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Molecular Phenotyping of Mannosyltransferases-Deficient *Candida albicans* Cells by High-Resolution Magic Angle Spinning NMR

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The yeast *Candida albicans* is an opportunistic pathogen that causes infections in immunocompromised individuals with a high morbidity and mortality levels. Recognition of yeasts by host cells is directly mediated by cell wall components of the yeast, including a wide range of abundantly expressed glycoconjugates. Of particular interest in *C. albicans* are the β-mannosylated epitopes that show a complex expression pattern on *N*-glycan moiety of phosphopeptidomannans and are absent in the non-pathogenic species *Saccharomyces cerevisiae*. Being known as potent antigens for the adaptive immune response and elicitors of specific infection-protective antibodies, the exact delineation of β-mannosides regulation and expression pathways has lately become a major milestone toward the comprehension of host-pathogen interplay. Using the newly developed HR-MAS NMR methodology, we demonstrate the possibility of assessing the general profiles of cell-surface-exposed glycoconjugates from intact living yeast cells without any prior purification step. This technique permitted to directly observe structural modifications of surface expressed phosphodiester-linked β-mannosides on a series of deletion strains in β-mannosyltransferases and phospho-mannosyltransferases compared with their parental strains

Key words: *Candida albicans*, high-resolution magic angle NMR, β-mannosylation, mannosyltransferase.

Abbreviations: HR-MAS NMR, high-resolution magic angle spinning nuclear magnetic resonance; Man, mannose; Man*p*, mannopyranoside, Glc*p*NAc, *N*-acetylglucopyranoside, PPM, phosphopeptidomannan; HSQC, heteronuclear single quantum coherence; BMT, β-mannosyltransferase.

The cell surfaces of yeasts are covered with a dense glyco-shield whose biogenesis is tightly controlled by environmental stimuli. The outermost layer of the yeast cell wall consists of mannoproteins with O- and N-linked polysaccharide moieties including so called phosphopeptidomannans (PPM). The unravelling of basic N- and O-mannosylation processes by using different temperature-sensitive mutants of the eukaryotic yeast model *Saccharomyces cerevisiae* took several decades but permitted to achieve a workable structural model partially applicable to other yeast model species (*1*). While O-mannosylation seems restricted to relatively homogeneous short stretches of mannose residues (DP < 8), N-glycosylation consists of $Man_8GlcNAc_2$ core structures elongated by a very large polymannosylated domain commonly referred as mannan. Detailed structural analysis on a wide range of yeast species established that mannans are extremely heterogeneous molecules containing a linear α-1,6 mannan substituted by a wide range of α-1,2-, α-1,3-linked mannose residues containing oligomannans. In many species, including *S. cerevisiae*, the mannan is further substituted by phosphodiester linked α-Man containing oligomannosides commonly referred as acid-labile domain.

The mannan structure of *Candida albicans* is characterized by the additional presence of unusual β-1,2-linked oligomannans, compared with non-pathogenic species *S. cerevisiae* (*2*). In serotype A strains, they substitute both mannan core (acid-resistant domain) and acid-labile domain whereas in serotype B, they exclusively substitute the acid-labile domain (Fig. 1). These epitopes have been first identified by Suzuki and co-workers in 1980s and then extensively characterized both for their distribution on different molecules and their contribution to *C. albicans* virulence (*3*). More recently, we have established its presence on a glycolipidic carrier which structure is so far strictly specific to *C. albicans* (*4*). Being known as potent antigens for the adaptive immune response and elicitors of specific infection-protective antibodies, the exact delineation of β-mannosides regulation and expression pathways has lately become a major milestone toward the comprehension of host-pathogen interplay (*5*, *6*). However, decisive progresses are still impaired by the length, and to a lesser extend by the lack of standardization, of cell

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wall molecules preparation and analysis procedures. Moreover application of harsh and selective extraction methods may give a poor physicochemical view of the cell wall molecules actually accessible to the host tissues and immune system. In the present report, we have adapted the newly developed HR-MAS technology to the analysis of yeast cell surface as a mean for connecting genetic and immunologic background knowledge to structure and ultra-structure of *C. albicans* cell wall. We used as model of studies a wide variety of wild-type strains and mutant strains deficient in the synthesis of α-mannosyltransferases and recently discovered putative β-mannosyltransferases (BMT) (*7*) (Supplementary Table 1). All the NMR parameters used during the course of this study are summarized in Supplementary data.

