Terr. Atmos. Ocean. Sci., Vol. 18, No. 2, 395-412, June 2007

Cultivation-Based Characterization of Microbial Communities Associated with Deep Sedimentary Rocks from Taiwan Chelungpu Drilling Project Cores

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(Manuscript received 7 January 2006, in final form 20 October 2006)

ABSTRACT

The Taiwan Chelungpu Drilling Project (TCDP) has provided an unprecedented opportunity to reveal a terrestrial subsurface microbial ecosystem that has possibly experienced continuous disturbance by the arc-continental collision since 5 Ma. The drilling penetrated Pliocene-Pleistocene sedimentary rocks to a depth of 2000 meters below the land surface (mbls) and encountered two major fault zones. Sixteen samples retrieved from the drilled cores at depths between 464 to 1451 mbls were examined to characterize microbial community structures through cultivation-based approaches. Cultivation experiments were performed with various media at temperatures ranging from 30 to 50°C. The results indicate that fermenters and heterotrophic sulfate reducers, using complex organic carbon, were ubiquitously present in most samples. Acetate-utilizing and H_2-utilizing sulfate reducers were restricted to shallower intervals along the depth profile. Iron reducers and methanogens were only cultivated in a few shallow samples. Twelve pure strains including 8 fermenters, 3 iron reducers and

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doi: 10.3319/TAO.2007.18.2.395(TCDP)

1 sulfate reducer originally enriched at 30 or 40°C were isolated and identified with 16S rDNA sequence analyses. They were phylogenetically affiliated with *Firmicutes*, *Bacteriodes*, *Actinobacteria*, and *Proteobacteria* in various degrees of similarity. The presence of metabolism was not correlated with lithology, depth, temperature and the appearance of fracture throughout the core. The ubiquitous appearance of fermentation and organotrophic sulfate reduction suggests that organic carbon sources were readily accessible in this deep terrestrial subsurface environment. These results infer that the foreland sedimentary strata disturbed by tectonic activities over a geological time scale might support a heterotrophy-dominated deep terrestrial subsurface microbial ecosystem.

(Key words: Taiwan Chelungpu Drilling Project, Microbial community, Cultivation, Metabolism)

1. INTRODUCTION

Subsurface ecosystems have been postulated to possess more than 70% of the biomass on Earth (Whitman et al. 1998). Among various subsurface environments, biomass in the terrestrial component is considered to be comparable with that in the oceanic component (Whitman et al. 1998). While sub-seafloor environments have been subject to relatively intense exploration, an exponential decline of biomass or cell density as a function of depth has been constantly observed in sediments distributed down to a depth of 1000 meters below the seafloor (Parkes et al. 2000). The biomass distribution corresponds to a successive development of metabolic zonation at which sulfate reduction is followed by anaerobic methane oxidation and methanogenesis as depth increases (D'Hondt et al. 2004). Such a stratified distribution of dominant metabolic pathway is basically consistent with diffusive substrate fluxes across highly porous media (Chapelle et al. 1995). In contrast, terrestrial subsurface environments receive far less attention and remain largely unknown in part due to the lack of appropriate access to samples at great depths. The fact that terrestrial subsurface environments are primarily composed of consolidated rocks makes sample acquisition even more difficult and costly.

Earlier studies on terrestrial subsurface microbial ecosystems focused on groundwater retrieved from shallow unconsolidated sedimentary aquifers at depths less than 1000 meters below the land surface (mbls) (Fredrickson and Onstott 2001). Geochemical and metabolic characteristics are generally consistent with those in sub-seafloor sediments with exceptions that nitrate and ferric iron appear to be more abundant in terrestrial environments and hence nitrate reduction and iron reduction are stimulated at depths above sulfate reduction zones (Lovley and Goodwin 1988). Only a few studies are related to deep consolidated or crystalline rocks (such as granite, basalt and meta-sandstone) (Stevens and McKinley 1995; Pedersen 1997; Haveman and Pedersen 1999; Chapelle et al. 2002; Cowen et al. 2003; Onstott et al. 2003; Lin et al. 2005a). The low porosity of consolidated rocks (<1%) renders groundwater confined in fracture networks and potentially isolated from surface recharge during a geological time scale (Torgersen and Clarke 1985; Colwell 2001). While organic content associated with

