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Cloning and Characterization of a Gene Cluster for Cyclohexanone Oxidation in *Rhodococcus* sp. TK6

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Abstract A gene cluster for cyclohexanone oxidation was cloned from *Rhodococcus* sp. TK6, which is capable of growth on cyclohexanone as the sole carbon source. The 9,185-bp DNA sequence analysis revealed seven potential open reading frames (ORFs), designated as *ssd-chnR-chnD-chnC-chnB-chnE*-partial *pcd*. The *chnBCDE* genes encode enzymes for the four-step conversion of cyclohexanone to adipic acid, catalyzed by cyclohexanone monooxygenase (ChnB), ε-caprolactone hydrolase (ChnC), 6-hydroxyhexanoate dehydrogenase (ChnD), and 6-oxohexanoate dehydrogenase (ChnE). Furthermore, the presence of a regulatory element in the downstream region of the *chnD* gene supports the notion that *chnR* is a putative regulatory gene. Among them, the activity of ChnB was confirmed and characterized, following their expression and purification in *Escherichia coli* harboring the modified *chnB* gene (*chnB* gene with 6 successive codons for His at the 3' terminus).

Key words: Cyclohexanone oxidation, cyclohexanone monooxygenase, *Rhodococcus* sp. TK6

A number of microorganisms are capable of oxidizing cyclic alcohols to corresponding dicarboxylic acids [24]. The biochemical metabolism of cyclohexanol, a cyclic alcohol, to adipic acid, a dicarboxylic acid, in organisms including *Acinetobacter*, *Pseudomonas*, and *Xanthobacter* has been studied [14, 28, 29]. Biological oxidation of cyclohexanol normally results in the formation of cyclohexanone, a cyclic ketone, and cyclohexanone is successively metabolized as ε-caprolactone, 6-hydroxyhexanoate, 6-oxohexanoate, and adipate (Fig. 1). Consequently, the final metabolite adipate enters the central carbon metabolism (β-oxidation) in the cell [14]. Most of the enzymes, 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), which are required for the oxidation of cyclohexanol, have been characterized biochemically [7, 9, 11, 17, 19]. Although both biological and chemical methods have been suggested for removing environmentally toxic organic compounds, such as cyclohexanol, the biological treatment of toxic organic compounds (bioremediation), using microorganisms or enzymes produced from microorganisms or plants, is usually considered to be more environmentally friendly [1, 3, 12, 13,

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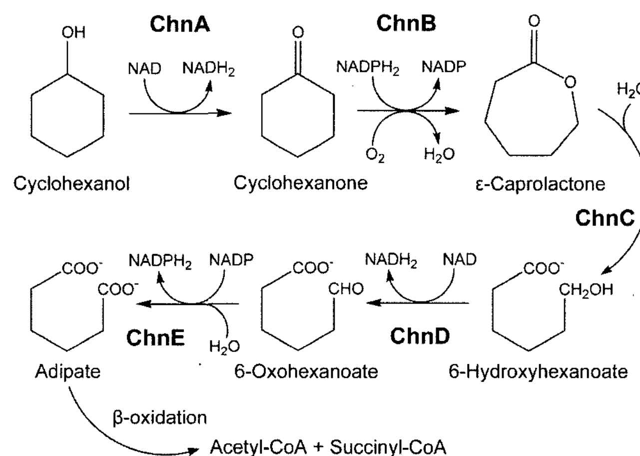

Fig. 1. Degradation pathway of cyclohexanol by *Acinetobacter* sp. strain NCIMB 9871 [17].
ChnA, cyclohexanol dehydrogenase; ChnB, cyclohexanone 1,2-monooxygenase (CHMO); ChnC, ε-caprolactone hydrolase; ChnD, 6-hydroxyhexanoate dehydrogenase; ChnE, 6-oxohexanoate dehydrogenase. Further oxidation of adipate to acetyl coenzyme A (acetyl-CoA) and succinyl coenzyme A (succinyl-CoA) proceeds via β-oxidation.

20]. Until recently, however, very little is known about the genes (*chnA*, *B*, *C*, *D*, and *E*, respectively) and their organization for the cyclohexanol oxidative pathway.

