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Cloning and Characterization of the Beer Foaming Gene *CFG1* from *Saccharomyces pastorianus*

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ABSTRACT: Foam production is an essential characteristic of beer, generated mainly from the proteins present in the malt and, to a minor extent, from the mannoproteins in brewer's yeast cell walls. Here, we describe the isolation and characterization of the novel fermentation gene *CFG1* (Carlsbergensis foaming gene) from *Saccharomyces pastorianus*. *CFG1* encodes the cell wall protein Cfg1p, a 105 kDa protein highly homologous to *Saccharomyces cerevisiae* cell wall mannoproteins, particularly those involved in foam formation, such as Awa1p and Fpg1p. Further characterization of Cfg1p revealed that this novel protein is responsible for beer foam stabilization. This report represents the first time that a brewing yeast foaming gene has been cloned and its action fully characterized.

KEYWORDS: *yeast, foam, beer, Saccharomyces pastorianus, mannoprotein*

INTRODUCTION

Foam quality is an important organoleptic property of beer that directly correlates to consumer appeal.[1] Foam formation depends on several factors, such as the presence of proteins, hop iso-α-acids, metallic ions, polysaccharides, and melanoidines.[2] The proteins originate mainly from the barley and include LTP1 (9.7 KDa lipid-transfer protein) and Z (40 KDa protein) as well as hordein-derived polysaccharides with molecular weights ranging from 10 to 30 KDa.[1,3,4] The yeasts involved in beer fermentation, either *Saccharomyces cerevisiae* or *Saccharomyces pastorianus*, release cell wall mannoproteins to the medium, mostly as a result of the action of β-glucanases,[3,5] which exhibit high foaming ability.[6−8] These yeast mannoproteins are tightly bound to either 1,6-β-D-glucan, through glycosylphosphatidylinositol (GPI), or to 1,3-β-D-glucan, through alkali-sensitive bonds, and the innermost layer of *S. cerevisiae* cell wall is formed by a polysaccharide net of 1,3-β-D-glucan strands, linked by hydrogen bonds, bound to 1,6-β-D-glucans and chitin.[9−14]

The yeast cell wall mannoproteins are released to the medium during fermentation, and they confer foam stability as a result of their hydrophobic nature. When gas bubbles are formed, the mannoproteins orient their hydrophobic protein moiety toward the inner side of the bubble, whereas their hydrophilic glycosylated moiety faces toward the surrounding liquid. The hydrophobic molecules increase the surface tension of the bubbles, whereas the glycosylated ones increase the liquid viscosity, thus increasing the foam stability and retarding the drainage of the liquid.[6,15−18] The presence of hydrophobic polypeptides in the brew favors both production and stability of foam, and it has been shown that an increase in these polypeptides coincides with higher amounts of yeast cells and also with foam production levels.[19,20]

The involvement of yeasts in foam formation was first established by fermenting synthetic must, in which foam was present in higher levels than in nonfermented must because of the presence of the released yeast mannoproteins. From these studies, it was concluded that yeast mannoproteins were involved mainly in the maintenance of foam, rather than in foam formation.[8,20,21] Traditionally, brewers have classified yeast into two types: ale and lager yeasts. This classification was based on the yeast's ability to flocculate; accordingly, ale strains tend to float on the fermentation tank and are usually known as "top-fermenting yeasts", whereas lager strains are identified as "bottom-fermenting yeasts". Another difference relates to their fermentation temperature: ale yeasts ferment between 15 and 26 °C, and lager yeasts do so between 8 and 15 °C.[22]

Both ale and lager yeasts belong to the *Saccharomyces* genus, although both physiologic and genetic analyses have shown them to belong to different species. Originally, the ale strains were classified as *S. cerevisiae*, whereas the lager strains belonged to the *S. pastorianus* species.[22−24] Later, DNA−DNA hybridization experiments revealed that the lager strains were a hybrid between *S. cerevisiae* and *S. bayanus*. They were shown to possess three types of chromosomes: (i) chromosomes from *S. cerevisiae* (Sc), (ii) *S. bayanus* chromosomes (also known as lager type chromosomes or Lg), and (iii) recombinant chromosomes originating from both yeasts Sc/Lg. These findings were later confirmed when the complete *S. pastorianus* Weihensntephan 34/70 genome was sequenced.[24−26]

In 1974, Kasahara et al.[27] were the first to identify the genes of a *S. cerevisiae* sake strain responsible for foaming and concluded that foam production was under the control of at least two genes. Thornton (1978)[28] also studied foaming in a *S. cerevisiae* strain and concluded that two genes, *FRO1* and *FRO2*, located on the right arm of chromosome VII, were responsible; he concluded that both genes were nonadditive dominants and allelic to others found in *S. cerevisiae* sake strains,[27−29] but these gene sequences, as well as the proteins they encode, remain yet unknown.

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Table 1. Yeast Strains Used in This Study, Their Genotype, Their Foam Production Ability and Their Origin

yeast strain	species	genotype	foam production	origin
Weihenstephan 34/70	*S. pastorianus*	lager, wild	+	Hijos de Rivera S.A.
SP5	*S. pastorianus*	*MATa, hoΔ*	+	this study
SPH5	*S. pastorianus*	*MATa, hoΔ, cfg1::loxPKanMXloxP*	−	this study
SPHA1	*S. pastorianus*	*MATa/MATα, HO/HO, cfg1::loxPKanMXloxP*	−	this study
SPHA1b	*S. pastorianus*	*MATa/MATα, HO/HO, cfg1Δ*	−	this study
MI-2B	*S. cerevisiae*	*MATα, ura3-52, trp1*	−	Benítez T
CSH84L	*S. cerevisiae*	*MATα, spo11, ura3, can1, cyh2, ade2, his7, hom3*	−	Benítez T
CSH89L	*S. cerevisiae*	*MATa, spo11, ura3, ade1, his1, leu2, lys7, met3, trp5*	−	Benítez T

Only two *S. cerevisiae* proteins have so far been shown to be responsible for the fermentation foaming phenotype of this species: protein Awa1p and protein Fpg1p. These proteins were identified in the K7sake strain and the 145A211 wine strain, respectively.[30,31] Both Awa1p and Fpg1p were shown to be cell wall mannoproteins, and both the genes and the proteins they encode are highly homologous to each other and to yeast cell wall mannoproteins such as Hpf1p (haze protein factor) used in the clarification of white wines.[30,32]

The above-described scarcity of information on yeast genes involved in foam-generation and maintenance prompted us to investigate the gene(s) generating foamy phenotypes in the brewery yeast strain *S. pastorianus* Weihenstephan 34/70. Here, we describe the isolation and characterization of the novel fermentation gene *CFG1* (Carlsbergensis foaming gene), which encodes the cell wall protein Cfg1p. The mature Cfg1p polypeptide has a molecular weight of 105 kDa and a pI of 4.86 and is highly homologous to *S. cerevisiae* mannoproteins, in particularly to those involved in foam formation, such as Awa1p and Fpg1p. Further characterization of Cfg1p demonstrated that this novel protein is responsible for beer foam stabilization by *S. pastorianus* Weihenstephan 34/70.

MATERIAL AND METHODS

Cell Culture. *Escherichia coli* TOP10 cells (Invitrogen Corporation, Carlsbad, CA, U.S.A.) were grown in Luria−Bertani (LB) medium at 37 °C for 12 h, supplemented with ampicillin (50 μg/mL) when required. All yeast strains used in this study belong to either the *S. pastorianus* or the *S. cerevisiae* species and are listed in Table 1. Yeasts were regularly cultured in YPD medium, supplemented with the antibiotics G418 (200 μg/mL), cycloheximide (2 μg/mL), or tunicamycin (1.5 μg/mL), as required. Auxotrophic yeast strains were grown in SD medium supplemented with the appropriate amino acid. When the carbon source had to be modified to incorporate galactose, the usual YPD or SD growth media were replaced by YPGal and SGal, respectively; they contained galactose instead of glucose as their carbon source. Although most yeast cultures were incubated at 30 °C overnight, those grown on sporulation media (SPOI) required incubation at 23 °C for 5 days. In all cases, solid media was prepared by supplementing the liquid media with agar (2% w/v).

