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The first acidobacterial laccase-like multicopper oxidase revealed by metagenomics shows high salt and thermo-tolerance

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Abstract Metagenomics is a powerful tool that allows identifying enzymes with novel properties from the unculturable component of microbiomes. However, thus far only a limited number of laccase or laccase-like enzymes identified through metagenomics has been subsequently biochemically characterized. This work describes the successful bio-mining of bacterial laccase-like enzymes in an acidic bog soil metagenome and the characterization of the first acidobacterial laccase-like multicopper oxidase (LMCO). LMCOs have hitherto been mostly studied in fungi and some have already found applications in diverse industries. However, improved LMCOs are in high demand. Using molecular screening of a small metagenomic library (13,500 clones), a gene encoding a

three-domain LMCO (LacM) was detected, showing the highest similarity to putative copper oxidases of *Candidatus Solibacter* (Acidobacteria). The encoded protein was expressed in *Escherichia coli*, purified by affinity chromatography and biochemically characterized. LacM oxidized a variety of phenolic substrates, including two standard laccase substrates (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), $k_{\text{cat}}/k_M = 8.45 \text{ s}^{-1} \text{ mM}^{-1}$; 2,6-dimethoxyphenol (2,6-DMP), $k_{\text{cat}}/k_M = 6.42 \text{ s}^{-1} \text{ mM}^{-1}$), next to L-3,4-dihydroxyphenylalanine (L-DOPA), vanillic acid, syringaldazine, pyrogallol, and pyrocatechol. With respect to the latter two lignin building blocks, LacM showed the highest catalytic activity ($k_{\text{cat}}/k_M = 173.6 \text{ s}^{-1} \text{ mM}^{-1}$) for pyrogallol, with ca. 20% activity preserved even at pH 8.0. The enzyme was thermostable and heat-activated in the interval 40–60 °C, with an optimal activity on ABTS at 50 °C. It was rather stable at high salt concentration (e.g., 34% activity preserved at 500 mM NaCl) and in the presence of organic solvents. Remarkably, LacM decolorized azo and triphenylmethane dyes, also in the absence of redox mediators.

Luka Ausec and Francesca Berini contributed equally to the work.

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Keywords Metagenomics · Laccase · LMCO · Acidobacteria · Heterologous expression · Dye decolorization

Introduction

Laccases are oxidoreductases that couple the oxidation of a substrate to the reduction of molecular oxygen to water. The majority of them are multicopper oxidases that can oxidize diverse substrates, such as monophenolic and polyphenolic molecules as well as non-phenolic organic and some inorganic compounds. They are usually characterized as monomeric enzymes that have three cupredoxin-like domains and four characteristic histidine-rich copper-binding sites in the first

and third domain. This description has, however, become insufficient with several recent discoveries, most notably with the thorough characterization of two types of two-domain laccases that lack the second domain and are only active as a homo-trimer (or sometimes as homodimer) (Nakamura et al. 2003). Moreover, some enzymes were recently called laccases based on their abilities to oxidize typical laccase substrates, even though they were structurally unrelated to multicopper oxidases (Beloqui et al. 2006).

Laccases and laccase-like enzymes have been found in plants, fungi, and bacteria, but have been most extensively studied in fungi. Several laccases are already applied at industrial scale, especially in the wood, biofuel, paper, textile, fine chemical, and food sectors (Roth and Spiess 2015). Recent discoveries indicated that bacteria encode a diverse array of genes showing high homology to three-domain fungal laccases (Ausec et al. 2011a). Some of them were purified and their activities studied in vitro (Tonin et al. 2016a; Callejón et al. 2016; Ausec et al. 2015). While their substrate specificity varies widely, bacterial laccases usually prove to be robust in terms of their pH and temperature stability, and they often remain active at industrially relevant concentrations of chloride anions. These reasons justify further searches for novel bacterial laccases. This paper describes a novel bacterial three-domain enzyme that is active as a monomer and is thus similar to the “traditional” laccases that were initially described in fungi. However, we will refer to it with the term laccase-like multicopper oxidase (LMCO) rather than laccase, to avoid ambiguity when referring to this extremely diverse group of enzymes (Reiss et al. 2013).

Several approaches to isolate new LMCO have been successfully implemented, most notably (I) screening and direct isolation of the enzyme from laccase-like enzyme-producing bacterial strains (e.g., Devasia and Nair 2016; Sondhi et al. 2014) and (II) heterologous expression of LMCO-encoding genes from bacterial strains (e.g., Ausec et al. 2015; Moghadam et al. 2016; Kim et al. 2015). A radically different approach is offered by metagenomics, which aims at discovering the natural genetic diversity that would otherwise be inaccessible due to poor cultivability of the vast majority of microorganisms. The way to achieve this is by direct extraction and cloning of total DNA produced from a microbial community (Handelsman 2004). In general, there are two ways of screening the resulting clones, each with its benefits and limitations (Schmeisser et al. 2007). First, screening can be based on the functional traits that are expressed in the clones. Thus, as-yet-unknown enzymes with desired properties can be unlocked. However, our poor understanding of expression conditions often limits the successful outcome of this approach. Alternatively, based on the fragment's DNA sequence, screens can be performed either by bioinformatics analysis of large amounts of sequencing data or by a PCR-based search-and-sequencing approach. While this approach

may somewhat limit the scope of novelty, it is warranted especially when a heterogeneous group of enzymes such as bacterial LMCOs is explored. Less than a handful of studies has successfully implemented metagenomic approaches for bacterial laccase and laccase-like enzyme discovery (Beloqui et al. 2006; Ye et al. 2010; Fang et al. 2011, 2012a, b). The reason for this lies in the difficulties brought about by the available screening methods, be it those based on activity (due to poor expression of foreign genes in *Escherichia coli* and similar hosts) or sequence (daunting amount of manual work or sequencing). Still, the promise of metagenomics is too great to ignore. Here, we show how metagenomics applied to DNA from an acid bog soil can yield full-length LMCO genes. In this, we were guided by the previous finding of fragments of similar genes in a natural wetland, where a great diversity of bacterial LMCO genes was found. These were to a large part phylogenetically affiliated to the highly diverse but still poorly understood phylum Acidobacteria, namely to subdivision 3 (e.g., containing *Candidatus Solibacter usitatus*-like sequences) and subdivision 1 (showing similarity to *Granulicella malensis* sequences) (Ausec et al. 2011b).

This paper extends the previous diversity study and reports on the finding of full-length bacterial LMCO genes in the wetland soil DNA. One full-length gene for an *Acidobacterium*-affiliated LMCO was cloned and expressed in *E. coli*, after which the enzyme was characterized. This paper contextualizes the biochemical properties of the enzyme and discusses the difficulties in finding and purifying an enzyme derived from metagenomics.

