The Neutral N-linked Glycans of the Basidiomycetous Yeasts *Pseudozyma antarctica* and *Malassezia furfur* (Subphylum Ustilaginomycotina)

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Ronilo Jose D. Flores¹, Takao Ohashi¹, Kanae Sakai², Tohru Gono³, Hiroko Kawasaki³ and Kazuhito Fujiyama¹,*

¹International Center for Biotechnology, Osaka University, Japan; ²Medical Mycology Research Center, Chiba University, Chiba, Japan; ³NITE Biological Resource Center (NBRC), National Institute of Technology and Evaluation (NITE), Chiba, Japan

*To whom correspondence should be addressed. Email: fujiyama@icb.osaka-u.ac.jp. Tel: +81-6-6879-7453. Fax: +81-6-6879-7454.

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R. J. D. Flores is an assistant professor at the Institute of Biological Sciences, College of Arts and Sciences, University of the Philippines Los Banos.
ABSTRACT

Pseudozyma antarctica and Malassezia furfur are basidiomycetous yeasts under the subphylum Ustilaginomycotina. P. antarctica is a commensal organism found in certain plant species, while M. furfur is associated with several skin diseases of animals including humans. N-linked glycans of P. antarctica and M. furfur were prepared, digested with glycosidases, and structurally analyzed using high performance liquid chromatography (HPLC) and mass spectrometry (MS). Analyses revealed the presence of neutral N-linked glycans ranging in length from Man₃GlcNAc₂-PA to Man₀GlcNAc₂-PA. The two species shared the most abundant neutral N-linked glycan: Man₁-2Man₁-6(Man₁-3)Man₁-6(Man₁-2Man₁-2Man₁-3)Manβ₁-4GlcNAcβ₁-4GlcNAc (M8A). The second and third most abundant neutral N-linked glycans for P. antarctica were Man₁-2Man₁-6(Man₁-2Man₁-3)Man₁-6(Man₁-2Man₁-2Man₁-3)Manβ₁-4GlcNAcβ₁-4GlcNAc (M9A) and Man₁-6(Man₁-3)Man₁-6(Man₁-3)Manβ₁-4GlcNAcβ₁-4GlcNAc (M5A), respectively. In the case of M. furfur, Man₁-2Man₁-6(Man₁-3)Man₁-6(Man₁-2Man₁-3)Manβ₁-4GlcNAcβ₁-4GlcNAc (M7A) was the second most abundant, while both M8A and M9A were tied for the third most abundant. The presence of putative galactose residues in the hypermannosylated neutral N-linked glycans is also discussed. This report is the first to analyze the neutral N-linked glycans of P. antarctica and M. furfur.
Asparagine (N)-linked glycosylation is found both in eukaryotes and prokaryotes and is an extensive process which results in the attachment of an oligosaccharide to the asparagine residue of a polypeptide (Schwarz and Aebi, 2011). In eukaryotic cells, almost 50% of cellular proteins and 90% of secreted proteins are found in glycosylated forms, making glycosylation one of the most abundant forms of post-translational modification (Agard and Bertozzi, 2009). Glycosylation affects a range of functions, including protein folding and trafficking (Mitra et al., 2003) and organ development (Grobe et al., 2002). In addition, glycans have been revealed to play a role in various biological processes, such as cell adhesion, receptor activation, signal transduction, endocytosis, and disease development (Ohtsubo and Marth, 2006). Recognition of the glycan moieties on proteins of invading microorganisms and viruses is essential in categorizing these as foreign molecules in the human body (Herrmann et al., 1997; Suzuki, 2005; Upreti et al., 2003). The N-linked glycosylation machinery, especially the early steps, is conserved throughout the eukaryotic domain (Kukuruzinska and Lennon, 1998). In the cytoplasmic face of the endoplasmic reticulum (ER), the synthesis of the N-linked glycan precursor is initiated by the transfer of N-acetylglucosamine phosphate (GlcNAc-P) from UDP-GlcNAc onto the dolichol phosphate to form GlcNAc-PP-dolichol, which is then further modified with one GlcNAc and 5 mannose (Man) residues, yielding Man$_3$GlcNAc$_2$-PP-dolichol. This intermediate is then flipped into the ER lumen, where it is further extended by the additions of glucose (Glc) and Man residues to form the Glc$_3$Man$_9$GlcNAc$_3$-PP-dolichol, before being transferred en bloc to a nascent polypeptide through the action of the oligosaccharyltransferase complex (Hamilton and Gerngross, 2007). The sequential actions of glucosidase I, glucosidase II, and ER α1,2-mannosidase allow for the removal of three Glc residues and one Man residue.
resulting in the Man$_8$GlcNAc$_2$ glycan structure, which is then transported to the Golgi, where a diverse process proceeds through the remaining secretory pathways involving either the addition or removal of certain sugar moieties. Therefore, the size, components, and branching patterns of the mature glycans differ among the different types of cell, tissue and species (Kornfeld and Kornfeld, 1985; Paulson and Colley, 1989).

