Discovery of lignin-transforming bacteria and enzymes in thermophilic environments using stable isotope probing

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Characterizing microorganisms and enzymes involved in lignin biodegradation in thermal ecosystems can identify thermostable biocatalysts. We integrated stable isotope probing (SIP), genome-resolved metagenomics, and enzyme characterization to investigate the degradation of high-molecular weight, 13C-ring-labeled synthetic lignin by microbial communities from moderately thermophilic hot spring sediment (52 °C) and a woody "hog fuel" pile (53 and 62 °C zones). 13C-Lignin degradation was monitored using IR-GCMS of 13CO2, and isotopic enrichment of DNA was measured with UHLPC-MS/MS. Assembly of 42 metagenomic libraries (72 Gb) yielded 344 contig bins, from which 125 draft genomes were produced. Fourteen genomes were significantly enriched with 13C from lignin, including genomes of Actinomycetes (Thermoleophilaceae, Solirubrobacteraceae, Rubrobacter sp.), Firmicutes (Kyrpidia sp., Alicyclobacillus sp.) and Gammaproteobacteria (Steroidobacteraceae). We employed multiple approaches to screen genomes for genes encoding putative ligninases and pathways for aromatic compound degradation. Our analysis identified several novel laccase-like multi-copper oxidase (LMCO) genes in 13C-enriched genomes. One of these LMCOs was heterologously expressed and shown to oxidize lignin model compounds and minimally transformed lignin. This study elucidated bacterial lignin depolymerization and mineralization in thermal ecosystems, establishing new possibilities for the efficient valorization of lignin at elevated temperature.

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INTRODUCTION

Lignin is the second most abundant terrestrial biopolymer after cellulose, and can comprise 10–30% of a plant’s dry weight [1]. While biological conversion of plant biomass to fuels and chemicals can reduce fossil fuel consumption, few processes exist to valorize lignin to value-added chemicals [2]. This is due in part to its recalcitrance and heterogeneity: lignin is a complex heteropolymer containing diverse ether and carbon-carbon bonds linking phenylpropanoid aromatic subunits. A promising approach to divert lignin from waste streams for production of valuable bioproducts is biological lignin valorization, involving lignin depolymerization and biocatalysts that funnel lignin-derived aromatic compounds (LDACs) into commercial chemicals [2]. There is a need to develop thermotolerant biocatalysts for efficient conversion of lignin derivatives produced by industrial processes [3].

In nature, fungi are thought to be mainly responsible for lignin depolymerization, with white rot fungi utilizing lignin peroxidases (EC 1.11. 1.14) and laccases (benzenediol oxygen oxidoreductases, EC 1.10.3.2) to do so. However, bacteria are increasingly recognized for their contributions to this process [4–6]. Investigation of bacterial lignin depolymerization has focused on two enzyme classes: dye-depolymerizing peroxidases (DyPs) and laccase-like multi-copper oxidases (LMCOs). Bacterial LMCOs carry out myriad reactions. Importantly, LMCOs in two-domain superfamilies, including K-type small laccases (SLACs), are capable of efficient lignin depolymerization [7, 8]. Further, bacteria have catabolic pathways that funnel diverse LDACs into catabolic intermediates (e.g., protocatechuate and catechol). These diols are typically degraded via either meta or ortho ring-cleavage pathways [6, 9]. Characterization of novel thermotolerant lignin-degrading bacteria therefore requires (1) evidence for their involvement in lignin degradation, (2) identification of enzymes that depolymerize lignin, and (3) identification of pathways for catabolic funneling of LDACs.

To facilitate identification of novel lignolytic organisms and biocatalysts, we undertook genomic bioprospecting in thermal environments. Massive piles of wood residue, known as “hog fuel,” can reach temperatures sufficient for spontaneous combustion due to biological activity. We hypothesized that thermophilic microbes within hog fuel are adapted to use lignin and LDACs as carbon sources. Likewise, we hypothesized that geothermal hot springs with regular inputs of woody biomass harbor thermophiles capable of catabolizing lignin. To test these hypotheses, samples of hog fuel and hot spring microbial communities were incubated with a synthetic 13C-ring-labeled lignin dehydrogenation polymer (DHP) to facilitate stable-isotope probing (SIP). 13C-Enriched genomes resolved from metagenomic libraries encoded a variety of enzymes with the potential for depolymerization of lignin and catabolism of LDACs. Subsequently, we heterologously expressed a two-domain LMCO and characterized...
its ability to transform β-aryl ether lignin model compounds and Eucalyptus milled wood lignin.

