Pichia pastoris ‘just in time’ alternative respiration

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Alternative oxidases (Aox or Aod) are present in the mitochondria of plants, fungi and many types of yeast. These enzymes transfer electrons from the ubiquinol pool directly to oxygen without contributing to the proton transfer across the mitochondrial membrane. Alternative oxidases are involved in stress responses, programmed cell death and maintenance of the cellular redox balance. The alternative oxidase gene of the methylotrophic yeast *Pichia pastoris* was isolated and cloned to study its regulation and the effects of deregulation of the alternative respiration by overexpression or disruption of the gene. Both disruption and overexpression had negative effects on the biomass yield; however, the growth rate and substrate uptake rate of the strain overexpressing the alternative oxidase were slightly increased. These effects were even more pronounced when higher glucose concentrations were used. The occurrence of free intracellular radicals and cell death phenomena was investigated using dihydrorhodamine 123 and the TUNEL test. The results suggest a major contribution of the alternative oxidase to *P. pastoris* cell viability. The negative effects of deregulated alternative respiration clearly indicated the importance of precise regulation of the alternative oxidase in this yeast.

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INTRODUCTION

Mitochondrial alternative oxidases (Aox or Aod) are key enzymes for a shortcut to the standard respiratory pathway in plants, many fungi and yeasts (Veiga *et al.*, 2000, 2003a), *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* being striking exceptions. These terminal oxidases directly transfer electrons from the mitochondrial ubiquinol pool to oxygen. This allows respiration even in the presence of complex III and IV inhibitors like antimycin A or cyanide. The resulting free energy gets released as heat. In contrast to the cytochrome *c* oxidase, which is the terminal oxidase of the standard respiratory pathway, an alternative oxidase does not pump electrons through the mitochondrial membrane. Thus the supply of small metabolic intermediates by the central metabolic pathways gets uncoupled from cellular energy production. Respiratory ATP production then relies on the activity of complex I (Berthold *et al.*, 2000; Joseph-Horne *et al.*, 2001; Siedow & Umbach, 2000). Cyanide-resistant respiration (CRR) is common among

Crabtree-negative yeasts, which are not capable of aerobic fermentation (Veiga *et al.*, 2000, 2003a).

The presumed biological functions of alternative oxidases in different organisms seem to be as diverse as the respective modes of induction. For example, osmotic stress, chilling (Gonzalez-Meler *et al.*, 1999; Veiga *et al.*, 2003b), wounding, pathogen attack (Simons *et al.*, 1999), and treatment with H₂O₂ or inhibitors of the main respiratory chain, such as cyanide and antimycin A (Kirimura *et al.*, 1996), have been reported to induce alternative respiration. There is substantial experimental support for the role of alternative oxidases as protecting the cell from reactive oxygen species or providing some metabolic flexibility (Joseph-Horne *et al.*, 2001). Alternative oxidases are generally considered to allow control of ATP synthesis to maintain growth rate homeostasis and an ongoing turnover of the TCA cycle under a high energy charge (Joseph-Horne *et al.*, 2001; Moore *et al.*, 2002). A special function of alternative oxidases is thermogenesis in floral tissues of plants, where heat-produced foul-smelling compounds attract insect pollinators (Meeuse, 1975).

The pathogen *Trypanosoma brucei*, in the blood-borne stage, even lacks the cytochrome pathway activity and completely relies on alternative oxidase activity for respiration (Van Hellemond *et al.*, 1998). The mammalian respiratory chain

Abbreviations: CRR, cyanide-resistant respiration; ROS, reactive oxygen species; SHAM, salicylhydroxamic acid; TUNEL, TdT-mediated dUTP nick end labelling.

The GenBank/EMBL/DBJ accession number for the *AOD* gene from *P. pastoris* is DQ465985.

does not contain an alternative oxidase, which therefore represents a potential pharmaceutical target against pathogenic organisms, e.g. trypanosomes (Kita *et al.*, 2003; Nihei *et al.*, 2003; Yabu *et al.*, 2003), *Aspergillus fumigatus* (Tudella *et al.*, 2004) or *Histoplasma capsulatum* (Johnson *et al.*, 2003), which may rely on alternative oxidase activity at least to survive the environmental stresses during host infection. On the other hand plants and fungi need alternative oxidases to employ their cyanide- or NO-based pathogen defence strategies, which may be essential for survival (Huang *et al.*, 2002; Zottini *et al.*, 2002).

The structure and function (Affourtit *et al.*, 2002) of alternative oxidases from plants and fungi have been studied intensively for several decades. However, no stable and active form of an alternative oxidase has been purified so far (Berthold & Siedow, 1993; Chaudhuri *et al.*, 1995; Huq & Palmer, 1978). Two structural models based on di-iron enzymes have been discussed, both putting alternative oxidases into the di-iron carboxylate protein family. The older model described the alternative oxidase as a transmembrane protein possessing two membrane-spanning helices connected via another helix in the intermembrane space and containing a non-haem di-iron centre (Siedow *et al.*, 1995). A revised model considered the enzyme to be an interfacial rather than a transmembrane protein (Andersson & Nordlund, 1999). Plant alternative oxidases are believed to be dimeric whereas fungal alternative oxidases are thought to have a monomeric structure. Additionally, fungal alternative oxidases have been found to display different modes of regulation (Joseph-Horne *et al.*, 2000; Umbach & Siedow, 2000). In both cases, plants and fungi, the monomeric structure of the enzyme was found to be the catalytically active subunit. The dimeric structure of plant alternative oxidases, detected by chemical cross-linking experiments, was therefore discussed to be related to regulatory features rather than to catalytic activity (Umbach & Siedow, 2000).

In *Pichia pastoris*, as a Crabtree-negative yeast, CRR was detected by Veiga *et al.* (2003c). However, forced aeration of resting cells was necessary to induce measurable CRR. A gene encoding an alternative oxidase of *P. pastoris* or its expression has not yet been described. No alternative oxidase activity was detected in a recent attempt to characterize the energetic properties of isolated *P. pastoris* mitochondria (Gonzalez-Barroso *et al.*, 2006). The closely related yeast *Pichia angusta* (*Hansenula polymorpha*) was described to lack CRR under the conditions studied (Veiga *et al.*, 2000). We have identified a potential alternative oxidase gene (AOD) in *P. pastoris* and studied the effects of its disruption and overexpression. Alternative oxidase activity was measurable with a Clark electrode during the transition between exponential and stationary growth without pre-treatment of the cells with an inducing agent. A GFP fusion protein was successfully used to characterize the time-course of *P. pastoris* alternative oxidase expression. Here we show that the AOD gene is expressed in measurable amounts only

during a certain growth phase. Deregulation of the alternative respiration either by overexpression or by disruption of the alternative oxidase gene negatively affected the biomass yield and cell viability, whereas growth rates and glucose uptake rates were slightly increased.

METHODS

Strains and media. *Pichia pastoris* X-33 (Invitrogen) was used as a platform strain for all engineered *P. pastoris* strains. *Escherichia coli* XL-1 Blue (Stratagene) was used for all *E. coli* cloning procedures. All components for *E. coli* media were purchased from Carl Roth GmbH, and all components for *P. pastoris* media from Becton Dickinson. Media and electrocompetent *P. pastoris* cells were prepared according to the Pichia Expression kit manual (Invitrogen).

