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Reconstruction of lactate utilization system in *Pseudomonas putida* KT2440: a novel biocatalyst for L-2-hydroxy-carboxylate production

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As an important method for building blocks synthesis, whole cell biocatalysis is hindered by some shortcomings such as unpredictability of reactions, utilization of opportunistic pathogen, and side reactions. Due to its biological and extensively studied genetic background, *Pseudomonas putida* KT2440 is viewed as a promising host for construction of efficient biocatalysts. After analysis and reconstruction of the lactate utilization system in the *P. putida* strain, a novel biocatalyst that only exhibited NAD-independent D-lactate dehydrogenase activity was prepared and used in L-2-hydroxy-carboxylates production. Since the side reaction catalyzed by the NAD-independent L-lactate dehydrogenase was eliminated in whole cells of recombinant *P. putida* KT2440, two important L-2-hydroxy-carboxylates (L-lactate and L-2-hydroxybutyrate) were produced in high yield and high optical purity by kinetic resolution of racemic 2-hydroxy carboxylic acids. The results highlight the promise in biocatalysis by the biotechnologically important organism *P. putida* KT2440 through genomic analysis and recombination.

In recent years, biocatalysis has been attracting much attention as an environment-friendly method for the production of enantiomerically pure building blocks[1–3]. Both whole cells and the related isolated enzymes have been used in biocatalytic production of chiral chemicals, which have emerged as important pharmaceutical and agrochemical intermediates. In comparison with the isolated enzymes, whole-cell catalysts can be prepared readily and inexpensively and thus offer economical and environmental advantages over isolated enzymes[3]. However, some problems, such as the unpredictability of reactions, the utilization of opportunistic pathogen, and the side reactions causing degradation of products, prevent the large-scale use of whole-cell catalysts[3,4].

Pseudomonas putida is one of the best studied species of the metabolically versatile, genetic plastic and ubiquitous genus of *Pseudomonads*[5]. The typical strain of *P. putida*, KT2440 (ATCC47054), is a microorganism Generally Recognized as Safe (GRAS certified), and thus has been extensively used in a wide range of biotechnological applications including bioremediation of contaminated areas, production of bioplastics, biocatalytic production of chiral chemicals, and so on[5,6]. Since the complete sequence of *P. putida* KT2440's genome has been released[7], the knowledge related to the strain has significantly increased, and various "-omics" data sets such as transcriptomic[8–10], proteomic[10,11], and fluxomic data[12,13] have also become available. Genome-scale reconstruction and analysis of the *P. putida* KT2440 metabolic network based on those data have facilitated the applications of the strain in biocatalysis[6].

Lactate has 2 optical isomers: L-lactate and D-lactate. Compared with D-lactate, L-lactate is an organic acid with more versatile applications in food, pharmaceutical, textile, and chemical industries. More importantly, L-lactate is also an indispensable monomer for the synthesis of poly-L-lactic acid, a bio-degradable polymer. Many *Pseudomonas* strains, such as *P. aeruginosa*, *P. putida* and *P. stutzeri*, can use L-lactate and/or D-lactate as the sole carbon and energy source for growth[14–16]. The lactate utilization system involves three membrane bound proteins: LldP (a lactate permease), NAD-independent L-lactate dehydrogenase (L-iLDH), and NAD-independent D-lactate dehydrogenase (D-iLDH). As a lactate permease, LldP takes up L-lactate and/or D-lactate into the cells[17,18]. L-iLDH and D-iLDH catalyze the oxidation of L-lactate and D-lactate to pyruvate via a flavin-dependent mechanism, respectively[18]. L-iLDH and D-iLDH are induced coordinately in all of the reported

Pseudomonas strains including *P. putida*. The two enzymes are not constitutively expressed; the enantiomer of lactate can induce the expression of both enzymes[18]. Thus, in addition that *P. putida* KT2440 is regarded as the most promising catalyst for the production of L-2-hydroxy-carboxylates such as L-lactate through D-iLDH catalyzed kinetic resolution, the co-present L-iLDH in the strain would also catalyze the oxidation of L-2-hydroxy-carboxylates and produce 2-oxo-carboxylates as the end product.

In this study, the lactate utilization system in the GRAS *P. putida* strain KT2440 was analyzed and further reconstructed (Fig. 1). A recombinant strain of *P. putida* KT2440 was obtained through disruption of L-iLDH encoding gene *lldD*. Whole cells of the recombinant strain were confirmed to exhibit only D-iLDH activity (catalyzing the oxidation of D-2-hydroxy-carboxylates). Two chiral 2-hydroxy-carboxylates: L-lactate and L-2-hydroxybutyrate were then produced from their racemic mixtures by using the recombinant *P. putida* KT2440 as the catalyst.

Results

Identification of the lactate utilization operon in *P. putida* KT2440. In a previous study, the *lldPDE* operon, which encodes lactate permease (LldP), L-iLDH, and D-iLDH, was studied in *P. aeruginosa* XMG[18]. Comparative genomics revealed that this operon was also found in most *Pseudomonas* strains including *P. putida* KT2440[7]. A BLAST search of the genome sequence of *P. putida* KT2440 with the *lldPDE* operon of *P. aeruginosa* XMG as the probe revealed a sequence encoding three proteins with strikingly high homology to LldP (84% sequence identity), L-iLDH (88% sequence identity), and D-iLDH (82% sequence identity). As shown in Fig. 2A, *lldD* (encoding L-iLDH), *lldP* (encoding lactate permease), and *lldE* (encoding D-iLDH) are located adjacent to the regulator LldR encoding gene *lldR*. Since the L-iLDH- and D-iLDH-encoding genes are in the same lactate utilization operon and are controlled by the same regulator, reconstruction of the lactate utilization operon *lldPDE* in *P. putida* KT2440 thus become a feasible method for utilization of the GRAS strain in L-2-hydroxy-carboxylates production.

