Genetic organization and characteristics of the 3-(3-hydroxyphenyl)propionic acid degradation pathway of Comamonas testosteroni TA441

Hiroyuki Arai, Takako Yamamoto, Tohru Ohishi, Takeshi Shimizu, and Toshiaki Kudo

Author for correspondence: Toshiaki Kudo. Tel: +81 48 467 9544. Fax: +81 48 462 4672.

Comamonas testosteroni TA441 degrades 3-(3-hydroxyphenyl)propionate (3HPP) via the meta pathway. A gene cluster required for degradation of 3HPP was cloned from strain TA441 and sequenced. The genes encoding six catabolic enzymes, a flavin-type hydroxylase (mhpA), extradiol dioxygenase (mhpB), 2-keto-4-pentenoate hydratase (mhpD), acetaldehyde dehydrogenase (acylating) (mhpF), 4-hydroxy-2-ketovalerate aldolase (mhpE) and the meta cleavage compound hydrolase (mhpC), were found in this cluster, encoded in this order. mhpD and mhpF were separated by two genes, orf4 and orf5, which were not necessary for growth on 3HPP. The gene mhpR, encoding a putative transcriptional activator of the IclR family, was located adjacent to mhpA in the opposite orientation. Disruption of the mhpB or mhpR genes affected growth on 3HPP or trans-3-hydroxycinnamate. The mhpB and mhpC gene products showed high specificity for 3-(2,3-dihydroxyphenyl)propionate (DHPH) and the meta cleavage compound produced from DHPH, respectively.

Keywords: 3-(3-hydroxyphenyl)propionate, trans-3-hydroxycinnamate, meta pathway, biodegradation, Comamonas testosteroni

INTRODUCTION

Phenylalkanoic acids and their hydroxylated derivatives are ubiquitous in the environment as breakdown products of lignin or other plant-derived phenylpropanoids and flavonoids. Bacterial degradation of such compounds is important for recycling of carbon sources on earth; however, little is known about the genes required for degradation of phenylalkanoic acids. The genes for degradation of 3-(3-hydroxyphenyl)propionate (3HPP) in Escherichia coli K-12 (Ferrández et al., 1997) and Rhodococcus globerulus PWD1 (Barnes et al., 1997), and the genes for degradation of 4-hydroxyphenylacetate in E. coli W (Prieto et al., 1996) have been reported. Although the ability of pseudomonads and related bacteria to degrade aromatic compounds has been well studied, the existence of genes for degradation of natural phenylalkanoic acids in pseudomonads has not been reported. Comamonas [formerly Pseudomonas (Tamaoka et al., 1987)] testosteroni TA441 was isolated from a homogenate of the gut of the wood-feeding termite Reticulitermes speratus together with a biphenyl-degrading bacterium Rhodooccus erythropolis TA431 (Arai et al., 1998; Chung et al., 1994). Strain TA441 was found to display extradiol dioxygenase activity, as colonies on LB plates turned yellow after being sprayed with 2,3-dihydroxybenzyl (DHB), but this strain is unable to grow on biphenyl as sole carbon source (unpublished data). Because there is an abundance of ligninolytic compounds in the gut of wood-feeding termites, bacteria in the termite ecosystem are expected to display high activity in degrading phenylalkanoic acids. Actually, strain TA441 is capable of growth on 3HPP, 3-hydroxycinnamate (3HCl) or 3-hydroxyphenylacetate (3HPA). 3HPP was found to induce extradiol dioxygenase activity in strain TA441, indicating that 3HPP is degraded by the meta pathway. In this study, we cloned and sequenced a gene cluster.

Abbreviations: 3HCl, trans-3-hydroxycinnamate; 3HPA, 3-hydroxyphenylacetate; 3HPP, 3-(3-hydroxyphenyl)propionate; 3MC, 3-methylcatechol; 4MC, 4-methylcatechol; DHPP, 2,3-dihydroxybiphenyl; DHCl, trans-2,3-dihydroxyacetamine; DHPP, 3-(2,3-dihydroxyphenyl)propionate; CFE, cell-free extract.

