Pyruvate Accelerates Palladium Reduction by Regulating Catabolism and the Electron Transfer Pathway in *Shewanella oneidensis*

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ABSTRACT *Shewanella oneidensis* is a model strain of electrochemically active bacteria (EAB) because of its strong capability of performing extracellular electron transfer (EET) and its genetic tractability. In this study, we investigated the effect of carbon sources on EET in *S. oneidensis* by using reduction of palladium ions [Pd(II)] as a model and found that pyruvate greatly accelerated Pd(II) reduction compared with lactate by resting cells. Both the Mtr pathway and hydrogenases played a role in Pd(II) reduction when pyruvate was used as a carbon source. Furthermore, in comparison with lactate-feeding *S. oneidensis*, the transcriptional levels of formate dehydrogenases involved in pyruvate catabolism, the Mtr pathway, and hydrogenases in pyruvate-feeding *S. oneidensis* were upregulated. Mechanistically, the enhancement of electron generation from pyruvate catabolism and electron transfer to Pd(II) explains the pyruvate effect on Pd(II) reduction. Interestingly, a 2-h time window is required for pyruvate to regulate transcription of these genes and profoundly improve Pd(II) reduction capability, suggesting hierarchical regulation for pyruvate sensing and response in *S. oneidensis*.

IMPORTANCE The unique respiration of EET is crucial for biogeochemical cycling of metal elements and diverse applications of EAB. Although a carbon source is a determinant factor of bacterial metabolism, research into the regulation of the carbon source of EET is rare. In this work, we report pyruvate-specific regulation and improvement of EET in *S. oneidensis* and reveal the underlying mechanism, which suggests potential targets for engineering and improvement of the EET efficiency of this bacterium. This study sheds light on the regulatory role of carbon sources in anaerobic respiration in EAB, providing a way to regulate EET for diverse applications from a novel perspective.

KEYWORDS *Shewanella*, pyruvate, palladium, extracellular electron transfer, transcriptional regulation

Shewanella isolates are distributed in diverse environments and can utilize more than 20 electron acceptors, such as oxygen, fumarate, nitrate, dimethyl sulfoxide (DMSO), and iron oxides. These bacteria possess a powerful respiration capability of performing extracellular electron transfer (EET), which permits them to reduce electron acceptors, such as iron oxides and azo dyes, outside cells (1, 2). Such a capability can

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be harnessed to recover energy from wastewater when an electrode is used as an electron acceptor (3). Since EET is essential for versatile applications of the genus *Shewanella*, tremendous efforts have been devoted to elucidating the EET pathway and improving EET efficiency, such as recently developed strategies to engineer *Shewanella* biofilm (4).

EET in *Shewanella oneidensis* mainly depends on the Mtr pathway, which includes four *c*-type cytochromes, CymA, MtrA, and the OmcA/MtrC complex, and a barrel protein, MtrB (5). CymA in the cytoplasmic membrane is a quinol dehydrogenase that functions as a hub to connect catabolism of carbon sources and respiration of some electron donors, such as arsenate (6–8). Depending on available electron acceptors under anaerobic conditions, CymA interacts with different electron transfer proteins or terminal reductases in the periplasm, such as MtrA and FccA (7, 9). MtrA transfers electrons to outer membrane-located OmcA/MtrC, which further transfers electrons to acceptors outside cells. The Mtr pathway builds an electron transfer bridge across the periplasm and outer membrane and is active not only in *Shewanella* but also in *Escherichia coli* (10–12). Besides the Mtr pathway, a pathway for DMSO respiration is also involved in EET, although DMSO is a readily cell-permeable compound. DmsE receives electrons from CymA and transfers them to outer membrane-located DmsABF, which reduces DMSO outside cells (7).

S. oneidensis is a potent reducer and recovery bacterium of palladium (Pd), which is a precious metal and has applications in chemical synthesis and automobile catalytic converters (13). The reduction of Pd(II) in *S. oneidensis* depends on the Mtr pathway when lactate is used as a carbon source and electron donor, while hydrogenases, but not the Mtr pathway, contribute to Pd(II) reduction when formate is used as an electron donor (14, 15). During anaerobic respiration in *S. oneidensis*, lactate is oxidized by lactate dehydrogenase (LldEFG and Lld-II) to pyruvate, which is further cleaved to formate by pyruvate formate-lyase (PFL; PflAB). Electrons from formate oxidation by formate dehydrogenases (FDH) are transferred to menaquinone, of which hydrogenases and the Mtr pathway are downstream. The difference in terminal reductases of Pd(II) reduction suggests that the carbon source and electron donor have regulatory roles in the choice of electron transfer pathway, including EET in *S. oneidensis*.

