Polarized growth in filamentous fungi needs a continuous supply of proteins and lipids to the growing hyphal tip. One of the important membrane compounds in fungi is ergosterol. At the apical plasma membrane ergosterol accumulations, which are called sterol-rich plasma membrane domains (SRDs). The exact roles and formation mechanism of the SRDs remained unclear, although the importance has been recognized for hyphal growth. Transport of ergosterol to hyphal tips is thought to be important for the organization of the SRDs. Oxysterol binding proteins, which are conserved from yeast to human, are involved in nonvesicular sterol transport. In *Saccharomyces cerevisiae* seven oxysterol-binding protein homologues (OSH1 to -7) play a role in ergosterol distribution between closely located membranes independent of vesicle transport. We found five homologous genes (*oshA* to *oshE*) in the filamentous fungus *Aspergillus nidulans*. The functions of OshA-E were characterized by gene deletion and subcellular localization. Each gene-deletion strain showed characteristic phenotypes and different sensitivities to ergosterol-associated drugs. Green fluorescent protein-tagged Osh proteins showed specific localization in the late Golgi compartments, puncta associated with the endoplasmic reticulum, or diffusely in the cytoplasm. The genes expression and regulation were investigated in a medically important species *Aspergillus fumigatus*, as well as *A. nidulans*. Our results suggest that each Osh protein plays a role in ergosterol distribution at distinct sites and contributes to proper fungal growth.

Filamentous fungal growth by continuous tip elongation and branching and form hyphae and mycelium. Hyphal growth requires continuous transport of the proteins and lipids necessary for the extension of the cell wall and cell membrane. Polarized growth of filamentous fungi depends on the microtubule and actin cytoskeletons, along with their associated motor proteins (1–5). Apical membrane-associated landmark proteins, called “cell end markers,” link these two cytoskeletons in *Aspergillus nidulans* (6–9). Furthermore, apical membrane domains play an important role in polarized growth and the localization of cell end markers (8, 10, 11).

Membranes in eukaryotic cells are differentiated into different functional areas (12, 13). Sterols and sphingolipids can cluster into domains within mixtures of glycerophospholipids. These domains, termed “lipid rafts,” contribute to specific protein localization at specific sites, such as glycosylphosphatidylinositol-anchored and lipid-associated proteins, and play important roles in cell signaling and cell polarity (14–16). One type of domain, characterized by a high sterol content, is found in fungi (17). Sterol-rich membrane domains (SRDs) were visualized using the sterol-binding fluorescent dye filipin. The most abundant sterol found in fungi is ergosterol (18). Filipin stained the tips of mating projections in *Saccharomyces cerevisiae* (19) and *Cryptococcus neoformans* (20), cell ends in *Schizosaccharomyces pombe* (21), and hyphal tips in *Candida albicans* (22) and *A. nidulans* (11). Recently, it has been shown that SRDs contribute to polarized growth in C. albicans (22) and *A. nidulans* (8, 10, 11); however, the roles and formation mechanism of SRDs remain elusive (17). The size of SRDs in fungi is around a few microns, depending on the organism, which is much bigger in size than lipid rafts (10 to 200 nm) (23). SRDs are specific to fungi and have not been found in mammalian cells, suggesting not only fungal specific roles in membrane organization, but also potential targets for antifungal drugs (24).

From yeast to human sterols are mainly synthesized de novo in the ER membrane and are transported to the plasma membrane by vesicular and nonvesicular transport (25, 26). Oxysterols are defined as oxygenated derivatives of cholesterol. Oxysterol-binding protein (OSBP) and OSBP-related proteins (ORPs) are lipid-binding proteins identified from several eukaryotes. They are involved in many cellular processes, including cell signaling, vesicular trafficking, lipid metabolism, and nonvesicular sterol transport (27, 28). All ORPs contain an OSBP-related domain (ORD) that comprises a hydrophobic pocket and binds a single sterol molecule (29). ORDs also contain additional membrane-binding domains, some of which bind phosphoinositides and regulate sterol binding (30). Many lines of evidence suggest that OSBP and ORPs play a direct role in sterol transport between donor membrane and acceptor membrane (27, 28).

*S. cerevisiae* possesses seven ORP proteins, oxysterol-binding protein homologues (Osh1 to -7), which appear to have distinct but overlapping functions with regard to maintaining intracellular sterol distribution and homeostasis (31). Individual OSH genes were not essential for yeast viability (32). The disruption of any of the six OSH genes showed minor affects on cellular growth.
however, inactivation of all seven OSH genes results in lethality, indicating that Osh proteins most likely share an overlapping essential function (32). Osh4 (Kes1), the most well-analyzed protein of this family, is suggested to inhibit vesicular trafficking at the trans-Golgi in a phosphatidylinositol 4-phosphate-dependent manner (30, 33–36). In vitro, Osh4 and other Osh proteins have been demonstrated to transfer sterols between membranes as direct nonvesicular sterol transporters (35, 36). On the other hand, recent in vivo analysis revealed that Osh proteins regulate membrane sterol organization rather than sterol transport (37).

Membrane contact sites (MCs) are close appositions between two organelles. Ultrastructural studies revealed a distance between two membranes in the order of the size of a single protein (38). These zones of apposition are highly conserved in evolution but not much is understood about their biological meaning (39). MCs may be particularly important for the endoplasmic reticulum (ER) function, since MCs are observed between the ER and many organelles, including mitochondria, Golgi bodies, endosomes, lysosomes, peroxisomes, chloroplasts, and the plasma membrane (27). They can also form between the cell nucleus and the vacuole in yeast (nucleus vacuole junction) (40). Importantly, there are several evidences for lipid trafficking across these sites (41–44).

In yeast, Osh proteins showed different localization patterns based on the different protein structures. Osh1 localization was observed in the cytoplasm, Golgi, and the nucleus-vacuole junction (45–48). Osh2, Osh3, Osh6, and Osh7 were enriched in regions of the ER that are closely apposed to the plasma membrane (PM) (36). Osh4 showed localization patterns at Golgi membranes, cytoplasm and sites of polarized growth (49–51). In addition, Osh4p associated with exocytotic vesicles targeted to sites of polarized growth (49).

Transport of ergosterol to the apical PM is most likely important for SRD organization in A. nidulans. We characterized five homologous ORPs (OshA to -E) in A. nidulans by gene deletion, subcellular localization, and analysis of the deletion strains toward changed sensitivities against ergosterol-associated drugs. In addition to broaden our knowledge for medical research, we analyzed expression of osh orthologue genes in a medically important species Aspergillus fumigatus. Our results are the first report about the roles of oxysterol-binding protein homologues in hyphal growth of filamentous fungi.

**MATERIALS AND METHODS**

**Strains, plasmids, and culture conditions.** Supplemented minimal medium for *A. nidulans* was prepared as described, and standard strain construction procedures were used (52). Glucose (2%) was used as carbon source, and 70 mM sodium nitrate and 0.9 μM ammonium molybdate were used as nitrogen sources. Growth assays on different carbon or nitrogen sources were performed with minimal medium with 2% carbon or nitrogen sources. Growth assays on different carbon or nitrogen sources were performed with minimal medium with 2% carbon or nitrogen sources. Growth assays on different carbon or nitrogen sources were performed with minimal medium with 2% carbon or nitrogen sources. Growth assays on different carbon or nitrogen sources were performed with minimal medium with 2% carbon or nitrogen sources. Growth assays on different carbon or nitrogen sources were performed with minimal medium with 2% carbon or nitrogen sources. Growth assays on different carbon or nitrogen sources were performed with minimal medium with 2% carbon or nitrogen sources. Growth assays on different carbon or nitrogen sources were performed with minimal medium with 2% carbon or nitrogen sources. Growth assays on different carbon or nitrogen sources were performed with minimal medium with 2% carbon or nitrogen sources. Growth assays on different carbon or nitrogen sources were performed with minimal medium with 2% carbon or nitrogen sources. Growth assays on different carbon or nitrogen sources were performed with minimal medium with 2% carbon or nitrogen sources. Growth assays on different carbon or nitrogen sources were performed with minimal medium with 2% carbon or nitrogen sources. Growth assays on different carbon or nitrogen sources were performed with minimal medium with 2% carbon or nitrogen sources.

**Molecular techniques.** Standard DNA transformation procedures were used for *A. nidulans* and *E. coli*. For PCR experiments, standard protocols were used. DNA sequence analysis of the deletion cassettes was determined by Southern hybridization.

**Tagging with GFP, gene deletion, and complementation.** To tag OshA-E with green fluorescent protein (GFP) at the C terminus, the full length of oshE was amplified with primers OshE-FW and OshE-Rev. The fragment coding GFP was amplified with primers GFP-link-GFP-Fw and GFP-Rev. The two amplification products were fused together with primers OshE-FW and OshE-Rev via a PCR. The Km-PacI fragment was subcloned into digested pCBM17apx with KpnI and PacI, yielding pH87 [alca(p)-gfp-oshA].

