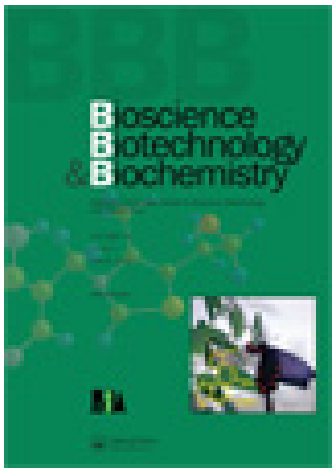

Bioscience, Biotechnology, and Biochemistry
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/tbbb20

In vitro reconstitution of the catabolic reactions catalyzed by PcaHG, PcaB, and PcaL: the protocatechuate branch of the β-ketoadipate pathway in Rhodococcus jostii RHA1

Tomoya Yamanashi[a], Seung-Young Kim[a], Hirofumi Hara[b] & Nobutaka Funa[a]
[a] Graduate Division of Nutritional and Environmental Sciences, University of Shizuoka, Shizuoka, Japan
[b] Department of Environmental Engineering and Green Technology, Malaysia-Japan International Institute of Technology, Universiti Teknologi Malaysia, Kuala Lumpur, Malaysia
Published online: 03 Jan 2015.

To cite this article: Tomoya Yamanashi, Seung-Young Kim, Hirofumi Hara & Nobutaka Funa (2015): In vitro reconstitution of the catabolic reactions catalyzed by PcaHG, PcaB, and PcaL: the protocatechuate branch of the β-ketoadipate pathway in Rhodococcus jostii RHA1, Bioscience, Biotechnology, and Biochemistry, DOI: 10.1080/09168451.2014.993915

To link to this article: http://dx.doi.org/10.1080/09168451.2014.993915

Bioscience, Biotechnology, and Biochemistry, 2014

In vitro reconstitution of the catabolic reactions catalyzed by PcaHG, PcaB, and PcaL: the protocatechuate branch of the β-ketoadipate pathway in *Rhodococcus jostii* RHA1

Tomoya Yamanashi[1], Seung-Young Kim[1], Hirofumi Hara[2,*] and Nobutaka Funa[1,*]

[1]*Graduate Division of Nutritional and Environmental Sciences, University of Shizuoka, Shizuoka, Japan;*
[2]*Department of Environmental Engineering and Green Technology, Malaysia-Japan International Institute of Technology, Universiti Teknologi Malaysia, Kuala Lumpur, Malaysia*

Received September 29, 2014; accepted November 25, 2014
http://dx.doi.org/10.1080/09168451.2014.993915

The β-ketoadipate pathway is a major pathway involved in the catabolism of the aromatic compounds in microbes. The recent progress in genome sequencing has led to a rapid accumulation of genes from the β-ketoadipate pathway in the available genetic database, yet the functions of these genes remain uncharacterized. In this study, the protocatechuate branch of the β-ketoadipate pathway of *Rhodococcus jostii* was reconstituted *in vitro*. Analysis of the reaction products of PcaHG, PcaB, and PcaL was achieved by high-performance liquid chromatography. These reaction products, β-ketoadipate enol-lactone, 3-carboxy-*cis*,*cis*-muconate, γ-carboxymuconolactone, muconolactone, and β-ketoadipate, were further characterized using LC-MS and nuclear magnetic resonance. In addition, the *in vitro* reaction of PcaL, a bidomain protein consisting of γ-carboxy-muconolactone decarboxylase and β-ketoadipate enol-lactone hydrolase activities, was demonstrated for the first time. This work provides a basis for analyzing the catalytic properties of enzymes involved in the growing number of β-ketoadipate pathways deposited in the genetic database.

Key words: β-ketoadipate pathway; *Rhodococcus jostii* RHA1; protocatechuate

Aromatic compounds, one of the most abundant classes of naturally occurring compounds, are primarily found in microbial cells as degradation products of plant-derived molecules such as lignins.[1)] Microorganisms have developed the ability to use an impressive variety of aromatic compounds as carbon and energy sources.[2,3)] The β-ketoadipate pathway is one of the main biodegradation pathways for aromatic compounds in aerobic soil bacteria and fungi.[2)] This pathway includes the protocatechuate and catechol branches, which catabolize protocatechuate (**1**) and catechol, respectively. Both branch converges at β-ketoadipate enol-lactone (**2**) (Fig. 1). **2** is then further catabolized to the tricarboxylic acid cycle intermediates, acetyl-CoA and succinyl-CoA, by downstream enzymes of the β-ketoadipate pathway.[2)] Since the 1940s, the enzymology of the β-ketoadipate pathway of *Pseudomonas* and *Acinetobacter* has been extensively studied,[4)] leading to the unraveling of this complex reaction pathway and the chemical structures of the intermediates involved, including **2**, 3-carboxy-*cis*,*cis*-muconate (**3**), γ-carboxymuconolactone (**4**), and muconolactone (**5**) (Fig. 1).[5)] The protocatechuate branch of the β-ketoadipate pathway begins with an *ortho*-cleavage of **1** by PcaHG, a protocatechuate 3,4-dioxygenase (Fig. 1),[6,7)] resulting in the formation of **3**. PcaB, a 3-carboxy-*cis*,*cis*-muconate cycloisomerase, catalyzes the lactonization of **3** to form **4**.[8,9)] Decarboxylation of **4** is catalyzed by PcaC, a γ-carboxy-muconolactone decarboxylase,[10)] but this reaction can also occur spontaneously.[11)] The resulting decarboxylated product, **2**, can be isomerized to form **5**, and the two exist in equilibrium.[11)] PcaD, β-ketoadipate enol-lactone hydrolase, also known as 3-oxoadipate-enol lactonase, hydrolyzes **2** to form β-ketoadipate (**6**).[12,13)]

