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Existence of cerebroside in *Saccharomyces kluyveri* and its related species

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Abstract

Sphingolipids are ubiquitous compounds derived from ceramide that consist of a sphingoid long-chain base with a 2-amino group amide linked to fatty acid and are present in the membranes of many organisms. As a principal sphingolipid, *Saccharomyces cerevisiae* contains a free ceramide and its inositol-phosphorylated derivatives (acidic types) but not a neutral glycosylated ceramide, glucosylceramide (cerebroside), which usually appears in eukaryotic cells. When 31 strains accepted in the genera *Saccharomyces*, *Torulaspora*, *Zygosaccharomyces*, and *Kluyveromyces* were analyzed for sphingolipids, cerebrosides were found in *S. kluyveri*, *Z. cidri*, *Z. fermentati*, *K. lactis*, *K. thermotolerans*, and *K. waltii*. The cerebrosides of *S. kluyveri* and *K. lactis* included 9-methyl 4-*trans*, 8-*trans*-sphingadienine and its putative metabolic intermediates. A unique characteristic of *S. kluyveri* was the presence of a trihydroxy sphingoid base, which rarely occurs in fungal cerebrosides. A polymerase chain reaction with primers targeted to the glucosylceramide synthase gene of other microorganisms amplified the fragments of the expected size from *S. kluyveri* and *K. lactis* and further extended to the adjacent regions. The presumed protein of *S. kluyveri* had 54.4% similarity to that of *K. lactis*, higher than the glucosylceramide synthases from *Candida albicans*, *Pichia pastoris*, and other organisms. From these observations, the divergence of *S. kluyveri* from the lineage of *K. lactis* in their evolution is discussed.

Keywords: Cerebroside; *Saccharomyces kluyveri*; *Kluyveromyces lactis*; Glucosylceramide synthase

1. Introduction

The genus *Saccharomyces* is divided into at least 22 species [1–3], including *S. cerevisiae*, which is used in various industries and as a model for the eukaryotic cell in biochemistry, physiology, genetics, and molecular biology [1]. The phenotypic characteristics of the *Saccharomyces* species have a close relationship with those of the genera *Torulaspora* and *Zygosaccharomyces* [4]. The species of these three genera are phylogenetically intermixed with some species of the genus *Kluyveromyces*, according to the analyses of chromosomal DNA [5]. A common feature is observed in the chemical composition of ubiquinones in an electron transport system. *Saccharomyces*, *Torulaspora*, *Zygosaccharomyces*, and *Kluyveromyces* contain the coenzyme Q6, while Q7–Q10 occur in other genera [4].

Glycosylceramide, or cerebroside, is a typical membrane lipid of plants, fungi, and animals that is synthesized by the transfer of a sugar moiety to a ceramide containing a sphingoid long-chain base and a fatty acid. The reaction is catalyzed by a UDP-glucose ceramide glucosyltransferase (glucosylceramide synthase (GCS), EC 2.4.1.80). Among yeasts, cerebroside is found in *Candida albicans* [6], *Pichia pastoris* [6], *Pichia anomala* [7], and the *Cryptococcus* species [8] but is lacking in *S. cerevisiae*, which instead synthesizes inositol phosphorylceramides as essential components for viable growth [9]. These differences suggested that the presence of cerebroside is useful to delimit and classify ascomycetous yeasts. In the present study, we have extensively surveyed the species of *Saccharomyces* and its related genera and found cerebrosides in *S. kluyveri*, *Z. cidri*, *Z. fermentati*, *K. lactis*, *K. thermotolerans*, and *K. waltii*. Successively, the putative genes encoding GCS

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were isolated from *S. kluyveri* and *K. lactis* and compared to those from other organisms.

