Report Title

Recovery Act: Understanding the Impact of CO_2 Injection on the Subsurface Microbial Community in an Illinois Basin CCS Reservoir: Integrated Student Training in Geoscience and Geomicrobiology

Final Scientific/Technical Report

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ABSTRACT

An integrated research and teaching program was developed to provide cross-disciplinary training opportunities in the emerging field of carbon capture and storage (CCS) for geobiology students attending the University of Illinois Urbana-Champaign (UIUC). Students from across the UIUC campus participated, including those from the departments of Geology, Microbiology, Biochemistry, Civil and Environmental Engineering, Animal Sciences and the Institute for Genomic Biology. The project took advantage of the unique opportunity provided by the drilling and sampling of the large-scale Phase III CCS demonstration Illinois Basin - Decatur Project (IBDP) in the central Illinois Basin at nearby Decatur, Illinois. The IBPD is under the direction of the Illinois State Geological Survey (ISGS, located on the UIUC campus) and the Midwest Geological Sequestration Consortium (MGSC).

The research component of this project focused on the subsurface sampling and identification of microbes inhabiting the subsurface Cambrian-age Mt. Simon Sandstone. In addition to formation water collected from the injection and monitoring wells, sidewall rock cores were collected and analyzed to characterize the cements and diagenetic features of the host Mt. Simon Sandstone. This established a dynamic geobiological framework, as well as a comparative baseline, for future studies of how CO_2 injection might affect the deep microbial biosphere at other CCS sites. Three manuscripts have been prepared as a result of these activities, which are now being finalized for submission to top-tier international peer-reviewed research journals.

The training component of this project was structured to ensure that a broad group of UIUC students, faculty and staff gained insight into CCS issues. An essential part of this training was that the UIUC faculty mentored and involved undergraduate and graduate students, as well as postdocs and research scientists, at all stages of the project in order to develop CCS-focused classroom and field courses, as well as seminars. This program provided an excellent opportunity for participants to develop the background necessary to establish longer-term research in CCS-related geology and microbial ecology. Further, the program provided an ongoing dynamic platform to foster long-term collaboration with the regional ISGS and MGSC sequestration partnership, while offering hands-on, applied learning experiences.

TABLE OF CONTENTS

EXECUTIVE SUMMARY

The specific integrated research and training tasks of this project were as follows:
• **Task 1 –** To characterize the subsurface microbial community structure and metabolic activity of the Cambrian-age Mt. Simon Sandstone of the Illinois Basin.
• **Task 2 –** To establish the paragenetic and diagenetic history of Cambrian-age Mt. Simon sandstone at the IBDP site.
• **Task 3 –** To fully involve and mentor undergraduate and graduate students, postdocs and research scientists in all microbiological and geological aspects of the research in Tasks 1 and 2.
• **Task 4 –** To develop and present a CCS seminar series and a formal UIUC undergraduate course in collaboration with researchers at the ISGS, both of which focused on the microbiology, geoscience, and technological challenges of CCS technology and approaches.

Accomplishment of Tasks 1, 2 and 3
IBDP Injection Well: Integrated analyses were completed of formation pore water, microbe and rock samples collected from the IBDP injection well to determine the composition and activity of native microbial communities within subsurface sandstone reservoirs. The target reservoir was the lower-Paleozoic Cambrian-age Mt. Simon Sandstone, which reaches a maximum thickness of 494 m (1620 ft) and a modern-day burial depth of 2.2 km (7236 ft). A 35-cm diameter well bore was drilled in Decatur, Illinois (39°52'36.58" N, 88°53'36.00" W), which penetrated the entire Mt. Simon Sandstone section. A comprehensive suite of seismic lines, wire-line logs, full rock core and sidewall rock core were done upon completion of the well. A breakthrough in low-contamination sampling was made possible for this study by deploying the newly developed Schlumberger ®Quicksilver Probe (part of the Schlumberger ®Modular Formation Dynamics Tester and ®*In Situ* Family of downhole sensors). Microbes detected in these ®Quicksilver Probe formation water samples were rigorously scrutinized for contamination by microbes from the drilling mud. Metagenome-enabled analyses of the microbes collected from the ®Quicksilver Probe water samples were then coupled with a comprehensive evaluation of the subsurface environmental conditions, rock properties and geological history of the Mt. Simon Sandstone at a depth of 1.8 km (5872 ft).

Results indicate that a low-diversity microbial community, dominated by the γ-Proteobacterium *Halomonas sulfidaeris*, inhabit the samples of warm saline formation pore water collected from the Mt. Simon Sandstone (1.8 km-5872 ft. burial depth, 50^{o} C, pH 8, 181 bars pressure). These highly porous and permeable quartz arenite sandstones are directly analogous to reservoirs around the world targeted for large-scale hydrocarbon extraction, as well as subsurface gas and carbon storage. Multiple lines of evidence suggest that this *H. sulfidaeris*-dominated subsurface microbial community is indigenous and not derived from drilling mud microbial contamination. Data to support this includes V1-V3 pyrosequencing of formation water and drilling mud, as well as comparison with previously published microbial analyses of drilling muds in other sites. Metabolic pathway reconstruction, constrained by the geology, geochemistry and present-day environmental conditions of the Mt. Simon Sandstone, implies that *H. sulfidaeris*-dominated subsurface microbial community may utilize iron and nitrogen metabolisms and extensively recycle indigenous nutrients and substrates. The presence of aromatic compound metabolic

pathways suggests this microbial community can readily adapt to and survive subsurface hydrocarbon migration.

IBDP Monitoring Well: In addition, "swabbing" techniques were used to collect samples at 1.72 km and 2.02 km from a monitoring well that was drilled near the injection well at the IBDP site. The two horizons exhibit similar temperatures (47-50 ^{o}C), formation pressures (170-210 bars), high concentrations of dissolved Fe(II) (1.05-1.5 mM) and salt (152-191 ppt). Active microbial enrichments with distinct physiological properties were successfully grown from both horizons at 45° C. The 1.72 km enrichment yielded five 16S rRNA gene OTUs (97 % cutoff). The dominant OTU exhibited 95 % similarity with *Vulcanibacillus modesticaldus*, an isolate from deep-sea hydrothermal vents that utilizes iron-reduction metabolisms at temperatures of 20-60 ^{o}C (optimal at 40 ^{o}C) and salinities of 25-75 ppt (optimal at 25 ppt). It was also capable of dissimilatory nitrate-reduction, sulfate-reduction and fermentation. In comparison, the 2.02 km enrichment contained only one 16S OTU, whose closest cultured representative is *Orenia marismortui* (94 % identity), a halophile from the Dead Sea. Unlike the 1.72 km culture, it exclusively uses hydrogen and pyruvate as the electron donors for iron reduction, tolerates a wide range of salinity (≤ 200 ppt), and exhibited lower nitrate- and sulfate-reduction rates. *Orenia sp* Strain Z6 fermentation on glucose was studied in the presence and absence of ferric iron oxide. Genome sequencing revealed a 3.5 MB genome for strain Z6 and its fermentation-related pathways consist of glycolysis, pentose phosphate pathway (PPP) and subsequent pyruvate metabolism, consistent to the products identified in glucose fermentation by this organism. The unique capacity of strain Z6 to use H_2 as the sole electron donor for iron reduction enabled qualitative and quantitative modeling of carbon and hydrogen flux during glucose fermentation with or without added hematite as a representative natural ferric iron oxide. Results showed that reduction of hematite was coupled to and significantly enhanced glucose fermentation by strain Z6 compared to that from fermentation alone.

Accomplishment of Task 4

A new course was formally offered at the University of Illinois Urbana-Champaign entitled "GEOL 497 Geology and Microbiology of CO2 Sequestration". There were 20 undergraduate and graduate students enrolled, representing 7 different departmental units and 3 colleges across the UIUC campus. In addition, a successful lecture series was offered in the UIUC Departments of Geology and Microbiology, entitled "***Subsurface Microbial Biosphere and CO_2 Sequestration".*** **L**eading scientists in subsurface geomicrobiology, molecular microbial ecology, CO_2 sequestration and hydrocarbon exploration were brought to UIUC to provide this lecture series on the UIUC campus during the Fall 2011 and Spring 2012 semesters. The lecture series included the following speakers: (1) Dr. Matthew Kirk, Sandia National Laboratory, spoke November 2011; (2) Professor Terry Engelder, Columbia University, spoke February 2012; (3) Professor Vincent Bulone, Royal Technical University (KTH) Stockholm, spoke April 2012; (4) Professor Kenneth Nealson, University of Southern California, spoke April 2012; and (5) Professor Mike McInerney, University of Oklahoma, spoke May 2012. In addition, UIUC undergraduate students, graduate students, postdocs and research scientists were actively trained and involved in all aspects of the ongoing field and laboratory research that were required for the successful completion of this project.