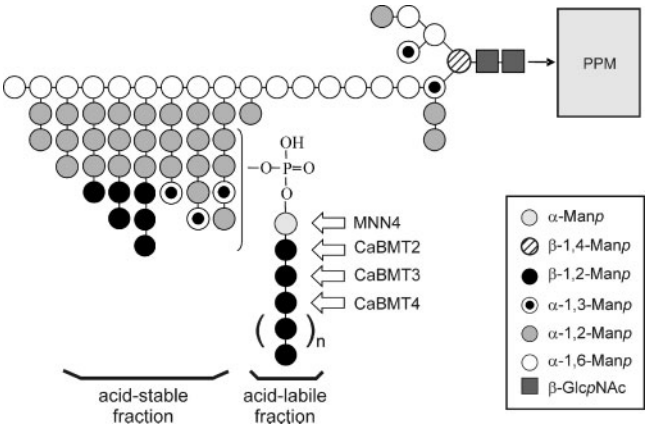

Fig. 1. **Structural model of serotype A *C. albicans* PPM *N*-glycan moiety.** *In vitro* analysis of PPM purified from *C. albicans* deletion mutants strongly suggested that the acid-labile β-mannosides is initiated by the sequential activities of three putative BMTs names CaBmt2-4p onto the Manα1-phosphate of the phosphodiester-linked side chain of PPM (*7*).

Determination of Soluble PPM Parameters by Liquid NMR—We have based our studies on the work of Suzuki and Okawa who have extensively studied the structure of *C. albicans* mannan by conventional multidimensional NMR experiments (*8–11*). By analysing hydrolysis and acetolysis mannan fragments, they showed that shear heterogeneity of polysaccharide generates a wide variability in the ^{13}C and ^{1}H NMR parameters of individual mannose residues, depending on their exact location within the polysaccharide chain. As a prelude for assessing the general glycosylation pattern of cell-associated mannans, we established the NMR parameters of individual mannose residues of PPMs purified from control serotype A *C. albicans* strain. To do that, we have recorded a series of homonuclear ^{1}H and heteronuclear $^{1}H/^{13}C$ liquid NMR experiments from PPMs, and compared our data to NMR parameters previously obtained from fragments of *C. albicans* mannan (*8*, *11*). We used both intact PPM containing phosphodiesterified β-oligomannosides (phosphomannosides) and mildly hydrolysed PPM devoid of phosphomannosides. The anomeric region of $^{1}H/^{13}C$ HSQC spectrum of phosphomannoside free PPM showed 12 different main signals (Fig. 2A). Total absence of phosphomannoside is assessed by the absence of H-1 Manα1-phosphate associated

