J. Microbiol. Biotechnol. (2011), **21**(3), 305–316
doi: 10.4014/jmb.1006.06022
First published online 14 January 2011

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Selection of Bacteria Capable of Dissimilatory Reduction of Fe(III) from a Long-term Continuous Culture on Molasses and Their Use in a Microbial Fuel Cell

Sikora, Anna[1]*, Justyna Wójtowicz-Sieńko[1], Piotr Piela[2], Urszula Zielenkiewicz[1], Karolina Tomczyk-Żak[1], Aleksandra Chojnacka[1], Radosław Sikora[3], Paweł Kowalczyk[4], Elżbieta Grzesiuk[1], and Mieczysław Błaszczyk[5]

[1]*Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Pawińskiego 5a, 02-106 Warsaw, Poland*
[2]*Industrial Chemistry Research Institute, Rydygiera 8, 01-793 Warsaw, Poland*
[3]*Institute of Radioelectronics, Warsaw University of Technology, Nowowiejska 15/19, 00-665 Warsaw, Poland*
[4]*Interdisciplinary Centre of Mathematical and Computational Modeling, Warsaw University, Pawińskiego 5a, 02-106 Warsaw, Poland*
[5]*Faculty of Agriculture and Biology, Warsaw University of Life Sciences, Nowoursynowska 159, 02-787 Warsaw, Poland*

Received: June 15, 2010 / Revised: November 10, 2010 / Accepted: December 22, 2010

Ferric ion-respiring microorganisms (FRMs) are a group of prokaryotes that use Fe(III) as well as other metals as terminal electron acceptors in the process of anaerobic respiration. Special attention is paid to a biotechnological significance of FRMs because of their potential role in electricity production in microbial fuel cells (MFCs) where the terminal acceptor of the electrons during anaerobic respiration is not a ferric ion but the anode. One of the best known FRMs is the Shewanellaceae family. Most of the *Shewanella* species have been isolated from marine environments. In this report, sugar beet molasses and ferric oxide were successfully used in the selection of a bacterial consortium capable of dissimilatory Fe(III) reduction in a long-term continuous culture. The inoculum was a sample of eutrophic lake bottom sediment. Among the bacteria present in this culture were representatives of the Enterobacteriaceae, and the genera *Pseudomonas*, *Arcobacter*, and *Shewanella*. Two non-marine Fe(III)-reducing *Shewanella*-related clones named POL1 and POL2 were isolated. The abilities of the POL1 and POL2 isolates to metabolize a panel of 190 carbon sources were examined using a BIOLOG assay. The results confirmed the abilities of the shewanellas to utilize a broad range of carbon substrates. The utility of the POL1 and POL2 isolates in H-type MFCs operating on pyruvate or molasses was demonstrated. The operation of the MFC with shewanellas cultured on molasses was shown for the first time. A two-stage character of the fuel cell polarization curves, not previously noted in *Shewanella* MFC studies, was observed.

*Corresponding author
Phone: +48 22 592 3337; Fax: +48 22 658 46 36;
E-mail: annaw@ibb.waw.pl

Keywords: *Shewanella*, molasses, microbial fuel cell, microbial Fe(III) reduction, anaerobic respiration, 16S rRNA

The ferric ion (Fe^{3+}), a common form of iron in the Earth's crust, can serve as an exogenous electron acceptor during microbial respiration. This process is called dissimilatory Fe(III) reduction. When Fe(III) is the dominant or exclusive terminal electron acceptor and the process leads to energy conservation, this is known as ferric ion respiration or Fe(III) respiration. Ferric ion-respiring microorganisms (FRMs) may also use other metals and compounds as terminal electron acceptors in the process of anaerobic respiration. Numerous bacteria are able to reduce Fe(III), but this process does not lead to energy conservation. Such dissimilatory iron reduction often accompanies fermentation and is thought to be a secondary respiratory pathway where ferric ions serve as a sink for excess reducing power [8, 25, 27, 29].

FRMs belong to different phylogenetic groups and include members of the two domains of Prokaryota: Bacteria and Archaea. The best known ferric ion-respiring bacteria are the Geobacteraceae and Shewanellaceae families that belong to the δ-Proteobacteria and γ-Proteobacteria, respectively. In natural environments, particularly those rich in Fe(III) compounds, the end-products of fermentation represent sources of carbon and energy for FRMs [25, 27, 29, 33].

Shewanella is the most frequently recognized genus of the Shewanellaceae family. These facultative anaerobes are widely distributed in marine and freshwater environments [15, 38], although most isolates come from the former. Currently, there are 55 known species of *Shewanella*, only

4 of which (*Shewanella oneidensis*, *S. putrefaciens*, *S. amazonensis*, and *S. decolorationis*) were isolated from non-marine environments (http://www.bacterio.cict.fr/s/shewanella.html). It has been hypothesized that some freshwater shewanellas may be of marine origin; for example, *S. oneidensis* isolated from Oneida Lake (New York, USA) [15]. Bacteria assigned to the genus *Shewanella* are Gram-negative, motile rods, 0.4–0.7 μm wide by 2–3 μm long, that possess a single polar flagellum. Among members of the genus *Shewanella*, there are mesophiles and psychrotolerant species, psychrophiles, piezophiles, piezotolerants, and halophiles [15, 38, 40]. Shewanellas use a wide range of exogenous terminal electron acceptors to receive electrons produced by the oxidation of organic substrates in the process of anaerobic respiration: Mn(IV), U(VI), Tc(VII), Co(III), Cr(VI), Hg(II), sulfur, nitrate, nitrite, sulfate, thiosulfate, selenite, arsenite, iodite, fumarate, glycine, trimethylamine oxide (TMAO), and dimethylsulfoxide (DMSO) [8, 15, 27, 33, 38]. The genomic sequences of *S. oneidensis* MR-1 and other *Shewanella* species combined with DNA microarray data indicate that members of this genus can utilize various organic compounds as sources of energy: organic acids, fatty acids, amino acids, peptides, nucleotides, DNA, and sugars. Previously, it was thought that *S. oneidensis* MR-1, the most highly studied representative of the genus *Shewanella*, was able to respire a restricted range of substrates such as lactate, pyruvate, and formate. It is now known that the list of substrates metabolized by shewanellas is extensive and far from complete [6, 11, 15, 16, 41].

FRMs have received special attention with regards their biotechnological significance because of their potential roles in (i) bioremediation of anaerobic environments contaminated by organic compounds and toxic heavy metals or radionuclides [15, 26, 28, 38] and (ii) electricity production in microbial fuel cells (MFCs). MFCs convert the chemical energy of natural, organic compounds directly into electrical energy with the aid of living microorganisms. One type of MFC involves the use of FRMs that cover the anode and utilize it as the terminal electron acceptor instead of ferric ions. Bacteria possessing such abilities have been named exoelectrogens [23], electricigens [30], electrochemically active bacteria [4], or anode respiring bacteria [43]. The electrochemical activity of shewanellas has been confirmed in many studies [*e.g.*, 17, 18, 23, 39, 50]. The list of genera and species of exoelectrogenic bacteria is still growing [24].

In this report we have (i) shown that sugar beet molasses and ferric oxide were successfully used in the selection of a consortium of bacteria capable of dissimilatory Fe(III) reduction; (ii) isolated two non-marine Fe(III)-reducing *Shewanella*-related clones named POL1 and POL2; (iii) examined the abilities of the POL1 and POL2 isolates to metabolize a panel of 190 carbon sources using a BIOLOG assay; and (iv) presented the operation of an H-type MFC based on shewanellas cultured on molasses and observed a two-stage character of the fuel cell polarization curves, not previously noted in *Shewanella* MFC studies.

