Metabolic engineering of *Pseudomonas putida* for increased polyhydroxyalkanoate production from lignin

Davinia Salvachúa,1,† Thomas Rydzak,2,† Raquel Auwae,2 Annette De Capite,2‡ Brenna A. Black,1 Jason T. Bouvier,2 Nicholas S. Cleveland,1 Joshua R. Elmore,2,§ Anna Furches,2 Jay D. Huennemann,2 Rui Katahira,1 William E. Michener,1 Darren J. Peterson,1 Holly Rohrer,1 Derek R. Vardon,1 Gregg T. Beckham1,2,‡ and Adam M. Guss2,‡

1National Bioenergy Center, National Renewable Energy Laboratory, Golden, CO 80401, USA.
2Biosciences Division, Oak Ridge National Laboratory, Oak Ridge, TN 37831, USA.

Summary

Microbial conversion offers a promising strategy for overcoming the intrinsic heterogeneity of the plant biopolymer, lignin. Soil microbes that natively harbour aromatic-catabolic pathways are natural choices for chassis strains, and *Pseudomonas putida* KT2440 has emerged as a viable whole-cell biocatalyst for funneling lignin-derived compounds to value-added products, including its native carbon storage product, medium-chain-length polyhydroxyalkanoates (mcl-PHA). In this work, a series of metabolic engineering targets to improve mcl-PHA production are combined in the *P. putida* chromosome and evaluated in strains growing in a model aromatic compound, *p*-coumaric acid, and in lignin streams. Specifically, the PHA depolymerase gene *phaZ* was knocked out, and the genes involved in β-oxidation (*fadBA1* and *fadBA2*) were deleted. Additionally, to increase carbon flux into mcl-PHA biosynthesis, *phaG*, *alkK*, *phaC1* and *phaC2* were overexpressed. The best performing strain – which contains all the genetic modifications detailed above – demonstrated a 53% and 200% increase in mcl-PHA titre (g l⁻¹) and a 20% and 100% increase in yield (g mcl-PHA per g cell dry weight) from *p*-coumaric acid and lignin, respectively, compared with the wild type strain. Overall, these results present a promising strain to be employed in further process development for enhancing mcl-PHA production from aromatic compounds and lignin.

Introduction

Lignocellulosic biomass offers a source of renewable carbon that can reduce reliance on fossil fuels, reduce greenhouse gas emissions and build a foundation for a sustainable bioeconomy. Recent studies have demonstrated that the co-production of hydrocarbon fuels from sugars and chemicals from lignin streams increases the value proposition for biorefinery processes (Davis et al., 2013; Ragauskas et al., 2014; Corona et al., 2018). By leveraging natural host metabolic capabilities and applying genetic engineering techniques, carbon from complex and heterogeneous substrates, such as lignin, can be funnelled into single products (Linger et al., 2014; Beckham et al., 2016). Of particular relevance to this work, the production of oleochemicals by native and engineered microbes has gained increased attention in the last decade due to the demand for more sustainable fuels and consumer and industrial products (Pfleger et al., 2015). An example of these oleochemicals is medium-chain-length polyhydroxyalkanoates (mcl-PHAs). These polymers can be used in the production of biodegradable plastics, medical devices and chemical and material precursors (Philip et al., 2007; Linger et al., 2014; Mozejko-Ciesielska and Kiewisz, 2016; Prieto et al., 2016; Chen and Jiang, 2018).

