Research Article

Sterol glycosides and cerebrosides accumulate in *Pichia pastoris, Rhynchosporium secalis* and other fungi under normal conditions or under heat shock and ethanol stress

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Abstract

The occurrence of glycolipids such as sterol glycosides, acylated sterol glycosides, cerebrosides and glycosyldiacylglycerols was examined in the three yeast species *Candida albicans, Pichia pastoris* and *Pichia anomala*, as well as in the six fungal species *Sordaria macrospora, Pyrenophora teres, Ustilago maydis, Acremonium chrysogenum, Penicillium olsonii* and *Rhynchosporium secalis*. Cerebroside was found in all organisms tested, whereas acylated sterol glycosides and glycosyldiacylglycerols were not found in any organism. Sterol glycosides were detected in *P. pastoris* strain GS115, *U. maydis*, *S. macrospora* and *R. secalis*. This glycolipid occurred in both yeast and filamentous forms of *U. maydis* but in neither form of *C. albicans*. This suggests that sterol glycoside is not correlated with the separately grown dimorphic forms of these organisms. Cerebrosides and sterol glycosides from *P. pastoris* and *R. secalis* were purified and characterized by mass spectrometry and nuclear magnetic resonance spectroscopy. The cerebrosides are β-glucosyl ceramides consisting of a saturated α-hydroxy or non-hydroxy fatty acid and a $\Delta4,8$-diunsaturated, C9-methyl-branched sphingobase. Sterol glycoside from *P. pastoris* was identified as ergosterol-β-D-glucopyranoside, whereas the sterol glucosides from *R. secalis* contain two derivatives of ergosterol. The biosynthesis of sterol glucoside in *P. pastoris* CBS7435 and GS115 depended on the culture conditions. The amount of sterol glucoside in cells grown in complete medium was much lower than in cells from minimal medium and a strong increase in the content of sterol glucoside was observed when cells were subjected to stress conditions such as heat shock or increased ethanol concentrations. From these data we suggest that, in addition to *Saccharomyces cerevisiae*, new yeast and fungal model organisms should be used to study the physiological functions of glycolipids in eukaryotic cells. This suggestion is based on the ubiquitous and frequent occurrence of cerebrosides and sterol glycosides, both of which are rarely detected in *S. cerevisiae*. We suggest *P. pastoris* and two plant pathogenic fungi to be selected for this approach. Copyright © 2001 John Wiley & Sons, Ltd.

Introduction

The yeast *Saccharomyces cerevisiae* serves as a model organism for studies of various aspects of the biochemistry and cell biology of eukaryotic cells (Botstein *et al.*, 1997). The knowledge of its entire genome, in combination with new powerful approaches such as mRNA profiling and monitoring of

protein expression in different physiological situations and in the massively produced mutants, will provide data of unprecedented comprehensiveness. They are expected to help in understanding the complexity and interdependence of biochemical networks at a cellular level. These expectations give also fresh impetus to the field of lipid metabolism, with the chance to recognize as yet unknown links of this metabolic area to other aspects of cellular activity (Daum *et al.*, 1999; Parks and Casey, 1995; Henry and Patton-Vogt, 1998; Dimster-Denk *et al.*, 1999).

In particular, *S. cerevisiae* appears to be an ideal organism for studies in the rapidly growing field of sphingolipid metabolism (Dickson, 1998). Baker's yeast synthesizes ceramide, sphinganine phosphate, phytosphingosine phosphate and different species of inositol-containing sphingolipids (recently reviewed by Daum *et al.*, 1998; Dickson and Lester, 1999a; Dickson and Lester, 1999b). In addition, there are rare reports on cerebrosides isolated from *S. cerevisiae* (Wagner and Zofscik, 1966). On the other hand, subsequent investigations have never found cerebrosides in baker's yeast, which demonstrates that these glycolipids are only minor compounds in this organism under usual growth conditions. This situation has prevented further investigations on the functions of cerebrosides, and therefore *S. cerevisiae* cannot be used as a model organism when studies are extended to these important components of the plasma membrane and endomembrane system of plant and animal cells.

In addition, plant cells produce sterol glycosides (SGs) as constitutive components of these membrane systems. In *S. cerevisiae* this glycolipid has only rarely been detected despite the fact that this organism contains a functional and recently cloned gene encoding a sterol glucosyltransferase (Warnecke *et al.*, 1999). Its expression in suitable hosts resulted in the formation of significant quantities of sterol glucoside. *S. cerevisiae* mutants with a disrupted version of this gene did not show any phenotype under normal growth conditions (Warnecke *et al.*, 1999). In addition, a survey of the above-mentioned expression data from baker's yeast did not reveal any peculiarity in the expression of this gene (*UGT51 = YLR189C*) under various conditions which might give a clue to understand the function of this membrane glycolipid. Because of the very low proportion of cerebrosides and SG, *S. cerevisiae* is not suitable for

studying the functions of these typically eukaryotic glycolipids.

In contrast to *S. cerevisiae*, other yeasts and fungi do not only contain different inositol phosphoryl ceramides, but additionally synthesize significant amounts of cerebrosides (Dickson and Lester, 1999a). However, there are only a few reports on SGs from these organisms (Kastelic-Suhadolc, 1980; McMorris and White, 1977; Ghannoum *et al.*, 1986, 1990; Deven and Manocha, 1976) and structural data are completely lacking except for an ergosterol glucoside, which was isolated from *S. cerevisiae* heterologously expressing a sterol glucosyltransferase gene from *Pichia pastoris* (Warnecke *et al.*, 1999). Consequently, there is a need for a survey of the occurrence and structure of these glycolipids in various species, which will enable systematic studies on their biological functions.

Therefore, we screened a series of lower eukaryotic organisms such as yeasts and fungi for the presence of both cerebrosides and SG. Criteria for selection were the possibility to genetically engineer these organisms and, if possible, their ability to grow in purely synthetic media. By this means we wanted to exclude a possible incorporation of exogenous glycolipids that may be present in complex culture media. We expect that the genetic manipulation of the content of both glycolipids, in organisms which contain significant quantities of them, will result in phenotypes that offer an insight into the functions of these, at least in the plant kingdom, ubiquitous membrane components. An example of a morphological phenotype occurring in parallel with different proportions of these glycolipids is the switch from yeast to mycelial growth, as observed in three different organisms (Buffo *et al.*, 1984; Ghannoum *et al.*, 1986; Toledo *et al.*, 1999, 2000). Therefore, the genetic manipulation of glycolipid biosynthesis was expected to show interesting effects on this dimorphic switch. The inclusion of phytopathogenic fungi in this survey offered the additional chance to generalize the involvement of free sterols, cerebrosides and SGs in pathogenesis. There is increasing evidence that free ergosterol and cerebrosides act as elicitors of plant defence mechanisms (Granado *et al.*, 1995; Koga *et al.*, 1998; Umemura *et al.*, 2000). Additionally, Sweigard *et al.* (1998) described pathogenicity genes from *Magnaporthe grisea*, among which one (*PTH8*) showed sequence similarities to

sterol-glucosyltransferase genes, which we identified recently (Warnecke *et al.*, 1999).

Materials and methods

Organisms and growth conditions

Candida albicans (strains 3153 and SC5314, provided by Dr B. Hube, University of Hamburg), precultured in YPD medium at 37°C overnight, was inoculated into Lee's medium (Lee *et al.*, 1975) with pH adjusted to 4.5 and cultured at 25°C for 2 days for yeast-type growth. The yeast cells were collected by centrifugation, then inoculated into Lee's medium at pH 6.5 at a concentration of 2×10^7 cells/ml and cultured at 37°C for 16 h to induce mycelial-type growth. Six *Pichia pastoris* strains (GS115 and GS115 β-Gal from Invitrogen, Carlsbad, USA; CBS2612 and CBS7435 from Centraalbureau voor Schimmelcultures, Utrecht, NL; DSM70382 and DSM70872 from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) and *Pichia anomala* (DSM70255 from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) were cultured in minimal dextrose medium (20 g/l glucose, 6.7 g/l Yeast Nitrogen Base without amino acids, supplemented with 40 mg/l L-histidine if necessary) at 30°C for 24 h. *Sordaria macrospora* [wt k(hell) 3346] and *Acremonium chrysogenum* (ATCC 14553), both provided by Professor Dr U. Kück, Universitiy of Bochum, were cultured in CCM medium at 27°C, as described earlier (Walz and Kück, 1993, 1995). *Penicillium olsonii* (P50, provided by Dr F. Maier and Professor Dr W. Schäfer, University of Hamburg) was cultured at 28°C in minimal medium according to Pontecorvo *et al.* (1953) with 1.5% sucrose. *Pyrenophora teres*, isolate Stefan (net blotch pathogen of barley, provided by Dr F. Maier and Professor Dr W. Schäfer, University of Hamburg) was cultured at 24°C in CM complete medium, as described previously (Leach *et al.*, 1982). *Ustilago maydis* (corn smut pathogen of maize) yeast form (FB1) and short filamentous form (HA103, obtained from Dr C. Basse and Professor Dr R. Kahmann, University of Munich) were cultured in minimal medium, as described previously (Holliday, 1974). *Rhynchosporium secalis* (leaf scald pathogen of barley) was cultured as described previously (Wevelsiep *et al.*, 1991).

