The *Rhodococcus erythropolis* DCL14 limonene-1,2-epoxide hydrolase gene encodes an enzyme belonging to a novel class of epoxide hydrolases

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Received 8 September 1998; received in revised form 7 October 1998

Abstract Recently, we reported the purification of the novel enzyme limonene-1,2-epoxide hydrolase involved in limonene degradation by *Rhodococcus erythropolis* DCL14. The N-terminal amino acid sequence of the purified enzyme was used to design two degenerate primers at the beginning and the end of the 50 amino acids long stretch. Subsequently, the complete limonene-1,2-epoxide hydrolase gene (*limA*) was isolated from a genomic library of *R. erythropolis* DCL14 using a combination of PCR and colony hybridization. The *limA* gene encoded a 149-residue polypeptide with a deduced molecular mass of 16.5 kDa. It was functionally expressed in *Escherichia coli*. The amino acid sequence of *limA* contains neither any of the conserved regions of the α,β-hydrolase fold enzymes, to which most of the previously reported epoxide hydrolases belong, nor any of the conserved motifs present in leukotriene A_4 hydrolase. The structural data presented in this paper confirm previous physical and biochemical findings [van der Werf et al. (1998) J. Bacteriol. 180, 5052–5057] that limonene-1,2-epoxide hydrolase is the first member of a new class of epoxide hydrolases.

Key words: Limonene-1,2-epoxide hydrolase; Terpene metabolism; α,β-Hydrolase fold superfamily; Leukotriene A_4 hydrolase; *limA*; *Rhodococcus erythropolis*

1. Introduction

Epoxide hydrolases are enzymes catalyzing the hydrolysis of epoxides forming the corresponding diol. They have been found in mammals [1], insects [2,3], plants [4–6], protozoa [7], and microorganisms [8,9]. Three functions for epoxide hydrolases are recognized. The best studied epoxide hydrolases, i.e. microsomal and cytosolic epoxide hydrolase from eukaryotes (for review see [1]), are involved in the detoxification of epoxides. Other epoxide hydrolases are involved in the biosynthesis of hormones, such as leukotrienes and juvenile hormone [3,10], and plant cuticular elements [4]. In microorganisms, epoxide hydrolases are involved in the degradation of epoxides formed during growth on alkenes or halohydrins as carbon and energy source (e.g. [11–13]).

Remarkably, the prokaryotic and eukaryotic epoxide hydrolases described so far form a remarkably homogeneous group of enzymes. They belong to the α,β-hydrolase fold superfamily [14,15] based on the observation that they show low, but significant, sequence similarity with haloalkane dehalogenase from *Xanthobacter autotrophicus* GJ10, of which the three-dimensional structure has been solved [16]. Epoxide hydrolases do not contain a prosthetic group. The α,β-hydrolase fold epoxide hydrolases have a two-domain organization. Domain I consists of an α,β-sheet that forms a catalytic pocket and domain II, which splits domain I, sits like a lid over the catalytic cleft [14]. The catalytic activity of α,β-hydrolase fold epoxide hydrolases depends on a catalytic triad consisting of Asp, His, and Asp(Glu) residues [15,17]. The α,β-hydrolase fold epoxide hydrolases show low overall amino acid similarities [14]. Homology is limited to a few conserved regions involved in catalysis and stabilization of the reaction intermediate [14,15,18].

Up to now, only two epoxide hydrolases have been described which do not belong to the α,β-hydrolase fold superfamily and do not show mutual homology: leukotriene A_4 hydrolase and cholesterol-epoxide hydrolase. Leukotriene A_4 hydrolase is a cytosolic enzyme involved in the production of the hormone derivatives of arachidonic acid in eukaryotes [10]. It is a bifunctional enzyme with both epoxide hydrolase and aminopeptidase activities, and it belongs to the metallohydrolase superfamily [19,20]. Mammalian cholesterol-epoxide hydrolase has not yet been characterized very well, but this membrane-bound enzyme does not form a covalent intermediate in the course of epoxide hydrolysis, as is the case with α,β-hydrolase fold epoxide hydrolases, suggesting a fully different reaction mechanism [21].