Tetrads were obtained from either homothallic or heterothallic diploid strains by mating the appropriate strains. The diploids were then maintained in SPOI medium until more than 75% of asci containing four ascospores were generated. Asci were treated with lyticase for 20 min at 30 °C, and spore separation was accomplished with the help of a Micromanipulator II (Allen Benjamin Inc.) coupled to a Nikon SE microscope.

***CFG1* Cloning and Expression.** The starting material for cloning the *CFG1* gene was *S. pastorianus* Weihenstephan 34/70 strain genomic DNA. The *CFG1* gene was PCR-amplified using the high fidelity polymerase Accuzyme DNA polymerase (Bioline, Taunton, MA, U.S.A.) and the primer pair awa11 and awa12 that correspond to nucleotides 1 to 30 and 5113 to 5142 in the *AWA1* gene sequence (AB071164.1).[30] The amplified DNA thus obtained was cloned into the plasmid pCRBluntII TOPO (Invitrogen) and transformed into competent *E. coli* TOP10 cells. From the resulting, ampicillin-resistant recombinant plasmids, we selected TOPO−CFG1. which was purified using a Miniprep Express Matrix kit (MP Biomedicals, Vista, CA, U.S.A.). *CFG1* was then sequenced, using the primer pair M13F and M13R, corresponding to DNA sequences present in the original TOPO plasmid. DNA sequence analysis was performed using the VectorNTI.9 program (Invitrogen) as well as the BLAST program, whereas the protein sequence data was analyzed with the tools provided by de Expasy proteomic server.

The entire *CFG1* DNA fragment was released from the TOPO−CFG1 plasmid by digestion with the restriction enzyme BstxI and subcloned into the yeast expression vector pYES2 (Invitrogen), giving rise to expression plasmid pYES2-CFG1. This plasmid was transformed into *S. cerevisiae* competent cells by the lithium acetate method.[33]

All the PCR primers and vectors mentioned here are included in Tables 2 and 3, respectively.

Table 2. Oligonucleotide Primers Used in This Study for the Purpose of PCR Amplification, Mutation, or Sequencing[a]

primer	primer sequence 5′ to 3′
awa11	ATGTTCAATCGCTTTAATAAACTTCAAGCC
awa12	TTAGTTAAAGAAAGCAAGAACGAAAATACC
FPGfint	ATGTTCAATCGCTTTAATAAACTTCAAGCCGCTTTGG **CTTTGACCCTTAATATAACTTCGTATAATG**
FPGrint	TTAGTTAAAGAAAGCAAGAACGAAAATACCGACCAA **TGCCCTAATAACTTCGTATAGCATAC**
HO1f	AAAA*CTGCAG*CGACTATTCTGATGGCTAACGG
HO1r	ACGC*GTCGAC*GTCCATCTGCGCACATAACG
HO2f	CGC*GGATCC*TGC*GATATC*TGCAAGTATGTACCAGAAGC
HO2r	CCG*GAATTC*CACTCTGGTCCTTTAACTG
loxPF	CGC*GGATCC*GCGGAG*GTCGAC*AACCCTTAATATAAC
loxPR	CGC*GGATCC*GCG*GATATC*ACCTAATAACTTCGTATAGC
M13F	GTAAAACGACGGCCAG
M13R	CAGGAAACAGCTATGAC

[a]The loxP-Kan and KanMX-loxP sequences are depicted in bold letters, whereas the restriction enzyme recognition sequences are in italics.

Chromosome Loss Mitotic Mapping. Before this assay could be carried out, *S. pastorianus* Weihenstephan 34/70 strain had to be made heterothallic, and this was accomplished using the disruption cassette HO-KanMX, present in vector pDHO, as previously described in Blasco.[34] Chromosome loss was then induced by treatment of the yeast with methylbenzimidazol-2-il-carbamate (MBC) following the procedure described by Wood.[35]

PCR-Mediated CFG1 Gene Disruption. *CFG1* gene disruption was accomplished by PCR, using the FPG1-KanMX cassette, which confers resistance to G418. This was possible because of the high homology that the 5′ and 3′ ends of *FPG1* share with those of *CFG1*.[31] The cassette was PCR-amplified by 30 cycles of 95 °C for 1 min, 55 °C for 30 s, and 68 °C for 2 min using Accuzyme DNA polymerase (Bioline, Taunton, MA, U.S.A.).

FPG1-KanMX cassette integration was confirmed by PCR, using the primer pair awa11 and awa12, whereas primers awa11/loxP2 and

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Table 3. Summary of the DNA Plasmids Used or Constructed in This Study, Their Characteristics, and Their Origin

vector	description	source or reference
PCRBluntIITOPO	Kan^R	Invitrogen
TOPO−CFG1	Kan^R, *CFaG1*	this study
pYES2	Amp^R, URA3, GAL promoter	Invitrogen
pYES2-CFG1	Amp^R, URA3, GAL promoter, *CFG1*	this study
pUG6	Amp^R, KanMX	Güldener et al. (1996)
YEp351-Cre-Cyh	Amp^R, CYH^R, LEU2, GAL promotor Cre recombinase	Delneri et al. (2000)
pDHO	Amp^R, lacZα, ho::KanMX	Blasco et al. (2011)

awa12/loxP1 were used to confirm removal of the cassette. The sequence in the cassette conferring G418 resistance was deleted from the recombinant yeast by means of the plasmid YEp351-Cre-Cyh[36] that contains a galactose-inducible cre-loxP system.

Isolation of Yeast Cell Walls and Mannoprotein Extraction. The recombinant yeast cells were grown on YPD, supplemented with tunicamycin, for 48 h. After centrifugation at 3000*g* for 5 min, cells were disrupted with a Braun MSK homogenizer, and cell walls were obtained by an additional centrifugation as above. Mannoproteins were extracted from the cell walls by treatment with SDS and 400 lyticase (μg/mL). The resulting proteins were then analyzed by protein gel electrophoresis (PAGE) on 7.5% SDS−PAGE gels.

Yeast Phenotype Analyses. To test sensitivity to lyticase, yeast cells were cultured overnight on YPD at 30 °C, for 48 h, recovered by centrifugation at 3000*g* for 5 min, and washed twice with water. The cells were then resuspended in 0.1 M sodium phosphate buffer (pH 7.5) containing lyticase (200 μg/mL; Sigma-Aldrich) and incubated at 30 °C for 2 h. Cellular density was measured by reading the optical density (OD) of the yeast culture at 600 nm. To determine osmotic sensitivity, the yeast cultures were grown on YPD solid medium, supplemented with increasing sorbitol concentrations (1, 1.5, 2, 2.5, or 3M). Five microliters of a yeast culture, containing 10^6 cells/mL, was grown on YPD plates for 3 days at 30 °C.

Sensitivity to sodium dodecyl sulfate (SDS) was tested by the ability of the yeast cells to grow on YPD plates supplemented with 10% SD. The plates were grown at 30 °C for 4 days. To test calcofluor sensitivity, the YPD plates contained 0.1% of this compound and were grown at 30 °C for 2 days.

Exponentially growing yeast cells were collected by centrifugation, washed first with sterile water and then with PBS and stained with calcofluor as described by Cabib.[37] The calcofluor-stained cells (100 μL aliquots containing 10^7 cells) were visualized on a transilluminator (Molecular Imager Gel Doc XR; Bio-Rad, Hercules, CA, U.S.A.).

For the ethanol tolerance test, 5×10^6 yeast cells were used to inoculate 5 mL of YPD medium, supplemented with either 12% or 15% ethanol. The cells were allowed to grow at 30 °C for 24 h, and their cell density was assessed by an OD reading at 600 nm.

