the function of their orthologs in *Saccharomyces cerevisiae*, appear to have roles in adaptation to nutrient deprivation, calcium signaling, cell wall remodeling and adherence. Future studies combining the use of *in vitro* and animal models of biofilm formation, omics approaches and forward or reverse genetics are needed to expand the current knowledge of *C. glabrata* biofilm formation and reveal the mechanisms underlying their antifungal tolerance.

Keywords: *Candida glabrata*; *Candida albicans*; biofilm; Epa adhesins; antifungal tolerance; subtelomeric silencing; Swi/Snf chromatin remodeling complex; Mediator; PKA pathway; glucose repression

INTRODUCTION

Biofilms are 3D communities of microorganisms that develop on living or inert surfaces, embedded in a self-synthesizing extracellular matrix and with distinctive biological properties (Costerton, Stewart and Greenberg 1999; Donlan and Costerton 2002). Biofilm growth (by comparison with planktonic growth where cells grow in suspension) is now regarded as the predominant mode of microbial life, with up to 80% of all microorganisms in the environment living as sessile (Donlan and Costerton 2002). Notably, it has been estimated that 65%–80% of human infections are associated with the formation of biofilms (Davies 2003). Indeed, biofilm formation occurs in chronic infections such as caries, stomatitis, otitis, endocarditis, penumopathies in cystic fibrosis patients, urinary tract infections and wound infections. Moreover, pathogenic microorganisms form biofilms on medical devices such as venous and urinary catheters, artificial joints, stents, shunts, implants, endotracheal tubes and pacemakers, whose use is constantly increasing, with an estimated 10 million new recipients every year (Darouiche 2004; Kojic and Darouiche

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2004). A distinctive feature of biofilms is their intrinsic tolerance to antimicrobials (biofilm cells can survive antimicrobial concentrations more than 1000 times the MICs defined for planktonic cells) and immune attack. Antimicrobial tolerance is distinct from antimicrobial resistance as it is not genetically inherited and involves alternative mechanisms such as the sequestration of drugs by the biofilm extracellular polymeric matrix and the occurrence in the biofilm of a subpopulation of so-called persister cells with a dormant-like physiology (Lewis 2007; Hall-Stoodley and Stoodley 2009). Hence, biofilms often represent a source for relapse following apparently successful antimicrobial treatments and the development of antimicrobial strategies to prevent the formation of biofilms or their eradication is an ongoing challenge.

Candida species, predominantly *Candida albicans*, *C. glabrata*, *C. parapsilosis*, *C. dubliniensis* and *C. tropicalis*, are responsible for diverse forms of infections that are associated with the formation of biofilms (Kojic and Darouiche 2004; Ramage and Williams 2013). Mucosal infections, such as oropharyngeal candidiasis and vaginal candidiasis, are per se associated with biofilm formation as they involve the development on epithelia of 3D communities of *Candida* cells in association with commensal bacteria and host components (Dongari-Bagtzoglou *et al.* 2009; Ganguly and Mitchell 2011). However, whether they share all the features of biofilms formed on inert surfaces is unclear, as they generally appear to be sensitive to antifungal treatments. In disseminated infections due to *Candida* species, biofilm formation is considered as a major risk factor (Rex *et al.* 2000; Filler and Kuhlberg 2002). Indeed, these nosocomial infections occur principally in hospitalized individuals with severe underlying diseases, complicated surgical abdominal procedures or critical illnesses, and where the use of various implants is constant (Kojic and Darouiche 2004). *Candida* biofilms are especially resistant to azoles and amphotericin B while sensitive to echinocandins (d'Enfert 2006; Ramage *et al.* 2012; Mathe and Van Dijck 2013). Hence, antifungal treatment without implant removal in patients with disseminated *Candida* infections is rarely successful, leading to relapse in a significant number of cases and even to complications such as endocarditis or distant infections. Consequently, catheter withdrawal is considered a prerequisite to a successful antifungal treatment (Mermel *et al.* 2001).

As mentioned above, biofilm formation by several *Candida* species has been documented in different clinical contexts. Yet, *C. albicans* is arguably the species that is the most frequently associated with biofilm formation (Kojic and Darouiche 2004). Consequently, most of our knowledge on biofilm formation in *Candida* species results from the study of *C. albicans* as a model. A sequence of events that yield mature *C. albicans* biofilms has been defined that involves the adherence of yeast cells to an artificial surface, the formation of a microcolony of yeast cells from which a dense network of hyphae emerges and the production of an extracellular matrix (Douglas 2003; Finkel and Mitchell 2011). Mature biofilms can release cells in the surrounding medium in a process that is referred to as dispersal (Blankenship and Mitchell 2006; Finkel and Mitchell 2011). Functional genomic approaches have now revealed key aspects of the formation and antifungal tolerance of *C. albicans* biofilms on inert surfaces both *in vitro* and in animal models and these have been reviewed recently (Ramage *et al.* 2009; ten Cate *et al.* 2009; Finkel and Mitchell 2011; Fox and Nobile 2012; Bonhomme and d'Enfert 2013; Mathe and Van Dijck 2013; Taff *et al.* 2013; Desai, Mitchell and Andes 2014). Briefly, comparison of the transcript profiles of planktonic cultures and biofilms formed in a variety of contexts has in particular revealed a consistent upregulation of glycolysis and sulfur aminoacid genes, similar to what is observed when *Candida* is grown under hypoxia, giving credit to the idea that *Candida* biofilms constitute heterogeneous environments with hypoxic niches (Garcia-Sanchez *et al.* 2004; Stewart and Franklin 2008; Rossignol *et al.* 2009; Bonhomme *et al.* 2011). The establishment of a hypoxic niche in *C. albicans* biofilms was recently demonstrated (Fox *et al.* 2014) and it is notable that impairing adaptation to hypoxia through inactivation of the Tye7 positive regulator of glycolytic genes yields defects in biofilm formation (Bonhomme *et al.* 2011). The systematic characterization of *C. albicans* transcription factors for their role in biofilm formation has revealed an interwoven network of nine transcription factors, namely Bcr1, Brg1, Efg1, Flo8, Gal4, Ndt80, Rfx2, Rob1 and Tec1, that is at the core of biofilm development, regulating the expression of about 1000 genes (i.e. 15% of the *C. albicans* gene repertoire) (Nobile and Mitchell 2005; Fox and Nobile 2012; Nobile *et al.* 2012; Fox *et al.* 2015). Notably, a number of these transcription factors—Brg1, Efg1, Flo8, Ndt80, Tec1—are also controlling the yeast-to-hypha transition (Sudbery 2011) and mutations affecting this process are almost always impairing biofilm formation, consistent with the occurrence of hyphal cells in *C. albicans* mature biofilms (Baillie and Douglas 1999; Ramage *et al.* 2002; Finkel and Mitchell 2011). Further characterization of the targets of Bcr1 has highlighted the major contribution of cell surface proteins to biofilm formation, among which the hyphal adhesins Als1, Als3 and Hwp1 that appear to establish heterotypic interactions that are necessary for maintaining cell–cell contacts within the biofilm (Nobile *et al.* 2006, 2008). Additional cell wall glycosyl phosphatidylinositol (GPI)-anchored proteins have now been identified that appear to contribute to the cooperative behavior of biofilm cells and are important participants during *C. albicans* biofilm growth (Cabral *et al.* 2014), and a network of adherence regulators contributing to biofilm formation *in vitro* has been defined (Finkel *et al.* 2012). Biochemical characterization of the *C. albicans* biofilm matrix has revealed that it includes proteins, carbohydrates, lipids and nucleic acids (Baillie and Douglas 2000; Martins *et al.* 2010; Zarnowski *et al.* 2014). In particular, soluble β-1,3-glucans produced by the β-1,3-glucan synthase Fks1 and subsequently released in the matrix have been shown to sequester azoles and amphotericin B thus contributing to the tolerance of *C. albicans* biofilms to these drugs (Nett *et al.* 2007b, 2010a,b; Nobile *et al.* 2009; Taff *et al.* 2012). Yet, other components of the matrix such as a mannan–glucan complex and extracellular DNA are likely to participate in drug sequestration and antifungal tolerance (Martins *et al.* 2010; Zarnowski *et al.* 2014).