**MATERIALS AND METHODS**

**Sampling thermal environments**

Lakelse hot spring (54°21’30.7”N, 128°32’28.0”W), near Terrace, Canada, is a concrete-enclosed pool 1–5 m deep, fed by geothermally warmed spring water (53.2 °C, pH 7.5) [10, 11]. About 500 ml of the top ~5 cm layer of organic-rich sediment was sampled at four equidistant locations on August 24th, 2017 using a manual pump. Sediment and spring water were placed in autoclaved 1 L Nalgene bottles and placed on ice. Two additional sediment samples per location for DNA extraction were placed in 5 ml screw-top vials and placed immediately on dry-ice. A 1.93 ha hog fuel pile in Crofton, Canada (48°52’31.9”N, 123°39’08.1”W) containing sub-boreal spruce, western redcedar and Douglas-fir residue was sampled on September 27, 2017. Three 1 m pits were dug at 40 m intervals along the perimeter of the pile and ~500 ml samples were removed from 20 and 80 cm depths (52.9 °C and 58.7 °C, respectively) with an ethanol- and distilled H2O-washed trowel. Bulk samples were stored on ice and 5 ml aliquots were stored on dry ice.

13C-DHP lignin microcosms

Sediment was separated from spring water using Steritop Filter bottles (Sigma-Aldrich, St. Louis, U.S.A.). Two sets of three replicate sediments from lakelse, and hog fuel samples from 20 and 80 cm equivalent to 1 g dry weight were added to autoclaved 50 ml serum bottles with 0.1 g of 10% 13C-DHP lignin or 12C-DHP lignin plus 5 ml M9 buffer [12], and the bottles were crimp-sealed. 13C-DHP lignin was synthesized as in [4] and in the Supplementary Methods. Lakelse sediment and 20 cm hog fuel were incubated at 53 °C, while 80 cm hog fuel was incubated at 62 °C. Incubations were in rotary shakers at 150 rpm.

13C-CO2 respiration analysis

We monitored 13C-CO2 production as an indicator of 13C-DHP lignin mineralization. In total, 0.5 ml of serum bottle headspace air was manually-injected into an Isoprime gas chromatograph isotope ratio mass spectrometer (GV Instruments, Wythenshawe, U.K.) using a 1.0-ml glass syringe. Headspace CO2 concentrations were calculated using a standard curve of 1.0-ml glass syringe.

DNA extractions and fractionation

DNA was extracted from 0.5 g of three replicate in situ thermal hot spring sediment and hog fuel samples using NucleoSpin Soil kits (Macherey-Nagel, Düren, Germany). After 24 days incubation, three replicate microcosms with each of the three inocula were emptied into sterile 15 ml Falcon tubes and centrifuged for 10 min at 4000 rpm at 4 °C. The ISME Journal (2022) 16:1944 – 1956

**RESULTS**

**13C-lignin catabolism**

In this study, we investigated the ability of thermophilic bacteria to mineralize synthetic lignin and assimilate lignin derivatives via stable isotope probing. We first synthesized ~3.5 g 13C-DHP with a mean molecular weight of 19.3 ± 0.2 kDa, approximately equivalent to 100 aromatic nuclei per DHP polymer. This synthetic lignin was recovered in this fraction from 13C-DHP lignin microcosms. Fraction six (F6, ~1.727 g ml⁻¹) was used as the “light” fraction for both. One ng DNA from light and heavy fractions were used to generate metagenomic sequencing libraries using the Nextera XT DNA Library Prep Kit (Illumina, San Diego, U.S.A.). In total, 12 libraries from hog fuel microcosms and 12 libraries from hot spring microcosms (Supplementary Table 1) were multiplexed separately on two runs of NextSeq (Illumina) using 150-bp paired-end sequencing in High Output mode at the UBC Sequencing and Bioinformatics Consortium (Vancouver, CAN).
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Evolved about 245 ± 45 µmol ¹³C-CO₂ g⁻¹ sediment or ground hog fuel, respectively, while Lakelse sediment to 24 days. We monitored the mineralization of ¹³C-DHP to CO₂ incubated with 2 g hot spring sediment or ground hog fuel for up to 150 h. (A) ¹³C-DHP Lignin Structure. (B) ¹³C-DHP Lignin Incorporation. (C) ¹³C-DHP Lignin Mineralization. (D) ¹³C-DNA Incorporation. (E) ¹³C-DNA Mineralization. (F) ¹³C-DNA Resolution.

