1Title:

2Iron Chelator-Mediated Anoxic Biotransformation of Lignin by Novel sp.,
3*Tolomonas lignolytica* BRL6-1

4

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16Abstract:

17 Lignin is the second most abundant carbon polymer on earth and despite having more
 18fuel value than cellulose, it currently is considered a waste byproduct in many industrial
 19lignocellulose applications. Valorization of lignin relies on effective and green methods of de-
 20lignification, with a growing interest in the use of microbes. Here we investigate the physiology
 21and lignin biotransformation mechanisms of the novel facultative anaerobic bacterium,
 22*Tolomonas lignolytica* BRL6-1, under anoxic conditions. Physiological and biochemical changes
 23were compared between cells grown anaerobically in either lignin-amended or unamended
 24conditions. In the presence of lignin, BRL6-1 had a higher biomass and shorter lag phase
 25compared to unamended conditions, and 14% of the proteins determined to be significantly
 26higher in abundance by log₂ fold-change of 2 or greater were related to Fe(II) transport in early
 27exponential phase. Ferrozine assays of the supernatant (<10 kDa fraction) confirmed that Fe(III)
 28was bound to lignin and reduced to Fe(II) only in the presence of BRL6-1, suggesting redox
 29activity by the cells. LC-MS/MS analysis of the secretome showed an extra band at 20 kDa in
 30lignin-amended conditions. Protein sequencing of this band identified a protein of unknown
 31function with homology to enzymes in the radical SAM superfamily. Expression of this protein
 32in lignin-amended conditions suggests its role in radical formation. From our findings, we
 33suggest that BRL6-1 is using a protein in the radical SAM superfamily to interact with the Fe(III)

34bound to lignin and reducing it to Fe(II) for cellular use, increasing BRL6-1 yield under lignin-
35amended conditions. This interaction potentially generates organic free radicals and causes a
36radical cascade which could modify and depolymerize lignin. Further research should clarify the
37extent to which this mechanism is similar to previously described aerobic chelator-mediated
38Fenton chemistry or radical producing lignolytic enzymes, such as lignin peroxidases, but under
39anoxic conditions.

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41**Keywords:** lignin depolymerization, lignin degradation, anaerobic bacteria

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43Introduction:

44 The industrial processing of lignocellulosic material produces 5×10^6 metric tons of lignin
45annually worldwide (1). Lignin is the largest renewable source of aromatics that can be used for
46products such as flavors, fragrances, dyes, and other valuable secondary metabolites (2,3).

47However, it is considered an “untapped” resource due to the recalcitrant nature of the
48polymer, making it difficult to separate and process for valuable downstream products (4,5).

49 Investigation of microbial mediated processes for the depolymerization of lignin have
50focused predominantly on aerobic fungi and bacteria (4,6–9). Under oxic conditions, enzymes
51such as laccases and peroxidases produce oxidants that diffuse into and reduce the lignin
52complex (9,10), causing bond scission reactions between lignin subunits. For organisms that
53lack lignolytic enzymes such as brown-rot fungi as well as bacteria like *Pantoea ananatis* Sd-1
54and *Cupriavidus basilensis* B-8, chelator-mediated Fenton chemistry (CMF) is used to
55depolymerize lignin (11–14). In this mechanism, the microorganism produces an iron reducer
56molecule, a chelator molecule, and H_2O_2 . Once the chelator binds to Fe(III) in the environment,
57it then reacts with the iron reducer molecule to reduce Fe(III) to Fe(II). Fe(II) then reacts with
58 H_2O_2 to create $\bullet OH$ radicals. Similarly to oxidants formed by laccases and peroxidases, the $\bullet OH$
59radicals disrupt the lignin structure, causing bond scissions of subunits (14). Both lignolytic
60enzyme and chelator mediated lignin depolymerization are promising for industries that rely
61lignocellulosic feedstocks (8). For example, the use of Fenton chemistry for lignocellulosic
62processing has been studied using the aerobic bacterium, *C. basilensis* B-8 grown on rice straw,
63showing a synergistic relationship in lignin depolymerization and cellulose yield (13). However,
64limitations to these processes hinder them from being competitive on the market. Both aerobic
65fungi and bacteria require constant aeration and mixing, making it very costly to maintain the
66cultures (15). Mass production of fungal or bacterial lignolytic enzymes are also not possible
67due to lacking a method of recycling the enzymes after one use, low substrate specificity, and
68low redox potential (16). For example, both laccases and manganese-dependent peroxidases
69cannot degrade 80-90% of lignin due to the presence of non-phenolic structures (17).