Amplification and cloning of the *P. pastoris* alternative oxidase gene. Standard molecular-biology procedures were performed according to Ausubel *et al.* (2003). The ORF of the *P. pastoris* AOD gene was amplified by PCR from genomic DNA of strain X-33 employing the primers 5'-cagaattcaaaacaatgttataaaactgtacgcaataagg-3' (*EcoRI*-PpAOD-f) and 5'-cagaattcactcagagttataaaacgagctcatctcttccc-3' (*XhoI*-PpAOD-r) (restriction sites are underlined). The stop codon of the gene was changed from TGA to TAA (bold). The gene was cloned into the pGAPZ-A vector (Invitrogen) via the restriction sites *EcoRI* and *XhoI* under the control of the constitutive GAP promoter. The resulting plasmid pGAPZ-A(PpAOD) was transformed into *E. coli* XL-1 Blue. Plasmid DNA was isolated from transformants and controlled by restriction analysis and sequencing. This construct was used for constitutive expression of AOD in *P. pastoris*.

Construction of the alternative oxidase disruption cassette.

The AOD disruption cassette was constructed by overlap-extension PCR. All overlap-extension PCR reactions were performed as follows. A 50 µl PCR reaction mixture without primers was prepared. This solution contained the templates with the overlapping regions at equimolar concentrations. A 35 cycle PCR programme was devised according to the specifications of the polymerase used. The annealing time was set to fit the T_m of the overlapping regions as well as the T_m of the primers. The extension time was set for amplification of the full-length product. After 10–12 cycles, 10 µl of a mixture containing the primers (0.2 pmol µl⁻¹ final concentration), buffer, polymerase and dNTPs was added to the PCR reaction and the final cycles were performed.

The zeocin resistance cassette from pGAPZ-A was amplified using the primers 5'-gttcgattgatcgtagtctcaggccacacaccatagctcaaaatg-3' (TefZeo1-f) and 5'-ggtagtgtaagtatacacagcttctcagtcgtctcctcgccacg-3' (TefZeo2-r). A 5' flanking region (the 5' 374 bp of the AOD gene) was amplified from genomic DNA with the primers 5'-ctcaaatgatgttaaaactgtacgcaataaggcc-3' (PpAOD-atg-f; the start codon of the alternative oxidase gene is in bold) and 5'-ctatttgagctatggtgtgtggccctgagactacgcatcaatccgaac-3' (TefZeo1-r). A 3' flanking region consisting of the 3' 328 bp of the alternative oxidase gene and an additional 145 bp downstream of the stop codon was amplified with the primers 5'-cgtggccgaggagcaggactgaggaagctgtgtatactactacc-3' (TefZeo2-f) and 5'-tagttgacgttgcggacatag-3' (PpAOD-Δ-r). The TefZeo primers had complementary sequences and were used to introduce homologous regions for the following overlap-extension PCR with the three PCR products. The two outer primers 5'-ctcaaatgatgttaaaactgtacgcaataaggcc-3' (PpAOD-atg-f) and 5'-tagttgacgttgcggacatag-3' (PpAOD-Δ-r) were added after cycle 10 to allow amplification of the full-size product. Gel electrophoresis showed a product of 1701 bp. The purified product was cloned into the plasmid pCR4Blunt-TOPO according to the Zero Blunt TOPO PCR Cloning Kit for Sequencing

manual (Invitrogen) and transformed into *E. coli*. Plasmid DNA was isolated from transformants and controlled by restriction analysis and sequencing.

Construction of a PpAOD-GFP fusion. The alternative oxidase gene was amplified from the plasmid pGAPZ-A(PpAOD) with the primers 5'-cagaattcaaaacaattgtaaaactgtacgaataagg-3' (*EcoRI*-PpAOD-f) and 5'-agcaccacaacaactttgatcaacagcagcagctaaacagagctcatc-tctttccc-3' (Linker-PpAOD-r). The second primer contained an additional 33 bases encoding an 11 amino acid linker region (underlined) and was also used to delete the stop codon of the alternative oxidase gene. The gene encoding GFP (Cramer *et al.*, 1996) was amplified with the primers 5'-gctgctgctgttgatccaaagtgttggtgctatggc-tagcaaaggagaagaac-3' (Linker-GFP-f; the 11 amino acid linker region is underlined) and 5'-cactcgagtttaacatgccatgtgtatcccc-3' (wtGFP-*XhoI*-r). The two PCR products were employed in an overlap-extension reaction. Finally the full-length product was purified and cloned into pGAPZ-A via *EcoRI* and *XhoI*. The resulting plasmid pGAPZ-A(PpAOD-GFP) for constitutive expression of the AOD-GFP fusion in *P. pastoris* was isolated from *E. coli* transformants and sequenced. For integration behind the native AOD promoter the PpAOD-GFP integration cassette was amplified from this plasmid using the primers 5'-cgttggttacttagaggaggaagctg-3' (PpAOD-infor) and 5'-agcttgcaaatgaagccttcgagc-3' (Cyc1TT-r). The primer PpAOD-infor is located within the ORF of the AOD gene. The Cyc1TT-r primer binds at the end of the *CYC1* transcription terminator of the vector pGAPZ-A.

Analysis of the alternative oxidase gene sequence. The *P. pastoris* alternative oxidase derived from the gene sequence was analysed for mitochondrial targeting sequences according to Claros & Vincens (1996); protein parameters were analysed as described by Gasteiger *et al.* (2005). It was aligned to other known sequences of alternative oxidases from three yeasts, three fungi and two plants using CLUSTALW (Thompson *et al.*, 1994).

Transformation of *P. pastoris* and *E. coli*. Transformation of *P. pastoris* and *E. coli* was performed by electroporation according to the Electrocomp kits (version G) from Invitrogen with a Gene Pulser and Gene Pulser Cuvettes (0.2 cm) from Bio-Rad. The plasmids pGAPZ-A(PpAOD) and pGAPZ-A(PpAOD-GFP) were linearized with *AvrII* (*XmaI*); *BlnI*) before transformation to facilitate integration into the GAP locus. The disruption and the PpAOD-GFP integration cassettes were amplified by PCR, purified and used directly for transformation. Integration of pGAPZ-A(PpAOD) and disruption of the AOD gene were confirmed by colony PCR and oxygen consumption measurements. The resulting strains were designated X-33 PpAOD (the strain overexpressing the alternative oxidase) and X-33 ΔPpAOD (the strain with the disrupted alternative oxidase), and X-33 PpAOD-GFP_(P-GAP) (the strain overexpressing the PpAOD-GFP construct under the control of the constitutive GAP promoter) and X-33 PpAOD-GFP_(P-AOD) (the strain expressing the PpAOD-GFP construct under the control of the native alternative oxidase promoter).

Shake-flask cultures of *P. pastoris* strains. Buffered minimal medium (50 ml) containing 1% or 5% glucose (BMD1% or BMD5%, respectively) in 250 ml baffled, wide-necked shake flasks was inoculated with overnight cultures to OD₆₀₀ ~0.1. Optical densities were measured against BMD1% on a Beckman Coulter Counter DU800. The cultures were incubated at 28 °C and 80% relative humidity with constant shaking (140 r.p.m.) in an Infors Multitron II shaker. *P. pastoris* X-33 was used for induction experiments to study the time-course of the alternative oxidase activity after induction with antimycin A (Sigma-Aldrich). The strain was grown in BMD2% until an OD₆₀₀ between 5 and 6 was reached. Antimycin A was then added to the exponentially growing cultures

to a final concentration of 10 µg ml⁻¹. Antimycin A was only used for this experiment; all other growth experiments were performed without antimycin A induction.

