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Understanding the Limitations in the Biosynthesis of Polyhydroxyalkanoate (PHA) from Lignin Derivatives

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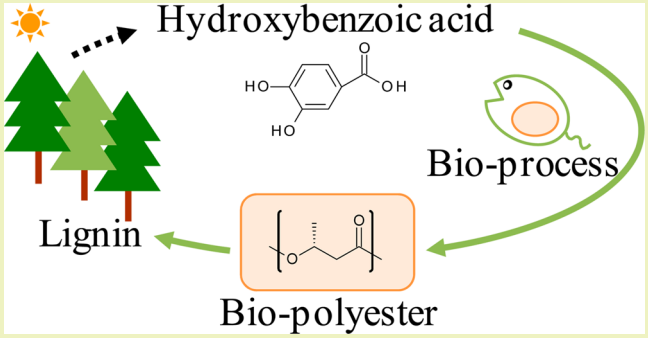
Supporting Information

ABSTRACT: To investigate the conversion of lignin derivatives to biopolyesters, 11 polyhydroxyalkanoate (PHA)-accumulating strains were cultured on mineral salt media containing each of the 18 lignin derivatives and hydroxybenzoic acids, including intermediates derived from the metabolism of lignin derivatives in bacteria. Most of the strains grew poorly in media containing lignin derivatives such as *p*-coumaric acid, caffeic acid, ferulic acid, and sinapinic acid. One of the strains, *Pseudomonas putida* Gpo1, grew in the presence of *p*-coumaric acid, ferulic acid, vanillic acid, 4-hydroxybenzoic acid (4-HBA), and 3,4-dihydroxybenzoic acid (3,4-DHBA). *Pseudomonas putida* JCM 13063 also grew in the presence of 4-HBA, 3,4-DHBA, and vanillic acid. Another strain, *Ralstonia eutropha* H16, synthesized PHA from 4-HBA, 2,5-DHBA, and 3,4-DHBA. On the basis of the data obtained from these experiments, we suggest that the conversion of lignin derivatives into intermediates such as 4-HBA, 2,5-DHBA, 3,4-DHBA, and vanillic acid represents the major bottleneck in the synthesis of PHA from lignin derivatives.



KEYWORDS: *Polyhydroxyalkanoate, Lignin derivatives, Aromatic compound, Microbial production*

INTRODUCTION

The use of biomass resources to produce energy and chemicals is currently recommended for the reduction of fossil fuel utilization and carbon dioxide emissions that are implicated in global warming. Lignin, which consists of *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol as its fundamental structural components, is a constituent of plants and one of the most abundant polymers found in nature.[1] Lignin is a largely discarded waste product from the paper industry and is not used to synthesize chemicals, with the exception of vanillin and dimethyl sulfoxide synthesis.[2] To enable the application of lignin as a raw material, its degradation has been studied using physical, chemical, and biological methods.[2−4] In nature, lignin is degraded by lignin peroxidases, manganese peroxidases, and laccase from white and brown rot fungi.[4] The degradation of lignin produces derivatives such as *p*-coumaric acid, caffeic acid, ferulic acid, and sinapinic acid, which can be metabolized by various bacterial strains like *Sphingomonas paucimobilis*, *Delftia acidovorans*, *Pseudomonas putida*, and *Bacillus megaterium*.[5−8] Extensive studies on lignin degradation pathways in *S. paucimobilis* SYK-6 and *P. putida* KT2440 revealed that lignin derivatives are metabolized to oxaloacetic acid and pyruvic acid, with aromatic carboxylic acid intermediates such as vanillic acid, 4-hydroxybenzoic acid (4-HBA), 2,5-dihydroxybenzoic acid (2,5-DHBA) (gentisic acid), 3,4-dihydroxybenzoic acid (3,4-DHBA) (protocatechuic acid), and 3,4,5-trihydroxybenzoic acid (3,4,5-THBA) (gallic acid) (Figure 1).[5,7] Pyruvic acid is converted by the glycolytic enzyme pyruvate dehydrogenase to acetyl coenzyme A (acetyl-CoA), a precursor for the biosynthesis of a biodegradable and biocompatible thermoplastic known as polyhydroxyalkanoates (PHAs).[9]

PHA is synthesized as a carbon and energy storage material by various microorganisms such as *Ralstonia eutropha*, *P. putida*, and *B. megaterium*.[9] The enzymes that play a role in PHA biosynthesis include 3-ketothiolase (PhaA), nicotinamide adenine dinucleotide phosphate (NADPH)-dependent acetoacetyl-CoA reductase (PhaB), and PHA synthase (PhaC). PhaA and PhaB produce (*R*)-3-hydroxybutyryl-CoA (3HB-CoA) from acetyl-CoA, and then PhaC polymerizes the 3HB moiety of 3HB-CoA to generate poly[(*R*)-3-hydroxybutyrate] [P(3HB)], which is the most common type of PHA.[9] Meanwhile, monomers such as the (*R*)-3-hydroxyacyl-CoA (3HA-CoA) are supplied from the beta-oxidation and fatty acid *de novo* biosynthesis pathways.[10−12] The composition of PHAs depends on the monomers supplied as well as on the substrate specificity of PhaC. For instance, PhaC from *R. eutropha* can polymerize