Yeasts are phylogenetically classified into two phyla, the Ascomycota and the Basidiomycota. The ascomycetous yeasts are taxonomically grouped under subphyla Saccharomycotina and Taphrinomycotina while the basidiomycetous yeasts are taxonomically grouped under the subphyla Ustilaginomycotina, Agaricomycotina and Pucciniomycotina (Hibbett et al., 2007). The Ustilaginomycotina yeasts are further classified into four classes, namely Exobasidiomycetes, Malasseziomycetes, Moniliellomycetes and Usitilaginomycetes (Wang et al., 2015). Although some basidiomycetous yeasts possess both the yeast and mycelial stages in their life cycles, sexual stages of the others such as P. antarctica and M. furfur have never been found (Morita et al., 2014; Velegraki et al., 2015). Some basidiomycetous yeasts—such as smuts and rusts—are known to be plant pathogens, while others are animal pathogens such as the genera Cryptococcus and Malassezia. In addition, some basidiomycetous yeasts, including Pseudozyma, are commensals of different plant species. P. antarctica, just like any other species of Pseudozyma, is frequently isolated in plant material such as leaves, flowers and stems (Boekhout and Fell, 1998). In contrast, M. furfur is naturally found on the skin surface of mammals and can potentially become pathogenic due to some predisposing factors such as the health state of the host. Although there are several studies on the biology, physiology, ecology and biotechnological applications of these species, their N-linked glycans have almost never been studied. To our knowledge, the only exceptions are a study of M. furfur, in which the linear
β1-6 linked galactofuranose polysaccharides carrying around 30 residues were assumed to be attached to the canonical N-linked core high-mannose glycans interlinked by the yeast-type mannan polysaccharides (Shibata et al., 2009), and a study of *Symphidiomycopsis paphiopedili*, in which high-mannose glycans with slight amounts of putative hypermannosylated glycans were detected (Flores et al., 2017). Elucidating the glycan structures of these yeasts is important not only for understanding the biological roles that these glycans play in their normal yeast physiology and in infecting host cells, as in the case of *M. furfur* and several other non-pathogenic and pathogenic yeasts, but also in realizing the tremendous biotechnological potential of these yeasts as being suitable alternatives to mammalian cell cultures in heterologous protein production (Gerngross, 2004). *P. antarctica* had been shown to be an excellent producer of mannosylerythritol lipids (MELs) that are of great biotechnological interest as a biosurfactant, anti-tumor agent, cell-differentiation inducer, moisturizer and hair-repairer (Kitamoto et al., 1990; Kitamoto et al., 2002; Kitamoto et al., 2009). Enzymes produced by *P. antarctica* have also been used to degrade biodegradable polymers (Shinozaki et al., 2013). Moreover, *P. antarctica* itself had been used as a host for high-level recombinant protein production of biodegradable plastic-degrading enzymes, which clearly underscores the biotechnological potential of this yeast (Watanabe et al., 2016).

Therefore, we selected the two basidiomycetous yeasts *P. antarctica* and *M. furfur* from the order Ustilaginales (class Ustilaginomycetes) and the order Malasseziales (class Malasseziomycetes), respectively, and report their neutral N-linked glycan structures. Our present analysis of the neutral N-linked glycan structures of these two species together with our previous report on the N-linked glycan structures of *S. paphiopedili* in the order Microstomatales (class Exobasidiomycetes) (Flores et al., 2017) constitute the initial N-linked glycan baseline.
information in the Ustilaginomycotina yeasts. High performance liquid chromatography (HPLC) and mass spectrometry (MS) analyses were carried out following the extraction and processing of the neutral N-linked glycans from the three species. High mannose-type glycans were detected in the neutral N-linked glycan fraction. The Man\(\alpha_1\)-2\(\alpha\)Man\(\alpha_1\)-6(Man\(\alpha_1\)-2\(\alpha\)Man\(\alpha_1\)-3)\(\beta\)1-4GlcNAc\(\beta\)-1-4GlcNAc (M8A) glycan structure was found to be the most abundant type of glycan in the two species studied. In \textit{P. antarctica}, this was followed by Man\(\alpha_1\)-2\(\alpha\)Man\(\alpha_1\)-6(Man\(\alpha_1\)-2\(\alpha\)Man\(\alpha_1\)-3)\(\beta\)1-4GlcNAc\(\beta\)-1-4GlcNAc (M9A) and Man\(\alpha_1\)-6(Man\(\alpha_1\)-3)\(\beta\)1-4GlcNAc\(\beta\)-1-4GlcNAc (M5A), whereas, in \textit{M. furfur}, Man\(\alpha_1\)-2\(\alpha\)Man\(\alpha_1\)-6(Man\(\alpha_1\)-3)\(\beta\)1-4GlcNAc\(\beta\)-1-4GlcNAc (M7A) was the second most abundant, and M8A and M9A were tied for third most abundant.

**MATERIALS AND METHODS**

**Yeast strains and medium**

The \textit{P. antarctica} NBRC 10750 and \textit{M. furfur} IFM 48685 strains were provided by the National Bioresource Research Center, NITE (Chiba, Japan) and the Medical Mycology Research Center of Chiba University (Chiba, Japan), respectively. \textit{P. antarctica} was sub-cultured in 10 mL of yeast-malt extract broth (YMB) for 48 h at 24°C and then cultured on 100 mL of YMB with 10% glycerol. In the case of \textit{M. furfur}, the culture was performed on yeast-peptone-dextrose (YPD) broth supplemented with 1% olive oil at 30°C. After 72 h of incubation, the yeast cells were harvested via centrifugation at 6,000 rpm for 5 min. The yeast pellet was then subjected to glycoprotein extraction.
2-Aminopyridine-tagged glycan preparation