The individual monomers comprising mcl-PHAs can range from C6 to C14 in chain length. mcl-PHAs are biosynthetic polyesters that can be produced from a
Fig. 1. The mcl-PHA production pathway in *P. putida* KT2440 via fatty acid biosynthesis and competing fatty acid β-oxidation pathway. Red boxes indicate genes targeted for deletion, and green boxes indicate genes targeted for overexpression. AccA-D, acetyl-CoA carboxylase; FabD, malonyl CoA-ACP transacylase; FabH, 3-ketoacyl-ACP synthase; FabG, 3-ketoacyl-ACP reductase; FabA and FabZ, 3-hydroxyacyl-ACP dehydratase; FabI and FabV, enoyl-ACP reductase; FabB and FabF, 3-oxoacyl-ACP synthase; PhaG, hydroxyacyl-ACP acyl-transferase; AlkK, acyl-CoA-specific enoyl-CoA hydratase; PhaC1 and PhaC2, PHA polymerases; PhaZ, PHA depolymerase; PhaJ, R-specific enoyl-CoA hydratase; FadB, enoyl-CoA hydratase; FadD, long-chain acyl-CoA synthetase; FadE, acyl-CoA dehydrogenase; FadA, 3-ketoacyl-CoA thiolase; FadF, acyl-CoA dehydrogenase.

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wide range of carbon sources in many bacteria. Some of the most well studied mcl-PHAs producers are fluorescent Pseudomonads (Madison and Huisman, 1999; Prieto et al., 2007). Among these Pseudomonads, Pseudomonas putida KT2440 naturally produces mcl-PHAs as a carbon storage compound in scenarios of carbon excess and nutrient limitation (de Eugenio et al., 2010). This bacterium is genetically tractable and metabolically diverse, with many advantageous features for biorefinery processes (Nikel and de Lorenzo, 2014). P. putida can produce mcl-PHAs from multiple carbon sources such as fatty acids, which directly undergo β-oxidation, or from sugars and aromatic compounds, which are subjected to fatty acid de novo biosynthesis (Prieto et al., 2016) (Fig. 1). Specifically, metabolic intermediates in the fatty acid biosynthetic pathway are converted to (R)-3-hydroxyacyl-ACP, which is hydrolyzed to a free hydroxy-fatty acid and subjected to CoA ligation, via PhaG and AlkK, respectively, to produce (R)-3-hydroxyacyl-CoA. Similarly, the PhaG and AlkK, respectively, to produce (R)-3-hydroxyacyl-CoA can be hydrated to generate (R)-3-hydroxyacyl-CoA. Another strategy to enhance PHA accumulation is to eliminate the degradation of the polymer through the deletion of the PHA depolymerase gene, phaZ. This deletion was evaluated in P. putida and resulted in a 1.9-fold increase in mcl-PHA titre (g L⁻¹) and 1.3-fold increase on PHA production (wt.% basis) when grown on octanoate under nitrogen-limited conditions (Cai et al., 2009) and in a 47% PHA increase (wt.% basis) when grown on glycerol as a sole carbon source (Poblete-Castro et al., 2014). De Eugenio et al. (de Eugenio et al., 2010) also reported a similar result in a comparable background strains after 48 h of incubation utilizing octanoate as carbon source while Cai et al. (Cai et al., 2009) showed a significant improvement in the knockout strain after 5 days of cultivation.

Metabolic engineering has been applied to improve mcl-PHA production through various routes (Chen and Jiang, 2018) such as (i) shutting down competing pathways (β-oxidation), (ii) overexpressing the PHA synthesis operon (via plasmid or chromosomal integration) with different ribosome binding sites (RBS) and/or promoters, (iii) enhancing NADH or NADPH supply for PHA synthesis, (iv) engineering cell morphology to increase cell size and (v) eliminating the ability to consume PHAs. For instance, to decrease the flux of PHA pathway intermediates to the fatty acid β-oxidation pathway, the genes fadA and fadB were deleted in P. putida which resulted in a 2.5-fold increase in mcl-PHA production (wt.% basis) when grown on nitrogen-rich medium supplemented with heptanoate and octanoate (Wang et al., 2011). In route (ii) above, the overexpression of phaG in the PHA synthesis operon of Pseudomonas jessenii resulted in a fourfold increase in mcl-PHA accumulation (wt.% basis) from phenylacetic acid (Tobin et al., 2007). The effect of overexpressing other genes in combination with phaG on mcl-PHA production was also tested in E. coli. The expression of phaG and phaC1(STQK) resulted in minimal mcl-PHA production from glycerol (0.9 mg L⁻¹), while the expression of phaG, phaC1 (STQK) and alkK increased mcl-PHA accumulation to 25 mg L⁻¹ when grown in the same conditions (Wang et al., 2012).
Table 1. Literature describing mcl-PHA production from lignin-derived aromatic compounds and lignin streams by native and engineered bacteria

