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Microbial nanowires: Is the subsurface "hardwired"?

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[1] The Earth's shallow subsurface results from integrated biological, geochemical, and physical processes. Methods are sought to remotely assess these interactive processes, especially those catalysed by micro-organisms. Using saturated sand columns and the metal reducing bacterium *Shewanella oneidensis* MR-1, we show that electrically conductive appendages called bacterial nanowires are directly associated with electrical potentials. No significant electrical potentials were detectable in columns inoculated with mutant strains that produced non-conductive appendages. Scanning electron microscopy imaging revealed a network of nanowires linking cells-cells and cells to mineral surfaces, ''hardwiring'' the entire length of the column. We hypothesize that the nanowires serve as conduits for transfer of electrons from bacteria in the anaerobic part of the column to bacteria at the surface that have access to oxygen, akin to a biogeobattery. These results advance understanding of the mechanisms of electron transport in subsurface environments and of how microorganisms cycle geologic material and share energy. **Citation:** Ntarlagiannis, D., E. A. Atekwana, E. A. Hill, and Y. Gorby (2007), Microbial nanowires: Is the subsurface ''hardwired''?, *Geophys. Res. Lett.*, *34*, L17305, doi:10.1029/2007GL030426.

1. Introduction

[2] Dissimilatory metal reducing bacteria directly control redox transformation of heavy metals and radionuclides in sediments where they principally use solid phase iron and manganese oxides as electron acceptors [*Lovley and Phillips*, 1988; *Myers and Nealson*, 1990]. Previous reports suggest that metal reducing organisms, such as *Shewanella* and *Geobacter*, reduce metals via multiheme cytochromes embedded in their outer membrane surfaces [*Myers and Nealson*, 1990]. Recent research suggests that these bacteria produce electrically conductive appendages called bacterial nanowires that facilitate electron transfer to solid phase electron acceptors [*Gorby et al.*, 2006; *Reguera et al.*, 2005]. In *Geobacter*, nanowires are composed of a unique pilin protein. The ''geopili'' are electrically conductive, presumably in the absence of traditional electron transport proteins such as cytochromes. In *Shewanella*, nanowires require multiheme cytochromes to be electrically conductive, as evidenced by the fact that mutants lacking genes for a pair of extracellular decaheme cytochromes (MtrC and OmcA) produced non-conductive appendages [*Gorby et al.*, 2006]. Moreover, these mutants were unable to reduce solid phase electron acceptors or transfer electrons to electrode surfaces in microbial fuel cells (MFCs) [*Gorby et al.*, 2006]. Admittedly, the complete composition and mechanisms of electron transfer in nanowires produced by either *Shewanella* or *Geobacter* are ill defined. Although we have recorded electrical signals associated with the microbial activity in the subsurface, the generating source mechanisms are poorly understood. However, the availability of *Shewanella* and mutants that produce nonconductive nanowires provide an opportunity to investigate (1) the role of fully functional nanowires in developing detectable electrical signals in near subsurface environments, and (2) to provide additional insight into the electrical signal source mechanisms.

[3] Here we report the results of experiments designed to utilize a common field geophysical technique, the self potential (SP) method, to evaluate the geo-electrical signatures of saturated sand columns inoculated with either *S. oneidensis* MR-1 known to produce conductive nanowires or with a mutant strain producing similar appendages that are non conductive. The experiment was designed to use oxygen as the sole electron acceptor and lactate as the electron donor utilized in microbial respiration.

2. Methods

2.1. Set Up and Materials

[4] Four polyvinyl chloride columns were wet packed with quartzitic sand ($SiO_2 \geq 99.8\%$, 20–30 Mesh, porosity $37 \pm 1\%$). Columns were saturated with nutrient medium (non inoculated) for 24 hrs to allow the columns to reach equilibrium conditions. We introduced inoculated medium directly from a chemostat (section 2.3) using a peristaltic pump. To ensure complete homogeneous saturation we pumped at least 10 pore volumes of the inoculated medium through the columns. After inoculation, no medium was added or removed from the columns.

