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ORIGINAL ARTICLE

Characterization of a protocatechuate catabolic gene cluster in *Rhodococcus ruber* OA1 involved in naphthalene degradation

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Abstract In this study, we investigated a protocatechuate catabolic gene cluster involved in naphthalene degradation in *Rhodococcus ruber* OA1. *Rhodococcus ruber* OA1 was isolated from the pharmaceutical wastewater treatment plant of Xinhua Pharmaceutical Co., Ltd. (Zibo, China). Substrate utilization tests showed that OA1 utilizes naphthalene, phenol, benzoate, salicylate, and protocatechuate as the sole carbon and energy sources for growth. A degradation assay revealed that phthalate is an intermediate in naphthalene degradation and that the protocatechuate pathway plays an important role in naphthalene degradation. To determine the genetic basis and regulation of protocatechuate catabolism in OA1, a fosmid genomic library was constructed and a positive clone carrying the protocatechuate degradation gene cluster was isolated. Sequencing and a bioinformatics analysis identified the complete gene cluster, *pcaJIGHBARC*, responsible for protocatechuate degradation. Based on this gene cluster, the genes *pcaGH* (encoding the α and β subunits of protocatechuate 3,4-dioxygenase, 3,4-PCD) were coexpressed and the expressed products showed 3,4-PCD activity. This study illustrates a potential pathway of naphthalene degradation and identifies a protocatechuate pathway in *Rhodococcus ruber* OA1 for the first time, thus extending our understanding of polycyclic aromatic hydrocarbon degradation and the related aromatic compounds degraded in the process.

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Introduction

Aromatic compounds, such as polycyclic aromatic hydrocarbons (PAHs) and aromatic acids, are spread widely by the combustion of fossil fuels, chemical production, pharmaceutical processes, etc. They are raising increasing concerns, not only because they have toxic effects on ecosystems and human health, but also because the high thermodynamic stability of the benzene moiety makes them relatively persistent in the environment (Zhu et al. 2011). With their long-term bioaccumulation and biomagnification, some are even mutagenic or carcinogenic (Burchiel and Luster 2001; Jones et al. 2011; Lehner et al. 2014). These properties have prompted researchers to find efficient ways of eliminating aromatic contaminants from the environment.

Biodegradation is considered the most efficient way of eliminating aromatic pollutants (Habe and Omori 2003; Seo et al. 2007, 2009; Haritash and Kaushik 2009; Kanaly and Harayama 2010). The microbial degradation of PAHs is well documented in Gram-negative bacteria, such as *Pseudomonas putida* OUS82 (Kiyohara et al. 1994; Takizawa et al. 1994; Tay et al. 2014), *Burkholderia* sp. RP007 (Laurie and Lloyd-Jones 1999), *Acidovorax* sp. NA3 (Singleton et al. 2009), and *Novosphingobium pentaromativorans* US6-1 (Yun et al. 2014). Most Gram-negative PAH degraders express similar PAH catabolic pathways. In the first step of catabolism of the model compound naphthalene, an oxygen molecule is introduced at the 1,2 position of the aromatic nucleus by naphthalene dioxygenase (NDO) to produce *cis*-naphthalene dihydrodiol (Kauppi et al. 1998; Parales et al. 1999). *cis*-Naphthalene dihydrodiol is transformed into 1,2-

dihydroxynaphthlene and subsequent intermediates, resulting in the production of salicylaldehyde, which is then transformed to salicylate by salicylaldehyde dehydrogenase. Salicylate is then catabolized via catechol or gentisic acid to tricarboxylic acid (TCA) cycle intermediates. The catabolic enzymes in the pathway are encoded by similar gene clusters in the Gram-negative degraders, which have been identified with molecular biological techniques (Meyer et al. 1999; Moser and Stahl 2001).

Some Gram-positive bacteria, such as *Rhodococcus* NCIMB12038, *Nocardioides* KP7, and *Mycobacterium* PYR-1, also degrade PAHs such as naphthalene, phynanthrene, anthracene, fluoranthene, pyrene, benzo[a]pyrene, etc. (Iwabuchi and Harayama 1997, 1998; Iwabuchi et al. 1998; Larkin et al. 1999; Uz et al. 2000; Kim et al. 2008). However, the genetic organization of the PAH catabolic enzymes is more diverse in Gram-positive degraders than in Gram-negative degraders, and have diverse substrate profiles. Naphthalene is mainly degraded through the catechol or gentisic acid pathway (Kulakov et al. 2005; Kweon et al. 2007). It was recently reported that a homologue of the *nidA* gene is amplified in *Rhodococcus wratislaviensis* IFP 2016, which degrades 11 compounds, including naphthalene, but the genetic background and degradation pathway remain unclear (Auffret et al. 2009).

