Characterisation of multicopper oxidase CopA from *Pseudomonas putida* KT2440 and *Pseudomonas fluorescens* Pf-5: involvement in bacterial lignin oxidation

Rommel Santiago Granja-Travez and Timothy D.H. Bugg*

Department of Chemistry, University of Warwick, Coventry CV4 7AL

*Author for correspondence: Prof Timothy D.H. Bugg, Department of Chemistry, University of Warwick, Coventry CV4 7AL, U.K. Tel 44-2476-573018 email T.D.Bugg@warwick.ac.uk

Abstract

CopA is a protein formed as part of a copper resistance operon in *Pseudomonas syringae* pv tomato, but CopA has also been identified from gene library screening as a potential lignin-oxidising enzyme. Few bacterial homologues for bacterial multi-copper laccases have been identified that can assist in lignin degradation. Bioinformatic analysis revealed that *copA* and *copC* genes were found in the genomes of bacterial strains capable of lignin oxidation. In this study, CopA enzymes from bacterial strains with lignin oxidation activity, *Pseudomonas putida* and *P. fluorescens*, were heterologously expressed and characterised kinetically, and expression of bacterial CopC proteins was also investigated. Purified CopA enzymes were dependent upon exogenous copper (II) ions for activity when expressed under fully aerated conditions, however after expression under microaerobic conditions with copper reconstitution, the activity was independent of copper addition. The CopA enzymes showed activity towards the laccase substrates 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS); syringaldazine (SGZ); guaiacol; 2,6-dimethoxyphenol (DMP) and 2,4-dichlorophenol (DCP). Moreover, CopA proteins were able to oxidise the lignin model compounds guaiacylglycerol-beta-guaiacyl (GGE) and 2,2'-dihydroxy-3,3'-dimethoxy-5,5'-dicarboxybiphenyl (DDVA), giving oxidised dimerised products; and they were active towards Ca-lignosulfonate, giving vanillic acid as product. A double gene deletion of *copA-I* and *copA-II* genes in *Pseudomonas putida* KT2440 was constructed, and this mutant showed diminished growth capability on different small aromatic compounds related with lignin degradation, when copper salts were present in the media.

Introduction

Multicopper oxidases (MCOs) are a family of copper containing enzymes, divided into laccases (EC 1.10.3.2), ascorbate oxidase (EC 1.10.3.3), ferroxidase (EC 1.16.3.1) nitrite reductase (EC 1.7.2.1) and ceruloplasmin (EC 1.16.3.1), subgroups [1]. MCOs usually contain four atoms of copper, which are arranged in two different copper centres, one containing a single atom of type 1 copper (T1), responsible for a characteristic blue colour of these proteins; and a trinuclear copper centre (TNC), containing three atoms of copper: one type 2 copper, and a binuclear type 3 copper (T3). Some MCOs can oxidize a given substrate by coupling the electron transfer to a four-electron reduction of dioxygen into water [2-5]. The amino acid residues involved in copper attachment and electron flow are very well conserved in MCOs, although their sequence homology is rather low. The conserved motifs in MCOs are as follows: HXHG, HXH, HXXHXH and HCHXXXHXXXXM/L/F [6, 7]. The most important and largest subgroup of MCOs, laccases, are widespread in nature, and show a variety of biological functions, depending on their sequence, host and environmental conditions [7, 8].

Fungal laccases are the most well studied so far and they have been reported commonly in white-rot fungi, suggesting an important role of laccases in lignin degradation, although the detailed mechanism is not yet well understood [9]. White-rot fungi are the most competent lignin degraders, being the only organisms known to entirely mineralize lignin to CO_2 and H_2O [10, 11]. Laccases could require a mediator molecule to oxidise some substrates, either because their large size prevents the diffusion of the substrate into the enzyme active site or because their high redox potential. A mediator can change its oxidation state and serve as an electron transporter between the substrate and the enzyme [12]. Laccases can oxidase a wide range of substrates, among them, syringaldazine is considered to be a unique laccase substrate as long as the reaction occurs in absence of hydrogen peroxide [13]. A lack of T1 copper in some laccases, which causes loss of the characteristic blue colour of these proteins, consequently these are known as “yellow” or “white” laccases. Fungal laccases of this type have been reported in *Pleurotus ostreatus Panus tigrinus*, *Phlebia radiata* and an unidentified basidiomycete [10, 14-17].

In the recent years, bacterial laccases have been discovered, but their classification and biological function is not clear, therefore some of them have been referred as laccase-

like multicopper oxidases (LMCOs) or pseudo-laccases. The function of some bacterial LMCOs include: for CotA protein from *Bacillus subtilis*, the formation of a spore pigment [18], and activity towards bilirubin [19]; while other LMCO proteins have been involved in copper resistance mechanisms, such as CopA from *Pseudomonas syringae* and *Xanthomonas campestris* [20], or CueO and PcoA from *Escherichia coli* [21, 22]. A CueO multicopper oxidase has recently been characterised from a lignin-degrading *Ochrobactrum* sp. strain, which shows activity for oxidation of lignin model compounds and Ca lignosulfonate [23].

All of the bacterial multicopper oxidases mentioned above, have shown oxidase activity for some common laccase substrates, although some of them require addition of exogenous copper ions to trigger or enhance their activity [22, 24-27]. The role of bacterial laccases in lignin degradation is not clear [28, 29]; even so, it has been reported that deletion of the *Streptomyces* laccase gene leads to reduced amounts of acid-precipitable lignin (APPL) formation from lignocellulose, implying a role for this laccase in lignin oxidation [30]. A predicted CopA protein having 100 % and 80% amino acid sequence identity with CopA in *Pseudomonas stutzeri* ATCC 14405 and *Pseudomonas putida*, respectively, has been identified from a bacterial metagenomic fosmid library using a biosensor developed for sensing lignin degradation products [25]. The selected fosmid harbouring this sequence, diminished the biosensor activation and lost lignin transformation capability, when it was disrupted [25].

The *copA* gene is commonly found in bacterial genomes as part of a copper resistance operon, containing *copABCDRS* genes [31, 32]. We noticed that although *copA* was present in lignin degrader *Pseudomonas putida* KT2440, this strain contained no *copC* gene. Conversely, we observed that two isolated *copC* genes were present in the genome of lignin-degrading *Microbacterium phyllosphaerae*, in the absence of other copper resistance genes, but situated close to other genes implicated in lignin degradation in other bacteria, as described below. These observations prompted us to characterise bacterial CopA and CopC proteins, and study their ability to oxidise lignin model compounds and polymeric lignin. Here we report expression and kinetic characterisation of CopA from *Pseudomonas putida* KT2440 and *Pseudomonas fluorescens* Pf-5; and CopC from *Burkholderia xenovorans* LB400.

Methods

Cloning of copA and copC genes

CopA proteins in *Pseudomonas putida* KT2440 and *Pseudomonas fluorescens* PF-5, and CopC proteins in *Burkholderia xenovorans* were found by a search in the online database UniProt (https://www.uniprot.org/), using the amino acid sequence of CopA-II (UniProt code Q88C03) as a probe for the Basic Local Alignment Search Tool (BLAST), performed in the NCBI online database (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

CopA encoding genes from *P. putida* KT2440 and *P. fluorescens* PF-5 were amplified by PCR from chromosomal DNA. Genomic DNA was isolated using a Wizard Genomic DNA Purification Kit (Promega). Primers used for amplification were as follows: Forward (5' CACC GTG ACC AGC CCT GGC CAA CCG 3') and reverse (5' TCA TTC GTC CAC GCG GAC TTC 3') for *copA-II* from *Pseudomonas putida,* whose expressed protein was named as Pp-CopA. Forward (5' CACC GTC AGC AGC CCG GGC CAG 3') and reverse (5' TCA AGC TTC ATG GTG GCG TTC CTC 3') for *copA* from *Pseudomonas fluorescens*, whose expressed protein was named as Pf-CoPA. The designed primers excluded a predicted TAT signal sequences found in these genes. After gene amplification by polymerase chain reaction (PCR) using Pfx Taq polymerase, PCR products were purified and used for cloning with the Champion™ pET Directional TOPO® Expression Kit (Invitrogen). The resulting vectors were transformed into TOP10 *E. coli* competent cells for maintenance, and into BL21 *E. coli* competent cells for protein expression, according to manufacturer's protocol.

Microbacterium phyllosphaerae was isolated from woodland soil and was identified as a bacterial lignin degrader in a previous study [33]. Two CopC encoding genes, *copC886* (accession number MH924843) and *copC1032* (accession number MH924844) were selected for cloning and protein expression under two different systems: an *E. coli* BL21 based system, using the pET151 vector; and an *R. jostii* RHA1 based system, using the pTipQC-II vector. The primers used are listed in

Table 1. The online tools Phobius (http://phobius.sbc.su.se/), and SignalP (http://www.cbs.dtu.dk/services/SignalP/) identified the presence of a signal peptide sequence in these genes. Hence, for expression in *E. coli*, two set of primers were designed, one including the predicted signal peptide sequence, and another excluding it. The primers used for expression under the *R. jostii* system contained the restriction sites *NdeI* and *HindIII* in the forward and reverse primers, respectively. In addition, the PCR products of these primers contained a TEV recognition sequence. For cloning into *R. jostii*, the PCR products and pTipQC-II vector were subjected to a restriction digestion and a T4 DNA ligation, using FastDigest restriction enzymes (Thermo Scientific) according to manufacturer's instructions. Resulting vectors were transformed into Top10 *E. coli* chemically competent cells for maintenance, and into *R. jostii* competent cells for protein expression. *R. jostii* competent cells were transformed by electroporation by mixing 50 µL of competent cells with 6 µL of pTipQC-II containing the gene of interest, in an electroporation cuvette. An electric potential of 1.8 V for 5 seconds was used in a pulse electroporator. The resulting reaction was incubated with 200 µL of Luria Bertani medium at 30 °C for 1 hour and then spread on a Luria Bertani agar plate supplemented with chloramphenicol (25 µg/mL).

Two genes encoding CopC from *Burkholderia xenovorans*, *copC-bx1* and *copC-bx2*, were ordered from Genscript. The synthetic genes were designed to include a His_6 tag at the C-terminus position and were cloned directly in the expression vector pET28b+ with the restriction sites: *NcoI* at the 5' end, and *XhoI* at the 3' end. The resulting vectors were transformed into TOP10 *E. coli* competent cells for maintenance, and into BL21 *E. coli* competent cells for protein expression, following the Champion™ pET Directional TOPO® Expression Kit (Invitrogen) protocol. All resulting plasmids were sent for sequencing to the GATC biotech Company, to confirm the correct structure and integrity of the cloned genes.

Protein Expression & purification of recombinant CopA and CopC

The antibiotic used for protein expression varied depending on the organism and intended protein, as follows: for Pp-CopA from *P. putida*, Pf-CopA from *P. fluorescens*, and CopC886 and CopC1032 from *M. phyllosphaerae*, all expressed in *E. coli*, ampicillin was used (100 µg/mL); for CopC886 and CopC1032 from *M. phyllosphaerae* expressed in *R. jostii*,

Chloramphenicol was used (25 μg/mL); and for CopC-Bx1 and CopC-Bx2 from *B. xenovorans*, expressed in *E. coli*, kanamycin was used (50 μg/mL).

