Mechanisms of copper loading on the *Schizosaccharomyces pombe* copper amine oxidase 1 expressed in *Saccharomyces cerevisiae*

Julie Laliberté and Simon Labbé

Correspondence

Simon Labbé

Simon.Labbe@USherbrooke.ca

Département de Biochimie, Université de Sherbrooke, 3001 12e Avenue Nord, Sherbrooke, Québec J1H 5N4, Canada

Copper amine oxidases (CAOs) are found in almost every living kingdom. Although *Saccharomyces cerevisiae* is one of the few yeast species that lacks an endogenous CAO, heterologous gene expression of CAOs from other organisms produces a functional enzyme. To begin to characterize their function and mechanisms of copper acquisition, two putative *cao*⁺ genes from *Schizosaccharomyces pombe* were expressed in *S. cerevisiae*. Expression of *spao1*⁺ resulted in the production of an active enzyme capable of catalysing the oxidative deamination of primary amines. On the other hand, expression of *spao2*⁺ failed to produce an active CAO. Using a functional *spao1*⁺–GFP fusion allele, the SPAO1 protein was localized in the cytosol. Under copper-limiting conditions, yeast cells harbouring deletions of the *MAC1*, *CTR1* and *CTR3* genes were defective in amine oxidase activity. Likewise, *atx1*Δ null cells exhibited no CAO activity, while *ccc2*Δ mutant cells exhibited decreased levels of amine oxidase activity, and mutations in *cox17*Δ and *ccs1*Δ did not cause any defects in this activity. Copper-deprived *S. cerevisiae* cells expressing *spao1*⁺ required a functional *atx1*⁺ gene for growth on minimal medium containing ethylamine as the sole nitrogen source. Under these conditions, the inability of the *atx1*Δ cells to utilize ethylamine correlated with the lack of SPAO1 activity, in spite of the efficient expression of the protein. Cells carrying a disrupted *ccc2*Δ allele exhibited only weak growth on ethylamine medium containing a copper chelator. The results of these studies reveal that expression of the heterologous *spao1*⁺ gene in *S. cerevisiae* is required for its growth in medium containing ethylamine as the sole nitrogen source, and that expression of an active *Schiz. pombe* SPAO1 protein in *S. cerevisiae* depends on the acquisition of copper through the high-affinity copper transporters Ctr1 and Ctr3, and the copper chaperone Atx1.

Received 15 March 2006

Revised 16 May 2006

Accepted 18 May 2006

INTRODUCTION

Copper is an essential transition metal required by most organisms (reviewed by Puig & Thiele, 2002a). This redox-active metal is a key cofactor for several enzymes, including cytochrome c oxidase, copper, zinc-superoxide dismutase, dopamine-β-hydroxylase and lysyl oxidase. These cupro-enzymes are involved in diverse biological processes such as respiration, free-radical defence, catecholamine formation and maturation of connective tissue (reviewed by Peña *et al.*, 1999). Another important copper-dependent enzyme, copper amine oxidase (CAO), catalyses the oxidation of various amine substrates to their corresponding aldehydes,

with the subsequent release of NH₃ and H₂O₂ (reviewed by Brazeau *et al.*, 2004). In prokaryotes and fungi, CAOs allow the organisms to utilize amine substrates as sources of carbon and nitrogen (Samuels & Klinman, 2005). In higher eukaryotes, their biological functions are less well defined, with suggested roles in detoxifying xenobiotic amines, wound healing, metabolism of glucose, cell–cell recognition and cell growth (reviewed by Yu *et al.*, 2003). CAOs are homodimers of ~70–95 kDa monomers. Each monomer contains a copper and an organic cofactor, 2,4,5-trihydroxyphenylalanine quinone (TPQ), which is post-translationally derived from a tyrosine residue that is highly conserved within the protein. The formation of TPQ has been shown to be a self-processing event requiring both copper and oxygen (reviewed by Dawkes & Phillips, 2001). Copper is coordinated by three His residues, conserved in all CAO primary sequences, from bacteria to eukaryotes (Kumar *et al.*, 1996; Li *et al.*, 1998; Matsunami *et al.*, 2004). Currently, the mechanism for copper acquisition by CAOs is unclear.

Abbreviations: BCS, bathocuproine disulfonic acid; CAO, copper amine oxidase; GFP, green fluorescent protein; HPAO, *Hansenula polymorpha* amine oxidase; PGK, phosphoglycerol kinase; PTS1, peroxisome targeting signal type 1; PTS2, peroxisome targeting signal type 2; TPQ, 2,4,5-trihydroxyphenylalanine quinone; TTM, ammonium tetrathiomolybdate.

Copper is also a potentially toxic metal due to its proclivity to engage in Fenton-like reactions that generate highly destructive hydroxyl radicals, thus, most organisms have developed mechanisms to assimilate copper in a highly controlled manner (reviewed by Rees & Thiele, 2004). In the yeast *Saccharomyces cerevisiae*, high-affinity copper uptake and distribution within cells have been characterized at the molecular level. Copper is first reduced from Cu^{2+} to Cu^+ by the cell-surface reductases Fre1 and Fre2 (Dancis *et al.*, 1990; Georgatsou & Alexandraki, 1994; Hassett & Kosman, 1995; Martins *et al.*, 1998). Following reduction, copper is transported across the plasma membrane by two distinct high-affinity copper transporters, Ctr1 and Ctr3 (Dancis *et al.*, 1994a; Knight *et al.*, 1996; Peña *et al.*, 2000; Puig *et al.*, 2002b). Both proteins function independently in high-affinity copper uptake (Peña *et al.*, 2000). Within the cell, copper is specifically delivered to the late secretory compartment, mitochondria and cytosolic copper, zinc-superoxide dismutase by the copper chaperones Atx1, Cox17, and Ccs1, respectively (reviewed by O'Halloran & Culotta, 2000). Within the secretory pathway, Ccc2 accepts copper from Atx1, and incorporates copper into cuproproteins, such as the multicopper ferrioxidase Fet3, as they mature in the pathway (Lin *et al.*, 1997; Pufahl *et al.*, 1997). In the mitochondria, copper delivered by Cox17 is incorporated into cytochrome c oxidase (possibly in cooperation with Sco1 and Cox11) (Abajian *et al.*, 2004; Carr & Winge, 2003; Glerum *et al.*, 1996; Horng *et al.*, 2004). Ccs1 delivers copper to copper, zinc-superoxide dismutase in the cytosol (Culotta *et al.*, 1997). Consistent with their function in discrete pathways in intracellular copper distribution, mutations in any one of the copper chaperone genes give rise only to specific defects in their respective pathways, while overproduction of one copper chaperone cannot complement the loss of function in another (Lin *et al.*, 1997). In contrast, mutations in Ctr1 and Ctr3, which act upstream of the copper chaperones, result in pleiotropic phenotypes due to copper deficiency in all compartments (Dancis *et al.*, 1994a, b; Knight *et al.*, 1996).

Curiously, *S. cerevisiae* is one of the few yeast species that does not have an endogenous CAO (Cai & Klinman, 1994b; Large, 1986). However, heterologous expression of a CAO from another organism in *S. cerevisiae* produces a functional enzyme (Cai & Klinman, 1994a). Thus, it can serve as an excellent host for the expression and characterization of genes encoding CAOs from other organisms. Furthermore, as elegantly shown by Klinman and colleagues when they studied heterologous expression of the CAO from *Hansenula polymorpha* (HPAO) in *S. cerevisiae*, this latter organism must have a 'molecular pathway' to activate HPAO, since these authors have been unable to perform *in vitro* reconstitution studies by addition of copper to the apo-form of HPAO (Cai *et al.*, 1997). In the fission yeast *Schizosaccharomyces pombe*, two candidate CAO molecules, SPAC2E1P3.04 and SPBC1289.16c, have been annotated from the *Schiz. pombe* Genome Project. We designated these proteins SPAO1 and SPAO2. Their function and

regulation in *Schiz. pombe* are currently unknown. To begin to characterize these proteins, we expressed the *Schiz. pombe* CAOs in *S. cerevisiae*, and determined the requirement of the known copper transporters and chaperones for their activity. Interestingly, SPAO1, but not SPAO2, is capable of catalysing ethylamine oxidation. SPAO1 is localized in the cytosol. Using mutant *S. cerevisiae* strains, we determined that a functionally active SPAO1 was dependent on the copper transporters Ctr1 and Ctr3 when cells were grown under conditions of copper starvation, Atx1 for delivery of copper within the cell, and Ccc2, whose deletion resulted in partial loss of SPAO1 activity. In contrast, deletion of *cox17Δ* and *ccs1Δ* had no effect on SPAO1 activity. Taken together, these results define components of the copper homeostatic pathway that are required for the physiological activation of CAOs when heterologously expressed in *S. cerevisiae*.

