NAD-Independent L-Lactate Dehydrogenase Is Required for L-Lactate Utilization in Pseudomonas stutzeri SDM

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Abstract

Background: Various Pseudomonas strains can use L-lactate as their sole carbon source for growth. However, the L-lactate-utilizing enzymes in Pseudomonas have never been identified and further studied.

Methodology/Principal Findings: An NAD-independent L-lactate dehydrogenase (L-iLDH) was purified from the membrane fraction of Pseudomonas stutzeri SDM. The enzyme catalyzes the oxidation of L-lactate to pyruvate by using FMN as cofactor. After cloning its encoding gene (lldD), L-iLDH was successfully expressed, purified from a recombinant Escherichia coli strain, and characterized. An lldD mutant of P. stutzeri SDM was constructed by gene knockout technology. This mutant was unable to grow on L-lactate, but retained the ability to grow on pyruvate.

Conclusions/Significance: It is proposed that L-iLDH plays an indispensable function in Pseudomonas L-lactate utilization by catalyzing the conversion of L-lactate into pyruvate.

Introduction

Pseudomonas strains are Gram-negative rod-shaped bacteria commonly found in soil, water, and plant and animal tissues [1,2]. They have very simple nutritional requirements and can grow well with a single organic molecule such as lactate as the sole carbon and energy source. In the lactate utilization processes of Pseudomonas aeruginosa, P. putida, and P. stutzeri, lactate is first converted to pyruvate, and then metabolized through the tricarboxylic acid cycle [3–5]. However, the enzymes responsible for the oxidation of lactate to pyruvate in these Pseudomonas strains have not been identified and further studied.

NAD-Independent l-lactate dehydrogenases (l-iLDHs), which catalyze the oxidation of l-lactate to pyruvate by an FMN-dependent mechanism, are widely distributed among bacteria, yeast, and protists [6–8]. These enzymes have been studied extensively in Escherichia coli and Saccharomyces cerevisiae [7,9–11]. In E. coli, l-iLDH is a peripheral membrane protein, while in S. cerevisiae, it is located in the mitochondrial intermembrane space [6,10,11]. l-iLDHSs in E. coli and S. cerevisiae have been purified and further characterized [6,10,11]. They catalyze the oxidation of L-lactate to pyruvate through the respiratory electron transport chain in vivo and allow these strains to grow well in medium containing L-lactate as the sole carbon source [6,9,12].

Previous works have also confirmed the presence of l-iLDHSs in P. aeruginosa, P. putida, and P. stutzeri [3–5,13,14]. However, l-iLDHSs are only induced when these Pseudomonas strains are grown aerobically with L-lactate as the carbon source [3–5,13]. Based on this observation, involvement of l-iLDHSs in Pseudomonas l-lactate utilization has been speculated [3–5,13]. As in E. coli, l-iLDHSs in Pseudomonas strains are membrane-bound proteins [13,14] and it is difficult to purify them. Therefore, there is a lack of information on the properties and functions of l-iLDHSs in Pseudomonas strains.

In this study, a membrane-bound l-iLDH from P. stutzeri strain SDM was purified, and its encoding gene, lldD, was cloned, expressed, and characterized. A mutant of strain SDM was constructed by knockout of the lldD gene. The mutant was unable to grow with L-lactate as the sole carbon source, providing evidence for an indispensable role of l-iLDH in l-lactate utilization in this Pseudomonas strain.

Results and Discussion

Purification of l-iLDH

Purification of membrane-bound l-iLDHs in Pseudomonas species has never been reported. In this study, the membrane-bound l-iLDH in P. stutzeri SDM was solubilized with Triton X-100 and purified. The results of a typical purification procedure are summarized in Figure 1 and Table 1. The specific activity at the final step was 83.0 U mg⁻¹ of protein, which was a 364.3-fold increase over that of crude cell extract. The molecular mass of l-iLDH was found to be 42.8±0.6 kDa (Figure 1), using sodium...
dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 12.5% gel.

Cofactor analysis of L-iLDH

The purified L-iLDH has an intense yellow color. The absorption spectrum of L-iLDH in the visible region has peaks at around 460 nm and 380 nm, suggesting that L-iLDH is a flavoprotein (Figure 2). The flavin was released from the protein by boiling. The released flavin was confirmed to be FMN based on its identical migration with authentic FMN on high-performance liquid chromatography (HPLC) (Figure S1). Analysis of the flavin content of several preparations of homogeneous enzyme identified the ratio of L-iLDH to FMN as 1.03. Therefore, the native enzyme contains one FMN per subunit (Figure S2).

