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Cloning of Four Genes Involved in Limonene Hydroxylation from *Enterobacter cowanii* 6L

YANG, EUN JU, YEON JIN PARK, AND HAE CHOON CHANG*

Department of Food and Nutrition, Chosun University, Gwangju 501-759, Korea

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Abstract Genes encoding proteins responsible for limonene catabolism were cloned from a limonene-degrading microorganism, *Enterobacter cowanii* 6L, which was isolated from citron (*Citrus junos*) peel. The 8.6, 4.7, and 7.7 kb fragments (CD3, CD4, and CD6) of *E. cowanii* 6L chromosomal DNA that confer to *E. coli* the ability to grow on limonene have been cloned and their corresponding DNA sequences were determined. Nine open reading frames (ORFs) were identified, and the four ORFs (921 bp of CD3-2; 1,515 bp of CD4-1; 1,776 bp of CD6-1; and 1,356 bp of CD6-2) that encode limonene hydroxylase were confirmed by independently expressing these genes in *E. coli*. FAD and NADH were found to stimulate the hydroxylation reaction if added to cell extracts from *E. coli* recombinants, and multiple compounds (linalool, dihydrolinalool, perillyl alcohol, α-terpineol, and γ-terpineol) were the principal products observed. Our results suggest that the isolate *E. cowanii* 6L has a broad metabolic capability including utilization of limonene. This broad metabolic ability was confirmed by identifying four novel limonene hydroxylase functional ORFs in *E. cowanii* 6L.

Keywords: *Enterobacter cowanii*, limonene, limonene hydroxylase, broad metabolic capability

Terpenes are natural unsaturated hydrocarbons derived from isoprene units, and are widely distributed in nature. Moreover, their oxygenated derivatives, commonly called terpenoids, are important flavor compounds. Many of these terpenoids are considered as GRAS (Generally Recognized As Safe) compounds and are frequently used as food additives or fragrances [27]. Microbial conversion of low value monoterpenes to higher value derivatives has been recognized for their attractive commercial potential [17]. Furthermore, because of its low cost and extensive availability as a waste citrus product [5], the monoterpene R-(+)-limonene has been selected as a target for directed microbial bioconversions.

Progress towards the bioproduction of monoterpene products from limonene has been impeded by the multiplicity and the microbial toxicity of the limonene metabolites [25]. Biocatalytic conversion of R-(+)-limonene was considered as early as the 1960s [12, 13], and numerous R-(+)-limonene-transforming microorganisms have been described. A number of limonene biotransformations have been studied by several groups [6, 7, 9, 14, 18]. Most of these studies have suggested the existence of R-(+)-limonene degradation pathways, which were based on metabolite structures and simultaneous induction experiments. Relatively little works have been undertaken on the cloning and expression of the genes that are required for a functional microbial monoterpene degradation [3, 8, 10, 18].

If a target compound is an intermediate in a pathway, the use of null mutant constructs may allow the biocatalytic production of this compound. In the absence of supportive data from blocked mutants or genetic constructs, the application of this strategy for R-(+)-limonene has met with only limited success. Molecular studies of the limonene pathway would enable a better understanding of the enzymes participating in monoterpene catabolism and the biological origin of the observed metabolites. This would allow us to better understand and control their formations in biological applications.

In our previous reports [19, 20], we described the isolation of a bacterium, *Enterobacter* sp., which was able to grow well on limonene or citron peel oil as a sole carbon source, without any additional nutrient supplementation, and it reached a high biomass level (A_{600}: 2.5–3.0) after 120 h of incubation. *E. coli* TG1, which was used as a control, was not able to grow on limonene or citron oil minimal media. Our current objectives were to clone the functional limonene pathway genes from the isolate using *E. coli* as a host, to characterize these genes, and to identify the major conversion products.

*Corresponding author
Phone: 82-62-230-7345; Fax: 82-62-222-8086;
E-mail: hcchang@mail.chosun.ac.kr

MATERIALS AND METHODS

16S rRNA Sequence Determination of the Isolate 6L

In a previous report, microorganisms capable of growing on limonene were isolated from citron (*Citrus junos*) peel. Ultimately, one colony designated 6L, which grew well on limonene as the sole carbon source was chosen as a limonene-degrading strain. The isolate produced small creamy colonies on M9 salts agar plates. Morphologically, cells were rod shaped, and Gram-negative. Metabolic fingerprinting of the 6L isolate was carried out, and 6L was 82.3% identical with *Enterobacter agglomerans* [19]. To ensure the identification of the 6L isolate, the 16S rRNA gene sequence was determined in this study. The 16S rRNA gene sequence analyses were performed using a Big Dye Terminator Cycle Sequencing Kit with an automatic DNA kit from Applied Biosystems (Foster City, CA, U.S.A.) [15, 16]. The 16S rRNA gene sequence of the strain was aligned with those of other related bacteria and the level of similarity was determined [11, 21].