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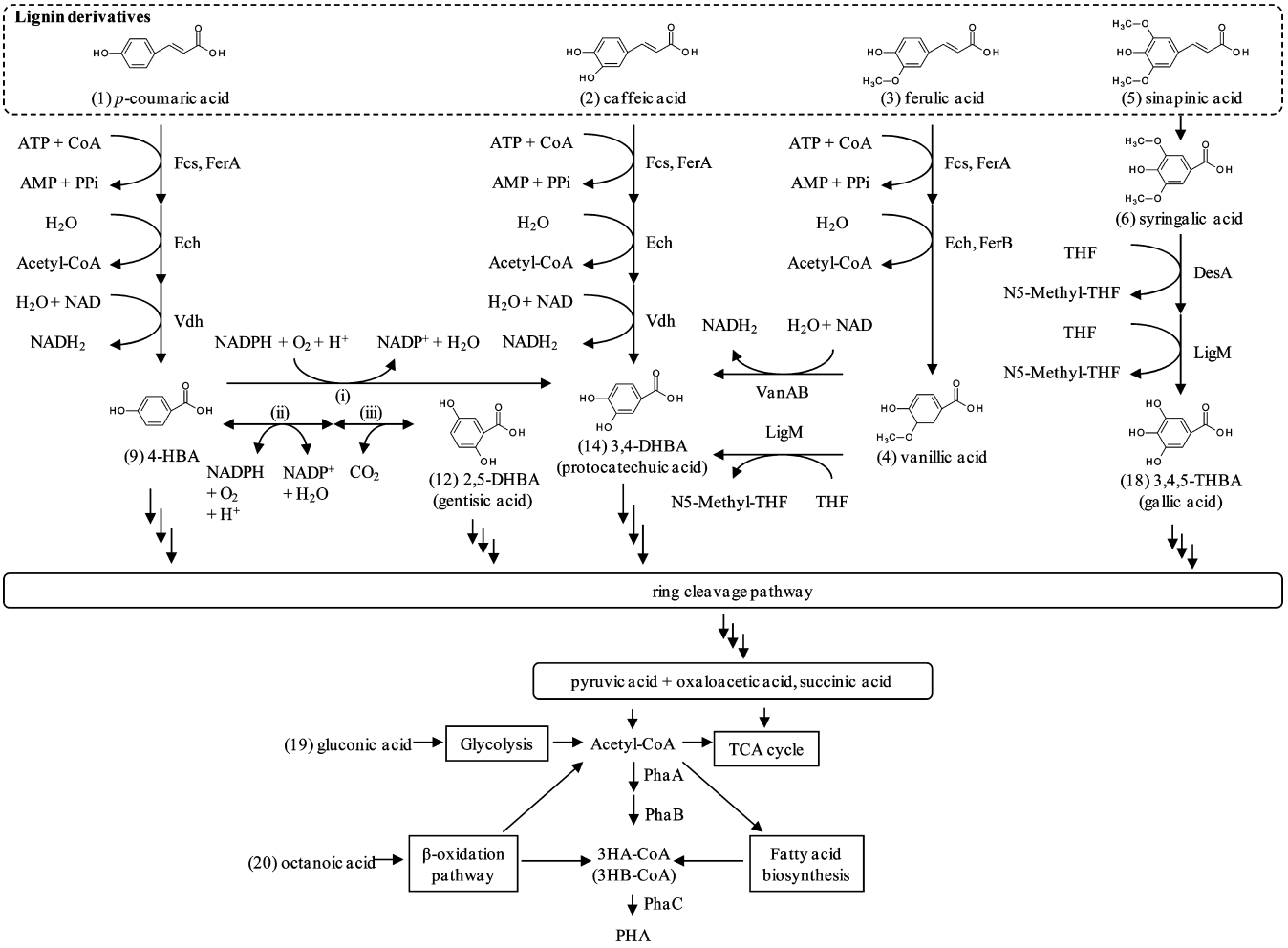

Figure 1. Degradation pathways of lignin derivatives based on the KEGG (Kyoto Encyclopedia of Genes and Genomes) for *Pseudomonas* sp. HR199, *Pseudomonas putida* KT2440, and *Sphingomonas paucimobilis* SKY-6.[5,7,30,32] Abbreviations: Fcs and FerA, feruloyl-CoA synthase; Ech, *p*-hydroxycinnamoyl-CoA hydratase/lyase; Vdh, vanillin dehydrogenase; VanA, vanillate-*O*-demethylase oxygenase subunit; VanB, vanillate-*O*-demethylase reductase subunit; FerB, feruloyl-CoA hydratase/lyase; LigM, vanillate/3-*O*-methylgallate *O*-demethylase; DesA, syringate *O*-demethylase; PhaA, beta-ketothiolase; PhaB, acetyl-CoA reductase; PhaC, polyhydroxyalkanoate synthase; ATP, adenosine triphosphate; CoA,cCoenzyme A; AMP, adenylic acid; PPi, pyrophosphoric acid; NAD and $NADH_2$, nicotinamide adenine dinucleotide; NADP and NADPH, nicotinamide adenine dinucleotide phosphate; THF, tetrahydrofolate; N5-Methyl-THF, 5-methyltetrahydrofolate; and (i), 4-hydroxybenzoate 3-monooxygenase; (ii), 4-hydroxybenzoate 1-hydroxylase; (iii), gentisate decarboxylase. Lignin derivatives: (1) *p*-coumaric acid, (2) caffeic acid, (3) ferulic acid, and (5) sinapinic acid. Hydroxybenzoic acids intermediate compounds: (4) vanillic acid, (6) syringalic acid, (9) 4-HBA, (12) 2,5-DHBA, (14) 3,4-DHBA, and (18) 3,4,5-THBA.

short-chain-length (3−5 carbon atoms) 3HB monomers to yield P(3HB) but not medium-chain-length (mcl) 3HA monomers. In contrast, mcl PHA can be produced efficiently from 3HA monomers with acyl chain lengths of 6−14 carbon atoms by PhaC from *P. putida* JCM 13063. The diversity in PhaC substrate specificities among known PHA producers suggests the possible existence of PHA-accumulating strains with the unique ability to convert lignin derivatives to acetyl-CoA or fatty acids for the biosynthesis of PHA. Several components of wood hydrolysate (e.g., vanillic acid, vanillin, syringaldehyde, and syringic acid) were reported to inhibit cell growth and PHA production.[13]

The aim of this study is to evaluate the conversion of lignin derivatives to PHA by PHA-accumulating bacterial strains. In order to synthesize PHA from lignin derivatives as sole carbon sources, 11 PHA-accumulating strains were cultured in media supplemented with each of the 18 lignin derivatives and hydroxybenzoic acids including the intermediates from bioconversion of lignin derivatives. On the basis of the results, we showed the limitations as well as optimal metabolic pathways in bacterial PHA synthesis from lignin derivatives, which will open the door to biopolymer production from lignin.

