Exploring the lignin catabolism potential of soil-derived lignocellulolytic microbial consortia by a gene-centric metagenomic approach

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ABSTRACT

An exploration of the ligninolytic potential of lignocellulolytic microbial consortia can improve our understanding of the eco-enzymology of lignin conversion in nature. In this study, we aimed to detect enriched lignin-transforming enzymes on metagenomes from three soil-derived microbial consortia that were cultivated on "pre-digested" plant biomass (wheat straw - WS1-M, switchgrass - SG-M and corn stover - CS-M). Of 60 selected enzyme-encoding genes putatively involved in lignin catabolism, 20 genes were significantly abundant in WS1-M, CS-M and/or SG-M consortia compared with the initial forest soil inoculum metagenome (FS1). These genes could be involved in lignin oxidation (e.g. superoxide dismutases), oxidative stress responses (e.g. catalase/peroxidases), generation of protocatechuate (e.g. *van*AB genes), catabolism of gentisate, catechol and 3-phenylpropionic acid (e.g. gentisate 1,2-dioxygenases, muconate cycloisomerases and *hca*AB genes), the beta-ketoadipate pathway (e.g. *pca*IJ genes) and tolerance to lignocellulose-derived inhibitors (e.g. thymidylate synthases). The taxonomic affiliation of 22 selected lignin-transforming enzymes from WS1-M and CS-M consortia metagenomes revealed that Pseudomonadaceae, Alcaligenaceae, Sphingomonadaceae, Caulobacteraceae, Comamonadaceae and Xanthomonadaceae are the key bacterial families in the catabolism of lignin. We sketched out a predictive "model" where each microbial population has the potential to metabolize an array of aromatic compounds through different pathways, suggesting that lignin catabolism can follow a "task division" strategy. Here, we have established an association between functions and taxonomy, allowing a better

understanding of lignin transformations in soil-derived lignocellulolytic microbial consortia, and pinpointing some bacterial taxa and catabolic genes as ligninolytic trait-markers.

Keywords: lignocellulose, lignin, metagenomics, microbial consortia, soil

INTRODUCTION

Agricultural residues are an abundant source of sugars used for production of commodity chemicals (e.g. biofuels). However, the high complexity of plant biomass decreases the saccharification (i.e. release of monosaccharides) rates in biorefineries [1]. This drawback has been attributed to the recalcitrant nature of plant cell walls and to strong linkages (e.g. ester linkage) between its main components (lignin, cellulose and xylan) [2]. Lignin is a highly heterogeneous aromatic polymer network formed via radical coupling reactions involving the three major monolignols: p-coumaryl, coniferyl, and sinapyl alcohol, linked by C–C and C–O bonds [3]. Delignification of agricultural residues could increase enzymatic access to plant polysaccharides, enhancing the saccharification rate [4]. Although lignin does not contain sugars, valorization of its aromatic constituents is a relevant topic for bioenergy, food and cosmetics industries [5, 6, 7]. Lignin mineralization requires two main steps: 1) extracellular lignin depolymerization (carried out mainly by peroxidases, multicopper oxidases and/or laccases) to yield mono, di and oligomers; and 2) ring fission of the resulting aromatic compounds. Lignin-derived aromatic compounds are metabolized intracellularly by different type of microbes with specific metabolic capabilities. Here, a niche partitioning of lignin-degrading microbes might be a common feature. During lignin catabolism, aromatic compounds are typically shunted through a number of reactions referred to as “funneling pathways”. Eventually, they converge on a couple of conserved “ring fission pathways” (e.g. beta-ketoadipate) where aromatic rings of the intermediate compounds (e.g. protocatechuate, gentisate and/or catechol) are cleaved. The subsequent catabolic products (e.g. pyruvate, acetyl-coA, oxaloacetate, fumarate and/or succinate) can enter the tricarboxylic acid cycle (TCA) to generate energy [8, 9, 10, 11, 12].

In terrestrial and aquatic ecosystems, different types of microbes transform lignin. These are typically white rot fungi (e.g. *Phanerochaete chrysosporium*), filamentous fungi, yeast, and bacteria mostly belonging to Actinobacteria (e.g. *Streptomyces*, *Arthrobacter* and *Rhodococcus* species) and Proteobacteria (e.g. *Pseudomonas*, *Sphingobium*, *Enterobacter* and *Sphingobacterium* species) [13, 14]. Apparently, bacterial populations, instead of fungal ones, contribute significantly to degradation of native forms of lignin in forest soils and seawater [15, 16, 17]. However, scientific knowledge about the eco-enzymology (defined here as the study of enzymes and their role in

68 microbial interactions and the modification of surrounding environments) of lignin degradation is
69 very limited. Here, we provide evidence that a complete catabolism of lignin and its derived
70 aromatic compounds is a complex process carried out by microbial communities (and their
71 enzymes) instead of a single species, similar to (hemi)cellulose bioconversion [16, 18].
72 Lignocellulolytic microbial consortia have been constructed using the dilution-to-stimulation
73 approach in which natural communities (e.g. from soils) are selected based on a unique carbon
74 source (e.g. agricultural residues) [19]. This strategy allows enrichment of different microbial
75 populations that have the capability to deconstruct plant polymers [20, 21]. In recent years, multi-
76 omic strategies (i.e. metasecretomics and metagenomics) have been used to identify enzymatic
77 mechanisms for polysaccharide degradation functioning in these lignocellulolytic microbial
78 consortia [22, 23, 24, 25]. However, a comprehensive understanding of lignin transformation in
79 these systems is still missing. Recently, Moraes et al. [18] explored the ligninolytic potential of a
80 soil-derived microbial consortium cultivated on soluble lignin. This lignin was obtained after the
81 acidification of black liquor generated from delignification of steam-exploded sugarcane bagasse.
82 Other environments, such as mangrove sediments, redwood compost, decay wood and
83 Mediterranean Sea, have also been used as initial inoculum to enrich lignin-adapted microbial
84 populations [17, 26, 27, 28]. In these latter studies, bacterial 16S rRNA amplicon sequencing and
85 shotgun metagenomic analyses were performed to evaluate the microbial composition and
86 ligninolytic potential within the consortia. A metagenomic exploration on lignin-enriched and low
87 diversity systems (i.e. lignocellulolytic microbial consortia) can help to improve our understanding
88 of lignin catabolism in nature. These approaches can be useful to identify novel pathways, key
89 microbes, genes and/or enzymes as ligninolytic trait-markers. In addition, such studies could inform
90 further lignin valorization strategies.
91 In this study, we explored the lignin-transforming potential of three lignocellulolytic
92 microbial consortia obtained from forest soil and cultivated on “pre-digested” plant biomass (wheat
93 straw, switchgrass and corn stover) under aerobic and mesophilic conditions. We hypothesized that
94 the use of biological-pretreated plant biomass can favour the presence of bacteria able to degrade
95 recalcitrant plant polymers (e.g. lignin) [23]. In order to evaluate the ligninolytic potential of these
96 engineered microbial consortia, we have used a metagenome “gene-centric” approach, selecting 60
97 enzyme-encoding genes that are putatively involved in lignin depolymerization and metabolism of
98 its derived aromatic compounds. Moreover, the taxonomic affiliation of some genes allowed us to
99 link specific functions with particular taxa. Our results suggest the catabolism of lignin in these
100 microbial systems is a specialized process in which each taxon has its own niche and can perform a
101 specific job, following the “task division” strategy.

MATERIALS AND METHODS

Construction of plant biomass-degrading microbial consortia

The soil-derived lignocellulolytic microbial consortia were developed following a dilution-to-stimulation approach [23, 29]. Briefly, soil suspension was added to triplicate flasks containing mineral salt medium with 1 % of plant biomass, trace mineral and vitamin solutions. Flasks were incubated at 28°C in oxic conditions (with shaking at 150 rpm). Once systems reached high bacterial cell density (7–8 log cells/ml, between 5 and 6 days and determined by microscopical cell counting), aliquots of microbial suspension were transferred to fresh medium (diluted 1000 fold). These procedures were repeated ten times. Five different consortia were analyzed in this study: 1) cultivated on fresh and heat-treated wheat straw (10-RWS and 10-TWS; transfer 10) [22], and 2) cultivated on "once used" or "pre-digested" (highly recalcitrant) wheat straw, corn stover and switchgrass (WS1-M, CS-M and SG-M, respectively). The "pre-digested" substrates were obtained after a biological pretreatment with different soil-derived lignocellulolytic microbial consortia [23].

