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Identification and Characterization of a Novel Plasmid-Encoded Laccase-Like Multicopper Oxidase from *Ochrobactrum* sp. BF15 Isolated from an On-Farm Bio-Purification System

Running head: *Ochrobactrum* Laccase Identification

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SUMMARY

Research background. In recent decades, laccases (p-diphenol-dioxygen oxidoreductases; EC 1.10.3.2) have attracted the attention of researchers due to their wide range of biotechnological and industrial applications. Laccases can oxidize a variety of organic and inorganic compounds, making them suitable as biocatalysts in biotechnological processes. Even though the most traditionally used laccases in the industry are of fungal origin, bacterial laccases have shown an enormous potential given their ability to act on several substrates and in multiple conditions. The present study aims to characterize a plasmid-encoded laccase-like multicopper oxidase (LMCO) from *Ochrobactrum* sp. BF15, a bacterial strain previously isolated from polluted soil.

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Experimental approach. We used *in silico* profiles Hidden Markov Models to identify novel laccase-like genes in *Ochrobactrum* sp. BF15. For laccase characterization, we performed heterologous expression in *E. coli*, purification and activity measurement on typical laccase substrates.

Results and conclusions. Profiles Hidden Markov Models allowed us to identify a novel LMCO, named Lac80. *In silico* analysis of Lac80 revealed the presence of the three conserved copper-oxidase domains characteristic of three-domain laccases. We successfully expressed Lac80 heterologously in *Escherichia coli*, allowing us to purify the protein for further activity evaluation. Of thirteen typical laccase substrates tested, Lac80 showed discrete activity on 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), pyrocatechol, pyrogallol, and vanillic acid, and higher activity on 2,6-dimethoxyphenol.

Novelty and scientific contribution. Our results point out Lac80 as a promising laccase for use in industrial applications. The present work shows the relevance of bacterial laccases and highlights the importance of environmental plasmids as valuable sources of new genes encoding enzymes with potential use in biotechnological processes.

Key words: laccase-like multicopper oxidases, *Ochrobactrum,* biopurification system, plasmid, biodegradation, heterologous expression

INTRODUCTION

Enzymes traditionally known as laccases are multicopper oxidases that catalyse the oxidation of a wide range of substrates and the simultaneous reduction of molecular oxygen to water. Due to their ability to oxidize a wide variety of phenolic and non-phenolic compounds, they have been extensively used in biotechnological processes as biocatalysts (reviewed in (*1,2*). They were recently renamed “laccase-like multicopper oxidases” (LMCOs), following the nomenclature revision of this enormously diverse group of enzymes (*3*). LMCOs are produced by a wide range of organisms such as bacteria, higher plants, insects, and fungi, the latter being those more broadly studied and characterized, in part due to the development of efficient expression systems in yeasts that have potentiated their industrial use, particularly in the textile and food industry (*4-7*). However, much attention has been given more recently to prokaryote-sourced LMCOs. Bacterial laccases usually display higher thermal and alkaline pH stability in comparison to their eukaryotic counterparts and are active on a broad range of substrates, unlike most fungal laccases (*3,8*) making them suitable for different industrial processes. A computational study analyzing over 2,000 bacterial genomes predicted the presence of laccase-like encoding genes in 36 % of the screened organisms (*9*) including autotrophic, alkalophilic, and even anaerobic bacteria (*10*),

highlighting the extraordinary potential of bacteria as a source of novel LMCOs and pointing out the need to continue exploring the genome of these microorganisms.

Multiple approaches have been applied for the identification of novel LMCOs in bacteria, including classical functional- and/or sequence-based screenings of culturable microbiota (*11*), or metagenomic analyses (*10,12*). In addition, the generation of chimeric laccases using bioinformatic approaches has also generated new hybrid enzymes with promising activity (*13*)]. Interestingly, the use of bacterial consortia has been proved to be a good alternative for screening of multicopper oxidase activity (*14,15*).

Despite the increasing interest in bacterial LMCOs, little attention has been paid to plasmid-encoded laccases. We previously published a bioinformatic analysis in which we analyzed the presence of laccase-encoding genes in a variety of bacterial genomes from different species (*9*). We found that, of the 749 genes identified in finished genomes, 10 % were encoded on plasmids; 68 % of these genes codify three domain laccases and 32 % two domain laccases. These findings highlight the importance of plasmids – and other mobile genetic elements (MGE) – as reservoirs of laccases and other enzymes.

In an attempt to identify novel laccases for potential use in industry or bioremediation, we screened a plasmid metagenome obtained from over 50 plasmids purified from bacterial strains carrying high-molecular weight plasmids. This bacterial collection consists of 35 organisms that include both Gram-negative and Gram-positive bacteria belonging to 14 genera, all of them isolated from a biopurification system (BPS) used for the remediation of pesticide-contaminated waters in Kortrijk, Belgium (*16*). Exposure of these indigenous bacteria to mixtures of pollutants have fostered their adaptation responses via horizontally acquired MGE. Of the MGE, plasmids are the most abundant and promiscuous ones, representing the main vehicles for horizontal gene transfer via conjugation in bacterial communities in polluted environments (*17,18*).

Considering the relatively great abundance of laccase-encoded genes in plasmids predicted *in silico* (*9*), we hypothesized that the plasmids from the 35 strains are a possible resource of laccases. To identify novel LMCOs, we computationally screened this plasmid data set. We were able to identify one full-length gene for a putatively novel LMCO, herein named *lac80*. The heterologous expression of the codon-optimized version of *lac80* in *Escherichia coli* followed by His-Tag purification allowed us to assess its activity on different substrates, exploring its potential use in bioprocesses.

MATERIALS AND METHODS

Strains and vectors used in this study

Bacterial isolates used in this study were previously obtained from a BPS used for pesticide removal from contaminated waters located in Kortrijk, Belgium, operational since 2008 (*16*). All strains were routinely grown on LB (Luria-Bertani) agar plates or in liquid LB medium at 37 °C and 200 rpm. The BPS composition, analysis of pesticide types and concentrations (*19*), high molecular weight (HMW) plasmid DNA purification, high-throughput sequencing, and computational analysis were previously reported (*20*). *E. coli* DH5α (Promega, Madison, USA) and *E. coli* BL21 Star™ (DE3) (Invitrogen Life Technologies, Carlsbad, USA) strains were employed for *lac80* cloning and heterologous expression, respectively. They were routinely cultured in LB medium Sigma–Aldrich (St Louis, USA) supplemented when appropriate with 100 μg/mL of ampicillin Sigma–Aldrich (St Louis, USA). pUC59 (Genscript, Piscataway, USA) and pET22b(+) (Novagen, Darmstadt, Germany) plasmids were used for *lac80* cloning and expression, respectively. The latter plasmid enabled us to introduce a hexahistidine tag (His_6-Tag) at the C-terminus of the protein. Molecular biology techniques were performed using the standard protocols (*21*).

All the reagents were purchased from Sigma–Aldrich (St Louis, USA) unless otherwise stated.

