A Novel Gene Cluster Is Involved in the Degradation of Lignin-Derived Monoaromatics in *Thermus oshimai* JL-2

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ABSTRACT A novel gene cluster involved in the degradation of lignin-derived monoaromatics such as *p*-hydroxybenzoate, vanillate, and ferulate has been identified in the thermophilic nitrate reducer *Thermus oshimai* JL-2. Based on conserved domain analyses and metabolic pathway mapping, the cluster was classified into upper- and peripheral-pathway operons. The upper-pathway genes, responsible for the degradation of *p*-hydroxybenzoate and vanillate, are located on a 0.27-Mb plasmid, whereas the peripheral-pathway genes, responsible for the transformation of ferulate, are spread throughout the plasmid and the chromosome. In addition, a lower-pathway operon was also identified in the plasmid that corresponds to the *meta*-cleavage pathway of catechol. Spectrophotometric and gene induction data suggest that the upper and lower operons are induced by *p*-hydroxybenzoate, which the strain can degrade completely within 4 days of incubation, whereas the peripheral genes are expressed constitutively. The upper degradation pathway follows a less common route, proceeding via the decarboxylation of protocatechuate to form catechol, and involves a novel thermostable γ -carboxymuconolactone decarboxylase homolog, identified as protocatechuate decarboxylase based on gene deletion experiments. This gene cluster is conserved in only a few members of the *Thermales* and shows traces of vertical expansion of catabolic pathways in these organisms toward lignoaromatics.

IMPORTANCE High-temperature steam treatment of lignocellulosic biomass during the extraction of cellulose and hemicellulose fractions leads to the release of a wide array of lignin-derived aromatics into the natural ecosystem, some of which can have detrimental effects on the environment. Not only will identifying organisms capable of using such aromatics aid in environmental cleanup, but thermostable enzymes, if characterized, can also be used for efficient lignin valorization. However, no thermophilic lignin degraders have been reported thus far. The present study reports *T. oshimai* JL-2 as a thermophilic bacterium with the potential to use lignin-derived aromatics. The identification of a novel thermostable protocatechuate decarboxylase gene in the strain further adds to its significance, as such an enzyme can be efficiently used in the biosynthesis of *cis,cis*-muconate, an important intermediate in the commercial production of plastics.

KEYWORDS *Thermus*, lignin-derived aromatics, biodegradation, thermophiles, thermostable enzyme

Lignin, the most abundant biopolymer in plants, is a component of lignocellulosic biomass and is primarily made up of three different methoxylated monomeric units: coniferyl alcohol (G units), coumaryl alcohol (H units), and sinapyl alcohol (S units) (1). Lignin is a rich and renewable source of value-added chemicals and pharmaceuticals, and new technologies are emerging to extract such compounds in high yields (2–5).

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However, the heterogeneity of aromatic compounds generated during lignin depolymerization has made it challenging to derive pure lignin-derived chemicals from lignin polymers (6). Thus, unlike cellulose and hemicellulose fractions of the biomass, which are used extensively to produce sugar and biofuels, a major part of the lignin fraction remains unused and is eventually wasted as a by-product, which has raised environmental concerns (7).

Biological lignin valorization offers an efficient solution for deriving a range of value-added chemicals. The channeling of aromatic compounds into central carbon metabolism via microbes, referred to as “biological funneling,” often uses two or more pathways (i.e., upper and lower pathways). Engineering this funneling approach can address issues related to heterogeneity in lignin valorization, as successfully demonstrated in *Novosphingobium aromaticivorans* as well as *Rhodococcus jostii* RHA1 (8–10). Among various linkages found in lignin, β -O-4 linkages are the most abundant ones (11, 12). Lignin-derived mononuclear aromatics, referred to here as “lignoaromatics,” are methoxy and/or phenolic hydroxyl compounds such as guaiacol, syringol, or carboxylated derivatives such as ferulate (FER), vanillate (VAN), *p*-coumarate (COU), and *p*-hydroxybenzoate (PHB), to name a few (13). Lignin-depolymerizing enzymes have been identified in both bacteria and fungi (14–17). Although extensively studied among fungi, actinomycetes and proteobacteria (especially those belonging to the alpha- and gammaproteobacterial subdivisions) are known to be the major decomposers of lignin among bacteria, which has been attributed to their ability to produce extracellular enzymes (18). Organisms belonging to the genera *Streptomyces*, *Rhodococcus*, *Brucella*, *Klebsiella*, *Comamonas*, *Burkholderia*, *Ochrobactrum*, *Microbacterium*, *Arthrobacter*, *Paenibacillus*, *Pseudomonas*, *Enterobacter*, *Sphingobacterium*, *Sphingomonas*, and *Sphingobium* have been reported to have lignin-degrading potential under aerobic and/or anaerobic conditions (19, 20). The degradation of lignoaromatics normally follows the β -ketoadipate pathway involving ring cleavage of either protocatechuate (PCA) or catecholic intermediates (14, 20, 21). *Sphingobium* sp. strain SYK-6 is one of the most well-studied organisms capable of degrading lignin-derived compounds and is considered a model organism for studying the catabolism of lignoaromatics (21, 22).

Although mostly reported from mesophilic bacteria, a few thermophilic microorganisms have also been found to possess lignoaromatic-degrading potential (23–26). Interestingly, immobilized protocatechuate 3,4-dioxygenase from *Pseudomonas putida* and dehydrogenases (DesV and LigV) from *Sphingobium* sp. SYK-6 were found to have optimum activities at around 60°C and 50°C, respectively (27, 28). Many industrial processes take place efficiently at elevated temperatures; the time and cost of precooling the system before subsequent downstream processes take place can be reduced with the use of thermostable enzymes (29). Delignification is usually performed at temperatures ranging from 140°C to 500°C and in the presence of solvents to yield small-molecule aromatics (30–32). Effective valorization is thus expected to be achieved with the use of downstream enzymes resistant to high temperatures as well as to solvents. Thus, it is always desirable to search for more thermostable enzymes to be used for efficient lignin valorization. Thermophiles capable of transforming aromatic hydrocarbons have been extensively studied in the last 2 decades (33). Exploring the unculturable microbial population in search of novel thermozymes and mining the large pool of genomic data already present in public databases might lead to the discovery of enzymes with desired properties. Following the latter strategy, we previously identified several Rieske nonheme iron oxygenase (RO) (34, 35) homologs in thermophilic bacteria, which indicates the possibility of transforming a wide array of aromatic hydrocarbons (36). One such organism was *Thermus oshimai* JL-2, an aerobic thermophile isolated as a nitrate reducer from U.S. Great Basin hot springs (37, 38). The RO genes in this organism were found in a unique cluster of oxidoreductase genes located in a 0.27-Mb plasmid, pTHEOS01. Although the plasmid is known to harbor genes for the *nar* operon, involved in denitrification by strain JL-2 (38), a preliminary inspection of genes vicinal to the RO genes showed a possible association with the beta-ketoadipate

pathway of aromatic hydrocarbons along with the presence of genes involved in protocatechuate metabolism in the chromosome. The aim of the present study was to explore the genome of strain JL-2 to find any possible correlation with lignoaromatic degradation and to identify the gene(s) therein with novel functions if any.