To express GFP-OshA, GFP-OshB and GFP-OshE under the native promoter, the alca promoter of pNB1, pNB2 and pNB6 was replaced by -1 kb of the putative promoter of oshA, oshB, and oshE, respectively. The promoter regions were amplified with the primers OshA(-p)-FW and OshA(-p)-Rev, OshB(-p)-FW and OshB(-p)-Rev, and with the primers OshE(-p)-FW and OshE-Rev. The amplified products were digested with EcoRI and KpnI and subcloned into EcoRI-KpnI digested pNB1, pNB2, and pNB6, yielding in pNB8 [oshA(p)-gfp-oshB], pNB9 [oshB(p)-gfp-oshE], and pNB10 [oshE(p)-gfp-oshA].

For tagging Sec63 (AN0834) with mCherry at the C terminus, the -1 kb C-terminal region of the gene was amplified with primers Sec63-3'-LB-Fw and Sec63-3'-LB-Rev. In addition, the 1-kb downstream sequence of sec63 was amplified with the primers Sec63-3'-FB-Fw and Sec63-3'-RB-Rev. The PCR products were cloned into plet1.2 (Thermo Scientific), yielding pH92 (sec63-mCherry). All plasmids were transformed into the TN02A3 strain (ku70 deletion) to favor homologous integration of the constructs at the corresponding gene loci. Primary transformants were screened microscopically for GFP fluorescence and by PCR for correct integration of the constructs. Integration events were further confirmed by Southern blotting.

To delete oshA, oshC, and oshD, 1-kb upstream and downstream of the corresponding osh open reading frames were amplified for oshA with the primers oshA-FW-LB, oshA-Rev-LB, oshA-FW-RB, and oshA-Rev-RB, for oshC with the primers oshC-LB-del-FW, oshC-LB-del-Rev, oshC-RB-del-FW, and oshC-RB-del-Rev, and for oshD with the primers oshD-LB-del-FW, oshD-LB-del-Rev, oshD-RB-del-FW, and oshD-RB-del-Rev. A fragment of the pyrG marker was amplified with the primers Sfr-linker-FW and Sfr-linker-Rev. The primers OshA-Rev-LB, OshA-FW-RB, oshC-LB-del-FW, oshC-RB-del-Rev, oshD-LB-del-FW, and oshD-RB-del-Rev were designed with the KpnI and PacI sites. The borders and cassettes of oshA, oshC, and oshD were fused together with the primer pairs oshA-FW-LB and oshA-Rev-RB, oshC-LB-del-FW and oshC-RB-del-Rev, and oshD-LB-del-FW and oshD-RB-del-Rev, respectively. The oshA deletion cassettes were cloned into plet1.2, yielding pH13. The oshC and oshD deletion PCR products and pH13 were transformed into TN02A3. Furthermore, oshB and oshE deletion cassettes were obtained from FGSC (plate number 20091230B) with pyrG as a selectable marker. To amplify the deletion cassettes of oshB and oshE, FGSC primers 39 and 40 were used, respectively.

Both PCR products were transformed into the TN02A3 strain. Primary transformants were screened by PCR for correct integration of the deletion cassettes. Integration events were further confirmed by Southern blotting.

Recomplementation of the oshC and oshD deletion strains was achieved by transformation of the corresponding gene coding regions. The oshC coding region was amplified with the primer set OshC Prim FW and OshC 3UTR Rev. For the amplification of the oshD coding re...
gion, the primer set OshD-Prom-FW and OshD-3UTR-Rev were used. The forward primer included the AcrII cutting site and the reverse primer included the Ascl cutting site, respectively. The AcrII-Ascl fragments were subcloned into digested pCMB17apx with AcrII and Ascl, yielding pNB11 (oshC coding region::pyr-4) and pNB12 (oshD coding region::pyr-4). The plasmid pNB11 was transformed into the strains SNB9 and pNB12 in SNB10, respectively. Transformants were screened by PCR for correct integration.

**Growth assay with calcofluor white.** Calcofluor white (Sigma) was used at 0 to 100 μM on agar plates. Solutions with 2.5 × 10^5 spores from wild-type and oshA-E deletion strains were inoculated on plates with different calcofluor white concentrations. Each plate was incubated at 37°C for 3 days.

**Biomass and total ergosterol extraction.** A total of 2.5 × 10^5 spores of the wild-type and osh deletion strains were inoculated in 50 ml of liquid minimal media with glucose, followed by incubation for 20 h at 37°C with 180 rpm. The mycelia were harvested by filtration, washed with sterile water, and then dried for 1 day. The fungal dry weight was determined, and the ergosterol extraction was done as previously described (54). The analysis of the ergosterol content was done with the system Flexar LC HPLC from Perkin-Elmer. This system includes a FXPB-Pump-2, a HPLC Pecosphere5/HPLC reversed-phase Peco HCODS C18 column (150 mm by 4.6 mm, 5 μm), a manual injector (20 μl). Filtered methanol eluent was established with a flow rate of 1 ml/min.

The UV light detector recorded the peak heights/areas at 282 nm, and quantification was done using the software Chromera. The total concentration of ergosterol was expressed as mAU/min (i.e., milliabsorbsion units per minute) of ergosterol, which was converted into concentration by milligrams of fungal semidry weight.

**Light/fluorescence microscopy.** For live-cell imaging of germings and young hyphae, cells were grown on coverslips in 0.5 ml of minimal media plus 2% glucose. When we observed strains expressing a gene of interest under the control of the inducible alcA promoter, minimal media plus 2% glycerol (derepression of the alcA promoter) was used. Cells were incubated at 28°C overnight. Coverslips were mounted on microscope slides. Images were captured using an Axiohot microscope with a Planapochromatic ×63 oil immersion objective lens, a Zeiss AxioCam MRM camera (Zeiss), and an HBO 101 mercury arc lamp (Osmar) or HXP 120 (Zeiss) possessing faster speed wavelength switching. Images were collected and analyzed using the AxioVision and Zen system (Zeiss). Kymograms were made using Imagej software (http://rsb.info.nih.gov/ij/).

For filipin staining, hyphae were stained with filipin at 1 μg/ml for 5 min from a 10-mg/ml dimethyl sulfoxide (DMSO) stock solution (55).

**Quantitative real-time PCR (qRT-PCR).** For RNA isolation, mycelia were collected and analyzed using the AxioVision and Zen system (Zeiss), resulting in a plasmid pUC119-srbA::ptrA, which was used for transformation to construct the AsrbA mutant. A. fumigatus transformation was performed according to conventional methods for protoplast-polypeylene glycol transformation for Aspergillus as performed previously (56). Homologous recombination and gene replacement were confirmed by PCR of the genomic DNA, and the absence of mRNA of the target gene was verified using RT-PCR.

**RNA isolation and cDNA preparation.** AF293 and the srbA deletion mutant of a concentration of 10^6 conidia per ml were cultivated in 40 ml of liquid YGMM at 37°C for 17 h. The mycelia were harvested 2 h after incubation at 37°C. RNA samples were extracted from the mycelia and total RNA was isolated using a FastRNA Pro Red kit (MP Biomedicals). To obtain cDNA pools from total RNA, removal of the possible contaminant DNA was performed according to conventional methods for protoplast-polypeylene glycol transformation for Aspergillus as performed previously (56). Homologous recombination and gene replacement were confirmed by PCR of the genomic DNA, and the absence of mRNA of the target gene was verified using RT-PCR.

**Ergosterol-associated drug treatment.** Voriconazole (Sigma) was used at 0.1 to 1 μM on agar plates and at 0.1 μM for microscopy analyses. To test the sensitivity of the wild-type and osh deletion strains against different ergosterol-associated drugs, microtiter plate assays were used (24). A 96-well microtiter plate, 200 μl of supplemented minimal medium was loaded per well with 2.5 × 10^5 conidiospores of each strain. Ampthotericin B (Am; Roth) solved in the stock solution of 0.1 to 10 mM in DMSO was tested at 0.5 to 200 μM in the medium. Methyl-β-cyclo-dextrin (MBD; Sigma) was tested at 0.5 to 10 mM in the medium from 100 mM stock solution solved in double-distilled H2O. As a redox indicator, we used resazurin (7-hydroxy-3H-phenoxazin-3-one-10-oxide) at a final concentration of 100 μM (24).