2.2. Self Potential (SP) Measurements

[5] SP measurements were performed with a Keithley 2700 DMM system. SP measurements at different locations along the columns (Figure 1) were made relative to a reference electrode located at the bottom of the column. SP measurements were obtained every 15 minutes for the duration of the experiment (400 hours). We used non polarizing Ag-AgCl electrodes installed on the outer surface

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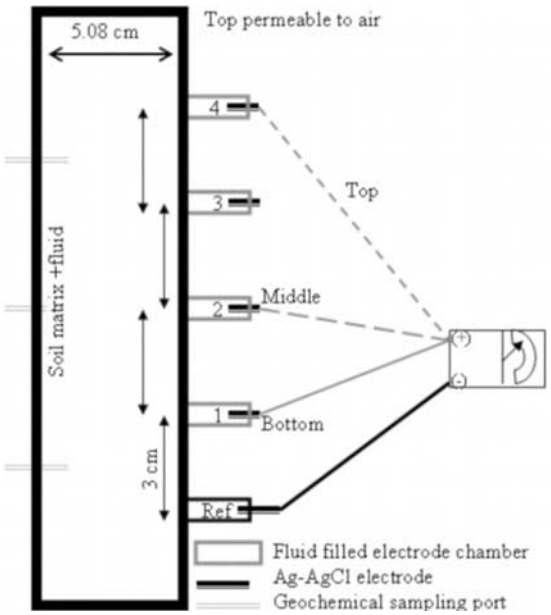

Figure 1. Schematic of the column design, dimensions, and measurement scheme used during the experiment. Ag-AgCl electrodes were in chambers saturated with column fluid. Mutant and active columns were identical.

of the column wall (Figure 1). Ideally, this setup provides continuous electrolytic contact between the electrodes and the target area and prevents spurious signals due to residual fluid flow local to the electrode and direct contact between the electrode and the microbial mass. The experiment was conducted under temperature controlled conditions (25°C).

2.3. Bacterial Strains and Culture Conditions

[6] Cells of *S. oneidensis* strain MR-1 (wild type) and mutant strains, described in detail elsewhere [*Gorby et al.*, 2006; *Myers and Nealson*, 1990; *Zachara et al.*, 1998] were used for this experiment. Cells were continuously cultured using a New Brunswick Scientific BioFlo 110 fermentor until they reached steady state. DO, pH, agitation, temperature, acid (2M HCl) and base addition (2M NaOH), antifoam addition, and gas mixture (Nitrogen vs. Air) were continuously monitored by AFS-BioCommand Bioprocessing Software in conjunction with the BioFlo 110 controller. During continuous culture, the pH was maintained at pH 7, the DO tension was controlled at 2% of air saturation, and the media was delivered with a dilution rate of 0.05 h^{-1}. Cells were transferred from the bioreactor to columns using sterile silicone tubing and a peristaltic pump. Initial lactate concentration in the columns was 10 mM.

2.4. Scanning Electron Microscopy

[7] Sand grains and attached bacteria were carefully removed from the column and transferred to a fixative solution containing 2.5% glutaraldehyde. Fixed samples were then subjected to a graded ethanol dehydration and critical point dried, coated with evaporated carbon, and viewed using a Zeiss-LEO 982 field emission-scanning electron microscope (FE-SEM) equipped with an Oxford Energy Dispersive System (EDS) detector (Oxford Instruments, Oxon, 9 UK).

3. Results

3.1. Self Potential

[8] Column 1, which was inoculated with *S. oneidensis* MR-1 showed no change in SP for the initial 220 hours (Figure 2). After 220 hours, SP rapidly increased and reached a maximum of 602 mV (±4 mV) at ~280 hours for all electrode pairs with respect to the reference electrode. The SP values remained stable for ~30–40 hours. Subsequently, SP values for the top electrode (1T; Figure 2) started to decline at around 320 hours, followed by the middle electrode (1M; Figure 2) with a ~25 hour delay (Figure 2). The SP values measured for the bottom electrode (1B; Figure 2) did not decrease until termination of the experiment (~400 hours) (Figure 2).

[9] In contrast to the above results no significant voltages (average value was 0 ± 10 mV) were recorded in Column 2 that was inoculated with the mutant strain (Figure 2). No SP anomalies were recorded for an abiotic control column under the same experimental conditions (data not shown).

3.2. Scanning Electron Microscopy (SEM)

[10] SEM imaging of sand surfaces from the *S. oneidensis* MR-1 column revealed significant development of pilus-like appendages (Figure 3a). These appendages appeared to be long and thick with an intricate pattern of appendages merging and splitting, connecting cells to cells and cells to mineral surfaces (Figure 3b) throughout the entire length of the column. In contrast, nanowires of the mutant strain appeared thin and frail (Figures 3c and 3d) with no oriented and structured arrangement.