REPORT DETAILS

Experimental Methods

IBDP Injection Well: The CO_2 injection well of the Illinois Basin-Decatur Project (IBDP), Decatur, Illinois, was sampled to analyze formation pore water chemistry, microbial ecology and host rock lithology. Sidewall cores were collected by Schlumberger during the drilling process. A deep subsurface formation water sample containing a high content of drilling mud was collected from a drill stem test (DST). In addition, a clean sample with minimal drilling mud contamination was sampled from 1.8 km (5872 feet) in depth using the newly-developed Schlumberger ®Quicksilver probe. Immediately upon return of the probe to the surface, microbial cells were then centrifuged from this formation water sample for molecular analyses. Culturing and isolation was attempted on a variety of substrates, however no cell growth was observed. Petrographic analyses of sidewall core thin sections were carried out under an Axiovert 200M Fluorescent Microscope with Apotome (Carl Zeiss Company, Oberkochen, Germany). Physical and geochemical parameters of the target horizons were determined *in-situ* (e.g., temperature, pressure, porosity and permeability), in the field (e.g., pH, conductivity, dissolved oxygen and redox potential) and on the filtered water samples after they were transported back into the laboratory at UIUC (e.g., total organic carbon, cations, anions, trace metals and isotopic analyses). Microbes were collected from the formation water by centrifugation and quantified with Fluorescence In Situ Hybridization (FISH). The full-length 16S rRNA clone libraries and pyrosequencing targeting V1-V3 hypervariable region of 16S rRNA genes were developed on the extracted genomic DNA to understand microbial composition. In addition, *D5872 Metagenome* and its reference genome *H. sulfidaeris* Esulfide1 were obtained via a combination of 454 shotgun, 454 paired-ends (3-5kb inserts) and Illumina shotgun sequencing. Metabolic reconstruction of the indigenous microbial community was performed with the aid of theSEED subsystem database (Aziz et al., 2008; Meyer et al., 2008). For a full description of the methods, see Supplementary Information.

IBDP Monitoring Well: "Swabbing" techniques were used to collect formation samples at 1.72 km and 2.02 km from a monitoring well that was drilled near the injection well at the IBDP site. A similar suite of water and rock analyses conducted for the injection well (described above) was applied to the monitoring well samples. Batch culture iron-reduction and fermentation experiments were carried out in crimp-sealed serum bottles containing sterile and anaerobic basal media under a headspace containing N_2. Bicarbonate as the buffering reagent in the original medium was replaced by 10 mM 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) with the final pH 6.5. The pH was adjusted with 1 N HCl. Approximately 200 mM Na_2S was added to maintain anaerobic condition of the cultures.

Under fermentation alone condition, 5 mM glucose was added. Iron-reduction-facilitated-fermentation was created by adding hematite (approximately 7 mM) and glucose (5 mM). Hematite was selected because it has been identified as one of a major indigenous minerals within the host Mt. Simon Sandstone reservoir (Fig. 1). Results indicate that the cultures can reduce. Hematite was synthesized following the method of Schwetmann and Cornell (2000) and washed with nanopure water for at least 6 times before suspended as 1 M slurry, degased and autoclaved. Confirmation of mineral purity and composition was testified by X-ray diffraction (XRD) at the Frederick Seitz Materials Research Laboratory Central Facilities, UIUC. After inoculation, the cultures was stored still and in dark at 42 °C and manually shaken daily.

Results and Discussion

The lithology of the Mt. Simon Sandstone at the sampling horizon of 1.8 km depth is composed of quartz arenite sands coated with iron oxides (e.g., hematite and goethite) and syntaxial quartz overgrowths (Driese et al., 1981; Bowen et al., 2010) (Fig. 1). The sandstone at this depth (17 % porosity, 30 mD permeability; Bowen et al., 2010) is saturated with warm saline formation waters (50˚C, pH 8, 127,100 ppm TDS, 181 bars).

Cell enumeration and phylogenetic analyses, conducted on microbial DNA recovered as centrifuged pellets from the ®Quicksilver Probe water sample, indicated low biomass ($1.46[\pm 0.39]\times 10^6$ cells/L) and low microbial diversity. The 16S rRNA gene sequences were analyzed with three approaches, including: (1) full-length 16S rRNA gene sequence clone libraries; (2) pyrosequencing targeting the V1-V3 hyper-variable region of 16S rRNA genes; and (3) prediction of 16S rRNA genes from a thorough metagenome analysis. All of these approaches consistently indicate that the 1.8 km depth water sample contained a low diversity microbial community dominated by operational taxonomic units (OTUs) closely affiliated with the genus *Halomonas* (Figs. 2 and 3). Furthermore, these sequences indicate that more than one *Halomonas* species and strain were present. Of these, the most abundant OTUs exhibited 97-100 % similarity with *Halomonas sulfidaeris Esulfide1* (Fig. 3), which is a facultative anaerobe isolated from hydrothermal vents at seafloor spreading centers (Kaye et al., 2004). In addition, other low abundance OTUs were detected that exhibited 95-97 % similarity with *H. neptunia* and *H. variabilis* (Fig. 3), which were enriched and isolated from hydrothermal vents and saline lake environments (Arahal et al., 2002; Kaye et al., 2004)

Evaluation of Drilling Mud Contamination

To determine whether or not the *Halomonas*-related microorganisms in the ®Quicksilver probe water sample (D5872) were native or introduced from contamination, the microbial communities within drilling mud collected from a Schlumberger drill stem test (DST) were analyzed. This DST drilling mud sample had already been circulated within the subsurface, but only to shallower depths within the stratigraphically overlying Ordovician St. Peter Sandstone. This allowed the drilling mud itself to be used as an "internal tracer" of microbial contamination in the deeper Cambrian Mt. Simon Sandstone. Our integrated analyses indicate that the composition of this DST drilling mud reference sample was dramatically different from that of the D5872 ®Quicksilver probe water sample, not only in physical and geochemical properties (Table S1) but also in microbial diversity and community composition (Figs. S2 and S4). OTUs within the genus *Halomonas* account for less than 3 % of the V1-V3 sequence reads detected in the DST drilling mud slurry sample (Table S2). Furthermore, none of the dominant *Halomonas* species- and strain-level OTUs, observed in D5872, were detected in the DST sample (both 16S rRNA clone library and V1-V3 pyrosequencing datasets; Figs. 3).

Halomonas OTUs similar (but not identical) to those identified in D5872 have been detected in pore waters of globally distributed deep subsurface terrestrial and marine sediments and rock reservoirs (Orphan et al., 2000; Kaye, 2003; Biddle et al., 2005; Zhang et al., 2005; Inagaki et al., 2006; Batzke et al., 2007; Morozova et al., 2010; Kaye et al., 2011). These *Halomonas* organisms exhibit unique metabolic traits that enable them to survive in the deep subsurface, including facultative anaerobic, heterotrophic, halophilic (or halotolerant) and thermophilic (or themotolerant) physiologies (Oren, 2006; de la Haba et al., 2011). This allows *Halomonas* to adapt to and survive the physical and geochemical conditions (e.g., anoxic, dark, oligotrophic, saline and warm) of the deeply buried Mt. Simon Formation in the Illinois Basin.

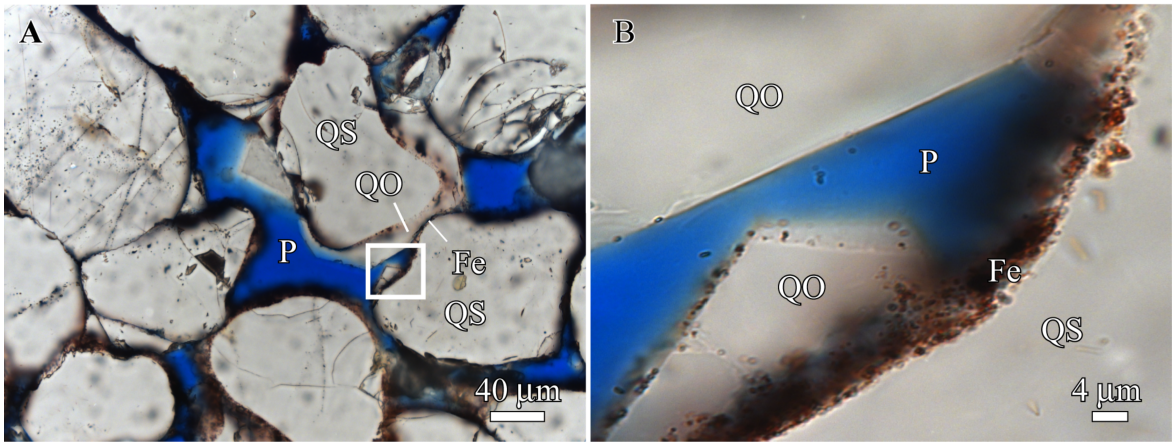


Figure 1. Plane-light photomicrographs of the Mt. Simon Sandstone quartz arenite at a burial depth of 1.8 km. The lithology is composed of high porosity (P) quartz sands (QS) and multiple intermittent events of quartz overgrowth (QO) cementation and dark brown to red iron oxide cementation (Fe).

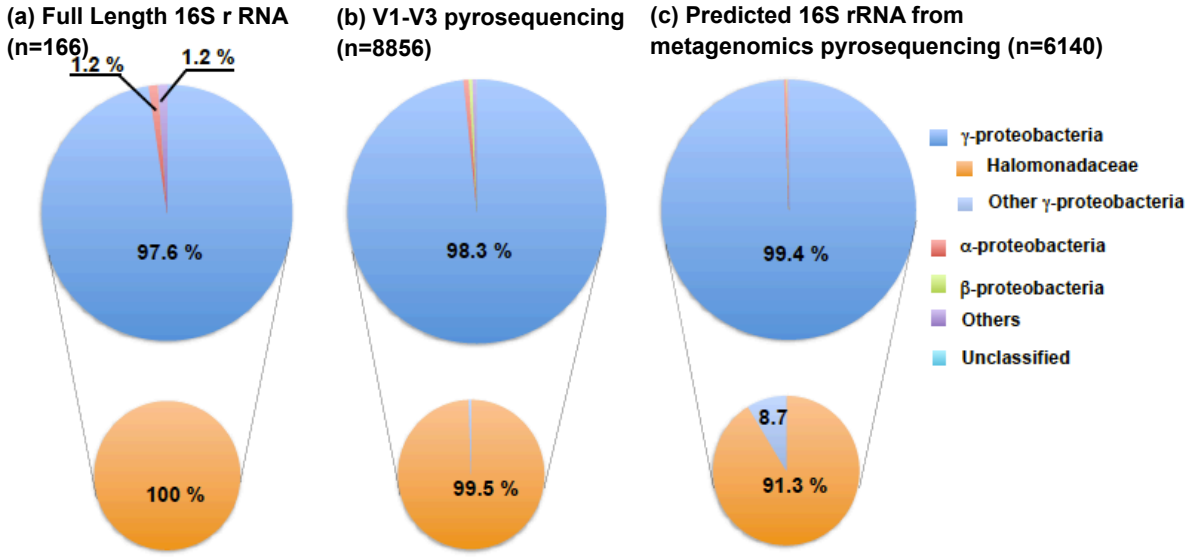


Figure 2. Composition of the microbial community inhabiting pore water collected using the Schlumberger ®Quicksilver probe at a burial depth of 1.8 km (5872 ft) from the Mt. Simon Sandstone saline sandstone reservoir in the Illinois Basin. (a) Classification of the full-length 16S rRNA gene clone library. (b) Proportions of 454 pyrosequencing reads targeting V1-V3 hypervariable region of 16S rRNA gene sequences. (c) Predicted 16S rRNA genes among the metagenomic reads. The lower small pie charts illustrate the dominance of the family *Halomonadaceae* in the class γ-Proteobacteria that was classified by the three approaches. N values represent the number of sequences or reads that were analyzed. Percentage values labeled within the pie charts denote the fraction of a phylum that was present in the indigenous subsurface microbial community.

