ISOLATION AND CHARACTERIZATION OF MANNAN MUTANTS*

(Received for publication, February 22, 1973)

WILLIAM C. RASCHKE, KENNETH A. KERN, CARYL ANTALIS, AND CLINTON E. BALLOU‡

From the Department of Biochemistry, University of California, Berkeley, California 94720

SUMMARY

The yeast Saccharomyces cerevisiae X2180, the strain most commonly used for genetic studies on yeasts, was treated with the mutagen ethyl methanesulfonate, and mutants defective in the synthesis of cell wall mannan were isolated. The initial selection of mutants was made on the basis of their failure to agglutinate with antiserum that was specific for the mannotetraose side chain of the mannan, the immunodominant group of this yeast strain, which has the structure αMan(1→3)αMan(1→2)αMan(1→2)Man. Nonagglutinating cells were assumed to have modified mannan structures, and this was confirmed by chemical analysis of the isolated mutant mannans.

Two classes of mutants were obtained. One was apparently defective in the formation of the α-1→3-mannosyltransferase (mnn1) which is presumed to be involved in the addition of the terminal α-1→3-linked mannose unit to form the mannotetraose side chain. The other class was defective in one of two α-1→2-mannosyltransferases that must be involved in adding the two α-1→2-linked mannose units, one of them (mnn2) directly to the α-1→6-linked mannose units in the backbone of the polysaccharide and the other (mnn3) to this first side chain mannose unit. No mutant was obtained that was defective in forming the backbone. Since such a mutation would yield a cell completely lacking mannan, it might be lethal.

Complementation studies on diploid crosses of representatives of the different mutant classes gave results that were consistent with the interpretations based on chemical analysis of the mutant mannans. Thus, diploids obtained from crosses between mnn1 × mnn2, mnn2 × mnn3, or mnn1 × mnn3 mutants all made mannan of the X2180 chemotype. We conclude that all of the mutations involve structural genes for the various mannosyltransferases that are required for mannan synthesis and not regulatory genes that control expression of a particular mannan chemotype.

Relatively little is known about the genetic control of yeast mannan structure or the mechanism of its biosynthesis. Being a polysaccharide-protein, it is not surprising that mannan formation is inhibited by cycloheximide (1). The mechanism by which the mannose units are added to the polypeptide chain is still open to speculation (2, 3), and this process may occur by the stepwise addition of monosaccharide units or in a manner similar to that involved in the O-antigen lipopolysaccharide biosynthesis in which oligosaccharide units are constructed on a lipid carrier and then are polymerized (4). Strong circumstantial evidence exists for the participation of a lipid carrier in yeast mannan synthesis (5, 6), but oligosaccharide intermediates have not been detected.

The structure of many yeast mannans is such that the latter mechanism is particularly attractive. For example, Saccharomyces cerevisiae S288C mannan has a linear α-1→6-linked backbone (7→9) to which mannose units are attached by α-1→2 and α-1→3 linkages (10, 11), the average length of the side chains being 2 mannose units (not counting the mannose unit in the backbone). It would seem feasible for the side chain units, including the backbone mannose unit, to be built on a carrier and then polymerized with the formation of the 1→6 linkage. A second feature of the mannan structure recommends such a mechanism, namely that the different side chain units in a particular mannan occur in fairly regular ratios regardless of the growth conditions or the age of the culture (3), whether haploid or diploid (12). This suggests some control of the amounts of the side chains and perhaps even their sequence in the polysaccharide chain, both features being easily understood if the biosynthetic mechanism involved the sequential action of a series of specific polymerases. The relevant published work (2, 13) does not resolve this question.