The yeast pellets were resuspended on citrate buffer (20 mM citrate-NaOH, pH 7.0) and autoclaved at 121°C for 90 min (Ohashi et al., 2010). The released glycoproteins were then precipitated in methanol at 4°C for 1 h. Methanol was removed from the suspension by centrifugation and the glycoprotein pellets were dissolved in hot water, and subjected to dialysis and lyophilization. Hydrazinolysis using hydrazine anhydrate at 100°C for 10 h was then used to release the N-linked glycans from the glycoprotein as previously described (Yoshizawa et al., 1966). The reducing ends of the released glycans were fluorescently tagged by 2-aminopyridine (PA) (Hase et al., 1978). The PA-tagged glycans (PA-glycan) were then lyophilized and dissolved in ultrapure water.

High performance liquid chromatography

Anion-exchange HPLC was employed using a TSKgel DEAE-5PW column (7.5 mm × 75 mm, Tosoh, Tokyo). Ultrapure water adjusted to pH 9.0 using aqueous ammonia was used as solvent A, while 0.5 M AcOH adjusted to pH 9.0 using aqueous ammonia was used as solvent B. The glycans were eluted by increasing the solvent B concentration to 100% over a period of 20 min with a flow rate of 1 mL/min. The acidic PA-glycans containing sialic acids from human α1-acid glycoprotein (Sigma-Aldrich, St. Louis, MO, USA) were used as the standard to determine the elution positions of the neutral and acidic PA-glycan fractions (Fournier et al., 2000). Neutral PA-glycans that were eluted in the flow-through fraction were collected and lyophilized. The neutral PA-glycan samples were dissolved in ultrapure water and were subjected to normal-phase (NP) HPLC using a TSK Amide-80 column (2.0 mm × 150 mm; Tosoh). HPLC-grade acetonitrile (solvent C) and 20 mM formic acid adjusted to pH 4.0 with aqueous ammonia.
(solvent D) were used. The solvent D concentration was linearly increased to 100% for 50 min at a flow rate of 0.2 mL/min after initial column equilibration with 100% of the solvent C for 20 min at a flow rate of 0.2 mL/min. The eluted peaks were collected, lyophilized, and re-dissolved in ultrapure water for subsequent analyses.

The PA-glycans collected in NP-HPLC were subjected to reversed-phase (RP) HPLC using a Cosmosil 5C18-AR-II waters column (4.6 mm × 250 mm; Nacalai Tesque, Kyoto, Japan). Equilibration was carried out using 0.02% trifluoroacetic acid (TFA) in ultrapure water (solvent E) with a flow rate of 0.7 mL/min. Increasing the concentration of 20% HPLC-grade acetonitrile in 0.02% TFA (solvent F) to 20% for 50 min allowed for the elution of peaks. Some standard isomeric forms of high-mannose-type PA-glycans ranging from Man$_3$GlcNAc$_2$-PA to Man$_9$GlcNAc$_2$-PA (Takara, Shiga, Japan) were used as standards. Peaks eluted in RP-HPLC were collected, lyophilized, and stored for the subsequent analyses.

**Glycosidase digestions**

The PA-glycans were subjected to digestion with endoglycosidase H (Endo H, New England Biolabs, Beverly, MA, USA) according to the manufacturer’s protocol or 100 munits of jack bean α-mannosidase (Sigma-Aldrich) in 50 mM citrate-NaOH at pH 4.5 for 12 h at 30°C. PA-glycans that did not co-elute with standard PA-glycans were first digested with 2.5 munits of α1-2 mannosidase (Seikagaku Co., Tokyo) in 100 mM AcOH-NaOH at pH 5.0 for 16 h at 30°C. The resultant peaks were then digested separately with 40 munits of α1-6 mannosidase (New England Biolabs) and with 32 munits of α1-2/1-3 mannosidase (New England Biolabs) according to the manufacturer’s protocol.
Mass spectrometry

Lyophilized samples collected in RP-HPLC corresponding to peaks which did not co-elute with standard PA-glycans were subjected to liquid chromatography-MS/MS (LC-MS/MS) using an HCT-Plus ion-trap mass spectrometer (Bruker Daltonics, Bremen, Germany) connected to an Agilent Technologies 1200 HPLC Series unit (Agilent Technologies, Santa Clara, CA, USA) with the following parameters: scan range of 350–2750 mass-to-charge ratio (m/z); nebulizer flow of 40 psi; dry gas flow rate of 5.0 L/min; dry temperature of 300°C; target count of 200,000; and MS/MS fragmentation amplification of 1.0 V in the positive-ion mode. Ten picomoles of each PA-glycan were dissolved in MS-grade acetonitrile to make a total of 50 µL.

For the LC analysis, acetonitrile/acetic acid (solvent G: 98/2, v/v) was used to equilibrate the column. The PA-glycans were then separated using a Shodex Asahipak NH2P-50 2D column (2.0 mm × 150 mm; Showa Denko Co., Ltd., Tokyo) and by increasing the solvent H (water/AcOH/triethylamine; 92/5/3, v/v/v) concentration from 20% to 55% over 35 min at a flow rate of 0.2 mL/min.

