Supporting information for

A bacterial multi-domain NAD-independent D-lactate dehydrogenase utilizes FAD and Fe-S clusters as cofactors and quinone as electron acceptor for D-lactate oxidization

Tianyi Jiang[a,c], Xiaoting Guo[a], Jinxin Yan[a], Yingxin Zhang[a], Yujiao Wang[a], Manman Zhang[a], Binbin Sheng[a], Cuiqing Ma[a], Ping Xu[b,a], and Chao Gao[a*]

[a]State Key Laboratory of Microbial Technology, Shandong University, Jinan 250100, People's Republic of China

[b]State Key Laboratory of Microbial Metabolism and School of Life Sciences and Biotechnology, Shanghai Jiao Tong University, Shanghai 200240, People's Republic of China

[c]School of Municipal and Environmental Engineering, Shandong Jianzhu University, Jinan 250101, People's Republic of China

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[*]Address correspondence to Chao Gao, State Key Laboratory of Microbial Technology, Shandong University, Jinan 250100, People's Republic of China. Ph: (+86) 531 883 64003; Fax: (+86) 531 883 69463; E-mail: jieerbu@sdu.edu.cn

Table S1. Purification procedure of heterologous expressed Fe-S D-iLDH.

	Specific activity (U/mg)	Fold purification	Yield (%)
Crude cell extract	1.25	1	100
His-trap	34.39	27.61	75.92
DEAE Sepharose FF	42.48	34.11	68.21
Superdex 200	44.51	35.74	44.67

The activities were determined in 1 mL of 50 mM Tris-HCl (pH 7.4) with 1.0 mM D-lactate as substrate and 0.2 mM MTT as electron acceptor.

Table S2. Strains and plasmids used in this work.

Strain or plasmid	Relevant characteristics[a]	Source or reference
Strains		
P. putida KT2440	Wild-type, capable of DL-lactate utilizing	ATCC[b]
P. putida KT2440 (Δ*lldE*Δ*glcD*)	*P. putida* KT2440 mutant obtained by deletion of the *lldE* gene and *glcD* gene	(1)
P. putida KT2440 (Δ*lldE*Δ*glcD::lldE*)	*P. putida* KT2440 (Δ*lldE*Δ*glcD*) harboring the plasmid pBBR-*lldE*	(1)
P. putida KT2440 (Δ*lldE*Δ*glcD::lldEFAD2*)	*P. putida* KT2440 (Δ*lldE*Δ*glcD*) harboring the plasmid pBBR-*lldEFAD2*	This study
E. coli DH5α	F^- φ80*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *recA1 endA1 hsdR17*(r_K^-, m_K^+) *phoA supE44 thi-1 gyrA96 relA1* λ^-, used for gene clone	Invitrogen
E. coli DH5α (*pEASY*-Blunt-*lldEFeS3*)	*E. coli* DH5α harboring the plasmid *pEASY*-Blunt-*lldEFeS3*	This study
E. coli C43 (DE3)	Mutant of *E. coli* BL21 (DE3), used for protein expression	(2)
E. coli C43 (DE3) (pETDuet-*lldE*)	*E. coli* C43 (DE3) harboring the plasmid pETDuet-*lldE*	This study
E. coli C43 (DE3) (pETDuet-*lldEFAD1*)	*E. coli* C43 (DE3) harboring the plasmid pETDuet-*lldEFAD1*	This study
E. coli C43 (DE3) (pETDuet-*lldEFAD2*)	*E. coli* C43 (DE3) harboring the plasmid pETDuet-*lldEFAD2*	This study
E. coli C43 (DE3) (pETDuet-*lldEFAD3*)	*E. coli* C43 (DE3) harboring the plasmid pETDuet-*lldEFAD3*	This study
E. coli C43 (DE3) (pETDuet-*lldEFeS1*)	*E. coli* C43 (DE3) harboring the plasmid pETDuet-*lldEFeS1*	This study
E. coli C43 (DE3) (pETDuet-*lldEFeS2*)	*E. coli* C43 (DE3) harboring the plasmid pETDuet-*lldEFeS2*	This study
E. coli C43 (DE3) (pETDuet-*lldEFeS3*)	*E. coli* C43 (DE3) harboring the plasmid pETDuet-*lldEFeS3*	This study
E. coli C43 (DE3) (pETDuet-*lldEFeS3M1*)	*E. coli* C43 (DE3) harboring the plasmid pETDuet-*lldEFeS3M1*	This study
E. coli C43 (DE3) (pETDuet-*lldEFeS3M2*)	*E. coli* C43 (DE3) harboring the plasmid pETDuet-*lldEFeS3M2*	This study
E. coli C43 (DE3) (pETDuet-*lldEFeS3M4*)	*E. coli* C43 (DE3) harboring the plasmid pETDuet-*lldEFeS3M4*	This study
E. coli C43 (DE3) (pETDuet-*lldEFeS3M6*)	*E. coli* C43 (DE3) harboring the plasmid pETDuet-*lldEFeS3M6*	This study
E. coli C43 (DE3) (pETDuet-*lldEFeS3M7*)	*E. coli* C43 (DE3) harboring the plasmid pETDuet-*lldEFeS3M7*	This study
Plasmids		
pBBR1MCS-5	Plasmid for gene complementation, Gm^R	Biovector Science