consolidated rocks is generally low and groundwater transport is extremely slow, microbial communities residing at the interface between fracture water and rock matrix have to rely on substrates derived from geological processes (Stevens and McKinley 1995). Deep terrestrial subsurface ecosystems in intra-cratonic continents are also characterized by a lessened degree of substrate exchange between different reservoirs when compared with those in shallow sedimentary aquifers (Onstott et al. 1998). The lack of ongoing tectonic processes, such as thrusting or normal faulting, reduces bulk permeability across structural features or geological strata. Solute transport would be largely restricted to the diffusion from rock matrix to fracture network (Colwell 2001; Pedersen 2001). Substrate availability, metabolic activity and community structure, therefore, can be highly heterogeneous in geographically proximal fractures, depending on water-rock interaction, groundwater residence time, and local source rock (Onstott et al. 2006). On the contrary, a deep terrestrial subsurface ecosystem would be disturbed by geological processes in a tectonic active region. Stacking of geological strata by thrusting or normal faulting would change the hydrological regime and hence affect substrate availability to certain portion of microbial communities. However, biomass distribution, community structure, and metabolic function in these terrestrial subsurface ecosystems and their relationships to geological parameters are poorly constrained.

The indigenous subsurface microbial community is difficult to recover primarily due to contamination associated with sample access. At a low biomass ($<10^4$ cell g^{-1} or ml^{-1}), it can be readily altered by manned activity that introduces and often stimulates the development of microbial communities with much greater biomasses (several orders of magnitude) (Fredrickson and Phelps 1996). Of various methods used for sample retrieval, surface coring provides many advantages over the others in that: (1) artificial contamination can be carefully monitored through drilling fluid and tracer; (2) microbial communities and metabolic pathways can be correlated with targeted geological features, temperature gradient, and lithology along a depth profile. The attached microbial community recovered from cores is also considered to represent the dominant component of a subsurface ecosystem that is responsible for observed geochemical signatures in groundwater (Pedersen et al. 1997).

To investigate the extent and function of indigenous, attached subsurface microbial communities associated with consolidated rocks in active tectonic environments, drilled cores with careful monitoring of artificial contamination would be required (Smith et al. 2000). The Taiwan Chelungpu Drilling Program (TCDP) has provided a unique opportunity to address these questions. The coring penetrated Pliocene-Pleistocene strata with ongoing tectonic activity in Taiwan. In this study, core samples were inoculated to various media in order to assess the cultivability of microorganisms residing within the pore space of consolidated rocks. The objective was to assess the metabolic diversity of viable microbes through the cultivation-based method and whether the cultivability of certain types of metabolism can be correlated with any geological parameter such as temperature, depth, and lithology. Four types of metabolism including iron reduction, sulfate reduction, methanogenesis and fermentation commonly observed in shallow sedimentary aquifers, were targeted for media design. Although a cultivation-based approach may unavoidably underestimate diversity, it offers direct assessment on physiological characteristics of a portion of the active microbial community.

2. GEOLOGICAL BACKGROUND

Taiwan is located at the boundary between the Philippine Sea Plate and the Eurasian Plate. Continuous convergence between these two plates has resulted in an arc-continent collision that further induced the uplift of the Eocene to Pleistocene strata above the sea level along a series of east-dipping thrusts since 5 Ma (Teng et al. 2000). The horizontal convergence has been estimated at a rate of around 80 km Ma^{-1} since late Miocene based on plate reconstruction and GPS measurements (Suppe 1981; Yu et al. 1997). Such a substantial shortening and uplifting occurred in sedimentary strata that were originally deposited at the same horizon but later displaced from each other by tectonic movement. The shearing further enhances the development of numerous fracture zones associated with the major fault planes and, therefore, changes the hydrological circulation pattern.

The coring conducted by the TCDP was designed to retrieve cores from an active fault zone that triggered an earthquake at a magnitude of 7.6 in 1999 (Wang et al. 2000). The coring penetrated through Cholan, Chinshui, and Kueichulin formations to a depth of 2000 mbls. These Pliocene-Pleistocene strata are mainly composed of sandstone and shale in different proportions. Two major fracture zones were encountered: one located within the Chinshui formation at around 1110 mbls and the other at the boundary between the Kueichulin formation and the underlying Cholan formations at around 1750 mbls. At depths greater than 1750 mbls, the Cholan formation was displaced underneath the Kueichuling formation by overthrusting along the deeper fracture zone. The temperature at the bottom of the borehole was ~60°C.

3. METHODS

3.1 Sample Handling

Coring was performed with a diamond-coated hollow bit at an inner diameter of 8.3 cm. The drilling fluids consisted of water pumped from an adjacent river and bentonite and barite ($BaSO_4$) to reduce heat, remove rock fragments generated during pulverization, and prevent groundwater intrusion. Rhodamine dye (B-type, Sigma Aldrich, St. Louis, MO, USA) was added to the drilling fluid at a concentration of 50 ppm (in weight) in order to monitor contamination introduced during the coring. Each core section with a maximum length of around 3 meters was retrieved to the surface using a wireless core catcher. Intact cores without lithological interclasts and structural features were chosen and sectioned into lengths of 20 - 30 cm for geomicrobiological study. The sectioned core was immediately wrapped with a sterilized aluminum foil and placed into an anaerobic box together with three Gas Paks (Becton Dickson, Franklin Lakes, NJ, USA) used for O_2 absorption and CO_2 production and an O_2 detection strip (Becton Dickson, Franklin Lakes, NJ, USA). The anaerobic box was continuously flushed with high-grade N_2 or Ar at a rate of 500 ml min^{-1} to accelerate the removal of O_2. Once the O_2 detection strip turned colorless for 5 min, the anaerobic gas stream was shut off and core samples were transported back into an anaerobic glove chamber (COY Laboratory Product, Inc., USA) in the laboratory within 3 hours.

