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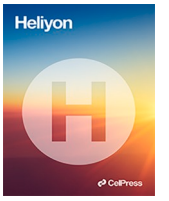

Review article

Fungal biofilm formation and its regulatory mechanism

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ABSTRACT

Fungal biofilm is a microbial community composed of fungal cells and extracellular polymeric substances (EPS). In recent years, fungal biofilms have played an increasingly important role in many fields. However, there are few studies on fungal biofilms and their related applications and development are still far from enough. Therefore, this review summarizes the composition and function of EPS in fungal biofilms, and improves and refines the formation process of fungal biofilms according to the latest viewpoints. Moreover, based on the study of *Saccharomyces cerevisiae* and *Candida albicans*, this review summarizes the gene regulation network of fungal biofilm synthesis, which is crucial for systematically understanding the molecular mechanism of fungal biofilm formation. It is of great significance to further develop effective methods at the molecular level to control harmful biofilms or enhance and regulate the formation of beneficial biofilms. Finally, the quorum sensing factors and mixed biofilms formed by fungi in the current research of fungal biofilms are summarized. These results will help to deepen the understanding of the formation process and internal regulation mechanism of fungal biofilm, provide reference for the study of EPS composition and structure, formation, regulation, group behavior and mixed biofilm formation of other fungal biofilms, and provide strategies and theoretical basis for the control, development and utilization of fungal biofilms.

1. Introduction

Fungi can naturally aggregate at various interfaces to form fixed communities [1,2], such as single or mixed fungal biofilms, showing adaptability to the environment. Fungal biofilm is usually defined as a three-dimensional structure composed of fungal cells and their extracellular polymeric substances (EPS) [3]. EPS contains macromolecules and small molecules [4–7], which have the functions of adhesion, cell aggregation, protection of antimicrobial agents and host defense mechanisms, provision of nutritional sources, stabilization of cell communities, and exchange of genetic information [8–11]. Due to differences in biology, environmental conditions, physical stress and nutrient availability, cells secrete varying components and ratios of EPS, and the composition, three-dimensional structure and function of the biofilm substrates formed may vary greatly [8,10,12]. It is the protection of the EPS

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from the environment that makes biofilm-related infections more difficult to control [13]. it is also the production of EPS that enhances cell surface adhesion and strengthens the expectoration of some heavy metals [14,15]. Therefore, understanding the important roles played by the components of EPS in biofilms is important for studying the structure and function of biofilms.

The formation of biofilms with certain structures and functions by fungi occurs through several stages such as initial adhesion, aggregation, growth, maturation, and dispersion [16]. Mature fungal biofilms have a variety of important functions (Fig. 1). Since some fungi can produce hyphae or pseudohyphae, the aggregation and growth of hyphae are involved in the production of biofilms. The first step in biofilm formation is the attachment of individual planktonic cells at the initial interface, which is thereafter accompanied by the continuous development and maturation of EPS production and accumulation to dispersion. However, a growing number of studies have shown that surface attachment is not necessary for biofilm formation. Many aggregated flocs in the planktonic state also have similar properties to biofilms, which are more prominent in aggregation characteristics [17]. Hence, it is noteworthy to update and refine the process of fungal biofilm formation according to the latest research perspectives.

Furthermore, the formation of biofilm cannot be separated from the regulation of internal genes, but most of the studies on the regulation of fungal biofilm genes have been concentrated in *Saccharomyces cerevisiae* and *Candida albicans*, which involve a large number of genes, complex regulatory pathways, and dispersed studies. Therefore, it is necessary to systematize the genes and metabolic pathways related to biofilm formation in *Saccharomyces cerevisiae* and *Candida albicans* to grasp the molecular mechanism of biofilm formation in these two fungi and to provide theoretical reference for the study of the internal regulatory mechanism of biofilm formation in other fungi. Meanwhile, quorum sensing plays an important role in regulating the formation of fungal biofilms. Quorum sensing factors are related to the behavior of fungal communities and the regulation of morphogenesis. Due to the complex and changeable environment, fungi can interact with other microorganisms to form a mixed fungal biofilm. Mixed fungal biofilms bring some advantages to other members, including substrate exchange, antimicrobial resistance, environmental adaptability, adhesion ability, and cell communication [18]. Therefore, summarizing the research content of fungal mixed biofilms is crucial for understanding the adhesion and signal transduction mechanisms of multi-species interactions.

Therefore, this review summarizes the main components in fungal EPS and describes the role that each component plays in the biofilm. This is of great significance for the study of the internal structure, cellular connection mode and function of other fungal biofilms. Meanwhile, the biofilm formation process was further improved according to the latest research viewpoints, which provides a reference for the subsequent study of the fungal biofilm formation process. Moreover, the basic understanding of fungal biofilms is still obtained from single-species biofilms formed by model organisms. We summarize the biofilm regulatory networks of two well-studied fungi to provide molecular theoretical foundations and methodological references for the study of biofilm-forming mechanisms in other fungi. Finally, this review also summarizes the current relevant studies on mixed fungal biofilms, which is of great significance for the subsequent study of the interactions between mixed fungi and other microorganisms, and for revealing the ecological value of fungal biofilms.

2. Extracellular polymeric substances in biofilm

In nature, most microorganisms exist in the form of biofilms. Biofilm formation is accompanied by the production of the extracellular polymer EPS. In general (Fig. 2), EPS can be categorized into two types: loosely -bound EPS (LB-EPS) and tightly -bound EPS (TB-EPS) [19]. LB-EPS can be easily released into the surrounding environment, whereas TB-EPS is tightly adhered to the surface of

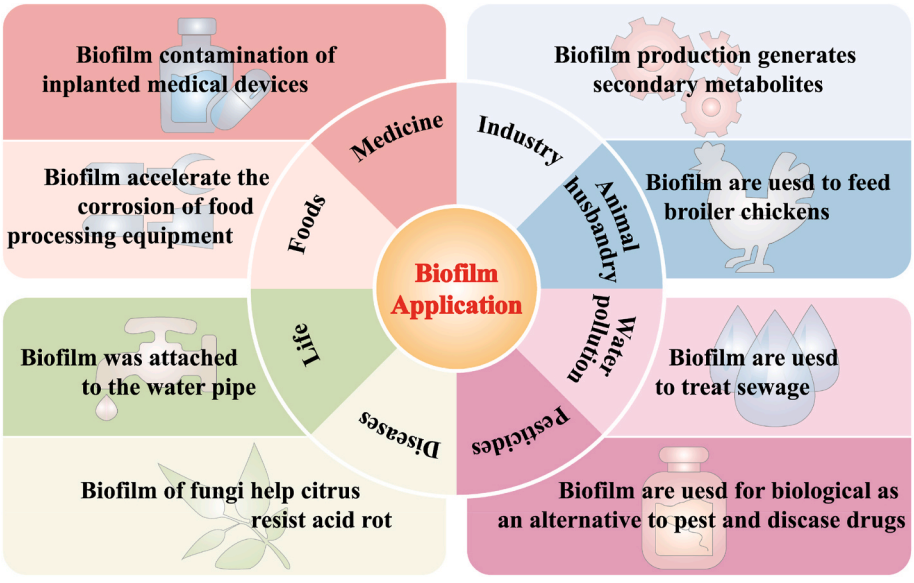

Fig. 1. Functions of fungal biofilm.

microbial cells [20,21]. The attachment of EPS to the surface of microbial cells enhances the protection of microbial cells and thus greatly improves the adaptability to the environment.

