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Flocculation protein structure and cell-cell adhesion mechanism in *Saccharomyces cerevisiae*

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Abstract

Cell-cell adhesion occurs in a broad spectrum of biological processes, of which yeast flocculation is an area of interest for evolutionary scientists to brewers and winemakers. The flocculation mechanism is based on a lectin-carbohydrate interaction but is not yet fully understood, although the first model dates back to the nineteen fifties. This review will update the current understanding of the complex mechanism behind yeast flocculation. Moreover, modern technologies to measure the forces involved in single carbohydrate-lectin interactions, are discussed. The Flo1 protein has been extensively described as the protein responsible for strong flocculation. Recently, more research has been directed to the detailed analysis of this flocculin. Due to the advances in the field of bioinformatics, more information about Flo1p could be obtained via structurally or functionally related proteins. Here, we review the current knowledge of the Flo1 protein, with a strong emphasis towards its structure.

Keywords: cell-cell adhesion, Flo1p, flocculation, *FLO* genes, Flo proteins, *Saccharomyces cerevisiae*,

The Flo adhesin family is involved in cell-cell and cell surface adhesion

A broad spectrum of biological processes requires controlled cell adhesion. In microbiology, this includes fungal, viral and bacterial infections or the formation of organised cell structures such as biofilms. Other examples are the assembly of tissues and the nervous system, cell culturing, embryonic development, cellular communication and tumor metastasis. Cell adhesion in general is commonly defined as the binding of a cell to a substrate, which can be another cell, a surface or an organic matrix. The process is regulated by specific cell adhesion molecules.

Many fungi contain a family of cell wall glycoproteins, called "adhesins" that confer unique adhesion properties (Teunissen and Steensma 1995, Guo et al. 2000, Hoyer 2001). These molecules are required for the interactions of fungal cells with each other (flocculation and filamentation) (Teunissen and Steensma 1995, Lo and Dranginis 1998, Guo et al. 2000, Vyas et al. 2003), inert surfaces such as agar and plastic (Gaur and Klotz 1997, Lo and Dranginis 1998, Reynolds and Fink 2001) and mammalian tissues (Cormack et al. 1999, Li and Palecek 2003). They are also crucial for the formation of fungal biofilms (Baillie and Douglas 1999, Reynolds and Fink 2001, Green et al. 2004).

The adhesin protein family in *Saccharomyces cerevisiae* strain S288C can be subdivided into two groups. The first group of proteins is encoded by genes including *FLO1*, *FLO5*, *FLO9* and *FLO10*. These proteins are called flocculins (Caro et al. 1997) because they promote cell-cell adhesion by interacting with the cell wall of adjacent cells. This binding event leads to the formation of multicellular clumps (flocs), which sediment out of solution. The *FLO1*, *FLO5*, *FLO9* and *FLO10* genes share considerable sequence homology. The second group of the Flo family, including Flo11p, Fig2p and Aga1p, has quite unrelated amino acid sequences. These last two proteins are induced during mating (Roy et al. 1991, Erdman et al. 1998), while Flo11p is required for diploid pseudohyphal formation and haploid invasive growth (Lambrechts et al. 1996, Lo and Dranginis 1998). In haploid invasive growth, cells adhere to the agar surface, so that they do not wash off (Roberts and Fink 1994). In diploid pseudohyphal growth, cells adhere to each other after division and form long chains or filaments (Gimeno et al. 1992). When yeast cells are grown on semi-solid (0.3% agar) medium, they form “mats”: complex multicellular structures composed of yeast cells (Reynolds and Fink 2001, Reynolds et al. 2008). Yeast mat formation, as well as the attachment of cells to plastic surfaces, requires Flo11p.

These morphogenetic events (flocculation, filamentation and invasive growth) are tightly regulated (Verstrepen and Klis 2006, Fichtner et al. 2007, Dietvorst and Brandt 2008). Flocculation often occurs upon depletion of sugar during late-exponential or stationary phases of growth (Stewart and Russel 1981), whereas filamentation requires starvation for nitrogen (Gimeno et al. 1992, Braus et al. 2003, Granek and Magwene 2010). The *FLO8* gene is a transcriptional activator of *FLO1* (Kobayashi et al. 1996) and *FLO11* (Rupp et al. 1999) and essential for their expression. In commonly

used laboratory strains a nonsense mutation in the *FLO8* gene leads to transcriptionally silencing of the *FLO* genes. Moreover, it was shown that one *FLO* gene may compensate for another in diverse morphogenetic events (Guo et al. 2000).

The dominant *FLO1* gene was identified and localised at the very end of the right arm of chromosome I (Russell et al. 1980, Teunissen et al. 1993b). Flo1p is a cell wall protein responsible for flocculation of Flo1-expressing cells and its presence is directly related to the degree of flocculation (Bidard et al. 1995, Bony et al. 1997, Bony et al. 1998). The *FLO5* gene is responsible for the strong flocculation of Flo5-expressing cells (Johnston and Reader 1983). This gene is not allelic with the *FLO1* gene but shows 96% identity (Jin and Speers 1998). It is located on the very end of chromosome VIII (Teunissen et al. 1995). When the *FLO5* gene is incorporated in a non-flocculent yeast strain, this strain acquires flocculation properties (Vezinhet et al. 1991, Bidard et al. 1995). Flo5p is, like Flo1p, a cell wall protein and the expression of Flo5p is correlated with the degree of flocculation (Bony et al. 1998). Not all yeast strains contain the dominant *FLO9* gene, which is located on chromosome I, but at the other end of the chromosome, when compared to *FLO1* (Teunissen and Steensma 1995). This gene shows 94% identity with *FLO1* and 74% identity with the *FLO5* gene (Bossier et al. 1997). The *FLO10* gene product has 58% identity with *FLO1* and is located at chromosome XI (Teunissen and Steensma 1995). Overexpressing the *FLO10* gene in *S. cerevisiae* leads to weak flocculation compared to the overexpression of the *FLO1* gene. Flo10p is also responsible for filamentation and the adhesion to agar or plastic (Guo et al. 2000). The Flo11p protein has the same domain structure as the other Flo proteins, but has a totally different amino acid sequence. The protein is responsible for flocculation, pseudohyphae formation, agar invasion, adhesion to substrates as well as for filamentation (Lambrechts et al. 1996, Lo and Dranginis 1996, 1998, Guo et al. 2000).

