Characterization of the gene family encoding alternative oxidase from *Candida albicans*

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Candida albicans possesses a cyanide-resistant respiratory pathway mediated by alternative oxidase (AOX), which seems to be encoded by a gene family with two members. Cloning and expression of *AOX1a*, one of the genes encoding alternative oxidase from *C. albicans*, has previously been reported [Huh and Kang (1999) J. Bacteriol. **181**, 4098–4102]. In the present study we report the isolation of another gene coding for alternative oxidase, designated *AOX1b*. *AOX1b* contains a continuous open reading frame that encodes a polypeptide consisting of 365 amino acids. Interestingly, *AOX1a* and *AOX1b* were found to be located in tandem on one of the chromosomes of *C. albicans*. The presence of cyanide in the culture medium remarkably retarded the growth of the *aox1a/aox1a* mutants. The growth of the *aox1b/aox1b* mutants and the *aox1a/aox1a aox1b/aox1b* double mutants was almost completely inhibited in the same medium. β -Galactosidase reporter assays indicated that, whereas *AOX1a* was expressed constitutively, the expression of *AOX1b* was

dependent on growth phase and was induced by treatment with cyanide, antimycin A, H_2O_2 , menadione and paraquat. Growth of the cells in media with non-fermentable carbon sources also enhanced the expression of *AOX1b*. *CaSLN1*, which encodes a histidine kinase, seems to be involved in the regulation of *AOX* expression in *C. albicans* on the basis of the observation that the activity of cyanide-resistant respiration and the expression level of *AOX* in the *casln1/casln1* mutants were found to be significantly low under normal conditions and slightly increased in the presence of respiratory inhibitors compared with the wild-type strain. Like *AOX1a*, *AOX1b* could also be functionally expressed in *AOX*-deficient *Saccharomyces cerevisiae* and confer cyanide-resistant respiration on the organism.

Key words: cyanide-resistant respiration, electron transfer, histidine kinase, reactive oxygen species.

INTRODUCTION

The synthesis of ATP in respiring organisms is coupled to the flow of electrons through the electron transport chain to a terminal electron acceptor. This process is performed by the cyanide-sensitive and cytochrome-involved respiratory pathway. In addition to this conventional pathway, higher plants, some protists and many fungi are known to possess an alternative cyanide-resistant respiratory pathway (reviewed in [1]). Cyanide-resistant respiration appears to be mediated by alternative oxidase (AOX), which accepts electrons from the ubiquinone pool of the main cytochrome pathway and reduces oxygen to water. Electron flow through AOX is not coupled to ATP synthesis, therefore, the free energy released from this pathway is thought to be lost as heat.

The precise physiological roles of cyanide-resistant respiration are still poorly understood. Cyanide-resistant respiratory activity correlates well with the level of the AOX protein. And the increase in the AOX protein level is directly associated with the increase in the steady state amount of the corresponding transcripts, suggesting that activation of gene expression is important in regulation of cyanide-resistant respiration. Cyanide-resistant respiration has been shown to be involved in thermogenic inflorescence [2], ripening of fruits [3] and cell-type proportioning during *Dictyostelium* development [4]. Increases in AOX activity have been observed in plant cells after exposure to a number of stress conditions, including low temperature, wounding, inhibition of the cytochrome pathway of electron transport and inhibition of macromolecule synthesis (reviewed in [5]). Some reports suggest that reactive oxygen species (ROS),

such as superoxide radical anions and H_2O_2 , also induce the expression of *AOX* [6,7]. These observations suggest that cyanide-resistant respiration may be related to defence systems against oxidative stress, minimizing the generation of ROS in mitochondria.

In addition to the transcriptional control, reversible covalent modification and allosteric control are also involved in the regulation of plant AOX activity. The plant AOX is believed to exist in the mitochondrial inner membrane as a dimeric form. It can either be covalently linked (less active oxidized form) or non-covalently linked (more active reduced form), indicating that the activity of AOX is sensitive to the sulphhydryl/disulphide redox poise in plants [8]. When the covalently linked disulphide bond is reduced to the constituent sulphhydryls, the plant AOX can then be stimulated by α -keto acids, such as pyruvate, probably through the formation of a thiohemiacetal [9]. Recently, the cysteine residue associated with the formation of the regulatory disulphide bond has been identified and the same cysteine residue has proved to be the site of α -keto acid stimulation [10]. This regulatory cysteine residue corresponds to the more N-terminal of the two highly conserved cysteine residues found in the plant AOX.

Biochemical regulatory features of AOX from microorganisms are quite different from those of the plant AOX. Purine nucleotides, such as AMP, ADP, GMP and IMP, have been shown to stimulate the activity of AOX from *Moniliella tomentosa* [11], *Neurospora crassa* [12], *Paramecium tetraurelia* [13] and *Hansenula anomala* [14], whereas AOX from higher plant mitochondria is not stimulated by purine mononucleotides. A recent study reported that, in contrast with the plant AOX,

Abbreviations used: AOX, alternative oxidase; ROS, reactive oxygen species; ORF, open reading frame.

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fungal AOX has a monomeric structure without the redox-sensitive disulphide bond system and its activity is independent of α -keto acids [15]. These differences in regulatory properties between the two types of AOXs suggest that they may play different physiological roles.

In plants, it is common that AOXs are encoded by multigene families, whose members are differentially regulated in a tissue- or developmental stage-dependent manner. Three *AOX* genes have been identified in soya bean [16], and two genes have been identified in tobacco [17] and rice [18]. In *Arabidopsis thaliana*, the *AOX* gene family, with four members, has been characterized [19]. In accordance with the presence of multigene families for AOX, multiple immunoreactive AOX protein bands have been detected on immunoblots of mitochondrial proteins from plants [16]. In micro-organisms, however, the presence of multigene families for AOX is controversial. It has been reported that AOX is encoded by a single gene in both *Trypanosoma brucei* [20] and *H. anomala* [21]. But, AOX from *Candida albicans* has been suggested to be encoded by a gene family with two members [22].

In our previous study, we reported the isolation of the *AOX1a* gene from *C. albicans* and functional expression of the gene in *Saccharomyces cerevisiae* [22]. In the present study, we report the characterization of the *AOX* gene family from *C. albicans*. We have identified another member of the *AOX* gene family, designated *AOX1b*, and found that *AOX1b* is located 1.3 kb upstream of *AOX1a*, in the same orientation. β -Galactosidase reporter assays showed that, whereas *AOX1a* is expressed constitutively, the expression of *AOX1b* is dependent on growth phase and is induced by respiratory inhibitors and oxidants. We also report that *CaSLN1*, a histidine kinase gene, is involved in the regulation of AOX expression in *C. albicans*. This is the first report describing the presence of a multigene family for a fungal AOX and the construction of null mutants of the *AOX* gene.

MATERIALS AND METHODS

Strains and growth conditions

C. albicans and *S. cerevisiae* strains used in the present study are listed in Table 1. For routine growth of cells, YPD medium [1% (w/v) yeast extract/2% (w/v) peptone/2% (w/v) glucose] was used. Cells containing plasmids or disrupted genes were cultured in minimal defined medium containing 0.67% yeast nitrogen base without amino acids (Difco, Irvine, CA, U.S.A.), 2% (w/v) glucose and appropriate supplements (e.g. 40 mg/ml uridine and 40 mg/ml histidine) [23]. Ura⁻ auxotrophs were selected on minimal defined medium supplemented with 5-fluoro-orotic acid (625 mg/l) and uridine (30 mg/l). Solid medium were prepared by adding 1.8% agar to the liquid broth. Unless specified, all cultures were incubated at 28 °C.

