Adaptive evolution by mutations in the FLO11 gene

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In nature, Saccharomyces yeasts manifest a number of adaptive responses to overcome adverse environments such as filamentation, invasive growth, flocculation and adherence to solid surfaces. Certain Saccharomyces wild yeasts, namely “flor yeasts,” have also acquired the ability to form a buoyant biofilm at the broth surface. Here we report that mutations in a single gene, identified as FLO11, separate these “floating” yeasts from their nonfloating relatives. We have determined that the capability to form a self-supporting biofilm at the liquid surface is largely dependent on two changes in the FLO11 gene. First, we identified a 111-nt deletion within a repression region of the FLO11 promoter that significantly increases FLO11 gene expression. Secondly, we found rearrangements within the central tandem repeat domain of the coding region that yield a more hydrophobic Flo11p variant. Together, these mutations result in a dramatic increase in cell surface hydrophobicity, which in turn confers these yeasts the ability to float by surface tension, an adaptive mechanism to gain direct access to oxygen within oxygen-poor liquid environments.

When microbial cells are subjected to environmental stress, evolutionary models hold that natural selection favors genetic changes that give cells an advantage in an adverse environment. Microbes predominantly follow asexual modes of reproduction that may limit genetic variability and their ability to adapt to new environments. However, random mutations, coupled with large populations and short generation times, may explain how microbial cells are able to overcome this restriction to adaptation (1). Their adaptive flexibility is evident in the speed at which microbial populations respond to selection in the laboratory (2–4). However, mechanisms concerning the origin of adaptive responses in a natural environment are poorly documented.

Saccharomyces yeasts have been exploited for baking and alcohol production by virtually every human society. For centuries, sherry wines have been produced in southern Spain, from where they have been exported worldwide. Originally, barrels were often left in long-term storage before being shipped, during which time a yeast velum developed upon the wine’s surface. The formation of the velum or “flor” is now an integral part of sherry wine production.

It has been shown that the velum in sherry wines is exclusively formed by wild Saccharomyces cerevisiae “flor yeasts,” with a permanent presence in these wines dating back to at least the 19th century (5–7). Saccharomyces flor yeasts are among the most ethanol-tolerant organisms known in nature (8–10). These yeast strains are able to proliferate in broth with 14–16% (vol/vol) ethanol containing only traces of fermentable sugars (sherry wine). Velum formation and the resulting occupation of the air–liquid interface provides these microbial cells with a great selective advantage in such an adverse environment, where access to oxygen is a critical factor (5, 7, 10–12). In turn, the velum is a yeast biofilm that has acquired the ability to float, possibly as an adaptation to the extreme selective pressures imposed by the conditions inside sherry wine barrels (6, 7). This characteristic makes flor yeasts particularly useful for genetic studies on adaptive evolution. The acquisition of very specialized phenotypes, such as velum formation, and the asexual reproduction shown by flor yeasts, as a consequence of the sexual isolation among different strains at this environment (8, 9), provide an excellent opportunity to study processes related to microbial adaptation and specialization.

Saccharomyces yeasts possess a range of responses to enable survival in deleterious circumstances including filamentation, invasive growth, flocculation, and biofilm development (13–21). However, buoyancy, a widespread mechanism in planktonic microorganisms, is not a response typically possessed by Saccharomyces yeasts. The question of how the naturally occurring flor yeasts acquired their ability to “float” is therefore intriguing. Here, we reveal the molecular mechanism underlying the ability to form a self-supporting yeast biofilm at the air–liquid interface, and the evolutionary origin of this adaptive response in wild Saccharomyces yeasts.

Results and Discussion
Identification of the Gene Conferring Floatability to Saccharomyces Flor Yeasts. The ability of flor yeasts to form a velum at the wine surface has been widely exploited in the biological aging of sherry wines (Fig. 1 and Movie 1, which is published as supporting information on the PNAS web site). Conventional genetic analysis has determined that it is a dominant (gain of function) characteristic, resulting from changes in only one or two genes (8, 10, 22). Thus, the identification and characterization of this gene (or genes) may help to understand the molecular nature of buoyancy in this nonmotile unicellular microorganism, and reveal mutations leading to this adaptive mechanism in wild yeasts.

