

## Localization and Cell Surface Anchoring of the *Saccharomyces cerevisiae* Flocculation Protein Flo1p

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**The *Saccharomyces cerevisiae* FLO1 gene encodes a large 1,536-amino-acid serine- and threonine-rich protein involved in flocculation. We have assessed the localization of Flo1p by immunoelectron microscopy, and in this study we show that this protein is located in the external mannoprotein layer of the cell wall, at the plasma membrane level and in the periplasm. The protein was also visualized in the endoplasmic reticulum and in the nuclear envelope, indicating that it was secreted through the secretory pathway. The protein was detected by Western blotting in cell wall extracts as a high-molecular-mass (>200 kDa) polydisperse material obviously as a result of extensive N and probably O glycosylation. Flo1p was extracted from cell walls in large amounts by boiling in sodium dodecyl sulfate, suggesting that it is noncovalently anchored to the cell wall network. The membranous forms of Flo1p were shown to be solubilized by phosphatidylinositol-phospholipase C treatment, suggesting that Flo1p is glycosyl phosphatidylinositol (GPI) anchored to this organelle. The expression of truncated forms with the hydrophobic C-terminal domain deleted led to the secretion of the protein in the culture medium. The hydrophobic C terminus, which is a putative GPI anchoring domain, is therefore necessary for the attachment of Flo1p in the cell wall. Deletion analysis also revealed that the N-terminal domain of Flo1p was essential for cellular aggregation. On the whole, our data indicate that Flo1p is a true cell wall protein which plays a direct role in cell-cell interaction.**

Yeast flocculation is an asexual, calcium-dependent, and reversible aggregation of cells into flocs. This phenomenon is thought to involve cell surface components. It is widely accepted that it results from a lectin-like interaction between a cell wall sugar-binding protein and cell surface mannan (22, 30).

Yeast flocculation is under genetic control, and three dominant flocculation genes have been defined by classical genetics, *FLO1*, *FLO5*, and *FLO8* (13). The *FLO1* gene is the gene that has been most studied, and it has been cloned and sequenced by different groups (3, 35, 39). Systematic sequencing of the yeast genome has recently led to the identification of other open reading frames which are homologous with the *FLO1* gene (5, 14). It is likely that two of them correspond to the already genetically defined *FLO5* and *FLO8* genes, while others are new putative flocculation genes (34).

The predicted Flo1 protein is a large, 1,536-amino-acid (aa) serine- and threonine-rich polypeptide which contains numerous repeated sequences and a potential signal peptide for secretion (39). In addition, the Flo1 product possesses hydrophobic C-terminal sequences which are characteristic of signals for glycosyl phosphatidylinositol (GPI) anchor addition (23). These features are consistent with a cell surface localization of the Flo1 protein. We have reported immunological evidence of this type of localization with the Flo1 protein in a previous work (4). A Flo1 homologous protein has also been found associated with fimbria-like structures localized at the yeast cell surface (32).

The *FLO1* gene product is thought to play a major role in flocculation. However, the exact function of this protein in the cell-cell interaction is still unclear. Despite the isolation of

different cell wall lectins described as associated with flocculation (29, 33), the lectin which mediates this interaction has not yet been clearly identified. It is still unclear whether Flo1p corresponds to the flocculation lectin.

In order to investigate the role of Flo1p in flocculation, we examined its distribution in the subcellular compartments associated with the cell surface and began a functional analysis of the protein. By using immunoelectron microscopy and cellular fractionation, we obtained data which show that Flo1p is a cell wall protein. Truncations of the polypeptide revealed that the hydrophobic C-terminal region is necessary for the anchoring of Flo1p in the cell wall, suggesting that GPI anchor addition is necessary for its cell surface attachment. In addition, we show that the N-terminal domain of the protein is essential for the cellular aggregation. The implications of these results for the possible role of Flo1p in flocculation are discussed.

### MATERIALS AND METHODS

**Materials.** Laminarinase, lyticase (partially purified), and alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G were obtained from Sigma. Endo- $\beta$ -*N*-acetylglucosaminidase H (endo-H), phosphatidylinositol-phospholipase C (PI-PLC), phenylmethylsulfonyl fluoride (PMSF), pepstatin, aprotinin, leupeptin, and pefabloc were obtained from Boehringer Mannheim Biochemicals. Protein A-Sepharose 6MB was purchased from Pharmacia Biotech, Inc.

**Strains and growth conditions.** *S. cerevisiae* ABXL-1D (*FLO1 MATa*) was obtained from the Yeast Genetic Stock Center. Strain V5 (*MATa ura3*) (Institut National de la Recherche Agronomique, Montpellier Collection) was used as the recipient strain for transformations. Yeasts were cultivated at 28°C on synthetic minimal medium (0.67% [wt/vol] yeast nitrogen base, 2% [wt/vol] dextrose), which was complemented when necessary.

**DNA manipulation and plasmid construction.** Standard protocols were used for recombinant DNA manipulations (27). The plasmids pRSFLO5, pECFLO5, and YEpFLO5 have been previously described (4). The constructs obtained in this study are presented in Fig. 1. The plasmid pRSFLO5 containing the *FLO1* gene on the pRS316 vector has been used as the starting material with which to delete the 36 aa at the C terminus of Flo1p. For this purpose, the region located between the *Bgl*II site (position 3661) and the *Hind*III site (position 4,856) was first replaced by a fragment covering from the *Bgl*II site to position 3814 and obtained after amplification of the corresponding region with the oligonucleotides 5'-GGAGCTGCTGAGACGAA-3' and 5'-TGAAGCTTATCCTACCAT