A 10 mL culture of the microorganism harbouring the vector with the gene of interest was used as starter culture to inoculate 1 L of Luria Bertani media, supplemented with the corresponding antibiotic. The culture was grown at 37 °C for *E. coli* and 30 °C for *R. jostii*, until OD reached ≈0.6 (about 4 h for *E. coli* and about 24 h for *R. jostii*), and then the inductor was added: IPTG 1 mM for expression on pET vectors (vectors used in *E. coli* cells) and thiostrepton (1 μg/mL) for pTipQC-II vector (vector used in *R. jostii*). At this point, cultures were supplemented with $CuSO_4$ (80 μM), and were incubated overnight at 15 °C and 200 rpm of agitation.

Cell harvesting was performed by centrifugation at 6000 x g, then the cells were re-suspended in 15 mL of lysis buffer (50 mM sodium phosphate, 300 mM NaCl, 10 mM imidazole, pH 8.0), lysed with a cell disruptor at 21 kpsi and then the cell lysate was centrifuged at 15000 x g for 30 min. The recombinant proteins were produced with a His_6 tag; hence the supernatant was loaded onto a nickel affinity column for IMAC purification. The flow through was discarded and the column was washed with wash buffer (50 mM sodium phosphate, 300 mM NaCl, 20 mM imidazole, pH 8.0). Finally, the protein was eluted from the column with 5 mL of elution buffer (50 mM sodium phosphate, 300 mM NaCl, 250 mM imidazole, pH 8.0).

Pp-CopA and Pf-CopA were subjected to a further step involving the cleavage of the His_6 tag with TEV protease, and a second IMAC was performed to remove TEV enzyme. The protein solutions containing untagged Pp-CopA and Pf-CopA were desalted using a PD10 column and transferred to a 20 mM Hepes, 100 NaCl buffer, pH 7.0. Protein quality was assayed by SDS/PAGE (**Figure 5**, **A**).

Copper reconstitution

Pp-CopA and Pf-CopA were produced as apoenzymes in fully aerated conditions. Expression under microaerobic conditions (agitation 0 rpm after induction) was performed to produce copper loaded proteins [34]. In addition, once these proteins were purified, Pp-CopA and Pf-CopA were concentrated by Centricon tubes and then 3 μL of sterile $CuSO_4$ 400 mM were added to each protein solution (≈1 mg/mL). The mixture was incubated overnight

at room temperature and then buffer exchanged twice to a 20 mM Hepes, 100 NaCl buffer, pH 7.0, by PD10 columns, to remove the excess of copper ions.

Enzyme activity and biochemical characterization

The laccase substrates 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS, λ 420 nm, ε 36,000 M^{-1} cm^{-1}); syringaldazine (SGZ, λ 530 nm, ε 65,000 M^{-1} cm^{-1}); guaiacol (λ 436 nm, ε 26,600 M^{-1} cm^{-1}); 2,6-dimethoxyphenol (DMP, λ 468 nm, ε 49,600 M^{-1} cm^{-1}) and 2,4-dichlorophenol (DCP, λ 510 nm, ε 18,000 M^{-1} cm^{-1}) in the presence of 4-amino antipyrine, were used for CopA activity assays. The substrate final concentration was 1 mM, except for SGZ, whose concentration was 0.05 mM. Enzyme mixture reaction contained the substrate, a buffer (50 mM sodium acetate buffer, pH 4.0, for ABTS and DMP; or 50 mM Hepes buffer, pH 7.0, for SGZ, DCP and guaiacol), enzyme (0.05 mg/mL) and $CuSO_4$ (0.5 mM). Activity was determined spectrophotometrically by measuring the change of the absorbance at the corresponding substrate wavelength. Kinetic parameters were established for ABTS, SGZ and DMP. One activity unit was defined as that amount of enzyme that catalyzes the conversion of 1 µmol of substrate per minute.

The pH-activity profile with ABTS and SGZ was obtained by using the same enzyme mixture reaction, using a Britton and Robinson Universal buffer, with a pH varying from 2.3 to 10.4 [35]. For comparison of activity and stability of Pp-CopA, the commercial fungal *T. versicolor* laccase (TvL, ≥ 0.5 U mg 1), purchased from Sigma-Aldrich (Gillingham, UK), was used using ABTS and SGZ as substrates. Thermal stability was assessed at 50 °C using only ABTS (1 mM) as substrate.

The lignin model compounds guaiacylglycerol-beta-guaiacyl ether (GGE) and 2,2'-dihydroxy-3,3'-dimethoxy-5,5'-dicarboxybiphenyl (DDVA), were also used as substrates for enzyme activity assays. GGE was dissolved in methanol whereas DDVA was dissolved in DMSO. The concentration of both substrates was 2 mM. Enzymatic reactions consisted of a substrate (GGE or DDVA), 20 mM ammonium bicarbonate buffer, enzyme (0.1 mg/mL) and $CuSO_4$ (0.5 mM), in presence and absence of ABTS (1 mM) as mediator. The reactions were incubated for 6 hours and then subjected to solvent extraction. Product formation was measured by HPLC and LC-MS.

For polymeric lignin activity assays, eight different lignin preparations were used, including organosolv lignins, alkali lignins, lignosulfonate, and industrial Kraft lignin. Reaction mixture consisted of 1.2 mL of buffer (20 mM ammonium bicarbonate), 0.2 mL of protein solution (≈0.7 mg/mL), 5 mg of lignin, 0.15 mL of 7 mM $CuSO_4$ and 0.15 mL of 10 mM ABTS as mediator. Reactions were incubated overnight. Samples were centrifuged (4000 x g for 2 minutes) and then subjected to solvent extraction. Product formation was measured by HPLC and LC-MS.

HPLC and LC-MS assays

The formation of products upon reaction of Pp-CopA and Pf-CopA with lignin model compounds and polymeric lignin was analysed by HPLC and LC-MS. Firstly, a solvent extraction of the samples was performed, by adding 2 volumes of ethyl acetate to one volume of the reaction mixture, then the samples were acidified with 100 µL of 1 M HCl, gently mixed and the organic phase was collected. The solvent was removed by rotary evaporation and the samples were re-suspended in 200 µL of 50% methanol, centrifuged at 11000 x g and filtered for HPLC or LC-MS injection. HPLC analysis was carried out on a Hewlett-Packard Series 1100 HPLC, using a Phenomenex Luna C18 reverse phase column (250 x 4.6 mm, 5 µ, 100 Å); whereas for LC-MS, a Bruker Amazon X system (Bruker, Coventry, UK) was used. The parameters used were as follows: flow rate, 0.5 mL/min; wavelength, 270 nm; and gradient whose percentage is referred to methanol (buffer B) in water (buffer A), 0 – 5% for 10 min, 5 – 10% for 10 min, 10 – 30% for 15 min, 30 – 40% for 5 min, 40 – 70% for 5 min, 70 – 100% for 15 min and 100 – 5% for 10 min, giving a total time of 70 min per each run.

Gene deletion

The method reported for Huang & Wilks was used for gene deletion [36], with some variations. *P. putida* KT2440 was selected for gene deletion experiments. Two *copA* genes were identified in its genome, named as *copA-I* and *copA-II*. The upstream and downstream regions (about 1 kbp) of each gene, were amplified by using a phusion high-fidelity DNA polymerase M0530, according to manufacturer's protocol. The primers used can be seen in

Table 2. A two-step PCR was performed, including an initial denaturation at 98 °C for 30 seconds, followed by 32 cycles of denaturation (98 °C for 10 seconds) and annealing (72 °C for 30 seconds), and a final extension at 72 °C for 10 minutes. PCR products were analysed in a 1% agarose gel and purified. Then, PCR products were ligated into the pJET1.2/blunt vector and cloned into Top10 *E. coli* chemically competent cells for maintenance, according to CloneJET PCR Cloning Kit (Thermo Fisher Scientific) protocol.

Plasmids harbouring the upstream and downstream fragments of the target gene, either *copA-I* or *copA-II*, and the vector pK18mobSacB were subjected to a double restriction digestion with the corresponding restriction enzymes. For upstream and downstream fragments, the restriction enzymes used can be seen in **Table 2**; while for pK18mobSacB vector intended for *copA-I* deletion, EcoRI and HindIII were used; and for *copA-II* deletion, XbaI and PstI were used. Then, the digested upstream and downstream fragments and the corresponding vector for *copA-I* and *copA-II* were ligated to generate the allelic exchange vector. FastDigest restriction enzymes (Thermo Scientific) were used according to manufacturer's protocol. 5 µL of ligation reaction was used to transform 50 µL of Top10 *E. coli* chemically competent cells. Transformed cells were grew on LB plates supplemented with Kanamycin (50 µg/mL). A screening was performed by double restriction digestion and sequencing, with the GATC Biotech Company to confirm the correct structure of the construct. These allelic exchange vectors were used to transform competent *P. putida* KT2440 cells.

1 µL of plasmid harbouring the allelic exchange vector was mixed with 100 µL of competent *P. putida* cells in an electroporation cuvette. The cuvette was placed in electroporator at a voltage of 1.8 kV for 5 seconds. Then, 500 µL of LB media was added to the reaction, it was incubated at 30 °C for 1.5 hours, plated on kanamycin plates and incubated overnight. Colonies able to grow on kanamycin plates were spread on 10% sucrose plates (counter selection marker), and they were incubated at 30 °C for 24 hours. The resulting colonies were subjected to PCR to confirm the deletion of the gene. The primers used to confirm the gene deletions were, for *copA-I* deletion (Δ*copA-I*), Fw TCGACTACGGCCTGCTGAT, and Rev CTCATGTTTGGCTCGACG; and for *copA-II* deletion (Δ*copA-II*), Fw CCACTGTGACTTGCGCTTTC, and Rev ACCAAAAGCTCCCCATGTCT. These primers were designed to be complementary with sequences located from ≈70 bp upstream of the targeted gene, to ≈70 beyond of the downstream flanking region (outside of the

target gene and the flanking regions construct). PCR products of wild type strains (gene not deleted), should show a bigger size (≈2.5 kbp) than strains whose genes have been deleted (≈1 kbp), since the amplified region of wild type strains would include the target gene (≈1.5 kbp) and the downstream flanking region (≈1 kbp). For the generation of the double mutant (Δ*copA-I-II*), the same steps were followed. In this case, the allelic exchange vector containing the construct to delete the *copA-I* gene was used to transform a *P. putida* strain that had already lost the *copA-II* gene. A sequence analysis by GATC Biotech Company was used to confirm all gene deletions.

Results

Identification and genetic context of copA and copC genes

The genome sequences of some bacterial lignin degraders were analysed to identify putative *copA* and *copC* genes. *Pseudomonas putida* KT2440 contains two different *copA* sequences, named as *copA-I* and *copA-II*. Gene *copA-II* was found located from nucleotide 6.131.843 to nucleotide 6.133.852, in the genome. Close to *copA-II* are putative genes that have been previously reported as part of the copper resistance operon: *copB-II*, *copR-II* and *copS* [31, 32]; and also copper and silver efflux system: *cusA*, *cusB* and *cusF* [37], were found. Gene *copA-1* was found located from nucleotide 2.510.006 to nucleotide 2.511.730, in genome, adjacent to a putative *copB-1* gene. No other putative genes related with copper resistance were found near this gene. No *copC* genes were found in *P. putida* KT2440. Concerning *Pseudomonas fluorescens* PF-5, a single putative *copA* gene was found, located from nucleotide 3.237.717 to nucleotide 3.239.456 in the genome. Near this gene, a putative *copB* gene was found to be the only one related with copper resistance. A putative *copC* gene was found in *P. fluorescens* PF-5, although it was not close to *copA*, but located from nucleotide 2.814.512 to nucleotide 2.814.880 in the genome, and adjacent to a *copD* gene.