Soil-derived lignocellulolytic microbial consortia metagenomes

Total DNA from the lignocellulolytic microbial consortia (10-RWS, 10-TWS, WS1-M, CS-M and SG-M) was extracted using the UltraClean Microbial DNA Isolation Kit (MoBio Laboratories Inc., Carlsbad, CA, USA). Metagenome sequencing was performed using the Illumina MiSeq v2 (2 x 250 bp paired-end reads) at LGC Genomics (Berlin, Germany). Forest soil (initial inoculum) metagenome (FS1) was sequenced using the same platform [22, 23]. For the purpose of comparison, we have also used a dataset retrieved from a ligninolytic microbial consortium (denoted LigMet). This metagenome was sequenced using the Illumina HiSeq 2500 (2 x 100 bp paired-end reads) [18]. The total metagenomic information retrieved was approximately 136 Mb for FS1; 112 Mb for 10-RWS; 198 Mb for 10-TWS; 1.5 Gb for WS1-M; 1.6 Gb for SG-M; 1.8 Gb for CS-M; and 18 Gb for LigMet.

Metagenomic analysis ("gene-centric" approach)

All metagenomes (unassembled sequences reads) were uploaded to the MG-RAST v3.1.2 server [30]. Overlapping sequence pairs were matched, and non-overlapping reads retained as individual reads, after which dereplication was performed. Duplicate read-based inferred sequencing error estimation and quality trimming (phred score <20) used default settings. Gene predictions were done using the FragGeneScan software and subsequently the proteins were annotated based on BLASTX searches against the RefSeq and KEGG databases using an e-value cutoff of 1e-5, a

minimum alignment length of 50 amino acids and a minimum identity of 50%. Data from MG-RAST annotation were statistically analyzed using the STAMP package [31]. This software was used also to obtain correlation values of the taxonomic (genus level) and functional (KO level) profiles across the metagenomes. All metagenome datasets are publicly accessible on the MG-RAST server IDs 4547279.3 (10-TWS); 4547280.3 (10-RWS); 4547285.3 (FS1); 4790808.3 (LigMet); 4579477.3 to 4579479.3 (CS-M); 4579485.3 to 4579487.3 (SG-M); 4579476.3, 4579480.3 and 4579481.3 (WS1-M).

Selection and analysis of genes encoding lignin-metabolizing enzymes

Based on an in-depth comprehensive bibliographic search [7, 9, 10, 11, 27, 32, 33, 34, 35], we have selected 60 enzyme-encoding genes (KO IDs) that could be involved in lignin depolymerization, oxidative stress response, catabolism of lignin-derived aromatic compounds (e.g. funneling and fission pathways) and tolerance to lignocellulose-derived inhibitors. For each enzyme, EC numbers, KO IDs, names and functions are shown in supplementary information Table S1. A matrix of read counts per gene in all the metagenomes was analyzed using the STAMP software [31]. To evaluate the relative abundance of reads per selected enzyme-encoding gene, the counts were normalized to hits, or unique matches, per million reads, thereby accounting for differences in metagenome sizes [36]. Heat maps were constructed in the web server Heatmapper using row Z score for each enzyme [37]. Differential abundance analysis was used to determine which lignin-metabolizing enzymes were highly enriched in WS1-M, CS-M and SG-M consortia compared with forest soil inoculum (FS1). This analysis was conducted using the R package DESeq2 with a Log Fold change > 1 [17, 38]. Unassembled sequences reads (in FASTA format) belonging to the selected 60 lignin-metabolizing enzymes were extracted from WS1-M and CS-M using the MG-RAST webserver. The taxonomic assignment was performed using the Lowest Common Ancestor (LCA) algorithm within the Kaiju web server [39]. These sequences were also clustered at 97 and 99% of nucleotide identity using the CD-HIT software [40]. Using the taxonomic affiliation and read counts in each gene, we have built a PCA in the software R.

RESULTS AND DISCUSSION

In this study, we aimed to unveil the ligninolytic potential of different soil-derived lignocellulolytic microbial consortia. For this purpose, we evaluated 60 catabolic genes that are putative involved in lignin depolymerization, oxidative stress response and catabolism of lignin-derived aromatic compounds (supplementary information Table S1). The selected gene list (i.e. KO IDs) could be a useful input to explore the lignin-degrading potential in other meta-omic surveys. Here, we have

used a metagenomic "gene-centric" approach, instead "genome-centric" approach, as this is known to be useful to evaluate gene differential abundance profiles and cause minimal disturbance with respect to representation of sequences within the abundant taxa [41]. Using relative abundance matrixes of annotated enzyme-encoding sequences (by KEGG Orthology database) and the R package DESeq2, we unveiled which enzymes were significant enriched in the lignocellulolytic microbial consortia compared to the initial soil inoculum. It is important to note that our study has a drawback inherent to any metagenomic study, and unfortunately we cannot claim enzymatic activity based on similarity and/or functional annotation of DNA sequences. However, our approach allowed us to link potential functions with particular taxa, improving our understanding of the eco-enzymology of the lignin transformation process in these engineered microbial systems.

Clustering of lignocellulolytic microbial consortia based their taxonomic/functional profile

In a previous study, we selected and characterized three soil-derived lignocellulolytic microbial consortia, denoted WS1-M (cultivated on wheat straw), CS-M (cultivated on corn stover) and SG-M (cultivated on switchgrass). These consortia were obtained using a new approach in which the plant biomass used to cultivate them was first partially degraded ("pre-digested") by another lignocellulolytic microbial consortium [21, 23]. This strategy allows microbes to be selected with high capacities to degrade highly complex plant polysaccharides, as well as lignin. The compositional profile obtained by Fourier transform infrared (FTIR) spectroscopy showed that the "pre-digested" wheat straw contained approximately 16% lignin, while switchgrass and corn stover only 11% [23]. Previous metagenomic sequence annotation showed that bacteria, rather than fungal communities, dominate in the WS1-M, CS-M and SG-M consortia [23]. Here, based on a PCA using RefSeq (taxonomic) annotation profile, we observed that SG-M and CS-M consortia clustered together ($R^2 > 0.88$) (Fig. 1a). A similar result was observed using the KEGG database (Fig. 1b). These findings were already reported in the previous study [23]. In terms of taxonomic composition, SG-M and CS-M are very similar, showing high abundance of sequences affiliated to species belonging to Pseudomonadaceae and Caulobacteraceae families. In contrast, Bacteriodetes species were preferentially selected in WS1-M [23]. Wheat straw-degrading consortia (WS1-M, 10-RWS and 10-TWS) showed a similar functional profile based on total KEGG annotation ($R^2 > 0.75$) (Fig. 1b). It is important to highlight that all consortia evaluated in this study were retrieved from the same forest soil inoculum, except LigMet. Interestingly, SG-M and CS-M showed the same functional profile when lignin-transforming enzymes were used to build the clustering ($R^2 > 0.97$). However, this ligninolytic profile was highly dissimilar compared to the LigMet consortium and the forest soil inoculum (FS1 metagenome) (Fig. 1c). The consortium LigMet was retrieved from

agricultural tropical soils and cultivated on soluble lignin [18]. Thus, these conditions can explain its dissimilar taxonomic and functional profile compared with the other forest soil-derived lignocellulolytic microbial consortia. The results indicated that the substrates used to develop the consortia are key factors to shape the taxonomic and ligninolytic potential in these engineered microbial systems.

