Identification and functional expression of ctaA, a P-type ATPase gene involved in copper trafficking in Trametes versicolor

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Here the identification and characterization of a gene encoding a copper-trafficking enzyme, ctaA (copper-transporting ATPase), from the basidiomycete Trametes versicolor are described. This P-type copper ATPase gene has two alleles, differing primarily in the length of the second, unusually long intron, and encodes a 983 aa protein with 40% sequence identity to yeast Ccc2p. Overexpression of ctaA in yeast grown in the presence of copper led to a 15-fold increase in laccase yields, while overexpression of ctaA and tahA, a previously identified copper homeostasis gene of T. versicolor, was additive, leading to a 20-fold increase in laccase production. In T. versicolor, overexpression of ctaA and tahA led to an eightfold increase in laccase expression, and a cotransformant still expressed laccase at 3000 μM copper when hardly any laccase activity is detected in the wild-type strain. Apparently, at low to moderate levels of copper tahA and ctaA overexpression disturbs the normal hierarchy of copper distribution, resulting in more being directed to the Golgi, while with high copper amounts that normally switch on the copper detoxification processes, tahA and ctaA gene products seem to out-compete the metallothionein copper chaperones, meaning laccase is still supplied with copper. These results may lead to a better understanding of copper trafficking and the hierarchy of copper distribution in the cell, and possibly be useful for constructing laccase-overproducing strains for biotechnological purposes.

INTRODUCTION

Copper is an essential cofactor in numerous enzymes that participate in redox reactions, including proteins involved in the detoxification of oxygen radicals, such as the (cytoplasmic) copper/zinc superoxide dismutase (Cu/Zn-SOD); electron-transport proteins, such as the (mitochondrial) cytochrome c oxidase; and proteins with oxidase activity, such as fungal laccase. However, copper can be toxic even at low concentrations, so organisms possess several mechanisms to maintain intracellular copper concentrations at adequate levels. These include various copper homeostasis factors that control the uptake, distribution and sequestration of this metal inside the cell. For example, under high copper concentrations, cells express metallothionein-like proteins that bind the metal tightly. Under normal cellular copper concentrations, cells express copper metallochaperones that guide and protect the copper ions, facilitating their proper delivery to their different destinations, e.g. mitochondria, secretory pathways and cytoplasmic enzymes (O’Halloran & Culotta, 2000). Under copper-starvation conditions, these copper homeostasis factors impose a hierarchy of copper distribution inside cells (mitochondria > cytoplasm > Golgi) by a process that is not yet fully understood.

In contrast to filamentous fungal systems, copper metabolism in yeast systems is well characterized: after being taken up from the medium by copper permeases, copper ions destined for insertion into secreted enzymes are bound by the copper chaperone Atx1p and transported to the post-Golgi compartment of the secretory pathway. There, Atx1p interacts with Ccc2p, a copper-transporting P-type ATPase, located in the membrane of the post-Golgi compartment. Ccc2p pumps the copper ions delivered by Atx1p into the lumen of the Golgi where copper is then inserted into secreted copper-dependent enzymes such as the iron oxidase Fet3p. The protein factors involved in cellular copper homeostasis are highly conserved between all eukaryotes analysed so far: copper metallochaperones homologous to Atx1p and to the copper-transporting P-type ATPases have been described in yeast, mammalian, nematode and plant species.

Abbreviations: ABTS, 2,2’-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diaminonium salt; Cu/Zn-SOD, copper/zinc superoxide dismutase; Ferrozine, 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4’-amine-2’-disulfonic acid. The GenBank accession number for the Trametes versicolor ctaA gene sequence reported in this article is AY210894.
systems (Himelblau et al., 1998; Hung et al., 1997; Klompe et al., 1997; Payne & Gitlin, 1998; Hirayama et al., 1999; Wakabayashi et al., 1998). However, these factors seem to be regulated differently in the various systems.

Yeast mutants have facilitated the identification of genes from heterologous sources by functional complementation. Applying this to the secretory copper-containing laccases, we recently described the isolation, identification and characterization of a homologue of the yeast gene anti-oxidant 1 (ATX1), the gene tahA (Trametes ATX homologue) from Trametes versicolor encoding a protein of 72 aa with 56 % sequence identity to yeast Atx1p (Uldschmid et al., 2002). We observed that tahA was up-regulated when copper was present and down-regulated when no copper was available, and that this copper chaperone protein efficiently provided the secretory copper-containing laccases with copper.

Apart from providing a useful marker for monitoring copper trafficking to secretory pathways via the Golgi apparatus, the laccases are involved in a wide variety of different cellular reactions. Besides detoxification of toxic compounds, morphogenesis and melanin formation, laccases are regarded as potential pathogenic factors of fungi. They are also of practical interest because they can oxidize a wide variety of phenolic compounds, and can be used in paper-bleaching processes without chlorine (Call & Mücke, 1997). The production of functional laccases depends on sufficient/elevated copper concentrations in the medium (Feldmann et al., 1996; Sariaslani, 1989), detoxification of xenobiotic compounds, organic synthesis (Rosenau et al., 1996; Sariaslani, 1989), dye bleaching, or binding of wood composites (Felby et al., 1997). The production of functional laccases depends on sufficient/elevated copper concentrations in the medium (A. Uldschmid, R. Dombi & K. Marbach, unpublished data).

