Candida albicans sphingolipid C9-methyltransferase is involved in hyphal elongation

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C9-methylated glucosylceramide is a fungus-specific sphingolipid. This lipid is a major membrane component in the cell and is thought to play important roles in the growth and virulence of several fungal species. To investigate the importance of the methyl branch of the long-chain base in glucosylceramides in pathogenic fungi, we identified and characterized a sphingolipid C9-methyltransferase gene (*MTS1*, C9-MethylTransferase for Sphingolipid 1) in the pathogenic yeast *Candida albicans*. The *mts1* disruptant lacked (*E,E*)-9-methylsphinga-4,8-dienine in its glucosylceramides and contained (*E*)-sphing-4-enine and (*E,E*)-sphinga-4,8-dienine. Reintroducing the *MTS1* gene into the *mts1* disruptant restored the synthesis of (*E,E*)-9-methylsphinga-4,8-dienine in the glucosylceramides. We also created a disruptant of the *HSX11* gene, encoding glucosylceramide synthase, which catalyses the final step of glucosylceramide synthesis, in *C. albicans* and compared this mutant with the *mts1* disruptant. The *C. albicans* *mts1* and *hsx11* disruptants both had a decreased hyphal growth rate compared to the wild-type strain. The *hsx11* disruptant showed increased susceptibility to SDS and fluconazole, similar to a previously reported *sld1* disruptant that contained only (*E*)-sphing-4-enine in its glucosylceramides, suggesting that these strains have defects in their cell membrane structures. In contrast, the *mts1* disruptant grew similarly to wild-type in medium containing SDS or fluconazole. These results suggest that the C9-methyl group of a long-chain base in glucosylceramides plays an important role in the hyphal elongation of *C. albicans* independent of lipid membrane disruption.

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INTRODUCTION

Glycosphingolipids, which consist of a ceramide with various sugars, are ubiquitous membrane lipids in eukaryotic organisms. They are classified by their characteristic polar headgroups. One type of glycosphingolipid, glycosylinositolphosphorylceramide (GIPC), is linked with inositol via a phosphodiester bond and is present only in fungi and plants. On the other hand, glucosylceramides (GlyCers), which are directly linked to a glucosyl or galactosyl residue, can form the base for further glycosylations. Glucosylceramides (GluCers), which contain a directly linked glucose residue, are unique glycosphingolipids that are found in animals, plants and fungi, whereas galactosylceramides, which are linked with galactose, are found only in animals and fungi (reviewed by Warnecke & Heinz, 2003).

In animals, GlyCers are not only essential for growth but are also thought to play a crucial role in cell differentiation and cell–cell interactions (reviewed by Merrill *et al.*, 1997; Ternes *et al.*, 2002). Plant GlyCers and their components such as ceramide and long-chain bases (LCBs) have been

suggested to function in signal transduction and host–pathogen interactions (reviewed by Sperling & Heinz, 2003; Thevissen *et al.*, 2004; Ramamoorthy *et al.*, 2007). Although two yeast species, *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, have been used as models to study the metabolism and function of sphingolipids in higher eukaryotic cells, these species are not capable of synthesizing GlyCers. Therefore, our understanding of the molecular mechanisms that contribute to the function of GlyCer in fungi is still limited.

Recently, a number of fungal pathogens have been reported to contain GluCers that play functional roles in growth and differentiation. The addition of antibodies against a purified fungal GluCer inhibited cell budding and growth in *Cryptococcus neoformans*, and antibodies against a purified *Pseudallescheria boydii* GluCer inhibited mycelium formation in *P. boydii* and germ-tube formation in *Candida albicans* (Rodrigues *et al.*, 2000; Pinto *et al.*, 2002). Inhibiting glucosylceramide synthase, which catalyses the final step in GluCer synthesis, influences the growth and differentiation of *Aspergillus nidulans* and *Aspergillus fumigatus* (Levery *et al.*, 2002). Moreover, additional evidence has emerged that GluCers play important roles in

Abbreviations: CFW, Calcofluor white; GluCer, glucosylceramide; GlyCer, glycosylceramide; LCB, long-chain base.

fungus pathogenicity. A $\Delta gcs1$ strain of *Cr. neoformans*, which lacks GluCer, is avirulent, and the virulence of a *Fusarium graminearum* $\Delta gcs1$ ($\Delta Fggcs1$) strain in wheat is highly compromised (Ramamoorthy *et al.*, 2007). However, it is still unknown what part(s) of the GluCer molecular structures cause(s) these biological phenomena in fungi.

GluCer biosynthesis starts with the condensation of palmitoyl-CoA and serine by serine palmitoyltransferase to form 3-ketosphinganine (reviewed by Warnecke & Heinz, 2003). 3-Ketosphinganine reductase catalyses the conversion of 3-ketosphinganine to sphinganine. Ceramide synthase catalyses the condensation of sphinganine to saturated acyl-CoA for the synthesis of ceramides. Next, sphingolipid Δ^4 -desaturase catalyses the Δ^4 -desaturation of LCB in ceramides (Ternes *et al.*, 2002). In many plants and fungi, sphingolipid Δ^8 -desaturase catalyses the Δ^8 -desaturation of LCB in ceramides, while sphingolipid C9-methyltransferase introduces a methyl group at C9 of LCB in ceramides (Sperling *et al.*, 1998; Takakuwa *et al.*, 2002; Ternes *et al.*, 2006). The desaturation of fatty acyl groups in ceramides has also been detected in some organisms (reviewed by Warnecke & Heinz, 2003). Finally, these ceramides react with UDP-glucose, catalysed by glucosylceramide synthase, resulting in GluCer (Leipelt *et al.*, 2001) (Fig. 1). Since it has been suggested that *Sac. cerevisiae* and *Sch. pombe* lack most of the genes that encode the enzymes for GluCer synthesis, there is currently little knowledge about the fungal genes that are involved in GluCer synthesis. It was recently reported that a *Cr. neoformans* strain that lacks the GluCer synthase gene and cannot synthesize GluCer was incapable of growing in neutral and alkaline solutions *in vitro*, which reduces its pathogenicity in its host (Rittershaus *et al.*, 2006). In contrast, disruption of the GluCer synthase gene (*HSX11*) in *C. albicans* does not affect the growth of its unicellular or filamentous forms (Leipelt *et al.*, 2001).

