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Ciclo XXVI

Phenomic analysis and cell wall remodulation of *Saccharomyces cerevisiae* flor strains in the presence of different nitrogen sources

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Abstract

Flor yeasts of *Saccharomyces cerevisiae* are the main actors in the biological ageing of Sherry wines by developing an air-liquid biofilm, called *velum* at the surface of wine at the end of fermentation. The air-liquid biofilm occurs due to a complex mechanism which is similar to many microbial biofilms. The *velum* phenotype is mainly regulated by the expression of a highly O-glycosylated cell wall protein Flo11 which by turn contributes to the hydrophobicity and electrostatic charge of the cell wall which are crucial for unspecific interactions and adhesion capacity of yeasts. In this study we characterized the interactions of flor yeasts with a series of nitrogen compounds known for their hydrophobicity and/or charge. We find that, different flor yeast strains characterized by different degree of functional *FLO11* are diversely affected in terms of biofilm formation and adhesion capacity when interact with different nitrogen sources. The positive role of the synthetic hexapeptide PAF26 in enhancing the biofilm formation was first discovered. On the contrary, cationic amino acids were able to inhibit biofilm formation and adhesion ability. Results together highlight on the involvement of the Flo11p in hydrophobic and electrostatic interactions and open to new investigations, addressed to the control of microbial adhesion and biofilm formation.

To my beloved Parents and Brothers

Marc Bou Zeidan, Phenomic analysis and cell wall remodulation of *Saccharomyces cerevisiae* flor strains in the presence of different nitrogen sources, Tesi di dottorato in Biotecnologie Microbiche Agroalimentari, Università degli studi di Sassari.

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INTRODUCTION

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Yeast have been used spontaneously by human from approximately 10.000 B.C in wine and bread production (Piskur *et al.*, 2006), and recently also as producers of bio-ethanol, vitamins and pharmaceutical products like hormones and protein drugs. Despite these "good" purposes, several yeast species are pathogenic to animals and plants. The most studied yeast is *Saccharomyces cerevisiae*, a unicellular eukaryotic, very well known for its ability to ferment, under fully aerobic conditions, glucose to ethanol and carbon dioxide, thus been classified as a Crabtree positive yeast (Gelinas, 2009). Besides its common usage in wine production, brewing and baking products, *S. cerevisiae* is intensively used as a model system to study numerous cellular processes such as eukaryotic gene regulation and evolution, cell cycle, metabolic pathways, apoptosis and ageing (Giaever *et al.*, 2002; Petranovic and Nielsen, 2008).

Yeasts also organize into multicellular communities which is critical for their surviving in harmful environments. Natural *Saccharomyces cerevisiae* strains form complex structured colonies, which share many typical properties with biofilms infections of *Candida albicans* and *Candida glabrata* strains in the human body (Lionakis and Netea, 2013). Microbial biofilms are widespread in nature and can develop on biotic or abiotic surfaces and are enclosed in an extracellular matrix, the whole forming a complex three-dimensional architecture. The formation of biofilms, whether bacterial or fungal, consists of cell-cell aggregation, adhesion of cells to a surface, initial formation of colonies and secretion of extracellular polymeric matrices (Stoodley *et al.*, 2002; Mowat *et al.*, 2009). Adhesion and biofilm formation mechanisms are considered as an adaptive response to adverse environmental conditions (Hall-Stoodley and Stoodley, 2009; Ning *et al.*, 2013).

At the biotechnological level, the multicellular behavior of *S. cerevisiae* is applied in the sedimentation and the removal of the biomass at the end of fermentations processes (Verstrepen and Klis, 2006; Bauer *et al.*, 2010). Flor yeasts are natural *S. cerevisiae* strains, and are the main actors in the biological ageing of Sherry and

Sherry-like wines. In Sardinia, sherry-like wine is produced traditionally, and flor yeasts found are endogenous (ex: Vernaccia di Oristano) (Budroni *et al.*, 2000; Budroni *et al.*, 2005; Zara *et al.*, 2008).

1. Peculiarities of flor yeasts

Flor yeasts belong to *S.cerevisiae* species and have the ability of cell-cell and cell-surfaces aggregation, invasive and pseudohyphal growth and in particular, they can form biofilm at air-liquid interfaces (Fig.1). The dimorphic unicellular to multicellular growth swift occurs when flor yeasts are exposed to critical environmental conditions, such as depletion of favorable carbon and/or nitrogen sources. Indeed, in wine making, at the end of alcoholic fermentation, when nutritional resources are depleted, the further growth becomes dependent on the access to oxygen (Freiberg and Cruess, 1955; Zara *et al.*, 2005; Fidalgo *et al.*, 2006). The formation of the air–liquid biofilm allows the yeast cells to grow aerobically thru the uptake of preferably glycerol, acetic acid and ethanol respectively, as non fermentable carbon sources (Zara *et al.*, 2010).

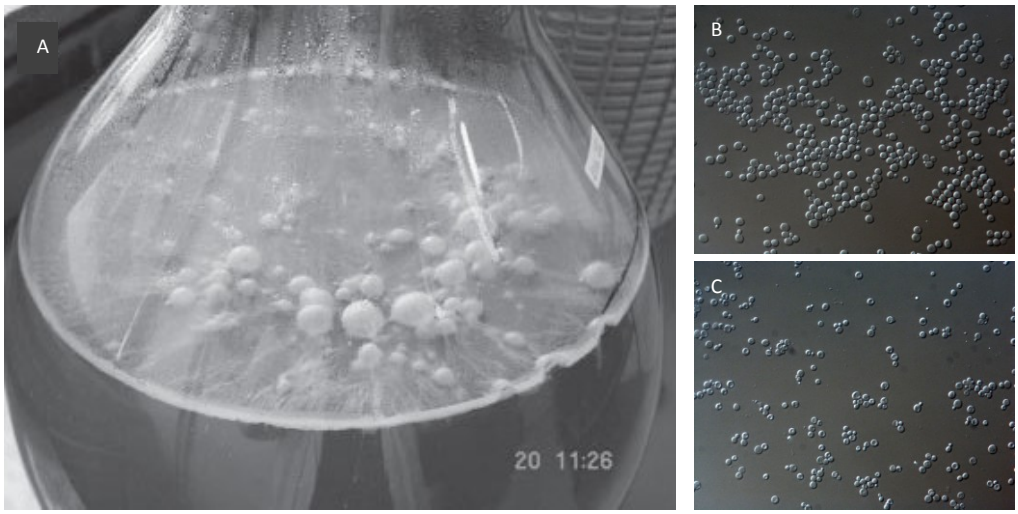

FIG 1 A) Air-liquid biofilm formation by *S. cerevisiae* flor strain on Vernaccia sherry-like wine (Zara *et al.*, 2005). B) Microscopic view on the multicellular tendency of flor yeasts. C) Microscopic view on the unicellular tendency of laboratory yeast S288c.

The formation of the air–liquid biofilm allows the yeast cells to grow aerobically thru the uptake of preferably glycerol, acetic acid and ethanol respectively, as non fermentable carbon sources (Zara *et al.*, 2010). This aerobic growth induces the substantial production of acetaldehyde, and activates complex metabolisms of amino acids, such as Proline, which are used directly as nitrogen sources or electron acceptors (Mauricio *et al.*, 2001). Moreover, studies evidenced a high accumulation of unsaturated long-chain fatty acids in flor yeast cells in fermentation phase, which subsequently increases in biofilm formation phase. The oleic acid was shown to be the most copious in flor yeasts (Mannazzu *et al.*, 2008; Marques *et al.*, 2008). It was suggested that the excessive presence of these unsaturated fatty acids is related to the enhanced ethanol tolerance and hydrophobicity of flor yeasts, and to the biofilm formation process in general, by increasing the flor yeast cell density, which enable them to grow on air-liquid surfaces (Zara *et al.*, 2012).

The emergence of molecular techniques has greatly improved the genetic characterization of flor yeasts. Restriction analysis of the intergenic region of 5.8S rDNA has identified a 24 base pair deletion in all analyzed flor strains (Esteve-Zarzoso *et al.*, 2001). In the last decade, a series of studies uncovered the key factor in biofilm formation. These studies revealed that the *FLO11* gene, which encodes for a cell wall mannoprotein, Flo11p is the main factor in biofilm formation and multicellular growth. Besides to *FLO11* gene, it was shown that the whole cell wall is involved in the multicellular response to threatening environments (Cid *et al.*, 1995; Galitski *et al.*, 1999; Reynolds, 2001; Zara *et al.*, 2005; Dranginis *et al.*, 2007).

2. Cell wall role and molecular structure

The cell wall is a principal compartment in yeast and is largely involved in the dimorphic shift of the cell (Verstrepen *et al.*, 2004). It has main functions toward mechanical and osmotic stresses. *i)* It provides protection from osmotic shock by limiting the influx of water to avoid bursting and to maintain cell the intracellular water activity (Hohmann, 2002); *ii)* it is essential for the strength and elasticity required to maintain the shape of the cell, as well as it provide an effective barrier against sheer and compression forces (Klis *et al.*, 2006); *iii)*it regulates the permeability of solutes (Lipke and Ovalle, 1998).

Apart of the protective role of the cell wall, it also serves as a tool for cell-cell and cell-environment interactions. In fact, one of the most important functions of the cell wall is the ability to adhere to other cells, biotic and abiotic surfaces (Zara *et al.*, 2005). This includes adhesion of sexual partner cells as well as vegetative adhesion. Sexual adhesion of budding yeast is well understood and is mediated by cell-type-specific adhesins called agglutinins, which are produced by mating partners after exchange of pheromones and confer cell–cell adherence by high-affinity heterotypic protein–protein interactions (Lipke and Kurjan, 1992; Chen and Thorner, 2007; Dranginis *et al.*, 2007). On the other hand, the vegetative adhesion includes cell-cell and cell-surfaces bindings, flocculation, biofilm formation and multicellular growth, which leads to an increased resistance to unfavorable chemical and physical conditions (Guo *et al.*, 2000; Kojic and Darouiche, 2004; Stovicek *et al.*, 2012).

All these crucial functions attributed to the cell wall reflect its complexity and high specificity. Therefore, yeast cells use considerable energy in the construction of the cell wall, which comprises some 10–25% of the cell mass depending on growth conditions (Smits *et al.*, 1999; Aguilar-Uscanga and Francois, 2003; Levin, 2011). It is mainly composed of chitin, β-glucans and mannoproteins arranged into two layers.

The inner layer is a load-bearing polysaccharides, acting as a scaffold for a protective outer layer of mannoproteins that extend into the medium. The mechanical strength of the wall is mainly due to the inner layer, which consists of β-1,3-glucan and chitin, and represents about 40-50% of the wall dry weight. The outer layer, which consists of heavily glycosylated mannoproteins emanating from the cell surface, is involved among others in cell-cell recognition events (Cappellaro *et al.*, 1994; Teunissen and Steensma, 1995; Reynolds, 2001). It also limits the accessibility of the inner part of the wall and the plasma membrane to foreign enzymes such as cell wall-degrading enzymes in plants tissues (Fig.2) (de Nobel *et al.*, 1990; Lipke and Ovalle, 1998; Klis *et al.*, 2002; Yin *et al.*, 2005). This macromolecular confirmation confers an electron-transparent internal layer and an electron-dense outer layer (Osumi, 1998).

2.1. Chitin. Chitin is a linear, insoluble homopolymer composed of β-1,4-linked subunits of the acetylated amino sugar N-acetylglucosamine. After cellulose, chitin is the second most abundant polymer found in the biosphere. It is the main compound of invertebrate exoskeletons and an essential structural component of the cell walls of yeast and filamentous fungi (Rabea *et al.*, 2003; Tharanathan and Kittur, 2003).

Even though it is considered as minor component of the yeast cell wall, it is structurally important for cell surviving. Chitin forms in normal growth conditions 1–2% of the yeast cell wall by dry weight (Klis, 1994; Klis *et al.*, 2002) whereas the cell walls of filamentous fungi, such as *Neurospora* and *Aspergillus,* are reported to contain 10–20% chitin (de Nobel *et al.*, 2000).

In *Saccharomyces cerevisiae*, the synthesis of chitin is mediated by expression of chitin synthases *CHS1*, *2* and *3* genes, which encode for an integral membrane enzymes that catalyze the transfer of N-acetylglucosamine from uridine diphosphate (UDP)-N-acetylglucosamine to a growing chitin chain (Roncero, 2002).

Chs1p functions in regenerating chitin polymers lost during cytokinesis and Chs2p is required for the formation of the primary septum within the dividing yeast cell (Shaw *et al.*, 1991; Latge, 2007). Chs3p generates approximately 80–90% of the total cellular chitin and its activity includes the *i)* synthesis of the bulk chitin of the cell wall, *ii)* increase of chitin synthesis as a response to cell wall stress, *iii)* chitin ring formation during bud emergence as well as *iiii)* the chitin linked covalently to the β-1,3-glucan fraction of the cell wall, and particularly, to β-1,6- glucan, as a response to certain cell wall stress (Bulawa, 1992; Kollar *et al.*, 1995; Latge, 2007).

Mutants affected in the Chs3p chitin synthase have vastly reduced chitin levels and rates of growth, accompanied by defects in cell wall integrity. The deletion of all three genes results a lethal phenotype, due to a high disorder in cell wall, cell malformation and osmotic instability, demonstrating that chitin is an indispensable component of the cell wall of *S. cerevisiae* (Bulawa, 1993). This is appropriated to the inter-chain hydrogen bonding between chitin microfibrils which forms polymers with high tensile strength and contribute to the overall integrity of the cell wall. Such hydrogen bondings occur mainly between the newly formed polymers of chitin, leading to the formation of microfibrils and subsequent crystallization of chitin in the extracellular space immediately adjacent to the plasma membrane. In yeast and filamentous fungi, this occurs mostly in sites of active growth and cell wall remodeling such as the bud tip during polarized growth and the bud neck during cytokinesis, cell wall synthesis and hyphal apex areas (Bowman and Free, 2006; Latge, 2007).

2.2. β-glucans. β-glucans are naturally occurring polysaccharides and are prevalent among the *Saccharomyces cerevisiae* cell wall by β-1,3 or β-1,6-links. β-1,3-glucans consist of chains with a degree of polymerization of almost 1,500 glucose units/chain, found integrally in a variety of bacteria, plants and fungi. β-1,3-glucans share a coiled

spring-like structure that confers elasticity and tensile strength to the cell wall. They constitute the 30-45% of the dry weight of the cell wall and 80-90% of the inner part of the cell wall. β-1,3-glucans network are characterized by their non-reducing ends, which are cross-linked to the reducing ends of chitin and β-1,6-glucans respectively at the lower and outer sides of the network (Kollar *et al.*, 1997; Lesage and Bussey, 2006). β-1,6-glucans represent the 10–15% of the total yeast cell wall polysaccharides, with an average size of 350 glucose units/chain. β-1,6-glucan polymers are amorphous in structure, and acts as a flexible join by forming covalent cross-links to β-1,3-glucan, to chitin and most importantly to cell wall mannoproteins (Kollar *et al.*, 1997; Shahinian and Bussey, 2000; Lesage and Bussey, 2006).

The synthesis of β-1,3-glucans occurs in the plasma membrane through the 1,3-β-D-glucan synthase (GS) enzymatic complex. The GS complex consists of a catalytic subunit and a regulatory subunit, both of which are essential for the complex activity. The regulatory subunit is a GTP-binding protein encoded by *RHO1*, which also regulates protein kinase C and acts as an activator responsive to cell morphogenesis signals (Mazur and Baginsky, 1996; Qadota *et al.*, 1996). *FKS1* and *GSC2* encode the catalytic subunit with the activity of UDP-glucose:1,3-β-D-glucan 3-β-glucose transferase, which catalyzes the transfer of a glucose moiety from UDP-glucose to the glucan chain. *FKS1* is mainly expressed during vegetative growth, whereas *GSC2* is induced under starvation, during sporulation, and in response to mating pheromones. Single mutation of each of these genes is not lethal, but the double null mutant *fks1 gsc2* is not viable, indicating that the GS function is essential.(Douglas *et al.*, 1994; Mazur *et al.*, 1995; Qadota *et al.*, 1996; Lesage *et al.*, 2004; Levin, 2011). Regarding the β-1,6-glucans, the mechanism and the genes involved in their synthesis seem to be more complex respect to other polysaccharides and still not well understood (Cabib and Arroyo, 2013).

Many genes throughout the secretory pathway to the cell wall were found to be involved indirectly in β-1,6-glucan synthesis, which has prevented identification of the gene(s) encoding the β-1,6-glucan synthase (Page *et al.*, 2003). Interestingly, the β-1,6-glucan formed in *Knh1* and/or *kre9* mutants is significantly smaller than its wild type equivalent and have an abnormal structure (Brown and Bussey, 1993; Klis *et al.*, 2006).

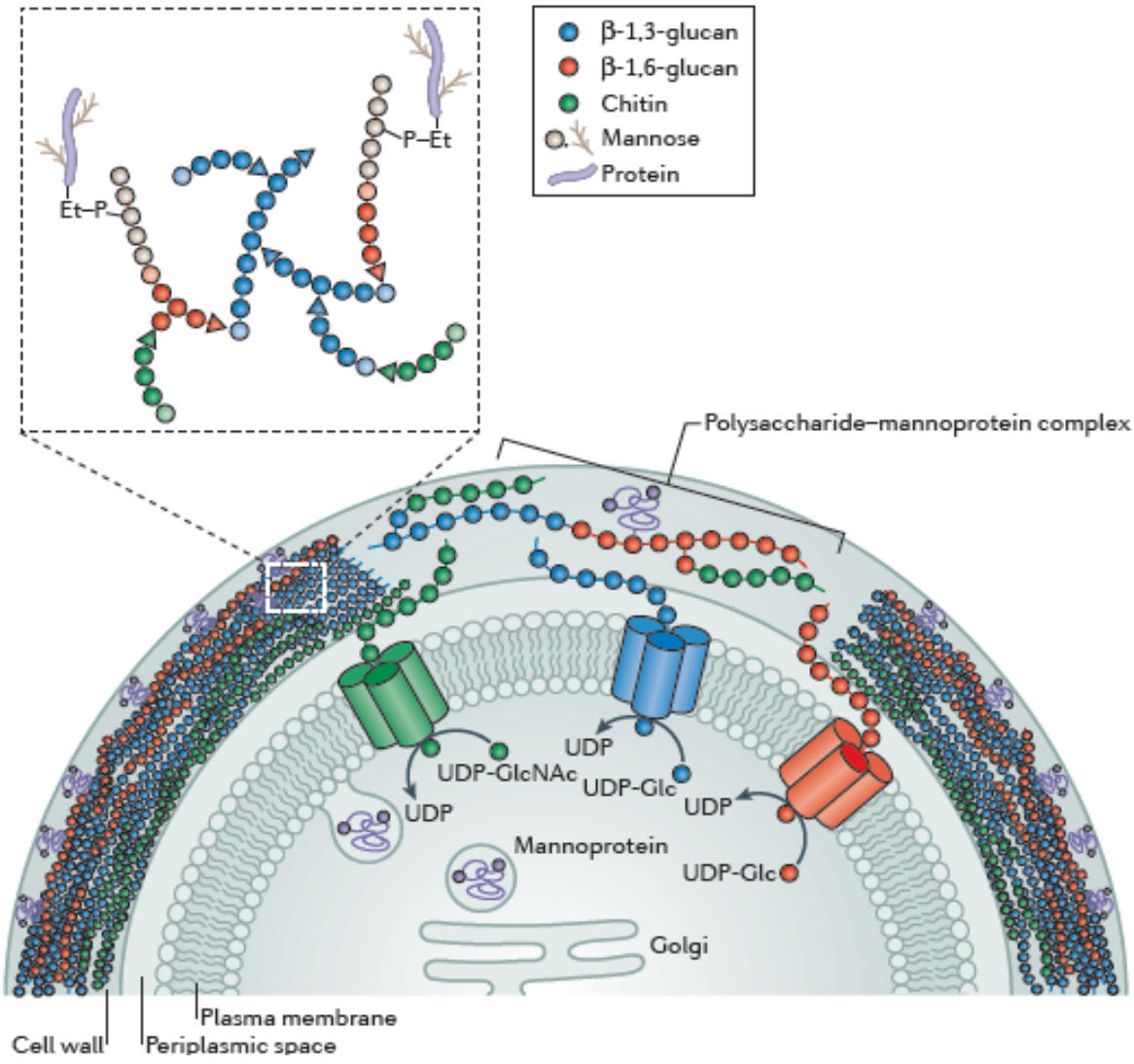

FIG 2 General structure and cell wall assembly in *Saccharomyces cerevisiae*. After synthesis in the ER and Golgi, mannoproteins are packed in vesicles and transported to the plasma membrane, whereas polysaccharides, such as chitin, β-1,3-glucan, and hypothetically β-1,6-glucan, emerge from the plasma membrane into the periplasmic space. Chitin is attached to β-1,6-glucan through a β-1,3-linked glucose branch and to the non-reducing terminal glucose of β-1,3-glucan (in the box, lighter-coloured circles represents non-reducing ends and reducing ends are shown as triangles). Mannoproteins are linked to β-1,6-glucan by the glycosylphosphatidylinositol remnant. The Pir proteins which are attached in the inner part of the wall to β-1,3-glucan through an alkali-labile linkage are not shown. Et, ethanolamine; Glc, Glucose; GlcNAc, *N*-acetylglucosamine; P, phosphate (Cabib and Arroyo, 2013).

3. Cell wall mannoproteins.

To the cell wall β-glucans are linked a varied set of highly glycosylated mannoproteins which represent the electron-dense and fibrillar outer layer of the wall (Lesage and Bussey, 2006). Cell wall mannoproteins form the 30-50% of the cell wall dry weight. In fact, the actual protein content is about 4–5%; the remaining mass is from protein-linked, mannose-containing side-chains. These carbohydrate side chains are added to cell wall proteins by N-glycosylation or O-mannosylation which are the two types of oligosaccharidic post-transcriptional protein modifications that have been described in *S.cerevisiae* so far. Both N- and O-linked carbohydrate side chains of yeast mannoproteins contain phosphodiester groups (Jigami and Odani, 1999). The outer surface of yeast contains abundant negatively charged groups at a pH ≥3.0 (Klis *et al.*, 2007).

N-glycosylated cell wall proteins receive an oligosaccharide through an N-glycosidic bond between a GlcNAc and an asparagine residue in the ER, than extensively mannosylated in the Golgi, with a final structure of α-1,6-linked mannose chain of up to 50 mannose residues extending from the N-glycan core and to which are attached shorter chains of α-1,2 residues terminating in α-1,3-linked mannose residues, forming a highly branched structure containing as many as 200 mannose residues (Dean, 1999; Dempski and Imperiali, 2002).

O-mannoslyation consists of attaching by glycosidic bonds, short oligomannose chains of up to five mannose units, to the hydroxyl group of serine and Threonine residues of cell wall proteins. The first two mannose residues being α-1,2 linked and subsequent ones α-1,3 linked. Mannosyltransfer takes place in the ER and is catalyzed by a conserved family of protein O-mannosyltransferases, encoded by *PMT* genes family (Loibl and Strahl, 2013). Despite the small size of the O-linked chains, the abundance of cell wall proteins with rich tandem repeats of serine and Threonine residues leads to heavy O-mannosylation, and so the number of O-linked

chains per protein can be high and the amount of O-linked mannose in the cell wall significant (Strahl-Bolsinger *et al.*, 1999).

The wall of *S. cerevisiae* contains nearly 20 different covalently linked mannoproteins (Yin *et al.*, 2005). The majority of cell wall proteins responsible for vegetative adhesions are GPI-anchored and are thus indirectly linked to the β-1,3-glucans network thru a glycosidic linkage with branched β-1,6 glucans (Kollar *et al.*, 1997; Cabib and Arroyo, 2013). In addition, a minor group of cell mannoproteins is directly linked to the β-1,3-glucan fibril chain through an unidentified linkage which is sensitive to mild alkali. These proteins are called ASL (alkali-sensitive linkage) and include the *PIR* genes family of cell wall mannoproteins (Pir, proteins with internal repeats) (De Groot *et al.*, 2005). ASL and GPI mannoproteins are distributed throughout the inner and the outer cell wall skeletal layer respectively, which is consistent with their being directly connected to β-1,3-glucan and β-1,6-glucan macromolecules (Kapteyn *et al.*, 1999b). Last, some cell wall proteins, such as Aga2p, are not directly linked to cell wall polysaccharides but are linked to other cell wall proteins through disulphide bridges (Cappellaro *et al.*, 1994; Moukadiri *et al.*, 1999).

3.1. GPI anchored cell wall mannoproteins. GPI-anchored proteins are conserved among yeast, protozans, plants and animals (Ferguson, 1999). They are proteins post transcriptionally modified with glycosyl-phosphatidyl-inositol. In *S.cerevisiae*, around 50 genes encode for GPI-anchored proteins, found attached to the plasma membrane or as an intrinsic part of the cell wall. Also, they are functionally diverse and important for signal transduction, cell-cell interaction, cell adhesion and host defense (Kapteyn *et al.*, 1999a; Ikezawa, 2002). *FLO* genes family involved in cell-cell and cell-surface adhesions are GPI-anchored proteins (Cid *et al.*, 1995; Teunissen and Steensma, 1995). The structure of a GPI anchor consists of an ethanolaminephosphate (EthN-P) linked to the Manα1-2Manα1-6Manα1-4GlcN tetrasaccharide, which in

turn is linked to *myo*14 inositol in α1-6 linkage. On the ER membrane, the protein C-terminal is covalently linked to EthN-P, by the putative GPI-protein transamidase complex (Caro *et al.*, 1997). Than GPI mannoproteins are directed to cell surface by their N-terminal through the secretory pathway. This modification enables GPI contained proteins either the anchor the membrane by incorporation of the GPI moiety into the lipid bilayer, or by conferring covalent attachment to the β-1,6-glucan of the cell wall, which is in turn, linked glycosidically to β-1,3-glucan or to chitin (Frieman and Cormack, 2004; Verstrepen and Klis, 2006; Brückner and Mösch, 2012).

3.2. Cell wall Adhesins. Multicellular growth of *S. cerevisiae* has been observed since the 19th century, but the molecular basis for adhesion remained unclear until the isolation of the involved genes and proteins. To date, molecular techniques helped to isolate and identify at least eight different adhesins that confer adhesions, from different industrial and laboratory strains. These adhesins are all GPI-anchored cell wall mannoproteins, sharing similar molecular core structure with some differences between adhesins. Mainly, the core structure consists of a C-terminal GPI-anchor, a large and highly O-mannosylated central domain rich in serine and threonine, and an N-terminal secretion signal domain (Teunissen and Steensma, 1995). These adhesins are composed mainly of *i)* sexual adhesins families *AGA1, AGA2* and *FIG2* responsible of adhesions during mating (Lipke and Kurjan, 1992; Cappellaro *et al.*, 1994; Zhang *et al.*, 2002; Chen and Thorner, 2007; Dranginis *et al.*, 2007) *ii)* and vegetative adhesions family, divided into sugar sensitive adhesins *FLO1, FLO5, FLO9* and *FLO10* responsible of flocculation and cell-cell interactions (Goossens *et al.*, 2010) and sugar-insensitive adhesin *FLO11* also involved in adhesion to biotic and abiotic surfaces, biofilm formation and interaction with hosts (Dranginis *et al.*, 2007; Van Mulders *et al.*, 2009). While mating adhesions arise as a pheromone sensing response, vegetative adhesions are reactions to affront environmental stresses.

