Supporting Information

Pinchuk *et al*. 10.1073/pnas.0806798106

SI Materials and Methods

Materials and Reagents. Enzymes for PCR and DNA manipulations were purchased from New England Biolabs Inc.. Plasmid purification kits were obtained from Promega. PCR purification kits and nickel-nitrilotriacetic acid (NTA) resin were from Qiagen. Oligonucleotides for PCR and sequencing were synthesized by Sigma Genosys. All other chemicals, including D- and L-lactate, phenazine methosulfate (PMS), 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT), 2,6-dichloroindophenol (DCIP), and potassium ferricyanide, 2,4-dinitrophenylhydrazine, were purchased from Sigma–Aldrich.

Regulon Reconstruction. To identify candidate regulatory motifs, we started from sets of potentially co-regulated lactate dehydrogenase (LDH) genes from the genomes that have orthologous transcriptional factors. An iterative motif detection procedure implemented in the program SignalX was used to identify common regulatory DNA motifs in a set of upstream gene fragments and to construct the motif recognition profiles. For every set of genomes with one particular orthologous regulon, we started from a training set of the upstream regions of LDH genes and their orthologs. Weak palindromes were selected in each region. Each palindrome was compared with all other palindromes, and the palindromes most similar to the initial one were used to make a profile. The candidate site score was defined as the sum of the respective positional nucleotide weights (1). These profiles were used to scan the set of palindromes again, and the procedure was iterated until convergence. Thus, a set of profiles was constructed. A profile with largest information content (1) was used as the recognition rule.

The constructed group-specific recognition rules were used to scan the group of genomes that contain the respective regulator. Positional nucleotide weights in the recognition profile and *Z*-scores of candidate sites were calculated as the sum of the respective positional nucleotide weights as previously described in ref. 2. Genome scanning for specific regulatory motifs by the Genome Explorer software (3) produced gene sets with candidate regulatory sites in the upstream regions (Table S2). The threshold for the site search was defined as the lowest score observed in the training set. The threshold choice was adequate in our cases, because very few clear false-positives were encountered and, on the other hand, most functionally relevant genes were found to belong to at least one of the studied lactate regulons. Sequence logos for the derived group-specific regulatory motifs were drawn using the WebLogo package v.2.6 (4) (http://weblogo.berkeley.edu/).

Deletion Mutagenesis in *S. oneidensis* MR-1. In-frame deletion mutagenesis of *dld-II* (SO_1521), *lldE* (SO_1520), *lldF* (SO_1519), *lldG* (SO_1518), and *ldhA* (SO_0968) was performed using a previously published method (5) with minor modifications. Upstream and downstream fragments flanking the target locus were PCR-amplified by using *S. oneidensis* MR-1 genomic DNA and fused through overlap extension PCR (6). The fusion PCR amplicon was ligated into XcmI-digested pDS3.0 (5). The resulting recombinant plasmids were used to transform *E. coli* β-2155 (7) or WM3063 (8) and subsequently transferred to *S. oneidensis* strain MR-1 by conjugation. The primary integrants were selected by plating on Luria-Bertani (LB) medium containing 7.5 μg/ml gentamycin. The screening for a second round of homologous recombination to remove the plasmid sequence was accomplished by counterselection on LB medium with 5% sucrose (9). Sucrose-resistant colonies were screened for sensitivity to gentamycin and then screened for deletion of the gene of interest by using PCR. The resulting PCR amplicon was then used as the template for DNA sequencing of the deleted and flanking regions involved in the recombination events (ACGT, Inc.). To validate that the observed phenotype could be attributed to the targeted deletion, complementing plasmids were constructed by inserting appropriate genes downstream to the *lac* promoter encoded in pBBR1MCS-5 (10).