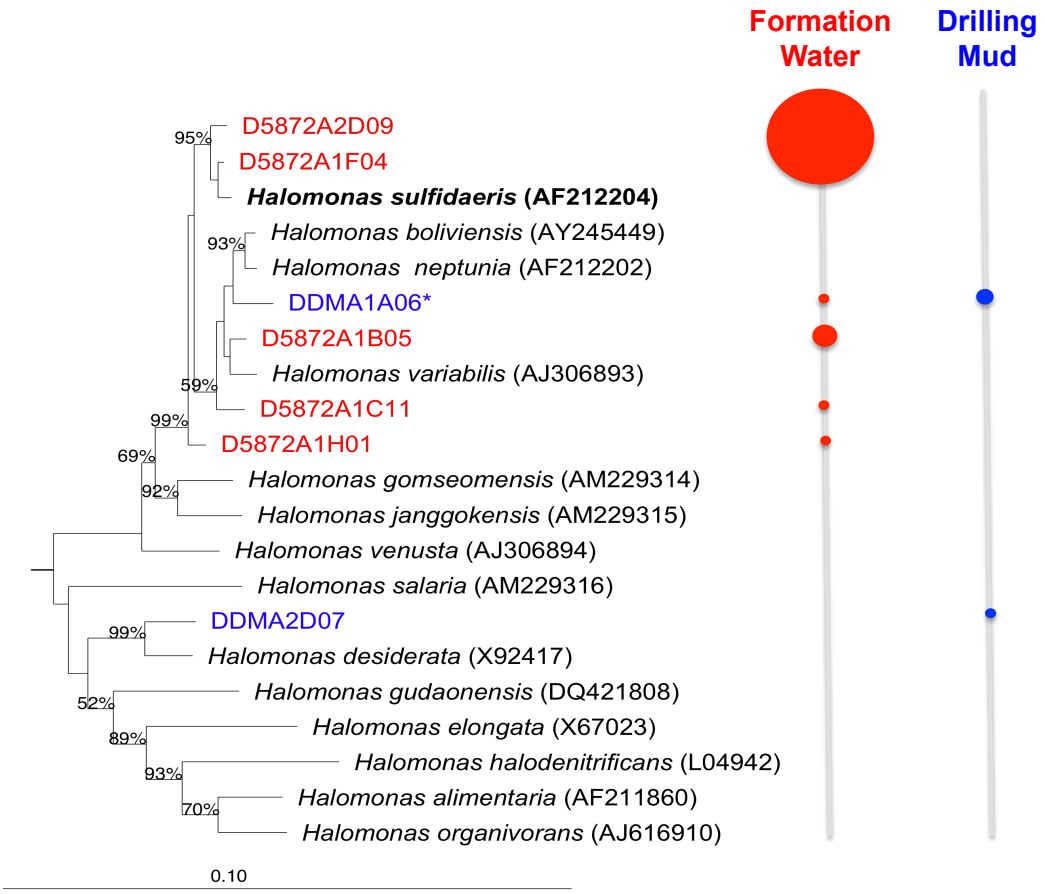


Figure 3. Phylogenetic comparison of *Halomonas* OTUs detected in 1.8 km-depth ®Quicksilver probe formation water and the DST drilling mud. *Escherichia coli* (X80725) was used as the outgroup and the scale bar indicates 0.1 change per nucleotide position. The accession numbers for the type strains are shown in parentheses. Statistical confidence for the evolutionary tree was assessed by bootstrap (1,000 replicates) and shown as bootstrap values in percentage.

A previous oil field study off the Shimokita Peninsula of Japan found *Halomonas* OTUs in drilling fluids after they had been circulated into the deep subsurface. However, these same *Halomonas* OTUs were not detected in either the drilling fluids prior to their subsurface circulation or xanthan gum powder added to increase riser fluid viscosity (Masui et al., 2008). Masui et al. (2008) interpreted these post-circulation *Halomonas* OTUs to be derived either from seawater or subsurface volcanic ash layers, but not directly from the pre-circulation drilling fluids themselves. In the present study, the dominance of a distinct *Halomonas* OTU (Fig. 3) in D5872, the absence of this same *Halomonas* OTU in the DST drilling mud sample (Fig. 3) and the consistency with results from the Masui et al. (2008) study, combine to suggest that the *Halomonas sulfidaeris*-dominated microbial community is indigenous to the subsurface Mt. Simon Sandstone and not a result of contamination from drilling mud infiltration.

Metabolic reconstruction and prediction of the native subsurface microbes

The metagenome library constructed from the *H. sulfidaeris*-dominated subsurface microbial community sampled from a depth of 1.8 km (5872 ft, herein referred to here as the *D5872 Metagenome*) contained approximately 3 million high-quality reads that were assembled into 3,239 contigs >500 bp and 4,603 contigs ≥200 bp (Table 1). Among the 4,603 contigs with an average GC content of 58.6±5.8 % and 59,702 singletons, 46,331 unique protein-coding genes (14,477 from contigs, and 31,854 from singletons with lengths >100 aa) were predicted (Table 1). In order to better capture all core pathways for the *D5872 Metagenome*, proteins derived from both contigs and singletons were included for the metabolic reconstruction (Rho et al., 2010). Using the *H. sulfidaeris Esulfide1* (American Type Culture Collection (ATCC) BAA-803) (Kaye et al., 2004) genome as a reference (Kumar et al., 2011), 55 % of the contigs showed similarity to the reference genome (blastn E-value $\leq 10^{-5}$) and exhibited an average GC content of 56.5±4.1%, which is close to the average GC-content of 54% for the reference genome (Fig. S5). Of the contig proteins, 77% show similarity to those predicted in the reference genome (blastp E-value $\leq 10^{-5}$). The high similarity between the proteins predicted in *H. sulfidaeris Esulfide1* and those in *D5872 Metagenome* is consistent with the phylogenetic relationships from the analysis of 16S rRNA sequences (Figs. 2 and S3). This was further supported by taxonomic assignment of predicted open reading frames as dominantly affiliated with the phylum Proteobacteria (Fig. S6). Comparison of the *D5872 Metagenome* and the *H. sulfidaeris Esulfide1* reference genome (Kumar et al., 2011) led to the identification of comparable genes involved in translation, transcription and replication within the metagenome (Tables S3-S5). Meanwhile, transfer RNAs enabling translation of all amino acids were also detected in the *D5872 Metagenome* (Table S6). Assuming that the dominant *H. sulfidaeris*-like bacterium detected in the ®Quicksilver probe water sample has a genome of similar size to that of *H. sulfidaeris Esulfide1* (4.44 Mb) (Kumar et al., In preparation) the *D5872 Metagenome* reads represent more than 8× genome recovery of this predominant organism in the environment (Table 1).

Functional annotation and metabolic reconstruction of the *D5872 Metagenome* was carried out by comparing the predicted proteins with those in theSEED Subsystems database (Aziz et al., 2008; Meyer et al., 2008). TheSEED subsystems (Aziz et al., 2008) represent collections of functional roles that make up a metabolic pathway, a complex (e.g., the ribosome), or a class of proteins. These subsystems are then grouped into broader functional categories, such as 'Virulence'. As illustrated in Fig. 4, the major categories observed within the *D5872 Metagenome* contain a complete suite of metabolic functions sufficient for survival in the

subsurface. Metabolic reconstruction from the *D5872 Metagenome* based on these enriched subsystems implies a cryptic lifestyle in which flexible stress response, broad nutrient sources and high extents of recycling (Morita, 1997) are utilized. Compared with the genome of *H. sulfidaeris Esulfide1* (Kumar et al., 2011), five broad metabolic categories from theSEED (Overbeek et al., 2005) were significantly enriched, including: 1) virulence; 2) carbohydrates; 3) virulence, disease and defense; 4) amino acids and derivatives; as well as 5) metabolism of aromatic compounds (Fig. 4). Pathway enrichment analysis (Curtis et al., 2005) against the microbial protein database within theSEED (Aziz et al., 2008; Meyer et al., 2008) exhibited enrichment of similar pathways, suggesting the importance of these metabolic categories for survival of the *H. sulfidaeris*-dominated microbial community in its deep habitat (Table S7).