Moreover, the genetic diversity and background between distinct *S. cerevisiae* strains and the different environmental conditions, highly influence the expression rate of *FLO* genes and on the potential adhesion of flocculins, which create very different phenotypes (Zara *et al.*, 2009a). Microbial cell development and survive along with their metabolic activity are strongly affected by cell adhesion, which represents the initial step in biofilm formation (Baror, 1990; Dague *et al.*, 2012).

3.3. Sugar-sensitive flocculins and flocculation. Genes which encode these flocculins are *FLO1 FLO5, FLO9* and *FLO10* and are carried in subtelomeric loci at chromosomes *I*, *VIII*, *I* and *XI* respectively (Teunissen and Steensma, 1995). Generally, in a sugar-depleted medium, *S. cerevisiae* encodes for adhesins Flo1p, Flo5p, Flo9p and Flo10p, which contain a lectin-like domain on their N-terminal, and can bind to mannose-containing carbohydrate structures present at the cell surface of neighboring cells, causing flocculation (Guo *et al.*, 2000; Govender *et al.*, 2008; Van Mulders *et al.*, 2009). This phenomenon was extensively studied in industrial *S. cerevisiae* strains because its importance as an environmental friendly way to sediment and remove yeast cells at the end of fermentation processes in the production of beer, wine, ethanol and biodiesel (Verstrepen and Klis, 2006; Bauer *et al.*, 2010). The classic definition of flocculation is the asexual, reversible and Ca^{2+} - dependent aggregation of thousands of vegetative cells into flocs (Lindquist, 1952; Bony *et al.*, 1997). Recent molecular and bioinformatic studies suggest that the N-terminus of sugar-sensitive flocculins contain approximately 250 amino acids, with highly similar sequence identities and conserved domains, such as Ca^{2+} and carbohydrates binding motifs, and a conserved PA14-like domain (Fig 3) (Veelders *et al.*, 2010).

PA14 domain is shared by a wide variety of bacterial and eukaryotic proteins, which include many glycosidases, proteases, amidases and bacterial toxins such as anthrax protective antigen (PA), and are involved in carbohydrate binding (Rigden *et al.*, 2004). Moreover, the study suggested that the particular conformation of the N-termimus implicates a lectin mode of carbohydrate binding via Ca^{2+} mediated recognition of the 2′- and 3′-hydroxyl groups, where the calcium ion is bound between sugar and protein in a distorted pentagonal bi-pyramidal coordination.

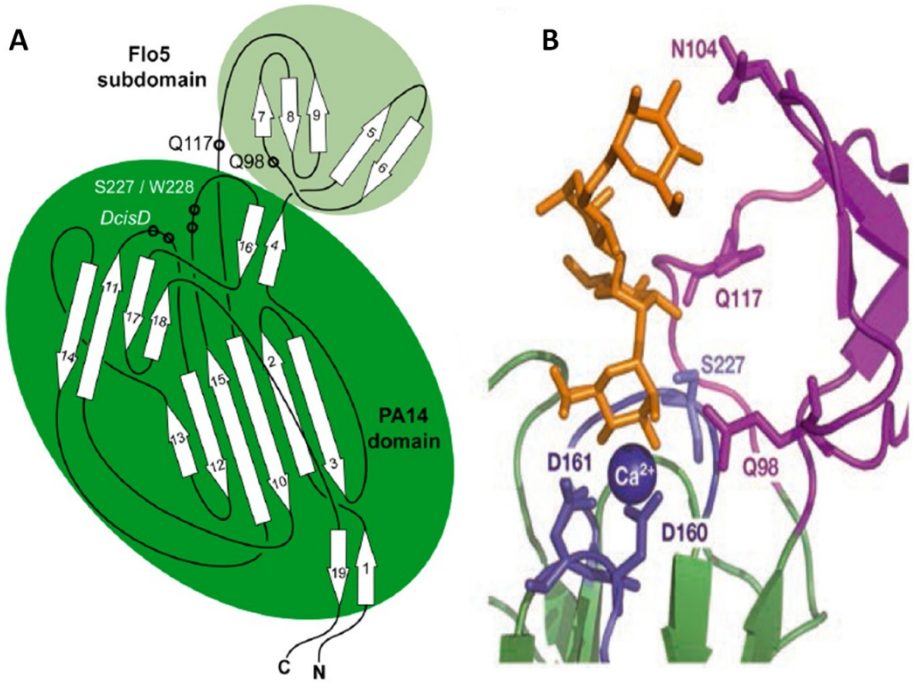

FIG 3 The novel flocculation molecular basis. These designs represent the novel molecular mode of action of flocculation, based on studying the N-terminus of Flo5p. A) Protein topology diagram showing the PA14-like domain (green) and the five-stranded Flo5 subdomain (light green). B) The Flo5-mannose complex and the conserved domains involved in the interaction (Veelders *et al.*, 2010).

In details, two calcium binding sites were noted in the PA14-like domain, and identified as Carbohydrate Binding Loops, CBL1 and CBL2 which consist of a conserved Asp160-Asp161 motif and the two carbonyl groups the side chain of Asp24 respectively. In parallel, the conserved motif Val226-Ser227-Try228-Gly229-Thr230, and precisely the residue Try228, recognizes the 2-hydroxyl group of

mannose and not of glucose or other sugars (Fig.3) (Kobayashi *et al.*, 1998; Goossens *et al.*, 2010; Veelders *et al.*, 2010).

Similar conserved motifs in the human pathogenic *Candida glabrata*, on the N-terminus domain of the epithelial adhesin Epa1, were found to be functionally homologous to these motifs found in *S.cerevisiae* flocculins (Zupancic *et al.*, 2008; Ielasi *et al.*, 2012).

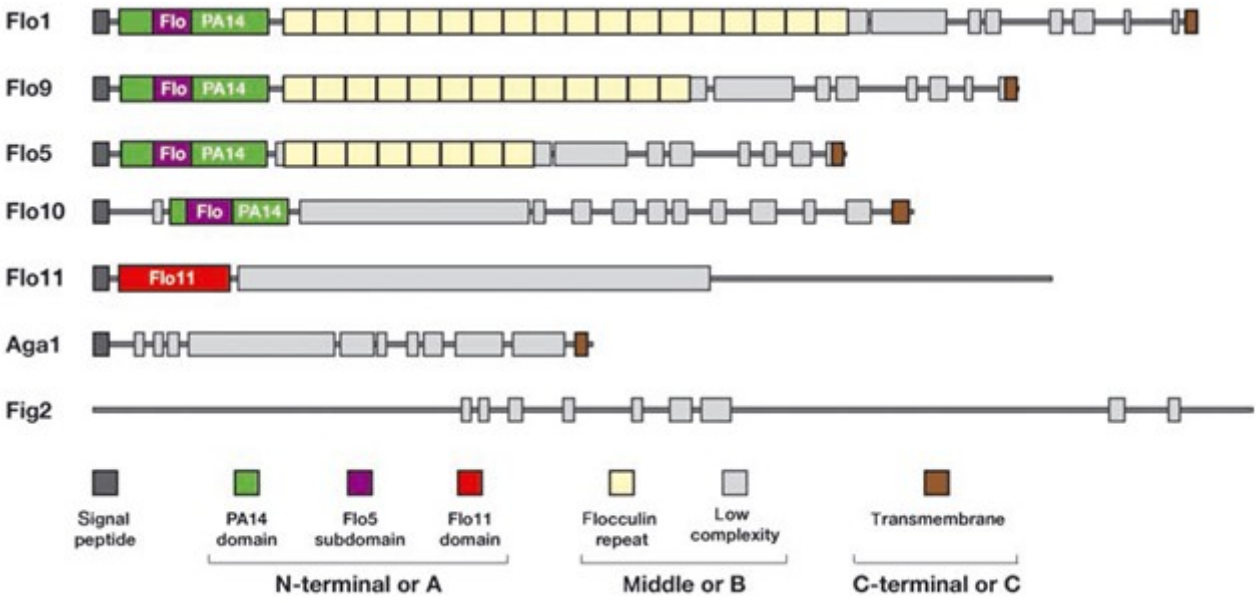

FIG 4 Domain organization of Saccharomyces cerevisiae adhesins. Proteins were analyzed using the Pfam protein families' database at http://pfam.sanger.ac.uk/ (Finn *et al.*, 2010). Known domains are shown in different colors. The broad partition into N-terminal (A), middle (B) and C-terminal (C) domains is indicated (Brückner and Mösch, 2012).

3.4. Sugar-insensitive flocculins. Unlike other flocculins, Flo11p is a mannose insensitive and its N-terminal domain was shown to handle homotypic interactions, with affinity to peptides instead of sugar, and exerts hydrophobic interactions between cells and abiotic surfaces, leading to biofilm formation (Reynolds, 2001; Goossens and Willaert, 2012b). At the molecular level, *FLO11* gene is located far away from telomere region on the chromosome *IX* and the N-terminus of Flo11p is less similar to the rest of flocculins. It doesn't possess a PA14 ligand-binding domain,

but contains a unique domain called Flo11 domain, covering residues 42-195 (160 amino acids) and responsible of homotypic interactions.

Finally, Flo11p N-terminal showed a high self-binding capacity, high O-glyosylation and its secondary structure contains almost 40% of β-sheets and 3% α-helices (Goossens and Willaert, 2012a). Despite its essential role in the multicellular growth phenotypes of *S. cerevisiae*, the real mode of action of this flocculin remains unclear and less studied, respect to other flocculins. In parallel, *FLO11* gene is being extensively studied at the genetic level, because it possesses the longest promoter in the *S. cerevisiae spp.* genome.

Therefore, many scientists study *FLO11* at different levels, from cell sensing of external environment to the signaling pathways, transcription regulation, epigenetic and post transcription, to arrive to the final product Flo11p and all the phenotypes related.

4. Genetic characteristics and regulation of *FLO11*

FLO11 gene has the largest promoter described in *S.cerevisiae* (≈ 3kb) (Steffen Rupp *et al.*, 1999). In general, this large promoter is shown to be a target for many regulatory pathways and transcription factors, which influence directly or indirectly the final phenotype. For now, it was uncovered the involvement of the cAMP-protein kinase A (PKA), Snf1-Nrg1/Nrg2, the mitogen-activated protein (MAP) kinase cascade as well as the Target of Rapamycin (TOR) signaling pathways as a response to glucose and/or nitrogen sources depletion (Kuchin *et al.*, 2002; Vinod *et al.*, 2008). *FLO11* gene expression was shown to be influenced by various transcription factors. At the level of flor yeasts of *S. cerevisiae*, the *FLO11* promoter carries several mutation points and a deletion of 111 bp, respect to laboratory strains, which was related to their high

level of expression of *FLO11*, leading to the formation of air-liquid biofilm (Fidalgo *et al.*, 2006; Zara *et al.*, 2009b).

4.1. *FLO11* signaling pathways and transcription factors. MAPK signaling pathway is conserved among all eukaryotes. In *S. cerevisae*, it is crucial for the response to environmental stresses. It regulates mating, filamentous and invasive growth and cell wall integrity, in response to pheromone, nutrient limitation and osmotic stress respectively (Elion, 2000; Granek and Magwene, 2010). When it comes to nutrients depletion, the MAPK pathway is known to activate the transcription factor Ste12-Tec1 complex that binds on the *FLO11* promoter. The GTP-binding protein Ras2 activates the Rho-type GTPase Cdc42, which along with Ste20 activate MAPK

cascade comprising of Ste11, Ste7 and Kss1. The MAPK Kss1 activates Ste12-Tec1 to

bind to the promoter of *FLO11* (Mosch et al., 1996) (Fig.5). In parallel, the MAPK Fus3, which is involved in mating, phosphorylates Tec1 and triggers rapid ubiquitin-mediated degradation of the transcription factor and inhibits the transcription of *FLO11* (Bao et al., 2004; Chou et al., 2004).

In addition to the MAPK cascade, the activation of *FLO11* gene as a response to nutrient depletion also requires a functional Ras-cAMP-PKA pathway. cAMP-PKA activates the transcription factor Flo8 which binds to a specific region on the *FLO11* promoter. In details, Ras2 and Gpa2 (nucleotide binding alpha subunit of Ras2 and other GTP-binding proteins) activate adenylate cyclase Cyr1 to synthesize cAMP, which in turn relieves the catalytic subunits Tpk1, Tpk2 or Tpk3 from the regulatory subunit Bcy1 (Tamaki, 2007). Tpk2 was shown to stimulate *FLO11* expression by activating Flo8 and inhibiting Sfl1, whereas Tpk1 and Tpk3 were shown to inhibit the synthesis of cAMP, by activating the repressing transcription factor Sfl1and as a result, the inhibition of *FLO11* expression (Gancedo, 2001; Sengupta *et al.*, 2007)

(Fig.5). Tpk1 phosphorylates Yak1 at high glucose levels (Zhu et al., 2000; Malcher et al., 2011), which targets Sok2 for binding and repression of the *FLO11* promoter (Borneman et al., 2006). At low glucose levels, this Tpk1 repression is relieved and *FLO11* activated.

Moreover, some studies demonstrated that both MAPK and cAMP-PKC pathways could be also triggered by Mep2, a high affinity ammonium permease, when low concentration of ammonium sulphate is available as nitrogen source (Lorenz and Heitman, 1998a). Genetic evidences also indicated the involvement of the Snf1 in connecting nitrogen starvation to *FLO11* expression by targeting the transcription factors Flo8, Sfl1, Nrg1 and Nrg2 (Lorenz and Heitman, 1998b, a; Van Nuland *et al.*, 2006). Studies also confirmed the contribution of the target of rapamycin complex (TORC1), which generally controls the nitrogen discrimination pathway (NDP) and senses theintracellular nutrients to regulate growth, in the signaling of *FLO11* (Schmelzle and Hall, 2000; Cutler et al., 2001; Vinod et al., 2008).

However, the connection between TOR and the *FLO11* promoter still not clear, but recent studies evidenced the involvement of the transcription factor Gln3 and its regulator Ure2, controlled by TOR pathway, in regulating positively the expression of *FLO11* and the filamentation, under nitrogen starvation (Lorenz and Heitman, 1998a, b; Cooper, 2002).Promoter analysis identified one upstream activation sequence and one repression site that confer regulation by amino acid starvation, which are mainly regulated by the Ssy1-Ptr3-Ssy5 (SPS) and General Amino Acid Control (GAAC) pathways (Braus, 2003; Brückner and Mösch, 2012). Further transcription factors with unknown mode of action as Mss11, Mga1, Phd1 and Haa1 were noted to control the expression of *FLO11*. Studies showed that Mss11 is required for the activation of *FLO11* by many other regulators including Tec1, Flo8, Phd1, Nrg1, Nrg2, Sok2 and Sfl1 (van Dyk *et al.*, 2005). In parallel, Haa1 transcription factor is required for adaptation of yeast to acidic stress (Aranda and del Olmo, 2004; Fernandes *et al.*, 2005). Even more, Mga1 and Phd1 were find to bind in vivo to

FLO11 promoter and to have a key role in the complex regulatory network for adhesion and filamentation (Borneman *et al.*, 2006).

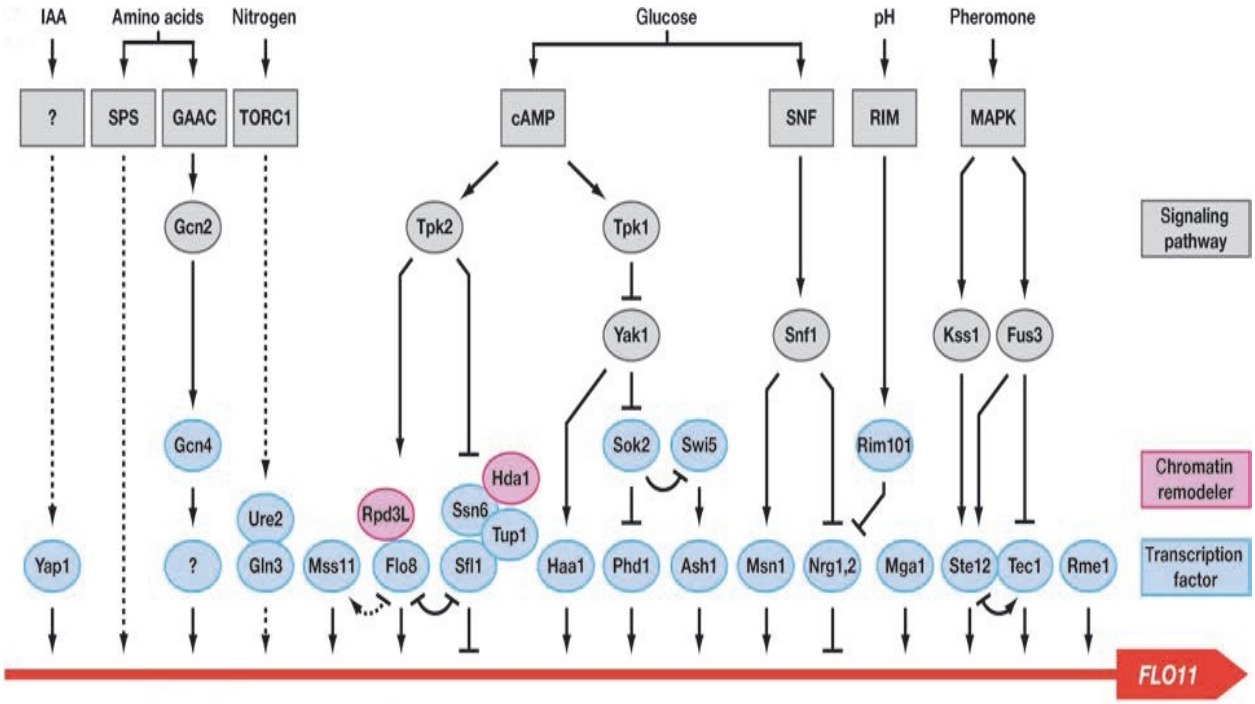

FIG 5 Regulation of *FLO11* expression. Wiring diagram showing the complex regulation of the *FLO11* promoter by conventional and epigenetic mechanisms. Arrows indicate positive regulation and inhibition is shown by bars. Different environmental stimuli and corresponding signaling pathways targeting *FLO11* are indicated at the top (Iaa= indole acetic acid; ?= unknown pathway). Downstream acting protein kinases are shown in gray. Chromatin remodelers found in the *FLO11* region are shown in magenta. Transcription factors targeting the *FLO11* promoter (red line) are shown in light blue (? = unknown transcription factor). The input of the different transcription factors is only shown schematically and does not correspond to the positions of known binding sites. (Bruckner and Mosch, 2012).

4.2. Elongation and tandem repeats. Besides the effects of expression signaling and/or silencing of *FLO11* gene on the biofilm formation process, it was also shown that the tandem repeats and the core length of its central domain, are also involved in affecting this phenotype. Indeed, the intragenic repeats are residues of Serine and Threonine, which in turn, are sites for O-mannosylation and N-glycosylation.

The central domain of *FLO11* gene varies between strains and results in a variation of the final product, in terms of gene length, glycosylation sites and high phenotype

variability (Verstrepen *et al.*, 2005) (Douglas *et al.*, 2007). Therefore, length variations in this single gene provide a combinatorial diversity, which may contribute to a very rapid adaptation to fluctuating environments (Fidalgo *et al.*, 2008).

Indeed, the cloning of two *FLO11* alleles from two different flor strains, of 3.1 and 5.0 kb length, into their appropriate locus in a lab strain BY4742 resulted in a significant correlation between biofilm-forming ability and *FLO11* length both in different and in the same genetic backgrounds (Zara *et al.*, 2009).

5. Adhesion, biofilm formation and phenotypic variation

- All these described peculiarities related to *FLO11* gene, along with the whole cell wall complex, generate a high variability in the multicellular process of *S. cerevisiae* yeasts. In *S. cerevisiae* flor yeasts, the innate diversity of *FLO11* and cell wall related genes background, generate variable adhesion ability between strains. These diversities comprise the *FLO11* gene length, expression level and the frequency of tandem repeats of serine and threonine residues resulting as O-mannosyled sites on the cell wall (De Groot *et al.*, 2005; Klis *et al.*, 2006). All these parameters influence crucially the physiochemical characteristics and the adhesion forces of yeasts cell wall, which affect directly the cell-cell and cell-surface adhesion and biofilm formation. These adhesion forces involve specific and non-specific interactions; flocculation phenotype is considered as a specific interaction due to lectin-carbohydrate binding motifs, meanwhile biofilm formation and adhesion to substrates are known to be non-specific interactions. Non-specific interactions include mainly electrostatic and hydrophobic interactions (van Oss, 1990; Bos *et al.*, 1999).

In aqueous solutions, the cell wall chemical groups are ionized and confer an electric charge to the cell surface which enables electrostatic interactions between the cell and the charged ions or molecules present in the external medium (Vu *et al.*, 2011). Electrostatic interactions are short-ranged repulsive or attractive interactions that occur between ions and charged surfaces and are subdivided into van der Waals interactions, permanent diapoles, and hydrogen bonding.

These individual subdivisions are relatively weak and transitory forces, but when aligned with each other and depending on the size of the molecule; the larger the molecule the stronger the force; electrostatic interactions could modulate significantly the total adhesion ability (Leckband and Israelachvili, 2001).

Hydrophobic interactions are considered as the predominant interactions mediating microbial binding to biotic and abiotic surfaces, of molecules dissolved in polar liquids. They are strong and long-ranged interactions, with a factor of 5-10 times efficiency respect to the rest of adhesion interaction forces (Vu *et al.*, 2011). Mechanisms behind hydrophobic interactions are still not well understood, even though, the classical description regarding the hydrophobicity is the rapid aggregation of non-polar liquid *i.e.* oil, when dissolved in polar liquid *i.e.* water, as the exclusion of hydrophobic molecules from water results in a squeezing effect of theses surfaces (Van Oss, 1995).

The mechanism leading to microbial aggregation is triggered by these adhesion forces when two cells start attracting and interacting thru the hydrophobic long-range forces, than reinforced by the electrostatic short-range forces, polar interactions, hydrogen bonds, and specific interactions (Van Oss, 1995; Holder *et al.*, 2007). In *S. cerevisiae*, accordingly with these interacting forces, flo11 mutant strains present a fall in cell wall hydrophobicity and electrostatic charge, which lead to a loss of their adhesion capacity and their ability to form biofilm (Reynolds, 2001; Zara *et*

al., 2005; Fidalgo *et al.*, 2006; Barrales *et al.*, 2008). This includes also flocculation mechanism in brewing, where strains with low cell wall hydrophobicity are less flocculants (Azeredo *et al.*, 1997; Holle *et al.*, 2012). Even more, at the biomedical level, the hydrophobicity of invasive *Candida* species is much higher than non invasive spp (Borghi *et al.*, 2011).

These physiochemical characteristics generate a high variability in adhesion and biofilm formation and structure. This is the reason why many recent studies are focusing on finding molecules which affect directly or indirectly the cell hydrophobicity, thru the interaction with cell wall components, mainly cell wall mannoproteins. The aim of such interactions studies is to intercept the hydrophobic ligands, which can lead to the collapse of adhesion and invasion capacity of yeast strains. Antimicrobial peptides (AMPs) are leader components in this approach. Many AMPs were found to modulate the adhesion and biofilm formation of some yeasts and fungi, thru hydrophobic and electrostatic interactions. For example, Histidine rich glycoproteins greatly inhibits *Candida albicans* biofilm formation thru binding and rupturing cell wall components (Rydengard *et al.*, 2008). In contrast, histatin-5 and LL-37 AMPs were shown to be sheded by the cell wall mucin Msb2 of *Candida albicans*, enhancing the cell resistance toward such AMPs (Szafranski-Schneider *et al.*, 2012).

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AIM OF THIS STUDY

The multicellular growth and the dimorphic shift of the unicellular *S. cerevisiae* are phenotypes of biotechnological interests. *FLO11* gene is directly involved in these phenotypes, by contributing fundamentally in the high hydrophobicity and electrostatic charge of the cell wall. These features particularly influence the adhesion ability and the air-liquid biofilm formation in *S. cerevisiae* flor strains.

In the frame of a project that aims to better elucidate the peculiar air-liquid biofilm and adhesion phenotypes of flor yeasts of *S. cerevisiae*, all the experimentations of this thesis were handled on a series of natural flor strains, previously isolated from different "Sherry-like" wineries in Sardinia, and characterized at *FLO11* genetic and phenotypic level. We focused in particular on the effect of nitrogen based molecules like peptides and amino acids, known for their hydrophobicity and/or charge, on the biofilm formation process of flor yeast strains. The aim of these studies is to characterize and better understand the cellular interactions of flor strains with these compounds, and to find molecules able to positively or negatively modulate the biofilm formation. This research has been divided into three parts:

i) Study of the effect of 3 antimicrobial peptides with different mode of action, on different flor strains, each with a previously characterized *FLO11* gene background. Antimicrobial peptides (AMP) are in general, high cationic and hydrophobic molecules; they can be natural, isolated from a vast number of organisms, from bacteria to humans or synthetic. These characteristics confer them the capability to bind to cell wall, permeate biological membranes and/or lyse living cells (Marcos and Gandia, 2009). Results showed the potential affinity between Flo11p and the synthetic hexapeptide PAF26 (Bou Zeidan *et al.*, 2013).

ii) High throughput analysis of nitrogen uptake and metabolism by flor strains with different backgrounds using the phenotype microarray © techniques. Results uncovered an interesting effect of Histidine contained dipeptides. Despite their high capacity to metabolize different nitrogen sources, flor strains were unable to grow on

dipeptides containing Histidine. To better understand this phenotype, we handled a series of subsequent analysis including an haploid flor strain and its *flo11* isogenic mutant. Results highlighted on the effect of L-histidine in inhibiting the adhesion and biofilm formation ability of flor strains. Results showed also a potential role of *FLO11* in regulating the cell surface net charge, which is in turn involved in cell-environment interactions.

iii) Study of the effect of different amino acids, on the cellular growth, biofilm formation and adhesion capacity of flor strains. Cellular growth screenings were handled in nutrient-rich or nutrient-poor media, in the presence of serial dilutions of amino acids with different physiochemical properties. Results showed that the presence of amino acids affect the biofilm formation and the adhesion capacity of flor yeasts.