Complementation studies. *E. coli* K12 Δ*dld* and Δ*lldD* knockout mutants expressing *dld-II* and *lldEGF in trans*, respectively, were precultured on LB media to mid-exponential growth phase, harvested by centrifugation, and washed three times with M9 minimal medium without any carbon sources. The cultures were started with the same optical density at 600 nm (OD_{600} = 0.04), and performed at 37 °C in triplicates in 200 μl of M9 minimal medium supplemented with 0.15% of L-arabinose and 20 mM of D- or L-lactate. *S. oneidensis* MR-1 Δ*dld-II*, Δ*lldEGF*, and Δ*dld-II*Δ*lldEGF* mutants carrying the pBBR1MCS-5 *dld-II* and *lldEGF* constructs were pregrown on tryptic soy broth (TSB) medium to a late exponential phase. All experiments were started by adding 2% of inoculum to M1 medium supplemented with D- or L-lactate as sole carbon and energy source to final concentration 18 mM. The growth of cultures was monitored spectrophotometrically at 600 nm.

His-tag Pull-Down Assays and Analysis of Protein–Protein Interactions. *S. oneidensis* MR-1 genes SO_1518, SO_1519, and SO_1520 were amplified with the proofreading polymerase *Phusion* and cloned into pBAD202/D-Topo (Invitrogen). The resulting constructs, including the native ribosome-binding site (RBS), were fused C-terminally to the V5 epitope and hexa-histidine tags, and expression was controlled by the arabinose promoter (*ara*P). Plasmid constructs were isolated from *E. coli* Top10 and transferred via electroporation into the respective *S. oneidensis* MR-1 mutants.

The resulting strains (Δ*lldE*/pBAD202::*lldE*, Δ*lldF*/pBAD202::*lldF*, and Δ*lldG*/pBAD202::*lldG*) were grown in modified M1 media (M1 without amino acids supplemented with 30 mM Pipes, 50 mM DL-lactate, 50 mM Na_2HPO_4, 0.02% TSB, 25 μg/ml kanamycin) supplemented with 0.02% DL-arabinose. All cultures were incubated for 18 h at 30 °C with 150 rpm agitation. Cells were collected by centrifugation at 8,000 × *g* (4 °C for 15 min) and resuspended in 25 ml ice-cold Metal Chelate Affinity Chromatography buffer (MCAC; 20 mM sodium phosphate pH 7.8, 0.5 M NaCl, 15% glycerol, 0.1% Triton X-100 and protease inhibitor) containing 20 mM imidazole (MCAC-20). Cells were lysed by being passed through a French press 5 times at 5,000lb/in^2 and the lysate was clarified by centrifugation at 35,000 × *g* at 4 °C for 30 min. The clarified lysate (25 ml) was diluted in MCAC-0 to a final volume of 100 ml and loaded into a chromatography column (Invitrogen) containing 5 ml Ni^{2+}-NTA agarose (Invitrogen), which was preequilabrated with 2 bed volumes (BV) of MCAC-10 (MCAC containing 10 mM imidazole). The column was then washed with 10 BV each of MCAC buffer containing an ascending series of imidazole concentrations (10 mM, 30 mM, 40 mM, 50 mM, 60 mM) and both 0.05% Tween-20 and Triton X-100. The column was then washed with 2 BV of MCAC containing 80 mM imidazole before the recombinant protein was eluted in 5 BV MCAC containing 200 mM

imidazole. All procedures were carried out at 4 °C with ice-cold buffers.

The elution fraction was concentrated by spinning in an Amicon Ultra 15 10,000 MWCO filter at 4,500 × *g* at 4 °C. During concentration, the buffer was exchanged by adding 15 ml of MCAC and spinning back down to 5 ml. This was repeated 3 times, with the third time ending in a total volume of 2.5 ml. Fifteen-milliliter aliquots of the concentrated samples were analyzed by SDS/PAGE and immunoblotting by using 4–12% NuPAGE Novex Bis-Tris denaturing gels and NuPAGE Mes buffer system (Invitrogen). Immunoblot visualization was carried out using a monoclonal Anti-His alkaline phosphatase-conjugated antibody (Invitrogen) that recognizes a polyhistidine amino acid sequence fused to the carboxyl-terminus of the target protein.

