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 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.

adaptive mechanism | buoyant biofilm | yeast hydrophobicity

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 abilty 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 interfacial 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.

Data deposition: The sequence reported in this paper has been deposited in the EMBL database (accession no. AM262523).

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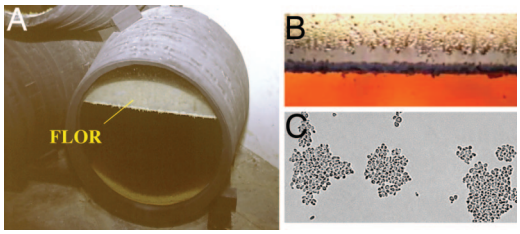

Fig. 1. *S. cerevisiae* ''flor yeasts'' are naturally able to grow on the sherry wine surface. (*A*) Oak barrels filled to 3/4 with sherry wine spontaneously form a self-supporting yeast biofilm on the wine surface, a velum known as ''flor.'' (*B*) This thick biofilm floats and allows flor yeast to gain 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 *KAN*^{R} marker in the laboratory YNN295 strain (*flo11*::*KAN*^{R}), and the meiotic products of 133d *FLO11*$^+$/YNN295 *flo11*::*KAN*^{R} diploid cells analyzed by tetrads dissection. The absence of floating *KAN*^{R} spores in these meiotic products strongly suggests that velum formation is conferred by an allelic variant of *FLO11* (Fig. 3*A*).

To test this hypothesis, DNA fragments (PCR generated) containing the *FLO11* gene from either the YNN295 laboratory strain

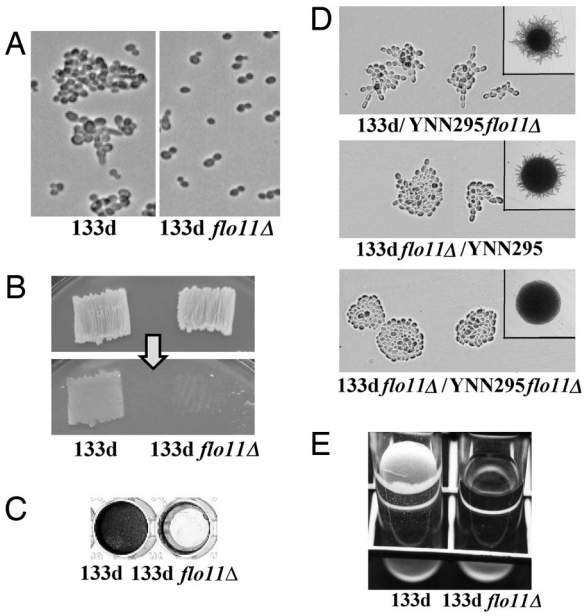

Fig. 2. Phenotypes related to *FLO11* in 133d flor yeast cells and in 133d *flo11*Δ cells, deleted for the endogenous *FLO11* gene (*flo11*::*KAN*^{R}). (*A*) Microphotograph of yeast cells. (*B*) Invasive growth in solid YPD media. (*C*) Biofilm formation in solid surface monitored by crystal violet staining of cells fixed to plastic. (*D*) Pseudohyphal growth of diploid *FLO11F*/*flo11L*Δ, *flo11F*Δ/*FLO11L*, and *flo11F*Δ/*flo11L*Δ strains developed in synthetic low-ammonia dextrose media. (*E*) Air–liquid interfacial biofilm formation in flor medium.