70 Though originally thought to be impossible 30 years ago (18), anaerobic bacteria could
71offer a solution to issues presented for aerobic microorganisms. For example, based on genome
72analysis, *Klebsiella sp.* strain BRL6-2 is hypothesized to use lignin as an electron acceptor for
73energy production (19). Support for this mechanism is also seen with humic substances, which
74are lignin rich (20), acting as extracellular electron acceptors for bacteria in sediments and
75anoxic waters (21). *Enterobacter lignolyticus* SCF1 has been studied comparing the growth in
76lignin-amended and unamended conditions under anaerobic conditions. RNAseq analysis
77suggested various enzymes that may be responsible for lignin depolymerization, including

78alcohol dehydrogenases (22,23). However, the exact mechanism has yet to be elucidated. By
79identifying additional anaerobic bacteria capable of degrading lignin, mechanisms and their
80regulation can be uncovered and further utilized for lignin depolymerization and valorization
81applications.

82 *Tolomonas lignolytica* BRL6-1 is a novel, facultative anaerobic bacterium that was
83isolated from the El Yunque experimental forest, Puerto Rico, on lignin as the sole carbon (C)
84source under anoxic conditions (24). Previously, BRL6-1 has demonstrated to have a shorter lag
85phase and a higher biomass in the presence of lignin (24). However, the mechanism of lignin
86modification and how it benefits cell growth is not well understood. We hypothesize that when
87grown anaerobically in the presence of lignin, BRL6-1 produces an extracellular protein that
88acts as both iron chelator and redox agent. This protein potentially generates organic free
89radicals and causes a radical cascade that modifies and depolymerizes lignin.

90

91Materials and methods:

92Culturing *Tolomonas lignolytica* BRL6-1

93 To study lignin modification under anoxic conditions, *Tolomonas lignolytica* BRL6-1 was
94grown in 0.04% D-glucose as the primary C source amended or unamended with 0.1% alkali
95lignin, low sulfonate (Sigma Aldrich, CAS Number 8068-05-1). Cultures grew on modified CCMA
96media consisting of (per liter) 2.25 g NaCl, 0.5 g NH₄Cl, 0.227 g KH₂PO₄, 0.348 g K₂HPO₄, 5 mg
97MgSO₄•7H₂O, 2.5 mg CaCl₂•2H₂O, 0.01 mL SL-10 trace elements, and 0.01 mL Thauer's
98vitamins (25–27) . The D-glucose concentration was 0.2% for the ferrozine assays, Arnow
99assays, proteome and secretome analysis, described in more detail below. Cultures grew at
10030°C anaerobically in triplicate and uninoculated bottles served as abiotic controls. Iron
101amended cultures had an additional 38 ppb Fe(II) added to the media as FeCl₂•4H₂O.

102 To study the physiological response of BRL6-1 in the presence of lignin, growth in lignin-
103amended medium, lignin unamended medium, and lignin unamended medium supplemented
104with the additional Fe (II) were monitored by measuring cell concentration by adsorption
105(OD₆₀₀). Bacterial growth curves were analyzed with gcFit function via *grofit* package in R (28).
106Calculated average lag phase, maximum growth rate (μ Max), and maximum cell growth (A)
107were based on the Gompertz Model.

108

109Proteomic Analysis for Cell Pellet and Secretome

110 To identify proteins differentially expressed during lignin-amended growth, biomass was
111collected at early and late logarithmic growth phase from cultures grown in the presence or
112absence of lignin. Briefly described, proteins were extracted from cell pellet fractions using
113methanol/chloroform and analyzed with LC-MS/MS. Raw mass spectrometry data were
114searched with MS-GF+ against NCBI RefSeq *Tolomonas* sp. BRL6-1 database (October 2014
115version) in addition to bovine/porcine trypsin and other common contaminants such as keratin
116sequences (3164 total sequences). Searching parameters required tryptic digestion of at least
117one of the peptide ends (partially tryptic), <10 ppm peptide mass tolerance and methionine
118oxidation as variable modification. The identified MS/MS spectra were filtered with an MS-GF+
119score of 1e⁻⁰⁹ resulting in $\leq$ 1.0% false discovery rate (FDR) at the protein level. The count of
120spectra attributed to each individual protein within each experimental condition was used as a
121proxy for relative quantitative values.

