NAD-Independent L-Lactate Dehydrogenase Required for L-Lactate Utilization in *Pseudomonas stutzeri* A1501

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ABSTRACT

NAD-independent L-lactate dehydrogenases (L-iLDHs) play important roles in L-lactate utilization of different organisms. All of the previously reported L-iLDHs were flavoproteins that catalyze the oxidation of L-lactate by the flavin mononucleotide (FMN)-dependent mechanism. Based on comparative genomic analysis, a gene cluster with three genes (*lldA*, *lldB*, and *lldC*) encoding a novel type of L-iLDH was identified in *Pseudomonas stutzeri* A1501. When the gene cluster was expressed in *Escherichia coli*, distinctive L-iLDH activity was detected. The expressed L-iLDH was purified by ammonium sulfate precipitation, ion-exchange chromatography, and affinity chromatography. SDS-PAGE and successive matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) analysis of the purified L-iLDH indicated that it is a complex of LldA, LldB, and LldC (encoded by *lldA*, *lldB*, and *lldC*, respectively). Purified L-iLDH (LldABC) is a dimer of three subunits (LldA, LldB, and LldC), and the ratio between LldA, LldB, and LldC is 1:1:1. Different from the FMN-containing L-iLDH, absorption spectra and elemental analysis suggested that LldABC might use the iron-sulfur cluster for the L-lactate oxidation. LldABC has narrow substrate specificity, and only L-lactate and DL-2-hydrobutyrate were rapidly oxidized. Mg²⁺ could activate L-iLDH activity effectively (6.6-fold). Steady-state kinetics indicated a ping-pong mechanism of LldABC for the L-lactate oxidation. Based on the gene knockout results, LldABC was confirmed to be required for the L-lactate metabolism of *P. stutzeri* A1501. LldABC is the first purified and characterized L-iLDH with different subunits that uses the iron-sulfur cluster as the cofactor.

IMPORTANCE

Providing new insights into the diversity of microbial lactate utilization could assist in the production of valuable chemicals and understanding microbial pathogenesis. An NAD-independent L-lactate dehydrogenase (L-iLDH) encoded by the gene cluster *lldABC* is indispensable for the L-lactate metabolism in *Pseudomonas stutzeri* A1501. This novel type of enzyme was purified and characterized in this study. Different from the well-characterized FMN-containing L-iLDH in other microbes, LldABC in *P. stutzeri* A1501 is a dimer of three subunits (LldA, LldB, and LldC) and uses the iron-sulfur cluster as a cofactor.

Lactate, one of the most important 2-hydroxy acids in nature, plays important roles in many biological processes of microbes (1–4). Lactate utilization is present in both prokaryotic and eukaryotic microbes (4). This process can allow the survival of some microbes through the use of lactate in habitats and has also been found to induce pathogenicity of several microbes (5–8). NAD-independent L-lactate dehydrogenases (L-iLDHs), the key enzymes involved in L-lactate utilization, are widely distributed among bacteria, yeasts, and protists (9–11). A few L-iLDHs, such as L-lactate oxidase in *Aerococcus viridans*, membrane-bound L-iLDH in *Escherichia coli*, and flavocytochrome *b*₂ in *Saccharomyces cerevisiae*, have been purified and further characterized (10, 12, 13). Those enzymes catalyze the oxidation of L-lactate by a flavin mononucleotide (FMN)-dependent mechanism and share remarkable similarities with other flavoproteins catalyzing the oxidation of L-2-hydroxy acids, such as glycolate oxidase, long-chain α -hydroxy acid oxidase, and L-mandelate dehydrogenases (13–18). Many biocatalysts and L-lactate determination techniques have been developed based on these enzymes (19–23).

In some organisms, the involvement of FMN-dependent L-iLDHs in L-lactate utilization has been studied. For example, the inactivation of the *lldD* gene, encoding the membrane-bound FMN-dependent L-iLDH, impairs the growth of *Escherichia coli* in minimal medium with L-lactate as the sole carbon source (24). Based on comparative genomics analysis and gene knockout re-

sults, the presence of another kind of L-iLDH, encoded by a gene cluster (named *lutABC*), has been identified in *Bacillus subtilis*, *Campylobacter jejuni*, and *Shewanella oneidensis* (25–28). However, the L-iLDHs in those strains have never been purified and further studied.

Utilization of lactate has also been studied in different *Pseudomonas* strains (29, 30). The genes similar to *lldD* of *E. coli* exist in the genomes of most *Pseudomonas* species (Fig. 1) (31–35). For example, an *lldRPDE* operon containing *lldD* and other three lactate utilization-related genes, including *lldP* (encoding a lactate permease), *lldE* (encoding an NAD-independent D-lactate dehydrogenase [D-iLDH]) and *lldR* (encoding an FadR-type regulator,

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FIG 1 An alignment of the lactate utilization operons in different *Pseudomonas* species, including *P. aeruginosa* PAO1 (33), *Pseudomonas entomophila* L48 (34), *P. fluorescens* Pf-5 (37), *Pseudomonas mendocina* DLHK (35), *Pseudomonas putida* KT2440 (32), *P. stutzeri* SDM (31), and *P. stutzeri* A1501 (38). Genes were functionally annotated following the color code indicated.