Abundance of lignin-transforming enzyme-encoding genes

Although the WS1-M consortium showed a different functional profile compared to CS-M and SG-M, some lignin-transforming enzymes were highly abundant in all three consortia ($p \leq 0.05$). For instance, alcohol dehydrogenases, glutathione S-transferases (GST) and catalases (*kat*E) (Fig. 2). Proteins involved in tolerance to lignocellulose-derived inhibitors (S-(hydroxymethyl)glutathione dehydrogenase - *frm*A) and catabolism of gentisate (gentisate 1,2-dioxygenase) were highly abundant in CS-M and SG-M compared to WS1-M. In contrast, a vanillate monooxygenase (*van*B) was highly abundant in WS1-M compared to CS-M and SG-M. Some specialized types of GST (e.g. beta-etherase) are involved in lignin depolymerization by cleaving beta-aryl ether linkages [42]. Its high abundance in the consortia can be correlated with the number of gene copies in *Pseudomonas* species. For instance, 14 GST genes have been identified in *Pseudomonas putida* KT2440 [14]. However, not all GST proteins have beta-etherase activity and in-depth functional analysis of these sequences would be worthwhile. Enzyme-encoding genes putative involved in lignin depolymerization and/or oxidative stress responses (e.g. GST, *kat*G, superoxide dismutase - SOD2, glutathione peroxidase and glycolate oxidase - *glc*D) were highly abundant on the three consortia (Fig. 2). Manganese superoxide dismutases from *Sphingobacterium* sp. T2 have been identified as novel lignin-oxidizing enzymes, which are able to solubilize organosolv and kraft lignin to generate a mixture of polymeric and monocyclic aromatic products [43]. Our results showed that GST, *glc*D and (S)-2-hydroxy-acid oxidase (HAO) were also abundant in the forest soil inoculum (Fig. 3). In particular, *glc*D and HAO could be accessory enzymes for lignin degradation, by generating hydrogen peroxide for peroxidase enzymes, and potentially detoxifying aldehyde by-products [44]. These findings can be an indication of the large enzymatic potential to breakdown lignin in the soil-derived consortia. In fact, WS1-M, CS-M and SG-M showed values of lignin degradation of 25.3 ± 1.8%, 24.7 ± 1.2% and 58.6 ± 1.0% over 6 days, respectively, obtained by FTIR spectroscopy [23]. The high value observed on SG-M can be explained by the lower lignin recalcitrance in switchgrass, compared to wheat straw and corn stover. Similarly, Wang et al. [45] have reported a bacterial consortium that could break down 60.9% of lignin in reeds (i.e. grass-like plants of wetlands) over 15 days.

Unfortunately, multicopper oxidases and dye-decolorizing peroxidases (DyPs) (e.g. K15733; EC: 1.11.1.19) involved in bacterial lignin oxidation processes were not identified in the metagenomic annotation, suggesting a low copy numbers within bacterial genomes and/or due to the relative low number of representative sequences available in the KEGG database. In fact, only three genes putative encoding for DyPs has been found in *P. fluorescens* [46] and around 100 DyPs sequences from bacterial origin are available in a specialized database (http://peroxibase.toulouse.inra.fr/) [14]. As was mentioned, the extracellular depolymerization of lignin releases a mixture of aromatic monomers that can be converted into metabolic intermediates via catechol and protocatechuate pathways. These routes can be divided in three blocks: 1) the branch of catechol intermediate (ortho-cleavage), which involves the following enzyme-encoding genes: *cat*A, *cat*B and *cat*C; 2) the branch of protocatechuate (meta-cleavage), which involves *pca*G, *pca*H, *pca*B and *pca*C; and finally, 3) the reactions common for both branches, catalyzed by *pca*D, *pca*I, *pca*J and *pca*F [11, 18]. In this regard, we found that the metagenomes from the consortia 10-RWS and 10-TWS contained a high abundance of *pca* genes (e.g. *pcaBC*GHIJ) compared with the other consortia (Fig. 3), suggesting a high proportion of low molecular weight aromatic compounds in these systems. These microbial consortia were cultivated on untreated and heat-treated wheat straw [22], where lignin proportion is higher, but probably with lower recalcitrance compared to substrates used on the selection of WS1-M, SG-M and CS-M. In a recent proteomic-based study, Park et al. [47] demonstrated that *P. putida* KT2440 significant induces the production of *pca*-derived proteins after growth on plant-derived lignolysate.

Differential and significant enrichment of lignin-transforming enzyme-encoding genes

Using the abundance values of the 60 selected catabolic genes in each metagenome and DESeq2 package, we identified genes that were significantly abundant in WS1-M, CS-M and/or SG-M compared with the initial forest soil inoculum (FS1 metagenome) (Fig. 3). Moreover, in Fig. 4, we present a scatter plot that shows the most highly abundant genes in each microbial consortium compared with FS1. Based on the results, we found that 20 genes (~33%) were highly abundant (*p*adj-value ≤ 0.05, Wald test; and Log2 FC ≥ 1) on WS1-M, CS-M and/or SG-M compared to FS1 (Fig. 3). These genes could be involved in oxidative stress response (e.g. *kat*E), lignin depolymerization/oxidation (e.g. superoxide dismutase - SOD1), generation of protocatechuate/gallate (e.g. vanillate monooxygenases - *van*AB and methylenetetrahydrofolate reductase - *met*F), catabolism of catechol (e.g. muconate cycloisomerase - *cat*B and muconolactone D-isomerase - *cat*C), gentisate (e.g. gentisate 1,2-dioxygenase) and 3-phenylpropionic acid (e.g. *hca* genes). Additionally, genes involved in the beta-ketoadipate pathway (*pca*I and *pca*J), thymidylate

synthases (*thy*A) and aryl-alcohol dehydrogenases (K00055) were also highly abundant compared to FS1. Interesting, the *pca* genes in *P. putida* A514 are upregulated in response to lignin [48] and thymidylate synthases conferred tolerance to lignin monomers such as furfural, ferulic acid, vanillic acid and syringic acid [49]. The *van*A, *van*B, *kat*E, *cat*C and *met*F genes were highly abundant in the LigMet consortia. In addition, *cat*C was highly abundant in the 10-RWS metagenome (Fig. 3). In particular, the O-demethylation of vanillate is catalyzed by the operon *lig*M-*met*F-*lig*H. This process can generate catecholic compounds, protocatechuate and gallate. However, the O-demethylation steps are also important for the production of 5-methyl-H_4folate, which is a C_1-H_4folate derivative in one-carbon (C1) metabolism [32]. Moreover, Ceballos et al. [27] have developed a lignin-degrading microbial consortium under high-solids and thermophilic conditions. In terms of enzyme-encoding genes that were identified by a predictive metagenomic approach, they found an enrichment of *van*A, protocatechuate 3,4-dioxygenase (*pca*G), catechol 1,2-dioxygenase (*cat*A), catechol 2,3-dioxygenase (*dmp*B), *cat*B and aryl-alcohol dehydrogenase. In a study reported by Carlos et al. [50], eight filter paper and wood chips-degrading microbial consortia were "perturbed" in alkali lignin as the sole carbon source. They found a significant enrichment of enzyme-encoding genes involved in catechol ortho-cleavage, especially *cat*A that cleaves the bond between the phenolic hydroxyl groups of catechol generating *cis*, *cis*-muconic acid. These type of catabolic genes (i.e. *cat*A), and other genes involved in the phenylacetyl-CoA pathway, were highly abundant on a lignin-adapted consortia retrieved from Eastern Mediterranean seawater [17]. Although *cat*A and *dmp*B were not significantly enriched in WS1-M, CS-M and/or SG-M compared to FS1, they were highly abundant in the LigMet and 10-RWS consortia, suggesting that they could be key genes involved in lignin transformation through catechol catabolism. Notably, the gene *dmp*B (catechol 2,3-dioxygenase) has been found in a plasmid from a phenol-metabolizing *Pseudomonas* strain CF600. This enzyme catalyses the conversion of catechol to 2-hydroxymuconic semialdehyde [51]. Moreover, alcohol dehydrogenases were highly abundant in the three consortia (e.g. *adh*) (Fig. 2) and one type of aryl-alcohol dehydrogenases was significant abundant in CS-M and SG-M compared to FS1 (Fig. 3). We suggest that these two proteins could be involved in oxidation of low molecular weight alcohols (e.g. coniferyl alcohol) that can be released after lignin depolymerization/oxidation [32, 52]. However, an in-depth functional analysis may be required to support this statement.

Taxonomic affiliation of lignin-transforming enzyme-encoding genes

From the 60-ligninolytic genes selected, 22 were used for the taxonomic affiliation, in the WS1-M and CS-M metagenomes, using the LCA algorithm (Fig. 5). We have selected these consortia due to

two main reasons: 1) CS-M and SG-M were highly similar between them and 2) we wanted to explore the consortia cultivated on "pre-digested" agricultural residues, instead of switchgrass. Otherwise, enzyme-encoding gene selection was done taking in account three parameters: 1) genes that contained more than 20 assigned metagenomic reads; 2) genes that were differentially enriched compared to FS1 (Fig. 3); and 3) genes that are putative involved in extracellular depolymerization of lignin and the catabolism of different aromatic intermediates (e.g. protocatechuate, gallate, gentisate and catechol). Two genes putatively involved in oxidative stress response (*kat*G and *kat*E) and one in lignin oxidation (superoxide dismutase - SOD1) were selected. In a recent study, Rashid et al. [53] have found that extracellular MnSOD1 protein from *Sphingobacterium* sp. require two mutations to have lignin demethylation activity. Apparently, these mutations are only found in Bacteroidetes phylum. In our two consortia, the SOD1-encoding genes were mostly affiliated to Pseudomonadaceae, Xanthomonadaceae, Alcaligenaceae and Caulobacteraceae. Thus, we are doubtful whether these proteins can have ligninolytic activity. Moreover, in order to obtain a proxy of sequence diversity within each enzyme-encoding gene, we have used values of operational functional units (OFUs) per thousands of reads. The OFUs values were obtained by clustering the sequences (with size average of 250 bp), affiliated within each catabolic gene, using a cutoff of >97% nucleotide sequence similarity. These analyses were carried out using the CD-HIT software (http://weizhongli-lab.org/cd-hit/) [40]. It is important to clarify that we did not assigned taxa to the predicted OFUs. The results of OFUs/thousands of reads showed that genes *kat*E and *kat*G were highly diverse in WS1-M and CS-M consortia (≥ 0.5). This indicates that the metabolism of reactive oxygen species (by action of catalases/peroxidases), which could be correlated with lignin degradation, is a process where different taxa (mostly Pseudomonadaceae, Xanthomonadaceae, Alcaligenaceae, Caulobacteraceae, Flavobacteriaceae, Sphingobacteriaceae and Sphingomonadaceae species) can be involved (Fig. 5). In contrast, low values of sequence diversity in catabolic genes involved in intracellular conversion of lignin-derived aromatics compounds were observed (< 0.2 OFUs/thousands of reads), except *met*F and *thy*A.