For some time, we have been investigating the structures of yeast mannans by chemical (10, 11, 14, 15), enzymatic (7-9), and immunological (15-17) methods. It is established that the mannan structures are species-specific, and we have defined several distinct chemotypes and serotypes (18). It has also been demonstrated that one can form hybrid diploids of haploid strains which have different mannans, and that the kind of mannan made by the hybrid is not an average mixture of the two kinds made by the two different haploid strains (18, 19). Thus, there appears to be an epistatic relationship in the genetic control of mannan structure. Recently, it was shown (20) that the hybrid between S. cerevisiae X2180-1A(a) and S. cerevisiae 4484-24D(a), the chemotypes of which are shown in Fig. 1, had the serotype of the X2180 strain. On sporulation of the hybrid diploid, the mannan chemotypes segregated 2:2, and the gene
designated now underway in this laboratory on the mechanism of mannan biosynthesis in S. cerevisiae.

Whatman No. 1 filter paper with ethyl acetate-pyridine-water investigation (22), a mutant of strain 4484-24D which lacks the mannosylphosphate side chain, and is presumed to have a defective mannosylphosphate transferase, was obtained and has been strain S288C. Both haploid strains of S. cerevisiae 4484-24D mutant in mannan biosynthesis. The two principal classes of mutants appear to be defective either in the α-1→3-mannosyltransferases (designated mnn1) or in one of two α-1→2-mannosyltransferases (designated mnn2 and mnn3). In a subsequent investigation (22), a mutant of strain 4484-24D which lacks the mannosylphosphate side chain, and is presumed to have a defective mannosylphosphate transferase, was obtained and has been designated mnn4. These mutants should be helpful in studies now underway in this laboratory on the mechanism of mannan biosynthesis in S. cerevisiae.

EXPERIMENTAL PROCEDURE

General Procedures—A column (2 × 200 cm) of Bio-Gel P-2 (200 to 400 mesh) was used for gel filtration and was eluted with water. Descending paper chromatography was carried out on Whatman No. 1 filter paper with ethyl acetate-pyridine-water (5:3:2, v/v). Oligosaccharides were detected on paper chromatograms with the silver nitrate-sodium hydroxide reagents (23). Total carbohydrate was measured by the phenol-sulfuric acid method (24) and total phosphate by the procedure described (16). Rabbit antisera for two of the mannan mutants, Oligosaccharides with α-1→6 linkages were also provided by Mr. Rosenfeld and were prepared by partial acetylation of the mannan backbone (9).

Methylation of Mannan—Methylation was performed by the Hakomori procedure (28), and was repeated 4 times to assure complete methylation. The methylleufibril anion was prepared according to Sandford and Conrad (29), while the methylation conditions followed those of Hellingqvist et al. (30). Methylated mannans were dialyzed extensively against water, lyophilized, and then hydrolyzed with 90% formic acid at 100°C for 2 hours. After rotary evaporation at 30-35° to remove the formic acid, the residue was dissolved in 0.27 N HCl and kept at 100°C for 12 hours. For analysis by gas chromatography-mass spectrometry, the products of hydrolysis were converted to alditol acetates by reduction with sodium borohydride followed by acetylation with acetic anhydride and pyridine.

Gas chromatography of the partially methylated alditol acetates was carried out at 205° on a 2.5-foot Carbowax 20M column on a Varian Aerograph 1400 instrument, equipped with a DuPont 21-491 mass spectrometer operating at an ionizing voltage of 70 eV.

Immunological Method—Antiserum against S. cerevisiae S288C was obtained by repeated intravenous injections as described (16). Rabbit antiserum for two of the mannan mutants were formed by injecting intradermally 2 mg of lyophilized cells in 1 ml of incomplete Freund's adjuvant. The emulsion was deposited over three positions on the back of the rabbit. After 1 month, the animals received three intravenous injections at 2-day intervals, with each injection containing 1 mg of dry cells in 1 ml of sterile 0.9% NaCl solution. One week later the ani-
mals were bled. The two above immunization procedures gave equivalent results. The procedures for precipitation and inhibition reactions are described elsewhere (17).

