Influence of outer region mannosylphosphorylation on N-glycan formation by *Candida albicans*: Normal acid-stable N-glycan formation requires acid-labile mannosylphosphate addition

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The pathogenic yeast *Candida albicans* produces large N-glycans with outer regions containing only mannose residues. The outer region comprises a primary branch with multiple secondary and tertiary branches. Tertiary branches are linked to secondary branches by phosphodiester bridges. In the current model of outer chain elongation in the genetically related yeast *Saccharomyces cerevisiae*, the initial step in tertiary branch formation, should not affect primary or secondary branch formation. Compared to its wild-type parent, a *C. albicans* mutant defective in tertiary branch mannosylphosphorylation (mnn4Δ/mnn4Δ) made outer regions with reduced susceptibility to low acid acetolysis treatment, suggesting that the secondary or primary region had been modified. Higher acid acetolysis conditions were required to release the secondary branches from the primary branches. The released secondary branches constitute the subset of the wild-type secondary branches that lack a phosphate group. In contrast, the acid-stable region of both wild-type and mnn4Δ *S. cerevisiae* strains required high acid acetylation conditions to release the secondary branches, despite having smaller and less complex secondary and tertiary branches. These results suggest that the complex and longer secondary and tertiary branches of *C. albicans* affect the conformation of the acid-stable region to render it more susceptible to acetylation which implies secondary and tertiary branch formation in *C. albicans* are interdependent events and occur concurrently, rather than sequentially.

**Keywords**: *Candida albicans*/cell wall protein/N-glycosylation/mannosylphosphorylation

**Introduction**

The fungal genus *Candida* contains multiple species that are pathogenic to humans. Infections caused by these species, especially *Candida albicans*, can be life threatening to immunocompromised individuals. *C. albicans* is unusual among the pathogenic *Candida* species in that it is a member of the normal microbiota of most humans, its natural habitat is the human body, and it maintains a long-term commensal relationship with the human host. To maintain this relationship, *C. albicans* must be able to withstand potentially deleterious host products and remain anchored to host surfaces. The cell wall contributes to both of these functions (Vecchiarelli et al. 1991; Jouault et al. 1995; Tada et al. 2002; Netea et al. 2006).

The surface of the *C. albicans* cell wall is populated with high molecular mass mannoproteins (HMMPs) in which the glycan may represent greater than 50% of the mass of the mannoprotein. Because of their location, the HMMPs are the structures of the cell that are in direct contact with the host. How the different HMMPs further contribute to the natural life cycle of the organism is unknown, but evidence suggests that one or more of the HMMPs organize into fibrillar structures (Cassone et al. 1978; Poulin et al. 1978; Ponton and Jones 1986; Tokunaga et al. 1986; Hazen et al. 1990) which radiate from the cell surface. Variations in protein N-glycosylation affect the conformation and relative abundance of the fibrils (Hazen and Hazen 1992; Singleton, Fidel, et al. 2005; Netea et al. 2006). As a consequence, the relative hydrophilicity of the surface changes, and those changes affect the organism’s virulence (Antley and Hazen 1988). In addition to affecting surface fibril structure, the *N*-glycans directly influence pathogenesis by stimulating production of inflammatory cytokines through interaction with the mannose receptors on macrophages (Masuoka 2004; Netea et al. 2006).

To better understand how N-glycosylation participates in pathogenesis and the life cycle of *C. albicans*, it is necessary to not only dissect the structure of the N-glycans it produces, but also determine how their synthesis is regulated. The general structure of the *N*-glycans of *C. albicans* and other *Candida* species has been elucidated primarily through the extensive nuclear magnetic resonance studies of Suzuki and colleagues (Kobayashi et al. 1989; Shibata et al. 1989; Kobayashi et al. 1991, 1992; Shibata et al. 1992, 1995). Like other eukaryotic *N*-glycans, the *C. albicans* N-glycans contain a core region and an outer region. The core region is structurally similar to the core region of other eukaryotes. The outer region differs from mammalian cells and can be structurally subdivided into two groups based on resistance to mild acid hydrolysis. The acid-stable portion consists of a linear homoanomeric oligosaccharide that branches directly from the core region and is composed of multiple α-1,6-linked mannose residues (Figure 1). Attached to this primary branch are secondary branches which consist predominantly of α-1,2-linked oligomannosides. An
The structure is similar to the outer region of Saccharomyces cerevisiae and C. albicans produced by S. cerevisiae N-glycosylation (Dean 1999; Jungmann et al. 1999). The outer region glycan synthesis occurs in a sequential fashion, similar to what is observed in higher eukaryotes. Support for a sequential model was obtained by Bringle et al. (2000) whose results suggested that α-1,6-, α-1,2-, and α-1,3-mannosyltransferase activity occur in the cis, medial and trans Golgi compartments, respectively. Sequential synthesis appears more likely to occur in S. cerevisiae versus C. albicans because fewer distinct mannosyltransferase reactions are required and only a limited number of N-glycan outer chain mannosyltransferases are needed to perform those reactions. Few glycosyltransferases are required to achieve synthesis of a full-length primary branch, as a specific enzyme is not needed for each sequential residue addition. Secondary branch formation in S. cerevisiae involves only three enzymes, each of which is responsible for a particular mannose residue addition. S. cerevisiae apparently lacks the enzymes to add β-oligomannosides onto the secondary and tertiary branches.