Recently, Moraes et al. [18] have developed a lignin-degrading microbial consortium composed of 355 bacterial types. Based on 16S rRNA amplicon sequencing data, they demonstrated that around 50% of the total consortium comprised *Achromobacter* (Alcaligenaceae family), *Paenarthrobacter* (novel Actinobacteria able to transform lignin), *Pseudaminobacter* and *Paenibacillus* species. These taxa were enriched in the consortium compared to sugarcane soil inoculum. Based on the metabolic functional profile unveiled by metagenomics, they showed that species from Actinobacteria and Proteobacteria contain putative novel enzyme-encoding genes involved in the metabolism of lignin. For instance, peroxidases and laccases were frequently found

in Actinobacteria. In contrast, both taxa have the potential to metabolize lignin-derived phenolic compounds. Moreover, Granja-Travez et al. [14] state that exist a high diversity of mechanism for lignin oxidation process in different bacteria and not just one class of enzyme carry out this function in all microbial systems. These observations suggest that lignin transformation is a process in which multiple microbes are involved. However, the intracellular catabolism of its derived aromatic compounds could be a specialized job, in which particular taxa can metabolize specific lignin-derived intermediates.

Based on the taxonomic affiliation of sequences affiliated to genes *hca*A1 (3-phenylpropionate/cinnamic acid dioxygenases) and *hca*B (2,3-dihydroxy-2,3-dihydrophenylpropionate dehydrogenases), we suggest that, in both consortia, species from Caulobacteraceae, Alcaligenaceae and Enterobacteriaceae could be involved in the catabolism of 3-phenylpropionic acid (Fig. 5, Fig. 6 and supplementary information Fig. S1). There are two branches at the start the 3-phenylpropionate catabolic pathway: one branch is from phenylpropionic acid, which is dihydroxylated via the *hca*AB genes to 2,3-dihydroxyphenylpropionic acid. In this regard, phenylpropionic acid has been found in anaerobic microbial systems treating lignocellulose [54, 55]. In this case, we hypothesized that 3-phenylpropionic acid could be generated from anaerobic lignin degradation processes, probably via dehydroxylation of hydroxycinnamic acids, occurring in the lignocellulolytic consortia (Fig. 6). Moreover, sequences assigned to catabolic genes *van*A, *van*B, *cat*C, *cat*B, *pca*B (3-carboxy-*cis*, *cis*-muconate cycloisomerase), *ben*D-*xyl*L (dihydroxycyclohexadiene carboxylate dehydrogenase) were mostly affiliated to Pseudomonadaceae (Fig. 5), suggesting that species within this family play key roles in the metabolism of protocatechuate, gallate and catechol in both consortia. Similarly, Carlos et al. [50] found an enrichment of genes involved in catechol ortho-cleavage that were mostly affiliated to *Pseudomonas* species in a lignin-adapted consortium.

Interestingly, the taxonomic assignment of sequences affiliated to the enzyme gentisate 1,2-dioxigenase shows that the catabolism of gentisate is a process where Caulobacteraceae species can play a unique and pivotal role, especially in CS-M consortium (Fig. 5b and supplementary information Fig. S1). DeAngelis et al. [15] have reported enrichments of Caulobacteraceae bacterial types in lignin-amended tropical soils compared to unamended ones. Similarly, Woo and Hazen [17] reported an increase of Caulobacteraceae species (along with others) in a seawater-derived ligninolytic microbial consortium, compared to xylan and unamended microcosms. In an outstanding study, Wilhelm et al. [16] using SIP microcosm-based experiments with ^{13}C-labeled lignin, coupled with shotgun metagenomics, have demonstrated that species from Caulobacteraceae and Comamonadaceae families are the most relevant microbes for lignin degradation on coniferous

forest soils across North America. They found that members of Caulobacteraceae family could degrade all three lignocellulosic polymers, providing new evidence for their importance in plant biomass degradation. In addition, some of these species contained genes predicted to encode the entire beta-ketoadipate pathway. It is reported that this pathway appears to be the most common route for aromatic metabolism in lignin-degrading bacteria. However, some lignin-degrading bacteria such as *Sphingobacterium* and *Paenibacillus* apparently lack these gene clusters [14]. In the current study, we observed that, based on taxonomic affiliation of sequences within the gene *pca*I (3-oxoadipate CoA-transferase), the protocatechuate catabolism via beta-ketoadipate pathway appears to be carried out primarily by species from Pseudomonadaceae and Alcaligenaceae families (Fig. 5 and Fig. 6). Observing the taxonomic affiliation of sequences within the gene *des*B (gallate dioxygenase), we suggest that the catabolism of gallate could be performed mostly by Yersiniaceae species in WS1-M and Sphingomonadaceae species in CS-M. For catabolism of catechol, species affiliated to Pseudomonadaceae and Alcaligenaceae families appear to be key players (Fig. 5 and Fig. 6). However, we observed a high proportion of sequences within the gene *dmp*B that were affiliated to Actinobacteria, indicating that this taxon could play an important role as well within the catechol catabolism, especially in the consortium CS-M (Fig. 5b and supplementary information Fig. S1). Overall, these findings suggest that particular bacterial taxa could do specific functional jobs in each microbial consortium.

The genes *pca*B, *pca*H, *lig*B, *lig*C, *lig*I and *lig*J (supplementary information Table S1) were not significantly enriched in WS1-M, CS-M and SG-M compared with FS1 (Fig. 3). However, their taxonomic affiliation shows that the catabolism of protocatechuate, by fission pathways (2,3-cleavage and 4,5-clevage), is a process probably carried out by species belonging to the taxa Pseudomonadaceae, Alcaligenaceae, Actinobacteria, Sphingomonadaceae, Xanthomonadaceae and Comamonadaceae (Fig. 5 and Fig. 6). In a previously reported thermophilic lignin-degrading consortium, the family Xanthomonadacea*e* was highly selected along the enrichment, while species from family Alcaligenaceae survives in moderately lignin-rich environments [27]. In addition, Fang et al. [28] have reported that *Stenotrophomas* (Xanthomonadaceae member) is an abundant microbe in a consortium retrieved from decaying wood and cultivated in guaiacol and tree trimmings, suggesting that Xanthomonadaceae species could be associated with lignin-abundant environments. The *lig*AB genes are less common than the *pca* genes for protocatechuate degradation. They have been studied in *Sphingobium* SYK-6 (formerly known as *Sphingomonas*) [56]. Looking in detail the protocatechuate 4,5-cleavage fission pathway (Fig. 5), we observed that sequences from *lig*B were mostly affiliated to Sphingomonadaceae and Comamonadaceae in WS1-M. However, some sequences were affiliated to Actinobacteria in the consortia CS-M (Fig. 5). Regarding genes *lig*I and

*lig*J, the data showed that sequences were mostly affiliated to Xanthomonadaceae in the consortium CS-M, whereas in WS1-M the sequences were mostly affiliated to Sphingomonadaceae and Comamonadaceae. Finally, we observed that Flavobacteriaceae family could be a key taxon in the WS1-M consortium, based on the high proportion of sequences of genes *kat*G, *met*F, *pca*H and *thy*A) affiliated to this family (Fig. 5 and supplementary information Fig. S1).

