Changes in growth conditions affect the structure of mannan in *C. albicans*

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Abstract

Several studies have investigated the general structure of mannan, the carbohydrate portion of mannoprotein in the cell wall of *C. albicans*. Many of the previous studies that have characterized the structure of mannan from *C. albicans* used synthetic rich or minimal media such as YPD or SD for passage and propagation of *C. albicans* cells. We have hypothesized that cultivation of *C. albicans* on different media and at different temperatures will contribute to significant changes in the cell wall structure of mannan. In this study, strain SC5314 was inoculated from frozen stock onto YPD, blood and 5 % serum agar media. The samples were passed on the agar-based media three successive times at 30°C and 37°C, then were cultivated for 24 hr at 30°C in YPD. Cells were harvested and subjected to a standard mannan extraction method. We accomplished the detailed structural characterization of mannan using ^{1}H and ^{13}C 1D and 2D NMR techniques on a 950 MHz NMR. These results supported structural characterization of the mannans in order to define structural changes as a function of growth media at 30°C vs 37°C. Under 30°C growth conditions and relative to growth in YPD medium, the acid-stable portion of mannan grown in blood and serum contains less (1-2)-linked β-Man chain termination and (1-2)-linked α-Man-containing side chains while the acid-labile portion of mannan grown in blood contains less Manβ1-2Manα1- side chains. At 37°C, the reduction in these side chains for the acid-stable portion of the mannan is greater than at 30°C, while the level of side chains in the acid-labile portion remains unchanged relative to growth in YPD. In addition, for cells grown at 37°C in blood medium, -6Manα1- repeat units of the acid-stable polymer backbone are present at a lower level. These results indicate that the mannan undergoes structural modification when grown on blood or serum compared to a synthetic medium like YPD. These structural changes can easily be followed with NMR without the necessity of extensive sample degradation and isolation of individual side chain structures for detailed structural study. This study also demonstrates that recognition of structural changes in cell wall carbohydrates, as a function of the growth conditions, is critical to our understanding of the interaction of these carbohydrates with their host receptors.

Introduction

One of the major contributors to *C. albicans* virulence is its cell wall. The cell wall serves a major function of protecting the organism from osmotic stress as well as a support structure for attachment of adhesion molecules and hydrolytic enzymes on the surface of the cell. A complex matrix of chitin, glucan, mannan and mannoprotein, the cell wall of *C. albicans* has a layer of 1,4-linked glucosamine (chitin), to which is crosslinked 1,3-β-D-glucan that has several sidechains of 1,6-β-D-glucan that serve as attachment points for glycophosphoinositol-linked mannoproteins (Chauhan et al, 2009). Mannan, collectively, is both the carbohydrate component and carbohydrate-linked protein, but is often referred to as carbohydrate alone without the mannoprotein. In this study we have characterized the carbohydrate structure of mannan and the changes that occur in relation to how *C. albicans* is cultivated. We have applied nuclear magnetic resonance spectroscopy (NMR) to the structural characterization of mannan isolated by a previously reported method (Li et al, 2009). Cells were cultured at 30°C and 37°C on different growth media including blood, serum, and YPD. In a parallel study, we observed that when cells were grown on blood or serum the overall molecular mass increases at 37°C vs 30°C (Kruppa and Williams, 2010 Candida meeting poster). In addition to the shift in molecular mass, 1D and 2D NMR demonstrates that there are several changes in the overall structure of the mannan. We observed that there is less β- and α-1,2-linked mannan sidechains in both the acid-stable and acid-labile fractions of the molecule. Additionally, we observed that there is a significant reduction in the sidechains of β- and α-1,2-linked sidechains when grown at 37°C versus 30°C and a reduction of the length of α-1,6-linked mannan backbone in the acid-stable fraction while the level of acid-labile sidechains remained unchanged when material was isolated from cells grown on blood or serum compared with YPD. The results of this study represent a major insight into how *C. albicans* modulates the complexity of its mannan component when grown under different conditions including media and temperature. These results may help to explain why some fungal species become less virulent when cultivated for extended periods on synthetic media at 30°C.