Putative *copC* genes were found in the genomes of the bacterial lignin degraders *Microbacterium phyllosphaerae* and *Burkholderia xenovorans*. Besides CopC, CopD putative genes were found in these genomes as the only genes forming part of the copper resistance operon. The predicted CopC proteins show a lack of a rich methionine region involved in

copper attachment, reported for the *Pseudomonas syringae* pv. tomato CopC [38, 39]. *Microbacterium phyllosphaerae* contained two *copC* genes, named as *copC886* and *copC1032*. Gene *copC886* (accession number MH924843) was adjacent to a dihydrolipoamide dehydrogenase gene, while *copC1032* (accession number MH924844) was situated close to a DyP-type peroxidase gene; both of these gene families have been implicated in lignin degradation [40, 41]. The genome of polychlorinated biphenyl degrader *Burkholderia xenovorans* LB400 contains two *copC* genes, named as *copC-bx1* and *copC-bx2*, with no other copper resistance genes nearby.

Expression & purification of recombinant CopA and CopC

Pseudomonas putida KT2440 *copA-II, Pseudomonas fluorescens* Pf-5 *copA*, *Microbacterium phyllosphaerae copC885* and *copC1032*, and *Burkholderia xenovorans copC-bx1* and *copC-bx2*, were selected for cloning and protein expression. The genes encoding *P. putida* CopA-II (Pp-CopA) and *P. fluorescens* CopA (Pf-CopA) were amplified from chromosomal DNA, excluding their predicted TAT signal peptide sequences. Amplicons were cloned into the expression vector pET151. The recombinant proteins were then expressed as N-terminal His_6 fusion proteins in *E. coli*, after induction with 1 mM IPTG, grown in Luria Bertani media supplemented with 80 µM $CuSO_4$ and ampicillin. The recombinant proteins were purified by immobilized metal ion chromatography (IMAC), which gave the expected protein bands at 70.8 kDa and 60.4 kDa for Pp-CopA and Pf-CopA, respectively (**Figure 5, A**). The His_6 fusion tag was then removed by proteolytic digestion with TEV protease, followed by further purification by a second IMAC, to give recombinant proteins free of impurities. Yields of ≈25 mg Pp-CopA and ≈3 mg Pf-CopA per litre of culture were obtained.

The purified proteins were colourless, even after copper reconstitution, in contrast to multi-copper oxidase CueO from *Ochrobactrum* [23], which is blue (**Figure 5**, **B**). Both proteins were found dependent upon exogenous Cu^{2+} salts for activity; however, when expressed under microaerobic conditions [34], and after copper reconstitution, the enzymes were active without exogeneous copper. Despite its activity, they remained colourless and did not shown the typical UV/vis spectrum for laccase enzymes (λ_{max} 612 nm, see **Figure 5**, **C**). Attempts to crystallise Pp-CopA and Pf-CopA were unsuccessful. The purified enzymes were analysed by inductively coupled plasma optical emission spectroscopy (ICP-OES), giving a stoichiometry of 9.1 ± 1.5 mol Cu per mol protein for Pp-CopA; and 8.7 ± 1.3 mol Cu

per mol protein for Pf-CopA; which is similar to the copper content of 10.9 ± 1.2 atoms of copper per protein reported for CopA from *Pseudomonas syringae* [31].

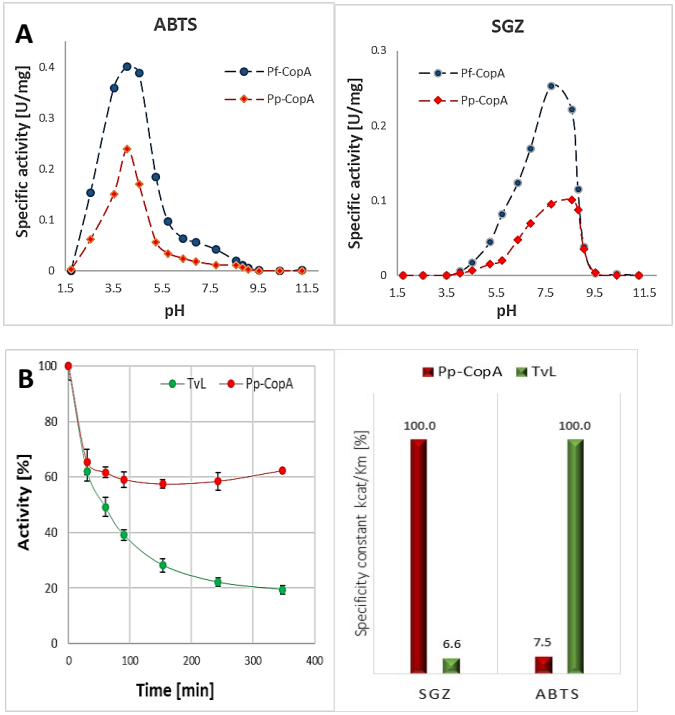
Microbacterium phyllosphaerae copC885 and *copC1032* were amplified from chromosomal DNA and cloned into the expression vector pET151, however, attempts to express recombinant protein either under a range of conditions either in *E. coli* or in Gram-positive host *Rhodococcus jostii* RHA1 (using expression vector pTipQC-II) were unsuccessful. Synthetic codon-optimised genes *copC-bx1* and *copC-bx2* from the Gram-negative *Burkholderia xenovorans* LB400 were cloned into expression plasmid pET-28b(+), and were expressed in *E. coli* BL21. Luria Bertani media supplemented with 150 µM $CuSO_4$ and kanamycin was used for recombinant protein expression, using 1 mM IPTG for induction. After cell harvesting, recombinant proteins were purified by immobilized metal ion chromatography (IMAC), which gave the expected protein bands at 10-15 kDa for both CopC-bx1 and CopC-bx2, together with a protein band at 30-40 kDa (see Supporting Information Figure S1), although in relatively low yield, approximately 0.3 mg protein per litre of culture in each case. The identities of purified CopC-bx1 and CopC-bx2 were confirmed by trypsin digestion and electrospray mass spectrometry: the protein CopC-Bx1 (uniport code Q13UF7) was identified with a coverage of 49%, whereas the protein CopC-Bx2 (I2IK44) was identified with a coverage of 68%.

Kinetic characterization of Pp-CopA, Pf-CopA, CopC-bx1 and CopC-bx2

All expressed proteins were assayed for activity with the regular laccase substrates ABTS; SGZ; DCP; DMP and guaiacol. Pp-CopA and Pf-CopA were active with all substrates, although only in the presence of exogenous copper ions. Neither CopC-bx1 nor CopC-bx2 showed activity with any of these substrates, and addition of CopC proteins to Pp-CopA or Pf-CopA did not enhance their activity. For Pp-CopA and Pf-CopA, copper-reconstituted protein and enzyme expressed under microaerobic condition was found to be active in the absence of exogenous Cu^{2+} addition, however, with a reduced specific activity and with a lower yield of purified protein. Addition of hydrogen peroxide gave no enzyme activity, indicating that CopA enzymes show oxygen-dependent oxidase activity, and not peroxidase activity. Since both enzymes were active with SGZ, and required addition of exogenous copper ions, these proteins could be classified as pseudo-laccases [6, 20]. Steady state kinetics parameters were measured for Pp-CopA and Pf-CopA using ABTS, SGZ and DMP as

substrates (**Table 3**), and the two enzymes showed similar kinetic parameters, for example for ABTS, k_{cat} values of 2.4 s^{-1} for Pp-CopA and 2.2 s^{-1} for Pf-CopA were determined. Lower values of K_M for SGZ resulted in a higher catalytic efficiency of these enzymes towards SGZ.

The pH activity profile was determined for Pp-CopA and Pf-CopA using ABTS and SGZ



as substrates (

Figure 6, A). An optimal pH value of ≈4 with ABTS, and ≈7.5 with SGZ, were determined. Both enzymes showed optimal pH values of approximately 4.0 with ABTS, and 7.5 with SGZ. Pp-CopA stability was evaluated at 50 °C and compared with the commercially available fungal *Trametes versicolor* laccase (TvL). Following an initial decay in activity for both proteins, results shown that Pp-CopA was more thermostable, as it was able to retain

their residual activity for over 6 hours, in contrast to TvL (

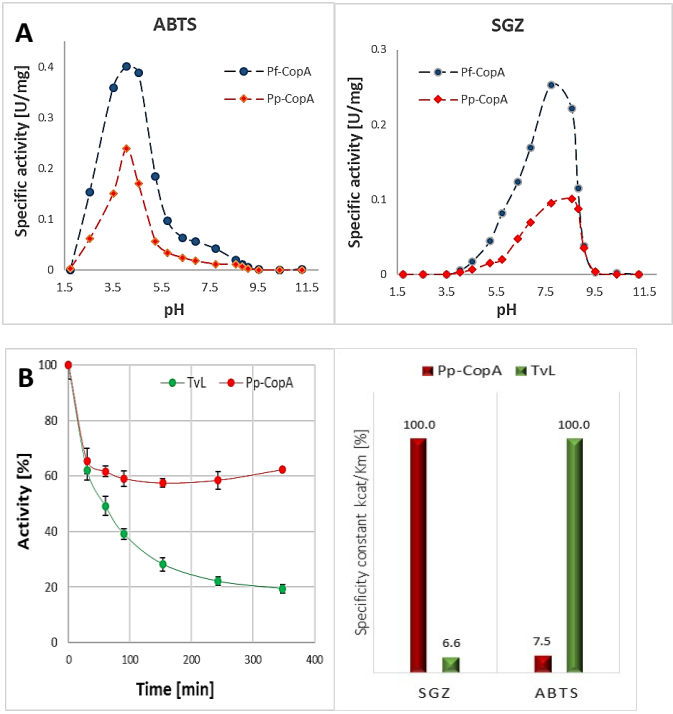
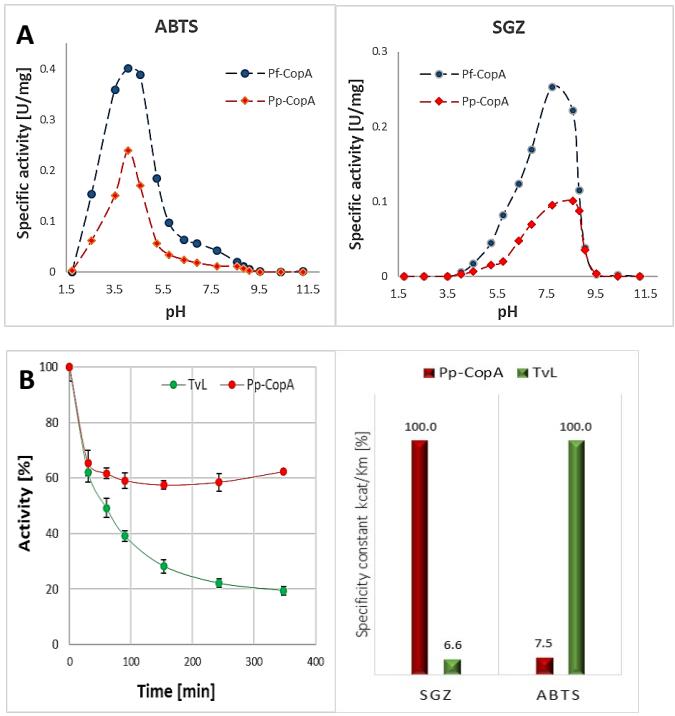


