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EXPERIMENTAL ARTICLES

Mutants of an Electrogenic Bacterium *Shewanella oneidensis* MR-1 with Increased Reducing Activity

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Abstract—The mutants of *Shewanella oneidensis* MR-1 resistant to fosfomycin, a toxic analogue of phosphoenolpyruvate, were obtained. The mutants exhibited increased reducing activity and higher rates of lactate utilization. A correlation was shown between the rates of metabolism of oxidized substrates and the rate of reduction of methylene blue, a mediator of electron transport. The mutants of *S. oneidensis* MR-1 may be used in microbial fuel cells for intensification of energy production from organic compounds.

Keywords: electrogenic bacteria, methylene blue, reducing activity, microbial fuel cells.

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Bacteria of the genus *Shewanella* are considered promising electron generators for microbial fuel cells (MFC), in which electricity is produced as a result of the activity of microorganisms oxidizing organic compounds under anaerobic conditions, efficiently generating electrons, and transferring them to acceptors, including the MFC electrodes [1]. Electron transport from the cytoplasmic membrane of *Shewanella* cells occurs either by direct contact of the cells or their nanowire appendages (pili) with the acceptors, or via the mediators synthesized by the cell or added into the medium [2, 3].

Apart from natural mediators (riboflavin, quinones, etc.), such synthetic dyes as methylene blue, alizarin brilliant blue, N,N-dimethyl disulfonate thionine, phenothiazine, toluidine blue, brilliant cresol blue, gallocyanine, resorufin, etc., may act as electron acceptors [4]. In the case of synthetic dyes, which may differ in both molecular mass and hydrophobicity, apart from the specific terminal cytochromes, other reduced cell compounds may act as direct electron donors. Methylene blue (MB), a traditional electron acceptor, is reduced (discolored) by a number of microorganisms lacking the Mrt-like cytochromes exposed at the surface of the cytoplasmic membrane. Since the rate of MB discoloration depends on dehydrogenase activity and the intracellular content of reduced NAD(P)H equivalents and their derivatives, MB may be used to assess the culture density and the degree of its reduction [5].

The goal of the present work was to obtain the mutants of *S. oneidensis* MR-1 with enhanced reducing activity. For this purpose, the mutants resistant to fosfomycin (FM), a toxic analogue of phosphoenolpyruvate, were obtained. The rate of MB discoloration (reduction) was used to assay the level of reducing activity of the strains. Determination of the rates of MB discoloration by cell suspensions of unified optical density was used to select FM-resistant mutants with increased reducing activity and capacity for reduction of the electron transfer mediator, i.e., the mutants with elevated levels of electron generation.

MATERIALS AND METHODS

Strains. Strain *S. oneidensis* MR-1 was obtained by the Russian National Collection of Industrial Microorganisms (VKPM), State Research Institute for Genetics and Selection of Industrial Microorganisms, from the Pasteur Institute microbial collection (no. CIP106686, France). Strains FRS1 (Fosfomycin-Resistant Small) and FRB1 (Fosfomycin-Resistant Big), the mutants of strain MR-1 resistant to 1000 μg/mL FM and forming on the TSB agar the colonies 2–3 and 5–6 mm in diameter, respectively, were obtained in the present work.

Media and cultivation conditions. Liquid TSB medium (Tryptic Soy Broth, Sigma), 40 g per 1 L of distilled water, and solid TSB (1.7% agar) were used, as well as the liquid synthetic MM medium [6].

The strains were grown under aerobic conditions in 35-mL test tubes with 5 mL of TSB or MM medium or in 750-mL flasks with 100 mL of the medium on a shaker (220 rpm) at 30°C.

Mutagenesis of strain *S. oneidensis* MR-1. The strain was grown for 18 h in liquid TSB medium. The culture was used to inoculate (1% vol/vol) the test tubes containing MM medium with 2.0 g/L lactate.