We have previously reported the isolation of *Rhodococcus* sp. TK6, capable of growth on cyclohexanol as a sole carbon source [22], the purification and characterization of cyclohexanol dehydrogenase (ChnA), which oxidized cyclohexanol to cyclohexanone [21, 23], and the cloning and characterization of the cyclohexanol dehydrogenase gene (*chnA*) in *Rhodococcus* sp. TK6 [12]. Biological oxidation of cyclohexanol by the *chnA* gene normally results in the formation of cyclohexanone, a cyclic ketone. However, as in the cases of *Arthrobacter* sp. BP2, *Rhodococcus* sp. Phl, and *Rhodococcus* sp. Ph2 [6], there has been no *chn* genes found related to cyclohexanone degradation in the *chnA* surrounding regions. Therefore, we tried to clone genes required for cyclohexanone oxidation in *Rhodococcus* sp. TK6, using the Baeyer-Villiger monooxygenase (BVMO) sequences of various strains reported previously [6, 7, 10, 17, 30]. In this paper, we report the cloning and genetic analysis of a gene cluster involved in the degradation of cyclohexanone in *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. *Rhodococcus* sp. TK6 was grown at 30°C in Luria-Bertani (LB) broth or basal medium [22] containing 0.4% cyclohexanol. *Escherichia coli* was routinely cultured in LB medium at 37°C. When necessary, media were supplemented with ampicillin (100 μg/ml).

Construction of *Rhodococcus* sp. TK6 Phage Library

Chromosomal DNA from *Rhodococcus* sp. TK6 was prepared by the method of Hopwood *et al.* [16], and then partially digested with Sau3AI to yield fragments with an average size of 15 to 20 kb. These fragments were ligated in the λBlueSTAR phage (Novagen, U.S.A.), which had been completely digested with BamHI and dephosphorylated with alkaline phosphatase. *In vitro* packaging and infection into *E. coli* ER1647 were carried out according to the recommendations of the manufacturer (Novagen, U.S.A.). The packaged genomic DNA library of *Rhodococcus* sp. TK6 contained a titer of 1.5×10^5 pfu per ml, as determined by transfecting of *E. coli* ER1647. Phage DNA, which was isolated from five randomly chosen *E. coli* transformants, was found to contain large inserts of DNA (15 to 20-kb).

Screening of a Genomic Library of TK6 for the *chnB* Gene

To screen the *chnB* gene from the phage library of *Rhodococcus* sp. TK6, we prepared a probe using polymerase chain reaction (PCR) with a forward primer of 2CMf with a start codon and NdeI site (5'-GGA ATT CCA TAT GAC CGC ACA GAC CAT CCA CAC C-3'), and a reverse primer of 2CMr with a stop codon and EcoRI (5'-CCG GAA TTC CGT CAG ACC GTG ACC ATC TCG GC-3'), designed from the *chnB* gene sequences of *Rhodococcus* sp. Phi2 [6]. PCR was performed at 94°C for 5 min, and then cycled

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

Strain or plasmid	Relevant characteristics	Reference
Strains		
Rhodococcus sp. TK6	Cyclohexanol degrader	[22]
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*] *λimm*^{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*	[15]
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	[31]
pET21a(+)	*E. coli* overexpression vector, Ap^r *lacI*^{q} P_{T7}	Novagen
pETCM2	1,623-bp NdeI, EcoRI fragment containing *chnB* in pET21a(+), Ap^r	This study
pCMC1 and pCMD31	About 15-kb Sau3A fragment containing *chnB* derived from λBlueSTAR^{TM} vector system, Ap^r	This study
pETCM-His	1,620-bp NdeI, HindIII fragment containing *chnB* in pET21a(+), Ap^r	This study

30 times at 94°C for 1 min, at 55°C for 1 min, at 72°C for 1 min, followed by incubation at 72°C for 5 min. In order to construct pETCM2, the PCR product was digested with NdeI and EcoRI and ligated into the same sites of the expression vector pET21a(+). About 1.6 kb of NdeI and EcoRI fragment in pETCM2 was labeled with ^{32}P-dCTP, using the random primer DNA labeling kit as recommended by the manufacturer (Takara, Japan). The hybridization was performed as described by Sambrook *et al.* [26], using Hybond-N$^+$ nylon membrane (Amersham-Pharmacia Biotech., England). Positive signal plaques, obtained from the phage library of *Rhodococcus* sp. TK6, were automatically subcloned by the *Cre-lox*P-mediated excision of plasmid from λBlueSTAR in *E. coli* BM25.8 (Novagen, U.S.A.). Two of the plasmids were selected and designated as pCMC1 and pCMD31.