Preparation of Specific Antiserum to S. cerevisiae Mannotetraose Side Chain—The major specificity of anti-S. cerevisiae S288C serum is directed against the terminal α-1→3 linkage of the tetrasccharide side chain. Other antibodies in this serum were removed by adsorption with Klöckerera brevis cells. Forty milliliters of anti S. cerevisiae S288C serum were added to 40 g wet weight of K. brevis cells (killed by heating at 70° for 90 min) in 100 ml of NaCl solution. The mixture was left overnight at room temperature with azide added as a preservative. The cells were centrifuged and the supernatant adsorbed again with another 40 g of K. brevis cells. After centrifugation, the serum was dialyzed against NaCl solution and finally concentrated to 34 ml by pressure dialysis and sterilized by passage through a Millipore filter. The serum agglutinated S. cerevisiae X2180 cells well but failed to show any agglutination of K. brevis cells, indicating that the adsorbed serum was specific for the α-1→3-tetrasaccharide side chain.

Isolation of Mannan Mutants—Mutagenesis of S. cerevisiae haploid strains X2180-1A-adel and X2180-1B-adel was accomplished with the alkylating mutagen ethyl methanesulfonate. For each strain, 50 μl of mutagen were added to approximately 2 × 10⁶ cells suspended in 1 ml of sterile 0.1 M sodium phosphate buffer, pH 7.0. After the suspension had shaken at 30° for 1 hour, 0.2 ml of the mutagenized culture was transferred to 8 ml of sterile 5% thiosulfate which terminated the reaction. Sixty per cent survival is expected from this procedure. The mutagenized cells were centrifuged, reaccumulated in 5 ml of complete medium, and grown at 30° for 2 days. Cells with mannan containing the α-1→3-mannotetraose linkage were agglutinated by the addition of 0.5 ml of sterile adsorbed S. cerevisiae antiserum. After 1 hour at room temperature, the agglutinated cells settled rapidly, and after 5 min only very small clusters of agglutinated K. brevis cells, indicating that the adsorbed serum was specific for the α-1→3-tetrasaccharide side chain.

Characterization of Mannan Mutants—Mutants of S. cerevisiae X2180 with altered mannan were selected by their failure to agglutinate with adsorbed S. cerevisiae S288C serum which was specific for the α-1→3 linkage of the mannotetraose side chains (16). The titers of the mutants (Table I) demonstrate that most failed to agglutinate with this serum, while others showed intermediate activity. The identification of the mating type and adenine markers in the mutants (Table I) confirmed that most were derived from the original parents.

The nonagglutinating mutants presumably lacked the mannotetraose antigenic structure. To determine the actual structural changes in the mutants, the mannan from each was isolated and subjected to acetolysis. Fig. 2 shows the gel filtration patterns obtained for four of the mutant mannans, compared with that of the parent. Three types of patterns were observed. Mutant X2180-1A-4 (mutant 4 from X2180-1A-adel) and X2180-1B-4 (mutant 4 from X2180-1B-adel) exhibited similar patterns with both lacking the mannolactose side chain. These strains presumably had a mutation in the gene coding for the α-1→3-transferase activity, and were designated mnn1 mutants since we conclude they involved the same gene mapped in our earlier study (20). Table II shows that mannone and mannobiose were present in approximately the same quantity as in wild type X2180 mannan. The mannotriose content, however, was higher in each case, perhaps from the inclusion of the precursor of mannotetraose side chains. Table II shows that mannone and mannobiose were present in approximately the same quantity as in wild type X2180 mannan. The mannotriose content, however, was higher in each case, perhaps from the inclusion of the precursor of mannotetraose side chains.

Mutant X2180-1A-5 lacked almost completely the ability to agglutinate with adsorbed S. cerevisiae S288C antiserum, diluted 1:20. This inability is directed against the terminal α-1→3 linkage of the mannotetraose side chain. A third type of genetic alteration, observed in mutant X2180-1B-2, led to a decreased amount of mannotriose side chains and pos-
FIG. 2. Acetolysis fingerprints of the isolated mannans from *Saccharomyces cerevisiae* X2180 and several of the mutants listed in Table I. The peaks are, from right to left, mannose, manno-biose, mannotriose, and mannotetraose. The inset for the X2180-IA-5 mannan was enlarged eight times, and covers the di- to tetra-saccharide region.

