Cell surface structures in osmotically fragile mutants of *Saccharomyces cerevisiae*

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Mutants of *Saccharomyces cerevisiae* characterized by osmotic fragility showed a marked fibrillar structure on the inner wall surface when studied by two electron microscopic techniques, i.e. freeze-etching of whole native cells and metal shadowing of isolated cell walls. The walls of the mutant cells were more permeable to macromolecules than were those of the wild-type parental strain. The synthesis and assembly of (1→3)- β -D-glucan wall microfibrils studied in protoplasts of mutant cells were not impaired. It is suggested that the osmotic fragility of the mutant cells is related to the deficiency of the wall structure as a consequence of the *srb1* mutation affecting biogenesis of the amorphous (glucan) component.

Introduction

The cell wall of *Saccharomyces cerevisiae* consists basically of β -glucans, chitin and mannoproteins. Microfibrils of β -(1→3)-glucan make up the wall fibrillar skeleton (Kopecká *et al.*, 1974; Houwink & Kreger, 1953), which is masked with amorphous β -glucans (Kopecká *et al.*, 1974) and mannoproteins (cf. Bacon, 1981; Kopecká, 1985). Chitin is largely found in bud scars (Bacon *et al.*, 1966; Cabib *et al.*, 1974, 1982). The wall is an essential component of the yeast cell. Because of its rigidity, it determines cellular shape, enables cell division and prevents lysis of the inner (cytoplasmic) compartment in a hypotonic environment.

Certain mutants of *S. cerevisiae* (in this work referred to as osmofragile cells) are characterized by osmotic fragility, i.e. they grow only in media containing osmotic stabilizers and lyse when transferred into distilled water (Venkov *et al.*, 1974). These mutants share their common characteristic – osmotic fragility in hypotonic media – with plant protoplasts. The osmotic fragility of yeast protoplasts is generally caused by the absence of the cell wall; this also prevents protoplast division (cf. Nečas, 1971) in liquid media even if these contain an osmotic stabilizer. The osmofragile mutants, on the other hand, divide in the presence of osmotic stabilizers. In contrast to *S. cerevisiae* protoplasts, flattened protoplasts of *Schizosaccharomyces japonicus* var. *versatilis* (Gabriel,

1983, 1984), although showing osmotic fragility, can divide in media with osmotic stabilizers. Electron microscopy of the cell walls of *Sch. japonicus* digested by purified enzymes reveals β -(1→3)- and α -(1→3)-glucan fibrils incompletely masked with amorphous matrix (Gabriel & Kopecká, 1988).

In osmofragile mutants of *S. cerevisiae*, the fragility is not caused by external conditions affecting the phenotype, as is the case for *Sch. japonicus* protoplasts (Gabriel, 1984), but is due to mutations in genes whose normal functions are not known. The osmofragile mutant strain VY1160 used in this study has three recessive nuclear mutations: *srb1*, *ts1* and *ts2*. The *srb1* mutation, which determines osmotic fragility, is present in the isogenic, single-mutation strain 193*srb1*. The *ts1* mutation, which increases the tendency of *srb1* cells to lyse, is present alone in the isogenic, single-mutation strain 191*ts1*. The mutation *ts2* is not related to cell fragility but has been found to render the mutant cell sensitive to rifampicin (Venkov *et al.*, 1974, 1975; Kozhina *et al.*, 1979; Stateva & Venkov, 1981).

The reason for the osmotic fragility of the mutants has been sought in differences in the quality and quantity of wall polymers, as compared to their parental strain. In the osmofragile cells of strain VY1160, an analysis of mannoproteins showed a twofold reduction in mannan. This was due to a significant reduction in the polysaccharide part of the mannoprotein complex and considerably

shorter mannan side chains (Märkisch *et al.*, 1983; Reuter *et al.*, 1979). A structural analysis of glucan revealed markedly less alkali-soluble glucan and more insoluble glucan which had a higher number of β -(1 $\rightarrow$ 6)-linkages and a lower molecular mass than that present in the parental cells (Blagoeva & Venkov, 1990).