Materials and Methods

Source of Microorganisms and Enrichment Technique

The inoculum was collected from a eutrophic, meromictic lake (Kluczysko Lake, Poland; area approx. 0.5 ha, average depth 4 m), at a site where the bottom sludge is not mixed during the spring and autumn homotherms. Direct counting using 4′,6-diamidino-2-phenylindole (DAPI) and fluorescence microscopy demonstrated that 1 cm^3 of the sludge contained 10^{10} bacterial cells. The cultivation medium was M9 medium [36] supplemented with trace elements (1 mg/l $FeSO_4$, 70 μg/l $ZnCl_2$, 100 μg/l $MnCl_2$, 6 μg/l H_3BO_3, 2 μg/l $CuCl_2$, 24 μg/l $NiCl_2$, 36 μg/l Na_2MoO_4, 238 μg/l $CoCl_2$), 1 g/l molasses Ropczyce Sugar Factory, Poland), and 10 mM Fe_2O_3 (POCh, Gliwice, Poland). The medium was boiled and saturated with a stream of pure N_2 or mixture of N_2:CO_2 (80:20) (Air Products, Poland). The bacterial culture was maintained for 30 months at room temperature in a 3 l-packed bed reactor (PBR) made of plexiglass (Fig. 1A). The medium was exchanged at a rate of 1 l per day. In the 21st week of cultivation, the bioreactor was filled with granitic stones (Ø2–3 cm) to act as a solid phase to permit biofilm development on their surface. The working volume of the bioreactor was 1.5 l. In the 60th week of

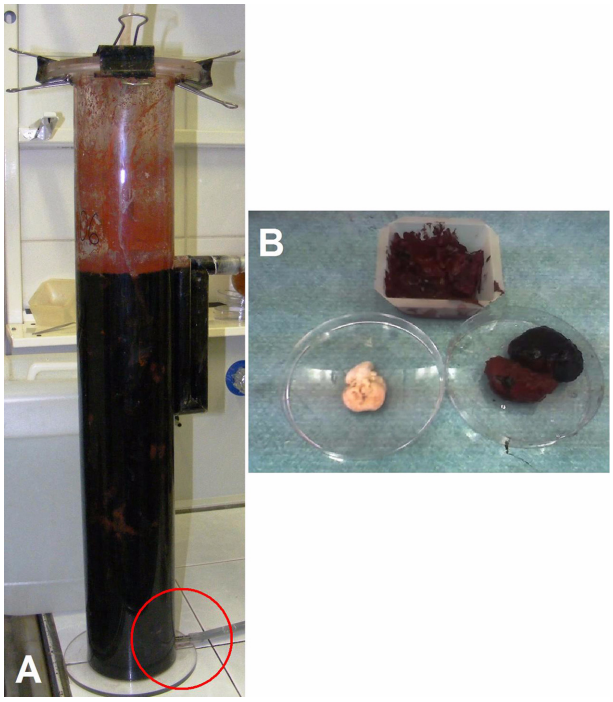


Fig. 1. A culture system for selecting bacteria capable of dissimilatory Fe(III) reduction when grown on M9 medium containing molasses and insoluble ferric oxide.
A. The packed bed bioreactor, with the area from which samples of liquid phase were taken indicated by a circle. **B**. Samples taken from the bioreactor in the 60th week of cultivation: mud in the square dish and two granitic stones taken from the bioreactor in the right Petri dish; a stone that was not put into the bioreactor is shown in the left Petri dish.

cultivation, half of the stones were removed in order to discourage mud formation in the bioreactor, which inhibited the flow of medium. The working volume of the bioreactor was enlarged to 2.2 l.

Analytical Methods

The circle in Fig. 1A shows where samples of liquid phase were taken from the culture for the analyses described below. Samples of the mud that developed after adding stones to the bioreactor were also analyzed. To enumerate bacteria capable of dissimilatory Fe(III) reduction, a 3-tube most probable number (MPN) technique was used. Samples of the culture taken from the bioreactor on selected days of cultivation were diluted in M9 medium containing 1.5 g/l peptone, 1.84 g/l Fe(III) EDTA [ethylenediaminetetraacetic acid, iron (III) sodium salt hydrate] [14], and 200 mg/l acetate. A 10-fold dilution series was prepared in an anaerobic chamber (Coy Laboratory Products, USA) and incubated at room temperature for 3–4 weeks under anaerobic conditions. To detect the reduction of Fe(III), 0.1% ferrozine in 50 mM HEPES was added to each tube. A purple color disclosed the presence of Fe(II), which indicated Fe(III) reduction. MPN values were calculated using McCrady's table [12].

To determine the content of Fe(II) in samples, extracts prepared in 1 M HCl were assayed using the ferrozine colorimetric method according to Lovley and Philips [32]. The absorbance at 562 nm was measured and compared with a calibration curve prepared using dilutions of $FeSO_4$ solution.

The pH of the effluent was measured using a standard pH meter (WTW, inoLab). The chemical oxygen demand (COD) of the medium and the effluent was determined using the dichromate method [35].

Isolation of Culturable Bacteria

To isolate culturable bacteria, selecting those capable of Fe(III) reduction, samples were taken from the bioreactor in the 60th, 124th, and 132nd weeks of cultivation. The samples taken in the 60th week were plated on solid M9 medium saturated with a stream of N_2 supplemented with trace minerals, 1 g/l glucose, 20 mM sodium acetate, and 10 mM ferric citrate. The samples taken in the 124th and 132nd weeks of cultivation were plated on solid NB medium according to Daniel Bond (personal communication) (0.38 g/l KCl, 0.2 g/l NH_4Cl, 0.6 g/l $NaH_2PO_4 \cdot H_2O$, 0.04 g/l $CaCl_2 \cdot 2H_2O$, 0.2 g/l $MgSO_4 \cdot 7\ H_2O$, 2.0 g/l $NaHCO_3$, 1.66 g/l sodium acetate) supplemented with trace minerals (1 mg/l $MnCl_2 \cdot 4H_2O$, 5 mg/l $FeSO_4 \cdot 7H_2O$, 1.7 mg/l $CoCl_2 \cdot 6H_2O$, 1 mg/l $ZnCl_2$, 0.3 mg/l $CuSO_4 \cdot 5H_2O$, 0.05 mg/l $AlK(SO_4) \cdot 12H_2O$, 0.05 mg/l H_3BO_3, 0.9 mg/l Na_2MoO_4, 0.5 mg/l $NiCl_2$, 0.2 mg/l $Na_2WO_4 \cdot 2H_2O$, 1 mg/l Na_2SeO_4) and 100 mM ferric oxyhydroxide (FeOOH). FeOOH was prepared by neutralizing a solution of $FeCl_3$ as described by Lovley and Philips [31]. The medium was saturated with a stream of N_2 and N_2:CO_2 (80:20), respectively, for the isolation of bacteria in the 124th and 132nd weeks of cultivation. All solid media contained Difco agar at 1.5%. All dilutions and plating were performed in an anaerobic chamber and all plates were incubated at room temperature in the anaerobic chamber for one week. Selected colonies were then plated on separate selection plates. All isolates were Gram stained and examined using an optical microscope (Nikon Microphot S.A.).

DNA Manipulations, Sequencing, and Sequence Analysis

Bacterial genomic DNA was extracted using a Genomic Mini isolation kit (A&A Biotechnology) according to the manufacturer's instructions. Approximately 100 ng of DNA was used as the template for PCR amplification of nearly full-length bacterial 16S rRNA gene fragments using the universal primers 27F (5'-AGAGTTTGATCCT GGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). AmpliTaq polymerase (Invitrogen) was used for the isolates obtained in the 60th and 124th weeks of cultivation, whereas MARATHON polymerase (A&A Biotechnology) was employed for isolates obtained in the 132nd week. The reactions were performed using a PTC-200 thermal cycler (MJ Research, Inc., USA) under optimized conditions: 95°C for 5 min; 20 cycles of 95°C for 30 s, 53°C for 30 s, 72°C for 90 s; followed by 15 cycles of 95°C for 30 s, 46°C for 30 s, 72°C for 1.5 min; and a final extension at 72°C for 10 min. Amplification products were purified using a NucleoSpin Extract II kit (Macherey-Nagel).

The PCR products were directly sequenced on an ABI3730 DNA Analyzer (Applied Biosystems) using the primers F27, 1492R, F357 (5'-GCCTACGGAGGCAGCAG-3'), 519R (5'-ATTACCGCGGCTG CTGG-3'), and 926R (5'-CCGTCAATTCCTTTGAGTTT-3'). DNA sequences were assembled using the Linux programs phred/phrap/ consed and checked manually. The obtained 16S rDNA sequences were then compared with those in the NCBI database using BLAST.

A multiple alignment with 43 shewanellas 16S rRNA sequences retrieved from the NCBI Reference mRNA and Microbes Assembled Genomes databases was generated using the program MUSCLE [7] and edited manually using BioEdit (http://www.mbio.ncsu.edu/ BioEdit/ BioEdit.html). Phylogenetic analysis was performed using Phylip package version 3.67 [9]. The bootstrap probabilities were calculated from 1,000 replications and consensus trees were constructed using the methods of neighbor-joining, maximum-likelihood, and parsimony.

