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L-Pantoyl lactone dehydrogenase from *Rhodococcus erythropolis*: genetic analyses and application to the stereospecific oxidation of L-pantoyl lactone

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Abstract The 1,2-propanediol (1,2-PD) inducible membrane-bound L-pantoyl lactone (L-PL) dehydrogenase (LPLDH) has been isolated from *Rhodococcus erythropolis* AKU2103 (Kataoka et al. in Eur J Biochem 204:799, 1992). Based on the N-terminal amino acid sequence of LPLDH and the highly conserved amino acid sequence in homology search results, the LPLDH gene (*lpldh*) was cloned. The gene consists of 1,179 bases and encodes a protein of 392 amino acid residues. The deduced amino acid sequence showed high similarity to the proteins of the FMN-dependent α-hydroxy acid dehydrogenase/oxidase family. The overexpression vector pKLPLDH containing *lpldh* with its upstream region (1,940 bp) was constructed and introduced into *R. erythropolis* AKU2103. The recombinant *R. erythropolis* AKU2103 harboring pKLPLDH showed six times higher LPLDH activity than the wild-type strain. Conversion of L-PL to ketopantoyl lactone was achieved with 92% or 80% conversion yield when the substrate concentration was 0.768 or 1.15 M, respectively. Stereoinversion of L-PL to D-PL was also carried out by using the combination of recombinant *R. erythropolis* AKU2103 harboring pKLPLDH and ketopantoic acid-reducing *Escherichia coli*.

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Introduction

D-Pantoyl lactone (D-PL) is an important chiral intermediate for the production of calcium D-pantothenate (Shimizu et al. 1987; Shimizu and Kataoka 2010). A racemic mixture of PL (DL-PL) is used as the starting material in industrial production of D-PL, while only the D-isomer is useful in the downstream step, and thus a problematic resolution is unavoidable. Although we have constructed an effective ketopantoyl lactone (KPL) or ketopantoic acid (KPA) reduction system for an alternative D-PL production method (Kataoka et al. 2004; Si et al. 2012), DL-PL is still the most common and readily available starting material for D-PL production at present. Therefore, numerous studies have concentrated on the enzymatic resolution of DL-PL to obtain enantiopure D-PL. The main approach has been stereoselective hydrolysis of the D-isomer or L-isomer in DL-PL to corresponding pantoic acid (PA), followed by separation of the PL from PA by solvent extraction, etc. Filamentous fungi, such as *Fusarium oxysporum*, *Gibberella fujikuroi*, and *Cylindrocarpon didymium*, have been shown to catalyze the stereospecific hydrolysis of D-PL to D-PA (Kataoka et al. 1995a, b, 1996; Shimizu et al. 1992), while *Agrobacterium tumefaciens* catalyzed the stereoselective hydrolysis of L-PL (Kataoka et al. 2000; Kesseler et al. 2002). Lactonohydrolase of *F. oxysporum* has been commercially exploited for the optical resolution of DL-PL. After the hydrolysis of the D-isomer in the racemic mixture, which was used in the following step, the remaining L-isomer must be re-racemized or discarded (Sakamoto et al. 2005).

Over the past few years, driven by the demand for enantiopure and optically active alcohols, some effective and practical methods for conversion of racemate to a single stereoisomeric product have been developed (Azerad and Buisson 2000; Faber and Kroutil 2005; Gruber et al. 2006; Stecher and Faber 1997; Strauss et al. 1999). An ideal approach for the resolution of DL-PL is to transform it into enantiopure D-PL by techniques such as stereoinversion or an enantio-convergent process (Faber 2001). In that case, the theoretical molar yield from DL-PL would overcome the 50% limitation and reach 100%. A novel D-PL production process, i.e., stereoinversion of L-PL to D-PL via KPL or KPA, has been proposed for this purpose (Fig. 1). In this process, an efficient reduction system of KPL or KPA has been established using *Escherichia coli* transformant cells overexpressing the KPL or KPA reductase gene (Kataoka et al. 2004; Si et al. 2012); however, an effective system for the oxidation of L-PL to KPL was not constructed. The membrane-bound L-PL dehydrogenase (LPLDH), which catalyzes the stereospecific dehydrogenation of L-PL to KPL without any modification of the remaining D-PL, was found in *Rhodococcus erythropolis* AKU2103 (formerly *Nocardia asteroides*; Kataoka et al. 1992). The LPLDH activity is induced by addition of 1,2-propanediol (1,2-PD) in *R. erythropolis* AKU2103, but not at a level sufficient for practical use. If the LPLDH gene (*lpldh*) could be overexpressed in a recombinant microorganism, stereoinversion of L-PL to D-PL might be achieved by combination with a KPL or KPA reduction system. We describe here the cloning of *lpldh*, its overexpression in *R. erythropolis* AKU2103, and its application to conversion of L-PL.

Materials and methods

Chemicals and reagents

D-PL and L-PL were obtained from Tokyo Chemical Industry Co. (Japan) and Daiichi Fine Chemicals (Japan), respectively. KPL was purchased from Sigma-Aldrich (USA). KPA was prepared from KPL by alkaline hydrolysis (King et al. 1974). Restriction enzymes, Ex-Taq DNA polymerase and PrimeSTAR Max DNA polymerase, were purchased from Takara-Bio (Japan). All other reagents used in this work were of analytical grade and commercially available.

