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SOIL MICROBIOLOGY

Microbial and Mineralogical Characterizations of Soils Collected from the Deep Biosphere of the Former Homestake Gold Mine, South Dakota

Gurdeep Rastogi · Shariff Osman · Ravi Kukkadapu · Mark Engelhard · Parag A. Vaishampayan · Gary L. Andersen · Rajesh K. Sani

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Abstract A microbial census on deep biosphere (1.34 km depth) microbial communities was performed in two soil samples collected from the Ross and number 6 Winze sites of the former Homestake gold mine, Lead, South Dakota using high-density 16S microarrays (PhyloChip). Soil mineralogical characterization was carried out using X-ray diffraction, X-ray photoelectron, and Mössbauer spectroscopic techniques which demonstrated silicates and iron minerals (phyllosilicates and clays) in both samples. Microarray data revealed extensive bacterial diversity in soils and detected the largest number of taxa in *Proteobacteria* phylum followed by *Firmicutes* and *Actinobacteria*. The archael communities in the deep gold mine environments were less diverse and belonged to phyla *Euryarchaeota* and *Crenarchaeota*. Both the samples showed remarkable similarities in microbial communities (1,360 common OTUs) despite distinct geochemical characteristics. Fifty-seven phylotypes could not be classified even at phylum level representing a hitherto unidentified diversity in deep biosphere. PhyloChip data also suggested considerable metabolic diversity by capturing several physiological groups such as sulfur-oxidizer, ammonia-oxidizers, iron-oxidizers, methane-oxidizers, and sulfate-reducers in both samples. High-density microarrays revealed the greatest prokaryotic diversity ever reported from deep subsurface habitat of gold mines.

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G. Rastogi · R. K. Sani (✉)
Department of Chemical and Biological Engineering,
South Dakota School of Mines and Technology,
Rapid City, SD 57701, USA
e-mail: Rajesh.Sani@sdsmt.edu

S. Osman · G. L. Andersen
Ecology Department, Earth Sciences Division,
Lawrence Berkeley National Laboratory,
Berkeley, CA 94720, USA

R. Kukkadapu · M. Engelhard
WR Wiley Environmental Molecular Sciences Laboratory,
Pacific Northwest National Laboratory,
Richland, WA 99352, USA

P. A. Vaishampayan
California Institute of Technology, Jet Propulsion Laboratory,
Pasadena, CA 91109, USA

Present Address:
G. Rastogi
Department of Plant Pathology, University of California,
Davis 95616, USA

Introduction

Ultra-deep mines provides a unique access to examine the microbial communities in deep terrestrial subsurface environments where hostile life conditions such as extreme temperature, pH, pressure, low oxygen concentration, no light and toxic metals persist. Several investigations, both culture-based and culture-independent, on gold mines in Japan [13, 14, 23], South Africa [3, 7, 12, 16, 21, 25, 38], Canada [24], and the USA [27, 30] have demonstrated active diverse microbial populations with bewildering metabolic capabilities. These studies also shed light on the spectacular biogeochemistry that governs microbial community composition in deep biosphere where contemporary photosynthetically derived carbon may be absent.

Most of the studies discussed above used 16S rRNA cloning-and-sequencing methods to examine the microbial

community composition in deep gold mine biosphere. Culture-independent methods, in particular polymerase chain reaction (PCR) and the sequencing of clone libraries are considered the "gold standard" for studying microbial diversity [9]. However, a clone library approach is tedious, time-consuming, and limited by the number of clones sequenced primarily because of the high sequencing cost. Thus, a clone library approach is impractical for generating comprehensive microbial molecular inventory in a given sample. While 16S rRNA clone libraries permit an initial survey of diversity, studies have shown that clone libraries with <400 sequences represent only an incomplete sampling of microbial populations and extremely low-abundance organisms remain un-represented [15]. Thus, most published reports on ultra-deep gold mines utilizing cloning-and-sequencing reveal only a small portion of the microbial diversity present in a given sample.

Recently with unprecedented progress in microbial ecology, hybridizing PCR products directly (without cloning) to a 16S rRNA gene microarray (PhyloChip) has emerged as a rapid, reproducible, and more comprehensive way to analyze microbial diversity in soil, water, and air samples [4, 9, 28]. One of the greatest advantages of using high-density microarrays is their capability to detect individual taxa from an environment that may contain as many as 10,000 different microbial types [40]. In addition, by using sequence-specific PCR, studies have validated that low abundant lineages captured by PhyloChips were indeed present in the original environment, despite their absence in corresponding clone libraries [9]. This highlighted the potential superiority of PhyloChips compared to classical clone libraries. Literature suggests that to date such comprehensive microbial census methods were not applied to explore the microbial diversity in mining-impacted deep biosphere of gold mines and hence these microbial communities remain largely uncharacterized.

The Homestake gold mine (44°35′2074″N, 103°75′082″W; Lead, SD) is the deepest mine (2.4 km deep) in the North America and had largest gold deposit ever found in the Western Hemisphere. A full description of the mine can be located at Lawrence Berkeley National Laboratory, CA, website http://www.lbl.gov/nsd/homestake/Reference.html). The mine was closed in December 2001 after more than 125 years of mining. On 10th July 2007, the National Science Foundation (USA) announced this mine as a site for the Deep Underground Science and Engineering Laboratory (DUSEL). This former gold mine offers a unique opportunity for direct deep subsurface exploration. In recent studies, we have cultured the cellulose-degrading bacteria [27] and evaluated the microbial diversity in soil samples collected from the Homestake mine using typical 16S clone libraries [30]. Our results showed that majority (>95%) of the sequences retrieved in clone libraries were most closely related to environmental sequences from yet-uncultured bacteria representing a hitherto unidentified microbial diversity. In addition, rarefaction analysis of clone library generated non-asymptotic plots which indicated that diversity was not exhaustively sampled due to insufficient clone sequencing, a common problem when assessing environmental microbial diversity by using cloning approaches. Thus, a more sensitive method such as microarrays were required for a comprehensive microbial community composition investigation in deep subsurface habitat of the Homestake mine. Therefore, the purpose of the research was to elucidate the microbial community composition in the soil samples collected from the Homestake mine by applying high-density 16S PhyloChips. Furthermore, for the first time, the detailed mineralogical characteristics of soil samples were analyzed using high-resolution techniques such as X-ray diffraction, X-ray photoelectron, and Mössbauer spectroscopy. The phylogenetic features of microbial community present in the Homestake mine were compared with corresponding 16S clone libraries constructed earlier from the same samples [30] and with those of communities from other deep subsurface environments including ultra-deep gold mines.