METHODS

Yeast strains and growth conditions. *S. cerevisiae* strains used in this study were the wild-type BY4741 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) (Brachmann *et al.*, 1998), and the *atx1Δ* (isogenic to BY4741 plus *atx1Δ::KAN^r*), *ccc2Δ* (isogenic to BY4741 plus *ccc2Δ::KAN^r*), *cox17Δ* (isogenic to BY4741 plus *cox17Δ::KAN^r*) and *ccs1Δ* (isogenic to BY4741 plus *ccs1Δ::KAN^r*) disruption strains. *S. cerevisiae* isogenic strains BR10 (*MATa gal1 trp1-1 his3 ade8 CUP^r*) (Rymond *et al.*, 1983), SLY12 (*MATa gal1 trp1-1 his3 ade8 CUP^r ura3::KAN^r leu2::HISG mac1::LEU2*) and MPY20 (*MATa gal1 trp1-1 his3 ade8 CUP^r ctr1::TRP1 ctr3::LEU2 leu2::KAN^r*) were also used in this work. All strains were cultured in yeast extract/bacto peptone/dextrose (YPD) medium or synthetic complete (SC) medium lacking the appropriate amino acid for plasmid selection (Sherman, 1991). For detection of peroxisomes, cells were grown in synthetic minimal (SD) medium containing 50 mg l⁻¹ methionine, histidine and leucine; 0.2% oleic acid; and 0.2% Tween 80 (adjusted to pH 7.0 with NaOH) (Gurvitz *et al.*, 1997; Veenhuis *et al.*, 1987). Copper starvation was carried out by adding the indicated concentration of ammonium tetrathiomolybdate (TTM) (323446; Aldrich) or bathocuproine disulfonic acid (BCS) (14662-5; Aldrich) to cells grown to mid-exponential phase ($\text{OD}_{600} \sim 1.0$). Similarly, copper repletion was performed by the addition of 10 or 100 μM CuSO_4 to cells grown at $\text{OD}_{600} \sim 1.0$. After treatments at 30 °C for 8 h, 20 ml samples were withdrawn from the cultures for subsequent detection of CAO activity, steady-state mRNA or protein analysis.

RNA analysis and plasmids. The *spao1⁺* gene was isolated by PCR using primers that corresponded to the initiator and stop codons of the ORF from *Schiz. pombe* strain FY254 (Forsburg *et al.*, 1997) genomic DNA. Because the primers contained *EcoRI* and *Sall* restriction sites, the purified DNA fragment was digested with these restriction enzymes and cloned into the corresponding sites of p416ADH or p416GPD (Mumberg *et al.*, 1995). Using an identical approach, we cloned *spao2⁺* in both p416ADH and p416GPD. The integrity of the DNA sequence of the *EcoRI*–*Sall* fragment from each respective PCR-amplified fragment was verified by dideoxy sequencing. For RNase protection assays, two plasmids were created to generate antisense RNA probes. pSK*spao1⁺* was constructed by inserting a 170 bp *NotI*–*EcoRI* fragment from the *spao1⁺* gene into the same sites of pBluescript SK (Stratagene). The antisense RNA hybridizes to the first 170 ribonucleotides of the *spao1⁺* transcript. To construct pSK*spao2⁺*, a 162 bp fragment of the *spao2⁺* gene was isolated by PCR and cloned into the *BamHI* and *EcoRI* sites of pBluescript SK. This fragment hybridizes to the region between

+2083 and +2245 downstream from the initiator codon of *spao2*⁺. The riboprobe derived from pKSACT1 (Labbé *et al.*, 1997) was used to probe *ACT1* mRNA as an internal control. Analysis of gene expression by the RNase protection protocol was carried out as described previously (Beaudoin & Labbé, 2001). The *GFP* coding sequence derived from pSP1*pccs*⁺*I-II-III-IV-GFP* (Laliberté *et al.*, 2004) was isolated by PCR using primers designed to generate *SpeI* and *Bam*HI sites at the 5' and 3' termini of the *GFP* gene, respectively. The resulting DNA fragment was used to clone the *GFP* gene into p416*ADH* or p416*GPD* vectors at compatible *SpeI* and *Bam*HI sites. To create green fluorescent protein (GFP) that harbours a C-terminal tripeptide SKL, the primers GFPKLEND (5'-AAGGAA-AAAAGCGGCCGCGGATCCTTATAATTTAGATAGTTCATCCATGCCATGTG-3') and GFPSTART (5'-GGACTAGTATGGGCCGCA-GTAAAGGAGAAGAAGACTTTTC-3') were made, corresponding to the beginning and the end of the *GFP* gene with three extra amino acid residues (underlined) after the leucine at position 238 (Leu²³⁸-Ser-Lys-Leu-Stop) of GFP. The PCR product obtained was digested with *SpeI* and *Bam*HI and cloned into the corresponding sites of p416*ADH* or p416*GPD*. To generate the *spao1*⁺-*StuI*-*Bsp*EI allele, a 12 bp *StuI*-*Bsp*EI linker was inserted in-frame and downstream of the last codon of the *spao1*⁺ gene by the overlap extension method (Ho *et al.*, 1989). This allele was found to be functional, based on its ability to encode a protein that catalysed the oxidative deamination of ethylamine. We used the restriction sites that *StuI* and *Bsp*EI created within *spao1*⁺ to insert a copy of the *GFP* gene. The plasmid, named p416*ADHspao1*⁺-*GFP*, was used to determine the localization of SPAO1-GFP fusion protein in *S. cerevisiae* by fluorescence microscopy. Fluorescence and differential interference contrast images of the cells were obtained on an Eclipse E800 epifluorescent microscope (Nikon) equipped with an ORCA ER digital cooled camera (Hamamatsu) as described previously by Beaudoin *et al.* (2006).

CAO assay. To determine the presence of CAO activity (Bruun & Houen, 1996), spheroplasts were obtained from transformed cells and lysed as described by Harding *et al.* (1995). Cell lysates were quantified using the Bradford assay, and equal amounts of cellular protein were subjected to electrophoresis on a 1% Tris/Tricine calcium lactate agarose gel. A Tris/Tricine calcium lactate stock solution [80 mM Tris base, 24 mM Tricine (T-7911; Sigma), 2 mM calcium lactate, pH 8.5] was utilized to make a 1% agarose solution. Gels were pre-equilibrated at 4 °C in cold Tris/Tricine (pH 8.5) calcium lactate buffer prior to electrophoresis, which was carried out at 75 V for 1 h. After electrophoresis, gels were blotted on to nitrocellulose membranes (Hybond-ECL; Amersham Biosciences). Transfer of proteins was performed by gravitational pressure for 90 min at 4 °C. The membrane was removed from the gel and placed in 5 mM phosphate buffer, pH 7.2, for 5 min at 4 °C, briefly pressed between two filter papers, and then layered on top of a filter paper that was prewetted with the chemiluminescence detection solution [10 mM ethylamine, 5 mM phosphate buffer, pH 7.2, 2 ml luminol reagent (ECL chemiluminescent detection reagent 2, Amersham Biosciences), and 10 µg ml⁻¹ horseradish peroxidase C]. The assembly was placed in a plastic sheet protector, and exposed to film (ECL hyperfilm, Amersham Biosciences) for 1 min to 1 h. Oxidation of the ethylamine to its corresponding aldehyde by an active CAO on the nitrocellulose membrane releases NH₃ and H₂O₂. The horseradish peroxidase and luminol present in the detection solution cause dismutation of H₂O₂ into water and oxygen, and subsequent light emission from the oxidation of luminol.

