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# Deficiency of D-Erythroascorbic Acid Attenuates Hyphal Growth and Virulence of *Candida albicans*

WON-KI HUH,[1] SEONG-TAE KIM,[1] HYUNGSOO KIM,[2] GAJIN JEONG,[2] AND SA-OUK KANG[1]*

*Laboratory of Biophysics[1] and Laboratory of Immunology,[2] School of Biological Sciences and Institute of Microbiology, Seoul National University, Seoul 151-742, Republic of Korea*

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**In some lower eukaryotes, D-erythroascorbic acid, a five-carbon analog of L-ascorbic acid, is present instead of L-ascorbic acid. We have cloned *ALO1*, the gene encoding D-arabinono-1,4-lactone oxidase, which catalyzes the final step of D-erythroascorbic acid biosynthesis in *Candida albicans*. The *ALO1* gene contained a continuous open reading frame of 1,671 bp that encodes a polypeptide consisting of 557 amino acids with a calculated molecular mass of 63,428 Da. To investigate the functional roles of D-erythroascorbic acid in *C. albicans*, we disrupted or overexpressed the *ALO1* gene. In the *alo1*/*alo1* null mutants, the activity of D-arabinono-1,4-lactone oxidase was completely lost and D-erythroascorbic acid could not be detected. When *ALO1* on a multicopy plasmid was transformed in *C. albicans*, the enzyme activity and the intracellular D-erythroascorbic acid level were increased up to 3.4-fold and 4.0-fold, respectively. The *alo1*/*alo1* null mutants of *C. albicans* showed increased sensitivity towards oxidative stress. Overexpression of *ALO1* made the cells more resistant to the same stress. The *alo1*/*alo1* mutants showed defective hyphal growth and attenuated virulence. Taken together, our results suggest that D-erythroascorbic acid functions as an important antioxidant and can be considered one of the virulence factors enhancing the pathogenicity of *C. albicans*.**

L-Ascorbic acid (ASC) is produced in all higher plants and in nearly all higher animals except human, other primates, guinea pig, some birds, and fish (1, 3). In animals, a microsomal L-gulono-1,4-lactone oxidase catalyzes the final step of ASC biosynthesis (15, 29). Koshizaka et al. (17) isolated and characterized a cDNA encoding L-gulono-1,4-lactone oxidase from rat liver. Recently, a biosynthetic pathway for ASC involving L-galactose and L-galactono-1,4-lactone in plants has been proposed (39). It is believed in plants that the final step of ASC biosynthesis is catalyzed by L-galactono-1,4-lactone dehydrogenase (25, 30). The cDNAs encoding L-galactono-1,4-lactone dehydrogenase in cauliflower (31) and sweet potato (11) have been isolated and analyzed. In some eukaryotic microorganisms, ASC is rare or absent but D-erythroascorbic acid (EASC), a five-carbon analog of ASC, is present (5, 24, 27, 28). In *Candida albicans* and *Saccharomyces cerevisiae*, the biosynthetic pathway of EASC from D-arabinose by D-arabinose dehydrogenase and D-arabinono-1,4-lactone oxidase (ALO) has been established (9, 10, 13, 14). ALO can also catalyze the production of ASC when L-galactono-1,4-lactone is supplied as a substrate (20).

ASC is known to carry out a number of biochemical functions that are a consequence of its ability to donate one or two electrons. Some known or proposed functions of ASC include its utilization as a free radical scavenger, a cofactor for a number of enzymes, and a controlling factor in plant cell development (26). However, many other functions of the ASC system as well as the precise mechanisms of its functions are still elusive. According to Shao et al. (33), EASC is almost as readily oxidized as ASC in an aqueous system and has reducing power similar to that of ASC. In a bioassay using tobacco hornworm (*Manduca sexta*) to determine the vitamin C activity of EASC, EASC supported the larval growth of the hornworm almost as well as ASC. This report suggests that EASC has biological properties similar to those of ASC. Considering that some eukaryotic microorganisms produce EASC instead of ASC, it is presumed that EASC may take the place of ASC in these microorganisms. In our previous study, EASC has been proved an important antioxidant molecule in *S. cerevisiae* (10), like ASC in animals and plants.

*C. albicans* is a well-known opportunistic fungal pathogen of humans that does not usually cause disease in immunocompetent hosts but causes serious diseases in immunocompromised patients. A number of factors have been implicated to be associated with the virulence properties of *C. albicans*, such as adhesion to the host tissues, secretion of proteases, and reversible morphological transitions between yeasts, pseudohyphae, and hyphae (4). Recent studies have led to the identification of several genes involved in the transition from yeast-like growth to hyphal growth in *C. albicans*. Deletion of the *Candida* genes in a MAPK pathway, such as *CST20, HST7*, and *CPHl*, results in impairment of the ability to make hyphae under some conditions, albeit not in response to serum (16, 19, 22), suggesting that there is more than one pathway controlling hyphal growth. Another gene, *EFG1*, a homolog of *S. cerevisiae PHD1*, has been found in *C. albicans*, and its reduced expression causes loss of hyphal growth (36). The *cphl*/*cphl efgl*/*efgl* double mutants of *C. albicans* are unable to form hyphae under almost all laboratory conditions tested and are avirulent in a mouse model (23). These studies demonstrate the importance of the transition from yeast-like to hyphal growth in the virulence of *C. albicans*. The ability to adhere to the host tissues has been also proved important in the pathogenicity of *C. albicans*. Re-

* Corresponding author. Mailing address: Laboratory of Biophysics, School of Biological Sciences, and Institute of Microbiology, Seoul National University, Seoul 151-742, Republic of Korea. Phone: (82) (2) 880 6703. Fax: (82) (2) 888 4911. E-mail: kangsaou@plaza.snu.ac.kr.

