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

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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), Te(VII), Co(III), Cr(VI), Hg(II), Sulfate, thiosulfate, selenite, arsenite, iodite, fumarate, glycine, U(VI), Tc(VII), Co(III), Cr(VI), Hg(II), sulfur, nitrate, nitrite, 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), Te(VII), Co(III), Cr(VI), Hg(II), Sulfate, thiosulfate, selenite, arsenite, iodite, fumarate, glycine, trimethylamine oxide (TMAO), and dimethylsulfoxide (DMSO) [8, 15, 27, 33, 38]. The genomic sequences of Shewanella MR-1, the most highly studied representative of the genus Shewanella, were 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 cm² of the sludge contained 10⁹ bacterial cells. The cultivation medium was M9 medium [36] supplemented with trace elements (1 mg/l FeSO₄, 70 µg/l ZnCl₂, 100 µg/l MnCl₂, 6 µg/l H₂BO₃, 2 µg/l CuCl₂, 24 µg/l NiCl₂, 36 µg/l Na₂MoO₄, 238 µg/l CoCl₃), 1 g/l molasses Ropczyce Sugar Factory, Poland), and 10 mM Fe₂O₃ (POCh, Gliwice, Poland). The medium was boiled and saturated with a stream of pure N₂ (Air Products, Poland). The bacterial culture was maintained for 30 months at room temperature in a 3l-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...
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 OD600 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 OD600 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 OD₆₀₀ 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 N₂:CO₂. The same media were used to fill the anode chamber of the MFC. The anode, a 24 cm² 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 K₃Fe(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 resistance 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 CaCO₃ 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 O₂/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 O₂/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

![Fig. 2](image-url)
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 \(4.5 \times 10^7 - 4.5 \times 10^8\)/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

<p>| Table 1. Phylogenetic affiliation of bacteria isolated from the bioreactor culture selecting bacteria capable of Fe(III) reduction. |
|--------------------|------------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Phylogenetic group</th>
<th>Number of clones</th>
<th>Nearest relative (GenBank Accession No.)/most related taxon*</th>
<th>% Similarity</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A. Selection of bacteria on solid M9 medium supplemented with trace minerals, glucose, acetate, and ferric citrate in the 60th week of cultivation</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gammaproteobacteria</td>
<td>5</td>
<td>Klebsiella oxytoca</td>
<td>99–100</td>
</tr>
<tr>
<td>Enterobacteriaceae</td>
<td>1</td>
<td>Pantonea sp. NJ-11 (AM396912.1)</td>
<td>99</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Citrobacter freundii</td>
<td>99</td>
</tr>
<tr>
<td><strong>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</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alphaproteobacteria</td>
<td>1</td>
<td>Ochrobactrum anthropi</td>
<td>100</td>
</tr>
<tr>
<td>Rhodospirillaceae</td>
<td>1</td>
<td>Azospirillum brasilense</td>
<td>100</td>
</tr>
<tr>
<td><strong>Betaproteobacteria</strong></td>
<td>1</td>
<td>Uncultured Betaproteobacteria bacterium clone QEDQ1DA08</td>
<td>99</td>
</tr>
<tr>
<td>Comamonadaceae</td>
<td>1</td>
<td>Aquaspirillum metamorphum (Y18618.1)</td>
<td>99</td>
</tr>
<tr>
<td><strong>Epsilonproteobacteria</strong></td>
<td>2</td>
<td>Arcobacter butzleri</td>
<td>99</td>
</tr>
<tr>
<td><strong>Gammaproteobacteria</strong></td>
<td>2</td>
<td>Pseudomonas putida</td>
<td>99–100</td>
</tr>
<tr>
<td>Pseudomonadaceae</td>
<td>1</td>
<td>Acinetobacter sp.</td>
<td>98</td>
</tr>
<tr>
<td>Enterobacteriaceae</td>
<td>1</td>
<td>Enterobacter sp.</td>
<td>100</td>
</tr>
<tr>
<td>Shewanellaceae</td>
<td>1</td>
<td>Shewanella sp. S-4 (FJ589031.1)</td>
<td>99</td>
</tr>
<tr>
<td><strong>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</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Betaproteobacteria</td>
<td>1</td>
<td>Massilia timonae H2P8 (EU221406.1)</td>
<td>99</td>
</tr>
<tr>
<td>Epsilonproteobacteria</td>
<td>1</td>
<td>Arcobacter butzleri</td>
<td>99</td>
</tr>
<tr>
<td>Gammaproteobacteria</td>
<td>1</td>
<td>Enterobacteriaceae bacterium M005 (AB461591.1)</td>
<td>94</td>
</tr>
<tr>
<td>Enterobacteriaceae</td>
<td>3</td>
<td>Klebsiella oxytoca</td>
<td>99</td>
</tr>
<tr>
<td>Pseudomonadaceae</td>
<td>1</td>
<td>Pseudomonas putida</td>
<td>99</td>
</tr>
<tr>
<td>Moraxellaceae</td>
<td>1</td>
<td>Acinetobacter sp. BHSN (EU293155.1)</td>
<td>100</td>
</tr>
<tr>
<td>Shewanellaceae</td>
<td>1</td>
<td>Shewanella sp. S-4 (FJ589031.1)</td>
<td>100</td>
</tr>
</tbody>
</table>

*aNearest 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\textsubscript{2}:CO\textsubscript{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

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**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 shown in Fig. 4. Scanning electron micrographs of the anode surface in the virgin state (A) and covered by the cells of *Shewanella* strain POL1 (B).
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, Pantoaea 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 signals for all the amino acids in the BIOLOG test. Interestingly, both isolates showed very weak growth on the amino acids but these substrates gave very weak positive or negative BIOLOG results for POL1 and POL2, respectively.

*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 α-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)₆³⁻/Fe(CN)₅⁴⁻ 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.

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