In this study, a Gram-positive PAH degrader, *Rhodococcus ruber* OA1, was isolated from the activated sludge of the wastewater treatment plant of Xinhua Pharmaceutical Co., Ltd (Zibo, China). The research revealed that OA1 can degrade a variety of aromatic compounds, including naphthalene. Interestingly, phthalate, whose downstream product is usually protocatechuate in the phenanthrene degradation pathways of other PAH degraders, was shown to be an intermediate of naphthalene degradation in *R. ruber* OA1, suggesting that the protocatechuate pathway plays a role in naphthalene degradation. With the purpose of determining the actual pathway(s) of naphthalene degradation and the molecular mechanism in OA1, the strain was identified, the degradation metabolites were determined, the related enzymes activities were analyzed, and the gene cluster encoding the key enzyme (protocatechuate 3,4-dioxygenase) involved in naphthalene degradation in OA1 was characterized. We identified the protocatechuate pathway and the *pca* gene cluster in *R. ruber* OA1, advancing the research into protocatechuate and naphthalene degradation by *Rhodococcus* and other Gram-positive bacteria.

Materials and Methods

Strain isolation and identification and substrate utilization

To isolate the strain, activated sludge was sampled from the pharmaceutical wastewater treatment plant of Xinhua Pharmaceutical Co., Ltd. (Zibo, China), which produces amidopyrine, analgin, aspirin, hydrocortisone, ibuprofen, pipemidic acid, theophylline, etc. We created a tenfold dilution series of the sludge, and the diluted solutions were spread on naphthalene-supplemented mineral medium plates: 1.0 g of NH_4NO_3, 0.5 g of KH_2PO_4, 0.5 g of K_2HPO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.02 g of $CaCl_2$, 15 g of agar, and 1 ml of trace element solution per liter (pH 7.0–7.2). The trace element solution contained 1 g of $FeSO_4 \cdot 7H_2O$, 1 g of $MnSO_4 \cdot H_2O$, 0.25 g of $Na_2Mo \cdot 2H_2O$, 0.1 g of H_3BO_3, 0.25 g of $CuCl_2 \cdot 2H_2O$, 0.25 g of $ZnCl_2$, 0.1 g of NH_4VO_3, 0.25 g of $CoCl_2 \cdot 6H_2O$, 0.1 g of $NiSO_4 \cdot 6H_2O$, and 5 ml of H_2SO_4 per liter of distilled water. Naphthalene was dissolved in hexane, filter sterilized, and added to the medium as the sole carbon source at a concentration of 500 mg/l. To eliminate the solvent, the solution was incubated overnight before inoculation. Bacterial strain OA1 was isolated from one of the plates. OA1 was further purified with the streaking plate method on a naphthalene-supplemented mineral medium plate.

To identify the isolate, its genomic DNA was extracted with the phenol–chloroform–isopentanol method from OA1 grown in LB medium. A 16S rRNA gene fragment was amplified and sequenced with primers 27 F 5′-AGAGTTTGATCCTGGCTCAG-3′ and 1541R 5′-AAGGAGGTGATCCAGCCGCA-3′ (Weisburg et al. 1991; Dastgheib et al. 2011). The morphological characteristics of the strain were evaluated under a normal optical microscope. The physiological and biochemical properties were determined with the API Coryne system (BioMerieux S.A., Marcy l'Etoile, France, version 4.0), according to the manufacturer's instructions.

Liquid mineral medium containing a 500 mg/l carbon source was inoculated with the isolated OA1 to test its substrate utilization. The substrates for these tests included naphthalene, phynanthrene, pyrene, phenol, benzoate, salicylate, protocatechuate, and 2-chlorobenzoate. Substrate utilization was determined by measuring the substrate degradation and cell growth. All determinations were made in triplicate with 10 % inoculum.

Phenol, benzoate, salicylate, protocatechuate, and 2-chlorobenzoate were detected with an UV–visible spectrophotometer (U-3310, Hitachi) at wavelengths of 225 nm, 225 nm, 298 nm, 252 nm, and 215 nm, respectively. Naphthalene was detected with a gas chromatograph (GC-2010, Shimadzu) (Hedlund et al. 1999). Phynanthrene and pyrene were detected with a high-performance liquid chromatography (HPLC) system (Waters) with a reversed-phase C18 column (5 μm, 4.6×150 mm) (Dastgheib et al. 2012). Cell growth was determined by monitoring the optical density of the cultures at 600 nm (OD_{600}).

Naphthalene degradation assay

On days 0, 6, and 16, 100 ml of culture grown in naphthalene-supplemented (2000 mg/l) mineral medium was extracted with three equal volumes of ethyl acetate. The aqueous fraction was acidified to pH 2.0 with HCl and extracted again with three equal volumes of ethyl acetate. The residual extract was dried over anhydrous Na_2SO_4 and concentrated at room temperature.

Naphthalene and the extracted metabolites were analyzed with gas chromatography–mass spectrometry (GC–MS; QP-2010plus, Shimadzu) and HPLC. For the GC–MS analysis, the prepared extracts were dried and dissolved in methanol. The GC temperature program was 80 °C (isothermal for 2 min), 80–280 °C (15 °C/min), and 280 °C (isothermal for 2 min). Helium was used as the carrier gas, at a flow rate of 1 ml/min. The following conditions were set for the mass analysis: ionization mode, EI+; ionizing electron energy, 70 eV; source temperature, 230 °C; and mass range, m/z 30–450. For the HPLC analysis, a reversed-phase C18 column (5 μm, 4.6×150 mm) was used. Elution was performed with a mobile phase consisting of eluent A (1 % acetic acid in water) and eluent B (1 % acetic acid in methanol) with an HPLC pump system (Waters 600 Controller). Gradient elution was performed as follows: linear gradient from 90 % to 50 % A at a flow rate of 0.8 ml/min at 0–30 min; 50 % A at a gradient flow rate from 0.8 ml/min to 1.0 ml/min at 30–35 min; 50 %–10 % A at a flow rate of 1.0 ml/min at 35–55 min; 10 % A at a gradient flow rate from 1.0 ml/min to 0.8 ml/min at 55–60 min; and a linear gradient from 10 % to 90 % A at a flow rate of 0.8 ml/min at 60–65 min. A Waters 2996 Photodiode Array Detector was used for UV detection at 275 nm. The biomass of OA1 grown in naphthalene-supplemented mineral medium was determined with dry weight measurements (Jackson et al. 1999).