**A. fumigatus strains and growth media.** A. fumigatus strain AF293 and the srbA deletion mutant were used for expression analysis. The strains were cultivated in 0.1% yeast extract containing glucose minimal media (YGM) at 37°C (56). Itraconazole (Wako Pure Chemical Industries) and amphotericin B (Sigma-Aldrich Co) were commercially obtained.

To construct the A. fumigatus srbA deletion mutant, a plasmid harboring deletion cassette targeting srbA gene and ptrA as a selection marker was generated. ptrA marker was obtained from pPTRI vector (TaKaRa Bio) using the primers ptrA-F and ptrA-R. 5’- and 3’-flanking regions of the srbA gene were obtained using the primers srbA-U-f (pUC119E) and srbA-U-r (pUC119B) for the 3’-flanking region. These flanking regions and the ptrA marker were fused into pUC119 using the GeneArt system (Invitrogen), resulting in a plasmid pUC119-srbA::ptrA, which was used for transformation to construct the AsrbA mutant. A. fumigatus transformation was performed according to conventional methods for protoplast-polypeylene glycol transformation for Aspergillus as performed previously (56). Homologous recombination and gene replacement were confirmed by PCR of the genomic DNA, and the absence of mRNA of the target gene was verified using RT-PCR.

**RIPA buffer.** A. fumigatus was cultivated in liquid YGMM for 18 h, and the mycelia were transferred onto a YGMM plate (set as 0 h of asexual stage), followed by incubation at 37°C. RNA samples were extracted from the mycelia harvested at 0, 6, 12, and 24 h.

**Quantitative RT-PCR.** RT-PCR was performed using the 7300 system (Life Technologies Corp.) with SYBR green detection as described previously (56). Briefly, the Thunderbird SYBR qPCR mix was used for reaction mixture preparation (Toyobo). Primer sets for the osh genes—RT-oshA-F/RT-oshA-R, RT-oshB-F/RT-oshB-R, RT-oshC-F/RT-oshC-R, RT-oshD-F/RT-oshD-R, and RT-oshE-F/RT-oshE-R—were used to quantify the expression. The relative expression ratios were calculated by the ΔΔCt method. The actin gene was used as a normalization reference (internal control) for target gene expression level determination, and wild type at 0 h or without drug treatment was set as the calibrator in each experiment. Each sample was analyzed in triplicate.
RESULTS

Structure and phylogeny of Osh proteins. We found five genes encoding putative orthologues of Osh in the database of A. nidulans genomic sequences and named them oshA (AN9063), oshB (AN3424), oshC (AN2877), oshD (AN3807), and oshE (AN3452), respectively. All of them possess an oxysterol-binding protein related domain (ORD; 350 to 380 amino acids) (Fig. 1A). OshA consists of 1243 amino acids and contains three ankyrin repeats, which is known to mediate protein-protein interaction, and one PH (pleckstrin homology) domain at the N-terminal half and the ORD at the C-terminal region, as based on the results of an InterProScan analysis (http://www.ebi.ac.uk/interpro/). OshB consists of 935 amino acids and contains a Golgi dynamics domain and the PH domain at the N-terminal half and the ORD in the C-terminal region. The Golgi dynamics (GOLD) domain is known to mediate protein-protein interaction (57, 58). OshC, OshD, and OshE possess no extra domain besides the ORD.

A phylogenetic tree of Osh proteins, including the ones from other fungi was constructed (Fig. 1B). Phylogenetic analysis revealed that Osh proteins are classified into five groups (groups 1 to 5). OshA, OshB, OshC, OshD, and OshE are classified into groups 1 to 5, respectively. In the Osh orthologues of group 1, the ankyrin repeats and one PH domain are conserved at the N-terminal half. In the Osh orthologues of group 2, the Golgi dynamics domain and the PH domain are conserved at the N-terminal half. Although OshC, OshD, and OshE of A. nidulans have no specific domain except the ORD, they are classified into distinct three groups (groups 3 to 5).

The genome of S. cerevisiae contains seven OSH genes (osh1 to osh7), but they are classified into only four groups (groups 1 to 4) (Fig. 1B and C). Osh1 and Osh2 of S. cerevisiae classified into group 1 showed 41 and 59% identity per 251 and 242 amino acids with E values of e−145 and e−148 to OshA of A. nidulans, respectively. Osh3 of S. cerevisiae in group 2 showed 49% identity per 185 amino acids and an E value of e−100 to A. nidulans OshB. Osh4 and Osh5 of S. cerevisiae in group 3 showed 48% identity per 194 and 193 amino acids with E values of e−118 and e−115 to A. nidulans OshC, respectively. Osh6 and Osh7 of S. cerevisiae in group 4 showed 49 and 50% identity per 111 and 115 amino acids and E values of e−140 and e−58 to A. nidulans OshD, respectively. The putative orthologue of A. nidulans OshE was not found in the genome of S. cerevisiae and Ashbya gossypii, whereas other filamentous fungi and the fission yeast Schizosaccharomyces pombe possess five to six Osh genes, which are classified into five groups, respectively (Fig. 1C).

Deletion of osh genes. In order to examine the functions of Osh proteins in A. nidulans, oshA-E gene deletion strains were constructed (see Materials and Methods). Each single gene deletion of oshB, oshC, and oshD caused slower growth. Although the colony diameter of the oshB deletion strain was smaller than that of the wild type (70%), the colonies of the oshC and oshD deletion strains were more compact than those of the wild type (50%) (Fig. 2A and B). Furthermore, a reduction of conidiospore formation was observed in all osh deletion strains. The numbers of conidiospores produced from strains grown on agar surfaces for 1 week were reduced to 70, 60, 40, and 30% in the oshA, oshB, oshC, and oshD deletion strains, respectively (Fig. 2C). Interestingly, the oshE deletion strain, whose orthologue was not conserved in bud-
did not show obvious differences in the colony diameter, whereas the formation of conidiospores was reduced to 30% compared to the wild type (Fig. 2A to C), suggesting a more specific role of OshE in the formation of conidiospores.

Hyphae in the oshA, oshB, and oshE deletion strains did not exhibit clear morphological changes compared to the wild type (data not shown), whereas hyphae in the oshC and oshD deletion strains formed more branches than did wild-type hyphae (Fig. 2D to H). Although the morphologies of hyphae in the oshC and oshD deletion strains were nearly identical, the oshD deletion strain sometimes also showed cell lysis at the hyphal tips (Fig. 2I, arrows). The lysin phenotype of oshD deletion strain was restored on minimal medium with 1 M sucrose as an osmotic stabilizer (data not shown). All phenotypes of oshC and oshD deletion strains were restored after complementation of the mutant with a wild-type copy of the genes (see Fig. S1A in the supplemental material).

The slower growth in the oshB, oshC, or oshD deletion strains was more moderate at 28°C compared to 37°C, and more severe at 42°C (see Fig. S2 in the supplemental material). Since cell lysis was observed at hyphal tips in the oshD deletion strain, the response to a cell wall-compromising agent of the osh deletion strains was investigated by using calcofluor white. However, no obvious difference was found in the osh deletion strains compared to the wild type (see Fig. S3 in the supplemental material).

**Localization of GFP-Osh proteins.** To examine the subcellular localization of OshA–E, GFP was fused to the N termini of all Osh proteins. Each Osh tagged with GFP was expressed under the control of the alcA promoter, which is regulated through the carbon source. Under repressed conditions with glucose as the carbon source, the strains repressing GFP-OshB, GFP-OshC, or GFP-OshD showed a growth delay like that of the corresponding deletion strains. Under derepressed conditions with glycerol as the carbon source, GFP-OshB, GFP-OshC, or GFP-OshD strains showed no growth delay, and the hyphae did not show abnormal hyphal morphology, as observed in the osh deletion strains (see Fig. S1B and C in the supplemental material). The strain expressing GFP-OshE produced fewer conidiospores under repressed conditions compared to the wild type, whereas the conidiation