To further investigate copper trafficking to the laccases, we employed complementation strategies in yeast. Here we describe the identification of a second copper homeostasis factor, the gene ctaA (copper-transporting ATPase) from the basidiomycete T. versicolor encoding a protein of 983 aa with 40 % sequence identity to yeast Ccc2p. We characterized this gene and its product at the structural, biochemical and functional levels. We also studied the effect of ctaA overexpression on the amount of active laccase produced in yeast and Trametes, as well as the effect of coexpressing ctaA and the previously identified tahA gene. Interestingly, the effects of these gene products proved additive in both organisms, leading to elevated amounts of active laccase production, even under elevated copper conditions where, normally, copper systems are repressed and copper detoxification processes are switched on.

METHODS

Organisms and culture conditions. The Escherichia coli strain used in this study was XL-1 Blue MRF’. The Saccharomyces cerevisiae strains used were: CM3260 (MATa, leu2-3,122, his3-69, trp1-63, ura3-52, gen4-101) obtained from Dr Dennis Winge, Salt Lake City, UT, USA; YPH250 (MATa, ura3-52, lys2-801, ade2, trp1-D1, his3-A200, leu2-D1) and Strain 3 (isogenic to YPH250 with Δcct2::::URA3) obtained from Dr Valeria Culotta, John Hopkins University School of Public Health, Baltimore, MD, USA. S. cerevisiae strains were grown in liquid yeast nitrogen base (YNB) and on solid media. Carbon sources were used at a concentration of 2 %. Ferrozine [3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4'-,4'-′-disulfonic acid] was added up to a concentration of 2:5 mM on solid media.

Monokaryotic strains Trametes F6-79B11 (AppyRg), used for cotransformation, and Trametes F2-100, used for Southern blot analyses, were as derived from fruiting bodies of dikaryotic strains. DNA for library construction (cDNA and genomic) was obtained from dikaryotic TV-1 (Uldschmid et al., 2002). Culture conditions for T. versicolor were as described by Uldschmid et al. (2002).

Plasmids and DNA libraries. A genomic DNA library from T. versicolor was obtained from Dr Rupert Pfaffer from the Consortium für elektrochemische Industrie, München. The library was constructed as described by Uldschmid et al. (2002). A Trametes cDNA library for yeast complementation was constructed in the yeast centromere plasmid pAH (Feldmann et al., 1996), essentially as described by Uldschmid et al. (2002). Restriction analysis of inserts from different clones revealed that the cDNA library contains inserts ranging in size from 0.1 to 3.5 kb. For expression of Trametes genes in S. cerevisiae, the yeast expression vectors pAH, pAT (Feldmann et al., 1996) and pYEX-S1 (Clontech) were used.

Isolation of T. versicolor ctaA cDNA by complementation. Yeast Strain 3 was transformed with the pAH cDNA library (above) and, after plating out on His− selection medium, the 2 × 106 yeast clones obtained were washed off the plate, purified and thoroughly mixed before plating on selection plates containing 1 mM Ferrozine to create an iron deficiency and incubation for 3 days at 30 °C. From the 1000 colonies that grew on the selection plates, 70 were re-plated on His plates. The plasmids isolated from these clones were amplified in E. coli and the DNA analysed by restriction digests using HindIII.

Isolation of T. versicolor ctaA structural gene. A complete genomic structural gene of ctaA-1 was obtained by PCR with the primers gcta-fw (5′-GAA TGC TGA AGT CCG GAG AAG C-3′) and gcta-rv (5′-GAA GTT GAA CAT CCC GTG TGA C-3′) and 500 ng of genomic DNA from T. versicolor with the Expand High Fidelity PCR system (Roche). The PCR conditions used were: 3 min at 94 °C, followed by 30 cycles of 10 s at 94 °C, 10 s at 60 °C and 4 min at 68 °C. The 4·9 kb product was separated on a 1 % agarose gel, extracted from the agarose gel using a Gel-Extraction Kit (Qiagen), ligated into the vector pCR2.1 and transformed into E. coli XL-1 Blue MRF’. Plasmid DNA was isolated from transformants and a correct clone (pCR2.1gcta) identified by restriction analysis.