Monosaccharide component analysis

Monosaccharide component analysis was performed essentially as previously described (Hase et al., 1992). Peaks eluted later in NP-HPLC (peaks p-h and m-j) that might correspond to higher-sized high-mannose-type glycans were collected and lyophilized. Two milligrams of the lyophilized fraction was dissolved in 100 µL of 4 M hydrochloric acid at 100°C for 3 h followed by repeated lyophilization (3 times). Re-N-acetylation was performed using 40 µL of pyridine:MeOH (1:9, v/v) and 10 µL of acetic anhydride at room temperature for 30 min followed by lyophilization. The reducing ends of the released monosaccharides were
fluorescently tagged by PA (Hase et al., 1978). The PA-monomosaccharides (PA-Mono) were then lyophilized and dissolved in 100 µL ultrapure water. Two microliters of dissolved PA-Mono was isocratically eluted by HPLC-grade acetonitrile:0.8 M H$_3$BO$_3$-KOH at pH 9.0 (Solvent I, 1:9, v/v) using a TSKgel Sugar AXI column (4.6 mm × 150 mm; Tosoh, Tokyo) at 65°C for 160 min.

**RESULTS AND DISCUSSION**

PA-glycans prepared from *P. antarctica* and *M. furfur* are composed of neutral *N*-linked glycans of varying sizes.

Anion-exchange HPLC revealed peaks (Fig. 1) eluted within the elution times at which the acidic glycans of the standard human α1-acid glycoprotein, which may contain one, two, or three sialic acid residues, are also eluted (Fournier et al., 2000). The structural complexity of the glycans of some yeasts may be brought about by acidic groups such as pyruvates and phosphates (Gemmill and Trimble 1999). The largest peak eluted around 5 min did not contain any PA-glycans, confirmed by NP-HPLC analysis as described elsewhere (Natsuka et al., 2006). The neutral PA-glycan-containing fractions were further separated by NP-HPLC (Fig. 2). The presumptive neutral Man$_3$GlcNAc$_2$PA-sized glycans, which represented well-separated peaks, were collected, freeze-dried, and dissolved for RP-HPLC analysis.

**Alpha-linked mannoses are attached to the non-reducing ends of the *N*-linked glycans.**

Jack bean α-mannosidase has the ability to hydrolyze the α1-2/3/6 Man linkages in the non-reducing termini of the glycans (Li, 1967; Snaith and Levy, 1968). It was therefore used to confirm whether the peaks contain α-linked Man residues at the non-reducing end. *P. antarctica* and *M. furfur* PA-glycan peaks later eluted from the standard Man$_3$GlcNAc$_2$-PA position were
digested, resulting in a $\text{Man}_3\text{GlcNAc}_2$-PA (M1-PA) glycan as analyzed using NP-HPLC (Fig. 2). The digested M9A-PA glycan standard was used to determine the elution position of the resultant M1-PA glycan resultant peak. Based on the elution position shifts to the peak at the M1-PA glycan position, it can be said that $\alpha$-linked Man residues are present at the non-reducing termini of the neutral PA-glycans. The seven PA-glycan peaks of *P. antarctica* were then collected and designated accordingly as p-a, p-b, p-c, p-d, p-e, p-f and p-g, while *M. furfur* also had nine peaks collected and designated as m-a, m-b, m-c, m-d, m-e, m-f, m-g, m-h and m-i. All of these peaks were eluted at positions close to those of the standard $\text{Man}_3\text{GlcNAc}_2$-PA glycans. Moreover, the peaks that were eluted later in the chromatograph (designated as p-h and m-j for *P. antarctica* and *M. furfur*, respectively) may correspond to other higher-sized high-mannose-type glycans.

**Galactose residues were detected in the fraction corresponding to higher-sized N-linked glycans**

Shibata et al. (2009) previously reported the presence of $\beta_1$-6 galactofuranose-containing galactomannan antigens in the cell wall of *M. furfur* NBRC 0656. In their study, cell wall polysaccharides were determined to be the $\beta_1$-6 galactofuranose-containing yeast-type mannan structure composed of an $\alpha_1$-6 linked Man backbone decorated with $\alpha_1$-2 linked Man residues based on a battery of analyses such as nuclear magnetic resonance (NMR) and antibody reactivity analyses. However, $\beta_1$-6 galactofuranose-containing galactomannan has never been detected in the *N*-linked glycan-rich fractions of either *M. furfur* NBRC 0656 or IFM 48685. We examined the monosaccharide composition of the later eluted peak (peak m-j) of *M. furfur* in NP-HPLC, which was susceptible to Endo H, and found the presence of galactose (Gal)-PA.
(Figs. 2 and 3), suggesting that galactofuranose residues might also be present in the putative larger neutral N-linked glycans. Hence, the detection of a Gal-PA peak corroborates the previous conjecture of Shibata et al. that galactofuranose residues are present on the N-linked glycans in *M. furfur*. Furthermore, the large amounts of Glc-PA as well as the small amounts of N-acetylgalactosamine (GalNAc)-, rhamnose-, and xylose (Xyl)-PA were found in the later eluted peak of *M. furfur*. They might have been partially derived from the cell wall polysaccharides, because some of the later eluted peak was resistant against Endo H. In addition, the presence of the GalNAc residues in the exopolysaccharides in *Aspergillus fumigatus* was also reported (Lee et al., 2015). Interestingly, the Xyl-PA and Gal-PA were also found in the later eluted peak (peak p-h) of *P. antarctica* in NP-HPLC as well as GlcNAc-, Glc- and Man-PA (Fig. 3). To unveil the detailed structures of the later eluted peaks from both species, the glycosidase digestion analysis using the galactofuranose-specific glycosylhydrolases followed by the HPLC or NMR analysis would be highly needed.