Sequence analysis of L-iLDH

Edman degradation analysis revealed that the N-terminal amino acid sequence of L-iLDH from P. stutzeri SDM is MIISASTDYRAAA. Two internal peptides were obtained by electrospray ionization-tandem mass spectrometry (ESI-MS/MS) of the peptides resulting from trypsin digestion (AAGVTTEVFITDMPGAR and DAVTFGADGIIYVSNHGGGR). The three peptides exhibit high similarity to the putative FMN-dependent L-lactate dehydrogenases in P. putida KT2440 (AAN70308.1) [15], P. aeruginosa PA01 (AAG08157.1) [16], and P. entomophila L48 (CAK13685.1) [17]. The L-iLDH coding gene (denominated as ilID in this work) was cloned from the genome of P. stutzeri SDM by PCR. Its sequence also exhibited high similarity to the genes encoding the putative L-lactate dehydrogenases from P. putida KT2440 [15], P. aeruginosa PA01 [16], and P. entomophila [17]. These results implied that the putative proteins might also exhibit L-iLDH activity in these Pseudomonas strains.

Considerable sequence identity exists between L-iLDH in P. stutzeri SDM and the proteins in the family of the L-α-hydroxyacid-oxidizing flavoproteins, including flavocytochrome b2 in S. cerevisiae (29% sequence identity) [18], L-iLDH in E. coli (85% sequence identity) [10], L-lactate oxidase in Aerococcus viridans (30% sequence identity) [19], long-chain α-hydroxy acid oxidase in rat kidney (32% sequence identity) [20], l-mandelate dehydrogenase in P. putida (38% sequence identity) [21], and glycolate oxidase in Spinacia oleracea (36% sequence identity) [22]. Based on the resolved crystal structure of flavocytochrome b2 from S. cerevisiae, six conserved amino acid residues required for flavin binding and enzymatic catalysis were identified. As shown in Figure 3, according to bioinformatic analysis with the program CLUSTAL X [23], the residues are highly conserved in all the members of the L-α-hydroxyacid-oxidizing flavoproteins family, including L-iLDH from P. stutzeri SDM.

Among the proteins compared in Figure 3, l-mandelate dehydrogenase in P. putida and l-iLDHs in P. stutzeri SDM and E. coli are all membrane-bound proteins [10,13,21]. In an earlier

Table 1. Purification of L-iLDH from P. stutzeri SDM.

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Specific activity (U mg⁻¹)</th>
<th>Purification (fold)</th>
<th>Yield (%)</th>
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<tr>
<td>Crude cell extract</td>
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<td>100</td>
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<tr>
<td>Membrane fraction</td>
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<td>2.9</td>
<td>61.0</td>
</tr>
<tr>
<td>Triton X-100 extract</td>
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<td>11.1</td>
<td>48.2</td>
</tr>
<tr>
<td>Ammonium sulfate fractionation</td>
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<td>28.3</td>
<td>21.5</td>
</tr>
<tr>
<td>DEAE-Sepharose</td>
<td>12.0</td>
<td>52.7</td>
<td>8.1</td>
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<tr>
<td>Source 30Q</td>
<td>83.0</td>
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<td>2.6</td>
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study, it was shown that L-mandelate dehydrogenase has an internal insertion which is responsible for membrane association [24,25]. L-iLDHs from P. stutzeri SDM and E. coli also have an internal insertion very similar to that found in L-mandelate dehydrogenase, in contrast to the soluble L-α-hydroxyacid-oxidizing flavoproteins (Figure 3). Therefore, like L-mandelate dehydrogenase, L-iLDH from P. stutzeri SDM might also be anchored in the membrane through an internal membrane-anchoring segment (Figure 3).

**Properties of L-iLDH**

L-iLDH activities of various Pseudomonas strains have been characterized using the crude cell extract [3–5,13,14]. L-iLDH was expressed in E. coli C43 (DE3) and purified to homogeneity, as described in the “Materials and Methods” (Figure S3). The purified recombinant L-iLDH showed a UV-visible absorption spectrum and cofactor ratio similar to that of the native protein. Effect of temperature on activity of the recombinant L-iLDH in P. stutzeri SDM was investigated over a range of 20–80°C; maximum activity was observed at 55°C (Figure 4a). The pH dependence of the recombinant L-iLDH was also investigated over a range of 4.0–13.0. The results showed that L-iLDH exhibited maximum activity at pH 9.0 (Figure 4b).

Substrate specificity of the recombinant L-iLDH from P. stutzeri SDM was examined with 20 mM α-hydroxy acids (L-lactate, D-lactate, glycollate, D-mandelate, L-mandelate, L-3-phenyllactate, L-3-phenylglycollate, L-3-phenylglyoxylate).