EPS has a complex composition, which mainly consists of polysaccharides, proteins, lipids, extracellular DNA (eDNA), and small molecules [4–6]. EPS polysaccharides are related to the adhesion of biofilm and constitute the basic skeleton of biofilm [7]; proteins are related to the hydrophobicity of biofilm, and in general, biofilms with high protein content are highly hydrophobic [5,6]. Lipids can interact with polysaccharides to form lipopolysaccharides with surfactant properties, helping to stabilize the biofilm structure [22]. The two ends of the eDNA chain can be connected to different cell surfaces to enhance cell-to-cell adhesion and contribute to the integrity of the biofilm structure [23–26]. It has been shown that eDNA induces adhesion to the surface of *Aspergillus fumigatus* conidia and participates in polysaccharide localization during the early stages of *Aspergillus fumigatus* biofilm development. eDNA has also been suggested to serve as an important source of nutrients for fungal biofilms, but the exact mechanisms of these are not known [25,27]. In addition, small molecules can be involved in the exchange of information within biofilms. The interaction of small molecules with macromolecules determines the internal chemical environment, physical structure, and mechanical strength of biofilms and provides biofilm cells with living conditions that are quite different from those of planktonic cells [28].

The formation of biofilm is affected by external and internal multiple factors. In the face of external complex and changeable environment, cells can adjust the proportion of each component of EPS in time, increase or decrease the secretion of certain substances, form a specific structure, and improve the survival probability of cells themselves. For example, the material composition of different stages of biofilm formation will also be different. The most obvious is that in the mature stage of biofilm, in order to strengthen the material exchange, the water content rises sharply [29]. Therefore, studying the composition and percentage of microbial biofilm EPS is crucial for clarifying the biofilm structure and function. It will help us to gain insight into the reasons for the structural changes of the same fungal biofilm in different periods and the differences in the composition of different fungal biofilms, and better interpret the ecological functions that different biofilm structures play in different periods and different species.

3. Formation of fungal biofilm

Fungal biofilm formation is a multi-stage process, and it can be categorized into yeast cell biofilms and hyphal biofilms based on whether or not they contain hyphae. The formation of fungal biofilm consists of four stages: interfacial adhesion, growth, maturation, and diffusion, as in the case of *Saccharomyces cerevisiae* and *Candida albicans* [16,30].

The first step in the process of fungal biofilm formation is adhesion at the interfacial surface, which is the basic condition for biofilms to acquire external nutrients [31]. In this stage, fungal cells synthesize sugars, proteins, and lipids to increase cell adhesion and promote adhesion or aggregation [30,32], which provides cell cohesion for the pre-biofilm stage and reduces cell motility to prepare for the next step of promoting biofilm diffusion and growth. However, there is growing evidence that biofilms do not necessarily require surface attachment to form, and that aggregates can form in fluids induced by asexual growth, co-aggregation, or

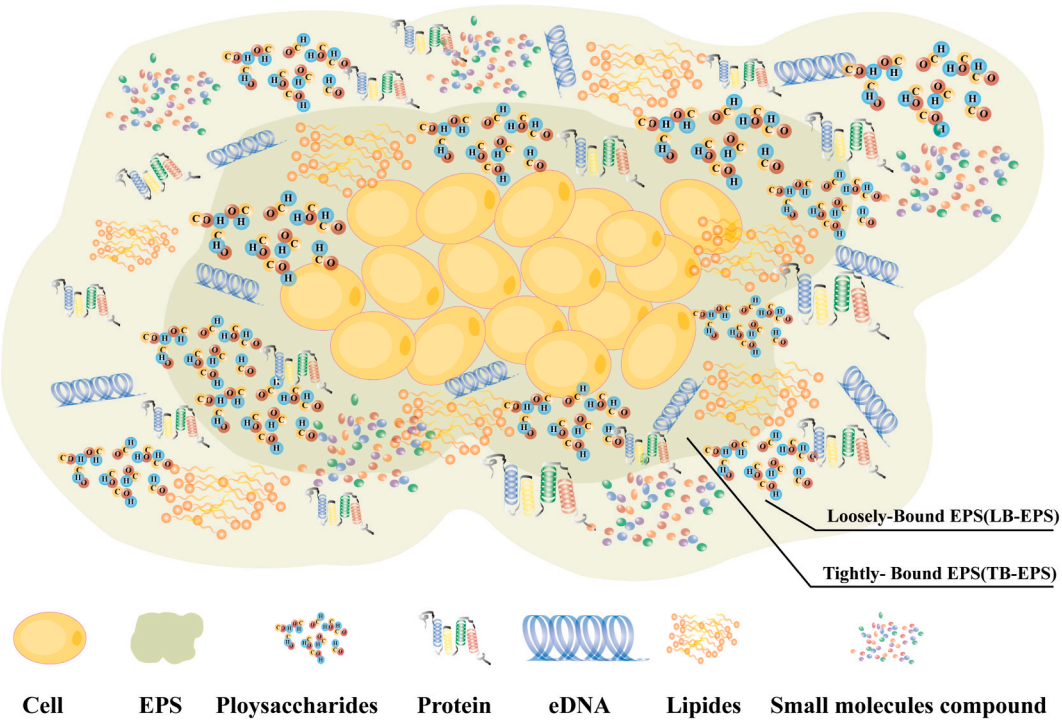

Fig. 2. Composition and function diagram of EPS.

host fluids [33–35]. For example, during wine fermentation, once all the sugars have been converted to alcohol, the biologically aged wine must spontaneously form a natural biofilm called a "flour velum" [36]. Cell aggregates formed in fluids also have the nature of biofilms, but it is unclear what drives aggregation in fluids, whether aggregates can attach to surface biofilms, and whether fusion with surface biofilms can occur, and these related mechanisms need to be further investigated and supplemented (As shown by the green arrow in Fig. 3).

With the continuous growth and expansion of the biofilm, the biofilm will enter the mature stage. The most obvious sign of mature biofilm is the formation of three-dimensional structure, which is closely related to the production of EPS. These components are interconnected to polymerize cells, making the colony present a special appearance structure and enhancing the stability of the biofilm. For example, *Candida albicans*, *Candida neoformans* and *Saccharomyces cerevisiae* will form wrinkled biofilms under the influence of EPS [37]. The formation of wrinkled colonies is a common form of biofilm formation, and it is also a basis for judging whether the strain can produce biofilm [38,39]. Fungal biofilms are covered by EPS at the mature stage, and the three-dimensional structure is dense, accompanied by the formation of water channels and microcolonies, such as filamentous fungal biofilms at this stage [22,30,40]. It is precisely because of these special three-dimensional structures in the biofilm that the biofilm can help the cell resist the adverse environment and greatly improve the cell survival rate.

Biofilm disperson is the final stage of the biofilm life cycle and the beginning of another life cycle [41]. Biofilm disperson is divided into initiative disperson and passive disperson, and the former is characterized by the promotion of disperson through gene regulation, which is mainly manifested in the inhibition of fungal production of extracellular matrix, the promotion of the production of enzymes that degrade extracellular components, and the enhancement of cellular motility, which facilitates the diffusion of planktonic cells to the surrounding environment [42–44]. Passive disperson, also known as environmentally induced dispersal, is mainly through environmental influences, and is manifested by the gradual increase in the area and thinning of the thickness of the biofilm as it continues to spread. With the lack of oxygen and the scarcity of nutrients, the biofilm will be forced to disintegrate, and the cells under the membrane will gradually disperse to the outside world [45]. After biofilm separation, individual fungal cells attach to new areas of tissue or similar surfaces and continue the process of biofilm formation [46]. As cells continue to adapt to their environment, the biofilm structure changes and reorganizes continuously [47].