2. Materials and methods

2.1. Yeast strains and culture

The following strains accepted in the genera *Saccharomyces*, *Torulaspora*, *Zygosaccharomyces*, and *Kluyveromyces* were used for the analysis of cerebroside: *S. barnettii* IFO 10849, *S. bayanus* IFO 1127, *S. castellii* IFO 1992, *S. cerevisiae* IFO 10217, *S. dairenensis* IFO 0211, *S. exiguus* IFO 1128, *S. kluyveri* IFO 1685, *S. kunashireneis* CBS 7662 (=IFO 10915), *S. martiniae* CBS 6334 (=IFO 0752), *S. paradoxus* IFO 10609, *S. pastorianus* IFO 1167, *S. rosinii* IFO 10008, *S. servazzii* IFO 1838, *S. spencerorum* IFO 10851, *S. transvaalensis* IFO 1625, *S. unisporus* IFO 0316, *T. delbrueckii* SANK 50188 (=IFO 0955), *T. globosa* IFO 1160, *T. pretoriensis* IFO 10218, *Z. baillii* IFO 1098, *Z. bisporus* IFO 1131, *Z. cidri* IFO 1684, *Z. fermentati* IFO 0479, *Z. florentinus* IFO 1088, *Z. mellis* IFO 1615, *Z. microellipsoides* IFO 1740, *Z. mrakii* IFO 1835, *Z. rouxii* IFO 1130, *K. lactis* IFO 1090, *K. thermotolerans* IFO 1985, and *K. waltii* IFO 1666. All of the strains can be obtained from the Institute for Fermentation, Osaka (IFO, http://www.ifo.or.jp).

Yeast cells were grown in 100 ml of a medium containing 1% yeast extract, 2% polypeptone, and 2% glucose in a 500-ml Erlenmeyer flask at 25°C for 1 day with shaking (220 rpm). Harvested cells were lyophilized after being washed twice with distilled water and then stored at −20°C.

2.2. Extraction, purification, and analysis of cerebrosides

The lyophilized cells were treated in chloroform–methanol (2:1, v/v) for 5 min by an ultrasonic disrupter (UD-200, Tomy Seiko, Tokyo, Japan). Total lipids were then extracted each time with five volumes of chloroform–methanol (2:1, v/v) and chloroform–methanol (1:2, v/v) and hydrolyzed in 0.4 M KOH–methanol at 37°C for 2 h. After washing, the organic phase was dried on a rotary evaporator to yield the alkali-stable lipids [10]. Cerebroside was isolated on preparative thin-layer chromatography (TLC) developed by chloroform–methanol (95:12, v/v) from the lipids and degraded with methanolic 1 M HCl and aqueous 1 M HCl [11]. Fatty acid methyl esters and sphingoid bases were obtained in both cases and analyzed by gas–liquid chromatography, according to the methods previously described [12–14].

2.3. Polymerase chain reaction (PCR)

Two degenerate primers, 5′-AAYCCNAARRTNMRNAAYHT-3′ and 5′-ADRAACATYTCNTCNAR-3′, were synthesized according to the sequences in *C. albicans* (gene *HSX11*, on contig 6-2503; information available on the Stanford Genome Technology Center Website at www-sequence.stanford.edu/group/candida/), *Pneumocystis carinii* (AP338415), and *Neurospora crassa* (AL-353820), which is homologous to human GCS (D50840). Based on the fragments amplified from the genomic DNA of *S. kluyveri* and *K. lactis*, the entire regions spanning the open reading frame (ORF) were sequenced by thermal asymmetric interlaced PCR [15] and assigned the DDBJ/EMBL/GenBank accession numbers AB080772 and AB080773, respectively.

2.4. Expression in S. cerevisiae

A PCR fragment corresponding to the GCS gene was inserted into pYES2 (Invitrogen, Carlsbad, CA, USA) as the expression vector. The cells of *S. cerevisiae* strain INVSc1 (*MATα his3-Δ1 leu2 trp1-289 ura3-52*, Invitrogen) transformed with the plasmid were grown in a medium composed of a 0.67% yeast nitrogen base without amino acids (Difco) (Becton Dickinson, Sparks, MD, USA), a 0.192% yeast synthetic drop-out medium supplement without uracil (Sigma, St. Louis, MO, USA), and 2% raffinose as the sole carbon source at 30°C. The medium containing cells in the mid-exponential growth (OD_{600} = 1) was supplemented with 2.0% galactose as the final concentration and incubated for up to 24 h. Cerebroside formed in the cells was extracted, purified, and analyzed as described above.