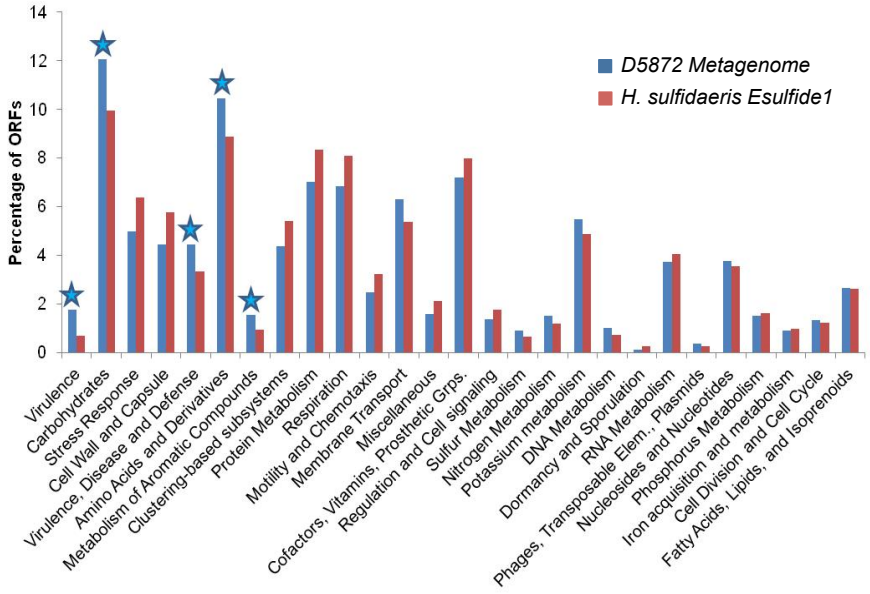
Comparison of the enriched metabolic pathways in the *D5872 Metagenome* with the present-day geochemical environment of the Mt. Simon Sandstone suggests genetic adaptation to environmental conditions of subsurface anoxia, high temperature and pressure, hypersalinity and heavy metal enrichment (Tables S1 and S7). A number of functions were identified that are essential to the survival of the *H. sulfidaeris*-dominated microbial community within subsurface burial brines. Transmembrane transporters and ion efflux pumps maintaining ion gradients and fluxes account for the majority of predicted genes within the transporter-related categories, such as Ton- and Tol-transport systems, antibiotic and toxic resistance, uni-, sym- and anti-porter and potassium homeostasis (Tables S7-S9). It suggests their importance to facilitate dynamic equilibrium of the intracellular and environmental ion concentrations, especially for those with high concentrations in the D5872 formation water (e.g., Na^+ and K^+; Table S1, S7 and S8). Antiporters exporting monovalent and divalent cations (e.g., Na^+, K^+ and Ca^{2+}) in exchange of H^+ (Fig. 5) may be used to export excessive salts and maintain osmotic and pH equilibrium, or perform energy transduction (Batista et al., 2012). A parallel strategy to protect the *H. sulfidaeris*-dominated microbial community from salt stress may be production of ectoine as an osmotic stabilizer for enzymes and proteins (Table S7 and S10). Other organic osmotic protectants, such as glycine betaine, may also be synthesized from imported choline and betaine (Ates et al., 2011). Thus, the *H. sulfidaeris*-dominated microbial community may be similar to *Halomonas elongata* (Schwibbert et al., 2011) in transporting and synthesizing organic osmolyte. Other environmental stresses, such as heavy metals, may be alleviated by a series of metal specific transporters to pump excess toxic heavy metals out of cells (Table S7 and S8).

The porewater of the Mt. Simon Sandstone contains relatively low concentrations of available carbon substrates (Table S1). Subject to this limited accessibility of nutrients, the *H. sulfidaeris*-dominated microbial community likely act as a group of metabolic opportunists capable of sensing and utilizing diverse types of nutrition for survival and growth. The enriched central metabolic pathways observed in the *D5872 Metagenome* (e.g., glycolysis, pentose phosphate pathway [PPP] and Entner-Doudoroff reactions [ED]), which produce precursors for biosynthesis and metabolisms (White, 2000), may also obtain some important metabolites from parallel catabolic reactions of a variety of organic materials. This hypothesis is supported with the enriched inventory of transporter and chemotaxis genes (Table S9), which imply sensing and the acquisition of diverse organic substrates from environment. For instance, nearly complete anaerobic degradation of benzoate and halogenated benzoate via benzoyl-CoA pathway and production of acetyl-CoA could be reconstructed in *D5872 Metagenome*, suggesting ability to degrade natural aromatic compounds by these indigenous microorganisms (Carmona et al., 2009) (Fig. 5 and S8, Tables S7 and S9). Meanwhile, the indigenous *H. sulfidaeris*-dominated microbial community appears to possess a variety of transporters

Table 1. General features of the metagenome established from the subsurface *H. sulfidaeris*-dominated microbial community (*D5872 Metagenome*) and the reference *H. sulfidaeris* genome.

Assembly statistics	***D5872 Metagenome***	***Halomonas sulfidaeris***[22]
Metagenome/genome size (Mb)	40.5	4.44
Total number of reads (million)	3	1.69
Number of assembled reads (million) (percentage of the expected whole genome)	2.8 (95%)	1.60 (94.7%)
Contigs (>500 bp)	4,603 (3,239)	61 (46)
N50 contig size	12.5 Kb	246.5 Kb
Number of unique singletons	59,702	-
Number of genes from contigs	14,477	4,143
Number of genes (>300 bp) from unassembled reads	31,854	-
GC content (%)	58.6	54.0
Protein coding genes	46,331	4,143
Genes coding for enzymes (active sites)	10,954 (2,163)	1,362(368)
Genes with Pfam domain/family	29,616	3,390
tRNA	116	66
rRNA (5S/16S/23S)	8/9/24	6/6/6

(a)



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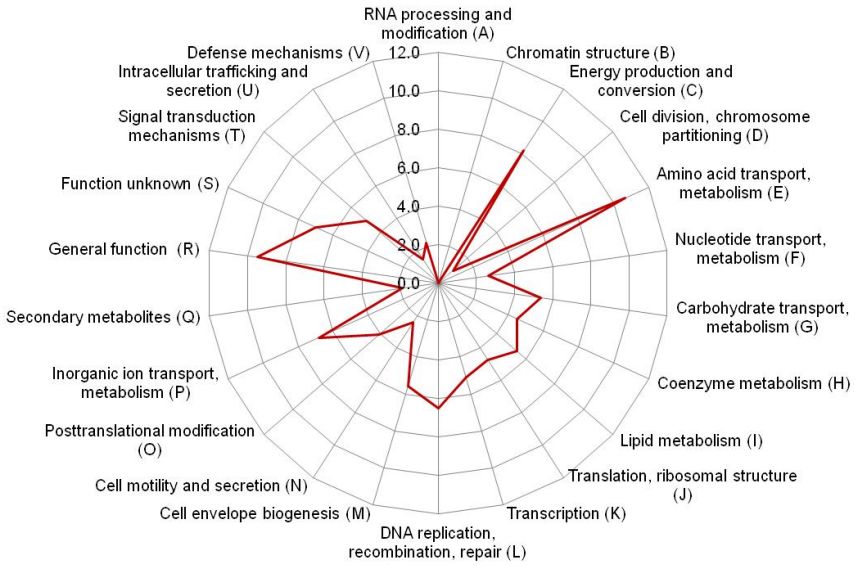

Figure 4. *D5872 Metagenome* annotation using SEED enrichment and Cluster of Orthologous Group (COG) classifications. (a) Comparison of the relative abundance of theSEED class2 categories assigned to the ORFs of the *D5872 Metagenome* versus the *H. sulfidaeris Esulfide1*. Categories enriched in *D5872 Metagenome* relative to those in the *H. sulfidaeris Esulfide1* at an FDR corrected P-value ≤ 0.05 are indicated with stars. (b) Radar plot depicts the percentage distribution of *D5872 Metagenome* ORFs assigned to COG functional categories. Letters in parentheses indicate the COG category.