TABLE 1. *C. albicans* strains used in this study

| Strain | Genotype | Source or reference |
|---|---|---|
| ATCC 10231 | Wild-type isolate | ATCC[a] |
| SC5314 | Wild-type isolate | 6 |
| CAI4 | Δ*ura3*::*imm434*/Δ*ura3*::*imm434* | 6 |
| JKC19 | Δ*ura3*::*imm434*/Δ*ura3*::*imm434 cphl*::*hisGlcph1*::*hisG-URA3-hisG* | 22 |
| HLC52 | Δ*ura3*::*imm434*/Δ*ura3*::*imm434 efg1*::*hisGlefgl*::*hisG-URA3-hisG* | 23 |
| WH201 | Δ*ura3*::*imm434*/Δ*ura3*::*imm434* Δ*alo1*::*hisG-URA3-hisG*/*ALO1* | This study |
| WH202 | Δ*ura3*::*imm434*/Δ*ura3*::*imm434* Δ*alo1*::*hisG*/*ALO1* | This study |
| WH203 | Δ*ura3*::*imm434*/Δ*ura3*::*imm434* Δ*alo1*::*hisG*/Δ*alo1*::*hisG-URA3-hisG* | This study |
| WH204 | Δ*ura3*::*imm434*/Δ*ura3*::*imm434* Δ*alo1*::*hisG*/Δ*alo1*::*hisG* | This study |
| WH205 | Δ*ura3*::*imm434*/Δ*ura3*::*imm434* (pRC2312) | This study |
| WH206 | Δ*ura3*::*imm434*/Δ*ura3*::*imm434* (pWK203) | This study |
| WH207 | Δ*ura3*::*imm434*/Δ*ura3*::*imm434* Δ*alo1*::*hisG*/Δ*alo1*::*hisG*::*ALO1*::*URA3* | This study |

[a] American Type Culture Collection.

cently, Int1p, a surface protein with limited similarity to vertebrate integrins, has been found in *C. albicans*. Disruption of *INT1* in *C. albicans* suppresses hyphal growth, adhesion to epithelial cells, and virulence in mice (8). Another hypha-specific surface protein, Hwplp, with similarities to small mammalian proline-rich proteins, has been found in *C. albicans* and shown to serve as a substrate for mammalian transglutaminases. The *hwpl*/*hwpl* mutants of *C. albicans* are unable to form stable attachments to human buccal epithelial cells and have a reduced capacity to cause systemic candidiasis in mice (35).

To fully understand the pathogenicity of *C. albicans*, survival traits should also be taken into consideration, in addition to virulence traits. Survival indicates the ability of *C. albicans* to defend itself against the host immune system and grow in the host successfully. In the present study, we describe the isolation and characterization of the gene encoding ALO (*ALO1*), which catalyzes the final step of EASC biosynthesis in *C. albicans*. We show that EASC serves as an important antioxidant, contributes to hyphal growth, and is essential for *C. albicans* to exhibit full virulence, presumably by enhancing survival of the organism in the host.

## MATERIALS AND METHODS

**Yeast strains and culture conditions.** *C. albicans* strains used in this study are listed in Table 1. The strains were routinely cultured on YPD medium (1% yeast extract, 2% peptone, and 2% glucose) at 28°C. Cells containing plasmids or disrupted genes were cultured in minimal defined medium containing 0.67% yeast nitrogen base without amino acids (Difco), 2% glucose, and appropriate supplements (34). Solid media were prepared by adding 1.8% agar to liquid broth. To assess filamentation on solid media, $10^4$ cells in 2 μl of water were spotted onto the plates and incubated for 3 to 4 days.

**Isolation, subcloning, and sequencing of *ALO1* from *C. albicans*.** To construct a *C. albicans* genomic library, the genomic DNA from *C. albicans* ATCC 10231 was partially digested with *Sau*3AI and DNA fragments of 10 to 23 kb were ligated into dephosphorylated λEMBL3 vector (Stratagene) generated by *Bam*HI cleavage. The ligated DNA was packaged using Gigapack II packaging extracts (Stratagene) and replicated according to the manufacturer's instructions. Then, degenerate oligonucleotide primers corresponding to residues 52 to 58 (VGSGHSP) and 444 to 450 (GGKPHWA) of *S. cerevisiae ALO1* (10) were synthesized: 5′-GTTGGTTCYGGCCAYTCYCC-3′ and 5′-GGCCCARTGTG GCTTACCTCC-3′, respectively, where Y represents C or T and R represents A or G. PCR amplification was carried out using the genomic DNA from *C. albicans* ATCC 10231 as a template under the following conditions: denaturation at 94°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 2 min. Among the amplified DNA products, a DNA fragment of 1,359 bp was cloned into pGEM-T vector (Promega). The insert DNA fragment was labeled with digoxigenin (Roche Molecular Biochemicals) and used as a probe to screen the λEMBL3 genomic library. Four positive clones were selected, and the common 3.8-kb *Hin*dIII fragment giving a positive signal was isolated and cloned into pGEM-7Zf(+) (Promega) at the *Hin*dIII site, yielding pCALO. Both stands of the cloned DNA were sequenced by dideoxy chain termination method with an automatic sequencer (ALFexpress; Amersham Pharmacia Biotech).

**Disruption, overexpression, and reintegration of *C. albicans ALO1*.** Both alleles of *ALO1* were disrupted by using the URA blaster technique (6). A 4.1-kb fragment containing the *hisG-URA3-hisG* gene disruption cassette from p5921 (6) was inserted in place of a portion of *ALO1* within the genomic clone (see Fig. 2A). The resulting plasmid, pWK202, was cut with *Apa*I and *Sac*I to remove the vector and transformed into the *ura3*/*ura3 C. albicans* strain CAI4 (6). $Ura^+$ transformants were selected on uracil-deficient medium, and the integration of the cassette into the *ALO1* locus was verified by either PCR or Southern blot analysis. Spontaneous $Ura^-$ derivatives of the heterozygous disruptants were selected on minimal defined medium supplemented with 625 mg of 5-fluoroorotic acid and 30 mg of uridine per liter. This procedure was repeated to delete the remaining functional allele of *ALO1*.

In order to overexpress *ALO1*, a 3.8-kb *Hin*dIII fragment containing the entire coding sequence of *ALO1* and its 5′ and 3′ flanking regions was isolated from pCALO and inserted into the *Hin*dIII site of pRC2312, which contains *LEU2* and *URA3* from *C. albicans* as selectable markers and an autonomously replicating sequence from *C. albicans* for replication in *C. albicans* and *S. cerevisiae* (2). The resulting plasmid pWK203 was transformed into CA14 strain and selected for the $Ura^+$ phenotype.