Materials and methods

Metagenomic library construction

High molecular weight DNA was extracted from Ljubljana Marsh bog soil- Slovenia (soil described in Ausec et al. 2011b) using a modified protocol of van Elsas et al. (2008). Briefly, 10 g of soil were suspended in 10 ml extraction buffer (100 mM Tris-HCl, 100 mM Na-EDTA (ethylenediaminetetraacetic acid sodium salt), 100 mM NaPO₄, 1.5% w/v NaCl, 1% w/v CTAB (cetyl trimethylammonium bromide) pH 8.0), and sonicated (water bath sonicator) for 15 min. Proteinase K (100 µl of 10 mg/ml solution) was then added and incubated for 2 h at 37 °C with gentle shaking (200 rpm). DNA extraction was performed with phenol/chloroform/isoamylalcohol (25:24:1) in a water bath at 60 °C for 30 min. The metagenomic DNA was precipitated with isopropanol and embedded in agarose plugs (1% w/v low melting point agarose). Removal of phenolic contaminants and separation of 30–40 kb size DNA fractions was performed by pulsed field gel electrophoresis (PFGE) on 1% w/v agarose gel supplemented in the upper part with 2% w/v polyvinyl pyrrolidone

(PVP). Electrophoresis was run in 0.5X Tris-borate-EDTA (TBE) at 14 °C using a PFGE DRIII System (BioRad, Hercules, CA, USA) with the following parameters: gradient 6 V/cm, included angle 120°, initial switch time 0.5 s, final switch time 8.5, linear ramping factor, 20 h. The 2-cm agarose fragments containing DNA in the range of 30–40 kb were cut out of gel without staining and exposing to UV radiation. DNA was recovered using β -agarase (New England Biolabs, Ipswich, MA, USA) according to manufacturer's protocol.

The metagenomic library was constructed using the CopyControl Fosmid Library Production Kit (Epicenter, Madison, WI, USA). The metagenomic DNA was blunt-ended, ligated into pCC1Fos fosmid vector and transformed into *E. coli* EPI300-T1^R (Epicenter). Positive selection of fosmid clones was performed on LB (Luria Bertani) agar supplemented with 12.5 μ g/ml chloramphenicol. The metagenomic library was stored at –80 °C according to manufacturer's recommendations. The clones were pooled into 27 pools with approximately 500 EPI300 colonies each.

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

Metagenomic library screening

Molecular PCR-based method was used to screen 27 metagenomic library pools for potential novel LMCO genes. In the initial step, PCR was employed to amplify the putative metagenomic LMCO genes using the primers Cu1AF and Cu4R (Table 1) and the protocol described in Ausec et al. (2011a). These primers supported amplification of three-domain and two-domain bacterial LMCO genes with expected size of 1000–1500 bp for three-domain and ~600 bp for two-domain LMCO genes, respectively. The pools giving rise to PCR amplicons of desired sizes were considered as potentially positives and further analyzed. First, the PCR amplicons of 1000–1500 bp and ~600 bp were gel purified, sequenced and sequences verified by Blast-P (<http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins>). The pools that carried a clone with a verified LMCO gene were first diluted to 10^{–5} and cells plated on LB agar supplemented with 12.5 μ g/ml chloramphenicol. Approximately, 800 clones per pool were picked randomly for further screening and distributed in 96-well microtiter plates. A single pool was created from each plate, and this was used directly as a template for PCR using the primers R1F and R1R (Table 1), specific for the metagenomic LMCO gene fragment identified during the initial screening of the 27 pools. For each positive pool, pools of clones were generated from each of the rows in the original microtiter plate and the PCR was repeated. This process allowed the identification of a specific individual clone that carried a putative LMCO gene referred to hereafter as *lacM*.

Table 1 Primer pairs used in this work

Primer	Nucleotide sequence (5'-3')	Use	Reference
Cu1AF	ACM WCB GTY CAY TGG CAY GG	First step screening of metagenomic library pools	Ausec et al. (2011a)
Cu4R	TGC TCV AGB AKR TGG CAG TG		
R1F	GCG TTA GTT TTA CCC CGA AGC	Second step screening of selected metagenomic library clones	This work
R1R	TTC CCG ACG TCC TCT TAT CG		
R1FrC	GCT TCG GGG TAA AAC TAA CGC	Reconstruction of <i>lacM</i> sequence (outward seq.)	This work
R1RrC	CGA TAA GAG GAC GTC GGG AA		
R3D40up	ACG ACG AGA TCC GCT TCC C	Reconstruction of <i>lacM</i> sequence (inward seq.)	This work
RD40down	AAT GTC TGC CCG AGG TGC C		
RocHisF	ATA CAT ATG TGT AGA ATT GTG CAT AAT CTC GTA ACG G	<i>lacM</i> cloning into pET21c	This work
RocHisR	TTT GAG CTC CGA GTA CTT CAC CAA AGT CTT GAA GC		
LacHisF	TTT GAA TTC CAA TGC CAG AGA ATT TCT ACT CAC	<i>lacM</i> cloning into pET-DUET	This work
LacHisR	TTT AAG CTT TTA CGA GTA CTT CAC CAA AGT C		

Sequence analysis

To obtain the whole gene of the putative LMCO LacM, the fosmid DNA was extracted from the identified clone and subjected to outward sequencing using primers R1FrC/R1RrC (Table 1). The *lacM* sequence was also confirmed with the inward sequencing of the fragment covering the complete *lacM* gene using primers R3D40up and R3D40down (Table 1) outside the *lacM* gene. The sequence was compared with known protein sequences using BlastP. Multiple sequence alignment of LacM with related sequences was performed using Clustal Ω (<http://www.ebi.ac.uk/Tools/msa/clustalo/>). Protein's module structure was predicted using SMART (Simple Modular Architecture Research Tool; <http://smart.embl-heidelberg.de/>). Presence and location of a putative signal peptide was estimated with the following bioinformatics tools, using default parameters: Phobius (<http://phobius.sbc.su.se/>), PRED-TAT (<http://www.compgen.org/tools/PRED-TAT/>), Signal Blast (<http://sigpep.services.came.sbg.ac.at/signalblast.html>), TatP 1.0 Server (<http://www.cbs.dtu.dk/services/TatP/>), SignalP 4.1 (<http://www.cbs.dtu.dk/services/SignalP/>).

www.cbs.dtu.dk/services/SignalP/). The 3D structure model of LacM was inferred using I-TASSER (Yang et al. 2015). Neighbor-joining phylogenetic tree was constructed using Mega6 (Tamura et al. 2013) to compare LacM with some references retrieved from the public databases as well as with putative LMCOs codified by gene fragments obtained in our previous study (Ausec et al. 2011b). The complete fosmid fragment was sequenced using 454 technology. Predicted protein-encoding genes (coding DNA sequences (CDSs)) were manually verified by similarity searches against the non-redundant protein database (<http://www.ncbi.nlm.nih.gov>) using Blast-P with optimized parameters, as in Cretoiu et al. (2015). Start and stop codons were identified for all annotated Open Reading Frames (ORFs). The nucleotide sequence of the annotated fosmid was deposited in the GenBank database under the accession number KY345412.