Materials and Methods

Subsurface Soil Sampling

A schematic cross section and locations of sampling sites in the former Homestake gold mine has been shown in Rastogi et al. [30]. In May 2008, two soil samples were collected corresponding to the Ross shaft and No. 6 Winze of the Homestake mine at a depth of 1.34 km. One sample was directly across the landing from the Ross shaft, one of two primary shafts from the surface into the mine, and one was outside the No. 6 Winze hoist room. Both samples were collected along the junction of the drift wall and the floor, where a small accumulation of soil debris had built up through the years of mining. These areas were not disturbed by any type of activities including human trafficking from June 2003 to May 2008. The outer surfaces of the soil debris built up were discarded and only inner surfaces were collected for microbial diversity analyses using sterile spatulas. This was done to minimize the chances of contamination from exogenous microbes. The temperature at the time of sampling was 26°C which was measured using a mercury thermometer. The samples were transported to South Dakota School of Mines and Technology laboratory (1 h drive) in sterile polypropylene bottles on ice and stored at −20°C until analysis. Soil samples were homogenized in sterile pestle and motor inside a laminar flow hood, and then used for geochemical characterization and DNA extraction.

Mineralogical Characterization of Ross and Winze Soil Samples

The structural characterization of Ross and Winze soils was carried out by Powder X-ray diffraction (XRD) technique for crystalline mineral phases, X-ray photoelectron spectroscopy (XPS) for surface chemical composition and oxidation states, and ^{57}Fe-sensitive Mössbauer for Fe-mineralogy. XRD was carried out with a Philips PW3040/00 X'Pert MPD system, using CuK_{α} radiation with a variable divergent slit and a solid-state detector. The Jade+, V5 (Materials Data, Inc., Livermore, CA) software package was used for data analysis. XPS measurements of soil samples were performed using a Physical Electronics Quantum 2000 Scanning ESCA Microprobe. The X-ray beam used was a 100 W, 10-μm diameter beam that was rastered over a 1.3 mm by 0.2 mm rectangle on the sample. Wide scan data was collected using 117.4 eV pass energy. For the $Ag3d_{5/2}$ line, these conditions produced FWHM (full width at half maximum) of better than 1.6 eV. High-energy resolution spectra were collected using 46.95 eV pass energy. For the $Ag3d_{5/2}$ line, these conditions produced FWHM of better than 0.98 eV. The binding energy (BE) scale was calibrated using the $Cu2p_{3/2}$ feature at 932.62±0.05 eV and Au 4f at 83.96±0.05 eV for known standards. The samples experienced variable degrees of charging and low-energy electrons at ~1 eV, 20 μA and low-energy Ar^{+} ions were used to minimize this charging.

Mössbauer spectra were collected at room temperature using a 50-mCi (initial strength) ^{57}Co/Rh source. The velocity transducer MVT-1000 (WissEL) was operated in a constant acceleration mode (23 Hz, ±12 mm/s). An Ar-Kr proportional counter was used to detect the radiation transmitted through the holder. Data were folded to give a flat background and a zero-velocity position corresponding to the center shift of a metal Fe foil at room temperature. Calibration spectra were obtained with a 25-μm-thick Fe (m) foil and Mössbauer data were modeled with Recoil software (University of Ottawa, Canada) [26].

DNA Extraction, PCR, and PhyloChip Hybridization

Total DNA was extracted from 200 mg of soil samples in duplicate using a PowerSoil™ DNA Isolation Kit (MO Bio, Carlsbad, CA) according to the manufacturer's instructions. This DNA isolation kit was used by majority of the previous microbial diversity studies on deep subsurface gold mines [12–14, 21, 23, 24, 30, 38]. Thus, results obtained in our study could be compared with earlier studies with greater confidence. For PhyloChip hybridization, almost full-length bacterial 16S genes were amplified using bacteria-specific (63f/1392r) primers [17]. Archael 16S genes were amplified using an archaea-specific (Arch 21f/Arch 958r) primer set which generated a partial amplification of ~950 bp [29]. Efforts to amplify full-length archael PCR products from Ross and Winze soil samples were unsuccessful despite variation in several parameters (e.g., annealing temperature, alternative primer sets, DNA concentration). Therefore, partial archaeal-16S PCR products were used for microarray hybridization. A control PCR without DNA was set up to check for any contaminants associated with PCR reagents.

Bacterial (~500 ng product) and archaeal (~100 ng product) PCR products amplified from a soil sample were mixed and then concentrated for PhyloChip hybridization. PCR products were prepared for PhyloChip hybridization as described earlier [9]. In brief, PCR products were purified and concentrated to a volume of 40 μl using MinElute columns (Qiagen, Valencia, CA). The PCR products were then spiked with known amounts of amplicons derived from prokaryotic metabolic genes. The mixture was then fragmented to 50–200 bp fragment using DNase I (Invitrogen, Carlsbad, CA) followed by labeling with a GeneChip DNA labeling kit (Affymetrix, Santa Clara, CA) as per manufacturer's protocol. The labeled DNA was hybridized to PhyloChips (Affymetrix GeneChips), washed and stained as per standard Affymetrix protocol. For detailed information on microarray design, fabrication, and analytic procedures (background subtraction, detection and quantification criteria, and array normalization) see DeSantis et al. [9]. Operational taxonomic units (OTUs) were classified in phylum, class, order, family, subfamily, and species at sequence similarity cut-off values of 80%, 85%, 90%, 92%, 94%, and 97%, respectively [9]. An OTU was considered present in the sample when 90% or more of its assigned probe pairs for its corresponding probe set were positive (positive fraction of >0.90) [9].

