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Life in the Absence of Oxygen: Alternative Electron Acceptors for Anaerobic Microorganisms in a Petroleum Environment

Melike BALK

Laboratory of Microbiology, Wageningen University, Hesselink van Suchtelenweg 4, 6703 CT, Wageningen, The Netherlands

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Abstract: Anaerobic microorganisms derive energy by transferring electrons from an external source or donor to an external electron sink or terminal acceptor and often have the capacity to reduce 2 or more terminal electron acceptors. The well-known type of microbial respiration, in which oxygen serves as an electron acceptor for the oxidation of organic carbon and/or hydrogen, has been studied elsewhere in detail. Anaerobic microorganisms are widely distributed in oil-producing vents, hydrothermal vents, volcanic hot springs, non-volcanic geothermally heated subsurface aquifers, and soil. In this study, anaerobic, thermophilic, and fermenting microorganisms in a petroleum sample from the Adıyaman region of Turkey were examined for their ability to use different electron acceptors. The temperature range for growth of the enrichment culture (TP1) was between 40 and 65 °C and the optimum pH ranged from 4.5 to 8.0. TP1 had the ability to use a wide variety of mono-, di-, and polysaccharides to form acetate, lactate, ethanol, H_2, and CO_2. No sulfate-reducing or methanogenic microorganisms were found. As an electron acceptor, TP1 reduces thiosulfate, elemental sulfur, sulfite, Fe(III), anthraquinone-2,6-disulfonate (AQDS), arsenake, and MnO_2, but not sulfate, nitrate, (per)chlorate, or selenate. Herein, we show that the enrichment culture from the petroleum environment was able to reduce multiple electron acceptors. The utilization of these electron acceptors by TP1 also indicated their presence in this area. The results presented suggest that TP1 may occupy a niche as an environmental opportunist by taking advantage of diverse electron acceptors.

Key Words: Anaerobic bacteria, electron acceptor utilization, petroleum environment, degradation of organic compounds

Oksijensiz Yaşam: Bir Petrol Bölgesindeki Anaerobik Mikroorganizmalar İçin Alternatif Elektron Akseptörleri

Özet: Anaerobik mikroorganizmalar enerjilerini elektronların bir başka kaynaktan veya bir donörden bir terminal electron akseptörüne transfer edilmesi yoluyla kazanmaktadırlar ve çoğunlukla iki veya daha fazla elektron akseptörünü indirgeme özelliğine sahiptirler. Oksijenin elektron akseptörü olarak kullanıldığı ve böylece organik karbonun ya da hidrojenin okside edildiği tür olan mikrobiyel aerobik metabolizma konusunda pek çok çalışma bulunmaktadır. Anaerobik mikroorganizmalar petrol üretimi yapılan bölgelerde, hidrotermal kaynaklarda, volkanik veya jeotermal bölgelerdeki su veya topraklarda yaygın olarak bulunmaktadırlar. Bu araştırmada Türkiye'nin Adıyaman ilinde bulunan bir petrol bölgesinden örnek alınmış ve anaerobik, termofilik, fermentasyon yapabilen mikroorganizmaların çeşitli elektron akseptörlerini kullanma yetenekleri incelenmiştir. Zenginleştirilen kültürün (TP1) optimum gelişme için sıcaklık aralığı 40-60 °C ve pH optimumu ise 4,5-8,0 aralığında bulunmuştur. TP1 oldukca farklı mono-, di- ve polisakkaritleri kullanma yeteneğinde olup asetat, laktat, etanol, H_2 ve CO_2 oluşturmaktadır. Kültürde sülfat indirgeyen ve metan oluşturan mikroorganizmalar bulunmamıştır. TP1 elektron akseptörü olarak tiyosulfat, elementel sülfür, sülfit, Fe(III), antrakinon-2,6-disülfonat (AQDS), arsenat ve MnO_2'i indirgeyebiliyorken, sülfat, nitrat, (per)klorat ve selenatı kullanamadığı gözlenmiştir. Bu çalışma ile bir petrol bölgesinden elde edilen zenginleştirilmiş kültürün cok farklı özelliklerdeki elektron akseptörlerini kullanabilme yeteneğinde oldugu ortaya çıkarılmıştır. Elektron akseptörlerinin TP1 tarafından kullanılması, bu minerallerin söz konusu bölgedeki varlığını da işaret etmektedir. Buradaki sonuçlar, TP1 kültürünün bulunduğu ortamda değişik elektron akseptörleri kullanabilmesi açısından avantajlı ve oportunist olabileceğini göstermektedir.