Enzyme assays

Enzyme activities were analyzed with a spectrophotometer (1510, Thermo Fisher) at 30 °C. Intradiol catechol 1,2-dioxygenase (C12O) activity was assayed by measuring the increase in absorbance at 260 nm (Dorn and Knackmuss 1978). Extradiol catechol 2,3-dioxygenase (C23O) activity was monitored under the same conditions, except that the increase in product absorbance was monitored at 375 nm (Strachan et al. 1998). The activity of gentisate dioxygenase (GDO) was assayed by measuring the increase in absorbance at 334 nm, attributable to the appearance of maleylpyruvate. The activity of protocatechuate dioxygenase (PCD) was assayed by measuring the increase in absorbance at 270 nm, attributable to the production of 3-carboxy-cis, cis-muconate (Strachan et al. 1998). The reactions were performed in 50 mM potassium phosphate buffer (pH 7) with 300 μl of enzyme solution, and were initiated by the addition of 10 μl of 10 mM substrate. One unit of enzyme activity was defined as the amount of enzyme catalyzing the release of 1 nmol of product per minute under the specified assay conditions. The protein concentrations were determined with the method of Bradford (1976).

Genomic library construction

Genomic DNA was extracted from *R. ruber* OA1 cells grown in LB medium with the method of Marmur (1961). The DNA was sheared to about 40-kb fragments by pipetting with a 200 μl pipette tip (Singleton et al. 2009), end-repaired with the End-Repair Enzyme Mix in the CopyControl™ Fosmid Library Production Kit (Epicentre), ligated into pCC2FOS, packaged with MaxPlax™ Lambda Packaging Extract (Epicentre), titered, and plated in the Phage T1-Resistant TransforMax™ EPI300™-T1^R *Escherichia coli* EPI300 strain, according to the kit instructions (Epicentre). For long-term storage, the packaged DNA, constituting the primary library in *E. coli* EPI300, was frozen at −80 °C after an equal volume of 20 % glycerol was added.

In situ hybridization

Based on the DNA sequences of the protocatechuate dioxygenase genes reported in Gram-positive bacteria, a pair of primers was designed for the PCR amplification of the protocatechuate dioxygenase gene of OA1: forward primer 2F 5′-TGCCCNTACCACGGCTG-3′ and reverse primer 2R 5′-GCDCCGAKCTTCCAGTT-3′. The genomic DNA of *R. ruber* OA1 was used as the template for PCR. The PCR products were purified with the EZ-10 DNA Gel Extraction Kit of Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China) and sequenced by Shanghai Biosune Biotechnology Co., Ltd. (Shanghai, China). A digoxigenin (DIG)-labeled nucleotide probe was generated using the dioxygenase gene amplified from *R. ruber* OA1 as the template and the DIG High Prime DNA Labeling and Detection Kit (Roche), according to the manufacturer's instructions.

Colonies of the OA1 genomic library were transferred to nylon membranes (Roche), according to the manufacturer's instructions, then sequentially soaked in denaturing solution (0.5 M NaOH, 1.5 M NaCl), buffer solution (1 M Tris–HCl, pH 7.4), and neutralizing solution (1.5 M NaCl, 0.5 M Tris–HCl, pH 7.4). The nylon membrane was dried at room temperature for 20 min and the DNA was fixed at 80 °C in a vacuum oven for 2 h.

Hybridization with the DIG-labeled DNA probe was performed according to the manufacturer's instructions for the DIG High Prime DNA Labeling and Detection Kit (Roche). Genes homologous to the *pcd* gene were detected with nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP), part of the DIG High Prime DNA Labeling and Detection Kit.

Bioinformatics analysis and functional prediction of positive fosmid DNA

Positive colonies were selected according to the locations of the blue spots on the nylon membranes. Positive colonies were transferred to LB broth containing chloramphenicol and induced to high copy numbers at 37 °C overnight with the CopyControl™ Fosmid Autoinduction Solution (Epicentre). The plasmids were then extracted from the positive colonies with the alkaline lysis method. The plasmid DNA was sequenced with high-throughput next-generation sequencing on an Illumina HiSeq 2000 sequencer (Pareek et al. 2011). Sequencing and the subsequent bioinformatics analysis were performed by BerryGenomics Co., Ltd., Beijing.