Each core sample was split into three approximately equal portions in the anaerobic glove chamber within one day. Two of these portions were archived in either an anaerobic jar at 4°C or freezer at -80°C. The rim of the third portion (potentially contaminated by drilling fluid) was peeled off with sterilized hammers and chisels. Only the central part of the core with a diameter of 2 to 3 cm was kept and ground into powders with a sterilized stainless steel bowl and pestle. A core sample for negative control was autoclaved at 121°C for 20 min and processed in the same procedure.

3.2 Cultivation

Rhodamine dye was measured for slurry consisting of ground powders and deionized water with a UV-spectrophotometer to assess the drilling-introduced contamination. For samples without detectable contamination, the powders (~1 g) were inoculated into sterilized media (15 ml) designed for fermentation, iron reduction, sulfate reduction and methanogenesis. The depths for sample inoculation ranged from 464 to 1451 mbls. All media were composed of basal salt solution, trace metal solution, and vitamin solution. The ingredients of the basal salt solution included 1.17 g of NaCl, 0.4 g of $MgCl_2 \times 6H_2O$, 0.3 g of KCl, 0.15 g of $CaCl_2 \times 2H_2O$, 0.27 g of NH_4Cl, and 0.2 g of KH_2PO_4 per liter. The trace metal solution consisted of 1.5 g of nitrilotriacetic acid, 3 g of $MgSO_4 \times 7\ H_2O$, 0.5 g of $MnSO_4 \times 2\ H_2O$, 1 g of NaCl, 100 mg of $FeSO_4 \times 7\ H_2O$, 180 mg of $CoSO_4 \times 7\ H_2O$, 10 mg of $CaCl_2 \times 2\ H_2O$, 180 mg of $ZnSO_4 \times 7\ H_2O$, 10 mg of $CuSO_4 \times 5\ H_2O$, 20 mg of $KAl(SO_4)_2 \times 12\ H_2O$, 10 mg of H_3BO_3, 10 mg of $Na_2MoO_4 \times 2\ H_2O$, 25 mg of $NiCl_2 \times 6\ H_2O$, and 0.3 mg of $Na_2SeO_3 \times 5\ H_2O$ per liter. The vitamin solution was a mixture of 2 mg of biotin, 2 mg of folic acid, 10 mg of pyridoxine-HCl, 5 mg of thiamine-HCl $\times$ 2 H_2O, 5 mg of riboflavin, 5 mg of nicotinic acid, 5 mg of D-Ca-pantothenate, 0.1 mg of vitamin B_{12}, 5 mg of p-aminobenzoic acid, and 5 mg of lipoic acid in one liter deionized water. One liter of media was added with 10 ml of trace metal solution and 1 ml of vitamin solution. Electron donors and acceptors used in each desired metabolism are listed in Table 1. Resazurin at a concentration of 1 mg per liter was added to monitor the presence of O_2. A reducing agent such as $Na_2S \times 9\ H_2O$ or cystein-HCl at a concentration of 0.05% was used to decrease the redox potential and to remove any trace amounts of O_2. Ingredients for media were mixed thoroughly with pH adjusted to ~7.2. The resultant solution was boiled and dispensed into borosilicate vials under anoxic conditions. Vials were sealed with butyl rubber stoppers and autoclaved at 121°C for 20 min. Headspace was flushed with desired 0.2 μm-filtered gases (Table 1) for 5 min before use. Incubation temperature was set at 30°C, 40°C and 50°C for samples retrieved from different depths (Table 2). Parallel negative control without inoculation was performed.

Enrichments were constantly checked with a phase-contrast microscope and assayed with aqueous or gas chemistry. DAPI staining technique with a fluorescence microscope was also applied for enrichments without distinguishable or dividing cells under a phase-contrast microscope. Fermentation was detected by an increase of cell density. Positive reduction of ferric iron to ferrous iron was indicated by an increase in cell density and a color change from orange to colorless. Concentrations of ferrous iron were measured with ferrozine assay (Lovley and Phillips 1987). Enrichments with an increase in cell density but no change of color were

attributed to positive fermentation given that the iron reduction medium consisted of citrate and lactate that can be used by fermenters. Positive sulfate reduction was reflected in an increase in cell density and substantial formation of H_2S, which was precipitated as ZnS while the enrichment was mixed with Zn-acetate solution. Methanogenesis was indicated by an increase in cell density with the production of methane over time. Once positive growth was confirmed, the enrichment was transferred to a fresh media with a dilution factor of 1 : 10 to reduce the contribution of original substrate derived from rocks. Metabolic capability was ascertained after at least 3 transfers.