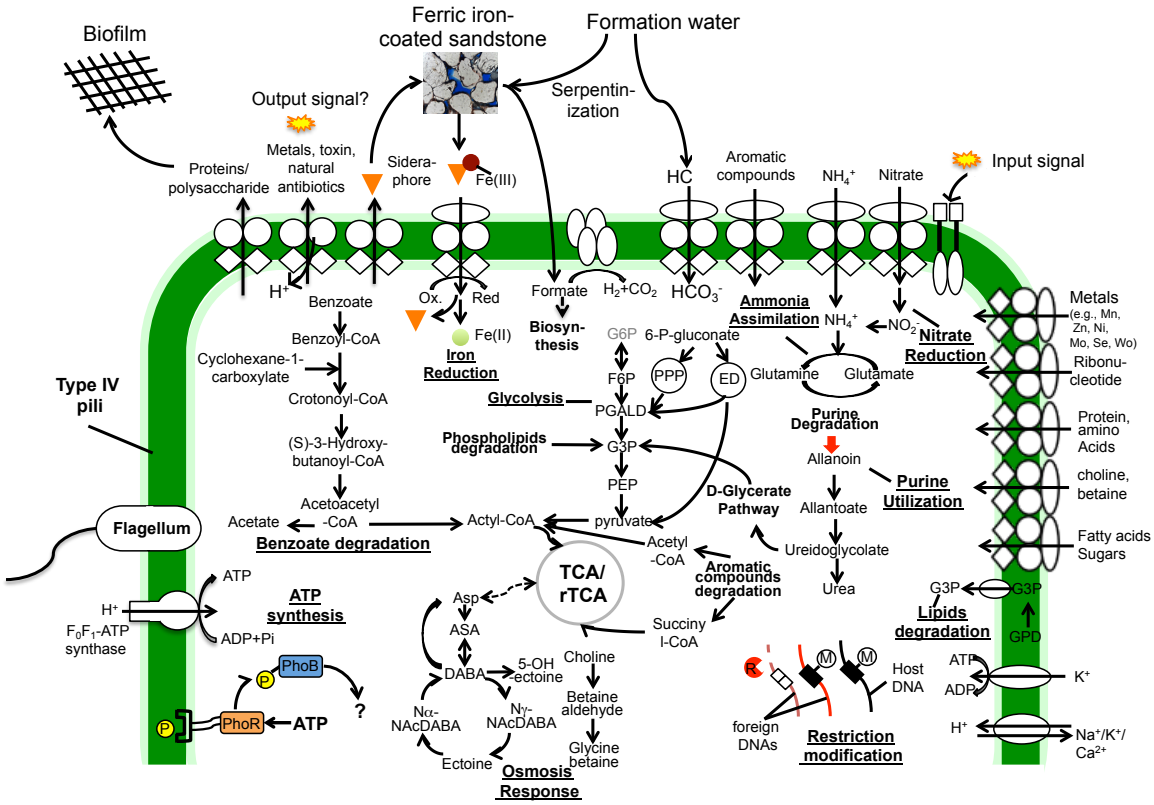

Figure 5. Metabolic reconstruction of enriched subsystems within the subsurface *H. sulfidaeris*-dominated microbial community. Transporters include approximate substrates. Citric acid cycle (TCA cycle) and reverse -TCA cycle (rTCA) is shown in gray because the genes coding these pathways are present but not enriched. The dashed arrow that connects Asp in osmosis response and TCA cycle is predicted based on previous study of osmolyte metabolisms in *H. elongata* (Schwibbert et al., 2011). PPP: pentose phosphate pathway; ED: Entner-Doudoroff pathway. For glycolysis, G6P: glucose-6-phosphate; F6P: fructose-6-phosphate; FGALD: 3-phosphoglyceraldehyde; G3P: glycerol-3-glycerate; PEP: phosphoenolpyruvate. For osmosis response, Asp: L-aspartate; ASA: L-aspartate-β-semialdehyde; DABA: diamino butyric acid; NαNAcDABA: Nα-acetyl-L-2,4-diaminobutyrate; NγNAcDABA: Nγ-acetyl-L-2,4-diaminobutyrate. For the lipid degradation pathway, GPD: glycerophosphodiesters.

(Forward et al., 1997; Fischer et al., 2010) to facilitate the uptake of diverse exogenous machinery (e.g., amino acids, peptides and DNA) (Tables S11 and S12). Biodegradation of these exogenous substrates can be initiated with proteases, peptidases and hydantoinase (Syldatk et al., 1999), respectively, and forms important intermediates (e.g., glycerol-3-phosphate) for glycolysis and the subsequent metabolic processes (Fig. 5 and Tables S7, S11-S12).

Other than exogenous compounds, intracellular components may also act as a potential carbon pool. The genes encoding glycerophosphodiester phosphodiesterase that catalyze the hydrolysis of lipids and release alcohol are enriched in the *H. sulfidaeris*-dominated microbial community, suggesting membrane phospholipids or byproducts of membrane phospholipid modification (e.g., alcohol) may be among the nutrients that could be used (Zhang and Rock, 2008) (Tables S7 and S13). Meanwhile, ectoine as an osmolyte (as discussed above) may be degraded and produce fatty acids (e.g., L-aspartate; Fig. 5 and Table S10). Biodegradation of ectoine has been predicted and experimentally confirmed in *H. elongata*, not only to mediate the concentration of this osmolyte but also to act as a carbon source for host cells (Ates et al., 2011). Since similar pathways can also be reconstructed for *D5872 Metagenome*, it is speculated that catabolism of osmolyte by the indigenous microorganisms may occur. Based on the exogenous and intracellular carbon sources inferred from the metagenome, we suggest that these *Halomonas*-related indigenous organisms may use limited carbon source from the environment and/or recycle substrates within their cells to carry out chemoheterotrophic metabolisms under oligotrophic conditions.

Unlike the single-species ecosystem identified in the deep subsurface of a South African gold mine (Chivian et al., 2008), *D5872 Metagenome* lacks nitrogen-fixation-related subsystems, suggesting that nitrogen gas detected in Mt. Simon Sandstone may not be a source of nitrogen for the indigenous microbes. Instead, in addition to amino acids, peptides, and DNA discussed above, nitrate in pore water inhabited by the subsurface *H. sulfidaeris*-dominated microbial community may provide another important nitrogen source (Fig. 5 and Table S1). Abundant nitrate transporters and genes encoding nitrate- and nitrite-reductase suggest that nitrate may act as an alternative electron acceptor for the *H. sulfidaeris*-dominated microbial community and produce ammonia for subsequent ammonia assimilation (Fig. 5 and S9, Tables S7 and S14). This observation is similar to nitrate-reducing capacity for *H. sulfidaeris Esulfide1*, although this closely related representative lacks the capacity for ammonification and produces nitrite from nitrate (Kaye et al., 2004).

High-resolution petrographic analyses indicate that goethite and hematite cements ubiquitously coat quartz grains throughout the Mt. Simon Sandstone reservoir (Fig. 1), which could be metabolically utilized by the *H. sulfidaeris*-dominated microbial community. Microbial iron transformation can be initiated by salvaging solid ferric iron using siderophores, a group of strong chelating agents that increase the solubility and bioavailability of ferric iron minerals (Schalk et al., 2011). In this study, a major fraction of genes affiliated to categories Ton- and Tol-transporter, ABC transporter and siderophore are specific for transporting the siderophore-Fe(III) complex into cytoplasm (Fig. 5). Enhancement of siderophores transporters in the *H. sulfidaeris*-dominated microbial community suggests potential utilization of solid iron minerals for biosynthesis and/or respiration. This observation is similar to studies on Soudan Mine, Minnesota, which is an iron-abundant ecosystem that contains microbes with enhanced

siderophore transporters for iron scavenging (Edwards et al., 2006). Biosynthesis of siderophore has also been seen in other *Halomonas* species (Homann et al., 2009), while the genome of *Halomonas elongata* contains pathways involving synthesis of two siderophore compounds, aerobactin and vibriobactin. Thus we speculate that iron may be of physiological importance to the *H. sulfidaeris*-dominated microbial community, such as being involved in fundamental enzymatic processes (e.g., electron transfer, DNA and RNA synthesis (Schalk et al., 2011)) or acting as an alternative electron acceptor for redox reactions catalyzed or mediated by c-type cytochrome (Schroder et al., 2003) (Fig. 5).

Other factors may also contribute to the fitness of the *H. sulfidaeris*-dominated microbial community inhabiting the deep subsurface environment of the Mt. Simon Sandstone. Protection of the native *H. sulfidaeris*-dominated microbial community against invading exogenous genetic elements may be mediated by Type I restriction-modification system (RM) and RM-induced DNA repair system (Fig. 5). RM can degrade or restrict invading DNA elements (e.g., plasmid, phage, conjugative elements/genome islands, transposons and integrons) by maintaining sequence-specific methylation of host DNA (Murray, 2000). It was also found that for some genetic elements beneficiary to host cells, RM fragments as mobile elements *per se*, can selectively transfer to or with the adjacent loci and thus maintain their stability (Furuta and Kobayashi, 2012) (Fig. 5). Meanwhile, the *H. sulfidaeris*-dominated microbial community appears capable of synthesizing Type IV pilli and flagella (Fig. 5), which may aid mobility of the organisms in response to environmental signals (e.g., nutrients or favorable geochemical conditions) (Wall and Kaiser, 1999), or enable them to form biofilm by stabilizing on or directionally exploring (Gibiansky et al., 2010) sandstone grain surfaces (Fig. 5).

IBDP Monitoring Well Microbial Cultures

A fermentative iron-reducing bacterium, *Orenia sp.* Strain Z6, was successfully isolated and cultured from the IBDP monitoring well formation water. Tests were then undertaken to understand its capacity to transfer electron equivalents produced from fermentation to crystalline ferric iron minerals in the host Mt. Simon Sandstone (e.g., hematite, Fig. 1). Strain Z6 is a restrictive anaerobic microorganism affiliated with the *Halobacteroidaceae* family of the Firmicutes. Strain Z6 exhibits a number of important physiological features common to the *Orenia* species (e.g., fermentation, tolerance to a broad range of geochemical environments (pH 5-9, temperature 20-60 °C and salinity 2-20 %). In addition, Strain Z6 is a unique iron-reducing organism in that it actively reduces both poorly- and well-ordered crystalline ferric oxides using H_2 on fermentative organic substrates as the electron donors (e.g., glucose, cellulobiase, fructose and sucrose). In contrast to many iron-reducers, Strain Z6 exhibits a low capacity to use most fatty acids (e.g., acetate) for redox reactions.