Coexpression of tahA, ctaA and laccase in S. cerevisiae. The laccase III gene (lacIII) from T. versicolor was coexpressed with ctaA, tahA or ctaA plus tahA in S. cerevisiae strain CM3260. Strain CM3260, which lacks endogenous laccase, was transformed with the laccase expression construct pLacP2 (lacIII cloned in the vector pYEX-S1 from Clontech; Fig. 2) where the lacIII cDNA was under transcriptional control of the yeast PGK promoter. This laccase-expressing yeast derivative was transformed with the pAH and pAT derivatives (Feldmann et al., 1996) that harboured either the Trametes ctaA or tahA cDNA under control of the yeast ADH1 promoter (Figs 2 and 3). The four strains were inoculated into 200 μl YNB selection medium (His−, Trp−, Ura−) containing 1 mM ABTS [2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt], supplemented with 0, 10, 100, 200, 400, 600, 800 or 1000 μM CuSO4, and grown in microtitre plates overnight, with shaking on a thermosaker (Eppendorf) at room temperature. Laccase activity was then determined by measuring the OD420 value.
Construction of ctaA and tahA expression plasmids for *T. versicolor*. First, a single disturbing BspLU111 restriction site was removed from pUC19 by cutting it with this enzyme, filling in the overhanging ends with Klenow subunit and re-ligating the vector. An *Sphl* fragment of about 1 kb containing the *T. versicolor* *gapDH* promoter was cloned in both orientations into the *Sphl* site of the multiple cloning site in the modified pUC19 vector to produce two vectors (pUC-Pgap— and pUC-Pgap+).

The *gapDH* promoter from pUC-Pgap— was excised with *EcoRI* and BspLU111 and inserted upstream of the ATG start codon by PCR using the High Fidelity PCR System (Roche), pCR2.1gcta as template and the primers gcta-ATG and gcta-rv. The primers were chosen so that a mismatch (C instead of T) at the second nucleotide position in the PCR creates an *NcoI* site. The PCR fragment was then cloned in pCR2.1 (Invitrogen) and the new plasmid called pATGcta was created, from which a 1236 bp fragment containing the 5′-region of the ctaA gene was excised using *NcoI* and inserted into the compatible BspLU111 restriction site in pUC-Pgap+ to give pgapCTA-A. The 3421 bp 3′-region of ctaA-1 was also isolated from pATGcta via *SphI* and *NcoI* and ligated into the vector pgapCTA-A cut with *SphI* and *Smal*, resulting in the expression plasmid pUC-gapCTA (Fig. 5).

To clone the ctaA expression vector, an *NcoI* restriction site was introduced upstream of the ATG start codon by PCR with the High Fidelity PCR System (Roche), pCR2.1gcta as template and the primers gcta-ATG and gcta-rv. The primers were chosen so that a mismatch (C instead of T) at the second nucleotide position in the PCR creates an *NcoI* site. The PCR fragment was then cloned in pCR2.1 (Invitrogen) and the new plasmid called pATGcta was created, from which a 1236 bp fragment containing the 5′-region of the ctaA gene was excised using *NcoI* and inserted into the compatible BspLU111 restriction site in pUC-Pgap+ to give pgapCTA-A. The 3421 bp 3′-region of ctaA-1 was also isolated from pATGcta via *SphI* and *NcoI* and ligated into the vector pgapCTA-A cut with *SphI* and *Smal*, resulting in the expression plasmid pUC-gapCTA (Fig. 5).

**Cotransformation of ctaA and tahA in *T. versicolor***. Plasmids pUC-gapCTA and gPuragapTAH were mixed in a 1:1 ratio, transformed into *T. versicolor* F6-79B11 according to the protocol of Bartholomeuw *et al.* (2000) and plated out on minimal medium (MM) (Uldschmid *et al.*, 2002) containing 0·6 M sucrose. After 1–2 weeks incubation at 28 °C, the fungal colonies that appeared were inoculated on MM plates and kept at 28 °C until they were fully grown (>7 days). From the circumference region of the plates, mycelia were again inoculated on MM plates and incubated again until they had grown.

Some mycelium was scraped from each of the transformants and resuspended in 50 μl TE and prepared for a PCR with the following programme: 5 min at 65 °C → 2 min at 96 °C → 4 min at 65 °C → 1 min at 96 °C → 1 min at 65 °C → 0·5 min at 96 °C → 20 °C. Subsequently, the cells were mechanically squashed with an inoculating loop and then centrifuged. Aliquots (1 μl) of supernatant were mixed with the primer pairs Pgap/tahE or Pgap/ctaA and Taq polymerase. The primer Pgap hybridizes in the *gapDH* promoter, tahE in the *tahA* gene and ctaA in the *ctaA* gene (see Fig. 5). Thus the primer combinations were specific for the expression constructs. The PCR product with Pgap/tahE was 690 bp in size and that with Pgap/ctaA was 460 bp in size. As a positive control for the PCR, a 260 bp fragment was amplified out of the chromosomal *T. versicolor* *gapDH* gene with the primer pair Pgap/gapA.

**Preparation of Trametes DNA and Southern blot analysis**. Preparation of genomic DNA from *T. versicolor* and analyses of yeast or *T. versicolor* transformants by Southern blotting were carried out as described by Uldschmid *et al.* (2002). DNA from cotransformants Ko7, Ko15 and Ko19 and the recipient strain F6 were cut with *BamH*/*NsiI*, *KpnI*, *SacI* and *XhoI*, and the blot was hybridized with a *tahA* probe (NcoI–NsiI fragment from p2gTAH-N) or a *ctaA* probe (Clal–NdeI fragment from pgATGcta). The *BamH*/*NsiI* digest generated a 1·5 kb band containing the complete expression cassette with the *gapDH* promoter and *tahA* gene.