Cloning and heterologous expression of the novel LMCO gene

The *lacM* gene was amplified from the metagenomic clone using a standard PCR protocol (as described in Ausec et al. 2015), followed by excision from 1% w/v agarose gel and purification using PureLink Quick Gel Extraction Kit (Invitrogen Life Technologies, Carlsbad, CA, USA). For cloning into the expression vector pET21c (Novagen Inc., Merck Millipore, Kenilworth, NY, USA), which introduced a polyhistidine tag (His₆-Tag) at the C-terminal of the enzyme, the primers RocHisF and RocHisR (Table 1), containing *Nde*I and *Xho*I restriction sites, respectively, were employed. *lacM* was also cloned with the help of primers LacHisF and LacHisR (Table 1) into the expression vector pET-DUET (Novagen Inc.) between restriction sites *Eco*RI and *Hind*III, which introduced the His₆-Tag at the N-terminal of the enzyme. Both vectors were used in parallel to transform *E. coli* BL21(DE3) pLysE (Invitrogen Life Technologies). Details on the expression protocols followed to produce active LacM from recombinant *E. coli* strains are reported in the Online Resource. In brief, (I) for growing cells, conventional LB medium was compared with Terrific Broth (TB), the latest being richer and not containing sodium chloride, which might inhibit laccase activity (Xu 1997; Baldrian 2006), (II) copper (0.25 or 0.5 mM CuSO₄) was added to the growth medium before cell inoculum or at the moment of induction to facilitate proper folding and incorporation of the metal into active sites of the enzyme (Durão et al. 2008), (III) protein expression was induced by adding 0.1, 0.5 or 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) to cells at the early exponential phase of growth (OD_{600nm} ~0.6 for LB or ~1 for TB) or at the late exponential phase of growth (OD_{600nm} ~4 for LB), (IV) temperature after IPTG addition was kept at 37 °C or reduced to 25 or 18 °C, and (V) following IPTG addition, the cells were incubated for different time intervals (0, 2, 4 h or

overnight (O.N.)) before harvesting, and using different aeration regimes (at 200 or 100 rpm or without shaking) since it was reported that creating microaerobic conditions may facilitate the production of fully copper-loaded multicopper oxidases (Durão et al. 2008; Gunne et al. 2013). Total protein concentration in the fractions was calculated by the Biuret assay (Gornall et al. 1949), whereas LacM production was estimated through sodium dodecyl sulfate polyacrylamide (10% w/v) gel electrophoresis (SDS-PAGE), western blot with Anti-HisTag antibody and laccase activity assay.

LacM purification

The following protocol was used to produce the active enzyme for characterization. O.N. cultures of *E. coli* BL21(DE3) pLysE/pET-DUET::*lacM* were prepared by inoculating 2 ml of glycerol stocks into 80 ml of LB medium containing 100 µg/ml ampicillin and 20 µg/ml chloramphenicol. Cells (OD_{600nm} of 0.1) were inoculated in 2 l Erlenmeyer flasks containing 750 ml LB medium supplemented with 0.5 mM CuSO₄, 50 µg/ml ampicillin and 20 µg/ml chloramphenicol, and grown at 37 °C and with 200 rpm shaking. At the OD_{600nm} of 0.6, 1 mM IPTG was added and cells incubated for further 4 h. Cells were harvested by centrifugation at 8000×g for 10 min. The cell pellets were resuspended in 20 mM phosphate buffer (KPi) pH 6.7, containing 10 µg/ml deoxyribonuclease I (DNaseI), 0.19 mg/ml phenylmethylsulfonylfluoride (PMSF), 0.7 µg/ml pepstatin, and then disrupted by sonication for 5 cycles of 30 s each (with a 30-s interval on ice) using a Branson Sonifier 250 (Danbury, CT, USA). After centrifugation at 34,000×g for 60 min at 4 °C, N-terminus His₆-tagged LacM was purified from the soluble cytoplasmic fraction by loading *E. coli* crude extracts, heated for 20 min at 50 °C and centrifuged at 17,000×g for 30 min, onto a 5-ml Hitrap chelating affinity column (GE Healthcare Sciences, Little Chalfont, UK), loaded with 0.1 mM CuSO₄ and equilibrated with 20 mM KPi pH 6.7, 20 mM imidazole, and 300 mM Na₂SO₄. The recombinant protein was eluted with increasing concentrations of 20 mM KPi pH 6.7, 300 mM Na₂SO₄ and 250 mM imidazole, and loaded onto a size-exclusion PD10 Sephadex G25 column (GE Healthcare Sciences) equilibrated with 20 mM KPi pH 6.7. Pure protein concentration was estimated by the use of the theoretical extinction coefficient at 280 nm ($\epsilon_{280\text{ nm}} = 61.88\text{ mM}^{-1}\text{ cm}^{-1}$) based on amino acid sequence.

SDS-PAGE electrophoresis, western blot, and zymogram

Protein purity was assessed by SDS-PAGE and staining with Coomassie Brilliant Blue (Schägger and van Jagow 1987). For western blot analysis, the protein was identified by anti His-Tag antibody HRP conjugate (Novagen Inc.) and detected by chemoluminescence (ECL Western Blotting Detection

System, GE Healthcare Sciences). Molecular weight markers were from the same supplier. For the zymogram (semi-denaturing SDS-PAGE), the purified enzyme was diluted in a sample buffer lacking any reducing agent and incubated for 10 min at room temperature before loading onto a 10% w/v SDS-PAGE gel. After electrophoresis running at 4 °C, the gel was rinsed several times in distilled water to remove SDS and equilibrated for 1 h at 50 °C in 100 mM sodium acetate buffer pH 4.0, containing 50 μ M CuSO₄ and 10 mM 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) or 1 mM 2,6-dimethoxyphenol (2,6-DMP).

Standard ABTS assay

Laccase activity was routinely measured with a spectrophotometer (V460, Jasco, Easton, MD, USA) using ABTS ($\epsilon_{420\text{nm}} = 36 \text{ mM}^{-1} \text{ cm}^{-1}$) as a substrate: assay mixtures (1 ml) contained 1 mM ABTS, 10 μ l of purified protein or 100 μ l of crude extract, 50 μ M CuSO₄ in a multi-component buffer at pH 4.0 (10 mM Trizma base, 15 mM sodium carbonate, 15 mM phosphoric acid and 250 mM potassium chloride) (Harris et al. 2001). Laccase from *Trametes versicolor* (Sigma-Aldrich) was used as positive control. All assays were performed in triplicate. The oxidation of the ABTS was followed as the change in absorbance at 420 nm and 25 °C for 5 min. One activity unit (U) was defined as the amount of enzyme required for oxidizing 1 μ mol of ABTS per min.

Substrate range

The ability of LacM to oxidize phenolic and nonphenolic compounds was tested with a spectrophotometer using the following substrates at the designed final concentration and wavelength: 1 mM 2,6-DMP ($\epsilon_{468\text{nm}} = 49.6 \text{ mM}^{-1} \text{ cm}^{-1}$), 1 mM pyrocatechol ($\epsilon_{450\text{nm}} = 2.21 \text{ mM}^{-1} \text{ cm}^{-1}$), 1 mM pyrogallol ($\epsilon_{450\text{nm}} = 4.4 \text{ mM}^{-1} \text{ cm}^{-1}$), 1 mM vanillic acid ($\epsilon_{316\text{nm}} = 2.34 \text{ mM}^{-1} \text{ cm}^{-1}$), 1 mM K₄Fe(CN)₆ ($\epsilon_{405\text{nm}} = 0.9 \text{ mM}^{-1} \text{ cm}^{-1}$), 1 mM tyrosine ($\epsilon_{475\text{nm}} = 3.6 \text{ mM}^{-1} \text{ cm}^{-1}$), 0.5 mM L-3,4-dihydroxyphenylalanine (L-DOPA, $\epsilon_{475\text{nm}} = 3.7 \text{ mM}^{-1} \text{ cm}^{-1}$), 1 mM guaiacol ($\epsilon_{468\text{nm}} = 12 \text{ mM}^{-1} \text{ cm}^{-1}$), 1 mM syringic acid ($\epsilon_{300\text{nm}} = 8.5 \text{ mM}^{-1} \text{ cm}^{-1}$), 1 mM ferulic acid ($\epsilon_{287\text{nm}} = 12.4 \text{ mM}^{-1} \text{ cm}^{-1}$), 10 μ M syringaldazine ($\epsilon_{525\text{nm}} = 65 \text{ mM}^{-1} \text{ cm}^{-1}$), 1 mM syringaldehyde ($\epsilon_{320\text{nm}} = 8.5 \text{ mM}^{-1} \text{ cm}^{-1}$). All assays were performed in the range of pH from 2.0 to 8.0 in the multi-component buffer adjusted to appropriate pH with HCl or KOH.