Results

Mineralogical Characteristics of Ross and Winze Soil Samples

The Powder XRD identified major crystalline phases. Both sample spectra were qualitatively similar to each other (Fig. 1). They were rich in quartz (SiO_2), chlorite-chamosite [$(Fe_5Al)(AlSi_3)O_{10}(OH)_8$], and annite [$KFe_3AlSi_3O_{10}(OH)_2$] minerals; only the major peaks (100% intensity) of each mineral phase are indicated by arrows in the Fig. 1. Other minor peaks were due to other reflections of annite, chamosite, and quartz phases and the difference in the peak intensities merely reflect different phase composition. Chamosite was detected in relatively higher amounts than annite in the Ross sample while its amount was lower than annite in the Winze sample.

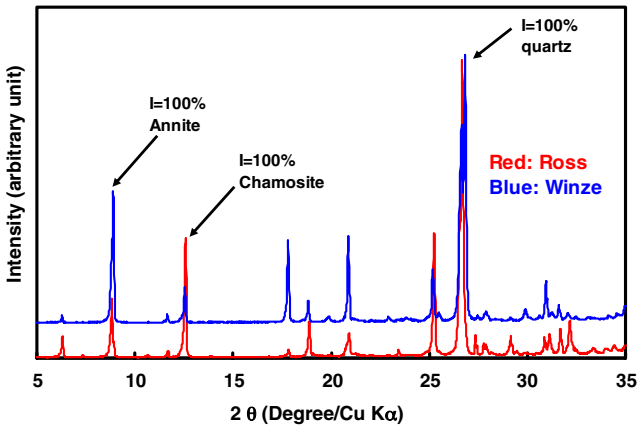

Figure 1 X-ray diffraction patterns of Ross and Winze soils showing major mineral phases of annite, chamosite, and quartz (shown by *arrows*). XRD patterns identified similar features in both samples. Only 100% intense peaks were identified by *arrows*

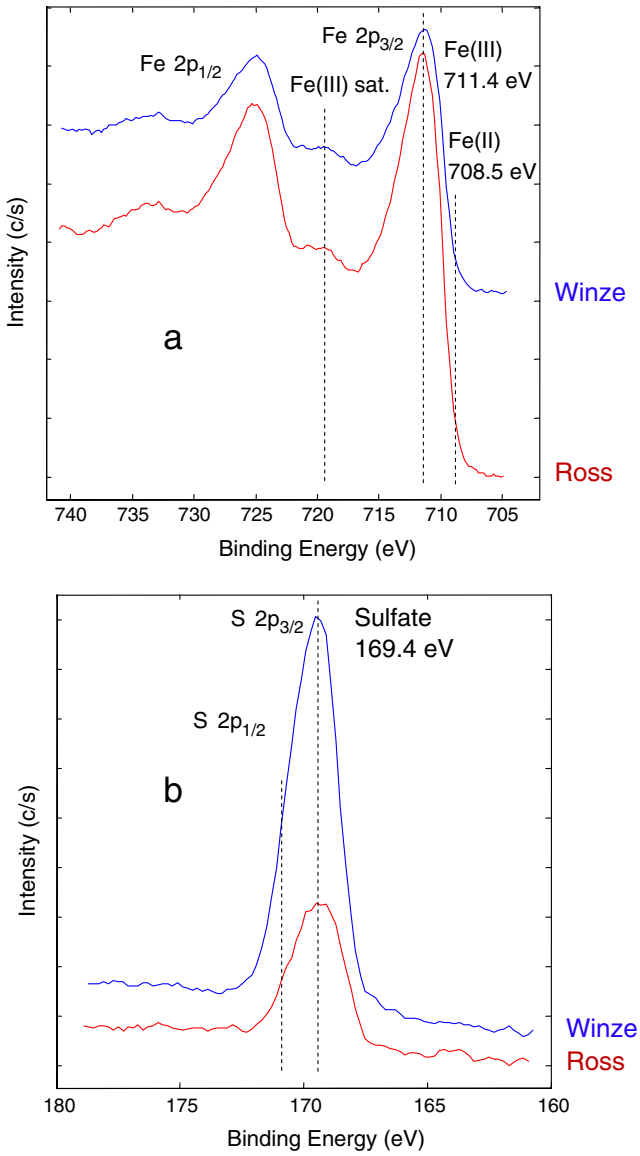

Figure 2 High energy resolution X-ray photoelectron spectra of Ross and Winze soils **a** Fe 2p region and **b** S 2p region showing the presence of Fe(II), Fe(III), and sulfate

XPS can quantify surface chemistry and composition by probing a maximum depth of ~10 nm. High-energy resolution photoemission spectra of the Fe2p region (Fig. 2a) and S2p (Fig. 2b) of both the soil samples were obtained to compare their surface characteristics. The spectra were qualitatively similar to each other, consistent with XRD data. The binding energies of the primary Fe $2p_{3/2}$ at (711.4 eV) were consistent with Fe(III) and the lines at (708.5 eV) indicated a small amount of Fe(II) [6]. The Fe satellite peak position (719.0 eV) and line shape was also consistent with mostly Fe(III). The binding energy for the $S2p_{3/2}$ lines (169.4 eV) was consistent with sulfate. The atomic sulfate concentration in the Winze sample was 3.1 atomic percent as compared with the Ross at 1.3 atomic percent (Fig. 2b).

More detailed insights in the composition of iron minerals were revealed by transmission ^{57}Fe-Mössbauer technique (a 57Fe-specific *bulk* technique; natural abundance of ^{57}Fe is 2.2%). Room temperature Mössbauer spectra of the soil samples were similar to each other, hence, only modeled spectrum of Ross soil was shown in Fig. 3a. The similarity of the spectral features was evident from the comparison (Fig. 3b inset); samples mostly differed from each other in relative composition of phyllosilicate minerals, in agreement with XRD. The Mössbauer spectra were rather complex with a wealth of complimentary information. For example, (a) modeling revealed 20% (Winze) to 45% (Ross) of the total Fe as Fe (II) contributed by chamosite and annite, was significantly different from the surface Fe(II) and Fe(III) composition derived from XPS, (b) presence of Fe(II) and Fe(III) various environments. The various Fe-environments in phyllosilicates was particularly apparent from the modeling. The Fe(II) doublets parameters were similar to Fe(II) in various environments in Fe-rich chlorite mineral (chamosite; [32]), and most probably annite [26]. The outer Fe(II) doublet's Mössbauer parameters (blue trace) were characteristic of *trans*-octahedral Fe(II) in chlorite (or *M2*-site). The inner Fe(II) doublet (red trace), however, appeared to be a mixture of *cis*-octahedaral Fe(II) in chamosite (or *M1* site), and most probably some from annite. This assignment was in good agreement with: (a) annite's Mössbauer parameters, (b) inner-to-outer doublet Fe(II) intensity ratios (~1 in Winze; 0.73 in Ross), which were higher than the pure chamosite (~0.5; [32, 41] and (c) apparent line-shape of the inner Fe(II) doublet in the Winze sample. The inner Fe(II) may also have contributions from Fe present in the interlayer brucite-like sheets of chlorite [36]. Similarly, the assignment of the Fe(III) doublet (black trace) was also