Strains X-33, X-33 ΔPpAOD and X-33 PpAOD were grown to study the effects of overexpression and disruption of the alternative oxidase on the growth behaviour and viability of *P. pastoris*. Strains X-33, X-33 PpAOD-GFP_(P-AOD) and X-33 PpAOD-GFP_(P-GAP) were used to study the regulation of expression of the alternative oxidase. Expression and degradation of the protein were followed by fluorescence measurements. The background fluorescence values of untransformed *P. pastoris* X-33 were subtracted from those of X-33 PpAOD-GFP_(P-AOD) and X-33 PpAOD-GFP_(P-GAP). Differences in the optical density of the cultures at the given time points were negligible and therefore not taken into consideration. All shake-flask experiments were performed in duplicate.

Sample preparation and measurements. Samples (1 ml) were taken from shake-flask cultures at set time points. After centrifugation for 1 min at 13 200 r.p.m. in an Eppendorf 5415R centrifuge at 4 °C, ethanol and glucose concentrations in the supernatant were determined. Glucose and ethanol concentrations in the media were measured using the Glucose UV Hexokinase method (DIPROmed, Weigelsdorf, Austria) and the Ethanol UV method (Hoffmann-La Roche), respectively. Both protocols were downscaled to a total volume of 200 µl to enable measurements with a SPECTRAMax Plus384 plate reader (Molecular Devices) in UV microplates (Greiner Bio-One). Depletion of glucose in the media during the cultivation of cells was defined as the glucose uptake rate (q_s) of the cells. Fluorescence measurements were performed in black microtitre plates from Greiner Bio-One containing 200 µl cells per well with a SPECTRAMax Gemini XS spectrofluorometer from Molecular Devices. The fluorescence was measured at 507 nm, the excitation wavelength was 395 nm and the cut-off filter was set at 495 nm. All measurements and the corresponding calibration curves were performed at least in duplicate.

Oxygen consumption measurements. For oxygen consumption measurements cells were grown to an OD₆₀₀ between 4 and 6 in BMD2%. Samples (200 µl) of the cell solution were used for oxygen consumption measurements in a total volume of 1 ml. The volume was adjusted with 100 mM potassium phosphate buffer (pH 6). Oxygen consumption was measured with a Dual Digital Clark electrode model 20 from Rank Brothers. CRR was assessed by adding potassium cyanide (KCN) or salicylhydroxamic acid (SHAM) to a final concentration of 2 mM. Both chemicals were prepared fresh for all measurements; SHAM (Fluka) was dissolved in dimethylsulfoxide (Roth), KCN (Sigma-Aldrich) in water with the pH adjusted to 10.5. Measurements of the X-33 and the X-33 PpAOD strains were performed in triplicate, measurements of the ΔPpAOD strain in duplicate.

Tests for apoptotic markers. The TdT-mediated dUTP nick end labelling (TUNEL) test was applied to determine the effects of overexpression and disruption of the alternative oxidase gene on cell viability of the recombinant strains by assaying apoptotic phenomena (Fröhlich & Madeo, 2003) with the *In situ* Cell Death Detection kit, POD (Roche Diagnostics). Dihydrorhodamine 123 (Sigma-Aldrich) was used to detect free intracellular reactive oxygen species (ROS). Cells were treated as described previously (Madeo *et al.*, 1997, 1999; Weis *et al.*, 2004). The total number of cells and the number of cells giving a positive signal per picture were counted and used to calculate the percentage of cells showing signs of ROS accumulation. At least four pictures per strain and time point from two independent slides were used for the calculations.

RESULTS

Induction of the *P. pastoris* alternative oxidase with antimycin A

Respiration of the exponentially growing *P. pastoris* control strain was impaired by the addition of cyanide; however, a low level of cyanide-resistant respiration (CRR) was observable. This was observed in the untreated control strain (Fig. 1a) as well as immediately after the addition of antimycin A (Fig. 1b), indicating that at least a small amount of active alternative oxidase is present during exponential growth on glucose. Addition of antimycin A abolished respiration to a large extent and induced the expression of the alternative oxidase. The effect of cyanide on the total respiratory activity decreased over time: 2 h after induction, full respiratory activity was restored (Fig. 1). Addition of SHAM to the control had virtually no effect on the respiratory activity.

Cloning and sequence analysis of the *P. pastoris* alternative oxidase gene

The deduced amino acid sequences of the *Candida albicans* alternative oxidase genes *AOX1* (AF031229) and *AOX2*

(AF116872) and the *Pichia stipitis* SHAM-sensitive terminal oxidase (*STO1*) gene (AY004212) were sent to Integrated Genomics to search for homologues in the preliminary genome sequence of *P. pastoris* IG66. A BLAST search delivered matches for all three queries in the same contig (contig3604). We analysed contig3604 for putative ORFs and found one ORF which showed high sequence similarity to the full coding region of other alternative oxidases. The ORF was amplified from genomic DNA of *P. pastoris* X-33 by PCR as described in Methods. The resulting PCR product was directly sequenced and found to be identical to the coding sequence in contig3604 of the *P. pastoris* genome sequence.

The uninterrupted alternative oxidase ORF (AOD) from *P. pastoris* had a length of 1089 nucleotides. The nucleotide sequence was deposited in the GenBank database under the accession number DQ465985. The translated protein sequence consists of 362 amino acids and contains the characteristic conserved regions NERMHL, LEEEA and RADEA.H (Berthold *et al.*, 2000). Multiple protein sequence alignment of the *P. pastoris* alternative oxidase with sequences from other yeasts, fungi and plants showed significant identity in the C-terminal part (Fig. 2), whereas the N-terminal part displayed very low identity, most probably due to the degenerate nature of mitochondrial targeting sequences. The protein has a predicted molecular mass of 41 833 Da. A predicted cleavable mitochondrial targeting sequence consists of the first 26 N-terminal amino acids. The processed protein therefore has a predicted molecular mass of 38 776 Da.

Reporter fusion proteins have been applied with great success to study the expression and localization of proteins (Zaslaver *et al.*, 2004). In order to verify the predicted targeting of the alternative oxidase into the mitochondria we constructed a fusion protein, linking green fluorescent protein (GFP) C-terminally to the alternative oxidase. This AOD-GFP fusion was expressed employing the strong constitutive GAP promoter. Fluorescence microscopy of the resulting transformants revealed local fluorescence, as is usually observed for a typical mitochondrial localization in yeast (data not shown). Measurements of oxygen consumption rates of strain X-33 PpAOD-GFP_(P-GAP) showed that the alternative oxidase part of the fusion protein was fully functional (data not shown). Results were similar to those obtained with strain X-33 PpAOD, which constitutively overexpressed the *P. pastoris* alternative oxidase.

Expression analysis of AOD-GFP fusions

We employed green fluorescent protein as a reporter to follow the time-course and level of expression of the *P. pastoris* alternative oxidase under standard laboratory conditions in shake-flask cultures. The fusion protein was expressed under the control of the native *P. pastoris* alternative oxidase promoter and also under the control of P_{GAP}. Fluorescence was measured at given time points and compared to the data obtained from untransformed *P.*

Fig. 1. Oxygen consumption measurements of exponentially growing *P. pastoris* X-33 cells. (a) Control measurement. (b) Addition of antimycin A (10 µg ml⁻¹ final concentration) at time zero. The values represent results from single measurements (relative standard deviation from three independent measurements was ~15% of the mean).