Physiological and Biochemical Characterization of *Shewanella* Isolates

For physiological and biochemical characterizations, the *Shewanella* isolates were precultivated on LuriaBertani (LB) broth [36] plates at 30°C and stored at 4°C under anaerobic conditions. Tests for the utilization of carbon sources were performed using Phenotype Microarray BIOLOG plates (OmniLog ID System, USA). Bacterial growth was collected using sterile cotton-tipped applicators from LB plates and suspended in M9 medium supplemented with 0.1 mM potassium ferricyanide and Dye mixA according to the manufacturer's instructions. The cell concentration was adjusted to an OD_{600} of 0.2 measured using a turbidimeter (BIOLOG, USA). This cell suspension was applied to BIOLOG PM1 and PM2A MicroPlates containing 190 separate carbon sources (95 each). A volume of 100 µl of the cell suspension was added to each well and the plates were incubated at 30°C under anaerobic conditions for 72 h before reading the result. The tests were performed twice for each isolate and the results were confirmed in tube tests using M9 medium containing selected carbon sources: 0.5% glucose, 0.5% maltose, 0.5% sucrose, 0.5% starch, 50 mM sodium pyruvate, 50 mM lactic acid, 1 g/l molasses, 1.5 g/l peptone, 2 g/l 2′-deoxyadenosine, 20 mM sodium acetate, 2.6 mM isoleucine, or 2.8 mM valine. The bacteria were grown overnight with shaking at 30°C, or for 5 days without shaking at room temperature, under aerobic or anaerobic conditions, respectively. For tests performed under anaerobic conditions, the medium contained 100 mM FeOOH, and 0.1% ferrozine in 50 mM HEPES was added to the tubes at the end of the incubation. A purple color confirmed the presence of Fe(II), thus indicating microbial reduction of Fe(III). The bacteria were pelleted by centrifugation and the OD_{562nm} of the supernatant was measured.

The ability of the bacteria to grow at various temperatures and under different salinity levels was checked by cultivation of the isolates in LB medium under aerobic conditions with shaking for 16–18 h. To vary the salinity, the LB medium contained different concentrations of NaCl. At the end of each experiment, the OD_{600} of the culture was measured.

The Wilcoxon test (R package) was used to compare the bacterial cell size of POL1 and POL2 isolates.

MFC Study

The *Shewanella* isolates were grown overnight in LB medium with shaking under aerobic conditions at 30°C. The bacteria were then pelleted by centrifugation and suspended under anaerobic conditions in M9 medium containing 50 mM sodium pyruvate or 5 g/l sugar beet molasses that had been saturated beforehand with a stream of N_2:CO_2. The same media were used to fill the anode chamber of the MFC. The anode, a 24 cm^2 strip of carbon cloth (E-TEK, Inc.), was placed in the bacterial suspension and incubated for 3–5 days at room temperature under anaerobic conditions to impregnate it with cells. The cathode was a strip of the same carbon cloth without bacteria. The MFC used in this study was of a typical H-cell design (two bottles separated by a cation-exchange membrane). The cathode chamber was filled with M9 medium containing 0.1 M $K_3Fe(CN)_6$. For continuous MFC operation, the anode was connected to the cathode *via* a 1.6 kΩ resistor. Steady-state polarization curves were recorded at different times during operation. This was done by varying the resistor across the MFC electrodes, and for each resistance, stabilized voltage and current values were recorded. Control polarization curves were recorded for anodes not covered with bacteria. Following MFC experiments using pyruvate, its concentration in the anodic solution was determined by reaction with 200 mg/l 2,4-dinitrophenylhydrazine in 1 M HCl, according to the method of Anthon and Barrett [1]. The utilization of molasses in MFCs was calculated on the basis of the COD of the medium before and after the experiments.

Scanning Electron Microscopy

MFC anodes covered with bacteria were placed in a desiccator and fixed by treatment with 36–38% formaldehyde in the presence of pure $CaCO_3$ for 4 weeks. Samples were then sputter-coated with gold and examined using a scanning electron microscope (LEO 1430VP) at an accelerating voltage of 70 kV.

Sequence Data

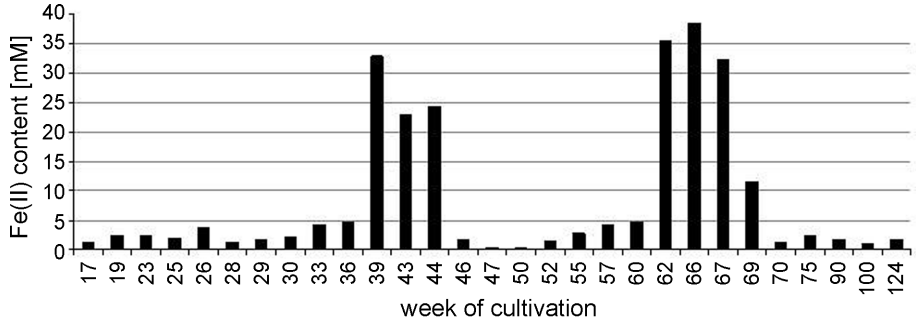
The EMBL accession number of the 16S rRNA sequence of POL1, 2 isolates is DS78813/183689.

RESULTS

Characteristics of the Culture Used in Selecting Fe(III)-Reducing Bacteria

An anaerobic flow-through continuous culture of bacteria was maintained in order to select microorganisms capable of Fe(III) reduction. Cultivation medium (2.5 l) containing 200 mg/l sodium acetate was inoculated anaerobically with a 30-ml sample of lake bottom sediment. The starting COD of the inoculated culture was 3,485 mg O_2/l with a pH of 7.0. The culture was initially incubated at room temperature for 17 days without any medium flow. During this period, the pH and COD of the culture were monitored regularly. No changes in pH were observed, whereas COD values decreased, and the medium flow was switched on when it reached 1,766 mg O_2/l. The bioreactor was filled with stones in the 21st week of cultivation, and over the next 22 weeks, formation of mud inside the bioreactor was observed. Fig. 1 (A and B, respectively) shows the PBR bioreactor and a sample of mud with stones taken from it in the 60th week of cultivation. The effect on granitic stones of 60-weeks incubation in the bioreactor is illustrated in Fig. 1B.

From the 17th week of cultivation, the content of reduced iron in the bioreactor and the number of bacteria capable of dissimilatory iron reduction were measured. Fig. 2 shows the content of Fe(II) in the liquid phase of the culture taken from the site circled in Fig. 1. The level of Fe(II) increased up to the 44th week of cultivation. Then, between the 44th and 46th weeks, the content of Fe(II) decreased dramatically and remained at a very low level owing to mud formation inside the bioreactor. After partial removal of the stones from the bioreactor, the Fe(II) content in the liquid phase



Content of Fe(II) in the fresh mass of mud taken from the bioreactor in the	
60th week of cultivation	124th week of cultivation
452 mM (50.3% of dry mass)	259 mM (45.1% of dry mass)

Fig. 2. Variation in the Fe^{2+} content of the liquid phase of the ferric oxide culture and in mud taken from the bioreactor at two sampling points during cultivation.

of the culture increased again. In comparison with the liquid phase, the concentration of Fe(II) in samples of mud taken from inside the bioreactor was 100-fold higher (Fig. 2). However, because of the bioreactor's construction, to avoid aeration of the culture, the ferrous ion content in the mud was determined only twice, in the 60th and 124th weeks of cultivation. The mud contained about 45–50% of dry mass.

The MPN of bacteria capable of dissimilatory Fe(III) reduction in the liquid phase of the culture was in the range of 4.5×10^7–4.5×10^8/ml, and there were no significant changes correlated with mud formation. There were also no differences in the MPN between samples taken from the liquid phase or the mud.

The presence of bacteria capable of Fe(III) reduction and the high content of the ferrous ion (Fe^{2+}) in the analyzed samples, pointed to intensive Fe(III) reduction processes occurring in the bioreactor. The pH of the supplied medium was 7, whereas the pH of the effluent was in the range of 6.8–7.5, indicating that the processes of iron reduction took place under neutral conditions. The COD of the supplied medium was 2,370±219 mg O_2/l and that of the effluent was 1,030±151 mg O_2/l. Since the COD of the M9 medium without molasses was around 1,000 mg O_2/l, it appears that all the molasses was utilized by the culture.