The DDBJ/EMBL/GenBank accession number for the sequence reported in this paper is AB024335.

1,2 Microbiology Laboratory and Synthetic Organic Chemistry Laboratory, The Institute of Physical and Chemical Research (RIKEN), Hirosawa 2-1, Wako, Saitama 351-0198, Japan

† Present address: Department of Biotechnology, University of Tokyo, Yayoi 1-1-1, Bunkyo-ku, Tokyo 113-8657, Japan.

Keywords: 3-(3-hydroxyphenyl)propionate, trans-3-hydroxycinnamate, meta pathway, biodegradation, Comamonas testosteroni
that encodes the enzymes for the complete degradation pathway of 3HPP and 3HCl.

**METHODS**

**Bacterial strains, plasmids and growth conditions.** The bacterial strains and plasmids used in this study are listed in Table 1. *C. testosteroni* strains were cultivated in LB medium or C-medium at 30 °C. C-medium was supplemented with 0.1% succinate, 3HPP, 3HCl or 3HPA as carbon source. Growth was monitored by measuring OD₅₅₀. *E. coli* JM109, used as the host for gene manipulation (Sambrook et al., 1989), was cultivated in LB medium at 37 °C. The medium composition and concentration of antibiotics were described previously (Arat et al., 1998).

**Chemicals.** 3HPP and 3-(2,3-dihydroxyphenyl)propionate (DHPP) were synthesized by the procedures described by Blakley & Simpson (1964) and Burlingame & Chapman (1983). Other chemicals were purchased from Tokyo Kasei or Sigma.

**Preparation of cell-free extract.** For preparation of cell-free extract (CFE), bacterial cells were cultured overnight. The cells were harvested by centrifugation, washed with 50 mM potassium phosphate buffer (pH 7.5), resuspended in the same buffer and disrupted by sonication. The cell debris was removed by centrifugation and the supernatant was used as CFE. Protein concentration was determined by the method of Bradfod (1976) using the Bio-Rad protein assay kit, with bovine serum albumin as standard.

**Enzyme assays.** Extradiol dioxygenase activity was determined spectrophotometrically by monitoring the increase in the yellow ring-fission products upon addition of CFE to 50 mM potassium phosphate buffer (pH 7.5) containing 0.8 mM diol substrate at 30 °C. The molar extinction coefficients (ε) for the ring-fission products were as follows (M⁻¹ cm⁻¹): DHBP, ε₄₃₈ = 13200; DHPP, ε₃₉₄ = 19150; catechol, ε₃₈₅ = 36000; 3-methylcatechol (3MC), ε₃₈₈ = 15000; 4-methylcatechol (4MC), ε₃₇₅ = 13500 (Barnes et al., 1997; Elitis et al., 1993).

The substrates for the assay of hydrolyase activity were prepared enzymically from diol compounds using recombinant extradiol dioxygenases. pYT103, pYT40, pUC18-xyfE and pUC19-todE express DHPP dioxygenase of strain TA441, DHBP dioxygenase of *R. erythropolis* TA421 (Maeda et al., 1995), catechol 2,3-dioxygenase of the TOL plasmid (Burlage et al., 1989) and methylcatechol dioxygenase of *Pseudomonas putida* F1 (Zylstra & Gibson, 1989), respectively. DHPP, DHBP, catechol or 3MC were converted to the ring-fission products in 50 mM potassium phosphate buffer (pH 7.5) using the CFE of *E. coli* JM109 harbouring the plasmid encoding the appropriate dioxygenase. After the ring-fission reaction was completed, the CFE for the hydrolyase assay was added to the reaction mixture. The activity was measured spectrophotometrically by monitoring the disappearance of the yellow ring-fission compounds.