**Isolation of mRNA from *T. versicolor* for library construction**. Total RNA from *T. versicolor* was prepared according to the method of Logemann *et al.* (1987), starting with 2 g semi-dried mycelium as described by Uldschmid *et al.* (2002).

**Isolation of genes from a *Trametes* genomic library**. The *Trametes* genomic library was screened as described by Uldschmid *et al.* (2002).

**Sequence analysis**. Clones were sequenced completely from their 5′- and 3′-ends by cycle sequencing on an automated sequencer (Laboratorium für molekuleare Biologie, Abt. Genomics, München). Database searches with DNA fragment-derived protein sequence data were performed with the program BLASTX (National Institutes of Health). Alignments were generated using the CLUSTAL_X, 63b Multiple Sequence Alignment Program, a multiple alignment tool developed by the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). Further sequence analysis and data processing were performed with the GENE INSPECTOR 1.5 program from Texco.

**Laccase expression in *T. versicolor***. Pre-cultures were prepared and 23 ml diluted into different 200 ml batches of expression medium containing 0, 100, 500, 1000, 2000 or 3000 μM CuSO4.

These were grown at 24 °C with shaking at 110 r.p.m., and the cultures were induced on the second day with 1·5 mM 2,5-xylylene. From the third day onwards, laccase production was measured for 10 days using an ABTS test as described below. The experiment was performed in duplicate and the activity values were averaged between the duplicate cultures.

**Measurement of laccase enzyme activity (ABTS test)**. Laccase activity was determined in MacIlvain buffer pH 4·5 (0·1 M citrate with 0·2 M disodium hydrogen phosphate to the desired pH) by monitoring the oxidation of ABTS (Sigma) at 420 nm with a specific extinction coefficient of 3·6 × 104 l M−1 cm−1. The test was performed at 37 °C in 990 μl MacIlvain buffer plus x ml enzyme and 10 μl of 10 mM ABTS. After incubation for 5–30 min, the reaction was stopped by adding 200 μl of 5 M NaCl. The volume activity was then calculated by $e (\text{U ml}^{-1}) = \frac{\text{AE}(1200/x)}{(36 \times t)}$, where $t$ is the time of incubation, $x$ is the amount (μl) of enzyme used in the reaction and $AE$ is the difference between the OD420 value of the ABTS test and that of the control (test without enzyme).

**RESULTS**

**Isolation of the gene for a copper P-type ATPase ctaA from *T. versicolor***. The ctaA gene from *T. versicolor* was isolated using functional complementation of a yeast Δccce2 strain (Strain 3) with the *T. versicolor* cDNA library cloned in the yeast expression vector pAH (Methods). This yeast mutant, with a deletion in the gene for P-type copper ATPase, cannot sequester copper to the Golgi compartment and is no longer able to activate the copper-dependent iron oxidase Fet3, which together with Fre1 comprises the 'high-affinity' iron uptake system. This strain cannot survive under iron-limiting conditions (Yuan *et al.*, 1995; Eide *et al.*, 1993).

Yeast transformants were isolated, further selected on Ferrozine medium and plasmids were isolated from selected
clones for analysis (Methods). Of the 70 plasmids tested, 62 contained a 3 kb cDNA insertion (the others contained no insert), proving that complementation of the Δccc2 mutant was very specific and probably involved one gene. To confirm this, a number of cDNA inserts from different clones were sequenced and a database search was performed on these sequences. All the clones showed clear homology to the known yeast, human and plant P-type copper ATPases Ccc2, Menkes and RAN1.

The cDNA sequences of clones 21 and 34 differ significantly from each other: comparison of their complete sequences revealed a total of 152 nt differences that result in 11 aa substitutions. To answer the question of whether these differences are due to allelic variations in ctaA or represent two different genes, Southern blot analysis was performed on restriction digests of diploid (dikaryon) or haploid (monokaryon) T. versicolor genomes. From the Southern blot analysis, it is clear that two fragments hybridize in the dikaryon TV1, while only one band is detected with the monokaryon F2-100 (data not shown). Therefore, clones 34 and 21 represent different alleles of ctaA, and are subsequently called ctaA-1 (cta34) and ctaA-2 (cta21).

To confirm that both ctaA-1 and ctaA-2 can complement the yeast Δccc2 mutant, the plasmids containing the two cDNA clones were transformed back into Strain 3. The transformants were subjected to a drop titre test on low iron medium (1 mM Ferrozine), to which some iron had been added (150 μM) to maintain better growth of the wild-type. As negative and positive controls, Strain 3 and the corresponding wild-type strain YPH250 were transformed with the empty pAH vector and similarly subjected to the drop titre test. The Δccc2 Strain 3 clearly showed no growth on the selection plate (1 mM Ferrozine, 150 μM iron), while the strains transformed with the constructs pAHcta21 and pAHcta34 – similar to the wild-type strain – grew well (Fig. 1). Thus, all the cDNAs cloned from T. versicolor show efficient complementation.