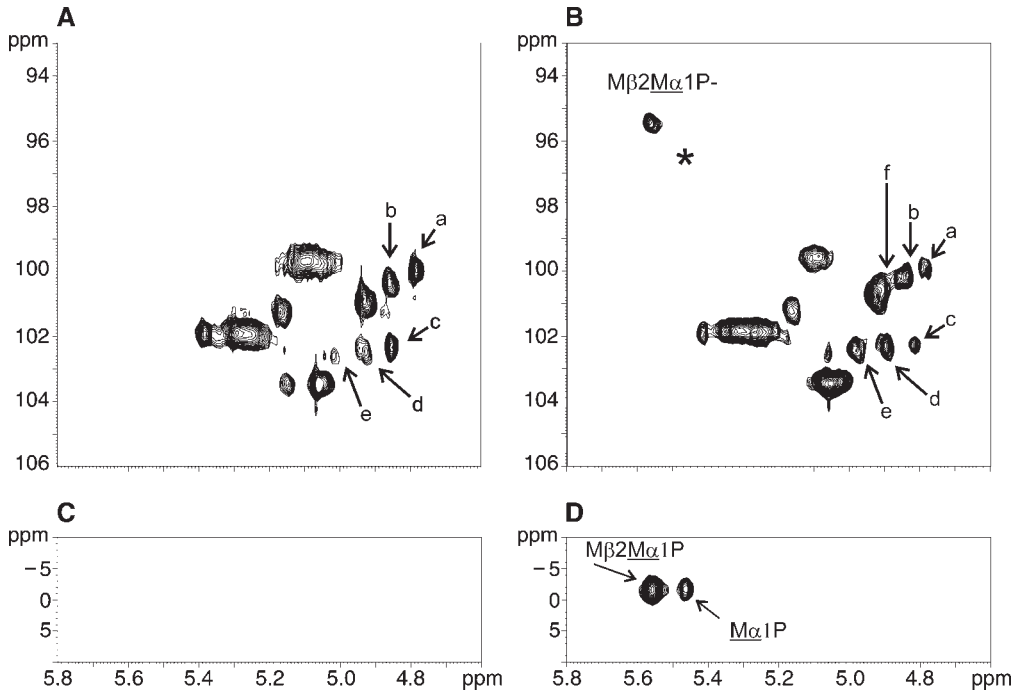

Fig. 2. **Liquid NMR analysis of purified PPM.** $^{1}H–^{13}C$ HSQC NMR spectra of (A), acid-stable domain of PPM and (B) native PPM isolated from serotype A *C. albicans* (BWP17) enable the identification of the β-mannoside moiety owing to six NMR signals **a**–**f** (Table 1) but do not permit to easily discriminate between acid-stable and acid-labile domains. $^{1}H–^{31}P$ HSQC NMR analyses confirm the presence of Manα1-phosphate and Manβ1-2Manα1-phosphate residues in native PPM (D), but their absence in acid-stable domain of PPM (C). Asterisk indicates H-1 Manβ1-2Manα1-phosphate signal which intensity is too low to be clearly distinguished on this spectrum.

signals on $^{1}H–^{31}P$ and $^{1}H–^{13}C$ HSQC heteronuclear spectra (Fig. 2A and C). Their associated spin systems were identified by $^{1}H/^{1}H$ TOCSY experiments at various mixing times (20–100 ms) which established that all anomers were associated to mannosyl residues, in accordance with literature (*12*). Then, their anomeric configurations were established by non-decoupled $^{1}H–^{13}C$ HSQC heteronuclear analyses. Seven out of twelve signals were assigned to α-anomers owing to direct coupling constant ($^{1}J_{H1,C1}$) of about 170 Hz, whereas five were assigned to β-anomers owing to $^{1}J_{H1,C1}$ of about 160 Hz. The β-anomer signals at 4.77/99.9, 4.85/100.3, 4.86/102.3, 4.94/102.4 and 5.04/102.5 ppm were labelled **a** to **e**, respectively (Fig. 2A). Based on spin systems and published data (*7*, *10*, *13*), individual signals were assigned to specific positions within the acid-resistant moiety (Table 1, panel A). These experiments established