

The cell wall galactomannan antigen from *Malassezia furfur* and *Malassezia pachydermatis* contains β -1,6-linked linear galactofuranosyl residues and its detection has diagnostic potential

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Lipophilic yeasts of the genus *Malassezia* are associated with several skin diseases, such as pityriasis versicolor, *Malassezia* folliculitis, seborrhoeic dermatitis and atopic dermatitis, and are also increasingly associated with catheter-related fungaemia. The cell wall components of pathogenic micro-organisms behave as an antigen and/or ligand of the innate immune response. Live cells of *Malassezia furfur* and *Malassezia pachydermatis* did not react with an anti- α -1,2-mannoside antibody. However, they showed a strong hydrophobicity and reactivity with an anti- β -1,3-glucan antibody compared to those of *C. albicans*. The cell wall polysaccharides of *M. furfur* and *M. pachydermatis* were isolated and their structures analysed by ^1H and ^{13}C NMR experiments. Both polysaccharides were shown to be β -1,6-linked linear galactofuranosyl polymers with a small amount of mannan. The presence of galactomannan on cells of *Malassezia* species has not been described previously. The galactomannan did not react with an anti-*Aspergillus fumigatus* monoclonal antibody which has specificity for β -1,5-linked galactofuranosyl residues. An anti-*M. furfur* antibody strongly reacted with the galactomannans of *M. furfur* and *M. pachydermatis*, but did not react with the galactomannans of *Trichophyton rubrum*, *A. fumigatus* or *Fonsecaea pedrosoi*. The characteristics of the anti-*M. furfur* antibody suggest that there is potential for diagnosis of *Malassezia* infections by antigen detection.

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INTRODUCTION

Members of the yeast genus *Malassezia* are important pathogens related to catheter-related fungaemia in premature neonates and other patients who are receiving parenteral nutrition supplemented with lipid emulsions (Dankner *et al.*, 1987; Devlin, 2006). Most *Malassezia* species, including *Malassezia furfur*, require a lipid for growth. Therefore, the administration of lipid infusions through a catheter is a risk factor for *Malassezia* sepsis (Marcon & Powell, 1992). A single non-lipid-dependent species, *Malassezia pachydermatis*, has been isolated from animals, including dogs, more frequently than from humans (Guillot & Bond, 1999). The incidence of otitis externa in dogs, caused by *M. pachydermatis*, is reported to be closely related to ear shape, breed, and lipids in the auditory canal (Guillot & Bond, 1999). *M. pachydermatis* has also been implicated in nosocomial systemic infections

(Larocco *et al.*, 1988; Welbel *et al.*, 1994). *Malassezia* species are part of the normal cutaneous microflora of homoiothermic animals. However, certain conditions, such as high relative humidity, greasy skin, corticosteroid treatment and immunodeficiency, can allow these yeasts to become pathogenic and cause skin diseases, such as pityriasis versicolor, *Malassezia* folliculitis and seborrhoeic dermatitis (Ashbee & Evans, 2002; Faergemann, 2002). *Malassezia* species are also regarded as exacerbating factors in atopic dermatitis and psoriasis. The genus *Malassezia* has been classified into eleven species based on molecular biological analysis of the nuclear rDNA/RNA (Hirai *et al.*, 2004; Sugita *et al.*, 2004).

The cell wall of pathogenic fungi represents the initial point of interaction between the host and the pathogen. Cell wall composition and structure are strongly associated with the adherence to and penetration into tissues. The dynamic nature of the fungal cell wall allows the yeast to overcome host defences. The cell wall of fungi is composed of a tight matrix of polysaccharides and proteins. In the case of *Candida albicans*, the representative opportunistic yeast pathogen, the cell wall polysaccharide is composed of mannan, β -glucan and chitin. Proteins within the cell wall

Abbreviations: ABEE, *p*-aminobenzoic acid ethyl ester; DEPT, distortionless enhancement by polarization transfer; Galf, galactofuranose; HMBC, heteronuclear multiple bond coherence spectroscopy; HSQC, heteronuclear single quantum coherence spectroscopy; Man, mannopyranose; NOESY, nuclear Overhauser enhancement spectroscopy; TFA, trifluoroacetic acid; TOCSY, total correlation spectroscopy.

matrix are cross-linked to the cell wall polysaccharides. Mannan, the outermost layer component of the wall, behaves as a potent antigen and has been utilized as the target for the diagnosis of invasive candidiasis. It has been shown that mannosyltransferase deletion mutants of *C. albicans* have reduced pathogenicity (Bai *et al.*, 2006; Bates *et al.*, 2006; Lengeler *et al.*, 2008). Furthermore, it is known that there are many mannose recognition molecules, such as the mannose receptor, dectin-2, the dendritic-cell-specific intercellular adhesion molecule-3 (ICAM-3)-grabbing non-integrin (DC-SIGN), galectin-3 and mannan-binding protein (Willment & Brown, 2008). These molecules are known to induce innate immune responses to microbial infection. However, there has been a limited number of studies of the antigenicity of the cell wall polysaccharide of the genus *Malassezia*. The cross-reactivity of IgE and IgG antibodies in atopic dermatitis patients to the mannans of *M. furfur*, *C. albicans* and *Saccharomyces cerevisiae* has been reported (Doekes & van Ieperen-van Dijk, 1993; Lintu *et al.*, 1999; Kosonen *et al.*, 2005). Although the structure of the mannans of the *C. albicans* serotype A and B strains (Shibata *et al.*, 1995, 2007) has been determined, there appear to be no reports of the structure of the cell wall polysaccharide of the genus *Malassezia*. The genus *Malassezia* has a lipophilic nature, and the surface of the cells seems to be different from that of many other yeasts or fungi. Electron microscopy has shown that the cell wall of *Malassezia* species is relatively thick and has a multilaminar ultrastructure along with characteristic invaginations of the innermost layer (Mittag, 1995).