Cloning and expression of target genes

Based on the sequencing results and gene function predictions for the positive fosmid clones, primers were designed with the Primer Premier 5.0 software to amplify the complete target gene. The positive fosmid DNA was used as the template for PCR. The PCR products were digested with a restriction enzyme and purified with the EZ-10 Spin Column Gel Extraction Kit. The purified products were ligated into the corresponding restriction site of pET-30a(+) with T4 ligase, and competent *E. coli* BL21(DE3) cells were transformed with the recombinant plasmids. The sequence was verified with DNA sequencing to ensure that no mutation had occurred during the process.

LB broth (2 ml) containing 50 μg/ml kanamycin was inoculated with the transformant carrying the recombinant plasmid, and incubated at 32 °C for 15 h at a shaking speed of 160 rpm. About 0.5 ml of the culture was transferred into 50 ml of LB containing 50 μg/ml kanamycin and incubated at 32 °C until an OD_{600} of 0.6–0.8 was reached. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the culture at a final concentration of 0.5 mM, and the culture was continuously incubated at 28 °C for 16 h. The cells were collected by centrifugation at 8000 rpm for 10 min, lysed by ultrasonication, and centrifuged at 12,000 rpm for 10 min. The expressed target proteins were identified with SDS-PAGE and an enzyme assay.

Results

Strain isolation and identification, and substrate utilization

The bacterium OA1 was isolated on a naphthalene-supplemented mineral medium plate. Gram staining and microscopic observation revealed that strain OA1 is a non-spore-forming Gram-positive short rod with round ends. The cells are around 0.8–1.2 μm in width and 1.5–3.0 μm in length. An API Coryne system analysis showed that OA1 shares the physiological and biochemical properties of the genus *Rhodococcus*. 16S rRNA gene sequencing and a phylogenetic analysis demonstrated that OA1 shares 99 % sequence identity with *R. ruber* M2 (Fig. 1) in tetrahydrofuran-degrading culture (Daye et al. 2003). These characters support the taxonomic identification of OA1 as a strain of *R. ruber*. The 16S rRNA gene sequence was submitted to GenBank under accession no. JQ687062.

Substrate utilization tests showed that not only naphthalene, but also phenol, benzoate, salicylate, and protocatechuate are utilized by OA1 as sole carbon and energy

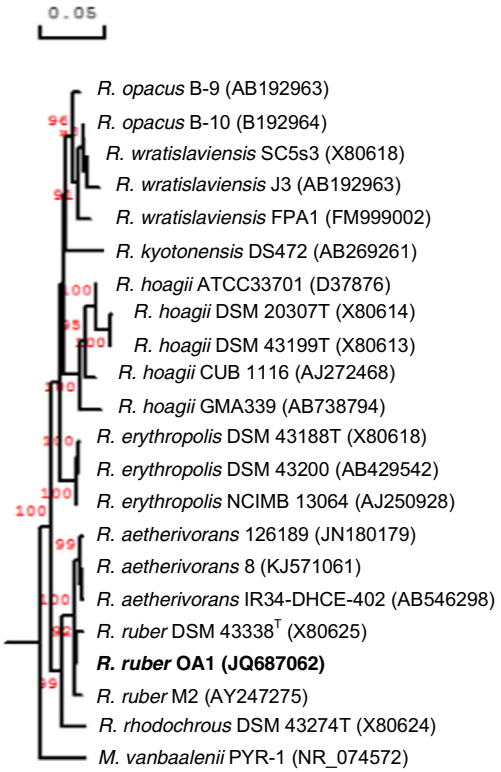

Fig. 1 Phylogenetic tree of *Rhodococcus ruber* OA1 and its relatives based on 16S rRNA gene sequences. The tree rooted with *Mycobacterium vanbaalenii* PYR-1 was constructed using the neighbor-joining method with bootstrap values based on 1000 replications. The numbers at branch points are the percentages supported by bootstrap, and those in parentheses are the GenBank accession numbers of the 16S rRNA gene sequences. Bar = 5 % sequence divergence

sources, whereas 2-chlorobenzoate, phynanthrene, and pyrene are not utilized by OA1.

Naphthalene degradation assay

Naphthalene and its metabolites were analyzed with GC–MS during the degradation of naphthalene by OA1. The GC–MS results showed that when naphthalene was degraded completely in 6 days, both salicylate and phthalate were present in the 6-day sample, whereas both salicylate and phthalate were substantially reduced in the 16-day sample, and neither salicylate nor phthalate was present on day 0. Then the concentrations of naphthalene, salicylate and phthalate in the samples were determined with HPLC. The HPLC results showed that the trends of these compounds were consistent with those in GC-MS analysis. The concentrations of naphthalene, phthalate and salicylate were displayed in Table 1. The dry weight measurement showed the biomass of OA1 increased with the degradation of naphthalene and the metabolites (Table 1). These results indicated that salicylate and phthalate were intermediates of naphthalene degradation in OA1.

Enzyme assays

Rhodococcus ruber OA1 cells were harvested by centrifugation from a late-exponential-phase cell culture in naphthalene-supplemented mineral medium. The enzyme solutions were obtained by ultrasonication and centrifugation, and were used to screen for different enzyme activities. The enzyme activity assays revealed that PCD, GDO, and C12O activities were present in the enzyme solution extracted from an *R. ruber* OA1 culture grown on naphthalene. The specific activities of the enzymes detected are shown in Table 2.