CHAPTER I

Applied and Environmental Microbiology

FLO11 Gene Is Involved in the Interaction of Flor Strains of Saccharomyces cerevisiae with a Biofilm-Promoting Synthetic Hexapeptide

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FLO11 Gene Is Involved in the Interaction of Flor Strains of *Saccharomyces cerevisiae* with a Biofilm-Promoting Synthetic Hexapeptide

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Saccharomyces cerevisiae **"flor" yeasts have the ability to form a buoyant biofilm at the air-liquid interface of wine. The formation of biofilm, also called velum, depends on *FLO11* gene length and expression. *FLO11* encodes a cell wall mucin-like glycoprotein with a highly O-glycosylated central domain and an N-terminal domain that mediates homotypic adhesion between cells. In the present study, we tested previously known antimicrobial peptides with different mechanisms of antimicrobial action for their effect on the viability and ability to form biofilm of *S. cerevisiae* flor strains. We found that PAF26, a synthetic tryptophan-rich cationic hexapeptide that belongs to the class of antimicrobial peptides with cell-penetrating properties, but not other antimicrobial peptides, enhanced biofilm formation without affecting cell viability in ethanol-rich medium. The PAF26 biofilm enhancement required a functional *FLO11* but was not accompanied by increased *FLO11* expression. Moreover, fluorescence microscopy and flow cytometry analyses showed that the PAF26 peptide binds flor yeast cells and that a *flo11* gene knockout mutant lost the ability to bind PAF26 but not P113, a different cell-penetrating antifungal peptide, demonstrating that the *FLO11* gene is selectively involved in the interaction of PAF26 with cells. Taken together, our data suggest that the cationic and hydrophobic PAF26 hexapeptide interacts with the hydrophobic and negatively charged cell wall, favoring Flo11p-mediated cell-to-cell adhesion and thus increasing biofilm biomass formation. The results are consistent with previous data that point to glycosylated mucin-like proteins at the fungal cell wall as potential interacting partners for antifungal peptides.**

Yeast cells possess a remarkable capacity to adhere to inert surfaces, other cells, and tissues. These adhesion properties are of medical and industrial relevance (1). The human fungal pathogen *Candida albicans* is able to form biofilms on the surfaces of medical implants such as prosthetic devices or catheters and on fragments of dead tissue. In the protected microenvironment of a biofilm, the pathogens are more resistant to antimicrobial therapies (2, 3). *Saccharomyces cerevisiae* also manifests a number of adaptive responses such as filamentation, invasive growth, flocculation, and adherence to solid surfaces in order to overcome adverse environments. At the industrial level, certain *S. cerevisiae* wine yeasts have also acquired the ability to form a buoyant biofilm at the air-liquid surface of wine (4, 5). The capacity of these so-called "flor" yeasts to form this biofilm (or velum) on wine mostly depends on components of the cell wall (CW).

The CW is an essential and dynamic structure and may account for up to one-third of the dry weight of the yeast cell. Major components of the yeast CW are glycoproteins, β-1,3-glucans, branched β-1,6-glucans, and chitin (6). Glycoproteins are extensively O- and N-mannosylated. Flo11p/Muc1p is a mucin-like protein that belongs to the family of flocculins and is involved in cell-substrate and cell-cell adhesion, invasive growth on agar, and biofilm formation on plastic and liquid surfaces (1, 5, 7–10). It is a CW glycoprotein with a C-terminal glycosylphosphatidylinositol (GPI) anchor, a large and highly O-glycosylated central domain rich in serine and threonine, and a N-terminal domain that mediates homotypic adhesion between cells (7, 11). *flo11* mutants of flor yeasts cannot form biofilms, demonstrating the role of Flo11p in biofilm formation on liquid surfaces (4, 5, 12–15). In addition, the absence of this protein drastically drops the affinity of yeast cells for hydrophobic solvents, whereas overexpression increases it (4, 10, 15). Additional *FLO* genes are *FLO1*, *FLO5*, *FLO9*, and *FLO10*, which are also involved in flocculation and invasive growth and code for proteins localized at the outer part of the CW (16, 17). On the other hand, *FLO8* serves as a transcription factor in the cyclic AMP (cAMP) pathway for the activation of *FLO11* (18). The members of the family of *PMT1* to -6 genes encode O-mannosyltransferases responsible for the attachment of the first mannosyl residue to serine/threonine residues of O-glycosylated proteins such as flocculins and other CW mannoproteins and are critical for their proper function (19). Additionally, the inner part of *S. cerevisiae* CW is composed of chitin which consists of a linear polymer of β-1,4-N acetylglucosamine and serves as a fibrous strengthening element responsible for CW rigidity. Chitin synthases are coded by *CHS1*, *CHS2*, and *CHS3* genes in yeast (20).

Antimicrobial peptides and peptide-related molecules are widespread in nature in organisms such as microbes, plants, or animals, including humans, and are considered part of an ancestral innate system of defense against pathogen attack or competition for nutrients (21). Most antimicrobial peptides contain cationic and hydrophobic residues, which confer amphipathic conformations. Antimicrobial peptides have been proposed as alternatives for the control of microorganisms in medicine, agriculture, and food preservation (21–24). Synthetic antimicrobial pep-

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tides have also been either designed *de novo* on the basis of properties of natural antimicrobial peptides or identified by means of combinatorial and nonbiased approaches. A detailed understanding of the antimicrobial mechanism of each antimicrobial peptide is needed for their development as useful antimicrobial agents. The mechanism of action of cationic and amphipathic antimicrobial peptides was initially correlated with their membrane permeation properties. However, more recent evidence indicates that the action of a number of antimicrobial peptides is more complex and involves specific interactions at cell envelopes, cell internalization, and/or intracellular targets (25–28).

Examples of antimicrobial peptides with antifungal activity and different modes of action are the synthetic PAF26, the human histatin 5, and the insect melittin. PAF26 is a tryptophan-rich cationic hexapeptide identified in a combinatorial chemistry approach (29) and belongs to the class of antimicrobial peptides with cell-penetrating properties (30). It has been proposed as model to study the mechanism of action of short antifungal cell-penetrating peptides, which can be divided into three stages: the initial interaction with fungal cell envelope, the internalization, and the intracellular killing (31). Histatin 5 belongs to the family of histatins, a group of histidine-rich cationic proteins produced by human salivary gland acinar cells that are components of the innate defense system in the oral cavity. Histatin 5 showed high potency against *C. albicans* and also cell penetration properties (32, 33). The P113 fragment is a 12-residue peptide derived from histatin 5 that is a minimal motif with antifungal activity (34). Melittin was originally isolated from the venom of the European honeybee *Apis mellifera*. It folds into an amphipathic α-helix that gives the intrinsic capacity to interact with cell membranes, leading to pore formation and nonspecific cytolysis and cell death (35, 36).

In this study, we explored the effect of these antimicrobial peptides of different mechanisms of action and properties on *S. cerevisiae* flor yeast viability and air-liquid biofilm formation ability. Interestingly, we found that the synthetic hexapeptide PAF26, but not P113 or melittin, enhanced biofilm formation without affecting cell viability under biofilm-forming conditions. This effect required the presence of a functional *FLO11* gene but was not accompanied by a change in its gene expression. Further, we demonstrate the interaction of PAF26 with *S. cerevisiae* flor strains and show that *FLO11* gene elimination abolishes the interaction with PAF26 but not with the P113 fragment derived from histatin 5.

MATERIALS AND METHODS

Yeast strains and media. The following wild-type *Saccharomyces cerevisiae* flor strains with different representative genetic backgrounds, *FLO11* gene lengths, and *FLO11* expression levels have been used in this study (14): A43, M25, A9, A68, V19, M12, V23, and V80. Particularly, A9 is a flor strain isolated from the Arvisionadu Sardinian winery. Strain 3238-32 (*MATα leu2-Δ1 lys2-801 ura3-52*) is a flor-forming haploid congenic to the diploid A9, and strain 3238-32Δ*flo11* (*MATα leu2-Δ1 lys2-801 flo11Δ::URA3 ura3-52*) is a non-biofilm-forming gene deletion mutant obtained from strain 3238-32 (5). All of the strains belong to the culture collection of the Dipartimento di Agraria (University of Sassari). Strains were cultured on YPD medium (1% yeast extract, 2% peptone, 2% glucose), 20% YPD medium (0.2% yeast extract, 0.4% peptone, 0.4% glucose), or synthetic complete (SC) medium (0.67% yeast nitrogen base without amino acids, 2% glucose, supplemented with bases and amino acids at standard concentrations) at 30°C. The flor medium was yeast nitrogen base (YNB), containing 4% ethanol as sole carbon source, which was supplemented when necessary with bases and amino acids at standard concentrations (5).

Synthetic peptides. Peptides used in this study were synthetic and purchased from GenScript (Piscataway, NJ) at ≥95% purity. The peptides were as follows: PAF26 (RKKWFW), which is a *de novo*-designed cell-penetrating antifungal peptide (29, 30); the active fragment P113 (AKRH HGYKRKFH) from human histatin 5, a natural cell-penetrating antifungal peptide isolated from human saliva (34); and melittin (GIGAVLKVL TTGLPALISWIKRKRQQ), a natural cytolytic antimicrobial peptide (35). Peptides PAF26 and P113 were also synthesized and labeled by covalent modification of their N terminus with fluorescein 5-isothiocyanate (FITC-PAF26) and tetramethyl-rhodamine (TMR-P113), respectively. Stock solutions of peptides were prepared at 1 to 5 mM in 10 mM 3-(*N*-morpholino)-propanesulfonic acid (MOPS; Sigma) (pH 7) buffer and stored at −20°C. Peptide concentrations were determined spectrophotometrically (29).

Antimicrobial activity assays. Growth inhibition assays were carried out in 96-well microtiter plates essentially as described previously (29) with some modifications. *S. cerevisiae* strains were cultured overnight in 5 ml YPD medium at 30°C with shaking. The following day, cultures were refreshed in YPD medium for 4 h at 30°C in order to reach the exponential phase (optical density at 600 nm [OD_{600}], 0.4 to 0.5). A volume of 90 μl of 10^4 cells/ml in 20% YPD medium was mixed in each microplate well with 10 μl of 10×-concentrated peptide solution from serial 2-fold dilutions; 10 μl of MOPS instead of peptide was added to the control well. All samples were prepared in triplicate. Microplates were statically incubated at 30°C for 48 h. Growth was measured every 2 h at OD_{600} using a Multiskan Spectrum microplate spectrophotometer (Thermo Electron Corporation, Finland). Dose-response curves were generated from measurements after 48 h.

Biofilm formation and measurement. For the air-liquid biofilm formation test, *S. cerevisiae* strains were grown overnight as described above. Cultures were washed twice with sterile water, and suspensions of 10^7 cells/ml were prepared in flor medium (see above); 900-μl aliquots were mixed in wells of 24-well microplates with 100 μl of 10×-concentrated peptides to reach a final concentration of 16 or 32 μM; and 100 μl of MOPS was added to control wells. Plates were incubated statically at 30°C for 5 days.

Biofilm dry weight measurement was carried out essentially as described previously (37), with minor modifications. After 5 days of static incubation at 30°C, biofilm biomass was harvested by pipetting ~1 ml of sample per well up and down 3 times (or more times, if necessary) without allowing the pipette tip to touch the bottom of the well, to avoid recovery of non-biofilm-forming cells that had settled at the bottom of each well. Once the biofilm-forming cells were disaggregated, the 1 ml of sample was transferred to a spectrophotometric cuvette. After that, 1 ml of sterile water was gently added to the well. The remaining biofilm cells were recovered as described above, without touching the bottom of the well, and transferred to the cuvette. The optical density of the final volume in the cuvette (2 ml) was measured spectrophotometrically and the OD_{600} value converted to dry weight (mg) (37).

Quantitative real-time PCR. The A9 yeast strain was grown overnight and refreshed as described above. Aliquots of 2.7 ml flor medium containing 10^7 cells/ml were mixed with 300 μl of 10× PAF26 (final concentration, 16 or 32 μM) or with MOPS buffer as a control and further incubated for 3 h at 30°C without agitation. Three independent biological replicate experiments were conducted for each treatment. Cells were collected by centrifugation and kept at −80°C until processing for RNA isolation was performed. Total RNA was extracted using an RNeasy mini-kit (Qiagen, Germany). Two micrograms of total RNA was treated with RNase-free DNase (Ambion Life Technologies) and retrotranscribed with SuperScript III reverse transcriptase (Invitrogen Life Technologies) essentially as described previously (38). Quantitative real-time PCR (qPCR) was performed according to the manufacturer's protocols using a LightCycler 480 real-time PCR system (Roche Diagnostics, Spain) and Light-

TABLE 1 Oligonucleotide primers used in this work

Gene	Primer	Sequence (5′–3′)
FLO5	Forward	5′-TGCTCTAAGACTGCTTCT-3′
FLO5	Reverse	5′-ATGTTGTGTATTCTGTGGTAA-3′
FLO8	Forward	5′-GCATCATCCCCATTAAGCAT-3′
FLO8	Reverse	5′-ATGTGGCGGCTTTATAACCA-3′
FLO10	Forward	5′-GCCTATCTTCAACTGTAAG-3′
FLO10	Reverse	5′-GCGTAATGCTATGTATGG-3′
FLO11	Forward	5′-AGGTTCAAATGGTGCCAAGA-3′
FLO11	Reverse	5′-AGCCACGCTAGAAGCAGAAG-3′
PMT1	Forward	5′-CTGCTTCTTTGGGGGCTCTT-3′
PMT1	Reverse	5′-CGAGTTTTCAACGGCAAAGC-3′
PMT2	Forward	5′-GGCTGATGACCGTGTTGG-3′
PMT2	Reverse	5′-GAGAACGGCTTGGACCTCTGG-3′
PMT4	Forward	5′-ACGTTCATCCCCCTTTTGCT-3′
PMT4	Reverse	5′-GCGTTGAAAGAACGGTACGC-3′
CHS1	Forward	5′-CCCCCACCAGCAACTGCAG-3′
CHS1	Reverse	5′-GTACGTTATTGTATTGATGTTC-3′
CHS2	Forward	5′-CCAAGGGTCTCTCGACTA-3′
CHS2	Reverse	5′-GCGTTGAATAGCCAACG-3′
CHS3	Forward	5′-GTCTTGATTCGACGATTATAC-3′
CHS3	Reverse	5′-GTCTAAAGTGGTTCTTAAACC-3′
ALG9	Forward	5′-CACGGATAGTGGCTTTGGTG-3′
ALG9	Reverse	5′-GGCAGCAGGAAAGAACTTGGG-3′
TAF10	Forward	5′-CAGGATCAGGTCTTCCGTAGC-3′
TAF10	Reverse	5′-GTAGTCTTCTCATTCTGTTGATG-3′
UBC6	Forward	5′-GATACTTGGAATCCTGGCTGG-3′
UBC6	Reverse	5′-GGGTCTTCTGTTTCATCACCTG-3′

Cycler 480 SYBR green I Master (Roche Diagnostics), with the following thermal profile: activation step, 95°C for 10 min; amplification step, 40 cycles of 95°C for 10 s, 56°C for 10 s, and 72°C for 10 s; melting curve program, 95°C for 10 s, 60°C for 15 s, and 95°C with a heating rate of 0.1°C/s; cooling step, 40°C for 30 s. Primers for the target genes *FLO1*, *FLO5*, *FLO10*, *FLO11*, *CHS1*, *CHS2*, *CHS3*, *PMT1*, *PMT2*, and *PMT4*, as well as *ACT1*, *ALG9*, *TAF10*, and *UBC6* as independent reference genes (38, 39), were designed for the same annealing temperature of 56°C (Table 1). The quantification cycle point (C_Q) for each transcript was obtained using LightCycler 480 SW 1.5 software (Roche Diagnostics). Three technical repeats of each of the three biological replicates were conducted. The algorithm geNorm (http://medgen.ugent.be/~jvdesomp/genorm/) (39) demonstrated the expression stability of the three reference genes *ALG9*, *TAF10*, and *UBC6* under our experimental conditions. The Relative Expression Software Tool (Multiple Condition Solver REST-MCS v2) was used to determine relative quantification and statistical significance data for the target genes normalized to the three reference genes (40).

Fluorescence microscopy. Yeast strains were grown overnight and refreshed as described above. Aliquots of 100 μl of flor medium containing 10^7 cells/ml were incubated with FITC-PAF26 (16 or 32 μM) for 2 h at 30°C in the dark. After incubation, cells were further incubated with 2 μM propidium iodide (PI) and 25 μM calcofluor white (CFW) for 5 min as described previously (38). In separate experiments, cells were incubated with or without PAF26 and then with 25 μM CFW. Cells were washed and examined with an epifluorescence microscope (E90i; Nikon, Japan) with excitation and emission wavelengths of 488 and 510 to 560 nm for FITC detection, 544 and 612 nm for PI detection, and 395 and 440 nm for CFW detection. Differential interference contrast (DIC) and fluorescence images were captured with 40× objectives using NIS-Elements BR v2.3 software (Nikon). Captured photos were merged using MacBiophotonics MBF ImageJ software. Total and stained cells were counted and percentages of stained cells were calculated using the same software. Essentially the same protocol was used with TMR-P113, using a final concentration of 16 μM, but without adding PI, because both dyes emit red fluorescence.

Flow cytometry. *S. cerevisiae* cells were prepared and incubated with either FITC-PAF26 or TMR-P113 (16 or 32 μM) as in the fluorescence microscopy experiments described above. After incubation, the cell suspension was made up to 1 ml with sterile water. Flow cytometry analyses were performed using an EPICS XL-MCL flow cytometer (Beckman-Coulter). An acquisition protocol (10,000 cells/sample) was defined after measuring background fluorescence from the nontreated A9 strain and fluorescence of A9 cells treated with 32 μM FITC-PAF26 or 32 μM TMR-P113, depending on the measured samples. Data were analyzed with the free FlowingSoftware v 2.5.0 (http://www.flowingsoftware.com/).

RESULTS

Activity of antimicrobial peptides against *S. cerevisiae* flor yeast strains. It has been shown that PAF26 is an antifungal cell-penetrating peptide that first interacts with the outer CW of fungi and that CW protein genes determine its activity toward *S. cerevisiae* (31, 38, 41). Since flor yeast strains offer a natural diversity of CW-related phenotypes associated with the abundant Flo11 protein, we decided to test the effect of PAF26 on a number of *S. cerevisiae* flor strains with distinct genetic backgrounds, lengths, and structure of *FLO11* gene and capabilities to form biofilm (see Materials and Methods) (14). Strains were tested for their susceptibility to PAF26 in growth inhibition assays on rich medium (20% YPD). Dose-response experiments performed using different concentrations of PAF26 did not show significant differences among the strains regarding their sensitivities to the peptide (data not shown). The MIC ranged between 32 and 48 μM, consistent with previous reported data of inhibitory activity of PAF26 for winemaking *S. cerevisiae* (42), but no correlation was found in these assays between the *FLO11* length or biofilm formation capability of the different strains and their susceptibility to PAF26.

Afterward, we decided to test the effect of the deletion of the *FLO11* gene on the susceptibility to PAF26 and also to compare peptides of different modes of action (melittin and the P113 fragment from histatin 5). To this end, we used the natural flor yeast A9 strain (which had been included in the experiments described above), its haploid derivative 3238-32, and its mutant 3238-32Δ*flo11* (5). Figure 1 shows representative dose-response curves from these experiments. The MIC of both PAF26 and P113 for the natural A9 strain was 32 μM, with a slightly increased resistance of both haploid strains 3238-32 and 3238-32Δ*flo11*. No difference was found between strains 3238-32 and 3238-32Δ*flo11* regarding their sensitivities to both peptides, demonstrating that the gene deletion did not have an impact on susceptibility to PAF26 or P113. The activity of melittin was higher than that of PAF26 or P113, with a MIC of 16 μM for both strains A9 and 3238-32 and a minor increase in resistance of strain 3238-32Δ*flo11* (MIC 32 μM).

Biofilm formation in the presence of antimicrobial peptides. We next tested the effect of the antimicrobial peptides on the biofilm-forming capability of the flor strains under biofilm-promoting experimental conditions (i.e., ethanol-rich medium). Biofilm formation in *S. cerevisiae* flor strains depends on the presence and expression of the *FLO11* gene (13). After depletion of sugars and nitrogen sources, growth slows and becomes dependent on access to oxygen and other nonfermentable carbon sources (37, 43).

Initial experiments evidenced that biofilm formation by strain A9 in flor medium was enhanced by the presence of PAF26 (Fig. 2A). We therefore set up a quantitative assay in 24-well microtiter plates to quantify this effect more precisely following previously

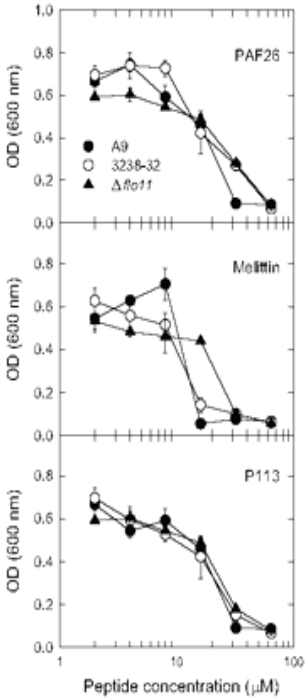

FIG 1 Growth inhibition of *S. cerevisiae* strains by peptides PAF26, melittin, and P113. Yeast cells (4×10^4 cells/ml) in 20% YPD were exposed to different concentrations of peptides and incubated at 30°C. Dose-response curves show mean OD_{600} ± SD after 48 h of inoculation. The *S. cerevisiae* flor strains represented are A9, 3238-38, and 3238-32Δ*flo11*.

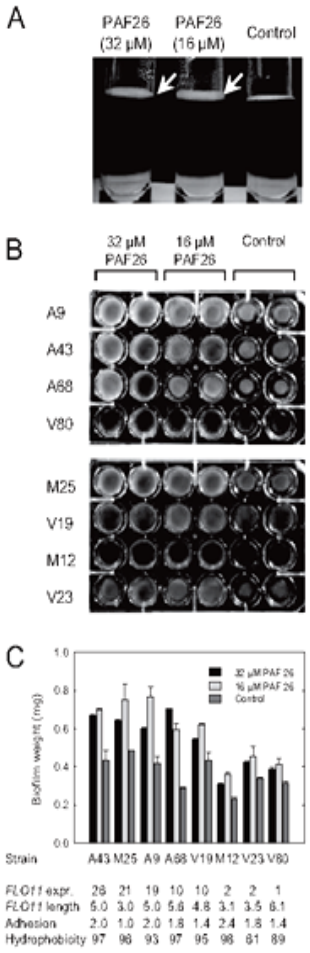

FIG 2 Air-liquid biofilm formation is enhanced by the presence of PAF26. (A) Biofilm formation at the air-liquid interface by the natural A9 strain in the absence (control) or presence (16 μM or 32 μM) of PAF26, after static incubation in 3 ml flor medium at 30°C in tubes. White arrows point to the thicker biofilm in the presence of PAF26. (B) Biofilm formation in 24-well plates by strains A9, A43, A68, V80, M25, V19, M12, and V23 after 5 days of static incubation in 1 ml flor medium at 30°C in the presence of PAF26 at different concentrations as indicated. The biofilm is visualized as opaque floating material at the top of each well. (C) Dry weight determination (*y* axis) of the biofilm formed by strains shown in panel B (*x* axis) after treatment with PAF26 at either 16 or 32 mM. Means and SD of the results from two replicate treatments are shown. The table under the strain names shows phenotypic properties of each strain as described in our previous publication (14). expr., expression.

described protocols (37) (Fig. 2B). We determined the biofilm formation in the presence of peptide of eight representative flor strains. As shown in Fig. 2C, our experiments demonstrated an enhancement of biofilm production of all the eight strains tested in the presence of PAF26 at either 16 or 32 μM, although subtle differences among the strains were observed in the total biofilm produced and the extent of enhancement in the presence of peptide. These peptide concentrations were chosen because they were considered the more informative based on the dose-response assays (Fig. 1). Overall, strains A43, M25, A9, A68, and V19 had the highest weight of dry biofilm (0.5 to 0.8 mg) among the tested strains, while strains M12, V23, and V80 presented a lower weight of dry biofilm (0.3 to 0.45 mg). This behavior correlated more closely with the level of *FLO11* gene expression than with the *FLO11* gene length, cell adhesion capability, or cell hydrophobicity of each strain.

The next logical step was to determine whether the enhancement of biofilm formation observed after exposure to PAF26 depended of the presence of the *FLO11* gene. At this point, we also analyzed the specificity of this phenomenon by testing the peptides melittin and P113 (Fig. 3). Differences were observed in air-liquid biofilm formation after 5 days of incubation of strains A9, 3238-32, and 3238-32Δ*flo11* treated with PAF26, P113, or melittin at 16 or 32 μM compared with control treatments without peptide (Fig. 3A). In this experiment, 16 and 32 μM PAF26 led to a similar 2-fold enhancements of the dry biofilm weight in strain A9 (approximately 0.4 to 0.5 mg/well versus 0.2 mg/well in the control) and a minor but reproducible 1.5-fold enhancement in strain 3238-32 (Fig. 3A). As predicted, the non-biofilm-forming 3238-32Δ*flo11* strain did not form observable biofilm in control wells, and measurements rendered values below 0.1 mg of biomass per well. PAF26 treatment did not result in observable or measurable biofilm formation in this mutant.

Importantly, the presence of a P113 fragment of histatin 5 at either 16 or 32 μM did not affect the biofilm formation in any of the strains, and measured values did not differ significantly from those of the no-peptide controls (Fig. 3A). This differential behavior compared with PAF26 occurred despite the very similar growth inhibitory properties of PAF26 and P113 (Fig. 1). Finally, the presence of the cytolytic peptide melittin prevented biofilm formation in all the strains assayed, and no biofilm was observed after 5 days of incubation.

To further characterize these experiments, aliquots of each sample were plated on YPD medium to monitor CFU recovery.

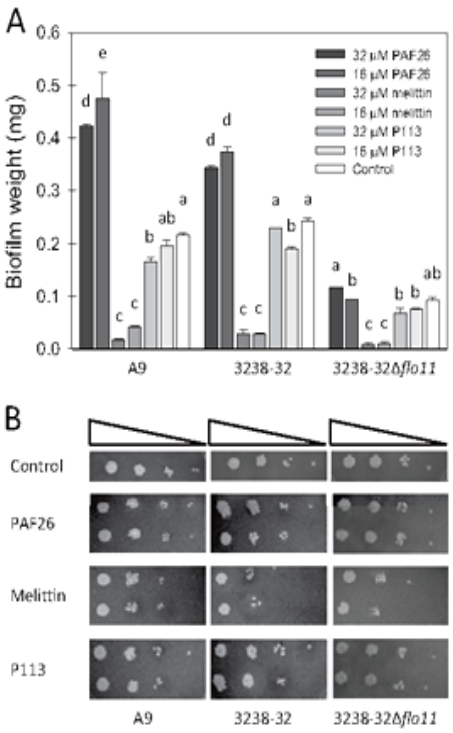

FIG 3 Enhancement of air-liquid biofilm formation by PAF26 is peptide specific and depends on the presence of the *FLO11* gene. (A) Dry weight determination (*y* axis) of the biofilm formed by strains A9, 3238-38, and 3238-32Δ*flo11* (*x* axis) after treatment with PAF26, melittin, and P113 at either 16 or 32 μM (see key in figure panel). Means and SD of the results from three replicate treatments are shown. Multiple comparison analysis was conducted, and bars with the same letter within each strain represent the absence of a statistically significant difference at 95% confidence. (B) CFU recovery after plating on YPD plates using serial dilutions of one of the replicates from each strain-peptide combination from the experiment described in the panel A legend.