3. Results and discussion

3.1. Detection of cerebroside

Total lipids extracted from the cells of 31 strains were separated on TLC and visualized by heating after spraying an anthrone reagent to detect cerebroside. In the alkali-stable polar lipids, the selected six strains had the component of an R_f value identical to the authentic cerebroside preparation (Fig. 1). These were *S. kluyveri* in the genus *Saccharomyces*, *Z. cidri*, and *Z. fermentati* in the genus *Zygosaccharomyces*, and *K. lactis*, *K. thermotolerans*, and *K. waltii* in the genus *Kluyveromyces*. In the phylogenetic tree depicted on the basis of the 18S rDNA of the species examined in the present experiments [2], most species of *Saccharomyces*, *Torulaspora*, and *Zygosaccharomyces* exhibited a genetic association within each genus. *S. kluyveri*, *Z. cidri*, and *Z. fermentati*, which were shown to contain cerebrosides, deviated from their accepted genera and related to the *Kluyveromyces* species tested instead.

3.2. Chemical composition of cerebroside

There are two different types of sphingolipids in higher

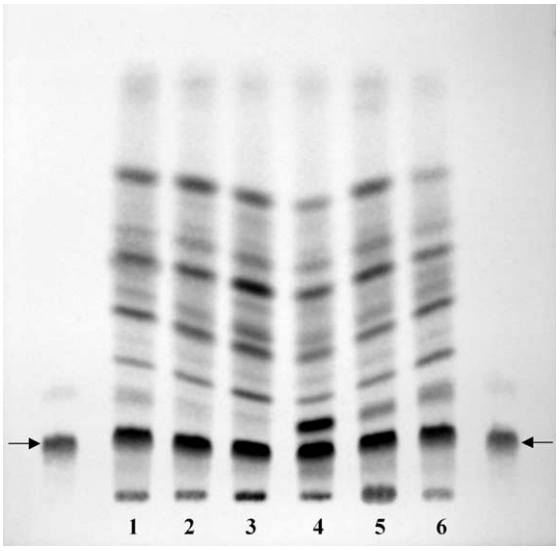

Fig. 1. TLC analysis of alkali-stable lipids from *S. kluyveri* (lane 1), *Z. cidri* (lane 2), *Z. fermentati* (lane 3), *K. lactis* (lane 4), *K. thermotolerans* (lane 5), and *K. waltii* (lane 6). The plate was developed with chloroform–methanol (95:12, v/v) and heated after spraying with an anthrone reagent. The lane at both ends is authentic cerebroside from soybeans [27].

fungi and yeasts [7]. The first type includes ceramide backbones with C24 to C26 fatty acids bound to trihydroxy sphingoid bases such as 4-hydroxysphinganine (t18:0) for the synthesis of the inositol phosphorylceramides; the second one includes ceramide backbones with saturated or 3-*trans*-unsaturated C16 or C18 2-hydroxy fatty acids attached to dihydroxy sphingoid bases such as 9-methyl 4-*trans*, 8-*trans*-sphingadienine (9-Me d18:$2^{4t,8t}$) for cerebroside synthesis [6,8,10,14,16,17]. Analysis of the cerebrosides extracted and purified from the cells of *S. kluyveri* and *K. lactis* showed that a C18 2-hydroxy fatty acid (18h:0) dominated in the fraction of fatty acids (Table 1). Cerebrosides of both strains included 9-Me d18:$2^{4t,8t}$ and its possible metabolic intermediates, 4-unsaturated and 4,8-diunsaturated sphingoid bases (4-*trans*-sphingenine, d18:1^{4t} and 4-*trans*, 8-*trans*-sphingadienine, d18:$2^{4t,8t}$). Interestingly, the trihydroxy sphingoid base, which consists exclusively of a free ceramide and its inositol-phosphorated derivatives in *S. cerevisiae* [18], covered 30% of the component sphingoid bases of the cerebroside in *S. kluyveri*. The proportion of 9-Me d18:$2^{4t,8t}$ was much lower than those in other fungi, suggesting that C9-methylation of the sphingoid base in ceramide is limited to form the usual acceptor for glucosyl transfer. A trihydroxy sphingoid base, which is rarely found in fungal cerebrosides, may be produced from sphinganine (d18:0) and used as a substrate for GCS. These observations mean that the overall activity of enzymes concerning cerebroside synthesis before GCS in *S. kluyveri* is smaller than that in *K. lactis*.