**Isomeric forms of the PA-glycan peaks isolated from NP-HPLC were detected using RP-HPLC.**

The NP-HPLC-collected PA-glycans were subjected to RP-HPLC to separate and possibly identify various isomeric forms co-eluting with the standard PA-glycan isoforms. Figures 4 and 5 show that some of the NP-HPLC peaks of *P. antarctica* and *M. furfur* were composed of various isomeric forms with correspondingly named chromatogram peaks. The structures of these abundant glycans were assigned by virtue of their co-elution with standard PA-glycans in both NP-HPLC and RP-HPLC. The presence of particular isomeric forms and their relative amounts differed among the species based on both the NP-HPLC and RP-HPLC
chromatograms. In *P. antarctica*, the most abundant PA-glycan detected was M8A glycan (32%), followed by M9A (14%) and M5A glycan glycans (10%). In the case of *M. furfur*, M8A was also the most abundant at 25%, followed by the M7A (16%) and M8B (15%) glycans. Interestingly, the M8A glycan structure was found to be the commonly most abundant neutral PA-glycan for both *P. antarctica* and *M. furfur*. This result also agrees with our previous report in which the M8A structure was shown to be the dominant structure in *S. paphiopedili*, which is also classified under the subphylum Ustilaginomycotina (Flores et al., 2017). Some peaks, however, did not co-elute precisely with the standard PA-glycans and were therefore subjected to additional glycosidase digestion and MS analyses to confirm their composition and presumptive structures. The standard PA-glycans used in this study and their determined structures are presented in Fig. 6.

**Glycosidase digestion and MS analyses of peaks p-ra1, m-rb1 and m-re1, which did not co-elute with standard PA-glycans, revealed other possible glycan structures**

Peaks p-ra1 and m-rb1—which co-eluted in RP-HPLC and NP-HPLC but did not co-elute with commercially available standard PA-glycans—could be identified as M4 glycan isomers other than M4B, while peak m-re1 might have been M6 glycan isomers other than M6A, M6B or M6C. These all three peaks were subjected to digestion with α1-2 mannosidase and α1-6 mannosidase or α1-2/1-3 mannosidase in a successive manner in order to determine the linkages by which the mannoses are attached (Fig. 7). The glycans for peaks p-ra1, m-rb1 and m-re1 glycans were first digested with α1-2 mannosidase and detected using NP-HPLC. Peaks p-ra1 and m-rb1 each yielded an M3-sized peak (p-ra1a and m-rb1a, respectively) after digestion with α1-2 mannosidase, indicating the presence of one α1-2 linked Man residue. Peaks p-ra1a and m-
rb1a were collected and separately digested with α1-2/1-3 mannosidase and α1-6 mannosidase. Both of these M3-sized peaks yielded an M2-sized peak after digestion with α1-2/1-3 mannosidase, indicating the presence of one α1-3 linked Man residue. But these peaks were not susceptible to α1-6 mannosidase, indicating the possibility that they consist of an α1-6 linked Man residue to which another α1-3 linked Man residue is attached (Manα1-3Manα1-6Manβ1-4GlcNAcβ1-4GlcNAc-PA), or they consist of an α1-6 linked Man residue attached to a β1-4 Man residue that is branched by an α1-3 linked Man residue (Manα1-3(Manα1-6)Manβ1-4GlcNAcβ1-4GlcNAc-PA). The α1-6 mannosidase enzyme was able to digest M4B-PA under the same enzymatic conditions (data not shown). On the other hand, the m-re1 peak yielded an M4-sized peak (m-re1a) after digestion with α1-2 mannosidase, indicating the presence of two α1-2 linked mannoses. The m-re1a peak yielded an M2-sized glycan after digestion with α1-2/1-3 mannosidase, indicating the presence of two α1-3 linked mannoses, but was not susceptible to α1-6 mannosidase after digestion. These M4-sized peaks (p-ra1 and m-rb1) and the M6-sized peak (m-re1) were co-eluted with the M4-sized peak (s-rb1) and M6-sized peaks (s-re1 and s-re2) from S. paphiopedili in NP- and RP-HPLC, respectively (Flores et al., 2017).