Isolation of Culturable Bacteria from the Bioreactor

Using classical microbiology methods, culturable bacteria were isolated from the bioreactor three times, in the 60th, 124th, and 132nd weeks of cultivation. Around 50–70 colonies

Table 1. Phylogenetic affiliation of bacteria isolated from the bioreactor culture selecting bacteria capable of Fe(III) reduction.

Phylogenetic group	Number of clones	Nearest relative (GenBank Accession No.)/most related taxon[a]	% Similarity
A. Selection of bacteria on solid M9 medium supplemented with trace minerals, glucose, acetate, and ferric citrate in the 60th week of cultivation			
Gammaproteobacteria	5	*Klebsiella oxytoca*	99–100
Enterobacteriaceae	1	*Pantonea* sp. NJ-11 (AM396912.1)	99
	2	*Citrobacter freundii*	99
B. Selection of bacteria on solid NB medium supplemented with trace minerals, acetate, and FeOOH sparged with pure nitrogen in the 124th week of cultivation			
Alphaproteobacteria			
Brucellaceae	1	*Ochrobactrum anthropi*	100
Rhodospirillaceae	1	*Azospirillum brasilense*	100
Betaproteobacteria	1	Uncultured Betaproteobacteria bacterium clone QEDQ1DA08	99
Comamonadaceae	1	*Aquaspirillum metamorphum* (Y18618.1)	99
Epsilonproteobacteria			
Campylobacteraceae	2	*Arcobacter butzleri*	99
Gammaproteobacteria			
Pseudomonadaceae	2	*Pseudomonas putida*	99–100
Moraxellaceae	1	*Acinetobacter* sp.	98
Enterobacteriaceae	1	*Enterobacter* sp.	100
Shewanellaceae	1	*Shewanella* sp. S-4 (FJ589031.1)	99
C. Selection of bacteria on solid NB medium supplemented with trace minerals, acetate, and FeOOH sparged with N_2:CO_2 (80:20) gas mixture in the 132nd week of cultivation			
Betaproteobacteria			
Oxalobacteraceae	1	*Massilia timonae* H2P8 (EU221406.1)	99
Epsilonproteobacteria			
Campylobacteraceae	1	*Arcobacter butzleri*	99
Gammaproteobacteria			
Enterobacteriales	1	Enterobacteriaceae bacterium M005 (AB461591.1)	94
Enterobacteriaceae	3	*Klebsiella oxytoca*	99
Pseudomonadaceae	1	*Pseudomonas putida*	99
Moraxellaceae	1	*Acinetobacter* sp. BHSN (EU293155.1)	100
Shewanellaceae	1	*Shewanella* sp. S-4 (FJ589031.1)	100

[a]Nearest relative (with GenBank Accession No.) was assigned when there was only one highest total score (BLAST).
Most related taxon (without GenBank Accession No.) was given when the BLAST total score was the same for more than two strains with different accession numbers.

per plate were obtained each time. The isolated bacteria were identified by analysis of their 16S rRNA gene sequences (Table 1). The second and the third isolations differed with respect to the gas used to sparge the culture medium. A mixture of N_2:CO_2 was preferred for the isolation of bacteria belonging to the *Geobacteraceae* family (Daniel Bond, personal communication). However, replacement of the medium-sparging gas did not significantly change the species composition of the isolated bacteria. The identified bacteria could be divided into three groups: (i) typical FRMs – *Shewanella* (obtained only in 2nd and 3rd isolations); (ii) bacteria for which iron(III) reduction is a secondary process not leading to energy conservation – *Enterobacteriaceae* and *Pseudomonas*; and (iii) bacteria that probably do not play any significant role in iron reduction (*Acinetobacter*, *Arcobacter*, *Aquaspirillum*, *Ochrobactrum*, *Azospirillum*, *Massilia*). Representatives of the *Enterobacteriaceae* family and the *Arcobacter* and *Pseudomonas* genera were the most abundant bacteria in all three isolations, constituting 35%, 25%, and 25% of isolates, respectively.

We assume that all the isolated bacteria came from the inoculum – a bottom sludge of the post-glacial eutrophic lake being a rich microbial community, which underwent long-lasting selection under specific conditions. The inoculum occurred to be a source of bacteria assigned to the genus *Shewanella*.

Characterization of the *Shewanella* Isolates

The two *Shewanella* isolates as potential FRMs were selected for further analysis. The 16S rRNA gene was amplified by

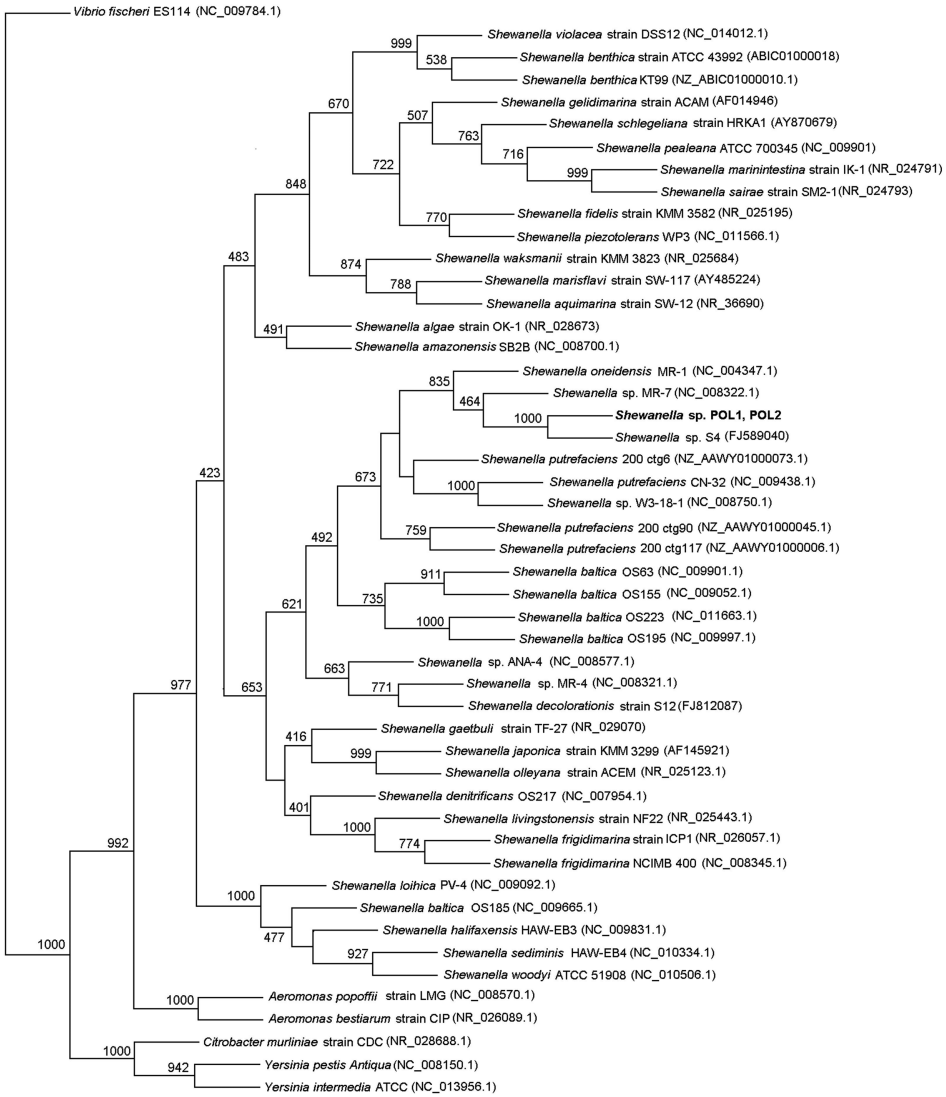


Fig. 3. Neighbor-joining tree based on 16S rRNA gene sequences, showing the phylogenetic positions of *Shewanella* strains POL1 and POL2 and related taxa.
Values on each branch represent the corresponding bootstrap probabilities obtained from 1,000 replications.