3.3 Pure Strain Isolation

Pure cultures were obtained by plating or serially diluting the positive enrichment cultures. After at least 5 transfers, enrichment was spread in 1.5% agar plates with various dilution factors and incubated under the same conditions until individual colonies were formed. Single colonies were picked up with sterilized pipette tips and re-suspended in liquid media to obtain pure strains. Pure strains were repeatedly purified in 1.5% agar plates. The purity of pure strains obtained by serial dilution was confirmed by restriction fragment length polymorphism patterns for clone PCR products obtained from at least 20 colonies in the clone library.

Table 1. Summary of ingredients used in the media.

Target metabolism	Electron donor	Electron acceptor	Headspace gas	Others
Fermentation	0.1% peptone, 0.1% yeast extract, 0.1% tryptone		100% N_2	0.05% sulfide
Iron reduction	10 mM lactate, 10 mM acetate	10 mM ferric citrate	10% H_2, 10% CO_2, 80% N_2	
Sulfate reduction – complex organics	0.1% yeast, 10 mM lactate	20 mM sodium sulfate	100% N_2	0.05% sulfide
Sulfate reduction – acetate	10 mM acetate	20 mM sodium sulfate	100% N_2	0.05% sulfide
Sulfate reduction – H_2	10% H_2	20 mM sodium sulfate	10% H_2, 10% CO_2, 80% N_2	0.05% sulfide
Methanogenesis	10% H_2, 10 mM acetate	20 mM sodium bicarbonate, 10% CO_2	10% H_2, 10% CO_2, 80% N_2	0.05% cystein-HCl or 0.05% sulfide

*All media consisted of basal salt solution, trace metal solution, and vitamin solution.

Table 2. Summary of incubation conditions and cultivation results.

Sample No.*	Lithology	T	Media type#&					
		(°C)	F	IR	SR-comp	SR-H_2	SR-Ac	M
TCDP464	Sandstone	30	+	+	+	n.d	n.d	n.d.
TCDP545.2SS	Sandstone	30	+	+	+	+	+	+
TCDP545.2SH	Shale	30	+	n.d.	+	n.d.	n.d.	n.d.
TCDP545.6	Sandstone	30	+	n.d.	+	+	+	n.d
TCDP690	Siltstone	40	+	n.d.	+	n.d.	+	n.d.
TCDP694	Sandstone	40	+	n.d.	n.d	n.d.	n.d	+
TCDP694FG	Gouge	40	+	n.d.	+	+	n.d	+
TCDP739	Siltstone	40	+	n.d.	+	+	n.d	n.d.
TCDP829	Shale	40	+	n.d.	+	n.d.	+	n.d.
TCDP833	Siltstone	40	n.d	n.d.	+	+	n.d	n.d
TCDP887	Siltstone	40	+	n.d.	+	n.d.	n.d	n.d.
TCDP1033	Shale	40	+	n.d.	+	n.d	n.d	n.d.
TCDP1187	Shale	40	+	n.d.	+	n.d	+	n.d.
TCDP1284	Siltstone	40	+	n.d.	n.d	+	+	n.d.
TCDP1450	Siltstone	50	+	n.d.	n.d	n.d.	+	n.d.
TCDP1451	Sandstone	50	+	n.d	n.d	+	n.d	n.d

* The number adjacent to TCDP in the column of "Sample No." represented the depth of the sample.

Media type: F: fermentation, M: methanogenesis, IR: iron reduction, SR-comp: sulfate reduction with complex organic carbon as electron donors, SR-H_2: sulfate reduction with H_2 as an electron donor, SR-Ac: sulfate reduction with acetate as an electron donor.

& +: positive; n.d.: not distinguishable.

