Cloning and Sequence Analysis of the 4-Hydroxybenzoate 3-Hydroxylase Gene from a Cyclohexanecarboxylate-degrading Gram-positive Bacterium, “*Corynebacterium cyclohexanicum*” Strain ATCC 51369

HIROAKI IWAKI¹, HIROSHI SAJI¹, KAZUYA ABE¹ and YOSHIE HASEGAWA^{1*}

¹ Department of Biotechnology, Faculty of Engineering, Kansai University, Suita, Osaka 564–8680, Japan

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A DNA fragment that carries the gene encoding 4-hydroxybenzoate 3-hydroxylase (*pobA*) together with genes encoding a potential regulator (*pobR*) and a potential transporter (*pobK*) was cloned from “*Corynebacterium cyclohexanicum*” strain ATCC 51369, which is herein reclassified as belonging to the genus *Arthrobacter*. Nucleotide sequencing revealed that the deduced amino acid sequence encoded by the *Arthrobacter pobA* gene exhibits 42.0–46.7% identity with that of gram-negative bacteria. The gene organization of the *pob* cluster differs from that of gram-negative bacteria. The *pobA* gene product (PobA), expressed in *Escherichia coli*, preferred NADH over NADPH similar to 4-hydroxybenzoate 3-hydroxylase of ATCC 51369. The *Arthrobacter pobA* gene was inactivated by insertion of pK19*mob*. The resultant mutant strain, POBA1, grew on neither cyclohexanecarboxylate nor 4-hydroxybenzoate. These results strongly suggest that the cloned *pobA* gene plays an essential role in the catabolism not only of 4-hydroxybenzoate but also of cyclohexanecarboxylate in strain ATCC 51369.

Key words: cyclohexanecarboxylate, biodegradation, 4-hydroxybenzoate 3-hydroxylase, *Arthrobacter*, “*Corynebacterium cyclohexanicum*”

Alicyclic acids such as cyclohexanecarboxylate (CHCA) and cyclohexylacetate are formed oxidatively from *n*-alkylcycloparaffins present in crude oils³⁴, and CHCA is also involved as an intermediate in the anaerobic degradation of aromatic rings^{8,9,36}.

The utilization of CHCA as a source of carbon for growth by a number of aerobic bacteria appears to follow two main routes: aromatization of the ring to yield hydroxybenzoate as an intermediate, which is a substrate for ring opening^{3,17,19}, or metabolism by a β -oxidation-like sequence, involving coenzyme A thioester-derivatives, before ring opening^{2,25}. “*Corynebacterium cyclohexanicum*” strain ATCC 51369 degrades CHCA through a pathway that includes aromatization (Fig. 1)^{19,20,33}. The degradation of CHCA has been found to be initiated by hydroxylation to yield 4-hydroxyCHCA, which is then dehydrogenated to 4-

oxoCHCA by a NAD⁺-dependent dehydrogenase²³. The next step, catalyzed by the third and fourth enzymes in the degradation pathway, which are two distinctive desaturases, is the aromatization of 4-oxoCHCA to 4-hydroxybenzoate (4-HBA)^{19,20}. 4-HBA is then converted to protocatechuate, a common intermediate in the biodegradation of various aromatic compounds, by 4-HBA 3-hydroxylase (PHBH) which utilizes NADH as well as NADPH as an electron donor¹⁶.

We have initiated a genetic analysis of strain ATCC 51369 to further characterize the pathway of CHCA's degradation and report here for the first time the cloning and sequencing of a gene cluster that contains the PHBH gene (*pobA*) of gram-positive bacteria.