PCR from the genomic DNA of both *Shewanella* isolates and their sequences were established by independent sequencing reactions performed using 5 primers. The sequences of the 1,421-bp fragments from the two isolates were found to be identical. However, because these strains showed some morphological and physiological differences (see below), they were designated as *Shewanella* sp. POL1 and POL2 (Fig. 3). Comparison with the 16S rRNA gene sequences from the sequenced genomes of other *Shewanella* species and strains revealed that POL1,2 share 99% identity with Shewanella sp. MR-7 and 98% with *S. putrefaciens* CN-32, *S. putrefaciens* 200, *S. oneidensis* MR- 1, *Shewanella* sp. W3-18-1, *Shewanella* sp. MR-4, and available *S. baltica* strains. In each case, the query coverage of the sequences was 99%. As shown in Fig. 3, isolates POL1,2 are most highly related to *Shewanella* sp. S4 (bootstrap value of 995) submitted directly by J. Huang from Anhui University of Science and Technology of China (unpublished), and form a clearly defined clade with *Shewanella* sp. MR-7 and *S. oneidensis* MR-1 (bootstrap value of 824). It should be noted that there are a dozen or so other 16S rRNA sequences in the Nucleotide Collection of the NCBI that are very similar to those of POL1 and POL2, but in most cases they have not been described or published.

Both isolates were Gram-negative rods and formed light salmon-colored colonies on LB medium; however, the shades were different. In addition, measurements taken from 30 cells at each growth phase demonstrated that there was significant variation in the average cell dimensions. At the stationary phase, cells of strain POL1 were 2.14 μm long and 0.53 μm wide, whereas those of POL2 were 1.88 μm long and 0.614 μm wide (statistical significance $P<0.0003$ and $P<0.00007$ for the length and width, respectively). In the logarithmic phase, POL1 cells were 2.48 μm long and 0.80 μm wide, whereas those of POL2 were 2.37 μm long and 0.86 μm wide ($P<0.04$ and $P=0.08$ for the length and width, respectively). The range of growth temperatures for the two *Shewanella* strains was 4–42°C, although growth at 4°C was very weak ($OD_{600}<0.1$ after 16–18 h of growth under aerobic conditions). At 42°C, the cells formed filaments, and stronger filamentation was observed for POL1. Both isolates grew in LB medium containing up to 5% NaCl. However, this NaCl concentration had an inhibitory effect on bacterial growth ($OD_{600}<0.5$ after 16–18 h of growth).

Using the Phenotype Microarray System (BIOLOG, USA), 190 different compounds were tested as potential carbon sources for isolates POL1 and POL2 growing under anaerobic conditions. POL1 could utilize a broader spectrum of compounds than POL2. The most intense signals observed in the BIOLOG test for both isolates were adenosine, 2′-deoxyadenosine, inosine, pyruvic acid, methyl pyruvate, L-lactic acid, formic acid, sucrose, maltotriose, α- and β-cyclodextrins, gelatin, maltose, L-arabinose, and *N*-acetyl-D-glucosamine. Tube growth tests were also performed for selected carbon sources under aerobic and anaerobic conditions (Table S1 in Supplemental Data). In most cases, the tube growth test confirmed the BIOLOG results. The isolates readily used different sugars and molasses as sources of carbon and energy under anaerobic and aerobic conditions. They also showed intensive growth on peptone, which was not included in the BIOLOG tests; however, single amino acids generally did not serve as carbon sources. Under anaerobic conditions, bacterial growth was accompanied by the presence of reduced iron in the medium. No acidification was detected when the examined isolates were grown in sugar-containing media under anaerobic conditions, which indicated that they were unable to ferment carbohydrates.

MFC Operation

Fig. 4 shows scanning electron micrographs of the MFC anode surface covered by *Shewanella* sp. POL1 after incubation with bacteria for one week. For comparison, a micrograph of the bare carbon cloth substrate is also shown. It can be seen that the bacteria have formed a dense biofilm in the void space of the cloth.

Representative steady-state polarization curves and the corresponding power density plots for the MFCs are

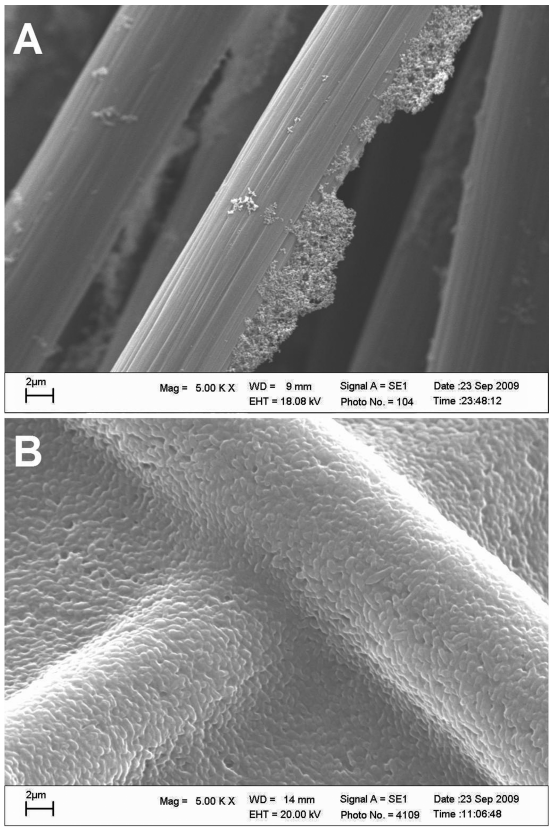


Fig. 4. Scanning electron micrographs of the anode surface in the virgin state (**A**) and covered by the cells of *Shewanella* strain POL1 (**B**).

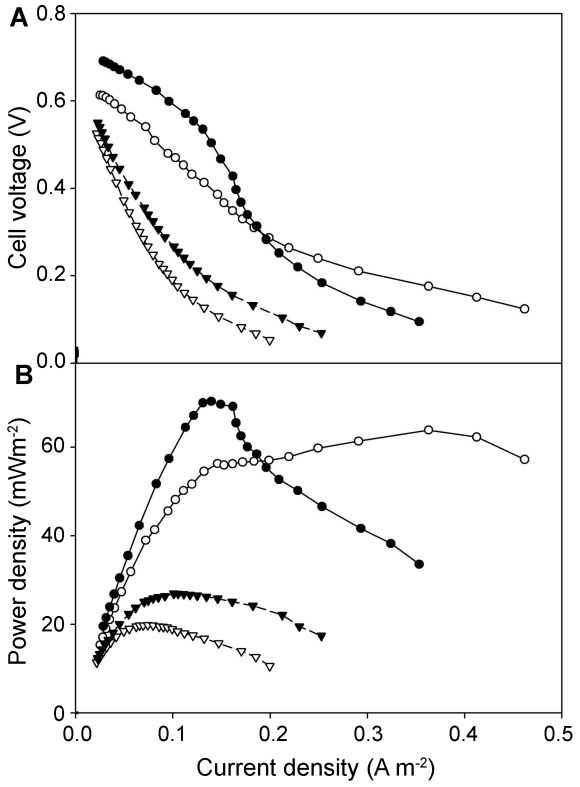


Fig. 5. Polarization (**A**) and power density (**B**) curves for MFCs with POL1 and sodium pyruvate (open circles), POL1 and molasses (open triangles), POL2 and sodium pyruvate (filled circles), and POL2 and molasses (filled triangles).
Polarizations of bacteria-free control MFCs filled with sodium pyruvate (solid lines) and molasses (dashed lines) media are included in the graphs, but are almost too small to be seen at this scale.

