Preferential Utilization of D-Lactate by *Shewanella oneidensis*

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***Shewanella oneidensis* couples oxidation of lactate to respiration of many substrates. Here we report that *llpR* (L-lactate-positive regulator, SO_3460) encodes a positive regulator of L-lactate utilization distinct from previously studied regulators. We also demonstrate D-lactate inhibition of L-lactate utilization in *S. oneidensis*, resulting in preferential utilization of the D isomer.**

Shewanella oneidensis strain MR-1 (here referred to as MR-1) (15) is a genetically tractable model environmental bacterium, and there is considerable interest in using it as a platform organism for a range of biotechnological applications (12). During anaerobic respiration, MR-1 couples the oxidation of organic carbon to the reduction of an impressive array of terminal electron acceptors, including insoluble extracellular metals and carbon electrodes (12, 16). In anoxic environments, MR-1 is thought to form syntrophic partnerships with fermentative microbes, oxidizing lactate, formate, hydrogen, and some amino acids for anaerobic respiration (16). Lactate is of particular interest, as it is used as a carbon and energy source for the majority of studies involving *Shewanella* spp. grown in defined medium. Under both oxic and anoxic conditions, MR-1 must oxidize lactate to pyruvate using membrane-bound quinone-linked lactate dehydrogenase complexes specific for the L or D isomer (*lldEFG* [SO_1520-1518] and *dld* [SO_1521], respectively) (17). While the enzymatic components of lactate utilization have been identified, the regulatory mechanisms controlling expression of these genes have not been characterized in MR-1. A better understanding of the regulation of carbon utilization under anoxic conditions is invaluable for engineering *Shewanella* spp. for specific applications in bioremediation, electrosynthesis, and synthetic biology.

The regulation of genes required for the uptake and subsequent oxidation of lactate has been studied in *Escherichia coli*, *Corynebacterium glutamicum*, *Bacillus subtilis*, and *Pseudomonas aeruginosa* (1, 4, 9, 11). Whereas lactate utilization genes in *P. aeruginosa* are regulated by an ortholog of the *E. coli* GntR family transcription factor LldR (1, 9), lactate utilization genes in *C. glutamicum* and *B. subtilis* are controlled by nonorthologous GntR family proteins (4, 11, 17). We are intrigued by the distinct regulatory schemes of lactate-utilizing microorganisms despite the high degree of conservation of the L- and D-lactate dehydrogenases. For example, the LldR protein of *E. coli* activates expression of L-lactate utilization genes in the presence of either lactate isomer and expression of D-lactate utilization genes is constitutive (1, 7). In contrast, the LldR protein of *P. aeruginosa* responds to either isomer and coordinately activates expression of L- and D-lactate utilization genes (9). LldR in *C. glutamicum* is a repressor of L-lactate utilization genes in the absence of L-lactate and is insensitive to D-lactate (11). SO_3460, originally named *lldR* in *S. oneidensis*, is present in 21 of the 25 sequenced *Shewanella* genomes and was predicted to regulate lactate utilization in MR-1 using comparative genomics (17); however, no formal characterization was performed. We propose the name *llpR* (L-lactate-positive regulator) for SO_3460, as this gene encodes a LysR family protein phylogenetically distinct from the characterized regulators of lactate uti-

lization genes in the bacteria described above. Moreover, LlpR regulates lactate utilization differently than previously characterized regulators.

