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MicroReview

Flocculation, adhesion and biofilm formation in yeasts

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Summary

Yeast cells possess a remarkable capacity to adhere to abiotic surfaces, cells and tissues. These adhesion properties are of medical and industrial relevance. Pathogenic yeasts such as *Candida albicans* and *Candida glabrata* adhere to medical devices and form drug-resistant biofilms. In contrast, cell–cell adhesion (flocculation) is a desirable property of industrial *Saccharomyces cerevisiae* strains that allows the easy separation of cells from the fermentation product. Adhesion is conferred by a class of special cell wall proteins, called adhesins. Cells carry several different adhesins, each allowing adhesion to specific substrates. Several signalling cascades including the Ras/cAMP/PKA and MAP kinase (MAPK)-dependent filamentous growth pathways tightly control synthesis of the different adhesins. Together, these pathways trigger adhesion in response to stress, nutrient limitation or small molecules produced by the host, such as auxin in plants or NAD in mammals. In addition, adhesins are subject to subtelomeric epigenetic switching, resulting in stochastic expression patterns. Internal tandem repeats within adhesin genes trigger recombination events and the formation of novel adhesins, thereby offering fungi an endless reservoir of adhesion properties. These aspects of fungal adhesion exemplify the impressive phenotypic plasticity of yeasts, allowing them to adapt quickly to stressful environments and exploit new opportunities.

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Introduction

Medical and industrial relevance of fungal adhesion

Apart from stabilizing and shielding cells from mechanical forces, the cell wall also serves as a tool for microbes to interact with their environment – and vice versa. One of the most critical functions of the cell surface is its ability to adhere to other cells and surfaces. Adhesion prevents cells from being washed away when they find themselves in a nourishing environment, and allows them to form biofilms that offer protection from hazardous conditions. Pathogenic yeasts exploit their capacity to adhere to abiotic surfaces such as plastic prostheses to gain access to the bloodstream and internal organs of patients (Kojic and Darouiche, 2004). Prostheses and catheters can also serve as carriers for fungal biofilms and thus provide an internal reservoir of highly drug-resistant infective cells (Kojic and Darouiche, 2004). In contrast to the mostly harmless superficial infections, disseminated fungal infections have a high mortality rate (> 40%) (Wisplinghoff *et al.*, 2004), placing fungal adhesion centre stage in today's medicine. Fungal adhesion is also of considerable economic importance for food-processing companies, because adherent fungi can form highly resistant biofilms in industrial installations. On the positive side, cell–cell adhesion of industrial brewing and wine yeast is often exploited as a convenient and cost-effective way to separate biomass from various fermentation products (Verstrepen *et al.*, 2003). At the end of the fermentation process, when all available sugars have been converted into ethanol and carbon dioxide, the yeast cells start adhering to each other to form macroscopic 'flocs' consisting of many thousands of cells. Cell–cell adhesion between yeast cells is therefore often called 'flocculation'. Depending on the yeast strains used, the yeast flocs rapidly sediment to the bottom ('lager' strains) or float to the surface ('ale' strains), thereby facilitating their removal from the medium.

One of the most remarkable features of yeast adhesion is the phenotypic variability and plasticity. Yeast cells are

able to quickly adapt their adhesion properties to new environments. Moreover, the adhesion behaviour of closely related strains can vary dramatically. In this paper, we first review the mechanisms of yeast adhesion. We then discuss the signalling pathways that regulate adhesion and the various genetic and epigenetic mechanisms that generate variability and plasticity of this crucial phenotype.

Fungal adhesion is conferred by specialized cell-surface proteins

Adhesion is conferred by specialized cell-surface proteins called 'adhesins' or 'flocculins' that bind specific amino acid or sugar residues on the surface of other cells or promote binding to abiotic surfaces (Fig. 1). All fungal adhesins share a common three-domain structure. The C-terminal part of adhesins contains a glycosylphosphatidylinositol (GPI)-anchor addition site and links the adhesin to the cell wall (Bony *et al*., 1997; Kapteyn *et al*., 1999). (Fig. 1). The N-terminal part of adhesins protrudes from the cell surface and often contains a carbohydrate or peptide binding domain (Kobayashi *et al*., 1998; Groes *et al*., 2002; Rigden *et al*., 2004). The large middle domain of adhesins is characterized by the presence of multiple serine- and threonine-rich repeats encoded by conserved DNA sequences that serve as a source of variability by triggering frequent recombination events through which new adhesin alleles are generated (see further). In many cases, calcium ions enable the adhesins to achieve their active confirmation (Stratford, 1992).

Despite their common features, there are many different yeast adhesins. First, each cell carries a family of (slightly) different specialized adhesins that provide the cells with an array of adhesion properties (Guo *et al*., 2000; Sheppard *et al*., 2004; Verstrepen *et al*., 2004a). Second, different yeast species carry different families of adhesins that reflect the species' lifestyle. The benign brewer's yeast *Saccharomyces cerevisiae*, for example, carries five *FLO* (flocculation) genes: *FLO1, FLO5*, *FLO9, FLO10* and *FLO11* (Teunissen and Steensma, 1995). *FLO1*, *5*, *9* and *10* confer cell–cell adhesion (flocculation), while *FLO11* is responsible for adhesion to substrates (Guo *et al*., 2000). In contrast, the adhesin families in pathogens like *Candida albicans* (*ALS* and *EAP* genes) and *Candida glabrata* (*EPA* genes) confer adhesion to mammalian host tissues (Hoyer, 2001; De Las Penas *et al*., 2003; Li and Palecek, 2003; Kumamoto and Vinces, 2005). Figure S1 shows a phylogenetic tree of the various adhesin genes as well as some related genes in higher eukaryotes.