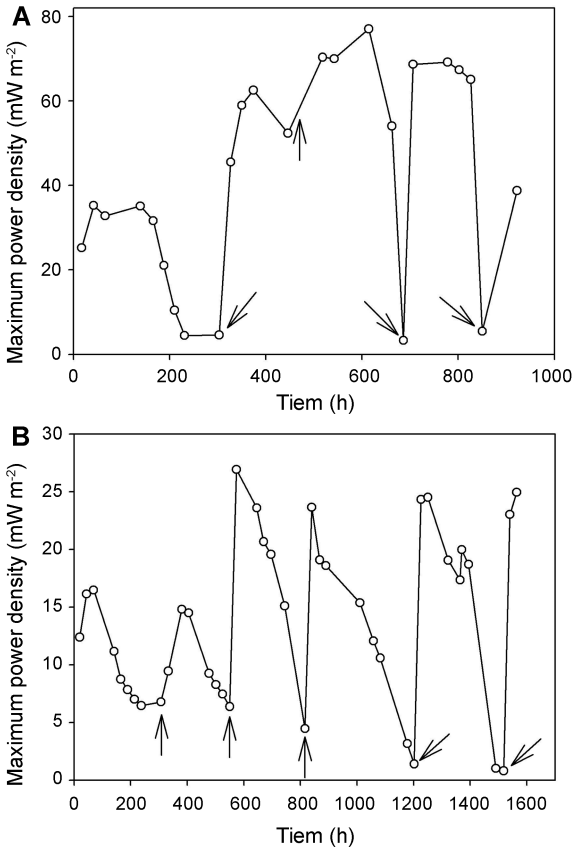


Fig. 6. Changes in the maximum power density of MFCs during continuous operation under 1.6 kΩ load: POL2 and pyruvate (**A**) and POL2 and molasses (**B**).
Arrows indicate the points at which the exhausted anodic and cathodic solutions were replaced.

presented in Fig. 5. Since control experiments gave virtually zero power under these conditions, the electricity generated by the MFCs can be unequivocally assigned to the activity of the bacteria. Both *Shewanella* strains were found to be more effective in generating electricity when utilizing sodium pyruvate as the carbon source rather than molasses, with the corresponding power densities differing by a factor of 3. Furthermore, the nonlinear shape of the polarization curves, generally similar for both strains and both media, was characteristic of processes controlled by charge transfer. In the case of the MFC with sodium pyruvate medium, and especially for the POL2 strain, the polarization curve could be divided into three regions. At high cell voltage values, the current increased rapidly with the lowering of the cell voltage, but at intermediate voltage values the rate of this increase was greatly diminished. At low values of the cell voltage, the rate of current increase became high again. A comparison of the electrode activities of POL1 and POL2 indicated that the former isolate worked better on sodium pyruvate (at least in the low voltage/high anode potential region), whereas the latter was a little better at utilizing molasses. These observations are further evidence that the two isolates are different.

The continuous operation of the H-type MFCs under constant load (1.6 kΩ) was examined over several hundred hours. Fig. 6 depicts the maximum power density vs. the operating time for the POL2 strain only (POL1 behaved analogously). Over the entire duration of the tests, deteriorating MFC performance could be restored to at least the previous level by replacement of the anodic and cathodic solutions. Operation on sodium pyruvate in the period between the renewal of the electrode solutions was steadier than operation on molasses, where the decline in performance started soon after the solutions had been replaced. Optimal overall performance of the MFCs occurred during the initial period of a few hundred hours operation.

In the case of operation on sodium pyruvate, the coulombic efficiency was calculated by comparing the charge generated in the MFC with the charge that could be obtained from complete conversion of the consumed pyruvate to carbon dioxide (10 electrons per molecule of pyruvate). Both the

POL1 and POL2 strains achieved a coulombic efficiency of 7.8±0.2% with sodium pyruvate. The coulombic efficiency for the conversion of molasses to electricity was calculated based on the change in the COD of the anodic solution upon operation of the MFC, according to Logan *et al*. [22]. Both strains gave the same coulombic efficiency of 7.1 ±0.1% with molasses.

DISCUSSION

Fe(III)-Reducing Bacterial Consortium

This is the first description of the use of sugar beet molasses and ferric oxide to select ferric-reducing bacteria. It was anticipated that utilization of molasses as a source of carbon to select bacteria capable of Fe(III) reduction would promote the development of a mixed population of bacteria analogous to those occurring in natural environments rich in Fe(III) compounds, such as sediments where products of fermentation act as sources of carbon and energy for FRMs. One model for the degradation of organic matter in anaerobic environments rich in Fe(III) involves symbiotic partnerships between fermentative microbes and FRMs [25, 27, 29]. Acetate is considered to be the most important fermentation product in Fe(III)-reducing sedimentary environments [26, 29]. For this reason, acetate as a source of carbon was added to the growth medium at the time of inoculation of the continuous culture and also to the solid media used to isolate culturable bacteria capable of Fe(III) reduction. Glucose was also added to the selection medium used in the first attempt at FRM isolation, because glucose-oxidizing Fe(III)-reducing bacteria were also expected. It should be noted that the agar used to solidify the medium could also represent a source of carbon and energy for the isolated bacteria.

Two observations indicated the occurrence of intensive Fe(III) reduction processes in the bioreactor: (i) the high content of the Fe^{2+} ion in samples taken; and (ii) the presence of bacteria capable of Fe(III) reduction, enumerated using a modified MPN method.

The identification of the selected bacteria does not give a full picture of the bacterial consortium formed in the long-term bioreactor culture because (i) only a small fraction of microorganisms can be routinely cultivated from natural bacterial consortia, and (ii) as explained above, acetate was used as a main carbon source in the solid selection media. However, the nature of the identified bacteria suggests that the observed high concentration of Fe^{2+} in the culture resulted from (i) microbial reduction (both the processes leading to energy conservation and those accompanying fermentation) and (ii) abiotic reduction (*e.g*., in the reaction of the ferric ion with the non-gaseous fermentation products).

Representatives of the *Enterobacteriaceae* and *Pseudomonas*- and *Arcobacter*-related bacteria were the most abundant groups among the isolated microorganisms. As expected, bacteria able to ferment carbohydrates developed in the bioreactor. Previously, Blothe and Roden [2] found an abundance of fermentative heterotrophs in a groundwater seep, whereas Lu *et al*. [34] identified mostly *Pseudomonas* and *Enterobacteriaceae* in irrigated tropical rice fields. Both of these sites are circumneutral-pH environments where Fe(III) reduction takes place. This means that the predominant species of Fe(III) reducers in these environments are bacteria that do not conserve energy from this process. However, *Pantoea agglomerans*, a representative of the Enterobacteriaceae, is able to respire by Fe(III) reduction [10]. One isolate from the continuous culture in the present study was identified as a bacterium related to *Pantoea* sp. *Arcobacter* species are mainly known as pathogens of humans and livestock. However, *Arcobacter*-related bacteria were found to be dominant among acetate-oxidizing manganese reducers in samples of Black Sea sediments, and they might represent an ecologically significant group of dissimilatory Mn reducers [46].

It is thought that the *Acinetobacter*-, *Aquaspirillum*-, *Ochrobactrum anthropi*-, *Azospirillum*-, and *Massilia timonae*-related isolates were unlikely to play any significant role in microbial iron reduction in the bioreactor. They are, respectively, saprophytes able to grow on a large variety of compounds [47], denitrifiers [37], opportunistic pathogens also found in the rhizosphere of diverse plants [52], plant growth-promoting rhizobacteria [5], and bacteria thought to be pathogens [21].

Phylogeny and Physiology of POL1 and POL2 Isolates

Two *Shewanella*-related isolates obtained in the 124th and 132nd weeks of cultivation – POL1 and POL2 – were the subject of further studies. These bacteria originate from very specific, non-marine environments, and their 16S rRNA gene sequences shared the highest similarity (99–98%) with various *S. putrefaciens*, *S. putrefaciens*-like, *S. oneidensis*, and *S. baltica* strains. The gene encoding 16S rRNA is a widely used molecular marker for the identification of bacterial strains and species. However, the variation in the 16S rRNA gene sequence among *Shewanella* species is not sufficient to define all members of the genus [20, 38]. Moreover, in the genomic era, it is now widely recognized that the definition of bacterial genus is frequently arbitrary and ambiguous. The comparison of whole genome sequences and the elucidation of ecophysiological and metabolic features are now necessary for the precise description of bacteria belonging to the same or closely related genera [19]. The identity of the examined 16S rRNA sequences of the POL1 and POL2 isolates versus the physiological and morphological differences between these strains confirm the above notion.

In this study, 190 different compounds were tested as potential carbon sources for the isolates POL1 and POL2

using the Phenotype Microarray System (BIOLOG, USA), which allows the simultaneous examination of many different substrates. The results were in agreement with data for *S. oneidensis* MR-1, with both isolates able to utilize the carboxylic acids pyruvate, lactate, and formate; the nucleosides adenosine and inosine; and *N*-acetylglucosamine, the main component of chitin [6, 38, 45, 51]. Uridine is regarded as a carbon source similar to adenosine and inosine, whereas α-ketobutyric acid is similar to pyruvate and lactate [6, 45], but these substrates gave very weak positive or negative BIOLOG results for POL1 and POL2, respectively.