To study lactate utilization in MR-1, strains containing deletions of the entire *lldEFG* gene cluster, *dld*, or *llpR* were generated by homologous recombination as previously described (5). Lists of strains/plasmids (see Table S1 in the supplemental material) and oligonucleotide primers (see Table S2 in the supplemental material) are provided. Strains were incubated at 30°C with vigorous shaking in *Shewanella* basal medium (SBM) (3) containing 40 mM DL-lactate, L-lactate, or D-lactate. Cultures for anaerobic growth were supplemented with 80 mM fumarate, stoppered with butyl rubber, and flushed with nitrogen gas for 15 min (2). Growth was periodically monitored by absorbance at 600 nm (OD₆₀₀), and all reported values are the averages of at least three independent experiments, with error bars showing the standard error of the mean (SEM). Two pieces of data suggest that the *llpR* gene product is a positive regulator of L-lactate utilization in MR-1. First, while growth on D-lactate was unaffected by the loss of *llpR*, strains lacking this gene exhibit a significant growth defect compared to MR-1 when grown on L-lactate (Fig. 1A). Second, the doubling time of the *llpR* mutant grown on a 1:1 DL-lactate mix in the presence of oxygen was the same as that for MR-1 grown on D-lactate alone (2.90 ± 0.03 h and 3.01 ± 0.20 h, respectively) (Fig. 2A). The growth rate is an indicator of lactate isomer utilization because aerobically grown MR-1 has a shorter doubling time on L-lactate (2.30 ± 0.25 h) than D-lactate (3.01 ± 0.20 h) (Fig. 1A), while under anoxic conditions, growth rate is the same regardless of the isomer (doubling time of 4.15 ± 0.15 h) (see Fig. S1 in the supplemental material) (17). The growth defect of *llpR* can be complemented with a wild-type copy of the gene expressed from the broad-host-range expression vector pBBR1MCS-2 (13) (Fig. 1B). We further determined that the L-lactate growth phenotype of the *llpR* mutant likely results from insufficient expression of *lldEFG*, as transformation of the *llpR* mutant with pBBR1MCS-2 expressing *lldEFG* completely restored growth on L-lactate (Fig. 1B). These data demonstrate that LlpR is a positive regulator of L-lactate utilization by MR-1 and are consistent

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FIG 1 Growth of MR-1 and the *llpR* mutant on lactate. (A) MR-1 (circles) and the *llpR* mutant (squares) were grown aerobically at 30°C in SBM with 40 mM L-lactate (closed symbols) or 40 mM D-lactate (open symbols). (B) MR-1 (●) and the *llpR* mutant (■) carrying pBBR1MCS-2 or pBBR1MCS-2 expressing *llpR* (▲) or *lldEFG* (◆) were grown aerobically at 30°C in SBM with 40 mM L-lactate. Reported values are the averages of three independent experiments, and error bars show the standard error of the mean (SEM).

with a model in which LlpR specifically controls expression of *lldEFG*. To date, characterization of proteins that regulate lactate utilization in bacteria has been limited to members of the GntR family of transcriptional regulators. While predictions have been made in a number of organisms (17), this is the first demonstration of a LysR-like transcriptional regulator controlling expression of genes involved in lactate metabolism. Proteins of the LysR family are the most abundant type of transcriptional regulator in bacteria (14), regulating diverse classes of genes, to which lactate utilization can now be added.

Routine propagation of lactate utilization mutants on a 1:1 DL-lactate mix led to the fortuitous discovery of another aspect of regulation in MR-1. The aerobic growth rate of the *llpR* and

TABLE 1 NAD-independent lactate dehydrogenase activity assays

Strain and growth substrate	Rate of substrate oxidation (nmol min ⁻¹ mg ⁻¹) for ^a :	
	L-Lactate	D-Lactate
<i>S. oneidensis</i>		
MR-1		
DL-Lactate	44.5 ± 15.3	229.3 ± 10.6
L-Lactate	107.8 ± 3.1	340.6 ± 27.3
D-Lactate	44.9 ± 6.2	204.2 ± 38.1
<i>E. coli</i> MG1655		
Glucose	14.4 ± 2.9	143.6 ± 1.2
DL-Lactate	137.3 ± 1.4	196.0 ± 9.4
L-Lactate	141.8 ± 4.9	206.0 ± 8.0
D-Lactate	125.7 ± 8.2	160.8 ± 14.6

^a Reported values are the means and SEMs of three independent experiments.