Temperature optimum and thermal stability

Enzyme activity at different temperatures was tested by following the oxidation of ABTS at pH 4.0 in the temperature

range from 10 to 70 °C. Enzyme stability was assessed by incubating LacM for 30, 60, 90, or 120 min at 40, 50, 60, or 70 °C; samples of the enzyme incubated at the different temperatures were withdrawn and residual activity on ABTS determined at pH 4.0 and at 25 °C.

Enzyme kinetics

Michaelis-Menten curves for ABTS, 2,6-DMP, and pyrogallol were obtained by using increasing concentrations of the substrates (0 to 20 mM), while maintaining constant enzyme concentration. Assays were performed at 25 °C and at pH 4.0 (for ABTS and pyrogallol) or 5.0 (for 2,6-DMP). Curve-fitting using Kaleidagraph (Synergy Software, Reading, PA, USA) was carried out to calculate k_M and k_{cat} value for LacM.

Effect of inhibitory compounds

Effect of salts (up to 1 M final concentration), enzyme inhibitors (25–150 μ M NaN₃, 10 mM dithiothreitol (DTT)), detergents (1–10% w/v Triton X-100, Nonidet P-40, Tween-20, and *N*-lauryl sarcosine (NLS)), organic solvents (1–10% v/v ethanol, isopropanol, and methanol), and a chelating agent (EDTA, 20–100 μ M) on LacM activity was investigated by adding each compound to the assay mixture and using the standard ABTS assay described above.

Dye decolorization

Decolorization experiments were conducted in 2-ml disposable cuvettes with 1-ml final reaction volume, over a panel of dyes: Remazol Brilliant Blue R (anthraquinone dye, maximum of absorbance at 495 nm), Direct Red 80 (azo dye, 528 nm), Reactive Black 5 (azo dye, 598 nm), Brilliant Green (triphenyl methane dye, 625 nm), Azure B (heterocyclic dye, 648 nm), Bromocresol Purple (triaryl methine dye, 719 nm). 3.5 μ g of purified LacM were added to the reaction mixture containing the dye solution (previously prepared by dissolving the dye in the multi-component buffer at pH 4.0, at the final concentration of 100 μ g/ml), and 50 μ M CuSO₄. To evaluate if ABTS can serve as a redox mediator in dye oxidation, some reactions were supplemented with ABTS at a final concentration of 10 μ M. Cuvettes were incubated at 200 rpm at 37 °C for up to 48 h. Decolorization was evaluated with a spectrophotometer at constant time intervals at the maximum of absorbance for each dye in comparison with controls without LacM. The percentage of decolorization was estimated by using the following equation (Zhuo et al. 2011): decolorization (%) = $[(A_i - A_t)/A_i] \times 100$, where A_i is the initial absorbance of the reaction mixture and A_t is the absorbance after incubation.

Results

Identification of putative LMCO gene-carrying clones in a metagenomic library using molecular screening

The metagenomic library was prepared from DNA extracted from an acidic bog peat, rich in soil organic matter (SOL), which was previously indicated for high diversity of bacterial LMCOs (Ausec et al. 2011b). An informed nested PCR-based method was used to screen the 13.5 k-clone (kC) bog soil metagenomic library for the presence of LMCO genes. The library was first divided into 27 pools, each composed of 500 clones, and the pools were screened using the previously-developed primers Cu1AF and Cu4R (Table 1). These primers amplify three-domain (1000–1500 bp) and two-domain bacterial LMCO (~600 bp) genes (Ausec et al. 2011b). Out of the five pools initially identified as “positive” (giving rise to PCR fragments of expected length) (Online Resource, Fig. S1a, b and c), only two were subsequently confirmed by sequencing to encode genes showing similarity to LMCO genes. These originated from pool P1 - that gave rise to a ~1000 bp PCR fragment - and from pool P25 - that gave rise to a shorter ~600 bp PCR fragment. The sequences thus produced provided information to design more specific primers for searching the clones carrying LMCO genes in both pools (primers R1F and R1R, see Table 1). By using these more specific primers, approximately 800 clones from each positive pool were screened. Screening of pool P25 to amplify the shorter fragment (potentially encoding a putative two domain laccase) was not successful, probably due to insert instability and loss during the repeated plating of the clones. The same approach applied to screen pool P1 led to identification of one stable fosmid clone carrying a 32.6 kb long genomic fragment containing the sequence for the putative three-domain LMCO gene, hereby named *lacM* (Online Resource, Fig. S1d).

Sequence analysis of the metagenomic fragment and of the *lacM* gene

Sequencing of the metagenomic fragment revealed the G + C content of the fosmid insert to be of 57.6%. A total of 28 ORFs was identified, with positive or negative transcription frames (Fig. 1a and Online Resource, Table S1): a predicted function was assigned to 21 of them, whereas seven were denoted as hypothetical proteins (Fig. 1a and Online Resource, Table S1). ORF lengths varied from 117 bp (ORF24, short-chain dehydrogenase) to 2979 bp (ORF7, partial sequence with the closest hit to an error-prone DNA polymerase from Acidobacteria). Most of the putative genes were predicted to be involved in housekeeping and cell replication functions, as well as in primary metabolism. Seventeen ORFs were affiliated to sequences from Acidobacteria: notably, seven of them

were affiliated, with a protein-based level of identity between 43% (ORF18) and 71% (ORFs 3 and 27), to proteins from *Candidatus Solibacter usitatus* (Subdivision 3 of Acidobacteria), making this candidate species the closest relative to the source organism (Online Resource, Table S1).

The sequence analyses also revealed the *lacM* gene (ORF18), which is 1359 nucleotides long and encodes a protein of 452 amino acid residues with a predicted molecular mass of 50.3 kDa and a theoretical isoelectric point of 6.54. LacM shares amino acid sequence identities lower than 30%—yet higher than the 21% cut-off—with well-known bacterial or fungal laccases: This enzyme shows 23% identity with the outer spore coat copper-dependent laccase from *Bacillus subtilis* subsp. *subtilis* str. 168 (GenBank accession number CAB12449), 28% with the multicopper oxidase of *E. coli* str. K12 substr. W3110 (BAB96698), 24% and 25% with those from the fungi *Phlebia radiata* (CAA36379.2) and *Trametes versicolor* (CAA59161.1), respectively. The phylogenetic tree (Fig. 1b) confirms the novelty of our finding, since LacM clusters most closely with uncharacterized LMCOs from Acidobacteria. Indeed, the full-length protein shows the highest sequence identity (66%) with a putative protein (ADV52204.1) identified in our previous study of bacterial LMCO genes in the same bog soil (Ausec et al. 2011b). LacM has a high identity with the two multicopper oxidases (MCO) WP_011684805.1 (47%) and WP_041857549.1 (43%) from *Solibacter usitatus* (Online Resource, Table S1).

