FINAL SCIENTIFIC REPORT

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PROJECT TITLE:	Bacterial Nanowires and Extracellular Electron Transfer to Heavy Metals and Radionuclides by Bacterial Isolates from DOE Field Research Centers
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EXECUTIVE SUMMARY

The work summarized here was aimed at understanding bacterial extracellular electron transport (EET), and the role of conductive nanowires produced by bacteria in this process. EET allow bacteria to utilize insoluble substrates outside the cell, opening up windows of metabolism unavailable to other bacteria that are incapable of EET. When these bacteria are electron-acceptor (e.g., oxygen) limited, they synthesize the EET system, and use other substrates like insoluble iron or manganese oxides. Alternatively, they can use soluble electron acceptors that become insoluble upon electrochemical reduction. It is this process that the work was focused on: the conversion of soluble oxidized uranium to insoluble uraninite as a mode of remediation, and the role of nanowires in this process. The work involved isolation of many bacteria from various environments that were capable of EET, and the examination of these isolates for the presence of nanowires. The work then progressed to the analysis of the nanowires, revealing that many of the isolates produced nanowires capable of long-range electron transfer. Finally, several of these isolates were examined for their ability to reduce (and render insoluble) uranium. This work, still in progress, sets the stage for a major effort in the bioremediation of uranium (and possible chromium, which is reduced (and rendered insoluble) in a similar way by many of these bacteria).

One point to emphasize is the importance of method development. The literature is not at all clear in terms of the conditions that favor the development of nanowires, and a considerable time was spent, especially with strains of *Geobacter* developing methods that could routinely produce abundant nanowires for analysis. These methods, along with several other new approaches are carefully described in the publication that is now in preparation (15). Other methods for the study of various organisms and ecosystems are described in detail in the publications listed in the bibliography.

SUMMARY FOR NON-EXPERTS:

This proposal involved the study of bacteria capable of transferring electrons from the bacterial cells to electron acceptors located outside the cell. These could be either insoluble minerals that were transformed into soluble products upon the addition of electrons, or they could be soluble salts like uranium or chromium, that become insoluble upon the addition of electrons. This process is called extracellular electron transport or EET, and can be done directly by cellular contact, or via conductive appendages called bacterial nanowires. In this work we examined a number of different bacteria for their ability to perform EET, and also looked at their ability to produce conductive nanowires that can be used for EET at a distance away from the EET-capable cells. In the work, new bacteria were isolated, new abilities of EET were examined, and many new methods were developed, and carefully described in the literature. These studies set the stage for future work dealing with the bioremediation of toxic metals like uranium and chromium. They also point out that EET (and conductive nanowires) are far more common that had been appreciated, and may be involved with energy transfer not only in sediments, but in symbioses between different bacteria, and in symbiosis/pathogenesis between bacteria and higher organisms.

MAJOR GOALS OF THE PROJECT:

The major goals of the proposed work were based on understanding the role(s) of bacterial nanowires in the reductive transformation of heavy metals and radionuclides. The work set forth 4 hypotheses that would be systematically tested to achieve the goal as stated above. These are:

1. Branched extracellular appendages produced by *Geobacter* FRC-32^T, *Geobacter* PCA (ATCC 51573), *Anaeromyxobacter* fw109-5 and *Desulfovibrio* FR1012B under conditions of electron acceptor limitation are electrically conductive.
2. Bacterial nanowires from ORFRC (Oak Ridge Field Research Center) isolates are protein assemblages containing a combination of structural proteins (pilin) and electron transport proteins (multi-heme c-type cytochromes).
3. Nanowires produced by ORFRC isolates can reduce and transform heavy metals and radionuclides over distances that exceed 10 microns.
4. Bacterial nanowires rapidly transform uranium into insoluble precipitates and mitigate its transport in porous subsurface media.

The tests of these hypotheses have involved the following research objectives:

1. confirm and characterize the conductivity of nanowires produced by metal-reducing and sulfate-reducing isolates from contaminated zones of the ORFRC;
2. Identify the genes and gene products involved in nanowire production;
3. Evaluate the enzymatic activity of these organisms with regard to Fe(III) and U(VI) reduction rates under electron acceptor-limited conditions;
4. Describe the location and stability of biogenic U(VI) precipitates; and
5. Evaluate the impact of U(VI) reduction and precipitation by targeted ORFRC isolates on the fate and transport of U(VI) through saturated porous media.