Reconstruction of the lactate utilization system in *P. putida* KT2440. To eliminate the oxidation of L-2-hydroxy-carboxylates by L-iLDH, the *lldD* gene in *P. putida* KT2440 was disrupted using homologous recombination (Fig. 2B). Suicide plasmid pK18mobsacB was utilized as described previously[19]. PCR was used to verify the disruption event of gene *lldD* using primer set LkF plus LkR. The mutant with a second crossover event (named *P. putida* KTM) was selected. The result in Fig. 2C shows that the PCR using the primer set generated products of the expected sizes.

The transcription of *lldP*, *lldD*, and *lldE* in *P. putida* KTM was also monitored by reverse transcription (RT)-PCR assays. RT-PCR fragments of the *lldP* and *lldE* were obtained from cells of *P. putida* KTM grown in MSM containing 5 g l^{-1} DL-lactate (Fig. 2E and Fig. 2F), thus indicating that the disruption of *lldD* in the *P. putida* KT2440 (Fig. 2D) would not influence the transcription of *lldP* and *lldE* (Table S1). LldP and D-iLDH still work in the transport and oxidation of D-2-hydroxy-carboxylates, respectively. With these considerations in mind, whole cells of *P. putida* KTM with reconstructed lactate utilization system could be used in the biocatalytic production of L-2-hydroxy-carboxylates (Fig. 1).

Utilization of DL-lactate by *P. putida* KT2440 and *P. putida* KTM. *P. putida* KT2440 and *P. putida* KTM were cultured with DL-lactate as the sole carbon source. As shown in Fig. 3A, both D-lactate and L-lactate in the medium would be utilized by *P. putida* KT2440. Since L-iLDH is required for L-lactate utilization in *Pseudomonas*, *P. putida* KTM with the mutant of *lldD* could not use L-lactate as the sole carbon source (Fig. S1). However, *P. putida* KTM still use D-lactate in the medium for growth (Fig. 3B).

P. putida KTM was complemented with a broad-host-range plasmid pBBR1MCS-5 harboring the *lldD* gene. The resulting complementary strain was designated as *P. putida* KTM (pBBR-*lldD*). The activities of L-iLDH and D-iLDH in crude extract of *P. putida* KT2440, *P. putida* KTM and *P. putida* KTM (pBBR-*lldD*) were also assayed. As shown in Table 1, when cultured in the medium containing DL-lactate, both L-iLDH and D-iLDH activities were detected in *P. putida* KT2440 and *P. putida* KTM (pBBR-*lldD*). However, *P. putida* KTM exhibited only D-iLDH activity when cultured with DL-lactate as the sole carbon source.

Substrate specificity of D-iLDH in *P. putida* KTM. Since *P. putida* KTM could use D-lactate in racemic lactate and left L-lactate in the medium (Fig. 3B), it might have potential for the production of other L-2-hydroxy-carboxylates. Substrate specificity of D-iLDH in *P. putida* KTM was then examined with different D-2-hydroxy-carboxylates as the test substrates. As shown in Table 2, only D-

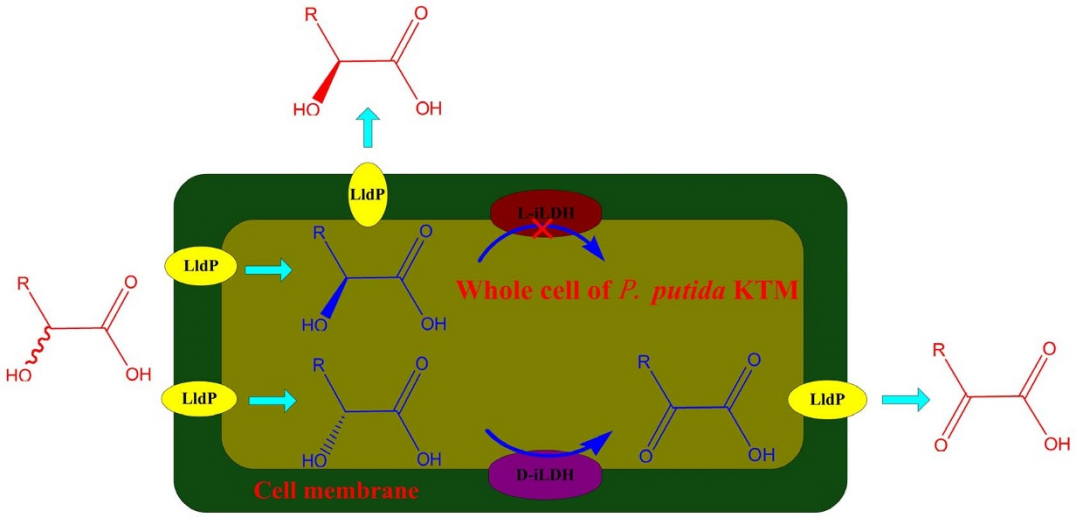

Figure 1 | Scheme of L-2-hydroxy-carboxylate production by the reconstructed lactate utilization system in *P. putida* KTM. The membrane bound LldP takes up L-2-hydroxy-carboxylate and D-2-hydroxy-carboxylate into the cells. The membrane bound L-iLDH and D-iLDH catalyze the oxidation of L-2-hydroxy-carboxylate and D-2-hydroxy-carboxylate to 2-oxo-carboxylates, respectively. Red cross indicates that the L-iLDH catalyzed oxidation of L-2-hydroxy-carboxylate was eliminated. R: CH_3, CH_2CH_3.