RESULTS

Genome analyses and identification of catabolic gene clusters. Analyses of the genome sequence of strain JL-2 using the KEGG and MetaCyc pathway databases resulted in the identification of three genes related to the degradation of PCA. Two genes, Theos_0322 and Theos_0385 (both present in the chromosome), encoded homologs of 3-carboxymuconate cycloisomerase and the β -subunit of protocatechuate 3,4-dioxygenase, respectively. A BLAST search further showed that the two genes shared 86.24% (100% coverage) and 66.49% (>99% coverage) identities with the adenylosuccinate lyase of *Thermus tengchongensis* and the intradiol ring cleavage dioxygenase of *Thermus brockianus*, respectively (Table 1). However, no homolog of the α -subunit of protocatechuate dioxygenase was found in the genome, which rules out the possibility of *ortho*-cleavage of PCA by the strain. Theos_2208, present in the large plasmid pTHEOS01, was predicted to encode a putative γ -carboxymuconolactone decarboxylase, which shared 94.07% identity (with 100% coverage) with the *Thermus igniterrae* gene (Table 1). The loci were further analyzed to identify a possible operon(s) based on gene order and intergenic spaces. This led to the identification of three possible gene clusters in pTHEOS01 (clusters I to III in locus 1) (Fig. 1A and Table 1), which are abundant in oxidoreductase genes and are collectively referred to as the “oxidoreductase cluster” in this study. According to conserved domain (CD) analyses and sequence homology, one cluster (cluster I) (Fig. 1A) corresponded to the *meta*-cleavage pathway of catechol (CAT) (Fig. 1B and Table 1) (39). The other two clusters, found in the upstream flanking region of cluster I, showed a novel organization of genes that could not be associated with known pathways. However, catabolic pathway mapping combined with CD analyses indicated that cluster II (Fig. 1A) may be involved in the transformation of monoaromatics such as PHB, PCA, and γ -carboxymuconolactone (Fig. 1B and Table 1). In the close vicinity of cluster III in locus 1, the gene encoding aldehyde dehydrogenase, which is required for the transformation of lignin-derived monomers such as FER or COU, was found, and two homologous genes were also found in the chromosome (locus 2) (Fig. 1A and Table 1). A gene encoding a putative *cis,cis*-muconate isomerase (locus 3) (Fig. 1A) and another gene cluster (locus 4) (Fig. 1A) corresponding to an incomplete hydroxyphenylacetate transformation pathway were also identified in the chromosome (Fig. 1B and Table 1). Based on pathway mapping, clusters I and II present in locus 1 are referred to as the “lower” and “upper” operons, respectively, of the lignoaromatic transformation pathway in strain JL-2. The function of cluster III, however, cannot be ascertained. Thus, it is proposed that the oxidoreductase cluster be recognized as the *phb* operon of lignoaromatic degradation and that the genes involved therein be (re)named as *phb* genes, as shown in Table 1.

Noticeable among the oxidoreductase cluster were genes encoding a four-component ATPase-binding cassette (ABC) transporter (*phbD1D2D3D4*) belonging to periplasmic binding protein (PBP) superfamily I (CDD identifier cd06268) and two putative transcription regulators, *phbR* (IcIR type) and *gntR* (GntR type). Deduced amino acid sequence similarity analyses of the periplasmic component (PhbD1) of the ABC transporter of strain JL-2 with biochemically studied lignoaromatic transporters (40) showed a possible preference of the protein for transporting carboxylated aromatic compounds such as PHB (see Fig. S1A in the supplemental material). The protein showed the highest identity (37.6%) to benzoate and the PHB transporter protein RPA0668 (GenBank accession no. CAE26112) of *Rhodopseudomonas palustris* CGA009 (40, 41). IcIR-type regulators are often associated with the degradation of compounds like PHB, PCA, and 3-(3-hydroxyphenyl)propionate (42–45). PhbR of strain JL-2 showed 33% identity with the PcbR regulator from *Acinetobacter* sp. strain ADP1 and is

TABLE 1 *Thermus oshimai* JL-2 genome loci predicted to be involved in the biotransformation of lignoaromatics and functional annotation of genes present therein^a

Locus tag (GenBank accession no.)	Proposed gene name	GenBank annotation	Predicted role in transformation of lignoaromatics	Closest homolog (organism) (% identity obtained by BLAST)	Description; UniProtKB/PDB accession no. (organism) (% identity with characterized proteins, obtained by BLAST) ^b
Locus 1 (pTHEO501)					
Cluster I (catechol meta-cleavage pathway)					
Theos_2467 (AFV77444)		Arabinose efflux permease family protein	Unknown	MFS transporter (<i>Thermus islandicus</i>) (77.31)	Quinolone resistance transporter; P0DPR4 (<i>Acinetobacter baumannii</i> ATCC 17978) (34.97)
Theos_2468 (AFV77445)		Hypothetical protein	Unknown	Hypothetical protein (<i>Thermogemmatipora tikiterensis</i>) (44.26)	
Theos_2469 (AFV77446)		2-Keto-4-pentenoate hydratase	Unknown	2-Hydroxypenta-2,4-dienoate hydratase (<i>Thermus thermophilus</i>) (80.71)	2-Keto-4-pentenoate hydratase; Q9KWS4 (<i>Pseudomonas</i> sp. strain AP-3) (44.30)
Theos_2470 (AFV77447)	<i>phbG</i>	NAD-dependent aldehyde dehydrogenase	2-Hydroxymuconic semialdehyde dehydrogenase	Aldehyde dehydrogenase (<i>Thermus brockianus</i>) (89.44)	2-Aminomuconic 6-semialdehyde dehydrogenase; Q9KW55 (<i>Pseudomonas</i> sp. AP-3) (42.56)
Theos_2471 (AFV77448)	<i>phbJ</i>	2-Keto-4-pentenoate hydratase	2-Oxopent-4-enoate hydratase	4-Oxalocrotonate decarboxylase (<i>Thermus filiformis</i>) (74.03)	2-Oxopent-4-enoate hydratase; P23107 (<i>Pseudomonas putida</i>) (31.63)
Theos_2472 (AFV77449)	<i>phbH</i>	4-Oxalocrotonate tautomerase	4-Oxalocrotonate tautomerase	4-Oxalocrotonate tautomerase (<i>Thermus brockianus</i>) (85.94)	2-Hydroxymuconate tautomerase; P70994 (<i>Bacillus subtilis</i> subsp. <i>subtilis</i> strain 168) (40.32)
Theos_2473 (AFV77450)	<i>phbK</i>	4-Hydroxy-2-oxovalerate aldolase	4-Hydroxy-2-oxovalerate aldolase	4-Hydroxy-2-oxovalerate aldolase (<i>Thermus thermophilus</i>) (92.51)	4-Hydroxy-2-oxovalerate aldolase; COZPX1 (<i>Rhodococcus erythropolis</i> PR4) (56.46)
Theos_2474 (AFV77451)	<i>phbQ</i>	Acetaldehyde dehydrogenase	Acetaldehyde dehydrogenase	Acetaldehyde dehydrogenase (acetylating) (<i>Thermus scotoductus</i>) (92.81)	
Cluster II (PHB degradation pathway)					
Theos_2475 (AFV77452)	<i>phbR</i>	Transcriptional regulator	Regulator of the <i>phb</i> operon	IcIR family transcriptional regulator (<i>Thermus thermophilus</i>) (91.02)	<i>p</i> -Hydroxybenzoate hydroxylase transcriptional activator; Q43992 (<i>Acinetobacter baylyi</i> ADP1) (33.01)
Theos_2208 (AFV77200)	<i>phbC</i>	γ -Carboxymuconolactone decarboxylase subunit-like protein	Protocatechuate decarboxylase	Carboxymuconolactone decarboxylase (<i>Thermus igniterrae</i>) (94.07)	Hypothetical protein Ttha0727, a CMD family member distinct from CMD and AhpD; 2CWQ (<i>Thermus thermophilus</i> HB8) (93.33)
Theos_2209 (AFV77201)	<i>phbE</i>	Catechol 2,3-dioxygenase	Catechol 2,3-dioxygenase	Catechol 2,3-dioxygenase (<i>Thermus thermophilus</i>) (94.34)	Manganese-dependent 2,3-dihydroxybiphenyl-1,2-dioxygenase; Q8GR45 (<i>Geobacillus</i> genomospecies 3) (40.98)

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TABLE 1 (Continued)

