Cell-wall Composition and Structure of Yeast Cells and Conjugation Tubes of *Tremella mesenterica*

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SUMMARY

Cell walls prepared from vegetative yeast cells and from hormone-induced conjugation tubes of the basidiomycete *Tremella mesenterica* had similar compositions. Evidence was found for 1,3- α -glucan (yeast 38 %, tube 25 %), 1,3- β -1,6- β -glucan (yeast 33 %, tube 48 %) and chitin (both < 3 %) in the walls. The walls also contained xylose (5 to 7 %), mannose (6 %), glucuronic acid (approx. 2 %), and traces of galactose. Protein amounted to less than 2 % of the wall weight. The cell capsule was very insoluble and could not be removed from the cell wall. The conjugation hormone did not appear to exert its effect on cell shape by causing gross changes in wall composition.

INTRODUCTION

Haploid cells of the basidiomycete *Tremella mesenterica* normally grow as yeasts, but can produce filamentous conjugation tubes when sexually compatible strains are mixed. The conjugation tubes are induced by hormones diffusing between the cells (Bandoni, 1965). The partially purified conjugation hormones from one mating type (Reid, 1974) can be used to induce conjugation-tube growth in single-strain cultures.

Fungal cell shape is largely determined by the pattern of wall synthesis. A gradient of wall growth restricted to a small apical region results in filamentous extension, whereas growth diffused over most of the cell surface leads to spheroidal bud expansion (Bartnicki-Garcia & Lippman, 1969). To understand the action of the conjugation hormones in *T. mesenterica* one must understand their effect on the wall-synthesizing enzymes and their three-dimensional distribution on the cell surface. This paper attempts to answer two preliminary questions – do the conjugation-tube walls have the same composition as the yeast walls, and what types of polysaccharides form these walls? Cameron & Taylor (1976) analysed the monomers of the *T. mesenterica* yeast wall; and the extracellular polysaccharides of *T. mesenterica* were studied by Fraser & Jennings (1971) and Fraser, Jennings & Moyna (1973). However, the cell-wall polymers of this fungus have not previously been characterized.

METHODS

Preparation of cells. Haploid cells of *Tremella mesenterica* Fr., strain RJB2259-6 (ATCC 24925) were grown in a medium containing (per litre distilled water): D-glucose, 10 g; urea, 1 g; KH_2PO_4 , 1 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.1 g; thiamine, 100 μg ; and 0.1 ml microelement stock solution (Reid, 1975). The urea was filter-sterilized as a 10 % (w/v)

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solution, and added aseptically after autoclaving the rest of the medium. Erlenmeyer flasks (1 l) containing 250 ml medium were inoculated with 5 ml of an exponentially growing culture of strain RJB2259-6 in the same medium, and incubated on a rotary shaker (140 rev. min⁻¹; 1 cm radius) at 20 °C.

Conjugation hormone from *T. mesenterica* strain RJB2259-7 (ATCC28783), partially purified by adsorption on Porapak Q (Reid, 1974), was added to half of the cultures 38 h after inoculation, when the turbidity (540 nm) of culture samples, diluted 1:10 with water, reached 0.02 (measured using a Bausch & Lomb Spectronic 100 spectrophotometer with a micro flow-through cell). Each treated culture received 0.25 ml of a solution containing crude hormone (5.7 mg ml⁻¹) in 60% (v/v) aqueous acetone; the other cultures received a control dose of 0.25 ml 60% aqueous acetone. Further doses of hormone solution or solvent blank were administered 47 h, 61 h and 71 h after inoculation. At 82 h after inoculation the cells were harvested by centrifuging for 10 min at 16000 g (max.) at 4 °C, and washed with distilled water.

A large batch of yeast cells was prepared by growing strain RJB2259-6 for 5 days in 15 l medium in a 19 l polypropylene bottle with sterile air bubbling through the culture.

Preparation of cell walls. All operations were performed at or below 4 °C. Washed cells were packed by centrifuging at 12000 g in 50 ml polycarbonate centrifuge tubes. The probe of a Biosonik III sonicator (Bronwill Scientific, Rochester, New York, U.S.A.) was immersed to a depth of 2 to 3 cm in the packed cells (vol. 35 ml) and the sonicator was operated at full power (300 W) for 2 min. The centrifuge tubes were kept in ice throughout, and the sonicator probe was cooled in ice between treatments. Each batch of cells received three 2 min sonications. The sonicated suspension was centrifuged for 15 min at 27000 g and the supernatant was discarded. The pellets were resuspended in 8 M-urea, sonicated for 15 s, and centrifuged for 10 min at 27000 g. This process was repeated five times. The pellet (crude wall preparation) was then washed five times with distilled water.

To separate the wall fragments from unbroken cells, the crude wall preparation was resuspended in water and centrifuged for 10 min at 1000 g. The supernatant was carefully decanted into a second centrifuge tube, and the pellet was resuspended in distilled water. After another centrifugation for 10 min at 1000 g, the supernatant from the second tube was decanted into a third tube, the supernatant from the first tube was mixed with the pellet in the second tube, and the pellet in the first tube was resuspended in water. The cycle of centrifugation and transfer of supernatants was repeated until four tubes were in operation. Then the supernatant from the fourth tube was set aside, and the procedure continued for three more rounds.

In the yeast wall preparation, the residual pellets had high contents of whole cells. The supernatants from the fourth centrifuge tube contained wall fragments, but very few unbroken cells. These wall fragments were recovered by centrifuging for 10 min at 27000 g, washed three times with distilled water, and freeze-dried.

