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Structure and Immunochemistry of the Cell Wall Mannans from *Saccharomyces chevalieri*, *Saccharomyces italicus*, *Saccharomyces diastaticus*, and *Saccharomyces carlsbergensis*

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The mannans of *Saccharomyces chevalieri*, *S. italicus*, *S. diastaticus*, and *S. carlsbergensis*, were acetolyzed, and the fragments were separated by gel filtration. All gave similar acetolysis fingerprints, which were distinguished from *S. cerevisiae* by the presence of a pentasaccharide component in addition to the mono-, di-, tri-, and tetrasaccharides. All oligosaccharide fragments were composed of mannose in α-linkage. From methylation analysis and other structural studies, the disaccharide was shown to be αMan(1 → 2)Man; the trisaccharide was shown to be a mixture of αMan(1 → 2)αMan (1 → 2)Man and αMan(1 → 3)αMan(1 → 2)Man; the tetrasaccharide was αMan(1 → 3)αMan(1 → 2)αMan(1 → 2)Man; and the pentasaccharide was αMan(1 → 3)αMan(1 → 3)αMan(1 → 2)αMan(1 → 2)Man. The ratios of the different fragments varied slightly from strain to strain. Mannanase digestion of two of the mannans yielded polysaccharide residues that were unbranched (1 → 6)-linked polymers, thus establishing the structural relationship between these mannans and that from *S. cerevisiae*. Antisera raised against the various yeasts cross-reacted with the mannans from each, and also with *S. cerevisae* mannan. The mannotetraose and mannopentaose acetolysis fragments gave complete inhibition of the precipitin reactions, which indicated that, in these systems as in the *S. cerevisiae* system, the terminal α(1 → 3)-linked mannose unit was the principal immunochemical determinant on the cell surface.

Yeasts have been shown to possess species-specific surface antigens which, in many cases, are based mainly on the cell wall mannan (8). The polymorphism of this somatic antigen is related to defined structural differences in the side chains of the polysaccharide component of the mannan-protein complex which coats the cell (2). In the initial survey of yeast strains for different mannan chemotypes, the acetolysis fingerprinting technique was employed (10), a procedure which reveals differences between mannan structures in the spectrum of oligosaccharides that is produced in the degradative reaction. Thus, *Saccharomyces cerevisiae* S288C, which is related to bakers' yeast, gives a characteristic four-peak pattern of mannose, mannobiose, mannotriose, and mannotetraose (11). *S. chevalieri*, *S. italicus*, *S. diastaticus*, and *S. carlsbergensis* give these same four fragments, but there is also an appreciable amount of a substance with the properties of a pentasaccharide. This report deals with the characterization of the acetolysis fragments of these latter four yeasts, in particular the pentasaccharide unit, and also describes some features of their immunochemistry. As expected from the close taxonomic relatedness of these yeasts, their mannans show close similarities in structure and properties.

MATERIALS AND METHODS

The yeast strains *S. chevalieri* Y32, *S. italicus* Y66, and *S. carlsbergensis* Y6 were obtained from Harlyn Halvorson and had those designations. The *S. chevalieri* strain sporulated readily and was homothallic, whereas the *S. italicus* strain did not sporulate. The *S. chevalieri* cells, when sporulated and mixed with *S. cerevisiae* cells, formed hybrid diploids as expected (14). *S. diastaticus* 2044 was obtained from Hermann Phaff.

The techniques for growth of yeast, isolation of mannan by citrate buffer extraction and Fehling's precipitation, immunochemical analysis, acetolysis, methylation analysis, and characterization by mass spectrometry followed published rocedures (12).