Previous research demonstrated connections between catabolism and EET in *S. oneidensis*. Heterologous expression of a NAD⁺-dependent formate dehydrogenase from methylotrophic bacterium *Moraxella* sp. in *S. oneidensis* results in increased current density in a bioelectrochemical reactor (16). Improvement in NAD⁺ biosynthesis accelerates the EET in *S. oneidensis* by increasing lactate oxidation (17). The electrode potential regulates pyruvate catabolism from an NADH-dependent pathway at high potential to a formate-dependent pathway at low potential (18). However, there is no systematic investigation into the effect of the carbon source on EET in *S. oneidensis*. In this study, we explore the regulation of the carbon source on the EET in *S. oneidensis*, using Pd(II) reduction as a model. The pathway of Pd(II) reduction was identified when pyruvate was used as a sole carbon source. The effect of pyruvate on EET was investigated and compared with that of lactate. Pyruvate supports a profoundly accelerated reduction of Pd(II) when *S. oneidensis* is allowed to sense and respond to pyruvate ahead of Pd(II) exposure. The formate oxidation and EET pathway are both upregulated in *S. oneidensis* fed with pyruvate versus lactate. This study sheds light on the regulatory role of carbon sources on EET in electrochemically active bacteria (EAB).

RESULTS

Pyruvate accelerates Pd(II) reduction in a time- and concentration-dependent manner. We first compared pyruvate, another common carbon source, with lactate and formate for their effects on Pd(II) reduction. We accidentally found that *S. oneidensis* reduced Pd(II) at a greatly increased rate when it was preincubated in pyruvate for 2 h ahead of Pd(II) addition, while lactate and formate did not show this effect (Fig. 1A). Incubation of resting cells with pyruvate, lactate, or formate before Pd(II) addition was later defined as preincubation. Cells without preincubation showed a similar

FIG 1 Pyruvate accelerated Pd(II) reduction in a time- and concentration-dependent manner. (A) Pd(II) reduction by resting cells of *S. oneidensis*, which were preincubated with pyruvate, lactate, or formate for 0 h or 2 h before the addition of Pd(II). (B) Pd(II) reduction by resting cells that were preincubated with pyruvate at different times. (C) Pd(II) reduction by resting cells that were preincubated with pyruvate at different concentrations. Error bars indicate standard deviations of results from four biological replicates.

capability of reducing Pd(II) when pyruvate or lactate was used as a sole carbon source and did not completely reduce $100 \mu\text{M}$ Pd(II) after 48 h (Fig. 1A). The incomplete reduction of Pd(II) might be attributed to Pd(II) toxicity that causes cell death or inactivation. Consistent with our assumption, cultivable cells were significantly decreased in cultures without preincubation compared to those preincubated with pyruvate (Fig. S1 in the supplemental material).

Based on the above findings, we proposed that preincubation in pyruvate might allow *S. oneidensis* to adjust metabolism and/or respiration before exposure to the toxic Pd(II). We then examined the time window of pyruvate preincubation that is required for the proposed adjustment for detectable acceleration of Pd(II) reduction. There was no obvious increase in Pd(II) reduction after cells were preincubated with pyruvate for 0.5 h, and there was a slight increase after preincubation for 1 h (Fig. 1B). An obvious increase in both reduction rate and efficiency was observed at 2 h preincubation, while there was no further increase when the preincubation was prolonged to

FIG 2 Terminal reductases of Pd(II) reduction in pyruvate-incubating *S. oneidensis*. (A) Pd(II) reduction by mutants lacking hydrogenases ($\Delta\text{hyaB } \Delta\text{hydA}$) and the Mtr pathway ($\Delta\text{omcA } \Delta\text{mtrC}$). (B) Pd(II) reduction by mutants deficient in menaquinone biosynthesis (ΔmenA) or lacking both hydrogenases and the Mtr pathway ($\Delta\text{hyaB } \Delta\text{hydA } \Delta\text{omcA } \Delta\text{mtrC}$). Pd(II) reduction by live and heat-killed cells of the wild-type strain (WT) was set as control to determine bioreduction of Pd(II). Pyruvate and Pd(II) were added to final concentrations of 20 mM and 100 μM, respectively. Error bars indicate standard deviations of results from four biological replicates.

3 h (Fig. 1B). This result suggests that *S. oneidensis* requires 2 h for physiological adjustment for complete reduction of 100 μM Pd(II). The reduction capability of such pyruvate-preincubated *S. oneidensis* cultures gradually dropped with the increase in Pd(II) concentration (Fig. S2), presumably due to the strong toxicity of a high concentration of Pd(II).

Besides the effect of time, we also examined whether pyruvate concentration affected the capability of *S. oneidensis* to reduce Pd(II), and we found that the acceleration effect of pyruvate preincubation was positively correlated with initial pyruvate concentration (Fig. 1C), while pyruvate consumption was comparable for all cultures supplemented with different concentrations of pyruvate (Table S1). *S. oneidensis* preincubated in 20 mM pyruvate for 2 h consumed 0.91 ± 0.18 mM pyruvate.