For reintegration of the *ALO1* gene into the genome, a 3.8-kb *Nsi*I/*Xho*I fragment with the *ALO1* gene from pCALO was inserted into pURA3, a plasmid containing the *C. albicans URA3* gene, at the *Nsi*I/*Sal*I sites to yield pWK204. pWK204 was linearized at the unique *Hpa*I site within the *ALO1* coding region and used to integrate into the *ALO1* locus in the $Ura^-$ *alo1*/*alo1* strain. The occurrence of the desired integration events in all the transformants was verified by Southern blot analysis.

**Measurement of ALO activity and intracellular EASC level.** The activity of ALO was measured spectrophotometrically in 0.2 M potassium phosphate (pH 6.1), 1 mM EDTA, 50 mM D-arabinono-1,4-lactone, and an aliquot of enzyme. The production of EASC ($\varepsilon_{265}$ = 13,150 $M^{-1} \cdot cm^{-1}$) was monitored by the increase in the absorbance at 265 nm during the first 1 min of the reaction at 36°C. One unit of the enzyme was defined as the amount of enzyme that produced 1 μmol of EASC per min. The lower limit for assay of ALO activity was 0.1 mU · mg of $protein^{-1}$.

The amount of EASC was measured as described previously (10). *C. albicans* cells (1 g [wet mass]) grown in liquid minimal defined medium were recovered by centrifugation at 6,000 × *g* for 5 min at 4°C, washed twice with distilled water, and resuspended in 2 ml of 10% trichloroacetic acid. This suspension was stored for 20 min at 4°C. The insoluble residue was removed by centrifugation at 10,000 × *g* for 10 min at 4°C, and the soluble extracts were subjected to analytical high-performance liquid chromatography using a Waters Associates liquid chromatography system linked to a Waters 460 electrochemical detector. The potential of the detector was set at +0.70 V versus the Ag/AgCl reference electrode. Ten microliters of trichloroacetic acid-soluble extracts was passed through two tandemly linked Hewlett Packard octyldecylsilane Hypersil columns (10 cm by 4.6 mm) and eluted with 0.1% trifluoroacetic acid at a flow rate of 0.7 ml · $min^{-1}$. The lower limit for detection of EASC was 0.1 nmol · g (wet wt) of $cells^{-1}$.

**Assay of resistance to oxidative stress.** The susceptibility of the cells to $H_2O_2$ and menadione was measured as described previously (10), with some modifications. Cells were grown in minimal defined medium to mid-logarithmic phase ($5 \times 10^6$ cells · $ml^{-1}$), harvested, and resuspended in 0.1 M potassium phosphate

buffer, pH 7.0, to obtain an initial optical density at 600 nm of 0.1. To observe the sensitivity of the cells to oxidants, various concentrations of $H_2O_2$ or menadione were added to the cell suspensions. After incubation for 1 h at 30°C, aliquots were taken from the cell suspensions, diluted appropriately in the same buffer, and plated onto solid minimal defined medium. Colonies were counted after incubation for 3 days at 28°C.

**Assay of *C. albicans* virulence.** Inbred female BALB/c mice (Seoul National University Laboratory Animal Center) weighing between 18 and 20 g were used for testing the virulence of *C. albicans* strains according to the method described previously (23). Statistical analyses of the differences in survival between paired groups were performed with the Kaplan-Meier log-rank test. A *P* value of 0.05 was taken to indicate statistical significance.

**Nucleotide sequence accession number.** The nucleotide sequence data of the *ALO1* gene have been deposited in the GenBank/EMBL/DDBJ database under accession no. AF031228.

## RESULTS

**Isolation and characterization of *ALO1*, which encodes ALO in *C. albicans*.** From the comparison of the predicted amino acid sequence of rat L-gulono-1,4-lactone oxidase (17) and that of *S. cerevisiae* ALO (10), two highly conserved regions were identified. PCR using the oligonucleotide primer pair corresponding to residues 52 to 58 (VGSGHSP) and 444 to 450 (GGKPHWA) of *S. cerevisiae* ALO could amplify a DNA fragment of 1,359 bp from the chromosomal DNA of *C. albicans* ATCC 10231. When cloned and sequenced, the fragment showed a high degree of amino acid sequence similarity to *S. cerevisiae* ALO upon BLAST searches of the GenBank database. The cloned PCR product was used as a probe to screen the λEMBL3 genomic library of *C. albicans*. From positive clones, the common 3.8-kb *Hin*dIII fragment was subcloned in pGEM-7Zf(+) and sequenced.

The cloned *ALO1* gene contains a continuous open reading frame of 1,671 bp that encodes a polypeptide consisting of 557 amino acids with a calculated molecular mass of 63,428 Da. When the nucleotide sequence of the cloned *ALO1* gene was compared with that of the same gene obtained from the *Candida* genome project (http://www-sequence.stanford.edu/group/candida), five mismatches were found within the coding region of the gene. However, the five mismatches of nucleotide sequence did not alter the amino acid sequence of the gene. *C. albicans ALO1* contained no CUG codon, which encodes serine in *C. albicans* but encodes leucine in *S. cerevisiae* and elsewhere (32). The nucleotide sequence of *ALO1* did not have the consensus sequence for splicing. The fact that the gene contains no intron was confirmed by reverse transcription-PCR (data not shown). The predicted amino acid sequence of *C. albicans* ALO shared 53, 32, and 24% identity with those of *S. cerevisiae* ALO (10), rat L-gulono-1,4-lactone oxidase (17), and cauliflower L-galactono-1,4-lactone dehydrogenase (31), respectively (Fig. 1). The hydropathy plot of ALO using the method of Kyte and Doolittle (18) predicted that the enzyme should be an integral membrane protein with a transmembrane segment corresponding to amino acid residues 188 to 204. This prediction agreed well with a previous report (9) in which ALO was suggested to be a mitochondrial membrane protein. Like *S. cerevisiae* ALO and rat L-gulono-1,4-lactone oxidase, *C. albicans* ALO also had a putative binding site for covalently bound flavin adenine dinucleotide (FAD) of oxygen-dependent oxidoreductases (7), corresponding to amino acid residues 29 to 62. The site has been found in some oxygen-dependent oxidoreductases, all of which contain a covalently bound FAD group that is attached to a histidine via an 8α-[*N*(1)-histidyl]FAD or 8α-[*N*(3)-histidyl]FAD linkage. Kenney et al. (12) reported that *S. cerevisiae* ALO contains a covalently bound FAD linked to the *N*(1) position of histidine. In case of *C. albicans* ALO, however, FAD proved to be covalently linked to the *N*(3) position of histidine (S.-T. Kim, W.-K. Huh, and S.-O. Kang, unpublished data). Based on these facts, we suggest that the region of amino acid residues 29 to 62 is the covalent FAD-binding site and that the histidine at position 62 is the amino acid covalently linked to FAD in *C. albicans* ALO.