Review of the annotated genome database for C. albicans (URL: www.candidagenome.org) suggests more and different mannosyltransferases are required to make the outer region compared to S. cerevisiae. In addition to the unique β-mannosyltransferases needed to cap secondary branches of serotype A cells and to make the tertiary branches, C. albicans appears to have multiple paralogs of two genes involved in outer region synthesis, CaMNN4 and CaMNN2, that are represented as single genes in S. cerevisiae. Curiously, C. albicans lacks homologs of other genes, such as MNN5 and MNN6, that are present in S. cerevisiae, although Bai et al. (2005, 2006) have evidence suggesting one of the CaMNN2 paralogs encodes a product with ScMnn5p-like activity. Mnn2p in S. cerevisiae initiates secondary branch formation while Mnn5p adds the subsequent mannose residue. Thus, elongation of the secondary branch in C. albicans likely involves one or more of the CaMNN2 paralogs. Similarly, evidence suggests that ScMNN6 encodes the structural enzyme mannosylphosphate transferase, while ScMNN4 encodes a regulator of Mnn6p activity (Wang et al. 1997; Jigami and Odani 1999). In C. albicans, expression of MNN4 is required for initiation of tertiary branch formation. Unlike MNN2 and MNN4, MNN1 is present as a single homolog in C. albicans. ScMNN1 encodes the α-1,3-mannosyltransferase that is responsible for capping the secondary branches.

We recently observed that mnn4 null mutants of C. albicans produce an altered acid-stable region that is not sufficiently explained by the current model of outer region maturation of S. cerevisiae. The mnn4 mutants were more hydrophobic than their cognate parent strain when grown at 37°C, suggesting that the N-glycan tertiary branch affects exomural fibril integrity (Singleton, Masuoka, et al. 2005). However, an unexpected
consequence of ablation of mannosylphosphorylation was a change in sensitivity of the N-glycan to acetolysis treatment involving a low concentration of acid. Acetolysis preferentially cleaves the α-1,6-glycosidic linkages in the primary branch of the acid-stable region (Rosenfeld and Ballou 1974). Enhanced resistance of the primary chain to acetolysis in the mutant could only occur if the acid-stable region has become altered in its structure compared to wild-type cells, implying tertiary branch formation or Mnn4p functional activity is required for normal acid-stable mannan formation. To begin to address how maturation of the outer region occurs in C. albicans, we further characterize how the loss of Mnn4p activity affects acid-stable mannan structure and suggest that N-glycosylation primary, secondary, and tertiary branch formation occur interdependently and concurrently, not sequentially.

Results

Cell surface architecture
Transmission electron microscopy of freeze fractured C. albicans wild-type and mnn4 mutant cells revealed differences in the surface fibril characteristics (for example, see Figure 2). Compared to wild-type cells, fibrils on mutant cells were less abundant, less uniformly distributed, and generally shorter. These differences are not evident when freeze substitution methods are used to prepare cells for transmission electron microscopy analysis (Netea et al. 2006). The architectural changes are consistent with the differences in cell surface hydrophobicity (CSH) between wild-type and mutant cells as previous studies have shown that surface hydrophobic proteins are masked by the long radiating fibrils seen on wild-type hydrophilic cells (Hazen and Hazen 1992).