Screening and lac80 sequence analysis

LMCO-encoding *lac80* gene was identified *in silico* in the present study from the plasmid dataset reported in (*20*). To retrieve novel LMCOs, profiles Hidden Markov Models (pHMMs) were used as previously described [9]. The protein module structure was analyzed using the Simple Modular Architecture Research Tool (SMART) (*22*). The presence and location of the signal peptide in *lac80* was checked using the Neural Networks and Hidden Markov Models trained on Gram-negative and Gram-positive bacteria with SignalP-5.0 (*23*). Multiple sequence alignment of *lac80* with related LMCO sequences was performed using ClustalΩ (*24*). Laccase gene was also blasted to the Laccase Engineering Database (*25*). Phylogenetic relationship was inferred by using the maximum likelihood phylogenetic method. The tree was constructed using MEGAX (*26*) using a multiple sequence alignment by MUSCLE of Lac80 sequence with a selection of previously characterized and some uncharacterized bacterial LMCOs, and 100 bootstrapping replications were used as a test of phylogeny.

A molecular PCR-based method was used to screen the bacterial collection for retrieving this novel LMCO-encoding gene. The strain harboring *lac80* was identified by using two sets of primers (set 1: 5'-CCACCGTCTGGGGTCTTG-3' and 5'-GTCGATGCGCCGTATTTC-3', amplicon size 549 bp; set 2:

5'-TCACCGGGCGATGCTGGC-3' and 5'-GAGGAGGTGATGGCCGAGATC-3', amplicon size 733 bp). PCR was performed as follows: an initial denaturation step at 94 °C for 4 min, followed by 35 cycles of denaturation at 94 °C for 20 s, annealing at 54 °C for 30 s and extension at 72 °C for 50 s. Then, a final extension at 72 °C for 2 min was used. The sequence of *lac80* was submitted to GenBank under the accession number MT130716.

Genetic localization of lac80

In situ lysis gel electrophoresis was performed to obtain further information on the genomic location of *lac80* by analyzing the DNA of the bands corresponding to plasmid(s) or chromosome, as previously described (*16*). The visualized bands were purified using the AccuPrep Gel Purification Kit (Bioneer, Daejeon, Korea). Then, a semi nested PCR for *lac80* was run to reduce nonspecific amplification of DNA template. Purified samples and primers Lac80F1 (5`-CCACCGTCTGGGGTCTTG-3`) and Lac80R2 (5`-TCACCGGGCGATGCTGGC-3`) were used in the first PCR and Lac80F1 and Lac80R1 (5`-GTCGATGCGCCGTATTTC-3`) in the second PCR run. To exclude the amplification of contaminating DNA, several controls were included in the PCR with DNA recovered from randomly selected positions of the agarose gel.

Codon optimization

The codon optimization strategy employed in the present study is referred to as 'one amino acid-one codon'. In this method, the most preferred codon of the *E. coli* expression system for a given amino acid is utilized in the target sequence (*27*). The sequence of *lac80* was obtained from the data set. The optimizer web server (*28*) was used for rare codon detection. The *E. coli* rare codon analyzer2 (http://www.faculty.ucr.edu /~mmaduro/codonusage/usage.htm) (*28*) was utilized for gene sequence optimization. GenScript web server (http://www.genscript.com) was applied to analyze the designed sequence Codon Adaptation Index (CAI).

Cloning and heterologous expression of Lac80 in E. coli

The codon-optimized sequence of *lac80* with the native signal peptide removed was synthesized by GenScript (Leiden, The Netherlands) and cloned into pUC59 plasmid (Leiden, The Netherlands) for DNA amplification. For protein expression, *lac80* was cloned from pUC59 into the expression vector pET22b(+) (Novagen Inc, Madison, USA), and introduced into *E. coli* BL21 Star™ (DE3) cells (Thermo Fisher Scientific, Waltham, USA). Multiple conditions were then evaluated for optimizing Lac80

expression. Briefly, 2 L unbaffled Erlenmeyer flasks, containing 750 mL of LB medium (Merck KGaA, Darmstadt, Germany) or richer Terrific Broth (TB, 12 g tryptone/L, 24 g yeast extract/L, 9.4 g K_2HPO_4/L, 2.2 g KH_2PO_4/L, 8 g glycerol/L) (components from Merck KGaA, Darmstadt, Germany) supplemented with 100 µg ampicillin/mL (Merck KGaA, Darmstadt, Germany) were inoculated with the starter culture ($A_{600\ nm}$ ~ 0.1), then incubated at 37 °C and 200 rpm (revolutions per minute). When the early exponential phase of growth was reached (corresponding to an $A_{600\ nm}$ of approx. 0.6 for LB medium and of approx. 1 for TB medium), different concentrations of isopropyl-β-D-thiogalactopyranoside (IPTG, from 0.4 to 1 mM) (Merck KGaA, Darmstadt, Germany) were tested to induce protein expression. At the same time none or 0.25 mM $CuSO_4$ (Merck KGaA, Darmstadt, Germany) was added to facilitate metal incorporation into the enzyme active site. Cells were then incubated at 37 °C or 20 °C and with different agitation regimes (200 rpm, 100 rpm, or no shaking), and harvested at 0 h, 2 h, 4 h, or overnight (O.N.) after induction, by centrifuging at 7500 x *g* for 10 minutes (Thermo Scientific Langenselbold, Germany). Multiple combinations of incubation conditions were also evaluated, such as incubation for 4 h after induction at 37 °C or at 20 °C and 200 rpm, followed by O.N. incubation at the same temperature but without shaking. Total proteins in the cell-free fermentation broths were concentrated by 10 % v/v trichloroacetic acid precipitation. Cell pellets were instead resuspended in 20 mM phosphate buffer pH=6.7 containing 10 µg deoxyribonuclease I (DNaseI)/mL (Merck KGaA, Darmstadt, Germany), 0.19 mg phenylmethylsulfonylfluoride (PMSF)/mL, and 0.7 µg pepstatin/mL (Merck KGaA, Darmstadt, Germany). Then cells were disrupted by sonication for 6 cycles of 30 seconds each on ice, followed by centrifugation at 34,000 x *g* for 60 minutes at 4 °C (Beckman Coulter Inc., California, USA). This enabled us to recover soluble (cytoplasmic) and insoluble (inclusion bodies) fractions, which were then analyzed through (i) sodium dodecyl sulfate-polyacrylamide (12 % *m/V*) gel electrophoresis (SDS-PAGE) and Coomassie Brilliant Blue staining, (ii) western blot with anti-His-Tag antibody HRP conjugate (Novagen Inc., Madison, USA) and detection by chemiluminescence (ECL Western Blotting Detection System, GE Healthcare Sciences, Little Chalfont, UK), and (iii) routine laccase activity assay with 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) (Merck KGaA, Darmstadt, Germany) as substrate (see below). Total protein concentration in the fractions was estimated by the Biuret assay.

Lac80 purification

Lac80 was purified starting from recombinant *E. coli* cells grown in the conditions allowing the highest soluble protein production. Hence, glycerol stocks of *E. coli* BL21 Star™ (DE3)/pET22(b)::*lac80* were inoculated into 80 mL of LB medium + 100 µg ampicillin/mL, grown O.N. at 37 °C and 200 rpm. This

pre-culture was then used to inoculate (at $A_{600\ nm}$ ~ 0.1) 750 mL of selective LB medium. Flasks were incubated as before until an A_{600nm} of 0.6 was reached. After induction of protein expression with 0.4 mM IPTG and addition of 0.25 mM $CuSO_4$, incubation was prolonged at 20 °C and 100 rpm O.N. Cells were disrupted as reported above and the soluble, cytoplasmic fraction recovered by centrifugation at 34,000 x *g* for 60 minutes at 4 °C (Beckman Coulter Inc., California, USA). C-terminus His_6-tagged Lac80 was purified by affinity chromatography on a 5-mL Ni^{2+}-HiTrap chelating affinity column (GE Healthcare Sciences, Little Chalfont, UK) equilibrated with 20 mM phosphate buffer pH=6.7, 20 mM imidazole, and 300 mM Na_2SO_4 (Merck KGaA, Darmstadt, Germany). The recombinant protein was eluted with 20 mM phosphate buffer pH=6.7, 250 mM imidazole, and 300 mM Na_2SO_4, then loaded onto a size-exclusion PD10 Sephadex G25 column (GE Healthcare Sciences, Little Chalfont, UK) equilibrated with 20 mM phosphate buffer pH=6.7. Lac80 was further concentrated with 30 K Amicon Ultra-2 centrifugal filter devices (Merck KGaA, Darmstadt, Germany).