---

**FIG 2** Deletion of osh genes. (A) Colonies of wild-type and oshA-E deletion strains. A total of $2.5 \times 10^5$ spores were inoculated on minimal medium glucose agar plates and incubated for 3 day at 37°C. (B) Colony diameters of wild-type and osh deletion strains were measured every day over 6 days. Error bars represent the standard deviations ($n = 3$). (C) Numbers of spores produced per square centimeter of wild-type and osh deletion strains. A total of $2.5 \times 10^5$ spores were spread on minimal medium glucose agar plates and incubated for 3 days at 37°C (S2). Asterisks represent statistical significance ($P < 0.05$). Error bars represent the standard deviations ($n = 3$). (D to I) Hyphal morphology of wild-type (D), ΔoshC (E and G), and ΔoshD (F, H, and I) strains. Hyphae with more branches in ΔoshC (G) and ΔoshD (H) strains are also shown. Cell lysis can be seen at the hyphal tips in the ΔoshD strain (I). Scale bar, 100 μm (D to F) or 20 μm (G to I).
Fig. 3 Localization of GFP-tagged OshA–E. (A) The strain expressing GFP-OshA under the oshA promoter was grown in minimal medium with glycerol. GFP-OshA localized to multiple puncta throughout the hyphae. A kymograph during 30 s shows that these puncta were immobile. Scale bars, 10 μm. (B) GFP-OshA colocalized with mRFP1-TlgB as the late Golgi marker. Scale bars, 5 μm. (C) The strain expressing GFP-OshB under the native promoter was grown in minimal medium with glycerol. GFP-OshB localized to several puncta at subapical regions. The kymograph during 30 s shows these puncta were immobile. Scale bars, 5 μm. (D) The strain expressing GFP-OshB under the alcA promoter and Sec63-mCherry was grown in minimal medium with glycerol. The GFP-OshB puncta appeared to contact the ER structures, especially colocalize at the edge of ER tubular structure (arrows). Scale bar, 2 μm. (E) GFP-OshC showed diffuse signals in the cytoplasm (upper). Some hyphae showed weak puncta at hyphal tips (lower). The kymograph during 1 min shows an immobile dot. Scale bars, 5 μm. (F) GFP-OshD showed diffuse signals in the cytoplasm. Scale bars, 5 μm. (G) The strain expressing GFP-OshE under the native promoter was grown in minimal medium with glycerol. GFP-OshE localized to several puncta through the hyphae except the apical region. The kymograph indicates that these puncta were immobile. Scale bars, 5 μm. (H) The strain expressing GFP-OshE under the alcA promoter and Sec63-mCherry was grown in minimal medium with glycerol. The puncta of GFP-OshE localized on the peripheral ER strands. Scale bar, 2 μm.

Defect in the strain expressing GFP-OshE was suppressed under derepressed conditions (see Fig. S1D in the supplemental material). These results suggest that the GFP fusion proteins are biologically functional. Repression and derepression of GFP-OshA did not show any obvious phenotype at the colony level and with regard to hyphal morphology. Localization of GFP-Osh proteins was investigated under derepressed conditions. In addition, localization of GFP-OshA, GFP-OshB, and GFP-OshE was analyzed in strains where each GFP-Osh protein was expressed from the native promoter (see below).

GFP-OshA expressed from the alcA promoter localized to multiple puncta throughout the hyphae. This localization pattern was confirmed in the strain expressing GFP-OshA under the native promoter (see Materials and Methods) (Fig. 3A). The kymograph constructed from a time-lapse analysis showed no active movement of these puncta. S. cerevisiae Osh1, which belongs to group 1, as OshA does, was shown to localize at the Golgi and nuclear vacuole junctions (45, 46). The localization of GFP-OshA appeared similar to late Golgi structures in A. nidulans (59, 60). To test whether OshA indeed localizes in late Golgi structures, the localization of GFP-OshA was compared to the localization of mRFP1-TlgB used as a late Golgi cisternae marker (60) (Fig. 3B). GFP-OshA colocalized with mRFP1-TlgB, indicating GFP-OshA localizes at the late Golgi compartment.

GFP-OshB expressed from the alcA promoter localized to several puncta close to the hyphal tip except the apical region. The localization of GFP-OshB was confirmed in the strain, which expresses GFP-OshB under the native promoter, although the GFP signal was much weaker than GFP-OshB expressed from the alcA promoter (Fig. 3C and data not shown). The kymograph constructed from a time-lapse analysis indicated no active movement of these puncta. Osh3 of S. cerevisiae, which belongs to group 2, as OshB does, is known to localize at the PM and ER contact sites (30, 36). To compare the localization of GFP-OshB with the ER, the translocon component Sec63 was tagged with mCherry as ER marker (61). The ER network structures in A. nidulans are shown to consist of peripheral PM-associated strands and nuclear envelope-associated strands. The ER is absent from apical regions (61). The GFP-OshB puncta appeared to contact the ER structures and especially colocalized at the edge of ER tubular structures (Fig. 3D, arrows). The localization pattern of GFP-OshB suggests the role at the plasma membrane and ER contact sites as it was shown for Osh3 in budding yeast.

Both GFP-OshC and GFP-OshD showed diffuse signals in the cytoplasm (Fig. 3E and F). The cytoplasmic signal was reduced under repressed conditions, whereas the signal increased under inducing conditions (data not shown). In some hyphae a weak GFP-OshC signal was seen as immobile puncta around the apical region (Fig. 3E, arrowhead). Osh4 of S. cerevisiae, which belongs to group 3, as OshC, is known to localize in the cytoplasm, the Golgi and exocytotic vesicles (49, 50). Osh6 and Osh7 of S. cerevisiae, which belong to group 4, as OshD, are observed at the ER near the PM and in the cytoplasm (36, 47).

GFP-OshE localized to several puncta throughout the hyphae, except the apical region in both cases GFP-OshE was expressed from the alcA and the native promoter (Fig. 3G and data not shown). The kymograph constructed from a time-lapse analysis indicates that these puncta were immobile. The GFP-OshE signal around hyphal tips appeared similar to that of GFP-OshB. To compare the localization of GFP-OshE with the ER, we con-
structured a strain expressing GFP-OshE and Sec63-mCherry. The puncta of GFP-OshE localized on the peripheral ER strands (Fig. 3H). OshE C-terminally tagged with GFP also showed similar immobile puncta throughout the hyphae (data not shown).

**Expression of osh genes.** The relative expression levels of all osh genes were investigated in the wild-type strain by real-time PCR (see Materials and Methods). oshA, oshC, oshD, and oshE were expressed at comparable levels (Fig. 4A, gray bars). The expression levels of oshC-E were 80 to 90% of that of oshA. oshB was expressed at much lower levels, with ca. 0.06% of oshA.

The relative expression levels of all osh genes were also investigated in the osh deletion strains (Fig. 4A and B). Various inductions of osh genes were observed in each osh deletion strain. The ratios of expression levels in each osh deletion strain compared to that in the wild type are indicated in Fig. 4C. The expression of oshD gene was induced 12-fold in the oshB deletion strain compared to the wild type (Fig. 4C). In comparison, the expression of oshD was increased in the oshA, oshC, or oshE deletion strains <2-fold. In the oshB deletion strain, the expression of oshA and oshE were increased 3-fold. The expression of oshC was increased <2-fold in the oshA, oshD, or oshE deletion strain. These results suggest that some osh genes are induced in the absence of certain Osh proteins probably to complement their functions. In particular, the expression of oshD was often increased in the other osh deletion strains.

The phenotype of oshE deletion suggested a role of OshE on the formation of conidiospores (Fig. 2A to C). Therefore, the expression of oshE was investigated during conidiation (see Fig. S4A in the supplemental material). At 24 h after the induction of conidiospore formation, the expression of oshE was increased <4-fold. The promoter region of oshE contains one consensus sequence for the transcription factor BrlA, which controls conidiophore morphogenesis (62). The localization of GFP-OshE was also analyzed during conidiophore development (see Fig. S4B in the supplemental material). GFP-OshE localized to several puncta throughout the stalk of conidiophores and inside the metulae, whereas no clear localization pattern was observed in phialides. These results support the hypothesis that OshE is specifically involved in conidiophore development.

**Sensitivity to ergosterol-associated drugs.** The antimycoticum voriconazole is generally used to treat serious invasive fungal infections (63). Voriconazole inhibits 14α-demethylase, which is one of the enzymes that convert lanosterol to ergosterol (34, 63–65). To compare the effect of voriconazole on wild-type and osh

---

**FIG 4** Expression profile of osh genes. (A) The relative expression of oshA-E genes in wild-type and oshA-E deletion strains was analyzed by qRT-PCR. Error bars represent the standard deviations (n = 3). The relative expression of oshB in the wild type is shown in panel B. (C) Ratio of the expression in the oshA-E deletion strains compared to that in the wild-type strain. Asterisks represent statistically significant (P < 0.01) differences. Error bars represent the standard deviations (n = 3).
deletion strains, they were grown in the presence of various concentrations of voriconazole (Fig. 5A and B). The oshB-deletion strain showed increased sensitivity toward voriconazole compared to wild type. The oshA and oshE deletion strains displayed almost the same sensitivity as wild type. In contrast, deletion of oshC or oshD caused slight resistance to the drug; whereas 0.1 and 0.5 μM voriconazole delayed the growth of the wild type, the oshC and oshD deletion strains were less affected (Fig. 5B).