Smith degradation of S. talicus pentasaccharide. About 17 mg of the pentasaccharide was reduced with $NaBH_4$-$NaBT_4$, and the isolated tritium-labeled product was added to 5 ml of 0.1 M

$NaIO_4$ in 0.1 M acetate buffer pH 4.0. After 2 days at 25 C, 2 drops of ethylene glycol was added to destroy the excess oxidant, and the entire solution was applied to a 100-cm Sephadex G-10 column which was eluted with water to separate the oxidized oligosaccharide from the salts. The major radioactive peak eluted from the column was collected and reduced with $NaBH_4$, the solution was treated with Dowex 50(H^+), and the reduced oligosaccharide was again purified by passage through the Sephadex G-10 column. The resulting solution of pentasaccharide that had been reduced, oxidized, and reduced was made 0.25 N in HCl and kept at 25 C for 24 h to allow for hydrolysis of the acylic acetals. The product, in addition to standards and mannobiose, was then chromatographed on Whatman no. 1 paper in the solvent ethyl acetate-pyridine-water (5:3:2 by volume). The sugars on the chromatogram were located by development with a NaOH-$AgNO_3$ dip reagent. The reaction product showed major components with R_f values the same as glycerol and mannobiose, whereas only traces of mannose and of other degradation products were visible on the chromatogram. Some streaking was observed owing to the formation of condensation products with the glycolaldehyde (7) that was released on acid hydrolysis of the oxidized and reduced oligosaccharide.

RESULTS

Chemical characterization of the acetolysis fragments. The acetolysis fingerprints of *S. chevalieri* and *S. italicus* mannans are shown in Fig. 1. A distinguishing feature of both mannans was the presence of the pentasaccharide unit; *S. chevalieri* mannan had an unusual amount of the disaccharide component. Bakers' yeast mannan gives a pattern much like that of *S. italicus*, but without the pentasaccharide unit; whereas *S. carlsbergensis* and *S. diastaticus* differed from *S. italicus* only in the relative amounts of the fragments (Table 1).

The linkages between the sugar units in the various fragments were determined by methylation (Table 2). In all cases, the disaccharide was exclusively (1 → 2)-linked. However, the trisaccharide component contained variable amounts of (1 → 2) and (1 → 3) linkages. In the *S. chevalieri* trisaccharide, these linkages occurred in equal amount, suggesting the presence of one isomer with the structure Man(1 → 3)Man(1 → 2)Man; in the other three yeasts the trisaccharide component was composed of a mixture of isomers, with approximately half having two (1 → 2) linkages and half having only one of each linkage. The tetrasaccharide gave methylation results indicating that it was similar to the mannotetraose found in *S. cerevisiae* and *Kluyveromyces lactis* mannans, namely Man(1 → 3)Man(1 → 2)Man(1 → 2)Man. The immunochemical cross-reactivity of the mannans from these *Saccharomyces* species with that from *S. cerevisiae* (see below) strongly supports the terminal placement of the (1 → 3) linkage in the tetrasaccharide unit. The pentasaccharide, in the two examples studied, had two (1 → 2) linkages and two (1 → 3) linkages.

The composition of the *S. italicus* trisaccharide was determined by methylation of the reduced sugar. The reactions are outlined in Fig. 2, which shows that the sugar at the reducing end of the oligosaccharide yields a pentamethyl ether, the sugar at the nonreducing end yields a tetramethyl ether, and the

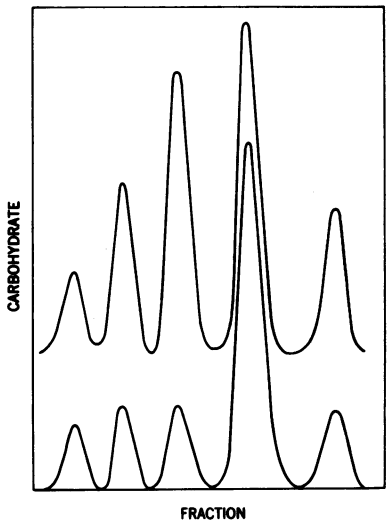

FIG. 1. *Acetolysis fingerprints of the mannans from S. italicus (top) and S. chevalieri (bottom), which show (from right to left) peaks for mannose to mannopentaose as separated on a column (2 by 200 cm) of Bio-Gel P-2 by elution with water. S. cerevisiae has a pattern very like S. italicus but without the pentasaccharide component. The acetolysis reaction was done under controlled conditions (11) such that only the (1 → 6) linkages of the backbone are cleaved and the (1 → 2) and (1 → 3) linkages of the side chains remain essentially intact.*