Protein purity was checked by 10 % *m/V* SDS-PAGE. For molecular mass determination, molecular mass markers (GE Healthcare Sciences, Little Chalfont, UK) were used. Protein concentration was estimated by densitometric analysis of SDS-PAGE bands using Quantity One analysis software (Bio Rad, Hercules, USA) and spectrophotometrically using Lac80 theoretical extinction coefficient at 280 nm calculated based on the amino acid sequence (ε_{280nm} = 58.44 $mM^{-1}cm^{-1}$).

Enzymatic activity of Lac80

Laccase activity was routinely measured at 25 °C in 1 mL reaction mixture containing 50 mM sodium acetate pH=5.0, 5 mM ABTS (ε_{420nm} = 36 $mM^{-1}cm^{-1}$), and 100 µL of protein sample. Oxidation of the substrate was followed by a spectrophotometer (V460, Jasco, Easton, USA) as the change in Absorbance at 420 nm for 5 min. All assays were conducted in triplicate and negative controls without enzyme were run in parallel. One activity unit (U) was defined as the amount of Lac80 required for oxidizing 1 µmol of ABTS per minute. The ability of Lac80 to oxidize various phenolic and non-phenolic compounds was checked by the following additional substrates at designed concentrations and wavelengths: 100 mM 2, 6-dimethoxyphenol (DMP) (ε_{468nm} = 49.6 $mM^{-1}cm^{-1}$), 100 mM pyrocatechol (ε_{450nm} = 2.21 $mM^{-1}cm^{-1}$), 100 mM pyrogallol (ε_{450nm} = 4.4 $mM^{-1}cm^{-1}$), 8 mM vanillic acid (ε_{316nm} = 2.34 $mM^{-1}cm^{-1}$), 10 mM $K_4Fe(CN)_6$ (ε_{405nm} = 0.9 $mM^{-1}cm^{-1}$), 2 mM tyrosine (ε_{475nm} = 3.6 $mM^{-1}cm^{-1}$), 5 mM L-3,4-dihydroxyphenylalanine (L-DOPA) (ε_{475nm} = 3.7 $mM^{-1}cm^{-1}$), 100 mM guaiacol (ε_{468nm} = 12 $mM^{-1}cm^{-1}$), 10 mM syringic acid (ε_{300nm} = 8.5 $mM^{-1}cm^{-1}$), 4 mM ferulic acid (ε_{287nm} = 12.4 $mM^{-1}cm^{-1}$), 1 mM syringaldazine (ε_{525nm} = 65 $mM^{-1}cm^{-1}$), 1 mM syringaldehyde (ε_{320nm} = 8.5 $mM^{-1}cm^{-1}$). The optimal pH for activity on

different substrates was evaluated at 25 °C in multi-component buffer (10 mM Trizma base, 15 mM sodium carbonate, 15 mM phosphoric acid, 250 mM potassium chloride (*29*), in the pH range 2.0–8.0. All substrates and buffer components were purchased from Merck KGaA (Darmstadt, Germany).

RESULTS AND DISCUSSION

Lac80 is a plasmid-encoded three-domain laccase

Polluted sites are hot spots of plasmids potentially carrying catabolic genes (*30*). To identify novel LMCO-encoding genes in the plasmid dataset from the 35 strains previously isolated from a biopurification system (*20*), a profile Hidden Markov models-based search was performed. One candidate gene, named *lac80*, was retrieved using this approach. This gene showed 89 % identity at nucleotide level with a yet uncharacterized multicopper oxidase from *Ochrobactrum anthropi* strain OAB (Accession number: CP008819). Lac80 is composed of 502 amino acids, with a predicted signal peptide of 20 amino acids at the N-terminal (Fig. 1a). Predicted isoelectric point and molecular mass of this protein, excluding the signal peptide, were 5.43 and 51.2 kDa, respectively. As reported in Table S1, full-length Lac80 protein showed the highest amino acid sequence identity with two putative multicopper oxidases, one from Alphaproteobacteria (WP_024899901.1, 100 % sequence identity) and the other from *Ochrobactrum rhizosphaerae* (WP_094574672.1, 99.8 %). In addition, Lac80 showed 30 % sequence identity with the Lac15 from a marine microbial metagenome (PDB: 4F7K_A) and 29.5 % identity with a laccase from *Pseudomonas thermotolerans* (PDB: 6VOW). These two latter proteins were crystallized and characterized as functional LMCOs. The phylogenetic tree shown in Fig. 1b, confirms the novelty of our enzyme since Lac80 clusters most closely with uncharacterized LMCOs from *Paracoccus* sp., *Ochrobactrum* sp., and *Rhizobiales*. These findings were also confirmed by the best protein matches identified by the Laccase Engineering Database (*25*) (LccED, https://lcced.biocatnet.de/) by the BLASTP algorithm for laccase-like protein.

In silico sequence analysis of Lac80 identified the three conserved copper-oxidase domains characteristic of laccases, with Pfam database accession numbers of PF07732, PF00394, and PF07731 (Fig. 1a). Multiple sequence alignment with bacterial laccases or multicopper oxidases sequences reported in Fig. S1, revealed that Lac80 contains metal-binding amino acid residues that are conserved in bacterial laccases.

To identify the strain containing *lac80*, a PCR-based screening (see Materials and Methods) was performed using gDNA from the 35 strains (*31*). Of all the strains tested, only one yielded PCR positive results. Identification of the PCR product was corroborated by sequencing. The strain carrying the

plasmid-encoded *lac80* gene was identified as *Ochrobactrum* sp. BF15. To determine the genomic localization of *lac80*, an additional PCR screening was performed using the DNA bands cut from the *in situ* lysis gel electrophoresis (Fig. 2a and Fig 2b). Positive amplification from plasmid bands (Fig. 2c) and further sequencing confirmed that *lac80* is encoded in a plasmid.

