Purification, characterization, and substrate specificity of two 2,3-dihydroxybiphenyl 1,2-dioxygenase from *Rhodococcus* sp. R04, showing their distinct stability at various temperature

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Abstract

The genes of two 2,3-dihydroxybiphenyl 1,2-dioxygenases (BphC1 and BphC2) were obtained from the gene library of *Rhodococcus* sp. R04. The enzymes have been purified to apparent electrophoretic homogeneity from the cell extracts of the recombinant harboring bphC1 and bphC2. Both BphC1 and BphC2 were hexamers, consisting of six subunits of 35 and 33 kDa as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, respectively. The enzymes had similar optimal pH (pH 9.0), but different temperatures for their maximum activity (30 °C for BphC1, 80 °C for BphC2). In addition, they exhibited distinct stability at various temperatures. The enzymes could cleave a wide range of catechols, with 2,3-dihydroxybiphenyl being the optimum substrate for BphC1 and BphC2. BphC1 was inhibited by 2,3-dihydroxybiphenyl, catechol and 3-chlorocatechol, whereas BphC2 showed strong substrate inhibition for all the given substrates. BphC2 exhibited a half-life of 15 min at 80 °C and 50 min at 70 °C, making it the most thermostable extradiol dioxygenase studied in mesophilic bacteria. After disruption of bphC1 and bphC2 genes, R04\textsuperscript{b}C1 (bphC1 mutant) delayed the time of their completely eliminating biphenyl another 15 h compared with its parent strain R04, but R04\textsuperscript{b}C2 (bphC2 mutant) lost the ability to grow on biphenyl, suggesting that BphC1 plays an assistant role in the degrading of biphenyl by strain R04, while BphC2 is essential for the growth of strain R04 on biphenyl.

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1. Introduction

Polychlorinated biphenyls (PCBs) have been of public and scientific concern for several decades because of their persistence in the environment, their ability to bioaccumulate, and their potential carcinogenicity. Studies showed that a number of biphenyl-degrading organisms are capable of transforming PCB congeners; accordingly, bioremediation has become a promising approach in dealing with PCB contamination.

Since 1973, a number of microorganisms that could degrade PCBs have been isolated and characterized [1–3]. The microbial degradation of PCBs has been intensively studied as a possible means to destroy these toxic, persistent environmental pollutants. Thereafter, particular attention has been focused on the *bph* pathway, which is responsible for the aerobic degradation of biphenyl in a wide range of microorganisms [4]. The upper *bph* pathway consists of four enzymatic activities which together transform biphenyl to benzoate and 2-hydroxy-penta-2,4-dienoate. This so-called upper pathway correspondingly consists of four enzymes: biphenyl 2,3-dioxynegase (BphA), 2,3-dihydro-2,3-dihydroxybiphenyl-2,3-dehydrogenase (BphB), 2,3-dihydroxybiphenyl 1,2-dioxygenase

Abbreviations: PCB, polychlorinated biphenyl; BphC, 2,3-dihydroxybiphenyl 1,2-dioxygenase; MM, minimal medium.

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Among these enzymes, BphC belongs to extradiol dioxygenases, which utilizes a mononuclear non-heme iron (II) center to cleave 2,3-dihydroxybiphenyl in an extradiol fashion, and plays a key role in the metabolism of aromatic compounds. In the upper pathway of PCB degradation, PCB transformation is limited in parts by the BphC enzyme, which is incapable of transforming certain chlorinated dihydroxy biphenyls [6]. Moreover, the enzyme is inhibited by 3-chlorocatechol and its aromatic substrates [7,8].

The information similarly indicated that the presence of multiple extradiol dioxygenases seems to be common in rhodococcal strains. For example, Rhodococcus sp. strain RHA1 contains two distinct PCB degradation systems [11]: one is preferentially involved in biphenyl degradation, whereas the other is involved in the degradation of ethylbenzene. Four 2,3-dihydroxybiphenyl dioxygenases were recently identified in this organism [12], and three of them were expressed when the organism was grown on biphenyl. A total of seven genes have been found in Rhodococcus erythropolis strain P6 [8]. Rhodococcus sp. R04, capable of utilizing biphenyl as the sole source of carbon and energy as well as transforming several PCB congeners, was also found to contain two bphC genes [14]. Here we present the cloning, characterization, and substrate specificity of the two extradiol dioxygenases (BphC1 and BphC2) involved in the meta-cleavage of the biphenyl ring. To the best of our knowledge, this is the first report regarding thermostable 2,3-dihydroxybiphenyl 1,2-dioxygenases (BphC2) isolated from mesophilic bacteria.