Glycoprotein distribution
A previous study showed that reactivity of cell wall protein extracts to mAb B6, which recognizes an undefined epitope within the acid-stable region, was significantly different between wild-type and mutant cells of C. albicans regardless of strain serotype (Singleton, Masuoka, et al. 2005). General glycoprotein staining of dithiothreitol (DTT)-extracted proteins of wild-type and mutant cells separated in 3–8% gradient gels indicated that the apparent masses of the glycoproteins from mutant cells were generally higher than wild-type cells and less variable compared to those obtained from wild-type cells (Figure 3A). Similar results (not shown) were seen when cell wall proteins were extracted by β-1,3-glucanase (Zymolyase) treatment (Singleton and Hazen 2004). The staining results for the C. albicans mnn4 mutants do not appear to be due to protein aggregation as silver stains demonstrated similar protein profiles for low-molecular weight nonglycosylated proteins of wild-type and mutant cells. The difference in glycoprotein staining was not seen with glycoproteins extracted from S. cerevisiae strain X2180 and the cognate mnn4 mutant, YKL201c (Figure 3B).

Susceptibility of the acid-stable region of mnn4 mutants to weak and strong acetolysis
Previous investigation revealed that the acid-stable mannan obtained from the mnn4 mutants of C. albicans, unlike the wild-type parent mannan, is relatively resistant to low acid acetolysis conditions (acetic acid:acetic anhydride:sulfuric acid, 100:100:1) resulting in a smear at the top of the fluorophore-assisted carbohydrate electrophoresis (FACE) gel (Singleton, Masuoka, et al. 2005). The acid-stable region of the mutant N-glycans could be fragmented into individual secondary branches by increasing the proportion of acid (i.e., strong acetolysis conditions, acetic acid:acetic anhydride:sulfuric acid, 10:10:1) (Figure 4). Fewer oligomannosides were present in the wild-type population versus the mutant, but a unique band was also present in the mutant population (Figure 4A, arrow). The banding pattern of the wild-type cells was similar between weak and strong acetolysis conditions suggesting strong conditions do not cause hydrolysis at glycosidic linkages other than α-1,6. Reintegration of CaMNN4 into its native locus in the mutant strain DSH4 restored normal susceptibility of its acid-stable N-glycans to low acid acetolysis conditions.

Mannan extracts from strain X2180 and the mnn4 mutant (YKL201c) of S. cerevisiae were also subjected to weak and strong acid acetolysis. Unlike C. albicans, the acid-stable region
Fig. 3. Comparison of general cell wall protein glycosylation profiles by electrophoresis and blotting of wild-type and associated mnn4 mutant of C. albicans (A) and S. cerevisiae (B). Extracted proteins were electrophoretically separated in Tris–acetate 3–8% gradient gels. Gels were stained with a general glycoprotein stain. A difference in migration is evident between the C. albicans, but not between the S. cerevisiae strains.

Fig. 4. (A) Comparison of the secondary branch FACE profiles of wild-type serotype B strain C. albicans LGH1095 and its isogenic MNN4 mutant DSH4 when prepared by low (LOW) and high (HIGH) acid acetolysis treatments. A similar result was seen with a second serotype B strain and a serotype A strain (not shown). High acid acetolysis was required to release the secondary branches from the primary branches produced by the mutants. The arrow indicates an oligosaccharide that is produced by the mutant but is not evident in wild-type cells. (B) Same as panel A except with wild-type (wt) S. cerevisiae strain X2180 and the isogenic mnn4 mutant YKL201c (designated mnn4).

Effect of calf intestinal phosphatase (CIP) treatment on C. albicans wild-type and mutant acid-stable acetolysis fragments

Release of tertiary branches by mild acid treatment results in breakage of the phosphoester bond, linking the tertiary branch, leaving the phosphate on the secondary branch. The retained phosphates affect the electrophoretic mobility of the oligosaccharides to which they are attached during FACE. To determine if the missing oligomannosides in the C. albicans mutant acid-stable region are due to loss of the phosphate group, acetolysis-released secondary branches were subjected to CIP treatment and then separated by FACE. CIP treatment not only simplified the banding pattern of wild-type cells, but also caused the oligomannoside profiles of wild-type and mutant cells to look similar (compare lanes 4 and 8, Figure 5). However, the mutant cells retained the unique band indicated by the arrow in Figure 4 (indicated as well by an arrow in Figure 5), while the wild-type cells appear to have a unique band upon CIP treatment (arrowhead in Figure 5).