Pd(II) reduction pathway in pyruvate-preincubated *S. oneidensis*. The Mtr pathway and hydrogenases are essential for Pd(II) reduction when lactate and formate are used, respectively (15). After preincubation with pyruvate for 2 h, a mutant lacking both hydrogenases ($\Delta\text{hyaB } \Delta\text{hydA}$) showed a severe defect in Pd(II) reduction and did not completely reduce 100 μM Pd(II) after 48 h (Fig. 2A). The OmcA/MtrC complex, the terminus of Mtr pathway, is responsible for direct contact with many extracellular electron acceptors. The $\Delta\text{omcA } \Delta\text{mtrC}$ mutant also displayed an impaired ability to reduce Pd(II) by pyruvate-preincubated *S. oneidensis* (Fig. 2A). The mutant lacking hydrogenases and OmcA/MtrC ($\Delta\text{hyaB } \Delta\text{hydA } \Delta\text{omcA } \Delta\text{mtrC}$) showed more severe defects in Pd(II) reduction than either the $\Delta\text{hyaB } \Delta\text{hydA}$ or $\Delta\text{omcA } \Delta\text{mtrC}$ mutants

TABLE 1 List of selected differentially expressed genes discussed in this study

Purpose and locus tag	Gene and annotation	Log ₂ FC ^a
Hydrogen production		
SO_2094	<i>hypF</i> , NiFe hydrogenase maturation protein	2.23
SO_2095	<i>hyaE</i> , NiFe hydrogenase assembly chaperone	2.16
SO_2096	<i>hyaD</i> , NiFe hydrogenase maturation protease	2.10
SO_2097	<i>hyaC</i> , periplasmic [NiFe] hydrogenase cytochrome b subunit	2.33
SO_2098	<i>hyaB</i> , periplasmic [NiFe] hydrogenase large subunit	1.52
SO_2099	<i>hyaA</i> , periplasmic [NiFe] hydrogenase small subunit	1.17
Extracellular electron transfer		
SO_1427	<i>dmsE</i> , periplasmic decaheme cytochrome c	1.54
SO_1429	<i>dmsA</i> , extracellular dimethyl sulfoxide/manganese oxide reductase molybdopterin-binding subunit	2.0
SO_1430	<i>dmsB</i> , extracellular dimethyl sulfoxide/manganese oxide reductase ferredoxin subunit	3.04
SO_1431	<i>dmsG</i> , extracellular dimethyl sulfoxide/manganese oxide reductase chaperone	2.71
SO_1432	<i>dmsH</i> , extracellular dimethyl sulfoxide/manganese oxide reductase accessory protein	1.30
SO_1776	<i>mtrB</i> , extracellular iron oxide respiratory system outer membrane component	1.10
SO_1777	<i>mtrA</i> , extracellular iron oxide respiratory system periplasmic decaheme cytochrome c component	0.57
SO_1778	<i>mtrC</i> , deca-heme c-type cytochrome	0.43
SO_1779	<i>omcA</i> , extracellular iron oxide respiratory system surface decaheme cytochrome c component	1.33
SO_4591	<i>cymA</i> , membrane anchored tetraheme cytochrome c	0.81
Formate oxidation		
SO_4507	<i>fdhT</i> , formate dehydrogenase chaperone	3.11
SO_4508	<i>fdhX1</i> , formate dehydrogenase accessory protein	3.16
SO_4509	<i>fdhA1</i> , formate dehydrogenase molybdopterin-binding subunit	3.22
SO_4510	<i>fdhB1</i> , formate dehydrogenase FeS subunit	2.08
SO_4511	<i>fdhC1</i> , formate dehydrogenase cytochrome b subunit	2.02
SO_4512	<i>fdhX2</i> , formate dehydrogenase accessory protein	1.78
SO_4513	<i>fdhA2</i> , formate dehydrogenase molybdopterin-binding subunit	1.19
SO_4514	<i>fdhB2</i> , formate dehydrogenase FeS subunit	1.21
SO_4515	<i>fdhC2</i> , formate dehydrogenase cytochrome b subunit	1.54

^aFC, fold change of transcription in groups of pyruvate compared with lactate.

(Fig. 2A and B). The reduction ability of the $\Delta hydA \Delta hyaB \Delta omcA \Delta mtrC$ mutant was similar to a mutant lacking *menA* ($\Delta menA$) and the heat-killed wild type (WT) (Fig. 2B).

menA, encoding a 1,4-dihydroxy-2-naphthoate octaprenyl transferase, is indispensable for menaquinone synthesis in *S. oneidensis* (19). Both hydrogenases and the Mtr pathway obtain electrons from the menaquinone (20, 21). The Pd(II) removed by heat-killed cells resulted from absorption but not reduction, which is indicated by analyses of transmission electron microscopy and X-ray photoelectron spectroscopy (Fig. S3). These observations indicated that when pyruvate is used as a carbon source, (i) menaquinone is indispensable for Pd(II) reduction and (ii) Pd(II) reduction only depends on two terminal reductases, hydrogenases and the OmcA/MtrC complex.