Although laccases have been already found in *Ochrobactrum* spp. (*32,33*), this is the first report of a plasmid-encoded LMCO in this genus. *Ochrobactrum* species are aerobic, non-fermenting, Gram-negative bacilli found in a variety of environmental sources such as water, soil, plants, and animals. Genome sequencing of *Ochrobactrum* strains has shown the presence of multiple circular chromosomes and large plasmids (*34,38*). In addition, plasmid conjugation machinery has been identified in *Ochrobactrum* plasmids (*20,34*), and horizontal gene transfer by conjugation was demonstrated in this genus (*39,40*). *Ochrobactrum* species was previously found to degrade a wide spectrum of recalcitrant and xenobiotic compounds, such as organophosphates (*41*), tetrachloroethene (*42*), para-dichlorophenol chloroethane (DDT (*43*), 2,4-D, endosulfan (*44*), and aniline (*45*), among many others. Interestingly, ligninolytic activity was also reported from *Ochrobactrum* species (*32,46*). However, this is the first report of a plasmid-encoded functional laccase in the *Ochrobactrum* genus. Granja-Travez and collaborators (*32*) reported the purification and characterization of a laccase, named OcCueO, from *Ochrobactrum* sp. Although this laccase has the typical three-domain multicopper oxidase structure and displays activity against different substrates, alignment of OcCueO with Lac80 showed a low percentage of identity at the amino acid level (25.75 %) (Fig. S2). This is not surprising, considering that OcCueO appears to be encoded in the chromosome, whereas lac80 is located in a plasmid, indicating a possible acquisition via horizontal gene transfer and consequently different evolution pathways.

Heterologous expression and purification of Lac80

The putative LMCO gene was synthesized optimizing its codon usage for *E. coli* expression and removing the signal peptide sequence. The synthetic gene was then cloned into the pET22b(+) plasmid, and expressed as a His_6-tagged protein in *E. coli* BL21 Star™ (DE3). Different expression conditions were explored, as detailed in Materials and Methods. Recombinant cells were grown in two dissimilarly composed cultivation media; increasing concentrations of IPTG were tested for inducing Lac80 expression; incubation at diverse temperatures and shaking regimes followed IPTG addition; with or without addition of $CuSO_4$. In most of the above conditions, SDS-PAGE and western blot analyses revealed that His_6-Lac80 (molecular mass of 52.5 kDa) accumulated into inclusion bodies (IBs) in an insoluble form. The recombinant protein was never detected in the extracellular broth, indicating that it

could not be secreted. Laccase activity assays, using ABTS as substrate proved that Lac80 accumulated into IBs was completely inactive, as generally expected for recombinant proteins packed into IBs in *E. coli* (*12,47*). This is not surprising, as other LMCOs expressed in *E. coli* were described to accumulate as biologically inactive into IBs. As previously reported, the addition of copper under microaerobic conditions was used to facilitate proper protein folding, a condition that facilitates copper incorporation into the LMCO active sites (*48*). Thus we grew *E. coli* BL21 Star™ (DE3)/pET22(b)::*lac80* cells in LB medium, supplemented with 0.25 mM $CuSO_4$ at the moment of induction, and incubated O.N. at 20 °C, at 100 rpm or without shaking. This procedure allowed us to obtain traces of active recombinant Lac80 in the soluble cytoplasmic fraction detectable by both western blot analysis and activity assay (Fig. 3). Although under these conditions most of the recombinant enzyme was still packed into IBs, approx. 50 µg of soluble Lac80/L of culture corresponding to approx. 10.6 µg/g cells was produced in the cytoplasmatic fraction. Activity assay (see below) confirmed that this soluble Lac80 was biologically active. Therefore, it was purified by HisTag affinity chromatography, following the procedure described in Materials and Methods. As a precaution, we prepared all the buffers with Na_2SO_4 rather than with NaCl, since chlorine ions were shown to inhibit laccase activity (*8*). Approx. 42 µg/L culture and 9 µg/g cells of highly pure Lac80 were recovered from the elution peak, a purification yield of approx. 84 %.

Lac80 displays activity on different substrates

We tested the ability of Lac80 to oxidize different phenolic and non-phenolic compounds that are typical LMCO substrates. Initial substrate screening was conducted at pH=5.0. Lac80 was active on five of the thirteen screened substrates: the non-phenolic ABTS (activity=1.25 U/mg) and the phenolic 2,6-DMP (activity=10.4 U/mg), pyrocatechol (activity=0.85 U/mg), pyrogallol (activity=3.2 U/mg), and vanillic acid (activity=0.87 U/mg). The highest specific activity at pH=5.0 was reported using 2,6-DMP as substrate, whereas oxidation of the other substrates was overall less efficient. Since for LMCOs the optimal pH for oxidation can vary among substrates, the activity of Lac80 on the five positive substrates was further evaluated at different pHs (from 2.0 to 8.0), as shown in Figs. 4a, 4b, 4c, 4d and 4e. In all cases, the pH-dependent activity showed a bell-shaped curve, with optimum activity in the acidic range. These results show that Lac80 was active in a wide pH range: on 2,6-DMP, for instance, the recombinant LMCO was active in the pH interval from 4.0 to 8.0, whereas on pyrocatechol Lac80 showed activity from pH=3.0 to pH=8.0. Notably, on this latter substrate, approx. 65 and 53 % of activity was also maintained at pH=7.0 and 8.0, respectively. A review of the literature shows that most of the thirteen substrates we tested with Lac80 were indeed oxidized by bacterial laccases in more than half of the cases reported

(*49,50*). Unlike other LMCOs, Lac80 was inactive on tyrosine, thereby excluding the possibility of being a tyrosinase.

Interestingly, the recently characterized laccase OcCueO from *Ochrobactrum* sp. showed activity against ABTS and DMP, two substrates that Lac80 was able to degrade (*32*). However, OcCueO displayed activity against guaiacol, while Lac80 showed no activity against this substrate. Given the differences at the amino acid level, these results are expectable. It is important to highlight here the prominence of *Ochrobactrum* species as a source of phenol oxidases.

CONCLUSIONS

Although purification of Lac80 yielded small amounts of active protein, we were able to characterize this laccase using typical substrates. Lac80 shows promising traits such as substrate flexibility and stability at different pHs, making it suitable for industrial purposes. Our further investigations will explore innovative platforms of heterologous expression coupled with using different constructs to overcome the limitations of *E. coli* system for producing Lac80. In subsequent trials, we will also test other soil-dwelling prokaryotic microbes such as bacilli or streptomycetes, known for their ability to secrete recombinant proteins, to facilitate Lac80 recovery and increase its purification yield. More comprehensive assessment of the biotechnological potential of Lac80 will be possible once the issue of its supply has been solved.

Finally, our study highlights the power of massive sequencing techniques for the discovery of novel oxidizing enzymes in still poorly investigated bacterial genera, especially those encoded in MGE. We show that *Ochrobactrum* species are a promising source of laccase-like enzymes. A still-open question is the physiological function and ecological role of this plasmid-carried LMCO and the way it contributes to the degradation capabilities of soil microbial communities exposed to pollution.

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CONFLICT OF INTEREST

The authors have no conflicts of interest to declare that are relevant to the content of this article.

AUTHORS' CONTRIBUTION

MCM, FB, CC, LA and CV conceived and performed the experiments and *in silico* analysis. MP, AL, IMM, FM and MFDP designed the experiments. MCM and MFDP wrote the manuscript. All authors read, corrected, and approved the final manuscript.

