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Identification of Two *Saccharomyces cerevisiae* Cell Wall Mannan Chemotypes

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We have obtained evidence for two structurally and antigenically different *Saccharomyces cerevisiae* cell wall mannans. One, which occurs widely and is found in *S. cerevisiae* strain 238C, is already known to be a neutral mannan which yields mannose, mannobiose, mannotriose, and mannotetraose on acetolysis of the (1 → 6)-linked backbone. The other, which was found in *S. cerevisiae* brewer's strains, is a phosphomannan with a structure very similar to that of *Kloeckera brevis* mannan. *S. cerevisiae* (brewer's yeast strain) was agglutinated by antiserum prepared against *Kloeckera brevis* cells. The mannan, isolated from a proteolytic digest of the cell wall of the former, did not react with *S. cerevisiae* 238C antiserum, whereas it cross-reacted strongly with *K. brevis* antiserum. Controlled acetolysis cleaved the (1 → 6)-linkages in the polysaccharide backbone and released mannose, mannobiose, mannotriose, and mannotriose phosphate. Mild acid treatment of the phosphomannan hydrolyzed the phosphodiester linkage, yielding phosphomonoester mannan and mannose. The resulting phosphomonoester mannan reacted with antiserum prepared against *K. brevis* possessing monoester phosphate groups on the cell surface. α-D-Mannose-1-phosphate completely inhibited the precipitin reaction between brewer's yeast mannan and the homologous antiserum. Flocculent and nonflocculent strains of this yeast were shown to have similar structural and immunological properties.

Mannan is a major constituent of the yeast cell wall. This polysaccharide, composed mainly of D-mannose, is normally associated with varying amounts of protein and phosphate, and may contain small amounts of *N*-acetyl-D-glucosamine as well as other sugars (7, 12, 14).

Many of the mannans possess a backbone of α(1 → 6)-linked D-mannose units which are substituted by side chains containing (1 → 2)- and (1 → 3)-linked D-mannose units (15). Since the structures of these side chains vary between species, the different yeast mannans can be recognized from the pattern of fragments obtained by a selective degradation reaction which cleaves the backbone linkages. This has been called an "acetolysis fingerprint" (8). The phosphate in *Saccharomyces cerevisiae* and in *Kloeckera brevis* mannans is present as α-D-mannosyl-1-phosphate units which are attached in diester linkage to position 6 of D-mannose units in the side chains (3, 4, 17).

The immunochemistry of yeasts is closely related to the fine structure of the mannan, since this polysaccharide is the main antigenic determinant on the yeast cell surface. For the *S. cerevisiae* strains that have been investigated, the tetrasaccharide side chain, which contains a terminal α(1 → 3)-linked mannose unit and two internal α(1 → 2)-linked units, is the most effective inhibitor of the homologous precipitin reaction (1, 16). Since the α(1 → 3)-mannobiose was a better inhibitor than the α(1 → 2)-mannobiose (16), the immunodominant group appears to be the terminal α(1 → 3)-linked unit of the tetrasaccharide. *K. brevis* mannan lacks this tetrasaccharide side chain and contains mainly α(1 → 2)-linked mannobiose and mannotriose side chains (15). Recently, however, it was shown that some of the mannotriose side chains of this mannan were substituted with α-D-mannosyl-1-phosphate units (17), and that this was the immunodominant group in the *K. brevis* mannan. Thus, the chemotypes which have been derived for the neutral *S. cerevisiae* mannan and the *K. brevis* phosphomannan can be represented as illustrated in Fig. 1.

We have now found that some strains of *S. cerevisiae* are agglutinated by anti-*K. brevis* serum, and that the mannan isolated from these yeasts cross-reacts strongly with this antiserum. We have investigated the structures of the mannans from these *S. cerevisiae* strains and find that they are very similar to that of *K. brevis* and possess a structure like that in Fig. 1B. Thus, one can distinguish two *S. cerevisiae* mannan chemotypes, one from the strains which contain mannan that is low in phosphate and which is characterized by the presence of the tetrasaccharide side chain, and the other in *S. cerevisiae* strains which contain phosphate in the cell wall mannan and which is characterized by the α-D-mannosyl-1-phosphoryl-mannotriose side chain in place of the tetrasaccharide side chain.