Locus tag (GenBank accession no.)	Proposed gene name	GenBank annotation	Predicted role in lignoaromatics	Closest homolog (organism) (% identity obtained by BLAST)	Description; UniProtKB/PDB accession no. (organism) (% identity with characterized proteins, obtained by BLAST) ^b
Theos_2210 (AFV77202)		Hypothetical protein	Unknown	Tripartite tricarboxylate transporter substrate-binding protein (<i>Thermus thermophilus</i>) (85.23)	
Theos_2211 (AFV77203)		Hypothetical protein	Unknown	Tripartite tricarboxylate transporter TctB family protein (<i>Meiothermus roseus</i>) (51.72)	
Theos_2212 (AFV77204)		Hypothetical protein	Unknown	Tripartite tricarboxylate transporter permease (<i>Meiothermus luteus</i>) (73.02)	2-Keto-3-deoxy-L-rhamnonate dehydrogenase; P0DOW0 (<i>Sulfobacillus thermosulfidooxidans</i> DSM 9293) (32.91)
Theos_2213 (AFV77205)		Zn-dependent alcohol dehydrogenase	Unknown	Alcohol dehydrogenase catalytic domain-containing protein (<i>Meiothermus ruber</i>) (58.31)	
Theos_2214 (AFV77206)		4-Hydroxyphenylpyruvate dioxygenase	Unknown	Sugar phosphate isomerase/epimerase and 4-hydroxyphenylpyruvate domain-containing protein (<i>Thermus brockianus</i>) (62.73)	
Theos_2215 (AFV77207)	<i>phbD1</i>	ABC-type branched-chain amino acid transport system, periplasmic component	Aromatic hydrocarbon transporter, periplasmic component	ABC transporter substrate-binding protein (<i>Thermus scotoductus</i>) (80.30)	
Theos_2216 (AFV77208)	<i>phbD2</i>	Branched-chain amino acid ABC-type transport system, permease component	Aromatic hydrocarbon transporter, permease component	Branched-chain amino acid ABC transporter permease (<i>Thermus igniterrae</i>) (82.24)	High-affinity branched-chain amino acid transport system permease protein; P0AEX7 (<i>Escherichia coli</i> K-12) (27.71)
Theos_2217 (AFV77209)	<i>phbD3</i>	Branched-chain amino acid ABC-type transport system, permease component	Aromatic hydrocarbon transporter, permease component	Branched-chain amino acid ABC transporter permease (<i>Meiothermus</i> sp.) (73.42)	
Theos_2218 (AFV77210)	<i>phbD4</i>	ABC-type sugar transport system, ATPase component	Aromatic hydrocarbon transporter, ATPase component	ATP-binding cassette domain-containing protein (<i>Meiothermus luteus</i>) (79.22)	Galactofuranose transporter ATP-binding protein YtfH; Q68EX0 (<i>Escherichia coli</i> K-12) (29.66)

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TABLE 1 (Continued)

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Theos_2219 (AFV77211)	<i>phbB1</i>	Ring-hydroxylating dioxygenase, large terminal subunit	Vanillate O-demethylase large subunit	Aromatic ring-hydroxylating dioxygenase subunit alpha (<i>Meiothermus luteus</i>) (85.14)	Naphthalene dioxygenase large subunit; AAD28100 (<i>Rhodococcus</i> sp. strain NCIMB 12038) (50.6)
Theos_2220 (AFV77212)	<i>phbB2</i>	Small subunit of phenylpropionate dioxygenase	Vanillate O-demethylase small subunit	3-Phenylpropionate/cinnamic acid dioxygenase subunit beta (<i>Thermus scotoductus</i>) (80)	Naphthalene dioxygenase small subunit; AAD28101 (<i>Rhodococcus</i> sp. NCIMB 12038) (54.8)
Theos_2221 (AFV77213)		Hypothetical protein	Unknown	Hypothetical protein (<i>Thermus igniterrae</i>) (83.33)	
Theos_2222 (AFV77214)	<i>phbA</i>	2-Polyprenyl-6-methoxyphenol hydroxylase-like oxidoreductase	4-Hydroxybenzoate 3-monoxygenase	4-Hydroxybenzoate 3-monoxygenase (<i>Thermus scotoductus</i>) (74.49)	4-Hydroxybenzoate 3-monoxygenase; P20586 (<i>Pseudomonas aeruginosa</i> PAO1) (4.62)
Cluster III					
Theos_2223 (AFV77215)		Putative nucleotidyltransferase	Unknown	Nucleotidyltransferase domain-containing protein (<i>Meiothermus taiwanensis</i>) (70.97)	
Theos_2225 (AFV77216)		6-Phosphogluconate dehydrogenase-like protein	Unknown	NAD(P)-dependent oxidoreductase (<i>Meiothermus</i> sp.) (73.39)	
Theos_2226 (AFV77217)		Demethylmenaquinone methyltransferase	Unknown	Dimethylmenaquinone methyltransferase (<i>Thermus scotoductus</i>) (69.61)	4-Carboxy-4-hydroxy-2-oxoadipate aldolase; G21QQ8 (<i>Sphingobium</i> sp. SYK-6) (42.50)
Theos_2227 (AFV77218)	<i>ImbE</i>	Putative LmbE-like protein	Unknown	PIG-L family deacetylase (<i>Thermus brockianus</i>) (85.17)	4-Oxalmesaconate hydratase; Q88JX8 (<i>Pseudomonas putida</i> KT2440) (42.86)
Theos_2228 (AFV77219)		Hypothetical protein	Unknown	Hypothetical protein (<i>Thermus igniterrae</i>) (72.43)	
Theos_2229 (AFV77220)	<i>gntR</i>	Transcriptional regulator	Regulator	GntR family transcriptional regulator (<i>Thermus thermophilus</i>) (80)	L-Lactate dehydrogenase operon regulatory protein; P0ACL7 (<i>Escherichia coli</i> K-12) (28.05)
Theos_2230 (AFV77221)	<i>catE</i>	Catechol 2,3-dioxygenase	Catechol 2,3-dioxygenase	Catechol 2,3-dioxygenase (<i>Thermus brockianus</i>) (82.41)	2,3-Dihydroxybiphenyl 1,2-dioxygenase; Q8GR45 (<i>Geobacillus</i> genomospecies 3) (36.91)

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TABLE 1 (Continued)

Locus tag (GenBank accession no.)	Proposed gene name	GenBank annotation	Predicted role in lignoaromatics	Closest homolog (organism) (% identity obtained by BLAST)	Description; UniProtKB/PDB accession no. (organism) (% identity with characterized proteins, obtained by BLAST) ^b
Theos_2231 (AFV77222)	<i>phbV</i>	NAD-dependent aldehyde dehydrogenase	Vanillin dehydrogenase	Aldehyde dehydrogenase (<i>Thermus brockianus</i>) (82.14)	4-Hydroxybenzaldehyde dehydrogenase; Q59702 (<i>Pseudomonas putida</i> NCIMB 9866) (38.75)
Locus 2 (chromosome) Theos_1789 (AFV76808)	<i>phbT</i>	Acyl-CoA synthetase (AMP forming)/AMP-acid ligase II	Feruloyl-CoA synthetase	Long-chain fatty acid-CoA ligase (<i>Thermus amyloliquefaciens</i>) (86.29)	Long-chain fatty acid-CoA ligase; P69451 (<i>Escherichia coli</i> K-12) (44.06)
Theos_1790 (AFV76809)		Hypothetical protein	Unknown	Dodecin domain-containing protein (<i>Thermus brockianus</i>) (88.24)	Dodecin; Q55IE3 (<i>Thermus thermophilus</i> HB8) (83.82)
Theos_1791 (AFV76810)		Hypothetical protein	Unknown	DUF2267 domain-containing protein (<i>Thermus thermophilus</i>) (86.71)	
Theos_1792 (AFV76811)		Hypothetical protein	Unknown	HD domain-containing protein (<i>Thermus igniterrae</i>) (92.78)	
Theos_1793 (AFV76812)	<i>phbU</i>	Enoyl-CoA hydratase/carnithine racemase	Feruloyl-CoA hydratase	Enoyl-CoA hydratase (<i>Thermus tengchongensis</i>) (91.58)	3-Hydroxypropionyl-CoA dehydratase; A4YI89 (<i>Metallosphaera sedula</i> DSM 5348) (50.97)
Locus 3 (chromosome) Theos_0322 (AFV75398)		Adenylosuccinate lyase	3-Carboxymuconate cycloisomerase	Adenylosuccinate lyase (<i>Thermus tengchongensis</i>) (86.24)	3-Carboxy- <i>cis,cis</i> -muconate cycloisomerase; P32427 (<i>Pseudomonas putida</i>) (32.28)
Locus 4 (chromosome) Theos_0385 (AFV75460)		Protocatechuate 3,4-dioxygenase beta subunit	Protocatechuate 3,4-dioxygenase beta subunit	Intradiol ring cleavage dioxygenase (<i>Thermus brockianus</i>) (66.49)	Catechol 1,2-dioxygenase; P95607 (<i>Rhodococcus opacus</i> 1CP) (33.58)
Locus 5 (chromosome) Theos_0969 (AFV76022)		Dihydrodipicolinate synthase	Unknown	2,4-Dihydroxyhept-2-ene-1,7-dioic acid aldolase (<i>Thermus sediminis</i>) (87.67)	