The nuclear genomes of Saccharomyces flor yeasts are very complex (involving both polyploidy and aneuploidy); therefore, the construction of a genetically tractable flor yeast strain is a prerequisite for the identification of “velum formation” genes. To this end, we used flor–laboratory yeast hybrids with the ability to sporulate that were produced in our laboratory (10). Spores harboring the “velum formation” characteristic were selected and backcrossed to the YNN295 haploid laboratory strain. After six rounds of backcross and selection, a genetically “domesticated” haploid flor strain, named 133d, was obtained (see Materials and Methods).

By crossing this 133d strain to the laboratory YNN295 strain, we determined that the ability to form an air–liquid interface biofilm segregated as a monogenic trait. This finding indicates that allelic differences in a single gene confer floatability. Functional cloning strategies, through the transformation of laboratory yeast cells with 133d genomic libraries to select “floating” clones, failed to identify this gene (see Materials and Methods). However, by using a standard set of genetic markers, we were able to map the “velum formation” gene to the right arm of chromosome IX, adjacent the centromere. FLO11 is located at this region (23). This gene is required for many cellular responses, including biofilm formation (17). Because the velum is a buoyant biofilm, we first decided to assess the role of

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Abbreviation: YPD, yeast extract/peptone/dextrose.
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Fig. 1. *S. cerevisiae* “flor yeasts” are naturally able to grow on the sherry wine surface. (A) Oak barrels filled to 3/4 with sherry wine spontaneously form a self-supporting yeast biofilm on the wine surface, a velum known as “flor.” (B) This thick biofilm floats and allows flor yeast to gain direct access to oxygen. (C) Cells in this buoyant biofilm are strongly aggregated, showing a flocculation phenotype.

*FLO11* in velum formation. As shown in Fig. 2, the deletion of *FLO11* in 133d yeast cells prevented all known Flo11p-dependent properties such as flocculation, filamentation, invasive growth, and biofilm formation on a solid surface, but also velum formation, indicating that this gene is required for this latter characteristic as well. Similar results were recently reported for velum formation in Sardinian wine yeasts (12).

Other genes such as *HSP12* and *NRG1* have also been implicated in this characteristic (11, 24). Therefore, we wished to determine whether *FLO11* is the only gene that differentiates 133d flor yeasts from the laboratory strain with respect to their ability to float. To this end, we replaced the *FLO11* gene with the *KANR* marker in the laboratory YNN295 strain (*flo11::KANR*), and the meiotic products of 133d *FLO11*+/YNN295 *flo11::KANR* diploids analyzed by tetrads dissection. The absence of floating *KANR* spores in these meiotic products strongly suggests that velum formation is conferred by an allelic variant of *FLO11* (Fig. 3).

To test this hypothesis, DNA fragments (PCR generated) containing the *FLO11* gene from either the YNN295 laboratory strain (FLO11L) or the 133d *flo11* (*FLO11F*) were cloned into the pRS316 centromeric plasmid, and their respective pRS316-*FLO11L* and pRS316-*FLO11F* constructs were used to transform YNN295 *flo11A* and 133d *flo11Δ* cells (both deleted for their endogenous *FLO11* gene by replacement with the *KANMX4* gene). The 133d *flo11Δ* strain recovered both invasive growth and biofilm formation on solid surfaces with either *FLO11L* or *FLO11F*, indicating that the constructs used were functional (data not shown). However, only plasmids containing the *FLO11F* allele restored the ability of mutant yeast to form a biofilm at the liquid surface (Fig. 3B). Interestingly, not only the 133d *flo11Δ* strain recovered this property, but the laboratory *flo11Δ* strain also gained this new function with the only acquisition of the *FLO11F* gene (Fig. 3B). This result, together with the monogenic segregation of velum formation and the linkage of *FLO11* to this character described above, demonstrates that *FLO11* is the only gene that separates *Saccharomyces* yeasts from their non-floating ancestors. Thus, our results suggest that floatability of flor yeast has arisen from the acquisition of additional functions for the *FLO11* gene via mutational changes rather than through the establishment of a new flor-specific “floatability” pathway.