Analysis of DNA Sequence

The inserted DNA (about 1.6-kb) of pETCM2 was sequenced by using the dideoxy chain termination method [27] with primer ETf (5'-TAC GAC TCA CTA TAG GGG-3') and primer ETr (5'-CTC AGC TTC CTT TCG GGC-3'). The inserted DNA (about 15 to 20-kb) of pCMC1 and pCMD31 were sequenced by the Out-PCR based technique, a primer walking method, with oligonucleotides constructed on the basis of a sequence known from the NdeI and EcoRI fragment in pETCM2. Database searches were performed using the BLAST (http://www.ncbi.nlm.nih.gov/BLAST) at the National Center for Biotechnology Information [2]. Multiple sequence alignments were generated using the ClustalW program (http://www.ch.embnet.org/software/ClustalW.html).

Expression of the *chnB* Gene in *E. coli*

To assess the activity of ChnB encoded by the *chnB* gene of *Rhodococcus* sp. TK6, the pETCM2 was constructed as described above. To purify ChnB, the *chnB*-[His-tag] fusion gene was subcloned by the PCR between the NdeI and HindIII sites of the expression vector pET21a(+). Two oligonucleotides were used to generate the unique NdeI and HindIII sites: 2CMf with start codon and the NdeI site described above, and 2CMr (5'-CCC AAG CTT GAC CGT GAC CAT CTC GGC GGA C-3') with the HindIII site and without stop codon at the 3'-terminus of the *chnB* gene. The PCR product was digested with NdeI and HindIII and ligated into the same sites of pET21a(+), in order to construct pETCM-His, in which the modified *chnB* gene (*chnB* gene with 6 successive codons for His at the 3' terminus) is located downstream from the T7 promoter. The inserted DNA in pETCM-His was sequenced, and the sequence confirmed that mutations resulting from PCR amplification were not present in the open reading frame (ORF). *E. coli* BL21(DE3) containing pETCM2 or pETCM-His was cultivated at 37°C in LB medium containing 100 μg/ml of ampicillin. When the culture reached A_{600} of 0.3 to 0.4, isopropyl-β-thio-D-galactoside (IPTG) was added to final 1 mM concentration in the medium. The cells were further cultured for 4 h. Then, the cells were harvested by centrifugation, washed in W buffer (50 mM sodium phosphate buffer containing 20 mM imidazole, pH 8.0), resuspended in the same buffer, and sonicated 3 times at 95 μA for 30 sec with an ultrasonicator (Ultrasonic Ltd, England). After centrifugation at 20,000 ×*g* for 10 min at 4°C, the supernatant of the cell extract was stored at 0°C for later use.

Purification of ChnB-[His-Tag] Fusion Protein

Affinity chromatography was used for the purification of the ChnB-[His-tag] fusion protein, containing 6 successive His sequences at the C-terminus. Four ml crude enzyme from BL21(DE3)/pETCM-His and 1 ml of Ni-NTA (Ni^{2+}-nitrilotriacetic acid) His·bind resin (Novagen, U.S.A.) were mixed by shaking gently at 4°C for 60 min. The mixture was loaded onto a column (ϕ 1.5×6.7 cm) and washed twice with 4 ml of W buffer. Protein was eluted four times with 0.5 ml of E buffer (50 mM sodium phosphate buffer containing 250 mM imidazole, pH 8.0). The elutate was then applied to a Superose 12 HR 10/30 column, operated with FPLC system (Pharmacia Biotech, Sweden), and protein was eluted with E buffer at a flow rate of 0.5 ml per min.

Enzyme Activity and Protein Concentration

ChnB activity was assayed using the protocol described by Brzostowicz *et al.* [7] with some modifications. The ChnB activity was spectrophotometrically assayed by monitoring the decrease of absorbance at 340 nm, which corresponds to the co-oxidation of NADPH. One unit of activity was defined as the amount of enzyme required to convert 1 μmol of NADPH in 1 min. Protein concentration was determined by the method of Bradford [4] using bovine serum albumin as the standard.

