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Spatially structured yeast communities: Understanding structure formation and regulation with omics tools

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ABSTRACT

Single-celled yeasts form spatially structured populations - colonies and biofilms, either alone (single-species biofilms) or in cooperation with other microorganisms (mixed-species biofilms). Within populations, yeast cells develop in a coordinated manner, interact with each other and differentiate into specialized cell subpopulations that can better adapt to changing conditions (e.g. by reprogramming metabolism during nutrient deficiency) or protect the overall population from external influences (e.g. via extracellular matrix). Various omics tools together with specialized techniques for separating differentiated cells and in situ microscopy have revealed important processes and cell interactions in these structures, which are summarized here. Nevertheless, current knowledge is still only a small part of the mosaic of complexity and diversity of the multicellular structures that yeasts form in different environments. Future challenges include the use of integrated multi-omics approaches and a greater emphasis on the analysis of differentiated cell subpopulations with specific functions.

Contents

1. Introduction

In nature, microbes occur predominantly in multicellular communities that positively or negatively affect the lives of other organisms, including humans. Yeast communities have long been used in the food industry for the production of bread and alcoholic beverages, and more recently for the production of various enzymes and chemicals. On the other hand, many yeasts are opportunistic pathogens that pose a risk, especially to immunocompromised patients, causing both skin and systemic infections. The formation of multicellular biofilms is an important risk factor for these infections. Understanding the mechanisms involved in

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the formation, characteristics and development of communities formed by different yeast species is a prerequisite for improving the beneficial and reducing the harmful effects of such communities.

Complex spatial organization and cell specialization are key features of structured microbial communities with attributes of multicellularity, including intercellular communication, coordinated development, and cell differentiation [1,2]. Microorganisms, including yeasts, in such communities cooperate by secreting extracellular components such as the extracellular matrix (ECM), adhesins, enzymes, siderophores, and signaling molecules [2] that can be used or sensed by other cells in the population. Community organization thus depends on interactions among microbial cells and with the environment. Cells within structured populations integrate information in the form of gradients of nutrients, metabolites, and signaling molecules, each of which contributes to cell differentiation and specialization. Spatial patterns are formed with specific differentiated cell types located in specific regions of the community [3,4].

Research on structured communities relies on a number of methodological approaches that allow direct study of cells in a population context or rapid separation of cells from the structure for further analysis [5–11]. Rapid manipulation is critical to minimize cellular changes when manipulation alters the "multicellular context" in which cells reside. Omics analyses play a vital role in providing initial insight into the processes that occur in differentiated cells of structured populations, whether single or multispecies structures. These methods are also key to identifying processes regulated by specific regulators and signaling cascades (Table 1 and S1).

Structured microbial communities can arise in different ways, depending on the properties of their cellular constituents. During community construction, motile cells may come together and then specialize, as in fruiting bodies formed by certain bacteria (e.g., *Myxobacteria*) or amoebae (e.g., *Dictyostelium*), and in multispecies bacterial biofilms. An alternative, typical of non-motile cells (e.g. yeast), is to form structures by "staying together" after cell division [1]. However, non-motile cells can also aggregate through a combination of passive movement and interaction via their surface adhesins [12]. Therefore, both strategies are often combined. Complex structures of colonies formed by the division of non-motile yeasts typify "staying together", whereas biofilms formed after yeasts attach to biotic or abiotic surfaces use a combination strategy, "coming together & staying together". Whether a colony or biofilm

