

## Ligninase Activity is not Consistently Predicted by the Presence of Manganese Coordinating Residues in Dyp-Like Proteins

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**DypB, a characterized bacterial lignin peroxidase from *Rhodococcus jostii* RHA1, is thought to modify lignin using oxidized manganese as a diffusible mediator. Structural analysis has determined that manganese is bound and oxidized through a pocket near the active site that is comprised of four residues. This study aimed to experimentally determine whether the manganese coordinating chemistry of DypB could be predictive of manganese oxidation activity and lignin modification of other dyp-like proteins. To do so a dyp-like protein from *Pseudomonas putida* F1 was cloned and its manganese oxidizing potential determined. Moreover, the ability to grow in the presence of lignin was also determined for seven environmental isolates which were predicted to contain dyp-like proteins of interest. The results suggest that the conservation of key residues responsible for manganese oxidation in DypB are not directly indicative of lignin modification. This study also found that a novel *Enterobacter* sp. and *Streptomyces viridochromogenes* DSM40736 may utilize lignin in a manner comparable to and greater than *R. jostii* RHA1.**

Lignin is the second most abundant terrestrial biopolymer and is the major component of lignocelluloses in plant cell walls (5). As a heterogeneous aromatic polymer, lignin is recalcitrant to chemical and biochemical breakdown and therefore there is a limitation in the production of bioethanol from plant biomass (6,13). The removal of lignin from lignocelluloses is currently carried out using energy intensive thermochemical methods, so there is considerable interest in discovering novel organisms and catalysts involved in lignin breakdown (2).

For the last 25 years, the study of lignin degradation has focused primarily on fungal enzymes such as lignin peroxidase (LiP) and manganese peroxidase (MnP) from *Phanerochaete chrysosporium* (6,1). However, these efforts are yet to be applied to a commercial process, partially due to difficulties in fungal genetics and protein expression (11).

Recently, a spectrophotometric assay involving chemically nitrated lignin has identified several bacterial strains as lignin degraders, including *Rhodococcus jostii* RHA1, a soil actinomycete (6). With the genome sequence available for this organism, a Dyp-type peroxidase, DypB, has been identified and is the first characterized recombinant bacterial lignin peroxidase (1,11). DypB has been shown to oxidize both polymeric lignin and lignin model compounds, and appears to oxidize manganese (11). Recent work suggests that DypB modifies lignin in a heme dependent manner using oxidized manganese as a diffusible mediator, similar to the fungal enzyme manganese peroxidase (1, 11). In DypB, manganese is

bound and oxidized through a pocket located beside the active site, a trait that may be indicative of lignin-modifying function in other Dyp-like proteins (Unpublished data, by Eltis, Grigg, Murphy, and Singh). Also, mutational analysis of DypB has shown an active site substitution that produced a more active protein (12). This could potentially be used to select enzymes that oxidize manganese more rapidly.

The manganese coordinating residues in DypB were heavily conserved among closely and distantly related homologues and the patterns of conservation of these residues were conserved among discrete clades of homologues (Unpublished data, by Strachan, VanInsberghe, and Williams). This study explored the hypothesis that the conserved residues within the binding pockets of DypB could be an indicator of manganese oxidation activity, and thereby predictive of lignin modification.

### MATERIALS AND METHODS

**Strains and media.** Strains used in the growth assay are described in Table 1. *Escherichia coli* DH5a and *Escherichia coli* BL21(DE3) were used in cloning (Eltis lab, University of British Columbia). Media used in the experiments included Luria broth (LB): 1% tryptone (w/v) (Sigma T7293), 0.5% yeast extract (w/v) (Sigma Y1625), and 1% sodium chloride (Sigma S7653) (w/v); and MG9: M9 mineral medium supplemented with goodies (0.0013% FeSO<sub>4</sub> and 1.3 mM MgSO<sub>4</sub> final concentration) (4).