Nucleotide Sequence Accession Number

The nucleotide sequence reported in this paper has been deposited in the GenBank under the accession number AY486161.

RESULTS

Cloning of Genes for Cyclohexanone Oxidation from *Rhodococcus* sp. TK6

In order to screen the *chnB* gene for cyclohexanone oxidation in *Rhodococcus* sp. TK6, the primer sets were constructed on the basis of various BVMO sequences reported earlier [6, 7, 10, 17, 30]. As a result of the PCR with each primer set and chromosomal DNA of *Rhodococcus* sp. TK6 as a template, most of the PCR products did not appear under

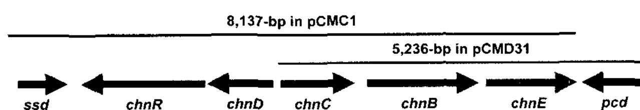

Fig. 2. Gene organization of the 9,185-bp cluster required for conversion of cyclohexanone to adipic acid and its flanking regions in *Rhodococcus* sp. TK6.
Exact locations of the ORFs are listed in Table 2. Black arrows indicate the direction of transcription of the genes, whose designations are listed below the ORFs. The names of proteins, homologous to the product of each ORF identified from the BLAST search, are shown in Table 2.

any of the PCR conditions. One of the PCR products, however, using a 2CMf-2CMr primer set based on the *chnB* gene of *Rhodococcus* sp. Phi2 [6], was selected because of its length. Finally, the PCR product was inserted in appropriate restriction enzyme sites of pET21a(+), in order to construct pETCM2. The inserted DNA fragment in pETCM2 was sequenced as described in Materials and Methods. Fortunately, the DNA sequence revealed one complete 1,623 bp ORF (putative *chnB* gene). The deduced amino acid sequence from the putative *chnB* gene showed 87, 82, and 76% homology (identity) to ChnB proteins of *Rhodococcus* sp. Phi2 (AY123972), *Rhodococcus* sp. Phi1 (AY123973), and *Arthrobacter* sp. BP2 (AY123974), respectively.

In order to clone the *chnB* gene and other genes required for cyclohexanone oxidation from *Rhodococcus* sp. TK6, its genomic DNA library was constructed using λBlueSTAR phage. Based on the results of plaque hybridization and automatic subcloning, two plasmids, pCMC1 and pCMD31, were constructed and further investigated. The inserted DNA fragments in both plasmids were sequenced by the primer walking method with oligonucleotides constructed on the basis of a sequence known from the putative *chnB* gene. A 8,137 bp nucleotide in pCMC1 and a 5,236 bp nucleotide in pCMD31, surrounding the putative *chnB* gene in each plasmid, were sequenced. Finally, a total 9,185 bp nucleotide sequence was determined.

Nucleotide Sequence of the *chnB* Gene and its Flanking Regions

The 9,185 bp DNA sequence analysis revealed that there were six complete ORFs and one partial ORF (Fig. 2). The DNA sequences were translated in all reading frames, and the putative products were compared, using the BLAST algorithm, with all publicly available protein sequences contained in the nonredundant database. Results of the homology search are shown in Table 2. It is seen that ORF1 (*ssd*), ORF2 (*chnR*), ORF3 (*chnD*), ORF4 (*chnC*), ORF5 (*chnB*), ORF6 (*chnE*), and partial ORF7 (pcd) have the greatest homology with putative succinic semialdehyde dehydrogenase, putative sigma 54-dependent transcriptional regulator, 6-hydroxyhexanoate dehydrogenase, caprolactone hydrolase, cyclohexanone monooxygenase, 6-oxohexanoate dehydrogenase, and protocatechuate dioxygenase from *Rhodococcus* sp. Phi2 [6], respectively. The organization of the gene cluster in *Rhodococcus* sp. TK6 appears to be identical to that of *Rhodococcus* sp. Phi2 with respect to the sequence and position of the metabolic genes. In particular, the ChnB, encoded by 1,623 bp ORF5 (*chnB*, encoding cyclohexanone monooxygenase), from *Rhodococcus* sp. TK6 was homologous to those from various strains described above (Table 2, Fig. 3). Furthermore, the *Rhodococcus* sp. TK6 ChnB sequence showed several notable features (Fig. 3): 1) A general feature of all BVMOs, which is an N-terminally located G(A, G, S, T)GX(A, G, S, T) G signature sequence for the coenzyme (FAD) binding at positions 16 to 21 (GAGFGG); 2) GG motif [FAD-binding Rossman fold; GGTWX(W)NXYPG] at positions 46 to 56 (GGTWYWNRYPG); 3) sequence of FEGETIHTAAWP, which fits to a so-called BVMO-identifying sequence motif, at positions 161 to 172; 4) NAD-binding Rossman fold [GKRV(G)XXIGTG] at positions 179 to 188 (GRRVGVIGTG); 5) amino acid residues involved in the adenosine binding signature (GXGXXG) at positions 186 to 191 (GTGSTG); and 6) a hexapeptide ATGFDA, a sequence that is described as

