Molecular Microbiology (2005) **58**(4), 984–998

doi:10.1111/j.1365-2958.2005.04890.x
First published online 10 October 2005

Candida albicans serotype B strains synthesize a serotype-specific phospholipomannan overexpressing a β-1,2-linked mannotriose

Pierre-André Trinel,[1] Florence Delplace,[2] Emmanuel Maes,[2] Jean-Pierre Zanetta,[2] Céline Mille,[1] Bernadette Coddeville,[2] Thierry Jouault,[1] Gérard Strecker[2] and Daniel Poulain[1]*

[1]*Inserm E0360, Physiopathologie des Candidoses, Faculté de Médecine, Pôle Recherche, 59037, Lille Cedex, France.*

[2]*Unité de Glycobiologie Structurale et Fonctionnelle, CNRS UMR n°8576, Université des Sciences et Technologies de Lille, 59655 Villeneuve d'Ascq Cedex, France.*

Summary

***Candida albicans* strains consist of serotypes A and B depending on the presence of terminal β-1,2-linked mannose residues in the acid-stable part of serotype A phosphopeptidomannan (PPM). The distribution of *C. albicans* serotypes varies according to country and human host genetic and infectious backgrounds. However, these epidemiological traits have not yet been related to a phenotypically stable molecule as cell surface expression of the serotype A epitope depends on the growth conditions. We have shown that *C. albicans* serotype A associates β-mannose residues with another molecule, phospholipomannan (PLM), which is a member of the mannoseinositol-phosphoceramide family. In this study, PLM from serotype B strains was analysed in order to provide structural bases for the differences in molecular mass and antigenicity observed between PLMs from both serotypes. Through these analyses, carbon 10 was shown to be the location of a second hydroxylation of fatty acids previously unknown in fungal sphingolipids. Minor differences observed in the ceramide moiety appeared to be strain-dependent. More constant features of PLM from serotype B strains were the incorporation of greater amounts of phytosphingosine C20, a twofold reduced glycosylation of PLM and overexpression of a β-1,2 mannotriose, the epitope of protective antibodies. This specific β-mannosylation was observed even when growth conditions altered serotype A PPM-specific epitopes, confirming the potential of PLM as a phenotypically stable molecule for serotyping. This study also suggests that the regulation of β-mannosyltransferases, which define specific immunomodulatory adhesins whose activity depends on the mannosyl chain length, are part of the genetic background that differentiates serotypes.**

Accepted 29 August, 2005. *For correspondence. E-mail dpoulain@univ-lille2; Tel. (+33) 3 20 62 34 20; Fax (+33) 3 20 62 34 16.

Introduction

Sphingolipids are ubiquitous and essential components of eukaryotic cells. In higher eukaryotic cells, the sphingolipid biosynthetic pathway leads through the formation of ceramides to gangliosides, cerebrosides and sphingomyelin. In fungi, the biosynthetic pathway of sphingolipids rapidly diverges from the former first by the use of phytosphingosine (PHS) instead of sphingosine to form phytoceramides, and then by the extensive addition of inositol-phosphate to these phytoceramides leading to the inositolphosphoceramide (IPC), mannoseinositolphosphoceramide (MIPC) and mannose (inositol phosphate)$_2$ ceramide [M(IP)$_2$C], the major plasma membrane sphingolipids of fungi also found in plants (Dickson, 1998; Dickson and Lester, 1999a). These complex sphingolipids are important for plasma membrane integrity, regulation of Ca^{2+} levels (Beeler *et al*., 1994), sensitivity to specific antifungal drugs (Mandala and Harris, 2000; Zhong *et al*., 2000; Desai *et al*., 2002) and improved resistance of fungal cell to stress conditions (pH or temperature) (Patton *et al*., 1992). They are also associated with ergosterols in lipid rafts, which play a key role in the delivery of specific proteins to the plasma membrane (Bagnat *et al*., 2000).

Most studies on sphingolipids have been performed with *Saccharomyces cerevisiae* and the sphingolipids of *Candida albicans* remain slightly explored (Wells *et al*., 1996; Levery *et al*., 2000; Barreto-Bergter *et al*., 2004). Previous studies by our group have shown that *C. albicans* strains and the closely related species *C. stellatoidea*, *C. tropicalis* (Cantelli *et al*., 1995) and *C. dubliniensis* (A.P. Trinel, unpubl. results) contain a glycolipid called phospholipomannan (PLM), whose glycan

moiety is composed exclusively of β-1,2-linked mannose residues (Trinel *et al*., 1999). This linkage anomery appears to be specific to pathogenic fungi and has not been demonstrated in mammals. β-1,2-linked mannose residues display adhesin (Li and Cutler, 1993) and strong immunomodulatory (Jouault *et al*., 1995; 2000) properties which depend on chain length and induce protective antibodies against *C. albicans* infection (Han *et al*., 1997; 2000). The PLM of *C. albicans* strain VW32 (serotype A) was recently shown to be a glycosphingolipid, and according to its structure was proposed to belong to the IPC family and to be derived from the usual biosynthetic pathway of complex sphingolipids at the MIPC level (Trinel *et al*., 2002a): instead of the addition of inositol-phosphate, which leads to $M(IP)_2C$, an end-product which is not further glycosylated, the addition of mannose phosphate to MIPC allowed further extensive β-mannosylation of the molecule. With regard to the present knowledge about glycosphingolipids, PLM appeared to correspond to a new family due to: (i) the importance of its glycosylation and the linkage anomery of the mannose residues which are both unusual in fungal glycosphingolipids (Dickson and Lester, 1999a), (ii) its specific spacer, P-Man-Ins-P-, used for linkage of the glycan moiety to the ceramide group and (iii) its strong heterogeneity, which arises from both the composition of the ceramide group and the degree of polymerization (DP) of the glycan moiety which can reach 19 units.

Western blot analysis previously revealed differences in the molecular mass and antigenicity of PLM from *C. albicans* serotypes A and B (Trinel *et al*., 2002b), and that these differences remained whatever the growth conditions. On the contrary, expression of the discriminating epitope (serum factor 6) used for serotyping *C. albicans* serotype A strains is unstable and depends on the growth temperature, pH and culture medium (Kobayashi *et al*., 1994a,b; 1997; Okawa *et al*., 1994; 1996). Moreover, serum factor 6 epitope may be expressed *in vivo* by serotype B strains infecting tissues and on germ tubes of serotype B strains *in vitro* (Poulain *et al*., 1983). In order to explain the serotype-related PLM differences which could represent an alternative to classical serotyping, PLM from *C. albicans* strain NIH B 792 (serotype B) was purified and analysed. Nuclear magnetic resonance (NMR) and mass spectrometry (MS) analysis revealed that the general structure of PLM from the NIH B 792 strain is similar to that of PLM from the serotype A strain but differs mainly by lower DPs of the β-oligomannosidic chains and an unexpectedly high ratio of DP=3, a β-1,2 mannotriose formerly described as the epitope of protective antibodies (Han *et al*., 1997; 2000). Analysis of the effect of growth temperature and investigation of additional strains of both serotypes confirmed that β-mannosylation of PLM appears to be the only fully described phenotypically stable molecule capable of differentiating *C. albicans* strains. It also suggests that the nature and/or regulation of β-mannosyl transferases could be among the genetic traits differentiating *C. albicans* serotypes and their characteristics.

Results

Analysis of the molar composition revealed that PLM-B from C. albicans NIH B 792 serotype B is a glycoinositolsphingolipid and pinpointed the position of the second hydroxylation of fatty acids in fungal sphingolipids

Analysis concerned PLM-B purified from broken cells according to our standard procedure including chloroform/methanol/water extractions, butanol/water partitions and phenyl-sepharose chromatography. As observed previously for PLM-A, gas chromatography (GC)-MS analysis of the methanolysis products of PLM-B revealed the presence of mannose, inositol, PHSs and hydroxylated fatty acids (FAs), and showed that the major FA and PHS of the ceramide moiety are always ramified in the position ω(n-3) whatever the growth temperature (Table 1, Fig. 1A). GC-MS analysis also revealed significant differences in lipid composition of PLM-28 and PLM-37, which mainly concerned the long-chain bases (LCBs): rC20:0 PHS is always predominant but its amount increases considerably at 37°C. rC18:0 PHS is also present in the two samples whereas trace of rC19:0 PHS was detected only in PLM 37°C (Fig. 1A).

The FAs detected were rC24:0 (major), rC25:0 and rC26:0, which are 2-hydroxylated as determined by the presence of the reporter ion at m/z = 286 (Pons *et al*., 2000). They are characterized by relatively intense molecular ions at m/z = 595, 609 and 623 respectively (Fig. 1C). Three other compounds with lower retention times showed high mass ions at m/z = 593, 607 and 621, respectively (Fig. 1D), suggesting that they were 2-hydroxylated mono-unsaturated fatty acid methyl esters (FAMEs) with 24, 25 and 26 carbon atoms but, in these compounds, the molecular ion was of low intensity compared with standard compounds (Pons *et al*., 2000). Their respective mass spectra also showed ions of higher mass suggesting that a second heptafluorobutyrate (HFB) group was present in these compounds. For the major compound of this series, the higher mass ion recovered systematically was the putative M-19 ion, corresponding to the loss of a fluorine atom as observed for HFB derivatives of LCBs and hydroxylated FAMEs. For the major C24 compound, the molecular ion (m/z = 806/807) was weak but detectable with amplification. Therefore, the ions at m/z = 593, 607 and 621 were not molecular ions, but resulted from a loss of an HFBA group (m/z = 214). It was

Table 1. Comparison of the composition of phospholipomannans synthesized by *C. albicans* NIH B 792 at 28°C and 37°C.