**Genomic DNA extraction.** Protocol was followed as previously described (3). Proteinase K (20 mg/ml stock, Promega V3021), Tris saturated phenol (Fisher BioReagents BP175), chloroform:isoamyl alcohol (24:1, Sigma C0549), sodium acetate (3M, Sigma S2889) were used. DNA content was read with the Nanodrop 2000c (Thermo Scientific).

**TABLE 1.** Strains used in growth assay and their shorthand identification

Strain ID	Strain	dyp-like protein genotype	Isolate Origin
Sequenced strains			
F1	<i>Pseudomonas putida</i> F1	N246S	Eltis lab, UBC
S.v.	<i>Streptomyces viridochromogenes</i> DSM 40736	N246A	DSMZ
RHA1	<i>Rhodococcus jostii</i> RHA1	Wild Type RHA1 sequence	Eltis lab, UBC
Environmental isolates			
69	<i>Pseudomonas</i> sp.	N246S	Mohn lab, UBC
132	<i>Burkholderia</i> sp.	None – control strain	Mohn lab, UBC
403	<i>Achromobacter</i> sp.	RHA1 residues conserved	Mohn lab, UBC
463	<i>Streptomyces</i> sp.	None – control strain	Mohn lab, UBC
466	<i>Burkholderia</i> sp.	RHA1 residues conserved	Mohn lab, UBC
474	<i>Streptomyces</i> sp.	RHA1 residues conserved	Mohn lab, UBC
620	<i>Enterobacter</i> sp.	E156A	Mohn lab, UBC

**Gene Amplification.** The Dyp genes from *Pseudomonas putida* F1 and *Streptomyces viridochromogenes* DSM 40736 were amplified using forward primers with NdeI restriction site (5'-GCGCATATGATGCCGTTCCAGCAAGGTC-3' and GCGCATATGATGCCGTTCCAGCAAGGTC, respectively) and reverse primer with HindIII restriction site (5'-GCGGGATCCTCAGGCCCGCAGCAAGG-3' and 5'-GCGGGATCCTCAGGCCCGCAGCAAGG-3', respectively). Amplification of Dyp genes from organisms were carried out using genomic DNA. Water for the PCR was purified using a Barnstead NANOpure UV apparatus (Barnstead International, Dubuque, IA) to a resistivity of greater than 17 MΩ cm. The PCRs contained 15.5 μl dH<sub>2</sub>O, 25 μl AccuMix (Bioline BIO-21051), 5 μl genomic DNA (4 ng/μl final DNA concentration), 1.5 μl of each forward and reverse primers (0.3 μM final concentration, each), and 1.5 μl of 100% DMSO (3% final concentration). The PCR cycling consisted of an initial denaturation stage at 95°C for two minutes; 30 cycles of 95°C for 30 seconds, 57°C for 40 seconds, and 68°C for three minutes; followed by a final extension step at 68°C for ten minutes.

**Competent Cell Preparation.** The same protocol was used for preparing both BL21 (DE3) and DH5α *E. coli* cells. 50 ml LB cultures were inoculated and grown to 0.5 OD<sub>600</sub>. Growth was stopped by chilling the cultures on ice. The cells were then centrifuged at 7000 xg for 15 min before being resuspended in 10% glycerol. The centrifugation and resuspensions were performed 3 times. The pellet was then resuspended in an equal volume of 10% glycerol and 40 μl aliquots were stored at -80°C.

**Plasmid Constructions.** Restriction double digests were set up with the PCR amplicon and pET28a+ plasmid vector (Eltis lab, University of British Columbia) using NdeI (Thermo Scientific FD0584) and HindIII (Thermo Scientific FD0504) using the following reaction conditions: 100 nM DNA, 0.25 U enzyme and 2 μl of 10x reaction buffer in a 20 μl reaction. This was incubated at 37°C for 3 hours before the reaction was heat inactivated at 80°C for 5 min. A ligation reaction was then set up with 500 nM of insert DNA, 100 nM vector DNA, 0.25 U of T4 DNA ligase (Thermo Scientific EL0011) and 2 μl of 10x reaction buffer in a 20 μl reaction. The reaction was incubated overnight at 16°C.

**Transformation.** 2 μl of the ligation reaction was used to electroporate (Micropulser electroporation apparatus, BioRad) competent DH5α cells. Transformed cells were grown for 24 hours on LB agar plates containing 25 μg/ml kanamycin (Sigma B5264) at

37°C. Candidate colonies were selected and inoculated in 3 ml LB cultures containing 25 μg/ml kanamycin and grown at 37°C for 24 hours. Plasmid miniprep was performed on these cultures using the biobasics DNA plasmid preparation kit according to the manufacturer's instructions. Plasmid digestion (the same protocol used in plasmid construction) and detection of the correct insert size on an agarose gel was performed. Confirmed plasmids were stored at -20°C before 2 μl was used to electroporate 40 μl of *E. coli* BL21 (DE3) competent cells.

**Expression.** *E. coli* BL21 (DE3) cells, grown in LB containing 25 μg/ml kanamycin at 37°C while shaking, were used for the production of His tagged Dyp proteins. When cultures reached an OD<sub>600</sub> of 0.5, Dyp expression was induced by adding isopropyl β-D-thiogalactopyranoside (IPTG) (Sigma I6758) to a final concentration of 0.5 mM. Cultures were then transferred to a 20°C shaking incubator for 12 hours. Cells were then harvested by centrifugation and frozen at -80°C in 20 mM MOPS (Sigma M1254), pH 7.5 containing 50 mM NaCl (buffer A).

**Protein Purification.** Dyp protein was purified according to the following protocol. Approximately 10 g of cells were thawed in 40 ml of buffer A and lysed at 4°C using an Emulsi Flex-C5 homogenizer (Avestin). Samples were then centrifuged at 45000 xg for 30 min to remove cell debris. The supernatant was loaded onto an 8 ml column of Ni-NTA resin (QIAGEN) and the Ht-Dyp protein was eluted as apoprotein according to the manufacturer's instructions. The protein was then exchanged from the elution buffer into buffer A by using a 30K NMWL Amicon Ultra-15 Centrifugal Filter Device (Millipore). The sample was then dialysed by incubating the sample at 4°C in molecular porous tubing (Spectra) while stirring in 1 L buffer A containing 1 mM EDTA. The DyP was then incubated in a 200:1 molar ratio of α-thrombin (Haematologic Technologies Inc.) for 12 h at room temperature in order to remove the affinity tag. The sample was loaded on a second IMAC column and eluted using buffer A containing 20 mM imidazole (Sigma I5513). To reconstitute heme, a 1:1 molar ratio of 20 mg/ml hemin chloride (Sigma H9039) was added drop wise with gentle stirring. The sample was centrifuged and loaded on a Sephadex G-25 fine (GE Healthcare) to remove excess heme. Sample was then concentrated to 2 ml and loaded onto 8 ml of Source 15Q anion exchange resin (GE Healthcare) packed in an AP-1 column (Waters Corp., Milford, MA). The column was equilibrated with buffer A before DyP was eluted using a linear gradient from 50 mM to 1 M NaCl over 20 minutes. The protein was then exchanged

into buffer A and purity was verified by sodium dodecyl sulphate-polyacrylamide gel electrophoresis. The purified protein was then concentrated to approximately 15 mg/ml, frozen as beads in liquid nitrogen, and stored at -80°C until use.

**Enzyme Activity.** Steady state reaction rates were determined spectrophotometrically for the oxidation of  $Mn^{2+}$ . The  $Mn^{2+}$  oxidation assay was performed in 200  $\mu$ l of 50 mM sodium malonate (Sigma M4795), pH 5.5 containing 10 mM  $MnSO_4$  (Sigma M8179), 1 mM  $H_2O_2$  (Sigma 216763) and 50 nM enzyme. The reaction was followed at 270 nm ( $\epsilon_{270} = 11.59 \text{ mM}^{-1}\text{cm}^{-1}$ ).

**Spectroscopy.** A Cary 5000 spectrophotometer (Varian) equipped with a thermostated cuvette holder maintained at  $25.0 \pm 0.5^\circ\text{C}$  was used to record electronic absorption spectra.

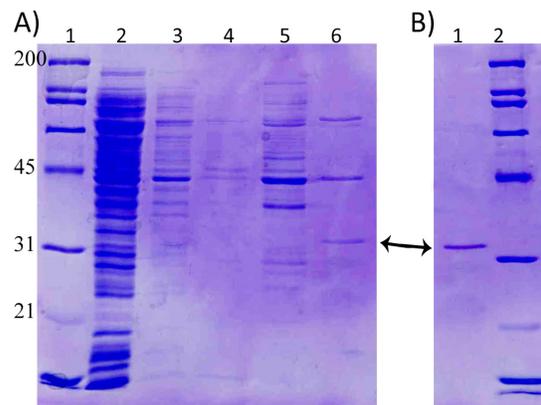
**Growth Assay.** M9G was prepared with 0.5 g/l hardwood kraft lignin (Lignoworks) and without lignin. 1 mM pyruvate (Sigma P2256) was supplemented to all media prior to use. Strains used in growth assay were grown up overnight at 25°C in M9G 1 mM pyruvate. Each strain was then inoculated into fresh M9G +/- lignin (inoculum size) and aliquoted (150  $\mu$ l) into a 96 well plate (Falcon). Each strain had 10 aliquots at each concentration of lignin. The plate was put in a tip box with moist paper towel to prevent evaporation and incubated at 25°C rotating at 200 rpm. At time points 5 hours, 12 hours, and day one through day seven, replicate samples from each media for each strain were removed from the microtiter plate and stored at -80°C prior to processing. When sampling was completed, samples were pelleted, washed twice with PBS (pH 7.4) and dried at 37°C overnight. The samples were then lysed with FastBreak cell lysis reagent (Promega V857C) and protein content was measured for each sample using a Micro BCA assay kit (Thermo Scientific) with a Tecan 96 well plate reader (PerkinElmer). In the large scale growth assays, a similar method was used but aliquot volumes were 5 ml and only a final time point (seven days) was measured to evaluate total accumulated protein content between 0 and 0.5 g/l lignin cultures in stationary phase.

**Data Analysis, Statistics, and Bioinformatics.** For the small volume growth assay, the 0 g/l lignin treatment was subtracted from the 0.5 g/l lignin treatment for each time point after the absorbance units were converted to protein concentration in  $\mu\text{g/ml}$  based on a standard curve. The two replicate values for these time points were then averaged. These average differences for each time point were in turn averaged to give the mean difference in biomass accumulation for each strain. The data processing for the large scale growth assay was identical, except only one time point was sampled (seven days), so the mean difference in biomass is representative only of cultures which have reached stationary phase. The significance of the trends observed was evaluated using calculated 95% confidence intervals.

The phylogenetic relatedness of the bacterial strains used in the growth assays was determined by constructing a maximum likelihood tree based off of a MUSCLE alignment of their V1-V3 16S rDNA sequences using FastTree (10).

## RESULTS

**Manganese coordinating residues in dyp-like proteins were not indicative of ligninase activity.** The protein from *P. putida* F1 was selected as it was relatively unrelated to DypB (32% identity), but contained the four conserved  $Mn^{2+}$  coordination residues found in DypB, a characterized ligninase. As for *S. viridochromogenes* DSM40736, the protein was selected as it was the only sequenced bacterial dyp-like protein identified to contain a natural mutation corresponding to an N246A substitution. This

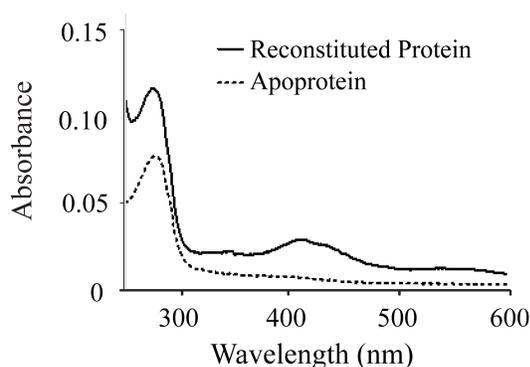


**FIG. 1. SDS-PAGE of protein fractions throughout purification.** Arrow shows the band representing the cloned protein from *P. putida* F1. A) Imidazole gradient used to elute the recombinant protein from the Ni-NTA affinity column. Lane 1 – Protein ladder, 2 – Crude protein extract, 3 – 20 mM Imidazole, 4 – 50 mM Imidazole, 5 – 100 mM Imidazole, 6 – 250 mM Imidazole. B) Protein product after ion-exchange chromatography. Lane 1 – Purified protein, 2 – Protein ladder. Molecular weights are displayed in kDa.

substitution was of interest because a N246A DypB variant was previously constructed, and this mutation resulted in a more active protein (12).

The dyp-like protein from *P. putida* F1 was successfully cloned and purified to apparent homogeneity as determined by SDS-PAGE (Fig. 1). The electronic absorption spectra of the reconstituted protein showed a feature at 408 nm, which was not seen in the apoprotein, suggesting heme incorporation (Fig. 2). However, the protein did not show any  $Mn^{2+}$  oxidation (data not shown). The dyp-like protein for *S. viridochromogenes* DSM40736 was not successfully cloned. High quality genomic DNA was prepared from *S. viridochromogenes* DSM40736; however, the gene was not successfully amplified. This was likely due to issues in primer design and the high GC content of the gene to be amplified, where the range of potential melting temperatures was within 5°C of the extension temperature for the reaction and all troubleshooting reactions were unsuccessful.

**Relative ability to catabolize, or grow in the presence of lignin is not directly correlated with manganese coordinating residue patterns or phylogenetic relationships between strains.** The environmental strains were taken from a bacterial culture collection, composed of isolates with their V1-V3 region 16S rDNA genes sequences. Four organisms (474, 403, 466, and 69 in Table 1) were selected because they were previously found to cluster closely in a phylogenetic tree to groups of organisms with genome



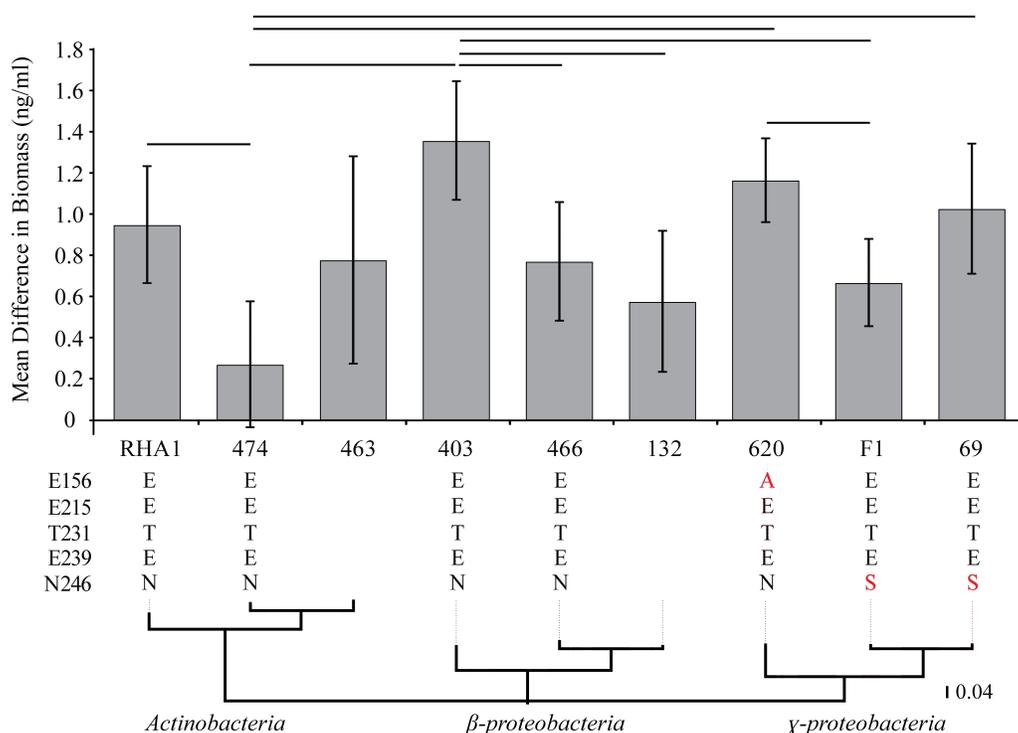
**FIG. 2. Electronic absorption spectra of the dyp-like protein from *P. putida* F1 before and after reconstitution with heme.**

sequences annotated to contain dyp-like proteins with variable conservation of the  $Mn^{2+}$  coordinating residues in the  $Mn^{2+}$  binding site (Unpublished data, by Strachan, VanInsberghe, and Williams). Isolates 463 and 132 were chosen as control strains because they did not cluster with any organisms known to contain dyp-like proteins in their genome sequences. The five residues considered in this study are conserved among members of the same species, and based on the annotated genomes of the sequenced strains and those of bacteria related to environmental isolates, 620 had a single E156A substitution, 69 and F1 had N246S substitutions, while the remaining isolates were predicted to have the same  $Mn^{2+}$  coordinating residues as *R. jostii* RHA1 (Fig. 3). This variation in residue conservation provided the opportunity to evaluate if these varied residues are necessary for the ability to degrade lignin.

The relationship between the phylogenetic relatedness of these isolates, their dyp-like protein residue conservation and their relative ability to degrade lignin was assessed by evaluating their relative ability to catabolize lignin. Pyruvate was included in the medium in addition to lignin as growth on lignin as a sole carbon source has yet to be reported; however, increases in biomass in the presence of lignin may be indicative of mineralization and consumption of lignin. Total protein of the cultures was measured to indicate relative accumulated biomass since some of the strains formed aggregates which would have led to inaccurate plate counts or turbidity readings. Initially, the experiment was conducted with samples collected at specific time intervals; however the protein data was too variable to identify any significant trends relating the strains based on their growth kinetics in the

presence of lignin. As a consequence, the mean difference in protein content between samples with and without lignin was calculated, which gave an approximation of the net increase in biomass which accumulated in the presence of lignin. Only isolates 403 and 620 were found to have a greater accumulation of biomass with respect to the two control strains, 463 and 132. Moreover, the ability to grow in the presence of lignin was not an attribute which *Actinobacteria*,  $\beta$ -*proteobacteria*, or  $\gamma$ -*proteobacteria* were more capable of doing (Fig. 3). Nor was possessing the  $Mn^{2+}$  coordinating residues found in DypB indicative of lignin tolerance since isolate 474 had no significant difference in biomass between growth in the presence and without lignin despite having the same  $Mn^{2+}$  coordinating residues as *R. jostii* RHA1 (Fig. 3). Likewise, there was no significant difference between the differences in biomass accumulation between the control strain 463 and any of the other tested isolates (Fig. 3). Similarly, specific clades of related dyp-like proteins had no significant relative ability to grow in the presence of lignin (data not shown).

The high amount of measured variation in the first growth assay was likely the result of the small culture volumes and incubation in microtiter plates, so the experiment was repeated with selected strains at larger culture volumes with one sampled time point. The amount of biomass was much larger in this assay because the growth volumes were much larger, so more biomass was able to accumulate (Fig. 3 and 4). The trends observed from this assay were very similar to those observed in the first growth assay, but these trends were significant (Fig. 4). That is, the differences between the mean difference in biomass among RHA1, 620, and F1 were similar – RHA1 and 620 had very similar difference in biomass accumulation and both had a greater accumulation with respect to F1, though these trends only became visibly significant with larger culture volumes (Fig. 3 and 4). As well, this assay gave the opportunity to include *S. viridochromogenes* DSM40736 which was received at a later time, and is most interesting because of its natural N246A mutation found to increase  $Mn^{2+}$  oxidation activity in DypB (Fig 4) (12). *R. jostii* RHA1 showed a comparable biomass increase in the presence of lignin to 620, which were approximately 7-fold higher than *P. putida* F1, and approximately 2-fold lower than *S. viridochromogenes* DSM40736 (Fig. 4). These results further suggest that the conservation of wild type  $Mn^{2+}$  coordinating residues in dyp-like proteins is not an important factor in predicting the capacity for lignin catabolism, though they suggest that the N246A mutation could facilitate it.



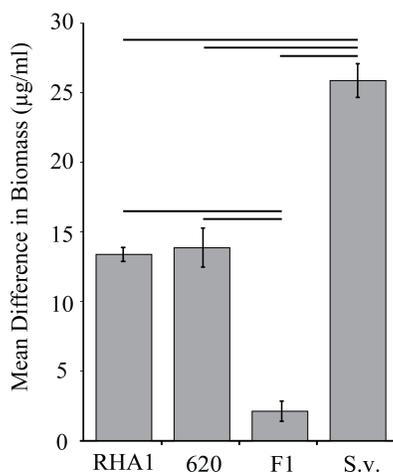
**FIG. 3. The relationship between growth in the presence of lignin, phylogeny, and the conservation of Mn<sup>2+</sup> coordinating residues in Dyp-like proteins.** Mean difference in biomass represents the difference in biomass accumulated between strains grown in 0.5 g/l and 0 g/l lignin; 95% confidence intervals are shown and horizontal bars indicate significant differences between the strains. The tree represents the phylogenetic relatedness of the strains based on their 16S rDNA sequences. The amino acid residues displayed (E156, E215, T231, E239, and N246) represent integral residues for Mn<sup>2+</sup> coordinating and oxidizing activity in DypB from *R. jostii* RHA1. No Dyp-like proteins were annotated in the genome sequences of any bacteria related to isolates 463 or 132 (control strains).

## DISCUSSION

The microbial degradation of lignin is poorly understood compared to white-rot and brown-rot fungi; however, bioinformatic analysis of the genome sequence of *R. jostii* RHA1 identified a dyp-type peroxidase which was found in other bacteria with lignin degradation activity (1). This enzyme, DypB, was the first characterized recombinant bacterial enzyme shown to degrade lignin. This lignin modification was found to be activated in the presence of Mn<sup>2+</sup> ions and, from the solved crystal structure, dependent upon a Mn<sup>2+</sup> binding pocket with four conserved residues (E156, E215, T231 and E239)(Unpublished, by Eltis, Grigg, Murphy, and Singh). This activity was reminiscent of a well characterized fungal lignin degrading enzyme, manganese peroxidase, which uses oxidized Mn<sup>2+</sup> as a diffusible mediator for oxidizing lignin. Therefore, lignin modification by bacteria may occur through a similar mechanism.

A phylogenetic analysis of bacterial dyp-like proteins indicated strong conservation of the four residues in protein sequences with homology to DypB, suggesting that these residues are important in a conserved function – potentially for the oxidation of Mn<sup>2+</sup> (Unpublished data, by Strachan, VanInsberghe, and Williams). As such, the present study attempted to see if these Mn<sup>2+</sup> coordinating residues could be indicators of lignin modification activity to begin to characterize lignin degradation physiologically and enzymatically.

The first part of this paper attempted to clone dyp-like proteins from both *Pseudomonas putida* F1 and *S. viridochromogenes* DSM40736. Characterizing these proteins would then give insight into whether Mn<sup>2+</sup> oxidation could be predicted in the environment based on the coordination chemistry of DypB. Although, all of the same residues which are important in Mn<sup>2+</sup> coordination and oxidation in DypB were present in the dyp-like protein of *P. putida* F1, there was no detectible Mn<sup>2+</sup> oxidizing activity by the purified protein (data not shown). This suggests that these residues alone are not



**FIG. 4. Relative ability of select strains to grow in the presence of lignin.** Mean difference in biomass represents the difference in biomass accumulated between strains grown in 0.5 g/l and 0 g/l lignin for seven days; 95% confidence intervals are shown and horizontal bars indicate significant between the strains.

solely important for  $Mn^{2+}$  oxidation activity. However, enzyme activity will need to be further assessed before  $Mn^{2+}$  is properly ruled out as a substrate.

The second part of this paper looked at growth of candidate strains on pyruvate in the presence of lignin. While there were certain statistically significant differences in the mean difference in biomass accumulation between some strains, these trends did not correlate with the conservation of  $Mn^{2+}$  coordinating residues, the phylogenetic relatedness of the organisms (Fig. 3), or the phylogenetic relatedness of the dyp-like proteins (data not shown). From the second growth assay, *R. jostii* RHA1 showed a comparable biomass increase in the presence of lignin as 620, which was approximately 7-fold higher than *P. putida* F1 (Fig. 4). As the most related, sequenced organisms to 620 did not have full conservation of the four  $Mn^{2+}$  residues which RHA1 has, while the *P. putida* F1 did, this is further evidence that the oxidation and metabolism of lignin is likely not dependent on the specific coordination chemistry found in DypB – supporting the results obtained from the protein cloning section of the present study.

Two organisms which showed increased biomass in the presence of lignin used in the study are of particular interest. First, *S. viridochromogenes* DSM40736 showed an approximately 2-fold increase in biomass in the presence of lignin compared to *R. jostii* RHA1. This was surprising as *R. jostii* RHA1 is an

organism that is currently receiving extensive attention for lignin degrading potential and that *S. viridochromogenes* DSM40736 was selected on the basis of conservation of certain residues with DypB. Also, certain strains of *S. viridochromogenes* have been suggested to degrade large aromatics, such as lignin, in a heme peroxidase dependent manner (9). Secondly, strain 620 is also of particular interest because this organism showed comparable biomass increases to *R. jostii* RHA1 and is a member of the *Enterobacter* genus. As well, several *Enterobacter sp.* have been shown to have lignolytic activity, to degrade lignin derivatives, and to have annotated lignin degradation pathways (7, 8). Taken together, these results may be indicative of the discovery of a novel lignin degrading *Enterobacter sp.* as well as attributing a dyp-like protein to significant growth in the presence of lignin in *S. viridochromogenes* DSM40736. However, given the success of the second growth assay in larger volumes, it is still possible that significant trends relating the phylogenetics of bacteria and the conservation of  $Mn^{2+}$  coordinating residues with lignin catabolizing activity.

Ultimately these results suggest that the conservation of the key residues found to be important for  $Mn^{2+}$  coordination and oxidation in DypB were not directly indicative of lignin modification or catabolism, enzymatically or physiologically. However, this study was too small in size to conclusively rule out that the characteristics measured here are not indicative of lignin modification or catabolism. Nevertheless, this study was successful in identify organisms from three distinct clades of life which have a comparable or greater capacity to catabolize lignin than the only bacterium with a characterized lignin modifying enzyme, *R. jostii* RHA1.

## FUTURE DIRECTIONS

As the dyp-like protein from *P. putida* F1 was successfully purified and reconstituted, various substrates could be screened for oxidation by the enzyme. This would confirm activity and begin the characterization of the enzyme. Furthermore, steady state kinetic constants could be determined for direct comparison to DypB. This data could possibility lead to a better understanding of the physiological roles of dyp-like proteins and their implications in lignin degradation. Also, the cloning and purification of the *S. viridochromogenes* DSM40736 dyp-like protein was not completed in this study. Future groups could optimize primer design and PCR conditions in order to reattempt cloning. This would be of particular interest as *S. viridochromogenes* DSM40736 showed the

greatest accumulation of biomass in the presence of lignin. Finally, the second growth curve design used in this study could be used to screen all the isolates used, as only four organisms were repeated after the first protocol. Ideally, once a sufficient number of dyp-like proteins have been characterized from organisms whose activity on lignin has been assessed, the implications of these proteins in lignin degradation will start to be understood.

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