Supernatant fractions from late logarithmic growth phase were collected to identify differentially expressed proteins in the secretome during lignin-amended growth. Cell-free supernatant was generated by collecting 100 mL cultures at the end of late logarithmic growth phase and vacuum filtrated with a 0.45 μ m filter. Cell-free supernatant was centrifuged using a 10 kDa filter & the >10 kDa fraction was further concentrated using trichloroacetic acid (TCA) precipitation (41). Samples were run on a 15% SDS-PAGE gel and silver stained. Bands of interest from both lignin-amended and unamended samples were cut out at 20 kDa, 37 kDa, and 50 kDa. Using an in-gel tryptic digest kit (Thermo Fisher, Catalog #89871), samples were prepared as described by the manufacturer for LC-MS/MS analysis. LC-MS/MS analysis was completed by the Mass Spectrometry Center at University of Massachusetts Amherst. Raw mass spectrometry data was search with MS/GF+ against the NCBI RefSeq *T. lignolytica* BRL6-1 database (2016 version).

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135 Statistical Analysis of Proteomic Data

Spectral counts from the cell pellet proteomics between lignin-amended and unamended conditions were compared using msms.edgeR function via *msms.Tests* package in R (29). The post-test effect size filter of msms.edgeR deemed proteins differentially expressed if proteins had p-values <0.05, absolute values of log₂ fold-change >1 or < -1, and total spectral counts >2 across biological replicates (29).

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142 Ferrozine and Arnow Assays

In order to confirm that iron was bound to lignin, Fe(II) and Fe(III) concentrations were measured with ferrozine assays as described with modifications (30). Supernatant from lignin-amended and unamended cultures were harvested by removing 15 mL aliquots of culture from serum bottles under anoxic conditions during lag phase, late logarithmic growth phase, and mid-stationary growth phase. Samples were filtered under anoxic conditions through a 0.45 μ m filter to remove biomass and then ultrafiltrated with a 10 kDa Amicon filter. Filtrate (<10 kDa) was tested in triplicate for total iron. Briefly, in a 96-well plate under anoxic conditions, 225 μ L of sample with 15 μ L 1 M ascorbic acid were added, followed by 60 μ L of 50 mg ferrozine mL⁻¹ and 500 mM potassium acetate buffer, pH 5.5. Plates were wrapped in tin foil and incubated for 15 min at 37°C before being measured at 562 nm with a plate spectrophotometer. A separate set of plates had ascorbic acid substituted with water to calculate Fe(II) in the media. For both ferrozine assays a standard curve of Fe(II) was completed as well as samples taken from the abiotic cultures to act as controls.

To determine if BRL6-1 is producing catechol-like siderophores known as catecholates, Arnow assays were completed as described (31). This assay was performed using the same fractions as the ferrozine assays. Briefly, 1 mL of a <10 kDa fraction sample was combined with 1 mL 0.5 M HCl, 1 mL nitrite-molybdate reagent, and 1 mL 1 M NaOH. Reactions were incubated for 5 min before being diluted 5-fold with water in a 96-well plate and light absorbance read at 510 nm. Samples from the biotic replicates as well as abiotic controls were completed in triplicate. A standard curve was produced using 3,4-dihydroxybenzoate (31).

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164 Inductively Coupled Plasma (ICP) Spectroscopy of Kraft Alkali Lignin Substrate

One gram of alkali lignin, low sulfonate (Sigma Aldrich, CAS Number 8068-05-1) was sent for analysis in triplicate to the University of Massachusetts Amherst Soil and Plant Nutrient Testing Laboratory. Lignin was acid wet digested in nitric acid, hydrochloric acid, and hydrogen peroxide in a block digester and measured with ICP Spectroscopy to determine the total P, K, Ca, Mg, Zn, Cu, Mn, Fe, and B.