Further in silico analysis of the predicted protein identified three conserved MCO domains: an MCO type-3 domain (PFAM accession number PF07732) between amino acids 39 and 159, an MCO type-1 domain (PF00394, residues 185–299), and an MCO type-2 domain (PF07731, residues 326–451) (Fig. 1c). Within these three domains, four copper binding regions were identified, corresponding to the highly conserved sequence motifs HxHG, HxH, HxxHxH, and HCHxxxHxxxx[MLF] (Reiss et al. 2013). Histidine and cysteine residues involved in the coordination of the T1, T2, and T3 copper ions are highlighted in Fig. 1c. Additionally, a putative signal peptide, with a cleavage site between amino acids 29 and 30, was identified by four out of the five programs employed (by Phobius, PRED-TAT, Signal Blast and TatP 1.0 Server, but not by SignalP 4.1). This signal peptide shows the typical features of TAT (twin-arginine translocation) system-secreted proteins (Natale et al. 2008): a positively charged n-region (residues 1–5), which includes the eponymous two-arginine motif, a hydrophobic h-region (residues 6–17), and a polar c-region (residues 18–29) (Fig. 1c). Finally, primary sequence alignment and 3D structure model comparison revealed that LacM does not have the methionine-rich region containing the fifth copper binding site reported in metallo-oxidases such as *E. coli* CueO (Fig. S2, Online Resource) and that is responsible for their higher affinity

Fig. 1 **a** Graphical representation of the genes detected and annotated in the fosmid insert selected by genetic screening approach. **b** Neighbor-joining phylogenetic tree of LacM protein sequence with reference sequences from NCBI Protein database and our previous work (Ausec et al. 2011b). LacM is different from previously identified sequences and clusters with other LMCOs from Acidobacteria. **c** LacM protein sequence includes a putative twin arginine translocation (TAT) system signal peptide (TATSP), a multicopper oxidase type 3 domain (MCO-3), a MCO type 1 domain (MCO-1), and a MCO type 2 domain (MCO-2). The amino acidic composition of the putative signal peptide (according to Phobius) is indicated in details. Amino acids corresponding to the four copper-binding regions are highlighted. Within them, residues involved in the coordination of the type 1 (T1 or blue) copper atom are indicated by *asterisks* (*), whereas residues responsible for binding the type 2 (T2 or normal) or the type 3 (T3 or coupled binuclear) coppers are indicated with the *section sign* (§) or with *hash* (#), respectively

towards metal ions than for larger organic substrates (Roberts et al. 2003; Kataoka et al. 2007).

Expression of LacM in *E. coli*

The gene for LacM was cloned under the control of the IPTG inducible *T7* promoter into two plasmids, pET-DUET and

pET21c, and expressed in the host *E. coli* BL21(DE3) pLysE, either as an N-terminus or a C-terminus His₆-tagged protein, respectively. Unfortunately, our first expression trials following conventional procedures for heterologous protein production did not yield active LMCO. Thus, different cultivation and protein expression conditions were unleashed to both strains (detailed in Online Resource). Immunoblotting

of soluble or insoluble cell extracts and of cell-free broths generally revealed an intense protein band at the expected molecular mass for the His₆-tagged LacM (51.2 kDa) only in insoluble fractions (Fig. S3 and S4, Online Resource). This band was absent in control strains transformed with the empty vectors. Activity assays of these fractions revealed the putative LMCO to be inactive, due to its accumulation in inclusion bodies (IBs), which is consistent with previous data (Sørensen and Mortensen 2005; Hjort et al. 2014; Cretoiui et al. 2015; Berini et al. 2017). Positively, traces of active LacM become detectable in the soluble cytoplasmic fractions when *E. coli* BL21(DE3) pLysE/pET-DUET::lacM cells were grown at 37 °C in LB medium supplemented with 0.5 mM CuSO₄, and 1 mM IPTG was added during early exponential growth phase (Fig. 2a). While approximately 95% of the LacM again accumulated into insoluble fractions using this approach, immunoblotting revealed faint bands of the enzyme in cytoplasmic fractions that were collected after cell

incubation for 4 h or overnight. These soluble fractions were then shown to contain an active enzyme: approximately 0.03 U/g cell corresponding to 0.10 U/l culture.

The soluble fraction containing LacM was purified using affinity chromatography. For this, *E. coli* cytoplasmic fractions were heated before being loaded onto a Hi-trap chelating affinity column as described in Tonin et al. (2016b). Subsequent SDS-PAGE analysis showed LacM to be an approximately 51 kDa protein (Fig. 2b) that could be detected also with ABTS or DMP-based zymogram electrophoresis in semi-denaturing conditions (Fig. 2c). Interestingly, when the Hi-trap column was loaded with CuSO₄ instead of NiCl₂, the activity of the purified enzyme on ABTS tripled, indicating the positive effect of copper addition for the enzyme activity at this stage. Finally, over 50% of the initial activity was recovered as partially purified protein, with a purification yield of 47 µg/g cell, corresponding to 180 µg/l culture. The amount and purity of this enzyme preparation was sufficient for the initial enzymatic characterization of LacM.

LacM characterization

Addition of copper increases LacM activity

LacM activity was increased if copper was added to the spectrophotometer reaction mixture containing the recombinant protein recovered from the chromatographic step (Fig. 3a). Adding 10, 50, and 100 µM CuSO₄ to the ABTS assay mixture increased the activity 5-, 9-, and 12-fold, respectively, indicating that the purified recombinant enzyme was still partially copper-depleted. This effect was confirmed by zymogram electrophoresis of purified LacM: with 50 µM CuSO₄ in the developing buffer (100 mM sodium acetate pH 4.0), the intensity of the activity band was significantly enhanced, both using ABTS and 2,6-DMP as substrates (data not shown). For this reason, 50 µM CuSO₄ was consistently added to the reaction mixture, denoted as “standard ABTS assay” throughout this study.

Substrate range and pH optimum

The capability of LacM to oxidize 13 phenolic and nonphenolic compounds was tested (see Materials and Methods). Since the activity of laccase and laccase-like enzyme on different substrates depends on pH, the LacM activity was assayed for each substrate in the pH range between 2.0 and 8.0. The enzyme oxidized seven out of the 13 substrates: the nonphenolic ABTS (Fig. 3b) and the phenolic 2,6-DMP, L-DOPA, pyrogallol, pyrocatechol, vanillic acid, and syringaldazine (Fig. 4). For each compound, the pH-activity profile was bell-shaped, with optima at acidic pH. The highest enzyme activities were detectable on pyrogallol (Fig. 4e) and pyrocatechol (Fig. 4f), i.e., phenolic substrates with two and

Fig. 2 SDS-PAGE analysis of LacM from *E. coli* BL21(DE3) pLysE/pET-DUET::lacM cells. **a** Western blot analysis of soluble and insoluble fractions of *E. coli* BL21(DE3) pLysE/pET-DUET::lacM cells grown at 37 °C and 200 rpm in LB supplemented with 0.5 mM CuSO₄; protein expression was induced by adding 1 mM IPTG during early exponential growth phase (OD_{600nm} of 0.6). The equivalent of 1 ml of culture was loaded in each lane. Protein concentration was calculated by densitometric analysis of SDS gel bands with the software Quantity One (Bio-Rad Laboratories) and His₆-glycin oxidase (His₆-GO) from *Bacillus subtilis* (kindly provided by Loredano Pollegioni, University of Insubria) (Job et al. 2002) as a standard (Std; 5 µg, 42.66 kDa). **b** SDS-PAGE analysis of chromatography fractions. C.E. crude extract, LacM purified protein, Std reference standard protein, His₆-GO (5 µg, 42.66 kDa). **c** Zymogram analysis in semi-native conditions of the purified LacM with ABTS (1) or 2,6-DMP (2) as substrates. In **a**, **b**, LMW are molecular weight markers, from GE Healthcare Sciences, Little Chalfont, UK. From **a–c** LacM is indicated by arrow