Generally, flocculation can be governed by the different *FLO* genes except by *FLO11*. But, when overexpressed, many of these proteins can substitute for each other in diverse morphogenic events: flocculation, mating, haploid invasion and filamentation (Guo et al. 2000). Indeed, yeast cells expressing Flo11p showed flocculation behaviour (Douglas et al. 2007) but the flocculation ability of yeast cells carrying the *FLO11* gene depends on the strain in which this gene is expressed. The yeast strain *S. cerevisiae* var. *diastaticus* exhibits Flo11 dependent flocculation and biofilm formation but did not invade agar or form pseudohyphae, whereas the strain with the ∑1278b background required Flo11p to form pseudohyphae, invade agar, adhere to plastic and develop biofilms, but did not flocculate (Douglas et al. 2007). These results clearly show that adhesin properties are influenced by the yeast strain in which they are studied. Specific glycosylation of the adhesin, or auxiliary factors influencing adhesin conformation on the cell wall may thus be responsible for differences in biophysical properties. This has also been investigated by Govender et al. (2010). They explain that the adhesion phenotype after expressing individual *FLO* genes is different depending on the yeast strain in

which they are expressed. This is due to the high allele heterogeneity of the *FLO* genes and to the fact that those genes are subjected to epigenetic regulation. So it can be concluded that care has to be taken when extrapolating data obtained in laboratory strains to industrial strains and that the optimization of the flocculation pattern of individual commercial strains will have to be based on a strain-by-strain approach (Govender et al. 2010).

Flo protein cell-cell adhesion mechanism

Flocculation was first defined as the phenomenon wherein yeast cells adhere in clumps, and sediment rapidly from the medium in which they are suspended (Stewart and Goring 1976). Further refinement led to the present definition: "flocculation is the asexual, reversible and calcium-dependent aggregation of yeast cells to form flocs containing thousands of cells that rapidly sediment to the bottom of the liquid growth substrate" (Stratford 1989, Bony et al. 1997). Other yeast strains, called ale yeasts, show a biofilm at the air-liquid interface at the end of the fermentation. The model for this biofilm formation is based on an increase in cell surface hydrophobicity and the fact that the multicellular aggregates effectively entrap carbon dioxide (Zara et al. 2005). The flocculation behaviour is of great importance for yeast cells as it is a way to escape from harsh conditions in the growth medium. It was suggested that cells in the middle of the floc could lyse and act as a source of new nutrients for the other cells (Stewart and Russell 1981, Iserentant 1996). Therefore, flocculation enhances the survival rate of yeast cells in starvation conditions.

In an attempt to elucidate the phenomenon of flocculation, Eddy and Rudin (1958) proposed the lectin hypothesis. In the presence of calcium, flocculins are able to bind highly branched mannose polymers located in the cell wall of adjacent cells (Eddy and Rudin 1958, Miki et al. 1982). Moreover, it was shown that flocculation could be reversibly inhibited by the presence of sugars (Taylor and Orton 1978, Miki et al. 1980, Nishihara and Toraya 1987, Stratford and Assinder 1991), which is validating the hypothesis. This flocculation model is now widely accepted.

The flocculation phenotypes of yeast strains can be divided into two groups: the Flo1-phenotype and the NewFlo-phenotype (Stratford and Assinder 1991, Sieiro et al. 1995). This partition is based on the type of carbohydrate that inhibits flocculation. Flocculation of the Flo1-phenotype can be inhibited by mannose, but not by glucose, maltose, sucrose or galactose. Therefore, Flo1p is considered as the most specific adhesion protein of the Flo-family (Kobayashi et al. 1998, Govender et al. 2008, Van Mulders et al. 2009). Flocculation of the NewFlo-phenotype can be inhibited by mannose, glucose, maltose and sucrose, but not by galactose. This flocculation phenotype is governed by a Flo1p homologue, named Lg-Flo1p. Considering the current research about the capacity of different sugars to inhibit the flocculation, the clear distinction of two flocculation phenotypes is questioned. Not all flocculation phenotypes can be

classified in either the Flo-phenotype or the NewFlo-phenotype. For example, the expression of *FLO5*, *FLO9* or *FLO10* does not lead to a specific flocculation phenotype making them less selective adhesins. They were strongly inhibited by mannose but also weakly inhibited by a range of sugars (Van Mulders et al. 2009). Although, based on the sequence homology with Flo1p, it was found that Flo5p and Flo9p were exclusively mannose-binding flocculins (Teunissen and Steensma 1995). More detailed research about the inhibition of flocculation by sugars has been performed. Miki et al. have quantified the inhibition of flocculation (1980). Up to 500 mM D-mannose was required to completely inhibit flocculation. Later, the sugars were ranked according to their inhibition capacity (Smit et al. 1992). It was shown that α-D-mannose could disrupt the flocs at a concentration of only 25 mM. This experiment has been repeated recently using a yeast strain overexpressing Flo1p (Van Mulders et al. 2009). It was observed that the flocculation mediated by Flo1p was only inhibited by mannose at the high concentration of 1 M.