Hyphal morphology was analyzed in wild-type and osh deletion strains grown using 0.1 μM voriconazole. The wild-type and the oshA, oshB, and oshE deletion strains exhibited swollen and depolarized hyphae (Fig. 5C), whereas the oshC and oshD deletion strains did not show the abnormal structure but grew identically to strains grown without voriconazole. These results suggest that deletion of oshC or oshD suppress the inhibitory effect of voriconazole.

The polyene macrolide antibiotic amphotericin B (AmB) is also used to treat serious invasive fungal infections, although the

---

**FIG 5** Sterol-related antibiotic sensitivity of osh deletion strains. (A) Colonies of wild-type and oshA-E deletion strains. A total of $2.5 \times 10^4$ spores were inoculated onto minimal medium glucose plates with different concentrations of voriconazole, followed by incubation for 6 days at 37°C. (B) Colony diameters of wild-type and osh deletion strains were measured at 6 days. Error bars represent the standard deviations ($n = 3$). (C) Hyphal morphology of wild-type and oshA-E deletion strains grown in minimal medium glucose with 0.1 μM voriconazole overnight at 28°C. Scale bar, 20 μm. (D and E) Antibiotic activities of AmB (D) and MBCD (E) to osh deletion strains in a 96-well microtiter plate. Each well contains $2.5 \times 10^4$ conidiospores of wild-type and osh deletion strains in 200 μl of minimal medium glucose and 100 μM resazurin. An antibiotic dilution series was conducted from the top to the bottom of the microtiter plate: 0 to 200 μM (D) and 0 to 10 mM (E). The microtiter plate was incubated statically at 37°C. This picture was taken after 32 h. (F) Biomass (in mg) from the wild-type and oshA-E deletion strains from inoculated $2.5 \times 10^5$ spores with per 50 ml from each strain, incubated overnight at 37°C and dried for 1 day. Error bars represent the standard deviations of the mean ($n = 3$). (G) Ergosterol concentration (mg/g mycelium). Spore solutions from wild-type and osh deletion strains were inoculated with $2.5 \times 10^3$ spores per 50 ml and incubated for 18 h at 37°C and 180 rpm. Ergosterol from each strain was isolated and measured by HPLC. Error bars represent the standard deviations of the mean ($n = 3$). Asterisks represent statistically significant ($P < 0.01$) differences.
mode of action of AmB differs from that of voriconazole. The classical model is that AmB binds to ergosterol in the fungal plasma membrane and forms a transmembrane channel that leads to K+ ion leakage, which finally causes cell death (66). Methyl-β-cyclodextrin (MβCD) does not affect the plasma membrane permeability; rather, it binds to ergosterol molecules and extracts ergosterol from the membrane into small unilamellar vesicles of MβCD (67, 68). To compare the effect of these drugs on wild-type and osh deletion strains, we tested concentrations ranging from 0.5 to 200 μM AmB, or 0.5 to 10 mM AmB in the case of MβCD, in microtiter plates (Fig. 5D and E).

We used resazurin as a color indicator for metabolic activity. Resazurin is a blue, nonfluorescent dye that is converted to pink by metabolic reduction to the wild type. oshD deletion strains showed higher sensitivity to AmB compared to the wild type, with MICs of 916 μM AmB, or 0.5 to 10 mM AmB in the case of MβCD, and minimally detectable in the case of oshC and oshB deletion strains, with MICs of ~6 mM (Fig. 5E), whereas the oshC and oshD deletion strains showed higher sensitivity to AmB compared to the wild type.

The wild-type and oshA deletion strains showed similar sensitivities to AmB, with a MIC of ~20 μM, whereas the oshB and oshE deletion strains were more resistant, with MICs of ~200 μM (Fig. 5D and data not shown). On the other hand, the oshC and oshD deletion strains showed higher sensitivity to AmB compared to the wild type.

The wild-type and the oshA and oshB deletion strains showed similar sensitivities to MβCD, with MICs of ~6 mM (Fig. 5E), whereas the oshC and oshD deletion strains showed higher sensitivities (MICs of ~2 mM). Intriguingly, the oshE deletion strain showed resistance, with an MIC of ~10 mM.

To find clues about the different sensitivities of the osh deletion strains to the ergosterol-associated drugs, the amounts of ergosterol in the cells were measured by high-pressure liquid chromatography (HPLC). After the wild-type and osh deletion strains were grown in liquid minimal medium for 20 h, the biomass of the mycelium was measured as semidry weight (Fig. 5F). Ergosterol was then extracted from the mycelium and analyzed by HPLC (Fig. 5G) (54). The amounts of biomass in the osh deletion strains were comparable to the sizes of the colonies. The biomasses of the oshC and oshD deletion strains were 60 to 70% of that of the wild type (Fig. 5F). The amounts of ergosterol were reduced to 70% in the oshA, oshC, and oshE deletion strains and reduced to 40% in the oshD deletion strain compared to the wild type (Fig. 5G).

Expression profile of osh genes in Aspergillus fumigatus. To broaden our knowledge of Osh protein function, we conducted expression analyses of the osh genes in the medically important species A. fumigatus. First, BLAST search analysis on AspGD identified the highly conserved OshA-E proteins in A. fumigatus. The orthologous proteins were OshA (Afu7g02480), OshB (Afu3g05880), OshC (Afu3g17500), OshD (Afu2g03790), and OshE (Afu3g05520) with 75.7, 66.5, 78.9, 73.7, and 76.3% amino acid identities to those of A. nidulans, respectively. The expression of the osh genes during asexual development was determined by RT-PCR analysis. A. fumigatus oshE was moderately upregulated in the asexual stage, whereas the other osh genes showed no increase in their expression levels (see Fig. S4C in the supplemental material).

Among clinically approved drugs, azoles inhibit ergosterol biosynthesis and polyene antifungycotics, e.g., AmB directly interacts with ergosterol present in the membrane (71, 72). We investigated the transcriptional effects of these drugs on osh genes in A. fumigatus. Although there were no influences due to itraconazole addition (Fig. 6A, red bars), polyene drug AmB treatment resulted in increased expression of oshA and oshD (Fig. 6A, green bars). Some genes for ergosterol biosynthesis are regulated by the SREBP (sterol regulatory element-binding protein) SrbA in A. fumigatus, and the total ergosterol level was reduced in the srbA-null mutant (73, 74). We examined osh expression in the srbA deletion strain and found that the increased expression of oshA in response to AmB was not observed in the srbA deletion mutant (Fig. 6A). These results suggest that the oshA gene was induced in the presence of AmB in an SrbA-dependent manner. The A. fumigatus SrbA DNA binding sequence (SRE) has been identified (75). We searched for the SRE element within 2-kb upstream of the translational start codon of the A. fumigatus oshA gene. There is no sequence perfectly matching with the element. This suggests that SrbA does not directly regulate the expression of A. fumigatus oshA upon AmB treatment.

In the A. fumigatus ergosterol biosynthesis pathway, several enzymes are oxygen dependent (76). The sterol profile might differ under hypoxic conditions, which would contribute to ergosterol-related drug susceptibility. To investigate whether the expression of osh genes is regulated in response to hypoxia conditions, we analyzed a set of RNA-Seq data (GSE30579) deposited by R. A. Cramer’s group, which showed only a slight transcriptional change for these genes (see Fig. S4D in the supplemental material). This suggests that at least at the transcriptional level hypoxic conditions do not affect OSH functions.

The azole susceptibility and resistant phenotype in the A. nidulans osh deletion strains might be related to the availability of the drug target proteins. In A. fumigatus, Cyp51A and Cyp51B encode a lanosterol α-14 demethylase enzyme and are known to be the target of azole drugs (77, 78). The expression of cyp51A and cyp51B is known to be regulated by SrbA in response to azole drugs and hypoxia (79). The cyp51A and cyp51B are conserved in A. nidulans (cyp51A, AN1901; cyp51B, AN8283). We examined the expression level of cyp51A and cyp51B in wild-type and osh deletion strains (Fig. 6B). The expression levels of cyp51A were increased 3-fold in the oshA and oshC deletion strains and 1.5-fold in the oshD and oshE deletion strains. The expression levels of cyp51B were increased 2- to 3-fold in the oshD and oshE deletion strains. The upregulation of cyp51A in the oshC deletion strain and the upregulation of cyp51A and cyp51B in the oshD deletion strain may be partly attributable to the voriconazole resistance phenotypes. The expression levels of both cyp51A and cyp51B in the oshB deletion strain were comparable to those in the wild type.

Roles of Osh on polarized growth and SRD organization. To investigate the roles of Osh proteins in apical sterol-rich membrane domain (SRD) formation (17, 55), we investigated SRD localization in the osh deletion strains by using filipin staining. The oshA, oshB, and oshE deletion strains showed the SRD signal at the hyphal tips (Fig. 7A and data not shown). Although the deletion of oshC and oshD caused more branches than with the wild-type strain, the SRDs were visible at the branched tips and, in addition, at subapical new branching sites (Fig. 7A, arrows). The signal intensities of SRDs were quantified in the osh deletion strains. The oshE deletion strain showed lower signals than that of the wild type (78%), however, there was no clear difference in the oshA, oshB,
oshC, or oshD deletion strains (Fig. 7B). These results suggest that OshE is involved in SRD organization.