Materials & Methods

Strains and media. *C. albicans* strain SC5314 was taken directly from frozen stock and passaged on YPD (1% yeast extract, 2% peptone, 2% dextrose, 2% agar), blood (5% sheep's blood, 4% Tryptic soy agar), and serum (5% serum, 2% agar) plate media at 30°C and 37°C. Cells were passaged every 48 hr a total of three times (**Figure 1**). For mannan extraction, cells were inoculated from the third passage of 30°C and 37°C plates into 2L of YPD for growth at 30°C for 18 hr. In addition, cells that were cultivated strictly at 37°C were also grown in 2L of YPD for mannan extraction at 37°C for 18 hr.

Mannan extraction. Mannan was isolated from cells using a modified extraction method as described previously (Li et al, 2009). Briefly, cells were delipidated with acetone, and the pellet disrupted by bead beating in dH_2O. The extracted cells were then autoclaved for 3 hr, and the extract centrifuged. The supernatant was saved and subjected to Fehling precipitation. The resulting precipitate was used for NMR analysis. From our cell preparations we generally recover 100-250mg of material from 10-15g cell wet weight.

NMR. Proton NMR spectra were collected on Bruker Avance II 600 and 950 NMR spectrometers using an inverse cryoprobe operating at 333°K (60°C) in 5-mm NMR tubes. Generally 20 mg of mannan was dissolved in ca. 600 µL D_2O. Proton 1D and 2D NMR spectra including COSY and NOESY were obtained in this study. Chemical shift referencing was accomplished relative to TMSP at 0.0 ppm.

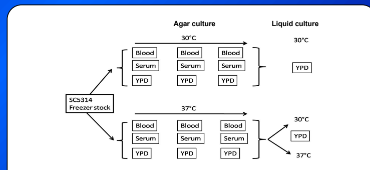


Figure 1. General scheme for growth of *Candida* for mannan extraction. *Candida albicans* was taken from a frozen stock and cultured on blood, serum and YPD agar plates and passaged in succession a total of three times before growth in liquid YPD culture.

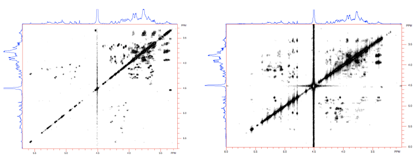
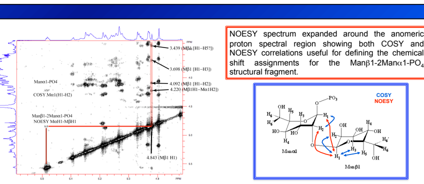


Figure 2. 950 MHz COSY (left) and NOESY (right) 2D NMR showing the entire carbohydrate spectral region.



NOESY spectrum expanded around the anomeric proton spectral region showing both COSY and NOESY correlations useful for defining the chemical shift assignments for the Manβ1-2Manα1-PO_4 structural fragment.
3.439 (Mβ1 [H1–H5?])
3.698 (Mβ1 [H1–H3])
4.092 (Mβ1 [H1–H2])
4.220 (Mβ1H1–Mα1H2])
Manα1-PO4
COSY Mα1(H1-H2)
Manβ1-2Manα1-PO4
NOESY MαH1-MβH1
4.843 (Mβ1 H1)
COSY
NOESY
Manα1
Manβ1

Figure 3. 950 MHz NOESY 2D NMR spectrum (left) expanded around the anomeric proton spectral region to assign inter-residue correlations and structures like Manβ1-2Manα1-PO_4 (right).