LldR) was identified in *Pseudomonas aeruginosa* (36). However, the *lldD* gene was replaced by a gene cluster (containing three genes denominated *lldA*, *lldB*, and *lldC* in this work) in genomes of *Pseudomonas fluorescens* Pf-5 and *Pseudomonas stutzeri* A1501 (Fig. 1) (37, 38). *P. stutzeri* A1501 could use L-lactate as the sole carbon source for growth. Because the L-iLDH-encoding gene *lldD* was replaced by the gene cluster *lldABC* (similar to the *lutABC* cluster in *B. subtilis*), we hypothesized that LldABC, encoded by *lldABC*, might also exhibit L-iLDH activity. The aim of the present study was to identify and characterize the novel type of L-iLDH, LldABC, in *P. stutzeri* A1501.

MATERIALS AND METHODS

Materials. Restriction enzymes and other DNA-modifying enzymes were obtained from TaKaRa Bio, Inc. (China). Chemicals were purchased from Fluka or Sigma and were of the highest quality available. The DEAE-Sephacrose fast-flow column, HisTrap HP column, and Superdex 200 10/300 GL column were from GE Healthcare Life Sciences.

Bacterial strains, plasmids, and media. All bacterial strains and plasmids used in this work are listed in Table 1. *E. coli* DH5 α (Novagen) was used for cloning procedures. *P. stutzeri* A1501 (CGMCC NO:0351 [a kind gift from Min Lin, National Key Facility for Crop Gene Resources and Genetic Improvement, Chinese Academy of Agricultural Sciences]) was used here to amplify the *lldABC* cluster by PCR. *E. coli* BL21(DE3)(pLysS) (Transgene) was used for expression of LldABC. The expression vector pETDuet-1 from Invitrogen was used to clone and overexpress the gene cluster *lldABC*. *P. stutzeri* A1501 was grown in minimal salt medium (MSM) supplemented with 5.0 g liter⁻¹ L-lactate or pyruvate at 30°C. *E. coli* was grown in Luria-Bertani (LB) medium at 37°C. LB medium was supplemented with 50 μ g ml⁻¹ ampicillin to select *E. coli* transformants.

Expression of LldABC. The gene cluster *lldABC* was amplified from genomic DNA of *P. stutzeri* A1501 through PCR with primers P1 and P2 (Table 2), which contained EcoRI and HindIII restriction enzyme sites, respectively. The amplified gene cluster was ligated into expression vector pETDuet-1 to obtain plasmid pETLDH. The constructed expression plasmid pETLDH was then introduced into *E. coli* BL21(DE3)(pLysS) for LldABC expression. *E. coli* BL21(DE3)(pLysS)(pETLDH) was cultured in LB medium at 37°C to an optical density at 600 nm (OD₆₀₀) of 0.6. Then, isopropyl- β -D-1-thiogalactopyranoside (IPTG) was added to reach a final concentration of 1.5 mM, and the cells were further incubated at 37°C for 6 h. Cells were harvested by centrifugation and resuspended in buffer A (pH 7.4, 20 mM sodium phosphate, 1 mM phenylmethylsulfonyl fluoride [PMSF], 10% glycerol, 0.1% Triton X-100). Cells were lysed by ultrasonics (Sonics 500 W; 20 kHz) in an ice bath for 20 min, and then the cell

lysate was centrifuged at 12,000 rpm for 20 min at 4°C to remove cell debris.

Enzyme assays. L-iLDH activity was assayed in a reaction mixture containing 50 mM Tris-HCl (pH 7.5), 0.2 mM 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 50 mM L-lactate, and the crude extract, in a final volume of 1 ml at 30°C. MTT reduction was monitored at 578 nm using a UV-visible spectrophotometer (Ultrospec 2100 pro; Amersham Biosciences). The extinction coefficient for reduced MTT was 16.2 mM⁻¹ cm⁻¹ (39). One unit of enzyme activity is defined as the amount of L-iLDH required to convert 1 μ mol of L-lactate to pyruvate per min. Protein was measured by the method of Lowry et al. (40).

Purification of LldABC. Solid ammonium sulfate was added in crude extract to 40% saturation in 30 min at 4°C. After being stirred at 4°C for 2 h, the suspension was centrifuged at 12,000 rpm for 20 min. Then, solid ammonium sulfate was added in the supernatant to give 50% saturation, followed by centrifugation of the suspension. The precipitate was dissolved in buffer A and loaded onto a DEAE-Sephacrose fast-flow column. The bound protein was eluted with a linear gradient of buffer B (pH 7.4, 20 mM sodium phosphate, 1 mM PMSF, 10% glycerol, 500 mM NaCl, 0.1% Triton X-100). The active fractions were concentrated by ultrafiltration and then loaded onto a HisTrap HP column preequilibrated with the binding buffer (pH 7.4, 20 mM sodium phosphate, 500 mM NaCl, 0.1% Triton X-100). The L-iLDH fractions were eluted with elution buffer (pH 7.4, 20 mM sodium phosphate, 200 mM imidazole, 500 mM NaCl, 0.1% Triton X-100). The pool with L-iLDH activity was concentrated by ultrafiltration, desalted with gel G-25, detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) performed using 12.5% polyacrylamide gels on a Mini-Protean III system (Bio-Rad), and then stored at 0 to 4°C.