**Recombinant DNA techniques.** DNA manipulations were performed by standard methods (Sambrook et al., 1989) or as described previously (Arat et al., 1998). An ABI 377 DNA sequencer was used for sequence determination (Applied Biosystems). A BigDye terminator cycle sequence kit (Perkin-Elmer) was used for dideoxy chain-termination reactions. Synthetic oligonucleotides used as primers for sequence determination were purchased from Sawady Technology.

**Cloning of the mhp gene cluster.** For isolation of the extradiol dioxygenase gene (*mhpB*), total DNA of strain TA441 was digested with Sall and ligated with the Sall digest of pUC19. *

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<tr>
<th>Table 1. Bacterial strains and plasmids</th>
<th>Relevant characteristics*</th>
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<td><strong>C. testosteroni</strong></td>
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<td>TA441</td>
<td>Wild-type (JCM9806)</td>
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<td>TTM101</td>
<td>mhpB mutant of TA441; Km*</td>
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<td>TTM107</td>
<td>orf4, orf5 mutant of TA441; Km*</td>
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<td>TTM112</td>
<td>mhpR mutant of TA441; Km*</td>
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<td>todE from <em>P. putida</em> F1 in pUC19</td>
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* Ap*, ampicillin resistance; Km*, kanamycin resistance.
coli JM109 was transformed with the ligation mixture and plated onto LB agar plates containing ampicillin (Ap) and IPTG. Colonies that expressed the extradiol dioxygenase were identified by spraying with a DHBP solution (20 mM in acetone). A 4.3 kb SalI fragment (pYT10) was obtained from the plasmid in a positive clone which turned yellow. A 5.5 kb BglI fragment (pYT11) and a 6.0 kb BglII fragment (pYT12), which hybridized with the upstream SalI–SacI and the downstream SacI–SalI fragment of pYT10, respectively, were cloned from the total DNA of strain TA441 and identified by colony hybridization.

Construction of plasmids and mutant strains. The mhpB gene of pYT10 was disrupted by insertion of a blunt-ended 1.5 kb HindIII–SalI fragment of pSUP5011, which contained a kanamycin (Km) resistance gene (Simon, 1984), into the EcoRV site (Fig. 1). The resultant plasmid, pYT101, was introduced into strain TA441 by electroporation and the transformed cells were plated on agar plates containing Km. The Km-resistant colonies were transferred to plates containing carbenicillin (Cb). A Cb-sensitive and Km-resistant colony, whose mhpB gene was exchanged for the disrupted mhpB gene by double crossover reactions, was selected and designated as strain TTM101. orf4 and orf5 were disrupted in the same way by use of pYT107, which was constructed by replacement of the BglII–Bpu1102I fragment with the Km resistance gene (Fig. 1). The resultant mutant was designated strain TTM107. The mhpR mutant strain TTM112 was constructed by use of pYT112, whose mhpR gene was disrupted by insertion of the Km resistance gene into the chromosome of the mutant strains was confirmed by PCR (data not shown).

pYT103 carries a PCR fragment containing the mhpB gene. The fragment was amplified with oligonucleotides YP4 (GGCCAGGGATCTGGCATCCAGCGACTTGG) and YP5 (CGGATGGAATTCTCATGAAAGGATGAG) from a fresh colony of strain TA441. The amplified 1.0 kb fragment was digested with BamHI and EcoRI, and ligated to the respective site of pUC19, resulting in pYT103. pYT121, which was used for expression of the mhpC gene in E. coli, was constructed by subcloning a 1.5 kb SalI–KpnI fragment into pUC19 (Fig. 1). pUC18-xyIE was constructed by ligation of a 1.8 kb BamHI–XbaI fragment of pTS1045 that carries the xyle gene (Inouye et al., 1986) with BamHI/SalI-digested pUC18. pUC19-todE was constructed by subcloning a BamHI/EcoRI digest of the 1.0 kb PCR fragment, which carries todE of P. putida F1 (Zylstra & Gibson, 1989), into pUC19. The fragment was amplified from a fresh colony of strain F1 with oligonucleotides TodE1 (GGCGCACGGATCCACAAGCACTTT) and TodE2 (CATATGGGAATTCAGGTAGAAGC). pYT101, pYT107 and pYT112 were used for construction of mutant strains TTM101, TTM107 and TTM112, respectively, by homologous recombination.