In this study, we isolated the cell wall polysaccharides from *M. furfur* and *M. pachydermatis*, and determined their chemical structures. Unexpectedly, the polysaccharide was galactomannan, which was mostly composed of galactofuranosyl residues with a small amount of mannosyl residues. The galactomannan was found to have linear β -1,6-linked galactofuranosyl polymer chains attached to the small core mannan. This structure is different from the galactomannan of *Aspergillus fumigatus*, which contains linear β -1,5-linked galactofuranosyl oligosaccharide side-chains (Latgé *et al.*, 1994), and that of *Trichophyton rubrum*, which has single terminal β -galactofuranosyl side-chains (Ikuta *et al.*, 1997). The galactomannan from *M. furfur* did not cross-react with anti-*A. fumigatus* and anti-*C. albicans* antibodies. Therefore, we postulate that the galactomannan acts as a *Malassezia*-specific antigen.

METHODS

Strains and culture conditions. *Malassezia furfur* NBRC 0656 (=CBS 1878), *Malassezia pachydermatis* NBRC 10064 (=CBS 1879), *Fonsecaea pedrosoi* NBRC 6733 and *Exophiala jeanselmei* NBRC 6857 were obtained from the NITE Biological Resource Center (NBRC) of the National Institute of Technology and Evaluation (NITE), Tokyo, Japan. Yeast cells were grown at 30 °C with shaking in YPD liquid medium supplemented with olive oil (1 % yeast extract, 2 % peptone, 2 % glucose, 1 % olive oil) for 48 h.

Materials. The *p*-aminobenzoic acid ethyl ester (ABEE) labelling kit was from Seikagaku. The factor 1 serum of *Candida* Check, which contains an anti- α -1,2-mannoside antibody, was from Iatron. The Platelia *Aspergillus* ELISA kit for detection of the *Aspergillus* galactomannan antigen was from Bio-Rad. The anti- β -1,3-glucan antibody, a mouse monoclonal IgG, was from Biosupplies (Australia). The cell wall polysaccharides of *C. albicans* J-1012 (serotype A) and *T. rubrum* NBRC 5467 were the same specimens as used in previous studies (Ikuta *et al.*, 1997; Shibata *et al.*, 2007).

Animals and immunization schedule. The anti-*M. furfur* antibody was prepared by immunizing two New Zealand White rabbits (weighing approximately 2.5 kg) with whole heat-killed *M. furfur* yeast cells. A pre-immune serum sample was collected as the negative control before the primary immunization. Each rabbit was subcutaneously immunized with a 4 ml portion of 10^8 *M. furfur* cells emulsified in Freund's complete adjuvant (1:1, v/v) at eight sites along the back. Two and four weeks later, the rabbits were subcutaneously boosted with a 4 ml portion of 10^8 *M. furfur* cells emulsified in Freund's incomplete adjuvant (1:1, v/v). The rabbits were bled 10 days after the last injection, and the sera were collected and stored at -20 °C. The ELISA titre of the anti-*M. furfur* antibody was 1/20 000.

Carbohydrate composition analysis. For analysis of the carbohydrate composition, the polysaccharides and dried cells were hydrolysed with 4 M trifluoroacetic acid (TFA) at 100 °C for 3 h. The resulting monosaccharide mixtures were converted into ABEE derivatives according to the manufacturer's instructions, and analysed by HPLC using a TSK-GEL ODS-120T column (4.6 × 150 mm) (Tosoh). Elution was done with a 93:7 (v/v) mixture of 0.2 M potassium borate buffer (pH 8.9) and acetonitrile, and the eluates were monitored at 305 nm. Specific optical rotation of the monosaccharide was measured using a P-2300 polarimeter (JASCO) for determination of the absolute configuration.

Preparation of fluorescein-conjugated stearyl dextran (FITC-stearyl dextran). The dextran was first stearoylated. A 200 mg sample of the dextran (70 kDa) was dissolved in 5 ml DMSO. Stearoyl chloride, 0.6 ml, was added dropwise to the solution and allowed to react overnight at 25 °C with stirring. Stearoyl chloride was decomposed by the addition of 1 ml water followed by evaporation to dryness. The reaction product was washed three times with 2-propanol, then dialysed and lyophilized. For the fluorescein labelling, the stearyl dextran was dissolved in 5 ml DMSO. To this solution, two drops of pyridine, 20 mg FITC and 4 mg dibutyltin dilaurate were added and allowed to react at 95 °C for 2 h. The reaction product, FITC-stearyl dextran, was recovered by ethanol precipitation (de Belder & Wik, 1975).

Flow cytometry. For preparation of the heat-killed yeasts, live *Malassezia* cells were suspended in PBS, and incubated at 90 °C for 20 min and washed with PBS. To analyse the cell surface polysaccharides, 10^6 cells were incubated for 30 min with 2 % BSA-PBS. After washing, the cells were incubated for 30 min with factor 1 serum (1:300 dilution in 1 % BSA-PBS), antibody to *M. furfur* cells (1:400 dilution in 1 % BSA-PBS), or antibody to β -1,3-glucans (1:50 dilution in 1 % BSA-PBS). After washing, the cells were incubated for 30 min with specific FITC-labelled secondary antibodies (1:100 dilution in 1 % BSA-PBS). The negative control was performed by adding a labelled second antibody at the same concentration. All processes were conducted at 4 °C. After washing, the cells were fixed in 0.4 % paraformaldehyde. Flow cytometry was performed using a FACSCalibur system (Becton Dickinson).

Cell surface hydrophobicity. Cell surface hydrophobicity was determined by staining the cells with FITC-stearyl dextran. Cells

grown at 25 and 37 °C were washed with PBS, suspended in PBS to a concentration of 2×10^6 cells ml^{-1} and stained with FITC-stearoyl dextran ($100 \mu\text{g ml}^{-1}$) for 1 h. As the control, FITC-dextran was used at the same concentration. All processes were conducted at 4 °C. After washing with PBS, the cells were fixed in 0.4 % paraformaldehyde and examined by flow cytometry. A hydrophobic microsphere assay developed by Hazen & Hazen (1987) was also conducted for comparison. From a stock 10 % solids suspension of polystyrene microspheres (0.8 μm diameter; Bangs Laboratories), 6 μl was taken and added to 2 ml buffer. Equal volumes (100 μl) of the cell and microsphere suspensions were combined in glass tubes and mixed in a vortex mixer for 30 s. Microsphere attachment was assessed by microscopy at $400\times$ magnification. The percentage of cells with three or more attached spheres was recorded as the percentage hydrophobicity.