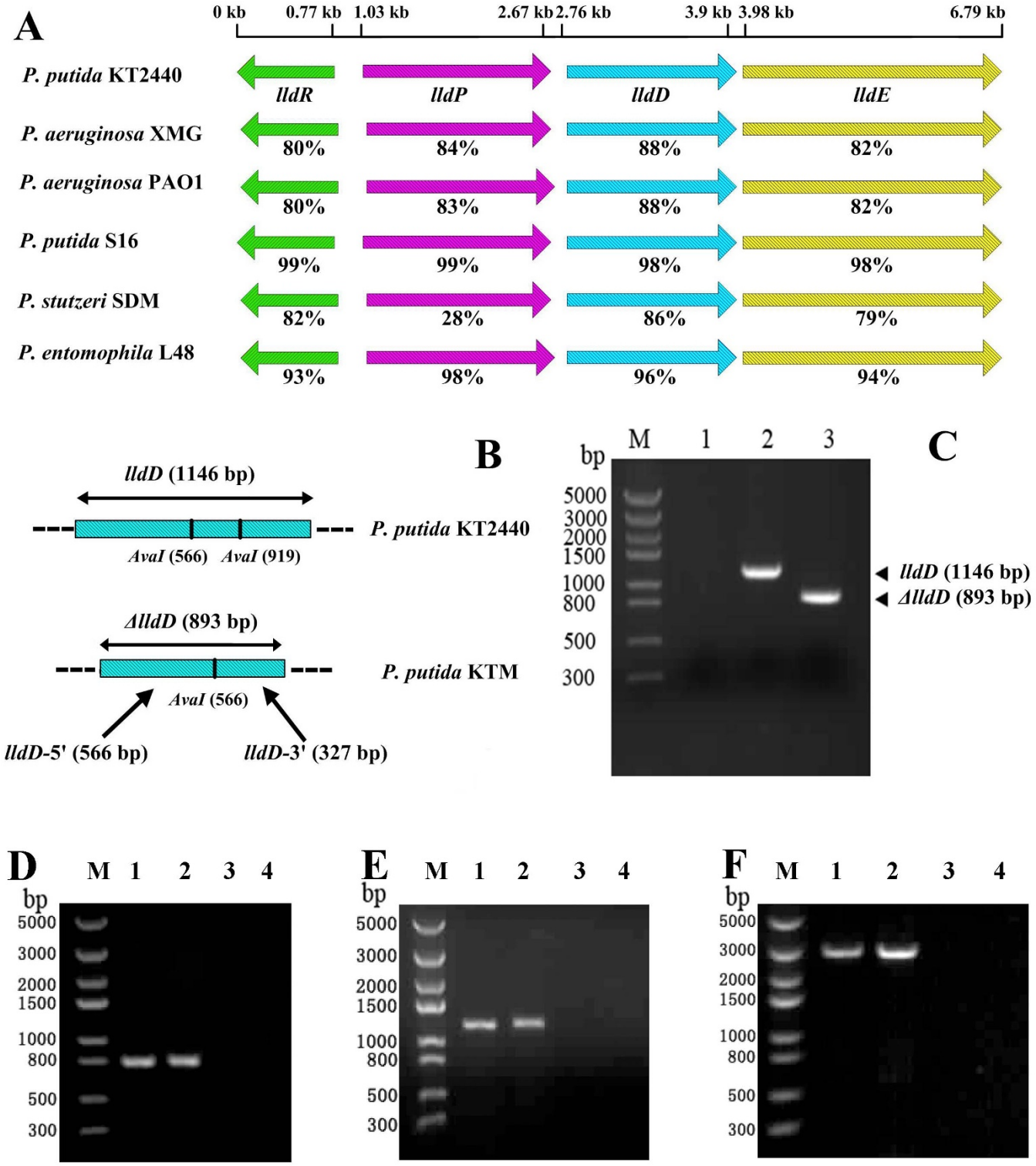

Figure 2 | Construction of *P. putida* KTM. (A) Organizations of lactate utilization operon in different *Pseudomonas* species including *P. aeruginosa* XMG[40], *P. aeruginosa* PAO1[41], *P. putida* KT2440[7], *P. putida* S16[42], *P. stutzeri* SDM[43] and *Pseudomonas entomophila* L48[44]. Genes were functionally annotated following the color code indicated. The numbers below the genes indicate the percentage similarities to the *P. putida* KT2440 protein sequences. The percentages represent amino acid homology of related enzymes. (B) Diagram illustrating the sequence analysis of the disruption of the *lldD*. (C) Analysis of PCR fragments to confirm *lldD* disruption. Lane M: molecular mass standard (λDNA/HindIII); Lane 1: product amplified with water as the template (negative control). Lane 2: product amplified with P. putida KT2440 genomic DNA as the template; Lane 3: product amplified with P. putida KTM genomic DNA as the template. The PCRs were performed with primers LkF and LkR. (D) Results of the RT-PCR assay in detecting the transcription of *lldD*. (E) Results of the RT-PCR assay in detecting the transcription of *lldP*. (F) Results of the RT-PCR assay in detecting the transcription of *lldE*. Lane 1, control reaction in which genomic DNA of *P. putida* KTM was used as the template; lane 2, reaction in which cDNA of *P. putida* KTM was used as the template; lane 3, control reaction in which mRNA of *P. putida* KTM was used as the template; lane 4, control reaction in which ddH_2O was used as the template. Lane M: molecular mass standard (λDNA/HindIII). Numbers on the left represent the sizes of the markers (in bp).

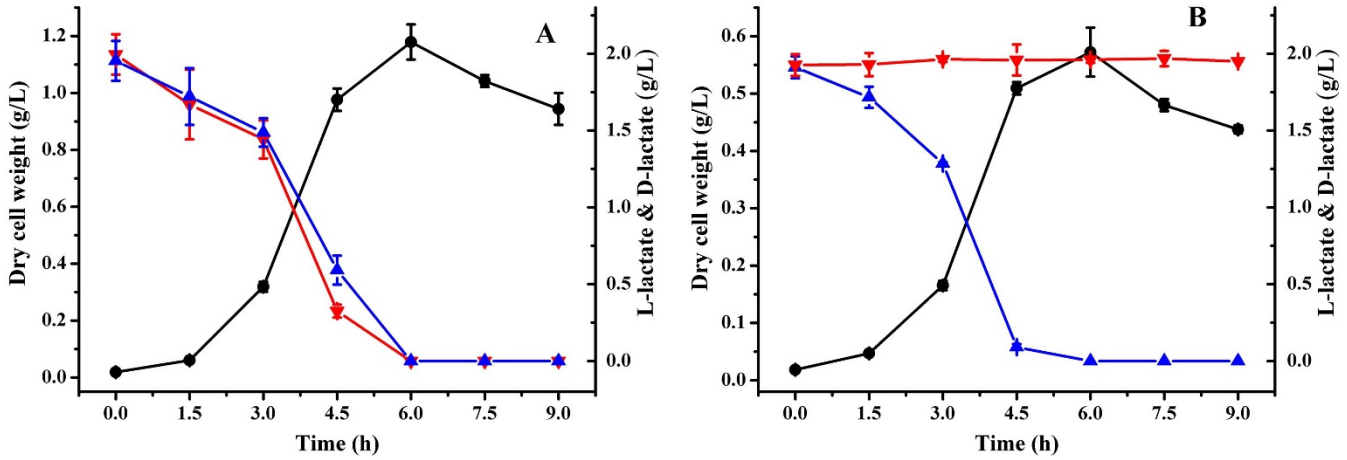

Figure 3 | Time-course study of *P. putida* KT2440 (A) and *P. putida* KTM (B) growth in medium with DL-lactate. Black solid circle, dry cell weight; blue up triangle, concentration of D-lactate; red down triangle, concentration of L-lactate. The initial DL-lactate concentration is 5 g l^{-1}. The performance was analyzed at 0, 1.5, 3, 4.5, 6, 7.5, and 9 h. Results are means ± SD of three parallel replicates.