TABLE 1. *Molar ratios of acetolysis fragments of different yeast mannans*[a]

Yeast strain	Molar ratio of acetolysis fragments				
	Mannose	Biose	Triose	Tetraose	Pentaose
S. chevalieri	1.0	2.1	0.37	0.37	0.14
S. italicus	1.0	1.1	0.9	0.22	0.07
S. diastaticus	1.0	1.4	1.1	0.72	0.11
S. carlsbergensis	1.0	2.4	2.0	1.4	0.44
S. cerevisiae S288C	1.0	1.7	1.1	0.7	0

[a] All ratios were calculated relative to the mannose.

TABLE 2. *Methylation analysis of acetolysis fragments*[a]

Oligosaccharide	Molar ratios relative to tetramethyl ether			
	S. chevalieri	*S. italicus*	*S. diastaticus*	*S. carlsbergensis*
Disaccharide				
2,4,6-	0	0	0	0
3,4,6-	1.1	0.9	1	1
2,3,4,6-	1	1	1	1
Trisaccharide				
2,4,6-	1.1	0.5	0.4	0.7
3,4,6-	1.1	1.5	1.6	1.3
2,3,4,6-	1	1	1	1
Tetrasaccharide				
2,4,6-	1.1	0.9	1	1
3,4,6-	1.8	2.1	2.2	1.9
2,3,4,6-	1	1	1	1
Pentasaccharide				
2,4,6-	1.9	2	[b]	[b]
3,4,6-	2	2.1		
2,3,4,6-	1	1		

[a] The methylated oligosaccharides were methanolyzed, and the products were separated and quantitated by gas chromatography. The peaks corresponded to the methyl glycosides of 2,4,6-tri, 3,4,6-tri-, and 2,3,4,6-tetra-*O*-methylmannose.

[b] Not determined, although the substances were chromatographically and immunochemically identical to the pentasaccharides from *S. chevalieri* and *S. italicus*.

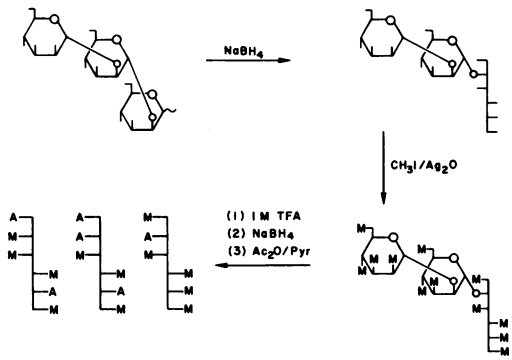

FIG. 2. *Steps for the methylation analysis of a (1 → 2)-linked mannotriose. The resulting substituted mannitol units were separated by gas chromatography and analyzed by mass spectrometry. M, Methyl; A, acetyl; TFA, trifluoroacetic acid.*

sugar in the middle yields a trimethyl ether. All of these fragments were converted to the alditol acetates and subjected to gas chromatography and mass spectrometry (12). As shown in Fig. 3, single peaks were obtained for the penta- and tetramethyl ethers, whereas the trimethylmannose component was a mixture. From the mass spectra, the pentamethylmannitol was identified as the 1,3,4,5,6-isomer, showing that the linkage to the reducing mannose unit in the trisaccharide was exclusively to position 2. The two trimethylmannitols were identified as the 2,4,6- and 3,4,6-isomers, in a ratio which indicated that the original trisaccharide was a mixture approximately of equal parts of Man(1 → 2)Man(1 → 2)Man and Man(1 → 3)Man(1 → 2)Man. This agrees with the results in Table 2, which suggest that *S. italicus*, *S. diastaticus*, and *S. carlsbergensis* have similar trisaccharide mixtures. On the other hand, the *S. chevalieri* trisaccharide appeared to be mainly of the latter type.