Figure 6, B). A comparison of the relative activity (percentage) between Pp-CopA and commercially available fungal *Trametes versicolor* laccase (TvL), was performed. The specific activity of Pp-CopA with ABTS was 13-fold less than TvL. Nevertheless, this relationship

inverted when SGZ was used as substrate, as the activity of Pp-CopA with SGZ showed to be

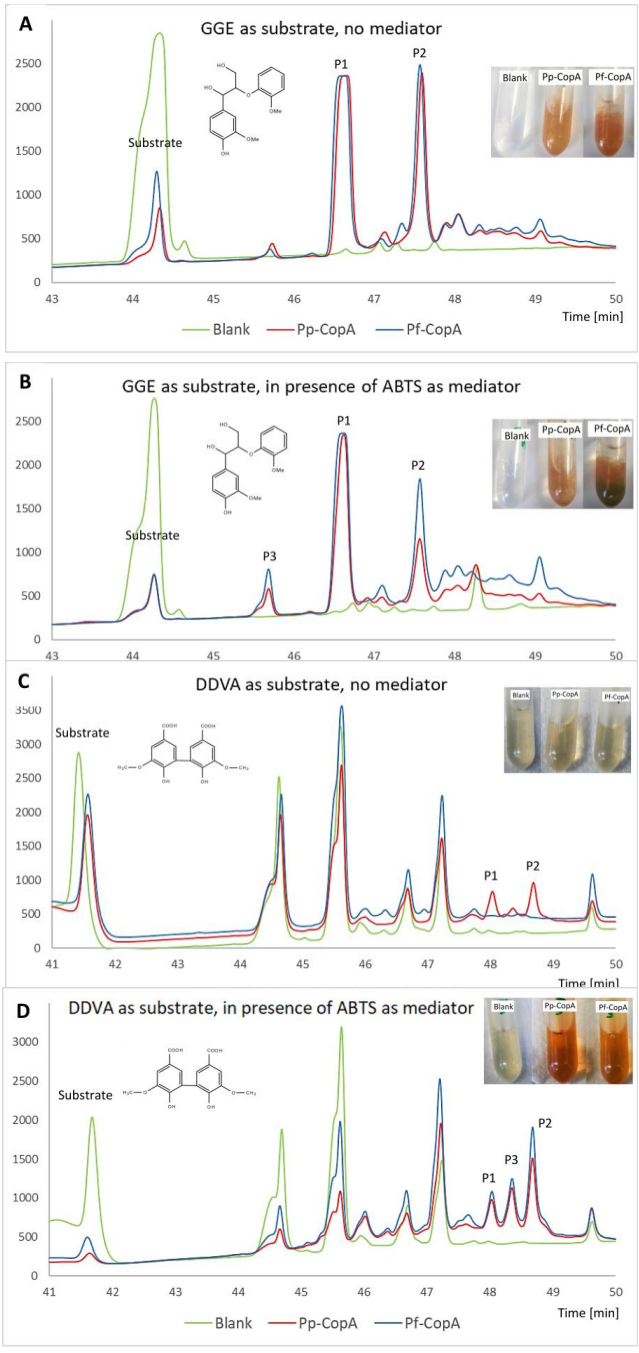


16-fold higher than TvL (

Figure 6, C), suggesting a potential of Pp-CopA for certain applications, as it might show a better activity for some specific substrates.

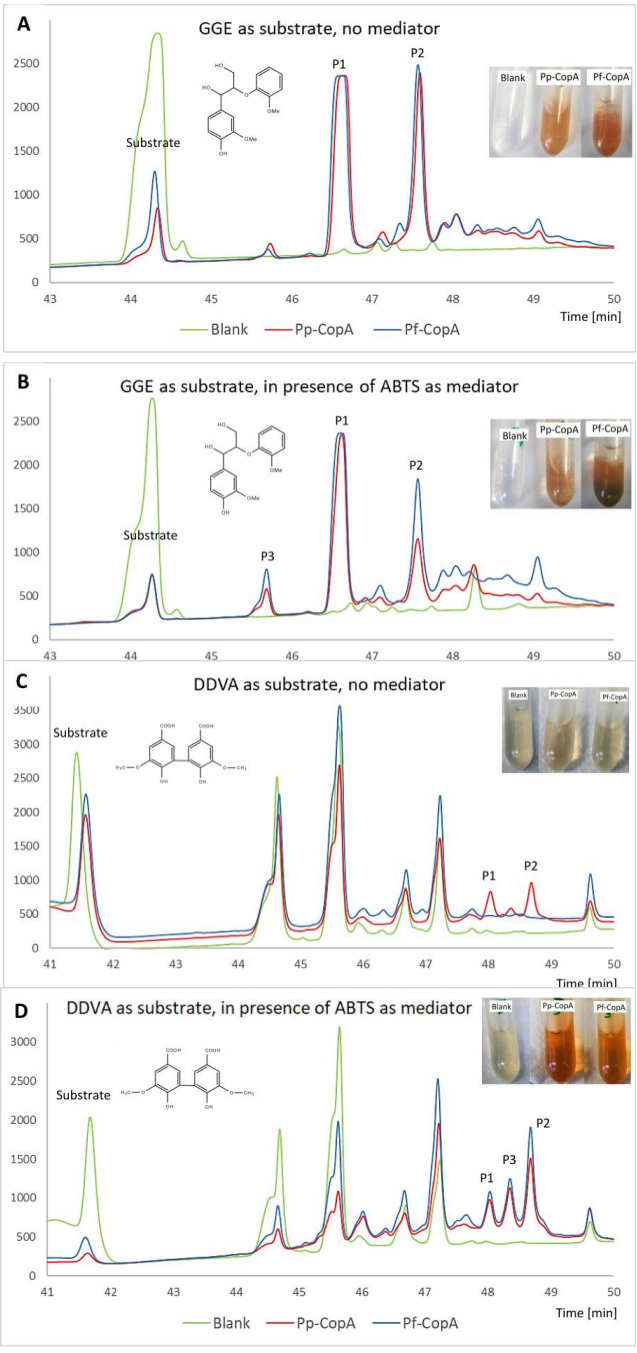
Activity of Pp-CopA and Pf-CopA with lignin model compounds (GGE and DDVA)

GGE and DDVA lignin model compounds were used as substrates to test the activity of Pp-CopA and Pf-CopA, in presence and in absence of 1 mM ABTS, or 25 µM SGZ, as mediators. Products were monitored by LC-MS. Both Pp-CopA and Pf-CopA showed activity towards GGE and DDVA lignin model compounds. Activity towards GGE was not dependant on the presence of mediators, however, slightly higher activity was observed when SGZ was used as mediator. Three new product peaks were observed by reverse phase HPLC analysis (

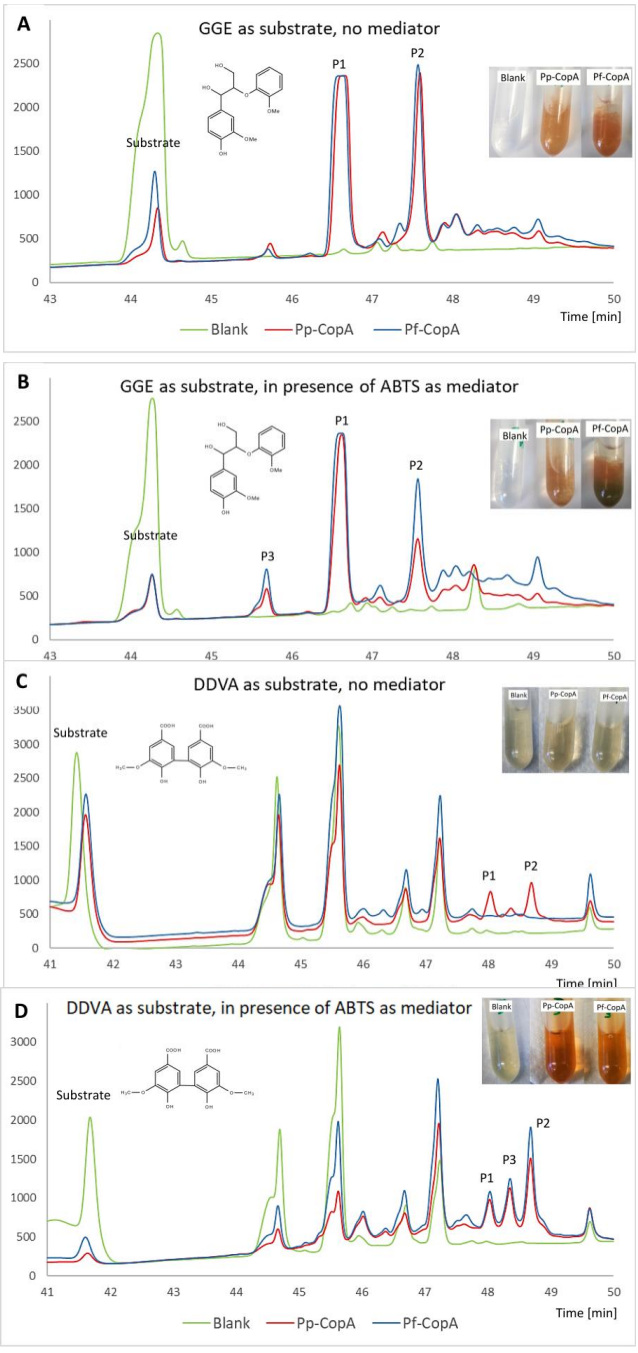


, A and B). The product profile and yields were slightly affected when a mediator was used: peak P2 was decreased, while P3 was increased in the presence of mediator.