<i>P.stipitis</i>	IAGVPGAVASFIRHLHSLRLLKRDKAWIETLLDEAFN	NERMHLLTFIKIGKPSWFTRTIY	220
<i>C.albicans</i>	VAGVPGSVAGFLRHLHSLRMLRRDKAWIETLLDEAY	NERMHLLTFIKIGKPSWFTRSIY	227
<i>P.anomala</i>	VAGVPGMVAAFIRHLHSLRLLKRDKAWIETLLDEAY	NERMHLLTFIKIGNPSWFTRFIIY	203
<i>P.pastoris</i>	IAGVPGSVASFRLRLQSLRLLKRDKAFTQLQDEAY	NERMHLLTFLEIGKPGPFMKVLLY	224
<i>A.niger</i>	VAGVPGMVGGMLRHLRSLRRMKRDNGWIETLLEEAY	NERMHLLTFLKLAEPGWFMRMLVL	215
<i>A.nidulans</i>	VAGVPGMVGGMLRHLRSLRRMKRDNGWIETLLEEAY	NERMHLLTFLKMAEPGWFMRMLVL	215
<i>N.crassa</i>	IAGVPGMVAGMLRHLHSLRLLKRDNGWIETLLEESY	NERMHLLTFMKMCEPGLLMKTLIL	224
<i>S.guttatum</i>	VAAVPGMVGGVLLHLKSLRRFEHSGGWIRALLEEA	NERMHLLTFMEVAQPRWYERLVI	239
<i>A.thaliana</i>	VAAVPGMVGGMLVHCKSLRRFEQSGGWIKALLEEA	NERMHLLTFMEVAKPNWYERLVI	215
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<i>P.stipitis</i>	VGQGVFCNLFFLFYLANPKYCHRFVGY	LEEEAVSTYTHFVHELQS-GKLPKF--ENIKIP	277
<i>C.albicans</i>	VGQGVFTNVFFLLYLLNPRYCHRFVGY	LEEEAVRTYSHLLDELAVPGKLPF--ETMKIP	285
<i>P.anomala</i>	MGQGVFANLFFLVYLIKPRYCHRFVGY	LEEEAVSTYTHLIKIDIDS-KRLPKF--DDVNL	260
<i>P.pastoris</i>	ASQGVFCNLFFFTYMLAPNACHRFVGY	LEEEAVYTYTTCLEDIER-GLLPDI--EHFKVP	281
<i>A.niger</i>	GAQGVFFNGFFLSYLSMRPRICHRFVGY	LEEEAVITYTRAIKEIEA-GSLPAW--EKTEAP	272
<i>A.nidulans</i>	GAQGVFFNGFFLSYLISPRTCHRFVGY	LEEEAVLTYTRAIKDLES-GRLPHW--EKLEAP	272
<i>N.crassa</i>	GAQGVFFNAMFLSYLISPKITHRFVGY	LEEEAVHTYTRCIREIEE-GHLPKWSDEKFEIP	283
<i>S.guttatum</i>	AVQGVFFNAYFLGYLLSPKFAHRMVGY	LEEEAIHSYTEFLKDIDN-GAIQDCP-----AP	293
<i>A.thaliana</i>	AVQGIFFNAYFLGYLLSPKFAHRMVGY	LEEEAIHSYTEFLKELDN-GNIENVP-----AP	269
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<i>P.stipitis</i>	TIWQYWPTELTSMSLDLILR	RADEAKHREVNHTLANLDQR-KDRNPFGLAIPDLKEP	336
<i>C.albicans</i>	EVAVQYWPTELTPKSSFKDLILR	RADEAKHREVNHTFANLEQK-TDRNPFALKIEGLNKP	344
<i>P.anomala</i>	EISWLYWTDLNEKSTFRDLIQR	RADESKHREVNHTLANLEQK-KDRNPFALKVEDVPKE	319
<i>P.pastoris</i>	KIAKDYW-HLSEDAKMRDLISY	RADEAKHREVNHTFANLKLDGTDRNPFALQVDSVPES	340
<i>A.niger</i>	EIAVQYWKMPQGRSMKDLLLY	RADEAKHREVNHTLGNLNQA-IDPNPYAAKYKDPTKA	331
<i>A.nidulans</i>	EIAVKYWKMPGNRTMKDLLLY	RADEAKHREVNHTLGNLKQA-VDVNPFVAVWKDPSKP	331
<i>N.crassa</i>	EMAVRYWRMPQGRSMKDLIHY	RADEAKHREVNHTLSNLDQK-EDPNPFVSDYKE-GEG	341
<i>S.guttatum</i>	AIALDYWRLPQG-STLRDVVTV	RADEAKHREVNHTFASDVHYQ-----	335
<i>A.thaliana</i>	AIAIDYWRLEAD-ATLRDVMV	RADEAKHREVNHTFASDVHYQ-----	311
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Fig. 2. Alignment of the conserved C-terminal part of the alternative oxidases from *Candida albicans* (AAF21993, *Aox1b*), *Pichia stipitis* (AAF97475), *Pichia anomala* (BAA90763), *Aspergillus niger* (O74180), *Aspergillus nidulans* (EAA64931), *Neurospora crassa* (AAN39882), *Sauromatum guttatum* (CAA78823), *Arabidopsis thaliana* (BAA22624) and *Pichia pastoris*. The highly conserved residues among alternative oxidases are boxed.

pastoris X-33. The resulting fluorescence curves followed the growth curves almost in parallel up to the point of complete glucose depletion in the medium (Fig. 3), again indicating a low expression level of the enzyme in the presence of glucose. Even after normalization of the fluorescence values by the optical density of *P. pastoris* X-33 PpAOD-GFP_(P-AOD) we saw increasing fluorescence per unit of optical density in the cells over time (Fig. 4), at least as long as glucose was not depleted. This implies that the expression level of the enzyme increased constantly during growth on glucose. Otherwise the increasing fluorescence could be explained by an increase of mitochondria, providing a higher hosting capacity for the fusion protein or the saturation of the degradation system.

Surprisingly, the fluorescence decreased very rapidly upon depletion of the carbon source. This could be explained by a glucose-induced expression in combination with a degradation mechanism switched on upon lack of glucose. Alternatively, degradation could be constitutive for alternative oxidase. This is supported by the instability index of the processed protein (41.40) calculated by the algorithm of Gasteiger *et al.* (2005), which classifies it as unstable. Addition of methanol (1% final concentration) to the

Fig. 3. Time-course of alternative oxidase expression with 5% glucose in the medium. The expression of the alternative oxidase-GFP fusion protein followed the same mode of expression and degradation with 1% and 5% glucose. Relative fluorescence values [mean values from three measurements given in relative fluorescence units (RFU)] rose continuously to a maximum shortly after the time point of glucose depletion. After this time point, fluorescence values declined due to the degradation of the fusion protein.

Fig. 4. Fluorescence normalized by optical density. *P. pastoris* was grown in medium containing 1% or 5% glucose at the time of inoculation. The fluorescence levels obtained (mean values from three measurements) were normalized to the OD_{600} of the respective strain (mean values from two measurements) at the given time points, thus reflecting the amount of enzyme per cell. Accumulation and degradation of the protein complex occurred at the same pace under the control of the alternative oxidase promoter, independent of the initial glucose concentration. The fusion protein kept accumulating when the constitutive GAP promoter was used. The fluorescence peak around the time point of glucose depletion reflects an expression characteristic of the GAP promoter.

culture with 5% initial glucose concentration 70 h after inoculation did not induce alternative oxidase expression (data not shown). The rising optical density of the cultures confirmed the use of methanol as carbon source, but no fluorescence was measured.

We also performed expression of the fusion protein under control of the GAP promoter (for 5% glucose only). This resulted in a constant increase of fluorescence up to 50 h of culture time (Fig. 4). We therefore propose that the alternative oxidase is permanently degraded and the level of alternative oxidase is controlled by regulation of protein formation. Interestingly, at the time point of substrate depletion, an increase of fluorescence became apparent during P_{GAP} forced expression. This is a feature of this 'constitutive' promoter, which we have observed in several cases before (data not shown). Upon depletion of glucose, the expression mediated by this promoter increases, resulting in a steeper slope in the respective product curves for a short period, usually two doubling times.