that all five **a-e** signals are associated to the acid-resistant moiety of serotype A PPM. Then, we have shown that $^{1}H/^{13}C$ HSQC spectrum of intact PPM was very similar to spectrum of hydrolysed PPM (Fig. 2B). It differed by the additional presence of two signals at 5.45/97.4 and 5.56/95.4, both associated to Manα1-phosphate residues (Fig. 2B), as confirmed by $^{1}H–^{31}P$ HSQC experiments (Fig. 2D), which confirmed the presence of a phosphomannoside moiety. It differed also by the presence of a signal at 4.90/100.1 ppm (signal **f**) that was typified as a β-mannose anomer according to its $^{1}J_{H1,C1}$ value. Overall, absence of a complete set of new β-man signals strongly suggests that acid-labile β-mannoside-associated signals partly overlap with those of acid-stable β-mannosides. Indeed, Shibata and collaborators recently showed that H-1 chemical parameters of β-man residues of acid-labile and acid-stable moieties are identical, except for the first β-Man residues that are shifted by a constant value of Δδ +0.08 in acid-labile compare with acid-stable oligomannanosides, due to the influence of phosphodiester link (*11*, *14*). In consequence, the H-1 values of Manβ1-2Manα1-phosphate and $[Manβ1\text{-}2]_{n}$Manβ1-2Manα1-phosphate (residues B) are deshielded to 4.83–4.86 ppm (signal **b**) and 4.89–4.90 ppm (signal **f**), respectively. This assumption was later confirmed by HR-MAS NMR analysis of BMT-deficient strains, which also showed a slight modification of the C-1 chemical shift (Δδ +0.3) of Manβ1-2Manα1-phosphate residue (Table 1, panel B). Thus, H-1/C-1 Manβ1-2Manα1-phosphate and Manβ1-2Manβ1-2Manα1-2Manα exhibit identical parameters