		Lab,Inc
pBBR-*lldE*	The *lldE* gene that encoding Fe-S D-iLDH was inserted into pBBR1MCS-5.	This study
pBBR-*lldEFAD2*	The *lldEFAD2* gene that encoding Fe-S D-iLDH 1-530 was inserted into pBBR1MCS-5.	This study
pEASY-Blunt	Ap^R, Km^R, cloning vector	TransGen
pEASY-Blunt-*lldEFeS3*	The *lldEFeS3* gene that encoding Fe-S D-iLDH 531-936 was inserted into *pEASY*-Blunt.	This study
pETDuet-1	Vector for protein expression, Ap^R	Novagen
pETDuet-*lldE*	The *lldE* gene that encoding Fe-S D-iLDH was inserted into pETDuet-1.	This study
pETDuet-*lldEFAD1*	The *lldEFAD1* gene that encoding Fe-S D-iLDH 1-519 was inserted into pETDuet-1.	This study
pETDuet-*lldEFAD2*	The *lldEFAD2* gene that encoding Fe-S D-iLDH 1-530 was inserted into pETDuet-1.	This study
pETDuet-*lldEFAD3*	The *lldEFAD3* gene that encoding Fe-S D-iLDH 1-538 was inserted into pETDuet-1.	This study
pETDuet-*lldEFeS1*	The *lldEFeS1* gene that encoding Fe-S D-iLDH 511-936 was inserted into pETDuet-1.	This study
pETDuet-*lldEFeS2*	The *lldEFeS2* gene that encoding Fe-S D-iLDH 520-936 was inserted into pETDuet-1.	This study
pETDuet-*lldEFeS3*	The *lldEFeS3* gene that encoding Fe-S D-iLDH 531-936 was inserted into pETDuet-1.	This study
pETDuet-*lldEFeS3M1*	The *lldEFeS3M1* gene that encoding Fe-S D-iLDH 531-936 (C540S) was inserted into pETDuet-1.	This study
pETDuet-*lldEFeS3M2*	The *lldEFeS3M2* gene that encoding Fe-S D-iLDH 531-936 (C540S/C594S) was inserted into pETDuet-1.	This study
pETDuet-*lldEFeS3M4*	The *lldEFeS3M4* gene that encoding Fe-S D-iLDH 531-936 (C540S/C594S/C751S/C752S) was inserted into pETDuet-1.	This study
pETDuet-*lldEFeS3M6*	The *lldEFeS3M6* gene that encoding Fe-S D-iLDH 531-936 (C540S/C594S/C751S/C752S/C540S/C594S) was inserted into pETDuet-1.	This study
pETDuet-*lldEFeS3M7*	The *lldEFeS3M7* gene that encoding Fe-S D-iLDH 531-936 (C540S/C543S/C594S/C751S/C752S/C540S/C594S) was inserted into pETDuet-1.	This study

[a] Km^R, kanamycin resistant; Ap^R, ampicillin resistant; Gm^R, gentamicin resistant.

[b] ATCC, American Type Culture Collection.

Tanle S3. Oligonucleotides used in this study.