Growth in Liquid Minimal Media

Triple-baffled 300-ml nephelo culture flasks with a cleanout port and depressed side arm (Bellco Inc., Vineland, NJ, U.S.A.) were used for microbial growth studies. These flasks allowed vapor introduction of R-(+)-limonene or citron oil into the culture from 1 ml of liquid contained in the side arm. Citron oil was prepared from pressed citron peel waste by steam distillation. Steam was generated using a steam generator (Miura Co., Seoul, Korea), and distillation was carried out for 30 min at 115°C (2 kgf/cm^2). The medium for growth contained 50 ml of M9 salts media either alone or supplemented with yeast extract (50 mg/l). Cultures were incubated at 28°C in a shaking incubator. Growth was measured by determining the colony forming unit (CFU) per milliliter.

Cloning and Subcloning

The bacterial strains and plasmids used in these experiments are listed in Table 1. Total genomic DNA from the isolate 6L was prepared as described by Saito and Miura [23]. Plasmid DNA from *E. coli* was isolated by the alkaline lysis method [4]. Following partial restriction digestion with BamHI restriction enzyme, the isolate 6L DNA fragments of larger than 5 kb were isolated from an agarose gel by electroelution (Exelutor PAK6, FinePCR Co., Seoul, Korea) and were treated with alkaline phosphatase prior to ligation into the vector pUC18 (Takara Bio Inc., Shiga, Japan), which was also cleaved with BamHI. For hybridization studies, cloned fragments conferring growth on limonene when expressed in *E. coli* TG1 were labeled by the random primed incorporation of digoxigenin (DIG)-labeled deoxyuridine-triphosphate (dUTP) and were detected using a DIG DNA labeling and detection kit (Boehringer Mannheim Co., Indianapolis, IN, U.S.A.). Hybridization was carried out using conditions recommended by the manufacturer using procedures described by Sambrook *et al.* [24]. The cloned DNA fragments were sequenced by the dideoxy chain termination method using an ABI prism bigdye terminator cycle sequencing ready reaction kit (Perkin-Elmer Biosystems, Foster City, CA, U.S.A.). Internal primer-utilizing sequences were constructed using the

Table 1. The bacterial strains and plasmids used in this study.

Strains or plasmid	Description	References
Strain		
Enterobacter cowanii 6L	Wild-type (limonene & citron oil degrading pathway), lim$^+$	This work
E. coli TG1	*supE hsdΔ5 thiΔ* (*lac-proAB*) F'[*traD36 proAB*$^+$ *lacZΔM15*], lim$^-$	
E. coli EC3	*E. coli* TG1 harboring pREC3, lim$^+$	This work
E. coli EC4	*E. coli* TG1 harboring pREC4, lim$^+$	This work
E. coli EC6	*E. coli* TG1 harboring pREC6, lim$^+$	This work
E. coli EC3-2	*E. coli* TG1 harboring pSREC3, lim$^+$	This work
E. coli EC4-1	*E. coli* TG1 harboring pSREC4, lim$^+$	This work
E. coli EC6-1	*E. coli* TG1 harboring pSREC6-1, lim$^+$	This work
E. coli EC6-2	*E. coli* TG1 harboring pSREC6-2, lim$^+$	This work
Plasmid		
pUC18	2.7 kb, Ap^r	
pREC3	pUC18+CD3 (8.6-kb limonene-degrading gene from *E. cowanii* 6L)	This work
pREC4	pUC18+CD4 (4.7-kb limonene-degrading gene from *E. cowanii* 6L)	This work
pREC6	pUC18+CD6 (7.7-kb limonene-degrading gene from *E. cowanii* 6L)	This work
pSREC3	pUC18+CD3-2 (921-bp ORF DNA fragment from CD3)	This work
pSREC4	pUC18+CD4-1 (1,515-bp ORF DNA fragment from CD4)	This work
pSREC6	pUC18+CD6-1 (1,776-bp ORF DNA fragment from CD6)	This work
pSREC6-1	pUC18+CD6-2 (1,356-bp ORF DNA fragment from CD6)	This work

lim$^+$, grows on M9 salts media with limonene oil as a sole carbon source.
lim$^-$, cannot grow with limonene minimal media.

