Geochemical Influence on Diversity and Microbial Processes in High Temperature Oil Reservoirs

V. J. ORPHAN
S. K. GOFFREDI
E. F. DELONG
Monterey Bay Aquarium Research Institute
Moss Landing, California, USA

J. R. BOLES
Department of Geological Sciences
University of California, Santa Barbara
Santa Barbara, California, USA

The diversity of thermophilic microbial assemblages detected within two neighboring high temperature petroleum formations was shown to closely parallel the different geochemical regimes existing in each. A high percentage of archaeal 16S rRNA gene sequences, related to thermophilic acetoclastic and hydrogenotrophic methanogens, were detected in the natural gas producing Rincon Formation. In contrast, 16S rRNA gene libraries from the highly sulfidogenic Monterey Formation contained primarily sulfur-utilizing and fermentative archaea and bacteria. In addition to the variations in microbial community structure, microbial activities measured in microcosm experiments using high temperature production fluids from oil-bearing formations also demonstrated fundamental differences in the terminal respiratory and redox processes. Provided with the same suite of basic energy substrates, production fluids from the South Elwood Rincon Formation actively generated methane, while thermophilic microflora within the Monterey production fluids were dominated by hydrogen sulfide producing microorganisms. In both cases, molecular hydrogen appeared to play a central role in the stimulation of carbon and sulfur cycling in these systems. In methanogenic production fluids, the addition of sulfur compounds induced a rapid shift in the terminal electron accepting process, stimulating hydrogen sulfide formation and illustrating the metabolic versatility of the subsurface thermophilic assemblage. The high similarity between microbial community structure and activity corresponding with the prevalent geochemical conditions observed in deep subsurface petroleum reservoirs suggests that the resident microflora have adapted to the subsurface physicochemical conditions and may actively influence the geochemical environment in situ.

Keywords methanogenesis, petroleum, subsurface, thermophile

Received 20 February 2002; accepted 7 April 2003.

We thank Peter Eichholtz and Steve Franks for generously sharing their unpublished work with us. We also are indebted to Prentice Patterson and the crew from Platform Holly (Mobil and Venoco Inc), Trent Taylor, Christa Schleper, and Chris Preston for assistance with sample collection and Jan Childress for technical advice and use of his analytical equipment. V. J. Orphan is currently supported by a National Research Council Fellowship at NASA Ames Research Center.

Address correspondence to Victoria Orphan, NASA Ames Research Center, Mail Stop 239-4, Moffett Field, California 94035, USA. E-mail: vorphan@mail.arc.nasa.gov
Introduction

Deep subsurface petroleum reservoirs are unique extreme environments characterized by high temperatures and pressures and widely varied geochemical conditions, typically associated with the depositional environment, source rock, and oil maturity. Previously thought to be too extreme to support life, studies over the last 10–15 years have now demonstrated the existence of a wide range of metabolically diverse hyperthermophilic microorganisms from high temperature petroleum reservoirs worldwide. Some of the most commonly cultured functional groups include sulfate and sulfur-reducing, Fe(III)-reducing, fermentative, and methanogenic microorganisms, with many of the thermophilic isolates capable of using multiple energy sources and electron acceptors (Ng et al. 1989; Stetter et al. 1993; Grassia et al. 1996; Greene et al. 1997; Slobodkin et al. 1999; Orphan et al. 2000). Like most anaerobic environments, the terminal mineralization processes within petroleum reservoirs appear to be driven by sulfide- and methane-producing microorganisms (Davydova-Charakhch’yan et al. 1993). The net production of these end products is largely influenced by the availability and concentration of energy sources, as well as by competitive or cooperative interactions between different microbial groups, such as sulfate-reducing bacteria and methanogenic Archaea (Holmer and Kristensen 1994).

Although diverse thermophilic microbes have been successfully cultured from both onshore and offshore high temperature stratal fluids, there still remains much uncertainty as to the level of microbial activity and the extent to which environmental conditions influence the naturally occurring diversity and community function in situ. Most deep subsurface microbial communities are thought to exist in a starvation state and, specifically within deep-seated oil reservoirs, fluids are thought to lack essential nutrients for growth. Microbial activity within these deep reservoirs has been typically attributed to either human disturbance from drilling, or the introduction of exogenous substrates through secondary production methods such as water flooding (Rozanova et al. 1993, 1997; Takahata et al. 2000, 2001). In addition, there exists much controversy as to the origin of thermophilic microbial assemblages in these geothermally heated petroleum reservoirs. Previous evidence supports both the existence of an indigenous deep subsurface biosphere (Ng et al. 1989; L’Haridon et al. 1995) as well as the notion that oil reservoir thermophiles, closely related to known hydrothermal vent genera, must have been introduced via human disturbance or natural conduits from the surface (Stetter et al. 1993).

To specifically examine the relationships between diversity, community structure, and geochemistry within these subsurface systems, we combined 16S rRNA gene surveys and domain-level rRNA quantification with measurements of metabolic potential (terminal end product production) in resident thermophilic assemblages from two geochemically distinct, high temperature oil-bearing formations. Although similar culturable groups of thermophiles are frequently recovered from California petroleum reservoirs and oil reservoirs worldwide, our findings suggest that the community structure of the free-living assemblage characterized with molecular methods and the metabolic potential of the resident community may differ dramatically. Compared with enrichment culture results reported earlier (Orphan et al. 2000), the culture-independent microbial diversity surveys and microcosm studies in this study were found to more closely reflect the dominant physicochemical conditions measured in the respective reservoirs.