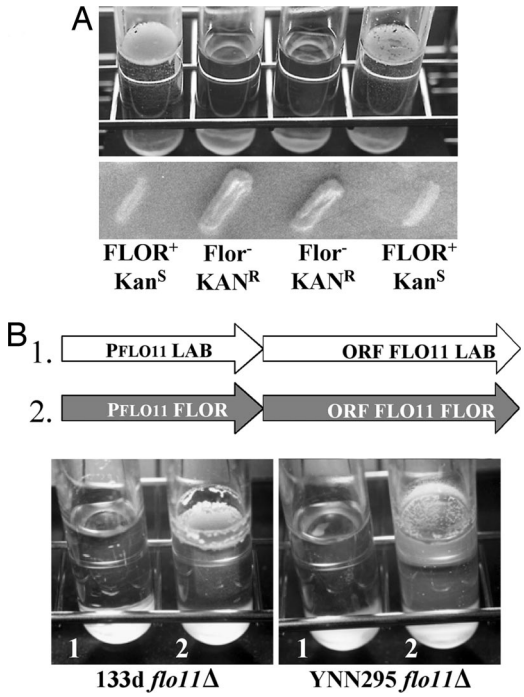

Fig. 3. The role of *FLO11* in the biofilm formation at the liquid surface (flor velum). (*A*) Dissected spores from a heterozygous 133d *FLO11*/YNN295 *flo11*::*KAN*^{R} strain were tested for air–liquid biofilm formation and geneticin sensibility. All tetrads produced 2 FLOR$^+$ Kan^S: 2 Flor$^-$ KAN^R (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 *flo11*Δ and YNN295 *flo11*Δ strains. Velum formation by these transformants was assessed in flor medium for 5 days at 28°C.

(*FLO11L*) or the 133d flor yeast (*FLO11F*) were cloned into the pRS316 centromeric plasmid, and their respective pRS316-*FLO11L* and pRS316-*FLO11F* constructs were used to transform YNN295 *flo11*Δ and 133d *flo11*Δ cells (both deleted for their endogenous *FLO11* gene by replacement with the *KANMX4* gene). The 133d *flo11*Δ 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. 3*B*). Interestingly, not only the 133d *flo11*Δ strain recovered this property, but the laboratory *flo11*Δ strain also gained this new function with the only acquisition of the *FLO11F* gene (Fig. 3*B*). 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 floating *Saccharomyces* yeasts from their nonfloating 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 ''floatablity'' 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*A*), suggesting that gene rearrangements could be involved in the functional differences between the two

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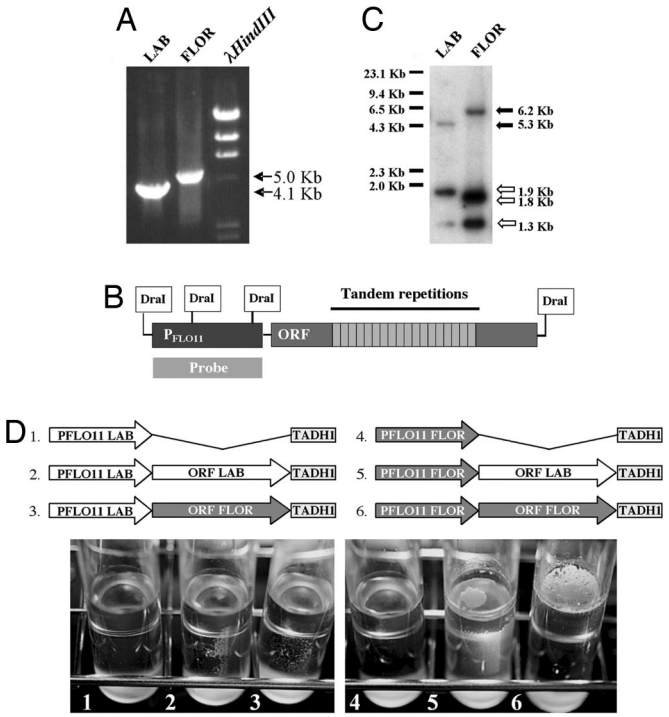

Fig. 4. Allelic differences in the *FLO11* gene of laboratory and flor yeast strains. (*A*) PCR amplification of *FLO11* ORF using genomic DNA from YNN295 (lane 1) or 133d (lane 2) strains as template. (*B*) DNA restriction map for the *FLO11* gene using DraI endonuclease. (*C*) DraI-digested genomic DNA from 133d and YNN295 was analyzed by Southern blot using the whole *FLO11F* 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 *flo11*Δ. Transformants were grown on flor medium.