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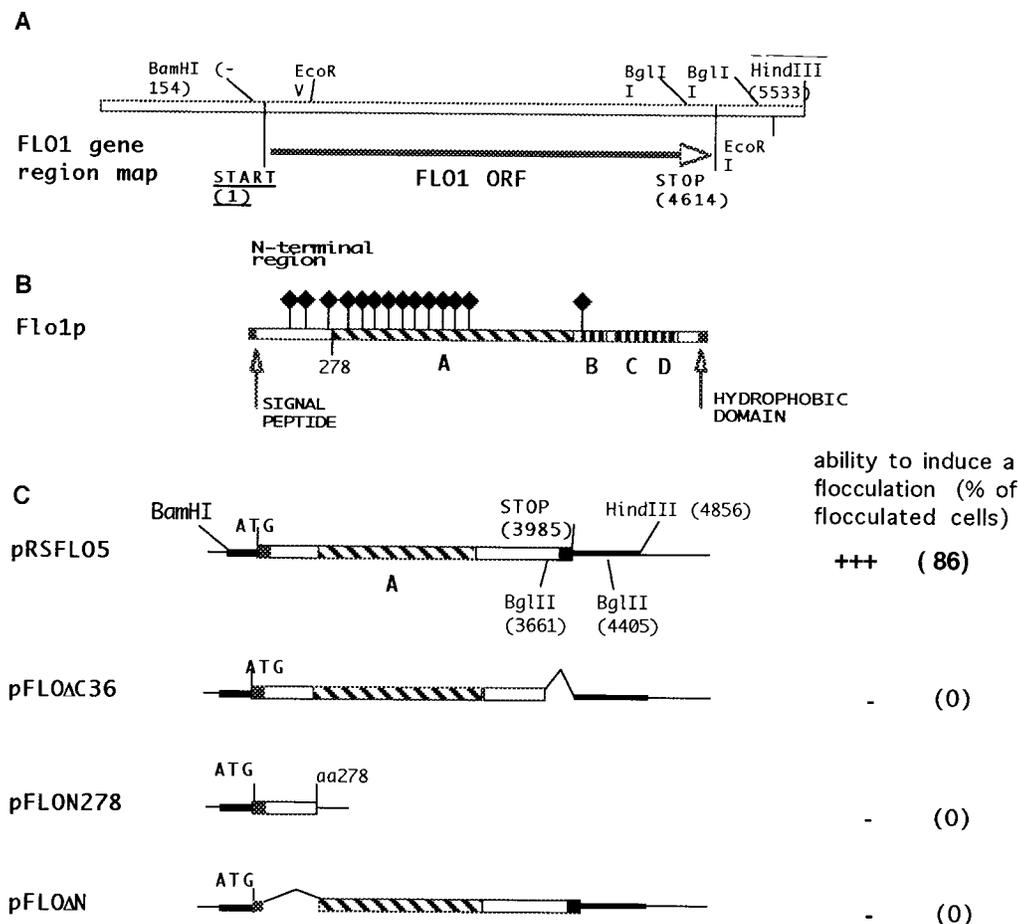


FIG. 1. Schematic representation of the *FLO1* gene, Flo1 protein, and constructs. (A) Restriction map of the *FLO1* gene region. (B) Schematic representation of the wild-type Flo1 protein. The regions corresponding to the repeated sequences (diagonal and vertical stripes) are indicated. The putative N glycosylation sites are represented as solid diamonds. (C) Schematic representation of the different constructs used. The plasmids pFLOΔC36, pFLON278, and pFLOΔN are derived from pRSFLO5. The abilities of the different constructs to trigger flocculation are indicated by +++ (full flocculation) and - (no flocculation). The values in parentheses represent the percentages of settled cells after 1 min.

GCT GCTT-3' (a *HindIII* site [underlined] is created), digestion by *BglII* plus *HindIII*, and ligation. The 3' region of the *FLO1* gene was recovered by amplification of a fragment (positions 4014 to 4445) with the oligonucleotides 5'-TAAAGCTTAACTGTTTCTCCTCCATA-3' (*HindIII* site underlined) and 5'-GTGGCATGTGCAGCAGA-3' and cloning after digestion by *HindIII* and *ClaI* in the preceding construct. The final plasmid was designated as pFLOΔC36.

A construct expressing only the first 278 N-terminal aa of Flo1p, pFLON278, was obtained as follows. A DNA fragment containing a portion of the 5' region, from the *BamHI* site (position -154) and the coding region to position 819, was cloned by amplification with the T7 primer (located on pRS316 at the 5' side of *FLO1*) and the oligonucleotide 5'-AAGCTTATGTAGTGGTACTG-3', digestion by *SacII* (site coming from pRS316 polylinker) and *HindIII*, and ligation in pRS426 (6). The *ADHI* terminator was added to the 3' site of the construct after isolation by amplification of the corresponding region from the pVT-100-U plasmid (38) with the oligonucleotides 5'-CTGCACAATATTTCAAGCTA-3' and 5'-ATGGTACCTACAGGAAAGAG-3', digestion by *HindIII* and *KpnI*, and ligation. The plasmid pFLOΔN expressing a Flo1 protein with a deletion from aa 50 to 278 was constructed as follows. A DNA fragment containing the promoter region starting at -154 and the first 147 nucleotides of the coding region was isolated by amplification from pRSFLO5 with the T7 primer and the oligonucleotide 5'-GAATTCCTTCAATGAATACTG-3', which creates an *EcoRI* site, and digestion by *BamHI* and *EcoRI*. The product was cloned in pRS426. The remaining part of the gene, from aa 278 to the C terminus, was recovered by amplification from the pECFLO5 plasmid (4) with the T3 primer and the oligonucleotide 5'-GAATTCACCTACAACGGAAC-3' (*EcoRI* site underlined), digestion by *EcoRI* and *ClaI*, and ligation to the former construct (the amino acids Glu-Phe are created at the *EcoRI* junction).

**Preparation of cell extracts.** Yeast cells grown to late exponential phase (optical density at 600 nm [OD<sub>600</sub>], 3) were collected and broken with glass beads

for 15 min in the lysis buffer (50 mM Tris-HCl [pH 7.4]; 5 mM EDTA; 1 mM PMSF; 10 μg of pepstatin, leupeptin, and aprotinin per ml; 1 mg of pefabloc per ml). Cell walls were collected by centrifugation for 5 min at 1,000 × g, and the resulting supernatant was centrifuged for 1 h at 100,000 × g. The pellet or membrane fraction was suspended in 50 mM Tris-HCl (pH 7.5)-1 mM EDTA-2% sodium dodecyl sulfate (SDS) and protease inhibitors, and the proteins were extracted by boiling for 5 min. Cell walls were washed three times in 20 mM triethanolamine (pH 4.0)-0.4 M KCl-1 mM MgCl<sub>2</sub>-1 mM PMSF and three times in 1 mM PMSF. Cell wall proteins were extracted by boiling in 50 mM Tris-HCl (pH 8.0)-100 mM EDTA-2% SDS-40 mM β-mercaptoethanol and protease inhibitors for the indicated time. The cell wall-extracted proteins were recovered by centrifugation at 10,000 × g for 5 min. Cell walls of 1.2 × 10<sup>9</sup> cells were treated with 0.2 U of laminarinase in 200 μl of 50 mM Na acetate (pH 5.4) containing a mixture of protease inhibitors (1 mM PMSF; 10 μg of pepstatin, leupeptin, and aprotinin per ml; 1 mg of pefabloc per ml) and incubated overnight at 37°C. After treatment, the extracted and insoluble materials were separated by spinning for 5 min at 10,000 × g.

**Protoplast preparation.** Exponentially growing cells were collected; washed twice in 250 mM EDTA, once in water, and once in 1 M sorbitol; and suspended at 2.10<sup>8</sup> cells/ml in 1 M sorbitol-50 mM Tris-HCl (pH 7.5)-40 mM β-mercaptoethanol-10 mM EDTA-protease inhibitors-400 U of lyticase/ml. The protoplasts were collected by centrifugation and washed three times with 1 M sorbitol-2 mM PMSF.

**endo-H treatment.** Cell extracts or polyethylene glycol (PEG)-precipitated secreted forms were diluted to 100 μl with the endo-H buffer (20 mM sodium acetate [pH 5.5]; 2 mM EDTA; 1 mM PMSF; 10 μg [each] of leupeptin, pepstatin, and aprotinin per ml; 1 mg of pefabloc per ml). endo-H (8 μl) was added, and the mixture was incubated overnight at 37°C.