2. Materials and methods

2.1. Chemicals

Catechol was purchased from BDH (BDH, Poole, UK), while 3-methylcatechol, 4-methylcatechol and 3-chlorocatechol were purchased from Aldrich (Sigma-Aldrich, MO, USA). 2,3-dihydroxybiphenyl was obtained from Wako (Wako, Osaka, Japan). All other chemicals were of analytical grade.

2.2. Strains, culture condition and vector

Rhodococcus sp. R04 was grown in minimal medium (MM) [20]. Biphenyl was added to MM to a final concentration of 0.2% (w/v) as the sole carbon and energy source, and the cultures were incubated in a reciprocal shaker at 200 rpm and 30 °C. On the other hand, Escherichia coli DH5α and TG1 were grown in LB medium at 37 °C. When necessary, 100 μg/ml ampicillin was used at the final concentrations. pBluescript II SK+ (Stratagene, La Jolla, CA, USA) was used as the cloning vector, whereas the pBV220 was used as the expression vector [15].

2.3. Construction of library and screening of bphC in strain R04

Rhodococcus sp. strain R04 genomic DNA was partly digested with SacI, and the 3–8 kb size of DNA fragments were fractionated by agarose gel electrophoresis. Obtained DNA fragments were subsequently joined to pBluescript II SK+, which was digested with SacI and treated with alkaline phosphatase. E. coli DH5α was transformed by the resulting plasmids, and plated onto LB agar plates containing 100 μg/ml ampicillin and 1 mg/ml IPTG. Colonies that expressed 2,3-dihydroxybiphenyl 1,2-dioxygenase activity were identified by spraying colonies with a solution containing 20 mM 2,3-dihydroxybiphenyl in acetone. Positive colonies turned yellow because they produced the yellow metabolite 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate (the meta-cleavage product) [16]. DNA sequencing of the inserted fragment in the positive colonies was then carried out by Invitrogen (Beijing, China), whereas sequence analysis and homology search were done with DNAMAN (Lynnon) and the BLAST programs in GenBank.

2.4. Expression of bphC

bphC1 and bphC2 were amplified from the plasmid DNA of the above mentioned positive colonies. The PCR product for bphC1 was digested by EcoRI and PstI, and inserted at the EcoRI–PstI site of pBV220 to obtain pEP1, whereas the PCR product for bphC2 was digested by Smal and SalI, and inserted at the Smal–SalI site of pBV220 to obtain pSM1. Resulting plasmids were then introduced into CaCl2-competent E. coli TG1. The recombinant E. coli TG1 containing pEP1 and pSM1 were grown at 30 °C in LB medium containing 100 μg ml−1 of ampicillin. When the A600 of the culture reached 0.6–0.7, E. coli TG1 cells were grown another 5 h at 42 °C for induction of BphC. The harvested cells were used for analysis of the enzyme production in E. coli.

2.5. BphC gene disruption

pEP1 was restricted with EcoRI and PstI to produce bphC1, which was restricted with XhoI, the resultant 5’ and 3’ end of bphC1 were ligated to create a 0.786 kb fragment with a deletion in bphC1. The ligated fragment was ligated into the EcoRI and PstI sites of pk18mobbacB to produce pK18mob-sacBΔC1. E. coli s17-1 was transformed with pK18mob-sacBΔC1 and conjugated into the strain R04. Screening for the first and second recombination events, as well as the confirmation of the chromosomal deletion, was performed as
described [17]. The resulting strain was designated Rhodococcus sp. R04ΔC1. Rhodococcus sp. R04ΔC2, a deletion mutant of bphC2 was constructed in a similar manner to the bphC1 mutant strain by double homologous recombination. The deletion of the respective genes in R04 mutants was verified by DNA sequencing. To verify the functions of BphC1 and BphC2, the wild-type strain R04, mutants R04ΔC1 and R04ΔC2 were incubated in 30 ml of MM with 10 mM biphenyl. In due course, the cell growth was monitored with a UV-visible spectrophotometer.