Immunoblotting. For Western blotting experiments, the protein extracts were resolved by SDS-PAGE, transferred to PVDF Hybond-P membranes (Amersham Biosciences), and the blots were analysed for steady-state levels of GFP and phosphoglycerol kinase (PGK) proteins using antisera B-2 (Santa Cruz Biotechnology) and 22C5-D8 (Molecular Probes), respectively. After a 2 h incubation, the

membranes were washed with TBS (10 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1% bovine serum albumin), incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences) and visualized by chemiluminescence.

RESULTS

SPAO1 is a *Schiz. pombe* CAO

Analysis of genomic DNA sequences from the *Schiz. pombe* Genome Project revealed two putative ORFs, *SPAC2E1P3.04* and *SPBC1289.16c*, encoding proteins related to the CAO family of enzymes (Jalkanen & Salmi, 2001). The *SPAC2E1P3.04*-encoded protein, denoted SPAO1, displays 48.6% amino acid sequence identity with an *SPBC1289.16c*-encoded protein, denoted SPAO2. Both SPAO1 and SPAO2 have predicted functional motifs that are highly similar to other microbial and metazoan CAOs. In SPAO1, a conserved Asn⁴⁰⁶-Tyr⁴⁰⁷-Glu⁴⁰⁸-Tyr⁴⁰⁹ sequence is present in which Tyr⁴⁰⁷ may serve as the precursor to TPQ (Fig. 1). The peptidyl Tyr³⁹⁴ residue of SPAO2 may play a similar role. Within the C-terminal halves of the two putative *Schiz. pombe* CAOs, three His residues (His⁴⁵⁸, His⁴⁶⁰ and His⁶²⁷ for SPAO1; His⁴⁴⁵, His⁴⁴⁷ and His⁶⁰⁴ for SPAO2) may act as a potential copper ligand. In addition to the above-mentioned residues, SPAO1 contains within its N-terminal region two amino acids (Tyr³⁰⁷ and Asp³²¹) that may promote the active conformation of TPQ during the enzymic reaction. To ascertain the potential role of *spao1*⁺ and *spao2*⁺ in nitrogen and carbon metabolism, we expressed these genes in *S. cerevisiae*, an organism that does not possess endogenous CAOs (Large, 1986), thereby providing an excellent background for their characterization. The *spao1*⁺ and *spao2*⁺ genes were placed under the control of the *ADH* or *GPD* gene promoter. We tested the ability of SPAO1 or SPAO2 expressed in *S. cerevisiae* cells to produce amine oxidase activity, using a peroxidase-catalysed chemiluminescent assay to detect the production of H₂O₂ (Bruun & Houen, 1996). Expression of SPAO1 under the control of the *GPD* promoter on a centromeric plasmid produced a stronger chemiluminescent signal than that observed in cells expressing *spao1*⁺ under the control of the *ADH* promoter (Fig. 2A). The CAO activity levels were consistent with the relative strength of the promoters, *ADH* being weaker than *GPD* (Mumberg *et al.*, 1995). Surprisingly, expression of *ADH-spao2*⁺ or *GPD-spao2*⁺ on a centromeric plasmid failed to produce detectable CAO activity. Expression of SPAO2 at higher levels using a multicopy plasmid still failed to generate CAO activity (J. Laliberté and S. Labbé, unpublished data). We verified the expression of *spao1*⁺ and *spao2*⁺ genes in the host cells by the RNase protection assay (Fig. 2B). These analyses clearly showed that readily detectable levels of both *spao1*⁺ and *spao2*⁺ mRNA were present in the host cells, indicating that the lack of CAO activity by SPAO2 was not due to lack of expression. Taken together, these data reveal that the orthologous *Schiz. pombe* SPAO1 protein, but not SPAO2, can produce an active enzyme when ectopically expressed in *S. cerevisiae*.

Fig. 1. Primary structural features of the SPAO1 and SPAO2 proteins. The putative precursor for TPQ is a peptidyl tyrosine (Y) residue (Y⁴⁰⁷ for SPAO1 and Y³⁹⁴ for SPAO2), which is part of a highly conserved sequence, N-Y-E-Y. The Y residue is converted to TPQ by a post-translational modification process that is dependent on both copper and oxygen. Three His residues located in the C-terminal halves of the *Schiz. pombe* CAOs (H⁴⁵⁸, H⁴⁶⁰ and H⁶²⁷ for SPAO1; H⁴⁴⁵, H⁴⁴⁷ and H⁶⁰⁴ for SPAO2) are potentially involved in the coordination of a single copper atom. The active conformation of TPQ is presumably modulated through interactions with Y³⁰⁷ and D³²¹ in SPAO1. In SPAO2, an F residue was found instead of Y at position 295. The amino acid sequence numbers refer to the position relative to the first amino acid of each protein.

Cellular localization of a functional SPAO1–green fluorescent protein (GFP) fusion protein in *S. cerevisiae*

To further characterize the physiological function of SPAO1 in *S. cerevisiae*, we determined its intracellular localization in living cells by fusing GFP to the C terminus of SPAO1. The amine oxidase activity of the SPAO1–GFP fusion protein, expressed from the *ADH* and *GPD* promoters, was ascertained using the peroxidase-catalysed chemiluminescent assay. The results in Fig. 3 show that the SPAO1–GFP

fusion protein was fully functional, demonstrating a level of CAO activity consistent with the strength of the promoter, and comparable to the untagged SPAO1.

Previous studies have indicated that the yeast HPAO protein is imported into the peroxisomal matrix when *H. polymorpha* cells are grown under nitrogen-limited conditions (Faber *et al.*, 1994). These were created by decreasing the concentration of (NH₄)₂SO₄ (0.5 %, w/v) in SD medium by two orders of magnitude (0.005 %, w/v). Furthermore, when cells are grown in the presence of oleic acid, the size

Fig. 2. Heterologous expression of SPAO1 and SPAO2 in *S. cerevisiae*. (A) *S. cerevisiae* BY4741 strain was transformed with an empty expression plasmid alone (p416*ADH*), p416*ADHspao1*⁺, p416*GPDspao1*⁺, p416*ADHspao2*⁺ or p416*GPDspao2*⁺. Extracts from cells transformed with plasmids expressing the indicated SPAO molecules were analysed for the presence of CAO activity using a chemiluminescent activity assay with ethylamine (10 mM) as a substrate (top panel, CAO activity). As an internal control, aliquots of total protein extracts were analysed by immunoblotting using anti-PGK antibody (bottom panel, PGK). (B) Total RNA was prepared from aliquots of *S. cerevisiae* cultures used in (A). Representative RNase protection assays of *spao1*⁺ (left panel) and *spao2*⁺ (right panel) are shown, indicating steady-state mRNA levels. Actin (*ACT1*) mRNA levels were probed as an internal control.

