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Cloning and Characterization of Cyclohexanol Dehydrogenase Gene from *Rhodococcus* sp. TK6

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Abstract The cyclohexanol dehydrogenase (ChnA), produced by *Rhodococcus* sp. TK6, which is capable of growth on cyclohexanol as the sole carbon source, has been previously purified and characterized. However, the current study cloned the complete gene (*chnA*) for ChnA and its flanking regions using a combination of a polymerase chain reaction (PCR) based on the N-terminal amino acid sequence of the purified ChnA and plaque hybridization from a phage library of *Rhodococcus* sp. TK6. A sequence analysis of the 5,965-bp DNA fragment revealed five potential open reading frames (ORFs) designated as partial *pte* (phosphotriesterase), *acs* (acyl-CoA synthetase), *scd* (short chain dehydrogenase), *stp* (sugar transporter), and *chnA* (cyclohexanol dehydrogenase), respectively. The deduced amino acid sequence of the *chnA* gene exhibited a similarity of up to 53% with members of the short-chain dehydrogenase/reductase (SDR) family. The *chnA* gene was expressed using the pET21a(+) system in *Escherichia coli*. The activity of the expressed ChnA was then confirmed (13.6 U/mg of protein) and its properties investigated.

Key words: Cyclohexanol dehydrogenase, *chnA* gene, *Rhodococcus* sp. TK6

A number of microorganisms are capable of oxidizing cyclic alcohols into dicarboxylic acids, and the biochemical metabolism of cyclohexanol, a cyclic alcohol, into adipic acid, a dicarboxylic acid, in organisms, including *Acinetobacter*, *Pseudomonas*, and *Xanthobacter*, has already been studied [8, 28, 30]. The biological oxidation of cyclohexanol normally results in the formation of cyclohexanone, a cyclic ketone, which is successively metabolized as ε-caprolactone, 6-hydroxyhexanoate, 6-oxohexanoate, and adipate (Fig. 1). Then, the final metabolite, adipate, enters the central carbon metabolism (β-oxidation) in the cell [8]. Some of the enzyme activities in this pathway have previously been demonstrated, including cyclohexanol dehydrogenase (ChnA), NADPH-linked cyclohexanone monooxygenase (ChnB), ε-caprolactone hydrolase (ChnC), NAD (NADP)-linked 6-hydroxyhexanate dehydrogenase (ChnD), and 6-oxohexanoate dehydrogenase (ChnE). Although biological and chemical methods have both been suggested for removing environmentally toxic organic compounds, such as cylohexanol, the biological treatment of toxic organic compounds (bioremediation) using microorganisms or

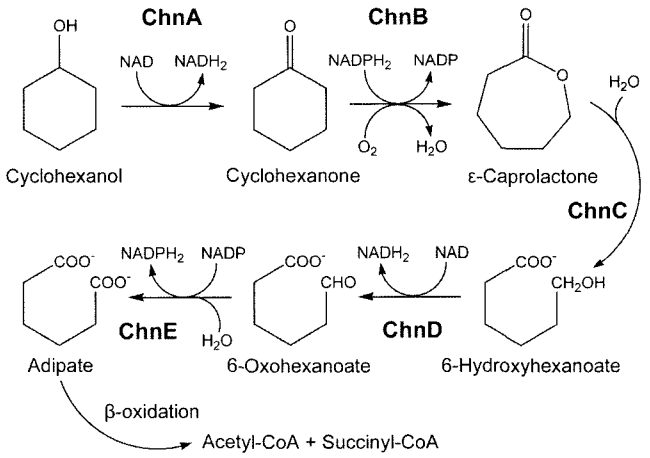

Fig. 1. Degradation pathway for cyclohexanol by *Acinetobacter* sp. strain NCIMB 9871 [11].
ChnA, cyclohexanol dehydrogenase; ChnB, cyclohexanone 1,2-monooxygenase (CHMO); ChnC, ε-caprolactone hydrolase; ChnD, 6-hydroxyhexanoate dehydrogenase; ChnE, 6-oxohexanoate dehydrogenase. Further oxidation of adipate into acetyl coenzyme A (acetyl-CoA) and succinyl coenzyme A (succinyl-CoA) proceeds via β-oxidation.