Recent studies have identified some of the subtle differences in specificity between the various adhesins. Guo *et al*. (2000) selectively overexpressed the *S. cerevisiae FLO1*, *FLO10* and *FLO11* genes and found that expression of *FLO1* results in strong flocculation, expression of *FLO10* confers weak flocculation, and *FLO11* expression does not produce flocculation at all (Fig. 2A). Instead, expression of *FLO11* results in adherence to agar and plastic (Fig. 2B). Similarly, subtle differences in the binding specificity of the *C. albicans* Als1 and Als5 adhesins to small heptapeptides have been described (Klotz *et al*., 2004). Furthermore, Als proteins heterologously produced in *S. cerevisiae* confer distinct adhesion profiles towards human proteins and cells (Sheppard *et al*., 2004). Another indication of the different specificities among the *Candida* adhesins is that the various adhesin genes are differentially expressed under different host and model conditions. More specifically, the *C. albicans ALS2*, *3*, *6*, *7* and *9* are highly expressed and *ALS4* and *ALS5* are repressed in models of vaginal candidiasis (Cheng *et al*., 2005). In contrast, oral specimens show strong expression of *ALS1*, *2*, *3*, *4*, *5* and *9*, while *ALS6* and *ALS7* are repressed (Green *et al*., 2004). This differential expression probably reflects the ability of cells to express only the appropriate, most effective adhesins in any given situation.

Different mechanisms of adhesion

While all fungal adhesins make cells bind to other cells or surfaces, their modes of action differ. Adhesion can be divided into two main groups: lectin-like adhesion (sugar sensitive) and sugar-insensitive adhesion. As the name suggests, lectin-like adhesion depends on the lectin-like binding of the adhesin to sugar residues on the surface of other cells. Adhesins of this group have a lectin-like carbohydrate binding domain (pfam no. 06660) in their N-terminus. Addition of certain sugars competitively inhibits adhesion and provides an easy way to determine the sugar specificity of the adhesins. Examples of lectin-like adhesins include the *S. cerevisiae FLO* gene products (except Flo11) (Stratford, 1992; Guo *et al*., 2000) as well as the *EPA* gene products in the human pathogen *C. glabrata* (Cormack *et al*., 1999). Pathogenic yeasts bind to glycosides on mammalian cells such as *N*-acetyl lactosamine (Cormack *et al*., 1999), while *S. cerevisiae* cells recognize mannose oligomers on their own surface, resulting in aggregation (Kobayashi *et al*., 1998). In *S. cerevisiae*, lectin-like adhesion is further divided into two sub-categories, Flo1 and NewFlo. The Flo1 group only binds mannose sugars, whereas the NewFlo type binds various sugars, including mannose, glucose and glucose oligomers such as maltose (Sato *et al*., 2002). Most industrial brewing strains are of the NewFlo type; competitive inhibition conferred by sugars in the medium prevents them from flocculating before all fermentable sugars are converted into ethanol, which is exactly what brewers and winemakers want.