The objective of this study was to find out if changes in the biochemistry of wall mannan and glucans would be reflected in an aberrant wall architecture of the mutants detectable at the ultrastructural level. A previous electron microscopic study of ultrathin sections of osmofragile mutant cells suspended in water showed ruptures in the walls through which cellular components leaked out (Mateeva *et al.*, 1976), but this was the only difference from the general ultrastructure of the cell wall typical of a wild-type yeast (Matile *et al.*, 1969). In the present work two additional approaches to electron microscopy were used, namely freeze-etching for studying native walls of whole cells, and metal shadowing for visualizing isolated cell walls. The properties of the glucan fibrillar wall component were investigated in protoplasts derived from osmofragile mutant cells because the yeast protoplast is known to produce a fibrillar glucan skeleton free from amorphous matrix (Kopecká *et al.*, 1965; Kopecká & Kreger, 1986; Kreger & Kopecká, 1973, 1976a, b).

Methods

Strains and media. The *S. cerevisiae* strains used in the study are listed in Table 1. The osmofragile mutant VY1160 was derived from the wild-type strain S288C (Venkov *et al.*, 1974). A previous genetic analysis of mutant VY1160 has identified three mutations, *srb1*, *ts1* and *ts2*, responsible for its phenotype (Venkov *et al.*, 1974, 1975; Kozhina *et al.*, 1979). Three single-mutation strains, 193*srb1*, 191*ts1* and 190*ts2*, were obtained from a cross between strains VY1160 and VY8, the latter being related to the S288C parental strain. Therefore, the mutant strains were isogenic to both the VY1160 and S288C strains. The genotypes of the isogenic single-mutation strains studied were ascertained by replica plating on appropriate selective media and further confirmed by complementation analysis with tester strains.

All yeast strains were cultivated in a nutrient medium consisting of 80% (v/v) malt extract medium and 20% (v/v) N1 medium (5 g glucose, 1.25 g asparagine, 1.25 g KH_2PO_4 , 0.425 g MgSO_4 , 50 ml distilled water; Kelletti *et al.*, 1954), osmotically stabilized with 10% (w/v) mannitol (pH 5.4). Growth was followed by measuring OD_{530} in a spectrophotometer at 29–30 °C. Cells growing exponentially were checked by phase-contrast microscopy.

Determination of osmotic lysis of mutants. Osmotic fragility was tested in the exponential phase of growth. Aliquots (1 ml) were centrifuged at 670 g for 5 min, the sediment was washed twice with 5 ml distilled water and, after shaking thoroughly for 1 min in a microshaker, centrifuged again at 670 g for 5 min. Methylene blue (5 ml, 0.01%) was added, the culture was stirred and the numbers of lysed (blue-coloured) and living (unstained) cells were counted under the microscope.

Cell wall isolation. For electron microscopic observation of the walls, lysed cells were washed three times with distilled water and repeatedly centrifuged at 670 g for 5 min. The cytoplasm was removed by a sodium

Table 1. *Saccharomyces cerevisiae* strains used

Strain	Genotype
S288C*	<i>MATα gal2 mal</i>
VY1160	<i>MATα gal2 mal srb1 ts1 ts2 leu2 tyr thr ade ura</i>
VY8	<i>MATα gal2 mal lys</i>
193 <i>srb1</i>	<i>MATα gal2 mal srb1 leu2</i>
190 <i>ts2</i>	<i>MATα gal2 mal ts2 leu2 ade</i>
191 <i>ts1</i>	<i>MATα gal2 mal ts1 leu2 lys</i>

* Strain S288C was originally obtained from Dr G. Fink, Cornell University, USA.

dodecyl sulphate (SDS) procedure developed by Kopecká (1976) and Kopecká & Kreger (1986).

Ultrasonic disintegration of cell walls. Treatment for 5 min at 22 kHz was used to fragment isolated cell walls of both the parental and mutant cells. Wall fragments were washed with distilled water and centrifuged at 670 g for 15 min.

