Characterization of Ccw7p cell wall proteins and the encoding genes of Saccharomyces cerevisiae wine yeast strains: relevance for flor formation

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Abstract
The specific flavour of Sherry-type wines requires aromatic compounds produced as by-products of the oxidative metabolism of yeasts that are able to form a biofilm (flor) at the wine surface. A similar yeast pellicle develops on the surface of ‘Tokaji Szamorodni’, one of the traditional Hungarian botrytized wines, during maturation. In this work, patterns of biotinylated cell wall proteins extracted from film-forming and nonfilm-forming Saccharomyces cerevisiae strains were compared. It was found that all the tested 23 film-forming ‘Szamorodni’ yeast strains had a decreased size of the Ccw7/Hsp150 protein, one of the members of the Pir-protein family. Sequencing of the encoding genes revealed that the strains were lacking three out of the 11 repeating sequences characteristic to this protein family. One of the film-forming strains contained CCW7 alleles of different length, which was generated by intragenic tandem duplication of a sequence containing two repetitive domains. Unlike the film-forming strains, 16 nonfilm-forming wine yeasts isolated from a different botrytized wine, ‘Tokaji Aszu’, showed pronounced polymorphism of the CCW7 locus. It is highly probable that the modified Ccw7 protein does not contribute to the increased hydrophobicity of film-forming strains but it may influence molecular reorganization of the cell wall during stress adaptation.

Introduction
Natural film-forming wine yeast strains are able to develop a pellicle (flor or velum) on the surface of certain high ethanol content dry wines like sherry (Martinez et al., 1997a) or vin Jaune (Pham et al., 1995). A similar yeast pellicle develops on the surface of ‘Tokaji Szamorodni’, one of the traditional Hungarian botrytized wines, during maturation. This wine speciality is produced from high sugar content must of noble-rotted grapes in a process wherein the main spontaneous fermentation is followed by a long maturation period. Depending on the original sugar content of the must dry or sweet Szamorodni, wine is produced. After the main fermentation is finished, a yeast pellicle develops on the surface of the dry and less frequently on the sweet Szamorodni wines.

Sherry flor yeasts were classified as four races of Saccharomyces cerevisiae (Martinez et al., 1995, 1997b). Esteve-Zarzoso et al. (2004), however, did not find any physiological and molecular differences among the flor strains studied; therefore, they suggested classifying them as a single race or variety inside the S. cerevisiae taxon.

Morphological changes in the shape, size and hydrophobicity of yeast cells were observed during velum formation of sherry yeasts (Alexandre et al., 1998), which is considered as an adaptive mechanism that allows yeast cells to utilize ethanol under aerobic conditions. The mechanism of the elevation of yeast cells to the wine surface, a process that is accompanied by cell–cell attachment, is not well understood – particularly at the molecular level. It has been proved in several cases that hydrophobicity of cells is a prerequisite for film formation (Iimura et al., 1980; Martinez et al., 1997a; Fidalgo et al., 2006; Ishigami et al., 2006). Martinez et al. (1997a) supposed that hydrophobicity causes the cells to aggregate and these aggregates retain gas bubbles originating from the respiration process. As a consequence, the cells will compose a multilayered floating biofilm. Based on this assumption, Zara et al. (2005) developed a FLO11-governed model for the air liquid interfacial biofilm formation.
The yeast cell wall is a surprisingly complex, layered structure (reviewed in Fleet, 1991; Klis et al., 2002, 2006). One of the most intriguing specificities of yeast cell walls is a variety of different wall proteins, many of which are highly mannosylated.

Yeasts have evolved at least three different ways of attaching proteins to the polysaccharide moiety. One group of proteins are bound to the β-1,3-glucan noncovalently (Valentin et al., 1984; Cappellaro et al., 1998) and can be released from the wall using hot sodium dodecyl sulphate (SDS). A second group contains proteins attached to the wall covalently through glycosylphosphatidyl inositol (GPI) anchoring to β-1,6-glucan, which is further linked to the β-1,3-glucan network (Klis, 1994; Montijn et al., 1994; De Groot et al., 2005; Klis et al., 2006). These proteins can only be released from the walls by either β-1,3- or β-1,6-glucanases. Finally, the third group, comprised of members of the Pir (proteins with internal repeats) protein family, is also attached covalently but directly to the β-1,3-glucan by an alkali labile ester linkage, formed between particular glutamine residues of the characteristic repetitive sequences and glucose (Ecker et al., 2006). Pir-proteins can be extracted from the cell wall either by mild alkali treatment or by β-1,3-glucanases (Mrša et al., 1997; Mrša & Tanner, 1999).