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enzymes produced from microorganisms or plants is usually considered to be more environmentally friendly [7, 14, 18, 21]. However, very little is still known about the genes (*chnA*, *B*, *C*, *D*, and *E*, respectively) involved in the cyclohexanol oxidative pathway and their organization. In this metabolic pathway, the dehydrogenation of cyclohexanol catalyzed by a specific alcohol dehydrogenase, cyclohexanol dehydrogenase, is the most recalcitrant and important reaction, yet the enzyme ChnA and its coding gene *chnA* have only been identified in a few species [4–6, 31].

The current authors previously reported on the isolation of *Rhodococcus* sp. TK6, which is capable of growth on cyclohexanol as the sole carbon source [16], and the purification and characterization of its cyclohexanol dehydrogenase (ChnA) that oxidizes cyclohexanol into cyclohexanone [15, 17]. Based on the N-terminal amino acid sequence of ChnA, the corresponding gene was then cloned by combining polymerase chain reaction (PCR) and plaque hybridization. Accordingly, this paper reports on the cloning and characterization of a novel cyclohexanol dehydrogenase gene (*chnA*) for cyclohexanol oxidation from *Rhodococcus* sp. TK6.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Culture Conditions

The bacterial strains and plasmids used in this study are listed in Table 1. The *Rhodococcus* sp. TK6 was grown at 30°C in a Luria-Bertani (LB) broth or basal medium [16] containing 0.4% cyclohexanol. The *E. coli* was routinely cultured in an LB medium at 37°C. When necessary, the media were supplemented with ampicillin (100 μg/ml).

Construction of *Rhodococcus* sp. TK6 Phage Library

The chromosomal DNA from *Rhodococcus* sp. TK6 was prepared using the method of Hopwood *et al.* [10], then partially digested with *Sau*3AI to yield fragments with an average size of 15 to 20 kb. Next, these fragments were ligated in a λBlueSTAR phage (Novagen, Madison, WI, U.S.A.), which had been completely digested with *Bam*HI and dephosphorylated with an alkaline phosphatase. The subsequent *in vitro* packaging and infection into *E. coli* ER1647 were carried out according to the manufacturer's recommendations (Novagen). The packaged genomic DNA library of *Rhodococcus* sp. TK6 contained a titer of 1.5×10^5 pfu/ml, as determined by transfecting the *E. coli* ER1647. The phage DNA isolated from five randomly chosen *E. coli* transformants was found to contain large inserts of DNA (15 to 20 kb).

Screening of Genomic Library of TK6 for a *chnA* Gene

To screen the *chnA* gene from the phage library of *Rhodococcus* sp. TK6, a probe was prepared by a PCR technique using a forward primer S3 (5'-ACCGGCGCC-GCGCGCGGAATHGG-3', where H=A, T or C) based on the N-terminal sequences of the purified ChnA [17] and reverse primer A3 (5'-CACGCCGTGTTTCGCGGCGCT-GTA-3') designed from the nucleotide sequences conserved in the short-chain alcohol dehydrogenases (SCADs) family.

Table 1. Bacterial strains and plasmids used in this study.