The SRDs are important for the localization of the cell end markers, TeaA and TeaR (8, 55). TeaA is anchored at the apical plasma membrane through interaction with TeaR after TeaA is transported at microtubule plus ends to the hyphal tip (8). We investigated the localization of both proteins in the wild type and in the oshC, oshD, or oshE deletion strains. mRFP-TeaA and GFP-TeaR expressed under their native promoters localized at the hyphal tip in the wild type (Fig. 7C). In the oshC and oshD deletion strains, TeaA and TeaR also showed signal accumulation at the hyphal tips, although the GFP signals of TeaR were often observed at the subapical regions (Fig. 7C). Since TeaA and TeaR have been shown to localize at the branching site (80), the TeaR accumulation at the subapical regions might induce more branch formation. The oshE deletion strain did not show any morphological defects, although the SRD signal was reduced. In the oshE deletion strain, almost all hyphae showed signal accumulation of mRFP-TeaA and GFP-TeaR at the hyphal tips, although 3% of the tips GFP-TeaR mislocalized to the subapical membrane regions (Fig. 7C, n = 100).

**DISCUSSION**

We analyzed five Osh orthologues (OshA to -E) in the filamentous fungus *A. nidulans*, since we expected that ergosterol transport would be important for SRDs organization at apical membranes and thereby for polarized growth. This is the first report of an analysis of Osh orthologues in filamentous fungi. To investigate their functions, osh deletion strains were constructed, their localizations were analyzed by fluorescent protein tagging, and their expression patterns were evaluated by qRT-PCR in several conditions. The sensitivities against ergosterol-associated drugs were also investigated. In addition, using the information of the ergosterol biosynthesis pathway in *A. fumigatus*, the regulation of related genes was analyzed in both *A. nidulans* and *A. fumigatus*.

*S. cerevisiae* possesses seven osh genes, whereas *A. nidulans* possesses only five. However, the osh genes in *S. cerevisiae* are classified into four groups, whereas the osh genes in *A. nidulans* are classified into five groups. Other filamentous fungi possess osh genes classified into five groups, with the exception of *A. gossypii*, with only four groups. Deletion of oshE in *A. nidulans*, whose orthologue was not found in the genomes of *S. cerevisiae* and *A. gossypii*, did not show any defect in hyphal growth but reduced the formation of conidiospores (30%). The expression of the oshE was induced during conidiophore development. These results support a specific role of OshE during conidiophore formation.

*S. cerevisiae* Osh4 transfers sterols between membranes as direct nonvesicular ergosterol transporters in vitro (35, 36). Since *S. cerevisiae* Osh1 to Osh7 share the oxysterol-binding protein-related domain, they have a potential to transfer ergosterol between membranes at distinct membrane contact sites depending on their
localization. *A. nidulans* OshA-E also possess the oxysterol-binding protein-related domain as sterol transporters. The GFP-tagged proteins showed different localization patterns. OshA co-localized with the late Golgi marker. OshB and OshE appeared at the contact sites of the PM and ER (PM-ER). OshC and OshD showed the signal in the cytoplasm. It is likely that OshA-E share the molecular mechanism of the enzyme activity as ergosterol transporters, although the functions are separated at different places.

On the other hand, functional redundancies of Osh proteins are known in *S. cerevisiae*. The disruption of any of the six *OSH* genes impaired growth mildly; however, inactivation of all seven *OSH* genes results in lethality (32). The functional redundancies of OshA-E in *A. nidulans* are not yet clear since no double- or multiple-deletion strains have been investigated. Our data, such as our findings regarding localization, gene deletion, and gene expression, however, led us to speculate about some functional redundancies. Both GFP-OshC and GFP-OshD showed signals in the cytoplasm. The deletion of oshC or oshD caused similar phenotypes, such as growth delay and more branching. The expression of oshD is highly induced in the oshB deletion strain and slightly induced in the oshC or oshE deletion strains. OshD may thus be induced to complement the functions of the deleted osh gene.

Deletion of oshE did not show morphological defects in hyphal growth; however, the oshE deletion strain showed only a reduced signal intensity of SRDs. The fact that SRDs are still visible could explain that TeaR localization is only slightly affected. GFP-OshE appeared at the contact sites of the PM-ER. The ergosterol transport by OshE at the PM-ER contact sites could be involved in SRD organization. However, the putative role of OshE is not essential for SRD organization. Since GFP-OshB also appeared at the PM-ER contact sites, at least OshB also might share the redundant function, although the deletion of oshB did not show any defect in hyphal growth or SRD formation. *S. cerevisiae* Osh4p associated with exocytic vesicles targeted sites of polarized growth and facilitated vesicle docking by the exocyst tethering complex at sites of polarized growth on the plasma membrane (49). In addition to the direct nonvesicular ergosterol transport function, Osh proteins are thought to regulate ergosterol transport through several steps at the vesicle biogenesis, transport, and fusion steps (81).

The oshC and oshD deletion strains showed resistance to the antifungal drug voriconazole, which inhibits the ergosterol biosynthesis pathway. Voriconazole affects growth by accumulation of toxic ergosterol intermediates in the ergosterol biosynthesis pathway. The amounts of ergosterol were reduced in the oshC (70%) and oshD (40%) deletion strains compared to that of wild type (Fig. 5G). Therefore, the reduction of ergosterol biosynthesis by voriconazole might affect growth of the oshC and oshD deletion strains less than it does the wild-type strain. Although the amount of ergosterol in the oshD deletion strain was less than that in the oshC deletion strain, the resistance to voriconazole is similar level in both strains. On the other hand, the oshE deletion strain showed resistance to AmB and MβCD, which attack ergosterol at the plasma membrane. Deletion of oshE reduced SRD signals partly. That could be a reason for the resistance of the oshE deletion strain to AmB and MβCD; however, the oshB deletion strain showed resistance to AmB without reduction of the SRD signal.

Our data revealed that the expression of *A. fumigatus oshA* gene was induced in the presence of AmB in an SrbA-dependent man-
ner; however, the regulation of oshA expression by SrbA seems to be indirect. Since SrbA regulates the ergosterol biosynthesis pathway in A. fumigatus, disruption of the ergosterol profile in the srbA deletion strain might affect a transcriptional response to the ergosterol-targeted antifungal drugs. Although there is no report on functional analysis for A. nidulans SrbA, A. fumigatus SrbA has been shown to regulate the expression of cyp51A and cyp51B directly (79). The varied expression levels of cyp51A and cyp51B in A. nidulans osh deletion strains suggest a possible disorder of the SrbA-related ergosterol biosynthesis pathway in the osh deletion strains. The upregulation of cyp51A in the oshC deletion strain and the upregulation of cyp51A and cyp51B in the oshD deletion strain might partly attribute to the voriconazole resistance phenotypes, whereas the oshA and oshE deletion strains, whose expression levels of cyp51A and cyp51B were similar to those of the oshC and oshD deletion strains, respectively, did not show the resistance. Although the oshB deletion strain was more sensitive to voriconazole, the expression levels of cyp51A and cyp51B were similar to those of the wild-type strains. Further analysis of the putative SrbA signaling pathway in A. nidulans is required to understand these results. The amount of ergosterol and expression of cyp51A and cyp51B were changed in the osh deletion strains. The distribution of ergosterol and by-products could be impaired at different organelle membranes in the osh deletion strains. Since there is no report discussing the relationship between the distribution of ergosterol in different cell membranes and sensitivity to ergosterol-related antifungal drug, the further analysis of Osh in Aspergillus is clinically relevant.

The molecular mechanism of polarized hyphal growth has been analyzed intensely over the last two decades (1–6). Whereas the role of the cytokinetics, motor proteins, RhoGTPase, land mark proteins, and membrane transport is understood quite well, the roles of lipid membrane domains remains less clear. The ergosterol distribution and apical SRDs, in addition, e.g., phosphatidylinositol-4,5-bisphosphate, on the plasma membrane are likely to serve as key regulators of fundamental biological processes. Analysis of membrane microdomains at hyphal tips still remains a widely open field and is likely to contribute to a comprehensive understanding of polarized growth in filamentous fungi, possibly opening new avenues for antifungal treatments.

ACKNOWLEDGMENTS

We thank Hanna Haag for the construction of Sec63-mCherry strain and Raphael Manck for the plasmid containing the mCherry-pyrG plasmid. This study was supported by the German Science Foundation (DFG TA819/2-1) and the Baden-Württemberg Stiftung.