Microorganisms and cultivation

R. erythropolis AKU2103 (Graduate School of Agriculture, Kyoto University, Japan), which could be obtained as NBRC12539 (NITE Biological Resource Center, Japan), was used as an enzyme and DNA source, or overexpression host. The culture medium comprised 1% polypepton (Nihon Pharmaceutical Co., Japan), 0.5% meat extract (Wako Pure Chemical Industries, Ltd., Japan), 0.1% yeast extract (Oriental Yeast, Japan), and 0.5% NaCl, pH 7.0. When necessary, 1.2% 1,2-PD, 10% sucrose, 225 μg/ml kanamycin, and/or 30 μg/ml nalidixic acid were added. Cultivation was carried out at 28°C with shaking at 300 rpm for 48 h. *E. coli* strains JM109, BL21(DE3), and S17-1 were used. All *E. coli* cells were grown in LB medium containing 1% NaCl, 0.5% yeast extract, and 1% tryptone (Becton Dickinson, USA), pH 7.0, at 37°C. When necessary, 100 μg/ml ampicillin, 50 μg/ml kanamycin, and/or 0.1 mM isopropyl-β-D-thiogalactopyranoside were added to the LB medium. Minimal medium comprising 0.2% $(NH_4)_2SO_4$, 0.02% $MgSO_4 \cdot 7H_2O$, 0.05% $NaH_2PO_4 \cdot H_2O$, 0.01% $CaCl_2 \cdot 2H_2O$, 0.05% K_2HPO_4, and a 1% carbon source, pH 7.0, was used for the growth test of the *lpldh* disruption mutant.

Purification of LPLDH and N-terminal amino acid sequence analysis

LPLDH was purified as described previously (Kataoka et al. 1992). The N-terminal amino acid sequence of LPLDH was determined by the automated Edman degradation method

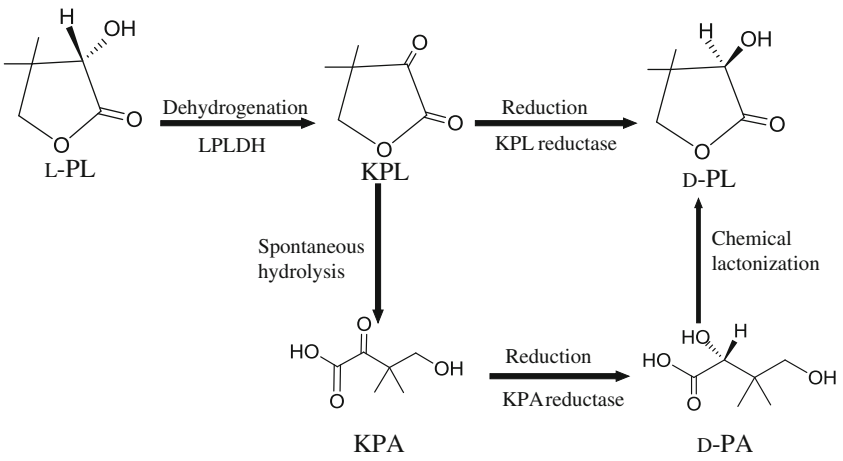

Fig. 1 Enzymatic process for D-PL synthesis from DL-PL via KPL and KPA

with a pulse-liquid protein sequencer model 491HT (Applied Biosystems, USA).

Preparation of genomic and plasmid DNA

The genomic DNA of *R. erythropolis* AKU2103 was isolated with a DNeasy Blood and Tissue Kit (QIAGEN, USA). Plasmid DNA was isolated with a QIAprep spin miniprep Kit (QIAGEN).

Cloning of a part of *lpldh* by degenerate PCR

The amino acid sequences, ETVAEAQR and YGEWMQTP, were used to design primers (LPLDH-partA and LPLDH-partB; Table 1) for degenerate PCR. The PCR mixture comprised 200 ng of genomic DNA, 25 pmol of each primer pool, 10 nmol of dNTPs, and 1.25 units of Ex-Taq DNA polymerase in 50 μl of reaction mixture. One thermal cycle consisted of 96°C for 20 s, 55°C for 30 s, and 72°C for 75 s. A total of 35 cycles were performed. The PCR products were ligated into the pT7Blue T-vector and sequenced.

Cloning of the full-length *lpldh* and flanking sequence

Oligonucleotide primers (LPLDH-fullA and LPLDH-fullB; Table 1) were designed based on the nucleotide sequence flanking a putative oxidoreductase gene in *R. erythropolis* PR4 (gene ID RER_18000). Using genomic DNA of *R. erythropolis* AKU2103 as template, the full *lpldh* and flanking region were amplified and sequenced in the same manner as described above.

Construction of the expression vector

Primers LPLDH-pK4F and LPLDH-pK4R (Table 1) for the cloning of *lpldh* with an upstream region (1,940 bp) were designed based on the nucleotide sequence around *lpldh* (unpublished data). The PCR mixture contained 150 ng of genomic DNA, 10 nmol of dNTPs, 25 pmol of each primer, and 1.25 units of Ex-Taq DNA polymerase in 50 μl. A program was performed in 30 cycles under the following conditions: 94°C for 30 s, 50°C for 30 s, and 72°C for 4 min. The PCR product was ligated into the pT7Blue T-vector and sequenced. Then the plasmid was digested with *Eco*RI and *Kpn*I and ligated into the pK4 (Hashimoto et al. 1992) digested with the same restriction enzymes. The resulting plasmid pKLPLDH was introduced into *R. erythropolis* AKU2103 by electroporation with the method of Urano et al. (Urano et al. 2011), except that medium containing 225 μg/ml kanamycin was used for selection of a successful transformant.

DNA sequencing

All the nucleotide sequences were determined by the dideoxy chain termination method (Sanger et al. 1977) with a CEQ dye terminator cycle sequencing kit (Beckman Coulter Inc., USA) and an automated sequencer DNA analysis system, CEQ2000XL (Beckman Coulter Inc.).

