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EXPERIMENTAL ARTICLES

Effect of NAD$^+$-Dependent Formate Dehydrogenase on Anaerobic Respiration of *Shewanella oneidensis* MR-1

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Abstract—An expression plasmid was constructed in order to carry out heterologous expression of the gene of the NAD$^+$-dependent formate dehydrogenase (FDH) from methylotrophic bacterium *Moraxella* sp. in the cells of *Shewanella oneidensis* MR-1 under aerobic and anaerobic conditions. In both modes of cell cultivation, recombinant FDH activity was revealed in the cell lysate of the transformants. In the medium with lactate as a carbon source, the rate of anaerobic respiration determined as the rate of conversion of fumarate (the electron acceptor) to succinate was higher in the transformant with recombinant FDH. Anaerobic cultivation of the FDH-containing transformant of *S. oneidensis* MR-1 in a microbial fuel cell (MFC) revealed increased current density.

Keywords: microbial fuel cells, NAD$^+$-dependent formate dehydrogenase, anaerobic respiration, *Shewanella oneidensis* MR-1

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The electrogenic facultative anaerobe *Shwanella oneidensis* MR-1 belongs to the class *Gammaproteobacteria*, order *Alteromonadales*. Since bacteria of this strain are able to reduce insoluble metal oxides, they are promising agents for bioremediation of contaminated soils and water [1]. Capacity of *S. oneidensis* MR-1 for electron transfer to the outer membrane in the course of anaerobic respiration due to numerous multiheme cytochromes and the broad spectrum of electron acceptors make *S. oneidensis* MR-1 an attractive component of microbial fuel cells (MFC) [2, 3].

Since the genome of *S. oneidensis* MR-1 has been completely sequenced, this organism may be considered a subject for genetic engineering manipulations aimed primarily at increasing its potential as the major MFC component [4]. Utilization of *S. oneidensis* MR-1 as a recipient for heterologous expression of various genes using the plasmid vectors developed for *E. coli* was recently reported [5, 6]. We established the possibility of using the promoter of the *E. coli udp* gene for heterologous expression in *S. oneidensis* MR-1 under aerobic and anaerobic conditions [7].

The goal of the present work was to intensify the process of anaerobic respiration by enhancing the biosynthesis of NADH equivalents. For this purpose, heterologous expression of the *fdh* gene encoding the NAD$^+$-dependent formate dehydrogenase (FDH, EC 1.2.1.2) from the methylotrophic bacterium *Moraxella* sp. under control of the promoter–operator region of the *E. coli* uridine phosphorylase gene (*udp*) was carried out under aerobic and anaerobic conditions. The rates of fumarate reduction to succinate were determined at different stages of anaerobic cultivation. The current density in MFC was determined for the FDH-containing transformant.

MATERIALS AND METHODS

Strains and plasmids. *E. coli* strain JM110 was obtained from the All-Russian Collection of Industrial Microorganisms, State Research Institute of Genetics and Selection of Industrial Microorganisms. The strain *S. oneidensis* MR-1 (no. CIP106686) was obtained from the collection of the Pasteur Institute (France). The pER plasmid containing the kanamycin resistance gene and the promoter–operator region of the *E. coli udp* gene has been constructed in our laboratory [7]. The pFDH vector containing the cloned sequence of NAD$^+$-dependent format dehydrogenase from *Moraxella* sp. was kindly provided by V. Tishkov (Moscow State University).

