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A PHYLOGENETIC COMPARISON AND CLASSIFICATION OF LMCO SEQUENCES RETAINED FROM SOIL METAGENOME

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Abstract

Laccases (EC 1.10.3.2) belong to the family of multicopper oxidases (MCOs) and have the ability to oxidise various phenolic and nonphenolic compounds. In recent years, interest in bacterial laccases has increased because they have more favourable reaction features when compared to fungal laccases, such as their thermostability, alkaline pH, short acquisition time and simple overexpression system, making them highly desirable for industry. There is increasing molecular evidence that laccase-like multicopper oxidases (LMCOs) are widely distributed in bacteria. In this study, a total of 228 sequences of 3-domain LMCOs from a metagenome were used for phylogenetic analysis. A BLAST analysis and the reconstruction of the phylogenetic tree allowed to distinguish several LMCO sequence clusters, such as *Alphaproteobacteria, Gammaproteobacteria, Deltaproteobacteria, Acidobacteria, Actinobacteria, Bacteroidetes* and a cluster of unclassified sequences. The results of the phylogenetic analysis also suggest that the bacterial class of *Deltaproteobacteria* may play an important role in the lignin degradation process.

Introduction

Laccases (EC 1.10.3.2) belong to the family of multicopper oxidases (MCOs), which are able to oxidise various phenolic and non-phenolic compounds by converting an oxygen molecule into water, with or without the presence of various mediators (CAÑAS and CAMARERO 2010, GIARDINA et al. 2010;). These enzymes are classified as monomeric enzymes that have three cupredoxin-like domains and four characteristic histidine-rich copper-binding sites in the first and third domains. However, recent studies

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have shown that some laccases from the phylum *Actinobacteria* can form a separate group of MCOs with two domains (FERNANDES et al. 2014). In addition, the cupredoxin superfamily consists of many other oxidase enzymes, such as manganese oxidase and ascorbate oxidase. Due to their slightly different biochemical properties and imprecisely defined biological functions, it also seems justified to distinguish the LMCO-laccase-like multi-copper oxidases from the MCOs (BRANDER et al. 2014).

Laccases have been known since the 19th century when they were first discovered in the lacquer of the Japanese tree *Toxicodendron vernicifluum* (YOSHIDA 1883). Due to their oxidative abilities, laccases and laccase-like LMCOs have become sought after in the textile, pulp and paper and food industries. They are also used in the development of biosensors and biofuel cells and can be used as medical diagnostic tools and in bioremediation. The industry mainly uses laccase from fungi, especially white rot fungi such as *Trametes versicolor* (SHRADDHA et al. 2011). However, the industrial extraction of laccases from fungi is hampered by the long fermentation time, the low laccase yield and their applicability only under mesophilic and acidic reaction conditions (VISWANATH et al. 2014). In recent years, bacterial laccases have gained increasing interest due to their more favourable reaction features when compared to fungal laccases. Their thermostability, alkaline pH, short acquisition time and the ease with which they can be cloned and expressed have made them highly desirable in industry (WANG and ZHAO 2016b).

The first bacterial laccase was found in the bacterium *Azospirillum lipoferum* associated with plant roots (GIVAUDAN et al. 1993). Since then, laccases have been found mainly in gram-positive and gram-negative bacteria (CHAUHAN et al. 2017), and a new laccase-like enzyme was developed by screening after it was isolated directly from bacterial strains (SIROOSI et al. 2016, REZAEI et al. 2017). Heterologous expression of laccase-encoding genes from bacterial strains was also successfully implemented (MATHEWS et al. 2016, SUN et al. 2017). Several bacterial laccases have already been used on an industrial scale, particularly in the fields of wood, biofuels, paper, textiles, fine chemicals and food (ZERVA et al. 2020).

The laccase and laccase-like oxidation reaction of MCOs, releasing water as a by-product, is extremely tempting for industrial waste-free technologies. However, its implementation is hampered by the high cost of obtaining large quantities of the active enzyme with the desired substrate specificity and by the fact that relatively few bacterial laccases have been characterised to date, reinforcing the need to search for new laccases (IHSSEN et al. 2015, SHARMA et al. 2018).

Metagenomics is a valuable tool to discover new LMCOs, and soil seems to be an ideal source for their identification. Studies on soil bacterial communities carried out in 150 forest and 150 grassland soils have revealed the complexity and variability of the microbiological consortium in these environments, suggesting a potential richness of bacterial laccases (KAISER et al. 2016, YANG et al. 2018). In turn, culture-independent metagenomics is a powerful tool for discovering new enzymes from uncultured microorganisms and for gaining knowledge about the entire microbiological community through the direct extraction and cloning of DNA (HANDELSMAN 2004). The metagenomics library can be searched in two different approach. In the first, fuctional-besed approach, screening is based on the detection of expression products, which facilitates the identification of completely new enzymes. However, the probability of identifying a gene of interest depends mainly on the host-vector system and its abundance in the metagenome. It is a major challenge to discover a new enzyme using this approach, so the number of clones that have the desired activity is extremely low. The second approach involves screening metagenomic libraries based on the sequence of a target gene. Although this approach may somewhat limit the scope of novelty, bioinformatic analysis based on searching for phylogenetic markers or other similar sequences in exclusively existing public databases is warranted – especially when studying a heterogeneous group of enzymes such as bacterial laccase and LMCO (BERINI et al. 2017). The use of the metagenome to screen laccase has been little explored (KIMURA and KAMAGATA 2016, AUSEC et al. 2017, YUE et al. 2017), mainly due to the limitations of screening methods mentioned above. Nevertheless, metagenomics remains an attractive approach to identify and study the diversity of laccases and LMCO genes in the environment.