Previously, we characterized the sphingolipid Δ^8 -desaturase gene (*SLD1*) from *C. albicans* and constructed a disruptant of this gene. Compared to wild-type, the *C. albicans* *sld1* disruptant had a decreased hyphal growth rate and was highly sensitive to SDS and fluconazole (Oura & Kajiwar, 2008). Therefore, it was suggested that (*E,E*)-sphinga-4,8-dienine and/or (*E,E*)-9-methylsphinga-4,8-dienine in ceramide and GluCer (Fig. 1) play a role in the morphogenesis of this fungus. More recently, two genes encoding a putative sphingolipid C9-methyltransferase in *F. graminearum* (named *FgMT1* and *FgMT2*) were characterized (Ramamoorthy *et al.*, 2009). Although the $\Delta Fgmt1$ mutant was able to produce GluCs, including (*E,E*)-9-methylsphinga-4,8-dienine (C9-methylated GluCer), similarly to the wild-type, the mutant had a reduced amount of this GluCer compared to the wild-type. The $\Delta Fgmt2$ mutant also exhibited a severe growth defect, produced abnormal conidia, and showed reduced disease symptoms in wheat and much delayed disease symptoms in *Arabidopsis thaliana*. These results suggested that C9-methylated GluCs play a functional role in the growth, differenti-

Fig. 1. Biosynthetic pathway of glucosylceramide in *C. albicans*. Gene symbols are according to the *C. albicans* nomenclature in the Candida Genome Database and are shown in *italics*.

ation and virulence of this fungus. Therefore, the structure of the LCB portion of GluCer was suggested to be responsible for the determination of biological phenomena in pathogenic fungi.

In this study, we focused on two other genes involved in GluCer biosynthesis, the *MTS1* and *HSX11* genes in *C. albicans*. We constructed a null mutant of *MTS1* to determine the function of this gene in *C. albicans*, and to investigate the effects of *MTS1* disruption on the morphogenesis of this fungus. We also constructed a *hsx11* null mutant of *C. albicans* and compared the phenotype of this mutant with that of the *mts1* null mutant. Both the *mts1* and *hsx11* mutants had an obvious delay in filamentous growth compared to the isogenic wild-type strains. The mutants were also found to differ in their susceptibility to some antifungal drugs and environmental stressors.

METHODS

Media, growth conditions, and basic techniques. The *C. albicans* strains used and constructed in this study are summarized in Table 1.

Strains were typically grown at 30 °C in YPD medium [1 % Bacto yeast extract, 2 % Bacto peptone, 2 % dextrose (glucose) (pH 5.6)] or complete minimal (CM) medium (Ausubel *et al.*, 1992) without uracil and/or arginine. The rate of growth was measured as OD₆₀₀ using an Ultrospec 3000 spectrophotometer (Pharmacia Biotech). The yeast-to-hypha transition was induced at 37 °C in both solid and liquid media. For the solid transition medium, we used 10 % fetal bovine serum medium (Hanaoka *et al.*, 2005). For the liquid transition medium, we used 10 % fetal bovine serum in YPD at pH 7.2. Filamentation in the liquid medium was induced by inoculating 2×10^6 – 5×10^6 cells ml⁻¹ and incubating at 37 °C. For spot tests, cells were grown overnight at 30 °C in YPD medium and then tenfold serial dilutions were spotted onto YPD agar plates and grown for 1–2 days at 30 °C. The antifungals used in this study were amphotericin B (Wako Pure Chemical Industries), fluconazole (LKT Laboratories), micafungin (Astellas Pharma) and terbinafine (LKT Laboratories).

Escherichia coli DH5 α and the cloning vector pBluescript II SK+ (Stratagene) were used to manipulate the DNA. General recombinant DNA procedures were performed as described by Sambrook *et al.* (1989). *C. albicans* was transformed following the method described by Umeyama *et al.* (2005).

Plasmid construction. The primers used in this study are listed in Table 2. The *MTS1* DNA fragment was PCR amplified using primers mts1-clo5 and mts1-clo3, with TUA4 chromosomal DNA as a template. The PCR products were cloned into the *EcoRV* site of pBluescript II SK+ to generate pBS-MTS1. The nucleotide sequence of the *MTS1* DNA was confirmed by DNA sequencing. The 1.5 kb *BamHI*–*XhoI* DNA fragment from pBS-MTS1 was inserted into the *BamHI*–*XhoI* sites of pFLAG-Act1 to generate pFLAG-Act1-MTS1. The *HSX11* DNA fragment was PCR amplified using primers hsx11-clo5 and hsx11-clo3, with TUA4 chromosomal DNA as a template. These products were cloned into the *EcoRV* site of pBluescript II SK+ to generate pBS-HSX11. The nucleotide sequence of this fragment was confirmed. The 1.6 kb *BamHI*–*XhoI* DNA fragment from pBS-

HSX11 was inserted into the *BamHI*–*XhoI* sites of pFLAG-Act1 (Umeyama *et al.*, 2002) to generate pFLAG-Act1-HSX11.