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TABLE 1 (Continued)

Locus tag (GenBank accession no.)	Proposed gene name	GenBank annotation	Predicted role in lignoaromatics	Closest homolog (organism) (% identity obtained by BLAST)	Description; UniProtKB/PDB accession no. (organism) (% identity with characterized proteins, obtained by BLAST) ^b
Theos_0970 (AFV76023)	Bifunctional isomerase/decarboxylase		Unknown	Fumarylacetoacetate hydrolase family protein (<i>Thermus scotoductus</i>) (90.24)	2-Hydroxyhepta-2,4-diene-1,7-dioate isomerase; Q46978 (<i>Escherichia coli</i> ATCC 11105) (47.83)
Theos_0971 (AFV76024)	5-Carboxymethyl-2-hydroxymuconate semialdehyde dehydrogenase		Unknown	5-Carboxymethyl-2-hydroxymuconate semialdehyde dehydrogenase (<i>Thermus</i> sp. strain CCB_US3_UF1) (91.46)	
Theos_0972 (AFV76025)	4-Hydroxyphenylacetate 3-monoxygenase, oxygenase component		Unknown	4-Hydroxyphenylacetate 3-monoxygenase, oxygenase component (<i>Thermus tengchongensis</i>) (93.18)	4-Hydroxyphenylacetate 3-monoxygenase oxygenase component; Q55JP8 (<i>Thermus thermophilus</i> HB8) (90.91)
Theos_0973 (AFV76026)	Conserved protein of DIM6/NTAB family		Unknown	Flavin reductase (<i>Thermus</i> sp. strain 2.9) (81.25)	4-Hydroxyphenylacetate 3-monoxygenase, reductase component; Q55JP7 (<i>Thermus thermophilus</i> HB8) (79.17)
Theos_0974 (AFV76027)	3,4-Dihydroxyphenylacetate 2,3-dioxygenase		Unknown	3,4-Dihydroxyphenylacetate 2,3-dioxygenase (<i>Thermus aquaticus</i>) (84.33)	Manganese-dependent 2,3-dihydroxybiphenyl 1,2-dioxygenase; Q8GR45 (<i>Geobacillus</i> genomospecies 3) (31.33)

^aMFS, major facilitator superfamily; CMD, carboxymuconolactone decarboxylase.^bFields were left blank where no significant similarity was found among characterized proteins upon a BLAST search.

phylogenetically closer to the PHB regulators of *Acinetobacter* and *Pseudomonas* than that of 3-(3-hydroxyphenyl)propionate from *Escherichia coli* (Fig. S1B). This indicates that the ABC transport system is involved in the transport of PHB and similar monoaromatics across the cell membrane, whereas the upper degradation pathway of PHB is regulated by PhbR.

When we searched the microbial genome database, we found that the oxidoreductase gene cluster was conserved among only a few closely related members of the *Thermales* (Fig. S2). Of the three predicted operons (gene clusters), the upper operon (in JL-2, cluster II) was present in all members and thus preserved a core set of genes. The lower operon (cluster I, corresponding to CAT *meta*-cleavage) was present in most members, with the exception of *Meiothermus* strains. *Meiothermus ruber* DSM 1279 instead contained genes corresponding to the CAT *ortho*-cleavage pathway. It is interesting that almost all organisms were isolated from hot springs located in geographically distant locations, except *Thermus thermophilus* JL-18, which has the same isolation source as strain JL-2 (Table S1). A search for homologs of individual genes present in cluster I revealed similarity with those present in taxonomically diverse bacterial taxa, including *Firmicutes*, *Proteobacteria*, *Actinobacteria*, and *Thermaceae* (Fig. S3). This suggests the possible acquisition of individual genes or gene clusters by strain JL-2 and related members of the *Thermales* from taxonomically diverse classes of bacteria. Among biochemically studied ROs outside the family *Thermaceae*, the RO genes PhbB1 and PhbB2 showed the closest homology (50.6% and 54.8%, respectively) to the well-studied naphthalene dioxygenase subunits from *Rhodococcus* sp. strain NCIMB 12038 (46). A BLAST search further resulted in hits from several corynebacteria, mostly among the genera *Rhodococcus* and *Mycobacterium*. The hypothetical gene (Theos_2221) adjacent to *phbB1B2* was also present in most of the genomes. This indicates that these three genes may have been acquired from corynebacteria. The unusual occurrence of RO genes within such a cluster in the *Thermales* as well as the low identity to biochemically studied homologs (naphthalene dioxygenase) further add to the uniqueness of the cluster.

Growth on lignoaromatics and their biotransformation. Attempts to formulate a suitable carbon-free growth medium for *T. oshimai* JL-2 for analyzing growth specificity on various lignoaromatics as a sole source of carbon and energy failed. As a result, growth was monitored in modified Castenholz medium (MCM) supplemented with PHB, VAN, COU, FER, and *p*-anisic acid (ANI) as individual substrates. Growth was monitored at different substrate concentrations, and 0.6 g/liter was chosen as the optimum concentration to compare growth in all the substrates as most of the substrates were found to be detrimental for the cells at higher concentrations (Fig. S4). Continuous monitoring of growth for 2 days showed that although growth was delayed in all cultures with supplementation of lignoaromatics compared to the control culture (without an additional substrate), enhanced growth was observed only in the presence of PHB (Fig. 2A). On the other hand, quantitative analyses of independent cultures showed that PHB was utilized completely by strain JL-2 within 4 days of incubation (Fig. 2B). However, no metabolite was identified in the culture extracts (data not shown). Although not very clear from these data, COU and FER also seem to be depleted to some extent compared to their respective abiotic controls (Fig. 2). Although ANI does not belong to lignin-derived aromatics, it was selected as one of the substrates to assess if the enzymes bringing about the transformation of VAN can act upon ANI as well, since both are structural analogs.

Elucidation of downstream pathways. Although PCA could not be detected in the extracts of PHB-grown cultures, it was expected to be the most probable transformation product of PHB owing to the presence of a possible 4-hydroxybenzoate 3-monooxygenase gene (*phbA*) in the oxidoreductase cluster. This left three possible steps for the further transformation of PCA: *ortho*-cleavage by protocatechuate 3,4-dioxygenase, *meta*-cleavage by a catechol 2,3-dioxygenase homolog (PhbE), or decarboxylation to yield CAT (Fig. 1B). As described above, *ortho*-cleavage of PCA seemed unlikely because of the absence of a protocatechuate dioxygenase α -subunit in the

FIG 2 (A) Growth of strain JL-2 on MCM medium in the absence (black dotted line) or presence of the additional substrates *p*-hydroxybenzoate (red), vanillate (blue), *p*-coumarate (purple), ferulate (orange), and *p*-anisic acid (green). The plot shows averages from two independent growth experiments. (B to F) Utilization of *p*-hydroxybenzoate (B), vanillate (C), *p*-coumarate (D), ferulate (E), and *p*-anisic acid (F) by strain JL-2. Black and gray bars correspond to the concentrations of each substrate in JL-2 cultures and abiotic controls (without cells), respectively. Vertical bars indicate means $\pm$ standard deviations.