In Vitro Enzyme Assays. The activities of fermentative D-LDH and lactate-oxidizing Dld-II and LldEFG enzymes were assayed in crude cell extracts of *S. oneidensis* MR-1 and *E. coli* DH10B carrying arabinose-inducible pBAD-TOPO *ldhA*, *dld-II*, and *lldEGF* constructs. *S. oneidensis* MR-1 extracts were prepared from cells grown aerobically on M1 minimal medium supplemented with 45 mM D- or L-lactate as the only carbon source to mid-log phase (OD_{600} of 0.4–0.6). Recombinant *E. coli* DH10B strains were grown on TB media (24 g/L yeast extract and 12 g/L tryptone) containing 1% glycerol and 50 mM Mops (pH 7.6). The expression was induced by the addition of 0.15% arabinose and additional incubation for 3 h at 37 °C. *S. oneidensis* MR-1 and *E. coli* DH10B cultures were harvested by centrifugation, washed twice, and resuspended in 2 ml of 20 mM Hepes buffer pH 7 containing 100 mM NaCl and 2 mM β-mercaptoethanol. Cell disruption was achieved by ultrasonic treatment (S3000; MISONIX) on ice for 5 min. Overexpression of both Dld-II (SO_1521) and LldEFG (SO_1518–SO_1520) in *E. coli* led to formation of inclusion bodies, and we did not pursue their refolding in this study. The resulting suspension was used for the in vitro enzyme assays. The total protein content of the extracts was determined by the Biuret method after hot NaOH treatment for complete extraction of proteins (11). In addition, the fermentative D-LDH activity was measured in partially purified protein fractions obtained by using Ni-NTA affinity chromatography (12).

The activity of the fermentative lactate dehydrogenase/pyruvate reductase was measured spectrophotometrically by following the NAD absorbance change at 340 nm by using a Shimadzu UV-2101PC scanning spectrophotometer equipped with a thermostat (Shimadzu). All respective cofactors (NAD^+, NADH, $NADP^+$, and NADPH) were tested while assessing the ability of this enzyme to catalyze the reaction in both directions: from pyruvate to lactate and from lactate to pyruvate. The reaction mixture (1.5 ml) contained 25 mM Tris-HCl (pH 7.5), 1 mM of either NAD^+, NADH, $NADP^+$, or NADPH and 10–50 μl of crude extract or protein fraction. Reaction was started by the addition of 10 mM D-lactate or pyruvate.

The D- and L-lactate oxidizing activity was assayed by 2 methods. In the continuous assay A, the oxidation of lactate was coupled to the reduction of the phenazine methosulfate (PMS) and 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) (13, 14). Briefly, 2 μl of the cell extract was added to 500 μl of reaction mixture containing 50 mM Tris buffer (pH 8.0), 120 μg/ml PMS, 60 μg/ml MTT, and 5 mM D- or L-lactate. The detergent Triton X-100 was added to 0.5% to enhance the LDH activity as reported previously (14–16). The increase in absorbance measured at 570 nm is because of the formation of formazan was monitored using a Beckman DU800 spectrophotometer. An MTT extinction coefficient of 17 $mM^{-1}cm^{-1}$ was used for rate calculation. In the endpoint assay B, applied to extracts of *E. coli* DH10B expressing *dld-II* and *lldEFG* genes, formation of pyruvate was directly monitored by using the reaction with 2,4-dinitrophenylhydrazine, extraction of the hydrazone from benzene by Na_2CO_3 solution, and reading the absorbance at 435 nm after addition of NaOH (17).