More detailed research regarding the flocculation of yeast cells has been performed recently. A high concentration of flocculins is found on the yeast cell surface (Bony et al. 1997). Where they can all interact with mannan chains of other cells, leading to multipoint attachments between yeast cells. When one interaction is in the range of millimolar affinities, it becomes micromolar affinity with two attachments. In this way the interaction strength of flocculation is increased dramatically (Dranginis et al. 2007). The first and only quantitative measurement of the affinity between a Flo protein and a carbohydrate was done for the interaction of Lg-Flo1p to mannose. The value for the dissociation constant was calculated to be 0.77 mM (Groes et al. 2002). Compared to the affinity of other lectin carbohydrate interactions, this is indeed a very low value. This two-way interaction has been confirmed by looking carefully at the cells part of a floc resulting from multiple stresses. While analysing the flocs, it was observed that most of the cells were *FLO1*-expressing cells. In other words, *FLO1*-expressing cells preferentially aggregate with other *FLO1* expressers (Smukalla et al. 2008). This was explained by the fact that the two-way interaction, which occurs when two *FLO1*-expressers are binding, is stronger than the one-way interaction of a *FLO1*-expresser with a non-*FLO1*-expresser. A more striking observation has been described about the Flo11 proteins. When secreted Flo11p was covalently attached to microscopic beads, it conferred the ability to specifically bind to *S. cerevisiae* var. *diastaticus*, but not to ∑1278b cells, which do not flocculate. They were not able to bind cells from another yeast strain expressing Flo11p or cells with a deletion of *FLO11* (Douglas et al. 2007). This Flo11p - Flo11p interaction points to a homotypic adhesive mechanism. A further quantitative investigation on the molecular level of the self-binding properties of Flo11p and the other Flo proteins is necessary to complete the flocculation mechanism.

Structural features of *S. cerevisiae* flocculins

The member proteins of the adhesin family have a modular configuration that consists of three domains (*N*-terminal, central and *C*-terminal domain) and an amino-terminal secretory sequence that must be removed when the protein moves to the plasma membrane through the secretory pathway (Hoyer et al. 1998, Hoyer 2001, Verstrepen et al. 2004). Until now, no atomic structure of any Flo protein has been determined. However, the *N*-terminal part of a homolog of Flo1p (Lg-Flo1p) has been crystallised and diffracted to high-resolution using X-ray radiation (Groes et al. 2002), but the structure has not been solved yet. However, some structural information about the Flo-proteins has already been obtained from structural bioinformatics. The prediction of the secondary structure of Flo1p shows that the protein is mainly composed of β-sheets alternated with coils (Nishikawa and Noguchi 1991). α-Helices are only found at the very end of the *N*- and *C*-terminal parts of the protein. The study of the hydrophobicity of the Flo1p protein showed that the *N*- and *C*-terminal parts of the protein were more hydrophobic than the middle part (Kyte and Doolittle 1982). An overview of the general gene pattern of the Flo family in strain S288C is represented in Fig. 1a. The length of each domain varies according to each *FLO* gene, which is visualised in Fig. 1b and described in Table 1. The Flo1p has been studied most extensively of all Flo proteins and will be discussed in more detail.

Modular configuration of the Flo proteins

The *C*-terminal domain contains a glycosylphosphatidylinositol (GPI)-attachment site. The adhesins are covalently linked to the β-1,6-glucans of the yeast cell wall through the GPI-remnant (De Nobel and Lipke 1994, Caro et al. 1997, Hamada et al. 1998a, Hamada et al. 1998b, De Groot et al. 2003).

The central domain of Flo1p contains many tandem repeats and 46% of the amino acids are serine and threonine residues (Teunissen et al. 1993a, Caro et al. 1997) (see Fig. 2). These amino acids are prone to extensive *O*-glycosylation during the post-translational modification of the protein and the *O*-linked oligosaccharide side-chains then enable the flocculins to attain a long, semi-rigid rod-like structure (Jentoft 1990) that might be stabilised by Ca^{2+} ions (Verstrepen and Klis 2006). The proline residues, which are common in this region, may also prevent the central domain to form a compact domain (Dranginis et al. 2007). All together, this leads to the general idea that the protein is attached to the cell wall and sticks out, reaching for mannose chains to interact with. Along the amino acid sequence, many Asn-Xaa-Thr/Ser sequences are found, where Xaa represents any amino acid except proline. This is the consensus sequence for *N*-

glycosylation (Kornfeld and Kornfeld 1985), hence, both *O*-glycosylation and *N*-glycosylation are present (Bony et al. 1997).

The *N*-terminal domain is the lectin domain, which interacts with mannose residues. This interaction is responsible for flocculation. This was discovered by expressing a truncated form of Flo1p (deletion of amino acids 50 to 278). It was observed that this truncated protein could not trigger flocculation (Bony et al. 1997). These results are in agreement with those of Kobayashi and co-workers (1998), who also reported that the *N*-terminal region of Flo1p contains the sugar recognition domain. Indeed, the replacement of the *N*-terminus of the Flo1 protein by the corresponding region of the Lg-Flo1 protein, caused conversion of the Flo1 flocculation phenotype to the NewFlo flocculation phenotype (Kobayashi et al. 1998).