**PI-PLC treatment.** Aliquots of membrane pellet (100,000 × g) corresponding to 5.10<sup>8</sup> cells were suspended in 60 μl of PI-PLC buffer (70 mM triethanolamine [pH 7.5], 0.16% Triton X-100, and protease inhibitors at the same concentration given above) and incubated overnight at 37°C with 0.6 U of PI-PLC (*Bacillus cereus*) for the assay and without enzyme for the control. The aqueous and membranous phases were separated by centrifugation at 13,000 × g for 30 min and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE).

**Protein precipitation with PEG.** The proteins were precipitated by addition of PEG 4000 to a final concentration of 30%. After 1 h of continuous stirring, the solution was centrifuged for 40 min at 15,000 × g, and the pellet was suspended in 10 mM Tris-HCl (pH 7.5).

**Western blot analysis.** Proteins separated by SDS-PAGE (4 to 15% gradient or 10% polyacrylamide) were electrophoretically transferred to a Hybond C membrane (Amersham). The membranes were incubated for 1 h in phosphate-buffered saline (PBS) containing 5% nonfat milk and for 2 h in the same solution with anti-Flo1p antibody added at a final dilution of 1/4,000. After being washed three times with PBS buffer (10 mM Na<sub>2</sub>HPO<sub>4</sub>, 2 mM KH<sub>2</sub>PO<sub>4</sub>, 0.15 M NaCl, 3 mM KCl [pH 7.4]) and once with N buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl), goat anti-rabbit antibody conjugated with alkaline phosphatase was added, and this mixture was then incubated for 1 h. The membrane was subsequently washed three times in N buffer, and the activity was visualized by addition of 5-bromo-4-chloro-3'-indolyl phosphate *p*-toluidine-nitroblue tetrazolium. The anti-Flo1p polyclonal antibodies specific for an *Escherichia coli*-expressed form of the protein were obtained as previously described (4).

**Electron microscopy and immunolabelling procedures.** Suspensions of yeast cells at 5 × 10<sup>7</sup>/ml were fixed with 3.7% formaldehyde and 0.5% glutaraldehyde in PBS overnight at 4°C. After 3 washes with PBS, the cells were suspended for 1 h in 1% NaIO<sub>4</sub> in PBS, washed again in PBS, and treated with 50 mM NH<sub>4</sub>Cl for 30 min. The cells were included in 1% low-melting-point agarose and fixed again overnight at 4°C. Dehydration was performed by increasing the ethanol concentrations from 50% to 100%. Small agarose blocks were then infiltrated with LR white resin in ethanol-resin mixtures at increasing resin concentrations and finally in pure resin for 1 night at 4°C and for 8 h at room temperature. Polymerization was carried on in oxygen-free capsules for 1 night at 60°C. Ultrathin sections (400 to 600 Å) were obtained with a diamond knife (Ultratome III; LKB) and collected on 300 mesh nickel grids. Anti-Flo1p antibodies were adsorbed on nonfloculating V5 cells, and the IgG was purified by chromatography on protein A-Sepharose 6MB (Pharmacia) according to the manufacturer's instructions. The sections were incubated in PBS-bovine serum albumin (BSA) (2% BSA, 0.1% gelatin, 0.05% Tween 20 in PBS buffer). Anti-Flo1p IgGs diluted 1/100 in Tris-buffered saline-BSA were incubated for 90 min with the sections, and after being washed with PBS-BSA, gold-coupled protein 2A was added for 90 min. After extensive washing with PBS-BSA and with water, the grids were dried and stained prior to observation with 2% uranyl acetate (3 min) and 0.4% lead citrate (2 min). Observations were made with a Phillips EM301 microscope at 80 kV.

**Flocculation measurement.** Yeast cells were deflocculated by two washes in 50 mM Na-citrate (pH 3.0)-5 mM EDTA buffer and suspended in the same buffer at 10<sup>8</sup> cells/ml (5 OD units). Flocculation was induced by addition of CaCl<sub>2</sub> (20 mM final concentration) to 5 ml of the cell suspension in a 10-ml test tube. Tubes were agitated at a rate of 50 oscillations per min for 5 min and then left standing vertically. After 1 min, 0.2 ml of suspension was removed just below the meniscus and mixed with 1 ml of 0.25 M EDTA (pH 8.0), and the OD<sub>600</sub> was measured. The proportion of flocculated cells was calculated by comparison with the OD of the fully deflocculated cell suspension (measured in 0.25 M EDTA).

## RESULTS

**Localization of Flo1p by immunoelectron microscopy.** The Flo1 protein has been previously localized at the yeast cell surface by immunofluorescence (4). To improve the resolution of the corresponding subcellular structures, we reassessed this localization by immunoelectron microscopy with anti-Flo1p polyclonal antibodies. Stationary-phase-grown cells of the V5 strain transformed with the multicopy plasmid YEpFLO5, carrying the *FLO1* gene, display an intense immunolabelling of their surface structures, as shown in Fig. 2A. No labelling was detected with the control strain carrying the YEp352 plasmid (data not shown). Gold particles are concentrated in two areas: at the vicinity of the plasma membrane and in the electron-dense outer part of the cell wall corresponding to the mannoprotein layer. The labelling is homogeneous all around the cell periphery, showing no preferential site for the incorporation of the protein. The intensity of labelling of the two layers varies from cell to cell, but the external mannoprotein layer is generally more reactive, as shown in Fig. 2B. The labelling of the inner layer can correspond to true plasma membrane forms or

to periplasmic forms of Flo1p. The labelling of some sections suggests that Flo1p is closely associated with the plasmalemma (Fig. 2B). However, when the cells display an intense labelling, the epitopes are essentially found in the periplasmic region (Fig. 2A). The accumulation of the protein in the periplasm seems to be restricted to cells actively synthesizing Flo1p and could result from overexpression of the protein.

Careful examination of the micrographs reveals the unusual organization of the Flo1p epitopes. As shown in Fig. 2C, gold particles form linear clusters expanding outside the cell apparently from the plasma membrane. Similarly, chains of gold particles can be observed in the mannoprotein layer (Fig. 2D). In some cases, larger epitope chains are formed which span the whole cell wall, suggesting a connection between the plasmalemma and the cell wall (Fig. 2B and D). Such epitope alignments may reflect either a structural property of Flo1p (an extended structure) or the association of the protein with cell wall fibrillar structures.

Although few endoplasmic reticulum (ER) tubules were visible on these cells, labelling of the lumen and the ER was observed (Fig. 2E). Gold particles are also found in the luminal space of the nuclear envelope (Fig. 2B and E). The latter compartment is thought to be part of the secretory process (21, 25), and these results strongly indicate that Flo1p is secreted through the secretory pathway. In other experiments aimed at analyzing the effect of cell wall removal by glucanases on the distribution of Flo1p, we observed a particularly intense labelling of the nuclear envelope and of the ER, which is abundant and adopts expanded forms in these cells (Fig. 2F). These protoplasts, obtained by the action of lyticase, have lost all of the Flo1p antigens of the external mannoprotein layer. The simultaneous removal of the cell wall and of Flo1p suggests that this protein is intimately associated with this cellular structure. The existence of an abundant, expanded ER (and labelling) could, therefore, be the cell's response to the elimination of the cell wall. The overexpression of Flo1p could also amplify this phenomenon.