Lipid extraction and separation

The cultured cells of *S. macrospora*, *A. chrysogenum* and *R. secalis* were collected, lyophilized and stored at $-20°C$ for up to 1 month. Prior to lipid extraction, cells from all organisms were boiled in distilled water for 10 min to inactivate any endogenous lipid-degrading enzymes (Christie, 1982, p. 17). Lipid extraction was performed with chloroform : methanol : water (1 : 2 : 0.8, v/v; according to Bligh and Dyer, 1959) three times.

For the determination of individual lipid classes, the total lipid extracts were directly separated by thin-layer chromatography (TLC) on silica gel 60 (Merck) with solvent systems as follows: sterol, sterol ester (SE), and triacylglycerol (TAG) in hexane : diethyl ether : acetic acid (60 : 20 : 2, v/v); acylated sterol glycoside (ASG) in chloroform : methanol (85 : 15, v/v); monoglycosyldiacylglycerol (MGD), SG, cerebroside and diglycosyldiacylglycerol (DGD) in acetone : toluene : water (91 : 30 : 8, v/v); phosphatidylinositol (PI) and phosphatidylserine (PS) in chloroform : methanol : acetic acid : water (85 : 15 : 10 : 4, v/v); phosphatidyl glycerol (PG) and an unknown phospholipid in chloroform : methanol : water (65 : 25 : 4, v/v) and chloroform : methanol : ammonia/isopropylamine (65 : 35 : 5 : 0.5, v/v); other phospholipids in chloroform : methanol : acetic acid (65 : 25 : 8, v/v). Lipids on TLC plates were visualized under UV light after spraying with 0.2% (w/v) anilinonaphthalene sulphonate. Additional spray reagents used for the detection of lipids on the plates were as follows: 50% (v/v) H_2SO_4 for general lipids, α-naphthol reagent for glycolipids, molybdenum blue reagent for phospholipids (Christie, 1982, p. 119f). The standard lipids cholesterol, cholesterol oleate, cerebroside, TAG and six phospholipids, phosphatidic acid (PA), phosphatidylcholine (PC), phosphatidylethanolamine (PE), PI, PS and cardiolipin (CL) were obtained from Sigma. SG, ASG, MGD and DGD had been purified from plant sources.

Total lipids were first fractionated by column chromatography on silica gel 60 (70–200 mesh, Merck) to purify appropriate amounts of lipids for NMR and MS analyses and also to detect minor lipid constituents. The lipids (50–100 mg dry weight) were loaded on the column (1.2 cm diameter × 3 cm high) and eluted successively with 20 ml 20% (v/v) acetone in chloroform, 40 ml 10% (v/v) methanol in acetone, and 20 ml pure methanol.

Sterol, SE, TAG and ASG in the first eluate were subsequently separated by TLC with hexane : diethyl ether : acetic acid (60 : 20 : 2, v/v). The separated lipids were recovered from silica gel scrapings by extraction with chloroform : methanol : water (5 : 5 : 1, v/v) and further purified by TLC with hexane : diethyl ether : acetic acid (90 : 10 : 1, v/v) for SE and TAG, with chloroform : acetone (9 : 1, v/v) for sterol, and chloroform : methanol (9 : 1, v/v) for ASG. To detect ASG, those fractions that, based on solvent polarity, should contain ASG were acetylated in 2 ml acetic anhydride : pyridine (1 : 2, v/v) at room temperature overnight. The resultant acetylated lipids were analysed by TLC with chloroform : acetone (95 : 5, v/v). The second eluate from the column that contained MGD, SG, cerebroside and DGD was also subjected to acetylation, and the resultant acetylated forms of cerebroside and SG were purified by preparative TLC with chloroform : acetone (95 : 5, v/v), followed by rechromatography with chloroform : acetone (9 : 1, v/v) or chloroform : methanol (97 : 3, v/v) for acetylated cerebroside. The occurrence of acetylated MGD was examined by TLC with chloroform : acetone (95 : 5, v/v).

Lipid quantification

Cerebroside and SG separated on TLC plates were detected with anilinonaphthalene sulphonate spray reagent and scraped off into test tubes for colorimetric determination, based on their sugar content, using anthrone reagent with glucose as standard (Heinz, 1996). Phospholipid classes were located with molybdenum blue reagent, scraped off and ashed by heating at 190°C for 30 min with 60% $HClO_4$. The resultant inorganic phosphate was measured colorimetrically with disodium phenyl phosphate as standard (Fiske and Subbarow, 1925). Phosphorus in the total lipid extracts was determined in the same way. Sterol was located on the plates under UV light after spraying with 0.03 (w/v) diphenylhexatriene in chloroform, scraped off and colorimetrically determined with *O*-phthalaldehyde reagent at 453 nm with ergosterol (Sigma) as a standard (Zlatkis and Zak, 1969).

For the determination of the fatty acid composition, TAG and SE separated on TLC plates were scraped off and transmethylated by heating with 5% (w/v) HCl in methanol at 85°C for 2 h. Phospholipids were first separated by TLC with chloroform : methanol : acetic acid (65 : 25 : 8, v/v).

The fractions of PC, CL and PA were directly transmethylated, whereas those of PE and PI plus PS were recovered and rechromatographed with chloroform : methanol : water (65 : 25 : 4, v/v) and chloroform : methanol : acetic acid : water (85 : 15 : 10 : 4, v/v), respectively, before transmethylation. The resultant fatty acid methyl esters were analysed by gas-liquid chromatography (GLC) using a capillary column (Thermon 3000B, 0.25 mm × 50 m; Shinwa Chemical Industries) and a temperature gradient from 170°C to 215°C at a rate of 1°C/min. To ensure the identification of fatty acid methyl esters, they were analysed on a second capillary column (CP-Sil 88, 0.25 mm × 50 m; Chrompack) with the temperature raised from 160°C to 200°C at a rate of 1°C/min. TAG and SE were determined from the amounts of the esterified fatty acids by gas-liquid chromatography, with pentadecanoic acid as internal standard (Sakaki *et al.*, 1985).

Combined gas–liquid chromatography–mass spectrometry (GLC–MS) analysis

Fatty acid and sugar analysis by GLC–MS was performed as described previously (Jorasch *et al.*, 2000). Peracetylated derivatives of SG and cerebrosides, as well as underivatized TAG and free sterols, were analysed by mass spectrometry on a HP 5989A instrument (Hewlett-Packard) using the direct insertion probe (DIP) mode, heating the sample through a temperature gradient starting at 80°C for 3 min, rising to 325°C at a rate of 30°C/min. Electron impact (EI) mass spectra were measured at 70 eV, and chemical ionization mass spectra (CI–MS) were recorded with ammonia as reactant gas (0.1 MPa).

Nuclear magnetic resonance (NMR) spectroscopy

One- (1D) [1]H- and two- (2D) dimensional homonuclear and H-detected heteronuclear ^{1}H, ^{13}C-correlation spectra were recorded on a 600-MHz spectrometer (Bruker Avance DRX-600). ^{13}C-NMR spectra were recorded at 90.6 MHz (Bruker DPX-360). Samples (0.1–5 mg) were dissolved in 0.5 ml $CDCl_3$ (99.96%, Cambridge Isotope Laboratories, Andover, MA, USA) and spectra recorded at 300 K, with $CDCl_3$ as internal reference ($\delta_H = 7.240$, $\delta_C = 77.0$). 1D- (^{1}H- and ^{13}C-NMR, DEPT) and 2D-homonuclear (^{1}H, ^{1}H-COSY) and H-detected heteronuclear (^{1}H, ^{13}C-HMQC, ^{1}H, ^{13}C-HMBC)

experiments were performed using standard Bruker software (XWINNMR, version 2.5).

