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$_2$O$_2$, menadione and parquat. 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 mutant was 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 path 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$_2$O$_2$, 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 sulphhydrys, the plant AOX can then be stimulated by z-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 z-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 micro-organisms 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,
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 soybean [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 [16]. In micro-organisms, however, the presence of multigene families for AOX is controversial. It has been reported that AOX [16].

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, Irving, 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). 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.

Isolation, subcloning and sequencing of AOX1b from C. albicans

To construct the C. albicans genomic library, the genomic DNA from C. albicans ATCC 10231 was partially digested with Sau3AI, and DNA fragments of 10–23 kb in size were ligated into the dephosphorylated λEMBL3 vector (Stratagene, La Jolla, CA, U.S.A.) generated by BamHI 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′-GCTGGTGTG-(C/T)GCC(A/T)GGTATGTTG-3′ and 5′-GCTTCTTC(T/C/T)TC-CAAA(G/T)TAAC-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 λEMBL3 genomic library. From positive clones, the common 4.1 kb XbaI fragment was subcloned into pGEM-7Zf(+) (Promega, Madison, WI, U.S.A.) at the XbaI site, yielding pCAOX1b. Both strands of the cloned DNA were sequenced by the dyeodeoxy 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 ApaI/SacI 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 μM 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
attenuance of 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'-TAAACAGCTCTATCTTTGAGAAAT-3' and 5'-AAACCATTATTACCTCAACTAACG-3', which were modified to carry HindIII and EcoRV sites (underlined) respectively. For amplification of 1.3 kb AOX1b promoter fragment, the primer pair of 5'-TTACCTTAAGCCCTATAGTGGCACAC-3' and 5'-TAAGAGATATGGAATTGAAAATCC-3' were used, which were also modified to carry HindIII and EcoRV sites (underlined) respectively. The 1.2 kb HindIII/EcoRV AOX1a promoter fragment or the 1.3 kb HindIII/EcoRV AOX1b promoter fragment was fused to the 4.2 kb PvuII/XbaI LAC fragment of pRS-LAC4 [27]. The resulting AOX1a promoter–LAC4 fusion products were inserted into pRC18 [27] at the HindIII/XbaI sites to generate pX1a-LAC4 and pXb1-LAC4 respectively. The resulting plasmids pX1a-LAC4 and pXb1-LAC4 were transformed into the ura3-52 strain CA44 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: \(A_{gm}/D_{mon}\) of assayed culture × volume of culture assayed (ml) × length of incubation at 30 °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 (GYLEEAA) 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 *S. cerevisiae* genomic library. From positive clones, the common 4.1 kb *XbaI* 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 41940 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 43975 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...
Figure 2 Disruption of the \textit{AOX1b} gene in \textit{C. albicans}

(A) Restriction map of the region containing the \textit{AOX} gene family and insertion of the \textit{hisG-URA3-hisG} cassette at the HpaI/HindIII sites in the \textit{AOX1b} coding sequence. The endonuclease restriction sites are as follows: B, BglII; E, EcoRI; ES, 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 (\textit{AOX1b}/\textit{AOX1b}); lane 2, WH311 (\textit{AOX1b}/\textit{hisG-URA3-hisG}); lane 3, WH312 (\textit{AOX1b}/\textit{hisG-URA3-hisG}); lane 4, WH313 (\textit{AOX1b}/\textit{hisG-URA3-hisG}); lane 5, WH314 (\textit{AOX1b}/\textit{hisG-URA3-hisG}).