These results strongly suggest that Flo1p is a true cell wall protein. It was previously proposed that on the basis of its predicted amino acid sequence, the protein could be attached to the plasma membrane and span the cell wall (4, 31, 35, 39). Our data suggest that this is not the case. Instead it is more likely that the protein is only transiently anchored to the plasma membrane before being incorporated in the cell wall, as shown by Lu et al. (19) for α-agglutinin.

**Detection of Flo1p in the subcellular fractions.** The distribution of Flo1p in different subcellular fractions (cell walls, membranes, and soluble [cytosolic plus periplasmic]) of the naturally flocculent yeast strain ABXL-1D (FLO1) was examined by Western blotting. As shown in Fig. 3A, Flo1p is detected in the soluble and membranous fractions with roughly similar patterns. The only difference between the membrane and soluble fractions is that in the soluble fraction, low-molecular-mass forms (<300 kDa) of the protein are overrepresented. The patterns are very fuzzy, but it is possible to distinguish several discrete bands (more visible with the soluble fraction): two in the range 210 to 250 kDa, one minor band estimated at 300 kDa, and two with molecular masses higher than 300 kDa.

According to the classically accepted behavior of cell wall proteins, noncovalently bound or bound through disulfide bridges (SDS and β-mercaptoethanol extractable) and covalently bound (extractable only with glucanase) (9, 24, 36), we performed protein extractions from purified cell walls with hot SDS-β-mercaptoethanol and with laminarinase. Flo1p is released from the cell walls by a short (5-min) hot SDS treatment

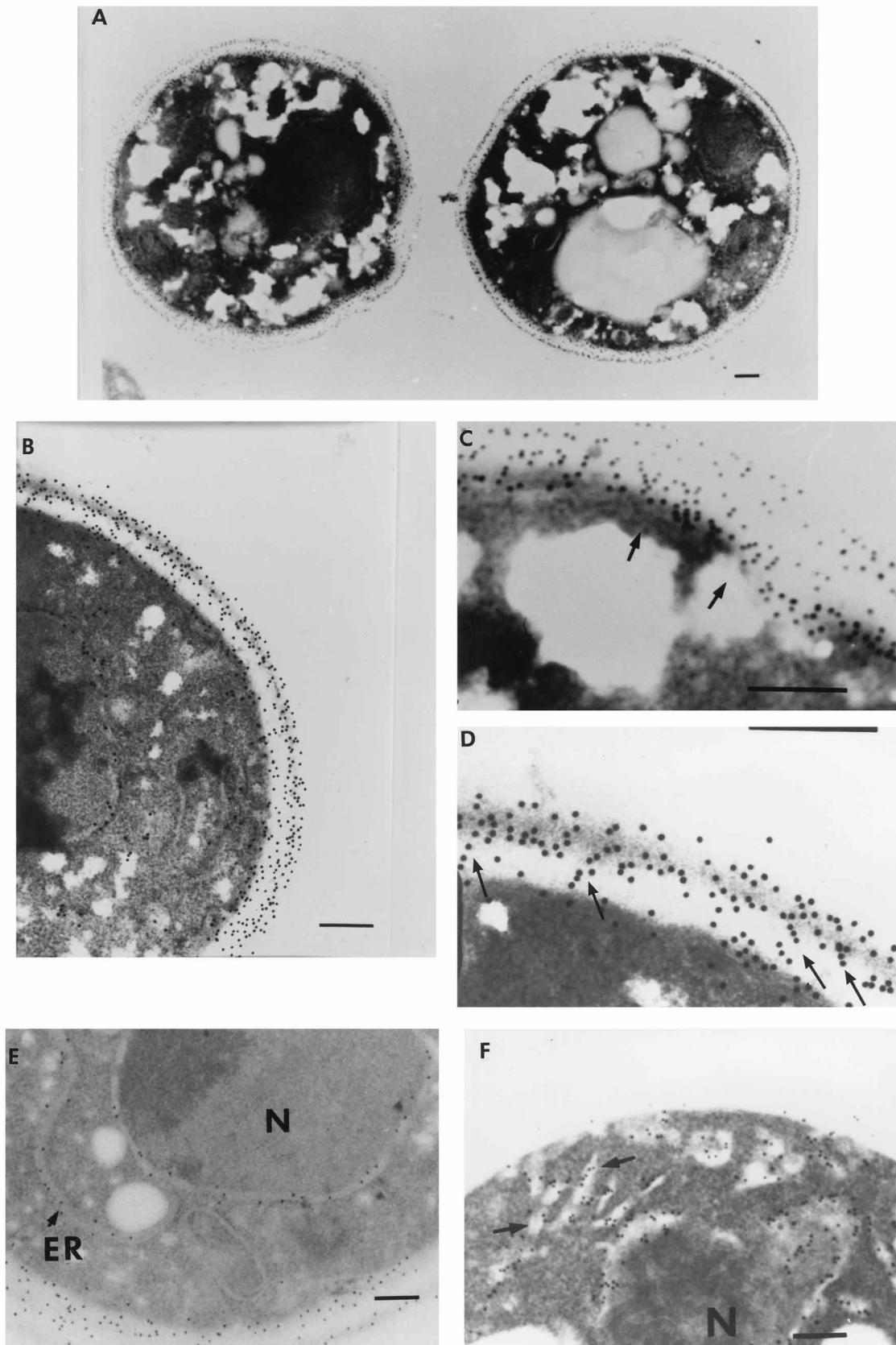


FIG. 2. Immunogold labelling with anti-Flo1p antibodies of V5 cells transformed with YEpFLO5. (A and B) Labelling of the cell wall, periplasm, and plasma membrane. (C) Linear organization of antigens expanding from the plasma membrane in the periplasmic space. (D) Antigens localized in the cell wall and spanning the whole cell wall. (E) Distribution of antigens in the ER and in the nuclear envelope (N, nucleus). (F) Distribution of antigens in the expanded endoplasmic reticulum of protoplasts and removal of the cell wall antigens. Bars, 0.25  $\mu$ m.