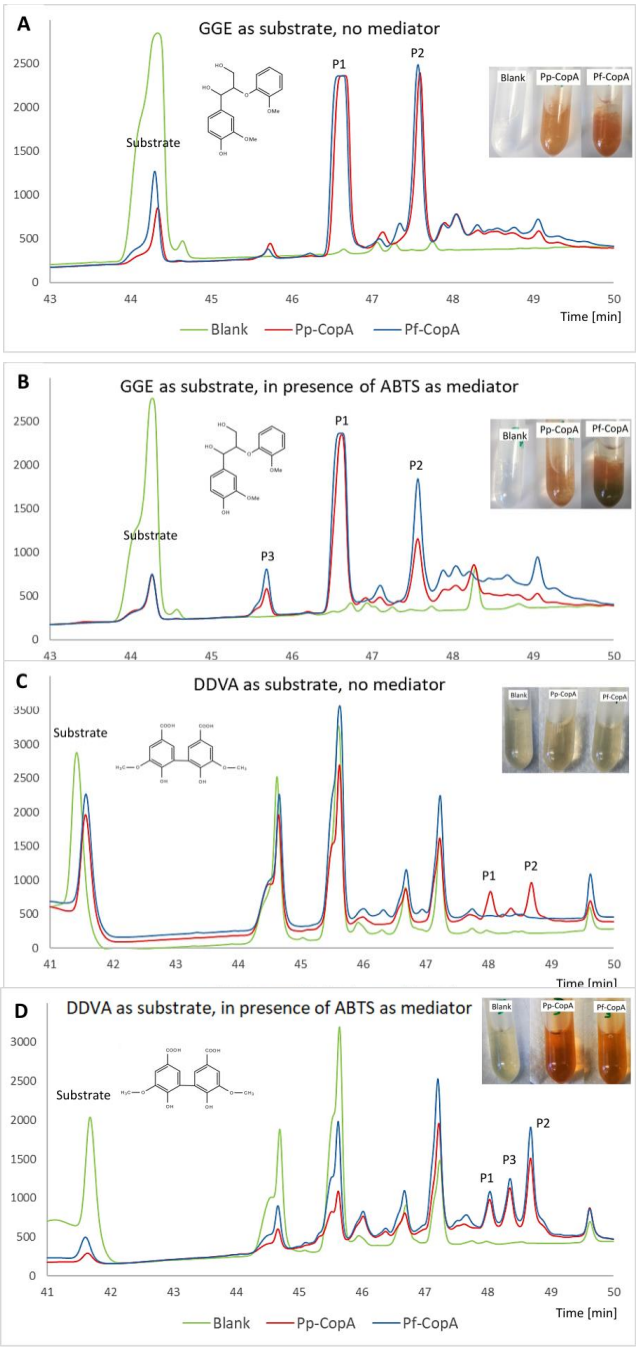
When DDVA was used as substrate, in absence of a mediator, Pp-CopA was able to form two new peaks, while Pf-CopA was unable to form any product (



, **C**). However, when either ABTS or SGZ was used as mediator, both Pp-CopA and Pf-CopA were able to catalyse the reaction, giving slightly increased peaks for P1 and P2, and also favouring the appearance of the third peak, P3 (



, **D**). In addition, substrate consumption was more evident in the presence of mediator. Incubation of Pp-CopA and Pf-CopA with GGE and DDVA gave a visible colour change in solution, as can be seen in



. Electrospray mass spectrometry was used to analyse peaks formation. For GGE, products P1, P2 and P3 showed higher molecular weight peaks with *m/z* 573.28, 695.32 and 479.22, compared with the substrate (343.28 Da for GGE). For DDVA, peaks P1, P2 and P3 also gave higher molecular weight peaks at *m/z* 621, 623 and 605, compared with the substrate (335 Da for DDVA). These results suggest that these proteins could catalyse an oxidative dimerization reaction, followed by additional modification reactions, which will be discussed further in the Discussion section.

Activity of Pp-CopA and Pf-CopA with polymeric lignin

A total of eight different lignin preparations were used to test the activity of Pp-CopA and Pf-CopA, in presence and absence of ABTS as mediator. Reactions were analysed by LC-MS. As was noted for lignin model compounds, incubation of Pp-CopA and Pf-CoPA with polymeric lignin substrates gave a visible colour change in solution, becoming somewhat darker, suggesting a possible oxidase activity of these enzymes towards polymeric lignin. Nevertheless, only when Ca-lignosulfonate was used as substrate, could a low molecular weight product be detected by HPLC analysis, at retention time 27 min. The new peak was produced more strongly by Pp-CopA, than by Pf-CopA (

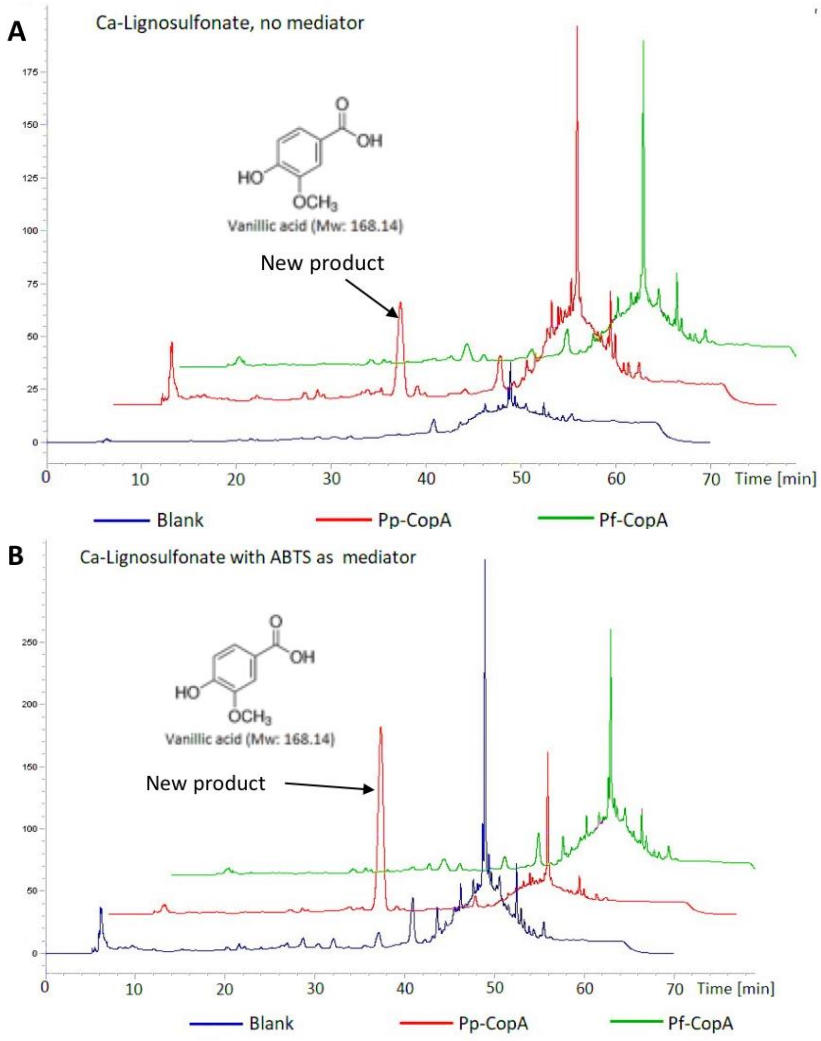
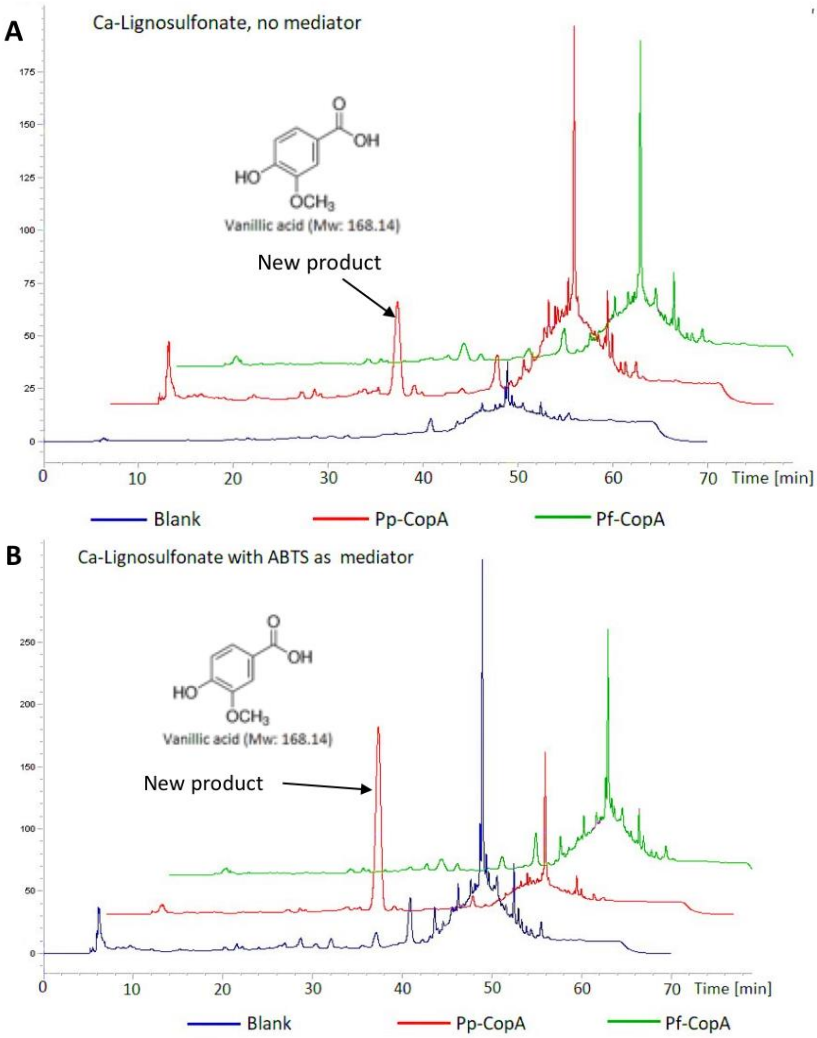


Figure 7, A). The addition of a mediator improved peak formation for Pp-CopA but had no



effect on Pf-CopA (

Figure 7, B). Electrospray mass spectrometry analysis suggested that the new peak contained the product vanillic acid (*m/z* 169 MH+, 191 MNa+)

Pseudomonas putida KT2440 copA-I and copA-II gene deletion

To evaluate the lignin degradation role of CopA proteins in *Pseudomonas putida*, deletion of individual genes, *copA-I*, *copA-II*, and a double deletion of both genes, were performed. A method for gene knockout in *Pseudomonas aeruginosa* [36] was used, with some variations. Briefly, upstream and downstream flanking regions of targeted genes were amplified by PCR from chromosomal DNA and assembled together into the pK18mobsacB suicide vector to produce the allelic exchange vectors for *copA-I* and *copA-II*. These vectors

were used to transform *P. putida* KT2440 competent cells and finally a sucrose counter-selection plates were used to select mutants that had deleted the gene. The double mutant strain (*ΔcopA-I-II)* was generated from an already confirmed *P. putida ΔcopA-II* mutant. Cells from this mutant were made competent, and the allelic exchange vector for *copA-I* was used for transformation. The PCR products verifying gene deletions are shown Supporting Information (Figure S3).

Effect on growth of Pseudomonas putida, after gene deletion of copA-I and copA-II

To evaluate the effect of *copA* genes in bacterial growth, four different conditions were used, with and without copper addition, since it has been reported that CopA proteins are inducible by copper [32]. The first group comprises growth on Luria Bertani media, supplemented with different copper concentrations, ranging from 0 mM to 4 mM of $CuSO_4$. The second group involved the utilization of Luria Bertani media supplemented with the well-known laccases substrates ABTS and SGZ. For the third group, minimal media was used with a single carbon source, consisting in a small aromatic molecule related with lignin degradation. For the fourth group, polymeric lignin was used in minimal media. In addition, for the second, third and fourth groups, the experiments were divided into two different subgroups, one in absence and another in presence of 0.5 mM $CuSO_4$.