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

ORF (Gene name)	Location (bp)	Homologous protein with *Rhodococcus* sp. Phi2 (Accession no. AY123973)	Identity[b] (%)	Similarity[c] (%)	E value[d]
1 (*ssd*)	68–856	Putative succinic semialdehyde dehydrogenase	84	89	e-109
2 (*chnR*)	1,024–2,850	Putative sigma 54-dependent transcriptional regulator	87	92	0.0
3 (*chnD*)	2,861–3,919	6-Hydroxyhexanoate dehydrogenase	80	83	e-147
4 (*chnC*)	3,969–5,147	Caprolactone hydrolase	81	85	e-175
5 (*chnB*)	5,190–6,812	Cyclohexanone monooxygenase	87	89	0.0
6 (*chnE*)	6.897–8.279	6-Oxohexanoate dehydrogenase	92	94	0.0
7 (Partial *pcd*)	8.357–9.185	Putative protocatechuate dioxygenase	89	93	e-104

[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 between the two proteins.
[c]Percentage of amino acids that is identical or conserved between the two proteins.
[d]Expected 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.

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TK6     MTAQTTHT-V DAVVIGAGFG GIYAVHKLHH ELGLTTVGFD KADGPGGTWY WNRYPGALSD TESHLYRFSF DRDLLQESTW
Phi1    MTAQISPTVV DAVVIGAGFG GIYAVHKLHN EQGLTVVGFD KADGPGGTWY WNRYPGALSD TESHLYRFSF DRDLLQDGTW
Phi2    MTAQTIHT-V DAVVIGAGFG GIYAVHKLHH ELGLTTVGFD KADGPGGTWY WNRYPGALSD TESHLYRFSF DRDLLQDGTW
SE19    ---MSQKMDF DAIVIGGGFG GLYAVKKLRD ELELKVQAFD KATDVAGTWY WNRYPGALSD TETHLYCYSW DKELLQSLEI

TK6     KSTYITQPEI LEYLEDVVDR FDLRRHFKFG TEVTSALYLD DENLWEVTTD HGEVYRAKYV VNAVGLLSAI NFPNLPGLDT
Phi1    KTTYITQPEI LEYLESVVDR FDLRRHFRFG TEVTSAIYLE DENLWEVSTD KGEVYRAKYV VNAVGLLSAI NFPDLPGLDT
Phi2    KNTYVTQPEI LEYLEDVVDR FDLRRHFRFG TEVTSAIYLD DENLWEVTTD GGDVYRATYV VNAVGLLSAI NFPNLPGLDT
SE19    KKKYVQGPDV RKYLQQVAEK HDLKKSYQFN TAVQSAHYNE ADALWEVTTE YGDKYTARFL ITALGLLSAP NLPNIKGINQ
                             ** ********
TK6     FEGETIHTAA WPEGKSLAGR RVGVIGTGST GQQVITSLAP EVEHLTVFVR TPQYSVPVGN RPVTPEQIAE IKADYDRIWE
Phi1    FEGETIHTAA WPEGKNLAGK RVGVIGTGST GQQVITALAP EVEHLTVFVR TPQYSVPVGN RPVTKEQIDA IKADYDGIWD
Phi2    FEGETIHTAA WPEGKSLAGR RVGVIGTGST GQQVITALAP EVEHLTVFVR TPQYSVPVGN RPVTPEQIDA IKADYDRIWE
SE19    FKGELHHTSR WPDDVSFEGK RVGVIGTGST GVQVITAVAP LAKHLTVFQR SAQYSVPIGN DPLSEEDVKK IKDNYDKIWD