The aim of this study was the phylogenetic analysis of LMCOs genes from the forest soil metagenome library. This bioinformatic analysis allows us to distinguish the laccase-like coding fragments of different bacterial taxa and to reveal the species diversity of laccases in soil. This knowledge could be useful to understand the role of laccase in soil bioremediation or provide a way to predict the full gene sequence of LMCO, its expression and characterisation of enzyme activity for industry in the future.

Material and Methods

DNA isolation, DOP-PCR fragments library construction and sequencing

In previous studies, two soil samples (forest and fen) collected in April 2009 from areas within the city of Olsztyn (Poland) were characterized for multicopper oxidase activity based on FLOCH et al. (2007), and the soil samples were used for DNA library construction. The DOP-PCR reaction was performed to amplify laccase-like gene fragments of about 1200 bp in size (AUSEC et al. 2011a, ZIELIŃSKA and ADAMCZAK 2013). Briefly, the procedure involved isolation of metagenomic DNA using a GeneMATRIX Soil DNA Purification Kit (EURx, Gdansk, Poland), followed by the DOP-PCR reaction performed with degenerate primers specific for conservative laccase sites: Cu1AF 5'ACM WCB GTY CAY TGG CAY GG3' and Cu4R 5'TGC TCV AGB AKR TGG CAG TG3'(AUSEC et al. 2011a) with the following protocol: an initial denaturation at 94ºC for 3 min, 30 cycles of 30 s at 94ºC, 30 s at 48ºC, 1 min at 72ºC, ending with 5 min at 72ºC, 25 ml reactions contained 2.5 ml of each primer (20 μM), 1 ml of dNTP (40 μM each), 3 ml of $MgCl_2$ (25 mM), approximately 200 ng of DNA template, 5 ml of PCR buffer and 1 U of Taq DNA polymerase (GoTaq ® Flexi DNA Polymerase, Promega, Madison, Wisconsin USA).

The amplified DNA fragments were cloned into the pGEM-T vector and a metagenome library was prepared in *E coli* JM109 cells following the manufacturing protocol (Promega, Madison, Wisconsin, USA). Recombinant pGEM-T plasmids were isolated using the Plasmid Mini AX Kit (A&A Biotechnology, Gdansk, Poland) and inserts were amplified using the GoTaq Colourless Master Mix Kit (Promega, Madison, Wisconsin, USA) with primer T7 5'TAATACGACTCACTATAGGG3' and primer SP6 5'TATTTAGGTGACACTATAG3' according to the following protocol: an initial denaturation at 94ºC for 3 min, 30 cycles of 30 s at 94ºC, 30 s at 50ºC, 1 min at 72ºC, ending with 5 min at 72ºC, 50 ml reactions contained 5.0 ml of each primer (10 μM), approximately 250 ng of DNA template, 25 ml of PCR 2X Colourless GoTaq® Reaction Buffer each containing 400 μM dNTP, 3mM $MgCl_2$ and Taq DNA polymerase.

DNA fragments were sequenced from both ends using the Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) on a ABI PRISM 3730 DNA analyser (Applied Biosystems, Foster City, CA). All sequences were manually proofread and assembled using DNA Baser v4.36.0. The sixteen metagenome sequences isolated from one of the forest soil samples were used for computational analysis in this study.

Sequences and Phylogenetic Analysis

For this work, the Polish soil LMCO sequences were stored in GenBank under accession numbers MN558914-MN558929, and one sequence artifacts was excluded from this study. The sequences with accession numbers: HM045759-HM045777, HQ286736-HQ286789, which were isolated from bog and fen soil samples using a non-culturing method, were also used in this study (AUSEC et al. 2011a). A total of eighty-six sequences potentially encoding LMCOs and derived from soil metagenome libraries were used for further computational analysis. These sequences were used for a similar search for sequences from the NCBI database (https://www.ncbi.nlm.nih.gov/) using BLASTn with a non-redundant nucleotide database, BLASTX and BLASTp with non-redundant protein sequences or a reference protein sequence database.

Sequence manipulation and phylogenetic tree reconstruction was carried out using two methods. In the first case, sequences from the Polish soil metagenome library and similar sequences from the NCBI database were used for phylogenetic tree reconstruction (about two hundred sequences). The nucleotide sequences containing LMCOs (LMCO sequences from the Polish soil metagenome and homologous sequences from the database) were translated into amino acid sequences using Geneious Prime (KEARSE et al. 2012). The sequences were then trimmed to contain two Cu-binding regions (cbr II and cbr III) and a nucleotide sequence occurring between them. The trimmed sequences were aligned using the software MUSCLE 3.8.425 (EDGAR et al. 2004). Based on the alignment, Bayesian analysis was performed using MrBayes 3.2.1 (RONQUIST and HUELSENBECK 2003). The MCMC algorithm was run for 5,000,000 generations (sampling every 500) with four incrementally heated chains. The first 25% of the trees were discarded as burn-in. The remaining trees were used to generate the consensus tree.

For the second phylogenetic tree reconstruction, nucleotide sequences from the Polish soil metagenome were translated using the Translate tool-ExPASy (https://web.expasy.org/translate/) and trimmed together with LMCO sequences from the database and from bog and fen soils (AUSEC et al. 2011a) trimmed to contain two Cu-binding regions, as had been done previously, and aligned as amino acid sequences using ClustalW implemented in MEGA7 v. 7.021 (KUMAR et al. 2016). MEGA7 v. 7021 software was used for sequence manipulation and phylogenetic reconstruction, and the tree was inferred from the protein sequence alignment. The evolutionary history was inferred using the Maximum Likelihood method based on the model of Whelan and Goldman (LE and GASCUEL 2008). An initial tree was first built using a Neighbor-Joining method and

its branch lengths were adjusted to maximise the likelihood of the dataset for this tree topology under the desired evolutionary model. The tree was drawn to scale, with branch lengths measured in the number of substitutions per site. All positions with gaps and missing data were eliminated.