Special cell surface molecules called adhesins are the main components responsible for cell–cell or cell–surface interactions. The adhesion genes of yeasts were found to be activated by diverse environmental adverse effects like carbon and/or nitrogen starvation. The switch of yeast cells from nonadherence to adherence probably allows adaptation to stress (Kapteyn et al., 1999, 2001; De Groot et al., 2005; Verstrepen & Klis, 2006).

Flo11p represents a special type of adhesins playing an important role in the determination of both cell surface hydrophobicity and the ability of cells to form biofilms (Ishigami et al., 2006). Moreover, Flo11p was also found to play a crucial role in pseudohyphal development, invasive growth and flocculation. The encoding gene FLO11 was demonstrated to be responsible for these diverse phenotypes in a strain-dependent manner. Flo11p/Muc1p in diastatic S. cerevisiae strains induced pseudohyphal differentiation and invasive growth (Lambrechts et al., 1996). Later on, it was proved that Flo11p induces a Ca\(^{2+}\)-dependent flocculation in such type of strains (Lo & Dranginis, 1996, 1998) and it belongs to the Flo1-type class flocculins (Bayly et al., 2005). In strains of \(\sum 1278b\) genetic background, Guo et al. (2000) found that the Flo11p-governed haploid invasion and diploid filamentation are insensitive to Ca\(^{2+}\) depletion and mannose inhibition, but instead Flo11p increases cell surface hydrophobicity, thereby promoting hydrophobic interactions among the cells or between the cells and certain abiotic surfaces. They also demonstrated that Fig2p can substitute Flo11p if overexpressed and vice versa. Recently, Douglas et al. (2007) showed that Flo11p molecules endowed with different phenotypic effects are completely identical and all of them were able to flocculate only the diastatic and not the \(\sum 1278b\) S. cerevisiae strains. It was interesting that binding of Flo11p molecules to Δflo1 mutants failed, thus demonstrating a homotypic adhesive mechanism.

Fidalgo et al. (2006) and Ishigami et al. (2006) proved independently that production of Flo1p was essential for flor formation. However, even the Δflo1/Δflo1 diploid cells retained the film-forming ability (Ishigami et al., 2006). Thus, this protein may be one but not the only prerequisite for flor formation. Moreover, Flo11p is a ubiquitous protein present in many S. cerevisiae strains, most of which do not form biofilms.

Low pH, high ethanol and acetaldehyde concentrations and oxidative stress during flor formation activate numerous heat shock genes. Gene expression analysis revealed the induction of HSP12, HSP82, HSP26 and HSP104 genes under acetaldehyde stress and it correlated well with the resistance to ethanol stress (Aranda et al., 2002). Expression of the aldehyde dehydrogenase-coding (ALD) genes was also found to be increased by the addition of acetaldehyde, especially in the case of a flor yeast strain (Aranda & del Olmo, 2003). Point mutations or complete deletion of a plasma membrane protein-coding gene HSP12 resulted in inability of Sardinian sherry strains to form flor (Zara et al., 2002). Expression of these genes could be a prerequisite for flor formation in wine although the gene products are not directly involved in the process itself.

The above-mentioned results indicate that even in the case of genetically well-characterized film-forming strains and mutants, several nonspecified genes and gene products are playing a role in flor formation and the activity and regulation of certain genes can increase or abolish flor formation.

These background results motivated us to characterize film formation of the ‘Szamorodni’ flor strains and screen for differences between the wall proteins of wine yeast strains, that were able to form flor and those that were unable to do so. The difference was expected to reveal potential cell wall proteins other than Flo11p having a role in cell–cell interactions during flor formation or being responsible for the reorganization of cell wall architecture under stressful conditions of wine fermentation and maturation. As we found a marked difference in the Ccw7/Hsp150/Pir2 proteins and the encoding genes of planktonic and film-forming wine yeast strains, we performed a structural analysis of these genes. We found that all the examined ‘Szamorodni’ and sherry film-forming strains harboured CCW7 alleles of identical nucleotide sequences and we concluded that a longer allele in a heterozygotic diploid strain was the consequence of an intragenic duplication. We may expect that a better understanding of the flor formation mechanism in a botrytized wine could facilitate the control
of the maturation process and creation of new yeast strains with the desired properties.