Preparation of cell-free extract and enzyme assay

R. erythropolis AKU2103 cells were cultured in 5 ml of medium supplemented with or without 225 μg/ml kanamycin, at 28°C for 48 h. For large-scale cultivation, the preculture (5 ml) was transferred to a 2-l shaking flask containing 300 ml of the medium, and then the culture was performed at 28°C with reciprocal shaking (100 strokes/min). The cultivated *R. erythropolis* AKU2103 cells were harvested by centrifugation, and then suspended in 1 ml of 10 mM Tris–HCl (pH 7.4) containing 0.1 mM dithiothreitol. One percent Brij 35 was added, and then the solution was stirred for 2.5 h after disruption with an ultrasonic oscillator (Insonater 201 M; Kubota, Japan). After centrifugation, the resulting supernatant

Table 1 Primer pairs used for cloning, overexpression, and disruption of *lpldh*

	Primer pairs	Application
LPLDH-partA	5′-GA(A/G)ACIGT(C/G/T)GCIGA(A/G)GC(A/T/G/C)CA(A/G)CG-3′	For cloning of a part of *lpldh*
LPLDH-partB	5′-GGIGT(T/C)TGCATCCA(T/C)TC(A/T/G/C)CC(A/G)TA-3′	
LPLDH-fullA	5′-TGCCACTGACACTGATGATGCGTCCG-3′	For cloning of the full *lpldh*
LPLDH-fullB	5′-GTCTCGTGCGGACCGAAAACAACCCT-3′	
LPLDH-pK4F	5′-GAATTCGTGCTGATTCCCTCCTTCG-3′ (*Eco*RI)	For amplification of *lpldh* and upstream sequence to construct overexpression vector pKLPLDH. Restriction enzyme sites were underlined.
LPLDH-pK4R	5′-ATCGGTACCGCAATCTCAGCTGG-3′ (*Kpn*I)	
LPLDH-A	5′-TGAATTCTTCGCGAGGACACAGCCATG-3′ (*Eco*RI)	For *lpldh* disruption. Restriction enzyme sites were underlined.
LPLDH-B	5′-CAGTTCGGTGATGGACGAGTGACCGGTCGGTGAAATCATGAC-3′	
LPLDH-C	5′-GTCATGATTTCACCGACCGGTCACTCGTCCATCACCGAACTG-3′	
LPLDH-D	5′-AAGCTTCACGCCGCTACGACGGCATTC-3′ (*Hin*dIII)	

was used for the LPLDH activity assay with the method described previously (Kataoka et al. 1992). One unit of LPLDH was defined as the amount catalyzing the formation of 0.5 μmol diformazan per minute under the standard assay conditions.

Construction of unmarked gene deletion mutagenesis of *lpldh* and growth test

To generate an *lpldh* disruption mutant, a 3-kb fragment AD containing Δ*lpldh* and the flanking region was amplified by overlap extension PCR (Horton et al. 1989; Fig. 2a). Using Prime STAR Max DNA polymerase and genomic DNA of wild-type *R. erythropolis* AKU2103 as template, fragments AB and CD were amplified with primers LPLDH-A/-B and LPLDH-C/-D, respectively (Table 1). After electrophoresis, the fragments were recovered with an illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, UK). Then, fragment AD was amplified with primers LPLDH-A/-D and the mixture of fragments AB and CD as template. In the amplified fragment AD, a part of *lpldh* was removed. After ligation into pT7Blue T-vector and digestion with *Eco*RI and *Hin*dIII, the fragment was ligated into broad-host-range plasmid pK18*mobsacB* (Schafer et al. 1994) digested with the same restriction enzymes to construct pK18ΔLPLDH. Deletion mutagenesis of *lpldh* in *R. erythropolis* AKU2103 was constructed through a two-step homologous recombination (Fig. 2b). Recombinant *E. coli* S17-1 cells harboring pK18ΔLPLDH were mixed with an equal amount of *R. erythropolis* AKU2103 cells and spread onto a non-selective plate to obtain conjugants containing pK18ΔLPLDH. The integration of the plasmid into the genomic DNA was selected with a selective-plate supplemented with 30 μg/ml nalidixic acid and 225 μg/ml kanamycin. Anti-kanamycin and anti-nalidixic acid colonies were picked up and spread onto a non-selective plate again to facilitate the second homologous recombination. Excision of the plasmid was carried out by transferring the cells from the second non-selective plate to a selective-plate supplemented with 10% sucrose and 30 μg/ml nalidixic acid.

R. erythropolis AKU2103 cells were cultured in complete medium for 24 h. After centrifugation, the cells were washed twice with minimal medium without a carbon source and suspended in the same solution. The washed cells were inoculated into 5 ml of minimal medium containing different sole carbon sources (glucose, acetic acid, lactic