The pentasaccharide from *S. italicus* was reduced and subjected to methylation analysis, as described above for the trisaccharide, and the results were compared with those from methylation of the unreduced compound. For the reduced sample, the amount of the 3,4,6-trimethyl ether was decreased by one-half owing to conversion of the reducing end mannose to a pentamethyl ether. Thus, the reducing mannose unit in the original pentasaccharide must have been linked at position 2. The location of the two (1 → 3) linkages with respect to each other was determined by Smith

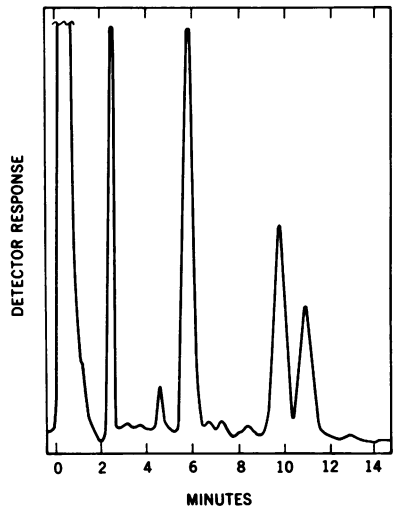

FIG. 3. *Gas chromatographic tracing of the methylation products from the S. italicus mannotriose. Following the solvent peak on the left, the major peaks correspond to 2-O-acetyl-1,3,4,5,6-penta-O-methylmannitol, 1,5-di-O-acetyl-2,3,4,6-tetra-O-methylmannitol, 1,2,5-tri-O-acetyl-3,4,6-tri-O-methylmannitol, and 1,3,5-tri-O-acetyl-2,4,6-tri-O-methylmannitol. A 5-ft (about 1.52 m) Carbowax 20M column was used at 180 C with nitrogen carrier gas.*

degradation which involved sequential periodate oxidation, borohydride reduction, and mild acid hydrolysis (7). The mannose units that are substituted at position 3 are protected from oxidation, whereas the glycosidic bond of those mannose units that do become oxidized is rendered highly susceptible to acid hydrolysis. If the two (1 → 3) linkages occur together, the degradation will yield 1 mol of intact (1 → 3)-mannobiose (Fig. 4); if they are separated by a (1 → 2) linkage, 2 mol of free mannose will be produced. As shown in the chromatogram reproduced in Fig. 5, the degradation yielded a substance with the properties of a disaccharide and gave only a trace of free mannose. Thus, the two (1 → 3) linkages in the pentasaccharide must be contiguous. Moreover, the immunochemical evidence below suggests that one of them must be at the nonreducing end, so we can assign the structure Man(1 → 3)Man(1 → 3)Man(1 → 2)Man(1 → 2)Man. The results do not rule out the presence of a small amount of other isomers because a trace of mannose was formed in the degradation reaction. Both the mannopentaose with a single terminal (1 → 3) linkage and the pentasaccharide we find here have been reported in other yeasts (6).