S. oneidensis transcriptomic response to pyruvate. To explore how pyruvate promotes Pd(II) reduction compared to lactate, the transcriptomic profiles of pyruvate-preincubated *S. oneidensis* were compared with lactate-preincubated *S. oneidensis*. We found that the transcription levels of [NiFe] hydrogenase (*hyaAB*) and the Mtr pathway (*cymA*, *omcA*, and *mtrCAB*) were upregulated close to or more than 2-fold in pyruvate-preincubated *S. oneidensis* (Table 1). We then examined the relative transcription level of all these genes by using reverse transcription-quantitative PCR (qRT-PCR). The result of qRT-PCR analyses was consistent with that of transcriptome sequencing (RNA-seq) (Fig. 3). Interestingly, transcription of *dmsEFAB* and *dmsGH* operons involved in DMSO respiration was also upregulated (Fig. 3). DMSO respiration in *S. oneidensis* is an extracellular respiratory process (7). Upregulation of *dms* operons was supposed to enhance DMSO respiration. Indeed, pyruvate supported faster anaerobic growth than lactate when DMSO was used as an electron acceptor (Fig. S4).

Genes involved in the catabolism of carbon sources were also differentially

FIG 3 Pyruvate upregulates expression of genes involving in Pd(II) reduction and formate oxidation. Expression ($\log_2$ fold change) of genes was examined using qRT-qPCR and compared with RNA-seq data. Resting cells were incubated with 20 mM pyruvate or 20 mM lactate for 2 h and then subjected to extraction of total RNA. Error bars indicate standard deviations of results from three biological replicates.

regulated in *S. oneidensis* preincubated with pyruvate or lactate. Transcription of L-lactate dehydrogenases (LldEFG) was increased in lactate-preincubated *S. oneidensis* (Table S2). The transcription level of pyruvate formate-lyase (PflAB), the first enzyme to perform pyruvate catabolism under anaerobic conditions, showed no significant difference between pyruvate-preincubated and lactate-preincubated *S. oneidensis* (Table S2). Instead, transcription of two formate dehydrogenases (FdhA1B1C1 and FdhA2B2C2) and associated proteins was increased by 3- to 9-fold in pyruvate-preincubated *S. oneidensis* (Fig. 3). Both FdhA1B1C1 and FdhA2B2C2 are active in formate oxidation and transfer electrons to the menaquinone (20). Overall, these results suggest that both electron generation from carbon source catabolism and electron transfer to Pd(II) were upregulated in *S. oneidensis* preincubated with pyruvate in comparison with lactate.

Expression of [NiFe] hydrogenase and hydrogen production. To examine the regulation of pyruvate on electron transfer, we chose [NiFe] hydrogenase HyaAB as a representative and monitored the expression dynamics of the *hyaAB* operon in *S. oneidensis* incubated with pyruvate, lactate, or formate. [NiFe] hydrogenase is a major hydrogenase contributing to hydrogen production in *S. oneidensis* (20). The expression of *hyaAB* remained low and almost unchanged within 4 h of incubation when lactate or formate was used (Fig. 4A). The expression of *hyaAB* in pyruvate-incubating *S. oneidensis* was as low as in lactate-incubating or formate-incubating *S. oneidensis* within 30 min; however, it robustly increased after 2 h of incubation (Fig. 4A), which is consistent with the above conclusion that *S. oneidensis* requires 2 h preincubation to sense and respond to pyruvate for Pd(II) reduction. Hydrogen was produced by pyruvate-incubating *S. oneidensis* after 30 min, while no hydrogen was detected either for lactate-incubating or formate-incubating *S. oneidensis*, even after 2 h (Fig. 4B). This result supports the transcriptional regulation of pyruvate on the *hyaAB* operon.

Next, we used the β -galactosidase reporter assay to examine whether the effect of pyruvate on the expression of *hyaAB* was in a concentration-dependent manner, and we found that the expression of *hyaAB* increased with the increase of pyruvate concentration (Fig. 4C). This result was consistent with the concentration effect of pyruvate on Pd(II) reduction.

Energy level of *S. oneidensis* after preincubation. Oxidation of pyruvate or lactate might change the energy level that can be evaluated by some bioindicators. The intracellular NADH and NAD⁺ are known as the crucial source of the intracellular electron pool. Previous studies demonstrate an increase in the EET rate by improving NAD⁺ biosynthesis or NADH regeneration (17, 22). To look into the possible contribution of

FIG 4 Expression of the hydrogenase HyaAB examined by LacZ (β -galactosidase) reporter. (A) Expression of *hyaAB* in resting cells incubated with lactate, pyruvate, or formate. (B) Hydrogen production by resting cells incubated with pyruvate or lactate. (C) Expression of *hyaAB* in resting cells incubated with pyruvate at different concentrations. Error bars indicate standard deviations of results from four biological replicates.

NADH and NAD⁺ to the pyruvate effect, we compared the levels of NADH and NAD⁺, as well as the ratio of NADH/NAD⁺ in cells after incubation in pyruvate, lactate, or none of the carbon sources. There was no significant difference in the level of NADH and NAD⁺ in cells no matter whether they were incubated with pyruvate, lactate, or none of the carbon sources for 2 h (Fig. 5A). The NADH/NAD⁺ ratios in cells incubated with pyruvate, lactate, or none of the carbon sources were also similar (Fig. 5B).