Strain or plasmid	Relevant characteristics	Reference or source
Strains		
Rhodococcus sp. TK6	Cyclohexanol degrader	[16]
E. coli ER1647	Host strain for plating libraries, amplification, *F' fhuA2 Δ(lacZ)r1 supE44 recD1014 trp31 mcrA1272*::Tn*10*(tet^r) *his$^-$1 rpsL104*(str^r) *xyl7 mtl2 metB1 Δ(mcrC-mrr)102*::Tn*10*(tet^r) *hsdS*($r_{K12}^-m_{K12}^-$)	Novagen
E. coli BM25.8	Host strain for automatic subcloning, *SupE thi Δ(lac-proAB)* [F' *traD36 proA$^+$B$^+$ lacI^{q}Z ΔM15*] λ*imm*^{434} (kan^r)P1(cam^r)*hsdR*($r_{K12}^-m_{K12}^-$)	Novagen
E. coli DH5α	Host strain for general DNA manipulation, *F' Ø80dlacZ ΔM15 Δ(lacZYA-argF)U169 deoR recA1 endA1 hsdR17* ($r_K^-m_K^-$) *supE44 λ$^-$ thi-1 gyrA96 relA1*	[9]
E. coli BL21(DE3)	Expression host strain for pET21a(+) vector, *F' ompT hsdS$_B$*($r_B^-m_B^-$) *gal dcm* (DE3)	Novagen
Plasmids		
pUC119	Cloning vector, Ap^r P_{lac} *lacZ* M13G	[32]
pET21a(+)	*E. coli* overexpression vector, Ap^r *lacI^q* P_{T7}	Novagen
pOLC	About 15-kb *Sau*3AI fragment containing *chnA* derived from λBlueSTAR™ vector system, Ap^r	This study
pOLC28	2.8-kb *Bgl*II fragment containing *chnA* in pUC119, Ap^r	This study
pOLC65	6.5-kb *Pvu*II fragment containing *chnA* in pUC119, Ap^r	This study
pETCDH	813-bp *Nde*I, *Eco*RI fragment containing *chnA* in pET21a(+), Ap^r	This study

The PCR was performed at 94°C for 5 min, then cycled 30 times at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, followed by incubation at 72°C for 5 min. A 508-bp PCR product was obtained and labeled with ^{32}P-dCTP using a random primer DNA labeling kit as recommended by the manufacturer (Takara, Tokyo, Japan). The hybridization was performed as described by Sambrook *et al.* [23] using a Hybond-N$^+$ nylon membrane (Amersham-Pharmacia Biotech., Little, Chalfont, England). Positive signal plaques obtained from the phage library of *Rhodococcus* sp. TK6 were automatically subcloned by *Cre-lox*P mediated excision of the plasmid from the λBlueSTAR in the *E. coli* BM25.8 (Novagen). One of the plasmids was selected and designated as pOLC.

Southern Hybridization and Subcloning

To subclone the DNA fragment containing the *chnA* gene from pOLC, the plasmid was digested with various restriction endonucleases. The preparation of the gene probe and hybridization conditions were the same as described above. As a result of the Southern hybridization, a positive single signal was obtained from a 2.8-kb DNA fragment digested with *Bgl*II and 6.5-kb DNA fragment digested with *Pvu*II. Each DNA fragment was cloned into the *Bam*HI and *Pvu*II sites of pUC119 and the resulting plasmids designated as pOLC28 and pOLC65, respectively.

Analysis of DNA Sequence

To analyze the sequence of the 2.8-kb *Bgl*II fragment in pUC119, the pOLC28 was digested with several different restriction endonucleases, then the DNA fragments less than 600-bp were subcloned into appropriate sites of pUC119. The DNA sequencing was performed by using the dideoxy chain termination method [24] using an M13-40 primer (5'-cyanine-CGCCAGGGTTTTCCAGTCACGAC-3') and M13 reverse primer (5'-cyanine-TTTCACACAG-GAAACAGCTATGAC-3'). The inserted pOLC65 DNA was sequenced by a PCR-based technique, Out-PCR, using oligonucleotides constructed based on the sequence of the 2.8-kb *Bgl*II. Database searches were performed using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST) at the National Center for Biotechnology Information [1]. Multiple sequence alignments were then generated using the ClustalW program (http://www.ch.embnet.org/software/ClustalW.html).