Recently, we described the purification and characterization of a third epoxide hydrolase which, based on physical and biochemical characteristics, clearly did not belong to the α,β-hydrolase fold superfamily: limonene-1,2-epoxide hydrolase from *Rhodococcus erythropolis* DCL14 [22]. This cytoplasmic enzyme is involved in the limonene degradation pathway of this microorganism [23], and catalyzes the conversion of limonene-1,2-epoxide into limonene-1,2-diol (Fig. 1). In this report, we describe the cloning, nucleotide sequencing, and functional expression in *Escherichia coli* of the *limA* gene encoding limonene-1,2-epoxide hydrolase from *R. erythropolis* DCL14.

2. Materials and methods

2.1. Bacterial strains, plasmids, and growth conditions

R. erythropolis DCL14 was previously isolated on (−)-dihydrocarveol [22]. *E. coli* DH5α [24] was used for cloning and expression studies. *E. coli* LE392 (Promega) was used to construct the genomic library. Plasmid vectors used were pLAFR5 [25], pBluescript (Stratagene) and pGEM-T (Promega).

R. erythropolis DCL14 was cultivated on (+)-limonene as described previously [22]. *E. coli* was cultivated in Luria-Bertani (LB) medium at 37°C for 16 h. When appropriate, tetracycline and ampicillin were added at 25 and 100 μg/ml, respectively. Isopropyl-β-D-thiogalactopyranoside (IPTG) was used at a concentration of 0.5 mM. For ex-

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PII: S0014-5793(98)01322-2

pression of the *limA* gene, *E. coli* [pLEH$_2$] was grown on LB at 28°C, and cells were harvested at an optical density at 660 nm of ~0.6.

2.2. Isolation of chromosomal DNA and library construction

Cells (17 mg dry weight) of (+)-limonene-grown *R. erythropolis* DCL14 were resuspended in 10 ml of TE buffer (10 mM Tris-hydrochloride, 50 mM EDTA, pH 8) and were incubated with 10 mg/ml lysozyme (Sigma). After 60 min at 37°C, sodium dodecyl sulfate was added to a final concentration of 2.5% (w/v) and the cell suspension was incubated for a further 30 min at 55°C. After complete cell lysis, this suspension was treated with pronase (Boehringer-Mannheim; final concentration 1 mg/ml, 50 min at 37°C). The resulting solution was subsequently treated with 3 volumes of −20°C absolute ethanol. Two phases were obtained and the lower one, containing chromosomal DNA, was collected and subjected to a second addition of absolute ethanol. After centrifugation (4°C, 5 min at 15000 rpm), the pellet was resuspended in TE (pH 8) buffer. This solution was then treated with DNase-free RNase (25 μg/ml) for 90 min at 37°C. Chromosomal DNA was purified by phenol/chloroform extraction [24] and dissolved in TE buffer.

R. erythropolis genomic DNA was partially digested with *Sau*3A and size fractionated on a 1.2% low melting temperature agarose gel, using pulsed field electrophoresis (Gene Navigator, Pharmacia). Fragments of 20–30 kb were isolated from the gel and ligated into *Bam*HI-digested cosmid pLAFR5. Transformation of *E. coli* LE392 with the recombinant cosmids was done by in vitro infection using a commercially available extract (Packagene, Promega).

2.3. DNA manipulations

Restriction analysis, ligation, electroporation, electrophoresis, colony and DNA hybridizations were performed with conventional techniques [24]. Probes used were prepared using the PCR DIG probe synthesis kit (Boehringer-Mannheim). Plasmids were routinely isolated using Spinn columns (Qiagen, Hilden, Germany). Small DNA fragments (<7 kb) were recovered from agarose gels with the Qiaex II gel extraction kit (Qiagen) while large fragments (>10 kb) were isolated as described by Sambrook et al. [24].

All PCRs were carried out with Super Taq polymerase and buffer (Sphaero Q, Leiden, The Netherlands) in a Perkin-Elmer 480 thermal cycler. DNA fragments were generated via touchdown PCR to minimize side reactions [26]. The annealing temperature of the reaction was decreased 1°C every second cycle from 56°C to 53°C and every fourth cycle from 53°C to 50°C, at which temperature 15 cycles were carried out. DNA fragments were purified from the PCR reaction mixture using the Wizard Minipreps DNA purification system (Promega). For synthesis of a homologous probe for colony filter hybridization, the degenerate primers, P1: 5′-AARATHGARCARCC-NCGRTGGGC-3′ (192 degeneracy) and P2: 5′-GTRTCYTCNG-CRAARTAYTCDAT-3′ (384 degeneracy; both primers are deduced from the N-terminal amino acid sequence of limonene-1,2-epoxide hydrolase [22]; Fig. 2), were used with *R. erythropolis* DCL14 chromosomal DNA as the template.