Isolation, subcloning and sequencing of *AOX1b* from *C. albicans*

To construct the *C. albicans* genomic library, the genomic DNA from *C. albicans* A.T.C.C. 10231 was partially digested with *Sau3AI*, and DNA fragments of 10–23 kb in size were ligated into the dephosphorylated λ EMBL3 vector (Stratagene, La Jolla, CA, U.S.A.) generated by *Bam*HI cleavage. The ligated DNA was packaged using Gigapack II packaging extracts (Stratagene), and was propagated according to the manufacturer's instructions. PCR using the oligonucleotide primer pair [5'-GCTGGTGT-(C/T)CC(A/T)GGTATGGT-3' and 5'-GCTTCTTC(C/T)TC-CAA(A/G)TAACC-3'] previously described for preparing the *AOX1a* probe [22], could also amplify a DNA fragment of approx. 0.3 kb from the chromosomal DNA of the *aox1a/aox1a* *C. albicans* strain WH304. The cloned PCR product was labelled

with digoxigenin (Roche Molecular Biochemicals, Indianapolis, IN, U.S.A.) and used as a probe to screen the λ EMBL3 genomic library. From positive clones, the common 4.1 kb *Xba*I fragment was subcloned into pGEM-7Zf(+) (Promega, Madison, WI, U.S.A.) at the *Xba*I site, yielding pCAOX1b. Both strands of the cloned DNA were sequenced by the dideoxy chain-termination method using an automatic sequencer (ALFexpress; Pharmacia, Piscataway, NJ, U.S.A.).

Disruption of *AOX1a* and *AOX1b* from *C. albicans*

Disruption of *AOX1a* has been previously described [22]. In order to disrupt *AOX1b*, a 4.1 kb fragment containing the *hisG-URA3-hisG* gene disruption cassette from p5921 [24] was inserted in place of a portion of *AOX1b* within the genomic clone (see Figure 2A). The resulting plasmid was cut with *Apa*I/*Sac*I to remove the vector, and was transformed into the *ura3/ura3* *C. albicans* strain CAI4 [24]. Ura⁺ transformants were selected on uracil-deficient medium, and integration of the cassette into the *AOX1b* 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 5-fluoro-orotic acid (625 mg/l) and uridine (30 mg/l). This procedure was repeated in order to delete the remaining functional allele of *AOX1b*. For construction of the *aox1a/aox1a aox1b/aox1b* double mutant strain, a disruption construct was prepared by replacing the region encompassing *AOX1b* and *AOX1a* with the *hisG-URA3-hisG* gene disruption cassette (see Figure 3A), and was transformed into CAI4, followed by the above procedure.

Measurement of respiration

Respiration of the cells in mid-exponential phase was measured polarographically at 25 °C using a YSI 5300 Biological Oxygen Monitor Micro System (Yellow Springs Instruments, Yellow Springs, OH, U.S.A.) as previously described [22]. Respiration proceeding by way of the cytochrome pathway was inhibited with 1 mM KCN, whereas respiration proceeding by way of the alternative pathway was inhibited with 2 mM salicylhydroxamic acid. Oxygen uptake rates remaining after the addition of these two inhibitors were subtracted from calculated rates to give the final values for cyanide-resistant respiratory activity. To induce cyanide-resistant respiration, 10 μ M antimycin A was added to the cell suspension, followed by incubation for 1 h at 28 °C with shaking. KCN was dissolved in distilled water and its pH was adjusted to 7.0. Salicylhydroxamic acid and antimycin A were dissolved in absolute ethanol.

Western-blot analysis

C. albicans mitochondria were isolated as previously described [25]. The purified mitochondria were added to SDS sample buffer [125 mM Tris/HCl (pH 6.8) containing 8% (w/v) SDS, 20% (v/v) glycerol and 0.004% Bromophenol Blue] and boiled for 2 min. A 10% (w/v) polyacrylamide gel was used for separating the mitochondrial proteins. The resolved proteins were transferred on to a nitrocellulose membrane (Schleicher & Schuell, Keene, NH, U.S.A.), which was subsequently probed with an antibody raised against the AOX protein from *Sauromatum guttatum* [26] at 1:1000 dilution.

Growth of *C. albicans* in the presence of KCN

After *C. albicans* cells reached the exponential phase in YPD medium, the cells were pelleted by centrifugation at 6000 *g* for 5 min. The cells were resuspended in distilled water at an

Table 1 *C. albicans* and *S. cerevisiae* strains used in the present study

Strain	Genotype	Reference
<i>C. albicans</i>		
A.T.C.C. 10231	Wild-type	A.T.C.C.
CAI4	$\Delta ura3::imm434/\Delta ura3::imm434$	[24]
WH303	$\Delta ura3::imm434/\Delta ura3::imm434 \Delta aox1a::hisG/\Delta aox1a::hisG-URA3-hisG$	[22]
WH304	$\Delta ura3::imm434/\Delta ura3::imm434 \Delta aox1a::hisG/\Delta aox1a::hisG$	[22]
WH311	$\Delta ura3::imm434/\Delta ura3::imm434 \Delta aox1b::hisG-URA3-hisG/AOX1b$	This study
WH312	$\Delta ura3::imm434/\Delta ura3::imm434 \Delta aox1b::hisG/AOX1b$	This study
WH313	$\Delta ura3::imm434/\Delta ura3::imm434 \Delta aox1b::hisG/\Delta aox1b::hisG-URA3-hisG$	This study
WH314	$\Delta ura3::imm434/\Delta ura3::imm434 \Delta aox1b::hisG/\Delta aox1b::hisG$	This study
WH321	$\Delta ura3::imm434/\Delta ura3::imm434 \Delta(aox1b-aox1a)::hisG-URA3-hisG/AOX1b-AOX1a$	This study
WH322	$\Delta ura3::imm434/\Delta ura3::imm434 \Delta(aox1b-aox1a)::hisG/AOX1b-AOX1a$	This study
WH323	$\Delta ura3::imm434/\Delta ura3::imm434 \Delta(aox1b-aox1a)::hisG/\Delta(aox1b-aox1a)::hisG-URA3-hisG$	This study
WH324	$\Delta ura3::imm434/\Delta ura3::imm434 \Delta(aox1b-aox1a)::hisG/\Delta(aox1b-aox1a)::hisG$	This study
WH331	$\Delta ura3::imm434/\Delta ura3::imm434$ (pRC18)	This study*
WH332	$\Delta ura3::imm434/\Delta ura3::imm434$ (pX1a-LAC4)	This study†
WH333	$\Delta ura3::imm434/\Delta ura3::imm434$ (pX1b-LAC4)	This study‡
SLN	$\Delta ura3::imm434/\Delta ura3::imm434 \Delta cas1n1::hisG/\Delta cas1n1::hisG$	[38]
<i>S. cerevisiae</i>		
WH117	<i>MATa/MATα ade2-1/ADE2 his3-Δ1/his3-Δ200 leu2-3/LEU2 leu2-112/LEU2 lys2-801a/LYS2 trp1-289/trp1Δ ura3-52/ura3-52</i> (pRS424)	[22]
WH119	<i>MATa/MATα ade2-1/ADE2 his3-Δ1/his3-Δ200 leu2-3/LEU2 leu2-112/LEU2 lys2-801a/LYS2 trp1-289/trp1Δ ura3-52/ura3-52</i> (pWK303)	[22]
WH120	<i>MATa/MATα ade2-1/ADE2 his3-Δ1/his3-Δ200 leu2-3/LEU2 leu2-112/LEU2 lys2-801a/LYS2 trp1-289/trp1Δ ura3-52/ura3-52</i> (pWK304)	This study§

* CAI4 + pRC18.

† CAI4 + pX1a-LAC4.

‡ CAI4 + pX1b-LAC4.