Rhodococcus jostii RHA1 was first isolated from γ-hexachlorocyclohexane-contaminated soil and initially characterized for its polychlorinated biphenyl-degrading properties.[14)] Genome sequence analyses of *R. jostii* RHA1 further indicated the potential ability of *R. jostii* RHA1 to degrade a broad range of aromatic compounds.[15)] For example, Hara et al. demonstrated the growth of *R. jostii* RHA1 on both phthalate and terephthalate through the use of *pad* and *tpa* genes, which encode the enzymes for the initial steps of phthalate and terephthalate degradation, respectively. These gene clusters were identified on two large linear plasmids of *R. jostii* RHA1, namely, pRHL1 and pRHL2.[16)] By proteomic analysis, Patrauchan et al. confirmed the translation of the *pad* genes, *pcaB*, *pcaL,* and *pcaF* of *R. jostii* RHA1 grown on phthalate.[17)] In addition, they detected the protocatechuate 3,4-dioxygenase activity from the cell-free extracts of *R. jostii* RHA1 grown on phthalate.[17)]

*Corresponding authors. Email: hhara@utm.my (H. Hara); funa@u-shizuoka-ken.ac.jp (N. Funa)

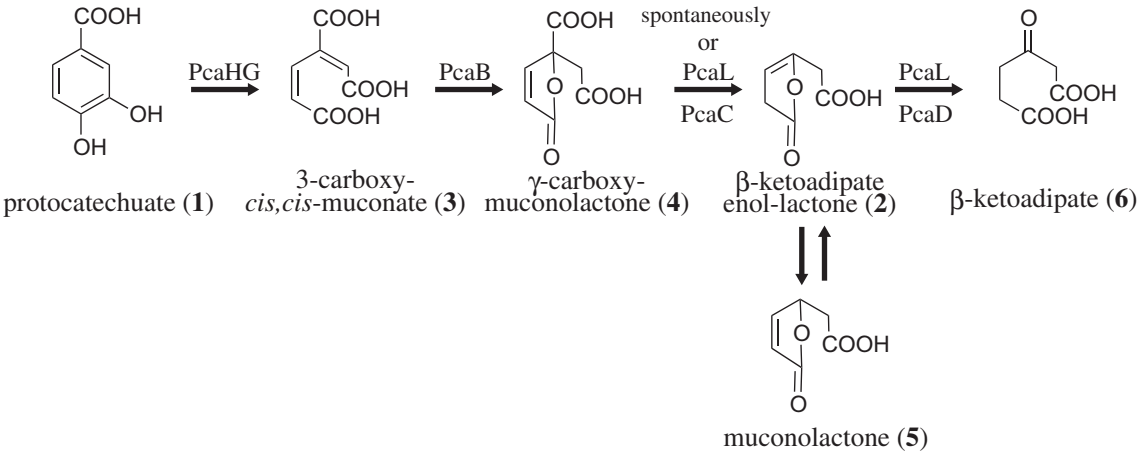

Fig. 1. The protocatechuate branch of the β-ketoadipate pathway from *R. jostii* RHA1.
Note: PcaL, instead of PcaC and PcaD, is responsible of transfer from **4** to **6** via **2** in *R. jostii* RHA1.

In *R. jostii* RHA1, the chromosomal *pca* cluster consists of genes predicted to encode the enzymes PcaJIHGBLF,[15,17] which are required to convert **1** to acetyl-CoA and succinyl-CoA. The genes *pcaH* and *pcaG* encode the two subunits of protocatechuate 3,4-dioxygenase,[15] and *pcaB* encodes a 3-carboxy-*cis*,*cis*-muconate lactonizing enzyme.[15] *pcaL* is a fused gene encoding for an enzyme with both γ-carboxy-muconolactone decarboxylase and β-ketoadipate enol-lactone hydrolase activities (Fig. 1).[15,17,18]

In this study, the protocatechuate branch of the β-ketoadipate pathway from *R. jostii* RHA1 was reconstituted *in vitro* using recombinant PcaHG, PcaB, and PcaL. The bifunctional nature of PcaL was characterized biochemically for the first time. All compounds of the protocatechuate branch, including the highly unstable compound, **3**, were characterized by high-performance liquid chromatography (HPLC), nuclear magnetic resonance (NMR), and liquid chromatography-electrospray ionization/high-resolution mass spectrometry (LC-ESI/HRMS) analyses.