Materials and Methods

Sample Collection and Preparation

The South Elwood oil field (34°23.4’N, 119°54.3’W), located 2 miles offshore Santa Barbara, California, produces from two geochemically distinct formations, the upper
Miocene Monterey Formation and the lower Miocene Rincon formation at an average production depth of 1300 m and 1500 m below the seafloor, respectively. The North Coles Levee (35°27'N, 119°33'E) and Yowlyume (35°04'N, 119°19'E) oil fields are situated in the San Joaquin Basin, California, with average production depths of 2,700 m and 3,400 m below ground level from the middle Miocene Monterey Formation. Detailed physicochemical characterization for each of the reservoirs has been previously reported (Fisher and Boles 1990; Orphan et al. 2000; Franks et al. 2001).

South Elwood field Rincon and Monterey production fluids (~30 l) were collected directly from the wellheads (well 3120-4 and well 3120-9, Platform Holly) in sterile Nalgene carboys on 5/28/97 and 6/19/97. Samples from North Coles Levee (N.C.L.) and Yowlyume fields Stevens Formation (wells 31-34 and 61x-3) were collected from onshore production wellheads on 2/11/98 and 3/6/98. Bulk samples were transferred within 6 h to the laboratory and subsampled for microcosm experiments, enrichment cultures, cell counts, and nucleic acid extractions.

**Microcosm Preparation**

Serum vials (120 ml) were filled with 45 ml untreated production fluids (oil and water) from either the Rincon (well 3120-4) or Monterey Formation (well 3120-9). Vials were then sealed with butyl rubber stoppers and flushed with nitrogen gas for 2 min before addition of substrates and headspace gas. Supplements to microcosm vials included H₂/CO₂, N₂/CO₂ (headspace pressurized to 10 psi), acetate (7.35 mM), elemental sulfur (0.5% w/v), and thiosulfate (20 mM). Microbial activity was considered positive if methane or sulfide production was above control levels, either without substrate additions or fixed with formalin.

**Chemical Analysis**

Four production wells, having temperatures in the range of 70–120°C, were selected for this study based on their unique geochemistries and preliminary evidence of viable thermophilic populations (Orphan et al. 2000). Sulfate and pH in formation water samples were measured according to Orphan et al. (2000). Methane, hydrogen sulfide, carbon dioxide, oxygen, and nitrogen were measured in headspace and liquid subsamples using a model 5890 Hewlett Packard gas chromatograph (Childress et al. 1984; Orphan et al. 2000). Unless otherwise noted, chemical concentrations in the headspace are given.

**16S rRNA Library Construction and Analysis**

Rincon Formation waters, sampled in May 1997, were separated from production fluids (~1.5 l) using a 2 l separatory funnel as previously described (Orphan et al. 2000). Microbial biomass from the water phase was concentrated onto a 0.22 μm sterivex filter using a peristaltic pump. The filter was subsequently filled with 1.8 ml lysis buffer (40 mM EDTA, 750 mM sucrose, 50 mM Tris-HCl) and stored at −80°C. Total nucleic acid extractions from the sterivex filter were conducted using a standard phenol-chloroform procedure (Murray et al. 1998) and purified on a small scale CsCl density gradient prior to PCR amplification (DeLong 1992).

PCR amplification using both universally-conserved (519ftra and 1390r tra) and archaeal-specific (20f and 958r) 16S rRNA gene primer sets were used in the construction of the Rincon Formation (O3) libraries. Four 50 μl reactions were amended with acetic acid to assist with amplification of rDNA with high G + C content (5% final concentration) and amplified for 20 cycles (universal library) and 30 cycles (archaeal library) using 3 min of denaturation at 92°C, 0.5 min of annealing at 55°C, 0.5 min of extension at 72°C,
and a final 7 min extension at 72°C. PCR reaction mixtures contained 0.2 µM of either universal or archaea-specific primers, 5 µl of PCR buffer containing 2 mM MgCl₂, 2.5 mM deoxynucleoside triphosphates, and 0.025 U of Taq polymerase (Promega).

DNA amplicons were pooled from 4 reactions, concentrated in a Centricon-YM100 and washed twice with 2 ml of TE buffer prior to library construction. SSU rRNA gene products were cloned using a TA cloning vector kit according to manufacturer's instructions. A total of 61 and 68 clones containing the correct sized insert were screened from the universal and archaeal libraries, respectively. Screening was conducted using RFLP digests with restriction enzymes Hae III and Rsa I and the unique clones were sequenced bidirectionally using a Thermo Sequenase Fluorescent Labeled Primer Cycle Sequencing Kit (Amersham, Braunschweig, Germany) and an automated Licor 4200 DNA sequencer. Archaeal and universal 16S rRNA gene surveys for the South Elwood Monterey Formation has been previously described in Orphan and others (2000).

Phylogenetic Analysis

Preliminary identification of closest phylogenetic relatives was determined for 16S rRNA gene sequences recovered from the Rincon (R) and Monterey (M) Formations using the "blastn" program from the National Center for Biotechnology Information (NCBI) site (http://www.ncbi.nlm.nih.gov). Sequences from the Monterey Formation (Genbank accession numbers: AF220303-AF220350) have been previously reported in Orphan et al. (2000). Sequences were aligned to the nearest neighbor with the automated "Fastaligner 2" tool of the ARB program package (Strunk and Ludwig [ed] ARB: a software environment for sequence data, 1999. [http://www.mikro.biologie.tu-muenchen.de]). A phylogenetic tree was constructed using parsimony insertion within the ARB program package to illustrate the general phylogenetic relationships of environmental Rincon archaeal sequences from this study to the previously reported uncultured and cultured 16S rRNA gene sequences from the S. Elwood Monterey Formation and related sequences in the GenBank database.