Fig. 1 Stable isotope probing (SIP) using ¹³C-DHP lignin polymer. A Detail of DHP lignin structure supported by GPC-MALS, 2D HSQC NMR and ¹³C-NMR analysis. Mn number average molecular weight (average molecular weight of all the polymer chains), PDI polydispersity index or Mw/Mn ratio. Mw, Mn and PDI determined by GPC-MALS. Red dots show possible positions of ¹³C-isotopes in coniferyl subunit rings. B ¹³C-CO₂ measured in the headspace of 50-ml sealed serum bottles containing 1 g (dw) of each sample incubated with 100 mg (10%) ¹³C-DHP lignin polymer. A ¹³C-DHP Lignin incorporating ¹³C-DHP to CO₂ measured in the headspace of 50-ml sealed serum bottles containing 1 g (dw) of each sample incubated with 100 mg (10%) ¹³C-DHP lignin polymer. A ¹³C-DHP Lignin Incorporation. Approximate 0.75% (w/w) ¹³C-DHP or ¹²C-DHP (control) was incubated with 2 g hot spring sediment or ground hog fuel for up to 24 days. We monitored the mineralization of ¹³C-DHP to ¹³C-CO₂ to determine if the lignin was mineralized (Fig. 1B). Measuring the incubation headspace using IR-GC-MS showed that, after 24 days, hog fuel from 20 and 80 cm depths evolved about 473 ± 18 and 681 ± 82 µmol ¹³C-CO₂ per gram of hot spring sediment or ground hog fuel, respectively, while Lakelse sediment evolved about 245 ± 45 µmol ¹³C-CO₂ g⁻¹. For comparison, ¹³C-DHP incubated without inoculum evolved 12 µmol ¹³C-CO₂ g⁻¹, and sediment incubated without ¹³C-DHP evolved 36 µmol ¹³C-CO₂ g⁻¹. Thus, microbial communities from thermal environments mineralized lignin at in situ temperatures.

Density fractionation was used to isolate DNA from microbes that incorporated ¹³C from the labeled synthetic lignin during incubation. To verify isotopic-labeling of DNA, UPLC-MS/MS was used to calculate atom% ¹³C in each of the 12 recovered fractions (Fig. 1C). Fractions 1–4 (F1–4) from ¹³C-DHP microcosms had a mean density of 1.738–1.745 g ml⁻¹ and contained about 20–60 atom% ¹³C. All fractions from ¹²C-DHP microcosms had a baseline mean density of 1.738 g ml⁻¹. Graphs show DNA concentration vs. fractional density and %¹³C-DNA for Hog Fuel 20 cm and Lakelse samples. %¹³C-DNA measured using UPLC-MS/MS.

Resolution of genomes from ¹³C-enriched metagenomes

To facilitate the identification and characterization of putatively lignolytic bacteria in thermal environments, we focused our investigation on genome assemblies resolved from shotgun sequencing of fractionated DNA. Fifty-two of these MAGs passing quality thresholds (>80% completion, <5% contamination) were assembled from hog fuel and 72 from hot spring sediment. DESeq2 was used to statistically compare MAG abundance between high-density fractions from ¹³C-DHP (F1–4) and ¹²C-DHP (F6) microcosms (Figs. 2 and 3), as well as between...
Actinobacteria, Steroidobacteraceae (Fig. 2). The remainder of enriched MAGs were Firmicutes (MB2.88, MB2.97, CON.104). While some Kyrpidia and Alicyclobacillus reference genomes encode catechol meta-degradation (Fig. 4), only the C.1-High and 12C-High for each genome with >L2FC 0 indicating enrichment in 13C-High libraries. Error bars represent standard error of L2FC. Bar plot provides cut-off estimates for significance at \( p_{adj} = 0.05 \) (individual \( p_{adj} \) values <0.05 provided).

High-density and low-density (F10) fractions from 13C-DHP microcosms (Figs. S2 and S3). MAGs with significantly higher (p < 0.05) abundance in 13C-F1-4 relative to 12C-F6 were considered 13C-enriched. There were four 13C-enriched MAGs in hog fuel 20 cm libraries, another three in the 80 cm libraries, and two in libraries from both depths. There were five 13C-enriched MAGs in hot spring sediment libraries. One gram-negative proteobacterial MAG enriched in hog fuel (MB2.51) was placed in family Thermaerobacteria (Fig. 2). The remainder of enriched MAGs were Gram-positive bacteria, including the phyla Chloroflexi, Actinobacteria and Firmicutes. MB2.64 from hog fuel was placed in the thermophilic actinobacterial family Solirubrobacteraceae, and was enriched over 300-fold in both 20 and 80 cm libraries.

While we were able to recover 125 MAGs with an average single-copy gene completeness of 87% (Supplementary Data 2), the full suite of metabolism-encoding genes was likely not recovered for all, potentially resulting in incomplete annotation of aromatic degradation pathways. Of the hog fuel MAGs, MB2.64 (99% completeness), encoded catechol and protocatechuate ortho-cleavage and 4-hydroxybenzoate monooxygenase (Fig. 4). A Thermophilobacteraceae MAG, MB2.39 was also highly enriched with 13C in the 80 cm hog fuel microcosm and like MB2.64, is a member of the thermophilic order Solirubrobacterales. Other 13C-enriched Actinobacteria include CON22 (Actinomadura rubrobrunnea), MAX.045 (Frankia sp.), MAX.030 (Micromonosporaceae), CON124 (Rubrobacter sp.) and MB2.74 (Rubrobacter sp.) (Fig. 3). These MAGs all encoded protocatechuate degradation. In addition, A. rubrobrunnea encoded a two-component vanillate O-demethylase, while the Micromonosporaceae MAG appeared to encode a LigM-type (aminomethyltransferase) vanillate O-demethylase based on pHMM results (Fig. 4). To account for incomplete annotation of our MAGs, their aromatic degradation pathways were compared with those annotated in closely related strains (Fig. 4), revealing that vanillate O-demethylation and protocatechuate ortho-cleavage are encoded widely in thermophilic Actinobacteria.