Fig. 3 LacM characterization. **a** Effect of increasing concentrations of CuSO_4 (10, 50, 100, 250, 500, and 1000 μM) on LacM activity. Enzyme activities were measured with ABTS as substrate, at pH 4.0 and 25 °C, and are reported as values relative to the activity of 0.27 U/mg recorded in the absence of copper. This value was set as 1 and – fold increase of the activity at different concentrations of copper is presented. **b** Effect of pH on LacM activity at 25 °C on ABTS, in the presence of 50 μM CuSO_4 . Activities are reported as values relative to the activity of 2.4 U/mg protein (set as 100%) at pH 4.0. **c** Effect of temperature on LacM activity using ABTS as a substrate at pH 4.0 in the presence of 50 μM CuSO_4 . Enzyme activities are expressed as relative to the highest activity of 8.0 U/mg (set as 100%) recorded at 50 °C. **d** Stability of LacM at different temperatures. The recombinant enzyme was incubated at

40 °C (circles, continuous line), 50 °C (triangles, continuous line), 60 °C (squares, dashed line), or 70 °C (rhombuses, dotted line) up to 120 min. At regular intervals, enzymatic fractions were cooled down, and the residual activity towards ABTS assayed at 25 °C, pH 4.0 in the presence of 50 μM CuSO_4 . The activity recorded at time 0 in standard assay condition (25 °C, pH 4.0, 50 μM CuSO_4) is set as 100% (2.4 U/mg). **e** Effect of increasing concentrations of NaCl on LacM activity was measured using ABTS. The activity measured at pH 4.0 and 25 °C, in the presence of 50 μM CuSO_4 and without NaCl (2.4 U/mg) was set as 100%. **f** LacM decolorization of Reactive Black 5 (circles) or Brilliant Green (squares) in the presence (black marks, continuous line) or absence (white marks, dotted line) of 10 μM ABTS. a–f values represent the means of three independent experiments (mean $\pm$ standard error)

three substituted hydroxyl groups, respectively. LacM optimally oxidized both substrates at pH 4.0 with a specific activity of 57.0 U/mg on pyrogallol and 39.1 U/mg on pyrocatechol. Other substrates were oxidized much less efficiently. For

ABTS, which is a reference nonphenolic substrate commonly used for laccase and laccase-like enzyme characterization, the maximum activity was recorded at pH 4.0 (2.4 U/mg), while 2,6-DMP was better oxidized at pH 5.0 (2.1 U/mg): LacM

Fig. 4 LacM activity versus pH on **a** vanillic acid, **b** L-DOPA, **c** 2,6-DMP, **d** syringaldazine, **e** pyrocatechol, and **f** pyrogallol. Enzyme activities were measured 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 means of three independent experiments (mean $\pm$ standard error)

oxidized 2,6-DMP in a wide pH interval from 3.0 to 8.0 (Fig. 4c). Finally, the highest activities on L-DOPA, syringaldazine, and vanillic acid, at pH 4.0, were respectively 2.9, 2.4, and 1.0 U/mg. Other methoxy-substituted phenolic compounds like guaiacol, syringic acid, ferulic acid, and syringaldehyde, as well as tyrosine and the non-phenolic $\text{K}_4\text{Fe}(\text{CN})_6$, were not oxidized by LacM.

Kinetic parameters

Kinetic parameters of LacM were determined for two reference laccase substrates, ABTS and 2,6-DMP, and for pyrogallol, which was the preferred LacM substrate. The initial reaction rates at various substrate concentrations were measured at the pH optimum for each substrate, as in Table 2. The results confirmed those described above: The lowest K_M value (0.13 mM) was found for pyrogallol; consistently, the turnover rate (k_{cat}) and the catalytic efficiency (k_{cat}/K_M) values on this substrate were the highest (22.4 s^{-1} and 173.6 $\text{s}^{-1} \text{mM}^{-1}$, respectively). Catalytic efficiency for pyrogallol was 20.5 times and 27 times higher than that for ABTS and 2,6-DMP, respectively.

Table 2 Kinetic properties of LacM on ABTS, 2,6-DMP, and pyrogallol

Substrate	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M ($\text{s}^{-1} \text{mM}^{-1}$)
ABTS	0.71	6.0	8.45
2,6-DMP	1.04	6.7	6.42
Pyrogallol	0.13	22.4	173.6

Temperature optimum and thermal stability

LacM could oxidize ABTS in a range of temperatures (Fig. 3c), with the highest oxidation rate recorded at 50 $^{\circ}\text{C}$ (3.5-fold higher than the one recorded at standard conditions, i.e., 25 $^{\circ}\text{C}$). At 40 and 60 $^{\circ}\text{C}$, the enzyme maintained 65 and 56% of the maximum activity, respectively, whereas at 30 $^{\circ}\text{C}$ and 70 $^{\circ}\text{C}$, the activity of LacM was slightly below 40%. Furthermore, activity of LacM was severely reduced below 20 $^{\circ}\text{C}$. LacM was not only thermostable in the range 40 to 60 $^{\circ}\text{C}$ (Fig. 3d), but it displayed a substantial heat activation, since after 2 h at 40, 50, or 60 $^{\circ}\text{C}$, its enzymatic activity increased, and it was approximately 1.2, 3.5, and 1.2-fold higher than the initial activity. However, after 2 h at 70 $^{\circ}\text{C}$ LacM retained only 28% of the initial activity.

Effect of potentially inhibitory compounds

The effect of several compounds on LacM activity was then investigated. Strong inhibition by halide anions is a common feature of laccase and laccase-like enzymes (Xu 1997; Baldrian 2006); hence, the effect of chloride on LacM activity was initially determined using NaCl as halide donor. Interestingly, between 95 and 85% of LacM activity was preserved in the presence of NaCl concentrations up to 100 mM, and 34 and 10% residual activities were still detectable after enzyme incubation with 500 and 1000 mM NaCl (Fig. 3e). Similarly, addition of 100 mM KCl reduced LacM activity by only 25% (Table 3). MgCl_2 , CaCl_2 , and ZnCl_2 were more powerful inhibitors than NaCl or KCl, probably because of the higher molar content of chloride anions. MgSO_4 and

Table 3 Characteristics of LacM. The enzyme activity is reported as relative to the activity of 2.4 U/mg protein (set as 100%), measured on ABTS at pH 4.0 and 25 °C, with 50 μ M CuSO₄