### TABLE II

**Acetolysis products of mutant mannans**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mole per cent of acetolysis products</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Man</td>
</tr>
<tr>
<td>X2180-1A-adel</td>
<td>28.4</td>
</tr>
<tr>
<td>X2180-1A-2</td>
<td>21.6</td>
</tr>
<tr>
<td>X2180-1A-4</td>
<td>33.2</td>
</tr>
<tr>
<td>X2180-1A-5</td>
<td>95.0</td>
</tr>
<tr>
<td>X2180-1B-2</td>
<td>68.1</td>
</tr>
<tr>
<td>X2180-1B-4</td>
<td>27.7</td>
</tr>
<tr>
<td>X2180-1B-7</td>
<td>30.0</td>
</tr>
</tbody>
</table>

Sensibly resulted from the defect similar to that in *mnn2* mutants, but involving the α-1→2-transferase that adds the second mannose unit of the side chain. We have called this mutant *mnn8*.

All other mutant mannans were examined by paper chromatography. Of these, X2180-1A-7 and X2180-1A-9 gave the same acetolysis products as X2180-1A-5. Like X2180-1A-5, both had a small amount of mannobiose and traces of mannotriose and mannotetraose. All three could have resulted from the same mutational event, although X2180-1A-7 was found to have accumulated an additional nutritional requirement during mutation. The remaining mutant mannans exhibited the loss of the tetrasaccharide side chain only.

The suspected structural changes in the mannans from mutants X2180-1A-4 and X2180-1A-5 were confirmed by methylation analysis (Fig. 3). The absence of 2,4,6-tri-O-methylmannitol in the products from the X2180-1A-4 mannan proved that this mutant lacked the ability to produce 1→3-mannosyl linkages. The X2180-1A-5 mannan gave essentially only 2,3,4-tri-O-methylmannitol, with very little tetra- and di-O-methyl derivatives, confirming that the mannan of *mnn2* mutants is a 1→6-linked linear polymer. The trace of 3,4,6-tri-O-methylmannitol indicated that the few side chains present were 1→2-linked. Apparently, the 1→2-transferase activity was not completely eliminated in this mutant, or else a second enzyme with a very low activity was able to act in the absence of the normal 1→2-transferase.

The ratios determined for the various methylated fragments relative to the 2,3,4-tri *O* methylmannitol were almost identical for X2180 mannan and X2180-1A-4 mutant, with the exception of the 2,4,6-isomer which was absent in the latter mannan. This indicates that the only modification in the X2180-1A-4 mannan was the absence of the terminal α-1→3-linked mannose unit of the mannotetraose side chain.

**Immunochemical Studies**—The mannans from mutants X2180-1A-4 and X2180-1A-5 cross-reacted poorly with anti-*S. cerevisiae* S288C serum (Fig. 4). The precipitation observed with the X2180-1A-5 mannan, in contrast to the backbone mannan, undoubtedly reflected the presence of a few tetrasaccharide side chains in the former, which was also apparent in the acetolysis pattern (Fig. 2).

Antisera were obtained against the two mutants, X2180-1A-4 and X2180-1A-5, to check for the presence of new surface determinants. In each case, the mutant mannans reacted well with the homologous antisera (Figs. 5 and 6). The anti-X2180-1A-5 serum cross-reacted well with the backbone mannan, indicating that this serum was directed against the α-1→6 linear sequence of mannose units. The poor precipitation of this antisera with the wild type X2180 mannan suggests that antibody
FIG. 4. Precipitin curves of anti-Saccharomyces cerevisiae X2180 serum with X2180 (- - -), X2180 1A-5 (---), X2180 1A-4 (-----) and backbone (........) mannans.

Fig. 5. Precipitin curves of anti-X2180-1A-5 serum with the various mannans, identified as in Fig. 4.

Fig. 6. Precipitin curves of anti-X2180-1A-4 serum with the various mannans, identified as in Fig. 4.