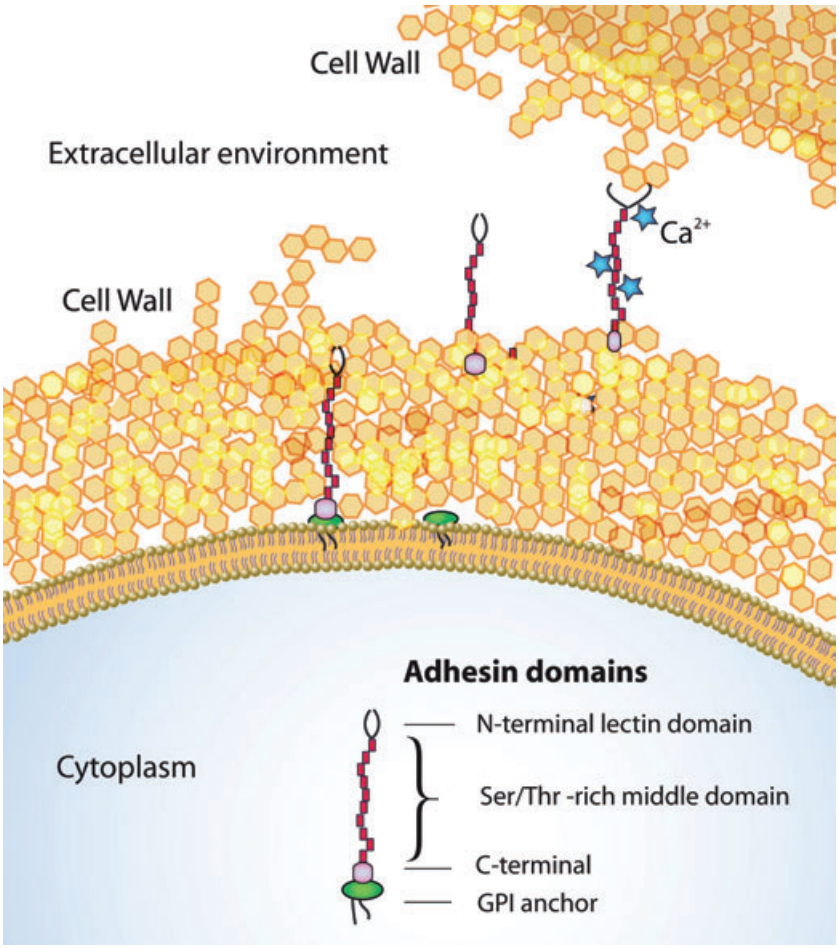

Fig. 1. Secretion and cell-surface anchoring of fungal adhesins. Mature fungal adhesins consist of three distinct domains (Hoyer *et al.*, 1998). During transport through the secretory pathway, adhesins undergo extensive post-translational modifications. In the endoplasmic reticulum the N-terminal signal peptide is removed and a C-terminal signal peptide is replaced by a GPI anchor; in addition, N-glycosylation and O-glycosylation (involving the abundant serine and threonine residues in the highly repetitive middle domain of the adhesins) are initiated. Further processing of the GPI anchor and the carbohydrate side-chains takes place in the Golgi (Udenfriend and Kodukula, 1995; Bony *et al.*, 1997; Tiede *et al.*, 1999; De Groot *et al.*, 2003; Frieman and Cormack, 2003). It is believed that the short *O*-linked oligosaccharide side-chains enable the adhesins to obtain a long, semi-rigid rod-like structure that is stabilized by Ca^{2+} ions (Jentoft, 1990). Upon arrival to the plasma membrane, the GPI anchor is cleaved off and the adhesin is ultimately linked covalently through a GPI remnant to the beta-1,6 glucans that are an integral part of the fungal cell wall (Lu *et al.*, 1995; Kapteyn *et al.*, 1999; Klis *et al.*, 2002).

Sugar-independent adhesion, on the other hand, is mediated by adhesins that bind peptides instead of sugars, or increase the cell-surface hydrophobicity, thereby promoting hydrophobic interactions between the cells and certain abiotic surfaces (Kang and Choi, 2005). Examples include the Flo11 adhesin in *S. cerevisiae*, which confers hydrophobicity-based adhesion to abiotic surfaces (Guo *et al.*, 2000) and the Als proteins in the pathogen *C. albicans*, which recognize certain peptides in host cells (Klotz *et al.*, 2004).

Adhesion is induced by various environmental triggers

As mentioned above, the adhesion-encoding genes are not constitutively expressed. Instead, adhesion is under tight transcriptional control by several interacting regulatory pathways. The adhesion genes are activated by diverse environmental triggers like carbon and/or nitrogen starvation, or changes in pH or ethanol levels (Verstrepen *et al.*, 2003; Sampermans *et al.*, 2005). The switch from non-adherence to adherence probably allows yeasts to adapt to stress. Activation of *FLO11* upon nitrogen starvation, or for example, allows fungi to adhere to and penetrate substrates in an attempt to forage for new nutrients (Kron, 1997; Gagiano *et al.*, 2002). Flocculation, on the other hand, might protect the cells in the middle of the flocs from the environment. In addition, the flocs sediment in the medium or float to the surface, and may therefore provide a means of passive transport in the medium, away from the stress.

Apart from being a stress-defence mechanism, adhesion is also crucial for fungal pathogenesis: fungi need to adhere to the appropriate host tissues in order to establish an infection site. Prusty *et al.* (2004) show that *FLO11* is also activated by the elevated concentrations of indole acetic acid (auxin) that occur at plant wound sites, suggesting that *FLO11* activation by indole acetic acid might be crucial for feral yeast cells to infect wound sites in plants. Similarly, activation of adhesins in animal pathogens occurs when the cells perceive an opportunity for infection, enabling cells to adhere to the appropriate tissue and establish a colony or biofilm of infectious cells (Verstrepen *et al.*, 2004a; Domergue *et al.*, 2005).

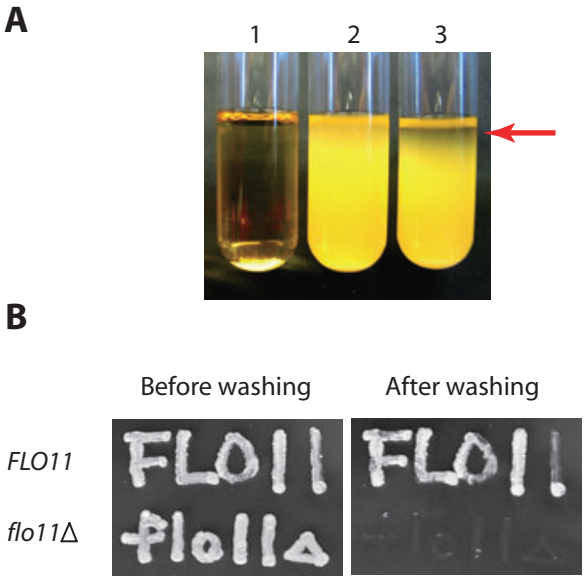

Fig. 2. Cell–cell and cell–surface adhesion associated with the *S. cerevisiae FLO* genes.
A. Three different *S. cerevisiae* S288C strains were shaken in liquid medium (YPD) and subsequently incubated without shaking for 5 min. Strain 1 expresses the flocculin gene *FLO1*, strain 2 does not express any *FLO* gene, and strain 3 overexpresses *FLO11*. Cells expressing *FLO1* show strong cell–cell adhesion (flocculation) and sediment to the bottom of the test tube. Strain 2 does not show significant flocculation, and the cells remain largely suspended in the medium. Strain 3 does not show extensive flocculation (no large flocs), but the cells do form microclumps of about 2–10 cells, resulting in some sedimentation (arrow).
B. *S. cerevisiae* S288C cells overexpressing *FLO11* and cells in which *FLO11* has been deleted are grown for 5 days on a standard YPD agar plate. Both strains grow equally well on the agar (before washing). However, when the plate is gently rinsed with water, the cells lacking *FLO11* wash off, while many of the cells overexpressing *FLO11* adhere to the agar surface.

Adhesion is controlled by several signalling pathways

Among the best-studied aspects of fungal adhesions are the various signalling cascades that translate environmental conditions into a proper transcriptional response of the adhesin genes. The pathways regulating adhesion were originally dissected for the *S. cerevisiae FLO11* gene, the only *FLO* gene that is active in the laboratory Σ1278b strain. It is generally expected that the other *S. cerevisiae FLO* genes as well as adhesins in pathogenic fungi are controlled by similar (but not identical) pathways to those that control *FLO11*. Indeed, recent studies indicate that at least in *Candida* spp., many *ALS* genes are regulated by orthologues of the pathways known to regulate adhesion in *S. cerevisiae* (Lengeler *et al*., 2000; Liu, 2001; Kaur *et al*., 2005; Maidan *et al*., 2005).

FLO11-mediated cell-surface adhesion is triggered by certain stress factors and/or nutrient limitation. At least three well-known signalling cascades regulate *FLO11* expression in response to environmental changes: the Ras-cAMP pathway, the MAP kinase (MAPK)-dependent filamentous growth pathway and the main glucose repression pathway (Madhani and Fink, 1997; Rupp *et al*., 1999; Gagiano *et al*., 2002; Vyas *et al*., 2003; Schwartz and Madhani, 2004). Figure 3 depicts these signal transduction pathways. A fourth pathway, the so-called 'Target of Rapamycin' (TOR) pathway, has recently also been implicated in *FLO11* regulation (not shown). This pathway is believed to respond to nitrogen starvation, but the exact details are not yet known (Cutler *et al*., 2001). A fifth (partial) pathway involves the transcription factors Sok2, Phd1 and Ash1, which seem to function in an epistatic pathway. However, the details are not yet understood (Gimeno and Fink, 1994; Ward *et al*., 1995; Pan and Heitman, 2000) (not shown). In addition, a plethora of other genes have been shown to affect yeast adhesion in large-scale genetic screens. Some of these genes may not be directly related to adhesion, but merely influence adhesion in an indirect way.

While at least some of the signalling pathways regulating adhesion are relatively well characterized, relatively little is known about the upstream sensors of the various signalling cascades. It is clear that these sensors must be triggered by conditions that activate adhesion, such as nutrient limitation, stress or the proximity of a suitable infection site or surface. Recently, the mucin-like transmembrane protein Msb2 has been shown to function as an upstream sensor for the MAPK-dependent filamentous growth pathway (Cullen *et al*., 2004). Msb2 might function as a sensor for stress, but the exact triggers of Msb2 remain unknown. The ammonium permease Mep2 has also been suggested as an upstream receptor for the MAPK pathway (Gagiano *et al*., 1999). Mep2 is believed to trigger the pathway in response to nitrogen starvation (Fig. 3A). Other reports indicate that activation of *FLO11* by nitrogen depletion relies on a MAPK-independent pathway, in which two of the known regulators of nitrogen metabolism, Gcn2 and Gcn4, play a key role (Braus *et al*., 2003). This pathway requires Flo8, indicating that the pathway merges or at least interacts with the cAMP/PKA pathway (Fig. 3B). In *C. albicans*, physical contact with a surface activates the mitogen-activated protein kinase Mkc1, which in turn actives adhesion and biofilm formation (Kumamoto, 2005). Hence, Mkc1 allows cells to determine their location within a host and sense whether they are in physical contact with a suitable carrier to adhere to and form a biofilm. This mechanism bears interesting similarities to the so-called 'contact inhibition' that regulates cell proliferation and behaviour in multicellular organisms.

Even when the sensors and signalling cascades for certain environmental factors are known, the picture is not straightforward. For example, adhesion is triggered by carbohydrate depletion (Sampermans *et al*., 2005). This

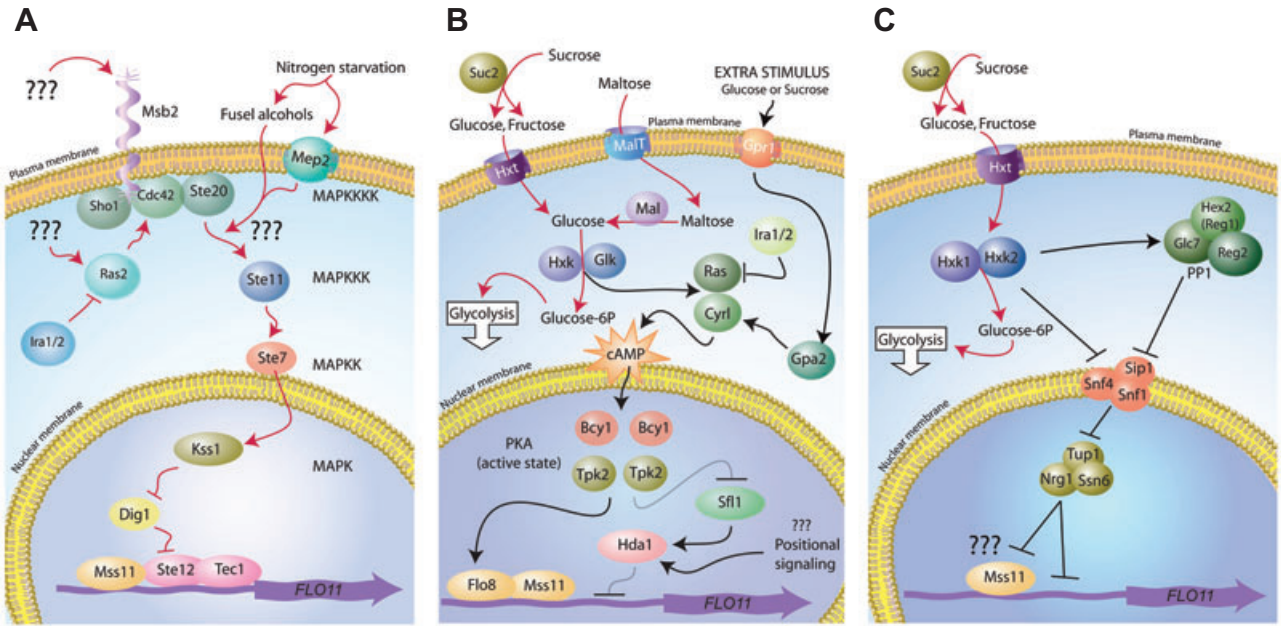

Fig. 3. *FLO11* is regulated by three signalling cascades.
A. The MAPK-dependent filamentous growth pathway. The core of this pathway in *S. cerevisiae* is formed by the central kinases Ste11 (MAPKKK) and Ste7 (MAPKK). These kinases are shared by other MAPK signalling cascades, such as the mating response pathway and the High Osmolarity Glycerol (HOG) pathway. The upstream receptors and kinases, and the downstream members of the pathway provide the necessary specificity. The specific upstream members of the MAPK pathway regulating filamentous and invasive growth in *S. cerevisiae* include the recently identified cell-surface mucin Msb2 and the MAPKKKK Ste20. Msb2 is thought to function as a sensor at the top of the pathway, but the conditions that trigger Msb2 have not yet been characterized. Other known triggers of *FLO11* that (at least partially) act through MAPK signalling include nitrogen starvation (which might be sensed through the ammonium permease Mep2) and elevated concentrations of certain fusel alcohols like butanol (Lorenz *et al*., 2000; Pan *et al*., 2000; Zeitlinger *et al*., 2003). Fusel alcohols are by-products of amino acid synthesis under conditions of nitrogen limitation. It is therefore possible that these alcohols act as signalling molecules for low nitrogen conditions. The specific downstream part of the MAPK-dependent filamentous growth pathway includes the MAPK Kss1 and the transcriptional regulators Dig1, Ste12 and Tec1.
B. The Ras/cAMP/PKA pathway. The Ras/cAMP/PKA pathway responds to the presence of glucose or sucrose in the medium. Targets include key proteins involved in the control of cell growth, glucose metabolism, stress resistance (including trehalose metabolism), flocculation and filamentous growth (Gagiano *et al*., 2002). The pathway is activated by two independent triggers. First, the intracellular phosphorylation of glucose enhances the activity of adenylate cyclase Cyr1. Second, a G protein-coupled receptor system, consisting of the receptor Gpr1 and the Gα protein Gpa2, senses extracellular glucose and sucrose (Rolland *et al*., 2000; Lemaire *et al*., 2004). Activation of the Gpr1/Gpa2 complex causes a further increase in Cyr1 activity, resulting in a transient cAMP peak (Colombo *et al*., 1998; Kraakman *et al*., 1999; Rolland *et al*., 2000; Versele *et al*., 2001). Subsequently, cAMP activates the protein kinase A complex (PKA), resulting in the dissociation of the Bcy1 subunits from the Tpk catalytic subunits of PKA (Toda *et al*., 1987a,b). The three different Tpk subunits, Tpk1, Tpk2 and Tpk3 have been shown to have distinct roles in *FLO11* regulation: Tpk2 mostly acts as an activator, while Tpk1 and Tpk3 function as inhibitors. Once released from the inhibitory Bcy1 subunits, the free Tpk2 kinase inactivates Sfl1 (suppressor of flocculation) and activates the positive regulator Flo8 (Gagiano *et al*., 2003; Kim *et al*., 2004; van Dyk *et al*., 2005). In strains lacking one of the *IRA* genes (*IRA1* or *2*), the pathway is overactivated, resulting in the expression of the normally silent *FLO10* gene (Halme *et al*., 2004). Apart from its role in the Ras/cAMP signalling cascade, together with certain histone deacetylases, Sfl1 also plays a role in the epigenetic silencing of *FLO* genes (see text). For details regarding Ras/cAMP signalling in yeast, see the reviews by Winderickx *et al*. (2003) and Verstrepen *et al*. (2004b).
C. The main glucose repression pathway. The hexose transporters (Hxt) allow glucose uptake from the medium. Once inside the cell, glucose is phosphorylated to glucose 6-phosphate by one of the hexokinases (Hxk). This phosphorylation process and/or the depletion of AMP due to the increase in ATP production inactivate(s) the central Snf1 protein kinase (Wilson *et al*., 1996). Inactivation of Snf1 allows the regulatory proteins Mig1 and Nrg1 bind to the *FLO11* promoter and recruit the general repressors Tup1 and Ssn6, resulting in repression of *FLO11*. When glucose levels drop, Snf1 is activated. The active Snf1 phosphorylates Mig1, resulting in its relocalization from the nucleus to the cytoplasm, where it can no longer repress *FLO11* transcription (not shown). For details on the main glucose repression pathway, see the reviews by Gancedo (1998) and Winderickx *et al*. (2003)

is explained by the role of the main glucose repression pathway, which represses *FLO11* as long as glucose is available in the medium (Fig. 3C). However, the Ras/cAMP/PKA pathway requires glucose or sucrose for activation (Fig. 3B). Hence, in a naïve implementation of the models, glucose would repress adhesion through the main glucose repression pathway, while on the other hand, glucose would be required for induction of the *FLO* genes because it is needed in order to activate the Ras/cAMP/PKA pathway. It becomes increasingly clear that our models of signalling are somewhat simplistic and that pathways should not be regarded as single, independent entities, but rather as integrated systems working together to control adhesion (Gagiano *et al*., 2002; Pan and Heit-

man, 2002; Schwartz and Madhani, 2004). The small G-protein Ras, for example, activates both the Ras/cAMP/PKA and the MAPK pathways. Another common component of various signalling cascades is Mss11. This protein seems to act as a central regulator, integrating the input of all pathways (van Dyk *et al*., 2005) (Fig. 3). Taken together, a more holistic study of the different pathways is needed to reveal the precise relationship between the environment, the signalling cascades and *FLO* gene expression.

Fungal adhesion is also controlled epigenetically

Apart from the different signalling cascades that regulate adhesin genes, the genes are often also under epigenetic control (Frieman and Cormack, 2004; Halme *et al*., 2004). For example, a homogeneous *S. cerevisiae* population will contain some cells in which *FLO11* is transcribed and some in which it is silent. The expression state of *FLO11* is metastable, and usually inherited from mother to daughter cells for several generations. However, the expression state is fully reversible, and cells regularly switch between the states. It is unknown if this only happens in a newly formed daughter cell, or if mature cells can also switch between expression states.

Replacing the *FLO11* promoter with another promoter or relocating *FLO11* with its endogenous promoter to another chromosomal location abolishes the epigenetic regulation. Hence, the epigenetic regulation of *FLO11* seems to be both promoter-specific and dependent on the subtelomeric location of *FLO11*. The promoter-dependent effect relies, at least partially, on Ras/cAMP target proteins, such as the transcriptional repressor *Sfl1*, and the histone deacetylase Hda1 (see Fig. 3). The latter is also believed to play a role in the position-dependent silencing of *FLO11*.

FLO11 is not the only adhesin gene that is under epigenetic control. A similar regulation has also been shown for the *S. cerevisiae FLO10* and the *C. glabrata EPA* genes (De Las Penas *et al*., 2003; Halme *et al*., 2004). In contrast to *FLO11*, silencing of *FLO10* does not depend on Hda1. Instead, other histone deacetylases (namely Hst1 and 2) and the telomeric silencing regulators Sir3 play a key role. This latter mechanism is similar to the transcriptional silencing of the *C. glabrata EPA* genes. Silencing of the subtelomeric *EPA* gene clusters is established by the Yak1 kinase and its effector, the Rap1/Sir3/Sir4 chromatin-remodelling complex (De Las Penas *et al*., 2003; Iraqui *et al*., 2005).

In addition to these silencing/de-silencing mechanisms, Halme *et al*. (2004) reported another way by which silent adhesins can be activated. The *IRA1* and *IRA2* genes show an unusually high rate of nonsense mutation (10^{-3}). Inactivation of these genes causes overactivation of the Ras/cAMP/PKA pathway, which in turn leads to de-silencing of the *FLO10* gene. The exceptionally high rates of *IRA* inactivation suggest that these mutations represent an additional mechanism that yeast cells employ to generate variability in the expression pattern of the adhesin family.

Epigenetic silencing and de-silencing is not always a stochastic switching mechanism. In a remarkable paper, Domergue *et al*. (2005) demonstrate that chromatin remodelling is used to induce expression of the *C. glabrata EPA6* in the host urinary tract. This de-silencing probably results from a reduction of NAD^+ levels in the environment, which in turn affects the activity of the NAD^+-dependent histone deacetylase Sir2. Due to the higher levels of nicotinic acid (the NAD precursor), activation of *EPA6* does not occur in cells during bloodstream infection. Hence, cells seem to use the low nicotinic acid/NAD^+ levels as a cue for establishing infection sites, a process requiring adhesion and thus *EPA* expression.

Epigenetic silencing of adhesins may serve multiple goals. First, silencing of adhesion in a subset of the population allows a balance between adhering, colonizing cells and non-adhering cells that can disseminate and find new sites for colonization. Epigenetic switching also helps cells to proactively anticipate new conditions in fluctuating environments (Kussell and Leibler, 2005). Furthermore, by only expressing a subset of adhesins, yeast cells can switch between appropriate adhesion phenotypes, allowing them to adhere to specific surfaces only. Lastly, for pathogenic fungi, stochastically variegated expression of different subsets of exposed cell-surface proteins may allow evasion of the host immune system.

Recombination of intragenic repeats generates novel adhesins

Each yeast strain only carries a limited number of slightly different adhesin genes. However, the variability in adhesins isolated from closely related strains is often much larger. Analysis of clinical isolates of *C. albicans*, for example, identified 60 different alleles of *ALS7* (Zhang *et al*., 2003). Various industrial *S. cerevisiae* brewer's strains carry sets of *FLO* genes that significantly vary in length (Verstrepen *et al*., 2005). This variability is also reflected in evolutionary studies: the adhesin genes were identified as some of the fastest expanding group of paralogues in the genomes of *S. cerevisiae* and the related *Kluyveromyces waltii* (Kellis *et al*., 2004). This genetic variability results in a remarkable phenotypic variation in adhesion phenotypes between different (but closely related) strains and species (Verstrepen *et al*., 2003).

Recent research shows that the highly repetitive DNA sequences in the middle part of the adhesin genes are

the driving force behind the creation of novel adhesins (Verstrepen *et al*., 2004a; 2005). Figure 4 shows a box plot of the *FLO1* gene, revealing the exceptional symmetry of the tandem repeats within the *FLO1* sequence. Because of their repetitive nature and their high sequence similarity, the repeats trigger frequent slippage and/or recombination events during DNA replication. This leads to removal or addition of repeat units, and thus contraction or expansion of the respective adhesin gene. Longer adhesins generally confer greater adherence, while smaller adhesins usually result in decreased adhesion, possibly because the N-terminal domain remains buried in the cell wall (Frieman *et al*., 2002; Verstrepen *et al*., 2005). Similar to epigenetic silencing, this mechanism may also allow fungi to generate variability within a population and attune their adhesion characteristics. In addition, recombination events between repeats of different adhesion genes could generate chimeric forms, which are differently regulated and might have other adhesion properties. In this way, the limited set of adhesins carried by one cell represents sort of a toolbox with which many other adhesins can be constructed to allow adherence to novel substrates. For pathogens such as *C. albicans* and *C. glabrata*, frequent recombination of cell-surface genes has the additional advantage of creating cell-surface variability (Verstrepen *et al*., 2004a; 2005). In combination with the variable expression of adhesins, the frequent recombination events provide fungi with an ever-changing outer coat, thereby keeping one step ahead of the host's adaptive immune system. The mechanism is different from that of trypanosomes, which continuously express different subsets of the hundreds of VSG (variant surface glycoproteins) genes through recombination and metastable gene silencing (Barry and McCulloch, 2001; Scherf *et al*., 2001).

Conclusion

Fungal adhesion is an unusually complex and variable phenotype. First, each cell possesses several slightly different adhesins. Differential expression of these various adhesin genes enables fungi to quickly adapt their adhesive properties to a particular environment. While the exact conditions that induce adhesion are not fully understood, many different genetic and epigenetic signalling cascades are employed to ensure proper regulation of

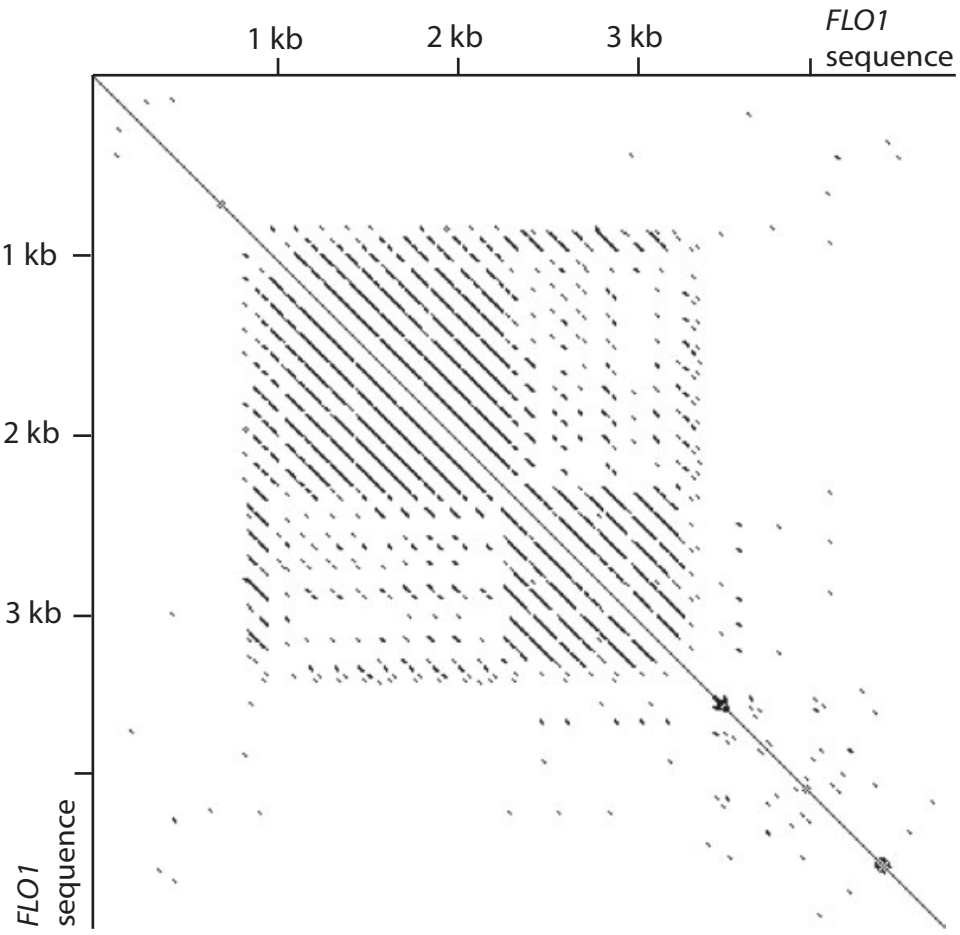

Fig. 4. Box plot of the *S. cerevisiae FLO1* nucleotide sequence. The *FLO1* nucleotide sequence was plotted against itself using the EMBOSS box plot program (Rice *et al*., 2000). The analysis shows the remarkable symmetry of the repetitive middle domain of the *FLO1* gene. These repeats trigger frequent recombination events, resulting in the formation of novel alleles (see text).

this vital phenotype. Some results indicate that these signalling cascades show significant differences between related species, adding to the complexity of the phenotype (Hoyer *et al*., 2001). Moreover, the genes encoding fungal adhesins are prone to recombination. The internal tandem repeats in these genes trigger frequent recombination events, leading to the formation of new adhesins with novel phenotypes. Together, these different mechanisms make fungal adhesion one of the most plastic and variable phenotypes, underscoring the need for fungi to adapt their adhesion behaviour to the highly variable environment and to constantly explore new opportunities for infection. It is important to note that the subtelomeric positioning of many of the adhesin genes is no coincidence. The relatively isolated position in the genome allows epigenetic switching and frequent recombination events without the risk of affecting other genes. Moreover, the numerous repetitive sequences in telomeric and subtelomeric regions help to recruit and position chromatin remodelling enzymes such as the Sir proteins, and may stimulate recombination events (Castano *et al*., 2005; Iraqui *et al*., 2005; Verstrepen *et al*., 2005). It would be interesting to see if other subtelomeric gene families, such as some of the *S. cerevisiae* carbohydrate uptake and metabolism genes, are also subjected to similar variability-inducing mechanisms as the adhesin genes.

Whereas constant switching and adaptation of adhesion may be crucial for survival in natural habitats, the instability of the flocculation profile is a true nightmare for industrial applications that rely on a constant and predictable yeast performance. Brewers and winemakers constantly struggle to get a grip on the flocculation behaviour of their little helpers, often without much success. In principle, recombinant DNA techniques make it possible to create more stable strains with an appropriate flocculation profile (Pretorius and Bauer, 2002). Yeasts carrying a specific *FLO* gene under transcriptional control of a late-fermentation promoter have proven useful in laboratory-scale fermentations (Ishida-Fujii *et al*., 1998; Verstrepen *et al*., 2001) but are currently not acceptable to consumers.

The remarkable plasticity of fungal adhesion also causes concern in medicine. Despite awareness and preventive measures to avoid the formation of highly resistant fungal biofilms, yeast cells are capable of adhering to many of the different materials used in today's hospitals, and as a consequence, yeast infections are becoming more prevalent. Given the variability of the fungal adhesins, directly targeting these cell-surface proteins or using them in vaccines seems a poor strategy. Better targets for new drugs are the less-variable features of adhesion, such as the biosynthesis of the GPI-anchor of the adhesins or the transcriptional and post-transcriptional regulatory mechanisms of adhesion. In a shorter term, the use of anti-fungal coatings on medical devices seems like the most realistic way of fighting disseminated yeast infections.

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Supplementary material

The following supplementary material is available for this article online:

Fig. S1. Phylogenetic tree of cellular adhesins and mucins. The figure shows the phylogenetic similarity of the best-known adhesin genes of *S. cerevisiae*, *S. pastorianus*, *C. albicans* and *C. glabrata.* Other genes that are not strictly adhesin family members, but do show homology to adhesins, are also included in the analysis. These include the *S. cerevisiae* **a**-agglutinin subunits encoded by *AGA1* and *2*, and the human mucin (*MUC*) genes. A surprising result of this analysis is the relative isolated position of *FLO11*, which shows more similarity to the yeast agglutinin subunit Aga1 and the human mucins than to the yeast flocculins encoded by *FLO1*, *5* and *9*. Another remarkable finding is the high similarity between the *C. albicans ALS4* gene and the *S. cerevisiae* uncharacterized open reading frame *YFL051C*. The tree was constructed using the CLUSTALX software; the robustness of the tree was confirmed with standard bootstrapping techniques.

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