Table 1. **Estimation of $^{1}H–^{13}C$ chemical shifts (δ) of β-mannosides associated residues substituting (panel A) acid-stable and (panel B) acid-labile domains of *C. albicans* PPM, according to 7, 8, 10, 12, 14 and personal data.**

Panel **A**

Sugar residues		Range of ^{1}H and ^{13}C chemical shifts							
E D C B A		E		D		C		B	
Mβ1-2 Mα1-2/3 Mα1-	^{1}H							**a**	4.76–4.79
	^{13}C								99.6–100.0
Mβ1-2 Mβ1-2 Mα1-2/3 Mα1-	^{1}H					**c**	4.80–4.86	**b**	4.83–4.86
	^{13}C						101.8–102.2		99.9–100.6
Mβ1-2 Mβ1-2 Mβ1-2 Mα1-2/3 Mα1-	^{1}H		**d**	4.89–4.94	**d**	4.89–4.94	**b**	4.83–4.86
	^{13}C			102.2–102.4		102.2–102.4		100.1–100.6
Mβ1-2 Mβ1-2 Mβ1-2 Mβ1-2 Mα1-2/3 Mα1-	^{1}H	**d**	4.89–4.94	**e**	4.96–5.04	**d**	4.89–4.94	**b**	4.83–4.86
	^{13}C		102.2–102.4		102.4–102.6		102.2–102.4		100.2–100.6

Panel **B**

Sugar residues		Range of ^{1}H and ^{13}C chemical shifts					
E D C B A		D		C		B	A
Mα1-phosphate	^{1}H						5.45
	^{13}C						97.4
Mβ1-2 Mα1-phosphate	^{1}H				**b**	4.83–4.86	5.56
	^{13}C					99.9–100.6	95.4
Mβ1-2 Mβ1-2 Mα1-phosphate	^{1}H		**c**	4.80–4.86	**f**	4.89–4.90	5.56
	^{13}C			101.8–102.2		100.1	95.4
Mβ1-2 Mβ1-2 Mβ1-2 Mα1-phosphate	^{1}H	**d**	4.89–4.94	**d**	4.89–4.94	**f**	4.89–4.90	5.56
	^{13}C		102.2–102.4		102.2–102.4		100.1	95.4

a to **f** correspond to the signals actually observed on liquid $^{1}H–^{13}C$ HSQC NMR and $^{1}H–^{13}C$ HSQC HR-MAS NMR spectra (Figs 2 and 3).

in liquid NMR analysis of total PPM (Table 1, panel B). In summary, although liquid $^{1}H–^{13}C$ HSQC NMR analysis of intact PPM permits to easily observe β-mannosylated epitopes owing to their very distinct H-1/C-1 parameters compared with those of α-mannosylated epitopes, it does not permit to efficiently distinguish the β-Man residues signals associated to acid-stable from those associated to acid-labile fraction due to a series of signal overlaps.