The 13C-enriched Lakelse hot spring MAGs represented a higher proportion of Firmicutes than the hog fuel MAGs (Figs. 2 and 3). These included Kyrpidia sp. (CON.60) and Alicyclobacillus sp. (MB2.88, CON.104). While some Kyrpidia and Alicyclobacillus reference genomes encode catechol meta-cleavage (Fig. 4), only CON.60 was found to encode this pathway in our MAG dataset. Three Alicyclobacillus genomes (MB2.88, CON25, and MB2.97) encoded LMCs with high amino acid identity (≥85%) to a homolog in phenolic- and polyphenolic-oxidizing Alicyclobacillus acidocaldarius DSM 446 [3]. Of these, MB2.88 contained two L-type 3-domain LMCs with 100% amino acid identity to those encoded by DSM 446.
Typically, DyPs and LCMOs are only broadly classified by existing pHHMs, or in sequence databases. We therefore applied phylogenetic profiling with TreeSAPP [39] to classify these enzymes into discrete sub-families (Fig. 5A). Sequences from FMMs, or in sequence databases. We therefore applied MAGs were placed only into A and B DyP types. Few DyPs were involved in lignin depolymerization has no detectable signal sequence [36]. In contrast to DyPs, 82 LCMOs were detected in hot spring MAGs, and 100 were detected in hog fuel MAGs. Of these, only four were detected in LCCED sub-family 11, corresponding to SLAC or K-type laccases, all of which contained TAT signal peptides (Fig. 5B). Additionally, an O-type two-domain LCMO was detected in MB2.51 (Steroidobacteraceae), also containing a leading secretion signal peptide (Fig. 5C). We named this enzyme LacOts1.

The classification of LCMOs into super-families, and comparison with enzymes of known function, may shed light on their functional roles. We applied structural alignment to assess the relationships between sequence, structure and function (Fig. 5D). Cu- and substrate-binding residues were highly conserved across K-type LMOs including those recovered in this study. However, a 15-amino acid sequence hypothesized to act as a “flap” covering one of the channels leading to the trinuclear cluster [40], which was present in all known SLACs, was absent in the recovered

**Fig. 3** Lakelse MAG abundance in $^{13}$C-High (F1–4) and and $^{12}$C-High (F6) SIP libraries. Phylogenetic tree, heatmaps, and bar plot as in Fig. 2.

**Identification of putative ligninases**

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Fig. 4 Predicted aerobic aromatic degradation pathways, dye-depolymerizing peroxidases (Dyps) and laccase-like multi-copper oxides (LMCOs) in 13C-DHP lignin enriched MAGs and select reference genomes. Phylogeny of MAGs as in Figs. 2 and 3. Aromatic degradation pathway genes were annotated using profile HMMs for KEGG orthologs (KO) with e < 0.01 and HMM scores above KO-specific thresholds. Syringate O-demethylase (LigM) orthologs annotated using the TreeSAPP reference package are shown with 50% opacity (i.e., light pink). Individual orders containing enriched MAGs are highlighted. H4F Tetrahydrofolate, PCA Protocatechuate acid, HB Hydroxybenzoate, BKA beta-ketoadipate.

thermophilic K-type LMCOs. Based on these sequence differences and the results of phylogenetic clustering, we categorize the LMCOs recovered from thermal systems as K2-type LCMOs, in contrast to the K1-type LCMOs found in mesophilic Actinobacteria such as Streptomyces coelicolor [46]. The lignin-degradation potential and thermostolerance of the K2 laccase clade remains uncharacterized.

Putative tetrahydrofolate-dependent O-demethylases

As our model lignin is comprised of 100% guaiaclyl-subunits, we hypothesized that mineralization of DHP requires O-demethylation. While we identified a small number of vanillate O-demethylases (Fig. 4), which are Rieske-type oxygenases, we also investigated the potential for tetrahydrofolate-dependent O-demethylation of methoxylated aromatic compounds. To interrogate MAGs for tetrahydrofolate-dependent aryl O-demethylases, we once again used phylogenetic placement. We categorized aminomethyltransferases by putative function and taxonomic identity, with LigM and DesA sequences partitioning into distinct clusters (Fig. 6A). Assembled sequences placed into the tree formed a separate clade emerging from the DesA branch, which we have labeled “DesA-like aminomethyltransferases” (Fig. 6B). Specifically, MB2.64 (Solirubrobacter), MB2.39 (Thermoleophilaeacea), CON124 (Rubrobacter sp.), and MB2.51 (Steroidobacteraceae) all contained what appear to be DesA-like aminomethyltransferases, with conservation of a methyl-transferring tyrosine residue verified by structure-guided protein alignment (Fig. 6C). While the metabolic function of these enzymes requires validation, it is intriguing that they may facilitate the O-demethylation of LDACs in thermophilic bacteria.