CONCLUSIONS

From examination of the results in this study, several conclusions can be made. Firstly, the SG-M and CS-M consortia are very similar, in terms of their taxonomical composition and ligninolytic gene profile, whereas the WS1-M consortium has different composition. Secondly, catabolic genes probably involved in lignin depolymerization or oxidative stress response (e.g. catalase/peroxidases and superoxide dismutases) were highly abundant in the three consortia, showing high sequence diversity. In contrast, we found less sequence diversity within catabolic genes involved in the intracellular metabolism of aromatic compounds, suggesting that these processes could have a degree of specialization. In general terms, we propose that lignin transformation follows a "task division" strategy, similar to that found for the degradation of cellulose and hemicellulose [57]. Thirdly, from the 60 enzyme-encoding genes involved in lignin catabolism, 20 were significant abundant in the three soil-derived lignocellulolytic consortia (WS1-M, CS-M and/or SG-M) compared to FS1, suggesting that these microbial communities have a huge potential to transform lignin. As a perspective, the use of spectroscopic analyses to reveal the molecular structure of lignin [58] will be very valuable to correlate gene relative abundances and taxonomy with specific lignin linkages in each plant biomass-degrading consortia. Moreover, based on the enrichment of some catabolic genes found in our study and the results reported in other lignin-adapted microbial consortia, we suggest that genes *van*A, *pca*I, *pca*J, *cat*A, *cat*B, *cat*C and *dmp*B can be key gene-markers for lignin transformation, especially those involved in catechol and protocatechuate metabolism. Interestingly, *hca* genes involved in 3-phenylpropionic acid metabolism were significant abundant in the lignocellulolytic consortia compared to FS1. We suggest that this aromatic compound can be released from the anaerobic lignin depolymerization and subsequently metabolized toward the TCA. Moreover, we conclude that the presence, in high abundance, of bacterial species belonged to Pseudomonadaceae, Caulobacteraceae, Xanthomonadaceae, Alcaligenaceae and Comamonadaceae families could be a strong indication of the high potential to depolymerize lignin and metabolize its derived aromatic compounds in any microbial community. In this regard, we are in accordance with Wilhelm et al. [16] to state that the variation in lignin-degrading activity could be better explained by the catabolic gene content and community structure.

Fourthly, we conclude that species belonged to Pseudomonadaceae family can be the most relevant ligninolytic members in these microbial consortia. They have the potential to participate in lignin depolymerization and in the metabolism aromatic compounds through the beta-ketoadipate pathway. Our predictive "model" (Fig. 6) allowed us to hypothesize that some bacterial populations could have a specific functional role within the lignin catabolism. For instance, some members have broad metabolic capacities (e.g. Pseudomonadaceae), while other ones could act as a specialist doing the catabolism of specific aromatic compounds (e.g. Caulobacteraceae and Sphingomonadaceae). Finally, we propose that by linking function and taxonomy, our metagenomic exploration has allowed us to better understand the lignin degradation process on soil-derived lignocellulolytic microbial consortia.

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DECLARATIONS

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Conflicts of interest/Competing interests

The authors declare that they have no conflict of interest.

Ethics approval

Not applicable

Consent to participate

Not applicable

Consent for publication

All authors read and approved the final manuscript.

476

477 **Availability of data and material**

478 Not applicable

479

480 **Code availability**

481 Not applicable

482

483 **Authors' contributions**

484 LDG carried out all bioinformatic and statistical analysis. TDHB helped in the data discussion and
485 drafting the text. DJJ conceived the study and drafted the manuscript.

486

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FIGURE LEGENDS

Figure 1. Taxonomic and functional clustering of lignocellulolytic microbial consortia based on metagenome annotation. A) Clustering based on taxonomic assignment (RefSeq database) of annotated sequences at genus level. B) Clustering based on functional assignment (KEGG databases) of annotated sequences at KOs level. C) Functional clustering based on the 60 selected enzyme-encoding genes involved in transformation of lignin and its derived aromatic compounds. Black squares correspond to initial forest soil inoculum (FS1) metagenome used to build the consortia WS1-M, CS-M, SG-M, 10-RWS and 10-TWS.

Figure 2. Number of normalized sequences (per million) that were annotated within the 60 enzyme-encoding genes involved in transformation of lignin and its derived aromatic compounds. The top 16 ligninolytic enzyme-encoding genes that were significantly abundant ($p \leq 0.05$) in WS1-M, CS-M or SG-M are shown at the bottom. Letters mean significant differences between them. *van*B (vanillate monooxygenase, in bold) was significant abundant only in WS1-M; whereas aryl-alcohol dehydrogenase (in bold) was significant abundant only in CS-M.

Figure 3. Heat map of normalized abundance values (Row Z-Score) obtained using the number of sequences annotated within the 60 enzyme-encoding genes involved in transformation of lignin and its derived aromatic compounds in each microbial consortium. Genes differentially and significantly enriched (*p*adj-value ≤ 0.05, Wald test; and Log2 FC ≥ 1) in WS1-M, CS-M and/or SG-M compared with FS1 are labelled in blue.

Figure 4. Comparison of ligninolytic profiles (percentage of relative abundance of genes involved in lignin transformation) between the soil inoculum (FS1) and metagenomes from the microbial consortia. Letters a to r indicate the most overrepresented gene functions in the observed metagenomes. In blue are the genes differentially and significantly abundant (*p*adj-value ≤ 0.05, Wald test; and Log2 FC ≥ 1) in WS1-M, CS-M and/or SG-M compared with FS1.

Figure 5. Taxonomic affiliation, using the Lowest Common Ancestor Algorithm (LCA), of 22 enzyme-encoding genes involved in transformation of lignin and its derived aromatic compounds in A) WS1-M and B) CS-M microbial consortia. Data in top panel are normalized functional diversity values (OFUs - operational functional units/thousands of annotated reads) obtained by clustering the sequences in each gene at 97% (line) and 99% (dashed line) similarity.

Figure 6. Schematic representation of transformation of lignin-derived aromatic compounds, showing genes/taxa (from WS1-M and CS-M microbial consortia) putatively involved in each catabolic pathway. This figure was build based on a figure reported by Brink et al. [11] that shows a schematic distribution of the known pathways for aromatic catabolism currently indexed in the eLignin database. In this figure, the 22 selected catabolic genes selected for taxonomic assignment were plotted based on their function within these pathways (bold arrows). Coloured circles represent the taxa that could be involved in each catabolic step. The size of each circle is an estimation of the data obtained from Fig. 5 and supplementary information Fig. S1. Asterisks represent genes that were significant abundant in WS1-M and/or CS-M compared with FS1.

SUPPLEMENTARY FIGURE AND TABLE LEGENDS

Table S1. The 60 enzyme-encoding genes involved in transformation of lignin and its derived aromatic compounds. Within the table, we showed the KOs number identifiers, gene or protein name, EC numbers and putative function or metabolic pathway within the lignin transformation processes.

Figure S2. PCA using the abundance values and taxonomic affiliation of 22 selected ligninolytic catabolic genes in WS1-M (up) and CS-M (down).