§ WH113 + pWK304.

attenuance at 600 nm (D_{600}) of 5 units. An aliquot of this suspension (0.2 ml) was added to 20 ml of YPG medium [1 % (w/v) yeast extract/2 % (w/v) peptone and 3 % (w/v) glycerol] with or without 0.5 mM KCN. Cell growth was monitored by measuring D_{600} .

Construction of plasmids for the β -galactosidase reporter assay

A 1.2 kb *AOX1a* promoter fragment was amplified by PCR using the primer pair, 5'-TAAAGCTTCATCTTTGAGAAAT-AGG-3' and 5'-AACCAATGATATCTAACTAACTCC-3', which were modified to carry *Hind*III and *Eco*RV sites (underlined) respectively. For amplification of a 1.3 kb *AOX1b* promoter fragment, the primer pair of 5'-TTACCTAAAGCTT-ATAGTTGCACAC-3' and 5'-TAAGGATATCGTAATTG-AATAAATC-3' were used, which were also modified to carry *Hind*III and *Eco*RV sites (underlined) respectively. The 1.2 kb *Hind*III/*Eco*RV *AOX1a* promoter fragment or the 1.3 kb *Hind*III/*Eco*RV *AOX1b* promoter fragment was fused to the 4.2 kb *Pvu*II/*Xba*I *LAC4* fragment of pRS-LAC4 [27]. The resulting *AOX1a* promoter-*LAC4* and the *AOX1b* promoter-*LAC4* fusion products were inserted into pRC18 [27] at the *Hind*III/*Xba*I sites to generate pX1a-LAC4 and pX1b-LAC4 respectively. The resulting plasmids pX1a-LAC4 and pX1b-LAC4 were transformed into the *ura3/ura3* CAI4 strain and colonies were selected for the *Ura*⁺ phenotype with high β -galactosidase activity.

β -Galactosidase assay

LAC4-specified β -galactosidase activity in *C. albicans* was measured by a permeabilized cell assay as previously described [28]. The specific activity of β -galactosidase was calculated using the following formula: $A_{420}/[D_{600} \text{ of assayed culture} \times \text{volume of culture assayed (ml)} \times \text{length of incubation at } 30^\circ\text{C (h)}]$.

RESULTS

Isolation and characterization of *AOX1b* from *C. albicans*

PCR using the oligonucleotide primer pair, which corresponded to amino acid residues 145–151 (AGVPGMV) and 229–235 (GYLEEEA) of *H. anomala* AOX [29] and which were used to prepare a probe for *C. albicans* *AOX1a* [22], could amplify a DNA fragment of approx. 0.3 kb from the chromosomal DNA of the *aox1a/aox1a* strain WH304. When cloned and sequenced, the fragment showed a high degree of amino acid sequence similarity to AOXs from other sources, including *C. albicans* *AOX1a* protein, upon BLAST searches of the GenBank® database. The cloned PCR product was used as a probe to screen the λ EMBL3 genomic library. From positive clones, the common 4.1 kb *Xba*I fragment was subcloned into pGEM-7Zf(+) and was sequenced.

The sequenced insert contained a continuous open reading frame (ORF) of 1098 bp that encoded a polypeptide consisting of 365 amino acids with a calculated molecular mass of 41 940 Da. This shows that the *AOX1b* ORF is slightly smaller than the *AOX1a* ORF, which consists of 379 amino acids with a calculated molecular mass of 43 975 Da [22]. *C. albicans* *AOX1b* contained no CUG codon, which encodes serine in *C. albicans* but encodes leucine in *S. cerevisiae* and all organisms that use the standard genetic code [30]. The nucleotide sequence of *AOX1b* did not have the consensus sequence for splicing. The fact that the gene contains no intron was confirmed by reverse-transcriptase PCR (results not shown). The predicted amino acid sequence of *C. albicans* *AOX1b* shared 65.2 %, 56.7 %, 36.2 %, 26.7 % and 25.8 % identity with the sequences of *C. albicans* *AOX1a* [22], *H. anomala* *AOX1* [29], *N. crassa* *AOX1* [31], *T. brucei* *AOX1* [32] and *S. guttatum* *AOX1* [33] respectively (Figure 1). Identities between the predicted amino acid sequence of *C. albicans* *AOX1b* and those of the four AOX proteins (*AOX1a*, *AOX1b*, *AOX1c* and *AOX2*) from *A. thaliana* were 24.0 %, 23.4 %, 26.4 % and 29.8 %, respectively (Figure 1), indicating that the *AOX*