**Mutations in the *FLO11* Gene Yielding Floatability.** In standard laboratory strains, *FLO11* has an ORF of 4.1 kb that encodes a putative cell-wall-associated glycoprotein involved in cellular adhesion (23, 25). Its expression is driven by a promoter of ≈3 kb, possibly the largest and most complex promoter found in the whole *Saccharomyces* genome (26, 27). PCR products resulting from the amplification of the *FLO11* gene from 133d and YNN295 strains differed in length (Fig. 4), suggesting that gene rearrangements could be involved in the functional differences between the two *flo11* alleles. However, we were unable to clone these amplified fragments into plasmids, possibly due to the complexity and size of the fragment (≈10 kb). Therefore, we chose to functionally characterize mutations that give rise to floatable strains by allelic exchange. 

Fig. 2. Phenotypes related to *FLO11* in 133d flor yeast cells and in 133d *flo11Δ* cells, deleted for the endogenous *FLO11* gene (*flo11::KANR*). (A) Microphotograph of yeast cells. (B) Invasive growth in solid YPD media. (C) Biofilm formation in solid surface monitored by crystal violet staining of cells fixed to plastic. (D) Pseudohyphal growth of diploid *FLO11F::flo11Δ, flo11ΔF*, *FLO11L*, and *flo11ΔF::flo11ΔL* strains developed in synthetic low-ammonia dextrose media. (E) Air–liquid interfacial biofilm formation in flor medium.

Fig. 3. The role of *FLO11* in the biofilm formation at the liquid surface (flor velum). (A) Dissected spores from a heterozygous 133d *FLO11*+/YNN295 *flo11::KANR* strain were tested for air–liquid biofilm formation and geneticin sensitivity. All tetrads produced 2 *FLOR*+/KanR; 2 *Flo*−/KanR (a sample tetrad is shown in the panel). (B) DNA fragments containing the *FLO11* complete gene (promoter plus ORF) from YNN295 (1) or 133d (2) strain were cloned into the pRS316 vector, and the resulting plasmids were transformed in 133d *flo11Δ* and YNN295 *flo11Δ* strains. Velum formation by these transformants was assessed in flor medium for 5 days at 28°C.
alleles. Southern blot analysis indicated that the FLO11F promoter region was \( \pm 0.1 \) kb smaller than that of FLO11L, whereas the coding region was \( \pm 1 \) kb larger (Fig. 4 B and C). This finding was confirmed by DNA sequence analysis of each allele, which also identified several point mutations, deletions, and rearrangements in both the promoter and the ORF of the FLO11F allele (see Figs. 8–10, which are published as supporting information on the PNAS web site).

To evaluate separately the relative importance that mutations in the promoter and in the ORF may have on velum formation, we identified several point mutations, deletions, and rearrangements in both the promoter and the ORF of the FLO11 promoter as probe. Filled arrows indicate ORF fragments, and open arrows indicate promoter fragments. (D) Chimeric FLO11 genes generated by using FLO11 promoter and ORF from 133d and YNN295 strains were cloned into pRS316 vector, and the resulting plasmids were transformed into 133d flos1. Transformants were grown on flor medium.

During the process of adaptation of \( \dot{S} \) cerevisiae flor yeasts to an oxygen-poor environment, it might be expected that increased fitness would be acquired by selecting these mutations allowing improved access to oxygen in a stepwise manner. In this context, it is interesting that the laboratory Flo11Lp protein was also able to generate a weak but detectable self-supporting biofilm when expressed under the FLO11F promoter, whereas no such interfacial biofilm was observed when the Flo11Fp protein was expressed by the FLO11L promoter (see Fig. 4D). On the basis of this observation, we speculate that, during the process of flor yeast adaptation, mutations triggering a weak access to the air–liquid surface in flor yeasts occurred first in the FLO11 promoter, followed by mutations in the coding region, which enhanced this ability.