The reference sequences of MCOs with the accession numbers, used in the phylogenetic analysis were as follows: PZN23754.1, RIK29390.1, WP_104023078.1, TML46446.1, PZR04526.1, WP_018614744.1, TAK25694.1, AKU93578.1, TMM19967.1, WP_137260238.1, WP_141196114.1, WP_136580480.1, WP_013768667.1, TMB38487.1, WP_123122544.1, WP_013891894.1., TMI72789.1, WP_139795072.1, MAY01904.1, TFG53223.1, RTM09228.1, RPI54963.1, WP_053239053.1, WP_044985630.1, WP_012233433.1, WP_129347183.1, WP_116036636.1, WP_086086790.1, WP_093734497.1, WP_004212985.1, WP_037491563.1, WP_035530640.1, WP_107978106.1, WP_080919611.1, WP_071900668.1, WP_015345738.1, WP_087868494.1, WP_141900028.1, WP_080522328.1, WP_050726212.1, WP_081950100.1, WP_093038291.1, WP_100095845.1, WP_067560161.1, WP_028464233.1, WP_012563321.1, WP_089851963.1, WP_040402904.1, WP_007602744.1, WP_009503858.1, WP_068241745.1, WP_102248111.1, WP_119461512.1, WP_109518985.1, WP_027238842.1, WP_094301476.1, WP_100161006.1, WP_119682026.1, WP_076695202.1, WP_029375169.1, WP_013901958.1, WP_046135170.1, WP_114910975.1, WP_048878272.1, WP_109923174.1, WP_094407154.1, WP_035228630.1, WP_062761965.1, WP_124964904.1, WP_103257142.1, WP_077546320.1, WP_071796151.1, WP_132474996.1, WP_141850878.1, WP_133034171.1, WP_119776634.1, HBD97996.1, WP_020404328.1, WP_070796186.1, WP_009021358.1, WP_121881975.1, WP_011642144.1, WP_109261381.1, WP_104832397.1, WP_009800481.1, WP_095158341.1, WP_053550716.1, WP_119376372.1, WP_060090832.1, WP_022698546.1, WP_022693166.1, WP_058178341.1, WP_031296247.1, WP_131153852.1, WP_091462888.1, WP_124235827.1, WP_119286241.1, WP_139795588.1, WP_116570295.1, WP_083962369.1, WP_093943049.1, WP_133787361.1, WP_083711762.1, WP_108046919.1, WP_130461760.1, WP_114958078.1, WP_119274986.1, WP_112661640.1, WP_140943417.1, WP_128779026.1, WP_073631571.1, WP_092863561.1, WP_073052225.1, WP_131834895.1, RPI54963.1, WP_136918250.1, WP_138380708.1, WP_012240709.1, WP_146645271.1, WP_129581229.1, WP_104023078.1, WP_136934632.1, WP_147205331.1, WP_146645271.1, WP_018614744.1, WP_136580480.1, WP_013768667.1, WP_023765434.1, WP_135908040.1, WP_053239053.1, WP_116036636.1, WP_013768667.1, WP_143177680.1, WP_015345738.1, WP_146979921.1, WP_073684142.1, WP_083812524.1, WP_046362600.1, WP_067983101.1, WP_078981544.1, WP_101368345.1, WP_062285860.1, WP_135116536.1, WP_114073056.1,

WP_071100875.1, WP_113682993.1, WP_019747846.1, WP_130461760.1, WP_148798198.1, WP_131153852.1, WP_111472834.1, WP_125483648.1, WP_068016701.1, WP_123490970.1, WP_086134294.1, WP_013031520.1, WP_006927672.1, WP_073377490.1, WP_086091162.1, WP_116460059.1, WP_086086790.1, WP_131569804.1, WP_119461512.1, WP_145809072.1, WP_110909913.1, WP_053206700.1, WP_130025932.1, WP_012173860.1, WP_102071196.1, WP_011633152.1, WP_106859220.1, WP_129206354.1, WP_082125471.1, WP_008974103.1, WP_006927672.1, WP_116717539.1, WP_073377490.1, WP_012173860.1, WP_116460059.1, WP_026014869.1, WP_148474672.1, WP_035353113.1, WP_026447326.1, WP_035353113.1, WP_024537120.1, WP_011633152.1, WP_013519563.1, WP_015056770.1, WP_095605214.1, WP_076441808.1, WP_047639815.1, WP_139717470.1, WP_123769215.1, WP_128739836.1.

Conserved domain analysis

A CD-search tool was used to find conserved multicopper oxidase domains in the protein sequences (https://www.ncbi.nlm.nih.gov/). The Pfam database is a large collection of protein families, each represented by multiple sequence alignments and Hidden Markov Models (HMMs) (https://pfam.xfam.org/) (MARCHLER-BAUER et al. 2017, EL-GEBALI et al. 2019). SMART (Simple Modular Architecture Research Tool) was used as a second approach for protein domain search (http://smart.embl-heidelberg.de/) (LETUNIC and BORK 2018).