Detection by PCR of sterol glucosyltransferase genes in different strains of *P. pastoris*

Oligonucleotide primers were derived from the sterol glucosyltransferase gene of *P. pastoris* GS115 (Warnecke *et al.*, 1999; GenBank Accession No. AF091397): forward 5′-CCATCTATGGAC TTTCCTC-3′ and reverse 5′-GAATGGCTGCA GAGACAC-3′. These primers were used for PCR experiments with genomic DNA from different strains of *P. pastoris*.

Results and discussion

We analysed the lipid composition of different yeasts and fungi to discover species that contain significant proportions of glycolipids such as cerebrosides, sterol glucosides, acylated sterol glucosides or glycosyldiacylglycerols. We selected *Pichia pastoris*, *Sordaria macrospora*, *Pyrenophora teres*, *Acremonium chrysogenum*, *Penicillium olsonii*, and *Rhynchosporium secalis* for this study. These organisms allow genetic manipulation. In addition, *Candida albicans* and *Ustilago maydis* are suitable to investigate a possible involvement of glycolipids in dimorphic growth.
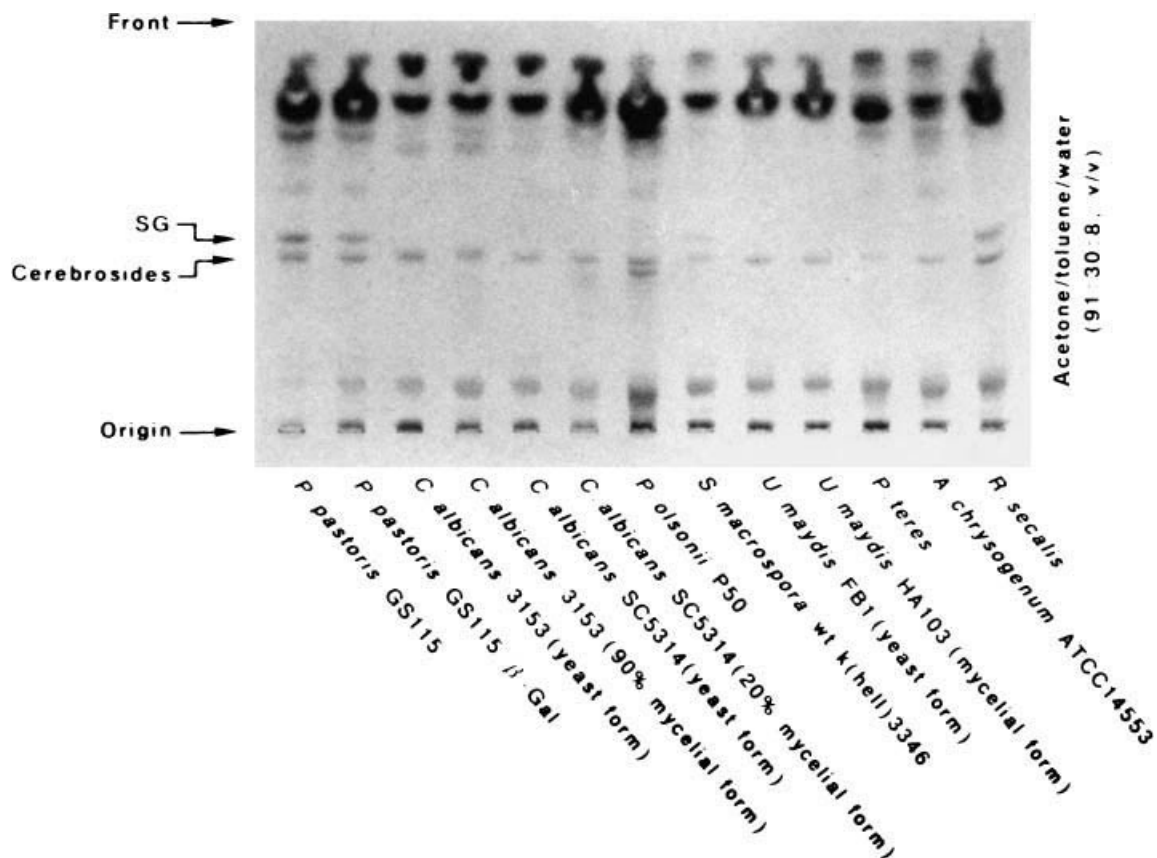

Figure 1. Thin-layer chromatography of total lipids from various yeast and fungal strains. Lipids equivalent to 40 nmol lipid phosphorous were separated and visualized by heating after spraying with 50% H_2SO_4. In this chromatogram, SG is not visible in the total lipids from *U. maydis*, whereas after fractionation of larger amounts of lipids by column chromatography we found low amounts of SG in the extract (data not shown)
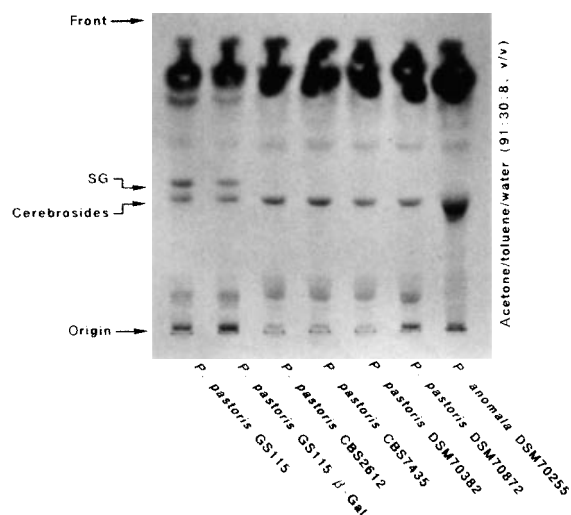
Figure 2. Thin-layer chromatography of total lipids from various strains of *Pichia*. Lipids equivalent to 20 nmol lipid phosphorous were separated from *P. pastoris* DSM70382, CBS7435 and CBS2612 and those equivalent to 40 nmol lipid phosphorous from the others. The separated lipids were visualized by heating after spraying with 50% H_2SO_4

Table 1. The occurrence of sterol glycoside and cerebroside in strains of several yeast and fungal species

Species	SG	Cer
Yeasts		
Candida albicans 3153 (yeast form)	−	+
Candida albicans 3153 (ca. 90% mycelial form)	−	+
Candida albicans SC5314 (yeast form)	−	+
Candida albicans SC5314 (ca 20% mycelial form)	−	+
Pichia pastoris GS115	+	+
Pichia pastoris GS115 β-Gal	+	+
Pichia pastoris CBS2612	−	+
Pichia pastoris CBS7435	−	+
Pichia pastoris DSM70382	−	+
Pichia pastoris DSM70872	−	+
Pichia pastoris DSM70255	−	+
Fungi		
Ustilago maydis FB1 (yeast type)	+	+
Ustilago maydis HA103 (mycelial type)	+	+
Penicillium olsonii P50	−	+
Sordaria macrospora wt k(hell)3346	+	+
Pyrenophora teres	−	+
Acremonium chrysogenum ATCC14553	−	+
Rhynchosporium secalis	+	+

SG, sterol glycoside; Cer, cerebroside; +, detected; −, not detected.

All examined yeasts and fungi contained cerebrosides

When total lipid extracts from the selected yeast and fungal cells were separated by TLC, cerebroside was detected in all organisms (Figures 1, 2; Table 1). We found this lipid in similar proportions in both the yeast and the mycelial form of *C. albicans* strains 3153 and SC5314, as well as in the two morphologically different forms of *U. maydis* (Figure 1). These results contrast with the findings of Ghannoum *et al.* (1986), that cerebroside occurred in considerable proportions in the yeast form of *C. albicans* ATCC10231 but only in trace amounts in its mycelial form. It has to be pointed out that we grew the different forms of both organisms under separate conditions, whereas Ghannoum and co-workers grew both forms together and separated them from a common culture. These differences in culture conditions may explain the inconsistent results. Since in our study all species contained cerebroside (Figure 1) regardless of whether the lipids were extracted from yeast or filamentous form, cerebroside synthesis is suggested to be independent of the growth type.