In the crude conjugation-tube wall preparation, the majority of the unbroken cells remained in the pellet in the first centrifuge tube. The pellets in the second, third and fourth centrifuge tubes were pooled with the supernatants, and centrifuged for 10 min at 27000 g to recover the wall fragments. This wall preparation was washed three times with water and freeze-dried. The total time from breaking the cells until freezing the wall preparation was 9 h.

Analytical methods. All analytical results were the mean of duplicate determinations. Carbohydrates were determined by Dreywood's anthrone method (Bartnicki-Garcia & Nickerson, 1962) and by the phenol method (Dubois *et al.*, 1956), with glucose as standard.

Total protein was estimated in the presence of amino sugar by a modified ninhydrin method (Bartnicki-Garcia & Nickerson, 1962) using bovine serum albumin as standard. Hexosamine was determined after hydrolysis with 2 M-HCl for 16 h at 105 °C by the method of Gatt & Berman (1966) using glucosamine as the standard, or after hydrolysis for 8 h with 6 M-HCl under N₂ at 105 °C by the method of Tracey (1955).

For monosaccharide analysis, samples (1 mg) were hydrolysed in 0.5 M-H₂SO₄ at 105 °C for 18 h. After cooling, each hydrolysate was supplemented with 200 µg 2-deoxy-D-glucose as an internal standard and neutralized by passing through a column containing Duolite ES-561 (Diamond Shamrock Corp., Redwood City, California, U.S.A.) weak-base anion-exchange resin in the free-base form. Sugars in the effluent were reduced by treating overnight with sodium borohydride, then converted into alditol acetates and separated by gas-liquid chromatography as described by Lippman, Erwin & Bartnicki-Garcia (1974).

For amino-acid analysis, samples were hydrolysed in 6 M-HCl under N₂ for 8 h at 105 °C. The amino acids were separated by the method of Spackman, Stein & Moore (1958) in a Beckman Spinco automatic amino-acid analyser, model 120 C.

To detect uronic acids, a 0.5 M-H₂SO₄ hydrolysate was neutralized with BaCO₃, and passed through a column containing 14 ml Dowex-1 anion-exchange resin (acetate form). The column was eluted with a linear gradient from 0 to 4 M-acetic acid. Fractions (5 ml) were collected and analysed for uronic acid by the carbazole method (Bitter & Muir, 1962). The carbazole-reactive material eluted from the column was separated further by thin-layer chromatography on silica gel (Eastman Chromagram sheets) in solvent A (pyridine/ethyl acetate/acetic acid/water, 36:36:7:21, by vol.), and on cellulose (250 µm layers of Adsorbasil, Applied Science Labs, State College, Pennsylvania, U.S.A.) in solvent B (n-butanol/acetic acid/water, 2:1:1, by vol.; two ascents). Spots were revealed with aniline phthalate or *p*-anisidine phthalate.

For quantitative estimation of uronic acids, wall samples were hydrolysed for 4 h at 105 °C with 1 M-trifluoroacetic acid. The acid was removed by evaporation *in vacuo* at 40 °C over NaOH. The hydrolysates were dissolved in 1 ml 0.01 M-NaHCO₃, left at room temperature for 20 min to saponify lactones, and applied to columns containing 5 ml Dowex-1 (acetate form). The columns were washed with 25 ml water, and uronic acids were eluted with 15 ml 4 M-acetic acid. The acetic-acid eluates were evaporated *in vacuo* and redissolved in water. Samples were taken for uronic acid estimation by the carbazole method and for neutral sugar estimation by the anthrone method. From the anthrone values, corrections for the contribution of neutral sugars to the carbazole readings were calculated.

Chemical fractionation. Duplicate samples of each wall type were carried through the fractionation procedure. Wall samples were extracted in polypropylene centrifuge tubes and were sedimented by centrifuging for 10 min at 24000 g after each extraction or washing. The walls were extracted three times for 15 min with 1 M-NaCl in a boiling water bath, and washed twice with water (hot-saline extract). They were then extracted three times for 30 min with 1 M-KOH at room temperature and washed twice with water. This cold-alkali extract was treated with 0.1 vol. Fehling's solution (Jones & Stoodley, 1965); the precipitated copper complex was collected by centrifuging at 2000 g, washed with water, decomposed by maceration in ethanol containing 1 % (v/v) concentrated HCl, and resuspended in water (copper-precipitated fraction). The supernatant from the precipitation with Fehling's solution was neutralized with glacial acetic acid. The resulting gelatinous precipitate was washed several times with water, and redissolved in 0.2 M-KOH (acid-precipitated fraction).

The residue from the cold-alkali extraction was extracted three times for 15 min with 1 M-HCl in a boiling water bath, and washed twice with water (hot-acid extract). The residue was extracted three times for 15 min with 1 M-KOH in a boiling water bath and washed twice with water (hot-alkali extract). The insoluble residue was washed three more times with water, and then suspended in water.

To obtain a better yield of the copper-precipitated fraction, the cold-alkali extract was mixed with 5 vol. copper tartrate reagent (Bartnicki-Garcia & Lindberg, 1972) and left overnight at 4 °C. The precipitate was collected by centrifuging at 1000 g, washed with copper tartrate solution, and dissolved in 0.5 M-acetic acid.

Enzymic digestion. Exo-1,3- β -glucanase was purified from the culture fluid of *Sporotrichum dimorphosporum* QM806 by the method of Huotari *et al.* (1968). Walls previously extracted with 1 M-KOH at room temperature, or solubilized polysaccharides, were incubated with 50 units of enzyme in 0.05 M-acetate buffer pH 4.8 for 48 h at 39 °C. The digestion mixture was heated in boiling water for 10 min and centrifuged. The supernatant was applied to a column of Sephadex G-15 (2.5 $\times$ 170 cm) and eluted with water at 40 ml h⁻¹. Fractions (4 ml) were collected, and analysed for carbohydrate by the phenol method.