2.6. Enzyme assay

2,3-Dihydroxybiphenyl 1,2-dioxygenase activity was determined in 50 mM K/Na-phosphate (pH 7.5) with 0.1 mM 2,3-dihydroxybiphenyl as the substrate. One unit of the enzyme was defined as the amount of enzyme that catalyzes the formation of droxybiphenyl as the substrate. One unit of the enzyme was defined as the amount of enzyme that catalyzes the formation of 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate at the rate of 1 μmol/min under the assay conditions mentioned above. The relative meta-cleavage activities were determined from the extinction coefficients of the ring fission products formed from the following substrates: catechol (ε375 = 36,000 M⁻¹ cm⁻¹), 3-methylcatechol (ε388 = 13,800 M⁻¹ cm⁻¹), 4-methylcatechol (ε382 = 28,100 M⁻¹ cm⁻¹), 3-chlorocatechol (ε379 = 33,000 M⁻¹ cm⁻¹) [18].

2.7. Purification of extradiol dioxygenases

For the preparation of the crude extract, the harvested E. coli TG1 cells containing pEP1 and pSMA1 were suspended in 50 mM Tris—HCl buffer (pH 7.5) containing 0.1 mM (NH₄)₂Fe(SO₄)₂ and were disrupted by sonication. Cell debris was removed by centrifugation (11,000 × g, 30 min). Purifications were performed under aerobic conditions, with cell extracts containing 400–550 mg of protein in 15–20 ml. All steps in the purification were carried out at 0–4 °C. Column chromatography was carried out on a Pharmacia fast protein liquid chromatography system (Amersham Biosciences, Uppsala, Sweden).

Cell extracts containing BphC1 and BphC2 were applied to a MonoQ HR10/10 column (Amersham Bioscience). BphC1 was eluted with an isocratic elution at 0.2 M of NaCl in Tris—HCl buffer (50 mM, pH 7.5) over 20 ml, followed by linear gradients of NaCl (0.2–0.5 M over 100 ml). BphC2 was eluted by linear gradients of NaCl (0–0.5 M over 100 ml). Active fractions (12 ml of BphC1-containing eluate with a specific activity of 6.8 U/mg of protein and a protein content of 30 mg; 8 ml of BphC2-containing eluate with a specific activity of 5.2 U/mg of protein and a protein content of 70 mg) were pooled for the next step of purification. BphC1 and BphC2 were precipitated from the active fractions by ammonium sulfate precipitation. The precipitates that were collected at 20–40% (BphC1) or 10–35% (BphC2) saturation were redissolved in 10 ml of Tris—HCl (pH 7.5) containing 0.4 M ammonium sulfate.

Proteins were next eluted from the HiLoad 26/10 phenyl-Sepharose high-performance column by a linear gradient of (NH₄)₂SO₄ (0.4–0.0 M) in Tris—HCl (20 mM, pH 7.5) over 80 ml, followed by 50 ml of Tris—HCl (20 mM, pH 7.5). All BphC-containing proteins were eluted in fractions without ammonium sulfate (after 100–120 ml). Active fractions (15 ml of BphC1-containing eluate with a specific activity of 13.5 U/mg of protein and a protein content of 21 mg; 10 ml of BphC2-containing eluate with a specific activity of 21 U/mg of protein and a protein content of 10 mg) were pooled and concentrated to final volumes of 1–2 ml with a Dialysis membrane (YM30, Amicon, MA, USA), and then stored at −20 °C until further use.

2.8. Analytic methods

The protein concentration was determined by the Bradford method with bovine serum albumin as a standard. The purity and size of the enzyme proteins were estimated by SDS-PAGE. Protein staining of the gel was performed with Coomasie Brilliant Blue R-250 whereas the molecular mass of the native enzyme was estimated by gel filtration with a Superose 12 10/300 GL column (23 ml; Amersham Pharmacia Biotech AB, Sweden). Feritin (440 kDa), catalase (230 kDa), lactate dehydrogenase (140 kDa) bovine serum albumin (67 kDa), and ovalbumin (45 kDa) were used as standards. Metal content of 2,3-dihydroxybiphenyl 1,2-dioxygenase was determined by inductively coupled plasma optical emission (ICP-OES) using an IRIS Intrepid II XSP spectrometer (Thermo Electron).