Comparison of Liquid and HR-MAS NMR Analyses of Soluble PPM—As a step forward to study the expression of β-mannosylated epitopes at the surface of intact yeast cells by NMR, we assessed the usefulness of high-resolution magic angle spinning (HR-MAS) NMR by analysing a range of wild type and *C. albicans* deletion strains (Supplementary Table 1). This recently developed technology, which enables the acquisition of NMR spectra directly from living intact cells (*15*), was previously shown to be effective to observe and analyse the structure of complex molecules present either at the cell surface or inside the cells as long as they are mobile enough and in sufficient quantity (*16–18*). In particular, it was effectively used to analyse the structure of cell-wall components, including mycobacterial polysaccharides (*19*) and complex lipids (*20*), lipooligosaccharides from *Campylobacter* cells (*21*), as well as intracellular metabolites (*22*) or periplasmic glucans (*23*). First, we analysed a solution of intact PPM purified from serotype A *C. albicans* (BWP17 strain) by HR-MAS NMR in order to standardize experimental procedure. $^{1}H–^{13}C$ HSQC experiments showed that HR-MAS NMR exhibited a comparable resolution to liquid NMR which permitted to readily discriminate all β-Man associated signals (Supplementary Fig. 1). Furthermore, all α- and β-Man residues on purified PPM analysed by HR-MAS NMR exhibited very similar parameters than those observed by liquid NMR, which validated the use of this technology to analyse PPM structure based on previously established chemical shifts.

Phenotyping of C. albicans Intact Cells by HR-MAS NMR—In a last step toward glycan phenotyping of whole cells, we acquired HR-MAS NMR spectra on intact cells from different *C. albicans* strains. As shown on Fig. 3A, anomeric region of $^{1}H–^{13}C$ HSQC HR-MAS NMR spectrum of living serotype A *C. albicans* (BWP17 strain) cells shows most of the anomeric signals of the mannose residues associated to PPM, including both α- and β-Man type residues. All major α-Man residues signals that have been identified on purified PPM are clearly observed on $^{1}H–^{13}C$ HSQC HR-MAS NMR spectrum with the noteworthy exception of Manβ1-2Manα1-2Manα at δ 5.16/101.3 ($^{1}H/^{13}C$) that completely disappeared. Furthermore, anomeric signal for branched Manα1-6(Manα1-2) Manα1-6 residue at δ 5.11/99.6 ($^{1}H/^{13}C$) was barely visible on intact cell compared with purified PPM. Then, very intense signals at δ 5.45/97.4 and 5.56/95.4 ($^{1}H/^{13}C$) assigned to Manα1-phosphate and [Manβ1-2]$_n$Manα1-phosphate of the PPM acid-labile fraction were also clearly observed. The α anomeric configuration of these residues was further confirmed by observing $^{1}J_{H–C}$ coupling constants at 171 Hz by non-decoupled $^{1}H–^{13}C$ HSQC, whereas their linkages to phosphorus atom were confirmed by $^{1}H–^{31}P$ HSQC experiments (data not shown). Unexpectedly, a third signal in the region of phosphorylated α-Man residues was identified at δ 5.56/96.0 ($^{1}J_{H–C}$ 164 Hz) exclusively on intact cells by HR-MAS NMR, whereas it never appeared on purified PPM analysed either by liquid NMR or by HR-MAS NMR. This strongly suggests that this yet unidentified signal is not associated to the purified PPM but to another cell-wall-related molecule. Of particular interest for the present study are signals previously associated to β-Man residues. They were easily identified, with the notable exception of **a**, at identical chemical shifts than those observed for purified PPM analysed by liquid and HR-MAS NMR. Signal **a** (Manβ1-2Manα1-2Manα-) being the only acid-stable fraction specific β-Man signal, its reproducible absence on whole-cell analyses suggests that β-Man residues associated to acid-stable moiety are somehow not detected by HR-MAS NMR analysis of intact *C. albicans* and thus only phosphodiesterified β-oligomannosides are observed. The transparency of cell-wall-associated acid-stable β-mannosides to HR-MAS NMR analyses was demonstrated by comparing data obtained from two strains deleted in the MNN4 gene which codes for the mannosylphosphate transferase involved in the initiation of phosphomannoside synthesis. Thus, serotype A *Ca*A-*mnn4Δ* strain is characterized by the presence of an acid-stable β-mannoside moiety but no acid-labile β-mannoside moiety, whereas serotype B *Ca*B-*mnn4Δ* strain is devoid of both β-mannoside moieties. Both strains showed identical HR-MAS HSQC NMR spectra that are characterized by the total absence of β-Man signals **a–e** associated to acid-stable moiety (Fig. 3B and C), which establishes that all β-Man signals observed on BWP17 strain are associated to acid-labile mannoside moiety. The transparency of core β-mannoside to HR-MAS NMR analysis is most probably the result of the specific feature of HR-MAS NMR that suppresses signal from constrain molecules exhibiting low transversal relaxation time (T2). Altogether, data established that HR-MAS NMR enables the *in situ* observation of cell-wall PPM directly from intact living yeasts without prior purification. Comparison of HR-MAS spectra from purified PPMs and from total cells also gives a clear indication that core β-mannosides exhibit a rigid conformation at the cell surface whereas phosphodiesterified β-oligomannosides are very mobile. One may speculate that the ordered conformation that β-mannosides adopt in solution favours the stacking of the core β-mannosides at the cell surface because of their high local density (*24*). On the contrary, phosphodiester linkage provides enough mobility to the acid-labile mannoside to be easily observed by HR-MAS NMR. Incidentally, the difference in respective mobility of both fractions gives the opportunity to specifically target the phosphodiesterified β-oligomannosides at the cell surface without interference from acid-stable β-mannosides.