Disruption of the \textit{C. albicans AOX} gene family

Disruption of \textit{AOX1a} has been described previously [22]. For the disruption of \textit{AOX1b}, a disruption construct was prepared by replacing a portion of the coding region of \textit{AOX1b} with the \textit{hisG-URA3-hisG} sequence (Figure 2A), which was then used to transform the \textit{ura3/ura3} \textit{C. albicans} strain CAI4. The resulting \textit{Ura}+ transformants were screened by PCR or Southern-blot analysis, and the spontaneous \textit{Ura}− “pop-out” revertants from them were selected on minimal defined medium containing 5-fluoro-orotic acid. A homozygous disruption of \textit{AOX1b} was generated by repeating the above procedure, and confirmed
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$_2$O$_2$ 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$_4$)$_2$SO$_4$/l, 0.2 g of MgSO$_4$·7H$_2$O/l, 2.5 g of anhydrous K$_2$HPO$_4$/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$_2$HPO$_4$] 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 CA14. The cyanide-resistant respiration rate of fresh WH314 was approx. 22 % of that of CA14. The aox1a/aox1a aox1b/aox1b mutant strain WH324 did not exhibit cyanide-resistant respiration under normal conditions. When incubated in the presence of 10 μM antimycin A for 1 h, the cyanide-resistant respiration rate of CA14 increased to 97 nmol O$_2$·h$^{-1}$·(mg of wet cells)$^{-1}$. Incubation of WH304 in the presence of antimycin A also greatly enhanced cyanide-resistant

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; F, 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 (ΔAOX1b-AOX1a/ΔAOX1b-AOX1a); lane 2, WH321 [Δaox1b-aox1a::hisG-URA3-hisG/ΔAOX1b-AOX1a]; lane 3, WH322 [Δaox1b-aox1a::hisG-URA3-hisG/ΔAOX1b-AOX1a]; lane 4, WH323 [Δaox1b-aox1a::hisG-URA3-hisG/ΔAOX1b-AOX1a]; lane 5, WH324 [Δaox1b-aox1a::hisG-URA3-hisG/ΔAOX1b-AOX1a].
respiration, the rate of which was approximately 89 nmol O$_2$.h$^{-1}$.mg wet cells$^{-1}$. Considering that WH304 is the $\text{aox1a/aox1a}$ mutant strain, the increased cyanide-resistant respiration of WH304 on treatment with antimycin A seems to be attributed to the induction of $\text{AOX1b}$. In accordance with this view, cyanide-resistant respiration of the $\text{aox1b/aox1b}$ strain WH314 did not respond to antimycin A. The differences in cyanide-resistant respiration between WH304 and WH314 treated with antimycin A suggest that $\text{AOX1b}$ can be induced by respiratory inhibitors, whereas $\text{AOX1a}$ is of constitutive character. Cyanide-resistant respiration of the $\text{aox1a/aox1a aox1b/aox1b}$ strain WH324 was scarcely observed, even on treatment with antimycin A.

The results of Western-blot analysis of the $\text{aox}$ 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, irrespective of treatment with antimycin A. The absence of the AOX signal in the $\text{aox1b/aox1b}$ 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 $\text{aox}$ mutants showed normal growth patterns indistinguishable from the wild-type strain in either minimal or complex medium. To investigate the effects of the $\text{aox}$ mutation on cell growth in the presence of a respiratory inhibitor, growth of the $\text{aox}$ 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 $\text{aox}$ mutants were also observed in the same medium containing 2 μM 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 $\text{AOX1a}$ and $\text{AOX1b}$**