2.9. Kinetic measurements

The Michaelis–Menten kinetics of the reaction was verified by plotting the reaction rates against the substrate concentration. The Kₘ and Vₘₐₓ values were calculated from the initial velocities with the Michaelis–Menten equation by non-linear regression by using KaleidaGraph (Synergy Software). Apparent kinetic parameters were determined among substrate ranges of 1–1000 μM of 2,3-dihydroxybiphenyl, 10–5000 of catechol, 3-methylcatechol, 3-methylcatechol and 3-chlorocatechol at 25 °C, and 0.1–200 μM of 2,3-dihydroxybiphenyl at 60 °C. An activity assay of the enzyme was performed as formerly mentioned. kₐₑ values were calculated based on deduced molecular masses of the BphC1 subunit of 35 kDa and the BphC2 subunit of 33 kDa [19]. Substrate inhibition was calculated as previously described [20]. The influence of different metal cations on the enzyme activity was tested by incubating samples (20 μg purified dioxygenase) in dilution buffer for 10 min at 0 °C in the presence of various concentrations of the metal cation prior to initiating the reaction. The influence of inhibitors was then investigated by incubating a similar amount of enzyme with the inhibitor for 10 min at 0 °C. In all cases, the reaction was initiated by the addition of substrate.

2.10. Secondary structure prediction

Secondary structure predictions were done by the Jpred methods [21]. The BphC protein was predicted in the secondary structure using the crystal structure of 2,3-dihydroxybiphenyl 1,2-dioxygenase (DHBD; Protein Data Bank code 1HAN) as the template [22].
2.11. Nucleotide sequence accession number

The nucleotide sequences for \textit{bphC1} and \textit{bphC2} determined in this study have been deposited in the GenBank databases under accession no. AY544582 and DQ403247, respectively.

3. Results

3.1. Screening of library for 2,3-dihydroxybiphenyl 1,2-dioxygenase expression

A plasmid library was prepared with pBluescript II SK as described in Section 2. Among approximately 15,000 candidates, 35 colonies immediately turned yellow when sprayed with 2,3-dihydroxybiphenyl solution, indicating that conversion of 2,3-dihydroxybiphenyl to the \textit{meta}-cleavage compound (2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate) had occurred. Plasmid DNA was prepared from these clones and classified into two types (pBS01 and pBS02) according to the sequence and size of the inserted DNA. pBS01 which contained a 8.7-kb fragment carrying the biphenyl catabolic genes \textit{bphABCD} was described previously [23], and pBS02 was identified to contain a 6.5-kb fragment carrying \textit{bphC} gene. \textit{bphC} in the pBS01 was named by \textit{bphC2} in this study. On the basis of the screening of the activity and sequence similarity with other \textit{bphC} genes reported previously, three ORFs (open reading frames) were identified in pBS02, the second as the \textit{bphC1} gene.

3.2. Purification of the recombinant 2,3-dihydroxybiphenyl 1,2-dioxygenase

A three-step protocol was used to purify the two recombinant 2,3-dihydroxybiphenyl 1,2-dioxygenases. BphC1 was purified 7.1-fold with an overall yield of 26%; BphC2 was purified 11.2-fold with an overall yield of 27%. The purified enzymes exhibited high specific activities of 21 and 13.5 U/mg for BphC1 and BphC2, respectively, to allow a subsequently detailed biochemical characterization. The SDS-PAGE of the purified enzymes showed one distinct band (Fig. 1).

3.3. Molecular mass of enzymes

The SDS-PAGE analysis showed that the purified enzymes exhibited a homogeneous band of 35 and 33 kDa for BphC1 and BphC2, respectively, which were in good agreement with the value calculated from the deduced amino acid sequence of the enzymes. The corresponding native molecular mass of BphC1 and BphC2 was estimated to be 220 ± 10 and 190 ± 20 kDa by gel filtration, respectively, indicating that both native enzymes are homohexamers.

3.4. General properties of the purified enzymes

The temperature and pH for the maximum activity of BphC1 and BphC2 were determined. The enzymes had a similar optimal pH of 9.0. BphC1 showed a maximal activity at 30 °C, whereas BphC2 was most active at 80 °C under standard assay conditions (Table 1). The thermostability of the enzymes was examined by measuring the remaining activity after incubation at various temperatures. BphC2 retained 62% and 41% of its activity after treatment for an hour at 60 and 70 °C, respectively. However, for BphC1, no activity was detected after treatment for 5 min at 60 °C. The thermostability of BphC2 at different temperature is shown in Fig. 2.