A recent study reported membrane potential as a bioenergetics indicator of EET in *S. oneidensis* (23). Therefore, we further examined the membrane potential in *S. oneidensis* preincubated with pyruvate, lactate, or none of the carbon sources. No obvious difference in polarization of membrane potential was observed in cells incubated with pyruvate, lactate, or none of the carbon sources for 2 h (Fig. 5C). These results suggest

FIG 5 Energy levels affected by incubation with carbon sources. (A) Quantitative measurement of intracellular NADH and NAD⁺. (B) Concentration ratio of NADH to NAD⁺ in resting cells after preincubation with pyruvate, lactate, or none of the carbon sources for 2 h. (C) Membrane potential of resting cells after preincubation with pyruvate, lactate, or none of the carbon sources for 2 h. Cells after preincubation were stained by DiOC₂(3) together with (green columns) or without CCCP (orange columns). The average intensity of green and red fluorescence was calculated based on data from flow cytometry analysis. Error bars indicate standard deviations of results from three biological replicates.

that the oxidation of pyruvate did not contribute to the acceleration effect of pyruvate on Pd(II) reduction.

DISCUSSION

Electron donors, some of which also function as carbon sources, show their unique effects on the physiology of *S. oneidensis*, a model EAB. Formate is the only electron donor that induces chemotaxis of *S. oneidensis* under anaerobic conditions (36). Lactate is a preferred carbon source and electron donor that supports the fastest

growth of most *Shewanella* isolates (24). As a more reduced compound, pyruvate is supposed to be a more preferable carbon source than lactate under electron acceptor-limited conditions (25), which is supported by this study. In this study, we found that pyruvate, but not lactate or formate, profoundly accelerated Pd(II) reduction in *S. oneidensis* after the bacterium was preincubated in pyruvate for at least 2 h, which we defined as the time window of pyruvate effect (Fig. 1). We proposed that *S. oneidensis* might experience transcriptional regulation and physiological adjustment during pyruvate preincubation. The pyruvate effect observed in this study is probably attributable to the activation of intracellular metabolism by pyruvate under electron acceptor-limited conditions or a specific sensing and responding mechanism for pyruvate. The 2-h time window suggests a hierarchical regulation in *S. oneidensis* for sensing and responding to pyruvate.

When looking into the transcriptional profile of *S. oneidensis* after incubation with pyruvate or lactate for 2 h, we found that genes responsible for the catabolism of carbon sources and electron transfer to Pd(II) are differentially regulated between pyruvate-incubating and lactate-incubating cells. Not surprisingly, lactate-incubating cells strengthened lactate catabolism by increasing transcription of lactate dehydrogenase (LldEFG). To our surprise, formate dehydrogenases FDH (FdhA1B1C1 and FdhA2B2C2), rather than pyruvate formate-lyase (PflAB), were upregulated by pyruvate compared with lactate (Table 1 and Fig. 5). PFL catalyzes the reversible conversion of pyruvate and CoA into acetyl-CoA and formate under anaerobic conditions, providing the sole source of acetyl-CoA for the citric acid cycle during bacterial fermentation. The level of PFL determines the carbon flux under anaerobic conditions. For example, it controls the shift between homolactic acid to mixed-acid fermentation in lactic acid bacteria (26). Formate formed from pyruvate is proposed to be the main redox intermediate that serves as an electron donor for anaerobic respiration in *S. oneidensis* (25). Our data suggest that FDH, rather than PFL, is the key enzyme of anaerobic catabolism, and formate oxidation rather than formate production is the rate-determining step of electron generation from the catabolism of pyruvate or lactate under anaerobic conditions. In this context, FDH might be a target for engineering and improving EET in *S. oneidensis*.

Electrons from the quinone pool are transferred to hydrogenases or quinone dehydrogenases depending on available electron acceptors, such as Pd(II) in this study (Fig. 6). Hydrogenases and the Mtr pathway, two branches of the electron transfer pathway involved in Pd(II) reduction, are both upregulated by pyruvate (Table 1, Fig. 5, and Fig. 6). The improvement in both electron generation and the electron transfer pathway largely explains the accelerated effect of pyruvate on Pd(II) reduction. Interestingly, the EET pathway for DMSO respiration is also strengthened by pyruvate. Consistently, pyruvate supports better growth when DMSO is used as an electron acceptor, while lactate supports better growth when fumarate is used (Fig. S4). In contrast, the terminal reductase of fumarate respiration (FccA) has no change in transcription levels in cells incubated with pyruvate or lactate (Table S2 in the supplemental material).