Primer	Sequence(5'-3')[a]	Use
Complementation		
lldE-pBBR-F	TGGATCCATGAGCCTGCCCGCCGCGTT (BamHI)	Amplification of fragment *lldE* for constructing pBBR-*lldEFAD2 (*forward)
lldEFAD2-pBBR-R	TCTCGAGTTACAGCGGCTTGAGGTTTT (XhoI)	Amplification of fragment *lldE* for constructing pBBR-*lldEFAD2* (reverse)
Overexpression		
lldE-F	TGAATTCATGAGCCTGCCCGCCGCGTT (HindIII)	Amplification of *lldE*, *lldEFAD1*, *lldEFAD2 or lldEFAD3* for constructing pETDuet-*lldE*, pETDuet-*lldEFAD1*,pETDuet-*lldEFAD2* or pETDuet-*lldEFAD*3 (forward)
lldE-R	ACTCGAGTTAGAGGCTGCGTGGCCGGG (XhoI)	Amplification of *lldE*, *lldEFeS1*, *lldEFeS2 or lldEFeS3* for constructing pETDuet-*lldE*, pETDuet-*lldEFeS1*, pETDuet-*lldEFeS2* or pETDuet-*lldEFeS*3 (reverse)
lldEFAD1-R	CGCTCGAGTTAGTCTTCGCTCAACACCACGT (XhoI)	Amplification of *lldEFAD1* for constructing pETDuet-*lldEFAD1* (reverse)
lldEFAD2-R	CGCTCGAG*TTA*CAGCGGCTTGAGGTTTTTCA (XhoI)	Amplification of *lldEFAD2* for constructing pETDuet-*lldEFAD2* (reverse)
lldEFAD3-R	TTCTCGAG*TTA*GTCGACGATTTTGTCGGCGG (XhoI)	Amplification of *lldEFAD3* for constructing pETDuet-*lldEFAD3* (reverse)
lldEFeS1-F	AGAATTCG*ATG*AACCCCGACGTGGTGTTGAGC (EcoRI)	Amplification of *lldEFeS1* for constructing pETDuet-*lldEFeS1* (forward)
lldEFeS2-F	AGAATTCG*ATG*CCGGATATCCACCTGAAAAAC (EcoRI)	Amplification of *lldEFeS2* for constructing pETDuet-*lldEFeS2* (forward)
lldEFeS3-F	AGAATTCG*ATG*AACCCCGACGTGGTGTTGAGC (EcoRI)	Amplification of *lldEFeS3* for constructing pETDuet-*lldEFeS3* (forward)
Site-directed mutagenesis		
C540S-F	CAAGTCCATCGAGTGCGGCT	Amplification of *pEASY*-Blunt-*lldEFeS3* for introducing C540S variation in

		Fe-S oxidoreductase domain (forward)
C540S-R	TCGACGATTTTGTCGGCGGC	Amplification of *pEASY*-Blunt-*lldEFeS3* for introducing C540S variation in Fe-S oxidoreductase domain (reverse)
C540SC543S-F	GACAAGTCCATCGAGTCCGGC	Amplification of *pEASY*-Blunt-*lldEFeS3* for introducing C540S and C543S variation in Fe-S oxidoreductase domain (forward)
C540SC543S-R	GACGATTTTGTCGGCGGCAG	Amplification of *pEASY*-Blunt-*lldEFeS3* for introducing C540S and C543S variation in Fe-S oxidoreductase domain (reverse)
C594S-F	ACACCTCCGCCGCTACCGGCCT	Amplification of *pEASY*-Blunt-*lldEFeS3* for introducing C594S variation in Fe-S oxidoreductase domain (forward)
C594S-R	CGATGCCCTGGTACTGGTAGCTTTGC	Amplification of *pEASY*-Blunt-*lldEFeS3* for introducing C594S variation in Fe-S oxidoreductase domain (reverse)
C751SC752S-F	CGACAGCCTGTCCTCCGGCCA	Amplification of *pEASY*-Blunt-*lldEFeS3* for introducing C751S and C752S variation in Fe-S oxidoreductase domain (forward)
C751SC752S-R	GCGTTGTCGGGGAACACCACC	Amplification of *pEASY*-Blunt-*lldEFeS3* for introducing C751S and C752S variation in Fe-S oxidoreductase domain (reverse)
C869SC870S-F	GTATTCATTCCTCCGGGTTT	Amplification of *pEASY*-Blunt-*lldEFeS3* for introducing C869S and C870S variation in Fe-S oxidoreductase domain (forward)
C869SC870S-R	CTTCCGGGATCACCACCTGT	Amplification of *pEASY*-Blunt-*lldEFeS3* for introducing C869S and C870S variation in Fe-S oxidoreductase domain (reverse)