		Phospholipomannan 28°C	Phospholipomannan 37°C
Fatty acids (FAMES)	Component	% total FA	% total FA
	rC24:0 OH2[a]	87.36	75.09
	lC24:0 OH2	0.24	0.8
	rC24:0 OH2,10	4.78	9.56
	rC25:0 OH2	2.77	7.76
	rC25:0 OH2,10	0.25	0.65
	rC26:0 OH2	4.31	5.03
	lC26:0 OH2	0	0.09
	rC26:0 OH2,10	0.28	0.55
	C16:0		1.28
Long-chain bases (LCBs)	Component	% total LCB	% total LCB
	rC18 PHS[a]	41.48	13.68
	lC18 PHS	0.44	1.56
	rC19 PHS	0.39	0
	rC20 PHS	55.69	82.96
	lC20 PHS	2.00	1.79

a. r refers to a ramification of the chain in the position ω(n-3); l: linear chain.
Samples were submitted to two consecutive runs of methanolysis/HFBAA acylation then analysed by GC-MS.

concluded that these compounds corresponded to di-hydroxylated saturated FAMEs, ramified in the ω(n-3) position. Another characteristic of the mass spectra of these compounds was the presence of relatively abundant ions at m/z = 423, 437 and 451 for the C24, C25 and C26 compounds respectively (Fig. 1D). This could only be explained by considering that the second hydroxyl group was on the C_{10} carbon atom (Fig. 1B). Therefore, these compounds were unambiguously 2,10-di-hydroxylated FAMEs (Fig. 1D).

NMR analysis of PLM-B revealed an heterogeneous β-1,2 mannose polymer anchored to the ceramide moiety through the same specific spacer as PLM-A

The ^{1}H-NMR spectra of the PLM fractions extracted from strain NIH B 792, serotype B, contained 14 signals in the anomeric region, which are labelled from **A** to **L** (Fig. 2). Owing to the spin-connectivity pathways delineated in the COSY and TOCSY spectra, almost all the atom resonances of the mannose units were assigned (Fig. 3 and Table S1). The α and β anomery of these mannose units were distinguished on the basis of the chemical shifts of their H-5 atoms resonance, which were largely shifted downfield in the case of the α anomers (Fig. 3A). Anomery of the mannose units was also identified by the observation of nuclear Overhauser effect (NOE) contacts H-1/H3, H-5 for β mannose units **C** to **L**, and H-1/H-2 for α mannose units **A** and **B**.

The cross-peaks observed on the ^{1}H-^{13}C heteronuclear (HMQC) spectrum were assigned by comparison with the H-1/H-1 relayed COSY spectrum observed previously (Fig. 3B). The relatively low-field position of some ^{13}C resonances, compared with the corresponding ^{13}C resonance position of the non-substituted units, arose from glycosylation or phosphorylation of the mannose and inositol units and revealed the substitution pattern in the polymer. These data showed that mannose residues **A, C, E, F, I, J, K, L** are 2-substituted, both **B** and **B*** mannoses are 6-substituted, inositols **IN(I)**, **IN(I*)** are 1,2-di-substituted, mannose residues **D, G, H** and inositol **IN(II*)** are terminal non-substituted units (Fig. 3B).

The ^{1}H-^{31}P HMQC and HMQC-HOHAHA spectra (Fig. 3C) clearly showed the presence of three ^{31}P atom resonances ($^{31}P^{SP}$, $^{31}P^{B}$, $^{31}P^{B*}$) relative to the phosphodiester bridges between carbons C-1 of PHS **SP** and inositol **IN(I,I*)**, carbon C-1 of α-mannose **A** (or **A′**) and carbon C-6 of α-mannose **B**, carbon C-1 of inositol **IN(II*)** and carbon C-6 of α-mannose **B*** as indicated by red signals in Fig. 3C. This precised the position of the phosphodiester bridges in the molecule.

The proton spin system for the PHS and 2-hydroxylated FA were assigned on the COSY and TOCSY spectra. The entry points were the downfield shifted CH_2 signals at δ= 1.47/1.32, 1.58/1.46 and 1.25/1.16 correlated to proton resonances at δ= 3.386, 3.852 and 4.028 respectively (data not shown). The ^{1}H signal of the ceramide amide group, at 7.26 parts per million (p.p.m.), showed correlation to signals at 4.021, 3.890, 3.732, 3.447 and 3.386 p.p.m. and the HMQC spectrum allowed correlation of the C-2 resonance at 51.62 p.p.m. to the H-2 proton at 3.890. Consequently, the spin connectivity pathway of **SP** delineated in the COSY spectrum was clearly established (Fig. 3 and Table S1). The ^{1}H atom resonance observed at δ= 3.386 was assigned to the proton H-2 of 2-hydroxylated FAs whereas the signal observed at δ= 4.028 (not shown), which correlated to the CH_2 resonances at δ= 1.25/1.16, was tentatively assigned to the proton H-10

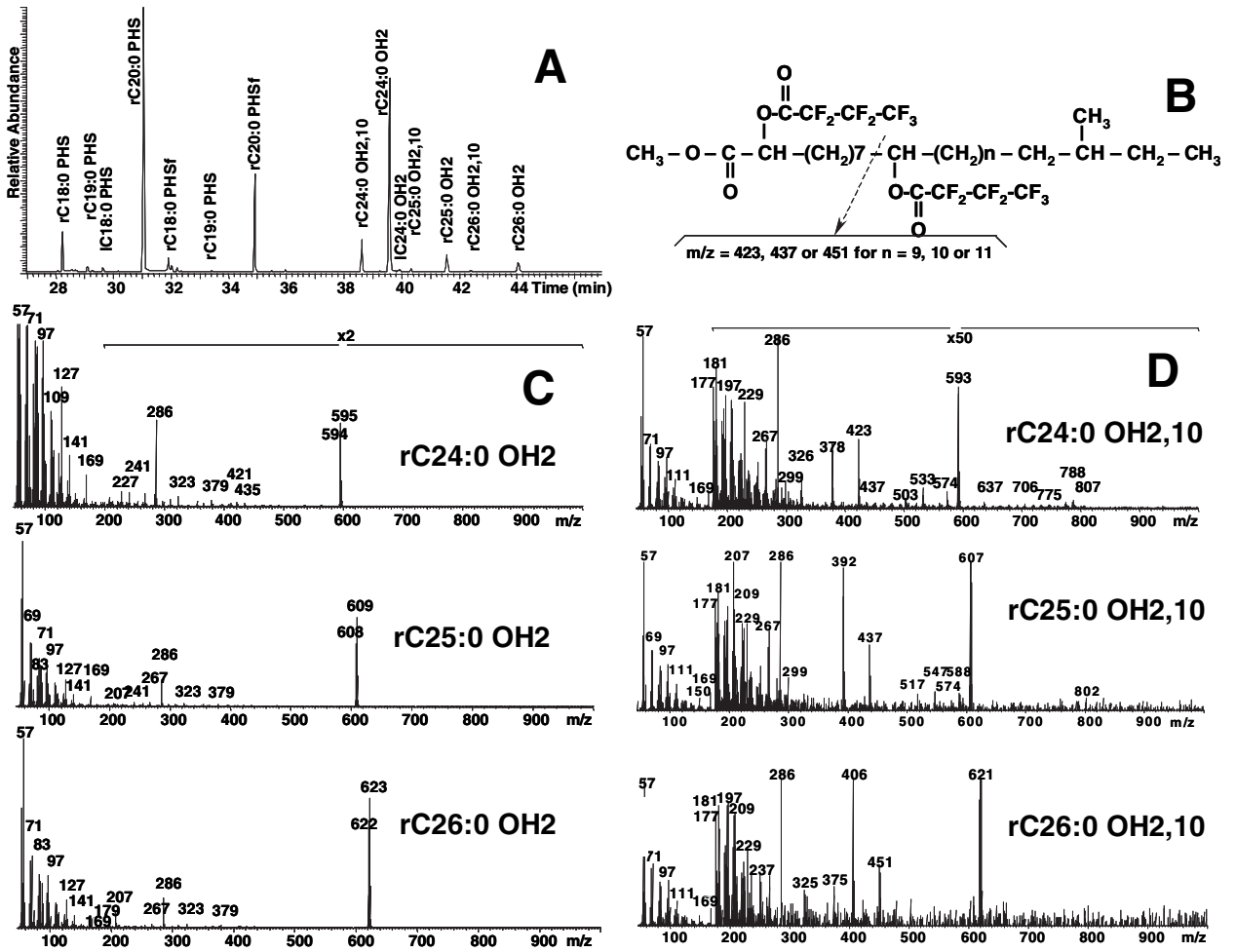


Fig. 1. GC-MS analysis of the lipid constituents of PLM 37°C samples.
A. GC-MS profile (total ion count of the area of elution of lipid constituents). The following nomenclature is used: rC18:0 PHS = ω(n-3) ramified C18:0 phytosphingosine; lC18:0 PHS = linear C18:0 phytosphingosine; rC24:0 OH2 FA = ω(n-3) ramified 2-hydroxy fatty acid with 24 carbon atoms; rC24:0 OH2,10 FA = ω(n-3) ramified 2,10-di-hydroxy fatty acid with 24 carbon atoms. It should be stressed that after acid-catalysed methanolysis, PHSs gave two peaks corresponding to the per-HFB derivative of the intact compound and the 2,3-di-HFB derivative of the dehydration product recovered as a furanic form (Pons *et al*., 2000; 2002).
B. Scheme of fragmentation allowing determination of the position of the second hydroxyl group of fatty acids.
C. Mass spectra of ramified 2-hydroxylated FAMEs with 24, 25 and 26 carbon atoms. The presence of a doublet ion for the molecular ion (e.g. 594 and 595 for rC24:0 OH2) was due to a limited resolution of the mass spectrometer necessary to increase the response of high mass ions, especially for derivatives of long-chain bases. The 2-hydroxy position was determined by the presence of the intense ion at m/z = 286 (Pons *et al*., 2000).
D. Mass spectra of ramified 2,10-di-hydroxylated FAMEs with 24, 25 and 26 carbon atoms. As an example, note that, in the rC24:0 OH2, 10 spectrum, the ion at m/z = 592/593 corresponded to a loss of a heptafluorobutyric acid group (m/z = 214) from the molecular ion (m/z = 806/807).

of 2,10-hydroxylated FAs identified above by GC-MS analysis.

Finally, the occurrence of transglycosidic NOEs between the proton pairs mannose **A** H-1/mannose **I** H-1 and mannose **A′** H-1/mannose **L** H-1 pinpointed the position of the mannose units in the molecule as shown at the top of Fig. 3: β-mannose **I** and **L** are attached to α-mannose-1-P **A** (or **A′**); **C, E, F, J, K** correspond to internal β-mannose units and β-mannose units **D**, **G** and **H** are located at a terminal non-reducing position. In the same way, the NOE contacts mannose **B** or **B*** H-1/inositol **IN(I)** or **IN(1*)** H-2 confirmed the attachment of mannose **B** at C-2 of **IN(I)**.