lldEFG mutants grown on a DL-lactate mix is similar to that of MR-1 grown on D-lactate (Fig. 2A). However, the growth of the *dld* mutant was significantly impaired on DL-lactate compared to that on L-lactate alone (Fig. 2B), suggesting an inhibition of L-lactate utilization in the presence of D-lactate. Growth of the *dld* mutant was indistinguishable from that of MR-1 on DL-lactate supplemented with 20 mM pyruvate (data not shown), indicating that a failure to grow on DL-lactate alone is due to an inability to oxidize L-lactate, not inhibition of another cellular process. Exogenous expression of *dld* from pBBR1MCS-2 restored the ability of the *dld* mutant to grow on D-lactate (data not shown) and DL-lactate (Fig. 2B). To determine the underlying cause of the growth defect of the *dld* mutant on DL-lactate, we examined lactate dehydrogenase activity in cell lysates derived from MR-1 or *E. coli* strain MG1655 grown in SBM or M9 minimal medium (18), respectively. The L- and D-lactate dehydrogenases (*lldD* and *dld*, respectively) of *E. coli* are not homologous to those of MR-1 (17), and inhibition of L-lactate utilization by D-lactate has not previously been reported. Aerobic cultures were grown on DL-, D-, or L-lactate to an OD₆₀₀ of ~0.5 (*E. coli* was also grown on 20 mM glucose as a control). Cultures were pelleted by centrifugation, washed once with fresh SBM or M9, and resuspended in ice-cold lysis buffer (50 mM Tris-HCl [pH 8.0], 100 mM NaCl, and 2 mM β-mercaptoethanol). Cells were lysed by sonication, and NAD-independent lactate dehydrogenase activity from 10 μl of extract was determined as previously described (17). D-Lactate dehydrogenase activity in MR-1 and *E. coli* was largely independent of growth substrate (Table 1). In *E. coli*, L-lactate dehydrogenase activity was low when grown on glucose and increased 10-fold when grown on DL-, L-, or D-lactate (Table 1), consistent with published reports (1, 6, 8). In contrast, L-lactate dehydrogenase activity in MR-1 increased only when grown on L-lactate, rising ~3-fold compared to the cells grown on D-lactate (Table 1). Growth on DL-lactate resulted in a low level of L-lactate dehydrogenase activity, similar to cells grown on D-lactate alone (Table 1). These results are consistent with the inability of the *dld* mutant to grow on DL-lactate (Fig. 2B) and represent a D-lactate-dependent regulation of L-lactate dehydrogenase activity that, to our knowledge, has not been previously reported. It should be noted that the lactate dehydrogenases have some activity against both enantiomers of lactate (17), accounting for some of the increased activities quantified in Table 1.

FIG 2 Growth of MR-1 and lactate utilization mutants on DL-lactate. (A) MR-1, the *llpR* mutant, and the *lldEFG* mutant were grown aerobically in SBM with 40 mM DL-, L-, or D-lactate. The growth curves of MR-1 grown on D-lactate (▲) and the *llpR* and *lldEFG* mutants grown on DL-lactate (◆ and ○, respectively) are overlaid (bottom set of lines). (B) *dld* mutant grown aerobically in SBM with 40 mM DL-, L-, or D-lactate. Reported values are the averages of three independent experiments, and error bars show the standard error of the mean (SEM).

The molecular mechanisms underlying regulation of lactate utilization genes vary dramatically, and we speculate that the nature of regulation is indicative of the role lactate plays in the ecophysiology of an organism. The data presented here demonstrate a clear preference by MR-1 for utilizing D-lactate, a curious result for two reasons. First, in the presence of oxygen, MR-1 grows faster on L-lactate. Second, the preference for D-lactate comes at the expense of L-lactate utilization, even in the presence of both isomers. We are also intrigued by the fact that, despite nearly identical enzyme activities in cells grown on D- and DL-lactate (Table 1), MR-1 grows slower on D-lactate in the presence of oxygen (see Fig. S1 in the supplemental material). If D-lactate inhibits the expression of L-lactate utilization genes, why does the growth rate of MR-1 on DL-lactate not mirror that on D-lactate alone? We speculate that the small amount of L-lactate utilization which occurs in the presence of D-lactate, as indicated by the slow growth of the *dld* mutant on DL-lactate (Fig. 2B), enables MR-1 to achieve the higher growth rate. A large number of fermentative microorganisms, including the majority of lactic acid bacteria as well as *E. coli*, produce D-lactate (10), and organisms adapted to oxidize fermentation end products may preferentially use the D isomer. MR-1 is adept at coupling the oxidation of fermentation end products to the reduction of a host of soluble and insoluble electron acceptors. We speculate that rapid oxidation of D-lactate may represent a niche occupied by *S. oneidensis* at redox interfaces.