170

171 Results and discussion:

172 Physiology and global expression changes in response to lignin

To determine the role of lignin in the anaerobic metabolism and growth of *T. lignolytica* BRL6-1, we compared cultures grown with glucose that were either amended or unamended with lignin. Lignin-amended cultures had shorter lag phase (5.0 ± 0.6 hrs) compared to cells grown in unamended conditions (11.0 ± 4 hrs, p -value = 0.03). Lignin-amended cultures also had higher yields (0.140 ± 0.013 OD₆₀₀) compared to cells grown in unamended conditions (0.124 ± 0.003 OD₆₀₀, p -value = 0.04). Maximum growth rate in unamended conditions was not significantly different from lignin-amended conditions (0.039 ± 0.03 OD₆₀₀ hr⁻¹ compared to 0.038 ± 0.008 OD₆₀₀ hr⁻¹). Our growth results support previous findings that under lignin-amended conditions, BRL6-1 fitness improves, experiencing a shorter lag phase and achieving a higher final biomass when lignin is amended (24).

To explain this change in growth, we originally hypothesized that lignin may serve as a secondary C source as well as a potential energy source (24). To explore this hypothesis, we compared protein production between lignin-amended conditions to unamended. EdgeR analysis uses the negative binomial distribution to detect proteins that are differentially abundant between the two growth conditions (29). The analysis resulted in a total of 41 proteins with significantly higher abundance and 101 lower abundance relative to unamended conditions in early exponential phase and a total of 9 proteins with significantly higher abundance and 9 proteins lower abundance in late exponential phase (Fig 1).

191

192 Fig 1. Proteomic analysis results of *T. lignolytica* BRL6-1 grown in lignin-amended versus unamended conditions.

Blue dots represent significant change in protein abundance in lignin-amended conditions (p -value < 0.05) whereas black dots denote represent proteins that did not change between lignin-amended and unamended conditions.

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198 Evidence of proton-relay system-related enzyme for lignin degradation

199

From our edgeR analysis results, we first investigated the roles of proteins that had significantly higher abundance in both early and late exponential phase in lignin-amended cultures relative to unamended. This subset of proteins included the protein with the highest abundance listed in lignin-amended conditions in both phases, which was annotated as a carboxymuconolactone decarboxylase (CMD) family protein (Pfam 02627). The CMD family of proteins is associated with aromatic degradation in aerobic bacteria, via the protocatechuate branch of the β -ketoadipate pathway, as well as antioxidant defense via peroxidase activity (32,33). For our protein, we identified an alkylhydroperoxidase (AhpD) domain and CXXC motif ($\log_2$ fold-change of 8 and 7, respectively) (Fig 2A). Based on the CXXC motif, it is thought that

the protein detected in BRL6-1 has AhpD-like activity (32). AhpD is part of an antioxidant defense system that forms a complex with peroxiredoxin, AhpC. The function of AhpD is to restore the enzyme activity of AhpC via reduction (34). Looking further into the genome, BRL6-1 contains a gene annotated as AhpF, which is an alternative alkyl hydroperoxide reductase to AhpD as seen in *Salmonella typhimurium* (63.5% sequence identity with NCBI BLASTp) (35). Based on this information, AhpC likely forms a complex with AhpF. To support this, BPROM program analysis, which predicts bacterial promoter sites, placed AhpC and AhpF downstream of the same predicted promoter in the BRL6-1 genome (36). Additionally, our edgeR analysis determined AhpCF protein complex expression was not significantly different between lignin-amended and unamended conditions. Therefore, this up-regulated AhpD-like protein in the presence of lignin could be serving another role with its reducing activity instead of restoring AhpC activity.

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Fig 2. Proteins with significant change in abundance ($\log_2$ fold change, p-value < 0.05) related to carbon metabolism and energy production by *T. lignolytica* BRL6-1 under lignin-amended conditions relative to unamended.

(A) Proteins of BRL6-1 with significantly high and (B) low abundance under lignin-amended conditions compared to lignin unamended (p-value < 0.05). Abbreviations are the following: phosphoenolpyruvate carboxykinase (PEPCK); 2-keto-3-deoxy-6-phosphogluconate (KDPG).