RNA Quantification

For samples containing significant concentrations of intact community RNA (Rincon well 3120-4 and N.C.L. well 31-34), quantitative hybridization experiments using 32P labeled domain specific probes for Bacteria (S-D-Bact-0338-a-A-18), Archaea (S-D-Arch-0915-a-A-20), and Eucarya (S-D-Euk-1209-a-A-16) were conducted. RNA extracts from formation water samples and control isolates were initially denatured with 1.5% gluteraldehyde, blotted onto Hybond-N nylon membranes (Amersham) in serial dilution (50, 25, 12.5, and 6.25 ng RNA), and immobilized on the membrane using a UV cross-linking apparatus according to Massana and others (1998). Membranes were subsequently hybridized at 45°C overnight with the radiolabelled RNA probes and then exposed to a high stringency wash at the appropriate temperature (Arch, 56°C; Bact, 45°C; Euk, 37°C; and Univ, 45°C) as described in Massana et al. (1998). The hybridization signal was quantified using a radioanalytic gas proportional counting system (Ambis Systems). Relative rRNA abundances of the three domains (Archaea, Bacteria, and Eucarya) in the formation water samples were determined by calculating the slope (counts per minute per ng of RNA) of the serially diluted natural sample that hybridized with each domain specific probe and dividing by the slope calculated for the total rRNA hybridizing with a Universal probe (S-1390-a-A-18) in the same sample (Massana et al.1998). Standard control microorganisms, representing the three domains, included Sulfolobus acidocaldarius, Pseudomonas aeruginosa, and Saccharomyces cerevisiae.
Results and Discussion

Physicochemical Variation Between Reservoirs

Analysis of production waters from four different high temperature petroleum reservoirs revealed significant differences in their geochemical conditions. A general summary of the physicochemical parameters from the South Elwood field and the two onshore production fields within the San Joaquin Basin, California are listed in Table 1. In the South Elwood field, located approximately 2 miles off the coast of Santa Barbara, California, the two neighboring oil producing formations, the lower Miocene Rincon Formation and the upper Miocene Monterey Formation produced oil at depths of approximately ~1300 and 1500 m, respectively and had similar temperature (~70–75°C) and pH (~7.75) regimes. Although these two reservoirs are close in proximity, the total dissolved solids (TDS), \( \Sigma \text{CO}_2 \), \( \Sigma \text{H}_2\text{S} \), and weight percent sulfur in the oil varied. Monterey-produced formation waters and oils, generally known for their high sulfur and organic acid concentrations (Fisher and Boles 1990; Franks et al. 2001), were characterized by elevated TDS concentrations (35,270 ppm),

<table>
<thead>
<tr>
<th>TABLE 1</th>
<th>Geochemical and biological characteristics of formation waters from three high-temperature California oil fields</th>
</tr>
</thead>
<tbody>
<tr>
<td>Field</td>
<td>South Elwood</td>
</tr>
<tr>
<td>Geological age:</td>
<td>Upper Miocene</td>
</tr>
<tr>
<td>Formation</td>
<td>Monterey</td>
</tr>
<tr>
<td>Well #</td>
<td>3120-9</td>
</tr>
<tr>
<td>Physicochemical conditions</td>
<td></td>
</tr>
<tr>
<td>Depth (m)</td>
<td>1295</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>70–75</td>
</tr>
<tr>
<td>TDS (ppm)*</td>
<td>35,270</td>
</tr>
<tr>
<td>pH</td>
<td>7.84</td>
</tr>
<tr>
<td>( \Sigma \text{H}_2\text{S} ) (mM)</td>
<td>11.2</td>
</tr>
<tr>
<td>( \text{SO}_4 ) (mM)*</td>
<td>0.47–0.9</td>
</tr>
<tr>
<td>wt% sulfur oil*</td>
<td>~4%</td>
</tr>
<tr>
<td>( \text{NH}_4 ) (mM)</td>
<td>8.4</td>
</tr>
<tr>
<td>( \text{NO}_2 ) (µM)</td>
<td>6</td>
</tr>
<tr>
<td>( \Sigma \text{CO}_2 ) (mM)</td>
<td>122</td>
</tr>
<tr>
<td>( \text{CH}_4 ) (µM)</td>
<td>60</td>
</tr>
<tr>
<td>Total Organic Acid (mg/L)*</td>
<td>3495</td>
</tr>
<tr>
<td>Direct counts (10^3 cells per ml)</td>
<td>5.45</td>
</tr>
</tbody>
</table>

Domain associated

16S ribosomal RNA

| | | |
| Archaea | ND | 5.4% | 16.3% | ND |
| Bacteria | 56.5% | 83% |
| Eucarya | 0.7% | 3.2% |