MATERIALS AND METHODS

Strains and Plasmids. *R. eutropha* H16 (currently designated as *C. necator* JCM 20644 = ATCC 17699), *D. acidovorans* DS-17 (JCM 10181), *P. putida* (JCM 13063^T), *P. aeruginosa* (JCM 14847^T = PAO1), *Sphingomonas paucimobilis* (JCM 7516^T), and *B. megaterium* (JCM 2506^T) were provided by RIKEN BRC through the National Bio-Resource Project of the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan. *Ralstonia eutropha* PHB-4 (DSM 541, a PHA-negative mutant strain of H16) and its transformants (harboring pBBRMCS2C$_{Re}$ and pBBREE32d13 expression vectors) were used for the production of PHA. The plasmid pBBRMCS2C$_{Re}$ carries the mutated [E11/S12 (I148 V/F420S)] PHA synthase gene from *R. eutropha* (PhaC$_{Re}$), and pBBREE32d13 carries the wild-type PHA synthase from *A. caviae* (PhaC$_{Ac}$).[14] *R. eutropha* NCIMB 11599 was provided by Techno Suruga Laboratory Co., Ltd.

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(Shizuoka, Japan) through the National Collection of Industrial and Marine Bacteria, NCIMB Ltd. (Aberdeen, Scotland).[15] Other strains include *P. putida* GPo1 (formerly known as *P. oleovorans* GPo1) ATCC 29347 and *Vibrio* sp. KN01, which was isolated from the seawater of Hizushi Beach (Okinawa, Japan).

Preparation of Carbon Sources. Approximately 2 g of *p*-coumaric acid, caffeic acid, ferulic acid, vanillic acid, sinapinic acid, syringic acid, 2,3-DHBA, 2,4-DHBA, 2,5-DHBA (gentisic acid), 2,6-DHBA, 3,4-DHBA (protocatechuic acid), 3,5-DHBA, 2,3,4-THBA, 2,4,6-THBA, and 3,4,5-THBA (gallic acid), individually, were neutralized by the addition of 30 mL of sodium hydrogen carbonate aqueous solution (50 g/L), and the solutions were stirred for 1 h at 25 °C. The resultant solution was subjected to wearing at 60 °C for the removal of carbon dioxide, and the pH of the solution was adjusted to 7 using 3N hydrochloric acid. The solution was then adjusted to a final volume of 100 mL using water and was sterilized by filtration through a 0.2 μm syringe filter (mixed cellulose ester, DISMIC-25AS, ADVANTEC, Tokyo, Japan).

Approximately 2 g of sodium salicylate, sodium 3-hydroxybenzoate (3HBA), sodium 4-hydroxybenzoate (4HBA), sodium gluconate, sodium octanoate, and sodium terephthalate, individually, were dissolved in 100 mL of water and sterilized by filtration through a 0.02 μm syringe filter.

Spectrophotometric Screening and Microscopic Observation of PHA-Accumulating Bacteria. All strains except for *Vibrio* sp. KN01 were grown for 24 h at 30 °C on nutrient-rich (NR) agar plates containing 10 g/L of meat extract, 10 g/L of polypeptone, 2 g/L of yeast extract, and 15 g/L of agar. A single colony was selected, transferred into 2 mL of a NR liquid medium in a test tube, and incubated at 30 °C (200 strokes/min). When the optical densities at 600 nm (Molecular Devices, Spectra Max M3, U.S.A.) (OD_{600}) reached 0.6, 20 μL of culture solution were transferred into 2 mL of mineral salt (MS) medium containing 9 g of $Na_2HPO_4{\cdot}12H_2O$, 1.5 g of KH_2PO_4, 0.5 g of NH_4Cl, 0.2 g of $MgSO_4{\cdot}7H_2O$, and 1 mL of trace element solution and supplemented with 10 g/L of each of the 18 lignin derivatives and hydroxybenzoic acids as the sole carbon sources. The trace element solution contained 9.7 g of $FeCl_3$, 7.8 g of $CaCl_2$, 0.218 g of $CoCl_2{\cdot}6H_2O$, 0.156 g of $CuSO_4{\cdot}5H_2O$, 0.118 g of $NiCl_3{\cdot}6H_2O$, and 0.105 g of $CrCl_3{\cdot}6H_2O$ per liter of 0.1 M HCl. The cells were incubated for 48 h at 30 °C.[16] *Vibrio* sp. KN01 was grown in marine broth (MB) agar, MB liquid medium (Difco, BD, Franklin Lakes, NJ), and MM (mineral medium) containing 2.80 g/L of KH_2PO_4, 3.32 g/L of Na_2HPO_4, 0.54 g/L of $(NH_2)_2CO$, 0.25 g/L of $MgSO_4{\cdot}7H_2O$, 19.45 g/L of NaCl, and 1 mL/L trace elements solution, supplemented with 10 g/L of each of the18 lignin derivatives and hydroxybenzoic acids as sole carbon sources.[17,18] The OD_{600} value was measured to determine cell growth. PHA accumulating cells were determined by visual inspection and stained with Nile blue sulfate (WALDECK) for detection of fluorescence by confocal laser scanning microscopy (CLSM) (Axio Observer Z1; Carl Zeiss, Oberkochen, Germany) using an excitation wavelength of 555 nm.[19] Prior to CLSM observation, cells were fixed on a glass plate, stained with a 1% aqueous solution of Nile blue sulfate following by incubation for 10 min at 60 °C, and finally washed with an 8% aqueous acetic acid.

Culture Conditions for PHA Accumulation. *R. eutropha* H16, *P. putida* JCM 13063^T, and *P. putida* Gpo1 were grown using two different culture conditions. In a one-phase culture, a single colony was selected, transferred into 2 mL of NR liquid medium in a test tube, and incubated at 30 °C (200 strokes/min). After 24 h, 1 mL of culture solution was transferred into a 500 mL shake flask containing 100 mL of MS medium supplemented with a carbon source, and the cells were grown for 72 h at 30 °C (130 strokes/min). The cells were then harvested by centrifugation at 15,900*g* for 10 min and then lyophilized. In a two-phase culture, a single colony was selected, transferred into 2 mL of NR liquid medium in a test tube, and incubated at 30 °C (200 strokes/min). After 24 h, 1 mL of preculture was transferred into a 500 mL shake flask containing 100 mL of NR medium, and the cells were grown for 24 h at 30 °C (130 strokes/min). The cells were then harvested under sterile conditions by centrifugation at 15,900*g* for 10 min and suspended using sterilized water. The cells were centrifuged once again at 15,900*g* for 10 min, resuspended, and transferred aseptically into 100 mL of MS medium containing 10 g/L of carbon source. The cell cultures were incubated for 48 h at 30 °C, harvested by centrifugation, and then lyophilized.[16] The lyophilized cells were weighed to determine the dry cell weight (DCW).