To avoid further confusion about our definition of the glycan chain and its DP, it should be pointed out that the true glycan chain of PLM is now considered to be the oligomannose chain linked to the second phosphate group (mannoses **A**, **A′** and **C** to **L** in Fig. 2), and therefore that mannose **B** (Fig. 3) is part of the spacer P-Man-Ins-P- linking this chain to the ceramide moiety, a structure common to PLM, MIPC and $M(IP)_2C$.

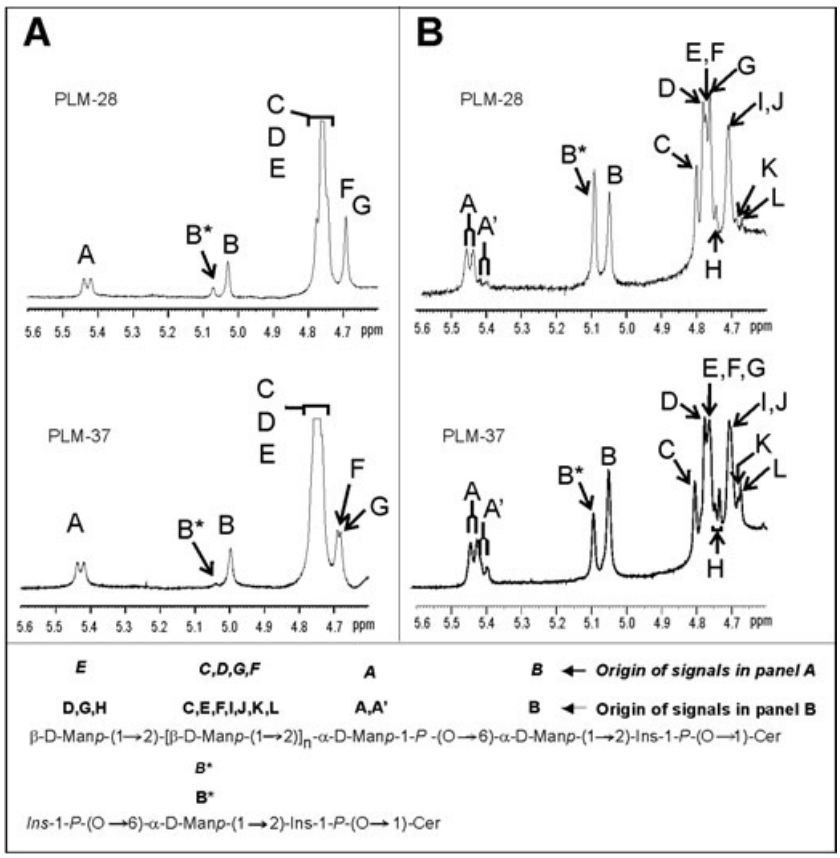
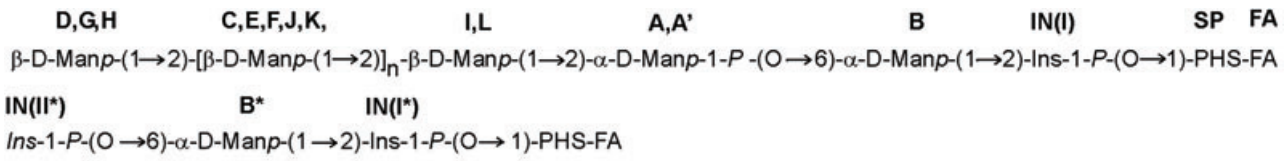


Fig. 2. Comparison of ^{1}H NMR spectra of PLM from *C. albicans* serotypes A and B strains grown at 28°C and 37°C.
A. The ^{1}H NMR spectra of PLM from serotype A (Trinel *et al*., 2002a) were used as reference spectra and to allow a better comparison of PLM-A and -B spectra.
B. In the PLM-B spectrum, signals relative to β-mannose (**C** to **L**) are more dispersed. This arises from the lower DP of the glycan chain of PLM-B. On the contrary, in PLM-A, the higher DP leads to uniformity in the values of the H-1 atom resonances of the inner β-mannose units (**C**, **D**, **E**) whatever their position and the DP of the glycan chain. The additional signal arising from α-Man-1-P observed in PLM-B spectra was labelled **A′**.
The general formula of phospholipomannan (upper formula) and $M(IP)_2C$ (lower formula) showed from which part of the molecules arise the signals labelled in (A) and (B).

Mass spectrometry analysis of PLM-B revealed an extensive heterogeneity arising from combinations of different ceramide moieties with various oligomannosidic chain lengths and evidenced a specific lower glycosylation

Electrospray mass spectra of PLM-28 and PLM-37 (Fig. 4A and B) purified from *C. albicans* strain NIH B 792, serotype B, demonstrated a lower heterogeneity than former MS spectra of PLM from a serotype A strain (Trinel *et al*., 2002a). These spectra revealed a series of groups of five peaks (sometimes six in PLM-37) spaced by an average m/z ratio of 81. These peaks correspond to (M-2H)/2 molecular-related ions and consequently to the DP of the glycan chain of PLM, which varies from 2 to 11. For example, peaks around m/z 840 corresponded to PLM with a mannotriose in its glycan chain. As observed formerly for PLM-A, the five peaks within each group arose from heterogeneity of the ceramide moiety of PLM which combines, according to molecular analysis of PLM (Fig. 1, Table 1), PHSs rC18 and rC20 to the hydroxylated FAs rC25:0, rC26:0 and rC24:0. For each group of peaks, these peaks are spaced alternatively at a m/z ratio of 8 and 6. This originates from the additional hydroxylation of the ceramide moiety of PLM present in peaks 2 and 4 of each group, which has not been observed previously in PLM-A. Complementary nano-ES-MS (Fig. 4C) specified that each peak 2 and 4 resulted in fact from the contribution of two PLM components with (major) and without (minor) this additional hydroxylation (Table 2). Therefore, in agreement with the ceramide hydroxylation described for sphingolipids of *C. albicans* (Wells *et al*., 1996) and according to molar composition analysis (Table 1), the ceramide moiety of these two components is most probably composed of rC24:0 OH2,10 FA and rC25:0 OH2 FA linked, respectively, to rC18 PHS in peak 2 and rC20 PHS in peak 4 (Fig. 4, Table 2). Whatever the growth temperature, the main peak of each group displays a ceramide moiety composed of rC20 PHS and rC24:0 OH2 FA, its ratio increasing at 37°C. A change in the relative importance of peaks 4 and 5 of each group is also observed between 28°C and 37°C. Major peaks with a m/z ratio between 650 and 700 may be attributed to (M-2H)/2 molecular-related ions of $M(IP)_2C$ which, as observed previously, is the main contaminant of PLM preparations. It is interesting to note that, as observed for *C. albicans* VW32, $M(IP)_2C$ and PLM display the same heterogeneity of their ceramide moiety and that this heterogeneity evolves in the same way when the growth temperature increases from 28°C to 37°C. At the other end of the spectrum, peaks around m/z 1357.2 correspond to a mix-