Fig. 3. SPAO1–GFP fusion protein is functional when heterologously expressed in *S. cerevisiae*. *S. cerevisiae* BY4741 cells were transformed with p416ADHspao1⁺, p416ADHspao1⁺–GFP, p416GPDspao1⁺ or p416GPDspao1⁺–GFP. As a control, these cells were also transformed with p416ADH vector alone (–). Extracts from lysed spheroplasts, prepared from BY4741 cells expressing the indicated plasmids, were tested for their ability to mediate the oxidative deamination of ethylamine using a chemiluminescent activity assay (top panel, CAO activity). Extracts used to detect CAO activity were also probed with anti-GFP (middle panel, SPAO1–GFP) or anti-PGK (bottom panel, PGK) antibody.

and abundance of peroxisomes increase, allowing visualization of peroxisomal proteins by microscopy (Veenhuis *et al.*, 1987). Under these conditions, a GFP harbouring the peroxisome targeting signal, the tripeptide SKL at its C terminus, is efficiently targeted into the peroxisomal lumen and exhibits the characteristic punctate peroxisomal staining pattern (Fig. 4A, lower third panel). Similarly, fusion of peroxisome targeting signal type 2 (PTS2) (Petriv *et al.*, 2004) to the N terminus of GFP specifically directed the protein to the peroxisomes (data not shown). Using these same conditions, we determined the localization of SPAO1–GFP, expressed under the control of the *ADH* promoter, by fluorescence microscopy. As shown in Fig. 4(A), the fusion protein accumulated in the cytosol of *S. cerevisiae* cells. Like GFP alone, SPAO1–GFP exhibited fluorescence throughout the cytoplasm and was excluded from the vacuole, which was detected as indentations by Nomarski optics (Fig. 4A). Importantly, under these conditions of growth, the vacuoles were morphologically predominant within the cells, most likely as a consequence of nutrient limitation. To ensure that the fluorescence observed was due to the SPAO1–GFP fusion protein rather than products from proteolytic cleavage of the linker between SPAO1 and GFP, total protein extracts from cells transformed with plasmids expressing the indicated GFP and SPAO1–GFP molecules were analysed by immunoblotting (Fig. 4B). These results showed that the SPAO1–GFP protein was present exclusively as a chimeric fusion (Fig. 4B). When the expression of SPAO1–GFP was driven by the *GPD* promoter, SPAO1–GFP was also found in the cytoplasm (J. Laliberté and S. Labbé, unpublished data). Under the conditions of nitrogen limitation used in our studies, SPAO1–GFP did not localize to the peroxisomes. Thus, SPAO1 is primarily a cytosolic protein when expressed in *S. cerevisiae*. Moreover, *S. cerevisiae* cells expressing SPAO1–GFP, GFP alone or GFP–SKL were also examined during growth under nitrogen-replete conditions in oleate-containing medium. Although the vacuoles were not detected under these conditions, like GFP alone, the SPAO1–GFP protein was mainly seen throughout the cells,

suggesting that it was localized to the cytosol. On the other hand, cells harbouring GFP–SKL exhibited the characteristic punctate peroxisomal pattern of small spots (see Supplementary Fig. S1).

***S. cerevisiae* cells harbouring a deletion of the *MAC1* gene or an inactivation of both *CTR1* and *CTR3* genes are unable to activate SPAO1**

In *S. cerevisiae*, *CTR1* and *CTR3* genes are known to encode two high-affinity copper transporters (Dancis *et al.*, 1994a; Knight *et al.*, 1996; Peña *et al.*, 2000; Puig *et al.*, 2002b). Although Ctr1 and Ctr3 are functionally redundant, these two proteins mediate copper uptake independently of each other. *CTR1* and *CTR3* genes are regulated at the level of gene transcription (Labbé *et al.*, 1997). They are induced under conditions of copper starvation and are repressed under conditions of copper repletion. Furthermore, it has been demonstrated that expression and copper-dependent regulation of *CTR1* and *CTR3* genes require the presence of a functional *MAC1* gene (Labbé *et al.*, 1997). To determine if Mac1, Ctr1 or Ctr3 are required for providing copper to SPAO1, CAO activity was assayed in whole-cell extracts from wild-type, *mac1Δ* and *ctr1Δ ctr3Δ* cells transformed with a wild-type copy of the *spao1⁺* gene expressed from a centromeric plasmid (Fig. 5). Under copper-limiting conditions, in the presence of 300 μM TTM in the growth medium, strains bearing the disrupted *mac1Δ* or *ctr1Δ ctr3Δ* alleles were devoid of detectable CAO activity (Fig. 5A, left panel). As expected, CAO activity could be restored by the addition of elevated copper concentrations (10 μM) to the growth medium (Fig. 5A, right panel). This is likely due to the fact that copper ions are taken up via the low-affinity copper-transport system, thereby bypassing any requirement for the high-affinity copper-transport pathway. To further investigate if the absence of CAO activity in the *mac1Δ* and *ctr1Δ ctr3Δ* mutant strains grown under conditions of copper deprivation was not due to a defect in SPAO1 expression, we transformed the *spao1⁺*–GFP

Fig. 4. Localization of SPAO1-GFP in *S. cerevisiae*. (A) Representative BY4741 cells expressing SPAO1-GFP, GFP alone and GFP-SKL proteins are shown. Cells were grown to mid-exponential phase in SC medium lacking uracil, washed twice, and then incubated in SD medium containing 0.2% oleic acid and 0.2% Tween 80 for 12 h. Cells expressing GFPs were viewed by direct fluorescence microscopy (left panels). Corresponding Nomarski images are shown on the right side of each panel. (B) Protein extracts prepared from cells transformed with *spa01*⁺-GFP (1), GFP alone (2) and GFP-SKL (3) were analysed by immunoblotting using either anti-GFP or anti-PGK (as an internal control) antibody. MW, molecular mass marker.

fusion allele into *S. cerevisiae* wild-type, *mac1*Δ and *ctr1*Δ *ctr3*Δ cells. As shown in Fig. 5(B) (left panel), in the presence of 300 μM TTM, the *mac1*Δ and *ctr1*Δ *ctr3*Δ mutant strains failed to exhibit measurable CAO activity, whereas the wild-type strain bearing functional *MAC1*, *CTR1* and *CTR3* genes exhibited high levels of CAO activity. CAO activity was restored by the addition of 10 μM CuSO₄ to the growth medium (Fig. 5B, right panel). To verify that the SPAO1-GFP fusion protein was expressed in the wild-type, *mac1*Δ and *ctr1*Δ *ctr3*Δ cells, total protein extracts from transformed cells were analysed by immunoblotting (Fig. 5B). These results showed that the SPAO1 protein was clearly produced in the *mac1*Δ and *ctr1*Δ *ctr3*Δ strains, indicating that the absence of activity in the mutant strains was not due to the lack of SPAO1 expression. Taken together, these results show that under conditions of copper starvation, the production of an active CAO in *S. cerevisiae* requires CTR-mediated copper transport, as well as the transcription factor Mac1, which is essential for the expression of the high-affinity copper uptake genes.

ATX1 is required for the synthesis of an active CAO

In *S. cerevisiae*, the intracellular distribution of copper after its uptake into the cells is carried out in a highly controlled manner. This is mediated, in part, by metalloproteins called copper chaperones that deliver copper to appropriate proteins and intracellular compartments. At low copper concentrations, we found that the high-affinity copper transporters were required for the production of an active *Schiz. pombe* SPAO1 enzyme in *S. cerevisiae* cells. We next determined which intracellular copper distribution pathway was responsible for the insertion of copper into the active CAO enzyme. We expressed the *spa01*⁺ gene in *S. cerevisiae* cells that harboured an *atx1*Δ, *ccc2*Δ, *cox17*Δ or *ccs1*Δ deletion and performed peroxidase-catalysed chemiluminescent assays on lysates from the transformed cells. As shown in Fig. 6(A) (left panel), the *atx1*Δ strain transformed with *spa01*⁺ exhibited no CAO activity; however, CAO activity was restored by the addition of 10 μM CuSO₄ to the growth medium (Fig. 6A, right panel). Furthermore, transformation of a wild-type *ATX1* on a centromeric vector restored CAO activity in the *atx1*Δ mutant cells expressing SPAO1 (data not shown). Deletion of the copper chaperones Cox17 and CCS1 did not affect the CAO activity in the transformed cells (Fig. 6A). In contrast, deletion of *CCC2* resulted in a partial loss of CAO activity that was corrected by the addition of copper to the growth medium (Fig. 6A). To ensure that the absence of CAO activity was not due to the lack of expression of the SPAO1 protein, we also transformed the mutant isogenic strains with a centromeric plasmid expressing the *spa01*⁺-GFP fusion gene. Under conditions of copper starvation, cells bearing a *cox17*Δ or *ccs1*Δ deletion displayed elevated levels of CAO activity (Fig. 6B, left panel) that were comparable to those found in the same mutant strains expressing the untagged SPAO1. In contrast, an *atx1*Δ mutant strain expressing the