Materials and Methods

Bacterial strains, culture conditions, and plasmids

The bacterial strains and plasmids used in this study are

* Corresponding author; E-mail: yoshie@ipcku.kansai-u.ac.jp, Tel: +81-6-6368-0909, Fax: +81-6-6388-8609

Fig. 1. Pathway for the degradation of cyclohexanecarboxylate (CHCA) by *Arthrobacter* sp. strain ATCC 51369. Poba, 4-hydroxybenzoate (4-HBA) 3-hydroxylase which converts 4-HBA to protocatechuate.

listed in Table 1. "*Corynebacterium cyclohexanicum*" strain ATCC 51369, reclassified as a member of the genus *Arthrobacter*, was grown at 30°C in Luria-Bertani (LB) medium²⁶ or mineral salt medium (MSM)¹⁸, pH 7.0, containing 0.1% CHCA, 4-HBA, or protocatechuate and 0.1 g of yeast extract per liter. Agar was added to 1.5% for plates. *Escherichia coli* strains were routinely cultured in LB media. When necessary, the media were supplemented with ampicillin (100 µg/ml) or kanamycin (100 µg/ml).

Genetic methods and sequence analysis

All of the DNA techniques used, including isolation of total DNA from strain ATCC 51369, plasmid isolation, Southern blot analysis, DNA labeling, DNA subcloning, nucleotide sequencing, electrotransformation (electroporation), and 16S rRNA gene analysis, were performed as described previously^{6,18,22}.

Cloning of the PHBH-encoding gene (*pobA*) and associated open reading frames (ORFs)

To clone the *pobA* involved in the degradation of CHCA, we purified the PHBH protein from CHCA-grown cells as described previously¹⁶, with the aim of determining an N-terminal amino acid sequence to design a primer for PCR amplification. As a result, a 20-amino-acid-residue sequence, GDRTVITTQVAIMGAGPAGL, was obtained by Edman degradation using a Perkin-Elmer model 477 automated protein sequencer. Two primers with the following sequences were designed from the N-terminal amino acid sequence (TTQVAIMG) and a conserved sequence (HIVPPTGA) of PHBH^{1,7,10,24,29,30,37}: 5'-ACIACICARGT-NGCNATHATGGG-3' and 5'-GCICIGTNGGIGGNAC-DATRTG-3'. PCR amplifications were carried out in 50 µl reaction mixtures containing 0.1 µg of strain ATCC 51369 genomic DNA as a template, 50 pmol of each primer, each

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s) ^a	Reference or source
<i>Arthrobacter</i> sp. strains		
ATCC 51369	CHCA degrader, Chc ⁺ , Pob ⁺ , Pca ⁺	33
POBA1	<i>pobA</i> mutant of strain ATCC 51369, Chc ⁻ , Pob ⁻ , Pca ⁺	This study
<i>E. coli</i> strains		
XL1-blue	<i>recA1 endA1 gyrA96 thi hsdR17 supE44 relA1</i> [F' <i>lacI</i> ^q ZM15 Tn10 (Tet ^r)]	4
BL21	F ⁻ <i>ompT hsdS_B</i> (r _B ⁻ m _B ⁻) <i>gal dcm</i>	Novagen
Plasmids		
pUC19	Cloning vector, Ap ^r	38
pK19mob	Mobilizable multi-purpose cloning vector, Km ^r	27
pSD80	Expression vector with <i>tac</i> promoter, Ap ^r	31
pXcmkn12	Direct cloning vector of PCR products, Ap ^r , Km ^r	5
pXcm- <i>pobA</i>	0.9-kb PCR product containing the partial <i>pobA</i> gene from <i>Arthrobacter</i> sp. strain ATCC 51369 genomic DNA in pXcmkn12	This study
pHBA1	7.3-kb <i>Pst</i> I fragment from <i>Arthrobacter</i> sp. strain ATCC 51369 in pUC19	This study
pPobA	1.2-kb <i>Eco</i> RI*- <i>Pst</i> I* fragment containing <i>pobA</i> in pSD80	This study
pK19- <i>pobA</i>	0.6-kb <i>Bgl</i> II fragment containing the internal region of <i>pobA</i> in pK19mob	This study

^a Chc⁺, growth on CHCA; Chc⁻, no growth on CHCA; Pob⁺, growth on 4-HBA; Pob⁻, no growth on 4-HBA; Pca⁺, growth on protocatechuate.