Immunochemical analysis. Antisera prepared against all four yeasts cross-reacted strongly with the isolated mannans from each other and with those from *S. cerevisiae* and *K. lactis*. This cross-reactivity is related to the common occurrence in these mannans of the tetrasaccharide side chain which possesses the immunodominant terminal α(1 → 3)-linked mannobiose unit (2). The precipitin curves in Fig. 6, and the typical inhibition curves shown in Fig. 7 confirm this point. In nearly all cases, the mannotetraose unit gave 100% inhibition of the precipitin reaction. Although the pentasaccharide also gave complete inhibition, it was not significantly better than the tetrasaccharide. This result is proof that the pentasaccharide has a terminal α(1 → 3) linkage, and since the Smith degradation indicated that the two (1 → 3) linkages must occur together, the results

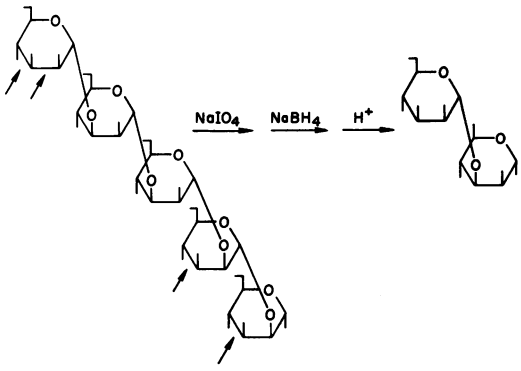

FIG. 4. *Smith degradation of the S. italicus pentasaccharide, in which the arrows indicate points of oxidative cleavage. Only if the two (1 → 3) linkages are contiguous is a disaccharide obtained.*

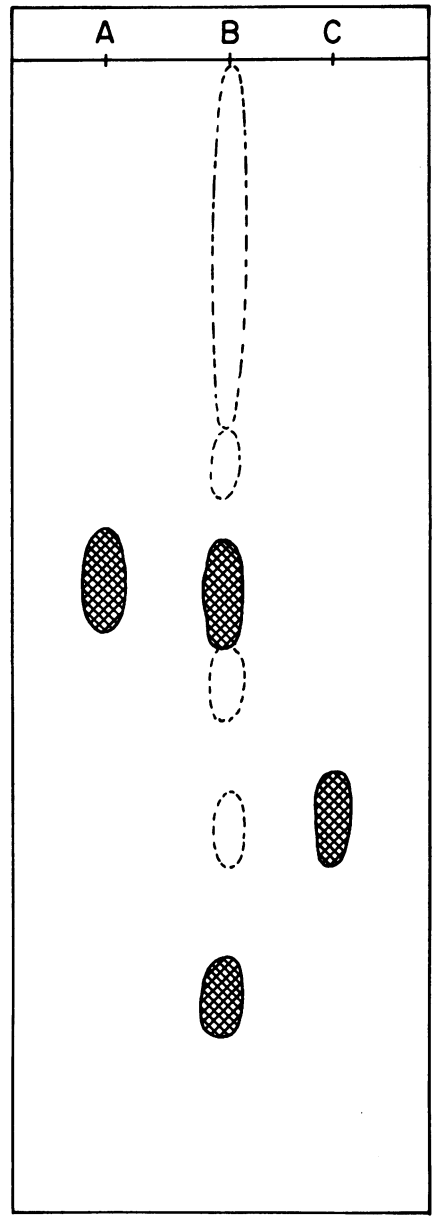

FIG. 5. *Chromatogram of the Smith degradation products obtained from the S. italicus pentasaccharide. A, reference mannobiose; C, mannose; and B, reaction product, with major spots for mannobiose and glycerol.*

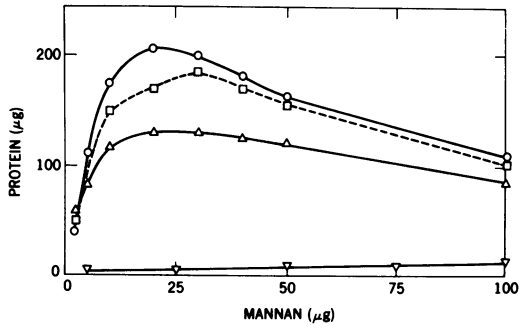

FIG. 6. *Precipitin curves showing the reaction of anti-S. chevalieri with mannan from S. chevalieri* (○), *S. cerevisiae* (□), *Kluyveromyces lactis* (△), *and Schizosaccharomyces pombe* (▽).