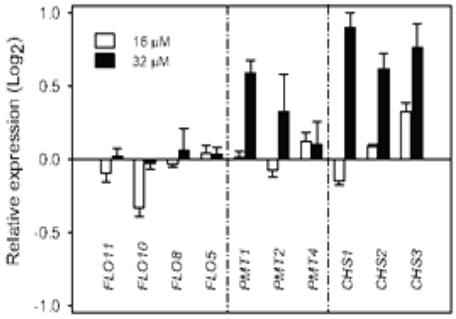

FIG 4 Quantitative real-time PCR analysis of gene expression after PAF26 treatment. Data represent mean relative expression ± SD (*y* axis, Log_2 values) of each individual gene (show at the bottom) upon treatment of A9 strain with PAF26 for 3 h in flor medium compared to the control treatment with no peptide. White bars indicate 16 μM PAF26 and black bars 32 μM PAF26. Genes *ALG9*, *TAF10*, and *UBC6* were simultaneously used as constitutive reference genes as determined by the geNorm algorithm (39). Relative expression was calculated using REST-MCS v2 software (40), which showed no statistically significant difference for any gene compared with the no-peptide control treatment.

Treatment with PAF26 did not significantly change the number of CFU recovered compared with the control cultures (Fig. 3B), demonstrating the absence of strong fungicidal activity under biofilm formation conditions. On the other hand, melittin treatment resulted in a minor reduction of CFU recovery from the corresponding samples.

Gene expression of *FLO*, *PMT*, and *CHS* genes in flor medium in the presence of PAF26. From the data reported above, we speculated that PAF26 treatment could result in increased expression of genes important for biofilm formation in flor yeast, similarly to the enhanced expression of CW-related genes observed in the laboratory strain of *S. cerevisiae* (38). We analyzed by qPCR the expression of the representative *FLO* genes *FLO5*, *FLO8*, *FLO10*, and *FLO11*; the *PMT* genes *PMT1*, *PMT2*, and *PMT4*; and the chitin synthase genes *CHS1*, *CHS2*, and *CHS3* on samples from the A9 strain after 3 h of incubation in flor medium with either 16 or 32 μM PAF26 (Fig. 4). Accumulation of mRNA from each gene was compared to that seen with control samples without PAF26 ($Log_2 = 0$ in Fig. 4). Our data showed no statistically significant differences between control and PAF26-treated samples in the expression of any of the tested genes, despite the observation of mean Log_2 values above 0.5 for some genes such as *PMT1* and the three *CHS* genes. *FLO* gene expression remained fairly constant for the four tested genes. Overall, the expression of chitin synthase genes was the most affected by the presence of PAF26, showing increased Log_2 values at 32 μM.

Interaction analysis of PAF26 with *S. cerevisiae* flor strains. The chitin binding stain CFW and fluorescence microscopy were used to evaluate the effect of PAF26 treatment on CW of flor yeast. Figure 5 shows the CFW staining of the A9, 3238-32, and 3238-32Δ*flo11* strains after 2 h of incubation with and without PAF26 in flor medium. Fluorescence microscopy observations showed a marked difference in staining among strains and peptide treatments. Strains A9 and 3238-32 showed very low staining that was substantially increased upon PAF26 treatment and was very similar to what has been found in *S. cerevisiae* laboratory strains (38). The high CFW fluorescence in treated cells could be related to the increased accumulation of chitin and/or changes in CW structure or composition, as a response to the peptide interacting with the CW.

In contrast, the CFW staining results seen with both treated and control cells of the 3238-32Δ*flo11* strain were very similar and were also comparable to the staining of the parental 3238-32 strain treated with PAF26 (Fig. 5). The high level of staining of untreated 3238-32Δ*flo11* cells could have been a consequence of overproduction of chitin in mutants affected in CW integrity (44, 45). At this point, our findings would indicate either that exposure to PAF26 does not cause further CW stress to the already-affected 3238-32Δ*flo11* cells or that PAF26 does not interact with 3238-32Δ*flo11* cells.

In order to determine whether the enhanced biofilm formation and increased CFW staining correlated with the interaction of PAF26 with our tested strains, we set up an assay in which cells were treated with labeled FITC-PAF26, followed by treatment with the cell death marker propidium iodide (PI) and CFW, and performed simultaneous three-color fluorescence detection experiments (Fig. 6). Previously, interaction with and internalization of fluorescently labeled FICT-PAF26 into FY1679 *S. cerevisiae* had been demonstrated (38). Figure 6 shows representative images of yeast incubated in flor medium for 2 h in the presence of 16 μM FITC-PAF26. CFW staining was consistent with data described above (Fig. 5), further indicating that the N-terminal addition of the FITC fluorophore does not substantially change the

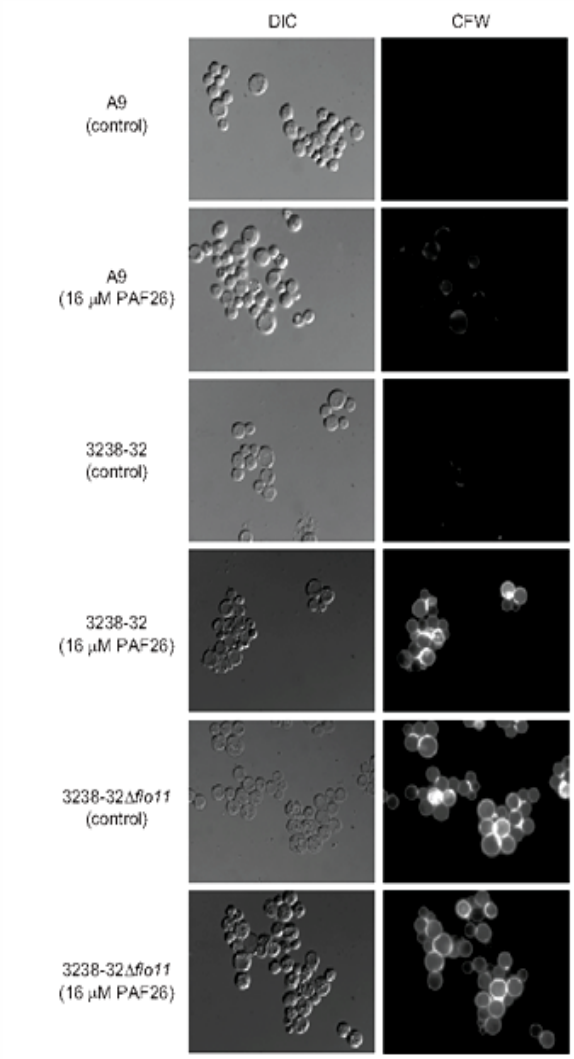

FIG 5 Fluorescence microscopy after CFW staining of different *S. cerevisiae* strains exposed to PAF26. Cells (10^7 cells/ml) of A9, 3238-32, and 3238-32Δ*flo11* strains were incubated for 2 h in flor medium with or without 16 μM PAF26. After incubation, samples were stained with 25 μM CFW for 5 min. DIC bright-field (left) and CFW (right) images of the same field are shown. All the images were captured under the same acquisition parameters and therefore reflect actual differences in CFW staining.

activity of PAF26 (38). Under these conditions, 18% ± 3% of A9 cells and 15% ± 3% of 3238-32 cells (mean values ± standard deviations [SD] of the results from three technical replicates) were positive for green fluorescence (FITC), in sharp contrast with the only 2.0% ± 0.8% of cells stained in the case of strain 3238-32Δ*flo11*. The latter result demonstrated that the presence of the *FLO11* gene determines most of the interaction with PAF26. PI staining used to indicate cell death showed 18% ± 2% positives in A9 cells, 20% ± 2% in 3238-32 cells, and 15% ± 5% in 3238-32Δ*Flo11* cells. Merged images indicate that in the A9 and 3238-32 strains, there was colocalization of green fluorescence (i.e., FITC peptide-labeled cells) and red fluorescence (PI, dead cells), while in strain 3238-32Δ*Flo11*, a proportion of PI-labeled cells did not show peptide staining. This observation indicates that the death of 3238-32Δ*flo11* cells could have been due to another factor(s) that was independent of peptide action, such as, for instance, the higher sensitivity to ethanol of this mutant.

The experiments were similarly carried out with 16 μM TMR-P113 (labeled with the red fluorophore TMR). As occurs in the case of PAF26 and FITC/TMR labeling, TMR labeling of P113 did not affect the antimicrobial activity of the peptide (data not shown). In sharp contrast with the PAF26 results, fluorescence microscopy after 2 h of incubation did not show any significant difference in peptide staining among the three strains (Fig. 7): 25% ± 6% of A9 cells stained red after TMR-P113 exposure compared to 42% ± 8% of 3238-32 and 45% ± 6% of 3238-32Δ*Flo11* cells. These results indicate an even greater interaction with P113 (which did not enhance biofilm formation; Fig. 3) than with PAF26 in these strains and, most importantly, no effect of the *FLO11* gene deletion on the interaction with P113. Moreover, CFW staining of TMR-P113-treated cells was lower than that of cells treated with FITC-PAF26 (compare Fig. 6 and 7, whose images were obtained using identical exposure and capture parameters), which indicates a CW effect caused by PAF26 that was higher than that caused by P113.

Flow cytometry analyses were conducted that confirmed the reduced interaction between PAF26 and the *flo11* mutant (Fig. 8). Fluorescence measurements of 10,000 events for both tested strains exposed to 16 or 32 μM FITC-PAF26 showed a shift to lower fluorescence, demonstrating a reduction in the amount of peptide bound to 3238-32Δ*flo11* cells (in the case of 32 μM, 6×10^2 versus 3×10^3 mean fluorescence arbitrary units [a.u.]) (Fig. 8A). This behavior was not observed in the case of P113, since the flow cytometry histograms of the two strains were not distinguishable (Fig. 8A). Determination of the number of positive single-cell counts demonstrated a statistically significant (P value = 0.034) 4-fold reduction in the number of 3238-32Δ*flo11* cells that interacted with FITC-PAF26 (Fig. 8B). Moreover, flow cytometry results confirmed that P113 interaction with flor strains was mostly independent of the presence of *FLO11*, because the levels of binding to the parental strains and binding to the mutant strains were similar and the differences were not statistically significant (Fig. 8B).

DISCUSSION

Most antimicrobial peptides are cationic and amphipathic molecules with the intrinsic capability to interact with and permeabilize biological cell membranes, which allows them to lyse and kill cells. However, some antimicrobial peptides may also have alternative nonlytic mechanisms of action. These include interactions with the CW, cell internalization, intracellular targets, disruption of nucleic acid and protein metabolism, or more subtle and specific signaling mechanisms (25–28, 46). The short PAF26 is a cationic and tryptophan-rich peptide with cell-penetrating and antifungal activity; it is selective against filamentous fungi (MIC values = 1 to 10 μM) and shows low activity against yeast (MIC > 32 μM) (31). We show in this report that PAF26 interacts with flor wine yeasts in ethanol-rich medium without substantial killing and also promotes biofilm formation. Remarkably, the P113 fragment of histatin 5, which is a different cell-penetrating antifungal peptide, also interacts with flor wine yeasts but does not enhance biofilm formation. Moreover, we demonstrate that the mucin-like Flo11p determines the interaction with PAF26 but has no effect on the interaction with P113.

Previous data had shown no activity of PAF26 and other PAF-derived peptides against wine spoilage yeasts and nonflor wine-making *S. cerevisiae* in wine (42), and the use of PAF26 as an

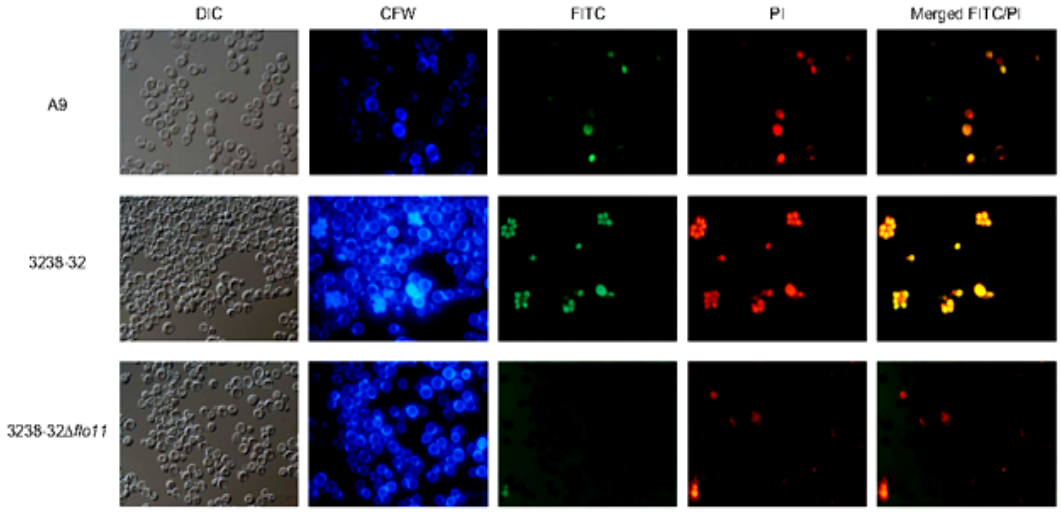

FIG 6 Fluorescence microscopy of different *S. cerevisiae* strains exposed to FITC-PAF26. Cells (10^7 cells/ml) were incubated in flor medium with 16 μM FITC-PAF26 at 30°C for 2 h and subsequently and simultaneously with 2 μM PI and 25 μM CFW at 20°C for 5 min. Representative DIC bright-field as well as CFW, FITC, PI, and FITC/PI-overlay fluorescence micrographs of the same field are shown, for the different strains, as indicated.

antifungal agent in wine production was discarded. Dose-response results presented here confirm that all yeast strains tested showed low susceptibility to PAF26. The variations in inhibition effects of antimicrobial peptides on different yeasts and fungi could be related to components of the cell wall, including glycoproteins from each microorganism (28, 47). Our results indicate that Flo11p is not a determinant of the susceptibility of *S. cerevisiae* to antimicrobial peptides in rich medium, because inhibition curves showed no significant differences between parental and mutant 3238-32Δ*flo11* cells. This may be related to the fact that the *FLO11* gene was induced at the stationary phase (43), when the cell OD is high, and after the peptides had completed their action in the experimental setup used in Fig. 1. The *flo11* mutant showed a higher tolerance to melittin than the A9 strain and its parental 3238-32 strain. Melittin must diffuse through the outer layers of the cell to reach the lipid cell membranes, where it is active through a cytolytic mechanism (36). Yeast CW is usually composed of approximately 2% chitin. In mutants affected in CW structure, chitin percentages may reach up to 20% of total CW polymers (44, 45, 48). The 3238-32Δ*flo11* mutant shows enhanced CFW staining (Fig. 5) consistent with increased chitin content and altered CW structure. It is hypothesized that in this mutant, melittin accessibility to the membrane could be hindered by chitin layers, thus explaining its reduced activity. An increased sensitivity to the human antimicrobial peptide LL-37 was recently observed in a *C. albicans* deletion mutant lacking another membrane glycoprotein, Msb2p (49). Msb2p is a mucin-like protein with sensor activity. Its protective effect against LL-37 was correlated with shedding and release of its large N-terminal and highly O-glycosylated domain, which is able to sequester the peptide when released to the cell environment and even to protect in *trans* bacterial cells from antimicrobial peptide action (49).

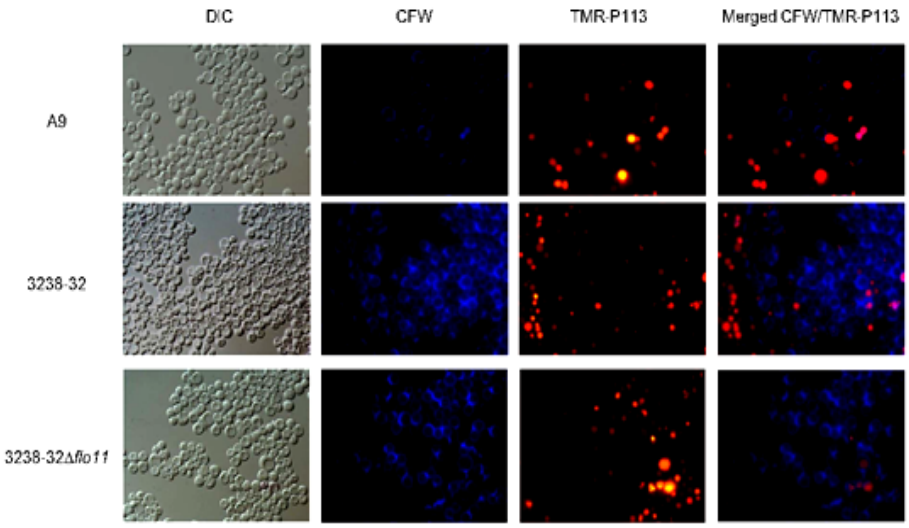

FIG 7 Fluorescence microscopy of different *S. cerevisiae* strains exposed to TMR-P113. Cells (10^7 cells/ml) were incubated in flor medium with 16 μM TMR-P113 at 30°C for 2 h and subsequently with 25 μM CFW at 20°C for 5 min. Representative DIC bright-field as well as CFW, TMR, and CFW/TMR-overlay fluorescence micrographs of the same field are shown, for the different strains, as indicated.

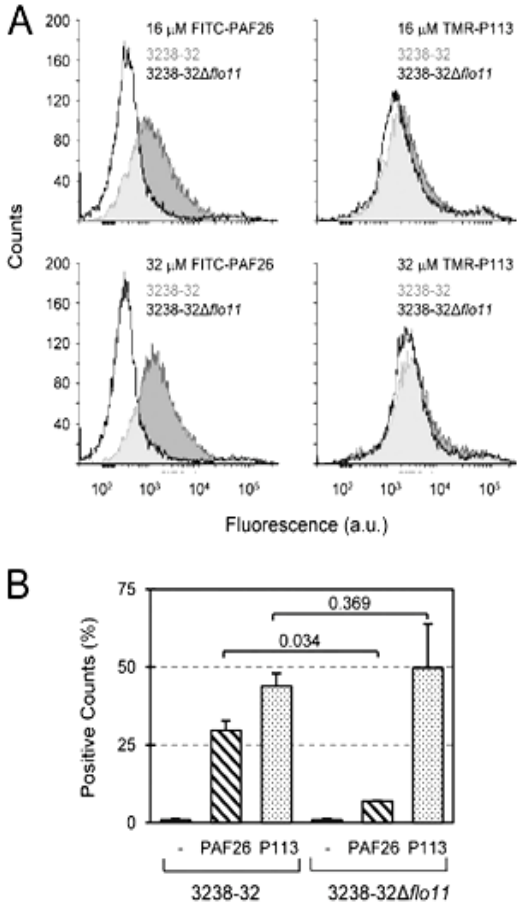

FIG 8 Flow cytometry analyses of the interaction of FITC-PAF26 and TMR-P113 with *S. cerevisiae* strains. (A) Representative histograms showing the intensity of fluorescence (*x* axis; arbitrary units [a.u.]) and cell counts (*y* axis) of a total of 10,000 cells from either strain 3238-32 (gray histograms and lines) or strain 3238-32Δ*flo11* (black lines) exposed in flor medium for 2 h to either a 16 μM (upper panels) or a 32 μM (lower panels) concentration of FITC-PAF26 (left panels) or TMR-P113 (right panels) peptide, as indicated. (B) Bar diagrams showing the percentages of positive (fluorescently labeled) cell counts of either the 3238-32 or 3238-32Δ*flo11* strain after control exposure to no peptide (−) and exposure to a 16 μM concentration of either FITC-PAF26 or TMR-P113, as indicated. Results show the means ± SD of the results from three replicate samples. *P* values are shown for the null hypothesis of no difference between the means of each strain (two sample *t* test, unequal variances).

The viability test indicated an enhanced resistance to antimicrobial peptides of flor strains in ethanol-rich flor medium (Fig. 3B) compared to rich YPD medium (Fig. 1). For instance, 32 μM peptide, which is a MIC in most peptide/strain combinations in YPD, permitted CFU recovery in flor medium. It has been previously reported that antimicrobial peptides reduce their activity against wine spoilage yeast when tested in wine (42). This reduced effect of peptides could be related to modifications of the CW that occur in ethanol-containing media or during the diauxic shift from fermentation to respiration (48).

Increase of biofilm formation in the presence of PAF26. The increased formation of biofilm of both A9 and 3238-32 in the presence of PAF26, but not of other peptides, and the absence of biofilm formation of control and PAF26-treated 3238-32Δ*flo11* cells (Fig. 3A) indicates a specific effect of PAF26 on biofilm formation that requires the presence of *FLO11*. The expression of the *FLO11* gene has been shown to be the key event that triggers biofilm formation in *S. cerevisiae* flor strains as a response to environment conditions (4, 5, 12, 15, 50). The biofilm enhancement was also confirmed in 8 natural flor yeast strains (including A9) that reach different levels of *FLO11* expression. The correlation of the amount of biofilm in the presence of PAF26 with high expression of *FLO11* in these strains is consistent with the requirement of a functional *FLO11*.

The expression of representative *FLO* genes was analyzed after PAF26 exposure in flor medium. We found that the increased biofilm formation observed after PAF26 exposure is not correlated to changes in *FLO11* transcription levels (Fig. 4). For this reason, we postulate that the biofilm enhancement by PAF26 is independent of *FLO11* gene regulation but requires the presence and expression of a functional *FLO11* gene. Indeed, it is well known that *FLO11* is highly expressed in flor medium but not in rich media such as YPD (16, 51). We confirmed in the experiment shown in Fig. 4 that the control samples, which do not contain peptide, express *FLO11* to high levels (data not shown). On the other hand, transcription of the CW-related genes, *PMT1*, a gene that codes for a O-mannosyltransferase, and the three *CHS* genes, was slightly enhanced in the presence of 32 μM PAF26. This increased gene expression did not reach statistical significance although it was consistent with increased staining with the chitin stain CFW (Fig. 5). We should notice that increased CFW staining could be also due to CW changes in cells exposed to PAF26. Previous independent results indicate the involvement of chitin synthase genes and chitin content in the mechanism of action of antifungal proteins (52–55). Nevertheless, the changes in CFW staining that occur during PAF26 exposure (Fig. 5) do not seem to be primarily related to the biofilm-promoting properties of the peptide, since they were also observed in the nonbiofilm mutant and in the absence of peptide.

Our conclusion is that the effect of PAF26 on biofilm is related to the ability of the peptide to enhance cell-to-cell aggregation under specific biofilm-forming conditions. Our microscopy data support this, since cells of strain 3238-32 showed a tendency to aggregate in the presence of PAF26 (Fig. 5). Similar data have been observed in *C. albicans* because the peptide LL-37 caused cell aggregation as well as prevention of cell adhesion (56).

***FLO11* is specifically involved in the interaction of PAF26 with *S. cerevisiae* flor strains.** The stability in *FLO* gene expression after treatment with PAF26 led us to study the interaction between flor yeast and labeled peptides. Studies with *Neurospora crassa* (30) and *S. cerevisiae* (41) have established that PAF26 (i) interacts first with cell outer layers prior to (ii) cell internalization and (iii) intracellular killing. Each of the three steps is necessary but not sufficient for the antifungal action of this peptide (31) and shows similarities to the previously reported antifungal mechanism of histatins (33). The experiments presented herein show that PAF26 and P113 (derived from histatin 5) also interact with yeast in flor medium (Fig. 6, 7, and 8), in which there is no significant cell killing (Fig. 3B), providing additional evidence that peptide interaction and killing are not necessarily linked.

Most importantly, two independent techniques (i.e., microscopy and flow cytometry) demonstrated different interactions with PAF26 between cells of the parental strains and the *flo11* mutant (Fig. 6 and 8): PAF26 showed a diminished interaction with 3238-32Δ*flo11* cells. It was noteworthy that P113 binding was not affected by the *flo11* mutation, thus providing additional sup-

port for the idea of a specificity component in the PAF26 interaction. Therefore, our results demonstrate that the *FLO11* gene selectively determines the binding of PAF26 to *S. cerevisiae* flor strains.

The large and highly glycosylated extracellular N-terminal domain of Flo11p confers high hydrophobicity and negative net charge to yeast CW and is responsible for cell-to-cell adhesion and biofilm formation (1, 4, 10, 11). Therefore, electrostatic and hydrophobic interactions could be established between the cationic and hydrophobic PAF26 and Flo11p. The absence of glycosylated Flo11p as in the case of the 3238-32Δ*flo11* strain reduces both CW hydrophobicity and the negative net charge (4). The lack of clear correlation between (i) the hydrophobicity or cell adhesion of the different strains used in our study and (ii) the enhancement of biofilm formation by PAF26 (Fig. 2) indicates that these cell properties (hydrophobicity and cell adhesion) are not uniquely responsible for the PAF26 interaction with cells and its effect on biofilm formation. Our results suggest an interaction between PAF26 and the highly glycosylated Flo11p. Following this hypothesis, PAF26 would act by facilitating and bridging the Flo11p-mediated interaction between cells and thus increasing biofilm formation. Previous studies have shown that protein glycosylation genes have a role in the sensitivity of fungi to antifungal peptides and proteins, through the glycosylation of mannosyl CW proteins (47, 57–59). As mentioned above, the glycosylated domain of the mucin-like Msb2p from *C. albicans* is capable of binding to histatin 5 and LL-37 and protects cells from the peptides (49). Also, in *C. albicans*, the CW chaperone Ssa2p has been shown to bind histatin 5/P113 (60, 61). However, parental and Δ*ssa2 C. albicans* cells bound similar amounts of P113 (34), in similarity to our findings determined with P113 and the Δ*flo11* mutant (Fig. 8B). This is in marked contrast to the diminished interaction of PAF26 with Δ*flo11* cells. It is clear that PAF26 and P113 might have similarities but also differences in their interactions with fungal cells. Unfortunately, various approaches designed to demonstrate a direct interaction between PAF26 and yeast proteins or CW components were not successful (data not shown). Other indirect alternatives are possible to explain our data. For example, PAF26 could interact with a component(s) of the *S. cerevisiae* outer cell layers whose presence is influenced by a functional Flo11p.