Isolation and Analysis of Polymers. Polymer was extracted from the lyophilized cells by stirring the cells in chloroform for 72 h at 25 °C and purified by reprecipitation with 10-fold volumes of methanol. The purified polymer was dried *in vacuo* before its weight was determined and expressed as a percentage of DCW. The polymer was analyzed by proton nuclear magnetic resonance (^{1}H NMR) (JNM-Excalibur 270; JEOL, Ltd., Tokyo, Japan) to determine its chemical structure, and the concentration of sample used was approximately 4 mg/mL in $CDCl_3$ with 0.05 v/v % of TMS (Wako Pure Chemical Industries, Ltd., JP). The molecular weight of PHA was determined by gel-permeation chromatography (GPC) (RI-2031, PU-2086, AS-2055, CO-2056; JASCO, Tokyo, Japan) with a Shodex K-806M, K802, and K-G column at 40 °C. Chloroform was used as mobile phase at a flow rate of 0.8 mL/min, and the concentration of the sample used was approximately 1 mg/mL. The molecular weight of the polymer was estimated by comparison with polystyrene standards of the following molecular weights: 1.32×10^3, 3.25×10^3, 1.01×10^4, 2.85×10^4, 6.60×10^4, 1.56×10^5, 4.60×10^5, 1.07×10^6, and 3.15×10^6.

RESULTS

Growth Assay of PHA-Accumulating Strains in the Presence of Hydroxybenzoic Acids as Sole Carbon Sources. To biosynthesize PHA from lignin derivatives, 11 PHA-accumulating strains were cultured in MS media containing 18 individual carbon sources that consist of lignin derivatives and hydroxybenzoic acids. Various strains were used because the PHA monomer biosynthetic pathway and PhaC substrate specificity differs for each strain. *R. eutropha* H16 has been extensively studied, and its PHA accumulation pathways are well understood. Its PHB-negative mutant strain, PHB-4, served as negative control as well as the host strain for heterologous expression of the PHA synthase genes from *R. eutropha* H16 (*phaC*$_{Re}$ containing the E11/S12 mutation) and *Aeromonas caviae* (*phaC*$_{Ac}$)[20,21] *R. eutropha* PHB-4 harboring the mutated PHA synthase gene from *R. eutropha* H16 (*R. eutropha* PHB-4/PhaC$_{Re}$ [E11/S12]) has been reported to show improved polymerization of the 5-hydroxyvalerate monomer compared to *R. eutropha* H16.[14] *R. eutropha* PHB-4 harboring the PHA synthase gene from *Aeromonas caviae* (*R. eutropha* PHB-4/PhaC$_{Ac}$) was able to synthesize the 3-hydroxyhexanoate unit.[20] *R. eutropha* NCIMB 11599, a glucose-utilizing mutant of *R. eutropha* H16, was used in this study because we expected its broad substrate specificity to enable the utilization of a wider range of carbon sources compared to other strains.[15] *D. acidovorans* (formerly *Comamonas acidovorans*) metabolizes lignin model compounds such as arylglycerol-*β*-aryl ether, ferulic acid, and vanillate.[4,6,22] It is known that *D. acidovorans* DS-17 synthesizes PHA containing 3HB and 4-hydroxybutyrate (4HB) units from sodium 4HB.[23] *P. putida* JCM 13063, *P. aeruginosa*, and *P. putida* GPo1 are able to cleave the aromatic rings of dimeric lignin compounds and dioxin.[24,25] Strains in the *Pseudomonas* genus are known for their PHA-accumulating abilities and express PHA synthases with wider substrate specificities than those of *R. eutropha* H16 and *D. acidovorans* DS-17. In addition to 3HA monomers, these PHA synthases have been found to polymerize monomers containing aromatic rings such as 3-hydroxy-5-phenylvalerate and 8-(*p*-methylphenoxy)-octanoate.[26,27] Another strain, *S. paucimobilis*, was previously reported to metabolize lignin derivatives such as 5,5′-