2.4. Nucleotide sequencing and sequence analysis of limA

DNA sequences of both strands of the pLEH$_2$ insert were determined by a combination of subcloning and primer walking. The nucleotide sequence of *limA* was determined by the DyeDeoxy Terminator Cycle Sequencing kit using AmpliTaq FC DNA polymerase (Perkin Elmer). Nucleotide sequence manipulations were performed with the PC-Gene software [27]. Protein sequences were screened against database sequence libraries using FASTA [28] and BLAST [29]. Multiple alignments were realized using the PILEUP program [30].

2.5. Limonene-1,2-epoxide hydrolase assay

Cells were collected by centrifugation (4°C, 10 min at 6000×*g*) and washed with 50 mM potassium phosphate buffer pH 7.0. The pellet was resuspended in the same buffer and cells were disrupted at 4°C by sonication (5 min, 30% duty cycle, output control 2.3) with a Branson sonifier 250. Cell debris was removed by centrifugation (4°C, 10 min at 20000×*g*). The supernatant was used as cell extract and kept on ice until used. Limonene-1,2-epoxide hydrolase activity was determined at 30°C by monitoring the (−)-limonene-1,2-epoxide conversion by chiral GC as described previously [22]. Protein was determined by the method of Bradford [31] with BSA as the standard.

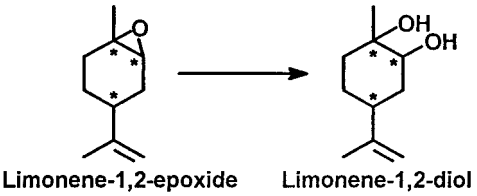

Fig. 1. Reaction catalyzed by limonene-1,2-epoxide hydrolase.

3. Results and discussion

3.1. Cloning of the limonene-1,2-epoxide hydrolase gene

Previously, 50 amino acids of the N-terminus of limonene-1,2-epoxide hydrolase were determined [22]. At the beginning and the end of this N-terminal amino acid sequence, stretches of eight amino acids were identified from which low degeneracy oligonucleotide primers were designed (Fig. 2). Using these primers and *R. erythropolis* DCL14 chromosomal DNA as the template, a PCR product of the expected size (144 bp) was synthesized (see Section 2.3). The nucleotide sequence of the PCR fragment was determined and the deduced amino acid sequence was identical to the N-terminal amino acid sequence of the purified enzyme.

A genomic library (7000 colonies, containing an average insert size of 29 kb) was constructed in cosmid pLAFR5 (Section 2.2). The 144-bp PCR product was used as a homologous probe to screen the *R. erythropolis* DCL14 genomic library. Of 3500 colonies, 15 hybridized strongly with the probe. After *Pst*I digestion and Southern blot analysis, only four of these cosmids exhibited a hybridization signal with a fragment of the same size as that of *Pst*I-digested chromosomal DNA of *R. erythropolis* DCL14. One of these four cosmids, cosmid pLEH$_1$, was used for further experiments. Southern blot analysis allowed the more precise location of the limonene-1,2-epoxide hydrolase encoding gene on a 1.4-kb *Bam*HI-*Hin*dIII fragment within this 6-kb *Pst*I fragment (Fig. 2). This 1.4-kb *Bam*HI-*Hin*dIII fragment was cloned in pBluescript and yielded pLEH$_2$.

3.2. Nucleotide sequence of the limA gene

The complete nucleotide sequence and the deduced amino acid sequence of the 447-nucleotide open reading frame present on this *Bam*HI-*Hin*dIII fragment are shown in Fig. 3. The N-terminal sequence determined from the purified enzyme [22] started next to the start codon Met, confirming that this open reading frame is the limonene-1,2-epoxide hydrolase gene. The gene was labeled *limA* (*lim* for limonene degradation).