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

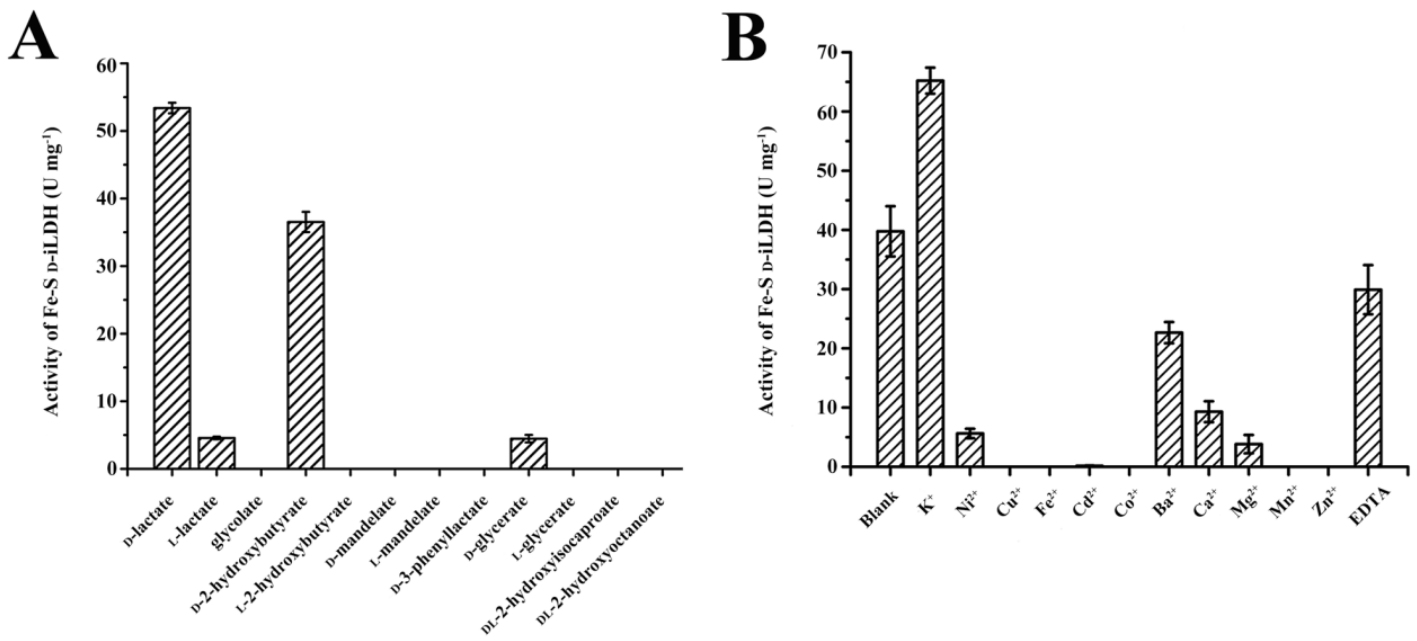


Figure S1. Characterization of Fe-S D-iLDH from *P. putida* KT2440. (A) Substrate specifity of the purified enzyme. (B) The effect of metal ions on activity of the purified enzyme. Values are the mean ± SD of three parallel replicates for each panel.