Ca-AOX1b	-----MLTASVYKQLPVLTITSTSTYSFIR-----LSSTLADPHSTTTTSPSSPAHPHPHP	53
Ca-AOX1a	-----MLTASVYKQLPVLTITSTSTSTYSFIR-----LSSTLADPHSTTTTSPSSPAHPHPHP	53
Ha-AOX1	-----MKTQVRSILHSMGIRFLK-----TSPSSPHS-----KDFSS	35
Nc-AOX1	-----KMTPKVNIHAPGAAQALSPALIS-----QCHTRFLILLAGSRVATSLHPTQNLIS	50
Tb-AOX1	-----MFEMHASRIATAAPVLRITACR-----QSDAKTIVWG-----HTQLNR	40
Sg-AOX1	-----NMSSRLVGTALCQLSHVPPVQVLPALRPTADTASLLHSCAAAPACRAQWPSWPSFPHKALSLAPQDGG	75
At-AOX1a	-----MMITRGGAAKSLVAGPRFSTVSTVSHALSAHSHKQVYTSANITSAITICIM-RFATITLIGERTM	74
At-AOX1b	-----MMHSRYGAKMETATVTHSHLNRVPLVTENITVDMGVV-RVFSTLCEKSKKTT	55
At-AOX1c	-----MITTLRSLDQAKTQNGHSHLQALAPKY-FRVAVDGL-RDFHMKCEKSKKTS	56
At-AOX2	-----MITTLRSLDQAKTQNGHSHLQALAPKY-FRVAVDGL-RDFHMKCEKSKKTS	17
Ca-AOX1b	-----TSVDVSTRIYQ-----EGINNNNDTKF-LTKAPYPHVFPQDCEBNVTVTHR	108
Ca-AOX1a	-----IDBNLEKPGTI-----PTKHKPTQTEVYVK-AGISANDDKF-LTKYTYSHEDFTRGVYVHVTNR	122
Ha-AOX1	-----KSLDQKTLKLN-----PPQK-MANNQY-VHSDPHKYSDECEAVHVR	82
Nc-AOX1	-----SPRMFTTSTVRKDFPAPKATVAVIKOTPDANPHGWTEBMTSVVPHR	102
Tb-AOX1	-----LSFLSTVPPVPLVLSDESSD-----RP-----TNSLPDINVAITHK	78
Sg-AOX1	-----KSKKATAGKVP-----PGEDSGARKEAVSVHAWP-PKVSFSPQSS-KRWTCFPPWSTIQDGLDHLHGH	141
At-AOX1a	-----KSDANQKTKTENESTGGAGKGNHSDKGLASWVE-PKATTEKAPR-KRWTCFPPWSTIQDGLDHLHGH	146
At-AOX1b	-----KSKKATAGKVP-----PGEDSGARKEAVSVHAWP-PKVSFSPQSS-KRWTCFPPWSTIQDGLDHLHGH	137
At-AOX1c	-----KSKKATAGKVP-----PGEDSGARKEAVSVHAWP-PKVSFSPQSS-KRWTCFPPWSTIQDGLDHLHGH	121
At-AOX2	-----KSKKATAGKVP-----PGEDSGARKEAVSVHAWP-PKVSFSPQSS-KRWTCFPPWSTIQDGLDHLHGH	74
Ca-AOX1b	-----ETTLGQMSISFISQFMKQCPDLVTGYAYP-----TNNDEPFGTSGKTCCKKLTLCPLASVAGVAGVAGFLR	180
Ca-AOX1a	-----EPPTGQMSISFISQFMKQCPDLVTGYAYP-----TNNDEPFGTSGKTCCKKLTLCPLASVAGVAGVAGFLR	194
Ha-AOX1	-----EPPTGQMSISFISQFMKQCPDLVTGYAYP-----TNNDEPFGTSGKTCCKKLTLCPLASVAGVAGVAGFLR	156
Nc-AOX1	-----KPTTQMSISFISQFMKQCPDLVTGYAYP-----TNNDEPFGTSGKTCCKKLTLCPLASVAGVAGVAGFLR	177
Tb-AOX1	-----KPTTQMSISFISQFMKQCPDLVTGYAYP-----TNNDEPFGTSGKTCCKKLTLCPLASVAGVAGVAGFLR	137
Sg-AOX1	-----VETTLGQMSISFISQFMKQCPDLVTGYAYP-----TNNDEPFGTSGKTCCKKLTLCPLASVAGVAGVAGFLR	192
At-AOX1a	-----VETTLGQMSISFISQFMKQCPDLVTGYAYP-----TNNDEPFGTSGKTCCKKLTLCPLASVAGVAGVAGFLR	197
At-AOX1b	-----VETTLGQMSISFISQFMKQCPDLVTGYAYP-----TNNDEPFGTSGKTCCKKLTLCPLASVAGVAGVAGFLR	168
At-AOX1c	-----VETTLGQMSISFISQFMKQCPDLVTGYAYP-----TNNDEPFGTSGKTCCKKLTLCPLASVAGVAGVAGFLR	172
At-AOX2	-----VETTLGQMSISFISQFMKQCPDLVTGYAYP-----TNNDEPFGTSGKTCCKKLTLCPLASVAGVAGVAGFLR	125
Ca-AOX1b	-----HUNHSMLEKPKAMETTLDEAYNEMHLLTFTKIGESWFTSLYVGGGVTVEVHLLMNRVCHRVGVL	255
Ca-AOX1a	-----HUNHSMLEKPKAMETTLDEAYNEMHLLTFTKIGESWFTSLYVGGGVTVEVHLLMNRVCHRVGVL	269
Ha-AOX1	-----HUNHSMLEKPKAMETTLDEAYNEMHLLTFTKIGESWFTSLYVGGGVTVEVHLLMNRVCHRVGVL	231
Nc-AOX1	-----HUNHSMLEKPKAMETTLDEAYNEMHLLTFTKIGESWFTSLYVGGGVTVEVHLLMNRVCHRVGVL	252
Tb-AOX1	-----HUNHSMLEKPKAMETTLDEAYNEMHLLTFTKIGESWFTSLYVGGGVTVEVHLLMNRVCHRVGVL	212
Sg-AOX1	-----HUNHSMLEKPKAMETTLDEAYNEMHLLTFTKIGESWFTSLYVGGGVTVEVHLLMNRVCHRVGVL	267
At-AOX1a	-----HUNHSMLEKPKAMETTLDEAYNEMHLLTFTKIGESWFTSLYVGGGVTVEVHLLMNRVCHRVGVL	272
At-AOX1b	-----HUNHSMLEKPKAMETTLDEAYNEMHLLTFTKIGESWFTSLYVGGGVTVEVHLLMNRVCHRVGVL	243
At-AOX1c	-----HUNHSMLEKPKAMETTLDEAYNEMHLLTFTKIGESWFTSLYVGGGVTVEVHLLMNRVCHRVGVL	247
At-AOX2	-----HUNHSMLEKPKAMETTLDEAYNEMHLLTFTKIGESWFTSLYVGGGVTVEVHLLMNRVCHRVGVL	200
Ca-AOX1b	-----SSGAVRTYSHLLDLAVPGK-LDAFETMKIRVAVQVWPELTPKSSFKDLILRPAKADAKREVTTFANLEQ	327
Ca-AOX1a	-----SSGAVRTYSHLLDLAVPGK-LDAFETMKIRVAVQVWPELTPKSSFKDLILRPAKADAKREVTTFANLEQ	341
Ha-AOX1	-----SSGAVRTYSHLLDLAVPGK-LDAFETMKIRVAVQVWPELTPKSSFKDLILRPAKADAKREVTTFANLEQ	302
Nc-AOX1	-----SSGAVRTYSHLLDLAVPGK-LDAFETMKIRVAVQVWPELTPKSSFKDLILRPAKADAKREVTTFANLEQ	325
Tb-AOX1	-----SSGAVRTYSHLLDLAVPGK-LDAFETMKIRVAVQVWPELTPKSSFKDLILRPAKADAKREVTTFANLEQ	283
Sg-AOX1	-----SSGAVRTYSHLLDLAVPGK-LDAFETMKIRVAVQVWPELTPKSSFKDLILRPAKADAKREVTTFANLEQ	333
At-AOX1a	-----SSGAVRTYSHLLDLAVPGK-LDAFETMKIRVAVQVWPELTPKSSFKDLILRPAKADAKREVTTFANLEQ	338
At-AOX1b	-----SSGAVRTYSHLLDLAVPGK-LDAFETMKIRVAVQVWPELTPKSSFKDLILRPAKADAKREVTTFANLEQ	309
At-AOX1c	-----SSGAVRTYSHLLDLAVPGK-LDAFETMKIRVAVQVWPELTPKSSFKDLILRPAKADAKREVTTFANLEQ	313
At-AOX2	-----SSGAVRTYSHLLDLAVPGK-LDAFETMKIRVAVQVWPELTPKSSFKDLILRPAKADAKREVTTFANLEQ	266
Ca-AOX1b	-----KTFNTEGRTSTGLNTRG-SH-HGHNWMEFTGWEKQSLQ-----	365
Ca-AOX1a	-----KTFNTEGRTSTGLNTRG-SH-HGHNWMEFTGWEKQSLQ-----	379
Ha-AOX1	-----KTFNTEGRTSTGLNTRG-SH-HGHNWMEFTGWEKQSLQ-----	342
Nc-AOX1	-----KTFNTEGRTSTGLNTRG-SH-HGHNWMEFTGWEKQSLQ-----	362
Tb-AOX1	-----KTFNTEGRTSTGLNTRG-SH-HGHNWMEFTGWEKQSLQ-----	345
Sg-AOX1	-----KTFNTEGRTSTGLNTRG-SH-HGHNWMEFTGWEKQSLQ-----	349
At-AOX1a	-----KTFNTEGRTSTGLNTRG-SH-HGHNWMEFTGWEKQSLQ-----	354
At-AOX1b	-----KTFNTEGRTSTGLNTRG-SH-HGHNWMEFTGWEKQSLQ-----	325
At-AOX1c	-----KTFNTEGRTSTGLNTRG-SH-HGHNWMEFTGWEKQSLQ-----	329
At-AOX2	-----KTFNTEGRTSTGLNTRG-SH-HGHNWMEFTGWEKQSLQ-----	282

Figure 1 Alignment of the deduced amino acid sequence of *C. albicans* AOX1b (Ca-AOX1b) with the sequences of *C. albicans* AOX1a (Ca-AOX1a), *H. anomala* AOX1 (Ha-AOX1), *N. crassa* AOX1 (Nc-AOX1), *T. brucei* AOX1 (Tb-AOX1), *S. guttatum* AOX1 (Sg-AOX1), and *A. thaliana* AOX1a (At-AOX1a), AOX1b (At-AOX1b), AOX1c (At-AOX1c) and AOX2 (At-AOX2)

The numbers on the right-hand side refer to the amino acid positions in each sequence. The regions where the sequences have been extended to allow optimal sequence alignment are indicated with dashes. Identical residues are indicated with shaded letters.

genes from *C. albicans* are slightly more similar to those of the AOX2/AOX3 group than those of the AOX1 group of higher plants. Like other AOXs, the AOX1b protein was predicted to be an integral membrane protein with two transmembrane segments corresponding to amino acid residues 162–179 and 225–243. According to the method of Gavel and von Heijne [34], the cleavage site for the mitochondrial presequence was predicted to be between Leu²⁶ and Ser²⁷. The molecular mass of the presumed mature form of *C. albicans* AOX1b protein, which consists of 339 amino acids, was calculated to be 39037 Da. When predicted by the same method, the molecular mass of the mature form of AOX1a protein, consisting of 337 amino acids, was calculated to be 39331 Da [22], which is very similar to that of the AOX1b protein.