Oxidation of pyruvate or lactate might result in a difference in metabolic state, especially energy level, which probably affects the ability of *S. oneidensis* to reduce Pd(II). Two indicators, membrane potential and the NAD(H) pool, are adapted to evaluate the energy level of *S. oneidensis* incubated with pyruvate or lactate. Both indicators are shown to correlate with an EET rate that largely depends on the Mtr pathway. Given the involvement of the Mtr pathway in Pd(II) reduction, membrane potential and/or the NAD(H) pool is probably involved in the pyruvate effect on Pd(II) reduction. The intracellular NAD(H) is an important redox intermediate in *S. oneidensis* under aerobic growth. Although *S. oneidensis* might not couple NADH oxidation to fumarate or Fe(III) citrate reduction (25), the expression of an NAD⁺-dependent formate dehydrogenase, improvement in NAD⁺ biosynthesis, and acceleration of NADH regeneration all improve the EET rate of *S. oneidensis* in bioelectrochemical reactors (16, 17, 22). In this context, the intracellular content of NADH and NAD⁺ is supposed to affect the Pd(II) reduction, at least through the Mtr pathway. However, no difference is observed either

FIG 6 Model of pyruvate regulation of catabolism and electron flows for Pd(II) reduction in *S. oneidensis*. Pyruvate upregulates transcription of formate dehydrogenases (FdhX1A1B1C1 and FdhX1A2B2C2), hydrogenase (HyaAB), and the Mtr pathway (CymA, MtrA, and MtrABC). Yet-unidentified proteins respond to and mediate regulation of pyruvate on the expression of these genes. Formate dehydrogenases oxidize formate and reduce menaquinone (MQ) to menaquinol (MQH₂), which further transfers electrons to HyaAB and CymA. Hydrogenase HyaAB directly reduces Pd(II), achieving intracellular reduction of Pd(II). Meanwhile, electrons from CymA transfer to MtrA, the OmcA/MtrC complex, and finally to Pd(II), achieving EET for Pd(II) reduction.

in content or in the ratio of NADH and NAD⁺ in cells fed with pyruvate, lactate, or none of the carbon sources for 2 h (Fig. 5). Although we cannot exclude its possible contribution to Pd(II) reduction, the NAD(H) pool does not play a role in the acceleration effect of pyruvate compared with lactate. Membrane potential, recently reported as an energetic indicator strongly correlated with EET rate, also has no difference in cells, no matter whether cells are allowed to oxidize a carbon source. Although Pd(II) reduction was positively correlated with pyruvate concentration (Fig. 1), pyruvate consumption levels were similar in cultures incubated with pyruvate at different concentrations (Table S1). In contrast, the expression of a representative operon (*hyaAB*) positively correlated with the pyruvate concentration. Therefore, these experimental data indicated that the acceleration effect of pyruvate on Pd(II) was primarily attributable to transcriptional regulation.

The high efficiency of electron transfer depends not only on the rate of electron transfer but also on the rate of electron generation from catabolism of carbon sources. Efforts have been devoted to improving one of the above aspects for better EET efficiency of *S. oneidensis* (17, 27). This study revealed that both EET pathway and catabolism of carbon sources were differentially regulated in *S. oneidensis* fed with pyruvate

TABLE 2 Strains, mutants, and plasmids used in this study

Strain, mutant, or plasmid	Description	Reference no. or source
<i>S. oneidensis</i> strain or mutant		
MR-1	Wild type	32
$\Delta omcA \Delta mtrC$ mutant	MR-1 derivative with double deletion of SO_1778 and SO_1779	33
$\Delta hydA \Delta hyaB$ mutant	MR-1 derivative with double deletion of SO_2098 and SO_3920	20
$\Delta hydA \Delta hyaB \Delta omcA \Delta mtrC$ mutant	MR-1 derivative with deletion of <i>hydA</i> , <i>hyaB</i> , <i>omcA</i> , and <i>mtrC</i>	This study
$\Delta menA$ mutant	MR-1 derivative with deletion of SO_1910	20
<i>E. coli</i> strain		
JM109	General cloning host for plasmid manipulation	Lab stock
WM3064	Auxotrophic to DAP; used for conjugation	34
Plasmid		
pRE112	Suicide vector, Cm ^r	35
pRE-SO2098	pRE112 derivative to delete <i>hyaB</i>	20
pRE-SO3920	pRE112 derivative to delete <i>hydA</i>	20

versus lactate (Fig. 6). As we discussed above, *S. oneidensis* required time to sense and respond to pyruvate in terms of regulation of electron transfer pathway and pyruvate catabolism. A previous publication reported that the increase in the level of bis-(3'-5') cyclic dimeric GMP (c-di-GMP), an intracellular secondary messenger, results in elevated expression of OmcA and MtrC (28). The transcription analyses in this study revealed that several genes presumably involved in c-di-GMP metabolism, sensing, or response were differentially regulated in *S. oneidensis* incubated with pyruvate versus lactate (data not shown), suggesting c-di-GMP is a signal mediating the pyruvate regulation on physiology discussed above. Further investigation is underway to explore whether and how *S. oneidensis* senses and transforms the signal of pyruvate into the signal of c-di-GMP that further regulates downstream targets. Improvements in electron generation and transfer enable *S. oneidensis* to reduce toxic Pd(II) efficiently and thus avoid Pd(II) toxicity. In this sense, the Pd(II) reduction might be a good model to deeply investigate the mechanism of such hierarchical regulation because toxic Pd(II) presumably stops transcriptional regulation at desired stages.