**Genetic Changes in the FLO11 Promoter.** Expression of FLO11 is controlled by signaling pathways activated in response to growth stage and nutritional conditions (18, 27–29). The fact that the FLO11F gene confers the velum-forming property to laboratory yeast cells (Fig. 3B), joint to the role that the FLO11F promoter itself plays in this character (Fig. 4D) indicate that cis-regulatory changes in the FLO11 promoter are essential for the acquisition of buoyancy in budding yeasts. To better characterize the nature and function of the changes that have occurred in the FLO11F promoter, we analyzed mRNA expression of the FLO11F coding sequence driven by either FLO11F or FLO11L promoters from the pRS316 centromeric plasmid in 133d flos1 cells (constructs 1 and 2).
2 in Fig. 5A) by Northern blotting. Transcripts were analyzed in cells subjected to nutritional stress (flor medium) where FLO11 function is essential for velum formation, and in optimal growth conditions (yeast extract/peptone/dextrose,YPD) where FLO11 expression is repressed in standard laboratory strains. As shown in Fig. 5A, although expression was induced from both promoters when the yeast cells were incubated in velum-forming media, FLO11 expression driven by the FLO11F promoter was several-fold higher than when driven by the FLO11L one. The difference was even more dramatic under optimal nutritional conditions, where the laboratory FLO11F promoter was strongly repressed as expected, whereas expression from the FLO11F promoter remained high (Fig. 5A). In agreement with this observation, we determined that a Flo11F:GFP fusion protein, expressed from the endogenous FLO11F promoter in the 133d flor yeast strain, was abundantly localized at the cell wall of yeast forming a buoyant biofilm (flor medium) as well as in cells exponentially growing in optimal conditions (YPD) (Fig. 5B).

A large number of factors with complex functional relationships have been shown to act on the FLO11 promoter (26–31). This promoter consists of at least four activating and nine repressing sequences (27). DNA sequence comparison between the laboratory and the flor FLO11 promoters (Fig. 10) revealed a number of point mutations and a 111-bp deletion (schematized in Fig. 5C). Interestingly, the deletion involves a large domain of a well characterized repression sequence (27). To determine whether this deletion plays a role in the stronger expression mediated by the FLO11F promoter, we engineered the same deletion in the FLO11L promoter (the flo11L-111Δ mutation) and assessed the level of expression of the mutant promoter by Northern blotting. As shown in Fig. 5D, the deletion in the flo11L-111Δ promoter partially relieved repression of the FLO11 mRNA expression. Therefore, this 111-bp region is required to repress FLO11 expression. Furthermore, expression of the Flo11Fp protein under the control of the flo11L-111Δ promoter was sufficient to induce a thin air–liquid interfacial biofilm (data not shown), suggesting that the 111-bp deletion was a significant event in the adaptation of flor yeasts to grow at the liquid surface.

The 111-bp deletion occurred between nucleotides −1313 and −1203 (inclusive), involving two direct repeats of the nucleotide sequence CAAATTAA. Short direct repeat sequences have been proposed as possible substrates for DNA intramolecular recombination (32). Thus, one such recombination event could be responsible for the evolutionary origin of the 111-bp deletion found in the FLO11F promoter of wild Saccharomyces flor yeasts.

**Variations in the Flo11p Protein.** In addition to increased expression, changes in the encoded Flo11p protein are also required to gain the floatability observed in the 133d flor yeast (Fig. 4D). The ORF of FLO11 in laboratory strains of S. cerevisiae is predicted to encode a protein of 1,367 aa (23, 25), similar in overall structure to flocculins in yeasts (33), and mucins in mammalian cells (34). The predicted product of FLO11 comprises of an N-terminal domain containing a hydrophobic signal sequence, and a C-terminal domain with homology to GPI (glycosylphosphatidylinositol) anchor-containing proteins, separated by a central domain of highly repeated serine/threonine-rich sequences. Flo11p is thought to be heavily O-glycosylated at specific serine and threonine residues within these repeated sequences (35, 36). By analogy to Flo1p, the integral membrane domain of the C-terminal region could anchor Flo11p to the plasma membrane, and the repeated sequences of the central domain could stretch out the protein to span the cell wall and to expose the N-terminal region at the surface (37). The glycosylation of the repeated sequences may help the laboratory strain Flo11p to adopt an extended conformation (38). However, whether the GPI anchor domain remains in Flo11p is an open question.