Results and Discussion

Over more than a decade, Ikura, Kobayashi, Lizicarova, Shibata and coworkers (Ikuta et al, 1997; Kobayashi et al., 1995, 2003; Lizicarova et al, 2007; Shibata et al, 1995, 2003, 2007) have made great strides in understanding the molecular structure of D-mannan isolated from fungal cell walls of several *Candida* species. They have elegantly used 2D NMR up to 600 MHz to examine the details of the structure of mannan side chains after carefully degrading the mannan and isolating the side chain fragments. Vinogradov and coworkers (Vinogradov et al, 1998) used 750 MHz NMR to extend these structural studies on intact mannan isolated from *Saccharomyces cerevisiae* without and with degradation followed by isolation of the fragmented side chains. Maes and coworkers (Maes et al, 2009) used solid-state magic-angle-spinning NMR at 800 MHz to examine mannan structures from intact cell walls of live *C. albicans* cells and observed the resonance for Manα1-Phosphate, not previously observed in solution-state NMR studies.

In this study we use results from 1D and COSY (**Figure 2**) and NOESY (**Figures 2-4**) 2D NMR studies at 950 MHz to detail structural changes in isolated cell wall D-mannan from *C. albicans SC5314* strain as a function of growth conditions – temperature and growth medium – based upon previous 1D and 2D NMR studies. From the 2D NOESY spectrum (**Figures 3 and 4**), various structural features can be discerned. For example, in the case of Manβ1-2Manα1-PO_4 (**Figure 3**), the anomeric proton, H1, of Manα1-PO_4 resonates at 5.56 ppm while H2 resonates at 4.220 ppm. The NOESY correlation across the glycosidic linkage of Manβ1-2Manα1-PO_4 between anomeric protons (ManαH1 [5.56 ppm]-ManβH1 [4.843 ppm]) is clearly evident. Manβ1 anomeric proton resonance correlates with Manα1 H2 (4.220 ppm) across the glycosidic link and to Manβ1 H2 (4.092 ppm), H3 (3.698 ppm), and H5 (3.439 ppm) within the Manβ1 repeat unit to define the Manβ1-2Manα1-PO_4 structural feature in the acid-labile fraction of mannan. Similarly other structural features involving both Manα and Manβ can be defined (**Figure 4**) where spectral regions for Manα-PO_4, Manα, and Manβ can be defined with subregions -2Manα1-, -6Manα1-, -2Manβ1-2Manβ1-2Manβ1- (β-β-β-), and -2Manβ1-2Manα1- (β-α-). Characterization of these structural features enables structural comparison of isolated mannans grown under different growth conditions of medium and temperature and definition of structural changes resulting from the different growth conditions (**Figures 5-7**).

Conclusions

1. **Our data indicate that *C. albicans* mannan undergoes significant structural changes as a function of growth conditions including temperature (30°C versus 37°C) and growth media (YPD, blood, and serum).**
2. **The structural differences impact both the acid-stable fraction as well as the acid-labile fraction of the carbohydrate structure.**
3. **We have demonstrated the ease of following these changes in mannan structural features using very high-field NMR (950 MHz) to develop an understanding of resonance assignments versus various structural features. However, we also employed 600 MHz NMR to characterize NMR spectra of mannans isolated from fungi grown under different growth conditions.**
4. **This approach to structural elucidation is fast and does not require extensive sample degradation and isolation of individual side chains.**
5. **Recognition of structural changes as a function of growth conditions in fungal cell wall carbohydrates, such as D-mannans and (1-3,1-6)-β-D-glucans, is critical to our ability to more accurately interpret the biochemistry of these complex carbohydrates.**
6. **These data also demonstrate that the response of *C. albicans* cell wall mannan to changing environmental conditions is dynamic and dramatic. How these cell wall structural changes benefit *C. albicans* remains to be elucidated.**

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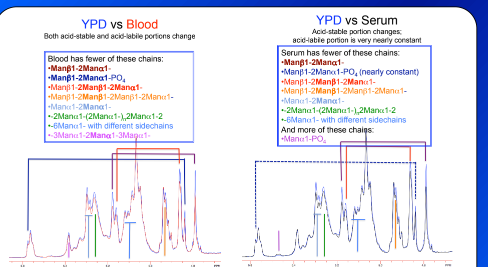
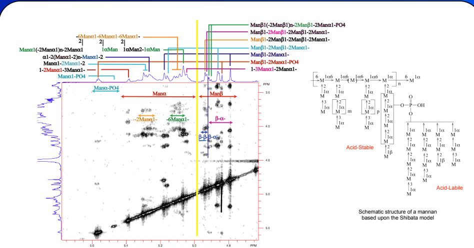