MS and NMR analysis of cerebrosides

Cerebrosides were purified from *C. albicans* 3153 (yeast form), *P. pastoris* GS115 and GS115 β-Gal, *S. macrospora*, *R. secalis* and *A. chrysogenum*. In their acetylated form, some of them were separated by TLC into several molecular species that were separately subjected to structural analysis by mass spectroscopy (MS) and nuclear magnetic resonance (NMR) spectrometry. The structures of the purified glycolipids from *C. albicans* 3153 (yeast form), *S. macrospora* and *A. chrysogenum* were confirmed to be glucosylceramides (data not shown). Only the analyses of the compounds from *R. secalis* and *P. pastoris* are exemplified in detail in the following. The peracetylated cerebroside from *R. secalis* was separated into two molecular species termed Cer-1 and Cer-2. Upon CI–MS analysis, Cer-1 showed a molecular ion of low intensity $[m/z = 981 \ (M + H^+)]$ compatible with a peracetylated ceramide monohexoside containing a hydroxylated hexadecanoic acid and a methyl-branched sphingadienine (C_{19}) as long-chain base [calculated molecular weight (M_r) 980.27 ($C_{53}H_{89}O_{15}N$)]. The base peak $[m/z = 921, (M + H^+ - HOAc)]$ was also in agreement with this

interpretation, since loss of acetic acid (HOAc) is a well-known feature in MS-fragmentation patterns of peracetylated cerebrosides (Boas *et al.*, 1994).

CI–MS analysis of Cer-2 also showed a pseudo-molecular ion signal of low intensity at $m/z = 923$ $(M + H^+)$ and an intense fragment at $m/z = 861$ $(M + H^+ - HOAc)$. This is consistent with $M_r = 922.23$ $(C_{51}H_{87}O_{13}N)$ and suggests Cer-2 to be structurally related to, but different from, Cer-1 by lack of one hydroxy group. Upon EI–MS

analysis, both peracetylated cerebrosides yielded fragments $(m/z = 331)$ characteristic of a terminal tetra-*O*-acetylated hexosyl residue.

The ^{1}H- and ^{13}C-NMR spectra of Cer-1 and Cer-2 (Tables 2, 3) were assigned based on ^{1}H, ^{1}H-COSY, 2D homonuclear and H-detected hetero-nuclear ^{1}H, ^{13}C-HMQC and HMBC spectros-copy. In the ^{1}H- and ^{13}C-NMR spectra of both cerebrosides, anomeric proton and carbon signals indicative of a glucopyranosyl residue (H-1″, 4.453

Table 2. [1]H (600 MHz) chemical shifts (ppm) and coupling constants J (Hz) for the peracetylated glucosylceramides Cer-1 and Cer-2 isolated from *Rhynchosporium secalis*; CDCl$_3$ ($\delta_H = 7.24$ ϑ), 300 K

	Cer-1			Cer-2		
	δ(ppm)	J	(Hz)	δ(ppm)	(Hz)	
β-D-Glc						
H-1″	4.453	$J_{1,2}$	7.9	4.447	8.0	
H-2″	4.928	$J_{2,3}$	9.5	4.943	9.4	
H-3″	5.160	$J_{3,4}$	9.5	5.177	9.5	
H-4″	5.051§	$J_{4,5}$	9.9	5.051§	9.7	
H-5″	3.665	$J_{5,6a}$	2.2	3.667	2.3	
H-6a″	4.116	$J_{6a,6b}$	12.3	4.134	12.2	
H-6b″	4.212	$J_{6b,5}$	4.5	4.207	4.6	
Sphingobase						
H-1a	3.586	$J_{1a,1b}$ 10.0	$J_{1a,2}$ 4.5	3.562	10.1	$J_{1a,2}$ 4.4
H-1b	3.909	$J_{1b,2}$	3.9	3.901	4.0	
H-2	4.278	$J_{2,3}$	6.9	4.306	6.8	
NH	6.299	$J_{NH,2}$	9.1	5.671	9.0	
H-3	5.287	$J_{3,4}$	7.3	5.250	7.3	
H-4	5.376	$J_{4,5}$	15.2	5.373	15.4	
H-5	5.799			5.764		
H-6	2.50*			2.05*		
H-7	2.50*			2.05*		
H-8	5.05§			5.05§		
H-9	–			–		
H-10	1.920			1.923		
H-11	1.328			1.332		
H-12–H-17	1.22–1.27			1.23–1.28		
H-18	0.855			0.859		
H-19	1.547			1.548		
Fatty acid						
H-2′	5.125			2.105		
H-3a′	1.748			1.55*		
H-3b′	1.796					
H-4′	1.796			1.230		
H-5′–H-15′	1.28–1.22			1.22–1.28		
H-16′	0.870			0.859		

Other signals: Cer-1; 6 OAc, 2.148, 2.069, 2.009, 1.998 (2×), 1.975; Cer-2; 5 OAc, 2.148, 2.070, 2.009, 1.999 (2×), 1.975 ppm. *Non-resolved multiplet. §Overlapping signals.

Table 3. [13]C (90.6 MHz) chemical shifts (ppm) for the peracetylated glucosylceramides Cer-1 and Cer-2 isolated from *Rhynchosporium secalis*; CDCl$_3$ ($\delta_C = 77.00$ ϑ), 300 K

	Cer-1 δ (ppm)	Cer-2
β-D-Glc		
C-1″	100.54	100.62
C-2″	71.22	71.36
C-3″	72.69	72.65
C-4″	68.27	68.34
C-5″	71.95	71.97
C-6″	61.83	61.86
Sphingobase		
C-1	67.17	67.32
C-2	50.67	50.62
C-3	73.10	73.63
C-4	124.54	124.78
C-5	136.76	136.54
C-6	32.57	32.59
C-7	27.39	27.47
C-8	122.94	123.06
C-9	136.24	136.29
C-10	39.71	39.74
C-11	28.03	28.07
C-12–C-15	29.3–29.7	29.3–29.7
C-16	31.81	31.93
C-17	22.66	22.69
C-18	14.08	14.11
C-19	15.96	16.00
Fatty acid		
C-1′	169.20	169.41
C-2′	73.97	36.83
C-3′	31.90	31.03
C-4′	24.70	25.71
C-5′–C-14′	29.3–29.7	29.3–29.7
C-15′	22.66	22.69
C-16′	14.08	14.11

Other signals: Cer-1; C=O, 170.6, 170.15, 169.76, 169.72, 169.61, 169.37, 169.23; OAc, 22.65, 20.98, 20.91, 20.66, 20.54 (2×); Cer-2; C=O, 172.7, 170.62, 170.17, 169.89, 169.41, (2×); OAc, 22.70, 21.06, 20.71, 20.58 (2×) ppm.

and 4.447 ppm, $J_{1,2}$ 7.9 and 8.0 Hz; C-1″, 100.52 and 100.62) expressed connectivities and coupling constants (>9 Hz) characteristic for a terminal β-glucosyl residue. A pronounced difference in the ^{1}H- and ^{13}C-NMR spectra of Cer-1 and Cer-2 was only seen for some signals derived from the fatty acids, i.e. H-2′ (5.125 vs. 2.105 ppm) and C-2′ (73.97 vs. 36.83 ppm), indicating that a C-2′-hydroxylated palmitic acid [16:0 (2-OH)] in Cer-1 and palmitic acid (16:0) in Cer-2 were amide-linked to the long-chain base. With the exception of the resonances assigned to the fatty acids, all other signals in the ^{1}H- and ^{13}C-NMR spectra were found to be identical to those published (Boas *et al.*, 1994). MS and NMR data together, therefore, identified Cer-1 from *R. secalis* as *N*-(*S*)-2′-hydroxyhexadecanoyl-1-*O*-β-D-glucopyranosyl-(*4E, 8E*)-9-methyl-sphingadienine and Cer-2 as *N*-hexadecanoyl-1-*O*-β-D-glucopyranosyl-(*4E, 8E*)-9-methyl-sphingadienine.

The cerebrosides isolated from the two strains of *P. pastoris*, GS115 and GS115 β-Gal, were analysed in a similar way and found to be structurally related to those identified from *R. secalis*. Upon CI–MS both compounds showed a pseudomolecular ion (M + H$^+$) at $m/z = 1009$, suggesting a molecular weight of $M_r = 1008$. Almost all of the fragments in the CI- and EI-mass spectra ($m/z = 271$, 331, 390) were found to be similar when compared to Cer-1 from *R. secalis*, indicating structural relatedness, since its molecular ion as well as all fragment ions were found to be shifted by $\Delta m/z = 28$ to higher masses. The base peak of both cerebrosides was detected at $m/z = 949$ and was assigned to (M + H$^+$ − HOAc). Therefore, the molecular weight of $M_r = 1008.32$ (C$_{55}$H$_{93}$O$_{15}$N) was assigned for both peracetylated cerebrosides from *P. pastoris* GS115 and GS115 β-Gal. Again, EI–MS analysis showed one fragment ($m/z = 331$) characteristic for a terminal hexose residue, confirming that these glycolipids are monoglycosyl ceramides.