4. Discussion

[11] The discovery of microbial nanowires suggests that our Earth's subsurface may be "hardwired" with electron conductive microbial appendages. This discovery has significant implications for geophysical methodologies, which have long been used to investigate near-surface geological environments. Although the geophysical response of microbial interactions with geologic media has been observed/measured in the field and in the lab,

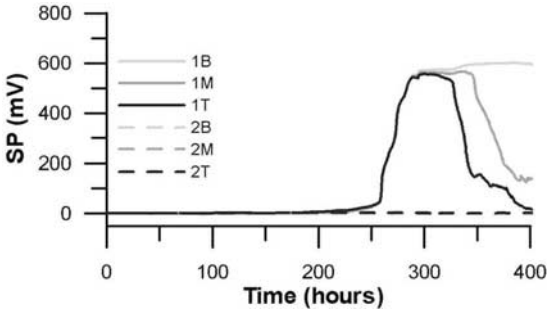

Figure 2. Self-potential values (voltage) measured in *S. oneidensis* MR-1 (1B, 1M, 1T) and mutant strain (2B, 2M, 2T) columns. Time 0 is the inoculation time. Letters indicate positions along the column where measurements were made: bottom, B; middle, M; top, T.

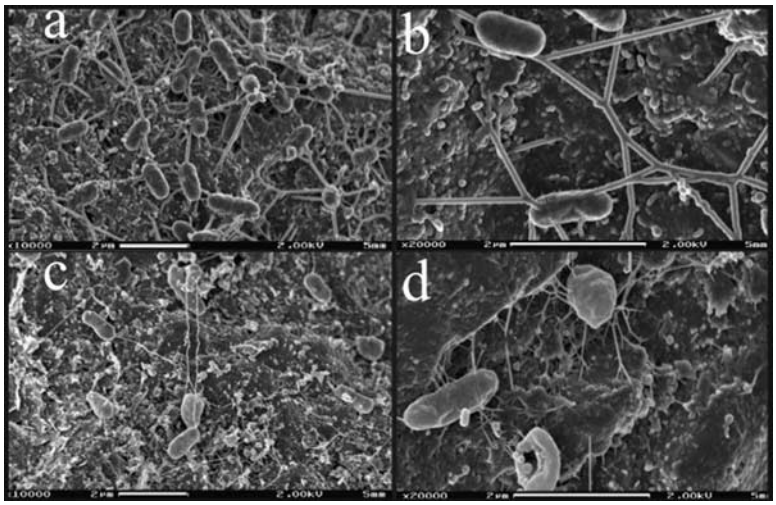

Figure 3. SEM images. (a) Pilus-like appendages from *S. oneidensis* MR-1. Appendages are long and thick. (b) Close up image showing a network of cell-cell and cell-mineral connection, merging and splitting of appendages. (c, d) Pilus-like appendages from *S. oneidensis* mutant strain showing thin and frail appendages and cells are visibly deteriorated.

the source mechanism(s) are neither well described nor understood. Currently, proposed mechanisms for geophysical changes in biostimulated geologic media include: (1) mineral weathering resulting from products of microbial metabolism [*Atekwana et al.*, 2004a; *Atekwana et al.*, 2004b]; (2) growth of microbial cells attached to sediment grains in biofilms that increase surface conductance [*Davis et al.*, 2006]; (3) biomineralization [*Ntarlagiannis et al.*, 2005], whereby the elevated electrical conductivity and polarization is the result of microbe-induced metal sulfide precipitation; and (4) redox processes [*Naudet and Revil*, 2005], whereby biofilms forming at the water table separate the oxic unsaturated zone from the anoxic saturated zone generating a geobattery effect.

[12] The SP method involves the passive measurements of the integral effect of naturally occurring electrical field (EF) generated over a designated path in geological materials. It is a common geophysical technique extensively used in the mineral and oil exploration industry. Major source mechanisms for SP signals include: electrokinetic potentials (due to groundwater flow) and electro-chemical processes (e.g., redox phenomena typically associated with massive sulphide deposits) [*Naudet et al.*, 2003]. However, until recently these potentials have not been directly associated with microbial activity [*Naudet et al.*, 2003; *Naudet and Revil*, 2005; *Sauck et al.*, 1998]. Temperature and ionic concentration gradients can contribute to the total SP signal.