*indicates restriction sites introduced by design.

dNTP at a concentration of 0.2 mM, 5 µl of *Taq* DNA buffer, and 2.5 units of *Taq* DNA polymerase (New England Biolabs, Beverly, USA). After 3 min of incubation at 94°C, the reaction mixture was subjected to 30 cycles of 15 s at 94°C, 30 s at 50°C, and 1 min at 72°C. The 0.9-kb PCR product was cloned directly in the vector pXcmkn12⁵⁾ and introduced into *E. coli* XL-1 blue. The resulting plasmid was designated pXcm-*pobA*. Before use of the amplified DNA as a hybridization probe, its nucleotide sequence was determined to confirm its identity. To clone the complete *pobA* gene, the DNA insert from pXcm-*pobA* was amplified, labeled with the digoxigenin-11-UTP system (Roche Diagnostics), and used for the Southern hybridization of genomic DNA digested with the following enzymes: *Bam*HI, *Eco*RI, *Hind*III, *Kpn*I, *Pst*I, *Sal*I, *Sph*I, and *Xba*I. A 7.3-kb *Pst*I fragment was cloned in *E. coli* XL1-blue cells by using pUC19 as a vector³⁸⁾. A clone containing the 7.3-kb insert was screened by colony hybridization using the PCR product as a probe; this recombinant plasmid was designated pHBA1, and both strands of the insert were sequenced.

Expression of the pobA gene in E. coli and enzymatic activity

The DNA fragment carrying *pobA* was amplified by using KOD polymerase (Toyobo, Japan, Osaka) with the following pair of PCR primers, with the desired restriction site (*Eco*RI and *Pst*I [underlined sequence]) to facilitate subsequent cloning: *pobAF* (5'-CGGAATTCATGGGCGATCG-CACCGTCAT-3') and *pobAR* (5'-AAAACTGCAGCT-TCTGGGTGTCATGTC-3').

The amplified DNA fragment was purified from an agarose gel, digested with *Eco*RI and *Pst*I, and cloned in the linearized pSD80³¹⁾ vector to yield pPobA. *E. coli* BL21 containing pPobA was cultivated in 20 ml of LB medium containing 100 µg/ml ampicillin at 30°C. When the culture reached an optical density at 600 nm of 0.5, IPTG was added to a final concentration of 0.5 mM in the medium. The cells were further cultured for 3 h. The resultant cells were harvested by centrifugation, washed in 50 mM phosphate buffer (pH 7.2), resuspended in the same buffer, and sonicated by two 30-s bursts with a Braun-Sonifier 250 apparatus. After centrifugation for 30 min at 18,000×g at 4°C, the supernatant was used for the determination of enzyme activity. PHBH activities were assayed spectrophotometrically by measuring the decrease in absorbance of 340 nm due to the substrate-dependent oxidation of NADPH or NADH as described by Fujii and Kaneda¹⁶⁾. The standard assay system contained 50 µmol phosphate buffer, pH 7.7, 0.15 µmol NADPH or NADH, 0.25 µmol 4-HBA, 0.025 µmol FAD,

enzyme solution and water. One unit of enzyme activity was defined as the amount catalyzing the oxidation of 1.0 µmol NAD(P)H/min under the conditions of the assay.

Inactivation of pobA in Arthrobacter

To disrupt the *pobA* gene, a 0.6-kb *Bgl*II fragment containing the internal region of *pobA* was inserted into the *Bam*HI site of pK19*mob*²⁷⁾. The resultant plasmid pK19-*pobA*, was introduced into strain ATCC 51369 by electroporation. A single crossover was expected to generate a tandemly duplicated *pobA* sequence, resulting in a vector between the sequences. Because the *pobA* gene in pK19-*pobA* was truncated at both termini, the upstream *pobA* sequence lacked the carboxyl terminus, and the downstream *pobA* sequence lacked the amino terminus. As a result, both of the *pobA* sequences had deletions, and neither of them was functional. Transformants were selected on LB agar plates containing 100 µg/ml kanamycin and were subjected to a PCR amplification analysis using primers *pobAF* and *pobAS* in order to examine the insertion of pK19-*pobA* into the *pobA* gene by single crossover.