lactate and D-2-hydroxybutyrate could be oxidized by D-iLDH in *P. putida* KTM. D-iLDH from *P. stutzeri* has been reported to show similar narrow substrate specificity[20]. Only D-lactate and D-2-hydroxybutyrate were attacked by the enzyme. Similar results have also been observed in other microorganisms including *E. coli*[21].

The rate of dehydrogenation of D-lactate and D-2-hydroxybutyrate catalyzed by D-iLDH in *P. putida* KTM followed Michaelis-Menten kinetics. Double-reciprocal plots of the initial rates plotted against the concentrations of D-lactate and D-2-hydroxybutyrate were linear at a fixed concentration of dichlorophenol-indophenol (DCPIP) (0.2 mM), and yielded K_m values of 0.16 ± 0.03 mM and 0.44 ± 0.03 mM, respectively. V_{max} was estimated to be 101.7 ± 18.2 nmol min^{-1} mg^{-1} for D-lactate and 96.7 ± 6.3 nmol min^{-1} mg^{-1} for D-2-hydroxybutyrate with DCPIP as the electron acceptor.

Production of L-lactate and L-2-hydroxybutyrate using whole cells of *P. putida* KTM. Although D-iLDH in *P. putida* KTM seemed to have narrow substrate specificity, it could attack the two most important members of 2-hydroxy-carboxylates, lactate and 2-hydroxybutyrate. The biocatalytic oxidation of the racemic lactate (100 mM) and 2-hydroxybutyrate (100 mM) with whole cells of *P. putida* KTM was carried out in distilled water in the presence of 20 mM ethylenediaminetetraacetic acid (EDTA). As shown in Fig. 4, the D-isomers in racemic lactate (100 mM) and 2-hydroxybutyrate (100 mM) could be oxidized into pyruvate and 2-oxobutyrate in 6 h. The final concentrations of L-lactate and L-2-hydroxybutyrate were 49.5 mM and 49.9 mM, respectively. These results revealed that the biocatalytic production of L-lactate and L-2-hydroxybutyrate proceeds in a high degree of enantioselectivity by preferential oxidation of D enantiomers in racemic mixtures with whole cells of *P. putida* KTM.