These results indicated that naphthalene induced the expression of PCD, GDO, and C12O in this culture, but not C23O. The expression of PCD was consistent with the protocatechuate pathway deduced above. This is the first proof that the protocatechuate pathway is involved in the degradation of naphthalene by *Rhodococcus*. The presence of GDO and C12O may confirm the presence of the gentisate and catechol pathways, respectively, in *R. ruber* OA1.

Table 1 Quantitative measurement of naphthalene and its metabolites as well as the biomass in the degradation of naphthalene by *Rhodococcus ruber* OA1

	Concentration (mg/l)*		
	0d	6d	16d
Naphthalene	1976.55±21.16	0	0
Phthalate	0	0.27±0.01	0.04±0.01
Salicylate	0	0.64±0.04	0.04±0.02
Biomass (dry weight)	10.00±2.89	955.56±19.25	1311.11±69.39

* The results were expressed as an average of three experiments

Table 2 Specific activities of enzymes in naphthalene degradation in *Rhodococcus ruber* OA1

Substrate	Specific activity (U/mg protein)*			
	PCD[a]	GDO[b]	C12O[c]	C23O[d]
Naphthalene	127.45±5.1	69.22±2.11	9.61±1.65	0

* The results were expressed as an average of three experiments

[a] Protocatechuate dioxygenase

[b] Gentisate dioxygenase

[c] Catechol 1,2-dioxygenase

[d] Catechol 2,3-dioxygenase

Genomic library construction and screening

A genomic library was constructed with the CopyControl™ Fosmid Library Production Kit using the pCC2FOS vector and the phage T1-resistant *E. coli* EPI300 plating strain. Titering the packaged CopyControl fosmid clones indicated that the library titer was 1×10^4 CFU/ml. The probability of the presence of every gene in the genome of *R. ruber* strain OA1 was 99.99 %, and the library coverage was at least tenfold genome equivalents.

With primers 2 F and 2R, a PCR product of about 300 bp was amplified from the genomic DNA of *R. ruber* OA1. The sequencing results showed that it was homologous to the protocatechuate dioxygenase gene. A DIG-labeled probe was generated based on the gene sequence, with the DIG High Prime DNA Labeling and Detection Kit (Roche), and was used to screen for the protocatechuate dioxygenase gene (cluster) with in situ hybridization. A positive colony was thus identified in the genomic library.

Sequencing and a bioinformatics analysis of the positive plasmid DNA

Sequencing and a bioinformatics analysis of the positive plasmid DNA revealed a 45,518-bp sequence containing 56 genes, including eight genes encoding proteins related to protocatechuate catabolism (Zhang et al. 2012). The eight genes were organized into the gene cluster *pcaJIGHBARC* (accession nos. KJ546148, KP057223), whose putative protein products were 3-oxoacid CoA-transferase β subunit, 3-oxoadipate CoA-transferase α subunit, protocatechuate 3,4-dioxygenase β subunit, protocatechuate 3,4-dioxygenase α subunit, 3-carboxy-cis, cis-muconate cycloisomerase, 4-carboxymuconolactone decarboxylase, transcriptional regulator PcaR, and acetyl-CoA acetyltransferase, respectively. The sizes, locations, and

directions of the genes are shown in Fig. 2. In the cluster, *pcaG* (720 bp) and *pcaH* (597 bp) encode the α subunit and β subunits of protocatechuate 3,4-dioxygenase (3,4-PCD), respectively. 3,4-PCD is the key enzyme in the cleavage of protocatechuate to 3-carboxy-cis, cis-muconate. The other genes in the cluster play roles in converting 3-carboxy-cis, cis-muconate to the subsequent products before they enter the TCA cycle of *R. ruber* OA1.

Heterologous expression of protocatechuate catabolic genes

To verify the function of the protocatechuate catabolic gene cluster, key genes (*pcaGH*) were cloned and expressed in *E. coli* BL21(DE3). Because these two genes share eight overlapping nucleotides (Fig. 3), the genes *pcaGH* were coexpressed in *E. coli* BL21(DE3), and formed the complete and active 3,4-PCD enzyme. A pair of primers was designed based on the complete *pcaGH* gene sequence: *pcaGH*F 5′-GGAATTCCATATGCTTCATCTGCCGCCC-3′ and *pcaGH*R 5′- CCCAAGCTTCTAGATCGCGAAGAAC-3′. The underlined nucleotides indicate *Nde*I and *Hin*dIII restriction sites, respectively. The plasmid DNA was used as the template for PCR amplification. The PCR product containing *pcaGH*, which was 1309 bp long, was purified and sequenced.

The purified and sequenced *pcaGH* gene was digested with *Nde*I and *Hin*dIII and ligated into the expression vector pET-30a(+) digested with the same enzymes. The *Nde*I and *Hin*dIII restriction enzymes were used to identify the recombinant pET-30a(+)–*pcaGH* construct. A sequencing analysis showed that the *pcaGH* sequence in the recombinant pET-30a(+)–*pcaGH* construct was correct. After induction with IPTG, the *pcaGH* gene efficiently expressed the α and β subunits of 3,4-PCD. An SDS-PAGE analysis confirmed the expressed α subunit (PcaG) and β subunit (PcaH) of 3,4-PCD (Fig. 4). The molecular weights of PcaG and PcaH were ~27.1 kDa and ~21.4 kDa, respectively.