In summary, our report identifies another biological activity of the synthetic peptide PAF26 that could be of biotechnological interest and also extends to other cationic and tryptophan-rich antifungal peptides. It also suggests that glycosylated mucin-like proteins such as Flo11p at the fungal CW could be interacting partners for cell-penetrating antifungal peptides. Future work will aim to explore the significance of this interaction in relation to the antifungal mechanism of action of PAF26 with other nonflor yeasts and filamentous fungi and to determine the importance of protein glycosylation for this mechanism.

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CHAPTER II

Inhibitory effect of L-histidine on biofilm formation by *Saccharomyces cerevisiae* flor yeasts with functional *FLO11* gene

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Saccharomyces cerevisiae flor yeasts are able to adapt to different environmental stresses. The adaptation mechanism is complex and includes the consumption of non-fermentable carbon sources and nitrogen sources and consequently, cell-cell adhesion and biofilm formation. Flo11p, which is a high hydrophobic and anionic cell wall glycoprotein, plays an essential role in biofilm formation. Within the frame of a project that aims elucidating the role of Flo11p in biofilm formation and cell wall remodulation, we analyzed by Phenotype Microarray technology the nitrogen metabolism ability of three natural *S. cerevisiae* flor strains that differ for genetic backgrounds, and harbor *FLO11* alleles that show different length and expression levels. S288c was utilized as reference strain due to its constitutive repression of *FLO11* gene. Flor strains were able to metabolize more amino acids and dipeptides as a sole nitrogen source. The only exception was for dipeptides containing L-histidine that inhibited flor strains but not S288c growth. To better understand the phenotypic effect of L-histidine, the three flor yeasts and S288c were subjected to further analyses together with a two isogenic haploids, one harboring a functional *FLO11* gene and one carrying a *FLO11* mutation. Air-liquid biofilm formation and the adhesion to polystyrene phenotypes of flor strains were dramatically decreased in the presence of L-histidine in flor medium. Moreover, in the presence of L-histidine, viability was

inversely related to *FLO11* expression. Accordingly, L-histidine did not affect viability of *flo11* mutant and S288c. Finally, strains showing higher *FLO11* expression increased chitin production in the presence of L-histidine thus suggesting that Flo11p plays a pivotal role in L-histidine cell response.

Keywords: Mannoproteins; velum formation; adhesins.

Introduction

Saccharomyces cerevisiae is a unicellular eukaryotic microorganism and it has been used as study model for higher eukaryotic cells. *S. cerevisiae* yeast is able to shift from anaerobic (fermentation) to aerobic (respiration) metabolism, depending on the surrounded environment. In general, the presence of an appropriate sugar (glucose) and nitrogen (ammonium) sources in a medium is followed by rapid fermentative growth, ethanol production and synthesis of all amino acids needed in protein folding (Ljungdahl and Daignan-Fornier, 2012). When such favorite nutrient sources are depleted from the medium, *S. cerevisiae* can shift their metabolism to aerobic respiration by the diauxic shift phenomenon (DeRisi *et al.*, 1997). Aerobic respiration growth consists of the catabolism of non fermentable carbon sources, such as ethanol, via the tricarboxylic acid cycle and oxidative phosphorylation in the mitochondria, to generate energy under form of Nicotinamide Adenine Dinucleotide (NADH) (Muller *et al.*, 2012). Nitrogen starvation was shown to trigger cells adhesion and multicellular growth in different yeast spp (Verstrepen and Klis, 2006; Granek and Magwene, 2010). In *S. cerevisiae* flor strains, nitrogen limitation induces the activation of *FLO11* gene and the formation of air-liquid biofilm or flor velum (Zara *et al.*, 2011). Molecular analysis showed that the General Amino Acids Control GAAC pathway and/or the plasma membrane localized Ssy1-Ptr3-Ssy5 (SPS) sensor, responsible for the nitrogen sensing and regulation, are also involved in the regulation of *FLO11* gene expression (Braus, 2003; Ljungdahl, 2009; Brückner and Mösch, 2012; Torbensen *et al.*, 2012).

The expression of *FLO11* gene encodes for an extensively O-mannosylated cell wall protein, which triggers the cell-cell and cell-surface adhesion and the air-liquid biofilm formation in flor yeast strains (Fidalgo *et al.*, 2006; Alexandre, 2013). The phosphorylation of the mannosyl side chains on the outer surface of yeast creates abundant negatively charged groups gives yeast its anionic surface charge at a pH ≥3 (Ovalle, 1998; Klis *et al.*, 2007). Therefore, forces that influence cellular adhesion and binding may also include non specific interactions such hydrophobic and electrostatic interactions (Caridi, 2006; Holle *et al.*, 2011; Kregiel *et al.*, 2012). Indeed, *flo11* mutants show a drastic decrease in cell wall O-mannosylation sites, loss of adhesion and biofilm formation capacity and loss of affinity to hydrophobic solvents (Reynolds, 2001; Zara *et al.*, 2005; Fidalgo *et al.*, 2006; Dranginis *et al.*, 2007; Barrales *et al.*, 2008; Fidalgo *et al.*, 2008; Goossens and Willaert, 2012a). These phenotypes seem also to be largely influenced by the gene length, tandem repeats and the expression level of *FLO11* gene (Zara *et al.*, 2009). Along with *FLO11* gene response to adverse environment, cell wall components such as chitin, β-glucan and mannosyl residues, are also involved in the adaptation process to environmental stresses, orchestrated mainly by the cell wall integrity pathway (Cid *et al.*, 1995; Levin, 2005).

In this work, we mainly explored the phenotypic response and metabolism rate of different flor yeast strains, each with a specific *FLO11* gene length and biofilm forming ability, respect to a laboratory strain, with a non-functional *FLO11* gene. For this, we used the Phenotype Microarray © (PM) techniques, to characterize the effects of various nitrogen sources on cellular growth of tested *S. cerevisiae* strains. Phenotype Microarray is a high-throughput technology used for phenotypic testing of microorganisms, in multi-well plates with different test components in each well, enabling to screen phenotypic characteristics and metabolism rate of tested culture, in up to 2000 different condition (Borglin *et al.*, 2012). Results showed a high variability in nitrogen metabolism among tested strains. Flor strains were able to metabolize a wide range of nitrogen sources, respect to the laboratory strain, but

were remarkably unable to metabolize dipeptides containing L-histidine. L-histidine is a cationic amino acid and the only to have an imidazole ring as a chain group (Shimba *et al.*, 2003). Subsequently, a series of analyses in the presence of L-histidine such as dose response, biofilm formation, adhesion to polystirene and microscopic observations, showed a novel role of L-histidine in biofilm and adhesion ability.

Materials and methods

Strains and media. Strains with different genetic backgrounds were used in this study (table 1). A9, M23 and V80 are natural flor strains, previously isolated from different wineries in Sardinia and genetically characterized. These natural strains posses a heterogeneous background at *FLO11* gene length and expression level (Zara *et al.*, 2009b). Strain 3238-32 is an haploid flor strain and 3238*Δflo11* is its mutant; both are derivatives of A9 (Zara *et al.*, 2005). S288c lab strain is knockout *flo8* gene mutant which disables *FLO11* expression (Mortimer and Johnston, 1986). Strains were cultured on YPD medium (1% yeast extract, 2% peptone, 2% glucose), 20% YPD medium (0.2% yeast extract, 0.4% peptone, 0.4% glucose), Biolog specific PM medium (20 mM of glucose, 5m M potassium phosphate and 2 mM sodium sulfate to buffer the medium at pH 5.6, 1x of a tetrazolium dye mix and 1x of IFY-0 medium, IFY-0 is a basal medium, lacking nitrogen, carbon, phosphate, and sulfur sources), and in Flor medium (0.17% of YNB without ammonium sulphate and amino acids, 0.5% ammonium sulphate, 4% of EtOH) supplemented when necessary with bases and amino acids at standard concentrations. Cell cultures were incubated at 30°C.

TABLE 1 ***Saccharomyces cerevisiae*** **strains used in this study.**

Strains	Genetic background	Reference
A9	Wild flor strains of S. cerevisiae isolated from Arvisonadu wine	Zara et al., 2009
M23	Wild flor strains of S. cerevisiae isolated from Malvasia wine	Zara et al., 2009
V80	Wild flor strains of S. cerevisiae isolated from Vernaccia wine	Zara et al., 2009
3238-32	*MATα leu2-Δ1 lys2-801 ura3-52*	Zara et al., 2005
3238-32*Δflo11*	*MATα leu2-Δ1 lys2-801 flo11Δ::URA3 ura3-52*	Zara et al., 2005
S288c	*MATα SUC2 gal2 mal mel flo1 flo8-1 hap1 ho bio1 bio6*	Mortimer and Johnston, 1986

Phenotype Microarray techniques. In order to screen the general metabolism of strains A9, M23, V80 and S288c upon different nitrogen sources, the Phenotype Microarray techniques were used. Specific micro-plates (PM3B, PM5, PM6, PM7 and PM8) were purchased from Biolog, Omnilog (Hayward CA, USA), enabling us to screen the metabolism of tested strains upon 380 different nitrogen sources, such as single amino acids, di/tripeptides, purines, pyrimidines and monoamines (Bochner, 2009). Strains were grown on YPD agar plates overnight, at 30°C , than suspended with sterile cotton swab in 15 ml of sterile water and cell density was adjusted to 62% transmittance (T) on a Biolog turbidimeter, equivalent to 0,22 of OD_{600} optical density (≈ 2-3x10^6 cells/mL). Cells suspensions were than inoculated into the Biolog specific PM medium to a final concentration ≈2-3x10^5 cells/mL, pipetted into adequate PM plates, than incubated statically at 30°C in an Omnilog Reader. Experiment was performed in duplicate. Quantitative color changes were recorded automatically every 15 min by a CCD camera for 96 h. The kinetic responses of the strains were analyzed by the Omnilog-PM software (Biolog, inc. hayward CA, USA). The data were filtered using average height as a parameter. Metabolism of control wells were considered as the zero point for other wells. Data was presented using a software by Bionumerics (http://www.applied-maths.com/bionumerics) and only wells with color change of at least one strain were presented.

Antimicrobial activity of L-histidine and L-histidine contained dipeptides. Dose response assays were carried out in 96-wells microplates. *S. cerevisiae* strains A9, M23, V80, 3238-32, 3238-32*Δflo11* and S288c were cultured overnight in 5 mL YPD at 30 °C with shaking. The following day, cultures were refreshed for 4 h before use to reach the exponential phase. A volume of 135 µL of 10^4 cells/mL in 20 % YPD was mixed in each microplate well with 15 µL of 10x concentrated L-histidine from serial two-fold dilutions. Fifteen µL of distilled water instead of L-histidine was added to control wells. All samples were prepared in triplicate.

Same test was repeated using the synthesized dipeptides Histidine-Methionine HM, Histidine-Valine HV and Histidine-Serine HS (GenScript, NJ, USA). Microplates were statically incubated at 30 °C for 48 h. Growth was automatically measured every 30 minutes at OD_{600} using a SPECTROstar nano microplate spectrophotometer (BMG Labtech). Growth rate and lag time of obtained curves were analyzed using DMFit software (Baranyi and Roberts, 1994).

Biofilm formation, adhesion capacity and cell viability in the presence of L-histidine and L-histidine contained dipeptides. Biofilm formation ability of strains A9, M23, V80, 3238-32, 3238-32*Δflo11* and S288c was tested in the presence of HM, HV, HS and L-histidine. Strains were cultured overnight in 5 mL YPD at 30 °C with shaking. The following day, cultures were refreshed in the same medium for 4 h before use to reach the exponential phase, washed twice, than $5x10^6$ cells/mL were prepared in flor medium. Aliquots of 1350 µL were mixed in 24-wells micro plates with 150 µL of 10x concentrated dipeptides or L-histidine stock to a final concentrationof 10 mM; distilled water was added to control wells. Plates were prepared in duplicate and were incubated statically at 30 °C for 5 days. Biofilm weight was measured and calculated as described by Zara et al., 2010 and the cell viability was tested by serial dilution spot test on YPD agar plates.

Yeast adherence to polystyrene was evaluated essentially as described by Reynolds & Fink (2001) with some modifications. Cells cultures were prepared as for the biofilm formation test, than 90 µL of $5x10^6$ cell/mL in flor medium were added in polystyrene microplate wells with 10 µL of 10x concentrated HM, HV, HS and L-histidine solutions, to a final concentrationof 10 mM. Cell suspensions were statically incubated at 30 °C for 48 h. An equal volume of 1% (w/v) crystal violet was then added to each well. After 30 min, the wells were washed with sterile water, and the adherence of the cells was quantified by solubilizing the retained crystal violet in 100 ml 10% (w/v) SDS and an equal volume of sterile water. After 30 min, 50 µL of these

solutions was transferred to fresh polystyrene 96-well plates, and A_{570} and A_{590} were measured spectrophotometrically.

Flow cytometry analysis of the mannose residues in the presence of L-histidine. Concanavalin A is a lectin with a specific binding activity to mannose residues (Touhami *et al.*, 2003). In order to estimate the abundance of mannose residues on the cell wall of strains treated with L-histidine, cells were grown overnight at 30°C in YPD medium with shaking. Next, cells were refreshed in YPD medium for 4 h, in order to reach the exponential phase, than $5x10^6$ cells/mL were incubated for 3 h in flor medium with or without 10 mM L-histidine. Subsequently, cells were washed and resuspended with PBS buffer, pH 7.2 (1.18 g/l of Na_2HPO_4-$2H_2O$, 0.22 g/l NaH_2PO_4, 8.5 g/l NaCl). A volume of 37.5 μL of Concanavalin A lectin labeled with Fluorescein isothiocyanate (ConA-FITC; FITC contents 3.6 mol/mol of lectin; Sigma-Aldrich Co., St. Luis; USA; stock solution 1 mg conjugate/ml) was added and cells were incubated for 20 min at room temperature, in dark. After incubation, samples were immediately analyzed by flow cytometer, by using a BD FACSCalibur™ (BD Biosciences, San Jose, USA). An acquisition protocol of 10.000 cells/sample was defined after measuring the background fluorescence and the maximum fluorescence of each strain, to standardize the fluorescence activity between them. Data were analyzed with the Expo32 software included in the cytometer.

Chitin staining for the detection of cell wall status after L-histidine treatment. Calcofluor white (CFW) is a dye with high affinity to chitin. In this part, CFW staining was used to detect the changing in chitin levels on the cell wall of L-histidine treated cells. Yeast strains were grown overnight in YPD and the following day refreshed in the same medium for 4 h at 30 C, in order to reach the exponential phase (OD_{600} 0.4-0.5). One mL of flor medium containing $5x10^6$ cells/mL was incubated with or without 10 mM L-histidine for 3 h at 30°C. Twentyfive μM of calcofluor white (CFW) were further added for 5 min.

Cells were washed and examined using a Monochrome Fluorescence CCD camera YM10 (BX61 motorized system microscope, Olympus, Tokyo, Japan) with excitation/emission wavelengths of 395/440 nm for CFW detection. Differential interference contrast (DIC) and fluorescence images were captured with 100x objectives using the imaging software Cell* for life science microscopy (Olympus, Tokyo, Japan).

Results

Monitoring of cellular metabolism upon the use of single nitrogen source through the Phenotype Microarray © techniques. Phenotype microarray techniques provide a metabolic high throughput analysis of tested strains. In normal growth conditions, cells must sense nutrients, transport, catabolize and reform them to produce essential small molecule components, polymerize these into macromolecules and create subcellular structures. If all of these processes are working normally, the cell can grow and there will be an actual physical flow of electrons from the carbon source to NADH, down the electron transport chain of the cell, and, ultimately, onto the tetrazolium dye to produce the purple color. If one of these processes is working at a subnormal rate it will become a pinch point, restricting this flow and resulting in an decrease in purple color (Bochner, 2001). Plates were photographed every 15 min, generating a growth curve for each well that primarily reflected dye reduction (Bochner, 2009). In this contest, PM techniques were initially used to make a wide-range screening on the metabolism activity upon 380 nitrogen sources, of flor strains A9, M23 and V80, each with a different *FLO11* genetic background, along with the laboratory strain S288c. The capacity of these strains to metabolize and grow in a minimal medium with different nitrogen substrates was tested in definite 96-wells microplates.

After 96 hr of static incubation, phenotype microarray analysis showed that flor strains A9 and M23 were able to grow, thought to metabolize more nitrogen sources respect to V80 and S288c. These strains metabolized 116, 139, 13 and 39 nitrogen source respectively (Fig. 1). The metabolism rate varied among different compounds and also among strains. Principal component analysis and clustering using the UPGMA (Unweighted Pair Group Method with Arithmetic Mean) of the metabolic capacity divided strains into two groups, A9 and M23 from a part and V80 and S288c from another part (Fig. 1). A9 and M23 but not V80 and S288c, grew slightly on different nucleotides such as Cytosine, Adenine and Allantoin. They also metabolized single L-amino acids such as L-arginine, L-glutamine, L-phenylalanine, L-serine and L-tryptophan. A high metabolism of A9 and M23 was also noted in wells containing dipeptides with mostly Alanine, Valine, Serine and Threonine on their N-terminus. In parallel, all tested strains showed a clear incapacity to metabolize dipeptides with Proline, Asparagine, Cysteine and Lysine at their N-terminus. Interestingly, A9 and M23 were clearly unable to grow in wells with L-histidine contained dipeptides at their C- and/or N- terminus, respect to strains V80 and S288c, which they showed a high and specific growth capacity toward these molecules.

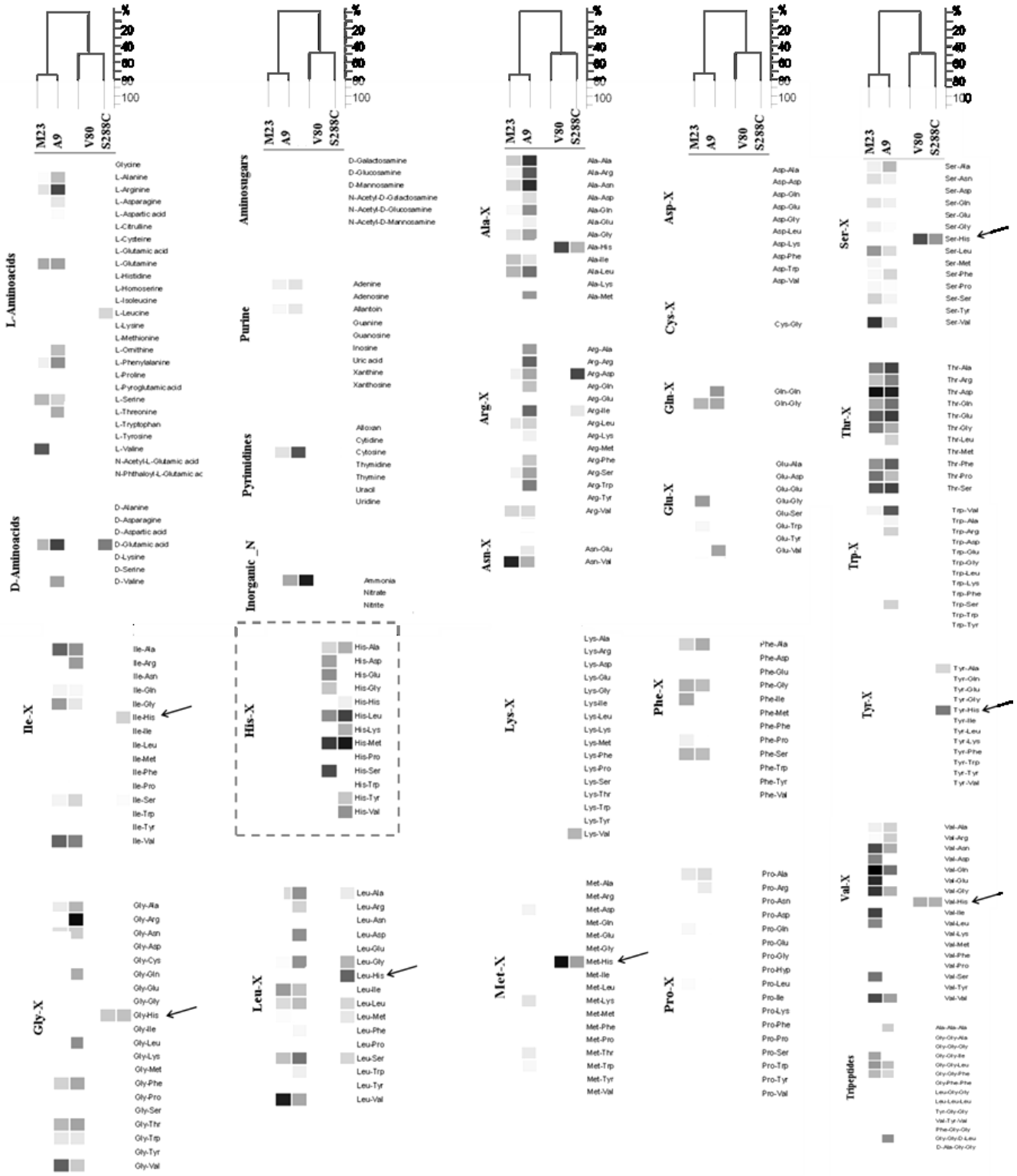
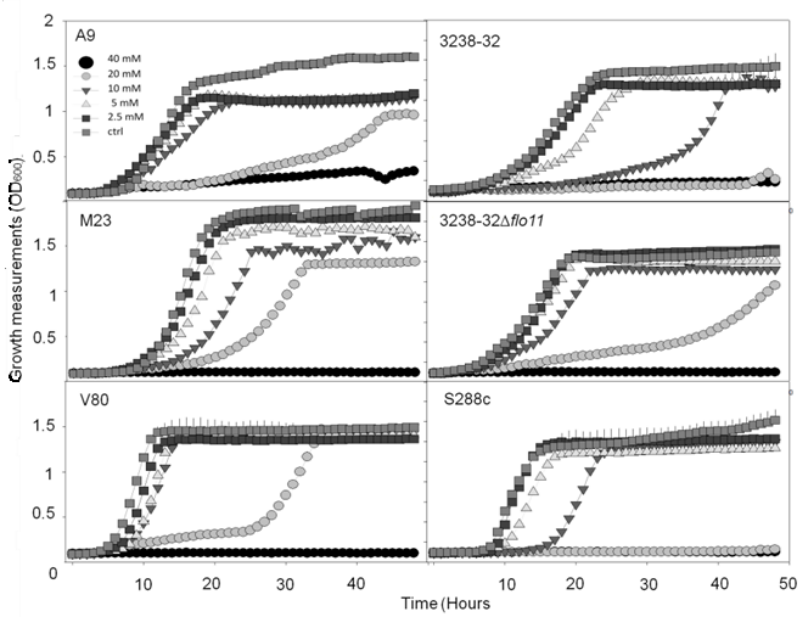


FIG 1 Hightroughput analysis of the nitrogen metabolism of different *S. cerevisiae*. The nitrogen uptake of strains A9, M23, V80 and S288c was measured using the Phenotype Microarray techniques. Each square represents the growth of one strain in the PM wells supplied with the indicated nitrogen source. The extent of growth was generated from the tetrazolium dye reduction during 96 h and represented by the intensity of coloration; white squares mean no growth and dark black squares mean abundant growth. Dipeptides are grouped respect to the N-terminus amino acid. Dashed rectangle and arrows indicate the most evidenced results.

Activity of L-histidine on *S. cerevisiae* yeast strains. Previous PM analysis showed that in the presence of nitrogen compounds, the growth and metabolism rate of strains A9, M23, V80 and S288c varied significantly, mainly in wells containing dipeptides with L-histidine located at both N- or C-terminals. In this part, we applied dye-independent measurements of growth of these strains in nutrient complete medium 20% YPD, with 2-fold serial dilutions of L-histidine or L-histidine contained dipeptides Histidine-Methionine, Histidine-Valine and Histidine-Serine which were synthesized by GenScrpit (NJ, USA) at ≥95 % purity. To better understand the phenotypic effect of L-histidine and the role of *FLO11* gene in response to such growth conditions, the three flor yeasts and S288c were subjected to further analyses together with 3238-32 and 3238-32*Δflo11*.

Growth curves showed that all strains were sensitive to L-histidine. The minimal inhibition concentration MIC of L-histidine was 20-25 mM and the half inhibitory concentration IC_{50} was 10-15 mM toward all strains. Natural flor strains A9, M23 and V80 were slightly more resistant to higher L-histidine concentrations respect to haploid strains 3238-32, 3238-32*Δflo11* and S288c. Notably, concentrations below the MIC value showed that strains V80, S288c and 3238-32*Δflo11* occurred a higher tolerance to L-histidine. At lower concentrations (2.5-10 mM), growth analysis using the DMFit software showed a notable effect of L-histidine on the specific growth rate (μ) and the lag time of treated cells respect to controls. Specific growth rate decreased notably and lag phase increased from 0 to 10 mM of L-Histidine in flor strains A9 (26.54%; 2.41 h), M23 (30.92%; 5.58 h), V80 (15.86%; 2.84 h). Specific growth rate also diminished in haploids strains from 0 to 10 mM; 3238-32 (73.87%; 18.29 h), 3238-32*Δflo11* (65.52%; 15.94 h) and S288c (-3.79%; 9.47 h). At concentrations below 10 mM of L-histidine, the growth rate of strains V80, 3238-32*Δflo11* and S288c was higher than in control wells (Fig.2). L-histidine contained dipeptides compounds had an inhibition effect on all tested strains, at higher concentrations (data not shown).

A



B

	Growth rate inhibition (%)	Lag phase retard (Hrs)	Growth rate inhibition (%)	Lag phase retard (Hrs)
mM	A9		3238-32	
2.5	6.41	0.97	(-2.01)	6.68
5	9.22	1.40	37.53	6.68
10	26.54	2.41	73.87	18.29
20	69.84	16.36	100.02	*
	M23		3238-32*Δflo11*	
2.5	8.40	1.65	(-15.31)	3.79
5	25.09	1.68	(-8.50)	12.17
10	30.92	5.58	71.52	17.94
20	63.27	7.65	100.02	*
	V80		S288c	
2.5	(-1.50)	1.38	(-25.89)	0.66
5	(-0.96)	2.84	(-5.31)	2.86
10	15.86	2.84	(-3.79)	9.47
20	72.14	14.68	98.35	32.00

FIG 2 L-histidine affects the growth of different S.cerevisiae strains in YPD rich medium. Yeast (10^4 cells/mL) in 20% YPD were exposed to different concentrations of L-histidine and incubated statically for 48 h at 30°C. **A)** Each growth curve represents the mean and the ±SD of a triplicate on a specific concentration of L-histidine; 40 mM (black circles), 20 mM (gray circles), 10 mM (black down-pointing triangle), 5 mM (white triangles), 2.5 mM (black square) and 0 mM (gray square). x-axis presents the time in hours and the y-axis presents the cell growth in OD_{600}. **B)** Table generated from growth curves analysis using the DMFit software, and shows the growth inhibition rate (%) and the lag phase retard (Hrs), in the presence of 2.5, 5, 10 and 20 mM of L-histidine. Results with no growth rate inhibition are shown as negative values inside parenthesis.