TDS: Total dissolved solids.
*Eichubl and Boles, unpublished data.
ND: not determined.
\( \Sigma \text{CO}_2, \text{NH}_4, \Sigma \text{H}_2\text{S}, \) and percent sulfur while the Rincon Formation, producing sweet (low sulfur) gas and oil, was found to contain higher \( \text{CH}_4 \) concentrations and lower TDS (20,530 ppm), \( \Sigma \text{CO}_2, \text{NH}_4, \Sigma \text{H}_2\text{S}, \) and percent sulfur. The North Coles Levee (N.C.L.) and Yowlumne oil fields within the San Joaquin Basin produced oil at higher temperatures in comparison to the South Elwood field, ranging between 93 to 120°C. Like the offshore Rincon Formation, the formation waters from N.C.L. well 34-31 contained high methane concentrations, low \( \text{CO}_2 \), and little to no \( \text{H}_2\text{S} \) and \( \text{SO}_4 \). N.C.L. fluids were also characterized by high levels of organic acids (up to 2345 mg/L; P. Eichhbul and J. Boles, unpublished data). Well 61x-3 from the Yowlumne field had a low TDS concentration (9,966 ppm) and lower organic acids (435 mg/L), likely explained by meteoric water breakthrough (J. Boles, unpublished data).

**Microbial Diversity: Ribosomal RNA Quantification and 16S rRNA Gene Surveys of Rincon Formation Assemblages**

Two 16S rRNA gene libraries were constructed from total community DNA from the Rincon Formation using 16S rRNA archaeal and universally conserved PCR primers. Sixty-eight archaeal clones were screened using restriction digests and 9 archaeal clones displaying unique RFLP patterns were sequenced. Unique archaeal phylotypes clustered within three distinct clades within the Euryarchaeota, all closely related to cultured thermophilic methanogens. The majority of recovered archaeal phylotypes from the Rincon production fluids were associated with aceticlastic (acetate-utilizing) lineages, with 69% closely related to *Methanosarcina thermophila* and *Methanosarcina siciliae* and 21% affiliated with *Methanoseta thermophila* (Figure 1). A small percentage of clones (3%), clustered with thermophilic *Methanoculleus* spp., included cultured strains previously recovered in 65°C enrichments amended with \( \text{H}_2/\text{CO}_2 \) from both the Rincon and Monterey Formations (Orphan et al. 2000). In addition to the predominance of methanogen-associated clones detected in the archaeal clone library, a low number of methanogen phylotypes were also recovered using universally conserved primers. Two archaeal phylotypes out of 61 total clones screened in the universal library were closely related to the hydrogenotrophic *Methanobacterium thermogibberans* and thermophilic (70°C) *Methanothermobacter* isolates recovered from the same Rincon production water sample (Figure 1; Orphan et al. 2000). Furthermore, PCR-independent, domain-specific ribosomal RNA quantification from Rincon Formation waters revealed that archaeal RNA comprised up to 5.4% of the total RNA. These archaeal rRNAs are likely attributed to viable populations of methanogens, affiliated with the methanogen 16S rRNA sequence types recovered from the same sample (Table 1). A second onshore site in the San Joaquin basin (N.C.L. production well 34-31, producing oil at 105°C) also contained a significant percentage of archaeal rRNA in the formation waters (up to 16%; Table 1) and viable thermophilic methanogen populations (detected with enrichment cultures). Although the methanogen-related 16S rRNA sequence types recovered from the Rincon Formation using the archaeal and universal primer sets represented distinctively different genera, these independent molecular surveys suggest that thermally adapted methanogens are an important component of the in situ microbial assemblage.

Bacterial rRNA from the offshore Rincon Formation comprised 56.5% of the total hybridized RNA. Bacterial 16S rRNA genes recovered using universally conserved primers were predominately affiliated with mesophilic gamma proteobacteria, with 47% and 18% of the clones related to heterotrophic *Pseudomonas* spp. and *Halomonas* spp., respectively. Other phylotypes represented in the universal library were related to known cultured oil-affiliated and thermophilic genera including *Thermotoga* (Grassia et al. 1996), *Deferribacter* (Greene et al. 1997), *Aminobacterium* (Buena et al. 1998), *Acetanaerobacterium*, and
FIGURE 1 Phylogenetic tree of archaean 16S rRNA genes recovered from the South Elwood field Rincon formation. Archaeal sequences recovered from the Rincon (R) and Monterey (M) formations are in boldface. Sequences were initially aligned to an ARB database of related 16S rRNA gene sequences and phylogenetic tree was constructed using parsimony insertion within the ARB program package. Monterey sequences with accession numbers are from Orphan et al. (2000). The scale bar represents the number of changes per nucleotide position.

*Anaerobaculum* (Rees et al. 1997). Similar thermophilic cultured isolates phylogenetically related to *Anaerobaculum thermoterrenum* (vp 184 and vp 14) and *Deferrribacter thermophilus* (vp 180) were previously obtained from the South Elwood Rincon and Monterey Formations in 80°C anaerobic marine enrichment cultures, suggesting these microbial...
groups may be a common component of the resident subsurface thermophilic microbial community (Orphan et al. 2000). Bacterial rRNA was also found to comprise the majority of intact rRNA recovered from the onshore North Coles Levee production waters; however, PCR-based diversity surveys have not yet been conducted at this site (Table 1). Additionally, a small percentage of rRNA recovered from both the North Coles Levee field and the South Elwood Rincon Formation showed positive hybridization with an Eucaryotic specific probe, totaling 3% and 0.7%, respectively. Eucaryotes have not been previously reported from high temperature oil-bearing formations and work is currently in progress to assess whether the measured signal is due to viable eucaryotic microorganisms associated with the production well, or derived from cross reactivity with the Euk.1209 probe.