LC-MS/MS analysis of M4-sized glycans (peak p-ra1 and peak m-rb1) and an M6-sized glycan (peak m-re1) was performed in order to confirm the composition of the individual RP-HPLC-isolated peaks (Fig. 8). The full length of the presumptive Man₄GlcNAc₂-PA-glycan peaks (p-ra1 and peak m-rb1) yielded m/z values of 1152.0 [M+H]+ and 1152.2 [M+H]+, respectively, with the loss of each successive fragment ion corresponding to one hexose residue, while the m/z of 503.2 corresponds to the GlcNAcβ1-4GlcNAc-PA residue (Figures 8A and 8B). On the other hand, the full length of the presumptive Man₆GlcNAc₂-PA glycan (peak m-re1) yielded an m/z of 1476.3 [M+H]+, wherein each preceding generated fragment ion corresponds to
a loss of one hexose residue, and an m/z of 503.3 corresponds to the GlcNAcβ1-4GlcNAc-PA residue (Fig. 8C). Considering the results of the mannosidase digestions of the PA-glycan samples, and the HPLC and LC-MS/MS analyses, it can be concluded that the N-linked glycans of the two species are of the high-mannose-type, which range from Man$_{4-9}$GlcNAc$_2$ in P. antarctica and Man$_{3-9}$GlcNAc$_2$ in M. furfur. Tables 1 and 2 summarize the composition, structure designation and percentage of the various peaks detected. The possible structures for these non-co-eluting peaks based on glycosidase digestions and MS analysis are presented in Fig. 9. As discussed in our previous paper, the M4- and M6-sized glycans may have been the results of N-linked glycan processing by putative α1-2, α1-3 and/or α1-6 mannosidases (Flores et al., 2017). As per the Carbohydrate Active Enzyme (CAZy) database (Lombard et al., 2014), there are a number of putative mannosidases present in other basidiomycetous yeast strains, it is therefore possible that certain α1-2/1-3 and/or α1-6 mannosidases belonging to any of three GH families, i.e., GH 38, GH 47 and GH 92, are partly or fully responsible for cleaving the mannose residues of the PA-glycan structures of P. antarctica and M. furfur. However, these mannosidases have not been completely characterized. An analysis of P. antarctica JCM 10317 strain using the Basic Local Alignment Search Tool (BLAST) revealed the presence of putative mannosidase genes or proteins that potentially belong to the GH 38, 47 and 92 families. Representative proteins within the multitude of hits annotated in the National Center for Biotechnology Information (NCBI) database include XP_014659398.1, XP_014654165.1, and XP_014658277.1 (Boratyn et al., 2013). The complete genome information of P. antarctica JCM 10317 and the partial genome sequence of the P. antarctica T-34 strain have already been published, and will be useful in studying the putative mannosidases of this strain (Morita et al., 2013; Saika et al., 2014).
In this study, we report the neutral N-linked glycan structures of *P. antarctica* and *M. furfur*. High-mannose-type N-linked glycans were detected in the neutral N-linked glycan fractions in the two species. Interestingly, *P. antarctica* and *M. furfur* share the most abundant glycan, M8A. Both of these yeasts also share the most abundant M8A structure with *S. pahiopedili* (Flores et al., 2017), which is also classified under Ustilaginomycotina. Moreover, the number of confirmed Man residues of neutral N-linked glycans of both species ranges from 3 to 9 residues, which differs from the numbers found in *Saccharomyces* and *Schizosaccharomyces* carrying hypermannosylated glycans, but is within the range of the number found in *S. paphiopedili* (Gemmill and Trimble, 1999; Flores et al., 2017). The analysis of the glycan structure for known commensals and pathogens such as *P. antarctica* and *M. furfur* may also provide initial insights into the roles of these glycans in adhesion, pathogenicity, and virulence. The mannosyltransferase Och1p is the key enzyme for the synthesis of hypermannosylated glycans, and the och1-deletion mutants of some ascomycetous yeast species, such as *S. cerevisiae*, *S. pombe*, *Pichia pastoris*, *Hansenula polymorpha*, *Kluyveromyces lactis*, *Yarrowia lipolytica*, and an ascomycetous filamentous fungus *Neurospora crassa*, exhibited altered morphological phenotypes under stress conditions, suggesting that the outer chain N-linked glycans are important for yeast and fungal cell wall integrity, a primary consideration in host-fungus interactions (Nagasu et al., 1992; Yoko-o et al., 2001; Choi et al. 2003; Kim et al., 2006; Uccelletti et al., 2006; Song et al., 2007; Maddi and Free, 2010). Moreover, the α1-6 Man-containing polysaccharides of some important fungal pathogens, including *C. albicans*, *Aspergillus fumigatus*, and *Cryptococcus neoformans*, have been shown to affect virulence by och1 deletion (Kotz et al., 2010; Park et al., 2012; Leach and Brown, 2012). Therefore, our study
may also serve as a starting point for future studies concerning the roles of these high-mannose-
type N-linked glycans in host-fungus interactions and other biological processes.

In conclusion, this baseline elucidation of the various neutral N-linked glycans provides a
necessary first step toward understanding the glycosylation of the biotechnologically important P.
antarctica and the medically relevant M. furfur. Even though the specific biological roles of
these glycans are yet to be elucidated, it can be noted that the detected range of neutral N-linked
glycans in P. antarctica (order Ustilaginales) and M. furfur (order Malasseziales) corresponds
with the range detected previously in S. paphiopedili (order Microstomatales). Interestingly, the
most abundant structure detected in the high-mannose N-linked glycan fraction in the 3 yeasts
from the different order under the same subphylum was M8A. Moreover, the unique glycan
structures detected in this study were similar to those detected in S. paphiopedili. This may have
been due to the close phylogenetic relationships among these species, since all three are
basidiomycetous yeasts. Analysis of the other representative species from the different orders
within the subphylum Ustilaginomycotina and of other species from other subphyla will expand
our current baseline information and might provide a starting point toward understanding of the
significance of N-linked glycans in biological functions, and even possibly, in evolution. The
evolution of the glycosylation machinery and the presence of similarly functioning glycosidases
across closely-related species may lead to certain similarities in the glycan fingerprints. It would
thus be interesting to explore these glycosylation-related enzymes to provide a better
understanding of Ustilaginomycetes glycosylation. While it would be advantageous to conduct
an intensive analysis of N-linked glycan structures in the Ustilaginomycotina, an analysis of the
putative mannosidases is also necessary. The search for these mannosidases, and their isolation
and characterization, is currently underway. Generally, the information presented in this study
should help to provide a clearer picture of the $N$-linked glycosylation pathway, and should pave the way for an analysis of related putative mannosidase enzymes with potential future use in biotechnological applications.