Changes in air-liquid biofilm formation of flor strains in the presence of L-histidine. After the detection of the inhibition activity and the MIC value of L-histidine, we next tested its effect on the biofilm forming capability of all strains, under biofilm-promoting experimental conditions (i.e., ethanol-rich medium, flor medium). Biofilm formation in *S. cerevisiae* flor strains depends on the presence and expression of *FLO11* gene (Nakagawa *et al.*, 2011). After depletion of sugars and nitrogen sources, further growth slows down and becomes dependent on access to oxygen and other non-fermentable carbon sources (Swinnen *et al.*, 2006; Zara *et al.*, 2010).

Preliminary experiment evidenced an inhibition effect of L-histidine on the formation of biofilm by strains A9, M23 and V80 in flor medium. We therefore set up a quantitative assays in 24-well microplates to exactly understand this effect, and to

measure the biofilm formation following previously described protocols (Zara *et al.*, 2010) (Fig. 3). We also tested the effect of the three dipeptides. Concentrations used of L-histidine and the dipeptides were chosen because were considered as the more informative based on the dose response assays (Fig. 2). Differences were observed in air-liquid biofilm formation after 5 days of incubation of strains A9, M23, V80, 3238-32 treated with 10 mM of L-histidine, when compared to controls wells without amino acid. The biofilm weights of these strains in control wells were respectively 0.78 ±0.043 mg, 0.642 ±0.051 mg, 0.416 ±0.02 mg, 0.562 ±0.073 mg, respect to 0.125 ±0.053 mg, 0.133 ±0.024, 0.112 ±0.047, 0.104 ±0.083 in L-histidine contained wells. Spot test showed a small reduction in colony forming unit (CFU). As expected, S288c and the *flo11* mutant were unable to form air-liquid biofilm in all wells and the CFU concentration was the same in both control and treated strains. Wells with 10 mM of dipeptides showed as well variations in biofilm formation after 5 days. Strains A9, M23 and 3238-32 couldn't form biofilm in the presence of all the three dipeptides, accompanied with a small reduction in the CFU (Fig. 3), similar to the reduction noticed in cells treated with L-histidine. Surprisingly, strain V80 showed, in contrast to other strains, an enhancement of biofilm weight in wells containing His-Met (0.525 ±0.03 mg), His-Val (0.611 ±0.045 mg) and His-Ser (0.64 ±0.037 mg) respect to the control wells (0.416 ±0.02 mg), and spot test showed a slightly enhancement in CFU in dipeptides treated cells respect to the control. S288c and the *flo11* mutant didn't form air-liquid biofilm in all wells and the CFU remained the same between both control and treated strains, with a slightly enhancement in CFU of the 3238-32*Δflo11* strain treated with 10 mM of His-Met.

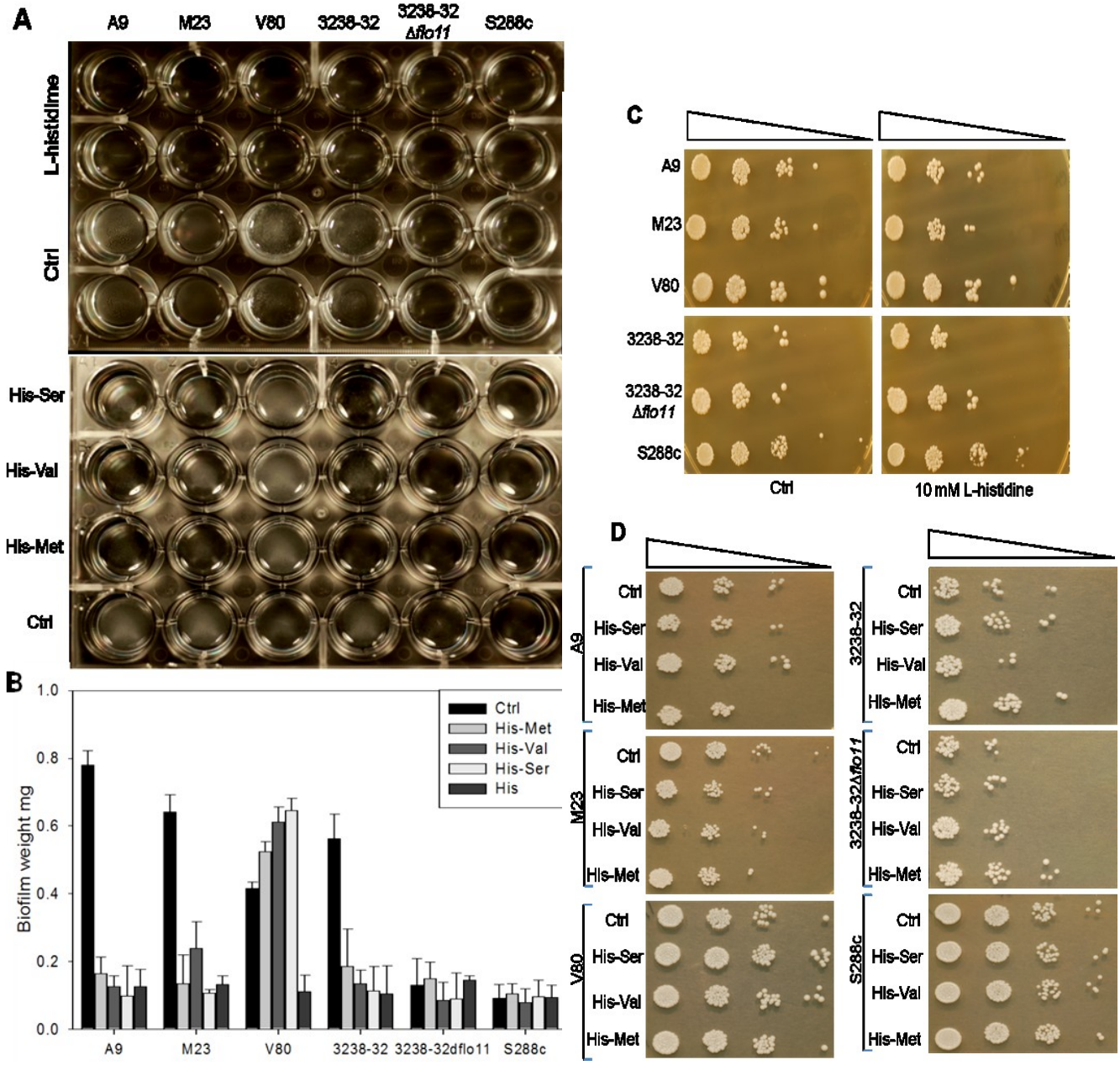


FIG 3 Biofilm formation of flor strains is inhibited in the presence of L-histidine. (A) Biofilm formation at the air-liquid interface in 24-well plates by strains A9, M23, V80, 3238-32, 3238-32*Δflo11* and S288c after 5 days of static incubation in 1.5 mL flor medium at 30°C in the presence of 10 mM of L-histidine and L-histidine contained dipeptides. The biofilm is visualized as opaque floating material at the top of each well. **(B)** Dry weight determination (y-axis) of the biofilm formed by strains in Fig 3 (A) (x-axis) after treatment with 10 mM of L-histidine and L-histidine contained dipeptides (see legend). Mean and SD of two replicate treatments are shown. **(C)** CFU recovery after plating on YPD agar using serial dilutions of a duplicate of all the strains/Histidine and **(D)** all strains/Dipeptides combinations.

Reduction in adhesion ability in flor medium in the presence of L-histidine and L-histidine contained dipeptides. Adhesion ability to polystyrene is an important behavior of yeast, when exposed to stress factors, and it is associated to a functional *FLO11* gene (Zara et al., 2009). Here, the adhesion ability on polystyrene of tested strains was evaluated after 48 h incubation in flor medium with or without L-histidine or dipeptides, in 96 wells polystyrene microplates. Results show a significant difference in adhesion capability between control and treated cells. In general, the adhesion ability of all treated cells was significantly reduced respect to control cells. Indeed, control wells of A9, M23, V80 and 3238-32 strains showed 0.895 ±0.1, 0.407 ±0.07, 0.3 ±0.03 and 1.01 ±0.02 adhesion values (A_{570} nm) respectively; the presence of 5 or 10 mM of L-histidne and L-histidine contained dipeptides induced a loss of adhesion ability (Fig. 4). Beside, both the *flo11* mutant and the S288c laboratory strains showed very low adhesion ability after 48 h in flor medium.

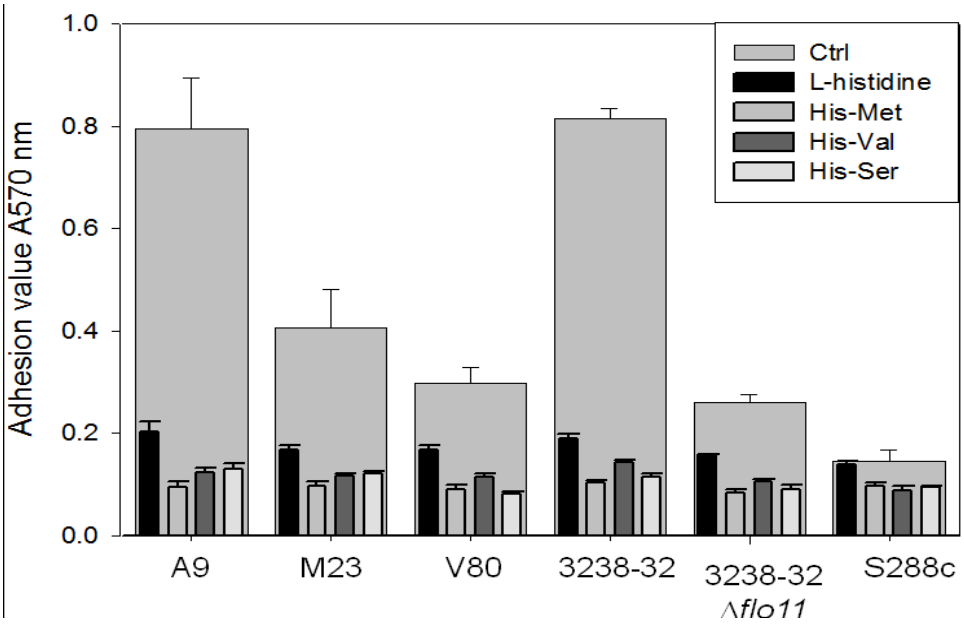


FIG 4 Lost of adhesion ability of tested strains in the presence of L-histidine and other dipeptides. Adhesion values (y-axis) are measured using the crystal violet dye after 48 h of incubation of $5x10^6$ cell/mL of *S. cerevisiae* strains in flor medium with or without 10 mM of L-histidine or L-histidine contained peptides (x-axis)

Remodulation of cell wall in the presence of L-histidine. Cell wall is essential in the adaptation process of yeast cells toward unfavorable environments, by activating the cell wall integrity pathway (Levin, 2005). Cell wall glycans consist of carbohydrate chains of mannose, attached mainly by O-mannosylation to serine and threonine residues of cell wall mannoproteins and known to regulate cell wall rigidity, permeability and biogenesis (Lipke and Ovalle, 1998; Loibl and Strahl, 2013). Here, flow cytometry techniques and the mannose-specific labeled lectin ConA-FITCwere used to estimate the mannosylation level of cell wall mannoproteins, of cells incubated in flor medium and treated with 10 mM of L-histidine, respect to their control without amino acids. Fluorescence measurements of 10,000 events showed an enhancement in concanavalin A binding to cells treated with 10 mM of L-histidine respect to their control without amino acids. Fluorescence intensity, measured by arbitrary units (a.u), of strains A9, M23, V80, 3238-32, 3238-32*Δflo11* and S288c changed between control cells versus L-histidine treated cells as followed: $4{,}6.10^2$ ± 8,5 a.u versus $6{,}2.10^2$ ± 7,2 a.u, $4{,}1.10^2$ ± 36,5 a.u versus $4{,}7.10^2$ ± 68,2 a.u, $2{,}7.10^2$ ± 56 a.u versus $4{,}1.10^2$ ±7,1 a.u, $6{,}7.10^2$ ±126,5 a.u versus $7{,}2.10^2$ ±34,8 a.u, $3{,}9.10^2$ ±18 a.u versus $3{,}7.10^2$ ±12,8 a.u and $1{,}5.10^1$ ± 46,5 a.u versus $3{,}5.10^2$ ± 39 a.u respectively (Fig. 5). The variation of fluorescence intensity reflects directly the variation in cell wall glycans, mainly mannose residues, thought, the enhancement of fluorescence of L-histidine treated cells could be directly proportional to the mannosylation strength of the cell wall compartment.

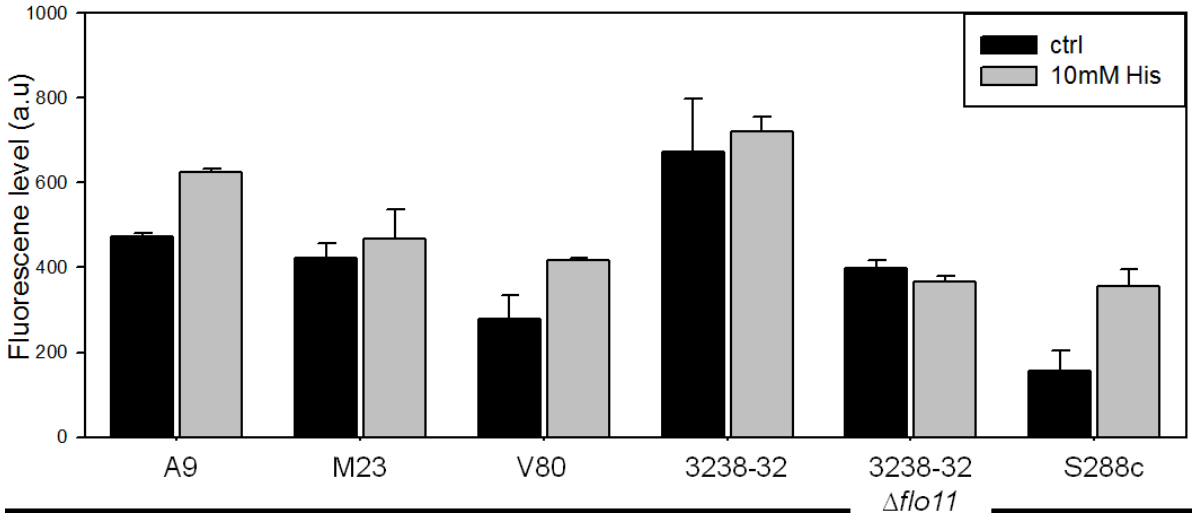


FIG 5 Modulation of cell wall glycans of *S. cerevisiae* strains in the presence of L-histidine. Bar diagrams showing the mean of positive (fluorescently labeled) cell counts of L-histidine treated and control strains A9, M23, V80, 3238-32, 3238-32Δ*flo11* and S288c, after exposure to ConA-FITC conjugate solution as indicated previously. Results show the means and SD of the results from three replicate samples.

Also chitin formation is modulated by the cell wall integrity pathway, as an adaptation response to environmental stresses (Valdivia and Schekman, 2003; Levin, 2011). The variation in chitin level between treated and control cells was microscopically observed using the calcofluor white which is a chitin-specific dye (Watanabe *et al.*, 2005). Under identical exposure and capture parameters, fluorescence microscopy observations showed remarkable differences in staining intensity among strains and between control and L-histidine treated samples. Control cells of strains A9, M23, V80, 3238-32 and S288c showed a low staining. In contrast, strains A9, M23 and 3238-32 showed a substantially increased staining when treated with L-histidine. This enhancement of staining upon L-histidine treatment was absent in strains V80, 3238-32*Δflo11* and S288c. Beside, CFW staining of both treated and control cells of 3238-32*Δflo11*strain was very similar, which could be due to the over production of chitin in mutants affected in CW integrity (Popolo *et al.*, 1997; García-Rodriguez *et al.*, 2000).

These variations in fluorescence levels of both CFW and ConA reveal *(i)* the chitin and glycans production specificity of each strain and *(ii)* the enhancement of their production rate in the presence of 10 mM of L-histidine in flor medium (Figs. 5, 6).

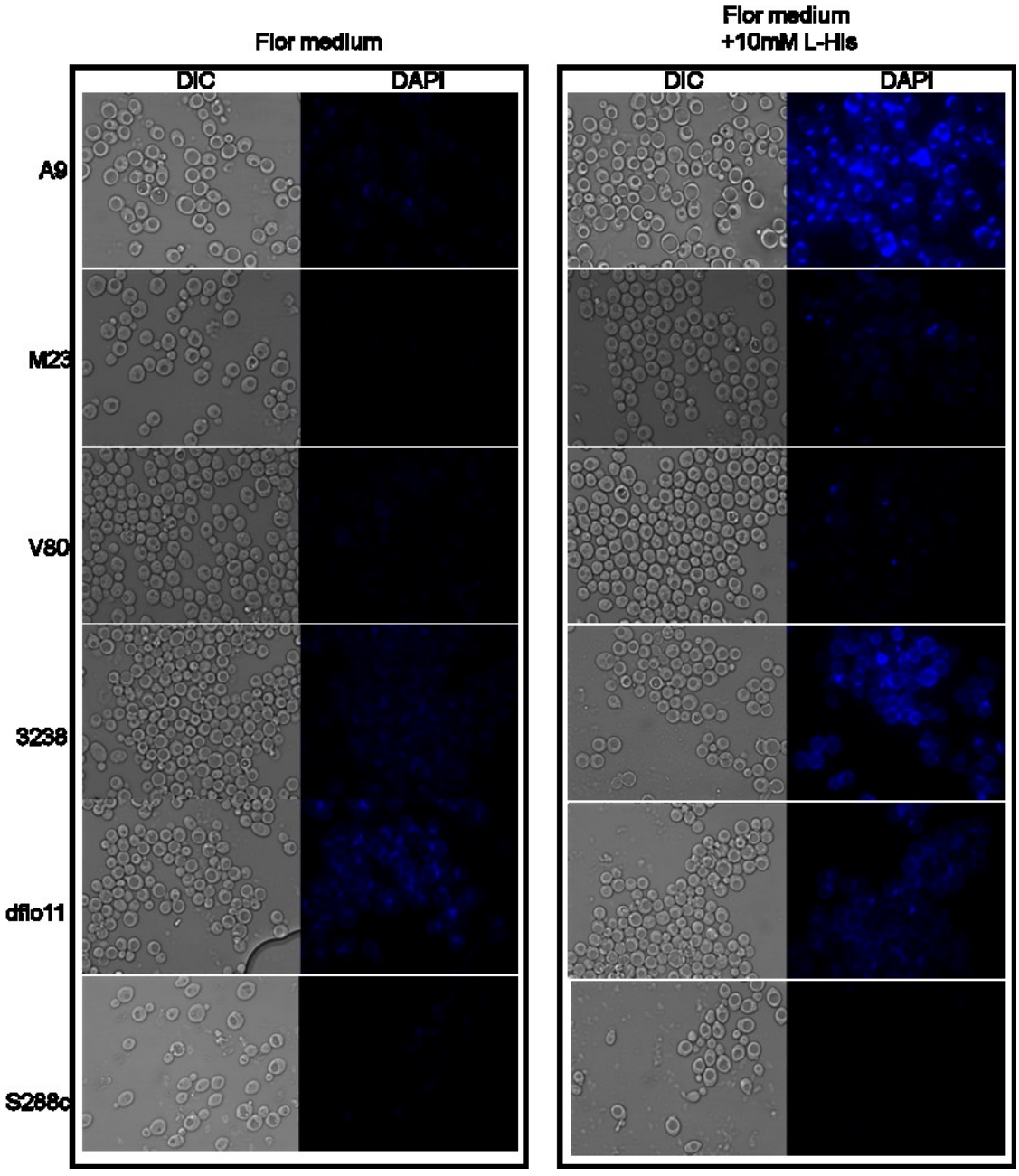


FIG 6 Fluorescence microscopy after CFW staining of different *S. cerevisiae* strains exposed to L-histidine. Cells (10^7 cells/mL) of A9, M23, V80, 3238-32, 3238-32Δ*flo11* and S288c strains were incubated for 2 h in flor medium with or without 10mM L-histidine. After incubation, samples were stained with 25 μM CFW for 5 minutes. DIC bright-field (left) and CFW (right) images of the same field are shown. All the images were captured under the same acquisition parameters and therefore reflect actual differences in CFW staining.

Discussion

S. cerevisiae can use either anaerobic or aerobic mode of metabolism of substrates to generate ATP and grow. Indeed, these metabolisms are shown to confer specific changes to the cell, at the level of cell wall organization, nutrients consumption and cellular interaction with the surrounded environment (Aguilar-Uscanga and Francois, 2003; Cartwright *et al.*, 2012). Flor strains of *Saccharomyces cerevisiae* species have a unique ability to form biofilm at the air-liquid interface of wine, at the end of fermentation, when the medium is depleted of nutrients and the further growth becomes dependent on oxygen. This multicellular growth is directly correlated with a series of rearrangement of the cell wall, hydrophobicity and adhesion (Zara *et al.*, 2009).

In this study, we initially used phenotype microarray analyses in order to observe the general behavior of *S. cerevisiae* strains with different genetic background, upon different nitrogen sources. These analyses, conducted in a minimal medium, highlighted on the high capacity of flor strains A9 and M23 to use different nitrogen compounds as sole nitrogen sources. Clustering of the phenotype microarrays data regrouped A9 with M23 and V80 with S288c. This observation was interestingly reversed in wells containing dipeptides with L-histidine at their N- or C-terminus, where A9 and M23 showed a clear inability to metabolize such molecules, respect to V80 and S288c strains. This particular behavior led to a series of subsequent analysis, concerning the effect of L-histidine on different phenotypic traits of a series of *S. cerevisiae* strains. Results observed reveal a novel role of this amino acid, which is its involvement in the inhibition of the air-liquid biofilm formation by flor yeasts.

Nitrogen is a vital metabolite in living cells and its metabolism is involved in major developmental decisions in *Saccharomyces cerevisiae*. Nitrogen sources are fundamental for vital purposes such as nucleotides and amino acids formation, thus,

S. cerevisiae is able to metabolize different nitrogen sources (Forsberg and Ljungdahl, 2001).

The capacity of flor strains A9 and M23 to metabolize a wide range of nitrogen sources, respect to the lab strain S288c, reflects the wild-type origins of these two strains and their adaptation capacity. Similar observations were reported in a previous study where wild-type isolates (clinical and vineyard) were able to grow on a wide range of nitrogen sources respect to laboratory strains (Homann *et al.*, 2005). This was not the case of V80 strain, which is also a wild-type flor strain. In a previous study Zara et al., 2009 observed that in nitrogen depleted medium, A9 and M23 strains expressed a high transcription level of *FLO11* gene, respect to V80 strain. This observation can potentially correlate with the two groups provided by the statistical analyses of the PM metabolism data, where V80 metabolism was similar to the S288c, which carries a *flo8* mutation, leading to the inactivation of its *FLO11* gene (Liu *et al.*, 1996). Beside, in nitrogen starvation environment, signaling pathways TORC1, SPS-sensor and GAAC, which are largely related to nitrogen and amino acids sensing and regulation in different *S. cerevisiae* spp, were also shown to be involved in *FLO11* gene expression and multicellular growth (Ljungdahl and Daignan-Fornier, 2012).

Despite they capacity to metabolize different nitrogen sources in minimal medium, flor strains A9 and M23 were clearly unable to metabolize dipeptides with L-histidine at their N- or C-terminus, respect to V80 and S288c, and all tested strains didn't grow even on L-histidine. This behavior was also previously observed in another PM study (Homann *et al.*, 2005). Subsequently, in order to better understand the effect of L-hisitidine, we handled a series of phenotypic analyses also using the isogenic strains 3238-32 and its flo11 mutant, both haploids and derivatives of A9, as a direct control for functional *FLO11* gene involvement. Dose response analysis in nutrient rich medium, showed that, L-histidine not only doesn't support the cellular growth as a nitrogen source, but its presence at high concentrations (≥10 mM)

reduces the growth rate, delays the lag-phase and finally inhibits the growth of tested strains.

These effects were absent in strains treated with same concentrations of L-histidine containing dipeptides. Recent study reported the effect of the L-carnosine (L-histidine containing dipeptide) in slowing the growth rates and increasing cell death of yeast cells in fermentative, but not oxidative metabolism (Cartwright *et al.*, 2012).

The most evidenced results of this study are related to the biofilm formation and adhesion capacity. In nutrient depleted media, *S. cerevisiae* can trigger a series of stress signaling pathways and responses, including the modulation of cell wall, expression of *FLO11* gene and to the formation of biofilm (Reynolds, 2001; Barrales *et al.*, 2008; Zara *et al.*, 2010). This phenomenon was observed in control wells of biofilm forming strains A9, M23, V80 and 3238-32 in flor medium, but not in wells containing L-histidine. The presence of 10 mM of L-histidine was sufficient to completely inhibit the biofilm formation and the adhesion capacity to polystyrene of all tested strains (Fig. 3A, 4). These major inhibition effects were accompanied by a minor reduction of cell viability (Fig. 3C, D). As mentioned before, cellular adhesion and binding are likely influenced by unspecific interactions such hydrophobic and electrostatic interactions (Caridi, 2006; Holle *et al.*, 2011; Kregiel *et al.*, 2012). Among the 20 amino acids, the particular physiochemical characteristics of L-histidine, being as a cationic charged amino acid with an imidazole motif at the side chain, make it a good candidate for unspecific interactions, mainly stacking and hydrogen bonds interactions, providing it a high affinity to cationic metals, aromatic amino acids and many other compounds (Shimba *et al.*, 2003; Liao *et al.*, 2013).

The inability of cell to grow on L-histidine as a nitrogen source (LaRue and Spencer, 1967; Homann *et al.*, 2005), the particular physiochemical features of L-histidine (Liao *et al.*, 2013), along with the loss of cells adhesion and biofilm formation capacity by flor strains in the presence of L-histidine (Fig. 3 and 4), may be explained by a

possible unspecific physical interactions between the single amino acid L-histidine and the high folded cell wall in general, and the highly O-mannosylated cell wall mannoprotein Flo11p in particular, which leads to the air-liquid biofilm formation failure. Indeed, the elevated fluorescence of cell wall glycans and chitin in cells treated with 10 mM of L-histidine, reflect the stress response of these cells upon its presence. Cell wall glycans and chitin are mainly responsible of the permeability of the cell and are related to the cell wall integrity pathway, as a response to adverse conditions (Lipke and Ovalle, 1998; Latge, 2007). The enhancement of these two cell wall compounds in L-histidine treated cells reveals its antimicrobial effect and reduces the permeability of the cell. The low permeability of the cell wall is in favor of the hypothesized unspecific interactions of L-histidine with the cell wall mannoproteins.