| Strain | Substrate | Antibiotic | Cultivation mode | Cultivation time (h) | CDW (mg ml\(^{-1}\)) | mcl-PHA (mg l\(^{-1}\)) | % mcl-PHA yield (g per g CDW) | References |
|--------|-----------|------------|------------------|----------------------|----------------------|-------------------------|-------------------------------|------------|
| Native strains | | | | | | | | |
| *P. putida* JCM13063 | Vanillic acid | – | Batch, flask | 72 | 210 | Traces\(^c\) | < 1 | Tomizawa et al. (2014) |
| *P. putida* GPO1 | p-Coumaric acid | – | Batch, flask | 72 | 270 | Traces\(^c\) | < 1 | Tomizawa et al. (2014) |
| *P. putida* KT2440 | p-Coumaric acid | – | Batch, flask | 72 | 378 | 160 | 41 | This study |
| *P. putida* KT2440 | p-Coumaric acid | – | Batch, flask | 48 | 470 | 160 | 34 | Linger et al. (2014) |
| *P. putida* KT2440 | Ferulic acid | – | Batch, flask | 48 | 436 | 170 | 39 | Linger et al. (2014) |
| *P. putida* KT2440 | Lignin-containing stream (corn stover)\(^3\) | – | Bioreactor, FB | 78 | 399 | 35 | 8.8 | This study |
| Engineered strains | | | | | | | | |
| *P. putida* A514 OVJ4C1 | Kraft lignin | T, G | Batch, flask | 40 | b | 70 | b | Lin et al. (2016) |
| *P. putida* A514 Aphas4AphaxC1 | Vanillic acid | T, G | Batch, flask | b | b | 73.5 | | Lin et al. (2016) |
| *P. putida* xyl_alkKphaGC1 | Vanillic acid | T | Batch, flask | 50 | 715 | 246 | 34 | Wang et al. (2018) |
| *P. putida* KT2440a | Lignin-containing stream (corn stover)\(^3\) | | | | | | | |
| *P. putida* AG2162 | p-Coumaric acid | – | FB, flask | 72 | 483 | 241 | 50 | This study |
| *P. putida* AG2162 | p-Coumaric acid | – | FB, flask, HCD | 85 | 1758 | 953 | 54.2 | This study |
| *P. putida* AG2162 | Lignin-containing stream (corn stover)\(^3\) | – | Flask, batch | 78 | 654 | 116 | 17.7 | This study |

CDW, cell dry weight; FB, fed-batch; G, gentamicin; HCD, high-cell density; T, tetracycline.

\(^a\) The strain is not specified. In the materials and methods section, the authors specify the use of a native strain (in batch mode) while in their results authors stress the use of an engineered strain (in fed-batch mode).

\(^b\) Not reported.

\(^c\) Not clear if authors analyzed mcl-PHAs or only polyhydroxybutyrate [P(3HB)].