Fig. 5. SPAO1 activity requires expression of genes involved in high-affinity copper transport when heterologously expressed in *S. cerevisiae*. (A) Wild-type (WT) strain was transformed with an empty vector (—) or a plasmid expressing the wild-type *spao1*⁺ allele. Similarly, the isogenic *mac1*Δ and *ctr1*Δ *ctr3*Δ disruption strains were transformed with the plasmid expressing *spao1*⁺. These strains were incubated in the presence of TTM (300 μM) or CuSO₄ (10 μM). Protein extracts from each culture were analysed for CAO activity (top panel). As a control, total extract preparations were also probed with anti-PGK antibody (bottom panel). (B) Strains described above in (A) were transformed with an empty vector (—) or the GFP epitope-tagged *spao1*⁺ allele. Whole-cell extracts were prepared from the indicated strains and analysed using an in-gel assay for CAO activity (top panel). Aliquots of total extract preparations were analysed by immunoblotting using either anti-GFP (SPAO1-GFP) or anti-PGK antibody (PGK) as an internal control.

spao1⁺-GFP allele failed to activate SPAO1 under copper-limiting conditions (Fig. 6B, left panel). The lack of CAO activity did not reflect the absence of protein since SPAO1-GFP fusion protein was stably expressed and readily detectable by immunoblotting in these cells (Fig. 6B). Analysis by peroxidase-catalysed chemiluminescent assay showed that a *ccc2*Δ mutant strain harbouring the *spao1*⁺-GFP allele appeared to have less CAO activity than the wild-type strain, even though the protein levels were comparable (Fig. 6B). This indicates that the Ccc2 protein may also participate in conferring CAO activity on SPAO1. Taken together, these results reveal that optimal activity of the *Schiz. pombe* SPAO1 protein expressed in *S. cerevisiae* requires functional *ATX1* and *CCC2* genes, with *ATX1* making a greater contribution than *CCC2*.

Expression of SPAO1 allows *S. cerevisiae* cells to utilize ethylamine as a nitrogen source, except in copper-starved *atx1*Δ and *ccc2*Δ null mutants

S. cerevisiae cells lack the ability to utilize primary amines such as ethylamine as a nitrogen source to support their growth (Large, 1986), an observation that was recapitulated

in the present study. As shown in Fig. 7, the wild-type strain expressing an empty vector failed to grow on minimal medium containing ethylamine as the sole nitrogen source. However, expression of the SPAO1 protein in the wild-type strain allowed the cells to grow on minimal medium prepared with ethylamine. SPAO1 catalysed the oxidative deamination of ethylamine to the corresponding aldehyde, with concomitant release of H₂O₂ and NH₃ that was sufficient to provide the cells with a nitrogen source. We examined the requirement of Atx1, Ccc2, Cox17 and Ccs1 for SPAO1 activity by testing the ability of mutant cells transformed with *spao1*⁺ to grow in medium containing ethylamine as the sole source of nitrogen. *atx1*Δ cells were unable to grow on ethylamine medium containing 100 μM BCS, a specific copper chelator, while cells harbouring a *ccc2*Δ deletion showed very limited growth (Fig. 7, lane 4). The growth deficiency of both mutants was efficiently reversed by the addition of exogenous copper (100 μM) to the ethylamine medium (Fig. 7). In contrast, insertional inactivation of *cox17*Δ or *ccs1*Δ had no effect on the growth of these mutant strains expressing *spao1*⁺ on ethylamine medium in the presence of 100 μM CuSO₄ or 100 μM BCS. Thus, the Cox17 and Ccs1 metallochaperones are not required for delivering copper to SPAO1. The results in this

Fig. 6. An *S. cerevisiae atx1Δ* mutant strain is defective in production of a functional SPAO1. (A) Wild-type (WT) strain was transformed with *p416ADH* (–, vector alone) or *p416ADHspao1⁺*. Isogenic mutant strains harbouring disrupted *atx1Δ*, *ccc2Δ*, *cox17Δ* and *ccs1Δ* were transformed with *p416ADHspao1⁺*. Whole-cell extracts from cells grown in the presence of TTM (300 μM) or CuSO₄ (10 μM) were prepared. The results of a representative in-gel assay for CAO activity (top panel) are shown. As an internal control, aliquots of cell lysates were probed by immunoblotting with anti-PGK antibody (bottom panel). (B) WT, *atx1Δ*, *ccc2Δ*, *cox17Δ* and *ccs1Δ* cells were transformed with an empty control plasmid (–) or a plasmid expressing the GFP epitope-tagged *spao1⁺* allele. Cultures were treated with TTM (300 μM) or CuSO₄ (10 μM) for 8 h. CAO activity was determined from the cell lysates (top panel). Aliquots of lysates were also analysed by immunoblotting to verify the presence of SPAO1–GFP (middle panel). As a control, all samples were subjected to immunoblotting with anti-PGK antibody (bottom panel).

section clearly establish that Atx1, and to a lesser extent Ccc2, participate in the production of an active recombinant CAO in *S. cerevisiae* cells.

DISCUSSION

In this study, we have identified two fission yeast proteins that are related to the CAO family of amine oxidases (Jalkanen & Salmi, 2001). To gain insight into their biological functions, we took advantage of the fact that the *S. cerevisiae* genome does not encode a CAO homologue. It has previously been demonstrated that an active enzyme

can be produced by the heterologous gene expression of HPAO in *S. cerevisiae* (Cai & Klinman, 1994a), thus, we utilized this host to ascertain if primary amines can be oxidized through the expression of either SPAO1 or SPAO2. Although wild-type *S. cerevisiae* strains expressing *spao1⁺* were competent in catalysing the oxidation of ethylamine to its corresponding aldehyde with the subsequent release of NH₃ and H₂O₂, we were unable to demonstrate CAO activity for SPAO2. This lack of activity may be due to the fact that the SPAO2 protein harbours a Tyr²⁹⁵→Phe amino acid substitution. Based on the predicted 3D model of active CAO, a conserved peptidyl Tyr residue is required for

Fig. 7. The effects of *atx1* and *ccc2* mutations on ethylamine-dependent growth of *S. cerevisiae* cells expressing *spao1*⁺. The indicated strains were spotted at a density of 3000 cells per 5 µl onto SD medium containing 38 mM (NH₄)₂SO₄ (left panel, lane 1), no nitrogen source (middle-left panel, lane 2), and 10 mM ethylamine supplemented with either 100 µM CuSO₄ (middle-right panel, lane 3) or with 100 µM BCS (right panel, lane 4). Cells were photographed after 7 days incubation at 30 °C. The isogenic parent strain (WT) was transformed with p416ADH*spao1*⁺ or the empty vector p416ADH (vector alone).

proper orientation of its redox cofactor TPQ (Mure *et al.*, 2005), thus, its substitution to Phe in SPAO2 might render the enzyme incapable of catalysing ethylamine oxidation. Another reason may be the nature of the substrates that we used. The primary amine substrates that we tested included monoamines (e.g. ethylamine), aromatic monoamines (e.g. benzylamine) and diamines (e.g. putrescine and 1,8-diaminooctane). None of these substrates was oxidized by SPAO2 (J. Laliberté and S. Labbé, unpublished data). In contrast, SPAO1 exhibited a broad range of substrate specificity. The best substrates for SPAO1 were ethylamine, putrescine and 1,8-diaminooctane, while the aromatic monoamine benzylamine was less efficiently oxidized (J. Laliberté and S. Labbé, unpublished data). Collectively, these data demonstrate that CAO activity was present in *S. cerevisiae* cells expressing *spao1*⁺, but not in cells expressing *spao2*⁺.

