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Microorganisms from deep, high temperature sandstones: constraints on microbial colonization

F.S. Colwell [a,*], T.C. Onstott [b], M.E. Delwiche [a], D. Chandler [c], J.K. Fredrickson [c], Q.-J. Yao [b], J.P. McKinley [c], D.R. Boone [d], R. Griffiths [e], T.J. Phelps [f], D. Ringelberg [g], D.C. White [g], L. LaFreniere [h], D. Balkwill [i], R.M. Lehman [a], J. Konisky [j], P.E. Long [c]

[a] *Biotechnologies Department, Idaho National Engineering Laboratory, P.O. Box 1625, Idaho Falls, ID 83415-2203, USA*
[b] *Department of Geology, Princeton University, Princeton, NJ 08544, USA*
[c] *Pacific Northwest Laboratories, Richland, WA 99352, USA*
[d] *Oregon Graduate Institute, 19600 Northwest von Newmann Drive, Beaverton, OR 97006-1999, USA*
[e] *Department of Forest Science, Forest Science Lab 020, Oregon State University, Corvallis, OR 97331, USA*
[f] *Oak Ridge National Laboratory, P.O. Box 2008, Oak Ridge, TN 37831-6036, USA*
[g] *Center for Environmental Biotechnology, University of Tennessee, 10515 Research Drive, Suite 300, Knoxville, TN 37932, USA*
[h] *Argonne National Laboratory, 9700 S. Cass Avenue, ER 203, Argonne, IL 60439, USA*
[i] *Florida State University, Department of Biological Science, 312 Nuclear Research Building, Tallahassee, FL 32306, USA*
[j] *Department of Microbiology, University of Illinois, 407 S. Goodwin, Urbana, IL 61801, USA*

Abstract

Cores were collected from Late Cretaceous and Early Tertiary rocks in the Piceance Basin of western Colorado, USA, to investigate the origins of subsurface microorganisms under geological conditions likely to constrain microbial transport and survival. The sampled strata from 856–862, 1996–1997 and 2091–2096 m recorded peak paleotemperatures of 120–145°C from 40–5 million years ago, while present temperatures range from 43 to 85°C. Cores were analyzed for culturable anaerobic bacteria (Fe(III)- and Mn(IV)-reducing bacteria, fermenters, sulfate reducers, nitrate reducers and methanogens), ester-linked phospholipid fatty acid and selected enzyme and physiological activities. Measurable but low biomass (total phospholipid fatty acid) and anaerobic bacteria, primarily Fe(III) reducers and fermenters, were present in samples from the 856–862 m core. Cores from greater depths yielded only a single positive enrichment and lower biomass values. Methanogens and sulfate reducers were not detected in any of the samples nor were bacteria that could grow with methane and any added electron acceptors. 16S rRNA genes cloned from products of PCR amplification of DNA extracted from an 858 m, 65°C, Fe(III)-reducing enrichment were most closely related to bacteria in the genus *Desulfotomaculum*, Gram-positive, spore-forming sulfate-reducing bacteria. Assuming the maximum temperatures would have eliminated any entrained bacteria, these anaerobic microorganisms likely migrated into the shallower Wasatch formation within the last 5 million years. However, the deepest stratum sampled was hydrologically isolated and lacked any indication of microbial colonization by all biological measures. Hydrologic connection to the surface, high maximum temperatures and the presence of fractures are probably the primary factors that control distribution of microorganisms in these deep rock environments.

Keywords: Subsurface; Microbial survival; Microbial transport; Sandstone; Iron-reducing bacteria; Fermenter

* Corresponding author. Tel.: +1 (208) 526-0097; Fax: +1 (208) 526-0828; e-mail: fxc@inel.gov

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1. Introduction

Recent microbial investigations of the terrestrial subsurface have revealed the presence of diverse and viable microbial communities at considerable depth [1–3]. To inhabit the most remote subsurface environments, microorganisms must survive burial or transport from the surface. The limits of long-term survival and transport in porous or fractured media are key aspects of subsurface microbial ecology, the knowledge of which will assist the use of microorganisms in technologies such as remediation of aquifers and recovery of fuel and mineral resources. If the hydrologic and geologic properties of the environment are adequately understood then it should be possible to predict the distribution and physiologies of microorganisms at depth and the mechanisms involved in their colonization.