Materials and methods

Strains and culture conditions

Saccharomyces cerevisiae laboratory, film-forming and other wine yeast strains used in this study are listed in Table 1.

For the isolation of cell surface proteins, yeast strains were cultured in liquid YPD1 (1% yeast extract, 2% peptone and 2% glucose) medium at 30 °C on a rotary shaker to obtain cultures of OD_{600 nm} = 4.

For DNA isolation, yeast strains were cultured on YPD2 (0.5% yeast extract, 0.5% peptone, 1% glucose) agar plates at 30 °C for 24 h.

Sporulation was induced on acetate medium (0.25% peptone, 0.062% glucose, 0.062% NaCl, 0.5% sodium acetate and 2% agar) for 3 days at 30 °C.

Film formation assay

Film formation assay was performed in YPDE (0.5% yeast extract, 0.5% peptone, 0.5% glucose, 6% ethanol) medium (pH 3.3). Five milliliters of liquid YPDE medium in a test tube was inoculated with stationary-phase (24 h old) cells at a concentration of 10^7 cells mL^-1. Static cultures were incubated at 20 °C and development of the film was controlled visually every 24 h. Different degrees of film formation were evaluated by estimating the ratio of film-covered and free surfaces of the medium as indicated in Table 2. These cultures were also used for the isolation of wall proteins from the film constituting yeast cells.

Cultivation of cells under nonfilm-forming (planktonic) conditions was performed by shaking the cultures at 180 r.p.m. at 30 °C in YPD2 medium.

Treatment of yeast films

Treatment of the isolated yeast films with EDTA-Na_2 (100 mmol) and different enzymes (cell wall lytic enzymes and proteases) was performed at 30 and 37 °C, respectively. The following enzymes were used: Trichoderma-lysing enzyme 200 μg mL^-1, Arthrobacter lyticase 10 μg mL^-1, proteinase K 200 μg mL^-1, pronase E 100 μg mL^-1 and α-chymotrypsin 100 μg mL^-1. All enzymes were purchased from Sigma. SDS and urea treatments were performed with 1% solutions.

Disorganization of the yeast films was observed under a microscope.

Cell hydrophobicity assay

Determination of cell surface hydrophobicity using the MATH test was performed as described by van der Mei et al. (1993), with slight modifications. This test is based on the measurement of the distribution of cells in a two-phase (aqueous and organic) solvent system. Cultures were prepared under film-forming and nonfilm-forming conditions as described previously. Twenty-four-to thirty-hour-old cultures were used for the hydrophobicity assay. Cells were washed in distilled water and the basic (A_0) suspensions

Table 1. Description of Saccharomyces cerevisiae laboratory, film-forming and other wine yeast strains used in the experimental work

<table>
<thead>
<tr>
<th>Strain</th>
<th>Type</th>
<th>Geographical origin</th>
<th>Sources</th>
</tr>
</thead>
<tbody>
<tr>
<td>S288c</td>
<td>Laboratory strain (nonfilm-forming)</td>
<td>Rotting fig</td>
<td>Culture Collection Szeged University, Hungary</td>
</tr>
<tr>
<td>SEY6210</td>
<td>Laboratory strain (nonfilm-forming)</td>
<td>Not known</td>
<td>Department of Biochemistry, University of Zagreb, Croatia</td>
</tr>
<tr>
<td>B1</td>
<td>Sherry flor strain</td>
<td>Spain</td>
<td>Department of Enology, CUB, Moldavia</td>
</tr>
<tr>
<td>B3</td>
<td>Sherry flor strain</td>
<td>Moldavia</td>
<td>Culture Collection, Kishinev, Moldavia</td>
</tr>
<tr>
<td>B8</td>
<td>Sherry flor strain</td>
<td>Commercial, CA, USA</td>
<td>Department of Enology, CUB</td>
</tr>
<tr>
<td>B4, B5</td>
<td>Szamorodni film-forming strains</td>
<td>Tokaj, Hungary</td>
<td>Culture Collection, Kecskemet, Hungary</td>
</tr>
<tr>
<td>B16, B22, TD01.1, TD01.2, TD03, TD04, TD05</td>
<td>Szamorodni film-forming strains</td>
<td>Tokaj, Hungary</td>
<td>Department of Enology, CUB</td>
</tr>
<tr>
<td>M2.1, M2.2, M4.1, M4.2, M7.1, M7.2, M10, M21, M17.1, M17.2, M12.1, M12.2, 02/62.1, 02/62.2</td>
<td>Szamorodni film-forming strains</td>
<td>Tokaj, Hungary</td>
<td>Department of Microbiology and Biotechnology, CUB</td>
</tr>
<tr>
<td>BAD1</td>
<td>Wine strain (nonfilm-forming)</td>
<td>Badacsony, Hungary</td>
<td>Culture Collection, Kecskemet, Hungary</td>
</tr>
<tr>
<td>PM322, V0/A, V0/B, V11/A, II/0, V3/10, V10/3, IV/8/z2, 34/A.03, IV/3, IV/12, V5/A, V8/D, II/0/D, II/4/6, II/5/1</td>
<td>Tokaj, Hungary</td>
<td>Department of Microbiology and Biotechnology, CUB</td>
<td></td>
</tr>
</tbody>
</table>