Despite their close proximity and similar temperature regimes, molecular surveys of the microbial assemblages in the S. Elwood Rincon and Monterey Formations contained dramatically different assemblages of thermophilic archaea and bacteria in the production fluids. In comparison to the large abundance of thermophilic methanogenic phylotypes recovered from the natural gas producing Rincon Formation, previous molecular characterization of production fluids from the neighboring sourced Monterey reservoir was shown to harbor a large percentage of sulfidogenic archaeal and bacterial thermophiles, including *Thermococcus* sp., *Thermoanaerobacter* sp., and *Desulfotignum* sp., all of which may actively produce sulfide in situ (Orphan et al. 2000). These fundamental differences in the diversity and community structure observed in the two reservoirs were only evident using 16S rRNA gene analysis, as prior enrichment culture experiments failed to recognize significant differences in methanogenic, sulfidogenic and fermentative functional groups between the Monterey and Rincon Formations (Orphan et al. 2000). Variations in microbial 16S rRNA gene diversity were found to most closely parallel the prominent geochemical conditions present in the two reservoirs, similar to diversity trends observed for microbial communities in shallow subsurface aquifer systems (i.e., Roling et al. 2001). The metabolic and taxonomic diversity of thermophilic anaerobic microorganisms represented in both oil producing reservoirs strongly suggests the potential for complex and unique microbial interactions and activities driven by, and perhaps contributing to, the in situ geochemical conditions.

**Activity and Temperature Optima of Microbial Assemblages in High Temperature Production Fluids**

Although thermophilic and hyperthermophilic microorganisms have been cultivated from high temperature petroleum reservoirs worldwide, it is still uncertain whether these microorganisms grow under the physical conditions and nutrient availability present in the reservoir. Detailed chemical analysis of production fluids and culture-based experiments with petroleum reservoir isolates previously suggested that key nutrients essential for microbial growth, such as nitrogen, phosphorous, and amino acids, may be limiting in situ (Adkins et al. 1992; Takahata et al. 2000, 2001). To determine if the Monterey and Rincon Formation fluids could support the growth of thermophilic assemblages at elevated temperatures, we conducted a series of microcosm experiments using raw production fluids (oil/water) incubated over a temperature range bracketing the in situ conditions found in the South Elwood reservoir. Eleven out of 18 microcosm treatments from four different oil-bearing strata (two from the offshore South Elwood field as well as two additional continental reservoirs), amended with simple substrates (e.g., H₂/CO₂, acetate), stimulated active production of either methane or hydrogen sulfide at temperatures equal to or greater than 70°C.

Two independent sets of microcosm experiments using South Elwood Rincon Formation waters (amended with both H₂/CO₂ and H₂/CO₂ and acetate) demonstrated greater methane production at the in situ reservoir temperature (70°C) than in parallel treatments
FIGURE 2 (a) Microbial production of methane at 70°C in Rincon production fluids (well 3120-4) supplemented with H₂/CO₂ (●) or H₂/CO₂ and acetate (●●). (■) represents formaldehyde fixed controls. Inset: Methane production in H₂/CO₂ + acetate treatments incubated at 70°C (●) and 50°C (○). (b) Methane production (▲) and CO₂ consumption (●) in North Coles Levee production fluids (well 34-31) amended with H₂/CO₂. Shaded symbols are treatments incubated at 75°C and open symbols were incubated at 85°C.

incubated at 50°C (Figure 2a inset). Methane production was accompanied by an increase of biomass in both the 50°C and 70°C incubations. Cell numbers increased significantly in a 15-day period from 2.84 × 10⁶ cells/ml to 1.82 × 10⁷ cells/ml in 70°C incubations, with lower densities (1.32 × 10⁶ cells/ml; day 15) recorded for those treatments incubated at 50°C (20°C below the in situ reservoir temperature). Production fluids obtained from the onshore N.C.L. field (well 34-31), with a bottom hole temperature of 105°C, also demonstrated active methane production and CO₂ consumption at 75°C upon the addition of H₂/CO₂ (Figure 2b). Microcosms from both the N.C.L. Stevens formation and South Elwood Rincon Formation incubated at approximately 85°C did not show significant methane production. The current upper temperature limit for cultured hyperthermophilic methanogens is 110°C.
(Kurr et al. 1991); however, our findings suggest that the thermophilic methanogen populations within these deep, geothermally heated petroleum systems are better adapted for optimal productivity at temperatures between 70–75°C. Similar temperature ranges (60–80°C) have been demonstrated for cultured methanogens such as *Methanobacterium* sp., *Methanococcus* sp., and *Methanoculleus* sp. recovered both from California reservoirs as well as from petroleum bearing strata worldwide (Ng et al. 1989; Mueller and Nielsen 1996; Orphan et al. 2000).

Production fluids obtained from the South Elwood Monterey Formation harboring viable thermophilic and hyperthermophilic sulfidogens produced measurable quantities of hydrogen sulfide with minimal substrate additions at both 73°C and 83°C over the course of 18 days. No significant difference in H₂S production was observed between the incubations amended with H₂/CO₂ at 73°C and 83°C, reaching total headspace H₂S concentrations of 212 µM and 245 µM, respectively (data not shown). Production of H₂S at temperatures up to 83°C is not surprising as many of the cultured sulfidogenic archaea and bacteria from deep petroleum reservoirs, including isolates from this study, have temperature optima between 85°C and 100°C (Stetter et al. 1993; Orphan et al. 2000). These findings from both offshore and continental subsurface systems suggest that production fluids from deep-seated reservoirs in California appear to contain biologically available nutrients and trace elements necessary to support microbial growth, and that viable, diverse thermophilic assemblages in these specialized deep subsurface environments, when exposed to the proper geochemical conditions, have the potential to actively produce methane and sulfide in situ at temperatures ranging between 70–83°C. This temperature range for microbial activity is consistent with observations of oil biodegradation predominately in reservoirs with burial histories ≥80°C (Wilhelms et al. 2001 and references therein).