On genomic Southern blot analysis, only one band was detected when the genomic DNA was digested with *Bgl*II, *Hin*dIII, *Nde*I, or *Xba*I, and two bands were detected when DNA was digested with *Eco*RI (data not shown). Since the nucleotide sequence of the *ALO1* open reading frame contained one *Eco*RI and no *Bgl*II, *Hin*dIII, *Nde*I, or *Xba*I site, the hybridization pattern indicates that there is a single copy of *ALO1* per *C. albicans* genome. On Northern blot analysis, a single band corresponding to a size of 2.0 kb was detected (data not shown). As in *S. cerevisiae* (10), the activity of ALO and the *ALO1* transcript level remained essentially unchanged whether *C. albicans* cells were treated with oxidants such as $H_2O_2$ and menadione (data not shown), suggesting that *ALO1* is constitutively expressed and not regulated in response to oxidative stress.

**Disruption and overexpression of *C. albicans ALO1*.** For the gene disruption, a disruption construct was prepared by replacing a portion of the coding region of *ALO1* with the *hisG-URA3-hisG* sequence (Fig. 2A) and used to transform the *ura3/ura3 C. albicans* strain CAI4. The resulting $Ura^+$ transformants were screened by PCR or Southern blot analysis, and the spontaneous $Ura^-$ "pop-out" revertants from them were selected on minimal defined medium containing 5-fluoroorotic acid. A homozygous disruption of *ALO1* was generated by repeating the above procedure and confirmed by Southern blot analysis (Fig. 2B). The *alo1/alo1* mutants did not show any auxotrophy and grew normally in minimal defined medium as well as in complex medium. They also showed normal growth patterns when grown in the media with nonfermentable carbon sources such as ethanol and glycerol. In order to overexpress ALO in *C. albicans*, we constructed the plasmid pWK203 by inserting the entire *ALO1* gene and its flanking sequences into the plasmid pRC2312, as described in Materials and Methods. *C. albicans* cells were transformed with the parental plasmid pRC2312 or pWK203, and transformants containing either plasmid were selected by plating on uracil-deficient medium. As originally reported by Cannon et al. (2), transformation with either pRC2312 or pWK203 resulted in small, slow-growing colonies at high frequency and larger, fast-growing colonies at a lower frequency. According to Cannon et al. (2), the small colonies are replicative transformants with a plasmid copy number of 2 or 3 per genome, and the larger colonies are integrative transformants, with the copy number of the integrated sequence being estimated to be 7 to 12 per diploid genome. For further experiments, we selected the larger, fast-growing colonies.

We measured the activity of ALO and the intracellular level of EASC in the strains in which the *ALO1* gene has been disrupted or overexpressed. As expected, the *alo1/alo1* mutant strain WH203 did not show any ALO activity. The ALO ac-

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Ca-ALO  ---------------------------------------------------------------------------     -
Sc-ALO  ---------------------------------------------------------------------------     -
Rn-GLO  ---------------------------------------------------------------------------     -
Bo-GLD  MLRSLLLRRSNARSLRPPFPPLRTLCTSGQTLTPAPPPPPPPPPPISSSASEKEFRKYAGYAALALFSGAATYFS    75

                                                                       *
Ca-ALO  ---------MTDIPESLKPFVTKKVIHSTWAGTFLCKPQAIFQPRNVEEIQELIKQARLHGKTIMTVGSGHSPSD    66
Sc-ALO  ---------MSTIP------FRKNYVFKNWAGIYSAKPERYFQPSSIDEVVELVKSARLAEKSLVTVGSGHSPSN    60
Rn-GLO  -----------------MVHGYKGVQFQNWAKTYGCSPEVYYQPTSVEEVREVLALAREQKKKVKVVGGHSPSD    58
Bo-GLD  FPFPENAKHKKAQIFRYAPLPEDLHTVSNWSGTHEVQTRNFNQPETLADLEALVKEAHEKKNRIRPVGSGLSPNG   150

Ca-ALO  LTMTTEWLCNLDKFNHVLLEEPYYAPKSPTDDTPEIKFVDLTVEAGTRIFELNEYLKRNNLAIQNLGSISDQSIA   141
Sc-ALO  MCVTDEWLVNLDRLDKVQKFVEY----------PELHYADVTVDAGMRLYQLNEFLGAKGYSIQNLGSISEQSVA   125
Rn-GLO  IACTDGFMIHMGKMNRVLQ--------------VDKEKKQITVEAGILLADLHPQLDEHGLAMSNLGAVSDVTVA   119
Bo-GLD  IGLSRSGMVNLALMDKVLE--------------VDKEKKRVRVQAGIRVQQLVDAIQEYGLTLQNFASIREQQIG   211

Ca-ALO  GLISTGTHGSTQYHGLVSQQVVSVKFLNSAGELITCSSVDKPEYFRAILLSLGKIGIITHVTLRTCPKYTIKSKQ   216
Sc-ALO  GIISTGSHGSSPYHGLISSQYVNLTIVNGKGELKFLDAENDPEVFKAALLSVGKIGIIVSATIRVVPGFNIKSTQ   200
Rn-GLO  GVIGSGTHNTGIKHGILATQVVALTLMTADGEVLECSESRNADVFQAARVHLGCLGIILTVTLQCVPQFHLQETS   194
Bo-GLD  GIIQVGAHGTGARLPPIDEQVIGMKLVTPAKGTIELSKDNDPELFHLARCGLGGLGVVAEVTLQCVERQELLEHT   286