3.3. Amplification of putative GCS

Entire regions spanning sequences homologous to GCS genes were amplified from the genomic DNA of *S. kluyveri* and *K. lactis*. The nucleotide sequences of the obtained fragments revealed a single ORF of 1662 and 1659 bp, sufficiently long to encode a polypeptide of 553 and 552 amino acids, with a deduced molecular mass of 62.7 kDa for *S. kluyveri* and 62.9 kDa for *K. lactis*. The presumed proteins of *S. kluyveri* and *K. lactis* had a conserved structure with 54.4% similarity that is higher than for GCSs from other organisms (Table 2). D1, D2, D3, and the Q/RXXRW motif, which also occur in other yeasts, may be involved in enzyme activity (Fig. 2), as shown by site-directed mutagenesis of mammalian GCS [19].

PCR fragments containing putative GCS genes of the two strains were introduced into *S. cerevisiae* after insertion into the expression vector. Cerebroside appeared by expression of the gene from *S. kluyveri* (Fig. 3) and not of that from *K. lactis* (data not shown). Similar reported findings have shown that GCS from *C. albicans* and not from *P. pastoris* was expressed in *S. cerevisiae* [16].

In mammalian systems, ceramide has been found to

Table 1
Composition of fatty acids and sphingoid bases in cerebroside from *S. kluyveri* and *K. lactis*

Component	Percentage	
	S. kluyveri	*K. lactis*
Fatty acid		
16h:0	3.7	3.0
18h:0	90.0	95.3
20h:0	1.0	0.4
22h:0	0.2	0.1
23h:0	< 0.1	< 0.1
24h:0	0.5	0.2
25h:0	0.1	< 0.1
26h:0	4.6	1.0
Sphingoid base		
d18:1^{4t}	4.3	19.5
d18:$2^{4t,8t}$	26.8	28.3
9-Me d18:$2^{4t,8t}$	38.8	49.0
t18:0	23.3	2.4
t19:0[a]	2.0	0.2
t20:0	4.8	0.6

[a]Including d18:0.

Fatty acid methyl esters were subjected to gas–liquid chromatography as trimethylsilyl ether derivatives and were identified by chromatography with authentic standards prepared from plant cerebrosides [10]. Sphingoid base fractions were subjected to periodate oxidation to obtain fatty aldehydes [11] and determined by capillary gas–liquid chromatography using mushroom sphingoid bases as the authentic standard [13].

Hydroxy fatty acids are abbreviated as *X*h:*Y*, where *X* and *Y* represent the number of carbon atoms and the number of double bonds, respectively. Abbreviations for sphingoid bases are shown as follows: d18:1^{4t}, 4-*trans*-sphingenine; d18:$2^{4t,8t}$, 4-*trans*, 8-*trans*-sphingadienine; 9-Me d18:$2^{4t,8t}$, 9-methyl 4-*trans*, 8-*trans*-sphingadienine; t18:0, 4-hydroxysphinganine; t19:0, 4-hydroxynonadecasphinganine; t20:0, 4-hydroxyeicosasphinganine; d18:0, sphinganine.