Anahtar Sözcükler: Anaerobik bakteriler, elektron akseptör kullanımı, petrol bölgesi, organik bileşiklerin parçalanması

Introduction

Respiring microorganisms inhabit much of the Earth's hydrosphere and lithosphere. Such microorganisms derive the energy they need to live, grow, and reproduce from their chemical environments by transferring electrons from reduced to oxidized chemical species. Anaerobic metabolism often occurs in the absence of oxygen and then organic contaminants can be transferred

or biodegraded anaerobically. Some anaerobic bacteria use nitrate, sulfate, iron, manganese, and carbon dioxide as their electron acceptors for the breakdown of organic chemicals, and carbon dioxide and methane are the main final products. Anaerobic microorganisms have dominated life on Earth for perhaps more than 2 billion years before the increasing oxygen content of the atmosphere forced them to retreat into niches hostile to oxygenic organisms. These environments were provided by geological conditions (1).

In 1926, the first bacteria were isolated from oil fields (2). Moderate to extreme thermophilic anaerobes belonging to Bacteria and Archaea inhabit oil reservoirs and have been isolated from or been detected in oil field samples (3-8). The presence of closely related bacteria in remote oil fields (9,10) supports the existence of a widespread anaerobic biosphere in oil reservoirs. Temperature is the main limiting factor for microbial growth in oil reservoirs because temperature increases with depth at a mean rate of 3 °C per 100 m. Different types of data suggest that the presence of indigenous bacteria in oil fields could be limited to a threshold temperature of 80-90 °C (8). Thermophilic and hyperthermophilic fermentative microorganisms also constitute an important microbial community of oil field environments. The members of the order *Thermotogales* (11-13), family *Thermoanaerobiaceae*, which include the genera *Thermoanaerobacter* and *Thermoanaerobacterium* (9,14), and fermentative *Archaea* (15), were isolated in different microhabitats within oil reservoirs. Natural environments for thermophilic microorganisms are widespread on Earth's surface. The existence of microorganisms in the deep terrestrial subsurface has been noted for a long time, but only in the past decade has there been an increasing interest in exploring anaerobic consortia (16).

Microorganisms have changed Earth in a number of ways. They have altered the chemistry of the atmosphere (17) and modified the compositions of oceans, rivers, petroleum environments, and pore fluids through control of mineral weathering rates or by inducing mineral precipitation. They have changed the speciation of metals and metalloids in water, soils, and sediments by releasing complexing agents and by enzymatically catalyzing redox reactions. They have shaped the physical world by binding sediments, precipitating ore deposits, and weathering rocks, and have sustained communities of higher organisms through primary production and by remineralizing organic carbon. Most remarkably, they perform these functions in every nook and cranny, from the Earth's near surface to its depths, including even the most extreme environments (18).

Many aquifers and sedimentary environments contaminated with hydrocarbons have limited oxygen. Thus, the resident microorganisms must use alternative electron acceptors when oxidizing the reduced molecules. Microbial communities associated with petroleum environments have attracted broad interest because of the unique properties of the constituent organisms. However, little attention has been given to petroleum environment communities as complete microbial ecosystems. In this research, in order to address the relative contributions of different geochemistries to the energy demands of these ecosystems, an enrichment culture from a petroleum environment in the Adıyaman region of Turkey was investigated. As a relatively simple and confined ecosystem setting, a petroleum environment was considered and a simple question was posed: what are the main electron acceptors for metabolic energy that drive such communities?

Materials and Methods

Study site and sample collection

The anaerobic sample was obtained from the formation water produced from the petroleum well, Ikizce-2, which is located in the Adıyaman region of southeast Turkey (lat 38°11'0"N, long 38°29'0"E) and operated by the Turkish Petroleum Corporation (TPAO), in May 2005. The culture was enriched from the formation water by serial dilution. Sampling depth was 2398-2443 m.

Enrichment

The sample (5 ml) was transferred to 50-ml sterilized bicarbonate-buffered medium in a 117-ml serum vial sealed with a butyl rubber stopper under a gas phase of N_2/CO_2 (80/20, v/v). Pyruvate (20 mM) was used as the electron donor. The enrichment culture was grown at 20-90 °C and after growth, cultures were transferred to liquid media containing 20 mM of pyruvate as a substrate.