Finally, we drew a model diagram of the fungal biofilm formation process based on the latest research results (Fig. 3), and the first step of biofilm formation in the new model not only covers interfacial attachment, but also non-interfacial cell aggregation is also reflected. At the same time, the final dispersion stage of biofilm was refined, and its perfected the process of fungal biofilm formation to provide theoretical reference for related research, but the model is not final.

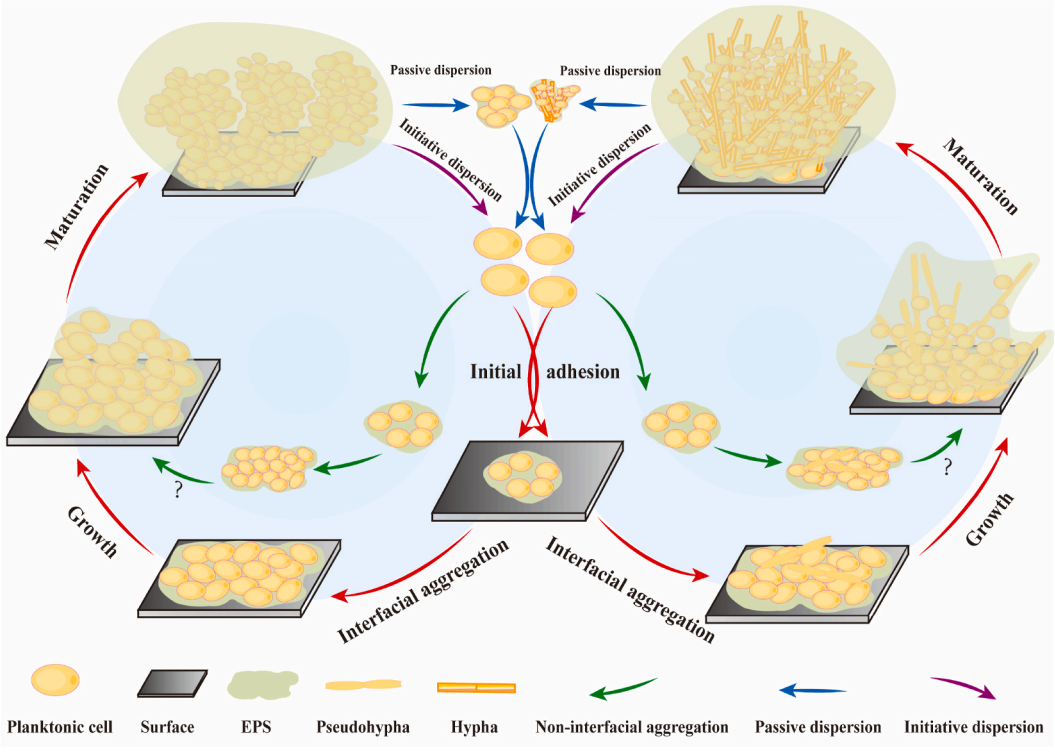

Fig. 3. The model for fungal biofilm development.

4. Regulation of fungal biofilm

The formation of fungal biofilms is closely related to the external environment, but the influence of signal transduction network regulation involving genes cannot be ignored. Although fungal biofilms have been studied for many years, the most studied in terms of gene regulation are *Saccharomyces cerevisiae* and *Candida albicans* [48]. In addition, the formation of biofilm is closely related to the external environment, but the influence of signal transduction network regulation involving genes cannot be ignored. Therefore, this review summarizes the regulatory network of biofilm synthesis in *Saccharomyces cerevisiae* and *Candida albicans*, which will help to understand the formation process, regulatory mechanism and signaling pathway of biofilm when fungi resist changes in the external environment, and develop effective methods from the molecular level to control harmful biofilms or enhance and utilize beneficial biofilms, providing a theoretical basis for the development and application of fungal biofilms.

4.1. Regulation of biofilm formation in Saccharomyces cerevisiae

The *flo11* gene is an important gene for biofilm formation in *Saccharomyces cerevisiae*, and it is also one of the most commonly

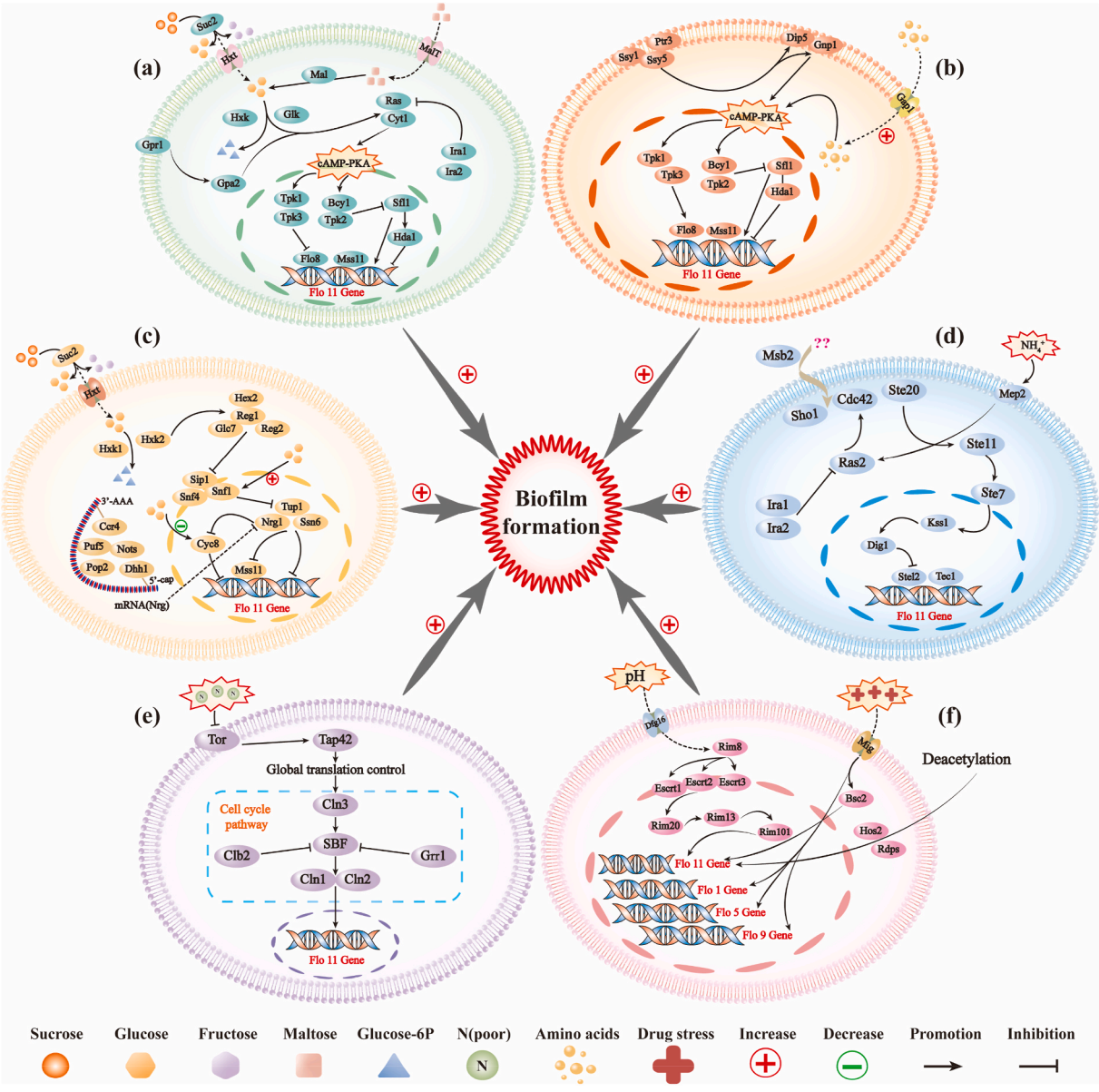

Fig. 4. Gene regulation of biofilm formation in *Saccharomyces cerevisiae*. (a) cAMP-PKA pathway; (b) Amino acid metabolic pathway; (c) GCN pathway and mRNA decay pathway; (d) MAPK pathway; (e) TOR pathway; (f) Rim pathway.