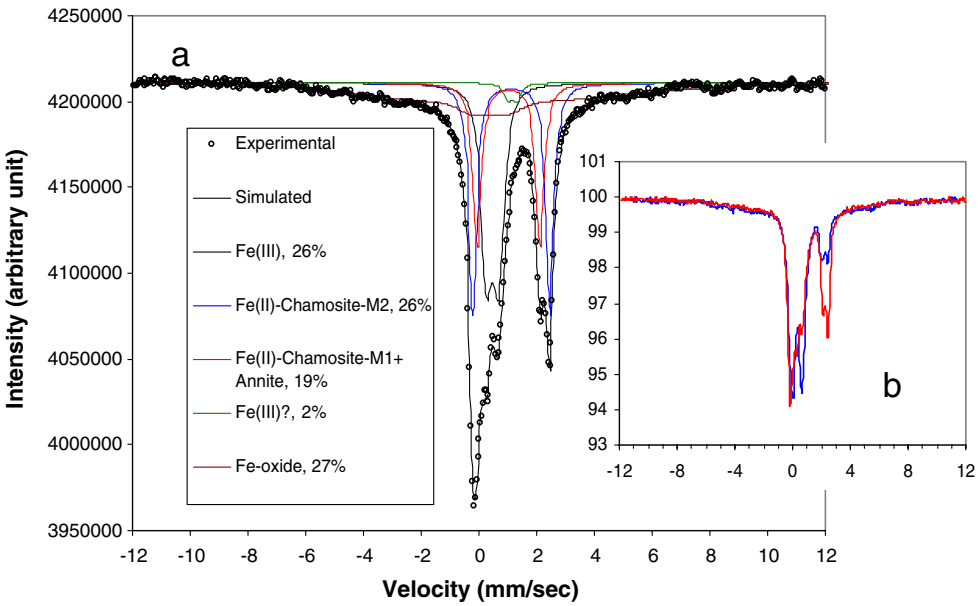

Figure 3 Room temperature Mossbauer spectra: **a** Modeled spectrum of Ross soil showing peaks due to Fe(II) and Fe(III) in chamosite and annite phyllosilicate minerals, and a broad sextet most probably due to small particle or metal-substituted Fe-oxides, and **b** a comparison of both the spectra (unmodeled; *inset*) showing similar Fe-mineralogy

complex, which may have contributions from both octahedral and tetrahedral Fe(III) sites of annite and chamosite, and small-particle (superparamagnetic) Fe-oxide [22]. The broad sextet (brown trace) which was one-third of the total Fe in both the samples was probably due to metal-substituted Fe-oxides (goethite and/or hematite substituted with Al) [22]. These iron oxides were either in amorphous forms or were present in small quantities, since they were not evident in XRD spectra. Absence of sulfide in XPS data also implied the absence of Fe-sulfide minerals in these soil samples.

Deep Biosphere Communities in the Ross Site of the Homestake Mine

PhyloChip proved extremely sensitive in capturing biosignatures at all taxonomic levels and detected a total of 1,511 OTUs positioned within 44 phyla which also included two archaeal phyla namely the *Euryarchaeota* and *Crenarchaeota* (Table 1). These 44 phyla contained diverse taxonomic lineages encompassing 49 classes, 91 orders, and 149 families. Among the 1,511 OTUs captured on the PhyloChip, a total of 25 OTUs were considered unclassified at the phylum level because of their low similarities (<80%) with reference probes on PhyloChip (Table 1). For a comprehensive distribution of bacterial and archaeal OTUs among different classes/orders/families/genera see supplementary tables (Electronic Supplementary Materials, Supplementary Class Table 1; Supplementary Order Table 2; Supplementary Family Table 3, and Supplementary Genera Table 4). PhyloChip data demonstrated that *Proteobacteria* was the most abundant taxa than other phyla, accounting for almost 49% of the total OTUs detected (Table 1). The *Firmicutes* (15% of total OTUs) and *Actinobacteria* (11% of total OTUs) represented the next most abundant phyla on PhyloChip.

Deep Biosphere Communities in the Winze Site of the Homestake Mine

The PhyloChip provided a comprehensive view of microbial diversity and captured a total of 1,678 OTUs spanning 44 phyla, 51 classes, 97 orders, and 154 families in Winze soil sample (Table 1). For a comprehensive distribution of these OTUs among different classes/orders/families/genera see supplementary tables (Electronic Supplementary Materials, Supplementary Class Table 1; Supplementary Order Table 2; Supplementary Family Table 3, and Supplementary Genera Table 4). Of the 1,678 bacterial OTUs detected, a total of 32 OTUs were considered as unclassified at the phylum level (Table 1). Like Ross soil, PhyloChip data for Winze soil indicated that *Proteobacteria* was far more abundant than other phyla, accounting for almost 47% of the total OTUs detected. The *Firmicutes* (15% of total OTUs) and the *Actinobacteria* (12% of total OTUs) represented the next most dominant phyla on the PhyloChip (Table 1).