Of course, the molecular mechanism of this novel role of L-histidine is still unknown. Many studies showed a similar mode of action of several small cationic peptides sequences, with antimicrobial effect toward different fungi species such as Human Histatins and Histidine rich glycoproteins which are directly involved in the host response to *Candida albicans* invasive growth, by binding to the cell wall glycoprotein Msb2p (Szafranski-Schneider *et al.*, 2012). A similar anti-adhesive behavior was reported of the filastatin compound against some *Candida* species (Fazly *et al.*, 2013). In contrast, hydrophobic interactions with the synthetic hexapeptide PAF26 served as a bridge between some *S. cerevisiae* flor strains, enhancing the biofilm formation (Bou Zeidan *et al.*, 2013). To our knowledge, no previous studies have reported this mode of action of L-histidine or any other amino acid. Interestingly, a recent study described a novel role of some D-amino acids in triggering different bacterial biofilm disassembly. These D-amino acids didn't affect the growth rate of bacterial cultures, and their mode of action was associated to their incorporation into the peptide side chains of the cell wall peptidoglycan (Kolodkin-Gal *et al.*, 2010). This highlights the important role of unspecific interactions in adhesion and biofilm formation process.

These findings reveal the importance of unspecific interactions of yeasts cell wall in cells interactions with their microenvironments, even with simple molecules like amino acids, which could be direct factors or cofactors in changing crucially considerable phenotypes, such as biofilm formation and adhesion.

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ANNEX

Considering the important physiochemical characteristics of Flo11p and L-histidine, and based on the results and discussions reported in the study above, we thought that it would be worth-full to observe the cellular behavior of the 3238-32 strain and its *flo11* isogenic mutant, grown in minimal medium, at pH 3, 4, 5 and 6, in the presence of 5 mM of L-histidine. The same experiment was replicated with the same growth conditions, and the cell surface net charge (Z-potential) was measured using a Zetasizer Ver. 6.20 "Malvern Instruments Ltd". A the same time, we used an Histidine-Histidine dipeptide labeled with Tetramethylrhodamine (TMR-HH) (GenScript, NJ, USA), and we observed its interaction with 3238-32 and 3238-32*Δflo11* using the fluorescence microscopy techniques. Taken together, results obtained highlight on *FLO11* in modulating the cell growth, surface charge and physical interactions with the embedded environment in response to pH variation.

In details, growth assays showed that, using L-histidine as a sole nitrogen source, cell growth and cell surface net charge of 3238-32 and its *flo11* isogenic mutant changed depending on the pH. At low pH, 3238-32 showed a low growth ability, which was gradually improved in correlation with the enhanced pH, to reach the maximum growth at pH 6. This was not the case of the *flo11* mutant, which showed stable and high growth ability along all tested pHs. We also noted that at pH 6, both strains showed similar growth (Fig. 1). The cell surface net charge of 3238-32 also varied; at pH 3, it was slightly positive (+0.1 ± 0.062 mV) respect to 3238-32*Δflo11* (-3.04 ± 0.142 mV). The cell surface net charge of 3238-32 got more anionic progressively with the pH increase, to reach a potential of -8.5 ±0.087 mV at pH 6, respect to a stable charge of 3238-32*Δflo11* cell surface (-4.22 ±0.081). So, the enhancement of the anionic charge occurs with an enhancement of the growth ability of 3238-32 strain along with the pH.

This could be explained by potential repulsive interactions between the cell surface of 3238-32 and L-histidine, which demolishes at higher pHs and leads to the attraction and assimilation of L-histidine. In the case of *flo11* mutant, the unvaried anionic charge of the cell surface was accompanied with unvaried growth ability among the pH variation, which could be related to the stable attractive interaction and assimilation of L-histidine. The charge of L-histidine also varies among different pH. According to the *Henderson-Hasselbalch* equation, the net charge of L-histidine is +1.06 at pH 3 and decreases among pH to +0.51 at pH 6.

Here, we used the TMR-HH labeled dipeptide to observe by fluorescent microscopy its interactions with 3238-32 and 3238-32*Δflo11* cells, in minimal medium, at pH 3 and pH 6. These pHs were considered as the most representatives. The idea was to label a single L-histidine, but we found to be very difficult to obtain a stable labeling of single amino acids, therefore, we proceeded with the TMR-HH. Fluorescence microscopy observations after 2 hours of incubation of strains with 1 mM of TMR-HH, and subsequent incubation with 25 μM CFW, showed that the staining emitted was low and constant between both strains at pH 3. This fluorescence intensity was notably enhanced in wild type strain, but not in the *flo11* mutant at pH 6. The CFW staining was used as a cell surface marker, to control the internalization or not of the TMR-HH.Taking in consideration *I)* the growth and cell surface charge of the 3238-32 strain with functional *FLO11* at low pH, *II)* the gradually enhancement of 3238-32 growth and anionic charge of the cell surface with the enhancement of the pH, *III)* the growth and cell surface charge stability of the *flo11* mutant along all tested pH, *IV)* the physiochemical of L-histidine and *V)* the enhanced/stable interactions of 3238-32/3238-32*Δflo11* with TMR-HH respectively, we speculate that *FLO11* gene is directly involved in the modulation of the cell surface net charge, depending on the pH variation, which leads to a modulation of cell adhesion and interaction abilities with the surrounded environments.

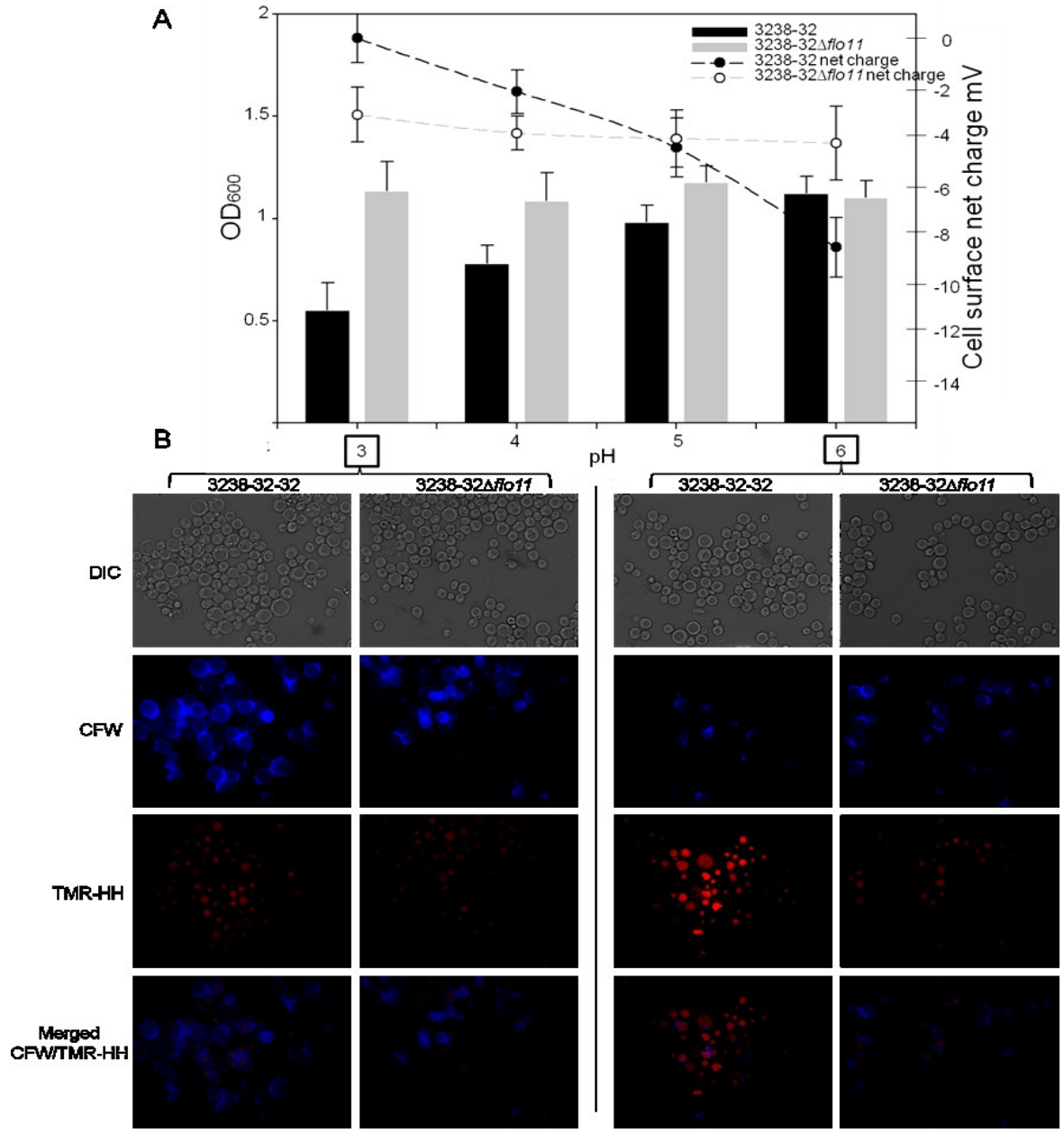

FIG 1 Cell growth, cell surface charge and cellular interactions of 3238-32 and *flo11* mutant in minimal medium on different pH. A) Growth and cell surface net charge measurements of *S. cerevisiae* strains 3238-32 and 3238*Δflo11* in minimal medium plus 5 mM of L-histidne, at different pH**.** Black/gray bars and black/gray dashed lines represent the growth and the cell surface net charge of 3238-32 and 3238*Δflo11* respectively, at pH 3, 4, 5 and 6. Cells (10^4 cells/mL) were incubated in minimal medium (0,17% Yeast Nitrogen Base w/o amino acids and ammonium sulfate; 0.5% ammonium sulfate and 20 mM of glucose; auxotrophic amino acids (Zara *et al*., 2005)) plus 5 mM of L-histidine. Media were buffered at pH 3, 4, 5 and 6 using aliquots of 0.1 M citric acid monohydrate ($C_6H_8O_7.H_2O$) and 0.2M sodium phosphate (Na_2HPO_4) stock solutions http://www.sigmaaldrich.com/life-science/core-bioreagents/biological-buffers/learning-center/buffer-reference-center.html. Cells were grown in 96 wells microplates, statically at 30°C for 48 h. Growth was monitored measuring the OD_{600} in a SPECTROstar nano microplate spectrophotometer (BMG Labtech, Germany). Cell surface net charge (Z-potential) was measured with same conditions using the "Zetasizer Ver. 6.20 Malvern Instruments Ltd" after 48 hr of treatment. All measurements represent the mean value and the SD of three replicates. **B)** Fluorescence microscopy of *S. cerevisiae* strains 3238-32 and 3238-32*Δflo11* exposed to TMR-labeled His-His. Cells (10^7 cells/ml) were incubated in minimal medium with 1 mM of TMR-His-His at 30°C for 2 h and subsequently with 25 µMCFW at 20°C for 5 min. Representative DIC bright-field as well as CFW, TMR, and CFW/TMR-overlay fluorescence micrographs of the same field are shown, for the different strains, as indicated.

CHAPTER III

Marc Bou Zeidan, Phenomic analysis and cell wall remodulation of *Saccharomyces cerevisiae* flor strains in the presence of different nitrogen sources, Tesi di dottorato in Biotecnologie Microbiche Agroalimentari, Università degli studi di Sassari.

The biofilm formation and adhesion ability of *Saccharomyces cerevisiae* flor yeasts are affected by the presence of amino acids

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Saccharomyces cerevisiae flor yeasts are able to form a particular air-liquid biofilm, called flor or velum, as a response to environmental stresses, mainly to nutrients depletion. Flor yeast strains offer an innate diversity of CW-related phenotypes like hydrophobicity, cell-cell and cell-surface adhesion, associated with a functional Flo11 protein. In this study, we tested the effect of 12 amino acids with different physiochemical characteristics, on cellular growth of flor yeast strains, characterized by different *FLO11* gene length and expression. Growth analysis showed variability in the effect of amino acids toward strains. Phenotypes related to biofilm formation and adhesion to polystyrene were the most influenced by the presence of amino acids. Cationic and sulphuric amino acids showed the highest inhibition activity. On the contrary, the presence of hydroxylic amino acids slightly enhanced the biofilm formation.

Keywords: Biofilm formation; amino acids metabolism;

Introduction

Saccharomyces cerevisiae yeast can shift from anaerobic to aerobic metabolism, depending on the surrounded environment. In general, sugar presence in a medium is followed by rapid fermentative growth of *S. cerevisiae*, with the production of ethanol. Yeast cells provided with an appropriate source of carbon and nitrogen can synthesize all L-amino acids used in protein synthesis (Ljungdahl and Daignan-Fornier, 2012). When the fermentable sugar is exhausted, yeast cells swift to ethanol as a carbon source for fully respiratory metabolism (Murray *et al.*, 2011).

This swift is known as diauxic shift, and is correlated with widespread changes in genes expression involved in fundamental cellular processes such as carbon metabolism, protein synthesis, and carbohydrate storage (Galdieri *et al.*, 2010). In some yeasts this shift is followed by a cell wall remodulation and the expression of *FLO11* gene, which encodes for a high hydrophobic and cell wall mannoprotein, leading to cell-cell and cell-surface adhesions and biofilm formation and higher resistance to physical and chemical stress agents (Verstrepen and Klis, 2006; Šťovíček *et al.*, 2010). In such conditions, flor yeast strains of *S. cerevisiae* have a particular ability to form an air-liquid biofilm and start using alternative nutrient sources for further growth (Ishigami *et al.*, 2006; Zara *et al.*, 2010). Therefore, the ability of yeast cells to grow and interact with different sources changes crucially, depending on their mode of metabolism (Barnett, 2003; Wu *et al.*, 2004). In general, the ability of cells to benefit of the biological role of amino acids requires the sensing and internalization of these compounds. In *S. cerevisiae*, it has been noted that extracellular amino acids are sensed by the plasma membrane localized Ssy1-Ptr3-Ssy5 (SPS) sensor, which was recently shown to be involved also in *FLO11* transcription, in amino acid starvation medium (Ljungdahl, 2009; Bruckner and Mosch, 2012). This sensor activates the transcription of general and/or specific permeases, which in turn facilitate the transport of amino acids across the plasma membrane. All the 20 amino acids could be internalized into *S. cerevisiae* cells and are qualified as preferred or good (Glutamate, Aspartate, Serine, Alanine, Arginine…), non-preferred or bad (Threonine, Leucine, Proline, Methionine…), or not nitrogen sources (Histidine, Lysine and Cysteine) (Homann *et al.*, 2005; Godard *et al.*, 2007). *S. cerevisiae* exhibits nitrogen catabolite repression (NCR) in which preferred nitrogen sources repress specific expression of genes required for uptake and catabolism, respect to less preferred nitrogen sources which are uptake by the general amino acid control GAAC (Magasanik and Kaiser, 2002; Hinnebusch, 2005).

Once internalized, the nitrogen of preferred amino acid is incorporated into glutamate, and the resulting carbon skeletons can yield pyruvate (Alanine and Serine), tricarboxylic acid cycle intermediates oxaloacetate (Asparagine and Aspartate) or α-ketoglutarate (Glutamate and Glutamine). In parallel, nitrogen from non-preferred, branched-chain, aromatic and/or sulphuric amino acids, is transferred to α-ketoglutarate by transaminases forming glutamate. The resulting deaminated carbon skeletons are converted to non-catabolic and growth-inhibitory fusel oils (Hazelwood *et al.*, 2008). Glutamate and Glutamine are very important compounds in amino acids biosynthesis, because ≈85% of the total cellular nitrogen is incorporated via the amino nitrogen of glutamate, and the remaining 15% is derived from the amide nitrogen of glutamine (Hans *et al.*, 2003; Ljungdahl and Daignan-Fornier, 2012).

In this work, we tested the effect of amino acids with different physiochemical properties, on the cellular growth of a series of *S. cerevisiae* strains upon glucose or ethanol and on biofilm formation and adhesion capacity of different flor strains. We found that the presence of different amino acids in flor forming medium may enhance or reduce the biofilm formation and adhesion ability of tested yeasts.

Materials and methods

Strains and media. Strains with different genetic backgrounds were used in this study (table 1). A9, M23 and V80 are natural flor strains, previously isolated from different wineries in Sardinia and genetically characterized. These tested natural strains have different *FLO11* gene length and expression level (Zara *et al.*, 2009b). A series of haploid strains derivatives of A9 were also included in this work; 3238-32 is an haploid wild-type flor strain and 3238-32*Δflo11* is its *FLO11* mutant (Zara *et al.*, 2005) . Other strains are 3238-32*Δras2* that carries a mutation on the *RAS2* gene which encodes for a GTP-binding protein involved in the nitrogen starvation response

(Zara *et al.*, 2011), 3238-32*Δsnf1* carries a mutation on the *SNF1* gene which encodes for an AMP-activated serine/threonine protein kinase, required for transcription of glucose-repressed genes (Sanz, 2003), S288c lab a strain that has a knockout *flo8* gene mutant which disables *FLO11* expression (Mortimer and Johnston, 1986). Strains were cultured at 30°C in YPD medium (1% yeast extract, 2% peptone, 2% glucose), 20% YPD medium (0.2% yeast extract, 0.4% peptone, 0.4% glucose) and in flor medium (0.17% of YNB without ammonium sulphate and amino acids, 0.5% ammonium sulphate, 4% of EtOH) which was supplemented when necessary with bases and amino acids at standard concentrations.

TABLE 1 *Saccharomyces cerevisiae* strains used in this study.

Strains	Genetic background	Reference
A9	Wild flor strains of S. cerevisiae isolated from Arvisonadu wine	Zara et al., 2009
M23	Wild flor strains of S. cerevisiae isolated from Malvasia wine	Zara et al., 2009
V80	Wild flor strains of S. cerevisiae isolated from Vernaccia wine	Zara et al., 2009
3238-32	*MATα leu2-Δ1 lys2-801 ura3-52*	Zara et al., 2005
3238-32*Δflo11*	*MATα leu2-Δ1 lys2-801 flo11Δ::URA3 ura3-52*	Zara et al., 2005
3238-32*Δras2*	*MATa leu2-Δ1 lys2-801 ura3-52 ras2::kanMx4*	Zara et al., 2011
3238-32*Δsnf1*	*MATa leu2-Δ1 lys2-801 ura3-52 snf1::kanMx4*	Dept. collection
S288c	*MATα SUC2 gal2 mal mel flo1 flo8-1 hap1 ho bio1 bio6*	Mortimer and Johnston, 1986

Dose response test of the effect of amino acids on cellular growth in glucose rich medium. As a preliminary test, the growth ability of tested strains was measured in glucose rich medium YPD20%, in the presence of serial dilutions of 12 amino acids. Amino acids used in this work have different physiochemical features. Lysine, Arginine and Histidine represent basic and positively charged amino acids. Serine and Threonine are hydroxylic amino acids. Methionine and Cysteine contain sulphur residue on their side chain. Proline, Tryptophan and Phenylalanine contain aromatic side chains and Leucine and Valine contain aliphatic side chains. Amino acids stocks were prepared at 10x (400 mM each) and diluted with H_2O before use. High temperatures were used for dissolving solutions if necessary.

Cellular growth assays were handled in 96-well micro-titer plates. *S. cerevisiae* strains were cultured for overnight in 5 ml YPD medium at 30°C with shaking. The following day, cultures were refreshed in YPD medium for 4 h at 30°C in order to reach the exponential phase ($OD_{600} \approx 0.4$ to 0.5). A volume of 180 µL of 10^4 cells/ml in 20% YPD medium was added to each micro-plate well with previously supplemented 20 µL of 10x-concentrated amino acids solution from serial 2-fold dilutions; 20 µL of H_2O instead of amino acids was added to control wells. All samples were prepared in triplicate. Micro-plates were statically incubated at 30°C for 48 h. Growth was measured every 2 h at OD_{600} using a SPECTROstar nano micro-plate spectrophotometer (BMG Labtech; Germany). Dose-response curves were generated from measurements after 48 h (Fig. 1).

Biofilm formation, adhesion capacity and cell viability in the presence of amino acids. For the air-liquid biofilm formation test, strains were grown overnight in YPD medium at 30°C with shaking. Next day, cultures were refreshed for 4 h before use to reach the exponential phase, washed twice with sterile water, and suspensions of $5x10^6$ cells/ml were prepared in flor medium. Nine hundreds µL aliquots were mixed in wells of 24-well micro-plates with 100 µL of 10x-concentrated amino acids to reach a final concentration of 5, 10 and 20 mM; 100 µL of sterile H_2O was added to control wells. Plates were incubated statically at 30°C for 5 days. All plates and conditions were prepared in triplicate. Biofilm dry weight measurement was carried out essentially as described previously (Zara *et al.*, 2010). Replicates of this experimental design were used to measure the cell viability of samples treated with 10 mM of different amino acids, after 5 days of incubation in flor medium. Samples were spotted on YPD agar plates to recover the colony forming units CFU. Yeast adherence to polystyrene was evaluated essentially using the method of Reynolds & Fink., (2001) with some modifications.

Cells cultures were prepared as for biofilm formation test. A volume of 90 µL of $5x10^6$ cell/mL in flor medium was added in polystyrene micro-plate wells with 10 µL of 10x concentrated amino acids, to have a

final concentrations of 5, 10 and 20 mM. Cell suspensions were statically incubated at 30 °C for 48 h. An equal volume of 1% (w/v) crystal violet was then added to each well. After 30 min, wells were washed with sterile water, and cells adherence was quantified by solubilizing the retained crystal violet in 100 µL of 10% (w/v) SDS and an equal volume of sterile water. After 30 min, 50 µL of these solutions was transferred to new polystyrene 96-well plates, and A_{570} and A_{590} were measured using a SPECTROstar nano micro-plate spectrophotometer (BMG Labtech; Germany).

Results

Activity of tested amino acids on *S. cerevisiae* growth in Glucose rich medium. In 20 % YPD medium with available carbon and nitrogen sources, such as glucose and ammonia, *S. cerevisiae* yeasts is able to growth and synthesize all the needed amino acids for protein folding (Ljungdahl and Daignan-Fornier, 2012). Many studies reported the effect of the supplementation of several amino acids on the final quality of fermented products, such as beer and wine (Krogerus and Gibson, 2013; Lei *et al.*, 2013). In this work, the growth ability of a series of *S. cerevisiae* strains was tested in a nutrient-rich medium, in the presence of amino acids at different concentrations. Cell growth showed a notable variability between strains and amino acids at different concentrations. Dose response analyses after 48 h of static incubation at 30°C, showed that the presence of amino acids whether inhibited, or not the cellular growth of treated strains. In details, dose response graphs (Fig. 1) showed that cationic amino acids L-arginine, L-lysine and L-histidine exerted a growth inhibition effect on all tested strains, with L-arginine showing the highest effect, at 2.5 mM for strains 3238-32 and isogenic mutans *3238-32Δflo11*, *3238-32Δras2*, *3238-32Δsnf1* and 5 mM for A9, M23, V80 and S288c.

L-lysine also showed an inhibitory effect of 5 mM on 3238-32 and isogenic mutants and 10 mM on A9, M23, V80 and S288c. L-histidine also inhibited the growth of tested strains but at higher concentrations, with a MIC of 10 mM on 3238-32 and isogenic mutants and 25 mM on A9, M23, V80 and S288c strains. Growth inhibition was also observed in wells including the sulphuric amino acid L-cysteine, at 10 mM for all strains, except A9, which growth was inhibited at 20 mM. L-methionine, which is also a sulphuric amino acid, inhibited the growth of 3238-32 and isogenics at 20 mM.

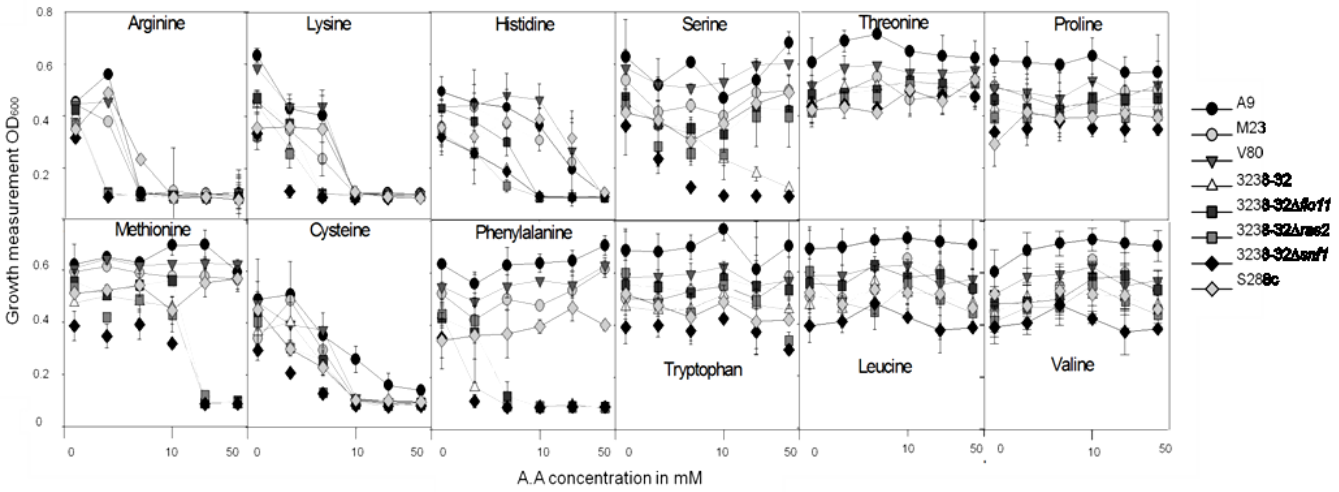

FIG 1 Dose response of *S.cerevisiae* strains upon different amino acids. Yeast cells (10^4 cells/ml) in 20% YPD were exposed to different concentrations of amino acids and incubated at 30°C. Dose-response curves show means and the standard deviations of three replicates of OD_{600} measurements after 48 h of inoculation. The *S. cerevisiae strains* represented are A9 (black circles), M23 (Gray circles), V80 (reversed triangle), 3238-38 (light gray triangle), 3238-32*Δflo11* (dark gray square), 3238-32*Δras2* (light gray square), 3238-32*Δsnf1* (black rhombus) and S288c (gray rhombus)

From another part, strains growth was stable among all concentrations tested of hydroxylic amino acids L-serine and L-threonine. Also aromatic amino acids such L-tryptophan and L-proline didn't affect the cellular growth respect to control cells. In particular, the presence of L-phenylalanine inhibited the growth of 3238-32 and isogenics at 5 mM, but not other strains.

Values in the table 2 reports the growth in OD_{600} of all tested strains in YPD20% with 10 mM of amino acids combinations. Effects of the amino acids on cell growth was divided into "growth inhibition effect" or "no effect".