During this study, we have also investigated the relation of the phosphomannan structure of strains of *S. cerevisiae* brewer's yeast to their flocculation property. Although the mechanism of flocculation is not understood, some evidence suggests that the cells are held together by salt bridges between the exposed phosphate groups on the cell surface, an interaction mediated by divalent cations (10). Since the main source of exposed phosphate groups is the phosphomannan, this hypothesis would suggest that flocculating strains should possess a greater concentration of the mannosyl-phosphate groups on the cell surface than nonflocculating strains. As a test of this hypothesis, we have compared the structure and the agglutinating ability with anti-*K. brevis* serum of flocculating and nonflocculating strains. All of the strains possessed mannan of the chemotype 1b, and they showed no significant differences in agglutinability with the antiserum specific for the α-D-mannosyl-1-phosphate group. This suggests that flocculence is not dependent solely on the surface location of these groups.

MATERIALS AND METHODS

Cultures of the following organisms were obtained from the Guinness Research Laboratory, Dublin, Ireland; *S. cerevisiae* 1406 (nonflocculent), and *S. cerevisiae* 1164 and 1338 (flocculent). The flocculation characteristic of these yeasts have been described by Gilliland (6). These yeasts and *S. cerevisiae* 238C (a strain which we believe is identical with *S. cerevisiae* 288C and which was obtained indirectly from R. K. Mortimer of the Univ. of Calif. several years ago for the study mentioned in reference 15) were grown as described by Stewart and Ballou (15). *S. cerevisiae* (baker's yeast) was obtained from the Red Star Yeast Company, Oakland, Calif. Yeast cell wall

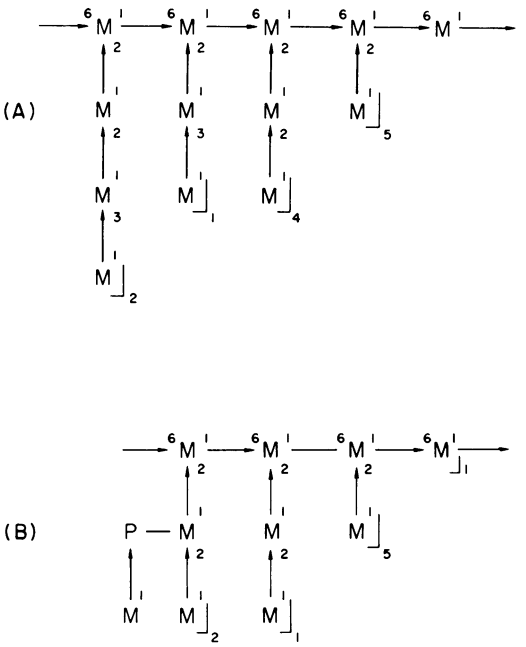

FIG. 1. *Structures which illustrate the chemotypes of neutral S. cerevisiae mannan (A) and of K. brevis phosphomannan (B). The S. cerevisiae brewer's strains studied here have chemotype 1B. M = mannose; P = phosphate.*

samples were supplied by R. Letters, Guinness Research Laboratory, Dublin, Ireland. *K. brevis* phosphomannan and mannotriose phosphate were obtained from T. R. Thieme, and α-D-mannose 1-phosphate and *K. brevis* antiserum from W. C. Raschke of this laboratory. Alkaline phosphatase (BAPC) was purchased from Worthington Biochemical Corp. and Bio-Gel P-2 (200 to 400 mesh) from Bio-Rad Corp.

Chemical procedures. Total carbohydrate was measured by the phenol-sulfuric acid method (5). Column effluents were monitored by the Refracto-Monitor model 1103, Laboratory Data Control. Total and inorganic phosphate were determined by the Bartlett procedure (2), whereas protein was measured by the Lowry method (9). Descending paper chromatography was carried out on Whatman no. 1 paper, using the solvent system ethyl acetate-pyridine-water (5:3:2, v/v). Sugars were detected on paper with a silver nitrate-sodium hydroxide reagent.

Phosphodiester mannans were isolated from whole cells (1) or from cell walls (5). Phosphomonoester mannans were prepared by heating the free acid form, obtained by Dowex 50 (H^+) treatment, at 100 C for 20 min, followed by dialysis for 48 hr against distilled water.

Acetylation and acetolysis of mannans were performed according to Kocourek and Ballou (8). In this procedure, the phosphorylated oligosaccharides are removed in the water wash of the benzene extract.

