The utilization of natural reservoir brine in an enrichment culture medium: An alternative approach for isolation of anaerobic bacteria from an oil reservoir

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Utilization of Natural Reservoir Brine in Enrichment Culture Medium: An Alternative Approach for Isolation of Anaerobic Bacteria from an Oil Reservoir

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Running Head Title: Utilization of Natural Reservoir Brine

Abstract

This study aims to suggest the new approaches of enrichment cultures using natural reservoir brine for enrichment culture medium in order to increase the bacterial population in the enrichment cultures and isolate novel thermophilic anaerobic bacteria from an oil reservoir. The results suggest that the brine-based culture medium should be sterilized by filtration to increase the number of bacterial population and CO_2 should be supplied to culture medium to increase the possibility of isolating novel bacteria from oil reservoirs. One of the specific bacteria isolated under the presence of CO_2 was a strain AR80 representing a novel bacterium within the genus *Petrotoga* on the basis of the phylogenetic analysis.

Keywords : reservoir brine, enrichment culture, isolation, CO_2, sterilization, novel bacteria

Introduction

The main focus of the previous studies of bacteria in oil reservoirs has primarily been related to culture-based approaches, such as those reported by Ollivier and Cayol (2005). However, according to Amann et al., (1995), approximately 85.0–99.9% of the total microbial community in oil reservoirs is viable but non-culturable (VBNC) bacteria. Thus, since the number of culturable bacteria is especially limited in oil reservoirs, culture-based approaches are insufficient for clearly describing the in situ microbial communities present. The roles of VBNC bacteria that have been detected in oil reservoirs by such techniques cannot be fully understood until these bacteria become available as pure culture for detailed physiological and molecular analyses. The lack of success in isolating not-yet-cultured bacteria from natural environments is due to the physiology of these bacteria. It is important to meet the requirements for the growth of these bacteria in enrichment cultures for the successful isolation; therefore, it is necessary to develop new approaches and modifications of conventional microbiological techniques to isolate not-yet-cultured bacteria from oil reservoirs.

In particular, the enrichment techniques are commonly used for isolating bacteria from natural environments, and it duplicates closely the conditions of particular habitats (Madigan et al., 2006). Enrichment techniques for isolating bacteria from oil reservoirs have been generally carried out using synthetic mineral media in previous studies; however, most of reservoir conditions can be easily duplicated using natural reservoir brine-based culture media (NBM) but synthetic mineral media. In addition, gases such as CH_4 and CO_2 dissolve in brine under reservoir conditions. The solubility of CO_2 in brine should be quite higher than that of CH_4 and dissolving CO_2 causes the brine to become more acidic; therefore, the presence of CO_2 should be taken into account as a reservoir condition in enrichment cultures.

This study aims to increase the bacterial population in enrichment cultures to isolate novel bacteria from an oil reservoir by new approaches using natural reservoir brine for the enrichment culture medium. For this purpose, the effect of the sterilization method for natural reservoir brine-based culture medium to the increase in the bacterial population was considered. In addition, the effect of the presence of CO_2, which was one of the reservoir conditions, to the isolation of novel bacteria from an oil reservoir was also considered in this study.

Materials and Methods

Enrichment Culture Experiments and Isolation of Thermophilic Anaerobic Bacteria

The reservoir brine was extracted from a wellhead of an oil well in the Yabase Oilfield in Akita, Japan. Enrichment culture experiments were carried out to isolate thermophilic anaerobic bacteria, which were applicable to petroleum industrial applications such as Microbial EOR, from the extracted sample. 0.1 ml of non-sterile brine was inoculated into 50 ml of culture medium that consisted of sterile brine supplemented with 0.1 g/l yeast extract as a nitrogen source and 2vol% of sterile crude oil as a carbon source for bacteria respectively. One set of brine supplemented with yeast extract was sterilized through 0.22 μm sterile membrane filters, whereas the other set was sterilized by autoclaving at 121ºC for 20 minutes. The crude oil was sterilized by autoclaving at the same conditions. Serum bottles containing the culture medium were sealed with butyl rubber caps and aluminum crimps, and then the headspaces were replaced with pure N_2, 10%-CO_2 (N_2 balanced), or pure CO_2. Enrichment culture experiments were performed at 50ºC without shaking.

After 1 week of incubation, bacteria were isolated from enrichment culture solutions by Hungate roll-tube technique (Hungate, 1969) with slight modification. The enrichment culture solution was inoculated into brine-based solid medium (BSM) solidified by 20 g/l of gellan gum.