MATERIALS AND METHODS

Strains and growth conditions. Bacterial strains were cultured in lysogeny broth (LB) medium (10 g/liter yeast extract, 5 g/liter tryptone, and 10 g/liter NaCl) or mineral medium (MM) [7.956 mM NaCl, 1.93 mM (NH₄)₂SO₄, 0.157 mM MgSO₄, 1.29 mM K₂HPO₄, 1.65 mM KH₂PO₄, 30 mM HEPES, 0.04 mM nitrilotriacetic acid, 34 μ M CaCl₂, 11.88 μ M MnSO₄, 3.2 μ M CoSO₄, 3.13 μ M ZnSO₄, 1.8 μ M FeSO₄, 0.526 μ M NiCl₂, 0.21 μ M KAl(SO₄)₂, 0.2 μ M CuSO₄, 0.8 μ M H₃BO₃, 0.2 μ M Na₂MoO₄, and 0.01 μ M Na₂SeO₃, pH 7.5] (20). Pyruvate, lactate, or formate was added into MM to a final concentration of 20 mM unless otherwise indicated. To prepare anaerobic mineral medium (AMM), the mineral medium with or without pyruvate, lactate, or formate was added into serum bottles, flushed with nitrogen gas for 10 min to purge oxygen, and finally sealed in bottles with aluminum covers and butyl rubbers. Stock solutions of Na₂PdCl₄ were treated similarly to AMM for the removal of oxygen and sealed in serum bottles until use. Strains of *Escherichia coli* and *S. oneidensis* listed in Table 2 were cultured at 37°C and 30°C, respectively.

Chemicals were added when needed at the following concentrations: 100 μ g ml⁻¹ diaminopimelic acid, 50 μ g ml⁻¹ kanamycin, 34 μ g ml⁻¹ chloramphenicol, and 20 μ g ml⁻¹ gentamicin for strains of *E. coli* and 17 μ g ml⁻¹ chloramphenicol and 10 μ g ml⁻¹ gentamicin for strains of *S. oneidensis* (20).

Strain construction. Mutants with an in-frame deletion of desired genes were constructed as described previously (29). Briefly, two flanking regions of a target gene were amplified, ligated, and inserted into a suicide vector, pRE112. The generated plasmid was transformed into *E. coli* WM3064 and then introduced into the wild-type strain (WT) or other mutants of *S. oneidensis* by conjugation. After a two-step selection, mutants were obtained with the deletion of a target gene and confirmed by sequencing. All strains and plasmids used in this study are listed in Table 2.

Pd(II) reduction by resting cells and quantification. Resting cells were obtained by cultivating strains of *S. oneidensis* in LB medium under aerobic conditions. After overnight growth, cells were collected by centrifugation (3,500 $\times$ g, 5 min) and washed three times with and suspended in MM. Resuspended resting cells were injected into AMM (supplemented with pyruvate, lactate, formate, or none of the carbon sources) to a final concentration of an optical density at 600 nm (OD₆₀₀) of 0.2. For preparation of heat-killed cells, resting cells sealed in serum bottles were incubated at 80°C for 60 min with occasional shaking. After resting or after heat-killed cells were incubated in AMM for the indicated

TABLE 3 Primers used in qRT-PCR analysis

Gene	Primer and sequence
<i>gyrB</i>	RT-SO0011-F, ACCAAGCGATTCTGCCACTTA RT-SO0011-R, AGCGTAGCCACTTCCTGAGA
<i>hyaC</i>	RT-SO2097-F, GAACCATCTGAAACCCGCATT RT-SO2097-R, CAGTGCTCGTAACCACTGGAA
<i>hyaB</i>	RT-SO2098-F, CAGCGATAGTGCGAATAGGTACAT RT-SO2098-R, CGTTATGGCGTGGTATTGAAGTCA
<i>omcA</i>	RT-SO1779-F, CGGCGTTGAAGATGTTGTAGC RT-SO1779-R, GGAATGGTTGGTCTTGATGTAAG
<i>mtrA</i>	RT-SO1777-F, CGCCAGCAACGCCTACTTA RT-SO1777-R, AAGCAGCTTCTTCCACCACTAA
<i>mtrB</i>	RT-SO1776-F, GCAACCGCCTTCTACAATTACC RT-SO1776-R, GCACCGACCACATCTACCTG
<i>mtrC</i>	RT-SO1778-F, AAGCCGACATGCCAGTGATT RT-SO1778-R, GAGCCTAAGCCTTGCCAGTT
<i>cymA</i>	RT-SO4591-F, TTGGTATCGTGATTGGTGTGTG RT-SO4591-R, GGCAAGACATACAGAACGCATC
<i>dmsB</i>	RT-SO1430-F, GTAAGGTGATGACCAAGTGTGATG RT-SO1430-R, AGTGCCCGTAAAGGACAAGAAT
<i>fdhA1</i>	RT-SO4509-F, CGCTCATGCCTGTGCCAAG RT-SO4509-R, TCGGTCTCAATCGTCGCAAT
<i>fdhA2</i>	RT-SO4513-F, ACGAGATTCTTCAACCACTT RT-SO4513-R, AGACGCTCCAAGAGAACGACTT

time, the stock solution of Na_2PdCl_4 was injected into serum bottles to a final concentration of $100\ \mu\text{M}$ unless otherwise indicated.