To investigate subsurface microbial populations under geologic conditions that constrain microbial transport and survival, cores were collected from 73–35 million year old Late Cretaceous and Early Tertiary rocks as deep as 2100 m below ground surface in the Piceance Basin of western Colorado, USA. The fluvial sandstones and shales that were sampled were deeply buried between 35 and 5 million years ago (Ma) when formation temperatures peaked between 120 and 145°C [4]. 5 Ma, the formation cooled to temperatures more permissible for microbial colonization and survival. Thus, it was reasoned that the presence of microorganisms in these rocks in the context of the past and present thermal regime would constrain the limits of microbial colonization of deep rock formations. This article describes the microbial biomass and viable anaerobic microorganisms, primarily Fe(III) reducers and fermenters, associated with intact cores taken from these deep sedimentary rocks.

2. Materials and methods

2.1. Sample site

The drill site was located about 10 km west of Rifle, Colorado, USA (39°30′ N latitude, 107°55′ W longitude). The targeted formations, the Molina Member in the Early Tertiary Wasatch Formation and the fluvial member of the Williams Fork Formation in the Late Cretaceous Mesaverde Group, contain sandstones interbedded with lower permeability shales. Sandstone lenses rich in methane and the encapsulating shales were deemed likely zones for microbial presence.

2.2. Sample collection

Cores were collected to establish the characteristics

of the indigenous microbial communities in the host rock. The downhole geophysical and drilling mud logs from a neighboring well were utilized to provide stratigraphic, methane and temperature data needed to select core points for the borehole sampled as part of this study. To minimize contamination of the core from drilling fluids or drilling tools, mud-rotary coring techniques and a hydro-lift core sampler (Baker-Hughes, Inc., Houston, TX) were used to encase the core in polypropylene glycol gel during retrieval of the core. Sample quality was determined from naturally occurring microbial tracers in the drilling muds [5]. Tool disinfection and sample handling procedures were adapted from previous coring efforts [6]. Cores were subsampled in an Ar-filled glove bag, refrigerated or frozen depending on analytical requirements and then distributed by overnight courier to various laboratories.

2.3. *Chemical and physical characterization of the core*

Formation gases in the drilling muds were determined on-site by Rocky Mountain Geo-Engineering Corp. (Grand Junction, CO, USA) using gas chromatography with a flame ionization detector. In situ temperatures and percent water saturation were determined by using downhole temperature and geophysical probes (Atlas Wireline Corp., Denver, CO, USA). Petrophysical characterization of the cores (e.g. helium porosities and air permeabilities) was conducted by Core Labs (Casper, WY, USA). Mercury injection porosimetry was used to determine pore throat dimensions (Core Petrophysics Inc., Houston, TX, USA).

2.4. *Microbial analyses*

Samples were analyzed for aerobic heterotrophic bacteria by plating onto PTYG, 1% PTYG and 5% TSA [7]. Several approaches were used for enrichment of anaerobic bacteria at incubation temperatures ranging from 45 to 85°C. Samples were maintained under anaerobic conditions, aseptically crushed and inoculated (2:1 (w/w) solution:solid) into media suited to grow Fe(III) and Mn(IV) reducers, sulfate reducers, and nitrate reducers [8], except that the enrichments lacked added electron donors. The absence of added electron donors was intended to encourage microbial growth on native electron donors in the rock. Native electron donors might include organics from shale and organic inclusions, H_2, or short chain alkanes. A duplicate series of these enrichments was prepared by purging the tubes with methane, a potential electron donor that may have been lost during core handling.

Another set of enrichments for anaerobic bacteria included electron donors. MS enrichment medium [9] was used, except peptones and yeast extract were each added at 0.5 g l^{-1} and mercaptoethanesulfonate was omitted. The MS medium:rock slurry (1:1 (w/w)) was shaken and used to inoculate seven different modifications of MS enrichment medium, in triplicate. Media for specific metabolic groups included the following substrates: 10 mM trimethylamine plus 10 mM acetate (methylotrophic methanogens); 1 atm H_2 and 10 mM formate (methanogens and acetogens); 10 mM lactate, 5 mM sulfate and 3 mM ferrous sulfate (sulfate reducers); 10 mM lactate and 5 mM sulfur (sulfur reducers); 20 mM lactate and 15 mM of MnO_2 (Mn(IV) reducers) [10]; 20 mM lactate and 20 mM ferric hydroxide (Fe(III) reducers) [10]. For fermentative bacteria, one set of enrichments used 1 g l^{-1} each of glucose, sucrose, fructose, and cellobiose as the electron donors [9] and another set of enrichments used 0.5 g l^{-1} each of glucose, casamino acids, pyruvate, acetate and formate and 1.7 g l^{-1} of lactate [8]. Methanogens were enriched at salinities ranging from 0.9 to 8% NaCl and pH values ranging from 5.5 to 7.4 [11].