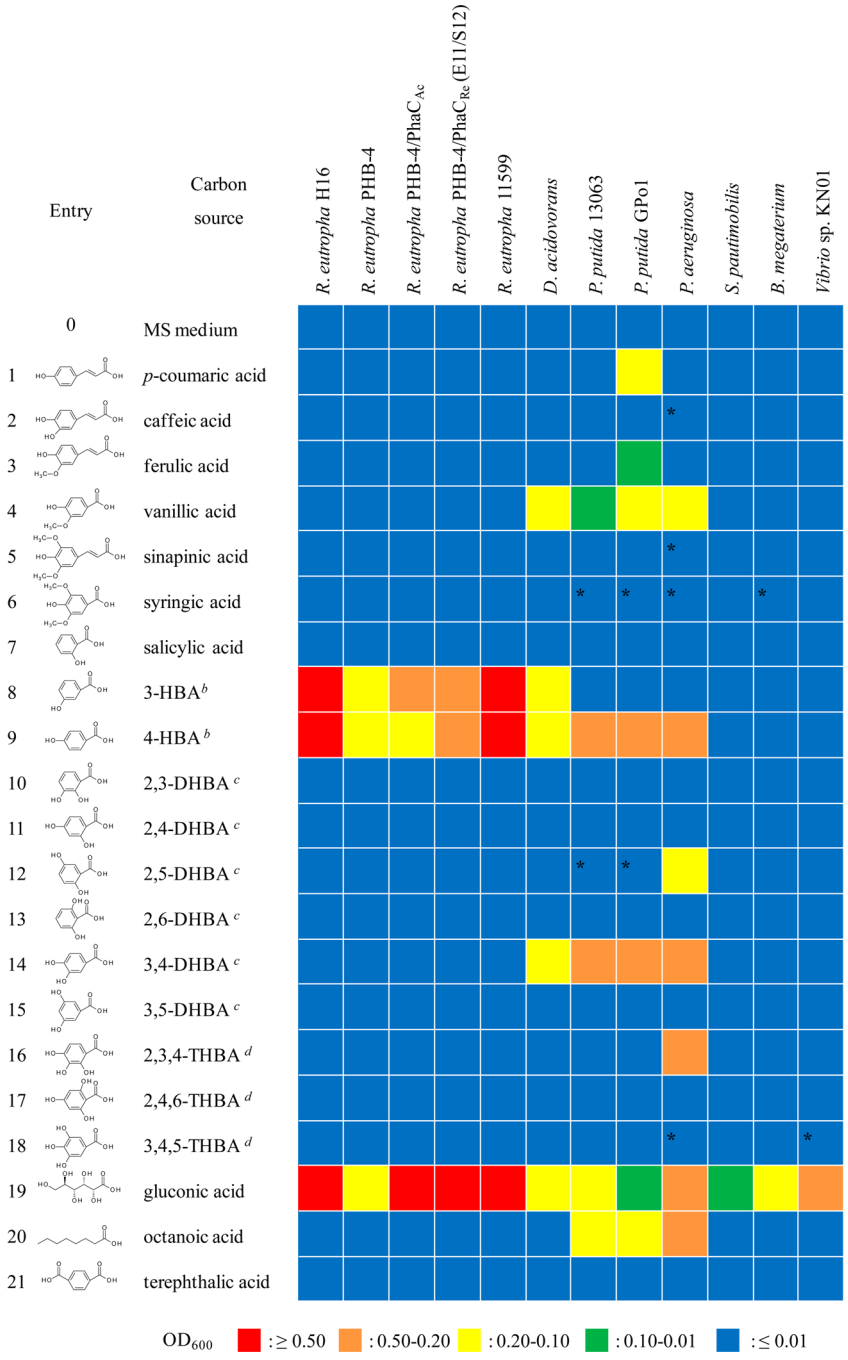

Figure 2. Effect of the carbon sources on the growth of various strains based on the optical densities at 600 nm (OD_{600}). [a]Control: nutrient-rich (NR) or marine broth (MB) medium. [b]HBA: hydroxybenzoic acid. [c]DHBA: dihydroxybenzoic acid. [d]THBA: trihydroxybenzoic acid. Asterisks (∗) indicate no cell growth but changes in OD_{600} readings. Lignin derivatives: (1) *p*-coumaric acid, (2) caffeic acid, (3) ferulic acid, and (5) sinapinic acid. Hydroxybenzoic acids intermediate compounds: (4) vanillic acid, (6) syringalic acid, (9) 4-HBA, (12) 2,5-DHBA, (14) 3,4-DHBA, and (18) 3,4,5-THBA. Hydroxybenzoic acids: (7) salicylic acid, (8) 3-HBA, (10) 2,3-DHBA, (11) 2,4-DHBA, (13) 2,6-DHBA, (15) 3,5-DHBA, (16) 2,3,4-THBA and (17) 2,4,6-THBA.

dehydrodivanillate and arylglycerol-*β*-aryl ether, and harbors a PHA synthase gene in its genome, while *B. megaterium* (AB733559, AB733549), the first PHA-producing strain to be discovered, produces vinyl derivatives from *p*-coumaric acid, ferulic acid, and sinapic acid.[5,8,28] Wood structures are known to be degraded in the marine environment, thus raising the possibility that marine bacteria are able to assimilate lignin derivatives.[29] Therefore, *Vibrio* sp. KN01 was selected as it is a PHA-accumulating marine bacterium that has the potential to assimilate lignin derivatives.[17]

Lignin derivatives such as *p*-coumaric acid, caffeic acid, ferulic acid, and sinapinic acid as well as hydroxybenzoic acids intermediate compounds such as vanillic acid, syringalic acid, 4-HBA, 2,5-DHBA, 3,4-DHBA, and 3,4,5-THBA were used as sole carbon sources for PHA production. In addition, another eight hydroxybenzoic acids such as salicylic acid, 3-HBA, 2,3-DHBA, 2,4-DHBA, 2,6-DHBA, 3,5-DHBA, 2,3,4-THBA and 2,4,6-THBA were added to the list of carbon sources to understand the effects of number and position of the hydroxyl groups on the polymerization activity of synthases (Figure S1,

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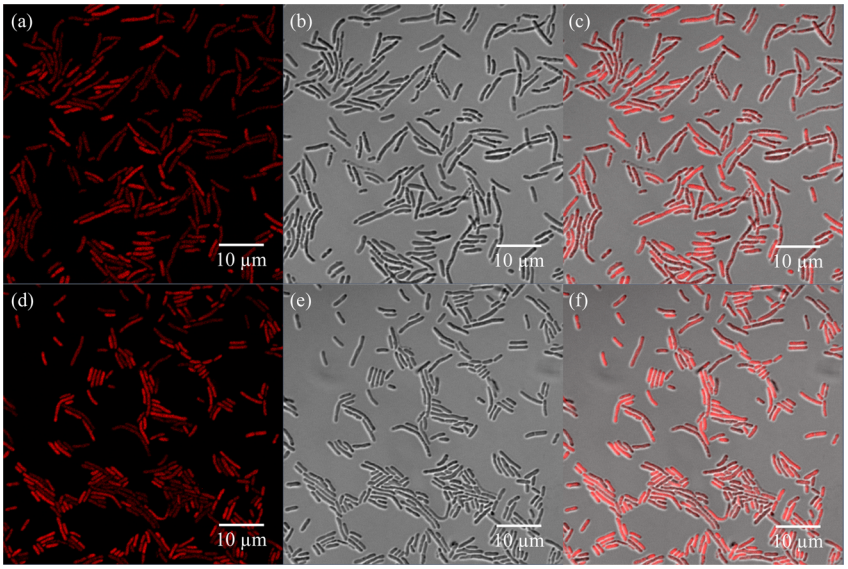

Figure 3. Confocal laser scanning microscopy (CLSM) images showing the accumulation of PHA in *R. eutropha* H16. The cells were grown for 48 h in a mineral salt (MS) medium (2 mL) containing 3-HBA (a–c) or 4-HBA (d–f). (a, d) Red fluorescence showing the accumulation of PHA, (b, e) differential interference contrast, and (c, f) overlay of the fluorescence and differential interference images. Fluorescence from Nile blue-stained PHA in the bacterial cells was imaged at an excitation wavelength of 555 nm.