Characterization of LacO25T51

A key question that emerged from metabolic reconstruction of 13C-DHP-enriched MAGs following the microcosm study was the mechanism for the observed lignin depolymerization. In the above analysis we focused on the LCMOs as a possible answer. To test this hypothesis, and potentially identify novel biocatalysts, we selected four two-domain LCMOs (two K2-type, one O-type, one N-type) for heterologous expression, with the objective of evaluating their role in depolymerizing lignin (Table 1). Of these
only "LacOST51," the O-type LCMO from MB2.51, proved soluble when expressed in E. coli (Table 1). Expression in Rhodococcus jostii RHA1 did not improve the solubility of the other proteins. SDS-PAGE indicated that the LacOST51 protein was purified to >99% apparent homogeneity and had a molecular mass of ~36 kDa. Purified LacOST51 exhibited the blue color typical of laccases, and had an absorption band at 620 nm, characteristic of a T1 blue copper site. The preparation had a molar copper content of 4.0 ± 0.2, indicating that the purified LacOST51 was loaded with a full complement of copper.

LacOST51 utilized 2,2′-azino-di-(3-ethylbenzthiazoline sulfonate) (ABTS) and 2,6-dimethoxyphenol (DMP) as reducing substrates with specific activities of 1.46 and 0.03 U mg⁻¹, respectively. The specific activity of LacOST51 for ABTS was of the same order of magnitude as that reported for other bacterial laccases (Table 1). However, specific activity for DMP was lower than the value for most other reported laccases. The oxidation of DMP was optimal at pH 8, and the enzyme retained ~90% activity at pH 9. These values are higher than the average reported for other bacterial laccases (Table 1). However, specific activity for DMP was lower than the value for most other reported laccases. The oxidation of DMP was optimal at pH 8, and the enzyme retained ~90% activity at pH 9. These values are higher than the average reported for other bacterial laccases (Table 1). However, specific activity for DMP was lower than the value for most other reported laccases.

Transformation of enzymatic mild acidolysis lignin by LacOST51

We tested the ability of LacOST51 to transform EMAL, a minimally altered form of lignin that contains little residual cellulose or hemicellulose from Eucalyptus wood [45]. A solution of EMAL incubated without enzyme for 6 days contained a significant quantity of vanillin (tR = 10.6 min) and oligomeric material that eluted as a broad band (tR = 13–22 min) (Fig. 7B). Incubation with 6 µM LacOST51 additionally resulted in the production of...
Aryl Tetrahydrofolate-Dependent O-Demethylase Reference Tree

**Fig. 6 Aminomethyltransferase family protein phylogeny using TreeSAPP reference package.** A Reference tree produced by TreeSAPP with 50 amino acid sequences using RAxML under the PROTGAMMALG model and 1000 iterations. Tree includes experimentally-validated green, and the primary methyl-transferring catalytic tyrosine residue shown in orange. B Placement of predicted MAG-encoded aminomethyltransferases into the reference tree. C Multiple sequence alignment with MAFFT using the ginsi setting under 1000 iterations for select sequences. Aromatic-binding residues derived from LigM structural model are shown in pink, folate-binding residues are shown in green, and the primary methyl-transferring catalytic tyrosine residue shown in orange. D Pathway diagram of tetrahydrofolate-dependent O-demethylation of methoxylated aromatic compounds.

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syringaldehyde and 2,6-dimethoxy benzoquinone (DMBQ) as well as a reduction in the amount of oligomeric material. To provide insight into how LacO<sub>ST51</sub> modified the lignin, the transformed lignin was isolated and characterized by NMR and GPC-MALS spectrometry. In these experiments, sLac was used as a positive control as it had previously been shown to transform lignin [7]. HSQC NMR spectra from laccase-treated lignin samples differed significantly from those of the no-enzyme controls (Fig. 7C).

Specifically, in samples treated with either LacO<sub>ST51</sub> or sLac, the secondary aliphatic hydroxyl groups of the lignin were oxidized to their benzylic ketone, as reflected by the increased in signals S', G', A', and A". This oxidation process leads to the ability to cleave the propyl side chain with oxidative reagents resulting in rupture of native linkages (β-O-4, A) as illustrated with model compounds [46]. The slightly modified reaction on the C-C linkages including β-5 (B) and β-β (C) in our study and elsewhere [47] require more investigation. Based on the amount of linkages modified, sLac modified the lignin more efficiently than LacO<sub>ST51</sub>.