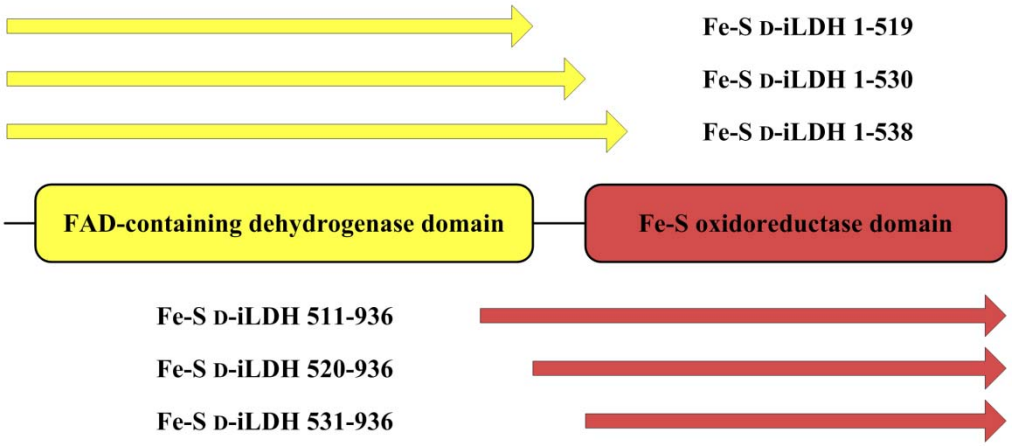


Figure S2. Scheme of expression procedure of FAD-containing dehydrogenase domain and Fe-S oxidoreductase domain. For the expression of FAD-containing dehydrogenase domain, three regions were cloned with different 3'-terminuses. The resulting genes *lldEFAD1*, *lldEFAD2* and *lldEFAD3* encode proteins Fe-S D-iLDH 1-519, Fe-S D-iLDH 1-530 and Fe-S D-iLDH 1-538, respectively. Fe-S D-iLDH 1-519 keeps the integral N-terminus and the predicted FAD-containing dehydrogenase domain without additional residues at the C-terminus. The hinge area was added in Fe-S D-iLDH 1-530, and 8 amino acids of the N-terminus sequence of Fe-S oxidoreductase domain were further added to construct Fe-S D-iLDH 1-538. Fe-S D-iLDH 1-530 and Fe-S D-iLDH 1-538 were soluble and have similar specific activities, while Fe-S D-iLDH 1-519 was expressed as inclusion body. So the following experiments were carried out using Fe-S D-iLDH 1-530 with less redundant sequence. Similarly, for the expression of Fe-S oxidoreductase domain, three regions were cloned with different 5'-terminuses. The resulting genes *lldEFeS1*, *lldEFeS2* and *lldEFeS3* encode proteins Fe-S D-iLDH 511-936, Fe-S D-iLDH 520-936 and Fe-S D-iLDH 531-936 with different N-terminus. All the three purified proteins had identical absorption spectrums,

so Fe-S D-iLDH 531-936 that has least redundant sequence was used in following experiments.

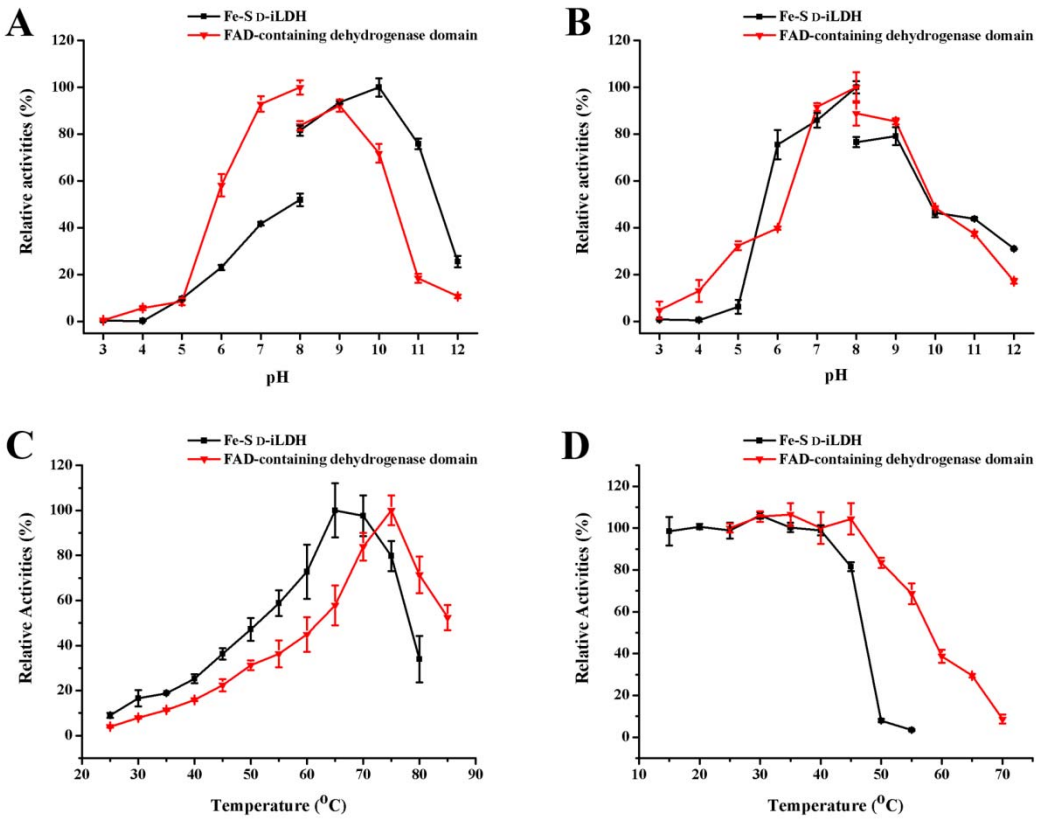


Figure S3. Enzymatic properties of Fe-S D-iLDH and FAD-containing dehydrogenase domain. (A) Effects of pH on activities of Fe-S D-iLDH and FAD-containing dehydrogenase domain. The highest activity obtained with each enzyme was defined as 100%, respectively. (B) The stabilities of Fe-S D-iLDH and FAD-containing dehydrogenase domain as a function of pH. The enzymes were incubated at different pH for 30 min and then assayed. The activity of each enzyme without pH treatment (stored in 100 mM sodium phosphate buffer, pH 7.4) was defined as 100%, respectively. (C) Effects of temperature on activities of Fe-S D-iLDH and FAD-containing dehydrogenase domain. The highest activity obtained with each enzyme was defined as 100%, respectively. (D) The stability of Fe-S D-iLDH and FAD-containing dehydrogenase domain as a function of temperature. The enzymes were incubated at different temperature for 30 min and then assayed. The enzyme activity without treatment (store at 4ºC)