These are then reduced to three central research questions as noted below. To summarize the work of this proposal as it relates to the three central research questions, I have presented a narrative below, followed by a list of the relevant publications and presentations that resulted from the work.

1. Are 'putative nanowires' from the ORFRC isolates *Geobacter* FRC-32^T, *Anaeromyxobacter* fr109-5, and *Desulfovibrio* FW1012B conductive? If so, what are their conductive (electron transport) and non-conductive components? How does conductivity of nanowires from FRC isolates compare with those from *Shewanella* and other *Geobacter* strains.

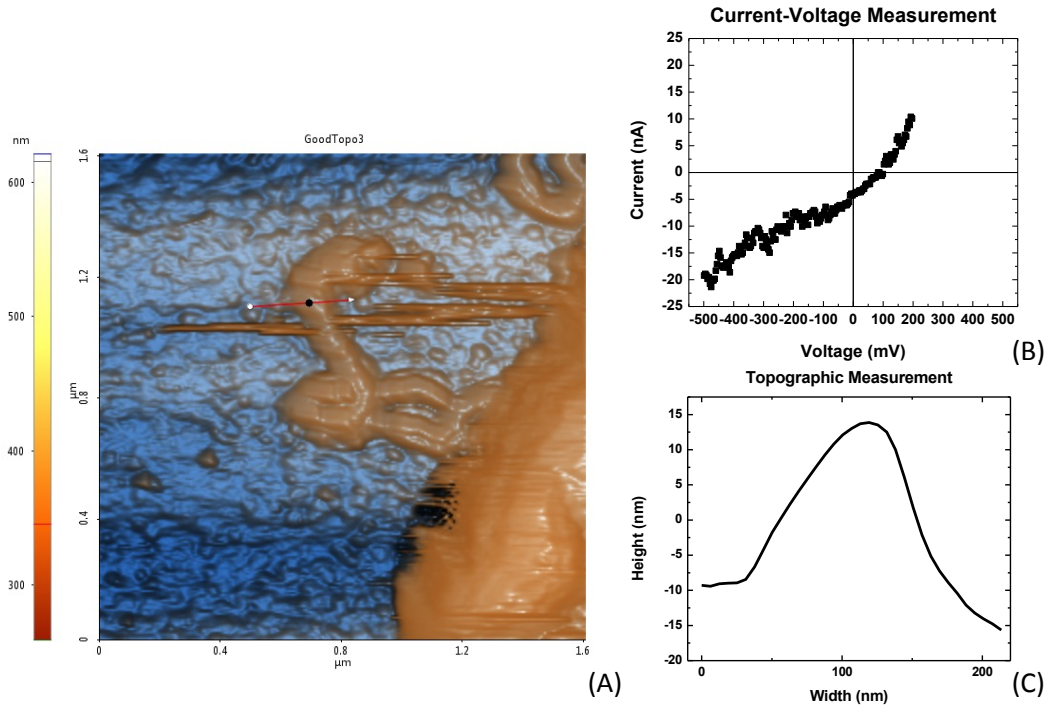
2. Do bacterial nanowires from FRC isolates conduct electrons along their length? If so, how far can nanowires conduct electrons? What is the maximum length of a conductive nanowire produced by FRC isolates? What

limits and controls growth of nanowires in porous subsurface media? Can nanowires access metals in pore spaces too small for cells to enter? If so, can our knowledge of nanowires be used to develop improved remediation strategies for heavy metals and radionuclides in contaminated sediments characterized by restricted pore spaces?

3. What metals are reduced and transformed by nanowires from FRC-32^T isolates? What effects do FRC-32^T isolates have on fate and transport of uranium in saturated porous media? Are nanowires involved in re-oxidation of reduced biogenic nanoparticles of uranium oxide? Can our improved knowledge of the range of metals transformed by nanowires be used to improve remediation approaches for sediments contaminated with mixtures of heavy metals and radionuclides?