Results and Discussion

Motif/Domain analysis

In previous studies, sequences were isolated from the soil metagenome by specific primers for laccase-like genes (AUSEC et al. 2011b, ZIELIŃSKA and ADAMCZAK 2013). In this study, sixteen fragments from a soil sample, each about 1200 bp in length, were used for bioinformatic analysis. The sequences could be translated into protein sequences without stop codons. The amino acid sequences derived from the soil metagenome DNA were used to identify protein domains. Identification of the conserved domains by CD-Search tools against the CCD database showed that eleven sequences (Lac3D4, Lac3D5, Lac3D8, Lac3D10, Lac3D30, Lac3D23, Lac3D25, Lac3D18, Lac3D32, Lac3D33, Lac3D15) contained the superfamily domain Sulf (COG2132) typical of multicopper oxidase with three cupredoxin domains, indicating that they belonged to the fragments of laccase genes.

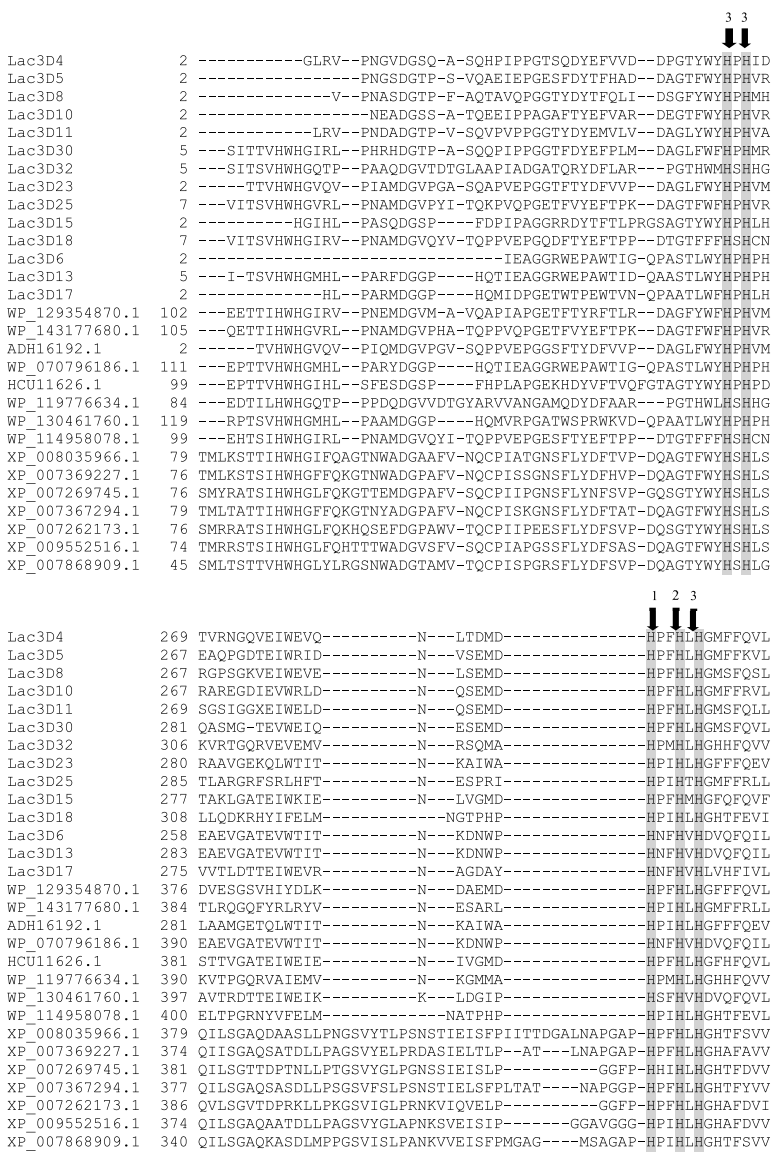

Fig. 1. Fragments of protein sequences alignment containing a two Cu-binding region (cbr II, cbr III) of sequences from Polish soil metagenome (Lac3D4-32), bacterial laccase: *Sorangium cellulosum* (WP_129354870.1), *Cystobacter ferrugineus* (WP_143177680.1), putative laccase partial uncultured bacterium (DH16192.1), *Rhodococcus erythropolis* (WP_070796186.1), *Gemmatimonadetes bacterium* (HCU11626.1), *Oleomonas sp.* K1W22B-8 (WP_119776634.1), *Plantactinospora* sp. CNZ321 (WP_130461760.1), *Rhizobiaceae bacterium* CPCC 101076 (WP_114958078.1); fungal laccases: *Trametes versicolor* FP-101664 SS1 (XP_008035966.1), *Dichomitus squalens* LYAD-421 SS1 (XP_007369227.1), *Fomitiporia mediterranea* MF3/22 (XP_007269745.1), *Dichomitus squalens* LYAD-421 SS1 (XP_007367294.1), *Fomitiporia mediterranea* MF3/22 (XP_007262173.1), *Heterobasidion irregulare* TC 32-1 (XP_009552516.1), *Gloeophyllum trabeum* ATCC 11539 (XP_007868909.1). Arrows and accompanying numbers indicate the amino acid residues for Cu-binding and the type of each ligand Cu. The histidine in copper-binding residues was marked on gray. The position of the first residue of each fragment in the polypeptide chain is indicated.

The sequences Lac3D6, Lac3D13 and Lac3D17 showed similarity to the multicopper oxidase domain belonging to the PRK10965 superfamily (cl35978). The domain of the cupredoxin superfamily domain (Cl19115) was identified for the sequence Lac3D33, while the domain of the copper_ res_A superfamily (cl36914) was found in the sequence Lac3D12.