Much less is known of the mechanisms that are important to the process of biofilm formation in other *Candida* species. In this review, we describe recent progresses that have been made in our understanding of the molecular requirements of biofilm development in *C. glabrata*. Indeed, functional genomic approaches in this species are now revealing key players of biofilm formation, with similarities and differences relative to what we have learnt from studying biofilm development in *C. albicans*.

Biofilm formation in *C. glabrata*: an introduction

Candida glabrata is now emerging as the second cause of disseminated *Candida* infections and has been shown to form biofilms on different medical devices such as vascular and urinary catheters and cardiac valves (Kojic and Darouiche 2004; Pfaller and Diekema 2007; Silva *et al.* 2012). Imaging of *C. glabrata* biofilms obtained from clinical samples as well as *in vitro* and animal models of biofilm formation have revealed a thick

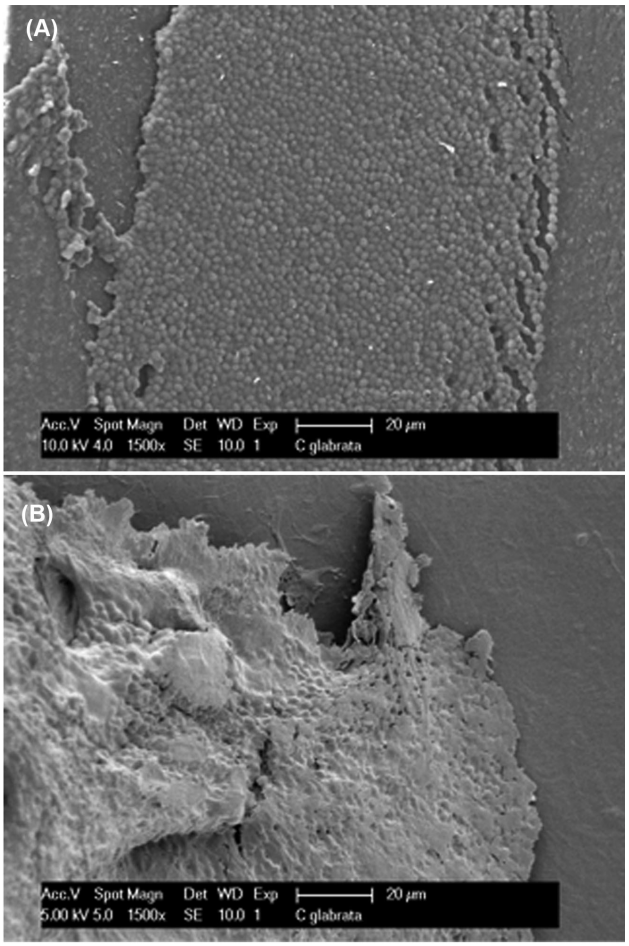

Figure 1. Ultrastructure of *C. glabrata* biofilms obtained *in vitro* and *in vivo*. Scanning electron micrographs of *C. glabrata* biofilms formed *in vitro* (**A**) or in a subcutaneous model of biofilm formation (**B**) for 6 days on polyurethane devices are shown. The occurrence of the extracellular matrix in which *C. glabrata* yeast cells are embedded is visible in (B). Scale bars = 20 μm. (Adapted from Kucharikova *et al.* 2015 with permission.)

network of yeast cells embedded in extracellular material (Iraqui *et al.* 2005; Nett *et al.* 2007a; Paulitsch *et al.* 2009; Seneviratne *et al.* 2009; Silva *et al.* 2011; Kucharikova *et al.* 2015), lacking the hyphal component that is seen in *C. albicans* biofilms (Fig. 1). While this is not surprising in a species that is apparently unable to undergo the yeast-to-hypha transition and seldom reported to form pseudo-hyphae (Glockner and Cornely 2015), it raises the question of the mechanistic similarities and peculiarities in *C. glabrata* and *C. albicans* biofilm formation, as hyphal differentiation plays critical structural and functional roles in the latter species biofilms (Finkel and Mitchell 2011). Little is known about the composition of the *C. glabrata* biofilm matrix except that it includes proteins and carbohydrates, among which β-1,3-glucans (Nett *et al.* 2007a; Silva *et al.* 2009). *Candida glabrata* biofilms formed *in vitro* or in an animal model show tolerance to azoles and polyenes but remain susceptible to echinocandins and lipid formulations of amphotericin B (Seidler, Salvenmoser and Muller 2006; Bernhardt, Knoke and Bernhardt 2011; Kucharikova *et al.* 2011, 2015; Toulet, Debarre, and Imbert 2012). Yet, the mechanisms that are involved in antifungal tolerance of *C. glabrata* biofilms remain elusive.