Molecular mass determination of LldABC. The molecular mass of the native LldABC in *P. stutzeri* A1501 was estimated by gel filtration with thyroglobulin (669 kDa), ferritin (440 kDa), catalase (250 kDa), aldolase (158 kDa), lactate dehydrogenase (140 kDa), bovine serum albumin (67 kDa), chymotrypsinogen A (21.2 kDa), and RNase A (13.7 kDa) as markers. L-iLDH was applied to a Superdex 200 10/300 GL column that had been equilibrated with buffer A supplemented with 150 mM KCl. The column was washed with the same buffer. The submolecular structure of the enzyme was studied under denaturing conditions by SDS-PAGE.

MALDI-TOF MS. SDS-PAGE was performed using a 12.5% polyacrylamide resolving gel and a 4% polyacrylamide stacking gel. Protein bands separated by SDS-PAGE were manually excised from the stained SDS-PAGE gels and subjected to in-gel tryptic digestion. Peptide mass fingerprinting (PMF) of tryptic peptides was detected by matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS), and the proteins were identified by comparing peak lists gen-

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype or description	Source or reference
Strains		
<i>E. coli</i>		
DH5 α	F [−] <i>endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG</i> ϕ 80 <i>dlacZ</i> Δ M15 Δ (<i>lacZYA-argF</i>) <i>U169 hsdR17</i> (r _K [−] m _K ⁺) λ [−]	Novagen
BL21(DE3)(pLysS)	F [−] <i>ompT gal dcm lon hsdS_B</i> (r _B [−] m _B [−]) λ (DE3)(pLysS)(Cm ^r)	Transgene
HB101	F [−] <i>mcrB mrr hsdS20</i> (r _B [−] m _B [−]) <i>recA13 leuB6 ara-14 proA2 lacY1 galK2 xyl-5 mtl-1 rpsL20</i> (Sm ^r) <i>glnV44</i> λ [−]	Lab stock
DH10B	F [−] <i>endA1 recA1 galE15 galK16 nupG rpsL</i> Δ <i>lacX74</i> ϕ 80 <i>lacZ</i> Δ M15 <i>araD139</i> Δ (Ara, Leu)7697 <i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) λ [−]	Lab stock
<i>P. stutzeri</i>		
A1501	Wild type, capable of L-lactate utilization	Chinese Academy of Agricultural Sciences
SDM	Wild type, capable of L-lactate utilization	Lab stock
Δ <i>lldA</i> mutant	Δ <i>lldA</i> in <i>P. stutzeri</i> A1501 background	This study
Δ <i>lldB</i> mutant	Δ <i>lldB</i> in <i>P. stutzeri</i> A1501 background	This study
Δ <i>lldC</i> mutant	Δ <i>lldC</i> in <i>P. stutzeri</i> A1501 background	This study
Δ <i>lldB</i> /pBBR:: <i>lldABC</i> mutant	Complementation of <i>lldABC</i> into <i>P. stutzeri</i> Δ <i>lldB</i> mutant	This study
Δ <i>lldB</i> /pBBR:: <i>lldD</i> mutant	Complementation of <i>lldD</i> in <i>P. stutzeri</i> SDM into <i>P. stutzeri</i> Δ <i>lldB</i> mutant	This study
Plasmids		
pETDuet-1	Expression vector containing two MCSs ^a ; Ap ^r	Invitrogen
pETLDH	<i>lldABC</i> in <i>P. stutzeri</i> A1501 inserted in pETDuet-1	This study
pK18mobsacB	Suicide plasmid for gene knockout; Km ^r	41
pRK2013	Helper plasmid for conjugation	Lab stock
pK18mobsacB- Δ <i>lldA</i>	Δ <i>lldA</i> cloned into pK18mobsacB	This study
pK18mobsacB- Δ <i>lldB</i>	Δ <i>lldB</i> cloned into pK18mobsacB	This study
pK18mobsacB- Δ <i>lldC</i>	Δ <i>lldC</i> cloned into pK18mobsacB	This study
pBBR1MCS-5	Plasmid for gene complementation; Gm ^r	Biovector Science Lab, Inc.
pBBR:: <i>lldABC</i>	<i>lldABC</i> in <i>P. stutzeri</i> A1501 inserted in pBBR1MCS-5	This study
pBBR:: <i>lldD</i>	<i>lldD</i> in <i>P. stutzeri</i> SDM inserted in pBBR1MCS-5	This study

^a MCSs, multiple-cloning sites.

erated from PMF with NCBI nr database of MASCOT using the bacterial subset (www.matrixscience.com).