Interestingly, the nucleotide sequence of the 3'-flanking region of AOX1b proved to be identical to that of the 5'-flanking region of AOX1a. When AOX1a and AOX1b were lined up on the basis of this identical sequence, AOX1b was found to be

Figure 2 Disruption of the AOX1b gene in *C. albicans*

(A) Restriction map of the region containing the AOX gene family and insertion of the *hisG-URA3-hisG* cassette at the *HpaI/HindIII* sites in the AOX1b coding sequence. The endonuclease restriction sites are as follows: B, *BglII*; E, *EcoRI*; E5, *EcoRV*; H, *HindIII*; Hp, *HpaI*; X, *XbaI*. (B) Southern-blot analysis with the sequence indicated in (A) used as a probe. The DNA digested with *EcoRI* was from the following strains: lane 1, CA14 (AOX1b/AOX1b); lane 2, WH311 (Δ aox1b::hisG-URA3-hisG/AOX1b); lane 3, WH312 (Δ aox1b::hisG/AOX1b); lane 4, WH313 (Δ aox1b::hisG/ Δ aox1b::hisG-URA3-hisG); lane 5, WH314 (Δ aox1b::hisG/ Δ aox1b::hisG).

located 1.3 kb upstream of AOX1a in the same transcriptional direction (Figure 2A). Genomic Southern-blot analysis also supported tandem arrangement of the AOX gene family. On genomic Southern-blot analysis using the entire AOX1a ORF as a probe, only one band was detected when the genomic DNA was digested with *Bam*HI, *Nde*I or *Xho*I, two bands were detected when digested with *Bgl*II, *Cl*aI, *Eco*RV or *Xba*I, and four bands were detected when digested with *Eco*RI or *Hind*III (results not shown). These blot patterns correlated well with the restriction map of the cloned region in the *C. albicans* genome including AOX1a and AOX1b (Figure 2A). On Northern-blot analysis of AOX1b, a single band corresponding to approx. 1.4 kb in size was detected (results not shown). However, as previously described [22], it was impossible to obtain the signal for AOX1a, even when the cells were incubated in the presence of antimycin A, to induce cyanide-resistant respiration, presumably because of the very low level of AOX1a mRNA.

Disruption of the *C. albicans* AOX gene family

Disruption of AOX1a has been described previously [22]. For the disruption of AOX1b, a disruption construct was prepared by replacing a portion of the coding region of AOX1b with the *hisG-URA3-hisG* sequence (Figure 2A), which was then used to transform the *ura3/ura3* *C. albicans* strain CA14. 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-fluoro-orotic acid. A homozygous disruption of AOX1b was generated by repeating the above procedure, and confirmed

Figure 3 Double disruption of the *AOX1a* and *AOX1b* genes in *C. albicans*

(A) Restriction map of the region containing the *AOX* gene family and insertion of the *hisG-URA3-hisG* cassette at the *HpaI* and *HindIII* sites in the *AOX1b* and *AOX1a* coding sequences respectively. The endonuclease restriction sites are as follows: B, *BglII*; E, *EcoRI*; E5, *EcoRV*; H, *HindIII*; Hp, *HpaI*; X, *XbaI*. (B) Southern-blot analysis with the sequence indicated in (A) used as a probe. The DNA digested with *EcoRI* was from the following strains: lane 1, CAI4 (*AOX1b-AOX1a/AOX1b-AOX1a*); lane 2, WH321 [$\Delta(aox1b-aox1a)::hisG-URA3-hisG/AOX1b-AOX1a$]; lane 3, WH322 [$\Delta(aox1b-aox1a)::hisG/AOX1b-AOX1a$]; lane 4, WH323 [$\Delta(aox1b-aox1a)::hisG/\Delta(aox1b-aox1a)::hisG-URA3-hisG$]; lane 5, WH324 [$\Delta(aox1b-aox1a)::hisG/\Delta(aox1b-aox1a)::hisG$].

by Southern-blot analysis (Figure 2B). To construct the *aox1a/aox1a aox1b/aox1b* double mutants, a disruption construct made by inserting the *hisG-URA3-hisG* gene disruption cassette in place of the region encompassing *AOX1b* and *AOX1a* ORF was used to transform CAI4 (Figure 3A). A homozygous disruption of both *AOX1a* and *AOX1b* was generated by

following the above procedure, and was confirmed by Southern-blot analysis (Figure 3B).

All of the mutant strains, i.e. the *aox1a/aox1a* strain, the *aox1b/aox1b* strain and the *aox1a/aox1a aox1b/aox1b* strain, 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 media with non-fermentable carbon sources, such as ethanol and glycerol. Furthermore, when treated with oxidants, such as H_2O_2 and menadione, the *aox* mutant strains showed cell viability similar to that of the wild-type strain (results not shown). To investigate the effect of the *aox* mutation on hyphal growth of *C. albicans*, isogenic Ura⁺ prototrophs were grown on liquid and solid media that induced hyphal growth, e.g. 20% (v/v) serum, Lee's medium [5 g of $(NH_4)_2SO_4$ /l, 0.2 g of $MgSO_4 \cdot 7H_2O$ /l, 2.5 g of anhydrous K_2HPO_4 /l, 5 g of NaCl/l, 12.5 g of glucose/l, 0.5 g of alanine/l, 1.3 g of leucine/l, 1.0 g of lysine/l, 0.1 g of methionine/l, 0.0714 g of ornithine/l, 0.5 g of phenylalanine/l, 0.5 g of proline/l, 0.5 g of threonine/l and 0.001 g of biotin/l], Spider medium [1% (v/v) nutrient broth, 1% (w/v) mannitol and 0.2% K_2HPO_4] and RPMI 1640. All of the mutant strains exhibited growth patterns little different from the wild-type strain in these media (results not shown). In addition, we could not observe any defect in the virulence of the *aox* mutants in a mouse model (results not shown). These results suggest that AOX is not needed for hyphal growth and virulence of *C. albicans*.