CUB, Corvinus University of Budapest, Budapest, Hungary.
Glucanase-extracted proteins were separated from the cell wall extract using centrifugation (3 min, 12 500 g). For the isolation of Pir-proteins, the SDS-treated cell walls were washed four times in 50-mM potassium phosphate buffer, pH 8.0, twice in distilled water, and incubated overnight in 30 mM NaOH at 4°C. NaOH-extracted proteins were separated from cell wall residues by centrifugation (3 min, 10 000 r.p.m.). All the protein samples were subjected to polyacrylamide gel electrophoresis (PAGE) and the cell wall proteins were detected by Western blot using a streptavidin–horseradish peroxidase (HRP) conjugate.

### SDS-PAGE, blotting and staining procedures

Electrophoresis was performed by the method of Laemmli (1970). In order to make biotin-labelled proteins visible, they were blotted onto nitrocellulose membranes, which were then incubated for 1 h in 10 mL of blocking buffer (50 mM Tris–HCl pH 7.5, 0.15 M NaCl, 0.1% Triton X-100) with 2% bovine serum albumin (BSA). After this step, membranes were incubated for 1.5 h in the same buffer with 1% BSA and a streptavidin–HRP conjugate (dilution 1:5000, Pierce Biotechnology), washed three times with the same buffer and developed using the ECL kit (Perkin-Elmer). To estimate the molecular mass, the following standard proteins were used: phosphorylase b (94 kDa), albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa). For protein sequence analysis, the extracted 87-kDa protein was separated using SDS-PAGE and blotted onto an Immobilon membrane (Millipore). Staining of proteins for carbohydrates in the gel was performed by the method of Zacharius et al. (1969).

### PCR amplification and restriction analysis

Total genomic DNA isolation was performed as described by Hoffman & Winston (1987), with slight modifications. The sequence of the S. cerevisiae gene encoding the Ccw7p cell wall protein was obtained from the http://www.ncbi.nlm.nih.gov site. Primers for the amplification of the CCW7 (HSP 150) gene were designed as described in the http://seq.yeastgenome.org/cgi-bin/web-primer site. The following are the sequences of those primers: the forward primer (C7-f) CTACTACATTGGCCGCCTATGCTC and the reverse primer (C7-r) CGATAGCTTCCAAGTGGACTGGAG.

The PCR reactions were performed in 30-μL reaction volumes using the PCR Sprint (Hybaid) apparatus. The reaction mixture contained 2.5-μL template DNA, 15 pmol of each of the primers (C7-f and C7-r), 0.16 mmol of each of the dNTPs, 1.6 U DNA polymerase (DyNAzyme, Finnzymes), 1× DNA polymerase buffer and 2.5 mmol of MgCl₂. PCR reactions were run as follows: DNA predenaturation for 5 min at 95°C; amplification (35 cycles): 95°C
for 30 s, 56 °C for 30 s and 72 °C for 1 min; and final extension: 72 °C for 7 min.

The amplification products were digested with the restriction enzymes HaellIII, RsaI and KpnI at 37 °C for 4 h.

The PCR products and the digested amplicons were separated by electrophoresis in 1.5% agarose gels in 0.5 × TBE buffer at 120 V for 2 h. The bands were visualized using ethidium bromide staining and UV transillumination.