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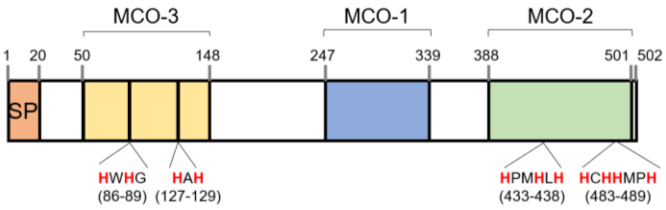
a)



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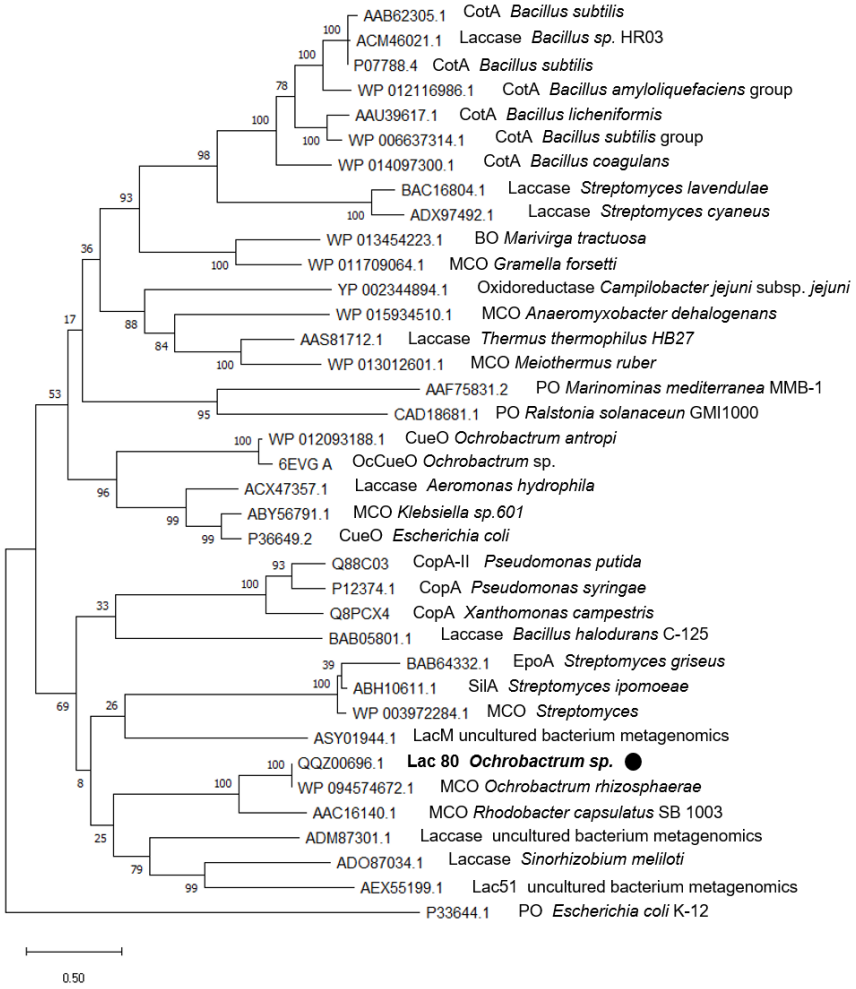
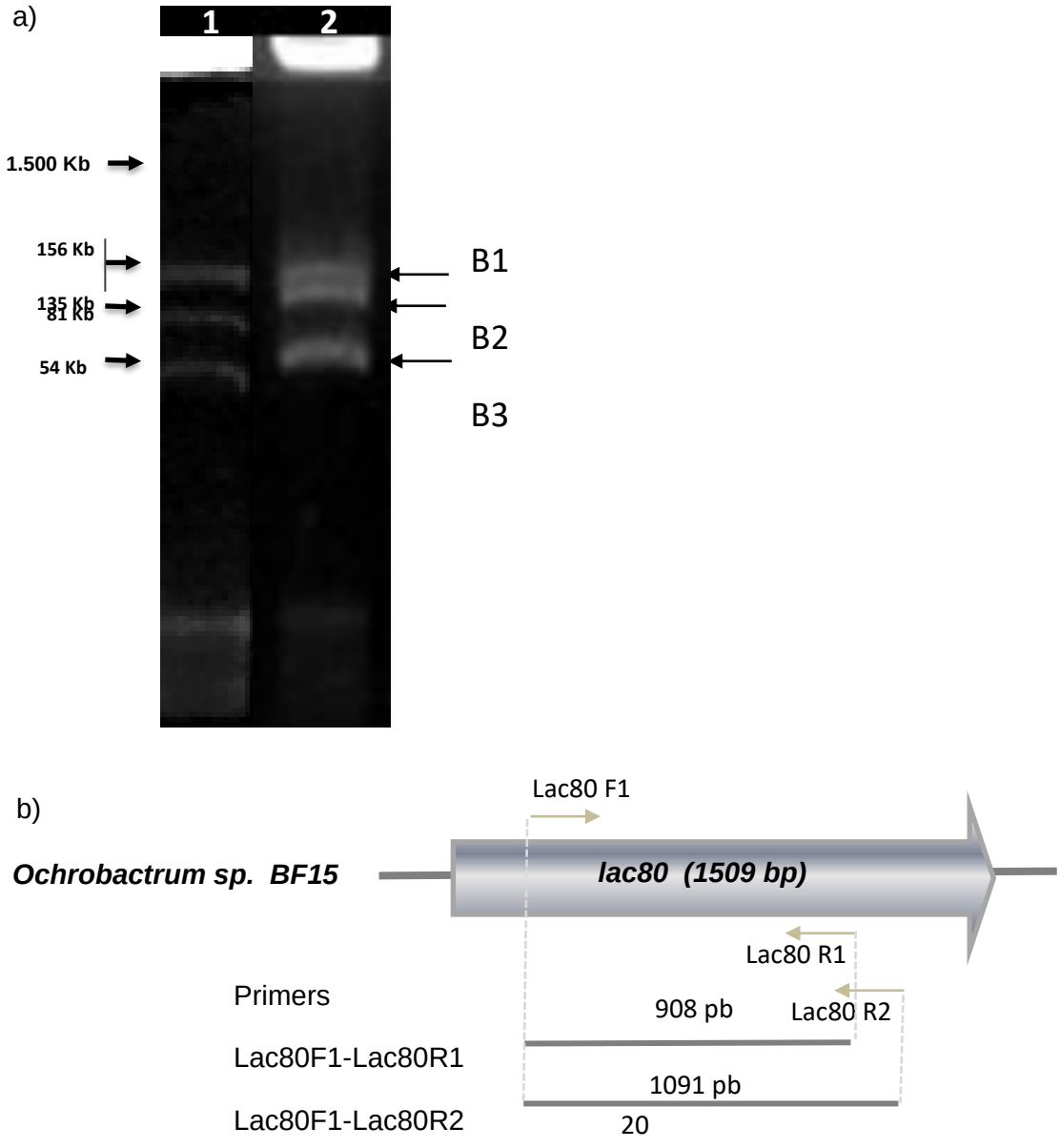


Fig. 1. *In silico* analysis of Lac80 protein sequence. a) Predicted signal peptide (SP), oxidase type 3 domain (MCO-3) PF07732, oxidase type 1 domain (MCO-1) PF00394 and oxidase type 2 domain (MCO-2) PF07731 are indicated. Residues involved in the coordination of the copper atoms are highlighted in red. b) Maximum likelihood phylogenetic tree of *Ochrobactrum* sp. BF15 putative LMCO based on reference protein sequences of characterized and some uncharacterized bacterial laccases from NCBI Protein Database. The percentage of trees in which the associated taxa cluster together is shown next to the branches. MCO multicopper oxidase, BO bilirubin oxidase, PO polyphenol oxidase. Solid black circle indicates the protein characterized in this study