Table 2: Growth mean values in OD_{600} ±SD of *S. cerevisiae* strains incubated in glucose-rich medium plus 10 mM of a series of amino acids

Ctrl	0.628± 0.083	0.537 ±0.105	0.583 ±0.067	0.455 ±0.122	0.471 ±0.075	0.442 ±0.089	0.365 ±0.073	0.589 ±0.11
Arg	0.096 ±0.182	0.111 ±0.014	0.093 ±0.003	0.087 ±003	0.092 ±0.002	0.084 *±0.004*	0.086 ±0.004	0.086 ±0.003
His	0.364 ±0.065	0.306 ±0.038	0.461 ±0.069	0.087 ±0.002	0.086 ±0.001	0.089 ±0.007	0.091 ±0.007	0.387 ±0.044
Met	0.696 ±0.034	0.575 ±0.01	0.625 ±0.002	0.459 ±0.014	0.56 ±0.004	0.429 ±0.067	0.318 ±0.01	0.446 ±0.053
Thr	0.648 ±0.03	0.463 ±0.005	0.566 ±0.099	0.531 ±0.004	0.5355 ±0.003	0.520 ±0.122	0.495 ±0.007	0.501 ±0.02
Phe	0.638 ±0.043	0.476 ±0.014	0.572 ±0.021	0.089 ±0.007	0.085 ±0.007	0.088 ±0.001	0.084 ±0.009	0.395 ±0.019
Leu	0.667 ±0.116	0.511 ±0.047	0.57 ±0.066	0.427 ±0.035	0.515 ±0.075	0.399 ±0114	0.382 ±0.084	0.367 ±0.026

Biofilm formation and biofilm weight in the presence of amino acids. Recent studies demonstrated that nitrogen and amino acids sensing pathways are also involved in the regulation of *FLO11* expression, thus, in biofilm formation process (Brückner and Mösch, 2012). Since flor yeast strains offer an innate diversity of CW-related phenotypes associated with the abundant Flo11 protein, the effect of 12 amino acids was tested on a series of *S. cerevisiae* flor strains with distinct genetic backgrounds, lengths, and structure of *FLO11* gene and capabilities to form biofilm under biofilm-promoting experimental conditions (i.e., ethanol-rich medium)(Zara *et al.*, 2009).

Results obtained from dose response tests (Fig. 1) provided a range of representative concentrations, of the effect of tested amino acids on cell growth. For biofilm formation tests, we initially screened the effect of a series of amino acids at 5, 10 and 20 mM on the biofilm formation ability of the flor forming strains, in flor medium (Fig. 2A). Wells with 10 mM were considered as the most representatives, so biofilm weights, cell viability and adhesion capacity were studied at this amino acids concentration. Results showed a variation in the biofilm formation phenotype between amino acids and control wells, after 5 days on static incubation (Fig.2A). Amino acids presence either reduced or enhanced or did not affect biofilm formation and weight. Biofilm weight graph represents the variation in the biofilm weight in percentage (%), respect to the control biofilm weight without amino acids, of each tested strain (Fig. 2B). Arginine, Lysine and Histidine inhibited the biofilm formation of all tested strains, respect to their controls without amino acids. The biofilm weight in wells containing these amino acids was reduced of 81%, 56% and 70% respectively, respect to the control. In addition, sulphuric amino acids L-cysteine and L-methionine reduced 83% and 40% respectively the biofilm weight of treated cells. Aromatic amino acids such L-tryptophan and L-phenylalanine, reduced the biofilm weight of all strains of 31% and 23% respectively, except in A9 strain where the L-phenylalanine presence slightly enhanced the biofilm weight of 12%. L-proline presence showed a notable and variable enhancement in biofilm weight of all treated cells, respect to their control without amino acids. This enhancement was less evident in wells with A9 and 3238-32 and 3238-32*Δras2* (20%, 15% and 18% respectively), respect to an average enhancement of 70% in other strains. This enhancement in biofilm weight was also observed in wells containing the hydroxylic amino acids L-serine and L-threonine, 36% and 13% respectively. At the end, the aliphatic amino acid L-leucine enhanced slightly the biofilm weight of all treated cells, about ≈15% respect to control wells, except for 3238-32*Δsnf1* which enhanced of 70% its biofilm weight. Valine presence didn't influence significantly the biofilm weight. Strain

3238-32*Δsnf1* enhanced remarkably (70%) the biofilm formation (weight) in the presence of L-serine, L-threonine, L-proline and L-leucine.

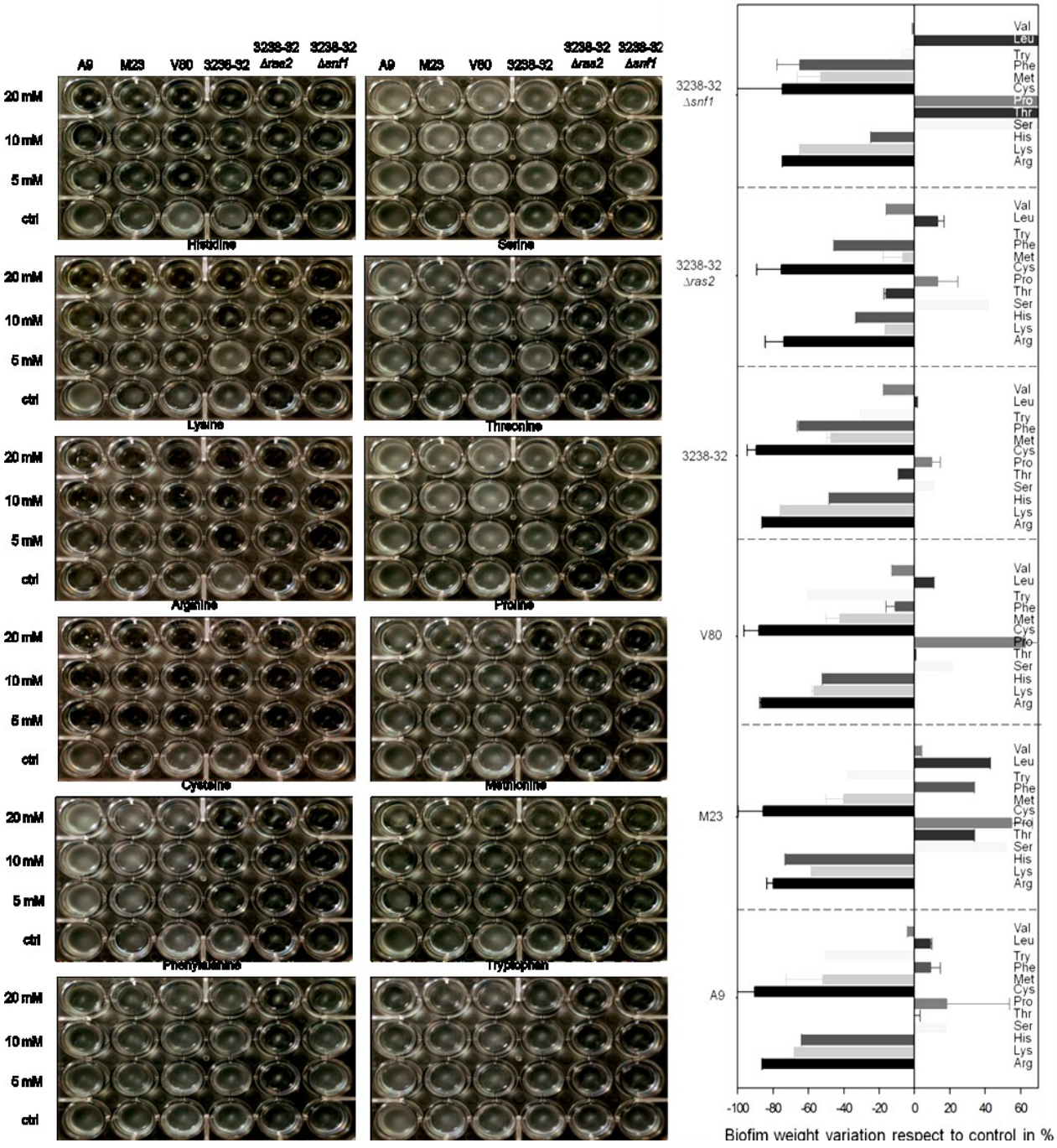

FIG 2 Variation in the air-liquid biofilm formation of S. cerevisiae flor strains in the presence of amino acids. (**A**) Biofilm formation in 24-well plates by strains A9, M23, V80, 3238-32, 3238-32*Δsnf1* and 3238-32*Δras2* after 5 days of static incubation in 1 ml flor medium at 30°C in the presence of Histidine, Lysine, Arginine, Serine, Threonine, Proline, Cysteine, Methionine, Phenylalanine, Tryptophan, Leucine and Valine at different concentrations as indicated. The biofilm is visualized as opaque floating material at the top of each well. (**B**) Biofilm dry weight variation in percentage (Horizontal axis), of strains A9, M23, V80, 3238-32, 3238-32*Δsnf1* and 3238-32*Δras2* in wells with 10 mM L-amino acids of tested strains (Vertical axis), each respect to the proper control well without amino acids; Means and SD of the results from three replicate treatments are shown.

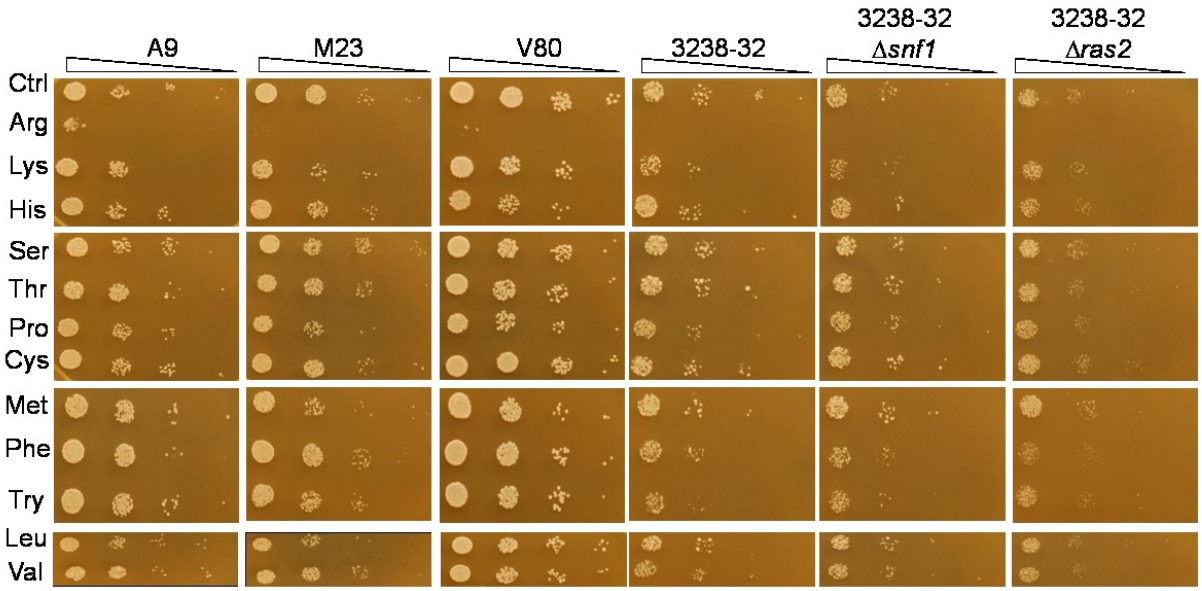

FIG 3. Viability of *S. cerevisiae* strains treated with a series of amino acids. CFU recovery by spot test on YPD agar plates, of serial dilutions of cells exposed to 10 mM of different amino acids. Samples used belong to replicates of plates from Fig. 2

To further characterize the obtained results, aliquots of each sample were plated on YPD agar plates to monitor CFU recovery. The variation in biofilm formation in the presence of 10 mM of different amino acids was also accompanied with variations in the viability of treated cells respect to their control without amino acids.

In general, the colony forming units was equal between treated and control samples. CFU recovery of all strains treated with L-arginine showed a remarkable decrease in cell count respect to control cells without amino acids. L-lysine and L-histidine decreased lightly the number of CFU respect to control cells. Flor strains A9, M23 and V80 showed no differences in colony forming units CFU between spots of cells treated with L-serine, L-threonine, L-proline, L-cysteine, L-methionine, L-phenylalanine, L-tryptophan and spots of control cells. Tested amino acids had likely the same effect on the vitality of haploids strains derivates of A9 strain (3238-32, 3238-32*Δras2* and 3238-32*Δsnf1*), similar to diploid strains but with lower CFU concentration. In particular, the CFU recovered from wells with 3238-32 cells in the presence of Proline, Methionine, Phenylalanine and Tryptophan, was slightly reduced respect to control (Fig. 3).

Cell adhesion to polystyrene in the presence of amino acids. Yeast adhesion is a crucial factor in biofilm formation and invasive growth. In *S. cerevisiae*, flocculins in general and Flo11p in particular are known to be the main responsible for cell-cell and cell-surface adhesions (Karunanithi *et al.*, 2010; Goossens and Willaert, 2012a). The screening for molecules which can modulate positively or negatively yeast adhesion is of a great biotechnological relevance. Here, the adhesion to polystyrene of some *S.cerevisiae* flor strains was studied in the presence of a series of amino acids. Cells in flor medium were inoculated in 96 wells micro-plates, in the presence of 10 mM of each amino acid. Crystal violet method was used, in order to detect cell adhesion after 48 hr of static incubation. Results showed noteworthy differences in adhesion to polystyrene between control and treated cells. Enhancement or reduction of the adhesion ability of each treated strain were randomized, respect to control wells of each strain and represented in percentage (%) (Fig.4). The presence of tested amino acids mainly reduced the cell adhesion to polystyrene. The reduction effect varied among amino acids. For example, cationic amino acids reduced between 40-80% the adhesion capacity of all strains. In details, L-arginine showed the highest reduction effect, respect to ≈30-60% of adhesion reduction by L-lysine and L-histidine. Also hydrophobic amino acids applied a reduction in adhesion capacity of cells. Indeed, sulphuric amino acids, L-cysteine and L-methionine reduced about ≈40-80% and ≈20-40% respectively, the cellular adhesion to polystyrene, depending on strains. In particular, L-cysteine presence enhanced slightly the adhesion value of 3238-32*Δras2* of 10%. Equally, aromatic amino acids such as L-phenylalanine and L-tryptophan showed a reduction in cellular adhesion, with a punctuated activity of L-tryptophan (≈80-95%) respect to L-phenylalanine (≈10-30%). At the end, aliphatic amino acids, mainly L-valine and L-leucine showed the highest inhibition effect on the adhesion of tested cells, with an average of ≈85-95% reduction, respect to control wells without amino acids. From another part, L-serine and L-threonine, hydroxylic amino acids, showed a variable effect on yeast adhesion.

A9 adhesion capacity was enhanced of 40% in the presence of Serine. The other strains showed no significant reduction or enhancement of adhesion in the presence of Serine. In contrast, Threonine presence generally reduced the amount of adhered cells to polystyrene of 20-50% respect to adhesion in control wells. At the end, Proline presence enhanced the adhesion values of V80, 3238-32 of 58% and 45% respectively.

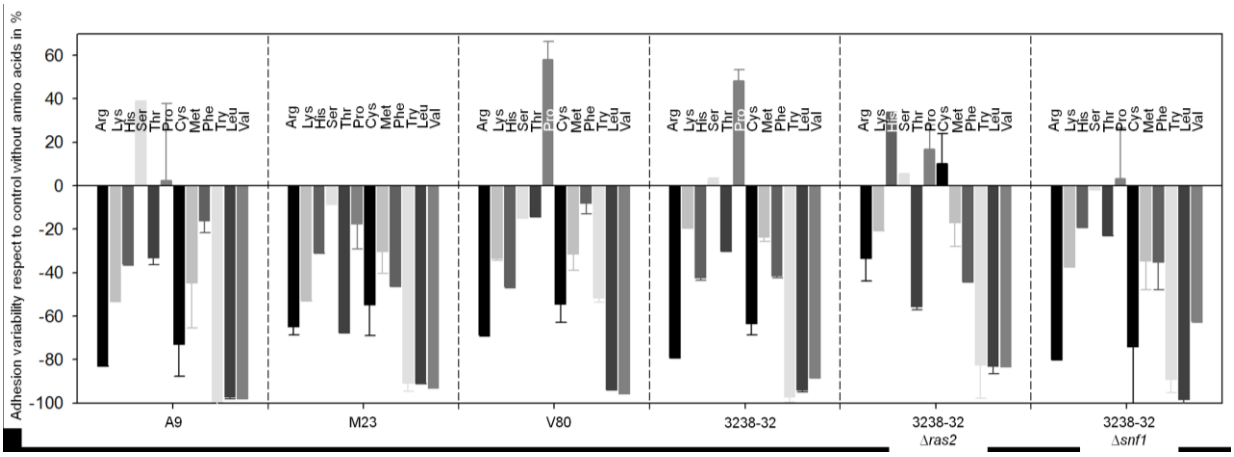

FIG 4 Variation of the adhesion ability of S. cerevisiae flor strains in the presence of amino acids. Flor strains A9, M23, V80, 3238-32, 3238-32*Δras2* and 3238-32*Δsnf1* were incubated in flor medium for 48 h at 30°C in the presence of amino acids at 10 mM. (x-axis). The variation of cell adhesions of the strain/amino acid combination was represented in percentage %, respect to the proper control cells without amino acids (y-axis). Means and SD of the results from three replicate treatments are shown.

Discussion

Amino acids are natural compounds which share the same general structure They consist of a carbon atom, bonded to one hydrogen atom, one amine and one carboxyl groups. The lateral chain R is the only variable which differs among amino acids. More than 300 amino acids were isolated from nature, but only 20 amino acids are reported to be as building blocks of proteins (Wu, 2009). A part of the well known roles of amino acids in nitrogen biosynthesis and being protein building blocks, they are also involved in a wide range of biological processes. In this work, we wanted to observe the effects of different amino acids, beyond their crucial role as fundamental components in biological processes. For that, we applied cellular growth tests, using

different *S. cerevisiae* flor strains in different growth media with the presence of high ranges of amino acids. These tests uncovered some observations which are previously un-described. Our results highlight the variation of biofilm formation and weight along with the adhesion capacity of flor yeasts of *S. cerevisiae* in the presence of different amino acids. This study reported that the presence of some amino acids, mainly L-arginine, L-lysine, L-histidine, L-cysteine and L-methionine at 10 mM, inhibited the biofilm formation and the adhesion to polystyrene of flor strains. In these conditions viability was not affected. In fact, except L-arginine, CFU recovery showed slight reduction viability of the tested strains respect to control. However, the growth inhibition effect of amino acids is barely studied, but we found some studies that undergo with our result, as the toxic effect of moderated concentrations of L-cysteine on *S. cerevisiae* strains, grown in both fermentative and oxidative conditions (Damberg and Blumberg Ia, 1983).

From data observations, we hypothesize two possible modes of actions of these amino acids on the biofilm formation and adhesion capacity of tested strains. The first speculation is that, after deamination of the internalized amino acids, the resulted carbon skeletons are used either as carbon sources to maintain a stable growth, or discarded out of the cells as higher alcohols and esters, also known as fusel oils (Hazelwood *et al.*, 2008). In our case, all diploid strains grew regularly in glucose and remained a steady viability in ethanol contained media at all tested concentrations of L-serine, L-threonine, L-methionine, L-proline, L-tryptophan and L-phenylalanine. In contrast, these amino acids affected biofilm formation and adhesion to plastic. L-serine, L-threonine and L-proline slightly enhanced biofilm formation and adhesion. Carbon skeletons of these amino acids could be possibly used as carbon sources to maintain the cellular growth of *S. cerevisiae* strains (Lee *et al.*, 2013; Pallotta, 2013). This enhancement was notable and interesting in the strain 3238-32*Δsnf1*. *SNF1* gene is required for the transcription of glucose-repressed genes and for cellular growth on carbon sources alternatives to glucose, thus its deletion

occurs a reduction in the biofilm formation capacity (Kuchin *et al.*, 2002). It is also directly involved in the negative regulation of fatty acids synthesis thru the repression of *ACC1* (Acetyl-CoA carboxylase) (Sanz, 2003). Since the presence of fatty acids is positively correlated to biofilm formation (Zara *et al.*, 2012), we suggest that in the presence of L-serine, L-threonine, L-proline and L-leucine, 3238-32*Δsnf1* could trigger the synthesis of fatty acids synthesis, thru the formation of Acetyl-CoA from the carbon skeleton of these amino acids, leading to an enhancement in biofilm formation.

Aromatic and sulphuric amino acids showed a reduction in biofilm formation and adhesion of tested strains. This could be related to the secretion of non-catabolic carbon skeletons, transformed to higher alcohols. Recently, some studies demonstrated that *Geotrichum candidum*, a fungus used in cheese fermentation, treated with aromatic amino acids, especially with L-phenylalanine, enhanced notably the production of phenyl-lactic acid, known for its antimicrobial effect on a range of fungi and bacteria species *(Naz et al., 2013)*. Besides, the filamentous growth of *Candida albicans* was inhibited in the presence of phenylethanol and tryptophol but not tyrosol, which are aromatic acids derivatives of L-phenylalanine and L-tryptophan ant L-tyrosine respectively (Chen, 2006). An opposite effect was observed by treating *Pichia fermentans* strain with high concentrations of L-phenylalanine (Sanna *et al.*, 2012). All the results show that the phenotypic behavior of cells toward such compounds may be species and sub-species specific.

As a second speculation, these effects could be even related to physical interactions between flor yeasts cell wall and amino acids, in particular electrostatic interactions with yeasts cell wall, inhibiting cell-cell and cell-surface adhesions. This hypothesis could be true for cationic amino acids. Indeed, cells treated with these amino acids in flor medium showed a reduction in CFU recovery, which indicates that these amino acids were not used as nitrogen sources. Indeed, previous studies confirm that

L-lysine and L-histidine, but not L-arginine, cannot be used as sole nitrogen sources by *S. cerevisiae* species (Homann *et al.*, 2005). The fact that cells treated with L-arginine grew less respect to their controls, reinforces the hypothesis that cationic amino acids could be sequestrated on yeasts cell wall. Recent studies showed a clear capacity of cationic amino acids in conferring electrostatic interactions with inorganic surfaces. The cationic side chain of Lysine was very crucial in maintaining high electrostatic interactions with defined surfaces (Razvag *et al.*, 2013). Moreover, a recent study noted that Histidine confers potent "coordinate bonds" with metals, cation-π, hydrogen-π and hydrogen-bond interactions and was highly attracted to cationic residues at neutral pH (Liao *et al.*, 2013). This intuits that at basic pH, L-histidine attraction to anionic residues should be stronger. From the part of yeast cell, many studies confirmed that the cell wall charge is highly anionic due to the presence of mannoproteins (Horisberger and Clerc, 1988; De Groot *et al.*, 2005). We suggest that these peculiarities of cationic amino acids could involve them in physical interactions with yeasts cell wall, especially in biofilm formation process, where the cell wall is heavily charged by anionic and hydrophobic mannoproteins, and as a result, these interactions reduce the cell hydrophobicity and charge, reducing biofilm formation and adhesion capacity. To our knowledge, the real interaction forces between simple molecules such as amino acids and fungal cell wall still not well characterized. This approach is very interesting, especially because of the dimorphic change capacity of fungi, between commensalism and invasion growth (Granek and Magwene, 2010; Brückner and Mösch, 2012). Indeed, in the last decade, many studies reported the high influence of the hydrophobic and electrostatic interactions between fungal cell wall and small peptides on general cell behavior (Marcos and Gandia, 2009; Bou Zeidan *et al.*, 2013; Fazly *et al.*, 2013).

Summarizing, this report identifies another biological activity of some amino acids which could be the positive or negative control of biofilm formation and adhesion of flor yeasts.

Even if no correlations were found between the tested strains each with a specific background of *FLO11* gene, a functional *FLO11* gene seems to be fundamental. The variation of phenotype in the presence of amino acids opens domain of new experiments, in order to better understand the molecular motifs behind this phenomenon, even considering its interest at the biotechnological level.

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GENERAL CONCLUSIONS

Research handled during this PhD thesis focused on the phenotypic characterization of natural flor strains with different genetic backgrounds, mainly at the level of *FLO11* gene length and expression. We studied the interactions between flor yeasts and nitrogen compounds, such as single amino acids, dipeptides and small peptides with particular physiochemical properties.

FLO11 gene is directly involved in multicellular phenotypic traits, like biofilm formation and adhesion, by conferring to yeasts cell wall high hydrophobic and electrostatic interactions. These characteristics influence directly the adhesion capacity and the air-liquid biofilm formation in *S. cerevisiae* flor strains.

In this thesis, we studied the effect of natural components such peptides and amino acids, known for their hydrophobicity and/or charge, on the biofilm formation process of some flor yeasts. In particular, the aim was to characterize and better understand the cellular interactions of flor strains with these nitrogen compounds, and among them to find some able to modulate positively or negatively the biofilm formation of flor yeasts.

Accordingly with this aim, results obtained in the three parts of this PhD revealed different interactions between *S. cerevisiae flor* and nitrogen compounds, which could be resumed as next:

- A novel role of the synthetic hexapeptide PAF26 in enhancing the biofilm formation of flor strains, in positive correlation with the specific *FLO11* gene expression of each strain.
- High interactions affinity between PAF26, and not other peptides, with flor strains having a functional *FLO11* gene.
- The ability of flor strains with high *FLO11* expression background to metabolize a wide range of nitrogen sources, except L-histidine and L-histidine contained dipeptides.

- A potential role of cationic amino acids, mainly L-histidine, and sulphuric amino acids in inhibiting the air-liquid biofilm formation and cell adhesion capacity of flor yeasts.

These major findings, along with the series of accompanied results, represent a new approach in the field of cell-cell and cell-environment interactions. It's worthy to note that in major part of handled experiments, the cell viability of flor strains treated with nitrogen compounds was constantly similar to control cells without treatments. Even though, the adhesion and biofilm formation ability were highly affected, positively or negatively, in the presence of these compounds.

Considering *I)* the innate cell wall diversity and the conferred high hydrophobicity and adhesion ability of flor strains; *II)* the enhancement of cell wall chitin and mannan in the presence of some of tested compounds (PAF26 and L-histidine) and *III)* the particular physiochemical characteristics of such molecules, we hypothesize that our results reflect potentially the unspecific interactions between the cell surface and its enclosed environment. We also showed the involvement of *FLO11* gene in such unspecific interactions with the cell environment and its role in modulating the cell surface net charge which potentially modulates the cellular growth.

Results in this PhD thesis could be of a high interest, because the microbial adhesion and biofilm formation is becoming a key factor in microorganisms control at the biotechnological level. Beside, Flo11p is a cell wall glycoprotein homologous to glycoproteins of other species, involved in the virulence of these species. In fact, investigations on the dimorphism in *S. cerevisiae* has been instrumental to uncover many of the signaling routes that control hyphal growth and virulence in a growing number of human pathogenic fungi. For this, further experiments at the molecular level are fundamental to understand and maybe lead to apply these results on other microbial biofilms.

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