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REFERENCES

1. Aguilera L, et al. 2008. Dual role of LldR in regulation of the *lldPRD* operon, involved in L-lactate metabolism in *Escherichia coli*. *J. Bacteriol.* **190**:2997–3005.
2. Balch WE, Wolfe RS. 1976. New approach to the cultivation of methanogenic bacteria: 2-mercaptoethanesulfonic acid (HS-CoM)-dependent growth of *Methanobacterium ruminantium* in a pressurized atmosphere. *Appl. Environ. Microbiol.* **32**:781–791.
3. Baron D, LaBelle E, Coursolle D, Gralnick JA, Bond DR. 2009. Electrochemical measurement of electron transfer kinetics by *Shewanella oneidensis* MR-1. *J. Biol. Chem.* **284**:28865–28873.
4. Chai Y, Kolter R, Losick R. 2009. A widely conserved gene cluster required for lactate utilization in *Bacillus subtilis* and its involvement in biofilm formation. *J. Bacteriol.* **191**:2423–2430.
5. Coursolle D, Baron DB, Bond DR, Gralnick JA. 2010. The Mtr respiratory pathway is essential for reducing flavins and electrodes in *Shewanella oneidensis*. *J. Bacteriol.* **192**:467–474.
6. Dong JM, Taylor JS, Latour DJ, Iuchi S, Lin EC. 1993. Three overlapping *lct* genes involved in L-lactate utilization by *Escherichia coli*. *J. Bacteriol.* **175**:6671–6678.
7. Futai M. 1973. Membrane D-lactate dehydrogenase from *Escherichia coli*. Purification and properties. *Biochemistry* **12**:2468–2474.
8. Futai M, Kimura H. 1977. Inducible membrane-bound L-lactate dehydrogenase from *Escherichia coli*. Purification and properties. *J. Biol. Chem.* **252**:5820–5827.
9. Gao C, et al. 2012. Lactate utilization is regulated by the FadR-type regulator LldR in *Pseudomonas aeruginosa*. *J. Bacteriol.* **194**:2687–2692.
10. Garvie EI. 1980. Bacterial lactate dehydrogenases. *Microbiol. Rev.* **44**:106–139.
11. Georgi T, Engels V, Wendisch VF. 2008. Regulation of L-lactate utilization by the FadR-type regulator LldR of *Corynebacterium glutamicum*. *J. Bacteriol.* **190**:963–971.
12. Hau HH, Gralnick JA. 2007. Ecology and biotechnology of the genus *Shewanella*. *Annu. Rev. Microbiol.* **61**:237–258.
13. Kovach ME, et al. 1995. Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. *Gene* **166**:175–176.
14. Maddocks SE, Oyston PC. 2008. Structure and function of the LysR-type transcriptional regulator (LTTR) family proteins. *Microbiology* **154**:3609–3623.
15. Myers CR, Nealson KH. 1988. Bacterial manganese reduction and growth with manganese oxide as the sole electron acceptor. *Science* **240**:1319–1321.
16. Nealson K, Scott J. 2006. Ecophysiology of the genus *Shewanella*. The prokaryotes, 3rd ed, vol 6. Springer Science, New York, NY.
17. Pinchuk GE, et al. 2009. Genomic reconstruction of *Shewanella oneidensis* MR-1 metabolism reveals a previously uncharacterized machinery for lactate utilization. *Proc. Natl. Acad. Sci. U. S. A.* **106**:2874–2879.
18. Sambrook J, Russell DW. 2001. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.