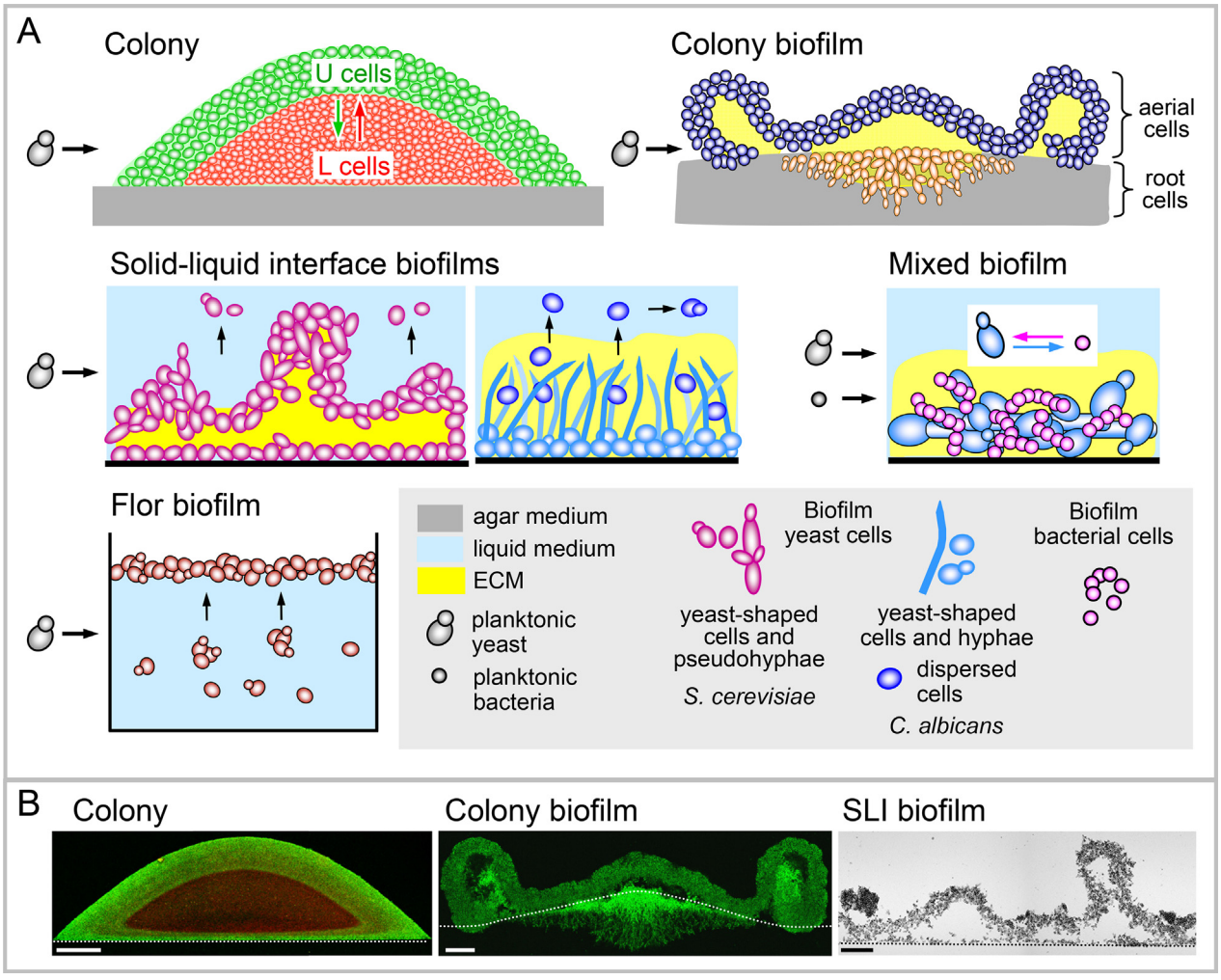

Fig. 1. Various types of yeast multicellular structures. A, schematic representation of the internal structures of colonies and different types of biofilms (side view of the middle part of the structure). Colored arrows indicate interactions between two cell types. B, cross sections of the structures visualized by 2-photon confocal microscopy (colony and colony biofilm) or wide-field microscopy (biofilm). Colonies: green, U-layer of cells expressing Cit3p-GFP; red, L-cells visualized as cell autofluorescence (adapted from [22]); colony biofilm: Rpa19p-GFP level differs in aerial and root parts (adapted from [7]); vertical structure of SLI biofilm (adapted from [30]). Dashed lines, surface of agar (white), polystyrene surface (black). Bars, 100 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

forms depends primarily on the properties of the yeast strain, with the ability to adhere to solid/semi-solid surfaces being one of the properties essential for biofilm formation (see Part 3). A comparison of the distribution of biofilm- and colony-forming *S. cerevisiae* cells shows that the decision is made at an early stage of structure development [12].

Here we summarize current knowledge on structure formation, cell differentiation and coordination in yeast colonies and biofilms, and also address changes and relationships that occur when yeast interact with bacteria in mixed species biofilms (Fig. 1). Each structure has advantages and disadvantages for studying cell interaction and differentiation. Biofilms are more complex structures that protect their cells from environmental influences, but some biofilm properties, such as the embedding of cells in extracellular matrix (ECM) and their connection by extracellular fibers, hinder some experimental manipulations. Biofilm research has become increasingly important in recent years, mainly because of biofilm key role in fungal infections [13–15]. Colonies are less structured and consist of precisely localized cell subpopulations that evolve synchronously over time, making specific cell types amenable to separation, relocalization, and other manipulation techniques. For these reasons, colonies have become an essential model for identifying mechanisms of multicellular structure formation, development, and regulation [3–4]. In this minireview, emphasis is placed on the use of omics methods (Table 1 and S1) in the study of different types of biofilms and colonies and their differentiated cell subpopulations, and on their combination with other techniques.