The ^{1}H-NMR spectra of both cerebrosides from *P. pastoris* GS115 and GS115 β-Gal were identical to each other and similar to that of *R. secalis* Cer-1, described above. Diagnostic signals from the anomeric region and ring protons were also found to be identical and thus the glycosyl part in both ceramides was identified to be a β-D-*gluco*pyranoside. In order to unravel the structural difference between Cer-1 from *R. secalis* and that of *P. pastoris* GS115 and GS115 β-Gal, fatty acids were isolated and analysed as their methyl esters by GLC–MS. Both cerebrosides from *P. pastoris* contained only α-hydroxystearic acid = 18:0 (2-OH). Since all other signals in both ^{1}H-NMR spectra were identical to those of Cer-1, it was concluded that the only structural difference between Cer-1 and the cerebroside from *P. pastoris* GS115 and GS115 β-Gal resides in the amide-linked fatty acid [16:0 (2-OH) vs. 18:0 (2-OH)]. Therefore, the cerebrosides GS115 and GS115 β-Gal isolated from *P. pastoris* can be described as *N*-(*S*)-2′-hydroxyoctadecanoyl-1-*O*-β-D-glucopyranosyl-(*4E, 8E*)-9-methyl-sphingadienine.

In conclusion, the cerebrosides from *R. secalis* and *P. pastoris* GS115 are monoglucosyl ceramides consisting of a saturated α-hydroxy or non-hydroxy fatty acid and a $\Delta 4,8$-diunsaturated, C9-methyl-branched sphingobase. We should point out that during acetylation and purification of the cerebrosides some minor components may have been lost. Therefore, we cannot exclude the existence of low amounts of cerebrosides that contain different fatty acids and sphingobases. A comparison of our results with data from other yeasts and fungi reveals that, with few exceptions, the $\Delta 4,8$-diunsaturated, C9-methyl-branched sphingobase forms the backbone of the cerebrosides. They differ either in the fatty acyl moiety, which is usually a saturated or 3-*trans*-unsaturated C16 or C18 α-hydroxy fatty acid, or in the sugar moiety, which is often glucose and rarely galactose [*Magnaporthe grisea* (Koga *et al.*, 1998); *Paracoccidioides brasiliensis*, *Aspergillus fumigatus* (Toledo *et al.*, 1999; Boas *et al.*, 1994); *Sporothrix schenkii* (Toledo *et al.*, 2000); *Lentinus edodes* (Kawai, 1989); *Candida albicans* (Matsubara *et al.*, 1987)]. These features contrast with the ceramide backbone of glycosyl inositol phosphoryl ceramide (GIPC) from yeasts and fungi, which contain phytosphingosine (4-hydroxysphinganine) and very long chain fatty acids [*S. cerevisiae* (Steiner *et al.*, 1969); *C. albicans* (Wells *et al.*, 1996); *Histoplasma capsulatum* (Barr and Lester, 1984); *Paracoccidioides brasiliensis* (Levery *et al.*, 1998)]. Although the isolation of both cerebrosides and GIPC have been reported for only a few organisms [*C. albicans*, *P. brasiliensis* (Matsubara *et al.*, 1987; Wells *et al.*, 1996; Toledo *et al.*, 1999; Levery *et al.*, 1998)], it seems likely that, except for *S. cerevisiae*, most yeasts and fungi contain both types of glycosphingolipids and therefore synthesize two different pools of ceramide backbones. As discussed previously (Toledo *et al.*, 1999), it is not

known whether the partitioning of the two ceramide pools results from compartmentalization or from distinct substrate specificities of enzymes downstream in the glycosphingolipid synthesis.

In contrast to the abundance of information on fungal cerebroside structures, knowledge of biosynthesis, intracellular location and function of these glycolipids is very limited. In plants two different pathways for cerebroside synthesis have been suggested that involve the transfer of a glucosyl residue from UDP-glucose (Nakayama *et al.*, 1995) or from sterol glucoside (Lynch *et al.*, 1997; Lynch, 2000) to ceramide. In mammals, cerebroside biosynthesis is catalysed by ceramide glucosyltransferases and galactosyltransferases, whose genes have been isolated (Ichikawa *et al.*, 1996; Schulte and Stoffel, 1993; Shayman and Abe, 2000; Marks *et al.*, 2000; Sprong *et al.*, 2000; Ichikawa and Hirabayashi, 2000). The corresponding genes from plants remain to be discovered, whereas the first yeast orthologues were recently cloned (Leipelt *et al.*, 2000).

Only a few of the yeasts and fungi contain sterol glycosides

SGs are well known lipids of the plasma membrane and tonoplast of plants (Warnecke *et al.*, 1994, 1997). However, to our knowledge this is the first report presenting structural data on SGs from fungi. SGs have occasionally been found in some yeasts and fungi (Kastelic-Suhadolc, 1980; McMorris and White, 1977; Ghannoum *et al.*, 1986, 1990; Työrinoja *et al.*, 1974; Baraud *et al.*, 1970; Deven and Manocha, 1976). In many of these studies, the fungi were grown on complex plant-based media that provided a potential source for fungal SG uptake. In our study, we avoided this problem by using minimal media. In addition, we carried out a detailed analysis of SGs showing the absence of phytosterols even in those organisms, that required complex media for culture (e.g. *R. secalis*). Thus, we conclude that SGs are genuine components of the lipid mixture found in *P. pastoris*, *U. maydis*, *A. macrospora* and *R. secalis*.

The occurrence of SG in total lipid extracts was restricted to *P. pastoris* GS115, *S. macrospora* and *R. secalis* (Figure 1; Table 1). Larger quantities of total lipids of the other organisms were fractionated by silica-gel column chromatography to possibly identify small amounts of SG not detectable on TLC plates of total lipid extracts. The SG-containing fractions were analysed by TLC directly or after acetylation. In this way, we were able to show that both strains of *U. maydis* FB1 (yeast form) and HA103 (mycelial form) contained low proportions of SG, whereas in the extracts of the other organisms, including both forms of *C. albicans* (strains 3153 and SC5314), SG was not detected (data not shown).

These results do not correspond to those obtained by Ghannoum *et al.* (1986), who showed that the mycelial form, but not the yeast form, of *C. albicans* strain ATCC10231 contained considerable proportions of SG. This previous result suggested that these glycolipids may be relevant to dimorphic growth and further to the virulence of this pathogen of human candidiasis, since the phenotypic switching to mycelia plays a role in the invasion of host epithelia (Mishra *et al.*, 1992). The difference from our results may be due either to the different method applied to induce mycelial growth (separate or common culture) or to the different strains used. In any case our results, that SG occurred in *U. maydis* but not in *C. albicans* (3153, SC5314), irrespective of yeast or mycelial growth forms, do not support the hypothesis that SG synthesis is correlated with dimorphic changes in these organisms.

Additional studies were carried out on the occurrence of SG in different strains of *Pichia pastoris* (Figure 2). Both GS115 and GS115 β-Gal, but none of the other strains, contained SG. In contrast to all other strains tested, the strain GS115 from Invitrogen (Carlsbad, CA) is haploid and, in addition, GS115 β-Gal, which was used as a control, is genetically manipulated to allow expression of recombinant β-galactosidase but with no intention to alter lipid metabolism. Under our culture conditions, expression of this protein was not induced and, therefore, we did not expect any difference between these two strains. Accordingly, we have no explanation for the quantitative differences in lipid composition of these strains (Table 4). Thus, we conclude that the ability to synthesize SG in strains GS115 may stem from the original wild-type strain, which is capable of synthesizing SG. Although the diploid *Pichia* strains examined in these experiments were devoid of SG (Figure 2), there may be other *Pichia* strains which are able to synthesize this glycolipid.

Table 4. Content of phospholipids, cerebroside, sterol glycoside, free sterol, sterol ester and triacylglycerol (TAG) in *P. pastoris* and *R. secalis*. All data are expressed as µmol lipid/g lipid dry weight and as mol% (in brackets)

	Phospholipids	Cerebroside	SG	Sterol ester	Sterol	TAG
P.p. GS115	227±4 (20)	9.8±0.1 (0.9)	13.5±1.2 (1.2)	75±1 (6.5)	144±18 (13)	680±12 (59)
P.p. GS115 *β*-Gal	369±5 (27)	12.5±2.0 (0.9)	7.0±0.7 (0.5)	107±1 (7.8)	94±13 (6.8)	789±4 (57)
R. secalis	170±3 (23)	15.0±1.0 (2.0)	6.1±0.4 (0.8)	16±0 (2.1)	85±2 (11)	463±5 (61)

From these results it is obvious that some yeasts and fungi are capable of synthesizing SG, whereas others are not. The recent cloning of genes coding for sterol glucosyltransferases (Warnecke *et al.*, 1999) from *P. pastoris* GS115, as well as from *S. cerevisiae* and *C. albicans* strain 1161, suggests a simple explanation of this difference: even among those yeasts and fungi which apparently do not synthesize SG, some may have sterol glucosyltransferase genes. They are silent under the growth conditions tested so far, but may become transcribed under different physiological conditions. Indeed, the application of abiotic stress, such as nutrient limitation or elevated temperature, resulted in the synthesis of SG (see below). In addition, we detected sterol glucosyltransferase genes in *P. pastoris* CBS7435, DSM70382 and DSM70872 by PCR experiments, using genomic DNA as template and primers derived from the recently cloned gene of *P. pastoris* GS115 (data not shown).