Functional genomic approaches in *C. glabrata* and their application to the study of biofilms

Candida glabrata is a haploid species with no demonstrated sexual cycle and diploid phase (Bolotin-Fukuhara and Fairhead 2014; Yanez-Carrillo *et al.* 2014). Even though these features prevent the use of classical forward genetic approaches based on chemical or physical mutagenesis, they allow for the implementation of insertional mutagenesis approaches to identify mutations that are associated with a phenotype of interest, an approach that is relatively ineffective in *C. albicans* because of its diploidy (Firon and d'Enfert 2007). Cormack and colleagues have pioneered insertional mutagenesis in *C. glabrata* (Cormack and Falkow 1999; Castano *et al.* 2003), and the resources developed by these authors have been applied to the identification of genes that are involved in biofilm formation: evaluation of ~5000 insertional mutants for their ability to form biofilms on a plastic surface using a quantitative colorimetric biofilm formation assay based on the reduction of 2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide (XTT) (Ramage *et al.* 2001) led to the identification of 72 mutants with decreased biofilm formation (<30% of the wild type) and 44 mutants with increased biofilm formation (>200% of the wild type) (Iraqui *et al.* 2005).

More recently, a large-scale reverse genetics approach has been applied to the study of biofilm formation taking advantage of a collection of 619 unique, individually barcoded *C. glabrata* deletion mutants established using gene replacement strategies (Schwarzmuller *et al.* 2014). This collection focuses on genes representing functional gene ontology categories such as environmental stress sensing and signaling, transcriptional regulation, antifungal drug resistance, cell wall structure and homeostasis, chromatin and histone modification, iron metabolism and metal sensitivity, and peroxisome biogenesis as well as genes apparently lacking a ortholog in *Saccharomyces cerevisiae*. These deletion mutants have been evaluated for their ability to form biofilms using an assay similar to that used for assessing insertional mutants except that a fluorescein diacetate accumulation assay was used to quantify the metabolic activity of biofilms (Honraet, Goetghebeur and Nelis 2005; Schwarzmuller *et al.* 2014). This has led to the identification of 14 gene deletions associated with significantly decreased biofilm formation and 11 gene deletions associated with significantly increased biofilm formation (Schwarzmuller *et al.* 2014). *Candida glabrata* genes that have been proposed to play a role in biofilm formation based on these two large-scale studies and other more focused studies are shown in Table 1 and discussed below. It is important to note that genes listed in this table that have not been individually reevaluated for their role in biofilm formation should be regarded as candidates rather than true players of biofilm formation. This is especially the case for genes identified from insertional mutations lying in intergenic regions as only one of the two genes flanking the insertion may be involved in biofilm formation.

The role of Epa adhesins and subtelomeric silencing in *C. glabrata* biofilm development

Screening of insertional mutants has in particular revealed the contribution of the *EPA6* gene to biofilm formation (Iraqui *et al.* 2005). Indeed, *C. glabrata* mutants with an insertion ~1000 bp upstream of *EPA6* or a deletion of *EPA6* showed a ~3-fold reduction of their biofilm. *EPA6* is one of ~20 genes that constitute the *C. glabrata EPA* gene family, encoding cell wall GPI-anchored

Table 1. *Candida glabrata* genes with a proposed role in biofilm formation.

Systematic name	Standard name[a]	Function[b]	Insertional (I)/ knockout (KO)/site specific (SS) mutant	Location of insertion	Effect on biofilm formation	Validated role in biofilm formation through detailed study	Reference
Cell-wall-related processes including adhesion							
CAGL0L06424g	*(CCW12)*	Predicted GPI-linked adhesin-like protein	KO		Increased	No	Schwarzmuller *et al.* (2014)
CAGL0G05896g	*(DSE2)*	Putative adhesin-like protein, with similarity to glucanases	I	Intragenic	Decreased	No	Riera *et al.* (2012)
CAGL0C04367g	*(ECM15)*	Orthologs have a putative function in cell wall biogenesis	I	Intergenic	Decreased	No	Riera *et al.* (2012)
CAGL0C00110g	*EPA6*	Subtelomerically encoded adhesin with a role in cell adhesion	I/KO	Intergenic	Decreased	Yes	Iraqui *et al.* (2005)
CAGL0C05643g	*EPA7*	Sub telomerically encoded adhesin with a proposed role in cell adhesion	NA[d]				Iraqui *et al.* (2005)
CAGL0M00132g[c]	*EPA12*	Putative adhesin-like cell wall protein	I	Intergenic	Increased	No	Riera *et al.* (2012)
CAGL0M13849g	*GAS2*	Putative glycoside hydrolase of the Gas/Phr family; predicted GPI anchor	KO		Decreased	No	Schwarzmuller *et al.* (2014)
CAGL0I10147g	*PWP1*	Putative adhesin-like protein	I	Intergenic	Increased	No	Riera *et al.* (2012)
CAGL0I10200g	*PWP3*	Putative adhesin-like protein	I	Intergenic	Increased	No	Riera *et al.* (2012)
CAGL0M03773g	*(TOS6)*	Predicted GPI-linked adhesin-like protein	KO		Decreased	No	Schwarzmuller *et al.* (2014)
CAGL0F00116g		Putative adhesin-like protein	I	Intergenic	Increased	No	Riera *et al.* (2012)
CAGL0M11726g		Putative GPI-linked cell wall adhesin-like protein	KO		Decreased	No	Schwarzmuller *et al.* (2014)
Polarity establishment							
CAGL0F06655g	*(AVO2)*	Ortholog(s) interact with the Tor2 kinase and have a role in establishment or maintenance of actin cytoskeleton polarity	KO		Decreased	No	Schwarzmuller *et al.* (2014)
CAGL0L08140g	*(BPH1)*	Protein with putative role in protein sorting	KO		Increased	No	Schwarzmuller *et al.* (2014)
CAGL0J00803g	*(MHP1)*	Putative microtubule-associated protein involved in microtubule organization	KO		Decreased	No	Schwarzmuller *et al.* (2014)
CAGL0F05335g	*(MSS4)*	Putative 1-phosphatidylinositol-4-phosphate 5-kinase activity with a role in actin cytoskeleton organization	I	Intergenic	Decreased	No	Riera *et al.* (2012)
CAGL0G01386g	*(SLM2)*	Putative phosphoinositide PI4,5P(2) binding protein involved in actin cytoskeleton regulation	KO		Decreased	No	Schwarzmuller *et al.* (2014)
Membrane transport and homeostasis							
CAGL0B02211g	*CCH1*	Putative calcium transporter	KO		Decreased	No	Schwarzmuller *et al.* (2014)
CAGL0J08613g	*(YVC1)*	Ortholog(s) have calcium activated cation channel activity	KO		Increased	No	Schwarzmuller *et al.* (2014)
CAGL0M00154g[c]	*CYN1*	Plasma membrane high-affinity cystine-specific transporter	I	Intergenic	Increased	No	Riera *et al.* (2012)