Cyanide-resistant respiration of the *aox* mutants

We measured the activity of cyanide-resistant respiration and the protein level of AOX in the *aox* mutant strains. As previously described [22], the *aox1a/aox1a* mutant strain WH304 showed slightly reduced cyanide-resistant respiration compared with CAI4 under normal conditions (Figure 4A). Cyanide-resistant respiration of the *aox1b/aox1b* mutant strain WH314 was much lower than that of CAI4. The cyanide-resistant respiration rate of fresh WH314 was approx. 22% of that of CAI4. The *aox1a/aox1a aox1b/aox1b* mutant strain WH324 did not exhibit cyanide-resistant respiration under normal conditions. When incubated in the presence of 10 μ M antimycin A for 1 h, the cyanide-resistant respiration rate of CAI4 increased to 97 nmol $O_2 \cdot h^{-1} \cdot (mg \text{ of wet cells})^{-1}$. Incubation of WH304 in the presence of antimycin A also greatly enhanced cyanide-resistant

Figure 4 Respiratory characteristics of the *aox* mutants of *C. albicans*

(A) The activity of cyanide-resistant respiration of *C. albicans* strains. Oxygen uptake rates of fresh cells (open bars) and antimycin A-treated cells (shaded bars) were measured in the presence of 1 mM KCN. Data shown represent the means $\pm$ S.E.M. of three independent experiments. (B) Western-blot analysis of AOX from fresh cells of CAI4 (*AOX1a/AOX1a AOX1b/AOX1b*; lane 1), WH304 (*aox1a/aox1a AOX1b/AOX1b*; lane 2), WH314 (*AOX1a/AOX1a aox1b/aox1b*; lane 3) and WH324 (*aox1a/aox1a aox1b/aox1b*; lane 4). (C) Western-blot analysis of AOX from antimycin A-treated cells of CAI4 (lane 1), WH304 (lane 2), WH314 (lane 3) and WH324 (lane 4). Numbers on the left-hand side of the immunoblots refer to molecular masses of the standards. Arrows on the right-hand side of each immunoblot indicate AOX.

Figure 5 Cyanide-resistant growth of the *aox* mutants of *C. albicans*

Cell growth of CAI4 (*AOX1a/AOX1a AOX1b/AOX1b*; ◆), WH304 (*aox1a/aox1a AOX1b/AOX1b*; ■), WH314 (*AOX1a/AOX1a aox1b/aox1b*; ▲) and WH324 (*aox1a/aox1a aox1b/aox1b*; ●) was monitored by checking D_{600} . Cells were grown at 28 °C in YPG medium containing 0.5 mM KCN.

respiration, the rate of which was approximately $89 \text{ nmol O}_2 \cdot \text{h}^{-1} \cdot (\text{mg of wet cells})^{-1}$. Considering that WH304 is the *aox1a/aox1a* mutant strain, the increased cyanide-resistant respiration of WH304 on treatment with antimycin A seems to be attributed to the induction of *AOX1b*. In accordance with this view, cyanide-resistant respiration of the *aox1b/aox1b* strain WH314 did not respond to antimycin A. The differences in cyanide-resistant respiration between WH304 and WH314 treated with antimycin A suggest that *AOX1b* can be induced by respiratory inhibitors, whereas *AOX1a* is of constitutive character. Cyanide-resistant respiration of the *aox1a/aox1a aox1b/aox1b* strain WH324 was scarcely observed, even on treatment with antimycin A.

The results of Western-blot analysis of the *aox* mutants were consistent with the measurement of respiration. The AOX protein level of WH304 was little different from that of CAI4 whether or not treated with antimycin A (Figures 4B and 4C). The signals for AOX could not be detected in WH314 and WH324, ir-

respective of treatment with antimycin A. The absence of the AOX signal in the *aox1b/aox1b* strain WH314 suggests that the level of AOX1a protein is too low to be detected. The very low level of AOX1a protein is also supported by the fact that it was impossible to detect the signal for AOX1a transcripts in Northern-blot analysis. Taken together, these results indicate that cyanide-resistant respiration mediated by the AOX1a protein makes a small contribution to the total cyanide-resistant respiration in *C. albicans*.

The *aox* mutants showed normal growth patterns indistinguishable from the wild-type strain in either minimal or complex medium. To investigate the effects of the *aox* mutation on cell growth in the presence of a respiratory inhibitor, growth of the *aox* mutants was monitored in YPG medium containing KCN (Figure 5). The presence of 0.5 mM KCN remarkably retarded the growth of WH304 compared with CAI4. The growth of WH314 and WH324 was almost completely inhibited in the presence of 0.5 mM KCN. These growth patterns of the *aox* mutants were also observed in the same medium containing $2 \mu\text{M}$ antimycin A (results not shown). These results suggest that cyanide-resistant respiration mediated by AOX may be critical in maintaining cell growth of *C. albicans* when the normal cytochrome pathway of electron transport is blocked.

Regulation of *AOX1a* and *AOX1b*

To precisely examine the regulatory features of *AOX1a* and *AOX1b* at the level of transcription, we constructed plasmids for the β -galactosidase reporter assay by fusing the *AOX1a* or *AOX1b* promoter to the *LAC4* gene from *Kluyveromyces fragilis*. The control strain WH331 containing empty vector pRC18 did not show any β -galactosidase activity under all of the conditions tested. We first investigated if *AOX* expression was affected by growth phase. The strain WH332 carrying the plasmid-borne *AOX1a-LAC4* fusion showed constant β -galactosidase activity irrespective of growth phase (results not shown), indicating that the expression of *AOX1a* is constitutive and independent of growth phase. In contrast, the β -galactosidase activity of the strain WH333 carrying the plasmid-borne *AOX1b-LAC4* fusion was largely dependent on growth phase (Figure 6A). Until early exponential phase, WH333 showed little β -galactosidase activity. As the cell culture grew mature, the β -galactosidase activity of WH333 increased gradually. The highest level of β -galactosidase

Figure 6 Regulatory characteristics of *AOX1b* from *C. albicans*

(A) Expression of *AOX1b-LAC4* (●) in response to cell growth phase monitored by D_{600} (■). (B) Effects of different treatments on the expression of *AOX1b-LAC4*. Cells were treated for 2 h with 1 mM KCN, 10 mM antimycin A, 5 mM H_2O_2 , 0.1 mM menadione, 5 mM paraquat, 1 mM *tert*-butyl hydroperoxide (*tert*-BHP), 1 mM diamide, low temperature (4 °C), heat shock (40 °C for 30 min) or osmotic stress (1 M NaCl). (C) Effects of various carbon sources on the expression of *AOX1b-LAC4*. Data shown represent the means $\pm$ S.E.M. of three independent experiments. U, units.

activity was achieved when the cell culture approached stationary phase. β -Galactosidase activity of WH333 then began to decrease as the cell culture grew old, and completely disappeared after 3 days. The rapid decrease in the β -galactosidase activity of the WH333 cells in stationary phase did not correlate with a reduction in cell viability, since the cell viability decreased very slowly, even after disappearance of the β -galactosidase activity. Western-blot analysis of AOX and measurement of the cyanide-resistant respiration rates against cell growth phase also coincided with the results of the β -galactosidase reporter assay (results not shown). These results suggest that cyanide-resistant respiration may play some roles in cell multiplication but it is not involved in the long-term survival of cells.

The activity of AOX has been reported to increase upon exposure to a number of stress conditions, including inhibition of the cytochrome pathway of electron transport and oxidant treatment. To examine the effects of various stress conditions on *AOX1a* and *AOX1b* expression, we determined the β -galactosidase activity of WH332 and WH333 after exposure to stress conditions. Like most AOXs from other sources, treatment with 1 mM KCN or 10 μ M antimycin A greatly enhanced the expression of *AOX1b*, as shown by an increase in the β -galactosidase activity (Figure 6B). The β -galactosidase activity of WH333 was also increased by treatment with oxidants, such as 5 mM H_2O_2 , 0.1 mM menadione and 5 mM paraquat; among which menadione was the strongest inducing agent. Menadione and paraquat are known as redox-cycling agents that generate superoxide radical anions when introduced into cells. Thus an increase in the β -galactosidase activity by menadione and paraquat suggests that superoxide radical anions are very potent inducers of *AOX* expression. However, other oxidants, such as *tert*-butyl hydroperoxide and diamide, did not change the β -galactosidase activity of WH333. Incubation of the WH333 cells at low temperature (4 °C) caused a slight increase in the β -galactosidase activity. But, the β -galactosidase activity of WH333 did not respond to heat shock (40 °C for 30 min) or osmotic stress (1 M NaCl). When the WH333 cells were grown in media containing non-fermentable carbon sources, such as glycerol or ethanol, a marked increase in the β -galactosidase activity was observed (Figure 6C), indicating that the expression of *AOX1b* is also influenced by the carbon source used for growth. In all the cases examined, the β -galactosidase activity of the strain WH332 carrying pX1a-LAC4 was essentially unchanged (results not shown), confirming that the expression of *AOX1a* is constitutive.