Cell growth in the enrichments was assessed by direct microscopic observation [8]. For some methanogenic enrichments, occurrence of methane in the headspace of the culture vessel was determined by using gas chromatography.

Anaerobic enzyme and cell activities were determined at 24°C with methods developed for aquatic environments [12,13] and previous investigations of microbial communities in subsurface environments [14]. Ester-linked PLFA was used to determine microbial community biomass in the cores [15].

2.5. *16S rRNA gene sequencing and analyses*

The types of microorganisms that grew in a 65°C, Fe(III)-reducing enrichment from 858 m were deter-

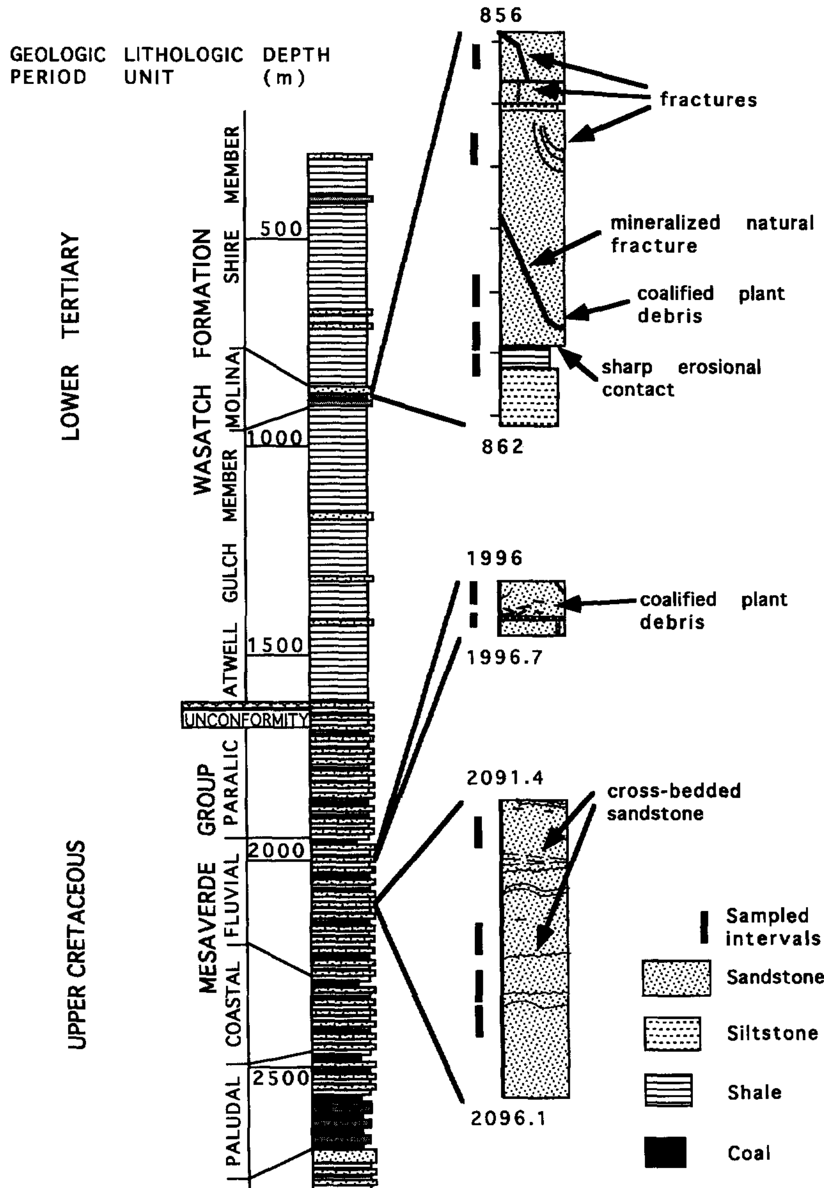

Fig. 1. Location and lithology of samples from the deep borehole in the Piceance Basin.