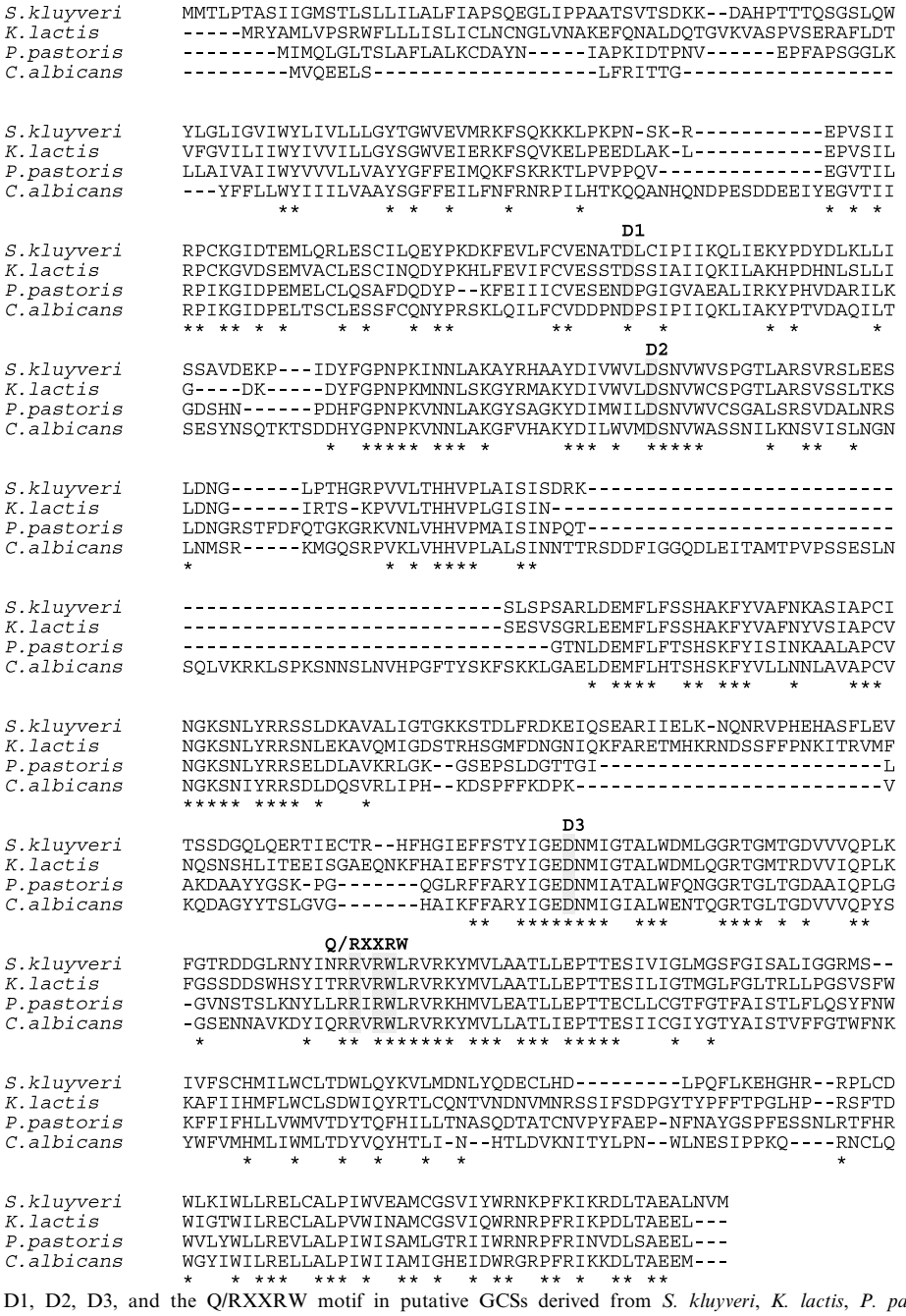

Fig. 2. Comparison of D1, D2, D3, and the Q/RXXRW motif in putative GCSs derived from *S. kluyveri*, *K. lactis*, *P. pastoris*, and *C. albicans*. Asterisks indicate identical amino acids in all sequences. Gray regions indicate D1, D2, D3, and the Q/RXXRW motif.

play important roles in signal transduction, regulation, cell homeostasis, and sphingolipid metabolism [20]. GCS is not only a first step in the synthesis of diverse glycosphingolipids but also, possibly, a key enzyme for the inactivation of excess ceramide and the maintenance of the intracellular level. Cerebroside may be required for cell-to-substratum adhesion but may not be essential for the survival and growth of animal cells. Sphingomyelin can be a substitute in the absence of cerebroside. Fungal cerebrosides are principally monohexosylceramides containing 9-Me d18:$2^{4t,8t}$ as the long-chain base, which is not found in mammalian sphingolipids. The presence of cerebrosides seems to be related to fructification and dimorphism, but the precise functions of cerebrosides and GCS are unclear in fungi and yeasts [17]. Cerebrosides are not indispensable for *P. pastoris* and *C. albicans* because mutants lacking the GCS genes were viable and grew similarly to the parental strains [16]. Most species of *Saccharomyces*, *Torulaspora*, and *Zygosaccharomyces* may require inositol phosphorylceramides for their growth and delete the GCS gene for cerebroside synthesis, as shown in *S. cerevisiae* [7].