1. Schneider TD, Stormo GD, Gold L, Ehrenfeucht A (2000) Information content of binding sites on nucleotide sequences. *J Mol Biol* 188:415–431.
2. Mironov AA, Koonin EV, Roytberg MA, Gelfand MS (1999) Computer analysis of transcription regulatory patterns in completely sequenced bacterial genomes. *Nucleic Acids Res* 27:2981–2989.
3. Mironov AA, Vinokurova NP, Gelfand MS (2000) Software for analyzing bacterial genomes. *Mol Biol* 34:253–262.
4. Crooks GE, Hon G, Chandonia JM, Brenner SE (2004) WebLogo: A sequence logo generator. *Genome Res* 14:1188–1190.
5. Wan XF, *et al.* (2004) Transcriptomic and proteomic characterization of the Fur modulon in the metal-reducing bacterium *Shewanella oneidensis*. *J Bacteriol* 186:8385–8400.
6. Ho SN, *et al.* (1989) Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene* 77:51–59.
7. Dehio C, Meyer M (1997) Maintenance of broad-host-range incompatibility group P and group Q plasmids and transposition of Tn*5* in *Bartonella henselae* following conjugal plasmid transfer from *Escherichia coli*. *J Bacteriol* 179:538–540.
8. Saltikov CW, Cifuentes A, Venkateswaran K, Newman DK (2003) The *ars* detoxification system is advantageous but not required for As(V) respiration by the genetically tractable *Shewanella species* strain ANA-3. *Appl Environ Microbiol* 69:2800–2809.
9. Blomfield IC, Vaughn V, Rest RF, Eisenstein BI (1991) Allelic exchange in *Escherichia coli* using the *Bacillus subtilis sacB* gene and a temperature-sensitive pSC101 replicon. *Mol Microbiol* 5:1447–1457.
10. Kovach ME, *et al.* (1994) pBBR1MCS: A broad-host-range cloning vector. *Biotechniques* 16:800–802.
11. Herbert D, Phipps PJ, Strange RE (1971) Chemical analysis of microbial cells. *J Microbiol Methods* 5B:209–344.
12. Osterman AL, *et al.* (1995) Domain organization and a protease-sensitive loop in eukaryotic ornithine decarboxylase. *Biochemistry* 34:13431–13436.
13. Erwin AL, Gotschlich EC (1993) Oxidation of D-lactate and L-lactate by *Neisseria meningitidis*: Purification and cloning of meningococcal D-lactate dehydrogenase. *J Bacteriol* 175:6382–6391.
14. Pratt EA, Fung LW, Flowers JA, Ho C (1979) Membrane-bound D-lactate dehydrogenase from *Escherichia coli*: Purification and properties. *Biochemistry* 18:312–316.
15. Fischer RS, Martin GC, Rao P, Jensen RA (1994) Neisseria gonorrhoeae possesses two nicotinamide adenine dinucleotide-independent lactate dehydrogenases. *FEMS Microbiol Lett* 115:39–44.
16. Reed DW, Hartzell PL (1999) The *Archaeoglobus fulgidus* D-lactate dehydrogenase is a Zn(2+) flavoprotein. *J Bacteriol* 181:7580–7587.
17. Friedemann T (1957) Determination of alpha-keto acids. *Methods Enzymol* 3:414–418.