The Flo protein at molecular level

Iterative database searches using a domain insert sequence from bacterial β-glucosidases revealed the presence of a conserved domain, called the PA14 domain. It is shared by a wide variety of bacterial and eukaryotic proteins, which include many glycosidases, glycosyltransferases, proteases, amidases, bacterial toxins such as anthrax protective antigen (PA), and also yeast adhesins (Rigden et al. 2004). This conserved domain is named PA14 after its location in the PA_{20} pro-peptide of the anthrax toxin protective antigen. The crystal structure of the anthrax toxin protective antigen (shown in Fig. 3a) indicates that the PA14 domain consists of a series of antiparallel β–strands. These correspond roughly to the conserved regions of the domain and are separated by loops that coincide with the variable region (Petosa et al. 1997). Most of the experimentally characterised PA14-containing proteins are involved in carbohydrate binding and/or metabolism. As mentioned before, in the *S. cerevisiae* flocculins, carbohydrate binding is associated with the N-terminal third of the protein. For Flo1p, this part has been assigned as a new domain (PF07691) in the Pfam database (Finn et al. 2010; http://pfam.sanger.ac.uk), covering residues 86-249, which overlaps the PA14 domain. The flocculin N-terminal domain might, therefore, be considered as one of the many PA14 domain variants. Based on the sequence alignment of the *N*-terminal domain of Flo1p and the anthrax toxin protective antigen, a model was proposed for the structure of *N*-Flo1p, considering amino acids 85 to 261 (Fig. 3b).

The amino acids that are responsible for the carbohydrate recognition in Flo1p and Lg-Flo1p, have also been described. It was suggested that the tryptophan residue on position 228 is involved in mannose-recognition in the Flo1 flocculation phenotype. This tryptophan 228 must be replaced by leucine to produce a flocculation phenotype inhibited by glucose. The following model for sugar recognition was proposed: tryptophan 228 in Flo1p recognises the C2-

hydroxyl group of mannose but does not recognise the C2-hydroxyl group of glucose. In the Lg-Flo1 protein, a leucine instead of a tryptophan at position 228 is required for the NewFlo flocculation phenotype. Also position 226 affects the flocculation phenotype: for NewFlo-type flocculation a glycine or arginine is needed at that position. Generally, the carbohydrate binding activity of Flo1p can be attributed to the VSWGT motif, a pentapeptide encompassing amino acids 226 to 230 (Kobayashi et al. 1998).

Recently, an EYDGA pentapeptide motif belonging to the PA14 domain was identified. The pentapeptide is involved in sugar recognition in the N-terminal domain of the Epa1p (epithelial adhesin) from *Candida glabrata*. This can be compared to the VSWGT pentapeptide in Flo1p (Zupancic et al. 2008). A multiple sequence alignment of various PA14 domains showed convincingly that the VSWGT motif of Flo1p and the EYDGA motif of Epa1p are found in precisely the same position, within a hypervariable region of PA14 (Rigden et al. 2004). The VSWGT/KVLAR motif of Flo1p/Lg-Flo1p, and the EYDGA motif of Epa1p, correspond to a surface loop between two β-strands (strands 9 and 10) in the anthrax toxin PA domain structure (Petosa et al. 1997) (see the blue loop in Fig. 3b).

The *FLO* genes posses internal tandem repeats located in the central region, that function as interchangeable modules (Watari et al. 1994). During DNA replication, the presence of internal tandem repeats is responsible for recombination events. Therefore, yeast cells show a highly variable adhesion phenotype as they are able to quickly adapt their adhesion properties to new environments. The removal or addition of repeat units results in a longer or shorter adhesin. This is immediately reflected in its flocculation behaviour: a longer adhesin showing a stronger flocculation phenotype. This can be explained by the fact that the *N*-terminal domain should be exposed clearly to the neighbouring cells to allow flocculation (Frieman et al. 2002, 2004, Verstrepen et al. 2005). Surprisingly, Liu et al. suggest that the deletion of tandem repeats can cause the conversion from Flo1- to NewFlo1-phenotype (Liu et al. 2007a, b). Moreover, for the Flo11 protein, the length of the gene affects the biofilm-forming ability of the cells expressing Flo11p. This was demonstrated by cloning two alleles of the *FLO11* gene with different sizes (3.1 and 5 kb) into *S. cerevisiae* strain BY4742 and analysing the corresponding phenotype (Zara et al. 2009). This is not in agreement with what is known so far about the specificity of the flocculin, which is believed to be on the *N*-terminal domain only (Bony et al. 1997, Kobayashi et al. 1998, Van Mulders et al. 2009). Not only Flo proteins, but several other yeast adhesins such as Als and Epa proteins from *C. albicans* and *C. glabrata* respectively, contain tandem repeat (TR) regions. So far, their structures are unknown, but recently *ab initio* modeling with either Rosetta or LINUS generated consistent structures of three-stranded anti-parallel β-sheet domains for the TR regions of Als5p (Fig. 3c) (Frank et al. 2010).

Searching the pool of all known yeast adhesins using the TANGO program (http://tango.crg.es/), many adhesins were found to contain sequences with high β–aggregation potential, including Flo1p and Flo11p (Ramsook et al. 2009). When

a peptide from the central domain of Flo1p (aa 305 to 315) was suspended in neutral buffer, it readily formed amyloids. It was proposed that amyloid formation plays a role in cell adhesion by increasing the avidity of the adhesins by "bundling" the adhesins. Therefore, amyloid formation can greatly increase the intercellular binding strength by increasing avidity (Otoo et al. 2008, Frank et al. 2010, Ramsook et al. 2010).