Materials and methods

Chemicals and bacterial strains. Protocatechuate was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Luria-Bertani (LB) Lennox medium was purchased from Sigma-Aldrich (St. Louis, MO, USA). *Escherichia coli* HST08, the pColdI plasmid, restriction enzymes, and other DNA-modifying enzymes used for DNA manipulation were purchased from Takara Bio Inc (Shiga, Japan). *E. coli* BL21 was purchased from the National BioResource Project (National Institute of Genetics of Japan). *R. jostii* RHA1 was obtained from Prof. Masao Fukuda. All other reagents were purchased from Wako Pure Chemicals.

Construction of pColdI-pcaHG, pColdI-pcaB, and pColdI-pcaL. Media, growth conditions, and general recombinant DNA techniques of *E. coli* were described by Sambrook et al.[19] The chromosomal DNA of *R. jostii* RHA1 was used as a template for PCR experiments. The accession numbers of the amino acid sequences of PcaG, PcaH, PcaB, and PcaL are ABG93160, ABG93159, ABG93161, and ABG93162, respectively. An NdeI site was introduced at the start codon of *pcaHG* by PCR with primer I: 5′-CGGAATTC*CATATG*CTGCATCTGCCAGCCC-3′ (the EcoRI site is underlined; the NdeI site is italicized), and primer II: 5′-CGCAAGCTTCGACACCTTTCTGCGTTGTG-3′ (the HindIII site is underlined). The amplified fragments were cloned between the EcoRI and HindIII sites of pUC19, resulting in pUC19-*pcaHG.* An NdeI site was introduced at the start codon of *pcaB* by PCR with primer III: 5′-CGGAATTC*CATATG*AACTCTCCGGAGCCTT-3′ (the EcoRI site is underlined; the NdeI site is italicized), and primer IV: 5′-CGCAAGCTTGTGTGCGAGTGCGACTG-3′ (the HindIII site is underlined). The amplified fragments were cloned between the EcoRI and HindIII sites of pUC19, resulting in pUC19-*pcaB.* An NdeI site was introduced at the start codon of *pcaL* by PCR with primer V: 5′-CGTCTAGA*CATATG*ACAGTCGCACTCGCA-3′ (the XbaI site is underlined; the NdeI site is italicized), and primer VI: 5′-CGCAAGCTTCGTCGGTTCGGTAGCGG-3′ (the HindIII site is underlined). The amplified fragments were cloned between the XbaI and HindIII sites of pUC19, resulting in pUC19-*pcaL*. DNA sequencing of pUC19-*pcaHG,* pUC19-*pcaB,* and pUC19-*pcaL* confirmed the correct sequence. Each NdeI-HindIII fragment excised from pUC19-*pcaHG,* pUC19-*pcaB,* and pUC19-*pcaL* was cloned between the NdeI and HindIII sites of pColdI, resulting in pColdI-*pcaHG,* pColdI-*pcaB,* and pColdI-*pcaL*, respectively.

Expression and purification of recombinant PcaHG, PcaB, and PcaL. *E. coli* BL21 harboring pColdI-*pcaHG,* pColdI-*pcaB,* or pColdI-*pcaL* was grown at 37 °C in 50 mL of LB broth containing 100 μg mL^{-1} of ampicillin. When an optical density at 600 nm reached 0.5, cells were incubated for 30 min at 15 °C and then protein expression was induced by the addition of 0.1 mM isopropyl β-D-thiogalactoside. Cells were then cultured for an additional 24 h at 15 °C. Cells were harvested by centrifugation and resuspended in 50 mM NaH_2PO_4 (pH 8.0), 300 mM NaCl, 10 mM

imidazole, and 10% glycerol. The resuspended cells were collected by centrifugation, and a crude cell lysate was prepared by sonication and cell debris was removed by centrifugation at 20,000 × *g* for 20 min. The cleared lysate was applied to Ni-nitrilotriacetic acid columns (Qiagen, Hilden, Germany), washed twice with 50 mM NaH_2PO_4 (pH 8.0), 300 mM NaCl, 20 mM imidazole, and 10% glycerol. The purified histidine-tagged protein was eluted with a buffer containing 50 mM NaH_2PO_4 (pH 8.0), 300 mM NaCl, 250 mM imidazole, and 10% glycerol and was dialyzed two times against 2 L of 10 mM Tris–HCl (pH 7.5) containing 10% glycerol. The protein concentration was measured by Bradford assay with a Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA) using bovine serum albumin as a standard. The purity of N-terminal His-tagged (MNHKVHHHHHHIEGRH) PcaHG, PcaB, and PcaL was verified by SDS analysis (Supplemental Fig. S1). The proteins were stored at −80 °C before use.

In vitro *reactions with protocatechuate.* The enzymatic reactions, containing 1 μg each of PcaHG, PcaB, and/or PcaL, 1 mM of protocatechuate, and 50 mM Tris–HCl (pH 7.5), were performed in a total volume of 300 μL. The boiled enzymes were used as negative controls. The reaction mixtures were incubated at 30 °C for 5 min and were stopped by filtration with Amicon Ultra-0.5 Centrifugal Filters (Merck Millipore, Darmstadt, Germany). Aliquots (5 μL) of the filtrate were directly analyzed via HPLC (JASCO International Co., Tokyo, Japan). Reverse-phase HPLC analysis was carried out using a COSMOSIL $5C_{18}$-PAQ column (4.6 × 150 mm; Nacalai Tesque Inc., Kyoto, Japan), and the analytes were subjected to an isocratic elution with 3% acetonitrile in water (both containing 0.1% trifluoroacetic acid) at a flow rate of 1 mL min^{-1} at 40 °C. UV absorbance was detected at 200 nm.