However, searching for protein domains using the CD-Search tools against the Pfam database allowed the identification of the cupredoxin superfamily domain (cl19115) for all sequences analysed. All analysed sequences, with the exception of Lac3D33, showed significant similarity to Cu-oxidase_2 (pfam07731). The Cu oxidase (pfam00394) domain was identified in four sequences (Lac3D5, Lac3D8, Lac3D10 and Lac3D 11). The three domains Cu-oxidase_2 (PF07731), Cu oxidase (PF00394) and Cu-oxidase_3 (PF07732) were identified by the software SMART in all sequences except sequences in which two domains were found (Lac3D 33 – domains PF00394 and PF07732; Lac3D13, Lac3D17 – domains PF07731, PF07732). Regardless of the method used to identify the domains, domains/motifs typical of MCOs were detected in each of the sequences analysed. Alignment of the amino acid sequences using MEGA7 software revealed that the Lac12 sequences did not contain a particular motif characteristic of the copper-binding site typical of laccase, and therefore this sequence was excluded from further analysis. The alignment of the remaining amino acid sequences made it possible to distinguish two copper-binding sites (Cu-binding region; cbr II, cbrIII) characteristic of laccase enzymes. These copper-binding regions are involved in the formation of the active enzyme centre, which contains four copper atoms divided into types 1-3. These three types are partially present in the sequences alignment (Figure 1 Type 1-1 his, type 2 and 3-4 his). The copper-binding region designated cbr III (Figure 1) is typical for laccase *sensu stricto* from fungi and was detected in 14 analysed sequences. The sequences derived from the Polish soil metagenome can therefore be classified as coding laccase genes or rather as laccase-like MCOs, according to the classification of HOEGGER et al. (2006), which is based on research into the diversity of bacterial laccase-like MCO genes.

The results of a domain analysis confirmed the usefulness of the selected tools such as CD-Search and SMART for identifying LMCO domains of genes isolated from a metagenome. CD-Search uses BLAST for a quick scan of a set of precalculated position-specific scoring matrices (PSSMs) with a protein query, and although the BLAST database search method is still the most widely used (GUPTA et al. 2017), it provides less specific results. In turn, the software SMART, which implements methods using probabilistic models called Profile Hidden Markov Models (pHMMs)

and was used in this study together with a Pfam profile database, has been shown to be more specific for identifying Cu-binding regions. Although the pHMMs model of AUSEC et al. (2011a) has been successfully used for searching databases and identifying laccase genes, it appears to be of little use for the analysis of bacterial LMCOs from metagenome data. The pHMMs-based search of Global Ocean Survey data yielded numerous hits for prokaryotic laccases that closely matched the copper-binding regions of the models (277 and 847 sequences showed similarities to laccase-like sequences with two and three domains, respectively), but only 33% of the putative laccases with three domains could be assigned to bacteria (AUSEC et al. 2011a).

Sequence analysis of LMCO from the Polish soil metagenome

BLASTn analysis of the Polish soil metagenome sequences showed that only five sequences in the database had significant similarities, i.e. Lac3D6 and Lac3D13 showed identity with *Rhodococcus* spp. (99% identity), Lac3D12 with *Maricaulis maris* (71% identity), while Lac3D23 and Lac3D18 showed identity with uncultured bacteria (76% identity). To see how limited the database is in this area of knowledge, note that the Entrez database has 2.5 billion records at the time of writing (SAYERS et al. 2019). A BLASTx search for protein sequences made it possible to characterise the tested sequences in more detail and to qualify them as proteins belonging to multicopper oxidase or copper oxidase from cultured and uncultured bacteria. The two sequences were associated with the gene encoding multicopper oxidase from *Rhodococcus* ssp. (*Actinobacteria*) (Lac3D6 – 100%, Lac3D13 – 99%), which is consistent with the results of BLASTN. This species is present in the soil, which was confirmed in the research carried out by AUSEC et al. (2011a), and the LMCOs from the genus *Rhodococcus* show interesting properties. For example, the actinomycete *Rhodococcus ruber* (designated C208; EC1.10.3.2.) was found to be able to recycle and degrade polyethylene at a high temperature (70°C), allowing recycling in view of the growing waste problem (SANTO et al. 2013).

The five sequences from the soil metagenome (Lac3D32, Lac3D15, Lac3D17, Lac3D18, Lac3D23) were associated with the gene encoding multicopper oxidase from *Oleomonas* sp., the bacterium *Gemmatimonadetes, Plantactinospora* sp. and the bacterium and uncultured bacterium clone, with a positive amino acid hit rate of over 58%. Six sequences were affiliated to a multicopper oxidase from *Sorangium cellulosum* with an amino acid hit rate below 40% in a BLASTX and BLASTp search. AUSEC et al. (2011a) came to similar conclusions. They found seven sequences

showing similarity to a multicopper oxidase from *Sorangium cellulosum*, but with a positive amino acid hit rate of about 60%. Bioinformatic analysis conducted in 2011 (AUSEC et al. 2011b) revealed the high diversity of bacterial genes for laccase-like enzymes and found that *Sorangium cellulosum* So ce 56 has eight genes in its chromosomes, with a two-domain laccase in each genome, and that the other enzymes are the three-domain laccases. Similarly, as mentioned earlier, two sequences of LMCO genes were obtained from *Rhodococcus* ssp., as mentioned earlier, which could also be related to the presence of multiple genes in this bacterial genus, as, for example, seven genes were detected in *Rhodococcus erythropolis* and five genes in *Rhodococcus opacus* and *Rhodococcus jostii* (AUSEC et al. 2011b).