Structural analysis of the sterol glycosides

For structural confirmation, SG was purified from two strains of *P. pastoris* GS115, *S. macrospora* and *R. secalis* and acetylated for NMR and MS analyses.

The peracetylated SG isolated from *R. secalis* were analysed by electron-impact (EI)– and CI–MS analysis in the DIP mode. Upon EI–MS, several characteristic fragments were obtained, one ($m/z = 331$) originating from a tetra-*O*-acetylated hexosyl residue and two from the steroid part ($m/z = 380$, 382), suggesting heterogeneity due to the absence of one double bond in the latter fragment ion. Consequently, in the CI–MS, two pseudomolecular ions [$(M + NH_4)^+$, $m/z = 746$ and 748] were detected that were compatible with the structures of tetra-*O*-acetylhexosyl-ergosta-diene ($C_{42}H_{64}O_{10}$; $M_r = 728.94$) and tetra-*O*-acetyl-hexosyl-ergosta-

ene ($C_{42}H_{66}O_{10}$; $M_r = 730.94$). This interpretation was confirmed by GLC–MS analysis of the free steroids obtained after acid methanolysis and peracetylation. Besides methyl 2,3,4,6-tetra-*O*-acetyl-glucopyranoside (t_R 15.4 min), two *O*-acetyl-steroids in equimolar proportions were identified ($t_R = 34.9$ and 35.6 min) with molecular ions (M^+) at $m/z = 440$ and 442 being compatible with 3-acetoxy-ergosta-dienol and 3-acetoxy-ergosta-enol, respectively. We did not try to identify the structure of the steroids in more detail, i.e. analysis of the position of the double bonds in the putative ergosterol derivatives.

The EI–MS of the peracetylated SG from *P. pastoris* GS115 and GS115 *β*-Gal also had the characteristic fragment for a terminal tetra-*O*-acetylated hexosyl residue ($m/z = 331$), whereas the steroid part showed a single fragment ion ($m/z = 378$), thus indicating homogeneity. The molecular ion $(M)^+$ ($m/z = 726$) in the EI mass spectrum was compatible with the calculated mass for tetra-*O*-acetyl-hexosyl-ergosterol ($C_{42}H_{62}O_{10}$; $M_r = 726.94$). Both EI and CI mass spectra were identical with that obtained from tetra-*O*-acetyl-hexosyloxyergosterol (Warnecke *et al.*, 1999).

In order to determine the glycosidic part in more detail, the peracetylated SGs were further analysed by ^{1}H- and ^{13}C-NMR spectroscopy. The anomeric signal (H-1) in all three SGs (*P. pastoris* and *R. secalis*) had coupling constants of $J_{1,2} \sim 8$ Hz, and the ring protons showed high coupling constant values ($J_{2,3}$, $J_{3,4}$ and $J_{4,5} > 9.5$) characteristic for a *β*-D-glucopyranoside. These findings were supported by the chemical shifts of the ring protons and the ^{13}C-NMR resonances, which for SG of *P. pastoris* were all identical to 3*β*-(2,3,4,6-tetra-*O*-acetyl-*β*-D-glucopyranosyloxy)ergosta-5,7,22-*E*-triene (Warnecke *et al.*, 1999).

The ^{1}H- and ^{13}C-NMR-spectra of the peracetylated SG from *R. secalis* had, as already evident

from the EI–MS analysis, a structurally related steroid part differing from ergosterol by the absence of one and two double bonds, respectively. Again, no further attempts were made to assign the position(s) of the double bonds in detail. This interpretation is also in agreement with MS and NMR data obtained from the free sterols and SE, as described below.

The SG from *S. macrospora* was confirmed to be a sterol β-D-glucopyranoside (data not shown).

SG accumulates under heat shock

We found that SG synthesis in *P. pastoris* depends not only on the strain but also on the culture conditions. When *P. pastoris* GS115 cells were grown in complete medium (YPD), SG remained below the detection limit, whereas in minimal medium SG accumulated to a level similar to that of cerebrosides. In contrast, SG was not detectable in other *Pichia* strains, e.g. CBS 7435, grown in minimal or complete medium. However, both *P. pastoris* strains GS115 and CBS 7435, growing in YPD, accumulated SG in quantities comparable to cerebrosides when subjected to stress conditions. For instance, a heat shock (from 28°C to 41°C) led to a strong increase in SG content within 6 h (Figure 3). Similarly, when YPD was supplemented with 3% or 6% ethanol, *P. pastoris* GS115 accumulated SG within 4 h (Figure 4). It is unknown whether these stresses induce the accumulation of SG directly or as a result of metabolic alterations.

We found two different SGs (SG-1 and SG-2) in *P. pastoris* CBS7435 under heat shock which differed after peracetylation by TLC-Rf values. Both EI and CI mass spectra of acetylated SG-1 were found to be identical to those obtained from the sterol glucoside accumulating in GS115 in minimal medium (see above). Although only a small quantity of SG-1 was available (100 µg), peracetylated SG-1 was further analysed by ^{1}H-NMR spectroscopy, showing signals identical to the SG isolated from *P. pastoris*, described above. Therefore, SG-1 was identified as 3β-(2,3,4, 6-tetra-*O*-acetyl-β-D-glucopyranosyloxy)ergosta-5,7, 22*E*-triene.

SG-2 showed the EI mass fragment characteristic of one terminal tetra-*O*-acetylated hexose residue ($m/z = 331$). In contrast, the sterol part of SG-2 was different, showing a fragment ($m/z = 392$) 14 mass units larger than that obtained from SG-1. How-

ever, since the CI mass spectrum showed several pseudomolecular ions [$(M + NH_4)^+$, $m/z = 823, 824, 881$], the sterol part of SG-2 appeared heterogeneous, which was not further analysed. Despite the heterogeneity in the sterol part, a β-D-*gluco*pyranoside could be unequivocally identified by ^{1}H-NMR spectroscopy as the glycosyl part in SG-2.

A peracetylated cerebroside isolated from *P. pastoris* CBS7435 under heat shock was analysed in a similar way. The CI–MS and EI–MS data showed that the cerebroside from the cells after heat shock was identical to the cerebroside from GS115 cells grown at 28°C.

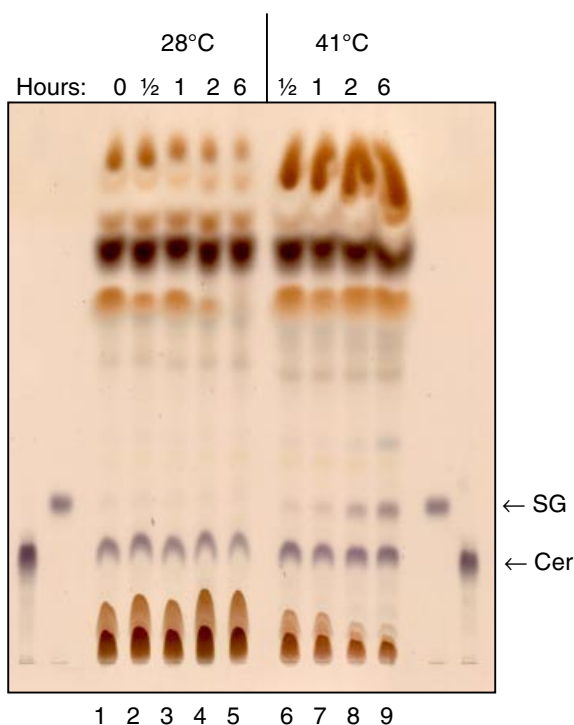Our data show that SG accumulates in *P. pastoris*