Expression of *chnA* Gene in *E. coli*

To overexpress the ChnA encoded by the *chnA* gene of *Rhodococcus* sp. TK6, the *chnA* gene was subcloned by a PCR between the *Nde*I and *Eco*RI sites of the expression vector pET21a(+). Two oligonucleotides were used to generate the unique *Nde*I and *Eco*RI sites in the PCR product of *chnA*: CDHf (5'-GGAATTCCATATGACGGA-CAACCTGCCC-3') with a start codon and *Nde*I site, and CDHr (5'-CCGGAATTCGGCTGTGG**CTA**CGAATATGT-3') with a stop codon and *Eco*RI. The PCR product was digested with *Nde*I and *Eco*RI and ligated into the same sites of pET21a(+), to construct pETCDH. The DNA inserted in pETCDH was sequenced to confirm that no mutations resulting from the PCR amplification were present in the open reading frame (ORF). The *E. coli* BL21(DE3) containing pETCDH was then cultivated in an LB medium containing 100 μg/ml of ampicillin at 37°C. When the culture reached an A_{600} of 0.3 to 0.4, isopropyl-β-thio-D-galactoside (IPTG) was added to the medium to a final concentration of 1 mM. The cells were further cultured for 4 h, then the cells were harvested by centrifugation, washed in a 50 mM sodium phosphate buffer (pH 7.0), resuspended in the same buffer, and sonicated at 95 μA for 30 sec 3 times using an ultrasonicator (Ultrasonic Ltd., England). After centrifugation at 20,000 ×*g* for 10 min at 4°C, the supernatant was used as the crude enzyme to determine enzyme activity.

Enzyme Activity and Protein Concentrations

The ChnA activity was assayed as described previously [15]. The protein concentrations were determined by the method of Bradford [2] using bovine serum albumin as the standard.

Nucleotide Sequence Accession Number

The nucleotide sequence reported in this paper was deposited in the GenBank under accession number AY394000.

RESULTS

Cloning of *chnA* Gene from *Rhodococcus* sp. TK6

To clone the *chnA* gene from *Rhodococcus* sp. TK6, which is capable of growth on cyclohexanol as the sole carbon source, the genomic DNA library of *Rhodococcus* sp. TK6 was constructed using the λBlueSTAR phage, as described in Materials and Methods. Five positive clones obtained from plaque hybridization were converted into autonomous replicating plasmids by infection of *E. coli* BM25.8. One of the plasmids, pOLC, was investigated further. The pOLC was digested with various DNA restriction endonucleases, and the digested fragment analyzed by Southern hybridization as described in Materials and Methods. Finally, a 2.8-kb DNA fragment and 6.5-kb DNA fragment containing the *chnA* gene were isolated from pOLC digested with *Bgl*II and *Pvu*II, and subcloned into the same sites of pUC119 to construct pOLC28 and pOLC65, respectively. Thereafter, the inserted DNA fragments were sequenced, as described in Materials and Methods.

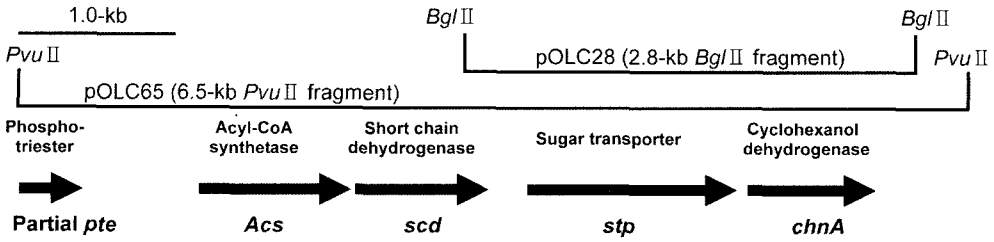

Fig. 2. Gene organization of the 5,965-bp cluster required for conversion of cyclohexanol into cyclohexanone and its flanking regions in *Rhodococcus* sp. TK6.

The exact locations of the ORFs are listed in Table 2. The arrows indicate the transcription direction of the genes, whose designations are listed below the ORFs. The names of the proteins homologous to the product of each ORF identified from the BLAST search are shown above the ORFs. CoA, coenzyme A.