described adhesin genes. The *flo11* gene also affects colony morphology, and its deletion will lead to the transformation of *Saccharomyces cerevisiae* colonies from biofilm state to smooth state [49]. The adhesion protein encoded by *flo11* gene helps yeast cells adhere to the interface, which is called adhesin. Adhesin plays an important role in the initial adhesion of biofilm. Studies have shown that the expression of *flo11* is stronger under nutrient deficiency conditions, and the cell adhesion of *Saccharomyces cerevisiae* is also enhanced [48–51]. The adhesin expressed by *flo11* has the ability of genetic identification, which can recognize homologous proteins, promote the interaction between homologous proteins, accelerate cell aggregation [52–55], and also play an important role in biofilm formation, invasive growth and pseudohyphae [52,53,56–60].

Cell surface adhesion mediated by *flo11* is triggered by specific stress factors and nutritional limitations. At present, the expression of *flo11* in *Saccharomyces cerevisiae* is mainly regulated by seven signaling pathways, including cAMP-PKA pathway (Fig. 4a), amino acid metabolism pathway (Fig. 4b), glucose inhibition pathway (GCN) (Fig. 4c), mRNA decay pathway (Fig. 4c), MAPK pathway (Fig. 4d), TOR pathway (Fig. 4e) and Rim pathway (Fig. 4f). In addition, transcription factors Sok2, Phd1 and Ash1 can also regulate the expression of *floll*, but the exact pathway related to it is still unknown [61–63].

The cAMP-PKA pathway (Fig. 4a) functions to induce *flo11* gene expression when sugars (e.g. sucrose) are present in the environment. Complete activation of this pathway requires dual signals to enhance Cyr1 activity. First, sucrose in the environment is converted to glucose and fructose by Suc2, a sucrose-converting enzyme secreted by yeast [64], and then enters the cell through the hexose transporter Hxt; maltose in the environment enters the cell through the transport protein MalT, and the hexose entering the cell is phosphorylated by one of the hexokinases Hxk to glucose 6-phosphate [65], which enhances the activity of adenylate cyclase Cyr1. Secondly, the g-protein coupled receptor system composed of Gpr1 and Gpa2 senses extracellular glucose or sucrose to further enhance Cyr1 activity [66]. Subsequently, cAMP activates the protein kinase A complex (PKA), resulting in the separation of the Bcy1 subunit from the Tpk catalytic subunit of PKA [67,68]. The three different subunits of Tpk, Tpk1, Tpk2 and Tpk3, play different roles in the regulation of Flo11: Tpk1 and Tpk3 are inhibitors, and Tpk2 is an activator [63,69,70]. Flo8 is located in the nucleus and has been considered as a transcriptional activator of *flo11* [69–71]. Tpk1 and Tpk3 inhibit the expression of *flo11* by inhibiting the expression of *flo8*. When the Bcy1 subunit is released, the free Tpk2 kinase inactivates the main inhibitor of *flo11* expression, Sfl1 (flocculation inhibitor), and activates the regulation of *flo11* expression [72–74]. Ira1 and Ira2 are two negative regulators that inhibit Cyr1 activity to silence the pathway, resulting in the silencing of the *flo11* gene [75]. In addition to the role of cAMP-PKA signaling pathway, Sfl1 can also use histone deacetylase Hda1 to silence *flo11* gene. Moreover, the transcription factor Mss11 regulates pseudohyphae differentiation, invasive growth and starch metabolism in response to nutrient utilization. Mss11 can positively regulate the expression of *flo11* gene through MAPK and cAMP-PKA pathways [74].

Amino acid metabolic pathway (Fig. 4b) can induce yeast cell adhesion, thereby regulating the expression of *flo11* gene. Extracellular amino acids induce the SPS (Ssy1-Ptr3-Ssy5) complex on the plasma membrane, and the activated complex induces the gene expression of amino acid transporters Dip5 and Gnp1, which activate Flo11 transcription in a cAMK-PKA-dependent manner. When the SPS (Ssy1-Ptr3-Ssy5) complex signal is not active, the transcription level of amino acid transporter Gap1 is increased, and the intracellular amino acid concentration is increased to induce the expression of *flo1*1 by triggering the cAMK-PKA pathway [57].

When intracellular glucose is excessive, the GCN pathway (Fig. 4c) has an inhibitory effect on the expression of *flo11* gene. Excessive intracellular glucose levels will stimulate the phosphatase complex Hex2-Reg1-Glc7-Reg2 to dephosphorylate Snf1 and inactivate it [75,76]. When Snf1 is inactivated, the DNA-binding protein Mig1 is translocated from the cytoplasm to the nucleus [77], and binds to the inhibitors Tup1 and Ssn6 to initiate the expression of related glucose-inhibiting genes, including *mal* and *gal* genes involved in gluconeogenesis, respiration and absorption and decomposition of alternative carbon sources, and negatively regulates the expression of *flo11* gene. In addition, Cyc8 can bind to Ssn6 and Tup1 as an inhibitor complex, and Cyc8 and Tup1 antagonize the regulation of *flo11* expression level and biofilm complexity. Cyc8 inhibits *flo11* gene and prevents biofilm formation, while Tup1 antagonizes Cyc8-mediated *flo11* inhibition and stabilizes its structure by preventing the degradation of *flo11* protein [78]. Moreover, the mRNA decay pathway (Fig. 4c) also inhibited the expression of *flo11* gene. The complex (Ccr4-Puf5-Nots-Pop2-Dhh1) binds to the mRNA of Nrg1, the deolefination of Ccr4 is used for the 3 ’ -AAA end of mRNA, and Dhh1 acts as an activator of decap and translation inhibitors on mRNA 5 ’ -cap, reducing Nrg1 translation, thereby inhibiting Miss11, inhibiting *flo11* expression, and reducing biofilm adhesion.

In the MAPK pathway (Fig. 4d), Dig1, Ste12 and Tec1 transcription factors regulate *flo11* to affect the biofilm synthesis of yeast cells. One of the receptor proteins at the top of the pathway, Msb2, was speculated to be a possible receptor for alcohols but was not confirmed [79–82]. Msb2 stimulates the cell surface adhesion protein complex Sho1-Cdc4-Ste20 by sensing extracellular signals. The complex positively regulates the central kinases Ste11 and Ste7, and further relies on Kss1 downstream of the MAPK pathway to positively regulate the transcription regulator Dig1, while Dig1 negatively regulates Ste12 and Tec1 to inhibit the expression of *flo11* [63].

The TOR pathway (Fig. 4e) can indirectly regulate the expression of *flo11* through the cell cycle pathway. Tor kinase can phosphorylate Tap42 under nitrogen-rich conditions, and Tap42 activated by Tor is involved in the entire translation process [83–86]. This process can affect the translation of G1 cell cycle proteins Cln3 mRNA [83], thereby further affecting SBF-mediated G1 cell cycle proteins Cln1 and Cln2 expression. The abundance and activity of G1 cell cycle proteins Cln1,2 are required for *flo11* polarization, cell elongation, and transcriptional activation [87]. The mitotic cell cycle protein Clb2 plays a negative regulatory role in this process.