Ca-ALO  EIINFETLLNNWDNLWLESEFIRIWWFPYTNKCVLWRANKSTDPLSDPR-PSWYGTKLGRFFYESLLWVSVHLFP   290
Sc-ALO  EVITFENLLKQWDTLWTSSEFIRVWWYPYTRKCVLWRGNKTTDAQNGPA-KSWWGTKLGRFFYETLLWISTKIYA   274
Rn-GLO  FPSTLKEVLDNLDSHLKRSEYFRFLWFPHTENVSIIYQDHTNKAPSSAS-NWFWDYAIGFYLLEFLLWTSTYL-P   267
Bo-GLD  YVSTLEEIKKNHKKLLSTNKHVKYLYIPYTDTVVVVTCNPVSKWSGAPKDKPKYTTEEALKHVRDLYRES-----   356

Ca-ALO  RLTPFVEKFVFGQQYGEVETLGKGDIAVQNSVEGLNMDCLFSQFVNEWSSPLNSGPEILTELKKIITDASQTGDF   365
Sc-ALO  PLTPFVEKFVFNRQYGKLEKSSTGDVNVTDSISGFNMDCLFSQFVDEWGCPMDNGLEVLRSLDHSIAQAAINKEF   349
Rn-GLO  CLVGWINRFFFWMLFNCKKESS------NLSHKIFTYECRFKQHVQDWAIPREKTKEALLELKAMLEA---HPKV   333
Bo-GLD  -----IVKYRVQDSSKKTPDSREPDINELSFTELRDKLIALDPLNDVHVGKVNQAEAEFWKKSEGYRVGWSDEIL   426

Ca-ALO  FVHAPIEVRCSNVTYSDEPFTDDKNQKSLYPSQEWLSNRSKTSAGPIPGNNLRPYLDNSPKL-PYSKDGKITNDQ   439
Sc-ALO  YVHVPMEVRCSNTTLPSEPLDT-------------SKRTNTSPGPVYGNVCRPFLDNTPSHCRFAPLENVTNSQ   410
Rn-GLO  VAHYPVEVRFTRGD---------------------------------DILLSPCFQRDSC-------------   360
Bo-GLD  GFDCGGQQWVSETCFPAGTLAKPSMKDLEYIEQLKELIQKEAIPAPSPIEQRWTGRSKSPMSPAFSTAEEDIFSW   501

Ca-ALO  LTLFINATMYRPFGTNVETHKWFQLFEDVMSKA----GGKPHWAKNFIGLTQDEKYDKQQDLKTQLEFGGKPFYT   510
Sc-ALO  LTLYINATIYRPFGCNTPIHKWFTLFENTMMVA----GGKPHWAKNFLGSTTLA----AGPVKKDTDYDD---FE   474
Rn-GLO  ---YMNIIMYRPYGKDVPRLDYWLAYETIMKKF----GGRPHWAKAHNC--------------TRKDFEEMY---   411
Bo-GLD  VGIIMYLPTADPRQRKDITDEFFHYRHLTQAKLWDQYSAYEHWAKIEIPKDKEELEALQERLRKRFPVD------   570

Ca-ALO  MLGFKPVMQDWFGKDLVAFNKVRKETDPDGVFLS---GKVWAERNGILLD-----   557
Sc-ALO  MRGMALKVEEWYGEDLKKFRKIRKEQDPDNVFLA---NKQWAIINGIIDPSELSD   526
Rn-GLO  -------------PTFHKFCDIREKLDPTGMFLNSYLEKVFY-------------   440
Bo-GLD  ----------------AYNKARRELDPNRILSNNMVEKLFPVSKTA---------   600
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FIG. 1. Alignment of the deduced amino acid sequence of ALO from *C. albicans* (Ca-ALO) with those of other enzymes with similar activity: ALO from *S. cerevisiae* (10) (Sc-ALO), L-gulono-1,4-lactone oxidase from rat (17) (Rn-GLO), and L-galactono-1,4-lactone dehydrogenase from cauliflower (31) (Bo-GLD). Numbers on the right are amino acid positions. The regions where the sequences have been extended to allow optimal sequence alignment are indicated with dashes. Identical residues are shaded. The asterisk indicates the histidine residue believed to be responsible for covalent attachment of FAD. A 17-residue putative transmembrane segment predicted according to the method by Kyte and Doolittle (18) is underlined.

tivity of WH206 carrying the plasmid pWK203 increased up to 3.4-fold compared with that of WH205 carrying the parental vector pRC2312 (Table 2). When the intracellular content of EASC was measured with electrochemical detector, it was impossible to detect EASC in WH203. On the other hand, WH206 showed a marked increase of EASC compared with the control strain WH205. The content of EASC in WH206 was estimated to be 4.0-fold higher than that of WH205 (Table 2). The *ALO1* reintegrant strain WH207 showed intermediate levels of ALO activity and EASC content.