Protoplast preparation. Exponential-phase cells were washed twice with citrate/phosphate buffer, pH 5.4, containing 0.6 M-KCl and 0.01 M-MgSO₄, then treated for 60–120 min at 29–30 °C with 40% snail enzyme solution in citrate/phosphate buffer, pH 5.5, containing 1 M-mannitol and 0.01 M-MgSO₄. The protoplasts obtained were washed twice by centrifugation at 670 g with 1 M-mannitol and 0.01 M-MgSO₄ in citrate/phosphate buffer, pH 5.4. They were transferred to malt extract/N1 medium (4:1, v/v) plus 1 M-mannitol and grown at 29–30 °C.

Isolation of glucan fibrillar component. After cultivation for about 24 h, the protoplasts were centrifuged at 670 g for 10 min and their cytoplasm removed by extracting with SDS (Kopecká & Kreger, 1986). Following three washes with distilled water, isolated fibrillar nets were placed on grids and prepared for examination by electron microscopy.

Freeze-etching of cells and protoplasts. Freeze-etched replicas were prepared from cells growing exponentially in nutrient medium with 10% (w/v) mannitol at 29–30 °C by centrifugation at 670 g for 6 min. The pellets of living yeast cells were transferred to copper grids, then frozen in Freon 22 and liquid nitrogen. Replicas were prepared after fracturing at –100 °C in a Balzers BA 360 M apparatus essentially according to the procedure of Moor & Mühlethaler (1963). The etching time was 1 min.

Electron microscopy. Washed isolated cell walls were applied to copper grids covered with Formvar films. After drying, the walls were shadowed with platinum under vacuum. Metal-shadowed preparations and freeze-etched replicas were studied and photographed with a Tesla BS 500 electron microscope.

Results

Cell proliferation, morphology and osmotic fragility testing

The cells of the parental strain S288C proliferated at a higher rate at 29–30 °C than the mutant strain VY1160; the generation times were 100 and 220 min, respectively (Fig. 1).

Fig. 1. Growth (OD_{530}) of the parental S288C and mutant VY1160 strains. The proliferation of VY1160 cells at a dilution of 1:100 (■) is much slower than that of S288C cells at the same dilution (□), which reach the stationary phase in 10 h while the VY1160 strain does so only after 22 h. ▲, VY1160, dilution 1:25; ○, VY1160, dilution 1:50; ●, VY1160, dilution 1:100; ●, VY1160, dilution 1:200.

Light microscopy observations showed that cells of the wild-type S288C in the exponential phase had the normal appearance of ovoid budding cells of *S. cerevisiae* (Fig. 2a) while cells of the mutant VY1160 were more rounded in shape (Fig. 2b). When tested for osmotic fragility, the parental S288C strain was stable and the majority of cells were viable after repeated washing with distilled water. The mutant VY1160 cells, on the other hand, were mostly dead and stained with methylene blue when examined after repeated washing with distilled water (Table 2).

Electron microscopy of cell walls

The fact that VY1160 cells died following transfer to distilled water opened up the possibility of isolating a pure wall fraction simply by washing the cells repeatedly with distilled water. It appeared, however, that this procedure was effective only in a small portion of the cell population, probably due to wall ruptures; most of the cells retained their cytoplasm either partially or completely.

Cells of VY1160 were treated with 0.5% SDS, which is reported to remove cytoplasm from regenerating protoplasts. The walls obtained were observed in the electron microscope and found to have amorphous surfaces typical of yeast cell walls (Fig. 2d). Both mother and bud walls were mostly isodiametrical in shape, and the bud scars had a normal appearance. Cell wall ruptures were observed rarely (not shown). Attempts to isolate cell walls from strain S288C under the same conditions failed. SDS concentrations of 5% and 10% had to be used

Table 2. Viability of cells of strains S288C, VY1160, 190ts2 and 193srb1 after repeated washing with distilled water and staining with methylene blue

Strain	No. of cells ml ⁻¹	Percentage of living cells (unstained)
S288C	2.3×10^7	98.0
	4.8×10^8	96.6
	8.25×10^7	82.0
VY1160	5.6×10^6	27.2
	4.75×10^6	6.6
	6.25×10^6	10.0
190ts2	1.75×10^7	91.1
193srb1	1.6×10^6	23.4

repeatedly (twice each) before a major proportion of walls could be purified (Fig. 2c). After treatment with 10% SDS the wild-type cells differed in shape from the mutant cells on electron microscopic examination, being ovoid as opposed to isodiametrical. Occasional cells of the mutant strain showed ruptures in their walls.