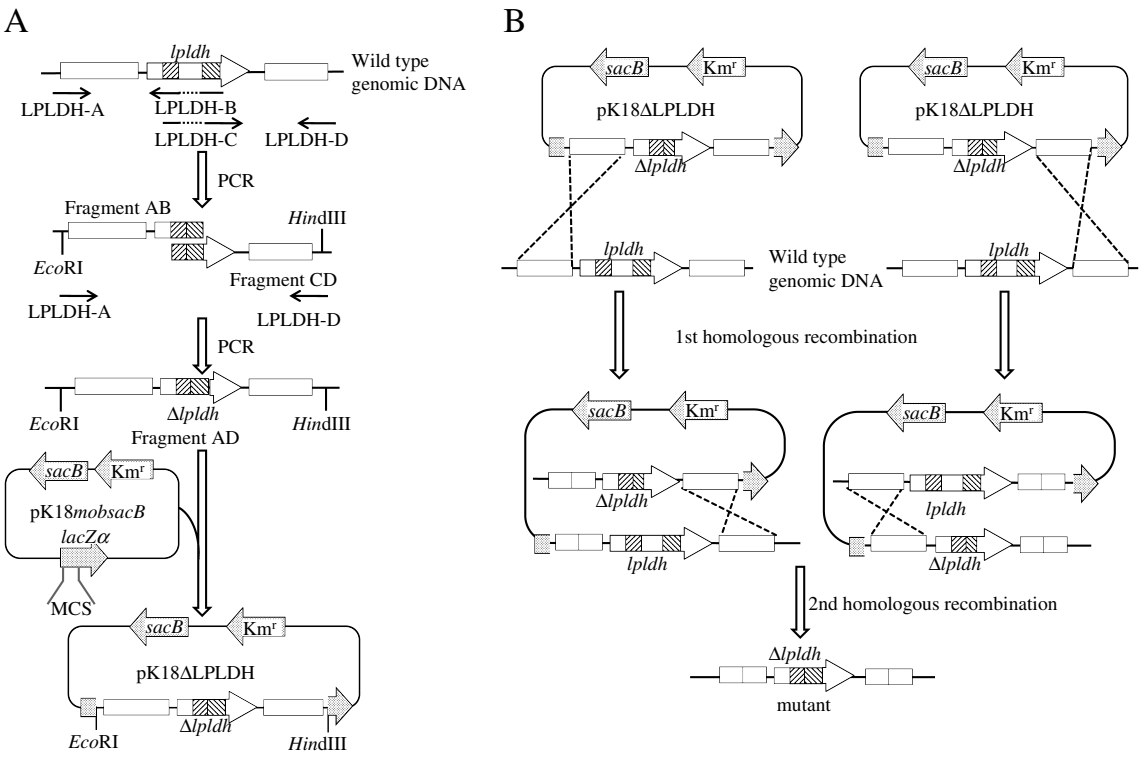

Fig. 2 Schematic representation of pK18ΔLPLDH construction by overlap extension (**a**), and disruption of *lpldh* via two-step homologous recombination in *R. erythropolis* AKU2103 (**b**). *Km*^{r} kanamycin-resistant gene, *sacB* modified levansucrase gene of *Bacillus subtilis*, *lacZα* 3′-truncated *lacZ* gene, containing a multiple cloning site (MCS). **a** 852 bp encoding 284 amino acid residues in the middle of *lpldh* were deleted. **b** Two types of integration were obtained after the 1st homologous recombination. Km^r was used to screen conjugants containing pK18ΔLPLDH. The *sacB* was used to anti-select the *lpldh* disruptant mutant after the 2nd recombination. Cells grown on the plate containing 10% sucrose underwent the second homologous recombination and lost the plasmid, thereby restoring the wild-type strain or leading to the mutant strain

acid, ethanol, 1,2-PD, 1-propanol, glycerol, 1-butanol, 1-hexanol, polyethylene glycol) and then cultured at 28°C with shaking at 300 rpm. The OD_{600} of the culture was measured at 24, 48, 60, and 72 h.

Conversion of L-PL to D-PA

Large-scale cultured cells were harvested by centrifugation, washed with physiological saline and stored at −20°C until use. The conversion rate of L-PL to KPL was measured in 5 ml of reaction mixture comprised of 0.2 M potassium phosphate buffer (pH 7.0), 15 mg/ml $CaCO_3$, 0.230–1.15 M (30.0–150 g/L) L-PL, and *R. erythropolis* AKU2103 cells. The mixture was incubated at 28°C with shaking at 300 rpm. After conversion of L-PL to KPL, 0.018 g lyophilized *E. coli* (pETSmKPR/pACGD; Si et al. 2012), 0.270–1.39 M glucose, and 0.1 mg/ml $NADP^+$ were added to the reaction mixture, and then the mixture was incubated at 28°C with shaking at 300 rpm for a further 24 h. During the reaction, the pH was adjusted to 7.0 with 12 M KOH periodically. A portion of the reaction mixture (250 μl) was pipetted out and analyzed as described previously (Si et al. 2012). The oxidation product, KPL, would be spontaneously hydrolyzed to KPA, and consequently the formation of acidic KPA should cause the pH drop of the reaction mixture. To avoid drastic pH drop of the reaction mixture, which brings slowdown of the LPLDH reaction, $CaCO_3$ was added to the reaction mixture.

Nucleotide sequence accession number

The nucleotide sequence of the LPLDH gene and flanking region of *R. erythropolis* AKU2103 was deposited in the DDBJ/GenBank/EMBL nucleotide sequence databases under accession number AB689131.

Results

N-terminal amino acid sequence of LPLDH

The N-terminal amino acid sequence of purified LPLDH was successfully analyzed as FFETVAEAQRRAKKRLPKSVY. A computer-aided homology search was carried out with the BLASTP program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and RefSeq database (NCBI, USA). The N-terminal amino acid sequence of LPLDH showed quite high homology (100% identities) to hypothetical proteins of *R. erythropolis* strains PR4 (RefSeq accession no. YP_002765247) and SK121 (RefSeq accession no. ZP_04384552), *Rhodococcus opacus* B4 (RefSeq accession no. YP_002783323), and *Rhodococcus jostii* RHA1 (RefSeq accession no. YP_706008) (McLeod et al. 2006).

Cloning of *lpldh*

For the amplification of a part of *lpldh*, the sense primer LPLDH-partA was designed based on the N-terminal amino acid sequence, ETVAEAQR, and the antisense primer LPLDH-partB was designed based on the highly conserved amino acid sequence, YGEWMQTP, in the alignment results of hypothetical proteins of *R. erythropolis* strains PR4 and SK121, *R. opacus* B4, and *R. jostii* RHA1. A DNA fragment (about 0.7 kbp) was amplified by degenerate PCR using the genomic DNA of *R. erythropolis* AKU2103 as a template. The deduced amino acid sequence of the cloned DNA fragment agreed with the N-terminal amino acid sequence of LPLDH. Therefore, this 0.7 kbp DNA fragment was considered to be derived from *lpldh*. The results of a homology search with the BLASTN program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) revealed that the nucleotide sequence of the amplified fragment showed the highest similarity to that of a putative oxidoreductase gene from *R. erythropolis* PR4 (RefSeq accession no. YP_002765247). Since the genome of *R. erythropolis* PR4 has been reported, the upstream and downstream sequences of the putative oxidoreductase of *R. erythropolis* PR4 were used to design primers (LPLDH-fullA/LPLDH-fullB) for cloning of the full *lpldh*.