organism. To assess the remaining two possibilities, we monitored spectrophotometric changes upon the incubation of the cell-free extract (CFE) (with a total protein content of 0.05 mg/ml) of JL-2 cells, grown in the presence of various lignoaromatics, with CAT. As shown in Fig. 3A and C to F, the majority of cultures, including those grown only on MCM, showed negligible catechol 2,3-dioxygenase activity, which was assessed by the formation of a yellow metabolite, 2-hydroxymuconaldehyde ($\lambda_{\text{max}} = 374$ nm), from CAT ($\lambda_{\text{max}} = 275$ nm). The activity, however, was markedly high for PHB-exposed cells (Fig. 3B). It is interesting that an identical spectral shift (corresponding to the formation of 2-hydroxymuconaldehyde from CAT) was observed even when the CFE was incubated with PCA (Fig. 3G to L). If *ortho*-cleavage of PCA had occurred, the spectra would have shown a decrease in the absorbance at 290 nm with an increase at 275 nm, indicating the disappearance of PCA and the formation of 3-carboxy-*cis,cis*-muconate, respectively (47, 48). Similarly, if there was *meta*-cleavage of PCA, an increase in the absorbance at 410 nm would have been noticed, indicating the formation of α -hydroxy-

FIG 3 UV-visible spectra of CFEs prepared from JL-2 cells exposed to various lignoaromatics upon incubation with catechol (A to F) or protocatechuate (G to L). The *meta*-cleavage reaction for catechol (A to F) and protocatechuate conversion to catechol followed by *meta*-cleavage (G to L) are also shown. JL-2 cells were exposed to the following lignoaromatics: *p*-hydroxybenzoate (B and H), vanillate (C and I), *p*-coumarate (D and J), ferulate (E and K), and *p*-anisic acid (F and L). Cells grown without any lignoaromatic supplement were used as controls (A and G). For each reaction, spectra were recorded at 0, 2, 4, 6, and 8 min. Chemical structures of the lignoaromatic compounds are shown in each panel. Arrows show increases in the absorbance at 374 nm, indicating the formation of 2-hydroxymuconaldehyde.

γ -carboxy- ϵ -semialdehyde (49). It is worth mentioning here that because we were unable to find any literature showing spectral changes upon *meta*-cleavage of catechol at 70°C, we performed a spectral assay for catechol 2,3-dioxygenase using the CFE obtained from *E. coli* BL21(DE3)(pUCA503) harboring the *meta*-cleavage enzyme for 2'-aminobiphenyl-2,3-diol from *Pseudomonas resinovorans* CA10, involved in carbazole degradation (50). The purified enzyme was reported to have *meta*-cleavage activity toward catechol. We subjected the CFE (corresponding to 0.2 mg protein) obtained from the recombinant *E. coli* cells to a spectral assay upon incubation with catechol, initially at 25°C, and spectra were recorded immediately after the addition of the substrate and subsequently at 1, 3, 5, and 10 min. The reaction temperature was then increased to 70°C, and further readings were taken at 15, 20, and 25 min. The peak at 374 nm kept increasing until 10 min, when the reaction was carried out at 25°C, indicating the formation of 2-hydroxy-*cis,cis*-muconate semialdehyde (Fig. S5A). When the temperature was increased to 70°C, no further transformation was observed, as the enzyme is reported to be completely inactive at 70°C (50). But interestingly, the peak at 374 nm was completely retained up to 25 min of incubation, indicating that the compound is stable at 70°C and shows similar spectra (Fig. S5B). This clearly indicates that the *meta*-cleavage enzymes for CAT were induced by PHB and that the pathway proceeded toward CAT through an initial decarboxylation of PCA, followed by ring cleavage by catechol 2,3-dioxygenase. A slight increase in the absorbance at 374 nm was observed in most of the cultures after the addition of PCA, including the uninduced culture (Fig. 3G), which indicates basal expression of the genes, although the activity was clearly higher in cells grown in the presence of VAN (Fig. 3I).

It is worth noting that PHB-grown cells showed such high catechol 2,3-dioxygenase activity only when grown initially in the presence of PHB for a significant period of time (e.g., at least four or five subcultures over a period of 1 month). The strain, at this point, was referred to as “PHB adapted.” Once adapted, the subsequent subcultures show similar activities even when grown overnight. The activity was much weaker when cells were transferred to PHB-containing medium for the first time (Fig. S6). However, no change in activity was observed when cells were grown in other substrates for the same period of time (data not shown). All the experiments were thus performed using the PHB-adapted strain. The generation of substrate-adapted strains was reported previously among phenol degraders, and mutations in the promoter region of degradative operons were responsible for this enhanced metabolic activity (51–53). In strain JL-2, three putative promoters were predicted around *phbR*: two (promoter 1 [P1] and P3) were upstream of the regulator gene, and one (P2) was at the 3' end (Fig. S7). The upstream region (365 bp) encompassing P1 and P3 and a 159-bp region at the 3' end (across P2) were amplified with two primer pairs (Table 2) and sequenced from both wild-type and PHB-adapted strains. However, no mutation was observed in this region (data not shown).

Regulation of expression. To understand the transcriptional control of the oxidoreductase cluster of strain JL-2 by PHB, we performed reverse transcription-PCR (RT-PCR) to amplify various genes using cDNA obtained from uninduced and PHB-induced cells grown for 8 h. As shown in Fig. 4, amplification was observed for all genes, and mRNA levels were significantly higher in the upper and lower operons when cells were induced in the presence of PHB. In the remaining putative operon (cluster III), relative induction ratios were almost negligible. Among the peripheral genes, only *phbV*, present in the close vicinity of the oxidoreductase cluster (in plasmid pTHEOS01), showed transcription; the other two genes (*phbT* and *phbU* in locus 2 on the chromosome) showed slightly enhanced expression in induced cells, which might be the result of either mild induction or constitutive expression.

Biotransformation of lignoaromatics by induced cells. To determine whether, upon induction, enzymes encoded in the oxidoreductase cluster can transform other lignoaromatics as well, we monitored the transformation of VAN, COU, FER, and ANI by resting-cell cultures of PHB-induced cells after 5 days of incubation. As expected, high-performance liquid chromatography (HPLC) showed the disappearance of PHB after 5 days of incubation (Fig. 5A). It is interesting that the concentrations of all substrates except ANI decreased, even in uninduced cultures (Fig. 5). This is consistent with spectrophotometric analyses in which basal expression of the genes was observed in uninduced cultures (Fig. 3G). The depletion of VAN in induced cultures suggests *O*-demethylation (Fig. 5B). However, PCA could not be detected in any of the culture extracts, most likely because of the high activity of downstream enzymes. In addition, FER was readily utilized by both induced and uninduced cultures, which further confirms the constitutive nature of the genes involved (Fig. 5D). The corresponding chromatograms are shown in Fig. S8. A peak corresponding to VAN was detected at 10 min upon incubation with FER for 5 days (Fig. S8D). The presence of VAN as an intermediate was further confirmed when a resting-cell culture of PHB-induced cells with a higher cell density was incubated for 3 days in the presence of FER (Fig. 6). This further confirms the transformation of FER by induced cells of JL-2, as had previously been observed (Fig. 2).