The genomic reads obtained from Illumina shotgun sequencing (~140-fold coverage) and 454 pair-end sequencing (~26-fold coverage) analyzed at UIUC led to assembly of 12 scaffolds for *Orenia sp.* Strain Z6. Genome sequencing and subsequent assembly revealed that the estimated genome size for *O. sp* strain Z6 is 3.2 Mb with the GC content 32.1 %. Annotation of the genome via RAST[37] revealed 3360 CDs, among which 1390 (38 %) were affiliated to the RAST subsystems. Metabolic reconstruction of the genome of strain Z6 revealed mix-acid fermentation by strain Z6 when glucose was provided as a substrate. Except for hexokinase that catalyzes glucose to glucose-6-phosphate and fructose-1,6-biphosphate aldolase that catalyzes fructose-1,6-biphosphate to the two isomer products, nearly complete glucose fermentation via glycolysis

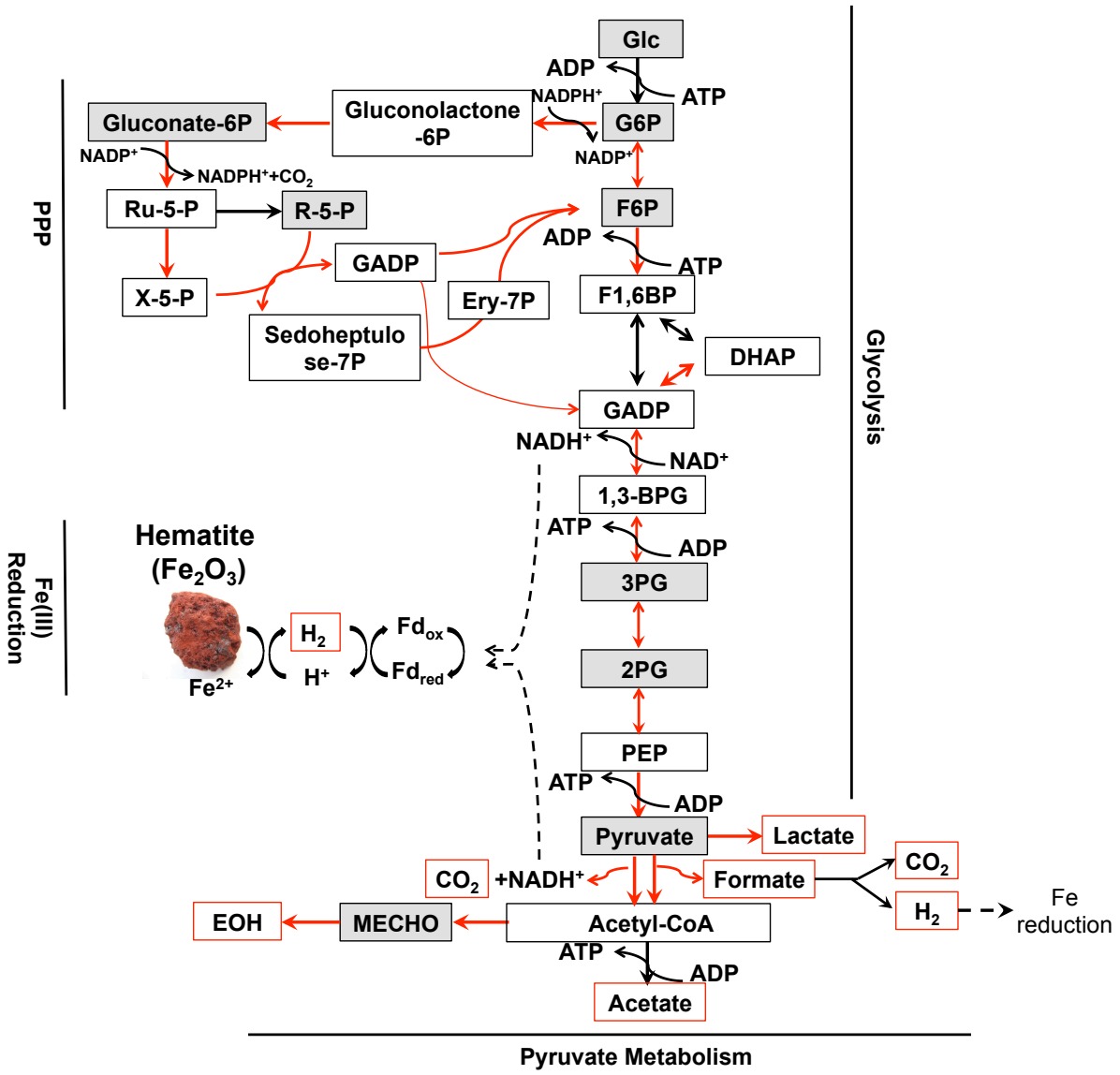

Figure 6. Predicted glucose fermentation pathways by *Orenia sp.* strain Z6 mainly consist of glycolysis, pentose phosphate pathway (PPP) and subsequent pyruvate metabolism. The intermediates detected using metabolomics and HPLC were highlighted with gray color and in red frames, respectively; the pathways with the appropriate functional enzymes predicted by genome annotation were illustrated in red arrows. Multiple pathways predicted for hydrogen production and subsequent iron reduction were shown in dashed lines. Glc: glucose; G6P: glucose-6-phosphate; F6P: b-D-fructose 6-phosphate; F1,6BP: Fructose-1,6-biphosphate; GADP: D-glyceraldehyde-3-phosphate; DHAP: hydroxyacetone-phosphate; 1,3-BPG: D-1,3-biphosphglycerate; 3PG: 3-phosphoglycerate; 2PG: 2-phosphoglycerate; PEP: phosphoenolpyruvate; Ru-5-P: ribulose-5-phosphate; X-5-P: xylulose-5-phosphate; Ery-7P: Erythrose-4-phosphate; MECHO: acetylaldehyde; EOH: ethanol.

and pentose phosphate pathway (PPP) and subsequent pyruvate metabolism were reconstructed based on the annotated functional genes (Fig. 6). Some important intermediate products (e.g., glucose-6-phosphate, 2-phosphoglycerate, 3-phosphoglycerate and ribulose-5-phosphate) were detected by metabolomics analyses on the cell extracts prepared from the cultures at log-phase of glucose fermentation (Fig. 6). Pyruvate produced from glycolysis and PPP was subsequently under parallel pathways, including transformation to lactate or acetate, production of ethanol via acetyl-CoA releasing formate as a C1 substrate or production of ethanol via direct formation of acetylaldehyde from pyruvate (Fig. 6).

Observed Strain Z6 metabolisms were consistent with previously studied fermentative iron-reducing organisms, such as *Clostridium saccarobutylicum* strain BS2, *C. butyricum* EG3, and *C. beijerinckii* and *Acidiphilium cryptum*, in terms of glucose consumption efficiency during fermentation alone versus in the presence of ferric iron oxide. Although reaction conditions varied in different studies, compared to fermentation alone, significantly enhanced glucose fermentation was observed when different forms of ferric iron substrates were provided (e.g., hematite for Strain Z6, ferric hydroxide for *C. saccarobutylicum* strain BS2 and *C. butyricum* EG3, ferric maltol for *C. beijerinckii* and ferric sulfate for *Acidiphilium cryptum* and under acidic condition). These indicate that the presence of ferric iron oxides can facilitate microbial degradation of fermentable organic substrates (Fe(III)-reduction-facilitated-fermentation).

However, Strain Z6 also exhibits unique attributes with respect to biomass production, iron-reducing capacity and product distribution under the defined experimental conditions. Enhanced growth yield in the presence of ferric iron than that of fermentation alone by *C. saccarobutylicum* strain BS2, *C. beijerinckii* and *C. butyricum* EG3, was not observed for Strain Z6. For *C. saccarobutylicum* strain BS2, a second growth phase was observed in the cultures amended with HFO after glucose was completely consumed in coupled to further iron reduction. It suggests two-step reactions by *C. saccarobutylicum* strain BS2 with concomitant glucose and ferric iron in the way that different fermented products from glucose fermentation may contribute to iron reduction. In comparison, significant cell number increase for strain Z6 only occurs in the exponential growth phase for both fermentation alone and fermentation in the presence of hematite, after which the concentrations of both substrates and products level off. However, slightly lower growth was observed in the culture with hematite. This might be due to the different cell quantification approaches used in the present study, since the previously published work used fluorescence stain enumeration or protein concentration analysis, while q-PCR was applied in the present study, which may be affected by DNA extraction efficiency for the cells attached on the surface of provided ferric oxide

Conclusion

IBDP Injection Well: In this study, a low diversity indigenous microbial community dominated by *H. sulfidaeris*-like bacteria has been discovered inhabiting saline pore water within the 1.8 km deep Cambrian-age Mt. Simon Sandstone deposits of the Illinois Basin. Metabolic reconstructions based on comparative environmental metagenomic analyses imply that this indigenous microbial community has evolved several strategies to cope with and survive subsurface environmental stress. Without primary phototrophic productivity, these organisms use multiple metabolic adaptations to access a wide variety of recycled extracellular and intracellular substrates as their carbon and nitrogen source. Fe(III) minerals and nitrate may act

as electron acceptors in this process. Among the enriched metabolic pathways, anaerobic aromatic compound biodegradation is of fundamental and practical importance.

Previous geological surveys have shown that the closest significant accumulations of subsurface hydrocarbons to the IBDP well site are stratigraphically shallower, residing in Mississippian and Pennsylvanian sedimentary reservoirs (Howard and Whitaker, 1988; Bethke et al., 1991b; Howard, 1995). Furthermore, lateral and up-section migration of these hydrocarbons has frequently occurred, influenced by tectonic uplift in the southern portion of the Illinois Basin, as well as the effect of Pleistocene glacial meltwaters on regional (Bethke et al., 1991a). It is therefore possible that the strong enrichment of hydrocarbon degradation pathways observed in the indigenous *H. sulfidaeris*-dominated community may have been derived from previous "stock" microbial communities residing in the subsurface, and are now utilized in the transformation of other natural organic substrates. Furthermore, if hydrocarbon migration eventually does occur, these indigenous microbes would have the adaptive edge of expressing these functional genes and recover their capacity to anaerobically degrade aromatic compounds. Knowledge of this native *H. sulfidaeris*-dominated microbial community provides a valuable glimpse into the mechanisms of adaptive microbial evolution within the deep subsurface, and illustrate the fundamental control of geological, physical and geochemical environmental constraints on the survival of life in the deep earth.