Comparison of CaMNN1 and CaMNN10 gene expression between wild-type and mnn4 mutants of C. albicans

C. albicans expresses a single homolog of the S. cerevisiae MNN1 and the MNN10 gene. In S. cerevisiae, MNN1 encodes the α-1,3-mannosyltransferase that terminates N-glycan secondary branch elongation (Ballou 1990). ScMNN10 encodes an α-1,6-mannosyltransferase involved in primary chain elongation.
Ca\textsuperscript{1056} albicans transferases (Jigami and Odani 1999). As mentioned earlier, C. \textit{shares significant similarities with families of known mannosyl-\textit{S. cerevisiae} in high acid acetolysis and CIP treatment. CIP treatment (\textit{to an oligosaccharide detected in the wild-type cells but not mutant cells after in the mutant but not the wild-type as shown in Figure 4. The arrowhead points to an oligosaccharide detected in the wild-type cells but not mutant cells after high acid acetolysis and CIP treatment. CIP treatment (+), mock treatment (−). Forward and reverse primer sets for CaMNN1 and CaMNN10 gene homologs were made and used to compare relative levels of expression by wild-type and \textit{mun4} mutant cells grown to mid-exponential phase. No difference in expression between wild-type and mutant cells was seen for both CaMNN10 and CaMNN1.

Discussion

In serotype B \textit{C. albicans} cells, the molar ratio of tertiary branches within the total population of mannoprotein secondary and tertiary branches is estimated to be less than 0.02 (Kobayashi et al. 1989, 1991; Shibata et al. 1989). Based on this minimal contribution of tertiary branches to the overall mannan structure and on the lack of direct linkage of tertiary branches to the primary branch, loss of tertiary branch formation in \textit{C. albicans} \textit{mun4} mutants derived from strains LGH1095 and A9 was expected to have an inconsequential effect on the remaining \textit{N}-glycan structure and on overall glycoprotein electrophoretic profile. However, both were dramatically affected in \textit{C. albicans}, but not \textit{S. cerevisiae} when \textit{MNN4} was deleted and mannosylphosphorylation of secondary branches did not occur. This observation suggests that Mnn4p activity has a global effect on \textit{N}-glycan formation in \textit{C. albicans}. Loss of Mnn4p activity and the \textit{β}-1,2-oligomannosyl tertiary structures also affected \textit{C. albicans} surface fibril conformation indicating these unusual branches may be critical for making the fibrils appear long and radiating.

The function of \textit{Mnn4p} in \textit{C. albicans} is unknown. Mnn4p in \textit{S. cerevisiae} appears to act as a regulator of Mnn6p, which shares significant similarities with families of known mannosyl-transferases (Jigami and Odani 1999). As mentioned earlier, \textit{C. albicans} lacks an \textit{MNN6} homolog. In \textit{C. albicans}, deletion of CaMNN4 led to loss of mannosylphosphate incorporation in cell wall mannoproteins (Hobson et al. 2004; Singleton, Masuoka, et al. 2005). Subsequent reintroduction of \textit{MNN4} at its native locus restored mannosylphosphorylation and susceptibility of the outer region to low acid acetolysis, indicating that the effect of Mnn4p is directly related to mannosylphosphate transferase function. The contribution of the multiple putative paralogs of \textit{MNN4} in \textit{C. albicans}, all of which are expressed (Singleton and Hazen, unpublished results), to \textit{N}-glycan synthesis, especially mannosylphosphorylation, is unclear. However, none of these appear to compensate for the loss of Mnn4p activity, suggesting their primary function is not mannosylphosphorylation.

Deletion of \textit{MNN4} in \textit{C. albicans} was expected to result in the simple loss of mannosylphosphorylation and tertiary branch formation with retention of normal primary and secondary branch production, and therefore, our standard mild acid acetolysis conditions used on wild-type cells should cause complete digestion of the primary branch produced by the mutants of \textit{C. albicans}. This expectation was not seen. Strong acid acetolysis conditions were required to release the secondary branches. In contrast, mild acid acetolysis conditions were ineffective at releasing the secondary branches from mannans produced by wild-type and mutant \textit{S. cerevisiae} cells, suggesting that the presence of \textit{β}-1,2-oligomannosyl groups on the tertiary branches somehow causes the acid-stable region to become susceptible to mild acid acetolysis. High acid acetolysis conditions were first used by Lee and Ballou (1965) in 1965 to release secondary branches of the acid-stable regions produced by \textit{S. cerevisiae}. No other conditions for acetolysis were tested.