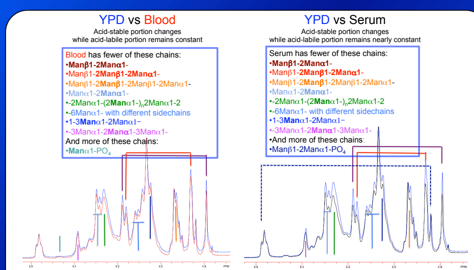
YPD vs Blood
Both acid-stable and acid-labile portions change
Blood has fewer of these chains:
•Manβ1-2Manα1-
•Manβ1-2Manα1-PO4
•Manβ1-2Manβ1-2Manα1-
•Manβ1-2Manβ1-2Manβ1-2Manα1-
•Manα1-2Manα1-
•-2Manα1-(2Manα1-)n2Manα1-2
•-6Manα1- with different sidechains
•-3Manα1-2Manα1-3Manα1-
YPD vs Serum
Acid-stable portion changes;
acid-labile portion is very nearly constant
Serum has fewer of these chains:
•Manβ1-2Manα1-
•Manβ1-2Manα1-PO4 (nearly constant)
•Manβ1-2Manβ1-2Manα1-
•Manβ1-2Manβ1-2Manβ1-2Manα1-
•Manα1-2Manα1-
•-2Manα1-(2Manα1-)n2Manα1-2
•-6Manα1- with different sidechains
And more of these chains:
•Manα1-PO4

Figure 5. Comparison of structural changes observed for mannan grown at 30°C on YPD, Blood, and Serum: YPD vs Blood (above left), YPD vs Serum (above right), and Serum vs Blood (below left). To compare these results to growth at 37°C, review Figure 6.



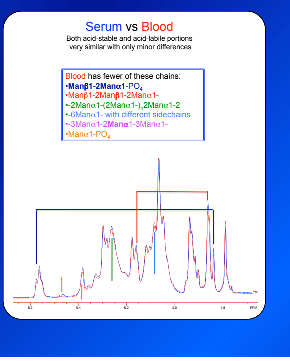
Manβ1(-2Manβ1)n-2Manβ1-2Manα1-PO4
Manβ1-2Manβ1-2Manβ1-2Manα1-
Manβ1-2Manβ1-2Manβ1-2Manα1-
Manβ1-2Manβ1-2Manα1-
Manβ1-2Manα1-
Manβ1-2Manα1-PO4
1-3Manα1-2Manα1-
-6Manα1-6Manα1-6Manα1-
Manα1(-2Manα1)n-2Manα1
1αMan
1αMan2-1αMan
α1-2(Manα1-2)n-Manα1-2
Manα1-2Manα1-2
1-2Manα1-3Manα1-
Manα1-PO4
Acid-Stable
Acid-Labile
Schematic structure of a mannan based upon the Shibata model

Figure 4. Assignment of anomeric ^{1}H resonances to specific structural features using the 950 MHz NOESY 2D NMR spectrum (left) and a schematic representation of the generic mannan carbohydrate structure (right) showing acid-stable and acid-labile structures based upon the Shibata model.