\(^d\) The origin and preparation of these lignin streams is different in each case.
Strain evaluation for mcl-PHA production from the model aromatic compound p-CA

To determine if mcl-PHA accumulation was affected by the genetic modifications, these strains were grown in nitrogen-limited medium containing 2 g l\(^{-1}\) p-CA (12.2 mM) and 0.13 g l\(^{-1}\) (NH\(_4\))\(_2\)SO\(_4\) (1 mM). AG2228 and AG2162 presented longer growth lags than KT2440 and AG2102 (Fig. 2A). Despite these initial growth profiles, p-CA maximum utilization rates were higher in the former strains (i.e. 0.15 ± 0.00 g l\(^{-1}\) h\(^{-1}\) in AG2162 and 0.10 ± 0.03 g l\(^{-1}\) h\(^{-1}\) in KT2440) and p-CA was nearly depleted at a similar time (48 h) in all the strains (Fig. 2B). mcl-PHA titres (mg l\(^{-1}\)) at the sample collection time (72 h) only increased significantly in the engineered strain AG2162 (242.0 ± 9.8 mg l\(^{-1}\)) when compared to the wild type (157.8 ± 10.2 mg l\(^{-1}\)) (Fig. 2C). In all cases, 3-hydroxydecanoate (C10) and 3-hydroxyoctanoate (C8) were the major mcl-PHA components produced, while 3-hydroxydodecanoate (C12) and 3-hydroxytetradecanoate (C14) were present in minor abundance, as expected (Fig. 2C). The proportions of the four constituents were similar in the tested strains as well (as a percentage of total mcl-PHAs produced, 22-26% C8, 66% C10, 7-10% C12 and 1-2% C14). The PHA yields (g mcl-PHA per g CDW) in AG2228 and AG2162 also exhibit significant increases compared with the wild type. Particularly, the yield increased from 41.9 ± 2.8% in wild type to 47.3 ± 1.2 and 49.8 ± 3.5% in AG2228 and AG2162, respectively.

Although the deletion of phaZ did not improve titres or yields, this genetic background will be still advantageous in further process development, which requires longer cultivations subjected to carbon-starvation. While the
Fig. 3. Production of mcl-PHA by AG2162 in fed-batch mode at different C (p-CA, g l⁻¹):N ((NH₄)₂SO₄, mM) ratios and concentrations in the batch phase, (1) 4:0, (2) 4:1, (3) 8:2, (4) 8:4, and fed-batch phase (1) 2.5: 0, (2) 2.5:0, (3) 5:0, (4) 5:0. (A) Consumption of p-CA and CDW, (B) mcl-PHA yields, and (C) mcl-PHA titres. The ‘inocula’ case corresponds to the seed culture data before inoculation. Results show the average of two biological replicates. Error bars present the absolute difference from the biological duplicate. These experiments were conducted in shake flasks. AG2162 was precultured from glycerol stocks in modified M9 medium containing 2 g l⁻¹ p-CA and non-limiting nitrogen (10 mM (NH₄)₂SO₄) for 24 h. The preculture was then washed twice in M9 medium (without carbon or nitrogen), and inoculated at an OD₆₀₀ of 4 in modified M9 medium containing different carbon (p-CA):nitrogen ((NH₄)₂SO₄) ratios in the combinations mentioned above. When p-CA was depleted (42 h), a pulse of 2.5 or 5 g l⁻¹ p-CA was also applied to the combination (1,2) or (3,4) respectively. Flasks were incubated at 30°C and 300 rpm for 85 h and samples were taken at 42 and 85 h to evaluate CDW and PHA production.

Enhancing PHA production from lignin

Further deletion of fadBA1 and fadBAE2 in AG2228 led to a statistically significant increase in yields when compared to the wild type, titres did not improve. A similar result was previously reported in a different P. putida strain when only deleting fadA and fadB, but utilizing fatty acids as a carbon source (Wang et al., 2011). The overexpression of phaG alone was previously reported not to affect P. putida mcl-PHA production from phenylactic acid (Tobin et al., 2007). Separately, overexpression of phaC1 combined with phaJ4 was sufficient to increase mcl-PHA accumulation from vanillic acid in a plasmid-bearing P. putida strain (Lin et al., 2016) (Table 1) while in E. coli, overexpressing the genes encoding PhaC and AlkK was necessary to enhance mcl-PHA accumulation from glycerol (Wang et al., 2012). Even though the present study has not evaluated single overexpressed genes, we demonstrate that the selected gene combination (gene knockouts and gene overexpression integrated into the genome) significantly improves carbon flux from p-CA into mcl-PHA biosynthesis in P. putida.