Table 1. (*Continued*).

Systematic name	Standard name[a]	Function[b]	Insertional (I)/ knockout (KO)/site specific (SS) mutant	Location of insertion	Effect on biofilm formation	Validated role in biofilm formation through detailed study	Reference
CAGL0F00187g	(*FET4*)	Putative copper ion transmembrane transporter	I	Intergenic	Increased	No	Riera *et al.* (2012)
CAGL0J07436g	*PDR16*	Putative phosphatidylinositol transfer protein	I	Intergenic	Decreased	Yes	Culakova *et al.* (2013)
CAGL0B04741g	*PGS1*	Phosphatidylglycerolphosphate synthase involved in cardiolipin synthesis	I	Intragenic	Decreased	Yes	Batova *et al.* (2009)
CAGL0E03674g	(*TPO1*)	Putative polyamine transporter	I	Intergenic	Decreased	No	Riera *et al.* 2012)
CAGL0G00242g	*YOR1*	Putative ABC transporter involved in multidrug efflux	KO		Decreased	No	Schwarzmuller *et al.* (2014)
Signaling							
CAGL0I05236g	*BCY1*	cAMP depedent protein kinase regulatory subunit	KO		Decreased	No	Schwarzmuller *et al.* (2014)
CAGL0L00605g	*CNB1*	Regulatory subunit of calcineurin, calcium/calmodulin-dependent Ser/Thr-specific protein phosphatase	KO		Decreased	No	Schwarzmuller *et al.* (2014)
CAGL0L00627g	(*GPB1*)	Putative regulator of Galpha protein and cAMP signaling	KO		Increased	No	Schwarzmuller *et al.* (2014)
CAGL0I07513g	(*PKH2*)	Putative protein kinase required for activation of the cell wall integrity signaling cascade	KO		Decreased	No	Schwarzmuller *et al.* (2014)
CAGL0F09075g	(*SCH9*)	Putative protein serine/threonine kinase	I	Intergenic	Decreased	No	Riera *et al.* (2012)
CAGL0K02673g	*STE20*	Putative signal transducing kinase of the PAK (p21-activated kinase) family; involved in maintaining cell wall integrity	KO		Increased	No	Schwarzmuller *et al.* (2014)
CAGL0I05896g	*YAK1*	Putative serine-threonine protein kinase	I	Intragenic	Decreased	Yes	Iraqui *et al.* (2005)
Subtelomeric silencing							
CAGL0K04917g	*RAP1*	Putative transcriptional regulator	SS		Increased	Yes	Iraqui *et al.* (2005)
CAGL0B03399g	*RIF1*	Protein involved in subtelomeric silencing, regulation of telomere length, silencing at *MTL3*	I/KO	Intergenic	Increased	Yes	Iraqui *et al.* (2005)
CAGL0M00770g	*SIR3*	Protein involved in subtelomeric silencing	KO		Increased	Yes	Iraqui *et al.* (2005)
CAGL0K11396g	*SIR4*	Protein involved in subtelomeric silencing	I/KO	Intergenic	Increased	Yes	Iraqui *et al.* (2005)
Transcriptional regulation							
CAGL0I05170g	*CST6*	bZIP domain-containing protein of the ATF/CREB family	I/KO	Intergenic	Increased	Yes	Riera *et al.* (2012)
CAGL0H06215g	(*MED15*)	Component of the transcriptional Mediator complex that provides interfaces between RNA polymerase II and upstream activator proteins	KO		Increased	No	Schwarzmuller *et al.* (2014)
CAGL0A01628g	*MIG1*	Transcription factor involved in glucose repression	KO		Increased	No	Schwarzmuller *et al.* (2014)
CAGL0B04895g	(*RFX1*)	Putative transcriptional repressor of DNA damage-regulated genes	I	Intragenic	Decreased	No	Riera *et al.* (2012)
CAGL0F09097g	*SKN7*	Transcription factor, involved in oxidative stress response	I	Intergenic	Decreased	No	Riera *et al.* (2012)

Table 1. (*Continued*).