The preparation of the purified enzyme contained determinate amounts of iron (1.0 ± 0.2 mol iron per mol subunit of BphC1, 0.8 ± 0.2 mol iron per mol subunit of BphC2) and traces of copper as determined by ICP-OES. The effects of the metal cations on the enzyme’s activity were measured as described in Section 2. Both BphC1 and BphC2 were significantly inhibited by Cu$^{2+}$ (0.1 mM caused 92% inhibition). 0.1 mM of Fe$^{3+}$ inhibited 21% and 12% of activity of BphC1 and BphC2, respectively. Interestingly, 0.1 mM of Mn$^{2+}$ stimulated the activity of BphC1 by 150% and BphC2 164%, respectively, but higher concentrations (1 mM) of this metal cation were inhibitory of BphC1 (13% residual activity), and had no effect on the activity of BphC2. Co$^{2+}$ (0.1 mM) stimulated the activity of BphC2 by 154%, whereas higher concentrations (1 mM) of this metal cation inhibited this enzyme (11% residual activity); however, both 0.1 and 1 mM of Co$^{2+}$ inhibited the activity of BphC1 by 100%. At a concentration of 50 μM, H$_2$O$_2$ inhibited all BphC1 and BphC2 activity.

![Fig. 1. SDS-PAGE of purified 2,3-dihydroxybiphenyl 1,2-dioxygenase. Mr, protein marker, from top to bottom (97, 67, 45, 35, 28.5, 20, 14 KD). Lane 1, purified BphC1; Lane 2, purified BphC2.](image-url)
3.5. Disruption of bphC gene in the strain R04

To examine whether the cloned bphC genes are responsible for the biphenyl catabolism in the R04, the bphC1 gene was inactivated through deleting its internal 0.141-kb XhoI fragment. Using the sacB counter selection system, we isolated a mutant strain, Rhodococcus sp. strain R04ΔC1. A 0.78 kb of fragment was amplified using the primers of the 5'-end and the 3'-end of bphA1 from the R04ΔC1 and confirmed by sequencing. In the resting cell assay, R04 could eliminate all of 10 mM biphenyl within 30 h, whereas R04ΔC1 degraded the same amount of biphenyl after 45 h. Moreover, the biomass of R04ΔC1 was lower than that of R04 in the time course of experiments, and the amount of HOPDA in the culture of R04ΔC1 was much less than that in the culture of R04. In the same way, we obtained a strain with disruption of bphC2, R04ΔC2. It could grow on LB, but failed to grow on biphenyl. These results indicate that BphC1 plays an assistant role in the degrading of biphenyl in strain R04, while BphC2 is essential for the growth of strain R04 on biphenyl.

3.6. Substrate specificities of purified enzymes

To determine the substrate specificity, the enzymes were tested for their abilities to oxidize 2,3-dihydroxybiphenyl, catechol, 3-methylcatechol, 4-methylcatechol, and 3-chlorocatechol. The kinetic constants of BphC1 and BphC2 were summarized in Table 2. BphC1 was shown to have significantly more Km than BphC2. Both isoenzymes exhibited substrate inhibition, although inhibition was pronounced only in the case of BphC2. The catalytic efficiencies (kcat/Km) of the two isoenzymes also differed significantly, with BphC2 approximately 36-fold more efficient than BphC1 with 2,3-dihydroxybiphenyl as the substrate.

Different from previously reported results [8,19], both isoenzymes displayed higher catalytic activity with catechol as the substrate. BphC1 and BphC2 showed a catalytic efficiency against catechol (kcat/Km), 1/10 and 1/5 that of 2,3-dihydroxybiphenyl by BphC1, respectively. In contrast, 3-methylcatechol, 4-methylcatechol, and 3-chlorocatechol were poor substrates for the two isoenzymes. In the case of 3-methylcatechol, BphC1 and BphC2 showed lower catalytic efficiency with three and four orders of magnitude than that observed with 2,3-dihydroxybiphenyl as the substrate, respectively. Similarly, 4-methylcatechol, compared to 3-methylcatechol, was transformed very inefficiently by BphC1 and BphC2, with kcat/Km values three and five orders of magnitude lower than the corresponding values with 2,3-dihydroxybiphenyl, respectively. BphC1 was only inhibited by 2,3-dihydroxybiphenyl, catechol, and 3-chlorocatechol, whereas BphC2 was inhibited by all give substrates. The Km of BphC1 for 2,3-dihydroxybiphenyl exhibited almost 1/10—1/300 that for catechol, 3-methylcatechol, 3-chlorocatechol and 4-methylcatechol. Conversely, the Km of BphC2 for 2,3-dihydroxybiphenyl was found to be lower, almost 1/33 that for catechol and 1/120—1/1000 that for 3-methylcatechol, 3-chlorocatechol and 4-methylcatechol. This implied that 2,3-dihydroxybiphenyl is a better fit of the enzymes in the given substrates.