Multiple laccase genes have been confirmed to be found in many organisms, including bacteria, fungi and plants, and this phenomenon is thought to be related to biological function (JANUSZ et al. 2020). For example, in the filamentous ascomycete *Gaeumannomyces graminis var. tritici*, three laccase genes (LAC1, LAC2 and LAC3) are able to oxidise or reduce polyphenolic compounds such as lignin or melanin precursors, depending on the redox potential of the environment (LITVINTSEVA and HENSON 2002). In addition, a multifunctional laccase gene family in cotton (*Gossypium* spp.) has been shown to play an important role in cotton fibre development (BALASUBRAMANIAN et al. 2016). However, it has been suggested that bacterial laccase-like genes are more involved in laccase activity than their fungal counterparts (CHAUHAN et al. 2017). The studies on multiple homologous laccase-encoding genes of *Achromobacter xylosoxidans* HWN16, *Citrobacter freundii* LLJ 16 and *Pleurotus nebrodensis* have shown that these genes might be responsible for the properties of laccases, such as optimal temperature, pH, thermal stability, pH stability or increased enzymatic activity under the influence of certain concentrations of fluoride (YUAN et al. 2016,UNUOFIN et al. 2019). However, the exact function of all bacterial laccase genes discovered so far has not yet been elucidated, as this requires time-consuming studies using an analysis of the overexpressed target genes, as was the case for the *Cot*A laccases from *Bacillus subtilis* or the recently characterised novel laccase gene (Lcc1) from *Ganoderma tsugae* (JIN et al. 2018). Nevertheless, LMCOs genes from multiplate bacteria have previously been linked to pigment synthesis, oxidation of phenolic compounds, sporulation, UV and H2O2 resistance, Cu^{2+} resistance and morphogenesis (CHAUHAN et al. 2017).

In summary, the sequence analysis of the LMCO genes suggests that most of the metagenome-derived partial genes belonged to unknown bacterial species. And several of the LMCO sequences isolated from

Polish soil metagenome, bog and fen soils (AUSEC et al. 2011b) showed similarity to the LMCO gene of *Sorangium cellulosum*. This could be evidence that these LMCO genes belong to a single bacterial group that is strongly associated with the lignin degradation process regardless of the environment.

Phylogenetic analysis of LMCO sequences from the Polish soil metagenome

To identify and assess the biodiversity of LMCO genes in environmental samples, several primers based on copper-binding regions (cbr) conserved in both bacterial and fungal LMCO have been designed (CHAUHAN et al. 2017). The oligonucleotide primer Cu2R was first designed for the detection of cbr II from basidiomycete LMCOs (LUIS et al. 2004). The first primer Cu1AF aligns to the sequence encoding two histidines of cbr that have enabled the detection of bacterial LMCOs and was designed by KELLNER et al. (2008). These primers proved useful in the detection of LMCO genes from the DNA of various bacterial taxa by amplifying a fragment of 140 bp (KELLNER et al. 2008). In contrast, the CuR4 primer design within the cbr IV region of LMCO sequences with three domains demonstrated the richness of diversity of LMCOs with 2 and 3 domains in soils. And the combination of Cu1AF and CuR4 primers with fractionated fragment sequencing allowed the detection of LMCOs with a sequence length of 1200 bp, which is almost the total length of the LMCO gene (AUSEC et al. 2011a). This strategy was used to obtain LMCO genes for the phylogenetic analysis presented in this article. However, the diversity of the LMCO amino acid sequence proved to be rather large to allow a logical alignment. This problem was also noted by other authors, and HOEGGER et al. (2006) performed the alignment of 350 multicopper oxidases (MCOs) from fungi, insects, plants and bacteria, but the sequences were trimmed to the most conserved parts of the sequences. However, this restriction also reduced the resolution of the phylogenetic analysis. In this work, the studies focused exclusively on bacterial LMCOs, and the sequences were trimmed to include two Cu-binding regions (cbr II and cbr III), but the intervening sequence was retained, which is a novelty compared to the method used by other authors.

For the preliminary phylogenetic tree reconstruction, all fifteen sequences of LMCO from the Polish soil metagenome and reference sequences from the NCBI database were used. The Bayesian phylogenetic method was chosen to distinguish the LMCO sequences isolated from soil samples.

Fig. 2. Phylogenetic tree obtained by Bayesian inference analysis of 110 LMCOs sequences from Polish soil metagenome and sequences from GenBank in NCBI, within two Cu-binding regions (cbr II and cbr III). Node numbers represent Bayesian posterior probability values

This statistical method is closely related to the Maximum Likelihood method, but could be a faster method to assess support for trees than Maximum Likelihood bootstrapping. However, in the preliminary study, it was not possible to obtain a phylogenetic tree with a good Bayesian posterior probability value (data not shown). To circumvent this problem, the Lac3D12 sequence with its similar reference sequences was excluded for further analysis, so that this sequence does not belong to the true laccase gene. Finally, a phylogenetic analysis of fourteen sequences from the Polish soil metagenome classified as laccase coding genes was performed, together with ninety-six reference sequences obtained from GenBank in NCBI. Most of the sequences from the Polish soil sample were affiliated to a specific cluster with a good Bayesian posterior probability value (> 0.9) – Figure 2.