IBDP Monitoring Well: Integrated genomic reconstruction, qualitative and quantitative intermediate geochemical analyses, and thermodynamics indicate that Strain Z6 fluxes electrons and carbon between fermentative organic substrates and ferric iron minerals in the Mt. Simon Sandstone via a novel anaerobic fermentative iron-reduction pathway. Strain Z6 uses fermented H_2 as the electron donor for simultaneous iron reduction, in a manner analogous to that observed in syntrophic methanogenesis as well as sulfate-reducing ecosystems. However, unlike the syntrophic biosystems in which intermediates exchanged between the partners are kept low for efficient cooperation among the partners to occur, Strain Z6 uses hydrogen as the intermediate between fermentation and ferric iron reduction. Therefore, it is not the intermediate concentration alone, but the iron-reducing reaction, that acts as the driving force for the enhanced glucose fermentation by the iron-reducing Strain Z6.

The favorable thermodynamics and enhanced energy production in the system with concurrent fermentation and iron reduction is useful in deep subsurface reservoirs. It has been postulated that the minor electron transfer of electron equivalents to Fe(III) during fermentation does not cause any increase in cell yield and Fe(III) reduction by fermentative bacteria is essentially a metabolic artifact. Although only a minor fraction of electron equivalents were previously oberserved to be transferred to Fe(III) as an alternative electron acceptor, this study suggests Strain Z6 may derive even more energy from enhanced fermentation. The capacity for this organism to reduce well-crystalline ferric iron mineral reflects its original habitat where hematite and goethite are the major components of ferric iron coatings on carbonate sandstone grains (Fig. 1). This capacity is likely responsible for the preservation of substantial amount of crystalline Fe(III) oxides in permanently reduced subsurface and subsurface sediments[56] and will ultimately mediate the degree to which crystalline Fe(III) oxides can serve as electron acceptors to couple to organic matter oxidation in deep subsurface sedimentary environments.

Project Training Conclusions and Accomplishments

Course Development

A new course was formally offered at the University of Illinois Urbana-Champaign entitled "GEOL 497 Geology and Microbiology of CO2 Sequestration". There were consistently 20 undergraduate and graduate students enrolled, representing 7 different departmental units and 3 colleges across the UIUC campus. The course description is as follows:

Course Title: Geol 497 Geology and Microbiology of Carbon Sequestration
1 Credit Hour, Section TY
Meeting Time and Place: 1-1:50 pm on Mondays in 259 NHB
Instructors: Bruce Fouke, Steve Marshak, Rob Sanford
Guest Instructors: Robert Finley (ISGS), Sallie Greenberg (ISGS), Hannes Leetaru (ISGS), Ivan Krapac (ISGS), Craig Bethke (UIUC Geology)

GEOL 497 Course Description:

The US Department of Energy has identified carbon sequestration (long-term carbon dioxide capture and storage, CCS) as a fundamental means by which to stabilize climate-forcing CO2 emissions. CCS is a broad field that addresses the capture of CO2 from point sources (power plants and industrial facilities), the compression and transport of this CO2, and the injection of liquefied CO2 into deep subsurface geological rock formations. A large-scale 2.1 km deep CCS demonstration project is currently underway at Decatur within the Illinois Basin, which is a prelude to several other CCS wells throughout the country. This course will investigate our current knowledge of the primary geological; geochemical and microbiological components of the subsurface that will control the effectiveness and impact of subsurface carbon sequestration. Topics to be covered will include: (1) global carbon cycles on the ancient and modern earth; (2) state-of-the-art CCS technologies and concepts; (3) the geological composition and history of targeted subsurface rock reservoirs; (4) site characterization and environmental impact; (5) the ecology and evolution of subsurface microbial communities inhabiting subsurface reservoirs and how they will be affected by CO2 sequestration; (6) the predicted effects of CO2 injection on subsurface rock porosity and permeability, formation water chemistry and microbial communities; and (7) the future economic and policy implications of carbon sequestration, as well as other alternative approaches, given these geological and microbiological constraints on subsurface reservoir ecosystems.

Lecture Series: Subsurface Microbial Biosphere and CO_2 Sequestration

We successfully completed a ***Subsurface Microbial Biosphere and CO_2 Sequestration*** lecture series in the UIUC Departments of Geology and Microbiology, in which leading scientists in subsurface geomicrobiology, molecular microbial ecology, CO_2 sequestration and hydrocarbon exploration came to provide lecture series on the UIUC campus during the Fall 2011 and Spring 2012 semesters. The lecture series included the following speakers:

(1) Dr. Matthew Kirk, Sandia National Laboratory, spoke November 2011;
(2) Professor Terry Engelder, Columbia University, spoke February 2012;
(3) Professor Vincent Bulone, Royal Technical University (KTH) Stockholm, spoke April 2012;
(4) Professor Kenneth Nealson, University of Southern California, spoke April 2012; and
(5) Professor Mike McInerney, University of Oklahoma, spoke May 2012.

Mentoring and Technique Training and Development

We actively involved and trained UIUC undergraduate students, graduate students, postdocs and research scientists in all aspects of the ongoing field and laboratory research that were required for the successful completion of this project. These participants included:

(1) UIUC Undergraduate Students - Joe Weber (UIUC Microbiology), Sheila Egan (UIUC Biochemistry), Francis Lee (UIUC Biochemistry), Annette Merkel (UIUC Microbiology) and Jiwon Kim (UIUC Microbiology)

Note that as a result of his research on this DOE project, Joe Weber has been awarded a 2013-2014 Fulbright Fellowship, which he will conduct in the labs of Professor Vincent Bulone, Royal Technical University (KTH) Stockholm.

(2) UIUC Graduate Students – Phil Miller (UIUC Geology), Samantha Dwyer (UIUC Geology), Kunal Shrungarkar (UIUC Civil and Environmental Engineering), Maria Jones (UIUC Civil and Environmental Engineering) and Brooke Eickhoff (UIUC Geology)

(3) UIUC Postdoc Researchers and Research Scientists – Dr. Raj Singh (UIUC Civil and Environmental Engineering), Dr. Yiran Dong (UIUC Institute for Genomic Biology), Dr. Rob Sanford (UIUC Geology)

All of the group members actively participated in the field sample collection and analyses. Based on their varying stages of molecular microbiology and geology training, background and research experience, all the students were provided with training for the lab work. The training program included: (1) field collection techniques; (2) aqueous geochemistry measurements (e.g., ferrous iron, ion concentration, extraction and identification of organic substrates in the formation fluid); (3) molecular and culturing approaches (mentored by Dr. Yiran Dong, postdoc in the Fouke lab); (4) ultra-high resolution fluorescence microscopy (*in situ* hybridization (FISH) and fluorescence techniques instructed by Dr. Sivaguru Mayandi, Microscopy Facility of IGB, UIUC); and (5) environmental scanning electron microscopy (SEM, mentored by Dr. Scott Robinson, Beckman Institute for Advanced Science and Technology, UIUC).

The main challenges of field sampling of deep subsurface samples were to develop appropriate methods for sampling samples for microbiology studies and avoid/minimize potential contaminations that can be introduced through the process of sampling. We closely collaborated with Schlumberger and ISGS Carbon Sequestration Group and combined our respective lab experience to develop the decontamination process that was applied in the sampling.

GRAPHICAL MATERIAL LIST(S)

Table 1. General features of the metagenome established from the subsurface *H. sulfidaeris*-dominated microbial community (*D5872 Metagenome*) and the reference *H. sulfidaeris* genome.

Figure 1. Plane-light photomicrographs of the Mt. Simon Sandstone quartz arenite at a burial depth of 1.8 km. The lithology is composed of high porosity (P) quartz sands (QS) and multiple intermittent events of quartz overgrowth (QO) cementation and dark brown to red iron oxide cementation (Fe).

Figure 2. Composition of the microbial community inhabiting pore water collected using the Schlumberger ®Quicksilver probe at a burial depth of 1.8 km (5872 ft) from the Mt. Simon Sandstone saline sandstone reservoir in the Illinois Basin. (a) Classification of the full-length 16S rRNA gene clone library. (b) Proportions of 454 pyrosequencing reads targeting V1-V3 hypervariable region of 16S rRNA gene sequences. (c) Predicted 16S rRNA genes among the metagenomic reads. The lower small pie charts illustrate the dominance of the family *Halomonadaceae* in the class γ-Proteobacteria that was classified by the three approaches. N values represent the number of sequences or reads that were analyzed. Percentage values labeled within the pie charts denote the fraction of a phylum that was present in the indigenous subsurface microbial community.

Figure 3. Phylogenetic comparison of *Halomonas* OTUs detected in 1.8 km-depth ®Quicksilver probe formation water and the DST drilling mud. *Escherichia coli* (X80725) was used as the outgroup and the scale bar indicates 0.1 change per nucleotide position. The accession numbers for the type strains are shown in parentheses. Statistical confidence for the evolutionary tree was assessed by bootstrap (1,000 replicates) and shown as bootstrap values in percentage.

Figure 4. *D5872 Metagenome* annotation using SEED enrichment and Cluster of Orthologous Group (COG) classifications. (a) Comparison of the relative abundance of theSEED class2 categories assigned to the ORFs of the *D5872 Metagenome* versus the *H. sulfidaeris Esulfide1*. Categories enriched in *D5872 Metagenome* relative to those in the *H. sulfidaeris Esulfide1* at an FDR corrected P-value ≤ 0.05 are indicated with stars. (b) Radar plot depicts the percentage distribution of *D5872 Metagenome* ORFs assigned to COG functional categories. Letters in parentheses indicate the COG category.