Nucleotide Sequence of *chnA* Gene and its Flanking Regions

The 5,965-bp DNA sequence analysis revealed one partial and four complete ORFs (Fig. 2). The DNA sequences were translated in all the reading frames, and the putative products compared as regards their homology with all publicly available protein sequences contained in nonredundant databases using the BLAST algorithm. The homology search results are shown in Table 2. The partial ORF1 (*pte*) exhibited the greatest homology with an ORF encoding a parathion hydrolase from *Mycobacterium tuberculosis* CDC1551. ORF2 (*acs*) had the greatest homology with an ORF encoding an acid-coenzyme A ligase from *Bacillus halodurans* strain C-125. ORF3 (*scd*) had the greatest homology with an ORF encoding a short-chain dehydrogenase from *Xanthomonas campestris* ATCC 33913. ORF4 (*stp*) had the greatest homology with an ORF encoding a sugar (and other) transporter from *Bacillus anthracis* A2012. The 813-bp ORF5 (*chnA*, encoding a cyclohexanol dehydrogenase) located 84-bp downstream of ORF4 and preceded by a consensus ribosome-binding sequence (GGAGA) was found to encode a polypeptide of 270 amino acid residues.

The homology search indicated that the ChnA belonged to a short-chain dehydrogenase/reductase (SDR) family [13]. The highest score from the BLAST search for the ChnA was the 3-oxoacyl-(acyl-carrier protein) reductase of *Bacillus halodurans* with a similarity of 53%. The ChnA of *Rhodococcus* sp. TK6 was 32.8, 33.6, 34.6, and 31.5% homologous to the ChnA of *Acinetobacter* sp. SE19, *Brevibacterium* sp. HCU, *Comamonas* sp. NCIMB9871, and *Xanthobacter* sp. ZL5, respectively, and 34.6% homologous to the cyclopentanol dehydrogenase (CpnA) of *Comamonas* sp. NCIMB9872 (Fig. 3). The TK6 ChnA sequence revealed several notable features (Fig. 3): Firstly, general feature of SCADs, i.e. an N-terminally located glycine-rich GXXXGXG sequence for coenzyme binding, was also exhibited on the TK6 ChnA. Secondly, there are six strictly conserved residues in SCADs according to Persson *et al.* [22]; three glycine residues at positions 16, 22, and 154 and three polar residues D-40, Y-176, and K-180. These residues were also conserved in the TK6 ChnA sequence. Thirdly, there are seven other residues conserved in most, yet not all, members of this family. These seven other residues were present in the TK6 ChnA sequence

Table 2. Homology of ORFs with proteins in the nonredundant protein databases[a].

ORF	Gene name	Location (bp)	Homologous protein (source species) [accession no.]	Similarity[b] (%)	Identity[c] (%)	E value[d]
1	Partial *pte*	1–403	Parathion hydrolase (*Mycobacterium tuberculosis* CDC1551) [NP334647]	67	50	1e-31
2	acs	1,099–2,046	Acid-Coenzyme A ligase BH1131 (*Bacillus halodurans* strain C-125) [C83791]	55	40	3e-56
3	scd	2,137–2,952	Short chain dehydrogenase (*Xanthomonas campestris* ATCC 33913) [NC003902]	63	47	2e-63
4	stp	3,134–4,456	Sugar (and other) transporter gene (*Bacillus anthracis* A2012) [NC003995]	49	34	2e-54
5	chnA	4,451–5,353	3-Oxoacyl-(acyl-carrier protein) reductase (*Bacillus halodurans*) [AP001515]	53	36	1e-32

a, Homology search was performed by the BLAST algorithm provided by the National Center for Biotechnology Information (NCBI).

b, Percentage of amino acids that is identical or conserved between the two proteins.

c, Percentage of amino acids that is identical between the two proteins.

d, Expect value, which estimates the statistical significance of the match by specifying the number of matches, with a given score, that are expected in a search of a database of this size absolutely by chance.