Evaluation of mcl-PHA production by AG2162 under different culture conditions

Carbon (C)-to-nitrogen (N) ratio (de Eugenio et al., 2010) and cell density (Davis et al., 2015) are known to affect mcl-PHA accumulation in P. putida. Thus, to obtain higher mcl-PHA titres and yields than those obtained in the previous experiment (Fig. 2C), we evaluated AG2162 for mcl-PHA production at different C:N ratios and concentrations in a high-cell density, fed-batch, shake flask experiment. The experimental setup consisted of a batch phase containing either 4 or 8 g l⁻¹ p-CA as a carbon source with (NH₄)₂SO₄ at different concentrations yet still nitrogen-limited and a fed-batch phase where the feeding contained only p-CA as a carbon source without any supplementary nitrogen (see Fig. 3 legend).

p-CA was depleted at the end of the batch phase and its concentration was < 0.75 g l⁻¹ at the end of the fed-batch phase in all the cultivation conditions (Fig. 3A). Maximum p-CA utilization rates (calculated after the feeding pulse) decreased at higher C:N ratios. Specifically, when nitrogen was absent from the media during the batch phase (case 1), p-CA utilization rate was 0.06 ± 0.00 g l⁻¹ h⁻¹ and, at the lowest C:N (case 4), the rate was 0.16 ± 0.01 g l⁻¹ h⁻¹. Utilization rates were the same (0.11 ± 0.01 g l⁻¹ h⁻¹) in cases 3 and 4, which correspond to the same C:N ratio but at different initial substrate concentrations. Regarding CDW (Fig. 3A), the highest values were observed in case 4, at the lowest C:N ratio. The mcl-PHA yields were similar at the end of the fed-batch phase in all cases (between 48% and 54% with errors up to 3.2%) (Fig. 3B), and case 4 presented the greatest CDW. Therefore, mcl-PHA titres were also higher in the latter case, up to 953 ± 44 mg l⁻¹ (Fig. 3B). The average yields at the end of the batch phase increased at the highest C:N ratio (case 1, without nitrogen added). However, since nitrogen starvation limits cell growth, the titres were ultimately similar to those found under other culture conditions (cases 3 and 4). These results suggest that the C:N ratios evaluated in this study do not have a critical effect on mcl-PHA yields if produced in fed-batch mode and high-cell density cultivations. However, that ratio is critical to enhance cell biomass and thus titres and productivity.
**Production of mcl-PHAs from a soluble and process-relevant lignin-rich stream**

As demonstrated above, wild type and engineered *P. putida* strains are able to convert the lignin-derived product p-CA to mcl-PHAs effectively. Thus, to finalize this study, we also tested the ability of both strains to convert a heterogeneous lignin stream that contains p-CA, ferulic acid and high molecular weight lignin as major carbon sources, to mcl-PHAs. This lignin comes from the solid fraction generated after enzymatic hydrolysis of pretreated corn stover (Chen et al., 2016). Then, it is further washed with water (to remove sugars) and solubilized via base-catalyzed depolymerization (Rodriguez et al., 2017; Salvachúa et al., 2018). Lignin solubilization was approximately 53% (lignin content in soluble stream/lignin content in initial solid stream) and contained ~4 g l⁻¹ of p-CA and 0.1-0.2 g l⁻¹ ferulic acid (as major aromatic compounds) from an initial total lignin content of approximately 22 g l⁻¹.