Temperature sensitivity was assessed by incubating the yeast on YPD plates for 4 days at 15, 23, 30, or 37 °C.

To determine cell surface hydrophobicity, yeasts were grown on YPD medium overnight at 30 °C. The cells were then collected by centrifugation, washed twice with sterile water, and resuspended in 3 mL of 10 mM KNO_3 buffer (pH 4.5). The yeast suspension was then treated with 1 mL of *n*-hexane, vigorously stirred for 25 s, and allowed to rest for 20 min. At this stage, the organic and aqueous phases had separated, and the decrease in absorbance at 570 nm was taken as the amount of cells transferred from the aqueous to the organic solvent. Cell surface hydrophobicity (CSH) was obtained according to the formula CSH = $(A_i570\ \text{nm} - A_f570\ \text{nm})/A_i570\ \text{nm}$; where A_i is the initial absorbance (before addition of *n*-hexane) and A_f is the final absorbance of the aqueous phase (after *n*-hexane extraction).

All assays were performed in triplicate.

Fermentations. To investigate if Cfg1p plays a role in fermentation, microfermentations of tyndalized beer must were conducted, in triplicate, at 15 °C. Yeast starter cultures were grown overnight at 30 °C on 100 mL of YPD medium. The cells were then recovered by centrifugation at 3000*g* for 20 min at 4 °C and resuspended in must at a final cell density of 10^6 cells/mL. The fermentation rate of each strain was gravimetrically evaluated by weight loss due to the CO_2 evolved. The CO_2 values thus obtained were used to estimate the fermentation rate (Vf_m), defined as the average of the initial fermentation rate (Vf_i: grams of CO_2 produced daily during the 5 initial days) and the total fermentation rate (Vf_t: grams of CO_2 produced during the overall fermentation). The fermentation power (Pf) was also evaluated and defined as the grams of CO_2 produced at the end of the fermentation. Samples of the culture media were taken at regular intervals; their pH was measured; and the ethanol, acetic acid, and glycerol content of the supernatant was analyzed using commercially available kits (Boehringer, Manheim, Germany)

The foam stability of the fermented beer was measured following the "shaking method" described by Kapp and Bamforth (2002).[38] For this purpose, 5 mL of beer was placed in a 15 mL test tube (1.5 cm diameter), and the tubes were capped. The beer samples were then hand-shaken 10 times, for 3 s each time, after which the stopper was removed. The height of the foam layer was measured twice, first 30 s after agitation and then 30 min later.

All the assays were performed in triplicate.

RESULTS

***CFG1* Gene Cloning and Sequencing.** Our strategy for cloning *S. pastorianus CFG1* gene was based on the homology shared by yeast cell wall mannoproteins, in particular, those involved in foam formation, such as Awa1. *CFG1* was obtained by PCR-amplification of *S. pastorianus* Weihenstephan 34/70 genomic DNA with the primer pair awa11 and awa12 (Table 1). These primers contained DNA sequences corresponding to the 5′ and 3′ ends of the *S. cerevisiae AWA1* coding region.[31] This PCR amplification produced a single DNA fragment of 3408 bp, which was then inserted into the PCRBluntII TOPO plasmid and sequenced. The cloned DNA contained the complete ORF sequence of a novel gene. We named the gene *CFG1* (Calsbergensis foaming gene) and deposited its sequence in the GenBank database (accession number EU414029.1). The *CFG1* gene displayed high DNA sequence homology (98% identity) to the *S. cerevisiae* K7 (sake strain) *AWA1*gene as well as to the SC288 strain *HPF1*, EC1118 strain *HPF1*, and wine strain 145A211 *FPG1* genes, but despite the high homology, these genes greatly differ in their size. Thus, the *AWA1* is 5141 bp long, whereas the *HPF1* and *FPG1* genes are only 2903 bp and 2313 bp long, respectively. These three genes encode yeast cell wall mannoproteins, and both *AWA1* and *FPG1* genes have been confirmed to produce foamy phenotypes that have been mapped to chromosomes XV and VII, respectively.[30,31,39]

The *CFG1* gene encodes an 1136 amino acid protein (named Cfg1p; Figure 1 A, GenBank accession number ABZ1081.1), with a molecular weight of 111 kDa and an isoelectric point (pI) of 4.3. The Cfg1p protein was characterized as a cell wall protein, since it contains the amino acid motifs characteristic of yeast cell wall mannoproteins (Figure 1 A). Cfg1p hydropathy analysis (Figure 1 B) revealed that both the amino and carboxyl terminal ends of the protein were hydrophobic. The polypeptide spanned a signal peptide (amino acids 1−24), a serine-rich domain (25.9%, amino acids 29−389), a threonine-rich region (17.80%, amino acids 686−1081), a possible GPI anchorage point (amino acid 1113), 755 putative O-glycosylation sites, and several N-glycosylation sites (amino acids 28, 35, 553, 661, and 698). Post-translational processing of the Cfg1p protein resulted in a polypeptide containing 1089 amino acids with a molecular weight of 105 kDa and a pI of 4.86.