Isolated cell walls were subjected to disintegration with ultrasound and their inner surfaces were investigated. In the parental strain S288C, these surfaces had the typical amorphous appearance of intact yeast cell walls (Fig. 2e). In the mutant VY1160, the inner wall surfaces revealed the fibrillar component exposed due to a decrease in the amount of amorphous matrix (Fig. 2f).

Freeze-etching of cells

The inner ultrastructure of cells shown in freeze-etched replicas did not differ between the parental S288C and the mutant VY1160 strains. It was in agreement with the normal picture of budding *S. cerevisiae* cells described by Moor & Mühlethaler (1963). Each cell possessed a nucleus with a nuclear envelope and pores, mitochondria, endoplasmic reticulum, vacuoles and also lipid droplets. The Golgi apparatus was not observed.

The cell surfaces, however, showed distinct differences in ultrastructure between the parental strain and the osmofragile mutant. In an attempt to relate these differences to one of the three mutations present in the osmofragile mutant VY1160, freeze-etched replicas of each of the isogenic, single-mutation strains were studied. Cells of S288C as well as both osmotically stable single-mutation strains 191ts1 and 190ts2 had ridges on the EF face of the plasma membrane (Fig. 3a), and invaginations and numerous hexagonally-arranged integral membrane particles densely spread over the PF face of the plasma membrane (Fig. 3b), as is typical for wild-type yeasts. The VY1160 mutant, on the other hand,

exhibited a net-like structure on the EF face of the plasma membrane (Fig. 3c) which penetrated through the EF face into the cell interior. The prominent network was reminiscent of the thick glucan fibrillar skeleton made by protoplasts (see Fig. 4) and of that seen on the inner surface of isolated cell walls (see Fig. 2f). The PF face bore invaginations and impressions (Fig. 3d) which corresponded to the prominent structures on the EF face (Fig. 3c). The osmofragile single-mutation 193*srb1* cells showed a marked net-like structure on the EF face of the plasma membrane (Fig. 3e), which was analogous to that in the VY1160 cells (Fig. 3c), and its imprint on the PF face (Fig. 3f), which could not be distinguished from that on the PF face of the plasma membrane in VY1160 cells (Fig. 3d).

Fibrillar glucan component of protoplast walls

To gain a deeper insight into the characteristics of the cell wall structures in osmofragile mutant cells, protoplasts were employed, because in the process of cell wall regeneration in liquid media *S. cerevisiae* protoplasts are known to produce a fibrillar glucan wall component free from amorphous matrix (Kopecká *et al.*, 1965; Kreger & Kopecká, 1973, 1976a, b; Kopecká & Kreger, 1986). Protoplasts of all four strains investigated, viz. S288C, VY1160, 193*srb1* and 190*ts2*, produced glucan-based fibrillar wall components. No differences were found at the ultramicroscopical level in either appearance or structure between the osmofragile mutants (Fig. 4a) and the parental strain S288C (Fig. 4b) or the osmotically resistant mutant 190*ts2* (not shown).

Discussion

The method of wall isolation used in this study was based on treatment with SDS. It appeared that the cell walls of mutant VY1160 were more permeable to cytoplasmic polymers than those of the wild-type, as the concentration of SDS required for their permeabilization (0.5%) was 20-fold lower. This finding was suggestive of differences in the wall characteristics. An increase in permeability of cell walls for cytoplasmic biopolymers has already been reported in strain VY1160 by Philipova & Venkov (1990). Electron microscopy after disintegration

with ultrasound showed that the inner surface of the cell wall in cells of the mutant VY1160 was markedly different from that of the parental strain. It was characterized by a fibrillar texture that was apparently due to a very low degree of masking by amorphous matrix (Fig. 2f). The inner surface of the S288C cell walls, on the other hand, had a typical amorphous appearance (Fig. 2e).