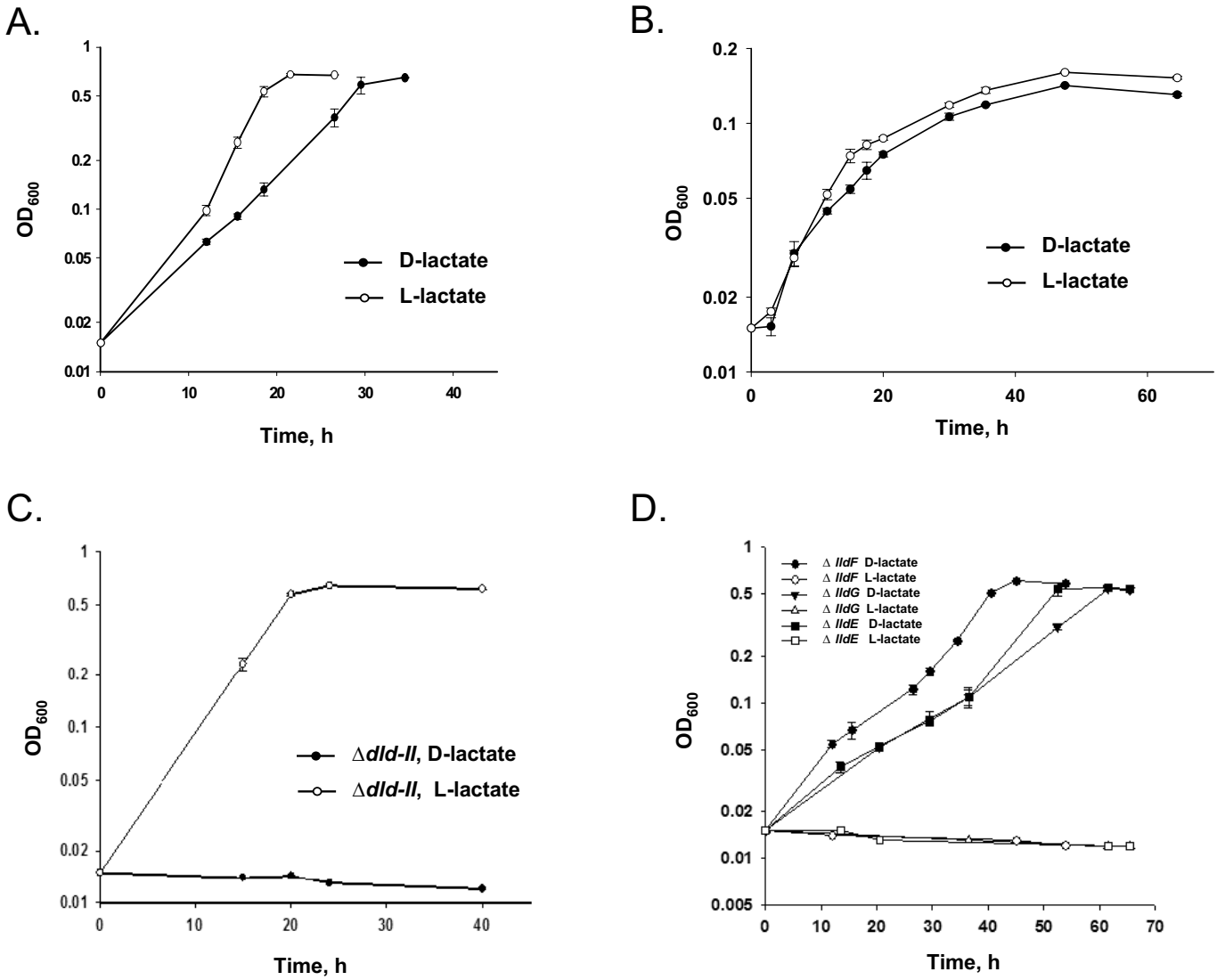

Fig. S1. Growth of *S. oneidensis* MR-1 wild type on D- and L-lactate as a sole carbon source under aerobic (*A*) and anaerobic (*B*) fumarate-reducing conditions. Growth of *S. oneidensis* MR-1 Δ*dld-II* (*C*), and Δ*lldE*, Δ*lldF*, and Δ*lldG* mutants (*D*) on D- and L-lactate under aerobic conditions. The cell growth was monitored by measuring the optical density at 600 nm. The data points and error bars represent the means and standard deviations of triplicate cultures, respectively.