ACKNOWLEDGEMENT

This study was partly supported by the Cooperative Research Program of Medical Mycology Research Center, Chiba University (11-9 and 12-7) to TO.
Table 1. Peak designation, confirmed identity, elution time in NP-HPLC and RP-HPLC, m/z and percentage quantity of neutral N-linked glycans of *P. antarctica*.

<table>
<thead>
<tr>
<th>Peak</th>
<th>Composition</th>
<th>Elution Time (min)</th>
<th>m/z</th>
<th>Identity</th>
<th>Abundance%</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-ra1</td>
<td>Hex$_4$GlcNAc$_2$</td>
<td>24.5</td>
<td>26.9</td>
<td>1152.0</td>
<td>M4*</td>
</tr>
<tr>
<td>p-rb1</td>
<td>Hex$_4$GlcNAc$_2$</td>
<td>25.0</td>
<td>27.0</td>
<td>1152.1</td>
<td>M4B</td>
</tr>
<tr>
<td>p-rc1</td>
<td>Hex$_5$GlcNAc$_2$</td>
<td>28.0</td>
<td>26.4</td>
<td>1314.4</td>
<td>M5A</td>
</tr>
<tr>
<td>p-rd1</td>
<td>Hex$_6$GlcNAc$_2$</td>
<td>29.3</td>
<td>23.7</td>
<td>1476.5</td>
<td>M6A</td>
</tr>
<tr>
<td>p-rd2</td>
<td>Hex$_6$GlcNAc$_2$</td>
<td>29.3</td>
<td>25.2</td>
<td>1476.5</td>
<td>M6B</td>
</tr>
<tr>
<td>p-re1</td>
<td>Hex$_7$GlcNAc$_2$</td>
<td>30.3</td>
<td>21.5</td>
<td>1638.4</td>
<td>M7A</td>
</tr>
<tr>
<td>p-re2</td>
<td>Hex$_7$GlcNAc$_2$</td>
<td>30.3</td>
<td>23.1</td>
<td>1637.9</td>
<td>M7B</td>
</tr>
<tr>
<td>p-rf1</td>
<td>Hex$_8$GlcNAc$_2$</td>
<td>31.5</td>
<td>20.2</td>
<td>1800.9</td>
<td>M8A</td>
</tr>
<tr>
<td>p-rf2</td>
<td>Hex$_8$GlcNAc$_2$</td>
<td>31.5</td>
<td>22.3</td>
<td>1800.7</td>
<td>M8B</td>
</tr>
<tr>
<td>p-rg1</td>
<td>Hex$_9$GlcNAc$_2$</td>
<td>32.5</td>
<td>21.2</td>
<td>1962.7</td>
<td>M9A</td>
</tr>
</tbody>
</table>

* Possible isomer other than M4B
Table 2. Peak designation, confirmed identity, elution time in NP-HPLC and RP-HPLC, m/z and percentage quantity of neutral N-linked glycans of *M. furfur*.

<table>
<thead>
<tr>
<th>Peak</th>
<th>Composition</th>
<th>Elution Time (min) (NP-HPLC) (RP-HPLC)</th>
<th>m/z</th>
<th>Identity</th>
<th>Abundance%</th>
</tr>
</thead>
<tbody>
<tr>
<td>m-ra1</td>
<td>Hex3GlcNAc2</td>
<td>22.5 26.8</td>
<td>990.1</td>
<td>M3B</td>
<td>3.0</td>
</tr>
<tr>
<td>m-rb1</td>
<td>Hex4GlcNAc2</td>
<td>24.5 26.9</td>
<td>1152.2</td>
<td>M4*</td>
<td>3.0</td>
</tr>
<tr>
<td>m-rc1</td>
<td>Hex4GlcNAc2</td>
<td>26.2 27.0</td>
<td>1152.0</td>
<td>M4B</td>
<td>3.2</td>
</tr>
<tr>
<td>m-rd1</td>
<td>Hex5GlcNAc2</td>
<td>28.0 26.8</td>
<td>1314.4</td>
<td>M5A</td>
<td>7.8</td>
</tr>
<tr>
<td>m-re1</td>
<td>Hex6GlcNAc2</td>
<td>28.5 25.0</td>
<td>1476.3</td>
<td>M6**</td>
<td>5.2</td>
</tr>
<tr>
<td>m-rf1</td>
<td>Hex6GlcNAc2</td>
<td>29.3 28.2</td>
<td>1476.9</td>
<td>M6C</td>
<td>6.8</td>
</tr>
<tr>
<td>m-rg1</td>
<td>Hex7GlcNAc2</td>
<td>30.3 21.5</td>
<td>1638.8</td>
<td>M7A</td>
<td>16</td>
</tr>
<tr>
<td>m-rh1</td>
<td>Hex8GlcNAc2</td>
<td>31.5 20.2</td>
<td>1800.5</td>
<td>M8A</td>
<td>25</td>
</tr>
<tr>
<td>m-rh2</td>
<td>Hex8GlcNAc2</td>
<td>31.5 22.3</td>
<td>1800.7</td>
<td>M8B</td>
<td>15</td>
</tr>
<tr>
<td>m-ri1</td>
<td>Hex9GlcNAc2</td>
<td>32.5 21.2</td>
<td>1962.7</td>
<td>M9A</td>
<td>15</td>
</tr>
</tbody>
</table>