Discussion

Optically active 2-hydroxy-carboxylates are important building blocks for glycols, halo esters, and epoxides compounds, which are important intermediates of pharmaceuticals[22,23]. Chemical processes for 2-hydroxy-carboxylates production result in a racemic mixture of both stereospecific forms. Many routes, such as high-performance ligand exchange chromatography[24], enzymatic resolution[25], and asymmetric hydrolysis[26], have been developed for the resolution of the racemic 2-hydroxy-carboxylates. Due to its excellent stereoselectivity, high product yield and environmental friendly process, biocatalytic oxidative resolution of the racemate has emerged as a desirable technique for the production of optically active 2-hydroxy-carboxylates[22,25]. Until now, all of the reported 2-hydroxy-carboxylates resolution processes utilized NAD-independent L-2-hydroxy-carboxylate dehydrogenases such as glycolate oxidase[25], L-lactate oxidase[27] and membrane bound L-iLDH[28,29] as the biocatalysts, and could only produce D-2-hydroxy-carboxylates. Thus, there is a demand for searching of biocatalysts that could be utilized in the resolution of racemic 2-hydroxy-carboxylates to produce L-2-hydroxy-carboxylates.

Although D-iLDH might catalyze the oxidation of D-2-hydroxy-carboxylates, the co-present L-iLDH would also catalyze the L-2-hydroxy-carboxylates into 2-oxo-carboxylates[30–32]. After analysis of the lactate utilization system through comparative genomics, the *lldPDE* operon was identified in *P. putida* KT2440. L-iLDH encoding gene *lldD* was disrupted using homologous recombination. As we expect, the resulting recombinant strain *P. putida* KTM exhibited only D-iLDH activity. The specific activity of D-iLDH in *P. putida* KTM was 65.0 ± 3.7 nmol min^{-1} mg^{-1}, which was higher than that of *P. aeruginosa* XMG (20 ± 4 nmol min^{-1} mg^{-1})[32] but lower than that of *P. stutzeri* SDM (132 ± 5 nmol min^{-1} mg^{-1})[20]. Similar to D-iLDH in other *Pseudomonas* strains such as *P. stutzeri* SDM[20], D-iLDH in *P. putida* KTM also exhibited narrow substrate specificity, and only catalyzed the oxidation of D-lactate and D-2-hydroxybutyrate. Optically active L-lactate and L-2-hydroxybutyrate could be used in production of polylactate and poly(2-hydroxybutyrate), which can be utilized as a biodegradable material for biomedical,

Table 1 | Activities of L-iLDH and D-iLDH in crude extract of *P. putida* KT2440, *P. putida* KTM and *P. putida* KTM (pBBR-*lldD*)[a]

Strain	L-iLDH (nmol min^{-1} mg^{-1})	D-iLDH (nmol min^{-1} mg^{-1})
P. putida KT2440	22.1 ± 0.6	66.6 ± 3.1
P. putida KTM	ND[b]	65.0 ± 3.7
P. putida KTM (pBBR-*lldD*)	34.2 ± 0.9	84.1 ± 1.5

[a]Activities of D-iLDH and L-iLDH were examined with 20 mM D-lactate or 20 mM L-lactate. DCPIP was used as the electron acceptor. Results are means ± SD of three parallel replicates.
[b]ND: not detected.

Table 2 | Substrate specificity of D-iLDH from *P. putida* KTM

Substrate[a]	K_m (mM)	V_{max} (nmol min^{-1} mg^{-1})
D-Lactate	0.16 ± 0.03	101.7 ± 18.2
D-2-Hydroxybutyrate	0.44 ± 0.03	96.7 ± 6.3
Other D-2-hydroxy-carboxylates	ND[b]	ND

[a]Substrate specificity of D-iLDH from *P. putida* KTM was examined with 20 mM 2-hydroxy acids (D-lactate, glycollate, D-mandelate, D-3-phenyllactate, D-2-hydroxybutyrate, DL-2-hydroxyisocaproic acid, DL-2-hydroxyoctanoic acid, D-glycerate, D-4-hydroxyphenyllactate) and DCPIP as the electron acceptor. Results are means ± SD of three parallel replicates.
[b]ND: not detected.

pharmaceutical, and environmental applications[33,34]. Then, whole-cells of *P. putida* KTM were used to catalyze the resolution of racemic 2-hydroxybutyrate and lactate. The D-lactate and D-2-hydroxybutyrate could be oxidized into pyruvate and 2-oxobutyrate, respectively. L-Lactate and L-2-hydroxybutyrate, which were not oxidized in the biocatalytic process, accumulated with enantiomeric excess higher than of 99% (Fig. S2 and Fig. S3).