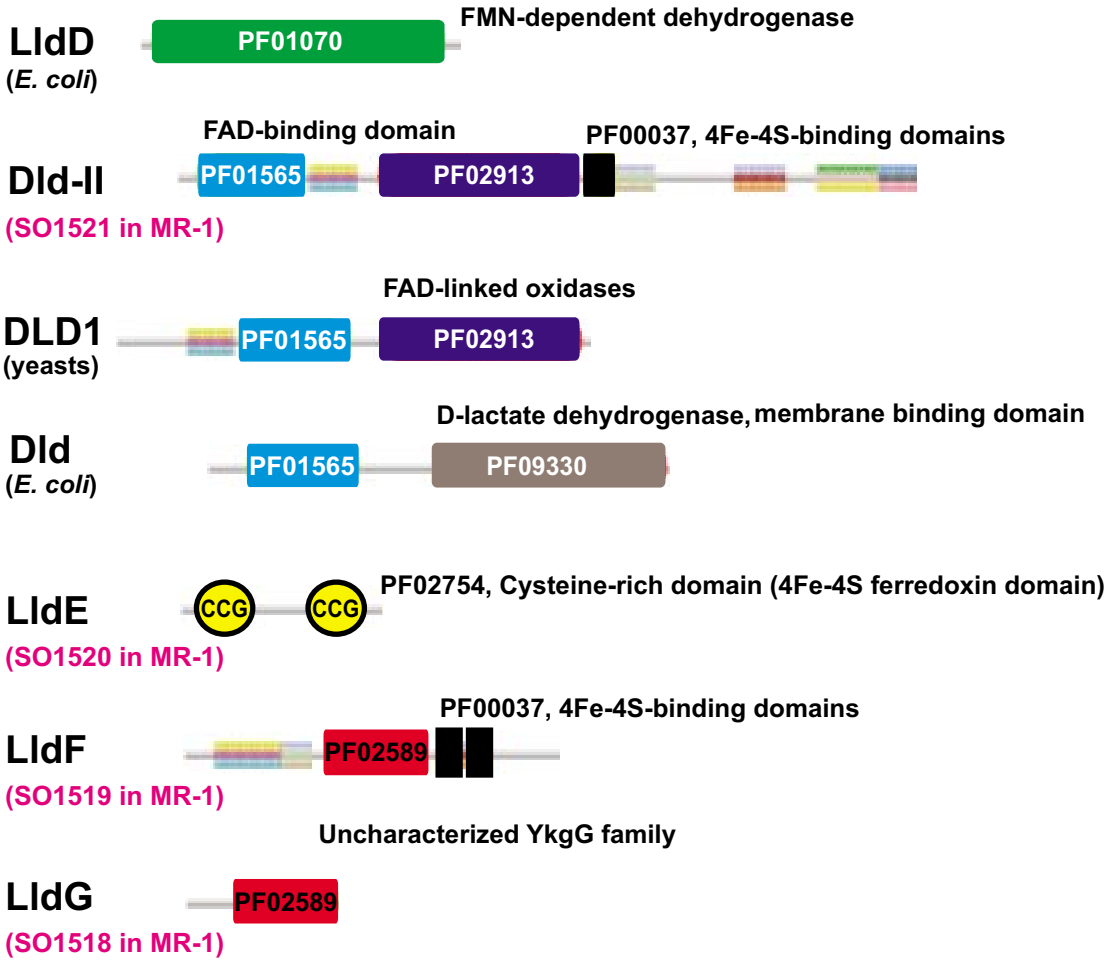

Fig. S2. Analysis of protein domains and motifs in lactate dehydrogenases (according to Pfam database).