NMR and MS analyses. The compounds, **2**, **3**, **5**, and **6**, used for NMR analyses were prepared from the *in vitro* reaction, which was scaled up to 10 mL. For preparation of **2**, **5**, and **6**, the reaction mixtures were acidified with 1 mL of 1 M HCl and extracted with ethyl acetate. The organic layers were evaporated until dry. The crude materials were dissolved in a small amount of methanol and purified using a reverse-phase preparative HPLC equipped with a COSMOSIL $5C_{18}$-AR-II column (20 × 250 mm; Nacalai Tesque Inc., Kyoto, Japan). The compounds were chromatographed using 10% methanol and 0.1% trifluoroacetic acid in water as a mobile phase, at a flow rate of 3 mL/min at ambient temperature. Because **3** was easily transformed into a geometical isomer under acidic conditions, the reaction containing **3** was filtrated with Amicon Ultra-0.5 Centrifugal Filters and the combined filtrate was lyophilized. The lyophilizate was used directly for NMR analyses. NMR data were collected on a Bruker AVANCE III 400 FT-NMR spectrometer (Bruker Corporation, Billerica, MA, USA). LC-ESI/HRMS analysis was carried out using a Q Exactive™ (Thermo Fisher Scientific Inc., Waltham, MA, US). For LC-ESI/HRMS analysis, a COSMOSIL $5C_{18}$-PAQ column (2.0 × 150 mm; Nacalai Tesque Inc., Kyoto, Japan) was used, and analytes were eluted under the isocratic condition of 3% acetonitrile in water (both containing 0.1% formic acid) at a flow rate of 0.2 mL min^{-1} at 40 °C.

NMR and MS data. β-ketoadipate enol-lactone (2). ^{1}H NMR (400 MHz, D_2O): δ 3.36 (d, 2H, J = 2.0 Hz, $C2H_2$), 3.49 (s, 2H, C3H), 5.58 (s, 1H, $C5H_2$). Negative mode LC-ESI/HRMS, $[M-H]^-$ = 141.01835 (calculated for $C_6H_5O_4^-$, 141.01933).

3-carboxy-cis,cis-muconate (**3**). ^{1}H NMR (400 MHz, CD_3OD): δ 6.01(dd, 1H, J = 0.9, 12.0 Hz, C5H), 6.50 (m, 1H, C2H), 6.77 (dd, 1H, J = 1.7, 12.0 Hz, C4H). Negative mode LC-ESI/HRMS, $[M-H]^-$ = 185.00800 (calculated for $C_7H_5O_6^-$, 185.00916).

γ-carboxymuconolactone (**4**). Negative mode LC-ESI/HRMS, $[M-H]^-$ = 185.00841 (calculated for $C_7H_5O_6^-$, 185.00916).

muconolactone (**5**). ^{1}H NMR (400 MHz, D_2O): δ 2.71 (dd, 1H, J = 8.2, 16.6 Hz, C1′Ha), 2.95 (dd, 1H, J = 4.8, 16.6 Hz, C1′Hb), 5.57 (dddd, 1H, J = 1.4, 1.9, 4.8, 8.2 Hz, C5H), 6.24 (dd, 1H, J = 1.9, 5.8 Hz, C3H), 7.81 (dd, 1H, J = 1.4, 5.8 Hz, C4H). Negative mode LC-ESI/HRMS, $[M-H]^-$ = 141.01831 (calculated for $C_6H_5O_4^-$, 141.01933).

β-ketoadipate (**6**). ^{1}H NMR (400 MHz, CD_3OD): δ 2.55 (t, 2H, J = 6.5 Hz, C5H), 2.86 (t, 2H, J = 6.5 Hz, C4H), 3.39 (s, 2H, $C2H_2$). Negative mode LC-ESI/HRMS, $[M-H]^-$ = 159.02898 (calculated for $C_6H_7O_5^-$, 159.02990).

Results

In vitro *catabolism of protocatechuate by PcaHG*

The first step of the catabolism of protocatechuate (**1**) in the β-ketoadipate pathway is a ring-opening reaction catalyzed by PcaHG.[6,7] An SDS PAGE-verified pure heterodimer of PcaHG (Supplemental Fig. S1) was incubated with **1** as described in the Methods section. HPLC analysis confirmed that **1** was readily converted to the product, **3**, which migrated at a retention time (RT) of 5.0 min. In control experiments with boiled, inactive PcaHG, **1** was not converted to product as evidenced by an RT of 10.2 min (Fig. 2(A)). The structure of the reaction product was confirmed by the negative mode LC-ESI/HRMS and ^{1}H NMR analyses (Fig. 2(B) and Supplemental Fig. S2). The unstable nature of the product hindered us from conducting ^{13}C NMR and two-dimensional NMR analyses. The *m*/*z* of the product, 185.00800, was in good agreement with the calculated theoretical value, 185.00916, of the $[M-H]^-$ ion of **3**. The geometrical isomerism of the product was characterized from the coupling constants calculated from the ^{1}H NMR spectrum (Supplemental Fig. S2). A coupling constant of 12.0 Hz characteristic of a *cis*-coupling across a double bond was observed for C4- and C5-protons (Supplemental Fig. S3), indicating that the geometrical isomerism of the olefin at C4–C5 position is in a *cis*-configuration. In addition, a long-range coupling was observed between the C2- and C5-protons, since the C5 proton appeared to be a