Nucleotide sequence accession number

The DNA sequences reported in this paper have been submitted to DDBJ, and the accession numbers are AB210281 (7.3-kb *Pst*I fragment), AB210282 (16S rRNA gene).

Results and Discussion

Reclassification of strain ATCC 51369

As the proper identification of a genus or strain is a prerequisite in a potential risk assessment scheme, strain ATCC 51369 was subjected to a 16S rRNA gene sequence analysis. As a result, analysis of the near-complete 1,453-bp 16S rRNA gene sequence of strain ATCC 51369 indicated that it belonged to the genus *Arthrobacter* with *Arthrobacter atrocyaneus* (DDBJ/EMBL/GenBank accession number, AY605543) as its closest relative (99.0% similarity). Based on this result, it is proposed that "*Corynebacterium cyclohexanicum*" strain ATCC 51369 is reclassified as an *Arthrobacter* sp.

Cloning of PHBH-encoding gene

As described in Materials and Methods, two primers were designed based on the N-terminal amino acid sequence of the purified protein and a conserved sequence of PHBH^{1,7,10,24,29,30,37)} and used to amplify the partial *pobA* gene. The amplified product was used as a probe for a

Fig. 2. Physical map of the *pob* gene cluster from *Arthrobacter* sp. strain ATCC 51369. The orientation of the arrows indicates the direction of gene transcription. The black arrows are genes involved in the degradation of 4-HBA. *pobR* is a putative regulatory gene and *pobK* encodes a putative transporter. Other ORFs are marked by open arrows.

Southern and colony hybridization, and a 7.3-kb *Pst*I fragment of strain ATCC 51369 genomic DNA was obtained. The plasmid was designated pHBA1. Using pHBA1 as a template, the insert was sequenced. On the basis of an analysis of the DNA sequence data, we predicted the presence of five complete and one incomplete open reading frames (ORFs) (Fig 2; Table 2). Three are on one strand, and the rest are on the opposite strand.

Based on a comparison of N-terminal amino acid sequence with purified PHBH from strain ATCC 51369, the 1,188-bp ORF preceded by a consensus ribosome-binding sequence, AAGGA, was identified as the PHBH-encoding gene (*pobA*). Its first 20 amino acids deduced from the nucleotide sequence were identical to the sequence determined in this study except for the N-terminal methionine. This result suggests that we have cloned the PHBH gene required

for degradation of CHCA.

Characteristics of *pobA* and flanking sequences

The deduced amino acid sequence of *pobA* consisted of 396 residues and had a calculated molecular mass of 44 kDa. It exhibited 42.0–46.7% identity with published PobA sequences of gram-negative bacteria and all active site residues in the novel sequence were strictly conserved^[1,7,10,24,29,30,37]. As expected, the most closely related sequence according to the phylogenetic analysis (data not shown) was PHBH from *Pseudomonas* sp. strain CSB3 which prefers NADH over NADPH, as well as PHBH of ATCC 51369. To the best of our knowledge, PobA from *Pseudomonas* sp. strain CBS3 was the only PHBH enzyme known to be active with NADH^[29]. The *pobA* gene product of strain ATCC 51369 represents the second PHBH that is active toward NADH to be cloned and sequenced, and the first from gram-positive bacteria. The model of coenzyme recognition by flavoprotein aromatic hydroxylases mainly comes from studies on the NADPH-specific PHBH of *Pseudomonas fluorescens*^[11–14]. The pyrophosphate moiety of NADPH most probably binds in a cleft between the FAD-binding domain and the substrate-binding domain, whereas the adenosine 2'-phosphate moiety is assumed to bind in the region around helix H2^[28,35] and Tyr38 may play an important role in the recognition of the adenosine 2'-phosphate moiety^[29]. The amino acid sequence of the helix