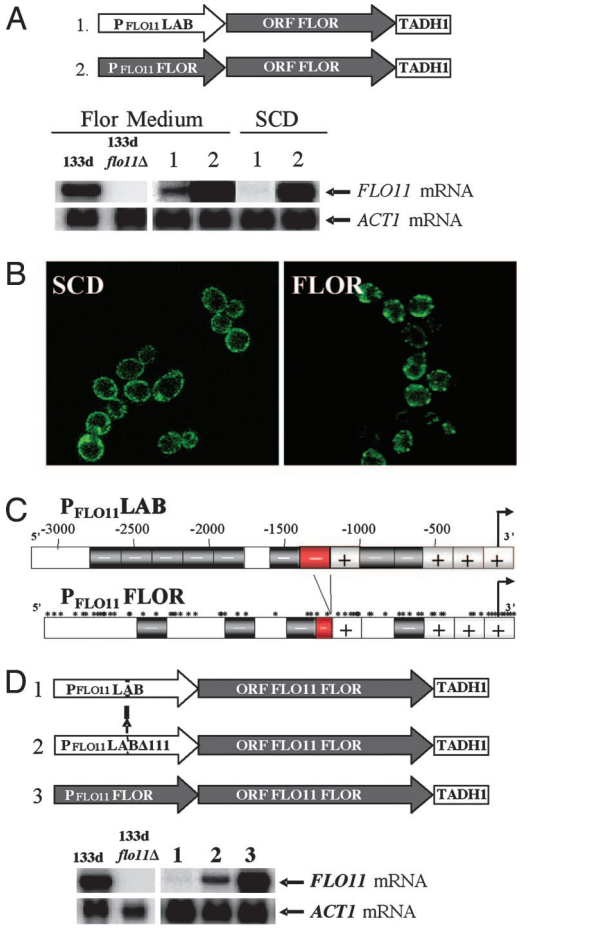

Fig. 5. Expression driven by the *FLO11* promoter. (*A*) The *FLO11F* ORFs under the control of the *FLO11L* (1) or the *FLO11F* (2) promoter were cloned into the pRS316 vector, and the resulting plasmids were transformed into 133d *flo11*Δ cells. *FLO11* expression was measured in comparison with 133d and 133d *flo11*Δ strains growing on flor or SCD medium. (*B*) Flo11p localization was monitored by using confocal microscopy in the 133d *FLO11:GFP* strain exponentially growing on liquid SCD media and during biofilm developing on flor medium. (*C*) The *FLO11F* promoter was sequenced, and the sequence was compared with the *FLO11L* promoter sequence. A scheme representing both promoters is shown. + indicates activator binding regions; − indicates repressor binding regions. Asterisks represent point mutation. (*D*) *FLO11F* ORF under the control of *FLO11L* (1), *flo11LΔ111* (2), or *FLO11F* (3) promoter were cloned into pRS316 vector, and the resulting plasmids were transformed in 133d *flo11*Δ strain. *FLO11* expression was measured in these transformants growing in SCD (133d is used as a control).

alleles. Southern blot analysis indicated that the *FLO11F* promoter region was ≈0.1 kb smaller than that of *FLO11L*, whereas the coding region was ≈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 constructed chimeric *FLO11* genes containing the *FLO11F* ORF under the expression of the *FLO11L* promoter, and *vice versa.* We then tested the capacity of these constructs to restore the floating phenotype to 133d *flo11*Δ cells. All of the chimeric constructs permitted normal invasive growth and biofilm formation in solid surfaces (data not shown); however, as shown in Fig. 4*D*, only the reconstructed *FLO11F* gene fully regenerated the floatability of 133d *flo11*Δ cells in flor medium. Thus, buoyancy in flor yeasts is an adaptive mechanism gained by mutations in both the promoter and the coding region of the *FLO11* gene.

During the process of adaptation of *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. 4*D*). 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. 3*B*), joint to the role that the *FLO11F* promoter itself plays in this character (Fig. 4*D*) 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 *flo11*Δ cells (constructs 1 and

2 in Fig. 5*A*) 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. 5*A*, 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 *FLO11L* promoter was strongly repressed as expected, whereas expression from the *FLO11F* promoter remained high (Fig. 5*A*). 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. 5*B*).

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. 5*C*). 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-111*Δ mutation) and assessed the level of expression of the mutant promoter by Northern blotting. As shown in Fig. 5*D*, the deletion in the *flo11L-111*Δ 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-111*Δ 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 *FLO11* 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. 4*D*). 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 *FL011* of the laboratory strain (Figs. 2 *A* and *B* and 8). However,