[13] The SP measurements in this study suggest that microbially induced transfer of charge developed in the *S. oneidensis* MR-1 column after a lag time of ~220 hours. Considering that the mutant did not produce conductive nanowires and/or cannot utilize solid state iron, we associate the large SP gradients developed in the *S. oneidensis* MR-1 column to the ability to transfer electrons through the nanowires. Thus, in the absence of fluid flow (our experiments were conducted under static conditions), temperature and concentration gradients, we attribute the generation of the observed electrical potentials to an electrochemical process akin to geobattery mechanism and/or galvanic cell.

[14] Our experimental setup can be modelled as an electrochemical cell (or a microbial fuel cell (MFC)) with the anode located at the lower part (reduced zone) and the cathode at the upper part (oxidized zone) (Figure 4) similar to the model suggested by *Naudet and Revil* [2005]. In typical MFCs, graphite or platinum electrodes are placed into anoxic chambers and connected to similar electrodes in oxic environments and a sustained electrical current is harvested [*Logan et al.*, 2006]. We note that our setup performed similar to a MFC but without the use of any direct wiring between cathode and anode.

[15] A conceptual model (Figure 4) is proposed showing the cathodic and anodic areas associated with the charge transfer mechanism in systems where only microbial activity occurs. In this model, electron donating (lactate oxidation) and accepting (O_2 reduction) processes that are solely microbially driven, serve as the anode and cathode reactions respectively (Figure 4). While ions move freely through the electrolytic medium, the presence of structured and oriented nanowires serve as electrical connections that link the oxidizing and reducing zones, thus permitting electron transfer, and producing an electrical field (EF). Such a "biogeobattery" model satisfies all the operating requirements of an electrochemical cell and is analogous to the geobattery model proposed for massive sulphide deposits [*Bigalke and Grabner*, 1997; *Sato and Mooney*, 1960] and complies with SP anomalies associated with microbial active redox fronts [*Naudet et al.*, 2003; *Naudet and Revil*, 2005]. Recent work suggest that extracellular appendages are essential for biofilm development [*Reguera et al.*, 2007] as well as serve as electron transfer pathways under electron acceptor limitation [*Gorby et al.*, 2006; *Reguera et al.*, 2005], a process that can generate an EF. This behaviour was recorded with the SP measurements (Figure 2) where no signal was recorded during the initial lag phase reflecting the time required to build the nanowires infrastructure; processes at work during this time could include: (1) dumping electrons from lactate using a proton motive

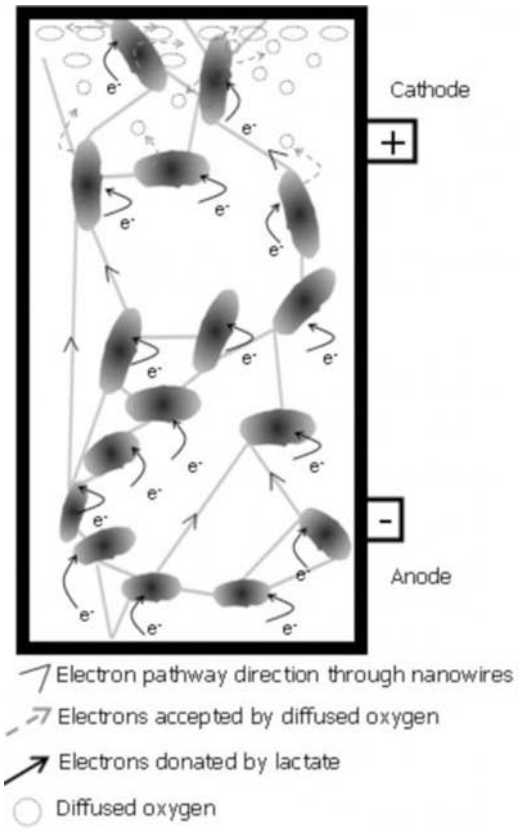

Figure 4. Conceptual bio-geobattery model describing generation of EF driven only by microbial activity. Lactate serves as the electron donor, oxygen as the electron acceptor, microbial nanowires as electronic conductors, and fluid as the ionic pathway. The role of nanowires is to transfer the electrons from the lower parts to the upper parts and an electric current then flows from the anode to the cathode. The difference in potential is then measured by a volt meter.

force-generating dehydrogenase into a vast reservoir of preassembled cytochromes c, (2) energy harnessed during this generator of at least partial capacitance for the cells may be used to assemble nanowires, and (3) still more energy could be stored in reduced carbon, especially polysaccharides as nanowires are being constructed. As electron acceptors become limiting in the system the *S. oneidensis* MR-1 grows the nanowire network to "reach" the electron acceptors. Subsequently, probably due to the development of nanowires within the column, we observed SP signal at all electrode locations.