Preparation of cell wall polysaccharide. The cells were washed and dehydrated with acetone, and the crude cell wall polysaccharide was extracted with water at 120 °C for 2 h. The extract was dialysed against running tap water and then lyophilized. The dialysate was dissolved in water and centrifuged at 13 000 g for 20 min to eliminate any insoluble material. The supernatant was applied onto a column (70×4 cm) of Sephacryl S-100 and eluted with water. Aliquots of the eluates were assayed for their carbohydrate and protein contents by the phenol/sulfuric acid method (Dubois *et al.*, 1956) and BCA protein assay kit (Pierce), respectively. Eluates corresponding to the top peak containing the polysaccharide were combined and lyophilized. The cell wall polysaccharide was fractionated using DEAE-Sepharose chromatography by stepwise elution with water, and 0.1 M and 0.2 M NaCl. The yields of these fractions were about 60, 20 and 20 %, respectively. Since the two latter fractions contained a large amount of β -glucan, judging from the ^1H -NMR spectra and carbohydrate composition analysis, a structural study of the polysaccharide was performed using the water-eluted fraction.

Hydrolysis of the polysaccharide. For the preparation of the fragment oligosaccharides, the cell wall polysaccharide was partially hydrolysed with 0.15 M TFA at 100 °C for 10 min. For the preparation of the core mannan, all of the galactofuranosyl residues were removed by treatment with 0.1 M HCl at 100 °C for 30 min. After neutralization, the reaction products were separated on a column (2.5×100 cm) of Bio-Gel P-2. The structure of the fragment oligosaccharides from dimer to pentamer was confirmed by NMR spectroscopy.

NMR spectroscopy. Samples were exchanged twice in $^2\text{H}_2\text{O}$ with intermediate lyophilization, then dissolved at 1 % (w/v) in $^2\text{H}_2\text{O}$ (99.97 % atom ^2H). The NMR spectra were recorded using JNM-LA600 spectrometers (JEOL) at 45 °C. The total correlation spectroscopy (TOCSY), 2D nuclear Overhauser enhancement spectroscopy (NOESY), distortionless enhancement by polarization transfer 135 (DEPT-135), 2D heteronuclear single quantum coherence spectroscopy (HSQC), and 2D heteronuclear multiple bond coherence spectroscopy (HMBC) were performed using the standard pulse sequence. The proton and carbon chemical shifts were referenced relative to the internal acetone at δ 2.225 and 31.07, respectively.

ELISA. The polysaccharide ($100 \mu\text{g ml}^{-1}$ in 50 mM carbonate buffer, pH 9.6) was immobilized overnight onto a microtitre plate at 4 °C. The non-specific binding sites were blocked with 2 % BSA-PBS for 2 h at room temperature. After incubation with the primary antibodies (diluted from 27- to 500 000-fold in blocking solution) for 2 h at room temperature, the wells were washed three times with PBS-T (PBS containing 0.05 % Tween 20). After washing with PBS-T, horseradish peroxidase-conjugated antibody (1:4000 dilution in blocking solution) was added and incubated for 2 h at room temperature. After washing, a substrate solution of 0.01 % *o*-

phenylenediamine and 0.03 % H_2O_2 in 150 mM citrate buffer (pH 5.0) was added. The reaction was stopped with 2 M H_2SO_4 and the A_{492} was measured using an ELISA plate reader (Tosoh).

Platelia Aspergillus ELISA. The reactivity of the galactomannans was determined according to the manufacturer's instructions. Briefly, 50 μl of a reaction mixture containing the horseradish peroxidase-conjugated anti-galactomannan monoclonal antibody was added to each well of a microtitre plate coated with the same monoclonal antibody and then 50 μl of the sample was added. After 90 min incubation at 37 °C, the plates were washed five times with washing buffer, then 200 μl of the buffer containing tetramethylbenzidine solution was added. The plates were incubated for another 30 min in the dark at room temperature, after which 100 μl of 0.75 M sulfuric acid was added to stop the reaction. The A_{450} was read.

Total carbohydrate content. This was determined by the phenol/sulfuric acid method (Dubois *et al.*, 1956) with D-mannose as the standard.

RESULTS

Antigenicity of *M. furfur* and *M. pachydermatis* cells

We first tested the reactivity of these cells with the anti- α -1,2-mannoside and anti-*M. furfur* antibodies. As shown in Fig. 1(a), a flow cytometric analysis revealed that the anti- α -1,2-mannoside antibody, factor 1 serum of Candida Check, did not cross-react with the *M. furfur* and *M. pachydermatis* cells, suggesting that the exposed mannan on the cell surface is very small. Similarly, the anti-*M. furfur* antibody showed weak reactivity to the *C. albicans* cells. We next examined whether the β -glucan layer was covered by other cell surface polysaccharides, using the anti- β -1,3-glucan antibody. Unlike in *C. albicans*, the β -1,3-glucan was constitutively expressed on the surface of the *Malassezia* cells (Fig. 1b). This result suggests that *Malassezia* cells have fewer polysaccharides on the outside of the β -glucan layer compared to *C. albicans* cells.

Comparison of cell surface hydrophobicity

Variation in growth temperature is one of the factors that is known to affect the hydrophobicity of *C. albicans* (Hazen *et al.*, 1991). Therefore, cells grown at 25 and 37 °C were stained with FITC-stearoyl dextran and analysed by flow cytometry. This assay method, which does not use polystyrene microspheres and microscopy, showed that the cell surface of the *Malassezia* species grown at 37 °C has a strong hydrophobicity compared to that of *C. albicans* J-1012 (Fig. 1c). In the case of *M. furfur*, hydrophobicity of the cells grown at 25 °C was slightly stronger than that of the cells grown at 37 °C. However, for *M. pachydermatis* the hydrophobicity was almost the same for the cells grown at 25 and at 37 °C. As a control experiment, the hydrophobicity of the same cells was also determined by a hydrophobic microsphere assay. As shown in Table 1, the results corroborated those obtained by the FITC-stearoyl dextran method.