228

The mechanism that AhpD uses to reduce its substrates is a proton relay system (37), which was originally described as protons being shuttled from the active site of a protein to bulk solvent molecules (38). This mechanism has been observed previously in the lignin degrading enzyme, LigL, found in *Sphingomonas paucimobilis* SYK-6. LigL catalyzes stereospecific oxidation of the benzylic alcohol as the first degradation step of lignin-derivative, (α S, β R)-GGE (39,40). The proton relay mechanism has also been described for p-Cresol methylhydroxylase (PCMH) in *Pseudomonas* species to degrade phenol p-cresol as well as p-hydroxybenzyl alcohol (37). Therefore, this AhpD-like protein could be reducing lignin-derived compounds in the cell using this mechanism. In support of cellular aromatic compound uptake, a C4-dicarboxylate ABC transporter protein in both early and late exponential phase ($\log_2$ fold-change of 1.2 and 4.9, respectively). This transporter has an 87% sequence identity to transporter, DctA, in *Pseudomonas chlororaphis* O6 that was found to be essential for benzoate uptake (41).

242

Lignin-amended cultures shift in carbon metabolism and energy production

With evidence that BRL6-1 is potentially transporting benzoate compounds into the cell and reducing them via AphD, we then compared changes in protein abundance relating to carbon (C) metabolism in the presence of lignin. In lignin-amended conditions, the HexR transcriptional factor was significantly lower in BRL6-1 by a $\log_2$ fold-change of -5 during early logarithmic growth phase (**Fig 2B**). HexR is a global transcription factor utilized for fine-tuning the carbohydrate catabolic pathways to adapt to variable C availability in the environment (42–25044). It has been previously shown that HexR is present in several groups of Proteobacteria and regulates a rather complex set of operons (44). Leyn and colleagues (2011) deleted *hexR*, which resulted in the de-repression of transcription of the central glycolytic genes as well as the

activation of genes involved in gluconeogenesis. In contrast to what would be expected, our results show that BRL6-1's glucokinase and pyruvate kinase, which are responsible for the first and last step of glycolysis, respectively, were lower in abundance by a $\log_2$ fold-change of -4, suggesting that the presence of lignin lead to the repression of glycolysis (**Fig 2**). This evidence is also supported by the fact that phosphoenolpyruvate carboxykinase (PEPCK), the rate limiting enzyme for gluconeogenesis, was also lower in abundance in lignin-amended growth conditions by a $\log_2$ fold-change of -4, indicating that PEP was not being funneled into gluconeogenesis, whereas a KDPG aldolase was significantly higher in abundance by a $\log_2$ fold-change of 5 (**Fig 2B12**). These findings suggest that in the presence of lignin, there is a higher conversion of KDPG to pyruvate by the KDGP aldolase. Very dynamic transcription regulation patterns on C metabolism pathways have been previously reported during metabolization of lignin of *E. lignolyticus* SCF1, another facultative anaerobic Gammaproteobacteria isolated from tropical rainforest soils (22).

Despite the observed shifts in C metabolism-related proteins described above, enzymes related to aromatic metabolism that were previously annotated in the genome did not significantly change in abundance in the presence of lignin relative to the unamended conditions (24). This lack in change may be due to the fact that either no monomers are being cleaved from lignin in the presence of BRL6-1 or that lignin was depolymerized into high molecular weight polymers and not directly consumed by BRL6-1.

To determine if lignin had a role in energy production, we searched for enzymes that were related to the electron transport chain that had differential abundance in lignin-amended conditions compared to unamended. During early exponential phase, 6% of all significantly lower abundance proteins ($\log_2$ fold-change >1, p-value <0.05) in the presence of lignin were NADH dehydrogenase subunits ($\log_2$ fold-change between -1.2 to -4) and one flavin mononucleotide ($\log_2$ fold-change of -4) (**Fig 2B**).

Iron redox by *T. lignolytica* BRL6-1 in the presence of lignin and hypothesized mechanisms

Of the proteins that were significantly higher in abundance by a $\log_2$ fold-change >2 in the presence of lignin during early exponential phase, 14% were related to Fe(II) uptake (**Fig 2B32A**). We hypothesized that BRL6-1 could be obtaining energy using iron redox and that lignin could play a role due to its strong affinity for iron (45). The complex between lignin and iron makes iron more soluble in the environment but not necessarily more bio-available for cellular use (46). This considered, BRL6-1 may have a mechanism that is disrupting the lignin-iron association. By doing so, BRL6-1 could obtain both iron and a potential C source faster than cells in unamended conditions, explaining the ability of BRL6-1 to exit lag phase more quickly in the presence of lignin (47).