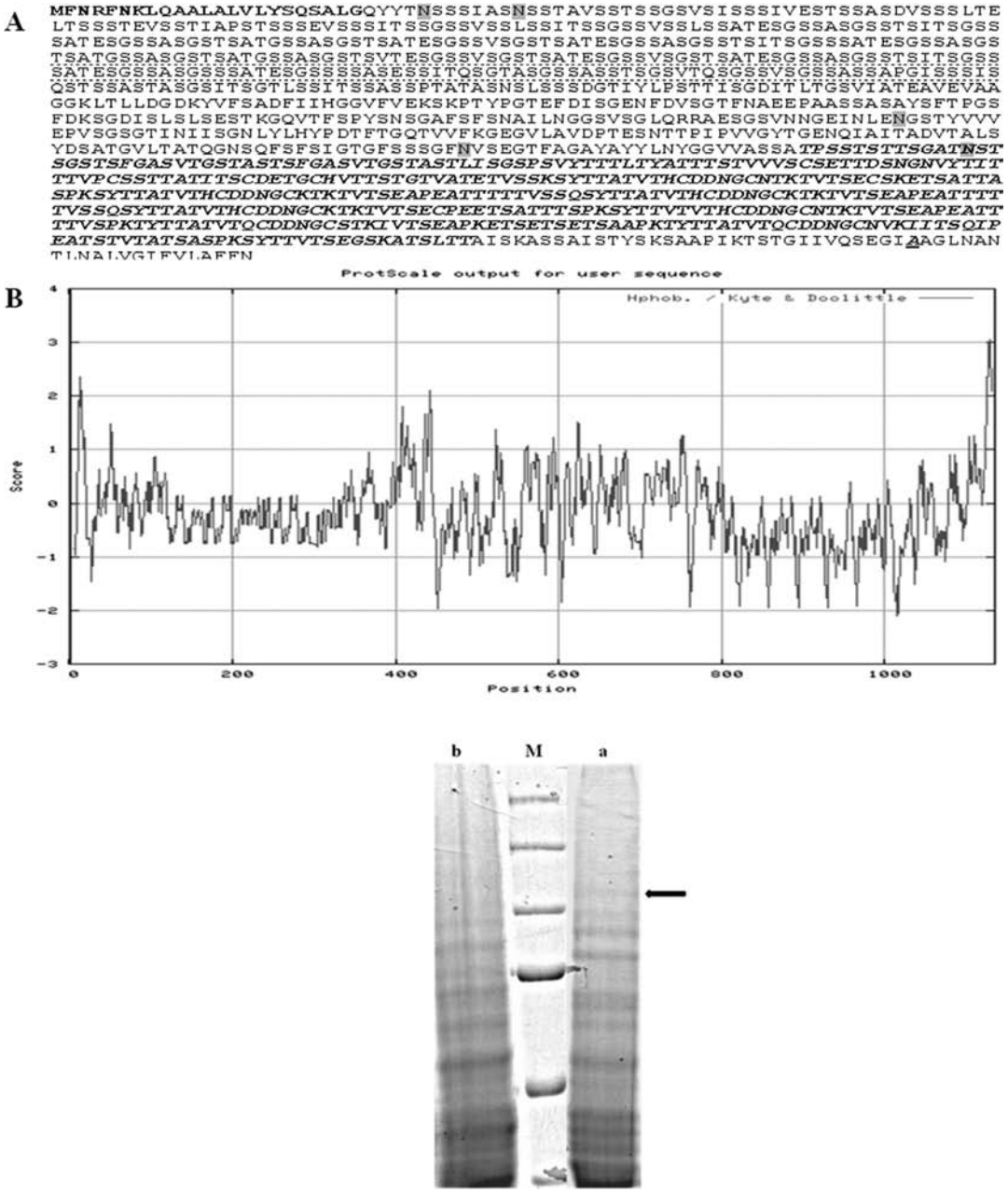

Figure 1. Cfg1p protein analyses. (A) Fpg1p amino acid sequence. The bold letters indicate the signal peptide, bold italic and underlined letters indicate the putative position of the GPI anchor. The serine-rich domain is indicated by a dotted underline, whereas the threonine-rich domain is indicated as italic bold letters, and the two N-glycosylation sites are shown as shaded letters. (B) Kyte and Doolittle[45] hydropathy plot for Fpg1p. (C) PAGE analyses of postalkali extracted mannoproteins. The arrow shows the position of the Cfg1p protein (105 KDa). Wild-type strain (lane a), SPDHA1b strain (lane b). Molecular weight markers (M; 200,150, 100, 75, and 50 KDa).

S. pastorianus Cfg1p was highly homologous to Awa1p, a foam-producing protein from *S. cerevisiae* sake strain K7. In addition, Cfg1p was similar to the Hpf1p protein from several *S. cerevisiae* strains (sc288, Fosters B, EC1118, Vin13, AWRI 796, Lanvin AQ23, and Fosters O).The Cfg1p polypeptide was also similar to all the published Hpf1p proteins from wine strains, except for those from strains SC288 (genetic strain) and Fosters B and O (brewer's yeast). In addition, Cfg1p was also homologous to YIL69CP, a protein isolated from the genetic strain SC288, which, like Hpf1p, is also rich in serine and threonine, and to Fpg1p, a foam-producing protein from the wine strain 145A211 (Figure 2). But despite the high homology shared by some areas of these proteins, between 90% and 100%, these polypeptides differ in size; thus, Cfg1p is 100 amino acids longer than Hpf1p, and the difference is even greater with Awa1p, which is 578 amino acids shorter that Cfg1p.

According to the structure and homology analysis, Cfg1p corresponds to a mannoproteins of the cell wall.

***CFG1* Located on Chromosome XV.** As a first approach, we used the Southern Blotting technique to attempt to determine *CFG1* chromosomal location. For this purpose, the *S. pastorianus* Weihenstephan 34/70 chromosomes were extracted, separated by pulsed field gel electrophoresis, and probed with a 1385 bp DNA sequence corresponding to a fragment of the *CFG1* gene. Unfortunately, these results were inconclusive because the probe hybridized with DNA sequences in several chromosomes (data not shown).

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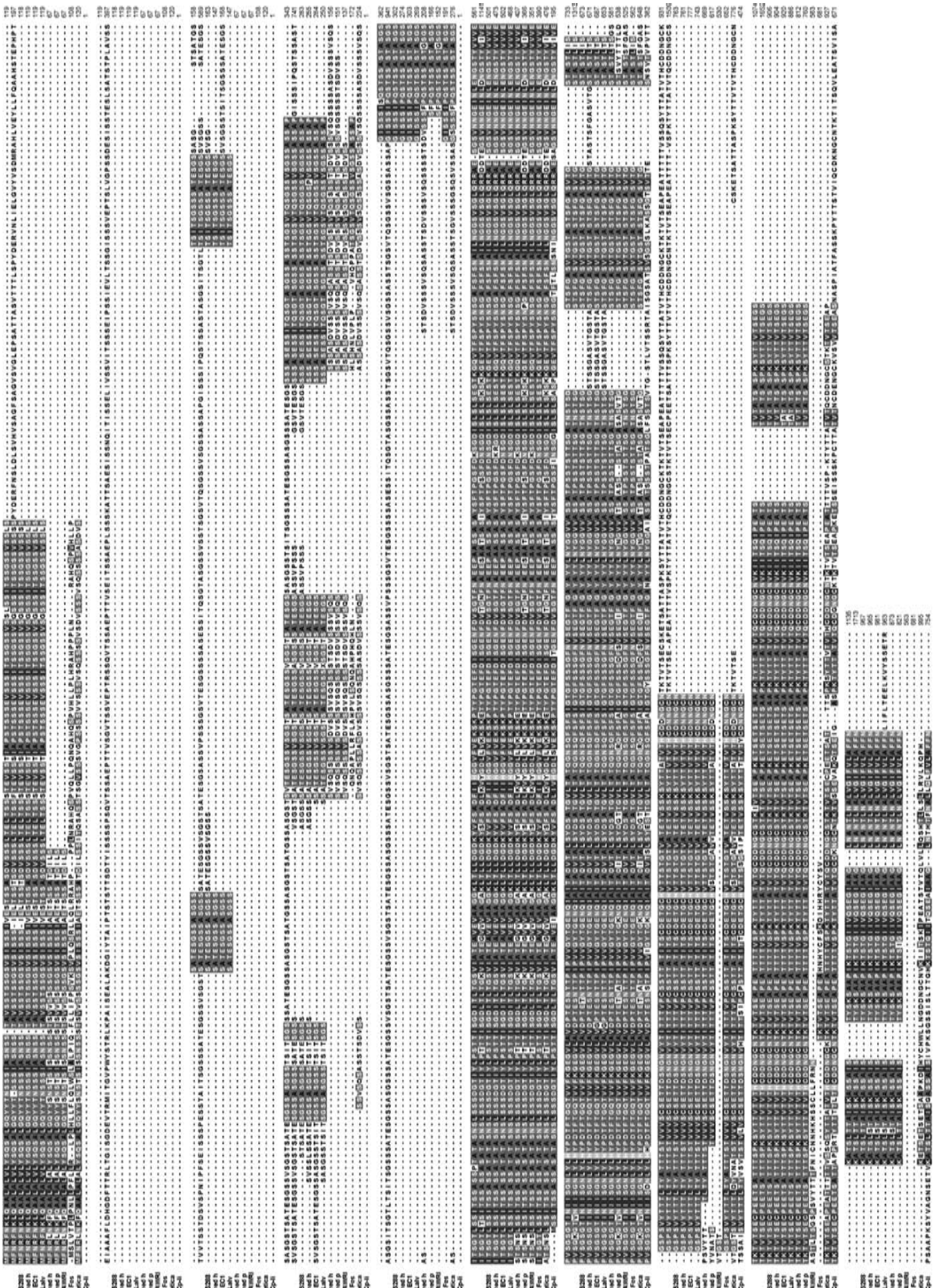

Figure 2. Homology of the protein Cfg1p. In descending order: Fpg1p; Awa1p (*S. cerevisiae* k7), Hpf1p (*S. cerevisiae* s288c), conserved hypothetical protein (*S. cerevisiae* YIL169C), Hpf1p (*S. cerevisiae* EC1118), Hpf1p (*S. cerevisiae* LalvinQA23), conserved hypothetical protein (*S. cerevisiae* RM11-1a), conserved protein (*S. cerevisiae* YJM789), Hpf1p (*S. cerevisiae* AWRI796), Hpf1p (*S. cerevisiae* Fosters O), Hypothetical protein SCY2333 (*S. cerevisiae* YJM789), and YIL169C like protein (*S. cerevisiae* Vin13).