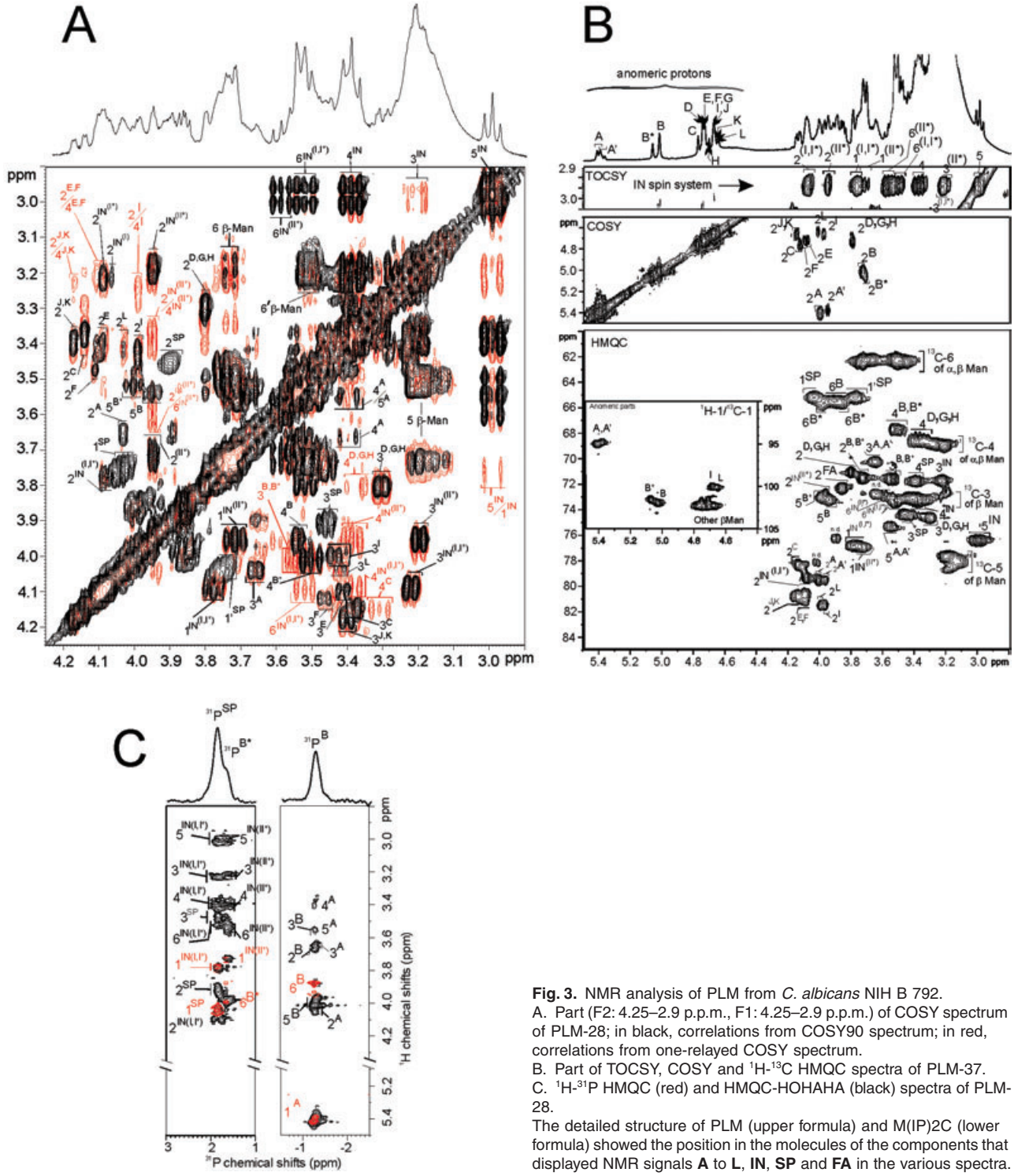


Fig. 3. NMR analysis of PLM from *C. albicans* NIH B 792.
A. Part (F2: 4.25–2.9 p.p.m., F1: 4.25–2.9 p.p.m.) of COSY spectrum of PLM-28; in black, correlations from COSY90 spectrum; in red, correlations from one-relayed COSY spectrum.
B. Part of TOCSY, COSY and ^{1}H-^{13}C HMQC spectra of PLM-37.
C. ^{1}H-^{31}P HMQC (red) and HMQC-HOHAHA (black) spectra of PLM-28.
The detailed structure of PLM (upper formula) and M(IP)2C (lower formula) showed the position in the molecules of the components that displayed NMR signals **A** to **L**, **IN**, **SP** and **FA** in the various spectra.

Table 2. Negative-ion ES-MS of PLM-37 from *C. albicans* strain NIH B 792: examples of masses and molecular ratios deduced from two groups of detected (M-2H)/2 molecular-related ions.

Average measured m/z	Calculated average mass value	Composition (number of residues)[a]				
		Man	Ins	PHS	FA	PO3H
826.30	1654.81	4	1	1 rC18	1 rC24:0 OH2	2
834.18[b]	1668.84 (minor)	4	1	1 rC18	1 rC25:0 OH2	2
	1670.81 (major)	4	1	1 rC18	1 rC24:0 OH2,10	2
840.32	1682.86	4	1	1 rC20	1 rC24:0 OH2	2
	1682.86	4	1	1 rC18	1 rC26:0 OH2	2
848.14[b]	1696.89 (minor)	4	1	1 rC20	1 rC25:0 OH2	2
	1698.86 (major)	4	1	1 rC20	1 rC24:0 OH2,10	2
854.77	1710.92	4	1	1 rC20	1 rC26:0 OH2	2
907.39	1816.95	5	1	1 rC18	1 rC24:0 OH2	2
915.27[b]	1830.98 (minor)	5	1	1 rC18	1 rC25:0 OH2	2
	1832.95 (major)	5	1	1 rC18	1 rC24:0 OH2,10	2
921.41	1845.01	5	1	1 rC20	1 rC24:0 OH2	2
	1845.01	5	1	1 rC18	1 rC26:0 OH2	2
929.10[b]	1859.03 (minor)	5	1	1 rC20	1 rC25:0 OH2	2
	1861.01 (major)	5	1	1 rC20	1 rC24:0 OH2,10	2
935.86	1873.06	5	1	1 rC20	1 rC26:0 OH2	2

a. Composition of the molecules was defined according to their mass and the results of molar composition analysis (Fig. 1 and Table 1).
b. As confirmed by additional nano-electrospray mass spectrometry (Fig. 4C), two components with and without an additive hydroxylation contribute to the peaks 2 and 4 from each group observed in Fig. 4B: the corresponding average mass values were calculated.

ture of peaks arising from a higher DP of PLM [(M-2H)/2 molecular-related ions] and (M-H) molecular-related ions of $M(IP)_2C$.

Mass spectrometry analysis of additional strains of both serotypes confirmed specific glycosylation of PLM-B and overexpression of a β-1,2 mannotriose, the epitope of protective antibodies

To confirm the differences observed between PLM-A and PLM-B, and whether these differences could arise from strain-to-strain variability rather than serotype, PLM was purified from other strains of both serotypes. ES-MS analysis of PLM-28 preparations revealed m/z values close to that of PLM-28 from strain NIH B 792 for a same DP of the glycan chain and an analogous heterogeneity within the same serotype (Fig. 5A and B). It also confirmed the importance of C24:0 OH2 FAs in *C. albicans* glycosphingolipids and the great differences in distribution of the β-oligomannose chains of PLM in serotypes A and B strains. A lower DP of the glycan chain, as well as the importance of DP=3 appeared to be a characteristic feature of PLM from serotype B strains (Fig. 5B) although their ratios varied from strain to strain (Fig. 6A). This higher expression of the DP=3, corresponding to a β-1,2 mannotriose previously shown to be the epitope of the protective antibody B6-1 (Han *et al*., 1997), explains the exclusive reactivity of PLM from serotype B strains to this monoclonal antibody (mAb) (Fig. 6B). The ratio PHS C20/C18 appears to be slightly higher in serotype B strains but does not reach that observed in PLM from NIH B 792 strain.

Discussion

In a previous study, the molecular structure of PLM from *C. albicans* strain VW32, serotype A, was shown to consist of highly mannosylated sphingolipids (Trinel *et al*., 2002a). The structure of this PLM, which combined glycan and lipidic heterogeneity, appeared to be unique among presently known glycosphingolipids due to the high DP of the glycan chain, the unusual β anomery of the mannose linkages and the composition of the spacer linking the oligomannosidic chain to the lipid moiety. From this structure, it was suggested that PLM biosynthesis follows the biosynthetic pathway of fungal sphingolipids up to MIPC where it diverges by the addition of mannose phosphate instead of the addition of inositol-phosphate leading to $M(IP)_2C$, the major fungal plasma membrane sphingolipid. This selective addition of mannose phosphate allows extensive β-mannosylation of the molecule, which confers on PLM its specific physicochemical and immunomodulatory properties (Jouault *et al*., 1998; 2003; Ibata-Ombetta *et al*., 2003a,b) along with its cell wall localization (Poulain *et al*., 2002). Both this biosynthesis hypothesis and the structure of PLM were confirmed by construction of a *C. albicans* mutant, *mit1*Δ, devoid of the IPC mannose transferase *CaMitp* homologous to *ScSur1p*. This mutant failed to synthesize MIPC, $M(IP)_2C$ and PLM (Mille *et al*., 2004). In another study, PLMs from *C. albicans* strains displayed different molecular weights and antigenic reactivities depending on the serotype of the parent strain (Trinel *et al*., 2002b). To explain these differences, the molecular structure of PLM from *C. albicans* strain NIH B 792, serotype B, has been characterized; this strain has been used

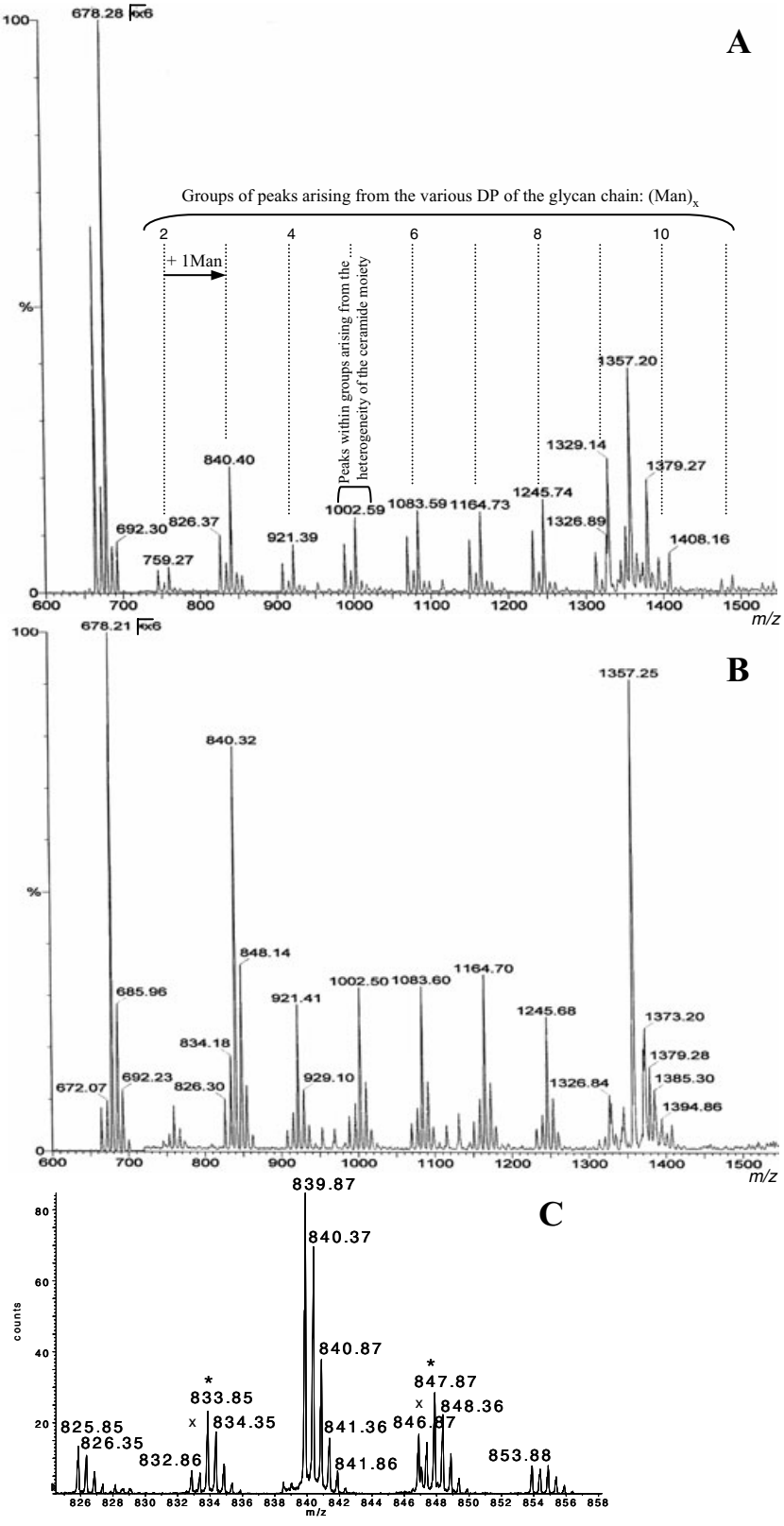


Fig. 4. Mass spectra of PLM synthesized by *C. albicans* NIH B 792.
A and B. Electrospray mass spectra of PLM 28°C (A) and 37°C (B). The groups of peaks correspond to M(IP)2C (peaks at m/z around 678) and to the various degrees of glycosylation of PLM (2–11 Man) and peaks within each group arise from heterogeneity of the ceramide moiety. Spectra mainly display (M-2H)/2 molecular-related ions except for the M-H molecular-related ions of $M(IP)_2C$ (peak at m/z 1357.2). The displayed m/*z* values resulted from smoothing of the spectra. Differences between PLM-28 and PLM-37 spectra mainly originate from a general increase in the incorporation of phytosphingosine C20 in the ceramide moiety of PLM and $M(IP)_2C$ at 37°C.
C. Selected part of the nano-electrospray mass spectrum of PLM-37 corresponding to the group of peaks with three mannoses in the glycan chain of PLM 37°C (m/z around 840.32 in Fig. 4B). This reveals (M-2H)/2 molecular-related ions and the presence of two compounds, mono-hydroxylated (x) and di-hydroxylated (*), in the second and fourth group of peaks.