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The mutagen N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) was dissolved in the phosphate–citrate standard buffer and added to the test tubes in required concentrations. After incubation with the mutagen for 2 h, the cells were centrifuged, washed twice with the physiological saline, and then plated on TSB medium to determine the titer of the suspension, and on TSB supplemented with antibiotics. The frequency of occurrence of antibiotic-resistant mutants was calculated for each antibiotic under different concentrations of the mutagen.

Determination of reducing capacity of *S. oneidensis* MR-1 by methylene blue reduction (discoloration). The strain was grown in liquid medium (TSB or MM with 2.0 g/L sodium lactate). To determine the time of MB discoloration, samples were taken at appropriate time intervals. The samples of different strains were adjusted to the standard optical density and their CFU titer was determined. For determination of the discoloration time, 1 mL of 0.01% MB solution was added to the sample (5 mL). The test tube was then stoppered and the discoloration time was determined under vigorous shaking.

Lactate consumption by strains *S. oneidensis* MR-1, FRS1, and FRB1. The strains were grown on solid TSB medium for 24 h at 30°C. The cells were then resuspended in the physiological saline and used to inoculate the flasks with MM medium (1% vol/vol). Lactate concentration in the medium was 2.0 g/L. The cultivation was carried out under the standard conditions, with samples collected at appropriate time intervals. The samples were centrifuged for 20 min at 6000*g*, and residual lactate was determined in the supernatant. The analysis was carried out using a Waters Allyans high-performance liquid chromatograph (HPLC) with a C18 column (250 ± 4.6 mm, 5 μm) at 1 mL/min flow rate. The mixture of phosphoric acid (0.1%), acetonitrile (0.5%), and methanol (0.5%) was used for elution. Detection was carried out at $\lambda = 210$ nm. Analysis duration was 10 min.

RESULTS AND DISCUSSION

Strain *S. oneidensis* MR-1 is often used as a model organism for investigation of production of electricity in MFC. Low power of the current produced is, however, the major drawback of all MFC. Rearrangement of the oxidative processes in *S. oneidensis* MR-1 in order to increase the rate of electron generation is one of the approaches to higher MFC efficiency.

For this purpose, selection of the mutants of *S. oneidensis* MR-1with higher capacity for reduction of exogenous substrate among the mutants resistant to the antibiotic fosfomycin was used. Fosfomycin (phosphonomycin, (L-*cis*-1,2-epoxypropylphosphonic acid) is synthesized by *Streptomyces* species and is a natural toxic analogue of phosphoenolpyruvate. Its antibiotic properties result from its ability to replace phosphoenolpyruvate in some enzymatic processes, thus blocking them. Fosfomycin irreversibly suppresses the activity of phosphoenolpyruvate: UDP-N-acetylglucosamine enolpyruvate transferase and therefore suppresses the initial stage of murein synthesis in microbial cell wall. Being a phosphoenolpyruvate analogue, fosfomycin also affects (apart from this major target) other phosphoenolpyruvate-dependent enzymes, such as plant phosphoenolpyruvate carboxylase [7]. Fosfomycin-resistant mutants unable to transport fosfomycin and the mutants with decreased affinity of phosphoenolpyruvate transferase to fosfomycin were found in *E. coli* [8, 9], while the mutants with deletions in the FM transport system genes are known in *Salmonella typhimurium* [10]. Since phosphoenolpyruvate plays a central role in the carbon metabolism and forms with modified or enhanced activity of the global enzymatic systems responsible for the transport and oxidation of carbohydrates, production of reduced NAD(P)H equivalents and ATP biosynthesis may be expected among fosfomycin-resistant mutants. In the present work, applicability of fosfomycin for selection of *Shewanella* mutants with increased level of substrate oxidation and more efficient generation of electrons was demonstrated.

Obtaining fosfomycin-resistant mutants. The original strain *S. oneidensis* MR-1 exhibited significant resistance to FM. Survival rates of this strain plated on the TSB medium with FM (100, 200, 400, and 1000 μg/mL) was 55.0, 30.0, 1.0, and 0.0001%, respectively. Spontaneous variants resistant to these concentrations of fosfomycin were unstable and produced sensitive variants in subsequent transfers with the frequency of up to 50%. The absence of stable spontaneous SF-resistant mutants made it necessary to develop a procedure of efficient mutagenesis for strain *S. oneidensis* MR-1.