Therefore, HR-MAS NMR analyses of intact yeasts were then used to determine the *in vivo* phenotypes of mutant strains *Ca*A-*bmt2Δ* *Ca*A-*bmt3Δ* and *Ca*A-*bmt4Δ* by assessing the structure of the phosphodiesterified β-oligomannosides expressed at their cell surfaces. Detailed structural analysis of the PPMs purified from these mutants by a combination of mass spectrometry,

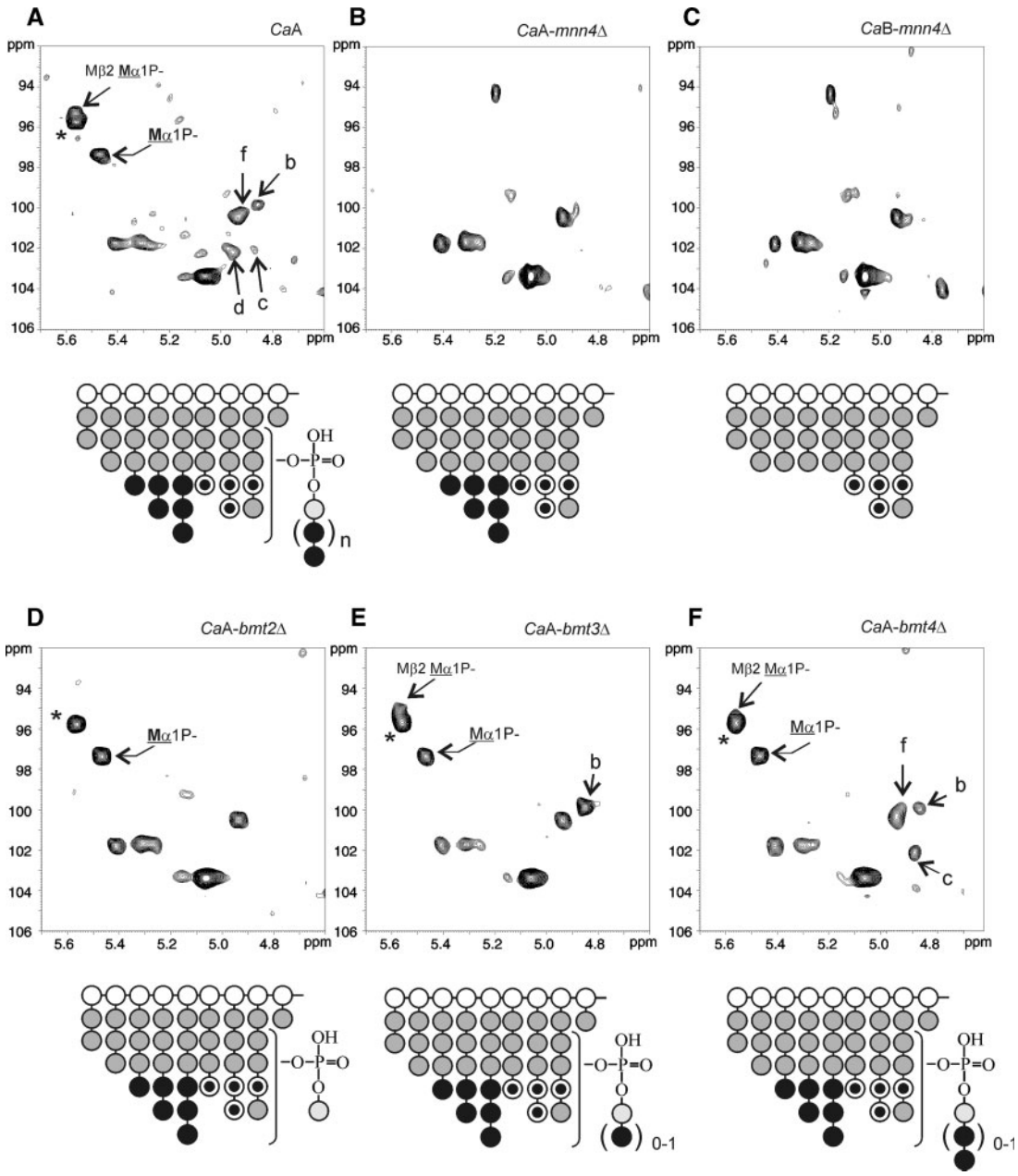

Fig. 3. **HR-MAS NMR analysis of six intact living *C. albicans* cells.** $^{1}H–^{13}C$ HSQC HR-MAS NMR experiments of intact cells permitted to establish the *in vivo* structure of phosphodiester-linked side chains of the cell wall associated PPMs of (A), serotype A (*Ca*A, BWP17); (B) serotype A devoid of acid-labile domain (*Ca*A-*mnn4Δ*); (C) serotype B devoid of acid-labile domain (*Ca*B-*mnn4Δ*); (D) *Ca*A-*bmt2Δ*; (*ΔCa*A-*bmt3Δ* and (F) *Ca*A-*bmt4Δ* strains. Under each spectrum is depicted the general sequence of mannan moiety of corresponding PPM. Asterisk indicates unidentified signal exclusively observed on $^{1}H–^{13}C$ HSQC HR-MAS NMR spectra of total cells.