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**Figure 3. Sequence alignment of L-iLDH from P. stutzeri SDM with other FMN-dependent α-hydroxy acid oxidases.** LOX, α-lactate oxidase in A. viridans [19]; LCHO, long-chain α-hydroxy acid oxidase in rat kidney [20]; MDH, mandelate dehydrogenase in P. putida [21]; GOX, glycolate oxidase in S. oleracea [22]; FC2B, flavocytochrome b2 (L-iLDH) in S. cerevisiae [18]; L-iLDHs, L-iLDH in P. stutzeri SDM; L-iLDHc, L-iLDH in E. coli [9]. Red arrows indicate the highly conserved residues important for FMN binding and enzymatic catalysis across this class of enzymes. The boxed segments in L-mandelate dehydrogenase represent the internal sequences that were implicated in membrane association. The lined segments in L-iLDH represent the peptides identified through Edman degradation analysis and ESI-MS/MS. The sequences were aligned with the program CLUSTAL X [23]. doi:10.1371/journal.pone.0036519.g003
2-hydroxybutyrate, L-2-hydroxyisovaleric acid, DL-2-hydroxyisocaproic acid, DL-2-hydroxyoctanoic acid) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as the electron acceptor (Figure 4c). The \textit{P. stutzeri} SDM L-iLDH seems to have narrow substrate specificity. Only L-lactate and L-2-hydroxybutyrate were clearly oxidized by the enzyme. The same results were observed in \textit{E. coli}, implying similar functions of L-iLDHs in L-lactate metabolism of \textit{E. coli} and \textit{P. stutzeri} SDM [9,10]. In contrast, L-iLDH in \textit{Neisseria gonorrhoeae} exhibited broader substrate specificity, which allowed the strain to catalyze the conversion of L-phenyllactate to phenylpyruvate [26].

The rate of dehydrogenation of L-lactate and L-2-hydroxybutyrate catalyzed by the recombinant t-iLDH from \textit{P. stutzeri} SDM followed Michaelis-Menten kinetics. Double-reciprocal plots of the initial rates plotted against the concentrations of L-lactate and L-2-hydroxybutyrate were linear at a fixed concentration of MTT (0.2 mM), and yielded $K_m$ values of 29±0.65 μM and 99±3.9 μM, respectively, at 30°C. $V_{\text{max}}$ was estimated to be 332.3±5.4 μmol min$^{-1}$mg$^{-1}$ for L-lactate and 305.4±7.9 μmol min$^{-1}$mg$^{-1}$ for L-2-hydroxybutyrate with MTT as the electron acceptor (Figure 4c). The $K_m$ for L-lactate of L-iLDH in \textit{P. stutzeri} SDM was lower than that of other homologous enzymes like L-iLDH in \textit{E. coli} [10], flavocytochrome b$_2$ in \textit{S. cerevisiae} [18], and L-lactate oxidase in \textit{A. viridans} [19], in spite of some differences in the experimental conditions (Table S2). Inhibition of L-iLDH by oxalate and oxamate, which are canonical inhibitors of iLDHs [8], was also studied. Both oxalate and oxamate competitively inhibited L-iLDH activity (Figure S5), and their $K_i$ values were 1.9±0.1 mM and 29±1.7 mM, respectively.

The role of metal ions on activity of the recombinant \textit{P. stutzeri} SDM t-iLDH was tested by adding metal salts (final concentration, 5 mM) to the assay buffer. As shown in Figure 4d, Ca$^{2+}$, K$^+$, Mg$^{2+}$, and Ba$^{2+}$ did not affect t-iLDH activity. However, Fe$^{3+}$, Co$^{2+}$, Cu$^{2+}$, and Cd$^{2+}$ partially decreased t-iLDH activity, while Zn$^{2+}$ and Ni$^{2+}$ completely inhibited t-iLDH activity. Experiments carried out with native protein gave similar results (data not shown).

**Figure 4. Basic enzymatic properties of l-iLDH from \textit{P. stutzeri} SDM.** The reaction mixture contained 20 mM L-lactate, 0.2 mM MTT, 50 mM Tris-HCl (pH 7.5), and 0.1 μg purified l-iLDH. (a) Effect of temperature on l-iLDH activity. Enzyme reactions were carried out at pH 7.5 at different temperatures (20–80°C). (b) Effect of pH on l-iLDH activity. The optimum pH was assessed in a buffer containing citric acid, KH$_2$PO$_4$, boric acid, and barbital (CKBB buffer) at various pHs (3.0–13.0) at 30°C. (c) Substrate specificity of l-iLDH from \textit{P. stutzeri} SDM. The sodium salts of different α-hydroxy acids were used at a concentration of 20 mM. (d) Effect of different metal ions on l-iLDH activity. The concentration of metal ions was 5 mM in 50 mM Tris-HCl (pH 7.5). Values are the average ± SD of three separate determinations. doi:10.1371/journal.pone.0036519.g004
Kinetic analysis of i-LDH