2. Yeast colonies - a model for research on cell differentiation and environmental adaptation

Environmental adaptation through metabolic reprogramming is essential for the survival of individual yeast cells and entire populations [3–4]. Yeast colonies on semisolid agar media undergo several metabolic phases throughout their development, during which metabolism is reprogrammed so that the entire population can cope with decreasing nutrients in the environment. Similar changes occur whether the colony is formed from a single cell (microcolony) or from a large number of cells placed close together on the agar medium (giant colony) [16]. Colonies on respiratory medium go through phases that can be monitored by changes in the pH of the colony environment - the acidic phase alternates with the alkaline phase and vice versa. The duration of each phase depends on the amount of nutrients and the number of colony cells consuming those nutrients [16,17]. The alkaline phase is associated with the production of volatile ammonia, which acts as a quorum-sensing molecule that synchronizes colony development in a given territory and is involved in metabolic reprogramming and differentiation of the colony [4,18,19]. Initial transcriptomic analyses provided a first glimpse into the metabolic changes that occur during each phase, showing that the transition from acidic to alkaline phase is associated with metabolic adaptation to nutrient deficiency and increasing stress in the colony [20–21]. Such metabolic reprogramming helps some cells escape stress and survive.

The introduction of methods for in situ analysis of internal colony structure [5,8,10,11] revealed further changes taking place during the transition to alkaline phase, in particular differentiation into two morphologically distinct cell subpopulations that are specifically localized and strictly demarcated. Thus, U cells were identified in the upper regions and L cells in the lower parts of the colony (Fig. 1) [5,16]. Previous transcriptomic analyses of whole colonies have helped identify marker genes that are turned on during metabolic adaptation [20]. *In situ* microscopic analyses of GFP-tagged variants of their proteins have shown that the two cell types differ significantly in the expression of these genes. The key question then was how these subpopulations differ in metabolism and regulation. Due to the demarcated location of both subpopulations it was possible to separate sufficiently pure U and L cells for omics analyses (transcriptomic & proteomic). Omics comparisons, along with other analyses, showed that both U and L cells have unique properties that differ from cells of unstructured populations (shaken cultures in liquid media) [5]. U cells are long-lived and stress-resistant, but they also activate a number of metabolic genes and adaptive mechanisms, including autophagy, and have some active nutrient-sensing signaling pathways, such as TORC1 [5]. Many of the metabolic (transcriptional) features in these cells correlate with the transcriptional changes identified in the entire colony population during the transition from acidic to alkaline phase [20]. Many activations of expression in U cells are evident at both the transcriptome and proteome levels [5,6,22,23], as is the most pronounced repressive feature - decreased expression (mRNA and protein) of mitochondrial respiratory chain components, resulting in decreased mitochondrial OxPhos activity and respiratory shutdown. This change was also observed during colony switching between phases and appears to contribute to stress reduction in U cells. Subsequently, several branches of the mitochondria-driven retrograde signaling pathway have been identified to regulate the expression of specific targets in differentiated colony subpopulations [9,22]. In contrast to U cells, L cells have the characteristics of starving, stressed cells, but lack some typical features of stress-adapted cells (such as accumulation of storage compounds and a strong cell wall), and partially resemble colony cells from "acidic" phase before differentiation [5,23].

Omics comparison of U and L cells not only revealed the basic metabolic properties of these cells, but also showed their relationships and possible interactions within the colony, including the existence of a nutrient and waste product flow [5], analogous to the Cori cycle and glutamine-ammonia cycle, as described between tumor cells and mammalian tissues [24]. This finding was consistent with the metabolic and regulatory similarities found between U cells and solid tumor cells [5]. The model based on omics data (including cycling of nutrients and waste products so that U cells are supplied with nutrients at the expense of L cells) was supported by metabolite and enzyme activity measurements that identified glycolytic activity in U cells and release of glucans from L cell walls [25]. However, not all transcriptomic changes and differences were also reflected in the proteome. A typical example is the expression of ribosomal proteins, whose mRNA is higher in U cells than in L cells, while protein differences could not be detected.

Some features of U and L cells were also identified in two cell types, termed "outside" and "inside" cells (producing/non-producing Cit1p-GFP), separated by FACS from microcolonies of the Cit1p-GFP strain grown on glucose medium for 4 days [26]. Comparison of the transcriptomes of these cells showed that "outside" cells, positioned similarly to U cells, also expressed genes for translational and ribosomal proteins, genes for amino acid metabolism, and genes for glycolytic enzymes and enzymes with functions in cell wall biosynthesis. "Inside" cells, positioned similarly to L cells, also increased expression of genes involved in mitochondrial respiration and genes for cell wall degrading enzymes [26].

3. Model and natural biofilms

Biofilms are another type of multicellular structures formed by cell-surface and cell–cell adhesion and cell proliferation at the solid–liquid interface (SLI biofilms) or at the liquid–air interface (flor biofilms). Special cases are colony biofilms and mats growing

on agar medium (at semi-solid surface-air interface), similar to colonies (Fig. 1) [4]. Common to all biofilm types is that only yeasts with specific properties (e.g. cell adhesion ability and ECM production) can form a biofilm. At the same time, biofilm formation is not so much a species property, as a property of specific strains, some of which can modify - deactivate and reactivate - this ability. Furthermore, the ability to form biofilms is affected differently by growth conditions in different yeast species/strains. For example, some natural strains of *S. cerevisiae*, as well as yeast strains belonging to important opportunistic pathogens such as *Candida albicans*, *C. glabrata*, *C. tropicalis*, *C. dubliniensis*, and *C. parapsilosis*, produce biofilms, but the conditions of biofilm formation and biofilm structure can vary considerably [27–30]. As with bacteria, biofilm formation is usually associated with increased virulence of the yeast pathogen. Typical features of yeasts necessary for biofilm formation include the ability of cells to adhere to biotic/abiotic surfaces, ECM formation, and in some cases the formation of hyphae/pseudohyphae. Other biofilm-related processes include activation of multidrug resistance (MDR) transporters and metabolic reprogramming leading to changes in cell wall components, among others. All these specific properties contribute to the high resistance of biofilm to the environment.