The expressed soluble proteins were extracted with ultrasonication and centrifugation. Enzyme assays revealed that the expressed proteins could convert protocatechuate to β-carboxy-cis, cis-muconic acid, indicating that they were the components of active 3,4-PCD. The specific activity of 3,4-PCD in *E. coli* BL21(DE3) cells was 81.37±4.32 nmol/min/mg protein, which was a little lower than its specific activity in *R. ruber* OA1.

Nucleotide sequence accession numbers

The 16S rRNA gene sequence of *R. ruber* OA1 was submitted to GenBank under accession number JQ687062. The *pca* gene cluster sequence, containing the *pcaJ*, *pcaI*, *pcaG*, *pcaH*, *pcaB*, *pcaA*, *pcaR*, and *pcaC* genes of *R. ruber* strain OA1, was submitted to GenBank under accession numbers KP057223 (*pcaJ*, *pcaI*) and KJ546148 (*pcaG*, *pcaH*, *pcaB* *pcaA*, *pcaR*, and *pcaC*).

Discussion

PAH contaminants are normally produced during the incomplete combustion of fossil fuels, oil treatments, chemical and pharmaceutical production, etc. (Cerniglia 1992; Seo et al. 2009; Braun et al. 2015). The study of PAH biodegradation has shown that different kinds of environments can be habitats of PAH degraders. A large number of PAH degraders have been isolated from PAH-contaminated or oil-contaminated soils and characterized (Ahn et al. 1999; Uz et al. 2000; Derz et al. 2004; Seo et al. 2007; Singleton et al. 2009), some of which were derived from sediments (Yu et al. 2005; Zhou et al. 2006), some from marine environments (Iwabuchi et al. 1998), and some from contaminated compost, consisting of lignite-treated wooden ties mixed with sugar beet mud (Annweiler et al. 2000). In this study, a PAH degrader, *R. ruber* OA1, was isolated from the activated sludge of a pharmaceutical wastewater treatment plant after the production of amidopyrine, pipemidic acid, etc. Biodegradation studies based on these strains should contribute to the biodecontamination or biotreatment of these kinds of environments.

Rhodococcus is a genus of the family Nocardiaceae, suborder Corynebacterineae, order Actinomycetales, subclass Actinobacteridae, phylum Actinobacteria, and domain Bacteria. The genus has a variety of degradation substrates, especially xenobiotic compounds that cause serious environmental problems throughout the world. *Rhodococcus* sp. strain RHA1 degrades polychlorinated biphenyls and benzoate as substrates (Kitagawa et al. 2001); *Rhodococcus* sp. strain DK17 uses indan as a growth substrate via the o-xylene pathway (Kim et al. 2010, 2011); *Rhodococcus wratislaviensis* IFP 2016 degrades 11 compounds, including naphthalene (Auffret et al. 2009); and *Rhodococcus*

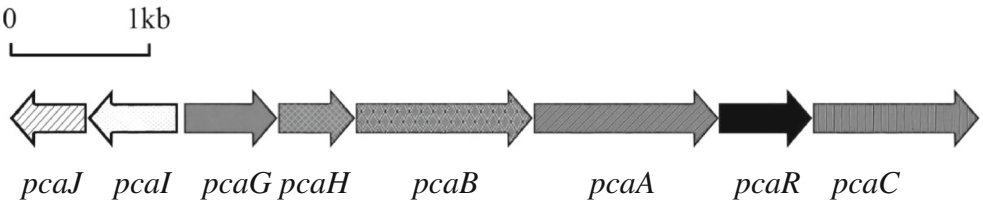

Fig. 2 Genes organization of the protocatechuate degradation pathway in *Rhodococcus ruber* strain OA1. The arrows indicate the sizes, locations, and directions of the genes

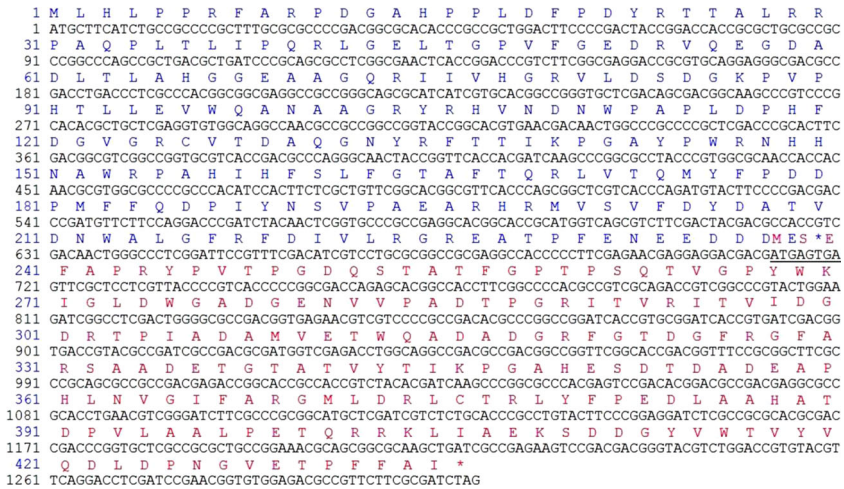

Fig. 3 The nucleotide sequence of *pcaGH* and the deduced amino acid sequence. The underlined nucleotides were the shared nucleotides of *pcaG* and *pcaH*. The asterisks (*) marked the stop codons of *pcaG* and *pcaH*