Physical techniques. Optical rotations were measured with an automatic polarimeter (Bendix Scientific Instruments, Cincinnati, Ohio, U.S.A.). X-ray diffraction patterns were obtained in a 114.6 mm Debye-Scherrer powder camera using Cu K α -radiation from a Norelco 2.5 kW generator. Intact and extracted wall samples were examined in a Hitachi HU-12 electron microscope after shadowing with palladium at an angle of 19°. Infrared spectra were measured with a Perkin-Elmer model 621 spectrophotometer on samples embedded in potassium bromide pellets.

Authentic S-glucan from *Schizophyllum commune* was a gift from Professor J. G. H. Wessels, University of Groningen, The Netherlands.

RESULTS

Preparation of cells

Cultures of *T. mesenterica* grown in the absence of hormone contained only yeast cells. Cells in the hormone-treated cultures had filamentous conjugation tubes (Fig. 1). At the time of harvest, about 10% of the conjugation tubes had stopped elongating and started budding. Indian-ink mounts showed that both the yeast cells and the conjugation tubes were sheathed in a transparent capsule (Fig. 2). The turbidity of the hormone-treated cultures increased more rapidly than that of the untreated cultures. By the time of harvest the hormone-treated cultures, after 10-fold dilution, had an extinction at 540 nm of 0.38 whereas the extinction of the untreated culture was only 0.21. One litre of hormone-treated culture yielded 3.96 g dry weight of cells with conjugation tubes, and 1 l of untreated culture yielded 2.72 g of yeast cells.

Preparation of walls

Sonication broke the cells of *T. mesenterica* more effectively than ballistic disruption in a Braun MSK cell homogenizer. A thick cell suspension seemed essential for efficient breakage by sonication, with three 2 min treatments breaking about 90% of the cells. The residual whole cells were removed by differential centrifugation after washing the walls. Inspection of the wall preparations by dark-field phase-contrast microscopy, or by bright-field microscopy after staining with lactophenol cotton blue, showed negligible numbers of unbroken cells, or partly broken cells with some cytoplasm (Fig. 3). From 1 l of yeast

Fig. 1. Dark-field phase-contrast microscopy of *T. mesenterica*: (a) yeast cells; (b) cells with conjugation tubes. Bar markers represent 20 μm .

Fig. 2. *Tremella mesenterica* cell with conjugation tube mounted in Indian ink to demonstrate capsule. Bar marker represents 10 μm .

culture, 158 mg of walls were recovered, and from 1 l of hormone-treated culture, 708 mg. Visual estimation of the relative areas occupied by yeast walls and conjugation-tube walls in several microscope fields indicated that the wall preparation from the hormone-treated cultures contained about 85 % conjugation-tube walls and 15 % yeast walls. This preparation will be referred to as 'conjugation-tube walls'. Yeast and tube wall fragments were encapsulated.

Chemical composition

The gross compositions of the yeast walls and the conjugation-tube walls were similar (Table 1). In both wall types, the phenol method indicated a greater carbohydrate content than did the anthrone method; this discrepancy was larger for the yeast walls than for the conjugation-tube walls. Xylose, mannose and glucose were the only neutral sugars that could be positively identified in hydrolysates of the whole cell walls, but small amounts of galactose were detected in two of the wall fractions. The recovery of neutral sugars after hydrolysis and gas-liquid chromatography was less than expected from the colorimetric

Fig. 3. Dark-field phase-contrast microscopy of isolated cell walls of *T. mesenterica*: (a) yeast walls; (b) conjugation-tube walls. Bar markers represent 10 μm .

Table 1. *Chemical composition of Tremella mesenterica cell walls*

Results are the mean of duplicate determinations.

Component	Method	Percentage of wall dry weight	
		Yeast	Conjugation tube
Total carbohydrate	Anthrone	72	70
	Phenol	88	78
Xylose	G.l.c.	7.1	4.9
Mannose	G.l.c.	6.3	5.8
Glucose	G.l.c.	47	53
Glucuronic acid	Carbazole	2.1	1.5
Protein	Ninhydrin	1.6	1.8
Amino sugar	Elson-Morgan	3.7	5.4

assays. The walls of *T. mesenterica* resisted hydrolysis. After 8 h hydrolysis with 0.5 M- H_2SO_4 , a visible undissolved residue remained; this residue gave a strong carbohydrate reaction with phenol. The prolonged hydrolysis (18 h) needed to liberate most of the glucose and mannose in the walls probably degraded considerable amounts of xylose, and glucose and mannose were possibly also degraded to a smaller extent. The yield of monosaccharides was not improved either by soaking the walls in 11 M- H_2SO_4 before hydrolysing for 8 h with 0.5 M- H_2SO_4 , or by hydrolysing with 2 M-trifluoroacetic acid for 4 h. Although the analytical values for the monosaccharides were not reliable in an absolute sense, they could be used to compare the two wall types. The conjugation-tube walls had a lower xylose content than the yeast walls. Although less glucose was recovered in hydrolysates of yeast walls than in those of conjugation-tube walls, the actual glucose contents were probably similar; the anthrone values for the two wall types were comparable. The discrepancy probably stems from the higher content of the acid-resistant α -glucan in yeast walls (see below).

Anion-exchange chromatography of wall hydrolysates demonstrated the presence of

Fig. 4. Separation of uronic acid from a hydrolysate of *T. mesenterica* yeast walls, by chromatography on Dowex-1 (acetate) anion-exchange resin, eluted with a gradient from 0 to 4 M-acetic acid, starting in fraction 17. Extinction values from the carbazole assay (O); acetic acid concentration (---).