The difference in acetolysis susceptibility between the wild-type and mutant \textit{C. albicans} strains is unlikely due to differences in secondary branch structure. FACE analysis of the secondary branches suggested that essentially all of the secondary branches, with one exception, of the mutant are a subset of the total present in wild-type cells. Secondary branches present in the wild-type cells and not present in the mutant appear to be those which contained phosphate groups. Because there is the potential for comigration of oligomannosides during FACE (Masuoka and Hazen 2006), especially oligomannosides with residue lengths of greater than three, we cannot completely rule out the possibility that there are some unique secondary branches present in the mutant acid-stable region which results in increased resistance to weak acetolysis conditions. Structural analysis of the FACE separated branches is needed to resolve this possibility.

An explanation for what causes wild-type \textit{C. albicans} acid-stable regions to be more susceptible than wild-type \textit{S. cerevisiae} and \textit{mun4} mutant \textit{C. albicans} acid-stable regions to acetolysis likely involves the contribution of secondary branches that are longer than those present on \textit{S. cerevisiae}. The difference in the tertiary oligomannosyl group between \textit{S. cerevisiae} and \textit{C. albicans} may also be a contributing factor, but the effect of this group must be exerted during synthesis of the outer region because the tertiary branches are released from the extracted mannans prior to acetolysis. The \textit{S. cerevisiae} results suggest that a high density of short secondary branches (three or less mannose residues) on the primary branch results in an acid-stable region resistant to low acid acetolysis. That is, the primary branch is not readily accessible to acid hydrolysis. The \textit{S. cerevisiae} results also indicate that the presence or absence of phosphate does not affect acetolysis susceptibility. In the case of \textit{C. albicans}, the long secondary branches, which may be
localized along the primary branch at certain regions due to the tertiary branch positional effects, may force the conformation of the acid-stable region to expose the primary branch α-1,6-glycosidic linkages allowing them to be hydrolyzed under low acid acetylation conditions. Loss of the phosphate along with its oligomannosyl group results in switching to a low-acid resistant conformation. To address this possible mechanism, mutants of *C. albicans* with defects in secondary branch elongation (e.g., *MNN2* knockouts) will be produced and their acid-stable regions assessed for acetylation susceptibility.

Jigami and Odani (1999) have reported that in *S. cerevisiae*, the activities of Mnn6p (mannosylphosphate transferase) and Mnnlp are competitive. Whether mannosylphosphorylation and outer region α-1,3-mannosylation are also competitive in *C. albicans* is unknown. Regardless of that possibility, an increased proportion of secondary branches with terminal or subterminal α-1,3-mannose units is unlikely responsible for the change in acetylation susceptibility of the mutant *C. albicans* strain's acid-stable regions. When expression of CaMNN1 (the putative outer region α-1,3-mannosyltransferase gene) at different growth times was compared between the *C. albicans* mnn4 mutant strains DSH4 and JMH1 and their respective wild-type parent strains LGH1095 and A9, no difference was observed, suggesting loss of MNN4 expression does not trigger a change in expression of MNN1. Preliminary experiments with *Galanthus nivalus* lectin, which specifically binds to terminal α-1,3-mannose, indicated that the mnn4 mutant cells did not demonstrate enhanced mannose-α-1,3 branch termination. While not definitive, as structural analysis is required, these results do not support the possibility that the mnn4 mutants produce increased levels of α-1,3-mannose capped secondary branches.

Our results show that loss of expression of Mnn4p and consequently mannosylphosphorylation of secondary branches causes a structural change in the remaining acid-stable region resulting in decreased susceptibility to low acid acetylation and in changes in general protein mannosylation in *C. albicans* but not *S. cerevisiae*. These observations raise the possibility that, at least in *C. albicans*, tertiary branch formation and secondary branch formation occur concurrently and are interdependent processes during N-glycan maturation. Additionally, the results raise the speculation that tertiary branch structures influence how secondary branches affect outer region conformation in *C. albicans*. The long residue length of some primary branches produced by *C. albicans* along with the need to decorate the residues with secondary branches without causing steric bulk that would interfere with additional secondary branch formation and tertiary branch syntheses could be accomplished if primary, secondary, and tertiary branch formation occur within the same Golgi compartments and occur concurrently with secondary and tertiary branches being added to the primary branch as the primary branch grows in length (Figure 6).