A set of parallel straight lines was obtained in the double-reciprocal plot of different concentrations of MTT plotted against the activity at fixed concentrations of L-lactate or different concentrations of L-lactate plotted against the activity at fixed concentrations of MTT (Figure 5a, 5b). This kinetic behavior is typical of a double-displacement kinetic (ping-pong) mechanism. A ping-pong kinetic mechanism for i-LDH in P. stutzeri SDM is in agreement with previous reports describing the same mechanism for other i-LDH oxidizing flavoproteins [19].

Function of i-LDH in vivo

Many microorganisms can use lactate as their sole carbon source for growth [6–9,27]. In these lactate utilization processes, lactate is first converted to pyruvate, which subsequently enters the tricarboxylic acid cycle [6–9,12,27]. Thus, the enzymes catalyzing lactate into pyruvate are very important for the survival of these microorganisms in habitats containing lactate [6–9,12,27]. In E. coli and Corynebacterium glutamicum, the lactate-induced i-LDHs oxidized lactate in vivo. Disruption of i-LDH resulted in the loss of ability to utilize lactate as the sole carbon source [9,28]. Constitutively expressed NAD-dependent lactate dehydrogenase would enable the i-LDH inactivation mutant of C. glutamicum to grow on lactate [29]. In the present study, i-LDH from P. stutzeri SDM was purified and characterized. The enzyme was able to catalyze the conversion of lactate into pyruvate in vivo (Figure S6). To identify the function of i-LDH in vivo, insertional inactivation of the i-LDH encoding gene, lldD, in P. stutzeri SDM was conducted using the pK18mobs system.

We investigated whether the mutant is impaired in growth on solid minimal salt medium (MSM) with lactate as the sole carbon source. The mutant P. stutzeri SDM-llldD exhibited little growth compared to the wild type (Figure S7a). As a control, both the wild-type and P. stutzeri SDM-llldD strains grew equally well on solid MSM with 0.5% pyruvate as the sole carbon source (Figure S7b).

As with growth on solid MSM, the wild-type and P. stutzeri SDM-llldD strains grew well in liquid MSM with 0.5% pyruvate as the sole carbon source (Figure 6a). However, compared with the wild type, P. stutzeri SDM-llldD exhibited no growth in liquid MSM with 0.5% lactate as the sole carbon source (Figure 6b).

Figure 5. Kinetic mechanism of i-LDH from P. stutzeri SDM. Purified i-LDH (0.1 μg) was incubated in a reaction mixture containing 50 mM Tris-HCl (pH 7.5) at 30 C. (a) The reaction was started with different lactate concentrations at variable MTT concentrations: ■, 0.2 mM MTT; ●, 0.25 mM MTT; ▲, 0.35 mM MTT; ▼, 0.75 mM MTT; ◆, 0.1 mM MTT. (b) The reaction was started with different MTT concentrations at variable lactate concentrations: ■, 0.02 mM lactate; ●, 0.0325 mM lactate; ▲, 0.05 mM lactate; ▼, 0.0825 mM lactate; ◆, 0.125 mM lactate; □, 0.1675 mM lactate; △, 0.25 mM lactate; ◇, 0.375 mM lactate.

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Growth in MSM with lactate as the sole carbon source could be restored when P. stutzeri SDM-llldD were complemented with a broad-host-range plasmid pBSP II SK(−) harboring the lldD gene. Altogether, these results suggest that the lldD gene encodes i-LDH, which catalyzes the indispensable transformation of lactate to pyruvate in the P. stutzeri SDM lactate utilization process.

Interestingly, the i-LDH inactivation mutant of P. stutzeri SDM also lost its ability to grow in liquid MSM with 0.5% lactate as the sole carbon source. Additionally, the expression of gene lldD was not able to complement the phenotype of lactate utilization (Figure 6c). In E. coli and C. glutamicum, the NAD-independent lactate dehydrogenase (o-LDH) that catalyzes lactate oxidation in vivo was constitutively expressed, while i-LDH was in the lactate utilization operon and controlled by the regulator LldR [9,28,30,31]. In a previous study, i-LDH activity in P. stutzeri SDM was studied. Both i-LDH and o-LDH were induced by either enantiomer of lactate in P. stutzeri SDM [13]. Because the insertion of pK18mobs into the genome of P. stutzeri SDM influenced the transcription of the i-LDH encoding gene and resulted in the inability of P. stutzeri SDM to use o-lactate as the sole carbon source, it was hypothesized that the i-LDH and o-LDH encoding genes might be in the same operon. Indeed, four adjacent genes (lldR, lldP, lldD, and dld-II) encoding a lactate-responsive regulator LldR, an o-lactate permease, i-LDH, and a predicted o-LDH, were annotated in the draft genome sequence of P. stutzeri SDM [32].