Constitutive expression and disruption of the AOD gene

Employing oxygen consumption measurements we demonstrated both constitutive expression of functional alternative oxidase and its disruption. The relative respiratory rates (taking non-inhibited respiration as 100%) of the recombinant strains and the control strain are shown in Fig. 5. The strong inhibitory effect of KCN on the respiration of the control strain *P. pastoris* X-33 indicated a marginal, yet

Fig. 5. Relative oxygen consumption rates (100% corresponds to an oxygen consumption rate of $33.25 \text{ nmol min}^{-1}$ per OD_{600} unit). The control strain X-33 displayed a low level of CRR; SHAM did not influence the respiratory activity. The strain overexpressing the alternative oxidase (X-33 PpAOD) was not influenced by either cyanide or SHAM. Both respiratory systems were capable of maintaining the total respiratory activity. Disruption of the alternative oxidase gene led to impairment of CRR. Measurements of the X-33 and the X-33 PpAOD strain were performed in triplicate; the reported values represent means $\pm$ SD. In the case of the disruption strain X-33 Δ PpAOD the bars indicate a similar deviation although measurements were made only in duplicate.

measurable commitment of the alternative oxidase to respiration during growth on glucose. The effect of SHAM on the total respiratory rate was negligible, providing further evidence of a low alternative oxidase activity making no significant contribution to total respiration during exponential growth on glucose. The disruption strain X-33 Δ PpAOD was not capable of CRR and consequently the addition of SHAM did not influence the respiratory activity. Since disruption of a single gene abolished CRR in *P. pastoris* it was evident that a single gene encoded the alternative oxidase. The disruption of the gene was also confirmed by colony PCR (data not shown).

The respiratory activity of the overexpressing strain X-33 PpAOD was not susceptible to either of the inhibitors applied. The final concentration of KCN was increased to 6 mM without significantly changing the total respiratory activity of this strain. Also, concentrations of 2 mM of each of the inhibitors SHAM and KCN were not sufficient to suppress respiration completely. Thus, the constitutively overexpressed alternative respiratory system was capable of maintaining full respiratory activity under conditions where respiration of the non-transformed *P. pastoris* strain was completely inhibited. The total respiratory activity of the recombinant strains before addition of the inhibitors was not significantly altered compared to the control strain (data not shown). No morphological changes due to the introduced genetic changes were observed by standard transmitted light microscopy. During growth, the engineered cells were the same size and shape as the wild-type cells.

Table 1. Physiological parameters of the *P. pastoris* strains

The values of q_s (glucose uptake rate) and $\mu_{\max}$ (maximum growth rate) were calculated from measurements during the early exponential growth phase. Overexpression of the alternative oxidase led to a slight increase of these parameters. The numbers are means $\pm$ SD from four measurements (cultures were grown in duplicate; two measurements per time point and culture were performed). The ethanol values represent the highest values measured during growth of the strains.

Strain	q_s (g g ⁻¹ h ⁻¹)		$\mu_{\max}$ (h ⁻¹)		Ethanol (g l ⁻¹)	
	1 % Glucose	5 % Glucose	1 % Glucose	5 % Glucose	1 % Glucose	5 % Glucose
X-33	0.39 $\pm$ 0.02	0.31 $\pm$ 0.03	0.237 $\pm$ 0.004	0.231 $\pm$ 0.003	1.6 $\pm$ 0.003	6.91 $\pm$ 0.22
X-33 Δ PpAOD	0.37 $\pm$ 0.025	0.32 $\pm$ 0.03	0.236 $\pm$ 0.003	0.231 $\pm$ 0.004	1.4 $\pm$ 0.019	3.47 $\pm$ 0.03
X-33 PpAOD	0.41 $\pm$ 0.015	0.40 $\pm$ 0.025	0.245 $\pm$ 0.004	0.241 $\pm$ 0.003	1.76 $\pm$ 0.035	7.97 $\pm$ 0.35

Growth characteristics of *P. pastoris* strains with deregulated AOD expression

Surprisingly the maximum growth rate ($\mu_{\max}$) and the substrate uptake rate (q_s) of the X-33 PpAOD strain were slightly increased (Table 1). Cultures of the strain expressing AOD constitutively entered the stationary growth phase earlier since also glucose was used up earlier in these cultures than in those of the other two strains. The similarity of growth behaviour of the control strain X-33 to that of X-33 Δ PpAOD in this stage of growth indicated that this phenomenon was a specific effect caused by AOD overexpression. Biomass yield was lowered as a consequence of constitutive or disrupted alternative oxidase activity under low as well as high glucose concentrations (Fig. 6). The AOD disruption strain showed only minor deviations from the control strain concerning the final optical density in 1 % glucose medium (data not shown), whereas the difference in 5 % glucose medium was more pronounced (Fig. 6). Since $\mu_{\max}$ and q_s were not significantly altered from the values of the wild-type, the difference in final biomass, indirectly determined by optical density, could not be attributed to these physiological parameters. Furthermore, the differences occurred at a later stage of growth than those observed in the

X-33 PpAOD strain. As the literature links alternative oxidase activity to stress response (Gonzalez-Meler *et al.*, 1999; Kirimura *et al.*, 1996; Ordog *et al.*, 2002; Robson & Vanlerberghe, 2002; Simons *et al.*, 1999; Vanlerberghe *et al.*, 2002; Veiga *et al.*, 2003b) and therefore to cellular viability we consider increased cell death phenomena as a possible explanation for this discrepancy.

In addition to the observed alterations in growth behaviour, overexpression of the alternative oxidase also led to increased ethanol production, whereas disruption of the gene led to lower ethanol concentrations. These differences were more pronounced in the experiments with the higher glucose concentration.

Alternative-oxidase-dependent cell death phenomena

Reactive oxygen species (ROS) are formed in any organism exposed to molecular oxygen and they are initiators of apoptosis. At least in plants, overexpression of the alternative oxidase was described to reduce intracellular ROS concentrations (Maxwell *et al.*, 1999). Reduced expression led to ROS accumulation. To test the effect of AOD

Fig. 6. Shake-flask cultures of X-33, X-33 PpAOD and X-33 Δ PpAOD in medium containing 5% glucose. Overexpression of the alternative oxidase resulted in lowered final OD₆₀₀ of the recombinant strain. The growth behaviour differed from that of the other two strains, characterized by a break in the exponential growth phase. This strain left the exponential phase earlier than the control or the X-33 Δ PpAOD strain. Upon reaching the stationary growth phase, the OD₆₀₀ of the X-33 Δ PpAOD strain decreased compared to the control strain. While overexpression of the alternative oxidase resulted in increased ethanol production, strain X-33 Δ PpAOD produced the lowest amount of ethanol. The same was observed in the experiments where 1 % glucose was used in the medium.

overexpression on ROS production and cell viability, we used the non-fluorescent dihydrorhodamine 123, which is oxidized intracellularly by ROS to the fluorescent rhodamine 123, to study the effect of alternative oxidase activity on ROS production. Furthermore we determined the fraction of apoptotic cells in liquid cultures of X-33, X-33 PpAOD and X-33 Δ PpAOD by the TUNEL test, which traces degradation of genomic DNA, a typical feature of apoptosis. At a glucose concentration of 1 % in the culture medium neither statistically relevant rhodamine 123-mediated fluorescence nor apoptotic cells in the TUNEL test were detected (data not shown). These results correlated well with the earlier findings of Weis *et al.* (2004), who reported 1 % as the optimal glucose concentration to minimize *P. pastoris* cell death phenomena in shake-flask and deep-well plate cultures.