The neutral fragments were dried by successive evaporation of the benzene solvent. The dried product was dissolved in 2 ml of dry methanol and deacylated by adding 1 ml of 1 N sodium methoxide. After 20 min at room temperature, the solution was neutralized with Bio-Rad cation exchange resin AG 50W-X12. The resin was filtered off, and the supernatant fluid was concentrated to 2 ml and applied directly to a Bio-Gel P-2 column. For isolation of the phosphorylated oligosaccharides, the procedure of Thieme and Ballou (17) was followed. Methylation and gas chromatography techniques have been described (15).

Immunological methods. Rabbit immunization with heat-killed yeast cells and the collection and storing of antisera were done as previously described (1). The serum from each rabbit was checked for antibody content with the respective phosphodiester mannan, using the agglutination assay. Since a given strain of yeast gave a similar response in each rabbit, the sera from pairs of rabbits injected with the same strain were combined. The precipitin reactions were performed as outlined by Raschke and Ballou (13). Inhibition of the precipitin reactions involved the incubation of serum with inhibitor at 37 C for 1 hr prior to the addition of antigen. The precipitate, formed on incubating anti-*K. brevis* serum with α-D-mannose 1-phosphate (13), was removed by centrifugation and washed with 0.5 ml of saline. The supernatant fluid and washing were combined and, after the addition of antigen, the total volume was adjusted to 1 ml. Preabsorption of serum with *K. brevis* phosphodiester mannan was performed by removing the initial antiserum-*K. brevis* phosphomannan precipitate by centrifugation and by washing it with 0.5 ml of saline. Antigen was added to the combined supernatant fluid and washing, bringing the total volume to 1.0 ml. Agglutination assays were performed by serial dilution on a plastic tray, using a Takatsy microtitrator (Cooke Engineering Company, Alexandria, Va.). Yeast suspensions were used which, when diluted 50-fold, gave an absorbance of 0.5 at 640 nm.

RESULTS

Composition and acetolysis of mannans. Pronase digestion of yeast cell wall solubilizes phosphomannan, which can be isolated and purified by anion exchange chromatography (3). Analysis of the products obtained from strains 1406, 1164, and 1338 by this procedure indicate that the overall compositions were similar. Acetolysis of 1406 phosphomannan yielded three neutral products (Fig. 2A). These were identified as mannose, mannobiose, and mannotriose. Mannans from strains 1164 and 1338 gave similar patterns with some variation in the relative amounts of the products.

In agreement with the structure shown in Fig. 1, *S. cerevisiae* strains previously studied (15) gave a mannotetraose in addition to mannotriose, mannobiose, and mannose (Fig. 2B). Since this pattern had been considered to be characteristic of *S. cerevisiae* strains (8), it seemed unusual that the brewer's yeast gave a different result. One explanation considered to account for this was that the mannans used in this study were isolated from a pronase digest of cell wall (3) which could have enriched a particular mannan fraction, whereas the previous work (15) was performed on mannan obtained by citrate extraction of whole yeast cells. However, this explanation proved invalid since mannan isolated from *S. cerevisiae* 1406 whole cells by citrate extraction, followed by precipitation with Fehling's solution (8), again yielded only mannotriose, mannobiose, and mannose on acetolysis. This phosphomannan had a mannose to phosphate ratio of 19. The acetolysis pattern shown in Fig. 2A is similar to that described for *K. brevis* (15) and, in agreement with this conclusion, methylation of the di- and trisaccharides showed that the sugars were (1 → 2)-linked (Table 1). No fragment with a (1 → 3)-linkage was detected. The