Supporting Information). Among the carbon sources employed in this study, gluconate and octanoate served as positive controls because *R. eutropha* H16, *R. eutropha* PHB-4/$PhaC_{Re}$ (E11/S12), *R. eutropha* PHB-4/$PhaC_{Ac}$, *R. eutropha* 11599, *D. acidovorans* DS-17, *S. paucimobilis*, *B. megaterium*, and *Vibrio* sp. KN01 can synthesize P(3HB) from gluconate, while *P. putida* JCM 13063, *P. aeruginosa*, and *P. putida* GPo1 can synthesize PHA from octanoate.

To synthesize PHA from 18 lignin derivatives and hydroxybenzoic acids, *R. eutropha* H16 and its mutant strains were cultured using MS medium containing various sole carbon sources. *R. eutropha* H16 grew in the presence of 3- and 4-HBA. *R. eutropha* mutants PHB-4/$PhaC_{Re}$ (E11/S12) and PHB-4/$PhaC_{Ac}$ were used to investigate the effect of PhaC substrate specificity and the effect of the carbon source on cell growth. The *R. eutropha* mutants were found to utilize similar carbon sources as *R. eutropha* H16 for growth (Figure 2). As for the other strains, *D. acidovorans* DS-17 grew in the presence of vanillic acid, 3-, 4-HBA, and 3,4-DHBA. While strains of the *Pseudomonas* genus grew in the presence of *p*-coumaric acid, ferulic acid, vanillic acid, 4-HBA, and 3,4-DHBA (Figure 2). *S. paucimobilis*, *B. megaterium*, and *Vibrio* sp. KN01 did not exhibit detectable growth on any of the carbon sources except for sodium gluconate. The aqueous solution of Nile blue sulfate is a lipophilic dye that is employed in a simple and highly sensitive staining method widely used to detect PHAs directly in bacteria.[19] To observe PHA accumulation in cells by CLSM, *R. eutropha* H16, *P. putida* JCM 13063, and *P. putida* Gpo1 were selected as we prioritize strains with the ability to convert lignin derivatives or intermediates derived from the metabolism of lignin derivatives in bacteria (Figure 2). *R. eutropha* H16 grew well in presence of 4-HBA, found in the *p*-coumaric acid metabolic pathway. *P. putida* JCM 13063 grew in the presence of vanillic acid and 3,4-DHBA, which are found in the ferulic acid metabolic pathway, while *P. putida* Gpo1 grew in the presence of lignin derivatives such as *p*-coumaric acid and ferulic acid. On the basis of our observation, cells of *P. putida* JCM 13063 and *P. putida* Gpo1 were not stained by Nile blue, whereas stained granules were found in the cells of *R. eutropha* H16 cells grown in the presence of 3-HBA, 4-HBA, and gluconic acid (Figure 3).

PHA Synthesized by *R. eutropha* H16, *P. putida* JCM 13063, and *P. putida* Gpo1. We focused on the cell growth with lignin derivatives such as *p*-coumaric acid, caffeic acid, ferulic acid, and sinapic acid because those four lignin derivatives are localized at the upstream of the lignin degradation pathways as show in Figure 1. *R. eutropha* H16, *P. putida* JCM 13063, and *P. putida* Gpo1 were selected based on the results of spectrophotometric screening (Figure 2) for cultivation at a larger scale (100 mL) using a one-phase culture condition in order to investigate the composition and structure of PHA. As a result of the 100 mL-scale cultivations, *R. eutropha* accumulated P(3HB) from 3-HBA and 4-HBA as the sole carbon sources, with a PHA content of 65 and 63 wt %, respectively (Table 1). Characterization by ^{1}H nuclear magnetic

Table 1. Production of PHAs from Aromatic Carbon Sources by *R. eutropha* H16, *P. putida* JCM13063, and *P. putida* GPo1 Using One-Phase Culture Condition

	carbon source[a]	dry cell weight (g/L)[b]	P(3HB) content (wt%)
R. eutropha H16	3-HBA	1.6 ± 0.1	65 ± 1
	4-HBA	0.69 ± 0.03	63 ± 1
P. putida JCM13063	vanillic acid	0.21 ± 0.01	trace[c]
	4-HBA	0.22 ± 0.01	trace[c]
	3,4-DHBA	0.16 ± 0.01	trace[c]
P. putida Gpo1	*p*-coumaric acid	0.27 ± 0.01	trace[c]
	ferulic acid	0.15 ± 0.03	trace[c]
	vanillic acid	0.14 ± 0.01	trace[c]
	4-HBA	0.22 ± 0.01	trace[c]
	3,4-DHBA	0.07 ± 0.01	trace[c]

[a]Sodium salt derivatives of the carbon sources were added at final concentration of 10 g/L. [b]Polymer content was determined based on the weight of the purified polymer calculated as the percentage of dry cell weight. [c]PHA content less than 1 wt %.

resonance (^{1}H NMR) revealed that P(3HB) was synthesized by *R. eutropha* H16 from 3-HBA and 4-HBA. The number average molecular weight and polydispersity index (PDI) of P(3HB) from 3-HBA and 4-HBA were 50×10^4 g/mol (PDI = 3.3) and 65×10^4 g/mol (PDI = 2.9), respectively (Figures S2 and S3, Supporting Information). The growths of *P. putida* JCM 13063 and *P. putida* Gpo1 were less in comparison with *R. eutropha* H16, and PHA was accumulated at trace amounts (less than 1 wt %) in these strains (Table 1).