REFERENCES

1. Berepiki A, Lichius A, Read ND. 2011. Actin organization and dynamics in filamentous fungi. Nat Rev Microbiol 9:876–887. http://dx.doi.org/10.1038/nrmicro2666.

2. Penalva MA, Galindo A, Abenza JF, Pinar M, Calcagno-Pizarelli AM, Arst HN, Pantazopoulos A. 2012. Searching for gold beyond mitosis: mining intracellular membrane traffic in Aspergillus nidulans. Cell Logist 2:2–14. http://dx.doi.org/10.4161/cell.19304.

3. Riquelme M, Yarden O, Bartnicki-Garcia S, Bowman B, Castro-Longoria E, Free SJ, Fleissner A, Freitag M, Lew RR, Mourino-Perez R, Plamann M, Rasmussen C, Richthammer C, Roberson RW, Sanchez-Leon E, Seiler S, Watters MK. 2011. Architecture and development of the Neurospora crassa hypha – a model cell for polarized growth. Fungal Biol 115:464–476. http://dx.doi.org/10.1016/j.funbio.2011.02.008.

4. Steinberg G. 2011. Motors in fungal morphogenesis: cooperation versus competition. Curr Opin Microbiol 14:660–667. http://dx.doi.org/10.1016/j.mib.2011.09.013.

5. Takeshita N, Manck R, Grun N, de Vega SH, Fischer R. 2014. Interdependence of the actin and the microtubule cytoskeleton during fungal growth. Curr Opin Microbiol 20:34–41. http://dx.doi.org/10.1016/j.mib.2013.09.005.

6. Fischer R, Zekert N, Takeshita N. 2008. Polarized growth in fungi–interplay between the cytoskeleton, positional markers, and membrane domains. Mol Microbiol 68:813–826. http://dx.doi.org/10.1111/j.1365-2958.2008.06193.x.

7. Higashitsuji Y, Herrero S, Takeshita N, Fischer R. 2009. The cell end marker protein TeaC is involved in growth directionality and septation in Aspergillus nidulans. Eukaryot Cell 8:957–967. http://dx.doi.org/10.1128/EC.00251-08.

8. Takeshita N, Higashitsuji Y, Konzack S, Fischer R. 2008. Apical sterol-rich membranes are essential for localizing cell end markers that determine growth directionality in the filamentous fungus Aspergillus nidulans. Mol Biol Cell 19:339–351. http://dx.doi.org/10.1091/mbc.E07-06-0523.

9. Takeshita N, Mania D, Herrero S, Ishitsuka Y, Niemans US, Podolski M, Howard J, Fischer R. 2013. The cell-end marker TeaA and the microtubule polymerase AlpA contribute to microtubule guidance at the hyphal tip cortex of Aspergillus nidulans to provide polarity maintenance. J Cell Sci 126:5400–5411. http://dx.doi.org/10.1242/jcs.129841.

10. Li S, Du L, Yuen G, Harris SD. 2006. Distinct ceramide synthases regulate polarized growth in the filamentous fungus Aspergillus nidulans. Mol Biol Cell 17:1218–1230.

11. Pearson CL, Xu K, Sharpless KE, Harris SD. 2004. MexA, a novel fungal protein required for the stabilization of polarity axes in Aspergillus nidulans. Mol Biol Cell 15:3658–3672. http://dx.doi.org/10.1091/mbc.E03-11-0803.

12. Lingwood D, Simons K. 2010. Lipid rafts as a membrane-organizing principle. Science 327:46–50. http://dx.doi.org/10.1126/science.1174621.

13. Rajendran L, Simons K. 2005. Lipid rafts and membrane dynamics. J Cell Sci 118:1099–1102. http://dx.doi.org/10.1242/jcs.01681.

14. Steuerman CA. 2010. The reggie/flotillin connection to growth. Trends Cell Biol 20:6–13. http://dx.doi.org/10.1016/j.tcb.2009.10.003.

15. Simons K, Toomre D. 2000. Lipid rafts and signal transduction. Nat Rev Mol Cell Biol 1:31–39. http://dx.doi.org/10.1038/35036205.

16. Schuck S, Simons K. 2004. Polarized sorting in epithelial cells: raft clustering and the biogenesis of the apical membrane. J Cell Sci 117:5955–5964. http://dx.doi.org/10.1242/jcs.01596.

17. Alvarez FJ, Douglas LM, Konopka JB. 2007. Sterol-rich plasma membrane domains in fungi. Eukaryot Cell 6:755–763. http://dx.doi.org/10.1128/EC.00008-07.

18. Xu X, Bittman R, Duportail G, Heissler D, Vilchez C, London E. 2001. Effect of the structure of natural sterols and sphingolipids on the formation of ordered sphingolipid/sterol domains (rafts): comparison of cholesterol to plant, fungal, and disease-associated sterols and comparison of sphingomyelin, cerebrosides, and ceramide. J Biol Chem 276:33540–33546.

19. Bagnat M, Simons K. 2002. Cell surface polarization during yeast mating. Proc Natl Acad Sci U S A 99:14183–14188. http://dx.doi.org/10.1073/pnas.172517799.

20. Nichols CB, Fraser JA, Heitman J. 2004. PKA kinases Ste20 and Pkh govern cell polarity at different stages of mating in Cryptococcus neoformans. Mol Biol Cell 15:4476–4489. http://dx.doi.org/10.1091/mbc.E04-05-0370.

21. Wachtler V, Rajagopalan S, Balasubramanian MK. 2003. Sterol-rich plasma membrane domains in the fission yeast Schizosaccharomyces pombe. J Cell Sci 116:867–874. http://dx.doi.org/10.1242/jcs.00299.

22. Martin SW, Konopka JB. 2004. Lipid raft polarization contributes to hyphal growth in Candida albicans. Eukaryot Cell 3:675–684. http://dx.doi.org/10.1128/EC.3.3.675-684.2004.

23. Pike LJ. 2009. The challenge of lipid rafts. J Lipid Res 50(Suppl):S323–S328.

24. Mania D, Hilpert K, Ruden S, Fischer R, Takeshita N. 2010. Screening for antifungal peptides and their modes of action in Aspergillus nidulans. Appl Environ Microbiol 76:7102–7108. http://dx.doi.org/10.1128/AEM.01560-10.

25. Urbani I, Simoni RD. 1990. Cholesterol and vesicular stomatitis virus G protein take separate routes from the endoplasmic reticulum to the plasma membrane. J Biol Chem 265:1919–1923.

26. Baumann NA, Sullivan DP, Ohvo-Rekila H, Simonet C, Pottekat A, Klaassen Z, Beh CT, Menon AK. 2005. Transport of newly synthesized Sterol Transporters (OSH) in Aspergillus nidulans.
sterol to the sterol-enriched plasma membrane occurs via nonvesicular equilibration. Biochemistry 44:5816–5826. http://dx.doi.org/10.1021/ 10648296z.

27. Holthuis JC, Levine TP. 2005. Lipid traffic: floppy domes and a super- highway. Nat Rev Mol Cell Biol 6:209–220. http://dx.doi.org/10.1038/ 10385919.

28. Yang H. 2006. Nonvesicular sterol transport: two protein families and a sterol sensor? Trends Cell Biol 16:427–432. http://dx.doi.org/10.1016/j. tch.2006.07.002.

29. Im YJ, Raychaudhuri S, Prinz WA, Hurley JH. 2005. Structural mechan- ism for sterol sensing and transport by OSBP-related proteins. Nature 437:154–158. http://dx.doi.org/10.1038/nature03923.

30. Stefan CJ, Manford AG, Baird D, Yamada-Handf J, Mao Y, Emr SD. 2011. A Golgi protein regulates phosphoinositide metabolism at ER-plasma membrane contact sites. Cell 144:389–401. http://dx.doi.org/10.1016/j.cell.2010.12.034.

31. Schulz TA, Prinz WA. 2007. Sterol transport in yeast and the oxyester binding protein homologue (OSH) family. Biochim Biophys Acta 1771: 769–780. http://dx.doi.org/10.1016/j.bbabio.2007.03.003.

32. Beh CT, Cool L, Phillips J, Rine J. 2001. Overlapping functions of the yeast oxysterol-binding protein homologues. Genetica 157:117–1140.

33. Drin G, Casella JF, Gautier R, Boehmer T, Schwartz TU, Antonyy B. 2007. A general amphiphilic alpha-helical motif for sensing membrane curvature. Nat Struct Mol Biol 14:138–146. http://dx.doi.org/10.1038/nsm1194.