N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) was used as a mutagen. This compound is often used to obtain mutants of the strains of the order *Alteromonadales*, to which *S. oneidensis* MR-1 belongs. Importantly, strain *S. oneidensis* MR-1 and the taxonomically related strains were highly sensitive to mutagens, so that even low doses resulted in significant cell death. For example, survival rates of *S. oneidensis* MR-1 after UV-irradiation were only ~5% of those for *E. coli* under the same conditions [11]. After treatment with MNNG (2.5 μg/mL) of the sulfate-reducer *Desulfovibrio desulfuricans* G20, which, like *S. oneidensis* MR-1, belongs to metal-reducing bacteria, the cell death rate was 99.7% [12].

Assessment of the sensitivity to MNNG revealed that low concentrations of the mutagen resulted in significant cell death. For example, at 5 μg/mL of the mutagen, the number of viable cells decreased by four orders of magnitude. We developed the conditions of MNNG treatment, the mutagen concentration, and growth medium composition and assessed the efficiency of mutagenesis with selective agents, antibiotics streptomycin (50 μg/mL), kanamycin (100 μg/mL),

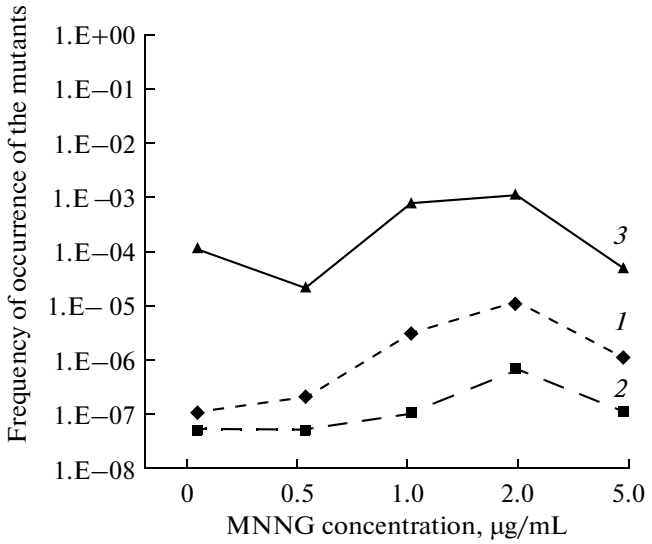

Fig. 1. Frequency of occurrence of the mutants of *S. oneidensis* MR-1 (with MNNG as a mutagen) resistant to different antibiotics. Selecting antibiotics were: streptomycin, 50 μg/mL (*1*), kanamycin, 100 μg/mL (*2*), and fosfomycin, 1000 μg/mL (*3*).

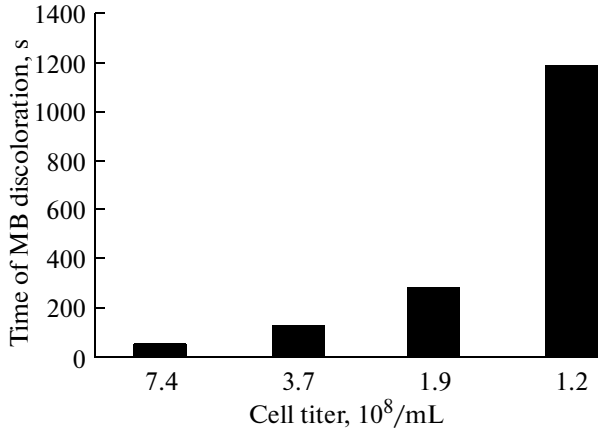

Fig. 2. Time of MB discoloration (reduction) depending on the concentration of *S. oneidensis* MR-1 cells grown in TSB medium.

and fosfomycin (1000 μg/mL). The optimal parameters for mutagenesis were determined, resulting in a 10–100-fold increase in the frequency of the mutants resistant to selective antibiotics (Fig. 1). The highest frequency of antibiotic-resistant mutants was observed at 2.0 μg/mL MNNG. These mutagen concentration and mutagenesis conditions were used for selection of FM-resistant mutants.