Media and cultivation conditions. For *E. coli* cultivation, liquid or solid Luria–Bertani medium (LB) was used at 37°C [8]. Selective cultivation of plasmid-

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Table 1. Oligonucleotides used in the work

No.	Name	Sequence, 5'–3'	Site
1	FDB	ATATAGATCTATGGCCAAGGTTGTTTGCGTT	*Bgl*II
2	FDX	ATATCTCGAGTCAGGCGTCGAGCTTTTCGT	*Xho*I

Note: The artificially introduced restriction sites are underlined.

containing *E. coli* cells was achieved using ampicillin (150 μg/mL). For cultivation of *S. oneidensis* MR-1, TSB medium (Tryptic Soy Broth, Sigma, United States) was used (40 g per 1 L of distilled water) at 30°C. Selective growth of the transformants was achieved using kanamycin (50 μg/mL). Anaerobic cultivation of *S. oneidensis* MR-1 was carried out in TSB medium or in minimal medium (MM) [9] supplemented with sodium lactate (2 g/L) as a carbon source and sodium fumarate (2.4 g/L) as an electron acceptor. Sterile plastic Falcon tubes (15 mL) were used for cultivation [9]. Prior to anaerobic cultivation, the transformants were grown aerobically for 18 h to accumulate the biomass, which was then transferred into fresh medium for anaerobic cultivation. Formate concentration varied from 0 to 10 g/L. In MFC, the cells were grown in MM medium with sodium lactate (4 g/L) and kanamycin.

Preparation of *S. oneidensis* MR-1 strains for MFC. The transformants containing the plasmids pERFDH and pER (K–) (further on *S. oneidensis* MR-1/pERFDH and *S. oneidensis* MR-1/pER) were grown aerobically on a rotary shaker (240 rpm) in 750-mL flasks containing 100 mL of TSB medium with kanamycin for 18 h. The cells were harvested by centrifugation at 6000 *g* for 20 min, washed with physiological saline, and centrifuged again under the same conditions. The biomass was resuspended in synthetic medium with the relevant supplements. The suspension was adjusted to equal OD and aseptically introduced into the MFC. The titer of viable cells was determined by plating the relevant dilutions onto TSB agar.

Determination of organic acids in the culture liquid. The samples for determination of the concentrations of organic acids (fumarate, lactate, formate, and succinate) were collected from anaerobic cultures at time intervals depending on the goals of the experiments. The aliquots (1 mL) were centrifuged for 10 min at 10000 *g*, and the supernatant was used for HPLC analysis on an Allyans liquid chromatograph (Waters, United States). The column used was C18 (250 × 4.6 mm, 5 μm); the eluent contained phosphoric acid (0.1%), acetonitrile (0.5%), and methanol (0.5%). The flow rate was 1 mL/min. The eluate registration was carried out at 210 nm. For each experimental series, the results of three independent experiments are presented as averages and standard deviations. Statistical treatment was carried out for three independent experiments using the StatPlus2007 software package.

DNA manipulations. DNA isolation and purification, as well as ligation and transformation of *E. coli* cells with plasmid DNA,were carried out according to [8]. Transformation of *S. oneidensis* MR-1 cells with the plasmid DNA was carried out as described in [6].

Polymerase chain reaction was carried out in an Eppendorf Mastercycler gradient amplifier (Eppendorf, Germany) in 20–25 μL, at 2–3 mM $MgCl_2$, 0.125–0.2 mM of each dNTP, 67 mM Tris–HCl (pH 8.3), 16.5 mM $(NH_4)_2SO_4$, 0.5 U *Taq* polymerase, 1–10 ng template DNA, and 5 pmol of each primer. The amplification was carried out as follows (°C/s): 1 cycle 95/120; 25–30 cycles 95/10, 60/10, 72/20; 1 cycle 72/180.

Oligodeoxyribonucleotide synthesis was carried out using an ASM-800 automatic sequencer (Biosset, Russia) and oligonucleotides were purified according to [10]. The structures of synthetic oligonucleotides used in the work are presented in Table 1.

DNA primary structure was determined by Sanger sequencing on a Beckman Coulter automatic sequencer (United States) in the Biotechnological Service Center, State Research Institute of Genetics and Selection of Industrial Microorganisms.

The cells were homogenized in an Ultrasonic Procession disintegrator (Cole Parmer, United States).