was defined as 100%. The activities were determined in 1 mL of 50 mM Tris-HCl (pH 7.4) with 1.0 mM D-lactate as substrate and 0.2 mM MTT as electron acceptor. For (A) and (B), the buffers used were: 0.2 M Na_2HPO_4-0.1 M citric acid buffer for pH 3.0-8.0; 50 mM Glycine-NaOH buffer for pH 8.0-12.0. Values are the mean ± SD of three parallel replicates for each panel.

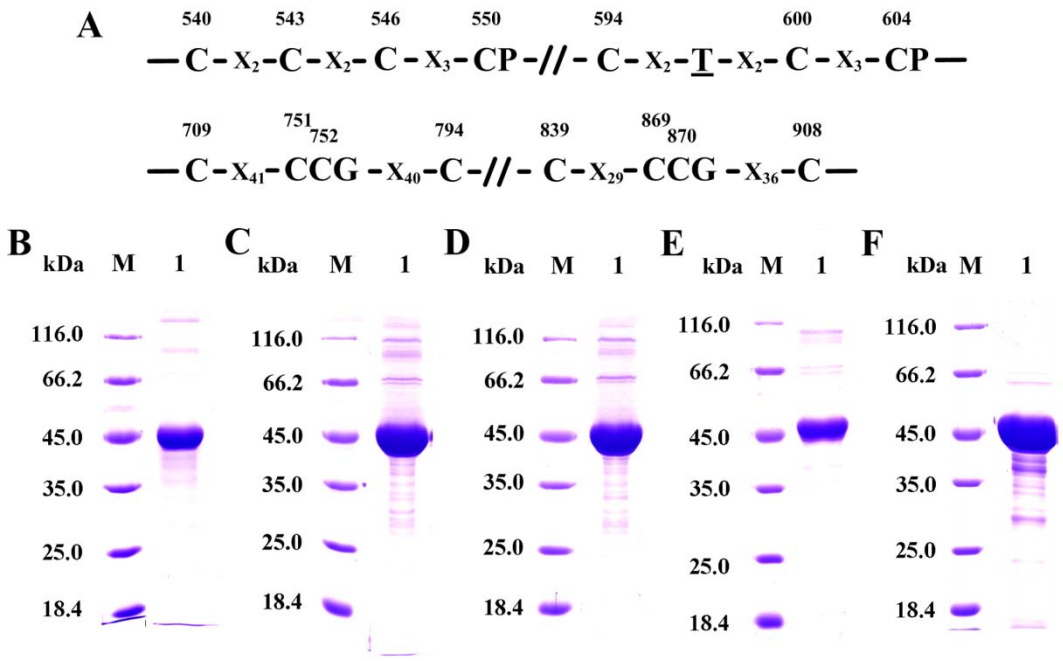


Figure S4. Construction of Fe-S oxidoreductase domain variants. (A) Scheme of the predicted cystines involved in Fe-S clusters binding in the sequence of Fe-S D-iLDH. (B) SDS–PAGE analysis of the purified C540S Fe-S oxidoreductase domain. (C) SDS–PAGE analysis of the purified C540S/C594S Fe-S oxidoreductase domain. (D) SDS–PAGE analysis of the purified C540S/C594S/C751S/C752S Fe-S oxidoreductase domain. (E) SDS–PAGE analysis of the purified C540S/C594S/C751S/C752S/C540S/C594S Fe-S oxidoreductase domain. (F) SDS–PAGE analysis of the purified C540S/C543S/C594S/C751S/C752S/C540S/C594S Fe-S oxidoreductase domain.