Stable mutants were obtained by efficient mutagenesis with subsequent plating of the cells of solid TSB medium with 1000 μg/mL FM. Stability of inheritance of antibiotic resistance in the individual colonies was determined twice. Initially, it was done by streak inoculation of TSB agar with FM. The surviving variants were then plated on TSB media with and without FM and the mutants with 100% survival on the medium with the antibiotic were selected. It should be noted that 20–30% of the examined colonies survived after the initial test of FM-containing medium. After the second test, ~50% of the mutants exhibited stable antibiotic resistance. Two groups of FM-resistant mutants were found, differing in their colony sizes, and designated FRS (Fosfomycin-Resistant Small, 2–3 mm in diameter) and FRB (Fosfomycin-Resistant Big, 5–6 mm in diameter). One typical member of each group was chosen and designated FRS1 and FRB1, respectively. Stability of inheritance of the morphological characteristics and FM resistance was analyzed. For FRS1, the colony size was found to increase insignificantly in subsequent transfers, although it remained less than in the case of FRB1. The efficiency of plating both groups of mutants on TSB medium with 1000 μg/mL FM being 100% and remaining at the same level without supporting selection on the medium with the antibiotic suggests stable inheritance of antibiotic resistance.

MB discoloration (reduction) time depending on the concentration of *S. oneidensis* MR-1 cells in suspensions. The method based on the time of MB discoloration by bacteria is generally used for assessment of microbial contamination in liquid foodstuffs, water, and biological fluids [13]. The higher the cell titer, the less time is required for discoloration of a known amount of MB. Determination of the differences in reducing activity between FM-resistant mutants and the original strain required investigation of MB discoloration time, depending on the number of *S. oneidensis* MR-1 cells in microbial suspensions. For this purpose, the strain was grown in liquid TSB medium under standard conditions. The grown culture was diluted 2-, 4-, and 6-fold with TSB medium. To determine discoloration time, each sample (5 mL) was mixed with 1 mL of methylene blue solution and shaken. The optical density of the sample and the CFU titer were determined. The cell titer in the original culture was 7.4×10^8 cells/mL. It can be seen that MB discoloration time depends significantly on the cell concentration (Fig. 2). For example, the titer decreased two- and fourfold resulted in the doubling of discoloration time. In the sixfold diluted culture, the discoloration time increased significantly. Assessment of potential electrogenicity of different cultures was therefore carried out in suspensions adjusted to the uniform optical density. Time intervals for sampling and analysis of reducing activity were determined. In a growing culture, a correlation was observed between its optical density and the number of living cells determined from CFU number. For example, the correlation was observed in 6-, 18-, and 24-h cultures grown in liquid media. Longer cultivation resulted in higher optical density, while the CFU titer decreased. Impor-

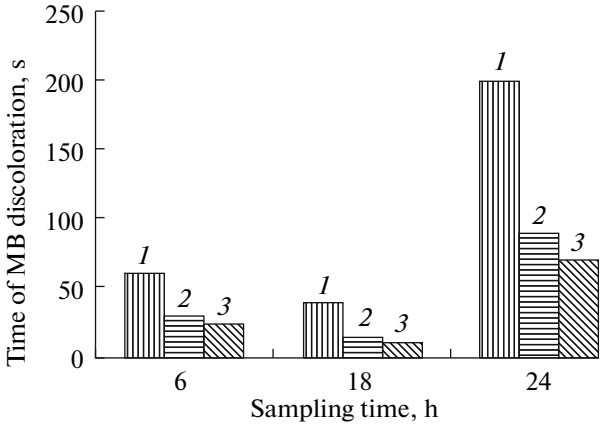

Fig. 3. Dynamics of MB discoloration (reduction) by the original strain *S. oneidensis* MR-1 (*1*) and the mutants FRS1 (*2*) and FRB1 (*3*) grown in TSB medium.