The Rim101 pathway (Fig. 4f) is closely associated with colony morphology in the Rim pathway, which is thought to function under alkaline conditions. Under these conditions, the Rim signaling pathway controls invasive growth and *flo11* expression and regulates mat formation [88,89]. The plasma membrane receptor protein Dfg16 in the Rim signaling pathway senses extracellular pH signals (e.g. neutral pH) and uses the inhibin protein Rim8 to enter the cell [88,90–92], and then uses the component transduction signal of the ESCRT-I, II and III complexes to activate the expression of *rim20*, *rim13*, *rim101*, and further regulate the expression of *flo11*, which is

crucial for the formation of biofilm matrix. In addition, in other pathways, Bsc2 can regulate the expression of *flo11* to promote biofilm formation and enhance the resistance of yeast to amphotericin (AMB) [93]. When extracellular ethanol accumulates, Mig1 enhances intercellular adhesion by regulating the overexpression of *flo1*, *flo5* and *flo9* genes, thereby protecting the cells in the outer layer of the biofilm from ethanol [71]. Hos2 and Rdps can also affect the expression of *flo11* by deacetylation [94].

4.2. Regulation of biofilm formation in Candida albicans

The most well-defined *Candida albicans* in terms of studies on the regulation of biofilm synthesis in pathogenic fungi [95]. The formation of *Candida albicans* biofilm includes four stages: adhesion (cell-interface adhesion, cell aggregation) (Fig. 5a), hyphal growth, aggregation (Fig. 5b), maturation (Fig. 5c) and dispersion (Fig. 5d). Biofilm formation and all stages are strictly regulated by genes.

The *Candida albicans* biofilm adhesion stage (Fig. 5a) is divided into two main parts: adhesion of fungal cells to biotic or abiotic

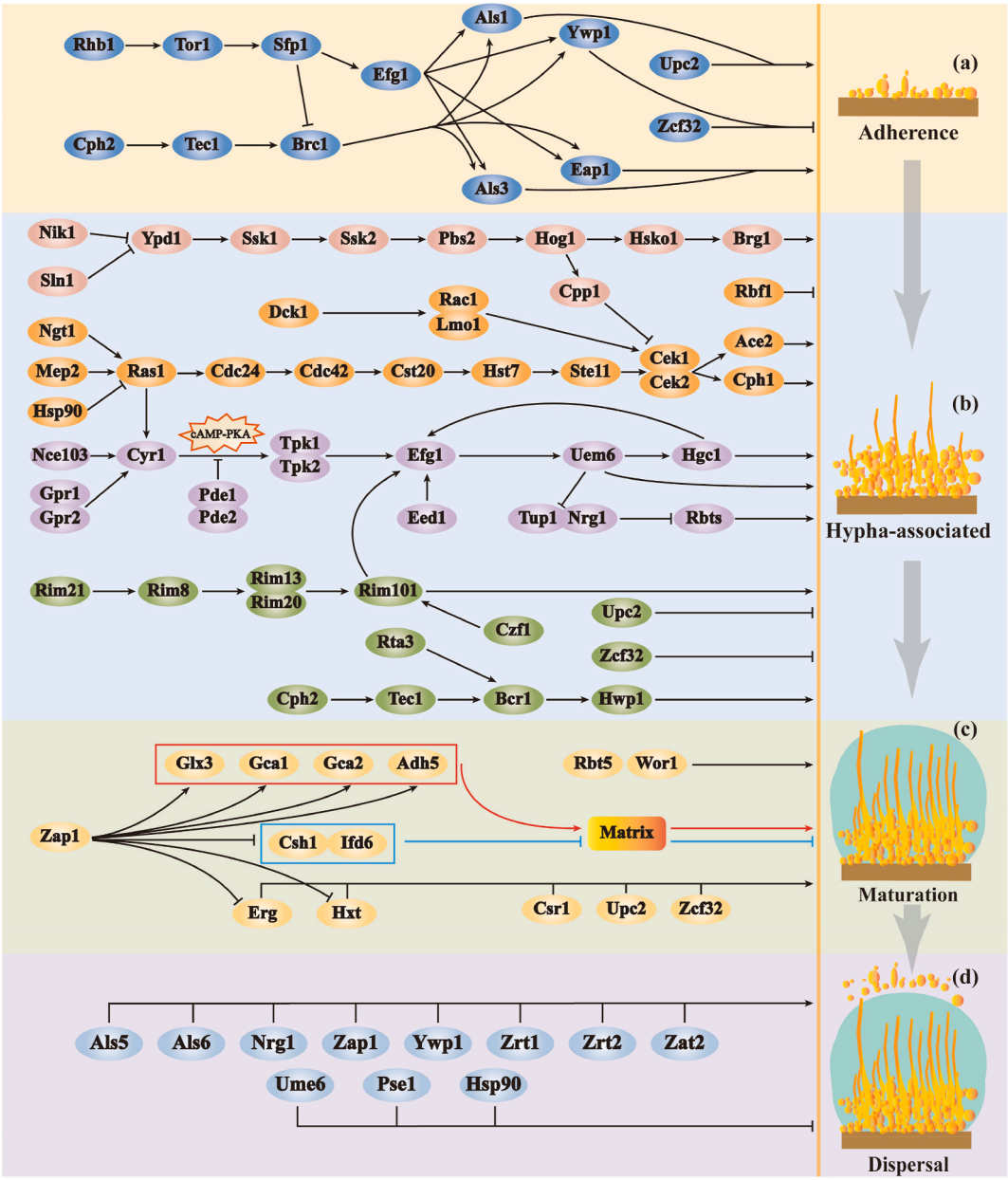

Fig. 5. Gene regulation of biofilm formation in *Candida albicans*. (a) adhesion stage; (b) hyphal growth and aggregation stage; (c) mature stage; (d) Dispersion stage.

surfaces, and tight adhesion and aggregation between fungal cells and fungal cells. Adhesion-related genes in *Candida albicans* include *als1*, *als3*, *ywp1*, and *eap1*. *als1* and *als3* are a class of cell wall glycoproteins from the Als protein family [96], which encode proteins that are highly similar in amino acid sequence to the cell surface adhesion protein-lectin of *Saccharomyces cerevisiae* [97]. In addition, the adhesion protein synthesized by *eap1* gene can enhance the adhesion between biofilm and medium. In this stage, *ywp1* gene plays a negative regulatory role. Ywp1 protein is a glycosylphosphatidylinositol-dependent cell wall protein (GPI-CWP protein), which is mainly expressed in yeast cells and inhibits the formation of biofilm. *als1*, *als3*, *ywp1* and *eap1* play a key role in the early stage of *Candida albicans* biofilm formation, and these genes are regulated by other genes. It has been found that the expression of *eap1* and *ywp1* genes is regulated by Efg1 protein encoded by *efg1* gene, which affects the colonization of biofilm [98]. In addition, Bcr1 is a zinc finger protein that can positively regulate the expression of *als1* and *als3*, and the expression levels of *als1* and *als3* are related to cell morphology [99]. Bcr1 can also directly regulate *ywp1* and *eap1* genes and enhance cell adhesion [100]. The stable formation of the adhesion stage can provide a basis for the subsequent development of the biofilm.