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We then resorted to mitotic mapping by the chromosome loss technique.[35] This approach required the strain Weihenstephan 34/70 to be converted to the heterothallic state, and this was accomplished by the use of the disruption cassette HO-KanMX, present in plasmid pDHO, following the procedure we previously described.[31] This resulted in the elimination of a copy of the Sc-*HO* gene, thus giving rise to the Weihenstephan 34/70 heterothallic strain SP5, containing a single copy of the Sc-*HO* gene.

The heterothallic strain SP5 was then successfully transformed with the disruption cassette FPG1-KanMX. This was possible because of the homology between *FPG1* and *CFG1*, giving rise to G418-resistant recombinant yeast colonies. Once the correct integration of the FPG1-KanMX cassette was confirmed by PCR analysis (data not shown), a recombinant colony was selected (named SPH5) and used to locate the *CFG1* gene on the basis of its resistance to G418. As indicated by the CHEF-Southern Blotting results (data not shown), *CFG1* was homologous to several genes located on chromosome XV. We further investigated this homology by yeast mating assays between yeast strains SPH5 and CSH84L (a *S. cerevisiae* strain that harbors auxotrophies in all its chromosomes and, in particular, in the ade2 marker in chromosome XV). The yeast diploids obtained were then treated with benomyl to eliminate homologous chromosomes and, hence, reduce chromosome numbers. This resulted in the generation of diploid colonies that were both resistant to G418 and contained the ade2 marker, hence, allowing us to confirm the location of the *CFG1* gene on the Sc type chromosome XV.

***CFG1* Gene Deletion.** To determine the function of the *CFG1* gene, we deleted the gene and analyzed the phenotypic and metabolic changes originating from this deletion. For this purpose, the wild-type *S. pastorianus* Weihenstephan 34/70 strain was transformed with the disruption cassette FPG1-KanMX and the recombinant colonies selected by their resistance to the antibiotic G418. The correct integration was then confirmed by PCR amplification using the oligonucleotide pair awa11/awa12. Successful integration of the disruption cassette resulted in the generation of an heterozygous yeast strain (*CFG1*/*cfg1*::FPG1-KanMX). To produce homozygous cells, the yeasts were induced to sporulate in the presence of the antibiotic G418. The resistant homothallic spores entered mitosis and originated diploid cultures homozygous for the disrupted *CFG1* gene. One of these diploids (named SPHA1) was selected for transformation with the Yep351-Cre-Cyh vector. This resulted in removal of the loxP-KanMX-loxP cassette, yielding a diploid yeast strain lacking *cfg1* and sensitive to G418, which was named SPDHA1b.

Characterization of *CFG1* Function. Sequence analyses of the *CFG1*DNA sequence already gave a clear indication that this gene encoded a cell wall mannoprotein (Figure 1), but our *CFG1* gene deletion allowed us to further characterize this protein and investigate the phenotypic effects of Cfg1p on the yeast cell wall. For this purpose, we carried out a series of assays comparing the *S. pastorianus* Weihenstephan 34/70 wild-type strain to the *cfg1*Δ SPDHA1b strain we generated (Figure 3). As shown in Figure 3A, although both strains are able to grow at the tested temperatures (15, 23, 30, and 37 °C), the *cfg1*Δ strain displayed a slower growth rate at 15 °C than the wild-type strain.

The anionic detergent SDS has the potential to be toxic to yeast cells, since it breaks the hydrophobic interactions between glycoproteins and the 1,3-β-D-glucan in the yeast cell wall. Hence, yeast cells containing cell wall mutations are normally more sensitive to SDS than wild-type strains.[40] Figure 3B shows that, although the wild-type strain was able to grow normally in the presence of SDS, SPDHA1b was unable to do so, even after 96 h of culture in these conditions. The wild-type and *cfg1*Δ strains displayed the same sensitivity to ethanol, being able to grow ethanol concentrations ranging from 12% to 15% (data not shown).

We were also unable to find any differences between the strains concerning their sensitivity to Calcofluor White, even after Calcofluor White staining.

Sensitivity to lyticase was determined by the decrease in cell density (measured by optical density at 600 nm during 120 min in the presence of lyticase) of the yeast cultures after 12 and 48 h of culture (Figure 3E). After 12 h of culture at 30 °C, the cell density of the wild-type strain decreased 80%, whereas that of the SPDHA1b was similarly decreased, but this decrease in cell density was less marked in the initial incubation stages. When the cultures were incubated for 48 h, an even more striking difference was apparent, with the wild-type strain cell density decreased by 42%, whereas the SPDHA1b strain was more resistant to lyticase, with a decrease of 18%. The above results, taken together, strongly support the hypothesis that Cfg1p is located in the yeast cell wall.

Additional support was provided by the hydrophobicity studies we carried out on the two strains, which revealed significant differences between the wild-type and SPDHA1b strains. Yet again, these results are consistent with the location of Cfg1p in the yeast cell wall, with *CFG1* deletion resulting in a relative hydrophobicity decrease of 40% at 24 h. This difference was even more marked after 48 h of culture (50%), even though the overall hydrophobicity had then increased in both strains (Figure 4).

***CFG1* Deletion Did Not Significantly Alter Fermentation.** The fermentation activity of yeast strains on brewer must is established by must weight loss due to CO_2 production. We found no significant differences in the fermentation rate or fermentative power of the SPDHA1b strain as compared with the wild-type, although CO_2 production was slighter higher in the former (Table 4, Figure 5E). In addition, the two strains displayed no significant differences in ethanol (Figure 5A), acetic acid (Figure 5B), or glycerol production (Figure 5C). The only significant difference detected was a slight pH increase in the beer fermented by the SPDHA1b strain (Figure 5D), as compared with that obtained in the presence of the wild-type strain. All fermentation compounds analyzed, as well as the pH value, were well within the acceptable range for beer.[41] These results indicated that Cfg1p was not included in the fermentation metabolism.

Purification and Characterization of Cfg1p. Cfg1p protein was purified from the cell walls of the Weihenstephan 34/70 wild-type yeast strain. To avoid protein N-glycosylation, both the wild-type and SPDHA1b strains were grown in the presence of tunicamycin. Although no differences between the two strains were observed during either the SDS extraction or the lyticase treatment, the postalkali treatment revealed a 105 KDa protein present only in the wild-type strain (Figure 1 C). This polypeptide had the molecular weight expected for the mature Cfg1p protein, having undergone the appropriate post-translational modifications.

Cfg1p Is Involved in the Yeast Foamy Phenotype. We carried out microbrewing assays with both *S. pastorianus* Weihenstephan 34/70 and SPDHA1b strains to study the effect of the Cfg1p protein deletion on foam formation and stabilization. No difference was found between the two strains concerning the amount of foam produced, but the foam generated by the SPDHA1b strain proved to be less stable (as determined by the "shaking method") than that produced by the wild-type strain (Figure 6 A). In addition, the bubbles generated