Thermostability assay. Because the transformation of both PCA and CAT could be detected spectrophotometrically in terms of the formation of 2-hydroxymuconaldehyde, the CFE of strain JL-2 was subjected to different temperatures to check the stability of the respective enzymes involved. Figure 7 shows the relative catechol 2,3-dioxygenase activity observed upon the incubation of the heat-treated CFE with PCA. The activity remained fairly high until 70°C, which is the optimum growth temperature of the strain (38), with the highest activity at around 60°C to 70°C. Although substantial activity (90%) remained after a 15-min incubation at 80°C, the CFE showed approximately 38% activity even at 90°C. Considering the fact that the thermostability assay

TABLE 2 Primers used in this study

Purpose and region or gene amplified	Primer name	Sequence (5'–3')
Amplification of putative promoter regions		
Regions P1, P3	Pdn_f	GCTTAGGGTGGCCAGGAG
	Pdn_r	GCCGTCTTCACGTCAAAG
Region P2	Pup_f	GAATGTAGGCGATCCCCTC
	Pup_r	CTTAAGCCTCTCCGTTCCC
RT-PCR		
<i>phbG</i>	phbg_RTf	CAACCTCATCTTCGCCAG
	phbg_RTr	CGTGGGCTCTAGGAAGTAGC
<i>phbR</i>	phbr_RTf	TGGTCTACGTGGAGAAGCTG
	phbr_RTr	AAGGGAACGAGAGGCTTAAG
<i>phbE</i>	phbe_RTf	GCTTCTCCAGGAGGTCC
	phbe_RTr	AACCAGATGGTGCTTCTC
<i>phbB1</i>	phbb1_RTf	AACCTCTCCATCCACAACCC
	phbb1_RTr	TAACGCCGGTGAAGTTG
<i>phbA</i>	phba_RTf	CATCTCCTCCACCGCATC
	phba_RTr	CGGCAAACCTCCGTCTGC
<i>lmbE</i>	lmbe_RTf	TTTCTGGCCCTGGACCTAG
	lmbe_RTr	CTTAAGCTTCTTCTCCACACC
<i>gntR</i>	gntr_RTf	TGGTGGAGAAGGACCTAAGC
	gntr_RTr	TCTTATGGCCGAAAGCTC
<i>catE</i>	xyle_RTf	AACCTAAGGCGGCTGGAC
	xyle_RTr	CAGGCGATCTCAAACCG
<i>phbV</i>	phbv_RTf	AGATCTGCATGTCCACGG
	phbv_RTr	GAAAGGCCGTACTCCGTG
<i>phbT</i>	phbt_RTf	GTGGCCTTCAACAACCTCC
	phbt_RTr	TTCCAGTAGCCTTTCATGACG
<i>phbU</i>	phbu_RTf	ATCTCCGCGAAGACCTGC
	phbu_RTr	TGGAGATTCCCGAGTTTGAG
Construction of <i>phbC</i> deletion mutant		
<i>phbC</i> upstream region	PHBC-UPf	CCGCTCGAGAGCTTGTACATGAGGTCCGTG
	PHBC-UPr	TACCATATCCGCCGTCAACGGCTGACCTCCTAAGCGGAC
<i>htk</i> cassette from pSK-HTK	PHBC-HTKf	GGTCCGCTTAGGAGGTGACGCCGTTGACGGCGGATATG
	PHBC-HTKr	ACCACATCAAACCCTTCCATCGTAACCAACATGATTAACAATTATTAG
<i>phbC</i> downstream region	PHBC-DNf	TGTTAATCATGTTGTTACGATGGAAGGGTTTGATGTGGTC
	PHBC-DNr	CCGCTCGAGCCTTGGCCGAGCTGCTTTAG

was performed with a CFE and not with purified enzymes, there is a strong indication that both enzymes (PCA decarboxylase and catechol 2,3-dioxygenase) should be active at temperatures as high as 90°C.

A novel PCA decarboxylase. From spectrophotometric analyses, it was inferred that the degradation pathway of PHB in strain JL-2 proceeds via the decarboxylation of PCA and not via the commonly observed ring cleavage of β -ketoadipate (Fig. 3). Although the decarboxylation of PCA was first reported as early as 1962 (54), few reports have described the occurrence of such a reaction step during the metabolism of aromatic compounds (55). Among the most studied PCA decarboxylases (EC 4.1.1.63) are AroY (GenBank accession no. [AB479384](#)) from *Klebsiella pneumoniae* subsp. *pneumoniae* ATCC 25597 (56), 4-hydroxybenzoate decarboxylase subunit C (GenBank accession no. [AF128880](#)) from *Sedimentibacter hydroxybenzoicus* JW/Z-1 (57), and PCA decarboxylase (GenBank accession no. [BAG24502](#)) from *Enterobacter cloacae* P241 (58), all of which belong to the ubiquitous UbiD family. Members of this family are known to catalyze the reversible decarboxylation of hydroxyarylic acids and are involved in the ubiquinone biosynthetic pathway (59). PCA decarboxylases work in association with a UbiX family flavoprotein. A search of the genome of strain JL-2 revealed a homolog of the UbiD family (annotated as menaquinone biosynthesis decarboxylase [GenBank accession no. [AFV75499](#)]) in the chromosome, with the closest homology (29.8% identity) to 4-hydroxybenzoate decarboxylase from *S. hydroxybenzoicus* JW/Z-1. A

FIG 4 Expression of lignoaromatic-degradative genes within the oxidoreductase cluster of strain JL-2. (A) Relative expression normalized to 16S rRNA in *p*-hydroxybenzoate (PHB)-induced (gray bars) and uninduced (black bars) cells. (B) Induction of lignoaromatic-degradative genes by PHB exposure (in PHB-induced cells). Vertical bars indicate means $\pm$ standard deviations. (C) Expression was quantified by semiquantitative RT-PCR analyses upon agarose gel electrophoresis. Genes are named as in Table 1.

flavoprotein homolog (43.4% identity; GenBank accession no. [AFV76951](#)) was also identified but in a different locus. The involvement of these UbiD/UbiX family proteins could not be predicted based on their respective loci and vicinal genes.

However, the only potential decarboxylase, *phbC*, present in the upper operon of PHB degradation in strain JL-2 (Table 1) was a homolog of γ -carboxymuconolactone decarboxylase. Owing to its presence in the cluster and its induction by PHB (Fig. 4), we attempted to ascertain its role in PHB degradation. The gene was replaced with the highly thermostable kanamycin nucleotidyltransferase gene (*htk*) (60) by homologous recombination, which resulted in the knockout mutant strain *T. oshimai* JL-2 Δ *phbC*::*htk*. Spectrophotometric analyses of the CFE (corresponding to 0.2 mg protein) obtained from PHB-induced JL-2 Δ *phbC*::*htk* cells revealed an increase in the absorbance at 374 nm upon incubation with CAT, which indicates the formation of 2-hydroxy-*cis,cis*-muconate semialdehyde (Fig. 8A), although the activity was much lower than that of the wild-type strain (Fig. 3B). However, when incubated with PCA, the spectral change detected after the addition of PCA (Fig. 8B) did not correspond to the formation of 2-hydroxy-*cis,cis*-muconate semialdehyde, as was observed with wild-type strain JL-2 (Fig. 3H). These results indicate that the mutant failed to produce CAT from PCA, which would have subsequently been transformed to 2-hydroxy-*cis,cis*-muconate semialdehyde by the catechol 2,3-dioxygenase. A small change was observed at approximately 350 nm, which is not characteristic of catechol 2,3-dioxygenase activity and might have resulted from the nonspecific transformation of PCA by another enzyme(s) present in the CFE. This confirms that *phbC*, annotated as γ -carboxymuconolactone decarboxylase, encodes a PCA decarboxylase in strain JL-2. Compared to the wild-type strain, the

FIG 5 Resting-cell transformation of *p*-hydroxybenzoate (PHB) (A), vanillate (VAN) (B), *p*-coumarate (COU) (C), ferulate (FER) (D), and *p*-anisic acid (ANI) (E) by PHB-grown cells of *T. oshimai* strain JL-2. Lignoaromatics incubated without cells are labeled “Abiotic,” while “Induced” and “Uninduced” correspond to resting cells prepared from cultures induced with or without *p*-hydroxybenzoate, respectively. Black and gray bars correspond to the concentrations of each substrate at day 0 and day 5, respectively. Vertical bars indicate means $\pm$ standard deviations.

mutant had a very low rate of utilization of PHB (Fig. 8C), which was almost negligible compared to that of the wild-type strain (Fig. S8A). This might have led to decreased growth in the presence of the PHB due to substrate toxicity (Fig. 8D).

DISCUSSION

Exploring the genome sequence of *Thermus oshimai* JL-2, followed by biochemical and molecular analyses, suggests that the oxidoreductase cluster present in the plasmid pTHEOS01 of the strain is responsible for the degradation of lignoaromatics such as PHB and VAN and involves an upper and a lower operon. Whereas the lower operon corresponds to a *meta*-cleavage pathway of CAT, the upper operon comprises a unique combination of genes that are conserved only among a few closely related members of the *Thermales*. Nevertheless, both operons are induced by PHB. *T. oshimai* JL-2 can efficiently degrade 0.6 g/liter PHB within 4 days of incubation, and the degradation follows the less common pathway of the decarboxylation of PCA to form CAT.