Fluorescence microscopy experiments revealed that a functional SPAO1–GFP fusion protein expressed in *S. cerevisiae* resides predominantly in the cytosol. Based on

previous data showing that HPAO is imported into the peroxisomes (Faber *et al.*, 1994), we examined the sequence of the SPAO1 protein for the presence of putative peroxisome targeting signals. The majority of peroxisomal matrix proteins are targeted to the peroxisome by a peroxisome targeting signal type 1 (PTS1) found at the extreme C terminus of the proteins (Neuberger *et al.*, 2003a; Petriv *et al.*, 2004). PTS1 is a tripeptide with the consensus sequence (S/C/A)(K/R/H)(L/M) (Petriv *et al.*, 2004). Using a PTS1 predictor program (Neuberger *et al.*, 2003b), we found no peroxisomal import motif within the C-terminal region of SPAO1, making it improbable that the C terminus is recognized by the soluble receptor molecule Pex5 that targets proteins to the peroxisome (Brocard *et al.*, 1994). A small subset of peroxisomal matrix proteins (including HPAO) is targeted by PTS2, which is composed of a nanopeptide located at the N termini of the target proteins (Faber *et al.*, 1994; Petriv *et al.*, 2004). The accepted consensus sequence for PTS2 is (R/K)(L/V/I/Q)XX(L/V/I/H/Q)(L/S/G/A/K)X(H/Q)(L/A/F) (Petriv *et al.*, 2004). Examination of the SPAO1 amino acid sequence revealed two potential N-terminal peroxisomal targeting signals, R²¹-L-S-D-P-L-D-P-L²⁹ and R⁴²-H-E-Y-P-S-K-H-F⁵⁰. However, both candidate signals harbour two important mismatches (underlined residues) at highly conserved positions, making it highly improbable that they will interact with Pex7, the receptor for PTS2-containing proteins (Rehling *et al.*, 1996). Consistent with these observations, analysis using the PSORT II program classified SPAO1 as a non-peroxisomal protein. Although we cannot exclude the possibility that signal peptides in *Schiz. pombe* proteins destined for the peroxisomal matrix differ from the currently accepted PTS1 and PTS2 consensus sequences, computational analyses have shown that these sequences are highly conserved from fungi to plants and mammals (Neuberger *et al.*, 2003b; Reumann, 2004). Consistent with the absence of a canonical PTS1 or PTS2 targeting sequence, our experiments failed to localize the SPAO1 protein into *S. cerevisiae* peroxisomes, even upon induction of the peroxisome proliferation response under conditions of nitrogen starvation, as determined for HPAO in the methylotrophic yeast *H. polymorpha* (Faber *et al.*, 1994). Instead, our data reveal that an active SPAO1 is primarily a cytosolic protein when expressed in *S. cerevisiae*.

Despite the fact that *S. cerevisiae* does not express a protein homologous to SPAO1 or to any of the members of the CAO family, copper is made available to the newly synthesized cytosolic SPAO1 protein when expressed in budding yeast. How does SPAO1 obtain copper? Under conditions of copper depletion and in the absence of the plasma-membrane-associated high-affinity copper transporters Ctr1 and Ctr3, mutant cells expressing SPAO1 did not exhibit CAO activity, even though the protein was efficiently expressed. Likewise, in the absence of Mac1, the transcription factor that activates high-affinity copper-transport genes under copper-limiting conditions, no CAO activity was observed. As expected, the requirement for Ctr1/3 or

Mac1 for CAO activity was bypassed by addition of copper to the growth medium. Therefore, lack of CAO activity observed in these mutant strains is consistent with a failure of the high-affinity copper-transport machinery to assimilate sufficient copper to provide the SPAO1 protein with the metal cofactor.

To further investigate the process by which copper is supplied to SPAO1, we tested the effects of deletions of the *ATX1*, *COX17*, *CCS1* and *CCC2* genes, which are involved in intracellular copper delivery, on SPAO1 activity. We found that production of active SPAO1 was dependent on Atx1 and to a lesser extent, Ccc2. However, the precise mechanism by which this process occurs is unknown. Previous studies have shown that the *S. cerevisiae* Atx1 resides in the cytosol (Lin *et al.*, 1997). Both SPAO1 and Atx1 co-localize in the cytosol, therefore, the possibility exists that SPAO1 and Atx1 physically interact with each other. Interestingly, X-ray crystal structures of Atx1 have revealed a region harbouring multiple Lys residues that may generate a positively charged patch on the protein surface (Banci *et al.*, 2001; Rosenzweig *et al.*, 1999). Furthermore, the Lys-rich face of Atx1 has been shown to be necessary for physical interaction with Ccc2 and subsequent delivery of copper to its target protein (Portnoy *et al.*, 1999). Analysis of X-ray crystal structures of CAOs indicates that the CAO homodimer has two identical active sites arranged along a molecular twofold symmetrical axis (Duff *et al.*, 2003; Li *et al.*, 1998; Lunelli *et al.*, 2005; Parsons *et al.*, 1995). Each active site contains one copper and one TPQ, both at a very close distance. Interestingly, the cavity leading to the active site is characterized by the presence of an area with a negative electrostatic potential, making it a potential zone for electrostatic interactions. Atx1 encodes a very small polypeptide of only 8.2 kDa, and exhibits multiple Lys residues that form a positively charged surface on the protein; therefore, it is possible that Atx1 docks on the active site of SPAO1, which is predicted to be negatively charged to allow the direct transfer of copper ions from one protein to the other. It is also possible that Atx1 delivers copper to an intermediate soluble factor which is then responsible for inserting copper into SPAO1; however, no such factor has yet been identified. It should be noted that no obvious Atx1-like domain was found in SPAO1 (J. Laliberté and S. Labbé, unpublished data). It would be interesting to assess the importance of the basic residues in Atx1 for the activation of SPAO1. The results from this study also show that, even though equal amounts of SPAO1 protein are synthesized, a *ccc2Δ* mutation results in reduced CAO activity compared to the total activity observed in a wild-type strain. The copper-transporting ATPase Ccc2 is required for delivery of copper to the late secretory pathway, therefore, the significance of this result is difficult to reconcile, unless the cytoplasmic N terminus of Ccc2 can serve as a source of copper for Atx1, in order to provide copper to the cytosolic SPAO1. Previous studies using two-hybrid analysis have shown that Atx1 can interact with the N-terminal metal-binding domains of Ccc2 (Portnoy *et al.*, 1999; Pufahl *et al.*, 1997). Perhaps,

under certain circumstances, Atx1 may acquire copper from Ccc2 for insertion into cytosolic proteins. It is noteworthy that previous data have suggested that Ccc2 can obtain copper in an Atx1-independent manner, possibly via copper-induced endocytosis of Ctr1 from the plasma membrane (Lin *et al.*, 1997). Thus, Ccc2 may have an effect on intracellular copper distribution and utilization. Alternatively, Atx1 and Ccc2 could act in parallel, as redundant mechanisms to independently supply copper to SPAO1. However, our unpublished data do not support this mechanism because overexpression of *CCC2* was incapable of suppressing the lack of SPAO1 activity in *atx1Δ* mutant cells.

The inability of *S. cerevisiae* to utilize amines is most likely correlated with the lack of endogenous CAOs in this organism. Thus, the wild-type *S. cerevisiae* strains used in this study were incapable of growing on minimal medium containing ethylamine as the sole nitrogen source, unless the SPAO1 protein from *Schiz. pombe* was ectopically expressed. Using this as a functional assay, we tested the ability of SPAO1 to allow yeast strains harbouring deletions in the *ATX1*, *CCC2*, *COX17* and *CCS1* genes to utilize ethylamine as a nitrogen source. Under conditions of copper deprivation, cells lacking *ATX1* cannot grow on medium with ethylamine. This is due to the lack of CAO activity in SPAO1 protein expressed in these cells. Deletion of the *CCC2* allele (*ccc2Δ*) resulted in weak growth of cells on medium containing ethylamine and the copper chelator BCS. As expected, both mutant phenotypes were corrected by the addition of copper to the growth medium. In contrast to the *atx1Δ* and *ccc2Δ* mutants, strains lacking Cox17 and Ccs1 grew robustly in the presence of ethylamine and BCS, suggesting that these copper chaperones are not involved in supplying copper to SPAO1. Collectively, these observations support a role for Atx1 and, to a lesser extent, Ccc2, in the shuttling of copper to SPAO1. It remains to be established whether the *Schiz. pombe* *SPBC1709.10c* gene that encodes a putative chaperone orthologous to *S. cerevisiae* Atx1 is in fact required for delivering copper to SPAO1 in fission yeast. Further studies on SPAO1 and SPAO2 in fission yeast cells will elucidate the function of these proteins and the mechanisms by which copper is loaded into CAOs in this organism.

