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Microbial community structure analysis of produced water from a high-temperature North Sea oil-field

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Abstract Molecular and culture-based methods were used to investigate the microbial diversity in produced water obtained from the high-temperature Troll oil formation in the North Sea. 16S rRNA gene libraries were generated from total community DNA, using universal archaeal or bacterial oligonucleotide primer sets. Sequence analysis of 88 clones in the bacterial library indicated that they originated from members of *Firmicutes* (48 sequences), *Bacteroidetes* (17 sequences), *δ-Proteobacteria* (15 sequences), *Spirochaetes* (5 sequences), *Thermotogales* (2 sequences) and *γ-Proteobacteria* (1 sequence). Twenty-two sequences in the archaeal library were close relatives to members of the genera *Methanococcus* (18 sequences), *Methanolobus* (3 sequences) and *Thermococcus* (1 sequence). Most of the bacterial sequences shared less than 95% identity with their closest match in GenBank, indicating that the produced water harbours a unique community of novel bacterial species or genera. Members of the thermophilic genera *Thermosipho*, *Thermotoga*, *Anaerophaga* and *Thermovirga* were isolated. The Troll formations are not injected with sea water. Thus, dramatic changes of the in situ conditions have been avoided, and a common source of continuous contamination from injection water can be excluded. However, the majority of the organisms detected in the gene libraries were most closely related to cultivated organisms with optimum temperatures for growth well below the in situ reservoir temperature (70°C), indicating that produced water from the Troll platform harbours a substantial amount of non-indigenous organisms. This was confirmed by the isolation of a number of mesophilic and moderately thermophilic organisms that were unable to grow at reservoir temperature.

Keywords Anaerobes · Deep biosphere · Formation water · Petroleum microbiology · Thermophiles · *Thermovirga*

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Introduction

A wide range of microorganisms have been isolated from samples of produced water obtained from oil reservoirs. These include fermentative organisms, methanogens, manganese and iron reducers, acetogens, sulphate reducers, aerobic organisms and nitrate reducers (for reviews see Magot et al. 2000; Birkeland 2004). Detection of an organism in a sample of produced water is, however, not evidence for this

organism being indigenous to the reservoir, i.e. being a natural inhabitant of the reservoir before the onset of oil exploitation. Samples from oil reservoirs are, for practical and economical reasons, normally obtained from the well-head or even at a subsequent point in the pipeline system, after the produced fluids have been transported in pipelines up to several kilometres from the reservoir. Possible sources of contamination are thus numerous (McInerney and Sublette 1997; Magot et al. 2000). Microorganisms can be introduced to the production system through drilling, well operations and damaged tubings or casings, which could lead to formation of biofilms inside the oil-transporting line, and cause a continuous supply of contaminants to the system (Basso et al. 2005). An additional source of contamination can occur in reservoirs subject to water-flooding, where contaminants from injected sea-water might be introduced directly to the formations. In many cases an indigenous nature of a detected organism can be excluded. This is the case for some mesophilic organisms detected in samples from high temperature oil wells (Magot et al. 1992; Tardy-Jacquenod et al. 1998). On the other hand, the widespread distribution of thermophilic organisms—e.g. members of the order *Thermotogales* and the genera *Thermoanaerobacter, Thermoanaerobacterium* and *Desulfotomaculum*—in geographically separated high-temperature oil reservoirs, is taken as evidence for the existence of a natural microbial community in these habitats (Magot et al. 2000). Members of the genera *Geotoga* and *Petrotoga*, in particular, might be considered as indigenous since they have been isolated only from oil reservoirs so far. Still, little is known about the diversity and ecophysiology of microbial communities in oil formations. The primary energy source for the microorganisms in oil reservoirs is not known. At temperatures up to about 80°C, petroleum in subsurface reservoirs is often biologically degraded, over geological timescales (Head et al. 2003). Nevertheless, organisms known to be able to degrade hydrocarbons anaerobically, are not known to be indigenous to oil reservoirs. Lacking is also general knowledge about nutrient flow, how organisms have entered the oil reservoirs and how they may have survived in the reservoirs for tens of millions of years.

Studies of the distribution of organisms in produced water from high-temperature oil reservoirs, by construction of 16S rRNA gene libraries, is so far restricted to a few Californian sulphur-rich reservoirs (Orphan et al. 2000,2003) and a long term water-flooded reservoir in China (Li et al. 2006). The Californian production waters are dominated by *Proteobacteria* and *Firmicutes*, while methanogenic organisms dominate among the archaea. Bacteria in the Chinese production water are dominated by *Proteobacteria*. Apparently, mesophilic organisms constitute a large fraction of organisms detected in both the Chinese and the Californian production waters. From the results it can be calculated that a total of 85% of the bacterial clones detected in the Chinese production water samples are most closely related (>92.7% identity) to mesophilic members of the genera *Pseudomonas* and *Serratia*. A total of 78% of the bacterial clones in the Californian production water samples are closely related (>94.6% identity) to mesophilic organisms within the genera *Pseudomonas*, *Halomonas*, *Acinetobacter*, *Sphingomonas*, *Brevundimonas*, *Methylobacterium* and *Acidaminococcus*, while 86% of the clones in the archaeal library were most closely related (>95.6% identity) to the mesophilic *Methanoplanus petrolearius*.

In this work we have analysed produced water from a non water-flooded high-temperature oil reservoir in the North Sea by construction of bacterial and archaeal 16S rRNA gene libraries combined with culture-based methods. Our results revealed that the produced water harbours unique microbial communities as most of the detected organisms probably represent novel species or genera, previously not detected in any other environments. Most of the detected bacteria seem to be thermophilic or moderately thermophilic fermenters, while the archaea are dominated by thermophilic methanogens. A comparison of the optimum growth temperature of the closest cultivated relatives to organisms detected in the clone libraries, suggested that most of the detected organisms, including organisms representing some of the most dominating groups, are probably not able to grow at reservoir temperature and therefore should be regarded as non indigenous. This was confirmed by cultivation experiments. Thus, our results present evidence for the view that the microbiology in produced water might only to a small extent reflect the indigenous microbiology in the respective oil formations, even when the formations are not water-flooded.

Materials and methods

Site description and sample collection

The Troll C platform is located at 60° 53′ 10.73″ N, 03° 36′ 41.41″ E. The formations are situated 1550–1600 m below the sea floor and have a temperature of 70°C. The velocity of the water transport from the formations to the platform is approximately 1–2 m per second. Produced water contained the following elements (per liter): Na (16.4 g), Ca (1.6 g), Mg (560 mg), Ba (160 mg), Fe (14 mg), Sr (300 mg), K (420 mg) and P (<0.1% (w/v)) (Malvin Sperre, personal communication). Sulfate and ammonium were present at concentrations of <2 and 110 mg/l respectively. The formations are not water-flooded.

Sterile Pyrex bottles filled with Argon gas were used to collect samples at the upper riser on the Troll C oil platform. The samples were a mixture of water produced from several wells. Lines were flushed for more than 20 min prior to sampling. The bottles were filled completely with a mixture of oil and water, and transported to our laboratory at ambient temperature.

Clone library construction

Immediately after arrival of the samples at our laboratory, cells from 3 ml of the water phase were harvested in Eppendorf tubes by centrifugation and subsequently lysed by repeated freezing at −70°C for 30 min and thawing at 30°C. No intact cells could be observed after 3 cycles of freezing and thawing. The lysate was further used as template for amplification of 16S rRNA gene sequences by PCR. The oligonucleotide primers, 21F (5-TCCGGTTGATCCTGCC-3), modified after Reysenbach and Pace 1995, and A976R (5′-YCCGGCGTTGAMTCCAATT-3′) (Reysenbach and Pace 1995) were used for amplification of archaeal 16S rDNA sequences, while primers 27f (5′-GAGTTTGATCCTGGCTCA-3′) (Rainey et al. 1992) and 1525r (5′-GAAAGGAGGAGATCCAGC-3′ (Rainey et al. 1992) where used for amplification of bacterial 16S rRNA genes. PCR was performed with 30 cycles of amplification as previously described (8). PCR products were cloned with the TOPO TA cloning kit version H using chemical transformation according to the manufacturer's instructions (Invitrogen).

Sequencing and phylogenetic analysis

Sequencing was performed on a ABI PRISM capillary sequencer according to the protocol of the ABI Prism BigDye Terminator kit (Perkin Elmer). Selected clones in the archaeal and bacterial 16S rRNA gene libraries were sequenced with primers 21F and 27f respectively, while clones representing a bacterial or archaeal OTU were additionally sequenced with reverse 16S rRNA gene primers 1525R or A976R, respectively. PCR products obtained by amplification of DNA extracted by use of a genomic DNA extraction Kit (Sigma) from liquid cultures were sequenced with one or more of the primers 27F, 1525R or 530F (5′-GTGCCAGCMGCCGCGG-3′) (Lane 1991). Sequences were checked for putative chimeric primary structures by use of the program Pintail (Ashelford et al. 2005) in combination with use of the BLAST service (Altschul et al. 1997), where identities between various fragments of a given sequence and sequences available in the GenBank database (Benson et al. 2005) were compared. Non-chimeric sequences were grouped into OTUs comprising sequences with more than 98% similarity. One representative of each OTU was sequenced in both directions. All sequences were compared with other sequences in the GenBank database using BLAST (Altschul et al. 1997) to identify closest relatives. Phylogenetic reconstructions, based on alignments created by CLUSTALX (Thompson et al. 1997), were produced using TreeView version 1.6.6 (Page 1996) with the Jukes-Cantor distance matrix (Jukes and Cantor 1969) and the neighbour-joining algorithms (Saitou and Nei 1987). Confidence in the tree topology was determined by using 100 bootstrapped trees (Felsenstein 1985). Calculation of identity between sequences was performed by use of the program BioEdit based on pairwise alignments subsequent to manual deletion of endgaps and positions with undetermined nucleotides ('N's).

Growth experiments

Enrichment and growth experiments of fermentative organisms were performed using an anaerobically prepared basal medium containing the following components (per liter distilled water): 20 g NaCl, 0.9 g $MgCl_2 \times 6H_2O$, 1.4 g $MgSO_4 \times 7H_2O$, 0.33 g KCl, 0.25 g NH_4Cl, 0.14 g $CaCl_2 \times 2H_2O$, 0.45 g KH_2PO_4, 1.0 ml trace element solution SL-10 (Widdel et al.

1983), and 0.5 ml resazurin (0.02%). After autoclaving in a dispenser (Lien and Beeder 1997) the medium was reduced with 4 ml 0.5 M Na_2S under a nitrogen atmosphere. 10 ml vitamin solution (Balch et al. 1979) was added and pH was adjusted to 6.8 with 1 M NaOH or 1 M HCl. 20 or 40 ml of medium was dispensed into 50 or 100 ml serum bottles, respectively, which were subsequently sealed with butyl rubber stoppers. Substrates were added from separate, anaerobically prepared, stock solutions. R1-medium contained yeast extract (0.1% w/v), peptone (0.25% w/v) and maltodextrin (0.25% w/v). Cas-medium contained 0.5% (w/v) Casamino-acids. The same basal medium as described above was used for enrichment of sulphate-reducing organisms and methanogenic organisms except for the addition of 4 g $NaHCO_3$ per liter medium as well as the use of a gas phase of H_2 and CO_2 (80:20) for enrichments of methanogens and a gas phase of N_2 and CO_2 (80:20) for the enrichment of sulphate-reducing organisms.

Enrichments were incubated at 6–70°C immediately after inoculation of produced fluids (a mixture of oil and water). Growth was detected by the use of a light microscope (Nikon, Eclipse E400). Isolation of pure cultures was performed by picking colonies grown in anaerobically prepared solidified medium as described elsewhere (8).

DNA–DNA hybridization

DNA–DNA hybridization experiments were performed at DSMZ. Genomic DNA from strain TCF52B and from *Thermosipho africanus* was prepared using a French pressure cell (Thermo Spectronic) and was purified by chromatography on hydroxyapatite as described (Cashion et al. 1977). DNA–DNA hybridization was carried out as described (Deley et al. 1970) under consideration of the modifications described by Huss et al. (1983) using a model Cary 100 Bio UV/VIS-spectrophotometer equipped with a Peltier-thermostated 6 × 6 multicell changer and a temperature controller with in-situ temperature probe (Varian).

Nucleotide sequence accession numbers

The gene sequences determined in this study have been deposited in GenBank under accession numbers DQ647058–DQ647185.

Results

Direct microscopy

Several morphotypes of microorganisms were observed by direct observation of the production water in a phase contrast microscope. Sheathed and unsheathed rods seemed to be dominating, but coccoid cells were also frequently seen. On one occasion a spirochaet-like cell was observed. Direct counts of microorganisms in production water samples using a Thoma chamber revealed the presence of approximately 10^6 cells/ml which is in agreement with samples from other oil reservoirs (Orphan et al. 2000).

16S rRNA gene libraries

One bacterial and one archaeal 16S rRNA gene library were constructed using PCR and DNA extracted directly from produced water as template. A total of 99 clones from the bacterial library were sequenced in the forward direction (700–800 bp). Among these, 11 sequences were found to be chimeric and were excluded from further analysis. The remaining 88 sequences were assigned to 25 distinct phylogenetic groups which clustered among *Firmicutes* (48 sequences), *δ*- and *γ*-*Proteobacteria* (16 sequences), *Thermotogales* (2 sequences), *Spirochaetes* (5 sequences) and *Bacteroidetes* (17 sequences) (Table 1). The samples of produced water appear to harbour unique microbial communities as the majority of sequences (83%) had less than 95% identity to closest matches in GenBank and probably represent novel genera or species (Figs. 1 and 2). A total of 29 operational taxonomic units (OTUs) were identified. A large fraction of the OTUs (62%) in the library were represented by only one sequence, while most (67%) of the sequences were assigned to OTUs comprising five or more sequences. One representative of each OTU was selected for almost full length sequencing (1300–1500 bp). Most organisms detected in the bacterial library cluster among fermentative organisms. The only exceptions are relatives to sulphate reducers in the genera *Desulfomicrobium* and *Desulfovibrio* as well as a relative to the aerobic *Agarivorans albus*. More than half of the OTUs in the bacterial library comprise sequences that cluster within the *Firmicutes* lineage, indicating the

Table 1 Closest relatives of clones obtained from the bacterial 16S rRNA gene library

Taxonomical group	Sequence representing OTU	No. of clones in OTU	Closest match	Closest cultivated species	% Identity to closest match (closest cultivated species)	Temperature optimum of closest cultivated species (°C)[a]	Source of closest match[a]
Firmicutes	TCB112x	2	*Halanaerobium saccharolyticum* X89069	*Halanaerobium saccharolyticum* X89069	87.7 (87.7)		
	TCB133x	1	*Clostridium celerecrescens* X71848	*Clostridium celerecrescens* X71848	87.1 (87.1)		
	TCB128x	1	Uncultured bacterium AY570599	*Sedimentibacter hydroxybenzoicus* L11305	87.7 (87.1)		
	TCB195x	1	*Orenia marismortui* X89073	*Orenia marismortui* X89073	94.5 (94.5)	36–45	The Dead Sea
	TCB166x	1	*Orenia marismortui* X89073	*Orenia marismortui* X89073	86.3 (86.3)		
	TCB170x	1	*Thermovirga lienii* DQ071273	*Thermovirga lienii* DQ071273[b]	89.9 (89.9)		
	TCB169x	1	*Thermovirga lienii* DQ071273[b]	Thermovirga lienii DQ071273[b]	91.4 (91.4)	58	Oil reservoir
	TCB152x	1	*Thermovirga lienii* DQ071273[b]	*Thermovirga lienii* DQ071273[b]	88.6 (88.6)		
	TCB197x	4	*Thermovirga lienii* DQ071273[b]	*Thermovirga lienii* DQ071273[b]	99.7 (99.7)	58	Oil reservoir
	TCB168x	1	*Thermovirga lienii* DQ071273[b]	*Thermovirga lienii* DQ071273[b]	97.8 (97.8)	58	Oil reservoir
	TCB6y	3	*Thermovirga lienii* DQ071273[b]	*Thermovirga lienii* DQ071273[b]	99.2 (99.2)	58	Oil reservoir
	TCB8y	9	*Thermovirga lienii* DQ071273[b]	*Thermovirga lienii* DQ071273[b]	99.4 (99.4)	58	Oil reservoir
	TCB198x	1	*Caminicella sporogenes* AJ320233	*Caminicella sporogenes* AJ320233	92.5 (92.5)	55–60	Hydrothermal vent
	TCB15y	3	*Caminicella sporogenes* AJ320233	*Caminicella sporogenes* AJ320233	92.8 (92.8)	55–60	Hydrothermal vent
	TCB136x	16	*Caminicella sporogenes* AJ320233	*Caminicella sporogenes* AJ320233	92.7 (92.7)	55–60	Hydrothermal vent

Table 1 continued

Taxonomical group	Sequence representing OTU	No. of clones in OTU	Closest match	Closest cultivated species	% Identity to closest match (closest cultivated species)	Temperature optimum of closest cultivated species (°C)[a]	Source of closest match[a]
	TCB199x	1	*Clostridium caminithermale* AF458779	*Clostridium caminithermale* AF458779	87.7 (87.7)		
	TCB1y	1	*Thermacetogenium phaeum* AB020336	*Thermacetogenium phaeum* AB020336	94.7 (94.7)	58	Methanogenic reactor
δ-Proteobacteria	TCB115x	1	Uncultured bacterium O11G2 AF220316	*Desulfomicrobium baculatum* AJ277894	99.4 (85.0)		Oil reservoir
	TCB124x	1	*Desulfovibrio indonesiensis* Y09504	*Desulfovibrio indonesiensis* Y09504	98.2 (98.2)	<37	Corroded oil storage vessel
	TCB4y	7	*Desulfomicrobium baculatum* AJ277894	*Desulfomicrobium baculatum* AJ277894	90.6 (90.6)	28–37	Manganese carbonate ore
	TCB131x	6	*Pelobacter carbinolicus* X79413	*Pelobacter carbinolicus* X79413	96.4 (96.4)	35–40	Anoxic marine mud
γ-Proteobacteria	TCB10y	1	*Agarivorans albus* AB076559	*Agarivorans albus* AB076559	95.9 (95.9)	Mesophilic	Marine animal
Thermotogales	TCB177x	1	*Thermosipho africanus* DQ647058	*Thermosipho africanus* DQ647058	99.4 (99.4)	75	Shallow hydrothermal system
	TCB116x	1	Strain TCEL2[b]	*Thermotoga maritima* M21774	99.4 (97.5)	80	Geothermal heated marine sediment
Spirochaetes	TCB129x	5	Uncultured bacterium AY667253	*Spirochaeta bajacaliforniensis* M71239	96.5 (83.8)		Deep basalt aquifer
Bacteroidetes	TCB179x	1	Uncultured eubacterium U81730	*Tannerella forsythensis* AB053943	92.3 (87.8)		Anaerobic digestor
	TCB123x	7	*Anaerophaga thermohalophila* AJ418048	*Anaerophaga thermohalophila* AJ418048	91.6 (91.6)	37–55	Oil saparator tank

Table 1 continued

Taxonomical group	Sequence representing OTU	No. of clones in OTU	Closest match	Closest cultivated species	% Identity to closest match (closest cultivated species)	Temperature optimum of closest cultivated species (°C)[a]	Source of closest match[a]
	TCB200x	8	Strain TC451[b]	*Anaerophaga thermohalophila* AJ418048	94.5 (92.0)	37–55	Oil reservoir
	TCB130x	1	Strain TC451[b]	*Anaerophaga thermohalophila* AJ418048	97.9 (94.5)	37–55	Oil reservoir

[a] Only 16S rRNA gene identities above 90% are considered

[b] Isolated in this study

presence of a large fraction of highly diverse Gram-positive organisms.

The archaeal library was less diverse than the bacterial library and only three OTUs were identified from 22 sequences of which none were chimeric (Table 2). One of the detected organisms is closely related to fermentative organisms within the *Thermococcus* genus while 21 sequences were closely related to members of the genera *Methanothermococcus* and *Methanolobus*. Thus, the archaeal community appears to be highly dominated by methanogens.

Culture-dependent analysis

In order to reveal the biological properties of the organisms detected in the culture-independent approach, we attempted to enrich and isolate organisms based on the physiological properties of close relatives to some of the organisms detected in the clone libraries. Most effort was put into enrichments of anaerobic fermentative bacteria because this physiological group seemed to be dominating according to the molecular analysis. Enrichments in anaerobically prepared medium, where a mixture of yeast extract, dextrin and peptone served as substrates (R1 medium), yielded growth in the temperature range of 6–70°C. Strain TC3 and strain TC2 were isolated from enrichments at 18 and 6°C respectively. Cells of strain TC2 were short, highly motile rods related to the recently characterized *Arcobacter halophilus* (95.8% 16S rRNA gene similarity; 801 bp), a gram negative, fermentative and facultative anaerobic ε-proteobacterium (Donachie et al. 2005). Strain TC3 exhibited 98% 16S rRNA gene (1435) similarity to *Alcalibacter saccharofermentans*, an alcaliphilic and mesophilic organism that ferments sugars and proteinous substrates (Garnova et al. 2004). Unlike what is reported for *A. saccharofermentans*, strain TC3 was able to grow at pH below 7.0. Two strains, strain TC371 and strain TC451 isolated from enrichments at 37 and 45°C respectively, had identical 16S rDNA sequences with 96% identity to the 16S rRNA gene from *Anaerophaga thermohalophila* (1438–1440 bp). Another strain isolated from an enrichment incubated at 45°C, strain TC452, had a 16S rRNA gene highly similar to *Geotoga petraea*. *Geotoga* spp. has only been recovered from oil reservoirs so far. Enrichments with R1 medium and with cellulose or fish

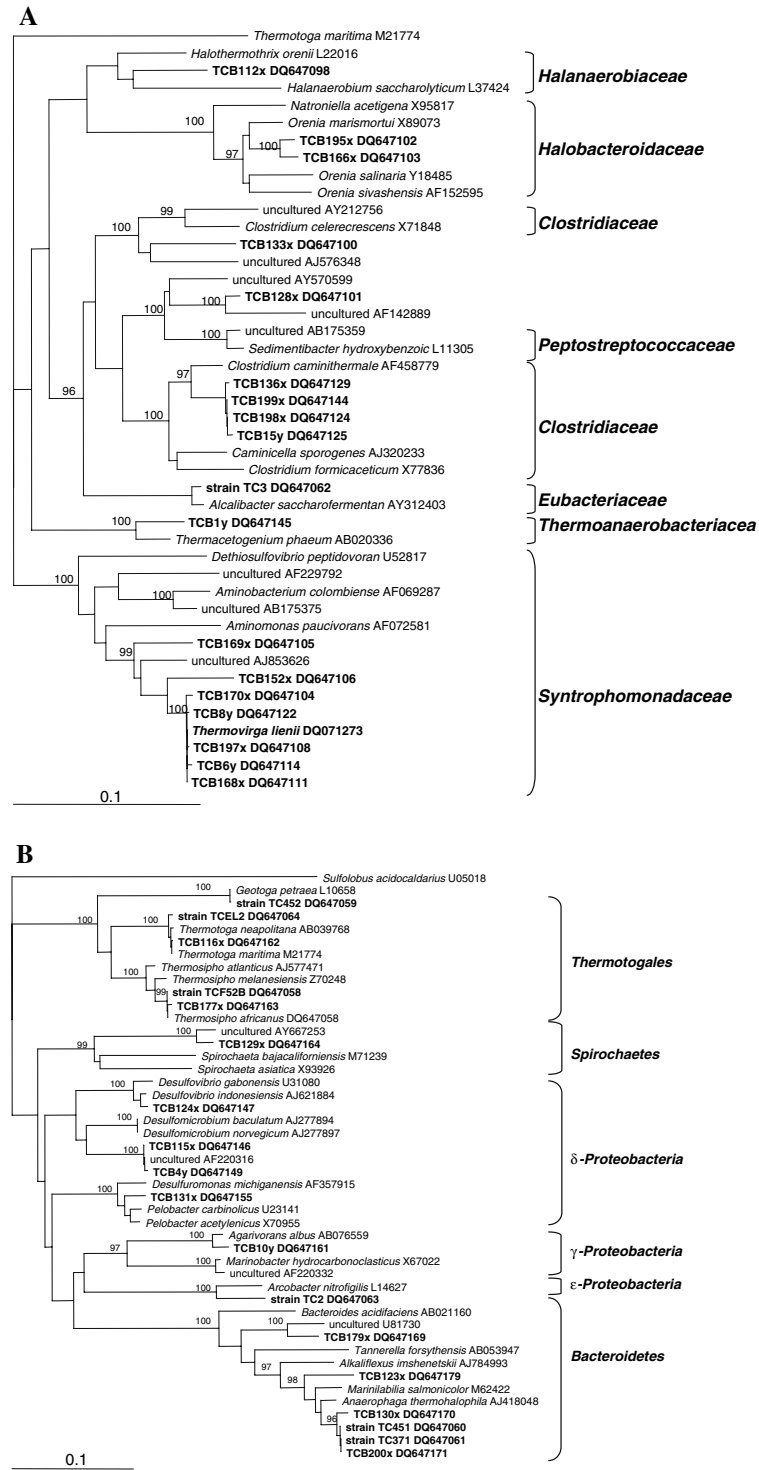

Fig. 1 Phylogenetic neighbour-joining trees based on 16S rRNA gene sequences. The position of the clones (TCB) and isolates (strain) obtained from the Troll formations are indicated in bold. Bootstrap values from 100 replications are shown at nodes. Only values above 96 are indicated. Bar indicates 10 substitutions per 100 bases. (**A**) Organisms within the *Firmicutes* lineage. *Thermotoga maritma* was used as outgroup. (**B**) Organisms within *Thermotogales*, *Proteobacteria*, *Spirocheates* and *Bacteriodetes*. *Sulfolobus acidocaldarius* was used as outgroup

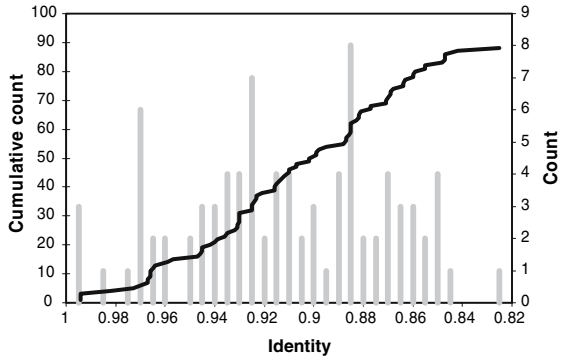

Fig. 2 Graphic representations of the novelty of organisms detected in the bacterial library. Bars indicate the distribution of identities to closest matches in GenBank, and are binned by 0.5% identity. The continuous line indicates the cumulative number of sequences that have closest match identities of at least the value defined by the *x*-axis. Primary *y*-axis (cumulative count) shows the cumulative number of sequences. Secondary *y*-axis (count) shows the number of counts in each bin in the histogram

waste as substrates at temperatures close to reservoir temperature (60–70°C) yielded cells with a morphology similar to members of the order *Thermotogales*. TCEL2, obtained from enrichments at 60°C on cellulose was related to *Thermotoga maritima* (98.5% rRNA gene sequence identity). Strain TCF52B was isolated from an enrichment incubated at 70°C where fish waste served as the only substrate, vitamin and carbon source. This strain had a 16S rDNA gene highly similar (99.9%) to 16S rDNA from *Thermosipho africanus* Ob7^T isolated from a marine hydrothermal spring in Djibouti, Africa (Huber et al. 1989). Interestingly, the genomes of strain TCF52B and strain Ob7^T were found to be identical as determined by DNA–DNA hybridization experiments.

In enrichments where Casamino acids served as the only substrate and carbon source (Cas-medium), growth was detected in the temperature range of 37–60°C, but not at 70°C. Isolates from enrichments at 37, 45 and 60°C possessed identical partial 16S rRNA genes. One of these strains, strain Cas60314, was further characterized and found to be a representative of the novel genus and species *Thermovirga lienii* (8). No growth was detected in non-reduced Cas- or R1 medium with a headspace of 0–50% air at 37–70°C. Enrichments for methanogenic and sulphate-reducing organisms were negative at 60 and 70°C.

Among the isolates, only the close relatives to *T. africanus* and *T. maritima* (i.e. strain TCEL2 and strain TCF52B) could grow at reservoir temperature (70°C). *T. lienii* strain Cas60314^T was able to grow at 60°C while for the remaining isolates (i.e. strain TC2, strain TC3, strain TC371, strain TC451 and strain TC452), no growth was detected at 60 or 70°C. As indicated in Table 1, close relatives to strain TC451 and strain Cas60314^T are among the organisms most frequently detected in the bacterial 16S rRNA gene library. Close relatives of strain TCEL2 and strain TCF52B were also detected. As indicated in Table 1, strain TCEL2 is a close relative to the organism detected by sequence TCB116x in the bacterial library. The 16S rRNA gene obtained from Strain TCF52B, had 99.4% identity to sequence TCB177x.

Discussion

The objective of this study was to investigate the microbiology of a high-temperature oil-bearing formation in the North Sea. The relatively low identity (<95%) between most of the sequences in the

Table 2 Closest relatives of clones obtained from the archeal 16S rRNA gene library

Taxonomical group	Sequence representing OTU	No. of clones in OTU	Closest cultivated species	% identity	Temperature optimum (°C)	Source
Methanococci	TCA8	18	*Methanothermococcus thermolithotrophicus* M59128	99.7	65	Geothermally heated sea sediments
Methanomicrobia	TCA2	3	*Methanolobus tindarius* M59135	97.2	25	Coastal sediments
Thermococci	TCA19	1	*Thermococcus alcaliphilus* AJ298872	99.4	85	Shallow marine hydrothermal system

bacterial 16S rRNA gene library and their respective closest matches among known species, indicate that produced water from the Troll formations harbours a unique microbial community comprising several novel genera or species. This was further indicated by the isolation of the novel genus and species *Thermovirga lienii* as well as isolation of organisms relatively distantly related (<95% 16S rRNA gene identity) to members of the *Anaerophaga* and *Arcobacter* genera.

Comparison between the culture dependent and culture independent approach

Several of the isolated strains clustered among sequences obtained from the bacterial library. Relatives of *T. lienii* strain Cas60314 apparently constitute a major component of the microbial community in the produced fluids. *T. lienii* has a 16S rRNA gene highly similar to the OTU representing sequences TCB197x, TCB168x, TCB6y and TCB8y (Table 1) while OTUs represented by TCB169x, TCB170x and TCB152x form phylogenetically distinct branches and probably represent novel genera. Another dominating group is apparently organisms clustering among strain TC451, strain TC371 and *A. thermohalophila*. Three OTUs representing sequences TCB123x, TCB200x and TCB130x, have one of these organisms as closest match (Table 1). Finally, close relatives to organisms isolated from enrichments incubated at reservoir temperature (70°C), were also detected in the bacterial clone library i.e. strains closely related to *T. africanus* or *T. maritima*. Negative enrichments for sulphate-reducing organisms close to reservoir temperature (60 and 70°C) is in agreement with the absence of sequences in the libraries with high degree of similarity to 16S rRNA from thermophilic sulphate reducers. The same reasoning holds for the negative enrichment of methanogens at 70°C, but at 60°C close relatives to *Methanothermococcus thermolithotrophicus* could have been expected to grow.

Comparison with organisms previously detected in oil-affiliated samples

Some organisms detected in the bacterial library clustered among sulphate-reducing organisms while most organisms clustered among fermentative organisms. This, together with the detection of close relatives to methanogens in the archaeal library, is consistent with previous reports on the microbiology of produced water from oil reservoirs (Orphan et al. 2000; Bonch-Osmolovskaya et al. 2003; Grabowski et al. 2005). Organisms previously detected in oil-related samples and in this study include representatives of the genera *Arcobacter*, *Anaerophaga*, *Desulfovibrio*, *Desulfomicrobium*, *Thermotoga*, *Thermosipho*, *Haloanaerobium* and *Spirochaeta*.

T. maritima strain M12597 has previously been isolated from an oil reservoir in Western Siberia (Slobodkin et al. 1999). Other members of this genus have been detected in oil reservoir samples around the world, suggesting that they belong to a natural microbial community in oil-bearing strata (Magot et al. 2000; Birkeland 2004). On the other hand, the only member of the genus *Thermosipho* previously isolated from an oil reservoir sample, is *T. geolei* (L'Haridon et al. 2001).

Recently, spirochaetes were detected in enrichment cultures inoculated with produced water from a low-temperature biodegraded oil reservoir in Canada (Grabowski et al. 2005). In the same study, mesophilic members the genera *Desulfovibrio* and *Desulfomicrobium* were, by growth experiments, found to dominate among the sulphate-reducing organisms while a bacterial 16S rRNA gene library, constructed from total DNA extracted directly from the formation fluids, was entirely composed of sequences highly similar to a 16S rRNA gene sequence from a member of the *Arcobacter* genus. The OTU represented by sequence TCB129x in the bacterial library, provides evidence for the presence of spirochaetes also in field water from the Troll formations. However, *Spirochaeta smaragdina*, isolated from an offshore oil well in Congo (Magot et al. 1997), still represents the only spirochaete isolated from the deep subsurface.

We also detected a close relative to *Desulfovibrio indonesiensis* (sequence TCB124x) while OTUs represented by sequences TCB4y and TCB115x are relatively distantly related to *Desulfomicrobium baculatum* and possibly represent novel genera of sulphate reducers. Mesophilic members of the genera *Desulfovibrio* and *Desulfomicrobium* have frequently been detected in samples from high-temperature oil reservoirs around the world, but are not believed to be indigenous in these habitats (Magot et al. 2000).

Members of the genus *Haloanaerobium*, one of a few genera of anaerobic fermentative bacteria that are adopted to high-salt conditions, have previously been isolated from various brines with salinities above 20% (Birkeland 2004). Organisms which are relatively distantly related to *Haloanaerobium saccharolyticum* (88% 16S rRNA gene identity), were detected in the bacterial library in this study (Table 1). They are possibly representatives of a novel genus within the extremely halophilic *Haloanaerobiaceae* family and indicate the presence of a subsurface saline.

The only described species within the genus *Anaerophaga* is so far *A. thermohalophila*, isolated from an oil separation tank (Denger et al. 2002). *A. thermohalophila* produces a surfactant that efficiently stabilises hexadecane/water emulsions and is therefore interesting as regards microbially enhanced oil recovery (Denger et al. 2002) and it would in this respect also bee interesting to investigate whether strain TC451 and strain TC371 share this ability.

Among archaeal organisms, members of the genus *Thermococcus* (Stetter et al. 1993; L'Haridon et al. 1995; Miroshnichenko et al. 2001; Bonch-Osmolovskaya et al. 2003) and members of several methanogenic genera (Orphan et al. 2000; Bonch-Osmolovskaya et al. 2003; Grabowski et al. 2005), have frequently been detected in oil reservoir samples. The strains vp183 and vp21, isolated from a high-temperature oil-well sample in California (Orphan et al. 2000) and strain ST22, isolated from produced water from the Statfjord formations in the North-Sea (Nilsen and Torsvik 1996) are all close relatives to *M. thermolitotrophicus*. Close relatives of this species are apparently dominating among archaeal organisms in the Troll formations (Table 2). Detection of the OTU represented by sequence TCA2 (Table 2), provides evidence for the presence of members of the *Methanolobus* genus in produced water from the Troll formations. No members of this strictly methylotrophic and halophilic genus have so far been isolated from oil reservoir samples.

Are the detected organisms indigenous to the Troll formations?

Great care has to be taken when inferences concerning the in situ microbiology of the Troll formations are to be drawn. First, there are a number of sources for bias in the construction of 16S rRNA gene libraries that can lead to a distorted view of the *de facto* distribution of microorganisms in the produced water (von Wintzingerode et al. 1997). Secondly, organisms detected in the production water sample are not necessarily indigenous in the reservoir. Although no gas or liquid is injected into the Troll formations, thereby excluding an assumingly important source of contamination, contamination cannot be excluded (Magot 2005).

One approach to assess whether a certain organism obtained from a production-water sample is indigenous to the reservoir or not, is to compare the growth characteristics with that of the in situ physico–chemical parameters of the oil reservoir in question (Magot 2005). Although growth characteristics cannot easily be ascribed to organisms based on 16S rRNA gene analyses only, it is interesting to note that most sequences in the bacterial and archael libraries display highest 16S rRNA gene identities to mesophilic or moderately thermophilic organisms with optimum temperatures for growth well below reservoir temperature (70°C). On the other hand, organisms obtained from enrichments at 70°C, were exclusively members of the order *Thermotogales*. Furthermore, several isolates were obtained from enrichments incubated at lower temperature, but none of these were able to grow at 70°C. Interestingly, two of these strains, strain Cas60314^T (the type strain of *T. lienii*) and strain TC451, related to *A. thermohalophila*, were highly similar to some of the most dominating groups of organisms detected in the bacterial 16S rRNA gene library.

From the observed genome identity of 100% between *T. africanus* strain TCF52B isolated in this study and the type strain of this species isolated from a hydrothermal system in Djibuti in Africa, it seems unlikely that strain TCF52B has survived and evolved in the Troll formations since reservoir deposition and consequently that these two *Thermosipho* strains have been separated for tens of millions of years. Based on the apparent massive contamination of moderately thermophilic organisms in our samples, among these a close relative to *Geotoga petraea*, it can not be ruled out that at least some of the thermophilic isolates still have a non-indigenous origin.

Taken together, no strong conclusions about the in situ microbiology of the investigated oil-formations can be drawn. Rather, our results suggest that

the produced water is heavily contaminated with non-indigenous organisms and that extreme care should be taken before claiming an indigenous origin of any isolate, even if its physiological characteristics match the in situ oil reservoir conditions. Improved sampling procedures are highly required for a better understanding of the microbiology of oil reservoirs.

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