Table 2. Homology of the *Arthrobacter* sp. strain ATCC 51369 gene products or ORFs

Gene designation	Function of gene product	Position in sequence ^a	Amino acid (aa)	Most similar gene products [species] (accession no.) [% aa identity (identical aa/overlapped aa)] ^b
<i>orf1</i>	Possible transposase	190–1,200	336	Putative transposase [<i>Corynebacterium efficiens</i> YS-314] (BAC18275) [55.4 (152/285)], Putative transposase [<i>Gloeobacter violaceus</i> PCC 7421] (BAC88274) [53.1 (135/254)]
<i>orf2</i>	Possible transposase	760–2,571	603	Putative transposase [<i>Mycobacterium ulcerans</i> AGY99] (CAE46838) [68.0 (185/272)], Putative transposase [<i>Azoarcus</i> sp. EbN1] (CAI09985) [44.9 (183/408)]
<i>pobK</i>	Possible 4-HBA transporter	2,672–3,991	439	Putative transmembrane transport protein [<i>Streptomyces coelicolor</i> A3 (2)] (CAB76354) [51.0 (224/439)], Permeases of the major facilitator superfamily [<i>Corynebacterium glutamicum</i> ATCC 13032] (BAB98469) [47.7 (195/409)]
<i>pobR</i>	IcIR-type regulator	4,105–4,851	248	Putative transcriptional regulator [<i>Corynebacterium glutamicum</i> R] (BAD83943) [39.5 (98/248)], Putative transcriptional regulator [<i>Streptomyces coelicolor</i> A3 (2)] (CAA19948) [37.0 (88/238)]
<i>pobA</i>	PHBH	5,033–6,223	396	Putative 4-hydroxybenzoate hydroxylase [<i>Bradyrhizobium japonicum</i> USDA 110] (BAC51685) [50.8 (197/388)], Putative 4-hydroxybenzoate 3-monooxygenase [<i>Corynebacterium efficiens</i> YS-314] (BAC17943) [49.0 (190/388)]
<i>orf3</i> (5'-truncated)	Possible transposase	6,379–7,287	303	Predicted transposase [<i>Corynebacterium glutamicum</i> ATCC 13032] (BAC00025) [37.4 (114/305)], Predicted transposase [<i>Corynebacterium glutamicum</i> ATCC 13032] (BAC00345) [40.8 (91/223)]

^a The numbers are nucleotide positions in the sequenced segment (DDBJ/EMBL/GenBank accession no. AB210281).

^b Nonredundant protein databases in the National Center for Biotechnology Information.

H2 region is highly conserved in NADPH-specific PHBH enzymes^{1,7,10,24,30,37}, but deviates in the NAD(P)H-dependent PHBH from strain CBS3 in which Tyr38 is replaced by glutamic acid²⁹. Based on this finding, it was proposed that helix H2 was involved in determining the coenzyme specificity. Actually, Eppink *et al.* succeeded in switching the coenzyme specificity of *P. fluorescens* PHBH by introducing multiple substitutions into helix H2 (33-RQTPDYVLGR-42) based on the sequence of PHBH from *Pseudomonas* sp. CBS3 (37-SRSRAEIEST-46) and concluded that specificity in *P. fluorescens* PHBH is conferred by interactions of Arg33, Tyr38 and Arg42 with the 2'-phosphate moiety of bound NADPH, and that introduction of an acidic group at position 38 potentially enables the recognition of the 2'-hydroxy group of NADH. As expected given these findings, this region is highly conserved in two PHBHs from strains CBS3 (37-SRSRAEIEST-46) and ATCC 51369 (39-IRSRAEISAT-48), providing new insight into the mode of coenzyme recognition by PHBH.