Identification of the L-lactate oxidation product of LldABC. To determine the L-lactate oxidation product of LldABC, biotransformation was carried out using L-lactate (10 g liter^{−1}) as the substrate and purified LldABC (1 mg ml^{−1}) as the biocatalyst in 50 mM Tris-HCl (pH 7.5) at 30°C. After 4 h of reaction, the mixture was centrifuged at 20,000 × g for 15 min. The supernatant was analyzed by high-performance liquid chromatography (HPLC) (Agilent 1100 series; Hewlett-Packard) equipped with an Aminex HPX-87H column (Bio-Rad) (20). The mobile phase (10 mM H₂SO₄) was pumped at 0.4 ml min^{−1} (55°C). The lactate oxidation product was detected using a refractive index detector.

Spectral characteristics of LldABC. Purified samples of LldABC were diluted with 20 mM Tris-HCl (pH 8.0) to a protein concentration of 1.98 mg ml^{−1}. UV-visible spectra were recorded on an Ultrospec 2100 pro UV/visible spectrophotometer (GE Healthcare Life Sciences) with a spectral resolution of 0.5 nm.

Elemental analysis of LldABC. The iron contents of LldABC were quantitated by inductively coupled plasma atomic emission spectroscopy (ICP-AES). The proteins were solubilized overnight in concentrated HNO₃ (65%) and then diluted with ultrapure water (Millipore) to 1 to 5% HNO₃ and further diluted for measurements using Iris Advantage 1000 ICP-AES (Thermo Electron). FeSO₄ solution (0.01 to 100 ppm) was used as a standard.

Knockout of *lldA*, *lldB*, and *lldC*. The primers used in the gene knockout procedure are listed in Table 2. Vector isolation, restriction enzyme digestion, agarose gel electrophoresis, and other DNA manipulations

were carried out by standard protocols. Knockout mutants of *P. stutzeri* A1501 lacking *lldA* were generated via allele exchange using the suicide plasmid pK18mobsacB (41). The mutant allele of *lldA* was generated as follows. The up- and downstream homologous arms (up- and down-fragments) of the target *lldA* gene were PCR amplified by using the primers *lldA*-uF/*lldA*-uR and *lldA*-dF/*lldA*-dR. The up- and down-fragments were fused together to get Δ *lldA* via recombinant PCR by using the primers *lldA*-uF and *lldA*-dR, which contained EcoRI and HindIII restriction enzyme sites, respectively. The PCR product was cloned into EcoRI- and HindIII-digested pK18mobsacB, a mobilizable plasmid that does not replicate in *Pseudomonas*, to form pK18mobsacB- Δ *lldA*. To transfer the plasmid pK18mobsacB- Δ *lldA* into *P. stutzeri* A1501, a triparental filter mating was performed as previously described using *E. coli* DH10B (pK18mobsacB- Δ *lldA*) as the donor strain, *E. coli* HB101 (pRK2013) as the helper strain, and *P. stutzeri* A1501 as the recipient strain (29). Integration of the plasmid pK18mobsacB- Δ *lldA* into the chromosome of *P. stutzeri* A1501 by the first crossover was selected on an LB plate supplemented with 50 μ g ml^{−1} kanamycin. The second crossover cells were selected by culture on LB plates containing 10% (wt/vol) sucrose. All of the constructed strains were validated by PCR and DNA sequencing. The *lldB* and *lldC* mutants of *P. stutzeri* A1501 were generated by the same procedure.

Complementation of genes in *P. stutzeri* A1501. The primers used in complementation of genes in *P. stutzeri* A1501 are listed in Table 2. For the complementation of *lldABC* and *lldD*, *lldABC* and *lldD* fragments were amplified by using *P. stutzeri* A1501 and *P. stutzeri* SDM genome as the templates and using *lldABC*-cF/*lldABC*-cR and *lldD*-cF/*lldD*-cR as the