Previous studies have shown that amino acids may be utilized by *S. oneidensis* MR-1 as sources of carbon [6, 42]. However, POL1 and POL2 gave very weak or no signals for all the amino acids in the BIOLOG test. These isolates showed very weak growth on the amino acids isoleucine and valine in a tube test. Interestingly, both isolates grew intensively when peptone was supplied as the source of carbon. BIOLOG results for *S. oneidensis* MR-1 showed that single amino acids do not serve as carbon sources for this strain, but positive signals were observed for a wide range of dipeptides [45].

S. oneidensis, *S. putrefaciens*, and *S. baltica*, the *Shewanella* species most closely related to isolates POL1 and POL2, do not possess essential glycolytic enzymes and are not fermentative microorganisms. However, the expression of enzymes of carbohydrate metabolism (*e.g.*, the pentose-phosphate or Entner–Doudoroff pathways) has been observed in *S. oneidensis* [6, 45]. It has also been demonstrated that sucrose, maltose, and glucose can be utilized by *S. baltica* and *S. putrefaciens*, and arabinose by *S. putrefaciens* [3, 44, 48]. The results of the BIOLOG tests for isolates POL1 and POL2 showed that maltose, maltotriose, sucrose, and L-arabinose as well as complex carbohydrates like α- and β-cyclodextrins or gelatin could serve as carbon sources for these bacteria. Glucose gave negative results in the same tests. In a previous study, similar negative results were observed for the utilization of glucose by *S. oneidensis* MR-1 [45]. Interestingly, weak growth on glucose was observed for POL1 and POL2 in tube tests. In comparison, both isolates showed intensive growth on medium containing molasses and starch. The main component of molasses is sucrose (50%), and amino acids constitute 10% of its content.

The temperature range for growth of isolates POL1 and POL2 was most similar to that of *S. oneidensis* and differed from that of *S. putrefaciens* and *S. baltica*. The ability to grow at different NaCl concentrations resembled that of *S. oneidensis* and *S. putrefaciens*, but differed from that of *S. baltica* [48].

Electrochemical Activity of POL1 and POL2 Isolates

The electrochemical activity of the POL1 and POL2 isolates was confirmed in MFCs operating on pyruvate or molasses. The use of a chemically complex nutrient (molasses) in MFCs utilizing pure cultures of representatives of the *Shewanella* genus was demonstrated for the first time. Although the performance on this type of nutrient was less than that obtained with a chemically pure and simple carbon substrate (sodium pyruvate), the operation of the MFCs was sustainable and was characterized by almost the same coulombic efficiency. The overall electrical performance of the MFCs operating on sodium pyruvate was very similar to that of an MFC of similar construction inoculated with *Shewanella oneidensis* MR-1 and utilizing sodium lactate [50].

The courses of the life tests for the studied MFCs were similar to those described in reports of analogous studies [39, 50]. As confirmed by titrations, the regular reversible drops in performance were caused by the depletion of nutrient in the anodic media. The initial increase in the overall performance of the bacteria to a constant average level may have been due to (i) a rise in the count of active bacteria in the anodic chamber, (ii) an increase in the number of electron transfer bridges from the bacteria to the anode [13], or (iii) the production of mediators in the form of electron carriers [49].

A novel feature not previously observed in *Shewanella* MFC studies was the two-stage character of the fuel cell polarizations, which must originate from two stages of anode operation with the POL1 and POL2 isolates (the cathode was fast and reversible). The quite rapid increase in current on the initial lowering of the cell voltage from the rest condition (which soon became slow at the intermediate voltage values) is evidence of a fast electron transfer at low anode potential, followed by the anode process entering a transport-limited region (or electrode passivation). The subsequent increase in the steepness of the polarization curve at low cell voltages suggests that another mechanism of electron transfer may have been switched on. At low cell voltages, the anode potential became close to the potential of the $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ redox couple, making the anode resemble – in an electrochemical sense – the natural electron acceptor used by these iron-reducing bacteria. It is probable that the anode electron transfer mechanism active under these conditions is similar to that occurring in the natural environment of the bacteria. This intriguing aspect of the MFC polarizations requires further investigation.

Acknowledgments

We acknowledge the support of the Polish Ministry of Science and Higher Education through grant 2-P04B-004-29 awarded for the years 2005–2009. We would like to thank Dr. Marek Rzepecki for supplying probes of the bottom sludge of Kluczysko Lake, Dr. Daniel Bond

for helpful remarks, and Dr. John Gittins for editorial assistance.

REFERENCES

1. Anthon, G. E. and D. M. Barrett. 2003. Modified method for the determination of pyruvic acid with dinitrophenylhydrazine in the assessment of onion pungency. *J. Sci. Food Agric.* **83:** 1210–1213.
2. Blothe, M. and E. E. Roden. 2009. Microbial iron redox cycling in a circumneutral-pH groundwater seep. *Appl. Environ. Microbiol.* **75:** 468–473.
3. Chang, I. S., H. Moon, O. Bretschger, J. K. Jang, H. I. Park, K. H. Nealson, and B. H. Kim. 2006. Electrochemically active bacteria (EAB) and mediator-less microbial fuel cells. *J. Microbiol. Biotechnol.* **16:** 163–177.
4. Chang, H. W., S. W. Roh, K. H. Kim, Y. D. Nam, C. O. Jeon, H. M. Oh, and J. W. Bae. 2008. *Shewanella basaltis* sp. nov., a marine bacterium isolated from black sand. *Int. J. Syst. Evol. Microbiol.* **58:** 1907–1910.
5. Couillerot, O., M. A. Poirier, C. Prigent-Combaret, I. P. Mavingu, J. Caballero-Mellado, and Y. Moenne-Loccoz. 2010. Assessment of SCAR markers to design real-time PCR primers for rhizosphere quantification of *Azospirillum brasilense* phytostimulatory inoculants of maize. *J. Appl. Microbiol.* **109:** 528–538.
6. Driscoll, M. E., M. F. Romine, F. S. Juhn, M. H. Serres, L. A. McCue, A. S. Beliaev, J. K. Fredrickson, and T. S. Gardner. 2007. Identification of diverse carbon utilization pathways in *Shewanella oneidensis* MR1 *via* expression profiling. *Genome Inform.* **18:** 287–298.
7. Edgar, R. C. 2004. MUSCLE: Multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* **32:** 1792–1797.
8. Ehrlich, H. L. 2002. Geomicrobiology of iron, pp. 345–428. *In* H. L. Ehrlich (ed.). *Geomicrobiology*. Marcel Dekker, New York.
9. Felsenstein, J. 1989. PHYLIP – Phylogeny Inference Package (Version 3.2). *Cladistics* **5:** 164–166.
10. Francis, C. A., A. Y. Obraztsova, and B. M. Tebo. 2000. Dissimilatory metal reduction by the facultative anaerobe *Pantoea agglomerans* SP1. *Appl. Environ. Microbiol.* **66:** 543–548.
11. Fredrickson, J. K., M. F. Romine, A. S. Beliaev, J. M. Auchtung, M. E. Driscoll, T. S. Gardner, *et al*. 2008. Towards environmental systems biology of *Shewanella*. *Nat. Rev. Microbiol*. **6:** 592–603.
12. Girard, H.and R. Rougieux. 1967. *Techniques de Mikrobiologie Agricole*. Dunod, Paris.
13. Gorby, Y. A., S. Yanina, J. S. McLean, K. M. Rosso, D. Moyles, A. Dohnalkova, *et al*. 2006. Electrically conductive bacterial nanowires produced by *Shewanella oneidensis* strain MR-1 and other microorganisms. *Proc. Natl. Acad. Sci. USA* **103:** 11358–11363.
14. Gould, W. D., M. Stichbury, M. Francis, L. Lortie, and D. W. Blowes. 2003. An MPN method for the enumeration of iron-reducing bacteria. *In* G. Spiers, P. Beckett, and H. Conroy (eds.). *Mining and the Environment*. Centre for Environmental Monitoring, Laurentian University, Sudbury.
15. Hau, H. H. and J. A. Gralnick. 2007. Ecology and biotechnology of the genus *Shewanella*. *Annu. Rev. Microbiol.* **61:** 237–258.
16. Heidelberg, J. F., I. T. Paulsen, K. E. Nelson, E. J. Gaidos, W. C. Nelson, T. D. Read, *et al*. 2002. Genome sequence of the dissimilatory metal ion-reducing bacterium *Shewanella oneidensis*. *Nat. Biotechnol.* **20:** 1118–1123.
17. Huang, J., B. Sun, and X. Zhang. 2010. Electricity generation at high ionic strength in microbial fuel cell by a newly isolated *Shewanella marisflavi* EP1. *Appl. Microbiol. Biotechnol.* **85:** 1141–1149.
18. Kim, H. J., H. S. Park, M. S. Hyun, I. S. Chang, M. Kim, and B. H. Kim. 2002. A mediator-less microbial fuel cell using a metal reducing bacterium, *Shewanella putrefaciens*. *Enzyme Microb. Technol.* **30:** 145–152.
19. Konstantinidis, K. T., A. Ramette, and J. M. Tiedje. 2006. The bacterial species definition in the genomic era. *Phil. Trans. R. Soc. B* **361:** 1929–1940.
20. Konstantinidis, K. T., M. H. Serres, M. F. Romine, J. L. M. Rodrigues, J. Auchtung, L. A. McCue, *et al*. 2009. Comparative systems biology across an evolutionary gradient within the *Shewanella* genus. *Proc. Natl. Acad. Sci. USA* **106:** 15909–15914.
21. Lindquist, D., D. Murrill, W. P. Burran, G. Winans, J. M. Janda, and W. Probert. 2003. Characteristics of *Massilia timonae* and *Massilia timonae*-like isolates from human patients, with an amended description of the species. *J. Clin. Microbiol.* **41:** 192–196.
22. Logan, B. E., B. Hamelers, R. Rozendal, U. Schröder, J. Keller, S. Freguia, P. Aelterman, W. Verstraete, and K. Rabaey. 2006. Microbial fuel cells: Methodology and technology. *Environ. Sci. Technol.* **40:** 5181–5192.
23. Logan, B. E. 2008. *Microbial Fuel Cells*. John Wiley & Sons, Hoboken, New Jersey.
24. Logan, B. E. 2009. Exoelectrogenic bacteria that power microbial fuel cells. *Nat. Rev. Microbiol.* **7:** 375–381.
25. Lovley, D. R. 1991. Dissimilatory Fe(III) and Mn(IV) reduction. *Microbiol. Rev.* **55:** 259–287.
26. Lovley, D. R. 1993. Dissimilatory metal reduction. *Annu. Rev. Microbiol.* **47:** 263–290.
27. Lovley, D. R. 2000. Fe(III) and Mn(IV) reduction, pp. 3–30. *In* D. R. Lovley (ed.). *Environmental Microbe–Metal Interactions*. ASM Press, Washington, DC.
28. Lovley, D. R. 2001. Anaerobes to the rescue. *Science* **293:** 1444–1446.
29. Lovley, D. R. 2006. Dissimilatory Fe(III)- and Mn(IV)-reducing prokaryotes. *Prokaryotes* **2:** 635–658.
30. Lovley, D. R. 2006. Microbial fuel cells: Novel microbial physiologies and engineering approaches. *Curr. Opin. Biotechnol.* **17:** 327–332.
31. Lovley, D. R. and E. J. P. Philips. 1986. Organic matter mineralization with the reduction of ferric iron in anaerobic sediments. *Appl. Environ. Microbiol.* **51:** 683–689.
32. Lovley, D. R. and E. J. P. Philips. 1987. Rapid assay for microbially reducible ferric iron in aquatic sediments. *Appl. Environ. Microbiol.* **53:** 1536–1540.
33. Lovley, D. R., D. E. Holmes, and K. P. Nevin. 2004. Dissimilatory Fe(III) and Mn(IV) reduction. *Adv. Microbiol. Physiol.* **49:** 219–286.