DNASIS program for both insert strands. Sequences were searched using the BLAST program at the NCBI (National Center for Biotechnology Information, Bethesda, MD, U.S.A.) database.

For subcloning of the limonene hydroxylase genes, each open reading frame (ORF) from the three DNA fragments (CD3, CD4, and CD6) conferring expression of limonene-degrading activity in *E. coli* was replicated by the polymerase chain reaction (PCR). Amplified ORF fragments were eluted using the Geneclean kit II (Bio 101, Irvine, CA, U.S.A.), and were then ligated with the pGEM T-easy vector (Promega Co., Madison, WI, U.S.A.). Ligation mixtures were transformed into *E. coli* TG1.

Nucleotide Sequence Accession Numbers

The DNA sequences reported here were deposited in the GenBank database under accession numbers DQ919062 (16S rRNA); DQ919063 (CD3-2); DQ919064 (CD4-1); DQ919065 (CD6-1); and DQ919066 (CD6-2).

Product Analysis

Chemicals standards and reaction products were analyzed with gas chromatography-mass spectrometry (GC-MS). The GC-MS system used consisted of a mass spectrometer coupled with gas chromatograph QP-5000 (Shimazu Co., Kyoto, Japan). A DB-Wax fused silica capillary column (60 m length×0.32 mm i.d.; J&W Scientific Co., Folsom, CA, U.S.A.) was used for the separation. The conditions used were the following: 1 μl injection; He carrier gas; injection port at 230°C and detector port at 250°C; and column temperature programmed from 40–230°C at 2°C/min up to 15°C and at 4°C/min up to 230°C with a 3 min initial hold time. Ethyl maltol was used as an internal standard, and positively identified compounds were quantified by using calibration of amount ratio (compound/internal standard) *vs.* peak area ratios (compound/internal standard) under identical experimental conditions. Compounds were identified by comparing GC-retention indices (RI) [22] and mass spectral data (Wiley 139 and NIST 12.62) with those of authentic standards.

Limonene Hydroxylase Assay

An activated colony of subcloned cells, *E. coli* EC3-2, EC4-2, EC6-1, or EC6-2, was inoculated into 50 ml of M9 salts solution supplied with 0.005% yeast extract, vapored limonene from the side arm, and 50 μg/ml ampicillin in a triple-baffled 300-ml nephelo culture flask with a cleanout arm and a depressed side arm, and then incubated at 28°C.

Cells were harvested, washed, and resuspended in M9 salts solution. The ice jacked cell suspension was sonicated (amplitude: 60; pulse: 2 sec; time: 10 min; Vibra-Cell VC130, Sonics & Materials Inc., Newton, CT, U.S.A.), and were then centrifuged at 9,950 ×*g* for 30 min at 28°C. Protein concentrations in cell-free extracts were determined by measuring the absorbance at 280 nm and 260 nm [2] using a UV spectrophotometer (Ultrospec 2100 pro, Amersham Biosciences, Uppsala, Sweden), and the extracts were adjusted to a final protein concentration of 0.064±0.001 mg/ml. These normalized extracts were used to determine the limonene hydroxylase activity. Three mM phenyl methyl-sulfonyl fluoride (PMSF, Sigma-Aldrich Co., St Louis, MO, U.S.A.) was added to the supernatant and this was used as a crude enzyme extract. The reaction mixture, which contained 500 μl of 0.2% limonene in 100 mM Tris-HCl buffer (pH 8.0) as a substrate, 1 mM flavin adenine dinucleotide (FAD, Sigma-Aldrich Co.), 1 mM nicotinamide adenine dinucleotide (NADH, Sigma-Aldrich Co.), or 1 mM FAD+ 1 mM NADH as a cofactor, 0.3 mM PMSF, 100 μl of sterilized distilled water, and 400 μl of crude enzyme solution, was incubated for 1 h at 28°C. The reaction mixture was extracted with ether (3×0.5 ml). The ether fraction was then evaporated and concentrated to 50 μl using a stream of nitrogen. The control reaction mixture, which contained the same mixture as above but with no addition of the crude enzyme extract or addition of *E. coli* TG1 (Luria-Bertani medium grown cell) extracts harboring pUC18 without the DNA insert, was extracted by using the same procedure. The reaction mixtures were analyzed for oxidized monoterpenes using GC-MS with reference to monoterpene standards.

RESULTS

Re-identification of the 6L Isolate-*Enterobacter* sp.