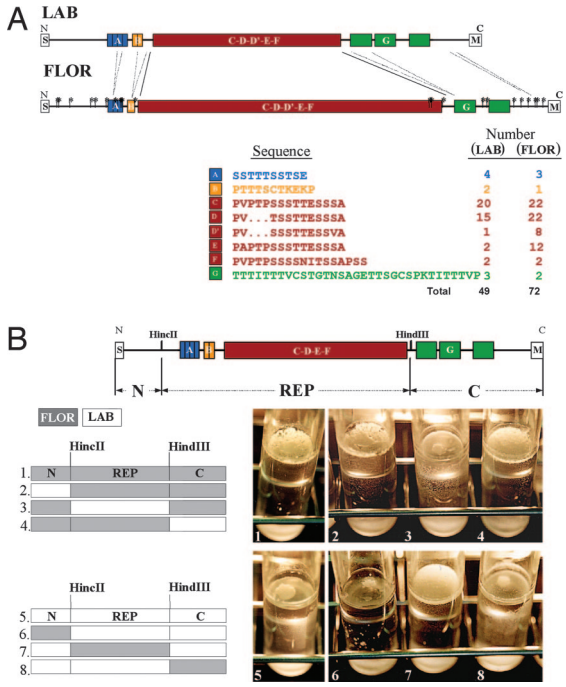

Fig. 6. Functional analysis of the *FLO11* coding region. (*A*) Schematic representations of the Flo11Lp and Flo11Fp primary structure are shown. Repeat domains are named according to ref. 25. Amino acid sequence for repeat domains and repeat number are indicated. (*B*) Chimeric *FLO11* genes that combined the three different domains defined by HincII/HindIII digestion were cloned into pRS316, and the resulting plasmids were transformed into 133d *flo11*Δ. Velum formation was assessed in these transformants in flor medium. S, signal peptide; M, GPI anchor domain; N, N-terminal domain; REP, repeat domain; C, C-terminal domain.

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. 6*A*.

To determine which region of the Flo11Fp protein contributes most to yeast floatability, we took advantage of the HincII 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. 6*B*). These constructs were expressed under the *FLO11F* promoter, and velum formation was assessed in 133d *flo11*Δ cells. As shown in Fig. 6*B*, 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

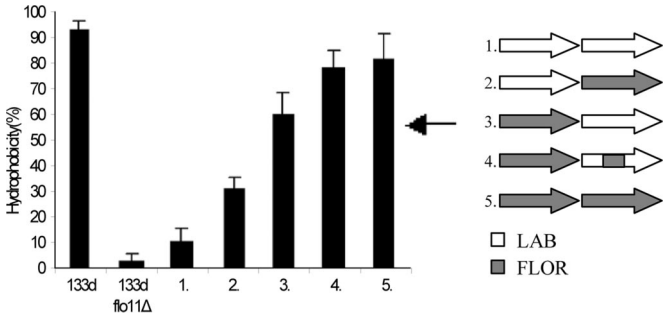

Fig. 7. Cellular hydrophobicity analysis. Chimeras for *FLO11L* and *FLO11F* promoters and ORFs were cloned into pRS316 vector, and the resulting plasmids were transformed in 133d *flo11*Δ. Transformants were grown on SCD media at 28°C, and hydrophobicity was measured as described in *Materials and Methods*. (Average value of at least three independent assays are shown. Error bars are indicated.) Filled arrows represent *FLO11F* gene fragments, and open arrows represent *FLO11L* gene fragments. Over a certain limit (an orientative value is indicated with an arrow), hydrophobicity is sufficient to self-maintain the yeast layer at the liquid surface.

hydrophobicity was extremely high in 133d yeast cells even in SCD media, whereas 133d *flo11*Δ 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 *flo11*Δ 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 hydrofobins 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 (*MATa 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 (*MAT*α *ura3 lys2 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 (*KAN*^{R}) transformants. Yeast nitrogen base containing 6% ethanol as a sole carbon source, supplemented when 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 10^6 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-P5D and FLO11-P6 and FLO11-P6D and simultaneously cloned into the EcoRI site of pRS316 vector. The combinations between promoters and ORFs were performed by cloning the promoters and the ORFs into EcoRI and SmaI/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 pBSSK vector without HincII and HindIII sites. So, the N-terminal domain is flanked by EcoRI/HincII sites, the central domain by HincII/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 OD_{600} of $\approx$0.8, collected, washed, and resuspended in YPD to an OD_{600} 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 OD_{600} of $\approx$0.8, then 1.2 ml of the culture was overlaid with 600 μl of octane and vortexed for 3 min. The OD_{600} of the aqueous layer was taken, and the relative difference with the initial OD_{600} 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 flor 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 pBSSK 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 dideoxy-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 Flor medium to OD_{600} of $\approx$0.05 and grown to an OD_{600} of $\approx$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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