Results are summarized in **Table 4** (photographs of plates are shown in Supporting Information Figure S4). No significant differences were found in bacterial growth for *ΔcopA-I* and *ΔcopA-II* single mutants, suggesting that these two genes encode proteins with similar functions, however, significant differences were observed with the double mutant *ΔcopA-I-II*. For the first group, copper concentrations used were 0, 0.5, 1, 1.5 and 4 mM $CuSO_4$. As expected, increasing copper concentrations had a negative effect on bacterial growth. Normal growth was observed for all mutants on Luria Bertani media without copper addition. When Luria Bertani media was supplemented with 0.5 mM $CuSO_4$, no *ΔcopA-I-II* colonies were found after 1 day of incubation, nonetheless after this delay, normal growth was visible for the following days. For copper concentrations of 1 and 1.5 mM, after 4 days of incubation, colonies were able to survive for all mutants and the wild type, although *ΔcopA-I-II* mutant presented a diminished growth. Finally, for 4 mM plates, after four days of incubation, colonies were able to growth only for the wild type and to a less extent for

ΔcopA-I, while *ΔcopA-II* and the double mutant *ΔcopA-I-II* showed no growth under these conditions. These results confirm the role of CopA proteins in copper resistance in *P. putida*.

For the second group, normal growth was observed on Luria Bertani media supplemented with ABTS and SGZ, although growth was diminished by about 50 % in presence of 0.5 mM $CuSO_4$, which was the only difference found. No effects were found upon deletion of *copA* genes. A greenish halo was formed around colonies growing on LB supplemented with ABTS, for mutant or wild type strains (Supporting Information Figure S3). No colour change was visible for SGZ plates.

Vanillin, vanillic acid, p-coumaric acid, p-hydroxybenzoic acid, ferulic acid and phenol were used as substrates for the third group, in presence and in absence of copper ions. *P. putida* KT2440 wild type and all *copA* deleted mutants were able to grow normally on these substrates, in the absence of copper ions. On the other hand, when $CuSO_4$ was added, no growth was visible for wild type, nor *copA* deleted mutants, when the same amount of inoculum was used. An increase of three orders of magnitude of the inoculum concentration was needed to promote growth on plates supplemented with $CuSO_4$ for these substrates. Under these conditions and after 4 days of incubation, a diminished growth was observed for the double deleted mutant, *ΔcopA-I-II*, growing on vanillic acid, p-coumaric acid, p-hydroxybenzoic acid and ferulic acid, as carbon source. No growth at all was observed for *ΔcopA-I-II* growing on vanillin as carbon source. No growth was observed for *P. putida* wild type nor *copA* mutants, on phenol as sole carbon source. Finally, no growth was achieved by either *P. putida* wild type, *ΔcopA-I*, *ΔcopA-II* nor *ΔcopA-I-II*, when polymeric lignins Ca-lignosulfonate or organosolv lignin (CIMV) was used as carbon source. However, *P. putida* was able to growth on polymeric lignin substrates when media was supplemented with glucose (0.1%), although no differences in growth were observed between the wild type strain and gene deleted mutants.

Conclusions

The function of CopA has been previously studied in *Pseudomonas syringae* in the context of copper resistance [31, 32]. In this organism, the *copA* gene is found as part of an operon that also includes the *copBCD* genes, and regulatory *copRS* genes. The proposed function of *P. syringae* CopA involves a methionine rich region in its amino acid sequence, which could bind several atoms of copper, preventing the entrance of toxic metal ions into

the cytoplasm, hence keeping toxic ions in the intermembrane space [31, 32]. On the other hand, a putative CopA has also been identified from a metagenomic fosmid library, using a biosensor detecting products of lignin degradation, suggesting a role of CopA in lignin degradation [25]. It is possible that these proteins can show more than one biological function, playing a role in both copper resistance and as catalyst in oxidative reactions. In multi-copper oxidases, it is thought that a one-electron Cu(II)/Cu(I) pair is used for substrate oxidation in the T1 copper site, through an outer sphere electron transfer mechanism, hence the substrate specificity of MCOs is defined by the nature of the substrate docking/oxidation site coupled with T1. Consequently, these proteins can catalyse a broad range of substrates and may have other cellular functions, therefore many of these enzymes have been called "moonlight proteins" [42, 43]. The amino acid sequence of CopA contains residues found in laccases that are ligands involved in 4 copper attachment, but also contains a methionine-rich region. The copper content of >8 atoms of copper per protein found in this and other work [31], and the lack of visible absorption, is different from laccase enzymes which contain 4 copper atoms per protein, implying a different mode of copper binding.

Purified recombinant Pp-CopA and Pf-CopA enzymes studied here normally required addition of exogenous copper to show oxidase activity. Nonetheless, if expressed under microaerobic conditions and after copper reconstitution, they were active without any addition of exogenous Cu^{2+} ions. Pp-CopA and Pf-CopA were active with common laccase substrates, lignin model compounds, and Ca-lignosulfonate polymeric lignin, consistent with a role in lignin degradation. The products obtained from oxidation of lignin model compounds GGE and DDVA suggest oxidative dimerization reactions. We suggest possible structures for two of these products in Supporting Information (Figure S2). Product P1 from GGE oxidation (*m/z* 573.28) corresponds to molecular formula $C_{33}H_{32}O_9$, which could be rationalised by oxidative dimerization, followed by loss of a formaldehyde fragment, and elimination of water. Products P1 and P2 from DDVA oxidation (*m/z* 621, 623) correspond to molecular formulae $C_{31}H_{26}O_{14}$ and $C_{31}H_{28}O_{14}$ which could be rationalised by oxidative dimerization, followed by decarboxylation (see Supporting Information Figure S2). Recombinant CopC-bx1 and CopC-bx2 showed no activity with laccase substrates, and no apparent synergistic effect with recombinant CopA, hence the CopC proteins appear not to be involved in phenolic oxidation.

Deletion of *copA-I* and *copA-II* in *Pseudomonas putida* has verified a role of CopA in copper resistance in this bacterium. The *copA-1/copA-II* double knockout showed a reduction in growth on aromatic compounds in the presence of copper ions, but not in the absence of Cu(II), indicating some effect on aromatic degradation in the presence of Cu(II). This behaviour could be explained either by the presence of copper ions causing stress on cells leading to a diminished growth, or repression of other oxidative pathways, or some biochemical role for CopA in the oxidation of small aromatic compounds. Although the *copA-1/copA-II* double knockout showed no phenotype for growth on lignin substrates, *P. putida* requires some additional carbon source in order to metabolise polymeric lignin, so it may be difficult in practice to observe a phenotypic effect on lignin metabolism, especially if multiple enzymes are used by *P. putida* to oxidise polymeric lignin. Our interpretation is that although there is no specific evidence for a primary role on lignin degradation from gene knockout studies, the biochemical studies herein suggest some accessory role in lignin oxidation by CopA in the presence of Cu(II) ions.

Acknowledgements

RSGT was supported by a PhD studentship funded by Secretaria de Educacion Superior, Ciencia, Tecnologia e Innovacion (SENESCYT), Ecuador. We thank Dr. James Williamson (University of Warwick) for assistance with genetic modification work, and Dr. Freddy Tjosås (Borregaard) for provision of samples of Ca-lignosulfonate.

References

[1] L. Ryden, L. Hunt, Evolution of protein complexity: The blue copper-containing oxidases and related proteins, Journal of Molecular Evolution 36(1) (1993) 41-66.

[2] A. Messerschmidt, Multi-copper oxidases, World Scientific1997.

[3] A. Messerschmidt, R. Huber, The blue oxidases, ascorbate oxidase, laccase and ceruloplasmin Modelling and structural relationships, European Journal of Biochemistry 187(2) (1990) 341-352.

[4] E.I. Solomon, U.M. Sundaram, T.E. Machonkin, Multicopper Oxidases and Oxygenases, Chemical Reviews 96(7) (1996) 2563-2606.

[5] A.C. Mot, R. Silaghi-Dumitrescu, Laccases: Complex architectures for one-electron oxidations, Biochemistry (Moscow) 77(12) (2012) 1395-1407.

[6] R. Reiss, J. Ihssen, M. Richter, E. Eichhorn, B. Schilling, L. Thöny-Meyer, Laccase versus laccase-like multi-copper oxidase: a comparative study of similar enzymes with diverse substrate spectra, PloS one 8(6) (2013) e65633.
[7] P. Giardina, V. Faraco, C. Pezzella, A. Piscitelli, S. Vanhulle, G. Sannia, Laccases: a never-ending story, Cellular and Molecular Life Sciences 67(3) (2010) 369-385.
[8] P.J. Hoegger, S. Kilaru, T.Y. James, J.R. Thacker, U. Kües, Phylogenetic comparison and classification of laccase and related multicopper oxidase protein sequences, FEBS Journal 273(10) (2006) 2308-2326.
[9] A. Leonowicz, N. Cho, J. Luterek, A. Wilkolazka, M. Wojtas-Wasilewska, A. Matuszewska, M. Hofrichter, D. Wesenberg, J. Rogalski, Fungal laccase: properties and activity on lignin, Journal of Basic Microbiology 41(3-4) (2001) 185-227.
[10] D.S. Arora, R.K. Sharma, Ligninolytic fungal laccases and their biotechnological applications, Applied Biochemistry and Biotechnology 160(6) (2010) 1760-1788.
[11] C.D. Boyle, B.R. Kropp, I.D. Reid, Solubilization and mineralization of lignin by white rot fungi, Applied and Environmental Microbiology 58(10) (1992) 3217-3224.
[12] S. Riva, Laccases: blue enzymes for green chemistry, TRENDS in Biotechnology 24(5) (2006) 219-226.
[13] J.M. Harkin, M.J. Larsen, J.R. Obst, Use of syringaldazine for detection of laccase in sporophores of wood rotting fungi, Mycologia (1974) 469-476.
[14] N. Pozdnyakova, O. Turkovskaya, E. Yudina, Y. Rodakiewicz-Nowak, Yellow laccase from the fungus Pleurotus ostreatus D1: purification and characterization, Applied Biochemistry and Microbiology 42(1) (2006) 56-61.
[15] A.A. Leontievsky, T. Vares, P. Lankinen, J.K. Shergill, N.N. Pozdnyakova, N.M. Myasoedova, N. Kalkkinen, L.A. Golovleva, R. Cammack, C.F. Thurston, Blue and yellow laccases of ligninolytic fungi, FEMS Microbiology Letters 156(1) (1997) 9-14.
[16] G. Palmieri, G. Cennamo, V. Faraco, A. Amoresano, G. Sannia, P. Giardina, Atypical laccase isoenzymes from copper supplemented Pleurotus ostreatus cultures, Enzyme and Microbial Technology 33(2-3) (2003) 220-230.
[17] J. Jordaan, B. Pletschke, W. Leukes, Purification and partial characterization of a thermostable laccase from an unidentified basidiomycete, Enzyme and Microbial Technology 34(7) (2004) 635-641.