mined by cloning the 16S rRNA genes from products of polymerase chain reaction (PCR) amplification of DNA extracted from the enrichment [16]. One microliter aliquots of undiluted or diluted nucleic acid extract were used as templates for PCR. Final PCR conditions were 10 mM Tris pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 200 μM of each dNTP, 0.2 μM of each primer and 1.25 units of LD-Taq polymerase (Perkin Elmer, Foster City, CA) in 50 μl total volume in 0.2 ml thin-walled tubes. The primers, 7f (a universal eubacterial primer): 5′-cua-cua-cua-cua-AGA-GTT-TGA-TCN-TGG-CTC-AG and Geo840r (a primer with known homology to *Desulfotomaculum* spp.): 5′-cau-cau-cau-cau-TAC-CCG-CRA-CAC-CTA-GT, were synthesized with 12 bp tails (lower case) to allow for directional clon-

ing with the CloneAmp system (Life Technologies, Gaithersburg, MD, USA). The template DNA, primers, $MgCl_2$ and water were preheated to 80°C (hot-start) before the final reaction components were added. A Perkin Elmer 9600 thermal cycler was used with cycling conditions of 5 cycles (94°C for 40 s, 60°C for 10 s, 72°C for 75 s) followed by 40 cycles (94°C for 12 s, 65°C for 10 s, 72°C for 80 s with a 2 s extension per cycle). A final extension at 72°C was performed for 20 min before the reactions were chilled to 4°C. PCR controls included no template, 1 μl of a 'blank' extract that was treated the same as enrichments, and 250 fg *Geobacter* GS-15 genomic DNA. Three replicate PCR reactions were performed on the undiluted and 1:10 diluted enrichment extracts. Subsequently, PCR products were purified and cloned, the cloned inserts reamplified and restriction fragment length polymorphism (RFLP) analysis was used to group like clones together [16].

One to two clones from each unique RFLP group were entirely sequenced. Plasmid DNA was isolated by a modified alkaline lysis/polyethylene glycol precipitation procedure and sequenced with DyePrimer (−21M13) chemistry and a cycle-sequencing protocol, all according to the manufacturer's instructions (Perkin Elmer). Sequences were resolved on 6% acrylamide gels run on an Applied Biosystems 377 sequencer (Perkin Elmer), using the gel-running service provided by Iowa State University (Ames, IA, USA). Full-length sequences were manually aligned to Fe(III) and sulfate reducer sequences in the Ribosomal Database Project (RDP) [17] to determine the relationship of cloned 16S rRNA molecules to those of known taxa. Phylogenetic trees were generated from conserved sequence positions (~700 bp total) using the PHYLIP package (version 3.5) available through the RDP. Parsimony analysis was performed using PAUP (version 3.1).

3. Results

3.1. Core characteristics

A single core in the Wasatch Formation (Paleocene-Eocene) and two cores in the Mesaverde Group (upper Cretaceous) were recovered in three coring efforts within the same borehole (Fig. 1). The shallowest core (856–862 m) consisted of well-cemented sandstone occasionally cross-bedded and interbedded with siltstones and shales. The core encountered two near-vertical, calcite-filled fractures. The porosity ranged from 1 to 12% and the permeability varied from < 0.001 to about 1 mD, with higher permeabilities likely in the fractures (Fig. 2). Maximum pore throat dimension approached 1.0 μm; however, most ranged between 0.01 and 0.1 μm. More than 2000 ppm methane was present in the strata adjacent to this core and the concentration of water in this interval was inversely related to the methane concentration (Fig. 2).

The second core, from the upper Mesaverde group (1996.4–1997 m), consisted of well-cemented, medium-grain sandstone, stylolitic organic laminations and coalified plant debris. The core encountered one near-vertical, quartz-filled fracture. The porosity was 5–6%, the air permeability was 0.02 mD, and the median pore throat dimension was 0.77 μm (maximum: 3 μm). Methane concentrations over this interval ranged from 7700 to 23 100 ppm. The deepest core (2091–2096 m) consisted of massive, unfractured sandstone with occasional cross-bedding and

Table 1
Natural microbial tracers for Piceance Basin cores, hydrolift gel and drilling muds

Sample type	CLPP (substrates used)	PLFA (pmol g^{-1})	Aerobic mesophiles (viable cells ml^{-1} or g^{-1})
Cores	0–12	≤10	BD
Gel	ND	ND	5.5×10^3
Drilling mud	ca. 90	> 3000	6×10^7

BD = below detection. BD for aerobic mesophiles is < 0.3 culturable cells ml^{-1} or g^{-1}.
ND = not determined.
CLPP = community level physiological profile, which uses a tetrazolium dye reduction assay (Biolog Inc., Hayward, CA, USA) to determine whole community use of 95 different carbon sources [5]. The sample from 858 m respired 12 of the 95 carbon sources provided. All other cores yielded no response (no measurable respiration) of any of the carbon sources.