Many Pseudomonas strains have been isolated as opportunistic human pathogens. Pseudomonas infections often elevate human fluid levels of lactate by catalyzing lactate to pyruvate and subsequent conversion to acetyl-CoA. Additionally, the concentration of lactate, the product of lactate catalysis by eukaryotic LDH-catalyzed pyruvate reduction, might also increase in human fluid during infection. Studies on some microorganisms such as Neisseria meningitidis and N. gonorrhoeae showed that, in addition to the general stimulation of metabolism, utilization of lactate in human hosts also promotes the production of some determinants of pathogenicity [33–38]. Thus, the utilization of lactate in human hosts has been found to increase the pathogenicity of these organisms [35]. As for the situation in different Pseudomonas strains, it has not been determined whether the metabolism of lactate would affect their pathogenicity. If a
mutant pathogenic Pseudomonas strain incapable of utilizing l-lactate is isolated, a comparison of the pathogenicity between the wild-type and mutant strains might provide useful information related to the pathogenic mechanisms of Pseudomonas strains.

In summary, l-iLDH in l-lactate utilization strain P. stutzeri SDM was purified and further characterized. This key enzyme was confirmed to be required for the l-lactate utilization of strain SDM. Considering the high sequence identity among the lldD genes in various Pseudomonas strains, l-iLDHs might also play an indispensable function in the l-lactate utilization of other Pseudomonas strains.

Materials and Methods

Chemicals

L-lactate, glycollate, d-mandelate, l-mandelate, l-3-phenyllactate, l-2-hydroxybutyrate, l-2-hydroxyisovaleric acid, DL-2-hydroxyisocaproic acid, DL-2-hydroxyoctanoic acid MTT, FMN, bovine serum albumin (BSA), isopropyl-ß-d-thiogalactopyranoside (IPTG), phenylmethanesulfonyl fluoride (PMSF), and dithiothreitol (DTT) were purchased from Sigma. D-Lactate was purchased from Fluka. Other chemicals were of the reagent grade.

Bacteria and culture conditions

Bacterial strains used in this study are listed in Table 2. The P. stutzeri SDM (CCCTC no: M206010), isolated from soil, was cultured in MSM supplemented with different sole carbon source (10 g L⁻¹) at 30°C [13]. E. coli strains were grown in lysogenic broth (LB) medium at 37°C. Antibiotics were used, when appropriate, at the following concentrations: ampicillin (100 µg mL⁻¹), chloramphenicol (50 µg mL⁻¹), and kanamycin (50 µg mL⁻¹).

Purification of l-iLDH

The membrane fraction of P. stutzeri SDM was prepared as described by Ma et al. [13]. Triton X-100 (10%, w/v) was added to the membrane fraction to a final concentration of 1 mg mg⁻¹ protein. The suspensions were stirred gently for 30 min, and then centrifuged at 140,000 × g for 180 min at 4°C. The supernatants (detergent extracts) were gently transferred into other containers. Solid (NH₄)₂SO₄ was slowly added to the detergent extracts to yield 30% saturation. When (NH₄)₂SO₄ was completely dissolved, the solution was stirred for another 30 min before being centrifuged at 140,000 × g for 30 min at 4°C. More (NH₄)₂SO₄ was added to the supernatant to reach 40% saturation, and the solution was stirred and centrifuged as before. The pellet was dissolved in buffer A: 50 mM Tris-HCl (pH 8.6) containing 1 mM DTT, 5 mM MgSO₄, 0.1% Triton X-100, and 1 mM EDTA, and applied to a column of DEAE Sepharose Fast Flow equilibrated with buffer A. The column was washed with buffer B: 50 mM Tris-HCl (pH 8.6) containing 1 mM DTT, 200 mM KCl, 5 mM MgSO₄, 0.1% Triton X-100, and 1 mM EDTA, at a flow rate of 5 mL min⁻¹. The fractions containing l-iLDH were concentrated by ultrafiltration and desalted with gel Sephadex G-25. The l-iLDH pool was then applied to a column of SOURCE 30Q pre-equilibrated with buffer A. The column was washed with a linear gradient of 0%–100% buffer B at a flow rate of 5 mL min⁻¹. The fractions containing l-iLDH were concentrated by ultrafiltration and analyzed by SDS-PAGE.