TABLE 2 Oligonucleotides used in this study

Primer	Sequence (5'→3') ^a	Use
Overexpression		
P1	GCGGAATTCGATGAGCGAACTCTTCTACAATGC (EcoRI)	Amplification of <i>lldABC</i> (forward)
P2	ATAAAGCTTTCATGCGTCCTCCAGGATCAG (HindIII)	Amplification of <i>lldABC</i> (reverse)
Gene knockout		
<i>lldA</i> -uF	AATGAATTCGATGAGCGAACTCTTCTACAAT (EcoRI)	Amplification of upstream homologous arm of <i>lldA</i> (forward)
<i>lldA</i> -uR	TTCGCTCTCGTGACCATACGTTGCGCGCCTCGTC	Amplification of upstream homologous arm of <i>lldA</i> (reverse)
<i>lldA</i> -dF	GACGAGGCGCGCAACGTATGGTCACGAGAGCGAA	Amplification of downstream homologous arm of <i>lldA</i> (forward)
<i>lldA</i> -dR	AATAAGCTTTCATCGGGCACCTCCGGTGCGT (HindIII)	Amplification of downstream homologous arm of <i>lldA</i> (reverse)
<i>lldB</i> -uF	GCGGAATTCGATGAACGCCGAACAGCGCAT (EcoRI)	Amplification of upstream homologous arm of <i>lldB</i> (forward)
<i>lldB</i> -uR	AGTGATGATCTTGCCGTCTGATTCCAGGCTCT	Amplification of upstream homologous arm of <i>lldB</i> (reverse)
<i>lldB</i> -dF	AGAGCCTGGAATCAGACGGCAAGATCATCACT	Amplification of downstream homologous arm of <i>lldB</i> (forward)
<i>lldB</i> -dR	ATAAAGCTTTCATCGCTTGCCCTCCAGGTG (HindIII)	Amplification of downstream homologous arm of <i>lldB</i> (reverse)
<i>lldC</i> -uF	GCGGAATTCGATGAGCGCCAAGGCCAATA (EcoRI)	Amplification of upstream homologous arm of <i>lldC</i> (forward)
<i>lldC</i> -uR	CGCTGGCCTTGAGGAATTCGGTGTGCACC	Amplification of upstream homologous arm of <i>lldC</i> (reverse)
<i>lldC</i> -dF	GGTGACACCCGAAATTCCTCAAGGCCAGCG	Amplification of downstream homologous arm of <i>lldC</i> (forward)
<i>lldC</i> -dR	ATAAAGCTTTCATGCGTCCTCCAGGATCAG (HindIII)	Amplification of downstream homologous arm of <i>lldC</i> (reverse)
Complementation		
<i>lldABC</i> -cF	GAAGCTTATGAGCGAACTCTTCTACAATGC (HindIII)	Amplification of fragment <i>lldABC</i> (forward)
<i>lldABC</i> -cR	GCTGCAGTCATGCGTCCTCCAGGATCAG (PstI)	Amplification of fragment <i>lldABC</i> (reverse)
<i>lldD</i> -cF	GAAGCTTATGATCATTTCCGCCTCT (HindIII)	Amplification of fragment <i>lldD</i> (forward)
<i>lldD</i> -cR	GCTGCAGTCAGACGTCAGCAGAC (PstI)	Amplification of fragment <i>lldD</i> (reverse)

^a Restriction sites are underlined, and the restriction enzymes are indicated in parentheses.

primers, respectively. The result PCR products were cloned into HindIII/PstI-cut pBBRMS-5, a broad-host-range plasmid. The resulting plasmids were, respectively, conjugated into *P. stutzeri* A1501 via a triparental mating method as previously described (29).

Comparative growth of *P. stutzeri* A1501 and its mutants. *P. stutzeri* A1501 and its mutant strains were cultivated in a Bioscreen C system (FP-1100-C; LabSystems Oy, Helsinki, Finland), in MSM with a single carbon source of 5.0 g liter⁻¹ L-lactate or pyruvate). The working volume in the Bioscreen plate was 200 μ l well⁻¹. The temperature was controlled at 30°C. Absorbance values at 620 nm were automatically read at regular intervals of 2 h. All experiments were performed in quadruplicate.

RESULTS

Overexpression and purification of LldABC. *lldABC* was cloned by PCR into the overexpression vector pETDuet-1 and expressed in *E. coli* BL21(DE3)(pLysS). Compared with the strain containing pETDuet-1 (4 U g⁻¹), distinct L-lDH activity (74 U g⁻¹) was detected in *E. coli* BL21(DE3)(pLysS)(pETLDH). Purification of LldABC was achieved by ammonium sulfate precipitation followed by ion-exchange chromatography using DEAE-Sepharose fast-flow and affinity chromatography using HisTrap HP (see Table S1 in the supplemental material).

After the purification process, three polypeptides of 25, 32, and 54 kDa were detected when aliquots of active fractions were resolved by SDS-PAGE (Fig. 2). PMF of tryptic peptides excised from the stained SDS-PAGE gels showed they corresponded to LldA (32 kDa), LldB (54 kDa), and LldC (25 kDa), respectively (see Table S2 in the supplemental material).

Although the three genes *lldABC* were expressed on one expression vector and put under one T7 *lac* promoter, only LldA had an N-terminus 6-histidine tag, which could be used to accelerate the protein purification process. However, the purification of the 6-His-tagged protein from the HisTrap HP column resulted in coelution of three polypeptides (LldA, LldB, and LldC). Thus, the

LldA, LldB, and LldC in *P. stutzeri* A1501 might comprise the protein complex LldABC, which exhibited L-lDH activity.

The specific activity of the purified LldABC was 147 U g⁻¹ protein, which was a nearly 2-fold increase compared to that in the crude extract. The purified enzyme was routinely stored at 4°C in buffer A. Under these conditions, the enzyme was stable for weeks with no apparent loss of activity and was used throughout the studies described below.

Molecular mass of LldABC. Gel filtration of the pure LldABC on a Superdex 200 10/300 GL column showed a molecular mass of

FIG 2 SDS-PAGE analysis of purified LldABC. LldABC was expressed and purified as described in Materials and Methods. Lane M, molecular mass standards; lane 1, active fraction after ammonium sulfate precipitation; lane 2, active fraction after purification on the DEAE-Sepharose fast-flow column; lane 3, the unbound protein of the HisTrap HP column; lane 4, purified LldABC; lane 5, supernatant of the crude extract of *E. coli* BL21(DE3)(pLysS) (pETLDH).