Figure 3. Thin-layer chromatography of lipids from *P. pastoris* GS115 grown in complete medium and subjected to heat stress. *Pichia* cells were grown in YPD with 2% glucose to an optical density of 1, when heat stress was initiated. Further growth of the stressed cells was retarded, while control cells reached an optical density of 4.7 during the 6 h of the experiment. (Left) Control cells were grown at 28°C. (Right) Cells were incubated for 30 min, 1 h, 2 h or 6 h at 41°C. *P. pastoris* contains glucosylceramide and small amounts of sterol glycoside. The proportion of sterol glycoside increases during heat shock, while cerebroside remains constant. Cer, cerebroside
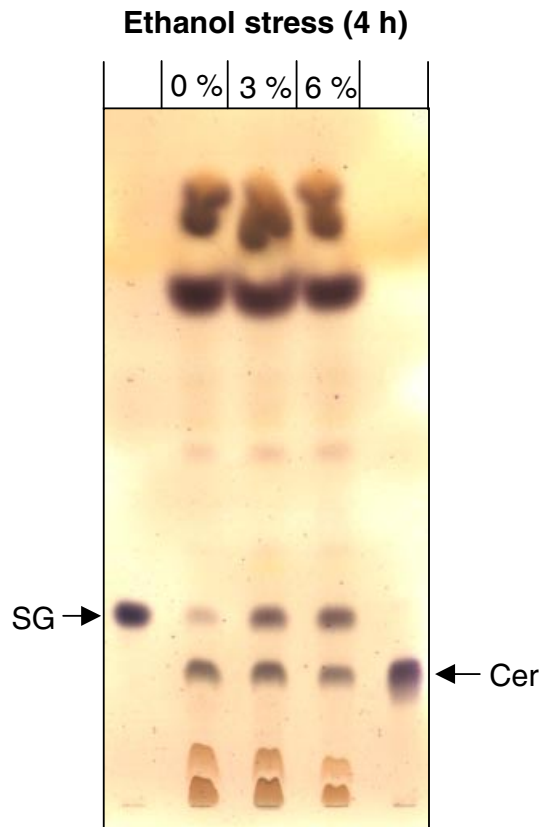
Ethanol stress (4 h)

Figure 4. Thin-layer chromatography of lipids from *P. pastoris* GS115 grown in YPD with 2% glucose and subjected to ethanol stress at an initial optical density of 5 (ethanol proportions given in percentage volume)

under different stress conditions, whereas cerebroside is constitutively synthesized. These findings are consistent with data from Murakami-Murofushi *et al.* (1997) and Kunimoto *et al.* (2000), who recently found a heat shock-induced production of SG in the slime mould *Physarum polycephalum* and in cultured human fibroblasts. The observation of stress-induced SG accumulation in different organisms suggests the involvement of these glycolipids in stress responses.

The availability of a gene or cDNA coding for a sterol glucosyltransferase seems to be desirable for further studies in this field. However, such a gene or cDNA has not been cloned from *P. polycephalum* and *Homo sapiens*. In contrast, a sterol glucosyltransferase gene has been isolated from *P. pastoris* (Warnecke *et al.*, 1999) and will be used to study the role of SGs in the stress response.

Acylated sterol glycosides and glycosyl diacylglycerols were absent from all strains tested

Additional efforts were directed towards the demonstration of ASG in the SG-containing species of *P. pastoris* GS115, *S. macrospora* and *R. secalis*. Since ASG was undetectable in the total lipids of any of these strains, we further tried to detect ASG after fractionation of larger quantities of total lipids by column chromatography followed by TLC, but also this approach gave no indication of the presence of ASG. Although ASG has been barely detected in fungi (Loesel, 1988), one exception is *C. albicans* ATCC 10231, which accumulated ASG in mycelial but not in yeast form after prolonged culture (Ghannoum *et al.*, 1986).

Glycosyl diacylglycerols (MGD, DGD) have been reported to occur in some yeast and fungal species (Loesel, 1988; Rattray, 1988). However, these glycolipids were undetectable in any of the organisms examined, even after further attempts to enrich these compounds by column chromatography from total lipid extracts of *C. albicans* 3153 (yeast and mycelial forms), *P. pastoris* GS115, *S. macrospora* and *R. secalis*.
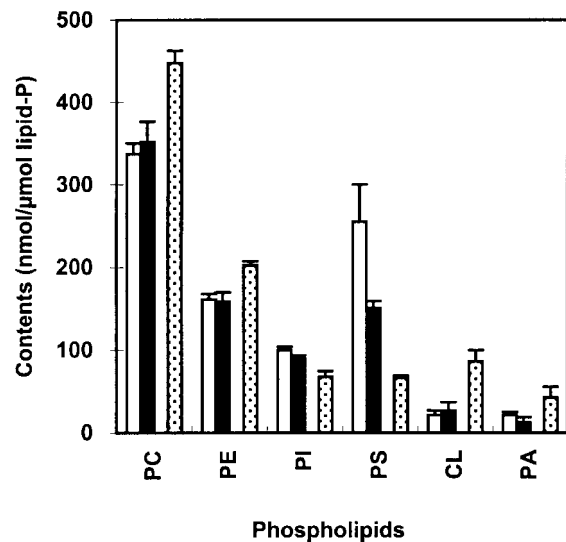

Figure 5. Proportions of individual phospholipids in *P. pastoris* GS115, GS115 β-Gal and *R. secalis*. Averages with SD of three independent preparations are presented. White bars, *P. pastoris* GS115; black bars, *P. pastoris* GS115 β-Gal; stippled bars, *R. secalis*

Composition of apolar and polar lipids in P. pastoris and R. secalis

We selected *P. pastoris* GS115 and *R. secalis* as representative SG-producing organisms to determine their polar and apolar lipids in more detail. Large proportions of TAG and six phospholipid classes were detected in these organisms (Table 4; Figure 5). In common with other yeasts (Rattrey, 1988) and fungi (Loesel, 1988), major phospholipid classes in *P. pastoris* were phosphatidyl choline, -ethanolamine and -serine, and those in *R. secalis* were PC and PE. The proportions of cardiolipin (CL) and phosphatidic acid (PA) were higher in *R. secalis* than in *P. pastoris*. In addition, trace amounts of PG and an unknown phospholipid were also detected in these three organisms.

On the basis of lipid phosphorous, they contained high levels of free sterols (Table 4), with proportions comparable to those of the most abundant phospholipid PC (Figure 5). SG and cerebroside occurred at relatively constant levels, 7–9% and 7–18% to those of free sterol, respectively. On the other hand, the proportions of SE were highly variable, as observed previously with other yeasts and fungi (Rattray, 1988; Loesel, 1988). Representative samples of free sterol, sterol ester and TAG from both organisms were subjected to analysis by NMR and mass spectroscopy for structural confirmation, as presented in the following.

Free sterols

EI–MS of free sterols from *R. secalis* indicated structural heterogeneity due to the absence of one or two double bonds as compared to ergosterol. This finding is in agreement with the data from the SGs (see above), where ergostadienol (dihydro-ergosterol) and ergosta-enol (tetrahydroergosterol) were detected in almost equimolar proportions.

The EI spectrum of free sterol isolated from *P. pastoris* was identical to that of ergosterol in the database (Hewlett-Packard). Since these findings were also supported by the data obtained from analyses of sterol glucoside (see above) and sterol ester (see below), ergosterol is considered to be the predominant sterol in all steroids (SG, SE and free sterols) found in *P. pastoris*.

Sterol esters

Here only the most relevant results of the spectroscopical confirmation of the structural identity of the SE fraction will be presented, whereas the GLC analysis gives a more precise profile of their fatty acids (Figure 6), which will be discussed below together with the fatty acid mixtures found in the phospholipids.

The results of CI–MS of sterol ester from *R. secalis* can best be interpreted by assuming that the SE is composed predominantly of stearic acid (18:0) or oleic acid (18:1) and ergostadienol or ergosta-enol, respectively. SE from *P. pastoris* GS115 was found to be ergostanol octadecanoic ester.

Triacylglycerols

In addition to the detailed and reliable fatty acid analysis of TAG by GLC (Figure 6), as discussed below, we have analysed representative samples of TAG from *R. secalis*, as well as from *P. pastoris* GS115 and GS115 β-Gal, by MS analysis in the direct insertion mode (DIP), with the intention of confirming their identity. We found a TAG containing three 18:1 fatty acid residues and two other TAGs in which one or two 18:1 residues were replaced by palmitic acid 16:0.

The TAG of *P. pastoris* GS115 was very similar to that of *R. secalis* but showed a slight predominance for the saturated fatty acids (16:0 and 18:0). The TAG of *P. pastoris* GS115 β-Gal was almost identical to that of *R. secalis*.