Discussion

Comparative Species Richness in the Ross and Winze Soils of the Homestake Mine

PhyloChip analysis confirmed the presence of all taxa detected in corresponding 16S clone libraries established earlier from the same samples and additionally demonstrated greater phylotype diversity extending into phyla not observed

Table 1 Bacterial and archael phyla detected in the Ross and Winze sites using PhyloChip analyses

Serial no.	Bacterial/archael phyla detected on PhyloChips	Distribution of OTUs among different phyla	
		Ross site	Winze site
1.	***Acidobacteria***	57	60
2.	***Actinobacteria***	162	198
3.	*AD3*	1	1
4.	***Aquificae***	4	3
5.	***Bacteroidetes***	77	76
6.	*BRC1*	2	1
7.	*Caldithrix*	2	2
8.	*Chlamydiae*	2	2
9.	***Chlorobi***	9	10
10.	***Chloroflexi***	41	38
11.	*Coprothermobacteria*	1	1
12.	***Crenarchaeota***[b]	2	3
13.	*Cyanobacteria*	33	46
14.	***Deinococcus-Thermus***	4	4
15.	*Dictyoglomi*	1	1
16.	*DSS1*	1	1
17.	***Euryarchaeota***[b]	1	3
18.	***Firmicutes***	231	260
19.	***Gemmatimonadetes***	9	9
20.	*LD1PA group*	1	1
21.	*Lentisphaerae*	3	3
22.	*marine group A*	1	2
23.	*Natronoanaerobium*	4	5
24.	*NC10*	1	1
25.	***Nitrospirae***	10	10
26.	*OD1*	1	1
27.	***OP10***	4	5
28.	*OP3*	3	3
29.	*OP8*	2	1
30.	*OP9/JS1*	5	2
31.	***Planctomycetes***	15	11
32.	***Proteobacteria***[c]	732	807
33.	*SPAM*	2	2
34.	***Spirochaetes***	24	35
35.	*Synergistes*	5	5
36.	*Termite group 1*	2	3
37.	*Thermodesulfobacteria*	1	1
38.	*Thermotogae*	1	1
39.	*TM6*	1	1
40.	***TM7***	5	5
41.	Unclassified[a]	25	32
42.	***Verrucomicrobia***	20	19
43.	*WS3*	2	2
44.	*WS5*	1	1

A total of 1,511 and 1,678 OTUs found at the Ross and Winze sites, respectively, were distributed in 44 phyla. Phylum printed in bold are those that have been reported earlier from deep subsurface gold mine environments [13, 23, 24, 28, 38]

[a] A total of 25 and 32 OTUs in the Ross and Winze sites, respectively, could not be assigned to any known phylum and were considered unclassified

[b] Phylum belonging to kingdom *Archaea*

[c] Most abundant phylum in the Ross and Winze sites

by cloning methods [30]. PhyloChip analysis of soil samples obtained from Ross and Winze sites demonstrated 1,511 and 1,678 OTUs, respectively. Comparative PhyloChip data analyses showed that Ross and Winze samples shared large number (1,360) of OTUs in common with only 151 and 318 OTUs exclusively present in Ross and Winze samples, respectively (Fig. 4a). Despite of differences in the physical and chemical composition, both sites had similar microbial communities. Our previous clone library results showed that Ross site had relatively more species richness (110 OTUs) than the Winze site (100 OTUs) as indicated by the number of phylotypes retrieved from the same library sizes (165 clones in each library) [30]. Contrary to earlier clone library data, microarray data showed that Winze site (1678 OTUs) had more species richness than the Ross site (1,511 OTUs). We assume that this was primarily due to the limited number of clones analyzed in our previous study and sequencing of additional clones would have presented more in-depth picture of microbial diversity present in these sites.

A strong linear correlation has been shown earlier between microarray probe set intensity and concentration of OTU-specific 16S rRNA gene copies, which allows relative abundance analysis between normalized PhyloChips [5]. Interestingly, relative abundance based on the fluorescence intensity of OTU probe sets for Ross and Winze samples identified specific bacterial groups which were significantly different between two sites. Bacterial taxa demonstrating significant changes in intensity between Ross and Winze are depicted in Fig. 4b. Though both samples, shared a majority of OTUs, differences in bacterial abundances were observed. We used same DNA extraction protocol for both soil samples therefore assuming the equal extraction efficiencies of various taxa in both soil samples, *Actinobacteria* and bacilli were relatively more abundant in Winze sample as compared to Ross sample. On the other hand bacteria belonging to β-proteobacteria, γ-proteobacteria, δ-proteobacteria, and clostridia were found to be more abundant in Ross sample compared to Winze (Fig. 4b).

Effects of Metals, pH, and Mineral Phases on Microbial Communities

To our surprise, microarray analyses detected a broad phylogenetic diversity in soil samples despite the presence of significant amount of toxic metals and absence of sunlight-irradiation. In our previous study [30], we showed the detailed geochemical characteristics of these soil samples using X-ray fluorescence spectroscopy, coupled plasma optical emission spectrometry, and inductively coupled plasma mass spectroscopy. Our results showed that a significant amounts of toxic metals such as As, Cd, Co, Cr, Cu, Ni, Pb, and Zn were present in Ross and Winze soil samples. However, the water soluble (bioavailable) concentrations of these toxic metals were very low (<0.3 mg/l). Metals are toxic only in their ionic form and pH has been shown a most crucial factor in determining the bioavailability [31]. Roane and Kellogg [31], while studying the bioavailability of Pb in mining-impacted soil samples, showed that soluble toxic Pb concentrations were only detectable in acidic soils. Both the soil samples analyzed in our study had near neutral pH (6.6–6.7) [30] due to which bioavailable metal concentrations will decrease resulting in to less or no toxic effects on the inhabitant deep subsurface microbial communities.

