Integrated microscopic and metagenomic analysis of subsurface microorganisms that contribute to carbon and metal cycling

Luis R. Comolli, Birgit Luef, Cindy J. Castelle, Sirine C. Fakra, Kyle R. Frischkorn, Sean W. A. Mullin, Kelly C. Wrighton, Brian J. Anderson, Michael J. Wilkins, Roseann Csencsits, Mary S. Lipton, Steven W. Singer, and Jillian F. Banfield

Our research is conducted at the Department of Energy's (DOE) Rifle Integrated Field Research Challenge (IFRC) site in Rifle, Colorado, USA. Microscopic analyses have advanced our understanding of well-studied bacteria that proliferate during initial subsurface acetate biostimulation and provided insight into the novel diversity that appears during secondary stimulation.

Geobacter species are important planktonic Fe-reducing bacteria (FeRB) abundant during primary stimulation (2010). We applied a new sample preparation method in which samples were cryo-plunged directly on site immediately after sampling (Comolli *et* al. 2011). Cells and cell-associated minerals were analyzed using 2D and 3D cryo-TEM, high resolution TEM (HRTEM), energy dispersive spectroscopy (EDS) and scanning transmission X-ray microscopy (STXM). Confocal laser scanning microscopy (CLSM) performed on cells labeled with *Geobacter*-specific fluorescence *in situ* hybridization (FISH) probes confirmed cell identities. This approach revealed that *Geobacter* accumulate remarkable aggregates of Fe-oxyhydroxide nanoparticles capable of providing sufficient electron acceptor to support planktonic growth and cell motility. To characterize relevant physiological states and expressed metabolic pathways more completely, correlated cryo-TEM and proteomic profiles associated with different growth stages and electron acceptors for *Geobacter* grown in laboratory experiments are under way. The development of antibody-based high-resolution image labeling is under way using *c*-type cytochromes detected in outer-membrane and extracellular protein fractions.

An experiment was designed partly to test the hypothesis that novel "Candidate Division" (CD) bacteria are active during secondary acetate biostimulation (2011). Clone library analysis of filtrate that passed through a 0.2 μm filter but is retained on 0.1 μm filter revealed that CDs OD1 and OP11 were dominant members of the community. Flash-frozen cryogenic groundwater samples (09/03/2011) were characterized via 2D and 3D cryo-TEM. We have identified and characterized highly unusual, ultra-small cells (200 - 250 nm in diameter), and are currently elucidating the 3D ultrastructure and associations. These data show median cell sizes below previously estimated lower size limits for life (NRC 1999). Evidence of unique spatial optimization strategies are evident in their 3D architecture, such as: a very compact packaged genome, distribution of ribosomes and polysomes, and cell wall-attached ribosomes. The cell wall has a novel architecture, with a remarkable and distinct surface layer, and several appendages. Higher resolution structure determination of the macromolecular components is under way. While uncultured, both OD1 and OP11 cells are inferred from Rifle community genomics data to have small genomes, lack a Gram-negative cell envelope, and encode pili, consistent with these observations (Wrighton et al., submitted). Work is ongoing to perfect fluorescence *in situ* hybridization probes that would correlate cell structure with identity. Metagenomic analysis suggests that both OD1 and OP11 are obligate fermentative bacteria, and likely play a carbon cycling role in the Rifle subsurface.