Analysis of N-terminal amino acid and microsequence

The purified l-iLDH was subjected to 12.5% SDS-PAGE for analysis. After electrophoresis, proteins in the gel were electrophoretically transferred to an Immunoblot-PSQ PVDF membrane (Millipore) by using 25 mM Tris, 192 mM glycine, and 0.1% SDS as the cathode buffer, and 25 mM Tris, 192 mM glycine, and 20% methanol as the anode buffer. The bands on the PVDF membrane were excised, and sent to the Shanghai GeneCore Biotechnologies Co., Ltd., for N-terminal sequencing by automated Edman degradation.

For microsequencing of the internal peptides, the protein bands were cut from SDS-polyacrylamide gels and washed 3 times with water before being digested with trypsin. The resulting peptides were sent to the Shandong Academy of Medical Sciences for ESI-MS/MS.

Cloning of the l-iLDH encoding gene

The plasmids used in this study are listed in Table 3. Degenerate primers P1, P2, and P3 were constructed according to the N-terminal and internal peptides resulting from the sequencing of P. stutzeri SDM l-iLDH. Primer P4 was constructed according to the conserved region of NAD-independent l-lactate dehydrogenase close to l-iLDH in Pseudomonas strains. The total genomic DNA of P. stutzeri SDM, extracted with the Wizard genomic DNA purification kit (Promega, Madison, WI), was used as the template for the first round of PCR with the primers P1 and P3. One microliter of the PCR product from the reaction mixture was used as the template for the second round of PCR with the primers P1 and P2. Using the genomic DNA of P. stutzeri SDM as the template, the third PCR was conducted with the primers P4 and P3. P5 was constructed according to the product of the second round of PCR. After sequencing of the three PCR products, the
gene sequence of l-iLDH in *P. stutzeri* SDM was identified (Figure S8).

**Purification of recombinant l-iLDH**

The l-iLDH encoding gene *lldD*, was amplified by PCR from the genome of *P. stutzeri* SDM by using the primers P1c and P2c. The PCR product was digested by *Xho*I and *Hind*III, and cloned into the *Hind*III/*Xho*I sites of pETDuet-1 to construct the plasmid pET-LDH. *E. coli* C43 (DE3) carrying the pET-LDH plasmid was used to induce the expression of l-iLDH. Cells were harvested by centrifugation at 14,000 × g for 5 min at 4°C and washed with 0.85% (w/v) sodium chloride (NaCl) solution. The cell pellets were subsequently suspended in the binding buffer (pH 7.4, 20 mM sodium phosphate, 20 mM imidazole, 500 mM NaCl, 1 mM PMSE, 0.1% Triton X-100, and 10% glycerol). Approximately 2–2.5 g of wet cells (suspended in 50 mL of binding buffer) were sonicated for 150 cycles (with 5 s at 40% of maximal output and 5 s rest in each cycle) with a Sonics sonicator (500 W/20 KHz, USA) in an ice bath. The sonicate was then centrifuged at 14,000 × g for 20 min at 4°C. The supernatant was loaded onto a HiTrap HP column (5 mL), and eluted with 80% binding buffer.

<table>
<thead>
<tr>
<th>Table 2. The strains used in this work.</th>
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<td><strong>Strain</strong></td>
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<td><em>P. stutzeri</em> SDM-dlldD</td>
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<td><em>P. stutzeri</em> SDM-dlldD(pBSP II SKlldD)</td>
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<td><em>E. coli</em> DH10B</td>
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<td><em>E. coli</em> HB101</td>
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<td><em>E. coli</em> C43 (DE3)</td>
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<sup>a</sup>Km<sup>a</sup> and Ap<sup>c</sup> indicate resistance to kanamycin and ampicillin, respectively.

<table>
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<th>Table 3. The primers and plasmids used in this work.</th>
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<td>pET-LDH</td>
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<sup>b</sup>Km<sup>a</sup>, Ap<sup>c</sup>, and Cm<sup>a</sup> indicate resistance to kanamycin, ampicillin, and chloramphenicol, respectively.

<sup>c</sup>W stands for one of A or T, S stands for one of C or G, R stands for one of A or G, and N stands for A, C, G, or T.

<sup>d</sup>*Hind*III, *Xho*I, *BamHI*, *KpnI* and *SacI* restriction sites introduced in the primers are underlined.

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and 20% elution buffer (pH 7.4, 20 mM sodium phosphate, 500 mM imidazole, 500 mM NaCl, 1 mM PMSF, 0.1% Triton X-100, and 10% glycerol) at a flow rate of 5 mL min⁻¹. The fractions containing i-LiLDH were concentrated by ultrafiltration, desalted with Sephadex G-25, and then stored in 100 mM sodium phosphate buffer (pH 9.0, containing 50% glycerol and 0.1% Triton X-100) at −20°C. Under these conditions, the enzyme was stable for months with no apparent loss of activity.