Fig. 7. Inhibition of the homologous X2180-1A-5 precipitin reaction with mannooligosaccharides. The α-1→6-linked backbone oligosaccharides are represented with open symbols, and are the mannotetraose (Ο), mannopentaoe (□), mannohexaose (△) and mannoheptaose (◊). The closed symbols are for side chain α-1→2-linked mannotriose (▼) and mannotetraose with both α-1→2 and α-1→3 linkages (●).

The specificity for 1→2- and 1→3-linked mannose units was not generated even though a few side chains exist in the X2180-1A-5 mannan, and that no extended lengths of unsubstituted backbone units were present in the X2180 mannan.

The specificity of the anti-X2180-1A-5 serum was further characterized by the hapten inhibition data shown in Fig. 7. The α-1→6-linked oligosaccharides, representative of the backbone structure, completely inhibited the precipitation reaction with X2180-1A-5 mannan, while the side chain fragments with α-1→2 and α-1→3 linkages were poor inhibitors. The maximum segment of backbone recognized by the antibody population was a penta- or hexasaccharide in agreement with the findings of Kabat (31).

The antiserum to X2180-1A-4 cells exhibited unusual properties. While the homologous X2180-1A-4 mannan precipitated well with this serum, the wild type X2180 mannan, which contains approximately the same number of trisaccharide side chain determinants, reacted significantly less (Fig. 6). Clearly, the specificity for a determinant other than the 1→2-linked mannotriose side chains was present. In subsequent studies (22), it has been shown that this reflects the formation of exposed mannosylphosphate determinants which appear in the absence of the α-1→3-mannosyltransferase. Considerable precipitation was observed with the X2180-1A-5 mannan as well as backbone mannan, indicating the presence in this serum of antibody with a specificity for the α-1→6-linked backbone units.

The precipitation of X2180-1A-4 antiserum with the homologous mannan was inhibited both by side chain oligosaccharides and 1→6-linked fragments (Fig. 8). The trisaccharide side chain together with the pentasaccharide backbone fragment gave a maximum inhibition of 70%. The greater inhibition by the tetrasaccharide side chain, compared with the trisaccharide, suggests that the antibody combining site was not restricted to α-1→2-linked mannose units and that a longer segment than the...
mannobiose side chain afforded greater stability for binding to antibody.

**Complementation Studies**—None of the mutations, with the exception of that in mutant X2180-lB-6, affected the mating process, since the mutants were able to form diploids with the wild type X2180 haploid of the opposite mating type (Table I). Therefore, the presence of side chains was not essential for mating.

**Wild Type Complementation**—The diploids formed by mnn1 x mnn1 crosses (i.e. X2180-lA-4 X X2180-lB-4, X2180-lA-4 X X2180-lB-7, and X2180-lA-2 X X2180-lB-7) failed to agglutinate with adsorbed anti-S. cerevisiae S288C serum, demonstrating that none of the mutations was phenotypically dominant.

**Crosses between the mutants** were obtained to determine complementation patterns. The diploids formed by mnn4 x mnn4 crosses (i.e. X2180-lA-4 X X2180-lB-4, X2180-lA-4 X X2180-lB-7, and X2180-lA-2 X X2180-lB-7) failed to agglutinate with adsorbed anti-S. cerevisiae X2180 serum, providing strong evidence that the same gene, controlling the \( \alpha-1\rightarrow3 \)-transferase, was affected in each. On the other hand, agglutination was observed for the diploid crosses mnn1 x mnn2 (i.e. X2180-lA-5 X X2180-lB-4 and X2180-lA-5 X X2180-lB-7). This successful complementation indicates that the \( \alpha-1\rightarrow3 \)-transferase activity was functionally independent of the side chain elongation enzyme, and two different genes must be involved.

The third type of mutant, represented by X2180-lB-2, appeared to represent a defect in a third gene, mnn3, also responsible for the correct assembly of the side chains. This conclusion comes from the complementation of this haploid with both mnn1 and mnn2 mutants to give wild type mannan. We interpret this mutation to involve a second \( \alpha-1\rightarrow2 \)-mannosyltransferase which attached the second \( 1\rightarrow2 \)-linked mannose unit in the side chains.