16S rRNA genes for pure strains were used to characterize their phylogenetic relationships with those deposited in Genbank (www.ncbi.nih.gov) and Ribosomal Database Project (rdp.cme.msu.edu, RDP 9 Release) (Cole et al. 2003). Genomic DNA was extracted using MoBio UltraClean Soil Kit (MoBio, Carlsbad, CA, USA) according to the manufacturer's instruction. Bacterial 16S rRNA genes were PCR-amplified with primers of 27F (5'-AGA GTT TGA TCM TGG CTC AG-3') with 1492R (5'-CGG TTA CCT TGT TAC GAC TT-3') (Lane 1991). Each PCR cycle (total 30 cycles) consisted of a 30-sec denaturation step at 94°C, a 45-sec annealing step at 54°C, and a 2-min elongation step at 72°C. Each PCR reaction (total of 50 μl) contained 5 μl of 10X PCR buffer, 4 μl of 2 mM deoxynucleioside, 4 μl of 25 mM $MgCl_2$, 1 μl of each 25 μM primer, and 1 U of Ex Taq (Takara, Shiga, Japan). PCR products were checked in 1.0% agarose gel stained with SYBR dye (Molecular Probes, Carlsbad, CA, USA). Positive PCR products were further purified using a MoBio PCR cleaning kit (MoBio, Carlsbad, CA, USA) and sequenced using an ABI 377 sequencer (Applied Biosystems, Foster, CA, USA). Sequences for 16S rRNA genes were compared and aligned with those in Genbank and RDP databases. A distances matrix for neighbor joining was calculated according to the algorithm of Jukes and Cantor (Felsenstein 1993) using the Bioedit program (Hall 1999). Neighbor-joining analyses were performed on the basis of ~1000 homologous bases.

4. RESULTS

Sixteen sets of inoculation were performed for six months after core samples were retrieved. A summary of cultivability for different samples is listed in Table 2. None of cultivable metabolisms has been detected in negative controls. Twelve isolates were obtained and characterized by their 16S rRNA gene sequences with their close affiliation (Table 3).

Fermentation bacteria appeared to be enriched positively for most inoculations. The incubation temperature for cultivated fermenters spanned from mesophilic to thermophilic range adopted from possible in situ temperatures (30 to 50°C) (Table 2). Several morphotypes, including cocci, short rod, and long rod, were observed. Six strains were isolated from the enrichment for the sample at 545.2 mbls. 16S rRNA gene sequences for these strains exhibited a close affiliation with *Clostriduim intestinale*, *Propionibacterium propionicum*, and some uncultured bacterium clone within *Firmicutes*, *Bacteriodes*, and *Actinobacteria* (Table 3). Fermenters were also enriched from iron reducing medium by using citrate and/or lactate as energy and organic sources without reducing ferric iron (no color change was observed). They appeared to be composed of cocci only, no matter the depth of the sample or incubation temperature. One of fermentative strains was isolated from the sample at 545.6 mbls. The 16S rDNA sequence of this fermenter cultivated in the iron reducing medium was a relative of *Propionibacterium acnes* within *Actinobacteria* at a similarity of 99% (Table 3). Sulfate reducing medium with yeast extract as an energy source yielded a fermentative isolate from 877 mbls; and its 16S rDNA sequence was related to an uncultured bacterium clone within *Actionbacteria* at a similarity of 99% (Table 3).

Positive iron reduction enrichments were obtained from two shallow depth intervals (464 and 545.2 mbls) (Table 2) with totally three strains isolated (Table 3). These strains exhibited two physiological characteristics with one producing brownish mineral precipitates during

Table 3. Summary of isolated strains.

Sample No.*	Medium type#	Closest sequence			
		Similarity (%)	Accession number	Phylogenetic affiliation	
				Group	Name of the closest sequence
TCDP464-IR-I1	IR	96	U23140	*δ-Proteobacteria*	*Pelobacter acetylenicus*
TCDP464-SR-comp-I1	SR-comp	98	AJ295679	*δ-Proteobacteria*	*Desulfovibrio* spp.
TCDP545.2SS-F-I1	F	94	AF407415	*Firmicutes*	Uncultured bacterium clone RB13C12
TCDP545.2SS-F-I2	F	91	AY188321	*Bacteriodes*	Uncultured bacterium clone KD6-12
TCDP545.2SS-F-I3	F	99	AY532555	*Firmicutes*	Uncultured bacterium clone 1013-1-CG43
TCDP545.2SS-F-I4	F	96	AY728067	*Firmicutes*	Uncultured bacterium clone S1-6-CL2
TCDP545.2SS-F-I7	F	94	AJ315953	*Actinobacteria*	*Propionibacterium propionicum*
TCDP545.2SS-F-I8	F	99	AY781385	*Firmicutes*	*Clostridium intestinale*
TCDP545.2SS-IR-I5	IR	99	AF016690	β–*Proteobacteria*	*Propionibacter pelophilus*
TCDP545.2SS-IR-I9	IR	95	X70955	δ-*Proteobacteria*	*Pelobacter acetylenicus*
TCDP545.6-IR-I3 &	IR	99	AY642054	*Actinobacteria*	*Propionibacterium acnes* WD1
TCDP887-SR-comp-I1 @	SR-comp	99	DQ336995	*Actinobacteria*	*Uncultured bacterium clone EV818CFSSAHH49*

* The number adjacent to TCDP in the column of "Sample No." represented the depth of the sample.