Fatty acid composition of glycerolipids in P. pastoris and R. secalis

Figure 6 shows the fatty acid composition of individual phospholipids, TAG and SE in these organisms, as determined by GLC. PC, PE, CL, TAG and SE are characterized by the predominance of C18 fatty acids of different degrees of unsaturation, as compared to the predominant C16 fatty acid palmitic acid, which in this group varied from 5.4% (PC) to a maximum of 22.9% (CL). High proportions of palmitic acid are characteristic for PS, PI and PA. It may be expected that palmitic acid is preferentially localized at the *sn*-1 position in these phospholipids. Figure 6 indicates the presence of significant proportions of linoleic and even linolenic acid, which is particularly high in *R. secalis* lipids, reaching 18%.
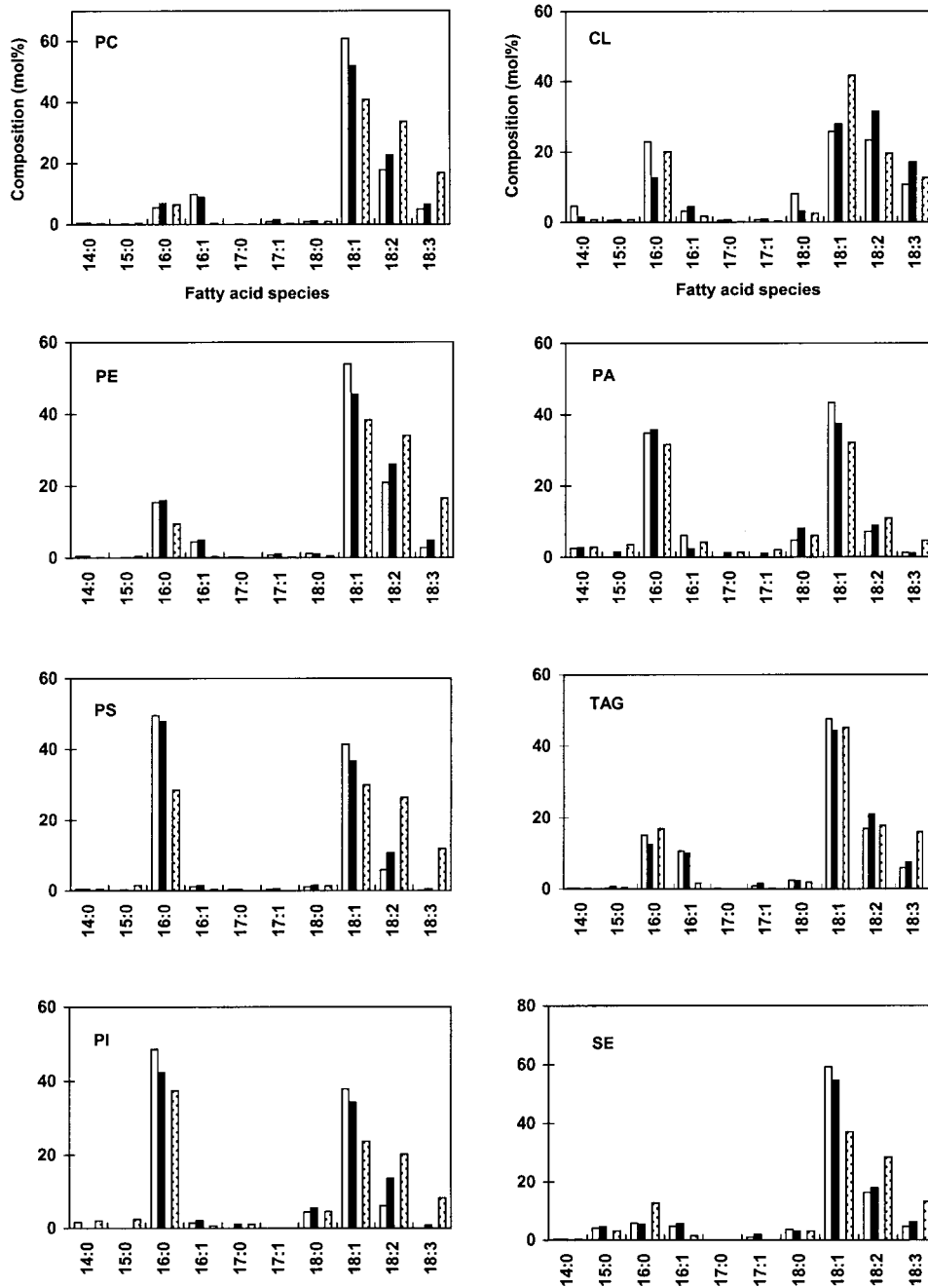
Figure 6. Fatty acid composition of individual phospholipids, triacylglycerol and sterol esters in *P. pastoris* GS115, GS115 *β*-Gal and *R. secalis*. Averages of two independent preparations are presented. White bars, *P. pastoris* GS115; black bars, *P. pastoris* GS115 *β*-Gal; stippled bars, *R. secalis*

The fatty acid composition of *P. pastoris* and *R. secalis* is more similar to other eukaryotes and in particular to higher plants (Heinz, 1996) than to *S. cerevisiae*. Baker's yeast is able to introduce only one double bond into C16 and C18 fatty acids, in contrast to *Pichia* and *Rhynchosporium*, which synthesize a complete set of C-18 fatty acids with up to three double bonds. Therefore, the fatty acid

profiles of the dominating phospholipids are very similar to those found in plant phospholipids.

Concluding remarks

This study shows that, with regard to glycolipids, *S. cerevisiae* is rather unusual as compared to other yeasts and fungi. *S. cerevisiae* contains different inositol-containing sphingolipids (Van Der Rest *et al.*, 1995; Dickson and Lester, 1999a, 1999b) and only minute amounts of SG (Warnecke *et al.*, 1999), whereas all yeasts and fungi that we examined were rich in cerebrosides and some of them even contain SG.

The elucidation of glycolipid functions requires the characterization of the enzymes responsible for the last step in their synthesis and will be promoted by the availability of the genes coding for the corresponding UDP-sugar:lipid glycosyltransferases. Genes for sterol glucosyltransferases have already been cloned and characterized from *S. cerevisiae* (*UGT51 = YLR189c*), *C. albicans* (*UGT51C1*, GenBank Accession No. AF091398), *P. pastoris* (*UGT51B1*, GenBank Accession No. AF091397), *Dictyostelium discoideum* (*ugt52*, GenBank Accession No. AF098916) and plants (Warnecke *et al.*, 1997, 1999). Putative sterol glucosyltransferase genes/cDNAs from *Magnaporthe grisea* (GenBank Accession No. AF027983), *Neurospora crassa* (found by a Blast search at the MIPS *Neurospora crassa* database, **http://www.mips. biochem.mpg.de/proj/neurospora**), and *Aspergillus nidulans* (GenBank Accession Nos AA966305 and AA965878) can be annotated by sequence homology.

A gene encoding a ceramide glucosyltransferase has recently been cloned from *C. albicans* (gene *HSX11* on contig 5-3259), based on its sequence homology to mammalian ceramide glucosyltransferases (Leipelt *et al.*, 2000). Other putative ceramide glucosyltransferase genes/cDNAs from *Neurospora crassa* (hypothetical protein 13E11.330 at the MIPS *Neurospora crassa* database, **http:// www.mips.biochem.mpg.de/proj/neurospora**), and from *Pneumocystis carinii* (GenBank Accession No. AW334716) can be annotated by sequence homology. It must be emphasized that there is no gene present in *S. cerevisiae* that is homologous to these sequences. Regarding the data from the lipid

analyses and the availability of glycosyltransferase sequences, it seems obvious to select organisms that, besides *S. cerevisiae*, should serve as alternative model organisms for the elucidation of glycolipid functions in eukaryotes. We suggest *P. pastoris* to play this role, due to its synthesis of cerebroside and SG, its importance as a protein expression system, its easy handling, and the increasing availability of tools for its genetic manipulation.

In a more particular view, we are looking for fungal pathogens of plants to analyse the role of their membrane glycolipids and free sterols in the host–pathogen interaction. Recent data indicate that cerebrosides, free ergosterol and SG may play a role during pathogenesis (Granado *et al.*, 1995; Sweigard *et al.*, 1998; Koga *et al.*, 1998; Umemura *et al.*, 2000), but the molecular mechanisms of these functions are far from being understood. Therefore, to study the function and mode of action of these compounds in more detail, *U. maydis* and *R. secalis* may be good candidates because they contain both cerebroside and SG.

Acknowledgements

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