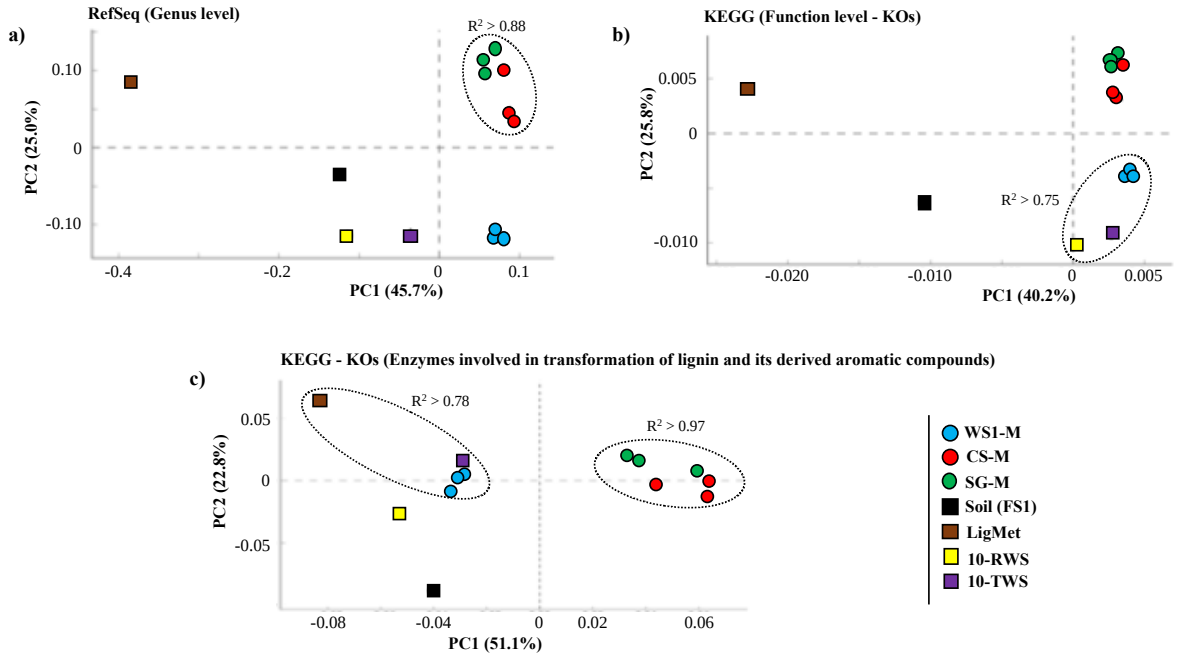

a)
RefSeq (Genus level)
R² > 0.88
PC2 (25.0%)
PC1 (45.7%)
b)
KEGG (Function level - KOs)
R² > 0.75
PC2 (25.8%)
PC1 (40.2%)
c)
KEGG - KOs (Enzymes involved in transformation of lignin and its derived aromatic compounds)
R² > 0.78
R² > 0.97
PC2 (22.8%)
PC1 (51.1%)
WS1-M
CS-M
SG-M
Soil (FS1)
LigMet
10-RWS
10-TWS

Figure 1

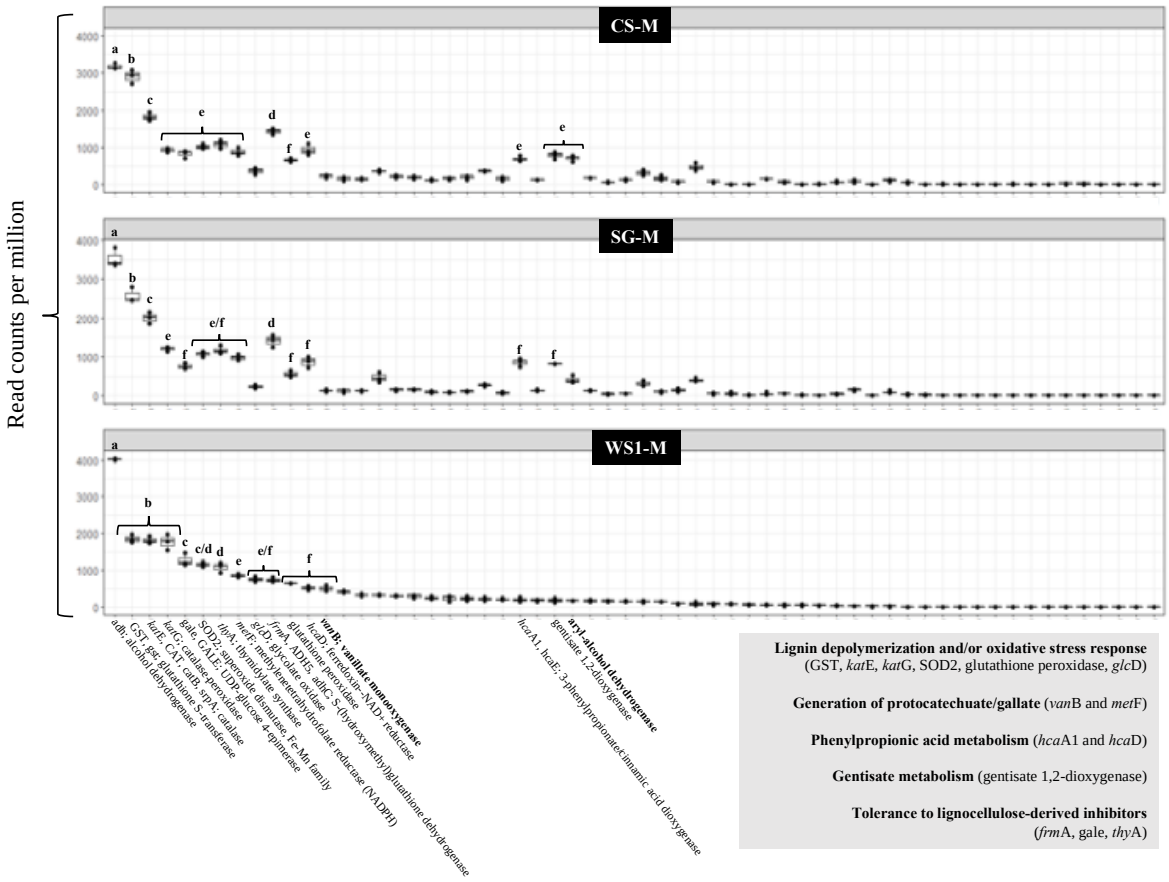

CS-M
SG-M
WS1-M
Read counts per million
adh; alcohol dehydrogenase
GST; gst; glutathione S-transferase
katE; CAT; catB; srpA; catalase
katG; catalase-peroxidase
gale; GALE; UDP-glucose 4-epimerase
SOD2; superoxide dismutase, Fe-Mn family
thyA; thymidylate synthase
metF; methylenetetrahydrofolate reductase (NADPH)
glcD; glycolate oxidase
frmA; ADH3; adhC; S-hydroxymethylglutathione dehydrogenase
hcaD; ferredoxin-NAD+ reductase
glutathione peroxidase
vanB; vanillate monooxygenase
gentisate 1,2-dioxygenase
ara1; alcohol dehydrogenase
hcaA1, hcaE; 3-phenylpropionate/cinnamic acid dioxygenase
Lignin depolymerization and/or oxidative stress response (GST, katE, katG, SOD2, glutathione peroxidase, glcD)
Generation of protocatechuate/gallate (vanB and metF)
Phenylpropionic acid metabolism (hcaA1 and hcaD)
Gentisate metabolism (gentisate 1,2-dioxygenase)
Tolerance to lignocellulose-derived inhibitors (frmA, gale, thyA)