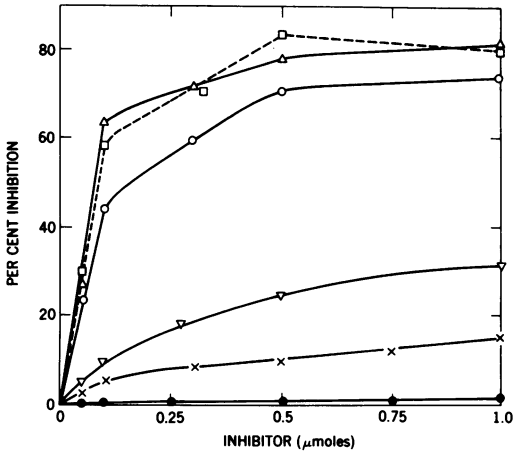

FIG. 7. *Inhibition of the homologous precipitin reaction of S. chevalieri by the pentasaccharide* (□), *tetrasaccharide* (△), *trisaccharide* (○), *disaccharide* (▽), *mannose* (×), *all from S. chevalieri mannan, and N-acetylglucosaminyl-mannotetraose* (●) *from Kluyveromyces lactis mannan.*

allow assignment of the structure given in the previous section. Although one might have predicted an antigenic difference between these two sidechains since Man(1 → 3)Man(1 → 3)Man unit has a shape that is quite different from that of Man(1 → 3)Man(1 → 2)Man unit, there was no suggestion from the immunochemical analysis for antibodies of these two specificities. Thus, the antibody combining site appears to encompass at most two mannose units and the linkage between them. In agreement with this conclusion, the disaccharide αMan(1 → 3)Man has been shown to be a very good inhibitor of the homologous *S. cerevisiae* precipitin reaction (13). The disaccharide used in Fig. 7 had a (1 → 2) linkage and was a poor inhibitor.

The cross-reactivity with the mannan from *K. lactis* also reflected the common occurrence of the terminal α(1 → 3)-linked mannose units in these mannans (2). The other determinant in this mannan, a mannotetraose unit substituted by *N*-acetylglucosamine, had no effect on the heterologous precipitin reaction (Fig. 7), although it is a good inhibitor of the homologous *K. lactis* precipitin reaction (12). There was no cross-reaction between the *Saccharomyces* species antisera with the mannan from *Schizosaccharomyces pombe*, which is known to consist of a linear α(1 → 6)-linked mannose backbone with short side chains of one or two galactose units (5).

Anomeric configurations. The anomeric configurations in many of the oligosaccharides were determined by nuclear magnetic resonance, and in all cases they showed the expected number of signals in the region characteristic of equatorial anomeric protons, τ4.5–5 (11). Since all of the mannose units are probably in the stable C1 conformation, this indicates that all had α-anomeric linkages. In confirmation, all of the fragments were completely hydrolyzed by an exo-α-mannanase (9).

This assignment of the α-anomeric linkage to the terminal nonreducing mannose units in the tetra- and pentasaccharides was confirmed by the immunochemical analysis (see above). Detailed studies (2) have already demonstrated that the immunodominant side chain in *S. cerevisiae* S288C mannan is the tetrasaccharide αMan(1 → 3)αMan(1 → 2)αMan(1 → 2)Man, and the tetrasaccharide components from the four yeast mannans in this study were immunochemically indistinguishable from that from *S. cerevisiae*. Moreover, the pentasaccharide fragment had the same inhibitory effect on the precipitin reactions as the tetrasaccharide, which is consistent with the conclusion that its nonreducing end was terminated by an α(1 → 3)-linked mannose unit.