While the general structure of the outer N-glycan regions of *C. albicans* and *S. cerevisiae* is similar, *C. albicans* produces a greater diversity of secondary branches and the unusual β-1,2-linked tertiary branch oligomannoside. A simple mechanism that achieves such diversity is the simultaneous function of the various mannosyltransferases within the same Golgi compartment(s). An additional mechanism is to utilize mannosyltransferases with similar function but different substrate specificities (such as possibly the products of the CaMNN2 paralogs). Both mechanisms may be operative in *C. albicans*, in contrast to what has been suggested by Brigance et al. (2000) for *S. cerevisiae*. It is possible that these differences in structure of the outer region and its synthesis are adaptations that contribute to the ability of *C. albicans*, unlike other yeasts, maintain a long-term communal relationship within the hostile human host environment.

**Materials and methods**

**Organisms and growth conditions**

Two serotype B *C. albicans* strains, LGH1095 (ATCC MYA-2719) and A9, and their respective mnn4Δ mutants, DSH4 and
JM1, were used throughout the study. The method for producing the C. albicans mutants, which involved a positive selection scheme is described elsewhere (Singleton, Fidel, et al. 2005). Strain DSH5, which has one copy of the gene MNN4 reintegrated into its native locus of strain DSH4 was also used (Singleton, Fidel, et al. 2005). Cells were typically grown in yeast nitrogen base broth supplemented with 2% (w/v) glucose for 24–26 h at 37°C with shaking at 150 rpm unless otherwise indicated (Masuoka and Hazen 2004). At this time point, the wild-type cell population typically consists of nearly 100% hydrophilic cells while the mnn4 mutant populations contained between 20 and 40% hydrophobic cells.

Saccharomyces cerevisiae strain X2180 (MNN4+) and the genetically related MNN4 knock-out strain YKL201c BY4742 (MATa his3Δ leu2Δ lys2Δ ura3Δ ΔMNN4 (ATCC 4017034)) were also used (Winzeler et al. 1999). The deletion mutant YKL201c is derived from strain S288C. Strain X2180 is also derived from strain S288C, but arose through spontaneous diploidization (Mortimer and Johnston 1986). Absence of phosphatase in the wall of strain YKL201c was confirmed by the inability of the strain to stain with Alcian Blue. They were grown at 30°C in YPD (yeast extract-peptone-glucose) for 24 h at 130 rpm.

Preparation of samples for rapid freezing, freeze drying, and rotary shadowing (FDS)
Yeasts were grown to early stationary phase, washed, pelleted by centrifugation, and shipped on ice to Frank Macaluso at the Albert Einstein College of Medicine for FDS. The procedure for FDS was based on the description of Hartwig (1992). A slurry of live yeast was placed on a thin gelatin cushion on a specimen mount of the rapid freezing apparatus (CF100; Life Cell Corp., Branchburg, NJ) and frozen by slamming them into a liquid nitrogen-cooled stage temperature raised to −196°C (CFE-50; Cressington, Watford, UK), the stage temperature raised to −90°C for 30 min, and rotary shadowed at a 45° angle with 1.2–1.3 nm tantalum-tungsten foil. After drying, samples were observed using a JEOL 100CX transmission electron microscope at 100 kV. By convention, images were viewed as negatives.

Cell wall protein extraction
Cell wall proteins were extracted using DTT essentially as previously described (Hazen et al. 1990). Briefly, yeast cells were washed three times with cold deionized water and suspended to 2.0 × 10^8 cells/mL in prewarmed (37°C) Tris HCl-EDTA buffer (0.2 M Tris, 0.005 M sodium ethylenediamine tetracetic acid, pH 8.6). DTT was added to a final concentration of 0.05 M, and the cell suspension was incubated with shaking (150 rpm) at 37°C for 60 min. Cells were removed by centrifugation and the supernatant fluid was dialyzed against four changes of distilled H2O. Proteins were concentrated by centrifugal ultrafiltration (Amicon Ultra, 5000 MWCO, Millipore, Billerica, MA). Preliminary experiments demonstrated that DTT treatment was not lethal to cells. Cell wall proteins were also extracted using β-1,3-glucanase (Zymolyase) as described elsewhere (Singleton and Hazen 2004).