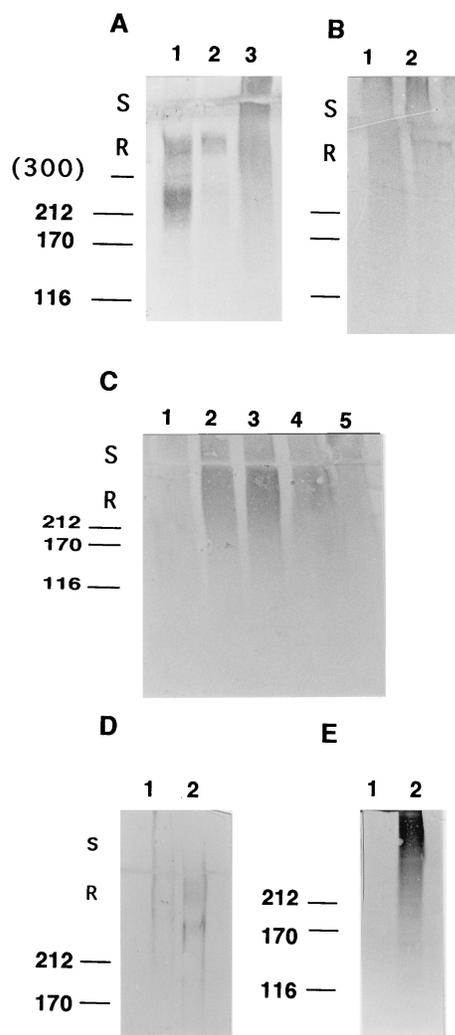


FIG. 3. Western blot immunodetection of Flo1p in the cellular fractions. The protein fractions of strain ABXL-1D were separated by SDS-PAGE with a 4 to 15% polyacrylamide gradient, blotted, and immunodetected with anti-Flo1p polyclonal antibodies. (A) Detection of Flo1p in the soluble fraction (lane 1), membrane fraction (lane 2), and cell wall hot SDS-extract (5 min) (lane 3). (B) Extraction of Flo1p from the cell walls by hot SDS treatment (5 min) (lane 1) and with laminarinase (following the SDS extraction) (lane 2). (C) Extraction of Flo1p from the cell wall by repeated hot SDS treatments. Cell walls were treated successively with hot SDS, once for 5 min and four times for 15 min, and then with laminarinase. The accumulated times of hot SDS treatment were 5 (lane 1), 20 (lane 2), 35 (lane 3), and 65 (lane 4) min, and the laminarinase extract treatment took 65 min (lane 5). (Note that the sample corresponding to the 50-min time is not presented.) (D) Hot SDS extract treatment (5 min) of the cell wall. Lanes: 1, untreated; 2, endo-H treated. (E) Detection of Flo1p in cell wall extracts (second SDS extraction, 5 min) of the V5 strain transformed with the control plasmid YEp352 (lane 1) and YEpFLO5 (lane 2). The amount of soluble and membrane fraction analyzed corresponded to  $0.3 \times 10^9$  cells, and that of the cell walls corresponded to  $1.2 \times 10^9$  cells. The molecular mass markers used were 212, 170, and 116 kDa, as indicated on the left. The 300-kDa size was estimated. S, stacking gel; R, running gel.

(Fig. 3A) and migrates as a highly heterogeneous material with a very high molecular mass. The fastest-migrating forms have molecular masses of 200 kDa. The largest forms migrate poorly in a 4 to 15% polyacrylamide gel, which makes estimation of their molecular masses difficult. Some forms clearly have higher molecular masses than the membrane-extracted ones (>350 kDa), but no discrete band can be distinguished. When,

after a short SDS extraction, the cell walls are subjected to digestion with laminarinase, Flo1p is also released (Fig. 3B). The patterns displayed by laminarinase and SDS-extracted protein are similar. They are very diffuse, and in both cases, not all material enters the resolving gel. We observed that two SDS extractions of 5 min each were not sufficient to remove all of the SDS-extractable material. To examine the extent of the release of Flo1p by hot SDS treatments, repeated extractions were performed before digestion with laminarinase. When successive hot SDS extractions were applied, Flo1p material was released in large amounts, as shown in Fig. 3C. Exhaustion of this material was slow, with some protein still being released in a fifth round of extraction (after one round of 5 min and three rounds of 15 min). If a laminarinase treatment is performed with the resulting SDS-extracted cell walls, only a small amount of Flo1p material is detected. This suggests that most of the protein was released by the repeated SDS treatments.

The fact that it is possible to solubilize a large amount of Flo1p by repeated SDS treatments (which are not expected to destroy covalent linkages) suggests that the protein could be noncovalently linked to the glucan network. Its release by laminarinase may result from nonspecific effects due to the solubilization of most of the cell wall structure. However, Flo1p is firmly anchored in the cell wall and is unusually difficult to extract compared to the classically SDS-extractable low-molecular-mass proteins. We observed proteins from this group that were, unlike Flo1p, totally released after the first two rounds of extraction (data not shown), which agrees with previous reports (9). The behavior of Flo1p also strongly differs from that reported for covalently linked cell wall proteins, such as  $\alpha$ -agglutinin (19). In the latter case, the SDS-extractable form was eliminated by a single SDS extraction and did not represent more than 30% of the cell wall-associated protein.

In all of the fractions, Flo1p has higher molecular masses than that calculated from the predicted amino acid sequence (160 kDa). This is probably due to a glycosylation of the protein, which contains 14 putative N glycosylation sites and a high content of serine and threonine (40%), which are potential O glycosylation sites (18). The detection of Flo1p as a highly polydisperse material is consistent with extensive glycosylation of the polypeptide. As shown in Fig. 3D, when an SDS-extracted fraction is treated with endo-H, a larger amount of the protein enters the resolving gel, and a pattern shift consistent with deglycosylation of the protein can be observed. Flo1p is therefore N glycosylated. However, since the molecular mass of the protein is still higher than the predicted one, Flo1p is also likely to be O glycosylated. In addition, other modifications might be associated with the incorporation of Flo1p in the cell wall because the cell wall forms extracted by hot SDS treatments have higher molecular masses than the membranous forms.

When an equivalent Western analysis was performed with the V5 strain overexpressing Flo1p the protein's distributions in the different fractions were similar. This suggests that the overexpression does not lead to mislocalization of Flo1p (data not shown). It is interesting that detection of Flo1p in the different subcellular fractions of the naturally flocculating strain ABXL-1D corresponds to the localization observed with immunoelectron microscopy after overexpression of the protein. It is likely that the forms extracted from the cell wall correspond to the epitopes localized in the mannoprotein layer. The membranous forms could correspond to those visualized at the level of the plasma membrane and in the secretory organelles. The origin of the soluble forms of Flo1p is less clear. We expected them to correspond to free, periplasmic

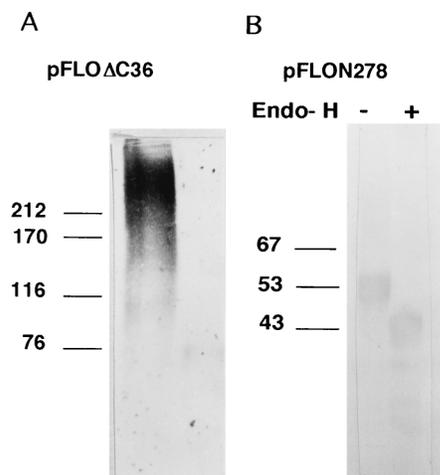


FIG. 4. Immunodetection of the truncated forms of Flo1p secreted in the culture media. After PEG precipitation, the proteins were separated by SDS-PAGE, blotted on a membrane, and immunolabelled with anti-Flo1p antibodies. (A) Detection of the truncated form secreted by the V5 strain transformed with pFLO $\Delta$ C36. (B) Detection of the truncated form secreted after transformation by pFLON278 without (-) or after treatment with (+) endo-H.

forms of the protein. However, pulse-labelling experiments suggested that they are early synthesized forms of Flo1p (data not shown). The soluble forms are likely to correspond to membranous forms solubilized during the breakage of the cells. The fact that these two fractions display similar patterns but with a larger amount of low-molecular-mass forms in the soluble one corresponds with this idea. The high level of glycosylation of the protein and the long grinding times used (15 min) may favor the mechanical solubilization of Flo1p; however, the contribution of a proteolytic action cannot be ruled out.