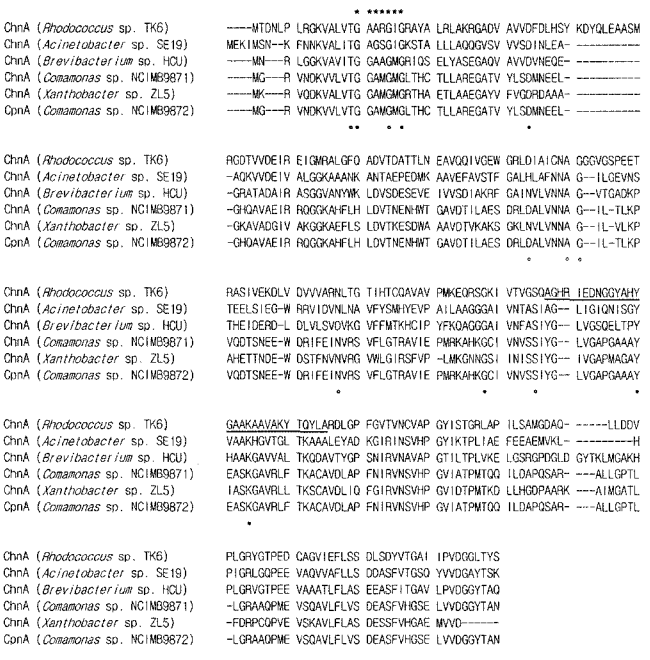

Fig. 3. Sequence alignment of ChnA from *Rhodococcus* sp. TK6 with GenBank cyclohexanol dehydrogenases from *Acinetobacter* sp. SE19 (accession no. AF282240), *Brevibacterium* sp. HCU (accession no. AF257214), *Comamonas* sp. NCIMB9871 (accession no. AJ418060), and *Xanthobacter* sp. ZL5 (accession no. AJ418061) and cyclopentanol dehydrogenase from *Comamonas* sp. NCIMB9872 (accession no. AB022102).
The alignment was performed using the ClustalW program. The amino acid residues involved in coenzyme binding are indicated by asterisks. The six strictly conserved residues are indicated by close circles below the residues. The seven other conserved residues common to most members of the SCADs superfamily are indicated by open circles below the residues. The consensus sequences of the short-chain dehydrogenases/reductase (SDR) family signature (accession no. PS00061) motif are underlined.

(T-15, G-20, D-100, A-106, G-107, N-133, and S-161). Fourthly, the polar residues, assumed to be of functional importance [22], were also conserved in the TK6 ChnA sequence (S-161, Y-176, and K-180). Finally, the TK6 ChnA sequence contained the PROSITE (accession no. PS00061) consensus pattern for SCADs, [LIVSPADNK] - X (12) - Y - [PSTAGNCV] - [STAGNQCIVM] - [STAGC] - K - {PC} - [SAGFYR] - [LIVMSTAGD] - X (2) - [LIVMFYW] - X (3) - [LIVMFYWGAPTHQ] - [GSACQRHM] at positions 163 to 191.

Expression of *chnA* Gene in *E. coli*

To verify the characteristics of the ChnA, pETCDH was constructed, as described in Materials and Methods. The *E. coli* BL21(DE3) cells containing pETCDH exhibited an increased intensity for the band corresponding to 28 kDa with IPTG induction (lane 5 in Fig. 4). The experimental M_r value agreed well with that for the native ChnA of *Rhodococcus* sp. TK6 [17] and predicted ChnA (28,376.1 Da). No enhanced protein band of this size was detectable