R. eutropha H16, *P. putida* JCM 13063, and *P. putida* Gpo1 were cultured using a two-phase culture condition (Table S1, Supporting Information). The cells were grown in NR medium, recovered by centrifugation, and subsequently transferred to a nutrient-limited environment supplemented with the aromatic compound as the carbon source.[16] Two types of control experiments were carried out. In the first control experiment, cells were cultured in NR medium for 24 h (C1). In another control experiment, cells were cultured in NR medium for 24 h and subsequently transferred to MS medium without a carbon source and cultured for 48 h (C2). The dry cell weight was found to be higher in C1 than in C2 suggesting that bacteriolysis occurred in the MS medium without a carbon source. Although *P. putida* JCM 13063 grew better in the presence of vanillic acid, 4-HBA, 2,5-DBHA, and 3,4-DBHA as the sole carbon sources rather than in the NR medium (C1), low amounts (less than 1 wt %) of PHA were accumulated. *P. putida* Gpo1 exhibited similar tendencies, showing growth in the presence of caffeic acid, ferulic acid, and 4-HBA but accumulating only trace amounts of PHA. Meanwhile, *R. eutropha* accumulated P(3HB) from 2,5-DHBA and 3,4-DHBA as sole carbon sources, with a PHA content of 26 and 13 wt %, respectively. When 2,4-DHBA, 2,6-DHBA, 3,5-DHBA, 2,3,4-THBA, and 2,4,6-THBA were added to the culture, *R. eutropha* H16, *P. putida* JCM 13063, and *P. putida* Gpo1 did not grow. On the basis of the dry cell weight and cell growth, the cell growth in the MS medium was not due to the remaining NR medium from the first phase cultivation. Characterization by ^{1}H NMR revealed that P(3HB) was synthesized by *R. eutropha* H16 from 2,5-DHBA and 3,4-DHBA (Figure S2, Supporting Information). The number average molecular weight and polydispersity index (PDI) of P(3HB) from 2,5-DHBA and 3,4-DHBA were 52×10^4 g/mol (PDI = 4.0) and 36×10^4 g/mol (PDI = 3.7), respectively (Figure S3, Supporting Information).

DISCUSSION

The focus of our research was to obtain a basic understanding on the conversion of lignin derivatives into PHA by using PHA-accumulating strains such as *R. eutropha*, *D. acidovorans*, *Pseudomonas* sp., *S. paucimobilis*, *B. megaterium*, and *Vibrio* sp. KN01. Lignin derivatives such as *p*-coumaric acid, caffeic acid, ferulic acid, and syringalic acid and various hydroxybenzoic acids were selected as sole carbon sources. In general, water-insoluble chemicals are not suitable for use as the carbon source in PHA biosynthesis because bacteria obtain carbon source from aqueous culture media. To enable the addition of insoluble chemicals to the culture media, carbon sources were neutralized using sodium hydrogen carbonate aqueous solution. As a result, we were successful in adding 18 lignin derivatives and hydroxybenzoic acids as the carbon source into the culture medium, which also allowed us to evaluate the conversion of lignin derivatives into PHA. In the first stage of the investigation, we evaluated cell growth in 2 mL of nutrient-limited media containing 18 lignin derivatives and hydroxybenzoic acids as the sole carbon sources using a one-stage cultivation strategy. *S. paucimobilis* did not show cell growth and PHA accumulation in the condition, indicating necessity to investigate various lignin-metabolizing strains under the other conditions in terms of PHA productivity. A noteworthy observation was that *Pseudomonas* strains, including *P. putida* JCM 13063, *P. putida* Gpo1, and *P. aeruginosa* grew better than other strains when hydroxybenzoic acids were employed as sole carbon sources (Figure 2). *P. putida* JCM 13063 and *P. aeruginosa* grew well in the presence of vanillic acid, 4-HBA, and 3,4-DHBA, whereas these strains did not grow in the presence of lignin derivatives such as *p*-coumaric acid, caffeic acid, ferulic acid, and syringic acid. Interestingly, *P. putida* Gpo1 grew in the presence of *p*-coumaric acid, ferulic acid, vanillic acid, 4-HBA, and 3,4-DHBA. Similar to a previous report, our results suggested that the metabolic conversion of lignin derivatives to aromatic carboxylic acids was critical in enabling the utilization of these compounds by the strains.[30,31]

The bacteria used in this study showed poor cell growth in the presence of the hydroxybenzoic acids such as 2,4-DHBA, 2,6-DHBA, 3,5-DHBA, and 2,4,6-THBA (Figure 2). PHA accumulation was investigated by Nile blue staining. We determined whether *P. putida* JCM 13063 and *P. putida* Gpo1 could produce PHA from *p*-coumaric acid, ferulic acid, vanillic acid, 4-HBA, 3,4-DHBA, and octanoic acid (control) as sole carbon sources at a 2 mL culture scale. Cells with PHA accumulation will fluoresce bright red when stained with Nile blue. Under CLSM observation, these strains did not show any red fluorescence. To investigate the accumulation of PHA at 100 mL scale, *P. putida* JCM 13063 and *P. putida* Gpo1 were cultured using a one-phase culture condition (Table 1). Although the cells grew, the PHA contents were trace (less than 1 wt %) under all conditions tested. Subsequently, *P. putida* JCM 13063 and *P. putida* Gpo1 were cultured at a 100 mL scale in the presence of 18 lignin derivatives and hydroxybenzoic acids using a two-phase culture condition (Table S1, Supporting Information). The two-phase culture condition is preferable for the synthesis of PHA from toxic carbon sources such as 4-hydroxy butanoic acid.[16] The dry cell weight of *P. putida* JCM 13063 obtained from cultures containing *p*-coumaric acid, caffeic acid, ferulic acid, sinapinic acid, and syringic acid as carbon sources ranged from 2.1 to 2.3 g/L and did not significantly differ from that obtained in the control experiment (2.3 g/L). This strain showed good growth in the presence of vanillic acid, 4-HBA, 2,5-DHBA, 3,4-DHBA, and 3,4,5-THBA, all of which are intermediates formed during the bioconversion of lignin derivatives. In contrast, *P. putida* Gpo1 showed good growth in the presence of caffeic acid, ferulic acid, and 4-HBA, with DCW ranging from 3.1 to 3.3 g/L. This strain showed poor growth in the presence of *p*-coumaric acid, vanillic acid, sinapinic acid, 3,4-DHBA, and 2,3,4-THBA. PHA levels of both strains, however, were independent of their dry cell weights and found to be less than 1 wt %. Despite the ability of *P. putida* JCM 13063 and *P. putida* Gpo1 to grow well in the presence of certain carbon sources, it is possible that the metabolized carbon substrates are not used in the generation of PHA, as evident from the low levels of accumulated PHA (less than 1 wt %). When octanoate was provided as the sole carbon source, *P. putida* JCM 13063 polymerized 3HA-CoA monomers, which were efficiently provided via the β-oxidation pathway, and produced PHA (Table S1, Supporting Information). When lignin derivatives

were used as substrates, the bioconversion of these compounds to PHA involves the conversion of aromatic compounds to acetyl-CoA, which is subsequently channeled through the fatty acid biosynthesis pathway (Figure 1). Although the aromatic compounds could be metabolized by *P. putida* JCM 13063 and *P. putida* Gpo1, the conversion of acetyl-CoA into PHA occurred less efficiently via the fatty acid biosynthesis pathway compared to more direct routes such as the *β*-oxidation pathway or the PhaA- or PhaB-involving P(3HB) biosynthetic pathway. This justifies the low amount of PHA produced, which is due to the low supply of mcl-3HA-CoA precursors. In this respect, *R. eutropha* H16 is a useful strain because it can metabolize 3-HBA and 4-HBA, intermediate products of lignin derivative bioconversion, to acetyl-CoA for growth as well as for P(3HB) production.