After the end of the adhesion stage, the biofilm began to develop to the colonization stage (Fig. 5b), which was characterized by the transformation of hyphal morphology and the aggregation of hyphae. This stage was related to the invasive growth of *Candida albicans*. The formation and growth of hyphae are the key to the improvement of pathogenicity of *Candida albicans*, gene regulation at this stage is carried out through four different pathways (Hog MAPK pathway, MAPK pathway, cAMP-PKA signaling pathway and the Rim101-pH sensing pathway). In *Candida albicans*, the Hog MAPK pathway (pink gene part) can regulate the formation of hyphae, thereby affecting the virulence of the strain and participating in the regulation of its oxidative stress response [101,102]. Nlk1 and Sln1 are membrane-bound sensing proteins that sense the progress of osmotic stress by reducing the activity of the complex Sln1-Ypd1-Ssk1 and silencing the expression of *hog1* gene. Hog1 stimulates yeast to transform into hyphae through downstream proteins Hsko1 and Brg1 [103]. *hog1* and *cpp1* genes are involved in the regulation of Cek1 phosphorylation, and Cpp1 phosphatase may negatively regulate the expression of *cek1* and *cek2* as a key mediator of Hog1 and Cek1 [104].

The MAPK pathway (orange gene part) is induced by matrix environment, low nitrogen and other factors [105,106]. The substrate environment stimulates Dck1, further stimulates Rac1, Lom1, Cek1 and Cek2, thereby regulating Ace2 and Cph1 to affect hyphal morphogenesis and biofilm formation. Low nitrogen environment stimulates Ngt1, Mep2, Hsp90, Ngt1 and Mep2 as positive regulators, while Hsp90 as a negative regulator stimulates Ras1 to interact with Cdc42 and Cst20, triggering Cek1 phosphorylation by stimulating Hst7 and Ste11 [107]. Recent studies have shown that Hst7 may be located between Cst20 and Cek1, Cek2 [105]. Cek1 and Cek2 positively regulate Ace2 and Cph1 to participate in the regulation of biofilm.

The cAMP-PKA signaling pathway (purple gene part) plays an important role in the hyphal elongation stage of *Candida albicans*. This pathway enhances the activity of adenylate cyclase Cyr1 through Nce103, Gpr1, and Gpr2 [108–110]. Subsequently, the cAMP-activated protein kinase A complex (PKA) stimulated complex Tpk1-Tpk2 acts on the transcription factor Efg1 to act on the hyphal elongation gene *ume6*, which further regulates the expression of downstream *hgc1* [105,111]. Furthermore, Hgc1 can inhibit the secretion of hypha cells, and Eed1 is necessary for *ume6*expression and plays an important role in maintaining hypha [112]. Eed1, Hgc1, and Ume6 play a key role in hyphal elongation. Ume6 and Eed1 negatively regulate Tup1 and Nrg1 and indirectly promote hyphal formation [113]. In solid medium, Tpk1 is involved in hyphal growth, while in liquid medium, Tpk2 is involved in hyphal growth [113–116].

Different cellular forms play different roles in infection and adaptation to different host niches. Acidic pH inhibits the transformation of yeast into filamentous cells, while neutral and alkaline pH promote the formation of filamentous cells [117,118]. *Candida albicans* can adapt to alkaline environment through the Rim101 pathway (green gene part). In alkaline environment, cells sense environmental signals through Rim21, and activate Rim101 through Rim8, Rim20 and Rim13 [118,119]. Studies have shown that the absence of any of these four proteins leads to the loss of the ability to form hyphae and affects the toxicity and adhesion to epithelial cells [120–123]. In addition, Hwp1 is a glycosylphosphatidylinositol-anchored cell wall protein (GPI). As a cell surface protein required for biofilm formation, Hwp1 can promote the contact and adhesion of *Candida albicans* with host cells, thereby accelerating the biofilm colonization process [119]. Hwp1 is regulated by upstream *bcr1*. Rta3 has a 7-transmembrane domain topology. Rta3 regulates the expression of *bcr1* target genes involved in cell surface characteristics, adhesion and hyphal growth. Bcr1 acts downstream of Rta3 and mediates the biofilm formation of *Candida albicans* [124]. Moreover, *bcr1* is also regulated by the upstream gene *tec1*. Studies have shown that the biofilm formed by *Candida albicans* lacking the *tec1* gene is thinner, and the content of hyphae in the biofilm is significantly reduced [117]. Zcf32 negatively regulates its adhesion ability and yeast to hyphae transformation by inhibiting the expression of many g-anchored proteins, while Upc2 negatively regulates the adhesion of biofilm development and positively regulates the maturation stage [125].

The mature biofilm of *Candida albicans* is a three-dimensional structure composed of hyphae, which is a highly structured microbial community. The mature biofilm has stronger resistance. The abundant proteomic components in the extracellular matrix of biofilm are mainly Glx3 proteins synthesized by *glx3* [126]. In the mature stage Fig. 5c), *glx3*, *adh5*, *gca2*, *gca1*, *csh1*, *ifd6* and *erg6* play an important role. *adh5*, *gca2*, *gca1*, *csh1*, *ifd6* and *erg6* genes are regulated by *zap1*, which encodes a zinc-sensitive transcription factor. In mature biofilms, Zap1 acts as a negative regulator in multiple pathways [127]. Zap1 inhibits the expression of downstream target genes *csh1*, *ifd6*, *gca1* and *adh5*, reduces the production of β-1,3 glucan in the extracellular matrix of biofilm, and inhibits the formation of extracellular matrix [127]. Zap1 can control the expression of *gca2* and *adh5* genes to promote the formation and accumulation of extracellular matrix. In addition, Zap1 inhibits the expression of *erg* and *hxt* genes, thereby inhibiting the maturation of biofilms [127].

Biofilms do not grow indefinitely after maturation, and when under pressure from the internal and external environment, such as excessive population density and nutrient deficiencies, biofilms will enter the final stage of their life cycle - dispersal (Fig. 5d) [128]. Dispersal of cells in a mature biofilm is an important part of the biofilm life cycle, and dispersed cells are highly adhesive, virulent and resistant to drugs. Some cells in the *Candida albicans* biofilm disperse into the surrounding environment in the form of yeast, forming a

new biofilm. Currently, there are few regulatory mechanisms for the diffusion stage of *Candida albicans* biofilm, and only *ume6*, *pes1* and *nrg1* genes have been reported to regulate the dispersion of cells in biofilm. *pes1* and *nrg1* genes can enhance cell dispersion, and *ume6* gene can promote cell release from biofilms. In addition, *hsp90* is also involved in the biofilm dispersion of *Candida albicans*. Loss of *hsp90* also induced hyphal formation by alleviating Hsp90-mediated inhibition of the cAMP-PKA signaling pathway [129]. Another protein identified to play a role in biofilm dispersion is the cell wall protein Ywp1, in which the absence of Ywp1 leads to a decrease in biofilm diffusion and an increase in adhesion [130]. Studies have shown that dispersed cells up-regulate adhesion genes (*als5*, *als6*, *ecm33*), resistance genes (*mdr1*, *qdr1*, *erg1*), nutrition acquisition genes (*zrt1*, *zrt2*, *zap*1) and pathogenesis gene (*sap*), which together determine the dispersion of biofilms [131].

The summary of the study on the molecular biology of *Candida albicans* biofilm can not only provide insight into its biofilm formation mechanism and its drug resistance mechanism, but also provide a theoretical reference for the regulatory mechanism of fungal biofilm formation. In addition, it can also provide new ideas and references for the study of profound fungal infections.