A potential ribosomal binding site (Shine-Dalgarno sequence), AGGGAG, precedes the ATG translational initiation site of *limA* by 7 bp (Fig. 3). The consensus sequence of the −35 promoter region, TTGACA, was present 66 bp upstream of the start codon. This region was separated by 18 bp from the putative −10 promoter region, TAGCGT (Fig. 3). The *limA* gene encodes a protein of 149 amino acids with a deduced molecular mass of 16520 Da. This molecular mass is in agreement with the value of 17 kDA obtained by SDS-PAGE for the purified enzyme [22].

The complete amino acid sequences of 17 α,β-hydrolase fold epoxide hydrolases, originating from a wide range of organisms, have been reported. They are at least 285 aa long [15,18], and more commonly in the range of 450–560

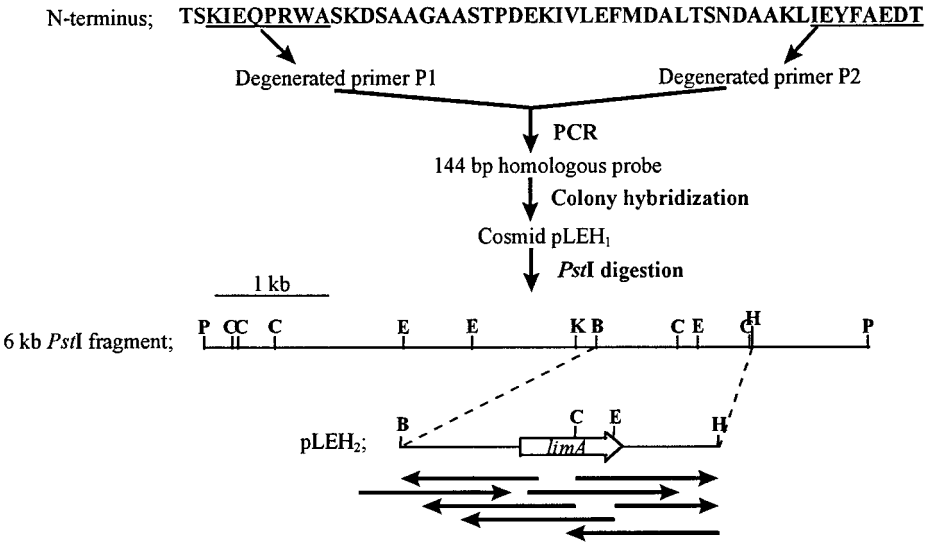

Fig. 2. Strategy for cloning and sequencing of the *limA* gene from *R. erythropolis* DCL14. The underlined amino acids (one letter code) of the N-terminal amino acid sequence of purified limonene-1,2-epoxide hydrolase [22] were used for primer design. B, *Bam*HI; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I and P, *Pst*I. The arrows show the sequencing strategy.

aa [14,32]. The leukotriene A_4 hydrolase gene was sequenced from four different mammals and these highly homologous genes were 610–611 aa long [33]. The *limA* gene is much smaller than the genes of members of these two classes of epoxide hydrolases. Moreover, the deduced and determined molecular mass for limonene-1,2-epoxide hydrolase is much lower than the estimated minimal length of 225 aa necessary to accommodate the reaction mechanism as used by the α,β-hydrolase fold epoxide hydrolases (Arand, personal communication).

3.3. Sequence comparison

The *limA* amino acid sequence was aligned with the previously reported epoxide hydrolase amino acid sequences, but no homology was found. Moreover, no functional equivalents for the catalytic triad common to α,β-hydrolase fold epoxide hydrolases were detectable in limonene-1,2-epoxide hydrolase, and also the other conserved motifs present in domain I of the α,β-hydrolase fold epoxide hydrolases [14], or the conserved motifs of leukotriene A_4 hydrolase [34], were not present in the *limA* gene. Remarkably, no significant functional homology was found with any other protein present in the databases.