Glycosylation of Flo proteins

Flo proteins are heavily *O*- and *N*-glycosylated (Straver et al. 1994a, Bony et al. 1997). Glycosylation is an enzyme-directed and site-specific process, which is required for proper functioning of the protein. It is a post-translational modification and the protein is glycosylated on its way through the endoplasmic reticulum and the Golgi apparatus. *N*-glycosylation is the addition of oligosaccharides to the amide group of asparagines and results in highly branched and extended high mannose structures (Dean 1999). *O*-glycosylation occurs at a later stage during protein processing and short linear chains up to five mannose residues are added to the β-hydroxyl group of serine and threonine residues (Tanner and Lehle 1987, Aikawa 1995, Goto 2007). Proteins of diverse yeast species contain other *O*- and *N*-linked oligosaccharide structures (glycans). The differences found in various yeast species, has been reviewed (Gemmill and Trimble 1999). Sugar analysis of a homolog of Flo1p revealed the presence of 64% carbohydrate, in the molar ratio of 60:27:1, which corresponds to mannose, glucose and *N*-acetylglucosamine respectively. However, the exact glycan profile of cell wall Flo proteins is not yet known (Straver et al. 1994b, Bony et al. 1997, Douglas et al. 2007).

The role of direct carbohydrate-carbohydrate interactions has not yet been explored in yeast flocculation. Recently, it was shown that these interactions can be important for cell adhesion phenomena since glycans occur on the outermost cell periphery and therefore are likely involved in the first intercellular contact (Bucior and Burger 2004, Bucior et al. 2009). Carbohydrate chains offer a rich supply of potential low-affinity binding sites, arranged in a polyvalent array that may create a flexible and versatile carbohydrate-carbohydrate recognition system (Misevic and Burger 1986). This then allows cells to explore surrounding surfaces, and to form or reinforce interactions before subsequent steps in the cell that are mediated by tight and stable covalent bonds (Mallinson et al. 2003). The interaction between individual carbohydrates is fairly weak, and biologically relevant high affinities are achieved by the organisation of cell surface proteoglycans, glycoproteins or glycolipids into clusters or superstructures (Hakomori 2003, Yoneda and Couchman, 2003, Todeschini and Hakomori 2008). These provide avidities that can attach cells to each other under physiological conditions. Carbohydrate self-recognition takes place through surfaces determined by carbohydrate epitopes and is based

on non-covalent bonds: van der Waals contacts, hydrogen bonds, electrostatic forces and interactions with cations (Spillmann and Burger 1996).

Carbohydrate self-interactions have been recently well described for sponge proteoglycans. Dissociated sponge cells from two different species have the capacity to recognise specifically their own species and reaggregate through cell-surface proteoglycans, termed aggregation factors (AFs) (Fernàndez-Busquets et al. 2003). In a calcium-independent process the AFs adhere to the cell surface, and in a calcium-dependent process they exhibit AF self-association (Jarchow et al. 2000, Haseley et al. 2001, Fernàndez-Busquets et al. 2003, Carvalho de Souza et al. 2009). AFs are large molecules (2×10^4 to 2×10^7 Da) that are composed of 30-60% carbohydrates. Atomic Force Microscope (AFM) visualisation has revealed either a linear or a sunburst-like core structure with 20-25 radiating arms (Dammer et al. 1995). Single-molecule force spectroscopy measurements reported equally strong adhesion forces between glycan molecules (190-310 pN) as between proteins in antibody-antigen interactions (244 pN) (Bucior et al. 2004). This observation suggested the existence of intermolecular carbohydrate adhesion domains (Garcia-Manyes et al. 2006).

Cell-cell adhesion biophysics

Molecular and genetic approaches have identified various cell adhesion molecules (CAMs) with their ligand specificities, and have determined the processes in which they are involved. However, the molecular mechanisms by which CAMs regulate different types of adhesion are open debates (Morgan et al. 2007, Ludwig et al. 2008). To understand cell adhesion, the vast amount of qualitative data that is available must be augmented with quantitative data of the biophysics of adhesion. Historically, the strength of cell adhesion to a substrate has been studied using simple washing assays (Klebe 1974). Washing assays have proven to be versatile and useful in identifying CAMs, important extra-cellular-matrix components and other proteins that are involved in various forms of cell adhesion. To estimate the force to which cells are subjected, various assays that are based on the regulated flow of media have been implemented, including flow chamber methods (Kaplanski et al. 1993). However, these assays only give estimates of adhesion forces, since the shear force that is exerted on the cells depends on parameters such as cell size, cell shape and how the cell is attached to the substrate. Recently, single-cell and single-molecule techniques have been developed to obtain more controlled and quantitative measurements of adhesion strength.

Single-cell force spectroscopy assays on living cells have been applied to measure the strength of cell adhesion down to single-molecule levels (Helenius et al. 2008). A living cell can be attached to a tipless cantilever of an AFM and the interacting partner (molecule or cell) on a substrate-coated surface. Alternatively, the living cell can be fixed on a surface

and the tip functionalised with the interacting molecule. AFM force spectroscopy with a single cantilever-bound cell can be used to investigate cell-cell and cell matrix interactions. The approach and withdrawal of this cell to and from its surface can be precisely controlled by parameters such as applied force, contact time and pulling speed by benefiting from the AFM's high-force sensitivity and spatial resolution. The data collected in these experiments include information on repulsive forces before contact, cell deformability, maximum unbinding forces, individual unbinding events, and the total work required to remove a cell from the surface (Table 2). Force spectroscopy can identify cell subpopulations and characterise the regulation of cell adhesion events with single-molecule resolution (Taubenberger et al. 2007). Analysis of discrete cell adhesion forces demonstrated a dynamic increase of adhesion over time.