Table 2. *Amino acids in Tremella mesenterica* cell walls

Amino acids were separated and determined as described in Methods. Results are the mean of duplicate determinations and are expressed as μg anhydroamino acid per mg wall.

Amino acid	Wall amino-acid content ($\mu\text{g mg}^{-1}$)	
	Yeast	Conjugation tube
Lysine	1.6	1.5
Histidine	Trace	0.3
Arginine	0.7	0.8
Aspartic acid	1.0	1.4
Threonine	0.6	0.9
Serine	0.9	1.2
Glutamic acid	1.1	1.5
Proline	0.6	0.8
Glycine	0.6	0.7
Alanine	0.9	0.9
Valine	0.6	0.6
Methionine	0.1	0.1
Isoleucine	0.4	0.5
Leucine	0.8	1.0
Tyrosine	0.4	0.3
Phenylalanine	0.5	0.7
Total	10.8	13.2

uronic acids. An acetic acid gradient eluted two peaks of carbazole-reactive material from the anion-exchange column (Fig. 4). Thin-layer chromatography of peak B gave spots with the same mobilities as glucuronic acid and glucuronolactone in two solvents, and a slower moving spot. Thin-layer chromatography of peak A showed only a slow moving spot, probably an aldobiouronic acid. There was not enough material for further characterization.

Quantitative analysis for glucuronic acid by the carbazole method after hydrolysis and anion-exchange purification indicated that the yeast walls contained 1.2% glucuronic acid and the conjugation-tube walls 0.97%. This procedure probably underestimated the uronic-acid content; recovery of uronic acid in samples of pure glucuronic acid treated in parallel

Table 3. *Distribution of carbohydrate, amino sugar and protein in fractions of Tremella mesenterica cell walls*

Carbohydrate was measured by the phenol method; amino sugar and protein were measured as described in Methods. All results are the mean of duplicate determinations and are expressed as a percentage of the wall dry weight.

Fraction	Carbohydrate (%)		Amino sugar (%)		Protein (%)	
	Yeast	Conjugation tube	Yeast	Conjugation tube	Yeast	Conjugation tube
Hot-saline extract	0.6	2.3	0.05	0.1	0.7	0.9
Cold-alkali extract:						
Copper precipitated	15.0†	8.0†	0.04	0.01	0.4	0.6
Acid precipitated	37.7	25.5	0.24	0.18	*	*
Acid soluble	1.4	2.9	0.00	0.00	0.0	0.0
Hot-acid extract	33.5	48.2	2.23	2.29	*	*
Hot-alkali extract	1.0	0.7	0.17	0.11	0.1	0.2
Insoluble residue	0.1	0.1	0.08	0.47	0.2	0.3
Total	89.3	87.7	2.81	3.16	1.4	2.0

* Excessive browning during hydrolysis prevented a valid ninhydrin reading.

† Data from a separate experiment.

with the wall samples was only 23.5%. Destruction by hot acid and incomplete saponification of lactones were probably the main reasons for poor recovery. However, the method did permit comparison of the two wall samples, and suggested that the yeast walls had a higher glucuronic acid content than the tube walls.

The conjugation-tube walls contained similar amounts of protein but more amino sugar than the yeast walls. The relative abundances of the amino acids in the two wall types were similar (Table 2). Glucosamine was the only amino sugar detected on the amino-acid analyser chromatograms.

Chemical fractionation

Most of the wall carbohydrate (as measured by the phenol method) appeared in the cold-alkali extracts and the hot-acid extracts (Table 3).

In the first fractionations, the yield of the copper-precipitable material was low. A subsequent experiment, in which the copper complex was washed with copper tartrate solution rather than water, gave much higher yields. The copper-precipitated fraction was the only cell-wall fraction which gave more colour in the carbazole assay than could be accounted for by its neutral sugar content. The values for uronic-acid content of the walls in Table 1 were calculated from the carbazole readings (corrected for neutral sugar interference) for these copper-precipitated fractions, on the assumption that they contained all the uronic acid of the walls. Uronic acid contents determined in this way were higher, and probably more accurate, than those estimated by hydrolysis and anion-exchange chromatography. The two methods were in agreement that the yeast walls contained more uronic acid than the conjugation-tube walls.

The yeast walls contained slightly more of the alkali-extracted, acid-precipitated fraction than the hot-acid-extracted fraction. In contrast, the conjugation-tube walls contained almost twice as much of the hot-acid-extracted fraction as of the alkali-extracted, acid-precipitated fraction.

Most of the amino sugar of the walls was found in the hot-acid extracts (Table 3). Significant amounts also occurred in the alkali-extracted, acid-precipitated fraction, the

Fig. 5. X-ray diffraction patterns: (a) *T. mesenterica* yeast walls; (b) *T. mesenterica* conjugation-tube walls; (c) α -glucan from *T. mesenterica* yeast walls; (d) S-glucan from *Schizophyllum commune*; (e) *T. mesenterica* yeast walls after extraction with cold 1 M-KOH; (f) *T. mesenterica* conjugation-tube walls after extraction with cold 1 M-KOH; (g) chitin prepared from *T. mesenterica* yeast walls; (h) purified crab chitin.

hot-alkali extract and the insoluble residue. Recovery of amino sugar in the fractionation was incomplete (compare Tables 1 and 3).

Almost half of the wall protein was extracted by hot 1 M-NaCl. The two fractions which contained large amounts of polysaccharides became deep brown during hydrolysis, which obscured any colour developed in the ninhydrin reaction and prevented a valid protein estimation. The high recovery of protein from the other fractions suggested that the protein content of these two fractions was low (Table 3).