Disruption of *MTS1* in *C. albicans* and construction of a *mts1* mutant expressing *MTS1*. To disrupt *MTS1* in a diploid *C. albicans* strain, two markers were used. The 0.5 kb DNA fragment corresponding to the 3' end of *MTS1* was PCR amplified using primers mts1-mut-Not5 and mts1-mut-Sac3, digested with *NotI* and *SacI*, and then cloned into the *NotI*–*SacI* sites of pBS-hph200-URA3 and pUC19-ARG4 to generate pBS-hph200-URA3-NS and pUC-ARG4-NS, respectively. The 0.5 kb DNA fragment corresponding to the 5' end of *MTS1* was PCR amplified using primers mts1-mut-Kpn5 and mts1-mut-Xho3, digested with *KpnI* and *XhoI*, and then cloned into the *KpnI*–*XhoI* sites of pBS-hph200-URA3-NS to generate pBS-hph200-URA3-disMTS1. The 0.5 kb DNA fragment corresponding to the 5' end of *MTS1* was PCR amplified using primers mts1-mut-Sph5 and mts1-mut-Kpn3, digested with *SphI* and *KpnI*, and then cloned into the *SphI*–*KpnI* sites of pUC-ARG4-NS to generate pUC-ARG4-disMTS1.

The 3.1 kb *KpnI*–*SacI* fragment of pBS-hph200-URA3-disMTS1 was used to transform the *C. albicans* Arg⁻ Ura⁻ strain TUA4 to generate a Ura⁺ transformant (MTS101). Then the 3.1 kb *SphI*–*SacI* fragment of pUC-ARG4-disMTS1 was used to transform the MTS101 strain (Arg⁻) to generate MTS102. The resulting Ura⁺ Arg⁺ transformant MTS102 was plated on medium containing 5-fluoroorotic acid to isolate the Ura⁻ segregant (MTS103). To confirm gene disruption, genomic DNA was isolated from each strain, digested with *EcoRV*, run on a 1 % agarose gel, and then transferred onto a Hybond-N+ nylon membrane (Amersham Biosciences). Southern hybridization was performed using a ³²P-labelled probe in a solution containing 5 × SSC, 6 × Denhart's solution and 0.1 mg herring sperm DNA ml⁻¹ at 65 °C according to the methods of Sambrook *et al.* (1989). The *MTS1* gene was reintroduced into the *Ca-RP10* locus of the null mutant by using *AatI*-digested pFLAG-Act1-MTS1 to transform strain MTS103,

Table 1. Yeast strains generated and tested in this study

Strain	Genotype	Reference
TUA4	<i>ura3Δ::imm434/ura3Δ::imm434 arg4Δ::hisG200/arg4Δ::hisG200</i>	NIID*
TUA6	<i>ura3Δ::imm434/ura3Δ::imm434 arg4Δ::hisG200/arg4Δ::hisG200 RP10::p3HA-ACT1</i>	NIID*
MTS101	<i>ura3Δ::imm434/ura3Δ::imm434 arg4Δ::hisG200/arg4Δ::hisG200 mts1Δ::hph200-URA3-hph200/MTS1</i>	This work
MTS102	<i>ura3Δ::imm434/ura3Δ::imm434 arg4Δ::hisG200/arg4Δ::hisG200 mts1Δ::hph200-URA3-hph200/mts1Δ::ARG4</i>	This work
MTS103	<i>ura3Δ::imm434/ura3Δ::imm434 arg4Δ::hisG200/arg4Δ::hisG200 mts1Δ::hph200/mts1Δ::ARG4</i>	This work
MTS104	<i>ura3Δ::imm434/ura3Δ::imm434 arg4Δ::hisG200/arg4Δ::hisG200 mts1Δ::hph200/mts1Δ::ARG4 RP10::pFLAG-ACT1</i>	This work
MTS105	<i>ura3Δ::imm434/ura3Δ::imm434 arg4Δ::hisG200/arg4Δ::hisG200 mts1Δ::hph200/mts1Δ::ARG4 RP10::pFLAG-ACT1-MTS1</i>	This work
HSX101	<i>ura3Δ::imm434/ura3Δ::imm434 arg4Δ::hisG200/arg4Δ::hisG200 hsx11Δ::hph200-URA3-hph200/HSX11</i>	This work
HSX102	<i>ura3Δ::imm434/ura3Δ::imm434 arg4Δ::hisG200/arg4Δ::hisG200 hsx11Δ::hph200-URA3-hph200/hsx11Δ::ARG4</i>	This work
HSX103	<i>ura3Δ::imm434/ura3Δ::imm434 arg4Δ::hisG200/arg4Δ::hisG200 hsx11Δ::hph200/hsx11Δ::ARG4</i>	This work
HSX104	<i>ura3Δ::imm434/ura3Δ::imm434 arg4Δ::hisG200/arg4Δ::hisG200 hsx11Δ::hph200/hsx11Δ::ARG4 RP10::pFLAG-ACT1</i>	This work
HSX105	<i>ura3Δ::imm434/ura3Δ::imm434 arg4Δ::hisG200/arg4Δ::hisG200 hsx11Δ::hph200/hsx11Δ::ARG4 RP10::pFLAG-ACT1-HSX11</i>	This work

*NIID, National Institute of Infectious Diseases, Japan.