**Fig. 8. Inhibition of the homologous X2180-lA-4 precipitin reaction with mannoooligosaccharides.** The closed symbols are for the side chain mannobiose (▲), mannotriose (▼) and mannotetraose (●); while the open symbols are for the backbone mannopentaose (□), and a mixture of side chain mannotriose and backbone mannopentaose (▼), or side chain mannotetraose and backbone mannopentaose (●).

**DISCUSSION**

In this study, we have selected and characterized two kinds of mutants of *S. cerevisiae* X2180 that are defective in mannan biosynthesis. The selection utilized antiserum specific for the \( \alpha-1\rightarrow3 \)-linked nonreducing terminal mannose in the mannotetraose side chain of wild type cells. Mutants of X2180 with altered mannan structure failed to agglutinate with this antiserum.

One type of mutant which lacked only the mannotetraose side chain, was apparently altered in a gene coding for the \( \alpha-1\rightarrow3 \)-mannosyltransferase activity. The other type had a defect in the formation of the shorter side chains, reflecting the loss of the \( \alpha-1\rightarrow2 \)-mannosyltransferase activity which adds the first mannose unit to the backbone. The two types complemented each other, which allowed the assignment of two genes responsible for mannan biosynthesis in *S. cerevisiae* X2180. A third type of mutant, which led to an altered ratio of mannosibiose and mannotriose side chains, complemented both of the other mutants. Since two \( \alpha-1\rightarrow2 \)-mannosyltransferases are probably involved in formation of the mannotriose side chain, this must represent a mutation in the \( 1\rightarrow2 \)-transerase that adds the second mannose unit in the side chains.

No mutant was detected which appeared to be defective in the presumed \( \alpha-1\rightarrow6 \)-mannosyltransferase for synthesis of the backbone. Since such mutants would lack mannan and might be lethal, they may best be obtained by selection of conditional mutants. A fourth type of mannan mutant, one defective in the addition of mannosylphosphate groups to the mannotriose side chains, is described in the following paper (22) and has been designated mnn4.

The ease of isolation of the mannan mutants and the apparently normal morphology and physiology of the cells suggest that the fine structure of the mannan component of the wall does not play a critical role. Because of the species specificity in structure, it was a reasonable speculation that the mannans might function in cell-cell recognition in processes such as sexual reproduction (12). However, the mannan mutants appear to mate normally, the diploid cultures proceed through meiosis, and the resulting spores germinate without any obvious difficulty. It has also seemed reasonable to postulate that the mannan component of mannan enzymes, such as invertase, might be involved in the specific orientation of the enzymes in the wall in association with the structural mannan-protein complex. Again, this seems unlikely because the structural mannan can be drastically modified without affecting the vegetative growth of the cells. It will be interesting to determine whether the structural mannan and the mannan component of the mannan-enzymes are modified in a coordinate fashion in the mutants. If so, it would suggest that these two polysaccharide fractions were formed by the same enzymatic machinery even though they end up in functionally differentiated cell wall macromolecules.

Our results have obvious significance with respect to the mode of mannan biosynthesis. Clearly, the polymerization of the 1→6-linked backbone is not dependent on the formation of carrier-bound oligosaccharides, since the mnn2 mutants made unbranched mannan molecules with no difficulty. While this does not rule out a mechanism of biosynthesis similar to that involved in Salmonella O-antigen formation, it does suggest that mannan is most likely made by the stepwise addition of single mannose units to the growing chain.

**REFERENCES**

15. RASCHKE, W. C., AND BALLOU, C. E., (1972) Biochemistry 11, 3807-3816
Genetic Control of Yeast Mannan Structure: ISOLATION AND CHARACTERIZATION OF MANNAN MUTANTS
William C. Raschke, Kenneth A. Kern, Caryl Antalis and Clinton E. Ballou


Access the most updated version of this article at http://www.jbc.org/content/248/13/4660

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/248/13/4660.full.html#ref-list-1