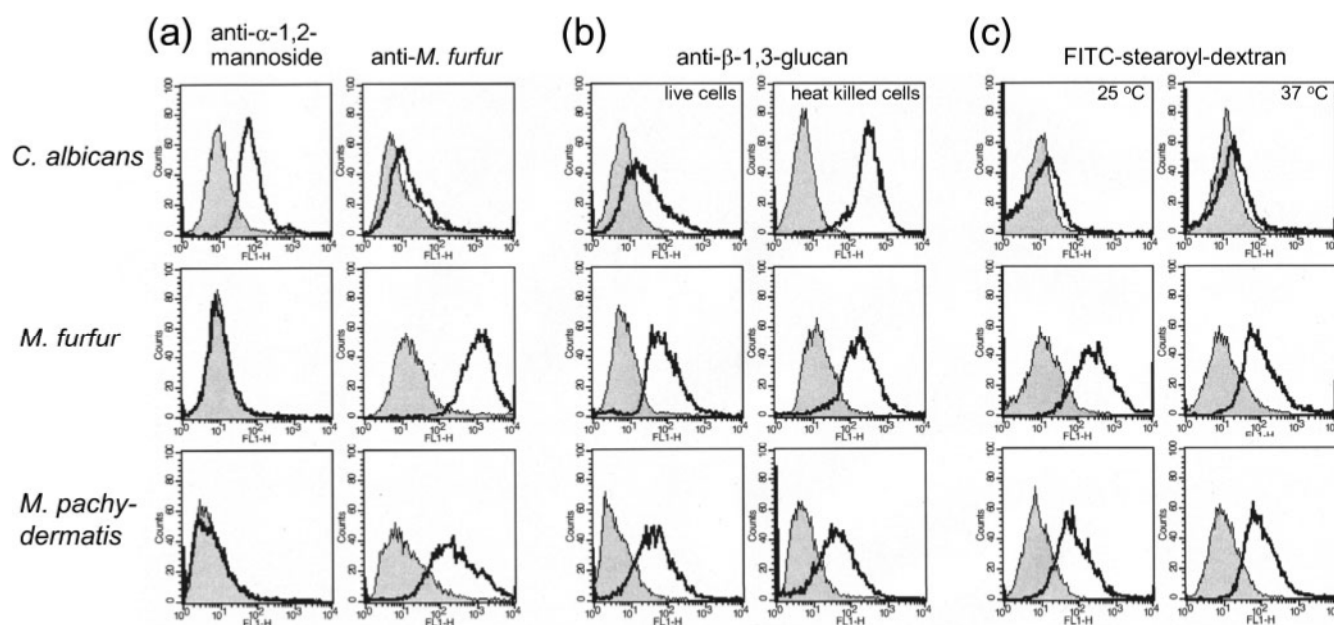


Fig. 1. Flow cytometry of *C. albicans*, *M. furfur* and *M. pachydermatis*. (a) Anti- α -1,2-mannoside antibody and anti-*M. furfur* antibody staining of live cells. (b) Anti- β -1,3-glucan antibody staining of live or heat-killed cells. (c) FITC-stearoyl dextran staining of live cells grown at 25 and 37 °C.

Structure of cell wall polysaccharide

The carbohydrate composition of the cells of *M. furfur*, *M. pachydermatis* and *C. albicans* is shown in Table 2. Compared with *C. albicans*, the amount of mannose in both *Malassezia* species was much lower, and, unlike *C. albicans*, the *Malassezia* species contained galactose. This carbohydrate composition may explain the lack of or reduced reactivity of the *Malassezia* cells to the anti- α -1,2-mannoside antibody. The cell wall polysaccharide of the *Malassezia* species was extracted with water at 120 °C for 2 h, and fractionated using Sephacryl S-100 and DEAE-Sephacryl column chromatography. Analysis of the carbohydrate composition of the main polysaccharide fraction, the water-eluted fraction from DEAE-Sephacryl, showed that the galactose:mannose ratio was around 15:1, indicating that the polysaccharide was galactoman-

nan. Since the galactose:mannose ratio of the 0.1 and 0.2 M NaCl eluted fractions from DEAE-Sephacryl was around 5:2 and 1:2, respectively (data not shown), the galactomannan seemed to have a high heterogeneity regarding the length of the galactosyl moiety. The absolute configuration of galactose was determined by analysis of the specific optical rotation. The result ($[\alpha]_D^{+80.2}$, c 0.2, water) indicated that the galactose had the D-configuration. The ^1H -NMR spectra of the galactomannans of *M. furfur* and *M. pachydermatis* were exactly the same (Fig. 2a, b). The extracellular polysaccharides isolated from the culture filtrate of these two species also exhibited the same ^1H -NMR spectra (data not shown). Fig. 2(c) and Fig. 2(d)

Table 1. Cell surface hydrophobicity of cells grown at 25 and 37 °C

Data are presented as the mean (sample standard deviation) ($n=5$).

	Hydrophobicity (%)	
	25 °C	37 °C
<i>C. albicans</i> J-1012	26.1 (2.4)	20.6 (5.6)
<i>M. furfur</i> NBRC 0656	83.0 (4.2)	85.5 (4.8)
<i>M. pachydermatis</i> NBRC 10064	86.8 (3.9)	87.3 (6.0)

Table 2. Carbohydrate composition of whole cells and cell surface polysaccharides

	Carbohydrate residue (%)		
	Galactose	Mannose	Glucose
Whole cells			
<i>C. albicans</i>	0	10	90
<i>M. furfur</i>	2	1	97
<i>M. pachydermatis</i>	2	1	97
Cell surface polysaccharide			
<i>C. albicans</i>	0	100	0
<i>M. furfur</i>	85	6	9
<i>M. pachydermatis</i>	84	11	5