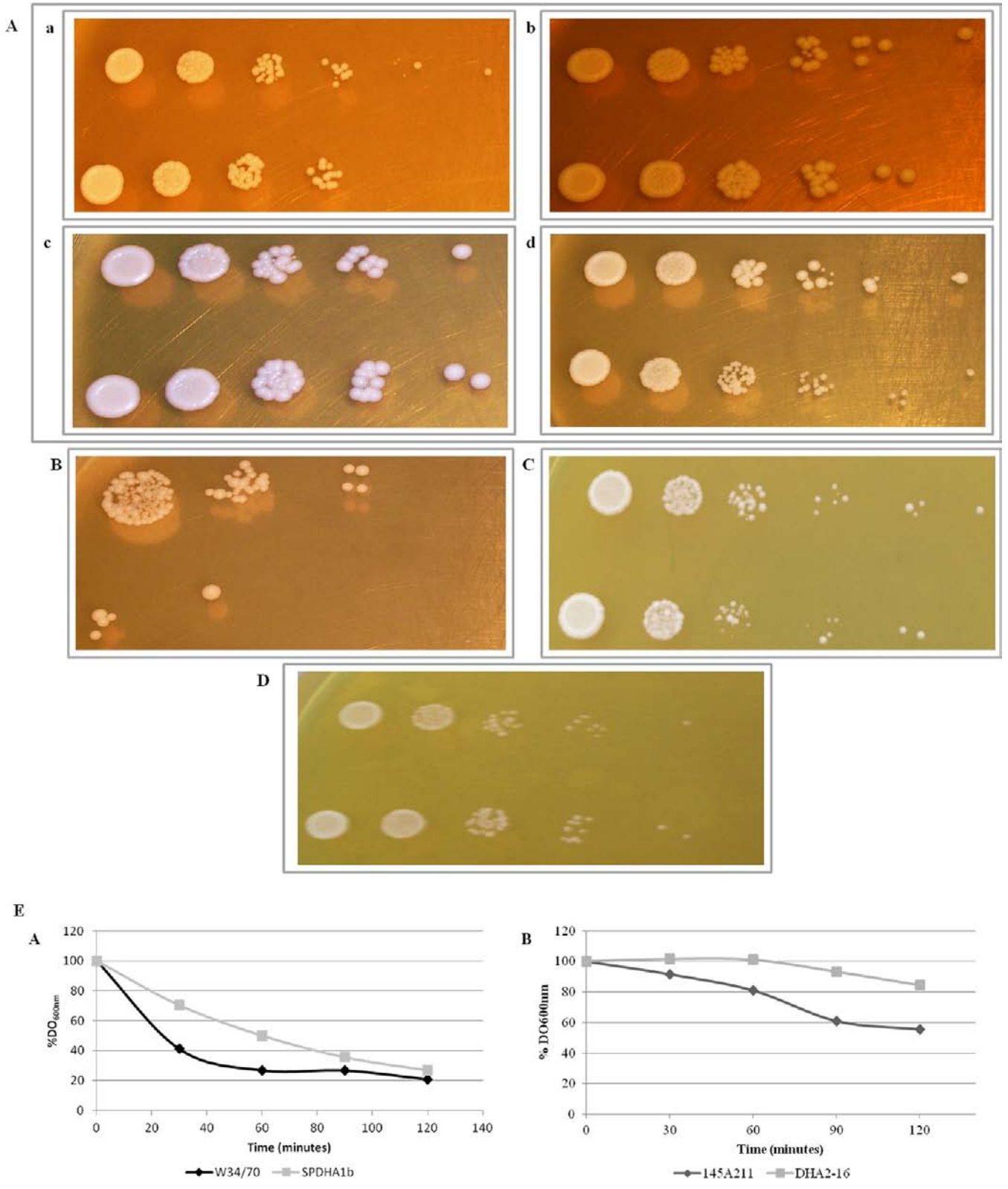

Figure 3. Phenotypic analyses of *S. pastorianus* yeast strains Weihenstephan 34/70 (top row) and SPDHA1b (lower row). (A) Growth at different temperatures: 15 (a), 23 (b), 30 (c), and 37 °C (d). (B) Growth in the presence of SDS. (C) Growth in the presence of Calcofluor White. (D) Growth in the presence of sorbitol. (D) Sensitivity of the two strains to the enzyme lyticase after either 12 (A) or 48 h (B) of incubation.

by the former strain were larger than those produced by the wild-type (Figure 6 B).

We further investigated the involvement of Cfg1p in the yeast foamy phenotype by cloning the *CFG1* gene into the foamless *S. cerevisiae* MI2B strain. This was accomplished by transforming the MI2B strain with the expression vector pYES2 containing the *CFG1* gene, thus generating the strain MI2B-CFG1. Cfg1p protein expression was induced by growing the yeast in YPGal

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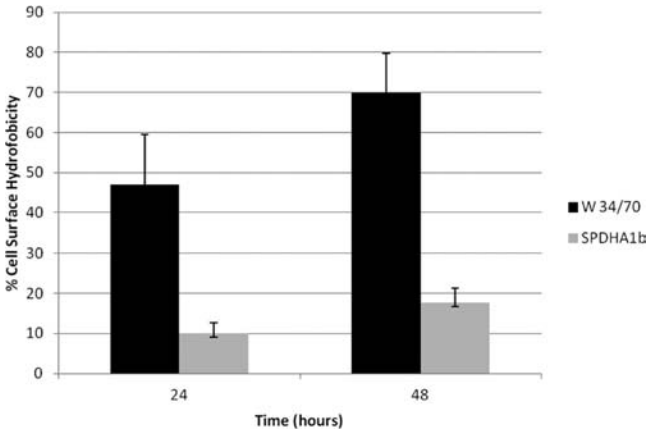

Figure 4. Cell surface hydrophobicity of strains Weihenstephan 34/70 y SPDHA1b.

Table 4. Fermentation Rate of the Weihenstephan 34/70 (W34/70) and SPDHA1b Strains[a]

strain	Vf_i	Vf_t	Vf_m	Pf
W34/70	0.017 33	0.012 18	0.014 76	0.72
SPDHA1b	0.024 67	0.015 15	0.019 91	0.83

[a] Vf_i is the amount (grams) of CO_2 produced daily during the first 5 days of fermentation, whereas Vf_t is the amount (grams) of CO_2 produced daily during the overall fermentation time. Vf_m is the fermentation velocity, taken as the average of Vf_i and Vf_t. Pf represents the fermentative power. This is the grams of CO_2 produced at the end of fermentation process.

induction medium for 48 h. After this, the cells were collected and used as a starting culture in microfermentations of brewer must. The rationale of this experiment was that, although Cfg1p protein expression could not be driven by the GAL1 promoter under the fermentation conditions, because of the lack of galactose in the brewer must used, the yeast cells used in the starting culture had the Cfg1p protein present and active in their cell walls, where the protein could keep on carrying out its action. The MI2B-pYES2 strain, containing a copy of the expression plasmid but lacking the *CFG1* gene, was used as a fermentation control, but because the MI2B strain and its derivatives were unable to ferment brewer must, these later fermentations were carried out with grape must (Figure 7). After 24 h of fermentation, practically no differences were observed between the MI2B-CFG1and MI2B-pYES2, as far as foam formation was concerned; however, after 48 h of fermentation, the foam level was far higher in fermentations with the MI2B-CFG1strain.

This suggests a role for the Cfg1p protein both in foam production and in foam stability.

DISCUSSION

Although there have been many publications concerning barley proteins involvement in beer foam production,[1,2] reports on yeast genes involved in beer foam generation and maintenance have been scarce. Indeed, no foaming gene from brewing yeast has yet been identified.[8,19−21] Here, we describe the cloning and characterization of gene *CFG1* as well as its identification as an essential foaming gene responsible for the stabilization of foam in the beer brewed with *S. pastorianus* wild-type strain Weihenstephan 34/70.