For the 16S rRNA sequence determination, the 16S rRNA gene was amplified by PCR, and the 1,384-bp DNA fragment was obtained and was sequenced. The 1,384-bp gene sequences containing the 16S rRNA gene were analyzed and compared using the BLASTN program of the NCBI BLAST server. The 1,384-bp DNA fragment was found to have 99% identity to the 16S rRNA gene of *Enterobacter cowanii* CIP 107300 (GenBank database Assession Number AJ508303). The nucleotide sequence was deposited in GenBank, with the accession number DQ919062. The 6L isolate, previously identified as *E. agglomerans* by using metabolic fingerprints [19], was finally identified as *E. cowanii*, and the phylogenetic relationship between the *E. cowanii* 6L and other related bacteria based on the 16S rRNA sequence is shown Fig. 1.

Cloning of the Limonene Pathway Genes

E. coli TG1 was transformed with a pUC18 vector containing *E. cowanii* 6L chromosomal DNA inserts. After growth on M9 salts agar plates containing ampicillin and incubation with limonene vapor, small colonies (designated lim^+) were observed at a low frequency after 2–3 days. Following verification of growth on limonene vapor by subsequent transfers, three lim^+ transformants designated *E. coli* EC3,

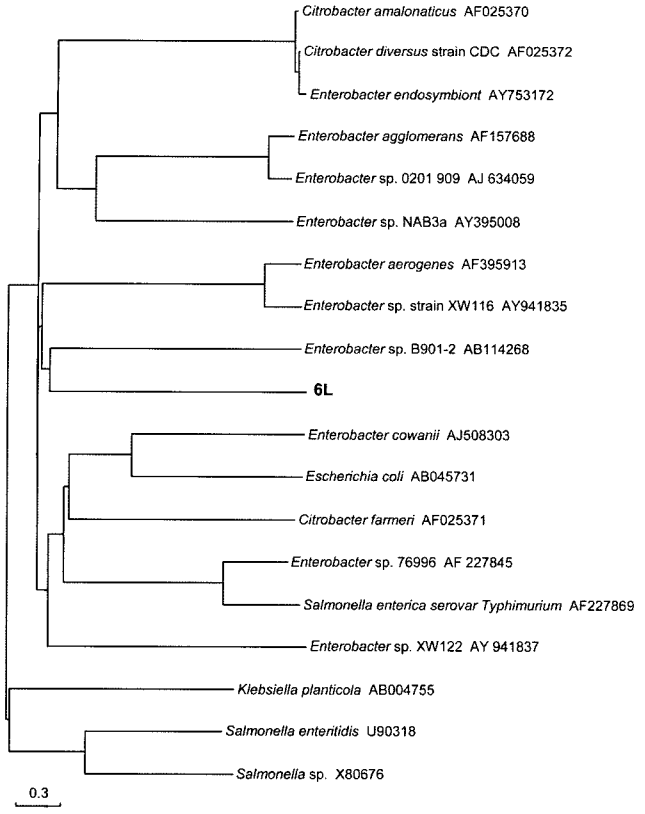

Fig. 1. Phylogenetic tree of *Enterobacter* species closely related to *Enterobacter cowanii* 6L.

EC4, and EC6 were selected. Growth on limonene vapor was not observed with *E. coli* TG1 harboring the pUC18 vector. The three transformants, EC3, EC4, and EC6, contained a 8.6, 4.7, and 7.7 kb insert, respectively. Hybridization experiments indicated that each insert was derived from the chromosome of the *E. cowanii* 6L parent (Fig. 2). However, the three inserts did not hybridize to each other (data not shown).

The nucleotide sequence of the loci encoding the limonene pathway was determined from the 8.6, 4.7, and 7.7 kb inserts, designated as CD3, CD4, and CD6, respectively. Sequence analysis of the cloned inserts suggested that CD3 has four open reading frames (ORFs), whereas CD4 has three, and CD6 has two (Fig. 3).

Subcloning of the Limonene Hydroxylase Gene

Each verified ORF (a total of nine ORFs), as shown in Fig. 3, was amplified by PCR and subcloned into the pGEM T-easy vector. Four kinds of *E. coli* transformants harboring ORF, CD3-2 (921 bp), CD4-1 (1,515 bp), CD6-1 (1,776 bp), and CD6-2 (1,356 bp), respectively, showed minimal colony growth on M9 salts agar plates with ampicillin and limonene. The other subcloned ORFs did not allow growth of transformants. The growth of transformants on limonene was confirmed by repeated culture on solid M9 salt media with limonene. Growth studies with the transformants EC3-2, EC4-1, EC6-1, and EC6-2 in M9 salts media with limonene vapor showed that these four transformants could utilize limonene as their sole carbon source in liquid culture, although biomass levels were very low (Fig. 4). No growth of EC3-2, EC4-1, EC6-1, or EC6-2 was observed in M9 salts media in the absence of limonene. Moreover, the addition of yeast extract alone, without limonene, did not increase cell growth (Fig. 4). However, the addition of yeast extract at a low concentration (50 mg/l) elevated biomass levels while retaining limonene-stimulated growth and was used in the limonene hydroxylation studies.