A 1.4-kbp fragment was amplified using primers LPLDH-fullA/LPLDH-fullB. The DNA sequencing data of this fragment showed that it included the 0.7 kbp fragment amplified above, and that only one complete open reading frame (ORF) with a length of 1,179 bp coding for 392 amino acid residues was found. The calculated molecular weight of the deduced peptide was 41,611 Da. This is in good agreement with the molecular weight of the subunit of LPLDH from *R. erythropolis* AKU2103 (Kataoka et al. 1992). Homology search analysis revealed that the LPLDH of *R. erythropolis* AKU2103 showed high similarity with proteins of the FMN-dependent α-hydroxy acid dehydrogenase/oxidase family (Cunane et al. 2005; Diêp Lê and Lederer 1991; Furuichi et al. 2008; Maeda-Yorita et al. 1995; Fig. 3).

Overexpression of *lpldh*

The ORF of *lpldh* was cloned into *E. coli* expression vector pET-21a(+) or *Rhodococcus* vector plasmid pREIT19 (Zhou et al. 2008), and then introduced into *E. coli* BL21(DE3) or *R. erythropolis* AKU2103, respectively. However, recombinant *E. coli* BL21(DE3) and recombinant *R. erythropolis* AKU2103 showed no obvious improvement in LPLDH activity or conversion ability of L-PL (data not shown).

From the results of exploring the ORFs around *lpldh*, an ORF showing high similarity to TetR family proteins of