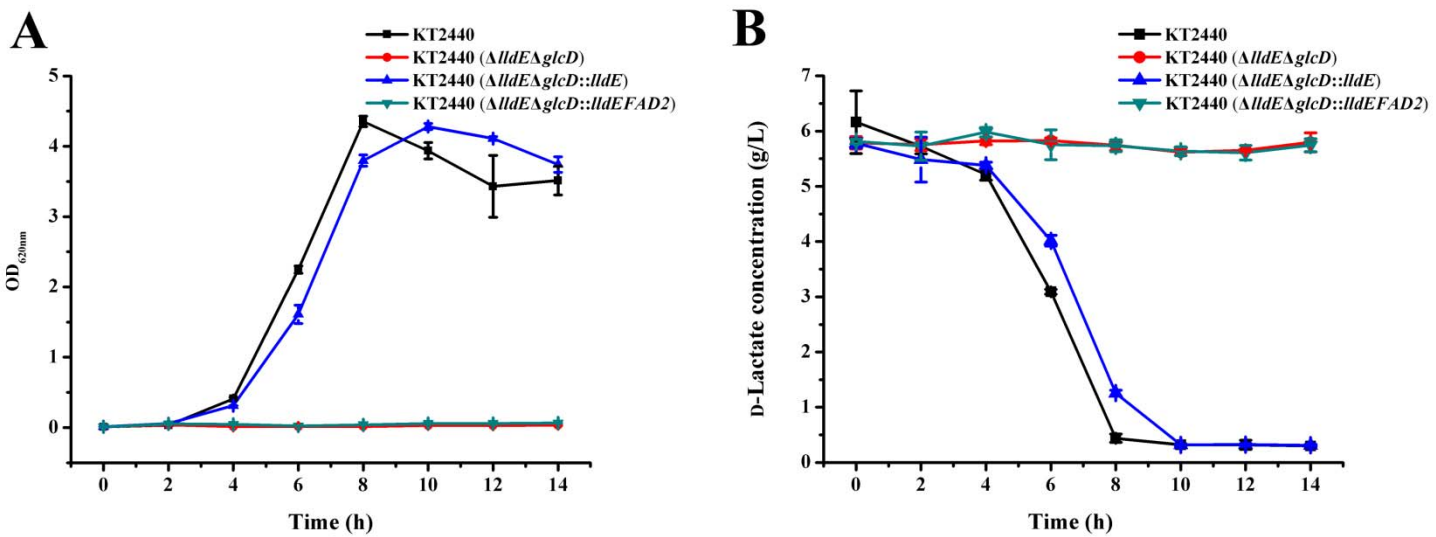


Figure S5. Time-course study of *P. putida* KT2440 and its derivatives growths with D-lactate as the sole carbon source. (A) Growth curves of *P. putida* KT2440 and its derivatives in minimal medium supplemented with 6 $g \cdot L^{-1}$ D-lactate as the sole carbon source. (B) D-Lactate concentrations measured during the growths of *P. putida* KT2440 and its derivatives. Results are means ± SD of three parallel replicates.