TK6     RAKNSAVAFG FEESTLPAMS VSEEERNRIF QEAWDHGGGF RFMFGTFGDI ATDEAANEAA ASFIRAKVAE IIEDPETARK
Phi1    SVKKSAVAFG FEESTLPAMS VSEEERNRIF QEAWDHGGGF RFMFGTFGDI ATDEAANEAA ASFIRSKIAE IIEDPETARK
Phi2    QAKNSAVAFG FEESTLPAMS VSEEERNRIF QEAWDHGGGF RFMFGTFGDI ATDEAANEAA ASFIRSKIAE IIEDPETARK
SE19    GVWNSALAFG LNESTVPAMS VSAEERKAVF EKAWQTGGGF RFMFETFGDI ATNMEANIEA QNFIKGKIAE IVKDPAIAQK

TK6     LMPTGLFAKR PLCDSGYYEV YNRPNVEAVA IKENPIREVT AKGVVTEDGV LHELDVLVFA TGFDAVDGNY RRIEIRGRDG
Phi1    LMPTGLYAKR PLCDNGYYEV YNRPNVEAVA IKENPIREVT AKGVVTEDGV LHELDVLVFA TGFDAVDGNY RRIEIRGRNG
Phi2    LMPTGLFAKR PLCDAGYHQV FNRPNVEAVA IKENPIREVT AKGVVTEDGV LHELDVLVFA TGFDAVDGNY RRIEIRGRDG
SE19    LMPQDLYAKR PLCDSGYYNT FNRDNVRLED VKANPIVEIT ENGVKLENGD FVELDMLICA TGFDAVDGNY VRMDIQGKNG
TK6     LHINDHWDGQ PTSYLGVSTA NFPNWFMVLG PNGPFTNLPP SIETQVEWIS DTIGYAERNG VRAIEPTPEA EAEWTETCTE
Phi1    LHINDHWDGQ PTSYLGVTTA NFPNWFMVLG PNGPFTNLPP SIETQVEWIS DTVAYAERNE IRAIEPTPEA EEEWTQTCTD
Phi2    LHINDHWDGQ PTSYLGVSTA NFPNWFMVLG PNGPFTNLPP SIETQVEWIS DTIGYAERNG VRAIEPTPEA EAEWTETCTA
SE19    LAMKDYWKEG PSSYMGVTVN NYPNMFMVLG PNGPFTNLPP SIESQVEWIS DTIQYTVENN VESIEATKEA EEQWTQTCAN

TK6     IANATLFTKG DSWIFGANIP GKKPSVLFYL GGLRNYRAVM AEVAADGYRG FEVKSAEMVT V-----
Phi1    IANATLFTRG DSWIFGANVP GKKPSVLFYL GGLGNYRNVL AGVVADSYRG FELKSAVPVT A-----
Phi2    IANATLFTKG DSWIFGANIP GKTPSVLFYL GGLRNYRAVL AEVATDGYRG FDVKSAEMVT V-----
SE19    IAEMTLFPKA QSWIFGANIP GKKNTVYFYL GGLKEYRSAL ANCKNHAYEG FDIQLQRSDI KQPANA
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Fig. 3. Sequence alignment of the ChnB from *Rhodococcus* sp. TK6 with other ChnB in the GenBank from *Rhodococcus* sp.
Phi1 (accession no. AY123974), *Rhodococcus* sp. Phi2 (accession no. AY123973), and *Acinetobacter* sp. SE19 (accession no. AF282240). An alignment was performed with the ClustalW program. The amino acid residues involved in the FAD-binding motif to the N-terminus and GG motif (FAD-binding Rossman fold) are indicated by open and closed circles, respectively. The boxed sequence is a so-called BVMO-identifying sequence motif. The amino acid residues involved in the NAD-binding Rossman fold are indicated by asterisks. The amino acid residues involved in the adenosine-binding signature are underlined. The common sequences among the FAD- and NAD(P)H-binding proteins are shaded.

common among the FAD/NADP(H)-binding proteins at positions 380 to 385.