The enrichment culture solution was also inoculated into a half concentration of solidified reinforced clostridium medium (0.5×RCM; RCM0149, Oxoid Limited, UK) for control. Approximately 50 ml of BSM or 0.5×RCM was poured in a serum bottle and the headspace was replaced by each gas as described above. These preparations were sterilized by autoclaving at 121ºC for 20 minutes. 0.1 ml of each enrichment culture solution was inoculated separately into both media after they had been cooled down to 70ºC, and was mixed before they had been solidified. Two bottles were prepared for each condition.

After the solutions had been solidified at a slant, they were incubated at 50ºC for 1–2 weeks. After this time, significant increase in the number of colonies hadn't been observed. Each colony whose size, pigmentation, margin and elevation were visually distinct were picked out from the solid medium using sterile Pasteur pipettes in an anaerobic chamber degassed with pure N_2 (> 99.99%). The picked colonies had been transferred into fresh NBM in the anaerobic chamber. The headspace of each serum bottle was replaced with each gas as described above. Each NBM was incubated at 50ºC for 3 weeks. The process of serial dilution was repeated at least twice in order to purify the culture.

Analyses of the Bacterial Populations in the Enrichment Culture Solutions

The enrichment culture solution was filtered through 0.22 μm sterile membrane filters to collect the bacterial cells on the filters. Total bacterial DNA was extracted from the filters and purified using the Ultra Clean Soil DNA Isolation Kit (MO BIO Laboratories, Inc., USA) according to the manufacturer's instructions.

A variable V3 region of the 16S rDNA gene of the purified DNA was amplified with nested polymerase chain reaction (PCR) using Premix Taq™ Hot Start Version (Takara Bio Inc., Japan) and primers. PCR conditions were carried out as described by Muyzer et al. (1993). The PCR

products were then applied to denaturing gradient gel electrophoresis (DGGE), and the bacterial diversities of the enrichment culture solutions were analyzed. The bacterial diversities of the enrichment culture solutions were evaluated based on the Shannon–Weaver diversity indices, which increase along with the increase in bacterial diversities, calculated using the DGGE results (Yeates et al., 2003). Homology searches of the DNA sequences were carried out using the GenBank DNA database (www.ncbi.nlm.nih.gov) with the BLAST program.

Identification of the Isolate

Preliminary characterization of the isolates was based on morphological observation of cells on an agar plate under both a phase contrast microscope (BH2; Olympus Corp., Japan) and a scanning electron microscope (JSM-6320F; JEOL Ltd., Japan). The isolate was also identified with molecular biology techniques. The identification of phylogenetic neighbors and pairwise 16S rRNA gene sequence similarities were calculated using the EzTaxon server (http://www.eztaxon.org/) (Chun et al., 2007). A phylogenic tree was constructed based on an alignment of the 16S rDNA with sequences of related genes from GenBank using the MEGA4 program (Tamura et al., 2007).

Results and Discussion

Microbial diversities of enrichment culture solutions are shown in Figure 1. The Shannon–Weaver diversity indices of the enrichment culture solutions that were sterilized by autoclaving and incubated in pure N_2, 10%-CO_2, or pure CO_2 atmosphere were 0.84, 0.68 and 0.37 respectively. On the other hand, those of the enrichment culture solutions that were sterilized by filtration and incubated under pure N_2, 10%-CO_2, or pure CO_2 atmosphere were 1.63, 1.22 and 0.96 respectively.

These results revealed that the diversity indices of filtrated enrichment culture solutions were higher than those of autoclaved culture solutions. Although the autoclaving is the most common and effective sterilization method, some elements and volatiles that are rich in natural reservoir brine usually are lost by the excessive heat in these experiments. The results observed in these experiments suggest that some composition of the natural reservoir brine was altered during autoclaving. These alterations inhibited the growth of several bacteria that had inhabited the brine originally; therefore, filtrated natural reservoir brine should be used for enrichment culture medium to increase the bacterial population.

In both sterilization cases, the Shannon–Weaver diversity indices of the enrichment culture solutions whose headspaces had been replaced by pure CO_2 were lower than those of other enrichment culture solutions. These results indicate that the number of bacterial strains decreased as the CO_2 concentration increased. This result was highly correlated with the pH of the enrichment culture medium, which became 9.0, 8.5, and 7.2 after their headspaces were replaced by pure N_2, 10%-CO_2, and pure CO_2 respectively. This result agrees with the results of a previous study (Morozova et al. 2010) that showed that the number of microbial cells was decreased from 2.4×10^6 cells/ml to 9.0×10^5 cells/ml just after CO_2 injection during a CO_2 storage project in a saline aquifer.