ACKNOWLEDGEMENTS

We are grateful to Maria M. O. Peña for critically reading the manuscript and valuable suggestions. We thank Kevin A. Morano for constructive comments. We are indebted to Maria M. O. Peña and Dennis J. Thiele for the *S. cerevisiae* *ctr1Δ ctr3Δ* double mutant disruption strain, and to Raymund J. Wellinger and Sherif Abou Elela for the *S. cerevisiae* *atx1Δ*, *ccc2Δ*, *ccs1Δ* and *cox17Δ* mutant strains. We would like to thank Richard Rachubinski and Rick Poirier for plasmids used in this study. J.L. is supported by the Natural Sciences and Engineering Research Council of Canada (NSERC). This work was supported by the Canadian Institutes of Health Research (CIHR) Grant MOP-36450 to S.L. and by the Fondation de la Recherche sur les Maladies Infantiles du Québec. S.L. is a New Investigator Scholar from the CIHR.

REFERENCES

- Abajian, C., Yatsunyk, L. A., Ramirez, B. E. & Rosenzweig, A. C. (2004). Yeast Cox17 solution structure and copper(I) binding. *J Biol Chem* **279**, 53584–53592.
- Banci, L., Bertini, I., Ciofi-Baffoni, S., Huffman, D. L. & O'Halloran, T. V. (2001). Solution structure of the yeast copper transporter domain Ccc2a in the apo and Cu(I)-loaded states. *J Biol Chem* **276**, 8415–8426.
- Beaudoin, J. & Labbé, S. (2001). The fission yeast copper-sensing transcription factor Cuf1 regulates the copper transporter gene expression through an Ace1/Amt1-like recognition sequence. *J Biol Chem* **276**, 15472–15480.
- Beaudoin, J., Laliberté, J. & Labbé, S. (2006). Functional dissection of Ctr4 and Ctr5 amino-terminal regions reveals motifs with redundant roles in copper transport. *Microbiology* **152**, 209–222.
- Brachmann, C. B., Davies, A., Cost, G. J., Caputo, E., Li, J., Hieter, P. & Boeke, J. D. (1998). Designer deletion strains derived from *Saccharomyces cerevisiae* S288C: a useful set of strains and plasmids for PCR-mediated gene disruption and other applications. *Yeast* **14**, 115–132.
- Brazeau, B. J., Johnson, B. J. & Wilmot, C. M. (2004). Copper-containing amine oxidases. Biogenesis and catalysis; a structural perspective. *Arch Biochem Biophys* **428**, 22–31.
- Brocard, C., Kragler, F., Simon, M. M., Schuster, T. & Hartig, A. (1994). The tetratricopeptide repeat-domain of the PAS10 protein of *Saccharomyces cerevisiae* is essential for binding the peroxisomal targeting signal-SKL. *Biochem Biophys Res Commun* **204**, 1016–1022.
- Bruun, L. & Houen, G. (1996). *In situ* detection of diamine oxidase activity using enhanced chemiluminescence. *Anal Biochem* **233**, 130–136.
- Cai, D. & Klinman, J. P. (1994a). Copper amine oxidase: heterologous expression, purification, and characterization of an active enzyme in *Saccharomyces cerevisiae*. *Biochemistry* **33**, 7647–7653.
- Cai, D. & Klinman, J. P. (1994b). Evidence of a self-catalytic mechanism of 2,4,5-trihydroxyphenylalanine quinone biogenesis in yeast copper amine oxidase. *J Biol Chem* **269**, 32039–32042.
- Cai, D., Williams, N. K. & Klinman, J. P. (1997). Effect of metal on 2,4,5-trihydroxyphenylalanine (topa) quinone biogenesis in the *Hansenula polymorpha* copper amine oxidase. *J Biol Chem* **272**, 19277–19281.
- Carr, H. S. & Winge, D. R. (2003). Assembly of cytochrome c oxidase within the mitochondrion. *Acc Chem Res* **36**, 309–316.
- Culotta, V. C., Klomp, L. W., Strain, J., Casareno, R. L., Krems, B. & Gitlin, J. D. (1997). The copper chaperone for superoxide dismutase. *J Biol Chem* **272**, 23469–23472.
- Dancis, A., Klausner, R. D., Hinnebusch, A. G. & Barriocanal, J. G. (1990). Genetic evidence that ferric reductase is required for iron uptake in *Saccharomyces cerevisiae*. *Mol Cell Biol* **10**, 2294–2301.
- Dancis, A., Yuan, D. S., Haile, D., Askwith, C., Eide, D., Moehle, C., Kaplan, J. & Klausner, R. D. (1994a). Molecular characterization of a copper transport protein in *Saccharomyces cerevisiae*: an unexpected role for copper in iron transport. *Cell* **76**, 393–402.
- Dancis, A., Haile, D., Yuan, D. S. & Klausner, R. D. (1994b). The *Saccharomyces cerevisiae* copper transport protein (Ctr1p): biochemical characterization, regulation by copper, and physiological role in copper uptake. *J Biol Chem* **269**, 25660–25667.
- Dawkes, H. C. & Phillips, S. E. (2001). Copper amine oxidase: cunning cofactor and controversial copper. *Curr Opin Struct Biol* **11**, 666–673.
- Duff, A. P., Cohen, A. E., Ellis, P. J., Kuchar, J. A., Langley, D. B., Shepard, E. M., Dooley, D. M., Freeman, H. C. & Guss, J. M. (2003). The crystal structure of *Pichia pastoris* lysyl oxidase. *Biochemistry* **42**, 15148–15157.
- Faber, K. N., Haima, P., Gietl, C., Harder, W., Ab, G. & Veenhuis, M. (1994). The methylotrophic yeast *Hansenula polymorpha* contains an inducible import pathway for peroxisomal matrix proteins with an N-terminal targeting signal (PTS2 proteins). *Proc Natl Acad Sci U S A* **91**, 12985–12989.
- Forsburg, S. L., Sherman, D. A., Otilie, S., Yasuda, J. R. & Hodson, J. A. (1997). Mutational analysis of Cdc19p, a *Schizosaccharomyces pombe* MCM protein. *Genetics* **147**, 1025–1041.
- Georgatsou, E. & Alexandraki, D. (1994). Two distinctly regulated genes are required for ferric reduction, the first step of iron uptake in *Saccharomyces cerevisiae*. *Mol Cell Biol* **14**, 3065–3073.
- Glerum, D. M., Shtanko, A. & Tzagoloff, A. (1996). Characterization of COX17, a yeast gene involved in copper metabolism and assembly of cytochrome oxidase. *J Biol Chem* **271**, 14504–14509.
- Gurvitz, A., Rottensteiner, H., Kilpelainen, S. H., Hartig, A., Hiltunen, J. K., Binder, M., Dawes, I. W. & Hamilton, B. (1997). The *Saccharomyces cerevisiae* peroxisomal 2,4-dienoyl-CoA reductase is encoded by the oleate-inducible gene SPS19. *J Biol Chem* **272**, 22140–22147.
- Harding, T. M., Morano, K. A., Scott, S. V. & Klionsky, D. J. (1995). Isolation and characterization of yeast mutants in the cytoplasm to vacuole protein targeting pathway. *J Cell Biol* **131**, 591–602.
- Hassett, R. & Kosman, D. J. (1995). Evidence for Cu(II) reduction as a component of copper uptake by *Saccharomyces cerevisiae*. *J Biol Chem* **270**, 128–134.
- Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K. & Pease, L. R. (1989). Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene* **77**, 51–59.
- Horng, Y. C., Cobine, P. A., Maxfield, A. B., Carr, H. S. & Winge, D. R. (2004). Specific copper transfer from the Cox17 metallochaperone to both Sco1 and Cox11 in the assembly of yeast cytochrome c oxidase. *J Biol Chem* **279**, 35334–35340.
- Jalkanen, S. & Salmi, M. (2001). Cell surface monoamine oxidases: enzymes in search of a function. *EMBO J* **20**, 3893–3901.
- Knight, S. A., Labbé, S., Kwon, L. F., Kosman, D. J. & Thiele, D. J. (1996). A widespread transposable element masks expression of a yeast copper transport gene. *Genes Dev* **10**, 1917–1929.
- Kumar, V., Dooley, D. M., Freeman, H. C., Guss, J. M., Harvey, I., McGuirl, M. A., Wilce, M. C. & Zubak, V. M. (1996). Crystal structure of a eukaryotic (pea seedling) copper-containing amine oxidase at 2.2 Å resolution. *Structure* **4**, 943–955.
- Labbé, S., Zhu, Z. & Thiele, D. J. (1997). Copper-specific transcriptional repression of yeast genes encoding critical components in the copper transport pathway. *J Biol Chem* **272**, 15951–15958.
- Laliberté, J., Whitson, L. J., Beaudoin, J., Holloway, S. P., Hart, P. J. & Labbé, S. (2004). The *Schizosaccharomyces pombe* pccs protein functions in both copper trafficking and metal detoxification pathways. *J Biol Chem* **279**, 28744–28755.
- Large, P. J. (1986). Degradation of organic nitrogen compounds by yeasts. *Yeast* **2**, 1–34.
- Li, R., Klinman, J. P. & Mathews, F. S. (1998). Copper amine oxidase from *Hansenula polymorpha*: the crystal structure determined at 2.4 Å resolution reveals the active conformation. *Structure* **6**, 293–307.
- Lin, S. J., Pufahl, R. A., Dancis, A., O'Halloran, T. V. & Culotta, V. C. (1997). A role for the *Saccharomyces cerevisiae* ATX1 gene in copper trafficking and iron transport. *J Biol Chem* **272**, 9215–9220.