Media type for maintain the isolated stains: F: fermentation, IR: iron reduction, SR-comp: sulfate reduction with complex organic carbon as electron donors.

& The strain is not capable to reduce ferric iron.

@ The strain is not capable to reduce sulfate.

incubation and the other one not generating any mineral precipitate. Phylogenetic analyses of 16S rDNA indicated that only two different sequences in accordance with their physiological characteristics were obtained. One strain capable of generating mineral precipitates was related to *Propionibacter pelophilus* (Meijer et al. 1999) at a similarity of 99%, whereas two strains not producing mineral precipitates during incubation were affiliated with *Pelobacter acetylenicus* (Schink 1985) at a similarity of 95 - 96% (Table 3). Both strains also grew in the fermentation medium.

Organotrophic sulfate reduction using yeast extract and/or lactate was positively enriched in most samples (Table 2). The incubation temperature also varied from the mesophilic to thermophilic range. One strain was further isolated from the enrichment for the sample at 464 mbls. Its 16S rDNA sequence exhibited a very close affiliation (at a similarity of 99%) with *Desulfovibrio* spp. within δ-*Proteobacteria*. Acetate-utilizing and H_2-utilizing sulfate reducers were cultivable in some samples, but they grew slowly and showed lower cultivability than coexisted sulfate reducers using complex organic carbon as electron donors.

Methanogens were enriched from two depth intervals (545.2 and 694 mbls) (Table 2). The cultivability for these enrichments was much lower than the other metabolisms. It generally took more than 2 months to observe significant production of methane. During six months of cultivation, methanogenesis was not detected for samples at the deeper region.

5. DISCUSSION

5.1 Substrate Source for Viable Microbes

Our cultivation work indicated a ubiquitous presence of heterotrophic fermenters and sulfate reducers to the depth of 1451 mbls. Autotrophic sulfate reducers and methanogens, however, were limited to certain depth intervals. The fact that heterotrophic fermenters and sulfate reducers required organic carbon for energy yield metabolisms suggests that organic matters associated with sedimentary particulates during deposition provided a direct energy source for this microbial ecosystem. Fermenters degraded refractory carbon molecules into smaller carboxylic acids and H_2 for heterotrophic and autotrophic metabolisms, respectively (Jackson and Mclnerney 2002). Since the sedimentary strata here are marine sediments in their origins, sulfate reducers may reply upon seawater sulfate preserved within pore space. Alternatively, re-oxidation of pyrite or iron mono-sulfide to sulfate by episodic oxygen penetration associated with tectonic processes may provide another source for sulfate reduction. Sporadic presence of iron reduction relied on ferric oxyhydroxide or goethite coated on the surface of sediments (Fredrickson et al. 1998). The abundance of ferric iron-bearing minerals may be low or limited in a local scale to inhibit the prevalence of iron reduction. Two possible methanogenic pathways, including H_2-utilization and acetate fermentation may persist in samples investigated. The most likely source for H_2 and acetate in sedimentary environments is derived from the fermentation of organic carbons (Boone et al. 1989). H_2-utilization methanogenesis, however, requires additional bicarbonate as an electron acceptor (Zinder 1993). This bicarbonate can be either produced from dissolution of fossils or carbonate minerals

filled within fracture surfaces or the byproduct of fermentation. The lack of methanogenesis for samples at the deeper region may be due to its low activity in limited cultivation time or the depletion of H_2/acetate sources through termination of H_2/acetate-producing mechanisms or competition by other H_2/acetate utilizers.

The metabolic pathways inferred from cultivation results were generally consistent with those from 16S rDNA analyses of the environmental DNA extracted from the same set of samples. For the samples at depths shallower than 1300 mbls, a single OTU (operational taxonomic unit) with a sequence highly similar (99%) with *Janthinobacterium* spp. dominated over the other OTUs (at least 60%) (Chang 2005). Given the high similarity, this OTU may share the same physiological characteristics with that of *Janthinobacterium* spp., which is capable of heterotrophically fermenting complex organic carbon or reducing nitrate (Zhang et al. 2005). The 16S rDNA analysis, however, didn't detect any sequence belonging to the iron reducer group or sulfate reducer group, such as *Geobacter*, *Geothrix*, *Desulfuromonas,* and *Desulfovirbrio* within δ-*Proteobacteria*, *Thermodesulfobacterium,* and *Archaeoglobus*. Possible explanation for this is that viable iron reducers and sulfate reducers are minor in the whole community, and hence not detected through molecular screening. More detailed FISH (fluorescence in-situ hybridization) are warranted to verify whether the fermenters enriched in the culture experiments are related with those detected in the environmental DNA.