To precisely examine the regulatory features of $\text{AOX1a}$ and $\text{AOX1b}$ at the level of transcription, we constructed plasmids for the $\beta$-galactosidase reporter assay by fusing the $\text{AOX1a}$ or $\text{AOX1b}$ promoter to the LAC4 gene from Kluyveromyces lactis. The control strain WH331 containing empty vector pRC18 did not show any $\beta$-galactosidase activity under all of the conditions tested. As the cell culture grew mature, the $\beta$-galactosidase activity of the $\text{AOX1b-LAC4}$ fusion showed constant $\beta$-galactosidase activity irrespective of growth phase (results not shown), indicating that the expression of $\text{AOX1a}$ is constitutive and independent of growth phase. In contrast, the $\beta$-galactosidase activity of the strain WH332 carrying the plasmid-borne $\text{AOX1a-LAC4}$ fusion showed constant $\beta$-galactosidase activity and was largely dependent on growth phase (Figure 6A). Until early exponential phase, WH333 showed little $\beta$-galactosidase activity. As the cell culture grew mature, the $\beta$-galactosidase activity of WH333 increased gradually. The highest level of $\beta$-galactosidase...
activity was achieved when the cell culture approached stationary phase. \( \beta \)-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 \( \beta \)-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 \( \beta \)-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 \( \beta \)-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 \( \beta \)-galactosidase activity of WH332 and WH333 after exposure to stress conditions. Like most AOXs from other sources, treatment with 1 mM KCN or 10 \( \mu \)M antimycin A greatly enhanced the expression of AOX1b, as shown by an increase in the \( \beta \)-galactosidase activity (Figure 6B). The \( \beta \)-galactosidase activity of WH333 was also increased by treatment with oxidants, such as 5 mM \( \text{H}_2\text{O}_2\), 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 \( \beta \)-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 \( \beta \)-galactosidase activity of WH333. Incubation of the WH333 cells at low temperature (4 °C) caused a slight increase in the \( \beta \)-galactosidase activity. But, the \( \beta \)-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 \( \beta \)-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 \( \beta \)-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 \( \text{CPH1} \) (a gene that encodes a transcription factor known as \( \text{Candida} \) pseudohypolphosphatase) [35] or \( \text{EFG1} \) (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 \( \text{CAP1} \) [37], which codes for a bZip transcription factor homologous to YAP1 involved in multidrug resistance and the response to oxidative stress in \( \text{S. cerevisiae} \), also had no effect on the activity of AOX. Interestingly, the AOX activity of the mutant strain with a deletion in \( \text{CaSLN1} \) [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 \( \text{casln1} \) mutant strain was much lower than that in CAI4 (Figure 7B). In contrast, when incubated in the presence of 10 \( \mu \)M antimycin A for 1 h, the activity and the protein level of AOX in the \( \text{casln1} \) mutant strain were slightly higher than those in CAI4. Taken together, these results suggest that \( \text{CaSLN1} \) may be involved in the expression of AOX1b. However, the mutant strains with null mutations in the other two histidine kinase genes, \( \text{CaNIK1} \) and \( \text{CaHK1} \) [38], showed normal cyanide-resistant respiration.

**Functional expression of AOX1a and AOX1b in \( \text{S. cerevisiae} \)**

In the previous study [22], we observed that \( \text{S. cerevisiae} \) is deficient of AOX-mediated cyanide-resistant respiration and showed that \( \text{C. albicans} \) AOX1a can be functionally expressed and confer cyanide-resistant respiration on \( \text{S. cerevisiae} \). To examine whether AOX1b can also be functionally expressed in \( \text{S. cerevisiae} \), we transformed \( \text{S. cerevisiae} \) 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 \( \text{O}_2\cdot\text{h}^{-1}\cdot(\text{mg of wet cells})^{-1} \). WH120, harbouring the plasmid pWK304, also showed enhanced cyanide-resistant respiration, although its cyanide-resistant respiration
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 H$_2$O$_2$ 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 H$_2$O$_2$ 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 H$_2$O$_2$ 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 soybean 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 mature form of the AOX1b protein (41940 Da).

It is interesting that the AOX activity and protein levels in the cas11m null 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. Snl1p of S. cerevisiae is a ‘two-component’ regulator involved in the osmoregulatory pathway and carries both histidine kinase and response regulator domains. Phosphorylation of Snl1p inhibits the HOG1 mitogen-activated protein kinase high-osmolality glycerol response pathway via a phosphorelay mechanism including the phosphorelay intermediate Ypd1p and the response regulator Ssk1p [46]. Snl1p 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 CasSN1, it is highly probable that expression of C. albicans AOX is regulated by CasSN1. 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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