To test the possibility that the expression of *AOX* may be under the control of one or more of the known transcriptional regulators, we measured the AOX activity of the corresponding null mutants. There was no difference in the cyanide-resistant respiration rate between the wild-type strain and the mutant strains with deletions in *CPH1* (a gene that encodes a transcription factor known as *Candida pseudohyphal* regulator) [35] or *EFG1* (a gene that encodes a putative transcription factor involved in enhanced filamentous growth) [36], which are known to play positive regulatory roles in hyphal development. Deletion of *CAP1* [37], which codes for a bZip transcription factor homologous to *YAP1* involved in multidrug resistance and the response to oxidative stress in *S. cerevisiae*, also had no effect on the activity of AOX. Interestingly, the AOX activity of the mutant strain with a deletion in *CaSLN1* [38], one of three putative histidine kinase genes, was markedly low compared with the wild-type strain under normal conditions (Figure 7A). Western-blot analysis showed that the AOX protein level of the *casln1* mutant strain was much lower than that in CAI4 (Figure 7B). In contrast, when incubated in the presence of 10 μ M antimycin A for 1 h, the activity and the protein level of AOX in

Figure 7 Respiratory characteristics of the *casln1* mutant of *C. albicans*

(A) The activity of cyanide-resistant respiration of *C. albicans* strains. Oxygen uptake rates of fresh cells (open bars) and antimycin A-treated cells (shaded bars) were measured in the presence of 1 mM KCN. Data shown represent the means $\pm$ S.E.M. of three independent experiments. (B) Western-blot analysis of AOX from fresh cells of CAI4 (*CaSLN1/CaSLN1*; lane 1) and SLN (*casln1/casln1*; lane 2), and antimycin A-treated cells of CAI4 (lane 3) and SLN (lane 4). Numbers on the left-hand side of the immunoblot refer to molecular masses of the standards. The arrow on the right-hand side of the immunoblot indicates AOX.

the *casln1* mutant strain were slightly higher than those in CAI4. Taken together, these results suggest that *CaSLN1* may be involved in the expression of *AOX1b*. However, the mutant strains with null mutations in the other two histidine kinase genes, *CaNIK1* and *CaHK1* [38], showed normal cyanide-resistant respiration.

Functional expression of *AOX1a* and *AOX1b* in *S. cerevisiae*

In the previous study [22], we observed that *S. cerevisiae* is deficient of AOX-mediated cyanide-resistant respiration and showed that *C. albicans AOX1a* can be functionally expressed and confer cyanide-resistant respiration on *S. cerevisiae*. To examine whether *AOX1b* can also be functionally expressed in *S. cerevisiae*, we transformed *S. cerevisiae* with the plasmid pWK304, which was constructed by inserting the entire *AOX1b* gene and its flanking sequences into the plasmid pRS424. As expected, the respiration of WH117 carrying the empty vector was completely inhibited on the addition of KCN (Figure 8A). The cyanide-resistant respiration rate of WH119 transformed with the plasmid pWK303 containing the *AOX1a* gene increased to approximately 133 nmol O₂·h⁻¹·(mg of wet cells)⁻¹. WH120, harbouring the plasmid pWK304, also showed enhanced cyanide-resistant respiration, although its cyanide-resistant respiration

Figure 8 Respiratory characteristics of *S. cerevisiae* strains over-expressing *C. albicans* AOX

(A) The activity of cyanide-resistant respiration of WH117 carrying an empty vector, WH119 expressing *C. albicans* AOX1a and WH120 expressing *C. albicans* AOX1b. Oxygen uptake rates of fresh cells were measured in the presence of 1 mM KCN. Data shown represent the means $\pm$ S.E.M. of three independent experiments. (B) Western-blot analysis of AOX from fresh cells of WH117 (lane 1), WH119 (lane 2) and WH120 (lane 3). Numbers on the left-hand side of the immunoblot refer to molecular masses of the standards. The arrow on the right-hand side of the immunoblot indicates AOX.

rate was much lower than that of WH119. In accordance with the measurement of respiration, Western-blot analysis demonstrated that both AOX1a and AOX1b could be expressed in *S. cerevisiae* with the level of AOX1a protein being much higher than that of AOX1b protein (Figure 8B). The difference between the levels of AOX1a protein and AOX1b protein suggests that the promoter of AOX1a is more strongly recognized than that of AOX1b by the transcriptional apparatus of *S. cerevisiae*, which is a circumstance opposite from that occurring in *C. albicans*. At any rate, these results indicate that *C. albicans* AOX1b, as well as AOX1a, can be functionally expressed in and confer cyanide-resistant respiration on AOX-deficient *S. cerevisiae*. However, functional expression of either AOX1a or AOX1b did not enhance cell growth of *S. cerevisiae* in the presence of 0.5 mM KCN (results not shown). Presumably, an unknown cyanide-resistant respiratory pathway inherent in *S. cerevisiae*, which is not mediated by AOX, may be sufficient to cause cyanide-resistant growth in *S. cerevisiae*.

DISCUSSION

In the present study, the gene family encoding AOX from *C. albicans* was characterized. The AOX gene family from *C. albicans* is composed of two members, AOX1a and AOX1b, which encode

polypeptides consisting of 379 and 365 amino acids respectively. Interestingly, AOX1b was located 1.3 kb upstream of AOX1a in the same transcriptional direction. The presence of a multigene family for AOX and tandem arrangement of some members of the gene family seem to be common in plants. For example, there are at least four AOX genes in *A. thaliana* and two of them are located in tandem on one of the chromosomes [19]. Tandem-arranged AOX genes have also been identified in rice [18]. To our knowledge, however, there is no report showing the presence of a multigene family for AOX in either fungi or protozoa. Therefore the present study is the first report describing that a multigene family for AOX is present in a fungus.

Many studies have shown that AOX gene expression is induced by respiratory inhibitors and ROS, such as H₂O₂ and superoxide radical anions [6,7]. Induction of AOX gene expression by respiratory inhibitors, such as antimycin A and cyanide, is also thought to be mediated by ROS arising from inhibition of the cytochrome-involved respiratory pathway. This study shows that AOX1b from *C. albicans* is also induced by respiratory inhibitors and ROS, like other AOXs. Induction of AOX by ROS contributed to the hypothesis that AOX may play a role as an antioxidant defence mechanism by keeping the ubiquinone pool sufficiently oxidized to prevent the autoxidation of reduced ubiquinone and the subsequent formation of ROS, when electron flow through the cytochrome pathway becomes limited [39]. Recent studies support the hypothesis advocating a role for AOX in preventing the generation of ROS. Studies with isolated plant mitochondria have shown that chemical inhibition of AOX increases the generation of ROS [40]. Also, it was found that antisense suppression of AOX results in cells with a significantly higher level of ROS compared with wild-type cells, whereas the overexpression of AOX results in cells with lower ROS abundance [41]. In addition, cells overexpressing AOX have been found to have consistently lower expression of genes encoding ROS-scavenging enzymes, including superoxide dismutase and glutathione peroxidase [41]. Recently, Parsons et al. [42] reported that mitochondrial AOX protein and the capacity for cyanide-resistant respiration increased dramatically in wild-type tobacco cells in response to growth under phosphate limitation, and transgenic tobacco cells unable to induce AOX during phosphate limitation displayed high rates of intracellular H₂O₂ generation. All these above studies support a role for plant AOX in preventing the generation of ROS.