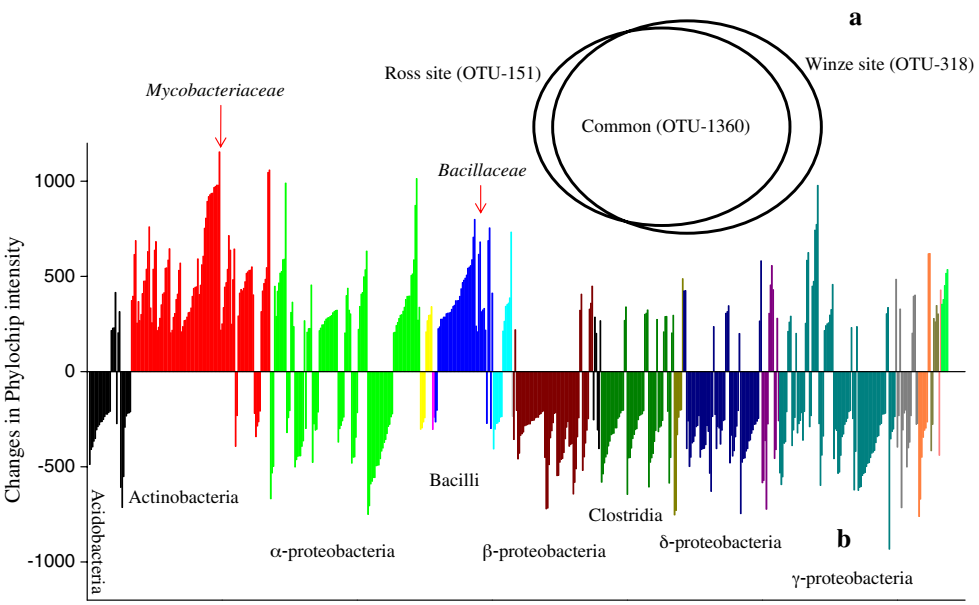

Figure 4 **a** Venn diagram showing fraction of similar OTUs observed between the Ross and Winze samples. The *number in the circle* indicates the estimated numbers of OTUs that are shared between two samples. **b** Comparison of relative abundance of taxa based on fluorescence intensity of OTU probes in Ross and Winze samples. OTUs were arranged based on their taxonomic affiliations. *Bars above the zero line* represent increased abundance in Winze sample relative in Ross sample while *bars below* represent relative decline in abundance in Winze sample

In addition to pH, another important factor controlling metal bioavailability in deep biosphere could be the presence of several complex mineral phases. XRD, XPS, and Mössbauer data indicated the presence of various minerals including chamosite, annite, and unidentified amorphous metal-substituted iron oxides. The surfaces of soil minerals have strong metal ions adsorptive capabilities and therefore may have reduced aqueous concentrations to a non-toxic levels, and decreasing overall metal bioavailability and toxicity [33, 34]. In subsurface soil, ferric oxides and oxyhydroxides (e.g., hematite, goethite, ferrihydrite) commonly exists as soil minerals. These minerals have strong affinity for a large number of cations and anions primarily due to their amorphous nature and high surface area [35]. Pertinent to this, our recent study on Pb toxicity to *Desulfovibrio desulfuricans* G20 also showed that in presence of goethite and quartz, Pb toxicity decreased significantly [34]. However, we acknowledge here that accurate effects of metal stress on microbial diversity can be predicted only when control soil samples from uncontaminated areas are also included in analysis. Such microbial diversity surveys with control samples will also help in identifying those taxa that are uniquely present or more dominant in mining-impacted soils.

Subsurface Microbial Community Composition in the Homestake Mine

To date, earlier studies on ultra-deep gold mines utilized clone libraries and culture-based approach to decipher the microbial community composition and so far no study has reported in-depth characterization of microbial diversity in deep subsurface of gold mines. PhyloChips captured lineages previously reported from ultra-deep gold mines and additionally helped recognize the presence of yet to be identified bacterial lineages at each taxonomic level in both samples. Compared to the microbial diversity assessments from other deep subsurface gold-mines [13, 14, 23, 25, 38], our results showed remarkable similarities at phylum level. The phyla in common were *Euryarchaeota, Crenarchaeota*, *Acidobacteria, Actinobacteria, Bacteroidetes, Chlorobi, Chloroflexi, Firmicutes, Gemmatimonadetes, Nitrospirae, Proteobacteria*, and candidate divisions OP10 and TM7 (Table 1). However, this is the first report for the presence of members of phyla AD3, BRC1, *Cladithrix*, *Coprothermobacteria, Cyanobacteria, Deferribacters, Dictyoglomi*, DSS1, LD1PA group, *Lentisphaerae,* marine group A, NC10, OP3,OP8, OP9, OP10, SPAM, SR1, *Synergists*, *Thermotogae*, termite group 1, TM6, TM7, WS3, and WS5 in ultra-deep gold mines (Table 1). Candidate phyla; OP10 and TM7 sequences were retrieved in 16S clone libraries constructed earlier from Homestake mine soil samples [30].

Earlier studies have shown that *Proteobacteria* constituted a major proportion of the clone libraries established from deep subsurface gold mines samples [13, 25, 30]. The PhyloChip results in this study agreed with earlier reports as *Proteobacteria*-related lineages constituted the most abundant group in both the samples. The ubiquitous nature of the *Proteobacteria* is mostly due to their capabilities to cope with hostile life conditions such as high temperature and pressure, extreme pH, oligotrophic environments, metal-reduction, and metal-resistance which are prerequisite to surviving in mining-impacted deep subsurface environments [1]. *Proteobacterial* genera documented in Ross and Winze sites such as *Acinetobacter*, *Burkholderia,* and *Ralstonia* (Electronic Supplementary Material, Supplementary Genera Table 4) have been reported earlier from metal-contaminated environments and are resistant to metals such as cadmium, copper, nickel, and zinc [1, 29] suggesting that abundance of toxic metals in mining-impacted Ross and Winze soils may have selected these genera. It is interesting to note that within the *Proteobacteria*, PhyloChips have identified genera belonging to *α-Proteobacteria (*e.g., *Sphingomonas, Rhodobacter, Caulobacter, Methylobacterium, Brevundimonas, Bradyrhizobium), β-Proteobacteria (*e.g., *Azoarcus, Acidovorax, Nitrosomonas, Thiobacillus, Comamonas), γ -Proteobacteria; (*e.g., *Pseudomonas, Thiocapsa, Nevskia, Methylococcus, Marinobacter, Stenotrophomonas, Halomonas,* and *δ- Proteobacteria (*e.g., *Desulfovibrio)* that have been described earlier from ultra-deep gold mines of Japan and South Africa [13, 14, 25]. Additionally, bacteria such as *Shewanella surugensis* detected in Ross and Winze sites were previously cultured from deep-sea sediments [20]. These genera indicate an original deep biosphere ecosystem in the Homestake mine. Noticeably, the Homestake mine is geographically distinct from previously studied ultra-deep gold mines or deep subsurface habitats. Thus it was interesting to note such similarities in retrieved phylotypes of the Homestake mine with other deep subsurface environments. In addition to retrieving deep biosphere microbial signatures, PhyloChip also indicated several bacteria (e.g., *Roseobacter*) from the Ross and Winze sites that have not been previously reported from deep subsurface or metal-contaminated environments (Electronic Supplementary Material, Supplementary Genera Table 4). Such genera have been widely shown in pristine environments [15] and probably would have been introduced in Homestake mine deep biosphere during mining operations.