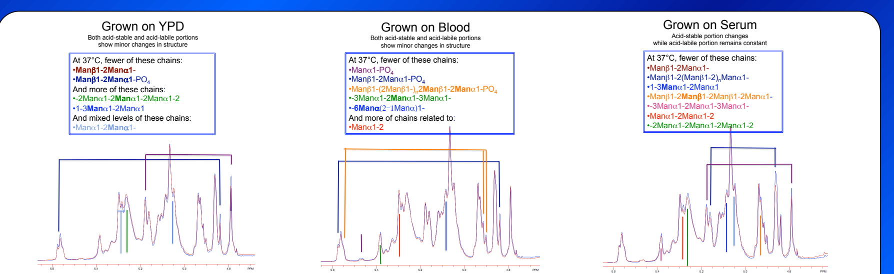


YPD vs Blood
Acid-stable portion changes
while acid-labile portion remains constant
Blood has fewer of these chains:
•Manβ1-2Manα1-
•Manβ1-2Manβ1-2Manα1-
•Manβ1-2Manβ1-2Manβ1-2Manα1-
•Manα1-2Manα1-
•-2Manα1-(2Manα1-)n2Manα1-2
•-6Manα1- with different sidechains
•1-3Manα1-2Manα1–
•-3Manα1-2Manα1-3Manα1-
And more of these chains:
•Manα1-PO4
YPD vs Serum
Acid-stable portion changes
while acid-labile portion remains nearly constant
Serum has fewer of these chains:
•Manβ1-2Manα1-
•Manβ1-2Manβ1-2Manα1-
•Manβ1-2Manβ1-2Manβ1-2Manα1-
•Manα1-2Manα1-
•-2Manα1-(2Manα1-)n2Manα1-2
•-6Manα1- with different sidechains
•1-3Manα1-2Manα1–
•-3Manα1-2Manα1-3Manα1-
•And more of these chains:
•Manβ1-2Manα1-PO4

Figure 6. Comparison of structural changes observed for mannan grown at 37°C on YPD, Blood, and Serum: YPD vs Blood (above left), YPD vs Serum (above right), and Serum vs Blood (below right). To compare these results to growth at 30°C, review Figure 5.

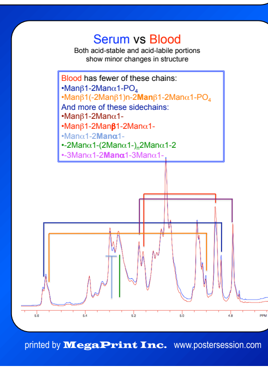


Serum vs Blood
Both acid-stable and acid-labile portions
very similar with only minor differences
Blood has fewer of these chains:
•Manβ1-2Manα1-PO4
•Manβ1-2Manβ1-2Manα1-
•-2Manα1-(2Manα1-)n2Manα1-2
•-6Manα1- with different sidechains
•-3Manα1-2Manα1-3Manα1-
•Manα1-PO4



Grown on YPD
Both acid-stable and acid-labile portions
show minor changes in structure
At 37°C, fewer of these chains:
•Manβ1-2Manα1-
•Manβ1-2Manα1-PO4
And more of these chains:
•-2Manα1-2Manα1-2Manα1-2
•1-3Manα1-2Manα1
And mixed levels of these chains:
•Manα1-2Manα1-
Grown on Blood
Both acid-stable and acid-labile portions
show minor changes in structure
At 37°C, fewer of these chains:
•Manα1-PO4
•Manβ1-2Manα1-PO4
•Manβ1-(2Manβ1-)n2Manβ1-2Manα1-PO4
•-3Manα1-2Manα1-3Manα1-
•-6Manα(2←1Manα)1-
And more of chains related to:
•Manα1-2
Grown on Serum
Acid-stable portion changes
while acid-labile portion remains constant
At 37°C, fewer of these chains:
•Manβ1-2Manα1-
•Manβ1-2(Manβ1-2)nManα1-
•1-3Manα1-2Manα1
•Manβ1-2Manβ1-2Manβ1-2Manα1-
•-3Manα1-2Manα1-3Manα1-
•Manα1-2Manα1-2
•-2Manα1-2Manα1-2Manα1-2

Figure 7. Comparison of structural changes observed for mannan grown at 30° vs 37°C on YPD (left), Blood (middle), and Serum (right).



Serum vs Blood
Both acid-stable and acid-labile portions
show minor changes in structure
Blood has fewer of these chains:
•Manβ1-2Manα1-PO4
•Manβ1(-2Manβ1)n-2Manβ1-2Manα1-PO4
And more of these sidechains:
•Manβ1-2Manα1-
•Manβ1-2Manβ1-2Manα1-
•Manα1-2Manα1-
•-2Manα1-(2Manα1-)n2Manα1-2
•-3Manα1-2Manα1-3Manα1-