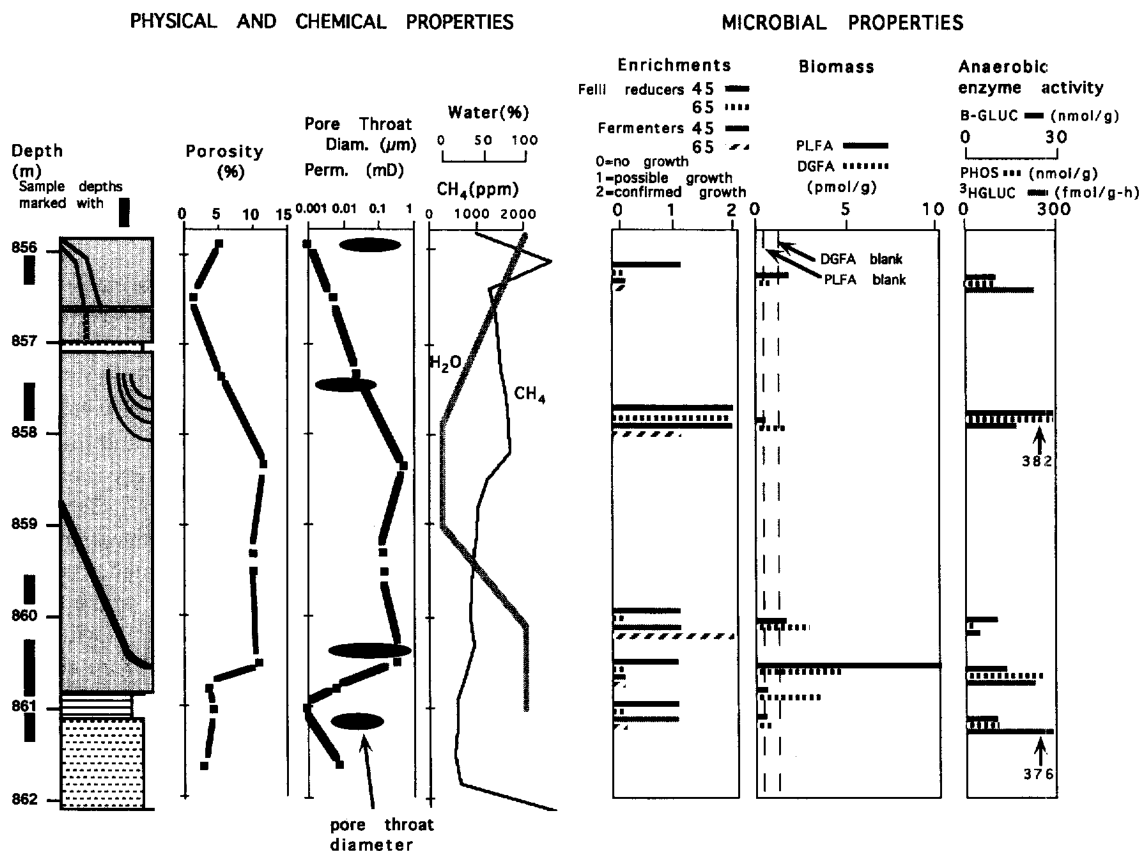

Fig. 2. Summary of abiotic and biotic properties along the vertical axis of the shallowest core obtained from the Piceance Basin. Only enrichments that showed some positive results are shown. DGFA = diglyceride fatty acid; PLFA = phospholipid fatty acid; B-GLUC = concentration of product (methylumbelliferon) after 5-day incubation as a measure of β-glucosidase activity; PHOS = concentration of product (methylumbelliferon) after 5-day incubation as a measure of phosphatase activity; ^{3}HGLUC = tritiated glucose uptake rates.

organic stylolites. The porosities were 6–7%, the air permeabilities ranged from 0.03 to 0.05 mD and the median pore throat dimension was 0.11 μm (maximum: 2 μm). Methane concentrations in this interval ranged from 751 800 to 983 500 ppm.

3.2. Microbiological quality of the samples

Indigenous tracers indicated that microbial biomass, cell numbers of specific physiological types and community functional characteristics of the cores were markedly different from those in the circulating drilling muds (Table 1). This indicated that the cores were minimally impacted during coring, retrieval and subsequent subsampling. The total PLFA in the samples was 300–10 000-fold lower than in the drilling muds. Aerobic, mesophilic heterotrophs were enumerated at levels up to 6×10^{7} ml^{-1}, yet no such microorganisms were cultured from the cores. This assay indicates a 10^{7}-fold decrease between the concentration of cells in the drilling fluids and those in the cores. Further, pre-combusted cores that were processed as authentic cores and then distributed to investigators showed no measurable biomass and yielded no positive enrichments.