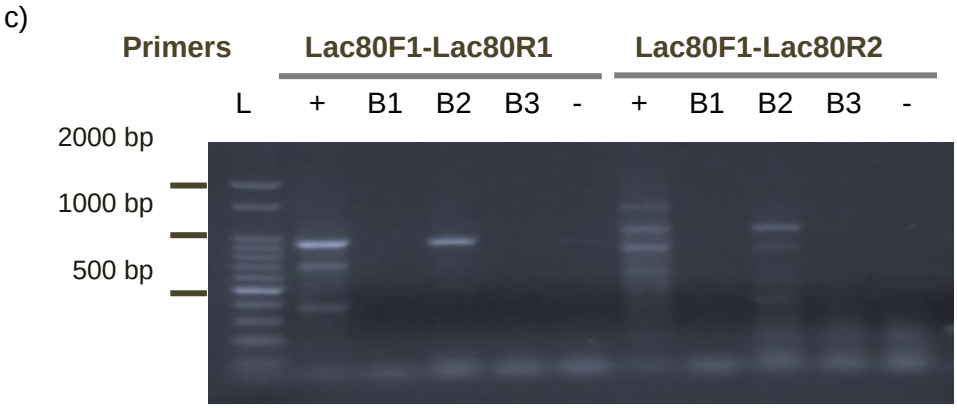


Fig. 2. Plasmid localization of *lac80* in *Ochrobactrum* sp. BF15. a) *In situ* gel electrophoresis showing plasmid profile from *Ochrobactrum* sp. BF15 (line 2). The well-known plasmid profile of *Sinorhizobium meliloti* MVII-1 (line 1) (46) was used to estimate plasmid sizes of *Ochrobactrum* sp. BF15 plasmids. b) Schematic localization of primers used for *lac80* plasmid localization and expected PCR products. **C)** Agarose gel showing the PCR products obtained using total DNA from *Ochrobactrum* sp. BF15 (positive control +) or from the different plasmids highlighted in a. B1, B2 and B3 indicate the different bands (plasmids) purified from a. Negative controls (-) were included. L: ladder

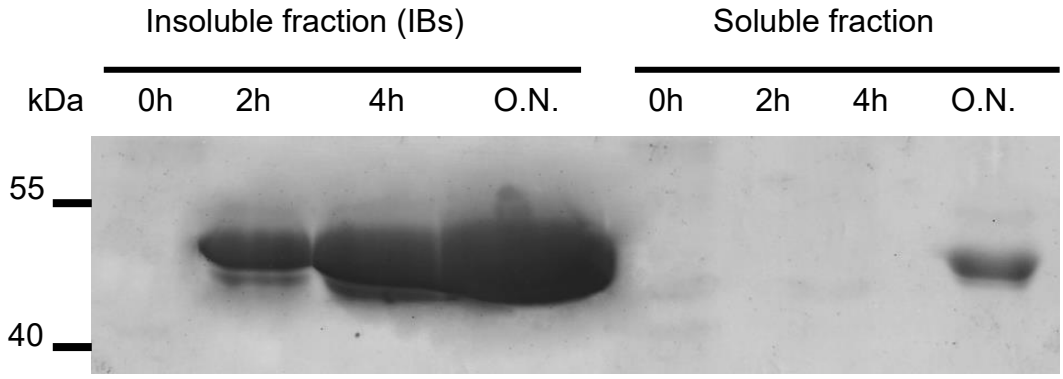


Fig. 3. Heterologous expression of Lac80. Western blot analysis of soluble and insoluble fractions from *E. coli* BL21 Star™ (DE3)/pET22(b)::*lac80* cells was performed. Cells were grown in LB medium,

supplemented with 0.25 mM $CuSO_4$ at the same time of IPTG induction, then incubated at 20 °C and 100 rpm and harvested 0 h, 2 h, 4 h, or O.N. after induction. The samples loaded correspond to the insoluble or soluble fractions from cells recovered from 1 ml or 5 ml of culture volumes, respectively

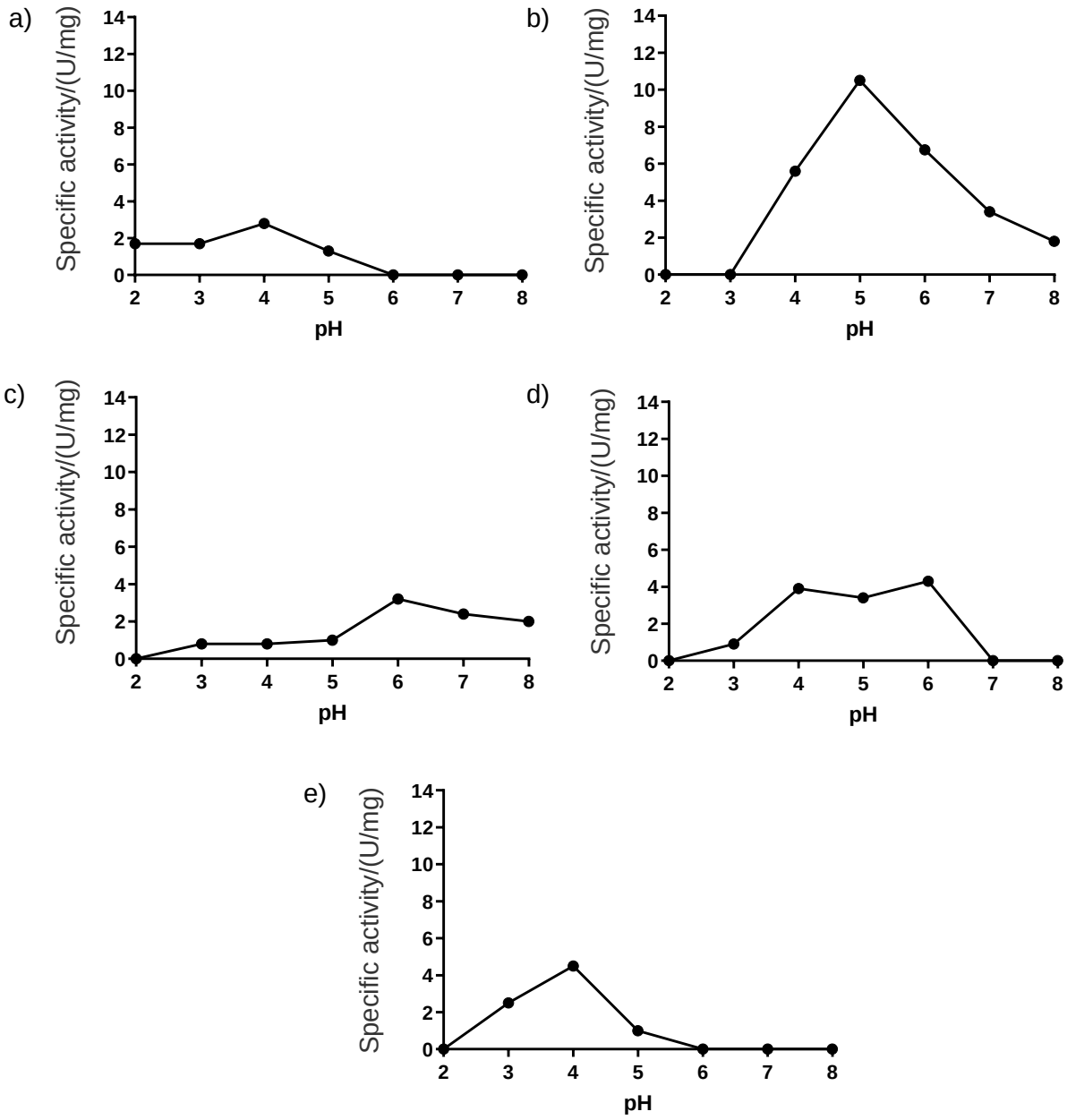


Fig. 4. Enzymatic activity of Lac80 at different conditions. Lac80 displayed activity against a) ABTS, b) DMP, c) Pyrocatechol, d) Pyrogallol, and e) Vanillic acid at 25 °C and in a multi-component buffer in the

pH range from 2.0 to 8.0, with 50 μM $CuSO_4$ supplemented to the assay mixture. Values represent the mean of three parallel measurements (mean ± standard error); when not shown, the error is smaller than the symbol used.