For nucleotide sequence determination, the amplified DNA was purified using the PCR-M/header Clean Up System (Viogene). Sequencing was performed by the ABI 3100 sequencer using PCR primers.

**Ascus dissection and characterization of the spores**

Diploid strains were streaked on sporulation medium. Four-spored asci were dissected using a micromanipulator (Carls Zeiss Jena) and the spores were allowed to germinate on YPD2 plates. The colonies produced were isolated and the presence of the HSP150 gene was determined by PCR amplification using the C7-f and C7-r primer pair.

**Results**

**Degree of film formation of sherry and Szamorodni strains**

Film formation of selected sherry and Szamorodni strains was followed for 7 days of incubation at 20 °C in YPDE medium. The results are shown in Table 2. The strains differed in the rapidity and intensity of film formation. Strains TD04 and TD01.2 were the best; by the 2nd day, they already established stable and continuous films. Film formation of the strain B1 was slightly delayed, while M4.1 was not even able to produce a continuous film by the 7th day. Strain 02/62.1 was the weakest; only small islands of aggregated cells were produced after 7 days of incubation.

**Cell surface hydrophobicity analysis**

Hydrophobic or hydrophilic characterization of four sedimenting and six film-forming yeast strains was performed by the application of the MATH test (van der Mei et al., 1993). The results shown in Fig. 1 revealed that all planktonic wine yeast strains had hydrophilic cell surfaces, while the film-forming yeasts proved to be highly hydrophobic. Hydrophobicity of film-forming strains did not depend on the growth phase, and there was no difference in the surface hydrophobicity when the strains were cultivated under planktonic or film-forming conditions. EDTA- Na2 did not affect the integrity of yeast films, which indicated that this type of aggregation did not belong to the Ca2+-dependent, lectin-mediated cell attachments. The integrity of the film, however, was ceased by the addition of proteases like proteinase K, pronase E, trypsin or chymotrypsin, which indicated the active participation of surface proteins in the film formation. SDS and urea treatments were ineffective in disorganization of the film.

**Comparison of cell wall proteins**

In order to reveal potential differences in the cell wall protein profiles of film-forming and nonfilm-forming strains, as well as between cells grown in planktonic state or under film-inducing conditions, the cell wall proteins were labelled by biotinylation and then extracted as described in Materials and methods. Noncovalently linked proteins were extracted using hot SDS, while covalently bound GPI-anchored and Pir proteins were released by β-1,3-glucanase or 30-mM NaOH treatments, respectively. Protein patterns were compared using Western blot analysis. No differences were observed in the protein patterns of the SDS-soluble and the GPI-anchored cell wall proteins of the strains tested (data not shown). A pronounced difference was found, however, in the NaOH-extracted Pir proteins. As can be seen in Fig. 2, the 117-kDa band corresponding to the Ccw7p/Pir2p/Hsp150p was missing in all the film-forming strains, but they all contained an additional 87-kDa protein band and, moreover, the TD04 strain possessed a 115-kDa band. This difference was, however, independent of whether the proteins were extracted from the film constituting or the sedimented cells of the film strains. To determine whether the 87-kDa band was a newly synthesized protein or whether it was a modified Ccw7p, the N-terminus of the isolated 87-kDa protein was sequenced. The AAXQIGDXEYQA amino acid sequence was obtained, which was identical to the N-terminus of the Ccw7p. Therefore, we supposed that the 87-kDa protein was a smaller variety of the Ccw7p, but
the decrease in the molecular mass could be the consequence of the lower level of glycosylation. Staining the separated proteins for carbohydrates, however, indicated that the smaller size of the protein was not due to significantly impaired glycosylation, because all the bands were stained to a similar intensity (Fig. 2b).

**PCR amplification of the CCW7/PIR2/HSP150 gene**

To check whether the smaller size of the Ccw7p was actually caused by the decrease in the size of the encoding gene, CCW7 was amplified from different film-forming and nonfilm-forming strains by PCR. The C7-f- and C7-r-specific primer pair, amplifying a 1185-bp long part of the CCW7 gene published in the *S. cerevisiae* genomic databank (http://www.ncbi.nlm.nih.gov), was designed and used for PCR amplification under highly stringent annealing conditions (see Materials and methods). PCR products generated from the *S. cerevisiae* S288c and SEY6210 laboratory strains, 26 film-forming and 17 nonfilm-forming wine yeast strains, are shown in Fig. 3. It was found that the amplicons obtained from the S288c and SEY6210 strains were identical (1190 bp) and corresponded to the size of HSP150/PIR2/CCW7 in the *S. cerevisiae* genomic databank (http://www.ncbi.nlm.nih.gov). In the case of film-forming wine strains, regardless of their origin, shorter, 1015-bp amplicons were generated. Two isolates, however, resulted in additional amplicons: B8, a shorter (890 bp), and TD04, a longer (1140 bp) one. In contrast to this, the nonfilm-forming wine yeast strains showed extended length polymorphism of the amplicons ranging from 1015 to 1500 bp. Several strains proved to be heterozygotic for the gene; they contained two CCW7 alleles.