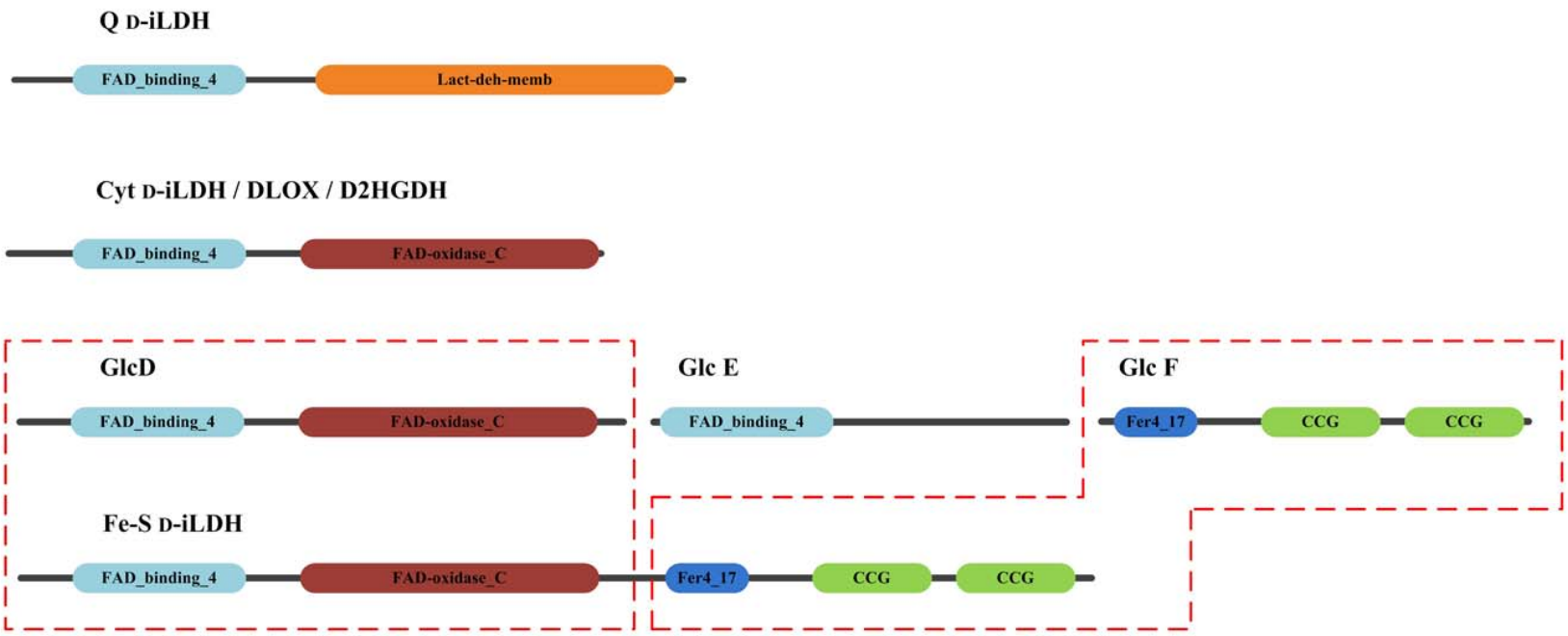
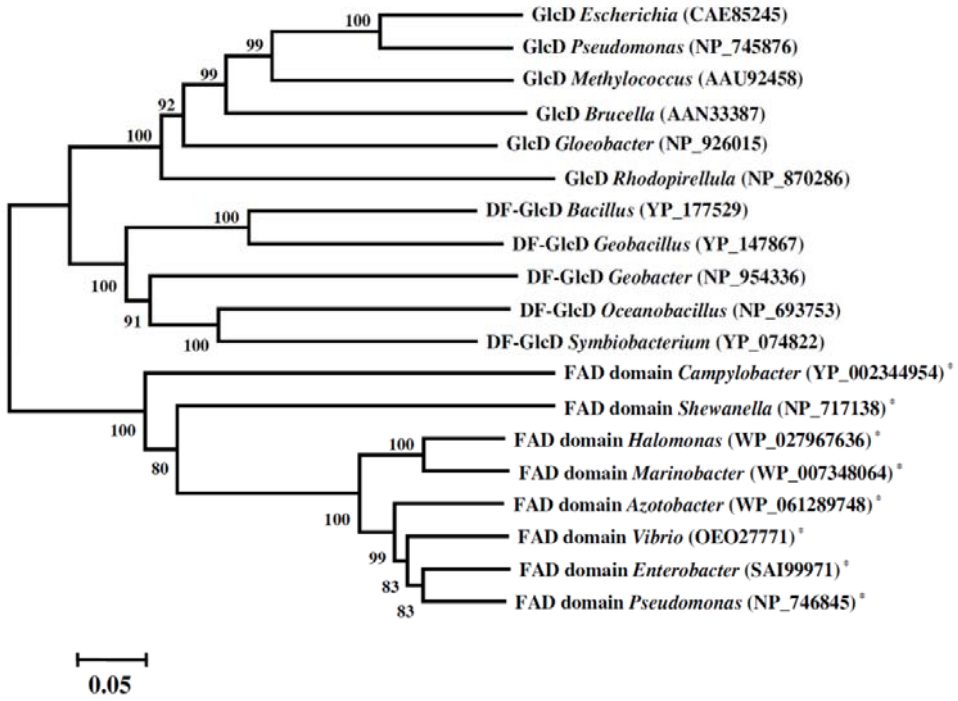


Figure S6. Sequence features of D-iLDHs and other related FAD-binding-4 family proteins. The analysis is based on the Pfam (protein family) database. The names of domains in the Pfam database are given. Detailed descriptions of the domains can be found in the Pfam database (http://pfam.sanger.ac.uk/). The dotted boxes indicate the similar domain organization of Fe-S D-iLDH and the GlcD, GlcF subunits of glycolate dehydrogenase.



Figuer S7. Evolutionary relationships of GlcD proteins and predicted FAD-containing dehydrogenase domains of Fe-S D-iLDHs. The phylogenetic tree was constructed using neighbor-joining method with Mega 5 software. Bootstrap values (%) are for 500 replicates. The scale at the bottom indicates sequence divergence. The accession numbers (from the National Center for Biotechnology Information, NCBI) are given in parentheses. Asterisks indicate the protein sequences analyzed are not full length of the sequences with the given accession numbers, but only the predicted FAD-containing dehydrogenase domains of them.

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