[18] L.O. Martins, C.M. Soares, M.M. Pereira, M. Teixeira, T. Costa, G.H. Jones, A.O. Henriques, Molecular and Biochemical Characterization of a Highly Stable Bacterial Laccase That Occurs as a Structural Component of the Bacillus subtilis Endospore Coat, Journal of Biological Chemistry 277(21) (2002) 18849-18859.
[19] S.-i. Sakasegawa, H. Ishikawa, S. Imamura, H. Sakuraba, S. Goda, T. Ohshima, Bilirubin oxidase activity of Bacillus subtilis CotA, Applied and Environmental Microbiology 72(1) (2006) 972-975.
[20] F. Solano, P. Lucas-Elío, D. López-Serrano, E. Fernández, A. Sanchez-Amat, Dimethoxyphenol oxidase activity of different microbial blue multicopper proteins, FEMS Microbiology Letters 204(1) (2001) 175-181.
[21] N.L. Brown, S.R. Barrett, J. Camakaris, B.T.O. Lee, D.A. Rouch, Molecular genetics and transport analysis of the copper-resistance determinant (pco) from Escherichia coli plasmid pRJ1004, Molecular Microbiology 17(6) (1995) 1153-1166.
[22] G. Grass, C. Rensing, CueO Is a Multi-copper Oxidase That Confers Copper Tolerance in Escherichia coli, Biochemical and Biophysical Research Communications 286(5) (2001) 902-908.
[23] R.S. Granja-Travez, R.C. Wilkinson, G.F. Persinoti, F.M. Squina, V. Fülöp, T.D. Bugg, Structural and functional characterisation of multi-copper oxidase CueO from lignin-degrading bacterium Ochrobactrum sp. reveal its activity towards lignin model compounds and lignosulfonate, The FEBS journal 285(9) (2018) 1684-1700.
[24] M.-F. Hullo, I. Moszer, A. Danchin, I. Martin-Verstraete, CotA of Bacillus subtilis Is a Copper-Dependent Laccase, Journal of Bacteriology 183(18) (2001) 5426-5430.
[25] C.R. Strachan, R. Singh, D. VanInsberghe, K. Ievdokymenko, K. Budwill, W.W. Mohn, L.D. Eltis, S.J. Hallam, Metagenomic scaffolds enable combinatorial lignin transformation, Proceedings of the National Academy of Sciences 111(28) 10143-10148.
[26] Y.-M. Hsiao, Y.-F. Liu, P.-Y. Lee, P.-C. Hsu, S.-Y. Tseng, Y.-C. Pan, Functional characterization of copA gene encoding multicopper oxidase in Xanthomonas campestris pv. campestris, Journal of Agricultural and Food Chemistry 59(17) (2011) 9290-9302.
[27] D.L. Huffman, J. Huyett, F.W. Outten, P.E. Doan, L.A. Finney, B.M. Hoffman, T.V. O'Halloran, Spectroscopy of Cu (II)-PcoC and the multicopper oxidase function of PcoA, two essential components of Escherichia coli pco copper resistance operon, Biochemistry 41(31) (2002) 10046-10055.

[28] C. Pezzella, L. Guarino, A. Piscitelli, How to enjoy laccases, Cellular and Molecular Life Sciences 72(5) (2015) 923-940.
[29] L. Munk, A.K. Sitarz, D.C. Kalyani, J.D. Mikkelsen, A.S. Meyer, Can laccases catalyze bond cleavage in lignin?, Biotechnology Advances 33(1) (2015) 13-24.
[30] S. Majumdar, T. Lukk, J.O. Solbiati, S. Bauer, S.K. Nair, J.E. Cronan, J.A. Gerlt, Roles of small laccases from Streptomyces in lignin degradation, Biochemistry 53(24) (2014) 4047-4058.
[31] J.S. Cha, D.A. Cooksey, Copper resistance in *Pseudomonas syringae* mediated by periplasmic and outer membrane proteins, Proceedings of the National Academy of Sciences 88(20) (1991) 8915-8919.
[32] S.D. Mills, C.A. Jasalavich, D.A. Cooksey, A two-component regulatory system required for copper-inducible expression of the copper resistance operon of Pseudomonas syringae, Journal of Bacteriology 175(6) (1993) 1656-1664.
[33] C.R. Taylor, E.M. Hardiman, M. Ahmad, P.D. Sainsbury, P.R. Norris, T.D.H. Bugg, Isolation of bacterial strains able to metabolize lignin from screening of environmental samples, Journal of Applied Microbiology 113(3) (2012) 521-530.
[34] P. Durao, Z. Chen, A.T. Fernandes, P. Hildebrandt, D.H. Murgida, S. Todorovic, M.M. Pereira, E.P. Melo, L.O. Martins, Copper incorporation into recombinant CotA laccase from Bacillus subtilis: characterization of fully copper loaded enzymes, JBIC Journal of Biological Inorganic Chemistry 13(2) (2008) 183-193.
[35] H.T.S. Britton, R.A. Robinson, CXCVIII.—Universal buffer solutions and the dissociation constant of veronal, Journal of the Chemical Society (Resumed) (1931) 1456-1462.
[36] W. Huang, A. Wilks, A rapid seamless method for gene knockout in Pseudomonas aeruginosa, BMC Microbiology 17(1) (2017) 199.
[37] S. Franke, G. Grass, C. Rensing, D.H. Nies, Molecular analysis of the copper-transporting efflux system CusCFBA of Escherichia coli, Journal of Bacteriology 185(13) (2003) 3804-3812.
[38] F. Arnesano, L. Banci, I. Bertini, A.R. Thompsett, Solution Structure of CopC: a cupredoxin-like protein involved in copper homeostasis, Structure 10(10) (2002) 1337-1347.
[39] F. Arnesano, L. Banci, I. Bertini, S. Mangani, A.R. Thompsett, A redox switch in CopC: An intriguing copper trafficking protein that binds copper(I) and copper(II) at different sites, Proceedings of the National Academy of Sciences 100(7) (2003) 3814-3819.

[40] M. Ahmad, J.N. Roberts, E.M. Hardiman, R. Singh, L.D. Eltis, T.D.H. Bugg, Identification of DypB from Rhodococcus jostii RHA1 as a Lignin Peroxidase, Biochemistry 50(23) (2011) 5096-5107.
[41] R. Rahmanpour, L.D. King, T.D. Bugg, Identification of an extracellular bacterial flavoenzyme that can prevent re-polymerisation of lignin fragments, Biochemical and Biophysical Research Communications 482(1) (2017) 57-61.
[42] Z. Xiao, A.G. Wedd, Metallo-oxidase enzymes: Design of their active sites, Australian Journal of Chemistry 64(3) (2011) 231-238.
[43] P. Bielli, L. Calabrese, Structure to function relationships in ceruloplasmin: a 'moonlighting' protein, Cellular and Molecular Life Sciences CMLS 59(9) (2002) 1413-1427.

Table 1. List of primers used for PCR gene amplification of *copC* genes from *Microbacterium phyllosphaerae*.

Expression system	***E. coli* BL21**	
Vector	pET151	
Gene	*copC886*	*copC1032*
Forward primer (5' to 3')	caccgtgaaaaccacagctcgccgcc	caccgtgaaaaccacagctcgc
Reverse primer (5' to 3')	ctatcgctccgtgggggcgtcg	ctatcgctccgtgggggcgtc
Forward primer (5' to 3') Excluding signal peptide	cacccacgacagcctcctcgcgtc	cacccacgatcagctggtctcgag
Reverse primer (5' to 3') Excluding signal peptide	ctatcgctccgtgggggcg	tcacagatcgtctgtcgtcc
Expression system	***R. jostii* RHA1**	
Vector	pTipQC-II	
Gene	*copC886*	*copC1032*
Forward primer (5' to 3') Excluding signal peptide	taagcacatatgcacgacagcctcctcgcgtc	taagcacatatgcacgatcagctggtctcgagc
Reverse primer (5' to 3') Excluding signal peptide	tgcttaaagctttccctgaaaatacaggtttctcgctccgtgggggcgtcg	tgcttaaagctttccctgaaaatacaggttttccagatcgtctgtcgtccgagg

Table 2. List of primers used for amplification of upstream and downstream regions of genes targeted for deletion. Hangover and restriction sequence bases are shown in lower case

Gene	**Location**	**Primer type**	**Restriction enzyme sequence**	**Primers (5' to 3')**
copA-I	Upstream	Forward	EcoRI	taagcagaattcTGTCGCTGGACGAAATCGAGGAAA
		Reverse	BamHI	tgcttaggatccTGTCAGGTAGCTGAGGTGCACATT AC
	Downstream	Forward	BamHI	taagcaggatccTGCTGACCTTGCTCGCCAGTGAAC
		Reverse	HindIII	taagcaaagcttTTGCAGCAGCCGCAGTTGGGGTC
copA-II	Upstream	Forward	XbaI	taagcatctagaACGCCTGCCGTTATCGGTAGCAATG
		Reverse	SalI	tgcttagtcgacTGCCGTAGTGTCCAGTCAGTCAGATAA
	Downstream	Forward	SalI	taagcagtcgacGAGATCCAAGATGAGCAAACCAATGA
		Reverse	PstI	taagcactgcagGAACGCCCATACAGAAGCTGCAA

Table 3. Steady-state kinetic constants for Pp-CopA and Pf-CopA. Enzyme assays were carried out in either 50 mM sodium acetate buffer, pH 4.0 (for ABTS and DMP) or 50 mM Hepes buffer pH 7.0 (for SGZ), in the presence of 0.5 mM $CuSO_4$, at 20 ^oC. Specificity constant is shown with the corresponding standard deviations.

Enzyme	K_M [µM]	Vmax [U/mg prot.]	k_{cat} [s^{-1}]	Specificity constant k_{cat}/K_M [M^1 s^{-1}]
ABTS				
Pp-CopA	488	2.0	2.40	4900 ± 90
Pf-CopA	214	2.2	2.20	10300 ± 160
Syringaldazine (SGZ)				
Pp-CopA	26	1.1	1.28	49000 ± 2000
Pf-CopA	51	1.2	1.22	23800 ± 500
2,6-Dimethoxyphenol (DMP)				
Pp-CopA	1459	3.4	3.97	2700 ± 100
Pf-CopA	822	1.4	1.42	1700 ± 20

Table 4. Qualitative growth of deleted mutants ΔcopA-I, ΔcopA-II, ΔcopA-I-II and wild type *Pseudomonas putida* KT2440, on different media ('+' means growth, the more '+' signs, the more growth was achieved. '-'means no growth was achieved).