One-dimensional protein electrophoresis and western blotting
Extracted proteins were separated by one-dimensional electrophoresis using Tris–acetate gradient gels (3–8% acrylamide, Invitrogen, Carlsbad, CA). Proteins (15 µg/lane) were solubilized in NuPage LDS sample buffer (Invitrogen) and heated to 100°C for 5 min. Electrophoresis conditions were 100 V for 1.5 h, and NuPage Tris–acetate SDS buffer (50 mM Tricine, 50 mM Tris base, 0.1% SDS, pH 8.24) was used as the running buffer. Following electrophoresis gels were stained with either a stain specific for glycoproteins (GelCode Glycoprotein staining kit, Pierce, Rockford, IL) in accordance with manufacturer’s directions or with silver (Hames 1990).

Mannan extraction and acetylation
Acetylation was used to release secondary branches from the primary branch and was performed on mannann extracts as described in our earlier studies (Masuoka and Hazen 2004). Acetylation selectively cleaves α-1,6-glycosidic linkages (Rosenfeld and Ballou 1974). Our standard acetylation conditions, herein referred to as weak acetylation, utilized a hydrolysis solution containing anhydride:acetic acid:sulfuric acid at a ratio of 100:100:1. Strong acetylation conditions use a 10-fold higher concentration of sulfuric acid (i.e., a reagent ratio of 10:10:1).

Carbohydrate electrophoresis
Concentration of the released secondary branches was determined using 3-methyl-2-benzothiazolinone hydrazone as described by Anthon and Barrett (2002). This assay detects reducing sugars under conditions mild enough so that oligosaccharides are not degraded into their monosaccharide components. For a given treatment or gel lane, 2 nmol of oligosaccharides were dried in a centrifugal vacuum evaporator (Virtis, Gardiner, NY) and labeled at the reducing end with the fluorophore 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS). Labeled oligosaccharides were then separated by FACE (Masuoka and Hazen 2004). Electrophoresis was carried out at 300 V for 30 min then 600 V until the ANTS front was approximately 1 cm for the bottom of the gel.

Calf intestinal phosphatase (CIP) treatment of secondary branches
ANTS-labeled oligosaccharides (2 nmol) were dried in a centrifugal vacuum evaporator. The dried oligosaccharides were then dissolved in 8 µL of distilled H2O. Calf intestinal alkaline phosphatase (New England BioLabs, Ipswich, MA, #M0290S, 10 000 U/ml, 1 µL) and 10X buffer (1 µL, buffer 3, New England BioLabs) were added to the oligosaccharide solution. Digestion was carried out at 37°C overnight (16–24 h). A control digest substituted 1 µL of distilled H2O for the enzyme. After digestion, the solution was centrifuged briefly, then dried. The dried samples were dissolved in 10 µL of 1X FACE loading buffer for separation by FACE.

Reverse transcriptase-polymerase chain reaction (RT-PCR)
RNA was isolated from exponentially growing and early stationary phase planktonic yeast cultures by extraction with hot phenol as described (Singleton and Hazen 2004). Following ethanol precipitation and quantification of dissolved RNA by UV spectrophotometry, equal amounts of total RNA were reverse transcribed to cDNA using oligo-dT primers and murine
Maloney leukemia virus (MMLV)-RT in a 20 µL reaction volume (Singleton, Fidel, Wozniak, et al. 2005). Synthesized cDNA was brought up to a 100 µL final volume, and 1 µL aliquots were used as templates for polymerase chain reaction (PCR) with the following primer sets: MNN1 (ACAGGGGAAGTTTGGGGTG and TGCTTGATGACA- GAAACGG), 18S (GCCAGCGAGTATAAGCCTTG and AG- GCCCTACTAGCTCTCA), and MNN10 (CGAAAAATAT- GCAAGGAGCAGAAC and TTGAGCCAGGAGGAC). PCR products were amplified using the following reaction conditions: initial denaturation at 94°C for 3 min, followed by either 20, 25, 30, or 35 cycles with an annealing temperature of 52°C, followed by a final 10 min extension at 72°C. PCR products were separated by agarose electrophoresis, stained with ethidium bromide, and band intensities quantified by densitometry using the NIH-Image program (http://rsb.info.nih.gov/nih-image/).

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**Conflict of interest statement**

None declared.

**Abbreviations**

ANTS, 8-aminonaphthalene-1,3,6-trisulfonic acid; CIP, calf intestinal phosphatase; CSH, cell surface hydrophobicity; DTT, dithiothreitol; FACE, fluorophore-assisted carbohydrate electrophoresis; HMMP, high molecular mass mannotetraoses; MMLV, murine Maloney leukemia virus; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase-polymerase chain reaction.

**References**