**Effect of EASC on resistance to oxidative stress.** We tested whether disruption or overexpression of *ALO1* affects the survival of the cells under oxidative stress conditions. For this purpose, exponentially growing cells were treated with various concentrations of $H_2O_2$ or menadione, a redox-cycling agent, and the viable cells were counted. As shown in Fig. 3A, the *alo1*/*alo1* mutant strain WH203, which is devoid of EASC, was more sensitive to $H_2O_2$ and menadione than the parental wild-type strain SC5314, and the susceptibility of WH207 to oxidative stress was intermediate. When WH206 with a high EASC content was challenged with the same oxidants, it showed increased resistance to oxidative stress compared with the control strain WH205 (Fig. 3B). These results indicate that EASC functions as an important antioxidant in *C. albicans*. However,

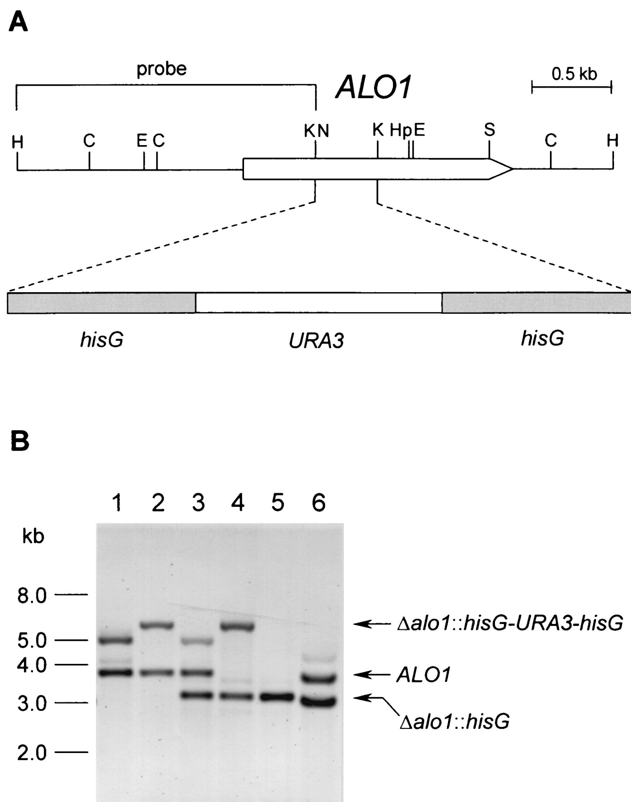


FIG. 2. Disruption of the *ALO1* gene in *C. albicans*. (A) Restriction map of the *ALO1* locus and insertion of the *hisG-URA3-hisG* cassette at the *Kpn*I sites in *ALO1* coding sequence. Endonuclease restriction sites: C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; Hp, *Hpa*I; K, *Kpn*I; N, *Nco*I; S, *Spe*I. (B) Southern blot analysis with the sequence bracketed in panel A used as a probe. The DNA digested with *Hin*dIII was from the following strains: CAI4 *ALO1*/*ALO1* (lane 1), WH201 Δ*alo1*::*hisG-URA3-hisG*/*ALO1* (lane 2), WH202 Δ*alo1*::*hisG*/*ALO1* (lane 3), WH203 Δ*alo1*::*hisG*/Δ*alo1*::*hisG-URA3-hisG* (lane 4), WH204 Δ*alo1*::*hisG*/Δ*alo1*::*hisG* (lane 5), and WH207 Δ*alo1*::*hisG*/Δ*alo1*::*hisG*::*ALO1*::*URA3* (lane 6).

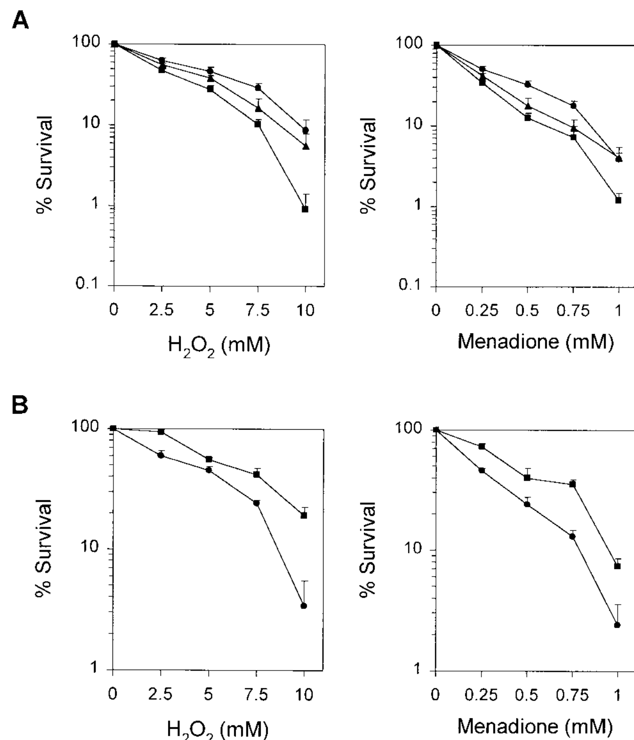


FIG. 3. Effect of disruption or overexpression of *ALO1* on the cell survival against oxidative stress. (A) Sensitivities of the *ALO1*/*ALO1* SC5314 strain (●), the *alo1*/*alo1* WH203 strain (■), and the *ALO1* reintegrant WH207 strain (▲) to $H_2O_2$ and menadione. (B) Sensitivities of the WH205 strain carrying parental vector pRC2312 (●) and the WH206 strain carrying pWK203 (■) to $H_2O_2$ and menadione. Exponentially growing cells were treated with each oxidant at various concentrations for 1 h at 30°C. Data are means plus standard errors of three independent experiments.

disruption or overexpression of *ALO1* did not affect the cell survival under other stress conditions, e.g., heat shock (40°C for 30 min) or osmotic shock (1 M NaCl).

**Effect of EASC on hyphal growth of *C. albicans*.** To investigate the effect of the *alo1* mutation on hyphal growth of *C. albicans*, isogenic $Ura^+$ prototrophs were grown on liquid and solid media that induce hyphal growth, e.g., 20% serum, Lee's medium (21), Spider medium (22), corn meal agar (Difco), and RPMI 1640 (Gibco BRL). When grown on solid Spider medium, the parental wild-type strain SC5314 formed extensive agar-invasive hyphae after 3 days (Fig. 4). The $Ura^+$ *alo1*/*ALO1* heterozygote strain WH201 showed a slight reduction in the extent of hyphal formation. The $Ura^+$ *alo1*/*alo1* strain WH203 showed little hyphal formation compared with SC5314. The hyphal growth of the *ALO1* reintegrant strain WH207 was similar to that of WH201, regaining the ability to form extensive hyphae. Growth on corn meal agar gave similar results (Fig. 4). These results indicate that EASC contributes to the hyphal growth of *C. albicans*. However, in spite of the defective hyphal growth of WH203 on solid Spider medium and corn meal agar, the mutant strain exhibited hyphal growth patterns little different from SC5314 in other liquid and solid media, suggesting that EASC is not needed for hyphal growth of *C. albicans* under all inducing conditions.