Through the regulation pathways of biofilm formation in two model fungi, *Saccharomyces cerevisiae* and *Candida albicans*, it was found that the MPKA pathway, cAMP-PKA pathway, and Rim pathway in the biofilm formation pathway existed in both fungi, and the biofilm synthesis pathway of *Saccharomyces cerevisiae* was ultimately attributed to the regulation of the key gene *flo11*, and the regulatory genes at each stage of the process of its formation were less studied. On the other hand, *Candida albicans* biofilm regulation is based on the study of related genes at different stages of its formation, and the study of related genes at the stage of hypha formation is predominant, because hypha is closely related to the pathogenicity of *Candida albicans*. By analyzing the research mode of both synthetic pathways, the study of biofilm formation and development from two perspectives of the synthetic pathway and formation stage can not only provide an in-depth understanding of its biofilm formation mechanism and its antireversal mechanism, but also provide theoretical references for the regulatory mechanism of fungal biofilm formation, and provide new ideas and references for in-depth study of the prevention and control of fungal infections.

5. Quorum sensing mediates fungal biofilm formation

Quorum sensing (QS) refers to the process in which cells sense each other through self-generated quorum sensing molecules (QSM) [132]. With the increase of cell density, increasing the concentration of QSM to a threshold can promote intraspecific or interspecific communication, which will lead to phenotypic changes in the whole population [39] and is also one of the important factors affecting biofilm formation [133]. QSMs play a role in regulating fungal pathogenesis, morphogenesis and hyphal formation [115]. Most of the QSM detection studies in fungi focused on *Saccharomyces cerevisiae* and *Candida albicans*.

In *Saccharomyces cerevisiae*, QS has been shown to regulate the expression of the *flo11* gene, affecting the production and development of biofilms in *Saccharomyces cerevisiae* [134]. Currently, the QSM found in *Saccharomyces cerevisiae* are ethanol, phenylethanol and 2-phenylethanol [119]. When cell density is high enough, ethanol production reaches a threshold that affects *Saccharomyces cerevisiae* biofilm development by activating *flo11* expression through the PKA pathway [135]. The contribution of QS to biofilm formation is also regulated by environmental factors such as nutrition [136,137]. For example, *Saccharomyces cerevisiae* forms smooth circular colonies under favorable conditions. When environmental conditions are unfavorable (e.g., nutrient deficiency), yeast can change its growth pattern and form complex structures through numerous cell interactions to produce visually striking wrinkled colony structures [1].

QSM found in *Candida albicans* include farnesol and tyrosol [117,138]. Farnesol was the first QSM reported in a fungal species [139], which blocks the transition from yeast to hyphae in *Candida albicans* through inhibition of the Ras1-Cdc35-PKA signaling pathway, and farnesol can also cause alterations in cell membranes by inhibiting ergosterol synthesis [126]. It affects other cellular metabolic processes to reduce biofilm formation [123]. However, the effect of farnesol on mature fungal biofilms depends on the species, as no inhibition of yeast biofilms was observed in the presence of farnesol [118]. The second fungal QSM is tyrosol, which promotes the transformation of *Candida albicans* from the yeast form to the hyphal form [140]. Additionally, tryptophan also has been reported.

Fungi use QS for cell communication to promote or inhibit biofilm formation, but the study of QS signal on the direction of movement has not been clear [132]. QS is considered to be a way of life that is more conducive to the survival of microorganisms and plays a vital role in biofilm regulation [141]. At present, there are still too few studies on the detection of QSM of fungi, and there are few studies on the role of biofilm formation. Therefore, the types of QSM of fungi and the mechanism of action on biofilm formation need to be further explored and studied.

6. Mixed fungal biofilms (MFB)

Microorganisms rarely exist as a single planktonic species. Most microorganisms live in complex communities, in the form of polymicrobial biofilms of fungi and fungi, bacteria and bacteria, and fungi and bacteria together. The microorganisms within these biofilms collaborate with each other to produce effects that are unachievable for individual species. The genetic diversity of biofilm communities increases the adaptive capacity of the community, allowing species to better adapt to environmental stresses, resulting in accelerated growth, enhanced resistance, immune evasion, passive resistance and metabolic cooperation.

The MFB is a biofilm formed by different fungi together. Studies have shown that fungi can coexist in different anatomical ecological niches and form MFB on different surfaces. However, little is known about the role of MFB, in particular the role that each species may play in them, including how they may be synergistically involved in pathogenesis [1]. Due to the lack of studies conducted on other fungi, the available information is practically limited to the genus *Candida*. The most common species in mixed fungal cultures

is *Candida albicans*, whose biofilm is a well-structured community of yeast cell pseudomycorrhizae and hyphae [142]. Many genes are differentially expressed in the formation of mixed biofilms in *Candida albicans* biofilms [143], with expression of the *hwp1* gene promoting aggregation and invasion with other fungal species, thereby accelerating the formation of more stable structures in MFB [143], which is key to the establishment of MFB.

Candida albicans and other different species of Candida (*Candida tropicalis*, *Candida parapsilosis*, *Candida glabrata*, and *Candida auris*.) can form mixed biofilms through co-aggregation and co-adhesion. Extracellular vesicles (EV) were found to be essential for mixed biofilm matrix formation [144]. Robert et al. studied the intra-vesicular composition of different *Candida* species and found that EV could carry substances such as proteins, nucleic acids, polysaccharides and lipids. For example, the EV of *Candida albicans* contained substances required for the assembly of the extracellular matrix of the biofilm (mannans and dextran), and the ratio of mannans to dextran in the vesicles corresponded to that required for the assembly of the matrix mannan-dextran complex [145,146]. The more mannose-glucan complexes there are, the greater the ability of *Candida albicans* biofilm formation and the greater the tolerance to antimicrobial drugs [147,148]. In mixed biofilms formed by *Candida albicans* and *Staphylococcus aureus*, the *Candida albicans* biofilm matrix produced symbiotic protection against *Staphylococcus aureus* and reduced the threat of vancomycin to *Staphylococcus aureus* [149,150]. Studies have shown that this protection is associated with glucan in *Candida albicans* and that this symbiotic protection is reduced when glucan synthesis is blocked [151]. In addition, a polysaccharide-modified glucanase was also found in EV of *Saccharomyces cerevisiae* [152]. In polymicrobial biofilms, the release of EV not only facilitates the communication of each species in a hybrid biofilm, but also facilitates the sharing of important resources among microorganisms in a community [153–155]. There is also group sensing of cell-to-cell communication in hybrid biofilms, relying on the secretion and diffusion of signaling molecules to influence the behavior of one species towards other species [156,157].

Cell-to-cell communication in mixed biofilms can also rely on the secretion and diffusion of signaling molecules to influence the behavior of one species towards other species [158,159]. In multi-microbial biofilms, the release of EV not only facilitates the exchange of each species in the mixed biofilm, but also facilitates the sharing of important resources in the community [144–146]. However, due to the lack of research on other fungi, the existing information is limited to *Candida* species.

MFB can also be formed between fungi. *Candida albicans* forms hybrid biofilms with other different species of *Candida* (*Candida tropicalis*, *Candida parapsilosis*, *Candida glabrata*, and *Candida auris*.) by co-aggregation and co-adhesion [1]. The formation of *Candida* mixed biofilms can increase the incidence of *Candida*, resulting in increased mortality after the disease. Coco [160]has found that *Candida albicans* and *Candida glabrata* can cooperate with each other to form a mixed biofilm from inflammatory patients infected with *Candida albicans* and *Candida glabrata*, and *Candida albicans* can also help *Candida glabrata* invade in vitro epithelial cells. The combined effect of *Candida glabrata* and *Candida albicans* can also cause significant tissue damage. In addition, Martinset et al. [147] reported a mixed biofilm formed by *Candida albicans* and *Candida rugosa* in vitro. *Candida rugosa* is a new fungal pathogen found in Brazil. Kirkpatrick et al. [161] also described the mixed biofilm formed by *Candida albicans* and *Candida dubliniensis*, indicating that *Candida albicans* had obvious competitive advantage over *Candida dubliniensis* under planktonic growth conditions. In contrast, under biofilm conditions, *Candida dubliniensis* was better able to withstand the severe competitive pressure from *Candida albicans* [161].