Group 1: Copper concentration on LB media						
Copper concentration		**0 mM**	**0.5 mM**	**1 mM**	**1.5 mM**	**4 mM**
Copper effect	WT	+++++	+++++	+++++	+++++	++++
	ΔcopA-I	+++++	+++++	++++	+++++	++
	ΔcopA-II	+++++	+++++	+++	+++	+
	ΔcopA-I-II	+++++	++++	++	+	-

Group 2: LB media supplemented with ABTS and SGZ							
		No copper added	**Copper added**			**No copper added**	**Copper added**
ABTS	WT	+++++	+++	**SGZ**	WT	+++++	+++++
	ΔcopA-I	+++++	+++		*ΔcopA-I*	+++++	+++++
	ΔcopA-II	+++++	+++		*ΔcopA-II*	+++++	+++++
	ΔcopA-I-II	+++++	+++		*ΔcopA-I-II*	+++++	++++

Group 3: M9 media with small aromatic compounds as sole carbon source							
		No copper added	**Copper added**			**No copper added**	**Copper added**
Vanillin	WT	+++	++	**Vanillic acid**	WT	+++++	+++++
	ΔcopA-I	+++	++		*ΔcopA-I*	+++++	++++
	ΔcopA-II	+++	++		*ΔcopA-II*	+++++	++++
	ΔcopA-I-II	+++	-		*ΔcopA-I-II*	+++++	+
p-coumaric acid	WT	+++++	+++++	**p-hydroxy-benzoic acid**	WT	+++++	+++++
	ΔcopA-I	+++++	++++		*ΔcopA-I*	+++++	++++
	ΔcopA-II	+++++	++++		*ΔcopA-II*	+++++	++++
	ΔcopA-I-II	+++++	++		*ΔcopA-I-II*	+++++	++
ferulic acid	WT	+++++	+++++	**phenol**	WT	-	-
	ΔcopA-I	+++++	++++		*ΔcopA-I*	-	-
	ΔcopA-II	+++++	++++		*ΔcopA-II*	-	-
	ΔcopA-I-II	+++++	++		*ΔcopA-I-II*	-	-

Group 4: M9 media with polymeric lignin as sole carbon source							
		No copper added	**Copper added**			No copper added	Copper added
Ca-lignosulfonate	WT	-	-	**Organosolv lignin (CIMV)**	WT	-	-
	ΔcopA-I	-	-		*ΔcopA-I*	-	-
	ΔcopA-II	-	-		*ΔcopA-II*	-	-
	ΔcopA-I-II	-	-		*ΔcopA-I-II*	-	-

Figure Legends.

Figure 1. CopA protein expression and copper content characterization. (A) SDS-Gel for the purified recombinant proteins Pp-CopA and Pf-CopA. (B) Purified proteins colour comparison of Pp-CopA, Pf-CopA and the blue bacterial laccase OcCueO [23]. UV–Visible absorption spectra of purified recombinant Pp-CopA (0·5 mg/mL).

Figure 2. Biochemical characterization of CopA. (A) Pp-CopA and Pf-CopA pH-activity profile with ABTS and SGZ. (B) Stability comparison between Pp-CopA and fungal laccase TvL, with ABTS as substrate; and specific activity comparison between Pp-CopA and TvL.

Figure 3. UV-Vis chromatograms showing products formation after reaction of lignin model compounds with Pp-CopA and Pf-CopA. In the right-upper side of each graphic, a mini-photo shows the actual reaction after incubation of each enzyme with the substrate. (A) GGE as substrate, in absence of mediator. (B) GGE as substrate, in presence of 1 mM ABTS as mediator. (C) DDVA as substrate, in absence of mediator. (D) DDVA as substrate, in presence of 1 mM ABTS as mediator.

Figure 4. UV-Vis chromatogram of Ca-Lignosulfonate reaction with Pp-CopA and Pf-CopA, and vanillic acid formation. (A) Reaction in absence of a mediator. (B) Reaction in presence of 1mM ABTS as mediator.

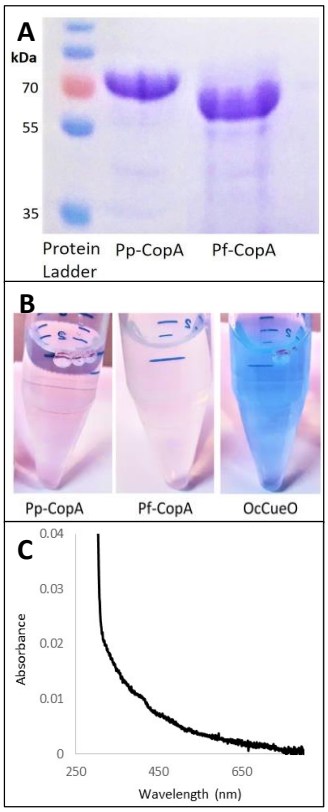


Figure 5. CopA protein expression and copper content characterization.

C

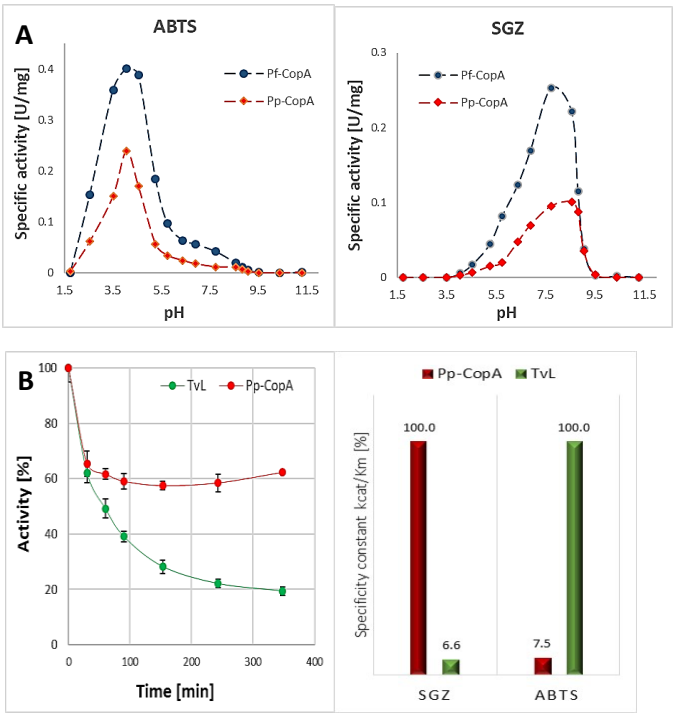


Figure 6. Biochemical characterization of CopA.

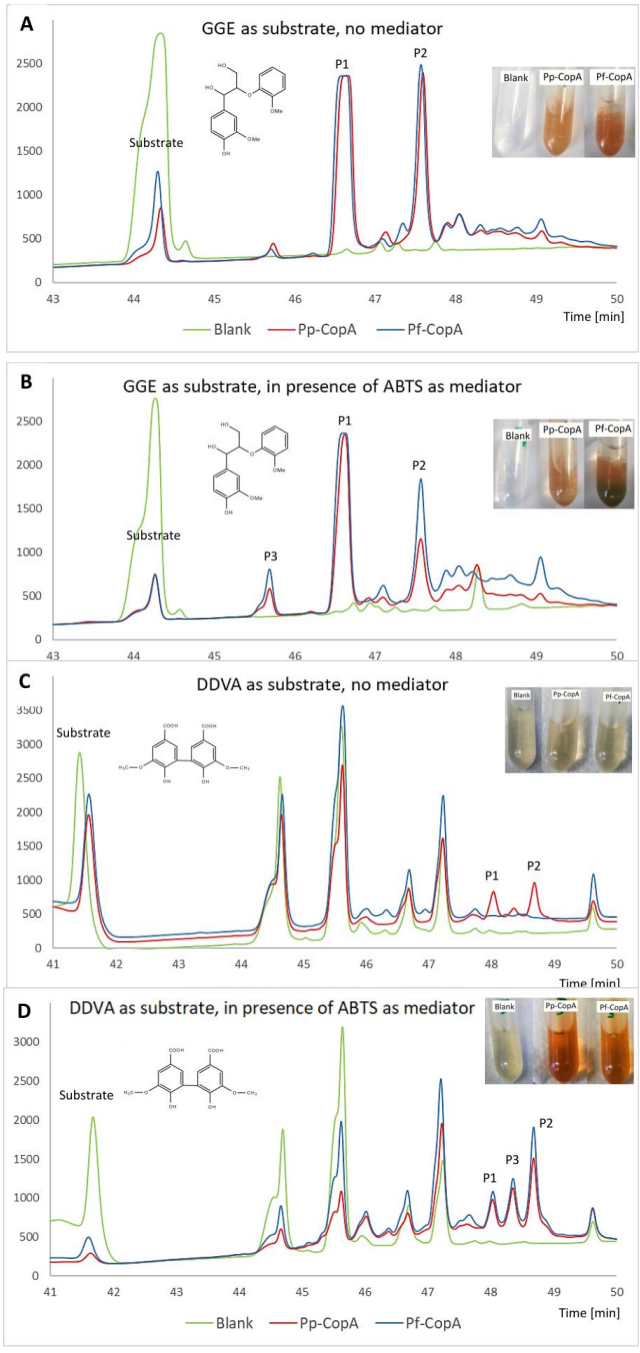


Figure 3. UV-Vis chromatograms showing products formation after reaction of lignin model compounds with Pp-CopA and Pf-CopA.

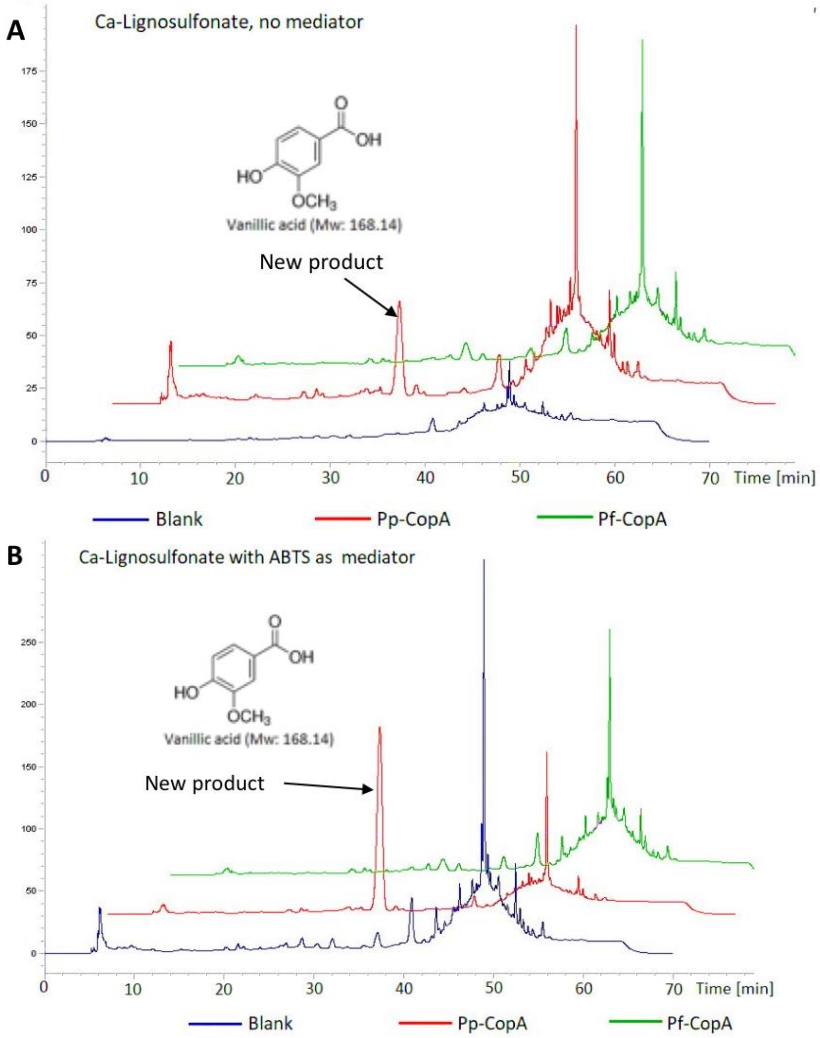


Figure 7. UV-Vis chromatogram of Ca-Lignosulfonate reaction with Pp-CopA and Pf-CopA, and vanillic acid formation.