The composition of basal medium used for routine growth and substrate utilization experiments contained the following (g l^{-1}); $Na_2HPO_4.2H_2O$: 0.53; KH_2PO_4: 0.41;

NH_4Cl: 0.3; $CaCl_2.2H_2O$: 0.11; $MgCl_2.6H_2O$: 0.10; NaCl: 0.3; $NaHCO_3$: 4.0; $Na_2S.9H_2O$: 0.48. Additionally, the basal medium contained acid and alkaline trace elements (1 ml l^{-1}) and vitamins (0.2 ml l^{-1}). The acid trace element solution contained the following (millimolar): $FeCl_2$: 7.5; H_3BO_4: 1; $ZnCl_2$: 0.5; $CuCl_2$: 0.1; $MnCl_2$: 0.5; $CoCl_2$: 0.5; $NiCl_2$: 0.1; HCl: 50. The alkaline trace element solution was composed of the following (millimolar): Na_2SeO_3: 0.1; Na_2WO_4: 0.1; Na_2MoO_4: 0.1; NaOH: 10.0. The vitamin solution had the following composition (g l^{-1}): biotin: 0.02; niacin: 0.2; pyridoxine: 0.5; riboflavin: 0.1; thiamine: 0.2; cyanocobalamine: 0.1; p-aminobenzoic acid: 0.1; panthothenic acid: 0.1. The medium was supplemented with 0.01% BBL yeast extract.

The samples were maintained by weekly transfer of a 1% (v/v) inoculum to fresh medium. Bottles were incubated in the dark without shaking.

The pH was adjusted to the desired pH with KOH. The medium was boiled and cooled to room temperature under a stream of O_2-free N_2 gas. The medium was anaerobically dispensed into 120-ml serum vials with 50 ml of medium and a 1.7-atm gas phase of N_2/CO_2 (80/20, v/v). The bottles were closed with butyl rubber stoppers sealed with crimp seals. The medium was autoclaved for 20 min at 121 °C. Prior to inoculation, the medium was reduced with sterile stock solutions of $Na_2S.7\text{-}9\ H_2O$ and $NaHCO_3$ to obtain final concentrations of 0.04% and 0.2%, respectively. The vitamin solution was filter sterilized and other compounds were sterilized by heat. Unless stated otherwise, substrates were added from 1 M stock solutions to give a final concentration of 20 mM. Routinely, pyruvate was used as the carbon and energy source.

Microscopy

Cell morphology was routinely examined under a phase-contrast Leica DC250 microscope (Rijswijk, The Netherlands) equipped with a digital camera system.

Analytical methods

Organic acids, sugars, glycerol, ethanol, and methanol were analyzed by HPLC using a Polyspher OA HY column (300-6.5 mm, Merck, Darmstadt, Germany) and RI SE-61 refractive index detector (Shodex, Tokyo, Japan). The mobile phase was 0.01 N H_2SO_4 at a flow of 0.6 ml/min at 60 °C. Hydrogen and methane were measured on a Chrompack CP9001 gas chromatograph fitted with a TCD detector. The injector and detector temperatures were 60 and 130 °C, respectively (19,20). Benzene, toluene, and xylene were determined with gas chromatography by injecting 0.2 ml of headspace gas into a 436 Chrompack gas chromatograph (GC) equipped with a flame ionization detector (FID) connected to a Sil 5CB column (25 m × 0.32 mm × 1.2 µm) and with split-injection (ratio: 1:50). Operating temperatures of the injector and detector were 250 and 300 °C, respectively. The oven temperature was 70 °C. Carrier gas was N_2 with an inlet pressure of 50 kPa. Thiosulfate, nitrate, (per)chlorate, arsenate, selenate, and sulfate were analyzed by an HPLC system equipped with an Ionpac AS9-SC column and an ED 40 electrochemical detector (Dionex, Sunnyvale, USA). The eluent consisted of 1.8 mM Na_2CO_3 and 1.7 mM $NaHCO_3$ at a flow of 1 ml/min at room temperature. The anions were detected with suppressed conductivity (21). Sulfide was analyzed as described by Trüper and Schlegel (22).

Results

Enrichment

The sample obtained from the formation water produced from the petroleum well was enriched and directly used to examine the metabolic diversity of the culture at temperatures ranging from 20 to 90 °C in a bicarbonate-buffered medium with pyruvate as the growth substrate.

pH, temperature, and sodium chloride concentration ranges for growth

The final optical density at 600 nm after 24-h incubation was used to determine the range and optimum temperature, pH, and NaCl concentration for growth of the enrichment culture, TP1. The pH of the medium was adjusted by injecting calculated amounts of sterile Na_2CO_3 or HCl from the sterile, anaerobic stock solutions. The effects of pH and NaCl concentration were determined at the optimal temperature for growth. Under these conditions, TP1 grew at 40-65 °C with optimum growth around 50 °C, while no growth was detected at 30 and 80 °C. Growth was observed between pH 4.5 and 9, with optimum growth around pH 6.5 (pH 6.3-6.5). Growth occurred at NaCl concentrations ranging from 0 to 20 g

l^{-1}, with optimum growth at 5 g NaCl l^{-1}. No growth was observed at 30 g l^{-1}. Under the optimal conditions of growth and in the presence of thiosulfate (20 mM), maximum cell density (OD_{600}) was about 1.20.

Morphology

In the exponential growth phase, the cells grown in a bicarbonate-buffered medium supplemented with 0.1% (w/v) yeast extract and lactate at 50 °C appeared to be mostly rod-shaped cells (Figure 1). Terminal spore formation was observed in some of the cells.

Physiological studies

The ability of TP1 to metabolize soluble substrates was tested in a bicarbonate-buffered medium. The growth was determined by measuring the optical density at 600 nm. TP1 grew on the following substrates (at a concentration of 20 mM, unless indicated otherwise) in the presence or absence of thiosulfate as the electron acceptor: yeast extract, peptone, formate, H_2/CO_2, lactate, fructose, pyruvate, glucose, galactose, lactose, maltose, mannose, ribose, sucrose, xylose, starch, pectin, cellobiose (10 mM), fumarate, and malate. In the presence of thiosulfate, very slow growth was also observed on propionate and benzoate (5 mM). No growth occurred with acetate, glycine, ethanol (5 mM), methanol, propanol, butanol, succinate, carboxymethylcellulose, cellulose, arginine, or lichenan because BTEX compounds [benzene (50 µM), toluene (50 µM) and o-, m-, and p-xylenes (0.8 mM)] were not utilized.

Although yeast extract was not required for growth of TP1, it did affect product formation. When the yeast extract concentration was raised to ≥ 0.1%, glucose fermentation was shifted in favor of lactate and acetate instead of lactate and ethanol.

In addition to thiosulfate (20 mM), elemental sulfur (2%, w/v), sodium sulfite (5 mM), $FeCl_3$ (10 mM), Fe(III)-NTA (10 mM), Fe(III)-citrate (10 mM), MnO_2 (5 mM), anthraquinone-2,6-disulfonate (AQDS) (20 mM), and arsenate (5 mM) served as electron acceptors with lactate as the electron donor. Sulfate (20 mM), nitrate (20 mM), perchlorate (10), and selenate (5 mM) could not be used. The use of the electron acceptors was determined in the presence of 20 mM of lactate by growth of the culture, detection of sulfide production (for sulfate, thiosulfate, sulfite, and elemental sulfur), change of color (for AQDS), and measurement of the reduction of Fe(III) or formation of white precipitates (for MnO_2) in the medium.

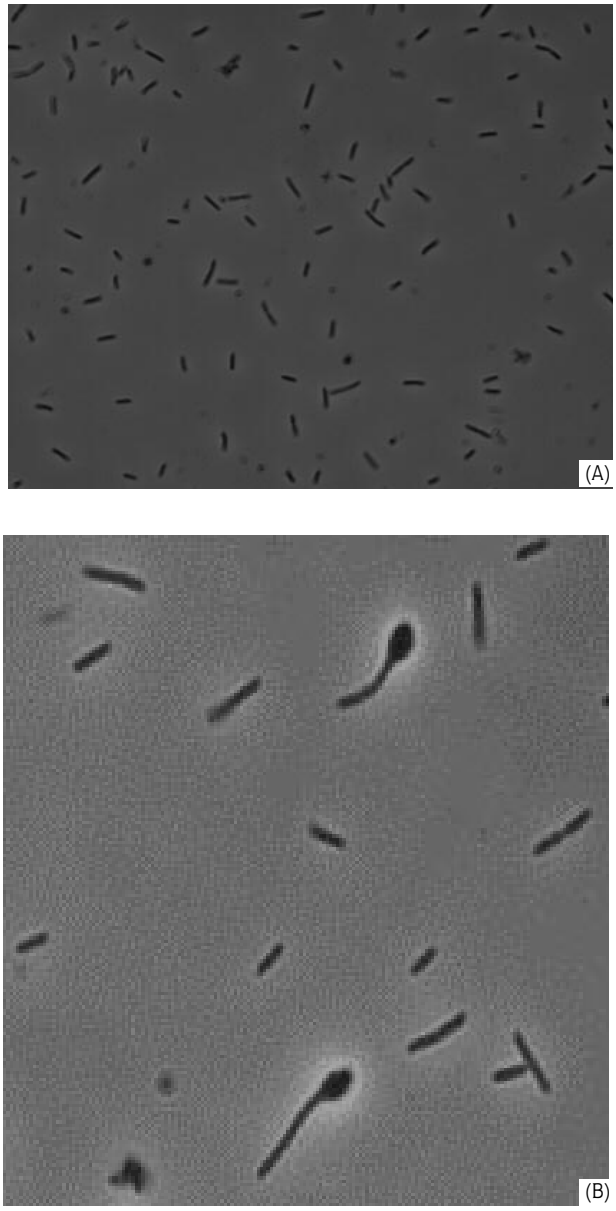

Figure 1. Phase contrast micrograph of culture TP1. (A) Vegetative cells from the culture growing on lactate and thiosulfate. (B) Vegetative cells and sporulated cells from the culture growing on lactate and sulfite.

The oxygen sensitivity of TP1 was tested under a gas phase replaced with 80% H_2, 19% CO_2, and 1% O_2. TP1 grew only under strictly anaerobic culture conditions and was sensitive to oxygen.

Discussion

The enrichment culture, TP1, was obtained from a petroleum well in the Adıyaman region of Turkey. TP1 was able to grow at 50 °C and optimum pH for growth was 7.0. Using different sugars and pyruvate, products formed by TP1 were mainly acetate, ethanol, lactate, hydrogen, and carbon dioxide, but no methane was formed.

Microorganisms capable of exploiting all the major terminal electron accepting processes, such as aerobic respiration, nitrate-, iron-, and sulfate reduction, methanogenesis, and fermentation, have been obtained from petroleum reservoirs and include isolates that range from mesophilic to hyperthermophilic (8). These reactions are of interest not only because they allow microorganisms to obtain energy, but also because they are involved in the natural biodegradation of organic contaminants (23). However, the involvement of microorganisms in petroleum environments is still poorly understood. Most of the common terminal electron acceptors that bacteria use for respiration, such as oxygen, nitrate, and sulfate, are soluble. This means they can freely diffuse to the cell to receive electrons from the membrane-bound molecules of the respiratory chain. Remarkably, in the batch cultures used in the present study, potential acceptors including sulfate, thiosulfate, sulfur, sulfite, and reduced metals such as As(III), Fe(II), and Mn(II) are used by TP1 (Table).

The prevailing physicochemical conditions and relatively high temperatures in this sample mean that novel microorganisms are likely to be present. Although culture TP1 was able to use a wide range of different substrates, BTEX compounds were not degraded. There are few reports on cultures of putative hydrocarbon degraders from petroleum reservoirs (24,25). On the other hand, hydrocarbons may not be the sole source of electron donors for microorganisms in petroleum reservoirs, and hydrogen or different organic acids might also serve as electron donors in production waters (26).

Utilization of sugars without the need of electron acceptors in TP1 also showed the presence of fermenting microorganisms. Fermentation occurs when an organic chemical acts as an electron acceptor. Although fermentative bacteria have been identified in petroleum reservoirs, there is as yet no evidence that any of the fermentative microorganisms in petroleum reservoirs serve as definitive proof of hydrocarbon degradation (27). TP1 was able to use thiosulfate, elemental sulfur, and sulfite (Table). Thiosulfate- and sulfur-reducing thermophilic fermentative bacteria have been reported to exist in oil field waters. The ability to reduce thiosulfate to sulfide is shared by a large number of fermentative bacteria from petroleum reservoirs (11,14). Sulfur (S) is biochemically and geochemically an important element. The average crustal abundance of sulfur is 260 mg g^{-1} and most sulfur on Earth is present as metal sulfide, gypsum, anhydrite, and dissolved sulfate. There are several intermediate valence forms of sulfur that can serve as both electron donors and acceptors for bacteria, depending on environmental conditions; the most important ones are elemental sulfur and thiosulfate (28). Thermophilic sulfidogens were frequently found at temperatures ranging from 70 to 85 °C and appear to be widespread in high temperature petroleum systems (14); however, the reason for the presence of thiosulfate in oil reservoirs has not been investigated in detail. Thiosulfate was reduced completely to sulfide by TP1. The ability to reduce thiosulfate to sulfide is a property of a large number of fermentative bacteria from petroleum reservoirs (8). Although sulfate-reducing bacteria were also found to be dominant in different oil fields (7),

Table. Coupled reactions involving lactic acid and electron acceptors by the enrichment culture TP1.

2 Lactic acid + $S_2O_3^{2-}$ + 2H+ → $2H_2S$ + 2 acetic acid + $2CO_2$ + H_2O
Lactic acid + 2S + H_2O → $2H_2S$ + acetic acid + CO_2
1.5 Lactic acid + SO_3^{2-} + $2H^+$ → 1.5 acetic acid + $1.5CO_2$ + H_2S +$1.5H_2O$
Lactic acid + $4Fe^{3+}$ + H_2O → $4Fe^{2+}$ + acetic acid + CO_2 + $4H^+$
Lactic acid + $2HAsO_4^{2-}$ + $4H^+$ → acetic acid + $2HAsO_2$(aq) + CO_2 + $3H_2O$

sulfate was not reduced by TP1. The amount of elemental sulfur was not quantified, but the concentration of sulfide increased during the growth, indicating the utilization of elemental sulfur.

In addition to thiosulfate and sulfur, TP1 was also able to reduce AQDS, Fe(III), and Mn(IV). In anaerobic environments, Fe(III) reduction predominates and Fe(III) is the most abundant alternative acceptor for anaerobic respiration in many sedimentary environments (29,30). Microorganisms reduce extracellular Fe(III) or Mn(III, IV) to support their metabolism or growth, and for the partial or complete oxidation of organic compounds or hydrogen (31,32). About 35% of the iron and 75% of the manganese in soils and sediments is in the form of free oxides (33-35). Iron and manganese oxides and hydroxides are common in the subsurface environment and can be present in oil-bearing rock (36). Electron transfer via humic acids is especially important during iron-reduction, and strongly enhances the rate of the redox process (37).

The metalloid contaminants As(V) and Se(VI) can also serve as electron acceptors for anaerobic respiration (38,39). Culture TP1 was able to use only As(V). Arsenic and selenium are 2 elements whose potential toxic and teratogenic effects outweigh their relatively low crustal abundances (40). Arsenate- and selenate-respiring bacteria have been shown to occur in a wide range of environments and are not confined to any specific genus. Arsenate concentration in petroleum is relatively low, ranging from 0.0024 to 1.63 mg kg^{-1} (41) with a mean of concentration of 0.26 mg kg^{-1} (42).

Culture TP1 was able to use H_2/CO_2 in the presence of 0.1% (w/v) yeast extract and acetate was the end product; however, when any electron acceptor was provided, the concentration of acetate did not decrease, indicating that acetate was not consumed during growth.

Although methane-producing microorganisms from the biodegradation of hydrocarbons have been demonstrated (43,44), methane was not formed during the growth of TP1 on different substrates. A syntrophic partnership that requires the interaction of hydrocarbon-degrading fermentative bacteria that produce hydrogen and short chain fatty acids for the growth of the methanogenic partner might be a significant process in petroleum environments; however, there is as yet no clear evidence to support this type of growth.

In conclusion, the presence of thermophilic microorganisms in a sample from the petroleum environment extends the known ecological habitats of such groups of organisms. The question that inspired this investigation was what kinds of minerals can be used in a petroleum environment by microorganisms. Life on Earth may have first evolved in a subterrestrial fashion and then migrated to the surface as the environment became more favorable. If so, the study of deep, subsurface microbial communities may provide another key to exploring our distant past. The subsurface microbial community constitutes a large portion of the Earth's biomass, yet only a small fraction of it has been characterized. Some subsurface microorganisms may be survivors or progeny of bacteria incorporated around the time the sedimentary strata were deposited (45). Persistence over millions of years would require adaptations for long-term survival in low-nutrient environments or the development of communities that are sustained through complementary interactions among members. Therefore, the widespread occurrence of TP1-like thermophilic microorganisms in deep-seated high-temperature petroleum reservoirs suggests the potential for closely coupled, active biogeochemical cycling of carbon and different electron acceptors in hot subsurface petroleum habitats, and therefore may have significant impacts on reservoir geochemistry.

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Corresponding author:
Melike BALK
Laboratory of Microbiology,
Wageningen University,
Hesselink van Suchtelenweg 4,
6703 CT, Wageningen,
The Netherlands
E-mail: melike.balk@wur.nl

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