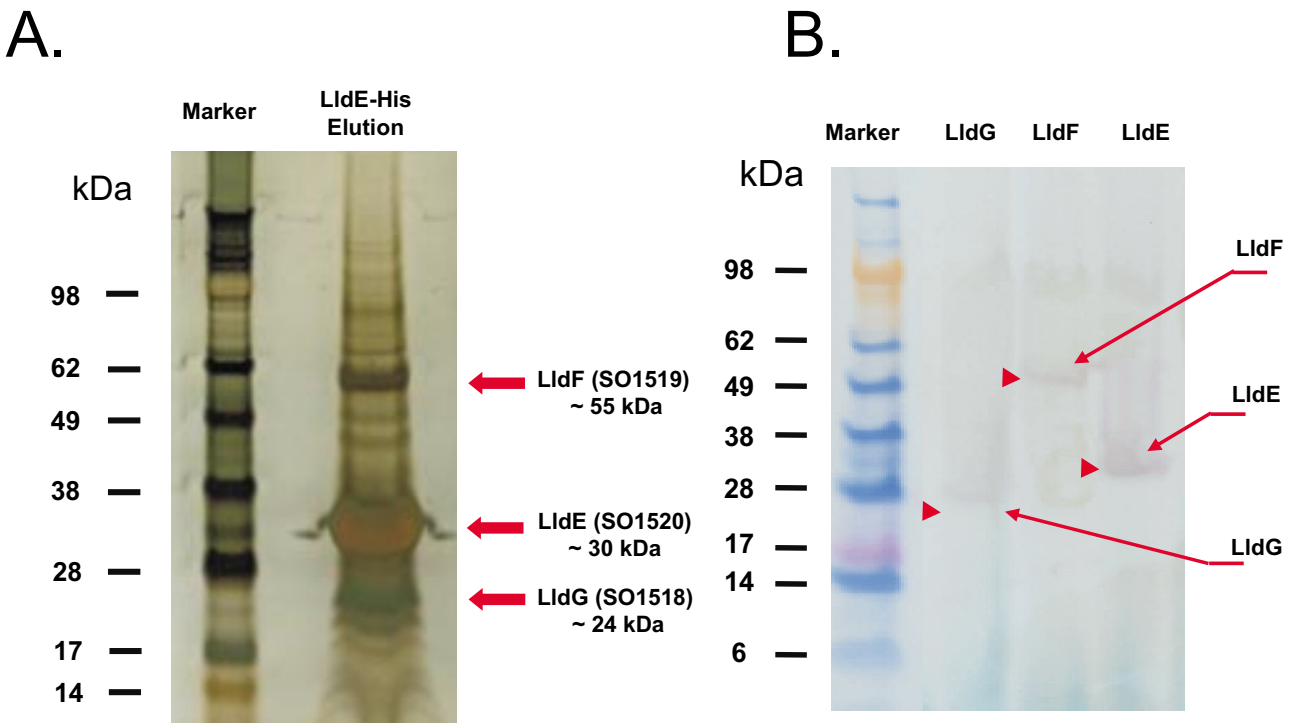

Fig. S3. Expression and affinity-based purification of the *S. oneidensis* MR-1 L-LDH components. (*A*) SDS/PAGE analysis of a pull-down reaction using His_6-tagged LldE. Recombinant LldE-His_6 expressed in the Δ*lldE* background was used as a bait protein for co-purification of two other predominant protein species with molecular weights corresponding to native LldF and LldG. (*B*) Western blot analysis of *S. oneidensis* MR-1 expressing His_6-tagged recombinant LldE, LldF, and LldG.

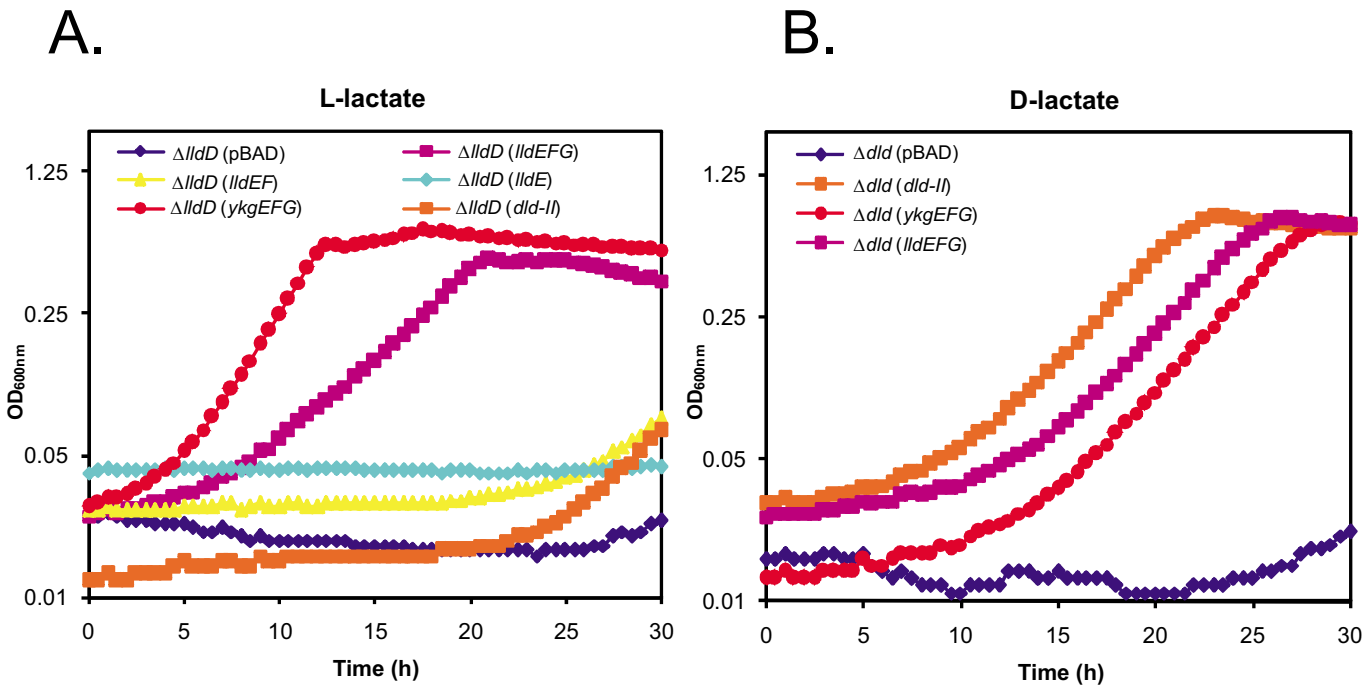

Fig. S4. Growth complementation of lactate dehydrogenase-deficient *E. coli* K12 Δ*dld* and Δ*lldD* strains by *S. oneidensis* MR-1 *dld-II* (SO_1521), *lldEFG* (SO_1520--SO_1518) genes, or *E. coli ykgEFG* operon. The empty vector was expressed in the same strain as a negative control. Cells were grown in a defined medium with (*A*) L-lactate or (*B*) D-lactate as carbon sources.