GPC analysis indicated that treatment of EMAL with either LacO<sub>ST51</sub> or sLac yielded lignin with a higher apparent molar mass (M<sub>w</sub> and M<sub>n</sub>) and increased the range of fragment size (Fig. 7B). However, sLac treatment resulted in a significant amount of insoluble material that was not included in this analysis. The higher molecular weight for both treated materials is presumably due to condensation reactions between the aromatic radicals [48]. The observed polymerization activity of LacO<sub>ST51</sub> and sLac is consistent with studies of other laccases (e.g. [49]) and depends on the reaction conditions, particularly the relative concentrations of lignin species of different molecular weight. Indeed, sLac catalyzes the depolymerization of lignin in steam-pretreated poplar in the presence of natural mediators [7]. Overall, these data demonstrate that LacO<sub>ST51</sub> oxidatively transforms lignin in the same manner as other laccases.

**DISCUSSION**

We hypothesized that lignin-degrading microorganisms occur in thermal environments that receive woody biomass inputs and would assimilate carbon from a synthetic lignin. The synthesis of 1<sup>3</sup>C-DHP lignin in our laboratory was essential for accurate assessment of such assimilation, as 1<sup>3</sup>C-labeled lignin of the purity used in this study is not commercially available. We verified lignin degradation in microcosms by monitoring 13C-CO<sub>2</sub> production and 13C-incorporation into recovered DNA. The incorporation of 13C molecules from labeled, high-molecular-weight synthetic 13C-DHP lignin in our laboratory was essential for accurate assessment of such assimilation.

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Several bacterial taxa were enriched with $^{13}$C from synthetic lignin. In hog fuel, these bacteria primarily belong to the proposed actinobacterial class Thermolephila—members of which are abundant in geothermal environments and soil but have poorly characterized metabolic potential [50]. Specifically, the genus *Rubrobacter* includes known thermophiles [51, 52], and *Rubrobacter* OTUs were strongly associated with lignocellulose degradation and tolerance of phenolic lignin metabolites at 55 °C [53]. Related actinobacterial MAGs, including from an *Actinomadura* rubrobrunea strain (CON22), were also $^{13}$C-enriched in hog fuel microcosms. CON22 contained one of few Rieske vanillate ortho-demethylases found in this study and encoded complete protocatechuate ortho-cleavage and partial meta-cleavage pathways (Fig. 4). While the lignin-degradation potential of *A. rubrobrunea* has yet to be characterized, other *Actinomadura* strains have been found to solubilize lignocellulose [54], and contribute to the degradation of the cellulose [55] or lignin [56]. The *Rubrobacter* and *A. rubrobrunea* strains identified in this study make compelling targets for further investigation. The MB2.64 MAG was placed in the actinobacterial family *Solinobacteraceae*. We previously identified putatively lignolytic *Solinobacteraceae* OTUs in forest soil through a similar stable isotope probing approach [14], but we were unable to resolve MAGs from lignin-assimilating *Actinobacteria* or identify enzymatic mechanisms that would explain their involvement in lignin degradation. We propose MB2.64 from hog fuel has robust lignin degradation potential. In contrast, in hot spring communities, $^{13}$C-enriched taxa were predominantly *Firmicutes* such as *Alicyclobacillus* spp. Therefore, this study expands the taxonomic range of bacteria associated with lignin degradation to include other thermophilic *Actinobacteria* and *Firmicutes*.

In addition to incorporation of $^{13}$C from synthetic lignin and presence of putative ligninases, we used the presence of genes encoding catabolism of aromatic compounds to evaluate each MAG for its lignin degradation potential. A key difference between MAGs from hog fuel and hot springs was not only taxonomy, but also the capacity for aromatic catabolism. Hog fuel MAGs encoded vanillate O-demethylation and the protocatechuate ortho-cleavage pathway, suggesting that LDACs liberated from lignin were funneled into specific degradation pathways. Although several cultured representatives of *Solirubrobacteriales* encode a two-component Rieske vanillate O-demethylase (Fig. 4), none were found in the genome of MB2.64 from the hot spring. This was at odds with our hypothesis that O-demethylation is critical to catabolism of LDACs. However, a methyltransferase with full-length amino acid identity of 69.5% to syringate O-demethylase (Des*$_{SYK}$), and with conserved substrate-binding residues, was identified through phylogenetic placement and sequence alignment. Accordingly, the 5,10-methylene-tetrahydrofolate reductase (*metF*) and formate-tetrahydrofolate ligase (*light*) genes, encoding the tetrahydrofolate-mediated C$_1$ metabolic pathway [57, 58], were also found in the MB2.64 genome (Fig. 5D). Genomes of other Gram-positive bacteria such as *Rubrobacter xylanophilus* DSM9941 [57], *Acetobacterium dehalogenans* and *Desulfotibetium hafniense* [59] (both *Firmicutes*) encode vanillate-demethylating methyltransferases. Thus, the MB2.64 genome provides strong evidence for lignin degradation mediated by bacterial thermophiles, facilitated by a novel one-component actinobacterial O-demethylase.