The amino acid identity between the limonene-degrading pathway sequence (CD3-2: 306 aa; CD4-1: 504 aa; CD6-1:

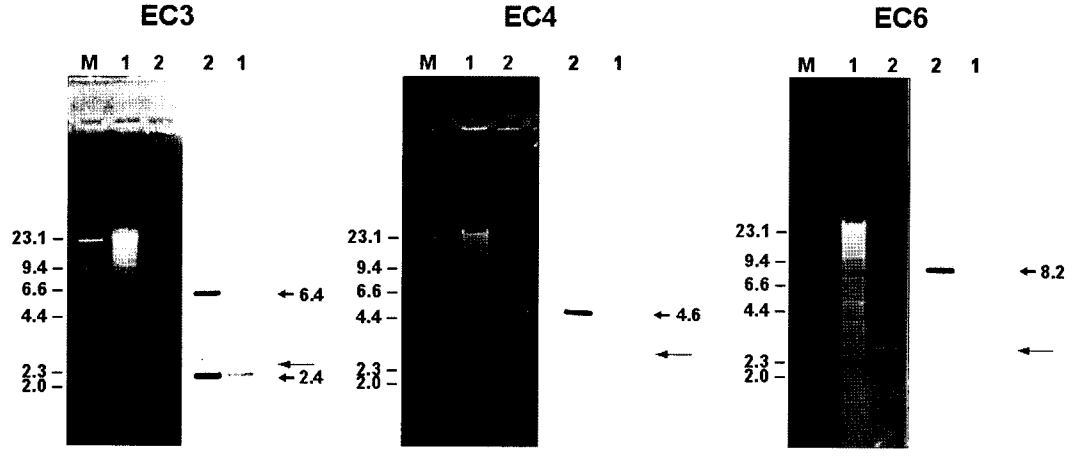

Fig. 2. Southern hybridization of pREC3, pREC4, and pREC6. Each cloned insert (8.6, 4.7, and 7.7 kb) from *E. cowanii* 6L was used as a probe.

The thin arrow indicates the vector position. Lane M: molecular weight of 6L DNA; lane 1: BamHI digest of 6L chromosomal DNA; lane 2: BamHI digest of recombinant plasmids, pREC3, pREC4, and pREC6.