Cofactor analysis of i-LiLDH

UV-visible spectra of i-LiLDH were recorded at 320–550 nm with an Ultrospec™ 2100 pro UV-visible Spectrophotometer (GE Healthcare Life Sciences). i-LiLDH was heated to 100°C for 3 min, and then centrifuged at 10,000 g for 10 min to remove denatured protein. The cofactor released from the purified protein was analyzed by HPLC (Agilent 1100 series; Hewlett-Packard, USA) using an ODS C18 column (4.6×150 mm, particle size: 5 µm) [43]. The eluent was 100 mM ammonium bicarbonate in 82%–18% methanol [43]. Standard FMN solutions of 0.05, 0.1, 0.15, 0.2, 0.25, and 0.3 mM were used for quantitative analysis and detected at 450 nm.

Knockout of gene lldD

To construct the P. stutzeri SDM-dlldD mutant strains, an internal fragment (322 bp) of the target lldD gene was PCR amplified by using the primers MF and MR, which contain HindIII and BamHI restriction enzyme sites, respectively. The PCR product was cloned into HindIII/BamHI-cut pK18mobL, a mobilizable plasmid that does not replicate in Pseudomonas, to form pK18moblldD [44]. To transfer plasmid pK18moblldD into P. stutzeri SDM, a triparental filter mating was performed as previously described by using E. coli DH10B (pK18moblldD) as the donor strain, E. coli HB101 (pRK600) as the helper strain, and P. stutzeri SDM as the recipient strain [44]. P. stutzeri SDM exconjugants harboring the disrupted lldD gene (P. stutzeri SDM-dlldD) were isolated on minimal medium plates containing kanamycin that was selected for the insertion of the suicide vector after incubation at 30°C for 16 h [44]. The mutant strain was analyzed by PCR to confirm disruption of the target gene (Table S1 and Figure S9).

Complementation of P. stutzeri SDM-dlldD

To achieve complementation of the P. stutzeri SDM-dlldD mutant, the lldD gene of P. stutzeri SDM was amplified by PCR using the primers CF and CR, which contain KpnI and SacI restriction enzyme sites, respectively. The PCR products were cloned into the KpnI/SacI restriction sites (indicated in the primers) of the vector pBSP II SK(−) [41], which contains an IPTG inducible promoter. The recombinant plasmid pBSP II SKlldD was then introduced into the P. stutzeri SDM-dlldD mutant by triparental filter mating [44]. The P. stutzeri SDM-dlldD harboring the plasmid pBSP II SKlldD was isolated on minimal medium plates containing citrate, ampicillin, and kanamycin.

Growth in MSM

To test bacterial growth on solid media, wild-type and mutant strains were first streaked on LB agar medium and grown at 30°C. The next day, cells from single colonies on LB agar medium were picked and streaked onto solid MSM. Solid MSM was supplemented with l-lactate, d-lactate, or pyruvate as the sole carbon source.

To test bacterial growth in liquid medium, cells were first grown in LB medium until log phase, and then diluted with 20 mL of minimal medium containing l-lactate, d-lactate, or pyruvate as the sole carbon source. Cells were then grown at 30°C with shaking. IPTG was added at a final concentration of 1 mM to the minimal media. At various time points, the cell densities (OD₆20nm) of the cultures were recorded with an Ultrospec™ 2100 pro UV-visible spectrophotometer.

Biochemical assays

The activity of i-LiLDH was determined at 30°C in 1 mL of 50 mM Tris-HCl (pH 7.5) and 0.2 mM MTT. The reaction was started by addition of l-lactate, and the rate of MTT reduction was determined by measuring the changes in absorbance in 578 nm [45]. One unit of i-LiLDH activity was defined as the amount reducing 1.0 µmol of electron acceptor per minute under the test conditions. Protein concentrations were determined by the Lowry method, with BSA as the standard [46].

Polyacrylamide gel electrophoresis

SDS-PAGE was performed using a 12.5% polyacrylamide resolving gel and a 4% polyacrylamide stacking gel. Electrophoresis was run at a constant voltage of 80 V when the samples were in the stacking gel. When the dye front reached the resolving gel, the voltage was increased to 120 V. The run was stopped when the dye front was 2–3 mm away from the bottom edge of the gel.

Nucleotide sequence accession number

The nucleotide sequence of the lldD gene has been deposited in the GenBank nucleotide sequence databases under the accession no. GU373722.