Systematic name	Standard name[a]	Function[b]	Insertional (I)/ knockout (KO)/site specific (SS) mutant	Location of insertion	Effect on biofilm formation	Validated role in biofilm formation through detailed study	Reference
CAGL0M04807g	*SNF2*	Component of the chromatin remodeling Swi/Snf complex involved in transcriptional regulation	KO		Decreased	Yes	Riera *et al.* (2012)
CAGL0E03718g	*SNF6*	Component of the chromatin remodeling Swi/Snf complex involved in transcriptional regulation	I/KO	Intergenic	Decreased	Yes	Riera *et al.* (2012)
CAGL0J10472g	*(MED13)*	Putative subunit of the RNA polymerase II mediator complex involved in transcriptional regulation	KO		Increased	No	Schwarzmuller *et al.* (2014)
CAGL0M06875g	*(MED12)*	Putative cyclin-like component of the RNA polymerase II holoenzyme	KO		Increased	No	Schwarzmuller *et al.* (2014)
CAGL0F06039g	*(SUB1)*	Putative transcriptional regulator	KO		Decreased	No	Schwarzmuller *et al.* (2014)
CAGL0I04246g	*(SUT2)*	Putative transcription factor involved in sterol uptake	I	Intergenic	Increased	No	Riera *et al.* (2012)
CAGL0F05357g	*(UME6)*	Putative transcription factor	I	Intergenic	Decreased	No	Riera *et al.* (2012)
CAGL0M08800g	*YAP6*	Putative bZIP transcription factor of the yAP-1 family	KO		Increased	No	Schwarzmuller *et al.* (2014)
CAGL0J01595g	*(YPR015c)*	Transcription factor, zinc finger, C2H2 type	I	Intergenic	Increased	No	Riera *et al.* (2012)
Others							
CAGL0I05148g	*DLD1*	D-lactate ferricytochrome C oxidoreductase	I	Intergenic	Increased	No	Riera *et al.* (2012)
CAGL0A02816g	*(GCY1)*	Putative 2-methylbutyraldehyde reductase	I	Intragenic	Decreased	No	Riera *et al.* (2012)
CAGL0A04829g	*(HXK2)*	Hexokinase isoenzyme 2 that catalyzes phosphorylation of glucose in the cytosol; involved in the regulation of glucose repression	I	Intergenic	Decreased	No	Riera *et al.* (2012)
CAGL0C04389g	*(HTB1/2)*	Core histone proteins required for chromatin assembly and chromosome function	I	Intergenic	Decreased	No	Riera *et al.* (2012)
CAGL0I04224g	*(RAD54)*	Member of the SWI/SNF family of DNA translocase involved in repair of double-strand breaks	I				
CAGL0B00440g		Protein of unknown function	KO		Decreased	No	Schwarzmuller *et al.* (2014)

[a]Gene names are according to those available at the Candida Genome Database (Binkley *et al.* 2014) when available or shown in parenthesis based on the ortholog in *S. cerevisiae*.
[b]Functions have been assigned based on data available at the Candida Genome Database (Binkley *et al.* 2014), the Saccharomyces Genome Database (Costanzo *et al.* 2014) and literature surveys.
[c]Riera *et al.* (2012) have described CAGL0M00132g and CAGL0M00154g as a homolog of *S. cerevisiae STA1* encoding an extracellular glucoamylase and a homolog of the *S. cerevisiae* Lyp1 lysine permease, respectively; in the current annotation of these ORF at the Candida genome database (Binkley *et al.* 2014), they appear as *EPA12* encoding a putative adhesion-like cell wall protein of the Epa family and *CYN1* encoding a plasma membrane high-affinity cystine-specific transporter, respectively.
[d]NA: not available.

lectins (Cormack, Ghori and Falkow 1999; De Las Penas *et al*. 2003; Weig *et al*. 2004; Kaur *et al*. 2005; de Groot *et al*. 2013). A majority of the *EPA* genes are located at subtelomeres and the expression of several of these subtelomerically located *EPA* genes is governed by chromatin silencing mediated by the Sir2-3-4 NAD-dependent histone deacetylase complex, whose recruitment at subtelomeres relies on interactions with Rap1 and involves Rap1-interacting factors such as Rif1 (De Las Penas *et al*. 2003; de Groot *et al*. 2013; Kueng, Oppikofer and Gasser 2013). Consistently, several *C. glabrata* mutants with an increased ability to form biofilms were found to have insertions near the *RIF1* or *SIR4* genes and inactivation of *SIR3* and *RAP1* also increased biofilm formation (Iraqui *et al*. 2005). Moreover, *EPA6* and its closest paralog *EPA7* were shown to be negatively regulated by Sir4 and Rif1 and induction of *EPA6* and *EPA7* upon biofilm growth to be dependent on the presence of an intact subtelomeric silencing machinery (Iraqui *et al*. 2005). Taken together, these results have provided evidence that in *C. glabrata* biofilm formation requires the negative regulation of subtelomeric silencing that in turns allows expression of *EPA6* and *EPA7*, the Epa6 and Epa7 adhesins being likely to contribute to adhesion processes necessary for the development of a mature biofilm. Yet, it should be noted that a direct demonstration of a role of Epa7 in biofilm formation is lacking as knockout mutants for the corresponding gene are lacking. A contribution of Epa3 to biofilm formation may also be proposed as this protein has been identified in biofilm extracts and expression of *EPA3* is induced upon biofilm formation (Kraneveld *et al*. 2011).

Inactivation of subtelomeric silencing appears to favor biofilm formation through cell–cell interactions rather than cell–surface interactions and has also been shown to promote adherence to epithelial cells (Castano *et al*. 2005; Iraqui *et al*. 2005). It is also notable that the Epa1-5 adhesins do not appear to play a significant role in biofilm formation despite their structural relationship to Epa6 and Epa7 and the contribution of Epa1 to interactions of *C. glabrata* with epithelial cells (Cormack, Ghori and Falkow 1999; Iraqui *et al*. 2005). This probably reflects variations in expression of the corresponding genes according to the conditions encountered by *C. glabrata* cells. Indeed, *EPA6* and *EPA7* are expressed in stationary phase cells (that are more prone to biofilm formation) and upon biofilm formation, *EPA1* is predominantly expressed in the exponential phase of growth, *EPA2* is expressed in response to oxidative stress, *EPA3* is expressed upon biofilm formation and conditions for *EPA4-5* expression have not been identified (Iraqui *et al*. 2005; Kraneveld *et al*. 2011; Juarez-Cepeda *et al*. 2015). This probably also reflects different substrate specificity of these adhesins. Indeed, Epa1, Epa6 and Epa7, while all recognizing a terminal galactose residue in their glycan ligand, have different ligand specificity, with Epa6 having a broader specificity range than Epa1 or Epa7 (Zupancic *et al*. 2008). Recent results using atomic force microscopy have additionally revealed that cell surface-exposed Epa6 conveys strong hydrophobic forces that contribute to *C. glabrata* adhesiveness to abiotic surfaces (El-Kirat-Chatel *et al*. 2015). Hence, modifying the levels of the different Epa adhesins in the cell wall may result in the ability of *C. glabrata* to interact with different biotic or abiotic surfaces and form the complex 3D structures of biofilms. In this regard, it is interesting to note that silencing at subtelomeres harboring *EPA* genes appears to vary from cell to cell as indicated by variegation in the expression of the *URA3* gene inserted at different location in the subtelomeric *EPA1-3, EPA4-5* and *EPA6* loci (De Las Penas *et al*. 2003; Iraqui *et al*. 2005). It is tempting to hypothesize that such cellular heterogeneity may in particular contribute to the establishment of the 3D biofilm structure. Variations in the efficiency of subtelomeric silencing and hence *EPA* gene expression may also underlie variations in the ability of different *C. glabrata* isolates to form biofilms. For instance, introducing an extra copy of the *SIR3* gene in *C. glabrata* strain ATCC2001 was shown to abolish spurious transcription from the *EPA1* promoter (Halliwell *et al*. 2012). Notably, this strain is characterized by elevated expression of several *EPA* genes and an increased ability to form biofilms, which may reflect inefficient subtelomeric silencing (Martinez-Jimenez *et al*. 2013).