The $^{13}$C-enriched MAG, MB2.51, from hog fuel was placed in the gammaproteobacterial family, *Thiobacillaceae*. As the name suggests, bacteria in this family can degrade steroidal hormones [60], but also polyvinyl alcohol [61] and rubber [62]. MB2.51 encoded full catechol and protocatechuate meta-cleavage pathways, as well as monooxygenases involved in 4-hydroxybenzoate and phenol hydroxylation. A methyltransferase from MB2.51 contained all conserved Lig*$_{SYK}$* residues involved in methyl-transfer, as well as aromatic substrate and folate binding, suggesting a role for this gene in degradation of methoxylated aromatic compounds. No thermophilic *Thiobacillaceae* strains have been previously

### Table 1. The specific activity of LacO$_{ST}$ and other bacterial laccases.

| Name         | Strain                  | Small laccase | ABTS (U/mg) | DMP (U/mg) |
|--------------|-------------------------|---------------|-------------|------------|
| LacO$_{ST}$  | Steriodobacteraceae MB2.51 | Y             | 1.46        | 0.03       |
| Lac$^a_{TOSS}$ | *Thermomonomatispora* MB2.59 | –             | –           | –          |
| LacK$_{TH39}$ | *Thermolephila* MB2.39 | Y             | –           | –          |
| LacK$_{R64}$ | *Solirubrobacterales* MB2.64 | Y             | –           | –          |
| sLac         | *Amycolatospora* sp. 7sv3 | Y             | 1.19        | 0.21       |
| SLAC$^a$     | Streptomyces coelicolor | Y             | 0.98        | na$^k$     |
| Sd1$^d$      | *Streptomyces sviceus*   | Y             | 21.7        | na         |
| SLAC$^c$     | Streptomyces coelicolor | Y             | 8           | na         |
| GeoLac$^c$  | *Geobacter metallireducens*| N             | 6.67        | 0.04       |
| CotA$^b$     | *Bacillus licheniformis* | N             | 16          | na         |
| Cort$^b$     | *Bacillus* sp. HR03      | N             | 0.15        | na         |
| LacM$^i$     | metagenome               | N             | 2.4         | 2.1        |
| ThioLac$^{c}$| *Thioalkalivibrio* sp. ALR | N             | 0.65        | na         |

$a$This study.

$^b$Reactions at 25 °C. For ABTS: 20 mM sodium acetate (l = 0.1 M), pH 5.0. For DMP: 20 mM sodium phosphate (l = 0.1 M), pH 8.0.

$^c$Sherif et al. [69]. Reactions at 60 °C. For ABTS: 50 mM sodium acetate, pH 4.0.

$^d$Gunne and Uralcher [70]. Reactions at 25 °C. For ABTS: 50 mM McIlvaine’s buffer, pH 4.0.

$^e$Dube et al. [71]. Reactions at 25 °C. For ABTS: 2-(N-morpholino)ethanesulfonic acid (MES)-glycine buffer 0.1 M, pH 4.0.

$^f$Berini et al. [41]. Reactions at 25 °C. For ABTS and DMP: 20 mM HEPES, pH 5.6.

$^g$Koschorreck et al. [72]. Reactions at 25 °C. For ABTS: citrate/phosphate buffer pH 4.0.

$^h$Mohammadjan et al. [73]. Reactions at 25 °C. For ABTS: 100 mM phosphate buffer, pH 4.0.

$^i$Ausec et al. [74]. Reactions at 25 °C. For ABTS: multi-component buffer (10 mM tris-maleate base, 15 mM sodium carbonate, 15 mM phosphoric acid and 250 mM potassium chloride, pH 4.0. For DMP: same buffer, pH 5.0.

$^j$Ausec et al. [75]. Reactions at 25 °C. For ABTS: 200 mM phosphate-citrate (McIlvaine), pH 5.0.

$^k$Not available.
reported. Yet this MAG yielded the only soluble, thermotolerant laccase found in this study, discussed below.

Lignin degradation mechanisms in 13C-enriched hot spring MAGs are less clear than in their hog fuel counterparts. One possible explanation for this is cross-feeding, the catabolism by one organism of LDACs produced by a different lignin depolymerizing organism. However, the two 13C-enriched Alicyclobacillus sp. MAGs from hot spring sediment encoded a suite of L- and O-type LCMOs that bore high sequence identity to LCMOs found in Alicyclobacillus acidocaldarius capable of non-specific cleavage of lignin-derived polyphenols [38].