3.3. Microbiological results

The shallowest core (856–862.2 m) yielded samples with significant PLFA concentrations. The highest PLFA concentration (10.1 pmol PLFA g^{-1}) was

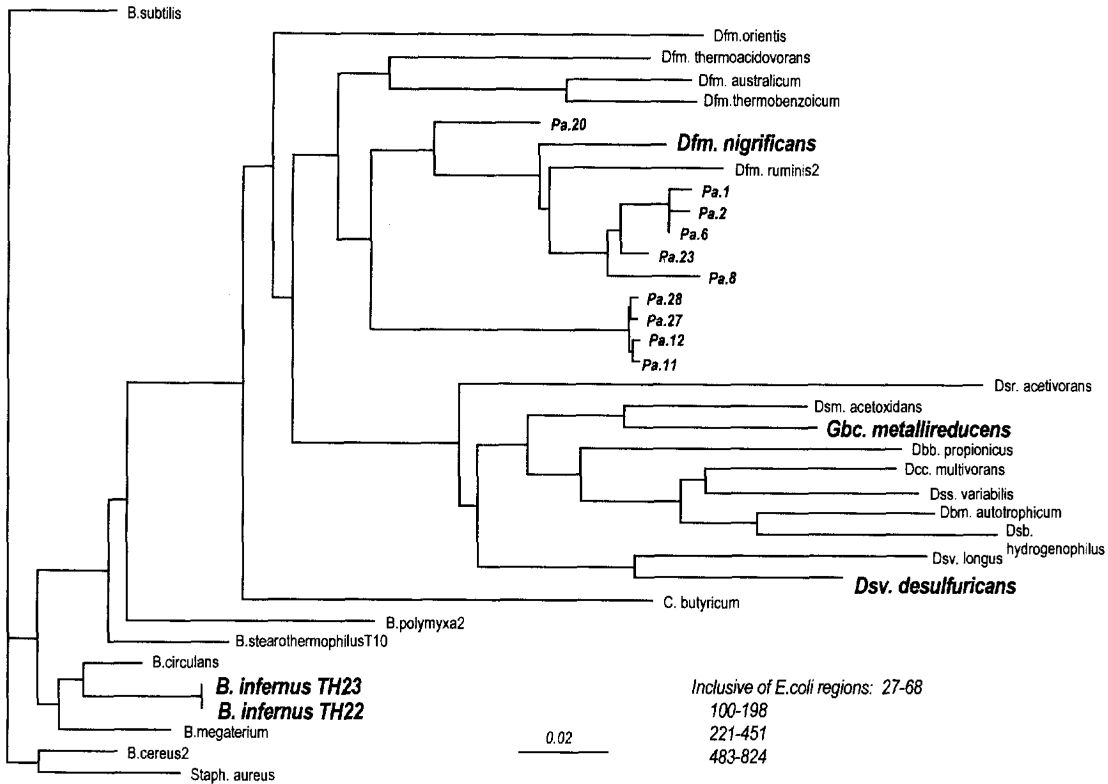

Fig. 3. Phylogenetic tree showing the relationship of microorganisms derived from the 858 m Fe(III) reducer enrichment (designated: Pa.#) to known Fe(III) reducers and sulfate reducers. Relationships are based on comparisons of 16S rRNA sequences obtained from the enrichment to those in the RDP. B = *Bacillus*; Dfm = *Desulfotomaculum*; Dsr = *Desulfurella*; Dsm = *Desulfuromonas*; Gbc = *Geobacter*; Dbb = *Desulfobulbus*; Dcc = *Desulfococcus*; Dss = *Desulfosarcina*; Dbm = *Desulfobacterium*; Dsb = *Desulfobacter*; Dsv = *Desulfovibrio*; C = *Clostridium*; Staph = *Staphylococcus*.

measured in the sample collected from 860 m (Fig. 2). This sample was located adjacent to a natural fracture. Samples collected from near the 1996 m depth also had PLFA concentrations above background levels (0.4 pmol g^{-1}) but samples from the deepest core did not have measurable PLFA. Many of the samples had PLFA values that were at the detection limit of the assay. Most samples contained diglyceride fatty acids (DGFA) as well and at total concentrations above background levels. DGFA are assumed to be a measure of dead cell abundance as they result from the dephosphorylation of phospholipids upon cell lysis.