In medium containing 5 % glucose, ROS production and the fraction of apoptotic cells were increased for both engineered strains in comparison to the untransformed strain X-33 (Fig. 7). The effects were more pronounced for the disruptant and appeared earlier. Both extremes of deregulated AOD expression obviously showed negative effects in *P. pastoris*. TUNEL positives, indicating apoptotic cells, appeared later than ROS positives, i.e. after 39 h of growth. Although showing the same trend as the ROS accumulation the differences between the analysed strains were more pronounced in the results of the more sensitive TUNEL test. Both viability tests and their correlation strongly suggest an important role for the alternative oxidase in preventing ROS production and programmed cell death. However, there are negative effects due to alternative oxidase overexpression or its disruption.

DISCUSSION

The alternative CRR system of plants and fungi is responsible for a wide variety of tasks and consequently follows different modes of expression. In *Pichia anomala* (formerly *Hansenula anomala*), the expression level of the alternative oxidase was found to be carbon source regulated. Expression was low in glucose-containing media but induced by glycerol, lactate or raffinose (Sakajo *et al.*, 1998). A very similar pattern was found for the *AOX1b* gene from *C. albicans*, which was up-regulated on non-fermentable carbon sources such as glycerol and ethanol (Huh & Kang, 2001). We identified the single gene encoding the *P. pastoris* alternative oxidase and found that it is expressed in the presence of glucose and is highly inducible by antimycin A, an inhibitor of complex III of the cytochrome pathway.

The effects caused by deregulation of alternative-oxidase-mediated CRR in *P. pastoris* were surprisingly diverse. The most noteworthy features of an up-regulated alternative oxidase activity were slightly increased growth and glucose uptake rates as well as higher ethanol concentrations. We do not believe that there is a direct influence of alternative oxidase activity on the hexose transporters. More reasonable

Fig. 7. Assessment of cell death phenomena associated with the overexpression or disruption of the *P. pastoris* alternative oxidase at high glucose concentration (5 % glucose). (a) Percentage of cells showing rhodamine-mediated fluorescence. (b) Percentage of TUNEL-positive cells. Disruption as well as overexpression of the alternative oxidase led to increased occurrence of apoptotic markers. The values are means $\pm$ SD from at least four pictures per time point from two independent slides.

is the assumption that an altered energy status of the cells forced increased glucose uptake. Thereby the lowered energy production efficiency was compensated by an increased glucose import, which in turn led to an increased growth rate.

Elevated ethanol production from xylose has been described before for a *cyc1 Δ* mutant of the yeast *Pichia stipitis*, which relies on alternative respiration (Shi *et al.*, 1999). Interestingly, disruption of the *P. stipitis* alternative oxidase also led to higher ethanol production on xylose (Shi *et al.*, 2002), whereas we found that disruption of the gene in *P. pastoris* led to decreased ethanol production, albeit with glucose as the carbon source. This might be an interesting feature for protein production since the most-used methanol-inducible *AOX1* promoter of *P. pastoris* is suppressed by ethanol. However, the ethanol which is produced by *P. pastoris* is usually used up during the switch of the carbon source from glucose to methanol.

In *Schiz. pombe*, growth rates were negatively affected upon overexpression of a plant alternative oxidase (Affourtit *et al.*, 1999). The authors found active competition between the alternative oxidase and cytochrome *c* pathway for reducing

equivalents. The alternative oxidase contributed up to 24 % of overall respiratory activity and the strong non-protonmotive force obviously lowered the efficiency of energy production and thereby growth (Affourtit *et al.*, 1999). Generally, bottlenecks in NAD^+ regeneration or ATP feedback inhibitions limit the flux through glycolysis and the citric acid (TCA) cycle. We assume that uncoupling of NAD^+ regeneration from ATP production by overexpression of the alternative oxidase abolished this limitation and increased growth rates, but lowered the growth efficiency in terms of biomass yield since alternative oxidase activity produces heat rather than supporting ATP formation for biomass production. To our knowledge, this is the first report of increased growth and substrate uptake rates due to elevated alternative oxidase activity. These desirable features came at the high price of reduced biomass yield. Thus, the hallmark ability of *P. pastoris* to grow to high cell densities, which is believed to be a positive feature for high-level protein expression, was impaired in our engineered strains. Being better than nature in optimizing the AOD expression level towards increased growth and substrate uptake rates without the overwhelming disadvantage of reduced biomass yields seems tempting but might be hard to achieve, if possible at all. At least the extremes of expression deregulation, i.e. gene disruption or constitutive overexpression, are not the tools to do so.

For many organisms, the protective role of the alternative oxidase against ROS has been investigated and connected to programmed cell death (PCD) phenomena. A contribution of alternative respiration to cell viability has been shown in *Trypanosoma brucei rhodesiense* (Tsuda *et al.*, 2005). The bloodstream form of this pathogen showed no PCD but up-regulated trypanosome alternative oxidase (TAO) activity. Inhibition of the TAO by ascofurane caused the development of PCD phenomena. Respiratory function is also a key factor in controlling the life-span of the filamentous fungus *Podospora anserina*. Several mutants of this organism, all related to the cytochrome pathway, displayed a strikingly increased longevity and have been described thoroughly (Borghouts *et al.*, 2001, 2002a, b; Dufour *et al.*, 2000; Lorin *et al.*, 2006, 2001; Osiewicz, 2002; Osiewicz & Stumpferl, 2001; Sainsard-Chanet & Begel, 1990; Schulte *et al.*, 1989). The phenotypic effects were traced back to almost exclusive respiration via the alternative pathway, and elevated levels of the alternative oxidase (PaAox) protein were found. Upon disruption of the gene encoding subunit V of the cytochrome *c* oxidase (COX5) the fungal respiration was completely cyanide resistant (Dufour *et al.*, 2000). In the case of the *cox5::BLE* mutant, reintroduction of the COX5 gene as well as overexpression of the PaAox re-established wild-type level growth rates and longevity. Surprisingly, direct overexpression of the alternative oxidase in *P. anserina* or its disruption did not influence the growth rate, longevity, ROS production or mtDNA stability (Lorin *et al.*, 2001). There was no direct link between the level of alternative oxidase protein and senescence; thus PaAox seemingly is not directly involved in the control of the

life-span in this fungus. A direct correlation was found between energy production, which the authors estimated from the growth properties, and longevity.

Our results suggest an important role of the *P. pastoris* alternative oxidase for cell viability, since disruption of the gene resulted in increased occurrence of ROS and apoptotic cells under high glucose concentrations. The fact that the opposite genetic modification yielded similar results indicates the importance of precise regulation of this enzyme. The ROS concentrations from the last measurement point should be considered with care. Due to the high standard deviation of this measurement the high ROS concentration of the AOD-overexpressing strain can not necessarily be seen as a trend reversal (Fig. 7a). This assumption is backed by the corresponding results of the more sensitive TUNEL test. The negative effects of the alternative oxidase disruption imply a more complex mode of regulation than was observable by oxygen consumption or fluorescence measurements. To do its best for a high cell viability, the alternative oxidase from *P. pastoris* has to be expressed in the right amount and exactly when needed. No alternative oxidase activity was found in the late stationary growth phase by oxygen consumption measurements. The fluorescence measurements did not show clear signals in the late phase. Levels of alternative oxidase at this stage, if present at all, must be very low and the fluorescence might be lost in the background. In *Neurospora crassa*, the *aod-1* gene is transcribed at a low constitutive level but it can be significantly induced by addition of antimycin A (Tanton *et al.*, 2003). A marginal amount of enzyme, not measurable with the methods we used, might also be present in *P. pastoris* all the time, suggesting very low constitutive expression of the enzyme, similar to the situation in *N. crassa*. Such a combination of constitutive and inducible expression also appears in *C. albicans*, although there this task is solved by two independently regulated genes: *AOX1a* is constitutively expressed while *AOX1b* expression is growth-stage dependent and inducible (Huh & Kang, 2001). Thus, *AOX1b* expression follows a similar mode to the *P. pastoris* AOD gene. To our knowledge, the *PpAOD-GFP* fusion used represents one of the most accurate methods to study the expression pattern of alternative oxidases, although the protein has to pass through membranes during targeting to mitochondria. Luckily, the C-terminal fusion with GFP did not negatively influence the alternative oxidase activity. The observed time-course of alternative oxidase expression in *P. pastoris*, which indicates a tight regulation of expression at the level of transcription in combination with a low half life of the Aod protein, explains why no alternative oxidase activity was found in isolated mitochondria (Gonzalez-Barroso *et al.*, 2006) or why forced aeration of cells had to be performed to induce CRR (Veiga *et al.*, 2003c). Therefore enhancing protein stability by engineering of the alternative oxidase is one of the major challenges for the isolation and purification of this enzyme for more detailed biochemical characterization.