In addition to identifying major taxa such as *Proteobacteria, Firmicutes*, and *Actinobacteria*, PhyloChips also captured other minor phyla such as *Gemmatimonadetes*, *Nitrospirae, Planctomycetes, Spirochaetes,* and *Verrucomicrobia* in both the sites. Although, these phyla have been reported earlier from ultra-deep gold mines [13, 14, 25, 30], their roles in the

ecology of deep biosphere remain unclear. Our study demonstrated very low archaeal diversity (only 3-6 OTUs in *Archaea* domain) in the Homestake mine deep biosphere which agreed well with our previous 16S clone library results which demonstrated only few archael lineages in Ross and Winze samples [30]. It is also likely that low archael diversity observed could be due to partial 16S amplicons used for microarray hybridization. The reference 16S probes attached on PhyloChips were based on sequence information from nearly complete (~1,325 bp) 16S rRNA genes. Therefore, PhyloChip analysis using partial archael 16S PCR products (as generated by Arch 21f/Arch 958r) would have missed hybridization of PCR amplicons with several 16S probes present on the PhyloChip and hence the "present" call for several archael OTUs.

Microarrays also indicated several phylotypes related to phyla for which no cultivated representatives are known. For example in the Ross and Winze sites, phylotype similar to sequences from candidate phyla, e.g., OP3, OP9, OP10, TM6, and TM7 were retrieved. To date no cultivable lineages belonging to these candidate divisions have been isolated in cultures [15]. These bacterial divisions are exclusively represented by environmental sequence data and are unstudied. Other phyla such as *Acidobacteria* and *Verrucomicrobia* contain only few cultured members [15]. Therefore, the physiological roles of any of these bacteria in natural environments including in deep subsurface ecology remain unknown. Our study has avoided over data interpretation based solely on PhyloChip data. Nevertheless, a species molecular inventory is an important initial step in describing unique and dynamic microbial communities, and forms basis for the development of improved culturing methods, and subsequently, elucidation of metabolic roles.

Metabolic Versatility in Deep Biosphere of the Homestake Mine

Deep subsurface environments are generally considered as oligotrophic due to the lack of photosynthetically derived electron donors [16]. Geochemical conditions in the subsurface biosphere such as nutrient availability and permeability, soil composition, redox potential, and a variety of other factors generally influence the resident microbial communities. In such environments the geothermal aquifer is the major source of carbon (e.g., methane, carbon dioxide) and energy (e.g., hydrogen, sulfide, reduced iron, and ammonium), and acts as a determinant for microbial community structures. Chemolithoautotrophic microorganisms in deep subsurface that could grow on hydrogen and carbon dioxide (e.g., acetogenic bacteria) can act as primary producers, initiating heterotrophic food chains independent of photosynthesis [16]. Active chemolithotrophic bacteria in the ultra-deep gold mines of South Africa and Japan have been reported earlier [3, 23, 38]. These studies have also demonstrated sulfate-reducing and methanogenic metabolic pathways in deep biosphere of gold mines. More interestingly, viable sulfate-reducers, methanotrophs, ammonia-oxidizers, nitrite-oxidizers, methane-oxidizers, and sulfur-oxidizers have been isolated from geothermal water sampled from deep Japanese gold mines [13].

Precambrian aquifer water was pumped continuously for dewatering during the operation of the Homestake mine and in June 2003, pumps were turned off. Current water inflow to the underground is about 2,839 L min^{-1} [8]. Geothermal water enters the Homestake mine from the surface primarily through the shaft and airways that intersect with the open pit. Our previous study, reported a variety of soluble ions (such as SO_4^- [9,237–15,156 mg L^{-1}], and NO_3^- [16–39 mg L^{-1}]) in soils collected from the Ross and Winze sites of the Homestake mine [30]. In present study, soil XPS analysis also identified considerable sulfur mainly in form of SO_4^- ions. These soluble ions can fuel the growth of various chemotrophic microorganisms in deep Homestake mine biosphere where the energy sources are limited. For example, PhyloChip has indicated several chemolithotrophic genera such as ammonia-oxidizers (e.g., *Nitrosomonas* sp.), nitrite-oxidizers (e.g., *Nitrospira* sp.), methane-oxidizers (e.g., *Methylosinus* sp.), sulfur-oxidizers (e.g., *Thiobacillus* sp), methanogens (e.g., *Methanosarcina* sp.), and sulfate-reducers (e.g., *Desulfosporoinus* sp.) in Ross and Winze soils samples. Interestingly, our laboratory enrichment studies for sulfate-reducing bacteria using acetate and sulfate as electron donor and acceptor, respectively, confirmed the existence of viable population of sulfate-reducers in Ross and Winze soil samples. A 16S clone library analysis of the enrichment culture revealed that *Desulfosporosinus* spp. were the dominant lineages in the sulfate-reducing enrichment (Rastogi et al., unpublished data).

The gold deposits at the Homestake mine are typically associated with the banded ironstone formation [2] therefore both soil samples contained very high amount of iron; 51,540–75,657 mg L^{-1} [30]. In this study, XPS and Mössbauer data clearly showed the presence of reduced iron [Fe(II)]. As expected, PhyloChip detected several Fe-oxidizing bacteria (e.g., *Leptothrix, Acidithiobacillus*) in both soil samples. These bacteria proliferate in habitats where anaerobic Fe(II)-rich water comes in the contact of air. During dewatering of the Homestake mine for the construction of DUSEL, the exposed oxygenated Fe(II)-rich surfaces served as a primary site for the growth of these bacteria, evident by the characteristic rust colored soil samples from insoluble Fe(III)-ions [30]. Room temperature Mossbauer spectra showed >25% of the iron in form of

oxidized Fe(III)-ions. In addition to Fe-oxidizing bacteria, microarrays also captured few Fe(III)-reducing bacteria such as *Shewanella* sp. in both site that can reduce variety of terminal electron acceptors, including nitrate, nitrite, thiosulfate, and elemental sulfur [11]. In a 16S clone library of Fe-reducing enrichment cultures initiated using Ross and Winze soils with acetate and hematite as electron donor and acceptor, respectively showed that *Clostridium* sp. dominated the iron-reducing communities (Rastogi et al. unpublished data). PhyloChips also indicated *Clostridium*-related OTUs in both soil samples (Electronic Supplementary Material, Supplementary Genera Table 4).