Fig. 7 Transformation of lignin by LacOSt51. A Reactivity of LacOSt51 with β-O-4 biaryl ethers. LacOSt51 (1 μM) was incubated for 6 h with 1 mM guaiacylglycerol-β-guaiacyl ether (left) or veratrylglycerol-β-guaiacyl ether (right) with 20 mM sodium phosphate and pH 8 at 55 °C. HPLC traces are of reactions with (solid line) and without (dotted) enzyme. B Treatment of EMAL with LacOSt51. LacOSt51 (6 μM) was incubated for 6 days with 0.5% (w/v) EMAL (12.5 mM sodium phosphate, pH 8, 10% DMSO, at 30 °C). HPLC traces are of reactions with (solid line) and without (dotted) enzyme. The identities of the indicated compounds were confirmed using authentic standards. DMBQ: 2,6-Dimethoxy benzoquinone. Inset: Effect of laccase treatment on molar mass distribution of Eucalyptus EMAL, where EMAL was treated with either LacOSt51, sLac or no enzyme (sLac treatment generated insoluble material that was not analyzed using GPC). C HSQC NMR spectra of laccase-treated EMAL. EMAL was incubated with no enzyme (a) and (d), LacOSt51 (b) and (e), or sLac (c) and (f). The top and the bottom panels show the aliphatic and aromatic regions, respectively, of the 2D-NMR spectra. Linkages and units are expressed as per 100 aromatic units (100 Ar), which represented the integration of the G2 + 1/2S2. Structures of the regions are shown to the right.

Lignin-degrading organisms can serve as a source of novel ligninases, including laccases. Laccases are multi-copper oxidases that oxidize a broad range of compounds including substituted phenols, arylamines and aromatic thiols [63]. Bacterial laccases are appealing and versatile catalysts due to their thermal stability [64], use of molecular oxygen as the final electron acceptor and production of only water as a by-product [65]. Here, we used newly published software, TreeSAPP [39], which places novel sequences into reference phylogenies. We designed a multi-copper oxidase reference phylogeny based on a model of 16 sub-families [66]. Specifically, we identified a possible thermophilic clade of the lignolytic two-domain K-type SLACs found in 13C-enriched Solirubrobacteraceae and Thermoleophilales, which we refer to as K2-type laccases. We also annotated a number of two- and three-domain LCMOs in 13C-enriched Steroidobacteraceae and Alicyclobacillus MAGs from sub-families O and L, which contain members capable of phenolic oxidation [67, 68]. Together, these results suggest that bacterial laccases are involved in lignin degradation in thermal environments.
To validate putatively lignin-degrading LCMOs identified with $^{13}$C-lignin SIP, we heterologously expressed a selection of these enzymes. Specifically, we attempted to express two K2-type, one O-type and one N-type laccase in *E. coli*. The two K2-type enzymes were expressed as insoluble forms, and expression of the N-type was not detected in either soluble or insoluble form. We also attempted to express the actinobacterial K2-type laccase in *Rhodococcus jostii* RHA1, but they were again insoluble—their laccin degradation potential of the K2- and N-type laccases remains uncharacterized. The O-type laccase originating from the gammaproteobacterial MB2.51 MAG was expressed in soluble form in *E. coli* and further purified and characterized. This enzyme (LacOST51) transformed a minimally transformed Eucalyptus lignin, and can be funnelled into the syringic acid *meta*-cleavage pathway in thermophilic *Alphaproteobacteria* [37]. Our results herein demonstrate that bacterial laccases, such as LacOST51 or the previously-characterized sLac, can generate lignin-derived mono-aromatic compounds at elevated temperature. We propose that these thermostable biocatalysts can be employed for bacterial bio-product production.

In this paper we characterized 14 genomes from bacteria in thermal hog fuel and hot spring sediment environments that incorporated synthetic $^{13}$C-labeled lignin. The results supported our hypothesis that these communities harbor thermophilic, lignin-degrading bacteria. These bacteria include members of the actinobacterial families *Sorribrobacterales* and *Thermobacteriaceae* that have a distinct clade of K2-type SLACs. These bacteria also included a *Gammaproteobacteria* (Steroidobacteraceae) from which we expressed a lignin-transforming O-type laccase. Overall, this study advanced our knowledge of how thermophilic bacteria can degrade lignin and LDACs and identifies enzymes potentially useful in biocatalysts for lignin valorization.

**DATA AVAILABILITY**

Sequence accessions are provided in Supplementary Data 1 and as part of NCBI BioProject PRJNA665309. Draft genome information and NCBI accessions are provided in Supplementary Data 2.

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AUTHOR CONTRIBUTIONS
DJLB conducted sampling, SIP experiments, genomic analyses, and wrote the paper. LEN performed enzymology experiments and co-wrote the paper. MMF synthesized DHP lignin substrates. LYL and SR performed lignin chemistry analysis and co-wrote the paper. TD performed SIP experiments. LDE and WWM designed experiments and co-wrote the paper.

COMPETING INTERESTS
The authors declare no competing interests.

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