34. Lu, W. J., H. T. Wang, C. Y. Huang, and W. Reichardt. 2002. Communities of iron (III)-reducing bacteria in irrigated tropical rice fields. *Microb. Environ.* **17:** 170–178.
35. Malina, J. 1979. *Chemical Oxygen Demand. Analytical Procedures and Methods*. Prepared for Poland Project, 26 WHO University of Texas at Austin.
36. Miller, J. H. 1972. *Experiments in Molecular Genetics*. Cold Spring Harbor Laboratory, New York.
37. Morgan-Sagastume, F., J. L. Nielsen, and P. H. Nielsen. 2008. Substrate-dependent denitrification of abundant probe-defined denitrifying bacteria in activated sludge. *FEMS Microbiol. Ecol.* **66:** 447–461.
38. Nealson, K. H. and J. Scott. 2006. Ecophysiology of the genus *Shewanella*. *Prokaryotes* **6:** 1133–1151.
39. Newton, G. J., S. Mori, R. Nakamura, K. Hashimoto, and K. Watanabe. 2009. Analyses of current-generating mechanisms of *Shewanella loihica* PV-4 and *Shewanella oneidensis* MR-1 in microbial fuel cells. *Appl. Environ. Microbiol.* **75:** 7674–7681.
40. Park, S. C., K. S. Baik, M. S. Kim, D. Kim, and C. N. Seong. 2009. *Shewanella marina* sp. nov., isolated from sea water. *Int. J. Syst. Evol. Microbiol.* **59:** 1888–1894.
41. Pinchuk, G. E., C. Ammons, D. E. Culley, S. M. W. Li, J. S. McLean, M. F. Romine, K. H. Nealson, J. K. Fredrickson, and A. S. Beliaev. 2008. Utilization of DNA as a sole source of phosphorus, carbon and energy by *Shewanella* spp.: Ecological and physiological implications for dissimilatory metal reduction. *Appl. Environ. Microbiol.* **74:** 1198–1208.
42. Ringo, E., E. Stenberg, and A. R. Strom. 1984. Amino acid and lactate catabolism in trimethylamine oxide respiration of *Alteromonas putrefaciens* NCMB 1735. *Appl. Environ. Microbiol.* **47:** 1084–1089.
43. Rittmann, B. E., R. Krajmalnik-Brown, and R. U. Haiden. 2008. Pre-genomic, genomic and postgenomic study of microbial communities involved in bioenergy. *Nat. Rev. Microbiol.* **6:** 604–612.
44. Scott, J. H. and K. H. Nealson. 1994. A biochemical study of the intermediary carbon metabolism of *Shewanella putrefaciens*. *J. Bacteriol.* **176:** 3408–3411.
45. Serres, M. H. and M. Riley. 2006. Genomic analysis of carbon source metabolism of *Shewanella oneidensis* MR1: Predictions versus experiments. *J. Bacteriol.* **188:** 4601–4609.
46. Thamdrup, B., R. Rossello-Mora, and R. Amann. 2000. Microbial manganese and sulfate reduction in Black Sea shelf sediments. *Appl. Environ. Microbiol.* **66:** 2888–2897.
47. Vallenet, D., P. Nordmann, V. Barbe, L. Poirel, S. Mangenot, E. Bataille, *et al*. 2008. Comparative analysis of Acinetobacters: Three genomes for three lifestyles. *PLoS ONE* **3:** e1805.
48. Venkateswaran, K., D. P. Moser, M. E. Dollhopf, D. P. Lies, D. A. Saffarini, B. J. MacGregor, *et al*. 1999. Polyphasic taxonomy of the genus *Shewanella* and description of *Shewanella oneidensis* sp. nov. *Int. J. Syst. Bacteriol.* **49:** 705–724.
49. Von Canstein, H., J. Ogawa, S. Shimizu, and J. R. Lloyd. 2008. Secretion of flavins by *Shewanella* species and their role in extracellular electron transfer. *Appl. Environ. Microbiol.* **74:** 615–623.
50. Watson, V. J. and B. E. Logan. 2010. Power production in MFCs inoculated with *Shewanella oneidensis* MR-1 or mixed cultures. *Biotechnol. Bioeng.* **105:** 489–498.
51. Yang, C., D. A. Rodionov, X. Li, O. N. Laikova, M. S. Gelfand, O. P. Zagnitko, *et al*. 2006. Comparative genomics and experimental characterization of *N*-acetylglucosamine utilization pathway of *Shewanella oneidensis*. *J. Biol. Chem.* **281:** 29872–29885.
52. Zuo, Y., D. Xing, J. M. Regan, and B. E. Logan. 2008. Isolation of the exoelectrogenic bacterium *Ochrobactrum anthropi* YZ-1 by using a U-tube microbial fuel cell. *Appl. Environ. Microbiol.* **74:** 3130–3137.