3.1. Colony biofilms and mats on semisolid agar - identification of specific cell subpopulations.

Biofilm-forming yeasts form colony biofilms when grown on semisolid agar, with 3D structure influenced by medium composition and agar density. A special type are "mats", flat structures formed by yeasts, including wine yeasts, that spread on low-density (0.3%) agar [31]. Mats exhibit similarities to flor biofilms and differ in morphology and size when formed by different strains [31,32]. Like colonies, colony biofilms and mats are easier to culture and manipulate than other biofilms and have therefore become an experimental model for studying the mechanisms of biofilm formation, including the identification of key regulators, particularly in *S. cerevisiae* [4]. These biofilms have also made it relatively easy to identify emerging subclones that have undergone "domestication," a process in which yeast turn off mechanisms important for biofilm formation and begin to form smooth colonies similar to those of laboratory strains [33]. This process is reversible and depends on nutrient sources and the presence of environmental stressors [34]. Most of the knowledge has been gained by analyzing the ability of *S. cerevisiae* mutants to form colony biofilms. For example, the surface adhesin Flo11p was identified as critical for both cell adhesion and colony biofilm formation [29,35,36]. Regulation of *FLO11* expression is complex [37], and most regulators identified by the reduced ability of their deletion mutants to form biofilms also affect *FLO11* gene expression [35]. An example is the antagonistic regulation of colony biofilm by Tup1p and Cyc8p, where Tup1p activates *FLO11* gene expression and contributes to Flo11p stability, while Cyc8p is a *FLO11* repressor [38]. In contrast to Flo11p, whose deletion completely abolishes the ability to form biofilms in various non-isogenic *S. cerevisiae* strains, the effect of the absence of other factors (including regulators unrelated to Flo11p) on biofilms is often strain-specific [4].

Several microscopic methods have been introduced to analyze the internal 3D organization of colony biofilms, allowing the identification of the presence and localization of differentiated cell subpopulations, such as cells with active MDR transporters on the surface and internal cells embedded in the ECM (Fig. 1) [11]. The internal arrangement of biofilms is more complex than that of colonies. Part of the biofilm consists of cells invading agar (the "root part"), and components such as ECM and extracellular fibers connecting biofilm cells complicate cell separation. Obtaining differentiated cells for subsequent omics analyses is therefore more difficult than for colonies. Identification of metabolic properties of colony biofilms by omics methods has so far mostly targeted the easier-to-isolate parts above the surface of the agar medium (the "aerial part") and differences between the wt and different mutants or between the wt and spontaneously domesticated yeast strains have been analyzed. For example, comparison of transcripts from colony biofilms of wild strains and colonies of domesticated isogenic strains, revealed complex reprogramming of metabolism between biofilm and domesticated colony, including activation of various glucosidases, maltases and glycosylation enzymes in the biofilm [33]. *S. cerevisiae* mutants in signaling pathways regulating filamentation (e.g., *ste12*, *ras2* and *rtg3*) form differentially structured colony biofilms and mats. Comparison of the transcriptomes of their colony biofilms with wt biofilms revealed an intertwining of these signaling pathways and indicated their role in co-regulation of target genes related to cell adhesion (e.g. *FLO11*) and cell wall structure [39].

The introduction of a method for separating "root" cells from agar allowed comparison of the transcriptomes of cells from the two main parts of the colony biofilm - the aerial and root regions [7]. Cells in aerial regions increase expression of genes related to metabolism of storage compounds, autophagy, stress response, and selected pathways of carbohydrate metabolism (e.g., fatty acid β-oxidation), whereas root cells express a range of metabolic genes (e.g., amino acid metabolism) and nutrient transporters [4,7]. Identification of specific target proteins for both regions in combination with microscopy then helped to identify additional smaller cell subpopulations in both the aerial and root biofilm parts [7].