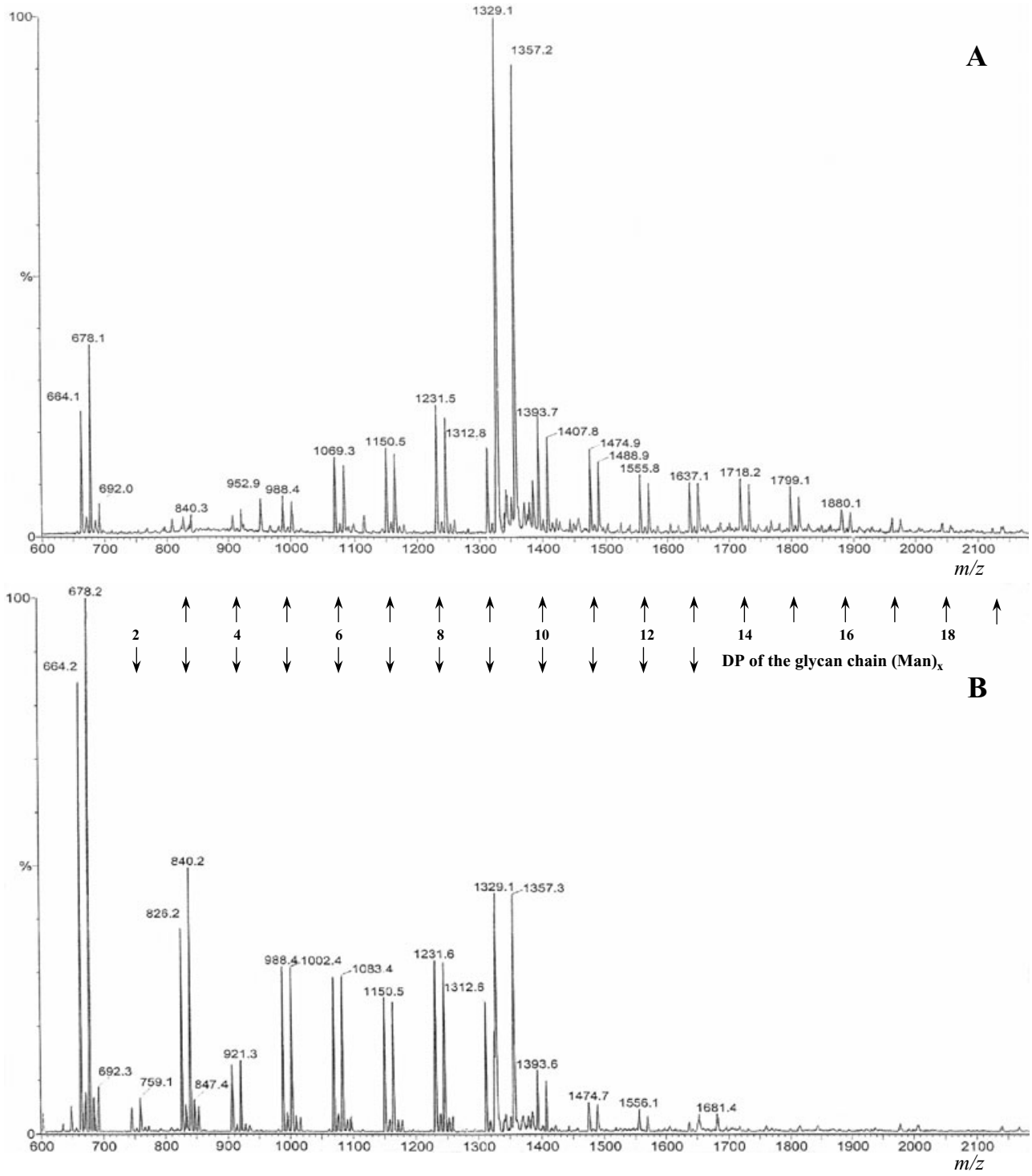


Fig. 5. Electrospray mass spectrometry of PLM synthesized by *C. albicans* strains ATCC 10261, serotype A (A), and ATCC 90028, serotype B (B), at 28°C. The general interpretation of mass spectrometry spectra follows that of Fig. 4. Arrows point out the degrees of polymerization observed in each strain. Spectra confirmed that the glycan chains of PLM from serotype A display higher degrees of polymerization than those of PLM from serotype B and the highly divergent expression of DP=3 in the two serotypes.

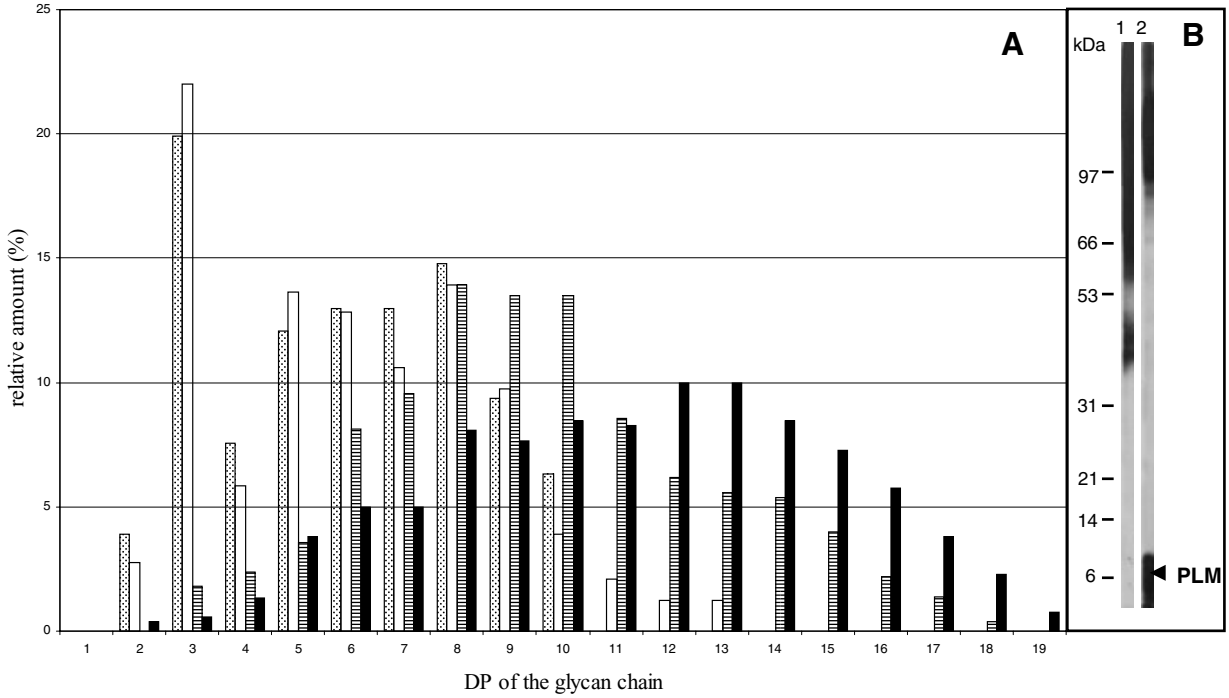


Fig. 6. Comparison of the glycosylation of PLM from *C. albicans* serotypes A and B.
A. Distribution and relative intensities of the degrees of polymerization (DP) of PLM-28 of strains NIH 8792 (⬚), ATCC 90028 (□), ATCC 10261 (▤) and VW32 (■). Relative intensities were estimated from the main peak observed in mass spectra for each DP of the glycan chain and must not be considered as true values. For strain VW32, values were calculated from a previous study (Trinel *et al.*, 2002a).
B. Western blot of whole cell extracts from *C. albicans*, strains VW32, serotype A (lane 1) and NIH B 792, serotype B (lane 2) stained with monoclonal antibody B6-1 according to our previously described procedure (Trinel *et al.*, 2002b). Extracts were obtained by the procedure of alkaline extraction under reducing conditions and analysed by SDS-PAGE on 5–15% acrylamide gels (Trinel *et al.*, 2002b): the absence of staining of PLM in lane 1 (smear around 6 kDa) is explained by the low concentration of β-1,2-linked mannotriose in PLM from serotype A strains (see A). Higher-molecular-weight materials stained in lanes 1 and 2 correspond to mannan and mannoproteins.

extensively for structural analysis of phosphopeptidomannan (PPM) (Shibata *et al.*, 1992; 1995). MS and NMR analyses revealed that PLM-B displays the same general structure and heterogeneity as PLM-A, but exhibits two-fold less mannosylation which explains its lower molecular mass on SDS-PAGE. The overexpression of the β-1,2 mannotriose in PLM-B provide a structural explanation for the previously observed elective reactivity of PLM from serotype B strains with mAb B6.1 (Trinel *et al.*, 2002b), an IgM that protects against experimental candidosis (Han and Cutler, 1995; Han *et al.*, 1998). It also confirms the nature of the epitope (Han *et al.*, 1997).