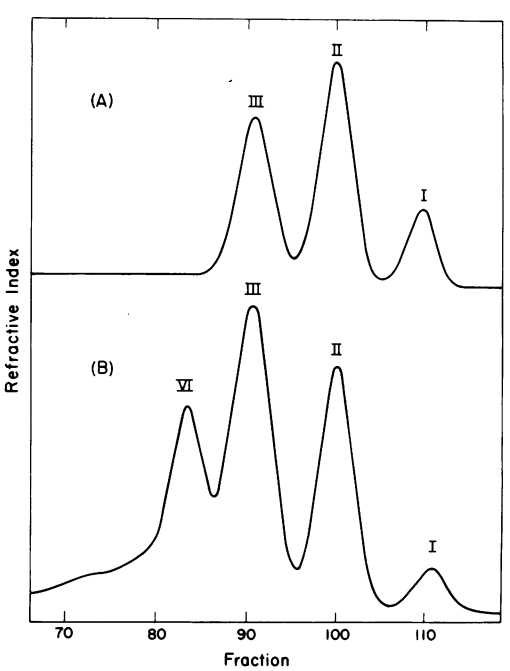

FIG. 2. *"Acetolysis fingerprints" of S. cerevisiae brewer's yeast phosphomannan (A) and of S. cerevisiae 238C neutral mannan (B). Peaks I, II, III, and IV correspond to mannose, mannobiose, mannotriose, and mannotetraose. The oligosaccharides, obtained by deacetylation of the acetolysis product, were separated on a 2 by 200 cm column of Bio-Gel P-2 by elution with water, and the solute peaks were detected by a flow-through refractive index monitor.*

TABLE 1. *Methylation analysis of mannan oligosaccharides*

Oligosaccharide[a]	Partially methylated mannose (moles/mole of oligosaccharide)		
	2,3,4,6-tetra-	3,4,6-tri-	2,4,6-tri-
S. cerevisiae 238C			
$(Man)_2$	1.0	1.1	0
$(Man)_3$	1.0	1.9	0.2
$(Man)_4$	1.0	2.2	1.1
S. cerevisiae 1406			
$(Man)_2$	1.00	1.01	0
$(Man)_3$	1.00	1.98	0
$(Man)_3$ from $(Man)_3P$	1.00	2.10	0
S. cerevisiae 1164			
$(Man)_2$	1.00	0.95	0
$(Man)_3$	1.00	2.10	0
$(Man)_3$ from $(Man)_3P$	1.00	2.05	0
S. cerevisiae 1338			
$(Man)_2$	1.00	1.05	0
$(Man)_3$	1.00	2.15	0

[a] Man = mannose; P = phosphate.

$\alpha(1 \rightarrow 3)$-linked mannose unit at the nonreducing end of the tetrasaccharide and some of the trisaccharide side chains from *S. cerevisiae* 238C have been shown to provide the basis of the precipitin reaction of this mannan with homologous antibody (1, 16). As expected, mannan from strains 1406 and 1164 did not cross-react with the antiserum (Fig. 3), confirming the absence of a common determinant in these mannans.

Phosphodiester linkage. The phosphate groups in *S. cerevisiae* 1406 phosphodiester mannan are present as α-D-mannosyl-1-phosphate groups, linked to position 6 of another mannose residue (3). The mannosyl-1-phosphate bond can be cleaved by heating the free acid form of the phosphomannan at 100 C for 20 min. Treatment of 1164 and 1338 mannans in this fashion released 1 mole of mannose per mole of phosphate in each mannan. Free mannose was separated from the larger mannan residue on a Bio-Gel P-2 column as shown in Fig. 4. Since α-D-mannose 1-phosphate is the immunodominant structure in the reaction of *K. brevis* phosphomannan with homologous antiserum, the presence of such a structure in other mannans should result in cross-reaction with this serum. This proved to be the case (Fig. 5).

The phosphorylated oligosaccharides, obtained from a 13-hr acetolysate of strain 1164 and 1406 phosphomannans, were isolated by gel filtration as described by Thieme and Ballou (17). Digestion with alkaline phospha-

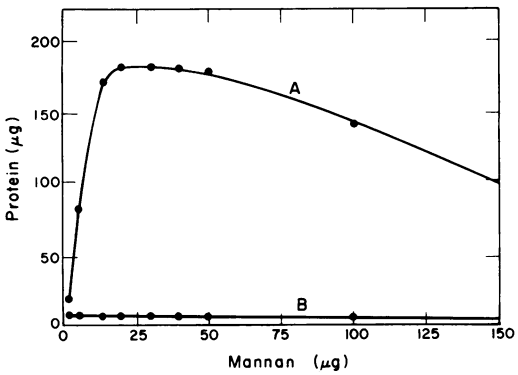

FIG. 3. *Precipitin curves for anti-S. cerevisiae 238C serum with S. cerevisiae 238C mannan (A), and with brewer's yeast mannans 1164 and 1406 (B). Each tube contained 0.1 ml of serum.*