RESULTS AND DISCUSSION

Induction of extradiol dioxygenase and hydrolase enzymic activities by 3HPP

3HPP is degraded to intermediates of the TCA cycle by the meta pathway in E. coli K-12 and in R. globerulus PWD1 (Barney et al., 1997; Burlingame & Chapman, 1983; Ferrández et al., 1997) (Fig. 2). 3HPP is initially converted to DHPP by 3HPP hydroxylase. Extradiol dioxygenase catalyses the meta fission of DHPP. The ring-fission product is converted to succinate and 2-keto-4-pentenoate by a hydrolase. The three downstream reactions catalysed by hydratase, aldolase and acetaldehyde dehydrogenase (acylating) are the same as the reactions in the pathway for meta degradation of other aromatic compounds such as biphenyl, phenol and toluene (Lau et al., 1994; Hofer et al., 1994; Shingler et al., 1992). When strain TA441 was cultivated with 3HPP as carbon source, DHPP dioxygenase and hydrolase activities were detected (0.16 and 0.35 µmol min⁻¹ mg⁻¹, respectively), whereas these activities were not detected when the strain was culturally with succinate as carbon
source. These results indicated that 3HPP is degraded by the meta pathway in strain TA441, as in E. coli K-12 and R. gilberul tus PWD1.

**Cloning and sequencing of the 3HPP degradation gene cluster**

A 4.3 kb SalI fragment encoding the extradiol dioxygenase was cloned from the total DNA of strain TA441 (pYT10, Fig. 1). Analysis of the nucleotide and deduced amino acid sequences revealed that the dioxygenase gene (*mhpB*) was accompanied by genes encoding proteins similar to the single-component hydroxylase (*mhpA*) and the 2-keto-4-pentenoate hydratase (*mhpD*). BgIII fragments encoding the regions upstream (pYT11) and downstream (pYT12) of the *mhpB* gene were cloned using two SalI–SacI fragments of pYT10 as probes. A putative regulatory gene, *mhpR*, was located adjacent to *mhpA*, in the opposite orientation. The *mhpFEC* genes, expected to encode acetaldheyde dehydrogenase (acylating), 4-hydroxy-2-ketovalerate aldolase and 2-hydroxy-6-ketona-2,4-dienoate hydratase, respectively, were located about 2 kb downstream of the *mhpD* gene. This gene arrangement is different from that of *E. coli* K-12 (*mhpABCDFE*) (Ferrández et al., 1997). The *mhpC* gene of strain TA441 is moved to the end of the cluster and two ORFs (*orf4* and *orf5*) encoding proteins of unknown function are moved to the end of the cluster and two ORFs (*orf4* and *orf5*) encoding proteins of unknown function are...
inserted between mhpD and mhpF. The arrangement is also different from that of the 3HPP catabolic genes (hppACBKR) of R. globulerus PWD1, in which the genes for the three downstream enzymes (hydratase, aldolase and acetaldehyde dehydrogenase) are not located within the cluster (Barnes et al., 1997). The gene corresponding to orfT of E. coli K-12 and hppK of strain PWD1, encoding a putative transporter, was not found in the fragments cloned from strain TA441. It is not certain at present whether strain TA441 expresses a specific transporter for intake of 3HPP.