The affiliation to *Actinobacteria* (NCBI:txid1760) was determined for three sequences, namely Lac3D6, Lac3D13 and Lac3D17. In turn, the sequences Lac3D25 and Lac3D32 were assigned to two different clades belonging to the *Alphaproteobacteria* cluster (NCBI:txid28211). Most sequences were identified as belonging to the *Deltaproteobacteria* cluster (NCBI:txid28221), but Lac3D25 and the six following sequences – Lac3D5, Lac3D10, Lac3D14, Lac3D8, Lac3D30, Lac3D11 – were grouped in different clades. Interestingly, these six sequences of LMCOs that showed homology to *Sorangium cellulosum* in the BLAST search were assigned to the *Deltaproteobacteria* cluster (NCBI:txid28221) but were not merged in the phylogenetic analysis (Figure 2). The sequence Lac3D23 showed the highest similarity to the unclassified *Acidobacteria* (NCBI:txid1978231) when the sequences BLAST were aligned against the non-redundant database.

Phylogenetic analysis of LMCO sequences from three soil metagenomes

Analysis of the 16S rRNA gene is commonly used to assess species diversity, but the recent study using a concatenated protein phylogeny has shown that this approach is not unreliable (PARKS et al. 2018). Therefore, phylogenetic tree reconstruction based on the alignment of the LMCO amino acid of the soil metagenome was applied in this study. This approach resulted in a classification of the LMCO sequences of the soil metagenome into individual bacterial classes and revealed some trends in the bacterial diversity of soils, which are familiar with the results of other authors (WANG et al. 2016a, YUN et al. 2016).

In the preliminary study, the fifteen LMCOs sequences from the Polish soil metagenome and seventy-two LMCOs sequences isolated by AUSEC et al. (2011a) and similar sequences from the GenBank database were

used for the phylogenetic analysis. The Maximum likelihood methods was used for phylogenetic reconstruction. Likelihood-based methods aim to find the best topology by maximising the likelihood function with respect to the topology and branch lengths by comparing the posterior probabilities for the different possible topologies (SVENNBLAD et al. 2006). However, when aligning all LMCO sequences, two groups of LMCO sequences were distinguished, one of which belonged to the "true" laccases with 3 domains and was used for further analysis (data not shown).

The phylogenetic tree was reconstructed for 132 LMCOs from the Polish soil metagenome, for 3-domain LMCOs from bog and fen soil (AUSEC et al. 2011a) and for sequences from the GenBank database in NCBI obtained by a BLAST search against reference protein sequences (Figure 3). Cluster membership was confirmed for most sequences from the Polish soil metagenome when they were compared with the phylogenetic tree reconstructed by Bayesian inference analysis. Only for the sequence Lac3D23 were the results different, and this sequence showed the highest similarity to the assigned cluster *Deltaproteobacteria* (NCBI:txid28221). The difference in sequence affiliation of Lac3D23 was likely due to the sequence having the highest similarity (amino acid hit rate 71%) to multicopper oxidase from unclassified *Acidobacteria* (RPI54963.1), which was newly identified and not included in the reference protein sequence database. This false affiliation was not found for other sequences. Therefore, all sequences derived from the Polish soil metagenome, as well as those obtained by AUSEC et al. (2011a), were checked against a non-redundant database by a BLAST search. In addition, studies on different bacteria with lignin-degrading potentials isolated from two cabbage species showed that LMCOs from both *Proteobacteria* (NCBI:txid1224) and *Actinobacteria* (NCBI:txid1760) had high similarities (85.00-100.00%), suggesting that these genes might be from the same ancestor (WANG et al. 2016a). Therefore, it is difficult to clearly determine the affiliation of this sequence.

Among the LMCO sequences obtained by AUSEC et al. (2011a), a large cluster of 17 sequences, for which no references were identified, was also listed and linked to the sequences of laccases from various *Proteobacteria* (NCBI:txid1224) using BLASTx (Figure 3). The phylogenetic analysis presented in this paper made it possible to match a part of the sequence to be assigned at the class level, and two clusters of *Alphaproteobacteria* (NCBI:txid28211) (I, II) and *Deltaproteobacteria* (NCBI:txid28221) (I, III) and one cluster of *Gammaproteobacteria* (NCBI:txid1236) (I) were highlighted (Figure 3). Similar to the study by AUSEC et al. (2011a), a large cluster of sequences was indicated to have similarity to *Acidobacteria* (NCBI:txid57723) LMCO (Figure 2). Four sequences were assigned to the

Fig. 3. Unrooted phylogenetic tree of the 132 amino acid sequences of LMCOs obtained from Polish forest soil metagenome, bog and fen metagenome (AUSEC et al. 2011a) and sequences from GenBank in NCBI. Tree was bootstrapped using 1000 replications

Bacteroidetes (NCBI:txid976) cluster, which contradicts the results of AUSEC et al. (2011a). Three sequences of LMOC resembled the laccase-like genes of *Actinobacteria* (NCBI:txid201174), although they were previously affiliated to *Verrucomicrobia* (NCBI:txid74201) (Figure 3) (AUSEC et al. 2011a). Of all sequences identified as LMCO, 7.57% were not assigned to any bacterial phylum, as confirmed by BLASTx and BLASTp analysis, and the sequences had amino acid similarity below 40%. The differences in the

phylogenetic analysis result from the fact that the GenBank NCBI database has been enriched in recent years, especially with the sequences belonging to the uncultured bacterium, which is due to the metagenomics studies (FERRER et al. 2010). A new approach using a concatenated protein phylogeny as the basis for a bacterial taxonomy that conservatively removes polyphyletic groups and normalises taxonomic ranks based on relative evolutionary divergence has resulted in changes to the Genome Taxonomy Database, i.e. 58% of the 94,759 genomes have had changes made to the existing taxonomy. These changes have not omitted the phylum *Proteobacteria* (NCBI:txid1224), within which the subdivision into the subphylum delta/epsilon has been formed, containing the class *Deltaproteobacteria* (NCBI:txid28221). There were also changes within the FCB group, *Bacteroidetes* (NCBI:txid976) and *Actinobacteria* (NCBI:txid1760) (PARKS et al. 2018).