ACCOMPLISHMENTS UNDER THESE GOALS:

1. **Are putative nanowires conductive:** A major effort was made to determine conductivity of putative nanowires from a number of different bacteria with the result that many of the tested samples contained nanowires that displayed substantial conductivity. This resulted in a publication describing the various bacteria, their ecological niches where known, and the conductivity of their appendages. (10). One very exciting result was the demonstration that the bacteria involved with jaw osteonecrosis had extensive conductive nanowires in contact with degrading jaw material (calcium phosphate) (8). Isolates were obtained using fuel cell methods for enrichment, using approaches similar to those employed by the PI from other environments (4,10). In our recent investigation, we found out that the nanowire produced by *Geobacter sulfurreducens* PCA is an outer-membrane-extension structure which can be distinguished from any known bacterial organelle, such as pilus. The conductivity of nanowire structure was measured using conductive-probe atomic force microscope. According to our results, the *Geobacter*-nanowire image was revealed by contact mode, in which a coiled structure could clearly be seen on the side of the bacterial cell (Fig. 1 (A)). Height profile (Fig. 1 (C)) exhibited that the appendage has a diameter of 23 nm. In addition, I–V curves (Fig. 1 (B)) showed a nonlinear ohmic behavior.



2.

3. Fig. 1. (A) AFM topography images, (B) I-V curve measurement (at the shown black dot), and (C) height profiles (along the shown red line) of *G. sulfurreducens* PCA (ATCC 51573) analyzed in this study.

4. **Do nanowires from FRC-32^T isolates conduct electrons along their length, and over what length can this conduction occur?**

It is clear from the analyses done in the Gorby laboratory that some of the FRC-32^T isolates produce nanowires that are capable of transmitting electrons along their length. The mechanism by which this occurs is still under study, and can't be specified at this time. The alternatives are that it is similar in mechanism to that of *Shewanella* species (1-3,6,11), or that it is, like *Geobacter*, distinctly different, but still under study (and strongly argued), or even similar to the (as yet uncharacterized) mechanism utilized by cable bacteria for long-range electron transport in sediments (9).

To verify the conductivity of nanowire produced by *Geobacter* FRC-32^T, electronic transport along a nanowire in contact with the Au elelctrode was measured by using the Pt-coated AFM tip as a second electrode. With the AFM tip at the position shown in Fig. 2A, ca. 600 nm away from the Au electrode and in contact with the nanowire, we obtained the I-V curve shown in Fig. 2C. According to the result of I-V curve, the current ramping (increasing from –9 to 9 nA with tip bias increasing from –1 to +1 V) measured in Fig. 4 (B) was found to be similar with the ones measured with Shewanella nanowires (Gorby et al. 2008; Leung et al. 2013). Combining the results in Fig. 1 and 2, it conclusively indicates that the nanowire structures produced by *Geobacter* sp. are eligible for extracellular electron transfer.

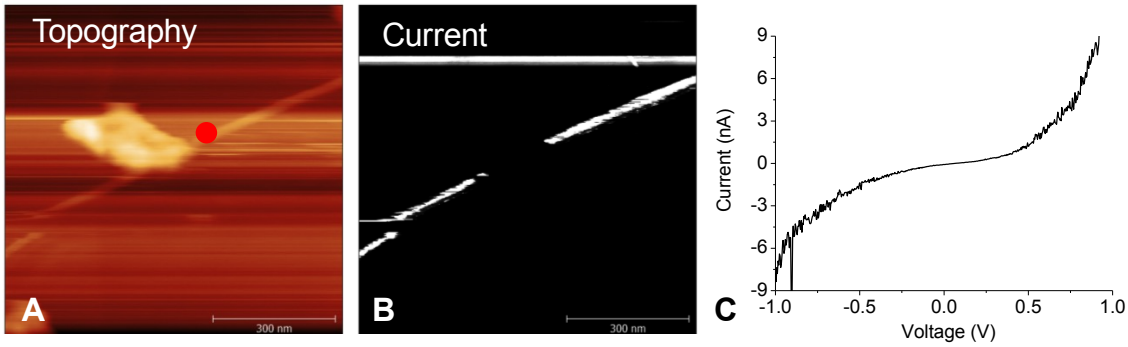


Fig. 2. CP-AFM of a bacterial nanowire. (A) Topographic AFM image showing air-dried *Geobacter daltonii* FRC-32^T cells and extracellular appendages deposited randomly on a SiO2/Si substrate patterned with Au electrode. (B) the simultaneously recorded current map obtained using a 100 mV sample voltage (C) An I-V curve obtained by probing the nanowire at a length of 600 nm away from the Au electrode (at the position marked by the red dot in A).