To further investigate the relationship between lignin, iron, and BRL6-1 fitness, we first asked if lignin was contributing to a higher iron concentration aside from the SL-10 minerals we added to the media. ICP Spectrophotometry showed that the lignin substrate contained 38 ppb iron (data not shown). To test whether the 38 ppb Fe was benefitting BRL6-1 fitness, we completed Fe addition growth curve experiments and monitored BRL6-1 growth. Compared to cells grown in lignin unamended conditions reported above, lignin unamended conditions with 38 ppb iron addition were not significantly different in lag phase, growth rate, or biomass yield:

29710.5 ±5 hrs, 0.072 ±0.03 OD₆₀₀ per hr, 0.124 ±0.007 OD₆₀₀, respectively. These results suggest
298that additional iron alone was not enough to benefit BRL6-1 growth.

299 To test if lignin has a strong affinity for iron in our system, we completed a ferrozine
300assay for <10 kDa fractions of supernatant from lag phase, late logarithmic growth phase, and
301mid-stationary growth phase. We expected that if the iron was binding to lignin in the media,
302we should see less bioavailable iron in the supernatant of lignin-amended compared to lignin
303unamended conditions. In lignin-amended conditions, Fe(III) was not detectable in the <10 kDa
304fractions throughout the entire growth curve whereas 292 ppb Fe(III) was detected during lag
305phase in lignin unamended conditions. As bacterial biomass increased over time, Fe(II)
306accumulated in both conditions to similar concentrations (**Fig 3**). There was no change in
307Fe(II/III) concentrations in abiotic controls. This suggests that Fe(III) was bound to the lignin and
308was reduced to Fe(II) by BRL6-1.

309

310**Figure 3. Bio-available Fe(II) (orange) and Fe(III) (yellow) concentrations in parts per million**
311**(ppm).**

312Concentrations of Fe(II/III) when BRL6-1 was cultured in (A) lignin-amended and (B) in
313unamended conditions at lag phase, late exponential phase, and late stationary phase.

314

315 With evidence for Fe(III) bound to lignin and being reduced to Fe(II) based on ferrozine
316assays, we next wanted to determine the mechanism that BRL6-1 may use to accomplish this
317redox reaction. One potential explanation was the use of siderophores, which are organic
318molecules used by bacteria to chelate Fe(III) under iron-limiting conditions (48). The most
319common siderophore used by bacteria are catecholates, which rely on hydroxyl groups of the
320catechol rings to form the iron chelation center (31). To see if BRL6-1 produces this type of
321siderophore, we completed Arnow assays on <10 kDa supernatant fractions from lignin-
322amended and unamended cultures during lag phase, late logarithmic growth phase, and mid-
323stationary growth phase. Catecholate detection was seen only in lignin-amended conditions;
324however, there was no change in concentration of catechol over the course of the growth curve
325(**Fig 4**). Additionally, abiotic controls of the lignin-amended conditions had similar
326concentrations to biotic replicates. This is likely due to Arnow assays being non-specific
327between catecholates and compounds containing catechol, such as soluble lignin (49), making it
328difficult to differentiate sources as well as any small changes in concentration of such
329metabolites. BRL6-1 may also be producing other groups of siderophores such as hydroxamates
330or carboxylates (48), which would need to be detected with a Csáky assay or the use of
331phenolphthalein and sodium hydroxide, respectively (50,51). Despite these limitations, initial
332results suggest that siderophores are unlikely to be the main explanation for the Fe redox being
333detected by the ferrozine assay and instead another mechanism may be used by BRL6-1.

334

335**Fig 4. Arnow assay of catechol-like compounds present in supernatant.**

336Catechol-like chelator concentrations (μg/mL) at lag, late exponential, and mid-stationary
337phase of BRL6-1 growth under lignin-amended (blue) and unamended conditions (gray). Abiotic
338controls are striped for both conditions.

339

We investigated to see if BRL6-1 secreted lignolytic enzymes or iron reducing proteins in the presence of lignin as seen for aerobic fungi and bacteria (11,12,14). Samples from late stationary phase were run on an SDS-PAGE and silver stained to detect differential banding between the two growth conditions. Differential banding was at 20kDa under lignin-amended conditions (**Fig. 5**). In addition, because BRL6-1 has had previously predicted peroxidases in its genome (24) and that lignin peroxidases are 35-48 kDa (52), we also were interested in the bands at 37 and 50kDa. Therefore, bands were cut out at 50 kDa, 37 kDa, and 20 kDa for both conditions to identify the proteins present by sequencing.