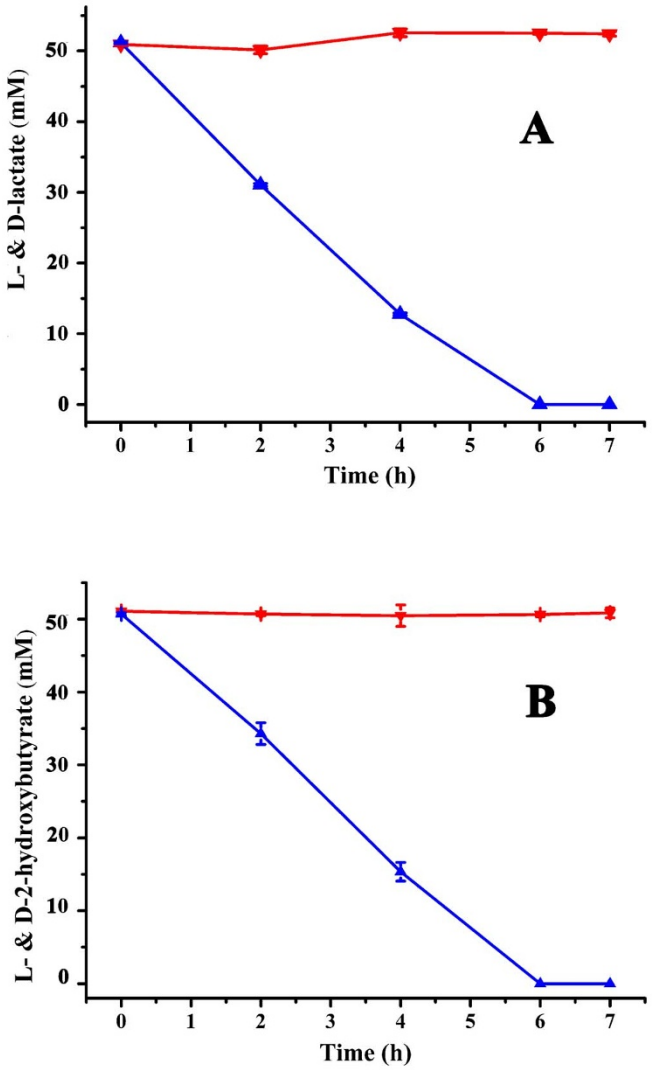

Figure 4 | Kinetic resolutions of racemic lactate (A) and 2-hydroxybutyrate (B) by whole cells of *P. putida* KTM. Red down triangle, concentrations of L-2-hydroxy-carboxylates; blue up triangle, concentrations of D-2-hydroxy-carboxylates. The initial DL-2-hydroxy-carboxylate concentration is 100 mM. The performance was analyzed at 0, 2, 4, 6, and 7 h. Results are means ± SD of three parallel replicates.

In a previous study, L-iLDH in *P. stutzeri* SDM was rationally re-designed on the basis of sequence alignment and the active site structure of a homologous enzyme; a new biocatalyst with high catalytic efficiency toward an unnatural substrate (L-mandelate) was successfully constructed[35]. Although the crystal structure of D-iLDH in *E. coli* was described[36], the position of the active site is still unknown. On the other hand, there is only 28% sequence identity between D-iLDHs from *P. putida* KT2440 and *E. coli*. Thus, at this stage, we did not attempt to expand the application range of D-iLDH in *P. putida* KTM due to the difficulty in the rational re-design of this membrane bound enzyme. However, if its structure is clarified, reconstruction of D-iLDH to improve its activity towards other straight long aliphatic or aromatic 2-hydroxy-carboxylic acids and synthesis of other valuable L-2-hydroxy-carboxylates might be successful in the future.

In summary, racemic 2-hydroxy carboxylic acids were first utilized in the production of L-2-hydroxy-carboxylates through enantioselective oxidation. A novel catalyst with lactate utilization system in *P. putida* KT2440 was reconstructed; it exhibited high bio-catalytic activities for production of L-2-hydroxy-carboxylates. The reconstruction of the lactate utilization system in *P. putida* KT2440 resulted in a novel catalyst that has exhibited high biocatalytic activities for L-2-hydroxy-carboxylates production. This study is a good example for the application of the GRAS *P. putida* KT2440 in the biocatalysis through genomic analysis and recombination. Other applications of *P. putida* KT2440 would also be possible through further understanding and reconstruction of this biotechnologically important organism.

Methods

Chemicals and biochemicals. L-Lactate (L7022), DL-lactate (71720), D-3-phenyllactate (376906), D-2-hydroxybutyrate (54917), DL-2-hydroxyisovaleric acid (219835), DL-2-hydroxyisocaproic acid (219819), DL-2-hydroxyoctanoic acid (H7396), DCPIP (33125), dithiothreitol (DTT, 43815), bovine serum albumin (BSA, B8667), and phenylmethanesulfonyl fluoride (PMSF, P7626) were purchased from Sigma (Sigma-Aldrich China Inc. Shanghai, China). D-Lactate (71716) was purchased from Fluka (Sigma-Aldrich China Inc. Shanghai, China). DL-2-Hydroxybutyrate (H0387) and DL-glycerate (G0232) were purchased from TCI (Tokyo, Japan). The restriction enzymes were obtained from Fermentas Bio Inc. (Beijing, China). FastPfu DNA polymerase was purchased from TransGen Biotech (Beijing, China). T4 DNA ligase was obtained from New England Biolabs (Beijing, China). All of the biochemicals mentioned above were of analytical grade reagents and are commercially available.