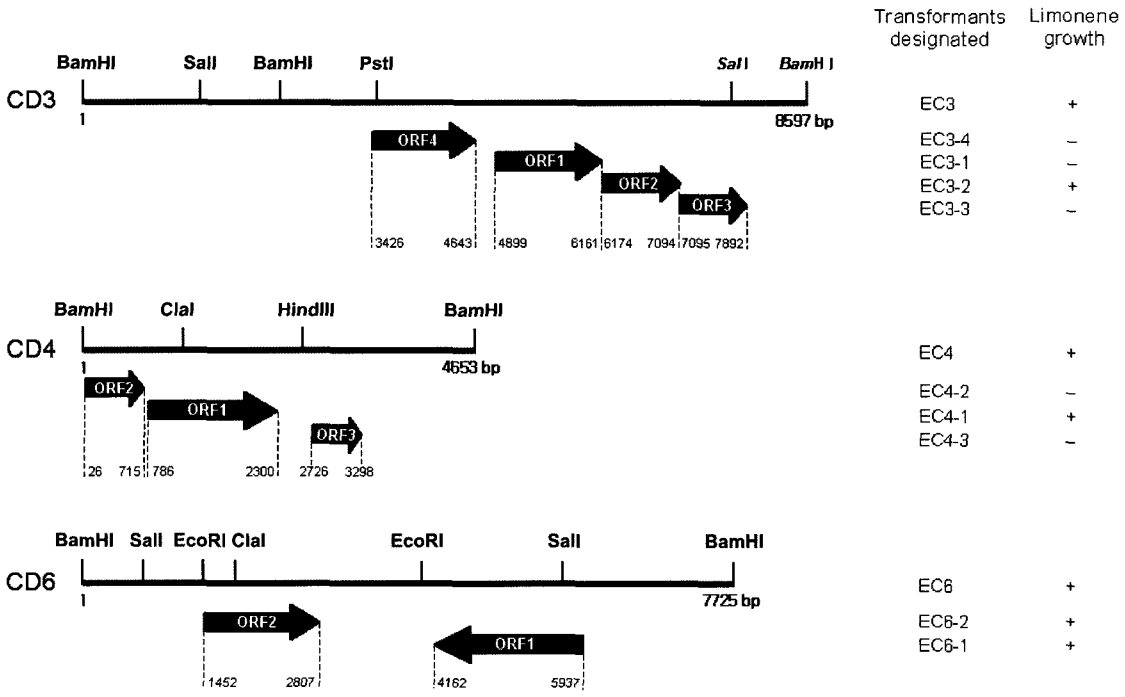

Fig. 3. Identification of ORFs in CD3, CD4, and CD6 and growth of the transformant harboring the subcloned ORF.

591 aa; and CD6-2: 451 aa) from *E. cowanii* 6L and the limonene hydroxylase sequence (543 aa; Accession Number AAC25032) from *Bacillus stearothermophilus* BR388 [10] was examined. CD3-2, CD4-1, CD6-1, and CD6-2 showed 16.1%, 16.3%, 17.3%, and 17.1% homology, respectively (Table 2). A comparison of the cloned limonene hydroxylase genes with sequences found in the GenBank database indicated that the CD3-2 gene showed a 70.3% identity with the nitrate transporter permease component (NTP) from *Erwinia carotovora* subsp. *atroseptica* SCRI1043, the CD4-1 gene showed a 86.1% identity with the L-arabinose transport ATP-binding protein araG (AT-ATP) from *Escherichia coli* CFT073, and CD6-1 and CD6-2 showed 39.4% and 36.9% identity with a putative signaling protein from *E. carotovora* subsp. *atroseptica* SCRI1043 and a hypothetical protein from *Yersinia pestis* biovar. *medievalis* 91001, respectively (Table 2).

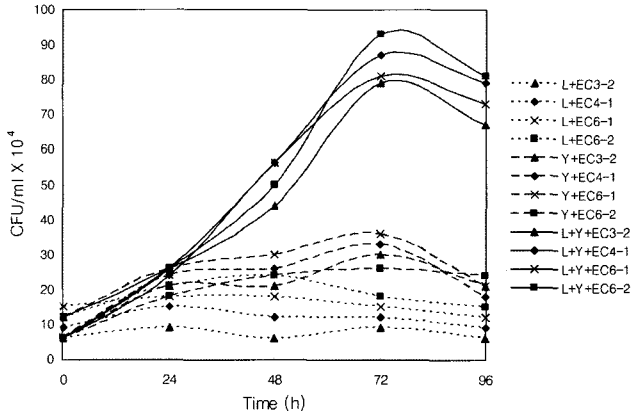

Fig. 4. Growth of *E. coli* transformants EC3-2, EC4-1, EC6-1, and EC6-2 on M9 salts media supplemented with limonene (L) and/or yeast extract (Y).

Limonene Hydroxylation Using Crude Enzyme Extracts

The hydroxylation of limonene by crude enzyme extracts of EC3-2, EC4-1, EC6-1, and EC6-2 was examined in the presence of various cofactors. Hydroxylation was stimulated in the presence of 1 mM NADH and 1 mM FAD as cofactors (Table 3). In this reaction, linalool was observed as the major product, although smaller amounts of dihydrolinalool were also formed. Perillyl alcohol was formed from the EC6-1 or EC6-2 reaction mixtures, but α-terpineol and γ-terpineol were formed only by the EC6-2 reaction mixture, although the reaction product levels were low.

DISCUSSION

The microbial conversion of low-value terpenes to high-value derivatives has been recognized as a commercially important process; however, enthusiasm has been jaded by the multiplicity of products produced and the low product yields caused by the relative toxicity of terpenes to most microorganisms.

Table 2. Amino acid identity between pairs of gene *E. cowanii* 6L CD3-2 (1), *E. cowanii* 6L CD4-1 (2), *E. cowanii* 6L CD6-1 (3), *E. cowanii* 6L CD6-2 (4), *B. stearothermophilus* BR388 LH (5), *E. carotovora* subsp. *atroseptica* SCRI1043 NTP (6), *E. coli* CFT073 AT-ATP (7), *E. carotovora* subsp. *atroseptica* SCRI1043 PSP (8), and *Y. pestis* biovar. *medievalis* 91001 HP (9).