5.2 Switch between Iron Reduction and Fermentation

Isolated iron reducing strains possessed 16S rDNA sequences affiliated with *Propionibacter pelophilus* within β-*Proteobacteria* and *Pelobacter acetylenicus*. within δ-*Proteobacteria* (Table 3). Of these two closest strains, *Propionibacter pelophilus* is capable of fermenting sugars to propionate with tolerance to the exposure of oxygen (Meijer et al. 1999). No iron reduction and sulfate reduction has been reported for this strain. A number of *Pelobacter* strains were firstly isolated from fermentation of various organic substrates with production of propionate and acetate (Schink and Stieb 1983; Schink 1984; Schink 1985). Subsequent tests have shown that they are capable of reducing ferric iron (dissolved Fe-citrate or iron oxyhydroxide), elemental sulfur and/or sulfate with completely mineralizing organic carbon to carbon dioxide (Lonergan et al. 1996). The fact that our isolates could reduce dissolved ferric iron and ferment complex organic carbon indicates that these two strains could adjust their physiological capability upon encountering various environments. When the ferric iron is in absence, these two strains conserve energy through incomplete degradation of organic carbon to carboxylic acids and bicarbonate (Boone 1984). Upon the presence of ferric oxyhydroxides, carboxylic acids are completely transformed to carbon dioxide with the reduction of ferric iron (Lovley and Anderson 2000). Whether these two isolates possess more metabolic capacity by reducing other electron acceptors warrants further experimentation.

5.3 Correlation with Geological Parameters

Our enrichment results suggest that the type and diversity of cultivable metabolisms did not correlate well in the whole-core scale with lithology, depth (from 464 to 1451 mbls), and

the presence of the major fault zone. Limited correlations, however, were revealed for certain sample sets.

The variation of lithology is directly related to porosity and pore throat, and hence affects the transport property of substrate and microorganisms between lithological units (Colwell 2001). Mercury porosimetry analyses of TCDP retrieved cores indicated that the sandstone possessed porosities ranging between 13% and 16% and pore throats clustering at ~10 μm, whereas the siltstone and shale had porosities ranging between 3% and 6% and pore throats distributed at ~0.2 and ~0.08 μm, respectively (Lin et al. 2005b). The porosity and pore throat are independent from depths for the same lithology (Lin et al. 2005b). Since the porosity of sandstone is greater than those of siltstone and shale by a factor of 2 to 4, the diffusion flux of substrates in sandstone would be enhanced with the same magnitude ($J = -\phi D \partial C/\partial z$ where J is the diffusive flux, C is the concentration of solutes, D is the diffusion coefficient for a given solute, ϕ is effective porosity, and z is the distance from a reference point) (Lin et al. 2005a). It would be expected that microorganisms residing within sandstone could have access to a greater flux of aqueous substrate than those within siltstone or shale. Such a porosity effect was only observed in the sandstone-shale samples from 545.2 mbls. In sandstone, all tested metabolisms were positively enriched, whereas in shale only fermentation and organotrophic sulfate reduction (using complex organic carbon) enrichments were obtained (Table 2).

The increasing depth corresponds to increasing temperature along the geothermal gradient. Within the depth range investigated, the temperature is expected to increase from 25 to 50°C, a temperature range within which a transition from mesophilic to thermophilic communities could survive. While the cultivated metabolism primarily consisted of fermentation and heterotrophic sulfate reduction, the temperature obviously didn't stimulate or inhibit either of these two metabolisms. The appearance of other metabolisms we examined has no correlation with depth, but both iron reduction and methanogenesis cannot be enriched at the deeper region.

Fracture zones have been considered to act as a channel for fluid circulated either downward or upward (Haneberg et al. 1999). The substrate flux within the fracture zone would be greater than that in the matrix, supporting microbial populations at a high activity and/or diversity. More cultivated metabolisms derived from the thin layer of fault gauge than those from its host rocks at 690 mbls were consistent with this general consideration (Table 2). The diversity of cultivable metabolisms for samples adjacent to the Chelungpu fault zone located at depths ranging from 1100 to 1220 mbls (Hung et al. 2007; Song et al. 2007; Yeh et al. 2007), however, neither increased nor decreased significantly. It seemed to suggest that either the fluid circulation was limited within a relatively narrow range or the fault zone had no effect on enhancing the diversity of metabolisms. Instead of relying upon substrates accompanying fluid circulation, microbial communities acquired their substrates preserved within the pore space or associated with sedimentary particulates. Alternatively, the fluid brought in chemistry similar to pore water. Therefore, no additional type of electron donor or acceptor is provided to stimulate any specific metabolism for samples near the fracture zone.