Overall, cell growth was not detected in > 97% of the enrichments. The deepest cores yielded no growth for all of the enrichments attempted. Methanogens and any microorganisms (aerobic or anaerobic) that were capable of using methane as an electron donor were not enriched from any of the samples. Methanogens were able of growing, however, in slurries composed of the cores indicating that there was nothing inherently toxic about the samples to such organisms. Confirmed growth occurred in enrichments for Fe(III) reducers (both 45 and 65°C incubations of 858 m core, 45°C incubations of 1996 m core), for fermenters (65°C incubation of 860 m core) (Fig. 2), and for Mn(IV) reducers (37°C incubation of 1996 m core). The enrichments that were positive for Fe(III) reducers used electron donors that were native to the parent rock.

Anaerobic enzyme activities were generally higher in the shallowest core (Fig. 2) than in the two deeper cores. Phosphatase, β-glucosidase and [^{3}H]glucose uptake activities in the two deeper cores ranged

from 1 to 160 nmol product generated g^{-1}, 3.16 to 18.52 nmol product generated g^{-1} and 0 to 102.1 fmol g^{-1} h^{-1}, respectively. While these values are all lower than the maximum values reported for the shallower core (Fig. 2), the combusted control core showed phosphatase and [^{3}H]glucose uptake activities of 189 nmol product generated g^{-1} and 91.4 fmol g^{-1} h^{-1}, respectively. These relatively high values for the combusted core suggest abiotic activity or low levels of contamination.

Characterization of 16S rRNA sequences derived from microorganisms in the 65°C, Fe(III)-reducing enrichment of the sample from 858 m, indicated that the nearest phylogenetically related organisms in the RDP were Gram-positive, spore-forming, sulfate-reducing bacteria in the genus *Desulfotomaculum* (Fig. 3). The microorganisms from this enrichment generally grouped together amongst low G+C Gram-positive bacteria, suggesting a relatedness to one another. The sequences from the enrichments were distinct from that of the metal-reducing bacterium *Geobacter metallireducens* and from *Bacillus infernus*, a thermophilic, strictly anaerobic metal-reducing bacterium isolated from the deep terrestrial subsurface.

4. Discussion

Microbial biomass, viability and activities were detected in select samples from the deep sandstone strata of the Piceance Basin, although for most of the cores, the measurements were below or close to the limits of detection. The levels of biomass and viable bacteria were comparable to those obtained in previous subsurface investigations (Table 2) and consistent with such a constrained environment in terms of space and nutrient flux. The low concentrations of PLFA in the samples were comparable to values obtained for cores from other sampling sites including the Taylorsville Triassic Basin in Virginia [5], the Ringold Formation near Richland, WA [23] and the Upper Colorado River Basin west of Albuquerque, NM [14].

The microorganisms enriched from these samples are consistent with the chemical and physical conditions of the habitat. That anaerobic bacteria, in this case metal reducers and fermenters, were cultivated from the samples is in agreement with the results of prior studies of deep terrestrial sedimentary deposits [10,23,24] and other deep anaerobic environments [1–3] (see Table 2). In most of these environments,

Table 2
Summary of results of selected investigations of the microbiology of deep subsurface environments

Location; geological medium	Depth (m)	Sample type	Microbiological results		References
			Numbers or biomass	Types of microorganisms present	
North Sea, North Slope; oil reservoir	3000	production fluids	10^1–10^4 viable cells ml^{-1}	hyperthermophilic archaea, sulphidogens	[3]
Virginia, USA; sandstones, shales	2800	core	16 pmol PLFA g^{-1}	Fe(III) reducers, sulfate reducers, anaerobic autotrophs and heterotrophs	[5,10,18]
Colorado, USA; sandstones, shales	860 and 1996	core	< 12 pmol PLFA g^{-1}	Fe(III) reducers, fermenters, possible sulfate reducers	this study
Paris Basin, France; oil reservoir	1670	production fluids	ND[a]	hyperthermophilic archaea, thermophiles	[1]
Washington, USA; basalts	316 and 1270	groundwater	10^3–10^5 total cells ml^{-1}	H_2-based autotrophic community, methanogens, Fe(III) reducers, sulfate reducers	[2,19]
Äpsö, Sweden; granite	≤1240	groundwater, core	10^3–10^7 total cells ml^{-1}	Fe(III) reducers, sulfate reducers, methanogens, aceticlastic methanogens, acetogens	[20]
Japan Sea; marine sediments	≤518	core	10^6–10^8 total cells cm^{-3}	methanogens, sulfate reducers, anaerobic heterotrophs	[21,22]
Washington, USA; lacustrine sediments	173–185	core	≤45 pmol PLFA g^{-1} or 10^5–10^7 total cells g^{-1}	fermenters, Fe(III) reducers, sulfate reducers	[8,23]

[a]ND = not determined.

recharge from the surface and hydrologic flux is very slow thus, the flux of soluble nutrients would be extremely limited. Any low-level metabolism of indigenous bacteria would rely upon endogenous electron acceptors such as sulfate, Fe(III) and organic compounds which can be fermented [23,24]. The apparent absence of methanogenic bacteria is consistent with carbon isotope data that indicate a thermogenic origin of the methane in this formation [25].