5. **What metals are reduced and transformed by nanowires from FRC isolates, and what are the interactions with U(VI)?** A complete suite of metals reduced by the FRC isolates was not possible to obtain, but several of the isolates were able to reduce U(VI) to uraninite, making them good candidates for useful organisms for uranium bioremediation.

   This project successfully developed and applied methods needed to optimize the production of bacterial nanowires in *Geobacter* species and used those techniques to demonstrate that nanowires in *Geobacter* are actually extensions of the outer membrane, similar to those produced by Shewanella. Tasks addressing the role of membranous nanowires from Geobacter for reducing U(VI) to U(IV) have yet to be completed. The Kinetics Phosphorescence Analyzer (KPA) that was purchased as capital equipment for this project is on loan from USC to Rensselaer Polytechnic Institute , where Dr. Gorby now instructs environmental engineering students to use the instrument for evaluation uranium at ultralow concentrations as part of his course in Remediation of Hazardous Compounds. Hence, students will use the KPA, transmission electron microscopy, and controlled cultivation techniques to evaluate the role of membranous nanowires from Geobacter for the reduction and precipitation of uranium under environmentally relevant conditions.

6. **How does the nanowire grow on *Geobacter* PCA?**
   To implement in-vivo observation of the nanowire growing on transparent cover slips, we incubated the *Geobacter* PCA in a perfusion chamber which was developed in our earlier investigation (Pirbadian et al., 2014). To obtain visible images, the membrane stain, FM4-64FX, served to enhance the

contrast under an epifluorescence microscope (Nikon Eclipas Ti-E, Tokyo, Japan). The best resolved image of conductive-appendage growth is shown in Fig 3 (A), which represents the result happening at 4 h after the fresh media streaming into the cell. As a result, the filament could grow up to ca. 10 μm long. The continuous images were also captured for filming a video as shown in Movie S1. The glass cover slips were soaking in a glutaraldehyde solution for fixing those *Geobacter* cells immediately after perfusion was stopped. The fixed specimens were observed with a better resolution using SEM. As a result shown in Fig. 3 (B), a number of vesicles aligned together and began to form a continuous filament.