FDH activity was determined according to [11]. Statistical treatment was carried out for three independent experiments using the StatPlus2007 software package.

Protein concentration was determined by the Bradford method [12].

Microbial fuel cell used in the work was an MTE1 element, a two-chamber cell with an MK-40 cation exchange membrane (Shchekinoazot, Russia). The volume of each cell was 295 cm^3; the areas of the cathode and anode were 15.5 and 64 cm^2, respectively. The electrodes were connected via an electric chain with a 150 kΩ resistive load. The parameters were monitored throughout the experiment using the LabVIEW software package (National Instruments, United States). The stability of the fuel cell and the reproducibility of the MTE1 parameters were described in [13].

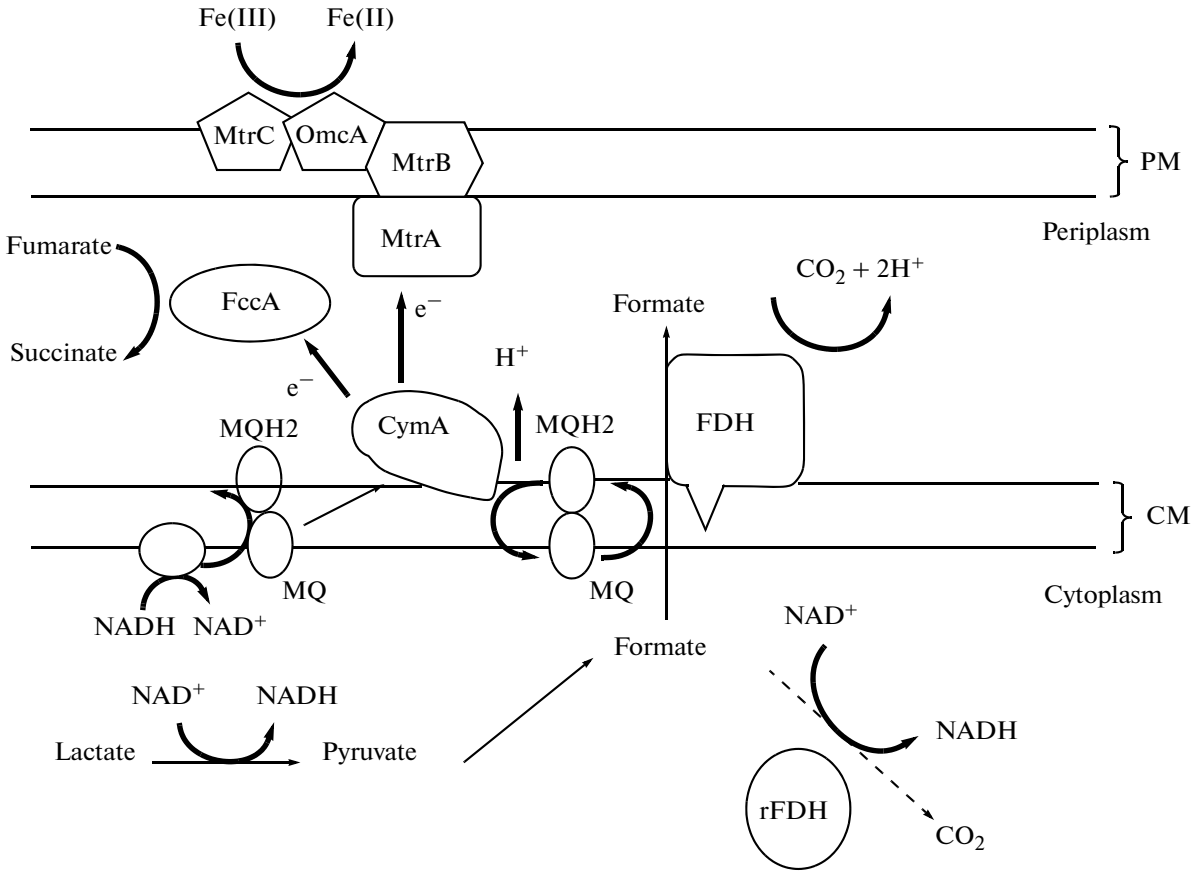

Fig. 1. Scheme of electron transfer in the electron transport chain of *S. oneidensis* MR-1 during anaerobic respiration coupled to lactate utilization. The alternative pathway of formate oxidation in the case of heterologous expression of NAD^+-dependent formate dehydrogenase is indicated by a dotted arrow. Designations: CM, cytoplasmic membrane; PM, periplasmic membrane; CymA, tetraheme cytochrome; FccA, fumarate reductase; MQ, menaquinones; FDH, NAD-independent formate dehydrogenase; rFDH, recombinant NAD^+-dependent formate dehydrogenase from *Moraxella* sp.

RESULTS AND DISCUSSION

S. oneidensis MR-1 is widely used for investigation of the mechanisms of anaerobic respiration and for electricity generation in MFC. Since the organism does not utilize glucose as a carbon source, it is grown on lactate, acetate, or pyruvate [14]. Formate produced in the course of lactate utilization is probably transported into the periplasmic space and is reduced to CO_2 and $2H^+$ by the periplasmic NAD^+-independent formate dehydrogenase, which is localized at the outer side of the cytoplasmic membrane (Fig. 1) [15]. In the course of anaerobic respiration, the electrons are transported to the terminal acceptors (nitrate, nitrite, DMSO, Fe(III), fumarate, etc.). Various cytochromes and their complexes are involved, depending on the type of the electron acceptor and its localization (outside