Figure 2

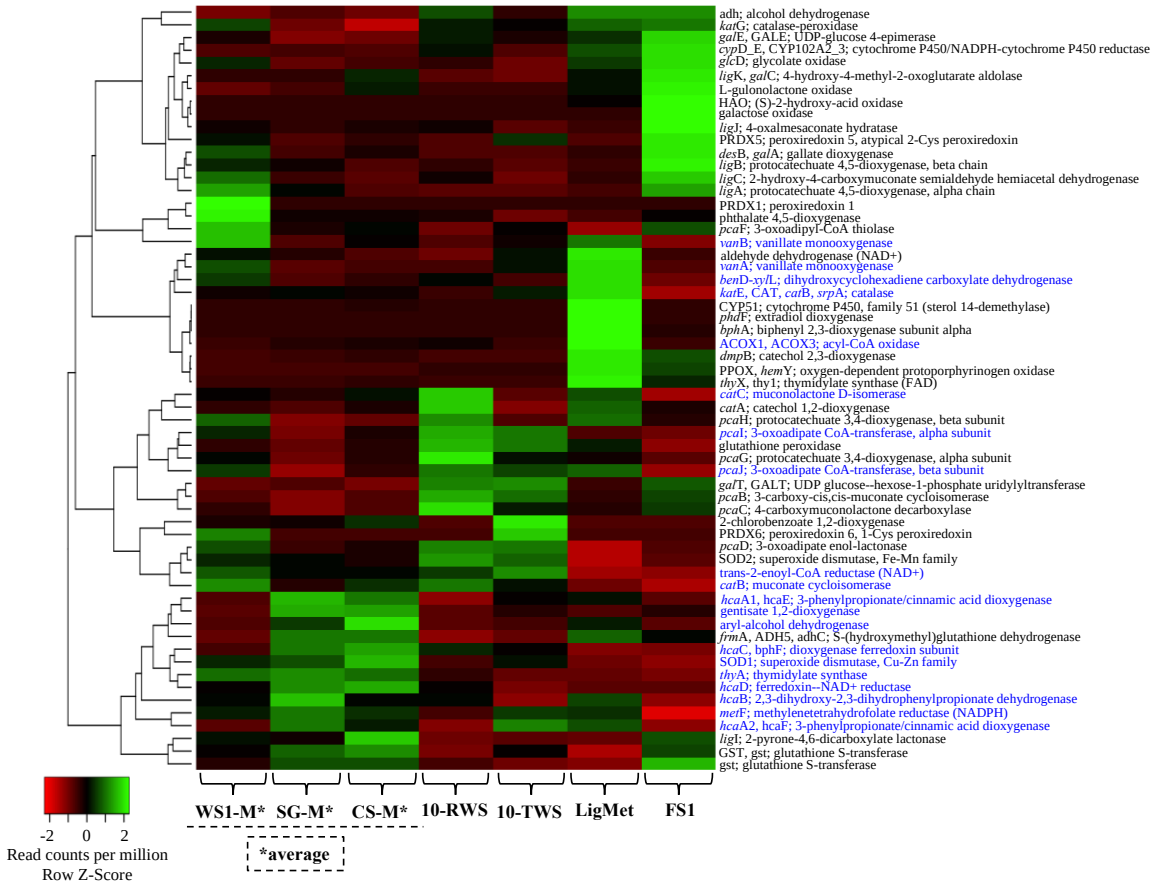
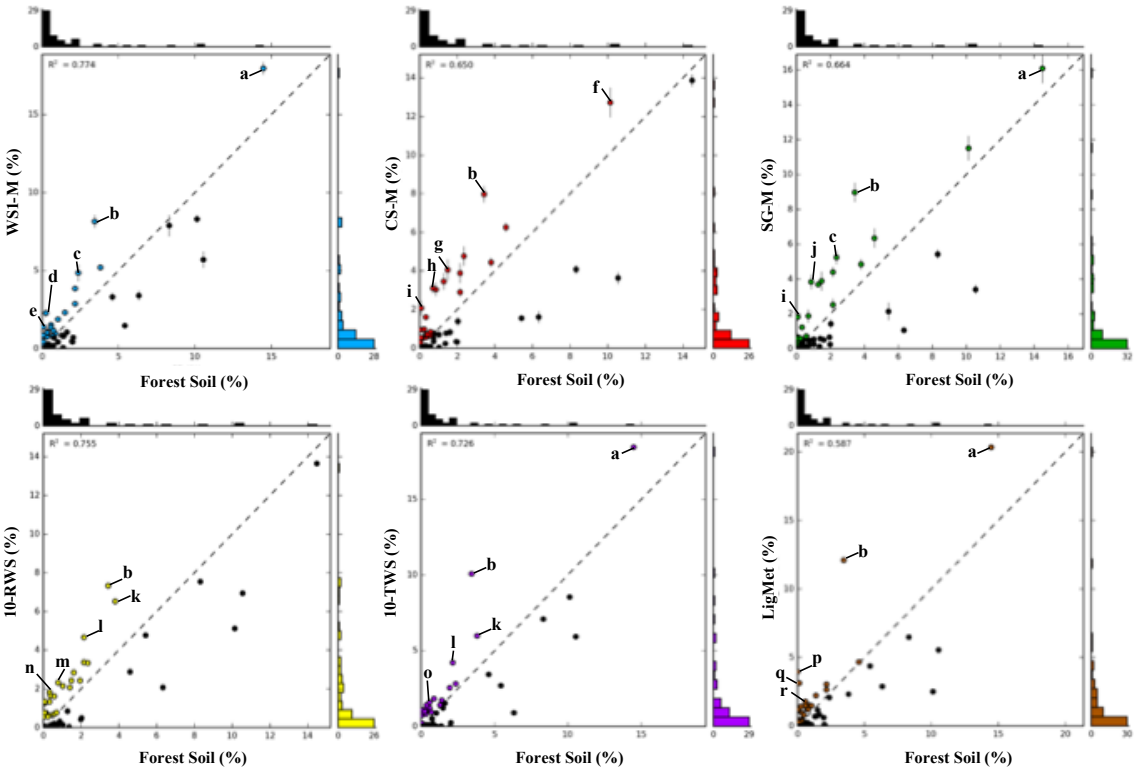

adh; alcohol dehydrogenase
katG; catalase-peroxidase
galE; GALE; UDP-glucose 4-epimerase
cypD_E, CYP102A2_3; cytochrome P450/NADPH-cytochrome P450 reductase
glcD; glycolate oxidase
ligK, galC; 4-hydroxy-4-methyl-2-oxoglutarate aldolase
L-gulonolactone oxidase
HAO; (S)-2-hydroxy-acid oxidase
galactose oxidase
ligJ; 4-oxalmesaconate hydratase
PRDX5; peroxiredoxin 5, atypical 2-Cys peroxiredoxin
desB, galA; gallate dioxygenase
ligB; protocatechuate 4,5-dioxygenase, beta chain
ligC; 2-hydroxy-4-carboxymuconate semialdehyde hemiacetal dehydrogenase
ligA; protocatechuate 4,5-dioxygenase, alpha chain
PRDX1; peroxiredoxin 1
phthalate 4,5-dioxygenase
pcaF; 3-oxoadipyl-CoA thiolase
vanB; vanillate monooxygenase
aldehyde dehydrogenase (NAD+)
vanA; vanillate monooxygenase
benD-xylL; dihydroxycyclohexadiene carboxylate dehydrogenase
katE, CAT, catB, srpA; catalase
CYP51; cytochrome P450, family 51 (sterol 14-demethylase)
phdF; extradiol dioxygenase
bphA; biphenyl 2,3-dioxygenase subunit alpha
ACOX1, ACOX3; acyl-CoA oxidase
dmpB; catechol 2,3-dioxygenase
PPOX, hemY; oxygen-dependent protoporphyrinogen oxidase
thyX, thy1; thymidylate synthase (FAD)
catC; muconolactone D-isomerase
catA; catechol 1,2-dioxygenase
pcaH; protocatechuate 3,4-dioxygenase, beta subunit
pcaI; 3-oxoadipate CoA-transferase, alpha subunit
glutathione peroxidase
pcaG; protocatechuate 3,4-dioxygenase, alpha subunit
pcaJ; 3-oxoadipate CoA-transferase, beta subunit
galT; GALT; UDP glucose--hexose-1-phosphate uridylyltransferase
pcaB; 3-carboxy-cis,cis-muconate cycloisomerase
pcaC; 4-carboxymuconolactone decarboxylase
2-chlorobenzoate 1,2-dioxygenase
PRDX6; peroxiredoxin 6, 1-Cys peroxiredoxin
pcaD; 3-oxoadipate enol-lactonase
SOD2; superoxide dismutase, Fe-Mn family
trans-2-enoyl-CoA reductase (NAD+)
catB; muconate cycloisomerase
hcaA1, hcaE; 3-phenylpropionate/cinnamic acid dioxygenase
gentisate 1,2-dioxygenase
aryl-alcohol dehydrogenase
frmA, ADH5, adhC; S-(hydroxymethyl)glutathione dehydrogenase
hcaC, bphF; dioxygenase ferredoxin subunit
SOD1; superoxide dismutase, Cu-Zn family
thyA; thymidylate synthase
hcaD; ferredoxin--NAD+ reductase
hcaB; 2,3-dihydroxy-2,3-dihydrophenylpropionate dehydrogenase
metF; methylenetetrahydrofolate reductase (NADPH)
hcaA2, hcaF; 3-phenylpropionate/cinnamic acid dioxygenase
ligI; 2-pyrone-4,6-dicarboxylate lactonase
GST, gst; glutathione S-transferase
gst; glutathione S-transferase
WS1-M*
SG-M*
CS-M*
10-RWS
10-TWS
LigMet
FS1
*average
-2
0
2
Read counts per million
Row Z-Score

Figure 3



a) aldehyde dehydrogenase (NAD+) [EC:1.2.1.3]
b) *kat*E, CAT, *cat*B, *srp*A; catalase [EC:1.11.1.6]
c) *thy*A; thymidylate synthase [EC:2.1.1.45]
d) *van*B; vanillate monooxygenase [EC:1.14.13.82]
e) *cat*B; muconate cycloisomerase [EC:5.5.1.1]
f) GST, gst; glutathione S-transferase [EC:2.5.1.18]
g) *hca*D; ferredoxin--NAD+ reductase [EC:1.18.1.3]
h) aryl-alcohol dehydrogenase [EC:1.1.1.90]
i) *hcaC*, *bphF*; dioxygenase ferredoxin subunit
j) *hca*A1, *hca*E; 3-phenylpropionate/cinnamic acid dioxygenase subunit alpha [EC:1.14.12.19]
k) SOD2; superoxide dismutase, Fe-Mn family [EC:1.15.1.1]
l) glutathione peroxidase [EC:1.11.1.9]
m) *cat*A; catechol 1,2-dioxygenase [EC:1.13.11.1]
n) *pca*G; protocatechuate 3,4-dioxygenase, alpha subunit [EC:1.13.11.3]
o) *pca*D; 3-oxoadipate enol-lactonase [EC:3.1.1.24]
p) CYP51; cytochrome P450, family 51 (sterol 14-demethylase) [EC:1.14.13.70]
q) ACOX1, ACOX3; acyl-CoA oxidase [EC:1.3.3.6]
r) *van*A; vanillate monooxygenase [EC:1.14.13.82]