Resting-cell analyses also showed that apart from PHB, induced cells of strain JL-2 could also use VAN. It is interesting that the upper operon of PHB degradation in the strain possesses genes encoding the large (α) (PhbB1) and small (β) (PhbB2) subunits of the oxygenase component of RO. ROs with vanillate *O*-demethylase activity are well documented (61–64). PhbB1 in strain JL-2 is phylogenetically closer to biochemically characterized heterohexameric ($\alpha_3\beta_3$) oxygenase components of polyaromatic hydrocarbon dioxygenases than to homotrimeric (α_3) oxygenase components of RO, including vanillate *O*-demethylases (see Fig. S9 in the supplemental material). Phylogenetically distant ROs catalyze similar reactions, such as angular dioxygenation of carbazole or monooxygenation of aryl groups by both α - and $\alpha\beta$ -type ROs (Fig. S9) (65, 66). Thus, because no other gene showing significant similarity to the known enzyme(s) responsible for the transformation of VAN was found in the genome of strain JL-2, the possibility that PhbB1B2 acts as vanillate *O*-demethylase, channeling the peripheral degradation pathway of FER toward PCA, cannot be ruled out. Two of the genes (*phbT* and *phbU*) required for FER degradation, in contrast, are constitutively expressed from the chromosome. Given these observations, the most probable lignoaromatic degradation pathway in strain JL-2 has been derived, as shown in Fig. 9.

FIG 6 HPLC chromatograms showing the presence of vanillate as an intermediate upon incubation of a resting-cell culture of strain JL-2 with ferulate for 3 days. Culture extracts were obtained from the abiotic control without cells (A), uninduced cells incubated with ferulate (FER) (B), and *p*-hydroxybenzoate-induced cells incubated with ferulate (C). The inset shows UV-visible spectra of the peak obtained at 10 min, identified as vanillate (VAN) after comparison with an authentic compound. mAU, milli-absorbance units.

The unique combination of genes in the cluster, as well as the homology of individual (or groups of) genes to taxonomically diverse classes of bacteria, points toward an interesting evolutionary aspect of this catabolic pathway. The *phb* gene cluster is present in the chromosome of all the closely related members of the *Thermales* (listed in

FIG 7 Relative catechol 2,3-dioxygenase activity measured at 70°C, upon incubation with protocatechuate, shown by the cell extract obtained from wild-type strain JL-2 subjected to room temperature (RT) or higher temperatures. The specific activity of catechol 2,3-dioxygenase at 70°C was measured to be 0.21 $\mu\text{mol}/\text{min}/\text{mg}$. Vertical bars indicate means $\pm$ standard deviations.

FIG 8 (A and B) Change in UV-visible spectra recorded upon the transformation of catechol (A) and protocatechuate (B) by the CFE of *T. oshimai* JL-2 $\Delta phbC::htk$. For each reaction, spectra were recorded at 0, 2, 5, 10, and 15 min. The rectangles within the spectra show the areas magnified in the insets. The dotted line shows the position of λ_{max} at 374 nm, corresponding to the desired product, 2-hydroxy-*cis,cis*-muconate semialdehyde. (C) HPLC chromatograms showing the utilization of *p*-hydroxybenzoate (PHB) by the mutant strain *T. oshimai* JL-2 $\Delta phbC::htk$. IS, internal standard. (D) Growth of the mutant in the presence (blue solid line) and absence (blue dotted line) of *p*-hydroxybenzoate, compared to that of the wild-type strain grown in the presence (red solid line) and absence (red dotted line) of *p*-hydroxybenzoate.

Table S1) except *Thermus oshimai* JL-2 and *Thermus thermophilus* JL-18, where it is present in a plasmid. Although the strains have been isolated from geographically different locations, they all have a similar landscape: hot springs in direct contact with vegetation. High-temperature steam treatment facilitates the release of polymer fiber from lignocellulosic biomass (67). The high temperature of these hot springs enhances lignin depolymerization, releasing a wide array of lignoaromatics in the ecosystem. This acts as a selection pressure in the microbiota that drives the vertical expansion of the catabolic potential of these organisms toward such compounds. In such phenomena, new peripheral routes are acquired that channel substrates into existing degradative pathways and often include the recruitment of entire operons or genes encoding *iso*-functional enzymes (68).

PCA decarboxylase is an important enzyme in the biosynthesis of *cis,cis*-muconate, which is industrially important and the subject of metabolic pathway engineering (55, 69, 70). It is worth noting that no thermostable PCA decarboxylase has been reported so far, and thus, the identification of such an enzyme in strain JL-2 may have important industrial implications. The present study reveals possibilities for further analyses of the individual genes, in particular those encoding RO subunits and the PCA decarboxylase, by heterologous expression followed by characterization of the enzymes in terms of substrate preferences and thermostability. In addition, this study demonstrates another example of extracting useful information from the large pool of genetic information stored in public repositories.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 3. Unless otherwise stated, bacteria were grown aerobically in MCM (composition provided in Table S2 in the supplemental material) (71) at 70°C (*T. oshimai* JL-2) or Luria-Bertani (LB) medium (72) at 37°C (*E. coli* DH5 α) with shaking (200 rpm). In order to understand the optimum substrate concentration, growth was measured at increasing substrate concentrations, *viz.*, 0.4, 0.6, 0.8, 1.0, 1.2, and 1.5 g/liter. An OD-Monitor C&T noncontact turbidimeter (Taitec, Japan) equipped with a shaker

FIG 9 Proposed pathway for the transformation of lignoaromatic compounds by *T. oshimai* JL-2. The pathways and corresponding gene clusters are highlighted in similar colors. Genes without any color fill within a cluster are those whose functions could not be predicted or correlated with the possible pathways. Dotted arrows indicate multiple steps.

incubator was used for continuous growth monitoring in the presence of individual substrates, while for biotransformation studies, different sets of cultures were grown on MCM supplemented with 0.6 g/liter of the appropriate aromatic hydrocarbon. Individual aromatic hydrocarbon stock solutions were made in dimethyl sulfoxide (DMSO). Kanamycin (50 μ g/ml) was added as a selection marker when required.

Resting-cell transformation. *T. oshimai* JL-2 cells were grown in MCM supplemented with PHB (0.6 g/liter) for ~24 h. The cells were harvested by centrifugation (8,000 $\times g$ for 5 min), washed twice with carbon-free mineral medium (73), and resuspended in the same medium at an optical density at 600 nm (OD_{600}) of approximately 6 to 7. They were then incubated for 5 days in the presence of the following substrates (0.6 g/liter each): PHB, VAN, COU, FER, and ANI. The cultures were acidified with 6 N HCl to pH 1 to 2 and extracted twice with equal volumes of ethyl acetate following the addition of phenanthrene (PHEN) (0.3 g/liter) as an internal standard. The organic extracts were dried with a vacuum centrifuge evaporator and redissolved in 4 ml methanol, followed by filtration. A total of 1.5 ml of each sample was transferred to HPLC vials, and 5 μ l of the sample was injected into a Hitachi Elite LaChrom L2455 HPLC system (Hitachi, Tokyo, Japan) equipped with a diode array detector, an autosampler injector, a thermostat column compartment, and a Pegasil-B ODS (octadecyl silica) analytical column (4.6 by 250 mm; Senshu Scientific, Tokyo, Japan). A gradient elution with 2% acetic acid and methanol, as the mobile phase, was used as follows: an increase of the methanol volume from 15% to 40% until 22.5 min, an increase to 100% until 25 min, a hold at 100% until 35 min, and then a gradual decrease to 15% until 45 min, continuing up to 60 min.