Single *S. cerevisiae* cells have been attached to a tipless AFM cantilever, and used as a living single-cell probe to perform single-cell force spectroscopy (Kang and Elimelech 2009). The contributions of several galectin family members in cell-substratum adhesion of Madin-Darby canine kidney cells have been studied using quantitative single cell AFM force spectroscopy (Friedrichs et al. 2007) (Table 2). Optical tweezers have been used to orient uropathogenic *Escherichia coli* (which present a FimH lectin at the tip of their type 1 pilus) relative to a mannose-presenting surface, and thus, limit the number of points of attachment (Liang et al. 2000) (Table 2). It was possible to quantify the forces required to break a single interaction between pilus and mannose groups.

The Flo proteins Flo1p, Lg-Flo1p, Flo5p, Flo9p and Flo10p are lectins, since they have an affinity towards specific sugar moieties. Recently, AFM force spectroscopy has been used to directly measure the forces involved in single carbohydrate-lectin interactions (Table 3). AFM force spectroscopy has been used to determine the unbinding force of oligoglucose carbohydrates and Lg-Flo1p present on the cell wall of industrial brewer's yeast strains (Touhami et al. 2003a). AFM force probes functionalised with carboxymethyl-amylose were used to record force-distance curves on living cells. Flocculation cells showed adhesion forces of 121 ± 53 pN. Unbinding forces of other lectin-carbohydrate interactions range from 30 pN to 200 pN (Table 2 and 3).

Medical and industrial significance of *S. cerevisiae* flocculation phenotype

In hazardous environmental conditions, *S. cerevisiae* cells possess the remarkable properties to adhere to other cells or to substrates like agar or plastics. Adhesion to surfaces is a mechanism that may lead to biofilm formation. It is often used as a model to study biofilm formation of pathogenic yeasts, responsible for 41% of the mortality rate in hospitals (Wisplinghoff et al. 2004). Biofilms give protection to yeast cells, e.g. by conferring resistance to antifungal drugs. Adhesion to abiotic surfaces as catheters or prostheses can serve as a reservoir of pathogenic cells ready to gain access to

the bloodstream of patients (Jabra-Rizk et al. 2001, Donlan and Costerton 2002, Jabra-Rizk et al. 2004, Kojic and Darouiche 2004). A detailed study of this mechanism is necessary in the search for an adequate antifungal treatment.

The flocculation phenomenon is exploited in the brewery industry as an easy, convenient and cost-effective way to separate the aggregated yeast cells from the beer at the end of the primary fermentation. The timing of flocculation is crucial for brewers as the quality of beer highly depends on it. When cells start to flocculate too early, the fermentation will be incomplete with undesirable aromas and too many residual sugars. On the other hand, when the flocculation is delayed, problems can arise during beer filtration (Willaert 2007a). A good understanding of the underlying mechanism and the factors affecting flocculation is of crucial importance for the performance and control of the brewing process (Verstrepen et al. 2003). Another application is the self-aggregation of yeast cells, which can be classified as a type of cell immobilisation (Willaert and Baron 1996, Willaert 2007b). Flocculent yeast strains can be selected to obtain a high cell density during the beer fermentation process (Nedovic et al. 2005, Willaert and Nedovic, 2006, Verbelen et al. 2010). Consequently, the volumetric productivity can be increased considerably.

Altogether, elucidating the structure and the physical binding properties of adhesins will allow controlling yeast adhesion more efficiently in medical and biotechnological applications.

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Tables

Table 1: Overview of the lengths of the important domains for each of the 6 Flo proteins; according the Pfam database (Finn et al. 2010; http://pfam.sanger.ac.uk). Numbers refer to the amino acid residues in the primary sequence.

Protein	Total length	Signal sequence	N-terminal domain	PA14	Transmembrane domain

Lg-Flo1	1300	1-24	25-245	59-222	nn
Flo1	1537	1-24	25-272	86-249	1519-1536
Flo5	1075	1-24	25-272	85-249	1057-1074
Flo9	1322	1-23	24-272	85-249	1304-1321
Flo10	1169	1-24	25-296	112-271	1144-1168
Flo11	1367	1-21	22-208	nn	nn

Table 2: Carbohydrate-lectin interactions studied by single-cell (SCFS) and single-molecule (SMFS) force spectroscopy.