We expected that, if *C. albicans* AOX is also involved in preventing the generation of ROS like plant AOX, the mutant strains deficient in AOX might exhibit increased sensitivity to oxidative stress. To assay the resistance of *C. albicans* cells against oxidative stress, the cells were treated with H₂O₂ or menadione, a redox-cycling agent, and cell viability was monitored. However, we could not detect any significant difference in resistance against oxidative stress between the *aox* mutant strains and the wild-type strain. In addition, the cells overexpressing AOX showed similar oxidative stress responses to the control strain (results not shown). Moreover, in contrast with the observation in plant cells [41], we found little difference in the intracellular levels of ROS between the *aox* mutant strains and the wild-type strain when using the ROS-sensitive probe 2',7'-dichlorofluorescein diacetate. These results suggest that AOX may make little, if any, contribution to preventing the generation of ROS in *C. albicans*. Another possibility is that unidentified factors, other than AOX, may be involved in the removal of excess reduced ubiquinone. Alternatively some antioxidant enzymes scavenging ROS may be induced in the *aox* mutants, making their resistance against oxidative stress comparable with that of the wild-type cells. Since there is no report on the assay

of resistance against oxidative stress using the plant cells deficient in AOX, it will be interesting to test oxidative stress responses of AS8 tobacco cells [43], which are the only reported plant cells with lowered AOX activity, and compare them with those of the *aox* mutants of *C. albicans*.

There are some reports suggesting that cyanide-resistant respiration may be involved in cell growth and differentiation. AOX genes have been found to be differentially regulated during the ripening of mango fruit [3] and during the postgerminative development of soya bean cotyledons [44]. A study with *Dictyostelium* showed that chemical inhibition of AOX induces the formation of unique cell masses, in which almost all of the cells differentiate into stalk-like cells with large vacuoles and thick cell walls [4]. A recent study by Parsons et al. [42] showed that there are striking differences in cell morphology between the wild-type cells and the AS8 cells that are unable to induce AOX. *C. albicans* is a well-known polymorphic fungus capable of reversible morphological transitions between yeasts, pseudohyphae and hyphae. We tested if cyanide-resistant respiration influences the morphology of *C. albicans*. In our preliminary study, we observed that treatment with salicylhydroxamic acid, known as a specific inhibitor of AOX, remarkably retarded hyphal growth of *C. albicans* grown in solid Spider medium (results not shown), suggesting that AOX activity may be involved in the morphogenesis of *C. albicans* as in plants. However, the cell shape and the hyphal growth of the *aox* mutant strains differed little from those of the wild-type strain. In addition, the β -galactosidase activities of WH332 and WH333 carrying pX1a-LAC4 and pX1b-LAC4, respectively, did not respond to growth conditions that induced hyphal transition (results not shown). Taken together, these results clearly demonstrate that cyanide-resistant respiration mediated by AOX has no correlation with cell differentiation in *C. albicans*.

It has been shown that the AOX mRNA level in *H. anomala* remarkably increases when the cells are grown in medium containing glycerol, lactate or raffinose as a carbon source [45]. In the present study we observed that the carbon source used for growth also influences AOX gene expression in *C. albicans*. The expression of AOX1b was greatly enhanced when the cells were grown in media containing non-fermentable carbon sources, such as glycerol or ethanol. We presume that growth of the cells in a medium containing a non-fermentable carbon source rapidly saturates the cytochrome-involved respiratory pathway, and as a response, AOX is induced to maintain the turnover of the tricarboxylic acid cycle so that carbon skeletons for biosynthetic demands can be supplied. However, considering the observation that the *aox* mutants showed normal growth patterns in the media containing non-fermentable carbon sources, it is unlikely that AOX is indispensable for normal operation of the tricarboxylic acid cycle. Presumably, another, as yet unidentified, factor may participate in relieving saturation of the cytochrome-involved respiratory pathway. Alternatively, limitation in supplying carbon skeletons from the tricarboxylic acid cycle may not be a rate-limiting step of cell growth in the media containing non-fermentable carbon sources.

Although the expression of AOX1b is induced under numerous conditions, the only condition we could identify under which AOX affects cell growth of *C. albicans* was complete inhibition of the cytochrome pathway of electron transport by respiratory inhibitors, such as antimycin A or cyanide. However, such a condition is not likely to be realized *in vivo*. This may explain why we could not observe any other phenotypic changes in the *aox* mutants except growth retardation in the presence of a respiratory inhibitor. Further investigation is required to elucidate the true physiological roles of AOX in *C. albicans*.

Curiously, two bands were frequently, if not always, observed in the Western-blot analysis of AOX, raising a possibility that they might represent the products of two AOX genes. However, we found that the appearance of two bands on the Western blots depended on cell homogenization conditions. For example, addition of PMSF, a protease inhibitor, led to a decrease in the lower band. Considering this fact, together with the observations that the two bands disappear simultaneously in the *aox1b/aox1b* mutants (Figures 4B and 4C) and appear even when AOX1b is expressed in *S. cerevisiae* (Figure 8B), it seems that the two bands do not represent two gene products but result from post-translational modification of the AOX1b protein. We presume that the lower band is the mature form of the AOX1b protein (39037 Da), which results from the cleavage of the mitochondrial presequence from the premature form of the AOX1b protein (41940 Da).

It is interesting that the AOX activity and protein levels in the *casln1* mutant strain are markedly low under normal conditions, whereas the mutant strain shows slightly increased AOX activity and protein levels in the presence of a respiratory inhibitor compared with the wild-type strain. Sln1p of *S. cerevisiae* is a 'two-component' regulator involved in the osmoregulatory pathway and carries both histidine kinase and response regulator domains. Phosphorylation of Sln1p inhibits the HOG1 mitogen-activated protein kinase high-osmolarity glycerol response pathway via a phosphorelay mechanism including the phosphorelay intermediate Ypd1p and the response regulator Ssk1p [46]. Sln1p has also been shown to regulate the activity of Skn7p (a response regulator involved in the osmoregulatory pathway and the response to oxidative stress) by a phosphorelay from Ypd1p [47]. Deletion of the SKN7 response regulator results in the sensitivity of yeast to oxidizing agents, indicating that Skn7p is involved in the response to oxidative stress [48]. Skn7p is known to cooperate with Yap1p to activate various kinds of H₂O₂-inducible target genes [49], e.g., catalase (CTT1), thioredoxin (TRX2) and thioredoxin reductase (TRR1). Considering these facts, together with our results showing that *C. albicans* AOX is also induced by H₂O₂ and its expression is influenced by CaSLN1, it is highly probable that expression of *C. albicans* AOX is regulated by CaSKN7. It is not known, however, whether the SLN1-SKN7 signal transduction system established in *S. cerevisiae* is also conserved in *C. albicans*. Therefore it will be interesting to identify the CaSKN7 gene and investigate its potential role in the regulation of AOX expression in *C. albicans*.

Subunit structures and biochemical regulation of fungal AOX are quite different from those of the plant AOX. Fungal AOX generally exists as a monomer and is not subject to disulphide bond formation or organic acid stimulation, but can be stimulated by purine nucleotides. Regulatory mechanisms of the redox-sensitive disulphide bond system and α -keto acid stimulation have been well established for AOXs from a variety of plant sources. On the contrary, the mode of action of purine nucleotides on fungal AOX has not yet been determined. Various approaches, including site-directed mutagenesis, will be required to identify the regulatory domain of fungal AOX that interacts with purine nucleotides. In this respect, the *aox* null mutants of *C. albicans* will prove very useful in elucidating the regulatory mechanisms of fungal AOX.

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