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REFERENCES

- Affourtit, C., Albury, M. S., Krab, K. & Moore, A. L. (1999). Functional expression of the plant alternative oxidase affects growth of the yeast *Schizosaccharomyces pombe*. *J Biol Chem* **274**, 6212–6218.
- Affourtit, C., Albury, M. S., Crichton, P. G. & Moore, A. L. (2002). Exploring the molecular nature of alternative oxidase regulation and catalysis. *FEBS Lett* **510**, 121–126.
- Andersson, M. E. & Nordlund, P. (1999). A revised model of the active site of alternative oxidase. *FEBS Lett* **449**, 17–22.
- Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidmann, J. G., Smith, J. A. & Struhl, K. (2003). *Current Protocols in Molecular Biology*. New York: Wiley.
- Berthold, D. A. & Siedow, J. N. (1993). Partial purification of the cyanide-resistant alternative oxidase of skunk cabbage (*Symplocarpus foetidus*) mitochondria. *Plant Physiol* **101**, 113–119.
- Berthold, D. A., Andersson, M. E. & Nordlund, P. (2000). New insight into the structure and function of the alternative oxidase. *Biochim Biophys Acta* **1460**, 241–254.
- Borghouts, C., Werner, A., Elthon, T. & Osiewacz, H. D. (2001). Copper-modulated gene expression and senescence in the filamentous fungus *Podospora anserina*. *Mol Cell Biol* **21**, 390–399.
- Borghouts, C., Scheckhuber, C. Q., Stephan, O. & Osiewacz, H. D. (2002a). Copper homeostasis and aging in the fungal model system *Podospora anserina*: differential expression of PaCtr3 encoding a copper transporter. *Int J Biochem Cell Biol* **34**, 1355–1371.
- Borghouts, C., Scheckhuber, C. Q., Werner, A. & Osiewacz, H. D. (2002b). Respiration, copper availability and SOD activity in *P. anserina* strains with different lifespan. *Biogerontology* **3**, 143–153.
- Chaudhuri, M., Ajayi, W., Temple, S. & Hill, G. C. (1995). Identification and partial purification of a stage-specific 33 kDa mitochondrial protein as the alternative oxidase of the *Trypanosoma brucei* bloodstream trypomastigotes. *J Eukaryot Microbiol* **42**, 467–472.
- Claros, M. G. & Vincens, P. (1996). Computational method to predict mitochondrially imported proteins and their targeting sequences. *Eur J Biochem* **241**, 779–786.
- Cramer, A., Whitehorn, E. A., Tate, E. & Stemmer, W. P. (1996). Improved green fluorescent protein by molecular evolution using DNA shuffling. *Nat Biotechnol* **14**, 315–319.
- Dufour, E., Boulay, J., Rincheval, V. & Sainsard-Chanet, A. (2000). A causal link between respiration and senescence in *Podospora anserina*. *Proc Natl Acad Sci U S A* **97**, 4138–4143.
- Fröhlich, K. U. & Madeo, F. (2003). *Genetics of Apoptosis: Regulators and Applications of Yeast Apoptosis*. Oxford: Oxford University Press.
- Gasteiger, E., Hoogland, C., Gattiker, A., Duvaud, S., Wilkins, M. R., Appel, R. D. & Bairoch, A. (2005). Protein identification and analysis tools on the ExPASy server. In *Proteomics Protocols Handbook*, pp. 571–607. Edited by J. M. Walker. Totowa, NJ: Humana Press.
- Gonzalez-Barroso, M. M., Ledesma, A., Lepper, S., Perez-Magan, E., Zaragoza, P. & Rial, E. (2006). Isolation and bioenergetic characterization of mitochondria from *Pichia pastoris*. *Yeast* **23**, 307–313.
- Gonzalez-Meler, M. A., Ribas-Carbo, M., Giles, L. & Siedow, J. N. (1999). The effect of growth and measurement temperature on the activity of the alternative respiratory pathway. *Plant Physiol* **120**, 765–772.
- Huang, X., von Rad, U. & Durner, J. (2002). Nitric oxide induces transcriptional activation of the nitric oxide-tolerant alternative oxidase in *Arabidopsis* suspension cells. *Planta* **215**, 914–923.
- Huh, W. K. & Kang, S. O. (2001). Characterization of the gene family encoding alternative oxidase from *Candida albicans*. *Biochem J* **356**, 595–604.
- Huq, S. & Palmer, J. M. (1978). Isolation of a cyanide-resistant duroquinol oxidase from *Arum maculatum* mitochondria. *FEBS Lett* **95**, 217–220.
- Johnson, C. H., Prigge, J. T., Warren, A. D. & McEwen, J. E. (2003). Characterization of an alternative oxidase activity of *Histoplasma capsulatum*. *Yeast* **20**, 381–388.
- Joseph-Horne, T., Babij, J., Wood, P. M., Hollomon, D. & Sessions, R. B. (2000). New sequence data enable modelling of the fungal alternative oxidase and explain an absence of regulation by pyruvate. *FEBS Lett* **481**, 141–146.
- Joseph-Horne, T., Hollomon, D. W. & Wood, P. M. (2001). Fungal respiration: a fusion of standard and alternative components. *Biochim Biophys Acta* **1504**, 179–195.
- Kirimura, K., Matsui, T., Sugano, S. & Usami, S. (1996). Enhancement and repression of cyanide-insensitive respiration in *Aspergillus niger*. *FEMS Microbiol Lett* **141**, 251–254.
- Kita, K., Nihei, C. & Tomitsuka, E. (2003). Parasite mitochondria as drug target: diversity and dynamic changes during the life cycle. *Curr Med Chem* **10**, 2535–2548.
- Lorin, S., Dufour, E., Boulay, J., Begel, O., Marsy, S. & Sainsard-Chanet, A. (2001). Overexpression of the alternative oxidase restores senescence and fertility in a long-lived respiration-deficient mutant of *Podospora anserina*. *Mol Microbiol* **42**, 1259–1267.
- Lorin, S., Dufour, E. & Sainsard-Chanet, A. (2006). Mitochondrial metabolism and aging in the filamentous fungus *Podospora anserina*. *Biochim Biophys Acta* **1757**, 604–610.
- Madeo, F., Frohlich, E. & Frohlich, K. U. (1997). A yeast mutant showing diagnostic markers of early and late apoptosis. *J Cell Biol* **139**, 729–734.
- Madeo, F., Frohlich, E., Ligr, M., Grey, M., Sigrist, S. J., Wolf, D. H. & Frohlich, K. U. (1999). Oxygen stress: a regulator of apoptosis in yeast. *J Cell Biol* **145**, 757–767.
- Maxwell, D. P., Wang, Y. & McIntosh, L. (1999). The alternative oxidase lowers mitochondrial reactive oxygen production in plant cells. *Proc Natl Acad Sci U S A* **96**, 8271–8276.
- Meeuse, B. J. D. (1975). Thermogenic respiration in aroids. *Annu Rev Plant Physiol* **26**, 117–126.
- Moore, A. L., Albury, M. S., Crichton, P. G. & Affourtit, C. (2002). Function of the alternative oxidase: is it still a scavenger? *Trends Plant Sci* **7**, 478–481.
- Nihei, C., Fukai, Y., Kawai, K., Osanai, A., Yabu, Y., Suzuki, T., Ohta, N., Minagawa, N., Nagai, K. & Kita, K. (2003). Purification of active recombinant trypanosome alternative oxidase. *FEBS Lett* **538**, 35–40.
- Ordog, S. H., Higgins, V. J. & Vanlerberghe, G. C. (2002). Mitochondrial alternative oxidase is not a critical component of plant viral resistance but may play a role in the hypersensitive response. *Plant Physiol* **129**, 1858–1865.
- Osiewacz, H. D. (2002). Mitochondrial functions and aging. *Gene* **286**, 65–71.
- Osiewacz, H. D. & Stumpf, S. W. (2001). Metabolism and aging in the filamentous fungus *Podospora anserina*. *Arch Gerontol Geriatr* **32**, 185–197.