The lower degree of mannosylation of PLM-B leads to increased $M(IP)_2C$ contamination in PLM-B preparations, because these molecules have closer physicochemical properties than those of serotype A. However, according to the extreme heterogeneity of the PLM and our results, we hypothesized that any additive purification step should lead to a partial selection of PLM molecules and thus to the study of an unrepresentative population of PLM molecules. This hypothesis was confirmed by attempts at improved purification using silicagel columns that have highly decreased $M(IP)_2C$ contamination but have also lowered the amount of the DP=3 of PLM (data not shown). Nevertheless, $M(IP)_2C$ contamination does not limit the interpretation of MS and NMR analyses and, moreover, revealed that PLM, whatever its DP, expresses the same heterogeneity as $M(IP)_2C$ in its ceramide moiety, and that this heterogeneity evolves in the same way when growth temperature increases from 28°C to 37°C. It therefore appeared that the heterogeneity of the ceramide moiety of *C. albicans* sphingolipids takes place in steps anterior to MIPC rather than from a change in the ceramide composition of the mature molecules as observed for remodelling of the ceramide moiety of GPI anchors (Reggiori *et al.*, 1997). MS analysis of $M(IP)_2C$, along with PLM composition, also confirmed that *C. albicans* preferentially integrates a C24:0 FA and C20 PHS in its sphingolipids (Wells *et al.*, 1996) instead of a C26:0 FA and C18 PHS seen in *S. cerevisiae* (Dickson and Lester, 1999b). A higher PHS C20/C18 ratio was observed in PLM-B together with minor second hydroxylation of the FAs. GC-MS analysis of the methanolysis products revealed that the latter was localized on carbon C10 of rC24:0 OH2,10 and rC25:0 OH2,10 FAs. The position of the second hydroxylation of fungal sphingolipid FAs was still unde-

fined for both *C. albicans* and *S. cerevisiae* sphingolipids (Uemura *et al*., 2003). However, analysis of PLMs from several strains of both serotypes showed that most of the observed differences in the ceramide moieties, including this additional hydroxylation, appeared to be strain-dependent rather than serotype-dependent. A more constant feature was the incorporation of higher amounts of PHS C20 in PLM from serotype B strains.

As told above, the most important and constant difference between PLM from the two *C. albicans* serotypes concerned the DPs of their glycan part. This serotype-specific difference observed in this study on a limited number of strains explains the results of Western blots of a larger number of strains of both serotypes (Trinel *et al*., 2002b) and subsequently confirmed in our laboratory (data not shown). Interestingly, as observed formerly for PLM-A, the specific β-mannosylation of PLM-B is not affected by growth at high temperature that inhibit PPM β-mannosylation (Okawa *et al*., 1994; 1996) as well as by growth at acidic pH also known to have the same effect (Kobayashi *et al*., 1994a,b; 1997). None of the culture conditions investigated, including serum-induced filamentation, have an effect on both PLMs serotype specificity assessed in Western blots (data not shown).This alteration of PPM β-mannosylation depending on growth conditions affects both the PPM acid-labile fraction (homopolymers of β-1,2-linked mannoses corresponding to antigenic/serum factor 5) and the PPM acid-stable fraction (β-1,2-linked mannoses at the non-reducing end of α-1,2 linkages) corresponding to antigenic/serum factor 6 (Shibata *et al*., 1992). Selective cell agglutination with serum factor 6 is the current reference method for serotyping *C. albicans*. In addition to the possible environmentally triggered absence of synthesis of the serotype A epitope by serotype A cells, it has also been shown that serotype B strains can synthesize the serotype A epitope when cells are triggered by environmental conditions encountered during tissue invasion (Poulain *et al*., 1983) or those promoting germ tube formation (Poulain *et al*., 1983).

Accurate identification of *C. albicans* serotype is a necessary step for the correct analysis of biochemical or biological data on wild or mutant strains (Hobson *et al*., 2004). The epidemiological relevance of serotyping has long been recognized and firmly established by numerous studies showing that *C. albicans* serotypes differ in their ability to colonize hosts with different genetic backgrounds or immune status (McMullan-Vogel *et al*., 1999; Hannula *et al*., 2001) and sensitivity to 5-fluorocytosine (Velegraki, 1995; Al-Hedaithy and Fotedar, 2002), even in countries where this drug is not currently available for therapy (Quindos *et al*., 2004). This leads to the concept that serotype A and B strains differ in their parasitic adaptation, justify different types of clinical management and suggest genetic differences as the basis for such important issues. Unfortunately, discrepancies have regularly been reported between serotyping methods (Brawner, 1991) and serotype is often difficult to define when antigenic factors are poorly expressed at the cell surface (either poor expression or masking by other epitopes) depending on the strain or growth conditions (Kobayashi *et al*., 1994b; Barturen *et al*., 1995; Okawa *et al*., 1996). Moreover, recent studies have also demonstrated that problems in serotyping could arise from the specificity of the rabbit antisera used since the β-1,2-linked mannose structures expressed in the acid-stable fraction do not react as antigenic factor 6 but are erroneously recognized as antigenic factor 5 (acid-labile part) when these structures express more than two β-mannose linkages at the non-reducing end of α-1,2-linked mannose residues (Kobayashi *et al*., 2003; Shibata *et al*., 2003). These problems with serotyping may explain why some 'serotype B' strains constantly display a PLM with serotype A properties (P.A. Trinel, unpubl. results). As recently stated in the discussion of a paper on the contribution of Ca*MNN4* gene disruption to the study the biological role of β-mannosides (Hobson *et al*., 2004), difficulties in serotyping (retesting of the CA1 strain considered as A and subsequently identified as B) provide unnecessary confusion in a domain already very complex. According to the stability of expression of PLM by *C. albicans* strains, which is not affected by growth conditions, PLM would appear to be a phenotypically more stable alternative for the accurate identification of *C. albicans* serotype. A study in progress on *C. dubliniensis*, a species closely related to *C. albicans* serotype A, has demonstrated a constant expression of type A PLM within a large number of reference strains and clinical isolates.

It is interesting to note that, as for PPM, β-mannosylation of a molecule is once more at the centre of this new serotype-related issue. The pathophysiological importance of these residues has been shown in a large number of studies, which have demonstrated their role in adhesion, signal induction in host cells and protective antibody generation. Such studies have involved β-mannosides associated with carrier molecules (i.e. PPM, PLM, PPM-derived β-mannosides or their synthetic analogues). Very close structural/activity relationships have been shown with the DP of β-1,2-linked oligomannoses (i.e. the ability to stimulate TNF-α production by cells of macrophage lineage; Jouault *et al*., 1995), or the induction/support of protective antibodies where the importance of DP=3, a β-1,2 mannotriose, was confirmed by the production of two different mAbs of different isotypes which presented both protective activity against experimental candidosis and the same specificity (Han and Cutler, 1995; Han *et al*., 1997; 2000). Therefore both serotypes, which have β-mannose differently expressed on PPM (Shibata *et al*., 1992), PLM (Trinel *et al*., 2002b) or mannoproteins (Mille

et al., 2004), probably interact differently with host adaptive and innate immunity (Kozel *et al*., 2004).

In conclusion, the present article proposes that PLM is a phenotypically stable molecule for the identification of *C. albicans* serotype, and suggests that the nature and/or regulation of β-mannosyl transferases could be among the genetic traits differentiating *C. albicans* serotypes and their established characteristics.

Experimental procedures

Strains and growth conditions

Candida albicans strains NIH B 792 (serotype B), ATCC 90028 (serotype B) and ATCC 10261 (serotype A) were used in this study. For large scale cell production, cells were pre-incubated on Sabouraud's agar for 18 h at 28°C or 37°C, and then inoculated at a concentration of 1×10^6 cells ml^{-1} in 1 l Erlenmeyer flasks containing 500 ml of Sabouraud's broth. Growth was performed to stationary phase at 28°C or 37°C on an orbital shaker (180 r.p.m.). Cells were finally collected by centrifugation.

Purification of PLM

Cells were broken with a French press (Aminco) at 20000 psi, dialysed and lyophilized. PLM was then extracted and purified as described previously (Trinel *et al*., 1999) except that care was taken to avoid micelle formation in order to improve PLM solubility for NMR studies (Trinel *et al*., 2002a). The main steps in PLM purification were: (i) successive extractions with chloroform/methanol and chloroform/methanol/water mixtures, (ii) extensive butanol/water partitions of the chloroform/methanol/water (10:10:3, v:v:v) extracts and (iii) purification of the water phases on phenyl-sepharose using increasing concentrations of ethanol (1–40%) for elution. Purification and control of PLM fractions was followed by thin-layer chromatography (Trinel *et al*., 1999) and Western blot analysis using the mouse IgM mAb, DF9-3 (Trinel *et al*., 1993).

Monosaccharide and lipid composition of PLM

Samples (1–5 μg) of purified PLM were lyophilized in conical heavy walled Pyrex tubes (2.0 ml) with Teflon lined screw caps. Methanolysis reagent (0.25–0.5 ml) was then added and the closed vessels were left for 20 h at 80°C. The methanolysis reagent was obtained by dissolving anhydrous gaseous HCl (up to 0.5 M) at −50°C in anhydrous methanol previously redistilled on magnesium turnings (Pons *et al*., 2000). Gaseous HCl was prepared by the dropwise addition of concentrated sulphuric acid onto crystallized sodium chloride. After methanolysis, samples were evaporated to dryness under a light stream of nitrogen in a ventilated hood, followed by the addition of 200 μl of acetonitrile and 25 μl of heptafluorobutyric anhydride (HFBAA) with a pipette with plastic tips. The closed vessels were heated for 15 min at 150°C in a sand bath. After cooling to room temperature, the samples were evaporated in a light stream of nitrogen in a ventilated hood in order to eliminate excess reagent, and samples were then submitted to a further methanolysis followed by acylation with HFBAA as described above. This second methanolysis and acylation step was found to be necessary for complete cleavage of phosphodiester bonds (and, when present, the GlcN-Ins linkage found in glycosylphosphatidylinositols). After drying, the sample was taken up in 200 μl of acetonitrile (previously dried on calcinated calcium chloride) and an aliquot was introduced into the Ross injector of the GC apparatus.