Table 2. Oligonucleotide primers used in this study

Restriction enzyme sites are underlined.

Oligonucleotide	Sequence (5'–3')
mts1-clo5	AAGGATCCAGGATGTTAGACGACGTTGCATTCATCAAG –3'
mts1-clo3	AACTCGAGTTAATCGTAAAAGTTGGTAAACTCCTTAGC –3'
mts1-mut-Sph5	AAGCATGCCCAACACCAATCAATTGACTTTCG –3'
mts1-mut-Kpn3	AAGGTACCGAAATGTTAAACTCGACAAAAGACGAAAAC –3'
mts1-mut-Not5	AAGCGGCCGCACTGGTTGAGCTTTTATGGTGTATTG –3'
mts1-mut-Sac3	AAGAGCTCAGAGTCAGAATGTCGTCATAGTG –3'
mts1-mut-Kpn5	AAGGTACCCAACACCAATCAATTGACTTTC –3'
mts1-mut-Xho3	AACTCGAGAAATGTTAAACTCGACAAAAGACGAAAAC –3'
hsx11-clo5	AAGGATCCAGGATGGTTCAAGAAGAATTATCCTTATTAGG –3'
hsx11-clo3	AACTCGAGTCACATTTCTTCAGCAGTTAAATCTTTCTTAATC –3'
hsx11-mut-Bam5	AAGGATCCAGTAATGGCACCTTATTGTAATGTATG –3'
hsx11-mut-Kpn3	AAAGGTACCGAAGAACAAAAGATTACGATATTTACAGTG –3'
hsx11-mut-Not5	AAAGCGCCGCTACGCCAATTGTTATAGATAGATAGACAGG –3'
hsx11-mut-Sac3	AAGAGCTCGCAATATCAGGATCATAATGTTTATACCATG –3'
hsx11-mut-Kpn5	AAGGTACCAAGTAATGGCACCTTATTGTAATGTATG –3'
hsx11-mut-Xho3	AACTCGAGAAGAACAAAAGATTACGATATTTACAGTG –3'

generating MTS105. The *AatI*-digested empty vector pFLAG-Act1 was integrated into strain MTS103 to generate MTS104 as a control.

Disruption of *HSX11* in *C. albicans* and construction of a *hsx11* strain expressing *HSX11*. To disrupt *HSX11* in *C. albicans*, the *ARG4* and *URA3* genes were used as markers. The 0.6 kb DNA fragment corresponding to the 3' end of *HSX11* was PCR amplified using primers *hsx11-mut-Not5* and *hsx11-mut-Sac3*, digested with *NotI* and *SacI*, and then cloned into the *NotI*–*SacI* sites of pBS-hph200-*URA3* (Oura & Kajiura, 2008) and pUC19-*ARG4* (Hanaoka *et al.*, 2005) to generate pBS-hph200-*URA3*-NS and pUC-*ARG4*-NS, respectively. The 0.6 kb DNA fragment corresponding to the 5' end of *HSX11* was PCR amplified using primers *hsx11-mut-Kpn5* and *hsx11-mut-Xho3*, digested with *KpnI* and *XhoI*, and then cloned into the *KpnI*–*XhoI* sites of pBS-hph200-*URA3*-NS to generate pBS-hph200-*URA3*-dis*HSX11*. The 0.6 kb DNA fragment corresponding to the 5' end of *HSX11* was PCR amplified using primers *hsx11-mut-Bam5* and *hsx11-mut-Kpn3*, digested with *BamHI* and *KpnI*, and then cloned into the *BamHI*–*KpnI* sites of pUC-*ARG4*-NS to generate pUC-*ARG4*-dis*HSX11*.

The 3.2 kb *KpnI*–*SacI* fragment of pBS-hph200-*URA3*-dis*HSX11* was used to transform the *C. albicans* Arg[–] Ura[–] strain TUA4 to generate the Ura⁺ transformant HSX101. Then the 3.2 kb *BamHI*–*SacI* fragment of pUC-*ARG4*-dis*HSX11* was used to transform HSX101 (Arg[–]) to generate HSX102. The resulting Ura⁺ Arg⁺ transformant, HSX102, was plated on a medium containing 5-fluoroorotic acid to isolate the Ura[–] segregant (HSX103). To confirm gene disruption, genomic DNA was isolated from each strain, digested with *SpeI*, run on a 1% agarose gel, and then transferred onto a Hybond-N+ nylon membrane (Amersham Biosciences). Southern hybridization was performed using a ³²P-labelled probe in a solution containing 5 × SSC, 6 × Denhart's solution, and 0.1 mg herring sperm DNA ml^{–1} at 65 °C according to the methods of Sambrook *et al.* (1989). The *HSX11* gene was reintroduced into the *RP10* locus of the null mutant by using *AatI*-digested pFLAG-Act1-*HSX11* to transform strain HSX103, generating HSX105. The *AatI*-digested empty pFLAG-Act1 vector was integrated into strain HSX103 to generate HSX104.