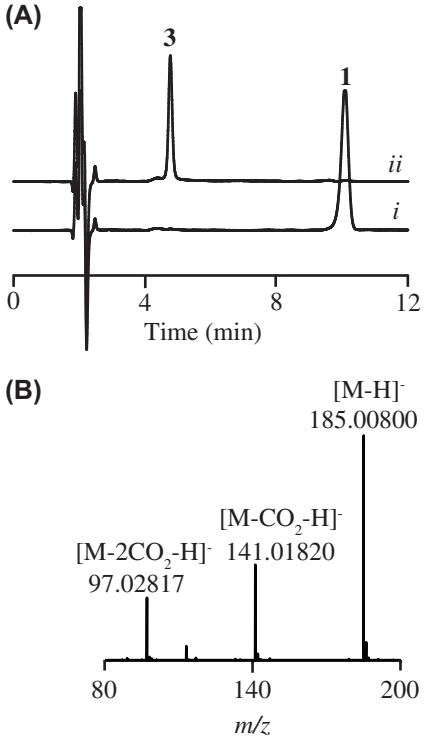

Fig. 2. HPLC and MS analyses of the product from PcaHG reaction.

Note: (A) HPLC chromatograms of *in vitro* reactions of PcaHG. Boiled PcaHG (*i*) and active PcaHG (*ii*) were used as enzymes. UV absorption was detected at 200 nm. (B) LC-ESI/HRMS spectrum of 3-carboxy-*cis*,*cis*-muconate (**3**). $[M-H]^-$, $[M-CO_2-H]^-$, and $[M-2CO_2-H]^-$ ions were observed.

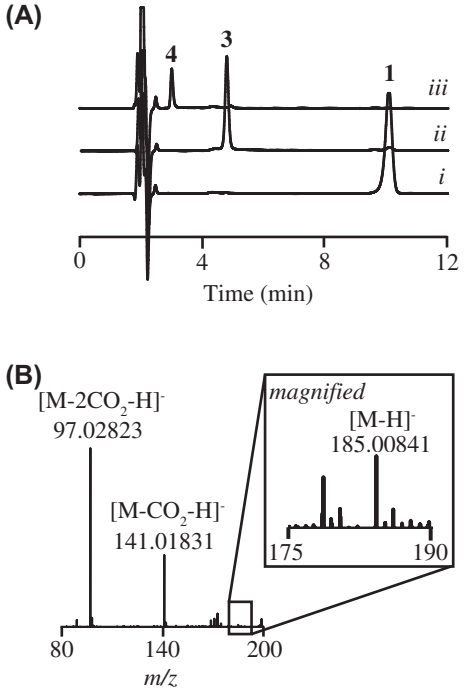

Fig. 3. HPLC and MS analyses of the product from PcaHG and PcaB reaction.

Note: (A) HPLC chromatograms of *in vitro* reactions of PcaHG and PcaB. All enzymes were active unless inactivated by boiling prior to use in assay. Boiled PcaHG and PcaB (*i*), PcaHG and boiled PcaB (*ii*), and PcaHG and PcaB (*iii*) were used as enzymes. UV absorption was detected at 200 nm. (B) LC-ESI/HRMS spectrum of γ-carboxymuconolactone (**4**). $[M-H]^-$, $[M-CO_2-H]^-$, and $[M-2CO_2-H]^-$ ions were observed. Decarboxylation of **4** occurred during ionization resulting in the observation of both $[M-CO_2-H]^-$ and $[M-2CO_2-H]^-$ as major ions.

doublet of doublets (Supplemental Fig. S2). The geometrical isomerism of the olefin at the C2–C3 position was deduced to be in a *cis*-configuration, since the coupling constant of the dienylic coupling was calculated to be 1.7 Hz (Supplemental Fig. S3). Taken together, molecule **3** was determined to be the product of the reaction of PcaHG with **1** via an enzyme catalyzed oxidation.