34. Fairn GD, Curwin AJ, Stefan CJ, McMaster CR. 2007. The oxyester binding protein Kes1p regulates Golgi apparatus phosphatidylinositol-4-phosphate function. Proc Natl Acad Sci U S A 104:15332–15337. http://dx.doi.org/10.1073/pnas.0705571104.

35. Raychaudhuri S, Im YJ, Hurley JH, Prinz WA. 2006. Nonvesicular sterol movement from plasma membrane to ER requires oxyester-binding pro- tein-related proteins and phosphoinositides. J Cell Biol 173:107–119. http:// dx.doi.org/10.1083/jcb.200510084.

36. Schulz TA, Choi MG, Raychaudhuri S, Mears JA, Ghirlando R, Hin- shaw JE, Prinz WA. 2009. Lipid-regulated sterol transfer between closely apposed membranes by an oxyester-binding protein. J Cell Biol 187:889–903. http://dx.doi.org/10.1083/jcb.200905007.

37. Georgiev AG, Sullivan DP, Dittman JS, Beh CT, Menon AK. 2011. Osh proteins regulate membrane sterol organization but are not required for sterol movement between the ER and PM. Traffic 12:1341– 1355. http://dx.doi.org/10.1111/j.1600-085X.2011.01234.x.

38. Levine T. 2004. Short-range intracellular trafficking of small molecules across the plasma membrane. Trends Cell Biol 14:483–490. http://dx.doi.org/10.1016/j.tcb.2004.07.017.

39. Levine T, Loewen C. 2006. Inter-organelle membrane contact sites: through a glass, darkly. Curr Opin Cell Biol 18:371–378. http://dx.doi.org/ 10.1016/j.cjb.2006.06.011.

40. Kvam E, Goldfarb DS. 2006. Structure and function of nucleotide-vacuole junctions: outer-membrane nucleosome targeting of Npt1p and a role in tryptophan uptake. J Cell Sci 119:3622–3633. http://dx.doi.org/10.1242/jcs.03093.

41. Achleitner G, Gaigg B, Krasser A, Kainersdorfer E, Kohlwein SD, Perktold A, Zellnig G, Perktold A, Daum G. 2013. Antifungal azoles: trace element and minimal medium salt stock solutions. Fungal Genet Newsl 48:20–21.

42. Beh CT, Cool L, Phillips J, Rine J. 2001. Overlapping functions of the yeast oxysterol-binding protein homologues. Genetica 157:117–1140.

43. Drin G, Casella JF, Gautier R, Boehmer T, Schwartz TU, Antonyy B. 2007. A general amphiphilic alpha-helical motif for sensing membrane curvature. Nat Struct Mol Biol 14:138–146. http://dx.doi.org/10.1038/nsm1194.

44. Fairn GD, Curwin AJ, Stefan CJ, McMaster CR. 2007. The oxyester binding protein Kes1p regulates Golgi apparatus phosphatidylinositol-4-phosphate function. Proc Natl Acad Sci U S A 104:15332–15337. http://dx.doi.org/10.1073/pnas.0705571104.

45. Raychaudhuri S, Im YJ, Hurley JH, Prinz WA. 2006. Nonvesicular sterol movement from plasma membrane to ER requires oxyester-binding pro- tein-related proteins and phosphoinositides. J Cell Biol 173:107–119. http:// dx.doi.org/10.1083/jcb.200510084.
65. Blatzer M, Barker BM, Willger SD, Beckmann N, Blosser SJ, Cornish Tizzard AC, Bergsma JH, Lloyd-Jones G. 2014. Amphotericin forms an extramembranous and fungicidal sterol sponge. Nat Chem Biol 10:400–406. http://dx.doi.org/10.1038/nchembio.1496.

66. Anderson TM, Clay MC, Cioffi AG, Diaz KA, Harris GS, Tuttle MD, Nieuwkoop AJ, Comellas G, Marym N, Wang S, Uno BE, Wildeman EL, Gonen T, Rienstra CM, Burke MD. 2014. Chlorhexidine: beta-cyclodextrin inhibits yeast growth by extraction of ergosterol. Braz J Microbiol 43:810–818. http://dx.doi.org/10.1590/S1517-83822012000200047.

67. Valitova J, Sulkarnayeva A, Kotlova E, Ponomareva A, Mukhitova FK, Murtazina L, Ryzhkina I, Beckett R, Minibayeva F. 2014. Sterol binding by methyl-beta-cyclodextrin and nystatin-comparative analysis of biochemical and physiological consequences for plants. FEBS J 281:2051–2060. http://dx.doi.org/10.1111/febs.12761.

68. O’Brien J, Wilson I, Orton T, Pogman F. 2000. Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. Eur J Biochem 267:5421–5426. http://dx.doi.org/10.1046/j.1432-1327.2000.01606.x.

69. Tizzard AC, Bergsma JH, Lloyd-Jones G. 2006. A resazurin-based biosensor for organic pollutants. Biosens Bioelectron 22:759–763. http://dx.doi.org/10.1016/j.bios.2006.01.011.

70. Arnold TM, Dotson E, Sarosi GA, Hage CA. 2010. Traditional and emerging antifungal therapies. Proc Am Thorac Soc 7:222–228. http://dx.doi.org/10.1513/pats.200906-048AL.

71. Watanabe Y, Umezawa Y, Takano T, Tsunekawa H, Matsumori N, Murata M. 2014. Effect of sterol side chain on ion channel formation by amphotericin B in lipid bilayers. Biochemistry 53:3088–3094. http://dx.doi.org/10.1021/bi500122c.

72. Blatzer M, Barker BM, Willger SD, Beckmann N, Blosser SJ, Cornish Tizzard AC, Bergsma JH, Lloyd-Jones G. 2014. Amphotericin forms an extramembranous and fungicidal sterol sponge. Nat Chem Biol 10:400–406. http://dx.doi.org/10.1038/nchembio.1496.

73. Willger SD, Grahl N, Cramer RA, Jr. 2009. Aspergillus fumigatus metabolism: clues to mechanisms of in vivo fungal growth and virulence. Med Mycol 47(Suppl 1):S72–S79. http://dx.doi.org/10.1080/13693780802455313.

74. Chung D, Barker BM, Carey CC, Merriman B, Werner ER, Lechner BE, Dhingra S, Cheng C, Xu W, Blosser SJ, Morohashi K, Mazurie A, Mitchell TK, Haas H, Mitchell AP, Cramer RA. 2014. ChIP-seq and in vivo transcriptome analyses of the Aspergillus fumigatus SREBP SrbA reveals a new regulator of the fungal hypoxia response and virulence. PLoS Pathog 10:e1004487. http://dx.doi.org/10.1371/journal.ppat.1004487.

75. Grahl N, Sheppardson KM, Chung D, Cramer RA. 2012. Hypoxia and fungal pathogenesis: to air or not to air? Eukaryot Cell 11:560–570. http://dx.doi.org/10.1128/EC.00031-12.

76. Bellado E, Garcia-Effron G, Bittagro MJ, Alazar-Fuoli L, Cuenca-Estrella M, Rodriguez-Tudela JL. 2005. Targeted gene disruption of the 14-alpha sterol demethylase (cyp51A) in Aspergillus fumigatus and its role in azole drug susceptibility. Antimicrob Agents Chemother 49:2536–2538. http://dx.doi.org/10.1128/AAC.49.6.2536-2538.2005.

77. Buied A, Moore CB, Denning DW, Bowyer P. 2013. High-level expression of cyp51B in azole-resistant clinical Aspergillus fumigatus isolates. J Antimicrob Chemother 68:512–514. http://dx.doi.org/10.1093/jac/dks451.

78. Blosser SJ, Cramer RA. 2012. SREBP-dependent triazole susceptibility in Aspergillus fumigatus is mediated through direct transcriptional regulation of erg11A (cyp51A). Antimicrob Agents Chemother 56:248–257. http://dx.doi.org/10.1128/AAC.05027-11.

79. Takeshita N, Fischer R. 2011. On the role of microtubules, cell end markers, and septal microtubule organizing centres on site selection for polar growth in Aspergillus nidulans. Fungal Biol 115:506–517. http://dx.doi.org/10.1016/j.funbio.2011.02.009.

80. Johansen J, Ramanathan V, Beh CT. 2012. Vesicle trafficking from a lipid perspective: lipid regulation of exocytosis in Saccharomyces cerevisiae. Cell Logist 2:151–160. http://dx.doi.org/10.4161/cl.20490.

81. Purschwitz J, Muller S, Kastner C, Schoster M, Haas H, Espso EA, Atoumi A, Calvo AM, Fischer R. 2008. Functional and physical interaction of blue- and red-light sensors in Aspergillus nidulans. Curr Biol 18:255–259. http://dx.doi.org/10.1016/j.cub.2008.01.061.