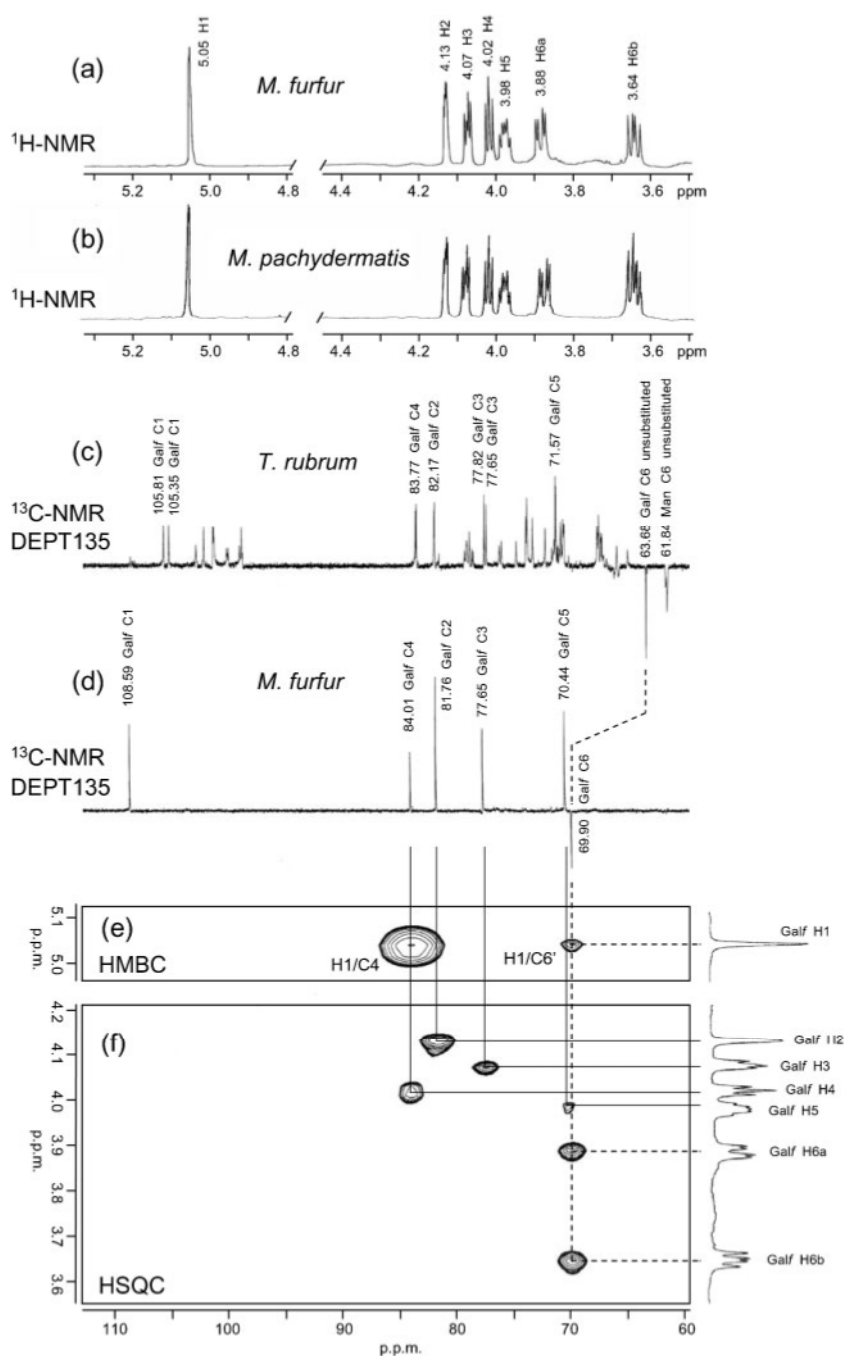


Fig. 2. NMR spectra of galactomannans: ^1H -NMR spectra of *M. furfur* (a) and *M. pachydermatis* (b); ^{13}C -NMR DEPT-135 spectra of *T. rubrum* (c) and *M. furfur* (d); HMBC (e) and HSQC (f) spectra of *M. furfur*. Dashed lines between the two ^{13}C -NMR DEPT-135 spectra indicate the downfield shift of the C-6 carbon of the galactofuranosyl residue caused by glycosylation with other galactofuranosyl residues. Galf, D-galactofuranosyl residue.

show the ^{13}C -NMR DEPT-135 spectra of the *T. rubrum* and *M. furfur* galactomannans, respectively. The negative signals in these spectra correspond to C-6 of the carbohydrates. As shown in Fig. 2(d), the *M. furfur* galactomannan had characteristic signals for galactofuranosyl residues (C-1, δ 108.59; C-2, δ 81.76; C-3, δ 77.65) (Table 3). However, the C-6 signal of the *M. furfur* galactomannan appeared at δ 69.90 instead of around δ 63.7, which corresponds to the C-6 of the terminal galactofuranosyl residues found in the galactomannan of *T. rubrum*. The downfield shift of the C-6 signal on *M.*

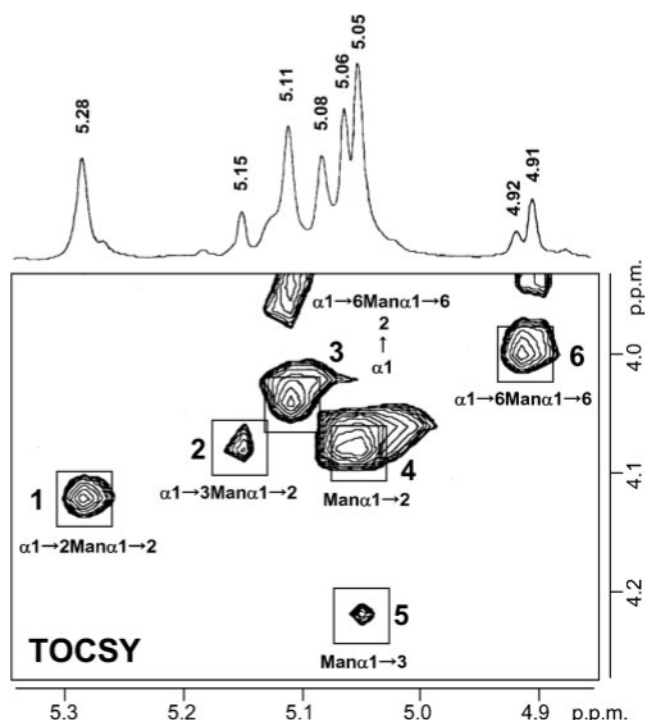
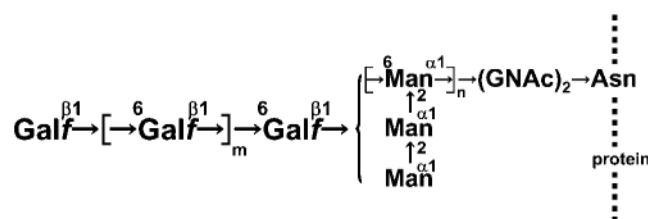
furfur galactomannan indicates that the polysaccharide consisted of linear 1,6-linked galactofuranosyl residues. The coupling constant of the anomeric proton of the galactofuranosyl residues was around 1.8 Hz, which corresponds to the β anomeric configuration (Cyr & Perlin, 1979). This is also supported by the chemical shifts of their anomeric carbons ($\delta > 106$ p.p.m.). To confirm this structure, an HMBC experiment was carried out. The presence of the intraresidue cross-peak H-1/C-4 shows the furanosidic character of the galactose, and the interresidue cross-peak H-1/C-6' demonstrates the β -1,6-linkage indi-