The 16S rRNA genes that were cloned from DNA extracted from the Fe(III) reducer enrichment of the 858 m sample indicate that the bacteria were closely related to known anaerobic bacteria (Fig. 3). Although the enrichment may contain other microorganisms whose sequences were not amplified by the primers that were used, those which were amplified most closely resemble *Desulfotomaculum nigrificans*, a Gram-positive, spore-forming, sulfate reducer. While it has not been determined whether the putative Fe(III)-reducing bacteria in the enrichment can reduce sulfate, many metal reducers have diverse metabolic strategies for deriving energy [10,26]. Furthermore, some sulfate reducers are capable of Fe(III) reduction [27] and sometimes derive energy for growth from this process. There would be selective advantages for microorganisms with metabolic diversity to survive with limited electron donors and acceptors [23,24].

Microbial biomass, activities and culturable cells decreased from the shallowest to the deepest intervals sampled. This suggests that the constraints on microbial colonization of and survival in the deep strata in the Piceance Basin are considerable. For the shallowest to deepest cores, the estimated in situ present temperatures were 43, 81 and 85°C and maximum paleogeothermal temperatures were 120, 140 and 145°C [28], respectively. The highest, generally accepted maximum temperature for growth of microorganisms is 110°C [29]. While in some cases, high hydrostatic pressures such as those at depth in the Piceance Basin can increase the upper temperature limits of microbial growth [30], this is not universal [31]. The endospores of thermophilic bacteria that are especially tolerant to wet heat are only able to survive for minutes at 121°C [32]. Thus, the maximum paleogeothermal temperatures probably killed any extant microorganisms even in the shallowest (856 m) core. Subsequently, the movement of relatively young, meteoric water into the Wasatch formation [28] through the fracture network in the sandstones [33] would have allowed recolonization by microorganisms. The cores from the deeper Mesaverde Group, especially the core from 2091–2096 m, existed in a zone of high methane concentration and the water in these strata is older [28]. The trapped methane and ancient water, together with the present day high temperatures, suggest that such zones represent a barrier to microbial colonization of the formation.

The shallowest core interval contained the highest biomass and yielded positive enrichments from samples at 858 m and 860 m which were proximal to fractures and within relatively high permeability (up to about 1 mD), high porosity (up to 12%) sandstone (Fig. 2). Pore throat diameters approached 1.0 μm near the 860 m sample, dimensions that would allow the entry of cells or spores that are likely to be small due to environmental stress [34]. The permeabilities (0.1–1.0 mD) are within the range of rock permeabilities that have been shown to allow bacterial penetration [35]. These strata that contain methane and water conducting fractures are likely the zones that would yield microbial biomass including viable cells or spores. In contrast, shale samples from approximately 861 m where porosity was $< 5\%$ and permeability was < 0.001 mD yielded no detectable biomass or positive enrichments, although they did have a high [^{3}H]glucose uptake rate.

In summary, this study confirms the existence of living microorganisms at great depths in the terrestrial subsurface. Collection and analysis of cores from a well-studied subsurface environment allows consideration of the origin of these microorganisms in the context of regional and core-scale processes which effect the distribution and survival of microorganisms. Microorganisms are sparse in the deep rocks of the Piceance Basin, perhaps more so than in other subsurface habitats. The absence of microorganisms in the deepest strata likely reflects past geothermal conditions which were hot enough to eliminate viable organisms. The recent, gradual cooling has allowed colonization by cell or spore migration through fractured horizons. Furthermore, the current high temperatures in the deepest strata sampled, the lack of access to and replenishment of nutrients, electron donors and electron acceptors as

well as physical barriers such as low permeability and limited pore space, all limit cell transport and survival. Metal-reducing bacteria were among the few types of microorganisms that were enriched from these samples. As a group, these cells often exhibit versatile physiologies that are well-suited for dealing with the constraints of survival in these harsh habitats.

Acknowledgments

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