GC-MS analysis

For GC-MS analysis, the GC separation was performed on a Carlo Erba GC 8000 gas chromatograph equipped with a 25 m × 0.32 mm CP-Sil5 CB low bleed/MS capillary column, 0.25 μm film phase (Chrompack France, Les Ullis, France). The temperature of the Ross injector was 260°C and the samples were analysed using the following temperature programme: 90°C for 3 min, then 5°C min^{-1} until 240°C. The column was coupled to a Finnigan Automass II mass spectrometer. The analyses were performed in the electron impact mode (ionization energy 70 eV; source temperature 150°C). As described previously (Pons *et al*., 2000; 2002), this method allowed the identification and quantification of all constituents of glycolipids in a single GC-MS analysis.

Mass spectrometry

Electrospray (ES) mass measurements were carried out in the negative-ion mode on a triple quadrupole instrument (Micromass, Altrincham, UK) fitted with an atmospheric pressure ionization ES source. A 5 pmol μl^{-1} solution of myoglobin in acetonitrile/water (50:50, v:v), 0.2% formic acid, was used to calibrate the quadrupole mass spectrometer. The samples were dissolved in dimethyl sulphoxide and further diluted in methanol to obtain a final concentration of about 0.5 μg μl^{-1}. Solutions were infused using a Harvard syringe pump at a flow rate of 3 μl min^{-1}. The quadrupole was scanned from 600 to 2200 Da with a scan duration of 5 s and a scan delay of 0.15 s. The samples were sprayed using a 3.5 kV needle voltage and the declustering cone was set at 70 V.

Nano-ES-MS analyses were performed using a Q-STAR Pulsar quadrupole time-of-flight (Q-q-TOF) mass spectrometer (Applied Biosystems, Toronto, Canada) fitted with a nano-ES ionization source (Protana, Odense, Denmark). The samples of PLM-B, dissolved in dimethyl sulphoxide and further diluted in methanol/water (80:20, v:v) to obtain a final concentration of about 0.2 μg μl^{-1}, were sprayed from gold-coated medium-length borosilicate capillaries (Proxeon, Odense, Denmark). A potential of −800 V was applied to the capillary tip and the focusing potential was set at −220 V, the declustering potential varying between −80 V and 120 V. Q-STAR spectra were acquired by scans accumulation during 1 min over the range m/z 700–2000 Da. Typically, the full width at half maximum was 7000 in the measured mass range. External calibration was performed using a 4 pmol μl^{-1} solution of taurocholic acid in acetonitrile/water (50:50, v:v) containing 2 mM ammonium acetate.

NMR experiments

Samples (PLM-28 and PLM-37) were treated repeatedly with ^2H_2O (99.97% ^2H atoms; Euriso-top, CEA, Saclay, France) and then dissolved in 250 µl of Me_2SO-d6 (99.98% ^2H; Euriso-top). ^1H and ^{13}C chemical shifts were expressed in parts per million (p.p.m.) downfield from the signal of DMSO-d6 methyl group ($\delta^1H = 2.50$ and $\delta^{13}C = 40.55$ p.p.m.) and ^{31}P chemical shifts were referenced to external H_3PO_4 ($\delta = 0.00$ p.p.m.). Samples were analysed in 200×5 mm BMS-005B Shigemi® tubes at 343°K on a Bruker® ASX-400 spectrometer (^1H: 400.33; ^{13}C: 100.66; ^{31}P: 162.5 MHz) equipped with a double resonance ($^1H/X$) broadband inverse z-gradient probe head. All NMR samples were recorded without sample spinning.

The one-dimensional proton ^1H spectra were measured using a 90° tipping angle for the pulse and 1.5 s as a recycle delay between each 32 acquisitions of 2.4 s. The spectral width of 4006 Hz was collected in 16 000 complex data points. One-dimensional ^{13}C was recorded using a spectral width of 20 161 Hz and 32 768 data points were collected to obtain a FID resolution of 0.6 Hz per point. The ^{31}P spectra of both compounds were acquired with a spectral width of 16 233 Hz collected in 16 384 data points. Both experiments were carried out with composite pulse decoupling during acquisition using GARP sequence at the carbon or phosphorus frequency. An exponential transformation (line broadening factor = 6 Hz for ^{13}C and 4 Hz for ^{31}P) was applied before processing data points in the frequency domain. Two-dimensional homonuclear (^1H-^1H) COSY, one step-relayed COSY, two step-relayed COSY and ROESY, and heteronuclear ^1H-^{13}C HMQC, ^1H-^{31}P HMQC, and HMQC-HOHAHA spectroscopy were carried out using standard Bruker pulse programmes (cosy, cosyrl, cosyr2, roesytp, inv4tp, inv4mlevtp respectively). All experiments were carried out in the time phase-proportional increment except COSYs, which were conducted in the magnitude mode. Finally, two-dimensional HMQC-HOHAHA was recorded using Malcom Lewitt-17 mixing sequences with a mixing time of 120 ms for both ^{13}C and ^{31}P nuclei. Two-dimensional ROESY spectra were obtained with mixing times of 400 ms. All parameters including special delays were carried out in each experiment.

Acknowledgements

The MS facility used in this study is funded by the European Community (FEDER), the Region Nord-Pas de Calais (France), the CNRS and the Université des Sciences et Technologies de Lille. We gratefully acknowledge Professor J. Cutler (Children's Hospital, New Orleans, USA) for providing mAb B6-1. We thank Mrs Annick Masset for her technical assistance. This work has been partly funded by the European programme Interreg IIIA.

References

Al-Hedaithy, S.S., and Fotedar, R. (2002) Recovery and studies on chlamydospore-negative *Candida albicans* isolated from clinical specimens. *Med Mycol* **40:** 301–306.

Bagnat, M., Keranen, S., Shevchenko, A., and Simons, K. (2000) Lipid rafts function in biosynthetic delivery of proteins to the cell surface in yeast. *Proc Natl Acad Sci USA* **97:** 3254–3259.

Barreto-Bergter, E., Pinto, M.R., and Rodrigues, M.L. (2004) Structure and biological functions of fungal cerebrosides. *An Acad Bras Cienc* **76:** 67–84.

Barturen, B., Bikandi, J., San Millan, R., Moragues, M.D., Regulez, P., Quindos, G., and Ponton, J. (1995) Variability in expression of antigens responsible for serotype specificity in *Candida albicans*. *Microbiology* **141:** 1535–1543.

Beeler, T., Gable, K., Zhao, C., and Dunn, T. (1994) A novel protein, CSG2p, is required for Ca^{2+} regulation in *Saccharomyces cerevisiae*. *J Biol Chem* **269:** 7279–7284.

Brawner, D.L. (1991) Comparison between methods for serotyping of *Candida albicans* produces discrepancies in results. *J Clin Microbiol* **29:** 1020–1025.

Cantelli, C., Trinel, P.A., Bernigaud, A., Jouault, T., Polonelli, L., and Poulain, D. (1995) Mapping of beta-1,2-linked oligomannosidic epitopes among glycoconjugates of *Candida* species. *Microbiology* **141:** 2693–2697.

Desai, K., Sullards, M.C., Allegood, J., Wang, E., Schmelz, E.M., Hartl, M., *et al.* (2002) Fumonisins and fumonisin analogs as inhibitors of ceramide synthase and inducers of apoptosis. *Biochim Biophys Acta* **1585:** 188–192.

Dickson, R.C. (1998) Sphingolipid functions in *Saccharomyces cerevisiae*: comparison to mammals. *Annu Rev Biochem* **67:** 27–48.

Dickson, R.C., and Lester, R.L. (1999a) Yeast sphingolipids. *Biochim Biophys Acta* **1426:** 347–357.

Dickson, R.C., and Lester, R.L. (1999b) Metabolism and selected functions of sphingolipids in the yeast *Saccharomyces cerevisiae*. *Biochim Biophys Acta* **1438:** 305–321.

Han, Y., and Cutler, J.E. (1995) Antibody response that protects against disseminated candidiasis. *Infect Immun* **63:** 2714–2719.

Han, Y., Kanbe, T., Cherniak, R., and Cutler, J.E. (1997) Biochemical characterisation of *Candida albicans* epitopes that can elicit protective and nonprotective antibodies. *Infect Immun* **65:** 4100–4107.

Han, Y., Morrison, R.P., and Cutler, J.E. (1998) A vaccine and monoclonal antibodies that enhance mouse resistance to *Candida albicans* vaginal infection. *Infect Immun* **6:** 5771–5776.

Han, Y., Riesselman, M.H., and Cutler, J.E. (2000) Protection against candidiasis by an immunoglobulin G3 (IgG3) monoclonal antibody specific for the same mannotriose as an IgM protective antibody. *Infect Immun* **68:** 1649–1654.

Hannula, J., Dogan, B., Slots, J., Okte, E., and Asikainen, S. (2001) Subgingival strains of *Candida albicans* in relation to geographical origin and occurrence of periodontal pathogenic bacteria. *Oral Microbiol Immunol* **16:** 113–118.

Hobson, R.P., Munro, C.A., Bates, S., MacCallum, D.M., Cutler, J.E., Heinsbroek, S.E., *et al.* (2004) Loss of cell wall mannosylphosphate in *Candida albicans* does not influence macrophage recognition. *J Biol Chem* **279:** 39628–39635.

Ibata-Ombetta, S., Idziorek, T., Trinel, P.A., Poulain, D., and Jouault, T. (2003a) *Candida albicans* phospholipomannan promotes survival of phagocytosed yeasts through modulation of bad phosphorylation and macrophage apoptosis. *J Biol Chem* **278:** 13086–13093.

Ibata-Ombetta, S., Idziorek, T., Trinel, P.A., Poulain, D., and Jouault, T. (2003b) Role of phospholipomannan in *Candida albicans* escape from macrophages and induction of cell apoptosis through regulation of bad phosphorylation. *Ann NY Acad Sci* **1010:** 573–576.

Jouault, T., Lepage, G., Bernigaud, A., Trinel, P.A., Fradin, C., Wieruszeski, J.M., *et al.* (1995) Beta-1,2-linked oligomannosides from *Candida albicans* act as signals for tumor necrosis factor alpha production. *Infect Immun* **63:** 2378–2381.