**Influence of Competitive Substrate Additions on Microbial Production of CH₄ and H₂S**

Characterization of the producing formations from the South Elwood field revealed significant differences in the chemical composition, total cell counts, and microbiological assemblages existing within the high-sulfur Monterey Formation and the low-sulfur Rincon Formation. These findings highlight potential variations in mineralization and carbon flow by thermophilic communities within the two adjacent reservoirs. Using a series of microcosm experiments amended with simple substrates (H₂/CO₂ and acetate) central to both carbon and sulfur-based pathways (Hoeher et al. 1998), potential differences in biogeochemical cycling of carbon and sulfur by indigenous microbial assemblages in these geochemically distinct, thermally heated reservoirs were investigated.

The response to H₂/CO₂ and acetate additions by the unique thermophilic microbiological assemblages within the South Elwood Rincon and Monterey Formations revealed significant differences in the dominant modes of carbon and energy flow within the respective reservoirs. Methanogenic processes were predominant in the Rincon production fluids, stimulated by both H₂/CO₂ additions as well as by H₂/CO₂ and acetate. Both 70°C and 50°C incubations demonstrated significantly greater methane production with the addition of acetate and H₂/CO₂ in comparison to treatments with H₂/CO₂ alone (Figure 2a). Concurrent with increased methanogenesis, final cell densities were also higher in acetate amended vials (1.82 × 10⁷ cells/ml at day 15) than in vials supplemented only with H₂/CO₂ (1.67 × 10⁷ cells/ml). Formaldehyde fixed controls amended with identical substrates and incubated at the same temperatures did not generate methane over the course of the experiment.

The increased production of methane with the addition of acetate in the Rincon Formation fluids is likely a result of aceticlastic methanogenesis and is supported by the predominance of 16S rRNA sequence types related to thermophilic members of the Methanosarcinales recovered from the same production fluid sample. Similar stimulation of
methanogenesis in the presence of acetate was also reported in enrichment cultures from North Sea production fluids incubated at a range of temperatures from 60–92°C; however, cultured methanogen isolates from the same formation had a temperature optima of 60°C (Nilsen and Torsvik 1996). Active acetoclastic methanogenesis (at 70°C) within deep subsurface petroleum formations has also been demonstrated using radiolabelled substrates; however, the microorganisms involved in this process were not described (Rozanova et al. 1997). In addition to acetoclastic methane production by members of the Methanosarcinales, the stimulation of methanogenesis in the presence of acetate may also have been due in part to syntrophic acetate oxidation (thermodynamically favorable at elevated temperatures, Zinder and Koch 1984; Hattori et al. 2000) or fermentation coupled with hydrogenotrophic methanogenesis. Although syntrophic acetate oxidation coupled to hydrogenotrophic methanogenesis has not yet been directly demonstrated in high temperature petroleum systems, the co-occurrence of both \( \text{H}_2/\text{CO}_2 \) utilizing *Methanoculleus* spp. and *Methanobacterium* spp. and \( \text{H}_2 \) producing, fermentative bacteria such as *Thermotoga* spp. and *Thermoanaerobacter* spp. within the acetate-containing treatments, as well as in selective media enrichment cultures derived from the microcosm experiments, suggest that this cooperative process may also have contributed to the elevated methane production observed.

Microbial activity potential within the neighboring Monterey Formation also corresponded with the dominant in situ geochemistry and microbial diversity. Microcosm experiments with the high temperature (73°C) Monterey production fluids were dominated by sulfur-based microbiological processes, with biological \( \text{H}_2\text{S} \) generation observed in treatments amended with the same suite of energy substrates shown to stimulate methanogenesis in the underlying Rincon Formation. In the Monterey stratal fluids, significant \( \text{H}_2\text{S} \) production was measured in all treatments containing \( \text{H}_2/\text{CO}_2 \) over an 18-day period, with 10-fold greater \( \text{H}_2\text{S} \) production over treatments amended with \( \text{N}_2/\text{CO}_2 \) or \( \text{N}_2/\text{CO}_2 \) and acetate (Figure 3). Acetate additions to Monterey production fluids did not significantly enhance \( \text{H}_2\text{S} \) production over those treatments lacking acetate (206 \( \mu \text{M} \) \( \text{H}_2\text{S} \) in \( \text{H}_2/\text{CO}_2 \) + acetate vials and 212 \( \mu \text{M} \) \( \text{H}_2\text{S} \) in the \( \text{H}_2/\text{CO}_2 \) treatment). Periodic analysis of dissolved \( \text{H}_2\text{S} \) in the water phase during the course of the experiment indicated approximately 11-fold higher \( \text{H}_2\text{S}/\text{HS}^- \) concentrations than measured in the headspace. Active \( \text{H}_2\text{S} \) production in the absence of added sulfur-based electron acceptors points to the existence of biologically available sulfur compounds within the formation waters or oil. Given the unusually high percentage of chemically bound sulfur associated with the immature Monterey sourced oils (8–14%; Baskin and Peters 1992), it is likely that thermophilic Monterey microflora are actively utilizing organic sulfur compounds in the oil itself. Previous studies have shown that some cultured hyperthermophilic archaea and bacteria, common in deep petroleum reservoirs, such as *Thermococcus* and *Thermotoga*, are capable of significant desulfurization of crude oil at elevated temperatures (Stetter 1998). Our findings support the potential for in situ biological \( \text{H}_2\text{S} \) production in high temperature petroleum systems using alternative sulfur respiratory pathways.