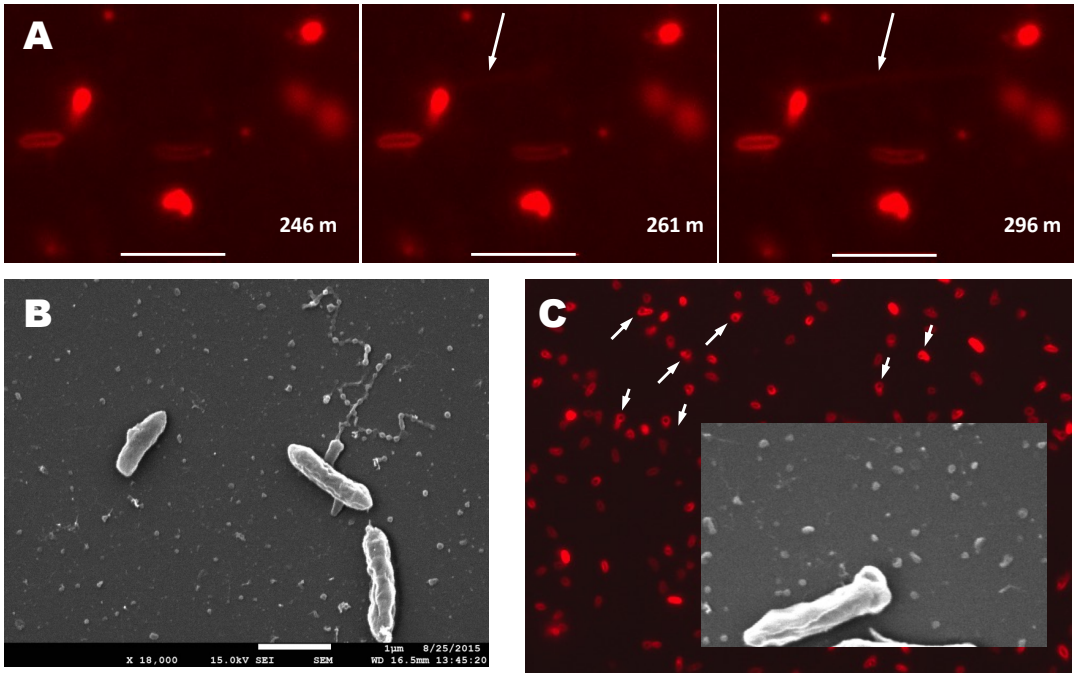


Fig. 3. Observation of nanowire-like-appendages formations and cell collapses of *G. sulfurreducens* PCA (ATCC 51573) with different compostions of acetate and fumarate in streaming media. (A) A nanowire-like filament observed with fluorescence from the membrane stain FM4 64FX. Extracellular structure formation was first observed in the t = 246 min frame. (Scale bars: 5 μm.) (B) produced vesicles incorporating to form a partially smooth filaments (Scale bars: 1 μm.) (C) formation of blister-like structures (white arrows) on cells which has been further observed using SEM (the inner graph). (A) and (B) were imaged under the condition of acetate : fumarate = 20 : 80 mM, while (C) was imaged under acetate : Fumarate = 20 : 0.25 mM

7. **What is the optimal nanowire-growing condition for *Desulfovibrio vulgaris* RCH-1, and how does the nanowire been used for depositing uranium (IV)?**

Desulfovibrio vulgaris RCH-1 produced nanowires under conditions of electron acceptor limitation, with sulfate serving as the electron donor (Fig. 4(A). *D. vulgaris* RCH-1 also produced membrane vesicles, revealed here by TEM (Fig. 4 (B)) . Vesicles appear to be connected to cells via extracellular appendages and/or in chains. When *D. vulgaris* RCH-1 was provided uranyl acetate as an alternative electron acceptor under these conditions, cells reduced and precipitated uranium along these extracellular filaments (Fig. 4 (C), (D)). Non-osmicated samples receiving uranyl acetate revealed an unstained thin core structure (Fig. 4(C)), which upon osmication becomes black (Fig. 4(D)), indicating that the thin structure is lipid-based. Note the number of vesicles nearby or docking to these extracellular structure, further supporting membrane lipid-based origin.