Determination of glucosylceramides. GluCers were extracted essentially as described by Saito *et al.* (2005) with the following modifications. The lyophilized cells (50 mg) were treated with

chloroform/methanol (1:1, v/v) and 2.0 ml 0.8 M KOH/methanol for 5 min with a subsonic homogenizer (HOM-100, Asahi Grass) and then incubated at 42 °C for 30 min. Organic salt was collected from the homogenate after mixing with 5.0 ml chloroform and 2.25 ml water, dried using a rotary evaporator, and dissolved in 100 µl chloroform/methanol (1:1, v/v). Ten microlitres of the extract was spotted onto a TLC plate (silica gel 60, Merck) and the chromatogram was developed using chloroform/methanol/water (65:16:2, by vol.) as mobile phase. After development, the plates were sprayed with orcinol/sulfuric acid reagent [0.1 g orcinol in 45 ml of a sulfuric acid/water/ethanol solution (5:13:27, by vol.)] and heated on a hotplate to detect the GluCers. GluCers prepared from the mushroom *Grifola frondosa* were used as standards.

Sphingoid base analysis. Analysis of the sphingoid base composition was performed essentially as described by Tanji *et al.* (2004) with the following modifications. The lyophilized cells were treated in chloroform/methanol (2:1, v/v) for 10 min with a subsonic homogenizer (HOM-100, Asahi Grass). Then the total lipids were extracted with 4 vols each of chloroform/methanol (2:1, v/v) and chloroform/methanol (1:2, v/v) and hydrolysed 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. GluCers were isolated on preparative TLC plates that were developed in chloroform/methanol (95:12, v/v) to separate the GluCers from other lipids. The GluCers obtained were degraded with 10% Ba(OH)₂/dioxane (1:1, v/v) and the released sphingoid bases were extracted with diethyl ether and reacted with 0.2 M sodium periodate for 2 h to obtain the fatty aldehydes. The fatty aldehydes were extracted in dichloromethane and analysed by GLC (GC-18A, Shimadzu) on a 0.25 mm × 50 m CP-Sil 88 capillary column (Varian). The initial column temperature of 150 °C was maintained for 2 min, increased by 2 °C min^{–1} to 190 °C, and then maintained for 6 min.

RESULTS

Construction of *mts1* and *hsx11* mutants

The *C. albicans* ORF (orf19.4831) encoding a homologue of the sphingolipid C9-methyltransferase of *Pichia pastoris* was

previously identified in the *C. albicans* genome (Ternes *et al.*, 2006). However, this ORF has not been further characterized. To determine whether this ORF (named *MTS1*) encodes the *C. albicans* C9-methyltransferase, we constructed *mts1* mutants as described in Methods. For the first allele, *MTS1* was replaced with an *hph200-URA3-hph200* cassette (MTS101) and the remaining allele was replaced with *ARG4*. The resulting strain was called MTS102. The *URA3* gene was excised from MTS102 to generate the Ura-auxotrophic derivative MTS103. Southern blot analysis was performed to confirm that the mutant obtained had a disrupted *MTS1* locus (data not shown). Next, to construct the *MTS1*-recovered strain, an *MTS* expression plasmid (pFLAG-Act1-*MTS1*) and its vector (pFLAG-ACT1), which contain the *URA3* gene as a selectable marker, were introduced into the *RP10* locus (Murad *et al.*, 2000) in the genome of the *mts1* mutant strain MTS103, yielding strains MTS105 and MTS104, respectively.

It was reported that disrupting the *HSX11* gene in *C. albicans* does not affect the growth of either the yeast or filamentous forms (Leipelt *et al.*, 2001). However, some researchers have suggested that GluCers play important roles in the growth, differentiation and pathogenicity of other fungi. Therefore, to investigate the function of *HSX11* in detail, we constructed *hsx11* mutants as described in Methods. For the first allele, *HSX11* was replaced with an *hph200-URA3-hph200* cassette (HSX101) and the remaining allele was replaced with *ARG4*. The resulting strain was termed HSX102. The *URA3* gene was excised from HSX102, generating the Ura-auxotrophic derivative HSX103. Southern blot analysis was performed to confirm that the mutant obtained had a disrupted *HSX11* locus (data not shown). Next, to construct the *HSX11*-recovered strain, a *HSX11* expression plasmid (pFLAG-Act1-*HSX11*) and the vector (pFLAG-ACT1), which contain the *URA3* gene as a selectable marker, were introduced into the *RP10* locus (Murad *et al.*, 2000) on the

chromosome of the *hsx11* mutant strain HSX103, yielding strains HSX105 and HSX104, respectively.

Sphingoid base analysis of the *mts1* mutants

To investigate the profile of sphingoid base residues in GluCers of the *C. albicans* transformants, GluCers were isolated from MTS104 (*mts1* mutant), MTS105 (*MTS1*-restored strain) and their corresponding wild-type strain, TUA6. The amounts of GluCers were highly similar among these strains (data not shown). Their sphingoid base compositions were also compared (Fig. 2). The GluCers in the TUA6 strain contained three sphingoid bases: (*E*)-sphing-4-enine, (*E,E*)-sphinga-4,8-dienine and (*E,E*)-9-methylsphinga-4,8-dienine. In contrast, the GluCers in the MTS104 strain lacked (*E,E*)-9-methylsphinga-4,8-dienine and contained (*E*)-sphing-4-enine and (*E,E*)-sphinga-4,8-dienine. The MTS105 strain recovered the ability to produce (*E,E*)-9-methylsphinga-4,8-dienine, although the proportion of this sphingoid base in the GluCers was lower than that of TUA6 (wild-type), making (*E,E*)-sphinga-4,8-dienine the most abundant molecule in the GluCers of MTS105. These results indicate that the *MTS1* protein (*MTS1p*) is the only enzyme that introduces a methyl group at the C9 position of the LCB of ceramides in *C. albicans*.