At indicated time points, aliquots were withdrawn from serum bottles using syringes and centrifuged at $13,000 \times g$ for 10 min to remove cells. Supernatants were subjected to Pd(II) quantification using the colorimetric 4-(2-thiazolylazo) resorcinol assay (30). The Pd(II) concentrations in samples were determined according to the standard curve of Pd(II) solution at concentrations over a range of 0 to $100\ \mu\text{M}$.

RNA extraction and transcriptome analysis. Resting cells were incubated in AMM containing 20 mM pyruvate or 20 mM lactate for 2 h. Five percent phenol solution (pH 4.5) chilled on ice was injected into serum bottles to a final concentration of 1%. Four biological replicates of preincubated cells were combined and collected by centrifugation ($12,000 \times g$, 4°C). Collected cells were subjected to extraction of total RNA using the TRIzol reagent (TaKaRa Biotechnology, Shandong, China) according to the manufacturer's instructions. Total RNA was further purified using RNA clean kit (Biotek, Beijing, China). The concentration, quality, and integrity of total RNA were determined using a NanoDrop spectrophotometer (Thermo Fisher Scientific, MA, USA) and an Agilent 2100 bioanalyzer (Agilent Technologies, CA, USA). Qualified total RNA was used to construct a sequencing library that is subsequently sequenced on a HiSeq platform (Illumina, New York, NY, USA), which was performed by Shanghai Personal Biotechnology Co. Ltd.

qRT-PCR. cDNA was synthesized using 500 ng of qualified total RNA and PrimeScript RT reagent kit with gDNA eraser (TaKaRa Biotechnology, Shandong, China). qRT-PCR was performed using the SYBR Premix Ex Taq kit (TaKaRa Biotechnology, Shandong, China) and LightCycler96 (Roche, Mannheim, Germany). *gyrB* was used as the reference gene. The relative expression value of target genes was obtained from three determinations with normalization against *gyrB* using the threshold cycle method ($2^{-\Delta\Delta\text{CT}}$). The primers used for qRT-PCR analysis are listed in Table 3.

Measurement of β -galactosidase activity. Aliquots of preincubated cells were withdrawn from serum bottles and used for determination of OD_{600} and activity of β -galactosidase. β -galactosidase activity in cells was determined as described previously (20).

Hydrogen quantification. We removed 0.9-ml gas samples from the headspace of serum bottles at indicated time points and injected them into a gas chromatograph equipped with a thermal conductivity detector, model SP-6800A6 (Lunan Inc., China), and a 2-m by 3-mm stainless steel packed column, TDX-01 (Jiedao Inc., China) for hydrogen quantification. The temperatures of the injector, column, and detector were kept at 100°C , 80°C , and 105°C , respectively, and argon gas was used as a carrier gas.

Quantification of intracellular NADH and NAD^+ . After incubation in AMM with or without a carbon source for 2 h, resting cells were transferred and sealed in centrifuge tubes in an anaerobic glove box and then collected by centrifugation ($12,000 \times g$ at 4°C for 5 min) at ambient conditions. The pellets were added with $600\ \mu\text{l}$ cold lysis buffer from an NAD^+/NADH assay kit with WST-8 (Beyotime Biotechnology, Shanghai, China) and lysed using an automatic freezer mill, JXFSTPRP-CL (Jingxin Industrial Development Co., Shanghai, China). The lysate was centrifuged ($12,000 \times g$ at 4°C for 20 min), and the resulting supernatant was used for quantification of NAD^+ and NADH levels using an NAD^+/NADH assay kit with WST-8 (Beyotime Biotechnology, Shanghai, China).

Quantification of protein concentration. The lysate used for NADH and NAD⁺ determination was also used to determine the protein concentration using a Pierce bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific, MA, USA) according to the manufacturer's instructions.

Measurement of membrane potential. Resting cells were incubated in AMM containing 20 mM pyruvate, 20 mM lactate, 20 mM formate, or none of the carbon sources. After 2 h, aliquots were withdrawn from serum bottles and added to 3,3'-diethyloxycarbocyanine iodide [DiOC₂(3)] (Merck KGaA, Darmstadt, Germany) to a final concentration of 100 μ M in an anaerobic glove box. For depolarization of cells, carbonyl cyanide 3-chlorophenylhydrazone (CCCP) (Merck KGaA, Darmstadt, Germany) was added, together with DiOC₂(3), to a final concentration of 250 μ M. After staining for 30 min, cells were analyzed on a Beckman flow cytometer, CytoFLEX (Beckman Coulter, IN, USA), to determine green and red fluorescence. Average fluorescence intensity was calculated from data of flow cytometer analysis using FlowJo software (BD, CA, USA). The ratio of average red to green fluorescence intensity is used as an indicator of membrane potential (31).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.7 MB.

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