The fibrillar wall component was also demonstrated in freeze-etched replicas of whole cells in the osmofragile mutant VY1160 and in the single-mutation strain 193*srb1*. However, only thick bundles of glucan microfibrils were prominent and seen on both the EF and PF faces of the plasma membrane. The finding of unmasked fibrillar structures in the walls of native cells has not, to our knowledge, been reported before in any other *S. cerevisiae* cells studied by freeze-etching.

However, no fibrillar wall structures were detected by freeze-etching in either the S288C strain or single-mutation strains 190*ts2* and 191*ts1*, both of which are resistant to osmotic changes (Table 2). These results imply a relation between an unmasked fibrillar network on the inner wall surface and the osmotic fragility of the mutant cell. The exposed fibrillar network in cells of VY1160, which bear *srb1*, *ts1* and *ts2* mutations, and in the single-mutation strain 193*srb1*, suggests that it is a phenotypic expression of the *srb1* mutation. It can be further speculated that this mutation is responsible for the synthesis of a mutant or aberrant glucan, and that consequently, this incomplete material cannot ensure the formation of a complete amorphous matrix. Blagoeva & Venkov (1990), studying the structure of glucans making up the cell wall of the VY1160 mutant, found a significant decrease in the amount of alkali-soluble glucan. This provides complementary biochemical evidence for a decreased quantity of amorphous matrix.

The quality of the glucan fibrillar network was studied in protoplasts prepared from both the wild-type and the mutant strains. No differences were found in the ultrastructure of the fibrillar wall component between the strains studied, indicating that the glucan microfibrils were undisturbed and similar in composition. Their electron microscopic images at the magnification used in the study showed no evidence of the chemical changes in insoluble glucans, namely a lower molecular mass and a higher number of β -(1 $\rightarrow$ 6)-linkages, found in cells of VY1160 cells by Blagoeva & Venkov (1990a, b). It

Fig. 2. Yeast cells and cell walls. (a, b) Phase-contrast micrographs of cells of S288C (a) and VY1160 (b) growing on the surface of 2% agar. (b-f) Electron micrographs of isolated cell walls of S288C (c, e) and VY1160 (d, f). In (c), cytoplasm was removed by treatment with 0.5% SDS for 10 min at 50 °C, followed by 5% and 10% SDS and washing with water. This procedure was repeated twice with each SDS concentration. In (d) the procedure was the same except that the treatments with 5% and 10% SDS were omitted. In (e) and (f) the cell walls were isolated by ultrasonic disintegration. All samples for electron microscopy were shadowed with platinum. Bars represent 10 μ m (a, b) and 1 μ m (c-f).

Fig. 3 (contd from previous page). Electron micrographs of freeze-etchings of the plasma membrane (freeze-etching terminology according to Branton *et al.*, 1975). (a) Exoplasmic fracture (EF) face of strain 190ts2; (b) protoplasmic fracture (PF) face of strain 190ts2; (c) EF face of strain VY1160; (d) PF face of strain VY1160; (e) EF face of strain 193srb1; (f) PF face of strain 193srb1. Bars represent 1 μ m.

Fig. 4. Electron micrographs of the fibrillar glucan wall component formed by protoplasts of strain S288C (a) and strain VY1160 (b). Shadowed with platinum. Bars represent 1 μ m.

can thus be concluded that the osmotic fragility manifested by cells of mutant VY1160 was related to a deficiency occurring, as a consequence of the *srbl* mutation, during biogenesis of the wall structure and affecting its amorphous (glucan) component.

Evidence that an incomplete cell wall can be related to osmotic fragility without interfering with cell division has been provided by studies of dividing *Schizosaccharomyces japonicus* protoplasts (Gabriel, 1984). The low amount of amorphous matrix found in their incomplete cell walls does not mask the fibrillar wall skeleton properly, and the resulting three-dimensional wall architecture lacks the rigidity necessary for protection against lysis. However, while in the flattened protoplasts of *Sch. japonicus* the wall fragility is due to the incomplete cell wall regeneration (Gabriel & Kopecká, 1988), in the osmofragile *S. cerevisiae* cells (VY1160 and 193*srbl*) it can be attributed to a genetically-coded deficiency in the wall ultrastructure.

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