GluCer analysis of *hsx11* mutants

To confirm the absence of GluCers in the *hsx11* disruptant, alkali-stable lipids were isolated from HSX104 (*hsx11* mutant), HSX105 (*HSX11*-restored strain) and the wild-type strain, TUA6. The amounts of GluCers were compared in these strains by TLC (Fig. 3). The HSX105 and TUA6 strains produced similar amounts of GluCers. In contrast, the HSX104 strain completely lacked GluCers. These results show that the *hsx11* mutants are incapable of synthesizing GluCers and that *HSX11p* is the

Fig. 2. GLC analysis of fatty aldehydes derived from sphingoid bases. Cells of strains TUA6 (*MTS1/MTS1*) (a), MTS104 (*mts1/mts1*) (b) and MTS105 (*mts1/mts1 MTS1*) (c) were grown overnight at 30 °C. Sphingoid base fractions were subjected to periodate oxidation to obtain fatty aldehydes and then identified by comparing their retention times to known standards prepared from *Grifola frondosa* sphingoid bases. The abbreviations for sphingoid bases are as follows: d18:1 Δ^4 , 4-*trans*-sphingenine; d18:2 $\Delta^{4,8}$, 4-*trans*,8-*trans*-sphingadienine; d18:2 $\Delta^{4,8-9m}$, 9-methyl,4-*trans*,8-*trans*-sphingadienine.

Fig. 3. TLC analysis of alkali-stable lipids. The alkali-stable lipids extracted from lyophilized cells of TUA6 (*HSX11/HSX11*) (lane 2), HSX104 (*hsx11/hsx11*) (lane 3) and HSX105 (*hsx11/hsx11+HSX11*) (lane 4) were dissolved in chloroform/methanol (1:1, v/v), spotted on a silica gel 60 TLC plate, and the chromatogram was developed using chloroform/methanol/water (65:16:2, by vol.) as mobile phase. After development, the plates were sprayed with orcinol/sulfuric acid reagent and heated on a hotplate to detect the GluCers. GluCers prepared from *Grifola frondosa* were used as standards (lane 1).

only enzyme involved in synthesis of GluCers in *C. albicans*, findings consistent with a previous report (Leipelt *et al.*, 2001).

Growth and morphogenesis of the *C. albicans* gene disruptants

The (*E,E*)-9-methylsphinga-4,8-dienine in GluCers is thought to be essential to maintain sufficient membrane fluidity in *Kluyveromyces lactis* in a low-temperature environment (Tanji *et al.*, 2004). Therefore, to investigate

the roles of fungal-specific GluCers during growth at low temperatures, we examined the growth rate of the *mts1* disruptant at 30 °C and 10 °C in liquid YPD medium (Fig. 4). There were no significant differences in growth rate between the *mts1* disruptant and the wild-type strain at either temperature. This result indicates that the C9-methylation of GluCer is not necessary for the vegetative growth of *C. albicans* at low temperatures.

To investigate the effects of the *mts1* and *hsx11* disruptions on the yeast-to-hypha transition in *C. albicans*, the gene-disrupted strains (MTS104 and HSX104) were incubated in either solid or liquid medium containing 10 % serum. On 10 % serum agar medium at 37 °C, the hyphal growth of both MTS104 and HSX104 was clearly slower than that of wild-type TUA6 (Fig. 5). These phenotypes were restored to wild-type by introducing *MTS1* and *HSX11* into the *mts1* and *hsx11* mutants, respectively (strains MTS105 and HSX105). On the other hand, there were no significant differences among these *C. albicans* strains in hyphal formation in liquid YPD medium (pH 7.2) containing 10 % serum (data not shown). These results suggested that not only GluCer synthesis but also the C9-methylation of the LCB play roles in hyphal formation of *C. albicans* on solid medium.

Susceptibilities of the *mts1* and *hsx11* disruptants to stressors and antifungals

To investigate the additional effects of the absence of GluCer and C9-methylated GluCer in *C. albicans*, the *mts1* and *hsx11* disruptants were examined for their susceptibilities to a wide range of stress conditions using dilution drop assays (Fig. 6). The stressors high temperature (42 °C), salt (1 M NaCl), 6 % ethanol and 50 µg ml⁻¹ Calcofluor white (CFW) did not affect MTS104 and HSX104 more than TUA6, MTS105 and HSX105. However, MTS104 was more susceptible than TUA6 and MTS105 to the β-1,3-glucan synthase inhibitor micafungin, while the other antifungals examined did not induce different effects on these strains. On the other hand,

Fig. 4. Growth of *C. albicans* strains in liquid YPD medium. Cells of strains TUA6 (wild-type, ●), MTS104 (*mts1/mts1*, ▲) and MTS105 (*mts1/mts1+MTS1*, ■) were pre-cultured overnight at 30 °C and then inoculated into 50 ml medium and grown to OD₆₀₀ 0.2 on a reciprocal shaker (130 r.p.m.) at 30 °C (a) and 10 °C (b). Vertical bars represent the standard errors of three independent experiments.