The DNA sequence of the 133d strain FLO11F ORF is predicted to encode a protein comprising 1,630 aa, with a similar structure to the FLO11 of the laboratory strain (Figs. 2A and B and 8). However, the FLO11F ORF contains several point mutations and deletions, mainly distributed throughout the N- and C-terminal domains. We also found that the number of the repeated sequences in the central domain was greatly increased. The amplified number of repeated sequences accounts for almost all of the increased size of the FLO11F gene. A schematic representation of the FLO11F and FLO11L alleles highlighting these differences is shown in Fig. 6A.

To determine which region of the Flo11Fp protein contributes most to yeast floatability, we took advantage of the HindII and HindIII restriction sites on the FLO11 ORF to construct chimeric Flo11p proteins that combined N-terminal, central, and C-terminal domains of Flo11Fp and Flo11Lp proteins (as illustrated in Fig. 6B). These constructs were expressed under the FLO11F promoter, and velum formation was assessed in 133d flo11Δ cells. As shown in Fig. 6B, the central domain containing the tandem repeated sequences was sufficient to confer the same floatability as the wild-type Flo11Fp protein, suggesting that changes in copy number of repeated sequences in the central domain is the main determinant of floatability conferred by the Flo11Fp protein. In general terms, increased repetition of modules within proteins is an important mechanism in evolution (33, 39), which, in the case of the FLO11F coding region, might account for the rapid adaptation that flor yeasts have undergone to acquire floatability.

**The Molecular Mechanism that Confers Floatability to Wild S. cerevisiae Flor Yeasts.** It has been reported that flor yeasts possess high levels of surface hydrophobicity (40). In laboratory strains, it has been shown that hydrophobicity is largely dependent on FLO11 (17). The absence of this protein drastically drops the affinity of yeast cells for hydrophobic solvents, whereas overexpression of this protein increases it (11). Consistent with this, we found that
hydrophobicity was extremely high in 133d yeast cells even in SCD media, whereas 133d fliΩΔ cells were mostly hydrophilic (Fig. 7). To determine whether changes in hydrophobicity might be the mechanism by which the FLO11F allele confers floatability, we assessed the hydrophobicity of 133d yeast fliΩΔ cells expressing different chimeric FLO11 constructs. We found that hydrophobicity was higher when the same Flo11p protein was expressed under the FLO11F promoter than under the FLO11L partner (Fig. 7), indicating that the higher FLO11 expression level mediated by the FLO11F promoter is at least partially responsible of the increased hydrophobicity of 133d yeast cells. On the other hand, we found that the 133d Flo11Fp protein also conferred higher cell hydrophobicity than that of the YN295 Flo11Lp variant when expressed under identical promoters (Fig. 7). Under these conditions, FLO11 mRNA levels were similar in all of the different constructions tested (see Fig. 11, which is published as supporting information on the PNAS web site), suggesting that Flo11p itself is a more hydrophobic variant. Moreover we determined that the increased hydrophobicity of the Flo11Fp protein is mediated primarily by its expanded central domain (Fig. 7). Because the glycosylation of this type of protein is supported by the repeated sequences found in the central region (41, 42), changes in Flo11Fp glycosylation due to changes in the copy number and/or distribution of these repeats may explain the gain of hydrophobicity found in the Flo11Fp protein (see Fig. 6).

From the above results, we conclude that as a mechanism of adaptive evolution, flor yeasts gained floatability through mutations in the FLO11 gene that conferred both a higher level of expression of this gene and a more hydrophobic Flo11Fp protein variant. As a consequence of these genetic changes, flor yeast cells increased cell hydrophobicity over a limit where the exclusion of water from the cell surface is sufficient to self-maintain the yeast biofilm at the air–liquid interface by surface tension in this aqueous environment. Thus, mutations in cis-regulatory sequences and variations in gene-associated tandem repeats, two of the main mechanisms that facilitate rapid morphological evolution (43), are the remarkable genetic events that account for the adaptation of flor yeasts to oxygen-limited liquid environments.