From the results (Table 3), the Km values with 2,3-dihydroxybiphenyl, catechol, 3-methylcatechol, 3-chlorocatechol and 4-methylcatechol as substrates were determined at 60 °C, and the decreases in all the Km were observed compared with those at 25 °C (Table 2). The kcat/Km values with 2,3-dihydroxybiphenyl, catechol, 3-methylcatechol, 3-chlorocatechol at 60 °C were remarkably higher than that at 25 °C, indicating that they were catalyzed compatibly by BphC2 at the higher temperature. However, with 4-methylcatechol as substrate, the kcat/Km value of BphC2 at 60 °C was similar to that at 25 °C, suggesting 4-methylcatechol is not a good substrate for BphC2 regardless of conditions of low or high temperatures.
Table 2
Kinetic constants of BphC1 and BphC2 at 25 °C

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$K_m$ (μM)</th>
<th>$K_{m*}$ (μM)</th>
<th>$k_{cat}/K_m$ (s$^{-1}$ μM$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,3-Dihydroxybiphenyl</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BphC1</td>
<td>17 ± 2$^b$</td>
<td>60 ± 20</td>
<td>6600 ± 600</td>
<td>280 ± 90</td>
</tr>
<tr>
<td>BphC2</td>
<td>12 ± 1</td>
<td>1.0 ± 0.2</td>
<td>540 ± 60</td>
<td>12,000 ± 3000</td>
</tr>
<tr>
<td>Catechol</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BphC1</td>
<td>15 ± 1</td>
<td>540 ± 50</td>
<td>7500 ± 600</td>
<td>30 ± 10</td>
</tr>
<tr>
<td>BphC2</td>
<td>24 ± 3</td>
<td>400 ± 30</td>
<td>10,000 ± 1000</td>
<td>60 ± 10</td>
</tr>
<tr>
<td>3-Methylcatechol</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BphC1</td>
<td>3.0 ± 0.1</td>
<td>14,600 ± 700</td>
<td>Not observed</td>
<td>0.2</td>
</tr>
<tr>
<td>BphC2</td>
<td>8 ± 1</td>
<td>2100 ± 50</td>
<td>4300 ± 1200</td>
<td>4.0 ± 1.0</td>
</tr>
<tr>
<td>4-Methylcatechol</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BphC1</td>
<td>2.0 ± 0.2</td>
<td>5600 ± 100</td>
<td>Not observed</td>
<td>0.3</td>
</tr>
<tr>
<td>BphC2</td>
<td>1.0 ± 0.1</td>
<td>11,000 ± 500</td>
<td>12,400 ± 1500</td>
<td>0.1</td>
</tr>
<tr>
<td>3-Chlorocatechol</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BphC1</td>
<td>4.0 ± 0.1</td>
<td>2100 ± 100</td>
<td>4200 ± 600</td>
<td>2.0 ± 0.3</td>
</tr>
<tr>
<td>BphC2</td>
<td>9 ± 2</td>
<td>1600 ± 100</td>
<td>6500 ± 800</td>
<td>6 ± 1</td>
</tr>
</tbody>
</table>

Kinetic constants represent apparent kinetic parameters.

$^a$ $k_{cat}$, substrate inhibition constant [20].

$^b$ Mean ± standard deviation.

3.7. Homology to other extradiol dioxygenases

The deduced amino acid sequences of BphC1 and BphC2 were compared with other extradiol dioxygenases. In a phylogenetic tree of extradiol dioxygenase (Fig. 3), BphC1 has a high homology with newly isolated BphC7 from R. rhodochrous K37 (85%) [24], moreover, exhibits 35%–83% identity with other extradiol dioxygenases. BphC2 exhibits 99% identity with the BphC8 of R. rhodochrous K37 [24], 62% identity with PhdF in the phthalate degradation pathway of Nocardoides sp. KP7 [25], but has no more than 42% identity with the other known extradiol dioxygenases; it even exhibits 33% identity with BphC1 from the same bacteria.

3.8. Conservation secondary elements

Secondary structure was predicted from the deduced amino acid sequence of BphC1 and BphC2 using Iprof methods. Some of the elements in the secondary structure of BphC1 (all of the α-helices; the first, second, fourth, fifth, sixth, ninth, sixteenth and seventeenth β-strands) were in close agreement with those of BphC2 (Fig. 4). The long loop present between the N- and C-terminal domains in BphC1 was also present in BphC2. However, the sequence between the third and fourth β-strands in BphC2 formed an extra β-strand, which was not found in the secondary structure of BphC1. In addition, the third, eighth, fifteenth, and nineteenth β-strands in the secondary structure of BphC2 extend to more than three amino acids compared with those in BphC1. The results revealed that amino acids with the secondary structure tendency in BphC2 were more than those in BphC1.