Fig. 5. Colony morphology of *C. albicans* strains grown on solid agar medium. Cells of strains TUA6 (wild-type), MTS104 (*mts1/mts1*), MTS105 (*mts1/mts1* + *MTS1*), HSX104 (*hsx11/hsx11*) and HSX105 (*hsx11/hsx11* + *HSX11*) were grown overnight at 30 °C. Then 10^5 cells were spotted onto agar medium containing 10 % serum and grown for 7 days at 37 °C. The results of a representative experiment are shown; four additional independent experiments showed the same results.

HSX104 was more sensitive than TUA6 to 0.05 % SDS, while MTS104 showed no sensitivity to 0.05 % SDS. Moreover, HSX104 was more susceptible than TUA6 and HSX105 to the lanosterol 14 α -demethylase inhibitor

fluconazole, while the other tested antifungals did not induce different effects among these three strains. Thus MTS104 had a different phenotype from HSX104 when treated with SDS and certain antifungals.

Fig. 6. Susceptibility of *C. albicans* strains to stressors and antifungals. Cells of strains TUA6 (wild-type), MTS104 (*mts1/mts1*), MTS105 (*mts1/mts1* + *MTS1*), HSX104 (*hsx11/hsx11*) and HSX105 (*hsx11/hsx11* + *HSX11*) were grown overnight at 30 °C; then tenfold serial dilutions of the cultures were spotted onto the indicated YPD agar plates and grown for 1 to 2 days at 30 °C. AMB, amphotericin B; CFW, Calcofluor white; FCZ, fluconazole; MFG, micafungin; TB, terbinafine.

DISCUSSION

In this study, we characterized the sphingolipid C9-methyltransferase gene (*MTS1*) from *C. albicans* and constructed a disruptant of this gene by replacing the two *MTS1* alleles with two different marker genes. To our knowledge this is the first report of the physiological characterization of a *C. albicans* mutant with a disrupted sphingolipid C9-methyltransferase gene. We also constructed a disruptant of the *HSX11* gene, encoding GluCer synthase, and compared its phenotype to that of the wild-type strain.

Based on the results of the LCB analysis of its GluCers, the *mts1* mutant contained only the sphingoid bases (*E*)-sphing-4-enine and (*E,E*)-sphinga-4,8-dienine, but not (*E,E*)-9-methylsphinga-4,8-dienine. The *MTS1*-restored strain contained all three sphingoid bases (*E*)-sphing-4-enine, (*E,E*)-sphinga-4,8-dienine and (*E,E*)-9-methylsphinga-4,8-dienine in its GluCers. These results showed that *MTS1p* is a unique enzyme that introduces a methyl group at the C9 position of the LCB in ceramides. It was previously shown that a *P. pastoris* strain lacking the sphingolipid C9-methyltransferase gene produced no (*E,E*)-9-methylsphinga-4,8-dienine (Ternes *et al.*, 2006), similar to the *mts1* mutant of *C. albicans*. Moreover the databases for *Cr. neoformans*, *Debaryomyces hansenii*, *Kluyveromyces lactis*, *Saccharomyces kluyveri* and *Yarrowia lipolytica* indicate that these yeast species have only one sphingolipid C9-methyltransferase gene homologue in their genomes. Therefore, most other yeast species producing GluCers may have only one sphingolipid C9-methyltransferase gene. On the other hand, the genomes of both the filamentous ascomycetes *A. nidulans* and *F. graminearum* contain two sequences that are homologous to the sphingolipid C9-methyltransferase gene (Ternes *et al.*, 2006). Moreover, it was shown that *F. graminearum* has two genes with sphingolipid C9-methyltransferase activity (Ramamoorthy *et al.*, 2009).

The amounts of GluCer in the *mts1* mutant and wild-type *C. albicans* strains are highly similar. This indicates that C9-methylation by *MTS1p* has little influence on subsequent GluCer synthesis. In contrast, disrupting the *HSX11* gene in *C. albicans* leads to the loss of GluCers, indicating that *HSX11p* is the only enzyme that combines glucose with ceramide for GluCer synthesis. This result is consistent with the first report on *hsx11* mutants of *C. albicans* (Leipelt *et al.*, 2001).

The wild-type and *mts1* disruptant strains of *C. albicans* had the same growth rate at 30 °C in liquid YPD medium. A *P. pastoris* strain lacking the sphingolipid C9-methyltransferase gene did not have impaired growth compared to the wild-type strain, whereas the *F. graminearum* Δ *Fgmt2* mutant exhibited severe growth defects (Ternes *et al.*, 2006; Ramamoorthy *et al.*, 2009). It therefore appears that sphingolipid C9-methyltransferase is not necessary for the vegetative growth of yeast but is necessary for the growth of filamentous fungi. Previously, (*E,E*)-9-methyl-

sphinga-4,8-dienine in the GluCers of *K. lactis* was assumed to be essential to maintain sufficient membrane fluidity in a low-temperature environment (Tanji *et al.*, 2004). However, the authors neither constructed any sphingolipid C9-methyltransferase gene mutant of *K. lactis* nor investigated the growth of any gene mutants of the yeast under low-temperature conditions. In our work, the *mts1* mutant of *C. albicans* had the same growth rate as the wild-type strain, indicating that C9-methylated GluCer is not necessary for the yeast growth of this fungus at 10 °C or 30 °C. Although the mechanisms that fungi use to adapt to low-temperature stress are complex and many factors are thought to affect the stress response, the loss of C9-methylated GluCers is suggested to not influence the yeast-like growth of fungi at low temperature.