Expression of the *chnB* Gene in *E. coli* and Purification

In order to verify the activity of ChnB, pETCM2 and pETCM-His were constructed as described in Materials and Methods. *E. coli* BL21(DE3) cells containing pETCM2 and pETCM-His showed an increased intensity of the band corresponding to 60 kDa after IPTG induction (data not shown). The experimental M_r value was in correlation with the predicted ChnB (59.9 kDa). No enhanced protein band of this size was detectable in the control cells containing the pET21a(+) vector only (data not shown). Thus, the 60 kDa band appears to represent the ChnB protein from *Rhodococcus* sp. TK6.

We also investigated the effect of a His-tag added to the C-terminus of ChnB. We compared the ChnB activity in *E. coli* BL21(DE3) cells containing the pET21a(+) (negative control possessing no ChnB), the pETCM2 (possessing wild-type ChnB), and the pETCM-His (possessing ChnB-[His-tag] fusion protein). *E. coli* BL21(DE3) cells containing the pETCM2 and the pETCM-His showed much higher activity than the cells containing the pET21a(+) (data not shown). The ChnB-[His-tag] fusion protein showed an enzyme activity as high as that of the wild-type ChnB (data not shown). Thus, we conclude that the His-tag at the C-terminus did not significantly influence the functional properties of the ChnB protein.

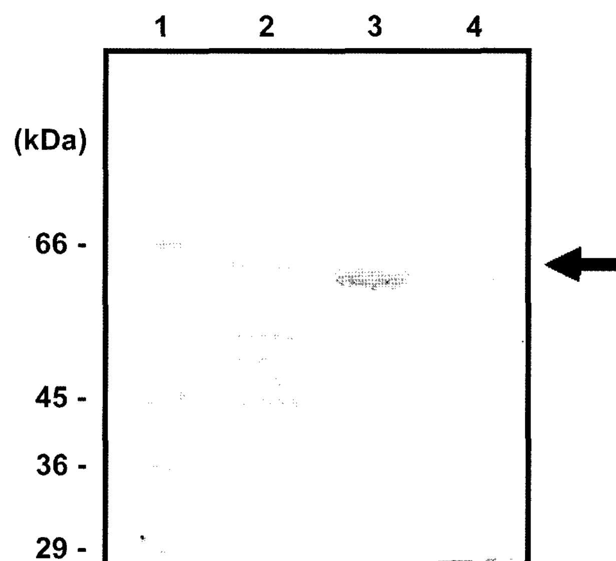

Fig. 4. Coomassie blue-stained protein profiles for the purification steps of ChnB-[His-tag] fusion protein from *E. coli* BL21(DE3)/pETCM-His, separated on a sodium dodecyl sulfate-10% polyacrylamide electrophoresis gel.
Lane 1, molecular size marker (kilodaltons); Lane 2, crude enzyme from *E. coli* BL21 (DE3)/pETCM-His; Lane 3, eluate of affinity chromatography; Lane 4, eluate of FPLC with Superose 12 HR. The arrow indicates the purified ChnB-[His-tag] fusion protein.

In order to purify the ChnB protein, the recombinant ChnB-[His-tag] fusion protein was expressed in *E. coli* BL21(DE3), which harbored the plasmid encoding the *chnB*-[His-tag] fusion gene, pETCM-His, by induction with 1 mM IPTG. The ChnB-[His-tag] fusion protein was purified by affinity (Ni-NTA His·bind resin) chromatography (Lane 3 in Fig. 4) and FPLC with Superose 12 HR (Lane 4 in Fig. 4). The experimental M_r value of the purified enzyme was estimated to be about 60 kDa, which correlated with

Table 3. Summary for the purification of ChnB-[His-tag] fusion protein.

Purification step	Total protein (mg)	Total activity (μmol/min)	Specific activity (μmol/min/mg protein)	Recovery (%)
Crude enzyme	3.777	21.74	5.8	100
Affinity chromatography	0.021	1.55	73.6	7.1
FPLC with Superose 12 HR	0.003	0.57	226.3	2.6

Table 4. Substrate specificity of the ChnB[a].