**Isolation of ctaA**

A sequence of ctaA was derived by aligning clones from a genomic phage library of T. versicolor, including five phages isolated by cross-hybridization with ctaA cDNA. These were initially sequenced from the phage vectors with T7 and T3 primers and then, as far as possible, with the primers specifically designed for sequencing the ctaA cDNA sequences (see below). New primers were created to close the sequencing gaps arising from the introns. A complete sequence could not be compiled from the two partial sequences obtained, and so the gaps in the genomic sequence were filled in by PCR using a collection of cta-fw and cta-rv primers based on known flanking sequences (not shown). The amplified fragments obtained were cloned into pZero0 and sequenced. All the individual sequences were aligned to produce a complete sequence using sequence comparison with the ctaA-1 allele. Only 2200 bp of the 5′-region were obtained from allele ctaA-2, of which 850 bp are promoter sequences. A complete genomic structural gene of ctaA-1 was obtained by PCR as described in Methods. One clone confirmed by restriction analysis, pCR2.1gcta, was completely sequenced and found to carry the sequence of the ctaA-1 allele (not shown).

**Characterization of ctaA**

Comparison of the structural gene (deposited in GenBank under accession number AY210894) of allele ctaA-1 with the cDNA showed that the gene is interrupted by three introns between the start codon ATG and the double stop codons TGA TAG, whereby intron II, with 643 nt, is unusually long for a fungus. A fourth and alternatively spliced intron is found between the stop codons and the putative polyadenylation signals AATATT and ATATT. Intron I is 50 nt long, intron III is 53 nt long and intron IV is 100 nt long. The consensus sequences for the splice sites correlated with those described for tahA and the fungi in general (Ballance, 1986). The isolated 2200 bp 5′-region of allele ctaA-2 stretches from the promoter region until shortly beyond intron II. Like intron II in allele ctaA-1, this intron is unusually large, although with 284 nt it is less than half the size of intron II in ctaA-1. However, analysis of both ‘giant’ introns revealed no unusual sequence homology that might indicate, for example, an insertion element, and the sequence of the 5′- and 3′-splice sites of the intron correlated with those described for tahA and the fungi (Ballance, 1986). Comparison of the promoter regions of both alleles showed that the first 430 nt upstream of the start codon (ATG) are highly conserved. Many sequences with...
homology to described elements that can play a role in transcription regulation were found in both alleles, including homologies to the binding sites for the transcription factors NIT2, GATA-1, PEA3, NF1, GCN4, GCR1 and Sp1. To what extent these elements influence the transcription of ctaA remains to be elucidated. A TATA box was not found, although a pyrimidine-rich sequence was detected directly before the start codon (ATG).

**Characterization of the protein sequence of ctaA**

The cDNA of ctaA encodes a protein of 983 aa, which shares up to 40% identity and up to 60% homology with other P-type copper ATPases. As with yeast Ccc2 and the human Menkes protein, eight transmembrane domains could also be predicted for CtaA since sequences in this region are highly conserved. Highly conserved motifs essential for the function of all copper-transporting P-type ATPases were also found in CtaA. For example, at positions 50 and 124 in the N terminus are two putative copper-binding sites with the peptide sequences GMTCGAC and GMTCSSC, which correspond to the consensus sequence GMTCxxC for copper-binding sites in proteins. A phosphatase site, TGEP, is found at position 407 and the APCPxLG motif important for copper transduction is located in membrane domain V at position 527, followed by a phosphorylation site, DKTGT (572), and an ATP-binding domain, GDGIND (816). The SEHPL motif, as yet not precisely characterized functionally, is found in CtaA at position 633. A methionine involved in copper transduction localized in membrane domain VIII is also conserved in CtaA. At the protein level the two CtaA alleles differ by 11 aa substitutions, none of which is located in any of these motifs.

**Coexpression of taha, ctaA and laccase in S. cerevisiae**

To investigate the effect on laccase expression of deregulated expression of ctaA plus another copper-trafficking factor, taha, which we identified previously (Uldschmid et al., 2002), the lacIII gene from *T. versicolor* was coexpressed with ctaA, taha or ctaA plus taha in the *S. cerevisiae* strain CM3260. In addition, strain CM3260 that already carries the laccase expression construct pLacP2 was transformed with different combinations of empty vectors (pAH and pAT) and expression constructs (pATtahA and pAHctaA: Methods, Fig. 2). The wild-type yeast strain CM3260 used in this test shows no laccase activity at all (data not shown), since yeast do not have an endogenous laccase gene. As shown in Fig. 3, overexpression of either taha or ctaA had a positive effect on heterologous laccase expression in yeast. Overexpression of taha showed the largest effect between 200 and 400 μM CuSO4, where up to three times as much laccase was produced compared to the parent strain. More dramatically, overexpression of ctaA resulted in an up to 15-fold increase in laccase expression compared to the control strain. Coexpression of ctaA and taha showed an additive effect, particularly at very low copper concentrations (≤ 10 μM), where up to 20-fold more laccase was expressed than in the control strain. Thus, overexpression of the *T. versicolor* genes taha and ctaA can clearly increase the expression of lacIII in yeast.