**Alternative Sulfur Compounds as a Source for Biological \( \text{H}_2\text{S} \) Souring in the Hot Subsurface**

Phylogenetic evidence from both enrichment cultures and culture independent 16S rRNA gene surveys from Monterey Formation fluids have previously indicated that biological \( \text{H}_2\text{S} \) production is likely driven by sulfur-based metabolic pathways other than sulfate reduction (Orphan et al. 2000). To further investigate the potential utilization of alternative sulfur sources by thermophilic reservoir assemblages, three independent sets of microcosm experiments were amended with elemental sulfur and thiosulfate. Elemental sulfur (and \( \text{H}_2/\text{CO}_2 \))
incubations with Monterey and Rincon production fluids from the offshore South Elwood field both stimulated H₂S production to varying degrees at the in situ reservoir temperature (71–73°C). In Monterey production fluids, the addition of elemental sulfur and H₂/CO₂ did not significantly increase H₂S generation over treatments containing only H₂/CO₂, supporting earlier indications of nonlimiting concentrations of biologically available sulfur in the production fluids (Figure 3a).

In contrast, elemental sulfur additions to the predominately methanogenic Rincon formation fluids invoked a shift from methanogenic processes (occurring during days 1–5) to a sulfidogenic system with significant H₂S production, reaching concentrations greater than 1.5 mM in the gas phase (days 6–24; Figure 3b). Headspace CO₂ concentrations also increased positively with H₂S concentration, indicating stimulation in remineralization of organic carbon sources present in the stratified fluids coupled to sulfur reduction. Sulfur amended organic enrichments, recovered from the H₂S-producing Rincon microcosms, contained both heterotrophic sulfur-utilizing Thermococcus and Thermotoga strains. Analysis of the microbial assemblages within sulfur amended enrichment cultures inoculated with fluids from the H₂S-producing Rincon microcosm (incubated at 75°C) stimulated the growth of both heterotrophic sulfur-utilizing Thermococcus and Thermotoga strains. Although low concentrations of SO₄²⁻ (470–900 μM; Eichhubl and Boles, unpublished data) and sulfur

**FIGURE 3** (a) Microbial H₂S production in South Elwood Monterey production fluids (well 3120-9) incubated at 73°C and amended with H₂/CO₂ (●), H₂/CO₂ + S⁻ (○), N₂/CO₂ (□), H₂/CO₂ + acetate (△). (b) Microbial production of CH₄ (△), H₂S (●), and CO₂ (○) in South Elwood Rincon production fluids (well 3120-4) amended with H₂/CO₂ + S⁻ and incubated at 72°C.
(0.2%) are present in the production fluids of the Rincon Formation, the concentrations apparently were not high enough to stimulate significant sulfidogenic activity by the associated thermophilic assemblage, as no H$_2$S production was detected in microcosm treatments lacking an exogenous sulfur source.

In the continental reservoirs located within the San Joaquin Basin, high temperature, low sulfur production fluids obtained from the Yowlumne oil field were also screened for thermophilic activity and response to sulfur intermediates. Like the offshore Rincon Formation, formation fluids from a Yowlumne production well (well 61x-3) provided with a H$_2$/CO$_2$ headspace and no external sulfur source, were also shown to be methanogenic. Significant quantities of methane gas were produced in 75°C incubations between days 6–14 with a concomitant decrease in headspace CO$_2$ (Figure 4a). Cell densities increased throughout the 14-day experiment, from the initial 1.0 × 10$^5$ cells/ml to 8.79 × 10$^6$ cells/ml (day 7) and 1.19 × 10$^7$ cells/ml (day 14). Of the estimated total cell abundance, over 87% of the cells were rod-shaped methanogens, similar to Methanobacterium sp., as determined by their morphology and characteristic blue-green autofluorescence under UV light.

**FIGURE 4** (a) Methane production (▲) and CO$_2$ consumption (●) in Yowlumne production fluids (well 61x-3) amended with H$_2$/CO$_2$ and incubated at 72°C. (b) H$_2$S production (■) and CO$_2$ (●) in Yowlumne production fluids amended with H$_2$/CO$_2$ + S$_2$O$_3$. Open symbols in both figures represent CH$_4$, H$_2$S, and CO$_2$ concentrations in fixed control.
In contrast to the methanogenic response with the addition of H₂/CO₂, Yowlumne production fluids amended with a sulfur source (thiosulfate) and H₂/CO₂ demonstrated significant H₂S production at 75°C, marking a shift in the dominant terminal electron accepting process. In thiosulfate-amended treatments, H₂S was detected in the headspace after 5 d of incubation (559 µM) and plateaued at 2.4 mM H₂S after 14 d (Figure 4b). Concentrations of H₂S in the liquid phase were approximately 14-fold higher than headspace H₂S, with biological H₂S production in excess of 36 mM. No methane production was detected in thiosulfate containing treatments. Despite the extremely high H₂S concentrations in the production fluids, cell counts increased over two orders of magnitude from 1.0 × 10⁷ cells/ml to 4.3 × 10⁷ cells/ml after 14 days. Similar metabolic shifts from methanogenesis to sulfidogenic processes by thermophilic microbial assemblages have also been reported in high temperature, sulfate-rich production fluids from Alaskan oil fields (Mueller and Nielsen 1996). These results suggest that the thermally-adapted microbial assemblages within the continental Yowlumne reservoir are sulfur limited, but, like the microbial communities found within the offshore Rincon Formation, are capable of exploiting exogenous sources of sulfur and actively respiring sulfur compounds.