In vitro *catabolism of protocatechuate by PcaHG and PcaB*

The second step of the β-ketoadipate pathway is a cycloisomerization of **3** catalyzed by PcaB, forming molecule **4**.[8] When active PcaB and PcaHG were simultaneously incubated with **1**, a product was observed at an RT of 3.0 min via HPLC analysis (Fig. 3(A)). This product was not generated in the reactions when boiled PcaB or PcaHG were used instead of active enzymes (Fig. 3(A)). The *m/z* of the product, 185.00841, was in good agreement with the calculated theoretical value, 185.00916, of the $[M-H]^-$ ion of **4** (Fig. 3(B)). A filtrate of the reaction was incubated at 30 °C to examine whether facile decarboxylation of the product occurred (Supplemental Fig. S4). As a result, a rapid decrease in the product with a concomitant increase in **5** (see below for a structural characterization) was observed. This suggests that the product of PcaHG and PcaB reaction is **4**, which then undergoes spontaneous decarboxylation to form **2**. It is known that **2** and **5** exist in equilibrium,[11] even though the form of **5** is more energetically favorable than **2** (Supplemental Fig. S4). Unfortunately, no NMR data of **4** were successfully obtained, because **4** proved to be extremely unstable.

The molecular formula for **5** was determined to be $C_6H_6O_4$ since the LC-ESI/HRMS analysis showed that the *m/z* of the product of decarboxylation of **4** was 141.01831—a value that was in agreement with the theoretical value, 141.01933, of the $[M-H]^-$ ion of **5** (Supplemental Fig. S5). Similarly, the molecular formula of **2** was also determined as $C_6H_6O_4$ (Supplemental Fig. S6). The ^{1}H NMR data of **5** were assigned as follows: the doublets at 6.24 and 7.81 ppm were assigned to the C4 and C3 protons of the dihydrofuran ring; the dddd at 5.57 ppm was assigned to the C5 proton; the doublet of doublets at 2.71 and 2.95 ppm were assigned to the germinal protons at C1′, since the coupling constant between these protons was 16.6 Hz (Supplemental Fig. S7). In addition, the ^{1}H NMR data of **2** were consistent with the structure of β-ketoadipate enol-lactone (Supplemental Fig. S8).

In vitro *catabolism of protocatechuate by PcaHG, PcaB, and PcaL*

PcaL is a fusion protein composed of PcaC and PcaD that catalyzes the decarboxylation of **4** and the hydrolysis of **2**, by their respective activities. When PcaL, PcaB, and PcaHG were incubated with **1**, a

product was observed at an RT of 3.3 min via HPLC trace (Fig. 4(A)). This product was not generated in reactions with boiled, inactive PcaL, PcaB, or PcaHG (Fig. 4(A)). Similar results were obtained from the two-step reaction in which PcaL was added to the filtrate of the reaction of PcaHG and PcaB, as seen with HPLC and LC-MS analyses (Supplemental Fig. S9). The *m/z* of the product, 159.02892, was in good agreement with the calculated theoretical value, 159.02990, of the $[M-H]^-$ ion of **6** (Fig. 4(B)). In addition, the ^1H NMR spectrum of **6** was identical to β-ketoadipate (Supplemental Fig. S10).[20] Furthermore, PcaL exhibited a β-ketoadipate enol-lactone hydrolase activity with **2** *in vitro*, resulting in the formation of **6**. HPLC analysis revealed that **5** was not utilized as a substrate in this reaction (Fig. 5). These results indicate that PcaL is a bifunctional enzyme, functioning as both γ-carboxy-muconolactone decarboxylase and β-ketoadipate enol-lactone hydrolase.

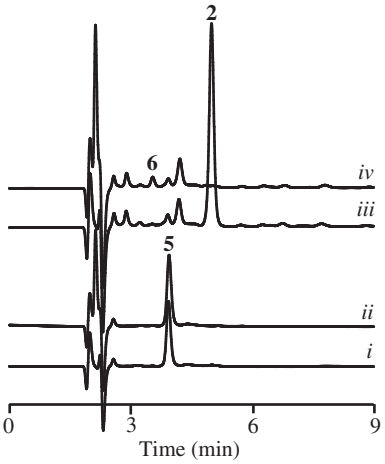

Fig. 5. HPLC chromatograms of *in vitro* reaction of PcaL.

Note: Reactions of boiled PcaL and **5** (*i*), active PcaL and **5** (*ii*), boiled PcaL and **2** (*iii*), and active PcaL and **2** (*iv*).

Discussion

Although a complete picture of the β-ketoadipate pathway was depicted by the Ornston group in the 1970s,[4] chromatographic, spectroscopic, and mass spectrometric studies of the intermediates in this pathway have not been thoroughly investigated. Although Ravi et al. reported the proton NMR spectrum of **3**, they did not observe the long-range couplings of **3**.[5] On the other hand, Ainsworth et al. reported the coupling constants of the dienylic ($J = 0.9$ Hz) and allylic ($J = 2.1$ Hz) couplings of **3**.[21] They explained that these "abnormal" coupling constants can be attributed to the nonplanarity of **3**. However, we have observed "normal" coupling constants for the dienylic and allylic couplings as 1.7 and 0.9 Hz, respectively (Supplemental Fig. S4). We suppose that the rapid sample handling allowed us to analyze the intact **3**.