3.2. Single-species biofilms on solid biotic or abiotic surfaces

Yeast biofilms (Fig. 1), like bacterial biofilms, form on natural substrates, including mammalian and plant tissues, as well as on a variety of synthetic polymeric materials. The basic cycle of biofilm formation involves 4 phases - i) cell adhesion to the substrate, ii) biofilm initiation (sometimes referred to as proliferative phase) in which adherent cells form a basal layer consisting of yeast-like cells, pseudohyphae and hyphae, iii) biofilm maturation into a complex structure, in which a large amount of ECM is formed, and iv) biofilm dispersion, in which yeast-like cells are released from the upper parts of the biofilm and can colonize new niches [15,40,41]. A special cell type in biofilm is the so-called persisters, a subpopulation of metabolically quiescent cells that manifest only in situations where most biofilm cells are killed, e.g. by antifungal drugs [42,43]. Persisters survive the effect of these drugs and can give rise to a new biofilm.

Although biofilms are produced by a number of yeast species, most current information relates to biofilms of *C. albicans*, which is responsible for the largest number of fungal infections and whose ability to form highly antifungal-resistant biofilms contributes significantly to its pathogenicity [41,44,45]. Different types of in vitro biofilms in different media are used for research, e.g. those adhering to polystyrene plates, silicone squares or, less commonly, cotton wool [42,46,47]. The second option is in vivo biofilms formed in the host - e.g. mouse and rat catheter models or prosthesis models [48–50]. Various techniques have been used to determine the structure and activity of in vitro biofilms, including microscopic analysis of cell viability, methods to determine biofilm biomass (including the effect of antifungal drugs), often in combination with analysis of specific mutants [15]. These approaches have provided important information on differences in biofilm structure under different nutrient source conditions or in different strains, and on the function of specific genes. However, for the identification of functional components of biofilms and regulatory mechanisms involved in biofilm formation, mutant strain libraries, biochemical metabolic assays (e.g. ECM and cell wall

analysis) and omics methods (transcriptomics, proteomics and ChIPseq) have become essential (Table 1 and S1).

An ECM composed of extracellular polymeric substances secreted by biofilm cells is critical for biofilm formation, its structural integrity, and its resistance to environmental factors, such as antifungal agents, some of which may bind directly to the ECM. The ECM of *C. albicans* is composed of proteins (55%), carbohydrates, mainly mannans associated with β-1,6-glucans (25%), lipids, neutral and polar glycerolipids and sphingolipids (15%) and nucleic acids - eDNA (5%) [15,51]. Proteomics identified more than 500 different proteins in the ECM, including enzymes that lack secretory sequences and likely enter the ECM from a small fraction of lysed biofilm cells, along with other components such as eDNA. Enzymes associated with carbohydrate (particularly TCA cycle, pyruvate metabolism and glycolysis/gluconeogenesis), amino acid and energy metabolism have been identified [15,51]. Several other identified proteins are related to ECM polysaccharide production and modification. In addition, proteomics of the ECM of in vivo biofilms has identified a number of host cell molecules [51–52]. Despite the evidence for the role of primary intracellular proteins/enzymes of the ECM in cell adhesion [53], placing them among "moonlighting" proteins, the functions of these proteins are still largely unknown. Since these proteins lack secretory sequences, they may either originate from disrupted cells (the proteins are re-adsorbed to the ECM) or reach the cell surface via an unconventional mechanism, such as extracellular vesicles [53]. Extracellular vesicles (EVs) are another component of biofilms that appear to play important roles in several processes, including ECM production and biofilm drug resistance [54]. EV biogenesis is thought to occur via the conventional secretory pathway (endoplasmic reticulum, Golgi apparatus, exocyst, plasma membrane), ESCRT-mediated MVB, or other pathways; however, mutant strains that cannot produce EVs have not yet been described [55,56]. Proteomics has identified numerous proteins in EVs from biofilms of various yeasts, but their protein composition varies widely and few studies have compared the abundance of proteins in EVs with their abundance in cell lysates or other types of vesicles [57]. Metabolic and proteomic analyses of *C. albicans* biofilm EVs compared to vesicles released from planktonic cells revealed major differences in protein composition: 34% of the proteome is specific to biofilm EVs and a number of other proteins are significantly more abundant in them [54]. The protein composition of biofilm EVs resembled the protein composition of ECMs, as did their polysaccharide content (predominantly mannan and glucan), suggesting that about 45% of ECM proteins might be transported there via EVs. Intracellular enzymes of biofilm ECM have not been identified in these EVs [54]. Although mutants with reduced EV production formed a biofilm, this biofilm was more sensitive to the antifungal drug fluconazole. The assumption that EVs transport specific components of the ECM was also supported by the finding that the EV defect can be complemented by EVs from the wt biofilm [54].