The evolution of *S. cerevisiae* and close relatives has been studied in detail since the sequencing of the whole genomic DNA of *S. cerevisiae* [21]. The large duplicated

Table 2
Sequence similarity of putative GCS amplified from *S. kluyveri* and *K. lactis*

Organism	Accession number	Similarity (%)	
		S. kluyveri	*K. lactis*
Saccharomyces kluyveri	AB080772	100	54.4
Kluyveromyces lactis	AB080773	54.4	100
Pichia pastoris	AF364403	43.7	43.9
Candida albicans	–[a]	39.0	38.1
Magnaporthe grisea	AF364402	28.5	28.6
Gossypium arboreum	AF367245	18.1	19.6
Caenorhabditis elegans 1	U53332	22.5	18.9
Caenorhabditis elegans 2	U58735	21.8	20.5
Rattus norvegicus	AF047707	25.2	23.4
Mus musculus	D89866	25.4	23.8
Homo sapiens	D50840	25.2	23.8

[a]Gene *HSX11*, on contig 6-2503; information available on the Stanford Genome Technology Center Website at www-sequence.stanford.edu/group/candida/.
The similarity values were calculated using the Genetyx-Mac computer program (Software Development, Tokyo, Japan).

chromosomal regions suggested that *S. cerevisiae* is a degenerate tetraploid generated by genome duplication approximately 10^8 years ago. *S. cerevisiae* and *S. kluyveri* are assumed to diverge from the lineage leading to *K. lactis* before genome duplication in *S. cerevisiae* [22]. Since an ancestral organism for these yeasts selected inositol phosphorylceramide as an essential constituent for growth, the biosynthetic pathway of cerebroside may have become narrow in *K. lactis* and *S. kluyveri* and finally may have been lost in *S. cerevisiae* during the process of divergence.

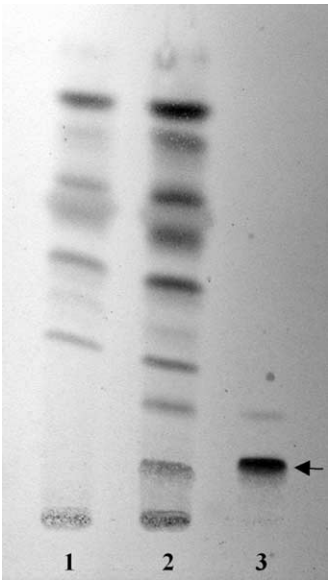

Fig. 3. Production of cerebroside in *S. cerevisiae* by the expression vector carrying the putative GCS of *S. kluyveri*. In this chromatogram, alkali-stable lipid fractions from the host strain INVSc1 (*MATα his3-Δ1 leu2 trp1-289 ura3-52*) with the empty vector pYES2 (lane 1) and the vector carrying the putative GCS gene from *S. kluyveri* (lane 2) and soybean cerebroside (lane 3) were developed by the method of Fig. 1.

4. Concluding remarks

S. kluyveri has been shown to be an outlying species of the genus *Saccharomyces* from its ecological, physiological, and genetic characteristics [23]. However, cells of *S. kluyveri* can be cultured as either stable haploids or diploids [24] and applied for classical genetic analysis, which has provided the essential background for the molecular biology of *S. cerevisiae*. *S. cerevisiae*-based plasmids have been replicated and maintained in auxotrophic mutants of *S. kluyveri* after transformation by the lithium method [25]. Recently, the genome of *S. kluyveri* has been extensively analyzed [26]. From these observations, *S. kluyveri* may be used as a model organism to investigate the metabolism of cerebrosides in mammals.

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