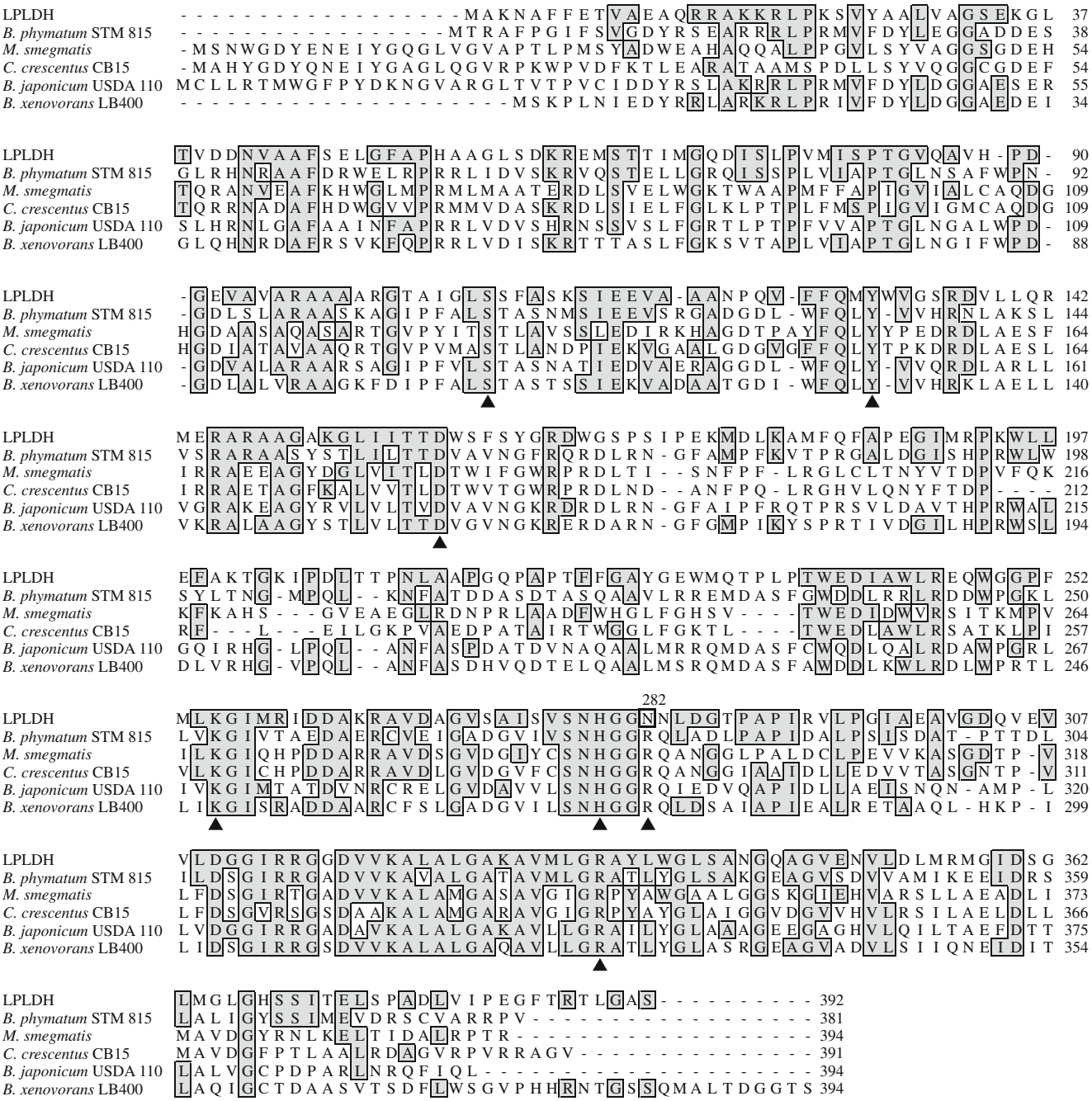

Fig. 3 Alignment of LPLDH with several proteins of the FMN-dependent α-hydroxy acid dehydrogenase/oxidase family. *B. phymatum* STM815 (FMN-dependent α-hydroxy acid dehydrogenase from *Burkholderia phymatum* STM815; RefSeq accession no. YP_001862260), *M. smegmatis* (Lactate 2-monooxygenase from *Mycobacterium smegmatis*; RefSeq accession no. P21795), *C. crescentus* CB15 (L-lactate 2-monooxygenase from *Caulobacter crescentus* CB15; RefSeq accession no. NP_420209), *B. japonicum* USDA 110 (L-lactate dehydrogenase from *Bradyrhizobium japonicum* USDA 110; RefSeq accession no. NP_773041), *B. xenovorans* LB400 ((*S*)-mandelate dehydrogenase from *Burkholderia xenovorans* LB400; accession no. RefSeq YP_555152). Gaps in the aligned sequences are indicated by *dashes*. Identical amino acid residues are enclosed in *boxes*. Putative FMN-binding sites are indicated by *triangles*. The sole different FMN-binding residue (N282) is indicated with a *thick box*

several *Rhodococcus* and *Mycobacterium* strains was found upstream of *lpldh* (unpublished data). TetR family proteins usually function as regulators of gene expression (Ramos et al. 2005). Therefore, *lpldh* and the 1,940-bp upstream region (between the TetR homologous gene and *lpldh*) were cloned into *Rhodococcus* plasmid pK4 to construct expression plasmid pKLPLDH. The recombinant *R. erythropolis* AKU2103 harboring pKLPLDH exhibited higher LPLDH activity (42.4 mU/mg) even without 1,2-PD induction during the cultivation period. The activity

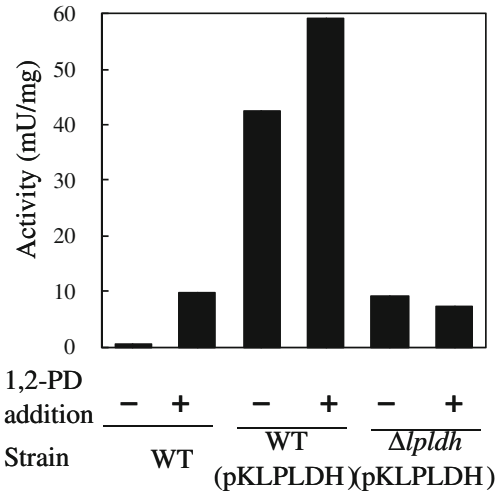

Fig. 4 LPLDH activities of each strain of *R. erythropolis* AKU2103. +/− with or without 1,2-PD induction, *WT* wild-type strain of *R. erythropolis* AKU2103, *WT (pKLPLDH)* recombinant wild-type *R. erythropolis* AKU2103 harboring pKLPLDH, Δ*lpldh (pKLPLDH)* recombinant Δ*lpldh* strain of *R. erythropolis* AKU2103 harboring pKLPLDH

was much higher than that of 1,2-PD-induced wild-type *R. erythropolis* AKU2103 (9.6 mU/mg; Fig. 4). Under the induction condition, higher LPLDH activity (59.2 mU/mg) was observed in recombinant *R. erythropolis* AKU2103 harboring pKLPLDH.

Effect of *lpldh* disruption

To confirm that *lpldh* is the only gene responsible for LPLDH activity, and to examine the physiological function of LPLDH in *R. erythropolis* AKU2103, a 852-bp region at the middle of *lpldh* was deleted, and an LPLDH-defective mutant strain, Δ*lpldh*, was constructed. The gene disruption in the Δ*lpldh* strain was confirmed with sequence analysis. No LPLDH activity was detected in the cell-free extract of the Δ*lpldh* strain. However, the recombinant Δ*lpldh* strain harboring pKLPLDH showed LPLDH activity even without 1,2-PD induction (9.0 mU/mg).

The Δ*lpldh* strain could not grow in minimal medium containing 1,2-PD, ethanol, 1-butanol or 1-propanol as the sole carbon source, while the recombinant Δ*lpldh* strain containing pKLPLDH and wild-type strain could grow. However, all 3 of the strains could grow in minimal medium containing glucose as the sole carbon source (Fig. 5).

Conversion of L-PL to D-PA

Conversion with different concentrations (0.230–1.15 M) of L-PL to produce KPL was performed with *R. erythropolis* AKU2103 cells. As shown in Table 2, in the case of 0.230 M (30.0 mg/ml), increasing the cell mass as the catalyst remarkably improved the conversion efficiency; 0.384 M L-PL (50.0 mg/ml) could be completely converted to KPL, in 60 h, by 0.18 g of recombinant *R. erythropolis* AKU2103 cultivated under the induced condition; 0.768 M L-PL (100 mg/ml) could be converted by 0.18 g of recombinant *R. erythropolis* AKU2103 cultivated under the induced condition, with a conversion yield of 91.9%. When the initial L-PL concentration was 1.15 M (150 mg/ml), 80% of added L-PL was converted to KPL with 0.36 g of recombinant *R. erythropolis* AKU2103, while only 13% of L-PL could be converted to KPL with wild-type *R. erythropolis* AKU2103.