The role of the Epa adhesins in *C. glabrata* biofilm formation is reminiscent of that of the Als proteins in *C. albicans* biofilm formation (Hoyer 2001; Nobile *et al*. 2006, 2008). Yet, the similarity between these two species does not extend to the regulation of biofilm formation by subtelomeric silencing as the *C. albicans ALS* genes are not located at subtelomeres and thus not the subject of such regulation (De Las Penas *et al*. 2003; Filler 2006). In *C. albicans*, chromatin remodeling through the Set3 NAD-dependent histone deacetylation complex has been shown to modulate the transcription kinetics of several key regulators of biofilm formation and promote biofilm dispersal and decreased drug tolerance (Hnisz *et al*. 2012; Nobile *et al*. 2014) while the contribution of the Sir2 NAD-dependent histone deacetylase to biofilm formation has not been tested. Importantly, the mechanism by which subtelomeric silencing operates in *C. albicans* is unclear as orthologs to the Sir3 and Sir4 proteins are not found in this species (Fabre *et al*. 2005).

A role for other adhesins and cell wall remodeling in *C. glabrata* biofilm formation

Changes in the *C. glabrata* cell wall necessary for biofilm formation do not appear to be restricted to the expression of the Epa6 and Epa7 adhesins. Indeed, mutations that affect biofilm formation positively or negatively have been identified within or close to genes for other predicted adhesins such as *EPA12*, *PWP1* and *PWP3* (Table 1). Moreover, some of the *C. glabrata AWP* genes encoding non-Epa adhesin-like wall proteins show differential expression during adherence and biofilm formation *in vitro* and in animal models (Weig *et al*. 2004; Kraneveld *et al*. 2011; de Groot *et al*. 2013; Kucharikova *et al*. 2015). Thus, intense cell wall remodeling and changes in cell surface physical properties is undoubtedly one of the key events associated to *C. glabrata* biofilm formation. In this respect, cell wall remodeling enzymes such as the glucanosyl transferase Gas2 and the glucanase Des2 are also candidates for a role in biofilm formation (Table 1), and modifications in cell surface hydrophobicity due to mutations in genes involved in phospholipid synthesis such as *PDR16* and *PGS1* appear to alter biofilm formation (Batova *et al*. 2009; Culakova *et al*. 2013). Moreover, orthologs of genes related to the response of *S. cerevisiae* cells to cell wall stress are candidates for a role in biofilm development (Table 1). This includes (1) *MSS4* involved in the biosynthesis of phosphoinositides that are important in mediating activation of the Rho1 GTPase in response to cell wall stress; (2) *AVO2* that encodes a component of the Tor complex 2 that, among other roles, contributes to cell wall integrity; (3) *SLM2* that encodes a phosphoinositide-binding protein that physically interacts with Avo2 and is a downstream effector of the Tor complex 2; (4) *PKH2* that encodes a kinase contributing to an alternative cell wall integrity pathway (Levin 2011). While *C. glabrata PKH2* appears required for both biofilm formation and growth at elevated temperatures, this is not the case for its paralogs *PKH1* (*CAGL0G04609g*) and *PKH3* (*CAGL0K06479g*), suggesting some specialization of the Pkh kinases in sensing cell wall

integrity or regulating cell surface homeostasis (Schwarzmuller *et al.* 2014). It is also notable that the Mpk1 kinase is not required for biofilm formation despite its known role in regulating cell wall integrity (Iraqui *et al.* 2005). Finally, it should be noted that calcium signaling appears to play important roles in biofilm formation as inactivation of the *CCH1* gene that encodes a calcium channel and of the *CNB1* gene that encodes the regulatory subunit of calcineurin yield defects in biofilm formation. Many of the stresses that activate Cch1 also activate cell wall integrity signaling and the requirement for calcium signaling in *C. glabrata* biofilm formation may be linked to cell wall processes. In this respect, one should note that the transcription factor Skn7 has been tentatively linked to biofilm formation (Riera *et al.* 2012) and that it is involved in linking cell wall stress responses and calcium signaling in *S. cerevisiae* (Levin 2011).

Regulatory pathways controlling *C. glabrata* biofilm formation

The mechanisms by which *C. glabrata* senses an environment conducive to biofilm formation are still poorly understood. Domergue *et al.* (2005) have shown that nicotinic acid limitation encountered in the urine results in inefficient subtelomeric silencing due to the decreased activity of the Sir2 NAD-dependent histone deacetylase. Indeed, *C. glabrata* is a nicotinic acid auxotroph due to the loss of the *BNA* genes involved in the production of nicotinic acid mononucleotide from tryptophan via the kynurenine pathway (Dujon *et al.* 2004; Domergue *et al.* 2005). Hence, the urinary tract environment, in contrast to other environments in the human body that are rich in nicotinic acid, promotes expression of the *C. glabrata EPA1, EPA6* and *EPA7* adhesins, adhesion to host cells and further colonization by *C. glabrata* and it has been hypothesized that nicotinic acid limitation in the urinary tract, or specifically on catheter surfaces, might increase *EPA6* expression and stimulate the formation of catheter-associated biofilms (Domergue *et al.* 2005). Other inducers of *EPA6* expression have been identified such as high cell density, biofilm formation, hypoxia, the preservatives paraben and sorbic acid (De Las Penas *et al.* 2003; Domergue *et al.* 2005; Iraqui *et al.* 2005; Mundy and Cormack 2009).