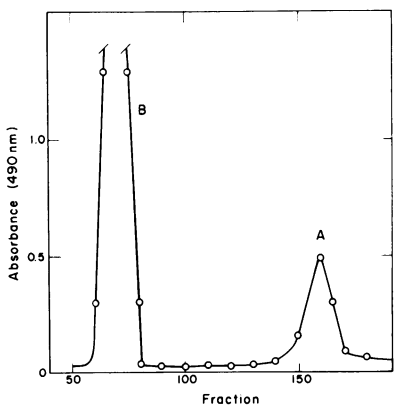

FIG. 4. *Gel filtration on a Bio-Gel P-2 column (2 by 200 cm) of 1406 phosphomannan after heating the free acid form at 100 C for 20 min. Peak A, mannose; peak B, phosphomonoester mannan.*

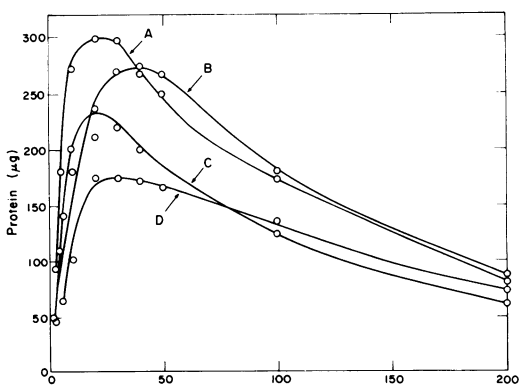

FIG. 5. *Precipitin curves for anti-K. brevis serum with K. brevis mannan (A), 1406 mannan (B), 1338 mannan (C), and 1164 mannan (D).*

tase (15) released all of the phosphate as inorganic phosphate, and the neutral oligosaccharides were separated on Bio-Gel P-2 as described in Fig. 6A. The products, identified by paper chromatography as mannotriose and mannobiose, were shown to contain only (1 → 2)-linkages on methylation analysis (Table 1). In the case of *K. brevis* phosphomannan, short-term acetolysis (6 hr) yielded only mannotriose phosphate, indicating that the mannobiose phosphate was a degradation product of the former (17). Acetolysis of strain 1406 phosphomannan for 6 hr, followed by alkaline phosphatase digestion, yielded mainly mannotriose and only a small amount of mannobiose (Fig. 6B), which again demonstrates that most of the phosphate was esterified to trisaccharide units in the native phosphomannan. This was confirmed by the strong cross-reaction shown by each phosphomonoester mannan with antiserum prepared against *K. brevis* cells having exposed monoester groups (13). The amount of antibody protein precipitated by 1406 phosphomonoester mannan was almost as large as that obtained with *K. brevis* phosphomonoester mannan. As anticipated, strain 1406 phosphodiester mannan reacted poorly with this antiserum.

Immunological properties. Strain 1406 phosphodiester mannan and homologous antiserum gave the strongest precipitin reaction. The nature of the antigenic determinants in each case was investigated by inhibition of the precipitin reaction with mannan fragments. Both the neutral mannobiose and mannotriose side chains were poor inhibitors. Each antiserum gave a precipitate when incubated with α-D-mannose 1-phosphate, a phenomenon already observed in the analogous *K. brevis* system (13). After removal of this precipitate and addition of mannan antigen, α-D-mannose 1-phosphate gave 100% inhibition. Confirmation that all of the antibody was directed against the α-D-mannose 1-phosphate structure was obtained by preabsorbing anti-1406 and anti-1338 with *K. brevis* phosphodiester mannan.

The amount of antibody precipitated by *K. brevis* phosphodiester mannan was much greater in the reaction with 1406 antiserum than that with 1338 antiserum. Addition of 1406 and 1338 phosphodiester mannan to the supernatant fluid from these reactions did not precipitate additional antibody (Fig. 7).

Agglutination tests. *S. cerevisiae* strains 1164, 1338, and 1406 were readily agglutinated in the presence of anti-*K. brevis* serum or with any of the antisera directed against these yeasts, whereas *S. cerevisiae* 238C and *S. cere-*