**The C-terminal hydrophobic domain is required for the attachment of Flo1p at the yeast cell surface.** The Flo1 protein possesses a hydrophobic C-terminal region (aa 1510 to 1536), which was first considered to be a putative transmembrane domain (39) but is more likely to correspond to a GPI anchor signal addition. Since GPI anchors have been implicated in protein anchoring in the cell wall (8, 19, 20, 40), we examined the effect of the deletion of this hydrophobic region on the localization of Flo1p. When the pFLO $\Delta$ C36 plasmid (Fig. 1), carrying the *FLO1* gene with 36 C-terminal codons deleted, was introduced into the V5 strain, no flocculation was observed, indicating that the protein was unable to drive the cell-cell interaction. Western blot analysis of the soluble, membranous, and cell wall fractions did not allow the detection of the Flo1-truncated protein (data not shown). Analysis of the culture supernatant revealed its presence (Fig. 4A) as a fuzzy band of high molecular mass (>160 kDa). Thus, the removal of the hydrophobic C-terminal region prevents the anchoring of Flo1p at the cell surface and leads to the secretion of the corresponding protein in the culture medium.

Similarly, the expression of the highly truncated form, pFLON278, containing only the first 278 N-terminal aa of Flo1p, leads to a nonfloculent phenotype and to the secretion of the corresponding polypeptide in the growth medium (Fig. 4B). Because of its reduced size, we were able to assess the effect of deglycosylation on this form more precisely (the secreted form has a molecular mass of 50 to 55 kDa). As shown in Fig. 4B, the molecular mass can be reduced to 40 to 45 kDa by endo-H treatment. The N-terminus region is therefore N

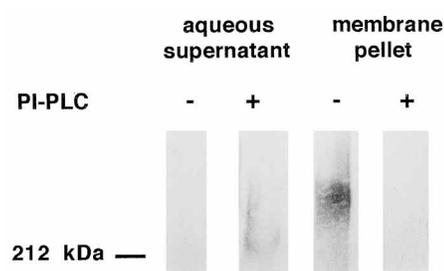


FIG. 5. Release of the membrane-anchored forms of Flo1p by PI-PLC. A crude membrane fraction (pellet [100,000  $\times$  g]) was suspended in the PI-PLC buffer and treated with *B. cereus* PI-PLC. The membrane and soluble fractions were analyzed by immunoblotting.

glycosylated, and this is consistent with the identification of three putative N glycosylation sites in this region (Fig. 1). However, since the expected molecular mass of the nonglycosylated polypeptide is 28 kDa and the one observed after endo-H treatment is 40 to 45 kDa, this polypeptide is also likely to be O glycosylated. This can be explained by a serine and threonine content of 22% for this region.

The secretion of these truncated forms indicates that the hydrophobic C-terminal domain of Flo1p is absolutely required for the attachment of Flo1p at the yeast cell surface. This domain is necessary not only for the retention of Flo1p in the plasma membrane but also for its anchoring in the cell wall.

**The membrane-anchored forms of Flo1p are released by PI-PLC.** The hydrophobic C-terminal domain which is involved in the cell surface attachment of Flo1p displays features of GPI anchor sequences (10, 23). To determine whether the attachment of Flo1p to the plasma membrane was mediated by a GPI anchor, we examined the ability of this protein to be released by PI-PLC. These enzymes are known to cleave specifically GPI anchors, leading to solubilization of the corresponding proteins (7, 10). A membrane pellet of the flocculent strain ABXL-1D was treated with purified bacterial PI-PLC, and the soluble and membrane fractions were analyzed by Western blotting (Fig. 5). After PI-PLC treatment, most of the Flo1 protein was recovered in the soluble phase, whereas in the control sample, it remained in the membrane fraction. This solubilization of the membrane form of Flo1p by PI-PLC is consistent with the GPI anchoring of this protein to the plasma membrane.

## DISCUSSION

**Localization of Flo1p.** Several lines of evidence indicate that Flo1p is a true cell wall protein. The Flo1 protein has been visualized in the cell wall mannoprotein layer by immunoelectron microscopy and has been detected by Western blotting in cell wall extracts. It is interesting that immunoelectron microscopy only succeeded after overexpression of Flo1p, whereas the Western approach, performed with a naturally flocculating strain, gave results which correspond with the immunoelectron microscopy data. This localization of Flo1p is also consistent with the observation that purified cell walls of flocculent yeasts display flocculation-like properties (1).

Similarly, the detection of Flo1p in the membranous fraction is in agreement with its visualization at the level of the plasma membrane and to a lesser extent in the secretory organelles. We have found evidence of secretion of Flo1p through the secretory pathway which corresponds to the existence of a signal peptide for secretion at the N terminus. The use of this

pathway fits with the classical model of protein secretion at the yeast cell surface (26, 28). As suggested by the Western analysis, Flo1p has higher molecular masses than those predicted from its amino acid sequence and is clearly extensively glycosylated during its secretion. This glycosylation seems to be of two types, N and O, as suggested by the effect of endo-H treatments on the full protein or on the secreted N-terminal region. These characteristics are consistent with the existence of 14 putative N glycosylation sites and Flo1p's high serine and threonine content (40%). These data also correspond with the report of a sugar content of 63% for a Flo1p homolog (32). A high level of glycosylation (N and/or O) is a common feature of cell wall mannoproteins (2, 11, 16), and Flo1p clearly belongs to this class of proteins.

The immunoelectron microscopy data suggested the existence of periplasmic forms of Flo1p. These forms are expected to behave as soluble proteins and were not clearly identified in our fractionation experiments. The soluble forms are most probably early synthesized forms and not periplasmic ones. The presence of many forms of Flo1p with low molecular masses in this fraction supports this hypothesis if molecular masses are considered to be relevant to the progression of Flo1p through the secretory pathway. Complementary experiments are necessary to determine the exact origin of the soluble forms and to establish the existence of a soluble periplasmic form of Flo1p.