Supporting Information

Figure S1 HPLC analysis of (a) authentic FMN, and (b) the cofactor released from the purified i-LiLDH in P. stutzeri SDM. For the identification of the cofactor of i-LiLDH, the purified i-LiLDH was heated to 100°C for 3 min and then centrifuged at 10,000 x g for 10 min to remove denatured protein. Cofactor released from purified protein was analyzed by HPLC (Agilent 1100 series, Hewlett-Packard, USA) using an ODS C18 column (4.6×150 mm, particle size: 5 µm). The eluent was 100 mM ammonium bicarbonate 82–18% methanol. As shown in Figure S1, a compound identical to authentic FMN was produced. Therefore, i-LiLDH in P. stutzeri SDM used FMN as the cofactor. (PDF)

Figure S2 Standard curve of FMN concentration related to peak area obtained by HPLC analysis. For the determination of the ratio between l-lactate and FMN, 0.1171 mM l-lactate was heated to 100°C for 3 min and then centrifuged at 10,000 x g for 10 min to remove denatured protein. Cofactor released from purified protein was analyzed by HPLC (Agilent 1100 series, Hewlett-Packard, USA) using an ODS C18 column (4.6×150 mm, particle size: 5 µm). The eluent was 100 mM ammonium bicarbonate 82–18% methanol. Standard FMN solutions of 0.05, 0.1, 0.15, 0.2, 0.25, and 0.3 mM were used for quantitative analysis and determined at 450 nm. The concentration of the cofactor released from purified protein was determined to be 0.1136 mM. Thus, the ratio between l-lactate and FMN was 0.1171/0.1136 = 1.03. Therefore, the native enzyme contains one FMN per subunit. (PDF)
to other reported t-iLDH, the t-iLDH in P. stutzeri SDM also catalyzes the conversion of t-lactate into pyruvate. (PDF)

Figure S7 The lldD gene is required for growth on t-lactate. (a), growth of wild-type and mutant strains of P. stutzeri SDM on solid minimal media containing 0.5% pyruvate as the sole carbon source. (b), growth of the same set of strains (shown in panels a) on solid minimal media containing 0.5% t-lactate as the sole carbon source. We constructed the P. stutzeri SDM mutants lacking the lldD. Whether the mutants were impaired in growth on solid minimal medium with 0.5% t-lactate as the sole carbon source was tested. As shown in Figure S7b, the mutant exhibited little growth compared to the wild type. As a control, both the wild type and the mutant grew equally well on solid minimal medium with 0.5% pyruvate as the sole carbon source (Figure S7a). (PDF)

Figure S8 Scheme for the lldD gene cloning procedure. (PDF)

Figure S9 PCR analysis for verification of the insertional inactivation of the gene encoding t-iLDH. t-iLDH encoding gene lldD. (A) Structure of pK18moblldD (left), the t-iLDH encoding gene lldD in P. stutzeri SDM genome (right), the insertional inactivated t-iLDH encoding gene lldD in P. stutzeri SDM genome (bottom). (B) PCR verification of the mutant strains (Figure S7) with the insertion of pK18mob in P. stutzeri SDM genome. In all cases, the primer pair VF1/VF2 (arrows; for the sequence, see Table S1) was used. (C) PCR verification of the mutant strains (Figure S7) with the homologous recombination between pK18mob and P. stutzeri SDM genome. In all cases, the primer pair VF1/VF2 (arrows; for the sequence, see Table S1) was used. (PDF)

Table S1 Primers used in the verification of the insertional inactivation of the t-iLDH encoding gene. (DOC)

Table S2 Comparison of \( K_m \) values estimated for different enzymes. (DOC)

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Author Contributions
Conceived and designed the experiments: PX CM CG. Performed the experiments: CG TJ PD. Analyzed the data: LL CG TJ. Contributed reagents/materials/analysis tools: JK CM PX. Wrote the paper: CG TJ CM PX.

References
2. Silby MW, Winstanley C, Godfrey SA, Levy SR, Jackson RW (2011) Purified L-iLDH (0.1 mM and 2.5 mM oxalate, 0.1 mM and 2.5 mM oxamate). The patterns of double-reciprocal plots indicate a competitive inhibition for both chemicals. The \( K_i \) values of oxalate and oxamate were estimated to be 1.9±0.1 mM and 29±1.7 mM, respectively. The dispersion values indicate the SEM of the linear regression analysis of one experiment. (PDF)

Author Contributions
Conceived and designed the experiments: PX CM CG. Performed the experiments: CG TJ PD. Analyzed the data: LL CG TJ. Contributed reagents/materials/analysis tools: JK CM PX. Wrote the paper: CG TJ CM PX.