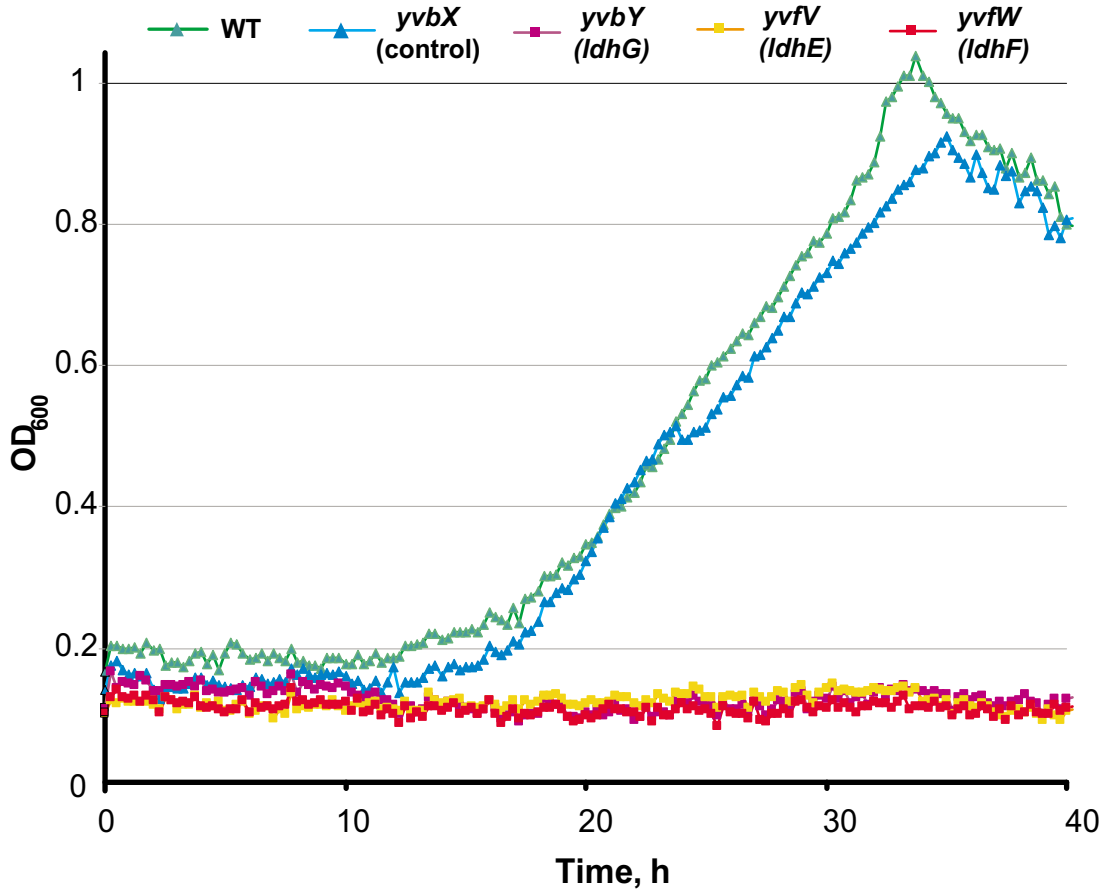

Fig. S5. Impairment in growth of *B. subtilis yvfV*(*lldE*)::pMUTIN2, *yvfW*(*lldF*)::pMUTIN2, and *yvbY*(*lldG*)::pMUTIN2 knockout strains on L-lactate as a sole source of carbon and energy. *B. subtilis* wild-type (str. 168) and *yvbX*::pMUTIN2 (knockout in a gene unrelated to lactate utilization) strains were used as positive controls.

Other Supporting Information Files

Table S1 (PDF)
Table S2 (PDF)
Table S3 (PDF)
Table S4 (PDF)