**Cell wall anchoring of Flo1p.** It is generally accepted that mannoproteins can be divided into two categories: those which are linked to the cell wall by covalent linkage to the  $\beta$ -glucans and those which are weakly associated with the cell wall components by noncovalent bonding, mainly hydrogen bonding, or S-S bridges (9, 16, 24, 36). As shown here, Flo1p can be released in large amounts by hot SDS- $\beta$ -mercaptoethanol treatments. This behavior is consistent with a weak association of the protein with the cell wall network. One possible explanation is that the SDS-extracted form corresponds to precursors before a covalent anchoring to the cell wall. However, because of the large amount of SDS-extracted Flo1 protein, this seems unlikely, because they would represent an unusually large proportion of the total cell wall forms. Actually, the amount of protein which might correspond to only-glucanase-extractable forms of Flo1p proved to be unexpectedly small. Although the existence of covalently anchored forms cannot be totally ruled out, our data strongly suggest that Flo1p is a true noncovalently stabilized cell wall protein. Nevertheless, Flo1p seems to be unusually firmly anchored in the cell wall, because it is less readily solubilized than other SDS-extractable proteins. The high stability of Flo1p in the cell wall might be related to its unusually high molecular mass (>350 kDa), because it is generally accepted that SDS-extractable proteins are small (9).

We have observed that truncation of the hydrophobic C-terminal domain resulted in a loss of cell surface anchorage and protein secretion. A biochemical characterization of the membranous form of Flo1p showed that it was released from the membrane by a treatment with PI-PLC, suggesting that the protein is linked to the membrane by a GPI. These results suggest that GPI anchoring is necessary for the attachment of Flo1p to the cell wall. A requirement for GPI anchor addition has been described for the linkage of  $\alpha$ -agglutinin to the cell wall (19, 40), and it has been proposed that it could represent a general mechanism for the covalent anchoring of proteins in the cell wall (20). Several covalently linked proteins were shown to possess a GPI anchor signal addition, which is consistent with this idea (15, 37). It is unclear whether Flo1p uses this established pathway, since the protein behaves like a noncovalently anchored protein. Our data suggest that Flo1p could

be an unusual member of the cell wall proteins, with a GPI-dependent cell wall anchoring. No GPI requirement has yet been identified for the noncovalent stabilization of proteins in the cell wall. It is generally accepted that noncovalently bound cell wall proteins are stabilized by direct, weak interactions with cell wall components (glucans and chitin) following their secretion in the periplasmic space. More experiments are necessary to establish whether the anchoring of Flo1p relies on a different GPI-dependent anchoring mechanism which leads to noncovalent stabilization of the protein in the cell wall.

**Structural organization and possible role of Flo1p.** The strong glycosylation of Flo1p could have important consequences for the structure of the protein. Most of the glycosylation is expected to be supported by the repeated sequences, which represent 70% of the overall amino acids of the protein (39). Their richness in serine and threonine (about 40%) suggests that they can bear most of the O glycosylation of Flo1p and adopt a stiff and extended conformation (12). Some of the unusual configurations of antigens and the alignments of gold particles, which were observed by immunoelectron microscopy, may be related to such an extended structure of Flo1p (Fig. 2C and D). However, the situation is complicated by the fact that an association of the protein with the glucan network can certainly lead to similar distributions of epitopes. The existence of these alignments of epitopes in the plasma membrane (Fig. 2C) is likely to be related to an extended structure, because the protein is not expected to be associated with the glucans at this level. Nevertheless, an association with the cell wall network could be responsible for the long alignments of epitopes, which span the whole cell wall, because it was observed that they were often surrounded by electron-dense material (not visible in the photographs presented here).

The localization of Flo1p in the outermost part of the cell wall makes it available to drive cell-cell interactions. The repeated sequences are thought to act as spacers to expose a reacting domain of Flo1p at the cell surface. We had previously shown that the efficiency of the interaction was dependent on the length of the repeated sequences (4). As we have shown here, Flo1p is stabilized in the cell wall from its C-terminal side, which suggests that the N-terminal region is the protein's reacting domain at the cell surface. We have assessed the role of this N-terminal region by expressing a truncated form of Flo1p, pFLOAN (Fig. 1), with aa 50 to 278 deleted. This N-terminal domain turned out to be essential for the cellular interaction. The truncated form could not trigger a flocculent phenotype, while the protein was detected in the cell wall (data not shown). This observation is consistent with the idea that this region corresponds to the active domain of the protein. It has already been mentioned that the N-terminal domain displayed features in common with legume lectins (4, 34), and the lectin-like sequences are removed in the construct pFLOAN. These results are also in agreement with those of Kobayashi et al. (17), who recently reported an indirect demonstration that the N-terminal region of Flo1p contains the sugar recognition domain. While no *in vitro* evidence has been provided that Flo1p possesses a lectin activity, Flo1p is likely to correspond to the flocculation lectin, this function being provided by the N-terminal domain of the protein. As a whole, our data are consistent with the theory that Flo1p is the structural protein of flocculation. Its cell wall localization and its probable structural organization are likely to allow it to interact with the adjacent cells and to drive the cellular aggregation. Because the characteristics and functions of very few cell wall proteins have been identified (16), the Flo1 protein could represent a valuable model for deciphering the mechanisms of mannoprotein incorporation in the yeast cell wall.