Bacterial strains and culture conditions. Bacterial strains, plasmids, and oligonucleotide primers used in this study were listed in Table 3. *P. putida* KT2440 was cultured in 500-ml baffled shake flasks containing 50 ml minimal salt medium (MSM) at 30°C and 120 rpm for 9 h[18]. MSM was supplemented with 5.0 g l^{-1} DL-lactate or pyruvate as the sole carbon source. *E. coli* DH5α was cultured in 500-ml baffled shake flasks containing 50 ml Lysogeny broth (LB) and maintained at 37°C and 120 rpm for 12 h. Isolation of vectors, restriction enzyme digestion, agarose gel electrophoresis and other DNA manipulations were carried out according to the standard methods[37].

Gene knockout procedure. Genomic DNA of *P. putida* KT2440 was extracted through the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA). A fragment from *P. putida* KT2440 genome containing whole length of *lldD* was amplified by PCR using LkF and LkR as the primers. The PCR reaction mixture contained 2.5 μl of each primer at 10 μM, 5.0 μl of 10 × PCR buffer, 2.0 μl of 25 mM $MgCl_2$, 2.5 μl of 2 mM dNTPs mix, 2 μl of enzyme solution containing 1 U FastPfu DNA polymerase in 33 μl of ddH_2O. Genomic DNA of *P. putida* KT2440 (0.5 μl at a concentration of 0.5 g l^{-1}) was used as the template. Thirty amplification cycles were performed on a Mastercycler® pro PCR thermal cycler (Eppendorf, Germany), each consisting of a denaturation of 30 s at 94°C, an annealing of 30 s at 50°C and an extension of 60 s at 72°C. Final extension was performed at 72°C for 5 min. The PCR product was digested by *EcoR*I and *Hind*III and then inserted in pK18mobsacB to form a new plasmid pKLK01[19]. Plasmid pKLK01 was completely digested by *Ava*I, and then the large fragment was self-ligated to form pKLK02. As a result, only partial lengths of *lldD* were inserted into pK18mobsacB in pKLK02. Plasmid pKLK02 was transformed into *P. putida* KT2440 by electroporation. Integration of the plasmid pKLK02 into the chromosome of *P. putida* KT2440 by the first crossover was selected on LB plate supplemented with 50 μg ml^{-1} kanamycin. The second crossover cells were selected by culture on LB plates containing 10% (w/v) sucrose. The resulting deletion mutant was designated as *P. putida* KTM.

All the constructed strains were validated by PCR and DNA sequencing.

Table 3 | Strains, plasmids, and oligonucleotide primers used in this study

Strain, plasmid, or primer	Relevant characteristics	Source or reference
strain		
E. coli DH5α	φ80 *lacZ*ΔM15 Δ(*lacZ*YA-*arg*F) U169 *recA*1 *endA*1 *hsdR*17 *supE*44λ- *thi*-1	Invitrogen
P. putida KT2440	Wide-type, capable of DL-Lactate utilizing	ATCC[a]
P. putida KTM	*P. putida* KT2440 mutant obtained by deletion of the *lldD* gene	This study
P. putida KTM (pBBR-*lldD*)	*P. putida* KTM harboring the plasmid pBBR-*lldD*	This study
Plasmid		
pK18mobsacB	Suicide plasmid for gene knockout, Km^R	19
pBBR1MCS-5	Plasmid for gene complementation, Gm^R	Biovector Science Lab,Inc
pKLK01	A fragment from KT2440 genome containing whole length of *lldD* was inserted in pK18mobsacB.	This study
pKLK02	pKLK01 was completely digested by *Ava*I, and then the large fragment was self-ligated; as a result, only partial lengths of *lldD* were inserted into pK18mobsacB.	This study
pBBR-*lldD*	A fragment from *P. stutzeri* SDM genome containing whole length of *lldD* was inserted in pBBR1MCS-5.	This study
Oligonucleotide primer	Sequence (5′→3′) and properties[b]	
LkF	CCGGAATTCGATGATCATTTCTGCCTCTACC (*EcoR*I)	This study
LkR	CCCAAGCTTTCAGGCGCCCAGTTCGCGTACCAG (*Hind*III)	This study
LcF	CCCAAGCTTATGATCATTTCCGCCTCT (*Hind*III)	This study
LcR	GCTGCAGTCAGACGTCAGCAGACGTTGT (*Pst*I)	This study

[a]ATCC, American Type Culture Collection; [b]recognition sites were introduced for restriction endonucleases (recognition sites underlined, restriction endonucleases indicated in parentheses).

Complementation of *P. putida* KTM. A fragment containing whole length of *lldD* of *P. stutzeri* SDM was amplified by PCR using the primers LcF and LcR, digested with *Hind*III and *Pst*I, and then inserted in pBBR1MCS-5 to form a new plasmid pBBR-*lldD*. Plasmid pBBR-*lldD* was transformed into *P. putida* KTM by electroporation. The resulting complementary strain was designated as *P. putida* KTM (pBBR-*lldD*).