5.4 Coexistence of Multiple Terminal Electron Accepting Processes

All the tested metabolisms were shown to be positive for the sample at 545.2 mbls. Iron

reduction and sulfate reduction coexisted in the sample at 464 mbls. Methanogenesis and sulfate reduction also coexisted in the fault gauge sample at 690 mbls (TCDP690FG). The concomitant presence of iron reducers, sulfate reducers and methanogen is contradictory to the conventional observation for shallow aquifers in that iron reduction, sulfate reduction, and methanogenesis developed successively along the groundwater transport pathway (Chapelle et al. 1995). Such a discrete metabolic and geochemical zonation resulted from the interplay of fermentation and terminal electron accepting processes (Lovley and Goodwin 1988). Because organic carbon is generally limited in shallow aquifer ecosystems in comparison with terminal electron acceptors, microorganisms with a certain metabolism have to maximize the utilization efficiency of bio-available organic acids (such as acetate) or H_2 produced by heterotrophic fermentation (McMahon and Chapelle 1991). The competition for organic acids or H_2, therefore, regulates the distribution of each terminal electron accepting process and geochemical characteristics along the groundwater flow path. Metabolic reaction with more free energy yield decreases organic acids or H_2 to a threshold level below which other metabolic reactions with less free energy yield would not be able to acquire enough substrate for minimum maintenance (Hoehler et al. 2001). Instead of being electron donor (presumably organic carbon) limited, the deep subsurface ecosystem might be limited by electron acceptors (Fredrickson and Onstott 2001). The stoichiometric excess of electron donor relative to electron acceptor would reverse the sequence of metabolism in the porous medium mentioned above (Coates and Achenbach 2002). While methanogenesis would be the dominant metabolism near the source of organic carbon, sulfate reduction and iron reduction zones would sequentially develop as the distance from carbon source increases (Smith 2002). This is because all electron acceptors, but bicarbonate, are depleted near the locus of organic carbon. Methanogenesis becomes advantageous over the other metabolisms. Such a reverse sequence of metabolic zonation, however, is still not consistent with the coexistence of multiple metabolisms for a given depth in our samples.

Possible explanations of the coexistence of multiple metabolisms here are twofold. The cultivated members only represented a small portion of the active population and might not necessarily be the dominant metabolism. Because the designed media were selective to certain metabolisms, any viable microorganism capable of utilizing the provided substrate could be enriched. Our results cannot conclusively infer that these cultivated members were numerically or metabolically dominant over the others, even the dominant member intuitionally could be easily cultivated. Therefore, the sequential appearance of various metabolisms shown in other subsurface ecosystems cannot possibly be evaluated with cultivation-based analyses only. The coexistence of multiple metabolisms observed here should not be interpreted further. Alternatively, the coexistence of multiple metabolisms may result from a microbial ecosystem without severe competition in nutrients. It's possible that microorganisms were universally distributed within the pore space that may not be interconnected to each other physically (Lin et al. 2005b). Each pore space harbored microorganisms capable of diverse metabolisms. Because electron donor is not a limiting factor, microorganisms trapped within the pore space would utilize any electron acceptor available within the pore space. Our current analysis doesn't allow for rejection of any speculation. Molecular screening of community structures based on the 16S rRNA gene variation and functional gene is warranted for future evaluation.

6. CONCLUSIONS

Sixteen cored samples retrieved by TCDP were analyzed with cultivation-based methods to reveal the metabolic diversity of microorganisms inhabiting a deep terrestrial subsurface environment composed of sedimentary strata with ongoing tectonic activities. The cultivation experiments indicated that mesophilic and thermophilic fermenters and organotrophic sulphate reducers (using complex organic carbon and acetate) dominated over the other investigated metabolisms and multiple terminal electron accepting processes coexisted at several depth intervals. The viable metabolic diversity was only correlated with lithology or fracture in some paired samples; however, such correlation cannot be extrapolated to the whole core. It appears that organic carbon associated with sedimentary particulates was accessible to but not a limiting factor to microorganisms. Instead, the availability of electron acceptors regulated the expression of terminal electron accepting processes within a potentially isolated niche (e.g., pore space). Unlike other deep terrestrial subsurface ecosystems (Fennoscandian Shield, Lidy Hot springs, and South African Witwatersrand Basin) where inorganically-derived H_2 plays an important role for autotrophic communities, the heterotrophic communities are fueled by photosynthetically-generated organic carbon stored in sedimentary strata in this ecosystem.

Acknowledgements This work was supported by NSC project grants to P. L. Wang, S. R. Song, H. T. Yu, and C. Y. Wang and by a distinguished postdoctoral fellowship to L. H. Lin. We also want to thank the TCDP drilling team and the sampling party for the assistance in field sampling, sample transportation and processing. Special thanks also to anonymous reviewers for providing considerable suggestions. This article is a TEC Contribution Number 00011.

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