Another important chemotrophic group of bacteria in the Homestake mine could be sulfur-oxidizers because of the significant amount of sulfur (as shown by XPS) present in soil samples. Chemical characterization of Ross and Winze soil also showed significant sulfur (1.79–2.66%; measured as total sulfur) which can act as energy source for these bacteria [30]. As a proof to this hypothesis, we have cultured sulfur-oxidizing bacteria belonging to genus *Thiobacillus* from Ross and Winze soil samples (Rastogi et al., unpublished data). PhyloChip data also demonstrated the presence of *Thiobacillus* sp. in soil samples. Interestingly, *Thiobacilli* are chemolithoautotrophic, sulfur-oxidizing bacteria that are restricted to habitats where both an electron donor (reduced sulfur compounds [S^0, H_2S, and $S_2O_3^{-2}$] or Fe (II) in some cases) and an electron acceptor (O_2 or NO_x) simultaneously exist. These bacteria produce sulfuric acid and Fe(III) as by-products of their metabolism and play a very important role in biomineralization. Southam et al. [37] further demonstrated a very strong physical association between *Thiobacillus* species and the sulfide minerals, which helps account for their prominence in tailings environments.

Alternative carbon sources such as lignocelluloses may also fuel the Homestake mine deep biosphere. During active mining-operations for over 125 years, lignocellulosic substrates were introduced into the Homestake mine [27]. Interestingly, we have isolated several strains of *Bacillus, Paenibacillus*, and *Geobacillus* from the Homestake mine that were able to grow on cellulose and sawdust (a fine powder of woodchips) as a source of carbon and energy [27]. All these genera have been captured by PhyloChips which indicates that Homestake mine deep biosphere harbors microbial communities that can use complex lignocellulosic materials as carbon and energy sources.

Molecular-based diversity methods (e.g., PCR, cloning-and-sequencing, PhyloChip) based on direct DNA/RNA-extraction are alternative to classical culture-based methods, and have provided great insights in to community composition, richness, and structure of microbial communities. However, like culture-based methods, these molecular methods have their own pitfalls and are associated with bias at every step. Bias associated with DNA extraction (e.g., incomplete/no lyses of microbial cells) can distort the revealed community composition, richness, and microbial community structure [10]. In addition, all PCR-based diversity studies have additional biases associated which include primer choice, annealing temperature, and preferential amplification of certain templates [39].

DNA extraction from environmental samples constitutes the first step in PCR-based community analysis. In fact, different DNA recovery methods have been shown to reveal different depth of microbial diversity due to variation in the ability to break open cells/spores [18]. Different studies have used different DNA extraction protocols in soil microbial diversity analysis. The primary reason so many methods for DNA extraction have been used is due to the fact that most procedures are optimized for a specific soil. This implies that any given procedure may not be universally applicable to all soils due to inherent spatial and microbial heterogeneity [42]. Use of a standard DNA extraction protocol which is efficient for a soil sample may or may not work for other samples sometimes even collected from the same site. DNA extraction efficiency is correlated with soil's physicochemical characteristics (texture, particle size), inhibitors (humic acids, metals, organic legends, etc.), and type of microbial communities.

Feinstein et al. [10] demonstrated that biases in community analysis can be reduced in many situations by pooling three successive DNA yields from a recovery method. Another study by McIlroy et al. [19] suggested use of several validated DNA extraction methods and pooled DNA extracts to minimize any risk of bias. Use of such validated protocols by all microbial ecologists could provide a more complete understanding of the soil microbial community composition and would offer a platform where quantitative inter-comparisons can be made [18]. In addition, development of a single method for purification of DNA from all soil samples will be a great step toward automating the procedures, and for standardizing results between laboratories. A procedure that is equally efficient for all soil samples, efficiently lyses all bacterial groups, requires little time to complete, and is easy to processing multiple samples simultaneously would be highly desirable. For example, MoBio PowerSoil™ DNA isolation kit which is also used in our study is very widely used method for isolating microbial DNA of the highest quality and purity from most environmental samples including deep subsurface gold mines. An advantage of using commercially available kits for DNA isolation is that they provide a rapid and standardized approach that can be quickly learned. Nonetheless, working with complex environmental samples, e.g., soil where >99% microorganisms have not been cultured yet; it is difficult to predict the efficiency of MoBio PowerSoil™ kit for those uncultured organisms. In

addition, it would be worth to acknowledge that although, microarray technology has been used for specific, quantitative, and high-throughput detection of microbial diversity in natural settings; it is unreliable in identifying and detecting novel prokaryotic taxa. The ecological importance of a species, which may be abundant and pivotal to the ecosystem under study, can be completely ignored if the species does not have a corresponding probe on the PhyloChip. Furthermore, community analysis using molecular methods alone is not sufficient for predicting the metabolic functions within the environment.

In summary, the present study provided a comprehensive microbial census on the bacterial and archael communities in the mining-impacted deep subsurface habitat of the Homestake mine. In comparison to previous studies, the use of high-density microarrays resulted in identification of enormous phylogenetic diversity from ultra-deep gold mines. We acknowledge here that molecular oxygen and surface microbes were introduced into the Homestake mine by human activities during mining-operations, thus the microbial community in soil samples does not necessarily reflect an indigenous deep subsurface microbial population. However, various environmental limitations in the deep biosphere of the Homestake mine would shape the microbial diversity as a novel and unique subsurface microbial ecosystem. The results on microbial diversity and geochemistry will serve as a vital comparison for future assessment of changes in microbial diversity and geochemistry as re-entry in Homestake mine continues and the deeper levels become exposed during the construction of the DUSEL.

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