erythropolis djl-11 degrades carbendazim for growth (Zhang et al. 2013). In this study, *R. ruber* OA1 was shown to degrade a variety of aromatic compounds, including naphthalene, phenol, benzoate, salicylate, and protocatechuate, extending our knowledge of *Rhodococcus* and its degradation properties. Compared to the naphthalene biodegradation, the more difficult thing with PAH is the biodegradation of congeners with a higher number of rings. However, when phynanthrene or pyrene was added to the mineral medium as the sole carbon source, it could not support the growth of OA1, nor be degraded by OA1. This might limit to some extent the study of OA1 degrading more complex PAHs.

Salicylate is a well-known intermediate in the degradation of naphthalene, phenanthrene, and anthracene by previously reported PAH degraders. Salicylate is further metabolized by catechol or gentisate to TCA cycle intermediates (Menn et al. 1993; Sanseverino et al. 1993; Kiyohara et al. 1994; Yang et al. 1994). Phthalate is regarded as an intermediate of phenanthrene, anthracene, and pyrene degradation by some PAH degraders (Heitkamp et al. 1988; Iwabuchi et al. 1998; Khan et al. 2001). Recently, phthalate was identified as an intermediate in naphthalene degradation by *Bacillus thermoleovorans* Hamburg 2 (Annweiler et al. 2000), but the pathway downstream from phthalate in *B. thermoleovorans* Hamburg 2 has not been determined or predicted. Kweon et al. (2011) proposed that phthalate is an intermediate in the degradation of naphthalene, fluorene, acenaphthylene, anthracene, phenanthrene, pyrene, and benzo[a]pyrene by *Mycobacterium vanbaalenii* PYR-1, and that protocatechuate is a downstream product of phthalate. In the present work, phthalate was identified as an intermediate in naphthalene degradation by *R. ruber* OA1, so *R. ruber* OA1 may degrade naphthalene via the protocatechuate pathway.

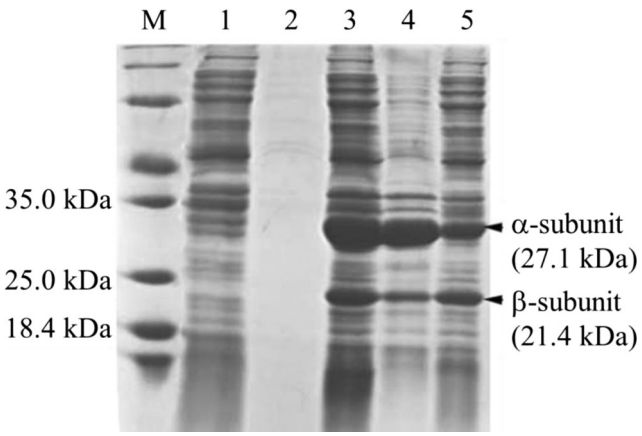

Fig. 4 SDS-PAGE analysis of the expressed proteins of recombinant pET-30a(+)-*pcaGH*. M, protein marker; 1, The total protein of non-deduced *E. coli* BL21 (DE3) containing pET-30a(+)-*pcaGH*; 2: The secretory protein in the medium; 3, The total protein of *E. coli* BL21 (DE3) containing pET-30a(+)-*pcaGH* induced with IPTG for 4 h; 4, The precipitate protein obtained by ultrasonication and centrifugation; 5: The supernatant protein obtained by ultrasonication and centrifugation. The arrows indicate the positions of the expressed α and β subunits

To confirm the protocatechuate pathway in *R. ruber* OA1, PCD, a key enzyme of protocatechuate metabolism, was assayed in OA1 grown on naphthalene-supplemented mineral medium. The high PCD activity of OA1 indicates that naphthalene is responsible for the PCD activity of OA1, and confirms the involvement of the protocatechuate pathway in naphthalene degradation. GDO and C12O were also present in OA1 grown on naphthalene-supplemented mineral medium, indicating that the gentisate pathway via GDO and the catechol pathway via C12O also have roles in naphthalene degradation by OA1. However, the catechol pathway via C23O might not be involved in naphthalene degradation by OA1, because no C23O activity was detected in OA1, although the catechol pathway via C23O is important in naphthalene degradation by *Rhodococcus rhodochrous* strains P200 and P400 (Kulakova et al. 1996). The probable pathways of naphthalene degradation in *R. ruber* OA1, based on these data, are shown in Fig. 5.