![Fig. 2.](image-url) Protein patterns of alkali-extractable cell wall proteins from different film-forming strains compared with the standard laboratory (nonfilm-forming) strain SEY6210. (a) Western blot of biotinylated cell wall proteins, (b) PAGE patterns of NaOH extracts stained for carbohydrates. The position of the molecular size marker proteins is indicated by arrows. Film-forming strains are marked with *.

![Fig. 3.](image-url) Amplicons of different laboratory and wine yeast strains obtained using PCR amplification of the CCW7 gene under the conditions described in Materials and methods. Film-forming strains are marked with *.
of different length. Interestingly, two nonfilm-forming 'Aszu' wine yeast strains (PM322 and 34/1.03) had 1015-bp amplicons that are characteristic of all film-forming strains.

**Restriction fragment length polymorphism (RFLP) analysis of CCW7 amplicons**

Partial sequence polymorphism of the CCW7 gene was determined by RFLP analysis of the CCW7 amplicons using the RsaI, HaeIII and KpnI restriction enzymes. A dendrogram was created from the RFLP patterns (Fig. 4) using the Molecular Analyst software (BioRad). RFLP analysis resulted in two main clusters around the 40% similarity level. All the film-forming strains belonged to cluster no. 1; further, they segregated into three different clusters at a similarity level of 68%. Except for the two film-forming strains that had two CCW7 amplicons (TD04 and B8), all strains had 100% identical patterns. All but two of the nonfilm-forming wine yeasts (PM322 and 34/1.03) belonged to cluster no. 2, while the 1/3/10 strain had a unique RFLP pattern. It is worth noting that the two wine yeast strains, which could not be separated from the film-forming ones by RFLP analysis, originate from the Tokaj Wine District.

**Segregation of CCW7 alleles of the TD04 strain**

All the 23 'Szamorodni' film strains were sporulating; the majority of them, however, produced irregular tetrads. The viability of the spores was extremely low; even from the regular tetrad-forming strains, we could hardly induce growth of segregants. Fortunately, the TD04 strain was the best in this respect; it contained two Ccw7 proteins of different sizes (see Fig. 2) and these proteins were shown to be encoded by two different CCW7 alleles (Fig. 3). One of the proteins was identical in size to that found in all film-forming strains, while the other was slightly smaller than the CCW7p of nonfilm-forming strains. Meiotic segregation of the CCW7 alleles was induced by sporulation and 21 asci, all containing four spores, were dissected. Even in this strain the viability of spores was very weak; it was not possible to grow even one complete tetrad, and only a maximum of three spores within one tetrad formed colonies. PCR amplification of the CCW7 gene from the segregants revealed that the CCW7 alleles segregated during meiosis, i.e. the spores contained either the longer (1140 bp) or the shorter (1015 bp) amplicons. Segregation patterns of the two alleles indicated that loss of spore viability within the tetrads was random in relation to the CCW7 alleles. We determined the film-forming ability and hydrophobic characteristic of 23 meiotic segregants and showed that independently of the alleles, all maintained both film-forming ability and hydrophobic cell surface character.

**Determination of polymorphism of CCW7 alleles by direct sequencing**

For determination of the sequence polymorphism of CCW7 alleles and the deduced proteins of film-forming and non-film-forming strains, we sequenced the CCW7 amplicons from the S288c, PM322, 34/1.03, B1, TD01.2 and M4.1 strains and the TD 04/1.a and TD 04/1.b meiotic segregants. CCW7 sequences of the seven wine yeast strains belonged to three different groups, that contained completely identical sequences within one group. The groups of strains are: (1) B1, PM 322, TD01.2; (2) 34/1.03, M4.1, TD04/1.b; and (3) TD04/1.a. Sequences of one representative strain in each group have been submitted to GenBank under the accession
numbers EU220722 (strain B1), EU220723 (strain TD04/1.b) and EU220724 (strain TD04/1.a).