- Lunelli, M., Di Paolo, M. L., Biadene, M., Calderone, V., Battistutta, R., Scarpa, M., Rigo, A. & Zanotti, G. (2005). Crystal structure of amine oxidase from bovine serum. *J Mol Biol* **346**, 991–1004.
- Martins, L. J., Jensen, L. T., Simon, J. R., Keller, G. L. & Winge, D. R. (1998). Metalloregulation of *FRE1* and *FRE2* homologs in *Saccharomyces cerevisiae*. *J Biol Chem* **273**, 23716–23721.
- Matsunami, H., Okajima, T., Hirota, S., Yamaguchi, H., Hori, H., Mure, M., Kuroda, S. & Tanizawa, K. (2004). Chemical rescue of a site-specific mutant of bacterial copper amine oxidase for generation of the topa quinine cofactor. *Biochemistry* **43**, 2178–2187.
- Mumberg, D., Muller, R. & Funk, M. (1995). Yeast vectors for the controlled expression of heterologous proteins in different genetic backgrounds. *Gene* **156**, 119–122.
- Mure, M., Brown, D. E., Saysell, C. & 7 other authors (2005). Role of the interactions between the active site base and the substrate Schiff base in amine oxidase catalysis. Evidence from structural and spectroscopic studies of the 2-hydrazinopyridine adduct of *Escherichia coli* amine oxidase. *Biochemistry* **44**, 1568–1582.
- Neuberger, G., Maurer-Stroh, S., Eisenhaber, B., Hartig, A. & Eisenhaber, F. (2003a). Motif refinement of the peroxisomal targeting signal 1 and evaluation of taxon-specific differences. *J Mol Biol* **328**, 567–579.
- Neuberger, G., Maurer-Stroh, S., Eisenhaber, B., Hartig, A. & Eisenhaber, F. (2003b). Prediction of peroxisomal targeting signal 1 containing proteins from amino acid sequence. *J Mol Biol* **328**, 581–592.
- O'Halloran, T. V. & Culotta, V. C. (2000). Metallochaperones, an intracellular shuttle service for metal ions. *J Biol Chem* **275**, 25057–25060.
- Parsons, M. R., Convery, M. A., Wilmoth, C. M., Yadav, K. D., Blakeley, V., Corner, A. S., Phillips, S. E., McPherson, M. J. & Knowles, P. F. (1995). Crystal structure of a quinoenzyme: copper amine oxidase of *Escherichia coli* at 2 Å resolution. *Structure* **3**, 1171–1184.
- Peña, M. M., Lee, J. & Thiele, D. J. (1999). A delicate balance: homeostatic control of copper uptake and distribution. *J Nutr* **129**, 1251–1260.
- Peña, M. M., Puig, S. & Thiele, D. J. (2000). Characterization of the *Saccharomyces cerevisiae* high-affinity copper transporter Ctr3. *J Biol Chem* **275**, 33244–33251.
- Petriv, O. I., Tang, L., Titorenko, V. I. & Rachubinski, R. A. (2004). A new definition for the consensus sequence of the peroxisome targeting signal type 2. *J Mol Biol* **341**, 119–134.
- Portnoy, M. E., Rosenzweig, A. C., Rae, T., Huffman, D. L., O'Halloran, T. V. & Culotta, V. C. (1999). Structure-function analyses of the Atx1 metallochaperone. *J Biol Chem* **274**, 15041–15045.
- Pufahl, R. A., Singer, C. P., Peariso, K. L., Lin, S. J., Schmidt, P. J., Fahrni, C. J., Culotta, V. C., Penner-Hahn, J. E. & O'Halloran, T. V. (1997). Metal ion chaperone function of the soluble Cu(I) receptor Atx1. *Science* **278**, 853–856.
- Puig, S. & Thiele, D. J. (2002a). Molecular mechanisms of copper uptake and distribution. *Curr Opin Chem Biol* **6**, 171–180.
- Puig, S., Lee, J., Lau, M. & Thiele, D. J. (2002b). Biochemical and genetic analyses of yeast and human high-affinity copper transporters suggest a conserved mechanism for copper uptake. *J Biol Chem* **277**, 26021–26030.
- Rees, E. M. & Thiele, D. J. (2004). From aging to virulence: forging connections through the study of copper homeostasis in eukaryotic microorganisms. *Curr Opin Microbiol* **7**, 175–184.
- Rehling, P., Marzoch, M., Niesen, F., Wittke, E., Veenhuis, M. & Kunau, W. H. (1996). The import receptor for the peroxisomal targeting signal 2 (PTS2) in *Saccharomyces cerevisiae* is encoded by the *PAS7* gene. *EMBO J* **15**, 2901–2913.
- Reumann, S. (2004). Specification of the peroxisome targeting signals type 1 and type 2 of plant peroxisomes by bioinformatics analyses. *Plant Physiol* **135**, 783–800.
- Rosenzweig, A. C., Huffman, D. L., Hou, M. Y., Wernimont, A. K., Pufahl, R. A. & O'Halloran, T. V. (1999). Crystal structure of the Atx1 metallochaperone protein at 1.02 Å resolution. *Structure* **7**, 605–617.
- Rymond, B. C., Zitomer, R. S., Schumperli, D. & Rosenberg, M. (1983). The expression in yeast of the *Escherichia coli galK* gene on *CYC1::GalK* fusion plasmids. *Gene* **25**, 249–262.
- Samuels, N. M. & Klinman, J. P. (2005). 2,4,5-trihydroxyphenylalanine quinone biogenesis in the copper amine oxidase from *Hansenula polymorpha* with the alternate metal nickel. *Biochemistry* **44**, 14308–14317.
- Sherman, F. (1991). Getting started with yeast. *Methods Enzymol* **194**, 3–21.
- Veenhuis, M., Mateblowski, M., Kunau, W. H. & Harder, W. (1987). Proliferation of microbodies in *Saccharomyces cerevisiae*. *Yeast* **3**, 77–84.
- Yu, P. H., Wright, S., Fan, E. H., Lun, Z. R. & Gubisne-Harberle, D. (2003). Physiological and pathological implications of semicarbazide-sensitive amine oxidase. *Biochim Biophys Acta* **1647**, 193–199.