Compound	Final concentration	Relative activity (%)	Compound	Final concentration	Relative activity (%)
Control		100			
Salts (mM)					
CaCl ₂	10	98.1 ± 1.0	K ₂ SO ₄	10	169.0 ± 2.0
	50	66.9 ± 1.5		50	131.0 ± 1.0
	100	15.3 ± 8.6		100	71.0 ± 2.0
MgCl ₂	10	58.7 ± 2.3	ZnSO ₄	10	81.0 ± 1.5
	50	24.1 ± 1.3		50	<i>n.d</i>
	100	0.0 ± 0.0		100	57.0 ± 1.5
MgSO ₄	10	123.0 ± 1.0	ZnCl ₂	10	63.0 ± 2.0
	50	<i>n.d</i>		50	35.0 ± 5.5
	100	72.0 ± 1.5		100	26.0 ± 5.0
KCl	10	81.0 ± 10.5	CoNO ₃	10	141.0 ± 3.0
	50	<i>n.d</i>		50	71.0 ± 1.0
	100	75.0 ± 7.5		100	63.0 ± 6.5
EDTA (μM)					
	20	96.0 ± 5.0			
	50	19.0 ± 4.5			
	80	9.0 ± 3.5			
	100	0.0 ± 0.0			
Enzyme inhibitors					
NaN ₃ (μM)	25	99.0 ± 4.5	DTT (mM)	10	1.4 ± 0.8
	50	62.0 ± 4.5			
	100	38.0 ± 5.0			
	150	0.0 ± 0.0			
Organic solvents (% v/v)					
Ethanol	1	117.0 ± 1.0	Methanol	1	143.0 ± 1.5
	10	95.0 ± 2.0		10	154.0 ± 3.0
Isopropanol	1	137.0 ± 1.5	DMSO	1	96.0 ± 1.5
	10	91.0 ± 4.0		10	48.0 ± 4.5
Detergents (% w/v)					
Nonidet P-40	1	80.0 ± 7.0	Triton X-100	1	39.0 ± 0.0
	10	26.0 ± 6.0		10	0.0 ± 0.0
Tween-20	1	85.0 ± 1.7	NLS	1	88.0 ± 3.5
	10	0.0 ± 0.0		10	15.0 ± 5.5

n.d not detected

ZnSO₄ inhibited LacM activity to a smaller extent than chloride salts, while at low concentrations K₂SO₄ and CoNO₃, as well as MgSO₄ even increased the enzyme activity. The chelating agent EDTA (at 0.1 mM) completely inhibited LacM (Table 3), indicating the reversible nature of copper coordination within the active site of the enzyme.

Known enzyme inhibitors like sodium azide and DTT strongly inhibited LacM. The inhibitory effect caused by NaN₃ may be due to the binding of N₃[−] to the metal ion sites of LacM, which can disrupt the internal electron transfer of the catalysis process (Chen et al. 2015). DTT, instead, is likely to inhibit LacM activity through the reduction of oxidized

substrate by their sulfhydryl groups (Chen et al. 2015). Among the surfactants tested, Nonidet P-40, Tween-20, and NLS considerably reduced LacM activity only at 10% v/v final concentration, while with 1% v/v detergent, more than 80% of the original activity was preserved. LacM was more strongly affected by Triton X-100: At 1% v/v final concentration, the activity was reduced to approx. 40%, and at 10% v/v it was completely absent.

Finally, in order to verify the potential for using LacM in the processes requiring organic solvents, for example for the solubilization of lignin, the effect of a panel of organic solvents on enzymatic activity was investigated (Table 3). At 1%

v/v ethanol, isopropanol, and methanol, the activity of LacM increased. Interestingly, the positive effect of methanol was even higher at the final tested concentration of 10% v/v. In the presence of DMSO, the activity decreased, yet almost 50% of the initial activity was retained at 10% v/v DMSO.

Dye decolorization

Enzymatic decolorization of six synthetic dyes by the purified LacM was assessed in the presence or absence of 10 μ M ABTS as a redox mediator. The highest decolorization was found for the azo dye Reactive Black 5: After 48 h of incubation, the decolorization percentage was 92 and 43% in the presence or absence of ABTS, respectively (Fig. 3f). In contrast, the decolorization of the triphenylmethane dye Brilliant Green was higher in the absence of redox mediator, being 45 and 18% after 48 h without and with ABTS, respectively (Fig. 3f). The other dyes were not decolorized.

Discussion

This study describes the discovery, production, and characterization of LacM, a novel bacterial LMCO obtained by metagenomics performed on acid bog soil. We succeeded in recovering a full-length gene directly from the soil, which was previously indicated as a rich source of bacterial laccase-like genes (Ausec et al. 2011b). Only a few bacterial laccases and laccase-like enzymes have been identified through metagenomics: Lac15 and Lac21 from a marine microbial metagenomic library (Fang et al. 2011, 2012b), RL5 and Lac51 from a bovine rumen microflora (Beloqui et al. 2006) and from a giant panda fecal microbiome (Fang et al. 2012a), respectively, and Lac591 from a mangrove soil metagenome (Ye et al. 2010). LacM originates from acidic and highly organic bog soil. Even more importantly, based on sequence similarity, the new enzyme comes from a bacterium affiliated to the ubiquitous and still elusive bacterial phylum Acidobacteria. Indeed, the LacM sequence showed the highest identity to an environmental fragment previously identified in molecular screening of the same soil (Ausec et al. 2011b), and its sequence is to some extent similar to that of putative copper oxidases from *Candidatus Solibacter usitatus*. This organism clusters to subdivision 3 of Acidobacteria and is the only representative of this subdivision whose genome has been sequenced (Ward et al. 2009). Acidobacteria are among the most abundant microorganisms found in soil ecosystems and are also widely distributed in high-latitude terrestrial ecosystems as well as in wetlands (Mannisto et al. 2007; Pankratov et al. 2008; Campbell et al. 2010). Although ubiquitous in the environment, the ecological role of the different members of the Acidobacteria is poorly understood. Members of this phylum are highly diverse. They

are also relatively difficult to cultivate, and thus only a few strains have been isolated and characterized (Nunes da Rocha et al. 2010; Mannisto et al. 2011; Janssen et al. 2006; reviewed in Kielak et al. 2016), or their genomes sequenced (Ward et al. 2009). Thus, LacM is the first LMCO and one of the very few enzymes of acidobacterial origin that have been extensively characterized.

The production of active LacM in sufficient amount for its biochemical characterization was not straightforward. *E. coli* has been predicted to readily express only 40% of environmental genes (Gabor et al. 2004). Furthermore, *E. coli* is not the most efficient microbe when it comes to secretion of proteins, and it tends to segregate heterologous proteins in inactive form into inclusion bodies (Hjort et al. 2014; Cretoiu et al. 2015). Consequently, relatively few enzymes from metagenomic sources have thus far been purified in milligram amounts (Ye et al. 2010; Beloqui et al. 2006; Berini et al. 2017), allowing only for a limited biochemical characterization. Here, the recombinant LacM often precipitated in inclusion bodies, making it inactive and thus not appropriate for downstream analyses. This was contrary to our expectations based on a preliminary *in silico* analysis of the LacM amino acid sequence using the model proposed by Diaz et al. (2010), which predicted a high chance of solubility (> 80%) if overexpressed in *E. coli*. Interestingly, Fang et al. (2014) reported that the addition of the His-Tag was evaluated as the main reason for fold disturbance and IB formation in the case of the marine metagenome-sourced bacterial laccase Lac15, when expressed in *E. coli*. Even if the His-Tag is the most popular fusion tag used in recombinant protein purification, and it has been often observed to prevent the formation of IBs (Sørensen and Mortensen 2005), we cannot rule out that it might contribute to IB formation in the case of LacM. Certainly, LacM is not the first problematic LMCO in this respect, as other examples of recombinant bacterial laccases accumulated in *E. coli* into IBs are reported by Martins et al. (2002), Fang et al. (2011), Mollania et al. (2013) and Ihssen et al. (2015). In spite of our efforts, including attempts for LacM solubilization from the inclusion bodies (data not reported here), the enzyme was poorly recovered in an active form, yet—in the end—sufficiently for allowing a biochemical characterization and comparison with other bacterial LMCOs.