Sequence comparisons

The deduced amino acid sequences of the mhp genes from strain TA441 were compared with the genes for other aromatic hydrocarbon degradation enzymes (Table 2). The mhpA gene encodes a protein consisting of 589 aa residues showing a high level of identity with the 3HPP hydroxylase of E. coli K-12 (MhpA) and that of R. globulerus PWD1 (HppA) (Barnes et al., 1997; Ferrández et al., 1997), suggesting that MhpA of strain TA441 is a 3HPP hydroxylase. MhpA also shows low similarity to single-component flavin-type hydroxylases, including phenol hydroxylase (PheA) from P. putida EST1001 (Nurk et al., 1991) and 2,4-dichlorophenoxyacetate hydroxylase (TfdB) fromRalstoniaeutrophaJMP134 (Perkins et al., 1990). The mhpB gene encodes an extradiol dioxygenase consisting of 321 aa residues categorized as a type II (class III) dioxygenase (Elitis & Bolin, 1996). The phylogenetic tree of this type of dioxygenase is shown in Fig. 3. MhpB from strain TA441 is closely related to dioxygenases with high specificity for DHP (MhpB from E. coli, HppB and Mpcl) (Barnes et al., 1997; Ferrández et al., 1997; Spence et al., 1996) and is distantly related to a subunit of the protocatechuate 4,5-dioxygenase (LigB) from Sphingomonas paucimobilis (Noda et al., 1990) and the 3,4-dihydroxyphenylacetate 2,3-dioxygenase (HpaD and HpcB) from E. coli strains (Prieto et al., 1996; Roper & Cooper, 1990; Roper et al., 1995). EdoD from Rhodococcus sp. II was most similar to MhpB of strain TA441 (Table 2); however, the physiological role of the protein has not been identified (Kulakov et al., 1998).

The mhpC gene encodes a protein consisting of 295 aa residues. The deduced sequence of the gene is homologous with the meta fission product hydrolases involved in degradation of aromatic compounds such as 3HPP (MhpC from E. coli and HppC), biphenyl (BphD), toluene (XylF) and phenol (DmpD). The mhpD, mhpE and mhpF genes encode proteins highly similar to hydratase, aldolase and acetaldehyde dehydrogenase (acylating), respectively. These three enzymic reactions are common to other meta degradation pathways for various aromatic compounds.

The mhpR gene encodes a protein consisting of 261 aa residues that belongs to the IclR family of transcriptional regulators. This family includes regulators for degradation of 4-hydroxybenzoate (PobR) and protocatechuate (PcaU and PcaR) (DiMarco et al., 1993; Harwood et al., 1994; Kowalchuk et al., 1994). The product of the corresponding gene from E. coli K-12 was identified as a transcriptional activator that regulates transcription of the mhp genes (Ferrández et al., 1997). Because expression of the enzymes for 3HPP degradation is responsive to 3HPP, it seems likely that MhpR acts as a regulator for the mhp genes in strain TA441, as in the case of E. coli.

The two ORFs, orf4 and orf5, encode proteins consisting of 325 and 278 aa residues, respectively. The deduced sequences of orf4 and orf5 are highly similar to each other, with 63% of the amino acid residues being identical. The orf4 gene product has a typical N-terminal signal sequence for membrane translocation, whereas the deduced sequence of orf5 lacks the N-terminal signal, indicating that the orf4 and orf5 gene products are probably located in the periplasm and in the cytoplasm, respectively. Genes similar to orf4 and orf5 have been found in the gene cluster for the ortho degradation pathway of chlorocatechol in Pseudomonas sp. P51 (ORF3 of the tcb gene cluster) (van der Meer et al., 1991) and in the catabolic plasmid pAC27 (ORF3 of
Table 3. Substrate specificity of MhpB and MhpC

*Hydrolase activities were measured by monitoring the decrease of the ring-fission product of the diol substrate, which was prepared enzymically as described in Methods. ND, Not determined.

Substrate specificity of the mhpB and mhpC gene products

The mhpB and mhpC gene fragments were cloned in pUC19, resulting in pYT103 and pYT121, respectively (Fig. 1). CFEs of E. coli JM109 harbouring the plasmids were prepared from cells grown in LB medium containing 0.5 mM IPTG. The extradiol dioxygenase activity in the CFE of JM109(pYT103) and the hydrolase activity in the CFE of JM109(pYT121) were measured with several substrates. The activities of DHPP dioxygenase and 2-hydroxy-6-ketonona-2,4-dienedioate hydrolase of strain JM109 harbouring pUC19 were not detected under the culture conditions used, indicating that the corresponding enzymes derived from the E. coli chromosome were not expressed (data not shown). Table 3 shows the substrate specificity of the recombinant enzymes. DHPP was the most preferable substrate for MhpB. MhpB also showed relatively high activity with DHBP as substrate, but the activity with catechol or methylcatechols was low. MhpC was highly specific for the meta cleavage compound produced from DHPP (2-hydroxy-6-ketonona-2,4-dienedioate) and showed very low activity with other substrates.