Table 3. ^1H and ^{13}C NMR chemical shifts of the galactomannan isolated from *M. furfur* (δ , p.p.m.) ($J=\text{Hz}$)

	Galf	
	^1H	^{13}C
H1 ($^3J_{1,2}$)	5.05 (1.8)	108.59
H2 ($^3J_{2,3}$)	4.13 (3.6)	81.76
H3 ($^3J_{3,4}$)	4.07 (6.0)	77.65
H4 ($^3J_{4,5}$)	4.02 (4.8)	84.01
H5 ($^3J_{5,6}$)	3.98 (3.6)	70.44
H6a ($^3J_{5,6b}$)	3.88 (7.2)	69.90
H6b ($^3J_{6,a}$)	3.64 (10.8)	—

cated above (Fig. 2e). The NOESY spectrum also contained the interresidue cross-peak H-1/H-6' (data not shown). The proton and carbon signals of the galactomannan were assigned by the HSQC (Fig. 2f) and COSY experiments.

In order to obtain information about the core mannan structure of the galactomannan, the polysaccharide was subjected to partial hydrolysis with dilute acid, which selectively removed the galactofuranosyl residues. Around 5% of a residual polysaccharide, composed only of mannose, was obtained. The ^1H -NMR spectrum of the

**Fig. 3.** NMR spectrum of the mannan core of the galactomannan obtained from *M. furfur*. The boxed regions in the TOCSY spectrum indicate the H-1-H-2-correlated cross-peaks of the α -mannosyl residues. These cross-peaks were common to those of the mannans from *C. albicans* and *S. cerevisiae*.**Fig. 4.** One of the possible structures of *M. furfur* and *M. pachydermatis*. Galf and Man denote the D-galactofuranosyl and D-mannopyranosyl residues, respectively. The m in the structure was estimated to be around 30 from the ratio of the signal dimensions of galactofuranosyl residues. The n indicates that the core mannan has a comb-like structure but we could not determine the number in this study. The long linear β -1,6-linked galactofuranosyl polymer chain is linked to a core mannan which has a comb-like structure.

mannan is shown in Fig. 3. The presence of the signal at δ 4.91–4.92, cross-peak 6, indicates that this mannan contains the linear α -1,6-linked backbone mannosyl residues. Furthermore, the signals at δ 5.05–5.06, cross-peak 4, and the signal at δ 5.28, cross-peak 1, correspond to the nonreducing terminal and intermediate α -1,2-linked mannosyl residues, respectively. The core mannan also contains a small amount of the nonreducing terminal α -1,3-linked mannosyl residues connected to the α -1,2-linked ones, judging from the presence of cross-peaks 2 (H1/H2, δ 5.15/4.08) and 5 (H1/H2, δ 5.05/4.23) (Shibata *et al.*, 2007). These results suggest that the core mannan has a comb-like structure similar to the mannans of *S. cerevisiae* and *C. albicans*.

On the basis of these results, in Fig. 4 we depict one possible chemical structure for the cell wall galactomannan of *M. furfur* and *M. pachydermatis*. The average degree of polymerization of the galactofuranose was estimated to be around 30 from the ratio of the total H-1 and nonreducing terminal H-6 signal dimensions of the galactofuranosyl residues (Wieneke *et al.*, 2007).

Antigenic cross-reactivity between galactomannans

Many fungi have galactofuranose-containing polysaccharides in their cell wall. Therefore, we tested the antigenic similarity of the galactomannan of *M. furfur* to other galactomannans using the anti- β -1,5-linked galactofuranosyl monoclonal antibody of the Platelia *Aspergillus* ELISA kit. At 10 ng ml^{-1} , the galactomannans from *M. furfur*, *M. pachydermatis* and *T. rubrum*, and from the dematiaceous fungi *F. pedrosoi* and *E. jeanselmei*, showed no reactivity, suggesting that the galactomannans from the *Malassezia* species do not contain β -1,5-linked galactofuranosyl residues and have a completely different antigenicity from that of *A. fumigatus* (Fig. 5).

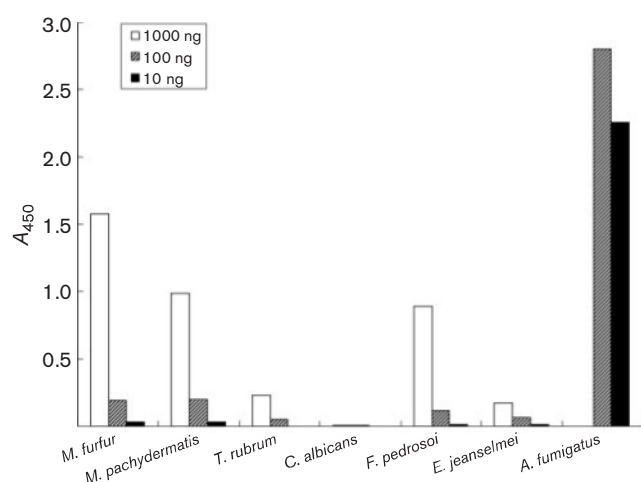


Fig. 5. Platelia *Aspergillus* ELISA of galactomannans and mannan obtained from various pathogenic fungi.