Interestingly, several LMCO sequences from Polish bog and fen metagenomes were assigned to the same clusters of *Deltaproteobacteria* (NCBI:txid28221) and *Alphaproteobacteria* (NCBI:txid28211), which might indicate the particular role of these bacteria in the soil environment and in the bioremediation process. This hypothesis was confirmed by the research carried out by WILHELM et al. (2019). The authors use metagenomic and quantitative stable isotope probes to identify and characterise the functional properties of lignin, cellulose and hemicellulose degrading fungi and bacteria in coniferous forest soils. Overall, high bacterial degradation of the model lignin substrate was found, particularly by gram-negative bacteria from the *Alphaproteobacteria* (NCBI:txid28211) and *Deltaproteobacteria* (NCBI:txid28221) groups. The existing evidence for bacterial lignin degradation by alpha and gamma-proteobacteria shows that it is necessary to characterise bacterial populations and determine their role in different soil environments in order to better understand the processes controlling the degradation of lignocellulose in situ.

Knowledge of the potential impact on soil bacterial diversity is also desirable so that the bacteria and their metabolites desired by industry can be identified in the future. A recent study highlighted that possible climate and land use changes (soil type, carbon source, precipitation patterns) affect the abundance and diversity of soil bacteria (BICKEL and OR 2020). A detailed study demonstrated the dominance of individual bacterial classes in relation to pH and soil type in karst areas, with surface soils dominated by *Acidobacteria* (NCBI:txid57723), *Verrucomicrobia* (NCBI:txid74201) and *Planctomycetes* (NCBI:txid203682). In turn, *Nitrospirae* (NCBI:txid40117), *Gemmatimonadetes* (NCBI:txid142182), *Firmicutes* (NCBI:txid1239) and *Chloroflexi* (NCBI:txid200795) occurred only in

cave sediments, while *Actinobacteria* (NCBI:txid1760) and *Proteobacteria* (NCBI:txid1224) dominated in weathered rock and drip water, respectively (YUN et al., 2016). Metagenomic analysis of 16S rRNA genes revealed that seven groups, including *Alphaproteobacteria* (NCBI:txid28211), *Gammaproteobacteria* (NCBI:txid1236), *Deltaproteobacteria* (NCBI:txid28221), *Betaproteobacteria* (NCBI:txid28216), *Acidobacteria* (NCBI:txid57723), *Actinobacteria* (NCBI:txid1760) and *Bacteroidetes* (NCBI:txid976) from the sediment of the polymetallic nodule fields dominated and consisted mainly of unclassified, uncultured bacteria (WANG et al. 2010).

It is also interesting that the LMCO sequences showing similarity to the laccase of *Bacteroidetes* were assigned to two different clusters, but too few sequences were analysed to draw further conclusions.

In this study, the universal primers Cu1AF and CuR4 were used to identify LMCO genes, but the entire biodiversity of LMCO genes could not be captured. This was described by FERNANDES et al. (2014), where primers specific for LMCO from actinomycetes belonging to LccED superfamilies I and K were designed and amplified LMCO fragments from isolates not recognised by the above primers, and where the fragments obtained were correctly assigned to the predicted superfamily. Another primer pair was designed by WANG et al. (2016a). This allowed for the first time the amplification of the LMCO gene from several genera, including *Massila*, which was detected in high expression using real-time PCR (qRT-PCR), confirming coal as a good seed bank. A new specific PCR primer pair targeting the two conserved copper-binding regions of the LMCO two-domain of *Streptomyces* was also developed. Most of the sequence clusters obtained in eight distinct clades are homologous with *Streptomyces* two-domain LMCO genes, but the sequences of clades III and VIII do not match any reference sequence of known *Streptomyces* (LU et al. 2014).

The studies described above and presented in this article have shown how limited the GenBank database is, especially for the taxa of the *Deltaproteobacteria*. For example, the sequence of LMCO from *Sporangium cellulosum* (*Deltaproteobacteria*) was most frequently identified as similar to the metagenome-derived LMCO sequences identified by various authors (AUSEC et al. 2011a; WANG et al. 2010, WANG et al. 2016a).

Soil metagenomics studies, which involve the isolation of soil DNA and the preparation and screening of clone libraries, can provide a cultivation-independent assessment of the largely untapped genetic reservoir of soil microbial communities. This approach has already led to the identification of new laccases (CHAUHAN et al. 2017, BERINI et al. 2018, KUMAR et al. 2018).

Conclusions

In this study, a total of 228 3-domain laccase-like multicopper oxidase LMCO sequences, including 66 sequences from the metagenome, were used for phylogenetic analysis. A BLAST analysis and phylogenetic tree reconstruction allowed distinguishing several LMCO sequence clusters belonging to different bacterial classes, such as *Alphaproteobacteria*, *Gammaproteobacteria*, *Deltaproteobacteria*, *Acidobacteria*, *Actinobacteria*, *Bacteroidetes* and a cluster of unclassified sequences. The results of the phylogenetic analysis also suggest that bacteria from the class of *Deltaproteobacteria* may play a significant role in the lignin degradation process. Trimming sequences to obtain two Cu-binding regions (cbr II and cbr III) and an intervening nucleotide sequence has been shown to be a good method for phylogenetic analysis of metagenome-derived LMCO sequences. However, since the limited information in the database could lead to false affiliations, extensive research using "omic" techniques should be conducted for a more comprehensive characterisation of bacterial laccase in different soil types.

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