Spectrophotometric analyses. *T. oshimai* JL-2 cells were grown on 5 ml MCM medium for ~18 h with or without (control) PHB (0.6 g/liter), washed with 50 mM sodium phosphate buffer (pH 7.5), and resuspended in 0.4 ml of the same buffer. The cells were lysed by ultrasonication with a Branson Sonifier 250 ultrasonic homogenizer (Branson Ultrasonics, Emerson, Japan) at a 50% pulse rate for 2 min and centrifuged for 15 min at 15,000 $\times g$. The supernatant was used as the CFE for spectral analyses. The protein concentration was measured using the Bradford method (74). We performed the enzymatic transformation of CAT and PCA by recording CFE-catalyzed changes in UV-visible spectra with a UV-2600 spectrophotometer (Shimadzu, Japan) equipped with a CPS-240A temperature controller (Shimadzu) using 1-cm-path-length quartz cuvettes. Spectra were recorded while the reaction mixture was incubated at 70°C unless stated otherwise. The CFE containing 0.05 mg protein was added to the buffer. The enzymatic reaction was initiated by adding the substrate (0.003% [wt/vol]) each of PCA or CAT to the reaction buffer. Spectra were recorded immediately after the addition of the substrate and subsequently at 2, 4, 6, and 8 min. To measure thermostability, the CFE corresponding to 0.05 mg protein was subjected to either room temperature (25°C) or heat treatment at 60°C, 70°C, 80°C, and 90°C for 15 min, followed by incubation on ice for another 15 min. The CFE was centrifuged, and the supernatant was incubated with PCA (0.003% [wt/vol]). Spectra were recorded as described above. For comparison of enzyme activities, 1 U of enzyme activity was defined as the amount of enzyme required to generate 1 μ mol of product per min.

TABLE 3 Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype and/or description	Reference(s) and/or source
Strains		
<i>T. oshimai</i> JL-2	Aerobic thermophile isolated as a nitrate reducer from the U.S. Great Basin hot springs	37, 38
<i>T. oshimai</i> JL-2 $\Delta phbC::htk$	<i>phbC</i> deletion mutant of <i>T. oshimai</i> JL-2	This study
<i>E. coli</i> DH5 α	F ⁻ $\Delta lacU169 \phi 80d lacZ \Delta M15 hsdR17 recA1 endA1 gyrA96 thi-1 relA1 supE44$	84
<i>E. coli</i> JM109	F ⁻ <i>traD36 proA⁺ proB⁺ lac^q lacZΔM15 recA1 endA1 gyrA96 (Nal^r) thi hsdR17 supE44 relA1 Δ(lac-proAB)</i>	85
<i>E. coli</i> DB3.1	<i>gyrA462 endA1 Δ(sr1-recA) mcrB mrr-hsdS20 glnV44 (supE44) ara14 galK2 lacY1 proA2 rpsL20 xyl5 leuB6 mtl1</i> ; HB101 derivative containing the <i>gyrA462</i> allele, which renders the strain resistant to the toxic effects of the <i>ccdB</i> gene	86, Invitrogen
Plasmids		
pSK-HTK	pBluescript KS(+) containing the <i>htk</i> gene as an insert	60
pZErO-2.1	3.3-kb vector for high-efficiency cloning of DNA inserts with sticky or blunt ends; contains kanamycin as a selection marker; a toxic <i>lacZα-ccdB</i> gene selects against clones lacking an insert	Invitrogen
pZHTKDC	pZErO-2.1 containing <i>htk</i> flanked by upstream and downstream sequences of <i>phbC</i> from <i>T. oshimai</i> JL-2	This study

RNA extraction and RT-PCR analyses. RNA extraction from JL-2 cells growing in the absence (uninduced) or presence (induced) of PHB was performed as described previously (75). Briefly, cells grown for 8 h were treated with RNAprotect bacterial reagent (Qiagen, Valencia, CA, USA) to stabilize the RNA before extraction. Cells were lysed by treatment with lysozyme followed by achromopeptidase. Total RNA from cells was extracted with one round of purification with the silica membrane spin columns available in the RNeasy bacterial minikit (Qiagen) according to the manufacturer's instructions, followed by treatment with RQ RNase-free DNase (Promega, Madison, WI, USA) and another round of RNA purification with the RNeasy minikit.

Reverse transcription was performed in 12- μ l reaction mixtures containing 1 μ g purified total RNA, 75 ng random primers (nonadeoxyribonucleotide mix; TaKaRa Bio Inc., Japan), 200 U SuperScript III (Invitrogen), 40 U RNase Out (Invitrogen), 1 $\times$ first-strand buffer (Invitrogen), 0.1 M dithiothreitol, and 10 mM deoxynucleotide triphosphates (Toyobo, Osaka, Japan). Following the denaturation of the RNA and random primers at 65°C for 5 min, the remaining reagents were added, and the mixture was incubated at 25°C for 10 min, followed by 50°C for 50 min, and then held at 85°C for 5 min to inactivate the enzymes. To degrade the RNA, we added 6.67 μ l 1 N NaOH, and the reaction mixture was heated to 65°C for 30 min. The mixture was neutralized with 6.67 μ l 1 N HCl. PCR was performed for 27 cycles with KOD-plus-Neo DNA polymerase (Toyobo) in a 20- μ l mixture containing 2 μ l of a diluted cDNA solution and 0.4 μ M primer set under the following conditions: denaturation at 98°C for 10 s, annealing at 55°C for 15 s, and extension at 68°C for 15 s. The PCR products were analyzed by 1.5% agarose gel electrophoresis, followed by quantification of the band intensities with ImageJ (76). The primer sequences for RT-PCR are shown in Table 2.

Construction of the *phbC* deletion mutant. The nucleotide sequences of oligonucleotides used in this study are shown in Table 2. The plasmid for *phbC* knockout was constructed as follows. Two independent PCRs using the primer pairs PHBC-UPf/PHBC-UPr and PHBC-DNf/PHBC-DNr to amplify ~1-kb regions upstream and downstream of the *phbC* gene, respectively, were performed with the chromosomal DNA of *T. oshimai* JL-2 as the template. An additional PCR to amplify the 1.1-kb *htk* gene was performed using PHBC-HTKf and PHBC-HTKr as primers and pSK-HTK as the template (60). The purified amplicons were used as the templates to perform overlap extension PCR using the primer pair PHBC-UPf/PHBC-DNr, and the resultant product was cloned into pZErO-2.1 to yield pZHTKDC. The transformation of *T. oshimai* JL-2 with pZHTKDC was performed by natural competency, as described previously by Hoseki et al. (60), with minor modifications. Briefly, JL-2 cells were grown overnight and diluted to 1:60 with fresh MCM supplemented with 0.4 mM CaCl₂ and 0.4 mM MgCl₂. The culture was grown until the OD₆₀₀ reached approximately 0.5, after which a 10- μ l plasmid solution (approximately 2 μ g plasmid DNA) was added to a 500- μ l culture, and the culture was incubated for 1 h at 70°C without shaking, followed by growth overnight at 70°C with shaking. The selection of double-crossover mutants was done by plating the cells on MCM agar (3% [wt/vol]) supplemented with 50 μ g/ml kanamycin and incubating the cells at 65°C until transformants appeared as visible colonies. Confirmation of gene disruption in the knockout mutant *T. oshimai* JL-2 $\Delta phbC::htk$ was verified by PCR and restriction enzyme digestion (Fig. S10).

Computational analyses. For pathway mapping, *in silico*-translated sequences from open reading frames present in the plasmid pTHEOS01 were functionally categorized as enzymes catalyzing bio-transformation steps, as proteins with other functions (e.g., regulator or transporter), or as proteins with unknown functions based on sequence homology, CD analyses, and prediction of their involvement in metabolic pathways with the KEGG pathway database (77) and MetaCyc (78). Promoter regions were predicted with BPROM (79). Protein similarity matrices were constructed with MatGAT (80) with Blosom 62 as the scoring matrix. Multiple-sequence alignments were obtained with ClustalX (81), with default parameters. Phylogenetic trees were constructed by the neighbor-joining (NJ)

method (82) from distance data with the NJ algorithm in ClustalX. The trees were visualized and manipulated with TreeExplorer v2.12 (83), and the layout was modified with a standard graphics program.

Data availability. The genome sequence of *Thermus oshimai* JL-2 is available in the NCBI BioProject database under accession no. [PRJNA63181](https://www.ncbi.nlm.nih.gov/bioproject/PRJNA63181).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 1.4 MB.

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