By screening libraries of mutants with deletions in transcriptional regulators in combination with transcriptomics and ChiP methods, more than 50 transcriptional regulators differentially involved in *C. albicans* biofilms have been identified to date [15]. Deletion of these regulators resulted, for example, in changes in biofilm structure (weakening or strengthening), loss of hyphal cells, reduced adherence to substrates, or changes in ECM and drug resistance. Nine "core" regulators (Bcr1p, Brg1p, Efg1p, Flo8p, Gal4p, Ndt80p, Rob1p, Rfx2p, and Tec1p) are required for biofilm formation both in vitro and in vivo [48,58]. ChIP and transcriptomics determined the functional association of the core regulators within the transcriptional interaction network, as well as target genes whose expression is similarly regulated by some of the core regulators [58]. These target genes include those encoding the adhesins Als1p and Hwp1p and the permease Can1p, whose deletion leads to partial disruption of biofilm formation and properties. Transcriptomics also identified the importance of individual core regulators at specific stages of biofilm development and the stepwise expression of their targets [48]. One example is the identification of a group of regulators involved in the first step of biofilm formation - adhesion - and their targets, such as the gene encoding the adhesin Als1p, which is regulated by the transcription factor Bcr1p [59].

Identification of core regulators and their targets in *C. albicans* biofilm raised the question of the extent to which the regulation and mechanisms of biofilm formation are universal with respect to biofilms of other yeast species and different growth conditions. A recent study focused on comparing biofilms of the evolutionarily related *C. albicans*, *C. tropicalis*, *C. dubliniensis* and *C. parapsilosis* (all belonging to the CTG clade, which decodes the codon CTG as serine instead of the usual leucine) in terms of biofilm formation on different media and analysis of the function and targets of 7 conserved core regulators of *C. albicans* (Bcr1p, Brg1p, Efg1p, Flo8p, Ndt80p, Rob1p and Tec1p) using ChIP seq and transcriptomics [28]. The comparison showed that only in *C. dubliniensis*, which is most closely related to *C. albicans*, all 7 regulators are involved in biofilm formation, whereas only 3 regulators (Bcr1p, Efg1p and Ndt80p) play a role in biofilm formation in all 4 yeast species. Correlation between biofilm transcriptomes was relatively low – 29% agreement between *C. albicans* and *C. dubliniensis* and 24% between the evolutionarily more distant *C. albicans* and *C. tropicalis*, consistent with significant differences in regulator-target interactions identified by ChiP seq. Similarly, a comparison of *C. parapsilosis* and *C. albicans* biofilms and their mutants grown under different conditions revealed significant differences in the importance and targets of individual transcription factors [60]. However, it cannot be excluded that "unproven" transcription factors also play important roles under other conditions of biofilm formation. The same is true for target genes, where current omics-based knowledge is insufficient to distinguish functions directly related to biofilm formation from metabolic functions, whose regulation also differs in different yeasts and conditions. For example, the conditions (medium composition) used to compare the 4 yeast species of the CTG clade did not lead to biofilm formation in *C. glabrata* and *S. cerevisiae* (not belonging to the CTG clade) [28], which form a structured biofilm under different conditions [27,30,61].

The natural *S. cerevisiae* strains that form colony biofilms (see above) also have the ability to adhere to a polystyrene surface and form a structured biofilm (Fig. 1) [30]. Apart from Flo11p, which is essential for cell adhesion and biofilm formation on polystyrene [62–64], little is known about metabolic changes and regulation behind the formation of these biofilms. Recent findings of antagonistic regulation of biofilm adhesion, formation, and dispersion by the regulators Cyc8p and Tup1p and environmental glucose (at concentrations similar to blood glucose) have suggested a possible mechanism for regulating the biofilm life cycle in host organism [30]. Transcriptomics of biofilms from wt and mutant *S. cerevisiae* strains on cotton fibers suggested a role for other FLO genes encoding the flocculins Flo1p, 5p, and 9p and the transcriptional regulator Mig1p at later stages of biofilm formation [47]. The formation of these biofilms is also potentiated by NO, where transcriptomics together with proteomics revealed the importance of Ctr1p (plasma membrane copper transporter) and its transcriptional regulator Mac1p. This regulation was independent of Flo11p and concentrations of Cu and Fe ions [65].

Although knowledge of biofilm formation as well as the global networks of its regulation has increased substantially in recent years, few studies have analyzed differentiated cell types that arise in the biofilm during its cycle. An exception is the analysis of dispersed cells and persisters of *C. albicans* biofilms [42,66]. Transcriptomics comparison revealed significant similarities between

Table 1
Selected omics studies of structured yeast populations and/or their differentiated cells.