It is known that two major signaling pathways, dependent on *CPH1* (22) and *EFG1* (36), activate hyphal growth of *C. albicans*. We compared hyphal growth of the *alo1*/*alo1* strain WH203 with that of the strain carrying a mutation on *CPH1* or *EFG1*. On solid Spider medium, the *cph1*/*cph1* mutant strain showed reduced hyphal formation comparable to that of the *alo1*/*alo1* strain WH203 (data not shown). As reported previ-

TABLE 2. Activity of ALO and amount of EASC in *C. albicans* strains of different genetic background[a]

| Strain | Activity of ALO (mU · mg of protein$^{-1}$) | Amt of EASC (nmol · g [wet wt] of cells$^{-1}$) |
|---|---|---|
| SC5314 (*ALO1*/*ALO1*) | 37.3 ± 5.1 | 427 ± 35 |
| WH203 (*alo1*/*alo1*) | <0.1[b] | <0.1[c] |
| WH205 (CAI4 carrying pRC2312) | 34.8 ± 4.2 | 336 ± 15 |
| WH206 (CAI4 carrying pWK203) | 119.0 ± 7.3 | 1,330 ± 52 |
| WH207 (*ALO1* reintegrant) | 19.4 ± 3.8 | 237 ± 26 |

[a] Activity of ALO and amount of EASC in *C. albicans* were measured from exponentially growing cells in minimal defined medium. Values are means ± standard errors ($n = 3$).
[b] Lower limit for assay of ALO activity.
[c] Lower limit of detection for EASC.

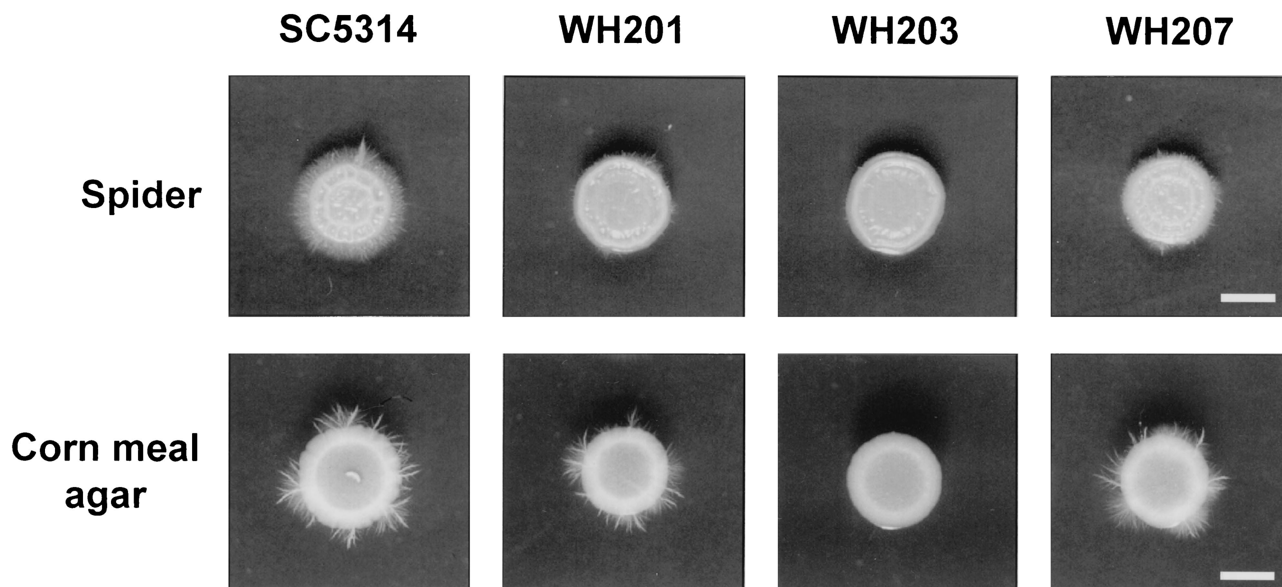


FIG. 4. Effect of *ALO1* mutation on hyphal growth of *C. albicans.* The indicated strains were spotted and incubated on Spider medium at 37°C for 3 days (bar = 3 mm) and on corn meal agar at 28°C for 4 days (bar = 5 mm).

ously (23), the *efg1*/*efg1* mutant strain did not form hyphae at all. Taken together with the report that the *cph1*/*cph1* strain shows defective hyphal formation on solid Spider medium but exhibits normal growth patterns in other liquid and solid media (22), these observations suggest that, as far as hyphal growth is concerned, the *alo1*/*alo1* strain is similar to the *cph1*/*cph1* strain.

To test a possibility that the expression of *ALO1* may be under the control of *CPH1* or *EFG1*, we measured the ALO activity and EASC content of the corresponding null mutants. However, there was no difference in the ALO activity and EASC content between the wild-type strain and the *cph1*/*cph1* or the *efg1*/*efg1* strain (data not shown), indicating that the expression of *ALO1* is not influenced by the signaling pathways dependent on *CPH1* and *EFG1*.

**Virulence studies in a mouse model.** To test the effect of EASC deficiency on the virulence of *C. albicans* in a mouse model, the wild-type strain SC5314, the *alo1*/*alo1* mutant strain WH203, and the *ALO1* reintegrant strain WH207 were intravenously injected into immunocompetent mice. Since the *ura3*/*ura3* mutants show decreased virulence, isogenic $Ura^+$ prototrophs were used to infect mice. As illustrated in Fig. 5, all the mice injected with SC5314 died within 10 days after infection. In contrast, 40% of the mice injected with the EASC-deficient strain WH203 survived to the end of the experiment. The survival difference between SC5314 and WH203 was significant ($P < 0.001$ by the Kaplan-Meier log-rank test). The *ALO1* reintegrant strain WH207 was more virulent than WH203 ($P < 0.05$ by the Kaplan-Meier log-rank test). These results indicate that EASC contributes to the virulence of *C. albicans* in a mouse model of intravenous infection.