Backbone structure. Digestion of *S. chevalieri* and *S. diastaticus* mannans with an exo-α-mannanase that is known to remove all side chains from *S. cerevisiae* mannan (9) yielded polysaccharide residues that gave only mannose on acetolysis. Thus, these two mannans have backbone structures that are exclusively (1 → 6)-linked, and from their other similarities it is reasonable to infer that *S. italicus* and *S. carlsbergensis* mannans have the same kind of structure.

DISCUSSION

A generalized structure for the yeast mannans studied here is summarized in Fig. 8. As demon-

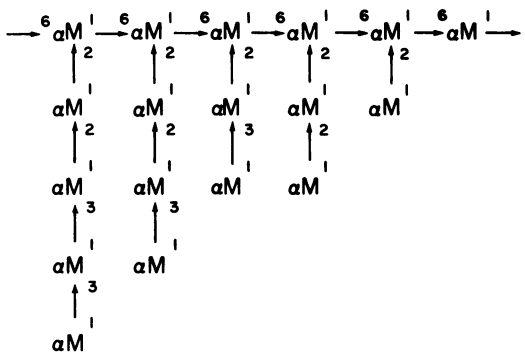

FIG. 8. *Generalized structure for the mannans from S. carlsbergensis, S. chevalieri, S. diastaticus, and S. italicus. The trisaccharide side-chain with two (1 → 2) linkages appears to be lacking in S. chevalieri mannan, although it must be formed as an intermediate in synthesis of the tetrasaccharide.*

strated by their interfertility (14), the four *Saccharomyces* species are closely related to each other and to *S. cerevisiae*, and this relatedness is confirmed in the similar structures of their cell wall mannans and surface antigens.

It is apparent that all of the longer side chains can be formed simply by a lengthening of the shorter chains, with the exception that the trisaccharide with a terminal (1 → 3) linkage is not converted to a tetrasaccharide with two (1 → 3) linkages. However, the tetrasaccharide with a terminal (1 → 3) linkage apparently does serve as the precursor of the pentasaccharide. Thus, all of the chains could be formed by the concerted action of four different mannosyltransferases, assuming that the trisaccharide with a terminal (1 → 3) linkage is the result of an "abortive" action of the first $\alpha(1 \rightarrow 3)$mannosyltransferase. From a study of *S. cerevisiae* mutants that make altered mannans, three of the enzymes that appear to be involved in this process have been identified (3). These are two $\alpha(1 \rightarrow 2)$-mannosyltransferases and an $\alpha(1 \rightarrow 3)$-mannosyltransferase. Apparently, *S. chevalieri* and the other three yeasts we have investigated possess a second $\alpha(1 \rightarrow 3)$-mannosyltransferase that is able to utilize the mannotetraose as an acceptor in forming the pentasaccharide side chain. The expression of this enzyme activity seems to be variable because a previous acetolysis pattern for a different *S. carlsbergensis* strain (10) did not show the pentasaccharide fragment. Thus, this structural feature of the mannan appears to be maintained only in certain of the *Saccharomyces* strains. Other *S. cerevisiae* strains are known that do not have the mannotetraose side chain in their mannans (4), and these appear to lack an $\alpha(1 \rightarrow 3)$-mannosyltransferase that is specific for adding the $\alpha(1 \rightarrow 3)$-linked mannose unit to the $\alpha(1 \rightarrow 2)$-linked trisaccharide sidechains (1). The physiological role for this natural variation in mannan structure among closely related yeast strains is not known.

Two other variable features deserve note. One is the unusually large amount of the disaccharide component in the mannan of *S. chevalieri* and the other is the variable amount of the two kinds of mannotriose units in the different mannans. These features imply that there is some regulation of the kind of mannan made in a particular yeast other than what might be determined simply by the presence or absence of a particular glycosyltransferase. Possibly, these differences could be determined by regulating the absolute activities of the different transferases. Alternatively, the enzymes could differ somewhat in acceptor specificity with the result that certain side chains would be formed in preference to others.

ACKNOWLEDGMENTS

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