* Possible isomer other than M4B
** Possible isomers other than M6A or M6B
FIGURE LEGENDS

Figure 1. Anion-exchange HPLC of PA-glycans from *P. antarctica* and *M. furfur.*

PA-glycans from *P. antarctica* and *M. furfur* were separated by DEAE anion exchange-HPLC. PA-glycans from human α1-acid glycoprotein were used as standard. The numbers and lines show the elution positions of standard PA-glycans containing 0–3 sialic acid residues.

Figure 2. Normal-phase HPLC of neutral PA-glycans.

Neutral PA-glycans from anion-exchange chromatography were separated by normal-phase HPLC. Peaks eluting closely to standard Man$_{3,9}$GlcNAc$_{2}$-PA glycans were collected, labeled, and lyophilized for further analysis. The numbers (1-9) on the chromatogram indicate the elution positions of standard M1-, M3-, M4-, M5-, M6-, M7-, M8-, and M9-PA glycans. Endo H and jack bean α-mannosidase-digested peaks are also shown alongside the undigested sample.

Figure 3. Monosaccharide component analysis of the later eluted peaks of *P. antarctica* and *M. furfur* isolated from NP-HPLC in Figure 2.

The PA-monosaccharides prepared from the later eluted peaks isolated from NP-HPLC in Fig. 2 were analyzed by borate-chelating anion-exchange HPLC. The arrowheads in the chromatograms show the elution positions of standard PA-monosaccharides.

Figure 4. Reversed-phase HPLC of peaks of *P. antarctica* isolated using NP-HPLC.

Peaks isolated from NP-HPLC were further separated by RP-HPLC. The arrowheads in the chromatograms show the elution positions of standard PA-glycans.
Figure 5. Reversed-phase HPLC of peaks of *M. furfur* isolated using NP-HPLC.

Peaks isolated from NP-HPLC were further separated by RP-HPLC. The arrowheads in the chromatograms show the elution positions of standard PA-glycans.

Figure 6. Abbreviations and structures of the N-linked glycan standards (PA-glycans) used in this study.

Figure 7. Sequential mannosidase digestion profile of unknown M4- and M6-sized glycans.

The NP-HPLC chromatogram of unknown M4 peak glycans (p-ra1 and m-rb1) and an unknown M6 peak glycan (m-re1) digested with α1-2 mannosidase. The resulting glycan peaks were collected and each was digested separately with α1-2/1-3 mannosidase or α1-6 mannosidase. The numbers (1-6) on the chromatogram indicate the elution positions of standard M1-, M2-, M3-, M4-, M5-, and M6-PA glycans. The MS analysis and proposed structures are shown in Figs. 7 and 8, respectively.

Figure 8. MS analysis of the peaks that did not co-elute with the standards in HPLC.

Mass spectrometric data show that fragmentation resulted in the loss of one hexose. The number of hexoses in the fragmentation pattern reveals the identity of the peaks presumptively identified in previous NP-HPLC and RP-HPLC analyses. Jack bean α-mannosidase digestion analysis confirms that the hexoses annotated in MS are mannoses. (A) peak p-ra1, (B) peak m-rb1, (C) peak m-re1. The rhombus (♦) indicates the m/z of the precursor ion. [M+H]^+ adduct ions were detected.
Figure 9. The possible structures of the p-ra1 peak of *P. antarctica* and m-rb1 and m-re1 of *M. furfur.*
REFERENCES


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Fig. 1

![Diagram showing retention times and fluorescence intensity for P. antarctica and M. furfur.](image-url)
Fig. 3
Fig. 4

- M9A p-rg1 p-g
- M8A p-rf1 p-rf2 p-f
- M7A M7B p-re1 p-re2 p-e
- M6A M6B p-rd1 p-rd2 p-d
- M5A p-rc1 p-c
- M4B p-rb1 p-b
- p-ra1 p-a

Fluorescence intensity (320 - 380 nm)

Retention time (min)
Fig. 7

![Fluorescence intensity graph](image)

Fluorescence Intensity (320 - 380 nm)

Retention time (min)

- **p-ra1 (M4-sized)**
  - p-ra1a
  - p-ra1 $\downarrow$ α-1,2 mannosidase
  - p-ra1a $\downarrow$ α-1,2-1,3 mannosidase
  - p-ra1a $\downarrow$ α-1,6 mannosidase

- **m-rb1 (M4-sized)**
  - m-rb1a
  - m-rb1 $\downarrow$ α-1,2 mannosidase
  - m-rb1a $\downarrow$ α-1,2-1,3 mannosidase
  - m-rb1a $\downarrow$ α-1,6 mannosidase

- **m-re1 (M6-sized)**
  - m-re1a
  - m-re1 $\downarrow$ α-1,2 mannosidase
  - m-re1a $\downarrow$ α-1,2-1,3 mannosidase
  - m-re1a $\downarrow$ α-1,6 mannosidase
Possible M4 structure

Possible M6 structure