- Robson, C. A. & Vanlerberghe, G. C. (2002).** Transgenic plant cells lacking mitochondrial alternative oxidase have increased susceptibility to mitochondria-dependent and -independent pathways of programmed cell death. *Plant Physiol* **129**, 1908–1920.
- Sainsard-Chanet, A. & Begel, O. (1990).** Insertion of an LrDNA gene fragment and of filler DNA at a mitochondrial exon-intron junction in *Podospora*. *Nucleic Acids Res* **18**, 779–783.
- Sakajo, S., Minagawa, N. & Yoshimoto, A. (1998).** Regulation of alternative oxidase gene expression by carbon source in the yeast *Hansenula anomala*. In *Plant Mitochondria: from Gene to Function, Proceedings of the International Congress, Aronsborg, Sweden, June 22–27, 1998*, pp. 515–518.
- Schulte, E., Kuck, U. & Esser, K. (1989).** Multipartite structure of mitochondrial DNA in a fungal longlife mutant. *Plasmid* **21**, 79–84.
- Shi, N. Q., Davis, B., Sherman, F., Cruz, J. & Jeffries, T. W. (1999).** Disruption of the cytochrome *c* gene in xylose-utilizing yeast *Pichia stipitis* leads to higher ethanol production. *Yeast* **15**, 1021–1030.
- Shi, N. Q., Cruz, J., Sherman, F. & Jeffries, T. W. (2002).** SHAM-sensitive alternative respiration in the xylose-metabolizing yeast *Pichia stipitis*. *Yeast* **19**, 1203–1220.
- Siedow, J. N. & Umbach, A. L. (2000).** The mitochondrial cyanide-resistant oxidase: structural conservation amid regulatory diversity. *Biochim Biophys Acta* **1459**, 432–439.
- Siedow, J. N., Umbach, A. L. & Moore, A. L. (1995).** The active site of the cyanide-resistant oxidase from plant mitochondria contains a binuclear iron center. *FEBS Lett* **362**, 10–14.
- Simons, B. H., Millenaar, F. F., Mulder, L., Van Loon, L. C. & Lambers, H. (1999).** Enhanced expression and activation of the alternative oxidase during infection of *Arabidopsis* with *Pseudomonas syringae* pv. tomato. *Plant Physiol* **120**, 529–538.
- Tanton, L. L., Nargang, C. E., Kessler, K. E., Li, Q. & Nargang, F. E. (2003).** Alternative oxidase expression in *Neurospora crassa*. *Fungal Genet Biol* **39**, 176–190.
- Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994).** CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.
- Tsuda, A., Witola, W. H., Ohashi, K. & Onuma, M. (2005).** Expression of alternative oxidase inhibits programmed cell death-like phenomenon in bloodstream form of *Trypanosoma brucei rhodesiense*. *Parasitol Int* **54**, 243–251.
- Tudella, V. G., Curti, C., Soriani, F. M., Santos, A. C. & Uyemura, S. A. (2004).** In situ evidence of an alternative oxidase and an uncoupling protein in the respiratory chain of *Aspergillus fumigatus*. *Int J Biochem Cell Biol* **36**, 162–172.
- Umbach, A. L. & Siedow, J. N. (2000).** The cyanide-resistant alternative oxidases from the fungi *Pichia stipitis* and *Neurospora crassa* are monomeric and lack regulatory features of the plant enzyme. *Arch Biochem Biophys* **378**, 234–245.
- Van Hellemond, J. J., Simons, B., Millenaar, F. F. & Tielens, A. G. (1998).** A gene encoding the plant-like alternative oxidase is present in *Phytomonas* but absent in *Leishmania* spp. *J Eukaryot Microbiol* **45**, 426–430.
- Vanlerberghe, G. C., Robson, C. A. & Yip, J. Y. (2002).** Induction of mitochondrial alternative oxidase in response to a cell signal pathway down-regulating the cytochrome pathway prevents programmed cell death. *Plant Physiol* **129**, 1829–1842.
- Veiga, A., Arrabaca, J. D. & Loureiro-Dias, M. C. (2000).** Cyanide-resistant respiration is frequent, but confined to yeasts incapable of aerobic fermentation. *FEMS Microbiol Lett* **190**, 93–97.
- Veiga, A., Arrabaca, J. D. & Loureiro-Dias, M. C. (2003a).** Cyanide-resistant respiration, a very frequent metabolic pathway in yeasts. *FEMS Yeast Res* **3**, 239–245.
- Veiga, A., Arrabaca, J. D. & Loureiro-Dias, M. C. (2003b).** Stress situations induce cyanide-resistant respiration in spoilage yeasts. *J Appl Microbiol* **95**, 364–371.
- Veiga, A., Arrabaca, J. D., Sansonetty, F., Ludovico, P., Corte-Real, M. & Loureiro-Dias, M. C. (2003c).** Energy conversion coupled to cyanide-resistant respiration in the yeasts *Pichia membranifaciens* and *Debaryomyces hansenii*. *FEMS Yeast Res* **3**, 141–148.
- Weis, R., Luiten, R., Skranc, W., Schwab, H., Wubbolts, M. & Glieder, A. (2004).** Reliable high-throughput screening with *Pichia pastoris* by limiting yeast cell death phenomena. *FEMS Yeast Res* **5**, 179–189.
- Yabu, Y., Yoshida, A., Suzuki, T., Nihei, C., Kawai, K., Minagawa, N., Hosokawa, T., Nagai, K., Kita, K. & Ohta, N. (2003).** The efficacy of ascofuranone in a consecutive treatment on *Trypanosoma brucei* in mice. *Parasitol Int* **52**, 155–164.
- Zaslaver, A., Mayo, A. E., Rosenberg, R., Bashkin, P., Sberro, H., Tsalyuk, M., Surette, M. G. & Alon, U. (2004).** Just-in-time transcription program in metabolic pathways. *Nat Genet* **36**, 486–491.
- Zottini, M., Formentin, E., Scattolin, M., Carimi, F., Lo Schiavo, F. & Terzi, M. (2002).** Nitric oxide affects plant mitochondrial functionality in vivo. *FEBS Lett* **515**, 75–78.

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