Figure 5. Metabolic reconstruction of enriched subsystems within the subsurface *H. sulfidaeris*-dominated microbial community. Transporters include approximate substrates. Citric acid cycle (TCA cycle) and reverse -TCA cycle (rTCA) is shown in gray because the genes coding these pathways are present but not enriched. The dashed arrow that connects Asp in osmosis response and TCA cycle is predicted based on previous study of osmolyte metabolisms in *H. elongata* (Schwibbert et al., 2011). PPP: pentose phosphate pathway; ED: Entner-Doudoroff pathway. For glycolysis, G6P: glucose-6-phosphate; F6P: fructose-6-phosphate; FGALD: 3-phosphoglyceraldehyde; G3P: glycerol-3-glycerate; PEP: phosphoenolpyruvate. For osmosis response, Asp: L-aspartate; ASA: L-aspartate-β-semialdehyde; DABA: diamino butyric acid;

NαNAcDABA: Nα-acetyl-L-2,4-diaminobutyrate; NγNAcDABA: Nγ-acetyl-L-2,4-diaminobutyrate. For the lipid degradation pathway, GPD: glycerophosphodiesters.

Figure 6. Predicted glucose fermentation pathways by *Orenia sp.* strain Z6 mainly consist of glycolysis, pentose phosphate pathway (PPP) and subsequent pyruvate metabolism. The intermediates detected using metabolomics and HPLC were highlighted with gray color and in red frames, respectively; the pathways with the appropriate functional enzymes predicted by genome annotation were illustrated in red arrows. Multiple pathways predicted for hydrogen production and subsequent iron reduction were shown in dashed lines. Glc: glucose; G6P: glucose-6-phosphate; F6P: b-D-fructose 6-phosphate; F1,6BP: Fructose-1,6-biphosphate; GADP: D-glyceraldehyde-3-phosphate; DHAP: hydroxyacetone-phosphate; 1,3-BPG: D-1,3-biphosphglycerate; 3PG: 3-phosphoglycerate; 2PG: 2-phosphoglycerate; PEP: phosphoenolpyruvate; Ru-5-P: ribulose-5-phosphate; X-5-P: xylulose-5-phosphate; Ery-7P: Erythrose-4-phosphate; MECHO: acetylaldehyde; EOH: ethanol.

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LIST OF ACRONYMS AND ABBREVIATIONS

CCS = carbon capture and storage

UIUC = University of Illinois Urbana-Champaign

IBDP = Illinois Basin - Decatur Project

ISGS = Illinois State Geological Survey

MGSC = Midwest Geological Sequestration Consortium

APPENDICES

CONFERENCE PAPERS/PROCEEDINGS AND JOURNAL ARTICLES

National Conferences

Dong, Y., Sanford, R. A., Locke, R. A. II, Weber, J. R., Egan, S. M., Cann, I. K. O., Mackie, R. I., Fouke, B. W. Bacterial Ferric Iron Reduction by a Fermentative Orenia sp. (Strain 6634) Isolated from 2.02 KM Depth Cambrian-age Mt. Simon Sandstone, Illinois Basin, USA, 113 ASM General Meeting, May 18-21, 2013, Denver, Colorado.

Dong, Y., Sanford, R. A., Werth, J. C., Fouke B. W., Understanding the Impact of CO_2 Injection on the Subsurface Microbial Community in an Illinois Basin CCS Reservoir: Integrated Student Training in Geoscience and Geomicrobiology, Carbon Storage R&D Project Review Meeting ⎯ Developing the Technologies and Building the Infrastructure for CCUS, U.S. Department of Energy, Pittsburgh, PA, August 21-23, 2012 (invited oral presentation)

Dong, Y., Sanford, R. A., Cann, I. K. O., Mackie, R. I., Locke, R. A. II, Fouke, B. W. W., Detection and Preliminary Characterization of Active Iron-Reducing Bacterial Populations in Deeply Buried (1.7-2.0 KM) Saline Sandstone Reservoirs of the Illinois Basin, Midcontinent, USA, 2012 General Meeting for the American Society of Microbiology, San Francisco, CA, June 16-19, 2012

Kumar, C. G., **Dong Y.**, Kim, P., Olsen, G. J, Cann, I. K. O., Mackie, R. I., Fouke, B. W., Price, N. D. Comparative metabolic predictions for *Halomonas sulfidaeris* inhabiting km-deep marine hydrothermal vents and subsurface sedimentary rocks. 11th Cold Spring Harbor Laboratory/Wellcome Trust conference on Genome Informatics, Cold Spring Harbor, New York, NY, November 2-5 2011

Dong, Y., Cann I. K. O., Mackie, R. I., Price, N. D., Flynn, T. M., Sanford, R. A., Miller, P., Chia, N., Kumar, C. G., Kim, P., Sivaguru, M., Fouke, B. W. Discriminating Native Subsurface Microbes from Drilling Mud Contaminants at Depths of 1.0-2.2 KM in the Illinois Basin Using 454 Pyrotag Sequencing, 3rd Annual Argonne Soil Metagenomics Workshop, Chicago, IL, October 5-7, 2011

Dong, Y., Flynn, T. M., Sanford, R. A., Miller, P. A., Chia, N., Kumar, C. G., Kim, P., Cann, I. K. O., Mackie, R. I., Price, N. D., Sivaguru, M., Nolte, M., Fouke, B. W. Phylogeneic Diversity, Stratigraphic Distribution of Indigenous Microbes Revealed from a 2KM Well in Decatur, IL. Midwest Geological Sequestration Consortium Annual Meeting and Workshop, Champaign, IL, November 7-9, 2011

Dong, Y., Cann, I. K. O., Mackie, R. I., Price, N. D., Flynn, T. M., Sanford, R. A., Miller, P. A., Chia, N., Kumar, C. K., Kim, P., Sivaguru, M., Fouke, B. W. Looking For a Needle in the Haystack: Deciphering Indigenous 1.79 km Deep Subsurface Microbial Communities from Drilling Mud Contaminants Using 454 Pyrotag Sequencing, AGU 2010 Annual Conference, San Francisco, CA, December 12-18, 2010

Other Invited Oral Presentations

Dong Y. Robert A. Sanford, Joseph Weber, Sheila M. Egan, Randall A. Locke and Bruce W. Fouke, Isolation and Characterization of a Novel Iron-reducing Bacteria Inhabiting Cambrian-age Mt. Simon Sandstone of the Illinois Basin, IL. 2013 Institute for Genomic Biology Symposium, University of Illinois, Urbana-Champaign, May 2, 2013

Dong Y., Robert A. Sanford, Joseph Weber, Sheila M. Egan, Randall A. Locke and Bruce W. Fouke, Detection, Isolation and Characterization of Iron-reducing Bacteria Inhabiting the Deep Subsurface of the Illinois Basin, Illinois State Geological Survey, March 4, 2013

Dong, Y., Fouke, B. W., Looking For a Needle in the Haystack: Deciphering Indigenous 1.79 km Deep Subsurface Metagenomic Microbial Communities Inhabiting Illinois Basin, IL., Mechanical Engineering Department, University of Illinois, Urbana-Champaign, September 27, 2012

Dong, Y., Flynn, T. M., Sanford, R. A., Miller, P. A., Chia, N., Kumar, C. G., Kim, P., Cann, I. K. O., Mackie, R. I., Price, N. D., Sivaguru, M., Nolte, M., Fouke, B. W. Discriminating Native Subsurface Microbes from Drilling Mud Contaminants at Depths of 1.0-2.2 KM in the Illinois Basin Using 454 Pyrotag Sequencing, 32th Microbiology Annual Conference, University of Illinois, Urbana-Champaign, September 24, 2011

Dong, Y., Sanford, R. A., Flynn, T. M., Fouke, B. W. Microbial Analysis of 2 Km Deep Subsurface Samples from Decatur Well, Illinois Basin, 30th Microbiology Annual Conference, University of Illinois, Urbana-Champaign, September 26, 2009

UIUC Campus Symposia and Seminars

Dong Y., Robert A. Sanford, Randall A. Locke, Joseph Weber, Sheila M. Egan, Cann, I. K. O., Mackie, R. I., and Bruce W. Fouke, Detection, Bacterial ferric iron reduction by a fermentative Orenia sp. (strain 6634) isolated at 2.02 KM in the cambrian-age Mt. Simon Sandstone, Illinois Basin, USA, Research review of the School of Earth, Society, and Environment, University of Illinois, Urbana-Champaign, February 28, 2013

Dong, Y., Sanford, R. A., Weber, J. R., Egan, S. M., Cann, I. K. O., Mackie, R. I., Locke, R. A. II, Fouke, B. W. Detection and Characterization of Active Iron-Reducing Bacterial Populations in Deeply Buried (1.7-2.0 KM) Saline Sandstone Reservoirs of the Illinois Basin, Midcontinent, USA, 33th Microbiology Annual Conference, University of Illinois, Champaign, IL, October 21, 2012

Dong, Y., Sanford, R. A., Weber, J. R., Egan, S. M., Cann, I. K. O., Mackie, R. I., Locke, R. A. II, Fouke, B. W. Iron Reduction Detected in the 1.8-2.0 km Deep Subsurface Microbial Biosphere of the Illinois Basin, 2012 Institute for Genomic Biology Symposium, May 3, 2012

Dong, Y., Cann, I. K. O., Mackie, R. I., Price, N. D., Flynn, T. M., Sanford, R. A., Miller, P. A., Chia, N., Kumar, C. K., Kim, P., Sivaguru, M., Fouke, B. W. Looking For a Needle in the Haystack: Deciphering Indigenous 1.79 km Deep Subsurface Microbial Communities from Drilling Mud

Contaminants Using 454 Pyrotag Sequencing, 31st Microbiology Annual Conference, University of Illinois, Champaign, IL, September 24, 2010