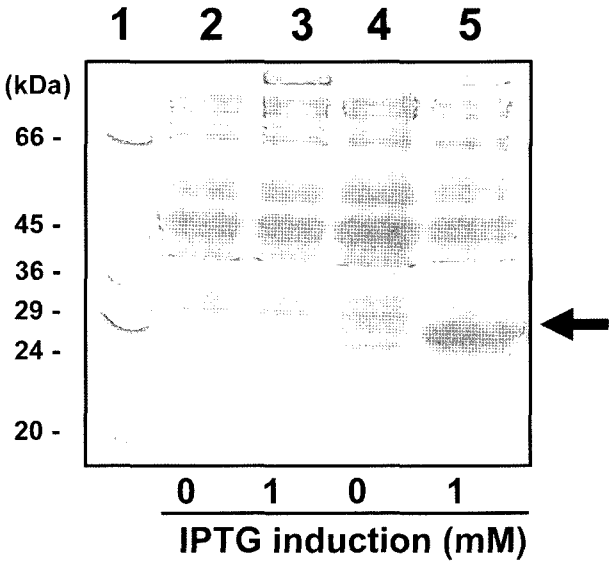

Fig. 4. Coomassie blue-stained protein profiles of recombinant *E. coli* BL21(DE3) crude extracts separated on sodium dodecyl sulfate-10% polyacrylamide electrophoresis gel.
To overexpress the ChnA, *E. coli* BL21(DE3) containing pETCDH was cultivated in an LB medium and 1 mM IPTG was added at an A_{600} of 0.3 to 0.4. The cells were further cultured for 4 h, then harvested and resuspended in a 50 mM sodium phosphate buffer (pH 7.0). The resulting solution was sonicated at 95 μA for 30 sec 3 times using an ultrasonicator. After centrifugation at 20,000 ×*g* for 10 min at 4°C, the supernatant was used as the crude enzyme. Lane 1 shows the molecular size markers as indicated alongside in kilodaltons. Lanes 2 and 3 are the controls with cells containing only the pET21a(+) vector. Lanes 4 and 5 are the crude enzyme from the cells containing pETCDH. The arrow indicates the position of the overexpressed ChnA protein.

in the control cells containing only the pET21a(+) vector (lanes 2 and 3 in Fig. 4) and IPTG-uninduced cells (lane 4 in Fig. 4). Crude extracts prepared from the *E. coli* BL21(DE3) cells containing the pETCDH were used to determine the enzyme activity, as described in Materials and Methods. For comparison, the respective activity in the control cells containing the pET21a(+) vector was also determined. As a result, the ChnA activity of the BL21(DE3) containing pETCDH (13.6 U/mg of protein) was 2.1-fold higher than that of the BL21(DE3) containing pET21a(+) (6.3 U/mg of protein). Thus, the 28 kDa band represented the ChnA protein from *Rhodococcus* sp. TK6.

Properties of TK6 ChnA

The optimal pH for ChnA activity was 8.0 in a 100 mM Tris-HCl buffer, where the activity was only 38% at pH 7.0 and 49% at pH 8.5 when the activity at pH 8.0 was defined as 100%. Furthermore, the activity dropped by more than 74% for all pHs after incubation for 12 h at 4°C. The ChnA activity was unstable at acidic and basic pHs and very difficult to maintain, which was consistent with the native ChnA purified from *Rhodococcus* sp. TK6 [17] and partially purified ChnA from *Nocardia* species [27].

Table 3. Substrate specificity of cyclohexanol dehydrogenase[a].

Substrate (13.3 mM)	Relative activity (%)[b]	Substrate (13.3 mM)	Relative activity (%)[b]
Methanol	71	2-Methyl-2-butanol	90
Ethanol	135	1,3-Propanediol	95
1-Propanol	126	1,2-Hexanediol	124
2-Propanol	71	1,6-Hexanediol	100
1-Butanol	126	Cyclopentanol	90
2-Butanol	76	Cyclohexanol	100
1-Pentanol	120	Cyclohexane-1,2-diol	90
1-Hexanol	136	Cyclohexane-1,3-diol	87
2-Methyl-2-propanol	90	Cyclohexane-1,4-diol	87

a, Assay was performed as described in Materials and Methods.
b, The activity with cyclohexanol was defined as 100%.

To investigate the substrate specificity of the ChnA, the enzyme's activity was tested with various aliphatic and alicyclic alcohols (Table 3). The activity toward cyclopentanol was 90% of that of cyclohexanol, whereas the activity toward cyclohexane-1,2-diol, cyclohexane-1,3-diol, and cyclohexane-1,4-diol was 90, 87, and 87%, respectively. Interestingly, in the case of the aliphatic alcohols, the primary alcohols (ethanol to 1-hexanol), except for methanol, were good substrates, whereas the secondary and tertiary alcohols were weak substrate under the same experimental conditions.