FIG 3 HPLC chromatograms illustrating the product-forming behavior (A) and UV-visible absorption spectra of LldABC in *P. stutzeri* A1501 (B). (A) Red line, authentic pyruvate; black line, the reaction mixture at 0 h; blue line, the reaction mixture after 4 h. nRIU, nano-refractive index units. (B) UV-visible absorption spectra of LldABC in *P. stutzeri* A1501 as purified (red line) and sodium dithionite reduced (green line). The protein concentration was 1.98 mg ml⁻¹, and the sample was reduced with a 10-fold excess of sodium dithionite. The arrows mark absorption bands with maxima at ~340 nm and 420 nm, which are typically seen in Fe-S proteins.

220,200 Da. Analysis by SDS-PAGE showed three protein bands of 32, 54, and 25 kDa, consistent with the predicted molecular masses of LldA, LldB, and LldC, respectively. We conclude that the native LldABC is a dimer of three subunits according to the molecular mass data (111 kDa).

LldABC proteins catalyze lactate oxidation. LldABC could catalyze the L-lactate oxidation with MTT as the electron acceptor. The oxidation product of lactate was identified by HPLC in this work. As shown in Fig. 3A, a compound identical to authentic pyruvate was produced. Similar to the reported FMN-dependent L-lDH in *E. coli* (14), LldABC also catalyzes the conversion of L-lactate into pyruvate.

Spectroscopic characterization of LldABC. L-lDHs, such as lactate oxidase and flavocytochrome *b*₂, were flavoproteins and used FMN as the cofactor. To investigate whether LldABC was

also a flavoprotein, spectroscopic characterization of LldABC was studied. As shown in Fig. 3B, no absorption spectra of FMN were detected. The UV-visible absorption spectra of the as-purified and dithionite-reduced LldABC are consistent with the iron-sulfur clusters (42, 43). The presence of the iron-sulfur cluster in LldABC was further identified by elemental analyses. The iron analysis on the purified protein revealed an iron content of 4.8 mol of iron per mol of protein.

Characterization of LldABC. After preincubation at different temperatures or pHs for 30 min, the effects of temperature and pH on LldABC stability were first studied. The purified LldABC could keep stable only at neutral pH and a temperature lower than 30°C (Fig. 4A and B). The optimum temperature and pH conditions for LldABC were also investigated. The effect of temperature on LldABC activity was determined within a range of 25 to 80°C. The optimum temperature was found to be 70°C (Fig. 4C). The dependence of L-lDH activity on pH was determined in buffers containing citric acid, KH₂PO₄, boric acid, and barbital (CKBB buffer). The buffer was adjusted to the appropriate pH with 200 mM NaOH. The optimum pH for L-lDH activity was observed at 11.0 (Fig. 4D).

Substrate specificities of LldABC were examined with L-lactate, DL-2-hydroxybutyrate, glycolate, DL-2-hydrocaproate, DL-2-hydroxycaprylate, DL-2-hydroxyvalerate, D-mandelate, L-mandelate, and L-phenyllactate as the test substrates and MTT as the secondary substrate (Fig. 5A). The LldABC seemed to have narrow substrate specificity. Only L-lactate and DL-2-hydroxybutyrate were rapidly oxidized by the component. The effects of various metal ions (5 mM) on L-lDH activity were also tested (Fig. 5B). Ca²⁺, Mn²⁺, K⁺, Cd²⁺, Mg²⁺, Zn²⁺, Ni²⁺, and Co²⁺ could activate L-lDH. Mg²⁺ exhibited the highest activation capability (6.6-fold) for L-lDH activity. Cu²⁺ and Fe³⁺ completely inhibited L-lDH activity. In the subsequent experiments, 5 mM Mg²⁺ was added to the L-lDH assay system.

Steady-state kinetics of LldABC. The dehydrogenation catalyzed by LldABC followed a Michaelis-Menten kinetic pattern. The apparent *K_m* values were determined (see Table S3 in the supplemental material). The apparent *K_m* of the purified LldABC for L-lactate was 55.45 ± 0.27 μM. L-lDH could use MTT as the electron acceptor. The apparent *K_m* of the purified LldABC for MTT was 4.76 ± 0.48 mM.

A set of parallel straight lines were obtained in the double-reciprocal plot of L-lactate versus activity at different fixed concentrations of MTT (Fig. 6). This kinetic behavior is typical of a double-displacement kinetic (ping-pong) mechanism. The double-displacement kinetic mechanism for LldABC is in agreement with those of previous reports describing the same mechanism for other FMN-dependent L-lDHs, including L-lactate oxidase (16).

LldABC is required for use of L-lactate. LldABC in *P. stutzeri* A1501 exhibited L-lDH activity, and thus, it might play a key role in the utilization of L-lactate *in vivo*. To test our hypothesis, we constructed mutants of *P. stutzeri* A1501 lacking *lldA*, *lldB*, and *lldC*, respectively. The growth of the mutant in minimal medium with 0.5% L-lactate as the sole carbon source was investigated. As shown in Fig. 7A, these mutants exhibited little growth with L-lactate compared to the wild type. As a control, both the wild type and the mutants grew equally well in minimal medium with 0.5% pyruvate as the sole carbon source (Fig. 7B).