All the examined strains showed the characteristic common feature of the Pir genes: they contained several tandemly arranged repetitive sequences comprising a long repetitive region within the CCW7 gene. Patterns of the repetitive sequences, however, varied among the strains, thus indicating the already published polymorphism of the CCW7 gene (Marinangeli et al., 2004). Figure 5 shows alignment of the deduced amino acid sequences of the CCW7 alleles from the examined strains, which illustrates the positions of deletions and insertions within the

![Fig. 5. Alignment of Ccw7p sequences from different film-forming and nonfilm-forming strains. Film-forming strains are marked with *.

The Kex2-processing site is marked with a grey box and the single conserved amino acid substitutions are presented in white boxes. Repetitive sequences characteristic of the Pir-protein family are written in bold. The duplicated part of the protein from TD04/1a is underlined.](https://academic.oup.com/femsyr/article-abstract/8/7/1115/493238)
replicative regions. As expected, all the proteins had the Kex2 processing site. The amino acid sequences of all the wine yeast strains (i.e. film-forming and nonfilm-forming) showed alteration from the S288c strain in the following positions: serine instead of threonine in the first and 11th repetition and isoleucine instead of valine in the sixth repetition. Moreover, variation of valine or isoleucine in the fifth repetitive sequence was observed.

Systemic examination of the amplified CCW7 genes and the deduced amino acid sequences revealed that all the wine yeast strains studied or their meiotic segregants (irrespective of their film-forming ability) lacked two segments within the repetitive region, which corresponded to the third plus fourth and the eighth repetitive domains of the S288c strain. It is interesting that the meiotic segregant of the film-forming TD04 heterozygotic strain (TD04/1), possessing a longer amplicon, contained 10 complete repetitive domains but the extra domains were in a different position from that in the S288c strain. The region of these extra domains was the continuous duplicate of a 135-bp sequence – extending from 250 to 385 nt. The nucleotide sequences of the duplicated regions were completely identical to each other and also to the same region of the homologous chromosome represented by the shorter amplicon of the TD04/1b segregant.

**Discussion**

'Tokaji Szamorodni' is a botrytized Hungarian wine speciality; its maturation is accompanied by the development of a yeast biofilm on the surface. In contrast to the sherry flor strains, some of the isolates formed a film not only on dry (below 10 g L⁻¹ sugar) but also on sweet (around 40 g L⁻¹ sugar) wine. It has been supposed that utilization of nonfermentable carbon sources (mainly ethanol) instead of fermentable ones (e.g. glucose) is a prerequisite for flor formation (Cantarelle & Martini, 1969; limura et al., 1980); therefore, it is expected that flor formation is suspended until utilization of ethanol is liberated from catabolite repression. According to the model suggested by Verstrepen & Klis (2006) for outlining the regulation of FLO11 – the key gene for flor formation – FLO11 is also under strong glucose repression. However, Ishigami et al. (2006) found that FLO11 was highly expressed in a flor yeast independent of whether glucose or ethanol was the sole carbon source. How the ‘Szamorodni’ flor yeasts adapted to form a film on sweet wine is an interesting question and this would require further studies.

On comparing the ‘Szamorodni’ biofilm with the sherry flor, we found high similarity in terms of several other aspects. All the film-forming strains belonged to *S. cerevisiae* and showed the sugar utilization patterns characteristic of the flor yeasts (Martinez et al., 1995, 1997b). Cells of the multilayered biofilm contained intracellular lipid drops and produced a high amount of acetaldehyde from ethanol. They could also use glycerol as a nonfermentable carbon source for the formation of a floating biofilm (data not shown). ‘Szamorodni’ film strains were weakly sporulating diploids having low spore viability, similar to sherry and sherry-like flor-forming strains (Esteve-Zarzoso et al., 2004; Budroni et al., 2005). The hydrophobicity of ‘Szamorodni’ and sherry strains belonged to the same range (Fig. 1). As regards the cell–cell attachment in the film, we showed that lectin-type protein–carbohydrate interactions are not likely to govern it, because availability of calcium or other bivalent metal ions has no influence on this property. Different proteases, however, disintegrated the film, indicating direct participation of the external cell wall proteins in the cell–cell attachment.