Structure	Yeast species/ strain	OMICS approach	Conditions/ medium	Comparison	Major finding	Part of the structure	Ref
Colony	*S.cerevisiae*/ BY4742	Transcriptomics	agar plates/ respiratory	time kinetics	activation of adaptive metabolism associated with ammonia signaling	whole	[20]
Colony	*S.cerevisiae*/ BY4742	Transcriptomics	agar plates/ respiratory	U × L cells	main features of differentiated U and L cells and their interactions	U & L cells	[5]
Colony	*S.cerevisiae*/ BY4742	Proteomics	agar plates/ respiratory	wt × *whi2* × *psr1psr2*	WPP complex involved in interference competition	whole	[6]
Colony biofilm	*S. cerevisiae*/ BRF	Transcriptomics	agar plates/ respiratory	aerial × root cells	main features of aerial and root cell metabolism	aerial & root cells	[7]
Biofilm	*C. albicans*	Transcriptomics	polystyrene/ Spider	biofilm × planktonic; wt × mutants in 6 TFs	mutual regulation of six regulators, 19 targets differentially regulated by all regulators in planktonic and biofilm cells	whole	[58]
Biofilm	*C. albicans*	Proteomics, metabolite analysis	polystyrene / RPMI-1640	ECM	macromolecular composition of biofilm ECM (exopolysaccharides, lipids, eDNA, proteins)	ECM	[51]
Biofilm	*C. albicans*	Transcriptomics	plastic plates, BSA/ Spider	biofilm × planktonic, 8–48 h	3 novel regulators Flo8p, Rfx2p and Gal4p identified and characterized	whole	[48]
Biofilm	*C. albicans*	Proteomics	polystyrene/ YNB, glucose	biofilm +/- amphotericin B	proteomics characteristics of persister cells	whole, persister cells	[42]
Biofilm	*C. albicans*	Transcriptomics	silicone (flow system) / YNB, glucose	biofilm × planktonic × dispersed cells	dispersed cells differ from planktonic cells and retain some of the expression characteristics of biofilm cells	whole, dispersed cells	[66]
Biofilm	*C. albicans*	Proteomics, metabolite analysis	polystyrene / RPMI-1640	ECM, EVesicles; biofilm × planktonic	biofilm EVs deliver cargo to the ECM, EVs play a role in biofilm resistance	ECM, EV	[54]
Biofilm	*C. albicans, C. tropicalis, C. dubliniensis, C. parapsilosis*	ChIP-seq, Transcriptomics	polystyrene/ Spider + glucose	biofilm × planktonic; wt × mutants in 7 master regulators	large differences in master TF target genes between *Candida* spp.; only 3 master TFs (Bcr1p, Efg1p, Ndt80p) required for biofilms in all 4 species.	whole	[28]
Mixed species biofilm	*C. albicans, S. mutans*	Transcriptomics Proteomics	polystyrene/ UFTYE, 1% sucrose	single × mixed species biofilms	*S. mutans* provides sugars *to C. albicans*; *C. albicans* lowers biofilm pH and helps *S. mutans* survive	whole	[68]
Mixed species biofilm	*C. albicans, S. aureus*	Transcriptomics	mouse catheter model	single × mixed species biofilms	*C. albicans* modulates transcription of *S. aureus* to increase eDNA and extracellular polysaccharides	whole	[50]

dispersed and biofilm cells and differences between dispersed and planktonic cells [66]. Dispersed cells retained a number of expression properties of biofilm cells, but also acquired other novel properties distinct from both biofilm and planktonic cells, such as expression of genes for various transporters and carriers, proteases and lipases, and lipid metabolism, which may help these cell to obtain nutrients such as zinc and amino acids and metabolize alternative carbon sources. Dispersed cells retain the ability to adhere, but preferentially express different adhesins (*ALS5* and *ALS6*) than biofilm cells (*ALS2*, *ALS3*, and *ALS4*). Proteomics of persister survivors in biofilms after amphotericin B treatment compared to cells from untreated biofilms identified more than 200 differentially expressed proteins [42]. In contrast to biofilm cells, persisters inhibited key pathways of C and N metabolism, such as glycolysis, TCA and pentose phosphate pathways, and enzymes associated with amino acid metabolism, and only key enzymes of the glyoxylate cycle (isocitrate lyase and malate synthase) and gluconeogenesis (fructose-1,6-bisphosphatase) were upregulated. On the other hand, persisters highly expressed proteins related to stress response (Hsp proteins), cell adhesion, morphogenesis and cell cycle, which are important for cell viability and virulence [42].

3.3. Mixed-species biofilms on solid surfaces

C. albicans often forms biofilms in host organisms, along with bacteria such as *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Streptococcus gordonii*, *Streptococcus mutans* and others, and the coexistence of both organisms often results in the formation of a stronger and more resistant biofilm, compared to single species biofilms [15,67]. Omics (especially transcriptomics and proteomics) play an irreplaceable role in revealing the mutual metabolic relationships of microbial species in biofilm and identifying their specific markers (Table 1, Table S1) [50]. For example, a recent transcriptomic study revealed the effect of *C. albicans* on another pathogen, *S. aureus*, in a mixed-species biofilm formed on a catheter [50]. There, the presence of *C. albicans* stimulates the biofilm formation network in *S. aureus* by down-regulating the lrg operon, the autolysis repressor, and up-regulating the ica operon to produce intercellular adhesin PIA, which together leads to increased production of eDNA and ECM essential for bacterial biofilm formation and stability. More than 100 differentially expressed genes have also been identified in *C. albicans*, but their role in mixed biofilm is unclear. Multi-omics analysis (transcriptomics and proteomics) of mixed-species biofilms of *C. albicans* and *S. mutans* identified metabolic changes in both microorganisms - a significant increase in proteins of various pathways of carbohydrate metabolism, including sugar transport, glyoxylate cycle and aerobic respiration in *C. albicans* and an increase in TCA cycle proteins and the pentose phosphate pathway in *S. mutans* [68]. There was also an increase in cell wall structure-associated proteins in *C. albicans* and various sugar transport systems and glucan and cell wall biosynthetic enzymes in *S. mutans*. A positive interaction between the two organisms was identified in the biofilm, where *S. mutans* produces glucosyltransferase B (GtfB), which binds to the surface of *C. albicans* and cleaves sucrose into glucose and fructose, facilitating sugar uptake by the yeast. The yeast in turn lowers the pH of the biofilm through its metabolism, thus helping *S. mutans* to survive [68].