348

Fig 5. SDS-PAGE of *T. lignolytica* BRL6-1 secretome cultured in lignin-amended and unamended conditions.

Arrows showing differential banding at 20kDa.

352

A protein originally annotated as hypothetical, WP_024871222.1 was detected in all three lignin-amended biological replicates with a predicted size of 20.8 kDa. There were no conserved domains detected in this protein, but Position-Specific Iterated (PSI) BLAST analysis of the protein identified homology to hypothetical protein from *Alteromonadales bacterium* BS08 (53% Identity; E-value 4e-61). BS08 was isolated from the gut of *Bankia setacea*, also known as the shipworm, that digests wood as a food source (53). Additionally, WP_024871222 had homology to enzymes in the radical SAM superfamily (37.7% Identity; E-value 0.06), a wide range of enzymes that support oxygen-independent alternatives to aerobic pathways (54). This may be relevant since *Tolomonas lignolytica* as other *Tolomonas* spp., are the only members of the *Aeromonadaceae* that are oxidase and catalase negative (24). Therefore, it is possible, that WP_024871222.1 has a role in lignin modification via radical formation.

364

365Conclusions

The presence of lignin has been previously shown to be beneficial for the growth of *T. lignolytica* BRL6-1 (24) with speculation that lignin was acting as a secondary C source or energy source. In our study, proteomic analysis revealed that 14% of the upregulated proteins by log₂ fold-change of 2 or greater relating to Fe(II) transport in early exponential phase. Transient iron accumulation in *Salmonella enterica* serovar Typhimurium is required in order for the cells to come out of lag phase (47) and so it was possible that the up-regulation of iron enzymes might be due to BRL6-1 having the same iron requirement or relied on iron redox for energy (55). However, lignin has a strong affinity for iron (45) and therefore we would expect that iron is less bioavailable to the cells in lignin-amended conditions (46). Ferrozine assays of the <10kDa supernatant fractions confirmed that Fe(III) was bound to lignin, but it was reduced to Fe(II) when BRL6-1 was present, suggesting redox activity by the cells. To explain this redox activity, we hypothesized that BRL6-1 is producing a protein that acts as both an iron chelator and redox agent under anoxic conditions to obtain the iron bound to lignin. Secretome (extracellular enzyme) analysis coupled with LC-MS/MS identified the presence of a protein of unknown function but had homology to enzymes in the radical SAM superfamily, suggesting that it may have a role in radical formation in lignin-amended conditions.

382

While our work illustrates a potential molecular mechanism for anaerobic lignin modification by *T. lignolytica* BRL6-1, protein isolation and characterization are needed to

confirm that this protein interacts with the Fe(III) bound to lignin and reduces it to Fe(II) for cellular use. Further analysis with electron paramagnetic resonance (EPR) as well as nuclear magnetic resonance (NMR) are necessary in order to confirm that organic radicals are being produced in the process of this enzyme obtaining iron and that these radicals alter the structure of lignin. By continuing research into the biochemical nature of microbial lignin transformations under anaerobic conditions, industries using lignocellulose as raw material will be one step closer to lignin valorization.

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393Acknowledgements:

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Mass spectral data were obtained at the University of Massachusetts Mass Spectrometry Center. This work was part of the DOE Joint BioEnergy Institute ([http:// www.jbei.org](http://www.jbei.org)) supported by the U. S. Department of Energy, Office of Science, Office of Biological and Environmental Research, through contract DE-AC02-05CH11231 between Lawrence Berkeley National Laboratory and the U. S. Department of Energy. This research was performed using resources at the DOE Environmental Molecular Sciences Laboratory (grid.436923.9), which is an DOE Office of Science User Facilities sponsored by the Office of Biological and Environmental Research and operated under Contract No. DE-AC05-76RL01830. This publication was also developed under Assistance Agreement No. FP-91782301-0 awarded by the U.S. Environmental Protection Agency to Gina Chaput. It has not been formally reviewed by EPA. The views expressed in this document are solely those of Gina Chaput *et al.* and do not necessarily reflect those of the Agency. EPA does not endorse any products or commercial services mentioned in this publication.

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