## DISCUSSION

In the present study, the *ALO1* gene, which encodes the enzyme (ALO) that catalyzes the final reaction of EASC biosynthesis, was identified and cloned in *C. albicans*. The *ALO1* gene is 1,671 bp in size and encodes 557 amino acids with a calculated molecular mass of 63,428 Da, which is comparable to the molecular mass of the enzyme purified from the mitochondrial fraction of *C. albicans* (66.7 kDa) (9). The results of sequence comparisons show that *C. albicans* ALO is more similar to L-gulono-1,4-lactone oxidase from animals than to L-galactono-1,4-lactone dehydrogenase from plants, which has also been suggested by investigating the substrate specificity of *C. albicans* ALO (9). Through disruption of *ALO1*, we could obtain *C. albicans* strains devoid of EASC. Also, we could make *C. albicans* strains with high intracellular levels of EASC by overexpression of *ALO1*. The *alo1*/*alo1* mutant strain was more sensitive to oxidative stress, and the strain carrying *ALO1*

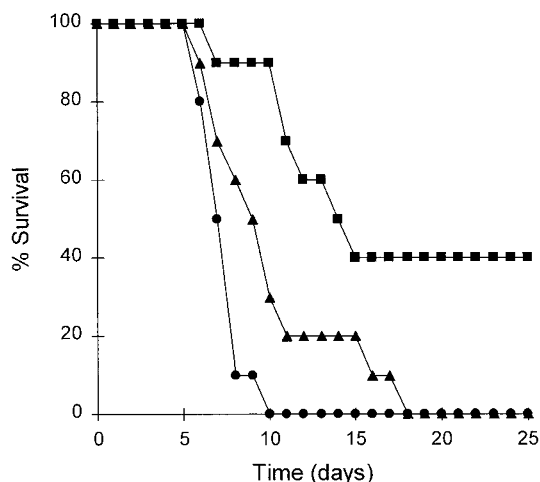


FIG. 5. Virulence assay of *C. albicans* in a mouse model. BALB/c mice were inoculated with $10^6$ cells of SC5314 (●), WH203 (■), and WH207 (▲) in a final volume of 100 μl through the lateral tail vein. Curves are the compiled results of two replicate experiments (five mice for each strain in each experiment).

on a multicopy plasmid showed a significant increase in survival under oxidative stress compared with the control strain. In *S. cerevisiae*, EASC has been reported to function as an important antioxidant (10), like ASC in higher animals and plants. The present study shows that it still holds true for *C. albicans*.

The *alo1*/*alo1* mutants show defective hyphal growth on solid Spider medium and corn meal agar, although not under all inducing conditions. Conditional defects in hyphal growth have been already observed in some other mutants, e.g., the *cph1*/*cph1* (22) and the *int1*/*int1* mutants (8). It is rather interesting that EASC affects the hyphal growth of *C. albicans* under some conditions. Some possibilities can be suggested: EASC may be required for proper operation of the components in a signal transduction pathway involved in the transition from yeast-like growth to hyphal growth, or a strong reductant activity of EASC may be needed in constituting normal cell wall structure in hyphal growth. It remains to be determined how EASC deficiency causes defective hyphal growth of *C. albicans*.

*C. albicans* is a member of the normal microbial flora and does not usually cause disease in immunocompetent hosts. However, *C. albicans* causes serious diseases in immunocompromised hosts such as leukemic, diabetic, organ transplant, and human immunodeficiency virus-infected patients. Elimination of *C. albicans* from an infected host requires the cooperation of many immune cells and several candidacidal mechanisms, among which oxygen-dependent killing mechanisms, mediated by a superoxide anion radical myeloperoxidase-$H_2O_2$-halide system, and reactive nitrogen intermediates, are crucial (37). Therefore, antioxidant defense systems are assumed to be essential for *C. albicans* to resist the host immune response and exhibit full virulence. In accordance with this view, exogenous antioxidants impair killing of *C. albicans* by neutrophils (38) and a catalase-deficient *C. albicans* strain is far less virulent for mice than the parental wild-type strain (40). The present study shows that the EASC-deficient *alo1*/*alo1* mutant strain exhibits attenuated virulence. These results, taken together with the proved function of EASC as an important antioxidant molecule in *C. albicans*, suggest that EASC may be essential for *C. albicans* to stand against the oxidant-mediated killing actions of the host immune system.

Nevertheless, there is a possibility that attenuated virulence of the *alo1*/*alo1* strain may be attributed to its defective hyphal growth, considering the well-established fact that the transition from yeast-like to hyphal growth is important to *C. albicans* virulence (23). However, this possibility does not seem to be acceptable for the following reasons. (i) Although the *alo1*/*alo1* strain exhibits suppressed hyphal growth on solid Spider medium and corn meal agar, it shows no difference from the wild-type strain when cultured on other media, including the one containing serum. This result strongly suggests that, when inoculated into the vein of a mouse, the *alo1*/*alo1* strain will show a normal transition from yeast-like to hyphal growth. (ii) The *cph1*/*cph1* strain shows defective hyphal formation similar to that of the *alo1*/*alo1* strain but does not suffer any damage in its virulence for mice (23). Therefore, it is not likely that attenuated virulence of the *alo1*/*alo1* strain is attributed to its defective hyphal growth.

The overall virulence of *C. albicans* can be defined as the sum of survivability and virulence. The former indicates the ability of *C. albicans* to defend itself against the host immune system and to grow in the host successfully. The latter allows *C. albicans* to adhere to and penetrate the host tissues and cause the symptoms of disease. Up to now, most studies on *C. albicans* have been focused on its virulence traits, including adhesion to the host tissues, secretion of proteases, and reversible morphological transitions between yeasts, pseudohyphae, and hyphae. The present study shows that EASC functions as an important antioxidant and is essential for *C. albicans* to exhibit full virulence, presumably by enhancing survival of the organism in the host. Therefore, we suggest that EASC can be regarded as an important virulence factor and that closer investigation of the defense mechanisms against the host immune system will broaden our understanding of the pathogenicity of *C. albicans*.

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