Plasmid constructs expressing *lldABC* in *P. stutzeri* A1501 and *lldD* in *P. stutzeri* SDM were also introduced into *P. stutzeri* defi-

FIG 4 Basic enzymology properties of LldABC in *P. stutzeri* A1501. The reaction mixture contained 20 mM L-lactate, 0.2 mM MTT, 50 mM Tris-HCl (pH 7.5), and 0.1 μ g purified LldABC. (A) Thermostability of the LldABC in *P. stutzeri* A1501. After the purified enzyme was preincubated at different temperatures (25, 30, 37, 40, 45, and 50°C) for 30 min, enzyme reactions were carried out in 50 mM Tris-HCl (pH 7.5). (B) Stability of LldABC at different pHs. After the purified enzyme was preincubated in CKBB buffer of various pHs (3.0 to 12.0) for 30 min, enzyme reactions were carried out. (C) Effect of temperature on L-lactate dehydrogenase activity. Enzyme reactions were carried out at pH 7.5 at different temperatures (25 to 80°C). (D) Effect of pH on L-lactate dehydrogenase activity. The optimum pH was assessed in CKBB buffer of various pHs (3.0 to 13.0).

cient in *lldB* (Δ *lldB*). Expression of *lldABC* completely restored the ability of the *P. stutzeri* Δ *lldB* mutant to grow on L-lactate (Fig. 7A). Similarly, expression of *lldD* encoding the FMN-dependent L-lactate dehydrogenase (L-lactate dehydrogenase) also successfully restored the robust growth of the *P. stutzeri* Δ *lldB* mutant in L-lactate (Fig. 7A).

DISCUSSION

L-lactate is an important end product of fermentation of glucose and other carbohydrates. NAD-dependent L-lactate dehydrogenases (L-lactate dehydrogenases), using NADH as the cofactor, could convert pyruvate to L-lactate (2, 3). Although the *in vitro* oxidation of L-lactate to pyruvate at high pH and a high substrate concentration was reported for L-lactate dehydrogenases, they are generally considered enzymes that catalyze only the reduction of pyruvate to L-lactate *in vivo*. L-lactate dehydrogenases, which catalyze the oxidation of lactate to pyruvate *in vitro*, have attracted researchers' attention for their essential roles in L-lactate utilization (2, 4).

Based on the comparative genomics analysis of different *Pseudomonas* species, a gene cluster, *lldABC*, which might encode the other kind of L-lactate dehydrogenase, was discovered in the genome of *P. stutzeri*

A1501. After expression of the gene cluster in *E. coli*, the novel L-lactate dehydrogenase was purified and further studied in this work. Based on the MALDI-TOF MS analysis of the purified gene cluster-encoded products, the LldA, LldB, and LldC encoded by *lldA*, *lldB*, and *lldC*, respectively, comprise a protein complex, LldABC, with L-lactate dehydrogenase activity. The native LldABC was a dimer of three subunits. The ratio between LldA, LldB, and LldC was 1:1:1. To our knowledge, this would be the first characterized L-lactate dehydrogenase comprised of different subunits.

Normally, L-lactate dehydrogenases catalyze the L-lactate oxidation into pyruvate. However, L-lactate monooxygenase, an FMN-dependent L-lactate dehydrogenase, catalyzes the oxidation of L-lactate with molecular oxygen, producing acetate, H₂O, and CO₂ as end products (15). In this work, the product of the LldABC-catalyzed reaction was determined to be pyruvate by HPLC. Thus, in the utilization of L-lactate in *P. stutzeri* A1501, L-lactate might be first converted to pyruvate by LldABC.

To test the putative role of the LldABC in *P. stutzeri* A1501 L-lactate utilization, the growth abilities of *P. stutzeri* A1501 mu-

FIG 5 Substrate specificity and effects of metal ions on LldABC in *P. stutzeri* A1501. The reaction mixture contained 20 mM test substrates, 0.2 mM MTT as the electron acceptor, and 0.1 μ g of purified LldABC in 50 mM Tris-HCl (pH 7.5). (A) Substrate specificity of LldABC in *P. stutzeri* A1501. Bars: 1, L-lactate; 2, glycolate; 3, DL-2-hydroxycaproate; 4, DL-2-hydroxybutyrate; 5, L-phenyl-lactate; 6, DL-2-hydroxycaprylate; 7, DL-2-hydroxyvalerate; 8, L-mandelate; 9, D-mandelate. (B) Effects of different metal ions on LldABC activity. The concentration of metal ions was 5 mM in 50 mM Tris-HCl (pH 7.5).

tants lacking the *lldA*, *lldB*, and *lldC* genes, respectively, were studied. None of the mutants grew in minimal medium with L-lactate as the sole carbon source, indicating that all three genes are indispensable for L-lactate utilization. On the other hand, both the wild-type strain and *lldABC* mutants grew well in minimal medium with pyruvate as the sole carbon source. Both FMN-dependent L-lactate dehydrogenase (LldABC) and LldABC catalyze pyruvate production *in vitro* and can restore the growth of the *P. stutzeri* Δ *lldB* mutant in L-lactate. Thus, we postulated that LldABC, like FMN-dependent L-lactate dehydrogenase (LldABC), oxidatively converts L-lactate to pyruvate *in vivo*.