	% Amino acid identity with gene from								
	1	2	3	4	5	6	7	8	9
1	100.0	17.4	16.5	19.1	16.1	70.3	17.6	14.0	16.2
2		100.0	17.6	20.3	16.3	15.2	86.1	17.3	20.5
3			100.0	19.8	17.3	15.9	21.7	39.4	17.7
4				100.0	17.1	16.3	19.4	16.9	36.9
5					100.0	14.9	17.1	16.9	17.8
6						100.0	14.9	12.7	14.8
7							100.0	17.4	16.7
8								100.0	18.0
9									100.0

LH, Limonene hydroxylase; NTP, nitrate transfer permease; AT-ATP, L-arabinose transport ATP-binding protein araG; PSP, putative signaling protein; HP, hypothetical protein YP1161.

The isolate *E. cowanii* 6L grew unexpectedly well (A_{600}: 2.5–3.0 after 120 h) on minimal medium with limonene or citron peel oil as the sole carbon source [19, 20]. The volatile components of citron peel oil, extracted by steam-distillation in this study, were R-(+)-limonene, γ-terpinene, β-myrecene, linalool, β-phellandren, and many others. R-(+)-limonene was the major component and accounted for 54% of the citron volatile components, and many other terpenes are known to be present in small quantities [20]. *E. cowanii* 6L can degrade not only limonene efficiently but also other terpene compounds. It has a broad metabolic capability and can be utilized for the degradation or conversion of terpenes.

The cloning of the genes involved in limonene degradation in *E. cowanii* 6L was achieved in this study. Three different inserts (CD3-8.6 kb, CD4-4.7 kb, and CD6-7.7 kb) from *E. cowanii* 6L chromosomal DNA allowed the *E. coli* EC3, EC4, and EC6 constructs to grow on R-(+)-limonene as the sole carbon source. Four different ORFs were characterized; CD3-2, CD4-1, CD6-1, and CD6-2; these genes encoded proteins with limonene hydroxylation activity, and *E. coli* cells carrying one of them were able to grow on limonene. Moreover, limonene hydroxylation activity was found for four different transformants, indicating the presence of four distinct hydroxylase genes in *E. cowanii* 6L.

A comparison study of the cloned four genes (CD3-2, CD4-1, CD6-1, and CD6-2) with those found in the GenBank database, including a limonene hydroxylase gene from *B. stearothermophilus* BR388 [10], was carried out. Homologies in amino acid sequence were quite low (less than 20%), but the amino acid sequences of CD3-2 and CD4-1 genes showed significant (70–86%) similarities with those of *E. carotovora* NTP and *E. coli* AT-ATP. A sequence comparison (shown in Table 2) between CD3-2 and CD4-1 sequences (the genes of which showed limonene hydroxylation activity in Table 3) and other known protein sequences raises a question; *i.e.*, what is the significance of a homology greater than 70% between the CD3-2 and CD4-1 genes from *E. cowanii* 6L and NTP from *E. carotovora* SCRI1043 and AT-ATP from *E. coli* CFT073? It is interesting that the genes in this comparison are all related to the electron transfer process. Further research is needed to explain the relationship between specific function and gene sequences that show high similarity.

Table 3. Limonene hydroxylation products by the subcloned *E. coli* cell extracts.

Compounds	Concentration of product (μM)														
	FAD					NADH					FAD+NADH				
	Control	EC3-2	EC4-1	EC6-1	EC6-2	Control	EC3-2	EC4-1	EC6-1	EC6-2	Control	EC3-2	EC4-1	EC6-1	EC6-2
Dihydrolinalool	3.2	–	–	62.2	5.1	3.2	71.8	85.3	–	10.3	2.4	126.9	64.1	10.3	6.4
Linalool	552.0	6,409.1	9,577.9	6,922.1	707.8	594.8	7,785.7	9,000.0	6,675.3	1,357.1	412.3	7,123.4	6,961.0	1,993.5	818.2
Perillyl alcohol	–	–	–	2,263.2	188.2	–	–	–	1,782.9	153.3	–	–	–	2,855.3	231.6
α-Terpineol	–	–	–	–	194.8	–	–	–	–	163.6	–	–	–	–	211.0
γ-Terpineol	–	–	–	–	23.4	–	–	–	–	16.9	–	–	–	–	26.0

–, Not detected.
All experiments were performed in duplicate.