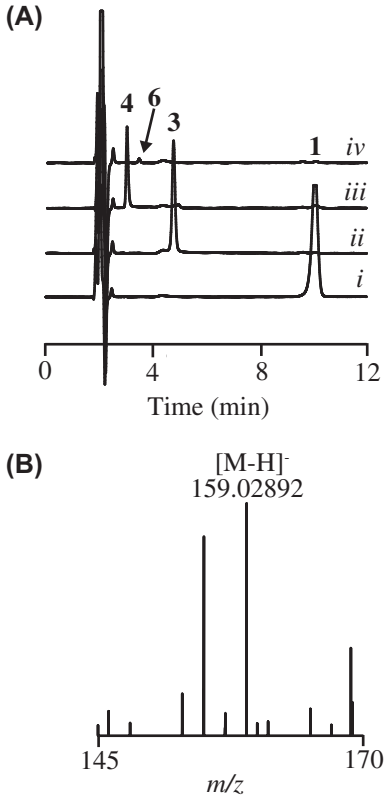

Fig. 4. HPLC and MS analyses of the product from PcaHG, PcaB, and PcaL reaction.

Note: (A) HPLC chromatograms of *in vitro* reactions of PcaHG, PcaB, and PcaL. All enzymes were active unless inactivated by boiling prior to use in assay. Boiled PcaHG, PcaB, and PcaL (*i*), PcaHG, boiled PcaB, and PcaL (*ii*), PcaHG, PcaB, and boiled PcaL (*iii*), PcaHG, PcaB, and PcaL (*iv*) were used as enzymes. UV absorption was detected at 200 nm. (B) LC-ESI/HRMS spectrum of β-ketoadipate (**6**). $[M-H]^-$ ion was observed.

In this study, enzymes of the protocatechuate branch of the β-ketoadipate pathway from *R. jostii*, whose functions were predicted based on sequence similarity, were reconstituted *in vitro*. We accomplished the structural determination of the reaction products of PcaHG, PcaB, and PcaL using HPLC, LC-MS, and NMR, leading to a biochemical characterization of these enzymes. PcaHG catalyzes the oxidative cleavage of the aromatic ring of protocatechuate to yield **3**. Following cycloisomerization by PcaB, the formation of the lactone ring gives **4**. PcaL, a bidomain protein consisting of PcaC and PcaD activities, catalyzes the sequential decarboxylation and hydrolysis reactions of **4** that result in the formation **6**. The Pca enzymes of *R. jostii* share moderate sequence similarity with the enzymes whose catalytic properties were characterized biochemically. For example, PcaG is 42% identical in amino acid sequence to the alpha chain of protocatechuate 3,4-dioxygenase[7] of *P. aeruginosa*. Similarly, PcaH is 50% identical to the beta chain of protocatechuate 3,4-dioxygenase[7] of *P. aeruginosa*. In addition, PcaB is 41% identical to 3-carboxy-*cis*,*cis*-muconate lactonizing enzyme[8] of *P. putida*. While the N-terminus (residues 1–261) of PcaL is 45% identical to β-ketoadipate enol-lactone hydrolase[11] of *P. putida*, the C-terminus (residues 272–400) of PcaL is 49% identical to γ-carboxy-muconolactone decarboxylase[10] of *P. putida*. Therefore, it is not surprising that the catalytic reactions of the Pca enzymes of *R. jostii* were identical to those of the homologous enzymes of *Pseudomonas* species. To our knowledge, this is the first report

demonstrating the *in vitro* reactions of the Pca enzymes from actinomycetes.

In the course of our study, **4** was found to be an extremely unstable compound that was readily transformed to **5** via spontaneous decarboxylation and isomerization. It is possible that the bifunctional PcaL is more advantageous than the separate enzymes of PcaC and PcaD in carbon and energy acquisition due to the necessity of PcaC to release intermediate **2**, allowing it to equilibrate with **5**, and resulting in the loss of the carbon source from the pathway. We also found that **3** was an unstable compound and was readily transformed into 3-carboxy-*trans*,*trans*-muconate (results not shown). There may be unidentified enzymes that scavenge these side products and bring them back to the β-ketoadipate pathway to recover the invested energy. Recent progress in genome sequencing has revealed that the β-ketoadipate pathway genes are distributed widely among microbial taxa. This work provides a basis for analyzing the catalytic properties of enzymes involved in the growing number of the β-ketoadipate pathway genes deposited in the seemingly continuously updated genetic databases.

Supplemental material

The supplementary material for this paper is available at http://dx.doi.org/10.1080/09168451.2014.993915.

Acknowledgment

We are grateful to Prof. M. Fukuda for providing us *R. jostii* RHA1.

Funding

This work was, in part, supported by a Grant-in-Aid for Scientific Research on Innovative Areas from the Ministry of Education, Culture, Sports, Science and Technology [grant number 23108521] (to N. F); The Naito Foundation (to N. F.); The Kato Memorial Bioscience Foundation (to N. F.); The Uehara Memorial Foundation (to N. F.).

References

[1] Bugg TD, Ahmad M, Hardiman EM, Rahmanpour R. Pathways for degradation of lignin in bacteria and fungi. Nat. Prod. Rep. 2011;28:1883–1896.

[2] Harwood CS, Parales RE. The β-ketoadipate pathway and the biology of self-identity. Annu. Rev. Microbiol. 1996;50:553–590.

[3] Wells T Jr., Ragauskas AJ. Biotechnological opportunities with the β-ketoadipate pathway. Trends Biotechnol. 2012;30:627–637.