DISCUSSION

In contrast to the biodegradation of aliphatic and aromatic hydrocarbons, there have been relatively few reports on the biodegradation of cycloaliphatics. Cyclohexanol, a cycloaliphatic alcohol, is an intermediate in the breakdown of cyclohexane in fossil fuels and the degradation of more complex alicyclic compounds, such as terpenes and sterols [16]. In the cyclohexanol degradation pathway, ChnA first catalyzes the dehydrogenation of cyclohexanol into cyclohexanone. The current authors previously reported on the isolation of *Rhodococcus* sp. TK6, which is capable of growth on cyclohexanol as the sole carbon source [16], and the purification and characterization of ChnA [15, 17].

Accordingly, this paper applied a reverse genetic approach to clone the *chnA* gene from *Rhodococcus* sp. TK6 using the N-terminal amino acid sequence of the purified ChnA. Finally, the complete ChnA gene and its flanking regions were cloned. The Out-PCR technique was used to sequence a 6.5-kb DNA fragment flanking the *chnA* gene. The genes upstream of the *chnA* gene were sequenced, yet despite various efforts the downstream genes were not. The DNA region downstream of the *chnA* was very difficult to sequence, as is often the case for *Rhodococcus* sp. Phi1 [3]. In another study, the current authors cloned and sequenced a gene cluster for cyclohexanone oxidation in *Rhodococcus* sp. TK6 and the sequence analysis revealed five potential ORFs as *chnR*, *D*, *C*, *B*, and *E* (unpublished data). Nonetheless, there was no *chnA* gene sequence in the surrounding regions. In *Acinetobacter* sp. strain SE19 [6] and *Brevibacterium* sp. strain HCU [5], the *chnA* gene is part of a gene cluster that includes all the genes required for the degradation of cyclohexanol. Meanwhile, in *Arthrobacter* sp. BP2, *Rodococcus* sp. Phi1, and *Rodococcus* sp. Phi2, the *chnA* gene is not found in a gene cluster required for the degradation of cyclohexanone [3], demonstrating that the *chnA* gene may not necessarily be in a gene cluster from the cyclohexanol degradation pathway.

To express the *chnA* gene of *Rhodococcus* sp. TK6, the present study used a T7 promoter in an *E. coli* system. The expression plasmid for the ChnA, pETCDH, provided a high level of recombinant ChnA production in *E. coli* BL21(DE3). Unfortunately, most of the expressed ChnA was inactive owing to formation of insoluble proteins as inclusion bodies (data not shown), as is often the case for *Rhodococcus* genes [31, 33]. The overproduction of heterologous proteins in *E. coli* often results in the formation of biologically inactive inclusion bodies [12, 20, 25, 29]. Although various approaches have been used to minimize the inclusion body formation in recombinant *E. coli* [26, 29], none of them were effective in the present study as regards optimizing the activity of the expressed ChnA (data not shown); controlling the IPTG induction conditions (i.e., temperature, concentration of IPTG) and *chnA* gene expression based on incorporating a his-tag in the C-terminus in a pET system using pMal-c2X (NEB, U.S.A.) with a maltose-binding protein fusion system and pSTV28 (Takara, Japan) with a low copy number. Another attempt to unfold and refold the insoluble ChnA using a refolding CA kit (Takara, Japan) also met with failure. Subsequently, it was demonstrated that the expression of the *chnA* gene from the *Rhodococcus* strain was more effective in a Gram-positive *Bacillus* expression system than in a Gram-negative *E. coli* expression system as regards maximizing the expression of foreign gene products, such as the ChnA of *Rhodococcus* sp. TK6, which is prone to form inclusion

bodies in a recombinant *E. coli* system. These results will be described elsewhere.

Finally, the 5,965-bp sequence obtained from *Rhodococcus* sp. TK6 revealed 5 potential ORFs; partial *pte*, *acs*, *scd*, *stp*, and *chnA*. In these ORFs, in addition to the ChnA, a 271-amino acid peptide (named SCD) encoded by the *scd* gene was identified as part of the SDR family from the BLAST searches. Thus, the *scd* gene was expressed in *E. coli* in the same way, and its activity confirmed using cyclohexanol as the substrate (data not shown). Further studies are currently underway to characterize SCD protein.

Acknowledgment

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