The results of CLSM showed that *R. eutropha* H16 accumulated PHA when grown on a MS medium containing 3-HBA and 4-HBA, which was in agreement with P(3HB) production levels of 65 ± 1 and 63 ± 2 wt % in one-phase 100 mL cultures containing 3-HBA and 4-HBA, respectively (Table 1). The number average molecular weights of P(3HB) synthesized from 3-HBA and 4-HBA were 50 × 10^4 g/mol (PDI = 3.3) and 65 × 10^4 g/mol (PDI = 2.9), respectively (Figure S3, Supporting Information). These results revealed the ability of *R. eutropha* H16 to accumulate PHA from intermediate compounds of lignin derivatives bioconversion, which led to subsequent examination of all 18 carbon substrates at a 100 mL scale using the two-phase culture condition (Table S1, Supporting Information). The dry cell weights of *R. eutropha* H16 cultured with 3-HBA and 4-HBA were 2.1 ± 0.3 and 2.0 ± 0.1 g/L, respectively. These values were comparatively lower than that in the control experiment (C1 in Table S1, Supporting Information, 2.7 ± 0.1 g/L). The dry cell weights and P(3HB) contents from 2,5-DHBA and 3,4-DHBA were 5.2 ± 0.2 g/L and 26 ± 1 wt % and 3.8 ± 0.1 g/L and 13 ± 2 wt %, respectively. The number average molecular weights of P(3HB) synthesized from 2,5-DHBA and 3,4-DHBA were 52 × 10^4 g/mol (PDI = 4.0) and 36 × 10^4 g/mol (PDI = 3.7), respectively, and these values were lower than that of P(3HB) synthesized from gluconic acid in the control experiment (81× 10^4 g/mol, PDI = 2.8) (Figure S3, Supporting Information). The relatively broad molecular weight distribution and low molecular weight of P(3HB) produced from 2,5-DHBA and 3,4-DHBA may be attributed to the decreased production of 3HB-CoA from 2,5-DHBA and 3,4-DHBA through the *β*-oxidation pathway compared to corresponding production of 3HB-CoA from other pathways. The feasibility of the two-phase cultivation condition for *R. eutropha* H16 is clearly demonstrated as the strain showed good growth and produced P(3HB) from 2,5-DHBA and 3,4-DHBA, although negative results were obtained using similar carbon substrates in a single-phase culture. According to the Kyoto Encyclopedia of Genes and Genomes (KEGG), the pathways involved in the conversion of lignin derivatives to hydroxybenzoic acids are not present in *R. eutropha* H16. These results and the examination of metabolic pathway of lignin derivatives bioconversion in *R. eutropha* H16 suggest that the metabolic reaction for the conversion of lignin derivatives (*p*-coumaric acid, caffeic acid) to hydroxybenzoic acids (4-HBA, 3,4-DHBA) is a bottleneck in the biosynthesis of PHA from lignin derivatives (Figures 1 and 2). Our finding provides a new insight to design metabolic pathways to produce PHA from lignin in *R. eutropha* H16. If the pathway for conversion of lignin to 2,5-DHBA or 3,4-DHBA is introduced into *R. eutropha* H16, the resultant recombinant strain would be able to accumulate P(3HB) from lignin derivatives more efficiently. Similarly, because *P. putida* JCM 13063 and *P. putida* GPo1 readily synthesize acetyl-CoA from lignin derivatives, the introduction of genes encoding the PhaA, PhaB, and PhaC enzymes into *P. putida* JCM 13063 would enable the conversion of acetyl-CoA to P(3HB).

CONCLUSION

The bioconversion of PHA from lignin derivatives and hydroxyl benzoic acid was investigated using 11 PHA-accumulating strains. In addition, insoluble compounds, though a neutralization step, were successfully added into the culture media for PHA biosynthesis. *R. eutropha* H16 could synthesize P(3HB) from 3-HBA, 4-HBA, 2,5-DHBA, and 3,4-DHBA as sole carbon sources. On the other hand, *P. putida* JCM 13063 and *P. putida* Gpo1 were able to grow in the presence of aromatic carboxylic acids, although PHA was accumulated at low amounts (less than 1 wt %). A plausible explanation for this observation is that *R. eutropha* H16 expresses a PhaC that polymerizes the 3HB-CoA converted from aromatic carboxylic acids, whereas *P. putida* JCM 13063 and *P. putida* GPo1 does not. The ability to convert lignin derivatives to intermediates such as 4-HBA, vanillic acid, 3,4-DHBA, and 3,4,5-THBA in bacteria is significant in enabling the biosynthesis of PHA from lignin derivatives. On the basis of these results, we propose two potential strategies for the conversion of lignin derivatives into P(3HB): (i) the introduction of the transduction pathway for the conversion of lignin derivatives to 4-HBA or 3,4-DHBA into *R. eutropha* H16, and (ii) construction of the P(3HB) biosynthesis pathway in recombinant *P. putida* JCM 13063 and *P. putida* GPo1. This understanding will provide new insights for the bacterial production of not only bioplastics but also biocompounds from lignin, which is the most challenging biomass to use efficiently.

ASSOCIATED CONTENT

Supporting Information

PHA production by *R. eutropha* H16, *P. putida* JCM 13063 and *P. putida* Gpo1 (Table S1). Additional degradation pathways of hydroxybenzoic acid (Figure S1). Analysis of PHA by ^{1}H NMR and GPC (Figures S2, S3). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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