Polysaccharides of the yeast cell wall

Yeast walls (500 mg) from a large batch of yeast cells were fractionated to provide enough material for characterization of the fractions.

Copper-precipitated fraction. After three cycles of precipitation with Fehling's solution and dissolution in 0.5 M-acetic acid, this fraction contained xylose, mannose, glucose, galactose and uronic acid in the ratio 1.2:1.0:0.2:0.04:0.7.

α -Glucan. The cold-alkali extract contained a large amount of a polymer which was not precipitated by Fehling's solution, but did precipitate on acidification. This polymer contained glucose with smaller amounts of xylose and mannose (glucose 11.7:xylose 1.6:mannose 1.0). The polysaccharide was strongly dextrorotatory, $[\alpha]_D^{27} +257^\circ$ (c 0.82 in 1 M-KOH). The acid-precipitated material gave an X-ray diffraction pattern (Fig. 5) which was similar to, but slightly sharper than, the X-ray diffraction pattern of the 1,3- α -glucan (S-glucan) from *Schizophyllum commune* (Wessels *et al.*, 1972). The α -glucan reflexions were also visible in the X-ray diffraction pattern of the unextracted walls (Fig. 5). The infrared spectrum

Fig. 6. Distribution of carbohydrate (phenol assay) after gel filtration on Sephadex G-15: (a) hot 1 M-HCl extracts of yeast walls (○) and conjugation-tube walls (●); (b) after digestion with *exo*-1,3- β -glucanase of the 'polysaccharide' fraction of the hot-acid extract from yeast walls (○) or of alkali-extracted yeast walls (●).

of the acid-precipitated material from *T. mesenterica* was identical to the infrared spectrum of the S-glucan from *S. commune*.

The mannose and xylose residues in the α -glucan fraction were not removed by three cycles of dissolution in alkali, treatment with Fehling's solution, and reprecipitation with acetic acid.

The α -glucan was resistant to acid hydrolysis, probably because of its crystallinity and insolubility. The undissolved residue after 8 h hydrolysis of whole walls with 0.5 M-H₂SO₄ gave the characteristic X-ray diffraction pattern of α -glucan. For this reason, the glucose content of the acid-precipitated fraction was probably underestimated by the acid hydrolysis procedure.

The acid-precipitated fraction from conjugation-tube walls also gave the characteristic X-ray diffraction pattern and infrared spectrum of α -glucan.

β -Glucan. Hydrolysis of the hot-acid extract yielded glucose, xylose and mannose (5.4:1.3:1.0). The non-dialysable portion of the neutralized extract was laevorotatory, $[\alpha]_D^{27} - 15.0^\circ$ (*c* 2.0 in water), indicating a β -linked polymer. Gel filtration on Sephadex G-15 separated the extract into 'polysaccharide' (excluded from the column), a complex mixture of oligosaccharides, and monosaccharides (Fig. 6a). The hot-acid extract from conjugation-tube walls gave a similar profile on gel filtration. The monosaccharide fraction from the yeast walls contained glucose, xylose and mannose in the ratio 15.3:34.3:1.0. The oligosaccharides were not investigated further.

The infrared spectra of the cell walls (both yeast and conjugation tube) after extraction

Fig. 7. Electron micrographs of shadowed wall preparations of *T. mesenterica*. Texture of isolated walls: (a) yeast walls; (b) conjugation-tube walls. Note the granular outer surface and microfibrils embedded in the inner surface. Bar markers represent 1 μm .

with cold 1 M-KOH, and of the polysaccharide fraction from the hot 1 M-HCl extracts, closely resembled the infrared spectrum of *Saccharomyces glukan*. However, the spectra of the *T. mesenterica* samples had a broad absorption band at 895 to 905 cm^{-1} , rather than the characteristic peak of β -glucans at 890 cm^{-1} (Michell & Scurfield, 1970).

When the polysaccharide fraction was digested with *exo*-1,3- β -glucanase and rechromatographed on the Sephadex G-15 column (Fig. 6b), there was a residual polysaccharide peak, one small oligosaccharide peak and a large monosaccharide peak. After hydrolysis, the residual polysaccharide yielded xylose, mannose, galactose and glucose in the ratio 0.4:1.0:0.07:0.7. The monosaccharide peak contained only glucose. *Exo*-1,3- β -glucanase digestion of 10 mg alkali-extracted yeast walls solubilized glucose (90%) and a disaccharide (10%) (Fig. 6b). The disaccharide was tentatively identified as gentiobiose by thin-layer chromatography on cellulose in the solvent butanol/pyridine/water (6:4:3, by vol.). The *exo*-1,3- β -glucanase digestion did not remove the capsule from the walls. The results of the enzyme digestions suggested the presence of a 1,3- β -1,6- β -glucan in the walls. The mannose, xylose and galactose were probably in a separate polymer.

Chitin. The hot-acid- and hot-alkali-insoluble residue from both the yeast and conjugation-tube walls gave an X-ray diffraction pattern characteristic of chitin (Fig. 5). The amount of chitin in the walls was hard to assess. Only a small fraction of the total amino sugar remained in the insoluble residue; most was in the hot 1 M-HCl extract. Hot 1 M-acetic acid did not dissolve appreciable amounts of amino sugar from the walls, so the acid-soluble amino sugar was probably not chitosan. X-ray diffraction of walls after extraction with cold 1 M-KOH, but before extraction with hot 1 M-HCl, gave a very diffuse pattern in which

Electron micrographs of shadowed wall preparations of *T. mesenterica*. All bar markers represent 1 μm .

Fig. 8. Conjugation-tube wall after extraction with cold 1 M-KOH.

Fig. 9. Conjugation-tube walls after extraction with hot 1 M-HCl.