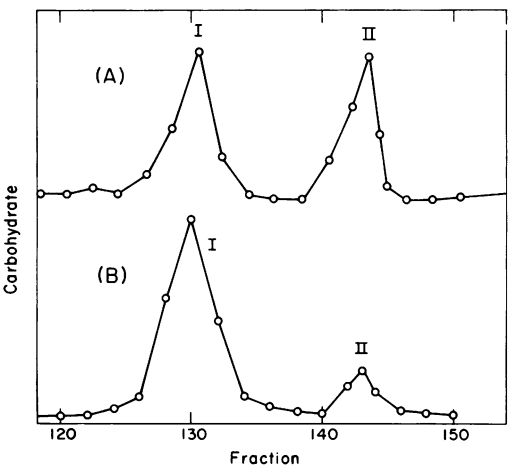

FIG. 6. *Gel filtration on a Bio-Gel P-2 column (2 by 200 cm) of the oligosaccharides obtained by alkaline phosphatase digestion of the phosphorylated products obtained from a 13-hr (A) and 6-hr (B) acetolysate of phosphomannan 1406. Peak I, mannotriose; peak II, mannobiose. Similar results were found with 1338 and 1164 phosphomannans.*

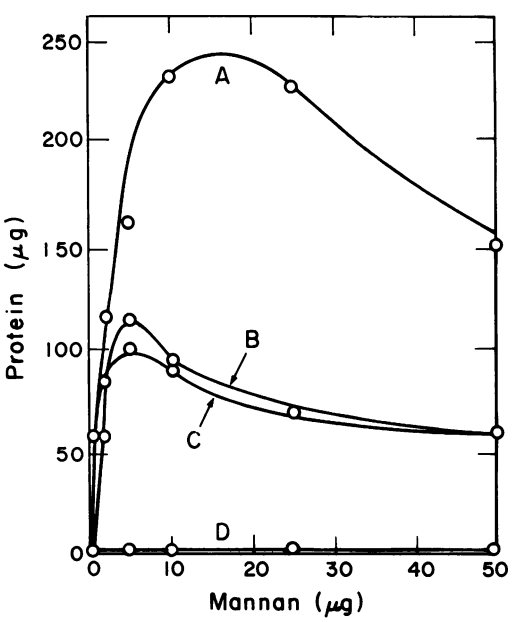

FIG. 7. *Precipitin curves showing the cross-reaction of K. brevis phosphodiester mannan with antiserum against S. cerevisiae strains 1406 (A), 1164 (B), and 1338 (C). Each tube contained 0.1 ml of serum. Curve D shows the reaction of the supernatants from curves A and B on addition of the homologous phosphodiester mannan.*

visiae baker's yeast were not. Conversely, anti-*S. cerevisiae* serum failed to agglutinate any of the brewer's yeast strains.

DISCUSSION

In this study, we have obtained evidence that *S. cerevisiae* (brewer's yeast) differs in structure and immunological properties from the *S. cerevisiae* strains previously described. The brewer's yeast phosphomannan structure is similar to that found in *K. brevis*, although it may not be as highly phosphorylated. It was surprising to find that these yeasts lacked the tetrasaccharide side chain previously observed in *S. cerevisiae* mannans. This may be the result of an inability to synthesize the α-D-mannosyl-(1 → 3) linkage. Concomitant with this change was the presence of much more phosphate than is found in the mannan from *S. cerevisiae* 238C or baker's yeast, and it was shown that this is present as α-D-mannosyl-1-phosphate units in diester linkage to trisaccharide side chains.

We have not determined whether these changes are related. McMurrough and Rose (11) have shown that the phosphate content of the cell wall increases under conditions of glucose and NH_4^+ limitations, and it may be possible to obtain a more highly phosphorylated mannan from *S. cerevisiae* 238C by modifying the growth conditions. The failure of whole cells of yeast with one mannan type to be agglutinated by the antiserum prepared against yeasts of the other mannan type is strong evidence that we have not lost any antigenic specificity during the isolation of the pure mannans. The simple agglutination test described in this paper should prove useful in identifying other strains with similar structures on the cell surface.

Flocculent and nonflocculent yeasts showed no significant difference in structure or in their cross-reaction with anti-*K. brevis* serum. The α-D-mannosyl-1-phosphate unit was the main antigenic determinant in all three yeasts. Some small, but possibly significant, differences were observed in the precipitin reactions of the mannans. Mannan from the nonflocculent yeast invariably precipitated more antibody than that from the flocculent strains. This was in keeping with the slightly higher phosphate content of the former. Since all three yeasts showed similar agglutination properties with anti-*K. brevis* serum, there does not appear to be a clear-cut difference in the mannan structure or in the distribution of phosphomannan between flocculent and nonflocculent yeast cells which can explain cell agglutination on the basis of salt bridges alone.

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