Table 3
Kinetic constants of BphC2 at 60 °C

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$K_m$ (μM)</th>
<th>$k_{cat}/K_m$ (s$^{-1}$ μM$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,3-Dihydroxybiphenyl</td>
<td>40 ± 8</td>
<td>0.3 ± 0.1</td>
<td>140,000 ± 18,000</td>
</tr>
<tr>
<td>Catechol</td>
<td>50 ± 9</td>
<td>120 ± 10</td>
<td>420 ± 40</td>
</tr>
<tr>
<td>3-Methylcatechol</td>
<td>14 ± 4</td>
<td>1100 ± 60</td>
<td>13 ± 3</td>
</tr>
<tr>
<td>4-Methylcatechol</td>
<td>10 ± 0.2</td>
<td>7600 ± 100</td>
<td>0.1</td>
</tr>
<tr>
<td>3-Chlorocatechol</td>
<td>15 ± 1</td>
<td>800 ± 90</td>
<td>19 ± 2</td>
</tr>
</tbody>
</table>

Kinetic constants represent apparent kinetic parameters.

4. Discussion

We cloned and characterized two 2,3-dihydroxybiphenyl 1,2-dioxygenases, BphC1 and BphC2. Sequence alignment verified that bphC2 is localized in a bph gene cluster, which consists of genes involved in biphenyl/PCB degradation, including bphA, bphB, bphC, and bphD [23], whereas bphC1 is localized out of the bph gene cluster. Although BphC1 and BphC2 are from Rhodococcus sp. R04, their characterization are very different.

As described previously, a multiplicity of extradiol dioxygenase genes have been reported in Rhodococci, such as R. sp. TA421, R. sp. RHA1, R. sp. K37 and R. globerulus P6 [8,12,13,24]. By now, there are also two extradiol dioxygenases (BphC1 and BphC2) to be found in R. sp. R04. In TA421, the bphA and bphB genes, which are required for degradation of PCB/biphenyl, accompany the bphC3_TA421 and bphC4_TA421 genes, but the role of the other five bphC genes is unknown. RHA1 are known to have fours bphC genes and two genes (bphC1_RHA1 and bphC5_RHA1) are induced by biphenyl, suggesting that both two genes are involved in biphenyl degradation [26]. K37 has eight genes encoding extradiol dioxygenase, which exhibits 2,3-dihydroxybiphenyl 1,2-dioxygenase activity, but only BphC8 is essential for the growth of K37 on biphenyl [24]. After disruption of bphC1 and bphC2 in R04, R04AC1 can utilize biphenyl as a growth substrate, while R04ΔC2 failed to grow on biphenyl, suggesting that BphC2 is essential for the growth on biphenyl rather
Fig. 3. Phylogenetic tree obtained from the alignment of BphC with related proteins. The protein sequences of the 18 2,3-dihydroxybiphenyl dioxygenases including the R04 BphC1 and BphC2 are classified. The multiple-alignment analysis and the construction of phylogenetic unrooted tree were performed with the MEGA. The scale bar indicates the percentage divergence. The sequence abbreviations, species, and GenBank references are as follows: BphC7 RK37, R. rhodochrous K37, AB271918; BphC7 RHA1, B. xenovorans M, D88017; BphC1 RK37, R. rhodochrous K37, AB271915; BphC1 RHA1, B. xenovorans M, D88018; BphC1 TA421, R. erythropus TA421, D88013; BphC2 RK37, R. rhodochrous K37, AB271916; BphC2 TA421, R. erythropus TA421, D88012; BphC4 RK37, R. rhodochrous K37, AB117722; BphC1 RRHA1, R. sp. RH1A1, D32142; BphC3 TA421, R. erythropus TA421, D88010; BphC CB-356, C. testosterone B-356, U91936; BphC POU83, P. putida OU83, X91876; BphC PKF715, P. putida 715, M33813; BphC BLB400, B. xenovorans LB400, AB37053; BphC4 TA421, R. erythropus TA421, D88016; PdbC NKF7, N. sp. KP7, AB031319; BphC8 RK37, R. rhodochrous K37, AB272984; BphC5 TA421, R. erythropus TA421, D88017; Etbc RRHA1, R. sp. RH1A1, AB120955; BphC5 RK37, R. rhodochrous K37, AB117723; BphC BJF8, B. sp. JF8, AB092521.

than BphC1 in R04. R04 with bphC1 gene deletion delayed the time of its degrading biphenyl, suggesting that BphC1 participates in the process of transformation of biphenyl at the presence of BphA, BphB, BphC2 and BphD in R04, but its primary physiological role has yet to be elucidated.