Cell type	Lectin	Carbohydrate	SCFS rupture force (pN)	SMFS rupture force (pN)	SCFS Technique	Reference
CHO	SGLT1 (SLC5A1)	Glucose	51	—	AFM	Puntheeranurak et al., (2006, 2007)
E. coli	FimH	Mannose			Optical tweezers	Liang (2000)
NIH3T3	Concanavalin A	Surface-expressed mannose residues	86	—	AFM	Chen (2000)
NIH3T3	Concanavalin A	Not specified/known	80	95	AFM	Baumgart and Offenhäusser (2003)
PMN	E-selectin	Not specified/known	140	—	AFM	Hanley (2004)
PMN	L-selectin	Not specified/known	80	—	AFM	Hanley (2004)
PMN and LS174T	P-selectin					
Red blood cells	*Helix pomatia* lectin	Saccharides from blood types O and A	65	—	AFM	Grandbois et al. (2000)
S. cerevisiae	Concanavalin A	Mannan	75-200	—	AFM	Gad et al. (1997)
S. cerevisiae	Concanavalin A	Mannan/mannose	117 ± 41	—	AFM	Touhami et al. (2003b)
S. cerevisiae	Lg-Flo1p	CM-amylose	121 ± 53	—	AFM	Touhami et al. (2003b)
Madin-Darby canine kidney cells	integrin	collagen-I coated substrate	86.42	—	AFM	Friedrichs et al. (2007)
Madin-Darby canine kidney cells	galectin-3 and -9	laminin-111 coated substrate	60.02	—	AFM	Friedrichs et al. (2007)

Table 3: Carbohydrate-lectin unbinding forces measured using single-molecule AFM force spectroscopy.

Lectin (origin)	Carbohydrate	Component attached to AFM probe	Interaction partner	Unbinding force (pN)	Reference
$(AB)_2$ agglutinin (*Ricinus communis*)	Lactose	Lactose (p-amino-phenyl lactoside)	Agglutinin bound to Sepharose 4B beads	58 ± 9	Dettmann et al. (2000)
$(AB)_2$ agglutinin (*Viscum album*)	Lactose	Lactose (p-amino-phenyl lactoside)	Agglutinin bound to Sepharose 4B beads	47 ± 7	Dettmann et al. (2000)
ConA[a] (Jack bean)	Amylose	CM[c]-amylose	Con A on gold surface	96 ± 55	Touhami et al. (2003a)
Con A (jack bean)	Mannose	Con A	Mannose bound to SAMs[d] on gold-coated silicon	47 ± 41	Ratto et al. (2004)
FimH (*E. coli*)	Mannose	BSA[b]-mannose	FimH on polysty-rene plates		
Galectin-1 (bovine heart)	Lactose	Lactose (p-amino-phenyl lactoside)	Galectin-1 bound to Sepharose 4B beads	34 ± 6	Dettmann et al. (2000)
Galectin-3 (human)	Pectin galactan	Galactan	Galectin-derivatised glass surface	79	Gunning et al. (2009)

[a] Con A: Concanavalin A; [b] BSA: Bovine serum albumin; [c] CM: carboxymethyl; [d] SAMs: self-assembled monolayers of 16-mercaptohexadecanoic acid.

Figures legends

Fig. 1 | (a) General domain structure of the flocculins from *S. cerevisiae* strain S288C, as explained in the text. The N-terminal domain contains the PA14 domain which is responsible for the interaction with the sugar. **(b)** Domain structure for each Flo protein from the Pfam database (Finn et al. 2010; http://pfam.sanger.ac.uk). Table 1 gives the length of the domains for each specific protein.

Fig. 2 | Schematic representation of Flo1p updated from Watari and co workers (1994), indicating the 3 domains and the signal sequence (in grey) as well as the beginning and the end of each domain marked as numbers on the line above the figure. The percentage of the serine (Ser) and threonine (Thr) content is given for each domain. The internal repeats and the PA14 domain are shown in different colors and their length is marked as numbers on the line under the domains. The amino acid sequence of the 135 nt repeat is shown. This repeat contains a sequence with high β-aggregation potential, which is underlined (Ramsook 2009). In the *N*-terminal domain, it was shown that the amino acids ranging from 197 to 240 are important for binding to sugar (Kobayashi et al. 1998). Therefore, this region was zoomed in at the amino acid level. The pentapeptide involved in glycan specificity is shown in bold (Zupancic et al. 2008) and the amino acids contributing to sugar recognition have bigger size (Kobayashi et al. 1998).

Fig. 3 | (a) Structure of the anthrax toxin protective antigen, revealing 5 domains (PDB 1ACC). The PA14 domain (amino acids 14-150) is part of domain 1 shown in blue. PA14 domain is part of domain 1 and is composed of beta sheets (Petosa et al. 1997). **(b)** Model proposed for the tertiary structure of the *N*-terminal domain of Flo1p based on a sequence alignment with the anthrax toxin protective antigen, using the Phyre server (Kelley and Sternberg 2009). Only amino acids 85-261 from N-Flo1p were considered. The pentapeptide responsible for the binding event as described by Kobayashi and co workers (1998), is shown in blue. **(c)** The tandem repeat region comprises multiple amino acid repeats that are thought to be arranged in antiparallel-sheets. A model was made with ROSETTA and LINUS and this consistently predicted independently folded three-stranded antiparallel-sheet domains for each repeat of the Als5 protein (Frank et al. 2009).

Figure 1:

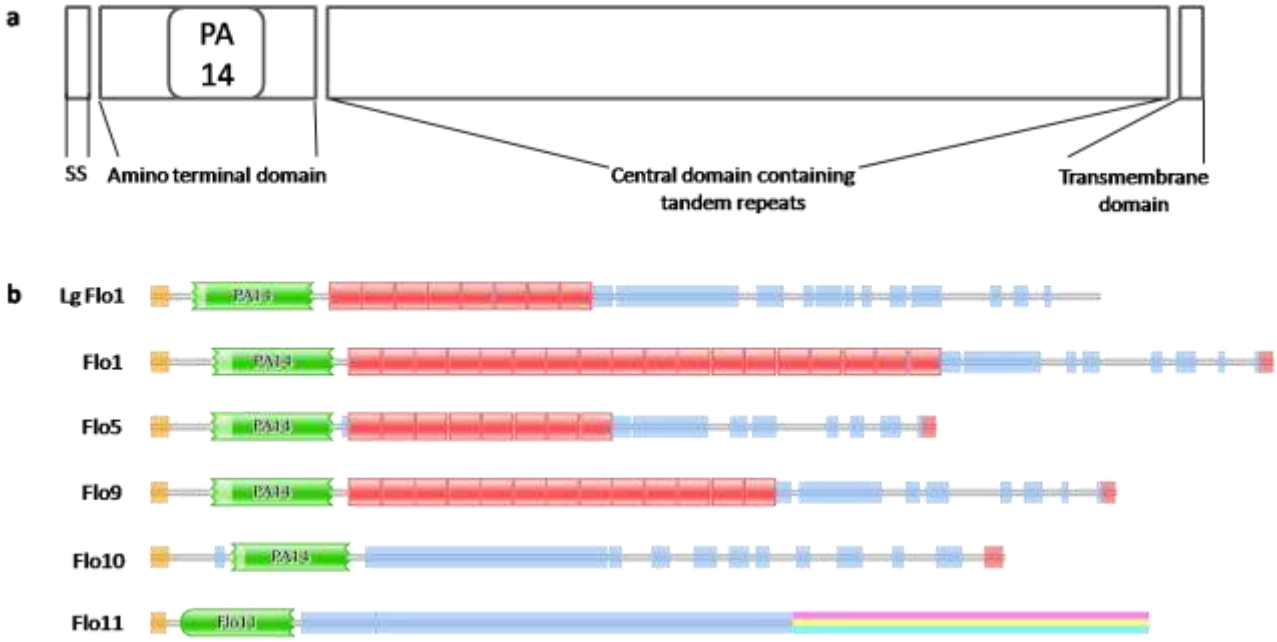
a
PA
14
SS
Amino terminal domain
Central domain containing
tandem repeats
Transmembrane
domain
b
Lg Flo1
Flo1
Flo5
Flo9
Flo10
Flo11

Figure 2:

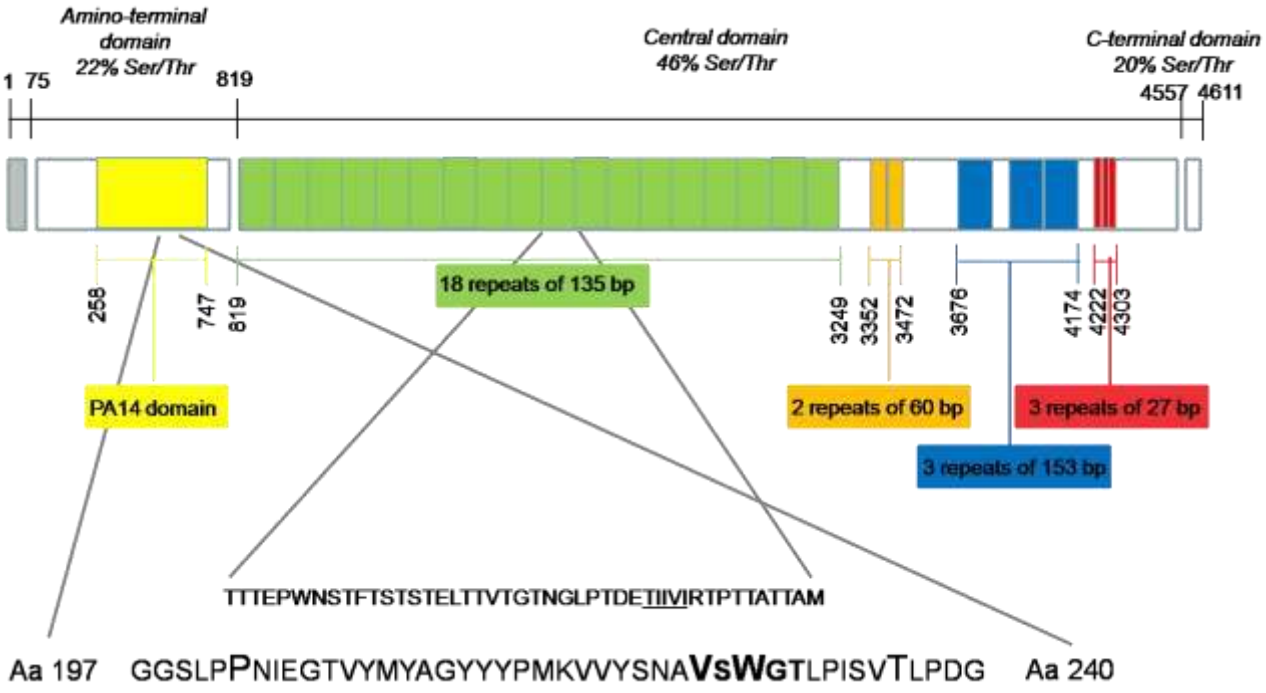
Amino-terminal domain
22% Ser/Thr
Central domain
46% Ser/Thr
C-terminal domain
20% Ser/Thr
1
75
819
4557
4611
258
747
819
18 repeats of 135 bp
3249
3352
3472
3676
4174
4222
4303
PA14 domain
2 repeats of 60 bp
3 repeats of 27 bp
3 repeats of 153 bp
TTTEPWNSTFTSTSTELTTVTGTNGLPTDETIIVIRTPTTATTAM
Aa 197
GGSLPPNIEGTVYMYAGYYYPMKVVYSNAVSWGTLPISVTLPDG
Aa 240

Figure 3: