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Overexpression of endogenous multi-copper oxidases *mcoA* and *mcoC* in *Rhodococcus jostii* RHA1 enhances lignin bioconversion to 2,4-pyridine-dicarboxylic acid

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Abstract

To improve the titre of lignin-derived pyridine-dicarboxylic acid (PDCA) products in engineered *Rhodococcus jostii* RHA1 strains, plasmid-based overexpression of seven endogenous and exogenous lignin-degrading genes was tested. Overexpression of endogenous multi-copper oxidases *mcoA*, *mcoB*, and *mcoC* was found to enhance 2,4-PDCA production by 2.5-, 1.4-, and 3.5-fold, respectively, while overexpression of dye-decolorizing peroxidase *dypB* was found to enhance titre by 1.4-fold, and overexpression of *Streptomyces viridosporus* laccase enhanced titre by 1.3-fold. The genomic context of the *R. jostii mcoA* gene suggests involvement in 4-hydroxybenzoate utilization, which was consistent with enhanced whole cell biotransformation of 4-hydroxybenzoate by *R. jostii* pTipQC2-mcoA. These data support the role of multi-copper oxidases in bacterial lignin degradation, and provide an opportunity to enhance titres of lignin-derived bioproducts.

KEYWORDS

lignin degradation, multi-copper oxidase, *Rhodococcus jostii* RHA1

The aromatic heteropolymer lignin constitutes 15%–30% of plant cell wall lignocellulose, and therefore represents a valuable source of renewable aromatic carbon that could potentially be valorized to aromatic chemicals (Zakzeski et al., 2010). Lignin presents a number of practical difficulties for bioconversion, such as difficult bond cleavages, insolubility, and repolymerisation of phenoxy radical intermediates, however, there are several examples of successful chemocatalytic and biocatalytic conversion of polymeric lignin to aromatic monomers (Bugg & Rahmanpour, 2015). In the biocatalytic field, the use of engineered strains of lignin-degrading bacteria such as *Rhodococcus jostii* RHA1 to generate vanillin (Sainsbury et al., 2013), and *Pseudomonas putida* KT2440 to generate muconic acid (Vardon et al., 2015), has been a successful strategy, due to the convergent metabolism of diverse lignin degradation metabolites into key intermediates such as protocatechuic acid, thereby removing the need for separation of complex mixtures of depolymerized monomers.

Previous work from our research group has established a biocatalytic route from protocatechuic acid to pyridine-dicarboxylic acid bioproducts in engineered strains of *Rhodococcus jostii* RHA1, by overexpression of either protocatechuate 4,5-dioxygenase *ligAB*, or protocatechuate 2,3-dioxygenase *praA*, followed by cyclisation of the resulting extradiol ring fission products with ammonia (Mycroft et al., 2015), as shown in Figure 1. Titres of 80–125 mg/L from wheat straw lignocellulose feedstock were obtained by plasmid-based expression of *ligAB* or *praA* genes in wild-type *R. jostii* RHA1 (Mycroft et al., 2015). Chromosomal integration of the *ligAB* genes, removal of the competing β-ketoadipate pathway via deletion of *pcaHG* genes, and increase in lignin degradation flux by overexpression of *Amycolatopsis*

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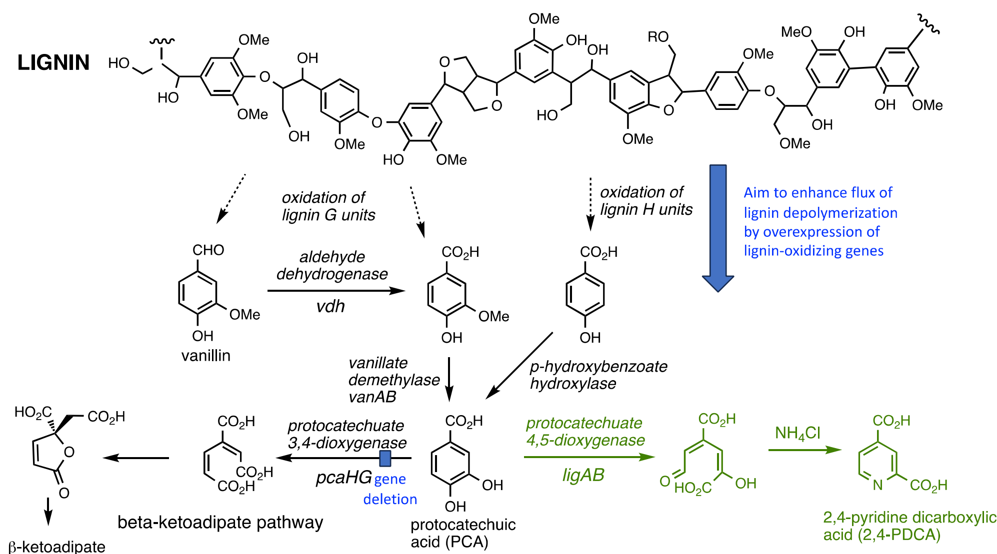

FIGURE 1 Catabolic pathway for generation of 2,4-PDCA from lignin, showing insertion of *ligAB* genes (Mycroft et al., 2015), and deletion of *pcaHG* genes (Spence et al., 2021). PDCA, pyridine-dicarboxylic acid.

dyp2, resulted in improved titres of 240–330 mg/L from either wheat straw lignocellulose or soda lignin (Spence et al., 2021).

To date the maximum titres for PDCA production represent conversion yields of 5%–12% from total polymeric lignin in the feedstock, which, although competitive with chemocatalytic lignin conversion methods, would need to be improved to bring the process towards commercial feasibility. Therefore, methods to enhance the titre of PDCA bioproducts are needed. We have previously demonstrated a 1.5-fold enhancement in PDCA titre by overexpression of *Amycolatopsis dyp2* (Spence et al., 2021), therefore, in this study we wished to examine whether further enhancements in titre could be achieved by overexpression of endogenous *R. jostii* lignin-oxidizing genes, or other exogenous lignin-oxidizing genes. Previous bioinformatic analysis has identified three multi-copper oxidase genes in *Rhodococcus jostii* RHA1, designated *mcoABC* (Granja-Travez et al., 2020). Dye-decolorizing peroxidase *dypB* is known from biochemical work and gene knockout to be active for oxidation of polymeric lignin (Ahmad et al., 2011), and the DypB protein is known to be targeted to an encapsulin nanocompartment (Rahmanpour & Bugg, 2013), whose precise role in metabolism is uncertain. Therefore, each of these genes was overexpressed, also *Streptomyces viridosporus* small laccase (SLAC, Majumdar et al., 2014) was tested, and the effect on titre of 2,4-PDCA was examined.

Each gene was separately cloned into expression vector pTipQC2, used previously for gene expression in *R. jostii* RHA1 (Mycroft et al., 2015). Each recombinant vector was then transformed into *R. jostii* pcaHG::ligAB(Ptpc5) in which the chromosomal *pcaHG* genes had been replaced with the *Sphingobium* SYK-6 *ligAB* genes, under the control of a constitutive Ptpc5 promoter, as published previously (Spence et al., 2021). Each recombinant construct was then grown on minimal M9 media containing 1% Green Value Protobind soda lignin for 7 days at 30°C, and supernatant analysed by C_{18} reverse phase HPLC for production of 2,4-PDCA.

As shown in Figure 2, enhanced production of 2,4-PDCA was observed in several cases. Highest production was observed via overexpression of endogenous *R. jostii mcoC* (3.5-fold) or *mcoA* (2.5-fold), corresponding to titres of 510 and 365 mg/L, respectively, higher titres than observed for overexpression of *R. jostii mcoB* (1.4-fold) or *Streptomyces* SLAC (1.3-fold). Overexpression of *R. jostii dypB* gave 1.4-fold increased titre, similar to that observed previously via overexpression of *Amycolatopsis dyp2* (Spence et al., 2021). Co-expression of *R. jostii dypB* and *encapsulin* genes gave no over-production of 2,4-PDCA, relative to a pTipQC2 empty vector control.

The genomic context of the *R. jostii mcoA* and *mcoC* genes was examined. As shown in Figure 3a, the *mcoA* gene (RHA1_ro02377) is colocated with phenol degradation genes such as phenol hydroxylase genes RHA1_ro02379 and RHA1_ro02380, and the *catABC* genes encoding the catechol intradiol cleavage pathway, and is located immediately adjacent to RHA1_ro02376 encoding a 4-hydroxybenzoyl CoA thioesterase. In contrast, the *mcoC* gene (RHA1_ro01580) is not colocated with any aromatic degradation genes (see Figure 3b). Phylogenetic analysis (see Figure 3c) shows that *R. jostii* RHA1 McoA and McoB are clustered together (49% sequence identity) as Group B bacterial multi-copper oxidases, of uncertain biological function, but containing an alkaliphilic laccase from *Bacillus halodurans* (Ruijssenaars & Hartmans, 2004), which shows 32% sequence identity to *R. jostii* McoA. Whereas *R. jostii* McoC is annotated as a Group A blue laccase (Granja-Travez et al., 2020), with 35% sequence identity to *B. subtilis* CotA (Martins et al., 2002), and 24% sequence identity to *Ochrobactrum* sp CueO (Granja-Travez et al., 2018).

Attempts to purify recombinant *R. jostii* McoA or McoC from the overexpressing strains were unsuccessful, indicating that the recombinant enzyme activities are unstable in vitro. However, to probe the function of *R. jostii* McoA, whole cell biotransformations were undertaken with the *R. jostii* pTipQC2-mcoA strain, using several low molecular weight substrates. Rate of consumption of 4-hydroxybenzoate,

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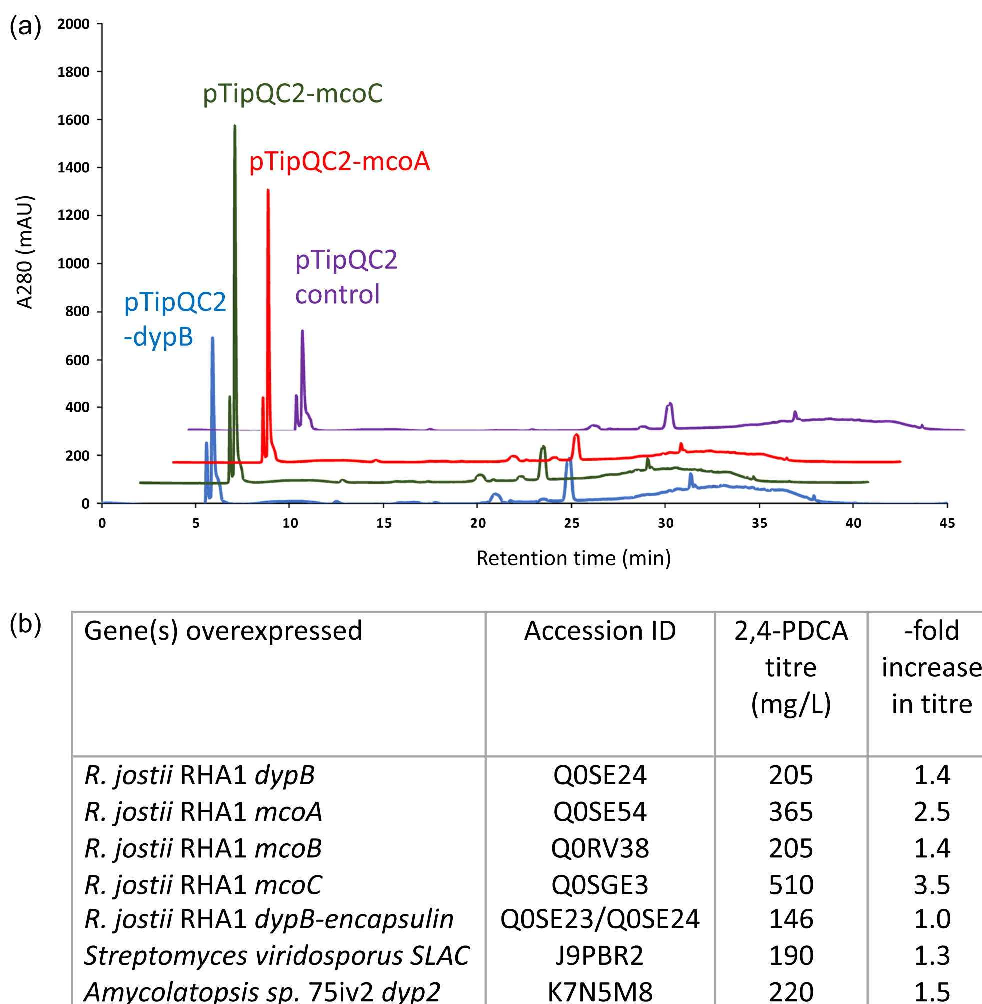

Gene(s) overexpressed	Accession ID	2,4-PDCA titre (mg/L)	-fold increase in titre
R. jostii RHA1 *dypB*	Q0SE24	205	1.4
R. jostii RHA1 *mcoA*	Q0SE54	365	2.5
R. jostii RHA1 *mcoB*	Q0RV38	205	1.4
R. jostii RHA1 *mcoC*	Q0SGE3	510	3.5
R. jostii RHA1 *dypB-encapsulin*	Q0SE23/Q0SE24	146	1.0
Streptomyces viridosporus SLAC	J9PBR2	190	1.3
Amycolatopsis sp. 75iv2 *dyp2*	K7N5M8	220	1.5

FIGURE 2 Enhanced production of 2,4-PDCA (peak at retention time 6.0 min) from M9 media containing 1% Green Value Protobind soda lignin using recombinant *R. jostii* pcaHG::ligAB(Ptpc5) strains transformed with pTipQC2 containing *dypB* (blue), *mcoC* (green), and *mcoA* (red), compared with *R. jostii* pcaHG::ligAB(Ptpc5) transformed with an empty pTipQC2 vector control. Data for *R. jostii* pTipQC2-mcoB is shown in Figure S2. PDCA, pyridine-dicarboxylic acid.

4-hydroxybenzaldehyde or vanillic acid by *R. jostii* pTipQC2-mcoA was increased by 40%–80%, compared with *R. jostii* pTipQC2 over 24 h, but no increase in rate of consumption of 4-hydroxyphenylacetic acid was observed (see Table 1, data in Figure S3). These data are consistent with a role of McoA in oxidation of 4-hydroxybenzoate or related low molecular weight phenols.

The results show that overexpression of lignin-oxidizing genes in *R. jostii* RHA1 can improve the titre of aromatic bioproducts, which is a useful strategy for maximizing yields of lignin bioproducts. The overall yield of 2,4-PDCA from Protobind lignin is 3.7% for *R. jostii* overexpressing *mcoA* and 5.1% for *R. jostii* overexpressing *mcoC*, compared 2.4% reported by Spence et al (Spence et al., 2021). It is interesting that overexpression of multi-copper oxidase genes *mcoA* and *mcoC* is more effective than dye-decolorizing peroxidase *dypB*, which is consistent with a role for multi-copper oxidases in lignin depolymerization, a point debated in the literature (Granja-Travez et al., 2020; Munk et al., 2015). Both *R. jostii mcoA* and *mcoC* genes have TAT signal sequences, implying that they are exported to the cell surface, where the initial phase of lignin oxidation takes place, although *mcoA* appears to be involved in small molecule phenolic oxidation. Co-expression of *R. jostii dypB* and encapsulin genes gave no enhancement in PDCA titre, implying that targetting of DypB to the encapsulin nanocompartment does not enhance polymeric lignin breakdown in whole cells (Rahmanpour & Bugg, 2013). Overexpression of fungal laccases in *Polyporus brumalis* (Ryu et al., 2013) and *Phanerochaete chrysosporium* (Linares et al., 2018) has also been reported to enhance rates of lignocellulose breakdown. The presence of three active multi-copper oxidase genes and two dye-decolorizing peroxidases in *Rhodococcus jostii* RHA1 may account for its relatively high activity for lignin depolymerization.

1 | MATERIALS AND METHODS

Gene cloning: *R. jostii mcoA* (accession Q0SE54), *mcoB* (accession Q0RV38), *mcoC* (accession Q0SGE3), dypB (accession Q0SE24) and *encapsulin* (accession Q0SE23) genes were amplified by polymerase

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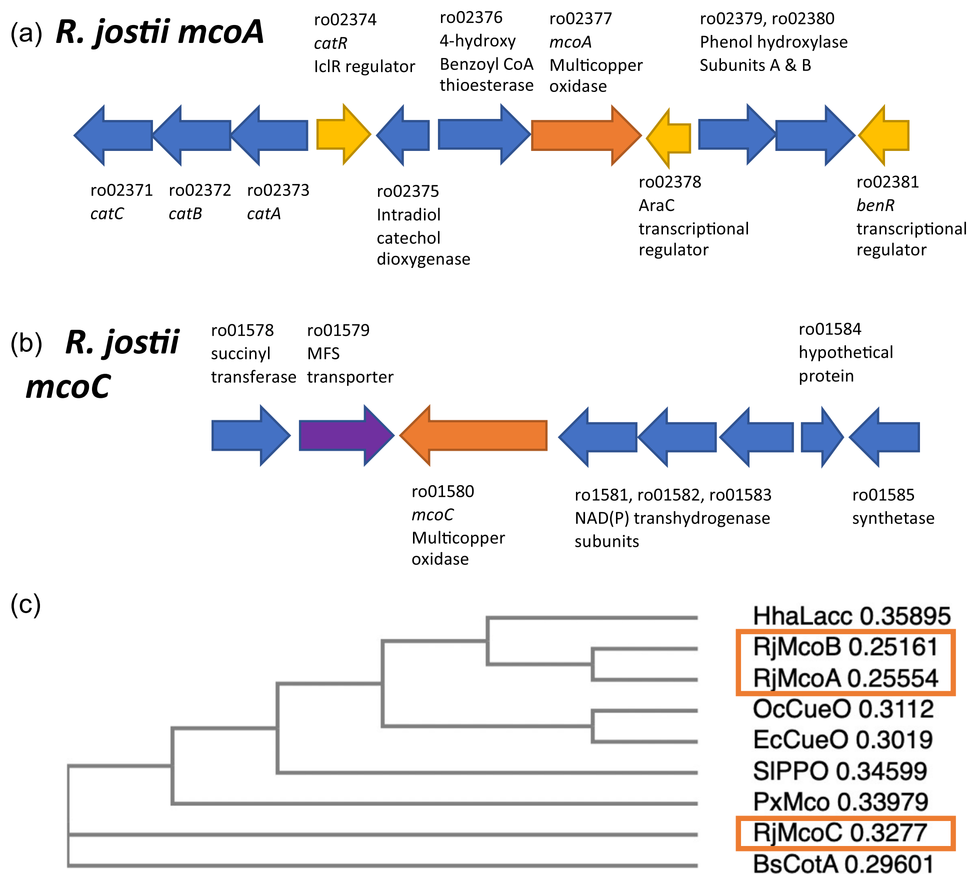

FIGURE 3 Genomic context of *R. jostii* RHA1 *mcoA* (a) and *mcoC* (b) genes, and phylogenetic tree (c, Clustal Omega) for *R. jostii* multi-copper oxidase sequences and other bacterial multi-copper oxidases. Sequence alignment shown in Figure S3.

TABLE 1 Rates of conversion of phenolic substrates via whole cell biotransformation by *R. jostii* pTipQC2-mcoA or *R. jostii* pTipQC2 (empty vector).

Substrate	Conversion by *R. jostii* pTipQC2-mcoA (μmol h^{-1} g $cells^{-1}$)	Conversion by *R. jostii* pTipQC2 (μmol h^{-1} g $cells^{-1}$)
4-hydroxybenzoic acid	1.42 ± 0.10	1.00 ± 0.10
4-hydroxybenzaldehyde	0.71 ± 0.05	0.39 ± 0.04
vanillic acid	0.68 ± 0.05	0.47 ± 0.05
4-hydroxyphenylacetic acid	0.33 ± 0.03	0.38 ± 0.03

Note: Biotransformations of 1 mM substrate carried out in PBS buffer at 20°C for 24 h. Conversion monitored by UV-vis spectroscopy (see Figure S4). Assays were run in duplicate.

Abbreviation: PBS, phosphate-buffered saline.

chain reaction from genomic DNA. *Streptomyces viridosporus* small laccase gene (accession J9PBR2) was synthesized by Genscript. Each gene was cloned into expression vector pTipQC2, used previously (Mycroft et al., 2015), and constructs checked by restriction digests (see Figure S1) and DNA sequencing. Recombinant plasmids were transformed into *Rhodococcus jostii* pcaHG::ligAB(Ptpc5) by electroporation (2.5 kV, 25 μF, and 400 Ω), and selected using 50 μg/mL chloramphenicol. A list of strains used is given in Table S1.

Lignin biotransformation: Cultures of each recombinant strain were grown on M9 minimal media containing 1% (wt/vol) Green Value Protobind soda lignin, containing 50 μg/mL chloramphenicol, and were induced by addition of 5 μg/mL thiostrepton every 48 h, at 30°C in an orbital shaker (180 rpm), for 7 days. Samples of culture supernatant were analysed by C_{18} reverse phase chromatography as described previously (Spence et al., 2021). Characterization of Green Value Protobind lignin has been reported previously (Constant et al., 2016).

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

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DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the Supporting Information material of this article.

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