In filamentous fungi, hydrophobins account for the hydrophobic coat that allows the formation of water-repelling aerial hyphae (44). Hydrophobins have not been identified in yeasts; however, both Flo11p and hydrophobins are glycoproteins that assemble at the cell wall to produce a hydrophobic coat, suggesting the possibility of a common molecular mechanism by which these two nonrelated proteins may facilitate growth at the water–air interface.

To extrapolate the conclusions obtained in this study with the genetically “domesticated” 133d strain to wild yeasts, we analyzed three independent Saccharomyces flor yeasts (MY91, MY138, and ET7) previously isolated in our laboratory from sherry wines (10). We determined that FLO11 was highly expressed in each strain, and that this gene was required for velum formation in each. Similarly, we found that each of these strains harbored a FLO11 allele with a increased number of tandem repeats within the central domain that conferred very high levels of surface hydrophobicity to these cells (Figs. 12 and 13, which are published as supporting information on the PNAS web site), suggesting that the conclusions of this study can be extended to wild S. cerevisiae flor yeasts.

Materials and Methods

Strains, Genetic Methods, and Media. Wild Saccharomyces flor yeast strains (MY91, MY138, and ET7) were isolated from the yeast film growing on the surface of sherry wines (10). The 133d haploid flor yeast (MATα ura3) was derived from a flor/laboratory yeast hybrid as described (10). The resulting flor/laboratory yeast hybrid was sporulated, and flor-forming spores were selected and backcrossed six times to the YNN295 laboratory haploid strain (MATα ura3 his2 ade1 ade2 his7 trp7) (Bio-Rad). Flor-forming spores were selected on the basis of their haploid constitution, determined by measuring relative DNA content/cell by flow cytometry analysis (10) and by the Mendelian segregation of genetic markers in all of the chromosomes as determined by crossing with standard laboratory strains. Tetrad analysis using these genetic markers was also used for mapping the flor velum character to chromosome IX near to the centromere (45).

FLO11 deletions were performed by PCR-mediated gene replacement (46), and the deletion was confirmed by PCR and Southern blot analyses. PCR was used to construct a GFP-tagged Flo11Fp protein as described (47). Yeast transformations were performed by the lithium acetate procedure as described (48). Bacterial transformations, bacterial DNA preparations, and plasmid constructions were performed by standard methods (49).

YPD and synthetic medium (SCD) supplemented when necessary with the appropriate base and amino acids at standard concentrations were used (45). The YPD medium was supplemented with 200 mg/liter geneticin for selection of geneticin-resistant (KAN⁴) transformants. Yeast nitrogen base containing 6% ethanol as a sole carbon source, supplemented where necessary with bases and amino acids at standard concentrations, was used as velum-forming medium (flor medium) (10). Synthetic low-ammonia dextrose (SLAD) medium used to induce pseudohyphae was prepared as described (14). Media used for sporulation contained 0.1% yeast extract, 1% potassium acetate and 0.05% glucose. Solid media contained 2% agar.

Primers and Plasmid Construction. All primers used in this study are listed in Table 1, which is published as supporting information on the PNAS web site. To identify the velum-forming gene by functional cloning, a number of 133d genomic libraries (containing 3- to 9-kb DNA inserts) were prepared in centromeric (pRS316) or multicopy (pYES2) plasmids. A reconstruction experiment allowed us to select a single “floating” yeast cell among 10⁶ nonfloating yeast cells. Because velum formation is a monogenic dominant character, direct cloning of laboratory yeast cells gaining velum formation due to the acquisition of the “velum” gene from the 133d genomic library should allow the direct identification of this gene. However, this cloning strategy failed several times. Once the FLO11 gene was identified as the velum-forming gene by positional cloning, we determined by Southern blot analysis that cloned FLO11 DNA was very unstable during bacterial amplification, making any functional cloning strategy extremely difficult.