Fig. 10. Insoluble residues after extraction with hot 1 M-HCl and hot 1 M-KOH: (a) conjugation-tube walls; (b) yeast cells.

the chitin reflexions could not be positively identified (Fig. 5). Perhaps most of the amino sugar was present as poorly crystalline chitin which was hydrolysed to soluble fragments by the hot-acid extraction. Highly crystalline regions of chitin would survive the hot-acid treatment and remain as the insoluble residue.

Cell-wall physical structure

The exterior surface of isolated walls was coarsely granular. The interior one was smoother, and showed some partially embedded microfibrils (Fig. 7). Extraction with 1 M-KOH removed the granular layer, and made the texture of the outer surface similar to that of the inner surface (Fig. 8). The wall fragments retained their shape after the cold-alkali extraction; light microscopy showed that they were still encapsulated. Hot-acid extraction completely removed the amorphous portion of the wall and left a network of microfibrils (Fig. 9). Subsequent extraction with hot alkali did not visibly alter the microfibrillar network. The initial shape of the wall fragments was largely destroyed, although it was possible in places to find the outline of a conjugation tube (Fig. 10a). Recognizable wall fragments could not be found on grids prepared from the yeast wall insoluble residues. However, the residues from hot-acid and alkali extractions of whole yeast cells showed microfibrils identical to those of the conjugation-tube walls (Fig. 10b).

DISCUSSION

When conjugation hormone was added to cultures of *T. mesenterica*, the cells stopped budding and produced conjugation tubes. After several hours, the conjugation tubes stopped growing and budding resumed, apparently when the conjugation hormone had been consumed; adding more hormone restarted conjugation-tube growth. We obtained a high ratio of conjugation tubes to yeast cells by adding hormone repeatedly over two days, so that only about 10% of the cells in the culture were budding at the time of harvest. The hormone-treated cultures received a total of 22.8 mg hormone preparation per litre. It is not known if any of the impurities in the hormone preparation affected the cell-wall composition. The hormone-treated cultures grew faster, as measured by both turbidity and dry weight, than the yeast cultures; this may have been caused by the hormone itself or by impurities. Thiamine, the only growth factor known to be required by this strain, was present in excess and complex natural nutrient mixtures do not generally accelerate the growth of yeast cells of this strain (Reid, 1975). Therefore, growth stimulation by nutrient impurities in the hormone preparation seems unlikely.

The presence of a capsule around the conjugation tubes of *T. mesenterica* is variable (Reid, unpublished observations). In these experiments the conjugation tubes were as thickly encapsulated as the yeast cells; however, under other conditions, for example with glutamine as nitrogen source, the conjugation tubes have no detectable capsule (Reid, unpublished observations). The environmental variables controlling conjugation-tube encapsulation have not yet been identified. In our experience, yeast cells always have a capsule except when grown at low pH (Reid, 1975). Cameron & Taylor (1976) reported the preparation of cell walls from cultures of *T. mesenterica* that were capsule-free. In some fungi, the wall is coated by mucilaginous polysaccharide which can be removed by repeated washing, for example, *Schizophyllum commune* hyphae (Wessels *et al.*, 1972). In contrast, the capsule of the *Tremella* cells was not removed by sonication and washing during wall isolation, nor by extraction with hot 1 M-NaCl. Even extraction with 1 M-KOH, which dissolved much

of the wall, did not remove the capsule. These results suggest that the capsule should be regarded as an integral part of the *T. mesenterica* wall rather than as a separate structure.

The discrepancies between the carbohydrate contents of the cell walls as measured with anthrone or with phenol can be attributed to the different specificity of these two reagents. The higher carbohydrate content of the yeast walls, as measured with phenol, is consistent with the higher content of xylose and glucuronic acid in these walls.

The polysaccharide precipitated from the cold-alkali extract by Fehling's solution had a gross composition similar to the extracellular acidic xylomannan from other strains of *T. mesenterica* (Fraser *et al.*, 1973; Slodki, Wickerham & Bandoni, 1966). We have not examined the wall polysaccharide in enough detail to establish a structural similarity. An acidic xylomannan was extracted with ethylenediamine from the walls of *Cryptococcus laurentii* by Ankel *et al.* (1970). The walls of *Polyporus tumulosus* contain an alkali-soluble neutral xylomannan that forms insoluble complexes with Cu^{2+} (Angyal, Bender & Ralph, 1974), and a similar polysaccharide can be extracted from the mycelium of *Armillaria mellea* (Bouveng, Fraser & Lindberg, 1967). The glucose present in the copper-precipitated fraction from *T. mesenterica* may be from contaminating glucan. Some of the glucitol hexaacetate detected by gas-liquid chromatography could have resulted from reduction of glucuronolactone formed during acid hydrolysis (Blake & Richards, 1970).

The polysaccharide precipitated by acid from the cold-alkali extract has the optical rotation, infrared spectrum and X-ray diffraction pattern of a 1,3- α -glucan. Such a polysaccharide has been characterized from the walls of *Schizophyllum commune* (S-glucan; Wessels *et al.*, 1972) and *Polyporus tumulosus* (Angyal *et al.*, 1974), and has been reported in many other fungi (see Wessels *et al.*, 1972). Wessels *et al.* (1972) showed that the α -glucan in *S. commune* forms microcrystalline rodlets, and is located on the outer surface of the wall. No rodlet pattern could be seen on the surface of the walls of *T. mesenterica*, but it could have been obscured by the capsule. The presence of a small number of linkages other than 1,3- α -glucosidic in the polysaccharide of *T. mesenterica* cannot be excluded without further investigation. *T. mesenterica* produces an extracellular glucan with 1,6- α and 1,4- α linkages (Fraser & Jennings, 1971). The polymeric state of the xylose and mannose in the α -glucan preparation from *T. mesenterica* walls is unknown.