The substrate range of laccases and laccase-like enzymes may vary due to differences in shape and chemical composition of the enzyme substrate-binding site. In the case of phenolic substrates, the type, number, and position of chemical substitutions may influence the activity of the enzymes (Margot et al. 2013; Ihssen et al. 2015). LacM was able to oxidize a variety of phenolic substrates with the highest specific activities recorded for pyrogallol and pyrocatechol, which are compounds with three and two hydroxyl groups, respectively. Among previously characterized bacterial laccases and laccase-like enzymes,

high specificity towards these two products (that could be linked to lignin degradation) was reported only for an enzyme from *Streptomyces lavendulae* (Suzuki et al. 2003). LacM catalytic efficiency on pyrogallol was $173.6 \text{ s}^{-1} \text{ mM}^{-1}$. Unfortunately, we could not compare this result to the one from *S. lavendulae*, since kinetic parameters for pyrogallol were not reported for the latter. The lack of activity on tyrosine indicated that LacM was a laccase and not a tyrosinase, which is important since substrate specificities in these two groups of enzymes can overlap. Furthermore, LacM could oxidize syringaldazine, which is commonly oxidized by laccases but not by tyrosinases or peroxidases (Fang et al. 2012a). Based on the amino acid sequence comparison and 3D structure model analysis, we conclude that LacM is not a metallo-oxidase such as CueO from *E. coli* (Roberts et al. 2003; Kataoka et al. 2007). LacM does not have the methionine-rich region that was suggested to be predictive for metallo-oxidases and whose absence helps distinguishing metallo-oxidases from true laccases (Fang et al. 2012b).

ABTS and 2,6-DMP are most commonly used for laccase and laccase-like enzyme activity determination. For this reason, we analyzed the kinetic parameters of LacM using these two substrates. Compared to metagenomic Lac21 (Fang et al. 2012a) that oxidized only 2,6-DMP, LacM oxidized both substrates. LacM, however, had lower catalytic efficiency (k_{cat}/k_M value of $6.42 \text{ s}^{-1} \text{ mM}^{-1}$) on 2,6-DMP compared to Lac21 (k_{cat}/k_M value of $82.5 \text{ s}^{-1} \text{ mM}^{-1}$). If compared to the metagenomics-derived Lac591 (Ye et al. 2010), LacM showed higher activity on the non-phenolic ABTS ($k_{\text{cat}}/k_M = 0.47 \text{ s}^{-1} \text{ mM}^{-1}$ for Lac591, $8.45 \text{ s}^{-1} \text{ mM}^{-1}$ for LacM), but lower activity on the phenolic 2,6-DMP ($k_{\text{cat}}/k_M = 283 \text{ s}^{-1} \text{ mM}^{-1}$ for Lac591). LacM was less active on both ABTS and 2,6-DMP than CotA-like laccases from *Bacillus coagulans* (Ihssen et al. 2015) and *B. pumilus* (Reiss et al. 2011). It showed lower catalytic efficiency on ABTS than the CotA-like laccase from *B. clausii* (Ihssen et al. 2015) and SilA from *Streptomyces ipomoea* (Molina-Guijarro et al. 2009), but on 2,6-DMP, its activity was comparable or even higher than the two last mentioned enzymes. LacM was more active on both ABTS and 2,6-DMP than the recently described one from the alkaliphilic bacterium *Thioalkalivibrio* sp. (Ausec et al. 2015) and less active than RL5 from the bovine rumen microflora (Beloqui et al. 2006). One should, however, be cautious when making direct comparisons of activity of recombinant multicopper oxidases, since it is known that their overexpression in *E. coli* frequently leads to copper-depleted proteins with important effects on enzyme activity (Durão et al. 2008). Probably only a fraction of the enzyme molecules produced had indeed four copper atoms in the active site, which is in our case supported by the fact that addition of copper was essential for active protein production and purification, and significantly enhanced its activity if added to the reaction mixture.

A general tendency of bacterial compared to fungal laccases and laccase-like enzymes is their activity in a wider pH range (Reiss et al. 2013; Brander et al. 2014). For LacM, this tendency was especially evident for 2,6-DMP, pyrogallol, and pyrocatechol, where activity remained high at pH 8.0. In contrast, LacM was active on ABTS in the pH range 4.0–5.0, similarly to what was found for *Bacillus* spp. (Brander et al. 2014; Guan et al. 2014; Martins et al. 2002; Reiss et al. 2011) and *Streptomyces* spp. laccases (Margot et al. 2013; Dubè et al. 2008; Reiss et al. 2013). The activity of LacM at broad pH range can be useful for future applications where neutral or slightly alkaline pH is desirable, including for instance oxidation of natural and synthetic dyes, bioremediation strategies at neutral pH, and organic synthesis reactions (Brander et al. 2014).

The thermo-stability of bacterial laccases is another key property making them attractive in diverse applications where protein lifetime is of major importance (Suzuki et al. 2003; Martins et al. 2002; Guan et al. 2014; Ihssen et al. 2015). LacM proved to be a thermo-tolerant enzyme with an optimum at 50 °C, maintaining a relatively high activity in a wide range of temperatures from 25 to 70 °C. Interestingly, LacM was activated by incubating it for 1 or 2 h at 50 °C, which differentiates it from some other thermostable bacterial laccases including CotA (Suzuki et al. 2003; Martins et al. 2002).

Additionally, LacM exhibited a high tolerance towards salts (e.g., preserving 34% activity at 500 mM NaCl). LacM activity was even increased by adding MgSO_4 , CoNO_3 , and K_2SO_4 , up to 10, 10, and 50 mM, respectively. Enzyme activation by some of these ions has been reported only in a few bacterial laccases (Molina-Guijarro et al. 2009; Fang et al. 2011; Sondhi et al. 2014). Eukaryotic laccases are generally sensitive to halides, being most fungal laccases inactivated by NaCl at concentrations higher than 100 mM (Guan et al. 2014; Jimenez-Juarez et al. 2005). Hence, bacterial laccases showing halide tolerance recently attracted much attention for their application in wastewater treatment (Molina-Guijarro et al. 2009; Jimenez-Juarez et al. 2005; Guan et al. 2014; Fang et al. 2011, 2012b). The high resistance of LacM towards NaCl may be advantageous for its application in the treatment of both municipal wastewater (where the NaCl concentration is typically 2.5–5 mM) and industrial wastewater or seawater (with NaCl concentrations up to 550 mM) (Margot et al. 2013). Interestingly, the addition of alcohols, in particular methanol, seemed to even increase LacM activity and that property might be relevant in applications that require getting access to insoluble substrates, such as the detoxification of persistent organic pollutants. Fungal laccases are generally inactivated in organic solvents, whereas bacterial solvent-resistant laccases are known. Less common is the activation by methanol seen in LacM (Guan et al. 2014). Considering the robustness of LacM, we tested its potential for bio-bleaching

of industrial textile dyes. Biological treatment of textile dyes in industrial wastewater requires thermostable enzymes to remain active even under alkaline pH conditions and/or in the presence of high concentrations of organic solvents (Guan et al. 2014). After a preliminary screening of synthetic dyes, LacM was found to constitute a potential candidate agent for decoloring azo dyes and triphenylmethane dyes in the presence or absence of redox mediators.

To conclude, in addition to the interesting biochemical characteristics (such as high salt and thermo- and solvent-tolerance) and possible “green” applications, the metagenomic origin and affiliation with Acidobacteria is at present one of the most interesting features of LacM. Even more, its sequence shows the highest resemblance to laccase-like gene fragments identified during the molecular screening of wetland soils (Ausec et al. 2011b) that served as the basis in this search for bacterial LMCOs by metagenomics. Therefore, the present study also makes a case of metagenomics fulfilling its promise.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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