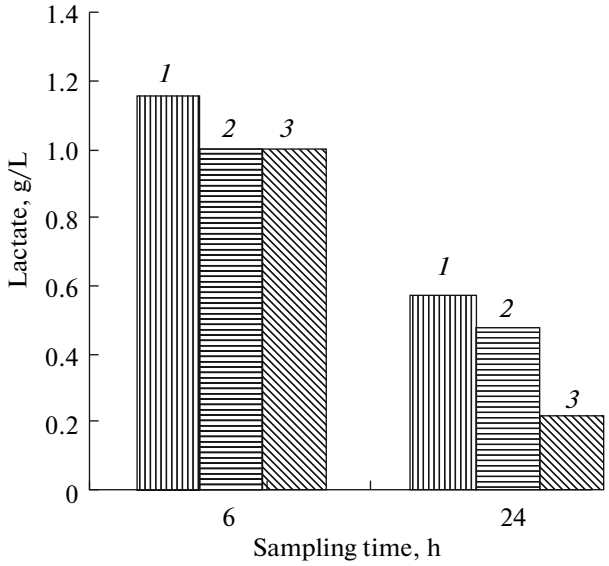

Fig. 4. Dynamics of lactate consumption by the original strain *S. oneidensis* MR-1 (*1*) and the mutants FRS1 (*2*) and FRB1 (*3*) grown in MM medium. Residual lactate concentrations in the culture liquid after 6 and 24 h of cultivation are shown.

tantly, MB discoloration time depended on the physiological state, cultivation conditions, duration of storage, and the pre-inoculation treatment of the strains. While MB discoloration time for each strain varied from experiment to experiment, the relative differences between the mutants and the parent strain were observed in each individual experiment.

Comparative analysis of reducing activity of the original *S. oneidensis* MR-1 strain and its mutants FRS1 and FSB1. Reducing activity of the strains was determined from MB discoloration time. The cultures were grown aerobically in TSB medium and sampled after 6, 18, and 24 h of cultivation. The samples were adjusted to uniform optical density, and MB discoloration time was determined. At all stages of cultivation, the time of MB discoloration by the mutants was at least half than in the case of the original strain (Fig. 3). Strain FRB1 exhibited a higher rate of MB discoloration than strain FRS1. Thus, the mutants resistant to fosfomycin had elevated reducing activity.

Efficiency of lactate consumption by the original *S. oneidensis* MR-1 strain and its mutants FRS1 and FRB1. Lactate, formate, and acetate are the usual carbon and electron sources for *Shewanella* strains under experimental conditions [14]. Lactate utilization was studied to characterize the mutants of *S. oneidensis* MR-1, FRS1 and FRB1. Lactate consumption was analyzed for the cultures grown in liquid MM medium with 2.0 g/L lactate. The results of HPLC analysis of residual lactate in the culture liquid in growth dynamics are shown on Fig. 4. It can be seen that the mutants had higher rates of lactate utilization than the original strain (10 and 30% higher for strains FRS1 and FRB1, respectively). Intensified lactate catabolism in the mutants correlated with increased rates of MB discoloration. For example, after 24 h of cultivation, the rates of MB discoloration by strains FRS1 and FRB1 was 2.2 and 4 times shorter, respectively, than by the original strain (table).

Thus, stable mutants of the electrogenic bacterium *S. oneidensis* MR-1 were obtained, resistant to the antibiotic fosfomycin (a phosphoenolpyruvate analogue), which exhibited high rates of lactate utilization and increased rates of discoloration of the stain methylene blue. These mutants will be used in microbial fuel cells for intensification of energy production from organic compounds.