Under the neutral pH condition, KPL can be hydrolyzed to KPA spontaneously. Therefore, *E. coli* (pETSmKPR/pACGD) cells coexpressing the KPA reductase and glucose dehydrogenase genes (Si et al. 2012) were added to the

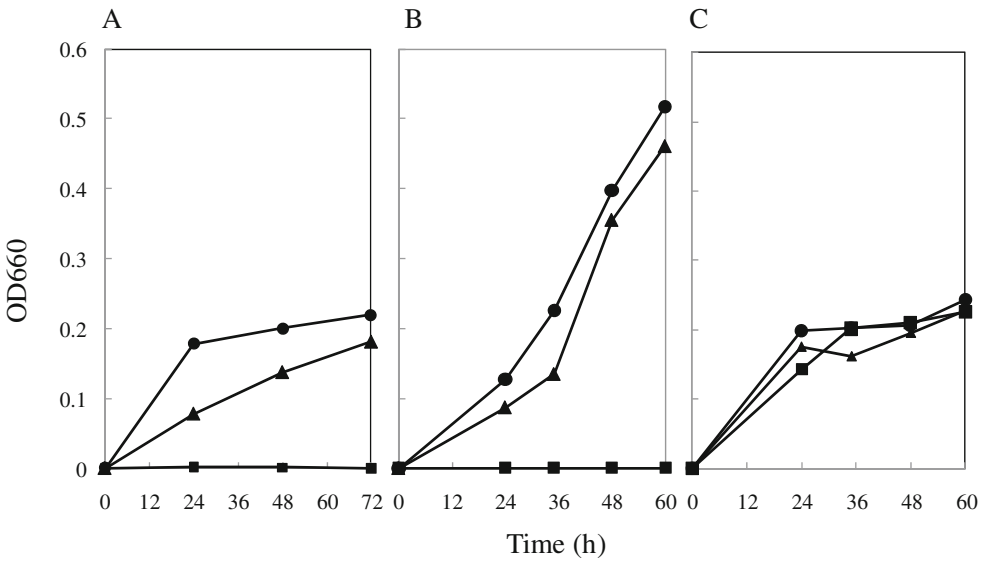

Fig. 5 Growth of *R. erythropolis* AKU2103 in minimal medium supplemented with 1% 1,2-PD (**a**), ethanol (**b**), or glucose (**c**) as the sole carbon source. *Filled circle* wild-type *R. erythropolis* AKU2103, *filled square* Δ*lpldh* strain of *R. erythropolis* AKU2103, *filled triangle* recombinant Δ*lpldh* strain of *R. erythropolis* AKU2103 harboring pKLPLDH

Table 2 Conversion of L-PL with wild-type strain (WT) and recombinant strain bearing pKLPLDH [WT (pKLPLDH)] of *R. erythropolis* AKU2103 cells

L-PL conc. (M)	Strain	1,2-PD	Time (h)	Cells added (g)	Conversion yield (%)
0.230	WT	−	12	0.045	2.8
0.230	WT	+	12	0.045	13.7
0.230	WT (pKLPLDH)	−	12	0.045	20.6
0.230	WT (pKLPLDH)	+	12	0.045	32.4
0.230	WT	−	12	0.09	5.1
0.230	WT	+	12	0.09	26.2
0.230	WT (pKLPLDH)	−	12	0.09	40.2
0.230	WT (pKLPLDH)	+	12	0.09	60.9
0.384	WT	+	60	0.18	56.4
0.384	WT (pKLPLDH)	+	60	0.18	>99.9
0.768	WT	+	144	0.18	67.8
0.768	WT (pKLPLDH)	+	144	0.18	91.9
1.15	WT	+	184	0.36	12.6
1.15	WT (pKLPLDH)	+	184	0.36	80.3

reaction mixture to reduce the resulting KPA derived from KPL to D-PA. After addition of *E. coli* (pETSmKPR/pACGD), all the accumulated KPA was reduced to D-PA (Fig. 6).

Discussion

Twenty-one amino acid residues of the N terminus of LPLDH were analyzed. A computer-aided homology search revealed that the N-terminal amino acid sequence showed quite high homology with that of hypothetical proteins of several *Rhodococcus* strains. So the highly conserved amino acid sequence, YGEWMQTP, in the alignment result of these hypothetical proteins, and the analyzed N-terminal sequence, ETVAEAQR, were used to design primers for degenerate PCR to clone a part of *lpldh*. The nucleotide sequence of the cloned part of *lpldh* showed 97% identity to a putative oxidoreductase from *R. erythropolis* PR4 (RefSeq accession no. YP_002765247), and *R. erythropolis* PR4 also showed LPLDH activity (data not shown). Therefore, the putative oxidoreductase gene in *R. erythropolis* PR4 should be the homologous gene of *lpldh*. We presumed that the nucleotide sequence around *lpldh* in both *R. erythropolis* AKU2103 and *R. erythropolis* PR4 share high similarity. Thus, the amplification of *lpldh* from *R. erythropolis* AKU2103 with primers designed based on the nucleotide sequence around the putative oxidoreductase gene of *R. erythropolis* PR4 proceeded successfully. This is the first L-PL dehydrogenase gene cloned from microorganisms.

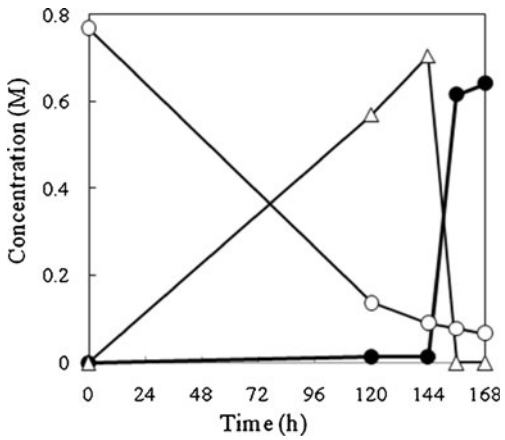

Fig. 6 Time course of the stereoinversion of 0.768 M L-PL using a combination of recombinant wild-type *R. erythropolis* AKU2103 harboring pKLPLDH and recombinant *E. coli* harboring pETSmKPR and pACGD. The recombinant *E. coli* cells were added at 144 h. *Filled circle* D-PL, *open circle* L-PL, *open triangle* KPL