DNA sequencing showed that the *CFG1* gene encodes a 111 kDa (Cfg1p) protein highly homologous to previously described yeast cell wall mannoproteins. The novel Cfg1p has a fibrillar structure and is oriented, through a GPI motif at the end of its C-terminal region, toward the cell wall β-glucans. This GPI also serves as an anchorage point of the glycoprotein to the cell wall, with the hydrophobic N-terminal region of the polypeptide exposed to the outside medium. The Cfg1p central region is rich in serine and threonine and displays multiple putative O-glycosylation sites with abundant lateral oligosaccharide chains.[42] The protein also contains several putative N-glycosylation sites. This is in agreement with what will be expected of such a protein, because heavy glycosylation of Cfg1p will confer limited porosity to the yeast cell wall, hence preventing the entry of lytic enzymes that could degrade the cell wall. In addition, this high glycosylation degree of protein Cfg1p would positively protect it from ethanol denaturation and increase its affinity toward the CO_2 bubbles generated during fermentation, thus favoring its role in foam stabilization.[6,12,13,17]

The novel *S. pastorianus* gene *CFG1* displays high homology to other *Saccharomyces* genes encoding yeast cell wall mannoproteins, in particular *AWA1*, a foam-producing gene from *S. cerevisiae* sake strain K7, and *FPG1,* a gene from *S. cerevisiae* wine strain 145A211, and this high homology also extends to the proteins encoded by these genes. Hence, Cfg1p shares a high similarity with Awa1p, a hydrophobic mannoprotein involved in foam formation during sake fermentation. In addition, Cfg1p also displays a high homology to Hpf1p, a protein involved in white wine clarification. Cfg1p is also homologous, but to a lesser extent, to Fpg1p, a mannoprotein involved in wine foam formation and stabilization.[30−32] The results concerning homology indicated the presence of highly conserved regions, such as the amino-terminal and threonine-rich domains. There was, however, an area in Cfg1p (spanning amino acids 822 to 932) that shared only homology with the foamy protein Awa1p.

The chromosomal location of the *CFG1* gene could not be directly established by Southern blotting because of the similarity of *CFG1* to other genes, probably also related to cell wall mannoproteins, present in other chromosomes. In addition, the hybrid nature of the *S. pastorianus* strain means that the *CFG1* probe we used for Western blotting could, in principle, hybridize with any gene coding for a cell wall mannoprotein in any of the three chromosome types (Sc, Lg, or Sc/Lg) this strain possesses.[25,26] Mitotic chromosome mapping allowed us to locate *CFG1* on chromosome XV, a location shared by its homologous gene (*AWA1*) from *S. cerevisiae* sake strain K7. In addition, our segregation analyses allowed us to characterize chromosome XV as a Sc-type chromosome, since the disruption cassette was modeled on *FPG1*, and *FPG1* is a typical *S. cerevisiae* gene.

Our studies on the phenotypic effect of Cfg1p on the *S. pastorianus* strain demonstrated that Cfg1p was not essential for the yeast, since its absence did not alter the essential characteristics of this strain. Similarly to the wild-type strain, SPDHA1b, the strain lacking Cfg1p, was able to grow at the tested temperatures (ranging from 15 to 37 °C) and in the presence of 15% ethanol, but the lack of this protein resulted in an increased sensitivity of the SPDHA1b strain to SDS. SPDHA1b was also more lyticase-resistant during the yeast exponential growth phase than was the wild-type strain, suggesting that the Cfg1p protein must play a protective role in the cell wall. Thickening of the cell wall during the stationary phase of growth explains the increase in lyticase resistance that both strains experience during this phase, but despite the changes, the difference between both strains does not ameliorate, but becomes more evident. As a general rule, an alteration in the cell wall such as that caused by the elimination of

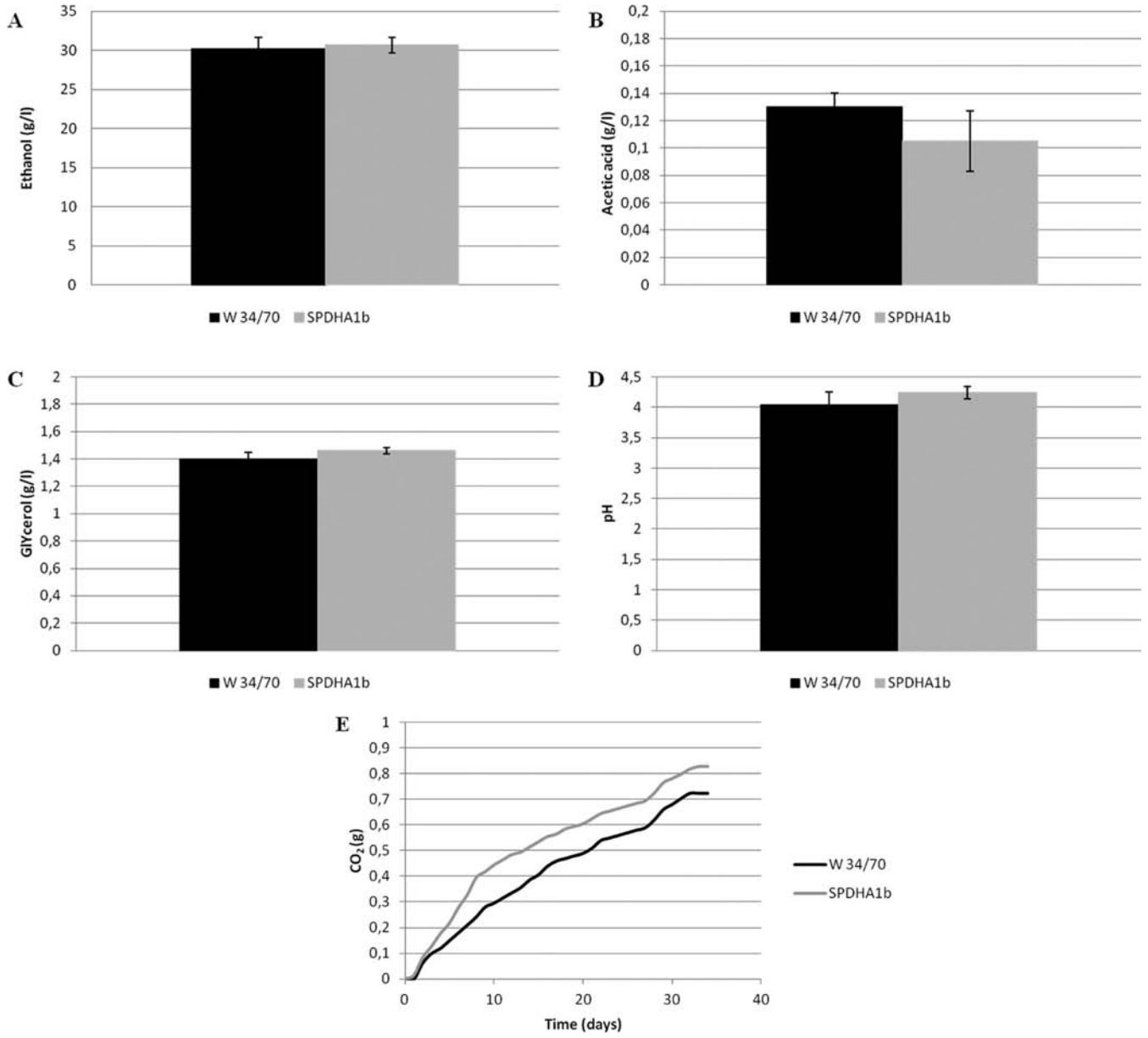

Figure 5. Organoleptic properties of wines obtained in fermentations with strains Weihenstephan 34/70 (W 34/70) or SPDHA1b: (A) ethanol concentration (g/L), (B) acetic acid concentration (g/L), (C) glycerol concentration (g/L), (D) pH, and (E) grams of CO_2 generated by the Weihenstephan 34/70 and SPDHA1b strains during the course of fermentations.

a protein should result in an increase in the wall's sensitivity to glucanases, but the absence of Cfg1p, as happens with proteins such as Fpg1p,Gas1p, Tos1p, or Scw4p, results in a decrease in sensitivity to glucanases.[14,43] This may probably be due to the synthesis of new cell wall components instead of the deleted protein. The results obtained from the Calcofluor White assays suggest that SPDHA1b did not compensate for the lack of Cfg1p by increasing the amount of chitin in its cell wall but that other cell wall glycoproteins had occupied the space previously filled by Cfg1p, as previously suggested by Yin et al.[14] for a *S. cerevisiae* strain protein Tos1p.