The presence of β -glucan in the Tremella walls was confirmed by digestion with *exo*-1,3- β -glucanase. Release of glucose and gentiobiose by the enzyme indicated both 1,3- β and 1,6- β linkages. This type of β -glucan is very widespread in fungi. Many fungal walls which contain 1,3- β -1,6- β -glucans produce, upon acid hydrolysis, an insoluble 'hydroglucan' fraction (Houwink & Kreger, 1953; Wang & Bartnicki-Garcia, 1970). There was no evidence for such an acid-insoluble β -glucan in the Tremella walls, which may indicate that the fine structure of the β -glucan from Tremella is different from that of other fungi that have been studied.

Although xylose and mannose have been commonly found in alkali-soluble wall polymers (O'Brien & Ralph, 1966), there have been no previous investigations of alkali-insoluble xylomannans from fungal cell walls. A small amount of chitin was detected in *T. mesenterica* walls and characterized by X-ray diffraction. Most of the glucosamine in the walls was solubilized by hot hydrochloric acid, but not by acetic acid. Other workers have found that hot dilute hydrochloric acid solubilized a significant fraction of the glucosamine in the walls of *Schizophyllum commune* (Wessels & de Vries, 1973) and *Rhodotorula* (Moulki & Bonaly, 1974). They assumed that chitin would be immune to such treatment, and that the glucosamine must have been released from some other polymer. We feel, however, that the hot-acid treatment could degrade some chitin to soluble fragments, particularly if the chitin

is only poorly crystalline. The yeast cell walls of *T. mesenterica* differ from those of other basidiomycetous yeasts, such as *Rhodotorula* and *Sporobolomyces*, in that the latter contain no xylose, have more mannose than glucose, and exhibit a higher chitin content (Crook & Johnston, 1962; Moulki & Bonaly, 1974).

Because of the similarity in their extracellular polysaccharides, nutritional requirements and morphology, Slodki *et al.* (1966) suggested that *Tremella* and *Cryptococcus* might be related; the presence of α -glucan and possibly xylomannan in the *Tremella* walls is another point of similarity between these two genera. α -Glucan was found in *Cryptococcus* by Bacon *et al.* (1968) and xylomannan was found in the wall of *Cryptococcus laurentii* by Ankel *et al.* (1970).

Walls prepared from yeast cells of *T. mesenterica* RJB2259-6 have previously been analysed by Cameron & Taylor (1976). Their results are in qualitative agreement with ours, but there are quantitative differences. We found less xylose and mannose, more glucose, less protein and more amino sugar than they report. The cells they used were grown in a complex, undefined medium, which may account for the differences.

The initial purpose of this study was to compare the walls of the yeast and conjugation-tube forms of *T. mesenterica*. The two wall types have very similar gross compositions – we did not find any component in conjugation-tube walls that was absent from the yeast walls. The conjugation-tube walls did contain less α -glucan than the yeast walls, but had more β -glucan and more chitin.

Kanetsuna and co-workers found more α -glucan and less β -glucan in the yeast forms than in the mycelial forms of the thermally dimorphic fungi *Paracoccidioides brasiliensis* (Kanetsuna *et al.*, 1972) and *Histoplasma capsulatum* (Kanetsuna *et al.*, 1974). They speculated that the change in the major wall polysaccharide was a cause of the change in morphology. At present we cannot ascertain whether observed differences in cell-wall composition between the two growth forms of *Tremella* were essential morphological determinants or incidental responses to the change in environment. The conjugation hormone of *T. mesenterica* does not appear to act by inducing major changes in the polysaccharides of the cell walls. Perhaps its main action is to control, in some unknown way, the distribution of wall synthesizing enzymes over the cell surface.

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REFERENCES

- ANGYAL, S. J., BENDER, V. J. & RALPH, B. J. (1974). Structure of polysaccharides from the *Polyporus tumulosus* cell wall. *Biochimica et biophysica acta* **362**, 175–187.
- ANKEL, H., ANKEL, E., SCHUTZBACH, J. S. & GARANCIS, J. C. (1970). Mannosyl transfer in *Cryptococcus laurentii*. *Journal of Biological Chemistry* **245**, 3945–3955.
- BACON, J. S. D., JONES, D., FARMER, V. C. & WEBLEY, D. M. (1968). The occurrence of a α -(1 $\rightarrow$ 3)-glucan in *Cryptococcus*, *Schizosaccharomyces* and *Polyporus* species, and its hydrolysis by a *Streptomyces* culture filtrate lysing cell walls of *Cryptococcus*. *Biochimica et biophysica acta* **158**, 313–315.
- BANDONI, R. J. (1965). Secondary control of conjugation in *Tremella mesenterica*. *Canadian Journal of Botany* **43**, 627–630.
- BARTNICKI-GARCIA, S. & LINDBERG, B. (1972). Partial characterization of mucoran: the glucuronomannan component. *Carbohydrate Research* **23**, 75–85.