The mhp gene cluster is involved in utilization of 3HPP and 3HCl

The mutant strains TTM101 (mhpB<sup>−</sup>), TTM107 (orf4<sup>−</sup> orf5<sup>−</sup>) and TTM112 (mhpR<sup>−</sup>) were constructed by insertion of the Km resistance gene by homologous recombination. The growth curves of the strains on 3HPP, 3HCl or 3HPA are shown in Fig. 4. Strains TA441 and TTM101 showed identical growth curves when 3HPA was used as a carbon source. They also
grew equally well on succinate or 4-hydroxybenzoate (data not shown). On the contrary, the growth of strain TTM101 was severely affected when 3HPP or 3HCl was used as carbon source (Fig. 4b). These results clearly indicate that the mhp gene cluster in strain TA441 is involved in utilization of 3HPP and 3HCl. The culture medium of strain TTM101 grown on 3HPP or 3HCl turned brown, which caused a gradual increase in optical density. Strain TTM101 could produce DHPP and 2,3-dihydroxybenzonic acid (DHCl) from 3HPP and 3HCl, respectively, because the gene for 3HPP hydroxylase (mhpA) is intact in this strain. The brownish compounds were probably spontaneously oxidized products derived from these catechols. A yellow meta cleavage product accumulated when strain TA441 was grown on 3HCl, indicating that the hydrolase (MhpC) has relatively low specificity for the meta cleavage compound produced from DHCl compared to that produced from DHPP.

Elimination of a region containing orf4 and orf5 had no effect on the utilization of 3HPP or 3HCl (Fig. 4c), therefore the role of these ORFs is still unclear. Strain TTM112 showed poor growth on 3HPP or 3HCl (Fig. 4d). This observation suggests that the putative transcriptional regulator encoded by the mhpR gene is an activator. Accumulation of the brownish compound, which was found with strain TTM101, was not observed, suggesting that the expression of 3HPP hydroxylase (MhpA) may be affected also by the mhpR mutation. The mhpR and mhpA genes are transcribed divergently. Probably, MhpR acts on a putative operator located in the mhpR–mhpA intergenic region.

We have reported another gene cluster (aphKLMNO-MhpA) encoding a set of enzymes, including a hydroxylase and an extradiol dioxygenase, which is required for adaptive growth of strain TA441 on phenol (Arai et al., 1998). The phenol hydroxylase encoded by the aphKLMNOP genes belongs to a family of multicomponent-type monooxygenases that differ from the flavin-type MhpA. The aphB gene encodes catechol 2,3-dioxygenase which has low specificity for substituted catechols. Möbus et al. (1997) reported that a protein (TIP1), whose N-terminal sequence was similar to that of the DHBP dioxygenase, was expressed when C. testosteroni was grown with testosterone as a carbon source. Comparing the N-terminal sequences, neither that of MhpB nor that of AphB was identical to that of TIP1. The extradiol dioxygenase activity was found to be induced when strain TA441 was grown on steroids such as testosterone or bile acids as a carbon source. The aphB mhpB double mutant of strain TA441 could also grow on steroids and expressed dioxygenase activity (unpublished data). These results indicate that strain TA441 has at least one more meta cleavage enzyme involved in steroid utilization.

ACKNOWLEDGEMENTS

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biphenyl genes involved in the last three steps of toluene degradation by Escherichia coli chlorinated substrates.

Sequence analysis of the gene cluster, which encodes metabolism of chlorinated catechols: 2-hydroxylase and dichlorocatechol oxidative operons of plasmid pUA2820.