A similar result was obtained using the yeast homologues of taha and ctaA, ATX1 and CCC2 (Fig. 4) in an analogous experiment. The overexpression of either ATX1 or CCC2 also had a positive effect on laccase expression. An additive effect with the coexpression of ATX1 and CCC2 was also
seen, since laccase activity could be increased twofold. However, with low copper concentrations, the effect of Atx1p and Ccc2p was not as strong as observed for the coexpression of the T. versicolor genes tahA and ctaA.

Coexpression of ctaA and tahA in T. versicolor

Since our yeast results (above) had demonstrated an additive effect, we also wanted to test the influence of the copper homeostasis factors TahA and CtaA on laccase expression in T. versicolor. Suitable expression vectors were constructed by cloning the genomic genes of both tahA and ctaA from the start codon ATG behind a T. versicolor gapDH promoter, which should lead to increased constitutive transcription of the genes in T. versicolor. Since both genes were to be cotransformed, the smaller tahA expression vector also carried a pyrG selection marker from Schizophyllum commune. The resulting plasmids pUC-gapCTA and gPuragapTAH (Methods, Fig. 5) were mixed

![Figure 3](image1.png)

**Fig. 3.** Heterologous coexpression of T. versicolor lacIII, tahA and ctaA in S. cerevisiae. (a, b) The laccase-expressing yeast derivative, i.e. CM3260+pLacP2, was transformed with pAH+pAT (●, control), pAHctaA+pAT (▲), pAHctaa+pATtahA (■), or pAH+pATtahA (▲). The strains were cultivated overnight in 200 µl media containing 0–1000 µM copper and 1 mM ABTS. The laccase activities of the cultures were then determined by measuring the OD420 values (Methods). (a) ABTS assay of the cultures in a 96-well plate. (b) Plots showing the mean value of three parallel experiments; laccase activity was determined by measuring the OD420 value. At low copper concentrations, the additive effect of tahA and ctaA expression is clearly visible, with an increase in laccase expression of up to 20-fold.

![Figure 4](image2.png)

**Fig. 4.** Coexpression of T. versicolor lacIII, yeast ATX1 and yeast CCC2 in S. cerevisiae. The laccase-expressing yeast derivative CM3260+pLacP2 was transformed with pAH+pAT (●, control), pAH+pATatx1 (■), pAHccc2+pAT (▲), or pAHccc2+pATatx1 (▲). The strains were cultivated overnight in media containing 0–1000 µM copper, and the laccase activities of the cultures were determined with an ABTS assay (Methods). Plots show the mean value of three parallel experiments; laccase activity was determined by measuring the OD420 value. The additive effect of CCC2 and ATX1 expression is clearly visible.

![Figure 5](image3.png)

**Fig. 5.** Constructs used for coexpression of tahA and ctaA in T. versicolor. In the tahA-expressing construct gPuragapTAH, the tahA gene (striped bar) is under the control of the T. versicolor gapDH promoter (Pgap). As a selection marker for T. versicolor this vector contains the pyrG gene of Schizophyllum commune. In the ctaA-expressing construct pUC-gapCTA, the genomic ctaA gene is also under the control of the T. versicolor gapDH promoter (Pgap). There is no selection marker in this vector as it is cotransformed with gPuragapTAH. The primers used for screening the cotransformants are indicated by black and grey arrowheads.
in a 1:1 ratio and transformed (Bartholomew et al., 2000) into T. versicolor F6-79B11 (also referred to as F6). After incubation, re-inoculation and further growth, 23 potential cotransformants were analysed for the presence of the expression constructs by PCR analysis (Methods). Of these transformants, 13 had taken up both plasmids and showed the appropriate specific PCR products, which meant a cotransformation rate of over 50%.

A more precise idea of the number of expression constructs integrated into the genome was obtained by isolating genomic DNA from the cotransformants Ko7, Ko15 and Ko19 and the recipient strain F6 and performing Southern blot analyses (Fig. 6). Hybridization of tahA in the recipient strain F6 showed only one cross-hybridizing band per digest, whereas the cotransformants had additional bands due to integration of the expression construct. The 1·5 kb band in the BamHI/NsiI digest corresponds to the complete tahA expression cassette with the gapDH promoter and tahA gene, since in the expression construct BamHI cuts before, and NsiI after, this cassette.