Next, we tested the reactivity of several fungal polysaccharides with the anti-*M. furfur* and anti- α -1,2-mannoside antibodies by ELISA (Fig. 6). The galactomannans of *M. furfur* and *M. pachydermatis* showed strong reactivity with the anti-*M. furfur* antibody. In contrast, the mannan of *C. albicans* and the galactomannans of *T. rubrum*, *F. pedrosoi* and *E. jeanselmei* showed no or less reactivity. Taken together, these results indicate that the cell surface of *M. furfur* and *M. pachydermatis* possesses galactomannan which has no cross-reactivity with other galactomannans from pathogenic fungi and behaves as a *Malassezia*-specific antigen.

Identification of epitopes

In order to identify the *Malassezia*-specific antigen, the effect of β -1,6-linked galactofuranosyl oligomers of increasing length, obtained by partial acid hydrolysis of the galactomannan, was studied in ELISA inhibition experiments using the anti-*M. furfur* antibody. As shown in Fig. 7, the tetramer and pentamer showed almost the same strong inhibitory effect. This result indicates that the anti-*M. furfur* antiserum contains a specific antibody which recognizes the β -1,6-linked galactofuranosyl tetramer moieties of the *M. furfur* galactomannan.

DISCUSSION

We could not obtain a mannan from crude hot-water extracts of *M. furfur* and *M. pachydermatis* by the Fehling's solution precipitation method. Furthermore, these cells did not react with the anti- α -1,2-mannoside antibody. A carbohydrate composition analysis of the whole cells indicated that there is a small amount of mannose in addition to galactose, suggesting that the *Malassezia* cells

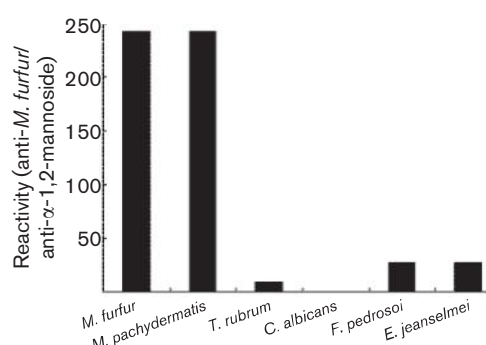


Fig. 6. Reactivity of the anti-*M. furfur* antibody with galactomannans and mannan obtained from various pathogenic fungi. One unit of reactivity was defined as an A_{492} of 0.25 in the ELISA. Reactivity is expressed as the ratio (reactivity units with anti-*M. furfur* antibody)/(reactivity units with anti- α -1,2-mannoside antibody). These results are representative of three independent experiments.

have fewer polysaccharides on the outside of the β -glucan and chitin layer compared to *C. albicans* cells. Mathov *et al.* (1996) reported that a psoriasis patient's serum reacted with 100 and 120 kDa bands of a French press extract of *M. furfur* cells. They showed that the reaction pattern was the same as that of a lectin which recognizes *N*-acetylglucosamine (GlcNAc), and that the reaction with the serum was inhibited by GlcNAc. Furthermore, one of the bands was digested by lyticase (β -1,3-glucanase), suggesting that these bands were glycoproteins containing cell wall β -glucan and chitin. Taken together, these results suggest that the cell wall chitin, a polymer of GlcNAc, in addition to β -glucan, is exposed at the cell surface and acts as an antigen. This would be consistent with our findings of the low mannose content of the cell wall polysaccharide and the reactivity of *Malassezia* cells with the anti- β -1,3-glucan antibody.

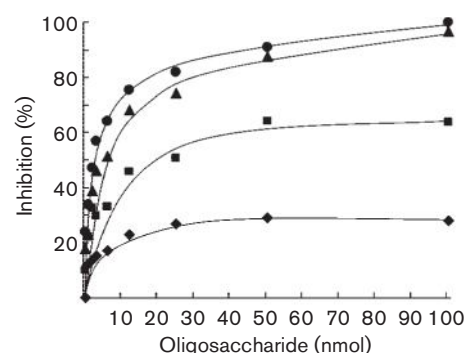


Fig. 7. Inhibition of ELISA by galactofuranooligosaccharides. To the *M. furfur* galactomannan-coated plate, anti-*M. furfur* antibody pretreated or not with haptenic galactofuranooligosaccharides (◆, dimer; ■, trimer; ▲, tetramer; ●, pentamer) was added, followed by incubation for 2 h at room temperature; the bound antibody was detected as described in Methods.

Cell surface hydrophobicity plays an important role in the pathogenicity of micro-organisms. Hydrophobic *C. albicans* cells are more adherent than hydrophilic cells to a variety of host tissues (Hazen *et al.*, 1991). For the detection of the hydrophobicity of a yeast cell surface, Hazen & Hazen (1987) developed an assay method involving two processes, adhesion of polystyrene beads to the yeast cells and counting of the bead-adhered cells by microscopy. Colling *et al.* (2005) introduced flow cytometry for the hydrophobicity assay. In this method, the fluorescence of blue-dyed polystyrene beads is utilized for counting the bead-adhered cells during flow cytometry, and a gate is applied to distinguish the bead-adhered cells from the nonbead-adhered cells. The FITC-stearoyl dextran method for determining hydrophobicity is simpler than these assay systems, and the hydrophobicity levels obtained by this method are similar to those obtained with the polystyrene microsphere method. *C. albicans* cells are hydrophobic when grown at 25 °C and hydrophilic at 37 °C (Hazen *et al.*, 1991). However, *Malassezia* cells did not show hydrophilicity when grown at 37 °C. It has been reported that the *Malassezia* cell wall has a multilaminar structure (Mittag, 1995). Since the lamellar structure varies with the different lipid sources in the medium and stains with Nile blue sulfate, it is suggested that the cell wall contains lipid. The cell wall lipid seems likely to be responsible for the hydrophobicity interaction of the FITC-stearoyl dextran in *Malassezia* species.