[16] The magnitude and the duration of the SP signals were controlled by the electron donor availability (lactate), electron acceptor availability (O_2, Fe^{3+}), nanowire building processes, and the charge accumulation within the electrolyte medium. We observed that the SP values remained stable at peak values of 602 mV for ~30–40 hours and subsequently declined (Figure 2). Lactate concentration in the column reduced during the experiment but never reached limiting conditions (6 mM final concentration); presumably lactate is not the limiting factor in the columns. As a result, the response we observed is likely dependent on the electron acceptor availability. The dominant electron acceptor in the column is atmospheric O_2 which diffused from the top of the column creating an oxidized zone that diminished towards the bottom of the column. The contrast between the oxic and anoxic zones stimulated extensive nanowire development observed by SEM (Figure 3). The electrical potentials generated in the sediments depend on the relative extent of the oxidized and reduced zones, as well as from charge accumulation in the electrolytic fluid which can impede the free ion movement in the columns. Due to lack of any proton removal mechanism we assume that as the experiment progressed, ions accumulated in the upper parts of the column (minimizing/terminating the charge imbalance), which combined with electron acceptor limitation caused the system to approach an equilibrium state leading to SP signal reduction. The SP response lies well within the theoretical reduction potential of the decaheme cytochrome and available oxygen [*Madigan et al.*, 2003]. Although we cannot definitively distinguish between atmospheric O_2 and Fe^{3+} as the dominant electron sink, electron transport pathways (nanowires) are needed in both cases.

[17] In the control column (*S. oneidensis* mutant strain), the absence of significant SP signals is consistent with earlier findings [*Gorby et al.*, 2006] that mutants deficient in genes for *c*-type decaheme cytochromes MtrC and OmcA display nanowires that are poorly conductive and unable to generate current in an MFC. Hence, although lactate oxidation was occurring and nanowire-like structures were produced, their inability to transport electrons prevented electron transfer between the anodic and cathodic regions. We note that at the end of the experiment, cells were visibly deteriorated (Figures 3c and 3d) presumably resulting from a lack of an appropriate electron-acceptor for re-oxidation of the cytochromes, limiting respiration.

[18] A subsequent replica experiment provided additional insights into the source mechanism for the SP signal, favouring a geobattery rather than a galvanic cell [*Nyquist and Corry*, 2002] model whereby the SP signal is the result of electrode reactions and the electrons are transferred through the instrumentation wiring. During this replica experiment, we observed Eh gradients in both the wild type (up to 450 mV) and mutant strain (up to 350 mV) columns, with a positive correlation between Eh and SP in the wild type column with no such correlation in the mutant column. This observation suggests that in the absence of electron conductive pathways (nanowires) within the columns, Eh gradients alone cannot drive SP signals. Moreover, no current flow was measured outside the column body (i.e., through instrument wiring). These observations suggest that the electrical potentials measured in our system cannot be explained by a galvanic cell mechanism.

[19] The fact that large electrical potentials were recorded for *S. oneidensis* MR-1 in contrast to the mutant strain is significant. Presumably the EF is associated with the electron flow in the conductive nanowires. Typically, conducting-probe atomic force microscopy [*Reguera et al.*, 2005] and scanning tunnelling microscopy [*Gorby et al.*, 2006] are used to determine the electrical conductivity of the nanowires. Because of the fragility of nanowires, such measurements are difficult to make. This problem is compounded in the presence of solid surfaces (i.e., sand grains).

Several attempts by our group to measure the conductivity of nanowires attached to sand grains were unsuccessful. Perhaps geophysical results such as presented in this paper provide an independent line of evidence confirming the functionality and conductivity of these nanowires. Thus, if nanowires "hardwire" the subsurface, the transfer of electrons across interfaces (electron acceptor poor/electron acceptor rich) to support cell growth and viability will generate a naturally detectable EF as evidence of their presence, permitting geophysical methods to be used as proxy indicators of the occurrence of microbial electron transport processes at field sites and for understanding the temporal and spatial changes in redox processes, microbe-mineral transformations, and for the investigation of life in remote (e.g., deep ocean basins) and extreme environments (e.g., alkaline, acidic, and extraterrestrial environments, etc.).

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