the cell or in the periplasmic space) (Fig. 1). The membrane-bound tetraheme cytochrome CymA localized at the outer surface of the cytoplasmic membrane plays the key role in electron transport from menaquinones to various chains [16]. According to McMillan et al. [17], the electrons are transported to CymA through the cytoplasmic membrane (CM) by menaquinones, which receive them from dehydrogenases localized at the CM inner surface. This process is coupled to proton transport into the periplasmic space and NAD^+ formation [17]. Heterologous expression of NAD^+-dependent FDH from methylotrophic yeast *Candida boidinii* in *E. coli* cells was previously shown to result in increased NADH content [18]. This phenomenon may be used to enhance the electrogenic capacity of *S. oneidensis* MR-1 by heterologous expression of the genes of NAD^+-dependent FDH.

Obtaining the transformant for FDH heterologous expression. The *fdh* gene encoding NAD^+-dependent FHD from the methylotrophic bacterium *Moraxella* sp. was amplified with the pFDH plasmid DNA using the FDB–FDX pair of oligonucleotide primers and cloned under control of the *E. coli udp* promoter at the sites *Bg*III–*Xho*I in the previously constructed pER plasmid [7]. The structure of the hybrid gene was confirmed by sequencing. The pERFDH plasmid was used to transform *S. oneidensis* MR-1. The transfor-

Table 2. Activity of recombinant FDH in the cell lysate of *S. oneidensis* MR-1 transformants grown under aerobic and anaerobic conditions at different formate concentrations

Formate, g/L	FDH activity, U/mg total protein			
	S. oneidensis MR-1/pERFDH		*S. oneidensis* MR-1/pER	
	Aerobic	Anaerobic	Aerobic	Anaerobic
0	0.11 ± 0.01	0.48 ± 0.01	*	
0.5	0.09 ± 0.02	–	*	–
5	–	0.51 ± 0.02	–	*
10	–	0.46 ± 0.02	–	

Note: Designations: *S. oneidensis* MR-1/pERFDH, *S. oneidensis* MR-1 transformant containing the pERFDH plasmid; *S. oneidensis* MR-1/pER, *S. oneidensis* MR-1 transformant containing the pER plasmid (control strain); "*", no activity found (>0.01 U/mg); "–", cultivation was not carried out under these conditions. The data obtained in three independent experiments are presented as averages and standard deviations for each experimental series.