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                                                -35
AGATGCAGAGTTCACTCGCCAGGTGTTGCGAACTGGGACATTGACAGTGACACAGGACAC    60
   -10                                  SD
ATAGCGTTGTGGCTCGAACAGAGTAGTCGTCAATCCACAGGGAGCCCTCACATGACATCA   120
                                                 M  T  S
AAGATCGAACAACCTCGCTGGGCGTCCAAGGACAGTGCCGCCGGCGCTGCCTCGACTCCG   180
 K  I  E  Q  P  R  W  A  S  K  D  S  A  A  G  A  A  S  T  P
GACGAAAAGATCGTTCTGGAGTTCATGGACGCACTGACCAGTAATGATGCTGCAAAACTC   240
 D  E  K  I  V  L  E  F  M  D  A  L  T  S  N  D  A  A  K  L
ATTGAGTACTTTGCAGAAGACACGATGTACCAGAACATGCCACTCCCCCCTGCATACGGC   300
 I  E  Y  F  A  E  D  T  M  Y  Q  N  M  P  L  P  P  A  Y  G
CGCGACGCCGTCGAGCAAACTCTGGCTGGCCTGTTCACCGTCATGAGCATCGATGCGGTG   360
 R  D  A  V  E  Q  T  L  A  G  L  F  T  V  M  S  I  D  A  V
GAGACGTTCCATATCGGCTCGAGTAACGGACTTGTGTACACCGAACGTGTCGATGTCCTA   420
 E  T  F  H  I  G  S  S  N  G  L  V  Y  T  E  R  V  D  V  L
CGCGCACTACCCACCGGCAAGAGCTACAACCTGTCAATCCTCGGAGTCTTCCAGCTCACC   480
 R  A  L  P  T  G  K  S  Y  N  L  S  I  L  G  V  F  Q  L  T
GAGGGCAAGATTACGGGTTGGCGTGACTACTTCGATCTGCGCGAATTCGAAGAAGCTGTC   540
 E  G  K  I  T  G  W  R  D  Y  F  D  L  R  E  F  E  E  A  V
GACCTTCCCCTCCGCGGCTAATCCCTTTTCCTTTAACATCTT                     582
 D  L  P  L  R  G  *
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Fig. 3. Nucleotide and deduced amino acid sequence of the *limA* gene. The putative Shine-Dalgarno (SD) and promoter sequences (−10 and −35 region) are underlined. The accession number of the sequence in EMBL is Y18005.

3.4. Expression of the limA gene in E. coli

E. coli containing $pLEH_2$ expressed limonene-1,2-epoxide hydrolase activity (Table 1). However, the limonene-1,2-epoxide hydrolase activity in cell extracts of *E. coli* [$pLEH_2$] is 30 times lower than in wild-type *R. erythropolis* DCL14 [22]. The addition of IPTG to the growth medium did not result in an increase in limonene-1,2-epoxide hydrolase activity, suggesting that *limA* expression is controlled by its own promoter. The stereochemical features of limonene-1,2-epoxide conversion by cell extracts of *E. coli* [$pLEH_2$] were the same as that of the purified enzyme (not shown). This proves that the conversion is indeed due to biological activity, and not to chemical hydrolysis of the relatively unstable epoxides.

3.5. Concluding remarks

This report describes the cloning and sequencing of *limA* from *R. erythropolis* DCL14, one of the first genes encoding an enzyme involved in microbial monoterpene degradation [35]. The unprecedentedly low molecular mass of limonene-1,2-epoxide hydrolase and the absence of any of the conserved motifs present in members of either the α,β-hydrolase fold [14] or the metallohydrolase [34] superfamilies suggest that this terpene-converting enzyme is completely different from known epoxide hydrolases. The structural data obtained in this study

Table 1
Limonene-1,2-epoxide hydrolase activity in cell extracts of *E. coli* [$pLEH_2$]

Plasmid	IPTG	Specific activity (nmol/min/mg protein)
None	−	<0.1
$pLEH_2$	−	54
$pLEH_2$	+	57

E. coli cultures were grown at 28°C in LB medium (pH 6.5) containing ampicillin to $OD_{660} \approx 0.6$.

confirm our previous physical and biochemical findings that limonene-1,2-epoxide hydrolase belongs to a novel class of epoxide hydrolases [22]. Moreover, as there is no significant functional homology between limonene-1,2-epoxide hydrolase and any other protein present in the databases, it is tempting to speculate that limonene-1,2-epoxide hydrolase belongs to a new protein family.

Acknowledgements: This work was supported by a grant from the European Community (BIO4-CT96-5132). We thank Martin de Wit for technical assistance. Michael Arand (Institute of Toxicology, University of Mainz, Germany) is acknowledged for his help with the sequence comparison studies.

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