Galactofuranose-containing glycoproteins or polysaccharides are known to be present in fungi, bacteria, protozoa, green algae and cyanobacteria. The galactofuranosyl residue makes a variety of linkages, such as β -1,2-, β -1,3-, β -1,5-, β -1,6-, and some α -linkages, and is highly immunogenic (Notermans *et al.*, 1988). The galactofuranosyl residue is essential for the survival or virulence of some pathogenic micro-organisms (Pan *et al.*, 2001). In fungi, galactofuranosyl residues have been found in *Aspergillus*, *Penicillium*, *Histoplasma*, *Paracoccidioides*, *Trichophyton* and *Fonsecaea* species (Barr *et al.*, 1984; Gander *et al.*, 1974; Ikuta *et al.*, 1997; Latgé *et al.*, 1994; Levery *et al.*, 1996; Suzuki & Takeda, 1977). The galactofuranosyl residues of *Aspergillus* and *Penicillium* are composed of β -1,5-linked oligosaccharide side chains. On the other hand, β -1,6-linked galactofuranosyl residues are present in the cell wall polysaccharide or glycolipid of *Mycobacterium tuberculosis* (Daffé *et al.*, 1990), *Paracoccidioides brasiliensis* (Levery *et al.*, 1996) and *Streptococcus oralis* (Abeygunawardana *et al.*, 1991). However, these carbohydrate moieties have a single terminal or single internal β -1,6-linked galactofuranosyl residue or alternating β -1,6-linked galactofuranosyl and β -1,5-linked galactofuranosyl residues. Although some galactomannans have consecutive β -1,6-linked galactofuranosyl residues on the main chain, these are almost fully substituted by side chains and have a comb-like structure (Ahrazem *et al.*, 2001). Recently, Wieneke *et al.* (2007) isolated intracellular β -1,6-linked galactofuranooligosacchar-

ides, up to the decamer, from the terrestrial cyanobacterium *Nostoc commune*. However, there appear to have been no reports of an antigenic cell wall polysaccharide consisting of consecutive β -1,6-linked galactofuranosyl residues without side chains. The mannan moiety of the *Malassezia* galactomannan has a comb-like structure. The side chain of the mannan mainly consists of α -1,2-linked mannosyl residues, with a small amount of terminal α -1,3-linked mannosyl residues, similar to the mannan of *S. cerevisiae*. Since the proportion of the mannan moiety on the galactomannan is small, it has not yet been determined to which mannosyl residues the galactofuranosyl polymer chains are attached. The anti-*M. furfur* antibody cross-reacted with *C. albicans* mannan at a high concentration in ELISA. This result suggests that the anti-*M. furfur* antibody contains an antibody against the α -1,2-mannosyl residues, which is consistent with the observation of Lintu *et al.* (1999).

In this report, we show the results for only two of eleven *Malassezia* species. However, the reactivity of *Malassezia sympodialis* cells with the anti-*M. furfur* antibody was comparable to that of *M. furfur* and *M. pachydermatis* cells by FACS analysis (data not shown). Therefore, we postulate that the β -1,6-linked linear galactofuranosyl polymer structure of the galactomannan is a common antigen in the genus *Malassezia*.

During the innate immune response, microbial carbohydrate chains are recognized by animal lectins. The mannose receptor and the mannan-binding protein, both of which have an affinity to mannose, are present on cells as phagocytosis receptors or in plasma as opsonins to activate the complement system. Although mannosyl residues are present in mammalian tissues as well as in micro-organisms, galactofuranosyl residues are not. Therefore, the recognition of galactofuranosyl residues can effectively induce pathogen-specific cellular responses and fluid-phase immune reactions for elimination. Recently, Tsuji *et al.* (2001) identified a human intelectin, which is a Ca^{2+} -dependent galactofuranose-binding lectin. Thus, the human intelectin would bind to various pathogens containing galactofuranosyl residues – *Malassezia*, *Aspergillus*, *Penicillium*, *Trichophyton*, etc. – and may play an immunological role. Most recently, Yamasaki *et al.* (2009) reported that a C-type lectin, Mincle, on activated macrophages specifically reacts with cells of the genus *Malassezia*, but does not react with cells of the genera *Aspergillus*, *Candida*, *Cryptococcus*, *Trichosporon* or *Saccharomyces*. They reported that the lectin binds to α -mannose, but not mannan, and speculated that it recognizes the specific geometry of α -mannosyl residues in the genus *Malassezia*. Although the carbohydrate recognition domain of Mincle contains an EPN (Glu-Pro-Asn) motif (Matsumoto *et al.*, 1999), which is a putative mannose-binding motif, it is possible that Mincle recognizes a *Malassezia*-specific cell surface component, such as the β -1,6-galactofuranosyl residue, some kind of hydrophobic molecule or a complex molecule of the α -mannosyl residue with these components.

There appears to be no report describing an identical or similar antigenic structure to the *Malassezia* galactomannan on the cell wall of pathogenic micro-organisms. Therefore, we can say that the antibody against the β -1,6-linked galactofuranosyl oligomer has potential as a diagnostic indicator for *Malassezia* infections of humans and animals by detection of the antigen or the fungal cells themselves. For the routine clinical diagnosis of *Malassezia* infection, skin scrapings are microscopically examined by KOH or KOH-Calcofluor white preparations, or by staining with periodic acid/Schiff or methylene blue. Since these stainings of the preparations are non-specific for the genus *Malassezia*, infection by *Malassezia* has to be diagnosed by its characteristic morphology. In veterinary medicine, *M. pachydermatis* is the most important species in the genus. *M. pachydermatis* is most frequently isolated from the skin, mucosa or ear canal of healthy dogs and cats; it acts as an opportunistic secondary pathogen within the ear canal and can cause otitis externa. We can stain *Malassezia* cells using the enzyme-conjugated antibody which is specific for the galactomannan of the *Malassezia* cell wall. Therefore, there is the possibility that we can semiquantitatively estimate the amount of *Malassezia* cells in specimens from the ear canal of a dog or cat by the colour reaction of the enzyme immunoassay, which would be clinically easier and more reliable for the diagnosis of *Malassezia* infection.

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