mants were grown in TSB medium with kanamycin for 18 h under aerobic conditions and for 24 h under anaerobic conditions. All selected transformants were stable. FDH activity was determined in order to confirm heterologous expression of the *fdh* gene. The transformants were grown in full TSB medium under anaerobic and aerobic conditions at sodium formate concentrations of 0–3 and 0–0.5%, respectively. Formate concentration for cultivation depended on survival of the transformants *S. oneidensis* MR-1/pERFDH and *S. oneidensis* MR-1/pER at different formate concentrations. No viable cells were found at formate concentrations of 0.2% (aerobically) and 3% (anaerobically). For further assessment of capacity for oxidation of exogenous formate, its concentration was decreased to 0.05 and 1% for aerobic and anaerobic cultures, respectively.

Activity of the recombinant NAD+-dependent FDH in the cell lysates of aerobic and anaerobic cultures at different formate concentrations are listed in Table 2. It can be seen that the heterologous expression of the *fdh* gene under control of the *E. coli udp* promoter occurred under both aerobic and anaerobic conditions. The differences in activity of the recombinant NAD+-dependent FDH under aerobic and anaerobic conditions probably resulted from significant differences in the biomass yield and therefore in the total protein. The levels of FDH activity in the total cell lysate were comparable to the literature data on heterologous expression of *C. boidinii fdh* gene in *E. coli* cells [18].

Aerobic and anaerobic cultivation of *S. oneidensis* MR-1/pERFDH at sublimiting formate concentrations (0.1 and 2%, respectively) did not reveal higher resistance to exogenous formate than in the transformant *S. oneidensis* MR-1/pER. This may indicate the absence of formate transport to the cytoplasm from the periplasm and, therefore, its unavailability for oxidation by recombinant FDH. Higher resistance to formate under anaerobic conditions may result from the presence of NAD-independent FDH, which oxidizes exogenous formate in the periplasm. Expressions of the *fdh* operon genes encoding the subunits of NAD-independent FDH was observed in *S. oneidensis* MR-1 only under anaerobic conditions [15]. Thus, recombinant FDH may oxidize (with formation of additional NADH) only the exogenous formate formed in the cytoplasm during lactate utilization under anaerobic conditions.

In *S. oneidensis* MR-1, transfer of an electron and a proton to fumarate (resulting in succinate production) is carried out in the periplasm by the soluble fumarate reductase FccA [9]. The concentrations of fumarate and succinate were measured in the culture liquid of *S. oneidensis* transformants MR-1/pERFDH and MR-1/pER grown anaerobically on lactate in the presence of fumarate during 3, 6, and 24 h. The data on fumarate and succinate concentrations in the culture liquid of the transformants are presented in Table 3. They indicate that conversion of fumarate to succinate occurred quicker in the *S. oneidensis* MR-1/pERFDH transformant than in *S. oneidensis* MR-1/pER, with a significant difference observed after 6 h of cultivation. This probably resulted from increased levels of intracellular NADH required for fumarate reduction. Succinate produced by fumarate reduction was found in the culture liquid; however, transport into the culture liquid of excessive succinate produced by the open TCA cycle under anaerobic conditions has been reported [9]. This may be the reason for the difference in the concentrations of fumarate and succinate in the culture liquid of the transformants (Table 3).