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Time of methylene blue discoloration (reduction) by the cells of strains *S. oneidensis* MR-1, FRS1, and FRB1 grown under aerobic conditions in MM medium with lactate

Sampling time, h	MB discoloration time, s		
	Original MR-1	FRS1	FRB1
0	410	430	400
6	170	150	130
24	360	160	90

REFERENCES

1. Debabov, V.G., Electricity from Microorganisms, *Microbiology*, 2008, vol. 77, no. 2, pp. 123–131.
2. Gorby, J.A., Janina, S., McLean, J.S., Rosso, K.M., Moyles, D., Dohnalkova, A., Beveridge, T.J., Chang, I.S., Kim, B.H., Kim, K.S., Culley, D.E., Reed, S.B., Romine, M.F., Saffarini, D.A., Hill, E.A., Shi, L., Elias, D.A., Kennedy, D.W., Pinchuk, G., Watanabe, K., Ishii, S., Logan, B., Nealson, K.H., and Fredrickson, J.K., Electrically Conductive Bacterial Nano-Wires Produced by *Shewanella oneidensis* Strain MR-1 and Other Microorganisms, *Proc. Nat. Acad. Sci. U. S. A.*, 2006, vol. 103, pp. 11358-11363.
3. Li, Z., Yao, L., Kong, L., and Liu, H., Electricity Generation Using a baffled Microbial Fuel Cell Convenient for Stacking, *Biores. Technol.*, 2008, vol. 99, no. 6, pp. 1650–1655.
4. Park, D.H. and Zeikus, J.G., Electricity Generation in Microbial Fuel Cells Using Neutral Red as an Electronophore, *Appl. Environ. Microbiol.,* 2000, vol. 66, no. 4, pp. 1292–1297.
5. Novikova, L.M. and Makarevich V.G., Relation between Dehydrogenase Activity of the *Streptomyces aureofaciens* Mycelium and Its Capacity for Tetracycline Biosynthesis, *Antibiotiki*, 1984, no. 2, pp. 735–739.
6. Tang, Y., Meadows, A., Kirby, J., and Keasling, J., Anaerobic Central Metabolic Pathways in *Shewanella oneidensis* MR-1 Reinterpreted in the Light of Isotopic Metabolite Labeling, *J. Bacteriol.*, 2007, vol. 189, no. 3, pp. 894–901.
7. Mujica-Jimenez, C., Castellanos-Martnez, A., and Munoz-Clares, R.A., Studies of the Allosteric Properties of Maize Leaf Phosphoenolpyruvate Carboxylase with the Phosphoenolpyruvate Analog Phosphomycin as Activator, *Biochim. Biophys. Acta*, 1998, vol. 1386, no. 1, pp. 132–144.
8. Kahan, F.M., Kahan, J.S., Cassidy, P.J., and Kropp, H., The Mechanism of Action of Fosfomycin (Phosphonomycin), *Ann. New York Acad. Sci.*, 1974, vol. 235, pp. 364–380.
9. Wu, J. and Venkateswaran, P., Fosfomycin-Resistant Mutant of *Escherichia coli, Ann. New York Acad. Sci.*, 1974, vol. 235, pp. 587–592.
10. Cordaro, J.C., Melton, T., Stratis, J. P., Atagen, M., Gladding, C., Hartman, P.E., and Roseman, S., Fosfomycin Resistance: Selection Method for Internal and Extended Deletions of the Phosphoenolpyruvate: Sugar Phosphotransferase Genes of *Salmonella typhimurium, J. Bacteriol.*, 1976, vol. 128, no. 3, pp. 785–793.
11. Qiu, X., Sundin, G.W., Cha, B., and Tiedje, J.M., Survival of *Shewanella oneidensis* MR-1 after UV Radiation Exposure, *Appl. Environ. Microbiol.*, 2004, vol. 70, no. 11, pp. 6435–6443.
12. Li, X., Lee, R., and Krumholz, L.R., Regulation of Arsenate Resistance in *Desulfovibrio desulfuricans* G20 by an arsRBCC Operon and an arsC gene, *J. Bacteriol.*, 2007, vol. 189, no. 10, pp. 3705–3711.
13. Belikov, V.G., *Uchebnoe posobie po farmatsevticheskoi khimii* (Teaching Aid in Pharmaceutical Chemistry), Moscow, Meditsina, 1979.
14. Serres, M. and Riley, M., Genomic Analysis of Carbon Source Metabolism of *Shewanella oneidensis* MR-1: Predictions versus Experiments, *J. Bacteriol.*, 2006, vol. 188, pp. 4601–4609.