The flor and laboratory FLO11 promoter and ORFs were amplified by PCR using primers FLO11-P5 and FLO11-P6 for promoter regions and FLO11-1 and FLO11-2 for ORFs. To reproduce in the laboratory FLO11L promoter, the 111-bp deletion...
found in the flor FLO11F promoter, the regions before and after the 111-bp sequence were amplified by PCR using primers FLO11-P5 and FLO11-PSD and FLO11-P6 and FLO11-P6D and simultaneously cloned into the EcoRI site of pRS316 vector. The combinations between promoter and ORFs were performed by cloning the promoter and the ORFs into EcoRI and Smal/NotI sites of the centromeric plasmid pRS316, respectively. In all cases, the ADH1 terminator was cloned into SacII site.

To carry out the FLO11 chimeric constructs, both laboratory and flor ORFs were cloned into EcoRI/XbaI sites of pBSKK vector without HincII and HindIII sites. So, the N-terminal domain is flanked by EcoRI/HindIII sites, and the C-terminal domain by HindIII/XbaI sites. Using these sites, the different domains were cloned in pRS316 containing FLO11 promoter and ADH1 terminator.

Assay for Adherence to Plastic, Hydrophobicity, and Air–Liquid Interfacial Biofilm Formation. Assays for adherence to the wells of a polystyrene 96-well microtiter plate and hydrophobicity were carried out as described (17) with minor modification. Briefly, for adherence to plastic assay, cells were grown in YPD to an OD600 of ≈0.8, collected, washed, and resuspended in YPD to an OD600 of 1. Cells (0.1 ml) were transferred to the wells of a microtiter plate and incubated for 1 h at 28°C. The cells were then stained with 1% crystal violet, and the wells were washed repeatedly with water. For hydrophobicity assays, cells were grown in SCD to an OD600 of ≈0.8, then 1.2 ml of the culture was overlaid with 600 μl of octane and vortexed for 3 min. The OD600 of the aequous layer was taken, and the relative difference with the initial OD600 was used to determine the percentage of hydrophobicity.

The air–liquid interfacial biofilm formation was assayed by inoculating cells of a fresh colony to glass tubes containing 5 ml of YEP medium. The cells were grown at 28°C for 5–8 days under static conditions (12).

Invasive and Pseudohyphal Growth. Invasive growth was determined by the plate-washing assay as described (15). To induce pseudohyphal growth, cells were streaked in synthetic low-ammonia dextrose plates, incubated for 2 days at 28°C, and then photographed.

DNA Sequencing. The complete DNA sequence of the FLO11F gene from the 133d strain is available at the EMBL database (accession no. AM262523). The FLO11F promoter was cloned into pRS316 vector and sequenced by using the primers listed in Table 1. The FLO11F ORF was cloned into pBSKK vector, digested by Exonuclease III to obtain suitable overlapping clones, and sequenced by using the primers Universal and Reverse. All sequences were obtained by the dye-oxo-dye terminator method with an Applied Biosystems Prism 310 Genetic Analyzer (PerkinElmer).

Analysis of DNA and protein sequences was performed by using the DNA Strider 1.2 software, and the sequences were compared by using the BLAST algorithm at the Saccharomyces Genome database (www.yeastgenome.org) and through the National Center for Biotechnology Information.

Northern and Southern blotting. For Northern blot analysis, overnight cultures of yeast strains in YPD were diluted with SCD or Flu medium to OD600 of ≈0.05 and grown to an OD600 of ≈1. RNA was isolated with the QIAGEN RNeasy Mini kit. A 10-μg sample of RNA was run on a gel, blotted, and hybridized with a 500-bp fragment of FLO11 (corresponding to the N-terminal ORF sequence) probe.

For Southern blot analysis, the genomic DNA of yeast cells was extracted following standard protocols (49) and digested with suitable restriction enzymes. Digested DNA was separated by agarose gel electrophoresis, blotted, and hybridized with radiolabelled probes.

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