Comparison of biotinylated cell wall proteins of a number of film-forming and nonfilm-forming yeasts revealed one difference regarding the size of Ccw7p/Pir2p/Hsp150p, a representative of the Pir-protein family. Film-forming strains contained uniform but much smaller size (87 kDa) proteins than the laboratory strains (117 kDa). Thorou...
In the case of adhesins other than Flo11p, high genetic variability is also attributed to the frequent recombination event between the intragenic repeats, thus resulting in a remarkable variation in adhesion phenotype. Creation of new adhesins and differential expression of various adhesin genes enables yeasts to quickly adapt their adhesion properties to a particular and changing environment (Verstrepen & Klis, 2006).

The extensive genetic polymorphism among the wine yeast strains (Bidenne et al., 1992; Rachidi et al., 1999) is considered as the result of the adaptive evolution governed mainly by stress adaptation (Bauer & Pretorius, 2000; Querol et al., 2003). We may expect that flor-forming strains also underwent such an adaptive evolution in different wine-producing districts (Budroni et al., 2005) but more genetic uniformity of sherry strains in Jerez (Spain) was revealed (Fernandez-Espinar et al., 2000;Esteve-Zarzoso et al, 2004). It has been reported (Fernandez-Espinar et al., 2000) and confirmed later (Esteve-Zarzoso et al., 2004) that all the tested flor yeast strains from the Jerez sherry region carried a 24-bp deletion in the ITS1 region, corroborating the genetic similarity of flor-forming yeasts. Our search for the presence of this 24 bp deletion in the ‘Szamorodni’ flor strains showed that most but not all carried this deletion (data not shown), which indicates that evolution of flor-forming strains in different wine-producing districts and/or in different types of wine might follow slightly divergent lines.

Botrytized wines are posing extremely stressful conditions for the fermenting yeasts in terms of different aspects, such as high sugar content, low pH, limitation in growth substances and the inhibitory effects of toxic compounds produced by Botrytis cinerea (Fleet et al., 1984;Doneche, 1993); therefore, self-selection of the most stress-tolerant yeasts during fermentation is expected. Covalently linked proteins of the yeast cell wall are believed to play an important role in the adaptation of cells to the changing environmental conditions (Klis et al., 2002, 2006). When the cells are exposed to stress conditions that affect the cell wall, it triggers specific transcriptional responses and will result in increased expression of putative cell wall assemblies and some potential cross-linking cell wall proteins.

Hsp150p/Ccw7p has been described as a constitutively expressed, extensively O-glycosylated protein, that is mostly secreted into the growth medium. Heat shock treatment substantially increased transcription of the encoding gene (Russo et al., 1992) but only a fraction of the secreted protein was retained in the cell wall. According to Kapteyn et al. (1999), the cell wall-retained fraction of the protein would remain directly attached to the 1,3-β-glucan. On the other hand, Moukadiri & Zueco (2001) presented evidence that a part of this Pir protein is bound to the cell wall through disulphide bridges. It has also been shown that cell wall weakening, caused by a low level of 1,6-β-glucan, induced increased incorporation of Pir2 protein into the cell wall (Kapteyn et al., 2001). Not only cell wall damage but also the decrease of pH from 5.5 to 3.5 caused most efficient retention of Pir2 protein by the cell wall. These structural changes were found to be dependent on a functional high osmolarity glycerol (HOG) pathway. These findings indicate the distinctive role of Pir2 (and probably other Pir proteins) in the molecular reorganization of the cell wall. Castillo et al. (2003) suggested that repetitive sequences of Pir proteins are mediating the linkage between 1,3-β-glucan, and Pir proteins with multiple repeats are able to interconnect two or even more 1,3-β-glucan chains, thereby strengthening the cell wall. Whether the number of repetitions within PIR2 can influence the structural reorganization and the strength of the wall architecture in the case of hyposmotic, ethanol or low pH shocks is an interesting question and natural variants in this respect may help answer it.

It is highly improbable that the modified Pir2p is directly involved in the establishment of the hydrophilic cell surface because the nonfilm-forming PM 322 and 34/1.03 strains having the same modified gene structure (Fig. 5) were found to be hydrophilic. Although evidence of the extended length polymorphism of PIR2 gene among the wine yeast strains has already been published (Marinangeli et al., 2004), this is the first time that a detailed sequence analysis of the alleles has been performed.

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References