Beyond this, the characterization of insertional mutants with alterations in biofilm formation has revealed several pathways that regulate biofilm formation in a manner that is dependent or independent of subtelomeric silencing (Iraqui *et al.* 2005; Riera *et al.* 2012). For instance, the Dual-specificity tyrosine-regulated (DYRK) Yak1 kinase appears to be required to repress the subtelomeric silencing machinery in response to an unknown biofilm formation signal and in turn allow expression of the *EPA6* and *EPA7* adhesin genes (Iraqui *et al.* 2005). Indeed, an insertion in *YAK1* results in decreased expression of *EPA6* and *EPA7* and biofilm formation, which are alleviated by impairing the subtelomeric silencing machinery. In *S. cerevisiae*, the Yak1 kinase is now regarded as a component of a nutrient sensing system that prevents growth upon nutrient deprivation, modifying the nucleo-cytoplasmic distribution and activity of the Pop2 subunit of the Ccr4–Not complex involved in mRNA deadenylation, of the Crf1 corepressor thus inhibiting transcription of ribosomal genes mediated by Fhl1, and of the Hsf1 and Msn2 stress-responsive transcription factors (Moriya *et al.* 2001; Martin, Soulard and Hall 2004; Lee *et al.* 2008). Under nutrient (glucose) sufficiency, phosphorylation of Yak1 by PKA prevents its translocation to the nucleus and hence its negative effect on growth (Lee *et al.* 2011) while under nutrient deprivation Yak1 promotes phosphorylation of the Bcy1 regulatory subunit of PKA and, consequently, its accumulation in the cytoplasm (Griffioen *et al.* 2001). Although a detailed investigation of the function of Yak1, its interplay with the PKA pathway and link to nutrient sensing has not been carried in *C. glabrata*, it is tempting to speculate that nutrient deprivation may to some extent favor biofilm formation in *C. glabrata* in line with the idea that this process constitutes a mean for *C. glabrata* to survive hostile environments. Interestingly, mutations in genes involved in glucose sensing have been identified that affect biofilm formation: (1) inactivation of the *BCY1* gene results in decreased biofilm formation consistent with a negative role of PKA in biofilm formation (Schwarzmuller *et al.* 2014); (2) inactivation of the *GPB1* gene that encodes a kelch repeat-containing protein acting in conjunction with the G alpha protein Gpa2 to mediate glucose sensing towards PKA results in increased biofilm formation as expected if glucose sensing is impaired (Harashima and Heitman 2002; Schwarzmuller *et al.* 2014); (3) lack of Mig1, a DNA-binding protein mediating glucose repression of gene expression, results in increased biofilm development as expected if glucose repression is abolished (Santangelo 2006; Schwarzmuller *et al.* 2014); (4) *HXK2* that encodes an hexokinase involved in mediating PKA activity towards glucose repression through modulation of the activity of the Snf1 kinase is one of the candidates for a role in biofilm formation identified by insertional mutagenesis (Santangelo 2006; Riera *et al.* 2012).

Insertional mutagenesis also identified Cst6, a basic leucine zipper (bZIP) transcription factor of the ATF/CREB family, as a negative regulator of biofilm formation and *EPA6* expression (Riera *et al.* 2012). As simultaneous inactivation of *YAK1* and *CST6* phenocopies the lack of *YAK1* while simultaneous inactivation of *YAK1* and *SIR4* phenocopies the lack of *SIR4*, it has been proposed that Yak1 and Cst6 control *EPA6* expression, and consequently biofilm formation, independently. There are several alternatives for a role of Cst6 in biofilm formation based on studies that have been conducted in *S. cerevisiae* and *C. albicans*. First, Cst6 has been implicated in chromosome stability and telomere maintenance and its absence may indirectly favor *EPA6* expression and biofilm formation, although in a manner that remains dependent on the Yak1 function (Ouspenski, Elledge and Brinkley 1999). Second, Cst6 is required for the activation of the *NCE103* gene encoding carbonic anhydrase in response to low CO_2 (Cottier *et al.* 2012). As CO_2 is a direct inducer of PKA in fungi (Klengel *et al.* 2005; Hall *et al.* 2010), inactivation of Cst6 may limit PKA activity and favor biofilm formation as discussed above. The implication of Cst6 in the utilization of non-optimal carbon sources in *S. cerevisiae* is harder to reconcile with the positive impact of its inactivation on *C. glabrata* biofilm formation (Garcia-Gimeno and Struhl 2000).

A number of other regulators of transcription are additional candidates for a role in biofilm formation in *C. glabrata* (Table 1). In this respect, it is especially notable that altering components of two key protein complexes associated with the regulation of transcription, namely the Swi/Snf chromatin remodeling complex and the Mediator complex, results in alterations of biofilm formation. Indeed, an insertional mutation near the *SNF6* gene resulted in decreased biofilm formation and *EPA6* expression and a similar phenotype was observed upon deletion of this gene and the *SNF2* gene encoding another component of the Swi/Snf complex (Riera *et al.* 2012). The Swi/Snf complex is involved in remodeling chromatin by destabilizing histone–DNA interactions, thereby modulating gene expression of a large number of genes in a positive or negative manner (Rando and Winston 2012). Recruitment of the complex at