Hydrophobicity studies revealed that Cfg1p is a cell wall mannoprotein, as is the case for its homologous protein Awa1p, a foamy polypeptide from *S. cerevisiae*.[30]

The beer fermented with the SPDHA1b *cfg1*Δ strain was similar to that produced with the wild-type strain Weihenstephan 34/70, indicating that Cfg1p does not have a metabolic role in fermentation. There were small differences in the pH of the beers produced with the two strains, with the wild-type strain generating a slightly more acid beer than the SPDHA1b strain.[44] Values obtained for ethanol, glycerol, acetic acid, and pH were within the recommended range for beer.[41]

The protein Cfg1p is a cell wall GPI-anchored mannoprotein, with high similarity to Awa1p and Fpg1p,[30,31] that could be extracted only by the postalkali treatment, thus confirming that the protein is bound to cell wall glucans and, therefore, involved in either the production or the stabilization of foam.[9,14] Due to its hydrophobicity, Cfg1p binds to the CO_2 bubbles produced during the fermentation process and orients its hydrophobic moiety toward the gas and aqueous layer; thus slowing the liquid drainage and stabilizing the gas bubbles.

Our results show that Cfg1p is involved in the production and stabilization of foam (Figure 6, Figure 7). This was further confirmed by expressing the *CFG1* gene in MI2B, a foamless

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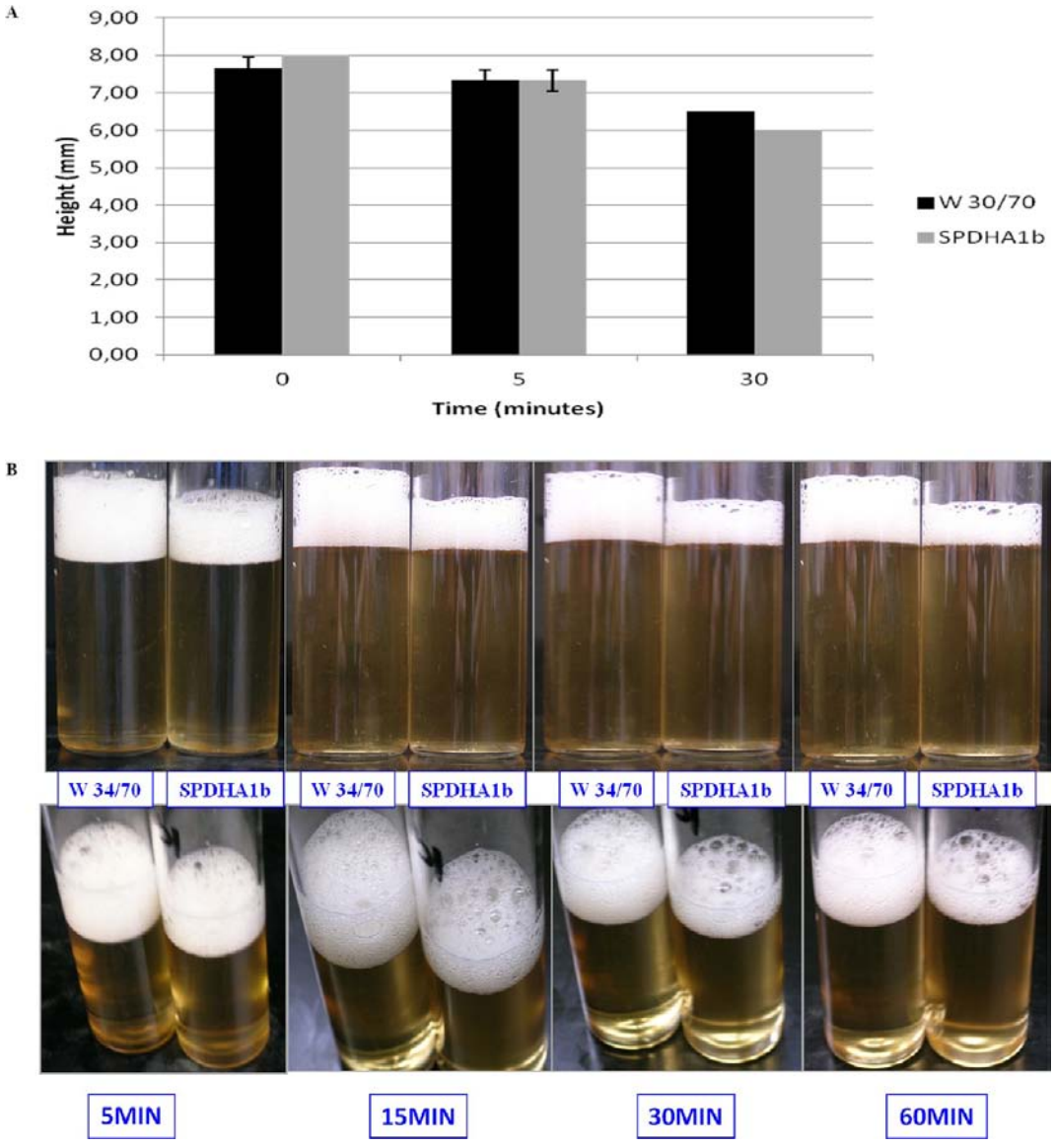

Figure 6. Foam generation and stability in beer fermented with either the Weihenstephan 34/70 (W 34/70) or the SPDHA1b strain by the shaking method. (A) Height of the foam produced by the two strains and foam stability after 5, 15, and 30 min. (B) Foam stability, as defined by the amount of foam present in the beer samples at 5, 15, 30, or 60 min after shaking (see Materials and Methods). The upper row shows a frontal view (allowing appreciation of the height of the foam layer) of the foam generated by the two yeast strains, whereas the lower row shows a top view of foam, making it possible to appreciate the size of the bubbles.

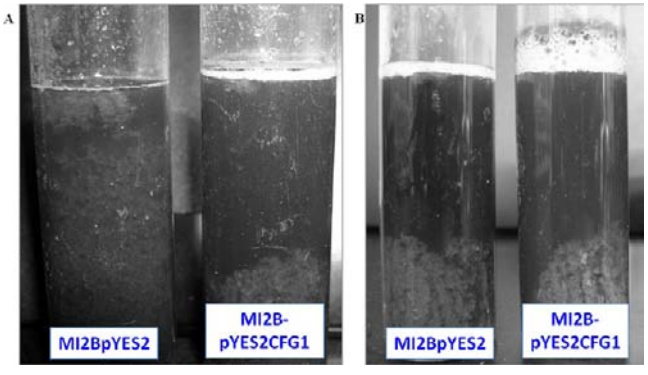

Figure 7. Fermentation vats inoculated with either the MIB-pYES2 (containing a copy of the expression plasmid, but lacking the *CFG1* gene) or the MI2B-pYES-CFG1 (containing the *CFG1* gene) yeast strains. The fermentations were carried out for either 24 (A) or 48 h (B).

S. cerevisiae strain, and showing that this resulted in the formerly foamless strain acquiring the foamy phenotype. Finally, the fermentations we carried out on brewer musts with either SPDHA1b (the strain we constructed, lacking Cfg1p) or the wild-type strain allowed us to conclude that the Cfg1p protein is directly involved in foam stabilization because the beer obtained with the Cfg1p-deficient strain produced a less stable foam of lower quality that rapidly collapsed in the beverages. In beer, unlike in wine, no differences were observed in the foam level during fermentation, thus suggesting that the foam production is determined by the gene copy number, because in wine fermentation, the gene was cloned in pYES2 and in beer fermentation, the gene was an indigenous one of the *S. pastorianus* strain Weihenstephan 34/70. In addition, the differences in the must composition do affect to the foam formation and stabilization.[1−5]

Taken together, all the results shown in the present paper show the *CFG1* gene to be a good candidate to improve foam character in the brewing industry.

AUTHOR INFORMATION

Notes

The authors declare no competing financial interest.

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