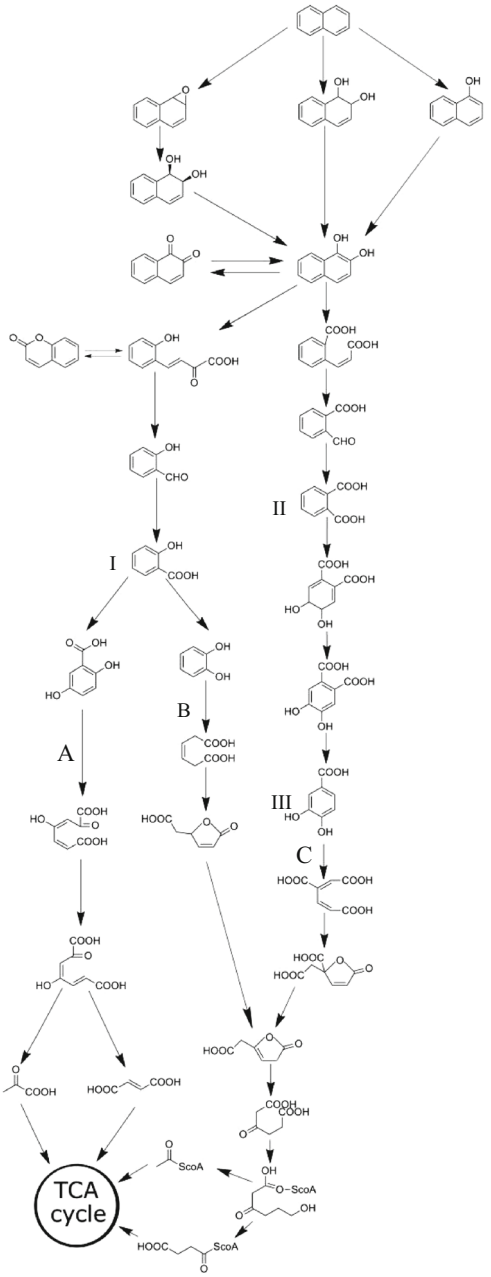

Fig. 5 Proposed pathways of naphthalene degradation in *Rhodococcus ruber* OA1. I, salicylate; II, phthalate; III, protocatechuate; A, gentisate dioxygenase; B, catechol 1,2-dioxygenase; C, protocatechuate 3,4-dioxygenase

In subsequent experiments, protocatechuate-degrading activity was detected in OA1 and a gene cluster related to protocatechuate degradation was cloned and identified. The genes in this cluster encoding protocatechuate 3,4-dioxygenase (3,4-PCD) were subcloned and expressed in *E. coli* BL21(DE3). Enzyme assays showed that the expressed heterodimeric protein had 3,4-PCD activity and successfully degraded protocatechuate. To date, three patterns have been identified in the catabolism of protocatechuate, which are catalyzed by three PCDs: protocatechuate 4,5-dioxygenase (4,5-PCD) reported in *Pseudomonas* (Dagley et al. 1968), protocatechuate 2,3-dioxygenase (2,3-PCD) reported in *Bacillus* (Wolgel et al. 1993), and 3,4-PCD, reported in *Pseudomonas*, *Burkholderia*, and *Stenotrophomonas maltophilia* (Ohlendoff et al. 1988; Romero-Silva et al. 2013; Guzik et al. 2013). The former two enzymes, which require ferrous iron as a cofactor, catalyze the metacleavage of the aromatic ring adjacent to the hydroxyl substituent (Arciero et al. 1983; Wolgel et al. 1993), whereas 3,4-PCD, which requires nonheme ferric iron as the sole cofactor, catalyzes the orthocleavage of the aromatic ring between the vicinal hydroxyls to form 3-carboxy-cis, cis-muconic acid (Ohlendoff et al. 1988; Guzik et al. 2013). The 3,4-PCD expressed in *R. ruber* OA1 displays the last pattern. This is the first report of 3,4-PCD in *R. ruber*. Protocatechuate 3,4-dioxygenase plays an important role not only in PAH degradation, but also in the degradation of other aromatic compounds. Romero-Silva et al. (2013) reported that *Burkholderia xenovorans* LB400 degrades 4-hydroxybenzoate via the protocatechuate pathway catalyzed by 3,4-PCD. Guzik et al. (2014) even found that 3,4-PCD from *S. maltophilia* KB2, immobilized in calcium alginate, increased the activities degrading 2,5-dihydroxybenzoate, caffeic acid, 2,3-dihydroxybenzoate, and 3,5-dihydroxybenzoate. Our results for 3,4-PCD in *R. ruber* OA1 should facilitate further research into 3,4-PCD in Gram-positive bacteria, such as *Rhodococcus*.

Besides the steps of protocatechuate catabolism, the naphthalene biodegradation activity of *R. ruber* involves many other steps catalyzed by other enzymes. The successful heterologous expression of 3,4-PCD in *E. coli* BL21(DE3) has paved the way for the heterologous expression of more gene products or enzymes involved in naphthalene degradation. This may be helpful in defining the gene functions, and the molecular mechanisms of degradation in *Rhodococcus*, and its potential application to bioremediation.

In conclusion, this study has demonstrated a new naphthalene degrader, *R. ruber* OA1, and the role of its protocatechuate pathway in naphthalene degradation. The gene cluster responsible for protocatechuate catabolism was identified, and the genes encoding the α and β subunits of 3,4-PCD were coexpressed. This is the first report of the protocatechuate pathway of naphthalene degradation in *Rhodococcus*. The study extends our knowledge of PAH biodegradation and should contribute to the efficient biomonitoring, biotreatment, and bioremediation of PAH pollution.

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Conflict of interest The authors declare that they have no conflicts of interest.

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