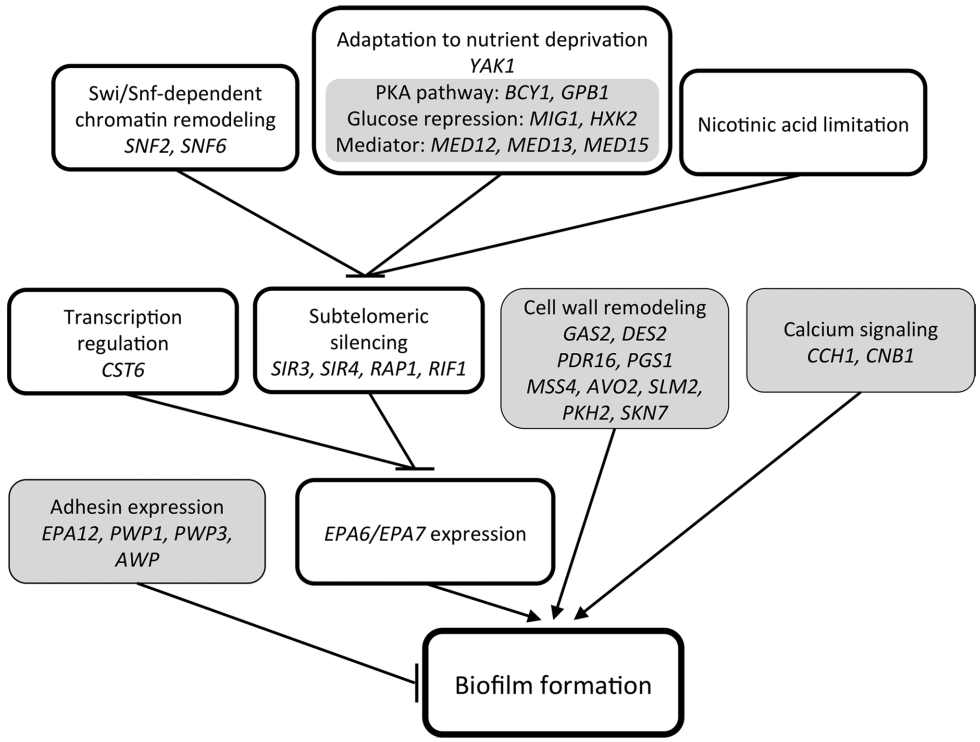

Figure 2. A model for the regulation of *C. glabrata* biofilm formation. The different processes and genes that have been implicated in biofilm formation have been integrated in a provisional model. An arrow indicates positive control of the subsequent process while a bar indicates negative control. Genes that have been tentatively associated with biofilm formation based on the sole phenotype of an insertional or deletion mutant are shaded in gray.

specific sites is mediated by interaction with transcription activators and repressors. As the function of the Swi/Snf complex in biofilm formation is only observed when subtelomeric silencing is intact, this complex must negatively modulate subtelomeric silencing either through interaction with components of the silencing machinery or transcription factors necessary for the activation of *EPA6* (Riera *et al.* 2012). Interestingly, the Swi/Snf complex does not appear to impact *EPA1* expression, which is consistent with individual transcriptional regulation of the different subtelomeres in *C. glabrata* (Rosas-Hernandez *et al.* 2008; Ramirez-Zavaleta *et al.* 2010).

Deletion in the *MED12*, *MED13* and *MED15* genes encoding components of the Mediator complex has been shown to lead to an increase in biofilm formation (Schwarzmuller *et al.* 2014). Mediator functions as a bridge between transcription regulators and the general transcription factors (Hahn and Young 2011). Specifically, Med15 (Gal11) is a component of the tail domain of Mediator involved in the interaction with several transcription factors while Med12 (Ssn8) and Med13 (Ssn2) are components of the kinase module of Mediator, the lack of which has positive and negative effects on transcription. Interestingly, lack of the kinase module allows the expression of genes induced by nutrient deprivation, which may explain the associated increase in biofilm formation in *C. glabrata* as discussed above for other regulators of nutrient adaptation.

OUTLOOK

Progress in the functional genomics of *C. glabrata* thanks to the development of collections of insertional mutants obtained by transformation of plasmid DNA or the systematic generation of deletion mutants by gene replacement technologies has now revealed a number of candidate genes for a role in biofilm formation (Table 1). Further investigation of a small subset of these genes, using epistasis assays, now provides for a model in which several regulatory pathways converge towards the release of the transcriptional silencing exerted on the *EPA6* and *EPA7* genes and their transcriptional activation, the Epa6 and Epa7 adhesin then contributing to cell–surface and/or cell–cell interactions necessary for biofilm formation (Fig. 2). This model provides a framework in which other genes that have been identified in the screens of Iraqui *et al.* (2005) and Schwarzmuller *et al.* (2014) should be investigated. Indeed, the tentative links that have been made in this review between several of these genes, processes such cell wall remodeling or adaptation to nutrient deprivation, the regulation of subtelomeric silencing and *EPA* gene expression, and biofilm formation need to be carefully assessed, especially as most of these hypotheses rely on knowledge gained in *S. cerevisiae* for the function of orthologs of the genes identified in *C. glabrata*. Although *S. cerevisiae* is phylogenetically close to *C. glabrata* (in contrast to the *Candida* species of the CTG clade such as *C. albicans*), important functional differences exist between these two species (Bolotin-Fukuhara and Fairhead 2014). In this respect, systematic evaluation of *C. glabrata* orthologs of genes that have been linked to biofilm formation in *S. cerevisiae* may be of significant value to assess commonalities and differences in biofilm formation between these two species (Ryan *et al.* 2012). *Candida glabrata* is unique among the pathogenic yeasts because of its phylogenetic position and haploid state. This facilitates reverse genetics approaches but also allows for forward genetics approaches that leverage the power of high-throughput sequencing (Brunke *et al.* 2014). This provides considerable opportunities for understanding the processes involved in biofilm formation and their tolerance to antifungals in this species. Notably, the search for genes contributing to the antifungal

tolerance of *C. glabrata* biofilms has not been undertaken yet, despite the importance of this phenomenon in the clinical setting. Whether mechanisms similar to those described in *C. albicans* are at play remains to be demonstrated.

The search for genes involved in biofilm formation in *C. glabrata* has taken advantage of a simple model whereby cells are allowed to adhere and form a biofilm on a polystyrene surface in 96-well plates. Although this model has been extremely valuable as outlined in this review, it does not directly address the formation of biofilms on clinically relevant materials and further genetic work should integrate an evaluation of mutant strains in *in vitro* and animal models of biofilm formation that use such materials. Notably, relatively little is known about the changes in physiology that are occurring during biofilm development, with only one study reporting the characterization of the *C. glabrata* biofilm proteome (Seneviratne *et al.* 2010). Further transcriptome and proteome studies are needed to provide a picture of the events that occur during biofilm formation and help in elucidating the mechanisms that govern biofilm formation, as has been done in *C. albicans*.

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