Supplementary materials for: Martini *et al,*. Identification and characterization of a novel plasmid-encoded laccase-like multicopper oxidase from *Ochrobactrum* sp. BF15 isolated from an on-farm bio-purification system.

Table S1. BLASTP alignment of the putative laccase-like multicopper oxidase protein sequence (Lac80) encoded by a plasmid-carried gene from *Ochrobactrum* sp. BF15. The ten best protein matches identified in NCBI by BLASTP algorithm for Lac80 protein are shown.

Accession	Description	Protein length	Organism	Identity	E value	Max Score	Total Score	Query Cover
WP_024899901.1	multicopper oxidase domain-containing protein	502	*Alphaproteobacteria*	100,00%	0	1000	1000	100,00%
WP_094574672.1	multicopper oxidase domain-containing protein	502	*Ochrobactrum rhizosphaerae*	99,80%	0	999	999	100,00%
WP_138787545.1	multicopper oxidase domain-containing protein	502	*Ochrobactrum haematophilum*	99,80%	0	999	999	100,00%
WP_036709831.1	multicopper oxidase domain-containing protein	502	*Paracoccus sanguinis*	99,60%	0	997	997	100,00%
WP_155044758.1	multicopper oxidase domain-containing protein	502	*unclassified Paracoccus*	98,80%	0	993	993	100,00%
WP_085378474.1	multicopper oxidase domain-containing protein	493	*Paracoccus contaminans*	91,83%	0	877	877	100,00%
WP_126156115.1	multicopper oxidase domain-containing protein	493	*Paracoccus haematequi*	91,63%	0	873	873	100,00%
WP_121583054.1	multicopper oxidase domain-containing protein	499	*Mesorhizobium* sp. YM1C-6-2	90,04%	0	870	870	100,00%
WP_114350933.1	multicopper oxidase domain-containing protein	493	*Paracoccus lutimaris*	91,43%	0	868	868	100,00%
WP_122520894.1	multicopper oxidase domain-containing protein	499	*Pannonibacter phragmitetus*	90,04%	0	867	867	100,00%

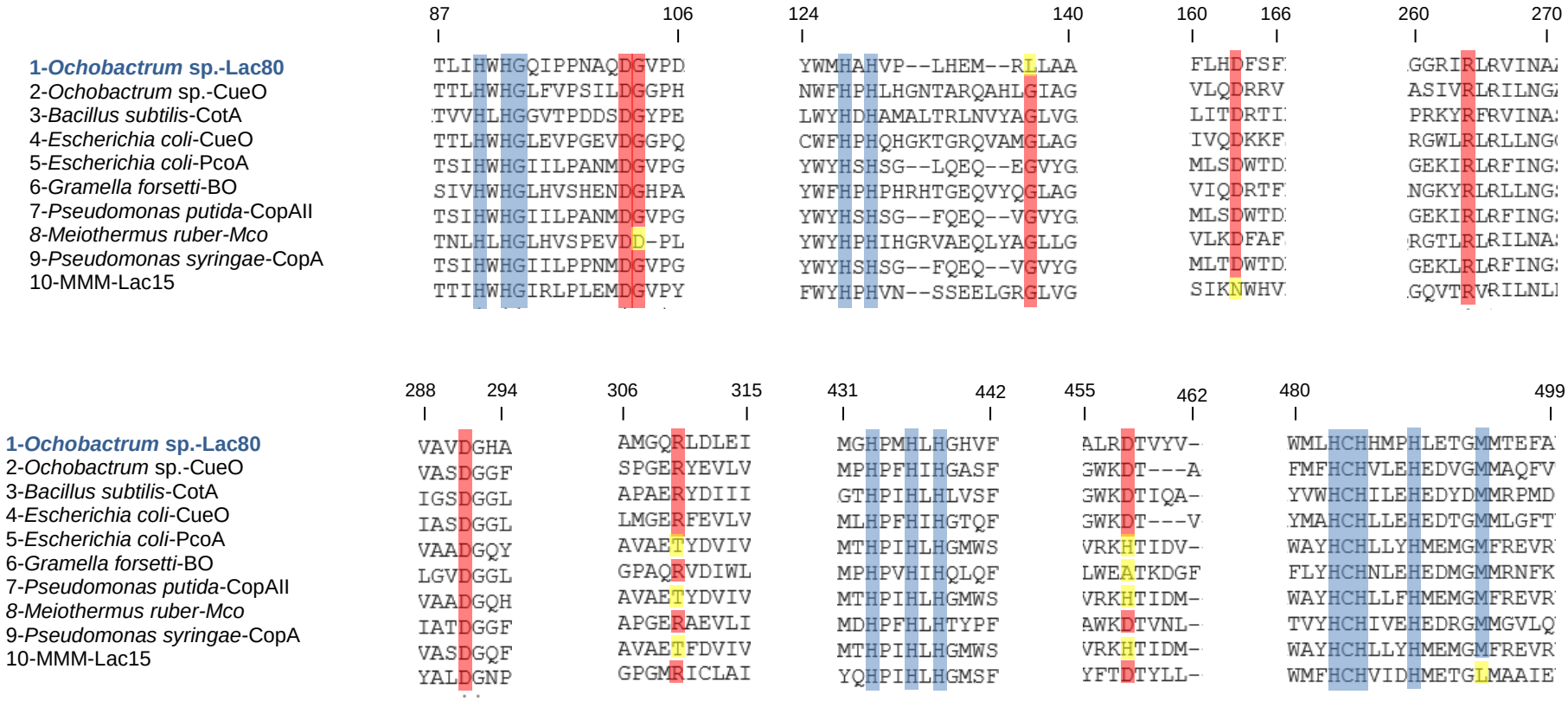


Figure S1. Partial amino acid multiple sequence alignment, showing 10 bacterial laccase-like multicopper oxidases. Metal-binding residues are coloured in blue; other conserved residues are highlighted in red, whereas mismatches are in yellow. Numbering is based on Lac80 sequence as reference. Uniprot or GenBank accession codes as follows: 1, QQZ00696.1; 2, 6EVG|A; 3, P07788; 4, P36649; 5, A0A0E0XT94; 6, (GB) WP_011709064.1; 7, Q88C03; 8, (GB) WP_013012601.1; 9, P12374; 10, E1ACR6 . BO: bilirubin oxidase, MMM: Marine microbial metagenome.