KpnI cuts once in the backbone of the 6·7 kb ctaA expression construct. The 6·7 kb band from KpnI digestion of Ko7 must therefore represent a tandem insertion of pUC-gapCTA. The wild-type band electrophoreses with a size of 4·8 kb. Densitometric comparison of the signal strength (Image Quant) between the 6·7 kb band and the 4·8 kb band showed that the ctaA construct is integrated at least five times in tandem in Ko7 (Fig. 6), and therefore at least four expression cassettes are intact. In Ko19 the KpnI restriction fragment is only 5·3 kb and thus it is uncertain whether the ctaA expression cassette is intact.

**Laccase expression in T. versicolor cotransformants**

In a preliminary test, the laccase expression of the T. versicolor cotransformants was compared to the wild-type F6 strain. For this purpose, strains Ko7, Ko15 and Ko19 and the control strain F6 were grown in liquid minimal medium without any additional copper. The laccase activity in the cultures was determined 7 days after induction with 2,5-xylidine. The cotransformants Ko7, Ko15 and Ko19 showed the highest laccase expression, ranging from fourfold for Ko15 (0·028 U ml⁻¹), to fivefold for Ko19 (0·035 U ml⁻¹), up to more than tenfold for Ko7 (0·092 U ml⁻¹), compared to F6 (0·007 U ml⁻¹).

Due to the promising result obtained with the Ko7 derivative, it was further characterized by analysing its laccase expression under different copper concentrations compared to the wild-type F6 strain. It was clear that all the Ko7 cultures supplemented with copper showed higher laccase expression than the F6 cultures. The difference was most obvious with 500 μM CuSO₄, where the Ko7 derivative showed up to six times more laccase activity than the F6 strain. The Ko7 culture without added copper showed no elevated laccase production and resembled the F6 culture without copper. At 3000 μM copper F6 produced hardly any more laccase than in the control culture without copper, while Ko7 still produced it well (Fig. 7).
The difference in laccase expression was not due to a difference in growth, since determination of the dry cell weight after the test showed that all the cultures had grown to the same extent despite the very different copper concentrations. The variation within one strain was not greater than 15–20 %, and between strains the differences were not much more. For example, the difference in cell mass between strains Ko7 and F6 at 500 μM CuSO₄ was only 14 %.

In conclusion, as in yeast, overexpression of the copper homeostasis factors tahA and ctaA leads to a significant increase in laccase expression.

**DISCUSSION**

**Isolation and characterization of ctaA**

Two different alleles of ctaA were isolated from a *T. versicolor* cDNA library by complementation of a *S. cerevisiae Δccc2* mutant. The coding sequence of the isolated ctaA gene is interrupted by three introns. The second intron is exceptional due to its length (intron II from allele ctaA-1 is 643 nt; intron II from allele ctaA-2 is 284 nt), since in higher fungi introns are mostly between 50 and 60 bp in length, or perhaps as long as 100 bp (Gurr, 1988). No unusual sequences, such as inverted repeats that might indicate an IS element, could be identified in these long introns.

The 983 aa protein predicted from the cDNA sequence of ctaA showed up to 60 % homology to the known copper P-type ATPases Ccc2 from yeast and human MNKP (Menkes Disease Protein). This family of proteins is highly conserved in all organisms from all kingdoms. There are always one to six metal-binding sites (MBSs) at the N terminus (Penn et al., 1999) with the consensus sequence GMTCxxC. Like Ccc2, CtaA also has two MBSs at its N terminus. For Ccc2 it was shown that Atx1 binds to this motif and passes on copper (Pufahl et al., 1997; Huffman & O’Halloran, 2000). Since ctaA fully complements the functionality of the Δccc2 yeast mutant, the two MBSs of the CtaA protein probably also interact with Atx1 to take up the copper. Forbes et al. (1999) could show by deletion analysis of the MBSs in WNDP (Wilson Disease Protein) that the MBS localized nearest to the first membrane domain is sufficient and also essential for copper transport. An additional highly conserved region is the CPC motif in membrane domain VI, which is essential for copper transduction. It could be shown that methionine 1386 in membrane domain VIII of the mouse WNDP is also essential (Voskoboinik et al., 2001). This methionine is conserved in all copper P-type ATPases and is also present in CtaA. The ATP-binding domains, the phosphorylation domains and the dephosphorylation domains, where the turnover of ATP to ADP that drives copper transport takes place, were all identified in CtaA. From this, one can conclude that CtaA in *T. versicolor* performs the same function as Ccc2 in yeast and that it is localized in the Golgi network.

A more precise description of the function of CtaA in *T. versicolor* might be obtained from a ctaA knock-out strain. However, despite repeated attempts we have been unable to generate this so far. One reason for this could be that one of the five laccase isoenzymes from *T. versicolor* might be essential for cell development and/or morphogenesis. If this laccase can no longer be loaded with copper in a ΔctaA strain, this would mean that a knock-out strain could not grow. In addition, a hydroxylation step in the biosynthesis of ceramides in *S. cerevisiae* has been described as being dependent on functional Ccc2 (Beeler et al., 1997; Haak et al., 1997). A similar effect could also influence cell development in *T. versicolor*. In support of this theory is the fact that transformation of *T. versicolor* with a ctaA antisense construct produced very few transformants. Antisense RNA expression may lead to the same lethal result as a potential ctaA knock-out. This question could be answered in the future by generating a ctaA null strain.
future by more detailed analysis of an antisense construct or by using RNA interference (RNAi).