LPLDH is an FMN-dependent enzyme (Kataoka et al. 1992). The results of the alignment showed that it belongs to the FMN-dependent α-hydroxy acid dehydrogenase/oxidase family (Cunane et al. 2005; Diêp Lê and Lederer 1991; Furuichi et al. 2008; Maeda-Yorita et al. 1995). This family occurs in both prokaryotes and eukaryotes. Members of this family include flavocytochrome b2, glycolate oxidase (GOX), lactate monooxygenase, mandelate dehydrogenase (MDH), and long chain hydroxy acid oxidase. The enzymatic reaction of this kind of enzyme proceeds in two stages (Maeda-Yorita et al. 1995). The substrate is oxidized to form a keto acid with flavin reduction in the reductive half-reaction. The reduced flavin is oxidized by an electron acceptor through a different pattern in the oxidative half-reaction (Cunane et al. 2005; Diêp Lê and Lederer 1991; Furuichi et al. 2008; Maeda-Yorita et al. 1995). A specific electron transfer chain should also be indispensable to L-PL dehydrogenation catalyzed by LPLDH. One of the reasons why the recombinant *E. coli* cells harboring pET21a(+)

carrying *lpldh* did not show sufficient activity as compared to wild-type *R. erythropolis* AKU2103 cells in dehydrogenation of L-PL might have been that there was no appropriate electron transfer chain suitable for LPLDH in *E. coli* cells. The FMN-binding sites of the family are conserved in LPLDH, except the N282 residue (Fig. 3). FMN-dependent α-hydroxy acid dehydrogenases from *Mycobacterium tuberculosis* CDC1551 (Fleischmann et al. 2002) and *Sphingomonas wittichii* RW1 (GenBank accession No. ZP_01607333) have the same Asn residue at the same FMN-binding site (Sukumar et al. 2001; Xia and Mathews 1990).

LPLDH is a membrane-bound enzyme, and therefore the correct binding to the membrane should be necessary for its activity. Another reason why recombinant LPLDH could not work in *E. coli* cells, even though a large amount of LPLDH protein was generated in recombinant *E. coli*, might have been the use of different conditions of the membrane in *E. coli* and in *R. erythropolis*. The (*S*)-MDH from *Pseudomonas putida*, which is a membrane-bound FMN-dependent α-hydroxy acid dehydrogenase, was rendered soluble by replacing its membrane-binding peptide segment with a corresponding segment from soluble GOX. The resulting soluble chimeric mutant enzyme expressed full activity like the wild-type MDH (Mitra et al. 1993; Sukumar et al. 2001). This is a potential method for improving *lpldh* overexpression in *E. coli*.

We also constructed a general expression system for *R. erythropolis* to overexpress *lpldh*; but unfortunately, the overexpression of *lpldh* was not successful using this construct. We therefore inferred that the nucleotide sequence around *lpldh* might be related to its expression. Based on nucleotide sequence exploration, *lpldh* and the 1,940-bp upstream region are presumed to be transcribed into the same mRNA. For this reason, the DNA fragment between the TetR homologous gene and *lpldh* was cloned into *Rhodococcus* plasmid pK4 and introduced into *R. erythropolis* AKU2103 to overexpress *lpldh*. Because the putative promoter/operator region of the operon containing *lpldh* was inserted into pKLPLDH, which was derived from multicopy plasmid pK4, the amount of putative regulator (the product of the TetR homologous gene) might be insufficient to completely repress the transcription of the operon in recombinant *R. erythropolis* AKU2103 harboring pKLPLDH. Therefore, even without 1,2-PD induction, its LPLDH activity was much higher than that of 1,2-PD induced wild-type *R. erythropolis* AKU2103 (Fig. 4). The detailed examination of the induction mechanism by this region (between the TetR homologous gene and *lpldh*) will be reported elsewhere.

As shown in Fig. 1, KPL can be spontaneously hydrolyzed to KPA under a neutral pH condition. KPA reductase from *Stenotrophomonas maltophilia* has been overexpressed in *E. coli* and could be used to convert 1.17 M KPA to D-PA (Si et al. 2012). Therefore, stereoinversion of L-PL to D-PL was carried out by a combination of stereospecific oxidation of L-PL to KPL with LPLDH and asymmetric reduction of KPA, which was formed from KPL under a neutral pH condition, to D-PA with KPA reductase. After addition of *E. coli* (pETSmKPR/pACGD) cells, all of the resulting KPL (actually KPA) was reduced to D-PA. As a result, 0.707 M D-PL could be obtained from 0.768 M (100 mg/ml) L-PL with a conversion yield of 92%. When *E. coli* (pETSmKPR/pACGD) cells were added together with *R. erythropolis* cells at the beginning of the conversion reaction of L-PL to KPL, regardless of L-PL concentration, the final conversion yield was lower than when *E. coli* cells were added after L-PL oxidation reaction as described above (data not shown).

To construct Δ*lpldh* strain of *R. erythropolis* AKU2103, 852 bp in the middle of *lpldh* was deleted. Since the deleted region coded V84-G367 residues of LPLDH, all the residues of putative FMN-binding sites required for LPLDH activity expression should be lost (Fig. 3). Furthermore, the ORFs upstream and downstream of *lpldh* were presumed to be in the same operon, thus there is some possibility that these ORFs were transcribed and/or translated with a part of *lpldh*. Therefore, to avoid affecting the transcription/translation of them, the 5′- and 3′-regions of *lpldh* were left in Δ*lpldh* (Fig. 2). The Δ*lpldh* strain of *R. erythropolis* AKU2103 showed no LPLDH activity. LPLDH should be encoded by the sole *lpldh* gene in *R. erythropolis* AKU2103. Thus, it is interesting to consider what role LPLDH plays in *R. erythropolis* AKU2103. Wild-type *R. erythropolis* AKU2103 grew in minimal medium containing 1,2-PD, ethanol, 1-butanol or 1-propanol as the sole carbon source, while the Δ*lpldh* strain did not. Therefore, LPLDH might be involved in certain types of alcohol catabolism in wild-type *R. erythropolis* AKU2103. For clarification of the physiological function of LPLDH, further investigations will be needed.

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