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## REFERENCES

- Amri, M. A., R. Bonaly, B. Duteurtre, and M. Moll. 1982. Yeast flocculation: influence of nutritional factors on cell wall composition. *J. Gen. Microbiol.* **128**:2001–2009.
- Ballou, C. E. 1982. The yeast cell wall and cell surface, p. 335–359. In E. W. Jones, J. N. Strathern, and J. R. Broach (ed.), *The molecular biology of the yeast Saccharomyces cerevisiae*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
- Bidard, F., B. Blondin, S. Dequin, F. Vezinhet, and P. Barre. 1994. Cloning and analysis of a FLO5 flocculation gene from *Saccharomyces cerevisiae*. *Curr. Genet.* **25**:196–201.
- Bidard, F., M. Bony, B. Blondin, S. Dequin, and P. Barre. 1995. The *Saccharomyces cerevisiae* FLO1 flocculation gene encodes for a cell surface protein. *Yeast* **11**:809–822.
- Bussey, H., D. B. Kaback, W. Zhong et al. 1995. The nucleotide sequence of chromosome I from *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. USA* **92**:3809–3813.
- Christianson, T. W., R. S. Sirkoski, M. Dante, J. H. Shero, and P. Hieter. 1992. Multifunctional yeast high-copy-number shuttle vector. *Gene* **110**:119–122.
- Conzelmann, A., H. Riezman, C. Desponds, and C. Bron. 1988. Myoinositol gets incorporated into numerous membrane glycoproteins of *Saccharomyces cerevisiae*: incorporation is dependent on phosphomannomutase. *EMBO J.* **7**:2233–2240.
- De Nobel, H., and P. N. Lipke. 1994. Is there a role for GPI in yeast cell wall assembly? *Trends Cell Biol.* **4**:42–45.
- Elorza, M. V., A. Murgui, and R. Sentandreu. 1985. Dimorphism in *Candida albicans*: contribution of mannoproteins to the architecture of yeast and mycelial cell wall. *J. Gen. Microbiol.* **134**:2393–2403.
- Ferguson, M. A. J., and A. F. Williams. 1988. Cell-surface anchoring of protein via glycosyl phosphatidylinositol structures. *Annu. Rev. Biochem.* **57**:285–320.
- Fleet, G. H. 1991. Cell walls, p. 199–277. In A. H. Rose and J. S. Harrison (ed.), *The yeasts*, vol. 4. Yeast organelles. Academic Press, London, United Kingdom.
- Jentoft, N. 1990. Why are the proteins O-glycosylated? *Trends Biochem. Sci.* **15**:291–294.
- Johnston, J. R., and H. P. Reader. 1983. Genetic control of flocculation, p. 205–224. In J. F. T. Spencer, D. M. Spencer, and A. R. W. Sait (ed.), *Yeast genetics, fundamental and applied aspects* Springer-Verlag, New York, N.Y.
- Johnston, M., et al. 1994. Complete nucleotide sequence of *Saccharomyces cerevisiae* chromosome VIII. *Science* **265**:2077–2082.
- Kapteyn, J. C., R. C. Montijn, E. Vink, J. De La Cruz, A. Llobell, J. E. Douwes, H. Shimoi, P. Lipke, and F. M. Klis. 1996. Retention of *Saccharomyces cerevisiae* cell wall proteins through a phosphodiester-linked  $\beta$ -1,3-/ $\beta$ -1,6-glucan heteropolymer. *Glycobiology* **6**:337–345.
- Klis, F. 1994. Cell wall assembly in yeast. *Yeast* **10**:851–869.
- Kobayashi, O., N. Hayashi, N. Unemoto, and H. Sone. 1997. The region of Flo1p responsible for sugar recognition. *Yeast* **13**(Special issue):S233.
- Lehle, L., and E. Bause. 1984. Primary structural requirement for N- and O-glycosylation of yeast mannoproteins. *Biochim. Biophys. Acta* **799**:246–251.
- Lu, C.-F., J. Kurjan, and P. N. Lipke. 1994. A pathway for cell wall anchorage of *Saccharomyces cerevisiae*  $\alpha$ -agglutinin. *Mol. Cell. Biol.* **14**:4825–4833.
- Lu, C. F., R. C. Montijn, J. L. Brown, F. Klis, J. Kurjan, H. Bussey, and P. N. Lipke. 1995. Glycosyl phosphatidylinositol-dependent cross-linking of  $\alpha$ -agglutinin and  $\beta$ -1,6-glucan in the *Saccharomyces cerevisiae* cell wall. *J. Cell Biol.* **128**:333–340.
- Matile, P., H. Moor, and C. F. Robinow. 1969. Yeast cytology, p. 219–302. In A. H. Rose and J. S. Harrison (ed.), *The yeasts*, vol. 1. Academic Press, New York, N.Y.
- Miki, B. L. A., N. H. Poon, A. P. James, and V. L. Selegy. 1982. Possible mechanism for flocculation interactions governed by the gene FLO1 in *Saccharomyces cerevisiae*. *J. Bacteriol.* **150**:878–889.
- Nuoffer, C., A. Horvath, and H. Riezman. 1993. Analysis of the sequence requirement for glycosyl phosphatidylinositol anchoring of *Saccharomyces cerevisiae* Gas1 protein. *J. Biol. Chem.* **268**:10558–10563.
- Pastor, F. I. J., E. Valentin, E. Herrero, and R. Sentandreu. 1984. Structure of the *Saccharomyces cerevisiae* cell wall, mannoproteins released by zymolase and their contribution to wall architecture. *Biochim. Biophys. Acta* **802**:292–300.
- Preuss, D., J. Mulholland, C. A. Kaiser, P. Orlean, C. Albright, M. Rose, P. W. Robbins, and D. Botstein. 1991. Structure of the yeast endoplasmic reticulum: localization of the ER protein by immunofluorescence and immunoelectron microscopy. *Yeast* **7**:891–911.
- Ruiz-Herrera, J. (ed.) 1992. Fungal cell wall: structure, synthesis and assembly. CRC Press, Boca Raton, Fla.
- Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: a laboratory manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
- Schekman, R., and P. Novick. 1982. The secretory process and yeast cell surface assembly, p. 361–393. In E. Jones and J. Broach (ed.), *Molecular biology of the yeast Saccharomyces*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
- Shankar, C. S., and S. Umesh-Kumar. 1994. A surface lectin associated with flocculation in brewing strains of *Saccharomyces cerevisiae*. *Microbiology* **140**:1097–1101.
- Strafford, M. 1992. Yeast flocculation: reconciliation of physiological and genetic viewpoints. *Yeast* **8**:25–38.
- Strafford, M. 1994. Another brick in the cell wall? Recent developments concerning the yeast cell envelope. *Yeast* **10**:1741–1752.
- Straver, M. H., G. Smit, and J. W. Kijne. 1994. Purification and partial characterization of a flocculin from brewer's yeast. *Appl. Environ. Microbiol.* **60**:2754–2758.
- Straver, M. H., V. M. Traas, G. Smit, and J. W. Kijne. 1994. Isolation and partial characterization of a mannose specific agglutinin from brewer's yeast involved in flocculation. *Yeast* **10**:1183–1193.
- Teunissen, A. W. R. H., and H. Y. Steensma. 1995. The dominant flocculation genes of *Saccharomyces cerevisiae* constitute a new subtelomeric gene family. *Yeast* **11**:1001–1013.
- Teunissen, A. W. R. H., E. Holub, J. Van der Hucht, J. A. van den Berg, and H. Y. Steensma. 1993. Sequence of the open reading frame of the FLO1 gene from *Saccharomyces cerevisiae*. *Yeast* **9**:423–427.
- Valentin, E., E. Herrero, F. I. J. Pastor, and R. Sentandreu. 1984. Solubilisation and analysis of mannoprotein molecules from the cell wall of *Saccharomyces cerevisiae*. *J. Gen. Microbiol.* **130**:1419–1428.
- Van Der Vaart, J. M., L. H. P. Caro, J. W. Chapman, F. M. Klis, and C. T. Verrips. 1995. Identification of three mannoproteins in the cell wall of *Saccharomyces cerevisiae*. *J. Bacteriol.* **177**:3104–3110.
- Vernet, T., D. Digniard, and D. Thomas. 1987. A family of yeast expression vectors containing the phage f1 intergenic region. *Gene* **52**:225–233.
- Watari, J., Y. Takata, M. Ogawa, H. Sahara, S. Koshino, M. Onnela, U. Airaksinen, R. Jaatinen, M. Penttillä, and S. Keränen. 1994. Molecular cloning and analysis of the yeast flocculation gene FLO1. *Yeast* **10**:211–225.
- Wojciechowicz, D., C.-F. Lu, J. Kurjan, and P. N. Lipke. 1993. Cell surface anchorage and ligand-binding domains of the *Saccharomyces cerevisiae* cell adhesion protein  $\alpha$ -agglutinin, a member of the immunoglobulin superfamily. *Mol. Cell. Biol.* **13**:2554–2563.