When the *mts1* and *hsx11* disruptants were grown in liquid medium to induce the hyphal form, they were able to elongate and produce their germ tubes normally. However, when grown on hyphal-inducing solid media, both the *mts1* and *hsx11* disruptants showed slower elongation than the wild-type. In addition, in our previous report, a *sld1* mutant of *C. albicans*, which is unable to produce not only GluCers including (*E,E*)-sphinga-4,8-dienine as a sphingoid base residue (Δ^8 -desaturated GluCer) but also C9-methylated GluCers, showed slower elongation than the wild-type, similar to our *mts1* and *hsx11* disruptants. These results suggest that GluCers, including C9-methylated LCB, are at least necessary for normal hyphal elongation of *C. albicans* on solid media. Filamentous growth of *C. albicans* is influenced by the physical environment. Several other *C. albicans* genes needed for filamentous growth on solid medium have been reported (reviewed by Kumamoto & Vines, 2005). Thus, several other C9-methylated GluCers also may be involved in the induction of the filamentous form of this fungus on solid media. Some GlyCers in fungi and plants are thought to be highly active in inducing fruiting body formation in *Schizophyllum commune* (Kawai & Ikeda, 1985; Kawai *et al.*, 1986). A structural analysis of these GlyCers revealed that C9-methylation in the sphingoid base moiety of fungal GlyCer is essential, while the sugar moiety has no effect. Moreover, the Δ *Fgmt2* mutant of *F. graminearum*, which has been shown to have reduced amounts of C9-methylated GluCers compared to the wild-type, was defective in mycelial growth (Ramamoorthy *et al.*, 2009). These results and our findings suggest that C9-methylated GluCers may be active in inducing hyphal morphogenesis of *C. albicans*.

The *C. albicans* *hsx11* disruptant was more sensitive to SDS than the wild-type, but the cell wall stress inducer CFW did not induce different growth effects on the *hsx11* disruptant or wild-type. In addition, the *C. albicans* *hsx11* disruptant was more susceptible than the wild-type to the lanosterol 14 α -demethylase inhibitor fluconazole. Fluconazole disrupts cell membrane structures by inhibiting the synthesis of ergosterol, which is a major membrane component. Our data suggest that the loss of GluCer leads to the disruption of cell membrane integrity in *C. albicans*. Moreover, the

sphingolipids that are enriched in detergent-resistant membrane microdomains, also known as lipid rafts, are known to be important for normal hyphal growth in *C. albicans* (Martin & Konopka, 2004). Martin & Konopka (2004) showed that myriocin, a specific inhibitor of serine palmitoyltransferase, which catalyses the first step of sphingolipid biosynthesis, affects hyphal elongation in *C. albicans*. Lipid rafts have been implicated in numerous cellular processes, including signal transduction, protein and lipid sorting, cellular entry of toxins and viruses, and viral budding (reviewed by Chazal & Gerlier, 2003; Bollinger *et al.*, 2005). Moreover, an antimicrobial peptide inhibitor of the fungal plasma membrane ATPase (Pma1p), which is known to be associated with lipid rafts, blocks the azole resistance of *C. albicans* (Monk *et al.*, 2005). GluCer is a major component of lipid rafts in most fungi. Therefore, the *C. albicans* *hsx11* disruptant may be sensitive to SDS and fluconazole due to defects in lipid rafts and thereby show abnormal hyphal growth in response to these agents. Future experiments should examine the lipid composition of membrane detergent-resistant microdomains and the membrane fluidity of the *C. albicans* *hsx11* mutant in detail.

On the other hand, the *C. albicans* *mts1* disruptant had susceptibility to SDS and fluconazole similar to the wild-type strain, although unlike the *hsx11* mutant it was more susceptible than the wild-type to the β -1,3-glucan synthase inhibitor micafungin. These results indicate that the membrane structure of the *C. albicans* *mts1* disruptant is not disturbed, suggesting that the abnormal hyphal growth of this disruptant is different from those of other GluCer synthesis gene disruptants. Micafungin disrupts the fungal cell wall structure by inhibiting cell wall synthesis. Although the detailed mechanisms of this process are still unclear, deleting the *MTS1* gene may affect fungal cell wall synthesis during hyphal growth.

The micafungin susceptibility of the single *mts1* mutant and the *MTS1*-restored strain was similar to that of the *mts1* knockout mutant. This result indicates that both alleles of *MTS1* are necessary for resistance to micafungin. On the other hand, the single disruptant of the *HSX11* gene showed SDS and fluconazole resistance similar to the wild-type while the *HSX11*-restored strain had high susceptibility to SDS and fluconazole, like the *hsx11* knockout strain. In this study, we used the constitutive *ACT1* promoter instead of the native *HSX11* promoter to restore the expression of the *HSX1* gene in the knockout strain. The difference in susceptibility to SDS and fluconazole between the single *hsx11* disruptant and the *HSX11*-restored strain may be due to the difference in the promoters used for the expression of the *HSX11* gene.

The findings of this study suggest that C9-methylated GluCer not only plays functional roles in hyphal morphogenesis but also contributes to cell wall synthesis in *C. albicans*. We have clarified the genes related to the biosynthetic pathway of GluCer in *C. albicans*. In contrast,

the entire metabolic pathway of GluCer, especially its degradation, remains unclear because no fungal genes for GluCer degradation, such as a glucosylceramidase gene, have been identified. To further understand the physiological function of GluCer, it will be necessary to analyse the degradation pathway of GluCer.

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