Summary

This comparative investigation of geochemically distinct, high temperature petroleum reservoirs using complementary molecular 16S rRNA gene surveys, microcosm experiments, and biogeochemical analyses has revealed specific new insights about the diversity and activity of thermophilic microorganisms residing in these extreme environments. The free-living thermophilic assemblages present in the production fluids from both offshore and continental petroleum reservoirs were diverse and comprised of both complementary and competitive groups of microorganisms, many of which may possess versatile metabolic capabilities. Thermophilic communities deep within subsurface petroleum reservoirs were shown to be capable of chemolithoautotrophy as well as fermentative or heterotrophic growth using various sulfur sources such as elemental sulfur, thiosulfate, and perhaps organosulfur compounds in the oil (Konishi et al. 1997; Stetter 1998). Furthermore, the high levels of organic acids associated with Monterey shales offshore and within San Joaquin Basin production fields may also be utilized or possibly even produced by these diverse thermophilic assemblages. Although some reports have suggested an abiotic origin for the Monterey organic acids (Franks et al. 2001), isotopic enrichment in ¹³C for the carboxyl carbon relative to the methyl group in these compounds may also be indicative of biological reworking. Similar isotopic patterns have been reported for acetate from West Siberian high temperature (70°C) petroleum reservoirs harboring active thermophilic populations of acetilastic and methylotrophic methanogens, acetogens, and sulfate-reducing microorganisms (Rozanova et al. 1997). Additionally, a number of fermentative and anaerobic thermophiles, many related to microorganisms recovered from oil reservoirs in this study, are capable of producing and consuming organic acids (Rees et al. 1997; Davydova-Charakhch’yan et al. 1992; Fardeau et al. 1997) and degrading hydrocarbon components of the oil directly (Rueter et al. 1994; Stetter et al. 1993).

Methanogenic, possibly sulfur-limited production fluids showed a relatively rapid response to introduced sulfur compounds, redirecting the terminal electron accepting process from methanogenesis to sulfur-based pathways. Additionally, some thermophilic isolates from California oil-bearing formations were related to microorganisms previously shown to possess versatile metabolisms. Strains of Methanothermobacter thermostophilicus (formerly Methanobacterium thermoautotrophicum) have been shown to be capable of switching from hydrogenotrophic methanogenesis to lithotrophic sulfur respiration in the
presence of elemental sulfur (Stetter and Gaag 1983) and many *Thermococcus*, *Thermotoga*, and *Thermoanaerobacter* species are able to ferment complex organic substrates as well as respire both sulfur compounds and iron (Fardeau et al. 1994; Slobodkin et al. 1999). The capacity for metabolic flexibility by these thermophilic assemblages may serve as an effective strategy for adapting to the geochemically heterogeneous subsurface environment.

The community structure of thermophilic assemblages as well as predominant potential pathways of carbon and energy cycling were directly correlated with the geochemical conditions existing in the reservoirs, with active methanogenic and sulfidogenic processes observed. Production fluids from the natural gas producing Rincon Formation was shown to support diverse thermophilic methanogens related to aceticlastic and hydrogenotrophic genera, optimized for methane production from both H₂/CO₂ and acetate at in situ temperatures. In comparison, the thermophilic microbial assemblage from the sulfur-rich South Elwood Monterey Formation was dominated by fermentative and heterotrophic sulfidogens which actively produced H₂S at the in situ temperature in the presence of H₂/CO₂ and acetate. In all microcosm experiments, molecular hydrogen appeared to be essential to the terminal metabolic pathways within these systems. In situ sources of hydrogen within high temperature petroleum reservoirs may be provided from biotic processes through fermentative and syntrophic interactions (Bonch-Osmolovskaya and Stetter 1991; Fardeau et al. 1997; Baena et al. 1998) or abiotically, possibly generated through mineralogical interactions such as pyrite formation under high temperatures (Drochner et al. 1990). The close correlation between the geochemical conditions within the reservoirs and the structure and potential activity of the resident microbial communities suggest that these unique thermophilic assemblages have actively adapted to their environment and have likely contributed to the current geochemical conditions.

A long-standing question in microbial ecology concerns the origin of thermophilic assemblages in the subsurface. Previous studies have suggested that the relation of cultured thermophilic microorganisms in offshore petroleum reservoirs to hyperthermophiles present in hydrothermal vent systems implies that these organisms were introduced through seawater flooding or perhaps via natural conduits such as seeps and faults (Stetter et al. 1993). Others argue that the recovery of similar hyperthermophilic microorganisms from continental formations previously uncontaminated by seawater is evidence for indigenous subsurface biosphere (Ng et al. 1989; Bernard et al. 1992; L’Haridon et al. 1995). Our observations of similar sulfidogenic and methanogenic hyperthermophilic microbial assemblages adapted to in situ temperatures in both offshore and continental deep-seated petroleum formations, and the emphasis of biological pathways which reflect the dominant physicochemical conditions, point to an active, potentially indigenous thermophilic subsurface community.

References