- BARTNICKI-GARCIA, S. & LIPPMAN, E. (1969). Fungal morphogenesis. Cell wall construction in *Mucor rouxii*. *Science, New York* **165**, 302-304.
- BARTNICKI-GARCIA, S. & NICKERSON, W. J. (1962). Isolation, composition and structure of cell walls of filamentous and yeast-like forms of *Mucor rouxii*. *Biochimica et biophysica acta* **58**, 102-119.
- BITTER, T. & MUIR, H. M. (1962). A modified uronic acid carbazole reaction. *Analytical Biochemistry* **4**, 330-334.
- BLAKE, J. D. & RICHARDS, G. N. (1970). A critical re-examination of problems inherent in compositional analysis of hemicelluloses by gas-liquid chromatography. *Carbohydrate Research* **14**, 375-387.
- BOUVENG, H. O., FRASER, R. N. & LINDBERG, B. (1967). Polysaccharides elaborated by *Armillaria mellea* (Tricholomataceae). II. Water-soluble mycelium polysaccharides. *Carbohydrate Research* **4**, 20-31.
- CAMERON, D. S. & TAYLOR, I. E. P. (1976). Quantitative microanalysis of cell walls of *Saprolegnia diclina* Humphrey and *Tremella mesenterica* Fries. *Biochimica et biophysica acta* (in the Press).
- CROOK, E. M. & JOHNSTON, I. R. (1962). The qualitative analysis of the cell walls of selected species of fungi. *Biochemical Journal* **83**, 325-331.
- DUBOIS, M., GILLES, K. A., HAMILTON, J. K., REBERS, P. A. & SMITH, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry* **28**, 350-356.
- FRASER, C. G. & JENNINGS, H. J. (1971). A glucan from *Tremella mesenterica* NRRL-Y6158. *Canadian Journal of Chemistry* **49**, 1804-1807.
- FRASER, C. G., JENNINGS, H. J. & MOYNA, P. (1973). Structural analysis of an acidic polysaccharide from *Tremella mesenterica* NRRL-Y6158. *Canadian Journal of Biochemistry* **51**, 219-224.
- GATT, R. & BERMAN, E. R. (1966). A rapid procedure for the estimation of amino sugars on a micro scale. *Analytical Biochemistry* **15**, 167-171.
- HOUWINK, A. L. & KREGER, D. R. (1953). Observations on the cell wall of yeasts. An electron microscope and X-ray diffraction study. *Antonie van Leeuwenhoek* **19**, 1-24.
- HUOTARI, F. I., NELSON, T. E., SMITH, F. & KIRKWOOD, S. (1968). Purification of an *exo*- β -D-(1 $\rightarrow$ 3)-glucanase from *Basidiomycete* species QM806. *Journal of Biological Chemistry* **243**, 952-956.
- JONES, J. K. N. & STOODLEY, R. J. (1965). Fractionation using copper complexes. In *Methods in Carbohydrate Chemistry*, vol. 5, p 36. Edited by R. L. Whistler. New York: Academic Press.
- KANETSUNA, F., CARBONELL, L. M., AZUMA, I. & YAMAMURA, Y. (1972). Biochemical studies on the thermal dimorphism of *Paracoccidioides brasiliensis*. *Journal of Bacteriology* **110**, 208-218.
- KANETSUNA, F., CARBONELL, L. M., GIL, F. & AZUMA, I. (1974). Chemical and ultrastructural studies on the cell walls of the yeast-like and mycelial forms of *Histoplasma capsulatum*. *Mycopathologia et mycologia applicata* **54**, 1-13.
- LIPPMAN, E., ERWIN, D. C. & BARTNICKI-GARCIA, S. (1974). Isolation and chemical composition of oospore-oogonium walls of *Phytophthora megasperma* var. *sojae*. *Journal of General Microbiology* **80**, 131-141.
- MICHELL, A. J. & SCURFIELD, G. (1970). An assessment of infrared spectra as indicators of fungal cell wall composition. *Australian Journal of Biological Sciences* **23**, 345-360.
- MOULKI, H. & BONALY, R. (1974). Etude des parois de levures du genre *Rhodotorula*. IV. Variation de la teneur en chitine. *Biochimica et biophysica acta* **338**, 120-134.
- O'BRIEN, R. W. & RALPH, B. J. (1966). The cell wall composition and taxonomy of some Basidiomycetes and Ascomycetes. *Annals of Botany, N.S.* **30**, 831-843.
- REID, I. D. (1974). Properties of conjugation hormones (erogens) from the basidiomycete *Tremella mesenterica*. *Canadian Journal of Botany* **52**, 521-524.
- REID, I. D. (1975). Growth of *Tremella mesenterica* haplonts with various nitrogen sources. *Canadian Journal of Botany* **53**, 73-77.
- SLODKI, M. E., WICKERHAM, L. J. & BANDONI, R. J. (1966). Extracellular heteropolysaccharides from *Cryptococcus* and *Tremella*: a possible taxonomic relationship. *Canadian Journal of Microbiology* **12**, 489-494.
- SPACKMAN, D. H., STEIN, W. H. & MOORE, S. (1958). Automatic recording apparatus for use in the chromatography of amino acids. *Analytical Chemistry* **30**, 1190-1206.
- TRACEY, M. V. (1955). Chitin. In *Modern Methods of Plant Analysis*, vol. 2, pp. 269-280. Edited by K. Paech and M. V. Tracey. Berlin: Springer-Verlag.
- WANG, M. C. & BARTNICKI-GARCIA, S. (1970). Structure and composition of walls of the yeast form of *Verticillium albo-atrum*. *Journal of General Microbiology* **64**, 41-54.
- WESSELS, J. G. H. & DE VRIES, O. M. H. (1973). Wall structure, wall degradation, protoplast liberation and wall regeneration in *Schizophyllum commune*. In *Yeast, Mould, and Plant Protoplasts*, pp. 295-306. Edited by J. R. Villanueva, I. Garcia-Acha, S. Gascon and F. Uruburu. London and New York: Academic Press.
- WESSELS, J. G. H., KREGER, D. R., MARCHANT, R., REGENSBURG, B. A. & DE VRIES, O. M. H. (1972). Chemical and morphological characterization of the hyphal wall surface of the basidiomycete *Schizophyllum commune*. *Biochimica et biophysica acta* **273**, 346-358.