Upstream of *pobA* and separated by a 181-bp intergenic sequence is a potential IclR-type transcriptional regulator³². This ORF, designated *pobR*, consists of 248 residues with a ribosome-binding sequence (GGA). PobR is most closely related to the putative transcriptional regulator of *Corynebacterium glutamicum* strain R (Table 2), and the PobR of *Acinetobacter* is the first regulatory protein for a PHBH-encoding gene to be identified in a BLASTP search, with identities of 39.5% and 23.7%, respectively. No significant homology was detected with other PobR using the Blast 2 program, which suggests that PobR from this strain and *Acinetobacter* belong to another family of regulatory proteins. The open reading frames of *pobR* and *pobA* are oriented in the same direction (Fig. 2). This orientation differs from published *pob* gene clusters in gram-negative bacteria, where *pobA* and *pobR* are oriented in opposing directions.

One hundred and thirteen base pairs upstream of *pobR*, but transcribed in the reverse orientation and preceded by a consensus ribosome-binding sequence, GAGGT, is a 1,317-bp ORF designated *pobK* that encodes a polypeptide of 439 residues which is a putative 4-HBA transporter. PobK is most closely related to a putative transmembrane transport protein of *Streptomyces coelicolor* strain A3 (2) (Table 2), and benzoate transport protein, BenK, is the first functional protein identified by a BLASTP search with 50.4 and 30.7% identity, respectively²¹. A PobK homolog was not found in flanking sequences of *pobA* and *pobR* in gram-negative bacteria. Also, three transposase-like genes are in the flanking region of the *pob* degradative cluster (Table 2, Fig. 2).

Expression of *pobA* in *E. coli*

A knowledge of the *pobA* nucleotide sequence was used to construct a IPTG-inducible clone of *E. coli* cells using the plasmid pSD80 as a vector in order to verify its protein production and enzymatic activity. Figure 3 shows the expression of the PobA protein (lane 2) and the observed M_r estimated at 45 kDa. No enhanced protein band of this size was detectable in the control cells containing pSD80 only.

Crude protein extract from the *E. coli* BL21 cells containing pPobA was used to determine the enzymatic activity as described in Materials and Methods. As a result, the specific activity of the PHBH enzyme was found to be 3.7 and 2.3 U/mg using NADH and NADPH as an external electron donor, respectively.

Disruption of the *pobA* gene in strain ATCC 51369

To examine if the cloned *pobA* gene is essential to the catabolism of CHCA and 4-HBA in strain ATCC 51369, the *pobA* gene was insertionally inactivated by homologous recombination. We constructed the plasmid pK19-*pobA* to inactivate *pobA* by single crossover and introduced it into strain ATCC 51369 by electroporation. A kanamycin-resistant transformant, POBA1, was recovered and PCR amplification was performed to confirm the expected arrangement of duplicated *pobA* sequences. The amplified fragment from strain POBA1 genomic DNA was 4.4-kb larger than that of ATCC 51369, indicating that insertion of the entire 4.4-kb pK19-*pobA* segment into the *pobA* sequence occurred (data

Fig. 3. Coomassie blue-stained protein profile of recombinant *E. coli* crude extracts separated by SDS-10% polyacrylamide gel electrophoresis. Lane 1 shows molecular size markers as indicated alongside in kilodaltons; the open circle in lane 2 indicates the positions of the expression of PobA in the clone pPobA (see Table 1). Lane 3 is a control with *E. coli* cells containing the pSD80 vector only.

not shown). Strain POBA1 did not grow on CHCA and 4-HBA. In contrast, when protocatechuate was used as the sole source of carbon, no significant difference was observed between the growth rates of POBA1 and ATCC 51369 (data not shown). This result strongly suggested that the cloned *pobA* gene was essential for the metabolism of CHCA and 4-HBA.

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