Several recognition motifs for transcription factors such as NIT2, GCN4 and NF1 were found in T. versicolor, although a transcript could not be detected by Northern blotting (not shown). It remains unclear whether the very large second intron plays a role in the very low transcription level of ctaA, although it is possible the length of the intron could have an indirect influence on the number of transcripts via splicing activity (Pukkila & Casselton, 1991). A second possibility could be that the intron forms a kind of terminator structure and thus full-length ctaA transcripts are kept at a low level. The transcriptional regulation of homologous proteins has yet to be investigated in any detail. For example, four metal response elements (MREs) detected in the promoter of the WND gene (Oh et al., 2002) have not yet been tested for copper induction. The P-type ATPases can probably be considered household genes that are often regulated at the protein level. However, to date, it could only be shown for the homologous copper P-type ATPases WNDP and MNKP that their localization changed according to copper concentrations. At low copper concentrations they are found in the Golgi and at high copper concentrations they are localized at the cell membrane where they pump copper out of the cells (Petris et al., 1996; Hung et al., 1997).

**Coexpression of tahA and ctaA in yeast and in T. versicolor**

We show here that deregulated coexpression of ctaA and tahA in a laccase III-expressing yeast could lead to increased laccase yields by up to 20-fold with a copper concentration of 10 μM. Analogous results were also obtained by overexpressing ATX1 and CCC2, the yeast homologues of tahA and ctaA. However, although the increase in laccase expression was not as high as that mediated by tahA and ctaA coexpression, it was nevertheless significant. We then determined that the deregulated coexpression of both these genes in T. versicolor also led to an increase in laccase expression of up to eightfold. This shows that the efficiency of laccase production depends on the supply of copper to the Golgi vesicle and that overexpression of the genes that supply this cell organelle with copper apparently increase the copper flow in this direction. Since overexpression of ctaA in yeast under low copper concentrations showed the more dramatic effect on laccase expression, and CtaA is probably regulated more at the protein level, one might suppose that the CtaA protein is the ‘bottleneck’ in copper transport to the Golgi lumen. Further investigations into the regulation of CtaA protein activity are needed to determine to what extent CtaA function is influenced post-translationally, for example, by phosphorylation. Examples described by Shatzman & Kosman (1978), analysing galactose oxidase, Cu/Zn-SOD and cytochrome c oxidase, showed that under limiting copper conditions the copper supply is first regulated in secreted proteins, then in Cu/Zn-SOD, followed by the respiratory chain proteins. In T. versicolor under copper-deficient conditions, the copper supply to the Golgi is minimized by down-regulation of TahA and laccase is no longer supplied with sufficient copper. The fungus, however, grows quite normally under these conditions, which shows that its respiratory chain proteins still receive copper. The overexpression of both genes (ctaA and tahA) probably results in a strong interference in this hierarchy of copper distribution to the advantage of the Golgi network. A kind of copper highway is achieved that directs the major part of the available copper pool to the Golgi. However, this has no effect on the growth of the fungus, which shows that an undersupply is not created for the important respiratory copper enzymes such as COX (cytochrome c oxidase). The increased copper transport to the Golgi described above could also be the reason why the cotransformant Ko7 still expresses laccase at 3000 μM copper while hardly any laccase activity is found in the wild-type F6 strain. Under these conditions all the copper systems are probably repressed in F6 due to the high amounts of copper, while all the copper detoxification proteins such as metallothionein, as well as copper chaperones and Cu/Zn-SOD, are highly expressed. The expression of phytochelatins under copper stress has been shown for Schizosaccharomyces pombe (Perego et al., 1996). Whether, and in what way, these mechanisms apply to T. versicolor must be elucidated by future experiments.

Since metallothionein and phytochelatin have a very high affinity for copper (e.g. >2 × 10^{-14} M^{-1} for human MT-3) (Hasler et al., 2000), they probably compete with the copper chaperone TahA for the available copper. This would undermine the supply of copper to the Golgi and laccase would no longer be supplied with copper. In Ko7 the overexpression of TahA and CtaA may relieve the competition with metallothioneins, meaning laccase is still supplied with copper. This might prove an important consideration if, for example, one was constructing laccase-overproducing strains for biotechnological purposes.

**ACKNOWLEDGEMENTS**

This work was supported financially by the Consortium für elektrochemische Industrie, München, Germany. We thank Dr Rupert Pfaller from the Consortium für elektrochemische Industrie for his very helpful cooperation. We warmly thank Dr Valeria Culotta for providing yeast strain YPH250 and Strain 3 used in the complementation assays. We also thank Dr Dennis Winge for providing yeast strain CM3260 and for very interesting discussions on copper metabolism.

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