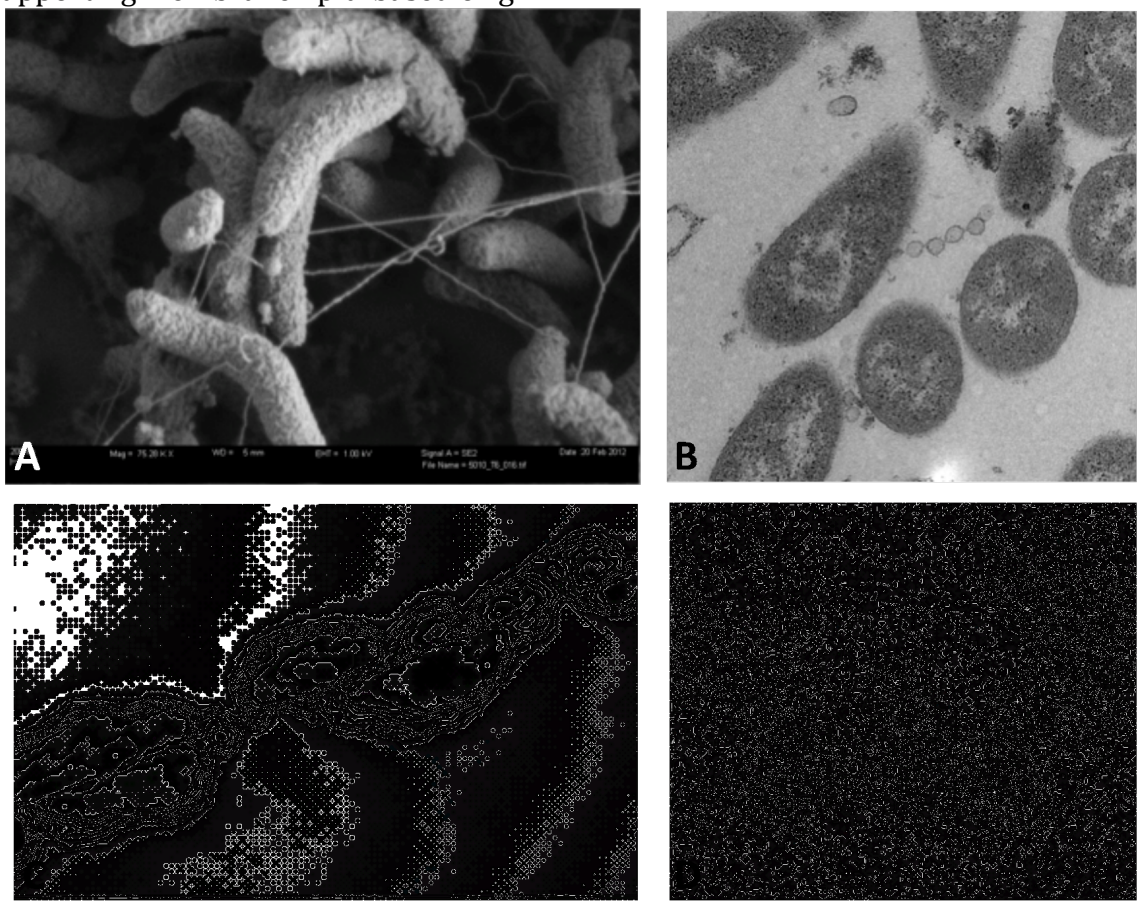


Fig. 4 (A) Scanning-electron micrograph of filamentous appendages observed on *Desulfovibrio vulgaris* RCH-1 cells (B) transmission electron micrograph showing the vesicle chain structure beginning to form a continuous filament (C) transmission electron micrograph (non-osmicated) of Desulfovibrio vulgaris RCH-1 cells incubated in a medium containing uranyl acetate which was transformed to nanocrystalline along nanowire structure (D) transmission electron micrograph obtained under the same cultivate condition but with osmium stain during sample preparation.

Publications Resulting from this Work:

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3. Pirbadian, S. S.E. Barchinger, K.M. Leung, H.S. Byun, Y. Jangir, R.A. Bouhenni, Y. Gorby. 2015. *Shewanella oneidensis* MR-1 nanowires are outer membrane and periplasmic extensions of the extracellular electron transport components. Proc. Nat. Acad. Sci. USA. 111: 12883-12888.

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12. Leung, K.M., G. Wanger, Q. Guo, Y. Gorby, G. Southam… 2011. Bacterial nanowires: conductive as silicon, soft as polymers. Soft Matter 7:6617-6621.

13. El-Naggar, M.Y., G. Wanger, K.M. Leung, T.D. Yuzvinsky, G. Southam, J. Yang, Y. Gorby. 2010. Electrical transporta along bacterial nanowires from *Shewanella oneidensis* MR-1. Proc. Nat. Acad. Sci. 107:18127-18131.

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15. Li., S.-L., Gorby, Y., Giordano, M., Lewis, K. M., Nealson, K. H., Conductive appendages Produced by Geobacter sulfurreducens PCA (ATCC 51573) Under High-Fumarate-Loading Conditions are Composed of Vesicles (in preparation)

Abstracts and Presentations:

Bauermeister, A., Y. A. Gorby, J. Schramm. 2014. Abstracts of the American Geophysical Union Fall Meeting. 1, 0135.

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Li, S. L., Nealson, K., Enriching Sulfate-Reducing Bacteria via an Electrochemical-Sulfide-Oxidizing Process on Carbon Electrodes. *Goldschmidt 2014,* Sacramento, United States. (2014). (Oral Presentation)

Li, S. L., Lis, L, Gorby, Y., Nealson K, Bacterial Nanowires and Extracellular Electron Transfer to Heavy Metals and Radionuclides by Bacterial Isolates from DOE Field

Research Centers. *Environmental System Science (ESS) PI Meeting,* Potomac, MD, United States. (2014). (Poster)

Li, S. L., Lis, L, Gorby, Y., Pirbadian, S., Leung, K. M., El-Naggar, M. Y., Lis, L., Nealson K. H., Investigating the Properties of *Geobacter sulfurreducens* DL-1 Extracellular Extensions: Determining a Optimum Condition of Chemostat Culturing to Reach the Best Extracellular-Electron-Transfer Performance. *The Fifth International Meeting on Microbial Electrochemistry and Technologies*, Tempe, AZ, United States. (Oral Presentation)