fluorophore-assisted carbohydrate electrophoresis (FACE) and liquid NMR previously strongly suggested that the CaBmt2, CaBmt3 and CaBmt4 proteins were involved in the sequential transfer of the first, the second and the third β-Man residues, respectively, onto the acid-labile mannoside of *C. albicans* PPM (Fig. 1) (*7*). However, the possibility that methodologies based on multiple purification and chemical degradation steps generate artefacts or overlook eventual alternative substrates can never be completely ruled out, which justifies the use of a non-destructive type of analysis on unprocessed samples. Anomeric region of the $^{1}H–^{13}C$ HSQC HR-MAS NMR spectrum of intact *Ca*A-*bmt2Δ* cells shows most α-Man signals previously identified on parental BWP17 strain as well as on *Ca*B-*mnn4Δ* and *Ca*A-*mnn4Δ* strains, but none of the β-Man signals (Fig. 3D). However, in contrast to *Ca*B-*mnn4Δ* and *Ca*A-*mnn4Δ* strains, spectra of *Ca*A-*bmt2Δ* exhibits

Manα1-phosphate H-1 signal at δ 5.45/97.4, which confirms the presence of an acid-labile domain on its PPM. Still, Manβ1-2Manα1-phosphate H-1 signal at δ 5.54/95.4 is absent, which establishes that Manα1-phosphate residue is not further elongated by β-Man residues. Altogether, these data unambiguously demonstrates that *Ca*A-*bmt2Δ* cells express at their surface PPMs substituted by phosphodiester side chains that are constituted by a single α-Man residue. Compared to *Ca*A-*bmt2Δ*, ^{1}H–^{13}C HSQC NMR spectrum of *Ca*A-*bmt3Δ* showed a single additional β-Man residue H-1 signal (signal **b**: Manβ1-2Manα1-phosphate) as well as a single additional α-Man residue H-1 signal at δ 5.56/95.4 (Manβ1-2Manα1-phosphate) (Fig. 3E). The assignment of this last signal as an internal 2-substituted Man was confirmed by the deshielding of its C-2 value to 78.8 ppm (data not shown). These data establish that *Ca*A-*bmt3Δ* PPMs are substituted by a mixture of Manα1-phosphate and Manβ1-2Manα1-phosphate. Then, spectrum of *Ca*A-*bmt4Δ* was characterised by the simultaneous presence of β-Man associated signals **b**, **c** and **f** (Fig. 3F). In association with the observation of both terminal and internal Manα1-phosphate H-1 signals and in accordance with previously established NMR parameters (Table 1), these signals established the presence of Manα1-phosphate, Manβ1-2Manα1-phosphate and Manβ1-2Manβ1-2Manα1-phosphate side chains at the surface of intact *Ca*A-*bmt4Δ* cells. In summary we have shown that the selective absence of some putative β-1,2 mannosyltransferases can be directly assessed by NMR analyses of intact cells. Indeed, *C. albicans* mutant strain deficient in *BMT2* synthesis was demonstrated to be totally devoid of β-mannose substituted phosphodiester side chains. It is noteworthy that we did not observe any compensatory effect by α-mannosyltransferase that would elongate the Manα1-phosphate motif. This demonstrates that Manα1-phosphate is not a potential substrate for a competing α-mannosyltransferase as observed in *S. cerevisiae*. Similarly, absence of *BMT3* and *BMT4* was directly assessed by the observation of truncated β-mannosylated phosphodiester side chains at the surface of intact cells.

Altogether, data established the validity of HR-MAS NMR technology for analysing glycosylation phenotypes of intact yeast cells. We have shown a perfect correlation between *in vitro* and *in vivo* analyses of β-mannoside structures by using a number of *C. albicans* serotypes strains as well as strains deficient in some mannosyltransferases. *In vivo* analysis has the enormous advantage over classical approach of combining low quantities requirements (50 μL of packed cells) and no need for any purification procedure, making it useful for quick, although structurally informative, phenotyping of mutant strains. A possible drawback of this approach is the selective signal suppression phenomenon associated to low mobility. However, in the case of *C. albicans*, we took advantage of it to specifically target mobile molecules by getting rid of overlapping, non-informative signals. We do believe that HR-MAS technology has an enormous potential for analysing the regulation of expression a wide range of yeast parietal molecules and could be extremely useful to the yeast community.

SUPPLEMENTARY DATA

Supplementary data are available at *JB* online.

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CONFLICT OF INTEREST

None declared.

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