3.4. Flor biofilms and other "positive" natural biofilms

The flor biofilm formed by certain *S. cerevisiae* strains during the production of sherry wines is a classic example of a "positive" biofilm associated with the food industry. Towards the end of alcoholic fermentation, when nitrogen and carbon sources become limited, yeast switch their metabolism from fermentative to oxidative (diauxic shift), begin to adhere and cell aggregates reach the surface with the help of CO_2 bubbles, where they form a liquid–air interface biofilm (velum) [69]. As with other *S. cerevisiae* biofilms, the formation of flor biofilm is dependent on Flo11p and its regulators [70]. Proteomics and metabolomics revealed metabolic changes during flor formation, including alterations in oxidative carbohydrate metabolism (e.g. glyoxylate and TCA cycle), cell respiration, cell wall biosynthesis, and glycosylation. Metabolic analyses identified components associated with the specific aroma of wine produced at the time of flor formation, such as acetaldehyde, isoamyl alcohols, 1,1-diethoxyethane, ethyl and isoamyl acetates, and 4-butyrolactone [69,71–73]. The relationship between metabolic changes and the production of specific metabolites and their possible role in biofilm formation is still unclear. The ability of some halotolerant yeasts to form resistant flor and SLI biofilms with a high content of enzymes for biosynthetic processes in the ECM could potentially be exploited for biocatalytic processes and applications [74].

Biofilm formation by so-called biocontrol yeasts could be an important strategy that allows effective competition for space and accumulation of biogenic products. Biocompetition prevents a pathogen from invading a host, such as a plant, by competing for space and nutrients, producing a specific toxin, enzyme or certain volatiles, or inducing resistance [75]. For example, flor-biofilm-yeast effectively colonize apple wounds, controlling development of the blue fungus *Penicillium expansum* [75]. The dependence of biocontrol function on environment can be documented by *Pichia fermentans*, whose biofilm formed in apple wounds prevents the development of other diseases. In contrast, biofilm formation by this yeast in pear wounds leads to the growth of hyphae and decay of inoculated fruit [76]. Despite the existence of a number of documented examples and proposed mechanisms of interaction between microorganisms leading to biocontrol - summarized in a recent review [75], molecular analyses (including omics) are still largely lacking.

4. Conclusions and perspectives

The development of omics techniques that provide comprehensive information on cellular expression at multiple levels (transcriptomics & proteomics), localize target genes of specific regulators (ChIP seq), and identify extracellular metabolites, along with the widespread use of deletion strains, has dramatically expanded knowledge of molecular mechanisms and regulation in structured yeast populations. Further development of integration tools that combine multi-omics data to better understand the interrelationships of the biomolecules involved and their functions at different levels will undoubtedly accelerate this progress. At the same time, however, genome-wide analyses have clearly demonstrated that the formation of multicellular structures, whether colonies or biofilms, is a highly complex process involving a number of parallel regulations that can vary depending on the conditions under which the structure develops (e.g., different nutrient sources) and the genetic makeup of the microorganism. Divergences in the processes and regulations involved in the formation of a given structure are evident not only between individual yeast species (e.g. biofilms of different *Candida* spp., [28]), but also between individual strains of the same species (e.g., differences in the ability to form biofilms in *S. cerevisiae*, [30]). Also, the question of which external signals contribute to or block biofilm development, which signaling pathways respond to them, and how the transition between the different developmental stages of multicellular structures is controlled is still almost completely open. Moreover, most omics analyses to date have examined whole structures, often in comparison to structures that have been altered, either by

changing growth conditions or by the absence of a gene, important for structure development. Thus, little information is available on the properties, regulation, and interactions of specific differentiated cellular subpopulations of multicellular structures. The development of microscopic techniques and fluorescent markers to visualize the presence of a specific cell subpopulation within the structure, as well as techniques to separate differentiated cells for subsequent omics analyses, is essential for development in this area. However, the development of these methods in biofilms is limited in part by the fact that biofilms are a complex structured population of tightly interconnected cells. Single cell sequencing methods, the development of which is also advanced in yeast [77], in combination with micromanipulation techniques, could partially circumvent this limitation.

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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Appendix A. Supplementary data

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