Importantly, after 24 h all fumarate in the medium was converted to succinate, even in the case of the control strain. Prolonged anaerobic cultivation (over 24 h) probably required higher concentrations of the electron acceptor or its addition to the culture in the feed-batch mode. Accelerated fumarate reduction by the cells containing the recombinant NAD+-depen-

Table 3. Fumarate and succinate concentrations in the culture liquid of anaerobically grown *S. oneidensis* MR-1 transformants

Transformant	Fumarate, mM				Succinate, mM			
	Cultivation time, h				Cultivation time, h			
	0	3	6	24	0	3	6	24
S. oneidensis MR-1/pERFDH	18.4 ± 1.1	14.6 ± 0.9	6.5 ± 0.6	0	0	21 ± 1.1	37.8 ± 1.4	44 ± 1.7
S. oneidensis MR-1/pER	18.4 ± 0.8	14.8 ± 0.6	11.2 ± 0.7	0	0	18.4 ± 0.9	30.8 ± 1.6	44 ± 1.5

Note: Designations: *S. oneidensis* MR-1/pERFDH, *S. oneidensis* MR-1 transformant containing the pERFDH plasmid; *S. oneidensis* MR-1/pER, *S. oneidensis* MR-1 transformant containing the pER plasmid (control strain). The data obtained in three independent experiments are presented as averages and standard deviations for each experimental series.

dent FDH suggested investigation of the transformants in MFC.

Cultivation in MFC. During anaerobic cultivation of the strains in MFC, the electrode acted as the terminal electron acceptor, making it possible to measure the current density directly in the course of anaerobic respiration. To determine the electrogenic characteristics of the transformant strains, they were grown in MM medium with lactate and kanamycin in the MTE1 cells until the current density reached 50% of the maximal value. Electrogenic properties of the recombinant strains (dependence of the current density on the cultivation time) are presented on Fig. 2. It can be seen that the maximal current density achieved with the transformant *S. oneidensis* MR-1/pERFDH was higher than in the case of the control strain. The highest current density was achieved on days 2–3 of the cultivation, as compared to days 6–7 for the control strain. The cultivation was carried out for 27 days.

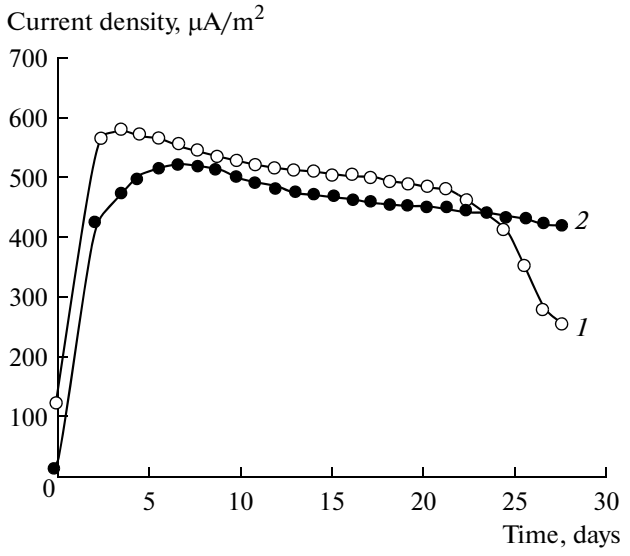

Fig. 2. Current density depending on the time of cultivation for transformants *S. oneidensis* MR-1/pERFDH (*1*) and *S. oneidensis* MR-1/pER (*2*) in an MFC.

After the cultivation, no residual lactate was found in the medium for *S. oneidensis* MR-1/pERFDH, while 0.245 g/L was present in the case of the control strain. While the efficiency of the MFC was higher with *S. oneidensis* MR-1/pERFDH than with the control strain, a drastic decrease in the current density occurred on days 22–23 of the cultivation of the transformant. This may be due to complete consumption of the carbon source (lactate), which was not completely utilized in the case of the control strain. Our data indicated elevated levels of intracellular NADH in the case of heterologous expression of recombinant NAD^+-dependent FDH, resulting in enhanced anaerobic respiration and therefore in higher current density when the strain was grown in an MFC. This tendency, as well as the data on the rates of fumarate conversion by the transformant strain with FDH, indicates the prospects for further investigation and research into approaches for the intensification of MFC operation. Optimization of the cultivation conditions of the transformant strain may result in higher MFC efficiency and stability, as well as to a significant increase in the current density.

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