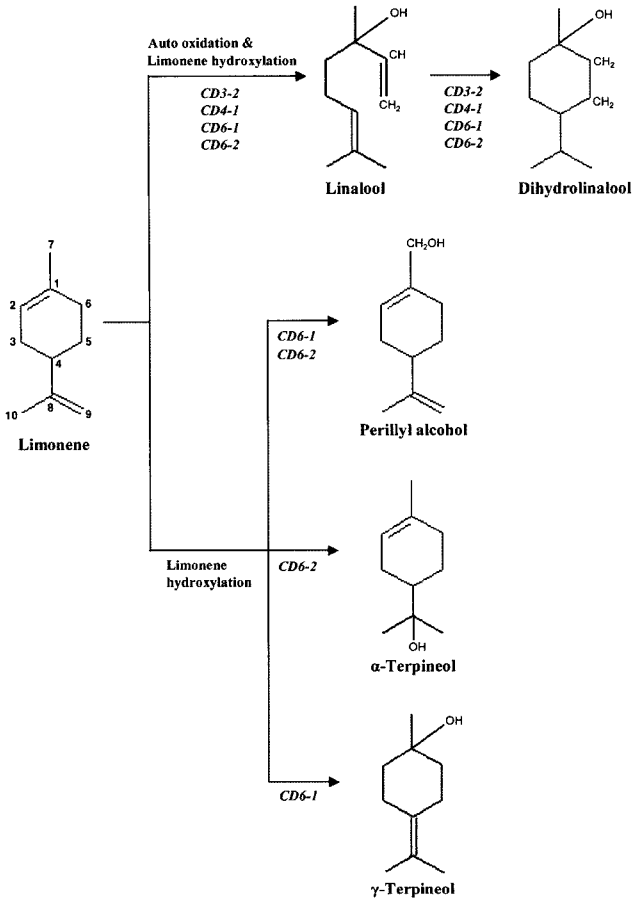

Fig. 5. Proposed pathway for limonene degradation and principal metabolic products of *E. coli* transformants containing cloned DNA fragments from *E. cowanii* 6L.

Four types of *E. coli* transformants harboring 921 bp (CD3-2), 1,515 bp (CD4-1), 1,776 bp (CD6-1), or 1,356 bp (CD6-2) were found to encode a limonene hydroxylase that hydroxylated limonene in the 1, 7, or 8 positions. A limonene-degrading pathway involving the four transformants (EC3-2, EC4-1, EC6-1, and EC6-2) is proposed in Fig. 5. Although biochemical examinations with purified enzymes are needed to elucidate the role of cofactors in the initial limonene oxidation step, it seems likely to require NADH and FAD.

Limonene hydroxylation products, linalool and dihydrolinalool, were also detected in the control reaction mixture, which contained substrate (limonene) and cofactors without enzyme extract. This finding suggests that the multiple products formed in this case result from the microbial enzymatic conversion of limonene and the auto-oxidation products of limonene. The results shown in Table 3 imply that linalool and dihydrolinalool are the combined results of the microbial enzyme reaction and auto-oxidation. The presence of these compounds in the control reaction shown in Table 3 was attributed to limonene auto-oxidation. It has also been reported [1] that limonene is relatively unstable, and some of the products identified in the culture media were also attributed to auto-oxidation and rearrangement of limonene. In addition, it has also been reported that, in many cases, products are only produced in small quantities (mg/l range), and that long incubation times are required (weeks); thus, it is uncertain if the products formed were the result of a biological activity [26]. In this study, the availability of the *E. coli* EC3-2, EC4-1, EC6-1, and EC6-2 recombinants should allow for the identification of the participating limonene pathway enzymes and metabolites. Moreover, the levels of the desired monoterpene products produced were moderately high (g/l range: *i.e.*, linalool 9,577.9 μM in Table 3 is equivalent to 1.475 g/l/h productivity) to be of commercial interest. The parent strain *E. cowanii* 6L and the four subcloned *E. coli* transformants described here should be of commercial interest and provide supportive information about the genetic make-up of a limonene degradation pathway.

Moreover, in this study, we cloned four different genes that have similar or overlapping functions for limonene hydrolysis, from *E. cowanii* 6L. We believe that the present study is the first to identify four different clones of functional microbial limonene degradation pathway genes in one parent strain. *E. cowanii* 6L, seems to have several different enzymes capable of breaking down limonene that act independently from other known activities. It can be deduced that the bacterium might have accumulated several independent catabolic pathways from different species by horizontal gene transfer, but this needs to be confirmed. Clearer evidence of the ecological significance of these findings should follow from further investigation.

Acknowledgments

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