Figure 4

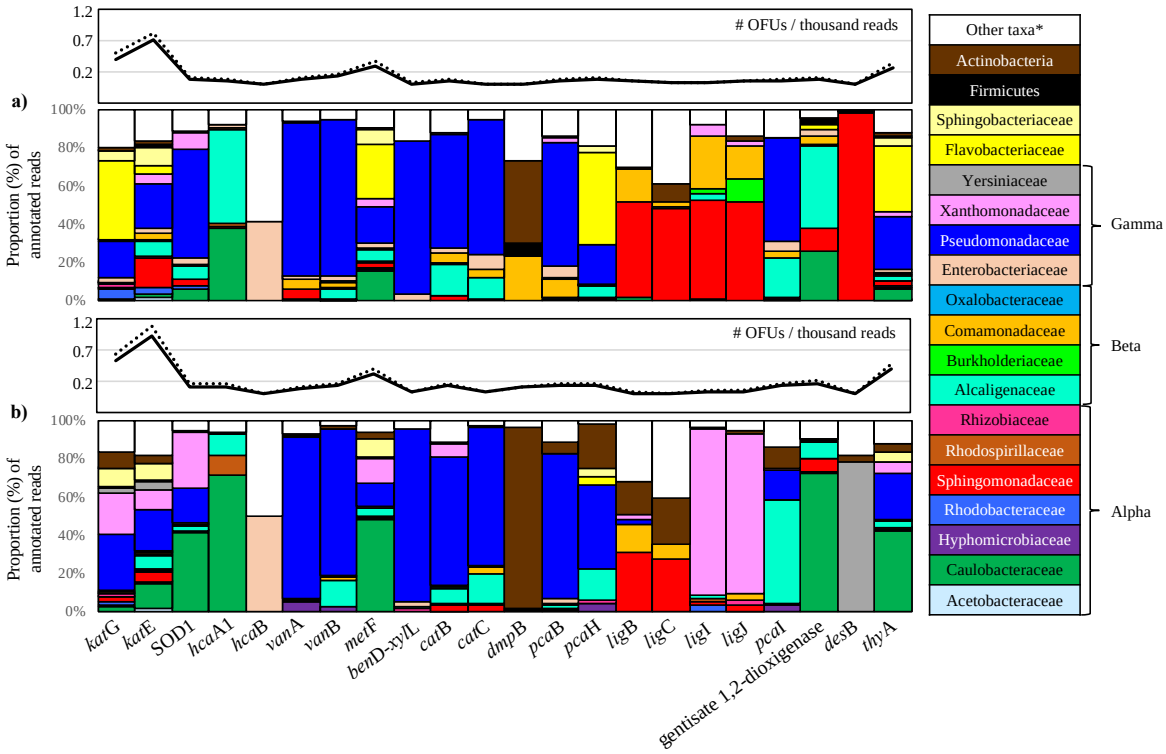

a)
b)
OFUs / thousand reads
1.2
0.7
0.2
Proportion (%) of annotated reads
100%
80%
60%
40%
20%
0%
katG
katE
SOD1
hcaA1
hcaB
vanA
vanB
metF
benD-xylL
catB
catC
dmpB
pcaB
pcaH
ligB
ligC
ligI
ligJ
pcaI
gentisate 1,2-dioxigenase
desB
thyA
Other taxa*
Actinobacteria
Firmicutes
Sphingobacteriaceae
Flavobacteriaceae
Yersiniaceae
Xanthomonadaceae
Pseudomonadaceae
Enterobacteriaceae
Oxalobacteraceae
Comamonadaceae
Burkholderiaceae
Alcaligenaceae
Rhizobiaceae
Rhodospirillaceae
Sphingomonadaceae
Rhodobacteraceae
Hyphomicrobiaceae
Caulobacteraceae
Acetobacteraceae
Gamma
Beta
Alpha

Figure 5

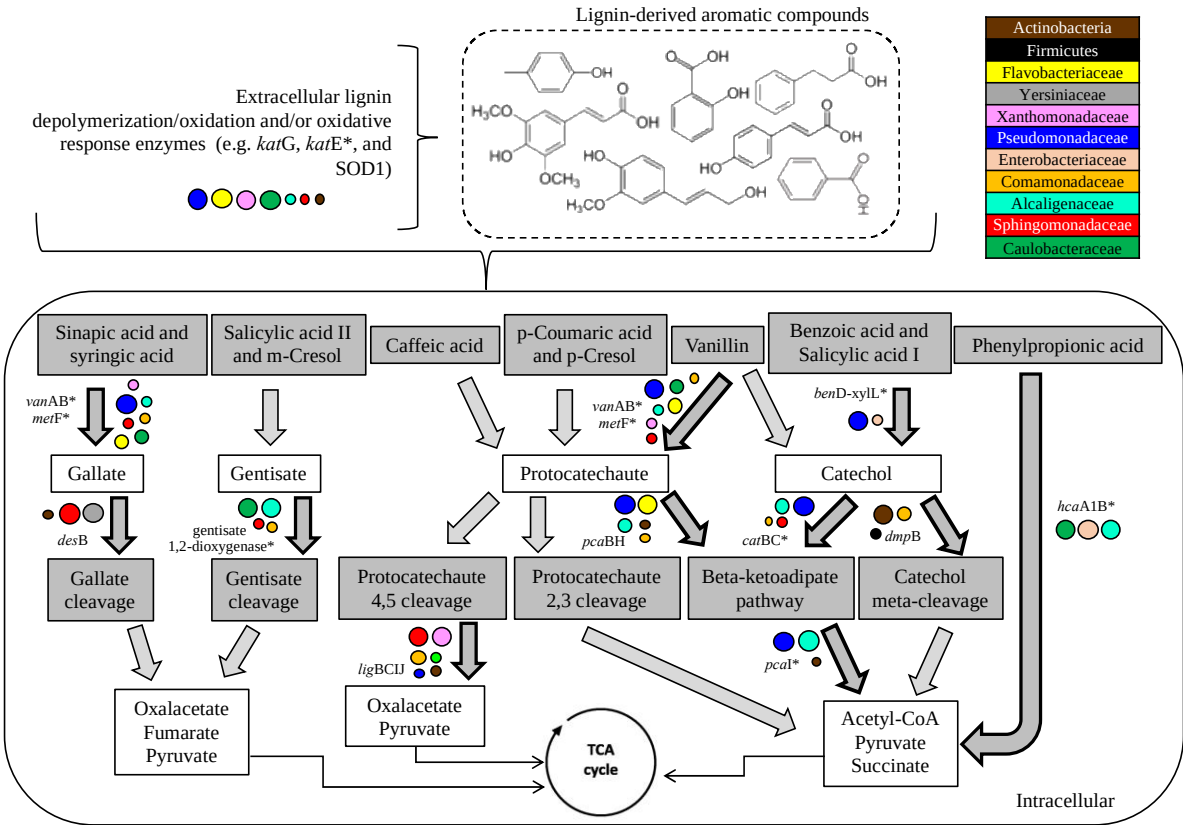

Lignin-derived aromatic compounds
Extracellular lignin depolymerization/oxidation and/or oxidative response enzymes (e.g. katG, katE*, and SOD1)
Actinobacteria
Firmicutes
Flavobacteriaceae
Yersiniaceae
Xanthomonadaceae
Pseudomonadaceae
Enterobacteriaceae
Comamonadaceae
Alcaligenaceae
Sphingomonadaceae
Caulobacteraceae
Sinapic acid and syringic acid
Salicylic acid II and m-Cresol
Caffeic acid
p-Coumaric acid and p-Cresol
Vanillin
Benzoic acid and Salicylic acid I
Phenylpropionic acid
vanAB* metF*
Gallate
desB
Gallate cleavage
Gentisate
gentisate 1,2-dioxygenase*
Gentisate cleavage
Protocatechaute
pcaBH
Protocatechaute 4,5 cleavage
ligBCIJ
Oxalacetate Pyruvate
Protocatechaute 2,3 cleavage
Catechol
catBC*
Beta-ketoadipate pathway
pcaI*
benD-xylL*
dmpB
Catechol meta-cleavage
hcaA1B*
Oxalacetate Fumarate Pyruvate
TCA cycle
Acetyl-CoA Pyruvate Succinate
Intracellular

Figure 6