Wild type *P. putida* and strain AG2162 were grown in the lignin liquor (75% v/v containing 1 mM (NH₄)₂SO₄) and reached stationary phase between 24 and 48 h, likely due to the total consumption of readily accessible carbon sources and/or nitrogen (Fig. 4A). Strain AG2162 increased the mcl-PHA yield by ~100% compared with the wild type (17.7 ± 0.2 vs. 8.9 ± 0.8% respectively) and titre by 3.3-fold (116 ± 35 vs. 35 ± 5 mg l⁻¹ respectively) (Fig. 4B) which demonstrates the robustness of AG2162 and the increased carbon flux into mcl-PHA biosynthesis even in complex lignin streams. The main hydroxycarboxylic acid species accumulated in both strains was again 3-hydroxyoctanoate. However, unlike the proportions observed in Fig. 2C, 3-hydroxyoctanoate was lower in these lignin cultures, representing 10% and 18% of the hydroxycids in AG2162 and KT2440, respectively (Fig. 4B), instead of 22-26%. We also analyzed the lignin molecular weight profile by gel permeation chromatography (GPC) at the end of the cultivations (78 h). Low molecular weight lignin (indicated as monomeric aromatic compounds in Fig. 4C) disappeared after the bacterial treatments, which aligns with the total p-CA depletion shown in Fig. 4A. As observed in previous work (Salvachúa et al., 2015), both strains also decreased the high molecular weight lignin content, although that decrease is more evident in AG2162 cultivations (Fig. 4C) which suggests the conversion of oligomeric lignin. To confirm if high molecular weight lignin is metabolized by strain AG2162 to a higher extent than KT2440, we also analyzed the lignin content at the end of the bacterial treatments. Lignin utilization was 23.5 ± 1.7% and 18.3 ± 1.5% in AG2162 and KT2440, respectively, which verifies the GPC observations. Overall, these results corroborate that AG2162 is a robust and improved mcl-PHA production strain compared with KT2440 from both pure aromatic compounds, such as p-CA, and a process-relevant lignin stream.

**Culture conditions (Davis et al., 2015), carbon sources (Cai et al., 2009) and volume ratios (v_media/v_liquor) (Poblete-Castro et al., 2014)** are critical parameters in mcl-PHA production. Considering the number of variables, quantitative comparison of mcl-PHA production studies on an equivalent basis is challenging. In fact, comparisons become more complicated when using a heterogeneous substrate as lignin since, in many cases, lignin streams contain carbon sources other than aromatic compounds (e.g. acetic acid and sugars) that can lead to the production of mcl-PHAs (Linger et al., 2014), or contain very different lignin
concentrations [e.g. 10 g l\(^{-1}\) (Salvachúa et al., 2015) to 30 g l\(^{-1}\) of lignin (Rodriguez et al., 2017)]. In addition, considering the increased titres obtained in fed-batch mode from p-CA (Fig. 3) as well as the mcl-PHA titres (1 g l\(^{-1}\)) achieved from a lignin stream in fed-batch mode in a recent publication (Liu et al., 2017) (Table 1), it is likely that mcl-PHA titres from the current lignin stream could be further improved by using a different feeding strategy. However, in this study we did not pursue optimizing titres from lignin because the main limitation currently faced in valorizing lignin is its low content of bioavailable monomeric aromatic species and carbon to the production hosts (Beckham et al., 2016). Nevertheless, it is worth highlighting that the lignin stream utilized in this study contains up to 15% of monomeric species (mainly p-CA) (Rodriguez et al., 2017; Salvachúa et al., 2018), which is already a reasonable concentration to be upgraded.

Overall, while there is extensive space to improve the conversion of aromatic compounds and lignin to mcl-PHAs through process development in bioreactors, our results suggest that strains developed here can be a reasonable starting platform to efficiently convert lignin-derived aromatic compounds into different value-added molecules that are derived from fatty acid biosynthesis (e.g. fatty alcohols, ketones, chemically-functionalized mcl-PHAs). Furthermore, the AG2162 background can also be utilized for further pathway engineering to increase mcl-PHA titre, rate and yield by increasing flux into fatty acid biosynthesis in future work.

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Conflict of interest
None declared.

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Supporting information
Additional supporting information may be found online in the Supporting Information section at the end of the article.
Appendix S1. Materials and methods.