Proteins similar to LldABC were found to exist in other organisms and to be required for L-lactate utilization in *B. subtilis*, *C. jejuni*, and *S. oneidensis* (25–28). The results in this work indicated the homolog of LldABC might also mediate L-lactate utilization in *P. stutzeri* A1501. On the other hand, the homologs of LldABC and FMN-dependent L-lactate dehydrogenase (LldABC) might be copresent in bacteria. *E. coli* relies on FMN-dependent L-lactate dehydrogenase (LldABC) for L-lactate utilization, even though it also has a homolog of the LldABC (26). In previous studies, the homolog of LldABC was regarded as being responsible for the catabolism of a metabolite other than (but perhaps related to) lactate in *E. coli*. Further studies, especially of the substrate

FIG 6 Kinetic mechanism of LldABC in *P. stutzeri* A1501. Purified LldABC (0.1 μ g) was incubated in a reaction mixture that contained 5 mM Mg²⁺ and 50 mM Tris-HCl (pH 7.5) at 30°C. The reaction was started with the indicated MTT concentrations (0.2, 0.35, 0.5, and 0.75 mM) at variable L-lactate concentrations (0.1 to 1.0 mM).

specificities of LldABC in *E. coli*, were needed to identify its biochemical roles (26).

Unlike flavin-dependent L-lactate dehydrogenase (LldABC), none of the LldA, LldB, and LldC proteins contained the structural domain involved in flavin binding. The UV-visible absorption spectra of L-lactate dehydrogenase in *P. stutzeri* A1501 also excluded the presence of the flavin cofactor. However, the presence of iron-sulfur clusters in L-lactate dehydrogenase of *P. stutzeri* A1501 was identified by UV-visible absorption spectra and elemental analyses. Those observations suggested that L-lactate dehydrogenase in *P. stutzeri* A1501 might oxidize lactate by using the iron-sulfur cluster as the cofactor.

The FMN-dependent L-lactate dehydrogenases used different natural electron acceptors for lactate oxidation. For example, the lactate oxidase catalyzes the oxidation of L-lactate using molecular oxygen, producing pyruvate and hydrogen peroxide as end products (12). Other L-lactate dehydrogenases, such as L-lactate dehydrogenase in *E. coli* and flavocytochrome *b*₂ in *S. cerevisiae*, could not use molecular oxygen as the electron acceptor (9, 24). In these species, the external electron acceptor is a membrane quinone (*E. coli*) or cytochrome *c* (*S. cerevisiae*). As for LldABC, it could not catalyze the oxidation of L-lactate with molecular oxygen. The iron-sulfur clusters containing dehydrogenases often interacted with respiratory chain to transfer electrons to oxygen (44, 45). Although the natural electron acceptor of LldABC in *P. stutzeri* A1501 has not been examined, by analogy with iron-sulfur cluster-dependent respiratory dehydrogenases, such as succinate dehydrogenase (44) and glycolate oxidase in *E. coli* (45), the aerobic utilization of L-lactate by LldABC might also be through the respiratory electron transport chain in *P. stutzeri* A1501.

The L-lactate dehydrogenase-catalyzed reaction involves oxidation of a bond between carbon and oxygen. This reaction can be described as hydride transfer, in that two electrons and a proton are transferred. The mechanisms of FMN-dependent L-lactate dehydrogenases have been studied extensively. Unlike the similar reactions catalyzed by NAD-dependent enzymes, the mechanisms of FMN-dependent L-lactate dehydrogenase-catalyzed oxidations have been controversial. Based on studies of the mechanism of FMN-dependent L-lactate dehydrogenases,

FIG 7 Growth of *P. stutzeri* A1501 and its mutants in MSM with a single carbon source of L-lactate (A) or pyruvate (B). The cell growth was monitored by measuring the optical density at 620 nm (OD₆₂₀). The data points and error bars represent the means and standard deviations from quadruplicate cultures, respectively. Black, *P. stutzeri* A1501; red, *P. stutzeri* $\Delta lldA$ mutant; green, *P. stutzeri* $\Delta lldB$ mutant; dark blue, *P. stutzeri* $\Delta lldC$ mutant; light blue, *P. stutzeri* $\Delta lldB/pBBR::lldABC$ mutant; pink, *P. stutzeri* $\Delta lldB/pBBR::lldD$ mutant.

and hydride transfer mechanisms have been proposed (46). As for LldABC in this work, only steady-state kinetics was studied. Just like most of the FMN-dependent L-lDHs, the kinetic pathway for LldABC is also a ping-pong mechanism. Because of the lack of structural information of LldABC, mechanistic studies are difficult to interpret now. However, this study has provided a research objective in terms of increasing our understanding of the L-lactate oxidation mechanism. Continuing the study of LldABC might provide new insights into the diversity of microbial lactate utilization mechanisms and then assist in the production of valuable chemicals and understanding of microbial infections.

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