Substrate	Relative activity (%)[b]	Substrate	Relative activity (%)[b]
Cyclobutanone	65	Cyclodecanone	ND
Cyclopentanone	48	Cycloundecanone	ND
2-Methyl cyclopentanone	81	Cyclododecanone	ND
Cyclohexanone	100	1,2-Cyclohexanedione	24
2-Methyl cyclohexanone	95	1,3-Cyclohexanedione	1
Cycloheptanone	90	1,4-Cyclohexanedione	91
Cyclooctanone	23	Cyclohexene oxide	ND

[a]Assay was performed as described in Materials and Method.
[b]The activity with cyclohexanol was defined as 100%.
ND, Not detected.

the predicted ChnB-[His-tag] fusion protein. The ChnB-[His-tag] fusion protein was then purified about 39.3-fold from the supernatant, and the specific activity of the purified enzyme was 226.3 μmol/min/mg of protein and the yield was 2.6% (Table 3).

Properties of the TK6 ChnB

The optimal pH of the ChnB activity was 7.5 in 100 mM Tris-HCl buffer, and the enzyme exhibited 80% activity at pH 7.0–8.0, when the activity at pH 7.5 was defined as 100%. More than 80% of the enzyme activity remained at the pH range of 5.0 to 9.0 for 24 h at 4°C. As shown in Table 4, the purified enzyme oxidized cyclohexanone as well as a large variety of cyclic ketones: The enzyme readily oxidized cyclobutanone, cyclopentanone, cycloheptanone, and cyclooctanone as well as 2-methyl cyclopentanone and 2-methyl cyclohexanone. In addition, 1,2-cyclohexanedione and 1,4-cyclohexanedione were good substrates under the same conditions. The chnB, however, did not oxidize cyclodecanone, cycloundecanone, cyclododecanone, and cyclohexene oxide.

Discussion

In contrast to the biodegradation of aliphatic and aromatic hydrocarbons, there have been relatively few reports regarding cycloaliphatics. Until recently, very little has been known about the genes required for cyclohexanol degradation. In a previous work [12], we cloned and sequenced a *chnA* gene and its flanking regions in *Rhodococcus* sp. TK6. There were, however, no *chn* genes related to cyclohexanone oxidation in the region surrounding the *chnA* gene. In this study, we cloned and sequenced a gene cluster for cyclohexanone oxidation in *Rhodococcus* sp. TK6, and confirmed again that there was no *chnA* gene sequence in their surrounding regions. In *Acinetobacter* sp. strain SE19 [10] and *Brevibacterium* sp. strain HCU [8], the *chnA* gene is part of a gene cluster that includes all the genes required for the degradation of cyclohexanol. Furthermore, the *chnA* gene was not found in a gene cluster required for the degradation of cyclohexanone, in *Arthrobacter* sp. BP2, *Rhodococcus* sp. Phil, and *Rhodococcus* sp. Phi2 [6]. Finally, therefore, we concluded that the *chnA* gene cannot be in a gene cluster of the cyclohexanol degradation pathway in *Rhodococcus* sp. TK6.

The BVMO-catalyzed reactions have become important tools in organic syntheses, and the BVMOs have been exploited as valuable biocatalysts in chemoenzymatic syntheses and biotransformations [5, 32]. This is mainly due to their high enantioselectivity and regioselectivity. The flavin-containing ChnB, which catalyzes the BV oxidation of cyclohexanone into ε-caprolactone, has been characterized from several bacterial species [7, 25, 29]. During the last 3 years, several BVMOs have been cloned using methods such as reverse genetics [18], transposon mutagenesis [10], and mRNA differential display [7]. Until recently, however, only a few BVMO genes have been cloned and sequenced, although considerable amount of effort has gone into the characterization of BVMO reactions for biocatalytic applications [32]. In this study, we cloned and expressed the *chnB* gene in order to produce the ChnB-[His-tag] fusion protein in *E. coli*. The purified protein oxidized cyclohexanone as well as a large variety of cyclic ketones (Table 4). The specific activities were in the general range of those previously reported for the BVMOs of various strains [6]. Although overall patterns of the activity on most substrates were similar, the ChnB enzyme from *Rhodococcus* sp. TK6 exhibited different specific activities on some of the substrates (data not shown). We hopefully expect that this enzyme will be of use in biocatalysis application. Currently, we are in the process of characterizeing the ChnB enzyme as a biocatalyst in BVMO-catalyzed reactions.

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