Mixed biofilms can also be formed between fungi and bacteria, which is a multi-microbial community composed of EPS of fungi and bacteria [149]. Studies have shown that in the mixed biofilm formed by *Pseudomonas aeruginosa* and *Candida albicans*, 12-acyl homoserinelactones secreted by *Pseudomonas aeruginosa* can be detected by *Candida albicans* as a QSM signal, thereby inhibiting the hyphal growth of *Candida albicans* [162,163]. When *Candida albicans* and *Pseudomonas aeruginosa* form mixed biofilms exposed to phenazines, *Candida albicans* produces large amounts of ethanol. In turn, in response to increased ethanol levels, *Pseudomonas aeruginosa* increases the production of phenazines and promotes the formation of its own biofilm [143,164]. Mixed biofilms composed of *Staphylococcus aureus* and *Candida albicans* have been extensively studied, and both microorganisms are often found together in different types of infections, exhibiting increased virulence and drug resistance. *Staphylococcus aureus* and *Candida albicans* are the two most common blood pathogens that cause severe morbidity and mortality in hospitalized patients [149]. Some evidence suggests that they are often co-infected, as both *Candida albicans* and *Staphylococcus aureus* have been isolated from microbial biofilms on various mucosal surfaces, including the vaginal and oral mucosa. Peters et al. [165] used a proteomic approach to identify proteins upregulated during the interaction between *Candida albicans* and *Staphylococcus aureus* and showed that both species induced a stress response during the initial interaction, and that this stress response was more intense when *Candida albicans* was in yeast form. Manavathu et al. showed that airways of patients infected with *Pseudomonas aeruginosa* and *Aspergillus fumigatus* isolated mixed microbial biofilms distinct from *Pseudomonas aeruginosa* biofilms [158]. Zheng et al. [159] found that derivatives of phenazines produced by *Pseudomonas aeruginosa* could act as a signal to influence biofilm development in *Aspergillus fumigatus* and that when phenazines concentrations were reduced, *Aspergillus fumigatus* shifted from weak trophic growth to asexual spore production (conidia) thereby affecting biofilm formation. Furthermore, the presence of multiple microbial biofilms between fungi and bacteria has been reported at other sites of infection [149]. Examples include *Candida albicans*, *Aspergillus fumigatus*, *Fusarium oxysporum*, *Pseudomonas aeruginosa*, and *Staphylococcal agr* at burn wounds and trauma sites; mixed biofilms formed by *Candida albicans* and *Cryptococcus neoformans* in the lower genital tract and *Escherichia coli* with *Candida albicans* present in the urethra [162,163]. Finally, mixed biofilms formed between *Candida albicans*, *Escherichia coli*, and *Salmonella entericasubsp. enterica* subspecies are also present in the gastrointestinal tract [162, 164]. However, the interaction between *Candida albicans* and bacteria is not limited to cooperation. For example, when *Pseudomonas aeruginosa* and *Candida albicans* form a mixed biofilm, *Pseudomonas aeruginosa* can kill the hyphae produced by *Candida albicans*, although the underlying mechanism is not yet clear [82].

There is no consensus as to whether the interaction of one strain within different mixed biofilms with other participating strains is beneficial or harmful, but what can be demonstrated is that mixed biofilms can form between different strains, adhering to both biotic and abiotic surfaces. Due to the complexity and versatility of these microorganisms in mixed biofilms, the mechanisms of interaction

between individual strains in mixed biofilms are not known.

7. Conclusion and perspective

Since the study of fungal biofilms started relatively late compared to bacteria, people tend to summarize and adopt known schemes about bacteria. It is worth noting that fungi and bacteria have completely different cell physiology and structure, so the generalization and summary related to fungal biofilms are particularly important [166]. Therefore, this review summarizes the EPS, formation, gene regulation, QS, and MFB of fungal biofilms, and provides a molecular theoretical basis for studying the gene regulation of other fungal biofilms. It is also of great value to study the intercellular interaction and cell signal transduction and differentiation in the structure of fungal biofilms. It has important reference value for comprehensively understanding the current research status of fungal biofilm development and clarifying the future research direction of fungal biofilms.

The function of biofilm is closely related to the production of EPS. Understanding the role of its components in biofilm is of great significance for further exploring the internal structure and connection mode of biofilm. Although the main substances in fungal biofilms have been known, other unique substances have also appeared in EPS of some fungi. For example, flavin derivatives exist in EPS of *Saccharomyces cerevisiae* [167]. However, whether these substances have other functions and strain specificity remains to be further studied. In addition, fungal biofilm formation is one of the most important factors that cause recurrent infections, because mature biofilms show increased pathogenicity, enhanced drug resistance, and improved host immune defense capabilities, posing a great threat to health and huge economic losses [168]. At the same time, fungal biofilm formation is beneficial and plays an important role in ecosystem functions. For example, extracellular polysaccharides in *Rhodotorula mucilaginosa* UANL-001L biofilm have antibacterial properties [169]. Moreover, due to the higher cell viability and density in fungal biofilms, the production and accumulation of secreted enzymes and fermentation products, such as acids and alcohols, can be sustained and effective [170], which is of important practical significance for production [171]. Therefore, it is important to update and refine the formation process and regulatory mechanisms of fungal biofilms to reduce or prevent harmful biofilm formation and provide strategies to control pathogenic fungal biofilm infections; it is also important for the development, utilization, and protection of beneficial fungal biofilms, and to play the role of fungi in the ecosystem.

At present, the research on fungal biofilm is mostly on single culture, and the related research on MFB is only limited to the clinical research on the formation of *Candida albicans*. For other fungi, the species that constitute a mixed biofilm and the interaction between these microorganisms are far from enough. The beneficial interactions in mixed biofilms have important environmental, industrial and clinical significance. Microbial communities formed by mixed biofilms of different flora are likely to play an important role in determining the development, structure, and function of these biofilms in terms of understanding how different cell types are arranged and organized in biofilms, the roles and positions of the different cell types in rows, and interspecies interactions. Intercommunication between different fungi in mixed fungal biofilms when they are in the same space plays an important role in studying cellular communication among microbial ecologies, among others. The protection provided between one species and another in two-species biofilms as well as multi-species biofilms is important for studies that reveal the ways in which distantly related microorganisms work closely together.

CRediT authorship contribution statement

Dandan Wang: Writing – review & editing, Writing – original draft, Investigation, Conceptualization. **Nan Zeng:** Writing – review & editing. **Chunji Li:** Writing – review & editing, Investigation, Conceptualization. **Zijing Li:** Investigation. **Ning Zhang:** Supervision, Project administration, Funding acquisition. **Bingxue Li:** Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Abbreviations

EPS	Extracellular Polymeric Substances
ECM	Extracellular Matrix
GCN	Glucose Inhibition Pathway
AMB	Amphotericin
QS	Quorum Sensing

QSMs Quorum Sensing Molecules
MFB Mixed Fungal Biofilm
EV Extracellular Vesicles

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