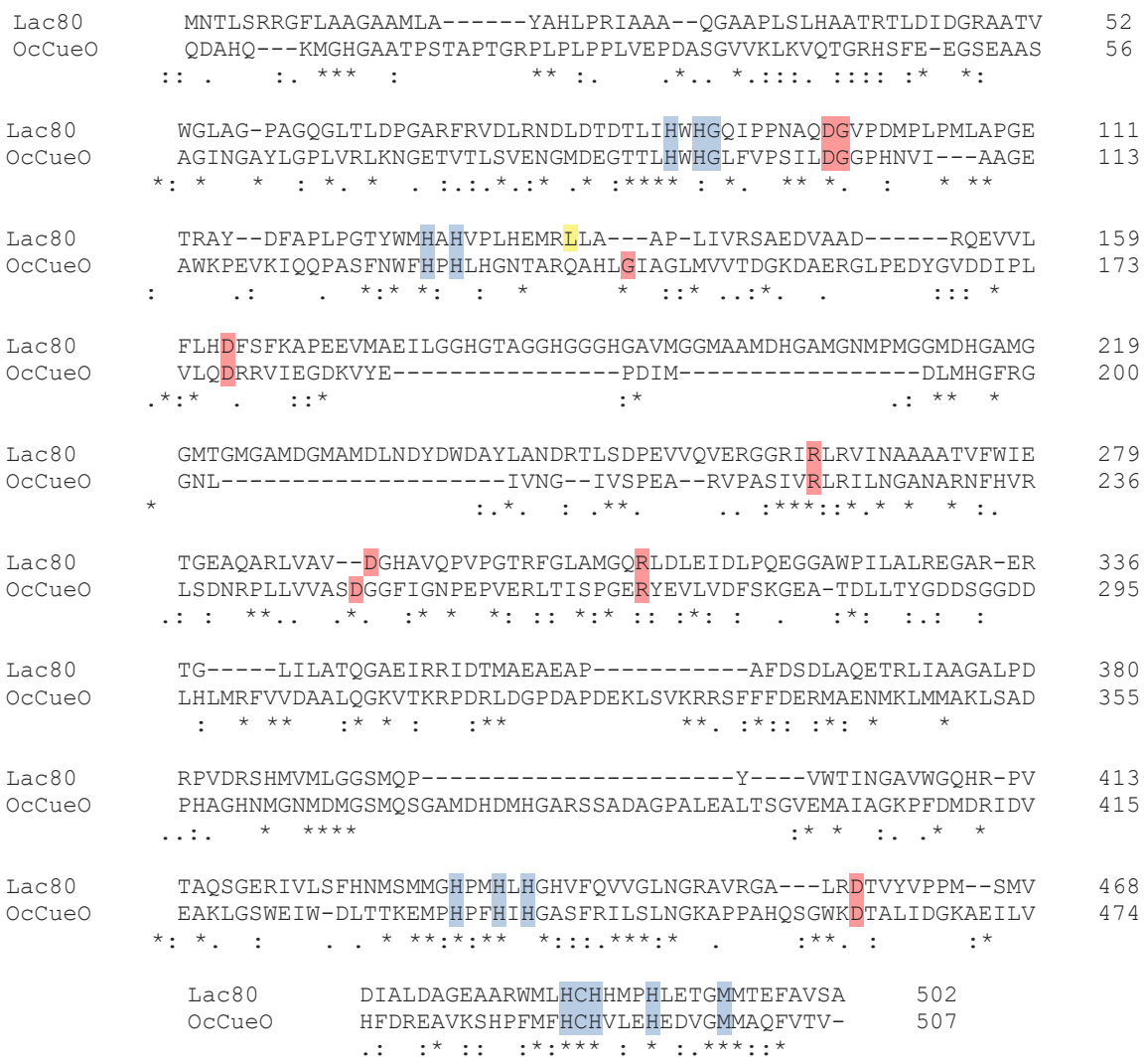
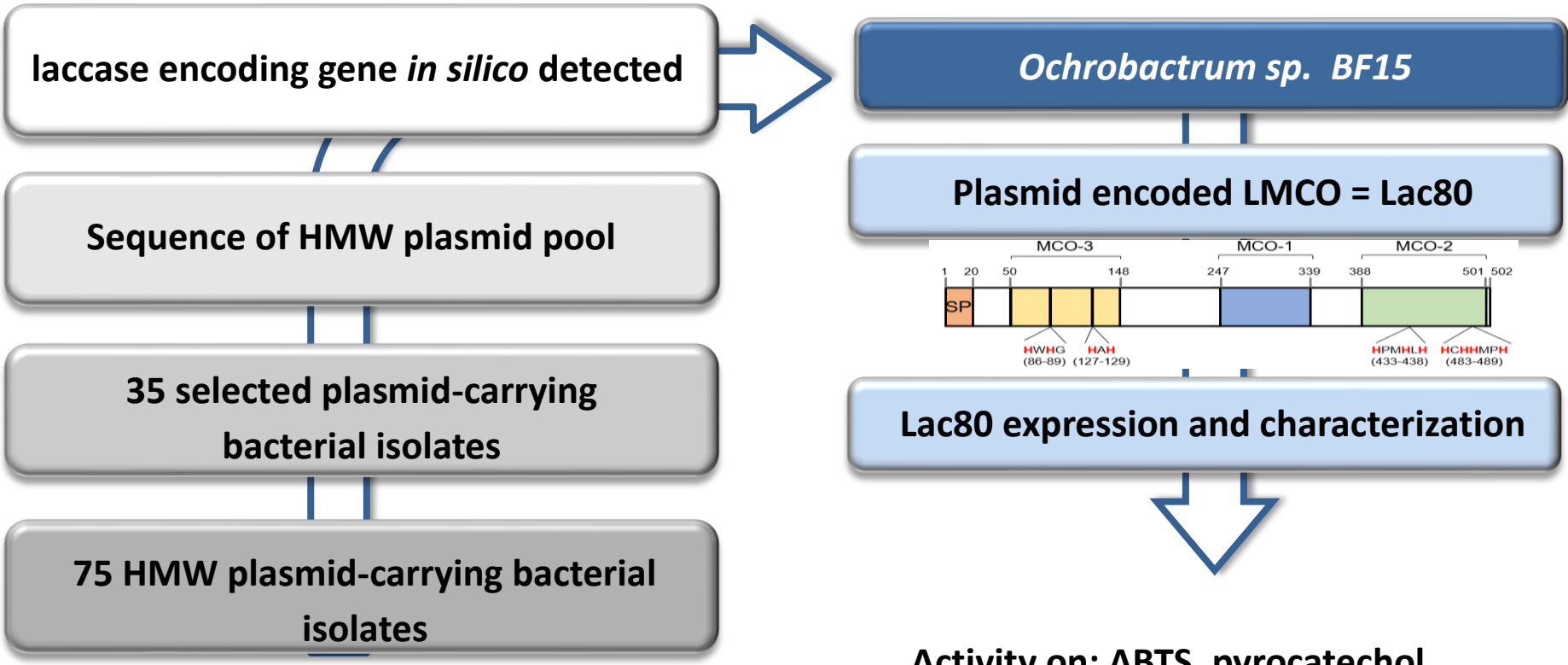


Figure S2. Sequence alignment of Lac80 with OcCueO from *Ochrobactrum* sp. Alignment was carried out using Clustal Omega online tool. The percentage of identity is 25.5 %. Metal-binding residues are coloured in blue; other conserved residues among the bacterial laccase-like multicopper oxidases shown in Supplementary Figure 1 are highlighted in red, whereas mismatches are in yellow. Uniprot or GenBank accession codes as follows: Lac80 (QQZ00696.1), OcCueO (6EVG|A)



Graphical Abstract