Jouault, T., Fradin, C., Trinel, P.A., Bernigaud, A., and Poulain, D. (1998) Early signal transduction induced by *Candida albicans* in macrophages through shedding of a glycolipid. *J Infect Dis* **178:** 792–802.

Jouault, T., Fradin, C., Trinel, P.A., and Poulain, D. (2000) *Candida albicans*-derived beta-1,2-linked mannooligosaccharides induce desensitisation of macrophages. *Infect Immun* **68:** 965–968.

Jouault, T., Ibata-Ombetta, S., Takeuchi, O., Trinel, P.A., Sacchetti, P., Lefebvre, P., *et al.* (2003) *Candida albicans* phospholipomannan is sensed through toll-like receptors. *J Infect Dis* **188:** 165–172.

Kobayashi, H., Shibata, N., Suzuki, A., Takahashi, S., Suzuki, M., Matsuda, K., *et al.* (1994a) Expression of alpha-1,3 linkage-containing oligomannosyl residues in a cell-wall mannan of *Candida tropicalis* grown in yeast extract-Sabouraud liquid medium under acidic conditions. *FEBS Lett* **342:** 19–22.

Kobayashi, H., Takahashi, S., Shibata, N., Miyauchi, M., Ishida, M., Sato, J., *et al.* (1994b) Structural modification of cell wall mannans of *Candida albicans* serotype A strains grown in yeast extract-Sabouraud liquid medium under acidic conditions. *Infect Immun* **62:** 968–973.

Kobayashi, H., Tanaka, S., Suzuki, J., Kiuchi, Y., Shibata, N., Suzuki, S., and Okawa, Y. (1997) Amended structure of side chains in a cell wall mannan from *Candida albicans* serotype A strain grown in yeast extract-Sabouraud liquid medium under acidic conditions: detection of the branched side chains corresponding to antigenic factor 4. *FEMS Microbiol Lett* **152:** 235–242.

Kobayashi, H., Oyamada, H., Matsuda, K., Shibata, N., and Suzuki, S. (2003) Distribution of antigenic oligomannosyl side chains in the cell wall mannans of several strains of *Candida tropicalis*. *Arch Microbiol* **180:** 76–80.

Kozel, T.R., MacGill, R.S., Percival, A., and Zhou, Q. (2004) Biological activities of naturally occurring antibodies reactive with *Candida albicans* mannan. *Infect Immun* **72:** 209–218.

Levery, S.B., Toledo, M.S., Doong, R.L., Straus, A.H., and Takahashi, H.K. (2000) Comparative analysis of ceramide structural modification found in fungal cerebrosides by electrospray tandem mass spectrometry with low energy collision-induced dissociation of Li+ adduct ions. *Rapid Commun Mass Spectrom* **14:** 551–563.

Li, R.K., and Cutler, J.E. (1993) Chemical definition of an epitope/adhesin molecule on *Candida albicans*. *J Biol Chem* **268:** 18293–18299.

McMullan-Vogel, C.G., Jude, H.D., Ollert, M.W., and Vogel, C.W. (1999) Serotype distribution and secretory acid proteinase activity of *Candida albicans* isolated from the oral mucosa of patients with denture stomatitis. *Oral Microbiol Immunol* **14:** 183–189.

Mandala, S.M., and Harris, G.H. (2000) Isolation and characterisation of novel inhibitors of sphingolipid synthesis: australifungin, viridiofungins, rustmicin, and khafrefungin. *Methods Enzymol* **311:** 335–348.

Mille, C., Janbon, G., Delplace, F., Ibata-Ombetta, S., Gaillardin, C., Strecker, G., *et al.* (2004) Inactivation of CaMIT1 inhibits *Candida albicans* phospholipomannan beta-mannosylation, reduces virulence, and alters cell wall protein beta-mannosylation. *J Biol Chem* **279:** 47952–47960.

Okawa, Y., Takahata, T., Kawamata, M., Miyauchi, M., Shibata, N., Suzuki, A., *et al.* (1994) Temperature-dependent change of serological specificity of *Candida albicans* NIH A-207 cells cultured in yeast extract-added Sabouraud liquid medium: disappearance of surface antigenic factors 4, 5, and 6 at high temperature. *FEBS Lett* **345:** 167–171.

Okawa, Y., Goto, K., Nemoto, S., Akashi, M., Sugawara, C., Hanzawa, M., *et al.* (1996) Antigenicity of cell wall mannans of *Candida albicans* NIH B-792 (serotype B) strain cells cultured at high temperature in yeast extract-containing sabouraud liquid medium. *Clin Diagn Lab Immunol* **3:** 331–336.

Patton, J.L., Srinivasan, B., Dickson, R.C., and Lester, R.L. (1992) Phenotypes of sphingolipid-dependent strains of *Saccharomyces cerevisiae*. *J Bacteriol* **174:** 7180–7184.

Pons, A., Popa, J., Portoukalian, J., Bodennec, J., Ardail, D., Kol, O., *et al.* (2000) Single-step gas chromatography-mass spectrometry analysis of glycolipid constituents as heptafluorobutyrate derivatives with a special reference to the lipid portion. *Anal Biochem* **284:** 201–216.

Pons, A., Timmerman, P., Leroy, Y., and Zanetta, J.P. (2002) Gas-chromatography/mass-spectrometry analysis of human skin constituents as heptafluorobutyrate derivatives with special reference to long-chain bases. *J Lipid Res* **43:** 794–804.

Poulain, D., Tronchin, G., Vernes, A., Popeye, R., and Biguet, J. (1983) Antigenic variations of *Candida albicans in vivo* and *in vitro* – relationships between P antigens and serotypes. *Sabouraudia* **21:** 99–112.

Poulain, D., Slomianny, C., Jouault, T., Gomez, J.M., and Trinel, P.A. (2002) Contribution of phospholipomannan to the surface expression of beta-1,2-oligomannosides in *Candida albicans* and its presence in cell wall extracts. *Infect Immun* **70:** 4323–4328.

Quindos, G., Ruesga, M.T., Martin-Mazuelos, E., Salesa, R., Alonso-Vargas, R., Carrillo-Munoz, A.J., *et al.* (2004) *In-vitro* activity of 5-fluorocytosine against 1021 Spanish clinical isolates of *Candida* and other medically important yeasts. *Rev Iberoam Micol* **21:** 63–69.

Reggiori, F., Canivenc-Gansel, E., and Conzelmann, A. (1997) Lipid remodeling leads to the introduction and exchange of defined ceramides on GPI proteins in the ER and Golgi of *Saccharomyces cerevisiae*. *EMBO J* **16:** 3506–3518.

Shibata, N., Arai, M., Haga, E., Kikuchi, T., Najima, M., Satoh, T., *et al.* (1992) Structural identification of an epitope of antigenic factor 5 in mannans of *Candida albicans* NIH B-792 (serotype B) and J-1012 (serotype A) as beta-1,2-linked oligomannosyl residues. *Infect Immun* **60:** 4100–4110.

Shibata, N., Ikuta, K., Imai, T., Satoh, Y., Satoh, R., Suzuki, A., *et al.* (1995) Existence of branched side chains in the cell wall mannan of pathogenic yeast, *Candida albicans*. Structure–antigenicity relationship between the cell wall mannans of *Candida albicans* and *Candida parapsilosis*. *J Biol Chem* **270:** 1113–1122.

Shibata, N., Kobayashi, H., Okawa, Y., and Suzuki, S. (2003) Existence of novel beta-1,2 linkage-containing side chain in the mannan of *Candida lusitaniae*, antigenically related to *Candida albicans* serotype A. *Eur J Biochem* **270:** 2565–2575.

Trinel, P.A., Borg-von-Zepelin, M., Lepage, G., Jouault, T., Mackenzie, D., and Poulain, D. (1993) Isolation and preliminary characterisation of the 14- to 18-kilodalton *Candida albicans* antigen as a phospholipomannan containing beta-1,2-linked oligomannosides. *Infect Immun* **61:** 4398–4405.

Trinel, P.A., Plancke, Y., Gerold, P., Jouault, T., Delplace, F., Schwarz, R.T., *et al.* (1999) The *Candida albicans* phospholipomannan is a family of glycolipids presenting phosphoinositolmannosides with long linear chains of beta-1,2-linked mannose residues. *J Biol Chem* **274:** 30520–30526.

Trinel, P.A., Maes, E., Zanetta, J.P., Delplace, F., Coddeville, B., Jouault, T., *et al.* (2002a) *Candida albicans* phospholipomannan, a new member of the fungal mannose inositol phosphoceramide family. *J Biol Chem* **277:** 37260–37271.

Trinel, P.A., Jouault, T., Cutler, J.E., and Poulain, D. (2002b) Beta-1,2-mannosylation of *Candida albicans* mannoproteins and glycolipids differs with growth temperature and serotype. *Infect Immun* **70:** 5274–5278.

Uemura, S., Kihara, A., Inokuchi, J., and Igarashi, Y. (2003) Csg1p and newly identified Csh1p function in mannosylinositol phosphorylceramide synthesis by interacting with Csg2p. *J Biol Chem* **278:** 45049–45055.

Velegraki, A. (1995) *In vitro* susceptibility to itraconazole and fluconazole of switch phenotypes of *Candida albicans* serotypes A and B, isolated from immunocompromised hosts. *J Med Vet Mycol* **33:** 83–85.

Wells, G.B., Dickson, R.C., and Lester, R.L. (1996) Isolation and composition of inositolphosphorylceramide-type sphingolipids of hyphal forms of *Candida albicans*. *J Bacteriol* **178:** 6223–6226.

Zhong, W., Jeffries, M.W., and Georgopapadakou, N.H. (2000) Inhibition of inositol phosphorylceramide synthase by aureobasidin A in *Candida* and *Aspergillus* species. *Antimicrob Agents Chemother* **44:** 651–653.

Supplementary material

The following supplementary material is available for this article online:

Table S1. ^{1}H and ^{13}C chemical shifts of structural reporter groups of the phospholipomannan isolated from *C. albicans* strain NIH B 792, serotype B grown at 28°C.

This material is available as part of the online article from http://www.blackwell-synergy.com