With 2,3-dihydroxybiphenyl as the substrate, kinetic data of BphC1 and BphC2 in Rhodococcus sp. R04 were in close conformity with those from purified 2,3-dihydroxybiphenyl 1,2-dioxygenases from Rhodococcus globulus strain P6 [27]. Either BphC1 or BphC2 of R. sp. R04 displayed good catalytic activity with catechol as the substrate (kcat/Km), but it was a poor substrate for all three 2,3-dihydroxybiphenyl 1,2-dioxygenases (BphC1, BphC2 and BphC3) from Rhodococcus globulus strain P6 [27]. 4-Methylcatechol was transformed inefficiently by the two isoenzymes and thus resembles BphC1, BphC2 and BphC3 of Rhodococcus globulus strain P6 [27]. 3-Methylcatechol and 3-chlorocatechol, like 4-methylcatechol, could not be transformed sufficiently by 2,3-dihydroxybiphenyl 1,2-dioxygenases from Rhodococcus sp. R04 along with the other bacteria. Both isoenzymes displayed poor catalytic activity with substituted catechols as the substrate and hence have a preference for bicyclic substrates. Moreover, BphC1 and BphC2 preferentially cleaved these substituted catechols in the order H > Cl > Me, suggesting that catalysis is affected by steric determinants.

Generally speaking, proteins from thermophilic organisms, which are functional at high temperatures, are in essence, particularly stable [28]. For example, 2,3-dihydroxybiphenyl 1,2-dioxygenase from a thermophilic bacterium Bacillus sp. JF8, is thermostable, retaining 75% of its activity after treatment at 70 °C for 60 min [29]. Furthermore, there has been no report that BphC from mesophilic organism are thermostable. For instance, BphC of Pseudomonas putida OU83 (which exhibits 94% identity to BphC LB400) lost 47% of its activity at 37 °C and all activity at 65 °C [30]. In the same way, BphCII from Rhodococcus globulus P6 retained only 10% activity after 10 min of incubation at 50 °C [19]. BphC1 of Rhodococcus sp. R04 likewise lost all activity after 5 min of incubation at 60 °C in the study. Interestingly, BphC2 from a mesophilic bacterium, Rhodococcus sp. R04, retained 50% of its activity after treatment at 70 °C for 70 min, implying it to be a thermostable 2,3-dihydroxybiphenyl 1,2-dioxygenase. Overall, increases in Km with temperature have previously been demonstrated for bacterial and eukaryotic enzymes [31]. However, each individual enzyme exhibited a distinct Km variation with the temperature change, the Km decreased with increase in temperature have previously been reported for various enzymes including the 3-phosphoglycerate kinase of Zymomonas mobilis, 2,3-dihydroxybiphenyl 1,2-dioxygenase of Bacillus sp. JF8 [29,31], which had a lower Km as the temperature approached the optimum temperature. The results similar to those reported for Km had been observed for BphC2 in the study.

Here we determined the stabilities for oxidation of BphC1 and BphC2 from R04. We found that their activities were reduced by 100% at a concentration of 50 μM of H2O2. The same results were observed by Asturias, at a concentration of 11 μM, H2O2 reduced the specific activity of 2,3-dihydroxybiphenyl 1,2-dioxygenase II from R. globulus P6 by 88% [27]. However, when incubated with 0.1 mM H2O2, the thermostable Mn (II)-independent 2,3-dihydroxybiphenyl 1,2-dioxygenase from Bacillus sp. JF8 (BphC_JF8) was not inhibited, although 1 mM H2O2 for 60 min resulted in weak inhibition (86% residual activity) [29]. BphC2 together with BphC_JF8 was the thermostable 2,3-dihydroxybiphenyl 1,2-dioxygenase, but their activity center differed, BphC2 contains Fe(II) as a catalytic metal center, whereas BphC_JF8 was manganese-dependent. Incubation with H2O2, Fe(II) was oxidized into Fe(III), thereby BphC2 lost its activity, however, Mn(II) was not oxidized easily by a lower concentration H2O2. Therefore, the ability for antioxidation of BphC_JF8 was stronger than that of BphC2 and other Fe(II)-independent mesophilic BphC.
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