[4] Stainer RY, Ornston LN. The beta-ketoadipate pathway. Adv. Microb. Physiol. 1973;9:89–151.

[5] Chari RV, Whitman CP, Kozarich JW, Ngai KL, Ornston LN. Absolute stereochemical course of muconolactone Δ-isomerase and of 4-carboxymuconolactone decarboxylase: A ^{1}H NMR "ricochet" analysis. J. Am. Chem. Soc. 1987;109:5520–5521.

[6] Frazee RW, Livingston DM, LaPorte DC, Lipscomb JD. Cloning, sequencing, and expression of the *Pseudomonas putida* protocatechuate 3,4-dioxygenase genes. J. Bacteriol. 1993;175:6194–6202.

[7] Ohlendorf DH, Orville AM, Lipscomb JD. Structure of protocatechuate 3,4-dioxygenase from *Pseudomonas aeruginosa* at 2.15 Å resolution. J Mol Biol. 1994;244:586–608.

[8] Yang J, Wang Y, Woolridge EM, Arora V, Petsko GA, Kozarich JW, Ringe D. Crystal structure of 3-carboxy-cis, cis-muconate lactonizing enzyme from *Pseudomonas putida*, a fumarase class II type cycloisomerase: enzyme evolution in parallel pathways. Biochemistry. 2004;43:10424–10434.

[9] Chari RV, Whitman CP, Kozarich JW, Ngai KL, Ornston LN. Absolute stereochemical course of the 3-carboxymuconate cycloisomerases from *Pseudomonas putida* and *Acinetobacter calcoaceticus*: analysis and implications. J. Am. Chem. Soc. 1987;109:5514–5519.

[10] Yeh WK, Fletcher P, Ornston N. Homologies in the NH_2-terminal amino acid sequences of γ-carboxymuconolactone decarboxylases and muconolactone isomerases. J Biol Chem. 1980;255:6347–6354.

[11] Ornston LN, Stainer RY. The conversion of catechol and protocatechuate to β-ketoadipate by *Pseudomonas putida*. I. Biochemistry. J. Biol. Chem. 1966;241:3776–3786.

[12] McCorkle GM, Yeh WK, Fletcher P, Ornston LN. Repetitions in the NH2-terminal amino acid sequence of β-ketoadipate enol-lactone hydrolase from *Pseudomonas putida*. J. Biol. Chem. 1980;255:6335–6341.

[13] Ornston LN. The conversion of catechol and protocatechuate to β-ketoadipate by *Pseudomonas putida*. II. Enzymes of the protocatechuate pathway. J. Biol. Chem. 1966;241:3787–3794.

[14] Seto M, Masai E, Ida M, Hatta T, Kimbara K, Fukuda M, Yano K. Multiple polychlorinated biphenyl transformation systems in the gram-positive bacterium *Rhodococcus* sp. strain RHA1. Appl. Environ. Microbiol. 1995;61:4510–4513.

[15] McLeod MP, Warren RL, Hsiao WW, Araki N, Myhre M, Fernandes C, Miyazawa D, Wong W, Lillquist AL, Wang D, Dosanjh M, Hara H, Petrescu A, Morin RD, Yang G, Stott JM, Schein JE, Shin H, Smailus D, Siddiqui AS, Marra MA, Jones SJ, Holt R, Brinkman FS, Miyauchi K, Fukuda M, Davies JE, Mohn WW, Eltis LD. The complete genome of *Rhodococcus* sp. RHA1 provides insights into a catabolic powerhouse. Proc. Natl. Acad. Sci. USA. 2006;103:15582–15587.

[16] Hara H, Eltis LD, Davies JE, Mohn WW. Transcriptomic analysis reveals a bifurcated terephthalate degradation pathway in *Rhodococcus* sp. strain RHA1. J. Bacteriol. 2007;189:1641–1647.

[17] Patrauchan MA, Florizone C, Dosanjh M, Mohn WW, Davies J, Eltis LD. Catabolism of benzoate and phthalate in *Rhodococcus* sp. strain RHA1: redundancies and convergence. J. Bacteriol. 2005;187:4050–4063.

[18] Eulberg D, Lakner S, Golovleva LA, Schlömann M. Characterization of a protocatechuate catabolic gene cluster from *Rhodococcus opacus* 1CP: evidence for a merged enzyme with 4-carboxymuconolactone-decarboxylating and 3-oxoadipate enol-lactone-hydrolyzing activity. J. Bacteriol. 1998;180:1072–1081.

[19] Sambrook J, Maniatis T, Fritsch EF. Molecular cloning: a laboratory manual. New York (NY): Cold Spring Harbor; 1989.

[20] Pieper DH, Pollmann K, Nikodem P, Gonzalez B, Wray V. Monitoring key reactions in degradation of chloroaromatics by *in situ* ^{1}H nuclear magnetic resonance: solution structures of metabolites formed from cis-dienelactone. J. Bacteriol. 2002;184:1466–1470.

[21] Ainsworth AT, Kirby GW. Stereochemistry of β-carboxy-and β-hydroxymethyl-muconic derivatives. J. Chem. Soc. C. 1968;1483–1487.