The higher variety of bacterial composition was observed in the enrichment culture solution that had been sterilized by filtration and incubated in pure N_2 atmosphere; however, two resultant bands: Nos. 9 and 10 in Figure 1 were detected only under the presence of CO_2. These results indicate that CO_2 may be useful in isolating novel bacterial strains from oil reservoirs. The pH range for the growth of *Petrotoga* species that No. 10 was determined to be closely related to

was 6.5 to 8.0. These results suggest that the pH reduction of the culture medium due to the dissolving CO_2 was effective in the screening of this kind of bacterium.

Nevertheless, the predominant bacterial population of the natural brine (i.e., Nos. 11, 12, and 13 in Figure 1) was not detected in the enrichment culture solutions. This result revealed that the new approaches of enrichment cultures using natural reservoir brine developed in this study effectively stimulated the growth of the bacteria that had naturally inhabited the reservoir as non-dominant species.

To identify the bacterial communities in the enrichment culture solutions, sequence analyses of 16S rDNA isolated from the DGGE gels were performed using the BLAST web program. The predominant bacterium detected in all the enrichment culture solutions was similar (represented by No. 6 in Figure 1), and was determined to be closely related to *Petrotoga mobilis* (92.5% similarity). *Petrotoga* species have been found exclusively in oil reservoirs (Ollivier and Cayol, 2005), suggesting that they are native to high temperature and anaerobic environments; therefore, the new approaches of enrichment cultures developed in this study could make a thermophilic anaerobic bacterium a dominant successfully.

Based on the morphological appearance of the bacterial colonies, 7 kinds of bacteria had been successfully isolated from the enrichment culture solutions after 1 week incubation (Table 1). The number of bacterial colonies isolated from incubation with 0.5×RCM was higher than those from incubation with BSM. Many kinds of bacteria could form colonies and be isolated with 0.5×RCM because it was abundant in simple carbon and nitrogen sources. On the other hand, because BSM was poor in those substrates, the bacteria that could grow under substrate-limited conditions could be isolated selectively with BSM. Furthermore, based on the previous report by Ueda et al., (2008), it has been indicated that the enrichment cultivation approach with

a high level of CO_2 may enable the isolation of novel bacterial strains. According to these considerations, only one isolated bacterium named AR80 could be expected to be a novel bacterium because it formed its colonies only with BSM in pure CO_2 atmosphere. AR80 was then further characterized.

As shown in Figure 2, the typical AR80 cell is rod-shaped, with a width of 0.25–0.75 μm and a length of 2.5–7.0 μm, and has a characteristic outer sheath-like structure and polar flagella. Complete nucleotide sequence of 16S rDNA of AR80 revealed that it is a member of the genus *Petrotoga* with a similarity of 94.0% (GenBank accession number: GQ385331). Its closest phylogenetic relationships were with *P. mobilis* (Lien et al., 1998), *P. sibirica*, *P. olearia* (L'Haridon et al. 2002), and *P. Mexicana* (Miranda-Tello et al. 2004), which showed sequences similarities of 94.7%, 94.2%, 94.4%, and 94.6% respectively (Figure 3). According to Stackebrandt and Goebel (1994), 94.0% is a sufficiently low similarity to establish a new species; therefore, AR80 is assumed to be a novel species within the genus *Petrotoga*.

Conclusions

This study aims to suggest new approaches of enrichment cultures using natural reservoir brine to increase the bacterial population in enrichment cultures and isolate novel bacteria from oil reservoirs. For this purpose, the effect of the sterilization method for natural reservoir brine-based culture medium to the increase in the bacterial population was considered. In addition, the effect of the presence of CO_2, which was one of the reservoir conditions, to the isolation of novel bacteria from an oil reservoir was also considered. The following conclusions have been reached in this study:

1) Natural reservoir brine-based culture medium should be sterilized by filtration to increase the bacterial population in enrichment cultures of natural reservoir brine.

2) Presence of CO_2 in enrichment cultures is effective in increasing the possibility of isolating novel bacteria inhabiting oil reservoirs as non-dominant species.

3) A thermophilic anaerobic bacteria AR80 was isolated successfully through the new approaches of enrichment cultures developed in this study. According to the phylogenetic analyses, AR80 is a novel species belonging to the genus *Petrotoga*.

Thus, the new approaches of enrichment cultures developed in this study increase the possibility of isolating novel bacteria from oil reservoirs.

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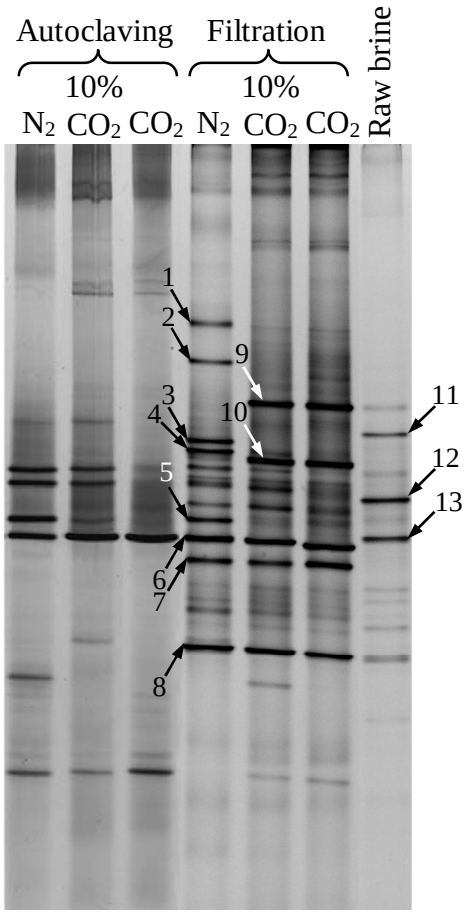
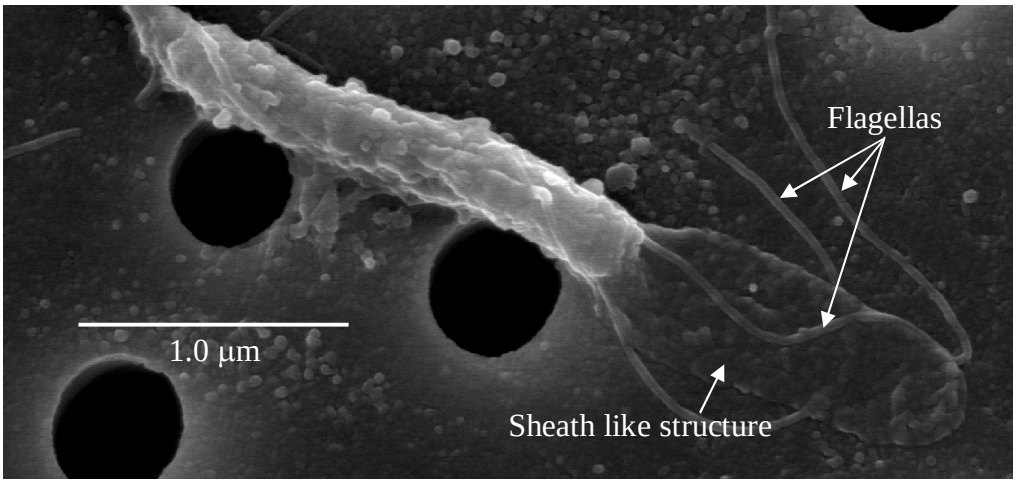

Autoclaving
Filtration
10%
10%
N2
CO2
CO2
N2
CO2
CO2
Raw brine
1
2
9
3
4
10
11
12
5
13
6
7
8


Flagellas
1.0 µm
Sheath like structure

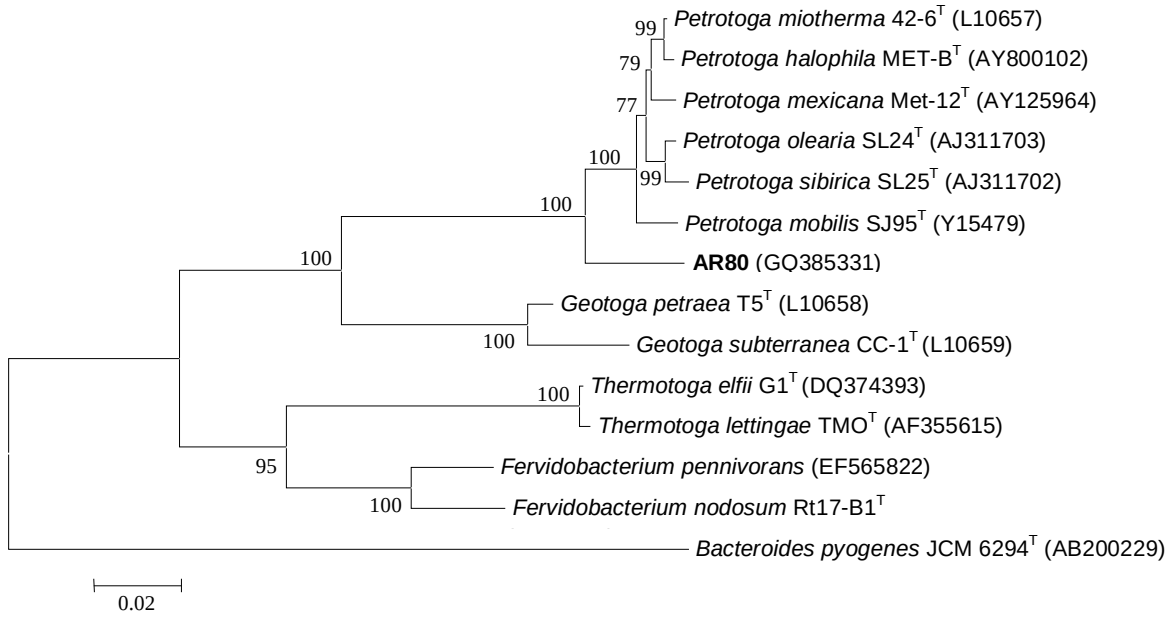

99
79
77
100
99
100
100
100
100
100
95
100
Petrotoga miotherma 42-6T (L10657)
Petrotoga halophila MET-BT (AY800102)
Petrotoga mexicana Met-12T (AY125964)
Petrotoga olearia SL24T (AJ311703)
Petrotoga sibirica SL25T (AJ311702)
Petrotoga mobilis SJ95T (Y15479)
AR80 (GQ385331)
Geotoga petraea T5T (L10658)
Geotoga subterranea CC-1T (L10659)
Thermotoga elfii G1T (DQ374393)
Thermotoga lettingae TMOT (AF355615)
Fervidobacterium pennivorans (EF565822)
Fervidobacterium nodosum Rt17-B1T
Bacteroides pyogenes JCM 6294T (AB200229)
0.02

Figure 1. Microbial community structures of enrichment culture solutions and the natural reservoir brine with different sterilization methods and different atmospheric conditions. Arrows indicate the bands that had been identified by sequencing analyses (identified similarity). [1. *Bacteroidales bacterium* (99.2%); 2. *Bacteroides* sp.(99.2%); 3. *Bacteroides* sp.(99.2%); 4. *Bacteroidales bacterium* (99.1%); 5. *Geobacter* sp.(81.8%); 6. *Petrotoga mobilis* (92.5%); 7. *Flexistipes* sp. (95.4%); 8. *Clostridiaceae bacterium* (100%); 9. *Bacteroidales bacterium* (93.9%); 10. *Petrotoga* sp. (92.5%); 11. *Bacteroidales bacterium* (99.1%); 12. *Acetobacterium* sp. (100%); 13. *Syntrophomonas Palmitatica* (92.4%)].

Figure 2. Scanning electron micrograph of AR80.

Figure 3. Phylogenetic dendogram based on 16S rDNA sequences showing the phylogenetic positions of AR80 and related species in the genus *Petrotoga* and other members of the family *Thermotogaceae*. *Bacteroides pyogenes* served as an outgroup. Accession numbers of 16S rDNA sequences of reference organisms are appeared in brackets. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branching points. Bar, 2 nucleotide substitutions per 100 nucleotides.

Table 1 The bacteria isolated from enrichment culture solutions by using two kinds of solid medium

Isolate	Colony morphology				0.5×RCM			BSM		
	Size	Pigmentation	Margin	Elevation	N_2	10%-CO_2	CO_2	N_2	10%-CO_2	CO_2
AR15	1-2 mm (Punctiform)	Translucent	Entire	Convex	+++	++	–	++	–	–
AR27	3-5 mm (Round)	White plus opaque	Filamentous	Raised	+	–	–	–	–	–
AR33	1 mm (Punctiform)	Cream	Entire	Raised	++	+	–	–	–	–
AR50	5 mm (Round)	Cream plus opaque	Undulate	Raised	–	+	–	–	–	–
AR61	3-5 mm (Round)	Cream plus opaque	Entire	Raised	–	++	–	–	–	–
AR73	3 mm (Round)	Cream	Entire	Raised	–	+	–	–	–	–
AR80	0.5-1 mm (Punctiform)	White	Entire	Raised	–	–	–	–	–	++++

Number of colonies (++++): >100 ; (+++): 50-100; (++): 20-30;(+):1-10; (−): Not detected

Enrichment culture solution that 0.1 ml of unsterile brine had been inoculated into was inoculated into both solid media