RT-PCR of *lldP*, *lldD*, and *lldE*. Cells of *P. putida* KT2440 and *P. putida* KTM grown in MSM containing 5 g l^{-1} DL-lactate were harvested when they reached the mid-exponential phase. Total mRNA was prepared using a Qiagen RNeasy total RNA kit. DNA contamination was eliminated by a DNase I treatment at 37°C for 1 h. RNA integrity was checked by agarose gel electrophoresis. After quantification, 0.2 mg of purified total RNA was used to prepare cDNA by using Superscript II RT (Invitrogen) and reagents supplied by the manufacturer (42°C, 50 min). PCR were carried out using FastPfu DNA polymerase (TransGen Biotech, Beijing, China). Primer sequences for the *lldP*, *lldD*, and *lldE* are listed in Table S1. Samples were first denatured by heating at 94°C for 3 min. Thirty amplification cycles were performed on a Mastercycler® pro PCR thermal cycler (Eppendorf, Germany), each consisting of a denaturation of 30 s at 94°C, an annealing of 30 s at 50°C and an extension of 60 s at 72°C. Final extension was performed at 72°C for 5 min. Control reactions in which mRNA was used as the templates ensured that DNA products resulted from the amplification of cDNA rather than from DNA contamination. PCR products were separated by electrophoresis at 80V on 1.3% Tris Borate EDTA agarose gel, followed by ethidium bromide staining.

Preparation of crude extracts of *P. putida* KT2440, *P. putida* KTM, and *P. putida* KTM (pBBR-*lldD*). *P. putida* KT2440, *P. putida* KTM, and *P. putida* KTM (pBBR-*lldD*) were maintained on LB agar slants. The slants were incubated at 30°C for 12 h and then stored at 4°C. Cells of *P. putida* KT2440, *P. putida* KTM, and *P. putida* KTM (pBBR-*lldD*) from freshly prepared slants were inoculated into 300-ml baffled shake flasks containing 50 ml of LB. The cultivation was conducted at 30°C and 120 rpm for 9 h. Then cells of *P. putida* KT2440, *P. putida* KTM, and *P. putida* KTM (pBBR-*lldD*) were collected by centrifugation at 4500 rpm for 10 min, washed twice with MSM, then diluted (at approximately OD_{620} 0.05) in MSM containing 5.0 g l^{-1} DL-lactate as the sole carbon source. The cultivation was conducted in 500-ml baffled shake flasks at 30°C and 120 rpm for 9 h. Cells of *P. putida* KT2440, *P. putida* KTM, and *P. putida* KTM (pBBR-*lldD*) were harvested from 500 ml MSM, washed, resuspended in 50 ml of 50 mM Tris–HCl (pH 8.0) containing 10 mM DTT, 20 mM KCl, 5 mM $MgSO_4$, and 1 mM PMSF and disrupted by sonication (Sonics 500 W/20 KHz, USA) in an ice bath. The disrupted cells were centrifuged for 10 min at 10,000 × *g*, and the supernatants were used as the crude cell extracts. Proteins were determined by the Markwell variation of the Lowry method, with BSA as standard[38].

Enzymatic activity assays. The activities of iLDHs were determined at 30°C in 1 ml of 50 mM Tris–HCl, pH 7.5, 0.2 mM DCPIP, and 50 μl of crude cell extract (0.2 mg ml^{-1}). The reaction was started by addition of 20 mM L- or 20 mM D-lactate, and the rate of DCPIP reduction was determined by measuring the absorbance changes at 600 nm using a UV/visible spectrophotometer (Ultrospec 2100 pro, Amersham Biosciences, USA)[39].

Kinetic resolution by whole cells of *P. putida* KTM. Optical density of *P. putida* KTM was assayed at 620 nm by a 2100 spectrophotometer (Shanghai Exact Scientific Apparatus CO., LTD, China), and was converted to dry cell weight using the following equations: *P. putida* KTM, dry cell weight (g l^{-1}) = 0.35 × OD_{620nm}. The reaction was carried out at 30°C and 120 rpm in distilled water (pH 7.4) containing 6 g dry cell weight l^{-1} of *P. putida* KTM, 20 mM EDTA, and 100 mM racemic 2-hydroxy-carboxylates. The concentrations of 2-hydroxy-carboxylates and 2-oxo-carboxylates in the reaction mixtures were quantitatively analyzed by high-performance liquid chromatography (HPLC).

HPLC analyses. Concentrations of 2-hydroxy-carboxylates and 2-oxo-carboxylates were measured by HPLC (Agilent 1100 series, Hewlett-Packard, USA) using an Aminex HPX-87H column (Bio-Rad) and the eluent (10 mM H_2SO_4) at 1 ml min^{-1} flow-rate. Stereoselective assays of 2-hydroxy-carboxylates were performed by HPLC equipped with a chiral column (MCI GEL CRS10W, Japan) and the eluent at 1 ml min^{-1} flow-rate. The mobile phase was 2 mM $CuSO_4$ for lactate and a mixture of water and acetonitrile (90 : 10), containing 2 mM copper sulphate for 2-hydroxybutyrate. The enantiomeric excess (ee) value of 2-hydroxy-carboxylates was defined as follows $\frac{(\text{L-2-hydroxy-carboxylate})-(\text{D-2-hydroxy-carboxylate})}{(\text{L-2-hydroxy-carboxylate})+(\text{D-2-hydroxy-carboxylate})} \times 100\%$.

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Author contributions

C.G., P.X. and C.M. conceived and designed the project and experiments. C.G., Y.W., T.J., W.Z., M.L., X.X., and C.H. performed the experiments. C.G., Y.W., Y.Z., and C.M. analyzed the data. C.G., P.X. and C.M. wrote the paper.

Additional information

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